METHIONINE SULFOXIDE REDUCTASE AND UBIQUITIN-DEPENDENT DEGRADATION OF ALPHA-SYNUCLEIN

By

Yue Deng

Submitted to the graduate degree program in Pharmacology and Toxicology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

_____

Chair: Jackob Moskovitz, Ph.D.

_____

Jeff Staudinger, Ph.D.

_____

Mark Richter, Ph.D.

Date Defended: May 9th, 2017

The Thesis Committee for Yue Deng
certifies that this is the approved version of the following thesis:

METHIONINE SULFOXIDE REDUCTASE AND UBIQUITIN-DEPENDENT DEGRADATION OF ALPHA-SYNUCLEIN

_____

Chair: Jackob Moskovitz, PhD.

Date approved: May 4th, 2017

ABSTRACT

Alpha-synuclein (αSyn) is one of the key proteins considered to be involved in the pathogenesis of the Parkinson`s disease (PD) and being the main component of Lewy bodies (LBs). Studies on the *αSyn* gene mutants and overexpression of αSyn in yeast and rodent brains indicate that the level of αSyn in neurons is linked to dopaminergic neurons degeneration, and impairment of αSyn degradation is directly related to its aggregation in PD. However, the exact mechanism by which αSyn dysfunction and its degradation are regulated are not well established.

Previous studies have suggested that both the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) are involved in the degradation of αSyn, in which the ubiquitin (Ub) system plays an important role. Moreover, αSyn was found to bind Ub and the molecular chaperone 14-3-3 proteins, leading to enhanced αSyn aggregation. Our lab recently reported on elevated dopamine and 14-3-3 levels in the methionine sulfoxide reductase A (*MsrA*) knockout mouse brain, as well as decreased αSyn degradation in *MsrA* KO yeast cells. Hence, this study will focus on the interactions among MsrA, αSyn degradation, 14-3-3 proteins, and Ub.

Here we show that *msrA* overexpressing (OP) yeast cells extract promotes αSyn aggregation in a Ub- and ATP-dependent manner. Moreover, ubiquitin could promote clearance of both native and A53T mutant αSyn monomers in the presence of *msrA* OP extract in comparison to a *msrA* null mutant yeast extract. Furthermore, MsrA was found to inhibit the auto-ubiquitination of Parkin (a known E3 ubiquitin ligase of αSyn). These data suggest that MsrA plays an important role in ubiquitin-dependent

αSyn degradation and may function as an E3 Ub ligase. To explore the interactions between αSyn, MsrA, and Ub-bound proteins, a mammalian *ex-vivo* system was designed using wild-type (W) and *MsrA* knockout (M) mouse brain extracts. Following pull-down experiments using His-tagged Ub and the mouse brain extracts, it was determined that 14-3-3 proteins were ubiquitinated with dependency on the presence of MsrA (W brain) and ATP. Immunoprecipitation experiments using anti-Ub antibody revealed that M brain exhibited lower levels of ubiquitinated and degraded band products of both αSyn and 14-3-3 proteins, compared with W brain. Complementary immunoprecipitation experiments using anti-14-3-3 antibody showed an enhanced αSyn degradation and ubiquitination in the presence of ATP in W versus M brain extracts, suggesting that 14-3-3 interacts with MsrA and the Ub-system to enhance αSyn degradation. Last but not least, an *ex-vivo* study performed in yeast cells (wild-type and *msrA* null mutant strains overexpressing A53T αSyn) showed that the *msrA* mutant strain possessed significantly much lower total and αSyn ubiquitination levels compared with the wild-type strain. These observations suggest that MsrA plays an important role in the ubiquitination and degradation of αSyn through the ubiquitin-dependent modification of 14-3-3.

ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the guidance and support from the following people:

- My advisor, Dr. Jackob Moskovitz – thank you for your continuous support. I met a lot of problems and made many mistakes in the lab, but you were always patient and willing to help me out. You are a great mentor both in academia and in daily life. It is always pleasant and inspiring to share thoughts with you.
- My committee members, Dr. Jeff Staudinger and Dr. Mark Richter – Dr. Staudinger gave me tons of good suggestions on my schoolwork, lab project, and Ph.D. application. Dr. Richter provided us with Parkin protein and corresponding enzymes. I appreciate your help and I learned a lot about the background experiment design from meeting with you as you are so experienced in doing research.
- My dear father and mother, Xueneng Deng and Shuxia Liu – thank you for your unconditional love, trust, and support. I will always try to make you proud.
- All the people in the Department of Pharmacology & Toxicology – We have wonderful professors there, we have excellent students there, and we have great stuff there. Thank you all for the help I got during the last 2 years!

CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

PD	Parkinson's disease
DA	dopamine
CNS	central nervous system
SNpc	substantia nigra pars compacta
ROS	reactive oxygen species
L-DOPA	L-3,4-dihydroxyphenylalanine
LB	Lewy Body
αSyn	alpha-synuclein
DLBD	diffuse Lewy body disease
LBD	Lewy body dementia
MSA	multiple system atrophy
NMR	nuclear magnetic resonance
NSF	soluble N-ethylmaleimide-sensitive factor
SNARE	NSF attachment protein receptor
VAMP2	vesicle-associated membrane protein 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
ER	endoplasmic reticulum
UPR	unfolded protein response
MAM	mitochondria-associated ER membrane
UPS	ubiquitin-proteasome system
ALP	autophagy-lysosomal pathway

ERAD	Endoplasmic-reticulum-associated protein degradation
Ub	ubiquitin
CMA	chaperone-mediated-autophagy
TH	tyrosine hydroxylase
Met	methionine
MetO	methionine sulfoxide
Msr	methionine sulfoxide reductase
IP	immunoprecipitation
WB	Western blot

CHAPTER 1: INTRODUCTION

1.1. Parkinson's Disease and Dopamine

1.1.1. Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the world. As many as 10 million people worldwide are living with PD, in which about 60% patients are male. The mean age of PD's onset is 55, and the incidence increases markedly with age [1]. In the United States alone, the combined direct and indirect costs of PD are estimated to be near $25 billion per year [2].

Parkinson's disease is now recognized to be a heterogeneous neurodegenerative syndrome with motor and non-motor symptoms [3]. The motor symptoms are characterized by a combination of rigidity, bradykinesia, tremor and postural instability. On the other hand, common non-motor symptoms in PD relate to cognitive dysfunction, affective disorders, abnormalities of autonomic function, personality changes, sleep disorders, and sensory complaints or pain [4]. Although it has been known for some time that genetic factors can be involved in some cases, there is no apparent genetic linkage in most of the PD cases [5]. Like other neurodegeneration diseases, PD is generally progressive, leading to increasing disability unless effective treatment is provided.

1.1.2. Dopamine and Parkinson's disease

Dopamine (DA) functions as a neurotransmitter in the central nervous system (CNS). DA pathways are involved in reward-motivated behavior, motor control and the release of various hormones. The loss of dopaminergic neuronal cells in the substantia

nigra pars compacta (SNpc), which results in DA loss in the basal ganglia (an area of the brain responsible for coordinating fine motor control) is one of the main features of PD [6]. One hypothesis proposes that mitochondrial dysfunction and the consequent oxidative stress, including toxic oxidized DA species, lead to the death of SNpc neuronal cells. Molecular studies found that dopaminergic neurons are very vulnerable to oxidative stress, as the metabolism of DA produces hydrogen peroxide and superoxide radicals [7]. Besides, mitochondria-related energy failure may disrupt vesicular storage of DA, leading to increasing free cytosolic DA concentration, allowing harmful DA-mediated reactions to damage cellular macromolecules [8]. Recent studies have revealed that DA oxidation to dopamine *o*-quinone, aminochrome, and 5,6-indolequinone seems to play an important role in the neurodegenerative processes of PD through (i) mitochondrial dysfunction, (ii) aggregation of misfolded proteins, (iii) protein degradation dysfunction and (iv) generation of reactive oxygen species (ROS) [9].

1.1.3. L-DOPA Therapy

Following the observation that the levels of DA are severely reduced in the striatum of patients with PD, researchers found that L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of DA that crosses the blood-brain barrier, can restore brain DA and motor function in an animal model [10]. Soon attempts were made to alleviate the symptoms of PD through the administration of L-DOPA, which gave birth to the L-DOPA therapy. To this day, this therapy remains the cornerstone of PD symptoms' control but it has a lot of limitations. For example, it is only a symptomatic treatment,

and its efficacy tends to decrease with the disease progression.

1.2. Lewy Body and Alpha-synuclein

1.2.1. Lewy Body and Parkinson's Disease

The pathological hallmarks of PD are the loss of the nigrostriatal dopaminergic neurons and the presence of insoluble intraneuronal proteinaceous cytoplasmic inclusions, termed "Lewy Bodies (LBs)". LBs are spherical eosinophilic protein aggregates composed of numerous proteins, including alpha-synuclein (αSyn), parkin, ubiquitin (Ub), and neurofilaments. The major component of Lewy bodies is αSyn [11]. One hypothesis for LB formation is that αSyn monomers become oligomers (protofibrils), which coalesce into fibrils and then aggregate into Lewy body inclusions [12]. Besides Parkinson's disease, LBs were also found in patients with diffuse Lewy body disease (DLBD), Lewy body dementia (LBD) and multiple system atrophy (MSA) [13]. These observations show that Lewy body pathology may affect different subtypes of neurons as well as glia, and they also indicate that additional factors other than Lewy pathology alone might be required for αSyn's toxicity in patients.

1.2.2. Alpha-synuclein (αSyn)

1.2.2.1. Structure

The first link between αSyn and PD was made when the identification of a missense mutation on chromosome 4 was linked to an autosomal-dominant inherited form of PD [14]. Since then, the research interest in this protein has grown rapidly. αSyn is expressed in all vertebrates. Human αSyn is a 140-amino acids protein (**Figure 1A**), and it belongs to the synuclein family that consists of three subtypes: alpha, beta,

and gamma. The synucleins are similar in sequence, with most of the similarity lying within the N-terminus of the proteins [15]. A structure of the full-length, membrane-bound form of human αSyn was revealed through nuclear magnetic resonance (NMR) spectroscopy (**Figure 1B**). Homology of αSyn across species is greater at the N-terminus of the protein, with more variability in sequence located toward the C-terminus. N-terminal point mutations in αSyn (A30P, E46K, H50Q, G51D, A53E and A53T) [11, 16-21] and genomic duplications or triplications that contain the αSyn locus result in autosomal dominant forms of familial PD [22, 23]. Within cells, αSyn is highly soluble and intrinsically disordered under normal conditions. However, in the presence of acidic lipid membranes or membranes with high curvature, the N-terminus of αSyn folds into an alpha-helix that interacts with membranes [24, 25]. Moreover, under certain circumstances, the protein can undergo a profound conformational transition to a β-sheet–rich structure that polymerizes to form toxic oligomers and or amyloid structure in the fibrils that compose Lewy bodies [26]. The β-pleated conformation binds other proteins such as synphilin-1 [27], parkin [28] and the anti-apoptotic chaperone 14-3-3 [29].

