FUNCTIONAL ANALYSIS OF NEUROPROTECTIVE GENES USING *CAENORHABDITIS ELEGANS* AND MAMMALIAN MODELS OF PARKINSON’S DISEASE

by

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A DISSERTATION

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ABSTRACT

Dysfunction of the protein degradation machinery has emerged as a leading cellular defect associated with age-onset proteotoxicity in neurodegenerative diseases, including Parkinson’s disease (PD). While the etiology of PD is not fully understood, the protein α-synuclein (α-syn) is thought to play a central role in the pathology associated with this disease, in that accumulation of α-syn is observed in both familial and sporadic forms of PD and mutations or overexpression of α-syn result in enhanced dopaminergic (DA) neurodegeneration. Without an effective therapeutic treatment for the progression of PD, we set out to identify and validate potential genetic and chemical modifiers of key pathological features of PD, including α-syn accumulation and DA neurodegeneration using Caenorhabditis elegans in vivo studies and mammalian cell culture in vitro assays. Using these approaches, we validated the lysosomal trafficking protein, VPS41, as a potential neuroprotective candidate, in that overexpression of VPS41 suppressed both α-syn and chemical neurotoxicity in C. elegans and mammalian cell culture, as well as suppressed α-syn accumulation in mammalian cells. Through a structure/function analysis, we identified the minimal domains required for the protective function of human VPS41. Using worms engineered to enable DA-neuron specific RNAi, we showed that post-Golgi trafficking of AP-3 vesicles to the lysosome affected α-syn-induced neurodegeneration, indicating that VPS41 may be mechanistically eliciting protection via Golgi to lysosome trafficking. Furthermore, we functionally analyzed two VPS41 single nucleotide polymorphisms (SNPs) that naturally occur in human populations and found that both SNPs prevent VPS41 from suppressing α-syn accumulation and neurotoxicity in mammalian cells and
C. elegans, respectively. These SNP data may represent additional genetic susceptibility factors for PD onset or progression. Additionally, using our C. elegans model of α-syn-induced DA neurodegeneration, we analyzed other genetic and chemicals for potential neuroprotective capacity. In this regard, the protein 14-3-3θ and the small molecule bafilomycin, originally identified in mammalian experiments, were functionally validated in C. elegans. Taken together, these studies support the predictive nature of C. elegans in validating potential modifiers of α-syn neurotoxicity, and highlight the importance of lysosomal function in maintaining α-syn homeostasis and its implications for suppressing the pathology associated with PD.
DEDICATION

I would like to dedicate this dissertation to everyone who helped me and supported me over the past years to achieve this masterpiece. In particular, this manuscript is dedicated to my parents, Johnny and Dottie Harrington, and my brother, Ben, who supported me in any way possible throughout the creation of this dissertation.
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADE</td>
<td>Anterior deirid neuron</td>
</tr>
<tr>
<td>ALP pathway</td>
<td>Alkaline phosphatase pathway</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>BafB1</td>
<td>Bafilomycin B1</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<td>bp</td>
<td>Base pair</td>
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<tr>
<td>°C</td>
<td>Celsius</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;++&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CEP</td>
<td>Cephalic neuron</td>
</tr>
<tr>
<td>CHCR</td>
<td>Clathrin heavy chain repeat domain</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COR</td>
<td>C-terminal of Roc</td>
</tr>
<tr>
<td>CORVET</td>
<td>Class C core vacuole/endosome tethering complex</td>
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<td>CPY pathway</td>
<td>Carboxypeptidase Y pathway</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
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<td>Control</td>
</tr>
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<td>δ</td>
<td>Delta</td>
</tr>
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<td>D2</td>
<td>Dopamine 2</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>Dimethyl sulfoxide</td>
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<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
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<td>Epsilon</td>
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<td>Ubiquitin ligase</td>
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<td>E4</td>
<td>Multiubiquitylation enzyme</td>
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<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
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<tr>
<td>f</td>
<td>Frequency</td>
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<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
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<td>γ</td>
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<td>GABA</td>
<td>Gamma aminobutyric acid</td>
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<td>GEF</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>GRO</td>
<td>Growth retardation</td>
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<td>hNSC</td>
<td>Human neural stem cell</td>
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<td>Full Name/Definition</td>
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<tr>
<td>HOPS</td>
<td>Homotypic fusion and protein sorting complex</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>MG-132</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>Δψm</td>
<td>Mitochondrial membrane potential</td>
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<tr>
<td>Mn⁺⁺</td>
<td>Manganese</td>
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<tr>
<td>MPP⁺</td>
<td>1-Methyl-4-phenylpyridinium</td>
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<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,5,6-tetrahydropyridine</td>
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<td>µg</td>
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<td>nm</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
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<td>Abbreviation</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PARK</td>
<td>Parkinson’s disease gene</td>
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<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDE</td>
<td>Posterior deirid neuron</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride membrane</td>
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<tr>
<td>RING</td>
<td>Really interesting new gene domain</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>shRNA</td>
<td>Small hairpin RNA</td>
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<td>Substantia nigra pars compacta</td>
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<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
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<td>θ</td>
<td>Theta</td>
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<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
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<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
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<tr>
<td>TPR domain</td>
<td>Tetratricopeptide repeat domain</td>
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<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
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<td>Unc</td>
<td>Uncoordinated movement</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td>WD40</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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<td>ζ</td>
<td>Zeta</td>
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**Proteins/Genes**

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<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
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<td>AP-2</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<td>CAT-2</td>
<td><em>C. elegans</em> tyrosine hydroxylase</td>
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<tr>
<td>CD</td>
<td>Cathepsin D</td>
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<tr>
<td>CHIP</td>
<td>C-terminus of Hsp70-interacting protein</td>
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<td>CHN-1</td>
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<tr>
<td>DAT-1</td>
<td>Dopamine transporter 1</td>
</tr>
<tr>
<td>DJ-1</td>
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<td>DJR-1</td>
<td>Oncogene DJ-1</td>
</tr>
<tr>
<td>DJR-2</td>
<td>Oncogene DJ-1</td>
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</table>
FTT-2  Fourteen-three-three (C. elegans homolog)
GADPH  Glyceraldehyde-3-phosphate dehydrogenase
GBA    Glucocerebrosidase
GIGYF2 GRB10-interacting GYF protein 2
GRPK2  G-protein coupled receptor kinase
HIF-1α Hypoxia inducible factor 1α
HTRA2  HTRA serine peptidase 2
HPRT-1 Hypoxanthine phosphoribosyltransferase
LAMP1  Lysosome associated membrane protein
Light  Drosophila VPS41 homolog
LDH    Lactate dehydrogenase
LRK-1  Leucine-rich repeats, Ras-like domain, kinase 1 (LRRK2)
LRRK2  Leucine-rich repeat kinase 2
NPC1   Niemann-Pick disease type C1
Pael receptor Parkin-associated endothelin receptor
PARK   Parkinson’s disease gene
PARP   Poly ADP ribose polymerase
PDR-1  Parkinson’s disease related 1 (parkin)
PGP-3  P-glycoprotein transmembrane protein
PINK1  PTEN-induced putative kinase 1
RAB1   Ras-associated protein 1
RAB3   Ras-associated protein 3
RAB5   Ras-associated protein 5
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<td>synT</td>
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<td>TOR-2</td>
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<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
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<td>UBC-2</td>
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<tr>
<td>VPS41</td>
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<tr>
<td>Yck3</td>
<td>Yeast casein kinase 3</td>
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ACKNOWLEDGMENTS

First and foremost, I would like to acknowledge my mentors, Drs. Guy and Kim Caldwell, for giving me the opportunity and support to continue my education and achieve this goal. Their help and guidance assisted in the maturation of me as a scientist and taught me how to critically analyze problems that need to be addressed in the science community. Working in their lab also taught me the skill of managing other students to achieve the goals in which we set out to achieve. With their continued support, I was able to explore various areas outside of my main dissertation projects to study various aspects of biology in which I have intense interest.

The progression of this dissertation would not have been possible without my graduate committee members, Dr. Janis O’Donnell, Dr. Perry Churchill, Dr. Stevan Marcus, and Dr. David Standaert. Their continued support, suggestions, and stimulating questions motivated and guided me through the trials and tribulations that occurred throughout my research. I would also like to acknowledge all faculty members who contributed to the progression of this masterpiece, especially Dr. Andy West, Dr. Talene Yacoubian, and Dr. Qingmin Ruan, for their insights into mammalian cell culture studies.

I would also like to acknowledge the general dynamic and cooperative nature of all members of the Caldwell lab, as well as discussions stemming from UA-UAB Parkinson’s Research Group meetings. The supportive nature of all Caldwell lab members assisted in the progression of this dissertation. In particular, I would like to thank Dr. Shusei Hamamichi, Dr. AJ Burdette, and Susan DeLeon for their continued support and guidance during the maturation
of my projects, and for the science-based discussions we shared weekly. I would like to acknowledge Wilhagan’s for always having cold beer on tap and a supportive atmosphere for us to conduct our scientific discussions.

Financial support for the PD research I conducted in the Caldwell lab over the past 5½ years came from the Michael J. Fox Foundation for Parkinson’s Research, QRxPharma, Ltd, the Department of Biological Sciences at the University of Alabama, as well as the UA Graduate Council. Without their support, the research described here within would not have been possible.

Lastly, this work would not have been possible without the support of my family and friends. The continued encouragement from them enabled me to focus on my goals and persist, despite the trials that I encountered throughout the years.
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CHAPTER 1
INTRODUCTION

The introduction covering the background of PD and the C. elegans models of PD was published in *Developmental Dynamics*, May, 2010 under the following citation: Harrington A.J., Hamamichi S., Caldwell G.A., Caldwell K.A. (2010) *Dev Dyn* 238:1282-1295. Adam Harrington, Dr. Shusei Hamamichi, Dr. Guy Caldwell, and Dr. Kim Caldwell co-wrote the manuscript.

*Parkinson's disease*

Since the seminal discovery of PARK1/α-synuclein (α-syn) as a genetic cause of familial PD in 1997 (Spillantini et al.), human geneticists have meticulously identified, in the span of just over 10 years, eight additional gene products that are linked to this disease. These studies determined a set of mutations, modifying functions as well as expression level and pattern of PARK proteins, which ultimately trigger two most prevalent PD pathological hallmarks: formation of the proteinaceous inclusions called Lewy bodies in PD patient brains and a selective loss of dopamine (DA) neurons in the *substantia nigra pars compacta*. Subsequently, mammalian researchers performed functional analyses of diverse PARK proteins, unveiling a snapshot of potential cellular defects in several common pathways. For example, PARK proteins are implicated in synaptic function (PARK1/α-synuclein), protein degradation (PARK2/parkin,
PARK9/ATP13A2, and PARK5/UCHL-1), signal transduction (PARK8/LRRK2 and PARK11/GIGYF2), and protection against mitochondrial/oxidative stress (PARK6/PINK1, PARK7/DJ-1, and PARK13/HTRA2). Collectively, malfunctions in these cellular mechanisms may prompt the onset and progression of PD; limited therapeutic interventions are available, despite being the second most common neurodegenerative disease (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Fahn, 2003).

While studies on familial PARK proteins have founded the basis for the current understanding of PD etiology, it has been reported that only 5-10% of all PD cases are associated with monogenic forms of PD. Consequently, multiple genetic and environmental susceptibility factors, singly or accumulatively, may contribute to idiopathic form of this disease. Nevertheless, human genetic analyses continue to play a pivotal role in terms of illuminating mechanistic insights into PD. Importantly, these findings have led to development of animal models that facilitate evaluation. These findings represent a laborious task of examining potential genetic or physical interactions, screening for susceptibility factors, and identification and validation of putative neuroprotective candidates that may also serve as diagnostic or therapeutic targets. Mammalian PD models remain invaluable tools, especially considering the wide range of clinical features associated with PD, such as muscle rigidity, tremors, depression, and dementia, (Lees et al., 2009) that may illustrate under-explored intra- and inter-neuronal misregulation beyond the DA system. Nevertheless, mammalian models may not recapitulate all PD pathological impairments (Fleming and Chesselet, 2006); moreover these models have limitations and are expensive. In this regard, despite their lack of evolutionary complexity, invertebrate model organisms, such as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, have been utilized as first pass screens for identifying genes and drugs...
that might be therapeutically relevant to PD processes (Gitler, 2008; Bilen and Bonini, 2005; Caldwell and Caldwell, 2008). These simple organisms share many conserved molecular pathways and cellular mechanisms with mammals and offer economical, yet strategic experimental paradigms that allow rapid analyses of susceptibility factors that can be predictive of PD pathological mechanisms.

*C. elegans as a model organism*

Among the various cellular and transgenic models available for use in studying the pathology of PD, *C. elegans* offers several distinct advantages enabling researchers to dissect avenues leading to this disease. While mammals have billions of neurons in their brain, and even fruit flies have ~10,000 neurons, the adult *C. elegans* hermaphrodite has exactly 302 neurons throughout its body, with fully mapped neuronal circuitry (Bargmann, 1998). Of the 302 neurons, 8 neurons are dopaminergic in the hermaphrodite (Figure 1.1A), which include 6 anterior dopaminergic neurons (4 CEP neurons and 2 ADE neurons) (Figure 1.1B) and 2 posterior DA neurons (PDE neurons) (Sulston et al., 1975). With *C. elegans* being a completely transparent animal, neurons are easily visualized by simply expressing a fluorescent protein (e.g., GFP), as previously described by Martin Chalfie and colleagues (1994), who later shared the 2008 Nobel Prize in Chemistry for his groundbreaking research using GFP. This is particularly useful when studying neurodegenerative diseases, such as PD, in that neuronal cell death can be readily observed and quantified within living organisms (Figure 1.1C,D). The capacity for immediate assessment for the presence or absence of specific neurons in a living organism is a key attribute in using this system to study neurodegenerative disorders.
Interestingly, the pathways involved in the processing, packaging and transport of DA have been conserved throughout evolution, enabling researchers to utilize the worm for studying various aspects of DA neurons. By using methods that alter DA signaling in *C. elegans*, several behavioral phenotypes have been identified as being specific for DA signaling. Upon exposure to exogenous DA, worms exhibit a decrease in egg laying, locomotion, and defecation. DA also functions in the basal slowing response, where worms normally decrease locomotion upon entering a bacterial lawn (Sawin et al., 2000). Identification of mutations in *cat-2*, the worm tyrosine hydroxylase which is the rate limiting enzyme for DA synthesis, resulted in no basal slowing response, indicating the role of DA in mechanosensation and food sensing (McDonald et al., 2006). While this mutant phenotype is a viable readout for DA neuronal function, this behavioral effect is difficult to quantify during high-throughput screening, due to the time involved in collecting data.

The most advantageous attribute that *C. elegans* offers is the ability to perform a large-scale genetic analysis. The nematode was the first multicellular organism to have its complete genome sequenced and made available to the public (*C. elegans* Sequencing Consortium, 1998). In addition, a large number of mutant strains are available within the *C. elegans* community allowing researchers to study the effect of various proteins and pathways. Another tool widely used in *C. elegans* research is reverse genetics. This approach entails the application of the intracellular mechanism of RNA interference (RNAi) to knockdown target genes by simply injecting, soaking, or feeding worms dsRNA which is complementary to the targeted and subsequently silenced gene. Fire et al. (1998) initially described this method to induce selective knockdown of genes in *C. elegans*, for which Andrew Fire and Craig Mello later received the 2006 Nobel Prize in Physiology or Medicine. Along with examining reduced gene expression
through RNAi or different loss of function strains, researchers are also able to easily generate transgenic animals to express a protein of interest within specific cells to further analyze the function of the protein. Lastly, a short generation time (3 days from egg to adult) and the availability of various methods to alter expression of protein levels have established *C. elegans* as a valuable organism to dissect different pathways involved in the pathology of disease.

Both genetic and environmental causes of PD and their associated cellular malfunctions are described within the context of *C. elegans* biology in this introduction (Table 1.1). As discussed below, these genetic and environmental factors share common pathways that lead to a selective loss of DA neurons. Most notably, α-syn has been studied extensively due to its central role in PD pathogenesis, and multiple disease models have been generated via expression of wild-type or mutant α-syn. Among them, *C. elegans* has been exploited to uncover genetic interactions among different worm orthologs of PD genes, discern genetic and environmental susceptibility factors, and discover putative neuroprotective genes against α-syn toxicity. Taken together, the use of this nematode has provided a novel set of therapeutic targets as well as mechanistic insights into PD pathogenesis. Identification of putative genetic susceptibility loci is imperative to determine diagnostic and therapeutic targets to ameliorate clinical symptoms and ultimately halt DA neurodegeneration.

*C. elegans* models of α-syn neurotoxicity

With the completion of the *C. elegans* genomic sequence, many worm orthologs of human genes have been identified including those that are linked to familial PD. Currently, six worm orthologs have been identified (Table 1.2), with the most notable exception being α-syn. This feature has allowed *C. elegans* researchers to readily overexpress wild-type or mutant α-syn
in the α-syn null genetic background without concerns for endogenous α-syn or a dominant negative effect. α-Syn, a main component of Lewy bodies, plays a central role in pathogenesis of both familial and idiopathic forms of PD. Mutations in α-syn, such as A30P and A53T, in addition to multiplication of the α-syn locus have been linked to increased α-syn aggregation, as well as DA neuronal death (Mezey et al., 1998; Conway et al., 2000; Singleton et al., 2003; Ross et al., 2008). In C. elegans models, both α-syn and GFP are selectively expressed in a subset of cells. For example, Lakso et al. (2003), Cao et al. (2005), and Karpinar et al. (2009) co-express α-syn and GFP in DA neurons and reported that overexpression of either wild-type or mutant α-syn under the control of dat-1 (DA transporter) promoter resulted in the loss of DA neurons.

Initial hypotheses on PD causality suggested that α-syn aggregation may be toxic, and that formation of Lewy bodies may induce neuronal death by disrupting normal cellular functions. While detection of α-syn aggregation is challenging in worm DA neurons, Kuwahara et al. (2006) observed accumulation of α-syn in the cell bodies and dendrites of DA neurons overexpressing wild-type or mutant α-syn. They also reported that a small fraction of worm DA neurons exhibited a positive immunoreactivity to phosphorylated α-syn, which is suggestive of α-syn deposited in Lewy body-like inclusions. Although the formation of inclusion bodies is widely accepted as a PD pathological hallmark by possibly disrupting normal cellular functions, its role in neurotoxicity remains controversial. However, subsequent studies demonstrated that the inclusion bodies remain undetected in some forms of PD. Furthermore, multiple articles have reported that intermediate protofibrils of α-syn are more toxic than those found in either the monomeric or oligomerized state (Conway et al., 2001; Lashuel et al., 2002), providing an alternative hypothesis that the accumulation of mature protein aggregates may not be solely responsible for neurotoxicity. For example, since α-syn is primarily found in presynaptic nerve
termini (Clayton and George, 1998; Recchia et al., 2004) with high propensity for lipid binding (Welch and Yuan, 2003), this natively unfolded protein has been proposed to associate with synaptic vesicle functions. Consistent with this view, Karpinar et al. (2009) elegantly characterized A56P and A76P \(\alpha\)-syn variants, both part of \(\beta\)-sheet rich core of \(\alpha\)-syn fibrils, which disrupted \(\alpha\)-syn aggregate formation \textit{in vitro} and \textit{in vivo}. Remarkably, when \(\alpha\)-syn variants were overexpressed in worm DA neurons, they observed more robust neurodegeneration than wild-type, A30P, and A53T \(\alpha\)-syn, indicating that soluble oligomers, not insoluble aggregates, as toxic species.

To evaluate the effect of \(\alpha\)-syn on neuronal activity, changes in worm behavior have been examined as a functional readout. For example, overexpression of both wild-type and A53T \(\alpha\)-syn under the control of pan-neuronal promoter (\textit{aex-3}) and motor neuron promoters (\textit{acr-2} and \textit{unc-30}) in \textit{C. elegans} resulted in significant reduction of motor movement (Lasko et al., 2003). Moreover, overexpression of A30P and A53T \(\alpha\)-syn in worm DA neurons resulted in modified food-sensing movement, a mechanosensory behavioral response that is specific to the DA neurons, illustrating the alteration of DA neuronal activity (Kuwahara et al., 2006). This behavior was further exacerbated by \(\alpha\)-syn with A56P and A76P mutations (Karpinar et al., 2009). It is conceivable that, similar to observations in mammalian neuronal culture, intermediate protofibrils may exert toxicity by physically disrupting vesicular membranes (Volles et al., 2001) and causing defective sequestration of DA into synaptic vesicles (Lotharius and Brundin, 2002). In \textit{C. elegans}, Kuwahara et al. (2006) reported A30P and A53T \(\alpha\)-syn overexpression resulted in reduced DA levels, but oxidized DA was not examined. Intriguingly, Ved et al. (2005) showed that pan-neuronal (\textit{unc-119}) overexpression of A53T \(\alpha\)-syn, after rotenone treatment, increased thioflavine-positive \(\alpha\)-syn aggregation and caspase activation
through enhanced protein oxidation. Given the fact that DA is readily oxidized, the presence of cytosolic DA may increase oxidative stress, ultimately leading to DA neurodegeneration. Alternatively, as described by Karpinar et al. (2009), A56P and A76P α-syn variants with a reduced ability to form β-sheets exhibited higher neurotoxicity than wild-type, A30P, and A53T α-syn, proposing that the formation of rigid β-structure may not be as critical for PD pathogenesis as previously considered. Additional mechanistic insights are needed to clarify the neurodegenerative capabilities of the α-syn species.

**PD pathogenesis: protein degradation machinery**

The accumulation and cellular stress induced by misfolded or aggregated proteins may underlie the neurodegenerative mechanisms leading to PD. Thus, intracellular mechanisms that mediate the clearance and degradation of proteins have been widely considered prospective targets for therapeutic intervention.

The primary protein degradation machinery of the cell is the ubiquitin-proteasome system (UPS) (Figure 1.2). This consists of a variety of proteins including the ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), ubiquitin ligases (E3), multiubiquitylation enzyme (E4), ubiquitin carboxyl-terminal hydrolases (UCHL), and proteasomal subunits. Briefly, E1, E2, E3, and E4 are involved in the processes of activating, transferring, and binding ubiquitin to target proteins that are subsequently degraded by proteasomes. After proteolysis, ubiquitins that are attached to the degraded products are recycled by UCHLs to maintain the cytoplasmic ubiquitin pool. Two PARK proteins, parkin (an E3 ubiquitin ligase) and UCHL-1 are UPS components.
In *C. elegans*, the availability of mutant strains has provided experimental platform for analyzing loss-of-function mutations and potential genetic and environmental interactions. Springer et al. (2005) demonstrated that PDR-1 physically interacted with worm E2 enzymes, UBC-2, UBC-18, and UBC-15, as well as the E4 enzyme, CHN-1, which is consistent with the mammalian findings whereby parkin interacted with Ubch7, Ubch8, and CHIP (Shimura et al., 2000; Zhang et al., 2000; Imai et al., 2002). They isolated one *pdr-1* mutant (*lg103*) that resulted in the expression of truncated PDR-1 and subsequent aggregation, similar to pathogenic *parkin* point mutations in PD patients. Using this mutant strain, they determined that the *pdr-1* mutation enhanced sensitivity to ER stress, and that PDR-1 is regulated by the unfolded protein response (UPR). Interestingly, one of the substrates of parkin is a G protein-coupled Pael receptor wherein accumulation of insoluble receptor leads to UPR-induced cell death in mammalian DA neurons (Imai et al., 2001; Wang et al., 2008). While a worm ortholog of the Pael receptor has not been identified, it is interesting to consider the same conserved pathway might be responsible for mutant parkin-induced toxicity. In a separate study, Ved et al. (2005) studied *pdr-1* knockout strain and observed a reduced level of basal ubiquitination, further establishing PDR-1 as an E3 ligase. They reported that *pdr-1* knockout enhanced vulnerability to mitochondrial complex I inhibitors including rotenone, fenperoximate, pyridaben, and stigmatellin, altering cellular respiration to generate more oxidative stress. Accordingly, in mammals, parkin has been shown to rescue DA neurons against DA or 6-hydroxydopamine (6-OHDA)-induced apoptosis (Jiang et al., 2004; Hasegawa et al., 2008).

Lysosomes are organelles that contain digestive enzymes including lipases, carbohydrases, nucleases, and proteases to break down organelles, macromolecules, and microorganisms. Most notably, recent findings indicate that the bulk of misfolded and
aggregated proteins, including α-syn, are degraded by lysosomes via macroautophagy and chaperone-mediated autophagy (Webb et al., 2003; Cuervo et al., 2004) (Figure 1.2). Further supporting a potential role of lysosomal function in PD pathogenesis is a strong association between PD and type I Gaucher disease (Bembi et al., 2003; Sidransky et al., 2009). Type I Gaucher disease is an autosomal recessive lysosomal storage disorder that is caused by reduced activity of glucocerebrosidase (GBA), a lysosomal enzyme that catalyzes the breakdown of glucosylceramide. Depletion of GBA in animal and cellular models of PD results in increased levels of soluble oligomeric and insoluble α-syn protein species as well as enhances DA neuron degeneration dependent on the elevated levels of α-syn oligomers (Mazzulli et al., 2011). One PARK gene product, ATP13A2 is a lysosomal P-type ATPase. Ramirez et al. (2006) determined that while wildtype ATP13A2 protein is localized in the lysosomes, PD-associated mutant forms are misfolded and retained in the ER to be degraded by proteasomes. Surprisingly, they observed approximately a 10-fold increase in ATP13A2 mRNA levels in the surviving DA neurons from the substantia nigra of human idiopathic PD post-mortem midbrains, suggesting the potential neuroprotective function of this protein.

The importance of lysosomal trafficking and function in suppressing PD pathology has been shown in several studies involving C. elegans. Overexpression of α-syn::GFP in body wall muscle cells (under the control of the unc-54 promoter) led to misfolding and accumulation of α-syn. Co-overexpression of TOR-2 (a worm ortholog of torsinA that is linked to another movement disorder called early-onset torsion dystonia), a protein with chaperone-like activity, ameliorated α-syn misfolding, and maintained the cellular threshold of α-syn at the diffused and soluble state. This genetic background (i.e., α-syn::GFP + TOR-2) has allowed rapid analysis of α-syn misfolding suppressors by scoring the return of α-syn aggregates following the RNAi
treatment (Hamamichi et al., 2008). An enhancement in α-syn misfolding was observed when catp-6/ATP13A2 was knocked down, while overexpression of catp-6 under the control of dat-1 promoter protected DA neurons against α-syn-induced neurodegeneration (Gitler et al., 2009). Importantly, this genetic interaction was also detected in mouse primary DA neuron culture, indicating the nematode data was predictive of relationships present within the mammalian system. Following the same procedure, another study showed that while RNAi knockdown of asp-4 (a worm ortholog of cathepsin D) enhanced α-syn misfolding, overexpression of human cathepsin D rescued worm DA neurons from α-syn-induced toxicity (Qiao et al., 2008).

