RAB PROTEIN EXPRESSION REDUCES DIMER/OLIGOMER ALPHA-SYNUCLEIN AND FACILITATES DEGRADATION

By

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To my future children, for all the times you complain about having to write a page-long book report

ACKNOWLEDGMENTS

I thank my family, friends, and most of all, my awesome boss who has had to deal with me for the past three years.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS....4

LIST OF FIGURES....6

ABSTRACT....7

CHAPTER

1 RABS AND α-SYNUCLEIN....9

Introduction....9
Materials and Methods....12
Plasmids and Antibodies....12
Cell Culture and Transfections....13
Gaussia Luciferase Protein-Complementation and MTS Assays....13
Size Exclusion Chromatography (SEC) to Separate Monomeric and Oligomeric α-Syn....14
Western Blot Analysis....15
Statistical Analysis....16

2 RESULTS....17

mRab1 and Rab8A Decrease α-Syn Oligomers....17
Rab8A Facilitates Degradation of α-Syn....19

3 DISCUSSION....21

LIST OF REFERENCES....27

BIOGRAPHICAL SKETCH....30

LIST OF FIGURES

Figure page

Figure 1-1. mRab1 and Rab8A decrease α-Syn oligomers. 25

Figure 1-2. Rab8A facilitates α-Syn degradation. 26

Abstract of Thesis Presented to the Graduate School
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The protein alpha-synuclein (α-Syn) is implicated in the etiology and pathogenesis of Parkinson's disease. Elevated levels of α-Syn can cause detrimental effects to cellular homeostasis, including deficits in mitochondrial maintenance and vesicle trafficking, such as ER-Golgi transport, depletion of reserve vesicle pool and reduced synaptic vesicle response. Recent data suggests that the trafficking deficits caused by α-Syn may be due to an interaction with Rab proteins. Rab proteins are small GTPases critical for membrane transport, including intracellular trafficking and neurotransmitter release. In several PD animal models, overexpression of murine Rab1 and human Rabs 3A and 8A can ameliorate the trafficking deficits caused by α-Syn overexpression. However, it is unknown whether this rescue is due to Rab protein interaction with the oligomeric forms of α-Syn thought to be the more toxic species in disease pathogenesis. To assay for the effects of these Rabs on oligomeric α-Syn, we utilized a previously-established bi-luminescent protein complementation in which fusion of *Gaussia* luciferase hemi-constructs to α-Syn results in an active luciferase enzyme upon α-Syn dimerization/oligomerization, allowing for direct quantification of these α-

Syn species. Supplementing this assay with size-exclusion chromatography and native immunoblot, we tested the effects of mRab1, Rab3A, and Rab8A to reduce the level of α-Syn oligomers. While Rab3A had no effect on reducing α-Syn oligomers, overexpression of mRab1 and Rab8A reduced the level of both intracellular and secreted α-Syn oligomers. Furthermore, Rab8A facilitated the degradation of α-Syn, reducing the level of both oligomeric and total α-Syn. Together, these data provide evidence that 1) toxic oligomeric α-Syn interferes with specific Rab pathways over others and that, 2) Rab8A, which has not been identified as a Rab involved in protein degradation, may have a previously unknown function upon increased α-Syn burden. Whether this new role affects normal Rab8A functioning remains to be seen.

CHAPTER 1
RABS AND α-SYNUCLEIN

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the United States, affecting approximately one million individuals (1). Pathological hallmarks of PD include the progressive loss of midbrain dopaminergic neurons essential for the control of movement and the presence of Lewy bodies, or intracellular accumulations enriched with the protein alpha-Synuclein (α-Syn). The implication for α-Syn as a potent factor in the pathophysiology of PD is evident from mutations in the *SNCA* gene, encoding for α-Syn, as well as duplications and triplications in the *SNCA* locus, all of which are known to cause early-onset, familial forms of PD (2). Elevated levels of α-Syn have also been found in the blood plasma and cerebrospinal fluid of PD patients, and it is thought that trans-synaptic transmission of small oligomeric forms of α-Syn cause the spreading pathology and disease progression (3-7).

