

Free ubiquitin: a novel therapeutic target for neurodegenerative diseases

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Neurodegenerative diseases are widespread and the increasing number of patients with these diseases can no longer be ignored. Dementia is a symptom of many neurodegenerative diseases, and Alzheimer's disease (AD), which is associated with memory and learning disabilities, accounts for approximately 60 to 80% of all dementia cases (Wyss-Coray, 2016). In the United States, it is estimated that 13.8 million people over the age of 65 years will be affected by AD in 2050 (Alzheimer's Association, 2020). Moreover, the onset of AD is closely related to aging, and as such, AD occurs at a frequency of 50% in people over the age of 95 years. Although progress in medical science has contributed to an increase in the average human lifespan, advances in AD treatment strategies have been unable to keep up with the increasing number of elderly individuals. Most drugs targeting AD merely delay the progression of the disease, without providing a *bona fide* treatment. Moreover, because the life expectancy of AD patients is 3 to 11 years after diagnosis, an effective therapy for AD is urgently required (Alzheimer's Association, 2020).

The majority of AD cases originate from abnormalities in at least three proteins: namely, amyloid precursor protein (APP), presenilin 1, and presenilin 2 (Chen et al., 2017). Early-onset familial AD is caused by mutations in genes encoding these proteins and late-onset sporadic AD is caused by genetic and environmental factors that affect the expression or function of these genes. The molecular features of AD are amyloid-beta (A β) aggregates and hyperphosphorylated tau neurofibrillary tangles in the brain. A β is produced by the sequential cleavage of APP by β -secretase and γ -secretase. Under normal conditions, A β monomers are released into the extracellular environment. These monomers may form aggregates, which can be taken up into the cytoplasm by receptor-mediated endocytosis. They are then cleared by autophagy or degraded by the proteasome. However, in the elderly, A β cannot be cleared or degraded properly due to decreased function of the autophagic process and the proteasome. Therefore, these A β remnants may come in contact with other cellular components via the vesicular transport system, resulting in damage to neuronal cells. In addition, levels of A β monomers in the extracellular environment are increased and these monomers form various types of aggregate structures, including oligomers, protofibrils, and amyloid fibrils, finally producing amyloid plaques. A β can also be taken up by astrocytes and microglial cells in the brain, and these cells secrete inflammatory cytokines that cause neuronal cells to be under inflammatory stress in a wide range of brain regions. For this reason, the brains of AD patients exhibit loss of neurons, due to extensive amyloid plaque formation and inflammatory stress. To counteract AD progression, numerous studies have focused on autophagy and proteasomal degradation in the neuronal system (Ciechanover and Kwon, 2015).

Autophagy and proteasomal degradation require many cellular components. Ubiquitin (Ub), a highly conserved eukaryotic protein, is involved in both processes. Ub is known to mediate its effects through the post-translational modification of its substrates (Park and Ryu, 2014). Ub is expressed by two different types of genes: monomeric Ub-ribosomal fusion genes, *UBA52* and *UBA80* (also known as *RPS27A*) and polyubiquitin genes, *UBB* and *UBC*, which are composed of three and nine tandem repeat coding units, respectively, in humans. Ub levels are normally maintained in a dynamic state of equilibrium, with "free" Ub, which is ready for the ubiquitination of substrates, and "conjugated" Ub, which is covalently linked to substrates or other Ub molecules via isopeptide bonds. Ub is conjugated to its substrates through sequential enzymatic reactions catalyzed by Ub-activating enzymes (E1), Ub-conjugating enzymes (E2), and Ub ligases (E3). Substrates can be modified by a single Ub molecule at one or multiple lysine residues (mono- or multi-monoubiquitination) or by Ub chains (polyubiquitination). Ub chains are formed through lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the N-terminal methionine residue (M1). Monoubiquitination has been shown to be required for endocytosis and histone modification, whereas polyubiquitination, especially by K48 and K63 chains, has been shown to play important roles in proteasomal degradation and autophagy, respectively. These physiological functions of Ub are especially prominent during developmental stages or under cellular stress conditions. The overall Ub levels are fluctuated and free Ub is conjugated to target proteins to induce proteasomal degradation or other cellular signaling through interaction with proteins containing Ub-binding domains. When Ub pools are reduced, protein turnover is not regulated in a timely manner, leading to abnormal development of embryos, failure of cell fate determination, or increased susceptibility to proteotoxic stress.

