Neurobiology of Disease

Functional Analysis of VPS41-Mediated Neuroprotection in *Caenorhabditis elegans* and Mammalian Models of Parkinson's Disease

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Disruption of the lysosomal system has emerged as a key cellular pathway in the neurotoxicity of α-synuclein (α-syn) and the progression of Parkinson's disease (PD). A large-scale RNA interference (RNAi) screen using *Caenorhabditis elegans* identified VPS-41, a multidomain protein involved in lysosomal protein trafficking, as a modifier of α-syn accumulation and dopaminergic neuron degeneration (Hamamichi et al., 2008). Previous studies have shown a conserved neuroprotective function of human VPS41 (hVPS41) against PD-relevant toxins in mammalian cells and *C. elegans* neurons (Ruan et al., 2010). Here, we report that both the AP-3 (heterotetrameric adaptor protein complex) interaction domain and clathrin heavy-chain repeat domain are required for protecting *C. elegans* dopaminergic neurons from α-syn-induced neurodegeneration, as well as to prevent α-syn inclusion formation in an H4 human neuroglioma cell model. Using mutant *C. elegans* and neuron-specific RNAi, we revealed that hVPS41 requires both a functional AP-3 (heterotetrameric adaptor protein complex) and HOPS (homotypic fusion and vacuole protein sorting)-tethering complex to elicit neuroprotection. Interestingly, two nonsynonymous single-nucleotide polymorphisms found within the AP-3 interacting domain of hVPS41 attenuated the neuroprotective property, suggestive of putative susceptibility factors for PD. Furthermore, we observed a decrease in α-syn protein level when hVPS41 was overexpressed in human neuroglioma cells. Thus, the neuroprotective capacity of hVPS41 may be a consequence of enhanced clearance of misfolded and aggregated proteins, including toxic α-syn species. These data reveal the importance of lysosomal trafficking in maintaining cellular homeostasis in the presence of enhanced α-syn expression and toxicity. Our results support hVPS41 as a potential novel therapeutic target for the treatment of synucleinopathies like PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease clinically characterized by the progressive loss of dopaminergic neurons in the substantia nigra and the presence of intracellular protein inclusions termed Lewy bodies. While the etiology of PD is not fully understood, the α-synuclein protein (α-syn) is thought to be a central component for the progression of this disease, in that mutations or multiplication of the α-syn locus are known genetic causes of autosomal-dominant PD (Polymeropoulos et al., 1997; Singleton et al., 2003). Furthermore, α-syn is a polypeptide that can oligomerize to form fibrils and aggregates, and is a primary component in cytoplasmic Lewy bodies (Spillantini et al., 1997). While most cases of PD have no known genetic defect and are thought to involve an environmental component, accumulation of α-syn is still observed (Irizarry et al., 1998). Both cellular and animal models also exhibit cellular toxicity through α-syn overexpression. With no effective treatment to slow the progression of this disease, the identification of potential therapeutic targets or pathways remains a high priority.

We have previously established *Caenorhabditis elegans* as a model for age-dependent α-syn accumulation and dopaminergic neuron degeneration (Cao et al., 2005; Hamamichi et al., 2008). Importantly, this model has proven predictive in the evaluation of genetic factors and small molecules that protect cells from α-syn accumulation and toxicity, with several candidates validated across other organisms, including mammalian systems (Cooper et al., 2006; Gitler et al., 2008, 2009; Su et al., 2010). Using *C. elegans* assays, Hamamichi et al. (2008) conducted a large-scale RNA interference (RNAi) screen to identify modifiers of α-syn pathology. Through this screen, the *C. elegans* VPS-41 protein was identified as being a top neuroprotective candidate. We subsequently demonstrated that human VPS41 (hVPS41) can also protect dopaminergic neurons from both α-syn- and 6-hydroxydopamine (6-OHDA)-induced dopaminergic neurodegeneration in *C. elegans*, as well as protect human SH-SY5Y neuroblastoma cells from PD-relevant neurotoxins, including rotenone and 6-OHDA (Ruan et al., 2010).

VPS41 is a highly conserved, multidomain protein that functions in lysosomal trafficking of Golgi-derived AP-3 (heterotetrameric adaptor protein complex) vesicles (Radisky et al., 1997; Rehling et al., 1999; Darsow et al., 2001). Given the conserved neuroprotective activity of hVPS41, we conducted a structure/function analysis of hVPS41 using the nematode model and show that both the AP-3 interaction and clathrin heavy-chain repeat (CHCR) domains are required to protect dopaminergic neurons from α-syn-induced degeneration. Through mutant and RNAi analysis, we discerned that both the AP-3 and HOPS (homotypic fusion and vacuole protein sorting) complexes are required for hVPS41-dependent neuroprotection. Interestingly, two human single-nucleotide polymorphisms (SNPs) found within hVPS41 abolish the neuroprotective function of the protein. Our results are further confirmed in a mammalian model of α-syn inclusion formation, where protective forms of hVPS41 identified in *C. elegans* significantly reduce α-syn accumulation in human neuroglioma cells. Additionally, expression of hVPS41 reduces the level of overexpressed α-syn, suggesting that protein clearance may function in the protective effect of hVPS41. These data provide mechanistic insights into a conserved neuroprotective pathway and support further development of hVPS41 as a prospective therapeutic target for PD.

Materials and Methods

Plasmid construction
Human *vps-41* cDNA was obtained from Open Biosystems. Truncated *hvps41* constructs were generated by using Phusion high-fidelity polymerase (Finnzymes) with primers specified for each truncation available upon request, with fusion of segments and site-directed mutagenesis conducted as previously described (Hobert, 2002). Plasmid entry vectors were generated using Gateway Technology (Invitrogen) to clone PCR-amplified constructs into pDONR221 by BP reaction, and the constructs were then further cloned into either Gateway expression vector pDEST-DAT-1 (Cao et al., 2005) or pcDNA3.2/V5-DEST (Invitrogen), via LR reaction. Gateway entry vectors were verified by DNA sequencing to validate the constructs. Genomic *sid-1* was amplified using Phusion high-fidelity polymerase from genomic DNA isolated from N2 Bristol nematodes and cloned into the pDEST-DAT-1 Gateway expression vector using Gateway Technology, as described above.

C. elegans *strains*
Neuroprotection assay. Transgenic *C. elegans* lines were generated by directly injecting plasmid constructs (P_{dat-1}::Gene X), along with a phenotypic marker [P_{unc-54}::mCherry (body wall muscle expression)], into N2 Bristol hermaphrodites to create at least three stable transgenic animals expressing the gene of interest in the dopaminergic neurons as detected by the phenotypic marker mCherry in the body wall muscle cells (Harrington et al., 2011). Transgenic animals expressing mCherry were crossed with isogenic UA44 [$baIn11$ (P_{dat-1}::α-syn, P_{dat-1}::GFP)] males, and the resulting heterozygous progeny expressing both GFP and mCherry were transferred and allowed to self-fertilize. Homozygous GFP (α-syn)-expressing worms were isolated and used in the dopaminergic neurodegeneration analysis (Table 1). AP-3 mutant animals were generated by crossing strain RB662 [$apb-3(ok429)$; provided by the *C. elegans* Gene Knockout Project at Oklahoma Medical Research Foundation (OMRF)] with either strain UA44 or UA126 [$baIn11$; $baEx98$ (P_{dat-1}::hvps41-1, P_{unc-54}::mCherry)] males to generate strains UA200 [$apb-3(ok429)$; $baIn11$] and UA194 [$apb-3(ok429)$; $baIn11$; $baEx98$], respectively. Homozygous progeny were verified by PCR amplification flanking the $apb-3(ok429)$ deletion region (Primer 1: 5′-gctcaattgaagtg-cactgtg-3′; Primer 2: 5′-ccgagaaatcaacgtcaatcagc-3′).

