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# p62在蛋白質堆疊物的降解作用之調控機制

# The role of the autophagic cargo receptor p62 in the clearance of aggregation-prone proteins

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# 國立臺灣大學博士學位論文
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## p62 在蛋白質堆疊物的降解作用之調控機制
## The role of the autophagic cargo receptor p62 in the clearance of aggregation-prone proteins

本論文係 董盈岑君（f93b41019）在國立臺灣大學動物學研究所完成之博士學位論文，於民國 101 年 12 月 13 日承下列考試委員審查通過及口試及格，特此證明

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# 中文摘要

神經退化性疾病的共同特徵，是在神經內或周圍有不正常的蛋白質堆疊，許多研究指出，這些異常堆疊的蛋白質，是導致神經退化的主因。目前已知選擇性的細胞自噬作用(selective autophagy)，可以特定清除這些堆疊物，做為一種保護神經的機制。有證據顯示，在哺乳動物細胞中，這樣的選擇性是經由 p62/sequestosome1 做為受體:它可以跟自噬體上的 LC3 結合，且被觀察到會共同沉積含特定致病蛋白的堆疊物中，例如亨丁頓舞蹈症(Huntington's disease, HD)的突變型 Huntingtin (Htt)，以及阿茲海默氏症(Alzheimer's disease, AD)的 Tau。

本論文的第一部分，延續了之前在酵母菌模式生物的發現，找出在 LC3 上負責與 p62 結合的胺基酸位點，顯示從酵母菌到哺乳動物都利用相同的分子機制，將受體連結到自噬體中。而我們的實驗也證實，當 LC3 與 p62 無法形成連結時，突變 Htt 型蛋白堆疊便無法經由選擇性細胞自噬作用所清除，進一步支持 p62 可作為細胞自噬作用降解特定蛋白質堆疊的受體。

p62 在神經退化性疾病的重要性，促使我們去研究它基因表現上的調控，與其蛋白質功能的相關性。本論文的第二部份，陳述我們發現 presenilin-1 (PS1)可經由活化 Akt/AP-1 訊息傳導途徑，來促進 p62 基因的轉錄表現。我們也測試了幾個在家族遺傳性 AD 病人中特有的 PS1 基因突變型，發現當細胞表現這些突變的 PS1 時，卻無法維持其 p62 基因的正常表現，導致細胞沒有足夠的 p62 來幫助清除 Tau 蛋白質。此研究找到了 PS1 調控 p62 表現進而影響 Tau 降解的新功能，也更加確認 p62 在神經退化性疾病中，作為清除蛋白質堆疊的重要分子。

本論文的研究成果，證實 LC3-p62 的結合，在選擇性細胞自噬作用中擔任舉足輕重的角色，負責將誘發神經退化性疾病的蛋白質堆疊送至自噬體中。另一方面，也找到調控 p62 表現的新途徑，幫助我們更進一步了解 AD 及其他神經退化性疾病的致病機轉。

**關鍵字**: 細胞自噬作用；p62/sequestosome-1；神經退化性疾病；presenilin-1

## Summary

The accumulation of certain misfolded protein aggregates in the brain is a common feature in various neurodegenerative diseases, and is accepted as a major causative factor of neurodegeneration. Aggrephagy, the process by which protein aggregates are selectively degraded through macroautophagy, plays an essential role in protecting neurons from aggregate-induced neurotoxicity. Recent findings have identified p62/sequestosome1 as a cargo receptor that interacts with the autophagosomal membrane associated protein LC3, and recruits ubiquitin-positive protein aggregates into autophagosomes. The finding that p62 is co-localized with inclusion bodies in the brains of patients with Huntington's disease (HD) and Alzheimer's disease (AD) suggests a critical role for p62 in neurodegeneration.

Previous findings have identified residues in a yeast LC3 homologue, Atg8, that are essential for interaction of Atg8 with the cargo receptor Atg19 in selective autophagic processes. In the first part of my thesis, I describe our attempts to determine whether such interaction is evolutionally conserved from yeast to mammals. By using an amino acid replacement approach, we determined that three residues in LC3 corresponding to those in Atg8 were essential for p62 binding. Furthermore, while disruption of the LC3-p62 complex formation did not alter overall autophagic activity, it was sufficient to impede the autophagy-mediated clearance of aggregation–prone mutant Huntingtin (Htt), the cytotoxic protein which induces the

pathological phenotypes of HD.

The protective role of p62 in the clearance of aggregation-prone proteins prompted us to investigate how p62 expression is regulated under pathological conditions. In the second part of my thesis, I describe our discovery that p62 expression is transcriptionally regulated by presenilin 1 (PS1), a protein which is mutated in the majority of patients with early-onset familial Alzheimer's disease (FAD). The PI3K/Akt/AP-1 pathway was found to be required for PS1-mediated regulation of p62 expression. Moreover, down-regulation of p62 by either PS1 deficiency or over-expression of FAD-linked PS1 mutants compromised clearance of aggregation-prone Tau, which forms intracellular neurofibrillary tangles in the AD brain; these findings thus confirm the essential role of p62 in the clearance of neurotoxic protein aggregates.

Together, our studies emphasize the importance of the LC3-p62 interaction in selective autophagy, and the requirement of p62 for the removal of neurodegeneration-associated protein aggregates. Furthermore, the identification of PS1-dependent transcriptional regulation of p62 expression uncovers a novel PS1/p62-mediated molecular mechanism underlying the pathogenesis of AD and related neurodegenerative diseases.

**Key words**: autophagy；p62/sequestosome-1；neurodegenerative diseases；presenilin-1

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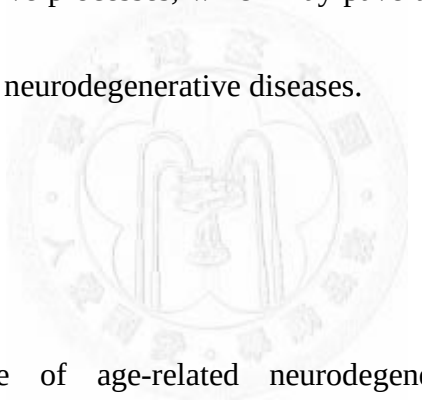
# Chapter 1

# Introduction

## 1.1 Neurodegenerative disease

Neurodegenerative disease is a general term for a number of disorders that are characterized by gradual loss of central or peripheral structures of the nervous system. As neurons are highly differentiated, non-proliferating cell types, recovery from their degeneration is difficult. Aging is a major risk factor for neurodegenerative diseases, as it makes patients more prone to neuronal atrophy, as well as interfering with the replacement of lost neurons. It is therefore important to uncover the mechanisms underlying neurodegenerative processes, which may pave the way for the prevention or treatment of detrimental neurodegenerative diseases.



Common features

A common feature of age-related neurodegenerative diseases is the accumulation of misfolded proteins within or around neurons. Different neurodegenerative diseases are characterized by aggregates composed of distinct protein components. For example, aggregates of α-synucleins in Parkinson's disease (PD); prions in Bovine spongiform encephalopathy (BSE); SOD1 in amyotrophic lateral sclerosis (ALS); huntingtin (Htt) in Huntington's disease (HD); and amyloid-β (Aβ) and Tau in Alzheimer's disease (AD) [1]. While the constituents of inclusions are disease specific, they share strikingly similar structural characteristics.

These disease-related proteins behave normally under physiological conditions, but may become aggregation-prone due to mutations that alter amino acid residues or translational modifications that alter secondary structures. Such mutations or modifications result in improper folding of the protein and exposure of hydrophobic patches, thereby enhancing self oligomerization via hydrophobic interaction. After formation of oligomeric (globular) intermediates, protofibrillar structures are assembled. The protofibrillar intermediates further elongate first into filaments and then fibrils, finally resulting in a helical assemblage with a cross β structure, visible under a light microscope [2]. In most cases these large inclusion bodies are ubiquitin-positive; however, they are usually resistant to degradation [3]

The accumulation of neurodegenerative disease-related protein aggregates has been shown to impair basic cellular functions. For example, aggregate formation can induce oxidative stress and mitochondrial dysfunction, which lead to neuronal death [4]. Synaptic activity and membrane integrity of neurons are also disrupted by aggregates [5]. Furthermore, proteasomal degradative activity is decreased when abnormal protein aggregates form inside cells [3, 6], thereby further promoting accumulation of inclusion bodies.

Interestingly, recent findings argue that oligomeric soluble species exert greater neurotoxic effects than large protein aggregates. This hypothesis emerged from the

observation that application of oligomers composed of 10~20 molecules induced cell death, while application of large aggregates (> 200mers) did not cause significant cytotoxicity [7, 8]. Animal models that express aggregation-prone proteins were treated with specific antibodies to remove soluble proteins, resulting in amelioration of behavior deficits, while the aggregates remained intact [9]. Hence it is possible that the formation of aggregates is a protective mechanism to sequester the toxic oligomeric species [10, 11]. Despite these findings, further investigations are required to elucidate which polymeric stage exerts the most detrimental effects on cellular functions; in addition, it remains widely accepted that degradative systems involved in the clearance of these aggregate-prone proteins play a key role in protecting against neurotoxicity.

In the following sections, I will first discuss specific protein aggregates implicated in two neurodegenerative diseases: HD and AD. I will subsequently introduce the degradative systems involved in these diseases, and describe the current understanding of the regulatory mechanisms involved in the degradation of aggregation-prone proteins.

### 1.2 Huntington's disease

Huntington's disease (HD) is named after Dr. George Huntington, who first

accurately described the symptoms of patients with this disease [12, 13]. It has been estimated by a systematic analysis that the world-wide prevalence of HD is about 3 per 100,000 people [14]. HD is an adult onset disease, the pathological changes of which start with neurodegeneration in the striatum of the basal ganglia, a brain region controlling the voluntary motor functions and the cognitive and emotional functions. The most common symptom of this disease is involuntary movement of head and limbs, which resembles dance-like movements. As such, HD is also called Huntington's chorea; chorea is derived from the Greek word choreia, meaning a type of dance. Patients also develop cognitive dysfunction and psychiatric problems during aging, which correspond with progressive neurodegeneration from the striatum to other brain regions, such as the cerebral cortex, the hippocampus, the hypothalamus and parts of the thalamus [15]. Patients usually present with severe dementia, mental illness and other complications around 15 to 20 years after the onset of HD, which finally leads to death. Treatments are now available to alleviate the symptoms, but no therapies can alter the course of this disease.

Huntintin (Htt)

It is now clear that HD is an autosomal dominant disease which occurs in patients carrying mutations in the Htt gene. This gene is located on human

chromosome 4 and contains poly(CAG) repeats within the exon 1 region. In healthy subjects, the number of CAG repeats in the Htt gene range from 3 to 25. Once the number of CAG repeats exceed 36, the resulting long polyQ-containing Htt proteins tend to aggregate inside neurons [16]. Direct intermolecular interactions between polyQ extensions of mutant Htt proteins, or intramolecular interactions between polyQ and the N terminus cause a dramatic conformational change from the α-helical structure of normal Htt to a misfolded parallel β-sheet form [17]. The misfolded confomation is more prone to form aggregates.

The length of the polyQ repeat has been shown to inversely correlate with the onset age of HD [18]. However, the number of neurons containing Htt inclusions do not positively correlate with neurodegeneration [19], suggesting that soluble monomers or, more likely, oligomers, contribute to neurotoxicity, while insoluble Htt deposition may be a protective response. In support of this view, microinjection of soluble β-sheet monomers and β-sheet oligomers of an expanded poly-Q protein into cell cultures caused severe cytotoxicity, which was rescued by a poly-Q binding peptide that prevents β-sheet conformation transition [20].

The cytotoxic effects of mutant Htt have been extensively studied. BDNF transportation is crucial for striatum neurons to maintain neuronal functions, but can be impaired by mutant Htt [21]. Htt can translocate between the cytosol and the

nucleus, and nuclear mutant Htt has been reported to attenuate expression of several genes [22]. Furthermore, mutant Htt was found to transfer between cells in culture [23], indicating that, like prions, mutant Htt is able to spread to neighboring cells, inducing formation of neurotoxic aggregates and contributing to progressive neuronal death [24].

The above evidence suggests that polyQ extension in Htt causes a gain-of-toxic function that promotes HD pathogenesis. On the other hand, genetic depletion of Htt expression in a mouse model caused early developmental lethality [25], which could be reversed by breeding knock-in mice that expressed Htt containing 50 polyQ repeats [26]. Therefore, Htt plays a role during development regardless of poly Q expansion. In addition, this protein is required for suppression of caspase-mediated apoptosis [27], and may also be involved in maintaining pro-survival Akt pathways [28]. Loss of the normal protective Htt function may act synergistically with mutant Htt to cause neurodegeneration in HD patients.

Htt can be modified via phosphorylation [29-31], ubiquitination [32, 33], acetylation [34] and sumoylation [35]. These post-translational modifications can regulate the stability of Htt or contribute to the toxicity of the mutant form. Enzymes involved in the above modifications are potential therapeutic targets for HD. On the other hand, Htt with a poly-Q extension has been reported to impede both proteasomal

and autophagy systems [36-38], leading to greater accumulation of toxic Htt species. Strategies to enhance cellular degradative systems may prove to be potent therapies.

### 1.3 Alzheimer's disease

Alzheimer's disease (AD) is named after Dr. Alois Alzheimer, who first reported the symptoms and pathologies of a patient with the disease [39]. As of the time of writing, more than 35 million people worldwide are affected by this disease. In Taiwan, the prevalence of AD in elderly people is around 2~4%, which means that more than 25,000 people suffer from AD [40]. This prevalence is estimated to increase dramatically in the next decade due to the ageing population. The enormous medical costs to patients' families and society will inevitably become an important social issue.

Progression of AD is accompanied by gradual neuronal loss [41]. The first region to undergo degeneration is the entorhinal cortex-II (EC-II), which is connected to the hippocampus, the main area in the brain for memory storage [41-43]. Other brain regions connected to EC-II subsequently shrink. Severe loss of the cortical regions becomes apparent within 7 to 10 years following disease onset, and accounts for cognitive and emotional deficits. Hence, patients in the late stage of AD not only lose their short-term memory, but also suffer from cognitive dysfunction and

psychiatric problems that dramatically disrupt their quality of life and reduce life expectancy.

Alois Alzheimer used a silver staining method to uncover two abnormal structures in the brain of the first patient identified with AD -- protein deposits scattered over the cortex (amyloid plaques) and intraneuronal bundles of fibrils (neurofibrillary tangles, NFTs) [39]. Both pathological hallmarks are now widely regarded as the primary causes of neurodegeneration in AD.

Amyloid-β (Aβ) and Presenilins (PSs)

Amyloid plaques are composed of fibrillar aggregates of amyloid-β peptide (Aβ). These 39-43 amino acid polypeptides are derived from an integral membrane protein called amyloid-β precursor protein (APP) [44]. Aβ deposits are closely associated with dystrophic neurites, active microglia, and reactive astrocytes [45]. The neurotoxicity of Aβ fibrils, which has been demonstrated in various *in vitro* systems, is closely correlated with the pathogenesis of AD, and blockage of Aβ fibril formation prevents Aβ toxicity [46]. A recent report showed that soluble Aβ oligomers exert neurotoxic effects by inhibiting hippocampal long-term potentiation (LTP) in rats *in vivo* [47], strongly suggesting Aβ-induced neurodegeneration in AD pathogenesis. Thus, understanding how Aβ is generated and how this pathogenetic process can be

regulated is extremely crucial.

Aβ is generated through sequential cleavages by two proteases: β- and γ-secretase [48]. First, APP is cleaved by β-secretase at a site 28 residues N-terminal to the transmembrane domain [49], releasing a large soluble fragment (sAPPβ) into the lumen/extracellular space, and retaining a 99-residue C-terminal fragment (C99-CTF) in the membrane. This membrane-tethered C99 can then be further processed by γ-secretase through an unusual cleavage that apparently occurs within the transmembrane domain of C99, thereby producing Aβ peptides.

γ-Secretase has been regarded as a prime therapeutic target, because it catalyzes the final proteolytic step in the generation of Aβ. The enzyme is a large membrane protein complex composed of presenilin (PS), nicasatrin (NCT), Aph1 and Pen2. Evidence compiled from pharmacological studies, mutagenesis, affinity labeling, and biochemical isolation strongly suggests that γ-secretase is an aspartyl protease with an active site located at the interface of the PS heterodimer [50], which is thought to be the active form of PS [51, 52]. Significant accumulation of γ-secretase substrates is found in ES cells derived from mice deficient in both PS homologues ($PS1^{-/-}$, $PS2^{-/-}$), concordant with the lack of detectable Aβ in these animals [53, 54]. Two highly conserved aspartate residues residing within the sixth and seventh transmembrane domains (TM6 and TM7) of presenilins are required for γ-secretase activity [55].

Research on familial AD (FAD) has identified several mutations in the genes encoding APP, PS1, and PS2. Approximately 90% of FAD mutations are found in PS1 and PS2, and the majority of these mutations alter the proteolytic activity of γ-secretase, leading to an increase in relative production of aggregation-prone Aβ42 peptides [56]. However, emerging evidence implies both secretase-dependent and -independent roles of PS1 in diverse cellular activities, which may also contribute to neurodegeneration in AD. The secretase-independent function of PS1 will be further discussed in chapter 3.

Tau

The NFTs are composed of Tau proteins, which are encoded by the human gene MAPT (microtubule-associated protein Tau). As suggested by the name of the encoding gene, Tau proteins were first co-purified with microtubules in 1975 [57], and later studies revealed a major role of this protein in stabilization of microtubules [58]. The region required for such interaction is located in the C-terminus of the Tau protein, and consists of either three or four 18 amino acid repeats that result from alternative splicing [59, 60]. The positively charged repeats allow Tau to bind directly to the negatively charged microtubule surface. The highly acidic N-terminal region contains zero to two negatively charged inserts (29 amino acids of each) which are

also derived from alternative splicing. Therefore, six Tau isoforms exist in the adult human nervous system. Between the C-terminal repeat regions and the N-terminal acidic domain is a proline-rich region with PPXXP or PXXP motifs; these motifs are believed to bind to plasma membrane-associated proteins, including the Fyn kinase [61, 62]. Association between Tau and Fyn kinase contributes to dendritic localization of Fyn and promotes Fyn-meditated phosphorylation of NMDA receptors, leading to exaggerated excitotoxicity in the presence of toxic Aβ species.

Tau proteins have been found to undergo several post-translational modifications. Phosphorylation of Tau is the most prominent modification in AD, and is thus a subject of extensive study [63]. There are 79 postulated sites of phosphorylation in the longest Tau isoform. Phospho-serine or -threonine surrounding repeat domains have been shown to disrupt the interaction between Tau and microtubules. Under normal conditions, the phospho-state of Tau is tightly regulated by kinases, which include glycogen synthase kinase 3β (GSK3 β), Cdk5, MAP kinase (p38), JNK, cyclic AMP-dependent kinase (PKA), $Ca^{2+}$/calmodulin-dependent kinase (CaMKII), and protein kinase C (PKC), and by phosphatases, including protein phosphatase (PP) 1, PP2A, PP2B (calcineurin), and PP2C [63]. However, the brains of most AD patients exhibit abnormal accumulation of hyper-phosphorylated Tau (eight or more phosphates per protein, as compared to only 2~3 phosphates in a

normal Tau protein), and the extent of phosphorylation correlates with the progression of the disease [64].

More than 45 phospho-residues have been identified in AD patients [65], and a subset of antibodies recognizing different AD-associated phospho-residues on Tau have been developed for diagnostic purposes [63]. Although the phosphorylation sites that underlie pathogenesis remain unclear, phospho-$Thr^{231}$, -$Ser^{235}$ and -$Ser^{262}$, all of which are observed at early pre-tangle stages, were shown to be critical for the dissociation of Tau from microtubules, leading to destabilization of the latter [66, 67]. Therefore, intracellular trafficking of neurotrophins and proteins essential for metabolism is blocked in the presence of hyper-phosphorylated Tau. Furthermore, formation of Tau aggregates through hyper-phosphorylation results in attenuation of proteasomal activity [68], supporting the pivotal role of hyper-phosphorylated Tau in neurodegeneration. It should be noted that spatial and temporal phosphorylation of Tau by diverse kinases contributes to the formation of hyper-phosphoryated Tau in AD, and promotes self-assembly of Tau into insoluble filament structures, thus complicating the development of stratagies targeting Tau phosphorylation through inhibition of protein kinases [69]. For example, PKA-mediated phosphorylation of Tau facilitates subsequent phosphorylation by GSK3β, which is accompanied by loss of memory in a rat model [70].

Conversely, there is also evidence suggesting protective roles for hyper-phosphorylated Tau, as there are reports that it renders neurons more resistant to apoptosis and oxidative stress [71]. This is consistent with the observation that Tau proteins in neurons from fetal brains possess more phosphorylated residues [72]. Whether these protective phosphorylated sites are the same as those detected in AD remains unaddressed. Nevertheless, the levels of total Tau and phophorylated Tau in cerebrospinal fluid is tightly correlated with cognitive deficits in AD, suggesting that Tau is an important factor in the disease.

Aβ versus Tau hypothesis

The formation of two pathological hallmarks, amyloid plaques and neurofibrillary tangles, in AD patients strongly implies that Aβ and Tau contribute to pathogenesis of the disease. However, which of these is the primary cause of AD has been debated among researchers for a long time. In the past decade, the amyloid hypothesis claiming that Aβ acts upstream of Tau to exert neurotoxicity and facilitate hyper-phosphorylation of Tau is predominant in the field of research [56]. This concept is substantially supported by the genetic discovery of FAD-linked mutations in APP, PS1, and PS2, but not in Tau. As mentioned earlier, the common feature of these mutations is to increase the relative production of Aβ42, which accelerates the

aggregation of amyloid peptides. Several transgenic mouse models expressing either a FAD-linked human APP mutant alone or in combination with a human PS1 mutant exhibit neurodegeneration and intraneuronal NFT accumulation, supporting the role of Aβ in driving AD pathogenesis.

Interestingly, mounting evidence suggests that Tau is indispensible for Aβ-induced neurotoxicity. Neurodegeneration was observed in Aβ42-treated primary neuron cultures derived from wild-type mice, but no toxic effects were detected in those from Tau KO mice that underwent the same treatment with Aβ42 [73]. Another study reported that mutant APP-expressing J20 mice exhibit Aβ plaque pathology accompanied by premature lethality and memory defects. Crossing J20 mice with Tau-null mice can ameliorate these pathological phenotypes [74]. Later, Ittner, L. M. *et al*. demonstrated that the interaction between Tau and Fyn leads to postsynaptic localization of Fyn and induces phosphorylation of NMDA receptors by Fyn [61]. Phospho-NMDA receptors tend to associate with postsynaptic density protein 95 (PSD95), forming a complex required for Aβ-induced excitotoxicity. According to the above evidence, Itner and Gotz proposed the “Tau axis hypothesis” [75], which suggests that formation of Aβ oligomers in dendrites results in synaptic dysfunction at the early stage of AD. As Aβ also induces accumulation of hyper-phosphorylated Tau (possibly through activation of NMDA receptors), neurons are rendered more

vulnerable to Aβ toxicity, contributing to neuronal loss during the progression of the disease.

Taken together, these results suggest that although Aβ is the principle causative factor for AD, excessive amounts of Tau are also required to mediate Aβ-driven toxicity. Strategies to prevent Tau accumulation are therefore potential therapeutic approaches to block neurodegeneration. In the following sections, I summarize the two main cellular degradative systems, both of which are involved in clearance of neurodegeneration-related proteins and are thus therapeutic targets for these diseases.

### 1.4 Proteasome

Both the ubiquitin-proteasomes and the autophagosome/lysosome systems are indispensible degradative complexes in our cells. Functional defects in these complexes can severely impair cellular protein quality control. While each is composed of different cellular elements, the two systems also share common elements, and have been recently shown to undergo cross-talk with each other [76, 77].

The 26S proteasome is a complex that resembles a tube with upper and lower covers composed of several subunits [78]. The tubule structure is the 20S core particle of the proteasome, consisting of two outer α-rings and two inner β-rings, each of which is formed by seven subunits. Catalytic activity is conferred by the β-ring.

Different β-subunits possess either trypsin-like, chymotrypsin-like, or peptidyl-glutamyl peptide-hydrolyzing activities, all locating to the inner face of the tube. Exchange between these subunits contributes to catalytic specificities of the proteasome. The α-rings connect with the 19S regulatory particles that constitute the upper and lower covers. The precisely-organized 19S regular particles control the opening of the α-rings, and are involved in substrate recognition, unfolding and translocation.

