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MICRORNA REGULATION AND CELLULAR PROTEOSTASIS IN PARKINSON'S DISEASE

A dissertation submitted in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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at

ST. JOHN'S UNIVERSITY
New York
by
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ABSTRACT

MICRORNA REGULATION AND CELLULAR PROTEOSTASIS IN PARKINSON'S DISEASE

Alberim Kurtishi

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder predominately affecting the aging population. It is the second most common neurodegenerative disorder after Alzheimer's disease (AD) affecting over 1 million individuals in the US alone. Recent studies have tried to understand the exact nature in which healthy neurons transition to a degenerative state in PD. There are a multitude of combinatory factors that can lead to the development of PD including environmental factors, genetic factors, and aging. The majority of PD cases are sporadic in nature, however familial cases account for 5-10% of total PD cases world-wide. The hallmark feature of PD is the formation of Lewy bodies, abnormal protein aggregates enriched in the protein α-synuclein (a-syn) in dopaminergic neurons of the substantia nigra. These aggregates affect the overall protein homeostasis (proteostasis) in neurons causing deleterious effects. The lack of accurate diagnostic biomarkers clearly represents a challenge for PD patients and their caregivers.

It has become increasingly apparent that microRNAs (miRNA), key regulators of gene expression, are involved in numerous disease processes including PD. We investigated miR-335-5p and miR-3613-3p, two microRNAs previously reported by our laboratory, to be significantly up-regulated in serum samples of PD patients as compared to healthy age-matched controls. We also corroborated those findings when we extracted

RNA from frontal cortex tissue of PD patients and age-matched controls finding that miR-335-5p, miR-3613-3p, and miR-6865-3p were all significantly upregulated in patients with PD. Using mass spectrometry and *in silico* prediction methods we identified a number of potential protein targets for both miR-335-5p and miR-3613-3p. Using several molecular approaches, we found that three proteins involved in PD pathogenesis are regulated by miR-335-5p and miR-3613-3p, respectively. Ataxin-3 (ATXN3), BCL2 Associated athanogene 5 (BAG5), and Autophagy related 5 (ATG5) are all proteins targeted and regulated by both miR-335-5p and miR-3613-3p. miR-335-5p and miR-3613-3p have not previously been characterized for their potential biological roles with respect to neurodegeneration or neuroprotection. In this study we further dissect the neuroprotective role of both miR-335-5p and miR-3613-3p in cells induced with stress. Our results significantly contribute to our understanding of PD and the contributing factors to neurodegeneration.

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ABBREVIATIONS

°C ……. Degrees Celsius

AD ……. Alzheimer's Disease

ALS ……... Amyotrophic Lateral Sclerosis

APP ……. Amyloid Precursor Protein

ATG5 …….. Autophagy Related Gene 5

ATXN3 ……. Ataxin 3

BAG5 …… Bcl-2-associated athanogene 5

BME ……. β-Mecaptoethanol

BSA ……. Bovine Serum Albumin

cDNA …….. Complementary DNA

CMA ……... Chaperone Mediated Autophagy

CSF …….. Cerebrospinal fluid

DA …… Dopaminergic

DIW …….. Deionized water

DLB …….. Dementia with Lewy Bodies

DMSO …… Dimethoxysulfoxide

DNA …… Deoxyribonucleic acid

GBA ……. Glucocerebrosidase

GD …….. Gaucher's disease

HD ……. Huntington's disease

Hsp70 ……... Heat shock protein 70

kDa …….. Kilodalton's

L …… Liter

L-DOPA ……... Levo-dopamine

LB ……. Lewy bodies

LB …… Lysogeny Broth

LRRK2 ……. Leucine-rich repeat kinase 2

M ……. Molar

MCS …… Multiple cloning site

Min …….. Minutes

miRNA …… microRNA

MPTP ……... 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine

mRNA …… Messenger RNA

mtDNA …… Mitochondrial DNA

ng ……... Nanograms

NIH ……. National institutes of Health

NTC …… No Template Control

O/N ……... Overnight

PAGE …… Polyacrylamide gel electrophoresis

PARKmiRs …… Diagnostic biomarker-miRNAs in PD

PBS ……... Phosphate buffered saline

PCR …….. Polymerase chain reaction

PD ……... Parkinson's disease

PINK1 …… PTEN-induced putative kinase 1

QC …… Quality Control

qRT-PCR …….. Real-time polymerase chain reaction

RNA ……. Ribonucleic acid

RNase ……. Ribonuclease

ROS …….. Reactive oxygen species

Rpm …… Revolutions per minute

RT ……. Room Temperature

RT-PCR …… Reverse Transcriptase polymerase chain reaction

SDS ……. Sodium dodecyl sulfate

SNc ……. Substantia nigra pars compacta

SNCA ……. Alpha-synuclein

TE ……. Tris-EDTA solution

TEMED …… N,N,N,N'-tetramethyl ethylene diamine

Tris …… Tris (hydroxymethyl) aminomethane

UV …….. Ultra-violet

v/v …… Volume to Volume

w/v …… Weight to Volume

WT ……. Wild-type

µg …….. Microgram

µl …… Microliter

µM …… micromolar

Introduction

1.1. Parkinson's Disease

Neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and Amyotrophic lateral sclerosis (ALS) are all caused by neuronal dysfunction and cell death. Neurodegeneration is a complex process often influenced by a combination of genetic, molecular, and environmental factors [1]. PD is an irreversible and slow progressing disorder involving both motor and non-motor symptoms such as sleep disorders, loss of smell, depression, and skin problems [2]. PD is the second most common neurodegenerative disorder associated, with selective degeneration of dopamine-producing neurons in the substantia nigra pars compacta (SNpc) [3]. The appearance of Lewy Bodies (LB) is a pathological hallmark of the disease and was first discovered by Frederic Lewy in 1912. These LBs are abnormal aggregation of proteins mainly alpha-synuclein (α-syn), that cause an array of issues for neuronal cells, ultimately leading to cell death [4]. PD affects over 5 million individuals worldwide, and over 1% of the US population over the age of 60. This number is grossly underestimated because of the limitations we currently have diagnosing this disease.

1.2. Symptoms

PD is characterized by both motor and non-motor symptoms, but most individuals are diagnosed when characteristic motor symptoms appear. It has been shown that by the time motor symptoms occur, 60-80% of dopaminergic neurons have already been compromised severely limiting treatment options [5]. Parkinsonism is a syndrome characterized by tremor, bradykinesia, rigidity, and postural instability [6]. These four cardinal signs are what physicians currently use to diagnose patients with suspected PD along with the clinical history of the patient and a possible DaTSCAN.

Tremor is the most common and well-known symptom of the four major motor symptoms. Tremor is defined as a rhythmical, involuntary oscillatory movement of a body part [7]. Resting tremor is developed at the onset of the disease and worsens as PD progresses [8]. Making an accurate diagnosis of tremor disorders is challenging, since similar disorders such as essential tremor (ET) and dystonic tremor can all cause misdiagnosis [9]. Tremors are more common in arms compared to legs and the frequency of these tremors is usually 4-6 hertz [10].

Bradykinesia is characterized by reduction in spontaneous movements giving an appearance of stiffness. This stiffness and reduction in movement affects a patients ability to perform daily tasks such as writing, brushing teeth, and getting dressed [10]. There are a range of issues that are related to bradykinesia, however in most cases the principal deficit is that movements are slow. Several factors contribute to bradykinesia such as muscle weakness, tremor, rigidity, and the primary factor is due to insufficient recruitment of muscle force during initiation of movement, this results in patients' movements undershoot their targets [11].

Rigidity is a hypertonic state characterized by constant resistance throughout range of motion that is independent of velocity [12]. Rigidity is one of the physician's telltale sign that helps them diagnose PD. It is caused by loss in muscle tone accompanied by extreme stiffness [8]. Further, rigidity is often asymmetric but affects the entire body as the disease progresses. This can lead to problems with achiness or pain in the muscles or joints affected. Many people with PD will have reduced arm swing when walking, more so on the most affected side. It has also been shown that rigidity negatively impacts sleep quality due to pain making it hard to fall or stay asleep [13].

Postural instability refers to imbalance and loss of ability to stand upright. This loss of reflex and imbalance leads to frequent falls, which may cause bone fractures [8]. Postural instability has been identified as a feature of late-stage PD, and considered one of the most important criteria for diagnosing and categorizing PD patients [14]. These motor impairments are caused by dopaminergic neuronal deficits [15]. One study found that about 38% of patients experience falls due to instability and the frequency of falls increases with severity of the disease [16]. These falls are the most common reason for emergency room visits and largest motor related contributor to health care costs in PD patients [17].

There are also some non-motor symptoms associated with PD, which include sleep disorders, psychosis, dementia, depression, hallucinations, and mood disorders to name a few [8]. Gastrointestinal (GI) issues such as constipation and excessive saliva, as well as loss of smell, are also observed in patients especially during the early stages of the disease, and usually antedate the first occurrence of motor signs [18]. In 2003, Braak et al. hypothesized that an unknown pathogen in the gut could be responsible for the

initiation of sporadic PD [3]. Braak postulated that sporadic PD starts in two places: the neurons of the nasal cavity and the neurons in the gut causing the spread of α-syn via the vagus nerve and olfactory bulb [19]. The Braak hypothesis has also received criticism due to the fact that not all PD patients follow the proposed staging system. The mean age of onset is 60, however some cases appear between the ages of 20-50 [10]. Some studies point to PD being more common in men as compared to women [20].

1.3. Four factors influencing PD pathogenesis

The exact cause for the onset of PD is still unknown, however there are many risk factors associated with developing PD. It is unlikely that a single factor contributes to PD, instead a multitude of factors such as age, genetics, environmental factors, and many molecular factors such as protein aggregation, autophagy, and mitochondrial dysfunction most probably act in concert.

Age is usually one of the major factors when it comes to neurodegenerative diseases [21], and PD is characterized as an age-related disorder. The risk of developing idiopathic PD after the age of 60 increases dramatically. This age-related risk is due to a multitude of factors which include the inherent weakening of the neuronal cellular repaid system [22], as well as increased genetic mutations. Neuronal protein aggregation represents a hallmark of PD pathogenesis and proteosome dysfunction may contribute to the increased susceptibility of developing PD [23]. Mitochondrial dysfunction also increases with age and can lead to increased levels of reactive oxygen species (ROS) [24] which has damaging cellular effects contributing to PD.

Genetics also play an important role in both idiopathic and familial PD. Most PD cases are sporadic, however approximately 10% of cases are familial or genetic [25]. Mutations in genes such as Leucine-rich repeat kinase 2 (Lrrk2), PTEN-induced putative kinase 1 (PINK1), a-syn and Htra serine peptidase 2 (Park13) are all associated with PD onset and progression [26, 27]. Autosomal recessive forms of early-onset PD are caused by mutations in Parkin (PARK2) [28], and PTEN-induced putative kinase 1 (PINK1) [29], both Parkin and PINK1 lead to mitochondrial dysfunction through the E3-ubiquitin ligase pathway [30]. Despite extensive studies the molecular and genetic pathways leading to the onset and progression of PD are poorly understood.

Environmental factors have also been shown to have a causative role in the onset of PD. For example, paraquat (1'-dimethyl-4-4'-bypyridinium dichloride) has been associated with PD where subjects, usually farmers associated with this pesticide, have an increased risk of developing PD [31]. Paraquat causes an increase in ROS levels leading to increased oxidative stress in neurons [32]. Insecticides and Organochlorides, such as dieldrin, have also been shown to increase the risk of developing PD [33]. Interestingly, some studies have also suggested that metal toxicity is correlated with the risk of developing PD [34]. Indeed, one finding showed that postmortem PD brains had increased iron levels [34], and that this elevation could in turn cause an in increase in oxidative stress ultimately leading to cell death. Metal toxicity alone does not necessarily increase the risk of developing PD, however coupled with other contributing factors it may increase the occurrence of PD.

Molecular factors contributing to the onset of PD include protein aggregation, oxidative stress, and autophagy and are described in more detail in the following sections.

However, as a high level summary all of these factors contribute to proteostasis dysfunction in cells.

1.3.1. Protein Aggregation

The aggregation of disease-specific proteins is a hallmark of many neurodegenerative disorders. The presence of pathological aggregates, often called bodies or inclusions, certainly classify both PD and AD. In most cases these pathological aggregates result from the accumulation of misfolded and damaged proteins. In PD, LBs are enriched in α-syn and this aggregation has been correlated with inappropriate protein misfolding, and protein accumulations. α-syn proteotoxicity has traditionally be attributed to LB formation in neurons. However, more recent studies have emerged suggesting that smaller non-fibrillar, multimeric species of α-syn are more significant to PD pathogenesis [35, 36]. These multimers interact with and damage cell membranes [37]. Inappropriate autophagy is also implicated in the abnormal protein accumulation and aggregation of proteins such as *SNCA* as cells age. Proteosome dysfunction is also responsible for accumulation of aggregated proteins such as a-syn due to its inability to clear misfolded proteins [38].

1.3.2. **Oxidative stress**

Oxidative stress is the major source of mitochondrial dysfunction, which is caused either by dysfunctioning mitochondria producing more ROS or the inabilty to sustain proper regulation of ROS [39]. The consequences of oxidative stress are overarching and devastating to the cell; due to its deleterious effects oxidative stress is a key element to

elucidating the link between neurodegenerative diseases and age [39]. Oxidative stress can alter transcription and translation of disease proteins by reacting with transcription factors or altering enzymes respectively. Neurons combat oxidative stress by employing a battery of antioxidant proteins called Peroxiredoxins involved in regulating H_2O_2 levels [40]. Oxidative stress contributes to neuronal cell death in PD due to a gradual decrease of the cells ability to cope with cellular damage over time [41].

Several key PD-associated proteins are linked to mitochondrial function and ROS regulation such as, PINK1, PARK13, Parkin, and α-syn. PINK1 along with Parkin regulates mitophagy in response to mitochondrial dysfunction. PARK13, also activated by PINK1, is a protease, which removes damaged mitochondrial proteins [42]. PARK13 mutations have been associated with oxidative stress and PD phenotypes, while overexpression and knockout mouse models have shown both neuroprotection and PD phenotypes [43, 44]. The accumulation of a-syn on the outer and inner membranes of mitochondria in dopaminergic neurons decrease mitochondria complex 1 activity and induced ROS levels [45]. Furthermore, mitochondria are fundamental regulators of apoptosis, which makes oxidative stress-induced mitochondrial dysfunction an important factor in relation to both PD and aging [46].

1.3.3. Autophagy

Autophagy is a cellular process that involves the efficient and selective degradation of misfolded/ aggregated proteins [47]. Autophagy is a lysosomal-mediated degradation pathway that is activated under stress conditions to degrade and eliminate damaged organelles and proteins [48]. The process of autophagy is driven by a group of

autophagy-related proteins (ATGs) that are responsible for autophagosome formation, vesicle expansion, infusion with the lysosome, and cargo recruitment [49]. A complex of protein kinases initiates the signaling mechanism that controls the activation of autophagy, one of which is the mammalian target of rapamycin (m TOR) [50]. Defects in autophagy are associated with the pathogenesis of many neurodegenerative diseases and controlled modifications of autophagy may prove very useful in the development of possible therapeutics [51]. Autophagy provides a clear link to PD and aging due to the decline in cells ability to properly remove misfolded and aggregated proteins [44]. The pathogenic mutations of α-syn A30P and A53T have been shown to block the uptake of damaged proteins or substances by the lysosome [52]. This inhibition is one of the key aspects in the pathogenesis and progression of PD. It appears therefore that as neurons age the susceptibility to develop PD increases based on reduced autophagy and clearing of misfolded proteins such as *SNCA*. Indeed, mice deficient in the Autophagy-related proteins ATG5 and ATG7 show accumulation of ubiquitinated aggregated proteins causing neuronal stress and ultimately leading to cell death [53]. Another example is illustrated by mitochondrial dysfunction where enlarged mitochondria do not get removed by mitophagy [54]. The loss of function of mitophagy is caused by elevated ROS levels and decreased lysosomal-autophagic degradation resulting from mutations in DJ-1, a protein involved in cellular protection against oxidative stress [55]. Indeed, enlarged mitochondria are often observed in PD subjects, possibly resulting from the fusion of damaged mitochondria and compromised mitophagy [54]. This enlarged mass is not observed in healthy subjects which show a 30% decrease in mitochondrial mass [56]. Combined these results point to the importance of autophagy, a regulated and orderly

recycling of unnecessary or dysfunctional components of the cell. This process needs to be tightly regulated by chaperones and proteins to maintain cellular homeostasis.

1.4. Clinical significance

Despite extensive research in the field the exact processes that contribute to the onset of PD is poorly understood. Similarly, there has been no test formulated to correctly diagnose PD. This presents a unique opportunity where combining research strategies such as cellular assays and miRNA technology may lead to the identification of new targets and pathways that could act as potential biomarkers for the identification of PD. There is no biochemical test available to accurately diagnose PD, and patients often have to visit multiple physicians and perform multiple tests to diagnose [57]. The most effective drug to treat PD symptoms is dopamine replacement with Levodopa (L-Dopa), which was discovered in the 1960s. This treatment is only effective for a short period of time and also has severe side effects such as motor dyskinesia and increased oxidative stress caused by metabolites of dopamine [58] . There is a great need to establish novel pathways for potential therapeutics and drug design, as well as the development of biomarkers for diagnostic purposes as well as monitoring disease progression.

1.5. miRNAs

miRNAs are a class of non-coding RNAs, 22 nucleotides in length, that play important roles in gene regulation. Among all regulatory molecules, miRNAs are the most studied, particularly as regulators in human diseases [59]. Most miRNAs are transcribed from DNA sequences into primary miRNAs (Pri-miRNA) and processed into precursor

miRNAs (Pre-miRNAs) and mature miRNAs [60]. Pri-miRNAs are transcribed from miRNA-encoding genomic sequences by RNA polymerase II [61, 62], and they are then processed into pre-miRNAs by the microprocessor complex, consisting of and RNA binding protein DiGeorge syndrome critical region 8 (DGCR8) and ribonuclease III enzyme drosha [63]. Once pre-miRNAs are generated they are exported to the cytoplasm by exportin 5 and then processed by dicer [63]. This processing involves the removal of the terminal loop, resulting in a mature miRNA duplex [64]. The directionality of the miRNA strand determines the name of the mature miRNA strand. For instance, the 3p strand originates from the 3' end and the 5p strand originates from the 5' end of the pre-miRNA hairpin [65]. miRNAs are proposed to be downregulators of gene expression via two mechanisms: A). mRNA cleavage and B). translational repression. Through mRNA cleavage the miRNA binds to complementary regions of protein-coding mRNA sequences resulting in RNA induced silencing complex (RISC) cleavage [66]. Alternatively, and in the absence of appropriate complementarity, miRNAs also have the ability to bind to 3'UTRs and block translation [66]. Intriguingly, miRNAs do not solely function as target-specific regulators but may play key roles in post-transcriptional reduction of expression [67].