Cell-specific RNAi. Transgenic *C. elegans* lines were generated by directly injecting the expression plasmid P_{dat-1}::sid-1 along with the phenotypic marker P_{myo-2}::mCherry (pharyngeal expression) (Addgene) into *sid-1(pk3321)* hermaphrodites to create stable transgenic animals expressing genomic *sid-1* in the dopaminergic neurons as well as the

Table 1. Transgenic *C. elegans* strains utilized

Strain	Plasmid construct	Genotype
UA44[a]	α-syn	$baIn11$ [P_{dat-1}::α-syn, P_{dat-1}::GFP]
UA126[b]	α-syn; VPS41 Isoform 1	$baIn11$; $baEx98$ [P_{dat-1}::hvps41-1, P_{unc-54}::mCherry]
UA128[b]	α-syn; VPS41 Isoform 2	$baIn11$; $baEx99$ [P_{dat-1}::hvps41-2, P_{unc-54}::mCherry]
UA177	α-syn; VPS41 A	$baIn11$; $baEx117$ [P_{dat-1}::hvps41-A, P_{unc-54}::mCherry]
UA179	α-syn; VPS41 B	$baIn11$; $baEx118$ [P_{dat-1}::hvps41-B, P_{unc-54}::mCherry]
UA181	α-syn; VPS41 C	$baIn11$; $baEx119$ [P_{dat-1}::hvps41-C, P_{unc-54}::mCherry]
UA183	α-syn; VPS41 D	$baIn11$; $baEx120$ [P_{dat-1}::hvps41-D, P_{unc-54}::mCherry]
UA185	α-syn; VPS41 E	$baIn11$; $baEx121$ [P_{dat-1}::hvps41-E, P_{unc-54}::mCherry]
UA187	α-syn; VPS41 F	$baIn11$; $baEx122$ [P_{dat-1}::hvps41-F, P_{unc-54}::mCherry]
UA189	α-syn; VPS41 G	$baIn11$; $baEx123$ [P_{dat-1}::hvps41-G, P_{unc-54}::mCherry]
UA191	α-syn; VPS41 T146P	$baIn11$; $baEx124$ [P_{dat-1}::hvps41-T146P, P_{unc-54}::mCherry]
UA193	α-syn; VPS41 A187T	$baIn11$; $baEx125$ [P_{dat-1}::hvps41-A187T, P_{unc-54}::mCherry]
UA200	α-syn	$apb-3(ok429)$; $baIn11$
UA194	α-syn; VPS41 Isoform 1	$apb-3(ok429)$; $baIn11$; $baEx98$
UA195	genomic *sid-1*	$sid-1(pk3321)$; $baIn33$ [P_{dat-1}::sid-1, P_{myo-2}::mCherry]
TU3401[c]	genomic *sid-1*	$sid-1(pk3321)$; $uIS69$ [$P_{unc-119}$::sid-1, $pCFJ90$ (P_{myo-2}::mCherry)]
UA196	α-syn; genomic *sid-1*	$sid-1(pk3321)$; $baIn11$; $baIn33$
UA197	α-syn; genomic *sid-1*	$sid-1(pk3321)$; $uIS69$; $baIn11$
UA49	α-syn::GFP	$baIn2$ [P_{unc-54}::GFP, P_{unc-54}::α-syn::GFP, $rol-6(su1006)$]

Transgenic *C. elegans* were generated by microinjecting plasmid constructs into N2 hermaphrodites and crossing into other transgenic or mutant animals (see Materials and Methods). Plasmid constructs expressed in each animal and the resulting genotypes are reported for each strain.

[a]Referenced in Hamamichi et al, 2008; Ruan et al, 2010; Yacoubian et al, 2010.

[b]Referenced in Ruan et al, 2010.

[c]Gift from Marty Chalfie (Columbia University, New York, NY); Referenced in Calixto et al, 2010.

phenotypic marker (mCherry) in the pharynx. The extrachromosomal array (P_{dat-1}::sid-1, P_{myo-2}::mCherry) was integrated into the *C. elegans* genome by UV irradiation (Inoue and Thomas, 2000) to create strain UA195 [$sid-1(pk3321)$; $baIn33$ (P_{dat-1}::sid-1, P_{myo-2}::mCherry)]. Integrated transgenic lines were outcrossed with $sid-1(pk3321)$ nematodes five times to remove any extraneous mutations. The isolated homozygous integrated lines were crossed into strain UA44 [$baIn11$ (P_{dat-1}::α-syn, P_{dat-1}::GFP)] to generate strain UA196 [$sid-1(pk3321)$; $baIn11$; $baIn33$], which expresses α-syn, GFP, and SID-1 in the dopaminergic neurons and is susceptible to RNAi selectively in these specific neurons.

Worm strain TU3401 [$sid-1(pk3321)$; $uIS69$ ($P_{unc-119}$::sid-1, $pCFJ90$ (P_{myo-2}::mCherry))] was generously provided by Marty Chalfie (Columbia University, New York, NY) for pan-neuronal-specific RNAi (Calixto et al., 2010). TU3401 hermaphrodites, which express *sid-1* pan-neuronally, were crossed into strain UA44 to generate strain UA197 [$sid-1(pk3321)$; $uIS69$; $baIn11$]; these worms are sensitive to RNAi specifically in the neurons. To isolate homozygous $sid-1(pk3321)$ worms, crossed progeny were grown for three generations in the presence of *par-2* (F58B6.3) RNAi, which inhibits embryonic development, allowing for only $sid-1(pk3321)$ worms to develop. After isolating homozygous $sid-1(pk3321)$ animals, worms expressing GFP and mCherry were sequestered and allowed to self-fertilize, and the progeny were examined for isogenic lines.

Dopaminergic neurodegeneration analysis in *C. elegans*
C. elegans dopaminergic neurons were analyzed for degeneration as previously described (Hamamichi et al., 2008; Harrington et al., 2011). Briefly, strain UA44 and experimental strains expressing hVPS41 constructs in the dopaminergic neurons were synchronized, grown at 20°C, and analyzed at specific time points in development, between day 6 and day 10 posthatching (3- and 7-d-old adults), as reported in Results and figure legends, for α-syn-induced dopaminergic neurodegeneration. On the day of analysis, the six anterior dopaminergic neurons [four CEP (cephalic) and two ADE (anterior deirid)] were examined in 30 adult hermaphrodite worms, which were immobilized on glass coverslips using 3 mM levamisole and transferred onto 2% agarose pads on microscope slides. Worms were considered normal when all six anterior neurons were present without any signs of degeneration, as previously

reported (Cao et al., 2005; Hamamichi et al., 2008; Ruan et al., 2010; Harrington et al., 2011). In total, at least 90 adult worms were analyzed for each independent truncated hVPS41 transgenic line (30 worms/ trial × 3 independent transgenic lines × 3 trials = 270 total animals/ construct) or RNAi treatment (30 worms/trial × 4 trials = 120 total animals/trial), with the scorer uninformed of the plasmid variant expressed.

RNA isolation and quantitation

Total RNA was isolated from 50 young adult hermaphrodite worms as previously described (Hamamichi et al., 2008). Briefly, the worms were transferred into 10 μl of 10% Single Worm Lysis Buffer (10 mM Tris, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, 0.01% gelatin, 60 μg of proteinase K) and frozen at −80°C for 1 h. After thawing, 100 μl of TRI Reagent (Molecular Research Center) was added to the samples and incubated for 10 min at room temperature (RT). The samples were freeze thawed five times in liquid N₂, vortexed for 15 s with 10 μl of 1-bromo-3-chloropropane (Acros Organics), incubated for 10 min at RT, and centrifuged for 15 min at 14,500 rpm at 4°C. The supernatant was transferred to an RNase-free tube, mixed with 1.5 μl of glycoblue (Ambion) and 50 μl of −20°C chilled isopropanol, and stored overnight at −20°C. After incubation, the sample was centrifuged for 15 min at 14,500 rpm at 4°C and supernatant discarded. The pellet was washed with 100 μl of RNase-free ethanol (75%) and resuspended in 10 μl of DEPC-treated water. The sample was treated for 15 min with 1 μl of DNase I (Invitrogen) at RT, then for 10 min with 1 μl of 25 mM EDTA at 65°C. RT-PCR was performed using SuperScript III RT (Invitrogen) using oligo dT primers following the manufacturers protocols. cDNA amplification was performed using Phusion polymerase (Finnzymes) with primers specified for each construct, available upon request. For each hVPS41 construct, the 5′ FLAG primer was used to specifically amplify the transgene. Amplified products were separated on 0.8% agarose gels and visualized using GelRed staining (Biotium).