A

```
        10         20         30         40         50
MDVFMKGLSK AKEGVVAAAE KTKQGVAEAA GKTKEGVLYV GSKTKEGVVH
        60         70         80         90        100
GVATVAEKTK EQVTNVGGAV VTGVTAVAQK TVEGAGSIAA ATGFVKKDQL
       110        120        130        140
GKNEEGAPQE GILEDMPVDP DNEAYEMPSE EGYQDYEPEA
```

B

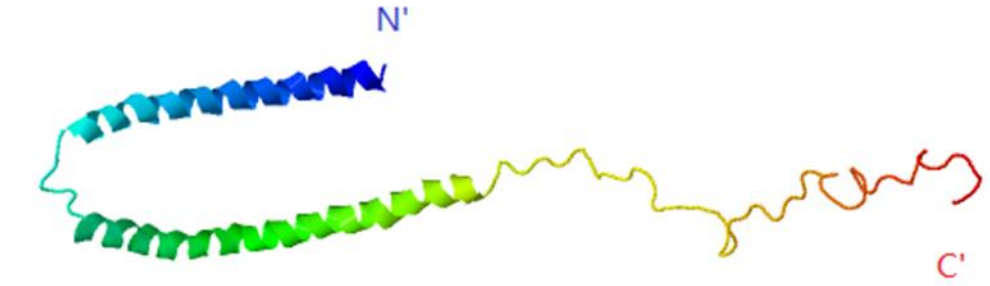


Figure 1. Human αSyn sequence and structure (A) Human αSyn sequence (accession number P37840.1) **(B)** Structure of human micelle-bound αSyn (determined by NMR, PDB ID: 1XQ8)

1.2.2.2. ASyn and Neurotoxicity

αSyn is expressed predominantly in the central nervous system and localizes to presynaptic terminals. The expression level of αSyn is up to 1% of the total protein in certain regions of the brain [30]. Within neuronal cells, αSyn has been detected in both the cytoplasm and nucleus [31]. Studies utilizing fractionated rat brains revealed that about 15% of αSyn is membrane-bound [32], and the protein was recently found associated with mitochondrial membranes in normal dopaminergic neurons [33]. The relative subcellular distribution of αSyn varies among different neuronal cell populations [34].

The exact function of αSyn has not been established, and the roles of the various physical forms of αSyn in PD pathogenesis are still controversial. αSyn-

knockout mice, are viable and have normal synaptic architecture and brain morphology, suggesting that other proteins may play a redundant role in the cell [35]. On the other hand, the high correlation between αSyn burden and PD has led to the hypothesis that αSyn aggregation produces toxicity through a gain-of-function mechanism. However, αSyn has been implicated to function in a diverse range of essential cellular processes such as the regulation of neurotransmission and response to cellular stress [36]. These findings add on to the complexity of the relationship between αSyn and dopaminergic neurons.

Despite the divergence on the actual biological function of αSyn, there is a consensus that the dysfunction of αSyn results in its toxicity. To be more specific, the misfolding and aggregation of αSyn are vital in the death of SNpc dopaminergic neurons (**figure 2**). Although αSyn normally localizes to the presynaptic terminal, its oligomers and aggregates distribute throughout the cell body and neurites, which suggests that αSyn might disrupt cellular function beyond the presynaptic terminal. Indeed, multiple organelles are implicated in αSyn toxicity, showing that aggregated or soluble misfolded αSyn can be neurotoxic through a variety mechanisms:

Synaptic-vesicle trafficking: αSyn regulates soluble N-ethylmaleimide-sensitive factor（NSF） attachment protein receptor (SNARE) complex assembly by binding the SNARE protein synaptobrevin-2/vesicle-associated membrane protein 2 (VAMP2) to promote synaptic vesicle fusion at the presynaptic terminal [37]. It also potentially regulates additional steps during synaptic vesicle trafficking [38].

Mitochondrial function: Mitochondria are crucial for ATP synthesis,

calcium storage, lipid metabolism and neuronal survival. αSyn toxicity might directly or indirectly disrupt mitochondrial homeostasis [39]. Besides, in αSyn knockout mice, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced degeneration of dopaminergic neurons is prevented [40], probably because αSyn oligomers promote mitochondrial dysfunction via increased calcium uptake [41].

Endoplasmic reticulum and Golgi function, and the endocytic pathway: The endoplasmic reticulum (ER) is essential for protein folding, trafficking to the Golgi, calcium buffering and the unfolded protein response (UPR). Both wild-type and mutant αSyn disrupt ER to Golgi trafficking in yeast [42]. Increased αSyn expression also disrupts endosomal transport events via the E3 ubiquitin ligase in yeast [43]. Moreover, increased αSyn levels also raise cytoplasmic calcium levels, which leads to the activation of a toxic calmodulin–calcineurin cascade [44], suggesting that calcium buffering in the ER might be disrupted by αSyn.

Autophagy or lysosomal pathway: Autophagy is a dynamic pathway involved in the degradation of damaged organelles and protein aggregates [45]. αSyn overexpression disrupts ER to Golgi trafficking of the autophagic transmembrane protein ATG9 and decreases the formation of omegasomes, a precursor for autophagosome biogenesis [46].

Dysregulation of inter-organelle contacts and organelle dynamics: In the past few years, multiple inter-organelle contacts have emerged as sites of cellular homeostatic regulation. Mitochondria-associated ER membrane (MAM), a subdomain of the ER tethered to mitochondria via a group of adaptor proteins, which serves as a

critical site for autophagosome biogenesis, mitochondrial fission, calcium homeostasis, phospholipid transport and fatty acid and cholesterol transfer [47], is significantly increased after αSyn overexpression, resulting in increased mitochondrial calcium uptake from the ER and mitochondrial fragmentation [48]. Moreover, αSyn fibrils were found to impair the axonal transport of autophagosomes and RAB7- and TrkB-receptor-positive endosomes [49]. Further investigation of αSyn in the dynamic organelle transport could provide us some new insights of αSyn's role in its toxicity and neurodegeneration.

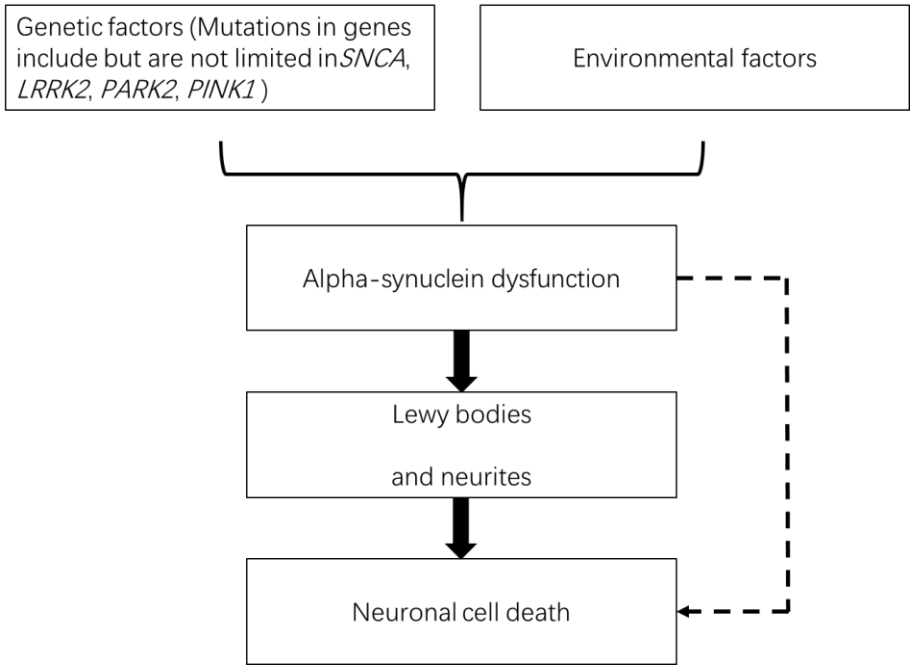


Figure 2. Simplified model of αSyn neurotoxicity

1.3. Ubiquitination and Protein Degradation Pathways in Parkinson's disease

Protein misfolding, aggregation, and deposition are common disease mechanisms shared by many neurodegenerative diseases. In PD, αSyn accumulates to

form Lewy bodies and Lewy neurites. Given that no consistent alternations in αSyn mRNA levels have been found in sporadic PD [50], impaired αSyn degradation is very likely to account for the accumulation of this protein in disease states. In neurons, the two major that participate in the clearance of altered proteins are the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal pathway (ALP). Both pathways are involved in the degradation of αSyn [51].

1.3.1. Ubiquitin-proteasome System Dysfunction

The UPS is the major pathway that mediates the degradation of soluble intracellular proteins in the cytoplasm, nucleus and endoplasmic reticulum (ERAD) [52]. The clearance of proteins by the UPS involves a certain sequence of events: First, ubiquitin (Ub), a small and stable globular protein, is activated in an ATP-dependent reaction by the E1 enzyme, which forms a thioester with the ubiquitin C-terminus, and is then transferred to the active site cysteine of the E2 enzyme to yield an E2~Ub thioester intermediate. The E3 ligase binds to both the E2~Ub thioester and the substrate, catalyzing the transfer of the ubiquitin from the active site cysteine of the E2 to the substrate lysine or N-terminus [53]. This ubiquitination process results in a covalent link between a chain of activated ubiquitin monomers and the lysine residues of the substrate protein. The ubiquitin chain serves as a signal for recognition by the proteasome. Proteasomal degradation yields short peptide fragments that are then subjected to further degradation by peptidases, allowing the recycling of amino acids for the synthesis of new proteins [52].

In some PD models, the inhibition of proteasome causes dose-dependent

neurodegeneration and the formation of Ub- and αSyn-positive inclusions [54-56]. *In-vivo* studies show that toxin-induced proteasome inhibition was found to cause neuronal cell loss in the SN, DA depletion in the striatum and even a disease phenotype. However, the UPS impairment was significantly alleviated in mice lacking αSyn, suggesting that αSyn exacerbates the deleterious effects of MPTP on the UPS [57]. Furthermore, a few studies showed that increased levels of αSyn can lead to impaired proteasome function. This mutual interaction between αSyn and proteasome function suggests that increased αSyn levels impair UPS, which can, in turn, lead to further αSyn accumulation.

1.3.2. Autophagy Dysfunction

The autophagy-lysosomal pathway or autophagy is the general term used to describe pathways that converge into the degradation of intracellular proteins or organelles in lysosomes [52]. The essential component of this proteolytic system are the lysosomes, single membrane vesicles that contain in their lumen the larger variety of cellular hydrolases including proteases, lipases, glycosidases, and nucleotidases. In ALP, the mechanism of target recognition and delivery can be divided into three distinct categories: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [58]. Although autophagy has long been viewed as a random cytoplasmic degradation system, with the identification of autophagic-ubiquitin receptors, the roles of Ub and Ub-binding proteins in selective autophagy are emerging.

In PD patients, the accumulation of autophagic vacuoles was found in the SN. This accumulation is consistent with either an overproduction (as would be the case following primary UPS failure or as a secondary phenomenon to neurodegenerative

changes) or impaired turnover of autophagic vacuoles (as would be the case with primary ALP dysfunction or secondary dysfunction due to neurodegenerative changes) [59]. Besides, an increase of the autophagosome marker LC3-II and a decrease of the lysosomal marker LAMP-1 were identified in both tissues of PD and DLB patients, suggesting the presence of abundant and dysfunctional autophagosomes and lysosomes in synucleinopathies (reviewed in [51]).

1.3.3. Degradation of Alpha-synuclein

Given the fact that αSyn aggregation plays a central role in the formation of LBs and PD pathogenesis, therapeutic strategies that aiming at the degradation of αSyn draws growing attention. However, the precise mechanisms of αSyn clearance in cells are not well established. Current studies suggest that both the UPS and the ALP are involved in the degradation of human wild-type αSyn. In addition, mutant A53T and A30P αSyn, as well as Serine129 phosphorylated αSyn were found to be degraded by both the UPS and macroautophagy (reviewed in [51]).

