14 March 2015

Identification of the Zeo1 Protein as a Candidate Structural Homolog of α-Synuclein in Budding Yeast

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ABSTRACT

Human α-synuclein (SNCA) is a 140-amino-acid protein belonging to the three-member synuclein family. It has been extensively studied due to its misfolding/aggregation in and genetic linkage to neurodegenerative diseases, especially Parkinson's disease (PD). To better understand its biology, models of SNCA toxicity have been developed in budding yeast over the past decade, which have yielded insights into the protein's modes of action in specific pathways and potential therapeutic targets. Given that the synuclein gene family is not present in yeast, an extensive homology search was undertaken to determine if any yeast protein may possess structural homology to SNCA and whose native biology may shed more light on SNCA's pathomechanism in eukaryotes. We identified Zeo1, a membrane-associated protein involved in the cell wall integrity (CWI) pathway, as a candidate structural homolog. We show that Zeo1 overexpression is toxic in yeast and, similar to SNCA, localizes to lipid membranes. A number of biochemical similarities between Zeo1 and SNCA also become apparent in light of this potential structural connection. Moreover, the yeast *PKC1* gene, a kinase acting as a downstream signaling hub in the CWI pathway, rescues both SNCA- and Zeo1-induced toxicities. Using the same homology search methods that identified Zeo1, we show that Pkc1 has a hybrid structural similarity to PINK1 and PARIS, two mitochondrial PD-implicated proteins not generally linked directly to synuclein-specific pathobiology. Overall, this proof-of-concept study shows the potential utility of hitherto uncharacterized cross-species structural homologs, identified using comparative proteome-wide structure prediction algorithms, in shedding light on abstruse connections among disease-relevant proteins.

INTRODUCTION

The α-synuclein gene in humans (*SNCA*) encodes a 140-amino-acid protein that has been implicated in groups of patients with familial [1] and sporadic [2] Parkinson's disease. SNCA's relatively short length and its procession of both structured and unstructured domains has made it a protein of choice in studying different aspects of protein dyshomeostasis in Parkinson's disease and neurodegeneration in general [3]. However, SNCA's precise function in normal and disease biology remains an active area of investigation with many unresolved questions, which is in part due to the intractability of complex mammalian model systems. As a complementary approach to these ongoing efforts, budding yeast (*Saccharomyces cerevisiae*) models of SNCA toxicity were developed more than a decade ago, which have thus far revealed specific pathways perturbed by SNCA [4,5] and potential therapeutic targets [6,7].

The overexpression of SNCA in yeast leads to highly specific phenotypes as opposed to merely an unspecific toxic response. This may lead one to speculate that perhaps overexpressed SNCA occupies the cellular space and function of an endogenous synuclein-like protein. However, no conventional homolog of the synuclein gene family exists in yeast (and non-chordates in general) [8]. Low sequence similarities to plant Late Embryo-Abundant (LEA) proteins have been suggested, but the sequence similarity is very minimal [9]. Another possibility could be the presence of shared localized sequence motifs between the synucleins and a more distant yeast homolog. This is because evolutionarily-linked gene families which diverged at the time of the Cambrian explosion, for example, can still possess shared domain-specific sequence motifs, as in the case of the mammalian prion gene family and ZIP zinc transporters [10]. But even a localized domain-specific search reveals no shared sequence motifs between any yeast protein and the synuclein protein family.

Because of the absence of a potential sequence homolog, an alternate consideration would be the possibility of structural homology: specifically, it is possible that one or more yeast proteins possess a three-dimensional structure similar to SNCA irrespective of any evolutionary linkages between the protein families. Such proteins may thus still perform similar or complementary functions as overexpressed SNCA in yeast. Prior indications of this possibility exist, for example, as reported with respect to structural connections between the small yeast heat-shock protein Hsp12 and SNCA [11,12].

Here we undertook an exhaustive structural homology search of the yeast proteome and identified the protein Zeo1 (Zeo1p; referred to as Zeo1 in this report), a structural paralog of Hsp12, as a candidate yeast protein with notable structural similarity to the synuclein family relative to other yeast proteins. By connecting

biochemical data presented here and in the literature, we show how knowledge of this structural similarity can lead to novel hypotheses regarding the biology of SNCA.

METHODS

Protein sequence alignments and structural searches

Reference protein sequences were retrieved from UniProt, except where noted otherwise. Sequence alignments were manually curated and highlighted based on an initial ClustalW alignment using Invitrogen's (Carlsbad, CA) AlignX program. For structural searches, the HHsuite program version 2.0.16 [13] was installed on a Debian GNU/Linux cluster, and the '02 Sep. 2011' UnitProt20 package was used as the main sequence database to generate multiple sequence alignments for each yeast protein (searches using the online public server of HHpred were performed using the pdb70_1Jun13 HMM database and the PSI-BLAST alignment method). At the time of the structural homology search, 10 SNCA structures existed in the Protein Data Bank (PDB). Of those, only 2 (1XQ8 and 2KKW) represented monomeric full-length α-synuclein. Of the two, only 1XQ8 [14] was present in the HHsuite PDB database as a hidden Markov model. This is due to the 70% maximum pairwise sequence identity threshold used for generating the 'pdb70' database, which seeks to avoid multiple very similar representations of a given protein in the database. 1XQ8 was therefore the benchmark SNCA structure used for the search.

It should be noted that due to different versions of sequence databases and alignment algorithms available for use with the local/offline HHpred program, the probability values of the local program and/or its search results may not exactly match those of the online server. Therefore, the values presented here should be compared relative to values obtained for other proteins in this same analysis.

Biochemistry

For microscopy and liquid growth assays, *ZEO1* and *HSP12* were cloned into 2-μm galactose-promoter-containing pAG425GAL plasmids (leucine auxotrophy) [15] carrying a C-terminal monomeric superfolder GFP (msfGFP) tag [16]. SNCA was tagged with the monomeric mKate2 sequence. These tags are thought to decrease protein oligomerization and reduce potential tag-specific artifacts. For growth assays, cultures were grown in a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific; Waltham, MA) at a starting OD_{600} of 0.001. Statistical significance was tested using a two-tailed Student's *t*-test (assuming equal variance).

Spot assay growth comparisons among SNCA, Zeo1 and amyloid-β (Aβ1-42) integrated yeast strains were performed as previously described [17]. Three independent transformants and spot assays were performed for each condition, and growth rates in galactose were normalized to possible slight differences in growth in glucose. A custom ImageJ macro (available upon request) was used to quantify the spots.

RESULTS

Absence of evolutionary homolog of α-synuclein in yeast

To begin to investigate the potential evolutionary conservation of SNCA in yeast, it is necessary to initially survey variability within the synuclein family itself, which is comprised of α- (SNCA), β- (SNCB) and γ-synuclein (SNCG) in humans. Human SNCA has 67% and 55% identity to SNCB and SNCG, respectively, and 80% and 66% amino acid conservation (**Fig. 1A**). The N-terminal lipid-binding domain (containing the repeat motif 'KTKEGV') and the following hydrophobic domain are highly conserved. The acidic C-terminal domain, however, is less conserved and is unfolded (**Fig. 1C**). How does the composition of synucleins vary across different phyla? We searched for all synuclein-domain-containing proteins using the SUPERFAMILY database of protein structural and functional annotations [18]. The distribution of domains in representative species is presented in **Fig. 1B**. The phylogenetically-earliest *bona fide* synuclein-like annotation was for the early metazoan sea lamprey, and annotations for nematodes or acidobacteria could not be verified as containing genuine synuclein-like sequence motifs. It appears from this tree that the potential synuclein ancestor gene was more similar to SNCG than SNCA or SNCB, and that this ancestor gene emerged in the metazoan branch of eukaryotes.

SNCA's structural homology to Zeo1

Since no sequence-based homolog of SNCA could be found in yeast, we sought to explore the possibility of the presence of one or more structural homologs in the yeast proteome. As described in the **Methods** section, we selected one SNCA structure, 1XQ8, as a benchmark for the search. However, given the small size of the 1XQ8 structure and its two α-helices, the false positive rate of similarity scores could be substantial. As a solution, we aimed to add a second dimension to the search results, whereby we would plot the similarity of each query to (i) 1XQ8 and (ii) the closest non-synuclein synuclein-like structure in PDB. To identify such a structure, the human SNCA sequence was used as a query in the online HHpred program [13], and in addition to its own structure (1XQ8), showed similarity only to one other available PDB structure, the *Leishmania major* SHERP (Lm_SHERP) protein (2X43). We were also able to find this similarity in a complimentary approach using

publically-available datasets from the Fold and Function Assignment server (FFAS) [19]. Specifically, one FFAS dataset using 1XQ8 as a query identified Lm_SHERP as the best-matching PDB structure, followed by a number of apolipoprotein structures (**Fig. S1**). Although only a few studies have investigated the properties of Lm_SHERP thus far, it possesses a number of functional similarities to SNCA [20], reinforcing its utility in our structural search. A comparison of the SNCA (1XQ8) and Lm_SHERP (2X43) structures reveals an RMSD of 2.55 and a TM-score of 0.36148 over an aligned length of 34 residues [21] (**Fig. 2A**). Furthermore, the presence of many apolipoproteins in the FFAS dataset is corroborated by previously-reported structural similarities between the synuclein protein family and apolipoproteins [22]. Lastly, in light of previous reports cited in the **Introduction**, the presence of yeast Hsp12 in the dataset is interesting and noteworthy.