Additionally, endogenous α-syn formed aggregates in the mouse cathepsin D knockout, further implicating the importance of protein clearance in preventing α-syn aggregation and neurodegeneration. Taken together, continued investigation of the role that the degradative processes play in PD is likely to reveal important insights into disease mechanism.

**PD pathogenesis: signal transduction**

LRRK2 is a signaling component, but precisely which pathways it alters is unclear (Figure 1.2). LRRK2 encodes a large, 2527 amino-acid protein with leucine-rich repeat, Roc GTPase, COR, MAPKKK, and WD40 domains, illustrating its putative function in the MAPK signaling pathway. Given the high frequency of G2019S mutation (in the MAPKKK domain) in autosomal dominant (Di Fonzo et al., 2005) and idiopathic (Gilks et al., 2005) PD patients, West et al. (2005) characterized LRRK2 G2019S expressed in HEK293 and SH-SY5Y cells, and determined that the mutant form exhibited significantly higher kinase activity compared to wildtype LRRK2. West et al. (2007) also analyzed 10 PD-linked LRRK2 mutations, and determined that LRRK2 GTPase activity regulates its kinase activity, and enhanced kinase
activity leads to neurodegeneration. This kinase activity was observed with dimeric LRRK2, and disruption of the GTPase or kinase activity prevented dimer formation (Sen et al., 2009). PD-associated mutants which promote the kinase activity showed an increase in dimeric LRRK2, further implicating the importance of dimer formation and kinase activity in LRRK2 toxicity.

In *C. elegans*, overexpression of either wildtype or G2019S LRRK2 under the control of pan-neuronal promoter (*snb-1* promoter) enhanced DA neurodegeneration accompanied by reduction of DA (Saha et al., 2009). Additionally, LRRK2 G2019S, compared to wildtype LRRK2, resulted in enhanced vulnerability to rotenone. Focusing on mitochondrial malfunction, one common PD pathological mechanism, Saha et al. (2009) also reported that reduction of *lrk-1* by RNAi and mutant analysis increased toxicity in the nematodes to rotenone treatment. Conversely, overexpression of wildtype LRRK2 significantly increased resistance against rotenone and paraquat. These findings illustrate an initial step in elucidating how LRRK2 alters cellular vulnerability to various forms of stress inducers.

**PD pathogenesis: mitochondrial/oxidative stress**

While cellular stress induced by misfolded or aggregated proteins may contribute to the neurodegenerative mechanisms leading to PD, defects in protein degradation machinery alone may not explain the selective loss of DA neurons. Another cellular defect associated with PD pathogenesis is insufficient response to mitochondrial/oxidative stress (Figure 1.2). Two PARK proteins, DJ-1 and PINK1 appear to combat cellular oxidative stress. For example, overexpression of wildtype DJ-1 significantly rescued DA neurons from hydrogen peroxide, DA, and MPP+ insults, demonstrating the anti-oxidant properties of DJ-1 (Junn et al., 2005). Compatible with the mammalian results, Ved et al. (2005) knocked down worm *djr-l.1* by
RNAi, and observed increased vulnerability to rotenone similar to pdr-1 knockout. Further, d-β-hydroxybutyrate and tauroursodeoxycholic acid treatment reversed the susceptibility of the RNAi-treated worms to altered complex I inhibition.

Valente et al. (2004) demonstrated that, after proteasome inhibitor treatment, wildtype PINK1 enhanced mammalian DA neuron survival without modifying mitochondrial membrane potential whereas a PD-associated mutant displayed no protection with decreased potential. Moreover, Pridgeon et al. (2007) reported that PINK1 protects the mammalian cells from oxidative stress-induced cell death by suppressing cytochrome c release from mitochondria. Using available C. elegans mutants, Sämann et al. (2009) reported modified mitochondrial morphology, enhanced sensitivity to paraquat, and defective axon guidance in pink-1 knockout strain, phenotypes which were rescued by expression of wildtype PINK-1. Interestingly, similar to Saha et al. (2009) demonstrating a functional link between LRRK2 and mitochondria, the same rescue was also observed by brk-1 knockout mutation, suggesting an antagonistic role of these two kinases. While the genetic interaction between PINK1 and LRRK2 needs to be confirmed in the mammalian models, this study clearly demonstrates the advantages of model organisms for uncovering genetic interactions among different orthologs of PD genes.

Environmental contributors to PD

With only ~10% of the PD patient population comprising known genetic mutations, it has been proposed that environmental factors largely contribute to the prevalence of this disease. The use of environmental toxins has been widely applied in a variety of model organisms to induce Parkinsonian symptoms. MPTP, which is metabolized to a neurotoxin, 1-methyl-4-phenylpyridinium (MPP+), has been shown to specifically induce DA cell death in both
mammalian and invertebrate models of PD (Langston et al., 1983; Braungart et al., 2004). MPTP is a highly lipophilic molecule which easily passes through the blood-brain barrier and cell membranes. DA neurons take up this molecule through the vesicular DA transporter, where MPP+ inactivates the mitochondrial complex I of the respiratory chain and induces cell death. The DA analogue, 6-OHDA, is another commonly used toxin to specifically induce monoaminergic neuron cell death, since this molecule requires the DA or noradrenergic transporter for uptake.

With many cases of PD being of sporadic nature, C. elegans is an excellent organism to examine environmental toxins that may induce the pathological features of PD, including DA neuron toxicity. Nass et al. (2002) showed that exposing C. elegans to the neurotoxin 6-OHDA induces specific degeneration of the DA neurons, which is both time- and dose-dependent. 6-OHDA is specifically taken up by monoaminergic neurons and causes free radical formation and oxidative stress, which in turn leads to cell death. Using this 6-ODHA model, several groups have identified genes and drugs that alter the sensitivity of DA neurons to 6-OHDA-induced toxicity (Cao et al., 2005; Nass et al., 2005). One group used forward genetics to identify the DA transporter (dat-1) as being a required component for this toxicity (Nass et al., 2005), indicating that 6-OHDA is taken up through the C. elegans DA transporter to induce toxicity in vivo. Cao et al. (2005) used this model to identify a human torsinA-related protein, TOR-2, that showed protection against several cellular insults shown to induce degeneration of the DA neurons, including exposure to 6-OHDA, excess DA synthesis, and transgenic overexpression of human α-syn in DA neurons. Maranova and Nichols (2007) extended application of this model to identify specific DA, GABA, and NMDA receptor agonists as neuroprotective in a dose-dependent manner in C. elegans. Other toxins that have been widely exploited to induce
Parkinson-like symptoms in mammalian models have also been reported to induce DA degeneration in worms, including rotenone and MPTP. Exposure of worms to the neurotoxin MPTP was shown to increase lethality and reduce mobility (Braungart et al., 2004), and treatment of these worms with pharmacological active substrates used in PD therapy reduced this toxicity, further proving this model system to be beneficial in studying PD.

Among the few known risk factors for PD is an association with a rural lifestyle (Priyadarshi et al., 2001). Although the underlying cause for this relationship is unknown, the prevalence of PD seen within the rural population has led to the discovery that several environmental toxins commonly used in farming communities can induce PD symptoms. The organic pesticide, rotenone, and the herbicide, paraquat, are two such chemicals that have been studied in both mammalian and invertebrate models and found to cause DA neurotoxicity by inhibiting the mitochondrial complex, similar to MPP+ and 6-OHDA. The aforementioned report from Ved et al. (2005) showed that exposing nematodes to rotenone enhanced the sensitivity of mitochondrial dysfunction in several models of PD including pan-neuronal α-syn expression and mutants in parkin and djr-1.1. Furthermore, this study identified several compounds, including an antioxidant (probucol), a mitochondrial complex II activator (d-β-hydroxybutyrate), and an anti-apoptotic bile acid (tauroursodeoxycholic acid), that protected against mitochondrial dysfunction in this model. The use of all of these environmental toxins has proven to be an effective method to induce DA degeneration in C. elegans, and these models enable screening for chemical and genetic modifiers of cellular toxicity.

Recently, several other environmental factors have been identified which promote dopaminergic cell death in C. elegans. Caldwell et al. (2009) identified a highly stable and lipophilic secondary metabolite produced by a species of common soil bacteria, Streptomyces
venezuelae. This neurotoxicity, when compared to other neuronal subtypes, was enhanced within the DA neurons. Additionally, the presence of DA exacerbated DA neuron toxicity because neurodegeneration was attenuated in cat-2 mutants, which were depleted for tyrosine hydroxylase, the rate-limiting enzyme in DA production. Upon further analysis, the secondary metabolite was shown to inhibit the degradation of proteins through the UPS, similar to that observed with the proteasome inhibitor MG-132. The neurotoxic bacterial excretion was further confirmed as being detrimental to mammalian DA neurons as well, suggesting an evolutionarily conserved mechanism involving protein degradation is disrupted by this metabolite. As another possible environmental contributor to PD, Settivari et al. (2009) developed a model in C. elegans to study manganese (Mn\(^{++}\)) toxicity with respect to DA neurodegeneration. Worms briefly exposed to Mn\(^{++}\) showed increases in reactive oxygen species, altered mitochondrial membrane potential, and DA neurodegeneration; moreover, the Mn\(^{++}\) toxicity was dependent on the divalent metal transporters, smf-1/2. Furthermore, Mn\(^{++}\) exposure enhanced degeneration of DA neurons expressing human \(\alpha\)-syn, consistent with mammalian observations, and indicating the importance of environmental exposure to heavy metals in exacerbating the pathology seen with \(\alpha\)-syn in the progression of PD. Taken together, C. elegans offers an elegant system to study environmental triggers that may play a pivotal role in the progression of PD and serves to accelerate the identification of molecular pathways involved in the toxicity of environmental factors.

Large-scale analysis for modifiers of \(\alpha\)-syn toxicity in C. elegans

Since formation of Lewy bodies is a central pathological feature of both familial and idiopathic forms of PD, much current research on PD focuses on \(\alpha\)-syn toxicity (protein aggregation, neuronal defects, and DA neurodegeneration) and cellular mechanisms involved in
ameliorating it. Presently, the modification of at least four cellular mechanisms has been linked to the enhanced α-syn toxicity including ER to Golgi trafficking, proteasomal and lysosomal protein degradation, signaling pathways, and mitochondrial function (Figure 1.2). For example, overexpression of α-syn blocks ER to Golgi trafficking (Cooper et al., 2006; Gitler et al., 2008), and overexpression of mutant α-syn induces ER stress (Smith et al., 2005). α-Syn is degraded by the UPS (Stefanis et al., 2001; Zhang et al., 2008; Nonaka and Hasegawa, 2009) and lysosomes via macroautophagy or chaperone-mediated autophagy (Webb et al., 2003; Cuervo et al., 2004); conversely, treatment with proteasome or lysosome inhibitors enhances its aggregation (Sawada et al., 2004; Lee et al., 2004). α-Syn has also been shown to regulate MAPK signaling and accelerate cell death by reducing the amount of active MAPK (Iwata et al., 2001). Additionally, α-syn may be targeted to mitochondria and impair complex I function via cryptic mitochondrial targeting signal (Devi et al., 2008). Functional analyses of the remaining eight PARK proteins highlight involvement of these same cellular mechanisms. As discussed below, large-scale screening for PD susceptibility factors using C. elegans has both confirmed and revealed additional pathways altered in PD pathogenesis.

Several groups have developed and used models for α-syn misfolding to screen for genetic modifiers of this process in C. elegans. Hamamichi et al. (2008) fused the green fluorescent protein (GFP) to wild type human α-syn and expressed the transgene in the body wall muscles of C. elegans, where significant accumulation of cytosolic α-syn::GFP puncta was observed over the course of development and aging. Upon co-expression of TOR-2, which exhibits chaperone-like activity, α-syn::GFP was found diffused and soluble throughout the cytoplasm. Through a reverse genetic strategy applying RNAi to knockdown gene expression, Hamamichi et al. (2008) undertook a hypothesis-based approach to identify putative genetic
modifiers of α-syn misfolding. This hypothesis-based approach entailed bioinformatic prioritization and subsequent screening of ~900 target genes involved in pathways related to PD, such as protein folding, degradation and trafficking, or co-expressed with known C. elegans PARK gene orthologs. Through an initial screen, 20 genes were identified as having the greatest effect on increasing α-syn misfolding when knocked down. Among these candidate genes were included several PD related genes, such as worm homologues of DJ-1 and PINK1, along with another gene, ULK2, that was previously reported as one of only six genes identified in a genome-wide polymorphism screen for candidate SNPs in PD patients (Fung et al., 2006). To further analyze the effect of these candidate genes in an assay with more direct implications for PD, several candidates from the initial RNAi screen for effectors of α-syn misfolding were co-expressed in DA neurons along with human α-syn, and evaluated for their impact on DA neurodegeneration. Strikingly, overexpression of five of seven genes tested protected worm DA neurons against α-syn-induced neurodegeneration. Two of the top neuroprotective candidates, atg-7 and vps-41, have been further validated in mammalian models, in that knockout of Atg7 (E1 ubiquitin activating enzyme) in mice results in neuronal degeneration (Komatsu et al., 2006), and overexpression of human VPS41 (lysosomal trafficking protein) in mammalian cell culture protects cells against several PD related neurotoxins, including 6-OHDA and rotenone (Ruan et al., 2010). Both of these proteins have been shown to be involved in the autophagy pathway for protein clearance in which cargo is delivered to the lysosome for degradation. Thus, the outcomes of this study exemplify the value of using C. elegans to study pathologies characteristic of PD, both α-syn misfolding and DA neurodegeneration, and include the identification of several genes that may represent novel therapeutic targets to combat the progressive degeneration associated with PD.
Another group performed an extensive RNAi screen to identify processes involved in age-dependent α-syn inclusion formation. For this screen, van Ham et al. (2008) expressed human wildtype α-syn fused to the yellow fluorescent protein (YFP) in the body wall muscles of *C. elegans*. Upon expression of the fusion protein, but not YFP alone, cytosolic inclusions were observed in young animals, and these inclusions increased in an age-dependent manner. Since one of the pathological hallmarks of PD is accumulation of electron-dense protein material with inclusion formation, fluorescence recovery after photobleaching (FRAP) was used to monitor the mobility of the inclusion contents, and led to the identification of two types of inclusions, one with mostly mobile material and one with immobilized material. The inclusions with immobilized material resemble inclusions seen with aggregated proteins, and these inclusions were not observed till late adulthood, similar to what is observed in human PD cases. Using this system, van Ham and coworkers (2008) conducted a genome-wide RNAi screen to identify genetic factors that, when knocked down, increased the number of cytoplasmic α-syn inclusions. They identified 80 suppressors of inclusion formation, with many having human orthologs, and further verified some of these hits by examining *C. elegans* mutant strains corresponding to three of their positive candidates, in which they observed a similar increase of inclusions. Of the 80 suppressors isolated, several were predicted to be involved in similar pathways that have been implicated in having roles in PD, including vesicular transport, lipid metabolism, and aging. Interestingly, one of the suppressors identified, *sir-2.1*, has also been shown by others to play a significant role in aging by regulating ER stress genes (Tissenbaum and Guarente, 2001; Viswanathan et al., 2005). Furthermore, the effect of knocking down another known modifier of α-syn toxicity, the G-protein coupled receptor kinase, Grpk2, confirmed data from *Drosophila* studies, wherein an increase in neuronal toxicity was seen when Grpk2 was overexpressed,
possibly through Grpk2 phosphorylating α-syn. Accordingly, RNAi of the *C. elegans* orthologs of Grpk2, *grk-1* or *grk-2*, reduced the accumulation of cytoplasmic α-syn inclusions, a result that was further validated by utilizing worm strains mutant in these two genes.

Kuwahara et al. (2008) conducted a similar RNAi screen with the readout for α-syn-neurotoxicity being behavioral defects. This screen involved overexpressing either WT or mutant human α-syn under a pan-neuronal promoter, and a systematic analysis of ~1700 genes involved in the nervous system or synaptic function was performed for evidence of altered behavior dependent on α-syn expression. Since neurons in *C. elegans* are not highly responsive to RNAi treatment, this screen was conducted in an *eri-1* mutant background, a neuronal RNAi supersensitive mutant, to enhance the genetic knockdown of target genes within neurons. Through this screen, 11 genes were categorized as altering the distinct behavioral phenotypes, including uncoordinated movement (Unc) and growth retardation (Gro), in pan-neuronal expressed α-syn nematodes. Of these 11 candidates, four are reported as being components of the endocytic machinery. Interestingly, one of these genes, *apa-2*, was also identified as a positive candidate that enhanced α-syn misfolding when knocked down by RNAi in the Hamamichi et al. (2008) study. Kuwahara et al. further validated their findings of the involvement of the endocytic pathway with α-syn toxicity by testing mutants within the AP-2 complex, involved in endocytosis, and found enhanced behavioral defects only in mutants expressing α-syn, thereby indicating the endocytic pathway is affected by α-syn. Using pharmacological assays that have been well established in *C. elegans* to study acetylcholine (Ach) neurotransmitter release at the neuromuscular junction, it was further shown that α-syn decreased presynaptic neurotransmitter release of Ach, similar to endocytosis-defective mutants. Furthermore, immunohistochemical analysis revealed an increase in phosphorylated α-syn within
the cell bodies of neurons upon RNAi knock-down of *apa-2*, resembling the accumulation of phosphorylated α-syn indicative of synucleinopathies. Collectively, these large-scale functional genomic analyses epitomize the substantial value of using *C. elegans* to identify novel proteins and pathways involved in disease mechanisms, specifically in the context of the dysfunction caused by α-syn accumulation and its impact on the pathogenesis of PD.

*Function of VPS41*

Through a high-throughput RNAi screen to find modifiers of α-syn accumulation, as well as DA neurodegeneration, Hamamichi et al. (2008) identified the *C. elegans* lysosomal trafficking protein, VPS-41, as a leading protective candidate. RNAi knockdown of *vps-41* resulted in enhanced α-syn misfolding and accumulation in *C. elegans* body wall muscles, and overexpression of VPS-41 in the DA neurons along with α-syn suppressed the level of α-syn-induced DA degeneration. VPS-41 is a highly conserved metazoan protein with multiple protein domains, including WD40 protein interaction, metal ion binding, RING-H2 zinc finger, microtubule binding, and clathrin-heavy chain repeat domains (CHCR) (Figure 1.3A).

VPS41 was initially identified in *Saccharomyces cerevisiae* as a protein involved in iron transport by promoting assembly and trafficking of the high-affinity iron transport system (Radisky et al., 1997). Disruption of the gene encoding Vps41p resulted in decreased growth on low-iron medium, defective post-Golgi trafficking, and abnormal vacuolar morphology. In yeast, post-Golgi trafficking of adaptor protein complex 3 (AP-3) transport vesicles to the vacuole (yeast equivalent of the lysosome) via the alkaline phosphatase (ALP) pathway requires Vps41p function (Rehling et al., 1999). Two pathways are involved in post-Golgi trafficking of proteins to the vacuole, the carboxypeptidase Y (CPY) and ALP pathway. The CPY pathway involves
trafficking cargo through an endosomal intermediate, while the ALP pathway bypasses these early, sorting endosomes to traffic cargo directly from the Golgi to the vacuole. For AP-3 vesicle trafficking in the ALP pathway, the WD40 protein interaction domain of Vps41p directly interacts with the δ-subunit of the AP-3 complex (apl-5), while the CHCR domain homooligomerizes to form the clathrin-like lattice which surrounds the AP-3 vesicles for post-Golgi trafficking (Darsow et al., 2001; Angers and Merz, 2009). The C-terminal RING domain was proposed to function in membrane association of hVPS41 with Golgi-derived vesicles, and in humans, a second isoform of hVPS41 is missing the RING domain (McVey Ward, et al., 2001). However, upon further sequence analysis, the difference in the two human isoforms occurs in the N-terminal WD40 domain, where 25 amino acids are missing in the second isoform (Ruan et al., 2010). It is unclear at the time if the different isoforms have alternative functions or expression patterns.

The interaction of Vps41 with the AP-3 δ subunit occurs preferentially in the presence of the homotypic fusion and protein sorting (HOPS) docking complex, which functions in vacuolar fusion of endosomes and vesicles (Angers and Merz, 2009). The HOPS complex consists of Vps39 and Vps41, which interact with the Vps-C core complex (Figure 1.3B). The Vps-C core complex includes Vps11, Vps16, Vps18, and Vps33 at the lysosome to promote fusion events (Nickerson et al., 2009). As vesicles approach the vacuole, the HOPS complex replaces the CORVET complex (class C core vacuole/endosomes tethering). The COVET complex is composed of the same components as the Vps-C core complex but has the accessory proteins Vps3 and Vps8 instead of Vps39 and Vps41 (Figure 1.3B). The CORVET complex is found on early endosomes and interacts with Rab5, while the HOPS complex localizes on vesicles at the vacuolar membrane and interacts with Rab7 (Nickerson et al., 2009).
As vesicles approach the vacuole, the HOPS component and a guanosine exchange factor (GEF) Vps39, activate vacuolar Rab7 (Ypt7). Activated Rab7 physically interacts with both Vps39 and Vps41 to promote vacuolar fusion (Brett et al., 2008). The lysosomal fusion event involving the HOPS complex is mediated by the phosphorylation state of Vps41 (LaGrassa and Ungermann, 2005). The vacuolar type 1 casein kinase in yeast, Yck3, phosphorylates Vps41 within the N-terminal WD40 domain to regulate the function of Vps41. Phosphorylated Vps41 functions in fusion of endosomes to the vacuole, while the non-phosphorylated Vps41 is associated with AP-3 vesicles trafficked from the Golgi to the vacuole (LaGrassa and Ungermann, 2005; Cabrera et al., 2009). Mutations associated with YCK3 result in AP-3 trafficking defects in yeast that mimic the vacuole trafficking defects seen in AP-3 complex mutants (Anand et al., 2009). The phosphorylation of Vps41 by Yck3 occurs in an amphipathic lipid-packing sensor motif that is inserted into the highly curved lipid bilayer of endosome membranes, resulting in masking of the AP-3 interaction domain of Vps41 (Cabrera et al., 2010). When this sensor motif is phosphorylated, the AP-3 binding domain of Vps41 is available to interact with the AP-3 complex to promote vacuolar fusion (Hickey et al., 2009; Cabrera et al., 2010). Intriguingly, by increasing the expression or activity of Rab7, the specific fusion event of either the phosphorylated or non-phosphorylated Vps41 can be promoted, indicating that Rab7 functions in both endosomal and AP-3 vesicular fusion with the vacuole (Cabrera et al., 2009). Interestingly, RAB7L1 was recently identified within the PARK16 locus (Tucci et al., 2010). The identification of RAB7L1 within the PARK16 locus may directly links the AP-3 trafficking pathway, which involves Vps41, to PD. More extensive genomic analysis to identify exactly which gene within the PARK16 locus may be contributing to PD needs to be conducted.
While most of the previous work on Vps41 has been done in yeast, several studies in multicellular organisms have further identified the molecular functions of Vps41. In *C. elegans*, inhibition of VPS-41 results in impaired formation of lysosome-associated gut granules in embryos (Hermann et al., 2005), and homozygous mutations with *vps-41* result in enhanced germline apoptosis and embryonic lethality (Lackner et al., 2005). A recent study using *Drosophila* identified Light as the Vps41 homologue; it functions in post-Golgi trafficking of lysosome associated proteins, including LAMP1 (lysosome associated membrane protein), V0-ATPase (vacuolar proton pump), and NPC1 (cholesterol transport protein) (Swetha et al., 2011). Depletion of Light resulted in defective lysosome acidification and degradation, abnormal lysosome morphology, and defects in sterol homeostasis, supporting the role that VPS41 functions in lysosomal trafficking of AP-3 vesicles from the Golgi, as seen in yeast.

Interestingly, in yeast, the vacuolar V1/V0-ATPase functions in releasing Vps41 from vacuoles after fusion (Takeda et al., 2008), supporting the role that a protein trafficked to the lysosome dependent on Vps41-mediated AP-3 vesicle transport functions at the lysosome to promote release of the complex after fusion. Taken together, post-Golgi trafficking of AP-3 vesicles to the lysosome is dependent on VPS41 function, and the cargos being transported to the lysosome in these vesicles maintain the function of the lysosome, therefore promoting protein homeostasis and cell survival.

*Current studies*

With *vps-41* being a highly conserved metazoan gene and *C. elegans* VPS-41 affecting α-syn accumulation and neurotoxicity in our worm models of PD, we set out to determine if human VPS41 (hVPS41) would show an evolutionarily conserved function in our *C. elegans* models of
DA neurodegeneration. By overexpressing hVPS41 in the DA neurons of our worms, we showed that hVPS41 was able to suppress both α-syn- and 6-hydroxydopamine (6-OHDA)-induced DA neurotoxicity, showing the neuroprotective function of VPS-41 is conserved amongst species. This protective function of hVPS41 was also observed in SH-SY5Y neuroblastoma cells stably transfected with hVPS41, where expression of hVPS41 suppressed toxicity from the PD-relevant neurotoxins 6-OHDA and rotenone. Expression of hVPS41 did not alter mitochondrial membrane potential, which is affected by these neurotoxins, but was able to suppress the activation of the apoptotic cascade following toxin treatment. With hVPS41 overexpression, a reduction in the level of detergent insoluble α-syn species was observed in SH-SY5Y cells exposed to rotenone, with the detergent insoluble fraction representing the oligomeric forms of α-syn. This study is described in detail in Chapter 2 (and published as Ruan et al., 2010).

Since hVPS41 showed a conserved neuroprotective mechanism, we conducted a structure/function analysis of hVPS41 to determine the minimal domains required for the neuroprotective activity of hVPS41. In this study, we constructed 7 truncated forms of hVPS41 with various domains of the protein absent and expressed these truncated hVPS41 constructs in our worm model of α-syn-induced DA neurodegeneration. Through this analysis, we identified that both the WD40 protein interaction and CHCR domains are required for hVPS41-mediated neuroprotection, while the TPR-like and RING finger domains are dispensable for this protective function. Interestingly, two SNPs have been identified within the WD40 domain of hVPS41 in the general human population, and the presence of either SNP in hVPS41 abrogates the neuroprotective function of the protein. The structural and SNP data for hVPS41 is supported in mammalian cells, in that overexpression of the protective hVPS41 constructs identified in C.
*C. elegans* are also able to suppress α-syn-inclusion formation in H4 human neuroglioma cells. Lastly, mutant analysis revealed that hVPS41 requires a functional AP-3 complex to suppress α-syn toxicity. Using a novel strain of *C. elegans* that facilitates, for the first time, DA neuron-specific RNAi, we show the importance of post-Golgi trafficking of AP-3 vesicles to the lysosome, in that genetic perturbation of genes involved in AP-3 vesicular trafficking enhanced α-syn-induced DA neurodegeneration. In all, these data validate the importance of lysosomal trafficking in maintaining α-syn homeostasis within cells and supports hVPS41 as a potential candidate for therapeutic intervention of PD. This study is described in detail in Chapter 3 (and submitted for publication as Harrington et al).

