

α -Synuclein is part of a diverse and highly conserved interaction network that includes PARK9 and manganese toxicity

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Parkinson's disease (PD), dementia with Lewy bodies and multiple system atrophy, collectively referred to as synucleinopathies, are associated with a diverse group of genetic and environmental susceptibilities. The best studied of these is PD. α -Synuclein (α -syn) has a key role in the pathogenesis of both familial and sporadic PD, but evidence linking it to other predisposition factors is limited. Here we report a strong genetic interaction between α -syn and the yeast ortholog of the PD-linked gene *ATP13A2* (also known as *PARK9*). Dopaminergic neuron loss caused by α -syn overexpression in animal and neuronal PD models is rescued by coexpression of *PARK9*. Further, knockdown of the *ATP13A2* ortholog in *Caenorhabditis elegans* enhances α -syn misfolding. These data provide a direct functional connection between α -syn and another PD susceptibility locus. Manganese exposure is an environmental risk factor linked to PD and PD-like syndromes. We discovered that yeast *PARK9* helps to protect cells from manganese toxicity, revealing a connection between PD genetics (α -syn and *PARK9*) and an environmental risk factor (*PARK9* and manganese). Finally, we show that additional genes from our yeast screen, with diverse functions, are potent modifiers of α -syn-induced neuron loss in animals, establishing a diverse, highly conserved interaction network for α -syn.

Compelling evidence implicates α -syn in the pathogenesis of PD¹, including the identification of point mutations and locus duplication^{2,3} and triplication⁴ in familial forms, the abundance of α -syn in Lewy bodies⁵ and neurodegeneration resulting from increased expression of α -syn in multiple animal models^{6–9}. Likewise, expression of α -syn in yeast cells results in dosage-dependent toxicity¹⁰. Several features of this toxicity, including production of reactive oxygen species (ROS), lipid droplet accumulation and vesicle trafficking defects, are reminiscent of α -syn toxicity in mammalian neurons¹⁰. Therefore, yeast cells afford the opportunity to rapidly screen for modifier genes with the hope that the identified genes will point to common cellular mechanisms of toxicity and suggest avenues for therapeutic intervention¹¹.

We recently reported the identification of a set of conserved genes functioning in vesicular trafficking between the endoplasmic reticulum (ER) and Golgi that are potent modifiers of α -syn toxicity in yeast. Rab1, the mammalian ortholog of one of the encoded proteins from the yeast screen, Ypt1, was tested in neuronal models of PD and was able to prevent dopaminergic neuron loss¹². We then expanded this screen to include 5,000 yeast genes. In addition to vesicular

trafficking genes, we identified several other categories of α -syn-toxicity modifiers, many with clear human orthologs, which reveal additional complexities to synuclein pathology and which complement numerous studies of human synucleinopathies¹³.

A key question in the field is whether the genetic loci linked to PD interact with each other or whether multiple independent insults simply happen to result in a common phenotype (dopaminergic neuron loss and resulting parkinsonism). There is emerging evidence for a genetic interaction between PD-linked genes *parkin* and *pink1* in *Drosophila*^{14–16} as well as interactions between α -syn and DJ-1, another PD-linked protein^{17–20}. Recently, the molecular nature of the gene responsible for early-onset parkinsonism with pyramidal degeneration and dementia (Kufor-Rakeb syndrome; MIM606693) was elucidated and found to encode *ATP13A2*, a predicted lysosomal P-type transmembrane cation transporting ATPase^{21–23}.

As part of our unbiased screen to find modifiers of α -syn toxicity^{12,13}, here we show that the yeast homolog of human *ATP13A2* (*PARK9*) can suppress α -syn toxicity in yeast. This genetic interaction between *ATP13A2* and α -syn is conserved in neurons because *ATP13A2* expression in animal models of PD is sufficient to rescue

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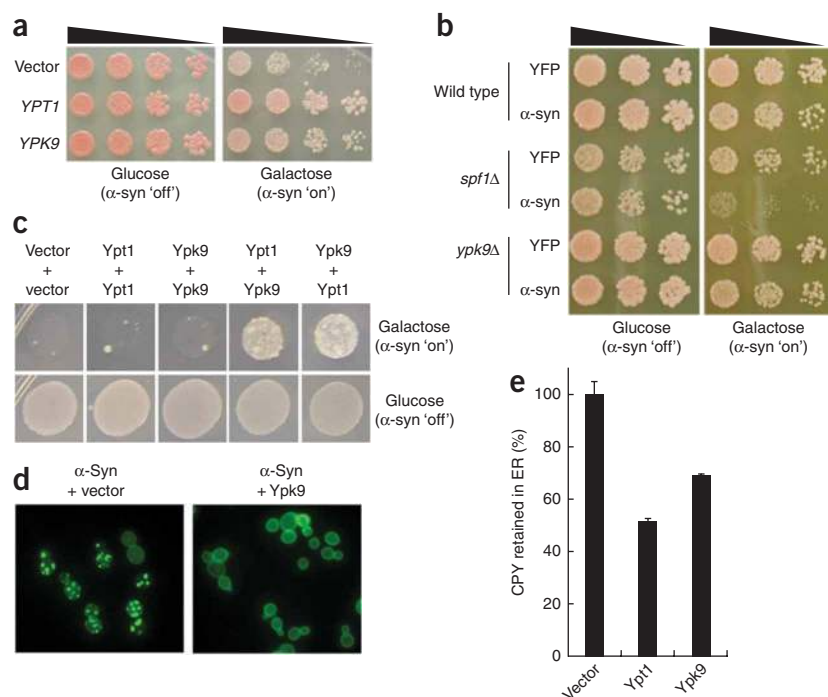


Figure 1 Interaction between α -syn and the yeast PARK9 homolog. **(a)** Spotting assays with yeast α -syn toxicity modifier genes *YPT1* and *YPK9* showing their ability to suppress toxicity compared to empty vector control. Fivefold serial dilutions of yeast cells were spotted onto glucose (α -syn expression repressed) or galactose (α -syn expression induced). **(b)** Deletion of *YPK9* has no effect on α -syn toxicity; however, deleting the closely related ATPase *SPF1* enhances α -syn toxicity. **(c)** Synergistic genetic interaction between α -syn toxicity modifiers Ypt1 and Ypk9. In a high toxicity (HiTox) two-copy α -syn yeast strain, expression of Ypt1 or Ypk9 alone is not sufficient to rescue toxicity. However, their coexpression restores growth to this strain. **(d)** Ypk9 overexpression eliminates α -syn inclusions. Cells expressing α -Syn-YFP contain many vesicular inclusions when transformed with an empty vector and these are greatly diminished in cells transformed with a Ypk9 expression plasmid. **(e)** The ability of Ypk9 to suppress the α -syn-induced block in ER-Golgi was measured by carboxypeptidase Y (CPY) maturation assay. Ypk9 considerably improved the trafficking of CPY from ER to Golgi. Values represent means \pm s.d.

neurodegeneration. We also show that the yeast ortholog of the *ATP13A2* gene can protect cells from manganese toxicity, suggesting an intimate connection between genetic and environmental causes of neurodegeneration. Finally, we find other diverse modifiers of α -syn toxicity from our screen, which are able to suppress α -syn-induced neurodegeneration in animal and neuronal models of PD. These include yeast genes and their human orthologs, encoding an E3 ubiquitin ligase (*HRD1/SYVN1*), ubiquitin protease (*UBP3/USP10*), phosphodiesterase (*PDE2/PDE9A*), polo-like kinase (*CDC5/PLK2*) and a casein kinase (*YCK3/CSNK1G3*). Thus, our data establish α -syn as part of a highly conserved, multifaceted pathway.