For mammalian cells, 30 μl of H4 (human neuroglioma) cells in lysis buffer were transferred to microcentrifuge tubes and frozen at −80°C. RNA isolation, RT-PCR, and cDNA amplification were performed as described above.

RNA interference

RNAi feeding clones (Geneservice) were isolated and grown overnight in LB media with 100 μg/ml ampicillin. NGM plates containing 0.25% β-D-lactose were seeded with RNAi feeding clones and allowed to dry. Eight, larval stage 1 (L1) worms (neuron-specific RNAi worm strains, UA196 or UA197) were transferred to the plates and incubated until adulthood at 20°C. Adult hermaphrodites were transferred to corresponding RNAi plates and allowed to lay eggs for 5 h to synchronize. The dopaminergic neurons in the F1 progeny of the RNAi-treated worms were analyzed at day 6 and day 8 for degeneration, as described above.

Immunostaining of hVPS41 in H4 cells

H4 cells were grown in four-chambered slides and cotransfected with FLAG-hVPS41 variants using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Twenty-four hours post-transfection, cells were fixed with 4% paraformaldehyde, permeated with 0.5% Triton X-100 for 20 min at RT, and immunostained using a primary rabbit monoclonal antibody against FLAG (Sigma) and Alexa Fluor 594 secondary rabbit antibody (Invitrogen). Images were acquired using a Nikon Eclipse E800 epifluorescence microscope equipped with a Texas Red HYQ filter cube (Chroma Technology) and a Cool Snap CCD camera (Photometrics) driven by MetaMorph software (Molecular Devices).

α-Syn inclusion assay

H4 cells were grown in four-chambered slides and cotransfected with α-syn/truncated green fluorescent protein fusion (synT), synphilin, and either empty vector control or hVPS41 variant using Lipofectamine 2000 (Invitrogen), as previously described (McLean et al., 2001; Yacoubian et al., 2010). Twenty-four hours post-transfection, cells were fixed with 4% paraformaldehyde, permeated with 0.5% Triton X-100 for 20 min at RT, and immunostained using a primary α-syn antibody (BD Biosciences) and Alexa Fluor 488 secondary antibody (Invitrogen). Every cell with

α-syn staining (>400 cells/well) was scored as positive or negative for α-syn inclusions, with the scorer uninformed of the plasmid variant expressed. Each of the four wells was scored independently with the experiment replicated in triplicate.

Western blotting

Transfected H4 cells were washed in PBS 24 h after transfection, collected in lysis buffer [150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 2% SDS, and Halt protease inhibitor cocktail (Thermo Scientific)], sonicated for 10 s on ice, and centrifuged at 16,000 × g for 10 min. Soluble protein was transferred to a clean tube, and the bicinchoninic acid assay used to quantify protein concentrations. Samples were boiled for 5 min in 2× Laemmli sample buffer (Bio-Rad) with 5% 2-mercaptoethanol, separated on 10% SDS-polyacrylamide gels (Bio-Rad), and transferred to PVDF membranes (GE Healthcare). Membrane was blocked for 1 h in 5% nonfat milk in TBST [20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20] at RT and incubated with primary mouse monoclonal antibody against α-syn (1:5000; Abcam), actin (1:5000; MP Biomedicals), or primary rabbit monoclonal antibody against FLAG (1: 400; Sigma). Blots were washed three times with TBST and incubated with HRP-conjugated mouse or rabbit secondary antibodies (1:10,000; GE Healthcare) for 2 h at RT. After four washes of 10 min with TBST, blots were incubated for 5 min with Immobilon Western chemiluminescent HRP substrate (Millipore) and developed using the Fujifilm Image Reader LAS-4000. Multigage software v3.0 (Fujifilm) was used to quantify protein levels.

Imaging and statistics

Fluorescent microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HYQ filter cube (Chroma Technology). A Cool Snap CCD camera (Photometrics) driven by MetaMorph software (Molecular Devices) was used to acquire deconvolution images. DNA gel and protein blot images were acquired using the Fujifilm Image Reader LAS-4000. Quantification of DNA or protein level was done using Multigage software v3.0 (Fujifilm). The Student's t test was used to statistically analyze all datasets.

Results

WD40 and clathrin domains of VPS41 are required for protecting *C. elegans* dopaminergic neurons against α-syn-induced neurodegeneration *in vivo*

Through a large-scale RNAi screen using *C. elegans* to identify modifiers of α-syn pathology, the nematode ortholog of VPS-41 was previously identified as a leading candidate. RNAi knockdown of *C. elegans vps-41* enhanced α-syn aggregation in body wall muscles, while transgenic overexpression of VPS-41 in the dopaminergic neurons protected these neurons against α-syn-induced neurodegeneration (Hamamichi et al., 2008). Since VPS-41 is a highly conserved metazoan protein (Radisky et al., 1997), we further showed that both human isoforms of VPS41 (hVPS41) were able to protect *C. elegans* dopaminergic neurons from both 6-OHDA-induced and α-syn-induced neurodegeneration, validating a preserved function of the VPS41 protein across species (Ruan et al., 2010).

Initially, we sought to determine which functional domains were required for the neuroprotective property of hVPS41 using our *C. elegans* model of α-syn-induced dopaminergic neuron degeneration (P_{dat-1}::α-syn; P_{dat-1}::GFP) (Fig. 1). Previous studies in yeast identified several functional domains within hVPS41, including AP-3 interaction (WD40), CHCR, RING finger, metal ion binding, and microtubule binding domains (Radisky et al., 1997). To functionally elucidate the minimal domains required for hVPS41-dependent neuroprotection, we created truncated forms of the protein (Fig. 1B) and generated transgenic animals expressing the truncated proteins specifically in the dopaminergic neurons. These transgenic animals were then crossed into an isogenic worm strain expressing human α-syn and GFP in the
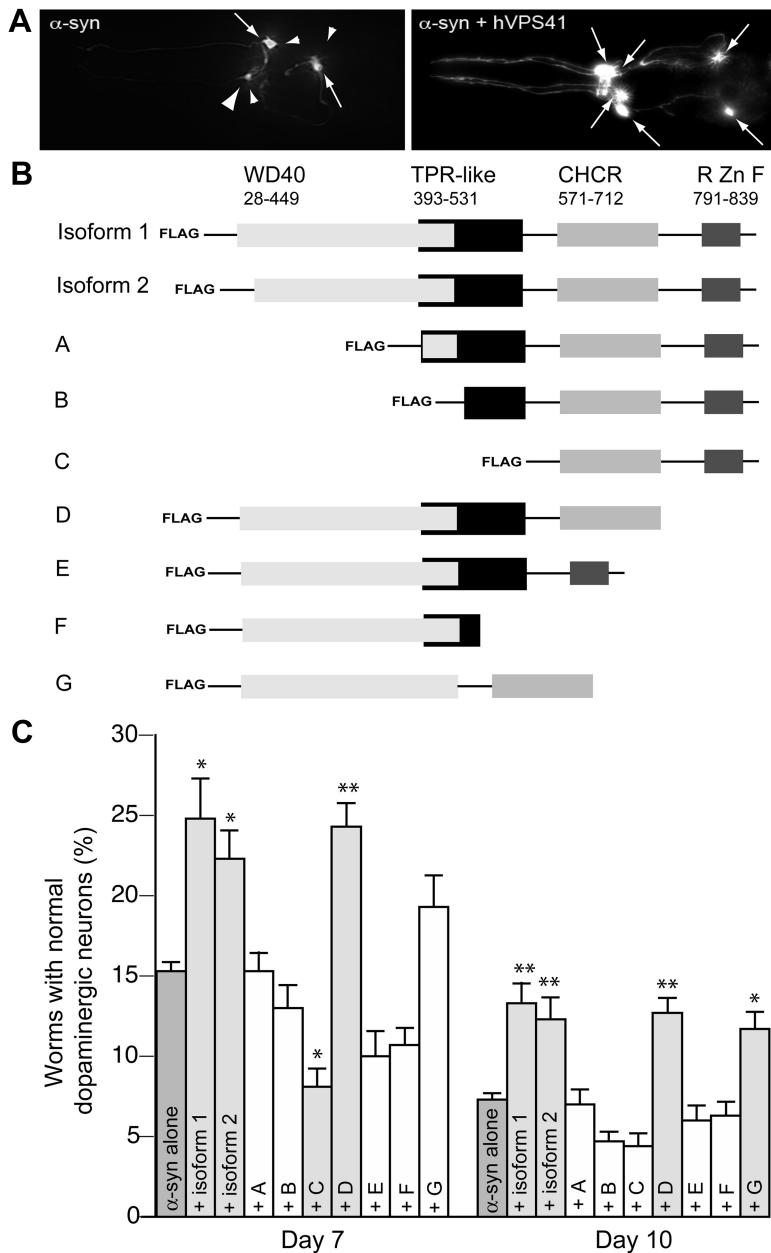
Figure 1. hVPS41 overexpression protects *C. elegans* dopaminergic neurons from α-syn-induced neurodegeneration. ***A***, Anterior dopaminergic neurons (4 CEP and 2 ADE) in worms expressing human α-syn and GFP. Left, A worm with only two normal neurons (1 CEP and 1 ADE), where the other four neurons (3 CEP and 1 ADE) have degenerated. Right, The full complement of six anterior dopaminergic neurons of *C. elegans* expressing hVPS41 Isoform 1 along with α-syn and GFP, showing no evidence of degeneration. Arrows show intact dopaminergic neuron cell bodies, large arrowhead shows a degenerating neuron, and small arrowheads indicate regions where neurons have degenerated. ***B***, Schematic diagram representing the various truncated hVPS41 protein constructs generated for functional analysis. Predicted functional protein domains were identified using bioinformatics and include the WD40, TPR-like, CHCR, and RING Zn (R Zn F) finger domains. These domains were truncated to determine the minimal regions required for hVPS41 function. The FLAG epitope tag was attached to the N terminus of each truncation. Transgenic animals were generated that express each construct in the DA neurons, and these animals were crossed into the isogenic α-syn strain, which displays age-dependent neurodegeneration. ***C***, Overexpression of either full-length hVPS41 or two truncated forms (D and G) protects dopaminergic neurons from α-syn-induced neurodegeneration. The common domains in all protective forms were the WD40 protein interaction and CHCR domains. Worms were scored as displaying normal dopamine neurons when there was no degeneration observed in the six anterior dopaminergic neurons. Homozygous worms were synchronized and analyzed at days 7 and 10 posthatching. Data are reported as the mean ± SD, $n = 270$ worms. $*p < 0.05$, $**p < 0.01$, Student's *t* test.