Lastly, we set out to evaluate and functionally validate both genetic and chemical modifiers of neurotoxicity identified in mammalian systems by using our worm model of α-syn-induced DA neurodegeneration (Yacoubian et al., 2010; Pivtoraiko et al., 2010). We were able to suppress α-syn toxicity in *C. elegans* by overexpressing human 14-3-3θ in our worm DA neurons, consistent with cell culture data showing this isoform can suppress toxicity caused by exposure to several PD-relevant neurotoxins. We were also able to protect DA neurons from degeneration by overexpressing *ftt-2* (a worm ortholog of 14-3-3). Furthermore, we showed that a low-dose of the macrolide antibiotic, bafilomycin, could suppress α-syn toxicity in the worms DA neurons, validating the findings from human neuroblastoma cells. These studies are described in detail in Chapter 4 (and published as Yacoubian et al., 2010; Pivtoraiko et al., 2010).

Concluding remarks and future directions resulting from this dissertation research are discussed in detail in Chapter 5. Taken together, these data show the predictable nature of *C. elegans* for identifying and validating potential neuroprotective targets. Furthermore, the
nematode model can be used to uncover neuroprotective mechanisms that can be translated to mammalian systems for further confirmation as potential therapeutic candidates. Overall, our data demonstrate that the promotion of lysosomal trafficking and function may suppress α-syn accumulation and toxicity; these data support and highlight a significant role for these processes in maintaining protein homeostasis in regulating neuronal survival in PD.

REFERENCES


### Table 1.1. Molecular, genetic, and chemical manipulations used in *C. elegans* models of PD and their corresponding phenotypes

<table>
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<tr>
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<th>Phenotype</th>
<th>Reference</th>
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<td>DA neurodegeneration; Reduced motor movement</td>
<td>Lakso et al., 2003</td>
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<td>Ved et al., 2005</td>
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### Mutant/RNAi Analysis

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### Chemical Treatment

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<td>Oxidative stress; Mitochondrial stress</td>
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<td></td>
<td><em>P</em>~ca:::0-sym::GFP; <em>P</em>~ca:::0-sym::α-sym</td>
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36
Table 1.2. Summary of PD genes and corresponding *C. elegans* orthologs.

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n/a: Not applicable
Figure 1.1
Figure 1.2
Figure 1.3

A

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<th>clathrin heavy chain</th>
<th>RING Zn finger</th>
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- isoform 1
  - 28-449
  - 393-531
  - 571-712
  - 791-839

- isoform 2
  - 28-449
  - 393-531
  - 571-712
  - 791-839

B

- Late Golgi
- AP-3 complex
- RAB
- Vps-C
- CSNK-1

- Lysosome
- Early Endosome
- Late Endosome
- HOPS

CORVET

AP-3 vesicle
FIGURE LEGENDS

**Figure 1.1.** The DA neurons of *C. elegans* hermaphrodites highlighted using GFP driven from the DA transporter promoter (P\textsubscript{dat-1}::GFP). A. Cell bodies and processes of the 6 anterior DA neurons include 2 pairs of cephalic (CEP) neurons (arrows) and 1 pair of anterior deirid neurons (ADEs; large arrowheads). There are also 1 pair of posterior deirid neurons in each hermaphrodite (PDEs; small arrowheads). B. Magnified view of the anterior region of *C. elegans*, detailing the 6 anterior-most DA neurons. The dendrites of the 4 CEP neurons and the axons of the 2 ADE neurons are labeled with arrows and arrowheads, respectively. C. A worm exposed to 6-OHDA exhibiting DA neurodegeneration; 2 of 4 CEP cell bodies are present, but degenerating (arrows). The two ADE neurons in this animal are still intact (arrowheads). D. A worm co-expressing both GFP and α-syn in DA neurons. Most worms within a population expressing α-syn within the DA neurons are missing anterior DA neurons when they are 7-day old adults. In this example, only 2 of the 4 CEP neurons (arrows) and 1 of 2 ADE neurons (arrowhead) remain.

**Figure 1.2.** α-syn misfolding leads to defective cellular mechanisms. PD-associated gene products have been shown to influence various aspects of cellular function and some of these gene products are directly affected by α-syn misfolding.

**Figure 1.3.** VPS41 is a multi-domain protein functioning in lysosomal trafficking. A. Human VPS41 contains several predicted protein domains, including a WD40 protein interaction, TPR-like, clathrin heavy-chain repeat, and RING Zn finger domain. hVPS41 isoform 2 differs from isoform 1 with isoform 2 missing 25 amino acids within the WD40 domain. B. Different complexes are present on vesicles being trafficked within the cell. The Vps-C core complex consists of Vps11, Vps16, Vps18, and Vps33 and has two accessory
subunits. The accessory proteins Vps3 and Vps8 are present on early endosomes along with Rab5, while Vps39 and Vps41 are present on these vesicles at the vacuole with Rab7. Vps41 is phosphorylated at the vacuole by casein kinase 1 (CSNK-1). Phosphorylated Vps41 functions in AP-3 vesicle transport from the Golgi to the lysosome, while the non-phosphorylated Vps41 functions in endosomal fusion with the lysosome.
CHAPTER 2
VPS41, A PROTEIN INVOLVED IN LYSOSOMAL TRAFFICKING, IS PROTECTIVE IN MAMMALIAN CELLULAR MODELS OF PARKINSON’S DISEASE

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ABSTRACT

VPS41 is a protein identified as a potential therapeutic target for Parkinson’s disease (PD) as a result of a high-throughput RNAi screen in *C. elegans*. VPS41 has a plausible mechanistic link to the pathogenesis of PD, as in yeast it is known to participate in trafficking of proteins to the lysosomal system and several recent lines of evidence have pointed to the importance of lysosomal system dysfunction in the neurotoxicity of alpha-synuclein (α-syn). We found that expression of the human form of VPS41 (hVPS41) prevents dopamine (DA) neuron loss induced by α-syn overexpression and 6-hydroxydopamine (6-OHDA) neurotoxicity in *C. elegans*. In SH-SY5Y neuroblastoma cell lines stably transfected with hVPS41, we determined that presence of this protein conferred protection against the neurotoxins 6-OHDA and rotenone. Overexpression of hVPS41 did not alter the mitochondrial membrane depolarization induced by
these neurotoxins. hVPS41 did, however, block downstream events in the apoptotic cascade including activation of caspase-9 and caspase-3, and PARP cleavage. We also observed that hVPS41 reduced the accumulation of insoluble high molecular weight forms of α-syn in SH-SY5Y cells after treatment with rotenone. These data show that hVPS41 is protective against both α-syn and neurotoxic-mediated injury in invertebrate and cellular models of PD. These protective functions may be related to enhanced clearance of misfolded or aggregated protein, including α-syn. Our studies indicate that hVPS41 may be a useful target for developing therapeutic strategies for human PD.

INTRODUCTION

Parkinson's disease (PD) is a disabling neurodegenerative disorder marked by progressive motor dysfunction and characterized by the loss of nigrostriatal dopamine neurons and cytoplasmic inclusions termed Lewy bodies (Lang and Lozano, 1998; Olanow and Tatton, 1999). While the pathogenesis of PD has not yet been established, previous studies have implicated both genetic as well as environmental contributions. The protein alpha-synuclein (α-syn) appears to have a central role; mutations or overexpression of this protein leads to autosomal dominant PD (Singleton et al., 2003), and accumulation of α-syn is observed even in sporadic cases of PD (Kotzbauer et al., 2004), possibly as a result of impaired protein clearance. No treatment has yet been demonstrated to slow the rate of the neurodegenerative process in PD, and the discovery of potentially protective pathways is a high priority (Yacoubian and Standaert, 2008).

Animal models of PD have been constructed by over-expression of α-syn, and several of these have been used to identify factors that protect against α-syn toxicity. In a recent study, a nematode model of α-syn-induced misfolding and age-dependent DA neurodegeneration was
used to screen a candidate list of ~900 starting targets, derived from analysis of proteins or pathways implicated in PD, as well as co-expressed and interacting partners (Hamamichi et al., 2008). From this screen, 20 candidate gene products were identified that when inhibited, reproducibly led to an enhanced misfolding of human α-syn in worms. In a secondary analysis, select candidates were expressed together with α-syn in *C. elegans* dopaminergic (DA) neurons, and loss of DA neurons was assessed. One of the most effective neuroprotective protein identified was VPS-41, encoding a conserved vesicular protein necessary for lysosomal biogenesis.

VPS41 was originally identified in yeast as a member of the “Class B” proteins involved in trafficking of proteins from the late Golgi to the vacuole (the yeast equivalent of the lysosome) (Bowers and Stevens, 2005). Subsequent work has shown that VPS41 has an unusual role in yeast metabolism, in that it is required for the “ALP” pathway, which bypasses the endosome and carries only a select set of protein cargoes to the vacuole (Rehling et al., 1999). VPS41 has metal ion binding, microtubule binding, RING finger, and AP3 interaction domains (Radisky et al., 1997). It was suggested that the RING-H2 domain associated with a class of intracellular vesicles that originated from the Golgi (McVey Ward et al., 2001).

Two isoforms of VPS41 are expressed in humans (McVey Ward et al., 2001), and there is a strong circumstantial case that these proteins may be relevant to α-syn related disease. Several recent lines of evidence have pointed to the importance of lysosomal system dysfunction in the toxicity of α-syn: the α-syn protein is degraded in part by the lysosomal pathway, under the regulation of the co-chaperone CHIP (Shin et al., 2005); augmentation of the lysosomal enzyme cathepsin D can accelerate the degradation of α-syn (Cullen et al., 2009; Qiao et al., 2008); lysosomal failure has been proposed as a mechanism underlying the age dependence of PD (Chu
and Kordower, 2007); modified forms of α-syn can block the chaperone-mediated component of autophagy (Bandhyopadhyay and Cuervo, 2007; Finkbeiner et al., 2006); PARK9, a hereditary form of parkinsonism with dementia, has been linked to a mutation of a lysosomal ATPase (Ramirez et al., 2006) that contributes to α-syn accumulation and manganese toxicity (Gitler et al., 2009); and recently knockout of the lysosomal protein ATG7 (also identified in the C. elegans screen along with VPS41) has been reported to produce a neurodegenerative phenotype (Komatsu et al., 2006). Furthermore, recent studies have described the ability of other vesicular trafficking proteins such as Rab1, Rab3 and Rab8 proteins to mitigate the toxicity of α-syn in animal models of PD (Cooper et al., 2006).

In this study, we show that the functions of VPS41 are evolutionarily conserved, in that overexpression of human VPS41 (hVPS41) in the C. elegans model of PD produces protection against both α-syn- and 6-hydroxydopamine (6-OHDA)-induced DA neurodegeneration. Furthermore, we set out to determine whether hVPS41 would have protective effects in mammalian cellular models of PD and explore the possible mechanism underlying its effect. Stably transfected cell lines were derived from SH-SY5Y neuroblastoma cells and later subjected to several PD-relevant neurotoxins, including rotenone and 6-OHDA. We found that the overexpression of hVPS41 was protective as it reduced the extent of cell death induced by these neurotoxins. Of note, overexpression of hVPS41 failed to alter the mitochondrial membrane depolarization induced by these neurotoxins. However, downstream events in the apoptotic cascade induced by rotenone or 6-OHDA, including caspase-9, caspase-3 activation and PARP cleavage, were attenuated by hVPS41 overexpression in these cell lines. In addition, we observed that the expression of hVPS41 reduced the accumulation of detergent-insoluble high-molecular weight forms of α-syn, suggesting that modulation of α-syn contributes to its protective actions.
Overall, our data support the hypothesis that hVPS41 represents a potential therapeutic target in PD, and that its protective effect is mediated at least in part through modulation of the apoptotic cell death pathway. Furthermore, the protective function of hVSP41 may result from its effect on modulating protein misfolding or aggregation. Our study strongly implicates hVPS41 as a potential target for therapeutic development and intervention to combat PD.

MATERIALS AND METHODS

Generation of transgenic *C. elegans*: Gateway Technology (Invitrogen) was used to generate expression plasmids, \( P_{dat-1::vps41 \text{ Isoform 1}}, P_{dat-1::vps41 \text{ Isoform 2}} \), along with the marker \( P_{unc-54::mCherry} \). These plasmids were injected into the gonads of N2 Bristol *C. elegans* to generate independent stable transgenic lines that were crossed into strains BY200 \( [P_{dat-1::GFP}] \) (Nass and Blakely, 2003), which expresses GFP in the DA neurons without degeneration, and UA44 \( [baIn11; P_{dat-1::\alpha\text{-syn}}, P_{dat-1::gfp}] \), which co-expresses human \( \alpha\text{-syn} \) and GFP in the DA neurons and exhibits age-dependent- \( \alpha\text{-syn} \) -induced degeneration. This resulted in 4 independent transgenic worm strains, UA127 \( [BY200; baEx98, P_{dat-1::vps41-1}, P_{unc-54::mCherry}] \), UA129 \( [BY200; baEx99, P_{dat-1::vps41-2}, P_{unc-54::mCherry}] \), UA126 \( [baIn11; baEx98, P_{dat-1::vps41-1}, P_{unc-54::mCherry}] \), and UA128 \( [baIn11; baEx99, P_{dat-1::vps41-2}, P_{unc-54::mCherry}] \).

Analysis of DA degeneration in *C. elegans*: Strains UA126 and UA128 were synchronized, grown at 20°C, and analyzed at both day 7 and day 10 post-hatching (4- and 7-day-old adults) for protection against \( \alpha\text{-syn} \)-induced DA degeneration. For each trial, 30 worms were immobilized with 3mM levamisole and placed on a 2% agarose pad on a microscope slide, where the 6 anterior DA neurons (4 CEP and 2 ADE neurons) were analyzed. In total, 90 worms from each of the 3 independent hVPS41 transgenic lines were analyzed (3 lines X 3 trials of 30...
animals/trial = 270 total animals scored). Worms displaying any degenerative changes in the neurons were scored as having degenerating neurons, as previously described (Cao et al., 2005; Hamamichi et al., 2008). Fluorescent microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HYQ filter cube (Chroma Technology, Rockingham, VT), and images were acquired with a Cool Snap CCD camera (Photometrics, Tucson, AZ) driven by MetaMorph software (Molecular Devices, Downington, PA). These data were statistically analyzed by the Student’s t-test.

Worm strains UA127 and UA129 were treated with 6-OHDA as follows. These worms were synchronized, grown at 25°C for 35 hours (until late L3 stage), and then treated with 10 mM 6-OHDA containing 2mM ascorbic acid for 1 hour with gently agitation every 10 minutes, as previously described (Nass et al., 2002). The worms were then washed and transferred to NGM plates seeded with bacteria (OP50), and DA neurons were scored at 24, 48, and 72 hours post-treatment. DA analysis was performed as described above.

Cell culture and generation of stable cell lines: cDNA clones of hVPS41 (including both isoforms) were obtained from Origene (Rockville, MD) and subcloned into the mammalian expression vector pcDNA 6/myc-His, which has a blasticidin selectable marker to allow selection for stably transfected cells. DNA constructs were verified by automated sequencing. For stable transfection, SH-SY5Y cells were electroporated with various pcDNA constructs mentioned. Two days after transfection, the cells were maintained with 5 µg/ml blasticidin to select stable transfectants, and then subsequently subcloned. SH-SY5Y cells stably transfected with vector only were used as controls. Cells were selected and maintained on Corning dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 µg/ml blasticidin, 10% fetal
bovine serum, 10 U/ml penicillin, 100 µg/ml streptomycin. Cells were grown in a humidified atmosphere containing 5% CO₂.

**Cell treatment paradigm:** Twenty-four hours after plating, cells were transferred to serum free media and were subjected to designated treatment thereafter. Rotenone or staurosporine was prepared freshly in DMSO, and cells were treated with 0.1–20 µM rotenone (Sigma, St. Louis, MO, USA) or 0.04 -1 µM staurosporine (Sigma, St. Louis, MO, USA), and the final concentration of DMSO in the media was 0.1%. For 6-OHDA, dilutions of 6-OHDA were made immediately before 6-OHDA addition in 0.1% ascorbic acid and added to fresh cell culture medium to achieve the required concentration. Control cells were treated with 0.1% ascorbic acid under the same conditions.

**LDH assay for cell viability:** The release of the intracellular enzyme lactate dehydrogenase (LDH) into the medium was used as a quantitative measurement of cell viability. The measurement of LDH was carried out as described previously (Decker and Lohmann-Matthes, 1988).

**Immunoblotting:** Cells were rinsed in ice-cold phosphate-buffered saline (PBS) and collected in lysis buffer, containing 0.5% NP-40, 150 mM NaCl, 10 mM Tris-Cl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 µM okadaic acid, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin. Samples were sonicated on ice for 10s and centrifuged at 16,000g for 10 min. Protein concentrations of supernatants were then determined by the bicinchoninic acid assay (BCA) and samples were diluted to a final concentration of 1 mg/ml with 2x reducing stop buffer (0.25 M Tris-HCl, pH 6.8, 5 mM EDTA, 5 mM EGTA, 25 mM dithiothreitol, 2% SDS, 10% glycerol, and bromophenol blue as the tracking dye). Samples (20 µg of protein) were resolved on 10% or 12.5% SDS-polyacrylamide gels, and transferred to
PVDF membrane. Blots were blocked in 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween 20) for 1 hr at room temperature. The blots were then incubated with an anti-cleaved caspase-3 polyclonal antibody, or with the anti-cleaved PARP polyclonal antibody in the same buffer overnight at 4°C. The membranes were then washed three times with TBST and incubated with HRP-conjugated secondary antibody for 2 hr at room temperature. The membranes were rinsed three times for 30 min with TBST, followed by four quick rinses with distilled water, and developed with the enhanced chemiluminescence method.

In situ caspase-3 activity: In situ caspase-3 activity was measured using a previously described protocol (Bijur et al., 2000). In brief, 200 µl of assay buffer (20 mM HEPES, pH 7.5, 10% glycerol, and 2 mM dithiothreitol) containing the peptide substrate for caspase-3 (AC-DEVD-AMC) was added to each well (final concentration of 25 ng/µl) of a 96-well clear bottom plate (Corning). Cell lysate (20 µg of protein) was added to start the reaction. Duplicate or triplicate measurements were performed for each sample. Background fluorescence was measured in wells containing assay buffer, substrate, and lysis buffer only. Assay plates were incubated at 37°C for 2h, and fluorescence was measured on a fluorescence plate reader set at 360 nm excitation and 460 nm emission.

Detection of the mitochondrial membrane potential (Δψm): Δψm was analyzed using 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a lipophilic cationic fluorescence dye. JC-1 is driven into mitochondria in a membrane potential-dependent manner. At high mitochondrial membrane potentials, JC-1 accumulates sufficiently in the mitochondria to form aggregates that fluoresce red. At lower mitochondrial potentials, less dye enters mitochondria resulting in monomers that fluoresce green (Nicholls and Ward, 2000; Smiley et al., 1991). For these studies, cells were grown on 24-well plates. After treatment, cells
were incubated with 5 µg/ml JC-1 for 30 min at room temperature in the dark. Then cells were washed twice with PBS and fluorescence was measured on a fluorescence plate reader set at 485 nm excitation and 528 nm emission for green monomer or 530 nm excitation and 590 nm emission for red aggregate.

**α-Syn solubility analysis:** This experiment was carried out as previously described with minor modification (Cantuti-Castelvetri et al., 2005). Briefly, cell lysates were collected in cold lysis buffer (Tris/HCl 50 mM pH 7.4, NaCl 175 mM, EDTA 5 mM pH 7.0, 1 mM phenylmethysulphonyl fluoride (PMSF), 1 µM okadaic acid, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin) and sonicated for 10 seconds to generate total cell lysates. These lysates were divided into Triton X-100 soluble and insoluble fractions by adding Triton X-100 to total cell lysates (final concentration 1%) and incubating for 30 minutes on ice followed by centrifugation (15,000 × g, 15 minutes, 4°C). The supernatant was designated as Triton X-100 soluble fraction. The pellet was dissolved in lysis buffer containing 2% SDS and sonicated for 10 seconds. This was designated the Triton X-100 insoluble fraction. 40 µg of Triton-insoluble fraction or 3 µg of Triton-soluble fraction was loaded onto 8% to 15% SDS-polyacrylamide gels, and subsequently transferred to nitrocellulose membrane. After blocking, blots were subjected to anti-α-syn antibody (BD Transduction Lab, San Jose, CA) overnight at 4°C and subsequently developed as described previously.
RESULTS

Expression of hVPS41 rescues DA neuron loss induced by α-syn overexpression and 6-OHDA neurotoxicity in C. elegans

Our prior study in C. elegans demonstrated that inhibition of the worm ortholog of VPS41 (the C. elegans vps-41 gene product) by RNAi increased the aggregation of α-syn, and that overexpression of this protein protected nematode dopamine (DA) neurons against α-syn induced degeneration (Hamamichi et al., 2008). As a first step, we sought to confirm that hVPS41 shared these neuroprotective properties in the C. elegans model. For that purpose, transgenic nematodes expressing hVPS41 cDNAs were generated and crossed into isogenic lines of worms expressing both α-syn and GFP in DA neurons as described before (Hamamichi et al., 2008). Worms were scored as wild-type (WT) when all six anterior DA neurons were intact. Overexpression of α-syn alone resulted in significant degeneration in DA neurons (Figure 2.1A). Strikingly, coexpression of hVPS41, either isoform 1 or isoform 2, significantly ameliorated DA neurodegeneration, both at day 7 and 10 post hatching (Figure 2.1A). Figure 2.1B is an image showing that worm expressing only α-syn and GFP in the DA neurons has degeneration in 3 CEP neurons, in contrast to the intact DA neuron seen in a WT worm co-expressing hVPS41 in addition to α-syn and GFP.

To further investigate the neuroprotective properties of hVPS41 in vivo, we also examined the effect of hVPS41 expression in the worm model on vulnerability to 6-OHDA. Prior studies have demonstrated that 6-OHDA causes progressive and selective degeneration of C. elegans DA neurons (Nass et al., 2002; Cao et al., 2005). Transgenic animals expressing either isoforms of hVPS41 were subjected to 6-OHDA treatment, as previously described (Nass et al., 2002; Cao et al., 2005). In control worms, 6-OHDA treatment resulted in substantial
degeneration of DA neurons (Figure 2.1C). Conversely, over-expression of either isoforms of hVPS41 significantly rescued DA neurodegeneration, at all times tested following exposure (Figure 2.1C). Figure 2.1D shows the anterior DA neurons in C. elegans after exposure to 6-OHDA. The top image shows a worm expressing only GFP in the DA neurons and has 3 degenerating CEP neurons, while the worm in the bottom image co-expresses hVPS41 with GFP and has the full complement of DA neurons still intact. Our experiments were performed in worm strains that have normal levels of endogenous CeVPS41. Thus, the effect observed here may reflect the combined action of CeVPS41 together with the induced expression of hVPS41. Worm strains that are null for CeVPS41 are maternal effect embryonic lethal (Lackner et al, 2005), therefore it is not possible to generate homozygous CeVPS41 knock-out worms to be used for the overexpression of hVPS41 in a CeVPS41 null background.

Construction of SH-SY5Y neuroblastoma cell lines stably expressing hVPS41 protein

In order to evaluate the cellular role that VPS41 plays in neuronal cell death and possible underlying mechanisms, human neuroblastoma SH-SY5Y cells were stably transfected with constructs containing hVPS41 cDNAs encoding either isoform 1 and 2. Since no reliable hVPS41 antibody has been identified so far, a vector encoding a C-terminal myc tag was used in these constructs for easy identification. Stable transfection with hVPS41 cDNA and subsequent subcloning resulted in significant VPS41 protein expression in SH-SY5Y cells, and these expression levels were compared using western blot against myc. In Figure 2.2A, the levels of VPS41 expression in several cell lines are shown. GADPH was used as protein loading control. We selected clones of SH-SY5Y cells overexpressing comparable protein levels of either hVPS41 isoform 1 or 2 for further study; these are referred to as hVPS41-1 and hVPS41-2,
respectively, while vector only cells are referred as vector. For some experiments indicated, multiple cell lines were used and representative blot showing various protein expression levels in these cell lines in Figure 2.2B.

Overexpression of hVPS41 in SH-SY5Y cells ameliorates cell death in cellular models of PD

We studied the effect of hVPS41 overexpression in several neurotoxin-based cellular models of PD. Chronic exposure of rodents or non-human primates to rotenone, a mitochondria complex I inhibitor, has been shown to recapitulate many of the pathological, biochemical, and behavioral features of PD (Greenamyre et al., 2001). In dopaminergic cell lines, it has also been shown to lead to aggregation of α-syn and gradual cell death. We subjected hVPS41 transfected cell lines to rotenone treatment at various concentrations (ranging from 0.1 μM to 20 μM) for 40 hours and cell death was measured via calculating the percentage of the intracellular enzyme LDH released into the medium. Rotenone induced dose-dependent increases in cell death as measured by LDH release in all cell lines tested. We found that the overexpression of hVPS41, either isoform 1 and 2, resulted in the attenuation of rotenone-induced LDH release in SH-SY5Y cells (Figure 2.3A). This effect was subsequently verified in several other hVPS41 overexpressing cell lines. Interestingly, the expression level of hVPS41, which varies in the different cell lines, was not clearly correlated with the extent of neuroprotection in the cell lines tested, suggesting that even modest hVPS41 expression was sufficient to confer protection (Figure 2.3A). Meanwhile, we observed no difference between cell lines overexpressing isoform 1 and 2 of hVPS41, suggesting both isoforms share similar neuroprotective properties.

We also tested the effect of hVPS41 overexpression in SH-SY5Y cells in a second cellular model of PD, 6-OHDA toxicity (Blum et al., 2001). Cell lines expressing either empty
vector or hVPS41-1 were subjected to 6-OHDA treatment for 16 hours, and cell death was measured via LDH assay. 6-OHDA induced dose-dependent increases in cell death as measured by LDH release in all cell lines tested. We found that overexpression of hVPS41-1 attenuated 6-OHDA-induced cell death in this model as well (Figure 2.3B). We confirmed our result in the 6-OHDA model using an alternative assay for cell death, based on fluorescence of the dye Calcein-AM. This dye, which accumulates intracellularly, fluoresces in response to increased cytosolic Ca$^{++}$, an early event in cell injury (Jonas, 2009). This approach also demonstrated a strong protective effect of hVPS41-1 against 6-OHDA treatment (data not shown).