The ubiquitin-proteasome system is tightly controlled to enable degradation of specific proteins. Such selectivity is achieved by the ubiquitin conjugation process [79, 80]. Proteins that fail to fold properly are tagged with 8 kDa ubiquitin molecules through covalent binding at Lys residues. Three enzymes are required for the ubiquitin conjugation process—first, ubiquitin is linked to an ubiquitin-activating E1 enzyme, which is accompanied by ATP hydrolysis to activate the ubiquitin through adenylylation. Second, the ubiquitin is transferred to an ubiquitin-conjugating E2 enzyme. Finally, an ubiquitin ligase E3 enzyme binds to both the E2 conjugase and the specific substrate, allowing the ubiquitin to form a covalent bond with a lysine residue on the substrate. While only two E1 and around one hundred E2 enzymes have been identified, it has been shown that more than one thousand E3 ligases are present in eukaryotic cells, which enables restricted and precise degradation of target

proteins.

Ubiquitin contains seven lysine residues, each of which can be conjugated with another ubiquitin, resulting in the formation of linear chain linkages or branched chain linkages on the substrate (polyubiquitination). K48 linkages, consisting of at least four ubiquitin molecules that are all linked through the lysine residues at position 48, are the most abundant form of polyubiquitin chains. It has been shown that proteins targeted with K48 linkages are the most rapidly degraded substrates of proteasomes. The K11 linkage was also demonstrated to be a tag for proteasomal degradation in some cases [81, 82]. Other types of lysine linkages are specifically responsible for different cellular activities. For example, the K33 linkage is involved in kinase modification [83], while K29 and K63 linkages are associated with endocytosis trafficking and autophagic degradation (to be discussed in later sections) [84, 85].

After polyubiqutination, most target proteins can directly bind to the ubiquitin receptors on the 19S regulatory particles of proteasomes. In some cases, they require specific shuttle factors such as Rad23, ubiquilin 1/2 and p62 for transportation to proteasomes [86]. Common features of these shuttle proteins are the presence of at least one ubiquitin associated (UBA) domain to interact with ubiquitin chains on the substrates, and a ubiquitin-like (UBL) domain that binds to proteasomal Ub receptors.

Once the substrate protein docks to a proteasome, it undergoes deubiquitination

and unfolding, as mediated by certain subunits of 19S regulatory particles. For example, Rpn11 of the lid subcomplex is required for removal of entire ubiquitinated chains [87]. Both deubiquitination and the unfolding reaction involve high energy demands, and therefore a subgroup of 19S regulatory particles possess ATPase activity to provide sufficient driving force [86]. Finally, the unfolded proteins without ubiquitin enter the catalytic tube for digestion. Short peptides (around 8 amino acids) are generated and released back into the cytosol for further use.

Proteasome dysfunction has been observed in aging brains and in the affected brain areas of a number of neurodegenerative diseases [88]. Frame shift mutations of ubiquitin were identified in AD [89], indicating a link between proteasome impairment and neurodegeneration. In support of this link, treatment of cell models with chemical inhibitors targeting S20 core particles induced formation of ubiquitin positive-inclusion bodies and caused cell death [90]. Furthermore, transgenic mice that lacked functional 26S proteasomes in the central nervous system developed intraneuronal inclusions and presented with neurodegeneration [91]. Overall, loss of quality control in protein turnover by proteasome damage can contribute to neuropathogenesis, and the proteasomal system is thus a potential therapeutic target for neurodegenerative diseases.

## 1.5 Autophagy

While the proteasome is required for degradation of short-lived and small soluble proteins, the relatively large sized membrane compartments of the autophagy/lysosome machinery allows the digestion of long-lived macromolecules and even organelles. Autophagy/lysosomal processes are highly conserved from yeast to mammals. Three types of autophagy have been identified, namely microautophagy, chaperone-mediated autophagy and macroautophagy.

Microautophagy

Microautophagy is the direct engulfment of molecules surrounding lysosomes by the dynamic activity of the lysosomal membrane. Although it was discovered over 40 years ago [92], the mechanisms are not yet completely understood. Nevertheless, the process of microautophagy can be divided into five steps, according to the involvement of distinct molecules and morphological changes of the lysosome [93]. The first step is the invagination of the lysosomal membrane and the resulting formation of autophagic tubes towards the interior of lysosome. This initial step can be induced by starvation or by inhibition of the mammalian target of rapamycin (mTor). Certain macroautophagy-related proteins (Atgs), including Atg3, Atg4, Atg5, Atg7, Atg8, Atg10 and Atg12, are required for organization of the autophagic tube

during the process of microautophagy (please refer to the “Macroautophagy” section for descriptions of Atgs). Additionally, vacuolar transporter chaperone (VTC) complexes were found localized to the invagination site in yeast, where they serve as scaffolds that interact with cytoskeleton proteins, membrane proteins and regulatory molecules [94]. Changes in the protein/lipid composition of the autophagic tube then leads to formation of a vesicle at the tip (vesicle formation step) with a lollipop shape. This vesicle undergoes expansion (expansion step) and finally pinches off into the lumen from the tube (scission step), which is driven by GTPase. At the final stage, the released vesicles are degraded by lysosomal hydrolases, and the digested products are transported back to the cytosol for recycling through the permease Atg22p.

Since microautophagy ends with consumption of the lysosomal membrane, and its regulatory machineries overlap with those of macroautophagy, it has been proposed that microautophagy is a compensatory mechanism for macroautophagy (which contributes the membrane source of lysosomes) to balance the size of lysosomes. However, microautophagy not only engulfs substrates non-selectively by invagination, but also specifically entraps damaged peroxosomes (micropexophagy), non-essential portions of the nucleus (piecemeal microautophagy of the nucleus, PMN) or impaired mitochondria (micromitophagy) in yeast models [93, 95], implying an active role of microautophagy in maintaining cellular physiological functions.

Chaperone-mediated autophagy (CMA)

Chaperone-mediated autophagy (CMA) requires specific chaperone proteins to transport cytosolic molecules directly into lysosomes. Proteins containing a pentapeptide (KFERQ) motif are recognized and bound by heat shock cognate protein of 70 kDa (hsc70) [96]. This interaction allows substrates to dock to a receptor, lysosomal associated membrane protein type 2A (LAMP2A), which is located on the lysosomal membrane. On the cytosolic side, several co-chaperones assist in the unfolding of the substrate [97], while a subgroup of hsc70 molecules on the luminal side provide a pulling force to facilitate translocation of the substrate through LAMP2A into the lysosome [98]. In contrast to microautophagy and macroautophagy, CMA is restricted to degrading soluble proteins (not large organelles), as substrates must be able to pass directly through transporters in the lysosomal membrane.

CMA can be induced by stress, including nutrient deprivation and oxidative damage [99]. During these conditions, nonessential proteins and oxidized proteins are removed partly through CMA. It has also been shown that CMA is required for presentation of endogenous antigens by MHC-II [100], suggesting diverse roles of CMA in physiological functions. Interestingly, the KFERQ-like motif is also present in proteins related to neurodegenerative diseases, such as α-synuclein, Htt and APP, mutations in all of which can cause conformational changes that may result in toxic

polymers. Among these potential substrates, wild-type α-synuclein has been proved to undergo CMA-mediated degradation, while mutant forms do not translocate into the lysosome through LAMP2A [101]. In addition, CMA has been shown to degrade endogenous wild-type Htt [102], and expansion of the polyQ tract in Htt may disrupt its transportation across lysosomal membranes.

Macroautophagy

Macroautophagy is characterized by the formation of a double-membraned autophagosome that fuses with a lysosome to degrade its contents. It is the most extensively investigated type of autophagy, and hereafter use of the term “autophagy” will refer to macroautophagy, if not otherwise indicated.

Macroautophagy is initiated by the formation of the phagophore assembly site (PAS); this encloses a portion of the cytoplasm, which becomes a double-membrane-limited autophagosome [103]. The degradation of contents within autophagosomes is subject to fusion with lysosomes [104] (Fig.1.1a). The process of autophagy is tightly governed by a series of autophagy-related proteins (Atgs), ranging from Atg1 to Atg36, that were first identified in yeast and are thought to be functionally conserved from yeast to human [105].

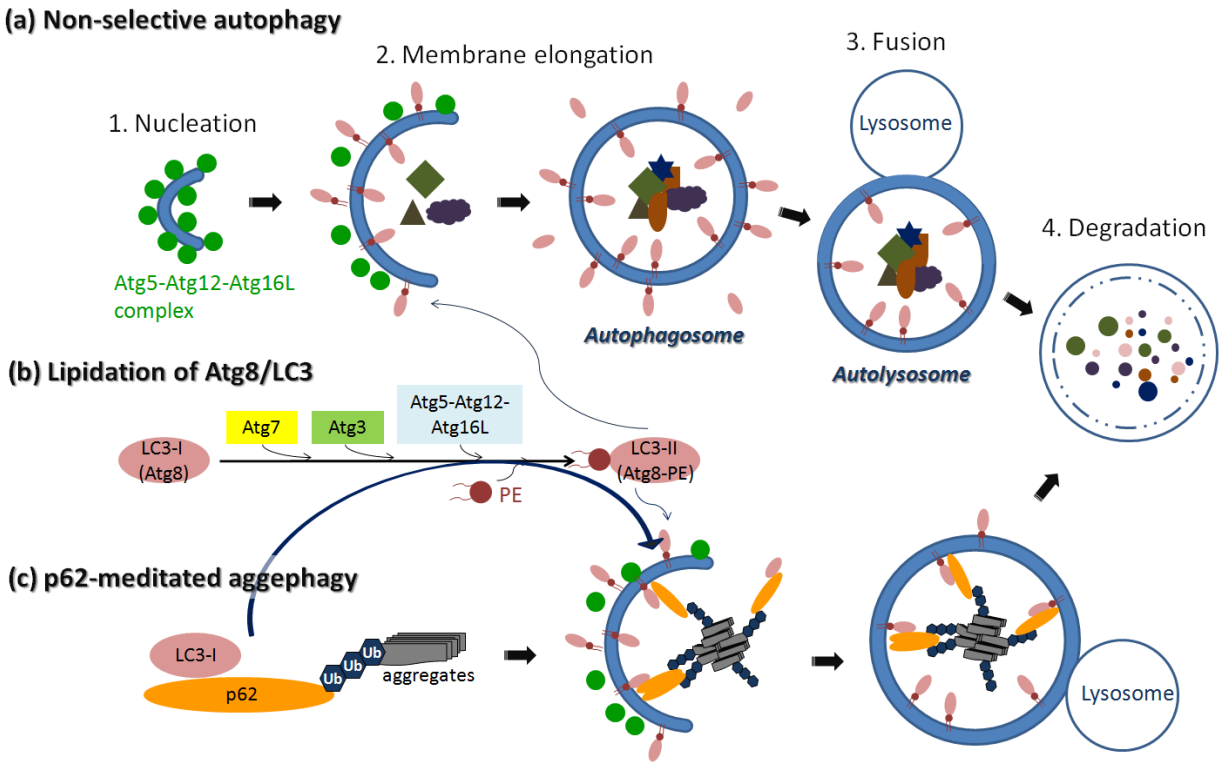



**Fig.1.1 Schematic depiction of macroautophagy, lipidation process and aggrephagy**.

(a) Non-selective autophagy: cytosolic material (bulk cytoplasm, protein aggregates, organelles or pathogens) is sequestered by an expanding membrane sac, resulting in the formation of a double-membrane vesicle, an autophagosome. The outer membrane of the autophagosome subsequently fuses with a lysosome, the cargo-containing membrane compartment is then lysed, and the contents are degraded. (b) Covalent binding of LC3-I/Atg8 to phosphatidylethanolamine (PE), mediated by Atg7, Atg3 and Atg5-Atg12-Atg16L complex, is essential for the translocation to autophagosome, which in turn mediates autophagosome formation. (c) p62-mediated aggrephagy: ubiquitinated proteins initially interact with the UBA domain within p62, and are selectively entrapped into autophagosomes through interaction between LC3 and p62 for their degradation.

One group of Atg proteins essential for the initial stages of autophagy is comprised of Atg1 and Atg13. The Atg1-Atg13 complex acts downstream of Target of Rapamycin (TOR), AMPK and the Ras/PKA pathway to regulate autophagy [106, 107].

Two mammalian homologs of Atg1, uncoordinated 51-like kinase 1 (ULK1) and ULK2, have also been shown to coordinate with mammalian Atg13 to induce autophagy [108, 109]. Upon activation, the ULK complex translocates to the PAS forming site (adjacent to the ER), where it may contribute to the relocalization of the phosphatidylinositol 3-OH kinase (PI3K) complex, BECLIN 1-VPS34 [110]. This promotes the generation of phosphatidylinositol 3-phosphate (PI3P) at the PAS, which is crucial for activating certain Atg complexes (namely Atg12-Atg5-Atg16L1).

The core Atg machinery, including Atg3, Atg4, Atg5, Atg7, Atg8, Atg10, Atg12, and Atg16, functions as a ubiquitin-like conjugation system during the early stage of autophagy [111]. The ubiquitin-like protein Atg12 is conjugated to Atg5 through the sequential action of Atg7 (ubiquitin-activating enzyme) and Atg10 (ubiquitin-conjugating enzyme) [112]. The Atg5-Atg12 conjugate then forms a large complex with Atg16-L.

The other ubiquitin-like protein, Atg8, is first synthesized as a precursor form, and its C-terminus is subsequently processed by the Atg4 cysteine protease to expose a glycine residue. The processed Atg8 is then transferred to phosphatidylethanolamine (PE) by Atg7 (the same ubiquitin-activating enzyme required by Atg12), Atg3 (a ubiquitin-conjugating enzyme) and the Atg5-Atg12-Atg16L complex (a ubiquitin E3 ligase) (Fig 1.1b). The PAS localization of the Atg5-Atg12-Atg16L complex therefore

determines the site at which Atg8 is converted from an unlipidated species (form-I) to a lipidated one (form-II), allowing the attachment of lipidated Atg8 to both sides of the forming autophagosome membrane. As Atg8 is the only molecule known to persist on autophagosomes throughout autophagy, it has been commonly used as a marker to identify autophagosomes [113].

At least six homologues of Atg8 have been identified in mammals, including three microtubule-associated protein 1 light chain 3 proteins (LC3A, B and C), γ-aminobutyrate receptor-associated protein (GABARAP), and two GABARAP-like proteins (GABARAPL1 and 2) [114, 115]. LC3B has been reported to be the major homologue involved in starvation-induced autophagy. However, cell type-specific distribution of LC3 isoforms, and the observation that all these homologues can be processed to conjugate with PE, suggest a complicated involvement of these proteins in the regulation of autophagy. It has recently been shown that the N-terminal α–helices of LC3 and GABARAPL2 are crucial for membrane fusion during autophagosome biogenesis [116]. Interestingly, overexpression of LC3 resulted in accumulation of larger Atg16-positive phagophores, while overexpression of GABARAPL2 reduced the number of phagophores, indicating that LC3s are required for expansion of phagophore membranes, and the GABARAP subfamily is involved in autophagosome maturation [117].

In order to catabolize the confined materials, the outer membranes of the autophagosome must first fuse with lysosomes or late endosomes, a process that requires the small GTPase Rab7A and LAMP1/2 [118]. By recycling the degraded products for sources of energy production and macromolecule synthesis, cells are able to survive under stress conditions. Interestingly, the released molecules can reactivate mTOR activity, which impedes autophagy and induces formation of proto-lysosomal tubules and vesicles; these vesicles form autolysosomes that finally mature into functional lysosomes. Hence, mTOR also participates in negative feedback regulation to maintain the homeostasis of lysosomal membranes [119].

The essential roles of autophagy in controlling the balance of protein metabolism have been intensively studied in various biological contexts. An increase in autophagic activity has been shown to expand the lifespan of various animal models [120-122]. Conditional deletion of Atg5 ot Atg7 in the central nervous system of mice results in loss of autophagic activity and concomitant accumulation of ubiquitinated proteins, leading to significant neurodegeneration [123, 124]. These findings clearly demonstrate that autophagy-mediated clearance of unwanted aggregates is crucial for neuronal survival, consistent with the notion that a decline in autophagy is tightly associated with normal aging [125]. Moreover, disruption of autophagy is also linked with a number of neurodegenerative diseases.

For HD, genetic blockage of autophagic activity hampered degradation of mutant Htt [126]. Activation of autophagy by the insulin signaling pathway promoted clearance of poly-Q-expanded Htt [127]. Rapamycin treatment, which inhibits mTor and in turn induces autophagy, has been reported to reduce Htt aggregates in cells overexpressing mutant Htt and in a mouse model of HD, and alleviate neurodegeneration [128, 129], strengthening the protective role of autophagy in HD.

Abnormal accumulation of autophagosomes and other pre-lysosomal autophagic vacuoles are found in dystrophic neocortical and hippocampal pyramidal neurons from AD patients [130]. This pathological phenotype can be simulated by inhibition of lyososomal proteolysis in neuronal cultures [131]. Autophagosomal-lysosomal dysfunction and memory deficits were observed in an APP transgenic mouse model, and these defects were rescued by enhancement of lysosomal activity [132]. These lines of evidence suggest that failures in the later stages of autophagy, rather than dysregulation of autophagic induction, account for the autophagosome/lysosome pathology in AD.

Accumulating evidence suggests that autophagy participates in the removal of detrimental Tau and Aβ peptides, and the APP-CTF precursor. For example, Aβ can be internalized and transported into the autophagic machinery for degradation via the Aβ-binding receptor α7nAChR [133], implying that enhancement of this degradative

pathway should exert neuroprotective effects. However, unlike HD, the role of autophagy is more complicated in AD. Convincing data reveal an unfavorable function of autophagy in facilitating the production of intracellular Aβ [134]. The two faces of autophagy in homeostasis of Aβ potentially place it in a unique and intriguing position for AD pathogenesis (refer to [135] for detailed information).

Selective autophagy

Although the autophagic pathway appears to non-selectively engulf cytosolic molecules, it can also trap certain cargos for selective turnover, which is pivotal for quality control of specific proteins and for removal of damaged organelles. Several types of selective autophagy have been identified and named for their distinct substrates, including mitophagy (turnover of mitochondria), ERphagy (turnover of ER), pexophagy (turnover of peroxisomes), nucleophagy (turnover of nucleus), lipophagy (turnover of lipid droplets), aggrephagy (turnover of aggregation-prone proteins), and xenophagy (turnover of pathogens) [136]. These forms of selective autophagy have been shown to have profound roles in the pathogenesis of diverse diseases.

These distinct forms of selective autophagy require precise regulatory systems, and emerging studies are making efforts to elucidate the molecules involved. It has

been reported that a cargo receptor is necessary to bring the substrates into autophagosomes. An adaptor may also be required to make a connection between the cargo-receptor complex and the Atg proteins on the forming phagophores.

The best characterized form of selective autophagy is the yeast cytosol-to-vacuole targeting (Cvt) pathway, a process specifically required for the maturation and vacuole transportation of the vacuolar aminopetidase 1 (Ape1), aminopetidase 4 (Ape4), α-mannosidase 1 (Ans1) and Ty1 transposon [137]. These substrates first form a Cvt complex to interact with the autophagy receptor Atg19. The adaptor protein Atg11 then binds to Atg19 and recruits the whole complex to the phagophore; Atg1/ULK1 at this site then regulates formation of the autophagic membrane. The cargo receptor Atg19 also interacts with Atg8, the only protein known to be retained on the autophagosome membrane, thereby allowing transportation of the Cvt complex to lysosomes via entrapment within autophagosomes.

In mammals, no Atg19 homologues have been thus far identified, but a few potential cargo receptors required for selective autophagy have been discovered. A common feature of these receptors is the presence of at least one consensus “LC3-interacting region (LIR)” [138, 139]. The protein p62/sequestosome1 was the first cargo receptor identified in mammals, and is currently the best characterized [140-142]. It is required not only for selective autophagy, and especially aggrephagy

(Fig 1.1c), but also for diverse cellular functions. Mammalian adaptor proteins are less understood; to date, only the ALFY adaptor is known to recruit the Atg5-Atg12 complex to phagophores and directly interact with the cargo-receptor complex (reviewed in [143]).

Interestingly, the cargo receptor p62 has been shown to be present in inclusion bodies within degenerating brain neurons of patients with various neurodegenerative diseases; these include aggregations of α-synuclein in PD, Htt in HD, TDP-43 in ALS, and Tau in AD [144-146]. In the next section, I will discuss in detail the role of p62 in diverse physiological functions and neurodegeneration-related pathological conditions.

**1.6 p62/sequestosome 1**

The structure of p62

The complementary (c)DNA of p62 was cloned in 1996 [147], and the encoding protein was subsequently named sequestosome 1, on account of its ability to form cytosolic inclusion bodies with ubiquitinated proteins, and to ”sequester” these proteins in specialized compartments [148]. p62 messenger (m)RNA is ubiquitously expressed in a variety of tissues [147], and the protein is conserved throughout metazoa. Multiple protein-protein interaction motifs have been characterized in the

p62 protein [149] (Fig 1.2). At the N-terminus of p62, there is a PB1 domain which mediates self interaction. As such, p62 can bind to other proteins containing PB1; these proteins are usually signaling molecules, including PKCζ, MEKK3, MEK5 and ERK, and p62 acts as a scaffold protein to regulate the various signaling pathways [150, 151]. In addition, p62 can assemble into homopolymers via the PB1 domain, which is crucial for the formation of inclusion bodies or sequestosome structures. It is interesting to note that this domain also interacts with the Rpt1 subunit of the proteasome, suggesting a role for p62 in the proteasomal degradative system.

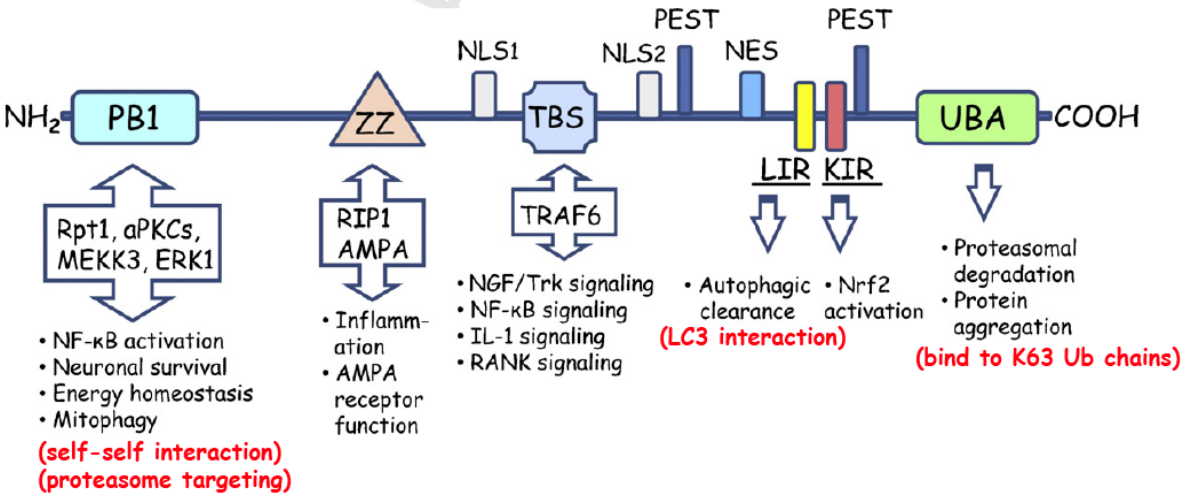



**Fig 1.2 Schematic layout of the domain organization of p62**

p62 dimerizes with another p62 molecule via binding of the PB1, Phox/Bem1 homology 1 (PB1) domain. This domain also interacts with the Rpt1 subunit of the proteasome. The LC3-interacting region (LIR) of p62 interacts with LC3, and the ubiquitin associated (UBA) domain binds K63-polyubiquitin chains. *(Adapted from [152])*

The C-terminus of p62 contains an ubiquitin-associated (UBA) domain which allows it to bind to ubiquitinated proteins, and in particular K63-polyubiquitinated proteins, with high affinity [153]. It has been reported that this domain enables p62 to co-localize with certain neurodegeneration-related polyubiquitinated protein aggregates, such as α-synuclein, Tau, and Huntingtin (Htt), in brain autopsies from patients [145, 146]. Interestingly, overexpression of p62 mutated in either the PB1 domain or the UBA motif significantly reduced the number of sequestosomes, as compared to those observed in cells expressing wild type p62 [154]. This finding indicates that both p62 self interaction and association of ubiquitin-positive proteins with p62 are required for the formation of inclusion bodies. Of note, the UBA domain is also required to recognize cargo for sorting to the autophagy or proteasome, which will be discussed in later sections.