We next searched the hidden Markov model profiles of all yeast proteins (6,434) against 1XQ8 and 2X43 using a local version of the HHpred homology tool (**Fig. 2B**). Based on a size exclusion criterion (**Fig. 2D-E**), four yeast proteins appeared as outliers in this analysis: Zeo1, Hsp12 and translation elongation factors Hyp2 and Anb1. Given the threshold similarity shown to Lm_SHERP by a panel of representative synuclein proteins (**Fig. 2C** and **2E**), Zeo1, followed by Hsp12, were the main outliers in this two-dimensional analysis. It should be noted that Zeo1 and Lm_SHERP share no particular sequence motifs. These unbiased results are significant in light of the previously-noted reports on structural similarities between SNCA and yeast Hsp12 [11,12], and the presence of annotated structural homology between Hsp12 and Zeo1 identified using the OrthoDB dataset of orthologous/homologous protein families [23].

This structural search was rerun using an alternative approach utilizing the online public server of HHpred, which was able to confirm Zeo1 (but not Hsp12) as the only candidate SNCA structural homolog in yeast (**Fig. S2**).

SNCA and Zeo1: similarities in annotations

Human SNCA and yeast Zeo1 are of a similar size (140 and 113 residues, respectively), but have no appreciable sequence motif similarities. SNCA has five ‘KxK’ repeat motifs, whereas Zeo1 has five ‘QxK’ motifs. SNCA has an acidic C-terminal domain, whereas Zeo1 has a mixed charge distribution throughout its length. However, both proteins undergo similar posttranslational modifications in yeast. Namely, unmodified, N-acetylated and N-acetylated/phosphorylated species of Zeo1 have been detected in the yeast cytosol [24], very similar to observations made in a yeast model of SNCA toxicity [25]. Moreover, a phosphoproteomic dataset

prepared for a high-toxicity SNCA-expressing yeast strain showed Zeo1 to have a 3-fold increase in phosphorylation and 3-fold decrease in abundance in that strain (Paola Picotti, unpublished data).

In view of SNCA's propensity for loosely attaching to the plasma membrane, Zeo1 is one of a few proteins in yeast which are annotated as being peripherally associated with the membrane in the *Saccharomyces* Genome Database. These include: Atg2 (1,592 residues; autophagy), Atg23 (453 residues; autophagy), Dsl1 (754; Golgi-to-ER retrograde trafficking), Inp1 (420 residues; peroxisome), Kes1 (434 residues; Golgi), Mon2 (1,636 residues; endosome), Pep3 (918 residues; endosome), Sec17 (292 residues; autophagy), Tip20 (701 residues; ER), Vid24 (362 residues; vacuole), Vma22 (181 residues; ER) and Vps17 (551 residues; endosome).

The only known physical and genetic interactor of Zeo1 is the cell wall stress sensing protein Mid2 [26]. Amongst Zeo1's annotated physical interactors is also the calmodulin protein Cmd1 [27], an important modulator of SNCA toxicity in yeast [28]. Furthermore, an identified genetic interactor of Zeo1 is the SNARE protein Sec9 [29], again a protein implicated in SNCA toxicity in yeast [30].

ZEO1 gene expression profile

Given that little is known about the function of Zeo1 in yeast and annotations on the protein are limited, a recently published yeast deletion mutant expression profile matrix [31,32] was utilized to determine if the *ZEO1* gene matches closely to other better-known yeast proteins in its response to specific gene deletions, thereby providing hints on its cellular function. The yeast deletion gene expression dataset contained 700 responsive mutants, defined as having ≥4 significant mRNA expression changes caused by the gene deletion. The highest outlying gene to correlate with *ZEO1* was *MRH1* (**Fig. 3A**), which encodes a plasma membrane protein that can also be detected in a phosphorylated state in mitochondria. We also plotted the expression profile correlation values for *HSP12* (**Fig. 3B**), which appears continuous and no particular gene stands out.

SNCA and Zeo1: shared cellular toxicity

To begin to assess the biochemistry of the candidate synuclein homolog Zeo1 in yeast, we chose to use Hsp12 alongside Zeo1 as a control. This is due to Hsp12's structural similarity to Zeo1 and previous reports of its potential connection to SNCA. Growth curves were generated for mock-, vector-, Hsp12- and Zeo1-transformed W303 *MAT*a yeast cells, the genetic background used for the generation of our yeast synuclein models (**Fig. 4A**). Two background strains differing in the production of an adenine-dependent red pigment were used for

comparison purposes (*ade2-1* and *ADE2*$^{+}$). Hsp12 and Zeo1 were C-terminally tagged with monomeric superfolder GFP (msfGFP). The results show a consistent negative effect of Zeo1-msfGFP expression on growth, with Hsp12-msfGFP not having a noticeable effect. Therefore, in growth-related assays, Hsp12 can be considered a suitable control for comparison with Zeo1. Growth in *ADE2*$^{+}$ cells appeared to start with a delay for all conditions relative to the *ade2-1* background, but reached a higher maximum OD_{600} value. These results were also confirmed using untagged versions of Hsp12 and Zeo1 (data not shown).

Hsp12-msfGFP or Zeo1-msfGFP were then co-overexpressed with SNCA-mKate2 in red-pigment-lacking W303 *MAT*a *ADE2*$^{+}$ cells and imaged using a confocal microscope 3 hours after galactose induction, confirming Yeast GFP Fusion Localization Database annotations of Hsp12's cytoplasmic and Zeo1's lipid membrane localizations (**Fig. 4B** and **C**). SNCA-mKate2 localized to the plasma membrane as previously reported in low to mild toxicity strains [33].

Moreover, given Zeo1's physical interaction with the yeast calmodulin protein (Cmd1) and the previous identification of FK506 as a SNCA-rescuing compound [28], integrated SNCA and Zeo1 strains (constructed in parallel) were grown in the presence of 25 µg/mL FK506, and their growth was quantified after 70 hours at late-log or early-stationary phase. A vector-integrated strain in the same background was used to control for FK506's possible independent effects on growth. The compound rescued SNCA and Zeo1 toxicities relative to FK506's effect on the control strain (**Fig. S3**).

Pkc1: a common denominator?

We next considered whether SNCA and Zeo1 might share one or more molecular pathways in their overexpression-driven toxicities in yeast. To begin to address this question, we used previously identified SNCA and amyloid-β (Aβ1-42) genetic modifiers in yeast [34,35] and performed a candidate-based genetic screen in Aβ1-42, SNCA and Zeo1 integrated yeast strains, each compared with a background-matched control (**Fig. 5**). The Aβ1-42 strain acted as a control. Although certain cellular aspects of SNCA and Aβ1-42 toxicities are shared in yeast, we were primarily interested in a genetic modifier whose effect on SNCA/Zeo1 would be in the same direction while being opposite in the Aβ1-42 strain. Amongst the modifiers tested, Mid2 (the Zeo1 binding partner through which Zeo1 was first described [26]) and the yeast protein serine/threonine kinase Pkc1 showed such a profile.

Similar to the SNCA structural homology search presented in **Figure 2**, we performed additional two-dimensional searches with other pairs of PD-related proteins. One such search with the human Parkin

interacting substrate PARIS (ZNF746), which is involved in mitochondrial dynamics [36], and the mitochondrial kinase PINK1 [37], revealed Pkc1 as the only outlier amongst all yeast proteins (**Fig. 6**). This hybrid homology of Pkc1 to two SNCA-independent PD-implicated human genes is noteworthy in light of Pkc1's connection to Zeo1 described earlier. No other yeast kinase has full-length homology to Pkc1. A close full-length human homolog of yeast Pkc1 is PKN3 (31% identity; 55% similarity). In turn, PKN3 has no appreciable sequence similarity to either PINK1 or PARIS.

DISCUSSION

Budding yeast cells have been greatly useful as models of proteotoxicity in neurodegenerative diseases [38] and broader aspects related to neurodegeneration, such as ageing [39]. An outstanding question in the context of the yeast SNCA model of proteotoxicity was the missing link to an endogenous SNCA-like protein in yeast itself, and our work here begins to answer that question by providing multiple pieces of evidence towards a link to the Zeo1 protein (**Table 1**). We relied on methods that look beyond sequence-based homologs in determining candidate structural homologs of SNCA in yeast. Although "true structural" search methodologies, where the real three-dimensional structure of a query protein is searched against the real or predicted structures of other proteins, do not exist, methods such as HHpred, which rely on hidden Markov model profiles of large multiple sequence alignments, are amongst the state-of-the-art approaches currently possible.