In the cell, cytoplasmic α-Syn exists primarily as a natively unfolded monomer and adopts an α-helical structure upon binding to lipid membranes (8). Although the exact physiological role of α-Syn remains unclear, it is thought that α-Syn may play a function in neurotransmitter release (9-12). α-Syn was originally identified as a presynaptic protein shown to associate with vesicles (11). Mice lacking α-Syn are grossly normal, but display functional deficits in activity-dependent dopamine release in the nigrostriatal system, significant impairments in synaptic response, and a depletion of the reserve vesicle pool upon repetitive neuronal stimulation (9, 10, 12). α-Syn has also been found to interact with the amino-terminus of the SNARE protein synaptobrevin-2

and to enhance SNARE-complex assembly *in vivo*, suggesting a role for α-Syn in modulating the SNARE-complex at the presynaptic terminal (13). Furthermore, the neurodegenerative phenotype in transgenic mice lacking CSPα, a molecular chaperone involved in SNARE-complex assembly, can be rescued by α-Syn (14). These studies suggest that the primary endogenous function of α-Syn is most likely involved in vesicle trafficking at the neuronal synapse.

However, elevated α-Syn levels have been shown to affect earlier stages of intracellular trafficking pathways, also (15, 16). Overexpression of α-Syn in yeast causes ER stress and inhibits ER-Golgi trafficking (15). Increased levels of α-Syn also promote the accumulation of small vesicle clusters at the peripheral ER and plasma membrane in a dose-dependent manner (16). A genome wide screen of yeast open reading frames revealed the largest class of suppressors for α-Syn toxicity to be those associated with early stages of membrane trafficking, including ER-Golgi COPII components, SNARE proteins, and— of particular importance here— Ypt1p, a Rab GTPase (15).

Rab GTPases represent the largest class of RAS-related small GTPases, and are vital to maintaining efficient vesicle transport. It was demonstrated that Ypt1p and Rab1, the murine *YPT1* ortholog and an early ER-Golgi Rab, rescued dopaminergic cell loss associated with α-Syn overexpression in several animal models of PD, including *Drosophila* and *Caenorhabditis elegans* (15). More importantly, Rab1 expression also rescued α-Syn-induced cell loss in mammalian dopaminergic neurons (15). Following the identification of Ypt1p/Rab1 as a potent inhibitor of α-Syn toxicity in yeast and PD animal models, additional Rab proteins were shown to rescue α-Syn-induced transport

deficits and toxicity. Rab8A, the paralog to human Rab1A, localizes to post-Golgi vesicles and has been shown to facilitate trafficking to the plasma membrane (17). Rab3A, a neuronal-specific Rab, is enriched at the synaptic membrane and is involved in neurotransmitter release (18, 19). In addition to Rab1, both Rab3A and Rab8A rescue α-Syn-induced DA neuronal cell loss in *C. elegans* and rat primary neurons (16). Furthermore, immunoprecipitation studies in mutant α-Syn transgenic mice suggest that Rab3A and Rab8A may either directly or indirectly interact with α-Syn (20). In several studies of the PD-related disorders diffuse Lewy body disease and multiple system atrophy—both of which contain aggregated α-Syn inclusions— Rab3A was found to immunoprecipitate with α-Syn in diseased brains but not in controls (21, 22). Together, these studies suggest that toxic levels of α-Syn can affect multiple transport pathways and that the trafficking deficits seen in disease may be a consequence of α-Syn interaction with Rab proteins.

It is thought that the primary toxic species of α-Syn is not the monomeric form or the fibrillar structures found in Lewy bodies, but small oligomers of α-Syn similar to those found in the blood plasma and CSF of PD patients (3, 4, 23, 24). In several PD model systems, Karpinar *et al*. (2009) expressed mutant forms of α-Syn with varying abilities to form fibrils and demonstrated that impairment of oligomeric α-Syn to form fibrils was correlated to a higher level of neurodegeneration (25). Although several Rab proteins have been shown to rescue the trafficking deficits caused by overexpression of α-Syn, the mechanism of reduced α-Syn toxicity remains unclear. We hypothesized that the mechanism by which Rab proteins may ameliorate trafficking deficits is by reducing “toxic” oligomeric species of α-Syn.

To examine the effects of Rab proteins on α-Syn oligomer formation, we utilized a protein complementation assay developed by fusion of the N- or C- terminal fragment of *Gaussia* luciferase (hemi-constructs) to full-length wild-type human α-Syn (26). Co-expression of both hemi-constructs and subsequent dimer/oligomerization of α-Syn results in reconstitution of an active luciferase enzyme that can be quantitatively measured with the addition of substrate, coelenterazine. Here, we demonstrate that α-Syn oligomers, as measured by changes in luciferase activity and high-molecular weight (HMW) native protein, are reduced by expression of either mRab1 or Rab8A. Furthermore, Rab8A also facilitates the degradation of α-Syn. Although we did not assay for direct interaction of Rab8A with α-Syn, the interaction between Rab8A and α-Syn shown in previous studies combined with the data presented here suggests that the ability of Rab8A to facilitate degradation of oligomeric α-Syn may provide a useful therapeutic target in future.