Other motifs for protein interaction in p62 include a ZZ domain for AMP receptor binding [155], a TBS domain for association with an E3 ligase, TRAF6 [156], and a KIR domain for association with Keap1, a negative regulator of Nrf2 [157]. A recent study also identified two nuclear localization signal domains (NLS1 and NLS2) and one nuclear export motif (NES) in p62 [158]. It also showed that nuclear p62 contributes to nuclear protein aggregate formation in frontotemporal lobar degeneration and spinocerebellar ataxia.

p62 as a shuttle factor for proteasomal degradation

The presence of a UBA domain, a TBS domain (for TRAF6 binding) and a PB1 domain (for direct targeting to the proteasome subunit Rpt1) strongly suggest involvement of p62 in proteasome degradation. In support of this hypothesis, depletion of p62 expression in a cell model system resulted in accumulation of polyubiquitinated proteins [153]. In addition, two substrates, TrkA and Tau, have been reported to be shuttled to the proteasome in a p62-dependent manner. Upon NGF stimulation, the NGF receptor, TrkA, is subject to K63-linked polyubiquitination by TRAF6, and subsequently interacts with the UBA domain of p62. This association targets TrkA to Rpt1 and facilitates deubiquitination of TrkA, which is essential for its turnover by the proteasome [159]. Therefore, depletion of p62 results in stabilization of TrkA in PC12 cells. On the other hand, Tau- and ubiquitin-positive inclusion bodies in AD brains were found to co-localize with the E3 ligase TRAF6 and p62. Depletion of p62 blocked the Tau-proteasome (Rpt1) interaction and Tau degradation [160]. Furthermore, neurofibrillary tangle-like structures containing hyperphosphorylated Tau were observed in the brains of $p62^{-/-}$ mice [161]. It remains possible that homeostasis of other substrates may also be regulated through the TRAF6/p62/proteasome axis, pending further investigation.

p62 as a cargo receptor in selective autophagy

In addition to its role as a shuttling factor for proteasome degradation, Bjorkoy et al. (2005) demonstrated that some p62 proteins are constrained inside LC3-positive autophagosomes. Blockage of autophagy increases the size and number of p62-containing inclusion bodies [154], suggesting a role for p62 in autophagic degradation. More recently, an LC3-interacting region (LIR) was identified within p62, N-terminally proximal to its UBA domain. Biochemical analyses showed that the LIR domain is required for p62-LC3 binding and the subsequent entrapment of p62 into autophagosomes for degradation [140, 141]. In cultured cells, overexpression of p62 mutated in either the LIR or UBA domain results in a significant increase in cytosolic ubiquitin-positive inclusion bodies [141], similar to those observed in neurodegenerative diseases. In the first part of my PhD project (described in chapter 2), I identified some of the residues in LC3 essential for LC3-p62 complex formation, and demonstrated the importance of this conserved p62-LC3 interaction in the turnover of cytotoxic Huntingtin aggregates.

It has become clear that the p62-LC3 complex not only mediates aggrephagy, but is also required for selective autophagic degradation of post-mitotic midbody rings, damaged mitochondria (mitophagy), excessive peroxisomes (pexophagy) and intracellular pathogens such as *S. enteric* (xenophagy) [138]. A recent study found that

during *Mycobacterium tuberculosis* infection, p62 recruits antibacterial proteins (rather than bacteria) into autolysosomes, which results in bacterial death when the bacteria-containing phagosomes fuse with autolysosomes [162]. In all of the selective autophagy processes described above, ubiquitination is a prerequisite for p62 to recognize specific substrates.

It should be noted that autophagic receptors other than p62 have been recently identified. These proteins, which include NBR1, Nix and NDP52, contain at least one LIR domain that interacts with LC3 [163-165]. These cargo receptors are believed to coordinately regulate substrate selectivity under different conditions, although elucidation of the underlying mechanisms awaits further investigation.

Regulation of p62 expression

Expression of the multifunctional p62 protein requires extensive regulation. Transcription of p62 has been shown to be rapidly activated by a variety of extracellular signals, including phorbol 12-myristate 13-acetate (PMA) and calcium ionomycin in peripheral blood mononuclear cells, serum and platelet-derived growth factor (PDGF) in serum-starved NIH3T3 cells [147], and prostaglandin J2 (PGJ2) in neuroblastoma SK-N-SH cells [166]. Analysis of the 5'-flanking region of the p62 gene revealed the presence of putative binding sites for the transcription factors AP-1,

Sp1, NF-κB, c-myc, Ets-1 family proteins, MyoD, and C/EBP [167], which may coordinately contribute to the early p62 induction response. It has been reported that the oncogene Ras induces binding of the activator protein (AP)-1 to the p62 promoter, thereby enhancing the production of p62 in human cancer cells [168]. In addition, several of the aforementioned putative transcription factor binding sites are embedded in CpG islands, which are sensitive to oxidative damage [169, 170]. Therefore, the expression of p62 can also be attenuated under conditions of chronic oxidative stress that commonly occur in aging. On the other hand, acute induction of oxidative stress by electrophile treatment also simulates p62 transcription, by activating nuclear factor (NF)-E2-related factor 2 (Nrf2) [171], a redox-sensitive transcription factor, indicating a pivotal role of p62 in response to oxidative and electrophilic stress.

The pathogenic functions of p62 in disease

Altered expression of p62 has been linked to various diseases. An abundance of p62 proteins is linked to breast tumors, liver cancers and liver cirrhosis [172, 173]. A mouse model with reduced levels of a key autophagic molecule, beclin 1, developed spontaneous liver and lung tumors with accumulation of p62 protein [174]. Furthermore, liver tumors developed spontaneously in a liver-specific ATG7-deficient mouse model; this was suppressed by depletion of p62 protein, suggesting that

increased levels of p62 contribute to tumorgenesis [175]. Additional studies have shown that p62 is an important mediator of Ras-induced lung adenocarcinomas. The interaction between p62 and TRAF6 triggers polyubiquitination of IκB kinase (IKK), which in turn activates NFκB signaling [176], a major signaling pathway downstream of Ras. Therefore, Ras induces excessive expression of p62, thereby activating the NFκB pathway in cancer cells; this in turn decreases the number of reactive oxygen species (ROS) and enhances tumor survival [168]. In addition, the KIR sequence of p62 also allows it to chelate Keap1, a negative regulator of the stress responsive transcription factor Nrf2 [157]. Up-regulation of p62 in tumor cells may therefore also alleviate oxidative stress by promoting Nrf2 activity, which in turn induces the expression of key ROS scavengers [177]. As mentioned in previous section, Nrf2 enhances p62 transcription, resulting in a positive feedback loop that prolongs the antioxidative response under conditions of stress.

Mutations of p62 are also implicated in Paget's disease of bone (PDB), a disease characterized by abnormal bone destruction and regrowth. Most of the mutations are within or surrounding the UBA region, indicating that impaired binding of p62 to ubiquitin contributes to the pathogenesis of PDB [178]. However, the most prevalent PDB mutation, P392L, does not alter the binding affinity of p62 to ubiquitin, and nor does it affect the formation of the sequestosome [179, 180]. Instead, the

P392L mutation reduces interaction between p62 and the tumor suppressor Cyclindromatosis (CYLD), a deubiquitinase enzyme that negatively regulates osteoclastogenesis [181]. The p62-CYLD complex may be the key for osteoclast development/bone resorption activity in PDB.

The presence of p62 in neuronal inclusions of a variety of neurodegenerative diseases implies a crucial role of this protein in neuropathy. The ability of p62 to serve as a scaffold and act as a shuttle factor in the proteasomal and selective autophagic systems supports the hypothesis that its protective function involves sequestering misfolded proteins and mediating their turnover. The case for this hypothesis was strengthened by a recent study demonstrating that p62 immunostaining co-localized with pathological inclusions in PD brains, but was homogenously distributed in healthy neurons of both control and PD brains [182].

Several studies have provided evidence that PD-associated PINK1 and parkin mutations might work together to impair mitophagy and promote the pathogenesis of PD [183, 184]. Depolarization of the mitochondrial membrane triggers accumulation of the mitochondrial kinase PINK1 at the outer mitochondrial membrane, which in turn induces translocation of the E3 ligase parkin to mitochondria. Parkin catalyses K27 and K63-linked ubiquitination of voltage-dependent anion channel 1 (VDAC1) on depolarized mitochondria, allowing binding of p62 and subsequent recruitment of

LC3 to accomplish mitophagy [183, 184]. Therefore, down-regulation of p62 may also contribute to defects in mitochondrial turnover in PD.

The role of p62 in AD has recently come under increased scrutiny. Du et. al. observed an age-dependent increase in oxidative damage to the p62 promoter in the brains of patients with sporadic AD, and this was inversely correlated with the level of p62 protein [169]. Overall, it seems that p62 functions as a neuroprotective protein in AD, although the regulatory mechanisms of this protein remain to be determined. The relationship between p62 and AD is discussed in detail in the second part of my PhD project (described in chapter 3). I also address the role of a novel transcriptional control pathway in regulating p62 expression in familial AD.

In summary, we are intrigued by the multifunctional and protective role of p62 in the clearance of aggregation-prone proteins. In the following chapters, we focus on the role of the evolutionarily conserved LC3-p62 interaction in the autophagic removal of neurodegeneration-associated Htt aggregates, and FAD-associated regulation of p62 expression.

# Chapter 2

# The evolutionarily conserved cargo-receptor binding site of LC3 interacts with p62 to mediate autophagy-dependent degradation of mutant Huntingtin

## 2.1 Abstract

Autophagy has been found to play a critical role in the clearance of various aggregated proteins that contribute to the pathogenesis of human diseases. In mammalian cells, p62/sequestosome-1 protein binds to both LC3 and polyubiquitinated cargo proteins destined to autophagy-mediated degradation. LC3, the mammalian homologue of yeast Atg8, is translocated to autophagosomes upon induction of autophagy and regulates autophagosome formation in cultured mammalian cells. Our previous findings have identified several hydrophobic or positively charged residues in Atg8 that are essential for its interaction with the cargo receptor Atg19 in selective autophagic processes of yeast. We thus sought to determine whether such interaction is evolutionally conserved from yeast to mammals. Using amino acid replacement approach, we demonstrated that cells expressing mutant LC3 (LC3-K30D, LC3-K51A, or LC3-L53A) all exhibit disrupted LC3-p62 interaction and impaired autophagic degradation of p62, suggesting that the p62-binding site of LC3 is localized within an evolutionarily conserved domain. While cells overexpressing LC3 mutants did not exhibit normal autophagic activity, the autophagy-mediated clearance of aggregation–prone mutant Huntingtin, the cytotoxic protein inducing pathological phenotypes of Huntintung's disease, was significantly defective in cells expressing LC3 mutants. These results suggest that p62

directly binds to the evolutionarily conserved cargo-receptor binding domain of Atg8/LC3 and selectively mediates the clearance of mutant Huntingtin aggregates.

## 2.2 Introduction

Autophagy, one of the main degradative pathways in eukaryotic cells, is tightly governed by a series of autophagy-related preoteins (Atgs) [185, 186]. Among them, Atg8 in yeast and its homologue, the microtubule-associated protein (MAP) light chain 3 (LC3), are the most reliable markers of autophagosome. The conversion of cytosolic Atg8/LC3-I to PE-conjugated Atg8-PE/LC3-II is tightly correlated with the numbers of autophagosome and the activation of autophagy [111, 187-189].

Selective autophagy has been better characterized in yeast. Atg8 can interact with a cargo receptor, Atg19, which binds to the precursor of aminopeptidase 1 (prApe1), a vacuolar enzyme [190]. The Atg8-Atg19 interaction is thus necessary for the specific delivery of prApe1 into vacuoles through autophagic transport vesicles (cargo sorting mechanism) and for the formation of mature Ape1 [191]. However, molecules governing the cargo-sorting mechanism of selective autophagy remain elusive in mammals.

A number of studies have indicated that the p62/sequestosome 1 protein may function as a cargo receptor to recruit particular proteins into autophagosomes. When

p62 mutants that lack LC3-binding ability are expressed in $p62^{-/-}$ cells, increased intracellular accumulations of polyubiquitinated proteins are only colocalized with p62, but not LC3 [141]. Of noted, p62 has been found to colocalize with certain neurodegeneration-related insoluble aggregates containing polyubiquitinated proteins, such as Tau, Huntingtin (Htt), and α-synuclein [145, 146]. These findings suggest that p62 could play a crucial role in the clearance of insoluble inclusion bodies by shuttling those aggregation-prone proteins to autophagy through its specific associations with both LC3 and polyubiquitin chain. Therefore, the identification of p62-binding residues within LC3 could delineate the role of the p62-LC3 interaction in selective autophagy-mediated clearance of neurodegeneration-related protein aggregates.

Previous studies have characterized several residues in Atg8, including $Arg^{28}$, $Tyr^{49}$, and $Leu^{50}$, which are required for both interaction with Atg19 and regulation of general autophagic activity [192]. Additionally, residue $Lys^{48}$ and $Leu^{55}$ of Atg8 are also essential for autophagic transport, but are not required for Atg19 binding. Sequence alignment of Atg8 and LC3 shows that these hydrophobic and positively charged residues are evolutionarily conserved, and are exposed to the surface. We thus hypothesize that the sorting mechanism of selective autophagy could also be evolutionarily conserved and mediated by sequences within LC3, which correspond to

essential residues in Atg8 for Atg8-Atg19 interaction. In the present study, we systematically examine the roles of conserved LC3 residues in its binding to p62 and its lipidation. The potential effect of LC3 mutants that lack p62 binding ability on the clearance of cytotoxic Huntingtin aggregates is also determined. Our findings provide the first direct evidence that the LC3-p62 interaction is pivotal for the selective recruitment of aggregation-prone proteins to autophagy, rendering neuroprotection against aberrant protein aggregates.

## 2.3 Materials and Methods

### Reagents

The QuikChange site-directed mutagenesis kit was from Stratagene. BCA protein assay reagent kit and ImmunoPure® Immobilized Protein A were purchased from Pierce. Mouse anti-p62 antibody was from BD Transduction Laboratories. Mouse anti-GFP (B-2) antibody, rabbit anti-GAPDH (FL-335) antibody, rabbit anti-calnexin (H-70) antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, HRP-conjugated anti-rat IgG, and HRP-conjugated anti-mouse IgG were from Santa Cruz Biotechnology, Inc. Mouse anti-Htt antibody and Immobilon Western Chemiluminescent HRP Substrate were from Millipore Corporation. Earle's balanced salt solution, saponin, poly-L-lysine, mouse anti-GFP antibody (clone GFP-20) (for

immunoprecipitation), and dimethyl pimelimidate dihydrochloride (DMP) were purchased from Sigma-Aldrich, Inc. Rapamycin was from CalBiochem. Lipofectamine 2000 transfection reagent, DMEM, Alexa Fluor® 633 goat anti-mouse IgG (H+L), and 4´,6-diamidino-2-phenylindole, dihydrochloride (DAPI) were from Invitrogen. Fetal bovine serum (FBS) was from Biological Industries Ltd (Kibbutz Beit Haemek, Israel). All other reagents were at least reagent grade and obtained from standard suppliers.

**Site-directed mutagenesis of GFP-LC3 mutants**

The pEGFP-C1-LC3 construct encoding wild-type LC3 was generated as previously described. Mutagenized pEGFP-C1-LC3 constructs encoding mutant LC3s (LC3-K30D, LC3-K51A, and LC3-K53A) were generated by QuikChange site-directed mutagenesis kit according to the manufacturer's instructions. Primers were designed by Primer-X website (http://www.bioinformatics.org/primerx/index.htm). The sequences of paired primers for each mutant were as follows: K30D-forward (5'- GAG CAG CAC CCC ACC GAC ATC CCA GTG ATT ATA G-3') and K30D-reverse (5'-C TAT AAT CAC TGG GAT GTC GGT GGG GTG CTG CTC-3'); K51A-forward (5'-GTC CTG GAC AAG ACC GCC TTC CTT GTA CCT GAT C-3') and K51A-reverse (5'-G ATC AGG TAC

AAG GAA GGC GGT CTT GTC CAG GAC-3'); K53A-forward (5'-G GAC AAG ACC AAG TTC GCC GTA CCT GAT CAC GTG-3') and K53A-reverse (5'-CAC GTG ATC AGG TAC GGC GAA CTT GGT CTT GTC C-3'). The underlined nucleotides denote the base changes made to incorporate the desired missense mutations. The mutagenized sequences were confirmed by DNA sequencing.

To generate wild-type and mutant DsRed-tagged LC3 constructs, LC3 sequences were excised from pEGFP-C1-LC3 constructs by Eco RI and Bam HI. Purified DNA inserts were ligated with and subcloned into Eco RI/Bam HI-digested pDsRed-Monomer-C1 vector (Clontech Laboratories, Inc. ).

**Cell culture**

Human embryonic kidney cells (HEK293) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were incubated in a humidified incubator at 37 ºC in 5% $CO_2$.

**Transient transfection and induction of autophagy**

Transient transfection of LC3 constructs into HEK293 cells was performed using Lipofectamine 2000 tansfection reagent as described by the manufacturers. HEK293 cells were seeded onto 6-well microplates and transfected with 1 μg of

wildt-type or mutant pEGFP-LC3 constructs for 24 or 48 h to allow protein expression. To induce autophagy, following the removal of growth medium, transfected cells were washed by Earle's buffered salt saline (EBSS) once and incubated in EBSS for 2 h. Cells incubated in fresh culture medium were included as controls. Alternatively, HEK293 cells transfected with different GFP-LC3s were treated with 0.2 μg/ml of rapamycin for various intervals as specified to induce autophagy. Clarified lysates derived from transfected cells were analyzed by SDS-PAGE and Western blotting.

To determine the autophagy-mediated clearance of Htt aggregates, HEK293 cells in 10-cm dish were transfected with 5 μg of pcDNA3.1-Htt-(Q)109-hrGFP (a generous gift from Dr. Yijuang Chern at Academia Sinica [193]) for 24 h. Htt-(Q)109-hrGFP-transfected cells were subcultured onto 6-well microplates and allowed to adhere overnight. Htt-(Q)109-hrGFP-expressing cells were then transfected with 0.5 μg/well of wild-type or mutant DsRed-LC3 constructs for 5h later. Following the removal of transfection mixtures, cells co-transfected with Htt-(Q)109-hrGFP and DsRed-LC3 were incubated with culture medium in the presence of 0.1% DMSO or 0.2 μg/ml rapamycin for additional 48 h to induce autophagy.

**The Generation of a tetracycline-inducible cell line that is stably transfected with YFP-tagged neomycin phosphotransferase II fusion construct**

To generate an inducible neomycin phosphotransferase II-YFP (NeoR-YFP) construct, a marker protein for constitutive autophagy degradation [194], the coding sequence of NeoR was first subcloned into pEYFP-C1 (Clontech) in-frame with YFP tag. The NeoR-YFP cDNA sequence was subsequently subcloned into pcDNA5 plasmid (Invitrogen), and the expression of NeoR-YFP fusion protein would be under the control of tetracycline operator sequences. The NeoR-YFP construct was transfected into T-REx293 cells by lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Transfected cells were cultured in DMEM supplemented with 10% FBS, 200 μg/ml hygromycin and 5 μg/ml blasticidin, and single colonies resistant to antibiotic selection were isolated individually. Each of independent cell lines was screened for the tetracycline-inducible expression of NeoR-YFP. Cell lines in which the accumulation of NeoR-YFP can be suppressed by rapamycin (an autophagy activator) and enhanced by pepstatin A/E64D (lysosomal protease inhibitors) were retained, and the one (T-REx-NeoRY) with maximal induction of NeoR-YFP expression that is responsive to rapamycin or pepstatin A/E64D treatment was chosen for subsequent experiments.

**SDS-polyacrylamide gel electrophoresis and Western blot analysis**

Transfected cells were lysed by 1% Triton X-100 lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 1 mM β-glycerophosphate, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM $Na_3VO_4$, and the Complete® protease inhibitor cocktail) on ice for 30min. Following removal of cell debris by centrifugation, protein concentrations of clarified lysates were determined by the BCA protein assay reagent kit.

Cell lysates containing equal amounts of proteins were mixed with 6x sample-loading buffer, and boiled at 100 °C for 10 min. Proteins were separated by 10% Tris-glycine polyacrylamide gels, then transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Pall). Membranes were blocked by 5% BSA in TBST (blocking buffer) at room temperature for 1 h, followed by incubation with appropriated first antibody in blocking buffer (1:5000) at 4 °C overnight. After being washed with TBST for three times, membranes were incubated with HRP-conjugated secondary antibody in TBST (1:5000) at room temperature for 1 h. Following extensive washes with TBST, antibody-reactive proteins were visualized by Immobilon Western Chemiluminescent HRP Substrate. Images were captured and processed with ChemiGenius2 (Syngene).

**Co-immunoprecipitation of GFP-LC3s and endogenous p62**

Anti-GFP (GFP-20) antibody (1.5 μl) and Immobilized Protein A (20 μl of 50% slur per well for lysates from 6-well microplates) were mixed in 1 ml PBS at 4 °C overnight. Following removal of unbound antibodies by PBS washes, the Protein A and bound antibodies were cross-linked in 1 ml borate buffer (0.2 M sodium borate, pH 9.0) containing 20 mM DMP at room temperature for 30 min. The coupling reaction was stopped by addition of 0.2 M ethanolamine (pH 8.0), and the antibody-protein A conjugates were further incubated in 1 ml of 0.2 M ethanolamine for 1 h. Clarified lysates containing equivalent amounts of proteins (about 200 μg) were applied to the coupled antibody-protein A complexes and mixed at 4 °C overnight to pull down target proteins. Following removal of unbound proteins and extensive wash by PBS, immunoprecipitated proteins were eluted by boiling at 100 °C in 3x sample loading buffer and analyzed by SDS-PAGE and Western blotting.

**Immunofluorescence staining and confocal image analysis**

To observe the starvation-induced formation of autophagsomes in GFP-LC3s transfected cells, cells were seeded on poly-L-lysine-coated coverslips overnight. Cells were incubated with EBSS for 24 h and fixed with 4% paraformaldehyde in PBS at room temperature for 1 h, followed by washes with PBS prior to mounting.

Approximately 150 cells in each group from four independent experiments were analyzed, and the numbers and sizes of GFP-LC3 punctas (i.e. autophagosomes) per cell were measured.

To visualize colocalization of GFP-LC3 and endogenous p62, cells were fixed at 48 h post-transfection. Following washes with PBS, transfected cells were permeablized by 10% formaldehyde in PBS containing 0.5% Triton X-100 at room temperature for 10 min, and blocked with PBS containing 2% BSA at room temperature for 1 h. After removal of blocking buffer, cells were stained with a mouse-anti p62 antibody in PBS-0.1% BSA at 4 °C overnight. Following PBS washes, cells were incubated with Alexa Fluor 633 goat anti-mouse IgG in PBS at room temperature for 1 h. GFP and Alexa Fluor 633 fluorescence images were captured separately by sequential scanning. Approximately, 100 cells in each group from three independent experiments were analyzed. The overlapped areas between GFP puncta and p62 signals were measured and shown as the percentage of the total number of GFP puncta.

To assess the clearance of huntingtin aggregates, cells co-transfected with Htt-(Q)109-hrGFP and DsRed-LC3 were fixed after 48 h rapamycin treatment. Nuclei were stained with DAPI for 5min. The coverslips were washed with PBS and mounted as described above. In 12~13 viewing areas from two independent experiments, total

numbers of Htt-(Q)109-hrGFP-expressing cells and Htt aggregate-containing cells were counted. Data were shown as the percentage of cells with aggregates.

All the immunofluorescence images were obtained by using a Leica TCS SP5 spectral confocal microscope. Quantitative analyses were processed by using the Metamorph 6.2 software package (Molecular Devices).

**Quantitative densitometry and statistical analysis**

The density of the immunoreactive bands on Western blots was quantitated by the TotalLab v2.01 program after acquisition of the blot image with ChemiGenius2 (Syngene). Results were expressed as the mean (±S.D.) of three to four independent experiments. Statistical analyses were done by a two-tailed Student's *t*-test. A value of $p < 0.05$ was considered significant.

## 2.4 Results

**Two basic (Lys$^{30}$ and Lys$^{51}$) and one hydrophobic (Leu$^{53}$) residues in the ubiquitin core of LC3 are required for the interaction with p62**

Atg8 and its mammalian ortholog LC3 share a high homology in their amino acid sequences and three-dimensional structures, with a hydrophobic patch on the surface surrounded by several basic residues [195, 196]. The hydrophobic patch

within the ubiquitin core of LC3 is well-conserved and resembles the E1-binding surface of ubiquitin. The notion that the hydrophobic patch of LC3 could be a recognition module of its interacting proteins prompted us to examine whether this evolutionarily conserved hydrophobic patch also plays an essential role in the interaction of LC3 and its cargo receptor p62. Several hydrophobic or positively charged residues in Atg8, including $Arg^{28}$, $Leu^{50}$, and $Lys^{48}$, have been shown to be essential for its interaction with the cargo receptor Atg19 in yeast [192]. To determine whether the corresponding residues in LC3 (Lys30, Lys51, and Leu53) are also essential for the interaction between LC3 and p62, we replaced $Lys^{30}$ with negatively charged aspartate (K30D), $Lys^{51}$ with alalnine (K51A), and $Leu^{53}$ with alanine (L53A) individually by site-directed mutagenesis. Both the wild-type and mutant LC3s were N-terminally tagged with GFP for fluorescence detection.