RESULTS

Yeast *ATP13A2* homolog suppresses α -syn toxicity

As part of our unbiased genetic screen for modifiers of α -syn toxicity¹², we discovered the yeast ortholog of human *ATP13A2* (*PARK9*), an uncharacterized yeast gene designated *YOR291W*, to be a suppressor of α -syn toxicity (**Fig. 1a**). We have therefore named this yeast gene *YPK9* (for Yeast P*PARK9*). This and other emerging work^{13–17,19} suggest the possibility of many more connections between α -syn and other known causes of PD.

Knockout of *YPK9* did not enhance α -syn toxicity (**Fig. 1b**). Reasoning that this might be due to redundancy in function, we carried out a BLAST search of the yeast genome and identified the *SPF1* (*COD1*) gene as encoding a highly related P-type ATPase (30% identical, 49% similar, e value = 3×10^{-80}). There are several P-type ATPases in yeast, but *SPF1* and *YPK9* are by far the most closely related to *PARK9* (**Supplementary Fig. 1** online). Overexpression of *SPF1* did not suppress α -syn toxicity (data not shown) and deletion of *SPF1* itself exerted a slight growth defect in yeast (**Fig. 1b**). However, the *SPF1* deletion was nearly lethal in cells expressing a single copy of α -syn, which is by itself below the dosage threshold for toxicity in our yeast model (**Fig. 1b**); the combination of the deletion of *SPF1* and the presence of a single copy of α -syn resulted in a growth defect that was far greater than the sum of their individual toxicities. The double knockout of *SPF1* and *YPK9* did not show a synthetic lethal phenotype

(**Supplementary Fig. 2** online); however, overexpression of *YPK9* was sufficient to rescue the increase in α -syn toxicity caused by deletion of *SPF1* (data not shown), clearly establishing a functional overlap between them. There are many possible explanations for the different effects of deletion and overexpression of these genes. For example, Spf1 expression or function could be feedback-regulated, preventing overexpression from being efficacious, but leaving cells still vulnerable to deletion. In any case, in yeast, the two genes most closely related to human *ATP13A2* have diverged such that *YPK9* suppresses α -syn toxicity when overexpressed whereas *SPF1* enhances toxicity when deleted.

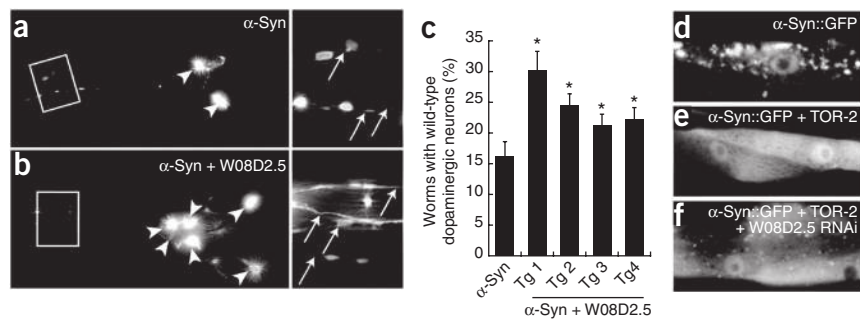
Next, we investigated the relationship between Ypk9 and another strong suppressor of α -syn toxicity from our screen, Ypt1 (ref. 12). Ypt1 is the yeast homolog of human RAB1A, a guanosine triphosphatase (GTPase) that regulates the trafficking of vesicles between the endoplasmic reticulum (ER) and the Golgi. As previously reported, overexpression of this protein rescues α -syn toxicity in both yeast and neuronal cells¹². To determine whether *YPK9* and *YPT1* suppress α -syn toxicity in a mechanistically similar manner, we used a yeast strain expressing higher levels of α -syn²⁴. This HiTox strain shows correspondingly higher toxicity and allows the detection of synergistic effects between different suppressors that would not be possible in the less toxic (IntTox) screening strain because *YPT1* fully rescues in that strain^{12,24}.

In the HiTox strain, neither Ypt1 nor Ypk9 could suppress the toxicity of α -syn. However, when Ypt1 and Ypk9 were both overexpressed, we observed strong, albeit not complete, suppression (**Fig. 1c**). Notably, this was not simply because the two proteins provided additional levels of a redundant function that had a strong threshold effect: cotransforming cells with two copies of either gene itself did not rescue toxicity at all (**Fig. 1c**). Thus, the genetic interaction between Ypt1 and Ypk9 is synergistic, indicating that Ypk9 and Ypt1 function in mechanistically distinct ways to suppress the toxic effects of α -syn accumulation.

In yeast and humans, the toxicity of α -syn is very dosage sensitive. One way that Ypk9 might affect α -syn toxicity, therefore, would be to

Figure 2 PARK9 antagonizes α -syn-mediated dopaminergic neuron degeneration in *C. elegans*.

(a,b) Anterior dopaminergic neurons in worms expressing $P_{dat-1}::GFP + P_{dat-1}::\alpha$ -syn at the day 7 stage. Arrowheads and arrows depict cell bodies and neuronal processes, respectively. Wild-type worms have six anterior dopaminergic neurons. (a) α -Syn toxicity is depicted by the loss of anterior dopaminergic neurons. (b) Dopaminergic neurons are protected when $P_{dat-1}::FLAG-W08D2.5$ cDNA is coexpressed. (c) Quantification of *C. elegans* PARK9 rescue of α -syn-induced neurodegeneration in four independent transgenic lines displaying all six anterior dopaminergic neurons. * $P < 0.05$, Student's *t* test. Values represent means \pm s.d. (d) Overexpression of α -syn in $P_{unc-54}::\alpha$ -syn::GFP results in misfolding and aggregation of α -syn in body wall muscle cells at the young adult stage. (e) Co-overexpression of TOR-2, a protein with chaperone activity, attenuates the misfolding of the α -syn::GFP protein. (f) The misfolding of α -syn::GFP is enhanced following RNAi targeting W08D2.5.



alter its accumulation. However, both immunoblotting and fluorescent quantification of the α -syn fusion protein established that overexpression of Ypk9 did not affect steady state levels of α -syn (Supplementary Fig. 3 online). Ypk9 did, however, markedly alter the localization of α -syn, largely restoring plasma membrane localization and reducing intracellular inclusions (Fig. 1d). Previously, immunoelectron microscopy established that the intracellular inclusions formed by α -syn in yeast are associated with clusters of mislocalized transport vesicles from various steps of the endocytic and exocytic pathways^{24,25}. Thus, these inclusions are a readout of vesicle trafficking defects elicited by α -syn accumulation and may well relate to early events in α -syn pathology seen in PD^{24,25}. Our data suggest that rescuing the vesicle trafficking block by overexpressing Ypt1 or Ypk9 results in a reduction in the number of intracellular inclusions.

We next asked whether the two proteins would have similar effects on the most immediate toxic defect that we have detected in cells expressing α -syn, a defect in ER-to-Golgi trafficking¹². We followed carboxypeptidase Y (CPY) as it was trafficked through this pathway by conducting a pulse-chase experiment. The subcellular location of CPY is easily determined by compartment-specific glycosylations and proteolytic cleavages that alter the molecular mass of the protein in a well-characterized manner. α -Syn inhibits ER-Golgi transport and prevents CPY from exiting the ER¹² (Fig. 1e). Ypk9 overexpression significantly rescued the ability of proteins to leave the ER and traffic to the Golgi (less protein in the ER, Fig. 1e), although the effect was not as strong as that of Ypt1. Thus, Ypk9 and Ypt1 have mechanistically distinct functions, but both converge on vesicular transport to antagonize α -syn toxicity.

