# Robust Sequence Determinants of α-Synuclein Toxicity in Yeast Implicate Membrane Binding

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**ABSTRACT**

Protein conformations are shaped by cellular environments, but how environmental changes alter the conformational landscapes of specific proteins *in vivo* remains largely uncharacterized, in part due to the challenge of probing protein structures in living cells. Here, we use deep mutational scanning to investigate how a toxic conformation of α-synuclein, a dynamic protein linked to Parkinson's disease, responds to perturbations of cellular proteostasis. In the context of a course for graduate students in the UCSF Integrative Program in Quantitative Biology, we screened a comprehensive library of α-synuclein point mutants in yeast cells treated with a variety of small molecules that perturb cellular processes linked to α-synuclein biology and pathobiology. We found that the conformation of α-synuclein previously shown to drive yeast toxicity—an extended, membrane-bound helix—is largely unaffected by these chemical perturbations, underscoring the importance of this conformational state as a driver of cellular toxicity. On the other hand, the chemical perturbations have a significant effect on the ability of mutations to suppress α-synuclein toxicity. Moreover, we find that sequence determinants of α-synuclein toxicity are well described by a simple structural model of the membrane-bound helix. This model predicts that α-synuclein penetrates the membrane to constant depth across its length but that membrane affinity decreases toward the C terminus, which is consistent with orthogonal biophysical measurements. Finally, we discuss how parallelized chemical genetics experiments can provide a robust framework for inquiry-based graduate coursework.

## INTRODUCTION

Proteins adopt multiple conformations to facilitate specific biological processes; conversely, protein misfolding can disrupt cellular homeostasis and drive disease. Protein conformation is sensitive to various factors including specific regulatory processes, general homeostatic mechanisms, and chemical/physical properties of the cellular environment. Understanding how cellular factors control protein conformation is critical for determining the basis for normal biological processes and aberrant disease pathologies; however, probing cellular determinants of protein conformation remains challenging due to a dearth of methods that report on protein conformation in living cells.

Deep mutational scanning (DMS) has emerged as a powerful approach for revealing protein conformation in living cells.[1, 2] In DMS, an exhaustive library of protein missense variants is screened for an activity of interest, and the activity of each variant is quantified by deep sequencing.[3] By determining the pattern of mutations that affects activity, one can build and/or test structural models of proteins. Moreover, this procedure can reveal the conformation(s) that are responsible for an activity of interest.

We recently used DMS to identify biologically active conformation(s) of α-synuclein,[4] which extensive genetic and pathological evidence implicates as a driver of Parkinson's disease (PD). Missense mutations[5] and increases in gene dosage[6] of α-synuclein are both sufficient to cause inherited, early-onset PD, and the brains of affected individuals accumulate misfolded and mislocalized α-synuclein.[7] Though predominantly unstructured in solution,[8] α-synuclein can form an amphiphilic helix,[9] which allows it to bind to the surface of synaptic vesicles and

mediate trafficking dynamics.[10] At high concentrations, membrane-bound α-synuclein can disrupt lipid membranes, which has been suggested as a mechanism of toxicity in synucleinopathies.[11] α-Synuclein can also adopt diverse β-sheet-rich aggregates, including amyloids,[12] which can propagate misfolding in a prion-like manner and drive cell death.[13] Because multiple structural states of α-synuclein demonstrate toxicity, the conformation(s) that are most responsible for α-synuclein's toxicity remain a matter of debate.

To address this question, we probed the toxic conformation(s) of α-synuclein in the yeast *S. cerevisiae*, a validated cellular model of α-synuclein pathobiology.[14] Expression of α-synuclein in yeast, which lacks synuclein homologs, recapitulates several features of its behavior in neurons, including membrane binding and dose-dependent, gain-of-function aggregation and toxicity.[15] In addition, the aggregates that develop in yeast consist of clustered, membrane-bound structures,[16] particularly ER/Golgi vesicles, reminiscent of the composition of Lewy bodies found in PD patients.[17] This system has also been widely used to identify genetic[18, 19] and chemical[20] modifiers of α-synuclein toxicity that have been subsequently validated in neuronal and animal models.

To identify the conformational state(s) that drive toxicity in yeast, we compared the relative toxicity of 2,600 single point mutants.[4] Our results were consistent with a membrane-bound helix with increasing dynamics toward the C terminus as the toxic conformation (Figure 1). Proline substitutions in the first ~90 residues disrupted toxicity, as did incorporation of polar residues within the membrane-binding face. Toxicity-disrupting mutations recurred with strict 3.67 residue periodicity, consistent with helix formation, and mutational sensitivity decreased toward

the C-terminal end of the helix, consistent with increased dynamics in this region.[21, 22] Interestingly, we did not observe any features that would suggest aggregation or β-sheet structure in the toxic conformation.

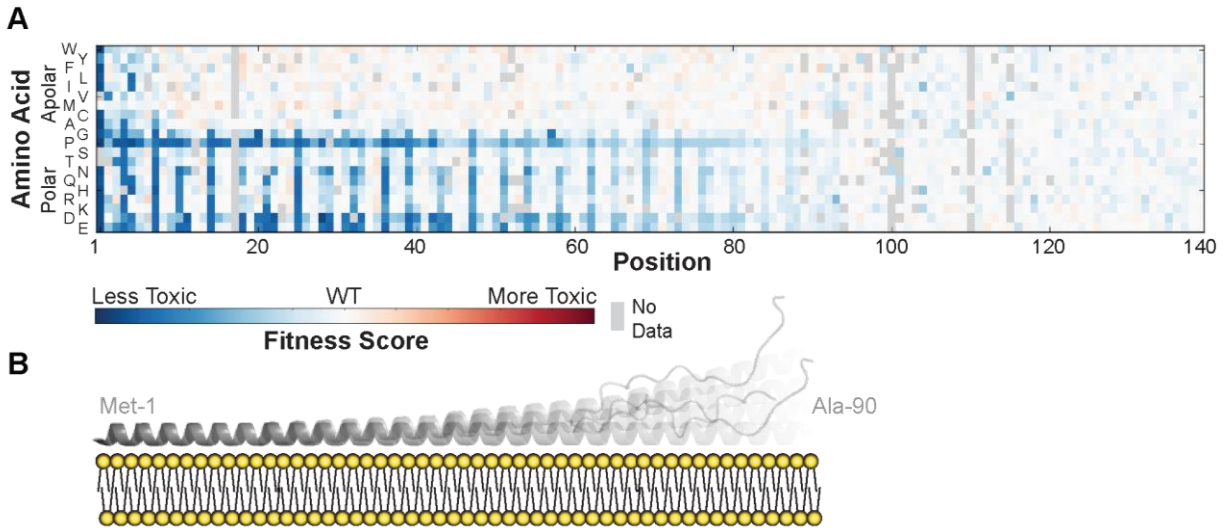


**Figure 1.** (A) Fitness scores (defined as the slope of the line describing change in log-transformed variant frequencies over time) for expression of α-synuclein point mutants in yeast.[4] (B) The structural model derived from the sequence–toxicity landscape in panel A: an extended, membrane-bound 11/3-helix with increasing dynamics toward the C terminus.[4]