dopaminergic neurons that exhibit age-dependent neurodegeneration (Fig. 1*A*, left) (Hamamichi et al., 2008) to determine which truncated forms could rescue α-syn-induced neurodegeneration. Due to the limited expression of the hVPS41 constructs

only in the dopaminergic neurons (8 of ~1000 cells within *C. elegans*), we were unable to detect the presence of the truncated proteins by Western blotting or immunostaining (data not shown). However, the expression of each hVPS41 construct was verified by RT-PCR using a 5′ FLAG primer and a 3′ primer specific for each hVPS41 construct (Fig. 2*A*,*B*).

In quantifying dopaminergic neurodegeneration, worms are considered "normal" when a full complement of all six anterior dopaminergic neurons is present with neuronal processes fully extended to their target areas (Fig. 1*A*, right). Expression of α-syn and GFP results in significant dopaminergic neurodegeneration, whereby only ~15% of the worm population retains the full complement of normal dopaminergic neurons at 7 d of development (Fig. 1*C*). In accordance with previous findings, overexpression of both isoforms of hVPS41 can significantly protect these neurons from degeneration (Fig. 1*C*). Interestingly, two truncated forms of hVPS41 (truncates D and G) were also able to significantly ameliorate α-syn-mediated neurotoxicity (Fig. 1*B*,*C*). These two truncated versions of hVPS41 share the AP-3 interaction domain (WD40 repeat) and the CHCR domain. In contrast to prior reports (McVey Ward et al., 2001), the RING-H2 finger does not appear to play a functional role in protecting *C. elegans* dopaminergic neurons from neurodegeneration, since removal of this domain does not impact the neuroprotective property of hVPS41, as demonstrated by the neuroprotective activity of truncate D. Removal of 107 aa within the tetratricopeptide repeat (TPR)-like domain (truncate G), which separates the WD40 and CHCR domains, also did not alter the overall function of hVPS41 in this assay.

All of the results described thus far have reported the percentage of individual animals within a population ($n \geq 270$) of worms that exhibit any neuronal degeneration (i.e., if even one of the DA neurons was degenerating, an animal was considered to show neurodegeneration). However, an unequivocal advantage of *C. elegans* is that detailed analyses of individual cells are achievable. Therefore, we also examined the severity of damage within dopaminergic neuron populations. The six dopaminergic neurons within the anterior region were individually scored for degeneration ($n \geq 1620$ neurons), and statistically identical correlations to what was found with animal percentages were also obtained with neuron populations for all hVPS41 constructs examined (data not shown). Together, these data indicate the WD40 protein interaction and CHCR domains are both essential for the conserved

function of hVPS41 in protecting dopaminergic neurons from α-syn-induced neurodegeneration *in vivo*.

VPS41 requires both AP-3 and HOPS complexes for neuroprotection

Previous studies in yeast have identified at least two independent pathways that regulate protein sorting and trafficking from the Golgi to the vacuole/lysosome, with VPS-41 functioning within one of these, the alkaline phosphatase (ALP) pathway (Darsow et al., 2001). Protein trafficking through the ALP pathway requires the AP-3 and transports cargo directly to the vacuole by bypassing early, sorting endosomes. The N-terminal WD40 domain of VPS-41 directly interacts with the large δ-subunit of the AP-3 complex, while the C-terminal CHCR domain of VPS-41 can homo-oligomerize to form a clathrin-like outer shell surrounding the AP-3 vesicle (Rehling et al., 1999; Darsow et al., 2001). As the vesicles approach the vacuole, VPS-41 functions as part of the HOPS docking complex along with VPS-39 to promote vacuole fusion, through activation of the Rab GTPase RAB-7 (Brett et al., 2008; Cabrera et al., 2009).

To determine whether hVPS41 is functioning to protect dopaminergic neurons from α-syn-induced degeneration via lysosomal trafficking of AP-3 transport vesicles, we used the availability of a *C. elegans* AP-3 complex mutant, *apb-3(ok429)*, which is a predicted null mutation that has defects in lysosome-related gut granule formation (Hermann et al., 2005). Our isogenic line of worms expressing α-syn, GFP, and hVPS41 in the dopaminergic neurons was crossed into *apb-3(ok429)* mutant worms, and the dopaminergic neurons were analyzed for the level of degeneration at days 7 and 10 (Fig. 3A). While overexpression of hVPS41 was significantly able to protect worms from dopaminergic neurodegeneration in a wild-type (N2) background, hVPS41 was unable to elicit the neuroprotective effect in the absence of a functional AP-3 complex, as observed in the *apb-3(ok429)* mutant background (Fig. 3A). This same correlation was obtained when comparing total individual neurons across animals (data not shown), instead of worm populations (Fig. 3A).