ATP13A2 homolog suppresses α -syn toxicity in *C. elegans*

To investigate the genetic relationship between α -syn and PARK9 in dopaminergic neurons, a cell type directly relevant to human PD, we turned first to the nematode model *C. elegans* (Fig. 2). Development in the nematode is highly stereotyped and wild-type animals invariably have exactly the same number of dopaminergic neurons. Expression of α -syn from the dopamine transporter (*dat-1*) gene promoter resulted in an age-dependent progressive loss of dopaminergic neurons⁷, with approximately 85% of animals having reduced numbers of dopaminergic neurons at the 7-d stage (Fig. 2a,c). Expression of W08D2.5 (*catp-6*, the *C. elegans* ATP13A2 ortholog) alone did not induce any change in the number of dopaminergic neurons (data not shown). Coexpression of W08D2.5 and α -syn, from the same promoter (*dat-1*), partially rescued this neurodegeneration in each of four independent transgenic lines (Fig. 2b,c).

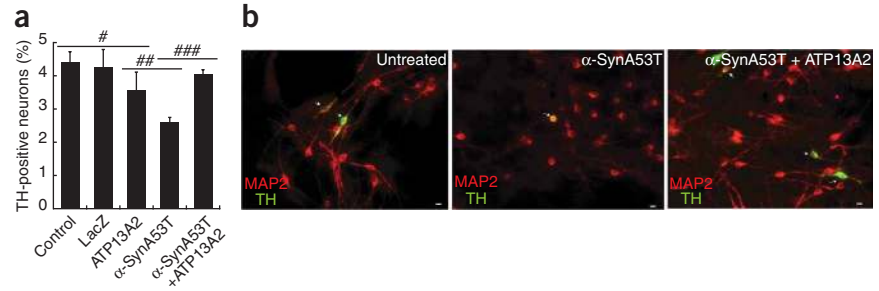
We also used *C. elegans* to explore the consequences of ATP13A2 loss of function. Unfortunately, neuronal cells of this organism are refractory to RNAi-mediated inhibition of gene expression²⁶. However, our work with yeast and neuronal model systems establishes that α -syn toxicity is the result of general cellular defects, to which neuronal cells are simply more sensitive²⁴. We therefore took advantage of another cell type that has been extensively exploited for studies of protein homeostasis in this organism^{27–30} and is readily affected by RNAi.

As previously described, body-wall muscle cells that express a human α -syn::GFP fusion show age-dependent α -syn aggregation (Fig. 2d). Coexpression of *tor-2*, which encodes a chaperone protein that reduces α -syn aggregation, provides a sensitized genetic background within which an enhancement of α -syn misfolding is readily visualized^{27,31,32} (Fig. 2e). In this sensitized background, we knocked down the expression of the *C. elegans* ATP13A2 ortholog (W08D2.5) by RNAi. This profoundly enhanced the misfolding of human α -syn and did so in an age-dependent manner (Fig. 2f). Notably, it did so without modifying the expression levels of α -syn or *tor-2* (Supplementary Fig. 4 online). These data provide further evidence for an intimate functional interaction between PARK9 and α -syn.

ATP13A2 suppresses α -syn toxicity in primary neuron cultures

To validate our findings in mammalian dopaminergic neurons, we used primary neuronal cultures prepared from the midbrain region of rat embryos at stage 17. Although this assay is far more laborious than those using stable tissue culture cell lines, the toxicity of α -syn in this setting is more robust and reproducible, likely because the cells retain apoptotic mechanisms that are lost in immortalized cell lines. Also, unlike the nematode model, these cultures provide an opportunity to assess toxicity to dopaminergic neurons relative to other neurons. Transduction of these cells with lentivirus encoding a PD-linked mutant α -syn (A53T) causes a reduction in the total number of neurons (MAP2-positive staining), including those using γ -amino butyric acid (GABA-positive) as a neurotransmitter. But tyrosine hydroxylase-positive dopaminergic neurons were even more severely affected^{12,17,33}. Co-transduction with a lentivirus encoding ATP13A2 (human PARK9) was potently protective. This was apparent from the increased percentage of tyrosine hydroxylase-positive cells (Fig. 3a), from the marked restoration of neuronal processes in cells expressing tyrosine hydroxylase and from the restoration of more normal neuronal morphology throughout the culture (Fig. 3b). ATP13A2 also increased the ratio of tyrosine hydroxylase-positive neurons compared to MAP2-positive neurons (Fig. 3a). Thus, the relationship

Figure 3 PARK9 antagonizes α -syn-mediated dopaminergic neuron degeneration in rat primary midbrain cultures. (a) Human PARK9 (ATP13A2) protects rat midbrain primary dopaminergic neurons from α -synA53T-induced toxicity. Primary rat embryonic midbrain cultures were either mock infected (control) or infected with lentivirus encoding LacZ, ATP13A2 alone, α -synA53T alone or α -synA53T and ATP13A2. Selective loss of dopaminergic neurons was assessed immunocytochemically by determining the percentage of MAP2-positive neurons that also stained positive for tyrosine hydroxylase (TH). $N \geq 3$, $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$, one-way analysis of variance with Newman-Keuls post-test (α -synA53T versus control is also $P < 0.001$). Values represent means \pm s.d. (b) ATP13A2 rescues α -synA53T-induced dopaminergic neuron loss in rat primary midbrain cultures. Representative micrographs of cells stained for MAP2 (red) and tyrosine hydroxylase (green). Arrows indicate dopaminergic neurons positive for both MAP2 and tyrosine hydroxylase. Scale bar, 20 μ m.



between PARK9 function and α -syn pathobiology that we had discovered in yeast is conserved in mammals.

Ypk9 subcellular localization and effect of *ATP13A2* mutations

Homozygous mutations in *ATP13A2* have been identified as causing a hereditary form of parkinsonism with dementia²³. That both alleles must be mutant to cause disease suggests that a recessive loss of function is the root cause. However, *ATP13A2* is expressed at a tenfold higher level in the surviving neurons of the substantia nigra of subjects with sporadic forms of PD²³. Therefore, it was also reasonable to suppose that the high expression of *ATP13A2* in sporadic PD and the two mutant alleles in familial forms represent a proteotoxic gain of function with, in the latter case, two alleles required to cross a disease-threshold burden²³. The fact that in yeast overexpression of *YPK9* suppressed α -syn toxicity supports the simpler view that it is a deficit of PARK9 function that leads to disease. To explore this further, we first determined the localization of the wild-type yeast and human proteins.

We used homologous recombination to chromosomally tag Ypk9 with the yellow fluorescent protein (YFP). YFP-Ypk9, expressed from its native promoter, localized strongly to the vacuole membrane (Fig. 4a), consistent with the localization of the human protein to the lysosome, the mammalian organellar equivalent of the yeast vacuole²³. We obtained similar results expressing GFP-Ypk9 fusion proteins from a low-copy (CEN) plasmid with a constitutive promoter (GPD) (Fig. 4a). Co-staining with a lipophilic dye, FM4-64, which

concentrates at the vacuole membrane, confirmed that this localization was vacuolar (data not shown).

The human protein ATP13A2 also localized to the vacuole membrane in yeast cells (Fig. 4a). However, even with a high-copy plasmid, it was expressed at lower levels than the yeast protein. This is common for multipass transmembrane proteins expressed across such large evolutionary distances (*ATP13A2* has ten predicted transmembrane domains). Not unexpectedly, the human protein was unable to protect against α -syn toxicity. Therefore, to test the effect of the human mutations on yeast PARK9 localization and function, we took advantage of the homology between the proteins to introduce equivalent mutations into the yeast ortholog. Both forms of yeast *YPK9* with mutations implicated in human familial PD ('subject-based' mutations) encoded proteins that were aberrantly localized. Ypk9 (Δ 833-1472) was expressed at lower levels than wild-type Ypk9 and was distributed throughout the cytosol, in a punctate pattern, whereas Ypk9 (Δ 1329-1472) was retained in the ER (Fig. 4a).

