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THE NEUROPROTECTIVE EFFECTS OF SUMO CONJUGASE, UBC9, IN DOPAMINERGIC CELLS AND NEURONS

by

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A THESIS

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DEDICATION

This thesis is dedicated to my grandmother Sarah Jane Faison-Evans, who suffers from dementia and sparked my interest to pursue my education in neuroscience. I would also like to thank my parents, siblings, Bishop and Lady Fowle, and all those who supported my graduate education.

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ABSTRACT

Parkinson's disease (PD) is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and related to alpha-synuclein mediated protein aggregation. These protein aggregates are known as Lewy bodies. The Small Ubiquitin Modifier (SUMO) is a form of post-translational modification that regulates protein function and stability. Although SUMOylation may increase the solubility of alpha-synuclein, the role of SUMOylation in dopaminergic neurons remains unclear for PD pathology. Our preliminary studies show that the SUMO conjugase, Ubc9, protects rat dopaminergic cells against 1-methyl-4-phenylpyridinium (MPP+) toxicity and prevents the protein degradation of dopamine transporter (DAT) (Cartier *et al.*, manuscript under review). We hypothesize that the overexpression of Ubc9 protects dopaminergic neurons against oxidative stress. For *in vitro* studies, N27 rat dopaminergic cells overexpressing Ubc9-EGFP showed higher cell viability and lower cytotoxicity against MPP+ induced toxicity, compared to EGFP only cells. *In vitro* studies include cell viability (MTT) and cytotoxicity assay (LDH), reactive oxygen species (ROS) detection, and mitochondrial respiration, to determine the protective role of Ubc9 in dopaminergic cells against oxidative stress (MPP+). We also established transgenic C57Bl/6 mice overexpressing Ubc9 or wildtype C57Bl/6 mice in the lab. Chronic intraperitoneal injection of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been utilized to induce parkinsonism for both transgenic and wildtype C57BL/6 mice. Using immunhistological staining of tyrosine hydroxylase (TH) as a marker for dopaminergic neurons. We found that the pan-Ubc9 overexpression protected dopaminergic neurons in the striatum and the Substantia

Nigra from MPTP-toxicities, compared to wildtype littermate mice. Our study supports that SUMOylation can be a potential therapeutic target to prevent oxidative stress-induced PD pathology. Currently we are assessing the neuroprotective mechanisms of Ubc9-mediated SUMOylation in PD model.

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LIST OF ABBREVIATIONS

ATP	Adenine Triphosphate
DA	Dopaminergic
DAT	Dopamine Transporter
EGFP	Enhanced Green Fluorescent protein
H_2O_2	Hydrogen Peroxide
MPP+	1-methyl-4-phenylpyridinuim
MPTP	1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PD	Parkinson's Disease
ROS	Reactive Oxygen Species
SENPs	Sentrin-Specific Proteases
SIM	SUMO interaction motifs
SNpc	Substantia Nigra pars compacta
SUMO	Small Ubiquitin-like Modifier
TH	Tyrosine Hydroxylase
Ubc9	Ubiquitin-like Conjugating Enzyme 9
VMAT	Vesicular Monoamine Transporter

Chapter I
INTRODUCTION

Parkinson's disease (PD) is an incurable neurodegenerative disease characterized by the aggregation of α-synuclein proteins forming Lewy bodies, initiating dopaminergic neuronal loss in the midbrain (Eckermann, 2013). Previous studies from our own lab demonstrated that SUMOylation regulates the dopamine transporter (DAT) and modifies proteins associated with PD. SUMOylation is thought to be involved in the regulation of aggregation-prone proteins in neurodegenerative disorders, particularly PD (Kim et al., 2011). The small ubiquitin-like modifier (SUMO) ligase, Ubc9 enhances the SUMO conjugation. SUMOs can be conjugated to substrate proteins, altering the protein's function and solubility (Krumova *et al.*, 2013). Preliminary results from the laboratory demonstrate that SUMOylation on DAT has been shown to enhance DAT function in plasma membrane (Cartier *et al.*, manuscript under review), however, SUMOylation of α-synuclein are not well understood (Eckermann, 2013). Additional studies are needed to clarify if SUMOylation can be a beneficial mechanism for protecting dopaminergic neurons from oxidative stress in PD. We tested the hypothesis that increased levels of Ubc9 elevate the level of SUMOylated α-synuclein, promoting proper folding and reducing aggregation of proteins. Our preliminary data support that Ubc9-mediated SUMOylation regulates the protein stability/solubility of α-synuclein (Krumova *et al.*, 2013).

The main hypothesis of my thesis is that Ubc9 overexpression is neuroprotective for dopaminergic neurons and cells against oxidative stress. To assess this hypothesis, we utilized mouse models including wild type and Ubc9 overexpressing transgenic mice (Ubc9-Tg) to study the role of SUMOylation in dopaminergic neurons. In addition, we used stable N27 cell lines, which overexpress Ubc9-EGFP or EGFP control.

Specific Aim 1: Is Ubc9 overexpression neuroprotective against oxidative stress in PD mouse models?

We studied the role of SUMOylation in PD models to determine whether it protects dopaminergic neurons from apoptosis. Several genetic mutations including alpha-synuclein are known to cause dopaminergic neuronal death which is resulted from the accumulation of α-synuclein-mediated protein aggregates in the midbrain, leading to disruption in neuronal synapses. Transgenic mice with the overexpression of Ubc9 were compared to wild type mice. Behavioral tests such as cross-beam analysis, pole test, and hindlimb clasping were performed on mouse models to measure motor-control ability. Biochemical analyses such as immunohistochemistry and Western blots were applied to measure the SUMO effects on dopaminergic neurons in the mouse striatum.

Currently there are limited treatments for PD. The enhancement of SUMOylation by Ubc9 overexpression may act as a neuroprotective agent and could possibly be a therapeutic target in PD.

Chapter II
LITERATURE REVIEW

Parkinson's Disease

Parkinson's disease is a common neurodegenerative disorder of the central nervous system, affecting 7- 10 million people worldwide (Eckermann, 2013). The disease was first described as shaking palsy by James Parkinson in 1817. Symptoms of PD include muscular rigidity, bradykinesia, resting tremor, and postural instability. PD is characterized by the loss of dopaminergic neurons in the SNpc which projects to the striatum. The loss of dopaminergic neurons leads to motor dysfunction. Initially Lewy bodies were considered as the hallmark of PD, but Lewy bodies are also seen in Alzheimer's disease and Lewy body with dementia. In 1997, it was revealed the main protein of Lewy bodies was α-synuclein (Polymeropoulos *et al.*, 1997).

Most PD cases are idiopathic, while probably less than 20% of PD cases are caused by genetic mutations which are listed in Table 1. LRRK2 is a multi-domain protein with kinase enzymatic activities. Patients with mutations in LRRK2 have a high risk for familial PD. Genetic mutations in LRRK2 increases mobility of alpha-synuclein and elevates accumulation of alpha synuclein in dopaminergic neurons in the midbrain, therefore inhibiting DA neurons survival and signaling (Zeng, X. *et al.*, 2018). Mutations in α-synuclein and LRRK2 gene are inherited in autosomal dominant manner. Mutations in PTEN-induced putative kinase 1 (PINK1), parkin and DJ-1 cause autosomal recessive early-onset Parkinsonism. PINK1 is a 581-amino acid protein that is highly expressed in neurons where it regulates mitochondrial homeostasis (Eckermann, 2013). PINK1 causes 1-3% early onset PD. Mutations in PINK1 lead to mitochondrial

dysfunction and increase kinase activity leading to high risk of PD (Zeng, X. *et al.*, 2018). Studies show that α-synuclein, DJ-1, and parkin can be modified by SUMOylation (Eckermann, 2013).

Locus	Chromosomal location	Protein	Function	SUMO relevance
PARK1/4	4q21–q23	SNCA (α-synuclein)/triplications	Not clear	Direct modification
PARK2	6q25.2–q27	Parkin	Ub E3 ligase	Direct modification/DJ-1
PARK3	2p13	Unknown	–	–
PARK5	4p14	UCH-L1 (ubiquitin carboxyl-terminal hydrolase L1)	Deubiquitination	None
PARK6	1p35–36	PINK1 (PTEN-induced putative kinase 1)	Kinase	Parkin, DJ-1
PARK7	1p36	DJ-1	Multifunctional	Direct modification
PARK8	12p11.2–q13.1	LRRK2 (leucine-rich repeat kinase 2)	Kinase	Several sites/α-synuclein, tau
PARK9	1p36	ATP13A2 (ATPase type 13A2)	ATPase	K804
PARK10	1p32	Unknown	–	–
PARK11	2q36–2q37	GIGYF2 (GRB10 interacting GYF protein 2)	Unknown	K346, K421, K736, K749, K922
PARK12	Xq21–q25	Unknown	–	–
PARK13	2p12	HTRA2 (Htra serine peptidase 2)	Protease	No
PARK14	22q13.1	PLA2G6 (phospholipase A2 group VI)	Phospholipase	K232, K305, K545, K565
PARK15	22q12–q13	FBXO7 (F-box only protein 7)	E3-Ub ligase complex	No
PARK16	1q32	Unknown	–	–
PARK17	16q11.2	VPS35 (vacuolar protein sorting 35)	Protein sorting	K403, K573, K662
PARK18	3q27.1	EIF4G1 (eukaryotic translation initiation factor 4γ)	Translation	K826, K881, K1222, K1452, K1556
	5q23.1–q23.3	Synphilin-1	α-Synuclein interactor	Several sites/α-synuclein
	2q22–q23	NR4A2	Nuclear receptor	K11, K28, K456, K514

Table 1. List of risk genes associated with PD. The table lists proteins that can be SUMOylated and their SUMOylation sites for SUMO interaction. The K denotes lysine residues where the protein may be SUMOylated (Eckermann, 2013).