To further validate the functional requirement of the AP-3 and HOPS complex, we took advantage of a recently reported innovation enabling us to conduct neuron-specific RNAi by generating a worm strain that overexpresses the dsRNA transporter SID-1 specifically in the dopaminergic neurons. Since *C. elegans* neurons are known to be highly resistant to RNAi, possibly through low expression of SID-1, Calixto et al. (2010) created a strain expressing genomic *sid-1* under the pan-neuronal promoter (P$_{unc-119}$) in a *sid-1(pk3321)* mutant background; this strain allowed for neuron-specific gene knockdown in worms, with all
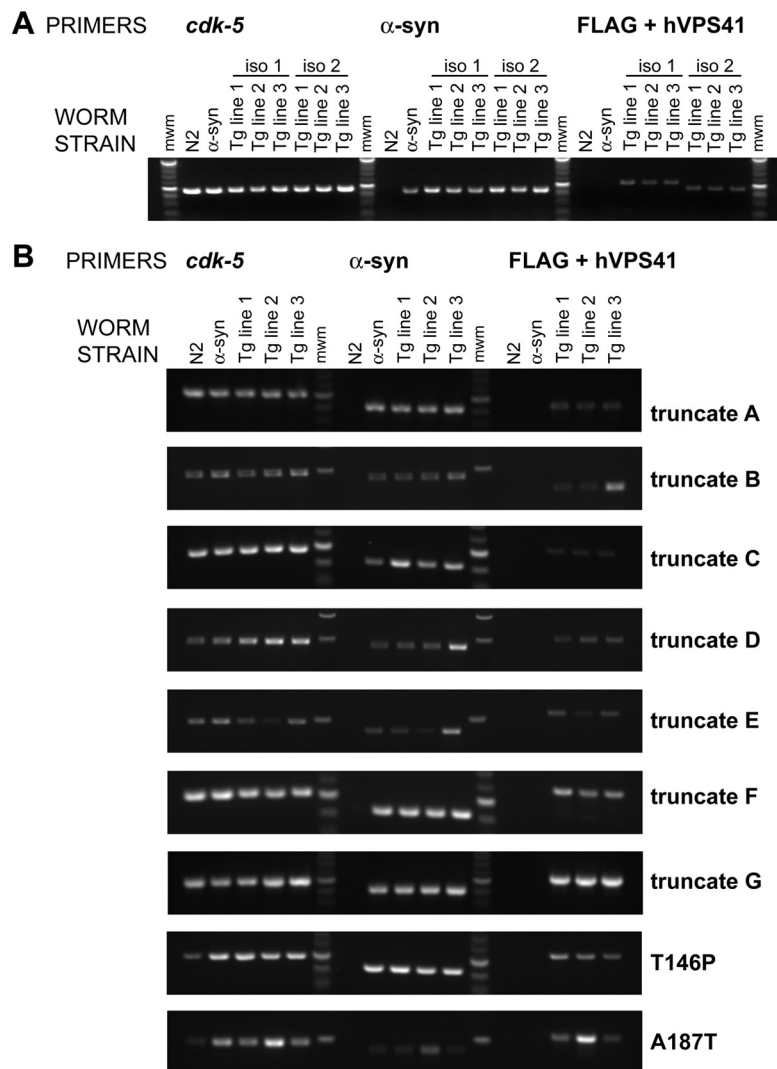

Figure 2. hVPS41 constructs are transcribed in *C. elegans*. RT-PCR of transgenic *C. elegans* lines expressing various hVPS41 constructs confirms expression of both α-syn and hVPS41. RNA was isolated from transgenic worms expressing α-syn and hVPS41 constructs, converted to cDNA, and amplified using primers specific for *cdk-5* (housekeeping gene; loading control), α-syn, or *hVPS41*. Primers were designed to amplify a ~500 bp region of each gene. Amplification of α-syn was detected at equivalent levels in all strains, excluding N2 Bristol (wild-type), which does not express α-syn (or *hVPS41*). Amplification of both isoforms of *hVPS41* (**A**), all *hVPS41* truncates (**B**), and both *hVPS41* SNPs (**B**) were detected using a 5′ FLAG primer and a 3′ primer specific for each *hVPS41* construct in each of three hVPS41 transgenic worm strains (Tg line). Molecular weight marker lanes are designated with "mwm."

other tissues/cells being resistant. Using a similar approach, we created a *sid-1(pk3321)* mutant strain of *C. elegans* that expresses wild-type SID-1 specifically in the dopaminergic neurons under control of the dopamine transporter promoter (P$_{dat-1}$), thereby enabling dopaminergic neuron-specific RNAi. The dopaminergic neuron-specific RNAi strain was crossed into our isogenic UA44 (P$_{dat-1}$::α-syn; P$_{dat-1}$::GFP) strain to examine the effect of neuronal gene knockdown to identify modifiers of α-syn toxicity selectively in dopaminergic neurons. With the expression of this additional transgene (P$_{dat-1}$:: *sid-1*), we observed slightly less dopaminergic neurodegeneration, yet this was still significant compared with worms not expressing α-syn and GFP (P$_{dat-1}$:: α-syn; P$_{dat-1}$::GFP).

Interestingly, knockdown of either of the HOPS complex components, VPS-39 and VPS-41 or the VPS-41-interacting δ-subunit of the AP-3 complex (APD-3), significantly enhanced the level of dopaminergic neurodegeneration elicited through
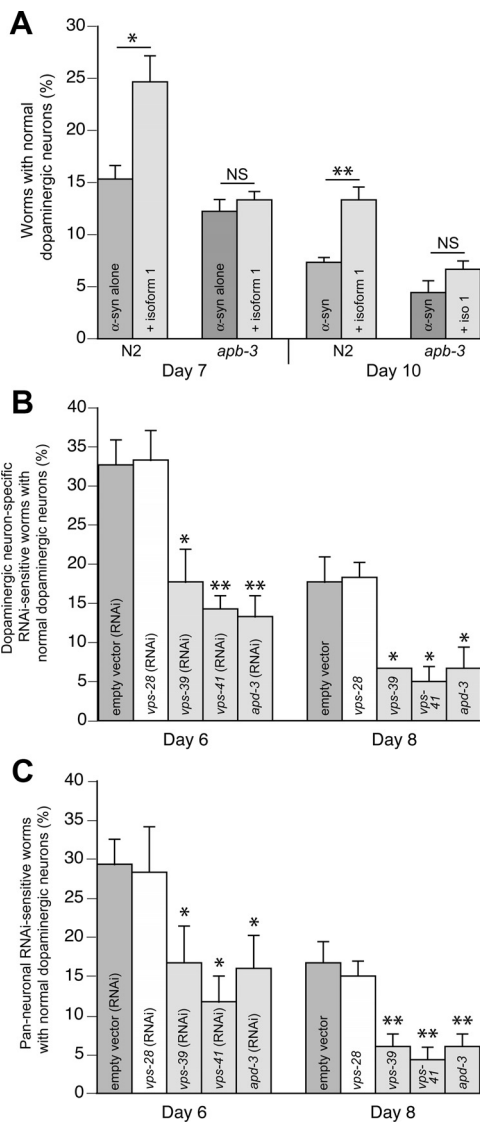
Figure 3. hVPS41 requires both a functional AP-3 and HOPS complex to protect dopaminergic neurons from α-syn-induced neurodegeneration. *A*, Worms expressing α-syn and GFP with or without hVPS41 expression were crossed into *apb-3(ok429)* mutant animals. Resulting homozygous animals were synchronized and analyzed at days 7 and 10 posthatching. Overexpression of hVPS41 protected dopaminergic neurons from degeneration in a wild-type (N2) background but failed to protect neurons from α-syn toxicity in an AP-3 complex mutant (*apb-3*). Data are reported as the mean ± SD, $n = 270$ worms. *$p < 0.05$, **$p < 0.005$, Student's *t* test. *B*, *C*, RNAi knockdown of components within the HOPS (VPS-39 and VPS-41) or AP-3 complex (APD-3) specifically in the dopaminergic neurons (*B*) {P$_{dat-1}$::*sid-1* in *sid-1* mutant [*sid-1(pk3321)*]} or pan-neuronally (*C*) {P$_{unc-119}$::*sid-1* in *sid-1* mutant [*sid-1(pk3321)*]} enhances α-syn-induced neurodegeneration in worms not overexpressing hVPS41. The F1 progeny of worms exposed to RNAi feeding bacteria were synchronized, and the dopamine neurons were analyzed for degeneration at days 6 and 8 posthatching. RNAi bacteria that do not express an RNAi clone (empty) or the VPS-28 RNAi clone were used as negative controls. Data are reported as the mean ± SD, $n = 120$ worms. *$p < 0.05$, **$p < 0.005$, Student's *t* test.

