Pathogenicity Assessment of Three Newer a-Synuclein Mutants Under Varying Expression Levels in Yeast Reveals Differential Toxicity

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Parkinson's disease is the second most common neurodegenerative disease, resulting from the misfolding and aggregation of the α- synuclein protein. The majority of Parkinson's cases occur sporadically; however, there are familial cases of PD resulting from genetic mutations which induce an early onset form of the condition. Additionally, research has shown that various conditions and alterations within the cellular environment can significantly alter the characteristics of α- synuclein toxicity and the rate of disease progression. There are six mutations to the α-synuclein gene that have been extensively evaluated by researchers, however there are three newer mutant variants of α- synuclein (A18T, A29S, and A53V) that haven't been assessed to the same degree. Here I investigate the intrinsic and extrinsic factors driving the toxicity of these three newer mutants using budding yeast as a model organism. I report that 1) these mutants show differential levels of toxicity in a concentration dependent manner; 2) the intrinsic property driving the toxicity of each mutant is more complicated than either loss or gain of the amino acid; 3) the A29S position dominates the phenotype of combined mutants; and 4) the mutants show differing degrees of sensitivity to altered cellular environments. This study reinforces the importance of understanding extrinsic and intrinsic factors that affect the toxicity of wildtype and mutant α- synuclein.

INTRODUCTION

Neurodegenerative diseases are conditions characterized by the death of neural cells and progressive degeneration of the brain. These disorders can vary in the age of onset although typically occur later in life and cause a variety of symptoms dependent on what brain region is degenerating. Synucleinopathies are one such group of degenerative disorders that includes conditions such as Multiple System Atrophy (MSA), Dementia with Lewy Bodies (DLB), and Parkinson's Disease (PD) which is the most common synucleinopathy in addition to being the 2nd most prevalent neurodegenerative disorder globally. PD is characterized by severe motor symptoms including tremors, rigidity, and bradykinesia as a result of degeneration of dopaminergic neurons within the substantia nigra (Jankovic, 2008) which has been linked with the presence of Lewy Bodies, aggregated plaques of the α- synuclein protein (Spillantini et al., 1998).

Most cases of PD are obtained sporadically late in life, the exact causes for which aren't well understood although it's been believed that environmental toxins are a factor (Chin-Chan et al., 2015), however there are familial cases of PD which are linked to genetic mutations. Mutation to the PINK1 gene for instance is shown to increase the expression of α- synuclein and cause an early onset form of PD that presents that same as the sporadic form (Gandhi et al., 2009). Through studying these familial cases researchers have gained significant insight into the mechanisms underlying the pathology of PD, improving our understanding of α- synuclein and the various intrinsic or extrinsic mechanisms that affect the pathology of the protein.

α- synuclein is protein expressed throughout the brain however it is more highly expressed in certain regions such as the substantia nigra (Taguchi et al., 2016). The exact function is unknown but research indicates that its potential involvement in neurotransmission and interacts with the SNARE complex (Burré et al., 2010) among various other potential roles such as plasticity and membrane trafficking (Bendor et al., 2013). In synucleinopathies like PD however α- synuclein misfolds and becomes pathological due to several environmental and genetic factors. Altered environmental conditions within neural cells have shown to affect the extent in which PD progresses and how α- synuclein aggregates. High levels of nitration and oxidation is shown to be heavily involved with α- synuclein fibrillation and toxicity (Giasson et al., 2000), while other processes such as

SUMOylation can be protective and reduce the accumulation of misfolded proteins (Krumova et al., 2011). As mentioned earlier, increasing levels of α- synuclein alone can cause PD, for instance a family with a gene duplication of the SNCA gene that codes for α- synuclein experienced the classic symptoms of PD at an earlier onset (Kara et al., 2014). Other genetic factors like mutations also can drastically alter the toxicity of α- synuclein, six mutations on α- synuclein itself (A30P, E46K, H50Q, G51D, A53T, and A53E) cause early onset PD.

Many PD researchers have evaluated mutants of α-synuclein to better understand its underlying mechanisms and the unique properties that contribute to pathogenicity, such as A53T having highest affinity for membrane binding while E46K has high affinity for negatively charged lipids in particular (Liu et al., 2021).

In contrast to these however, there are three newly identified mutants, two sporadic (A18T and A29S) and one familial (A53V), that haven't received as much attention, possibly due to the sporadic nature of A18T and A29S (Hoffman-Zacharska et al., 2013) while A53V is linked to later onset PD as opposed to early onset (Mohite et al., 2018).

Previous work in our lab has investigated these novel mutants within a yeast model system, two thesis students Carris Borland and Amanda Grassel comparatively evaluated the toxicities of these mutants and assessed how they are affected in various altered cellular environments. Both Carris and Amanda found that each mutant was more sensitive to a particular condition compared to the others, however their findings regarding the natural toxicities of the mutants were inconsistent. Carris reported that the new mutants were differentially toxic from one another while Amanda instead found no difference between them. Knowing that increased expression is a key component of α- synuclein toxicity, I aimed to determine how a higher level of expression would affect the toxicities of these mutants.

Most work in the lab, including the work done by Carris and Amanda, was done using a PYES2 vector, however more recent work in the lab uses a P426G vector provided by Dr. Tiago Outeiro that has a higher level of protein expression. I hypothesize that with P426G expression vector, the three new mutants will show differential levels of toxicity, using a yeast model organism. Yeast serves as a good model due to their manipulatable genome which can be used to induce altered cellular conditions like nitration through COX5A and COX5B knockouts (Chung et al., 2013; Costello et al., 2008). Furthermore yeast have conserved protein folding and modifying pathways as in our own cells which makes them useful to model protein accumulation and aggregation that's common in neurodegenerative diseases (Miller-Fleming et al., 2008).

To evaluate the toxicities of the three new mutants A18T, A29S, and A53V, I have four aims. First, I predict that the mutants will be differentially toxic when under increased expression, and that their toxicities are dependent on the level of expression. To do this I will create the mutants within the P426G high expression vector and then comparatively evaluate them with one another, and with the mutants in the PYES2 vector. I additionally will compare the new mutants to the older six mutants A30P, E46K, H50Q, G51D, A53T, and A53E, as well as within a second strain of yeast. Second, is to determine the intrinsic properties of each mutation that make them toxic, from which I predict that either the loss of the original amino acid (Alanine) or the gain of the mutant amino acid (Threonine, Serine, or Valine) is key. To do this I aim to create and evaluate various amino acid substitution mutations at those three positions, exchanging the amino acid for another that belongs to one of

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the four large categories of amino acids: hydrophobic, hydrophilic, acidic, or basic. My third aim was to see how the properties of the mutants might affect one another when combined and what that might reveal about the locations of these mutations, from which I expected the mutants toxicity to either be averaged or enhanced when combined. To assess this, I aimed to create and evaluate three double combinatorial mutants as well as one triple mutant and assess how the various combinations affect overall pathology. My fourth and final aim is to assess how these mutants are differentially affected in altered cellular conditions. Carris and Amanda had previously found each mutant to be particularly sensitive to certain conditions within the PYES2 vector so the goal for this aim is to reassess this within a higher expression vector, of which I've chosen to assess altered nitrative, SUMOylation, and mitochondrial dysfunction conditions.

METHODS

α- Synuclein Mutant Creation

α- synuclein constructs in PYES2.1 vector used for Amanda Grassel and Carris Borland's theses were reused for Aim 1. The WT, GFP tagged α-synuclein in the P426G vector used in this study was given to our lab by Dr. Tiago Outeiro (University Medical Center Goettingen). The three newer (A18T, A29S, A53V) and six older familial mutants (A30P, E46K, H50Q, G51D, A53T, A53E) in the P426G vector were created through GENEART Site-Directed Mutagenesis from Invitrogen Life Technologies, as were the substitution and combinatorial mutants. They were then transformed with TOPO TA Expression Kit into One Shot TM MAX Efficiency TM DH5αTM-T1R competent E. coli cells and stored.

Yeast Strains

W303 and BY4741 yeast strains were used for Aim 1 to assess the baseline toxicities of the mutants and serve as a control for other conditions, additionally both BY4741 and W303 were used for Aim 3. In Aim 2 knockout strains of cox5A and con5B were used to investigate the effects of nitrative stress, for investigating SUMOylation rather than knockouts a temperature sensitive strain was used such that the yeast grown at 25°C did not have the condition expressed and served as a control, when grown at 30°C the condition would be expressed and the effects of SUMOylation on mutant synuclein toxicity could be evaluated.

Serial Dilution Spotting

5-fold dilution spotting assays were done to test the toxicity and cell growth of each mutant. Cells inoculated in 5ml of SC-Ura glucose media were grown/incubated at 30˚C overnight then washed three times with 5mL of water and using a centrifuge. 10µl of cells is added to a centrifuge tube to make a 1ml suspension and the cells are counted on a hemocytometer. After calculations were made the appropriate amount of cells is taken and added to enough water to make a 1mL suspension, then the solution is diluted 5-fold in a 96-welled microtiter plate. 2µl of each dilution is spotted on SC-Ura glucose and SC-Ura galactose plates then placed inside the 30˚C incubator. Images of the plates were taken over the course of 1-4 days.

Fluorescence Microscopy

Fluorescent microscopy was used to observe α-synuclein mutant localization. The synucleins are tagged with GFP so when placed under blue light the protein will glow green. Yeast cells were grown overnight in 5 ml SC-Ura glucose media at 30˚C, then washed three times suspended in 5ml of water. The cells were then added to 20 ml of galactose media and incubated at 30˚C. 1mL of cells were removed and pelleted at 6, 12, and 24 hours timepoints, then images of approximately 1000 cells were taken under the TE2000-U Nikon fluorescent microscope. Cells were classified according to different established phenotypes below: cytoplasmic diffusion, membrane binding, and aggregates. Membrane binding and aggregation tended to associate with toxicity while cytoplasmic diffusion was associated with no toxicity.

Quantification

Fluorescing cells were quantified and compared statistically. Data is reported as the average percentage of the phenotype over four independent trials for the first three aims and three trials for the fourth aim. The amount of Foci present was compared between the α-synuclein variants using one-way ANOVA with Bonferroni post hoc analysis to determine significance. Significance threshold ($\alpha = 0.05$) was adjusted accordingly to the number of comparisons being made (α/n; n = number of comparisons) and graphs were generated in excel.