1.4. 14-3-3 Proteins, DA Synthesis and Neurodegeneration

14-3-3 proteins form a family of highly conserved, acidic, dimeric proteins with a subunit mass of approximately 28 kDa. This class of proteins has been found in all eukaryotic organisms studied so far [60]. The highest tissue concentration of 14-3-3 proteins is found in the brain (up to 1% of total soluble protein) and being present in the cytoplasmic compartment, plasma membrane and in intracellular organelles [61]. It is acknowledged that 14-3-3 proteins interact with various binding partners, including protein kinases, receptor proteins, enzymes, structural and cytoskeletal proteins,

proteins involved in cell cycle, transcriptional control and apoptosis [60]. In many but not all cases, 14-3-3 proteins bind to the phosphorylated forms of these proteins. The function of 14-3-3 proteins in these cellular processes is not entirely clear yet. However, more and more evidence suggests their role in enzyme regulation, control of protein subcellular localization and protein-protein interactions [60, 62]. Interestingly, immunohistochemical investigation of Lewy bodies showed intense positive 14-3-3 staining in PD patients. Here the involvement of 14-3-3 proteins in DA synthesis and neurodegeneration will be discussed.

The rate-limiting enzyme in DA synthesis is tyrosine hydroxylase (TH) [63]. Upon TH activation by phosphorylation, 14-3-3 proteins bind to TH and inhibit its dephosphorylation, leading to prolonged stimulation of TH [64, 65].

αSyn was also shown to bind to 14-3-3 proteins [66]. Normally, αSyn binds to dephosphorylated TH and reduce its activity, whereas the binding of 14-3-3 proteins to phosphorylated tyrosine hydroxylase enhances DA production [61]. In the case of a reduction of αSyn due to sequestration of the protein, 14-3-3 protein-induced DA production could overwhelm the cell with cytosolic DA, with the subsequent generation of quinones and ROS [67], which may, in turn, lead to the misfolding and aggregation of αSyn.

1.5. Methionine Sulfoxide Reductase A and αSyn degradation

In proteins, except cysteine, methionine (Met) is also a sulfur amino acid (Met), which is converted to methionine sulfoxide (MetO) following exposure to reactive oxygen species (ROS), in which their production is increased during oxidative

stress conditions. Methionine sulfoxide reductase (Msr) catalyzes the reduction of MetO in proteins to Met and it is proved to play a critical role in the antioxidant response. Two subtypes of Msr exist in nature, MsrA that is specific for *S*-MetO, and MsrB that is specific for *R*-MetO [68]. There is one type of MsrA and three types of MsrB and MsrA is a positive regulator of MsrB expression [69].

Oxidative stress mediated protein misfolding and aggregation is thought to play a major role in the pathogenesis of PD. It has been previously reported that the overexpression of αSyn could lead to a down-regulation of αSyn degradation in an *ex-vivo* system, in which αSyn forms are expressed in *msrA* null yeast cells [70]. In addition, oxidation of methionine/s of αSyn was shown to inhibit its phosphorylation, *in vitro,* which is required for αSyn UPS-dependent degradation [70]. Consistent with these findings, recent studies demonstrated that MsrA suppresses dopaminergic cell death and protein aggregation induced by the complex I inhibitor rotenone or mutant αSyn [71]. Moreover, in a *Drosophila* model, MsrA was shown to inhibit the development of locomotor and circadian rhythm defects caused by ectopic expression of human αSyn [72].

Interestingly, accumulative evidence from bacterial organism suggests that MsrA is involved in the regulation of Ub-dependent protein degradation (unpublished data). In addition, earlier studies in our lab revealed an elevated 14-3-3 level in a *MsrA* knockout mouse model [73]. Through the incorporation of every component discussed earlier (αSyn, ubiquitination, 14-3-3 proteins, MsrA), a hypothesis of MsrA and Ub-dependent degradation of αSyn is proposed in the next section.

1.6. Statement of Hypothesis

Hypothesis: Methionine sulfoxide reductase A plays an important role in the regulation of αSyn degradation through the ubiquitin-dependent clearance of 14-3-3 proteins.

CHAPTER 2: MATERIALS AND METHODS

2.1. MsrA-dependent αSyn mass modification

Three types of yeast strains (*Saccharomyces cerevisiae*, wild-type (WT), *msrA* null (KO) and *msrA* overexpression (OP)) were made and grown in synthetic minimal media as previously reported [74]. Mid-logarithmic cells were taken from the culture and resuspended in 1X PBS with yeast protease inhibitors cocktail (Cat# P8215-1ML, Sigma) and MG132 proteasome inhibitor (Cat# BML-P1102-0005, Enzo), then extracted by glass beads and a bead-beater homogenizer apparatus (BioSpec Products, Bartlesville, OK, USA). Bradford protein assay was performed to measure total protein concentration of the three yeast extracts. The optical density was read at 595 nm with an iMark microplate reader (BIO-RAD, USA). Equal amounts of extracted proteins of each yeast type were incubated for a period of 30 minutes at 37°C in the presence of one of the three synucleins (native αSyn (Cat# CF66, Novoprotein), A53T mutant of αSyn, or A30P mutant of αSyn), human ubiquitin (U5507-1MG, Sigma), 10mM ATP and $MgCl_2$ (In this study, ATP and $MgCl_2$ are always used as a combination). At the end of the incubation, the extracts were diluted with 5X SDS-PAGE sample buffer and subjected to Western blot analysis using αSyn polyclonal antibody (PA5-17239, Invitrogen). All the immunoblot assays in this study are conducted using following supplies: Novex WedgeWell 4-20% Tris-Glycine Gel (Cat# XP04200BOX, Invitrogen), BupH Tris-Glycine Buffer Pack (Cat# 28380, Thermo), other necessary buffer are made following lab protocols. Control experiments were performed with the same procedure in the absence of Ub and/or ATP to show dependency on these components for this

process.

2.2. Degradation of αSyn in an *ex-vivo* system of yeast

Yeast extracts of either *msrA* null (KO) or *msrA* overexpression (OP) were collected as described in 2.1 and incubated with two synuclein types: native (N) or A53T mutant (A53T) for a period of 30 minutes at 37°C, in the presence or absence of ubiquitin (Ub, which contained also ATP and $MgCl_2$). At the end of the incubation, the extracts were diluted with 5X SDS-PAGE sample buffer and subjected to Western blot analysis using αSyn polyclonal antibody (PA5-17239, Invitrogen).

2.3. Competition on Parkin auto-ubiquitination by MsrA

Recombinant proteins were added to a reaction mixture as follows: 5μM of yeast His-tag MsrA (previously expressed and purified in the lab), 10μM ubiquitin (U5507-1MG, Sigma), 0.9mg/mL Parkin, 22nM E1, 1.4μM E2 (Parkin and corresponding E1, E2 enzymes were gifts from Dr. Richter from the Department of Molecular Bioscience at KU), and 1μg native αSyn (Cat# CF66, Novoprotein). The reaction mixture buffer contained 50mM Tris-HCl (pH 8.0), 1mM DTT, 4mM ATP, 120mM $NaCl_2$, and 5mM $MgCl_2$. The reaction mixtures were incubated for a period of 30 min at 37°C. At the end of the incubation, the extracts were diluted with 5X SDS-PAGE sample buffer and subjected to Western blot analysis using ubiquitin monoclonal antibody (Cat# BML-PW0930-0100, Enzo).

2.4. Detection and identification of ubiquitinated proteins in wild-type and *MsrA* knockout mouse brains

Wild-type (W) and *MsrA* knockout (M) mouse whole brain extracts were

made in PBS in the presence of protease inhibitors cocktail (Cat# P8215-1ML, Sigma) and MG132 proteasome inhibitor (Cat# BML-P1102-0005, Enzo). Bradford protein assay was performed to measure total protein concentration of the three yeast extracts. The optical density was read at 595 nm with an iMark microplate reader (BIO-RAD, USA). Equal amounts of protein extracts were incubated with recombinant His-tagged human ubiquitin (U5507-1MG, Sigma) with 10mM ATP and $MgCl_2$ for or a period of 30 min at 37°C. A control experiment was performed with the same procedure but without adding ATP or $MgCl_2$. At the end of the incubation, the reaction mixture was added to 50µl of pre-washed His-Tag Purification Nickel Resin (Cat# 05893682001, Roche) for a 1 hour incubation at room temperature. Next, the binding mixture was centrifuged at 500rpm for 3 min at 4°C. The pellet was washed with 1X PBS for 5 times and eluted with elution buffer containing 100mm imidazole, 0.1M EDTA, and 5X SDS-PAGE sample buffer. The eluate was subjected to Western blot analysis using ubiquitin monoclonal antibody (Cat# BML-PW0930-0100, Enzo). Another gel (with same samples loaded) were stained using coomassie blue for 15 min and washed extensively with distaining solution (40% methanol and 10% glacial acetic acid). The band that corresponded to a 36kDa ubiquitinated band in Western blot analysis was carefully separated. In-gel tryptic digestion followed by mass-spectrometry analysis were performed on resulting peptides for the identification and sequence determination of the target protein band (these analyses were performed according to established protocols by the KUMC mass-spectrometry core lab).

2.5. Interactions between ubiquitin, αSyn, 14-3-3 proteins in wild-type and

MsrA knockout mouse brains

Immunoprecipitation was conducted using 500μg protein of whole brain extract from wild-type (W) or *MsrA* knockout (M) mouse brain, prepared as described above. The brain extracts were incubated with recombinant human ubiquitin, A53T αSyn, 1X PBS, 10mM ATP and $MgCl_2$ for 20 min. At the end of the incubation, the reaction was stopped on ice. Next, 10μl of anti-14-3-3 antibody (Cat# NB100-2140, NOVUS) was added to the reaction mixture and incubated at room temperature for 1 hour. Then the sample was added to 20μl of pre-washed protein G sepharose (Cat# 28-9440-08, GE Healthcare) and rotated at 4°C for overnight. The protein G beads-immune complex was centrifuged at 500rpm for 3 minutes at 4°C. The pellet was washed with 1ml ice-cold 1X PBS 5 times. After washing, the proteins were eluted from the immune complexes 5X SDS-PAGE sample buffer with β-mercaptoethanol. The eluate was loaded onto SDS-PAGE gels for Western blot analysis using anti-αSyn antibody (Cat# 610786, BD Transduction Laboratories) and anti-ubiquitin antibody (Cat# BML-PW0930-0100, Enzo). Immunoprecipitation was also conducted following the same procedure while using instead an anti-ubiquitin antibody (Cat# BML-PW0930-0100, Enzo) and detected with an αSyn polyclonal antibody (PA5-17239, Invitrogen) or anti-14-3-3 antibody (Cat# NB100-2140, NOVUS).

2.6. Expression and purification of A53T αSyn in yeast and its effect on the organism's protein ubiquitination

The mutant A53T αSyn were expressed in wild-type (WT) and *msrA* null (KO) *Saccharomyces cerevisiae* strains with the pYES6 expression vector used previously

[70]. This vector harbors the yeast GAL1 promoter for high-level inducible protein expression by galactose. It has a C-terminal peptide encoding a V5 epitope and a polyhistidine (6His) tag for detection and purification of the recombinant fusion αSyn. This vector contains the ampicillin and blasticidin resistance genes for selection of bacteria and yeast colonies harboring the plasmid, respectively. The yeast cells were grown in synthetic complete medium without leucine for 36 hours and then transferred to induction media containing 2% galactose and 1% raffinose for a two-hour induction. Next, cell extracts were made as described in section 2.1.