The protein α-synuclein is a protein consisting of 140 amino acids and expressed in the human brain (Eckermann, 2013). In 1988, α-synuclein was isolated and shown to be localized in presynaptic terminals of neurons (Maroteaux *et al.* 1988). Although the function of α-synuclein is not clear, it is proposed that α-synuclein is involved in synaptic vesicle processing and trafficking (Cooper et al., 2006). In 1997, the parkinsons disease 1, autosimal dominant gene (PARK1) mutation was observed, where genetic mutation was seen in α-synuclein on chromosome 4q21, mutating A53T (Polymeropoulos *et al.*, 1997). Alpha-synuclein can be ubiquitinated and SUMOylated (Krumova *et al.*, 2011).

Genetic mutations of DJ-1 account for 1-2% of early onset PD. DJ-1 is a homodimer located on chromosome 1p36 and consists of 189 amino acids. DJ-1 is expressed in neurons and glial cells, mainly localized in the cytoplasm, nucleus and mitochondria (Canet-Aviles *et al.*, 2004). DJ-1 is expressed in astrocytes of sporadic PD cases (Bandopadhyay *et al.,* 2004) and can be modified by SUMOylation.

Parkin is a ubiquitin ligase consisting of 465 amino acids which is localized in the cytosol and expressed in muscle tissues and the brain (Shimura *et al.,* 1999). Since parkin is a part of the E3 ubiquitin ligase family, it targets misfolded proteins for degradation and protects cells from cellular stress (Chen *et al.,* 2010). Parkin interacts with SUMO-1 and its interaction with SUMO1 was observed in neuronal cells.

Oxidative Stress

Parkinson's Disease is characterized by loss of DA neurons in the striatum. Loss of these DA neurons causes involuntary movements. Oxidative stress is proposed as the underlying mechanism for idiopathic and genetic cases of PD (Hwang, 2013). Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidants. DA neurons are more susceptible to ROS because of the enzyme, tyrosine hydroxylase, this enzyme produces ROS. Mitochondrial dysfunction can lead to oxidative stress in DA neurons. Aerobic respiration is needed by cells to generate ATP to essentially provide cellular energy. Oxidative stress triggers mitochondrial dysfunction in the presence of Complex I inhibitors such as MPTP. Inhibition of Complex I of the mitochondrial electron transport chain results in leakage of electrons and thus causing ROS production (Hwang, 2013).

In 1983, an incident occurred where users exposed themselves to neurotoxin 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) (Schapira *et al.*, 1990). MPTP crosses the blood brain barrier and is taken up by astrocytes where MPTP is converted to the toxic metabolite 1-methyl-4-phenylpyridinuim (MPP+) by monoamine oxidase-B (MAO-B) (Langston *et al.*, 1984). The MPP+ is taken up by DAT into dopaminergic neurons, inhibiting Complex 1 (Vila *et al.*, 2003). This case sparked the first correlation between PD and mitochondrial dysfunction. MPP+ is a Complex 1 inhibitor of the electron transport chain in the mitochondria. It was observed that there was less activity of Complex 1 in PD patient's brain. In Figure 1, inhibiting complex 1 is crucial to the pathogenesis of PD as it decreases ATP production and elevates ROS within the brain (Schapira *et al.*, 1990).

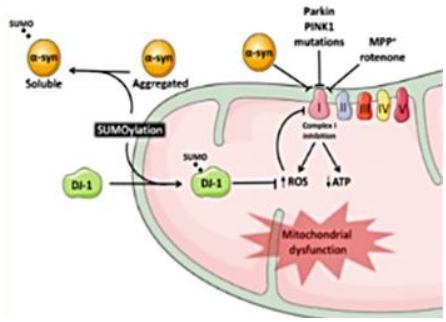

Figure 1. Mitochondrial dysfunction in PD. SUMOylated proteins such as DJ-1 can prevent mitochondrial dysfunction. Whereas genetic mutations and neurotoxins can inhibit Complex 1 of the ETC resulting in ATP depletion and increase of ROS (de Douza, 2016).

SUMOylation

SUMO (small ubiquitin-like modifier) proteins are members of ubiquitin-like protein family (Krumova *et al.*, 2013). SUMOylation modifies cellular processes such as cell signaling, plasma membrane depolarization and signal transduction. SUMOylation is the covalent binding of SUMO proteins to target proteins, but it can also bind non-covalently to SUMO interaction motifs (SIM). SUMOylation can be reversible, where target proteins can be de-SUMOylated.

Enzymes that are responsible for the de-SUMOylation of proteins are called sentrin-specific proteases (SENPs) (Shen, 2006). SUMOylation also plays a significant role in regulating synaptic transmission, plasticity and neuroprotection. Recent studies propose that SUMOylation regulates mitochondrial dynamics and mediates mechanisms causing PD. Proteins involved in the genetic mutations of PD can be modified by SUMO (de Souza *et al.*,2016).

The SUMO protein consists of 100 amino acids and covalently attaches to lysine residues of target proteins. The covalent attachment of SUMO helps to regulate cellular function, by acting as a transcription factor, stabilizing and localizing proteins. SUMO is activated by the E1 complex, which includes SUMO-activating enzyme subunit 1 and ubiquitin-like conjugating enzyme 9. Once SUMO is activated, it is transferred to the E2 conjugating enzyme, called ubiquitin conjugating enzyme (Ubc9), shown in Figure 2. Ubc9 can conjugate to SUMO motifs (Krumova *et al.*, 2013). Next, an E3 ligase catalyzes the conjugation of SUMO to a substrate and its deconjugation of SUMO requires SENP proteases. SUMO can be recycled for more conjugation to proteins multiple times (de Souza *et al.*, 2016).

The homologous mammalian proteins SUMO-1 to SUMO-4 are reversible protein modifiers. These SUMO homologues form covalent peptide bonds with lysine residues of target proteins and its modification of proteins by SUMO homologues is described as SUMOylation (Krumova *et al.*, 2013). There are three characterized SUMO isoforms: SUMO-1, SUMO-2, and SUMO-3. SUMO-1 is an 101-

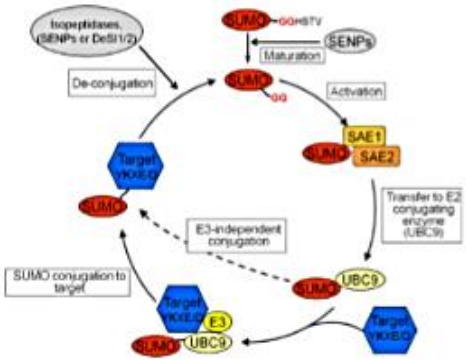

Figure 2. SUMO conjugation cycle (Krumova, 2013).

amino-acid proteins and about 11 kDa, which shares 18% sequence homology with ubiquitin. SUMO-1 is attached to target proteins as a single molecule (Krumova *et al.*, 2013). SUMO-2 and SUMO-3 differ by only N-terminal amino acids and due to their amino acid sequence similarity, they are denoted as SUMO-2/3 (de Souza *et al.* 2016).

The function of SUMO depends on the substrate that SUMO conjugates to. The protein α-synuclein is abundant in presynaptic regions of neurons and can be targeted by SUMO. However, the function of SUMOylated α-synuclein remains unclear (de Souza *et al.*, 2016). Studies have shown that the overexpression of α-synuclein in HEK293 cells is modified by SUMO-1 rather than SUMO-2/3 (Krumova *et al.*, 2011). The SUMOylation site of α-synuclein is located at amino acid K96 and K102. α-synuclein SUMOylation *in vivo* was studied by expressing His6-SUMO2 in transgenic mice. Transgenic mice were generated using His_6-SUMO2 as a model for sumoylated alpha-synuclein. Westrn blots revealed that endogenous alpha-synuclein is conjugated to SUMO2 (Figure 3). SUMOylated α-synuclein abolished fibril formation in contrast to non-SUMOylated α-synuclein which did not prevent fibril formation (Krumova *et al.*, 2011).

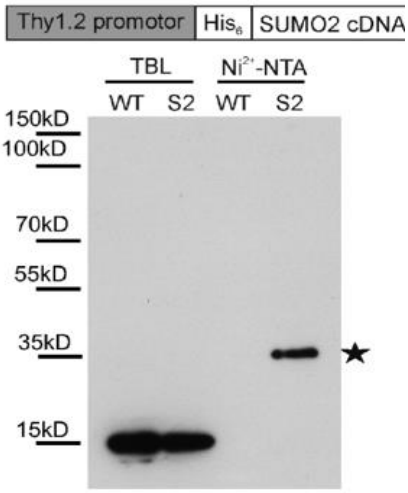

Figure 3. SUMOylated alpha-synuclein in mouse brain tissue. Alpha-synuclein conjugated to SUMO2 were isolated using Ni^{2+}-NTA affinity chromatography (Krumova *et al.*, 2011).