The notion of the candidate protein Zeo1 possessing a structure reminiscent of SNCA without any shared sequence motifs is similar to recent observations regarding the N-terminus of the transactivation response element (TAR) DNA-binding protein 43 (TDP-43) possessing a ubiquitin-like fold [40], even though the domain itself is clearly not a *bona fide* ubiquitin domain. There is little known about the Zeo1 protein's biochemistry and normal function in yeast. It was first identified in 2003 as an interacting partner of Mid2 [26], a Cell Wall Integrity (CWI) sensor protein that signals through the downstream Pkc1 / MAP kinase pathway [41,42]. Zeo1 is phosphorylated by the kinase Ime2 [43], a SNCA toxicity suppressor [34], and has been isolated in a phosphorylated form in purified yeast mitochondria [44]. Among Zeo1's known physical interactors, its interaction with calmodulin (Cmd1) is noteworthy given the established role of calcineurin and calmodulin on synuclein biology [28]. Lastly, a reported transcription factor regulating the expression of *ZEO1* is the gluconeogenesis and glyoxylate cycle factor Rds2 [45], which is notable given previous reports linking SNCA and glycation stress [46].

Most important to the analysis presented in this paper, a recent study utilizing a yeast α-synuclein model and focusing on mitochondrial cell death identified Zeo1 to be specifically downregulated at the outer mitochondrial membrane in yeast using immunoblotting and SILAC mass spectrometry approaches [47]. Moreover, the authors note that "the levels of additional outer mitochondrial membrane proteins such as components of the TOM (translocase of the outer membrane) complex were unaffected."

The proposal of Pkc1 as a potential common denominator of SNCA/Zeo1 toxicity in yeast and PINK1/PARIS mitochondrial Parkinson's pathobiology in humans (**Fig. 6**) is interesting for a number of reasons. First, it corroborates reports that have directly linked these two arms of PD research in other model systems, such as studies reporting Pink1 overexpression to be protective in a *Drosophila* model of SNCA toxicity [48] or *Pink1*-knockout mice to show increased α-synuclein toxicity [49]. Second, Mid2 and Pkc1 are on the two ends of the yeast CWI pathway, and were both reported to enhance Aβ1-42 toxicity in yeast [35]. In fact, Zeo1 itself is phosphorylated upon Pkc1 hyperactivation [50]. Furthermore, the ubiquitin protease Ubp3, which is a physical interactor of Zeo1 [27] and a yeast modifier of SNCA toxicity [51], downregulates Pkc1 signaling [52]. In budding yeast, the GTPase Rho1 transduces signals to Pkc1. This is in contrast to fission yeast, in which a greater number of protein modulators are involved in this process [53]. A specific Pkc1 inhibitor, cercosporamide, also exists for use in yeast experiments [54], which can facilitate studies pertaining to the linkage of SNCA and Pkc1 biologies in yeast, and its translation to other model organisms. An important example of this utilization was reported in the work of Wang and colleagues in 2012 [55] in which SNCA toxicity was shown to be mediated through Cdc5, Pkc1 and CWI sensors such as Mid2 in yeast.

ACKNOWLEDGEMENTS

This work was conducted as part of the research groups of Susan Lindquist (WIBR) and Bonnie Berger (MIT CSAIL). Jian Peng and Brooke J. Bevis provided technical assistance. We would like to thank Guri Giaever (University of British Columbia, Vancouver, Canada) for bringing the yeast molecular signatures database to our attention, George Bell (WIBR) for adapting and streamlining the database for correlational analysis, Wendy Salmon (WIBR) for assistance with confocal microscopy, Yanmei Huang (WIBR) for the ImageJ spot assay quantification macro and Johannes Söding (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany) for clarifying structural aspects of the HHpred algorithm. S.E. is supported by a Canadian Institutes of Health Research postdoctoral fellowship.

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FIGURE LEGENDS

Figure 1. Phylogenetic analysis of the synuclein gene family. (**A**) Schematic representation of human α- (SNCA), β- (SNCB) and γ-synuclein (SNCG) domain structures (SNCA domain model modified from Venda *et al.* 2010 [56]). (**B**) Phylogenetic tree representing the distribution of synuclein domains in the SUPERFAMILY database of protein structural and functional annotations. ‘C’ and ‘M’ represent chordates and metazoans,

respectively (the choice of species to represent transitions across different phyla is based on Schmitt-Ulms *et al.* 2009 [10]). (**C**) Multiple sequence alignment of representative synuclein proteins depicted in the phylogenetic tree demonstrates the high conservation of the lipid-binding and hydrophobic domains from mammals to fish. The C-terminal acidic domain shows greater variability.

Figure 2. Identification of yeast Zeo1 and Hsp12 proteins as candidate synuclein structural homologs. An alignment of the two search structures (human SNCA 1XQ8 and *Leishmania major* SHERP 2X43) used for this two-dimensional HHpred analysis is shown in panel **A**. All yeast proteins with non-zero scores to both structures are depicted in panel **B**. The values on the axes represent probabilities of secondary structure matches calculated by HHpred's *HHsearch* program. 3,103 proteins were identified to match either of the two structures, and 638 matched both structures to some degree. As a comparison, 17 representative synuclein sequences were added to the search (**C**), which matched the synuclein 1XQ8 structure 100%, and showed a distribution in matching the Lm_SHERP structure. Given that yeast proteins vary greatly in their lengths (**D**), proteins whose lengths were not within 5 standard deviations of the average length of the 17 representative synuclein proteins (127±(5×9) = 82 to 172) were excluded (**E**). Based on these criteria, Zeo1 and Hsp12 appear as two outlying protein hits.

Figure 3. Molecular signature of the *ZEO1* gene. (**A**) The correlation of the expression change of *ZEO1* was calculated one-by-one to the expression change of all yeast genes using an expression profile dataset of 700 deletion mutants, and sorted from the highest to lowest values. Three genes stand out from the rest of the *ZEO1* correlation trend (*MRH1*, *GPX2* and *FET3*), whereas such a 'falloff' is not apparent for *HSP12* correlations (**B**), which appear more as a continuum.

Figure 4. Zeo1 overexpression is toxic in yeast and localizes to lipid membranes. (**A**) Zeo1, expressed from a galactose-inducible 2-µm plasmid, is toxic in two yeast backgrounds previously used for the generation of reference SNCA toxicity strains. Mock-transfected strains (in leucine selective or CSM complete media) and Hsp12-expressing yeast were used as controls. The curves depict average values from three experimental replicates, with the error bars representing standard errors of the mean. To confirm cellular localizations, mKate2-tagged SNCA was co-overexpressed with Hsp12-msfGFP (**B**) or Zeo1-msfGFP (**C**) and imaged 3 hours after galactose induction. Hsp12 shows cytoplasmic localization, whereas Zeo1 and SNCA localize on the

plasma membrane. Additionally, Zeo1 appears to localize to the membranes of vacuoles, mitochondria and possibly the ER.

Figure 5. Pkc1, a common denominator of SNCA and Zeo1 toxicity. Three integrated yeast strains overexpressing (i) the human amyloid-β peptide (Aβ1-42), (ii) human SNCA and (iii) yeast Zeo1 were used for a candidate-based genetic modifier screen to determine commonalities between SNCA and Zeo1, with the Aβ1-42 strain serving as a control. The toxicities of the three strains relative to their background-matched controls are shown in **panel A**. Among the genes tested, *MID2* and *PKC1* showed a specific effect profile whereby they enhanced toxicity in the Aβ1-42 strain but rescued both SNCA and Zeo1 toxicities (**B**). Mid2 *P*-values: SNCA vs. Aβ1-42 = **0.006**; Zeo1 vs. Aβ1-42 = **0.002**. Pkc1 *P*-values: SNCA vs. Aβ1-42 = **0.018**; Zeo1 vs. Aβ1-42 = **0.012**.

Figure 6. Pkc1 and hybrid homology to two human PD-related mitochondrial proteins. (**A**) Pkc1 stands out amongst all yeast proteins as a candidate with a hybrid predicted structural homology to human PINK1 and PARIS (ZNF746) proteins. (**B**) Pkc1's homology to PINK1 is within residues 883-1084 (kinase domain) and matches to PARIS at residues 485 to 535 (membrane-targeting C1A domain).

Table 1. Similarities and differences of SNCA and Zeo1 biology in yeast.

Supplementary Figure 1. Selection of *Leishmania major* SHERP for structural homology search. To determine a second PDB structure to complement the micelle-bound SNCA structure (1XQ8) for our two-dimensional homology search, the results of a query using the 1XQ8 structure as input in the Fold and Function Assignment server (FFAS) tool [19] are illustrated. *L. major* SHERP appears as the closest structure to 1XQ8, followed by different apolipoprotein structures.