Because α-synuclein adopts a variety of structures, we were intrigued that the sequence landscape was consistent with a single toxic conformation; however, it is possible that additional species contribute to toxicity downstream of the membrane-bound helix. Moreover, several lines of evidence suggest that the cellular environment might alter α-synuclein's conformational landscape,[23] including the sensitivity of intrinsically disordered proteins to environmental conditions,[24] the effect of environmental stresses on α-synuclein toxicity,[25] the cell-type selective vulnerability in PD,[26] the age-dependence of α-synuclein pathology,[27] and the variety of α-synuclein species that demonstrate toxicity in other models.[13, 28, 29] Given the complex conformational landscape of α-synuclein and known environmental contributions to toxicity, we hypothesized that cellular stresses might create a less permissive folding environment that would

either perturb the structure of the toxic species or reveal contributions from species whose toxicity is not evident under unstressed conditions. For example, interventions by the proteostasis network might resolve otherwise disruptive species (e.g., oligomers or amyloids) or directly remodel the active conformation. Alternatively, the cell might harbor multiple sensitivities to α-synuclein, some of which might dominate in different environmental conditions. This would be consistent with the likely scenario that α-synuclein exerts toxicity through multiple mechanisms; because only a small percentage of synucleinopathy cases have clear genetic etiologies, environmental stresses likely predispose cells to particular mechanisms of toxicity.

To address these hypotheses, we used small molecules to alter cellular homeostasis and create distinct folding environments; we then used DMS to read out quantitative changes in the sequence determinants of the toxic conformational state of α-synuclein. DMS is an ideal approach for these questions because structural information can be inferred from phenotypic measurements obtained in living cells. The parallel nature of these experiments also offers an opportunity for authentic inquiry in a classroom-based setting; these studies provide training in critical thinking and creativity within a supportive environment that builds leadership and cross-disciplinary engagement.[30] We have previously undertaken similar course-based experiments examining environmental constraints on ubiquitin function;[31, 32] therefore, to sustain the educational impacts of this initiative and ensure authentic, open-ended inquiry, we undertook our experiments on α-synuclein in a project-based course for first-year biophysics and bioinformatics graduate students in the UCSF Integrative Program for Quantitative Biology. The resulting course thereby provided a unique training opportunity integrating experimental and computational approaches to enable both hypothesis-driven and unbiased inquiry.

## RESULTS AND DISCUSSION

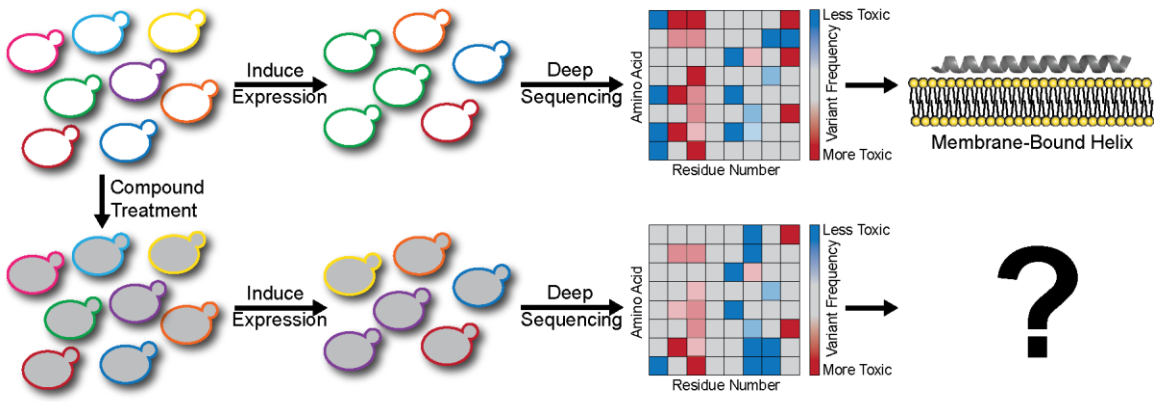


**Figure 2.** Chemical genetics approach to probe sequence and structural determinants of α-synuclein toxicity.

### Chemical genetics approach to revealing additional active states

To probe additional conformational states that contribute to α-synuclein toxicity, we perturbed a variety of cellular processes using small molecules and identified ensuing changes in the sequence–toxicity landscape using DMS (Figure 2). We selected cellular stresses relevant to α-synuclein pathobiology (Table 1), including perturbations to vesicle trafficking,[18, 19] oxidative stress,[25] the unfolded protein response,[33] protein degradation and autophagy,[34, 35] chaperone activity,[36] and membrane composition.[37]