In order to test whether the protection conferred by hVPS41 in SH-SY5Y cells also extends to other cell death models, we treated these cells with staurosporine, a general protein kinase inhibitor that has been used previously to induce cell death (Tamaoki et al., 1986). Stably transfected cell lines were subjected to staurosporine treatment for 5 hours and cell death was measured via LDH assay. The extent of staurosporine-induced cell death was also attenuated by expression of hVPS41 in SH-SY5Y cells (Figure 2.3C).

*hVPS41-1 expression does not prevent mitochondrial membrane depolarization in SH-SY5Y cells after rotenone treatment*

Rotenone is an inhibitor of mitochondrial complex I and has been reported to induce mitochondrial membrane depolarization (Moon et al., 2005), which could lead to cell death (Kong et al., 2001; Recchioni et al., 2002). To investigate whether hVPS41 overexpression attenuates mitochondrial depolarization resulting from rotenone treatment, hVPS41-1 cell lines, as well as the vector control lines, were incubated in the absence or presence of rotenone, and mitochondrial membrane potential ($\Delta\psi_m$) was measured with the fluorometric dye JC-1. The
ratio of JC-1 aggregates to monomer staining is independent of cell number or mitochondrial density, and thus can be used to quantitatively measure changes in Δψm (Chang et al., 2002). In all tested cell lines, rotenone induced a dose-dependent mitochondrial membrane depolarization as demonstrated by the decrease in the JC-1 ratio (Figure 2.4). Interestingly, hVPS41 had no effect on mitochondrial membrane depolarization induced by rotenone treatment; the extent of JC-1 ratio decrease was similar among all cell lines treated, whether or not hVPS41 was overexpressed (Figure 2.4). Similar effects were observed when cell lines were subjected to staurosporine treatment as the overexpression of hVPS41 also failed to alter the extent of mitochondrial membrane depolarization induced by that stressor (data not shown).

hVPS41-1 overexpression suppresses the activation of apoptosis induced by rotenone treatment in SH-SY5Y cells

Treatment with rotenone can result in the activation of mitochondrial-dependent apoptotic pathway (Potokar et al., 2006; Tada-Oikawa et al., 2003), which might contribute to the cell death in the pathogenesis of PD (Dauer and Przedborski, 2003). It has been shown that release of cytochrome c from the mitochondria results in the processing of pro-caspase-9 into its active fragment (Li et al., 1997), which subsequently converts pro-caspase-3 into its active form (Shi, 2002). Therefore it is important to determine whether the neuroprotection displayed by hVPS41 is related to inhibition of the apoptotic cell death pathway.

hVPS41-1 or vector control cells were incubated in the absence or presence of rotenone, and then lysed and immunoblotted to determine the presence of cleaved (active) caspase-9 or caspase-3. Incubation of all these cell lines with rotenone resulted in the appearance of cleaved caspase-9 and caspase-3, indicating that apoptotic cascade was activated in our cellular model.
The extent of caspase activation was, however, greatly reduced in hVPS41-1 overexpressing cells compared to the activation observed in vector cells (Figure 2.5A). We quantified this effect at a rotenone concentration of 0.5 µM (the concentration at which caspase-9 activation is maximal in both vector and VPS41 overexpressing cell lines), and found that the protein level of activated caspase-9 in hVPS41 overexpressing cell lines was only $17.5 \pm 12\%$ of that in the vector control cells (mean ± SEM, $P<0.05$, $n=3$).

Caspase-3 enzymatic activity was measured quantitatively using a fluorometric method in vector and hVPS41-1 cell lines incubated in the absence or presence of rotenone. These results revealed that rotenone treatment resulted in an increase in caspase-3 enzymatic activity in both cell lines, but the extent of caspase activity increase was significantly less in hVPS41-1 compared to vector cell lines ($P<0.05$) (Figure 2.5B).

We also tested the effect of hVPS41 overexpression on apoptosis induced by 6-OHDA as well as by staurosporine. We found that the overexpression of hVPS41 attenuates apoptosis in response to both these toxins, as the extent of caspase activation was much less in the hVPS41-1 cells, when compared to the vector control cells (Figure 2.5C, D).

**hVPS41-1 overexpression modulates the change in α-syn solubility induced by rotenone treatment in SH-SY5Y cells**

Accumulation of detergent-insoluble high-molecular weight forms of α-syn is a characteristic of human synucleinopathies (Cantuti-Castelvetri et al., 2005; Goedert, 2001). In cellular model systems, rotenone treatment induces a similar accumulation of insoluble α-syn (Lee et al., 2004; Sherer et al., 2002). We used this approach to examine the effect of hVPS41 on the accumulation of Triton-insoluble α-syn in SH-SY5Y cells treated with rotenone. In
agreement with previous studies, we found that rotenone treatment in vector cell lines induced an increase in the abundance of Triton-insoluble, which migrated predominantly with a size consistent with multimers (dimers and trimers) of α-syn, (Figure 2.6). The increase in these insoluble forms ranged from 3 to 6 fold across the range of rotenone concentrations tested, and was statistically significant at each of the concentrations studied. Interestingly, in hVPS41 overexpressing cells, the basal levels of Triton-insoluble α-syn were similar to vector cells, but the rotenone treatment paradigm failed to produce any significant increase in the abundance of Triton-insoluble α-syn (Figure 2.6A, B). Triton-soluble forms of α-syn were similar in hVPS41 and control lines, and not affected by rotenone (not illustrated). We also examined cathepsin D, an essential lysosomal protein, and found that while the abundance of this protein was increased after treatment with rotenone, this effect was not altered in the presence of hVPS41 (data not shown).

DISCUSSION

In this study, we have examined the potential neuroprotective effects of expression of hVPS41, a protein related to lysosomal trafficking, in both invertebrate and cellular models of PD. We have found that overexpression of hVPS41, in both C. elegans and SH-SY5Y neuroblastoma cells, attenuates cell death induced by several stimuli that model the pathophysiology of PD: overexpression of α-syn and 6-OHDA treatment in C. elegans, and rotenone and 6-OHDA in SH-SY5Y cells. Our results demonstrate a consistent and strong neuroprotective effect in each of these model systems. In cellular systems hVPS41 does not seem to modulate the loss of mitochondrial membrane potential (Δψm) induced by these neurotoxins, but it does seems to modulate several downstream events in the apoptotic cascade. We also
observed that the presence of hVPS41 reduced the accumulation of detergent insoluble forms of α-syn after rotenone treatment, supporting a role for hVPS41 in the prevention of protein misfolding or the clearance of misfolded proteins. Collectively, these data suggest that hVPS41 may be a useful target of therapy in PD.

VPS41 was initially identified as a potential modulator of α-syn neurotoxicity in the C. elegans model system (Hamamichi et al., 2008). An important advantage of the C. elegans system is that screening and identification of potential neuroprotective factors is relatively rapid and efficient. A limitation, however, is that the results obtained in this invertebrate model may not be generalizable to more complex mammalian systems. Interestingly, several other targets initially identified in the same C. elegans system have proven to have protective properties in more complex models. These include two genes (PINK1 and DJ-1) which have been shown to cause autosomal recessive PD when deleted, and a third gene (ULK2) recently identified in a whole-genome analysis of PD patient populations (Fung et al., 2006). In addition, the autophagy-associated gene ATG7, another target identified in this screen, has been functionally validated in mammals, as targeted disruption of this gene causes neurodegeneration in mice (Komatsu et al., 2006). We were able to observe protective effects of hVPS41 not only against α-syn, which was employed in the first phase of the C. elegans screen, but also against 6-OHDA and rotenone, two neurotoxins which are able to mimic many pathological hallmarks of PD, including neurodegeneration of dopaminergic neurons in substantia nigra and formation of structures similar to Lewy bodies (Blum et al., 2001; Bove et al., 2005; Uversky, 2004). These observations suggest that the C. elegans system has substantial predictive value with respect to effects in more complex organisms.
Our study provides insight into the mechanisms which may be responsible for the neuroprotective actions of hVPS41. Although both of the neurotoxins that we employed, 6-OHDA and rotenone, target the mitochondria, the overexpression of hVPS41 does not appear to alter the effect of the toxins on mitochondrial functions as measured using the JC-1 ratiometric dye. hVPS41 does, however, appear to block the activation of apoptotic mechanisms, with inhibition of activation of caspase-3 and caspase-9. hVPS41 was also able to attenuate cell death induced by staurosporine, which is commonly believed to be a general inducer of apoptosis (Lee et al., 2003). Apoptosis does seem to play a critical role in PD dopaminergic cell death (Dawson and Dawson, 2002). Increased levels of caspase-3 and BAX have been shown in nigral neurons in PD postmortem brain (Hartmann et al., 2001; Tatton, 2000). In adult mice, there is an up-regulation of BAX in the SNpc after MPTP administration and a decrease in Bcl-2, both of which are in parallel to MPTP-induced dopaminergic neurodegeneration (Vila et al., 2001). In human neural stem cells (hNSCs) and their differentiated cultures, rotenone was found to induce apoptosis, evidenced by ultrastructural characteristics and TUNEL staining. Time-dependent release of cytochrome c, apoptosis-inducing factor (AIF), and caspase 9/3-dependent apoptosis were also reported in that study (Li et al., 2005). Meanwhile, 6-OHDA infused in the striatum of adult rats induced the presence of apoptotic profiles, confirmed by electron microscopic studies (Marti et al., 2002). Intracellularly, 6-OHDA was also found to induce a caspase-3-dependent apoptotic cell death (Ding et al., 2004; Hanrott et al., 2006). Therefore, our results not only support a role for hVPS41 in protection against PD-relevant neurotoxins, but also shed light on the underlying molecular mechanisms resulting in this neuroprotection.

While apoptosis seems to be part of the process of cell death in PD and models of the disease, the triggers for cell death in PD are less clear. α-syn appears to have a central role:
mutations of α-syn (Kruger et al., 2001; Kruger et al., 1998; Polymeropoulos et al., 1997), or even overexpression of the normal protein as a result of gene multiplication (Ross et al., 2008; Singleton et al., 2003), cause dominantly inherited PD, while accumulation of aggregated α-syn in Lewy neuritis and Lewy bodies is a nearly universal feature of sporadic as well as genetic forms of the disease (Irizarry et al., 1998; Spillantini et al., 1997). Misfolding and accumulation of α-syn is observed in SH-SY5Y cells after treatment with rotenone (Sherer et al., 2003).

We found that the overexpression of hVPS41 markedly reduced the accumulation of detergent-insoluble high molecular weight forms of α-syn. This suggests that a primary effect of hVPS41 may be to segregate and target for degradation misfolded α-syn, a role which is consistent with its known function in yeast (Bowers and Stevens, 2005). Our observations do not, of course, exclude the possibility of additional effects of hVPS41. We did not find any clear effect of VPS41 overexpression on the basal level of cathepsin D protein level or the induction of this protein by rotenone treatment, suggesting that enhanced VPS41 expression does not lead to an increased number of lysosomes, but it is certainly possible that hVPS41 may alter the functional properties of the lysosomal system. In yeast, VPS41 has an unusual role in mediating a pathway which bypasses the endosome and carries a small set of selected protein cargoes directly to the vacuole (Rehling et al., 1999). Whether a similar pathway is present in mammalian cells or whether VPS41 participates in trafficking of a large spectrum of proteins in mammals is at present uncertain. In either case, the view that enhanced trafficking of misfolded proteins is important for the actions of VPS41 is supported by recent work on related trafficking proteins, including Rab proteins, which lie upstream in the pathway between the Golgi and the lysosome, and also protects against α-syn toxicity in a variety of animal and cellular models of PD (Gitler et al., 2008).
It is important to note that the predictive value of all of the existing animal models with respect to human disease is at present uncertain. Indeed, the lack of any therapy proven efficacious in human PD is a barrier to rigorous validation of such model systems. Here, we have taken the approach of evaluating the actions of hVPS41 against neurodegeneration in several different classes of models, which encompass both genetic (α-syn) and neurotoxic approaches. As discussed elsewhere (Yacoubian and Standaert, 2008), success across several different models with diverse mechanisms conveys a higher probability of successful translation to human disease.

Two forms of VPS41 have been described in human. Previous descriptions of these two isoforms based on sequence analysis, suggested that they were likely to differ substantially in structure, with isoform 2 lacking the C-terminal RING-H2 sequence motif, which can be responsible for membrane association (McVey Ward et al., 2001). Careful analysis of the sequence reported, however, suggest that this conclusion was not correct. Using the sequence provided in the prior publication, we performed an independent alignment, which demonstrated that the predicted amino acid sequence difference was much less significant, with isoform 2 lacking only amino acids 83-107 in the amino terminus. This finding was also verified by direct sequence analysis of cDNA plasmids purchased from Origene, and by our protein expression studies that showed an apparent size difference consistent with the N-terminal deletion. We also examined the properties of both human isoforms in our assays and found that they were similar, suggesting that the structural difference between hVPS41-1 and hVPS41-2 is not important for protection in models of PD.

In summary, our data support the neuroprotective efficacy of hVPS41 in a range of invertebrate and cellular models of PD. The protective effects appear to involve inhibition of
apoptotic cascades. The mechanism of action is likely to involve enhancement of the clearance of abnormal high molecular weight forms of α-syn, perhaps by targeting them to lysosomes for degradation. Further studies in intact mammalian models will be important to define the therapeutic potential of this target and may justify efforts to develop pharmacological strategies to induce the expression of VPS41 or to enhance its actions.

REFERENCES


Figure 2.1

A) Bar graph showing the percentage of worms with normal DA neurons over time. The x-axis represents days, with day 7 and day 10 shown. The y-axis represents the percentage of worms. The bars are labeled as follows: α-syn alone, α-syn + VPS41-1, and α-syn + VPS41-2. The asterisks indicate statistical significance.

B) Images showing α-syn alone and α-syn + VPS41. The images are labeled with arrows and arrowheads indicating specific areas of interest.

C) Bar graph showing the percentage of worms with normal DA neurons at 24 hours, 48 hours, and 72 hours. The bars are labeled as follows: 6-OHDA control, 6-OHDA + VPS41-1, and 6-OHDA + VPS41-2. The asterisks indicate statistical significance.

D) Images showing 6-OHDA alone and 6-OHDA + VPS41. The images are labeled with arrows and arrowheads indicating specific areas of interest.
Figure 2.2

(a) VPS41

-100KD

GADPH

-37KD

Vector  hVPS41-1  hVPS41-2

(b) VPS41

-100KD
Figure 2.3

(a) Graph showing LDH (percent release) against Rotenone Concentration (μM).

(b) Graph showing LDH (percent release) against 6-OHDA Concentration (mM).

(c) Graph showing LDH (percent release) against Staurosporine Concentration (μM).
Figure 2.4
Figure 2.5

(a) Western blot analysis of Caspase-9, Caspase-3, and GADPH in cells treated with different concentrations of Rotenone (µM) in the presence of Vector or hVPS41-1. 6-OHDA (mM) was used as a positive control.

(b) Graph showing Caspase-3 activity (fold to control) in response to Rotenone (µM) treatment. The bars represent the mean ± SEM of three independent experiments. *p < 0.05 compared to the Vector group.

(c) Western blot analysis of Caspase-9 and Caspase-3 in cells treated with different concentrations of 6-OHDA (mM) in the presence of Vector or hVPS41-1.

(d) Western blot analysis of Caspase-9, Caspase-3, and PARP in cells treated with different concentrations of Staurosporine (µM) in the presence of Vector or hVPS41-1.
Figure 2.6

(a) Western blot showing the expression levels of α-synuclein at different concentrations of rotenone.

(b) Bar graph showing the fold change in triton-insoluble α-synuclein oligomers under different rotenone concentrations.

* indicates statistical significance compared to control.
FIGURE LEGENDS

Figure 2.1. Expression of either isoform of hVPS41 rescues DA neuron loss in C. elegans. Plasmids that drive expression of different isoforms of hVPS41 were injected into C. elegans. After establishing stable lines, these transgenic animals were crossed into α-syn worms.

A. The F2 homozygous progeny for both genes were synchronized and analyzed for protection from DA neurodegeneration at Day 7 and Day 10 after hatching. Worms without any degeneration are considered as WT (wild type). Mean ± SD, n = 270 worms; * p < 0.05, ** p <0.01, Student's t-test. B. The anterior DA neurons of worms OE α-syn alone or with hVPS41 are shown. Top image shows degeneration of 3 CEP neurons, while the bottom image shows the full complement of anterior DA neurons when co-expressing hVPS41 with α-syn. C. Expression of either isoforms of hVPS41 rescues DA neuron loss induced by 6-OHDA in C. elegans. Plasmids that drive expression of different isoforms of hVPS41 were injected into C. elegans. After establishing stable lines, these worms were crossed into P-dat-1::GFP worms, subjected to 10 mM 6-OHDA for 1 hour, and analyzed for degeneration at 24, 48, and 72 hours post-treatment. Worms without visible degeneration are considered as wild type (WT). Mean ± SD, n = 270 worms; * p < 0.05, ** p <0.01, Student's t-test. D. The anterior dopaminergic neurons of C. elegans are shown after treatment with 6-OHDA. Top image is of a control worm expressing only GFP in the DA neurons and has degeneration in 3 CEP neurons. In contrast, the bottom image is of a WT worm co-expressing hVPS41 and GFP in the DA neurons and does not exhibit any degeneration (WT worms have all 6 anterior DA neurons) after 6-OHDA treatment. Arrows depict CEP neuronal processes while arrowheads depict ADE neuronal processes.

Figure 2.2. Levels of hVPS41 protein expression in SH-SY5Y neuroblastoma cells stably transfected with hVPS41. A. The representative immunoblot shows the expression of hVPS41 in the two cell lines used for the majority of the experiments described in this study. B.
The representative immunoblot illustrates a number of additional stably transfected cell lines, demonstrating variable levels of hVPS41 expression. Several of these additional lines were used in confirmatory studies, as indicated in the text.

Figure 2.3. Overexpression of hVPS41 in SH-SY5Y cells ameliorates cell death in cellular models of PD. A. SH-SY5Y stable cell lines were treated with rotenone for 48 hrs at indicated concentration, and cell death was measured by LDH release. B. SH-SY5Y stable cell lines were treated with 6-OHDA for 16 hrs at indicated concentration, and cell death was measured by LDH release. C. SH-SY5Y stable cell lines were treated with staurosporine for 5 hrs at indicated concentration, and cell death was measured by LDH release. * p <0.05, ** p <0.01, Student's t-test. Mean ± SEM, n=5, ANOVA test from SigmaStat.

Figure 2.4. hVPS41 overexpression does not prevent mitochondrial membrane depolarization in SH-SY5Y cells after rotenone treatment. SH-SY5Y stable cell lines were incubated under control conditions or in the presence of rotenone at indicated concentration for 48 hrs, and subsequently incubated with the mitochondrial membrane potential-sensitive dye JC-1 then quantitated with fluorescence plate reader. There is no significant difference in mitochondrial membrane potential between vector cells and hVPS41 overexpressing lines, with or without rotenone treatment. Mean ± SD, n = 3 experiments.

Figure 2.5. hVPS41 overexpression suppresses the activation of apoptosis induced by rotenone treatment in SH-SY5Y cells. Cells were incubated in the absence or in the presence of rotenone at the indicated concentrations for 48 h prior to cell collecting. A,B. Immunoblots showing that rotenone resulted in less pronounced caspase-9 and caspase-3 activation in hVPS41 overexpressing than in vector cell lines. Measurement of caspase-3 activity assay revealed that overexpression of hVPS41-1 in SH-SY5Y cells attenuated caspase-3 activation induced by
rotenone treatment. Data are expressed as fold increase over the activity in vector cells under control conditions. Mean ± SEM. n = 3. *p < 0.05. C. Cells were incubated in the absence or in the presence of 6-OHDA at the indicated concentrations for 6 h prior to cell collecting, and immunoblots showed that rotenone resulted in less pronounced caspase-9 and caspase-3 activation in hVPS41 overexpressing than in vector cell lines. D. Cells were incubated in the absence or in the presence of staurosporine at the indicated concentrations for 6 h prior to cell collecting, and immunoblots showing that staurosporine resulted in less pronounced caspase-9 and caspase-3 activation, as well as PARP cleavage in hVPS41 overexpressing than in vector cell lines. Immunoblots shown are representative of three independent experiments.

Figure 2.6. hVPS41 overexpression modulates the change in α-syn solubility induced by rotenone treatment in SH-SY5Y cells. Cells were incubated in the absence or in the presence of rotenone at the indicated concentrations for 48 h prior to cell collecting. These lysates were separated into Triton X-100 soluble and insoluble fractions. A. Immunoblots show that rotenone treatment induced an increase in the abundance of Triton-insoluble component in vector control but not hVPS41 overexpressing cells. Immunoblots shown are representative of four independent experiments. B. Summary of the quantified result of four independent experiments as outlined in Figure 2.6A. Mean ± SD, n = 4 experiments; * p < 0.05, Student's t-test.
CHAPTER 3

FUNCTIONAL ANALYSIS OF VPS41-MEDIATED NEUROPROTECTION IN C. ELEGANS AND MAMMALIAN MODELS OF PARKINSON’S DISEASE

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ABSTRACT

Disruption of the lysosomal system has emerged as a key cellular pathway in the neurotoxicity of α-synuclein (α-syn) and the progression of Parkinson’s disease (PD). A large-scale RNAi screen using C. elegans identified VPS-41, a multi-domain protein involved in lysosomal protein trafficking, as a modifier of α-syn accumulation and dopaminergic (DA) neuron degeneration (Hamamichi et al., 2008). Previous studies have shown a conserved neuroprotective function of human VPS41 (hVPS41) against PD-relevant toxins in mammalian cells and C. elegans neurons (Ruan et al., 2010). Here, we report that both the AP-3 interaction domain and clathrin heavy-chain repeat domain are required for protecting Caenorhabditis elegans DA neurons from α-syn-induced neurodegeneration, as well as to prevent α-syn-inclusion formation in an H4 neuroglioma cell model. Using mutant C. elegans and neuron-
specific RNAi, we revealed that hVPS41 requires both a functional AP-3 and HOPS-tethering complex to elicit neuroprotection. Interestingly, two non-synonymous SNPs found within the AP-3 interacting domain of hVPS41 attenuated the neuroprotective property, suggestive of putative susceptibility factors for PD. Furthermore, we observed a decrease in α-syn-protein level when hVPS41 was overexpressed in human neuroglioma cells. Thus, the neuroprotective capacity of hVPS41 may be a consequence of enhanced clearance of misfolded and aggregated proteins, including toxic α-syn species. These data reveal the importance of lysosomal trafficking in maintaining cellular homeostasis in the presence of enhanced α-syn expression and toxicity. This neuroprotective capacity of hVPS41 may result from increased clearance of misfolded and aggregated proteins, including toxic α-syn species. Our results support hVPS41 as a potential novel therapeutic target for the treatment of synucleinopathies like PD.

INTRODUCTION

Parkinson’s disease (PD) is the second most common neurodegenerative disease clinically characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra and the presence of intracellular protein inclusions termed Lewy bodies. While the etiology of PD is not fully understood, the α-synuclein protein (α-syn) is thought to be a central component for the progression of this disease, in that mutations or multiplication of the α-syn locus are known genetic causes of autosomal dominant PD (Polymeropoulos et al., 1997; Singleton et al., 2003). Furthermore, α-syn is a polypeptide that can oligomerize to form fibrils and aggregates, and is a primary component in cytoplasmic Lewy bodies (Spillantini et al., 1997). While most cases of PD have no known genetic defect and are thought to involve an environmental component, accumulation of α-syn is still observed (Irizarry et al., 1998). Both
cellular and animal models also exhibit cellular toxicity through α-syn overexpression. With no effective treatment to slow the progression of this disease, the identification of potential therapeutic targets or pathways remains a high priority.

We have previously established *C. elegans* as a model for age-dependent α-syn accumulation and DA neuron degeneration (Cao et al., 2005; Hamamichi et al., 2008). Importantly, this model has proven predictive in the evaluation of genetic factors and small molecules that protect cells from α-syn accumulation and toxicity, with several candidates validated across other organisms, including mammalian systems (Cooper et al., 2006; Gitler et al., 2008, 2009; Su et al., 2010). Using *C. elegans* assays, Hamamichi et al. (2008) conducted a large-scale RNA interference (RNAi) screen to identify modifiers of α-syn pathology. Through this screen, the *C. elegans* VPS-41 protein was identified as being a top neuroprotective candidate. We subsequently demonstrated that human VPS41 (hVPS41) can also protect DA neurons from both α-syn- and 6-hydroxydopamine (6-OHDA)-induced DA neurodegeneration in *C. elegans*, as well as protect human SH-SY5Y neuroblastoma cells from PD-relevant neurotoxins, including rotenone and 6-OHDA (Ruan et al., 2010).

VPS41 is a highly conserved, multi-domain protein that functions in lysosomal trafficking of Golgi-derived AP-3 vesicles (Radisky et al., 1997; Rehling et al., 1999; Darsow et al., 2001). Given the conserved neuroprotective activity of hVPS41, we conducted a structure/function analysis of hVPS41 using the nematode model and show that both the AP-3 interaction and CHCR domains are required to protect DA neurons from α-syn-induced degeneration. Through mutant and RNAi analysis, we discerned that both the AP-3 and HOPS complexes are required for hVPS41-dependent neuroprotection. Interestingly, two SNPs found within hVPS41 abolish the neuroprotective function of the protein. Our results are further
confirmed in a mammalian model of α-syn aggregation, where protective forms of hVPS41 identified in *C. elegans* significantly reduce α-syn accumulation in human neuroglioma cells. Additionally, expression of hVPS41 reduces the level of overexpressed α-syn, suggesting protein clearance may function in the protective effect of hVPS41. These data provide mechanistic insights into a conserved neuroprotective pathway and support further development of hVPS41 as a prospective therapeutic target for PD.

MATERIALS AND METHODS

*Plasmid Construction.* Human *vps-41* cDNA was obtained from Open Biosystems (Huntsville, AL). Truncated hVPS41 constructs were generated by using Phusion high-fidelity polymerase (Finnzymes) with primers specified for each truncation available upon request, with fusion of segments and site-directed mutagenesis conducted as previously described (Hobert, 2002). Plasmid entry vectors were generated using Gateway Technology (Invitrogen) to clone PCR amplified constructs into pDONR221 by BP reaction, and the constructs were then further cloned into the Gateway expression vectors, pDEST-DAT-1 (Cao et al., 2005) or pcDNA3.2/V5-DEST (Invitrogen), via LR reaction. DNA sequencing of entry vectors was used to validate the constructs. Genomic *sid-1* was amplified using Phusion high-fidelity polymerase from genomic DNA isolated from N2 Bristol nematodes and cloned into the pDEST-DAT-1 Gateway expression vector using Gateway Technology as described above.