SUMOylation has been suggested to act as a regulator of toxic proteins in neurodegenerative diseases. SUMO proteins are very soluble, which may help to prevent

misfolding or increase solubility of proteins (Krumova *et al.*, 2011). One study shows that the disease related mutation of DJ-1 protein inhibited SUMO conjugation and protein solubility (Shinbo *et al*., 2006). In *C. elegans*, the sequestering protein intermediate filament protein (IFb-1) is important for embryonic elongation. SUMOylated IFb-1 helps to translocate to cytoplasm, while non-SUMOylated IFb-1 causes cytoplasmic aggregation (Kaminsky *et al.*, 2009).

The mitochondria supply energy to cells in the brain. Mitochondrial fusion regulates DNA replication, while fission is responsible for redistributing mitochondrial DNA during cell division, in addition to helping to degrade damaged mitochondria using lysosomal system. There is supporting evidence that SUMOylation regulates the mitochondria fusion and fission (Eckermann, 2013). SUMOylation modifies proteins in the mitochondrial apoptosis pathway. Mitochondrial apoptosis requires mitochondrial fission. When mitochondrial fission is blocked, it prevents the release of apoptotic factors such as cytochrome c. Studies show that SUMO helps to translocate the mitochondrial protein GTPase dynamic related protein (DRP1) from the cytosol to the outer membrane of mitochondria. It was revealed that DRP1 interacts with SUMOylating enzyme Ubc9. SUMO is localized at sites of mitochondrial fission (Harder *et al.*, 2004). An experiment using Cos7 cells were used to determine SUMO function in

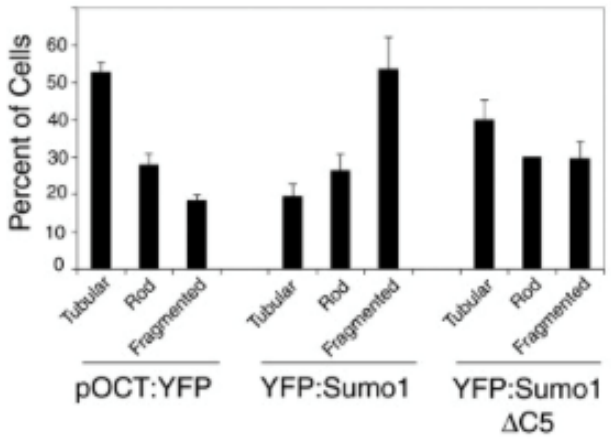

Figure 4. Overexpression of SUMO1 increases mitochondrial fragmentation. Cos7 cells transfected with overexpression of YFP:SUMO1 increases fragmented mitochondrial cells compared to control (Harder *et al.*, 2004).

tubular, rod-like and fragmented mitochondria. Cos7 cells transfected with overexpression of SUMO1 (YFP:SUMO1) shows increase of fragmented mitochondria when SUMOylated (Figure 4). It was also shown that apoptotic conditions help to stimulate the SUMOylation of DRP1 (Wasiak *et al.*, 2007).

Our preliminary data from the Kim lab show that the overexpression of Ubc9 protects rat dopaminergic N27 cells against MPP+ induced cell death (Figure 5). Our lab generated stable N27 parental cells that overexpress Ubc9-EGFP or EGFP (Cartier et al., under review). Ubc9-EGFP cells show higher cell viability at 320 µM of MPP+ compared to EGFP only control. This experiment was replicated using H_2O_2 to induce oxidative stress to determine the neuroprotective effects of Ubc9.

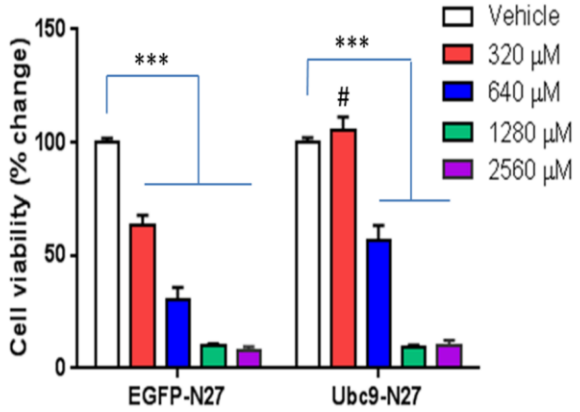

Figure 5. Ubc9 overexpression protects N27 rat dopaminergic cells against MPP+ toxicity. MTT assay was applied using stable cell lines (# = $p \leq 0.05$ vs. EGFP at the same concentration and *** = $p \leq 0.001$ vs. vehicle control by unpaired Student's t-test). Data credited to Eric Janezic.

Chapter III
RESEARCH METHODS

Cell lines and culture

N27 parental (N27P) cell (EMD Millipore) is a rat dopaminergic cell line. N27P cells were cultured in RPMI 1640 medium (Life Technologies) with 10% fetal bovine serum (Atlantic Bio) and 1% Penicillin-Streptomycin (Gibco) at 37°C in a 5% CO_2 incubator. N27 Ubc9-EGFP (enhanced green fluorescent protein) or EGFP overexpressing stable cell lines were developed by transfecting plasmids into parental N27 dopaminergic cells. N27P cells were transfected using Lipofectamine 2000 (Invitrogen), following manufacturer's protocol. Once cells were transfected for 48 hours the green fluorescence was visualized under EVOS fluorescent microscope. Following transfection, 500 µg/ml of Geneticin (Gibco) was added to media to select EGFP positive cells.

MPTP

All animal protocols were conducted in accordance with the United States Public Health Service Guide for the Care and Use of Laboratory Animals; all procedures were approved by the Delaware State University Animal Care and Use Committee. All efforts were made to minimize animal numbers and distress. C57/Bl6 mice were obtained from Charles River Laboratory (Wilmington, MA). Twenty-three mice were used in our treatment group. All mice received intraperitoneal (IP) injections of 25 mg/kg MPTP once a day for seven consecutive days. The negative control group was injected with saline.

Isolation and sectioning of mouse brains

Mice were sacrificed by perfusion with 150 mM NaCl/70% ethanol. Animals were anesthetized with isoflurane through a face mask. The abdominal cavity was revealed using surgical scissors exposing the heart. With a needle, the right atrium was punctured to release blood from the heart. Then animals were perfused with PBS and 150 mM NaCl/70% ethanol through the left ventricle. Then mice whole brains were divided sagittal in halves. The right half brains were preserved in 4% paraformaldehyde (PFA) and transferred to 30% sucrose solution at 4 °C. Then the right half brains were embedded in OCT (optimum cutting temperature compound), transferred to methanol with dry ice to quickly freeze them, then stored at -80 °C until its use. The left side of the brains were divided into the cerebellum, brainstem, striatum, olfactory bulb, and the rest of the brain and frozen at -20 °C for later use. The right half brains were sliced using a cryostat at 14 μm sections and placed on polarized slides (Midsci Scientific).

Mouse tissue extraction

Tissue samples of isolated brain regions were homogenized in the RIPA buffer, then centrifuged at 16,400g at 4°C for 30 min. The supernatant was collected to measure protein concentration using the BCA assay (Thermo Scientific).

Animals

C57BL/6 male mice (9 months of age) were obtained from Charles River Laboratory (Wilmington, MA). Mice were allowed ad libitum food and water and were housed 5 per cage in 12h light-dark cycle. For our *in vivo* experiments, mice aged 10 months or older were utilized to

study PD. We specifically used C57BL/6 mice due to its sensitivity to the neurotoxin MPTP. To analyze the overexpression of Ubc9 *in vivo*, our lab has obtained theUbc9 pan-overexpression transgenic mice from the NINDS. The Ubc9 gene is hemizygously expressed and is driven by the CMV early enhancer/chicken β-actin (CAG) promoter.

MTT assay

MTT assay was used to measure the cell viability of N27P, EGFP, and Ubc9-EGFP cell lines. Overall, the colorimetric assay shows the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT is reduced to purple formazan product localized on the mitochondria membrane. In a 96-well plate, 5000 cells/200 µl were plated in triplicate wells. Overnight incubation allowed cells to reach 70% confluency, then cells were treated with the toxin MPP+ at 0-1280 µM concentration and incubated at 37 °C, 5% CO_2 for 24 hrs. After MPP+ incubation, the media was removed. Next, 20 µl of MTT, a yellow tetrazole, was added to each well and incubated at 37°C for three hours. Next, the MTT reagent was removed from all wells, then 200 µl of dimethyl sulfoxide (DMSO) ,a solubilizing solution, was added to dissolve the insoluble purple formazan product into a colored solution. The absorbance was quantified by measuring at a sensitive wavelength (570nm), subtracted by the background level at 630 nm.

***In vitro* ROS in EGFP and EGFP-Ubc9 cells**

CellROX Deep Red kit (C10422, Molecular Probes) was used to measure the ROS in live cells. In its reduced state the reagent is non-fluorescent, but upon oxidation the reagent elicits a

red fluorescence. CellROX Deep Red reagent is localized in the cytoplasm. Cells were plated in glass bottom chambers (155409, Thermo) at 5,000 cells/200 µl, then placed in 37 °C, 5% CO_2 incubator overnight. Cells were treated with MPP+ and H_2O_2 for 16 hours. The media with the toxin was removed, then added CellROX reagent at final concentration of 5 µM and incubated at 37 °C for 30 minutes. After removing media and gently washing cells three times with PBS, 200 µl of Live Cell Imaging Solution was added to cells for microscopic imaging. Fluorescence intensity was measured using fluorescence microscopy and quantified using ImageJ image analysis software.