Materials and Methods

Plasmids and Antibodies

Fusion constructs α-Syn-hGLuc1 (S1) and α-Syn-hGLuc2 (S2) was generated by subcloning α-Syn into NotI/ClaI sites of humanized *Gaussia* luciferase and were kindly provided by Dr. Pamela McLean (Mayo Clinic, Jacksonville, FL; ref. 13). pcDNA-mRab1, pcDNA-Rab3A, and pcDNA-Rab8A were kindly provided by Dr. Susan Lindquist (Massachusetts Institute of Technology, Cambridge Massachusetts). FLAG-mRab1, FLAG-Rab3A, and FLAG-Rab8A were generated by subcloning the sequence GGATTACAAGGATGACGACGAT to the amino-terminus of pcDNA-Rab constructs. Antibodies were as follows: Millipore rabbit anti-α-Syn (1:2000), BD Transduction

Laboratories mouse anti-α-Syn (1:5000), Sigma mouse anti-FLAG M2 (1:500), Sigma mouse anti-β-actin (1:100,000).

Cell Culture and Transfections

Human H4 neuroglioma cells (HTB-148; American Type Culture Collection) were grown in Opti-MEM + GlutaMAX media (Life Technologies) containing 5% Fetal Bovine Serum and antibiotic (50U/mL penicillin, 50mg/mL streptomycin) in culture flasks under standard tissue culture conditions (37°C under 5% CO_2). Cells were plated 24 h prior to transfection, and transiently transfected at 80-90% confluency with SuperFect Transfection Reagent (Quiagen) according to the manufacturer's instructions. For conditioned medium (CM) experiments, media was collected 48 h post-transfection. To eliminate floating cells, media was centrifuged prior to use for 2 min, 3000rpm, at RT and analyzed immediately for luciferase assays, or 5 min, 3000rpm, at RT and stored at -20°C for size exclusion assays. For native and total α-Syn studies, cells were lysed 48 h post-transfection in a non-detergent lysis buffer (50mM Tris pH 7.4, 175mM NaCl, 5mM EDTA pH 8.0, protease inhibitor cocktail (Roche Applied Science). For cycloheximide studies, cells were transiently transfected in a 6-well plate. 24 h post-transfection, cycloheximide was added to new media at a final concentration of 3ug/mL. Cells were washed with PBS, lysed at indicated time points in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, 1% TritonX-100, 10mM N-ethylmaleimide [Fisher], protease [Roche complete mini] and phosphatase [Fisher] inhibitor), and stored at –20°C until all time points had been collected.

***Gaussia* Luciferase Protein-Complementation and MTS Assays**

To assay for changes in oligomeric α-Syn, H4 cells were transiently transfected in 96-well plates with fusion constructs S1, S2 and either pcDNA control vector or the

indicated FLAG-Rab plasmid. 48 hours post-transfection, culture medium was transferred to a new 96-well plate and centrifuged as indicated above. Cells were washed with PBS and replaced with serum- and phenol-red free Opti-MEM. Native coelenterazine (Nanolight), a cell-permeable substrate of *Gaussia* luciferase, was resuspended in methanol to 1 mg/mL and dispensed to a final concentration of 20µM onto live cells or CM by an automated plate reader, (Synergy HT, Bio-Tek). The bioluminescent signal generated by protein complementation of the luciferase enzyme was integrated over 2 seconds before measurement at 480nm.

Following luciferase assays, cell viability was measured using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit as per manufacture instructions. Briefly, media was removed following luciferase assay to a final volume of 100µL/well. 20µL MTS reagent was added and cells were incubated at 37°C for 1-3 hours. Absorbance was measured at 490nm with a microplate reader (SynergyHT, Bio-Tek). Control plasmid (pcDNA) was used to measure background luciferase signal and provide a baseline for cell viability assays. Each condition was transfected in 8 replicates per plate, and data from a minimum of 3 plates was collected for statistical analysis.

Size Exclusion Chromatography (SEC) to Separate Monomeric and Oligomeric α-Syn

SEC was performed as described previously (23). To assay extracellular α-Syn oligomerization, 500µL CM from H4 cells transfected with S1, S2, and pcDNA control or the indicated FLAG-Rab was thawed and filtered through an Ultrafree®-MC Centrifugal Filter (Millipore; PVDF 0.22µm) for 4 min, 10,000*g* at room temperature. 350µL filtered

CM was injected into a Superose 6 10/300 GL gel-filtration column (GE Healthcare) and eluted with PBS at a flow rate of 0.4mL/min. 200µL fractions were collected into microtubes (Fisher), and 100µL of each fraction was transferred to a 96-well plate and analyzed for *Gaussia* luciferase protein-complementation, as described above.