Supplementary Figure 2. Candidate-based confirmation of Zeo1 as a SNCA structural homolog. To reconfirm the identification of Zeo1 as the main structural synuclein-like protein in yeast, a candidate-based HHpred analysis was undertaken. To begin with, the three human synuclein proteins and the sea lamprey synuclein (representing an early confirmed synuclein) were queried against the yeast proteome (**Tier 1**) using the HHpred homology and structure prediction public server. Each protein name in a box on the flowchart is followed by the *E*-value and bit score of its structural alignment. Given that HHpred returns both structural (PDB)

and sequence-based similarity hits in addition to the identified yeast proteins, a number of protein structures from different species were also identified. These 11 hits were then each used as queries in HHpred to determine possible similarities with other yeast proteins (**Tier 2**). This step expanded the list of candidate yeast proteins to 99. In this tier, only yeast sequences were selected, and structural hits from other species were excluded. As a final step, each of the 99 yeast candidates was searched using HHpred against the human proteome to determine if any is synuclein-like (**Tier 3**). Only Zeo1 was predicted as having structural similarity to human SNCA. The PDB template identified in this step is the micelle-bound 1XQ8 structure of SNCA.

Supplementary Figure 3. FK506 rescues SNCA- and Zeo1-induced toxicity. The previously-reported SNCA toxicity-rescuing compound FK506 was added at its effective concentration (25 μg/mL) to the growth media of integrated SNCA- and Zeo1-overexpressing yeast strains and compared to a background-matched vector-integrated control strain. At the late-log phase, SNCA growth increased by approximately 200% and that of Zeo1 by about 100% relative to a no-compound condition (*P*-values of 0.001 and 0.027, respectively, compared to vector control).

Synuclein structural homology to yeast Zeo1p
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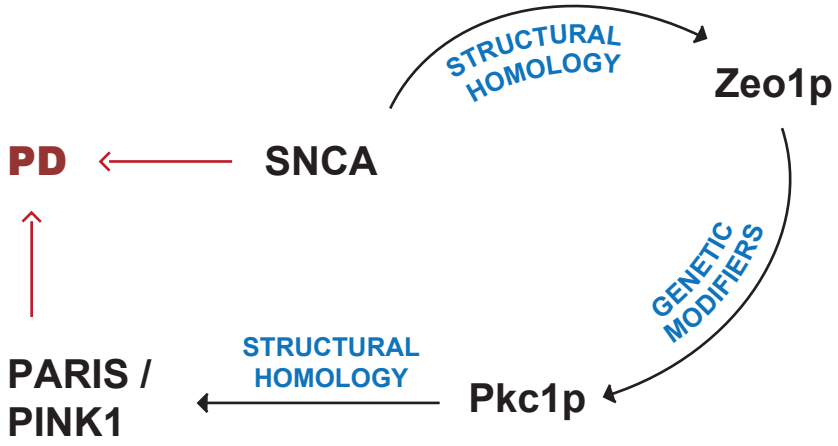
Graphical Abstract



Synuclein structural homology to yeast Zeo1p
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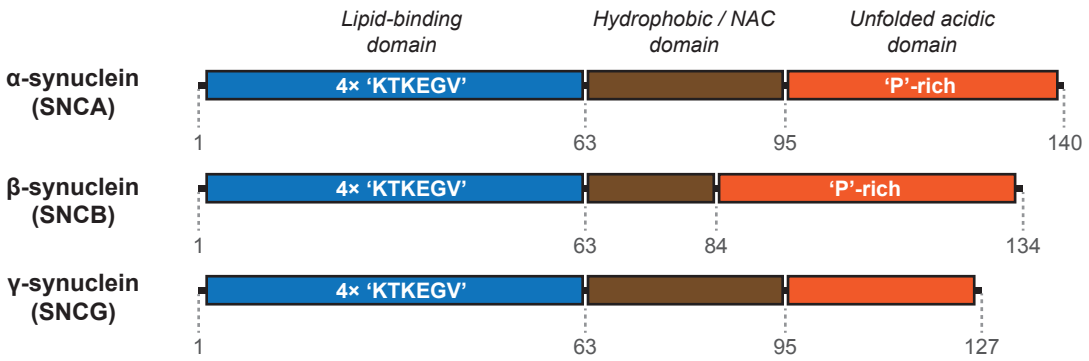
Figure 1

A



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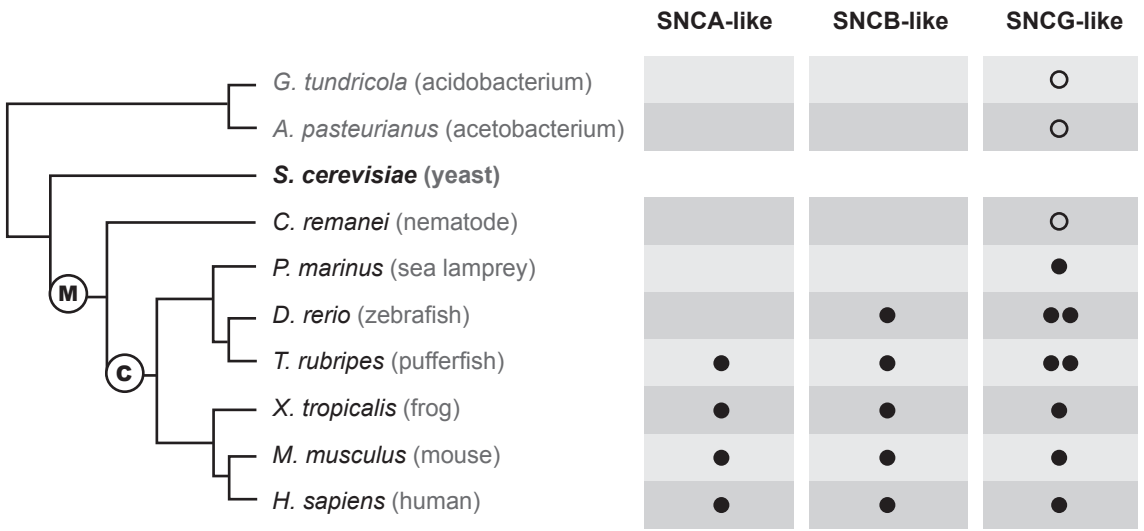

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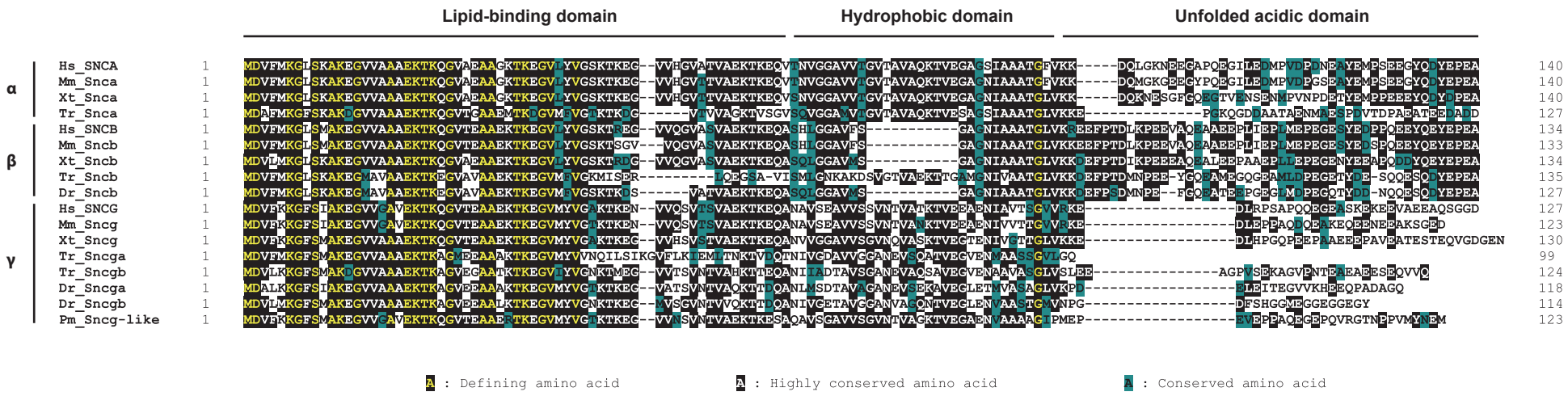
Figure 1

C



Species abbreviations and select accession #'s:

Hs	*Homo sapiens* (human)
Mm	*Mus musculus* (house mouse)
Xt	*Xenopus tropicalis* (Western clawed frog)
Tr	*Takifugu rubripes* (pufferfish)
Dr	*Danio rerio* (zebrafish)
Pm	*Petromyzon marinus* (sea lamprey); acc. #: ENSPMAP00000000053

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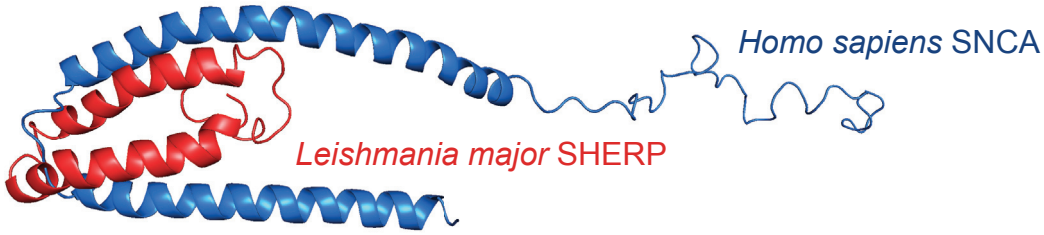
Figure 2