α-syn overexpression in the dopaminergic neuron-specific RNAi strain (Fig. 3*B*). Knockdown of *vps-28* did not enhance α-syn-induced neurodegeneration. Since the VPS-28 gene product encodes ESCRT-1 (endosomal sorting complex required for transport), and it is not involved in Golgi-to-lysosomal trafficking, but is required for cell surface receptor degradation, we chose this as a negative control. Dopaminergic neuron-specific RNAi data were confirmed using a pan-neuronal RNAi strain (Calixto

et al., 2010) (Fig. 3*C*), thus verifying that the dopaminergic RNAi strain did not alter the expression levels of proteins due to promoter saturation. All data obtained comparing worm populations (Fig. 3*B*,*C*) once again correlated with individual neuron data (data not shown). Additionally, RNAi knockdown of VPS-39, VPS-41, APD-3, or APB-3 in the absence of α-syn does not cause the dopaminergic neurons to degenerate, indicating that these genes play a role in protecting neurons from α-syn neurotoxicity (data not shown). Together, these data reveal the functional relevance of the AP-3 and HOPS complex in impacting α-syn-induced neurodegeneration as well as validate the requirement of a functional AP-3 complex for hVPS41 to function.

SNPs in VPS41 alter neuroprotective function
With advances in genomic sequencing, genetic variance within populations is an emerging field of intense interest, with identification of SNPs in specific genes being associated with human diseases. Several SNPs have been directly associated with the occurrence of PD, including mutations in the gene encoding α-syn (A30P and A53T). However, these mutations only account for a small population of patients with this disease, indicating that further genetic or environmental factors may play pivotal roles in the onset and progression of PD. Since we have shown the functional requirement of hVPS41 in protecting dopaminergic neurons from degeneration due to α-syn overexpression, we evaluated several SNP databases, including the NCBI SNP database (www.ncbi.nlm.nih.gov/snp), GeneCards (www.genecards.org), and SNPs3D (www.snps3d.org), where several SNPs have been identified within the general human population. Interestingly, the domain in which hVPS41 interacts with the AP-3 complex (WD40 domain) has several SNPs (T146P and A187T) reported within it that are predicted to be deleterious to the structure/function of the protein (Fig. 4*A*) (SNPs3D). These SNPs change functional groups of amino acids; there is a hydrophilic-to-hydrophobic amino acid change in the T146P SNP, while A187T changes this residue from hydrophobic to hydrophilic. In both cases, the structure and/or function of the WD40 domain could be altered. Moreover, both SNPs have a minor allelic frequency of $f = 0.02$ (2%), indicating that a small percentage of the population contains either of these SNPs.

Since these SNPs have not been functionally analyzed and are predicted to be deleterious to the activity of the protein (SNPs3D), we introduced these changes into hVPS41 and overexpressed the SNP mutants in the dopaminergic neurons to investigate their impact in our worm model of α-syn-induced neurodegeneration. The hVPS41 SNP transgenes were verified as being transcribed within the α-syn-expressing worms by RT-PCR (Fig. 2*B*). Strikingly, overexpression of hVPS41 T146P or A187T abrogated the dopaminergic neuroprotection provided by wild-type hVPS41 against α-syn-induced neurodegeneration (Fig. 4*B*). Data comparing worm populations (Fig. 4*B*) corroborated with collective individual neuron data (data not shown). While yet to be associated with human PD patient genomes, these data indicate that the T146P or A187T variants in the WD40 domain alter the neuroprotective property of hVPS41 in an *in vivo* animal model of PD.

hVPS41 constructs are localized within the cytoplasm of H4 cells
The neuroprotective capacity of the specific hVPS41 constructs may be due to those specific forms being expressed as proteins, while the truncated and SNP proteins may not be expressed, due to misfolding and/or degradation of the polypeptide. To ac-
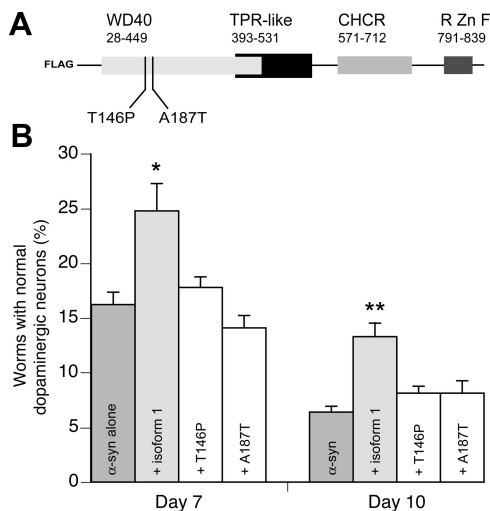
Figure 4. Two SNPs found within the WD40 domain of hVPS41 alter the neuroprotective property afforded against α-syn toxicity. **A**, Schematic diagram showing the location and amino acid changes associated with two SNPs within hVPS41. Both SNPs are located within the WD40 protein interaction domain, which is required for hVPS41-mediated neuroprotection in worms (Fig. 1C); the WD40 domain directly interacts with the δ-subunit of the AP-3 complex in yeast. Both SNPs have been reported to be present in a small percentage of the general human population and are predicted to be deleterious to the structure/function of the protein (www.snps3d.org). **B**, Overexpression of hVPS41 with either SNP T146P or A187T failed to protect DA neurons from α-syn-induced neurodegeneration. Transgenic animals expressing either wild-type or mutant hVPS41 were crossed into isogenic α-syn worms. Homozygous worms were synchronized and analyzed at days 7 and 10 posthatching. Data are reported as the mean ± SD, $n = 270$ worms. $*p < 0.05$, $**p < 0.005$, Student's t test.

cess the expression pattern of the various hVPS41 construct proteins, human neuroglioma (H4) cells were transfected with FLAG–hVPS41 constructs, and transfected cells were fixed and immunostained using a FLAG-specific antibody (Sigma) 24 h post-transfection. The intracellular localization of both hVPS41 isoforms showed diffuse expression of hVPS41 protein within the cytoplasm and perinuclear region of H4 cells, as detected by FLAG antibody staining (Fig. 5A). Most hVPS41 truncates (except truncate F) and both hVPS41 SNPs were localized in a similar manner and expression level when compared with hVPS41 isoforms 1 and 2, indicating that the truncated proteins can be expressed (Fig. 5A). Truncated hVPS41 construct F may not express a stable protein due to the substantially reduced size of the polypeptide encoded, which may be misfolded and degraded. hVPS41 truncate F also mimics the *C. elegans vps-41(ep402)* mutant allele, which is embryonic-lethal to the organism in the homozygous state and exhibits an increase in germline apoptosis (Lackner et al., 2005), suggesting that this truncated protein is defective in function. Additionally, most hVPS41 truncates (except B and F) and both hVPS41 SNPs were detected through Western blot analyses using FLAG antibody staining, with proteins detected at their predicted molecular weight (Fig. 5B,C). While immunostaining of transfected H4 cells detected hVPS41 truncate B, this truncated protein was not detected through Western blot analysis, possibly due to the instability of this protein. In agreement with immunostaining results, hVPS41 truncate F was not detected either, further supporting the lack of expression of this truncated protein. These results show that the protective hVPS41 isoforms and hVPS41 truncates, D and G, are expressed as proteins, while the nonprotective hVPS41 truncates A–C and E, and both hVPS41 SNPs are also able to express proteins.

VPS41 reduces cytoplasmic α-syn inclusion formation *in vitro*

A key pathological feature of PD is the formation of cytoplasmic protein inclusions, containing α-syn, which are thought to underlie the cellular toxicity and promote neuronal cell death. To further validate the results obtained in our evaluation of hVPS41 using *C. elegans*, we used a human cell culture model of α-syn inclusion body formation (McLean et al., 2001). Cotransfection of H4 cells with an α-syn/truncated green fluorescent protein fusion (synT) and synphilin results in the formation of cytoplasmic inclusions in ~50% of cells that immunostained for α-syn (Fig. 6A,B). Using this model, we cotransfected H4 cells with either an empty vector or an hVPS41 construct, and 24 h after transfection we fixed and immunostained the cells using an α-syn-specific antibody. Interestingly, both isoforms of hVPS41, as well as the two truncated forms (D and G) that reduced neurodegeneration in *C. elegans*, also significantly decreased the number of inclusion-positive cells by ~40% in H4 cells (Fig. 6A,B). Truncate B, which did not protect *C. elegans* dopaminergic neurons from α-syn toxicity, was also unable to alter the degree of α-syn inclusion formation in H4 cells (Fig. 6B). Additional correlative results between *C. elegans* and H4 cells were obtained when the hVPS41 T146P and A187T SNPs were examined. Similar to the lack of effect of these variants seen in *C. elegans*, the number of inclusion-positive H4 cells when transfected with either variant was similar to the empty vector control (Fig. 6B). The intracellular localization of hVPS41 was detected diffusely expressed within the cytoplasm and perinuclear region of H4 cells using FLAG immunostaining (Fig. 6C). These data confirm the *C. elegans* results in identifying functional domains and SNPs within hVPS41, which may alter cellular homeostasis in the presence of increased α-syn protein expression.