Next we tested the ability of the *YPK9* mutants to rescue α -syn toxicity in our yeast model. Overexpression of wild-type Ypk9 suppressed toxicity, but the two altered Ypk9 proteins did not (Fig. 4b). Moreover, expression of the altered Ypk9 proteins in wild-type yeast cells (without α -syn expression) did not affect growth (Fig. 4b), further supporting the notion that these are loss-of-function and not dominant negative mutations. Human *ATP13A2* and yeast Ypk9 are predicted P-type ATPases³⁴ (Supplementary Fig. 3). We also altered a conserved residue in Ypk9, predicted to abolish ATPase

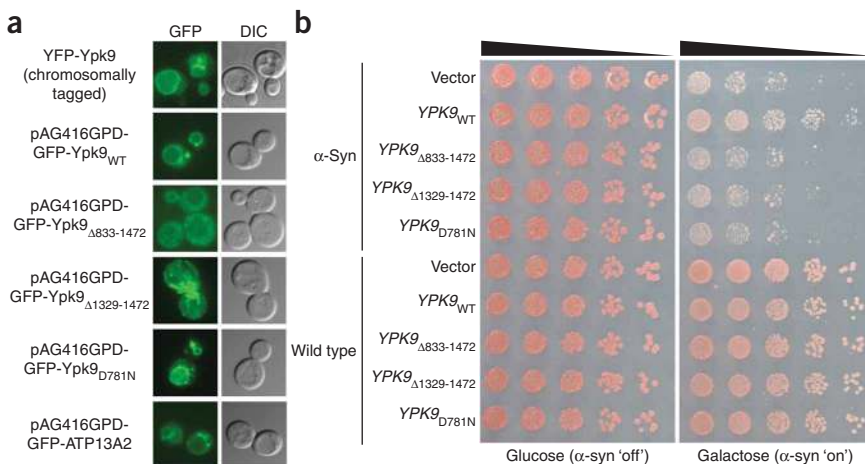


Figure 4 Ypk9 is localized to the vacuole in yeast and *ATP13A2* subject-based mutations affect its ability to rescue α -syn toxicity. (a) Fluorescence microscopy to visualize Ypk9 subcellular localization. A chromosomally tagged YFP fusion (Ypk9-YFP) localizes to the vacuolar membrane, as does wild-type GFP-Ypk9 expressed from the constitutive GPD promoter. *ATP13A2* mutations²³ alter Ypk9 localization, but the ATPase-dead mutant (D781N) does not. GFP-tagged human *ATP13A2* also localizes to the vacuole in yeast cells. (b) Spotting assays with wild-type or α -syn-expressing cells. Wild-type *YPK9* overexpression suppresses α -syn toxicity, but the two *ATP13A2*, subject-based mutant *YPK9* genes as well as the ATPase-dead mutant do not. Expressing mutant *YPK9* in wild-type cells does not inhibit growth, supporting the idea that these are loss-of-function and not dominant-negative mutations.

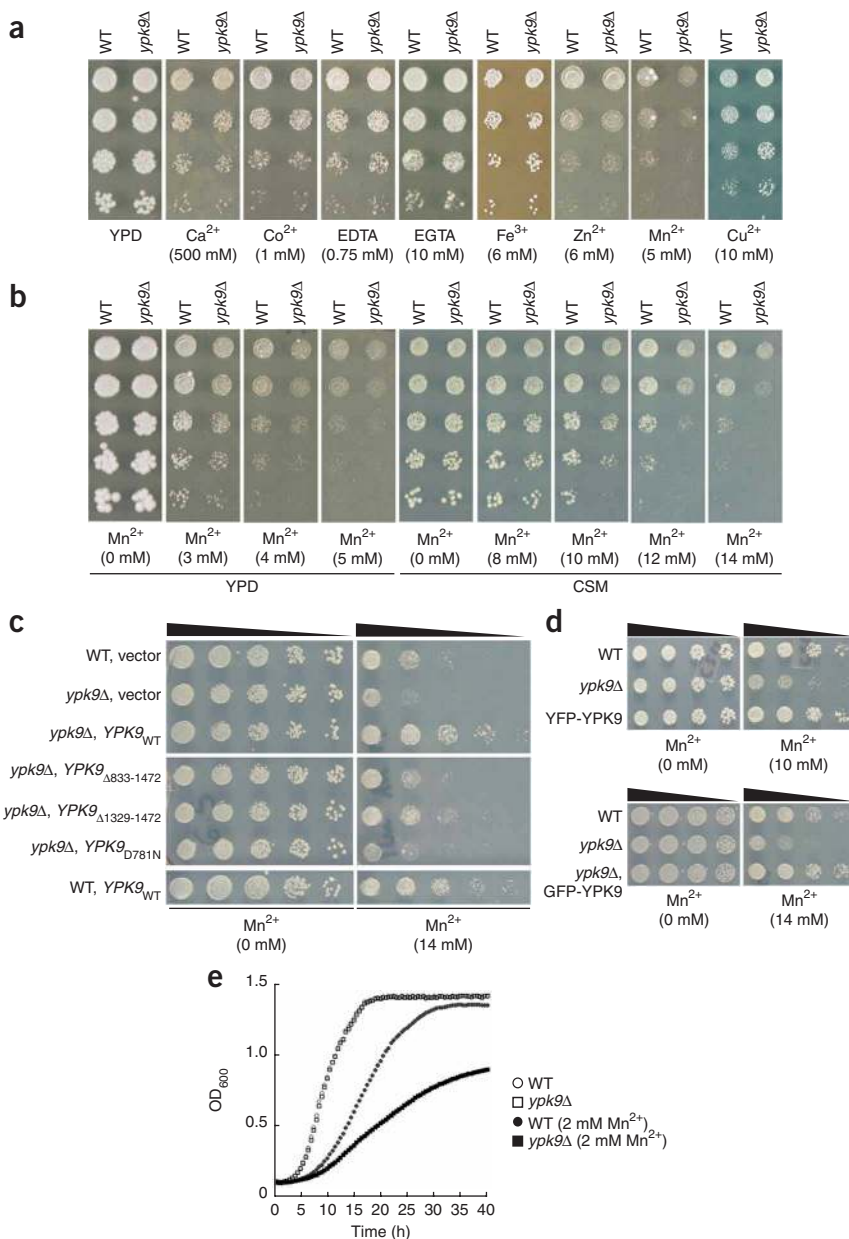


Figure 5 PARK9 protects cells from elevated manganese levels. **(a)** Examples of conditions used to identify the substrate specificity of YPK9. We identified *ypk9Δ* cells as being sensitive to manganese (Mn²⁺) relative to wild-type cells. **(b)** The effect of various Mn²⁺ concentrations on *ypk9Δ* cells grown on rich (YPD) or synthetic (CSM) media. **(c)** Expressing wild-type YPK9 in *ypk9Δ* cells is sufficient to rescue Mn²⁺ sensitivity but neither the ATP13A2 subject-based mutants nor the ATPase-dead mutant are able to rescue. Expressing YPK9 in wild-type yeast cells makes them more resistant to Mn²⁺ (compare top and bottom spottings). **(d)** YFP- (top panel) and GFP-tagged (bottom panel) Ypk9 fusion proteins used for localization studies are functional because they are able to protect against Mn²⁺ sensitivity. **(e)** *ypk9Δ* cells also show sensitivity to Mn²⁺ when grown in liquid culture.