*C. elegans strains.* Neuroprotection Assay: Transgenic *C. elegans* lines were generated by directly injecting plasmid constructs (P*dat-1::Gene X*), along with a phenotypic marker [P*unc-54::mCherry* (body wall muscle expression)], into N2 Bristol hermaphrodites to create at least 3 stable transgenic animals expressing the gene of interest in the DA neurons as detected by the
phenotypic marker mCherry in the body wall muscle cells (Harrington et al., 2011). Transgenic animals expressing mCherry were crossed with isogenic UA44 [baln11 (P_{dat-1}::α-syn, P_{dat-1}::GFP)] males, and the resulting heterozygous progeny expressing both GFP and mCherry were transferred and allowed to self-fertilize. Homozygous GFP (α-syn) expressing worms were isolated and used in the DA neurodegeneration analysis (Table 3.1). AP-3 mutant animals were generated by crossing strain RB662 [apb-3(ok429)] (provided by the C. elegans Gene Knockout Project at OMRF) with either strain UA44 or UA126 [baln11; baEx98 (P_{dat-1}::hyps41-1, P_{unc-54}::mCherry)] males to generate strains UA200 [apb-3(ok429); baln11] and UA194 [apb-3(ok429); baln11; baEx98], respectively. Homozygous progeny were verified by PCR amplification flanking the apb-3(ok429) deletion region (Primer 1- 5’-gtcaattgaagtgcactgtg-3’ and Primer 2- 5’-ccgagaatcaacgtcaatcagc-3’).

Cell-specific RNAi: Transgenic C. elegans lines were generated by directly injecting the expression plasmid P_{dat-1}::sid-1 along with the phenotypic marker P_{myo-2}::mCherry (pharyngeal expression) (Addgene) into sid-1(pk3321) hermaphrodites to create stable transgenic animals expressing genomic sid-1 in the DA neurons as well as the phenotypic marker (mCherry) in the pharynx. The extrachromosomal array (P_{dat-1}::sid-1, P_{myo-2}::mCherry) was integrated into the C. elegans genome by UV irradiation (Inoue and Thomas, 2000) to create strain UA195 [sid-1(pk3321); baln33 (P_{dat-1}::sid-1, P_{myo-2}::mCherry)]. Integrated transgenic lines were outcrossed with sid-1(pk3321) nematodes 5 times to remove any extraneous mutations. The isolated homozygous integrated lines were crossed into strain UA44 [baln11 (P_{dat-1}::α-syn, P_{dat-1}::GFP)] to generate strain UA196 [sid-1(pk3321); baln11; baln33], which expresses α-syn, GFP, and SID-1 in the DA neurons and is susceptible to RNAi specifically in the DA neurons.
Worm strain TU3401 [sid-1(pk3321); uIS69 (P_{anc-119}; sid-1, pCFJ90 (P_{myo-2}; mCherry))] was generously provided by Marty Chalfie for pan neuronal-specific RNAi (Calixto et al., 2010). TU3401 hermaphrodites, which express sid-1 pan-neuronally, were crossed into strain UA44 to generate strain UA197 [sid-1(pk3321); uIS69; baln11]; these worms are sensitive to RNAi specifically in the neurons. To isolate homozygous sid-1(pk3321) worms, crossed progeny were grown for 3 generations in the presence of par-2 (F58B6.3) RNAi, which inhibits embryonic development, allowing for only sid-1(pk3321) worms to develop. After isolating homozygous sid-1(pk3321) animals, worms expressing GFP and mCherry were sequestered, allowed to self-fertilize, and the progeny were examined for isogenic lines.

**DA neurodegeneration analysis in C. elegans.** C. elegans DA neurons were analyzed for degeneration as previously described (Hamamichi et al., 2008; Harrington et al., 2011). Briefly, strain UA44 and experimental strains expressing hVPS41 constructs in the DA neurons were synchronized, grown at 20°C, and analyzed at specific time points in development, between day 6 and day 10 post-hatching (3- and 7-day old adults) as reported in the results and figure legends, for α-syn-induced DA neurodegeneration. On the day of analysis, the 6 anterior DA neurons [4 CEP (cephalic) and 2 ADE (anterior deirid)] were examined in 30 adult hermaphrodite worms, which were immobilized on glass cover slips using 3mM levamisole and transferred onto 2% agarose pads on microscope slides. Worms were considered normal when all 6 anterior neurons were present without any signs of degeneration, as previously reported (Cao et al., 2005; Hamamichi et al., 2008; Ruan et al., 2010; Harrington et al., 2011). In total, at least 90 adult worms were analyzed for each independent truncated hVPS41 transgenic line (30 worms/trial x 3 independent transgenic lines x 3 trials = 270 total animals/construct) or RNAi treatment (30 worms/trial x 4 trials = 120 total animals/trial).
RNA isolation and semi-quantitative RT-PCR. Total RNA was isolated from 50 young adult hermaphrodite worms as previously described (Hamamichi et al., 2008). Briefly, the worms were transferred into 10 µl of 10% Single Worm Lysis Buffer (10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin, 60 µg proteinase K) and frozen at -80°C for 1 hour. After thawing, 100 µl of TRI Reagent (Molecular Research Center) was added to the samples and incubated for 10 minutes at room temperature (RT). The samples were freeze-thawed 5 times in liquid N2, vortexed for 15 seconds with 10 µl 1-bromo-3-chloropropane (Acros Organics), incubated for 10 minutes at RT, and centrifuged for 15 minutes at 14500 rpm at 4°C. The supernatant was transferred to an RNase-free tube, mixed with 1.5 µl glycoblu (Ambion) and 50 µl of -20°C-chilled isopropanol, and stored overnight at -20°C.

After incubation, the sample was centrifuged for 15 minutes at 14500 rpm at 4°C and supernatant discarded. The pellet was washed with 100 µl of RNase-free ethanol (75%) and re-suspended in 10 µl DEPC-treated water. The sample was treated for 15 minutes with 1 µl DNase I (Invitrogen) at RT, then for 10 minutes with 1 µl of 25 mM EDTA at 65°C. RT-PCR was performed using SuperScript III reverse transcriptase (Invitrogen) using oligo dT primers following the manufacturer protocols. cDNA amplification was performed using Phusion polymerase (Finnzymes) using primers specified for each construct, available upon request. For each hVPS41 construct, the 5’ FLAG primer and a 3’ primer specific for each hVPS41 construct was used to specifically amplify the transgene (Table 3.2). Amplified products were separated on 0.8% agarose gels and visualized using GelRed staining (Biotium).

For mammalian cells, 30 µl of H4 cells in lysis buffer were transferred to microcentrifuge tubes and frozen at -80°C. RNA isolation, RT-PCR, and cDNA amplification was performed as described above.
RNA interference. RNAi feeding clones (Geneservice, Cambridge, UK) were isolated and grown overnight in LB media with 100 µg/ml ampicillin. NGM plates containing 0.25% β-D-lactose were seeded with RNAi feeding clones and allowed to dry. Eight, larval stage 1 (L1) worms (neuron-specific RNAi worm strains, UA196 or UA197) were transferred to the plates and incubated until adulthood at 20°C. Adult hermaphrodites were transferred to corresponding RNAi plates and allowed to lay eggs for 5 hours to synchronize. The DA neurons in the F1 progeny of the RNAi-treated worms were analyzed at day 6 and day 8 for degeneration, as described above.

α-Syn inclusion assay. Human neuroglioma (H4) cells were grown in 4-chambered slides and co-transfected with α-syn/synT, synphilin, and either empty vector control or hVPS41 variant using Lipofectamine 2000 (Invitrogen), as previously described (McLean et al., 2001; Yacoubian et al., 2010). 24 hours post-transfection, cells were fixed with 4% paraformaldehyde, permeated with 0.5% TritonX-100 for 20 minutes at RT, and immunostained using a primary α-syn antibody (BD Biosciences, San Diego, CA) and Alexa Fluor-488 secondary antibody (Invitrogen). Every cell with α-syn staining (>400/well) was scored as positive or negative for α-syn inclusions, with the scorer uninformed of the plasmid variant expressed. Each of the 4 wells was scored independently with the experiment replicated in triplicate.

Western blotting. Transfected H4 cells were washed in PBS 24 hours after transfection, collected in lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 2% SDS, Halt protease inhibitor cocktail (Thermo Scientific)], sonicated for 10 seconds on ice, and centrifuged at 16000 x g for 10 minutes. Soluble protein was transferred to a clean tube and the bicinchoninic acid assay used to quantify protein concentrations. Samples were boiled for 5 minutes in 2X Laemmli sample buffer (BioRad) with 5% 2-mercaptoethanol, separated on 10%
SDS-polyacrylamide gels (BioRad), and transferred to PVDF membranes (GE Healthcare). Membrane was blocked for 1 hour in 5% non-fat milk in TBST [20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20] at RT and incubated with primary mouse monoclonal antibody against α-syn (1:5000; Abcam), actin (1:5000; MP Biomedicals), or primary rabbit monoclonal antibody against FLAG (1:400; Sigma). Blots were washed thrice with TBST and incubated with HRP-conjugated mouse or rabbit secondary antibodies (1:10000; GE Healthcare) for 2 hours at RT. After 4 washes of 10 minutes with TBST, blots were incubated for 5 minutes with Immobilon western chemiluminescent HRP substrate (Millipore) and developed using the Fujifilm Image Reader LAS-4000. Multigage software v3.0 (Fujifilm) was used to quantify protein levels.

**Imaging and statistics.** Fluorescent microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HYQ filter cube (Chroma Technology). A Cool Snap CCD camera (Photometrics) driven by MetaMorph software (Molecular Devices) was used to acquire deconvolution images. DNA gel and protein blot images were acquired using the Fujifilm Image Reader LAS-4000. Quantification of DNA or protein level was done using Multigage software v3.0 (Fujifilm). The Student’s t-test was used to statistically analyze all datasets.

RESULTS

*WD40 and Clathrin domains of VPS41 are required for protecting C. elegans DA neurons against α-syn-induced neurodegeneration in vivo*

Through a large-scale RNA interference (RNAi) screen using *C. elegans* to identify modifiers of α-syn pathology, the nematode orthologue of VPS-41 was previously identified as a
leading candidate. RNAi knockdown of *C. elegans vps-41* enhanced α-syn aggregation in body wall muscles, while transgenic overexpression of VPS-41 in the dopaminergic (DA) neurons protected these neurons against α-syn-induced neurodegeneration (Hamamichi et al., 2008).

Since VPS-41 is a highly conserved metazoan protein (Radisky et al., 1997), we further showed that both human isoforms of VPS41 (hVPS41) were able to protect *C. elegans* DA neurons from both 6-hydroxydopamine- (6-OHDA) and α-syn-induced neurodegeneration, validating a preserved function of the VPS41 protein across species (Ruan et al., 2010).

Initially, we sought to determine which functional domains were required for the neuroprotective property of hVPS41 using our *C. elegans* model of α-syn-induced DA degeneration (*P*<sub>dat-1</sub>::α-syn; *P*<sub>dat-1</sub>::GFP) (Figure 3.1). Previous studies in yeast identified several functional domains within hVPS41, including AP3 interaction (WD40), clathrin heavy-chain repeat (CHCR), RING finger, metal ion binding, and microtubule binding domains (Radisky et al., 1997). To functionally elucidate the minimal domains required for hVPS41-dependent neuroprotection, we created truncated forms of the protein (Figure 3.1B) and generated transgenic animals expressing the truncated proteins specifically in the DA neurons. These transgenic animals were then crossed into an isogenic worm strain expressing human α-syn and GFP in the DA neurons that exhibit age-dependent neurodegeneration (Figure 3.1A, left image) (Hamamichi et al., 2008) to determine which truncated forms could rescue α-syn-induced neurodegeneration. Each hVPS41 construct was validated to be expressed within the DA neurons by reverse-transcription PCR (RT-PCR) using a 5’ FLAG primers and a 3’ primer specific for each hVPS41 construct (data not shown).

Worms are considered normal when a full complement of all 6 anterior DA neurons is present with neuronal processes fully extended to their target areas (Figure 3.1A, right image).
Expression of α-syn and GFP results in significant DA neurodegeneration, whereby only ~15% of the worm population retains normal DA neurons at 7 days of development (Figure 3.1C). In accordance with previous findings, overexpression of both isoforms of hVPS41 can significantly protect these neurons from degeneration (Figure 3.1C). Interestingly, two truncated forms of hVPS41 (truncates D and G) were also able to significantly ameliorate α-syn-mediated neurotoxicity (Figure 3.1B, C). These two truncated versions of hVPS41 shared the AP3 interaction domain (WD40 repeat) and the CHCR domain. In contrast to previous reports (McVey Ward et al., 2001), the RING-H2 finger does not appear to play a functional role in protecting C. elegans DA neurons from α-syn-induced neurodegeneration, since truncation of this domain does not affect the neuroprotective property of hVPS41, as demonstrated by the neuroprotective activity from truncate D. Removal of 107 amino acids within the TPR-like domain (truncate G), which separates the WD40 and CHCR domains, also did not alter the overall function of hVPS41 in this assay. Together, these data reveal that both the WD40 protein interaction domain and CHCR domain are required for the conserved function of hVPS41 in protecting DA neurons from α-syn-induced neurodegeneration in vivo.

VPS41 requires both AP3 and HOPS complexes for neuroprotection

Previous studies in yeast have identified at least two independent pathways that regulate protein sorting and trafficking from the Golgi to the vacuole/lysosome, with VPS-41 functioning within one of these, the alkaline phosphatase (ALP) pathway (Darsow et al., 2001). Protein trafficking through the ALP pathway requires the heterotetrameric adaptor protein complex (AP-3) and transports cargo directly to the vacuole by bypassing early, sorting endosomes. The N-terminal WD40 domain of VPS-41 directly interacts with the large δ-subunit of the AP-3
complex, while the C-terminal CHCR domain of VPS-41 can homo-oligomerize to form a clathrin-like outer shell surrounding the AP-3 vesicle (Rehling et al., 1999; Darsow et al., 2001). As the vesicles approach the vacuole, VPS-41 functions as part of the HOPS (homotypic fusion and vacuole protein sorting) docking complex along with VPS-39 to promote vacuole fusion, through activation of the Rab GTPase RAB-7 (Brett et al., 2008; Cabrera et al., 2009).

To determine if hVPS41 is functioning to protect DA neurons from α-syn-induced degeneration via lysosomal trafficking of AP-3 transport vesicles, we utilized the availability of a C. elegans AP-3 complex mutant, apb-3(ok429), which is a predicted null mutation that has defects in lysosome-related gut granule formation (Hermann et al., 2005). Our isogenic line of worms expressing α-syn, GFP, and hVPS41 in the DA neurons was crossed into apb-3(ok429) mutant worms and the DA neurons were analyzed for the level of degeneration at days 7 and 10 (Figure 3.2A). While overexpression of hVPS41 was significantly able to protect neurons for degeneration in a wild-type (N2) background, hVPS41 was unable to elicit the neuroprotective effect in the absence of a functional AP-3 complex, as observed in the apb-3(ok429) mutant background.

To further validate the functional requirement of the AP-3 and HOPS complex, we took advantage of a recently reported innovation enabling us to conduct neuron-specific RNAi by generating a worm strain that over-expresses the dsRNA transporter, SID-1, specifically in the DA neurons. Since C. elegans neurons are known to be highly resistant to RNAi, possibly through low expression of SID-1, Calixto et al. (2010) created a strain expressing genomic sid-1 under the pan-neuronal promoter (P_{unc-119}) in a sid-1(pk3321) mutant background; this strain allowed for neuron-specific gene knockdown in worms, with all other tissues/cells being resistant. Using a similar approach, we created a sid-1(pk3321) mutant strain of C. elegans that
expresses wild-type SID-1 specifically in the DA neurons under control of the DA transporter promoter ($P_{\text{dat-1}}$), thereby enabling DA-neuron specific RNAi. The DA-neuron specific RNAi strain was crossed into our isogenic UA44 ($P_{\text{dat-1}}::\alpha$-syn; $P_{\text{dat-1}}::\text{GFP}$) strain to examine the effect of neuronal gene knockdown to identify modifiers of α-syn toxicity selectively in DA neurons. Interestingly, knockdown of either of the HOPS complex components, VPS-39 and VPS-41, or the VPS-41-interacting δ subunit of the AP-3 complex (APD-3), significantly enhanced the level of DA neurodegeneration elicited through α-syn overexpression in the DA-neuron specific RNAi strain (Figure 3.2B). Knockdown of $vps-28$ did not enhance α-syn-induced neurodegeneration. Since the VPS-28 gene product encodes ESCRT-1 (Endosomal Sorting Complex Required for Transport), and it is not involved in Golgi to lysosomal trafficking, but is required for cell surface receptor degradation, we chose this as a negative control. DA neuron-specific RNAi data were confirmed using a pan-neuronal RNAi strain (Calixto et al., 2010; Figure 3.2C), thus verifying that the DA-neuron specific RNAi strain did not alter the expression levels of proteins due to promoter saturation. Taken together, these data reveal the functional relevance of the AP-3 and HOPS complex in impacting α-syn-induced neurodegeneration as well as validate the requirement of a functional AP-3 complex for hVPS41 to function.

**SNPs in VPS41 alter neuroprotective function**

With advances in genomic sequencing, genetic variance within populations is an emerging field of intense interest, with identification of single nucleotide polymorphisms (SNPs) in specific genes being associated with human diseases. As for PD, several SNPs have been directly associated with the occurrence of PD, including mutations in the gene encoding α-syn (A30P and A53T). However, these mutations only account for a small population of patients
with this disease, indicating further genetic or environmental factors may play pivotal roles in the onset and progression of PD. Since we have shown the functional requirement of hVPS41 in protecting DA neurons from degeneration due to α-syn overexpression, we evaluated several SNP databases, including the NCBI SNP database (www.ncbi.nlm.nih.gov/snp), GeneCards (www.genecards.org), and SNPs3D (www.snps3d.org), where several SNPs have been identified within the general human population. Interestingly, the domain in which hVPS41 interacts with the AP-3 complex (WD40 domain) has several SNPs (T146P and A187T) reported within it that are predicted to be deleterious to the structure/function of the protein (Figure 3.3A) (SNPs3D). These SNPs change functional groups of amino acids; there is a hydrophilic to hydrophobic amino acid change in the T146P SNP, while A187T changes this residue from hydrophobic to hydrophilic. In both cases, the structure and/or function of the WD40 domain could be altered. Moreover, both SNPs have a minor allelic frequency of \( f = 0.02 \) (2%), indicating a small percentage of the population contains either of these SNPs.

Since these SNPs have not been functionally analyzed and are predicted to be deleterious to the activity of the protein (SNPs3D), we introduced these changes into hVPS41 and overexpressed the SNP mutants in the DA neurons to investigate their impact in our worm model of α-syn-induced neurodegeneration. The hVPS41 SNP transgenes were validated to be transcribed within the DA neurons of the α-syn expressing worms by RT-PCR (data not shown). Strikingly, overexpression of hVPS41 T146P or A187T abrogated the DA neuroprotection provided by wild-type hVPS41 against α-syn-induced neurodegeneration (Figure 3.3B). While yet to be associated with human PD patient genomes, these data indicate that the T146P or A187T variants in the WD40 domain alter the neuroprotective property of hVPS41 in an in vivo animal model of PD.
VPS41 reduces cytoplasmic α-syn inclusion formation in vitro

A key pathological feature of PD is the formation of cytoplasmic protein inclusions, containing α-syn, which are thought to underlie the cellular toxicity and promote neuronal cell death. To further validate the results obtained in our evaluation of hVPS41 using C. elegans, we utilized a human cell culture model of α-syn inclusion body formation (McLean et al., 2001). Co-transfection of human neuroglioma (H4) cells with an α-syn/truncated green fluorescent protein fusion (synT) and synphilin results in the formation of cytoplasmic inclusions in about 50% of cells which immunostained for α-syn (Figure 3.4A, B). Using this model, we co-transfected H4 cells with either an empty vector or an hVPS41 construct, and 24 hours after transfection, we fixed and immunostained the cells using an α-syn-specific antibody. Interestingly, both isoforms of hVPS41, as well as the two truncated forms (D and G) that reduced neurodegeneration in C. elegans, also significantly decreased the number of inclusion-positive cells by ~40% in H4 cells (Figure 3.4A, B). Truncate B, which did not protect C. elegans DA neurons from α-syn toxicity, was also unable to alter the degree of α-syn-inclusion formation in H4 cells (Figure 3.4B). Additional correlative results between C. elegans and H4 cells were obtained when the hVPS41 T146P and A187T SNPs were examined. Similar to the lack of effect of these variants seen in C. elegans, the number of inclusion-positive H4 cells when transfected with either variant was similar to the empty vector control (Figure 3.4B). The intracellular localization of hVPS41 is expressed in a diffuse pattern within the cytoplasm and perinuclear region of H4 cells, as detected by FLAG antibody staining (Figure 3.4C). Most hVPS41 truncates (except hVPS41 truncate F) and both hVPS41 SNPs were localized in a similar manner and expression level as compared to hVPS41 isoforms 1 and 2 (data not shown). Truncated hVPS41 construct F may not express a stable protein due to the reduced size of this
construct which may be degraded due to misfolding, and this hVPS41 truncate F mimics the *C. elegans vps-41(ep402)* mutant allele, which is embryonic-lethal to the organism in the homozygous state and has an increase in germline apoptosis (Lackner et al., 2005), indicating this truncated protein is defective in function. These data confirm the *C. elegans* results in identifying functional domains and SNPs within hVPS41, which may alter cellular homeostasis in the presence of increased α-syn protein expression.

**VPS41 promotes the clearance of α-syn in human neuroglioma cells**

Defects with lysosomal function in protein clearance are proposed to contribute to the progression of PD, with the α-syn protein being degraded, in part, though autophagy (Shin et al., 2005). Since α-syn accumulation may be associated with the neurotoxicity of DA neurons, we wanted to determine if hVPS41 could promote the clearance of α-syn under conditions of increased expression in human neuroglioma cells. We previously reported that hVPS41 overexpression could prevent the increase in Triton-insoluble (oligomeric) α-syn species in human neuroblastoma (SH-SY5Y) cells after rotenone treatment (Ruan et al., 2010), and here we report the ability of hVPS41 to decrease the number of cells with α-syn-positive cytoplasmic inclusions in H4 cells (Figure 3.4). Using the H4 cell model of α-syn-inclusion formation described above, we isolated total protein from cells after transfection with synT, synphilin, and either an empty vector control or hVPS41, and quantified the levels of synT protein expressed. While a high level of synT was observed in the control cells, a significant reduction (~45%) in synT was recorded when hVPS41 was also expressed (Figure 3.5A); this reduction was not due to transcriptional regulation of the transgenes (Figure 3.5B). In accordance with our synT inclusion formation results (Figure 3.4B), we show that both SNPs, T146P and A187T, found
within the WD40 domain of hVPS41 prevent the protein from reducing the level of synT protein (data not shown). These results validate that when either SNP is present in hVPS41, the function of hVPS41 is altered, preventing the protein from reducing α-syn accumulation. These results further support these two SNPs as being susceptibility factors for PD.

DISCUSSION

Increasing evidence supports a role for dysfunctional lysosomal trafficking and protein degradation as underlying the pathology of PD. Deleterious cellular consequences arising from lysosomal dysfunction have been proposed to underlie the age-related progression of the disease (Chu and Kordower, 2007). Lysosomal targeting of misfolded and aggregated proteins for degradation, including α-syn, has been shown to be largely mediated by macroautophagy and chaperone-mediated autophagy (CMA) (Webb et al., 2003; Cuervo et al., 2004). Mutations in α-syn that are associated with autosomal dominant forms of PD have been shown to block CMA (Cuervo et al., 2004), and overexpression of wild-type α-syn impairs macroautophagy (Winslow et al., 2010). Accumulation of α-syn is observed within Lewy bodies in post-mortem PD brains (Spillantini et al., 1997). In C. elegans, transgenic overexpression of α-syn leads to age-dependent α-syn accumulation and DA neuron degeneration (Lakso et al., 2003; Cao et al., 2005; Kuwahara et al., 2006; Hamamichi et al., 2008; van Ham et al., 2008; Karpinar et al., 2009). Depletion of the E1-like enzyme required for the initiation of autophagosome formation, atg-7, results in neurodegeneration in mice (Komatsu et al., 2006) as well as enhanced α-syn accumulation in C. elegans (Hamamichi et al., 2008). Augmentation of a lysosomal enzyme, cathepsin D, promotes α-syn clearance in vitro and protects C. elegans DA neurons from α-syn toxicity (Qiao et al., 2008). Furthermore, two PARK genes, PARK9 and PARK16, have been
identified which function in the lysosomal system. Mutations in the lysosomal P-type ATPase, ATP13A2 (PARK9), have been linked to inherited PD (Ramirez et al., 2006). Genetic knockdown of the C. elegans homologue of ATP13A2, catp-6, enhances α-syn misfolding and accumulation, while transgenic overexpression of catp-6 in worm DA neurons ameliorated α-syn-induced neurodegeneration (Gitler et al., 2009).

Recent identification of the PARK16 locus revealed a novel RAB7L1 mutation being associated with PD (Simón-Sánchez et al., 2009; Tucci et al., 2010). RAB7 is a member of the Rab GTPase superfamily involved in vesicle trafficking and functions at the lysosome to promote vesicle tethering and fusion. Interestingly, in yeast, RAB7 is activated by the HOPS complex component VPS39, which functions as a guanine nucleotide exchange factor (GEF), and activated RAB7 directly interacts with VPS41 in the HOPS complex to promote vesicle tethering and subsequent lysosomal fusion (Brett et al., 2008). Depletion of the Drosophila VPS41 homologue, Light, impairs lysosomal acidification and protein degradation (Swetha et al., 2011). Here, we show that overexpression of hVPS41 in human neuroglioma cells is able to reduce the number of α-syn inclusion-positive cells and decrease the level of α-syn protein, possibly through enhanced lysosomal trafficking of α-syn. Therefore, the significance of these data is further supported by identification of RAB7L1 as a genetic cause of PD.