Since Ub plays a variety of roles at the cellular level and there are many targets (2 E1 proteins, 30 to 50 E2 proteins, more than 600 E3 proteins, and more than 5000 substrates), Ub comprises 5% of the total cellular protein content. Therefore, cell survival requires the maintenance of cellular Ub pools above certain levels. The polyubiquitin genes, *UBB* and *UBC*, are known to be induced or upregulated under stress conditions, such as heat-shock, oxidative, and proteasomal stress. Under these conditions, the activity of deubiquitinating enzymes, which cleave Ub from Ub chains or ubiquitinated substrates, is also increased to recycle conjugated Ub to the free Ub pool. However, under chronic stress conditions or when cellular activity is reduced due to aging, the Ub pools are biased toward the conjugated state and the levels of free Ub decrease, despite increased expression levels of polyubiquitin genes. Huntingtin (Htt) aggregates, which are

a marker of Huntington's disease; α -synuclein (α -Syn) aggregates, which are a marker of Parkinson's disease; and neurofilament aggregates, which are a marker of amyotrophic lateral sclerosis, colocalize with Ub, suggesting that the levels of available free Ub may be depleted in various neurodegenerative diseases. In AD, proteasomal degradation and autophagy are important for the removal of A β aggregates, and Ub plays essential roles in both processes. Therefore, it is possible that neurons and brain tissues under A β stress have reduced free Ub levels.

Data from many recent studies have indicated that proteasomal and autophagic functions may be impaired when free Ub levels are reduced. In 2020, our laboratory found that levels of free Ub were reduced in A β 42-treated primary neurons and cultured brain tissue slices (Park et al., 2020b). Higher levels of neuronal death and inflammatory stress were detected in A β 42-treated samples than in A β 40-treated controls. In addition, the decreased proteasome activity observed in A β 42-treated samples was consistent with the AD phenotype. These results were not limited to cultured primary neurons. Similar effects of A β 42 were observed when brain slice cultures were used to mimic the brain tissue environment. A β , α -Syn, and Htt-Q53 have previously been reported to share a three-dimensional structure that significantly reduces proteasome activity by inhibiting 20S and 26S proteasome gate opening (Thibautaud et al., 2018). The monomers of A β , α -Syn, Htt-Q53 were shown to be degraded by the proteasome, but the common structures of these oligomers, which can be detected using A11 antibodies, impaired proteasome function by inhibiting the HbYX motifs of the 19S ATPases, which are required for the binding of 19S to the 20S catalytic core. However, it remains unclear whether the decrease in free Ub levels affects proteasome activity. To determine the relationship between free Ub levels and proteasome activity, we developed an innovative approach to diminish free Ub. We used the CRISPR/Cas9 system to simultaneously disrupt both *UBB* and *UBC*, to suppress their compensation of each other. When both polyubiquitin genes were disrupted in HEK293T and HeLa cells, free Ub levels were significantly reduced, with an associated decrease in proteasome activity and cellular proliferation (Park et al., 2020a). These results indicated that a decrease in free Ub levels alone was sufficient to affect proteasome activity. Therefore, in AD patients, reduced levels of free Ub may also inhibit proteasome activity, which may promote AD progression.

The effect of reduced levels of free Ub has also been extensively investigated in mouse models. In *Ubb* knockout (KO) mice, early-onset reactive gliosis and adult-onset neurodegeneration are observed in the hypothalamic region, but the locus coeruleus, another brain region expressing high levels of *Ubb*, is not affected. This discrepancy may be due to differences between these two brain regions. In the hypothalamic region, free Ub levels are significantly lower in *Ubb* KO mice than in wild-type mice. However, in the locus coeruleus region, free Ub levels do not differ between wild-type and *Ubb* KO mice, although Ub conjugate levels are reduced in *Ubb* KO mice (Park et al., 2012). Moreover, based on primary cultures derived from *Ubb* KO embryonic brains, abnormal differentiation of neural stem cells is observed when free Ub

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levels are reduced (Ryu et al., 2014), and these phenotypes are reversed by ectopic expression of Ub. By providing exogenous Ub using lentivirus-mediated delivery, reduced free Ub levels can be restored and neuronal phenotype can be improved.

Reduced free Ub levels are also observed in *Ubc* KO mouse embryonic fibroblasts (MEFs). In *Ubc* KO MEFs, cellular viability is significantly reduced under oxidative stress conditions (Kim et al., 2015). Nuclear factor erythroid 2-related factor 2 (Nrf2), which is the major cellular transcription factor protecting against oxidative stress, is polyubiquitinated via Kelch-like ECH-associated protein 1 (Keap1) and degraded by the proteasome under normal conditions. However, under oxidative stress conditions, it is stabilized and translocates into the nucleus to upregulate antioxidant genes. Although regulation of the Nrf2-Keap1 pathway is almost intact in *Ubc* KO MEFs, reduced levels of free Ub cause the inefficient polyubiquitination and degradation of misfolded protein aggregates, which accumulate as large aggregates, causing toxicity to cells. Therefore, reduced levels of free Ub may be closely related to the increased formation of misfolded proteins. When polyQ-expanded aggregates (Q103) are ectopically expressed in cells, they form inclusion bodies at the juxtannuclear region (Bae and Ryu, 2018). In *Ubc* KO MEFs, an increased accumulation of Q103 aggregates is observed due to delayed clearance by autophagy. Therefore, reduced free Ub levels in *Ubc* KO MEFs seem to impair autophagic flux as well as proteasomal activity. Based on these data, reduced free Ub levels may adversely affect neurodegenerative diseases through the impairment of both autophagic and proteasomal functions.