Of all the conditions we tested, *ypk9Δ* cells were more sensitive to manganese (Mn²⁺) than were wild-type cells (Fig. 5a,b,e). This was detectable in rich media (Fig. 5a), and even more so in minimal media (Fig. 5b), and occurred in cells grown either on plates or in liquid (Fig. 5e). Notably, *ypk9Δ* cells were also slightly resistant to copper (Fig. 5a). Expression of wild-type Ypk9 from an extra-chromosomal plasmid with a strong promoter was sufficient to rescue the Mn²⁺ sensitivity and indeed to make both *ypk9Δ* and wild-type cells more resistant to Mn²⁺ (Fig. 5c). We were unable to complement this phenotype with the human gene, which, as noted above, we attribute to our inability to express the human protein at sufficient levels in our yeast system. We therefore used YPK9 with subject-based mutations to test the effects of the human mutations on Ypk9's ability to protect against Mn²⁺ toxicity.

Whereas wild-type Ypk9 suppressed Mn²⁺ toxicity, expression of the disease-associated Ypk9 proteins did not (Fig. 5c). ATPase activity was also required to protect against Mn²⁺ toxicity because the ATPase-dead Ypk9_{D781N} protein failed to rescue the defect (Fig. 5c). Moreover, the GFP-tagged Ypk9 fusion proteins, which we used for the localization studies (Fig. 4a), were functional, because they were able to rescue Mn²⁺ sensitivity (Fig. 5d). Thus, yeast Ypk9, and possibly human ATP13A2, likely function as manganese transporters to protect cells from excess Mn²⁺ exposure.

activity (D781N). This resulting protein localized properly to the vacuole (Fig. 4a) but was unable to rescue α -syn toxicity (Fig. 4b). Taken together, our data indicate that both vacuolar localization and ATPase activity are required for Ypk9 to antagonize α -syn toxicity.

ypk9Δ cells are hypersensitive to manganese

Little is known about the normal function of PARK9 or how it might contribute to PD. To gain mechanistic insight into PARK9 function, we explored the function of the yeast homolog. Both the yeast and human proteins are predicted to be transmembrane cationic metal transporters, but their substrate specificity has remained elusive^{23,34,35}. We tested several metals to identify potential functions for Ypk9. We grew wild type and *ypk9Δ* cells in media containing a wide range of metals and metal chelators at various concentrations to determine the concentration that partially inhibited growth in our genetic background. This provided sensitized conditions to test the effects of Ypk9 (Fig. 5 and data not shown).

Mn²⁺ toxicity because the ATPase-dead Ypk9_{D781N} protein failed to rescue the defect (Fig. 5c). Moreover, the GFP-tagged Ypk9 fusion proteins, which we used for the localization studies (Fig. 4a), were functional, because they were able to rescue Mn²⁺ sensitivity (Fig. 5d). Thus, yeast Ypk9, and possibly human ATP13A2, likely function as manganese transporters to protect cells from excess Mn²⁺ exposure.

Validating additional genes from yeast screen in PD models

The other α -syn toxicity modifier genes we discovered in our yeast screen¹³ offer a multitude of promising possibilities for discovering new therapeutic strategies. But it is axiomatic that this approach will only work if hits from the yeast screen can be validated in neurons. As an initial step toward this goal, we chose a subset of genes from our screen for further analysis in neuronal PD models. Our sole criteria were to test representative genes (i) from diverse functional categories, (ii) with different strengths of suppression, (iii) with clear human orthologs (Table 1) and (iv) with readily obtainable gene clones. We

Table 1 α -Syn toxicity modifiers tested in neuronal PD models

Yeast Gene	Type	Predicted function	Human ortholog	Effect in Yeast	Effect in <i>C. elegans</i>	Effect in rat neurons
<i>YPK9</i>	Suppressor	Lysosomal ATPase	ATP13A2	Suppress	Suppress	Suppress
<i>HRD1</i>	Suppressor	E3 ubiquitin ligase	SYVN1	Suppress	No change	Suppress
<i>UBP3</i>	Suppressor	Ubiquitin protease	USP10	Suppress	No change	Suppress
<i>PDE2</i>	Suppressor	Phosphodiesterase	PDE9A	Suppress	Suppress	Suppress
<i>CDC5</i>	Suppressor	Polo-like kinase	PLK2	Suppress	Suppress	Suppress
<i>YCK3</i>	Suppressor	Casein kinase	CSNK1G3	Suppress	No change	No change

tested five suppressor genes, using human expression clones because of their availability at the time the experiments were done (yeast/human: *HRD1/SYVN1*, *UBP3/USP10*, *PDE2/PDE9A*, *CDC5/PLK2*, *YCK3/CSNK1G3*), in the rat primary neuron lentiviral model and the *C. elegans* α -syn model. Four out of five were efficacious. Two (*PLK2* and *PDE9A*) even suppressed α -syn-induced dopaminergic neuron loss in the nematode (**Supplementary Fig. 5** online). Because we used human expression clones for these studies, it is not surprising that the suppressors were more effective in rat neurons than in nematode, which is separated from human by ~ 800 million years. In any case, these studies establish a highly conserved genetic interaction network operating between α -syn and several genes of diverse function from yeast to mammals.

DISCUSSION

There are no clear homologs of α -syn in either yeast or nematode. How relevant, then, is it to study this human PD gene in yeast? Very. Work from our laboratory^{12,13,24} and others^{25,36,37} indicates that α -syn, likely through its ability to bind lipids and associate with membranes, is involved in the control of vesicle trafficking, a core function conserved in all eukaryotes. We have discovered an α -syn interaction network in yeast consisting of proteins with very diverse functions (for example, kinases, phosphatases, metal transporters, deubiquitinating enzymes)^{12,13} and demonstrated its functional conservation in neurons of the rat and nematode, organisms separated from yeast by a billion years of evolution. This notable and unexpected degree of conservation not only confirms a conserved and fundamentally important role for peripheral membrane proteins such as α -syn in normal vesicle trafficking, but also indicates that this function is deeply integrated with, and regulated by, other diverse and conserved cellular functions.

α -Syn is a very small (14 kDa) protein that binds lipids and is peripherally associated with membranes. Although it folds when associated with membranes^{38–40}, it is otherwise natively unfolded, with a propensity to form toxic oligomeric species⁴¹. We suggest that it is these very basic properties of α -syn that account for the conservation of its pathobiology from yeast to man. Yeast cells may have proteins with similar function. If so, they are likely constrained more by protein–lipid than protein–protein interactions and have simply diverged too greatly over the enormous evolutionary distances covered here to allow clear recognition of functional homologs by amino acid sequence.

The discoveries of single-gene mutations in familial forms of PD over the last ten years provides an opportunity for further elucidating the fundamental mechanisms of PD⁴². Our approach has revealed genetic interactions between human disease genes, encoding α -syn and PARK9, for which there was previously no known relationship. The fact that five of the six genes that we discovered in yeast also affect the toxicity of α -syn in neurons suggests that other modifiers recovered in our screen will also be relevant¹³. It may, therefore, be

useful to test polymorphisms in these genes for association in synucleinopathies. An additional challenge is to explore the complexities of gene–gene and gene–environment interactions in PD. Our identification of a connection between α -syn, PARK9 and manganese also provides a toehold for these investigations.

A unifying theme emerging from our work, as well as that of several other laboratories, is that α -syn sits at a nodal point, integrating a multitude of seemingly diverse genetic and environmental lesions^{13,43,44}. We hope the ability to dissect the nature of these interactions, and how they contribute to disease in a variety of model systems will forge new avenues for understanding disease mechanisms and suggest therapeutic approaches aimed at the fundamental biological lesions in the complex disorders associated with α -syn that intersect with diverse aspects of pathology.