Many studies have noted circulating miRNAs in biological fluids such as plasma, serum, cerebrospinal fluid, saliva, and breast milk to name a few [68-71]. These extracellular miRNAs are highly stable resisting degradation at room temperature, high or low pH, and multiple freeze thaw cycles [72]. Extracellular miRNAs can be either found in vesicles such as exosomes and microvesicles [73] or associated with proteins such as Argonaute (AGO2) [70]. The presence of miRNAs in vesicles or with proteins is thought

to protect miRNAs and increase stability in extracellular fluid [70]. It is now accepted that extracellular circulating miRNAs can play important roles in intracellular communication also serving as potential biomarkers for disease.

1.6. A-syn

A-syn is primarily expressed in neuronal tissues and is linked genetically and neuropathologically to PD. A neuropathological hallmark of PD is the presence of LB which are insoluble aggregates enriched in the a-syn protein [74]. The SNCA gene encodes for an 140 amino acid protein that does not have a defined structure and whose function is not fully understood. In 1997, *SNCA* became one of the first reported genetic aberration linked to PD [26]. The mutation corresponded to substitution in the *SNCA*, resulting in an A53T amino acid change in the a-syn protein [26]. The pattern of inheritance was autosomal-dominant and the disease was early-onset, generally around the age of 40. Soon after two more autosomal dominant pathogenic *SNCA* mutations were also discovered, A30P and E46K [75, 76]. In 2003, an *SNCA* triplication event was reported, resulting in a two-fold increase in a-syn protein levels, which could ultimately lead to development of PD [77].

A-syn may contribute to PD in multiple ways but it is generally thought that its protofibrils are the toxic species that mediate disruption of the cellular homeostasis and neuronal death [78]. Furthermore, secreted a-syn may exert deleterious effects on neighboring cells including seeding of aggregation, possibly contributing to the pathogenesis of PD [79]. Although genetic and biochemical studies are revealing

important aspects of a-syn with respect to PD, it is still unclear how changes in *SNCA*/a-syn influences disease onset and progression.

1.7. Parkin

Parkin is an E3 ubiquitin ligase which ubiquitinates mitochondrial proteins and targets them for proteasomal degradation [30]. Mutations in Parkin cause loss of E3 ligase function, and account for the second most common cause of PD [28]. Parkin plays an important role in Familial PD due to genetic defects in Parkin causing autosomal recessive PD. PINK1 acts upstream of Parkin and is required for Parkin activation and recruitment to depolarized mitochondria [80]. Parkin gets selectively recruited to damaged mitochondria and brings about ATG5-dependent mitophagy by recruiting ubiquitin and p62 to mitochondrial membrane [81]. Parkin overexpression acts as a protective agent reducing proteotoxicity in dopaminergic neurons [82].

Parkin has also been shown to prevent cell death through proteasomal degradation of certain aggregated proteins such as *SNCA*. The exact mechanism of how over expression protects against proteotoxicity is unknown, but it seems to be dependent on its E3 ligase activity [83]. Mitochondrial deficits are also very prevalent in Parkin deficient patients where decreased lymphocyte mitochondrial complex I is observed [84], and this has also been observed in Parkin-knockout mice which show oxidative stress and mitochondrial dysfunction [85]. Numerous mutations throughout the PRKN gene are linked to autosomal recessive PD which is associated with early-onset PD [86], making examination of Parkin crucial to understanding the pathogenesis of PD.

1.8. ATG5

Autophagy is a highly conserved homeostatic process from yeast to mammals that involves degradation of certain intracellular molecules and organelles [87]. Autophagy is a tightly regulated process with 41 Autophagy-related proteins [88]. The autophagy pathway plays an important role in the pathological process of neurodegenerative diseases like PD. Autophagy related gene 5 (ATG5) plays an important role in initiation and vacuole formation in autophagy [89]. Knocking down ATG5 can result in downregulation or inhibition of autophagy suggesting that ATG5 plays an essential role in autophagy, and thus ATG5 is one of the most targeted genes in autophagy gene editing assays [90]. ATG5 initiates the formation of the autophagosome membrane and the fusion of autophagosomes and lysosomes functioning in both canonical and non-canonical autophagy. ATG5 has been shown to play a protective role in neurons displaying synucleinopathies and ATG5 deficiencies led to deficits in motor function with accumulation of cytoplasmic inclusion bodies in the neurons of mice [91]. Autophagy is essential to the clearing of misfolded/aggregated proteins, therefore disruption in the initiation or any steps can lead to cell death. One finding displayed that ATG5 acted as a protective mechanism in zebrafish induced with the compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) a mitochondrial toxin [92]. MPTP is a standard compound used to establish PD models *in vitro* and *in vivo* causing damage to dopaminergic neurons [93]. ATG5 downregulation along with treatment impacted the progression of PD and PD-related markers, however when ATG5 was overexpressed it showed a restorative function and reversed the pathological progress of PD [92]. In

patients with PD, ATG5 protein levels were altered, which suggests that ATG5 may serve as a PD-related marker and risk factor [94].

1.9. Ataxin-3

Post-translational modification of proteins is central to regulating stability and activity. One important modification involved in cellular homeostasis is ubiquitination which involves tagging proteins for degradation [95]. Ataxin-3 (ATXN3) is a deubiquitinating enzyme involved in protein quality control and is involved in removing ubiquitin from proteins prior to degradation so that the ubiquitin may be recycled [96]. It's known activities include regulating the action of E3 ligases, participating in proteasomal substrate delivery, and regulating aggresome formation [97]. Mutations in *SCA3*, the gene encoding ataxin-3 causing repeat expansion causes spinocerebellar ataxia-3 (SCA3), the most common inherited ataxia worldwide [98]. PolyQ diseases are age-related, progressive disorders that typically first manifest in midlife, leading to death 15-30 years later [99]. A common neuropathological hallmark in SCA3 is the accumulation of ubiquitin-positive nuclear inclusions in neurons [100]. It has been reported that patients with SCA3 have been shown to present parkinsonian symptoms [101]. Recent studies have shown that ataxin-3 interacts with Parkin [102] and mutations in ATXN3 can deubiquitinate parkin directly and reduce the extent of parkin ubiquitination in cells [102]. The poly (Q) expansion mutant form of ataxin-3 promotes parkin degradation by autophagy. Ataxin-3 has not been directly linked to PD however its interaction with parkin and hinderance of parkin to properly ubiquitinate proteins for degradation has

been linked to parkinsonian phenotypes. Thus, a deeper understanding of Ataxin-3 function and relationship to PD is important in further explaining the pathogenesis of PD.

1.10. BAG5

Misfolding, aggregation, and aberrant accumulation of proteins are hallmarks of many neurodegenerative diseases including PD. Chaperones and cochaperones help guide and execute protein folding as well as shuttling proteins that are destined for degradation [103]. The Bcl-2 associated athanogene 5 (BAG5) is a co-chaperone known to act in both cell survival and cell death pathways. Bag5 has been shown to interact with HSP70 molecular chaperone as well as Parkin [104], and this interaction with Parkin hinders its function in mitophagy resulting in cell death [105]. It has been shown that BAG5 delays parkin recruitment to mitochondria following mitochondrial depolarization, therefore impairing mitophagy and enhancing cell death [105]. The mechanism by which BAG5 impedes Parkin recruitment to mitochondria is not clear however and is in direct contradiction to Bag2 which enhances Parkin's recruitment to mitochondria [104]. It has been reported that physiological stress increases BAG5 expression [106] however conflicting evidence has shown that BAG5 can either play a neuroprotective role as well as a neurotoxic role.

BAG5 has been demonstrated to directly interact with PINK1, and regulated PINK1 degradation via the UPS, and in addition protected mitochondria against MPP+ [107]. These findings suggested that BAG5 played an important role in stabilizing PINK1 by decreasing the ubiquitination of PINK1. Upregulation of BAG5 has also been shown to inhibit apoptosis and increase expression of anti-apoptotic proteins in PC12 cells

induced with MPP+ an apoptosis inducer [108]. Lastly, BAG5 has also been shown to interact with DJ-1 and reduces the level of DJ-1 dimers in the mitochondria, which suggests that BAG5 inhibits mitochondrial translocation of DJ-1 [109]. The role BAG5 plays in neurodegeneration is yet to be revealed due to conflicting evidence of its role within the cell.

2.0. Specific aims

The primary objective of this research is to achieve a deeper understanding of pathways that contribute to neuronal cell death in PD, examine the relationship between PD-related proteins, and discover the involvement of miRNAs in PD.

Specific aims include:

1. Evaluate and verify specific protein targets of miRNA candidates involved in PD.
2. Investigate what role miR-335-5p and miR-3613-3p play in the regulation of key PD related proteins.
3. Examine whether elevated levels of miRs in PD patients play a neuroprotective or neurotoxic role in neurons.
4. Investigate the regulation of ATG5, ATXN3, and BAG5 by miR-335-5p and miR-3613-3p, and how they relate to PD pathogenesis.

2.1. Materials and Methods

Buffers

Universal buffers and their composition used in various protocols are listed below.

Specific buffers are described together with the specific protocols.

Lysis buffers

The recipes for the lysis buffers used during this dissertation are given here.

Following is the list of protein localization and lysis buffer used:

Protein Localization	**Buffer recommended**
Whole cell	NP-40 or RIPA
Cytoplasmic (soluble)	Tris-HCl
Cytoplasmic (cytoskeletal Bound)	Tris-Triton
Membrane bound	NP-40 or RIPA
Nuclear	RIPA
Mitochondria	RIPA

Please consult separate protocols for sub-cellular fractionation.

Nonidet-P40 (NP40) buffer

150mM NaCl, 1% NP-40, 50mM Tris at pH8.0, and 2mM EDTA

Radio Immuno Precipitation Assay buffer (RIPA)

150mM NaCl, 1% NP-40, 50mM Tris at pH 8.0, 0.5% Sodium deoxycholate, and 0.1%

Sodium

Dodecyl sulfate (SDS).

Tris-HCl buffer

20mM Tris-HCl, pH 7.5

Tris-Triton buffer

100mM NaCl, 1% Triton X-100, 10mM Tris at pH 7.4, 0.5% Sodium deoxycholate, 0.1% SDS,

10% Glycerol, 10mM EDTA, and 10mM EGTA.

Western Blot analysis buffers

The recipes for buffers used for western blot analysis including buffers required for pouring

Polyacrylamide gels are listed below.

Table 1.1. SDS Polyacrylamide gel recipe

Component	Stacking gel	Resolving gels			
	4%	8%	12%	15%	20%
Deionized water (DIW)	6.15ml	4.73ml	3.4ml	2.4ml	730ul
30% Acrylamide	1.25ml	2.67ml	4ml	5ml	6.67ml
1.5M Tris Buffer pH 8.8	-	2.5ml	2.5ml	2.5ml	2.5ml
0.5M Tris Buffer pH 6.8	2.5ml	-	-	-	-
10% SDS	100ul	100ul	100ul	100ul	100ul
10% APS	50ul	50ul	50ul	50ul	50ul
TEMED	10ul	5ul	5ul	5ul	5ul

30% Acrylamide solution is 29.2% acrylamide and 0.8% N',N'-bis-methylene-acrylamide.

Tris Buffer (1.5M at pH 8.8, 0.5M at pH 6.8)

Tris base 54.46g and 18.15g was dissolved in DIW; pH was adjusted with 6N HCl to 8.8 and 6.8

Respectively to make up the volume to 300ml with DIW.

Running buffer (10X) and Transfer buffer (10X)

Stock solutions (10X) for running and transfer buffers were prepared with 3.84M Glycine and

0.5M tris base. For running buffer 2% SDS was added.

Tris Buffer Saline (TBS) (10X)

TBS stock solution (10X) was prepared by dissolving 160.1g NaCl and 48.45g tris-HCl; pH was

adjusted to 7.6 with HCl to make a 2L stock solution.

For **TBS-T:** 100ml of TBS (10X) + 900ml ultra-pure water + 1ml Tween20

Bacteriological Techniques

Bacterial Strains

***E.coli* DH5α;** (F^S/*endA1 hsdR17*($rk^S mk^+$), supE44, thi1, *r*ec*A1, gyrA*(Nal^r), *gyrA96*(Nal^r),

relA1, Δ(*lacZYA=argF*) U169, *deoR* (Φ80*dlac* Δ(*lacZ*) *M15*)) was used For propagation and

preparation of plasmid DNA.

Vectors

pJET 1.2/blunt (Appendix. A) (Thermo Fischer Scientific) was used for routine subcloning and

DNA manipulations.

pcDNA3 (Appendix A-) (Thermo Fischer Scientific) was used for heterologous protein

expression and stable cell line generation in mammalian cells.

Growth media and growth conditions

Bacteria were grown using Lysogeny broth (LB-1% Tryptone, 0.5% Yeast extract, and 1%

NaCl) in liquid cultures or LB-agar (1.5% agar added to LB) as colonies. Super optimal broth

With catabolite repression (SOC – 2% Tryptone, 0.5% Yeast extract, 0.02% KCl, 0.25% MgSO4

and 0.06% NaCl, and 036% Glucose; pH 7.0) was used during transformation for

recovery of transformants. Liquid cultures for DH5α were grown in a shaking incubator

at 250rpm at 37°C. Colonies were grown on LB-agar plates incubated overnight (O/N) in

an incubator at 37°C. Colonies were picked using sterile pipette tips and added to culture

tubes for growing liquid cultures with antibiotic selection.

Antibiotic Selection

Antibiotic selection was carried out to select colonies ad grow cultures of positive clones.

Antibiotics were added to medium prior to use (LB) or before pouring plates (LB-agar).

Ampicillin (100μg/ml) was used as selection marker for pJET 1.2/blunt, pcDNA3

vectors; Kanamycin (50μg/ml) was used for pET-28a(+) vector.

Cloning

Primers

Primers used for various cloning experiments as well as semi-quantitative PCR and qRT-PCR experiments are listed below (Table). Restriction endonuclease cutting sites are underlined and in italics, and mutations are in bold/codon is underlined, if applicable.

Table 1.2. List of Primers

No.	Name of Primer (RE site/Tag)	Sequence with RE site & Tag
1)	pcDNA3 forward (100bp MCS)	CGCAAATGGGCGGTAGGCGTGTACG
2)	pcDNA3 reverse (MCS100bp)	TGGCACCTTCCAGGGTCAAGGAAGG
3)	pJET1.2 forward (_MCS)	CGACTCACTATAGGGAGAGCGGC
4)	pJET1.2 reverse (MCS_)	AAGAACATCGATTTTCCATGGCAG
5)	scaRNA17 forward	AGAGGCTTGGGCCGCCGAGC
6)	RNA U6 forward	CGCTTCGGCAGCACATATAC
7)	cel-miR-39-3p forward	ATTCACCGGGTGTAAATCAGC
8)	hsa-miR-335-5p forward	ATTCAAGAGCAATAACGAAAAATGT
9)	hsa-miR-3613-3p forward	ATACAAAAAAAAAAGCCCAACC
10)	hsa-miR-6865-3p forward	ATACACCCTCTTTCCCTACC
11)	hsa-miR-4797-5p forward	ATGACAGAGTGCCACTTACTG
12)	hsa-miR-455-3p forward	ATGCAGTCCATGGGCATATAC
13)	hsa-miR-3910-1 forward	ATTGCTGTCAGTTTTTCTGTTGC
14)	hsa-miR-16-2-3p forward	ATCCAATATTACTGTGCTGC
15)	hsa-miR-937-5p forward	ATGTGAGTCAGGGTGGGGC

Polymerase Chain Reaction (PCR)

PCR was performed to amplify genes of interest from cDNA, and to screen for colonies of positive transformants by colony PCR.

PCR to amplify genes for subsequent cloning:

Phusion® DNA polymerase (Thermo Fischer Scientific) was used to amplify genes or gene products due to its high-fidelity and to get accurate, blunt ended PCR products which were first used for cloning into the shuttle vector pJET 1.2 vector.

PCR reactions were setup as follows:

Component	**1x reaction (µl)**
5x Phusion HF buffer	4
10mM dNTPs	0.2
Phusion DNA Polymerase	0.2
Forward primer	1
Reverse Primer	1
Vector	1
DIW	12

Reactions were setup with the following program:

Step	**Temp. (ºC)**	**Time**
1	98	1min.
2	98	30sec.
3	49	30sec.
4	72	30sec/kb of PCR product
5	Goto step 2, 30x	
6	72	7min
7	4	Forever

PCR to screen for bacterial colonies

Taq PCR Master mix kit (Qiagen, Inc.) was used to perform colony PCR for screening positive transformants. Colonies were grown on LB-agar were picked using sterile pipette tips and added to culture tubes with LB media and appropriate antibiotic selection. Cultures were grown O/N at 37°C in an incubator at 250rpm. 1µl of liquid culture was used as template in each PCR. Specific primer pairs (Table) were used to either confirm the presence and/or orientation of gene product. The reactions were setup as follows:

Component	**1x reaction (µl)**
PCR Master Mix (2X)	10
Forward Primer	1
Reverse Primer	1
Cultures/colony	1
DIW	7

Reactions were setup with the following program:

Step	**Temp. (ºC)**	**Time**
1	95	2min.
2	94	1min.
3	58	1min.
4	72	1min.
5	Goto step 2, 34x	
6	72	5min.
7	4	Forever

Nucleic acid isolations

All DNA isolations were performed using commercial kits following manufacturer's protocol. GeneJET plasmid miniprep kit (Thermo Fischer Scientific) was used for small scale routine isolation of plasmid DNA. QIAquick PCR purification kit (Qiagen) was used to clean up the PCR product to remove components of the PCR mix to obtain purified DNA product for further experiments. GeneJET Gel Extraction kit (Thermo Fischer Scientific) was used to clean up the PCR product to remove primer dimers, and other non-specific DNA bands.

Nucleic acid measurement

Nucleic acid in the form of plasmid DNA, genomic DNA, and cDNA were always, at various stages, ran on agarose gels or NanoDrop 2000 Fluorospectrometer (Thermo Fischer Scientific) for quality control (QC) purpose.