Based on previous studies by our group and others, we propose that reduced levels of free Ub are an important determinant of the progression of various neurodegenerative diseases, such as AD with A β aggregates and other diseases accompanied by protein aggregates. It is not currently known whether the administration of free Ub to patients is an effective treatment strategy for these diseases. However, we found that reduced levels of free Ub impair both proteasomal and autophagic functions; therefore, we cautiously suggest that increasing the amount of free Ub above certain levels may be a good candidate for a therapeutic strategy for neurodegenerative diseases (Figure 1). Currently, some drugs that have been developed to treat neurodegenerative diseases activate autophagy to remove aggregates. This is an effective strategy to delay the progression of disease symptoms, but there are concerns that activated autophagy may indiscriminately affect cellular organelles. However, restoring free Ub to normal levels may be a cell-friendly approach. We suggest that the supply of free Ub not only affects the clearance of aggregates, but also restores cellular signaling, which has been suppressed by Ub deficiency. When Ub levels are reduced, both proteasomal and autophagic functions are impaired, but free Ub supply may recover both functions only up to the cellular requirement or physiological relevance. Therefore, free Ub supply may be a more cell-friendly approach than the drug treatment that can activate proteasomal or autophagic pathway, which may affect other unwanted cellular systems. As more than 5000 proteins are regulated by ubiquitination, global protein pools are affected by Ub supply. Therefore, the global alteration of protein levels

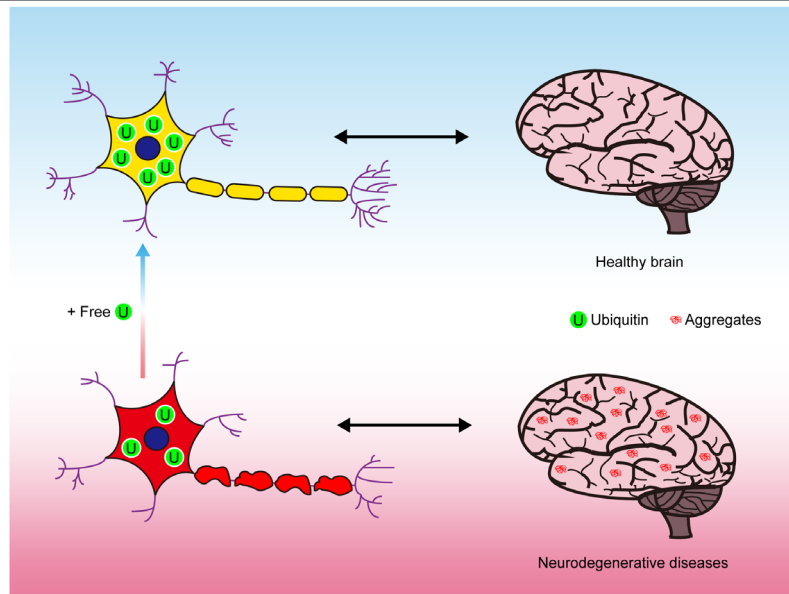


Figure 1 | A potential therapeutic approach for neurodegenerative diseases by increasing the levels of free ubiquitin in neurons.

In the brain of patients with neurodegenerative diseases, there is abnormal deposition of protein aggregates and damaged neurons with reduced free ubiquitin levels. After supplying these damaged neurons with free ubiquitin, they recover and the brain becomes healthier, most likely through the restoration of proteasomal and autophagic functions.

by free Ub supply can overcome the limitations of target-specific therapeutic strategy and may efficiently ameliorate the progression of neurodegenerative diseases.

It is not currently known whether ectopic expression of free Ub indeed increases the levels of endogenous free Ub, and not Ub conjugates. The best way to keep Ub as a free form is probably to increase the expression or activity of deubiquitinating enzymes, which will shift the equilibrium towards free Ub instead of Ub conjugates. Alternatively, overexpression of Ub mutant that cannot form any chains (K0 mutant) may be used, although there will be a limitation of this Ub mutant as it cannot extend Ub chains if needed. However, maintaining various forms of Ub, while keeping free Ub at normal levels, should be considered as a novel approach to treat neurodegenerative diseases and our research will serve as a milestone for this innovative treatment strategy.

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