The expressed His-αSyn protein in each yeast strain was purified by nickel resin chromatography. Soluble protein extract of each packed yeast cells was incubated with 50µl of pre-washed His-Tag Purification Nickel Resin (Cat# 05893682001, Roche) for 1 hour at room temperature. Then, the resin was washed several times with PBS for and the His-A53TαSyn was eluted with 50µl elution buffer containing: 100mm imidazole, 0.1M EDTA, and 5X SDS-PAGE sample buffer. The eluate was subject to Western blot analysis with anti-αSyn antibody (Cat# 610786, BD Transduction Laboratories) and anti-ubiquitin antibody (Cat# BML-PW0930-0100, Enzo). In addition, protein extracts of both yeast strains expressing His-A53T αSyn were also subjected to western blot analyses using the same antibodies.

CHAPTER 3: RESULTS

3.1. MsrA-dependent αSyn mass modification

To investigate the effects of MsrA on native αSyn and its point mutation variants (A30P, A53T), an *ex-vivo* system was designed in which the extracts of three types of yeast strains (wild-type (WT), *msrA* null (KO), and *msrA* overexpression (OP)) were incubated for a period of 30 minutes at 37°C in the presence of one of the synucleins, human ubiquitin, 10mM ATP and 10mM $MgCl_2$. The mass modification of αSyn was detected by Western blot analysis (**Figure 1**). Since the UPS is inhibited in this system by the protease and proteasome inhibitors, this figure shows the mass-accumulation pattern of the added synucleins. The *msrA* OP strain shows a high level of aggregation in all types of synuleins. However, only little aggregation is presented in *msrA* KO strain (**Figure 1A**), indicating that MsrA may play a role in the mass modification of αSyn. There is no significant difference between WT cells and *msrA* KO cells in the aggregation pattern of native αSyn or A30P αSyn (**Figure 1A**), presumably due to (i) low expression level and activity of MsrA in WT yeast cells [74] and (ii) native and A30P αSyn have a lower capacity of forming aggregates [42]. The relatively high MsrA level in *msrA* OP yeast cells may promote the aggregation of A30P αSyn either by enhancing its oligomerization due to ubiquitination or by strengthening its interaction with other cellular protein components. To evaluate the role of ubiquitination in the mass modification of αSyn, comparative experiments were done following similar idea but without adding ATP or Ub. *msrA* KO and *msrA* OP cells were used in these experiments because they share the same genomic background [74].

Strong aggregation is observed only in *msrA* OP strain incubated with both ATP and Ub (**Figure 1B, 1C**), suggesting that both ATP and ubiquitination are involved in the mass modification of αSyn.

3.2. Degradation of αSyn in an *ex-vivo* system of yeast

Given the fact that both UPS and autophagy pathways contribute to the degradation of soluble αSyn monomers and ubiquitination events may be directly or indirectly involved in αSyn clearance, we further investigated the effect of ubiquitin on αSyn degradation with an *ex-vivo* system. Western blot analysis reveals that ubiquitin promotes the clearance of both types of αSyn (native and A53T) monomers in *msrA* OP strain, while *msrA* KO strain only shows slightly enhancement of A53T dimer degradation (**Figure 2**). These findings are consistent with the previous report that MsrA suppresses protein aggregation induced by mutant αSyn in dopaminergic neuron cells [71], suggesting that (i) both ubiquitin and MsrA are involved in αSyn degradation, (ii) multiple degradation pathways are involved in the clearance of the different forms of αSyn. It is noteworthy that, compared with *msrA* KO extract, an enhanced αSyn dimer clearance was observed for both native and A53T αSyns in the presence of *msrA* OP extract without the addition of Ub and ATP (**Figure 2**). However, addition of Ub and ATP only increased the degradation of the A53T αSyn dimer in extracts of both yeast strains (**Figure 2**). This observation may suggest that the dimers of native and A53T αSyn are differently recognized by proteolytic pathways that may possess also different dependency on MsrA, Ub or both (**Figure 2**). Interestingly, in the *msrA* OP strain, decreased native αSyn dimer degradation was observed when external ubiquitin

was added to the reaction mixture. This phenomenon further supports the idea that the degradation pathways of αSyn monomers and dimers are not the same. A possible overall explanation for these data is that MsrA has dual function active site, as follows : in the absence of external ubiquitin, MsrA functions mainly as a reductase, which may promote the degradation of αSyn dimers; while when the system is supplied with ample ubiquitin and ATP, the active site of MsrA is mainly occupied with ubiquitin (instead of MetO) and this might contribute to the enhanced degradation of αSyn monomers (The MetO reduction and Ub binding of *Archaea* MsrA is carried out by the same active site, unpublished data).

3.3. Competition on Parkin auto-ubiquitination by MsrA

The data presented above suggest that both ubiquitin and MsrA are involved in the degradation pathways of αSyn. In order to determine if these two factors directly interact with each other, an *in-vitro* system was created in presence of the parkin protein. Parkin has an E3 ubiquitin ligase activity and is believed to directly or indirectly mediate proteasomal degradation of aggregation-prone proteins, including αSyn. Moreover, mutations in the *Parkin* gene are the most common cause of hereditary parkinsonism [75]. In agreement with previous finding [75], Parkin showed a strong tendency of auto-ubiquitination. Surprisingly, this auto-ubiquitination disappeared when MsrA is introduced to the system (**Figure 3**). This result suggests that MsrA inhibits the binding between Parkin and Ub, possibly through competing for Ub binding (auto-ubiquitination of MsrA was not observed (data not shown) in this *in-vitro* system). It is yet to be determined what are the other factors that are needed for stabilizing the

complex between MsrA and Ub. Overall, based on the data presented under sections 3.1 and 3.2, it is suggested that MsrA has a direct interaction with ubiquitin and may function as an E3 Ub ligase.

3.4. Detection and identification of ubiquitinated proteins in wild-type and *MsrA* knockout mouse brains

The potential relationships between ubiquitination and αSyn degradation sought us to examine if similar interactions exist also in mouse brain. Wild-type (W) and *MsrA* knockout (M) mouse whole brain extracts were made in the presence of proteasome and protease inhibitors. After incubation with His-Ub and ATP, the reaction mixture was added to His-tag purification nickel resin to pull-down ubiquitinated proteins. The ubiquitinated proteins were then detected following Western blot analysis. A unique ubiquitinated band of ~36kDa was detected mainly in the wild-type mouse brain, in which ATP was added to the reaction mixture (**Figure 4**), indicating that the presence of MsrA and ATP are important for the formation of this Ub-bound protein. Consequently, the corresponding band was subjected to mass spectrometry for identification after in-gel tryptic digestion. Surprisingly, the sequence determination suggests that this 36kDa ubiquitinated protein comes from the 14-3-3 protein family (**Figure 5**). Moreover, glycine residues were found on the lysine sites of the digested peptides, indicating that 14-3-3 was ubiquitinated and these lysine residues are indeed ubiquitin binding sites.

3.5. Interactions between ubiquitin, αSyn, 14-3-3 proteins in wild-type and *MsrA* knockout mouse brains

14-3-3 proteins are known binding partners of αSyn. Hence, we hoped to further investigate the role of MsrA in the ability of 14-3-3 to affect the ubiquitination and degradation of αSyn. Immunoprecipitation experiments followed by Western blot analyses were performed using brain extracts to identify the interaction among αSyn, Ub, and 14-3-3 proteins. *MsrA* knockout brain (M) showed lower levels of ubiquitinated and degraded band products of both αSyn and 14-3-3 proteins compared with wild-type brain (W) (**Figure 6A**). These results suggest that both αSyn and 14-3-3 proteins are ubiquitinated and degraded more efficiently in the presence of MsrA. Immunoprecipitation with anti-14-3-3 antibody confirmed that 14-3-3 is indeed more ubiquitinated and Ub-dependent degraded (as reflected by the presence of mid to low size molecular mass bands) in the wild-type strain. Furthermore, the fact that αSyn and ubiquitinated protein separation pattern in the wild-type strain looked very similar (following immunoprecipitation with 14-3-3 antibody) suggests that ubiquitination and/or associated degradation of αSyn are both MsrA and 14-3-3 depended. (**Figure 6B**). Next, to show the dependence on ATP in this process, a comparative experiment ± ATP was performed followed by 14-3-3 immunoprecipitation. The wild-type brain showed an enhanced αSyn degradation and ubiquitination in the presence of ATP. However, no change was observed in the levels of the monomer and the dimer forms of αSyn and no mid-to-low molecular mass ubiquitinated proteins were present in the *MsrA* knockout brain. This phenomenon suggests again that MsrA is important for the ubiquitination-dependent degradation of αSyn. An enhanced non-ubiquitinated αSyn band showed up when external ATP was added to the *MsrA* knockout mouse brain

extract, maybe due to an increased stability of αSyn phosphorylation caused by a decreased clearance of 14-3-3 proteins (**Figure 6C**). This correlates with a previous suggestion that 14-3-3 protect the phosphorylated αSyn from being dephosphorylated at the presence of high DA levels (like in the case of *MsrA* KO brain) [70, 73]. The expression levels of αSyn and 14-3-3 proteins were similar **(Figure 6D**), eliminating the possibility that lower expression of either of these proteins may account for their absence in the *MsrA* knockout brain.

3.6. Expression and purification of A53T αSyn in yeast and its effect on the organism's protein ubiquitination

To confirm the effect of MsrA on αSyn ubiquitination, we expressed the mutant A53T αSyn in wild-type (WT) and *msrA* null (KO) yeast strains. Nickel resin chromatography was conducted to pull down the His-tagged αSyn. Similar expression levels were found in both strains, and the quantity of the pulldown product was also the same in the two strains (**Figure 7A**). *msrA* null mutant strain showed significantly lower total ubiquitination level and αSyn ubiquitination level (**Figure 7B**) compared with wild-type expressing A53T αSyn cells. These results provide further evidence for the hypothesis that MsrA plays an important role in the ubiquitination of αSyn *in-vivo* (especially in yeast when one of the major αSyn E3 ligases, Parkin, is absent in yeast)