**Table 1.** Effects of chemical perturbations

| **Compound** | **Target and Mode of Action** | **Primary Cellular Effects** | **Connection to α-Synuclein** | **Hypothesized Effects on α-Synuclein Fitness Landscape** |
|---|---|---|---|---|
| Dopamine | Covalent modification of α-synuclein[38] | Neurotrans-mitter | α-Synuclein deficiency alters dopamine signaling;[10] dopaminergic neurons degenerate selectively in PD;[26] dopamine promotes oligomer formation, inhibits fibrillization[38] | Residues that interact with dopamine will show disruptive mutations selectively in the presence of dopamine; sequence signatures of oligomers (e.g., positions sensitive to steric hindrance) will emerge |
| Menadione | Quinone reduction by molecular oxygen generates superoxide radicals | Induces oxidative stress | Various α-synuclein species induce oxidative stress;[39] oxidative modification of α-synuclein is observed in patients[40] and inhibits fibrillization;[41] menadione exacerbates α-synuclein toxicity in yeast[42] | Residues whose oxidation contributes to α-synuclein toxicity will become more sensitive to mutation; compromised capacity to resolve oxidative stress might reveal conformations that drive toxicity by increasing oxidative stress |
| Melatonin | Scavenges free radicals | Relieves oxidative stress | Various α-synuclein species induce oxidative stress;[39] oxidative modification of α-synuclein is observed in patients[40] and inhibits fibrillization;[41] melatonin attenuates α-synuclein toxicity in yeast challenged by menadione[42] | Residues that become modified by oxidative stress will be less sensitive to mutation; enhanced resilience to oxidative stress might reveal conformations that drive toxicity through other mechanisms |
| Miconazole | Inhibits 14α-sterol demethylase, reducing ergosterol production[43] | Decreases membrane sterol content, affecting membrane fluidity and integrity[43] | Sterol content modulates the membrane-binding affinity of α-synuclein[44] | Membrane-contacting residues will be selectively affected; cells might maintain resistance only to variants with the weakest membrane affinity |
| Brefeldin A | Inhibits COP-I coat assembly | Disrupts Golgi–ER and other membrane transport processes; induces the unfolded protein response | ER–Golgi trafficking genes modify α-synuclein toxicity;[18] α-synuclein inclusions involve clustered ER/Golgi vesicles[16, 17] | Because cells will be more sensitive to agents that interfere with trafficking, cells should be more sensitive to all variants except those with the weakest membrane affinity, which are less likely to affect trafficking; sequence signatures of alternatively localized α-synuclein should emerge |

**Table 1.** Effects of chemical perturbations (continued)

| **Compound** | **Target and Mode of Action** | **Primary Cellular Effects** | **Connection to α-Synuclein** | **Hypothesized Effects on α-Synuclein Fitness Landscape** |
|---|---|---|---|---|
| MG-132 | Covalent inhibitor of the 20S proteasome | Reduces degradation of ubiquitin-modified proteins | Lewy bodies are enriched in ubiquitinated proteins, including α-synuclein;[45] ubiquitination is a common cellular strategy for mitigating misfolded proteins; α-synuclein impairs proteasome function[46] | Residues whose ubiquitination modifies α-synuclein toxicity will be less sensitive to mutation; species that are more efficiently ubiquitinated and cleared will become apparent |
| Tunicamycin | Inhibits glycosyl-transferases involved in glycoprotein synthesis[47] | Creates a burden of misfolded ER proteins; induces the unfolded protein response[47] | α-Synuclein accumulation induces the unfolded protein response;[19] α-synuclein interferes with glycoprotein maturation;[19] tunicamycin induces α-synuclein oligomerization in cellular models[33] | Cells will become less resistant to all variants except those with the weakest membrane affinity, which are less likely to affect glycoprotein maturation; additional sequence determinants of oligomerization might appear; sequence determinants of species resolved by the unfolded protein response will appear |
| Geldanamycin | ADP/ATP-competitive inhibitor of Hsp90[48] | Reduces proteostatic capacity[48] | Hsp90 reduces α-synuclein aggregation and toxicity by modifying oligomers[49] | Sequence signatures of conformations resolved by Hsp90 will appear; residues that directly interact with Hsp90 will be less sensitive to mutation |
| Rapamycin | Inhibitor of mTORC1 formation[50] | Inhibits translation; induces autophagy[50] | α-Synuclein aggregates are cleared by autophagy;[34] α-synuclein aggregation impairs autophagy[51] | Variants with sub-saturating toxicity will increase in fitness due to reduced translation; residues that mediate trafficking to autophagosomes will be more sensitive to mutation; sequence signatures of toxic species not resolved by autophagy will be revealed |
| Spermidine | Inhibits histone acetyltransferases, altering the epigenetic state of histone H3 and inducing transcription of autophagy-related genes[52] | Induces autophagy; suppresses oxidative stress[52] | α-Synuclein aggregates are cleared by autophagy;[34] synuclein aggregation impairs autophagy[51] | Residues that mediate trafficking to autophagosomes will be more sensitive to mutation; sequence signatures of toxic species not resolved by autophagy will be revealed |

We treated yeast with compound concentrations that reduced growth rate, where possible, to confirm that the compound was active in cells. Compounds that failed to induce a growth defect (dopamine, melatonin, and spermidine) were employed at high concentrations previously reported to perturb survival processes in yeast.[42, 52, 53] We then compared the resulting sequence–toxicity landscape to that obtained in untreated controls.

**Retention of structure upon diverse chemical treatments**

To determine the effect of chemical perturbations, we compared fitness scores for each mutant in the presence versus the absence of a given compound. If the compound had no effect on the conformation responsible for toxicity, the data would retain periodicity reflecting the α-helix, and the introduction of helix-breaking residues such as proline would suppress toxicity throughout the helical region. This is precisely what is observed (Figures 3, S1–S10).