VPS41 promotes the clearance of α-syn in human neuroglioma cells

Defects with lysosomal function in protein clearance are proposed to contribute to the progression of PD, with the α-syn protein being degraded, in part, though autophagy (Shin et al., 2005). Since α-syn accumulation may be associated with the neurotoxicity of dopaminergic neurons, we wanted to determine whether hVPS41 could promote the clearance of α-syn under conditions of increased expression in human neuroglioma cells. We previously reported that hVPS41 overexpression could prevent the increase in Triton-insoluble (oligomeric) α-syn species in human neuroblastoma (SH-SY5Y) cells after rotenone treatment (Ruan et al., 2010), and here we report the ability of hVPS41 to decrease the number of cells with α-syn-positive cytoplasmic inclusions in H4 cells (Fig. 6). Using the H4 cell model of α-syn inclusion formation described above, we isolated total protein from cells after transfection with synT, synphilin, and either an empty vector control or hVPS41, and quantified the levels of synT protein expressed. While a high level of synT was observed in the control cells, a significant reduction (~50%) in synT was recorded when hVPS41 was also expressed (Fig. 7A,D); this reduction was not due to transcriptional regulation of the transgenes (Fig. 7B). In accordance with our synT inclusion formation results (Fig. 6B), we demonstrate that SNPs (T146P and A187T) found within the WD40 domain of hVPS41 prevent the protein from reducing the level of synT protein, while both wild-type hVPS41 isoforms significantly reduced the synT protein level (Fig. 7C,D). These data confirm that when either SNP is present in hVPS41, the function of hVPS41 is altered, preventing the protein from reducing α-syn accumulation. Thus, these specific
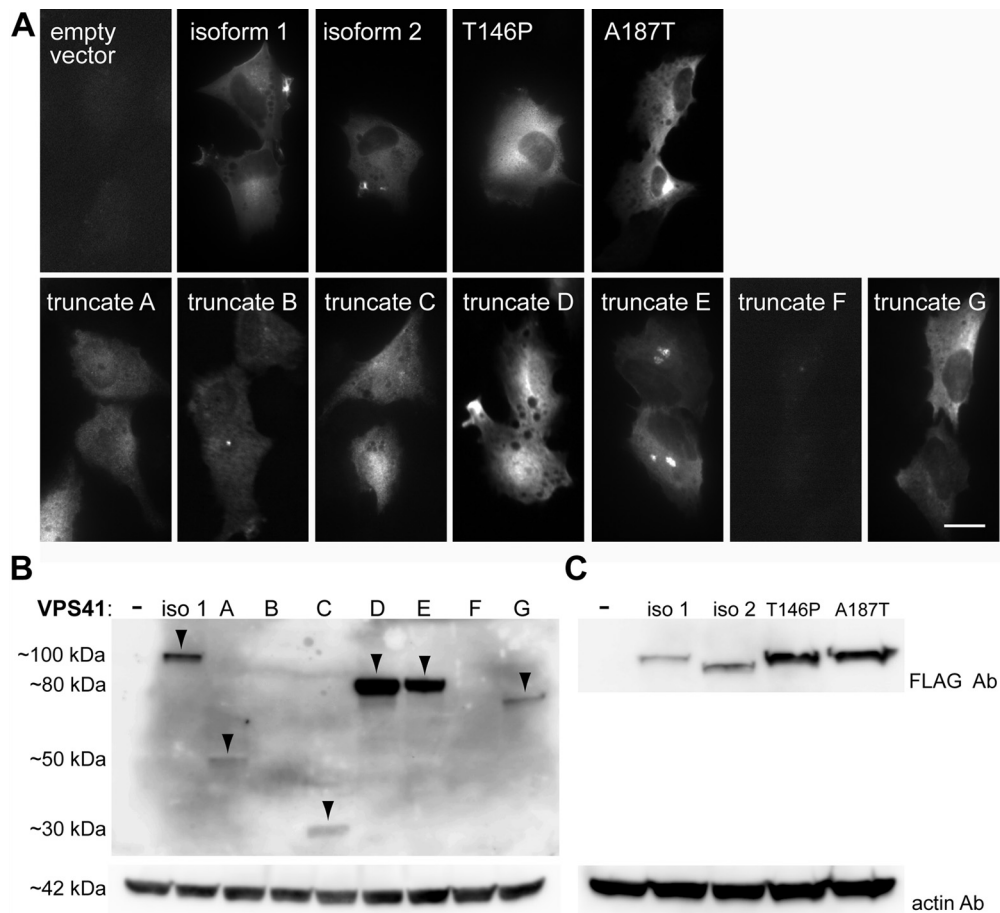
Figure 5. Expression of hVPS41 constructs in H4 cells. *A*, Immunostaining of transfected H4 neuroglioma cells using a FLAG-specific primary antibody with an Alexa Fluor 594-conjugated secondary antibody detects FLAG-hVPS41 protein diffusely in the cytoplasm and perinuclear region. Both human isoforms, the SNPs, and most of the truncates displayed similar expression patterns, except for truncate F, which was not expressed. The reduced size of hVPS41 truncate F may result in misfolding and degradation of this smaller protein. Scale bar, 20 μM. *B*, *C*, Western blot showing expression of hVPS41 constructs in H4 cell lysates. H4 neuroglioma cells were transfected with hVPS41 constructs, and total protein was isolated 24 h after transfection. Proteins were resolved using SDS-PAGE and transferred to PVDF membranes. *B*, Using a FLAG-specific antibody, full-length hVPS41 was detected at ~102 kDa in transfected cells. Proteins for truncated hVPS41 A (~48 kDa), C (~34 kDa), D (~82 kDa), E (~82 kDa), and G (~70 kDa) (arrowheads) were also detected using FLAG antibody staining. Truncated proteins for hVPS41 B and F were not detected through Western blot analysis, possibly due to instability of these proteins. Actin (~42 kDa) was used for a loading control. *C*, Both hVPS41 isoforms and hVPS41 SNPs were detected with a FLAG-specific antibody. hVPS41 isoform 2 is missing 25 aa compared with hVPS41 isoform 1, resulting in a protein of reduced size (~100 kDa). Both hVPS41 SNPs introduced into full-length isoform 1 were detected at ~102 kDa, similar to hVPS41 isoform 1. Actin (~42 kDa) was used for a loading control.

polymorphisms, or other currently unidentified but functionally equivalent SNPs in hVPS41, may represent putative susceptibility factors for Parkinson's disease.