Nucleic acid manipulation

pJET cloning

All genes were first cloned by blunt end cloning into the pJET 1.2 vector using the CloneJET PCR cloning kit (Thermo Fischer Scientific) according to the manufacturer's protocol. Ligated products were transformed into *E. coli* DH5-α cells and colonies were grown under ampicillin selection, and screened for positive transformants using ampicillin selection.

Restriction endonuclease digestions

Restriction endonuclease digests were performed as follows:

Component (per reaction)	**(µl)**
Water (Nuclease free)	3
10X FastDigest buffer	1
DNA	5
Enzyme 1	0.5
Enzyme 2	0.5
Time 30min	**Temp 37℃**

Vector	**Restriction Endonuclease**
pJET 1.2	BglII (2 sites)
pcDNA3	RE flanking the gene of interest

Ligations

Ligation (Thermo Fischer Scientific) reactions were setup as follows:

Component	(µl)
Digested insert	8
Digested Vector (10-50ng)	0.5
T4 DNA ligase	0.5
10X T4 DNA ligase buffer	1
Nuclease free water	0
Total	10

Ligation mix was incubated at RT for 20-30mins. Ligation mix was used for transformations.

Bacterial Transformations

DH5-α cells were thawed on ice and typically 5µl of the ligation reaction was added to the cells. The transformation mix was incubated on ice for 30min. At the end of the incubation, cells were subjected to heat shock at 42°C for 45sec. Immediately followed by incubation on ice for 2-3min. 900µl SOC or LB media was then added to the cells and the cells were incubated at 37°C for 1hr with shaking. Cells were plated onto appropriate LB-agar plates (antibiotic selection). The LB-agar plates were incubated at 37°C for at least 16hrs but no more than 20hrs.

Characterization of bacterial transformants

Bacterial transformants were generally analyzed by two methods mentioned below. Positively transformed colonies were verified by DNA sequencing at the Yale DNA analysis facility, CT. The sequencing data was analyzed using BLAST by NCBI.

Colony PCR for colony screening

1µl of O/N liquid bacterial culture were used for PCR reactions and the remaining was used for subsequent DNA isolation. PCR reactions were setup using the *Taq* PCR Master Mix Kit (Qiagen) and the primer pairs either flanking the MCS in vector, Flanking the gene of interest or one internal primer along with vector specific primer. Samples were run on a 1% agarose gel and colonies that gave the correct sized DNA fragment was used for further analysis.

Agarose gel electrophoresis

PCR products or restriction digests were analyzed on 1% agarose gel electrophoresis. The products were mixed with loading buffer (6X Loading buffer. 30% glycerol, 0.25% bromophenol blue) and ethidium bromide in order to visualize the separated DNA fragments on a UV transluminator. Tris-acetate-EDTA buffer was used to make the gels and also running buffer. The gels were prepared by adding the appropriate amount of agarose to 1X TAE buffer (40mM Tris base, 20mM Glacial acetic acid, and 1mM EDTA at pH 8.0) followed by melting the agarose by heating in a microwave oven, adding ethidum bromide, pouring into a gel cassette and allowing agarose gel to solidify. HiLo DNA marker (Minnesota Molecular) was used as a size reference (Appendix).

RNA processing and analysis

RNA isolation

Total RNA was extracted using specialized kits following the manufacturer's protocol. All the RNA samples were quantified and quality controlled on a Nanodrop 2000 (Thermo Scientific, MA).

RNA isolation (mRNA from mammalian cells)

All RNA isolations were performed 24hrs post-transfection or from stable cells in triplicate. Total RNA was extracted from mammalian cells by using the RNeasy Mini kit (Qiagen) and GeneJET RNA purification kit following manufacturer's protocol. Total RNA (enriched small RNA) was isolated using the miRCURY RNA isolation kit- cell and plant (Exiqon).

RNA isolation (Small RNA from mammalian cells)

All RNA isolations were performed 24hrs post-transfection in triplicate. Total RNA was extracted from mammalian cells using the miRCURY RNA isolation kit – cell and plant (Exiqon) following manufacturer's protocol.

cDNA synthesis

All RNA samples were treated with 1 unit/μg of RNA of DNase-1 (Thermo Scientific) for 30min at 37°C followed by 10min at 65°C with 50mM EDTA. The DNase 1-treated RNA was used to synthesize first strand cDNA using a variety of commercial kits.

cDNA synthesis (from mRNAs)

The DNase 1-treated RNA was used to synthesize first-strand cDNA, by using the RevertAid First Strand cDNA Synthesis kit and an oligo dT primer (Thermo Fischer Scientific) following the manufacturer's instructions. The cDNA samples were used for PCR amplifications of genes of interest for cloning, semi-qualitative PCR, and qRT-PCR.

cDNA synthesis (From small RNAs)

For small RNAs, cDNA was synthesized using DNase 1-treated RNA and the qScript™ miRNA cDNA synthesis kit (Quanta Biosciences) following manufacturer's protocol. The cDNA samples were used for semi-quantitative PCR and qRT-PCR.

Reactions were setup as follows in two steps:

Poly(A) Tailing reaction

Component	Per reaction (uL)
Poly(A) Tailing Buffer (5X)	2
RNA (up to 1ug total)	up to 7uL
Nuclease free water	Variable
Poly(A) Polymerase	1

Mix components from above and incubate at 37°C for 60mins followed by 5mins at 70°C. Centrifuge and keep on ice for cDNA synthesis.

First strand cDNA synthesis reaction

Component	Per reaction (uL)
Poly(A) tailing reaction (from previous step)	10
microRNA cDNA Reaction Mix	9
qScript Reverse Transcriptase	1

Incubate reaction mix for 20mins at 42°C followed by 5mins at 85°C, and allow to gradually come down to 4°C. Store at -20°C for future expression analysis.

Semi-quantitative RT-PCR (mRNA and small RNAs)

cDNA was used for semi-quantitative RT-PCR using appropriate primer pairs (Table) and PCR protocol for 25 cycles. The samples at the end of the reactions were run on 1% agarose (for mRNAs). The gel images were analyzed at the end, to semi-quantitatavely measure gene expression. *GAPDH* (mRNAs) and *U6* (small RNAs) were used as controls in all the semi-quantitative RT-PCR experiments.

Real-time qPCR

Equal amount of cDNA in each sample was used to perform qRT-PCR. All qRT-PCR were performed using PerfeCTa® SYBR® GREEN SuperMix for IQ™ containing AccuStart Taq DNA polymerase (Quanta Biosciences) for 45 cycles followed by dissociation steps with a MyiQ single color Real-time PCR detection system (Bio-Rad, CA). No template control (NTC) was employed.

Reaction was setup as follows:

Component	Per reaction (uL)
PerfeCTa SYBR Green Supermix (2X)	25
PerfeCTa microRNA Assay Primer (10uM)	1
PerfeCTa Universal PCR Primer (10uM)	1
Nuclease Free water	23

Real-time qPCR (mRNA)

For mRNA qRT-PCR, gene specific primers, at a concentration of 1uM were used. The qRT-PCR protocol was as follows: Intial denaturation at 95°C for 2mins followed by 35 cycles of denaturation at 95°C for 15s, annealing at 60°C for 45s and extension at 72°C for 40s. *GAPDH or Actin* was used as reference RNAs for normalizing Cq values to calculate relative expression.

Real-time PCR (Small RNA)

qRT-PCR for small RNA quantification were performed with reaction volume of 20µl with small RNA-specific forward primers and a PerfeCTa Universal PCR primer at a final concentration of 0.2uM. The qRT-PCR protocol involved initial denaturation at 95°C for 2min followed by 45 cycles of denaturation at 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 15s, which was followed by dissociation steps of 0.5°C increments. *scaRNA and U6* were used as reference small RNAs for normalizing Cq values whereas cel-miR-39-3p was used as spike-in control. The standard curve for cel-miR-39-3p was anaylzed in MS excel with $R^2 = 0.97882$ and PCR efficiency 92.96%.

Tissue culture and processing

Tissue culture guidelines:

Table 1.3 Tissue Culture Guidelines

Useful Numbers for Cell Culture

There are various sizes of dishes and flasks used for cell culture. Some useful numbers such as surface area and volumes of dissociation solutions are given below for various size culture vessels.

	Surface Area (mm^2)	Seeding Density	Cells at Confluency[1]	Versene (ml of 0.53 mM EDTA)	Trypsin (ml of 0.05% tryspin, 0.53 mM EDTA)	Growth Medium (ml)
Dishes						
35 mm	962	0.3×10^6	1.2×10^6	1	1	2
60 mm	2,827	0.8×10^6	3.2×10^6	3	2	3
100 mm	7,854	2.2×10^6	8.8×10^6	5	3	10
150 mm	17,671	5.0×10^6	20.0×10^6	10	8	20
Cluster Plates						
6-well	962	0.3×10^6	1.2×10^6	2	2	3–5
12-well	401	0.1×10^6	0.4×10^6	1	1	1–2
24-well	200	0.05×10^6	0.2×10^6	0.5	0.5	0.5–1.0
Flasks						
T-25	2,500	0.7×10^6	2.8×10^6	3	3	3–5
T-75	7,500	2.1×10^6	8.4×10^6	5	5	8–15
T-160	16,000	4.6×10^6	18.4×10^6	10	10	15–30

[1]The number of cells on a confluent plate, dish, or flask will vary with cell type. For this table, HeLa cells were used.

Conversion chart used for tissue culture experiments. Invitrogen.

Cell culture and stock information

Sh-SY5Y cells were cultured in a base medium (1:1 mixture of DMEM/Ham's-F12) (Invitrogen) supplemented with fetal bovine serum (FBS) (Atlanta biologics) at a final concentration of 10% and 2mM GlutaMax (Invitrogen) in a 5% CO_2 atmosphere at 37°C. BE-M17 cells were maintained in the same way. Cells were sub-cultured every 4-5 days

to maintain confluency. Full medium supplemented with 5% DMSO was used as freezing medium to cryopreserve cells.

Transfections

The transfection experiments were performed the day after seeding, for each plasmid transfection, 0.5µg plasmid DNA was diluted with 50µl Opti-MEM (Life technologies, Grand Island, NY) and 1.25µl Lipofectamine 2000 (Invitrogen) was diluted with 50µl of Opti-MEM and incubated for 5min at RT. After incubation both solutions were mixed and incubated for 15mins at RT. This transfection mix was diluted to 2ml with Opti-MEM and added to cells for transfection. Cells were incubated at 37°C in 5% CO_2 atmosphere for 4-6hrs. before replacing with full media. Appropriate empty vector controls were used with each transfection experiment. The cells were used 24hr (RNA) and 48hr (Protein) after transfection and incubation for downstream processing.

Transfection (miRs)

The day after seeding cells were transfected using scrambled control mimic/inhibitor and mimic/inhibitor of miR-335-5p, miR-3613-3p, and miR6865-5p obtained from mirVana™ (Life Technologies), at a final concentration of 20nM. 2µl of each µRNA (20µM stock), was diluted with 100µl of Opti-MEM and 7µl of Lipofectamine RNAiMax (Invitrogen) was diluted with 100µl of Opti-MEM and incubated for 5min at RT. The two solutions were then mixed together and incubated for 15min at RT. The transfection mix was diluted to 2ml with Opti-MEM, added to the cells and incubated at 37°C in 5% CO_2 atmosphere for 4-6hrs before replacing with full media. Cells were harvested after 24hr

for transcript analysis by qRT-PCR and after 48hr for protein analysis by Western Blotting.

Cell Treatments

All treatments were performed in serum-free complete media at 37°C for 6hr, unless indicated otherwise. Untreated cells were maintained in serum-free complete media at 37°C for 6hr as well. Various reagents used for treatments are given in the table below.

Table 1.4. Cellular Treatments

Reagent	Concentrations used	Supplier
H2O2	100µM	J.T. Baker, Center Valley, PA
L-3,4-dihydroxy-phenylalanine (L-DOPA)	100µM	Acros, Pittsburgh, PA
Rotenone	1µM, 10µM, 50µM	Sigma Aldrich
6 hydroxydopamine (6-OHDA)	1µM, 10µM, 100µM	Sigma Aldrich

Cell Harvesting

Cells growing at 85-90% confluency were trypsinized and removed from the plate. Cell suspension was spun down by centrifugation at 500xg at 4°C for 5min, supernatant was discarded and cells were washed with 1ml ice cold PBS and spun down again. Supernatant was discarded and lysis buffer (RIPA) was added to cell pellet, pipetting up and down to break up the pellet. Centrifuge tube was kept on ice for 10mins, followed by

a spin at max speed (25,000xg) for 10min. Supernatant was collected and placed in another labeled centrifuge tube and kept at -20°C for future processes.

Protein analysis

Western blot analysis

Western blot analysis was performed using the following protocol:

Sample preparation: Protein lysates were always thawed on ice and 5x reducing dye was added to samples followed by boiling at 95°C for 5min. Samples were than cooled on ice for 1min, and subjected to quick centrifugation to collect condensate.

SDS-PAGE and Blotting: Whole cell lysates were generally prepared using RIPA buffer. Protein estimation was performed using the DC Protein assay kit (Bio-Rad, Hercules CA) showing <10% variation in protein concentrations among the same fractions. 4µl of 5x Laemmli sample buffer (50mM Tris pH 6.8, 0.1% SDS, 20% glycerol, 0.2% bromophenol blue) with BME was added to 16µl of whole-cell lysates. Samples (20µl) were subjected to SDS-PAGE by using the mini-Protein Tetra system (Bio-Rad) with 12% polyacrylamide slab gels at 100V for 90min in running buffer (192mM glycine, 25mM Tris Base, 0.2% SDS). The separated proteins were transferred to PVDF membranes (Pall Life Sciences, Ann Arbor, MI) in transfer buffer (192mM glycine, 25mM Tris Base, 20% methanol, and 0.005% SDS) at 200mA for 45min, followed by blocking in 5% nonfat dry milk/TBS-T (137mM NaCl, 15.4 mM Trizma HCl, 0.1% Tween 20, pH 7.6) for 1 hour at RT. After blocking the membrane was incubated with the appropriate primary antibody at 1:1000 dilution (unless otherwise indicated) in 2.5%

nonft dry milk overnight at 4°C and washed with TBS-T three times for 10min. After wash appropriate secondary antibody (Jackson Immunoresearch, West Grove, PA) at 1:10,000 dilution for 1hr at RT. The membrane was washed with TBS-T three times for 10min each. The membrane was developed using the Pierce ECL Western blot substrate (Therno Fischer Scientific) and signal detection was recorded with the molecular imager Chemi Doc XRS1 imaging system (Bio-Rad). PageRuler™ Unstained Broad Range Protein Ladder, which has each protein containing an integral Strep-tag II Sequence, was used as a marker for all the gels and blots.

Table 1.5. List of Primary and Secondary antibodies

	Antibody (Dilution used 1:1000 for primary and 1:10000 used for secondary	**Vendor**
	Primary Antibodies	
1)	Rabbit polyclonal anti-GAPDH	Santa Cruz Biotechnology
2)	Mouse monoclonal anti-PARKIN	Santa Cruz Biotechnology
3)	Rabbit polyclonal anti-ATXN3	Abcam
4)	Mouse polyclonal anti-BAG5	Santa Cruz Biotechnology
5)	Rabbit polyclonal anti-ATG5	Santa Cruz Biotechnology
6)	Rabbit polyclonal anti-SOD1	Santa Cruz Biotechnology
7)	Rabbit polyclonal anti-GBA	Santa Cruz Biotechnology
8)	Goat polyclonal anti-Park13	Santa Cruz Biotechnology
9)	Rabbit polyclonal anti-SNCA	Abcam
10)	Rabbit polyclonal anti-LRRK2	Abcam
11)	Rabbit polyclonal anti-PINK1	Novus Biologicals
12)	Mouse monoclonal anti-DJ-1	Enzo Life Sciences
13)	Mouse monoclonal anti-Actin	Santa Cruz Biotechnology
	Secondary Antibodies	
1)	Goat anti-Rabbit IgG (H+L)-HRP	Jackson Immunoresearch
2)	Donkey anti-Goat IgG (H+L)-HRP	Jackson Immunoresearch
3)	Goat anti-Mouse IgG (H+L)-HRP	Jackson Immunoresearch
4)	Precision Protein StrepTactin-HRP	Bio-Rad

Cell Viability assay

Cell viability was measured using the neutral red uptake assay 48hr post transfection. Cells were washed with PBS, 100µl of neutral red working solution (40µg/ml) added to each well and plates were incubated for 2hr. Cells were then washed with PBS, neutral red extracted using 150µl of destain solution (50% ethanol, 1% glacial acetic acid, 49% deionized water) per well and the plates were subjected to shaking for 10min. Absorbance was measured at 540nm using an Epoch microplate spectrophotometer (Bioteck)

Breadford assay for protein estimation

Protein concentrations were measured using protein assay kit II and DC protein assay kit (Bio-Rad, Hercules, CA) Bovine serum albumin was used to prepare incremental standard solutions from 0.05-1.0mg/ml. Assays were performed in 96-well micro-plates and the colormetric reading were recorded using Epoch microplate spectrophotometer (Bioteck). Protein samples were diluted from 1:10 to 1:100 for the assay. Samples for loading were prepared based on the concentrations obtained by the assay.

Image analysis and statistical analysis

ImageJ software (NIH, Bethesda, MA) and IQTL software (GE healthcare) was used to analyze

Western blot as well as agarose gel images. All values represent mean values ± Standard error

unless indicated. Statistical analyses were performed using Microsoft excel unless otherwise

mentioned, with students' t-test, and $p<0.05$ is denoted as significant, unless mentioned

otherwise.

Main Project: microRNA regulation of key PD proteins

Introduction

PD is the most common, progressive neurodegenerative movement disorder characterized by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) [110]. The etiology of PD has been linked to age, environmental factors, and mutations in several genes including *SNCA*, Parkin, and PINK1 [111]. Aggregation of misfolded proteins causing inclusion bodies is a hallmark of PD [112]. The presence of LBs enriched in the protein a-syn plays a major role in neuronal cell death enhancing the progression of Parkinson's like neurodegeneration [113, 114]. The E3 ubiquitin ligase Parkin plays a prominent role in both familial and sporadic PD, although Parkin is neuroprotective and promotes neuronal cell survival its loss of function causes mitochondrial dysfunction[115, 116]. Parkin, along with its associated molecular chaperone HSP70, are found in LBs in sporadic PD [117]. BAG5 (bcl-2-associated athanogene 5) is a member of the BAG family and has been shown to form a complex with HSP70 [118], and interact with Parkin. This interaction has deleterious functional consequences by inhibiting HSP70 chaperone activity and Parkin E-3 Ligase activity causing an influx of protein aggregation [119].