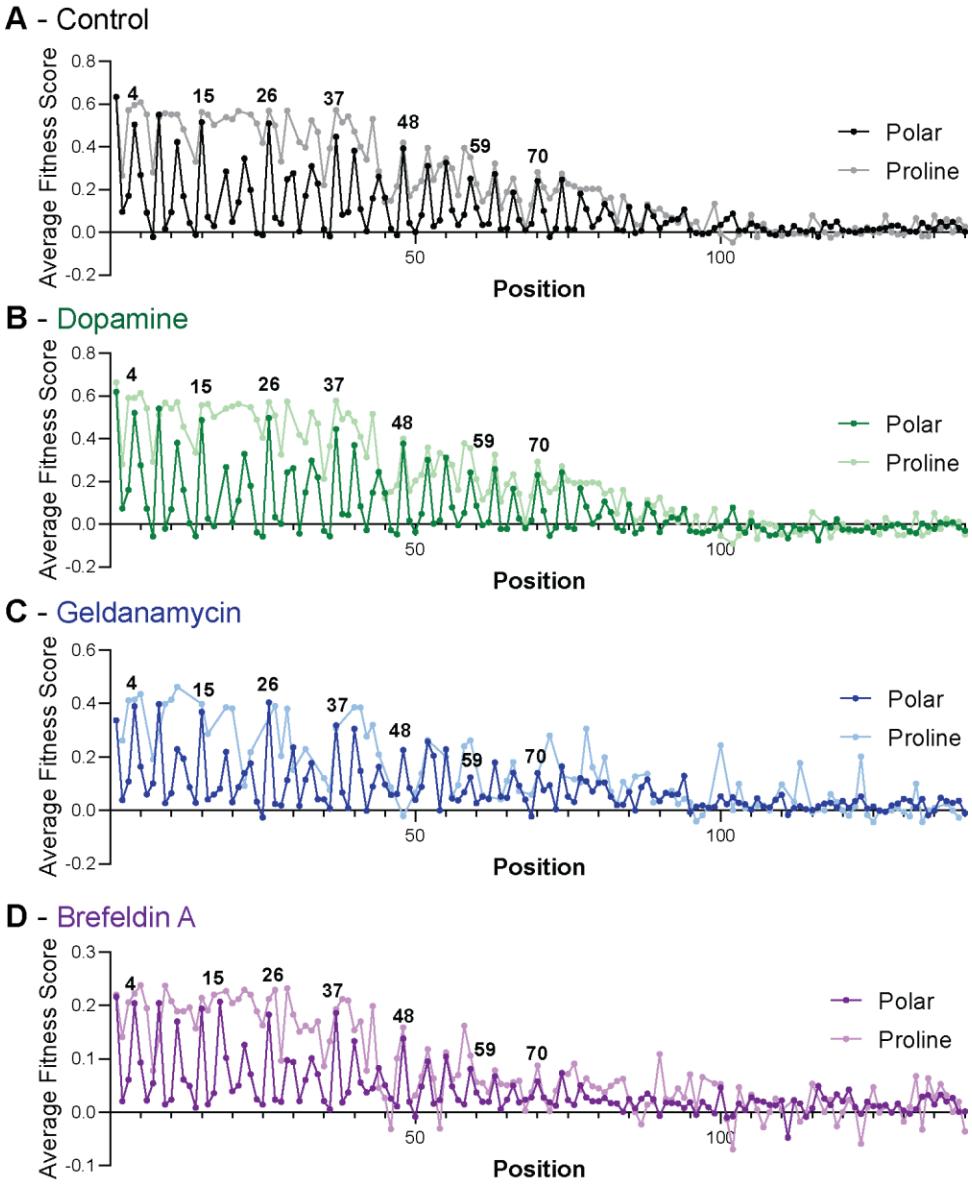


**Figure 3.** Average fitness scores of mutants with polar (S, T, N, Q, H, R, K, D, E) or proline residues in (A) untreated cells[4] or cells treated with (B) dopamine, (C) geldanamycin, or (D) brefeldin A. Fitness scores are calculated as described in "Methods". Data for other compounds are given in Figures S1–S10.

In each condition, polar residues continue to increase fitness when substituted on the apolar face of α-synuclein's membrane-binding helix (Figure 3). Moreover, the effects of apolar-to-polar and proline substitutions decrease along the chain in each condition. We therefore conclude that the same dynamic membrane-binding helix is the key driver of toxicity over a wide range of chemical perturbations.

**Chemical perturbation modulates efficiency of single-site suppressors of toxicity**

To determine the impact of chemical perturbation on the efficiency with which point mutations suppress α-synuclein toxicity, we compared the fitness scores measured in the presence and absence of each compound. If treatment places a stress on the system that globally increases sensitivity to α-synuclein, then it should become more difficult to suppress toxicity with otherwise beneficial substitutions (e.g., apolar-to-polar substitutions), resulting in a strong correlation between control and treatment, but with a slope less than one. Conversely, if the chemical perturbation induces processes (e.g., induction of beneficial chaperones or proteases) that rescue the toxic effects of α-synuclein, then a slope greater than one should be observed. Finally, outliers that deviate significantly from the linear trend would reveal any sequence-specific effects on toxicity caused by the treatment.

The data for DMS in the presence of menadione, melatonin, spermidine, dopamine, miconazole, and MG-132 were nearly perfectly correlated with the control and had slopes near one (Figure 4). Thus, these conditions had no global effect on the sensitivity of cells to the expression of α-synuclein. By contrast, the correlations between data for controls and data for tunicamycin, geldanamycin, brefeldin A, and rapamycin had significantly reduced slopes, indicating that suppression of toxicity by point mutations was uniformly less efficient under these growth conditions (Figures 4, 5A). A second effect seen in plots with the slopes significantly less than one was the emergence of a large cluster of mutants with a fitness score near zero for the drug-treated population (e.g., brefeldin A), indicating that these mutants had significant effects under normal conditions but were unable to impact fitness in the presence of this chemical.