Additional evidence supporting the role of α-syn impairment of protein trafficking has been previously reported. In yeast, overexpression of α-syn leads to profound defects in ER-Golgi trafficking that can be rescued through expressing of the Rab GTPase RAB1, which functions in the tethering and docking of ER transport vesicles to the Golgi (Cooper et al., 2006). Further analysis in mammalian and C. elegans neurons revealed two other Rab proteins, RAB3A and RAB8A, which can function to protect cells against α-syn toxicity (Gitler et al., 2008).
RAB3 functions in docking and tethering of neurotransmitter vesicles at the presynaptic terminal specifically in neurons, while RAB8 functions in post-Golgi trafficking. With VPS41 being involved in Golgi-lysosome protein transport, our data support the involvement of post-Golgi trafficking in mediating α-syn toxicity. Taken together, impaired protein trafficking throughout the cell may underlie the toxicity induced through increased α-syn expression, and by promoting protein transport machinery, cells are able to overcome this proteostatic imbalance.

Structural analysis of hVPS41 revealed that both the WD40 protein interaction and clathrin heavy-chain repeat (CHCR) domains are required for hVPS41 to protect from neurotoxicity (Figure 3.1). In contrast to previous studies (McVey Ward et al., 2001), truncation of the RING-H2 domain did not alter the neuroprotective function of hVPS41 in our assays, indicating this domain is dispensable for hVPS41-mediated neuroprotection. These structure/function data were further corroborated using a human cell culture model of α-syn inclusion formation, where both the WD40 and CHCR domain were required for hVPS41 to reduce the number of α-syn inclusion-positive H4 cells (Figure 3.4). In yeast, the WD40 domain of VPS41 has been shown to directly interact with AP-3 δ-adaptin subunit to facilitate protein trafficking between the late Golgi and vacuolar lysosome (Rehling et al., 1999), while the CHCR domain directs homo-oligomerization of VPS41 to form a clathrin-like lattice surrounding vesicles being trafficked to the vacuole (Darsow et al., 2001). As the vesicle approaches the vacuole, the HOPS (homeotypic fusion and vacuole protein sorting) docking complex, composed of VPS39 and VPS41, are recruited to the vesicle and function in vacuole fusion through activation of the yeast RAB7 homologue by VPS39 (Brett et al., 2008). Identification of the AP-3 interacting domain as being a required structural motif for hVPS41 function led us determine if the AP-3 complex was required for the neuroprotective property of hVPS41. With only one C.
elegans AP-3 complex mutant strain available, we generated worms expressing α-syn with or without hVPS41 in the DA neurons and showed that mutations in the AP-3 β-subunit (apb-3) attenuated the neuroprotective effect of hVPS41. Without worm mutants in the AP-3 δ-subunit or HOPS complex, we utilized a novel methodology to conduct cell specific RNAi through expression of the dsRNA transporter (SID-1), either in all neurons (Calixto et al., 2010) or specifically in the DA neurons, within the genetic background of RNAi resistant sid-1 mutant animals. The use of neuron-specific RNAi in animals resistant to RNAi in other cell types enables cell-specific depletion of genes that are otherwise lethal to the nematode when knocked down in all cell types. Genetic knockdown of the AP-3 δ-subunit or HOPS complex (VPS-39 and VPS-41) either pan-neuronally or selectively in the DA neurons significantly enhanced degeneration in worms expressing α-syn. In this manner, the pan-neuronal RNAi strain served to validate the results obtained using the DA neuron-specific RNAi, and also confirm that overexpression of an additional gene in the DA neurons does not simply alter the level of α-syn-induced neurodegeneration through decreased transgene expression. These data reveal the importance of Golgi-lysosomal trafficking in regulating cellular toxicity elicited by α-syn overexpression and identify the cellular pathway in which hVPS41 is acting to protect neurons against α-syn-induced neurodegeneration. Moreover, the development of transgenic nematodes enabling neuronal-specific RNAi allows for post-embryonic phenotypic evaluation of essential gene products, such as VPS-41, for which mutant animals are not viable (Lackner et al., 2005).

With the rapid advancement in genome sequencing technologies, numerous SNPs have been identified within the general human population, yet limited reports have emerged on the functional implications of such variation or their mechanistic implications for neurodegenerative, and other, diseases. In this context, the two SNPs, T146P and A187T that have been identified
within the WD40 domain of hVPS41 represent such an uncharacterized example. Here we report that when either SNP is introduced into the WD40 domain of hVPS41, the modified protein failed to protect cells against α-syn accumulation (in H4 cells) and toxicity (in C. elegans DA neurons). These results correlate to our structure/function analysis, wherein we also showed that the WD-40 domain was required to maintain neuroprotection against α-syn-induced DA degeneration. Both these SNPs have a minor allelic frequency of \( f = 0.02 \), indicating that only a small percentage of the general population contains either polymorphism. With only 5-10% of PD cases having a known genetic mutation, these SNPs may indicate susceptibility factors that possibly sensitize individuals to environmental or genetic cues promoting the onset or progression of PD. The molecular effect of these SNPs on hVPS41 may prevent hVPS41 from interacting with the AP-3 complex, recruiting of the HOPS complex, or activation of the vacuolar RAB7 to promote lysosomal fusion. Further protein interaction studies will need to be conducted to clarify the molecular mechanism underlying the functional effect of these variants. Likewise, as with the recent finding of RAB7 within the PARK16 locus, it will be interesting to determine if additional modifying polymorphisms are uncovered within this pathway as disease-associated genomic datasets expand among patient populations.

In this study, we exploited strengths of both invertebrate and cellular models of PD to functionally analyze the neuroprotective property of the lysosomal trafficking protein hVPS41. Together, these data reveal the cellular mechanism by which hVPS41 is acting to protect neurons from α-syn accumulation and toxicity and identify two SNPs in hVPS41 that may enhance the susceptibility of PD pathology within certain populations. Overall, our data support VPS41 as a potential therapeutic target for treating the progression of PD. This strategy serves as an
example by which the experimental paradigm of model systems can be expanded to evaluate functional consequences of human genomic variation and prospective disease-modifying factors.

REFERENCES


Table 3.1. Transgenic *C. elegans* were generated by microinjecting plasmid constructs into N2 hermaphrodites and crossing into other transgenic or mutant animals (See Methods). Plasmid constructs expressed in each animal and the resulting genotypes are reported for each strain.

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<th>Strain</th>
<th>Plasmid Construct</th>
<th>Genotype</th>
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<td>UA44&lt;sup&gt;A&lt;/sup&gt;</td>
<td>α-syn</td>
<td>baln11 [P&lt;sub&gt;dat-1&lt;/sub&gt;::α-syn, P&lt;sub&gt;dat-1&lt;/sub&gt;::GFP]</td>
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B: Referenced in Ruan et al, 2010.

C: A gift from Martin Chalfie (Columbia University); Referenced in Calixto et al, 2010.
Table 3.2. Primers used for cDNA amplification of hVPS41 constructs in *C. elegans*.

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Figure 3.1

A

α-syn

α-syn + VPS41

B

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<td>G</td>
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C

Worms with normal DA neurons (%)

Day 7
Day 10

α-syn alone
Isoform 1
Isoform 2
A
B
C
D
E
F
G

**

*
Figure 3.2

A

Worms with normal DA neurons (%)

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B

DA neuron-specific RNA-sensitive worms with normal DA neurons (%)

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<tr>
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C

Pan-neuronal RNA-sensitive worms with normal DA neurons (%)

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* p < 0.05
** p < 0.01
NS = not significant
Figure 3.4
Figure 3.5

A

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~102 kDa
~32 kDa
~42 kDa

B

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Figure 3.1. hVPS41 overexpression protects *C. elegans* DA neurons from α-syn-induced neurodegeneration. A. Anterior DA neurons (4 CEP and 2 ADE) in worms expressing human α-syn and GFP. Left image shows a worm with only 2 normal neurons (1 CEP and 1 ADE), where the other 4 neurons (3 CEP and 1 ADE) have degenerated. Right image shows the full complement of 6 anterior DA neurons of *C. elegans* expressing hVPS41 Isoform 1 along with α-syn and GFP, and show no evidence of degeneration. Arrows show intact DA neuron cell bodies, large arrowhead shows a degenerating neuron, and small arrowheads indicate regions where neurons have degenerated. B. Schematic diagram representing the various truncated hVPS41 protein constructs generated for functional analysis. Predicted functional protein domains were identified using bioinformatics and include the WD40, TPR-like, clathrin heavy-chain repeat (CHCR), and RING Zn finger domains. These domains were truncated to determine the minimal regions required for hVPS41 function. The FLAG epitope tag was attached to the N-terminus of each truncation. Transgenic animals were generated which express each construct in the DA neurons, and these animals were crossed into the isogenic α-syn strain, which displays age-dependent neurodegeneration. C. Overexpression of either full-length hVPS41 or two truncated forms (D and G) protects DA neurons from α-syn-induced neurodegeneration. The common domains in all protective forms were the WD40 protein interaction and CHCR domains. Worms were scored as displaying normal DA neurons when there was no degeneration observed in the six anterior DA neurons. Homozygous worms were synchronized and analyzed at days 7 and 10 post-hatching. Mean ± SD, *n* = 270 worms; *P* < 0.05, **P** < 0.01, Student’s *t*-test.

Figure 3.2. hVPS41 requires both a functional AP-3 and HOPS complex to protect DA neurons from α-syn-induced neurodegeneration. A. Worms expressing α-syn and GFP with or
without hVPS41 expression were crossed into *apb-3(ok429)* mutant animals. Resulting
homozygous animals were synchronized and analyzed at days 7 and 10 post-hatching.
Overexpression of hVPS41 protected DA neurons from degeneration in a wild-type (N2)
background, but failed to protect neurons from α-syn toxicity in an AP-3 complex mutant (*apb-
3*). Mean ± SD, *n* = 270 worms; *P* < 0.05, **P** < 0.005, Student’s *t*-test. B/C. RNAi knockdown
of components within the HOPS (VPS-39 and VPS-41) or AP-3 complex (APD-3) specifically in
the DA neurons (B) {P*dat-1::sid-1* in *sid-1* mutant [sid-1(pk3321)]} or pan-neuronally (C) {P*unc-
119::sid-1* in *sid-1* mutant [sid-1(pk3321)]} enhances α-syn-induced neurodegeneration in worms
not overexpressing hVPS41. The F1 progeny of worms exposed to RNAi feeding bacteria were
synchronized, and the DA neurons were analyzed for degeneration at days 6 and 8 post-hatching.
RNAi bacteria which do not express an RNAi clone (empty) or the VPS-28 RNAi clone were
used as negative controls. Mean ± SD, *n* = 120 worms; *P* < 0.05, **P** < 0.005, Student’s *t*-test.

*Figure 3.3.* Two SNPs found within the WD40 domain of hVPS41 alters the
neuroprotective property afforded against α-syn toxicity. A. Schematic diagram showing the
location and amino acid changes associated with two SNPs within hVPS41. Both SNPs are
located within the WD40 protein interaction domain, which is required for hVPS41-mediated
neuroprotection in worms (Figure 3.1C); the WD40 domain directly interacts with the δ-subunit
of the AP-3 complex in yeast. Both SNPs have been reported to be present in a small percentage
of the general human population and are predicted to be deleterious to the structure/function of
the protein (www.snps3d.org). B. Overexpression of hVPS41 with either SNP T146P or A187T
failed to protect DA neurons from α-syn-induced neurodegeneration. Transgenic animals
expressing either wild-type or mutant hVPS41 were crossed into isogenic α-syn worms.
Homozygous worms were synchronized and analyzed at days 7 and 10 post-hatching. Mean ± SD, n = 270 worms; *P<0.05, **P<0.005, Student’s t-test.

Figure 3.4. Overexpression of hVPS41 in human neuroglioma cells reduces the number of α-syn-inclusion positive cells. A,B. Cotransfection of H4 cells with syn/truncated GFP (synT), synphilin, and an empty vector control results in formation of cytoplasmic puncta (A, left image) which immunostain positive for α-syn in about 50% of transfected cells (B). Cells were fixed and immunostained 24 hours post transfection. Cotransfection of either isoform of hVPS41 or the two truncated constructs that were protective in C. elegans DA neurons (truncates D and G) significantly reduced the number of α-syn inclusion positive cells (A, right image), while control truncate B did not reduce the number of positive cells. When either SNP reported with the WD40 domain of hVPS41 (T146P or A187T) is present, hVPS41 is no longer able to reduce inclusion formation. Cells stained for α-syn were scored as either positive (A, left image) or negative (A, right image) for α-syn cytoplasmic inclusions, with the rater blind to each experimental condition. Each experimental condition was repeated three times with four replicas per experiment. Mean ± SD; **P<0.001, Student’s t-test. Scale bar = 20 µM. C. H4 cells transfected with synT, synphilin, and either empty vector control (left images) or FLAG-hVPS41 isoform 1 (right images) were immunostained using a primary mouse monoclonal antibody against α-syn with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (top images) or a primary rabbit monoclonal antibody against FLAG with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (bottom images). hVPS41 is expressed in cells co-transfected with synT and is diffusely localized within the cytoplasm and perinuclear region. Expression of hVPS41 reduces the number of α-syn inclusion-positive cells. Scale bar = 20 µM.
**Figure 3.5.** Overexpression of hVPS41 in H4 cells reduces the level of overexpressed α-syn/truncated GFP. A. Western blot analysis of protein levels in H4 cells transfected with synT, synphilin, and either empty vector control or hVPS41 isoform 1. 10 µg of total cell protein was resolved using SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibodies against FLAG (for hVPS41), α-syn, or actin (loading control). The normalized intensity values of synT (α-syn) are reported below the blot image. Data analysis using Multigauge software revealed ~45% decrease in synT (~32 kDa) levels when hVPS41 (~102 kDa) was being coexpressed. Mean ± SD; **P<0.001, Student’s t-test. B. Semi-quantitative rtPCR of transfected H4 cells reveal the transcriptional level of α-syn is not being altered between the empty vector control and hVPS41 expressing cells. HPRT-1 was used as an internal loading control. Each experiment was independently replicated three times.
CHAPTER 4

ANALYSIS OF NEUROPROTECTIVE GENES AND COMPOUNDS USING C. ELEGANS


Adam John Harrington collected all *C. elegans* data. Adam Harrington, Dr. Kim Caldwell, and Dr. Guy Caldwell co-wrote the manuscripts with the other authors.

ABSTRACT

Parkinson’s disease (PD) is characterized by the progressive loss of dopaminergic neurons in the brains of PD patients. While the etiology of this disease is not fully understood, misregulation of protein homeostasis as well as dysfunction of the protein degradation machineries has been implicated in the pathology of PD. Recent studies have revealed alterations of members of the 14-3-3 protein family in PD and the ability of 14-3-3s to interact with the PD-related protein alpha-synuclein (α-syn). In a transgenic α-syn mouse model, reduced expression of 14-3-30, -ε, and -γ was reported. These same isoforms functioned to
reduce α-syn inclusion formation in an H4 neuroglioma cell model as well as suppress toxicity from the PD-relevant neurotoxins rotenone and 1-methyl-4-phenylpyridinium (MPP⁺), while the other 14-3-3 isoforms were not protective against both toxins. Using a transgenic C. elegans model of PD, we confirmed the ability of both human 14-3-3 θ and a C. elegans 14-3-3 homolog (ftt-2) to protect dopaminergic neurons from α-syn toxicity, validating 14-3-3s as suppressors of neuronal cell death in vivo. Recent studies have identified that the plecomacrolide antibiotic bafilomycin can also attenuate neuronal cell death resulting from agents that disrupt lysosome function. Low-dose bafilomycin significantly attenuated SH-SY5Y human neuroblastoma cell death resulting from treatment with lysosomotropic agent as well as reduce the accumulation of detergent-insoluble α-syn oligomers resulting from chloroquine treatment. In addition, bafilomycin significantly and dose-dependently attenuated DA neuron death in C. elegans resulting from in vivo over-expression of human wild-type α-syn, supporting this small molecule as a potential mediator of neuronal cell death. Taken together, protecting neurons from cell death by maintaining protein homeostasis and/or promoting lysosomal function may provide therapeutic avenues for suppressing neurodegenerative diseases, such as PD.

INTRODUCTION

Parkinson’s disease (PD) is a neurodegenerative disorder affecting more than 4 million older individuals in the most populous nations (Dorsey et al., 2007). There is currently no treatment that slows the degenerative process. While the cause of most PD cases is unknown, substantial evidence points to a central role of the protein alpha-synuclein (α-syn). Families with point mutations or gene multiplication of α-syn exhibit autosomal dominant PD (Athanassiadou et al., 1999; Kruger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004; Singleton et
PD-related mutations in α-syn result in enhanced α-syn misfolding and accumulation, and these mutations have been reported to adversely affect protein degradation pathways, including autophagy (Conway et al., 2000; Cuervo et al., 2005; Shacka et al., 2008). In sporadic PD, α-syn aggregates are present in Lewy bodies (Irizarry et al., 1998; Spillantini et al., 1997). α-Syn overexpression in cellular and animal models leads to increased α-syn misfolding and accumulation as well as cellular injury and death (Kirik et al., 2002; Lo Bianco et al., 2002; Xu et al., 2002; Zhou et al., 2002), but the mechanisms of this toxicity are poorly understood.

In an effort to identify factors contributing to α-syn toxicity, alterations in gene expression in the substantia nigra of transgenic mice overexpressing human wildtype α-syn was previously examined (Yacoubian et al., 2008). The family of highly conserved 14-3-3 proteins was among genes altered in the brains of these α-syn overexpressing mice. There are seven mammalian 14-3-3 isoforms, comprising 1% of total brain protein, and these proteins participate in many cellular functions by mediating protein-protein interactions (Dougherty et al., 2004; Mackintosh et al., 2004). 14-3-3s play a central role in cell survival, and 14-3-3 depletion can lead to activation of pro-apoptotic factors (Porter et al., 2006). The observation that 14-3-3 expression was dysregulated in a rodent model of α-syn overexpression was of particular interest because of other evidence linking 14-3-3s to PD. 14-3-3s share structural homology with α-syn (Ostrerova et al., 1999), and co-aggregate with α-syn in Lewy bodies in human PD (Kawamoto et al., 2002) and in a mouse PD model (Shirakashi et al., 2006). 14-3-3 and α-syn can be co-immunoprecipitated from mammalian brains (Xu et al., 2002, Ostrerova et al., 1999), and co-immunoprecipitation is increased in PD brains (Sato et al., 2006). 14-3-3η is a negative regulator of the protein parkin (Sato et al., 2006), mutation of which leads to early onset PD (Kitada et al., 1998).
Together, these observations support the hypothesis that α-syn toxicity can arise from sequestration of 14-3-3 proteins, disrupting cellular signaling and liberating pro-apoptotic factors. To test this hypothesis, each 14-3-3 isoform was examined in α-syn transgenic mice and the effects of selective expression of the different isoforms in PD cellular models and in an invertebrate system was evaluated (Yacoubian et al., 2010). In α-syn transgenic mice, a significant reduction in 14-3-3θ and -γ was observed in transgenic mice as compared to wild-type mice. Using a mammalian cell culture model of α-syn inclusion formation, a significant reduction in α-syn-inclusion positive cells was reported when either 14-3-3θ, -ε, and -γ was overexpressed in these cells. Furthermore, overexpression of 14-3-3θ, -ε, and -γ suppressed toxicity from both PD-relevant neurotoxins rotenone and 1-methyl-4-phenylpyridinium (MPP⁺) in stably transfected M17 dopaminergic cells as well as reduced the increase of detergent-insoluble α-syn resulting from rotenone treatment. Interestingly, the other isoforms of 14-3-3 failed to suppress α-syn inclusion formation and rotenone toxicity to the extent of the protective 14-3-3 proteins. To validate the neuroprotective effect of 14-3-3s in an in vivo system, we overexpressed human 14-3-3θ, -ε, and -γ in our C. elegans model of α-syn-induced dopaminergic (DA) neurodegeneration. Here we report that overexpression of either 14-3-3θ or ftt-2, the closest C. elegans homologue to 14-3-3θ, was able to suppress α-syn neurotoxicity in our worm model of PD. These data reveal a potent and selective inhibition of toxicity by a subset of the 14-3-3 isoforms, especially 14-3-3θ, and support the role of certain 14-3-3 proteins in promoting cell survival in the presence of several PD-related cellular stressors.

Additionally, growing evidence suggests that the autophagy-lysosome pathway is also altered in age-related neurodegenerative diseases including PD. Alterations in autophagy were reported initially by the aberrant accumulation of autophagic vacuoles in substantia nigra
neurons of PD patients (Anglade et al., 1997). Mutations in several PD-specific genes, including α-syn, LRRK2, Parkin and ATP13A2, are known to adversely affect autophagy and/or lysosome function (Shacka et al., 2008; Pan et al., 2008). α-Syn is a major component of Lewy bodies in PD brain and α-syn accumulation is thought to play an important causal role in the onset and progression of PD. Lysosomes are important for α-syn clearance, and cathepsin D (CD) is the main lysosomal enzyme involved in the degradation of α-syn (Sevlever et al., 2008). Consistent with this hypothesis, CD deficiency has been reported to enhance α-syn toxicity (Qiao et al., 2008; Cullen et al., 2009), and several studies indicate the therapeutic potential for autophagy induction in promoting α-syn clearance in PD (Webb et al., 2003; Spencer et al., 2009; Yang et al., 2009; Yu et al., 2009). Together, these findings suggest that autophagy-targeted therapies may be effective in maintaining the proper clearance of α-syn and in inhibiting neurodegenerative disease-associated neuropathology.

Bafilomycin A1 is a member of the plecomacrolide subclass of macrolide antibiotics and was characterized initially by its selective inhibition of vacuolar type ATPase (V-ATPase) (Bowman et al., 1988). V-ATPases function to maintain the low pH of acidic vesicles through its regulation of proton pumping (Forgac et al., 2007). At concentrations $\geq 10$ nM, bafilomycin A1 effectively inhibits V-ATPase and in turn increases the pH of acidic vesicles (Yoshimori et al., 1991). However, at low concentrations ($\leq 1$ nM) which do not inhibit V-ATPase (Bowman et al., 1988) or induce autophagic vacuole accumulation (Shacka et al., 2006), the plecomacrolides bafilomycin A1, bafilomycin B1 and concanamycin all significantly attenuate chloroquine-induced death of cerebellar granule neurons (Shacka et al., 2006a; Shacka et al., 2006b), supporting bafilomycin-mediated neuroprotection is independent of its ability to inhibit V-ATPase. This result was extended in to SH-SY5Y human neuroblastoma cells, where sub-
lethal doses of bafilomycin suppressed chloroquine-induced apoptotic cell death as well as attenuated the chloroquine-induced decrease in the mature form of the lysosomal protease CD (Pivtoraiko et al., 2010). With the α-syn protein well documented to be degraded through the lysosome (Lee et al., 2004; Qiao et al., 2008; Selvever et al., 2008), an increase in detergent-insoluble α-syn was observed when the cells were exposed to chloroquine, which disrupts lysosome function and inhibits autophagy completion by raising the pH within lysosome (Zaidi et al., 2001; Boya et al., 2003; Shacka et al., 2006b). This chloroquine-induced accumulation of insoluble α-syn was attenuated when cells were exposed to bafilomycin, supporting bafilomycin in maintaining lysosome integrity and function. Additionally, we assessed the cytoprotective effects of bafilomycin in transgenic C. elegans that over-express human wild-type α-syn, which we have previously shown to induce both age- and dose-dependent DA neurodegeneration in vivo (Cao et al., 2005; Hamamichi et al., 2008). Acute exposure of these worms to sub-lethal doses of bafilomycin attenuated age-dependent α-syn-induced DA neurodegeneration. Our results indicate that bafilomycin attenuates neuronal cell death in a dose-dependent manner, consistent with its ability to maintain autophagy function and reduce α-syn neurotoxicity.

These collective studies support the predictive nature of the nematode in validating potential neuroprotective targets in an in vivo system. Using both genetic and chemical modifiers, we show the importance of maintaining protein homeostasis as well as lysosomal function in suppressing α-syn-induced DA neurotoxicity. These data demonstrate the cellular mechanisms that are affected by various PD-relevant molecules are evolutionarily conserved, supporting the nematode as a promising model for identifying and confirming potential protective candidates that can be further translated to mammalian systems.
MATERIALS AND METHODS

*Generation and analysis of transgenic 14-3-3 C. elegans.* Expression plasmids, P_{dat-1}::14-3-3\(\theta\), P_{dat-1}::14-3-3\(\gamma\), P_{dat-1}::14-3-3\(\epsilon\), and P_{dat-1}::ftt-2, as well as a marker, P_{unc-54}::mCherry, were constructed via Gateway Technology (Invitrogen) and microinjected into the gonads of *C. elegans* strain UA44 [baIn11; P_{dat-1}::\(\alpha\)-syn, P_{dat-1}::gfp] that already expresses \(\alpha\)-syn and GFP and exhibits age-dependent \(\alpha\)-syn-induced degeneration in the dopaminergic neurons (Cao et al., 2005). Three resulting independent transgenic lines for each strain UA113 [baIn11; baEx88, P_{dat-1}::14-3-3\(\theta\), P_{unc-54}::mCherry], UA114 [baIn11; baEx89, P_{dat-1}::14-3-3\(\gamma\), P_{unc-54}::mCherry], UA115 [baIn11; baEx90, P_{dat-1}::14-3-3\(\epsilon\), P_{unc-54}::mCherry], and UA131 [baIn11; baEx100, P_{dat-1}::ftt-2, P_{unc-54}::mCherry] were synchronized, grown at 20 °C, and analyzed for neuroprotection at days 7 and 10 (4- and 7-day-old adults). For each trial, 30 worms were transferred onto a 2% agarose pad and immobilized with 3 mM levamisole, and the six anterior dopaminergic neurons (4 CEP and 2 ADE dopaminergic neurons) were examined. In total, 90 animals from each of three 14-3-3\(\theta\), 14-3-3\(\gamma\), 14-3-3\(\epsilon\), and ftt-2 transgenic lines were analyzed (3 lines x 3 trials of 30 animals/trial = 270 total animals scored per 14-3-3 isoform). Worms displaying at least one degenerative change were scored as exhibiting degenerating neurons, as previously described (Cao et al., 2005; Cooper et al., 2006). The number of intact neurons per worm was also counted. To study a ftt-2 loss of function mutant, *C. elegans* knockout strain MT14355 [ftt-2(n4426)] was crossed into strain UA44 [baIn11; P_{dat-1}::\(\alpha\)-syn, P_{dat-1}::gfp] to generate the isogenic strain UA130 [baIn11; ftt-2(n4426)]. This strain was synchronized and analyzed as described above. Fluorescence microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with Endow GFP HYQ filter cube (Chroma Technology,
Rockingham, VT), and images were captured with a Cool Snap CCD camera (Photometrics, Tucson, AZ) driven by MetaMorph software (Molecular Devices, Downington, PA).