Western Blot

Cells are grown in 5ml of SC-Ura glucose media and incubated at 30° C overnight. The cells are washed twice and added to flasks of 20 mL galactose media which are incubated for 12 hours at 30°C. Cells are counted on a hemocytometer to calculate the volume needed for a total of $2.5x10^7$ cells to prepare lysates with. Cells are washed three times with 50mM Tris, 50mM NaN3 and resuspended with electrophoresis sample buffer (ESB). The lysates are run along with SeeBlue protein ladder on a 10-20% Tris-Glycine gel at 130 volts with 1x SDS running buffer. The gel is transferred onto a polyvinylidene fluoride membrane using a semi-dry transfer technique at 15 V for 45 minutes. The membrane is then washed with our target protein antibody, those being the anti-GFP antibody to target our synuclein or anti-PGK antibody to target phosphoglycerokinase as a loading control, and a secondary antibody. Chromogen substrate is used to detect the amount of the target proteins in samples.

AIM 1 mutants		AIM2 + AIM3 mutants	
New	Old	Substitution	Combinatorial
A18T	A30P	A18S	A18T+A29S
		A18G	
	E46K	A18E	A18T+A53V
		A18R	
A29S	H50Q	A29T	
		A29G	
	G51D	A29E	A29S+A53V
		A29R	
A53V	A53T	A53G	A18T+A29S+A53V
		A53N	
	A53E	A53D	
		A53R	

Table 1: Summary of Mutants Created
All mutants were created on the P426G vector and were transformed into W303 and BY4741 strains. The new mutants were additionally transformed into genetically modified strains for chapter 4.

Results

Mutating α-Synuclein

To begin my investigation of the new mutants (A18T, A29S, A53V) as well as the older mutants (A30P, E46K, H50Q, G51D, A53T, A53E) I first need to create them on the high expression P426G vector, using site directed mutagenesis reactions to do so. Through mutagenesis the desired mutation can be created by using designed forward and reverse primers roughly 30 nucleotides in length that match the target segment of the gene except for a single point at the center of the primer that alters the codon to read for the desired amino acid. As the PCR reaction proceeds the primer acts as a scaffold for the rest of plasmid to be replicated and once finished leaves a copy of the plasmid that contains a single nucleotide difference due to the primer. Once completed the results of the reaction are verified through gel electrophoresis, the reaction samples are run through the gel along with the starting amount of base template to confirm the DNA was successfully amplified, if so then the amplified product is used to transform a DH5a strain of E. Coli. Transforming into E. Coli allows us to effectively make more copies of our plasmid as the bacteria replicates, to ensure that they are replicating our plasmid the bacteria are grown

on Luria broth (LB) ampicillin plates as the plasmid contains a gene for ampicillin resistance so bacteria will only survive if they have our plasmid. The DH5a strain was used because in mutagenesis an additional reaction is used that methylates the template plasmid but leaves the newly created mutant plasmid unmethylated, the DH5a strain contains an enzyme that breaks down methylated DNA which ensures that the transformed E. Coli should only contain the new mutant plasmid. The mutant plasmid is then isolated and purified from a colony of E. Coli to be sent for sequencing to then verify if we've successfully created the correct mutation.

Sequencing α- Synuclein

Once we have our purified plasmid, we have to ensure that the reaction successfully induced the desired mutation and that no other mutations occurred. The plasmid is sent along with the Gal1 gene forward primer to the Genomics Facility at the University of Chicago to sequence the entire α- synuclein gene, the files of the results are shared with was and we can then determine the results of our mutagenesis reaction. Using the ApE (a plasmid editor) software we can check for differences between our sequenced α- synuclein and the wildtype version, determine whether the correct change in amino acid has occurred and ensure that there are no unintentional changes to the sequence. When the sequence for a given mutant is incorrect the isolated plasmid is discarded and another colony of E. Coli is selected to sequence, when the sequences are correct the isolated plasmid and the E. Coli colony it was isolated from are stored.

Substituting/Combining Mutants

For my second aim different sets of mutants have to be made. The substitution mutation project involves substituting the mutant amino acid of the three new mutants (A18T, A29S, and A53V) for a different amino acid belonging to one of four categories. Additionally we'll be creating combinatorial mutants of the three mutants to three double mutants and one triple mutants. The process for creating the substitution mutants was no different from how I created the natural mutants, utilizing designed primers to get the specific mutation however the combinatorial mutants required additional steps. The A18T, A29S, and A53V mutants I created would be put through mutagenesis again being the base template DNA this time instead of wildtype α- synuclein. The primers for a different mutant would then be used on the select plasmid (for instance using A29S primers with A53V mutant) and run the mutagenesis reaction for those to get mutants A18T+A29S, A18T+A53V, and A29S+A53V. Then the double combinatorial mutants will be used as the template plasmid to create the triple combinatorial mutant A18T+A29S+A53V using the primers for the last of the three mutants.

Transforming Yeast

Once all the mutants have been created we next have to have them expressed in yeast before we can properly study them. For aims 1 and 2 of the project two strains of yeast, W303 and BY4741, are used whereas for aim 3 specific strains are used, those being knockout strains cox5AD and cox5BD for studying the effects of nitrative stress as well as temperature sensitive $smt3^{ts}$ and $ulp1^{ts}$ strains for the effects of SUMOylation. To get the yeast to express our plasmid, the cells are put into a "transformation mix" that contains our purified mutant plasmid and other ingredients that will assist in the uptake of the plasmid. The cells are then heat shocked to disrupt the membrane and allow for the plasmid to enter the cells, after this the cells are washed and prepared to be plated onto SC-Ura Glucose media which contains essential molecules except for uracil. Both the W303 and BY4741 strains lack the URA3 gene, preventing them from synthesizing Uracil so if they were grown on SC-Ura glucose media they wouldn't survive however the plasmid contains the URA3 gene, ensuring that only the yeast that had successfully picked up the plasmid would survive. Once the colonies have grown after incubation we can begin studying the mutant proteins through the various assays described in the methods.

Comparative Evaluation of New Mutants

This work serves as a replication of the work previously done by Carris Borland and Amanda Grassel only using the newer P426G expression vector instead. I assessed the three new α- synuclein mutants (A18T, A29S, and A53V) with the P426G vector first in the W303 yeast strain. I assessed these mutants along with wildtype α- synuclein and GFP serving as my positive and negative controls respectively. Both Carris and Amanda used A30P and A53T as additional positive controls due to them having been extensively characterized and researched, however I exclude them here to later compare the three newer mutants to all the older mutants together.

Carris and Amanda had previously reported some inconsistency regarding the degree of toxicity observed between the three new mutants in the PYES2.1 vector. Carris had reported that in the BY4741 strain, the mutants were more toxic than GFP but less so than wildtype and were differentially toxic from one another. When Amanda replicated this work in both BY4741 and W303, she found that the mutants were more toxic than the negative control and less so than wildtype but there was little to no difference in toxicity between the three mutants in either strain. To further validate these findings, I would additionally perform serial dilution spottings with the PYES2.1 vector in the same way I perform the dilution assay with the P426G vector, as well as together so I can make direct comparisons to the mutants' degree of toxicity between both the vectors.

Next, I assess the three mutants together alongside the older mutants. Previous work has found that the degree of toxicity and localization between the older mutants vary in a way that corresponds to the degree of toxicity. Mutant A30P, for instance, has been characterized as the least toxic among the α- synuclein mutants, with a comparable level of growth to the negative control and diffuse localization. A53T on the other hand is highly toxic much like wildtype and develops a high quantity of foci. In Carris's findings the new mutants had a moderate degree of toxicity that was greater than A30P and less than A53T, when Amanda repeated this work, she instead found the degree of toxicity for the new mutants was closer to A30P although still more toxic and this was seen in both BY4741 and W303 strains. Here I continue this work by including the other four older mutants and evaluate the toxicity of the three mutants relative to all the older mutants in both the P426G and the PYES2.1 vectors.

Lastly, I repeat the work from the previous two experimental assessments using the BY4741 yeast strain, and comparatively evaluate the level of toxicity, localization, and expression of the three newer mutants to that of the results within the W303 strain. The purpose of using multiple strains is to replicate, to a certain degree, the genetic variability of a population where a condition may affect individuals differently. The effect of genetic variability on synucleins has been observed previously in our lab and others where W303 strains show slightly higher level of toxicity and protein expression in comparison to the BY4741 strain despite having the same vector. I performed the 3 functional assays as mentioned previously in the methods: the five-fold serial dilution spotting, live image fluorescent microscopy, and western blotting. The spotting assay allows for comparative evaluation of yeast growth in presence or absence of α- synuclein expression. Fluorescent microscopy allows for qualitative and quantitative assessment of α-synuclein localization patterns within the cell. Western blotting allows for comparative assessment of α- synuclein protein expression between yeast.

New Mutants are Differentially Toxic Dependent on Expression

I assessed the level of α- synuclein protein expression of these new mutants between the P426G and PYES2.1 vectors (Figure 1A). Cells were grown and transferred into liquid galactose media for 12 hours, after which cell lysates were prepared to run the western blot. Anti-GFP antibody was used to detect the amount of synuclein present and anti-PGK antibody was used for loading control, when the PGK bands for each sample are sized evenly, then the GFP membrane can be assessed. I found that the amount of α- synuclein protein present in samples with the P426G vector is roughly equal with one another and similarly between the samples in the PYES2.1 vector. When comparing the two vectors there is a much greater amount of protein present from the P426G

samples compared to samples in the PYES2.1 vector. From this We can conclude that the differing and increased degree of toxicity along with the increased presence of foci is correlated to the higher amount of protein.

To evaluate the toxicity of these mutants I performed a 5-fold serial dilution spotting, counting the cells and creating the appropriate dilutions then spotting 2 µl of each sample dilution onto both a glucose and galactose media plate and left in an incubator to grow. The yeast cells growing on the glucose plate won't express α- synuclein and if done correctly all sample rows should have an equal level of growth, then the cells grown on the galactose plate can be assessed as they will express α- synuclein and the differences in growth are a result of the expressed synuclein.

The growth of the newer mutants (A18T, A29S, and A53V) in both P426G and PYES2.1 vectors is shown with two set of plates. The glucose plate of each (left picture) shows a roughly equal level of growth allowing for assessment of the galactose plate. In the P426G vector (Figure 1B) all mutants show a high level of toxicity relative to the negative control. A53V being the most toxic of them, showing the same or greater toxicity as wildtype α- synuclein. A18T is the next most toxic, appearing only slightly less toxic than wildtype and A29S being the least toxic of the three. When repeating the spotting assays with the mutants in the PYES2.1 vector (Figure 1B), I obtained similar results to what Amanda found, the mutants were all less toxic than α- and had no noticeable difference in toxicity between them.