Non-coding RNAs are involved in many regulatory cellular processes and make up a significant portion of our genome. miRNAs are small non-coding RNAs which are 20-22 nucleotides in length and function in gene regulation and silencing [120]. miRNAs have also been extensively studied as potential biomarkers due to their extracellular stability and altered disease state expression [121] A recent study highlighted the differences in miRNA levels from PD and non-diseased brains stating that miRNAs are

differentially expressed in patients with PD allowing for classification of PD within a 5% error [122]. While this is taking a potential diagnostic approach there have been studies which show regulatory effects of miRNAs in PD. One specific study showed the regulatory effects of miR-34b/c on the key Parkinson's proteins Parkin and DJ-1 causing loss in cell viability [123]. A considerable number of miRNAs have been reported for their regulatory roles in PD [124]. We investigated miR-335-5p and miR-3613-3p which have been associated with an array of diseases from cancer to gestational diabetes, however there are no studies to date that show the relationship between miR-335-5p and miR-3613-3p and PD.

In this study we show that miR-335-5p and miR-3613-3p levels are significantly elevated in PD patients as compared to healthy individuals which further corroborated our previous findings [125]. This increase in miRNA expression was also prevalent in post-mortem frontal cortext samples for both PD and age-matched controls. We further report that miR-335-5p and miR-3613-3p overexpression may have a neuroprotective function as it increases cell viability in SH-SY5Y cells induced with oxidative stress. We also show that oxidative stress causes miR-335-5p and miR-3613-3p overexpression not seen under normal, non-stressed conditions. This increase leads to increased levels of ATG5 and BAG5 whilst decreasing levels of ATXN3.

Results

miR-335-5p and miR-3613-3p show upregulated expression in post-mortem PD brains

In our previously reported longitudinal study we identified, verified and validated miR-335-5p, miR-3613-3p, miR-6865-3p as differentially expressed in 370 PD (drug-naïve) and control serum samples from the Norwegian ParkWest study and in 64 PD (drug-naïve) and control serum samples from NY Parkinsonism in UMeå (NYPUM) study [125]. We further showed that that miR-335-5p, miR-3613-3p, miR-6865-3p (PARKmiRs), and any two combinations of the PARKmiRs, were robust classifiers of PD at baseline diagnosis [125].To expand on these findings and to gain insight into whether the increased miRNA levels in serum may reflect changes in brain miR profiles we isolated small RNAs from post-mortem frontal cortex brain samples of PD patients and age matched healthy control individuals. RNA isolation was performed on 9 PD brains and 6 age matched control brain samples, and expression levels were measured using a SYBR Green-based quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assay. scaRNA17 was used as a small RNA reference for normalizing the qRT-PCR Cq values and cel-miR-39-3p was used as a spike-in control. The spike in control was included to ensure that recovery of small RNA was consistent across the samples. The results from the qRT-PCR assay revealed that both miR-335-5p and hsa-miR-3613-3p levels were significantly upregulated in PD brains (Fig.1) compared to healthy age matched controls. We also tested six other PARKmiRs discovered in our previous study [121] revealing that hsa-miR-6865-3p was also significantly upregulated in PD brain samples, however for this study we will only focus on miR-335-5p and miR-

3613-3p. The finding that miR-335-5p and miR-3613-3p levels are elevated in frontal cortex brain samples of PD patients, complements our previous data showing elevated PARKmiRs levels in PD serum, possibly suggesting a mode of miR release into serum from the brain.

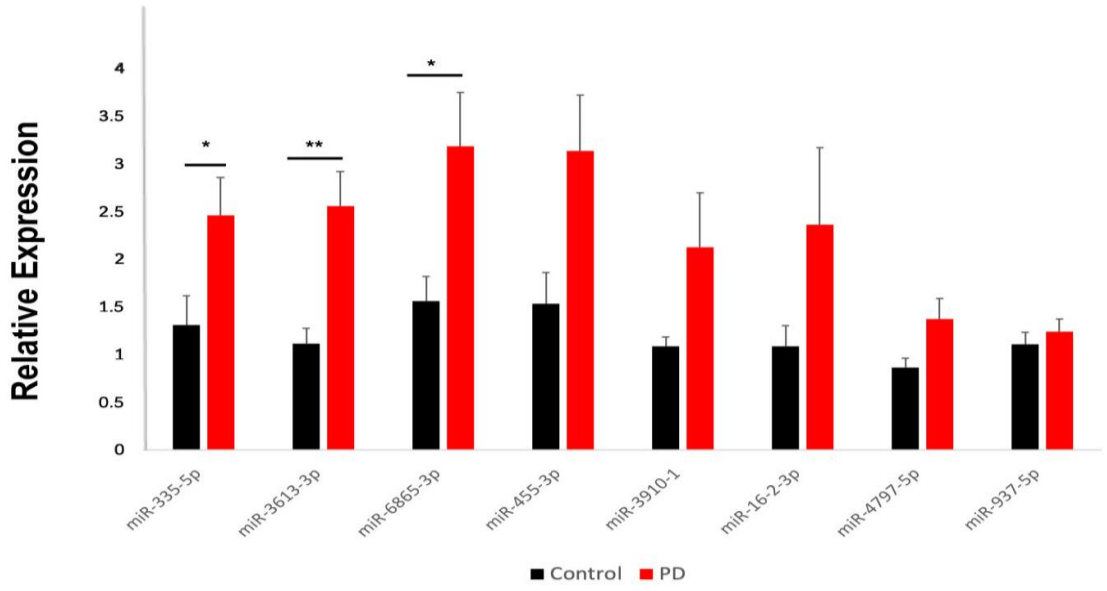


Figure 1.1 Upregulation of PARKmiRs in brain samples. qRT-PCR comparison of post-mortem frontal cortext brain samples of Parkinson's patients and healthy age-matched controls showing significant upregulation of PARKmiRs hsa-miR-335-5p, hsa-miR-3613-3p, and hsa-miR-6865-3p in PD brain samples compared to control. All above (n=4), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Overexpression of miR-335-5p and miR-3613-3p increase cell viability in response to oxidative stress

To investigate whether overexpression of miR-335-5p and miR-3613-3p has any significant effect on cell viability we performed a neutral red assay on SH-SY5Y cells overexpressing the two PARKmiRs, independently. To impose stress on the SH-SY5Y cells we also treated cells with 100μM H_2O_2 and 100μM L-Dopa for six hours followed by the neutral red assay. We observed no change in cell viability in SH-SY5Y cells overexpressing a control mimic or the miR-335-5p and miR-3613-3p mimics (miR-335M and miR3613M) in untreated cells as expected (Figure 2A). However, after six hours of treatment with 100μM H_2O_2 we observed a significant loss in viability in cells expressing the control mimic (Figure 2A). Interestingly, cells overexpressing miR-335-5p and miR-3613-3p showed a significant rescue in cell viability as compared to the control mimic expressing cells suggesting that both PARKmiRs may have a possible protective role (Figure 2A). A similar result was also observed in response to 100μM L-Dopa treatment (Figure 2A) suggesting that both PARKmiRs have a protective effect in response to oxidative stress, either directly by H_2O_2 treatment or indirectly in response to L-Dopa exposure.

This observed protective effects of both miR-335-5p and miR-3613-3p on SH-SY5Y cells, in response to oxidative stress, may explain the elevated levels of both PARKmiRs in PD serum [121] and PD brain samples (Figure 1). These elevated levels of both PARKmiRs may indeed contribute to mitigating neurodegeneration in PD. To further dissect this possibility, we first exposed wild-type SH-SY5Y cells to H_2O_2 and L-

Dopa in a dose dependent manner for six hours followed by cell viability analysis. We observed, as expected, a progressive loss in cell viability with increasing concentration of both H_2O_2 and L-Dopa (Figure 2B). We then performed qRT-PCR on the wild-type SH-SY5Y cells exposed to 100μM H_2O_2 and 100μM L-Dopa for six hours, which showed that both endogenous PARKmiRs are significantly upregulated in response to oxidative stress as compared to untreated cells (Figure 2C). Combined these results suggest that the elevated levels of miR-335-5p and miR-3613-3p in PD brains and PD serum may positively contribute to a neuroprotective pathway in PD.

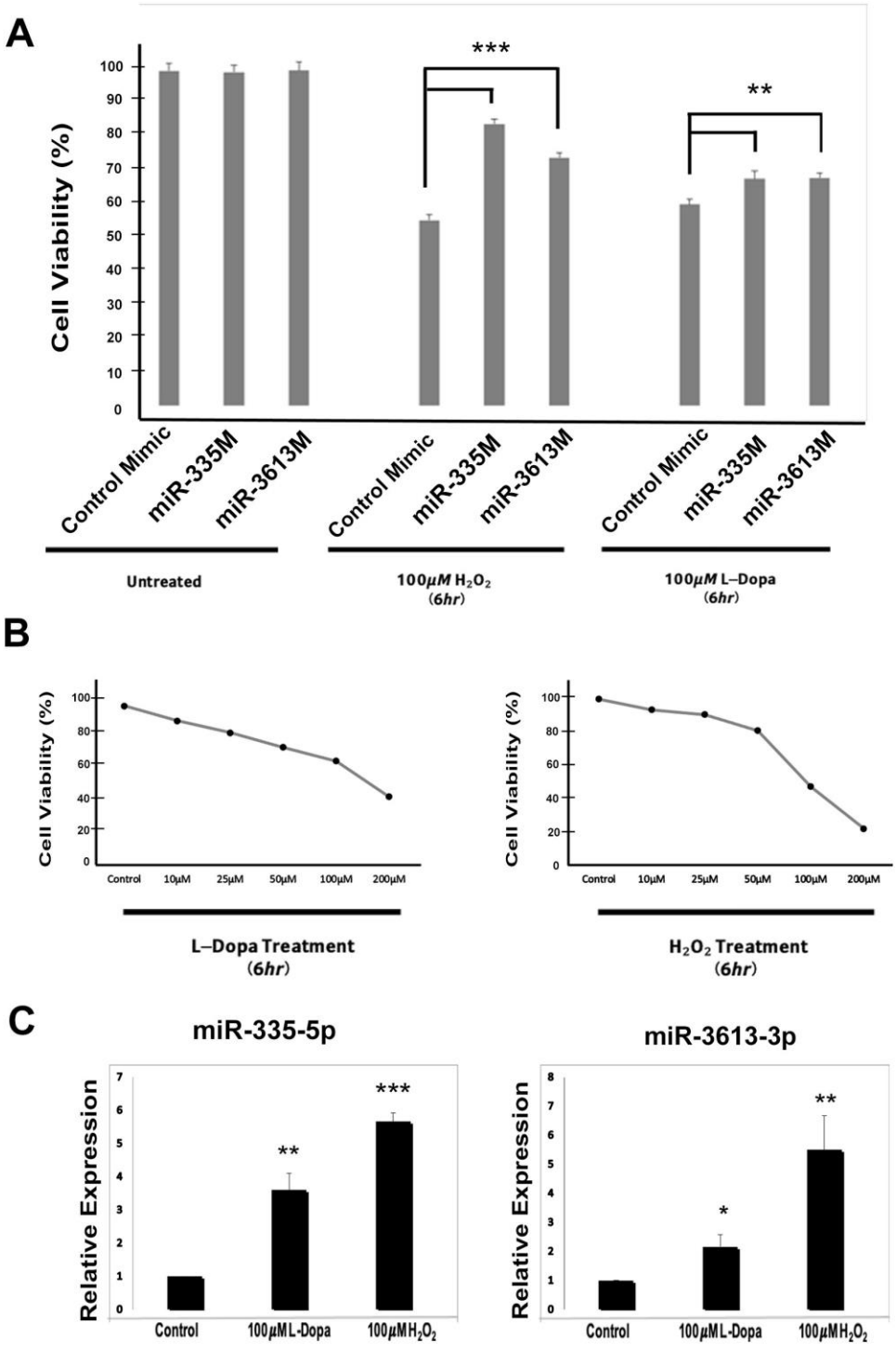


Figure 2.1 PARKmiRs significantly increase cell viability in response to oxidative stress.

Neutral red assay and fluorescence image analysis showing significant increase in cell viability in response to H_2O_2 and L-Dopa treatments in cells overexpressed with miRs. (A) miR-335-5p and miR-3613-3p mimic overexpression shows significant rescue in cell viability for SH-SY5Y cells treated with 100μM H_2O_2 or 100μM L-Dopa. (B) Dose-dependent curve of control SH-SY5Y cells treated with H_2O_2 and L-Dopa showing increased loss in cell viability with increasing concentrations of treatments. (C) qRT-PCR expression of miR-335-5p and miR-3613-3p respectively showing significant increase in expression in response to H_2O_2 and L-Dopa treatments. All above (n=4), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increased expression of miR-335-5p and miR-3613-3p has neuroprotective effects

As a hallmark of PD onset is the aggregation of a-syn and progression is directly associated with missense mutations, duplication and triplication events in the a-syn encoding gene *SNCA* [26] we wanted to more fully understand how miR-335-5p and miR-3613-3p function in a neurotoxic environment similar to that observed in PD. To this end we created SH-SY5Y cell lines that overexpressed wild-type a-syn triplication mutation and the two clinical a-syn variants a-syn-A53T and a-syn-E46K point mutations [76, 77]. To confirm overexpression of wild-type a-syn, a-syn-A53T and a-syn-E46K in our stable SH-SY5Y cell models we performed western blot analysis using a monoclonal anti-a-syn antibody. The western blot analysis showed that all three cell lines overexpressed a-syn, a-syn-A53T and a-syn-E46K to a similar level showing significant overexpression of the 14kDa a-syn monomer as compared to cells expressing the pcDNA empty vector control (Figure 3A). To further determine the levels of endogenous miR-

335-5p and miR-3613-3p in the three a-syn cell models we performed miR extraction from the cells followed by qRT-PCR analysis. Interestingly, we found that both miR-335-5p and miR-3613-3p were significantly elevated in cells overexpressing wild-type a-syn and in cells overexpressing the two clinical variants a-syn-A53T and a-syn-E46K as compared to cells expressing pcDNA3 alone (Figure 3B).

Closer analysis of the data also revealed that the expression profiles of miR-335-5p and miR-3613-3p differed between the different cell lines except for a-syn-A53T where the expression of both PARKmiRs was similar (Figure 3B). It is unclear why the expression profiles of miR-335-5p and miR-3613-3p differ between the different a-syn mutant cell lines but it may be due to differences in severity of cellular toxicity caused by the mutations. To further investigate whether overexpression of wild-type or mutated a-syn has an effect on cell viability we performed neutral red assays on the three cell lines and showed that overexpression of wild-type a-syn, a-syn-A53T and a-syn-E46K had no significant effect on SH-SY5Y cell viability under normal conditions (Figure 3C).

As overexpression of wild-type a-syn, a-syn-A53T and a-syn-E46K, coupled with exposure to oxidative stress such as H_2O_2 mimics the neurotoxic environment in PD, we wanted to determine whether miR-335-5p and miR-3613-3p can act as neuroprotectors in this PD simulated environment. To accomplish this we transfected miR-335-5p and miR-3613-3p into SH-SY5Y cell lines overexpressing wild-type a-syn, a-syn-A53T and a-syn-E46K, respectively. Two days after transfection we treated cells with 100μM H_2O_2 for six hours and performed a neutral red assay to quantify cell viability. Our results show that miR-335-5p and miR-3613-3p overexpression results in cell viability rescue for all mutants compared to control cells (Figure 3D). Indeed, we did not observe this cell

viability rescue with only endogenous levels of miR-335-5p and miR-3613-3p implying that overexpression of these two PARKmiRs is needed in order to positively effect cell viability under neurotoxic stress conditions. These data suggest therefore that miR-335-5p and miR-3613-3p are acting in a neuroprotective capacity perhaps by regulating genes that confer neuroprotection in neurons.

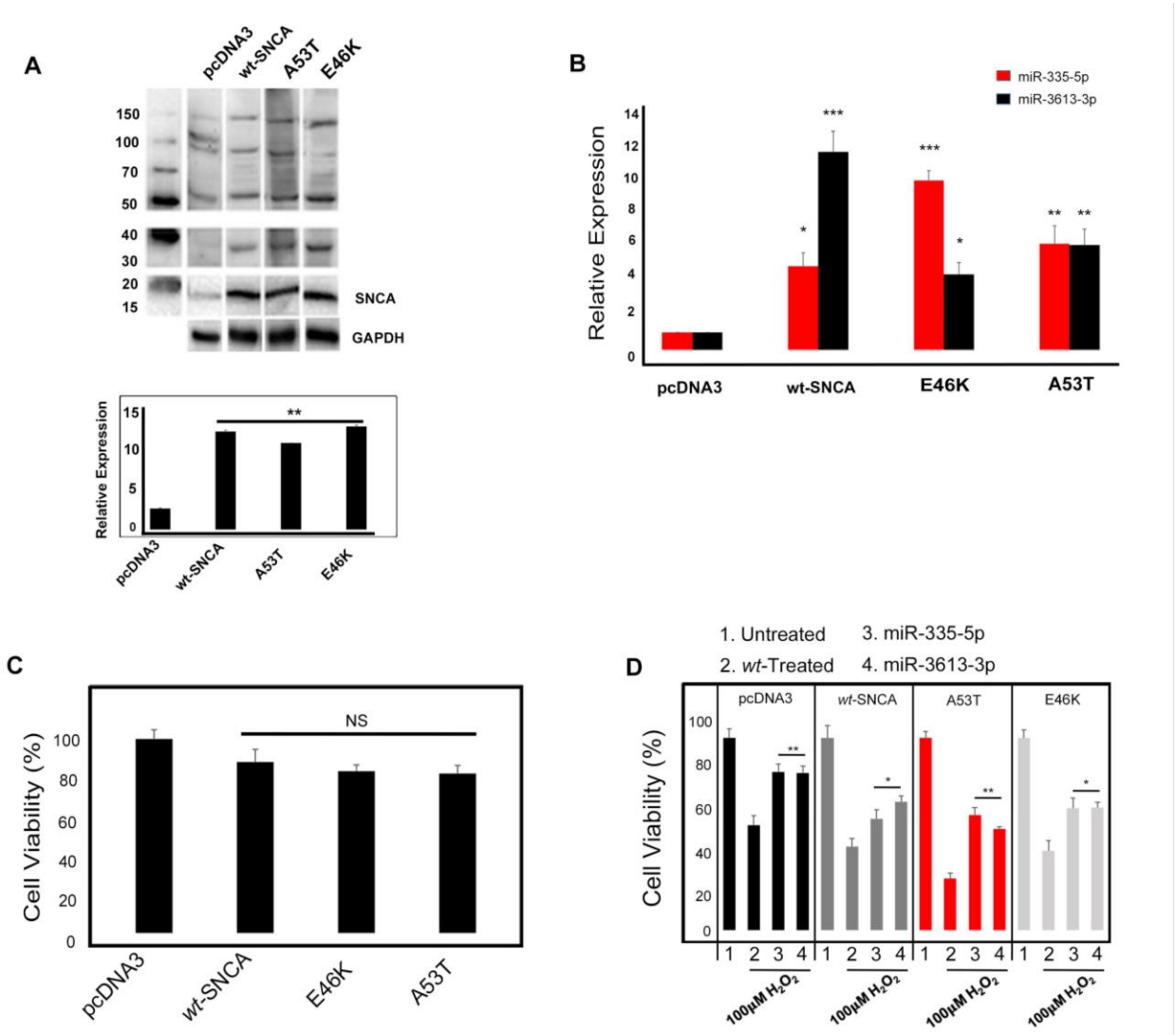


Figure 3.1 PARKmiRs show increased cell viability under neurotoxic conditions.