A



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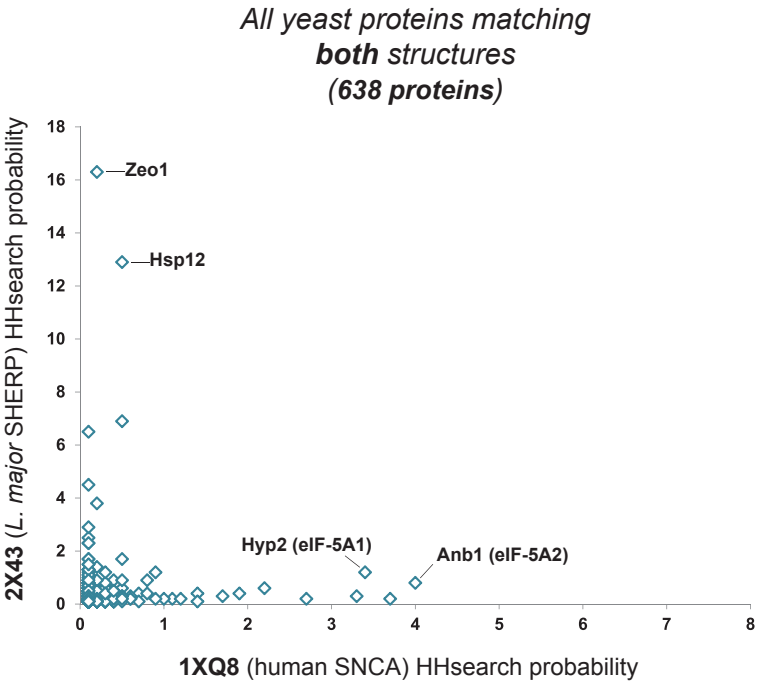

Synuclein structural homology to yeast Zeo1p
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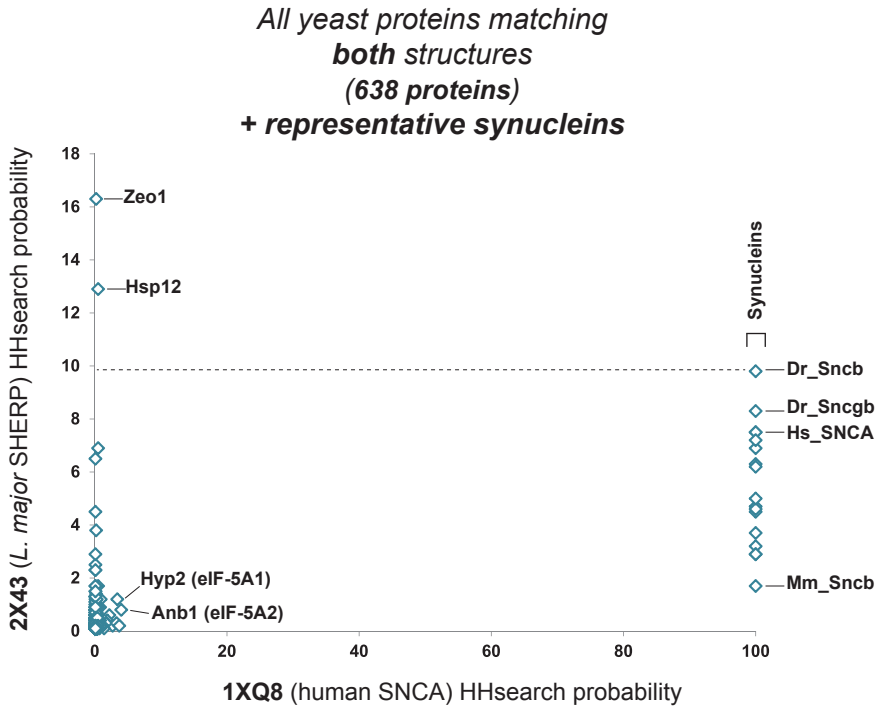
Figure 2

C



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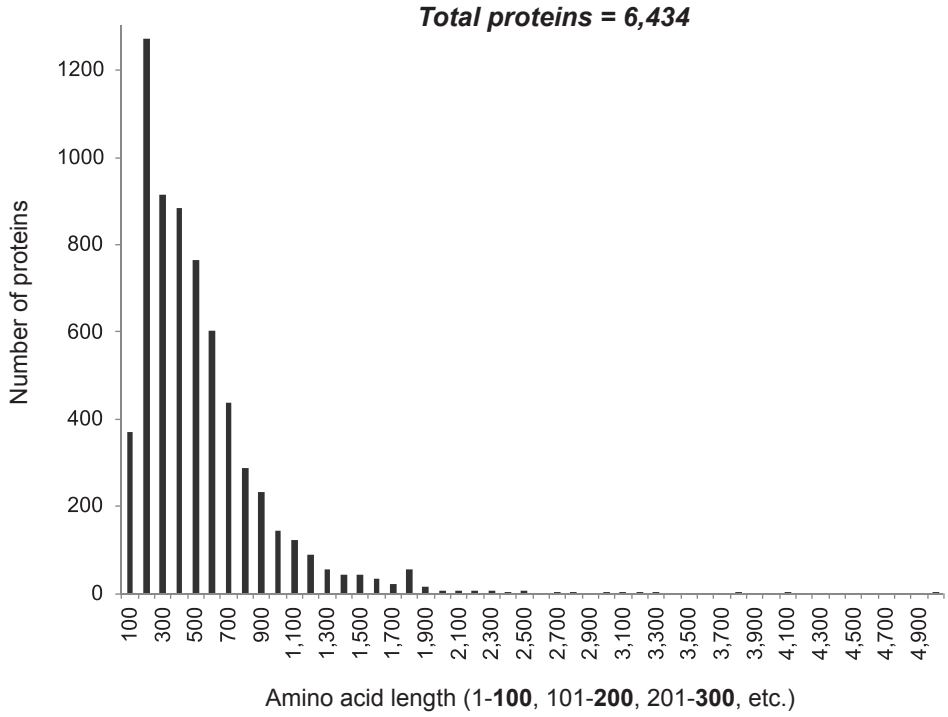