Immunofluorescence staining

For immunofluorescence staining, sections were washed in 0.1M PB three times for 10 minutes each. After wash, slides were post-fixed in 4% PFA for 20 minutes. Next, endogenous peroxidases were inactivated in a mixture containing 30% MeOH/0.2% Triton-X-100/1.5% H_2O_2 for 10 minutes and then washed in 0.2% PBT 3 times for a total of 30 minutes. Following the wash step, the slides were blocked in 3% goat serum for 1 hour. Sections were then incubated in rabbit anti-TH primary antibody (1:250, EMD Millipore) or anti-Synaptophysin (1:250, Santa Cruz) at 4ºC overnight. Following overnight incubation, sections were washed in 0.1% PBT 3 times for a total of 30 minutes. Alexa 555 goat anti-rabbit IgG secondary antibody (1:250, A21428, Thermo) or Alexa 488 goat anti-mouse IgG (1:250, A1101, Thermo) was applied and sections were incubated for 1 hour at room temperature. Following incubations, sections were washed in 0.1M PB two times for 15 minutes each, followed by brief wash in triple distilled

water to remove salt residue. Coverslips were mounted using Prolong diamond antifade mounting medium. Samples were imaged using LSM 510 confocal microscope.

Western Blot

Using extracted tissue samples from our MPTP and Saline injected mice, we measured protein concentrations using the BCA assay kit (Thermo) following the manufacturer's protocol. Samples (20 µg/µl) containing 10% β-mercaptoethanol in 1X Laemmli sample buffer were denatured at 97°C for 5 minutes and immediately placed on ice. Samples were separated in 4-20% SDS-PAGE gel (GenScript) and transferred to PVDF membranes using the Bio-Rad wet transfer system. The membranes were first blocked in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween 20) containing 5% dry milk for 1 h and then incubated with primary antibody (anti-TH from EMD Millipore, 1:1,000) in blocking solution overnight. Following primary antibody incubation, the membrane was washed three times for 10 min each using TBST and incubated with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit from, 1:10,000) followed by TBST washes. Next, 1.0 mL of ECL Western Blotting Substrate (Thermo) were added to plastic covers with membrane in between. The imaging substrate is used to detect HRP from antibodies. Protein expression were visualized using iBright CL1000 (Invitrogen). Membranes were then stripped and probed with mouse anti-β-actin (EMD-Millipore 1:15,000) antibody for loading control.

Behavioral Tests

Three behavioral tests were conducted once a day for three days, including pole test, crossbeam, and hindlimb clasp test. Prior to the behavioral assessments, mice were habituated for two days to get familiar with tasks.

To evaluate movement of the mice, the pole test was utilized. The mice were positioned onto the top of the pole and were expected to travel down faceward on the pole. Time traveled from the top of the pole to the bottom was recorded. The pole test was performed 5 times each for each animal. The cross-beam test was utilized to measure motor function. The crossbeam measurement length was twenty-four-inches. The start of the beam measured two inches wide and decreased by half inch every six inches. Mice were initially placed at wide end of the crossbeam. Mice traveled along the beam until it reached the narrow end of the beam. Mice cage with food were placed underneath the narrow end of crossbeam as reward. Cross beam analysis included five trials. The number of right and left leg slips per animal were counted using video recordings. Unfortunately, we were unable to retrieve recordings of right limb slips. Our behavioral analysis will only include left limb slips. For the hindlimb clasping test, mice were suspended in the air by the base of their tail for 15 seconds. A non-blinded analysis of hindlimb clasp scores ranged from 1-2; “0” represents “normal” hindlimb behavior where hindlimbs extended far from abdomen, “1” is for “moderate” clasping, and “2” represents “severe” clasping were hindlimbs close to abdomen for majority of the observance.

Data Analysis

All data were analyzed and graphed using Prism 7 (GraphPad Software, Inc.). For data obtained from MTT analysis, the absorbance at 630 nm was subtracted from the absorbance at 570 nm and compared to the average of the control wells to get the percent change from control. Student's t-test or a One-way ANOVA was applied to determine significance ($p < 0.05$), followed by Tukey's post-hoc test. All data were normalized to the control to measure the percentage change from controls. Behavioral test was non-blinded analyzed since the patterns.

Chapter IV
RESEARCH FINDINGS

PD is characterized by the loss of DA neurons in the midbrain due to the protein aggregation mediated by α-synuclein. The loss of DA neurons is a result of oxidative stress which produces free radicals leading to mitochondrial dysfunction (Hwang, 2013). The role of SUMOylation of α-synuclein in the PD pathology remains unclear. SUMO may modify proteins to prevent misfolding and regulates protein solubility (Krumova et al., 2011). In this study, we investigated the role of SUMOylation in our PD models by overexpressing the SUMO conjugase, Ubc9, to determine its protective effects against oxidative stress. We hypothesized that the overexpression of Ubc9 will protect DA cells and neurons from oxidative stress in PD models.

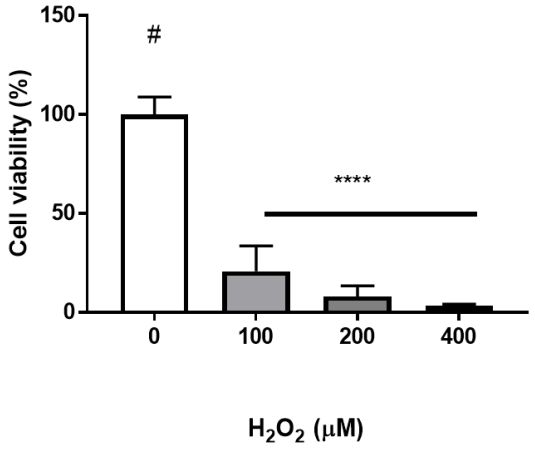

Figure 6. The oxidative stress induced by H_2O_2 decreased the cell viability of N27 dopaminergic cells in close dependent manner. EGFP cells were treated with 0-400 μM of H_2O_2 to induce oxidative stress. After 16 hour incubation of H_2O_2, MTT assay was utilized to measure cell viability. We observed more than 50% decrease in cell viability and very significant decrease of cell viability of EGFP cells at 100, 200, and 400 μM of H_2O_2. Overall, the figure demonstrates that H_2O_2 induced oxidative stress in EGFP cells. Data were presented in mean ± SEM, One-way ANOVA, Tukey's test was applied. (****) represents p-value ≤ 0.0001, compared to the vehicle (control #).

To test our hypothesis in *in vitro* model, we transfected N27P rat dopaminergic cells with EGFP and Ubc9-EGFP

constructs. We used EGFP-positive cells as a visual indicator to observe Ubc9 over-expression. We first tested the effect of H2O2 on inducing oxidative stress in EGFP and Ubc9-EGFP cells. In Figure 6, we observed a decrease in cell viability of EGFP cells in a dose dependent manner of H_2O_2 (100, 200, and 400 µM). Figure 6 shows that H2O2 does induce oxidative stress within EGFP cells, causing cell damage. Our data indicate that there is less than 25% of cell viability (more than 75% cell damage) for EFGP cells treated with 200 µM of H_2O_2. Next, we tested whether H2O2 had the same cell damaging effect for Ubc9-EGP cells.

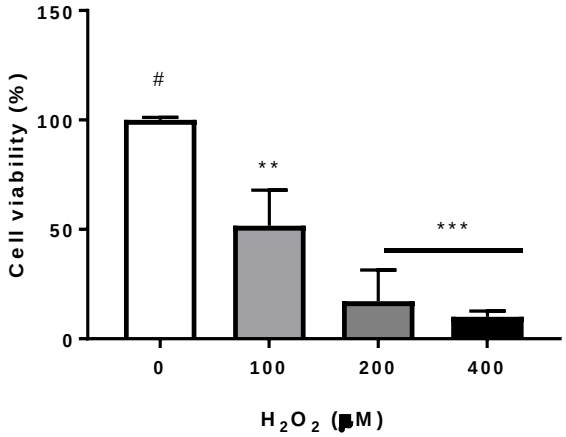

Figure 7. The oxidative stress induced by H_2O_2 decreased the cell viability of Ubc9-EGFP dopaminergic cells in dose dependent manner. Ubc9-EGFP cells were treated with 0-400 µM of H_2O_2 for 16 hours. MTT assay was used to measure cell viability for Ubc9-EGFP cells to analyze effects of induced oxidative stress. We observed ~50% decrease in cell viability for Ubc9-EGFP cells treated with 100 µM of H_2O_2. For Ubc9-EGFP cells treated with 200 and 400 µM of H_2O_2, observed less than 25% decrease in cell viability compared to non-treated Ubc9-EGFP cells. Data were presented in mean ± SEM, One-way ANOVA, Tukey's test was applied. (***) represents p-value ≤ 0.001 compared to 0 µM H_2O_2, (control #).

In Figure 7, we observed a significant decrease in the cell viability of Ubc9-EGFP cells in a dose dependent manner of H_2O_2. However, the Ubc9-EGFP cells treated with 400 µM H_2O_2 showed 50% decrease in cell viability, compared to control. Therefore, the Ubc9-EGFP cells showed 25% higher in cell viability, compared to control at 200 µM H_2O_2. Figures 6 and 7 show

that H_2O_2 induced oxidative stress decreases cell viability for both EGFP and Ubc9-EGFP in a dose-dependent manner.