(A) Western blot analysis showing overexpression of monomeric α-syn Lane's 1-4: pcDNA3, *wt-SNCA* (Triplication mutation), E46K, A53T. (B) Overexpression of endogenous miR-335-5p and miR-3613-3p in mutant cell lines using qRT-PCR. (C) pcDNA3, *wt-SNCA*, E46K, A53T cells were treated with neutral red and cell viability was assayed (n=3, ns- no significance). (D) Indicated cells were treated with 100uM H_2O_2 for 6 hours followed by neutral red assay for cell viability Lane 1-4, Untreated, Treated, miR-335-5p treated, miR-3613-3p treated (n=3, *p< 0.05, **p<0.01, t test).

ATG5, Ataxin-3, and BAG5 are all regaulated by miR-335-5p and miR-3613-3p

To identify potential targets of miR-335-5p and miR-3613-3p we used both *in silico* prediction analysis (miRTarBase, Partek Genomics Suite) and our previously reported LC-MS data [121]. From this analysis we identified 1,516 putative protein targets with high enrichment scores for miR-335-5p and miR-3613-3p. This putative protein target list was then further analyzed for proteins with known associations to neurodegenerative pathways, PD, and other fundamental neuronal processes. We found that ATG5, Ataxin-3, and BAG5 were all predicted targets of miR-3613-3p, however miR-335-5p did not target any of these. These targets were of interest because of their direct correlation with PD as well as other neurological diseases. We continued to investigate the regulation of ATG5, Ataxin-3 (ATXN3), and BAG5 with respect to miR-335-5p because of its previously recorded regulatory effects on Leucine Rich Repeat Kinase 2 (LRRK2) which BAG5 has been shown to directly interact with [126]. Recently, ATG5 has been shown to contribute to the protection of dopaminergic neurons in a MPTP zebrafish PD model where ATG5 downregulation leads to decreased autophagy flux further resulting in an

influx of aggregated proteins [92]. This was only alleviated when ATG5 was overexpressed in cells [111]. ATXN3 has also been shown to be involved in autophagy where mutant ATXN3 causes downregulation of BECN1 resulting in decreased autophagosome formation [127].

To further validate our *in silico* analyses we transfected the miR-335-5p and miR-3613-3p into SH-SY5Y cells and confirmed overexpression of miRs with qPCR (Figure 4A), followed by western blot analysis for ATG5, BAG5 and Ataxin-3. We found that both miR-335-5p and miR-3613-3p overexpression significantly upregulates the expression of Atg5 compared to the control (Figure 4C). Bag5 also showed significant upregulation in overexpressed miR-335-5p cells, however we did not observe any significant regulation of Bag5 in cells overexpressing miR-3613-3p (Figure 4D-E). Interestingly, miR-335-5p had a reverse regulatory effect on Atxn3 by significantly downregulating its expression (Figure 4F). We observed a moderate, but significant upregulation of Atxn3 in overexpressed miR-3613-3p cells (Fig 4G). It is certainly interesting to note that overexpression of miR-335-5p had an effect on all protein targets tested suggesting a possible expansive regulatory role miR-335 in these pathways. We did not find significant alteration of ATG5, ATXN3, and BAG5 at the mRNA level suggesting that a post-transcriptional mechanism was involved in the observed alterations of these proteins.

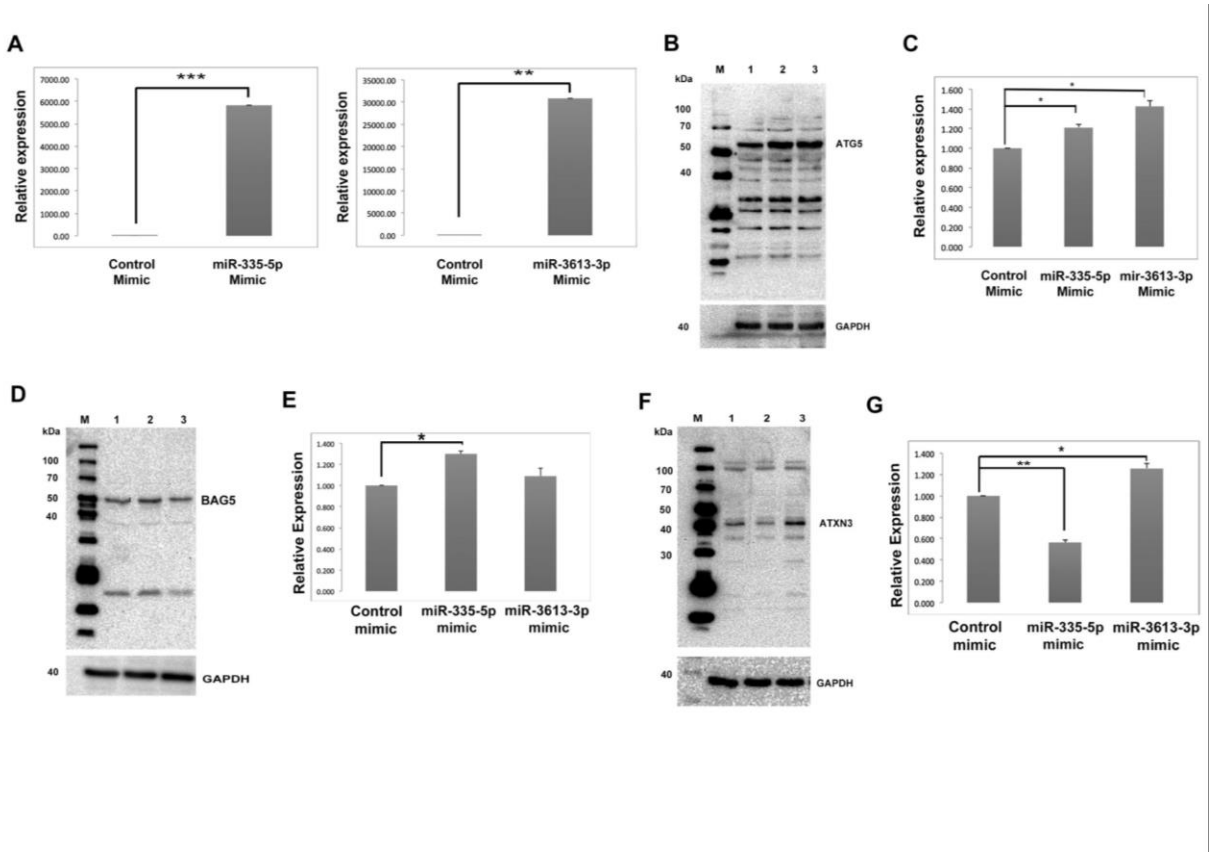


Figure 4.1 PARKmiRs 335 and 3613 significantly regulate expression of ATG5, BAG5, and ATXN3.

(A) qRT-PCR analysis showing miR-335-5p and miR-3613-3p in response to mimic microRNA transfection. Western blot analysis comparing Control mimic cells (1) with miR-335-5p mimic (2) and miR-3613-3p mimic (3). (B, C) Western blot analysis showing significant upregulation of ATG5 in both miR-335-5p & miR-3613-3p overexpression. (D, E) Western blot analysis showing significant upregulation of BAG5 in cells overexpressing miR-3613-3p. (F, G) Western blot analysis showing significant downregulation of ATXN3 in cells overexpressing miR-335-5p and significant upregulation of ATXN3 with cells overexpressing miR-3613-3p. All above (n=4), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Discussion

Mutations in ATG5, ATXN3, and BAG5 have been shown to enhance neurodegeneration due to misfolded proteins [128], and these mutations have been noted in idiopathic PD patients, suggesting that mutations in ATG5, BAG5, and ATXN3 are associated with PD [92]. Previous studies have demonstrated that BAG5 functions as a nucleotide exchange factor of Hsp70 and also interacts with Parkin to inhibit Parkin E3 ubiquitin ligase activity which in turn inhibits Parkin's ability to ubiquitinate misfolded proteins such as alpha synuclein [129]. ATG5 has been shown to play a neuroprotective role in dopaminergic neurons by increasing autophagy of misfolded and aggregated proteins. This clearance of aggregated proteins mitigated its cytotoxic effects in the neuron [92]. ATXN3 repeat expansion mutations have been shown mainly in Spinocerebellar ataxia type 3 (SCA3) a neurodegenerative disease in the cerebellum [130]. It has also been shown to cause Parkinson's like phenotypes and previous studies have found that repeat expansion mutations occur in early-onset PD [131].

In our study, we have demonstrated that miR-335-5p and miR-3613-3p are unregulated in PD post mortem brain samples corroborating our previous findings [125]. In cells over expressing miR-335 and miR-3613 we showed a neuroprotective function and a rescue of cell viability when inducing SH-SY5Y cells with hydrogen peroxide stress. Interestingly, we also found an increase in endogenous expression of miRs 335 and 3613 in our mutant cell lines over expressing a-syn variants further corroborating our claims that miR expression is increased in neurotoxic conditions. Taking it one step further when we overexpressed our miRs in mutant cell lines along with hydrogen

peroxide stress we also found an increase in cell viability compared to the control. This lead us to believe that miR-335 and miR-3613 are acting in a neuroprotective manner and this could be a reason why we see overexpression of both miRs in serum, brain, and mutant cell lines.

In conclusion, we report a novel role for miRs 335 and 3613 as modulators of ATG5, ATXN3, and BAG5. We have also shown that overexpression of both miRNAs resulted in an increase in cell viability under oxidative stress conditions. The understanding of how these miRNAs regulate expression of key targets and the pathways involved will lead to improved understanding of the pathogenesis of PD.

Future Work:

Future work would include analyzing the effect mutant versions of Ataxin-3 have on Parkin and how it's down-regulation by miR-335-5p ameliorates the negative effects Ataxin-3 has on the cell.

Review Project: Cellular Proteostasis in Neurodegeneration.

Introduction

Neurodegenerative disorders are progressive, debilitating diseases characterized by motor and cognitive symptoms caused by neuronal death and dysfunction. Neurodegenerative diseases such as Parkinson's Disease (PD), Alzheimer's Disease (AD), Huntington's Disease (HD), Amyotrophic Lateral Sclerosis (ALS) and prion diseases (pD) share implicated risk factors such as oxidative stress, aging, environmental factors and protein dysfunction [132]. These risk factors, however, manifest distinctly in each disease and produce unique pathologies.

Protein misfolding and aggregation is a common theme amongst many neurodegenerative diseases, therefore maintaining intracellular protein homeostasis (proteostasis) by balancing protein folding and misfolding is paramount in protecting the functionality of the proteome [133]. Misfolded proteins are generally inactive, but the accumulation of these inactive misfolded proteins causes stress responses in cells and organelles. The endoplasmic reticulum (ER) is a key organelle in the maintenance of proteostasis; ER stress via protein accumulation triggers the unfolded protein response (UPR). The UPR promotes correct protein folding and diminishes ER protein level by proteosomal degradation, translation mitigation and autophagy [134]. Autophagy plays an essential role in proteostasis because of its ability to degrade protein aggregates that cannot be processed by the proteasome [135]. Indeed, the autophagosome can break down and recycle whole organelles in an effort to promote cell survival. Protein degradation by autophagy and the proteasome utilize ubiquitination to recruit target proteins

[4]. Ubiquitination along with other covalent attachments such as phosphorylation, SUMOylation, and oxidation regulate normal proteome function. Post-Translational Modifications (PTMs) of proteins have been shown to facilitate aggregation in many neurodegenerative diseases (NDs) [136].

A hallmark of NDs is the toxic accumulation and proteotoxicity of disease-associated proteins. Protein accumulation can lead to aggregates or inclusions, which may be toxic or protective [137]. Proteotoxicity is especially problematic with respect to post-mitotic neurons, drawing a clear line between neurodegeneration and age. Mitochondrial proteostasis is also critical for cell survival. Its dysfunction can lead to accumulation of reactive oxygen species (ROS), which can be disruptive to cellular proteostasis. ROS can debilitate cellular processes by damaging DNA, RNA, lipids, and proteins; stress that disturbs mitochondrial proteostasis may lead to the irreversible induction of apoptosis. Apoptosis, a highly regulated set of pathways leading to cell death, is initiated in cells under stress to avoid the damaging of adjacent cells; this, while advantageous to organisms in most cases, is detrimental to the nervous system as differentiated neurons cannot be reproduced. Although neuronal loss cannot be fully attributed to apoptosis, it is a common culprit in many neurodegenerative disorders such as PD, AD, HD and ALS.

In this review, we describe important factors that can alter proteostasis and how proteostasis dysfunction ultimately affects neurodegeneration.

Aim of paper

To highlight the many factors that contribute to neurodegeneration such as post-translational modifications, protein aggregation, ER-stress, and autophagy. The review further aimed at describing how alterations in cellular proteostasis can lead to neuronal cell death, and highlight the correlation between many neurodegenerative disorders.

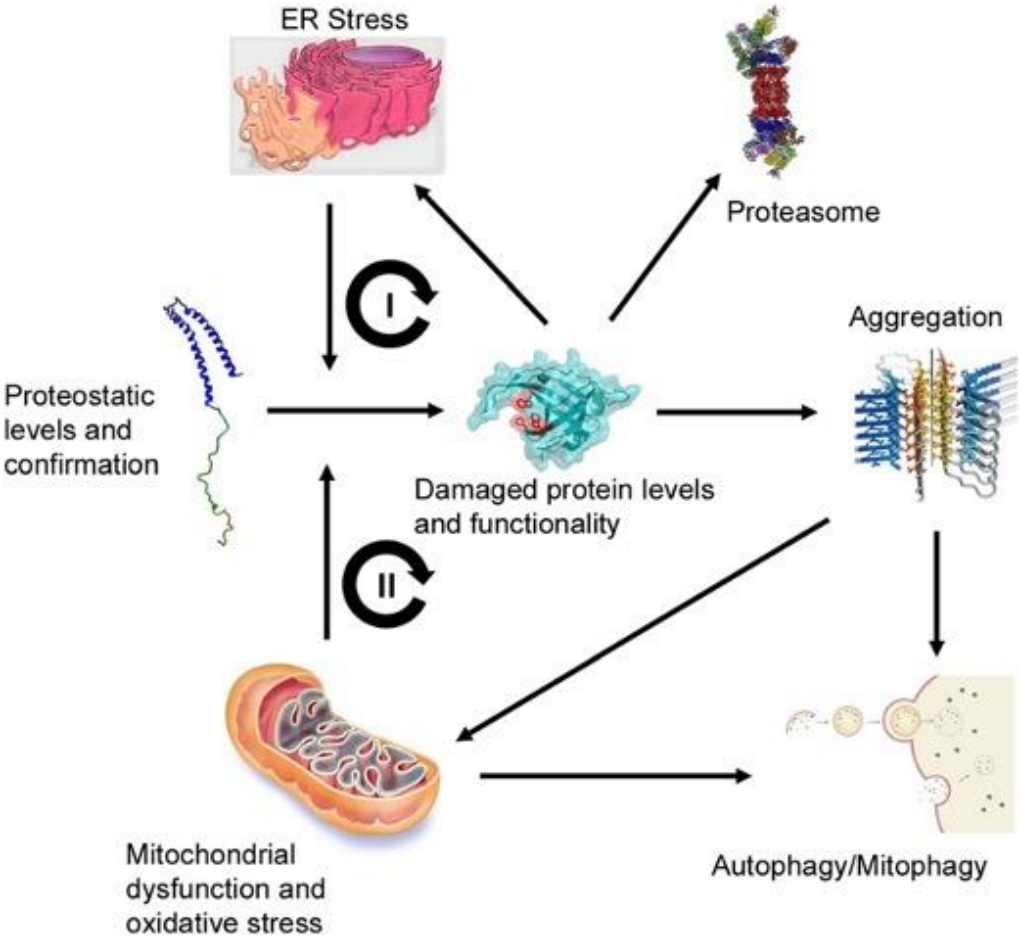


Figure 1.2 Altered proteostatic levels and protein conformations lead to hallmark pathological neurodegenerative pathways. Damaged and excessive protein directly causes ER stress and aggregation as well as mitochondrial dysfunction and oxidative stress indirectly. Additionally, feedback loops (I, II) highlight the destructive nature of damaged

proteins propagating ER stress and mitochondrial dysfunction leading to loss of functionality further altering proteostasis. The cellular defense response to these pathways Includes proteosome degradation, autophagy/mitophagy, and apoptosis