METHODS

Yeast strains and media. The α -synuclein-expressing yeast strain we used in the modifier screen was IntTox: α -syn-WT, *MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS303Gal- α -synWT-YFP pRS304Gal- α -SynWT-YFP. The α -synuclein expressing yeast strain we used for combinatorial gene analysis was HiTox: α -syn-WT, *MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS304Gal- α -synWT-GFP pRS306Gal- α -synWT-GFP. The Gal promoter reporter strain used to determine the effect of modifier genes on expression from galactose-regulated promoter was Gal-YFP, *MATa can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1* pRS303Gal-YFP. For Ypk9 localization studies, a cassette containing a *loxP*-flanked KanMX cassette followed by YFP (pDH22, a gift from Yeast Resource Center, University of Washington, Seattle) was inserted in frame at the N terminus of Ypk9 by homologous recombination in the BY4741 strain background. Correct insertion was checked by PCR and the KanMX gene was subsequently removed by transformation with a plasmid containing a GAL-inducible Cre recombinase (pSH47, a gift from Yeast Resource Center, University of Washington, Seattle). The *ypk9* Δ strain was obtained by replacing the *YPK9* coding region with the *HIS3* gene in the BY4741 strain background. Colony PCR was used to verify correct gene disruption. Strains were manipulated and media prepared using standard techniques.

Plasmids. For Ypk9 localization studies, pAG416GPD-EGFP-Ypk9 was constructed by Gateway cloning using the Ypk9 entry clone (pDONR221-YPK9) and pAG416GPD-EGFP-ccdB destination vector⁴⁵ in an LR reaction. *ATP13A2* subject-based mutations were introduced into pDONR221-YPK9 using the QuickChange Site Directed Mutagenesis Kit (Stratagene) and sequence-verified. Del_{833–1472} corresponds to human mutation 1632_1653dup22 and Del_{1329–1472} corresponds to human mutation 3075delC. The ATPase-dead mutation encodes D781N. Using Gateway cloning, we subcloned wild-type and mutant *YPK9* into pAG416GPD-EGFP-ccdB (for localization and Mn²⁺ rescue), pAG416GPD (Mn²⁺ rescue) or pBY011 (α -synuclein rescue). Primer sequences are available upon request. For studies with human PARK9, pDNA3.1V5-His-Topo-ATP13A2 was a gift from C. Kubisch (University of Cologne). The *ATP13A2* coding region was PCR-amplified and subcloned in pDONR221 to generate the entry clone pDONR221-ATP13A2. Subsequent LR Gateway reactions generated pBY011-ATP13A2, pAG416GPD-ATP13A2 and pAG416GPD-EGFP-ATP13A2.

Metals. Serial dilutions of wild-type (BY4741) or *ypk9Δ* cells were spotted onto YPD or CSM agar plates supplemented with excess concentrations of metals (Ca^{2+} , Fe^{3+} , Mn^{2+} , Zn^{2+} , Co^{2+} , Cu^{2+}) or metal chelators (10 mM EGTA, 0.75 mM EDTA) and growth was assessed after 2–3 d at 30 °C. For the Mn^{2+} toxicity rescue experiments, wild-type and *ypk9Δ* strains were transformed with the indicated plasmids and transformants spotted onto SD-URA plates containing different concentrations of MnCl_2 (8, 10, 12, 14 mM). To assess *ypk9Δ* Mn^{2+} sensitivity in liquid culture, we used the Bioscreen to monitor growth. Yeast cells were pre-grown in YPD to mid-log phase, diluted to $\text{OD}_{600} = 0.1$ and dispensed to individual wells. OD_{600} measurements were taken every 30 min in the presence of the indicated concentrations of MnCl_2 , and the plates were shaken every 30 s to aerate the cells. At least three independent runs were conducted for each growth condition, and each condition was tested in triplicate.

Phylogenetic tree. Protein sequences for all yeast and human P-type ATPases were retrieved from the UniProtKB/Swiss-Prot family/domain classification database (cation transport ATPase (P-type) family). A multiple sequence alignment was obtained using the ClustalW algorithm with default parameters. The phylogenetic tree was obtained using the PROML program (maximum likelihood algorithm with Jones-Taylor-Thornton probability model, constant rate of change among sites) in the PHYLIP package (v3.67).

α -Syn toxicity modifier screen. We carried out the high-throughput yeast transformation protocol as described previously for a smaller library of genes^{12,13}.

Combinatorial analysis. For the combinatorial analysis, pAG413GPD, pAG413GPD-YPK9 or pAG413GPD-Ypt1 was cotransformed along with pAG415GPD, pAG415GPD-YPK9 or pAG415GPD-Ypt1 into the HiTox α -syn yeast strain using the standard lithium acetate technique. The transformants were plated onto SD-His/Leu agar plates and grown for 2 d. Cells were then normalized and spotted onto SD-His/Leu and SGal-His/Leu plates. Suppressors of α -syn-induced toxicity were identified on the galactose plates after 3 d of growth at 30 °C.

ER-Golgi trafficking assay. We carried out the carboxypeptidase Y (CPY) maturation assay as described previously¹².

C. elegans experiments. Nematodes were maintained following the standard procedures⁴⁶. RNAi and fluorescent microscopy were done as described⁴⁷ by feeding UA50 (*baIn13*; *P_{unc-54}:: α -syn::gfp*, *P_{unc-54}::tor-2*, *rol-6 (su1006)*) worms with the RNAi clones (Geneservice) corresponding to the worm orthologs of YPK9 and its interactors. RNA isolation, cDNA preparation and semiquantitative RT-PCR were conducted as described⁴⁸ with the following modification. Total RNAs from 50 young-adult control (RNAi bacteria HT115(DE3) with empty vector) and RNAi-treated worms were isolated to generate cDNAs. PCR was then done using primers specific for amplifying *cdk-5* as loading control, α -syn and *tor-2*. For dopaminergic neurodegeneration analysis, strains UA51 (*baEx42*; *P_{dat-1}:: α -syn*, *P_{dat-1}::gfp*, *P_{dat-1}::FLAG-W08D2.5*, *rol-6 (su1006)*) and UA108 (*baEx83*; *P_{dat-1}::gfp*, *P_{dat-1}::FLAG-W08D2.5*, *P_{unc-54}::mCherry*) were generated by injecting 50 $\mu\text{g}/\text{ml}$ of each expression plasmid into integrated *P_{dat-1}:: α -syn*, *P_{dat-1}::GFP* as well as *P_{dat-1}::GFP* worms, respectively. The stable lines were analyzed for neurodegeneration as described previously^{7,12,24}.

Rat primary midbrain neuron culture experiments. Primary midbrain cultures were prepared, transduced with lentivirus and analyzed immunocytochemically, as described previously¹². All of the methods involving animal handling were reviewed and approved by the Purdue Animal Care and Use Committee. Relative dopaminergic cell viability was determined by counting MAP2- and tyrosine hydroxylase-immunoreactive neurons in randomly chosen observation fields. The data were expressed as the percentage of MAP2-positive neurons that were also tyrosine hydroxylase-positive (this ratiometric approach was used to correct for variations in cell density). Typically, 300–1,500 MAP2-positive cells were counted per experiment for each condition. In the control conditions, and in conditions where suppressors are efficacious, we typically count 500–1,500 MAP2-positive neurons, a range that corresponds to 20–60 tyrosine hydroxylase-positive neurons. It is more difficult to obtain

such high cell counts from cultures expressing A53T α -synuclein alone because the cell viability is markedly reduced. In these cases we typically count 300–500 MAP2-positive neurons.