However, in the comparison of the cell viability for both EGFP and Ubc9-EGFP cells shown in Figure 8, we found that the overexpression of Ubc9 significantly protected DA cells against oxidative stress induced by H_2O_2. In Figure 8, only 50% decrease in Ubc9-EGFP cells whereas EGFP cells has ~75% decrease in cell viability when treated with 100 µM of H_2O_2. Ubc9-EGFP cells have higher cell viability than EGFP cells treated with H_2O_2. Our data analysis indicates that there is a significant neuroprotective effect of Ubc9 overexpression from 400 µM H_2O_2 (p-value < 0.05).

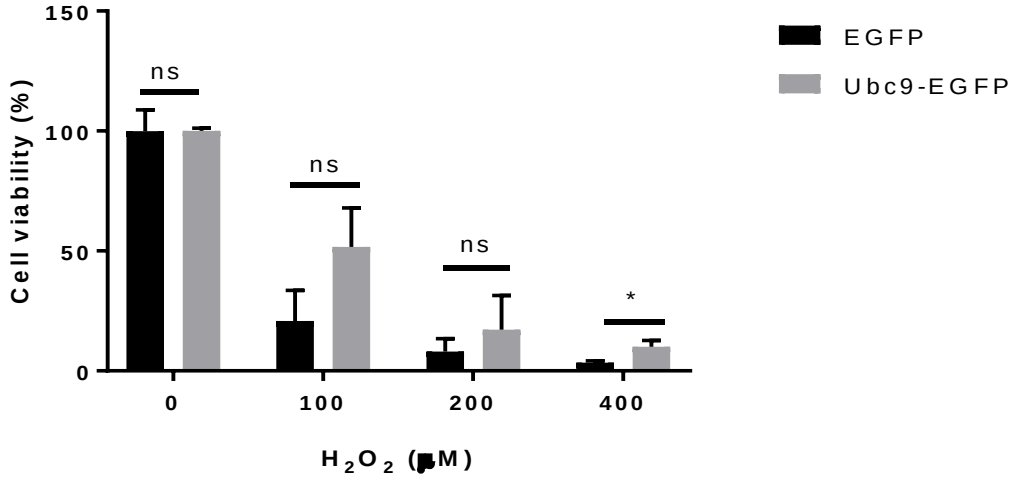

Figure 8. Overexpression of Ubc9 protects dopaminergic neurons at 400 µM H_2O_2 induced cytotoxicity. Both EGFP and Ubc9-EGFP cells were treated with 100, 200, and 400 µM of H_2O_2 to induce oxidative stress. MTT assay was utilized to measure cell viability. We observed increased cell viability for Ubc9-EGFP cells compared to EGFP cells. Student's t test. (ns) represents not significant, (*) represents p-value ≤ 0.05 compared to 0 µM H_2O_2, (control #).

For Parkinson's disease *in vitro* model, MPP+ was applied to N27 cells. MPP+ is a specific neurotoxin for damaging dopaminergic cells due to the uptake by DAT. The neurotoxin MPP+ induced oxidative stress in EGFP cells, which decreased cell viability (Figure 9). We observed ~70% decrease in cell viability for EGFP cells treated with 1,280 µM of MPP+ compared to control. It can be concluded that 1,280 µM of MPP+ is the optimal dosage for inducing oxidative stress in EGFP cells under the conditions (Figure 9).

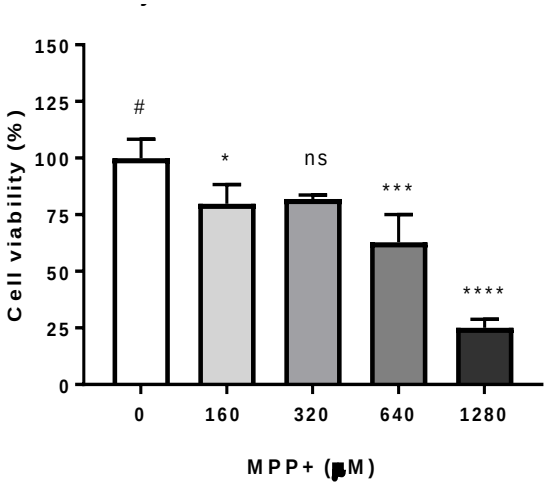

Figure 9. Neurotoxicity induced by MPP+ causes damage in DA cells. EGFP cells were treated with 160, 320, 640 1280 µM of MPP+ for 18 hours. We observed decrease in cell viability due to MPP+ induced toxicity. Data were presented in mean ± SEM and One-way ANOVA, Dunnett's test was applied. (ns) represents not significant, (*) represents $p \leq 0.05$, (***) represents $p \leq 0.001$, and (****) represents $p \leq 0.0001$ compared to 0 µM MPP+ (control #) (n= 3 wells per experiment).

Next, we assessed the MPP+ toxicity in Ubc9-EGFP cells. In Figure 10, oxidative stress induced by MPP+ decreased the cell viability of Ubc9-EGFP cells. At 1,280 µM of MPP+, we observed ~75% decrease in cell viability compared to control and the concentration appears to be the optimal dose for inducing cell damage in Ubc9-EGFP cells. In Figures 9 and 10, we observed a significant decrease in cell viability with application of 0-1,280 µM MPP+ in EGFP cells.

However, we did not observe a significant neuroprotective effect by the overexpression of Ubc9 in MPP+ induced cytotoxicity *in vitro* (Figure 11).

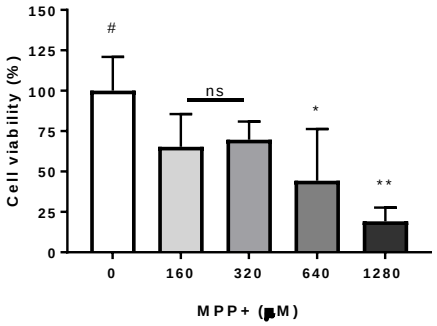

Figure 10. The neurotoxin MPP+ decreased the cell viability of Ubc9-EGFP cells. Ubc9 cells were treated with 160- 1280 µM of MPP+ for 18 hour incubation. We observed decrease of cell viability. At 1280 µM of MPP+ ~25% decrease in cell viability compared to control. Data were presented in mean ± SEM and One-way ANOVA, Tukey's test was applied. (*) represents $p \leq 0.05$, (**) represents $p \leq 0.01$ compared to control (#). (n= 3 wells per experiment)

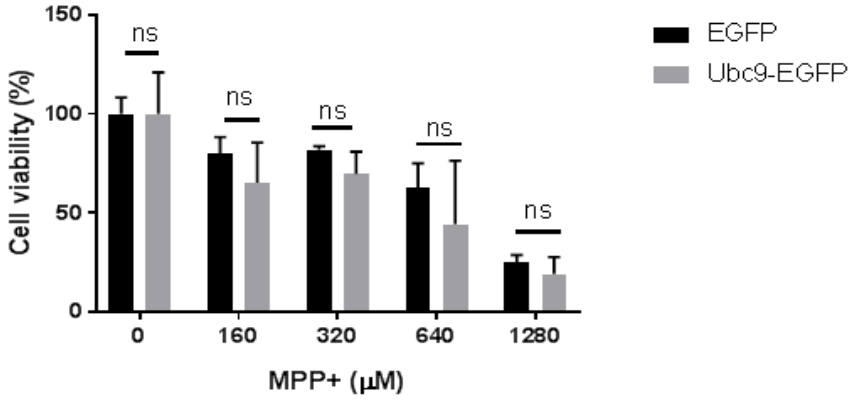

Figure 11. Overexpression of Ubc9 shows no neuroprotective effects in MPP+ induced cytotoxicity. MPP+ induces cytoxicity in EGFP and Ubc9-EGFP cells, however, the overexpression of Ubc9 did not significantly protect dopaminergic cells from MPP+ toxicity. EGFP cells had higher cell viability than Ubc9-EGFP cells at increasing concentrations of MPP+. Student's t-test was statistically applied. (n= 3 wells per experiment)

The underlying mechanism for MPP+ to cause neuronal damage is the production of ROS. ROS is produced when Complex 1 of the electron transport chain is interrupted, thus preventing the synthesis of ATP (Hwang, 2013). The neurotoxin MPP+ once taken up by the DAT, inhibits the function of Complex 1, therefore producing ROS. To test the production of ROS *in vitro,* we used CellRox Deep red reagent (Thermo) in which emits a red florescence in the presence of ROS. For both EGFP and Ubc9-EGFP cells, low levels of ROS were detected in the control group. The Ubc9-EGFP cells treated with 640 µM MPP+ had a substantial decrease of ROS production, compared with the EGFP cells treated with 640 µM MPP+ (Figure 12).