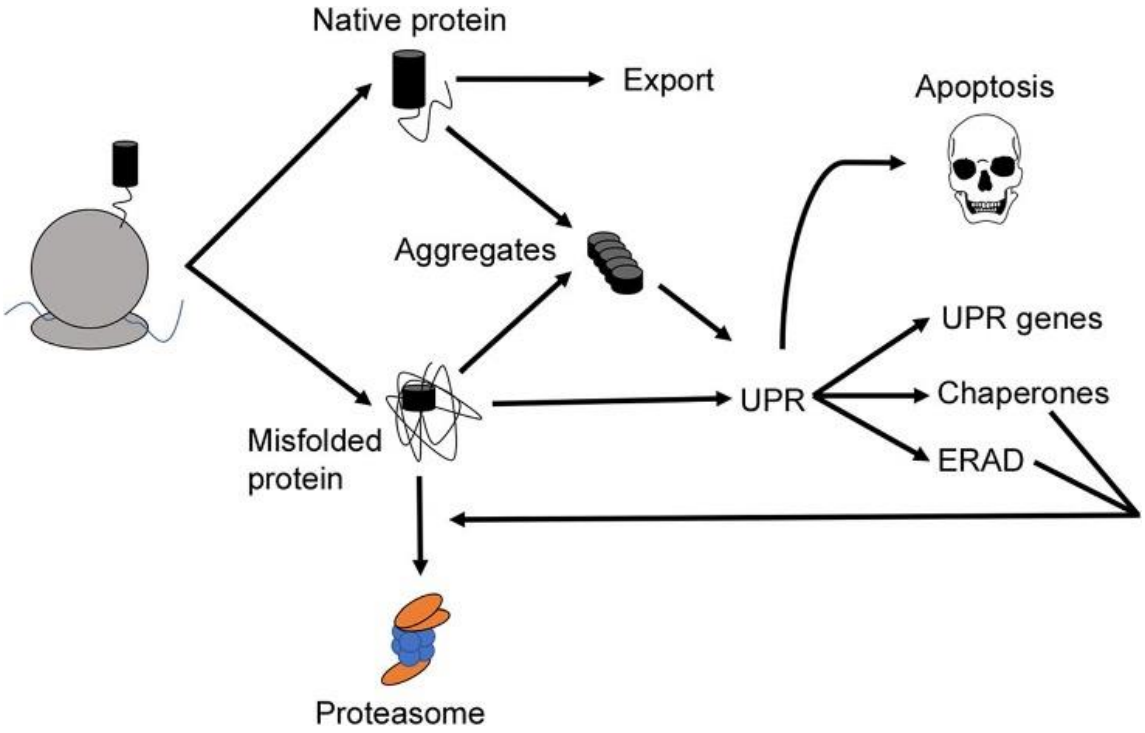


Figure 2.2 Pathway of protein throughout the ER. Protein is synthesized in the rough ER by ribosomes. Native protein is predominantly exported, while some can aggregate. Misfolded protein aggregates are sent to proteasome for degradation. Misfolded protein and aggregates promote the UPR,which includes UPR associated gene regulation, ERAD pathway, chaperone response, and apoptosis all in an effort to mitigate the effects of toxic aggregates

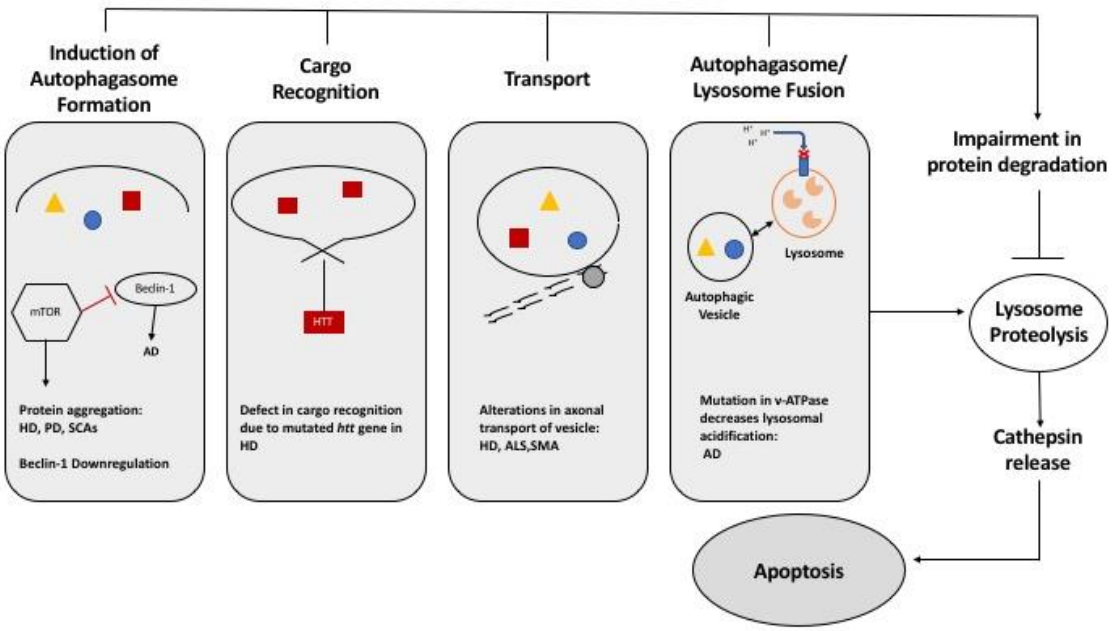


Figure 3.2 Neurodegenerative disorders alter specific steps in the autophagic pathway, which ultimately leads to neuronal cell death. The altered steps include reduced induction levels due to protein aggregation and defective mTOR inhibition; Defects in cargo recognition resulting in accumulation of toxic proteins; Mutation in VCP leads to inhibited transport of autophagic vesicle in ALS; Defects in lysosome/autophagasome fusion and acidification. All defective steps, leading to impairment of protein degradation by lysosomal degradation, ultimately leads to the release of cathepsin's and apoptosis. Huntington's disease (HD); Alzheimer's disease
(AD); Parkinson's disease (PD); spino-cerebelar ataxia (SCA); spino-muscular atrophy (SMA); dementia with Lewy bodies (DLB); Amyotrophic Lateral Sclerosis (ALS); Valsin-containing protein (VCP)

Collaborative Projects

Effects of SNCA and disease-causing mutations on the proteome of SH-SY5Y cells

SNCA aggregation and accumulation is a key part of PD pathology. It is however unknown at what stage *SNCA* changes its fate and translocates to Lewy bodies. The pathogenic mutations (A30P, E46K and A53T), in addition to duplication and triplication events, make it worthwhile exploring what effect the expression of these pathogenic forms of *SNCA* has on the proteome in dopaminergic neuronal SH-SY5Y cells. To this end we have transfected pcDNA4 empty vector, and the pcDNA4 vector, containing *SNCA* WT, *SNCA* A30P, *SNCA* E46K and *SNCA* A53T into SH-SY5Y cells. Stable cell lines were selected by supplementing full media with zeocin. At this stage we have extracted total protein from all the clones for 2D-gPAGE analysis and Western blot gels have already been run and analyzed. The manuscript is ready for submission to *Journal of Neuroscience Research*.

Contributions:

I worked with Rashed Abdullah on this project. I performed mammalian cell culture maintenance, I performed western blot analysis for multiple replicates as well as control samples. I also helped with image analysis of figures.

Figure contribution: Performed replicates of western blots from Whole cell lysates.

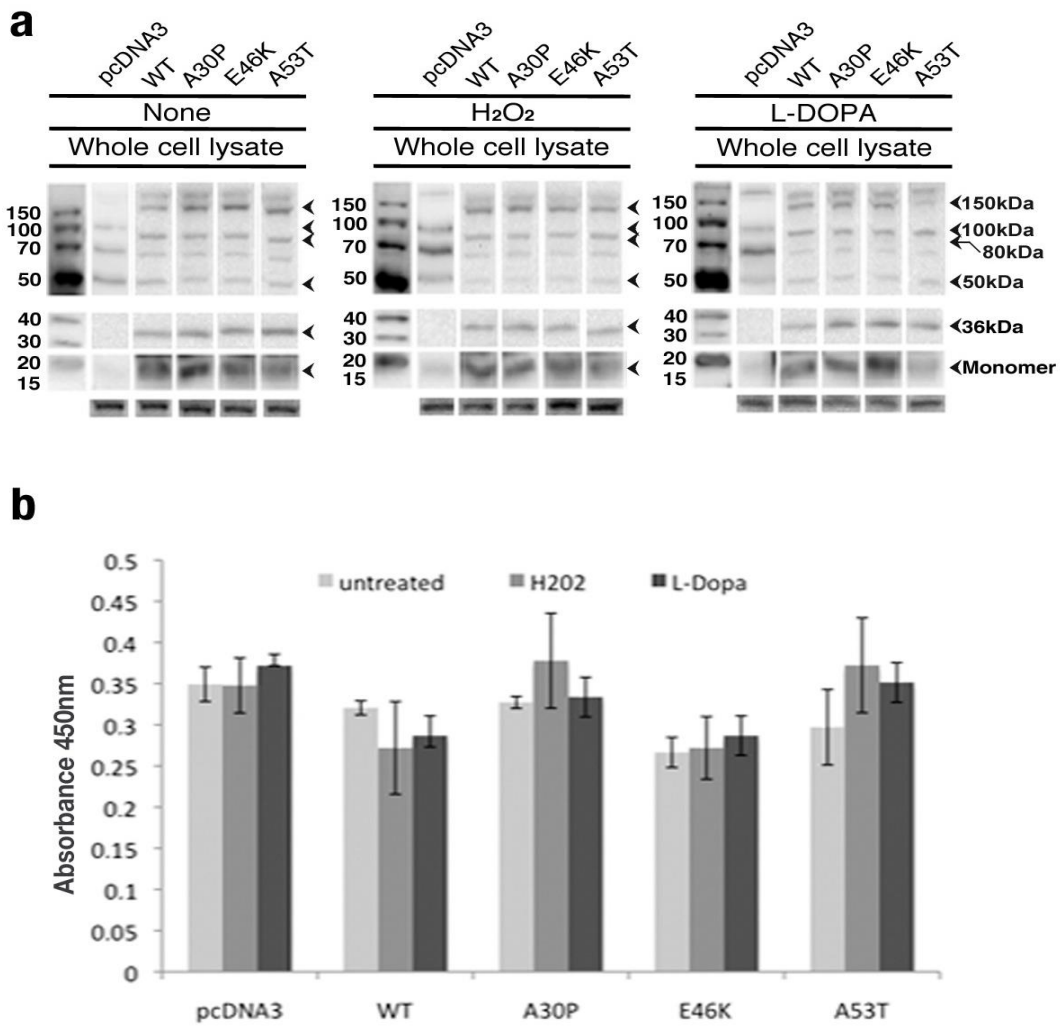


Figure 1.3 Whole cells lysate results of transfections of pathological variants of *SNCA* as well as wt-*SNCA* to mimic duplication/triplication event. Cells were transfected with pcDNA3 as a transfection control; wt-*SNCA* to mimic a duplication/triplication event; *A30P-SNCA, E46K-SNCA*, and *A53T-SNCA* to mimic the presence of the pathological form of a-syn in cells. Cells were measured for cell survival with and without treatments.

Western blot images are representative blots. a) Whole cells lysate of transfections of pathological mutations under no treatments, H_2O_2, and L-Dopa treatments (n=3). b) An assay of cell viability as measured by the neutral red assay. Cells were checked to determine if the viability was affected by transfections of pathological mutants and by H_2O_2 and L-Dopa treatments (n=3). We found no significant decrease in the viability of cell treated with H_2O_2, and L-Dopa.

Alpha-Synuclein Multimerization is Dependent on Structural Characteristics of Repeated KTKEGV Regions

A physio-pathological hallmark of PD is the presence of Lewy Bodies: aggregates which are highly enriched in α-syn. α-syn is an intrinsically disordered protein that forms soluble multimers, which may or may not contribute to its aggregation. Isolating critical components of alpha-synuclein's structure could lead to a therapeutic strategy of preventing its aggregation. The repeated KTKEGV motif has been implicated as critical to neurotoxicity and the presence of multimers or exclusive presence of monomers in neuroblastoma cells. Targeting the repeated motif. KTKEGV, is a sensible approach to studying the critical components of α-syn proteostasis simply due to its prevalence throughout the protein. We transfected BE(2)-M17 human neuroblastoma cells in order to overexpress at similar levels alpha-synuclein containing mutations in the KTKEGV motif. Western blotting was used to measure α-syn multimeric distributions in whole cell extracts. The data shows that physiological distribution of α-syn multimers throughout

neurons is cell-type specific and influenced by the sequence of repeated N-terminus KTKEGV motifs. The manuscript is ready for submission to *Journal of Biological Chemistry*.

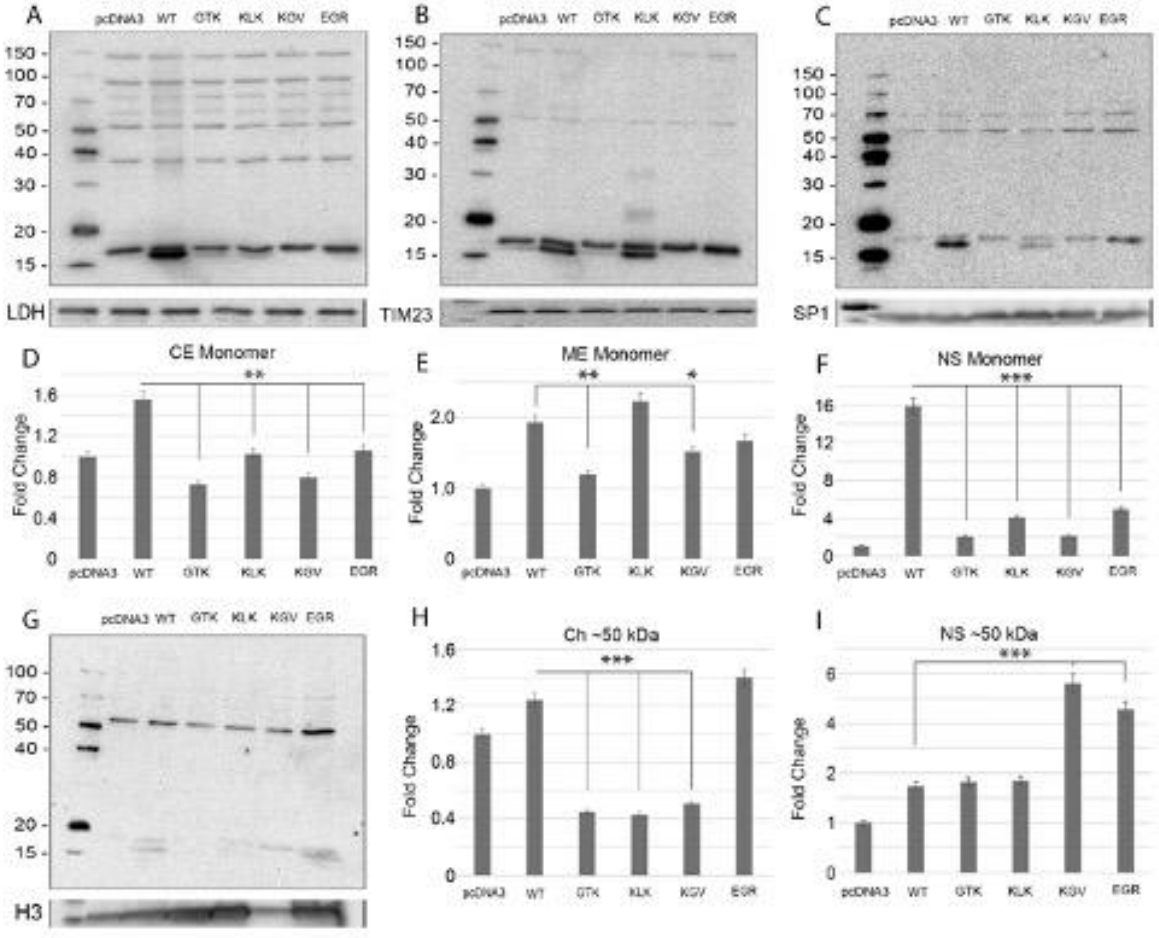
Contributions:

I worked closely with Benjamin Rosen. I assisted with subcellular fractionations of BE(2)-M17 cells, cell culture maintenance, and western blot analysis. I worked on multiple replicates and loading controls.

Concluding Remarks

This study aims to both clarify the importance of the repeats sequence to alpha-synuclein multimerization and aggregation in neurons as well as within subcellular compartments of neurons.

Figure 1.4 Subcellular fraction comparison of alpha-synuclein in M17D cells stably overexpressing repeat mutant SNCA. (A, D) Cytosolic (B, E Membrane bound (C, F, I) Nuclear soluble (G, H) Chromatin bound



Review Project: The Intersection of Parkinson's disease, viral infections and COVID-19

The novel SARS-CoV-2 coronavirus (COVID-19) pandemic has caused a global health crisis infecting millions worldwide. Along with flu-like symptoms (fever, chills, cough) a large number of infected individuals also experience a host of neurological issues including loss of smell and taste, dizziness, decreased alertness, and brain inflammation. A striking number of symptoms including gut issues are also prevalent in PD. A higher mortality rate has also been reported in patients with PD not only due to low age, but also through interactions with the brain dopaminergic system and the inflammatory response. The gut microbiota plays an important role in many processes outside of a healthy digestive system. It has been shown to be essential in protecting against host pathogens [138] and even plays a role in our psychological well-being [139]. Viral infections are among some of the most common pathogens invading our gut microbiome causing dysbiosis and a multitude of Gastrointestinal issues (GI)[140]. Patients with PD display an array of GI complications such as constipation, dysphasia, and dysbiosis (Table) [141] and these complications often occur years before hallmark motor symptoms arise [142]. The gut microbiota is also decreased in older adults due to a number of factors that include diet, genetics, and environmental factors [143]. It has also been noted that severe viral infections could possibly increase the risk of developing PD later in life [144] although not the primary cause but merely a risk factor. Evidence has suggested that SARS-CoV-2 RNA can be detected in the stool of some patients with COVID-19 [145]. This data suggests that there may be a link between the gut microbiota and COVID-19

severity, particularly as the higher COVID-19 mortality rate is seen within the elderly population that already have a decreased microbiota. This may also suggest an increased chance of COVID-19 mortality for a patient with PD due to an increase of underlying conditions which include a compromised Gut flora [146]. Revised manuscript resubmitted to Molecular Neurobiology.

Concluding remarks

In this review we have highlighted the intersections between PD, viral infections and COVID-19 with the emphasis on the many similarities between RNA viral pathways and neurodegeneration in PD. Indeed, the onset and progression of PD, as detailed in the Braak hypothesis, as well as the pathogenic nature, molecular mechanisms, and symptom development of the disorder share many similarities with the SARS-CoV-2 virus and COVID-19. As further research is conducted, more evidence of a possible correlation between PD, Viral infections and the current SARS-CoV-2 virus will become available.