Preparation of primary mesencephalic cultures. Whole brains were dissected from day 17 embryos obtained from pregnant Sprague-Dawley rats (Harlan). The mesencephalic region containing the *substantia nigra* and ventral tegmental area was isolated stereoscopically, and the cells were dissociated with trypsin (final concentration, 26 $\mu\text{g}/\text{ml}$ in 0.9% (w/v) NaCl). The cells were plated on coverslips pretreated with poly-L-lysine (5 $\mu\text{g}/\text{ml}$) in media comprised of DMEM, 10% (v/v) FBS, 10% (v/v) horse serum, penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). After a 4-d incubation, the cells were treated for 48 h with cytosine arabinoside (AraC) (20 μM) to suppress the growth of glial cells. Methods involving animal handling were approved by the Purdue Animal Care and Use Committee.

Preparation of lentiviral constructs. The ViraPower Lentivirus Expression System (Invitrogen) was used to generate lentiviruses encoding human α -syn (A53T), ATP13A2, CSNK1G3, USP10, PDE9A and PLK2 as described previously¹². The insert from a pENTR-based entry construct was transferred into the pLENTI6/V5 DEST lentiviral expression vector (Invitrogen) via recombination. The lentiviral construct was sequenced using an Applied Biosystems DNA sequencer and packaged into virus via transient transfection of the 293FT packaging cell line. We showed in a previous study that lentiviruses prepared using this method have similar transduction efficiencies for MAP2- and tyrosine hydroxylase-positive neurons (approximately 90% and 80%, respectively)¹⁷.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We are grateful to C. Kubisch (University of Cologne) for providing the human ATP13A2 cDNA and to the Yeast Resource Center for plasmids. A.D.G. was a Lilly Fellow of the Life Sciences Research Foundation and is currently a Pew Scholar in the Biomedical Sciences. A.D.G. is also supported by the US National Institutes of Health Director's New Innovator Award Program, part of the NIH Roadmap for Medical Research, through grant number 1-DP2-OD004417-01. A.C. is supported by a postdoctoral fellowship from the Parkinson's Disease Foundation. S.L. acknowledges support from the MGH/MIT Morris Udall Center of Excellence in Parkinson Disease Research, NS038372, and the Howard Hughes Medical Institute. M.L.G. was supported by a grant from the National Parkinson Foundation. *C. elegans* studies in the Caldwell laboratory were supported in part by grants from the Michael J. Fox Foundation, American Parkinson Disease Foundation and Bachmann-Strauss Dystonia and Parkinson Foundation. Research in the Rochet laboratory was supported by National Institutes of Health Grant NS049221 and a grant from the American Parkinson Disease Association.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturegenetics/>.

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**α -Synuclein is part of a diverse and highly conserved
interaction network that includes PARK9 and manganese
toxicity**

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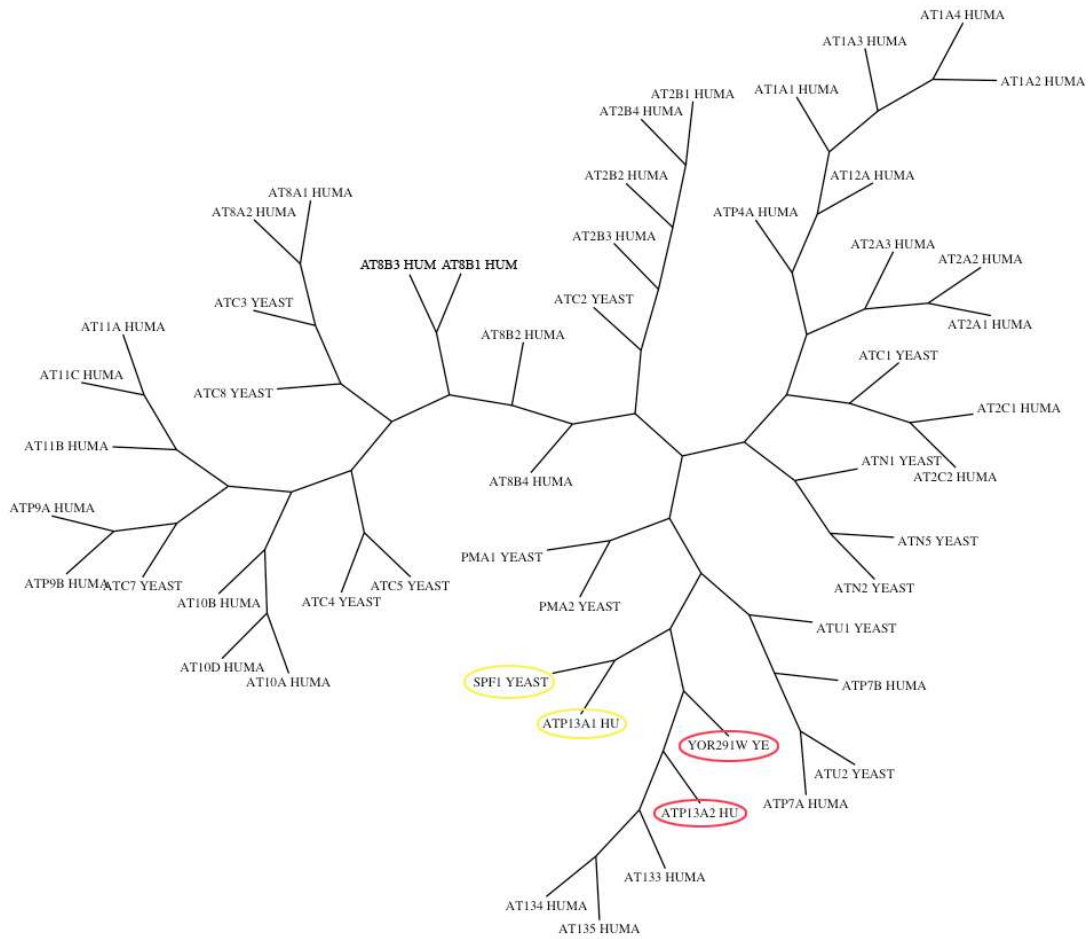


Figure S1. Phylogenetic analysis of yeast and human P-type ATPases. *YOR291W/YPK9* is the most closely related yeast P-type ATPase to human *PARK9/ATP13A2* (red circles). Yeast *SPF1* is most closely related to human *ATP13A1* (yellow circles).

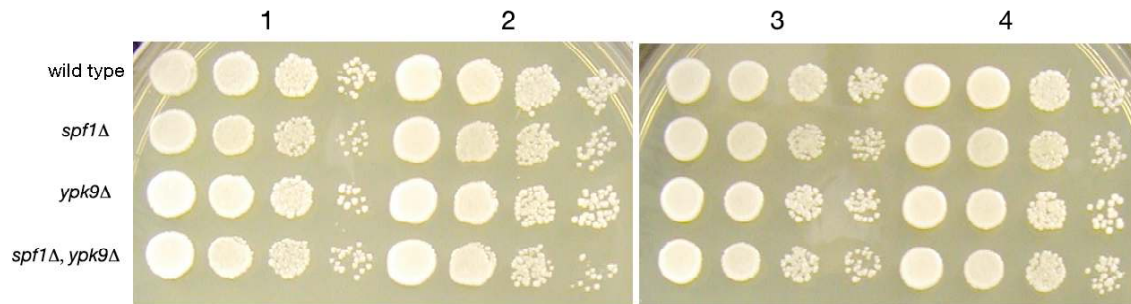


Figure S2. *ypk9Δ spf1Δ* double mutant is viable. *YPK9* was disrupted by replacing the coding region with *HIS3* and *SPF1* was disrupted by replacing the coding region with *KanMX*. Correct deletions were verified by colony PCR. Spotting assay with 4 independent colonies of each genotype (WT, *ypk9Δ*, *spf1Δ*, and *ypk9Δ spf1Δ*) shows no obvious growth defect.