Next, I assessed the three new mutants alongside with wildtype (in P426G vector) for patterns of α- synuclein localization within the cell across three timepoints. To do this the cells were grown and then transferred into a flask with liquid galactose media, at 6, 12, and 24 hours after this transfer some cells would be collected to take images under the fluorescent microscope. Over these timepoints I found that A53V and A18T showed a high number of foci, A53V appeared to have slightly more than A18T, and A29S remained primarily diffuse across the 24 hours, occasionally developing foci (Figure 1C). Running one-way analyses of variance revealed that there were significant differences in the number of foci between WT and the three new mutants across 6 and 12 hour time points (Figure 1D). At 6 hours the % phenotype of cells with foci between all comparisons was significant, A53V (M = 54.3, SD = 11.74) had significantly more foci than any of the mutants followed by WT (M = 44.3, SD = 6.24), A18T (M = 22.7, SD = 10.61), and A29S (M = 9.1, SD = 10.6), F (3, 12) = 1948, p < 0.0001. At 12 hours the trend remained the same however the difference between WT (M = 80.5, SD = 13.45) and A53V (M = 77.5, SD = 6.48) was less significant, F (3, 12) = 967.2, p < 0.0001 (Figure 1D).

New Mutants Comparable to Older Mutants

Next, I assess the toxicity of the new mutants in comparison to old ones, Carris and Amanda's work included mutants A30P and A53T together in their assessments of the three new mutants and here I expand this work by including the remaining older mutant variants. Assessing the toxicity of all the mutants together in the P426G vector showed that the toxicity of the newer mutants is more comparable to the more toxic older mutant variants (Figure 2A). Both mutants A18T and A53V show similarly high levels of toxicity like the toxic older mutants E46K, H50Q, A53T and A53E, and although mutant A29S is not as toxic as the other mutants but is still more toxic than both mutant G51D and A30P. In comparison to the PYES2.1 vector, the newer mutants are more comparable to the least toxic of the mutants, growing about the same amount as A30P, G51D, and GFP negative control.

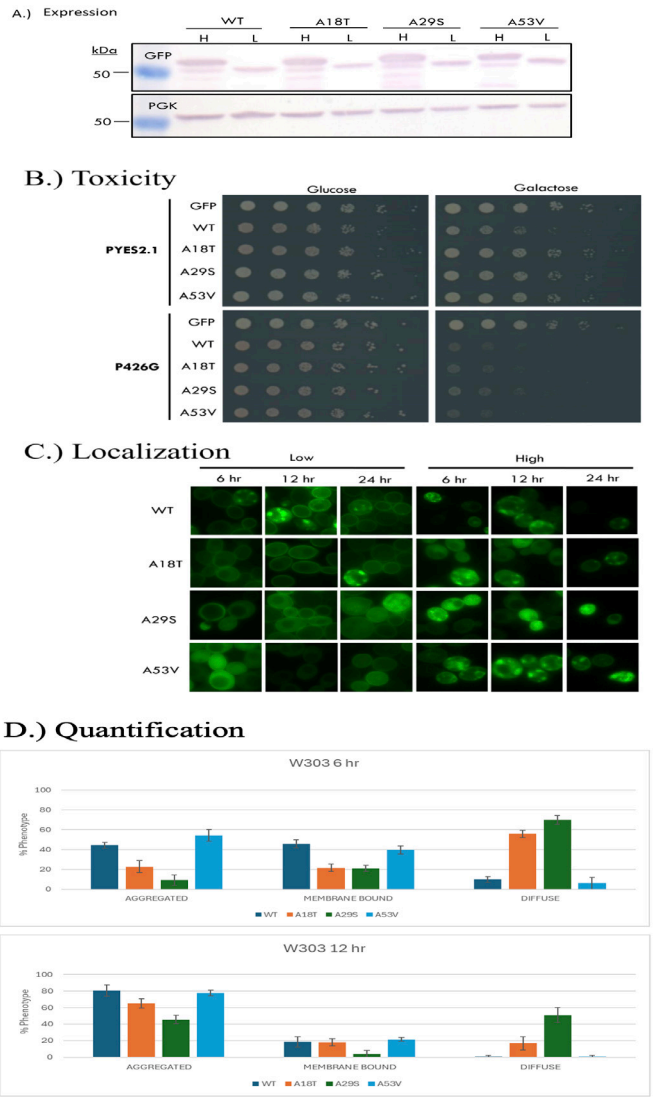


Figure 1: Comparative Evaluation of Newer Mutants in PYES2.1 and P426G Expression Vectors. Western blot of cell lysates prepared 12 hours post-induction in liquid SC-Ura Galactose media, showing expression levels of WT α-synuclein and the newer mutants (A18T, A29S, and A53V) in P426G and PYES2.1 vectors. Expression of α-synuclein was observed with an anti-GFP antibody and the expression of the PGK loading control was observed with an anti-PGK antibody. (n=3) Five-fold serial dilution spotting of yeast expressing GFP, WT α-synuclein, and mutants A18T, A29S, and A53V in either P426G or PYES2.1 expression vectors. Each dilution sample is spotted onto α-synuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). GFP serves as a negative control and WT α-synuclein serves as a positive control. Mutants A18T and A53V in P426G showed a higher degree toxicity comparable to WT α-synuclein with A29S being the least toxic of the three. In the PYES2.1 vector all three mutants were less toxic than wildtype and did not show a varying degree of toxicity as in the P426G vector. (n=5) Representative fluorescent microscopy images showing the localization for yeast expressing GFP or emGFP tagged WT α-synuclein and new α synuclein mutants (A18T, A29S, and A53V). Images were captured at 6, 12, and 24 hours post-induction in liquid SC-Ura Galactose media. (n=4) Quantification of the microscopy images for WT and each of the newer αsynuclein mutants (A18T, A29S, and A53V) were quantified according to the observed phenotypes at 6 and 12 hour time points. The percentage of cells exhibiting cytoplasmic diffusion, intracellular foci, and association with the membrane are displayed. A total of 1,000 cells displaying foci from each of four separate trials were quantified.

The localization of the new mutants compared to the older variants all shows a correlated amount of aggregation relative to their

toxicity (Figure 2B). Highly toxic mutants such as the A53 mutants had a significant number of foci much like wildtype α- synuclein, A18T has a somewhat lower amount of foci, followed by A29S that has very few foci, and then mutants A30P and G51D which are entirely diffused. Quantification of the number of cells containing foci in each mutant type across 6 and 12 hour timepoints revealed significant differences (Figure 2D). At 6 hours the difference in % cells displaying foci was significant between each variant however comparisons between A53V (M = 54.3, SD = 11.74), A53T (M = 59.6, SD = 10.75), and A53E (M = 55.4, SD = 27.6) revealed no significant difference between those three, F (9, 30) = 3217, p<0.0001. In comparison to 12 hours the result are more varied, with no significant difference in foci present between WT (M = 80.5, SD = 13.45) and A53V (M = 77.5, SD = 6.48), WT and A53T (M = 71.7, SD = 14.35), A18T (M = 64.9, SD = 11.16) and E46K (M = 58.6, SD = 30.52), A18T and H50Q (M = 65.4, SD = 14.93), A29S (M = 45.4, SD = 10.18) and A53E (M = 51.1, SD = 22.23), and between H50Q and A53T, while every other comparison revealed significance, F (9, 30) = 1024, p<0.0001.

Evaluating the protein expression between the new and old mutants showed that they had a similar level of expression and affirms that the differences in toxicity and localization between the mutants is a result of the mutations intrinsic properties (Figure 2C). Interestingly, the GFP bands for the less toxic mutants A29S, A30P, and G51D appear slightly larger than those of the more toxic mutants.

Strain Differences in Mutant Toxicity

Next the toxicity and localization of the three newer α- synuclein mutants in both the P426G and PYES2.1 vector are assessed in the BY4741 strain. In Amanda's work she reported no major difference in the degree of toxicity for the three mutants between the W303 and BY4741 strains, noticing only a minor difference in toxicity with wildtype α- synuclein and the older mutants A30P and A53T being slightly more toxic in W303. I repeated the spotting assay with the PYES2.1 vector and obtained the same results. The three mutants did not appear differentially toxic between each other as in the W303 strain, additionally the mutants showed a similar degree of toxicity relative to wildtype and the older mutants as in the W303 strain (Figure 3A). When repeating the assay using the P426G vector I also found that the results did not differ significantly between the strains. Much like in W303 the mutants showed the same differential levels of toxicity and the degree of toxicity was comparable to the more toxic older mutants. The localization of the mutants in BY4741 was mostly similar to what was observed in W303 however there are some differences despite. As is seen in the W303 strain, throughout all timepoints the more toxic mutants displayed a higher amount of aggregation while the less toxic mutants (A29S, A30P, and G51D) were largely diffuse (Figure 3B). At the 6 hour time point there were less significant differences in amount of foci between mutants as compared to W303. In W303 the only mutant that had no significant differences between one another at 6 hours were each of the A53 variants, in BY4741 however those three mutants were significantly different from one another statistically and there was less significant differences between less toxic mutants like A29S (M = 57.8, SD = 4.11) and G51D (M = 23.4, SD = 6.55), and between some of the other toxic mutants like E46K (M = 287.0, SD = 9.93) and A53T (M = 246.8, SD = 9.88), F (9, 30) = 620.7, p<0.0001 (Figure 3C). At the 12 hour timepoint, the amount of foci present between the A53 mutant variants was no longer significantly different, additionally other toxic variants like E46K (M = 569.8, SD = 11.50) and H50Q (M = 613.0, SD = 7.62) also did not differ significantly from the A53 mutants with the amount of foci present, F (9, 30) = 2047, p<0.0001.