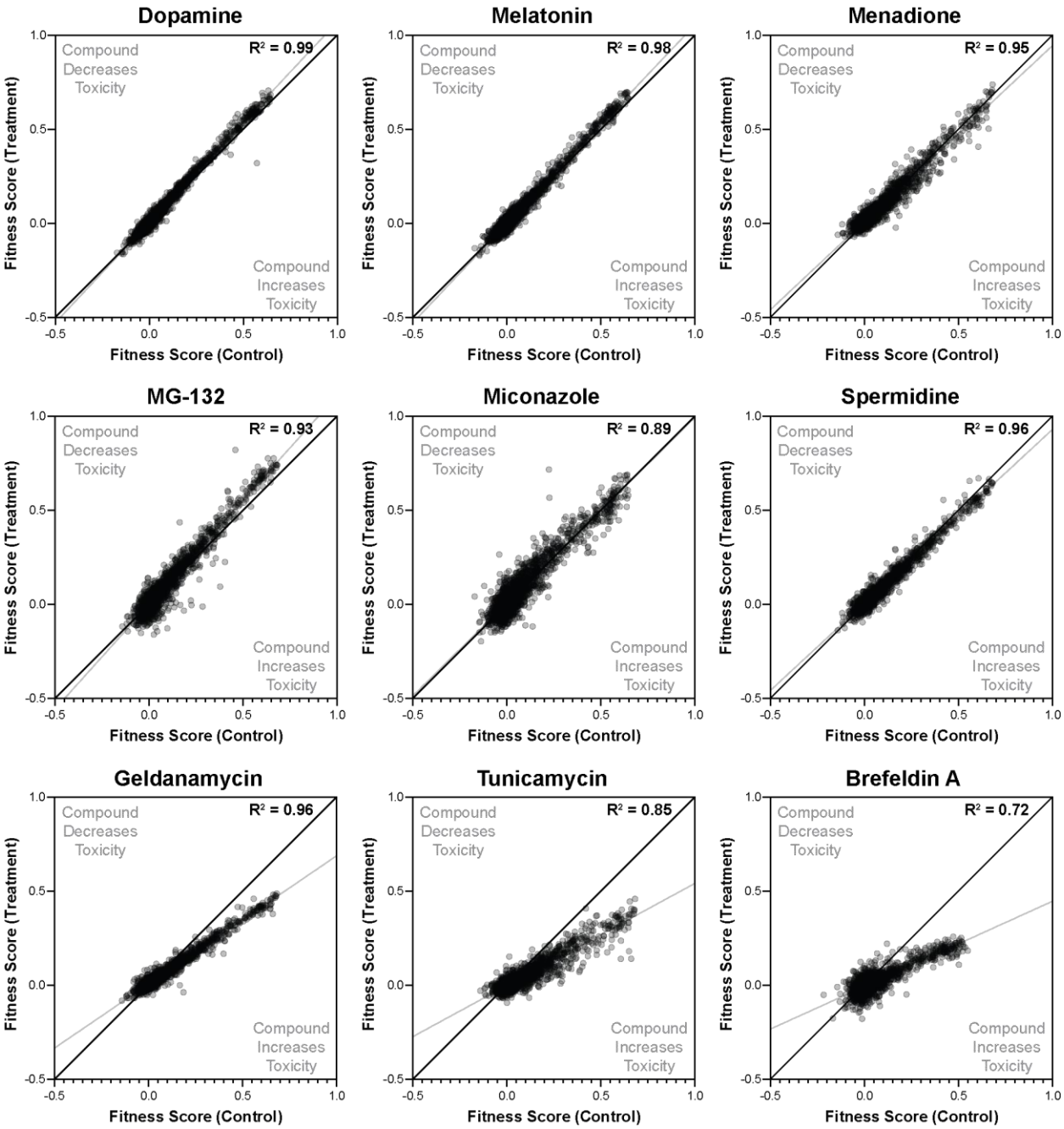


**Figure 4.** Fitness scores of yeast expressing α-synuclein point mutants obtained in the presence of the compounds in Table 1, relative to those obtained in untreated controls. Fitness scores and correlation coefficients are calculated as described in "Methods".

Among the ten chemical perturbations we tested, we identified one compound, rapamycin, that displayed non-linear behavior between treated and untreated conditions, suggesting the presence of mutant-specific effects of rapamycin treatment. Specifically, although the entire set of mutants

are poor suppressors of toxicity in the presence of rapamycin, two subsets of variants showed a small but significant positive deviation from the linear relationship between fitness scores observed in the presence versus absence of rapamycin: (1) those with substitutions of Met-1 (Figure 5A), which dramatically reduce expression of α-synuclein,[4] and (2) those with acidic residues on the hydrophobic face of the amphiphilic helix (Figure 5B/C). We showed previously that incorporation of polar residues on the hydrophobic face dramatically reduces membrane binding,[4] so one feature shared amongst the variants whose fitness was modified by rapamycin is decreased membrane-bound α-synuclein, either by reduced expression or reduced membrane affinity.

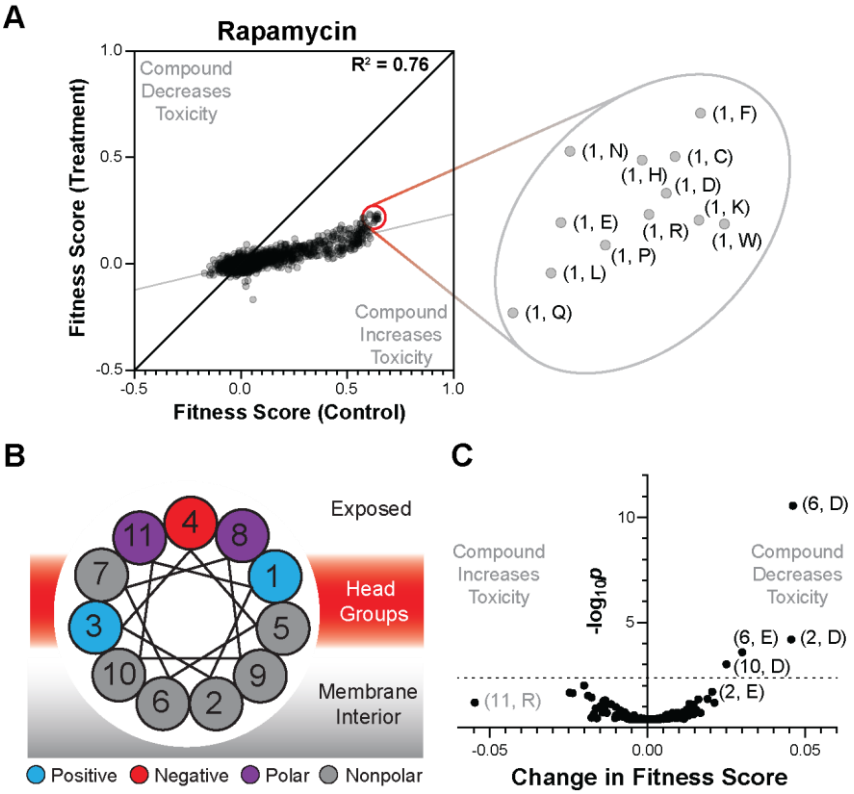


**Figure 5.** (A) Fitness scores of cells expressing α-synuclein mutants obtained in the presence of rapamycin relative to untreated controls. (B) Helical wheel of α-synuclein depicting the relative orientation of each residue in the 11-residue repeat. (C) Volcano plot describing changes in fitness score induced by rapamycin treatment, averaged over equivalent mutations in each of the seven repeating 11-residue segments. A 5% false discovery rate threshold is shown. Fitness scores, changes in fitness scores, and statistical significance (p-values, false discovery rate threshold) are calculated as described in "Methods".

We next sought a model to explain the overall trend for variants with decreased affinity for membranes to have smaller effects in the presence of many of the compounds, and how this might be specifically modulated in the presence of rapamycin. We previously showed that α-synuclein mutants with reduced toxicity also had reduced affinity for binding to membranes.[4] Moreover, the expression of α-synuclein in our system is tuned to maximize wild-type (WT) toxicity, so mutations that increase membrane affinity show little increase in toxicity. We hypothesize that a specific threshold of membrane-bound α-synuclein is necessary for toxicity, and that this threshold decreases under strong chemical stress.