To measure intracellular α-Syn oligomerization, cells from the same CM samples were scraped down, washed once with PBS, and mechanically sheered by passage through a 1mL 27-gauge needle in 170µL of a non-detergent lysis buffer (50mM Tris pH 7.4, 175mM NaCl, 5mM EDTA pH 8.0, protease inhibitor cocktail (Roche Applied Science). Lysates were spun at 13,000 rpm for 10 min at 4°C and stored at -20°C. For SEC, lysates were thawed and filtered through an Ultrafree®-MC Centrifugal Filter (Millipore; PVDF 0.22µm) for 4 min, 10,000*g* at room temperature. 140uL of filtered cell lysate was used for SEC under the same conditions as the CM and analyzed for protein-complementation.

Western Blot Analysis

Denatured cell lysates from cycloheximide or total α-Syn experiments were subjected to NuPAGE 4-12% Bis-tris SDS-PAGE electrophoresis (Life Sciences) followed by western blot analysis with antibodies described above. Protein bands were visualized by the ECL method (Amersham) or by the Odyssey system (Licor) using IR700/800 secondary antibodies (Rockland). Native western blot was performed under non-denaturing conditions using NativePAGE 4-16% Tris-glycine gels (Life Sciences) following manufacturer instructions.

Statistical Analysis

Statistical analyses were carried out using Prism 5.0 (GraphPad). One-way analysis of variance is used to evaluate groups with Turkey's multiple comparisons post-hoc. Values in the figures are expressed as means ± SEM.

CHAPTER 2
RESULTS

mRab1 and Rab8A Decrease α-Syn Oligomers

Expression of mRab1, Rab3A, and Rab8A has been shown to ameliorate toxicity and transport deficits caused by α-Syn overexpression in several PD animal models, including worms, flies, and rat midbrain dopaminergic neurons (15, 16). It has also been suggested that α-Syn may interact with Rab proteins in disease, providing a potential mechanism by which toxic forms of α-Syn can impair vesicle transport (21, 22). However, it is unknown whether Rab proteins can interact with different cytoplasmic forms of α-Syn, including small oligomeric forms that are thought to play a primary role in pathology. To determine the effects of Rab overexpression on α-Syn oligomerization, we utilized a protein complementation assay employing the bioluminescent protein *Gaussia* luciferase (26). In this model, two halves of the *Gaussia* luciferase enzyme are fused in frame to full-length, wild-type α-Syn. Co-expression of Syn-hGLuc1 (S1) and Syn-hGLuc2 (S2), but not S1 or S2 with either of the hGLuc hemi-constructs alone, results in high levels of luciferase signal indicative of α-Syn dimer/oligomer formation (26). This highly sensitive, quantitative assay can be used in both live cells and conditioned media (CM) to measure α-Syn.

We used transient transfection in H4 neuroglioma cells to evaluate the level of α-Syn dimer/oligomer formation in the presence mRab1 (a murine homolog of the human Rab1A), Rab3A, and Rab8A. We expressed either S1 or S2 constructs with a control plasmid (EGFP) to determine the background levels of the assay. To take into account cell death caused by the transfection process, we performed an MTS assay immediately following luciferase assay. The luciferase levels were then normalized to live cells and

are shown as the percent reduction in luciferase activity compared to the model condition (S1/S2 with EGFP) (Fig. 1A). After 2 d, expression of S1 or S2 constructs alone with control plasmid resulted in minimal luciferase signal (Fig. 1A). Co-expression of either mRab1 or Rab8A caused a significant reduction in luciferase activity (mRab1, 49%; Rab8A, 74%; n=4, $p<.001$) compared to control EGFP plasmid. Previous studies have indicated that Rab3A can rescue α-Syn-induced toxicity (16); however we did not detect any change in luciferase activity in the presence of Rab3A.

We hypothesized that the reduction in α-Syn luciferase signal by Rab proteins may be due to a shift of α-Syn into smaller molecular weight forms. Previous characterization of the S1/S2 model has shown that co-expression of S1/S2 in H4 neuroglioma cells results in a high molecular weight (HMW) smear of α-Syn under native conditions indicative of a wide range of α-Syn oligomers (26). Our data is consistent with this, and expression of S1, S2, or S1/S2 together results in a HMW smear of α-Syn ranging from 146kDa to over 700kDa (Fig. 1B, lane 1). Native-PAGE of wild-type α-Syn demonstrates a similar HMW smear and confirms that wild-type α-Syn behaves in a similar manner under our transfection conditions (Fig. 1B, lane 7).