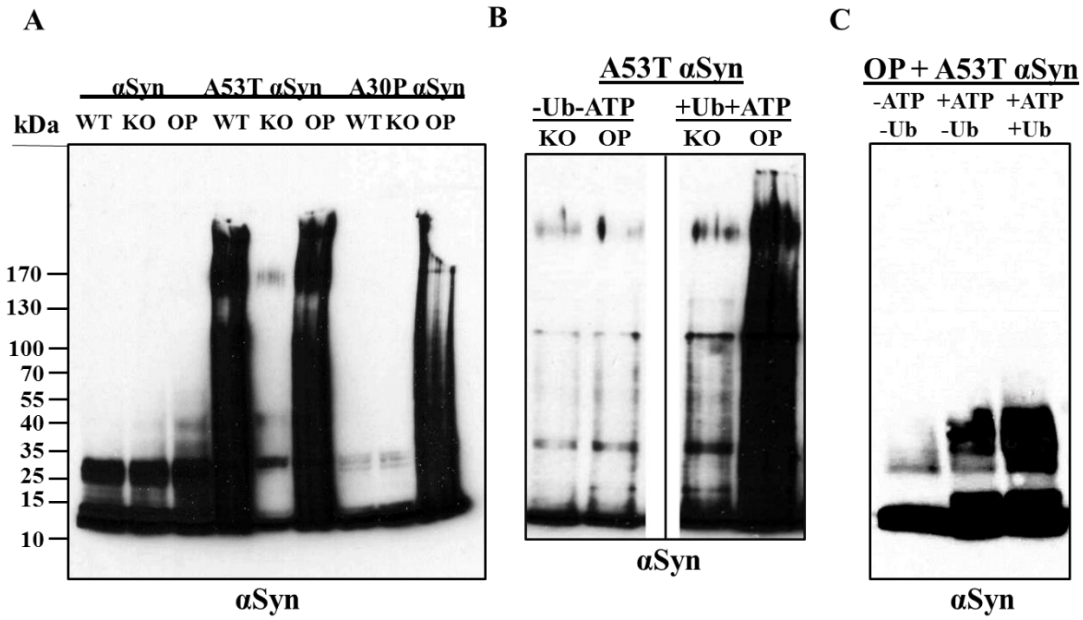


Figure 1. MsrA-dependent αSyn molecular mass modification by yeast extract. Three types of yeast strains, wild-type (WT), *msrA* null (KO), and *msrA* overexpression (OP) were made and grown in synthetic minimal media. Mid-logarithmic cells were harvested and extracted using PBS, yeast protease inhibitors cocktail, and MG132 proteasome inhibitor. **(A)** Equal amounts of extracted proteins of each yeast type were incubated for a period of 30 minutes at 37°C in the presence of either native αSyn, A53T mutant of αSyn, or A30P mutant of αSyn, ubiquitin (Ub), ATP and MgCl2. At the end of the incubation, the extracts were subjected to western blot analysis using anti α-syn antibody. **(B)** Showing the dependency of the external additions of both ATP and Ub on the enhancement of A53T α-syn molecular mass increase (as described in panel A) for the KO and OP extracts. Similar results were obtained for the native and A30P αSyn (data not shown). **(C)** Showing the net effect of ATP (that contains MgCl2) and ATP plus external Ub additions on the enhancement of A53T α-syn molecular mass increase (as described in panel A) for the OP extract only. Similar results were obtained

for the native and A30P αSyn (data not shown). The molecular mass markers fit with all three figures.

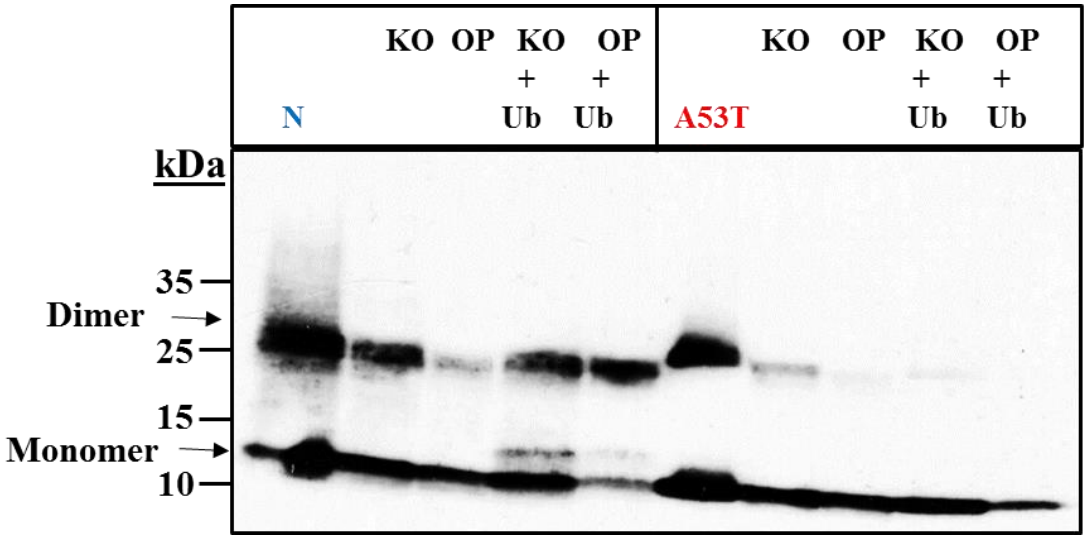


Figure 2. Degradation of α-synuclein in an ex-vivo system of yeast. The αSyn types: native (N) or A53T mutant (A53T), were incubated with yeast extracts of either *msrA* null (KO) or *msrA* overexpression (OP) for a period of 30 minutes at 37°C, in the presence or absence of ubiquitin (Ub, which contained also ATP and $MgCl_2$). At the end of the incubation, the extracts were subjected to western blot analysis using anti-αSyn antibody. kDa, molecular mass markers.

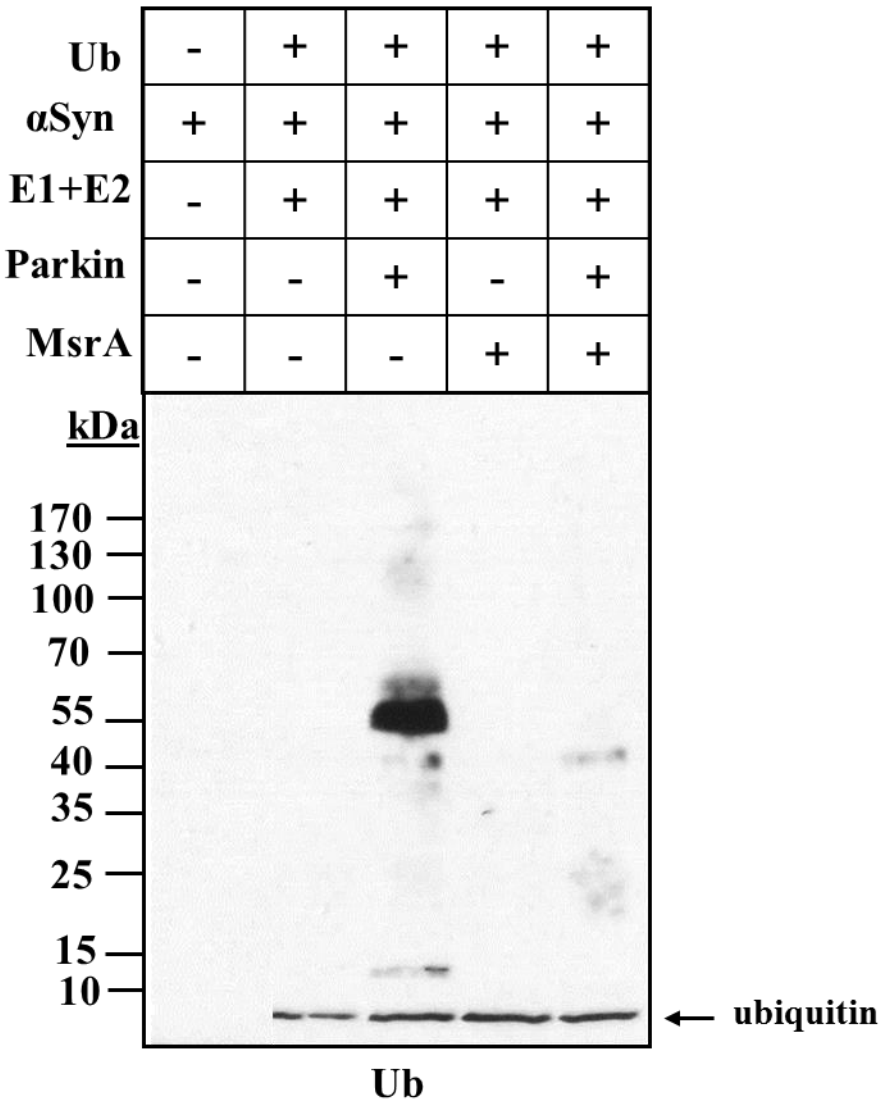


Figure 3. Competition on Parkin auto-ubiquitination by MsrA. Recombinant proteins were added to a reaction mixture as shown in the figure (details are described in Chapter 2). The reaction mixtures were incubated for a period of 30 min at 37°C. The reactions were stopped by adding SDS-gel-electrophoresis sample buffer, followed by western blot analysis using anti-Ub antibody. kDa, molecular mass markers.

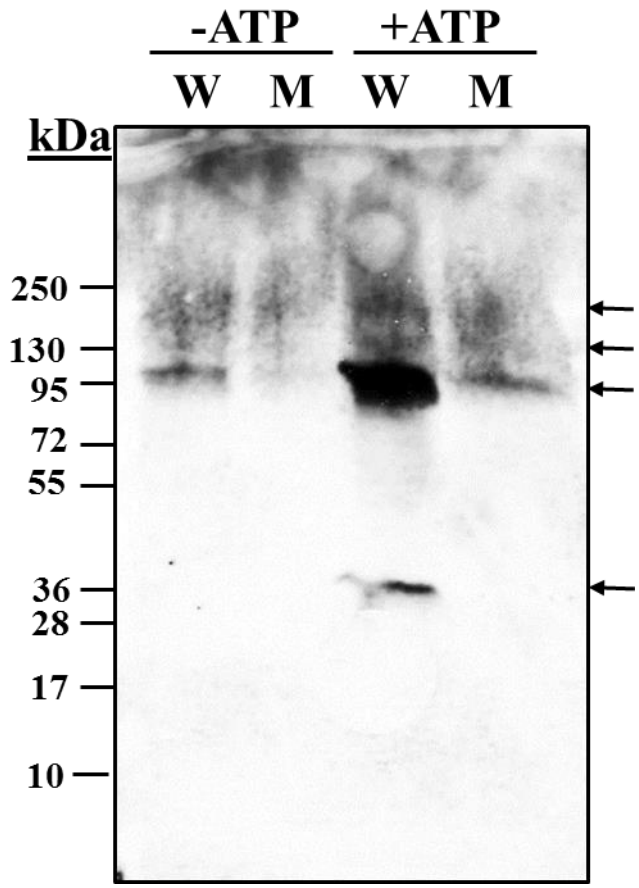


Figure 4. Detection of ubiquitinated proteins in extracts of wild-type (W) and *MsrA* knockout (M) mouse brains. Brain extracts were made in PBS in the presence of protease and proteasome inhibitors. Equal amounts of protein extracts were incubated with recombinant His-tagged ubiquitin (Ub) ± ATP and $MgCl_2$ for or a period of 30 min at 37°C. The reactions were stopped by adding SDS-gel-electrophoresis sample buffer, followed by western blot analysis using anti-Ub antibody. kDa, molecular mass markers. Arrows indicate the position of major ubiquitinated proteins.