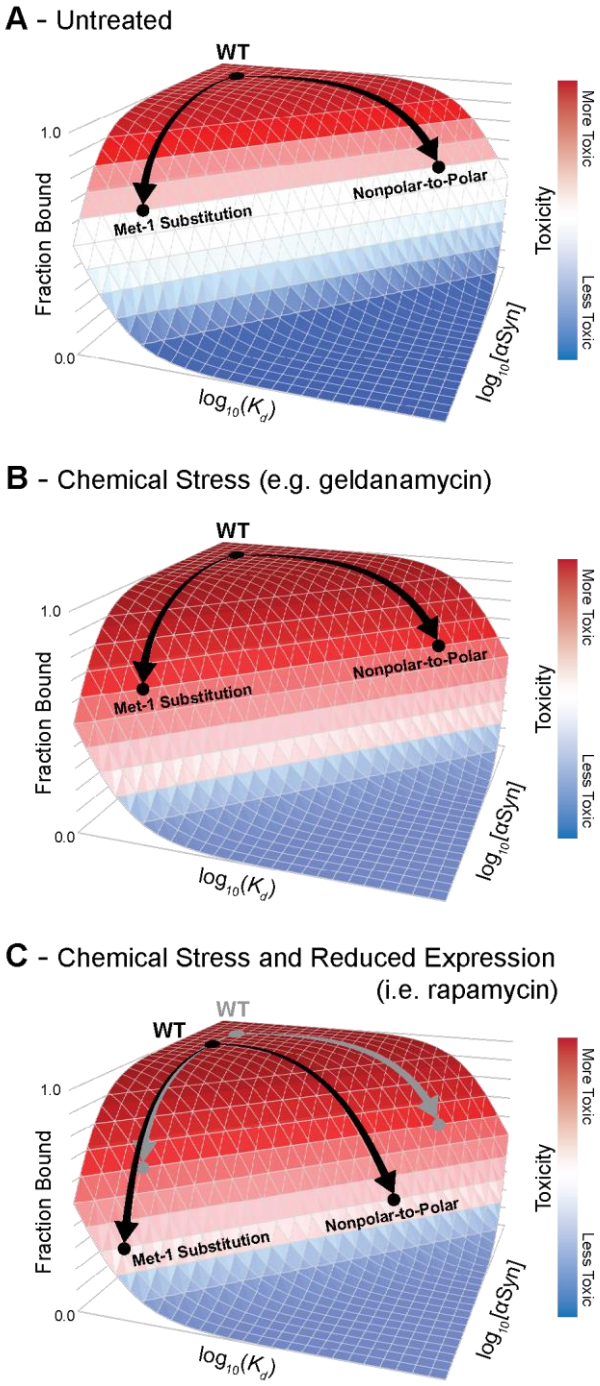


**Figure 6.** Cellular toxicity depends on the membrane-bound population of α-synuclein. (A) In untreated cells, WT α-synuclein binds to cellular membranes above a critical threshold for toxicity. Mutations can reduce toxicity by decreasing the membrane-bound population of α-synuclein, either by decreasing membrane affinity (e.g., by introducing polar amino acids on the membrane-binding face) or reducing α-synuclein expression (e.g., by substituting Met-1). (B) Chemical stresses increase sensitivity to α-synuclein toxicity, so mutations that reduce toxicity in untreated cells are less effective at reducing toxicity under general chemical stress. (C) Because rapamycin reduces protein expression (black arrows relative to gray arrows), the combination of rapamycin and specific α-synuclein mutations reduces membrane binding sufficiently to overcome the increased sensitivity caused by compound toxicity.

Figure 6 relates the cellular protein concentration and dissociation constant to the fraction of binding; toxic binding regimes are indicated by a color gradient. This graph explains how fit mutants with decreased affinity for the membrane can lead to decreased toxicity by lowering the overall surface coverage (Figure 6A). Similarly, reducing the expression of α-synuclein is expected to have the same effect (Figure 6A). If the fraction of binding required to achieve a toxic effect is decreased due to a pleiotropic chemical stress, then we see an expansion in the toxic region of the plot (Figure 6B), and a given suppressor will have a smaller effect on toxicity. Finally, treatment with rapamycin can be explained because it not only induces a pleiotropic effect on overall toxicity, but it also decreases the expression of the protein. Thus, the position of the WT shifts within the binding isotherm, resulting in a shift in the magnitude of the effect for the strongest suppressors (Figure 6C). Thus, the data are consistent with the hypothesis that α-synuclein exerts its toxicity in yeast through direct membrane interaction.

**Membrane Binding Predicts Yeast Toxicity**

In summary, chemical perturbation can affect the efficiency of disrupting mutations by changing the sensitivity of the cells to pleiotropic stress, while the pattern of mutations that alter toxicity remains unchanged. These findings provide strong support for the importance of a previously proposed structural model of α-synuclein, in which the N-terminal region serves as a membrane anchor, with increasing disorder and decreased membrane association along the chain. However, within this overall framework, there are two possible limiting possibilities that might account for the existing DMS and spectroscopic observations. In one model, the helix would become progressively less deeply submerged in the membrane and more dynamic toward the C terminus. A second limiting model would predict that any region of the chain could exist in an equilibrium

between two distinct states; a *membrane-inserted helical state*, in which the helix is buried to a similar depth of the membrane at each position throughout the membrane-binding region, and a second, *non-inserted state* in which the chain is located outside the hydrophobic region of the membrane, adopting a largely random-coil conformation. In the two-state model, the decrease in the effect size of mutations along the chain would be associated with changes in the local preference for the membrane-inserted helical state (transitioning from strongly favoring the inserted state near the N terminus to favoring the non-inserted state near the C terminus).

We expected that the two models would have different sensitivities to mutations, based on the known preferences of each of the amino acids types to occupy differing regions of the bilayer.[54] For example, Arg and Lys residues have a strong affinity for the headgroup region while hydrophobic residues have a strong preference for the apolar region of the bilayer, so substitutions should have predictable effects, depending on the depth of penetration of the helix within the bilayer.