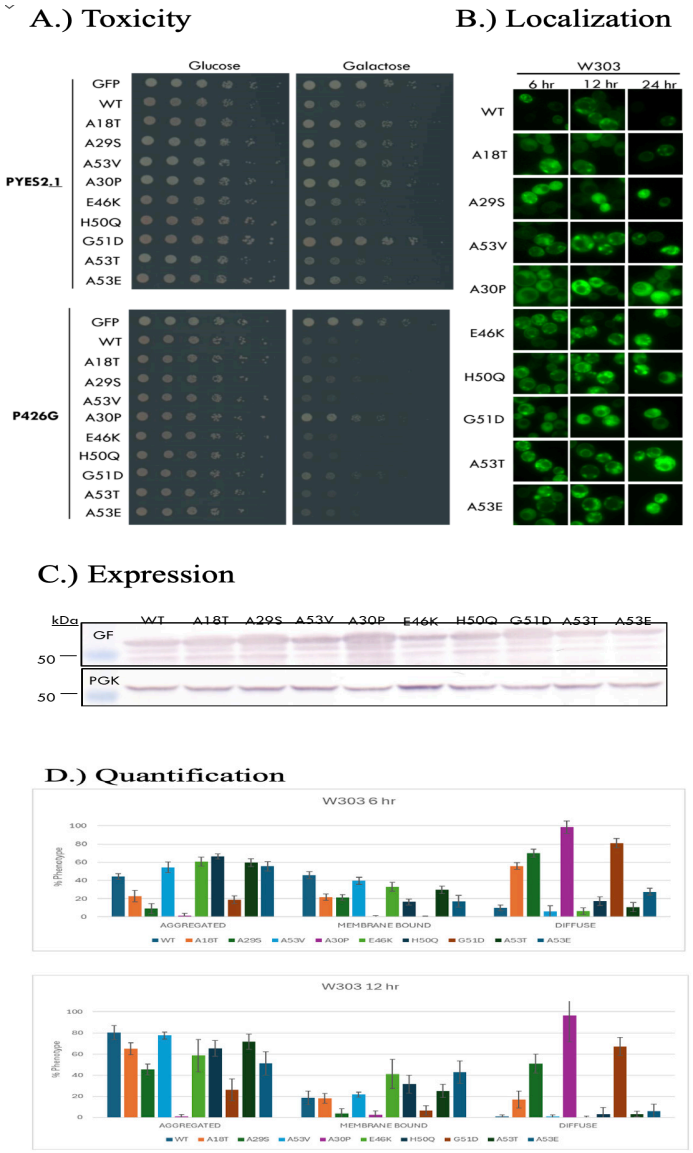


Figure 2: Comparative Evaluation of New Mutants to Older Mutants. Five-fold serial dilution spotting of yeast expressing GFP, WT α-synuclein, and mutants A18T, A29S, A53V, A30P, E46K, H50Q, G51D, A53T, and A53E in either P426G or PYES2.1 vectors. Each dilution sample is spotted onto α-synuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). GFP serves as a negative control and WT α-synuclein serves as a positive control. Newer mutants were more comparable to more toxic older mutants in P426G vector while in the PYES2.1 vector the new mutants were more comparable to the least toxic of the older mutants. (n=5). Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein α synuclein mutants A18T, A29S, A53V, A30P, E46K, H50Q, G51D, A53T, and A53E. Images were captured at 6, 12, and 24 hours post-induction in liquid SC-Ura Galactose media. (n=4) .Western blot of cell lysates prepared 12 hours post-induction in liquid SC-Ura Galactose media, showing expression levels of WT α-synuclein, the newer mutants (A18T, A29S, and A53V), and older mutants (A30P, E46K, H50Q, G51D, A53T, A53E) in P426G. Expression of α-synuclein was observed with an anti-GFP antibody and the expression of the PGK loading control was observed with an antiPGK antibody. (n=3) . Quantification of the microscopy images for WT, the newer mutants (A18T, A29S, and A53V) and older mutants (A30P, E46K, H50Q, G51D, A53T, and A53E) were quantified according to the observed phenotypes at 6 and 12 hour time points. The percentage of cells exhibiting cytoplasmic diffusion, intracellular foci, and association with the membrane are displayed and cells displaying foci across four separate trials were quantified.

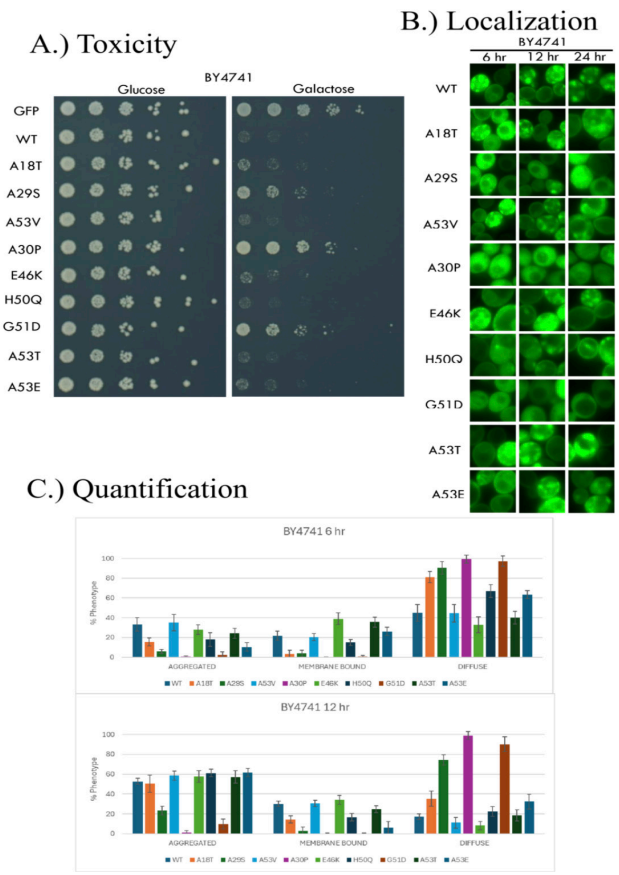


Figure 3: Comparative Evaluation of Mutants in BY4741 Strain. Five-fold serial dilution spotting of yeast expressing GFP, WT α-synuclein, mutants A18T, A29S, A53V, A30P, E46K, H50Q, G51D, A53T, and A53E in BY4741. Each dilution sample is spotted onto α-synuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). GFP serves as a negative control and WT α-synuclein serves as a positive control. Phenotype of all mutants matches that of what is seen in the W303 strain. (n=5). Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein and α synuclein mutants A18T, A29S, A53V, A30P, E46K, H50Q, G51D, A53T, and A53E. Images were captured at 6, 12, and 24 hours post-induction in liquid SC-Ura Galactose media. (n=4). Quantification of the microscopy images for WT, the newer mutants (A18T, A29S, and A53V) and older mutants (A30P, E46K, H50Q, G51D, A53T, and A53E) in BY4741 were quantified according to the observed phenotypes at 6 and 12 hour. The percentage of cells exhibiting cytoplasmic diffusion, intracellular foci, and association with the membrane are displayed and cells displaying foci across four separate trials were quantified.

Creating Substitution Mutations

I next want to investigate the intrinsic properties of these mutations that are contributing to toxicity, here focusing on the change in amino acid of each mutation. To investigate this, I aimed to answer whether it was the loss of the original amino acid (Alanine) or the gain of the mutant amino acid (Threonine, Serine, and Valine) that causes the toxicity of each, doing so by creating sets of amino acid substitution mutants with help from one of our Richter scholars, Kate Feist. These mutants will be assessed through the same assays as before, primarily using the W303 strain and performing serial dilution spotting for determining any differences in toxicity with GFP, wildtype, and natural mutant as controls, fluorescence microscopy to determine localization patterns, and western blotting to determine level of expression. The substitution mutations replaced the mutant amino acid of each of the new mutants with another amino acid belonging to one of four categories of amino acids (hydrophobic, hydrophilic, acidic, or basic). For A18T and A29S, I would exchange the natural mutation for serine or threonine as the hydrophilic amino acid, glycine as the hydrophobic, glutamic acid as the acidic, and arginine as the basic. With A53V since a natural mutant already exists using threonine and glutamic acid, asparagine and aspartic acid would be used instead for the hydrophilic and acidic substitutions. If the toxicity of the mutants was due to the loss of the original alanine amino acid then all the substitutions will be toxic regardless, however if it's the gain of the mutant amino acid that influences toxicity then the substitution that belongs to the same category as the natural mutant would likely be toxic as well while the rest are not toxic.

Loss or Gain?

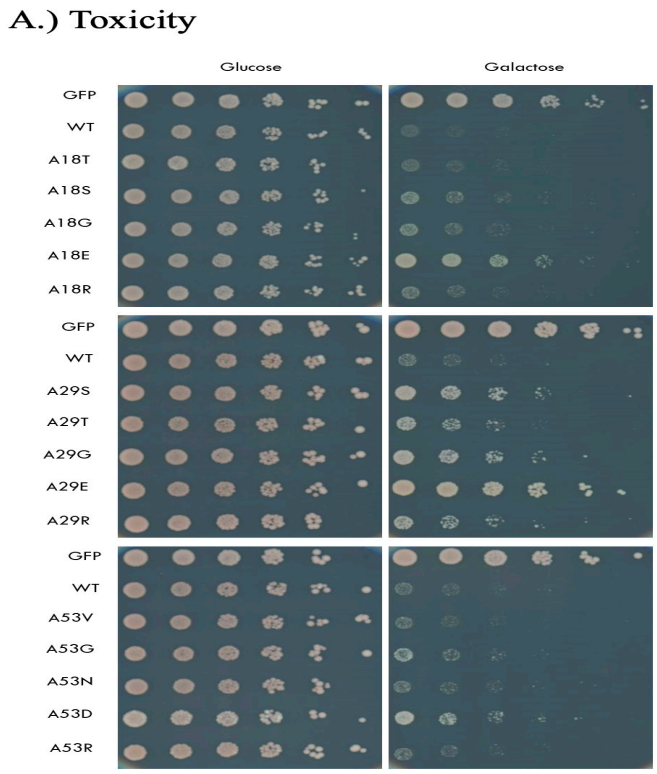
Assessing the toxicity of the substitution mutants didn't provide a clear answer as to whether it's the loss or gain of the amino acid that drives the toxicity of each mutant. As mentioned earlier if the loss of the original amino acid was the causing factor then all substitutions will be toxic and if the gain of the mutant amino acid is the contributing factor then the substitution of the same category would be toxic and the rest not toxic. The serial dilution spotting showed mixed results in this regard, with mutants A18T and A53V it mostly appeared to be loss driving the toxicity however the acidic substitution in both cases was less toxic (Figure 4A). A29S on the other hand was more complicated as each substitution varied in toxicity, the nonpolar substitution (G) appears just as toxic as the natural mutant while the polar (T) and basic (R) substitutions appear slightly more toxic, then the acidic substitution (E) is also significantly less toxic as with A18T and A53V.