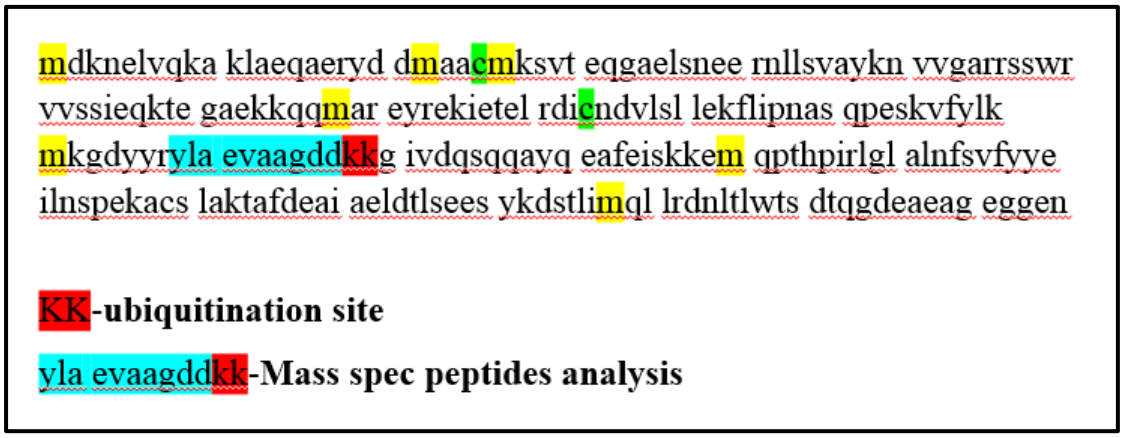
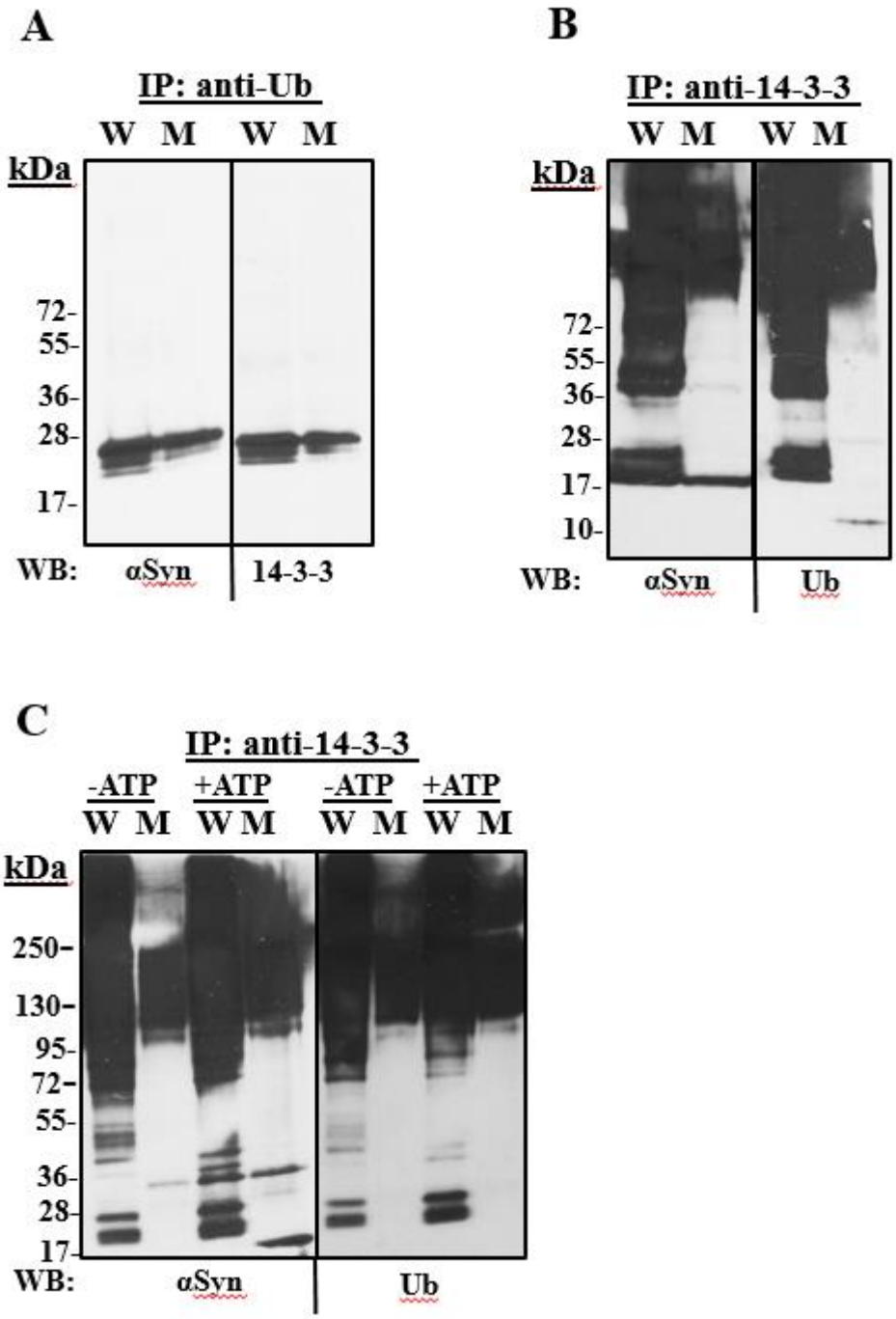


Figure 5. Identification of a protein that its ubiquitination is enhanced by MsrA.

The 36kDa ubiquitinated protein (~28kDa; + 1 Ub molecule = 36kDa) identity with descending priority is: 1) 14-3-3 zeta, also known as Ywhaz 2) 14-3-3 gamma 3) 14-3-3 theta 4) 14-3-3 eta.


A
IP: anti-Ub
W M W M
kDa
72-
55-
36-
28-
17-
WB: αSyn 14-3-3
B
IP: anti-14-3-3
W M W M
kDa
72-
55-
36-
28-
17-
10-
WB: αSyn Ub
C
IP: anti-14-3-3
-ATP +ATP -ATP +ATP
W M W M W M W M
kDa
250-
130-
95-
72-
55-
36-
28-
17-
WB: αSyn Ub

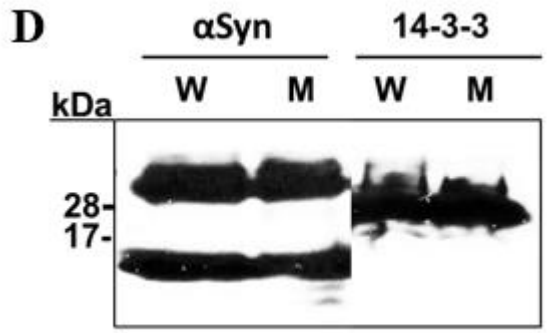


Figure 6. Interactions between Ub, αSyn, and 14-3-3 proteins in extracts of wild-type (W) and *MsrA* knockout (M) mouse brains. Immunoprecipitation (IP) experiments **(A-C)** were performed on brain extracts, followed by Western blot (WB) using the indicated protein antibodies, as described in Section 2. kDa, molecular mass markers. **(D)** Relatively high expression levels αSyn and 14-3-3 proteins in extracts of W and M mouse brains.

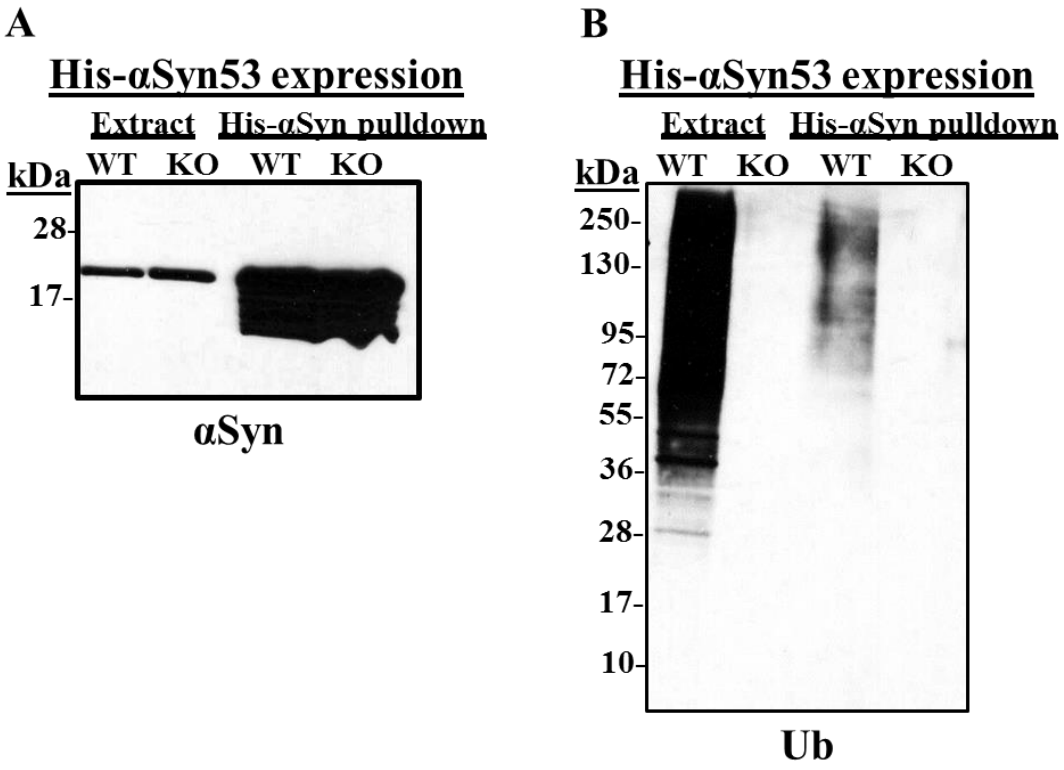


Figure 7. Expression and purification A53TSyn in yeast and its effect on protein ubiquitination of wild-type (WT) and *msrA* null (KO) yeast extracts. Yeast strains were grown and extracts were made according to the procedures described in Section 2. **(A)** Expression levels of αSyn in wild-type (WT) and *MsrA* knockout (KO) strains, as judged by western blot analysis using anti-αSyn antibody (note that the molecular mass of αSyn is higher than expected due to the presence of His-tag at the C-terminal region of the protein). **(B)** His-ubiquitin pulldown, purifying the proteins harboring His-Ub of the two yeast strains by nickel resin chromatography. kDa, molecular mass markers.

CHAPTER 4: DISCUSSION

Twenty years ago, αSyn was found to be the main component of Lewy bodies, the pathological hallmark of PD [11]. From then on, numerous efforts were made on the identification of αSyn's role in PD pathogenesis. However, the precise mechanisms of αSyn aggregation and degradation are still unclear. Recent studies revealed that both UPS and autophagy pathways are involved in the clearance of αSyn, and that the binding between 14-3-3 proteins and αSyn is contributed to the aggregation of αSyn [67]. Previously, our lab revealed an enhanced DA level and reduced αSyn clearance in *MsrA* knockout mouse brain and clearance of αSyn was also inhibited in *msrA* null mutant yeast cells expressing αSyn [70, 73]. The observations described above suggest potential interactions among αSyn, ubiquitin system, 14-3-3 proteins, and MsrA. Thus, our current study is focused on the molecular mechanism underlying the decreased αSyn clearance in *MsrA* knockout eukaryote models and the effect of MsrA on the interactions among αSyn, Ub system, and 14-3-3 proteins. Our studies have demonstrated for the first time that (1) MsrA is involved in the mass modification of αSyn in a Ub- and ATP-dependent manner (**Figure 1**). (2) MsrA is important for Ub-dependent clearance of both native αSyn and A53T mutant αSyn monomers (**Figure 2**). (3) MsrA plays an important role in the ubiquitination of αSyn and it inhibits Parkin's autoubiquitination (a known E3 ubiquitin ligase of αSyn), suggesting that it may function as an E3 ubiquitin ligase in the Ub-dependent degradation of αSyn (**Figure 3**). (4) Ubiquitinated 14-3-3 proteins are detected with dependency on the presence of MsrA and ATP (**Figure 4, 5**). (5) Both αSyn and 14-3-3 proteins are ubiquitinated and

degraded more efficiently in the presence of MsrA (**Figure 6**). (6) The ubiquitination and/or associated degradation of αSyn are both MsrA and 14-3-3 depended (**Figure 6**).