To address this question, we estimated the free energy associated with transferring α-synuclein from water to the surface of an implicit lipid bilayer with the helix axis parallel to the membrane surface (Figure 1B) and the hydrophobic residues projecting towards the hydrophobic region of the bilayer (Figure 5B). The transfer free energy was then estimated based on the resulting position of the sidechains and their known depth-dependent propensities for insertion into the bilayer.[54] Two parameters were allowed to vary: 1) the transfer free energy of the WT protein, and 2) the average depth of insertion (i.e., the depth of the helical axis). We estimated the WT transfer free energy and insertion depth for each 11-residue membrane-binding segment by

locally optimizing the correlation of predicted changes in the membrane-bound population (determined from changes in the transfer free energy caused by mutation) with the observed fitness scores for mutations in that segment. Given that over 200 variants are considered at each 11-residue segment, each parameter is well-defined.

The model predicts that the average depth of insertion was nearly identical (13.9 ± 0.6 Å from the bilayer center) for each the 11-residue segment (Figure 7A), which is consistent with orthogonal measurements by EPR spectroscopy.[55] Instead, we found that the predicted binding energy progressively decreased toward the C terminus (Figure 7A). Thus, the decrease in mutational sensitivity toward the C terminus likely results from reduced binding affinity rather than shallower insertion, resulting in partitioning between an ordered, membrane-bound helix and a disordered, dissociated coil, which is well-supported by spectroscopic studies.[21, 22]

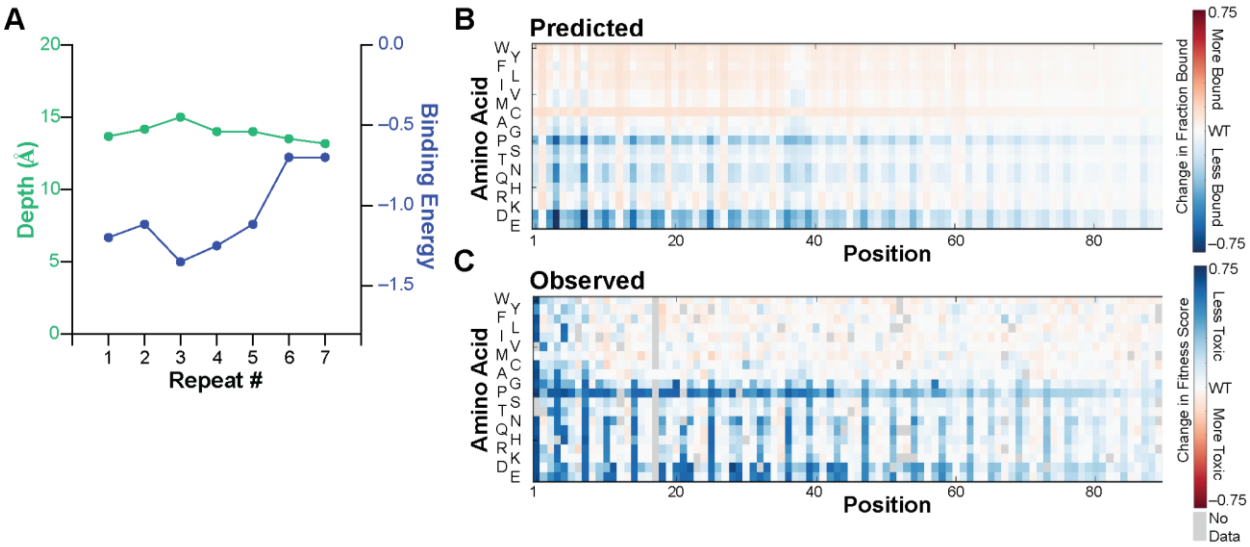


**Figure 7.** (A) Optimal predicted depth of insertion (green) and transfer free energy (blue) of each 11-residue membrane-binding segment. (B) Predicted changes in membrane-bound α-synuclein caused by mutation. (C) Observed changes in yeast toxicity caused by α-synuclein point mutations. The Pearson $R^2$ correlation coefficient between all predicted and observed scores is 0.57.

Having established that decreases in mutational sensitivity correlate with computed changes in population of the bound helix and not from changes in depth, we sought to develop a model that would describe the observed sequence–toxicity landscape. We therefore predicted changes in the bound fraction of α-synuclein for the optimal depth and average WT transfer free energy determined previously (Figure 7A) and scaled the predicted changes in bound fraction by the observed decay in mutational sensitivity to account for decreases in the population of the membrane-bound helix toward the C terminus. The resulting predicted changes in the membrane-bound fraction agree remarkably with the observed fitness scores (Figure 7B/C), providing strong additional evidence for the membrane-bound helix model.

This model is also consistent with the ability of membranes to induce α-synuclein amyloid formation.[56] The disordered C terminus of the membrane-bound helix contains the aggregation-prone non-amyloid component (NAC) segment that forms the core of α-synuclein amyloids. Membrane binding increases local concentration of α-synuclein monomers, so if the aggregation-prone region releases from the membrane locally, it would be primed for aggregation.[57]

**Parallelization provides authentic research experience in the classroom**

These studies were undertaken as an inquiry-based course for first-year biophysics and bioinformatics graduate students, which allowed the parallelized exploration of cellular mechanisms that mediate α-synuclein toxicity. In turn, these experiments provided training in experimental and computational skills, quantitative approaches to biological discovery and hypothesis testing, and key chemical biology concepts. (The complete syllabus for the α-

synuclein-focused course, which took place in two consecutive years, is available online: http://kampmannlab.ucsf.edu/pubs-2017 and http://kampmannlab.ucsf.edu/pubs-2018).

To adapt this study for the classroom, we divided each student cohort into teams of 3–4 with complementary academic backgrounds, ensuring computational and experimental expertise in each team, and assigned each group a teaching assistant who had completed the course previously. This course met for three four-hour sessions per week for six weeks. Although the nature of yeast growth makes it advantageous to schedule several sessions in the same week, other biological systems (e.g., *E. coli*) would be amenable to other course schedules. In particular, quarter- or semester-long adaptations would provide opportunities to structure entire class periods around particular topics of focus (e.g., statistical approaches, instrumentation, etc.), which we instead covered organically as questions arose.