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Figure 2

E

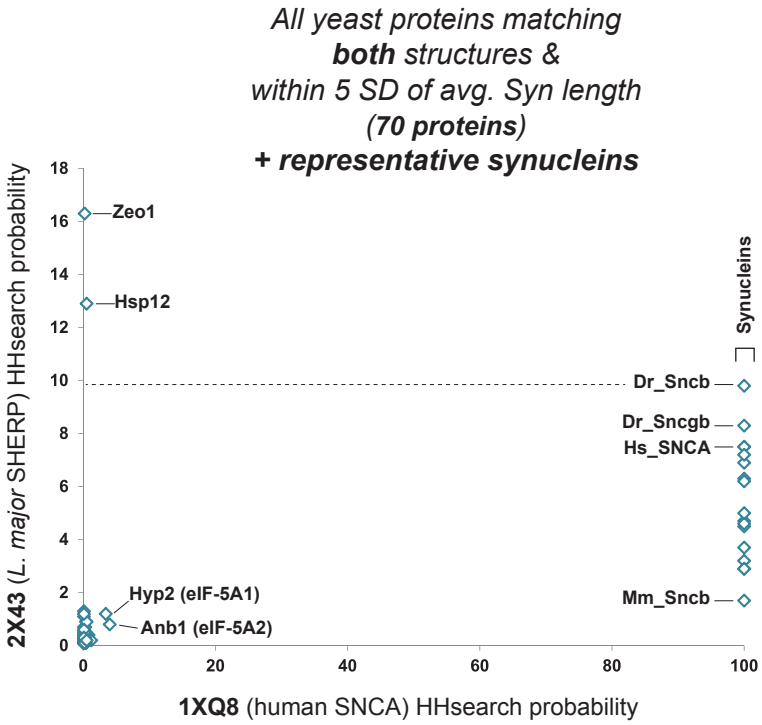

Figure 3

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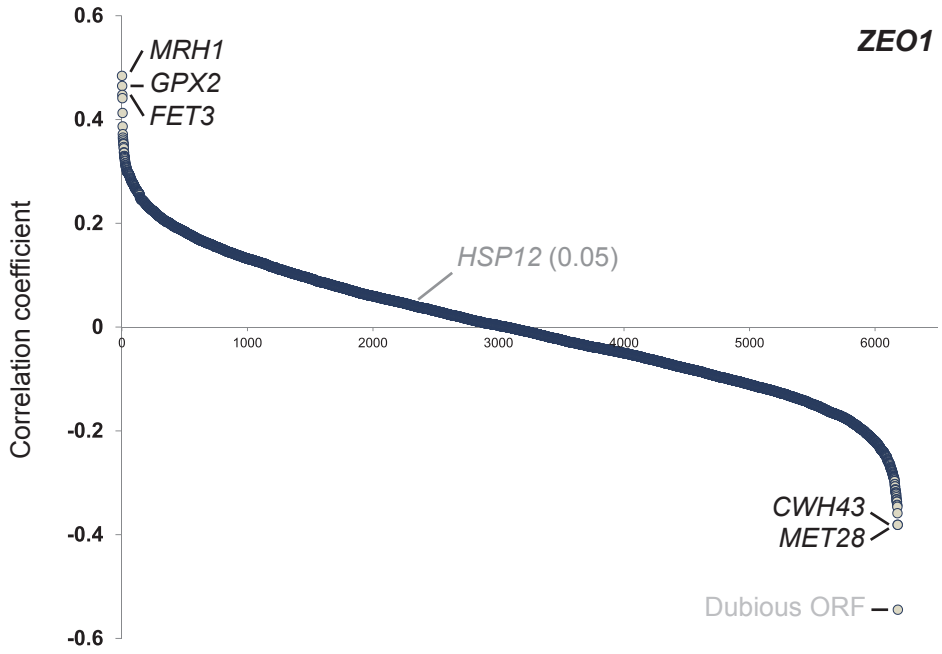
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MRH1
GPX2
FET3
HSP12 (0.05)
CWH43
MET28
Dubious ORF
Correlation coefficient
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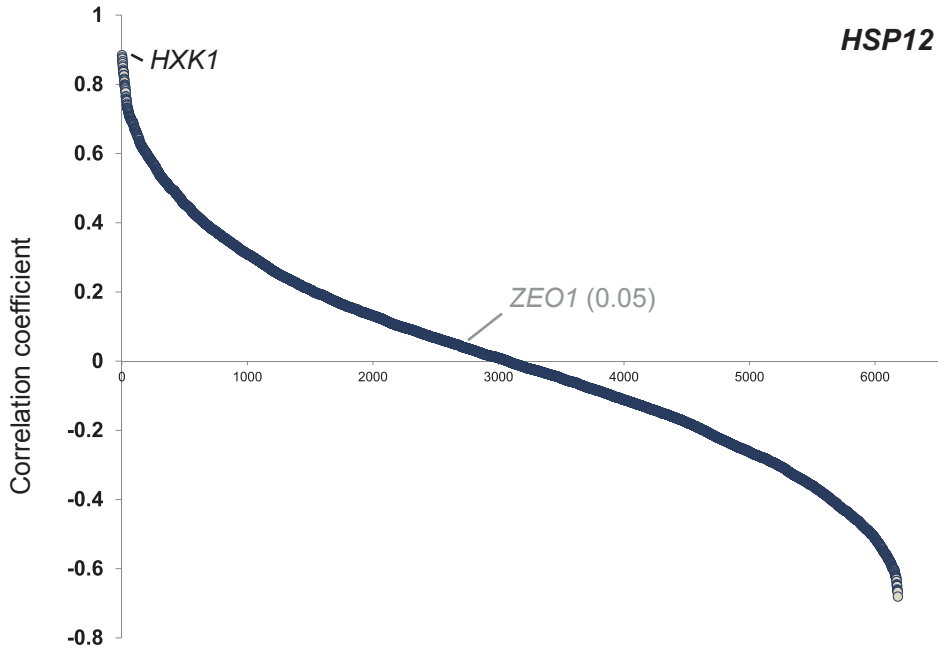
0.2
0
-0.2
-0.4
-0.6
0
1000
2000
3000
4000
5000
6000

B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HXK1
ZEO1 (0.05)
Correlation coefficient
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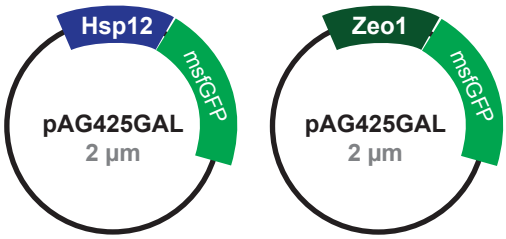
Figure 4

A



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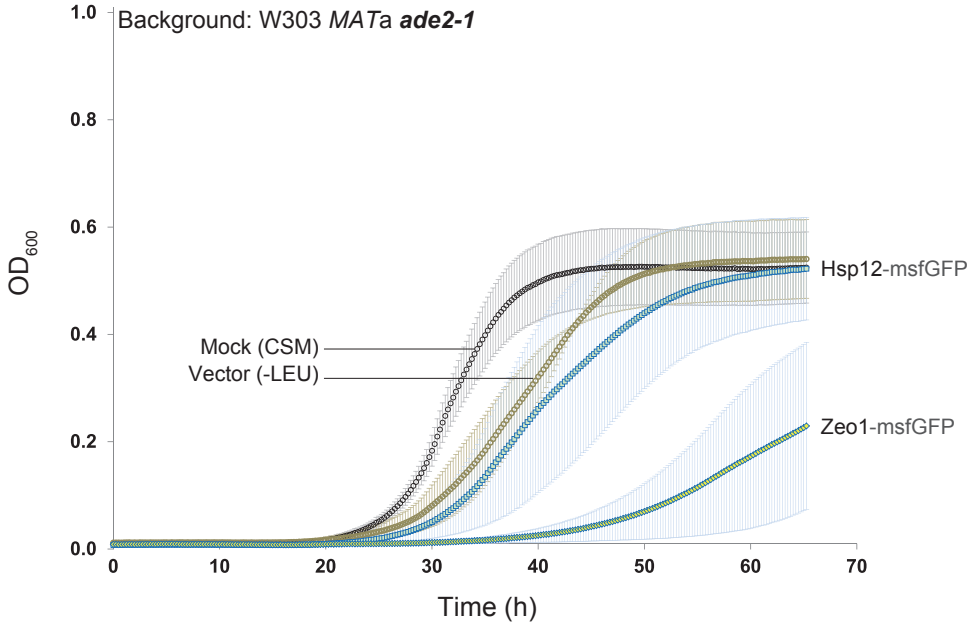

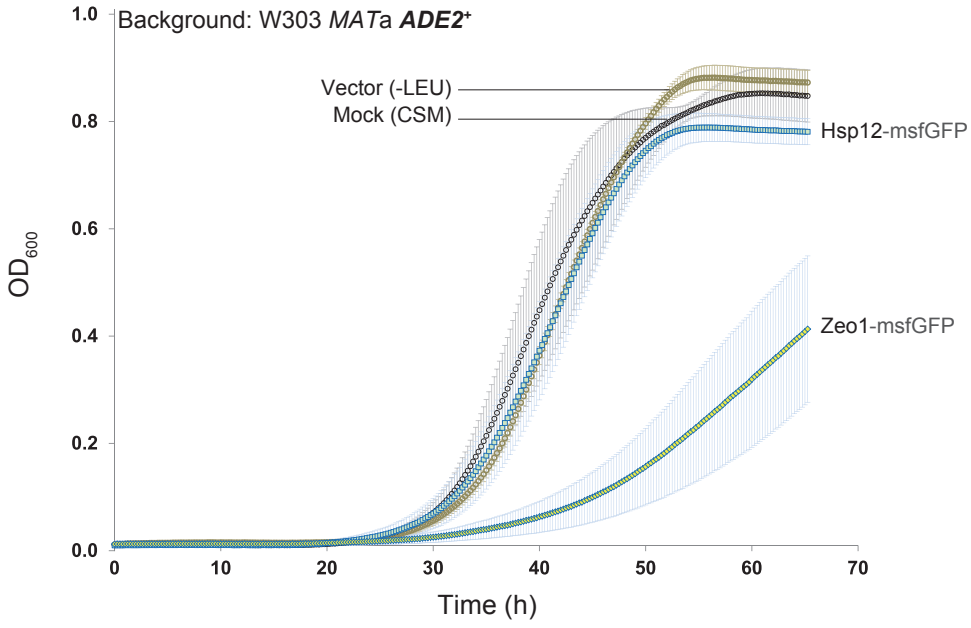

Synuclein structural homology to yeast Zeo1p
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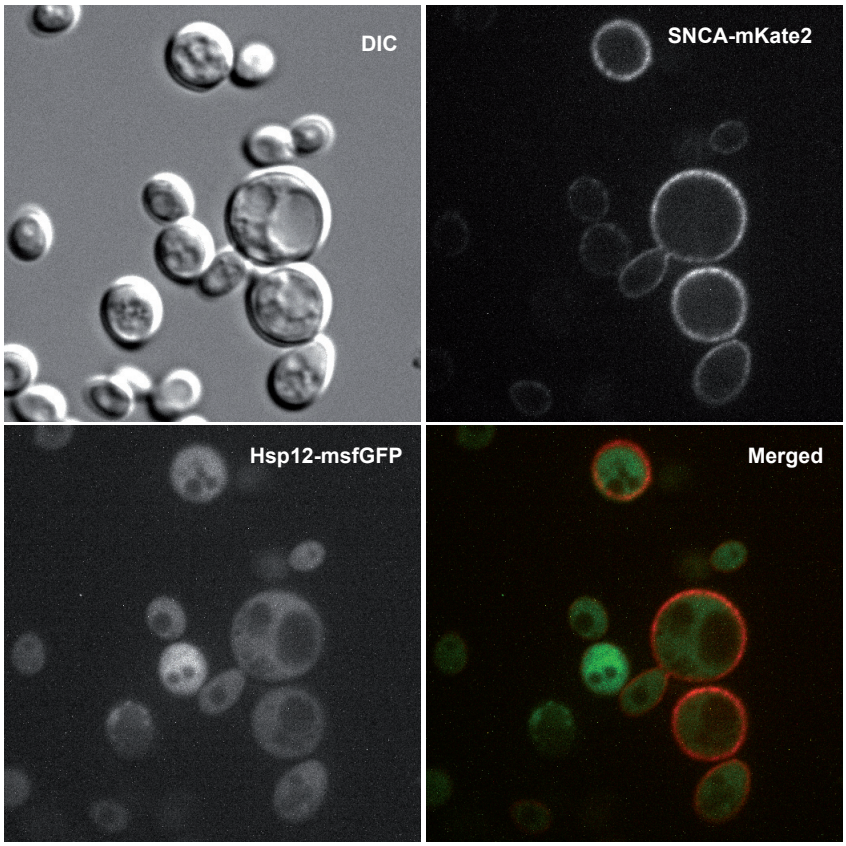
Figure 4