Yeast and humans share many key cellular pathways, such as membrane trafficking, protein aggregation, mitochondrial dysfunction, oxidative stress, transcriptional deregulation, and regulated protein turnover, allowing performing studies on the cellular potential to degrade protein aggregates. The budding yeast *Saccharomyces cerevisiae* is an established model to study αSyn aggregation and toxicity [76-78]. Various post-translational modifications were related to the aggregation, distribution, and degradation of αSyn, and several of these modification sites are conserved from yeast to human [79]. Thus, in our initial study, we used this yeast model to investigate the effect of MsrA on αSyn structural changes. The *msrA* OP strain shows a higher level of Ub- and ATP-dependent αSyn aggregation in all types of synucleins compared with *msrA* KO strain (**Figure 1**). However, there is much less difference between WT cells and *msrA* KO cells in the aggregation pattern of native αSyn or A30P αSyn, presumably due to relatively low MsrA activity in WT cells and a lower capability for native or A30P αSyn to form aggregates. Based on these considerations and the fact that *msrA* KO strain has an identical genomic background as *msrA* OP strain (which differs only by containing a MsrA overexpressing plasmid), we used these two strains to further investigate the effect of MsrA on αSyn, 14-3-3 and ubiquitination. Our results showed that ubiquitin promotes the clearance of both types of αSyn (native and A53T) monomers in *msrA* OP strain but not in *msrA* KO strain. Interestingly, in the presence of ubiquitin, *msrA* OP strain also showed reduced

degradation of the native αSyn dimer (**Figure 2**), suggesting that the degradation pathways are not always the same for the different formations of αSyn. It also indicates that MsrA may play different roles in multiple systems. The observation above and some accumulated evidence in *Archaea* (unpublished data) showed an inspiring possibility that MsrA may be involved in ubiquitination-associated degradation. Indeed, we found that MsrA inhibits the auto-ubiquitination of Parkin (**Figure 3**), possibly due to the competition for Ub binding between MsrA and Parkin. This result suggests a direct interaction between MsrA and Ub, as well as the similarity of function between MsrA and Parkin. The possibility that MsrA functions as an E3 ubiquitin ligase has been suggested to be the case for the Acraea MsrA (unpublished data). Although auto-ubiquitination of MsrA was not observed in this experiment, it is not ruling out the possibility that MsrA cannot function as an E3 ligase since this process is highly dynamic and other undetermined factors are needed for stabilizing the binding between MsrA and Ub (e.g. a suitable E2 type for MsrA).

The observations above clearly suggest that MsrA plays an important role in the Ub-dependent αSyn degradation. However, do 14-3-3 proteins also play a role in this process? To answer this question, we created an *ex-vivo* system using wild-type (W) and *MsrA* knockout (M) mouse brain extracts. Following His-ubiquitinated protein pull-down experiments in conjunction with mass spectrometry analyses, ubiquitinated 14-3-3 proteins were identified only in the presence of W extract and ATP (**Figure 4, 5**). This data fit very well to our anticipation that 14-3-3 proteins are ubiquitinated by MsrA for degradation. The protein 14-3-3 can bind αSyn in the presence of high levels

of DA and protect it from being degraded via the Ub system, through binding to a specific phosphorylated Ser129 of αSyn [67]. Thus, the presence of MsrA enhances αSyn degradation by eliminating this protective effect of 14-3-3 through ubiquitination and degradation. The aggregated forms of αSyn in the presence of MsrA (**Figure 1**) may be indicative of enhanced ubiquitination of αSyn by other ligases that otherwise is inhibited in the absence of MsrA, allowing a sustained protective effect of 14-3-3 against Ub-dependent degradation of αSyn. However, no ubiquitinated αSyn was detected in whole brain extracts of either W or M strain (**Figure 4**). This fact suggests that the condition favoring αSyn ubiquitination and degradation in the brain is affected by other factors besides MsrA, which is more complicated than in yeast. To further investigate the role of MsrA in the ability of 14-3-3 to affect the ubiquitination and degradation of αSyn, immunoprecipitation experiments followed by Western blot analyses were performed using brain extracts to identify the interaction among αSyn, Ub, and 14-3-3 proteins. It is clear that interactions exist among αSyn, Ub, and 14-3-3 proteins and MsrA plays an important role in the regulation of these interactions. (**Figure 6A, B**) Interestingly, an enhanced non-ubiquitinated αSyn band showed up when external ATP was added to the *MsrA* knockout mouse brain extract, probably due to enhanced αSyn phosphorylation that caused by the decreased clearance of 14-3-3 proteins (**Figure 6C**). These data confirm the previous suggestion that 14-3-3 protect the phosphorylated αSyn from being dephosphorylated at the presence of high DA levels (like in the case of *MsrA* KO brain) [70, 73]. At last, a complementary study in yeast showed that the deletion of *msrA* gene led to lower total ubiquitination level and

αSyn ubiquitination level, providing further evidence for the hypothesis that MsrA plays an important role in the ubiquitination of αSyn *in-vivo*.

To combine all the currently known information from previous studies and our observations, a hypothetical model for the effect of MsrA on αSyn function and ubiquitination processes involving 14-3-3 proteins the following schematic summary figure was created (**Figure 8**).This figure suggested events and their consequences could be summarized as follows: Compromised levels of the MsrA protein will lead to an impaired Ub-dependent 14-3-3 clearance, causing an upregulation of cellular 14-3-3 level. This will bring about the enhanced inhibition of αSyn dephosphorylation, resulting in reduced Ub-dependent αSyn degradation. The latter event will eventually lead to αSyn dysfunction and aggregation. On the other hand, the elevated 14-3-3 level will also cause an increase in TH activity, giving rise to upregulated DA synthesis. Overwhelming cellular DA will lead to increased production of quinones and ROS, causing further dysfunction and aggregation of αSyn.

According to this model, some future directions for this study could be: (1) further investigation on whether MsrA functions as an E3 ubiquitin ligase under certain conditions, establish the active site for Ub binding, and screen for its potential E2 enzyme/s. (2) To determine which subtypes of 14-3-3 proteins are involved in the regulation of αSyn degradation. (3) To determine how the UPS and the autophagy pathways are involved in the different formations αSyn (4) Complementary experiments are needed to confirm the relationship between αSyn clearance and 14-3-3 degradation. We think that the current study along with the future research directions

will provide more insights into the Ub-related function of MsrA in regulating αSyn oxidation state and degradation with relation to PD pathogenesis

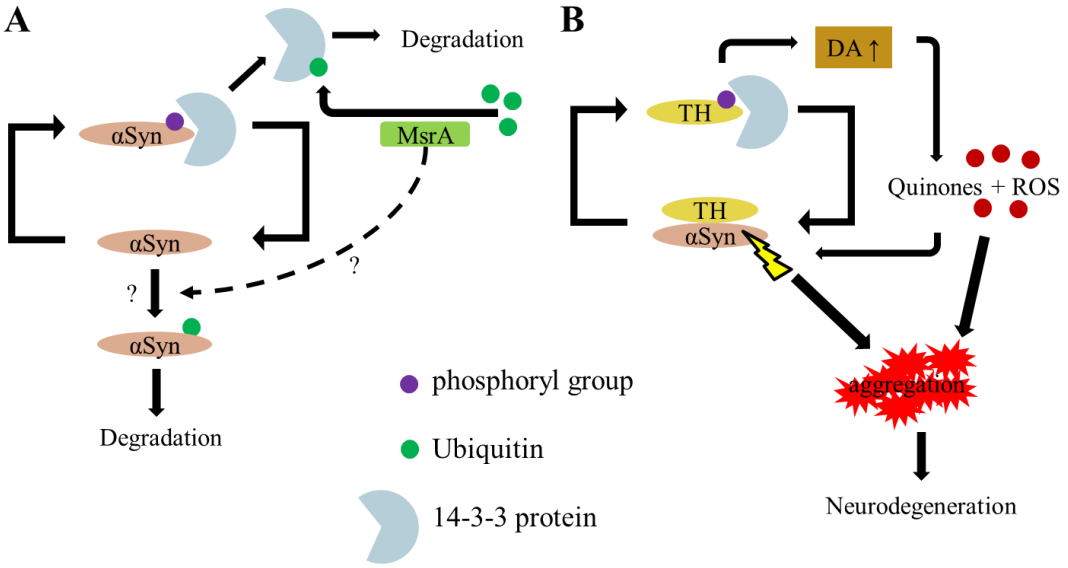


Figure 8. A schematic representation of hypothesized mechanism underlying the regulation of 14-3-3 clearance by MsrA and αSyn related PD pathogenesis. (A) MsrA plays an important role in the regulation of αSyn degradation through the ubiquitin-dependent clearance of 14-3-3 proteins. **(B)** Elevated levels of 14-3-3 will cause an increase in the activity of TH, leading to a synthesis upregulation of DA. Overwhelming cellular DA will lead to an increased production of quinones and ROS, causing further dysfunction and aggregation of αSyn, which will eventually result in the formation of protein aggregation and neurodegeneration.

References

1. Lee, A. and R.M. Gilbert, *Epidemiology of Parkinson Disease.* Neurol Clin, 2016. **34**(4): p. 955-965.
2. Foundation, P.s.D. *Statistics on Parkinson's*. 2016; Available from: http://www.pdf.org/parkinson_statistics.
3. Safarpour, D. and A.W. Willis, *Clinical Epidemiology, Evaluation, and Management of Dementia in Parkinson Disease.* Am J Alzheimers Dis Other Demen, 2016. **31**(7): p. 585-594.
4. Dauer, W. and S. Przedborski, *Parkinson's disease: mechanisms and models.* Neuron, 2003. **39**(6): p. 889-909.
5. Verstraeten, A., J. Theuns, and C. Van Broeckhoven, *Progress in unraveling the genetic etiology of Parkinson disease in a genomic era.* Trends Genet, 2015. **31**(3): p. 140-9.
6. Mhyre, T.R., et al., *Parkinson's disease.* Subcell Biochem, 2012. **65**: p. 389-455.
7. Graham, D.G., *Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones.* Mol Pharmacol, 1978. **14**(4): p. 633-43.
8. Przedborski, S., et al., *The parkinsonian toxin MPTP: action and mechanism.* Restor Neurol Neurosci, 2000. **16**(2): p. 135-142.
9. Segura-Aguilar, J., et al., *Protective and toxic roles of dopamine in Parkinson's disease.* J Neurochem, 2014. **129**(6): p. 898-915.
10. Carlsson, A., M. Lindqvist, and T. Magnusson, *3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists.* Nature, 1957. **180**(4596): p. 1200.
11. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies.* Nature, 1997. **388**(6645): p. 839-40.
12. Wood, S.J., et al., *alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease.* J Biol Chem, 1999. **274**(28): p. 19509-12.
13. Schulz-Schaeffer, W.J., *The synaptic pathology of alpha-synuclein aggregation in dementia with Lewy bodies, Parkinson's disease and Parkinson's disease dementia.* Acta Neuropathol, 2010. **120**(2): p. 131-43.
14. Polymeropoulos, M.H., et al., *Mutation in the alpha-synuclein gene identified in families with Parkinson's disease.* Science, 1997. **276**(5321): p. 2045-7.
15. Lavedan, C., *The synuclein family.* Genome Res, 1998. **8**(9): p. 871-80.
16. Kruger, R., et al., *Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease.* Nat Genet, 1998. **18**(2): p. 106-8.
17. Zarranz, J.J., et al., *The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia.* Ann Neurol, 2004. **55**(2): p. 164-73.
18. Appel-Cresswell, S., et al., *Alpha-synuclein p.H50Q, a novel pathogenic mutation for Parkinson's disease.* Mov Disord, 2013. **28**(6): p. 811-3.
19. Lesage, S., et al., *G51D alpha-synuclein mutation causes a novel parkinsonian-pyramidal syndrome.* Ann Neurol, 2013. **73**(4): p. 459-71.
20. Proukakis, C., et al., *A novel alpha-synuclein missense mutation in Parkinson*