	Alpha-synuclein	**Oxidative Stress**	**Inflammation**	**Metals**	**Gut Microbiome**	**Olfactory Tract**
Parkinson's Disease	Aggregation leads to neurotoxic Lewy Bodies [4]	Dysfunctional regulation of ROS by several genes including SOD1 leads to oxidative stress and apoptosis [147]	Protective short term, exacerbates non-motor symptoms long term [148, 149]	Exposure to heavy metals such as Mn is a risk factor for PD [150]	GI problems such as dysbiosis, constipation, and dysphasia [151]	Olfactory dysfunction is one of the earliest signs of PD [152, 153]
RNA Virus Infection	Upregulated in infected cells and restricts RNA virus replication [154, 155]	Can create ROS imbalance causing DNA damage and neuroinflammation [156]	Neuro-inflammatory response can be triggered by infection [154, 157]	RNA virus replication depends on and may cause increase in Mn and Fe [158, 159]	Microbiota depletion leads to GI issues causing inflammation and lack of gut flora [160]	Olfactory dysfunction is one of the earliest signs of COVID-19 infection [161, 162]

Table 1.6 Commonalities between Parkinson's Disease and RNA virus infection

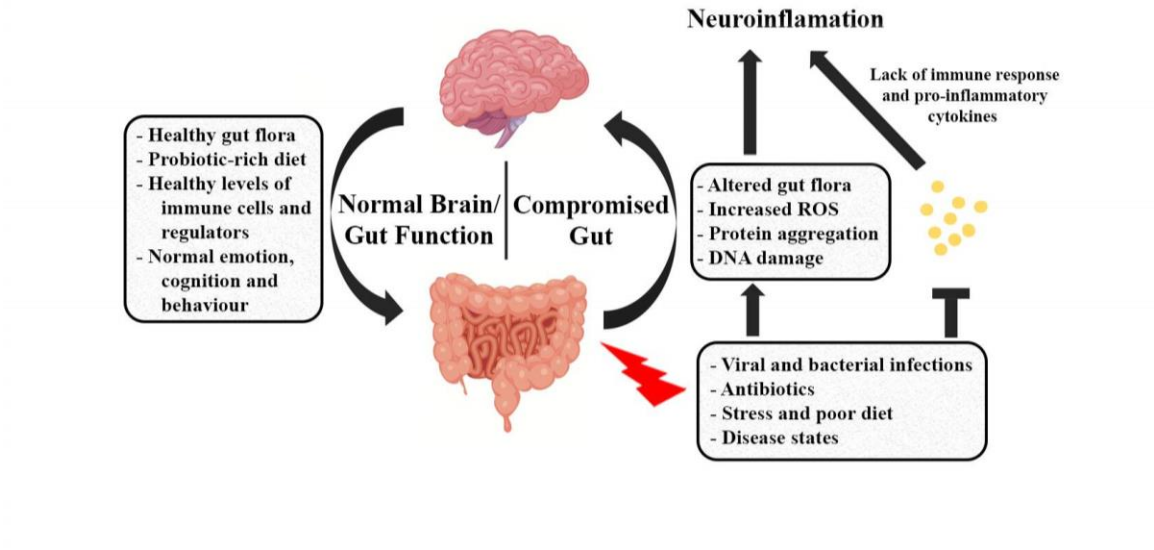


Fig. 1.5 The Brain-Gut microbiota axis: Schematic diagram highlighting the relationship between the brain and gut microbiota. A rich and diverse microflora allows for healthy immune and regulatory mediators, whereby a compromised gut microflora caused by viral infections, stress, and poor diet can cause a pro-inflammatory state with implications for neuroinflammation.

Publications

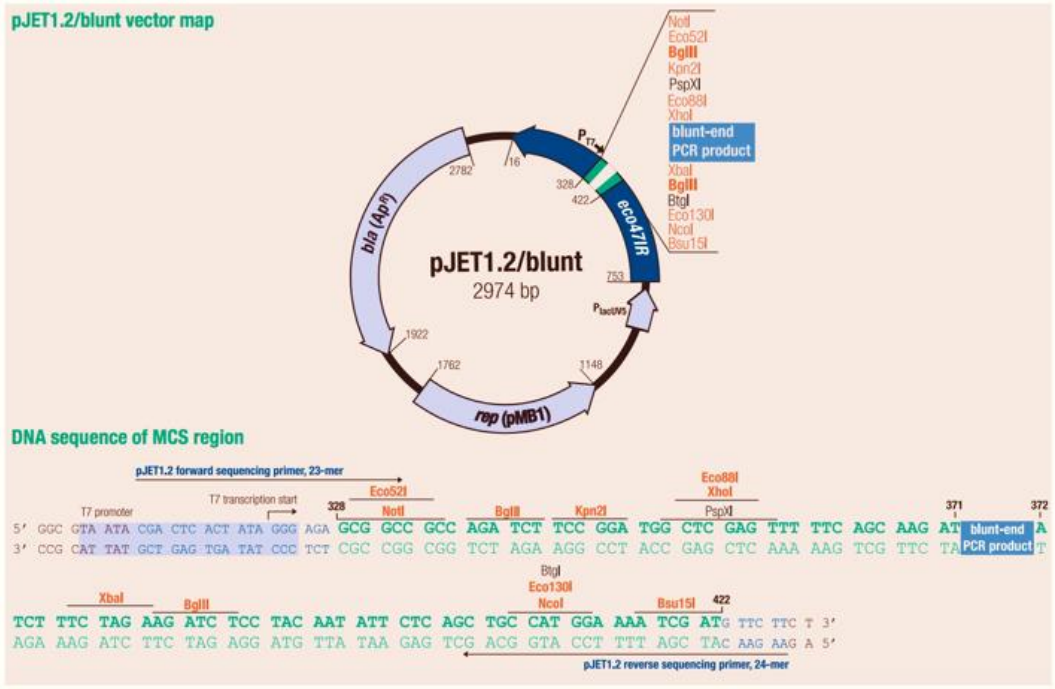
Published and submitted manuscripts resulting from the research described.

1. Alberim Kurtishi*, Benjamin Rosen*, Ketan S. Patil, Guido W. Alves, Simon Geir Møller (2018). Cellular Proteostasis in Neurodegeneration. *Molecular Neurobiology,* 56: 3676.
2. Alberim Kurtishi, Ketan S. Patil, Benjamin Rosen, Guido W. Alves, Simon Geir Møller (2021). Neuroprotection by miR335 and miR3613 may involve regulation of ATXN3, BAG5, and ATG5. *Journal of Neuroscience Research*. Submitted March 2021.
3. Benjamin Rosen, Alberim Kurtishi, Michael Padron, Ketan Patil, Francisco X. Vazquez, Simon Geir Møller (2021) Alpha-Synuclein Multimerization is Dependent on Structural Characteristics of Repeated KTKEGV Regions. *Journal of Biological Chemistry*, Submission March 2021
4. Rashed Abdullah, Ketan Patil, Benjamin Rosen, Alberim Kurtishi, James Young, Simon Geir Møller* (2021). Pathologic alpha-synuclein multimeric profiles in SHSY-5Y neuroblastoma cells. *Journal of Neuroscience Research*. In preparation.
5. Benjamin Rosen, Alberim Kurtishi, Gonzalo Vasquez, Simon Geir Møller (2021). The Intersection of Parkinson's Disease, Viral infections and COVID-19. *Molecular Neurobiology* submitted Jan. 2021

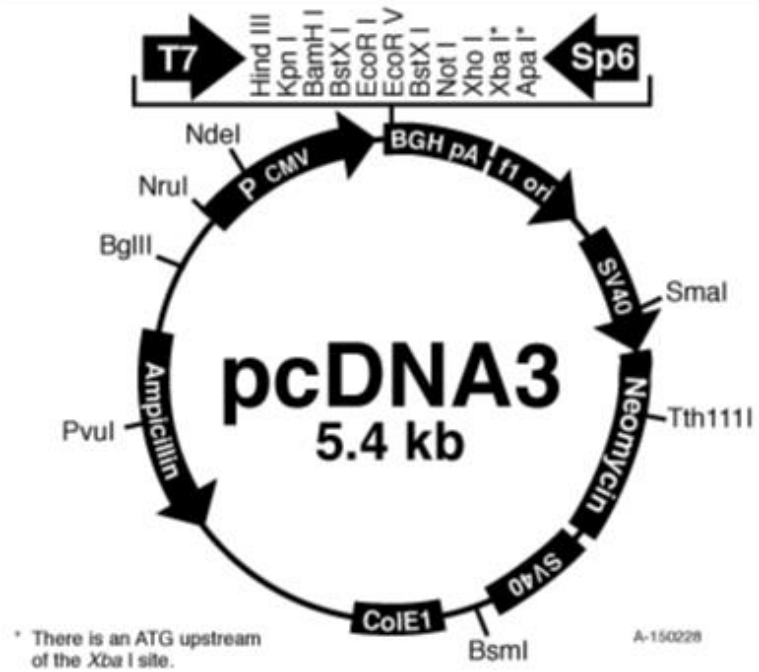
Appendix A: List of Vectors

Vector Maps were obtained from manufacturer's website.

A-1. pJET 1.2/blunt

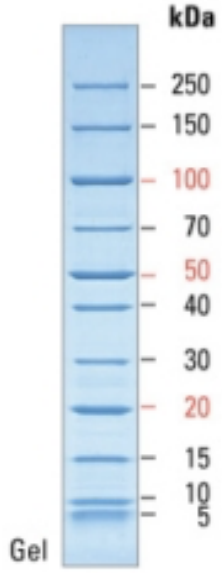


A-2 pcDNA3™


T7
Hind III
Kpn I
BamH I
BstX I
EcoR I
EcoR V
BstX I
Not I
Xho I
Xba I*
Apa I*
Sp6
NdeI
NruI
BglII
P CMV
BGH pA
f1 ori
SV40
SmaI
pcDNA3
5.4 kb
Amp
PvuI
Neomycin
Tth111I
SV40
ColE1
BsmI
A-150228
* There is an ATG upstream of the Xba I site.

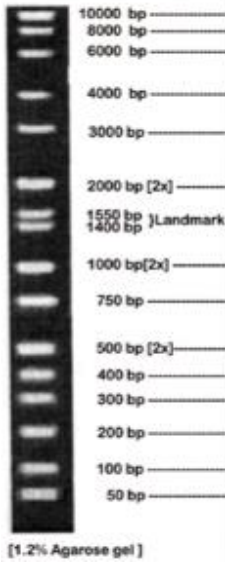
Appendix B: Molecular size markers

B-1. PageRuler™ Unstained Broad Range Protein Ladder



B-2. Hi-Lo DNA Marker

Hi-Lo DNA Marker has approximately 935ng/10ul with DNA concentration per band



REFERENCES

1. Bossy-Wetzel, E., R. Schwarzenbacher, and S.A. Lipton, *Molecular pathways to neurodegeneration.* Nat Med, 2004. **10 Suppl**: p. S2-9.
2. Hirsch, E., A.M. Graybiel, and Y.A. Agid, *Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease.* Nature, 1988. **334**(6180): p. 345-8.
3. Braak, H., et al., *Idiopathic Parkinson's disease: possible routes by which vulnerable neuronal types may be subject to neuroinvasion by an unknown pathogen.* J Neural Transm (Vienna), 2003. **110**(5): p. 517-36.
4. Tanaka, K. and N. Matsuda, *Proteostasis and neurodegeneration: the roles of proteasomal degradation and autophagy.* Biochim Biophys Acta, 2014. **1843**(1): p. 197-204.
5. Lang, A.E. and A.M. Lozano, *Parkinson's disease. First of two parts.* N Engl J Med, 1998. **339**(15): p. 1044-53.
6. Tuite, P.J. and K. Krawczewski, *Parkinsonism: a review-of-systems approach to diagnosis.* Semin Neurol, 2007. **27**(2): p. 113-22.
7. Deuschl, G., P. Bain, and M. Brin, *Consensus statement of the Movement Disorder Society on Tremor. Ad Hoc Scientific Committee.* Mov Disord, 1998. **13 Suppl 3**: p. 2-23.
8. Jankovic, J., *Parkinson's disease: clinical features and diagnosis.* J Neurol Neurosurg Psychiatry, 2008. **79**(4): p. 368-76.
9. Jain, S., S.E. Lo, and E.D. Louis, *Common misdiagnosis of a common neurological disorder: how are we misdiagnosing essential tremor?* Arch Neurol, 2006. **63**(8): p. 1100-4.
10. Samii, A., J.G. Nutt, and B.R. Ransom, *Parkinson's disease.* Lancet, 2004. **363**(9423): p. 1783-93.
11. Agostino, R., et al., *Analysis of repetitive and nonrepetitive sequential arm movements in patients with Parkinson's disease.* Mov Disord, 1994. **9**(3): p. 311-4.
12. O'Sullivan SB, S.T., Fulk G., *Physical rehabilitation.* 2019.
13. Berardelli, A., A.F. Sabra, and M. Hallett, *Physiological mechanisms of rigidity in Parkinson's disease.* J Neurol Neurosurg Psychiatry, 1983. **46**(1): p. 45-53.
14. Skeie, G.O., et al., *Differential effect of environmental risk factors on postural instability gait difficulties and tremor dominant Parkinson's disease.* Mov Disord, 2010. **25**(12): p. 1847-52.
15. Bohnen, N.I., et al., *Leucoaraiosis, nigrostriatal denervation and motor symptoms in Parkinson's disease.* Brain, 2011. **134**(Pt 8): p. 2358-65.

16. Koller, W.C., et al., *Falls and Parkinson's disease.* Clin Neuropharmacol, 1989. **12**(2): p. 98-105.
17. Ozinga, S.J., et al., *Objective assessment of postural stability in Parkinson's disease using mobile technology.* Mov Disord, 2015. **30**(9): p. 1214-21.
18. Poewe, W., *Dysautonomia and cognitive dysfunction in Parkinson's disease.* Mov Disord, 2007. **22 Suppl 17**: p. S374-8.
19. Hawkes, C.H., K. Del Tredici, and H. Braak, *Parkinson's disease: a dual-hit hypothesis.* Neuropathology and Applied Neurobiology, 2007. **33**(6): p. 599-614.
20. de Lau, L.M. and M.M. Breteler, *Epidemiology of Parkinson's disease.* Lancet Neurol, 2006. **5**(6): p. 525-35.
21. Abdullah, R., et al., *Parkinson's disease and age: The obvious but largely unexplored link.* Exp Gerontol, 2015. **68**: p. 33-8.
22. Hindle, J.V., *Ageing, neurodegeneration and Parkinson's disease.* Age Ageing, 2010. **39**(2): p. 156-61.
23. Saez, I. and D. Vilchez, *The Mechanistic Links Between Proteasome Activity, Aging and Age-related Diseases.* Curr Genomics, 2014. **15**(1): p. 38-51.
24. Reeve, A., E. Simcox, and D. Turnbull, *Ageing and Parkinson's disease: why is advancing age the biggest risk factor?* Ageing Res Rev, 2014. **14**: p. 19-30.
25. Martin, I., V.L. Dawson, and T.M. Dawson, *Recent advances in the genetics of Parkinson's disease.* Annu Rev Genomics Hum Genet, 2011. **12**: p. 301-25.
26. 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.
27. Zimprich, A., et al., *Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology.* Neuron, 2004. **44**(4): p. 601-7.
28. Kitada, T., et al., *Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism.* Nature, 1998. **392**(6676): p. 605-8.
29. Valente, E.M., et al., *Hereditary early-onset Parkinson's disease caused by mutations in PINK1.* Science, 2004. **304**(5674): p. 1158-60.
30. Shimura, H., et al., *Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase.* Nat Genet, 2000. **25**(3): p. 302-5.
31. Pezzoli, G. and E. Cereda, *Exposure to pesticides or solvents and risk of Parkinson disease.* Neurology, 2013. **80**(22): p. 2035-41.
32. Cole, N.B., et al., *Metal-catalyzed oxidation of alpha-synuclein: helping to define the relationship between oligomers, protofibrils, and filaments.* J Biol Chem, 2005. **280**(10): p. 9678-90.
33. Tanner, C.M., et al., *Occupation and risk of parkinsonism: a multicenter case-control study.* Arch Neurol, 2009. **66**(9): p. 1106-13.
34. Dexter, D.T., et al., *Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease.* J Neurochem, 1989. **52**(6): p. 1830-6.
35. Abdullah, R., et al., *Subcellular Parkinson's Disease-Specific Alpha-Synuclein Species Show Altered Behavior in Neurodegeneration.* Mol Neurobiol, 2017. **54**(10): p. 7639-7655.

36. Winner, B., et al., *In vivo demonstration that alpha-synuclein oligomers are toxic.* Proc Natl Acad Sci U S A, 2011. **108**(10): p. 4194-9.
37. Prots, I., et al., *alpha-Synuclein oligomers impair neuronal microtubule-kinesin interplay.* J Biol Chem, 2013. **288**(30): p. 21742-54.
38. Jellinger, K.A., *Basic mechanisms of neurodegeneration: a critical update.* J Cell Mol Med, 2010. **14**(3): p. 457-87.
39. Abdullah, R., et al., *Parkinson's disease and age: The obvious but largely unexplored link.* Experimental Gerontology, 2015. **68**: p. 33-38.
40. Neumann, C.A., et al., *Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression.* Nature, 2003. **424**(6948): p. 561-5.
41. Obeso, J.A., et al., *Missing pieces in the Parkinson's disease puzzle.* Nat Med, 2010. **16**(6): p. 653-61.
42. Kawamoto, Y., et al., *Accumulation of HtrA2/Omi in neuronal and glial inclusions in brains with alpha-synucleinopathies.* J Neuropathol Exp Neurol, 2008. **67**(10): p. 984-93.
43. Alvarez-Erviti, L., et al., *Influence of microRNA deregulation on chaperone-mediated autophagy and alpha-synuclein pathology in Parkinson's disease.* Cell Death Dis, 2013. **4**: p. e545.
44. Kiffin, R., et al., *Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age.* J Cell Sci, 2007. **120**(Pt 5): p. 782-91.
45. Scherz-Shouval, R. and Z. Elazar, *ROS, mitochondria and the regulation of autophagy.* Trends Cell Biol, 2007. **17**(9): p. 422-7.
46. Yamaguchi, R. and G. Perkins, *Dynamics of mitochondrial structure during apoptosis and the enigma of Opa1.* Biochim Biophys Acta, 2009. **1787**(8): p. 963-72.
47. Menzies, F.M., K. Moreau, and D.C. Rubinsztein, *Protein misfolding disorders and macroautophagy.* Curr Opin Cell Biol, 2011. **23**(2): p. 190-7.
48. Mizushima, N., et al., *Autophagy fights disease through cellular self-digestion.* Nature, 2008. **451**(7182): p. 1069-75.
49. Wong, E. and A.M. Cuervo, *Autophagy gone awry in neurodegenerative diseases.* Nat Neurosci, 2010. **13**(7): p. 805-11.
50. Sarkar, S., *Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers.* Biochem Soc Trans, 2013. **41**(5): p. 1103-30.
51. Vidal, R.L., et al., *Targeting autophagy in neurodegenerative diseases.* Trends Pharmacol Sci, 2014. **35**(11): p. 583-91.
52. Cuervo, A.M., et al., *Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy.* Science, 2004. **305**(5688): p. 1292-5.
53. Komatsu, M., et al., *Loss of autophagy in the central nervous system causes neurodegeneration in mice.* Nature, 2006. **441**(7095): p. 880-4.
54. Poole, A.C., et al., *The PINK1/Parkin pathway regulates mitochondrial morphology.* Proc Natl Acad Sci U S A, 2008. **105**(5): p. 1638-43.