**Generation and analysis of C. elegans for bafilomycin treatment.** Isogenic *C. elegans* strain UA44 (*baln11; P_{dat-1}::α-syn, P_{dat-1}::GFP*) that co-express α-synuclein and GFP in dopamine neurons and exhibit age-dependent α-synuclein-induced neurodegeneration were age synchronized by bleaching as previously described (Lewis and Fleming, 1995) and placed in 1 ml of water containing 5% methanol (with or without bafilomycin B1) for 24h at 20°C with gentle agitation. After incubation, worms were washed with M9 buffer three times, transferred to NGM plates, and grown at 20°C. For each trial, 30 worms were immobilized with 3 mM levamisole, transferred onto a 2.5% agarose pad, and analyzed for neuroprotection at both day 7 and day 10 post-hatchings. Worms were considered wild-type (WT) when there were four intact CEP type DA neurons and two ADE type DA neurons without any signs of degeneration. Each bafilomycin B1 treatment was analyzed in triplicate (90 worms per concentration).

To determine if increased concentrations of bafilomycin B1 induced DA neuron cell death, the *C. elegans* strain BY200 (*P_{dat-1}::GFP*) (Nass and Blakely, 2003), which express GFP in the DA neurons without degeneration, was synchronized, treated with high concentrations of bafilomycin B1, and analyzed as described above. To study the effect of chloroquine on DA neurons, *C. elegans* strain UA44 was crossed into knockout strain NL131 [pgp-3(pk18)], which has been shown previously to be sensitive to chloroquine (Broeks et al., 1995), to generate the isogenic strain UA146 [*baln11; pgp-3(pk18)*]. This strain was synchronized, treated with chloroquine using methods similar to bafilomycin B1, and analyzed as described above.
RESULTS

Effects of 14-3-3s on α-syn toxicity in vivo

Previous studies using α-syn transgenic mouse models identified a reduction in the expression of several 14-3-3 proteins within the brains of these animals, specifically 14-3-3θ and -γ. Overexpression of these two 14-3-3 isoforms, as well as 14-3-3ε, was able to suppress α-syn inclusion formation in human neuroglioma cells, while the other four isoforms failed to reduce this accumulation. Furthermore, these same isoforms, which reduced α-syn inclusion formation, were also able to suppress cell death in SH-SY5Y neuroblastoma cells from the PD-relevant neurotoxins rotenone and MPP⁺. These same isoforms reduced the level of α-syn aggregation resulting from rotenone exposure (Yacoubian et al., 2010). These findings support specific 14-3-3 proteins as playing pivotal roles in PD-like neurotoxicity (Table 4.1).

While both rotenone and MPP⁺ induce α-syn aggregation in mammalian cell culture, we wished to more directly assess the potential protective effects of 14-3-3 proteins against α-syn toxicity. We turned to a C. elegans model of PD, in which human wildtype α-syn and GFP are overexpressed in dopaminergic neurons under the control of the DA transporter (dat-1) promoter. Transgenic C. elegans overexpressing human wildtype α-syn demonstrate a predictable loss of dopaminergic neurons as indicated by alterations in cellular morphology and reduction in GFP expression (Cao et al., 2005). We created double transgenic worms in which a particular 14-3-3 isoform and α-syn were overexpressed, both under the dat-1 promoter (Figure 4.1). Three separate transgenic lines were created for each 14-3-3 isoform and analyzed. We focused on 14-3-3ε, -γ, and -θ, the three human isoforms that reduced neurotoxicity in response to both rotenone and MPP⁺. In worms that overexpressed only α-syn, we found that only 6.67% of the worms had all six anterior dopaminergic neurons present at 10 days, while 13.61% of
worms that overexpressed human 14-3-3θ along with α-syn had all six dopaminergic neurons at 10 days (Figure 4.1C; p < 0.001). Likewise, the average number of intact dopaminergic neurons was significantly higher in the double transgenic 14-3-3θ/α-syn worms compared to α-syn worms (Figure 4.1D; p < 0.001). [Similar results were seen when worms were analyzed at seven days (data not shown).] Transgenic 14-3-3ε or 14-3-3γ worms did not exhibit a significant change in dopaminergic neurodegeneration (Figure 4.1C).

We also investigated whether ftt-2, the closest C. elegans homolog to 14-3-3θ, can protect against α-syn toxicity. 15.19% of transgenic worms that overexpress ftt-2 along with α-syn retained all six dopaminergic neurons, compared to only 7.78% of α-syn worms at 10 days (Fig. 4.1E; p < 0.001). Average numbers of intact dopaminergic neurons were significantly higher in the ftt-2/α-syn double transgenic worms compared to α-syn worms (Figure 4.1F; p<0.001). We also tested whether knockout of ftt-2 affected α-syn toxicity. There was no significant difference in dopaminergic cell loss between α-syn worms and α-syn/ftt-2 knockout worms at 10 days (Fig. 4.1E, F). Semiquantitative RT-PCR was used to confirm lack of ftt-2 expression (data not shown).

*Effects of bafilomycin on α-syn-induced DA neurodegeneration in vivo*

Previous work identified the plecomacrolide antibiotic bafilomycin as a modulator of chloroquine-induced cell toxicity in human neuronal cell lines (Shacka et al., 2006a; Pivtoraiko et al., 2010). Chloroquine elicits toxicity by accumulating in the lysosome to increase lysosomal pH, therefore disrupting lysosomal function and inhibiting the completion of autophagy. With the PD-related protein α-syn degraded, in part, through the lysosome and impairment of lysosomes and autophagy being implicated in the progression of PD, this protein degradation
pathway remains a prime target for regulating neuronal toxicity. Exposure of neuronal cells to low-dose bafilomycin promoted lysosomal function after chloroquine treatment and reduced the accumulation of detergent insoluble α-syn resulting from chloroquine exposure *in vitro*.

To determine if treatment with the plecomacrolide bafilomycin protected against α-syn-induced dopaminergic neurodegeneration *in vivo*, isogenic worms over-expressing human wild-type α-synuclein in DA neurons were acutely exposed to bafilomycin B1 for 24h during larval development, and subsequently scored for dopaminergic neuron loss at either 7 or 10 days post-hatching (4 and 7 day adults, respectively). Animals that were acutely exposed to 50-150 µg/ml bafilomycin B1 showed significant protection against α-synuclein-induced dopaminergic degeneration (Figure 4.2). Exposure of these worms to lower concentrations of bafilomycin B1 did not significantly protect against dopaminergic neuron degeneration at day 7 (Figure 4.2A), whereas higher concentrations induced DA neuron death in a majority of the animals. At day 10, animals exposed to either 50 or 100 µg/ml bafilomycin B1 still exhibited significant protection against neurodegeneration (Figure 4.2B). Worms lacking α-syn over-expression were also treated with bafilomycin B1 and the percentage of animals exhibiting DA neurons at either 7 or 10 days following treatment was not significantly different from vehicle control at concentrations ranging from 100-300 µg/ml (Figure 4.2C). However, treatment of worms with 400-500 µg/ml bafilomycin B1 was lethal to the embryos, thus precluding neuron counts from these worms and suggesting the importance of intact V-ATPase function for worm survival. Together these results provide *in vivo* evidence that acute exposure of bafilomycin protects dopaminergic neurons against α-synuclein-induced neurodegeneration.

Since it is possible that low-dose bafilomycin may have exerted a “pre-conditioning effect” on DA neuron survival, we sought out to determine if treatment of worms with low doses
of chloroquine also exhibited a protective effect against DA neuron death. However, since worms are naturally resistant to some natural toxins, including chloroquine (Broeks et al., 1995), we crossed α-synuclein over-expressing worms into those that were mutant for pgp-3. PGP-3 encodes a P-glycoprotein transmembrane protein that has been predicted to export toxins such as chloroquine from cells and thus increase resistance to chloroquine toxicity (Broeks et al., 1995).

We treated worms with eleven concentrations of chloroquine ranging from 0.0005-10 mg/ml (as with bafilomycin-treated worms) and assayed for protection against α-synuclein-induced DA neurodegeneration (Figure 4.2D). We did not observe any significant difference between treated or non-treated DA neurons at day 7 or 10, although there was a general trend of DA neurodegeneration following treatment with the highest concentrations of chloroquine. These results suggest that bafilomycin is specific in exerting a possible pre-conditioned effect in attenuating α-synuclein cytotoxicity.

DISCUSSION

The nematode C. elegans has proven to be a valuable tool to determine the effect of various genetic and chemical modifiers on DA neurotoxicity in vivo. By over-expressing the human PD-related protein, α-syn, in the DA neurons, we observe both age- and dose-dependent DA neurodegeneration (Cao et al., 2005). Using this model, we were able to evaluate several potential neuroprotective targets that were initially identified in mammalian PD models. Many cellular pathways and mechanisms that are associated with PD neurotoxicity are evolutionarily conserved; this enables the nematode model system to recapitulate the pathology associated with the disease. Furthermore, these conserved mechanisms enable the worm to be used to identify and validate potential neuroprotective candidates in an in vivo system that may modify the
toxicity associated with PD. Here, we reported the use of the worm to validate both genetic (14-3-3) and chemical (bafilomycin) modifiers of α-syn-induced DA neurodegeneration, which reflected the results observed in human cell culture models of PD.

14-3-3 suppresses α-syn toxicity in C. elegans

Using a diverse set of cellular and animal PD models, a remarkably consistent neuroprotective effect of several 14-3-3 isoforms was observed. In α-syn transgenic mice, a prominent mRNA down-regulation of 14-3-3ε, -γ, and -θ in the cortex was reported (Yacoubian et al., 2010). These same isoforms reduced α-syn inclusions in neuroglioma cells and attenuated rotenone and MPP⁺ toxicity in stably transfected neuroblastoma cells. We found that both human 14-3-30 and its worm homolog ftt-2 reduced α-syn toxicity in our C. elegans model of PD. The effects of the other 14-3-3 isoforms were less consistent, although structurally similar, indicating that the isoforms may act on different effector systems.

Further support for the specific relationship between 14-3-3ε, -γ, and -θ and PD pathophysiology is provided by studies of Lewy bodies in human postmortem brain. Using isoform-specific antibodies, 14-3-3ε, -γ, and -θ, as well as 14-3-3ζ, were observed in human Lewy bodies, while the other isoforms were absent (Berg et al., 2003). In addition, proteomic analysis of phosphorylation-dependent α-syn interactions revealed that 14-3-3ε, -γ, and -ζ were among those proteins that interact specifically with phosphorylated α-syn (McFarland et al., 2008).

Currently, we do not know the mechanism for the differential 14-3-3 effects observed in our studies. The different isoforms may bind to distinct ligands that mediate the differential neuroprotective effects. An important ligand may be α-syn. As noted above, a proteomic study
showed that 14-3-3 ε, -γ, and -ζ interacted with a phosphorylated α-syn peptide, while other isoforms were not detected (McFarland et al., 2008). In contrast to the other isoforms, only 14-3-3ε, -γ, and -θ showed reduced α-syn inclusion formation. A reduced level of insoluble α-syn in 14-3-3θ cells treated with rotenone was also observed. Whether or not aggregation is important to α-syn toxicity, the ability of these isoforms to interact with α-syn may be key to their neuroprotective effects. Alternatively, the differential effects of 14-3-3s may arise from differing abilities to interact with downstream effectors. For example, 14-3-3ε, -θ, and -ζ interact with Bax, while 14-3-3β does not (Nomura et al., 2003). Differential interactions of 14-3-3 proteins with other apoptotic factors could also explain their differential effects.

While 14-3-3ε, -γ, and -θ showed protection in vitro, only human 14-3-3θ overexpression reduced toxicity in the C. elegans α-syn model. It is not clear why human 14-3-3ε and -γ were not protective in this worm model. One explanation is that 14-3-3θ is more effective at reducing toxicity. Alternatively, C. elegans may not be able to process 14-3-3 isoforms of a different species effectively. Human 14-3-3θ is more homologous with the C. elegans 14-3-3 orthologs than are 14-3-3ε and -γ. Indeed, we found that the worm 14-3-3 homolog fit-2 was also effective in reducing α-syn toxicity. It is also possible that only human 14-3-3θ can effectively interact with worm apoptotic factors.

Reduced RNA expression of 14-3-3ε, -γ, and -θ was observed in an α-syn transgenic mouse model. Since these are the same isoforms that were protective when overexpressed, this observation raises the possibility that reduction of 14-3-3 expression may contribute to the progression of PD. Reducing 14-3-3θ mRNA levels directly through shRNA inhibition had no clear effect on vulnerability to rotenone. Similarly, fit-2 knockout in the C. elegans model did not promote α-syn toxicity. In both cases, other 14-3-3 isoforms could compensate for the loss
of a single 14-3-3 isoform – six others in humans, and one other, par-5, in C. elegans. We are unable to test whether knockout of both fit-2 and par-5 would promote α-syn toxicity in the C. elegans model, as loss of par-5 function is embryonically lethal (Morton et al., 2002).

We hypothesize that α-syn can disrupt 14-3-3 function through several mechanisms that may contribute to α-syn toxicity. Previously it has been suggested that the ability of α-syn to sequester 14-3-3s would promote cell death by release of apoptotic factors normally inhibited by 14-3-3s (Xu et al., 2002). We propose that α-syn also acts at a transcriptional level to reduce the expression of functional 14-3-3 proteins; decreased 14-3-3 transcription would compound the effect of α-syn on 14-3-3 sequestration by preventing injured neurons from replenishing functional 14-3-3 stores. Functional genomic analysis of our microarray data has shown that genes whose expression is altered in α-syn transgenic mice are predominantly involved in transcription (Yacoubian et al., 2008). Together, our data point to the potential therapeutic utility of treatments that enhance 14-3-3 function or expression, especially 14-3-3θ.

**Bafilomycin-mediated protection against α-syn neurodegeneration**

The plecomacrolide subclass of macrolide antibiotics, including bafilomycin A1, bafilomycin B1 and concanamycin were defined originally by their selective inhibition of V-ATPase (Bowman et al., 1988), which effectively increases the pH of acidic vesicles. Previous studies have shown that bafilomycin-mediated inhibition of V-ATPase results in the inhibition of lysosomal enzyme function and/or processing (Ishidoh and Kominami, 2002; Singh et al., 2006), induction of lysosome membrane permeabilization (Nakashima et al., 2003), inhibition of autophagic vacuole-lysosome fusion (Yoshimori et al., 1991) as well as potent inhibition of macroautophagy completion followed by induction of cell death (Shacka et al, 2006b).
contrast, results of the present study suggest that bafilomycin significantly attenuate neuronal cell death caused by lysosomotropic agents and over-expression of wild-type human α-syn, when used at concentrations that reportedly do not inhibit V-ATPase (Bowman et al., 1988) or affect the pH of acidic vesicles (Shacka et al., 2006b). Importantly, the cytoprotective concentrations of bafilomycin used in human neuronal cell culture (≤ 1 nM) did not disrupt the autophagy-lysosome pathway when used alone, and actually attenuate markers of autophagy-lysosome pathway dysfunction (decrease in CD maturation and autophagic vacuole accumulation; inhibition of autophagic flux; increase in detergent-insoluble α-synuclein) caused by the lysosomotropic agent chloroquine (Pivtoraiko et al., 2010). Together, these findings delineate a potentially novel mechanism of action for bafilomycins as autophagy-lysosome pathway preservation agents, and may serve to identify future therapeutics capable of delaying the onset and/or progression of neurodegenerative diseases including PD through their maintenance of the autophagy-lysosome pathway.

With low doses of bafilomycin able to suppress lysosomal dysfunction associated with lysosomotropic agents in mammalian cell culture, we set out to determine if bafilomycin could affect α-syn neurotoxicity in vivo using the our C. elegans model of α-syn-induced DA neurodegeneration. Bafilomycin B1 significantly attenuated dopaminergic neuron death in C. elegans following the over-expression of wild-type human α-syn (Fig. 4.2) where an inverted “U” shaped dose response curve was observed ten days after initial treatment. The maximal protective concentration of bafilomycin B1 in C. elegans was approximated at 100 µg/ml or 161 µM, a 160-fold higher concentration for optimal cytoprotection (1 nM) in cultured cells (Shacka et al., 2006a; Shacka et al., 2006b; Pivtoraiko et al., 2010). However, C. elegans has a protective cuticle layer that most likely compromised diffusion and penetration of bafilomycin B1, an effect
that is well characterized for other compounds (Rand and Johnson, 1995; Holden-Dye and Walker, 2007). In addition, the amount of active bafilomycin B1 capable of affecting dopaminergic neurons in *C. elegans* may be further lowered upon metabolism within the worm, as has also been demonstrated previously with other compounds (Rand and Johnson, 1995), thus necessitating a higher effective concentration range than optimal for cultured cells. Over-expression of wild-type *ATP13A2*, a gene expressing a lysosomal ATPase and mutations in which are associated with a juvenile-onset hereditary parkinsonism (Klein and Lohmann-Hedrich, 2007), attenuates neuron death induced by α-syn over-expression in *C. elegans* (Gitler et al., 2009), further implicating the importance of intact lysosome function in regulating α-syn-induced neurotoxicity. Thus, it is conceivable that bafilomycin B1 protected against over-expression of wild-type α-syn in *C. elegans* in part through its preservation of lysosomal function and promotion of α-syn clearance. Importantly, results in *C. elegans* suggest that bafilomycin attenuates dopaminergic neuron death following a stimulus (α-syn over-expression) that is, on one hand distinct from treatment with lysosomotropic agents *in vitro* yet may produce the same end result (disruption of the autophagy-lysosome pathway).

The molecular target for low-dose bafilomycin-mediated neuroprotection remains unresolved, since previous findings predict that it is independent of V-ATPase inhibition (Shacka et al., 2006b; Pivtoraiiko et al., 2010). One potential target is hypoxia inducible factor (HIF)-1α, which has been shown previously to compete with Von Hippel-Lindau tumor suppressor protein for binding to the c subunit of the V0 sector of V-ATPase (ATP6V0C) in a pH-independent manner (Lim et al., 2007). HIF-1α is well known to regulate the trans-activation of many different genes, including heme oxygenase-1, an enzyme complex shown recently to play an important role in the degradation of wild-type α-syn (Song et al., 2009) and in regulating the
autophagy-lysosome pathway (Zukor et al., 2009). Thus bafilomycins may regulate neuronal survival through maintenance of the ALP that is in part independent of V-ATPase inhibition. Taken together, promoting lysosome function as well as maintaining the autophagy-lysosome pathway using low-doses of bafilomycin may support cell survival and protein homeostasis in the presence of increased expression of toxic proteins, such as α-syn.

This study using the nematode model system, C. elegans, provides further validation for gene and drug targets initially identified in human neuronal cell culture, in that the neuroprotective property of both 14-3-3θ and the small molecule bafilomycin are able to suppress α-syn-induced DA degeneration in vivo. While the neuroprotective mechanisms of these candidates have not been completely revealed, our data support these targets for further analysis as potential therapeutic avenues for suppressing the progression of PD.

REFERENCES


Table 4.1. Effects of 14-3-3 proteins on PD-related studies.

<table>
<thead>
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Figure 4.1

(a) Image showing nervous system with arrows indicating different structures.

(b) Image showing nervous system with arrowheads highlighting specific areas.

(c, d, e, f) Bar graphs showing percentage of wild-type worms and neurons across different conditions.

- (c) Comparison of α-syn, 14-3-3α, 14-3-3ε, and 14-3-3γ on percentage of wild-type worms.
- (d) Comparison of α-syn and 14-3-3ε on percentage of wild-type neurons.
- (e) Comparison of α-syn, fit-2 (OE), fit-2 knockout on percentage of wild-type worms.
- (f) Comparison of α-syn, fit-2 (OE), fit-2 knockout on percentage of wild-type neurons.
FIGURE LEGENDS

Figure 4.1. Overexpression of 14-3-3θ protects against dopaminergic cell loss in the α-syn transgenic C. elegans worm model. Expression plasmids, Pdat-1::human 14-3-3θ and Punc-54::mCherry, were constructed and microinjected into the gonads of C. elegans strain that already expresses α-syn and GFP (Pdat-1::α-syn; Pdat-1::GFP) and exhibits age-dependent α-syn-induced degeneration in dopaminergic neurons. Similar double transgenic worms were created to overexpress human 14-3-3ε, human 14-3-3γ, or the worm 14-3-3 homolog ftt-2. In addition, a mutant fit-2 knockout that overexpressed α-syn was also created. A. α-Syn worm shows loss of dopaminergic neurons (only 2 CEP neurons and 1 ADE neuron remain) at Day 7. Arrowheads show intact dopaminergic neuron cell bodies. Lined arrows indicate areas where dopaminergic neurons have degenerated. B. Overexpression of 14-3-3θ in the α-syn overexpressing line protects dopaminergic neurons from α-syn-induced cell death. Arrowheads show six intact dopaminergic neuron cell bodies. C,D. 14-3-3θ reduced dopaminergic cell loss, but 14-3-3ε and -γ did not. The numbers of α-syn transgenic worms and α-syn/14-3-3 double transgenic worms that had the full complement of six anterior dopaminergic neurons were scored at Day 10 (C). The percentage of intact dopaminergic neurons per worm was also scored at Day 10 for α-syn and α-syn/14-3-3θ transgenic worms (D). E,F. Overexpression (OE) of the worm 14-3-3 homolog fit-2 also reduced dopaminergic cell death, but fit-2 knockout did not enhance neurodegeneration. The percentage of worms that had the full complement of dopaminergic neurons (E) and the percentage of intact dopaminergic neurons per worm (F) were scored at Day 10. For each experiment, three independent transgenic lines were scored, with 30 worms per line analyzed in triplicate experimental trials. Plotted data reflects the average of these three separate
lines. ***p<0.001 (Bonferroni’s multiple comparison test). Error bars reflect standard deviation.

**Figure 4.2.** Bafilomycin attenuates the death of DA neurons in *C. elegans* following over-expression of wild-type human α-syn. A,B. Worms over-expressing α-syn in DA neurons were acutely exposed to bafilomycin B1 (BafB1, 0-200 µg/ml) for 24h during larval development, and then subsequently scored for DA neuron loss at either 7 days (A) or 10 days (B) post-hatching (4 and 7 day adults, respectively). C. Worms over-expressing GFP (but not α-syn) in DA neurons were acutely exposed to 0-500 µg/ml BafB1 for 24h and then scored for DA neuron loss as above (A,B). Note that the 400-500 µg/ml concentrations of BafB1 were lethal to *C. elegans* embryos thus precluding neuron counts from these worms. D. Worms over-expressing α-syn in DA neurons were crossed into worms mutant for *pgp-3*, a strain of worms that enhances CQ toxicity due to a predicted decrease in toxin export from cells. Worms were treated with 0.0005-10 mg/ml chloroquine and assessed for DA neuron loss as above for BafB1 (A,B). Treatment with chloroquine at any concentration did not alter the percentage of worms exhibiting WT DA neurons. Results represent mean ± SD obtained from at least three independent experiments, where 30 worms were analyzed for each experiment (n=90). *p<0.05 vs. 0 µg/ml vehicle CTL.
CHAPTER 5
CONCLUSION

The conclusion and future directions section was published in Developmental Dynamics, May, 2010 under the following citation: Harrington A.J., Hamamichi S., Caldwell G.A., Caldwell K.A. (2010) Dev Dyn 238:1282-1295. Adam Harrington, Dr. Shusei Hamamichi, Dr. Guy Caldwell, and Dr. Kim Caldwell co-wrote the manuscript.

The power of the C. elegans model system lies in the ability to genetically alter this organism to determine cellular effects of certain genes and/or chemicals in response to various environmental or cellular stresses. Through genetically overexpressing human α-synuclein (α-syn) and GFP specifically in the dopaminergic (DA) neurons of these microscopic worms, both age- and dose-dependent DA neurodegeneration is observed in vivo. Furthermore, exposure of worms to the DA neuron-specific toxin 6-hydroxydopamine (6-OHDA) induces DA neurotoxicity. These established neurodegeneration phenotypes provide a model for studying the pathology associated with Parkinson’s disease (PD) as well as identify factors that may suppress DA neurotoxicity in an in vivo animal model of PD. Through genetic manipulation of these animals using RNAi, mutant analysis, and transgenic overexpression, these models have identified and validated many candidates which are neuroprotective across species boundaries, strongly supporting the use of C. elegans as a powerful tool for studying the pathology
associated with PD. Recent studies using *C. elegans* PD models have uncovered new avenues that can be harnessed to examine neuropathology associated with PD.

*Neuroprotective mechanism of VPS41*

Through a large-scale RNAi screen to identify effectors of α-syn misfolding and α-syn-induced neurodegeneration, *C. elegans* VPS-41 was identified as a top candidate that modified α-syn toxicity (Hamamichi et al., 2008). As described in Chapter 2, we demonstrated that the neuroprotective function of VPS-41 is evolutionary conserved amongst species, in that overexpression of the human VPS41 (hVPS41) could also suppress DA neurotoxicity elicited by the neurotoxin 6-OHDA and overexpression of human α-syn. While Vps41 has been extensively characterized in yeast, few studies have addressed the functions of VPS41 in other systems, including *C. elegans*, *Drosophila*, and mammals, and especially not in a neuronal context. In *C. elegans*, mutations lead to defects in lysosome-related gut granule formation, increases in germline apoptosis, and embryonic lethality, indicating this protein is required for lysosome formation and organism survival (Hermann et al., 2005; Lackner et al., 2005). In yeast, Vps41 regulates the trafficking of AP-3 coated vesicles from the Golgi to the vacuole (yeast equivalent of lysosomes) via the alkaline-phosphatase (ALP) pathway (Rehling et al., 1999). The ALP pathway transports Golgi-derived vesicles directly to the vacuole, bypassing the early, sorting endosome (Darsow et al., 2001).