Using Native and SDS-PAGE Western followed by α-Syn immunoblot, we saw no change in HMW α-Syn with co-expression of mRab1 and Rab3A, but a significant reduction in HMW α-Syn was observed with Rab8A (Figure 1-1B, lane 6). Contrary to our hypothesis however, this does not appear to be due to a shift of α-Syn to smaller MW forms, but due to a reduction in the total level of α-Syn as shown when run under denaturing conditions.

To further characterize the oligomeric species of α-Syn, we used size-exclusion chromatography to measure the luciferase activity eluted from 1mL fractions of either cell lysate or culture medium. In cell lysate extracted under non-denaturing conditions, mRab1 and Rab8A, but not Rab3A, appear to reduce the level of HMW α-Syn. The peak seen at fraction 15 corresponds to an approximate molecular weight of about 440kDa, similar to the majority of α-Syn seen in the Native immunoblot. In conditioned media, primary SEC peak eluted at fraction 18 likely corresponds to α-Syn dimer and small oligomers of approximately 30kDa to 67kDa and is also significantly reduced by mRab1 and Rab8A, but not Rab3A (Figure 1-1C). Contrary to our hypothesis, however, no shift in MW from the α-Syn-containing fractions of both cell lysate and media occurred, indicating that although mRab1 and Rab8A reduce the level of oligomeric α-Syn (Figure 1-1A and C) and Rab8A appears to additionally reduce total α-Syn (Figure 1-1B), these Rab proteins do not affect α-Syn oligomerization.

Rab8A Facilitates Degradation of α-Syn

The reduction in total α-Syn by Rab8A suggests that Rab8A may facilitate degradation of α-Syn. To first rule out a transcriptional effect, we used PCR analysis of mRNA extracted from H4 cells co-transfected with α-Syn (both S1/S2 and untagged wild-type α-Syn) and either EGFP or Rab8A. We found no changes in the expression level of α-Syn mRNA, indicating that the effect of Rab8A on α-Syn is most likely post-translational (Figure 1-2A, B).

To determine whether Rab8A facilitates post-translational degradation of α-Syn, we used cycloheximide, which interferes with translational elongation, to block new protein synthesis following transfection of H4 cells with S1/S2 and either control plasmid (pcDNA) or Rab8A. 24 hours post-transfection, cycloheximide (3uM final concentration)

was added to the cells and lysates were collected over a 48 hour period. As expected, there was a marked reduction of α-Syn when co-transfected with Rab8A in cell lysate collected at Time 0 (Figure 1-2D, lanes 1 and 5). However, analysis of the relative α-Syn level reveals that degradation of α-Syn is significantly enhanced in the presence of Rab8A but not control plasmid (Figure 1-2C). To rule out a possible effect of the luciferase portion of the protein, we performed identical experiments with wild-type α-Syn with similar results (data not shown).

CHAPTER 3
DISCUSSION

Rab proteins have been shown to ameliorate toxicity caused by α-Syn overexpression in several PD animal models (15, 16). We hypothesized that one potential mechanism of Rab effects on α-Syn could be that Rab proteins reduce α-Syn oligomers. Using a previously established cell model for α-Syn dimer/oligomerization, we tested our hypothesis using mRab1, Rab3A, and Rab8A. In a bioluminescent protein complementation assay (PCA) utilizing co-expression of α-Syn-luciferase hemi-constructs, we found that mRab1 and Rab8A (but not Rab3A) cause a significant reduction in luciferase signal, with Rab8A decreasing luciferase activity by almost 75%. We further characterized this using size-exclusion chromatography to identify whether the reduction in luciferase activity corresponded to a decrease in high molecular weight α-Syn. We found that, in agreement with our luciferase assay, both mRab1 and Rab8A (but not Rab3A) reduce the luciferase signal of α-Syn eluted from SEC fractions in both cell lysate and conditioned media, suggesting that mRab1 and Rab8A have an effect on α-Syn dimer/oligomer formation. In contrast to our hypothesis however, we did not see a shift in the molecular weight of α-Syn, suggesting that although mRab1 and Rab8A may affect dimer/oligomer formation, they are not reduced into smaller molecular weight forms.