55. Krebiehl, G., et al., *Reduced basal autophagy and impaired mitochondrial dynamics due to loss of Parkinson's disease-associated protein DJ-1.* PLoS One, 2010. **5**(2): p. e9367.
56. Liang, C.L., et al., *Mitochondria mass is low in mouse substantia nigra dopamine neurons: implications for Parkinson's disease.* Exp Neurol, 2007. **203**(2): p. 370-80.
57. Rao, S.S., L.A. Hofmann, and A. Shakil, *Parkinson's disease: diagnosis and treatment.* Am Fam Physician, 2006. **74**(12): p. 2046-54.
58. Foster, H.D. and A. Hoffer, *The two faces of L-DOPA: benefits and adverse side effects in the treatment of Encephalitis lethargica, Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis.* Med Hypotheses, 2004. **62**(2): p. 177-81.
59. Ardekani, A.M. and M.M. Naeini, *The Role of MicroRNAs in Human Diseases.* Avicenna J Med Biotechnol, 2010. **2**(4): p. 161-79.
60. Ha, M. and V.N. Kim, *Regulation of microRNA biogenesis.* Nat Rev Mol Cell Biol, 2014. **15**(8): p. 509-24.
61. Carthew, R.W. and E.J. Sontheimer, *Origins and Mechanisms of miRNAs and siRNAs.* Cell, 2009. **136**(4): p. 642-55.
62. Kim, V.N., J. Han, and M.C. Siomi, *Biogenesis of small RNAs in animals.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 126-39.
63. Denli, A.M., et al., *Processing of primary microRNAs by the Microprocessor complex.* Nature, 2004. **432**(7014): p. 231-5.
64. Zhang, H., et al., *Single processing center models for human Dicer and bacterial RNase III.* Cell, 2004. **118**(1): p. 57-68.
65. Yoda, M., et al., *ATP-dependent human RISC assembly pathways.* Nat Struct Mol Biol, 2010. **17**(1): p. 17-23.
66. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
67. Siciliano, V., et al., *MiRNAs confer phenotypic robustness to gene networks by suppressing biological noise.* Nat Commun, 2013. **4**: p. 2364.
68. Chen, X., et al., *Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases.* Cell Res, 2008. **18**(10): p. 997-1006.
69. Cogswell, J.P., et al., *Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways.* J Alzheimers Dis, 2008. **14**(1): p. 27-41.
70. Gallo, A., et al., *The majority of microRNAs detectable in serum and saliva is concentrated in exosomes.* PLoS One, 2012. **7**(3): p. e30679.
71. Zhou, Q., et al., *Immune-related microRNAs are abundant in breast milk exosomes.* Int J Biol Sci, 2012. **8**(1): p. 118-23.
72. Mitchell, P.S., et al., *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10513-8.
73. Iftikhar, H. and G.E. Carney, *Evidence and potential in vivo functions for biofluid miRNAs: From expression profiling to functional testing: Potential roles of*

extracellular miRNAs as indicators of physiological change and as agents of intercellular information exchange. Bioessays, 2016. **38**(4): p. 367-78.
74. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies.* Nature, 1997. **388**(6645): p. 839-40.
75. Kruger, R., et al., *Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease.* Nat Genet, 1998. **18**(2): p. 106-8.
76. 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.
77. Singleton, A.B., et al., *alpha-Synuclein locus triplication causes Parkinson's disease.* Science, 2003. **302**(5646): p. 841.
78. Conway, K.A., et al., *Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 571-6.
79. Emmanouilidou, E., et al., *Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival.* J Neurosci, 2010. **30**(20): p. 6838-51.
80. Narendra, D.P., et al., *PINK1 is selectively stabilized on impaired mitochondria to activate Parkin.* PLoS Biol, 2010. **8**(1): p. e1000298.
81. Narendra, D., et al., *Parkin is recruited selectively to impaired mitochondria and promotes their autophagy.* J Cell Biol, 2008. **183**(5): p. 795-803.
82. Jiang, H., et al., *Parkin protects human dopaminergic neuroblastoma cells against dopamine-induced apoptosis.* Hum Mol Genet, 2004. **13**(16): p. 1745-54.
83. Perez, F.A. and R.D. Palmiter, *Parkin-deficient mice are not a robust model of parkinsonism.* Proc Natl Acad Sci U S A, 2005. **102**(6): p. 2174-9.
84. Muftuoglu, M., et al., *Mitochondrial complex I and IV activities in leukocytes from patients with parkin mutations.* Mov Disord, 2004. **19**(5): p. 544-8.
85. Palacino, J.J., et al., *Mitochondrial dysfunction and oxidative damage in parkin-deficient mice.* J Biol Chem, 2004. **279**(18): p. 18614-22.
86. Arkinson, C. and H. Walden, *Parkin function in Parkinson's disease.* Science, 2018. **360**(6386): p. 267-268.
87. Deter, R.L. and C. De Duve, *Influence of glucagon, an inducer of cellular autophagy, on some physical properties of rat liver lysosomes.* J Cell Biol, 1967. **33**(2): p. 437-49.
88. Yao, Z., et al., *Atg41/Icy2 regulates autophagosome formation.* Autophagy, 2015. **11**(12): p. 2288-99.
89. Gordy, C. and Y.W. He, *The crosstalk between autophagy and apoptosis: where does this lead?* Protein Cell, 2012. **3**(1): p. 17-27.
90. Pierdominici, M., et al., *Role of autophagy in immunity and autoimmunity, with a special focus on systemic lupus erythematosus.* FASEB J, 2012. **26**(4): p. 1400-12.
91. Hara, T., et al., *Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice.* Nature, 2006. **441**(7095): p. 885-9.

92. Hu, Z.Y., et al., *Up-regulation of autophagy-related gene 5 (ATG5) protects dopaminergic neurons in a zebrafish model of Parkinson's disease.* J Biol Chem, 2017. **292**(44): p. 18062-18074.
93. Yokoyama, H., et al., *Neuropharmacological approach against MPTP (1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine)-induced mouse model of Parkinson's disease.* Acta Neurobiol Exp (Wars), 2011. **71**(2): p. 269-80.
94. Chen, D., et al., *A novel and functional variant within the ATG5 gene promoter in sporadic Parkinson's disease.* Neurosci Lett, 2013. **538**: p. 49-53.
95. Kerscher, O., R. Felberbaum, and M. Hochstrasser, *Modification of proteins by ubiquitin and ubiquitin-like proteins.* Annu Rev Cell Dev Biol, 2006. **22**: p. 159-80.
96. Nijman, S.M., et al., *A genomic and functional inventory of deubiquitinating enzymes.* Cell, 2005. **123**(5): p. 773-86.
97. Durcan, T.M., et al., *The Machado-Joseph disease-associated mutant form of ataxin-3 regulates parkin ubiquitination and stability.* Hum Mol Genet, 2011. **20**(1): p. 141-54.
98. Kawaguchi, Y., et al., *CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1.* Nat Genet, 1994. **8**(3): p. 221-8.
99. Zoghbi, H.Y. and H.T. Orr, *Glutamine repeats and neurodegeneration.* Annu Rev Neurosci, 2000. **23**: p. 217-47.
100. Orr, H.T. and H.Y. Zoghbi, *Reversing neurodegeneration: a promise unfolds.* Cell, 2000. **101**(1): p. 1-4.
101. Klein, C., S.A. Schneider, and A.E. Lang, *Hereditary parkinsonism: Parkinson disease look-alikes--an algorithm for clinicians to "PARK" genes and beyond.* Mov Disord, 2009. **24**(14): p. 2042-58.
102. Durcan, T.M. and E.A. Fon, *Mutant ataxin-3 promotes the autophagic degradation of parkin.* Autophagy, 2011. **7**(2): p. 233-4.
103. Sousa, R., *Structural mechanisms of chaperone mediated protein disaggregation.* Front Mol Biosci, 2014. **1**: p. 12.
104. Qu, D., et al., *BAG2 Gene-mediated Regulation of PINK1 Protein Is Critical for Mitochondrial Translocation of PARKIN and Neuronal Survival.* J Biol Chem, 2015. **290**(51): p. 30441-52.
105. De Snoo, M.L., et al., *Bcl-2-associated athanogene 5 (BAG5) regulates Parkin-dependent mitophagy and cell death.* Cell Death Dis, 2019. **10**(12): p. 907.
106. Guo, K., et al., *Bag5 protects neuronal cells from amyloid beta-induced cell death.* J Mol Neurosci, 2015. **55**(4): p. 815-20.
107. Wang, X., et al., *BAG5 protects against mitochondrial oxidative damage through regulating PINK1 degradation.* PLoS One, 2014. **9**(1): p. e86276.
108. Ma, M., et al., *Protective effect of BAG5 on MPP+-induced apoptosis in PC12 cells.* Neurol Res, 2012. **34**(10): p. 977-83.
109. Qin, L.X., et al., *BAG5 Interacts with DJ-1 and Inhibits the Neuroprotective Effects of DJ-1 to Combat Mitochondrial Oxidative Damage.* Oxid Med Cell Longev, 2017. **2017**: p. 5094934.
110. de Rijk, M.C., et al., *Prevalence of Parkinson's disease in the elderly: the Rotterdam Study.* Neurology, 1995. **45**(12): p. 2143-6.

111. Pickrell, A.M. and R.J. Youle, *The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease.* Neuron, 2015. **85**(2): p. 257-73.
112. Farrer, M.J., *Genetics of Parkinson disease: paradigm shifts and future prospects.* Nat Rev Genet, 2006. **7**(4): p. 306-18.
113. Caggiu, E., et al., *Differential expression of miRNA 155 and miRNA 146a in Parkinson's disease patients.* eNeurologicalSci, 2018. **13**: p. 1-4.
114. Kurtishi, A., et al., *Cellular Proteostasis in Neurodegeneration.* Mol Neurobiol, 2018.
115. Dawson, T.M. and V.L. Dawson, *Parkin plays a role in sporadic Parkinson's disease.* Neurodegener Dis, 2014. **13**(2-3): p. 69-71.
116. Klein, C. and K. Lohmann-Hedrich, *Impact of recent genetic findings in Parkinson's disease.* Curr Opin Neurol, 2007. **20**(4): p. 453-64.
117. 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.
118. Song, J., M. Takeda, and R.I. Morimoto, *Bag1-Hsp70 mediates a physiological stress signalling pathway that regulates Raf-1/ERK and cell growth.* Nat Cell Biol, 2001. **3**(3): p. 276-82.
119. Kalia, S.K., et al., *BAG5 inhibits parkin and enhances dopaminergic neuron degeneration.* Neuron, 2004. **44**(6): p. 931-45.
120. McNeill, E. and D. Van Vactor, *MicroRNAs shape the neuronal landscape.* Neuron, 2012. **75**(3): p. 363-79.
121. Patil, K.S., et al., *Combinatory microRNA serum signatures as classifiers of Parkinson's disease.* Parkinsonism Relat Disord, 2019. **64**: p. 202-210.
122. Hoss, A.G., et al., *microRNA Profiles in Parkinson's Disease Prefrontal Cortex.* Front Aging Neurosci, 2016. **8**: p. 36.
123. Miñones-Moyano, E., et al., *MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function.* Hum Mol Genet, 2011. **20**(15): p. 3067-78.
124. Mouradian, M.M., *MicroRNAs in Parkinson's disease.* Neurobiol Dis, 2012. **46**(2): p. 279-84.
125. Patil, K.S., et al., *Combinatory microRNA serum signatures as classifiers of Parkinson's disease.* Parkinsonism Relat Disord, 2019. **64**: p. 202-210.
126. Beilina, A., et al., *Unbiased screen for interactors of leucine-rich repeat kinase 2 supports a common pathway for sporadic and familial Parkinson disease.* Proc Natl Acad Sci U S A, 2014. **111**(7): p. 2626-31.
127. Ashkenazi, A., et al., *Polyglutamine tracts regulate autophagy.* Autophagy, 2017. **13**(9): p. 1613-1614.
128. Kalia, S.K., et al., *BAG5 inhibits parkin and enhances dopaminergic neuron degeneration.* Neuron, 2004. **44**(6): p. 931-945.
129. Chung, K.K. and T.M. Dawson, *Parkin and Hsp70 sacked by BAG5.* Neuron, 2004. **44**(6): p. 899-901.
130. Hsu, J.Y., et al., *The Truncated C-terminal Fragment of Mutant ATXN3 Disrupts Mitochondria Dynamics in Spinocerebellar Ataxia Type 3 Models.* Front Mol Neurosci, 2017. **10**: p. 196.

131. Bettencourt, C., et al., *Parkinsonian phenotype in Machado-Joseph disease (MJD/SCA3): a two-case report.* BMC Neurology, 2011. **11**(1): p. 131.
132. Coppede, F. and L. Migliore, *DNA damage in neurodegenerative diseases.* Mutat Res, 2015. **776**: p. 84-97.
133. Hipp, M.S., S.H. Park, and F.U. Hartl, *Proteostasis impairment in protein-misfolding and -aggregation diseases.* Trends Cell Biol, 2014. **24**(9): p. 506-14.
134. Hetz, C., *The unfolded protein response: controlling cell fate decisions under ER stress and beyond.* Nature Reviews Molecular Cell Biology, 2012. **13**: p. 89.
135. Komatsu, M., et al., *Constitutive autophagy: vital role in clearance of unfavorable proteins in neurons.* Cell Death And Differentiation, 2007. **14**: p. 887.
136. Drazic, A., et al., *The world of protein acetylation.* Biochim Biophys Acta, 2016. **1864**(10): p. 1372-401.
137. Saudou, F., et al., *Huntingtin Acts in the Nucleus to Induce Apoptosis but Death Does Not Correlate with the Formation of Intranuclear Inclusions.* Cell. **95**(1): p. 55-66.
138. Wakabayashi, K., et al., *Parkinson's disease: the presence of Lewy bodies in Auerbach's and Meissner's plexuses.* Acta Neuropathol, 1988. **76**(3): p. 217-221.
139. Lee, S.H., et al., *Emotional well-being and gut microbiome profiles by enterotype.* Sci Rep, 2020. **10**(1): p. 20736.
140. Karst, S.M., *The influence of commensal bacteria on infection with enteric viruses.* Nat Rev Microbiol, 2016. **14**(4): p. 197-204.
141. Cersosimo, M.G. and E.E. Benarroch, *Pathological correlates of gastrointestinal dysfunction in Parkinson's disease.* Neurobiol Dis, 2012. **46**(3): p. 559-64.
142. Dutta, S.K., et al., *Parkinson's Disease: The Emerging Role of Gut Dysbiosis, Antibiotics, Probiotics, and Fecal Microbiota Transplantation.* J Neurogastroenterol Motil, 2019. **25**(3): p. 363-376.
143. Dhar, D. and A. Mohanty, *Gut microbiota and Covid-19- possible link and implications.* Virus Res, 2020. **285**: p. 198018.
144. Sulzer, D., et al., *COVID-19 and possible links with Parkinson's disease and parkinsonism: from bench to bedside.* NPJ Parkinsons Dis, 2020. **6**: p. 18.
145. Wu, Y., et al., *Prolonged presence of SARS-CoV-2 viral RNA in faecal samples.* Lancet Gastroenterol Hepatol, 2020. **5**(5): p. 434-435.
146. Sampson, T.R., et al., *Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease.* Cell, 2016. **167**(6): p. 1469-1480 e12.
147. Stolzenberg, E., et al., *A Role for Neuronal Alpha-Synuclein in Gastrointestinal Immunity.* J Innate Immun, 2017. **9**(5): p. 456-463.
148. Tansey, M.G., M.K. McCoy, and T.C. Frank-Cannon, *Neuroinflammatory mechanisms in Parkinson's disease: potential environmental triggers, pathways, and targets for early therapeutic intervention.* Exp Neurol, 2007. **208**(1): p. 1-25.
149. Wyss-Coray, T. and L. Mucke, *Inflammation in Neurodegenerative Disease—A Double-Edged Sword.* Neuron, 2002. **35**(3): p. 419-432.
150. Aslam, N., et al., *Effects of chelating agents on heavy metals in Hepatitis C Virus (HCV) patients.* Math Biosci Eng, 2019. **16**(3): p. 1138-1149.

151. Saito, Y., et al., *Lewy body pathology involves the olfactory cells in Parkinson's disease and related disorders.* Mov Disord, 2016. **31**(1): p. 135-8.
152. Braak, H., et al., *Staging of brain pathology related to sporadic Parkinson's disease.* Neurobiol Aging, 2003. **24**(2): p. 197-211.
153. Braak, H., et al., *Stages in the development of Parkinson's disease-related pathology.* Cell Tissue Res, 2004. **318**(1): p. 121-34.
154. Bantle, C.M., et al., *Infection with mosquito-borne alphavirus induces selective loss of dopaminergic neurons, neuroinflammation and widespread protein aggregation.* NPJ Parkinsons Dis, 2019. **5**: p. 20.
155. Beatman, E.L., et al., *Alpha-Synuclein Expression Restricts RNA Viral Infections in the Brain.* J Virol, 2015. **90**(6): p. 2767-82.
156. Paracha, U.Z., et al., *Oxidative stress and hepatitis C virus.* Virol J, 2013. **10**: p. 251.
157. Klein, R.S., et al., *Neuroinflammation During RNA Viral Infections.* Annu Rev Immunol, 2019. **37**: p. 73-95.
158. Ahn, D.G., et al., *Biochemical characterization of a recombinant SARS coronavirus nsp12 RNA-dependent RNA polymerase capable of copying viral RNA templates.* Arch Virol, 2012. **157**(11): p. 2095-104.
159. Montgomery, E.B., Jr., *Heavy metals and the etiology of Parkinson's disease and other movement disorders.* Toxicology, 1995. **97**(1-3): p. 3-9.
160. Rothschild, D., et al., *Environment dominates over host genetics in shaping human gut microbiota.* Nature, 2018. **555**(7695): p. 210-215.
161. Brann, D.H., et al., *Non-neuronal expression of SARS-CoV-2 entry genes in the olfactory system suggests mechanisms underlying COVID-19-associated anosmia.* Science Advances, 2020. **6**(31): p. eabc5801.
162. Passarelli, P.C., et al., *Taste and smell as chemosensory dysfunctions in COVID-19 infection.* Am J Dent, 2020. **33**(3): p. 135-137.

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