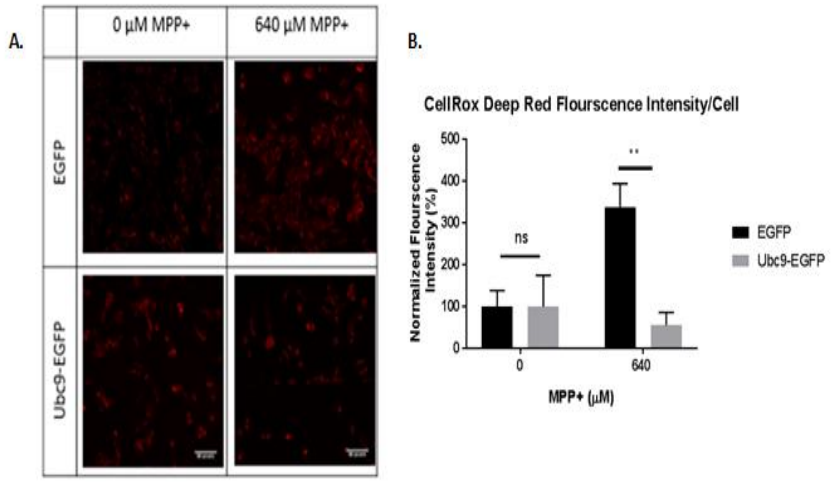

Figure 12. Overexpression of Ubc9 decreased ROS production in DA cells. (A) Red fluorescence represents ROS levels in cells using CellROX Deep Red reagent. EGFP and Ubc9-EGFP treated with 0 µM MPP+, we observed less ROS levels. At 640 µM of MPP+ increased ROS production compared to Ubc9-EGFP cells treated with 640 µM of MPP+. **(B)** Quantification of fluorescence intensity per cell from Figure 7A. Data were shown in mean ±SEM and analyzed in unpaired Student′s t-test. (ns) represents not significant. (**) represents $p \leq 0.01$. (n = 3 wells per experiment).

Therefore, we confirm that the expected neuroprotective effects derived from Ubc9 over-expression is mediated by reducing the ROS production in N27 cells. Although we found a

significant protective effect by Ubc9 over-expression against H_2O_2, we did not find a significant neuroprotective effect by Ubc9 over-expression from MPP+. Therefore, we need to repeat the cytotoxicity (LDH) assay in Ubc9-EGFP cells with exposure of MPP+.

For our *in vivo* studies, we used hemizygous transgenic C57/Bl6 mice that have pan-overexpression of Ubc9 in brain to determine the neuroprotective effects of Ubc9, and we used wild-type mice without overexpression of Ubc9 as controls. To induce Parkinsonian symptoms within our mice model, the neurotoxin MPTP was intraperitoneally injected to mice for seven consecutive days, once a day. We expect that MPTP-lesioned Ubc9 transgenic (Ubc9-Tg) mice will have more dopaminergic neurons in the striatum than MPTP-lesioned wild-type mice. We specifically quantified dopaminergic neurons in the striatum because the DA neurons project their axons to the striatum, while the cell bodies of DA neurons are localized in the substania nigra. Previously in our lab, we would perform immunhistological staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) using frozen brain sections. In order to quantify the DA neuron intensity within the striatum region, stereological analysis must be applied. In Figure 13, we

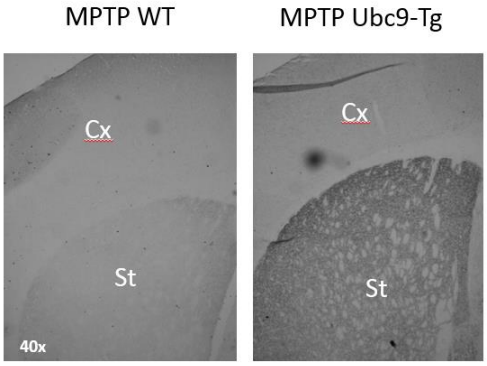

Figure 13. Immunhistological staining using DAB shows pan-overexpression of Ubc9 in mice protects dopaminergic neurons from MPTP. Overexpression of Ubc9 in mice model protects DA neurons against MPTP induced toxicity, whereas there is decrease of DA neurons in MPTP wild type group. Immunohistochemistry using DAB which yields an insoluble brown colored product conjugated to HRP secondary antibody. Using 14 µm thick brain sections, DA neurons were detected using anti-TH (1:100) conjugated to anti-rabbit (1:250). Images captured using EVOS FL Imaging System.

observed higher intensity of DA neurons in the striatum of MPTP Ubc9-Tg mice than MPTP WT. We applied the same principle for detecting DA neurons, to analyze the level of DA neurons in the striatum, an easier method immunofluorescence in confocal microscopy was applied (Shown in Figures 14 and 15). The primary antibody to tyrosine hydroxylase, a typical marker for DA neurons, was conjugated to anti-rabbit Alexa 555 (seen as red fluorescence in Figures 14 and 15). Saline was used as a control group to compare to the MPTP lesioned mice. In Figure 14A, we observed high intensity of DA neurons in striatum for the saline treated wild type mice. In contrast to Figure 14B, we observed decrease of DA neurons in the striatum region for the MPTP lesioned mice. Each column as seen in Figure 14 represents individual brain sections from separate animals within treatment group. In Figure 14C, with the overexpression of Ubc9 in our transgenic mice the DA neurons survived against MPTP induced toxicity. Figure 15, we have observed a significant depletion of DA neurons in the striatum from the MPTP treated wild type

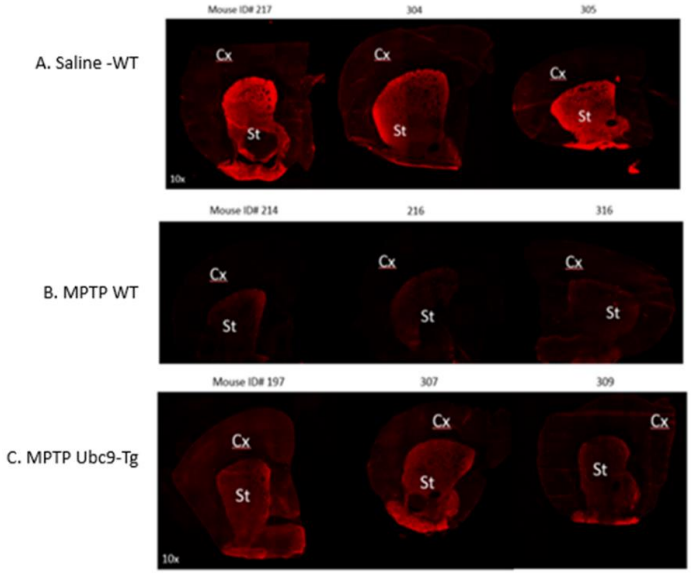

Figure 14. The over-expression of Ubc9 protects DA axon tips in the striatum from MPTP-induced toxicities. Using immunofluorescence DA neurons in the striatum region was detected using anti-TH (1:100). Figure 12A shows that Saline treated mice show high expression of DA neurons in contrast to 12B, the DA neurons were damaged by MPTP induced toxicity. However, with the overexpression of Ubc9, DA neurons remained intact in the striatum in presence of MPTP (12C).

mice, compared to the saline injected wild type mice. Our striking finding is that DA axon tips in the striatum from Ubc9 transgenic mice were significantly protected from MPTP toxicities, compared to those in WT striatum. Our data analysis indicates that the overexpression of Ubc9

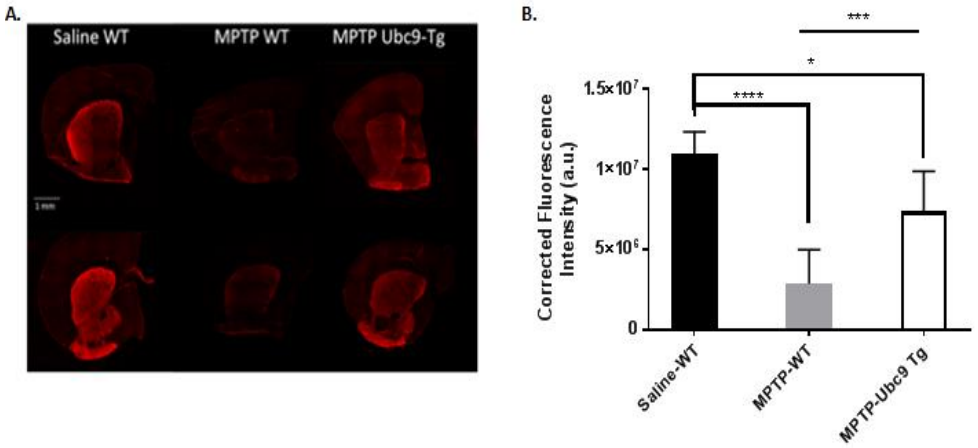

Figure 15. The overexpression of Ubc9 protects dopaminergic neurons from MPTP-induced toxicity in mouse striatum. (A) TH fluorescence intensity in striatum of C57/Bl6 mice using anti-TH (1:100) conjugated to Alexa Flour 555 (1:250). In our Saline control group, DA neurons are intact in the striatum. We observed decrease od DA neurons in MPTP WT model. The pan overexpression of Ubc9 in our MPTP lesion Tg model, we observed increase TH intensity in the striatum compared to MPTP WT. **(B)** Quantification of red florescence in striatum subtracted from cortex for Saline-WT, MPTP-WT, and MPTP-Ubc9 Tg. Statistical analysis using One-Way ANOVA, Tukey's Test. (*) represents p-value ≤ 0.05. (***) represents p-value ≤ 0.0004. (****) represents p-value ≤ 0.0001. Sample size: Saline-WT (n=3), MPTP-WT (n=6), and MPTP-Ubc9 Tg (n=4).

does protect DA neurons in the striatum from MPTP in average (see Figure 15B). So far, we have completed 4-6 animals per group, therefore, we expect to maintain the statistical significance after completing the analysis (n≥6 per group).