B



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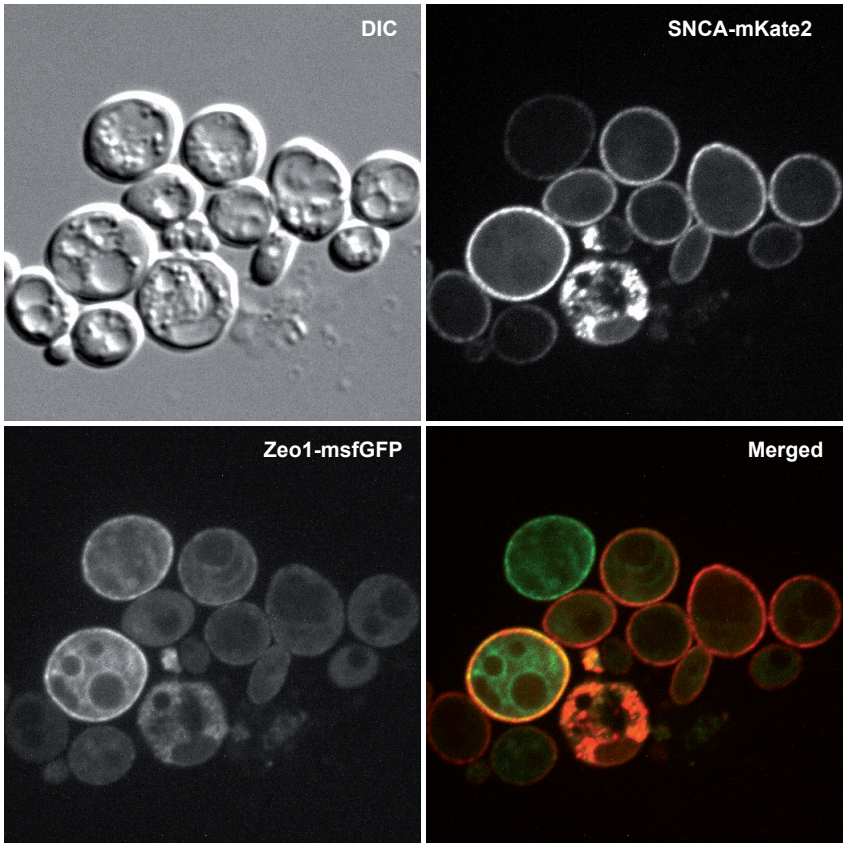

Synuclein structural homology to yeast Zeo1p
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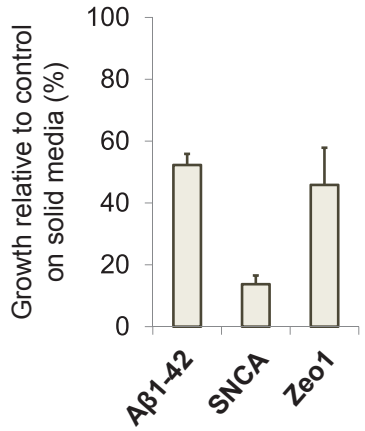
Figure 5

A



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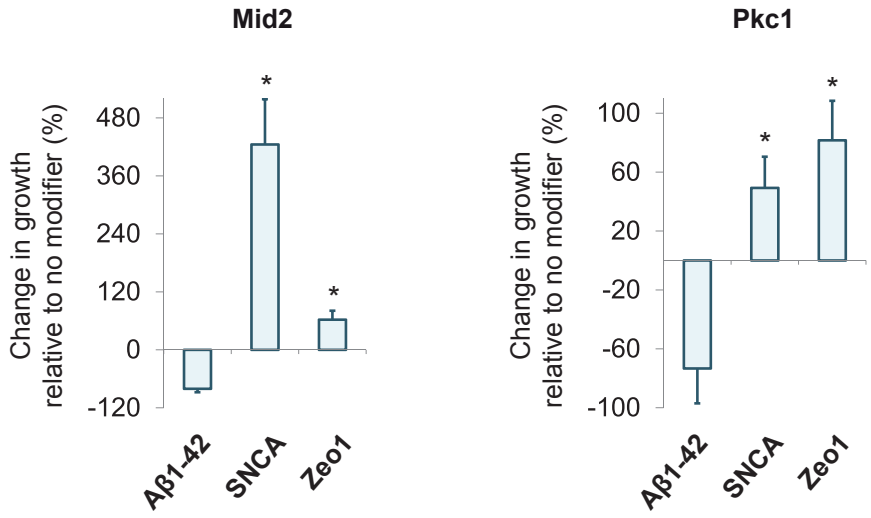

Synuclein structural homology to yeast Zeo1p
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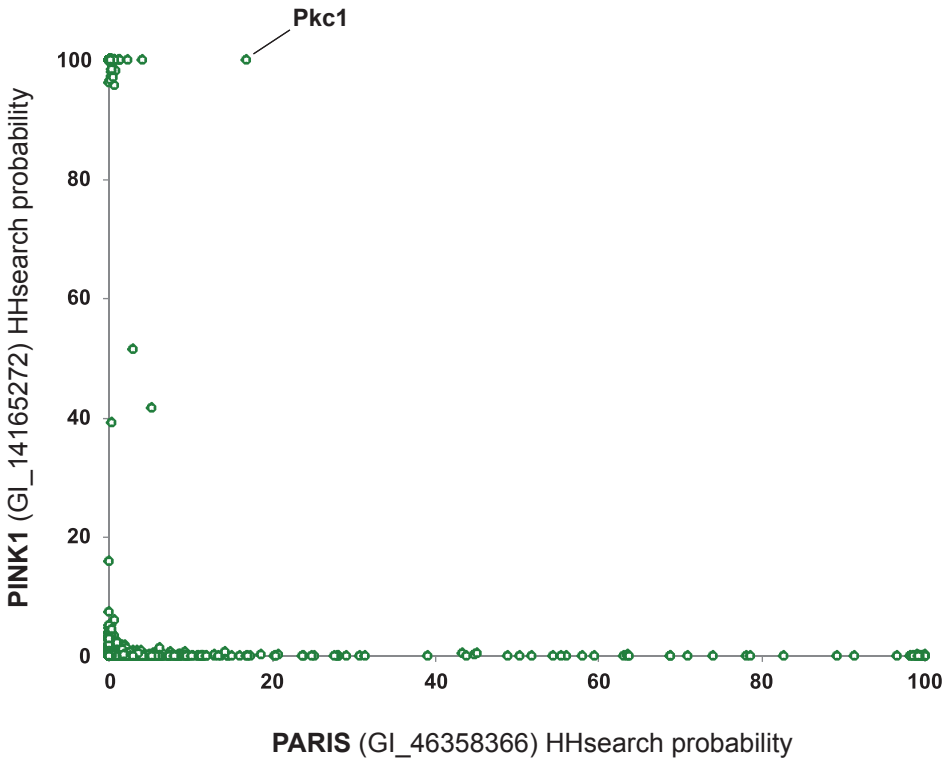
Figure 6

A



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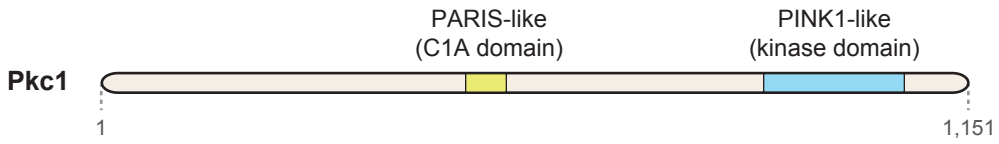