Of novel importance, we found that in addition to a decrease in luciferase signal by Rab8A in the PCA and SEC assays— presumably corresponding to a reduction in α-Syn dimer/oligomers— Rab8A also reduces the total level of α-Syn. This was not due to a transcriptional effect, as we did not see any change in α-Syn (both S1/S2 and wtSyn) mRNA expression in the presence of Rab8A. To determine whether Rab8A might have

a post-translational effect on α-Syn, we used cycloheximide to block translation and measure the degradation rate of α-Syn. At 24 h post-transfection there is already a marked decrease in α-Syn expression in the presence of Rab8A (Fig 1, lanes 1 and 5). However, by comparing the relative rate of α-Syn degradation, we found a significant increase in the rate of α-Syn degradation in the presence of Rab8A. This is most likely not due to an immediate turnover of α-Syn by Rab8A following transfection, as we have seen approximately equal levels of α-Syn as well as luciferase signal with or without the presence of Rab8A at 12 h post-transfection, suggesting that the effects of Rab8A on decreasing α-Syn levels are only effective once α-Syn reaches a certain level (unpublished observation). However, the mechanism by which this occurs and whether this is due to a direct or indirect interaction of α-Syn with Rab8A still remains to be seen.

We have demonstrated that although all three of the Rab proteins used in this study have previously been shown to reduce the toxicity and transport deficits caused by α-Syn overexpression, the effects they have on oligomeric forms of α-Syn are markedly different. One potential reason for this may be due to the different pathways in which each of the Rabs are involved. Rab3A, a neuronal-specific Rab, localizes to synaptic vesicles and most likely plays a role in vesicle release (27). One of the known effector proteins of Rab3A is rabphilin-3A, a synaptic vesicle Ca^{2+}/phospholipid-binding protein (28). Rabphilin-3A, which assists in synaptic targeting, depends on Rab3A for normal function. Immunoprecipitation studies of Rab-α-Syn interactions in diffuse Lewy body disease, a PD-related disorder with similar α-Syn inclusions, demonstrated that in control cases, rabphilin is pulled down with Rab3A (22). However, in diseased brains, rabphilin is no longer precipitated by Rab3A, which instead pulls down α-Syn (22).

Although this does not demonstrate a direct interaction of α-Syn and Rab3A, and rabphilin is certainly not the only Rab3A effector, it does suggest that in a disease state α-Syn can have abnormal interactions with Rab3A that might cause a disruption in vesicle release. While Rab3A overexpression can rescue the toxicity caused by α-Syn overexpression in PD animal models, we have shown that with transient transfection of H4 neuroglioma cells, Rab3A does not affect α-Syn oligomers (16). It is possible that the rescue in toxicity may be due more to compensation mechanism for Rab3A rather than an effect on α-Syn level.

While Rab3A is localized to the synapse, Rab1 and Rab8A play roles early in the vesicle transport pathway. Rab1 and Rab8A are localized to the ER-Golgi and *trans*-Golgi network, respectively (29, 30). In yeast, overexpression of α-Syn causes deficits in ER-Golgi transport which can be rescued by Rab1 (15). Additionally, both Rab1 and Rab8A can rescue dopaminergic cell loss in several different PD animal models (15, 16). We have shown that both mRab1 and Rab8A can reduce the level of α-Syn oligomers. Recently, Winslow *et al.* (2010) studied the effects of α-Syn overexpression on macroautophagy and found that both α-Syn overexpression and knockdown of Rab1A impaired early steps in the initiation of autophagosome synthesis (31). The impairment of autophagosome formation by α-Syn overexpression could be rescued by Rab1A, although the authors did not show whether this effect resulted in a decrease of α-Syn.

Interestingly, only Rab8A was able to reduce both α-Syn oligomers and total α-Syn and facilitate degradation. The method of α-Syn degradation remains controversial, and both the ubiquitin-proteasome system and the autophagy-lysosome pathway have

been implicated (32-36). However, as Rab8A was able to reduce high molecular weight α-Syn oligomers, it is unlikely that the ubiquitin-proteasome system is involved. A recent study by Ejlerskov *et al.* (2013) showed that overexpression of A30P α-Syn in the presence of Rab8A in PC12 cells redistributed α-Syn to the plasma membrane and resulted in increased levels of extracellular α-Syn (33). This was proposed to be due to unconventional secretion by exophagy, a process in which autophagosomal machinery is re-directed to the plasma membrane typically under conditions of cell starvation. While this is consistent with our finding of lower levels of total α-Syn in the presence of Rab8A, we did not find an elevated level of extracellular α-Syn. Mutant A30P α-Syn is known to cause a higher level of toxicity in the cell; it is possible that under transient conditions of increased α-Syn burden, such as that shown in our model, Rab8A directs α-Syn oligomers to the autophagy-lysosomal pathway for degradation. Then, under conditions of cell stress, Rab8A may be re-routed to assist in trafficking α-Syn to the plasma membrane (33).

In conclusion, we have demonstrated that three Rab proteins, all of which have been shown to rescue α-Syn toxicity and trafficking deficits in various PD models, have differential effects on oligomeric forms of α-Syn. The most important result of this study was a previously unidentified role for Rab8A in facilitating the degradation of α-Syn. Future studies will aim at identifying which degradation pathway is involved and whether this is correlated to a rescue in membrane trafficking and toxicity.