The significant findings observed from the confocal images reveal that the overexpression of Ubc9 protects DA neurons against MPTP induced toxicity. To confirm our findings, we measured protein expression of TH (1:1,000) in extracted striatum tissues of saline and MPTP-lesioned mice (Figure 16). Due to the lack of experience with Western blotting

technique, unexpected results were produced. For the Saline wild type and transgenic group, we expect to see high expression of TH, but unfortunately, we do not see bands at the 62 kDA molecular weight for TH. We also expected low expression of TH in the MPTP wildtype group. As shown in our Western blot results, there was high expression of TH in the MPTP WT compared to the Saline wild type and transgenic group, which does not support the damaging effects previously seen by MPTP. We expect a significant change in TH expression between

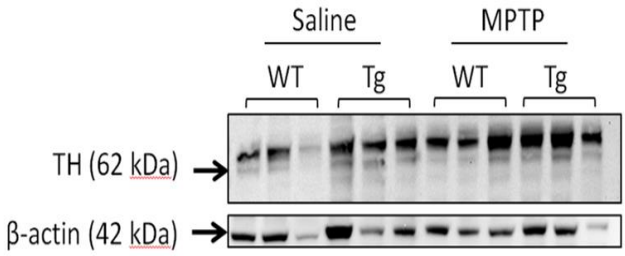

Figure 16. In Western blot, the levels of tyrosine hydroxylase (TH) were compared between Ubc9-Tg and WT siblings, with or without chronic MPTP.
Striatum tissue extract from Saline and MPTP treated mice were used. To observe effects of MPTP in DA neurons used anti-Tyrosine hydroxylase (1:1,000). β-actin serves as the loading control for samples. Western blot shows no difference in TH levels between Saline and MPTP. For the saline group, we expect high levels of TH because the DA neurons should not be damaged in the absence of neurotoxin. Repeat of experiment are needed in order to quantify results.

MPTP wildtype versus Ubc9-transgenic mice. Our data do not meet the expectation that TH expression is relatively the same as the fluorescent-staining results in Figures 14 & 15). The replication of Western blot using newer samples of MPTP or Saline-treated mice will be completed in future experiments in order to better quantify the TH protein levels.

To observe the effects of neurotoxin MPTP and its influence on motor function in mice, we performed three behavioral tests: the hindlimb clasping test, pole test, and the crossbeam test. Figure 17 represents examples of the hindlimb clasping scores ranging from 0-2.

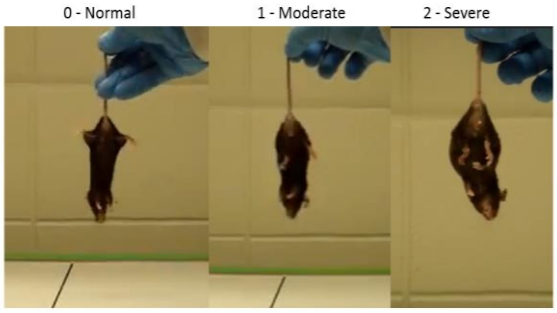

Figure 17. Hindlimb clasping score for Saline and MPTP lesioned mice. Mice were suspended by the base of their tail for 15 seconds. Non- blinded analysis of hindlimb clasp was rated on a scale from 1-2. "0" represents "normal", "1" is for "moderate" and "2" represents "severe."

We expect the MPTP-treated WT mice to demonstrate more severity in the hindlimb clasping, compared to saline treated control group. However, our behavioral data did not meet our expectations. In Figure 18, MPTP Ubc9-Tg mice had more severe hindlimb clasping, compared to MPTP WT, in which this finding does not support our previous data (Figure 15) that Ubc9 overexpression protects DA

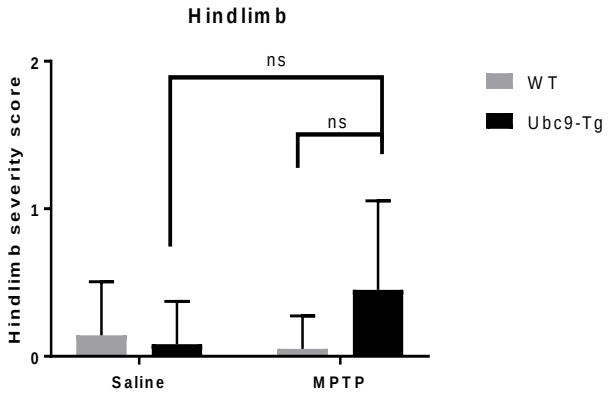

Figure 18. No significant in severity of hindlimb clasping for MPTP-WT and MPTP Ubc9-Tg. Data were presented in mean ± SEM, One-way ANOVA, Tukey's test was applied. (ns) represents not significant. Sample size: Saline-WT (n=5), Saline Ubc9-Tg (n=4), MPTP-WT (n=7), and MPTP-Ubc9 Tg (n=7)

neurons against MPTP in confocal imaging. Therefore, we expected in our behavioral analysis that the Ubc9 overexpression in the MPTP lesioned mice will have less severity in the hindlimb clasping, compared to MPTP WT. In Figure 19, we observed no significant difference in latency between MPTP Ubc9-Tg and MPTP WT. There is no significant difference in left leg slips recorded for the crossbeam analysis between MPTP-Tg and MPTP WT (Figure 20). Behavioral analysis must be replicated in order to reduce error bars and conclude motor functions between MPTP Ubc9-Tg and MPTP WT can be significantly different. To improve behavioral analysis, we will habituate the animals for three days instead of two days and perform behavioral analysis for five days instead of three days to eliminate outliers.

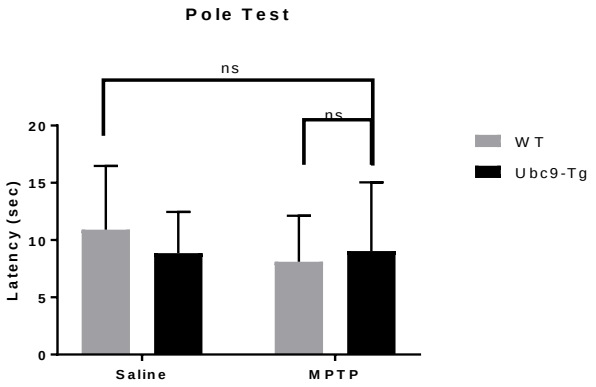

Figure 19. No significant difference in latency for MPTP-WT vs MPTP Ubc9-Tg mice. Data were presented in mean ± SEM, One-way ANOVA, Tukey's test was applied. (ns) represents not significant. Sample size: Saline-WT (n=5), Saline Ubc9-Tg (n=4), MPTP-WT (n=7), and MPTP-Ubc9 Tg (n=7)

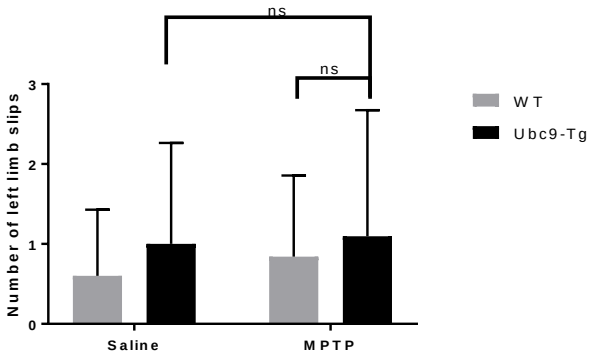

Figure 20. No significant difference in left limb slips between MPTP Ubc9-Tg vs MPTP WT. Data were presented in mean ± SEM, One-way ANOVA, Tukey's test was applied. (ns) represents not significant. Sample size: Saline-WT (n=5), Saline Ubc9-Tg (n=4), MPTP-WT (n=7), and MPTP-Ubc9 Tg (n=7)

Chapter V
DISCUSSION

Currently there is no cure for PD, and the only available treatments are to alleviate the symptoms of PD. The most common treatment, the administration of L-DOPA has been used to increase dopamine levels in the basal ganglia of the brain. L-DOPA can cross the blood bran barrier, whereas dopamine cannot. L-DOPA enters the central nervous system and is converted to dopamine by L-amino acid decarboxylase. However, this approach is far from curing or preventing PD pathology. Therefore, my thesis research focuses on identifying a potential regulatory target to prevent the PD pathology or at least slowing the pathological progress.

Our *in vitro* findings showed that the overexpression of Ubc9 in N27 cells protects dopaminergic cells against H_2O_2. However, Ubc9 overexpression did not significantly protect dopaminergic cells from MPP+ induced toxicity, although Ubc9 overexpression exhibits neuroprotective effects by reducing the levels of ROS (Figure 9). Therefore, we suspect technical problems in measuring the LDH levels reliably in N27 parental cells. Lately we have experienced that the MPP+ induced toxicities were varied in N27 cells, depending on the batch of experiments. Thus, we need to check the level of DAT expression from N27 cell lines, although we routinely checked the levels of TH and DAT from N27 parental cells. If the N27 cells do not express DAT heavily, we do not expect to see the significant damage by MPP+. Therefore, we may need to consider getting even earlier passage of N27 parental cells for rigor and reproducibility of the project. Currently, we are repeating the LDH assay with MPP+ treatment using early passaged N27 cells and freshly prepared MPP+ aliquot.