To confirm whether these conserved residues are also indispensable for the binding of LC3 to its cargo protein p62, individual GFP-LC3s (WT, K30D, K51A and L53A) were transiently transfected into HEK293 cells as previously described [197]. Using co-immunoprecipitation with a GFP antibody, we found that, while endogenous p62 can be pulled down with LC3-WT, all three mutant LC3s (K30D, K51A, and L53A) exhibit significant defect in the binding of p62 (Fig. 2.1a, b). Our data corroborated that the essential function of $Lys^{30}$, $Lys^{51}$, and $Leu^{53}$ in LC3 is

evolutionarily conserved as those corresponding residues in Atg8 of yeast for cargo protein binding.

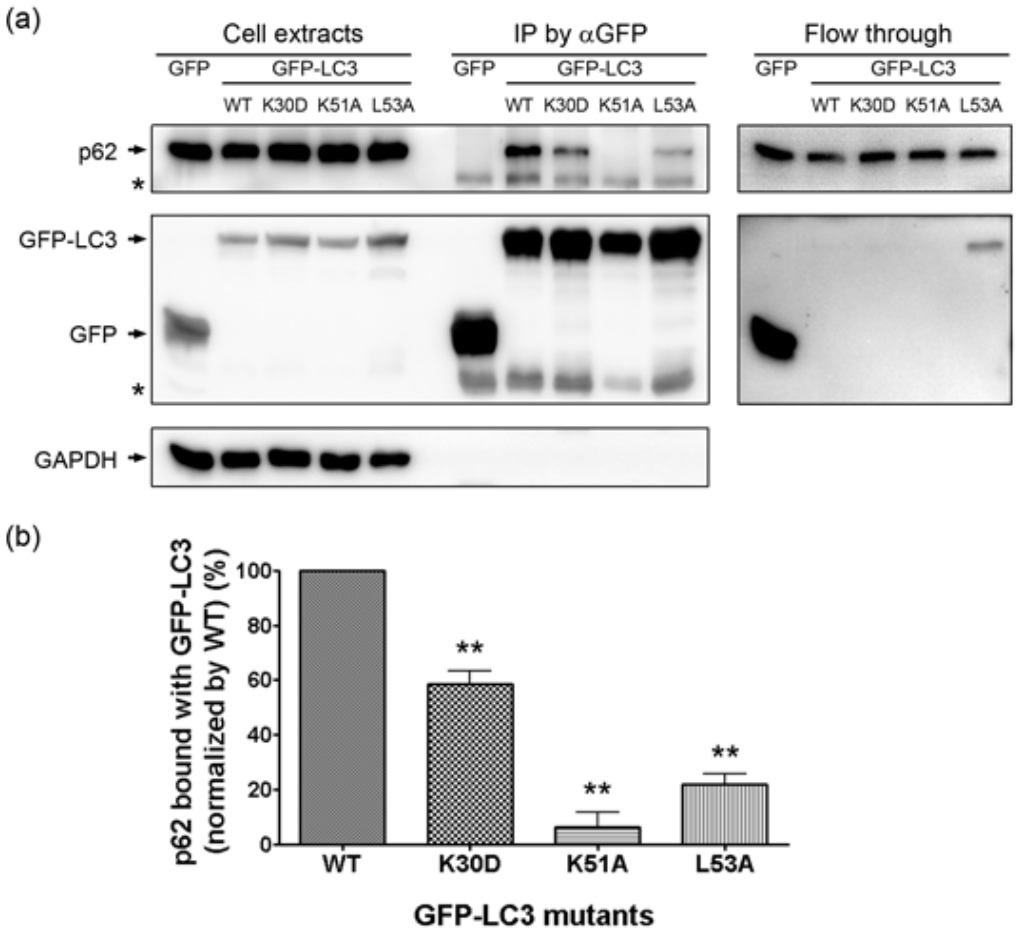



**Figure 2.1 Mutations in the ubiquitin core of LC3 attenuate its interaction with endogenous p62.**

**(a)** HEK293 cells were transfected with an empty vector expressing GFP tag alone (GFP) or a GFP-tagged LC3 expression vector that encodes wild-type (GFP-LC3-WT) or mutagenized LC3 (GFP-LC3-K30D, GFP-LC3-K51A, or GFP-LC3-L53A) for 24 h. Clarified lysates of transfected cells containing equivalent amounts of proteins were subject to co-immunoprecipitation by anti-GFP antibody. Levels of p62 (top panel) and LC3 (middle panel) in total lysates (5% of input), immunoprecipitated fractions, or the flow-throughs were analyzed by SDS-PAGE and Western blotting. GAPDH is shown as an internal loading control of cell lysates, and was not present in immunoprecipitated fractions. Asterisks indicate the heavy chain and light chain of IgG. **(b)** The band intensity of co-immunoprecipitated p62 and immobilized GFP-LC3 (WT, K30D, K51A, or L53A) was determined by densitometry, and the amount of p62 bound to GFP-LC3-WT was referred to as 100% p62-LC3 interaction. Quantitative data from three independent experiments are shown as the mean (± S.D.) p62-LC3 interaction and were analyzed by Student's t test. ** $p<0.01$, versus WT.

**Mutations at these p62-contacting residues of LC3 show processing defects but still can translocate to the membrane of autophagosomes**

The lipidated form of Atg8/LC3, Atg8-PE or LC3-II, is essential for membrane tethering during the process of autophagsome formation [187, 188]. The conserved residues $Lys^{48}$ and $Leu^{50}$ in yeast Atg8 (corresponding to $Lys^{51}$ and $Leu^{53}$ in mammalian LC3) were shown to regulate autophagic activity [195, 198], consistent with previous findings that the cargo-binding site of Atg8 also plays crucial roles in autophagy [192]. We thus sought to investigate whether the three p62-interacting residues of LC3 are required for PE-conjugation and its localization to autophagosomes. HEK293 cells overexpressing wild-type and mutant GFP-LC3s were treated with or without rapamycin, a commonly used autophagy inducer [199, 200], for various intervals. As rapamycin can successfully induce the conversion of wild-type LC3 from form-I (non-PE conjugated LC3-I) to form-II (PE conjugated LC3-II), our data showed that the autophagy-induced lipidation of LC3-K51A is significantly suppressed as comparing to that of LC3-WT, while the autophagy-induced lipidation of LC3-K30D and LC3-L53A are only partially compromised (Fig. 2.2a). To rule out the possibility that reduction in lipidated LC3-II is caused by enhancement of autophagic influx, LC3-transfected cells were treated with lysosomal inhibitors together with rapamycin. We found that level of mutant

LC3-K51A-II is still lower than that of wild-type LC3-II in the presence of lysosomal inhibitors (Fig. 2.2b), suggesting that the $Lys^{51}$ residue of LC3 is essential for its conjugation with membrane PE.

Given that the lipidation of all three LC3 mutants was not completely blocked, these recombinant LC3 mutant proteins, similar to LC3-WT, could be localized to autophagosomes to form punctate structures after autophagy induction by serum starvation (Fig. 2.2c). Interestingly, the average number of autophagosomes in HEK293 cells transfected with either of these three LC3 mutants was approximate to that in LC3-WT-expressing cells, which could be due to by the residual activity of endogenous LC3. Furthermore, the average size of autophasomes was slightly, but not significantly, decreased in LC3-K51A-expressing cells , further supporting the essential role of LC3-$Lys^{51}$ residue in the lipidation of LC3 and the formation of autophagosomes.

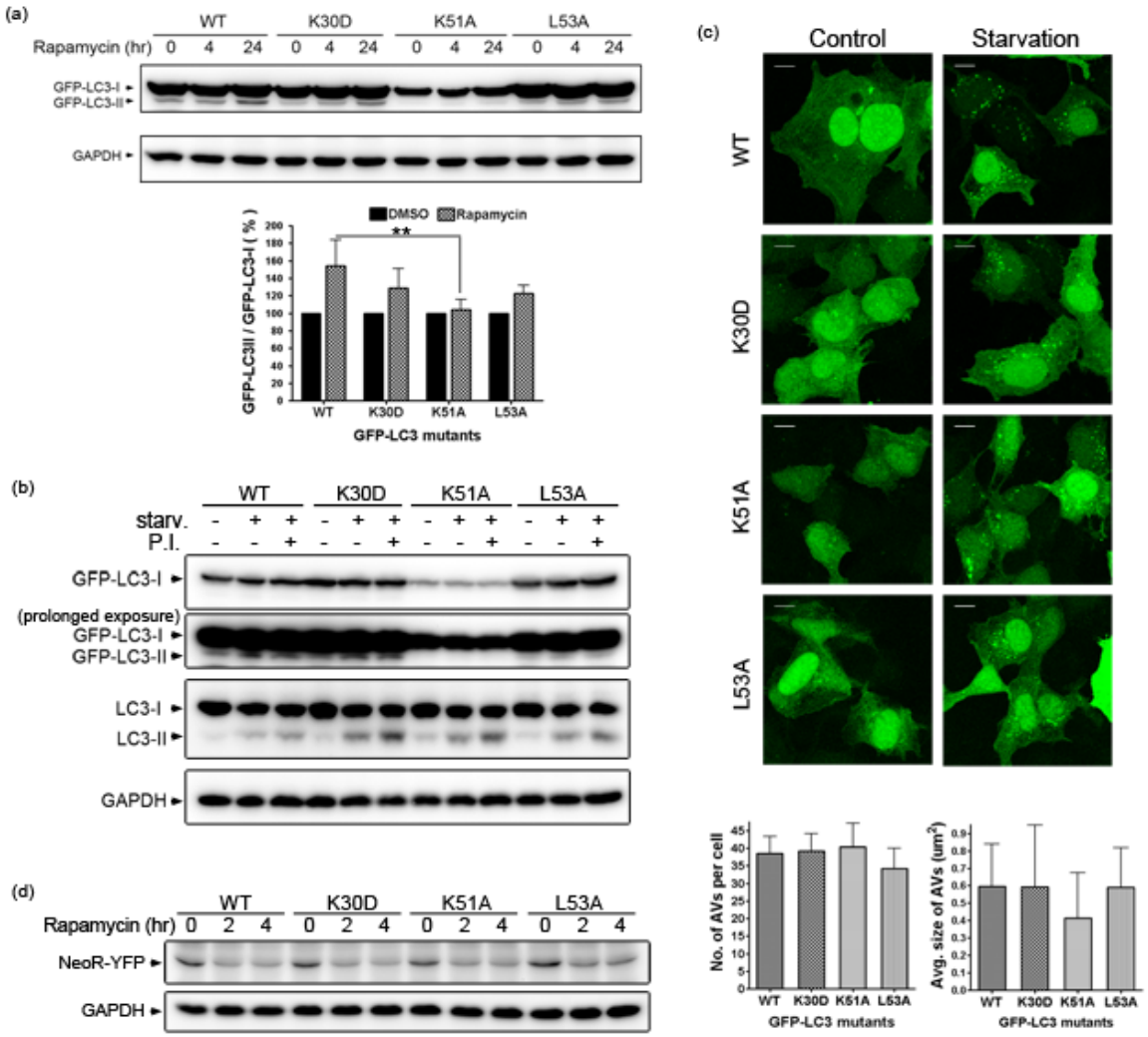



**Figure 2.2 Mutation within the ubiquitin core of LC3 results in reduced lipidation of LC3.**

(a) HEK293 cells were transfected with a wild-type (LC3-WT) or mutant GFP-LC3 construct (LC3-K30D, LC3-K51A, or LC3-L53A) for 48 h. Transfected cells were treated with 0.2 μg/ml rapamycin or vehicle alone (DMSO) for 4 h or 24 h. Clarified lysates of treated cells containing equivalent amounts of proteins were subject to SDS-PAGE and Western blotting with anti-GFP antibody to visualize cytosolic GFP-LC3-I and lipidated GFP-LC3-II (arrow, upper panel). GAPDH was included as a loading control (bottom panel). The levels of GFP-LC3-I and GFP-LC3-II were quantitated by densitometry. The ratio of GFP-LC3-II to GFP-LC3-I in rapamycin-treated cells (24 h) was determined, and that in DMSO-treated controls (24 h) was referred to as 100% LC3 lipidation (histogram). Data are shown as the mean (± S.D.) LC3 lipidation from three independent experiments and analyzed by Student's t test. ** $p<0.01$. **(b)** HEK293 cells transfected with WT or mutant GFP-LC3 were

subject to serum starvation for 2 h to induce autophagy in the presence or absence of lysosomal protease inhibitors (10 μg/ml pepstatin A and 10 μg/ml E64D). Clarified lysates of treated cells were analyzed by SDS-PAGE and Western blotting with anti-GFP (for GFP-LC3-I and -II, upper panel), anti-LC3 (for endogenous LC3-I and -II, middle panel), or anti-GAPDH (for loading control, bottom panel). The blot in upper panel was exposed for a longer duration to better visualize the GFP-LC3-II (prolonged exposure). **(c)** HEK293 cells were transfected with WT or mutant GFP-LC3 for 24 h, and were subject to serum starvation for 2 h to induce autophagy. After fixation, GFP puncta (regarded as autophagosomes) in starved cells were observed by confocal microscopy. Cells expressing either WT or mutant GFP-LC3 exhibited similar punctate structures and cellular distribution after starvation. Control groups are cells without the 2-h starvation. Scale bar, 10 μm. The average number and average size of autophagosomes (AVs, shown as GFP-puncta) in each cell from (c) were quantified and calculated by using MetaMorph software. Quantitative data are shown as the mean (± S.D.) from four independent experiments. **(d)** T-REx-NeoRY cells transfected with WT or mutant DsRed-LC3 were treated with 1 μg/ml of tetracycline for 48 h to induce the expression of the non-selective autophagic substrate, NeoR-YFP. Following the removal of tetracycline by the replacement of fresh culture medium, NeoR-YFP-expressing cells were treated with 0.2 μg/ml of rapamycin to induce autophagy for 2 h or 4 h. The degradation of NeoR-YFP in treated cells was subject to SDS-PAGE and Western blotting with anti-GFP (for NeoR-YFP, upper penal), or anti-GAPDH (for loading control, bottom panel).

To determine whether the defective LC3-p62 interaction could affect autophagic clearance of non-ubiquitinated substrates, such as neomycin phosphotransferase II (NeoR) [199, 201], we examined the degradation of NeoR-YFP fusion protein in the presence of either wild-type or mutant LC3. Using a cell line (T-REx-NeoRY) stably transfected with tetracycline-inducible NeoR-YFP cDNA construct, we demonstrated that the degradation of NeoR-YFP by rapamycin-induced autophagy in mutant LC3 transfectants is comparable to that in wild-type LC3-expressing cells (Fig. 2d), substantiating that constitutive autophagic activity is not impaired by the LC3 mutants. Taken together, these results suggest that $Lys^{30}$, $Lys^{51}$, and $Leu^{53}$ of LC3 are indispensable for p62 binding but that only $Lys^{51}$ is required for the lipidation of LC3. Our data also indicate that these three evolutionarily conserved residues are necessary but not sufficient for the full spectrum of LC3 function in autophagy.

**LC3 mutants defective in p62 binding impair the recruitment of p62 into autophagosome for degradation**

To determine whether the impairment of p62-LC3 association could affect the degradation of p62 by autophagy, we first examined the cellular localization of p62 in GFP-LC3-expressing cells after autophagy induction by confocal microscopy. Consistent with previous findings [154], endogenous p62 in cells expressing

recombinant GFP-LC3 was able to form cytosolic round bodies, while GFP-LC3, both wild-type and mutants, were recruited to autophagosome-like structures (Fig. 2.3a, b). Our data showed that these p62-positive puncta are associated closely with wild-type GFP-LC3 puncta, suggesting the successful engulfment of p62 into autophagosomes. On the other hand, all three mutant LC3s, K30D, K51A and L53A, displayed reduced colocalization with p62 and partially blocked the incorporation of p62 into autophagosomes (Fig. 2.3a, b). The level of p62 in cells expressing LC3-WT was reduced by 32% upon rapamycin-induced autophagy as comparing to non-treated cells, suggesting that autophagy promotes the degradation of p62 (Fig. 2.3c, d). Consistent with previous data, cells expressing GFP-LC3 mutants that are defective in p62 binding exhibited suppressed p62 degradation in rapamycin-induced autophagy. Since the autophagic clearance of the nonselective substrate, NeoR-YFP, was not affected by LC3 mutants (Fig. 2.2d), these results thus suggest that the interaction between p62 and LC3, mediated by an evolutionarily conserved surface motif (flanked by $Lys^{30}$, $Lys^{51}$, and $Leu^{53}$) of LC3, is crucial for recruitment and degradation of p62 in autophagosomes.

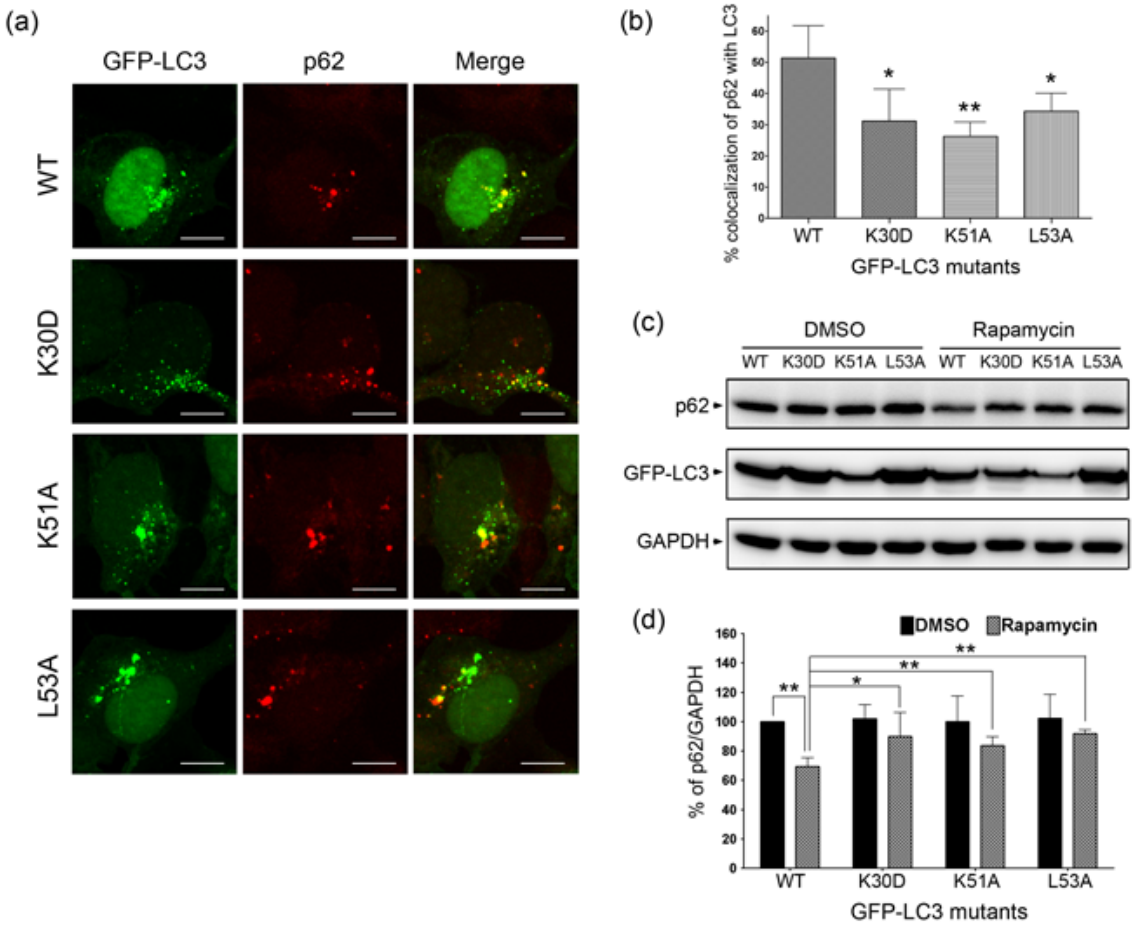



**Figure 2.3. Autophagosomal recruitment and degradation of p62 are impaired in cells expressing mutant LC3.**

**(a)** HEK293 cells were transfected with individual GFP-LC3 expression constructs (WT, K30D, K51A, or L53A) for 24 h, followed by serum starvation for an additional 2 h to induce autophagy. Transfected cells were fixed, permeabilized, and stained with anti-p62 antibody, followed by staining with an Alexa 633-conjugated secondary antibody. The colocalization between GFP-LC3 (green, left column) and p62 (red, middle column) was analyzed by confocal microscopy. Yellow in the merged images (right column) denotes the co-localization of LC3 and p62. Scale bar, 10 μm. **(b)** Fluorescent signals of GFP-positive puncta (green in left column, total LC3-positive autophagosomes) and that of p62-co-localized GFP granules (yellow in right column, p62-colocalized LC3) in each cell were quantified. The percentage of p62-colocalized LC3 (yellow) in proportion to total LC3 (green) per cell was then determined. Data are shown as the mean (± S.D.) from three independent experiments and analyzed by Student's t test. * $p < 0.05$, ** $p < 0.01$, versus WT. **(c)** HEK293 cells overexpressing

WT or mutant LC3 were treated with vehicle alone (0.1% DMSO) or 0.2 μg/ml rapamycin for 24 h. Clarified lysates containing equivalent amounts of proteins were analyzed by SDS-PAGE and Western blotting with anti-p62 (top panel), anti-GFP (for LC3, middle panel), or anti-GAPDH (for loading control, bottom panel). **(d)** The levels of p62 and GAPDH from (c) were quantified by densitometry. The level of p62 was normalized with that of GAPDH, and the normalized p62 level in DMSO-treated LC3-WT-expressing cells was referred to as 100% relative p62 level. Data are shown as the mean (± S.D.) from three independent experiments and analyzed by Student's t test. * $p < 0.05$, ** $p < 0.01$.

**Disruption of LC3-p62 interaction impedes the autophagy-mediated clearance of cytotoxic Htt**

Intraneuronal accumulations of ubiquitin-containing aggresomes are hallmarks of neurodegenerative disorders [202]. Although mechanisms underlying the formation of inclusion bodies have not been fully elucidated, accumulated evidence has revealed that autophagy plays an essential role in protecting cells from toxicity elicited by those polyubiquitinated aggregates [124]. Given that p62 can be associated with various types of ubiquitin-containing inclusion bodies, such as Tau, α-Synuclein and Huntingtin (Htt) [145], we thus reasoned that the impaired p62-LC3 interaction could significantly impact the formation of ubiquitin-positive inclusions in the pathogenesis of neurodegenerative diseases. To address this hypothesis, we first examined whether mutant LC3s deficient in p62 binding could affect the clearance of Htt aggregates. We cotransfected Htt-(Q)*109*-hrGFP and DsRed-LC3 mutants into HEK293 cells. The Htt-(Q)109 construct expresses a N-terminal fragment of Htt encoded by its exon 1 followed by a poly-Q expansion of 109 glutamines, and the recombinant Htt-(Q)109-hrGFP formed numerous aggregates in cytosol of transfected cells [193] (Fig. 2.4a & b). Our present data showed that cells transfected with mutant LC3, but not wild-type LC3, exhibit defective clearance of Htt aggregates by autophagy as evidenced by the unchanged number of cells with these aggregates

following the induction of autophagy by rapamycin, as earlier studies reported (Fig. 2.4a & b). Intriguingly, Western blotting analysis also revealed that the autophagy-mediated turnover of soluble Htt is also impaired in cells expressing mutant LC3 (Fig. 2.4c, d), corroborating that LC3-p62 interaction is essential for the autophagy-mediated clearance of both aggregated and soluble Htt.

Recent evidence has shown that autophagy-defective cells exhibit reduced activity of ubiquitin proteasome system (UPS) due to p62 accumulation [203], prompting us to determine whether the impaired Htt clearance might result from compromised UPS in mutant LC3-expressing cells. To address this possibility, we examined the degradation rate of a proteasome substrate, retinoic acid receptor-α (RARα)[204], in cells expressing mutant LC3. Our data showed that the clearance rates of RAPα among cells co-expressing both Htt-(Q)109 and exogenous LC3 (wild-type or mutant) are consistent (Fig. 2.4e), suggesting that compromised Htt clearance in mutant LC3-expressing cells is not due to impaired UPS. Together, the present findings suggest that the evolutionarily conserved cargo-receptor binding site of LC3 is required for the interaction with p62 and plays a critical role in mediating p62-dependent degradation of Htt through autophagy.