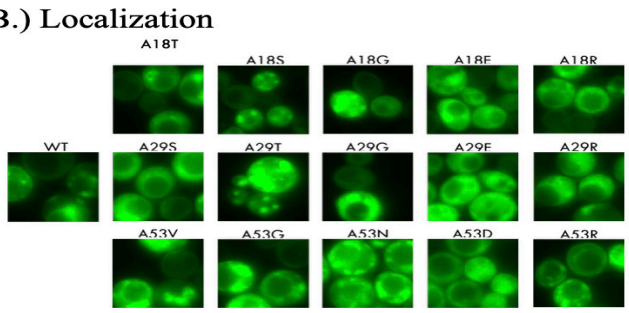
Localization patterns of the substitution mutants reflect the toxicity patterns, substitutions that more closely matched the natural mutant showed a similar pattern of localization as well. A18S, G, and R show a similar number of diffuse and aggregated cells as A18T, the A29G and A29R substitutions were similar in phenotype to A29S, with A29T appearing to have more cells with aggregates, and A53G, N, and R substitutions having many aggregated cells as A53V does (Figure 4B). The least toxic substitution mutants (A18E, A29E, and A53D) all showed significantly more phenotypically diffuse cells relative to the natural mutant, A53D still have many aggregated cells but significantly less relative to the other A53 substitutions, A18E had very few cells with aggregates and A29E was entirely diffuse. Quantifying the A18T substitutions revealed no significant difference of foci presence between A18S ($M = 508.3$, $SD = 18.93$), A18G ($M = 504.8$, $SD = 14.66$), and A18R ($M = 478.3$, $SD = 17.61$), while A18E was drastically different from each of them, $F(5, 18) = 1137$, $p<0.0001$ (Figure 4C). Similarly with A53V substitutions there was largely no significant differences in foci present for A53G ($M = 851.5$, $SD = 24.86$), A53N ($M = 850.0$, $SD = 18.07$), and A53R ($M = 853.0$, $SD = 19.78$), while A53D ($M = 185.3$, $SD = 17.59$) was significantly different, $F(5, 18) = 930.1$, $p<0.0001$ (Figure 4C). Comparing between A29S substitutions however showed significant differences between most substitutions, with no significant difference only between A29S ($M = 485.3$, $SD = 10.18$) and A29R ($M = 502.5$, $SD = 18.70$), $F(5, 18) = 1124$, $p<0.0001$ (Figure 4C). Western blotting showed that the amount of protein expression is consistent between the substitutions, the natural mutant and wildtype, indicating that the differences in toxicity for these substitutions is not a result of potentially altered gene expression.

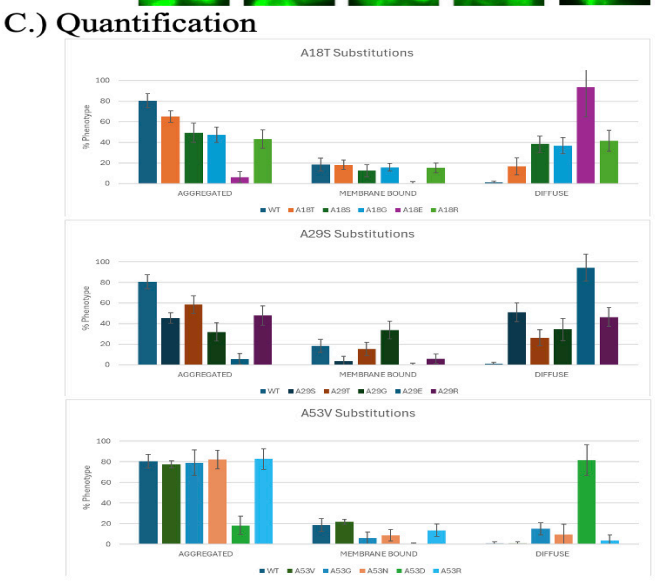
Creating Combinatorial Mutations

Next, I aimed to assess how combining these mutations would alter/contribute to toxicity and what might that reveal about the importance of each location regarding mutant toxicity. To answer this I created a set of combinatorial mutants with help from our second Richter scholar Mieng Chandavimol. These mutants will be assessed with the same assays as before, using the W303 strain to perform serial dilution spotting for evaluating toxicity using GFP, wildtype, and single mutants as controls, fluorescence microscopy to determine localization patterns, and western blotting to determine level of expression. Each of the single mutants are combined to create three double mutants (A18T+A29S, A18T+A53V, and A29S+A53V) and one triple mutant A18T+A29S+A53V), the phenotypes of each of these are then assessed relative to the single mutants. I expected to see that the toxicity of the combinatorial mutants would appear to be either additive to each other or averaged, with additive the toxicity of the mutants would be enhanced and indicates the locations of those mutant are independent

in relation to how they contribute to toxicity whereas if the toxicity was averaged between the mutants in combination it would indicate that those positions both have importance to structure/pathology and are interacting.







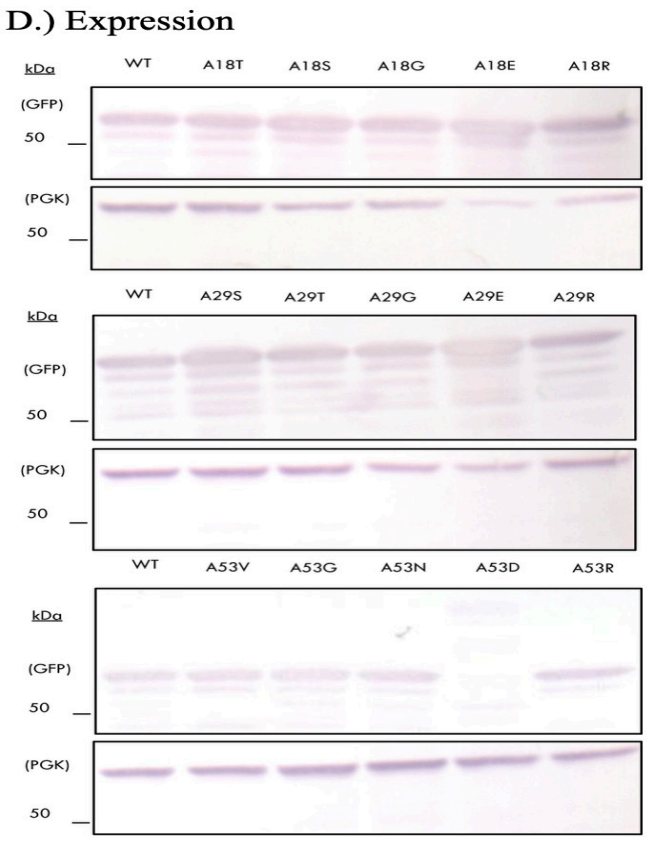


Figure 4: Comparative Evaluation of Amino Acid Substitution Mutants. Five-fold serial dilution spotting of yeast expressing GFP, WT α-synuclein, mutants A18T, A29S, A53V, and their amino acid substitutions: A18S, G, E, R; A29T, G, E, R; and A53G, N, D, R. Each dilution sample is spotted onto αsynuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). GFP serves as a negative control with WT α-synuclein and the natural mutants serving as positive controls. Toxicity of substitutions mutants varied depending on what amino acid was substituted. (n=5). Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein, α synuclein mutants A18T, A29S, A53V, and their substitutions. Images were captured at 12 hours post-induction in liquid SC-Ura Galactose media. (n=4). Quantification of the microscopy images for WT, the new mutants A18T, A29S, and A53V, and their respective substitution mutants were quantified according to the observed phenotypes at 12 hours. The percentage of cells exhibiting cytoplasmic diffusion, intracellular foci, and association with the membrane are displayed and cells displaying foci across four separate trials were quantified. Western blot of cell lysates prepared 12 hours post-induction in liquid SC-Ura Galactose media, showing expression levels of WT α-synuclein, mutants A18T, A29S, A53V, and their substitutions. Expression of α-synuclein was observed with an anti-GFP antibody and the expression of the PGK loading control was observed with an anti-PGK antibody. (n=2)

Location Dependent Influence on Mutation Toxicity

For the combinatorials I expected to either see an averaging between the toxicity and localization of the combined mutants or an additive effect in which the toxicity of the combinatorials would be greater than the that of the individual natural mutants. The spotting reveals a different story in which the phenotype of the combinatorial mutant appears to be dominated by the less toxic mutant (Figure 5A), this is seen most clearly with A18T+A53V and the A29S+A53V combinatorials where A18T+A53V toxicity closely resembles that of A18T and the A29S+A53V mutant toxicity closely matches that of A29S. For the other two combinatorial mutants, A18T+A29S and A18T+A29S+A53V, the result is somewhat different, instead of the toxicity matching that of only one of the mutants, the toxicity is significantly reduced such that they grow about as much as the GFP negative control.