disease. Neurology, 2013. **80**(11): p. 1062-4.
21. Pasanen, P., et al., *Novel alpha-synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology.* Neurobiol Aging, 2014. **35**(9): p. 2180 e1-5.
22. Singleton, A.B., et al., *alpha-Synuclein locus triplication causes Parkinson's disease.* Science, 2003. **302**(5646): p. 841.
23. Chartier-Harlin, M.C., et al., *Alpha-synuclein locus duplication as a cause of familial Parkinson's disease.* Lancet, 2004. **364**(9440): p. 1167-9.
24. Jao, C.C., et al., *Structure of membrane-bound alpha-synuclein studied by site-directed spin labeling.* Proc Natl Acad Sci U S A, 2004. **101**(22): p. 8331-6.
25. Jensen, M.B., et al., *Membrane curvature sensing by amphipathic helices: a single liposome study using alpha-synuclein and annexin B12.* J Biol Chem, 2011. **286**(49): p. 42603-14.
26. Olanow, C.W. and P. Brundin, *Parkinson's disease and alpha synuclein: is Parkinson's disease a prion-like disorder?* Mov Disord, 2013. **28**(1): p. 31-40.
27. Wakabayashi, K., et al., *Synphilin-1 is present in Lewy bodies in Parkinson's disease.* Ann Neurol, 2000. **47**(4): p. 521-3.
28. Schlossmacher, M.G., et al., *Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies.* Am J Pathol, 2002. **160**(5): p. 1655-67.
29. Kawamoto, Y., et al., *14-3-3 proteins in Lewy bodies in Parkinson disease and diffuse Lewy body disease brains.* J Neuropathol Exp Neurol, 2002. **61**(3): p. 245-53.
30. Iwai, A., et al., *The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system.* Neuron, 1995. **14**(2): p. 467-75.
31. Yu, S., et al., *Extensive nuclear localization of alpha-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody.* Neuroscience, 2007. **145**(2): p. 539-55.
32. Lee, H.J., C. Choi, and S.J. Lee, *Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form.* J Biol Chem, 2002. **277**(1): p. 671-8.
33. Li, W.W., et al., *Localization of alpha-synuclein to mitochondria within midbrain of mice.* Neuroreport, 2007. **18**(15): p. 1543-6.
34. Liu, G., et al., *alpha-Synuclein is differentially expressed in mitochondria from different rat brain regions and dose-dependently down-regulates complex I activity.* Neurosci Lett, 2009. **454**(3): p. 187-92.
35. Abeliovich, A., et al., *Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system.* Neuron, 2000. **25**(1): p. 239-52.
36. Benskey, M.J., R.G. Perez, and F.P. Manfredsson, *The contribution of alpha synuclein to neuronal survival and function - Implications for Parkinson's disease.* J Neurochem, 2016. **137**(3): p. 331-59.
37. Burre, J., et al., *Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro.* Science, 2010. **329**(5999): p. 1663-7.
38. Wislet-Gendebien, S., et al., *Cytosolic proteins regulate alpha-synuclein*

dissociation from presynaptic membranes. J Biol Chem, 2006. **281**(43): p. 32148-55.
39. Rocha, E.M., B. De Miranda, and L.H. Sanders, *Alpha-synuclein: Pathology, mitochondrial dysfunction and neuroinflammation in Parkinson's disease.* Neurobiol Dis, 2017.
40. Dauer, W., et al., *Resistance of alpha -synuclein null mice to the parkinsonian neurotoxin MPTP.* Proc Natl Acad Sci U S A, 2002. **99**(22): p. 14524-9.
41. Luth, E.S., et al., *Soluble, prefibrillar alpha-synuclein oligomers promote complex I-dependent, Ca2+-induced mitochondrial dysfunction.* J Biol Chem, 2014. **289**(31): p. 21490-507.
42. Outeiro, T.F. and S. Lindquist, *Yeast cells provide insight into alpha-synuclein biology and pathobiology.* Science, 2003. **302**(5651): p. 1772-5.
43. Tofaris, G.K., et al., *Ubiquitin ligase Nedd4 promotes alpha-synuclein degradation by the endosomal-lysosomal pathway.* Proc Natl Acad Sci U S A, 2011. **108**(41): p. 17004-9.
44. Luo, J., et al., *A calcineurin- and NFAT-dependent pathway is involved in alpha-synuclein-induced degeneration of midbrain dopaminergic neurons.* Hum Mol Genet, 2014. **23**(24): p. 6567-74.
45. Wong, Y.C. and E.L. Holzbaur, *Autophagosome dynamics in neurodegeneration at a glance.* J Cell Sci, 2015. **128**(7): p. 1259-67.
46. Winslow, A.R., et al., *alpha-Synuclein impairs macroautophagy: implications for Parkinson's disease.* J Cell Biol, 2010. **190**(6): p. 1023-37.
47. Decressac, M., et al., *TFEB-mediated autophagy rescues midbrain dopamine neurons from alpha-synuclein toxicity.* Proc Natl Acad Sci U S A, 2013. **110**(19): p. E1817-26.
48. Cali, T., et al., *alpha-Synuclein controls mitochondrial calcium homeostasis by enhancing endoplasmic reticulum-mitochondria interactions.* J Biol Chem, 2012. **287**(22): p. 17914-29.
49. Volpicelli-Daley, L.A., et al., *Formation of alpha-synuclein Lewy neurite-like aggregates in axons impedes the transport of distinct endosomes.* Mol Biol Cell, 2014. **25**(25): p. 4010-23.
50. Dachsel, J.C., et al., *The ups and downs of alpha-synuclein mRNA expression.* Mov Disord, 2007. **22**(2): p. 293-5.
51. Ebrahimi-Fakhari, D., L. Wahlster, and P.J. McLean, *Protein degradation pathways in Parkinson's disease: curse or blessing.* Acta Neuropathol, 2012. **124**(2): p. 153-72.
52. Wong, E. and A.M. Cuervo, *Integration of clearance mechanisms: the proteasome and autophagy.* Cold Spring Harb Perspect Biol, 2010. **2**(12): p. a006734.
53. Berndsen, C.E. and C. Wolberger, *New insights into ubiquitin E3 ligase mechanism.* Nat Struct Mol Biol, 2014. **21**(4): p. 301-7.
54. McLean, P.J., H. Kawamata, and B.T. Hyman, *Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons.* Neuroscience, 2001. **104**(3): p. 901-12.

55. McNaught, K.S., et al., *Impairment of the ubiquitin-proteasome system causes dopaminergic cell death and inclusion body formation in ventral mesencephalic cultures.* J Neurochem, 2002. **81**(2): p. 301-6.
56. Rideout, H.J., et al., *Proteasomal inhibition leads to formation of ubiquitin/alpha-synuclein-immunoreactive inclusions in PC12 cells.* J Neurochem, 2001. **78**(4): p. 899-908.
57. Fornai, F., et al., *Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein.* Proc Natl Acad Sci U S A, 2005. **102**(9): p. 3413-8.
58. Mizushima, N., Y. Ohsumi, and T. Yoshimori, *Autophagosome formation in mammalian cells.* Cell Struct Funct, 2002. **27**(6): p. 421-9.
59. Anglade, P., et al., *Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease.* Histol Histopathol, 1997. **12**(1): p. 25-31.
60. van Hemert, M.J., H.Y. Steensma, and G.P. van Heusden, *14-3-3 proteins: key regulators of cell division, signalling and apoptosis.* Bioessays, 2001. **23**(10): p. 936-46.
61. Berg, D., C. Holzmann, and O. Riess, *14-3-3 proteins in the nervous system.* Nat Rev Neurosci, 2003. **4**(9): p. 752-62.
62. Tzivion, G., Y.H. Shen, and J. Zhu, *14-3-3 proteins; bringing new definitions to scaffolding.* Oncogene, 2001. **20**(44): p. 6331-8.
63. Ichimura, T., et al., *Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases.* Proc Natl Acad Sci U S A, 1988. **85**(19): p. 7084-8.
64. Toska, K., et al., *Regulation of tyrosine hydroxylase by stress-activated protein kinases.* J Neurochem, 2002. **83**(4): p. 775-83.
65. Kleppe, R., K. Toska, and J. Haavik, *Interaction of phosphorylated tyrosine hydroxylase with 14-3-3 proteins: evidence for a phosphoserine 40-dependent association.* J Neurochem, 2001. **77**(4): p. 1097-107.
66. Ostrerova, N., et al., *alpha-Synuclein shares physical and functional homology with 14-3-3 proteins.* J Neurosci, 1999. **19**(14): p. 5782-91.
67. Perez, R.G., et al., *A role for alpha-synuclein in the regulation of dopamine biosynthesis.* J Neurosci, 2002. **22**(8): p. 3090-9.
68. Moskovitz, J., *Methionine sulfoxide reductases: ubiquitous enzymes involved in antioxidant defense, protein regulation, and prevention of aging-associated diseases.* Biochim Biophys Acta, 2005. **1703**(2): p. 213-9.
69. Moskovitz, J., *Prolonged selenium-deficient diet in MsrA knockout mice causes enhanced oxidative modification to proteins and affects the levels of antioxidant enzymes in a tissue-specific manner.* Free Radic Res, 2007. **41**(2): p. 162-71.
70. Oien, D.B., et al., *Clearance and phosphorylation of alpha-synuclein are inhibited in methionine sulfoxide reductase a null yeast cells.* J Mol Neurosci, 2009. **39**(3): p. 323-32.
71. Liu, F., et al., *Methionine sulfoxide reductase A protects dopaminergic cells from Parkinson's disease-related insults.* Free Radic Biol Med, 2008. **45**(3): p. 242-55.

72. Wassef, R., et al., *Methionine sulfoxide reductase A and a dietary supplement S-methyl-L-cysteine prevent Parkinson's-like symptoms.* J Neurosci, 2007. **27**(47): p. 12808-16.
73. Oien, D.B., et al., *MsrA knockout mouse exhibits abnormal behavior and brain dopamine levels.* Free Radic Biol Med, 2008. **45**(2): p. 193-200.
74. Moskovitz, J., et al., *Overexpression of peptide-methionine sulfoxide reductase in Saccharomyces cerevisiae and human T cells provides them with high resistance to oxidative stress.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14071-5.
75. Rankin, C.A., et al., *Parkin, A Top Level Manager in the Cell's Sanitation Department.* Open Biochem J, 2011. **5**: p. 9-26.
76. Flower, T.R., et al., *Heat shock prevents alpha-synuclein-induced apoptosis in a yeast model of Parkinson's disease.* J Mol Biol, 2005. **351**(5): p. 1081-100.
77. Witt, S.N. and T.R. Flower, *alpha-Synuclein, oxidative stress and apoptosis from the perspective of a yeast model of Parkinson's disease.* FEMS Yeast Res, 2006. **6**(8): p. 1107-16.
78. Cooper, A.A., et al., *Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models.* Science, 2006. **313**(5785): p. 324-8.
79. Popova, B., A. Kleinknecht, and G.H. Braus, *Posttranslational Modifications and Clearing of alpha-Synuclein Aggregates in Yeast.* Biomolecules, 2015. **5**(2): p. 617-34.