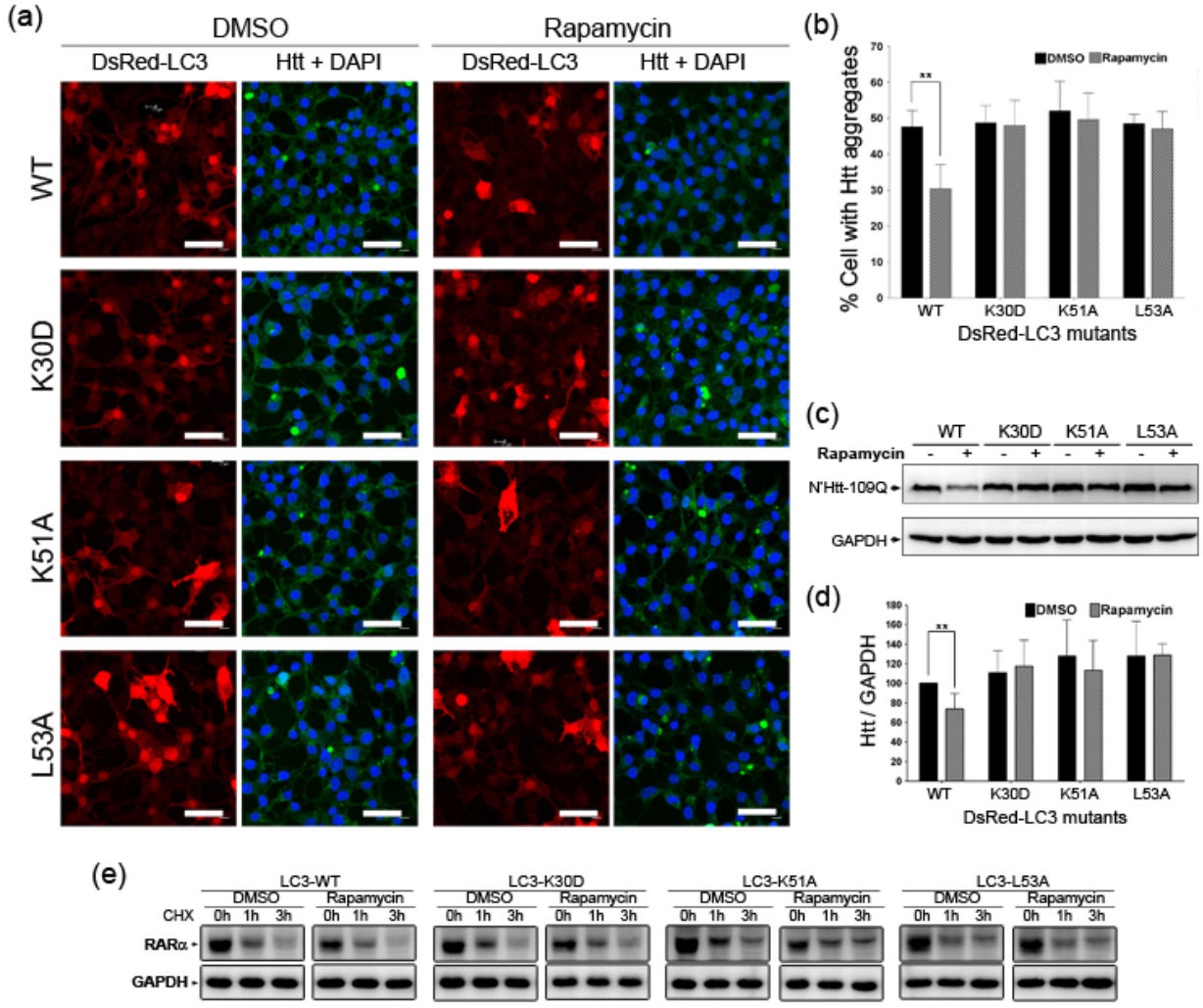



**Figure 2.4 Overexpression of mutant LC3 abolishes p62-dependent autophagic clearance of Htt.**

**(a)** HEK293 cells were transfected with pcDNA3.1-Htt-(Q)109-hrGFP (Htt-GFP) which encodes an aggregation-prone truncated form of Htt. Following 48 h incubation, Htt-GFP-expressing cells were further transfected with wild-type or mutant DsRed-LC3s. Htt-GFP/DsRed-LC3 double transfected cells were treated with or without 0.2 μg/ml rapamycin treatment for an additional 48 h. Treated cells were fixed, permeabilized, and stained briefly with DAPI to reveal cell nuclei. The levels and distribution of Htt-GFP and DsRed-LC3 were observed by confocal microscopy. Scale bar, 30 μm. **(b)** Numbers of cells containing Htt aggregates (green puncta) in DsRed-LC3-expressing cells (red) were counted from fluorescence images shown in (a). The percentage of cells with aggregates was calculated to denote the efficiency of clearance of Htt in each experimental group. Data are shown as the mean percentage (± S.D.) of Htt aggregate-containing cells from more than 10 viewing areas and

analyzed by Student's t test. ** p <0.01. **(c)** HEK293 cells were transfected with Htt-GFP and DsRed-LC3, followed by the treatment as described in (a). Clarified lysates containing equivalent amounts of proteins were analyzed by SDS-PAGE and Western blotting with anti-GFP antibody (for Htt) and anti-GAPDH (for loading control). **(d)** The levels of Htt were quantified by densitometry and normalized with those of GAPDH. The normalized level of Htt in DMSO-treated LC3-WT-expressing cells was referred to as 100% relative abundance. Data are shown as the mean (± S.D.) relative abundance of Htt from four independent experiments and analyzed by Student's t test. ** p <0.01. **(e)** Cells transfected with Htt-GFP and DsRed-LC3 were treated with DMSO or rapamycin for 24h, following by addition of 10 μg/ml cycloheximide (CHX) for 1 or 3 h to inhibit protein translation. Clarified lysates of treated cells were subject to SDS-PAGE and Western blotting with anti-RARα (for proteasome activity, upper penal), or anti-GAPDH (for loading control, bottom panel).

## 2.5 Discussion

Atg8 constitutively associates with autophagosomes after retrieval of other Atgs from the isolation membrane, and plays a critical role in the maturation of autophagosomes [205]. Residues on the surface of Atg8, especially those at the hydrophobic patch, mediate protein-protein interactions that are essential for autophagosome formation in yeast [192, 195, 198]. The existence of a conserved hydrophobic patch on the surface of LC3 has led to the hypothesis that LC3, like its yeast counterpart Atg8, could employ an evolutionarily conserved mechanism to govern cargo sorting and maturation of autophagosomes in mammalian systems [140, 141, 196]. Because selective autophagy has been suggested to play a pivotal role in the clearance of aberrant protein aggregates, elucidation of the molecular mechanism mediating the interaction between LC3 and its cargo receptor p62 could have profound implications in understanding the pathogenesis of various human diseases, including HD and other neurodegenerative disorders.

In this study, we investigate the LC3-p62 binding mechanism by overexpressing individual mutant LC3 cDNA constructs. Although the levels of exogenous GFP-LC3 protein were not always equivalent among transfectants, a phenomenon commonly seen in transient transfection of mammalian cells, the dominant-negative effects of these exogenous LC3 mutants on LC3-p62 interaction can still be vividly observed

even with the modest level of LC3-K51A, suggesting that these exogenous LC3 mutant proteins are sufficiently effective in substituting endogenous LC3 in autophagy. Given that $Phe^{52}$, $Leu^{53}$, and the first N-terminal α-helix of LC3 have been found to be essential for p62 binding [206], the present evidence strongly supports a model in which the ubiquitin core protruding from the surface of the LC3 protein is indispensable for the electrostatic and hydrophobic interactions between LC3 and p62 [141]. Our data, in accordance with X-ray crystallographic and NMR analyses of Atg8-Atg19 and LC3-p62 complexes [207], further support the notion that Atg8 homologues from yeast to higher eukaryotes could employ an evolutionarily conserved mechanism to bind and deliver specific cargo proteins into autophagosomes.

Before our studies, there was no documentation discussing the residue Lys30 of LC3. Unlike Lys51 and Leu53 which locate in the central core of the hydrophobic pockets, Lys30 resides at the head of beta1-sheet, slightly away from the central ubiquitin core, (and composing a protrusion on the surface of LC3). It is likely that Lys30 serves as a recognition site for p62 through electrostatic attraction, and then promotes later association of p62 with LC3. This assumption is in accordance with our previous results that substitution of Arg28 in Atg8 (which corresponds to Lys 30 in LC3) to Asp significantly impaired its binding with Atg19, while mutation to Ala

had no such blocking effect [192]. Interestingly, although K30D mutant only half attenuates the recruitment of p62, it almost completely, like K51A and L53A, suppresses the clearance of N'Htt109Q. The p62 protein utilizes a C-terminal UBA domain to bind with Lys 63-linked polyUb chains of its substrates (herein, Htt), and this associating process involves a conformational switch of UBA domain [208], indicating a high flexibility of this structure. Since the LC3 recognition sequence (LRS) locates adjacent to UBA domain of p62, it is plausible that replacing a positive charge with a negative one at Lys30 site on LC3 might cause a conformational change of its binding partner p62 which leads to reduce the interaction between p62 and Htt.

p62 has also been found in neuronal inclusion bodies of various neurodegenerative disorders. In cultured cells, RNAi-mediated down-regulation of LC3s or overexpression of p62 defective in the LC3-recognition sequence (LRS) or UBA can lead to a significant increase in cytosolic ubiquitin-positive inclusion bodies and cell death [145, 209, 210]. For the first time, we focused on a specific protein, the aggregate-prone mutant of Htt (N'Htt109Q), which is cytotoxic and induces pathological phenotypes of Huntintung's disease in mice models [211, 212]. Our results revealed that when LC3 mutants failed to trap p62 into autophagosomes, not only the degradation of p62 via autophagy was impaired, more accumulations of Htt aggregates in cells were observed, strongly prompting the pivotal function of p62 as a

specific cargo receptor involved in autophagy-mediated clearance of Htt, which greatly depends on the interaction between p62 and LC3. These findings are also correspondent with a previous finding that depletion of p62 levels by siRNA treatment or overexpressing p62 mutant lacking UBA domain which directly binds with Htt both results in severe cell death induced by mutant Htt [154]. Interestingly, some recent studies suggest that the deposition of inclusion bodies is rather a kind of protective mechanism to prevent the deleterious effects from toxic diffuse form of Htt [213]. We also observed the same inhibitory effects of LC3 mutants on the clearance of 1% Triton-soluble Htt detected by Western blot under denaturing condition, indicating that autophagy is also responsible for the degradation of monomeric or oligomeric soluble Htt proteins, mediated by interactions between LC3 and p62. We propose that rescue effect of autophagy on cell death induced by N'Htt-109Q could be reversed by expression of LC3 mutants.

Here, we displayed that mutant LC3s with less binding ability to p62 disrupt the transportation of aggregate-prone N'Htt109Q into autophagy. Developing strategies to upregulate autophgy or to maintain the level of functional p62 and LC3 would be beneficial for prevention and treatment of HD, and even other neurodegenerative diseases.

# Chapter 3

# Presenilin-1 regulates the expression of sequestosome-1/p62 and governs p62-dependent degradation of Tau independent of γ-secretase activity

## 3.1 Abstract

Mutations in the gene encoding presenilin-1 (PS1) account for most cases of early-onset familial Alzheimer's disease (FAD), which is characterized by extracellular amyloid plaques and the accumulation of intracellular Tau. In addition to being the catalytic subunit of γ-secretase to generate amyloid-β, PS1 has been shown to regulate diverse cellular functions independent of its proteolytic activity. We have found that cells deficient in PS1 exhibit reduced levels of p62 protein, a cargo receptor shuttling Tau for degradation in proteasomes or autophagosomes. The RNAi-mediated downregulation of PS1 also led to a significant decrease in both the protein and mRNA transcript of p62, concomitant with attenuated p62 promoter activity. This PS1-dependent transcriptional regulation of p62 was mediated through an Akt/AP-1 pathway that does not require the proteolytic activity of PS1/γ-secretase. In accordance with the role of p62 in mediating Tau degradation, the clearance of Tau was significantly impaired in PS1-deficient cells. Interestingly, such accumulation of Tau can be rescued by ectopic expression of either p62 or wild-type PS1 but not mutant PS1 containing FAD-linked mutations. Our data suggest that these FAD mutations of PS1 may cause a partial loss-of-function in PS1 to exacerbate tauopathies by suppressing p62 expression. Our study suggests a new role for PS1 in modulating the expression of p62 to control the degradation of aggregation-prone Tau

protein.

### 3.2 Introduction

Alzheimer's disease (AD), the most prevalent type of dementia in the elderly, affects more than 35 million people worldwide and imposes tremendous social costs in many countries. Extracellular plaques of amyloid-β (Aβ) and intracellular neurofibrillary tangles (NFTs) in the brains of AD patients are two major pathological hallmarks of the disease and are widely regarded as the main causes of neurodegeneration in AD [214]. Genetic studies on cases of familial AD (FAD) have found that the genes encoding presenilin-1 (PS1) and its homologue, PS2, harbor 90% of the mutations that cause AD [215]. PSs consist of the catalytic subunit of γ-secretase complexes, which cleave amyloid precursor protein (APP) to generate Aβ. The majority of FAD-linked PS mutations show a change in the proteolytic activity of γ-secretase, leading to increased relative production of aggregation-prone Aβ42 peptides that accelerate the formation of plaques and contribute to neurodegeneration [215].

PSs are also involved in diverse cellular functions independent of γ-secretase activity, such as calcium homeostasis and the modulation of intracellular signaling [216-218]. PS1 can bind the p85 subunit of phosphatidylinositol 3-kinase (PI3K) to

activate the pro-survival PI3K/Akt pathway [219]. Neurons from PS1-null ($PS1^{-/-}$) embryonic mouse brains have reduced levels of PI3K/Akt activity and increased caspase 3-dependent neuronal apoptosis [220]. Interestingly, such defects can be rescued by the reintroduction of wild-type (WT) PS1 but not the FAD mutant PS1, suggesting that the loss-of-function effects of FAD mutations on PS1 could alter the PI3K/Akt pathway to elicit neurodegeneration in AD. Consistent with this notion, the conditional double-knockout (DKO) of PSs in the forebrain of adult mice triggers robust AD-like neurodegeneration, including brain shrinkage, hippocampal atrophy, and NFT-like structures, further substantiating the correlation between the loss of PS function and the pathogenesis of AD [221]. The manifestation of NFT-like structures built up by hyperphosphorylated and ubiquitinated Tau in the brains of PS-DKO mice and FAD patients with PS1 mutations further suggests that PSs could play a role in the modulation of Tau aggregation [221-223].

The proteostasis of Tau can be mediated by both the ubiquitin/proteasome system (UPS) and the autophagosomal/lysosomal pathway [160, 224, 225]. Deregulation of both protein degradation systems has been shown to contribute to tauopathies in AD. Sequestosome-1/p62 colocalizes with certain polyubiquitinated protein aggregates related to neurodegeneration [145, 146] and associates with ubiquitin-dependent Tau clearance [160]. The binding of p62 to

K63-polyubiquitinated proteins and the proteasomal Rpt1 protein is mediated by its ubiquitin-associated (UBA) and PB1 domains, respectively [153, 159]. The LC3 interacting region (LIR) encoded within p62 mediates p62-LC3 binding and the subsequent entrapment of p62 into autophagosomes for degradation [140, 141]. These findings strongly suggest a critical role for p62 in governing the homeostasis of Tau.

Our preliminary study revealed a significantly reduced level of p62 protein in PS1-knockdown cells. Given that AD brains exhibit reduced expression of p62 and that genetic inactivation of p62 in a mouse model leads to tauopathy [161, 169], we hypothesize that PS1 modulates p62 expression to control p62-dependent Tau degradation and that FAD-linked mutations of PS1 manifest tauopathy by impairing the p62-dependent clearance of Tau. We report the first direct evidence that PS1 deficiency results in reduced p62 expression through Akt/AP-1-dependent transcriptional regulation. The downregulation of PS1 and expression of mutant PS1 harboring FAD-linked mutations significantly impair p62-dependent Tau degradation. Our results unveil a novel function of PS1 that may have profound implications in the pathogenesis of AD and the development of advanced therapeutics for AD.

## 3.3 Materials and Methods

### Reagents

Mouse anti-p62 (2C11) antibody was from Abnova. Rabbit anti-GAPDH (FL-335) antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG, HRP-conjugated anti-rat IgG, and HRP-conjugated anti-mouse IgG were from Santa Cruz Biotechnology, Inc. Rabbit anti-Akt, rabbit anti-phospho-Akt (Ser 473), and mouse anti-Tau (tau46) antibodies were from Cell Signaling Technology. N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-Butyl Ester (DAPT); rat anti-presenilin-1 monoclonal, rabbit anti-presenilin-1-loop, and rabbit anti-APP (C-terminal) antibodies; Immobilon Western Chemiluminescent HRP Substrate, and protease inhibitor cocktail (set III) were from Merck Millipore. Mouse anti-β-actin antibody (clone AC-15) and tetracycline were purchased from Sigma-Aldrich, Inc. Lipofectamine 2000 transfection reagent, *SuperScript® III* Reverse Transcriptase, Dulbecco's modified Eagle's medium (DMEM), DMEM/F12, fetal bovine serum (FBS), and TRIzol Reagent were from Invitrogen. SYBR Green I Master reagent was from Roche. The QuikChange Site-Directed Mutagenesis kit was from Stratagene. The BCA protein assay reagent kit was purchased from Pierce. Dual-Glo luciferase assay reagents and Steady-Glo luciferase assay reagents were from Promega. Pepstatin A, EST, U0126, LY294002, PI-103, SB202190, SB203580, SP600125,

MG132, and chloroquine were from EMD Millipore Bioscience. Cellulose acetate membranes (OE66, 200 nm pore size) were from GE Healthcare. All other reagents were at least reagent grade and obtained from standard suppliers.

**Cell culture**

Human embryonic kidney cells (HEK)293 and mouse embryonic fibroblasts (MEF) generated from $PS1^{+/+}PS2^{+/+}$ (WT), $PS1^{-/-}PS2^{+/+}$, $PS1^{+/+}PS2^{-/-}$, and $PS1^{-/-}PS2^{-/-}$ double-knockout (DKO) mice (kindly provided by Dr. Bart De Strooper, Flanders Interuniversity Institute for Biotechnology (VIB4) and K. U. Leuven, Belgium [226, 227]) were maintained in DMEM supplemented with 10% FBS. SH-SY5Y human neuroblastoma cells were maintained in DMEM/F12 medium supplemented with 10% FBS. Cells were incubated in a humidified incubator at 37 ºC in 5% $CO_2$.

**Virus preparation and infection**

Lentivirus encoding gene-targeting or control shRNAs were produced in 293T cells transfected with pLKO.1-shRNA-PS1 or pLKO.1-shRNA-LacZ (transducing vectors), pCMV-dR8.91 (packaging vector), and pMD2.G (a plasmid expressing vesicular stomatitis virus G (VSV-G) glycoprotein) by the calcium phosphate transfection method. Forty-eight to seventy-two hours post-transfection,

virus-containing supernatant was collected, concentrated, and stored at -80 ºC. Viruses were added to HEK293 or SH-SY5Y cells in growth media. After 24 h, the media were replaced with fresh growth medium, followed by further treatment or assay. Knockdown efficiency of the target gene was determined by Western blotting.

**Quantitative real-time PCR**

Total RNAs of transfected cells were prepared using TRIzol (Invitrogen) and were used to generate first-strand complementary DNA (cDNA) with the SuperScript™ III First-strand cDNA Synthesis Kit (Invitrogen). Equivalent amounts of cDNA were used in quantitative PCR on a LightCycler® 480 system (Roche) with the following isoform-specific primer pairs: for mouse p62, forward primer 5´-GCTGCCCTATACCCACATCT-3´ and reverse primer 5´-CGCCTTCATCCGAGAA-3´; for human p62, forward primer 5´-ATCGGAGGATCCGAGTGT-3´ and reverse primer 5´-TGGCTGTGAGCTGCTCTT-3´. The levels of mouse S18-rRNA (forward primer 5´-AGGGGAGAGCGGGTAAGAGA-3´ and reverse primer 5´-GGACAGGACTAGGCGGAACA-3´) and human S18-rRNA (forward primer 5´-CAGCCACCCGAGATTGAGCA-3´ and reverse primer 5´-TAGTAGCGACGGGCGGTGTG-3´) were determined as internal controls. For

quantification, the transcript level of p62 was individually normalized to that of S18-rRNA. Quantitative data were expressed as the average (± SD) of triplicate measurements from at least three independent experiments.

**Construction of reporter plasmids**

DNA encoding the SQSTM1/p62 promoter was isolated from human genomic DNA (Clontech). A reverse primer (5´-CCTCATGACGAGCGGCGAGCTGGCGGAA-3´), appended with a Bsp HI restriction site, and a forward primer (5´-CCAAGCTTCCCAAATCCTTCCACTTCAGCCCC-3´), affixed with a Hind III restriction site, were used to amplify a 2335-bp fragment of genomic DNA that encoded the 5´UTR (-2140 to +48) of the p62 allele. The PCR product was digested with Bsp HI and Hind III and subcloned into pGL3-Basic (Promega) to generate the full-length WT p62 promoter-driven luciferase reporter construct (pGL3.p62[FL]).

To generate p62 promoter reporters with truncation or point mutations within sequences corresponding to particular transcription-factor binding elements, site-directed mutagenesis using the pGL3.p62[FL] plasmid as a template was performed with the QuikChange Site-Directed Mutagenesis kit according to the supplier's instructions (Stratagene). The truncated pGL3.p62[0.3K] (-357 to +48)

promoter was generated by Kpn I digestion of pGL3.p62[FL] to remove 1700 upstream nucleotides, followed by self-ligation to recircularize the plasmid. All reporter constructs were verified by DNA sequencing.

**Luciferase reporter assay**

HEK293 or MEF cells were seeded onto 12-well microplates and transiently transfected with 0.5 μg of pGL3.p62[FL] or various mutant reporter constructs and 0.1 μg of pBud-Rrenilla (as a control for transfection efficiency) by Lipofectamine 2000 for 36-48 h. Expression of luciferase reporter genes in transfected cells was determined by using the Dual-Glo luciferase assay reagent kit. Luminescence emitted by the firefly luciferase reporter driven by p62 promoters (WT or mutant) was normalized to that of *Renilla* luciferase (pBud-Renilla). Relative promoter activity was expressed as the ratio of the normalized luminescence of the indicated p62 promoter reporter construct to that of pGL3-Basic. For kinase-inhibitor treatments, LY294002 (10 μM), PI-103 (10 or 20 μM), U0126 (10 μM), sp600125 (10 μM), SB202190 (5 μM), SB203580 (10 μM), or Go6976 (0.1 μM) were added to individual wells 24 h prior to harvesting the transfected cells.

**Generation of expression vectors**

The expression vector encoding the full-length WT Tau (containing exons 2, 3, and 10) *N*-terminally fused with EGFP (pEGFP-Tau) was kindly provided by Dr. Pei-Jung Lu (Graduate Institute of Clinical Medicine, Medical College, National Cheng Kung University, Tainan, Taiwan; [228]). The pEGFP-Tau-P301L mutant was generated from pEGFP-Tau by using the QuikChange Site-Directed Mutagenesis kit. The DNA fragment encoding EGFP-Tau-P301L was subcloned into pcDNA5 to generate a tetracycline-inducible pc5-EGFP-Tau-P301L construct.

The expression vector encoding the full-length WT human PS1 (pcDNA3.1-PS1) was kindly provided by Dr. Michael Wolfe (Brigham and Women's Hospital and Harvard Medical School, Boston, MA; [229]). FAD-linked mutations in mutant PS1 alleles, including A79V, M146L, G384A, and D257A, and ΔE9, were individually introduced into pcDNA3.1-PS1 by using the QuikChange Site-Directed Mutagenesis kit. The cDNA fragments encoding WT and mutant PS1 were subsequently subcloned into pAS2.puro or pAS3.zeo (National RNAi Core Facility, Academia Sinica, Taipei, Taiwan) separately for viral transduction.