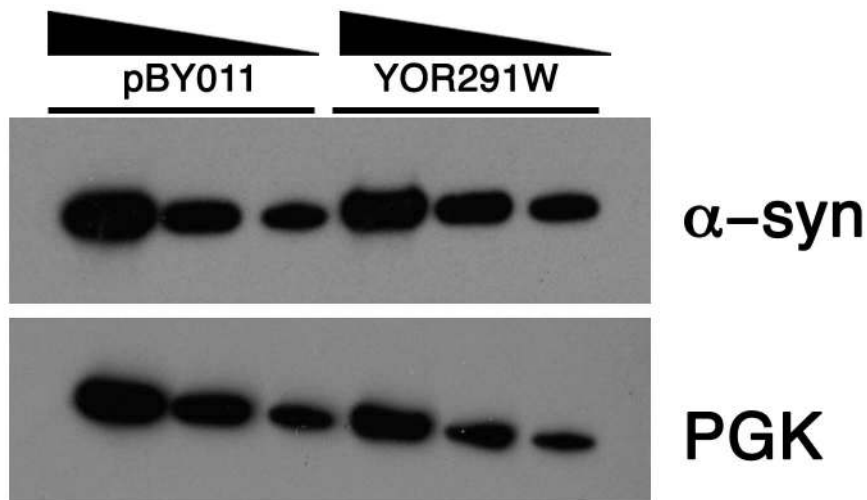


Figure S3. YOR291W (Ypk9) does not affect levels of α -syn protein. Yeast cells expressing α -syn were transformed with either empty vector (pBY011) or YOR291W and grown in galactose-containing media to induce expression of α -syn and YOR291W. Cell lysates were serially-diluted and subjected to immunoblotting with an anti- α -syn monoclonal antibody or an anti-phosphoglycerate kinase (PGK) monoclonal antibody (loading control). By flow cytometry with GFP reporters we also find that Ypk9 expression does not affect expression from the Gal promoter (data not shown).

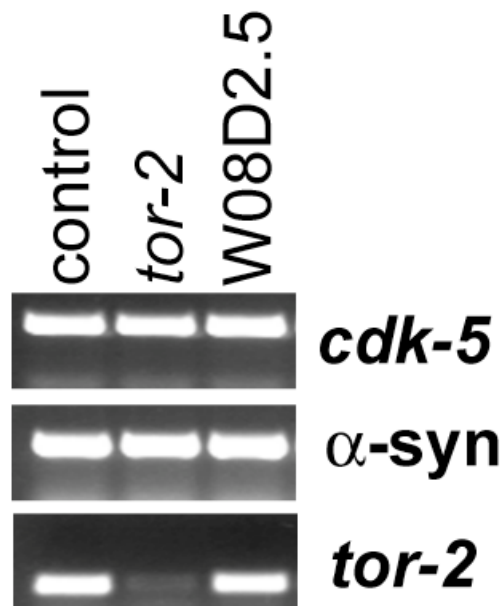


Fig S4. RNAi knock down of W08D2.5 does not reduce α -syn and *tor-2* expression level. Semi-quantitative RT-PCR was performed by using primers amplifying *cdk-5* (loading control), α -syn, and *tor-2*. Control UA50 [*baIn13*; *P_{unc-54}:: α -syn::gfp*, *P_{unc-54}::tor-2*, *rol-6 (su1006)*] worms were fed with RNAi bacteria HT115(DE3) with empty vector while RNAi-treated worms were fed with the bacteria producing the indicated dsRNA. Following total RNA isolation and cDNA preparation, semi-quantitative RT-PCR was performed by using primers specific for *cdk-5*, α -syn, and *tor-2*. Primer sequences are available upon request.

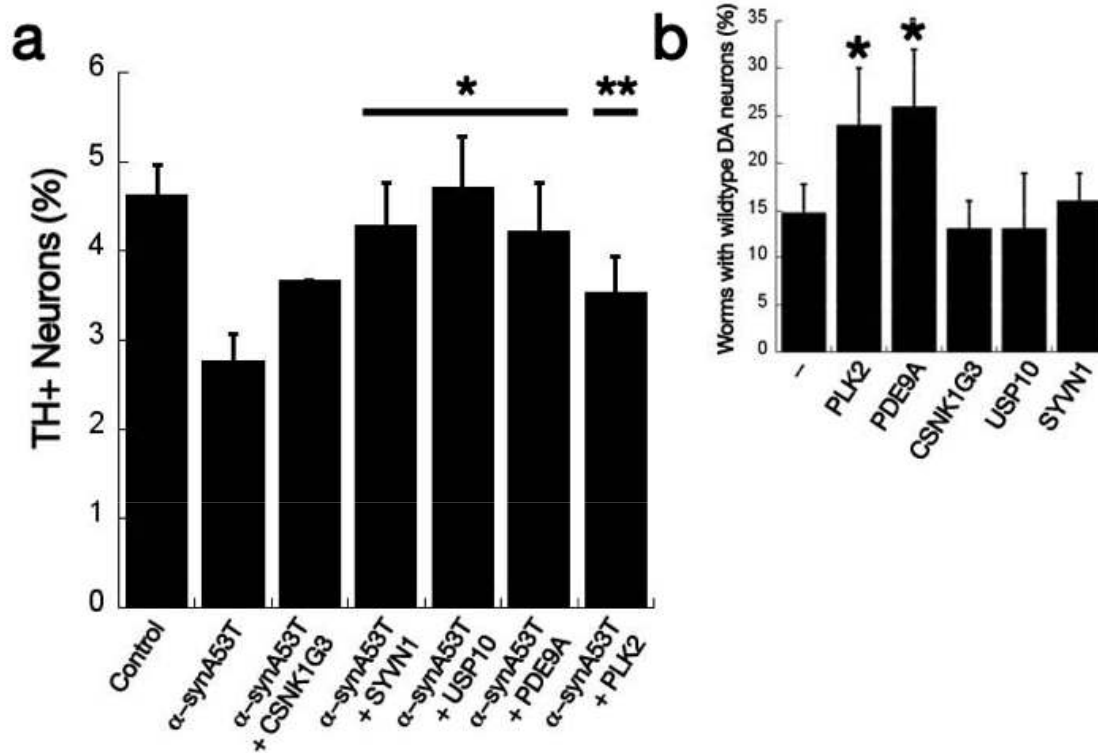


Figure S5. Additional human homologs of selected yeast α -syn toxicity modifiers were able to suppress or enhance α -syn-induced DA neuron loss in *C. elegans* and rat midbrain primary neuronal cultures. **(a)** Primary rat embryonic midbrain cultures were infected with lentivirus encoding α -synA53T alone or α -synA53T in combination with CSNK1G3, SYVN1, USP10, PDE9A, or PLK2. Preferential loss of DA neurons was determined immunocytochemically by comparing the percentage of MAP2-positive neurons that also stained positive for tyrosine hydroxylase (TH). N = 3, * P < 0.001 and ** P < 0.01 compared to cells expressing only α -synA53T, analysis of variance with Newman-Keuls post-test. **(b)** Quantification of α -syn-induced degeneration in DA neurons of *C. elegans* expressing cDNAs encoding human orthologs of representative α -

syn toxicity suppressors identified in yeast. Two suppressors, human polo kinase 2 (PLK2) and cAMP phosphodiesterase (PDE9A), a component of the cAMP-dependent protein kinase signaling system significantly suppressed a-syn toxicity in Day 7 adult nematode DA neurons , [$P < 0.05$, Student's t test (*)]. For each gene tested, 3-4 independent transgenic lines were analyzed; a worm was scored as wild type when all six anterior DA neurons were intact.