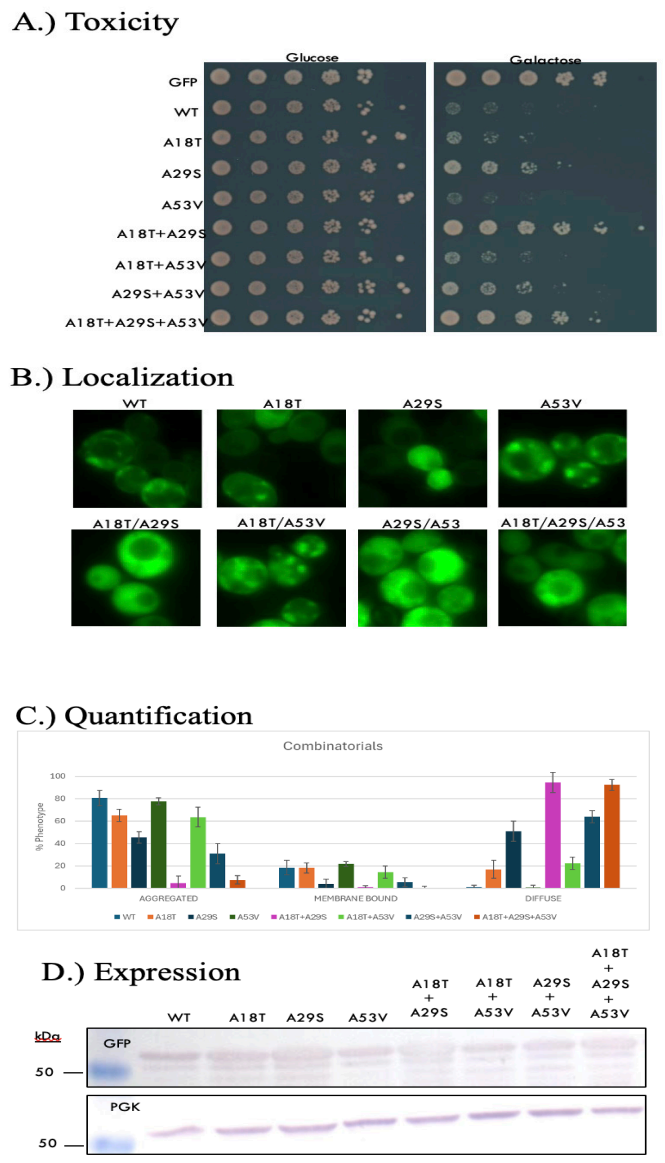


Figure 5: Comparative Evaluation of Combinatorial Mutants. Five-fold serial dilution spotting of yeast expressing GFP, WT α-synuclein, mutants A18T, A29S, A53V, and combined mutants A18T+A29S, A18T+A53V, A29S+A53V, and A18T+A29S+A53V. Each dilution sample is spotted onto αsynuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). GFP serves as a negative control with WT α-synuclein and the three new mutants serving as positive controls. Toxicity of combined mutants was varied but tended to be dominated by the less toxic of the combined mutants. (n=5) Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein, α synuclein mutants A18T, A29S, A53V, and the combined mutants. Images were captured at 12 hours post-induction in liquid SC-Ura Galactose media. (n=4) Quantification of the microscopy images for WT, the new mutants A18T, A29S, and A53V, and the combinatorial mutants were quantified according to the observed phenotypes at 12 hours. The percentage of cells exhibiting cytoplasmic diffusion, intracellular foci, and association with the membrane are displayed and cells displaying foci across four separate trials were quantified. Western blot of cell lysates prepared 12 hours post-induction in liquid SC-Ura Galactose media, showing expression levels of WT α-synuclein, mutants A18T, A29S, A53V, and the combined mutants. Expression of α-synuclein was observed with an anti-GFP antibody and the expression of the PGK loading control was observed with an anti-PGK antibody. (n=2)

For the localization of these combinatorials the pattern mostly matches with the results from the spotting assay, the least toxic mutants (those being combinations with A29S present) have mostly diffuse localization while the A18T+A53V mutant is largely aggregated (Figure 5B). Quantification mostly reflects this with no significant difference between presence of foci between A18T (M = 679.0, SD = 11.17) and A18T+A53V (M = 620.3, SD = 17.69), and between A18T+A29S (M = 46.8, SD = 12.09) and A18T+A29S+A53V (M = 73.5, SD = 7.42), $F(7, 24) = 2478$, $p<0.0001$ (Figure 5C). Western blotting of the mutants showed relatively equal levels of protein expression hence the toxicity of the mutants isn't a result of altered expression, interestingly the less toxic mutants appear to have slightly larger GFP.

Altered Cellular Conditions

For this chapter the focus is to assess how extrinsic factors such as altered cellular environments can affect the toxicity and pathology of these newer mutants. Amanda and Carris reported in their findings that the newer mutants exhibited different sensitivities to certain environmental conditions. Amanda reported that A29S was insensitive to nitrative stress in comparison to A18T and A53V, and work from both Carris and Amanda using SUMOylation report that A29S appears to be sensitive to low SUMOylation. Because the toxicity of the mutants was drastically affected by the level of expression, I wanted to see how the sensitivities of these mutants to altered cellular environments would be affected with higher expression. For this chapter I chose to evaluate the toxicity and localization of these mutants in altered nitrative stress and SUMOylation conditions to have a comparison with previous findings, as well as the effects of mitochondrial dysfunction as impaired mitochondrial function is a major characteristic of PD pathology. To do this the mutant plasmids are transformed into genetically altered strains of yeast that induce the desired condition, nitrative and mitochondrial conditions induced via gene knockouts (cox5AD and cox5BD for nitrative; sod2D, hsp31D, and hsp34D for mitochondrial dysfunction) and SUMOylation via modified temperature sensitive strains ($smt3^{ts}$ and $ulp1^{ts}$). Assessing the knockout yeast strains doesn't differ from how previous assessments were made, however the temperature sensitive SUMOylation strains require assessment of the cells grown at 25°C as a control and at 30°C for the experimental condition. At 25°C the amount of SUMOylation is still normal in both strains, however at 30°C the condition is induced with $smt3^{ts}$ exhibiting low SUMO and $ulp1^{ts}$ a high SUMO environment.

A29S Insensitive to Altered Nitrative Environment

Serial dilution spotting comparing the new mutants in a high nitrative stress vs low nitrative stress environment revealed different sensitivities between the mutants and reaffirms the previous findings of Carris and Amanda. A18T and A53V both show a similar level of sensitivity to high and low stress to that of wildtype, the high stress environment is toxic for each while the low stress environment significantly reduces their toxicity (Figure 6A). A29S on the other hand shows a lower degree of sensitivity to altered nitrative conditions, particularly the low stress condition, as the difference in toxicity between A29S in the high stress condition vs the low stress condition is noticeably less than the observed differences with A18T and A53V. The localization pattern of these mutants under the high and low nitrative stress is atypical to the trend where high toxicity is correlated to more aggregation. Interestingly, the mutants and WT appeared to show somewhat fewer aggregates under the high stress condition in comparison to the low stress condition (Figure 6B). Quantification of foci at 12 hour timepoint doesn't show significant differences between high and low stress conditions for mutants A18T or A53V but did show significance between WT and A29S. With the high stress condition, WT ($M = 477.3$, $SD = 24.00$) and A29S ($M = 282.3$, $SD = 11.51$) had slightly less foci compared to the low nitrative stress condition, WT ($M = 576.0$, $SD = 42.58$) and A29S (M = 403.0, SD = 13.23), $F(7, 16) = 55.92$, $p<0.0001$ (Figure 6C).

Mutant Sensitivity to Low SUMOylation

Next, I investigate the effects of SUMOylation on the new mutants. The toxicity of the mutants in the 25°C condition when both strains are exhibiting a normal level of SUMOylation shows the same level of toxicity as previously seen, with A53V being as toxic as wildtype, A18T being

slightly less toxic, and A29S being noticeably less toxic (Figure 7A). Under 30°C we can then assess how SUMOylation affects the mutants, the ulp1^{ts} strain (high SUMOylation) doesn't noticeably alter the toxicity of the mutants or wildtype however the smt3^{ts} (low SUMOylation) strain significantly enhances the toxicity of wildtype and all the mutants. A29S is the most sensitive to the effects of decreased SUMOylation as was noticed by Carris and Amanda in their assessments, the difference in toxicity between the smt3^{ts} and ulp1^{ts} strains is greatest with A29S as compared to the other mutants or wildtype (Figure 7A). The localization pattern of the mutants at 25°C is the same between both strains, supporting what was seen with the spotting assay in the 25°C condition. Under 30°C the localization of mutants in the smt3^{ts} strain shows slightly more aggregates in comparison to the ulp1^{ts} strain, with noticeably higher number of A29S mutants cells developing aggregates (Figure B). Images were taken at 12- and 24-hour timepoints as the number of expressing cells was too low at the 6-hour timepoint. Unexpectedly the number of expressing cells was considerably lower in both the strains in comparison to other strains.

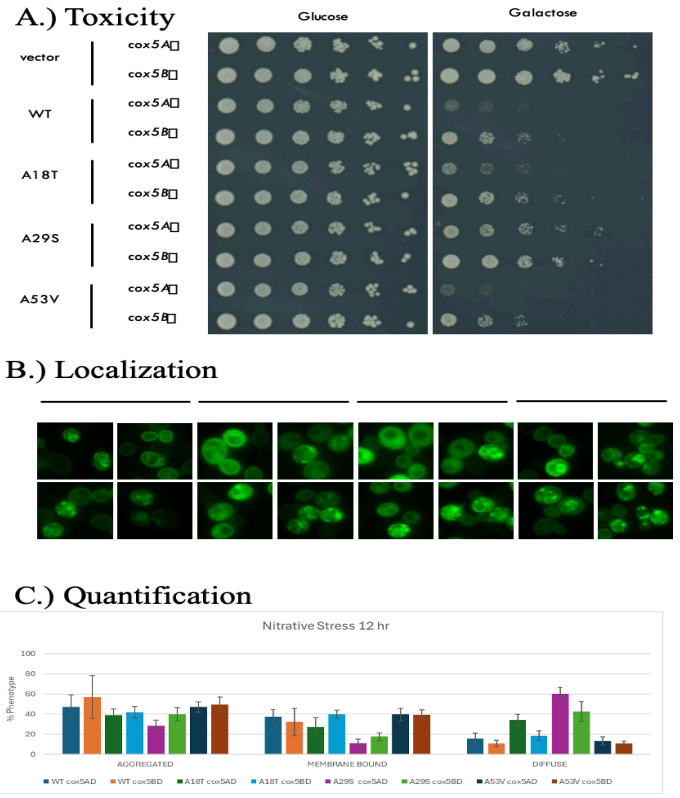


Figure 6: Assessment of New Mutant in Altered Nitrative Conditions. Five-fold serial dilution spotting of yeast with empty vector, WT α-synuclein, and mutants A18T, A29S, A53V in either cox5AD or cox5BD strains. Each dilution sample is spotted onto α-synuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). The empty vector serves as a negative control and WT α-synuclein as the positive control. Toxicity of all mutants was reduced in low stress although A29S appears less sensitive to this effect. (n=4). Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein and α synuclein mutants A18T, A29S, and A53V in either cox5AD or cox5BD strains. Images were captured at 6 and 12 hours post-induction in liquid SC-Ura Galactose media. (n=3).Quantification of the microscopy images for WT and the new mutants (A18T, A29S, and A53V) in high and low nitrative stress conditions were quantified according to the observed phenotypes at 12 hours. The percentage of cells exhibiting cytoplasmic diffusion, intracellular foci, and association with the membrane are displayed and cells displaying foci across four separate trials were quantified.