The cDNA amplimer encoding the ORF of p62 was isolated from cDNAs generated from HEK293 cells and subcloned into pAS2.puro for viral transduction. All constructs were verified by DNA sequencing.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis**

Cells were lysed in 1% Triton X-100 lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 50 μM β-glycerophosphate, 1 mM $Na_3VO_4$, and protease inhibitor cocktail set III) on ice for 30 min. Following removal of cell debris by centrifugation, protein concentrations of clarified lysates were determined by using the BCA protein assay reagent kit. Cell lysates containing equal amounts of proteins were mixed with the 6X sample-loading buffer and boiled at 100 °C for 10 min. To detect PS1 proteins, samples mixed with sample-loading buffer were directly subjected to SDS-PAGE without boiling to avoid aggregation of PS1 proteins. To examine the level of total Tau protein, SH-Tau cells were resuspended in 10 volumes (w/v) of PBS (37 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, and 1.47 mM $KH_2PO_4$) containing 5 mM EDTA and protease inhibitor cocktail set III, followed by sonication. The homogenate was spun for 10 min at 1500 *g* to remove nuclei and unbroken cells. Protein concentrations of clarified lysates were determined by using the BCA protein assay reagent kit. Cell lysates containing equal amounts of proteins were mixed with the 6X sample-loading buffer and boiled at 100 °C for 10 min. To detect insoluble Tau protein, clarified lysates were mixed with non-reducing sample-loading buffer and boiled at 100 °C for 10 min.

Proteins were resolved on 10% Tris-glycine polyacrylamide gels and then transferred electrophoretically to polyvinylidene difluoride membranes (Pall). Membranes were blocked by 5% BSA in TBST (blocking buffer) at room temperature for 1 h, followed by incubation with appropriate primary antibodies in blocking buffer (1:5000) at 4 °C overnight. After three washes in TBST, the membranes were incubated with HRP-conjugated secondary antibodies in TBST (1:5000) at room temperature for 1 h. Following extensive washes with TBST, antibody-reactive proteins were visualized by the Immobilon Western Chemiluminescent HRP Substrate. Images were captured and processed with ChemiGenius2 (Syngene).

**Generation of tetracycline-inducible SH-Tau stable cell line**

SH-SY5Y neuroblastoma cells were transfected with pcDNA6 (Invitrogen) encoding the Tet repressor by using Lipofectamine 2000 transfection reagent. Transfected cells were cultured in DMEM supplemented with 10% FBS and 5 μg□ ml□ blasticidin. Individual colonies resistant to blasticidin selection were isolated and tested for the tetracycline-inducible expression of EGFP reporter by transient transfection of the pcDNA5-EGFP plasmid. The clone (SH-pc6) with maximal induction of EGFP expression by tetracycline was chosen for subsequent experiments. SH-pc6 cells were further transfected with the pc5-EGFP-Tau-P301L

plasmid and cultured in DMEM supplemented with 10% FBS, 200 μg/ml hygromycin, and 5 μg/ml blasticidin. Colonies resistant to antibiotic selection were isolated individually and screened for the tetracycline-inducible expression of GFP-tagged $Tau^{P301L}$. The stable clone exhibiting maximal tetracycline induction of EGFP-$Tau^{P301L}$ expression was selected and named SH-Tau for subsequent experiments.

**Tau degradation assay**

Tetracycline-inducible SH-Tau cells were treated with 0.2 μg/ml tetracycline in growth medium for 3-5 d to induce Tau expression (tet-on). The media were replenished with fresh tetracycline-containing medium every 2-3 d. To terminate Tau expression, induced SH-Tau cells were washed twice with PBS and maintained in fresh culture medium without tetracycline (tet-off). Cells were harvested at various intervals after terminating the tetracycline-induced expression of nascent Tau protein. In some experiments, 0.2 μM MG132 or 20 μM chloroquine (CQ) were added to the tet-off media, and treated cells were harvested after 24 h. The level of nascent Tau protein in clarified lysates was analyzed by Western blotting, followed by densitometric quantification using Image J software (NIH). The intensity of Tau was normalized with that of actin protein, and the normalized level of Tau at tet-off day 0

was referred to as 100% relative abundance. The quantitative data were shown as the mean (± SD) from at least three independent experiments and were analyzed by nonlinear regression. The half-life of nascent Tau protein ($t_{1/2}$) was determined by the first-order decay constant ($k$) with Microsoft Excel software.

**Filter-trap assay**

SDS-insoluble Tau aggregates were detected by filter-trap assay as described previously [230]. In brief, SH-Tau cells were resuspended in 10 volumes (w/v) of PBS containing 5 mM EDTA and protease inhibitor cocktail set III, followed by sonication. Following removal of cell debris by centrifugation, clarified lysates containing equal amounts (200 μg) of proteins were diluted with 10% SDS to a final concentration of 1% in 200 μl PBS. Insoluble proteins were blotted onto cellulose acetate membranes (200 nm pore size, pre-equilibrated with 1% SDS in PBS) by vacuum. After three washes with 200 μl of 1% SDS in PBS, membranes capturing protein aggregates were incubated with a mouse anti-Tau antibody (1:5000) in blocking buffer (5% milk in TBST) at 4 °C overnight, followed by incubation with HRP-conjugated anti-mouse IgG antibody in TBST (1:5000) at room temperature for 1 h. Antibody-reactive aggregates were visualized by the Immobilon Western Chemiluminescent HRP Substrate. Images were captured and processed with the

BioSpectrum 600 Imaging System (UVP).

**Exogenous expression of WT or mutant PS1**

$PS1^{-/-}PS2^{-/-}$ DKO MEFs were transduced with lentivirus encoding WT or FAD-linked mutant human PS1. The cells were cultured in DMEM supplemented with 10% FBS and 200 μg/ml puromycin for 2-3 weeks. The expression of exogenous human PS1 protein in isolated clones was determined by Western blotting.

To establish PS1-knockdown cell lines, HEK293 and SH-SY5Y cells were infected by lentivirus encoding PS1-5′UTR-targeting shRNA (shPS1). Infected cells were cultured in DMEM supplemented with 10% FBS and 200 μg/ml puromycin for three weeks. Knockdown efficiencies of PS1-targeting shRNAs in shPS1-expressing cells were determined by Western blotting. Validated shPS1-expressing cells were further infected by lentivirus encoding WT or FAD-linked mutant PS1 and cultured with DMEM supplemented with 10% FBS and 150 μg/ml zeocin for an additional three weeks. The phenotypic changes in p62 expression and Tau degradation in response to exogenous PS1 were determined by Western blotting as specified.

## 3.4 Results

### PS1 deficiency reduces levels of p62 protein and mRNA

Previous studies have shown that p62 expression is decreased in AD brains, whereas genetic ablation of p62 leads to AD-like biochemical and cognitive deficits [161, 169]. These phenotypes associated with p62 deficiency resemble the pathological manifestations elicited by the conditional knockout of PSs in the adult cerebral cortex. These findings prompted us to examine whether p62 expression could be altered in response to the downregulation of PS1. Using an RNAi-based approach, we found that both neuronal (SH-SY5Y) and non-neuronal (HEK293) cells expressing PS1-targeting short-hairpin (sh) RNAs exhibit a significant reduction in the level of p62 protein (Fig. 3.1A, B). To rule out the RNAi-elicited off-target effects on p62 expression, we examined the expression of p62 in mouse embryonic fibroblasts (MEFs) derived from $PS1^{-/-}PS2^{+/+}$ ($PS1^{-/-}$), $PS1^{+/+}PS2^{-/-}$ ($PS2^{-/-}$), and $PS1^{-/-}PS2^{-/-}$ double-knockout (DKO) mice [226, 231]. The levels of p62 protein were significantly lower in $PS1^{-/-}$, $PS2^{-/-}$, and DKO MEFs compared to those in MEFs derived from WT littermates (Fig. 3.1C).

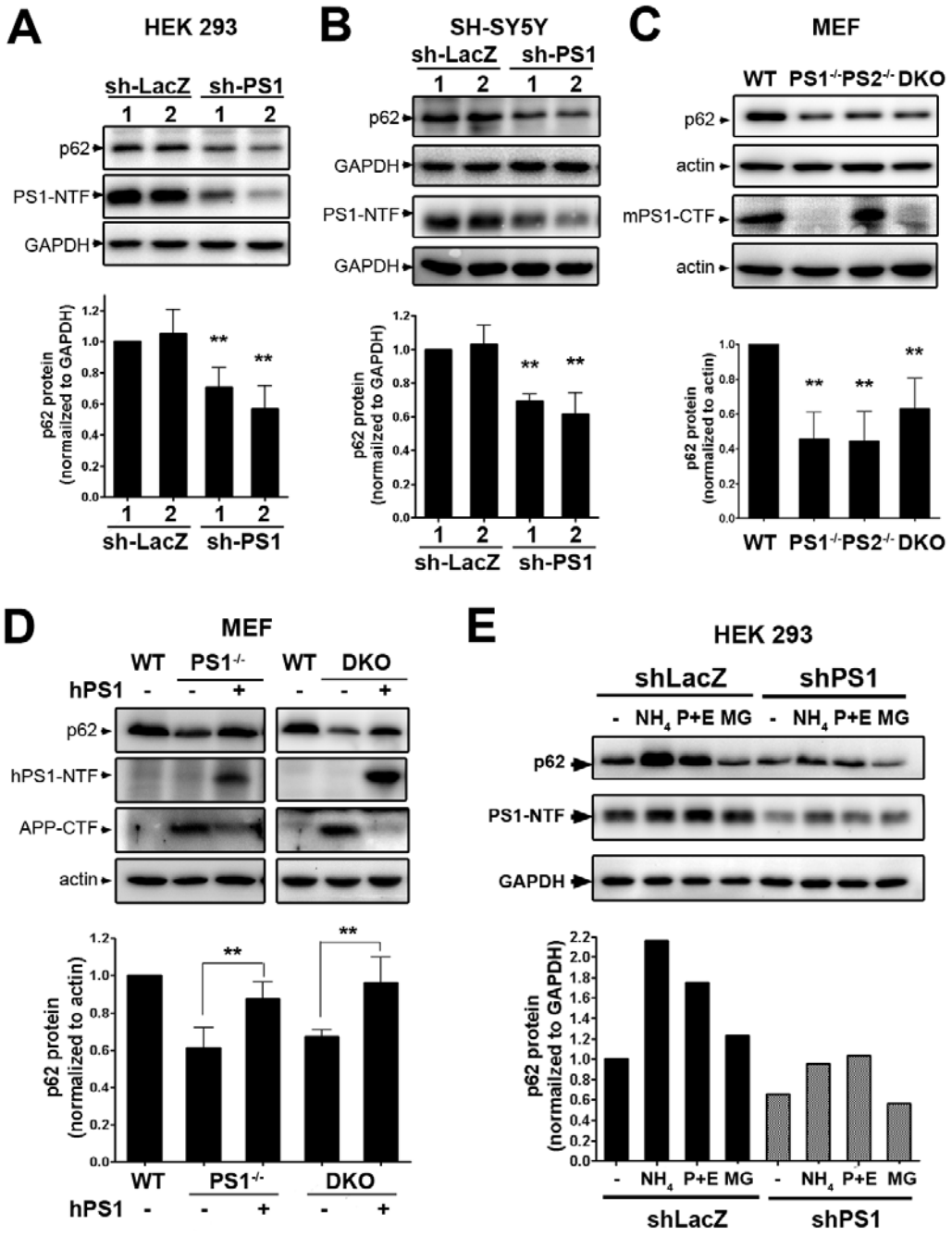



**Figure 3.1 PS1 deficiency reduces p62 protein levels.**

HEK293 cells (**A**) or SH-SY5Y cells (**B**) were individually transduced with lentiviral vectors encoding PS1-targeting shRNAs (shPS1-1 and -2) or LacZ-targeting control shRNAs (shLacZ-1 and -2) for 48-72 h. The shRNA-induced attenuation of PS1 and the levels of p62 and GAPDH were analyzed by SDS-PAGE and Western blotting. The level of p62 was normalized with that of GAPDH, and the normalized p62 level in shLacZ-1-transduced cells was referred to as 1-fold relative abundance. (**C**) Clarified lysates from wild type (WT), $PS1^{-/-}$, $PS2^{-/-}$, and $PS1^{-/-}PS2^{-/-}$ double-knockout

(DKO) MEFs were subjected to SDS-PAGE and Western blotting to detect endogenous mouse p62, mouse PS1-CTF and actin. The level of p62 was normalized with that of actin, and the normalized p62 level in WT-MEF cells was referred to as 1-fold relative abundance. (**D**) $PS1^{-/-}$ and DKO MEFs were individually infected by control virus or lentivirus expressing WT human PS1 (hPS1) for 48 h, followed by puromycin selection for more than two weeks. The expression of exogenous hPS1, endogenous mouse p62, mouse APP-CTF and actin was analyzed by SDS-PAGE and Western blotting. WT MEFs infected with control lentivirus were included as controls. The level of p62 normalized with that of actin in WT-MEF cells was referred to as 1-fold relative abundance. (**E**) HEK293 cells were transduced with hPS1-targeting shRNA-encoding lentivirus for 48 h, followed by treatment with either 10 mM $NH_4Cl$, 10 μg/ml pepstatin A plus 10 μg/ml EST (PI), or 2.5 μM MG132 for 20 h. The levels of hPS1, p62, and GAPDH were analyzed by SDS-PAGE and Western blotting. Quantitative data of (A, B, C, D) are shown as the mean (± SD) from at least three independent experiments and were analyzed by student's *t*-tests. * $p < 0.05$; ** $p < 0.01$. Quantitative data of (E) are shown as the mean from two independent experiments

Furthermore, the constitutive overexpression of human PS1 (hPS1) in either $PS1^{-/-}$ or DKO MEF cells concomitantly rescued γ-secretase activity and p62 expression, as evidenced by reduced accumulation of APP-CTFs, a substrate cleaved by γ-secretase, and increased p62 protein levels compared to mock-transfected PS1-null MEFs (Fig. 3.1D). These results strongly suggest that both PS1 and its homologue PS2 may be upstream modulators governing p62 expression in both non-neuronal and neuronal cells. Since expression of hPS1 alone profoundly restored p62 level in DKO MEF cells, we focused on the role of PS1 in regulation of p62 expression.

To determine whether PS1 modulates the level of p62 protein through an alteration in either the degradation of p62 protein or transcription/translation of the p62 gene, we examined the level of p62 protein in control and PS1-knockdown HEK293 cells in response to inhibition of proteasomes (MG132) or lysosomes ($NH_4Cl$ or EST+pepstatin A) (Fig. 3.1E). Blockage of lysosomal activity, but not proteasomal function, resulted in an increase of p62 protein in both control (shLacZ) and PS1-knockdown cells (shPS1), suggesting that autophagy is the primary degradative system for p62 turnover in HEK293 cells. Interestingly, MG132 treatment caused a slight reduction in p62 protein level, which is likely due to induction of autophagy by blockage of proteasome as reported previously [232, 233]. However, in

some cell lines (such as the neuroblastoma SH-SY5Y cells, data not shown), inhibition of proteasome instead enhances p62 transcription, leading to an accumulation of p62 proteins [234]. Given that the degradation of p62 in PS1-kncokdown cells cannot be fully restored by the inhibition of lysosomes or proteasomes, we thus reasoned that downregulation of the p62 protein in PS1-deficient cells could be mediated by an alteration in transcription or translation of the p62 gene rather than through enhanced p62 protein degradation.

It should be noted that the lysosome-dependent degradation of p62 is actually partially disrupted in PS1 knockdown condition, as NH4Cl treatment in control cells caused about 1 fold increase of p62 level (Fig. 3.1E, lane 1 versus lane 2) but only 0.5 fold increase of that in PS1 knockdown cells (Fig. 3.1E, lane 5 versus lane 6). The observation is consistent with previous reports that PS1 is required for maintaining functional lysosomes [235-238]. Nevertheless, the PS1-dependent transcriptional control is likely more prominent than lysosomal effect on p62 protein level in our system.

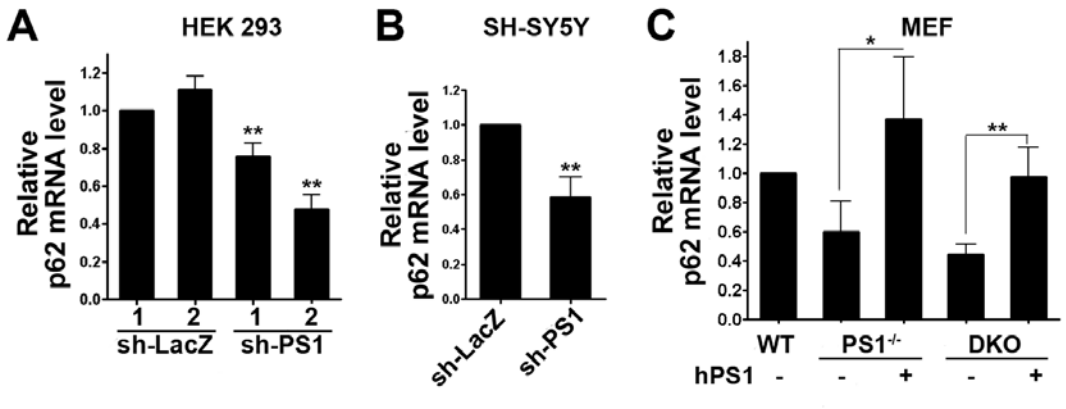



**Figure 3.2 PS1 deficiency reduces p62 mRNA levels.**

HEK293 cells (**A**) or SH-SY5Y cells (**B**) were individually transduced with lentiviral vectors encoding PS1-targeting shRNAs (shPS1-1 and -2) or LacZ-targeting control shRNAs (shLacZ-1 and -2) for 48-72 h. Infected cells were harvested and processed for total RNA isolation by TRIzol reagent. The levels of the p62 mRNA transcript and S18 rRNA (internal control) were determined by quantitative real-time PCR. The normalized level of the p62 mRNA transcript in sh-LacZ-1 control cells was referred to as 1-fold relative abundance. (**C**) MEFs were processed as described in Fig. 1D. The levels of p62 mRNA transcript and S18 rRNA (internal control) in infected MEFs were determined by quantitative RT-PCR. The normalized level of p62 mRNA transcript in mock-infected WT MEFs was referred to as 1-fold relative abundance. Quantitative data are shown as the mean (± SD) from at least three experiments and were analyzed by student's *t*-tests. * $p < 0.05$; ** $p < 0.01$.

We then determined the level of p62 mRNA transcripts in control and PS1-knockdown cells by quantitative real-time PCR and found that the level of p62 mRNA is significantly reduced in PS1-knockdown HEK293 and SHSY5Y cells (sh-PS1) compared to mock-infected cells (sh-LacZ) (Fig. 3.2A, B). A significant decrease in p62 mRNA was consistently observed in $PS1^{-/-}$ and DKO MEFs compared to WT MEFs, which could be restored to a comparable level to that in WT MEFs by exogenous expression of human PS1 (Fig. 3.2C). Our data clearly suggest that PS1 can transcriptionally modulate the expression of p62.

**PS1 regulates p62 expression at the transcriptional level independent of γ-secretase activity**

To determine whether p62 expression can be modulated by PS1, we generated luciferase reporter constructs containing genomic fragments of the 5' region of the p62 gene (-2140 to +48) that are fused in-frame to the firefly luciferase reporter gene (pGL3.p62[FL], Fig. 3.3A). Consistent with the role of PS1 in the transcriptional regulation of p62 expression, the activity of the p62 promoter was significantly suppressed by the downregulation of PS1 in HEK293 cells (sh-PS1 vs. sh-LacZ) or by genetic ablation of PSs in MEFs ($PS1^{-/-}$, $PS2^{-/-}$, and DKO vs. WT) (Fig. 3.3A and B). Our data strongly support a novel function of PS1 in the transcriptional regulation of

p62.

PS1-dependent γ-secretase can process its various substrates to generate intracellular domains (ICDs) that can then be translocated into the nucleus to modulate gene expression [239]. PS1 may thus possibly regulate p62 transcription indirectly through a certain γ-secretase-cleaved ICD. Activity of the p62 promoter, however, was not affected upon inhibition of γ-secretase by DAPT, a potent □ γ-secretase inhibitor (Fig. 3.3C)., at various concentrations that has been proved to block PS1-depedent cleavage in our previous studies [240]. The level of p62 mRNA consistently remained unchanged after DAPT treatment in either HEK293 (Fig. 3.3D) or SH-SY5Y cells (data not shown). We concluded that PS1 could transcriptionally regulate p62 expression independent of its γ-secretase activity and ICD-elicited transcriptional regulation.

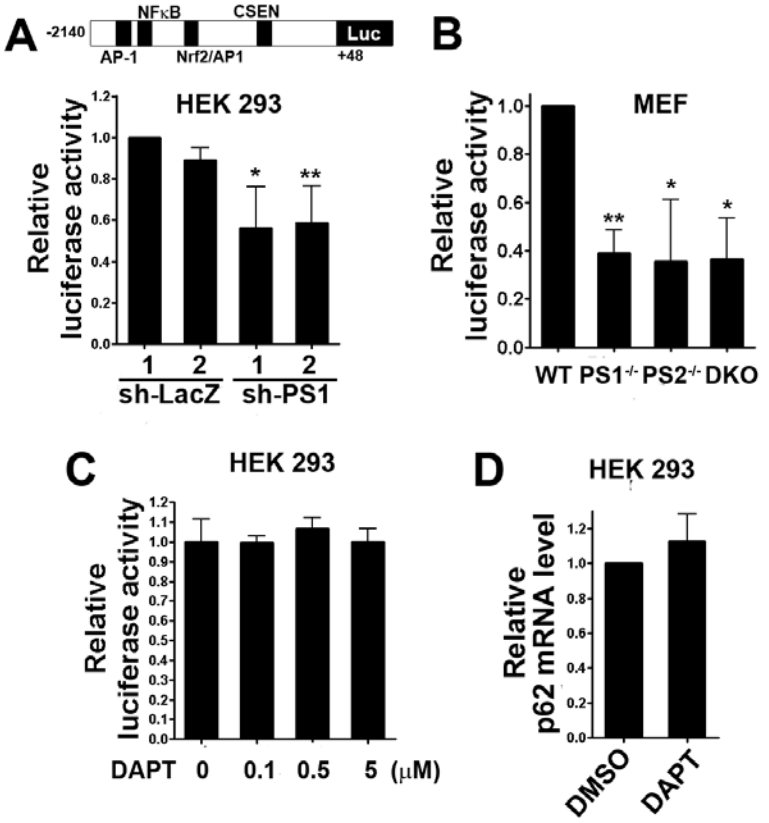



**Figure 3.3 PS1 transcriptionally regulates p62 expression independent of γ-secretase activity.**

shRNA-transduced HEK293 cells (**A**) or indicated MEF cells (**B**) were transfected with 0.1 μg of pBud-RLuc plasmid (a control of transfection efficiency) plus either pGL3-Basic (1 μg) or pGL3.p62[2k] (1 μg) reporter. Relative promoter activity was expressed as the ratio of the normalized luminescence derived from pGL3.p62[2k]-transfected cells to the normalized luminescence derived from pGL3-Basic-transfected cells. The luciferase signal of sh-LacZ-1-infected cells or of WT MEFs was referred to as 1-fold relative luciferase activity. (**C**) HEK 293 cells cotransfected with pGL3.p62[2k] and the pBud-RLuc control plasmid were treated with various concentrations of DAPT, a γ-secretase inhibitor, for 48 h. The p62 promoter activity was determined by Dual-luciferase assay reagents. The normalized luciferase signal of DMSO-treated cells was referred to as 1-fold relative luciferase activity. (**D**) The levels of p62 mRNA transcript and S18 rRNA (internal control) in HEK 293 cells that were treated with 5 μM DAPT for 48 h were determined by quantitative RT-PCR. The normalized level of p62 in DMSO-treated cells was referred to as 1-fold relative abundance. Quantitative data are shown as the mean (± SD) from at least three independent experiments and were analyzed by student's *t*-tests. * $p < 0.05$; ** $p < 0.01$.