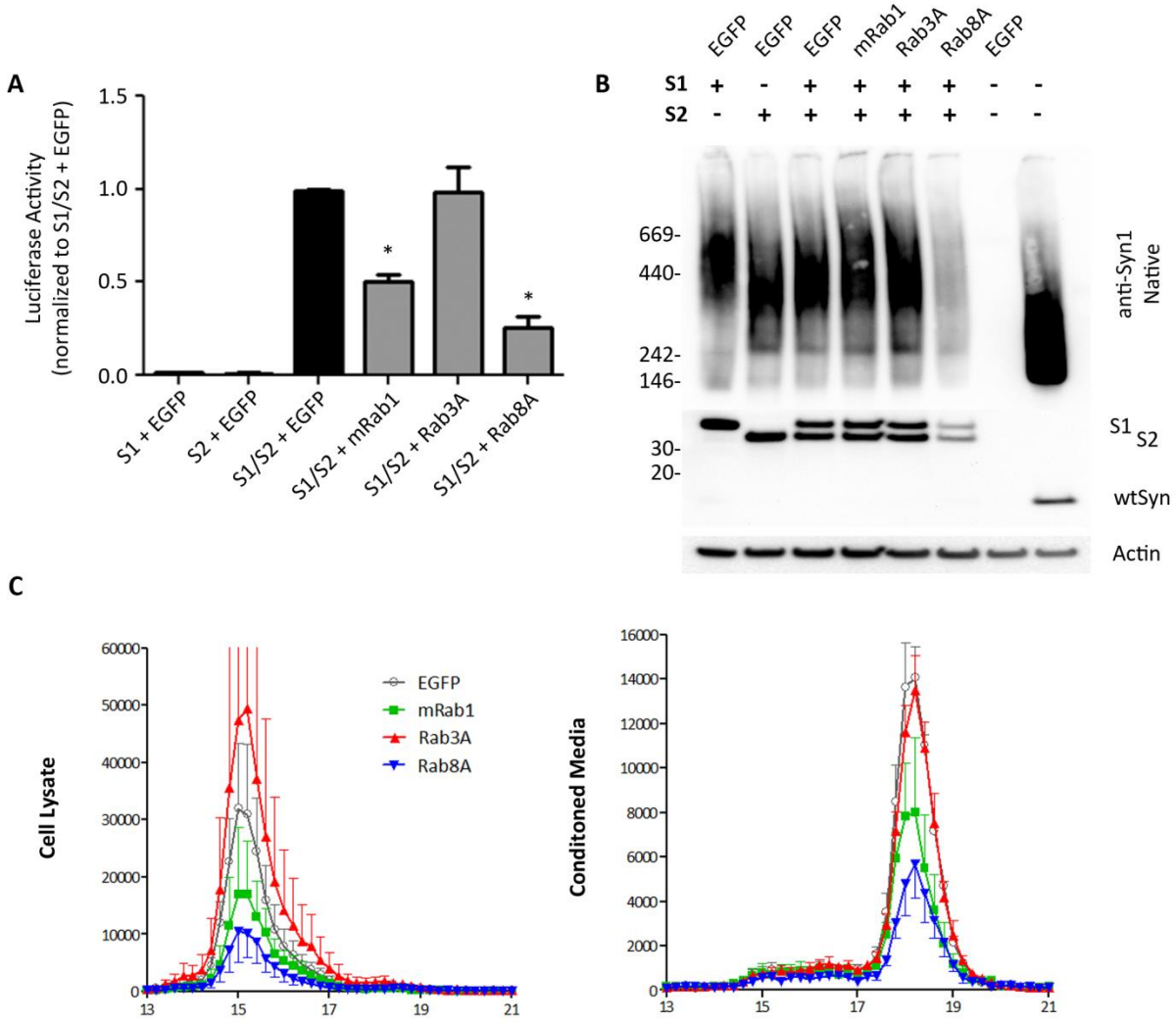

Figure 1-1. mRab1 and Rab8A decrease α-Syn oligomers. *A*) H4 cells transiently transfected with S1/S2 and mRab1, Rab3A, and Rab8A were assayed for luciferase activity 48 h post-transfection. Intact cells displayed a 49% reduction in luciferase activity when co-transfected with mRab1 and a 79% reduction when co-transfected with Rab8A. $n = 4$, $F = 46.56$, $^*P < 0.001$. *B*) Native and SDS-PAGE Western blot showing changes in high molecular weight and total α-Syn. *C*) Luciferase activity in SEC fractions of S1/S2 cell lysate and conditioned media co-transfected with control (EGFP), mRab1, Rab3A, or Rab8A.