Mutant Sensitivity to Different Causes of Oxidative Stress

The mutants are assessed in three different knockout strains that induce high oxidative stress and are compared to the BY4741 strain as a control for normal levels of oxidative stress. The spotting assays revealed that the mutants exhibited sensitivity to oxidative stress that was dependent on the specific strain used. It's been seen in many research labs that higher level of oxidative stress enhances the toxicity of wildtype α- synuclein, however from my assessments I find more mixed results concerning both wildtype and the mutants. I also find an odd but consistent issue in which the BY4741 control strain grows slightly less in comparison to the knockouts despite having repeated the experiment several times, making an accurate comparison more difficult. Looking solely at the knockout strains however, I can report that in wildtype and A18T the hsp34D most significantly reduces toxicity, in A29S the hsp31D and hsp34D strains potentially enhance the toxicity of the mutant, and in A53V the sod2D and hsp31D strains reduce the toxicity (Figure 8A).

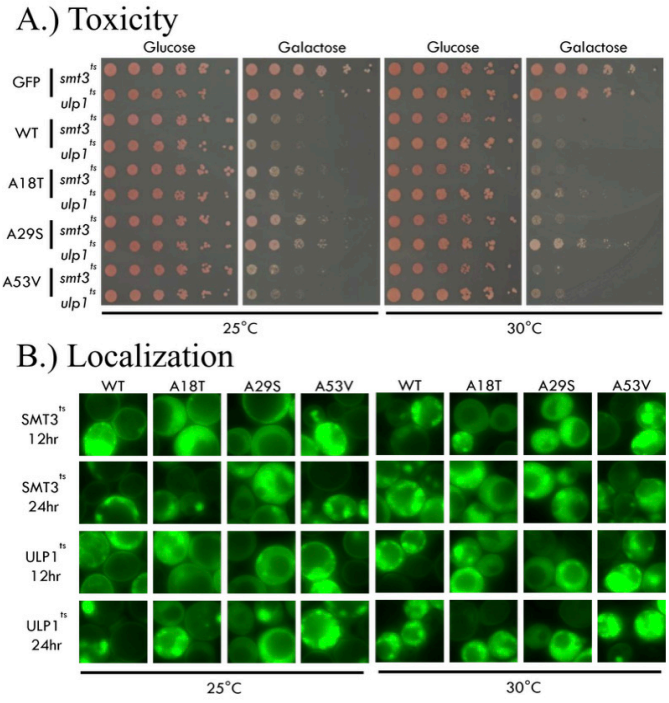


Figure 7: Assessment of New Mutants in Altered SUMOylation Conditions. Five-fold serial dilution spotting of yeast expressing GFP, WT α-synuclein, and mutants A18T, A29S, A53V in either smt3^{ts} or ulp1^{ts} strains. Each dilution sample is spotted onto α-synuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right) and grown in an incubator at 25° and 30°C. GFP serves as a negative control and WT α-synuclein as positive control. Toxicity of all mutants was enhanced in the smt3^{ts} with A29S being more sensitive to this effect. (n=4). Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein and α synuclein mutants A18T, A29S, and A53V in either cox5AD or cox5BD strains. Images were captured at 6 and 12 hours post-induction in liquid SC-Ura Galactose media. (n=3).

The localization of these strains follows a similar pattern to what is shown in the spotting assays and it helps determining whether the strains enhance or reduce the toxicity of these strains. The amount of aggregating cells remains fairly similar between BY4741, sod2D, and hsp31D for both wildtype and A18T, with hsp34D having a lower amount of aggregation (Figure 8B). With A29S the hsp31D and hsp34D strains show a higher degree of aggregation indicating that there is an increase in toxicity for the mutant, and for A53V the sod2D and hsp31D strains show a lower amount of aggregation as well (Figure 8B).

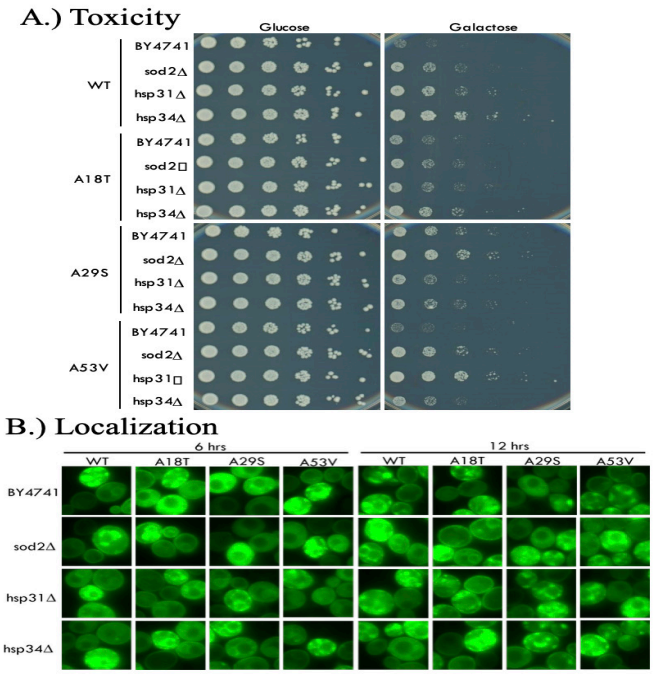


Figure 8: Assessment of New Mutants in Altered Mitochondrial Conditions. Five-fold serial dilution spotting of yeast with WT α-synuclein, and mutants A18T, A29S, A53V in either BY4741, sod2D, hsp31D, or hsp34D strains. Each dilution sample is spotted onto α-synuclein repressing (SC-Ura Glucose, left) and expressing media (SC-Ura Galactose, right). WT α-synuclein and the mutants in the BY4741 strain serve as positive controls. Toxicity of each mutant varied depending on the strain they were expressed. (n=4) Representative fluorescent microscopy images showing the localization for yeast expressing GFP tagged WT α-synuclein and α synuclein mutants A18T, A29S, and A53V in BY4741, sod2D, hsp31D, or hsp34D strains. Images were captured at 6- and 12-hours post-induction in liquid SC-Ura Galactose media. (n=3)

DISCUSSION

Parkinson's Disease is among the most debilitating diseases that exist today, with high levels and aggregation of a synuclein protein being correlated to the severity of degeneration. Studying the mutant forms of a synuclein has been an important focus of PD research as these mutants can reveal important aspects concerning a synuclein and its' role in PD, six mutants (A30P, E46K, H50Q, G51D, A53T and A53E) have already been extensively studied by researchers however three other variants (A18T, A29S, and A53V) haven't been evaluated to the same degree. Previous work in our lab has investigated the properties of these mutants found mixed results regarding the nature of these mutants' toxicity, leading me to reassess them using higher level of expression in addition to evaluating intrinsic properties influencing their toxicity. I found that 1) these mutants have differential level of toxicity dependent on their expression, 2) that the toxicity of the is not fully explained by loss of original nor gain of the mutant amino acid, 3) that mutations at certain locations have a larger influence over toxicity that others, and 4) that the mutants show unique sensitivities to certain cellular conditions.

New Mutants are Differentially Toxic

Both Amanda and Carris obtained different results regarding the toxicity of the mutants, Carris reported that the mutants were differentially toxic with A29S being least toxic of the three (Borland Thesis, 2021) while Amanda found that there was no discernible difference between them (Grassel Thesis, 2023). I repeated the spotting assay with the mutants in the PYES2.1 vector and came to the same conclusion Amanda had, however when evaluating the mutants in the P426G vector I instead find that the mutants are differentially toxic with A53V being the most toxic, A18T being slightly less toxic, and A29S being the least toxic of the three, similar to what Carris had seen. Interestingly, however the toxicity of the mutants A53V and A18T closely resembles that of WT which differs from what both Carris and Amanda had found where all three mutants appeared less toxic than WT. Western blotting confirmed that there was a difference in protein expression to explain the different results seen between the vectors, and then I assessed the localization of the proteins within live cells. The pattern of localization with the mutants in P426G reinforces my findings concerning their toxicity as A53V displays a near identical pattern of protein localization as WT, A18T being similar but having more cells with a diffuse pattern to explain the slightly lower level of toxicity, and A29S being the least toxic having the lowest number of cells with aggregation and highest amount of them being diffuse.

This result itself is not entirely surprising as a well-established model of neurodegenerative disorders is the nucleation polymerization model that ties the level of protein expression to how long it takes for aggregates to form, and different mutant form of a synuclein are shown to alter the rate of aggregate formation (Wood et al., 1999). So in the PYES2.1 vector where protein concentration is lower, Carris found differences while Amanda afterwords could not, potentially indicating that the level of expression of these mutants in PYES2.1 is bordering the threshold for nucleation and polymerization to translate into toxicity. Then in the P426G vector the concentration is well past threshold for rapid nucleation and polymerization to take place leading to the drastic increase in toxicity. The unique phenotypes of the mutants are consistent as well to the findings reported from another lab that investigated A18T and A29S in a yeast model as well (Joshi et al., 2023), finding that A18T had a fair amount of aggregates formed and A29S was largely diffuse in phenotype, as well as exhibiting different sensitivity to altered oxidative conditions.

New vs Old Mutants

I next looked at how the new mutants would compare to the older six mutants: A30P, E46K, H50Q, G51D, A53T, and A53E. I performed spotting assays of all mutants together with both the PYES2.1 and P426G vectors, then looked at localization of the mutants in P426G, and finally performed and assessed western blots of all mutants. Within the PYES2.1 vector all the new mutants are less toxic than WT and are very close to the GFP negative control in terms of growth, closely matching A30P and G51D of the older mutants which also appear to be nontoxic, the other mutants (E46K, H50Q, A53T, and A53E) however are roughly as toxic as wildtype. In the P426G vector the new mutants appear much more toxic with A53V as toxic as WT, A18T slightly less toxic and A29S being the least. The older mutants by comparison don't differ much regarding the pattern of toxicity seen in the PYES2.1 vector, both A30P and G51D remain the least nontoxic with G51D becoming perhaps slightly more toxic, and the other mutants retaining a high level of toxicity matching that of WT. Overall I observe that the old mutants E46K, H50Q, A53T, and A53E as well as new mutant A53V closely resemble WT in level of toxicity, A18T is slightly less, A29S is even less toxic than A18T, the other two older mutants A30P and G51D were the least nontoxic. The effects of high expression on the older mutants were not as drastic as compared to the newer mutants, and overall the findings regarding their toxicity is consistent with previous work in our lab.

The localization pattern of the old mutants is closely linked with their degree of toxicity, A30P being the least toxic is nearly entirely diffuse whereas A53T which is highly toxic has a similar localization pattern to that of WT and A53V. There is a small but noticeable presence of cells developing aggregates with the G51D mutant which appeared to become slightly more toxic in high expression. Additionally the other older mutants that are as toxic as wildtype, with exception of A53T, have significantly more cells with membrane bound localization in comparison to WT.