**PS1 modulates the transcription of the p62 gene through an Akt/AP-1-dependent pathway**

To further determine the mechanism underlying PS1-dependent regulation of p62 expression, we explored the possibility that PS1 regulates p62 expression by modulating the interaction between transcription factors and the p62 promoter. Using a computational approach, we identified putative binding sites for AP-1 (-1778 to -1771), NFκB (-1721 to -1712), Nrf2 (-1303 to -1291), and calsenilin (CSEN) (-649 to -639) within the p62 promoter (Fig. 3.4A) [167, 168, 241, 242]. Interestingly, CSEN was previously found to directly interact with PS1 [243]. To determine whether any of these transcription factors is required for PS1-dependent transcriptional control of p62 expression, we generated a series of mutant p62 promoter reporter gene constructs by individually ablating particular transcription-factor binding sites, including pGL3.p62[ΔA1], pGL3.p62[mA1], pGL3.p62[ΔNFκB], pGL3.p62[ΔA2], pGL3.p62[ΔCSEN], and pGL3.p62[mA1ΔA2] (Fig. 3.4A). A truncated 0.3K promoter (-357 to +48) reporter gene construct (pGL3.p62[0.3K]) containing none of the putative binding elements was also generated as a control. These p62 promoter reporter constructs were separately transfected into HEK293 cells that had been infected with lentivirus encoding shLacZ or shPS1. In shLacZ-expressing cells, deletions within AP-1 (A1), NFkB, and CSEN all resulted in a significant suppression

of luciferase reporter gene expression compared to that derived from the full-length p62 promoter (pGL3.p62[FL]), while the 0.3K truncated p62 promoter (pGL3.p62[0.3K]) only showed a basal luciferase activity, suggesting positive regulation of p62 expression by these transcription factors (Fig. 3.4B). Interestingly, the complete blockage of AP-1 binding to the p62 promoter (mA1ΔA2) resulted in the most dramatic reduction in expression of the luciferase reporter gene. Our findings thus support the notion that AP-1 plays an essential role in the transcriptional regulation of p62 expression [168].

To demonstrate how these transcription factors contribute to the PS1-dependent transcriptional regulation of p62 expression, expression of the luciferase reporter gene derived from various p62 promoter constructs was determined in shPS1-expressing cells in the absence or presence of exogenous expression of hPS1. Because the PS1-specific shRNAs targeted to the 5´UTR of the PS1 transcript, the exogenous expression of hPS1 in the shPS1-expressing cells would still be able to rescue the phenotypes resulting from PS1 knockdown. We found that the activity of the full-length p62 promoter (pGL3.p62[FL]) was significantly suppressed in PS1-knockdown cells, which could be fully restored by exogenous PS1 expression (Fig. 3.4B). Single deletions of the binding sites for NFκB, Nrf2, or CSEN in the p62 promoter ([ΔNFκB], [ΔA2], or [ΔCSEN], respectively) only partially attenuated

PS1-dependent transcriptional modulation. However, p62 promoters defective in AP-1 binding ([ΔA1] and [mA1]) showed a marked loss of their responsiveness to PS1-dependent modulation, suggesting that AP-1 could contribute to PS1-dependent regulation of p62 expression. Since another AP-1-responsive element (TGAGTCAC) was identified within the Nrf2 binding site (A2 site in Fig. 3.4A), we further generated a p62 promoter reporter construct containing both the mutagenized A1 site and the A2 deletion (pGL3.p62[mA1ΔA2], Fig. 3.4A). The activity of the p62 promoter was further suppressed by the simultaneous inhibition of AP-1 binding to both A1 and A2 sites compared to defective A1 or A2 sites alone. Most intriguingly, the PS1-dependent modulation of p62 transcription was completely abolished by the combination of defective A1 and A2 sites, strongly suggesting that AP-1 is the downstream effector of PS1 to govern p62 expression.

AP-1 is a complex transcription factor responsive to external stimuli through the regulation of phosphorylation by various kinases, including MAPK, Akt, and PKC [244, 245]. PS1 has been found to modulate these signaling pathways. We thus sought to determine whether MAPK-, Akt-, or PKC-dependent pathway could underlie the PS1/AP-1-dependent regulation of p62 expression. HEK293 cells transfected with the pGL3.p62[FL] promoter reporter construct were individually treated with various kinase inhibitors specific to PI3K/Akt (LY294002 and PI103), JNK (SP600125),

MEK/ERK (U0126), p38 (SB202190 and SB203580), and PKC (Go6976). We found that inhibition of the PI3K/Akt pathway by LY294002 or PI103 results in significant attenuation of p62 promoter activity, concomitant with a dramatic reduction in the level of p62 protein (Fig. 3.4C). Moreover, activity of the WT p62 promoter pGL3.p62[FL], but not of the promoter defective in AP-1 binding (pGL3.p62[mA1ΔA2]), was suppressed by the PI3K/Akt inhibitor LY294002 (Fig. 3.4D). Consistent with previous finding that PS1 enhances the upstream complex cadherin/PI3K to stimulate PI3K/Akt signaling [219], we also confirmed that Akt activation is significantly reduced in our PS1-knockdown cells, which can be rescued by the exogenous expression of WT PS1 (Fig. 3.4E). Our results thus provide the first direct evidence that PS1 transcriptionally modulates p62 expression by a PI3K/Akt/AP-1 pathway.

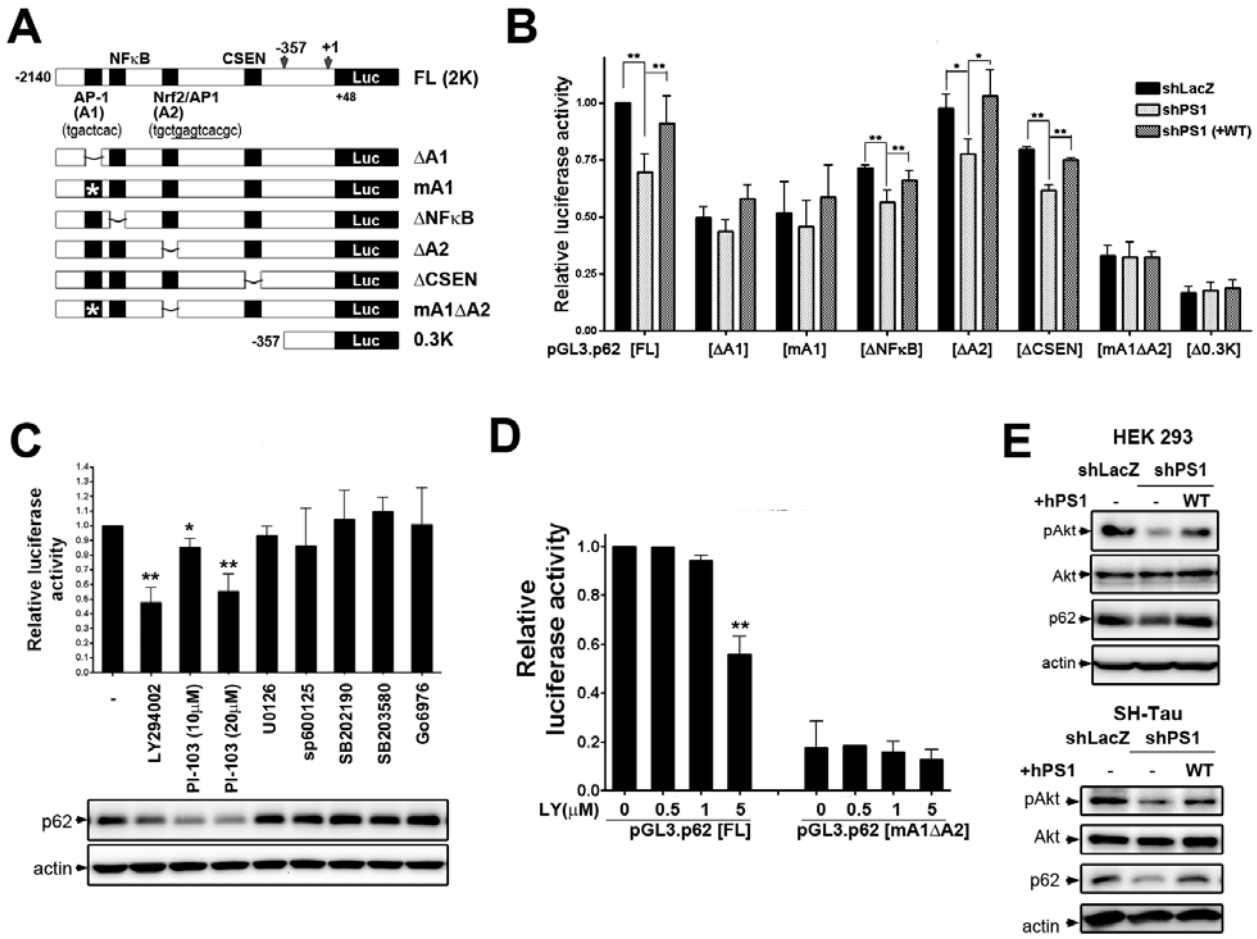



**Figure 3.4 The PS1-dependent regulation of p62 expression is mediated by an Akt/AP1 pathway.**

(**A**) Schematic representation of the full-length (pGL3.p62[2k]) and different truncations of p62 promoter reporter constructs. The putative binding sites for AP-1 (1778 to -1771), NFκB (-1721 to -1712), Nrf2/AP1 (-1303 to -1291), and CSEN (-649 to -639) are denoted as black rectangles. Angled lines denote deletions of transcription-factor binding sites, and asterisks denote point mutations. Numbers denote the nucleotide position relative to the transcription start site (+1) of the p62 gene. Luc denotes the firefly luciferase reporter gene. (**B**) HEK293 cells stably expressing LacZ control shRNA (shLacZ, solid bar) or PS1-targeting shRNAs in the absence (shaded bar) or presence (hatched bar) of exogenous PS1 expression were individually transfected with pGL3-Basic or various p62 promoter reporter constructs for 36-48 h. Relative promoter activity was defined as the ratio of the normalized luminescence of the indicated p62 promoter construct to the normalized luminescence

of pGL3-Basic in corresponding cells. The luciferase signal of shLacZ-expressing cells that were transfected with the full-length p62 promoter construct (pGL3.p62[FL]) was referred to as 1-fold relative luciferase activity. (**C**) HEK293 cells were individually transfected with pGL3.p62[FL] for 6-10 h, followed by treatments with kinase inhibitors (at concentrations described in Material and Methods) for an additional 24 h. The luciferase signal of DMSO-treated cells was referred to as 1-fold relative luciferase activity. The downregulation of p62 by treatments with Akt/PI3K inhibitors was shown by Western blotting. **(D)** HEK 293 cells were individually transfected with either WT p62 promoter reporter construct pGL3.p62[FL] or a mutant promoter construct pGL3.p62[mA1ΔA2] for 6-10 h, followed by treatments with various concentrations of LY294002 for another 24 h. The luciferase signal of DMSO-treated cells transfected with the pGL3.p62[FL] promoter reporter construct was referred to as 1-fold relative luciferase activity. (**E**) HEK293 cells or a SH-SY5Y-derived cell line (SH-Tau) that was stably transfected with a tetracycline-inducible Tau expression vector were additionally infected with lentivirus encoding LacZ control shRNA or PS1-targeting shRNAs in the presence or absence of exogenous PS1 (hPS1). Clarified lysates from infected cells were analyzed by SDS-PAGE and Western blotting to visualize phosphorylated Akt (pAkt), total Akt, p62, and actin. Immunoblots from one representative experiment are shown, and at least two independent experiments reveal reproducible results. Quantitative data are shown as the mean (± SD) from three independent experiments and were analyzed by student's *t*-tests. * $p < 0.05$; ** $p < 0.01$.

**Loss of PS1 function propagates Tau accumulation through impairment of p62-dependent Tau degradation**

Conditional knockout of PS1 and PS2 from the forebrain in mice triggers robust AD-like neurodegeneration, including Tau hyperphosphorylation and NFT formation [221]. It has also been reported that p62 interacts with ubiquitinated Tau through its UBA domain and shuttles Tau for proteasomal degradation [246]. We thus reasoned that PS1 could regulate Tau clearance through modulation of p62 expression. We first generated a SH-SY5Y-derived cell line (SH-Tau) that is stably transfected with a tetracycline-inducible expression construct encoding EGFP-tagged mutant Tau (EGFP-Tau-P301L). $Tau^{P301L}$ is an aggregation-prone mutant that accelerates tauopathies when expressed in mouse models [247]. To examine the role of PS1 in p62-dependent degradation of Tau, SH-Tau cells infected by lentivirus encoding PS1-targeting shRNAs or LacZ-targeting control shRNAs were incubated with culture medium containing tetracycline to induce $Tau^{P301L}$ expression for three days, followed by replacement with fresh tetracycline-free culture medium to trace the degradation of $Tau^{P301L}$ for various intervals. We found that downregulation of PS1 in SH-Tau cells markedly hindered p62-dependent Tau degradation (Fig. 3.5A). The impaired clearance of Tau due to PS1 knockdown in SH-Tau cells was fully rescued by exogenous expression of PS1 or p62 (Fig. 3.5B), substantiating the essential role of

PS1 in p62-dependent degradation of Tau.

The impairment of p62-dependent Tau degradation in PS1-kncokdown cells was evident, as exemplified by the significant accumulation of both soluble and insoluble Tau proteins (Fig. 3.5C, D), supporting the notion that enhanced Tau aggregation caused by PS1 deficiency primarily results from impairment of p62-dependent Tau degradation. Given that downregulation of p62 blocks Tau clearance [160], the exogenous expression of either PS1 or p62 in PS1-knockdown cells effectively restored the expression of p62 and rescued the p62-dependent Tau clearance, leading to overall decreases in both soluble and insoluble Tau (Fig. 3.5A, B, D).

The PB1 domain of p62 is required for interaction with the proteasomal Rpt1 protein to facilitate the degradation of p62-binding cargo proteins, including Tau [153, 160]. The newly identified LC3-interacting region (LIR) in p62 that mediates its binding to LC3, a key mediator of autophagic degradation [140, 141, 248], also plays an essential role in shuttling polyubiquitinated protein aggregates for selective autophagy. Consistent with previous findings [249, 250], we showed that p62-dependent Tau degradation could be processed through both proteasomal and autophagic pathways, as evidenced by the significant accumulation of Tau in response to either proteasomal inhibition by MG132 or lysosomal neutralization by chloroquine

(Fig. 3.5E). Downregulation of PS1 resulted in parallel impairment of p62-dependent Tau degradation through both proteasomal and lysosomal degradative systems, since no further augmentation in Tau accumulation was observed upon inhibition of either proteasomes or lysosomes. Consistent with this concept, overexpression of PS1 or p62 in PS1-knockdown cells was sufficient to restore p62-dependent Tau clearance by both degradative systems. These results thus favor a model in which p62 acts as the cargo-receptor protein to shuttle cargo protein Tau to both proteasomes and autophagosomes/lysosomes for efficient turnover. This novel function of PS1 in the transcriptional regulation of p62 expression underscores the delicate network for controlling the homeostasis of Tau.

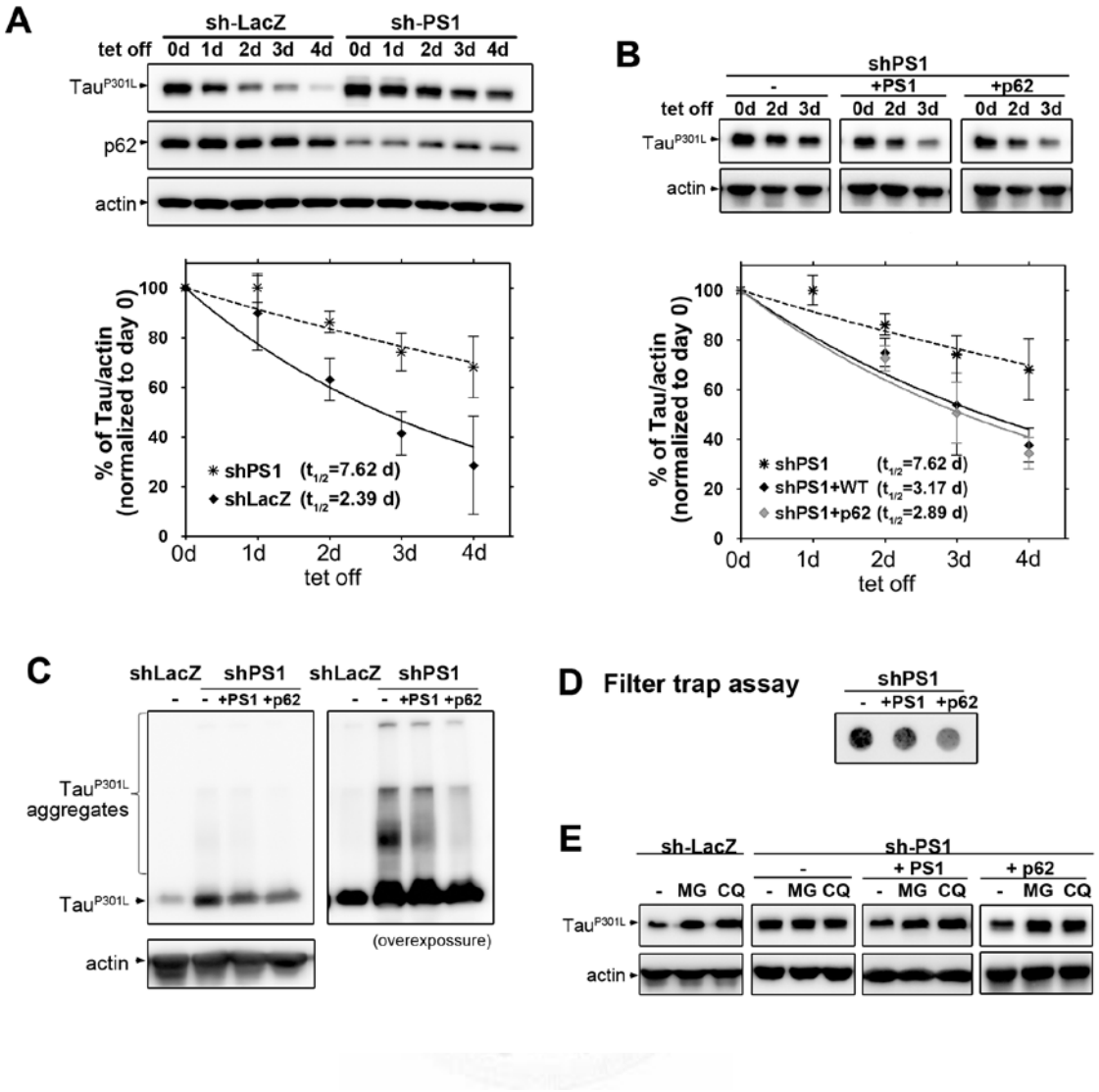



**Figure 3.5 Downregulation of PS1 enhances Tau accumulation through impairment of p62-dependent Tau degradation.**

(**A**) A SH-SY5Y-derived cell line (SH-Tau) that was stably cotransfected with a tetracycline repressor and a tetracycline-inducible GFP-tagged mutant Tau (GFP-Tau$^{P301L}$) expression construct was infected by lentivirus encoding LacZ control shRNA (sh-LacZ) or PS1-targeting shRNA (sh-PS1) and treated with 0.2 μg/ml of tetracycline for three days to induce Tau expression. Infected cells were then maintained in tetracycline-free media (tet-off) for various intervals to examine the degradation of exogenous Tau. Clarified lysates with equivalent amounts of proteins were analyzed by SDS-PAGE and Western blotting to visualize Tau (Tau$^{P301L}$), p62, and actin (protein-load control). The normalized level of Tau in cells harvested at tet-off day 0 was referred to as 100% relative abundance. The average abundance at each time point from at least three independent experiments was plotted and analyzed

by nonlinear regression. The half-life of nascent Tau protein ($t_{1/2}$) was determined by using a one-phase exponential decay equation. (**B**) SH-Tau cells infected with lentivirus encoding PS1-targeting shRNA (shPS1) were further transduced with an empty vector (-) or a PS1- or p62-expressing vector. Following incubation with tetracycline-containing media for 3 d (Tet-on), treated cells were maintained in tetracycline-free media (tet-off) for various intervals. Clarified lysates were analyzed by SDS-PAGE and Western blotting to visualize $Tau^{P301L}$ and actin. The normalized level of Tau in cells harvested at tet-off day 0 was referred to as 100% relative abundance. The average abundance at each time point from at least three independent experiments was plotted and analyzed by nonlinear regression. The half-life of nascent Tau protein ($t_{1/2}$) was determined by using a one-phase exponential decay equation. (**C** and **D**). PS1-knockdown SH-Tau cells (shPS1) were individually transduced with an empty vector (-) or a PS1- or p62-expressing vector and incubated with growth medium containing 1 μg/ml of tetracycline (Tet-on) for 5 d. SH-Tau-PL cells stably expressing LacZ-shRNA transduced with an empty vector were included as controls. Clarified lysates were prepared in the absence of β-mercaptoethanol and analyzed by either Western blotting (**C**) to visualize monomeric Tau and high molecular weight Tau aggregates or by a filter trap assay (**D**) to detect aggregated Tau. (**E**) Control (sh-LacZ) or PS1-knockdown (sh-PS1) SH-Tau cells transfected with an empty vector (-) or a PS1- or p62-expressing vector were incubated with growth medium containing 1 mg/ml tetracycline for 1 d. Tau-expressing cells were then maintained in tetracycline-free media and treated with vehicle alone (0.1% DMSO), the lysosomal inhibitor chloroquine (CQ, 20 μM), or the proteasomal inhibitor MG-132 (MG, 0.2 μM). Clarified lysates were analyzed by SDS-PAGE and Western blotting to visualize Tau ($Tau^{P301L}$) and actin.

**PS1 harboring FAD-linked mutations cannot restore p62 expression and p62-dependent Tau degradation**

Mounting evidence suggests that, despite the gain of toxic function from increased Aβ42/40 ratios and AD pathology caused by FAD-linked mutations in PSs, these effects could also be due to the biochemical loss of function of PSs [251], prompting us to examine whether FAD-linked mutations in PS1 could promote Tau aggregation by reducing p62-dependent Tau clearance. We first determined the expression of p62 in DKO MEFs upon overexpression of the WT or mutant PS1 harboring one of three FAD-linked mutations, A79V [252], G384A [253], or ΔE9 [254]. The levels of exogenous hPS1 were generally constant among transfected DKO MEFs (Fig. 3.6A). The levels of both p62 protein and mRNA transcript in DKO MEFs can be efficiently restored by WT hPS1 to a level comparable to WT MEFs, whereas all three mutant PS1s examined were significantly defective in rescuing p62 expression in DKO MEFs (Fig. 3.6A). PS1-A79V, -G384A, and -ΔE9 mutants all consistently failed to induce Akt activation and to rescue p62 expression in SH-Tau cells stably infected by lentivirus encoding PS1-5´UTR-targeting shRNA (Fig. 3.6B). We further confirmed that suppression of p62 gene transcription due to downregulation of PS1 could be effectively rectified by the overexpression of WT hPS1, but not by the PS1-A79V mutant (Fig. 3.6C). The partial loss-of-function effect

on p62 expression of PS1 harboring FAD-linked mutations was further substantiated by the evidence that the exogenous expression of PS1-A79V was insufficient to amend the impaired p62-dependent Tau degradation in SH-Tau cells that express PS1 5´UTR-targeting shRNA compared to the complete reversal of impaired Tau degradation by WT PS1 (Fig. 3.6D). Given that the $Asp^{257}$ residue of PS1 is essential for γ-secretase activity [55], we found that the catalytically inactive PS1-D257A mutant could act like WT PS1 to efficiently restore p62 expression (Fig. 3.7 A, B, C), further strengthening the notion that PS1-mediated transcriptional regulation of p62 expression is independent of γ-secretase activity. Our data strongly suggest that FAD-linked mutations in PSs exacerbate AD pathogenesis by impairing p62-dependent Tau degradation.