The first half of the course was devoted to data collection, with the remainder devoted to data analysis. In the experimental portion, each team selected a chemical perturbation from a curated list (Table 1). Teams then optimized treatment conditions, performed the library selection by outgrowth, and prepared DNA libraries for next-generation sequencing. During incubation periods, students formulated hypotheses for potential interactions of α-synuclein with the cellular processes perturbed by their compound. Students then wrote custom software in Python to process sequencing reads, build models, and test their hypotheses. During this process, instructors provided guidance in appropriate statistical approaches, computational tools, and visualization strategies. Throughout the course, students participated in a journal club covering papers relevant to the biological and technical foci of the course, which were supplemented by

topical lectures by guest faculty. Teams also provided oral updates to their classmates, and a final presentation was given in an open symposium.

The generation, analysis, and interpretation of large datasets not only illustrated the powerful intersection of experiment and computation but also provided training in quantitative methods and reasoning, which are becoming increasingly essential for modern biological research. The focus on large genetic libraries, which have become readily available, provided ample opportunities for students to propose their own biological hypotheses at the data analysis stage and formulate quantitative, testable predictions, given the information content in these datasets. This approach is particularly suited to advanced training (e.g., upper-division or graduate-level), as it provides a more complex and open-ended opportunity than those typical of lower-level course-based research experiences, where hypotheses and analytical approaches are often prescribed.

Our approach also provided practical benefits for open-ended inquiry in laboratory courses. First, conducting experiments in pools reduced the impact of variation between students or teams, since the fitness of individual variants are calculated relative to the entire population as a whole. Each group's experiment has WT as the internal control; thus, each group's results can be internally normalized to facilitate comparison between groups. In this way, experimental or sampling errors (e.g., pipetting) are minimized. Second, by combining chemical perturbations with a single genetic variant library, we were able to address a variety of questions without the need for multiple libraries or other specialized biological materials. This allowed to us to provide

each student or group of students with ownership of a complex project that relates to the broader goals of the course.

These particular experiments also illustrate many core biological concepts. Identifying the physical properties and chemical features of α-synuclein that mediate its activity illustrates the core concept of structure–function relationships. The DMS approach also offers a concrete example of the evolution of populations under various environmental constraints and illustrates gene–environment interactions that shape both normal and disease biology. Finally, by employing pleiotropic perturbations, we illustrate the interconnectedness of cellular systems. We have focused on proteostasis, but other biological topics are readily addressed.

This framework was based on a previous version of our course, in which we examined environmental constraints on the fitness landscape of ubiquitin.[31, 32] After thorough examination of the fitness of ubiquitin variants under many environmental stresses, we sought new subject matter to reinvigorate the course. Authentic, open-ended inquiry is central to achieving our learning goals of building independent critical thinking and fostering scientific creativity, which therefore required us to address previously unanswered questions. Without having to change the infrastructure of our course, which already featured yeast genetics and next-generation sequencing, we were able to sustain the course for more student cohorts. We therefore believe that this robust and adaptable approach provides a model for other graduate training experiences in classroom sizes typical of undergraduate laboratories.

## METHODS

### *Yeast Library Selection*

A yeast library expressing 2,600 barcoded missense variants of α-synuclein was cultured as described previously.[4] Briefly, α-synuclein expression was induced in the presence of compound, and cells were maintained in log-phase growth for 24 hours. Aliquots were sampled at 12-hour intervals. Each selection was performed in duplicate. Plasmids were isolated and amplified by PCR to isolate barcode DNA. Products were gel purified, pooled, and sequenced by Illumina sequencing. Additional details are available as Supporting Information.

### *Determination of Fitness Scores*

Barcode sequences were counted at each time point and normalized to counts of a representative WT barcode. These ratios were then log transformed and fit to a line over the three time points. Slopes for each barcode were averaged across replicates, and the average slopes for synonymous barcodes were averaged to determine a fitness score and associated error for each α-synuclein variant. Additional details are available as Supporting Information.

### *Prediction of Mutational Effects*

We modeled α-synuclein as a membrane-bound helix parallel to the surface of the lipid bilayer. The depth each amino acid penetrates into the membrane varies sinusoidally around the depth of the helix axis according to the observed periodicity. The energy associated with positioning each amino acid at a given depth was calculated using energy potentials derived from high-resolution membrane protein structures.[54] The energy difference between WT and each mutant was used to predict a change in the membrane-bound fraction resulting from substitution. The depth of the

helix axis and transfer free energy of the WT protein were then varied to optimize the linear correlation of predicted changes in bound fraction with observed changes in fitness score for each 11-residue membrane binding repeat (Figure 7A). The average optimal depth and WT transfer free energy were then used to predict the change in bound population for all mutants (Figure 7B/C). Additional details are available as Supporting Information.

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**Author Contributions**

R.W.N. and M.K. conceived the project and designed the course curriculum. R.W.N., E.D.C., and M.K. designed the library and sequencing strategy. R.W.N. constructed the variant library and yeast strains. R.W.N., T.A., J.C., G.C.H., A.M.M., Z.Z.C.N., M.P., N.R.R., D.M.C.S., D.R.W., T.S.W., D.B., C.C., A.C., T.B.C, L.S.E., B.F., E.A.F., M.A.G., Y.K.G., M.G.G., L.M.G., N.H., M.J.-G., M.C.J., M.G.J., A.F.K., K.E.L, J.L, C.M., E.E.M., L.E.M.-V., E.J.N., A.P., J.P., W.S., C.A.S., J.S., H.T., S.A.W., D.R.W., G.W., S.R., and E.D.C. contributed to data

collection. T.A., J.C., G.C.H., A.M.M., Z.Z.C.N., M.P., N.R.R., D.M.C.S., D.R.W., and T.S.W. served as teaching assistants and provided guidance to student teams. R.W.N. and W.F.D. developed the structural model. All authors contributed to data analysis and writing of the manuscript.

**Supporting Information**

Expanded methods; complete sequence–toxicity landscapes

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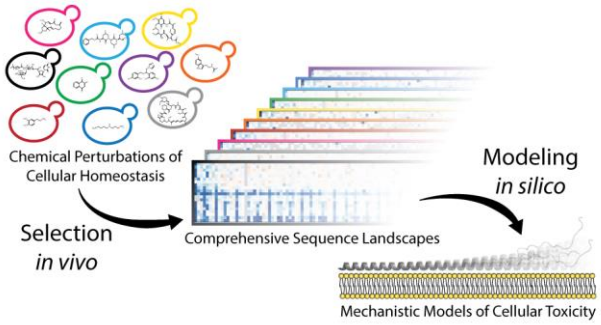
**TOC GRAPHIC**


Chemical Perturbations of
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