It is known that MPP+ causes mitochondrial damage, resulting in oxidative stress (Vila *et al.,* 2003). Our preliminary data suggest that Ubc9 over-expression protects dopaminergic cells from oxidative stress induced by MPP+. Our next experiment is to measure the mitochondrial respiration functions directly from EGFP and Ubc9-EGFP cells treated with MPP+, using recently established Seahorse XF-96 system (Agilent Biotech) in the department. We plan to measure the levels of oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and ATP synthesis using the Seahorse technology. We expect to see that Ubc9 over-expression prevents the mitochondrial dysfunction from MPP+ induced complex 1 damage, which would be novel findings since there is no studies executed in testing the hypothesis that Ubc9-mediated pan-SUMOylation protects mitochondrial functions against oxidative stress.

Our *in vivo* studies showed promising results that the overexpression of Ubc9 protects dopaminergic neurons in the striatum against oxidative stress. As a follow-up, we are also analyzing the TH+ DA neurons in the SN between Ubc9 overexpressing Tg mice and WT MPTP-treated mice. As seen in Figure 8, our finding of an increase of DA neurons in MPTP-lesioned Ubc9-Tg mice compared to MPTP-lesioned wildtype mice, is expected to be verified in the SN as well. A significant depletion of DA neurons in the striatum from MPTP toxicity was prevented by the overexpression of Ubc9, by which we can suggest that SUMOylation in the DA neurons can be a potential therapeutic target in PD pathology. Our in vivo results appear to support our hypothesis compared to the in vitro results due to experimental errors.

Future experiments will include the replication of Western blotting and behavioral tests. As seen in Figure 16, the loading control varied among the treatment groups. A new batch of saline and MPTP lesioned mice will be utilized for future experiments in hopes to improve

Western blot results and behavioral tests, in addition to increase the sample size for confocal imaging (n>6 per group). Due to the large error bars from our behavioral data, we cannot significantly conclude from the data. The inconsistency seen for the behavioral analysis may be derived from improperly habituated the animals. Future studies will include the interaction of MPTP/MPP+ with Ubc9 to determine the mechanisms of neuroprotection by Ubc9 or SUMOylation. Although more studies are needed to elucidate the potential neuroprotective mechanisms of Ubc9 overexpression and pan-SUMOylation, we hypothesize that oxidative stress induces deSUMOylation in numerous proteins, possibly including alpha-synuclein in DA neurons (Rott *et al.*, 2017). Furthermore, we plan to assess this hypothesis using human PD midbrain samples, compared with age-matched samples. Recently we have received 10 human PD post-mortem midbrain samples and age- and gender-matched (age range 75-89) 10 non-PD midbrain samples from the NIH NeuroBioBank (request #197) through Dr. Yoon-Seung Kim at the Central Florida University, school of medicine. Based on our promising preliminary data from Ubc9 overexpression mediated neuroprotective effects in PD models, our approach is to regulate the level of SUMO in DA neurons, which can be a potential regulatory target to prevent PD pathology. The overexpression of Ubc9 could be a possible regulatory target for PD. More studies are needed to demonstrate the behavioral effects in mice to confirm that the overexpression of Ubc9 prevents motor dysfunction. *In vitro* and *in vivo* studies strongly suggest that Ubc9 overexpression protects DA cells and neurons against oxidative stress in PD *in vitro* and *in vivo* models.

REFERENCES

Bandopadhyay, R., Kingsbury, A. E., Cookson, M. R., Reid, A. R., Evans, I. M., Hope, A. D., *et al.* (2004). The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain: A Journal of Neurology, 127(Pt 2), 420–430.

Blesa, J., Trigo-Damas, I., Quiroga-Varela, A., & Jackson-Lewis, V. R. (2015). Oxidative stress and Parkinson's disease. Frontiers in Neuroanatomy, 9.

Brieger, K., Schiavone, S., Miller, F. J., and Krause, K.-H. (2012). Reactive oxygen species: from health to disease. Swiss Med. Wkly 142.

Canet-Aviles, R. M., Wilson, M. A., Miller, D. W., Ahmad, R., McLendon, C., Bandyopadhyay, S., *et al.* (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cysteinesulfinic acid-driven mitochondrial localization. Proceedings of the National Academy of Sciences of the United States of America, 101(24), 9103–9108.

Cartier E, Garcia-Olivares J, Janezic E, Lin ML, Viana J, Moore M, Caplan JL, Torres G, and Kim YH. The SUMO conjugase Ubc9 delays the degradation of Dopamine Transporter, enhancing its cell surface level and dopamine uptake. Frontiers Cell Neuroscience. Under 3rd review after moderate critiques

Chandra, S., Fornai, F., Kwon, H. B., Yazdani, U., Atasoy, D., Liu, X., *et al.* (2004). Double-knockout mice for alpha- and betasynucleins: Effect on synaptic functions. Proceedings of the National Academy of Sciences of the United States of America, 101(41), 14966–14971.

Chandra, S., Gallardo, G., Fernandez-Chacon, R., Schluter, O. M., & Sudhof, T. C. (2005). Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. Cell, 123(3), 383–396.

Chen, D., Gao, F., Li, B., Wang, H., Xu, Y., Zhu, C., *et al.* (2010). Parkin mono- ubiquitinates Bcl-2 and regulates autophagy. The Journal of Biological Chemistry, 285(49), 38214–38223.

de Souza, A., Prediger, R., Cimarosti, H. (2016). SUMO-regulated mitochondrial function in Parkinson's disease. Journal of Neurochemistry. 137, 673-686.

Eckermann, K. (2013). SUMO and Parkinson's Disease. NeuroMolecular Medicine, 15(4), 737-759.

Harder Z, Zunino R, McBride H. (2004). Sumo1 conjugates mitochondrial substrates and participates in mitochondrial fission. Curr Biol 14.340–34

Hwang, Onyou. (2013) Role of Oxidative Stress in Parkinsons Disease. Experimental Neurobiology, 22(1).

Kaminsky R, Denison C, Bening-Abu-Shach U, Chisholm AD, Gygi SP, Broday L (2009). SUMO regulates the assembly and function of a cytoplasmic intermediate filament protein in C. elegans. Dev Cell 17:724–735

Krumova, P., & Weishaupt, J. H. (2013). Sumoylation in neurodegenerative diseases. Cellular and Molecular Life Sciences, 70(12), 2123-2138.

Krumova P., Meulmeester E., Garrido M. *et al.*(2011). Sumoylation inhibits a synuclein aggregation and toxicity. Journal of Cell Biology.194,49–60.

Kumaran, R., Vandrovcova, J., Luk, C., Sharma, S., Renton, A., Wood, N. W., *et al.* (2009). Differential DJ-1 gene expression in Parkinson's disease. Neurobiology of Disease, 36(2), 393–400.

Langston JW, Irwin I, Langston EB, & Forno LS (1984) 1-Methyl-4-phenylpyridinium ion (MPP+): Identification of a metabolite of MPTP, a toxin selective to the substantia nigra. Neurosci Lett, 48, 87-92.

Maroteaux, L., Campanelli, J. T., & Scheller, R. H. (1988). Synuclein: A neuron-specific protein localized to the nucleus and presynaptic nerve terminal. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 8(8), 2804–2815.

Muller, F. L., Liu, Y., & Remmen, H. V. (2004). Complex III Releases Superoxide to Both Sides of the Inner Mitochondrial Membrane. Journal of Biological Chemistry, 279(47), 49064-49073.

Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., *et al.* (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science, 276(5321), 2045–2047.

Rizzu, P., Hinkle, D. A., Zhukareva, V., Bonifati, V., Severijnen, L. A., Martinez, D., *et al.* (2004). DJ-1 colocalizes with tau inclusions: A link between parkinsonism and dementia. Annals of Neurology, 55(1), 113–118.

Ryan, B. J., Hoek, S., Fon, E. A., and Wade-Martins, R. (2015). Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. TrendsBiochem.Sci.40,200–210.

Schapira A. H., Cooper J. M., Dexter D., Clark J. B., Jenner P. and Marsden C. D. (1990). Mitochondrial complex I deficiency in Parkinson's disease. J. Neurochem. 54, 823–827.

Shen, et al., The structure of SENP1–SUMO-2 complex suggests a structural basis for discrimination between SUMO paralogues during processing. (2006). Biochemical Journal. 397, 279–288.

Shimura, H., Hattori, N., Kubo, S., Yoshikawa, M., Kitada, T., Matsumine, H., *et al.* (1999). Immunohistochemical and subcellular localization of Parkin protein: Absence of protein in autosomal recessive juvenile parkinsonism patients. Annals of Neurology, 45(5), 668–672.

Shinbo Y, Niki T, Taira T, Ooe H, Takahashi-Niki K, Maita C, Seino C, Iguchi-Ariga SM, Ariga H (2006). Proper SUMO-1 conjugation is essential to DJ-1 to exert its full activities. Cell Death Differ 13:96–108

Um, J. W., & Chung, K. C. (2006). Functional modulation of parkin through physical interaction with SUMO-1. Journal of Neuroscience Research, 84(7), 1543–1554.

Vera, Dias. (2013). The Role of Oxidative Stress in Parkinson's Disease. Journal of Parkinson's Disease.

Vila, M., & Przedborski, S. (2003). Targeting programmed cell death in neurodegenerative diseases. Nature Reviews Neuroscience, 4(5), 365-375.

Zeng, X., Geng. W., Jia, J., Chen, L., and Zheng, P. (2018). Cellular and Molecular Basis of Neurodegeneration in Parkinson Disease. Frontiers in Aging Neuroscience, (10).

Wasiak S, Zunino R, McBride HM (2007). Bax/Bak promote sumoylation of DRP1 and its stable association with mitochondria during apoptotic cell death. J Cell Biol.