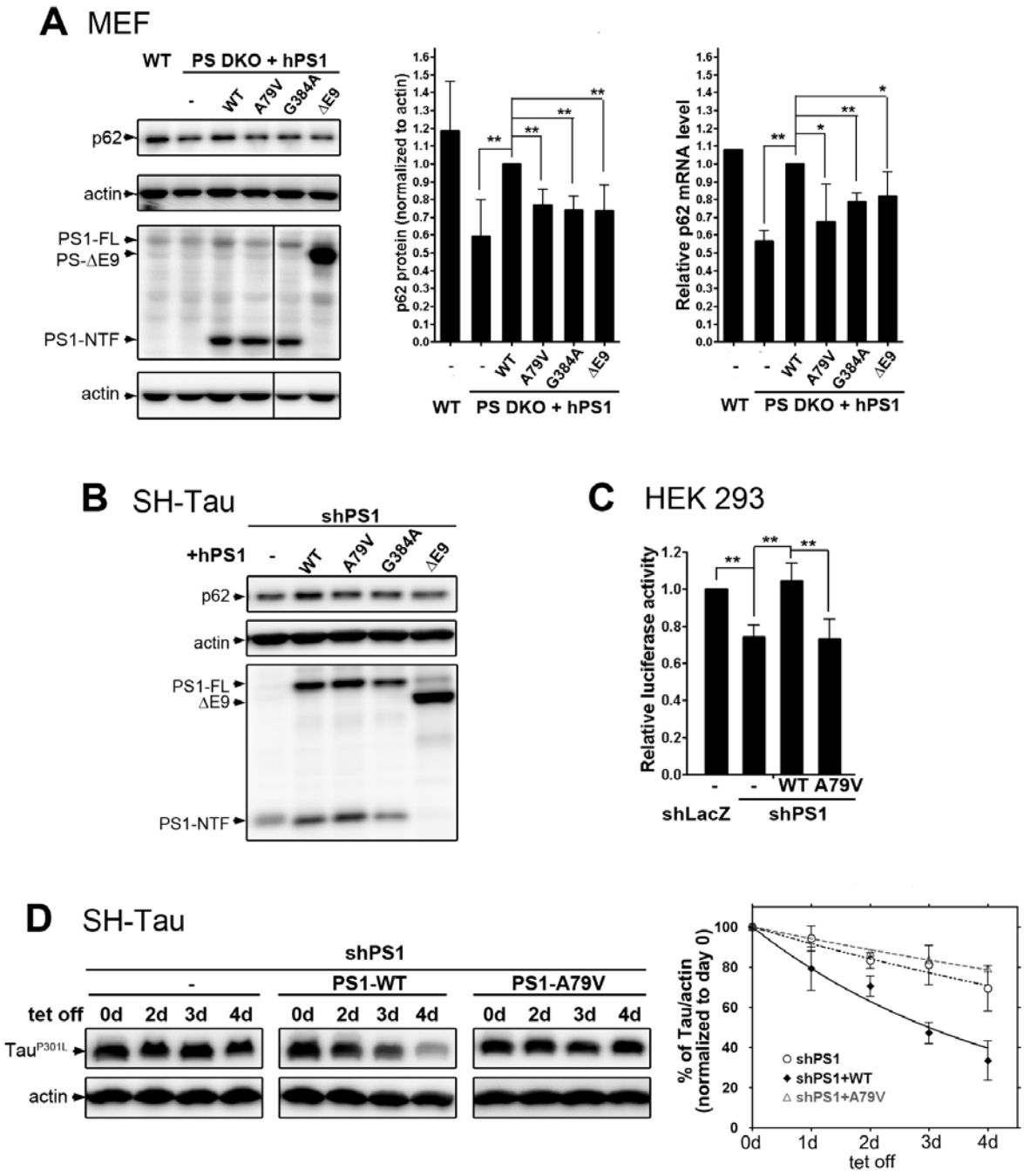



**Figure 3.6 Overexpression of PS1 containing FAD-linked mutations fails to restore the impaired p62 expression and p62-dependent Tau degradation in PS1-deficient cells.**

(**A**) Protein and mRNA levels of p62 in DKO MEFs stably expressing WT or mutant hPS1 (A79V, G384A, and ΔE9) were determined by Western blotting and quantitative real-time PCR, respectively. The expression of exogenous hPS1 was confirmed by Western blotting using an anti-hPS1 antibody. (**B**) SH-Tau cells that stably express PS1-targeting shRNAs (shPS1) were transduced with WT or mutant hPS1 (A79V,

G384A, and ΔE9), followed by antibiotic selection. The levels of p62, phosphorylated Akt (pAkt), total Akt, hPS1, and actin in clarified lysates were analyzed by SDS-PAGE and Western blotting. (**C**) HEK293 cells that stably expressed PS1-targeting shRNA (shPS1) were transduced with WT or mutant hPS1 (A79V), followed by antibiotic selection. Cells were then further transfected with either pGL3-Basic or pGL3.p62[FL] along with the pBud-RLuc plasmid (transfection-efficiency control). Relative promoter activity was expressed as the ratio of the normalized luminescence of pGL3.p62[FL] to the normalized luminescence of pGL3-Basic. The luciferase signal of shLacZ-expressing cells was referred to as 1-fold relative luciferase activity. (**D**) PS1-knockdown SH-Tau cells stably expressing WT or mutant hPS1 (A79V) were incubated with growth medium containing 0.2 μg/ml tetracycline (tet-on) to induce $Tau^{P301L}$ expression for 4 d, followed by replacement with fresh tetracycline-free growth medium (tet-off) and incubation for various intervals. Clarified lysates were analyzed by SDS-PAGE and Western blotting to visualize Tau and actin. The normalized level of Tau in cells harvested at tet-off day 0 was referred to as 100% of relative abundance. The average abundance at each time point from at least three independent experiments was plotted and analyzed by nonlinear regression. Quantitative data are shown as the mean (± SD) from at least three independent experiments and were analyzed by student's *t*-tests. * $p < 0.05$; ** $p < 0.01$. Immunoblots shown in (B), (C), and (D) are representative experiments of at least three reproducible experiments.

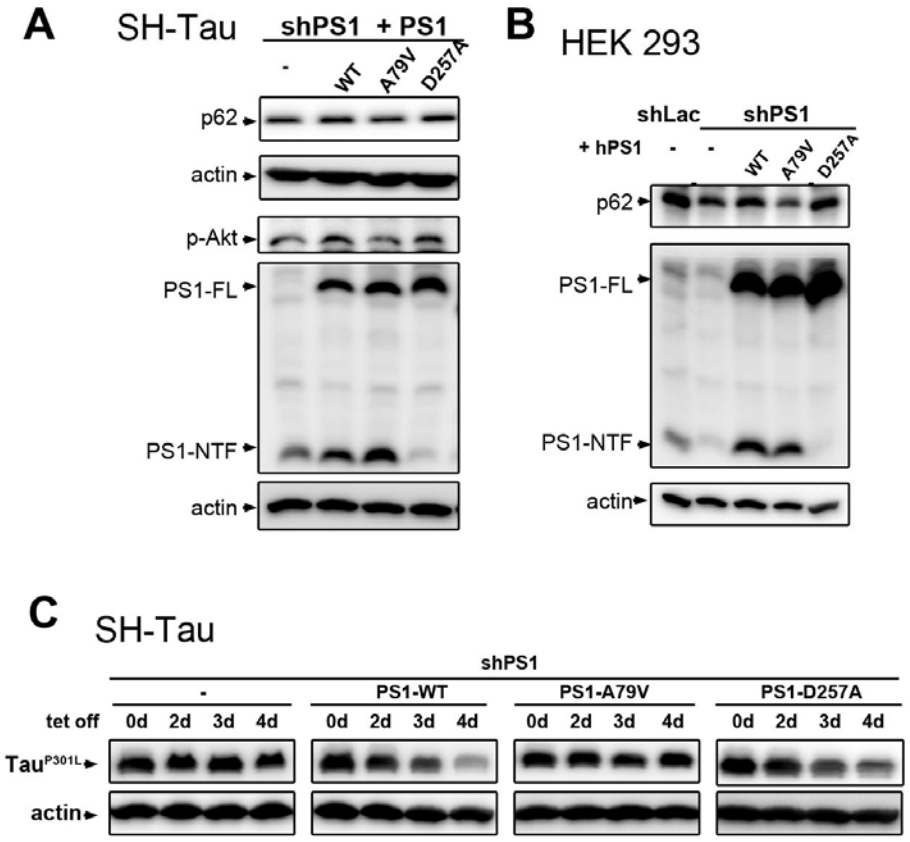



**Fig 3.7 Overexpression of secretase-inactive PS1-D257A mutant rescued the pAkt level, p62 expression and p62-dependent Tau degradation in PS1-deficient cells.**

(**A**) SH-Tau cells or (**B**) HEK cells that stably express PS1-targeting shRNAs (shPS1) were transduced with WT, FAD-linked mutant PS1 (A79V) or secretase-inactive mutant hPS1 (D257A) followed by antibiotic selection. The levels of p62, phosphorylated Akt (pAkt), hPS1, and actin in clarified lysates were analyzed by SDS-PAGE and Western blotting. (**C**) PS1-knockdown SH-Tau cells stably expressing WT, FAD-linked mutant PS1 (A79V) or secretase-inactive mutant hPS1 (D257A) were incubated with growth medium containing 0.2 μg/ml tetracycline (tet-on) to induce $Tau^{P301L}$ expression for 4 d, followed by replacement with fresh tetracycline-free growth medium (tet-off) and incubation for various intervals. Clarified lysates were analyzed by SDS-PAGE and Western blotting to visualize Tau and actin.

### 3.5 Discussion

In the present study, we delineate a new role for PS1 in the modulation of p62 expression through an Akt/AP-1 pathway and show that this novel function of PS1 is crucial for the homeostasis of Tau. The primary function of PS1 is to form the catalytic subunit of γ-secretase. The majority of FAD-linked mutations have been identified within the PS1 allele, which lead to the overproduction of the more toxic Aβ42 over Aβ40 [255-258]. Evidence has also demonstrated that PS1 directly participates in diverse cellular processes independent of γ-secretase activity, and the disruption of these functions severely affects homeostasis, expediting the pathogenesis of AD [217, 259].

The non-proteolytic function of PS1 in the regulation of p62 expression is corroborated by the downregulation of PS1 in non-neuronal (HEK293), neuronal (SH-SY5Y) cells and by MEFs derived from *PS1- and/or PS2-knockout* mice. Furthermore, deficits in p62 gene transcription can be fully ameliorated by exogenous expression of WT PS1.

Recent findings suggested a role of PS1 in lysosomal function. Lee et al. reported that lysosomal acidification is disrupted in PS1 null blastocysts [236]. Study from Neely et. al. revealed dysfunction of autophagy at the level of lysosomal fusion in PS DKO MEFs [237]. A more recent report by Coen et al. provided evidence that

lysosomal defect in PSs deficient cells is alternatively caused by imbalance of lysosomal calcium homeostasis [235]. On the other hand, Zhaung et. al. argued that loss of PSs instead affects the expression of genes involving in lysosomal biogenesis [238]. Although these findings seemingly contradict to our proposed model, we did observed that lysosomal inhibition resulted in a lower accumulation of p62 in PS1-deficient cells compared to control cells (Fig. 3.1E lane 1,2 compared to lane 5,6), indicating the requirement of PS1 to maintiain lysosomal activity, as previously found. However, the transcriptional effect seemed more prominent than lysosomal degradation in our system since level of p62 protein was still much lower in PS1-knockdown cells than in control cells in the presence of $NH_4Cl$ (Fig. 3.1E lane 2 compared to lane 6). We thus sought to investigate the transcriptional regulation of p62 and did not further determine machineries underlying PS1-dependent lysosomal activity. Results form qPCR analysis and promoter assay indeed support the positive role of PS1 in the transcriptional regulation of p62 expression. The discrepancy of p62 protein levels observed in previous studies and ours may be due to differential regulatory mechanisms participating lysosomal function and p62 gene expression in specific cell lines under different cultural conditions.

PS1 is a multi-transmembrane protein that is unlikely to be translocated into the nucleus to function as a transcription factor. As the catalytic subunit of γ-secretase,

though, PS1 could regulate gene expression by the actions of various ICDs derived from γ-secretase cleavages of membrane protein substrates. Since we observed no significant changes in the levels of p62 protein, mRNA, or promoter activity in the presence of a γ-secretase inhibitor (Fig. 3.3C), we concluded that PS1-associated γ-secretase activity was not required for PS1-dependent transcriptional regulation of p62 expression. The similar action of the secretase-inactive PS1-D257A mutant to the WT PS1 in rescuing the p62 expression in PS1-knockdown cells (Fig. 3.7) further supports this notion. We thus unveil a new nonproteolytic function of PS1 in the regulation of p62 expression.

Based on the promoter activity derived from various mutant constructs of p62 5´UTR, our data suggest that the two AP-1 binding sites, A1 and A2, are the primary regulatory motifs involved in the PS1-dependent modulation of p62 expression. Although the A2 binding site is identified as an antioxidant response element (ARE) responsible for Nrf2 binding under oxidative stress [241], the involvement of Nrf2 in the PS1-mediated modulation of p62 expression is ruled out by the nearly undetectable level of endogenous Nrf2 in HEK293 cells [241] and by the similar responses to PS1-elicited effects of the A2-truncated p62 promoter (pGl3.p62[ΔA2]) and the WT p62 promoter. Given that the mA1ΔA2 mutant completely loses its responsiveness to PS1-elicited regulation, our data thus suggest that AP-1 primarily

uses the A1 site to control p62 expression and resorts to the A2 site upon loss of A1 function to sustain p62 expression.

Our data also unveiled that Akt is involved in PS1-dependent p62 transcriptional control (Fig. 3.4C). Interestingly, it has been reported that inhibition of Akt signaling promotes autophagy induction in cancer cells [260]. It is possible that PI3K/Akt can affect p62 protein levels through modulating both autophagic degradation and AP-1 dependent transcription. In addition, p62 may fine-tune its own homeosatsis level since overexpression of p62 has been shown to activate Akt signaling [261]. It would be of interest to investigate this feedback regulation.

The UBA motif in p62 is the binding site for K63-linked polyubiquitin chains on Tau, and the PB1 domain of p62 can interact with proteasomes. These structural features allow p62 to be a transporter that can shuttle Tau to proteasomes for degradation [160]. Nevertheless, our data suggest that both proteasome and autophagy/lysosome pathways can contribute to p62-dependent Tau degradation. It has been demonstrated that the evolutionarily conserved interaction between p62 and LC3 is required for selective autophagic degradation of polyubiquitinated aggregates [140, 206, 248]. Another independent study showed that K63-linked polyubiquitinated Tau inclusions were preferentially removed by autophagy [84]. p62 could thus likely function as a dual transporter for Tau to be degraded through either selective

autophagy or proteasomes. When the ubiquitin-proteasome system is overwhelmed by excessive Tau, p62 could then shuttle ubiquitinated Tau aggregates for autophagic degradation through its binding to LC3. The level of p62 is thus pivotal for the proteostasis of neurotoxic Tau.

FAD patients carry mutations in PS1 develop tauopathies during early age [223, 262-264]. Studies using cell models or transgenic mice revealed that several FAD-linked PS1 mutants enhance tau phosphorylation partly via GSK3β and accelerate the aggregation of tau [219, 265-269]. Some reports also revealed concomitant increased level of total tau proteins [265, 267, 270]. These lines implicate that PS1 involves in modulating tau levels other than promoting tau phosphorylation, yet only rarely do studies address this issue. Using an inducible tau expressing system driven by tet-on promoter in SH-SY5Y cells to minimize the interference of protein synthesis or aggregates forming rates, we clearly showed that PS1-mediated p62 expression is required for tau degradation and that FAD mutations in PS1 caused tau accumulation due to impairment of tau turnover. However, we could not rule out the possibility that blockage of tau degradation by PS1 knockdown partly resulted from enhancement of tau phosphorylation. We detected tau phosphorylation at residue Ser202 and Thr205 by AT8 antibody and did not observe any significant difference in p-tau to total tau ratio between the control and PS1

knockdown SH-Tau cells (data not shown). It should also be noted that ablation of PS1 also partially attenuates lysosomal function (Fig. 3.1E) [235-238]. However, our data demonstrated that expression of p62 was sufficient to restore tau degradation, indicating that p62 dependent tau clearance is one of major mechanisms downstream of PS1 to regulate tau levels.

The current findings imply that p62 level could be downregulated in brains of FAD carriers, which is in line with a pervious study showing lower level of p62 expression in brains of sporadic AD patients than that of age-matched controls. Although it seems conflict with another earlier observation that p62 immunoactivity stably appears in NFT structures, we suppose that the presence of p62 at early stage of AD may be a protective response to sequester toxic tau and to mediate tau degradation. During the progression of the disease, accompanied lysosomal defect causes more p62-positive NTFs accumulated in cytosol and in unfused autophagosomes. Additionally, transcriptional downreulation of p62 further reduces the availability of this protein, leading to exaggerated impairment of tau turnover.

Recently proposed “Tau-axis hypothesis” suggests that accumulation of Tau could render neurons more sensitive to Aβ-elicited neurotoxicity [75]. Our results are thus in line with this hypothesis that FAD-linked mutations in PS1 not only increase the Aβ42/40 ratio to enhance plague formation, but also impede p62-dependent Tau

degradation, leading to the development of both amyloidopathies and tauopathies during early onset of AD. Given that impairment of p62 function has also been implicated for defects in mitophagy, trafficking of AMPA receptors, and the maintenance of TrkA receptors in neurons [155, 159, 271], deficiencies in p62 expression observed in AD brains could also associate with mechanisms other than tauopathies to induce neurodegeneration. The newly identified function of PS1 in modulating the expression of p62 could lead to the development of advanced AD therapeutics strategies.

# Chapter 4

# Concluding remarks and future perspectives

Adult neurons are characterized by an inability to self-renew, which is the major obstacle to neuron regeneration that makes nearly all neurodegenerative diseases incurable. The accumulation of misfolded protein aggregates, ubiquitously observed during the progression of various neurodegenerative diseases, has been widely accepted to be a major neuropathogenic culprit [10]. Elimination of aggregation-prone proteins by genetic approaches has been shown to reverse neurodegenerative symptoms in several mouse models [272-274]. Molecular mechanisms underlying the clearance of these neurotoxic oligomers or polymers are thus potential therapeutic targets for drug development. The process of p62-dependent aggrephagy, which recruits polyubiquitinated protein aggregates into autophagosomes for degradation, is gaining attention in the research field of neurodegenerative diseases [142, 275]. The dissertation presented here contributes to the elucidation of p62-LC3 binding machinery, and proves the importance of p62-LC3 assembly in specific neurodegenerative diseases. Additionally, the dual role of p62 in Tau clearance further substantiates p62 as a key factor in AD, and the discovery of transcriptional regulation of p62 expression via the PS1-Akt-AP1 axis provides additional information for the development of approaches to up-regulate neuroprotective p62 proteins.

In the first part of my thesis, I described the identification of two positively-charged residues, K30 and K51, and the hydrophobic L53 residue of LC3 essential for the LC3-p62 interaction [248]. During preparation of our published

manuscripts, similar findings were reported elsewhere [206, 207]. With the aid of X-ray and NMR structural studies, it is clear that the WXXL sequence in the LIR of Atg19 and p62 is the core for Atg8/LC3 binding [207, 276]. The conserved LIR domain has also been identified in several other proteins, including NBR1 [164, 277], Nix [278, 279], calreticulin [280], clathrin heavy chain [281], and FYCO [282]. All of these proteins have been shown to associate with LC3 or GABARAP, but to date only NBR1 has been confirmed to be a cargo receptor for aggrephagy. Recently, a proteomic screen performed by Wade Harper et. al. led to the identification of 67 proteins that associate with the six human Atg8 orthologues. Several of these proteins interact in a LIR-dependent manner, which was confirmed by using both wild-type and mutant Atg8 orthologues (Y49A/L50A for GABARAP or F52A/L53A for MAP1LC3B) as probes [283]. Such characterization of the evolutionarily conserved p62-LC3 binding site provides valuable information for the discovery of additional autophagic cargo receptors. Furthermore, the assembly properties of p62-LC3 enable the design of probes that target to autophagosomes. For example, a short peptide containing the LIR domain fused to a cell-penetrating peptide and a fluorophore can be used to detect autophagosomes when applied to culture media. This probe can be further modified to serve as a vehicle to bring specific compounds into autophagosomes or lysosomes.

In the second part of this thesis, the presented results again support the

important role of p62 in autophagic clearance of aggregation-prone proteins. However, in the case of Tau protein, p62 serves as a dual shuttle factor for both proteasomal and autophagic degradation. Our data demonstrates that all Tau proteins, including aggregated ones, accumulated upon treatment with either proteasomal or autophagic inhibitors. We were not able to determine whether there is any preference of p62 for binding to Tau complexes with various degrees of polymerization. It is possible that the accumulation of aggregates during proteasome blockage is caused solely by inhibition of monomer degradation; autophagy, on the other hand, may be required for clearance of polymers, with its impairment resulting in increased total Tau proteins. Several studies have addressed the mechanisms behind degradation of Tau species, but these remain incompletely understood [284]. Whether p62-dependent targeting for clearance is affected by different stages of Tau polymerization, post-translational modifications, or specific Tau binding proteins remains unanswered. Efforts are needed to optimize detection systems to differentiate between variously sized Tau polymers.

In addition, ALFY (autophagy-linked FYVE) protein has been reported to localize to autophagic machinery by interacting with Atg5, and to associate with ubiquitinated protein aggregates through binding to p62. ALFY is also required for efficient removal of polyglutamine inclusions via autophagy [232, 285]. This large protein (~400 kDa) is believed to promote formation of p62-containing inclusion

bodies that may facilitate aggrephagy. Hence, we speculate that ALFY may be involved in p62-dependent Tau turnover via autophagy, but not in p62-dependent proteasomal degradation of Tau.

The present study was performed mostly using human cell lines. To further confirm our hypothesis, establishment of *in vivo* systems is essential. Both PS1 and p62 are highly conserved among different species, and tauophathies have been established in several animal models [286]. The short lifespan and easy-to-manipulate characteristics of zebrafish make it an ideal model for our future studies [287]. Dr. Chang-Jen Huang (Institute of Biological Chemistry, Academia Sinica) has established a transgenic zebrafish line that expresses GFP-tagged Tau specifically in neurons, which causes neurotoxicity. If we were to use morpholinos to knock down endogenous PS1 or over-express FAD-linked PS1 mutant constructs in this model, we would expect to observe down-regulation of endogenous p62 in neurons, with enhancement of neuronal death caused by excessive human Tau accumulation.

Du et. al. reported that p62 is down-regulated in the elderly and sporadic AD patients due to oxidative damage in promoter regions of p62 [169]. The same study also discovered that a reduction of p62 levels in response to oxidative damage is a phenomenon present in a variety of neurodegenerative diseases, including AD, HD, PD, and FTD [288]. This finding is in concert with my observation that p62 is essential for removal of cytotoxic Htt in HD and Tau in AD. It should be noted that

the AP-1 sites in the p62 promoter region do not overlap with domains subjected to oxidative damage as revealed by Du et. al., suggesting that activation of the AKT/AP-1 pathway has the potential to restore p62 levels and to mitigate tauopathies in AD. Our p62 promoter assay system could therefore be a practicable screening platform to search for molecules that enhance p62 production.

In summary, my PhD project started with the conserved sorting basis of selective autophagy in the turnover of ubiquitinated aggregates, highlighting the role of autophagic cargo receptor p62 in this process. During the investigation, a serendipitous finding led us to study PS1-dependent regulation of p62 expression, resulting in the discovery of the underlying transcriptional mechanism. Exploring the involvement of such a multifunctional protein as p62 in various neurodegenerative diseases, and investigating how the evolutionarily conserved function of p62 can be delicately modulated, have been extremely rewarding processes. It is equally exciting to have discovered a new role of the well-characterized PS1 protein in Tau degradation. Although many questions remain unanswered, we believe that the studies presented here provide useful lines of evidence to aid in our understanding of these currently incurable neurodegenerative diseases.

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# Appendix: List of publications

1. **Presenilin-1 regulates the expression of p62 and governs p62-dependent degradation of Tau independent of γ-secretase activity.** Tung YT, Wan BJ, Hsu WM, Hu MK, Her GM, Huang WP, Liao, YF. (submitted)
2. **Establishment of a bioluminescence-based bioassay for the detection of dioxin-like compounds.** Wang BJ, Liao YF, Tung YT, Yih LH, Hu CC, Lee H. Toxicol Mech Methods. (in press)
3. **Autophagy: a double-edged sword in Alzheimer's disease**. Tung YT, Wang BJ, Hu MK, Hsu WM, Lee H, Huang WP, Liao YF., J Biosci. 2012 Mar;37(1):157-65.
4. **The evolutionarily conserved interaction between LC3 and p62 selectively mediates autophagy-dependent degradation of mutant huntingtin.** Tung YT, Hsu WM, Lee H, Huang WP, Liao YF., Cell Mol Neurobiol. 2010 Jul;30(5):795-806.
5. **Tumor necrosis factor-alpha-elicited stimulation of gamma-secretase is mediated by c-Jun N-terminal kinase-dependent phosphorylation of presenilin and nicastrin.** Kuo LH, Hu MK, Hsu WM, Tung YT, Wang BJ, Tsai WW, Yen CT, Liao YF., Mol Biol Cell. 2008 Oct;19(10):4201-12.
6. **Sodium selenite inhibits gamma-secretase activity through activation of ERK.** Tung YT, Hsu WM, Wang BJ, Wu SY, Yen CT, Hu MK, Liao YF., Neurosci Lett. 2008 Jul 25;440(1):38-43.
7. **New 1,2,3,4-tetrahydroisoquinoline derivatives as modulators of proteolytic cleavage of amyloid precursor proteins.** Hu MK, Liao YF, Chen JF, Wang BJ, Tung YT, Lin HC, Lee KP., Bioorg Med Chem. 2008 Feb 15;16(4):1957-6