*Drosophila* studies identified Light as the fly homologue of VPS41 and determined that it mediates post-Golgi trafficking of lysosomal proteins, including the lysosome associated membrane protein (LAMP1), a vacuolar proton pump (V0-ATPase), and the cholesterol transport protein NPC1 (Swetha et al., 2011). These proteins function in maintaining lysosomal
morphology/fusion, the acidic environment found within the lysosomes which promote protein degradation, and cholesterol homeostasis within cells. These data can be correlated with defects in protein degradation machineries, specifically the lysosomal system, that have been implicated in PD. For example, mutations in the lysosomal P-type ATPase, ATP13A2 (PARK9) have been linked to familial PD (Ramirez et al, 2006), and mutation in the α-syn locus are reported to inhibit the autophagy-lysosome pathway (Cuervo et al., 2004). Furthermore, overexpression of the C. elegans homolog of PARK9 suppresses α-syn toxicity in worm DA neurons (Gitler et al., 2009). With chaperone-mediated autophagy and macroautophagy being largely responsible for degrading misfolded and aggregated protein, including α-syn, promoting lysosomal function may aid in suppressing PD-related neurotoxicity. Emerging studies of mitochondria dysfunction associated with PD has supported the role of the autophagy-lysosome system in promoting the clearance of damaged mitochondria through mitophagy (Zhu and Chu et al., 2010). High cholesterol has also been recently linked to increased plaques within brain tissue of Alzheimer’s disease patients (Matsuzaki T, et al., 2011), suggesting that cholesterol homeostasis plays a role in neurodegenerative diseases. This further supports the hypothesis the involvement of VPS41-mediated trafficking of post-Golgi vesicles in the maintenance of cellular function. Since VPS41 has been shown to regulate trafficking of proteins required for regulating lysosomal function in Drosophila, VPS41 may suppress α-syn toxicity by promoting lysosomal integrity and function as well as maintaining sterol accumulation within cells to promote cell survival.

Further support for the hypothesis that VPS41-mediated lysosomal trafficking might perform a pivotal role in PD-related neurotoxicity was that RAB7L1 was identified within the PARK16 locus, with mutations in RAB7L1 identified in PD patients (Simón-Sánchez et al., 2009; Tucci et al., 2010). In yeast, VPS41-mediated lysosomal fusion of AP-3 vesicles requires
Rab7 (Cabrera et al., 2009; Hickey et al., 2009). As the AP-3 vesicles approach the vacuole, Rab7 is activated by the HOPS complex protein Vps39, and then activated Rab7 directly binds to Vps41 to promote t-SNARE complex formation and subsequent vacuolar fusion (Wurmser et al., 2000; Brett et al., 2008). Interestingly, when we knocked down rab-7 or the HOPS complex components vps-39 or vps-41 in our DA neuron-specific RNAi strain, we observed enhanced α-syn induced neurodegeneration (Chapter 3; unpublished observation). While the RAB7 interaction domain has not been identified in VPS41, it would be interesting to determine if the human SNPs alter the interaction of activated RAB7 with VPS41. This issue can be addressed by introducing the polymorphisms into RAB7 and determining if the proteins can still co-localize within cells. Alternatively, protein interaction can be accessed through co-immunoprecipitation (Co-IP) methods. When introducing the PD-associated SNP into RAB7, an epitope tag can be attached to the protein, which would allow for specific isolation of the mutated protein. With the epitope tag attached to RAB7, we can immunoprecipitated the protein from transfected cell lysates using epitope-specific agarose beads and immunoblot using a VPS41-specific antibody after separation by SDS-PAGE. It would be interesting if wild-type RAB7 was able to pull down VPS41 while the PD-related mutation in RAB7 prevents this interaction.

Previous studies using various model systems have identified several Rab proteins as functioning in α-syn proteotoxicity, including RAB1, RAB3, and RAB8 (Cooper et al., 2006; Gitler et al., 2008). These proteins function in various aspects of intracellular protein trafficking, including ER to Golgi transport (RAB1), exocytosis (RAB3), and post-Golgi transport (RAB8). These regulatory proteins may function to overcome the cellular defects elicited by increased expression of α-syn. With overexpressed α-syn affecting multiple intracellular pathways, these
trafficking proteins represent promising targets for maintain cellular homeostasis in the presence of toxic protein species. Identifying additional RAB proteins which can inhibit these cellular alterations would be beneficial to determine which additional pathways may be defective with high levels of α-syn, and introduce new avenues in which therapeutics may be developed to inhibit the progression of synucleinopathies.

As described in Chapter 3, we determined that two domains of VPS41 are required for the neuroprotective function of hVPS41 in our C. elegans model of α-syn-induced DA degeneration [WD-40 protein interaction and clathrin heavy-chain repeat (CHCR) domains], while the TPR-like and RING finger domains are dispensable for this neuroprotection. This result was validated in human cell culture where these same domains are required to reduce α-syn inclusion formation and accumulation. In yeast, the WD-40 domain was shown to directly interact with the δ-subunit of the AP-3 complex, while the CHCR domain homo-oligomerizes to form a clathrin-like lattice surrounding these AP-3 vesicles, which are transported to the vacuole (Darsow et al., 2001). Using both mutant analysis and a novel DA-neuron specific RNAi strain, we showed that the AP-3 complex was required for hVPS41-mediated neuroprotection and appears essential for functioning in suppressing basal levels of α-syn neurotoxicity. While the cargo being trafficked in these AP-3 vesicles has not been evaluated in the context of α-syn toxicity, it would be interesting to determine which proteins being trafficked to the lysosome via the AP-3 pathway are affecting α-syn toxicity. This can be achieved by isolating AP-3 vesicles from cells and analyzing the composition of the cargo being trafficked within these vesicles using mass spectrometry. This method will identify putative proteins that are transported within these vesicles. After determining potential candidates, these proteins can be further studied using co-localization techniques to determine if these proteins are found within these vesicles.
The identification of additional proteins being transported within the AP-3 vesicles may identify additional proteins required for maintaining lysosomal function and protein homeostasis.

Our results with VPS41 support the hypothesis that this protein can function to suppress α-syn toxicity via AP-3 vesicle trafficking of post-Golgi vesicles to the lysosome. In yeast, Vps41 is regulated by the phosphorylation of Yck3, the yeast type 1 casein kinase. The phosphorylation state of Vps41 alters the function of the protein, where phosphorylated Vps41 functions in AP-3 vesicle trafficking while the non-phosphorylated Vps41 functions in endosomal fusion with the lysosome (LaGrassa and Ungermann, 2005; Cabrera et al., 2009; Cabrera et al., 2010). Previous studies using worms showed that overexpression of casein kinase was able to suppress α-syn toxicity (Hamamichi and Caldwell, unpublished observation). Using our DA neuron-specific RNAi strain, we found that genetic knockdown of csnk-1 (C. elegans type 1 casein kinase) enhanced α-syn-induced DA neurodegeneration (unpublished observation). Bioinformatic databases [Scansite Motif Scanner (scansite.mit.edu/motifscan_seq.phtml); NetPhos 2.0 (www.cbs.dtu.dk/services/NetPhos/)] indicate a potential casein kinase 1 phosphorylation site within the WD-40 domain of hVPS41 at threonine 323 (T323), which is localized in the same region as the yeast casein kinase phosphorylation site. Using site-directed mutagenesis, we converted T323 to aspartate (T323D) or alanine (T323A) to create a phosphomimetic or non-phosphorylated hVPS41 construct, respectively. Interestingly, the phosphomimetic hVPS41 (T323D) construct was able to suppress both α-syn-induced degeneration in C. elegans, as well as α-syn accumulation in human neuroglioma cell lines (unpublished observation). While this potential phosphorylation site has not been verified, mutations at T323 affected the function of hVPS41 in our assays. It should be noted that the intracellular localization of hVPS41 was altered when T323 was changed. The hVPS41 T323A
construct showed similar cytoplasmic localization of wild-type hVPS41, while the hVPS41 T323D construct was found highly expressed in a juxta-nuclear region (unpublished observation). These results support the hypothesis that T323 is a potential phosphorylation site because the neuroprotective mechanism and the localization of hVPS41 are altered when this threonine is changed. In order to validate this site as a potential casein kinase 1 phosphorylation site, several different approaches can be taken. With the VPS41 construct having a FLAG-epitope tag attached to them, the protein can be isolated from transfected cell lysate by IP. After isolation, the protein should be separated by SDS-PAGE and transferred to a PVDF membrane. The membrane can then be probed using a phospho-threonine-specific antibody to detect proteins with phosphorylated threonines. If a band is present at the correct protein size, this would support that a threonine in VPS41 is phosphorylated. To confirm T323 is phosphorylated, the same method could be applied using the non-phosphorylated (T323A) mutant, where the phospho-threonine antibody would not recognize this protein. Alternatively, mass spectrometry can be utilized on the isolated protein to determine if there is an increase in the mass of the protein. To validate this site is phosphorylated by casein kinase 1, treating cells with casein kinase 1 inhibitors [CKI-7 (Sigma-Aldrich), D4476 (Tocris), or DRF053 (Tocris)] should inhibit this phosphorylation. Further verification of T323 as the casein kinase 1 phosphorylation site is currently being conducted in the Caldwell lab.

In Chapter 3, it was shown that the TPR-like and RING finger domains are not required for the neuroprotective function of hVPS41. While the yeast Vps41 does not contain this RING finger domain (Radisky et al., 1997), the C-terminal RING domain in human VPS41 was proposed to mediate membrane interaction, and this domain is present in only one isoform of hVPS41 (McVey Ward et al., 2001). However, upon further sequence analysis, we identified
that the two human isoforms of VPS41 differ by only 25 amino acids located within the N-terminal WD-40 domain (Ruan et al., 2010). While the function of the two isoforms has not been deciphered, we determined that both isoforms can suppress α-syn-induced neurodegeneration in our worm α-syn model, as well as suppress neurotoxicity in mammalian cell culture (Ruan et al., 2010). In contrast to the localization variance when the RING domain is absent (McVey Ward et al., 2001), we did not observe subcellular localization differences between the two hVPS41 isoforms or the hVPS41 constructs missing the C-terminal RING domain, indicating proper localization is maintained with these variants and deletion constructs.

With the advancement of human genome sequencing, multiple single nucleotide polymorphisms (SNPs) have been identified within the general human population, with certain SNPs being associated with disease. Some SNPs have been linked to PD, including mutations in α-syn, LRRK2, Parkin, and ATP13A2, to name a few. While most SNPs result in missense mutations that do not affect the function of the protein, some SNPs can alter protein structure and/or function. To determine if any potentially deleterious SNPs are present in hVPS41, we evaluated several SNP databases. Six SNPs have been identified across the domains of VPS41 (Figure 5.1), although most have not been correlated with changes in the human populations and might be sequencing artifacts. One SNP (H842R) was calculated to be present in ~42% of the population, but was present in the C-terminal RING domain, that I had previously determined to not be important for VPS41-mediated neuroprotection against a-syn-induced neurodegeneration. Therefore, I did not analyze this SNP. However, two other SNPs (T146P and A187T) were predicted to cause non-synonymous amino acid changes and both of these were found within the WD-40 domain of hVPS41, which we showed to be required for the neuroprotective function of hVPS41. These SNPs change functional groups of amino acids within the protein, and we find
that the presence of either SNP prevented VPS41 from suppressing α-syn toxicity or accumulation in either *C. elegans* or mammalian cell culture, respectively. While these SNPs have not been previously linked to disease, our functional data supports these mutations as potential genetic susceptibility factors for PD. Genomic analysis of hVPS41 in PD patients has been proposed to determine if there is a correlation of these SNPs with the prevalence of PD. If these particular SNPs are not correlated with PD, there could be other genetic lesions within VPS41 associated with PD. Thus, it would be prudent to examine the entire VPS41 genomic region for changes in PD populations versus controls. Although mutations in hVPS41 have not yet been associated with disease, mutations in the AP-3 complex are associated with Hermansky-Pudlak syndrome (HPS). HPS associated mutations in the AP-3 complex lead to defects in cell-type-specific organelles of the lysosomal system, specifically melanosomes and platelet dense granules, resulting in reduced pigmentation and prolonged bleeding in HPS patients (Starcevic et al., 2002), implicating this complex in maintaining lysosomal function. With the WD-40 domain interacting with the AP-3 complex, it would be informative to determine if the presence of either SNP inhibits hVPS41 from interacting with the AP-3 complex or if these SNPs are associated with lysosomal dysfunction diseases.

*Additional PD-related studies using C. elegans*

With the lysosomal system representing a prime target for maintaining protein homeostasis, in Chapter 4 we describe that low doses of the plecomacrolide antibiotic bafilomycin, which inhibits the function of V-ATPase at high concentrations (Ishidoh and Kominami, 2002), was able to suppress α-syn-induced neurodegeneration in our worms. While inhibition of the V-ATPase inhibits lysosomal function, the neuroprotective concentrations used
in our assays did not inhibit the V-ATPase, indicating this molecule has an alternative function that can suppress lysosomal dysfunction. In cultured neuroblastoma cells, low doses of bafilomycin promoted the maturation of cathepsin D (CD) (Pivtoraiko et al., 2010), which is the main lysosomal protease responsible for α-syn degradation (Sevlever et al., 2008). The relative expression level of CD significantly impacts the level of α-syn aggregation and toxicity observed in both mammalian cell culture and C. elegans (Qiao et al., 2008), further supporting lysosomal function as a regulator of α-syn toxicity. Furthermore, dysfunction of the lysosomal system promotes exosome-mediated release of α-syn in neuroblastoma cells, which can transmit pathological α-syn from affected neurons to healthy neurons (Alvarez-Erviti et al., 2011). With VPS41 reported to mediate the trafficking of V-ATPases to the lysosome, and the importance of the lysosomal system in regulation α-syn degradation, our results further support hVPS41 as functioning in maintaining lysosomal activity to suppress α-syn-induced neurotoxicity.

Additional experiments to address if VPS41 is functioning in a similar pathway as bafilomycin in regulating the function of V-ATPase would be to determine if by increasing VPS41 trafficking of AP-3 vesicles (including V-ATPases) we could alter the concentration of bafilomycin used to elicit protection against α-syn toxicity. Theoretically, by enriching V-ATPase at the lysosome through overexpressing VPS41, bafilomycin would elicit an enhanced protective response against lysosomal dysfunction. Also, determining if the acidity of lysosomes is affected by VPS41 overexpression and/or bafilomycin treatment would support lysosomal function in suppressing α-syn toxicity, which can be achieved by using lysosomal dyes [quinacrine; LysoSensor probes (Invitrogen)] which accumulate in acidic vesicles (lysosome) dependent on pH.
As reported in Chapter 2, hVPS41 suppressed the apoptotic cascade resulting from exposure of human neuroblastoma cells to neurotoxins commonly used to recapitulate PD-like neurotoxicity, including rotenone and 6-OHDA. With apoptosis resulting from lysosomal dysfunction, suppressing the apoptotic cascade may provide an alternative means for protecting cells from toxicity. Using our worm model of PD, we validated the role of 14-3-3 proteins in suppressing α-syn-induced DA neurodegeneration in vivo in Chapter 4 (Yacoubian et al., 2010). A proteomic study identified several 14-3-3 isoforms that can interact with phosphorylated α-syn peptide, as well as interact with pro-apoptotic factors to suppress their activity (Nomura et al., 2003). While differential neuroprotective effects were reported with the various 14-3-3 isoforms, certain isoforms which inhibit pro-apoptotic factors may be suppressed in the presence of α-syn, where these 14-3-3 proteins are bound to phosphorylated α-syn instead of pro-apoptotic factors, resulting in induction of apoptosis.

Conclusion and future directions

In this dissertation, we have provided evidence for the use of the model organism C. elegans to identify putative pathways involved in the cellular pathology seen within PD patients. Indeed, C. elegans provides immense opportunity for conducting chemical and genetic screens to identify factors that may be influencing certain pathways implicated in a variety of diseases (Kamath and Ahringer, 2003). Examples of large-scale RNAi screens, genetic mutant, and transgenic nematode analyses to identify and characterize genetic factors that influence several main pathological features of PD, described in the Introduction (Chapter 1), represent prime examples of the utility of the nematode system for advancing PD research (Hamamichi et al., 2008; Kuwahara et al., 2008; van Ham et al., 2008). Through these screens, several proteins
involved in lysosomal trafficking, autophagy, mitochondrial function, and aging have been identified to protect against α-syn accumulation and toxicity. While α-syn regulation and degradation may play a pivotal role in the pathogenesis of PD, alterations of the mitochondria have also been shown to play a significant role in the progression of this disease. Therefore, the application of *C. elegans* model toward identifying potential therapeutics that restore a homeostatic function to the mitochondria could be used to prevent the pathology seen with either environmental or genetic contributors to PD. Alternatively to restoring mitochondrial function, promoting the clearance and degradation of dysfunctional mitochondria through mitophagy could be a potential mechanism to suppress cytotoxicity. Our findings identify VPS41 for suppressing rotenone and 6-OHDA toxicity, which lead to mitochondrial dysfunction, supporting the role for lysosomal function in suppressing cell death due to malfunctioning mitochondria.

As PD research advances, a new and emerging field of biology is the identification of micro RNAs (miRNA), small 21-23 bp RNA fragments that function within cells to modify gene expression (Figure 5.2). These miRNAs are originally transcribed as ~1000 bp fragments which are processed by the RISC complex to form mature miRNAs (Moss and Poethig, 2002); these mature miRNAs have been shown in mammalian systems to regulate neuronal differentiation, neurite outgrowth, survival, and synaptic function. Interestingly, Kim et al. (2007) reported a decrease in *mir-133b* expression within the midbrain of patients with PD, which regulates the maturation and function of DA neurons through altering the transcription factor Pitx-3. Furthermore, *mir-7* was recently identified to target the 3’ UTR of α-syn and suppress α-syn protein expression (Junn et al., 2009). These findings only scratch the surface regarding the prospective importance of miRNA regulation of gene expression in PD, and are indicative of an exciting field for future exploration. Significantly, the nematode offers valuable tools in which
the regulation of genes by miRNAs can be examined, especially in respect to disease. With many genes identified through genetic screens in *C. elegans* to modify disease etiology and the availability of numerous bioinformatics databases that identify putative miRNA gene targets (miRBase, TargetScan, etc.), an interesting avenue to be pursued is the identification of miRNAs that may co-regulate several potential protective genes or molecular pathways in a coordinated manner. Another distinct advantage in studying the effect of miRNAs on the pathogenesis of disease in *C. elegans* is the rapidity with which creating transgenic animals overexpressing miRNAs or crosses with mutants of specific miRNAs may alter the toxicity seen with α-syn expression can be performed. Interestingly, several miRNAs have been predicted to regulate VPS41 expression, including miR-2, miR-251, and miR-797, in numerous miRNA prediction sites (TargetScan, MicroCosm, and MiRanda). Through mutant analysis, our lab has previously identified that knockout of miR-2 and miR-797 suppresses α-syn-induced DA degeneration, while knockout of miR-251 enhances α-syn neurotoxicity (Hamamichi, Dexter, and Caldwell, unpublished observation). With these miRNAs predicted to regulate numerous genes including VPS41, identification of the exact target transcripts which are being regulated by these miRNAs would be beneficial in determining which cellular pathways may be functioning in suppressing α-syn pathology.

While both genetic and environmental factors have been identified as being causative agents in the onset and progression of PD, the most common and undisputed risk factor for PD is aging (Figure 5.2). Currently, many advances in understanding the mechanisms of aging have come through work in *C. elegans* where several genes have been identified that significantly alter the lifespan of the worm. Interestingly, van Ham et al. (2008) identified the age-associated gene, *sir-2.1*, in their genome-wide screen for effectors of α-syn accumulation. This gene acts as
a histone deacetylase to regulate the expression of various genes, and it has been shown to function in the well-studied aging pathway involving the insulin-like receptor (*daf-2*) along with the downstream effector of the insulin-signaling pathway, the FOXO transcription factor, *daf-16*. Mutations within the *daf-2* gene promote the activation of DAF-16, which is transported to the nucleus where it, along with the SIR-2.1 protein, initiates transcription of genes that regulate a variety of cellular pathways including cellular stress-response, metabolic, and autophagy-related genes (Murphy et al., 2003; Lee et al., 2008). Additionally, proteolytic activity declines during aging, and increased levels of intracellular damaged proteins have been extensively observed in aged organisms due to decreased proteolysis (Martinez-Vincente et al., 2005). Inhibition of lysosomal proteases using leupeptin enhanced neurodegeneration in an AD mouse model, supporting the role of lysosomal function in age-related neurodegenerative disease (Nixon et al., 2001). However, the relationship between genes involved with the aging pathway and how they may be relevant to the progression of neurodegenerative disease has not been well defined and remains an open avenue to be further exploited in *C. elegans*.

With the sequencing of the human genome being complete, there has been identification of many single nucleotide polymorphisms (SNPs) throughout the general population, which accounts for 0.1% of the diversity within humans. These SNPs are single base pair changes within the genome which may significantly alter the function of the protein, by altering transcription, splicing, or protein coding or may have an effect on gene expression or transcript stability by being located outside the coding region of a gene. While many SNPs do not necessarily have an effect on an encoded amino acid sequence for a protein, several SNPs have been identified to be either causative agents or susceptibility factors for disease. With many non-synonymous SNPs identified in the population, the need to study the functional effect of these
mutations on the normal activity of a given protein is an avenue to further be explored. Previous genome-wide screening with *C. elegans* to identify effector genes of α-syn toxicity, either enhancers or suppressors, have revealed many candidates that may play a role in the pathogenesis of PD. Considering the wealth of candidate genes identified, an interesting direction of research would be to determine if these genes have verified SNPs within human populations, and if these SNPs alter the function of the respective protein, by either enhancing or reducing the functionality in *C. elegans* PD models (Figure 5.2). Identification of detrimental SNPs within genes, as well as screening PD patients for these polymorphisms, may represent genetic susceptibility factors for the occurrence of this disease.

While continued identification of genetic factors that may contribute to the progression of PD is critical, environmental factors have been shown to play an important role in the etiology of this disease. In this regard, in addition to recapitulation of pre-existing mammalian toxin models that enhance the progressive loss of DA neurons, including MPP+ and 6-OHDA, *C. elegans* represents an indispensable tool for potential identification of toxins which induce neuronal degeneration. The recent report by Caldwell et al. (2009) describing a novel secondary metabolite produced by a species of common soil bacteria that can induce neurodegeneration within *C. elegans*, an effect that is enhanced in the presence of dopamine, represents one such application of the model. Therefore, continued use of *C. elegans* to identify environmental toxins and study mechanisms by which these toxins contribute to neuronal cell death may provide greater understanding of the etiology of this disease (Figure 5.2). It would be interesting to determine if overexpression of VPS41 is neuroprotective against the bacterial toxin and/or if depletion of *vps-41* in the dopaminergic neurons exacerbates the neurodegenerative effect observed following exposure to the toxic species.
Additionally, as an intact microscopic animal, one of the greatest advantages of *C. elegans* is the ability to conduct large scale screening of chemical compounds which may alter disease state within a living organism (Figure 5.2). Previous studies with nematodes have identified small molecules that prevent or slow the onset and progression of disease through enhancing or inhibiting the activity of proteins or molecular pathways. Braugart et al. (2004) developed a *C. elegans* MPP+ model of PD to be used for high-throughput drug screening and verified their model by treating these worms with pharmacological compounds that are currently being used in therapy for treatment of PD and have protection against MPP+ toxicity. Marvanova and Nichols (2007) conducted a small molecule screen to identify modifiers of 6-OHDA-induced DA neurotoxicity and identified two D2 receptor agonists that protected against cell death. Furthermore, Cao et al. (2009) recently used a series of assays in *C. elegans* to identify an established drug which functions to enhance the molecular chaperone-like activity of human torsinA, a gene product that has previously been shown to protect against α-syn-induced DA neurodegeneration *in vivo* (Cao et al., 2005). As described in Chapters 2 and 3, overexpression of VPS41 is neuroprotective in three commonly studied PD models (6-OHDA and rotenone exposure as well as α-syn overexpression). Therefore, a logical step for PD therapeutic development is the identification of a small molecule that acts on VPS41 either by enhances activity or stabilizing endogenous levels of the protein. With VPS41 promoting the clearance of overexpressed α-syn protein, screening could be performed using cell culture models with stable overexpression of VPS41 to identify small molecules which enhance VPS41-mediated degradation of α-syn, compared to cells which are not overexpressing VPS41. Any potential molecules identified to modify α-syn levels in cells dependent on VPS41 can be further
examined *in vivo* using our *C. elegans* model of α-syn-induced DA neurodegeneration, as reported with bafilomycin in Chapter 4.

Of course, *C. elegans* has limitations with respect to its capacity for accurately predicting aspects of drug discovery, such as effective concentrations, penetration and bioavailability; these constraints are largely due to the thick cuticle of the animal and limited routes of molecular entry. Nevertheless, the means by which *C. elegans* can be readily, and inexpensively, exploited for high-throughput screening, rapid evaluation of cognate targets, and simultaneous testing of chemical modifiers across multiple genetic and/or transgenic backgrounds in an *in vivo* system all point to the value of using the nematode to identify small molecules which may provide promising therapeutics for the treatment of neurodegenerative diseases. While *C. elegans* has many distinct advantages, any potential candidate genes or molecules which modify PD-like neurotoxicity should be followed up in human cell culture and mammalian models to determine if these same molecules/genes perform similar protective functions in systems which can be directly translated to human health.

Through this dissertation, I have provided experimental evidence along with relevant findings from previous studies to support the role of the lysosomal system in protecting from PD-related neurotoxicity (Figure 5.3). With a bulk of misfolded and aggregated proteins being degraded through the lysosome, depletion of lysosomal function enhances the accumulation of toxic protein species leading to cell death. Overexpression of VPS41 may promote lysosomal function by increasing post-Golgi trafficking of lysosomal proteins required to maintain lysosome function, therefore reducing the level of toxic protein species. Additionally, the small molecule bafilomycin was identified to suppress α-syn neurotoxicity potentially through regulation of lysosomal function, as supported by findings in mammalian cell culture (Pivtoraiko
et al., 2010). Additionally, suppressing further downstream events of cell death, such as expression of 14-3-3 proteins to inhibit apoptosis, can promote cell survival in the presence of toxic protein species (Figure 5.3). Taken together, maintaining protein homeostasis by supporting the function of the protein degradation machinery, specifically the lysosome, may protect cells from accumulating toxic protein species which induce cell death, and provide an avenue in which to suppress the progressive proteotoxicity observed in neurodegenerative diseases during aging.

REFERENCES


Potential Applications of the *C. elegans* PD model

- Aging pathway
- Drug screening
- SNP analysis
- miRNA networks
- Environmental factors
FIGURE LEGENDS

*Figure 5.1.* Six SNPs have been reported to be found within VPS41. Three of these SNPs have minor allelic frequencies ($f$) reported within the general human population. None of these SNPs have been previously reported to be associated with disease.

*Figure 5.2.* There are numerous applications of *C. elegans* for PD research, including these potential directions using the dopaminergic system.

*Figure 5.3.* Suppressing proteotoxicity by promoting lysosomal function. Accumulation of misfolded and aggregated proteins, including $\alpha$-syn, leads to cellular dysfunction and cell death. Promoting the degradation of these toxic protein species through increasing the function of the lysosome, through VPS41 overexpression or low-dose bafilomycin treatment, may suppress cytotoxicity. Additionally, suppression of the apoptotic cell death pathway by overexpressing 14-3-3 proteins will promote cell survival in the presence of these toxic protein species.