Through western blotting I determined that the level of expression between the new and old mutants are roughly equal hence the differences in toxicity and localization is a result of the mutants' properties. There is an interesting trend regarding the mutants in which the less toxic

mutants (A29S, A30P, and G51D) appear to have slightly higher amounts of synuclein protein expressed in comparison to the other more toxic variants. A possible reasoning is that in response to stressful conditions, yeast enable mechanisms that lead to decreases the level of translation for proteins (Simpson & Ashe, 2012). It's possible that this difference in expression is a result of the more toxic mutant variants triggering stress responses that leads to slight decrease in expression or synuclein production, while the less toxic mutants, such as A30P, don't induce a stress response allowing for unaffected level of protein expression.

Two Strains

Genetic variability is a potential reason why some individuals are more susceptible to diseases like PD than others, making it an important variable to account for in research. To mimic this our lab uses the W303 and BY4741 yeast strains to see how the pathology of the synucleins might differ in different strains, my work is primarily focused on the W303 strain so here I evaluated how the new and old mutants appear in BY4741. The toxicity pattern of both the new and old mutants in the BY4741 however does not differ from that of W303, and similarly the localization pattern of the mutants remains the same between strains. Previous work in the lab has found that the W303 strain expresses the synucleins at a slightly higher level compared to BY4741, despite this I was unable to see a drastic difference hence the difference between the strains was too small to have a noticeable effect on the toxicities of the mutants.

Mutant Toxicity Influenced by Change of Amino Acid

To investigate the intrinsic factors that make the three new mutants toxic, substitution mutations for A18T, A29S, and A53V were created, changing the amino acid to another acid that was either hydrophobic, hydrophilic, acidic, or basic. Initially I expected that if loss of the original amino acid drove toxicity then any substitution would also be toxic whereas if instead the gain of the mutant amino acid was key then the substitution of the same category would be toxic and the other not toxic. Instead I find that the situation is more complex, A18T and A29S appeared driven by loss by the acidic substitutions were less toxic and A29S varied with each substitution. Localization of the substitutions was consistent with the degree of toxicity, and western blotting showed that level of expression between the mutants and substitutions were roughly equal with exception of the A53D acidic substitution.

The varied results from toxicity suggests that the toxicities of the mutants may be a result of both the loss and gain, where loss of alanine is enough to cause toxicity but that toxicity is further altered by what amino acid was exchanged in its place. Another likely possibility is that interactions between the mutant amino acid and neighboring acid in the sequence also affect the overall toxicity of the protein, with certain interaction decreasing or enhancing the rate in which these mutants aggregate. Interestingly the acidic substitutions A18E, A29E, and A53D were consistently less toxic, however the A53E natural mutant has an acidic amino acid but is still very toxic which is unusual and the only explanation might be the differences in structure between both acidic amino acids.

Combining Mutants Reveals Location Dominance

Through combining mutants I aimed to assess how the mutants would affect one another and what that indicates about the position expecting to find that the mutations might interact in a way such that the toxicity would either be averaged or enhanced. I instead found that the toxicity of the combined mutants was reduced when A29S was present and furthermore when A29S was combined with A18T the toxicity was drastically reduced. Localization of the was consistent with their toxicity, less toxic mutants with A29S present were mostly diffuse while the most toxic combination A18T/A53V had more aggregates. Western blotting of the mutants showed consistent expression, but much like the western blot between the new and old mutants, the least toxic combinations appeared to have slightly higher expression compared to the rest.

The findings suggest that of the mutants A29S is the most influential towards the overall phenotype of the mutant. This is not entirely surprising as previous work in our lab look at combining A30P with E46K and A53T (Kukulka Thesis, 2013) and combining G51D with H50Q and A53E (Tembo Thesis, 2013) found that the combinations were dominated by the less toxic A30P and G51D mutants. These results suggest that the positions of these less toxic mutant variants appear to have a greater importance to the misfolding and structure of a synuclein, as too why the least toxic variants happen to be more influential is yet to be understood. An additional point of interest is that combining A18T with A29S actually reduced toxicity drastically, suggesting that these mutant positions are interacting with each other and that may be a result of their relative proximity to one another.

Decreasing Nitrative Stress Reduces Toxicity

Previous studies have shown that high levels of nitrative stress enhances the toxicity of a synuclein while low levels of stress lessens toxicity. Assessing the new mutants under high and low nitrative stress conditions I find that both A18T and A53V were equally sensitive to the altered nitrative conditions as WT, however A29S was not affected by high levels of nitrative stress in comparison to the others and overall appears to be insensitive to alterations in nitrative conditions. This is consistent however with Amanda Grassel's findings where she evaluated the mutants in nitrative conditions as well, observing that A29S appear less sensitive in comparison to the other two mutants even in a lower level of expression. Localization of the mutants and WT in altered stress was interesting as unlike the previous findings, higher levels of aggregation for the mutants and WT were found in the low nitrative stress condition compared to high stress despite high stress being more toxic. Despite this there's research that indicates more soluble forms of a synuclein are more linked to toxicity and degeneration that the insoluble aggregates (Bridi & Hirth, 2018), which may be what is being observed here under altered nitrative conditions.

Lowering SUMOylation Enhances Toxicity

SUMOylation modifies the amount of ubiquitin tags are added to a protein which often regulates degradation of the protein. previous research suggests that higher levels of SUMOylation would reduce a synuclein's toxicity whereas less SUMOylation increases toxicity. The new mutants followed a similar trend, A18T and A53V show similar levels of sensitivity to altered SUMOylation as WT however A29S is much more sensitive and becomes highly toxic in low SUMO. This trend again is consistent with previous work, Carris Borland evaluated the toxicities of the mutants under altered SUMOylation and found that A29S appeared more sensitive under the PYES2 vector as well.

The localization pattern for the mutants is relatively consistent with the pattern of toxicity, however the differences weren't as drastic. The increase in toxicity for A29S under low SUMO may suggest that A29S is relatively easier for the cells to break down in comparison to the other mutations, thus when SUMOylation decreases the cell can't ubiquitinate and degrade the A29S mutant which increases toxicity.

Mitochondrial Stress Differentially Affects Mutant Toxicity

Work with oxidative stress is not as developed as all the previous projects I have discussed however there are some interesting trends. Mitochondrial dysfunction and resulting oxidative stress are a key features of PD pathology with research indicating that it worsens a synuclein aggregation and toxicity. Surprisingly when assessing the toxicity of WT and the mutants I found the opposite to be occurring. Unfortunately, there was an unusual but consistent trend where growth of the BY4741 control strain was lower than the knockout strains on the glucose control plate, making an accurate evaluation difficult although a rough comparison can still be made. Concerning WT, I find that none of the strains enhanced toxicity the hsp34D strain even reduced the toxicity. A18T appeared to share the same sensitivity to each strain as WT, A29S appeared to become more toxic in the hsp31D and hsp34D strains, and toxicity of A53V was reduced in sod2D and hsp31D strains.

Localization of WT and the mutants in these strains showed a pattern that's consistent with either an increase or decrease in toxicity, A29S develops more aggregates in the hsp31D and hsp34D and A53V has more diffuse cells in sod2D and hsp31D strains. Given that each strain increases mitochondrial stress it's interesting to see that each strain affects the mutants differently. A potential reason for this is that each knockout increases the concentration of certain reactive oxygen species, and each mutant is more susceptible to a particular species, either increasing or decreasing the toxicity.

Limitations

There are many limitations with my study. One limit is that at the moment the spotting assays and western blots are not quantifiable, making accurate assessment of varying levels of toxicity and expression difficult in situations where they appear to be close between different samples/mutants. The BY4741 control strain consistently growing less than the knockout strains is another limit more specific to the oxidative stress data. Most of the work is focused on the W303 strain so further assessments could be done with the substitutions and combinatorial in BY4741. Using yeast as a model organism is also a limitation, while yeast are a great model PD is ultimately a disease that affects neural cells which are vastly different from yeast cells. There are potential interactions between a synuclein and other neuron specific proteins that may contribute to the disease pathology but wouldn't be seen in yeast.

Future Studies

Future studies could address some of the limits, such as conducting more evaluations of the mutants in BY4741. I evaluated the effects of nitrative stress, oxidative stress, and SUMOylation on the new mutants but there are several other altered conditions that could be evaluated including PD genes, altered lipids, and phosphorylation. Western blotting for the altered cellular conditions should be done to determine whether certain conditions affect accumulation/expression of a synuclein and mutants. The substitution mutations project can be investigated further such as how differences in polarity or amino acid structures affect toxicity. Another future study could focus on creating other combinatorial mutants to get more insight into the role of mutation position in toxicity of a synuclein.

CONCLUSION

In this study I sought to evaluate the toxicity of the three new a synuclein mutants A18T, A29S, and A53V under a higher level of expression within a yeast model system, additionally evaluating intrinsic properties of the mutant that causes toxicity and the affects of altered environmental conditions. I find that while the results of previous assessments of the mutants under a lower expression system were mixed, at high expression each mutant has a distinct level of toxicity and localization pattern different from one another. By assessing the properties of various amino acid substitution mutants I found that neither loss nor gain of the amino acid fully explains the pathology of each mutant. Through combining the mutants, I find that mutations at certain positions within the amino acid sequence are more influential over the properties of the combined mutant than others. And lastly, I assessed the new mutants within high expression in various altered cellular conditions, reaffirming previous findings that each mutant was uniquely sensitive to a particular condition and under higher expression those differences are more dramatic.

Importance of Understanding New Mutations

Most PD cases are not a result of the individuals having mutant variants of a synuclein, however researching mutant variants can provide more insight into the properties of a synuclein and the conditions that make it toxic. The six older mutants A30P, E46K, H50Q, G51D, A53T, and A53E were extensively evaluated while the three newer mutants were largely ignored by most labs, my work as well as the work of Carris Borland and Amanda Grassel reveals that these three new mutants can also provide new insights into a synuclein toxicity.

Expression Matters

The most significant finding of my work is that the level of expression is central to the toxicity of a synuclein and its mutants. Accumulation of proteins is a central characteristic of PD and many neurodegenerative disorders, and the increased toxicity seen with high expression shown here is consistent with work done in other labs and established models of neurodegeneration like nucleation polymerization.

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