Table 1. Similarities and differences of SNCA and Zeo1 biology in yeast

	SIMILARITIES		DIFFERENCES
1.	Predicted structural homology	1.	Shared sequence motifs
2.	Similar sequence length	2.	Charge distribution and hydrophobicity
3.	Lipid binding and membrane affinity		
4.	Cell Wall Integrity pathway signaling		
5.	Modulation of metal biology (e.g., Mn^{2+})		
6.	Overexpression toxicity		
7.	Predicted *O*-glycosylation		
8.	Similar response to the SNCA-rescuing compound FK506		
9.	Increased phosphorylation and decreased abundance of Zeo1 in SNCA-expressing cells		
10.	SNCA expression specifically changes Zeo1 expression on outer mitochondrial membrane		
11.	Zeo1 identified as one of the few proteins phosphorylated by Ime2, a SNCA toxicity suppressor		

Synuclein structural homology to yeast Zeo1p
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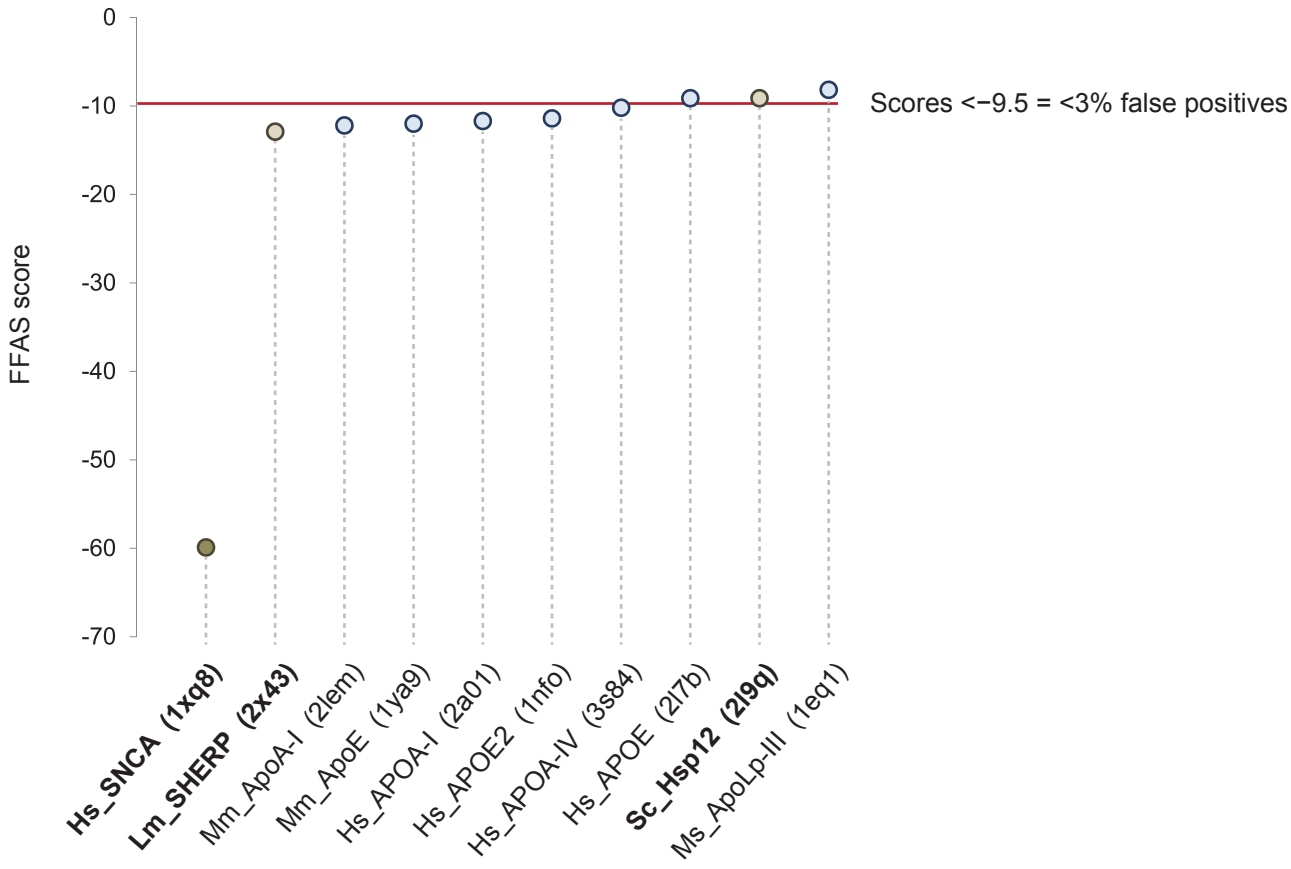
Supplementary Figure 1



Species abbreviations:

Hs: *Homo sapiens* (human)
Lm: *Leishmania major* (protozoan)
Mm: *Mus musculus* (house mouse)
Ms: *Manduca sexta* (hawk moth)
Sc: *Saccharomyces cerevisiae* (budding yeast)

Synuclein structural homology to yeast Zeo1p
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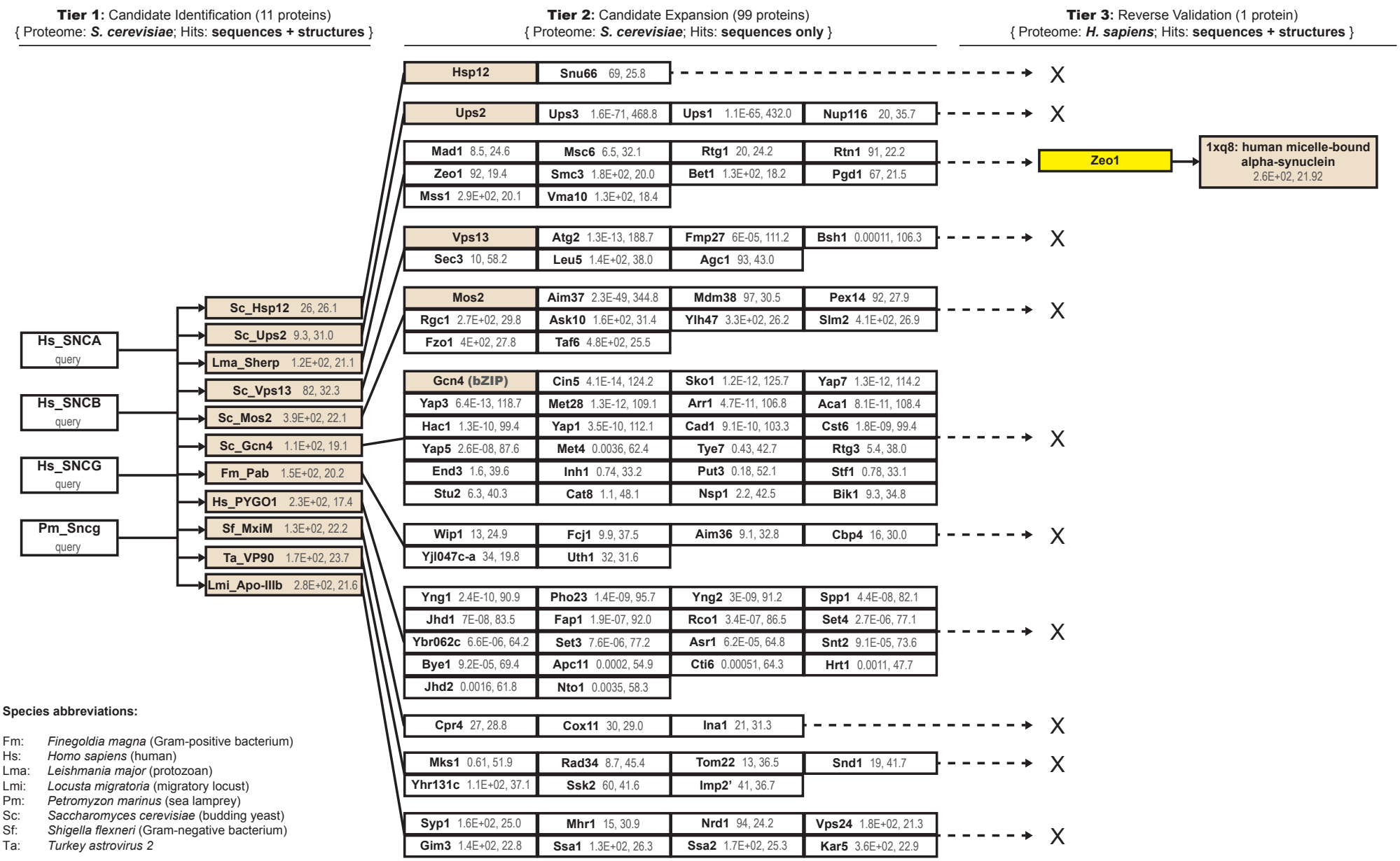
Supplementary Figure 2



Species abbreviations:

Fm: *Finegoldia magna* (Gram-positive bacterium)
Hs: *Homo sapiens* (human)
Lma: *Leishmania major* (protozoan)
Lmi: *Locusta migratoria* (migratory locust)
Pm: *Petromyzon marinus* (sea lamprey)
Sc: *Saccharomyces cerevisiae* (budding yeast)
Sf: *Shigella flexneri* (Gram-negative bacterium)
Ta: *Turkey astrovirus 2*

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Supplementary Figure 3