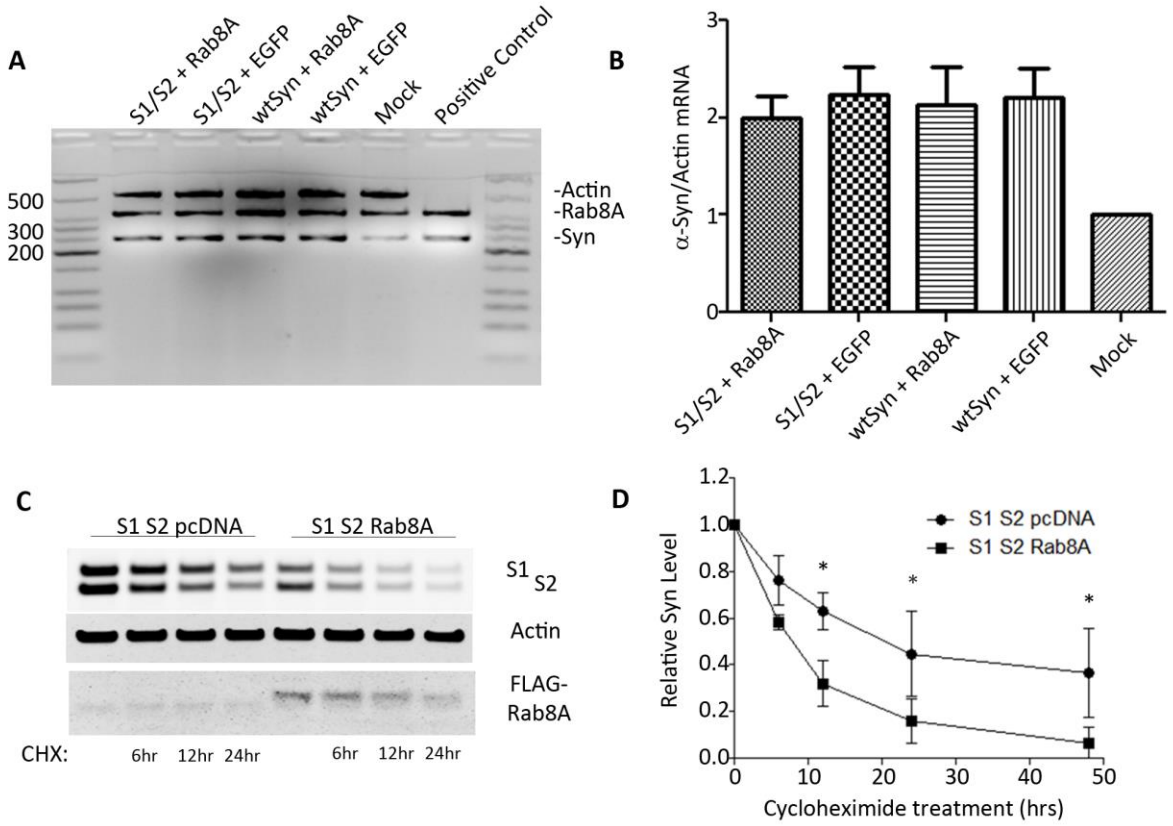

Figure 1-2. Rab8A facilitates α-Syn degradation. *A*) PCR of mRNA extracted from H4 cells transiently transfected with S1/S2 or wild-type (wt) α-Syn and Rab8A or control EGFP plasmid. Mock cells were exposed to transfection reagent only, and a combination of wtSyn and Rab8A plasmid were used as a positive control. *B*) Quantification of PCR in (*A)* reveals no change in mRNA expression of α-Syn when co-transfected with Rab8A. *C*) Westen blot showing α-Syn expression levels following exposure to cycloheximide 24 h after transfection with control (pcDNA) or Rab8A. *D*) Relative quantification of α-Syn expression level shown in (*C*) reveals a significant decrease in α-Syn expression at 12, 24, and 48 h following cycloheximide treatment in S1/S2 cells co-transfected with Rab8A compared to pcDNA. *P> 0.05, two-tailed *t* test.

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BIOGRAPHICAL SKETCH

Amanda Herring began her education at the University of South Florida as a chemistry major/voice minor. After a year, she transferred to the University of North Florida where she completed her Bachelor of Science in biology in 2009. During her undergraduate coursework, she started working at the Mayo Clinic Jacksonville as a research assistant in an Alzheimer's lab. She fell in love with research, and when she completed her undergraduate degree, she decided to pursue her Master of Medical Science at the University of Florida in neuroscience.