Discussion

Increasing evidence supports a role for dysfunctional lysosomal trafficking and protein degradation as underlying the pathology of PD. Deleterious cellular consequences arising from lysosomal dysfunction have been proposed to underlie the age-related progression of the disease (Chu and Kordower, 2007). Lysosomal targeting of misfolded and aggregated proteins for degradation, including α-syn, has been shown to be largely mediated by macroautophagy and chaperone-mediated autophagy (CMA) (Webb et al., 2003; Cuervo et al., 2004). Mutations in α-syn that are associated with autosomal-dominant forms of PD have been shown to block CMA (Cuervo et al., 2004), and overexpression of wild-type α-syn impairs macroautophagy (Winslow et al., 2010). Accumulation of α-syn is observed within Lewy bodies in postmortem PD brains (Spillantini et al., 1997). While the precise form of α-syn aggregates comprising the toxic species *in vivo*

remains unresolved, enhanced α-syn expression has been conclusively associated with neurotoxicity and PD (Singleton et al., 2003). In *C. elegans*, transgenic overexpression of α-syn leads to age-dependent α-syn accumulation and dopaminergic neuron degeneration (Lakso et al., 2003; Cao et al., 2005; Kuwahara et al., 2006; Hamamichi et al., 2008; van Ham et al., 2008; Karpinar et al., 2009). Depletion of the E1-like enzyme required for the initiation of autophagosome formation, *atg-7*, results in neurodegeneration in mice (Komatsu et al., 2006) as well as enhanced α-syn accumulation in *C. elegans* (Hamamichi et al., 2008). Augmentation of a lysosomal enzyme, cathepsin D, promotes α-syn clearance *in vitro* and protects *C. elegans* dopaminergic neurons from α-syn toxicity (Qiao et al., 2008). Furthermore, two PARK genes, *PARK9* and *PARK16,* have been identified that function in the lysosomal system. Mutations in the lysosomal P-type ATPase, ATP13A2 (*PARK9*), have been linked to inherited PD (Ramirez et al., 2006). Genetic knockdown of the *C. elegans* homolog of ATP13A2, *catp-6*, enhances α-syn misfolding and accumulation, while transgenic overexpression of *catp-6* in worm dopaminergic
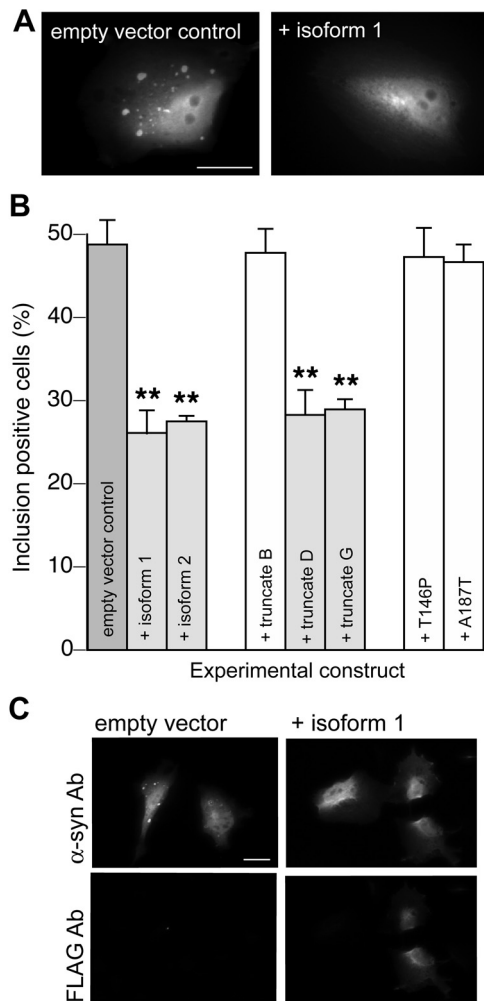
Figure 6. Overexpression of hVPS41 in human neuroglioma cells reduces the number of α-syn inclusion-positive cells. *A*, *B*, Cotransfection of H4 cells with synT, synphilin, and an empty vector control results in formation of cytoplasmic puncta (*A*, left) that immunostain positive for α-syn in ∼50% of transfected cells (*B*). Cells were fixed and immunostained 24 h post-transfection. Cotransfection of either isoform of hVPS41 or the two truncated constructs that were protective in *C. elegans* dopaminergic neurons (truncates D and G) significantly reduced the number of α-syn inclusion-positive cells (*A*, right), while control truncate B did not reduce the number of positive cells. When either SNP reported within the WD40 domain of hVPS41 (T146P or A187T) is present, hVPS41 is no longer able to reduce inclusion formation. Cells stained for α-syn were scored as either positive (*A*, left) or negative (*A*, right) for α-syn cytoplasmic inclusions, with the rater blind to each experimental condition. Each experimental condition was repeated three times with four replicas per experiment. Data are reported as the mean ± SD. **$p < 0.001$, Student's *t* test. Scale bar, 20 μM. *C*, H4 cells transfected with synT, synphilin, and either empty vector control (left panels) or FLAG-hVPS41 isoform 1 (right panels) were immunostained using a primary mouse monoclonal antibody against α-syn with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (top panels) or a primary rabbit monoclonal antibody against FLAG with an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody (bottom panels). hVPS41 is expressed in cells cotransfected with synT and is diffusely localized within the cytoplasm and perinuclear region. Expression of hVPS41 reduces the number of α-syn inclusion-positive cells. Scale bar, 20 μM.

neurons ameliorated α-syn-induced neurodegeneration (Gitler et al., 2009).

Recent identification of the *PARK16* locus revealed a novel RAB7L1 mutation as being associated with PD (Simón-Sánchez et al., 2009; Tucci et al., 2010). RAB7 is a member of the Rab GTPase superfamily involved in vesicle trafficking and functions at the lysosome to promote vesicle tethering and fusion. Interest-

ingly, in yeast, RAB7 is activated by the HOPS complex component VPS39, which functions as a guanine nucleotide exchange factor, and activated RAB7 directly interacts with VPS41 in the HOPS complex to promote vesicle tethering and subsequent lysosomal fusion (Brett et al., 2008). Depletion of the *Drosophila* VPS41 homolog, Light, impairs lysosomal acidification and protein degradation (Swetha et al., 2011). Here, we show that overexpression of hVPS41 in human neuroglioma cells is able to reduce the number of α-syn inclusion-positive cells and decrease the level of α-syn protein, possibly through enhanced lysosomal trafficking of α-syn. Therefore, the significance of these data is further supported by identification of RAB7L1 as a potential genetic cause of PD.

Additional evidence supporting the role of α-syn impairment of protein trafficking has been previously reported. In yeast, overexpression of α-syn leads to profound defects in ER–Golgi trafficking that can be rescued through expressing of the Rab GTPase RAB1, which functions in the tethering and docking of ER transport vesicles to the Golgi (Cooper et al., 2006). Further analysis in mammalian and *C. elegans* neurons revealed two other Rab proteins, RAB3A and RAB8A, which can function to protect cells against α-syn toxicity (Gitler et al., 2008). RAB3 functions in docking and tethering of neurotransmitter vesicles at the presynaptic terminal specifically in neurons, while RAB8 functions in post-Golgi trafficking. With VPS41 being involved in Golgi–lysosome protein transport, our data support the involvement of post-Golgi trafficking in mediating α-syn toxicity. Together, impaired protein trafficking throughout the cell may underlie the toxicity induced through increased α-syn expression, and by promoting protein transport machinery, cells are able to overcome this proteostatic imbalance.

Structural analysis of hVPS41 revealed that both the WD40 protein interaction and CHCR domains are required for hVPS41 to protect against neurotoxicity (Fig. 1). In contrast to previous studies (McVey Ward et al., 2001), truncation of the RING-H2 domain did not alter the neuroprotective function of hVPS41 in our assays, indicating that this domain is dispensable for hVPS41-mediated neuroprotection. These structure/function data were further corroborated using a human cell culture model of α-syn inclusion formation, where both the WD40 and CHCR domain were required for hVPS41 to reduce the number of α-syn inclusion-positive H4 cells (Fig. 6). In yeast, the WD40 domain of VPS41 has been shown to directly interact with AP-3 δ-adaptin subunit to facilitate protein trafficking between the late Golgi and vacuolar lysosome (Rehling et al., 1999), while the CHCR domain directs homo-oligomerization of VPS41 to form a clathrin-like lattice surrounding vesicles being trafficked to the vacuole (Darsow et al., 2001). As the vesicle approaches the vacuole, the HOPS docking complex, composed of VPS39 and VPS41, are recruited to the vesicle and function in vacuole fusion through activation of the yeast RAB7 homolog by VPS39 (Brett et al., 2008). Identification of the AP-3 interacting domain as being a required structural motif for hVPS41 function led us determine whether the AP-3 complex was required for the neuroprotective property of hVPS41. With only one *C. elegans* AP-3 complex mutant strain available, we generated worms expressing α-syn with or without hVPS41 in the dopaminergic neurons and showed that mutations in the AP-3 β-subunit (*apb-3*) attenuated the neuroprotective effect of hVPS41. Without worm mutants in the AP-3 δ-subunit or HOPS complex, we used a novel methodology to conduct cell-specific RNAi through expression of the dsRNA transporter (SID-1), either in all neurons (Calixto et al., 2010) or specifically in the dopaminergic neurons, within the
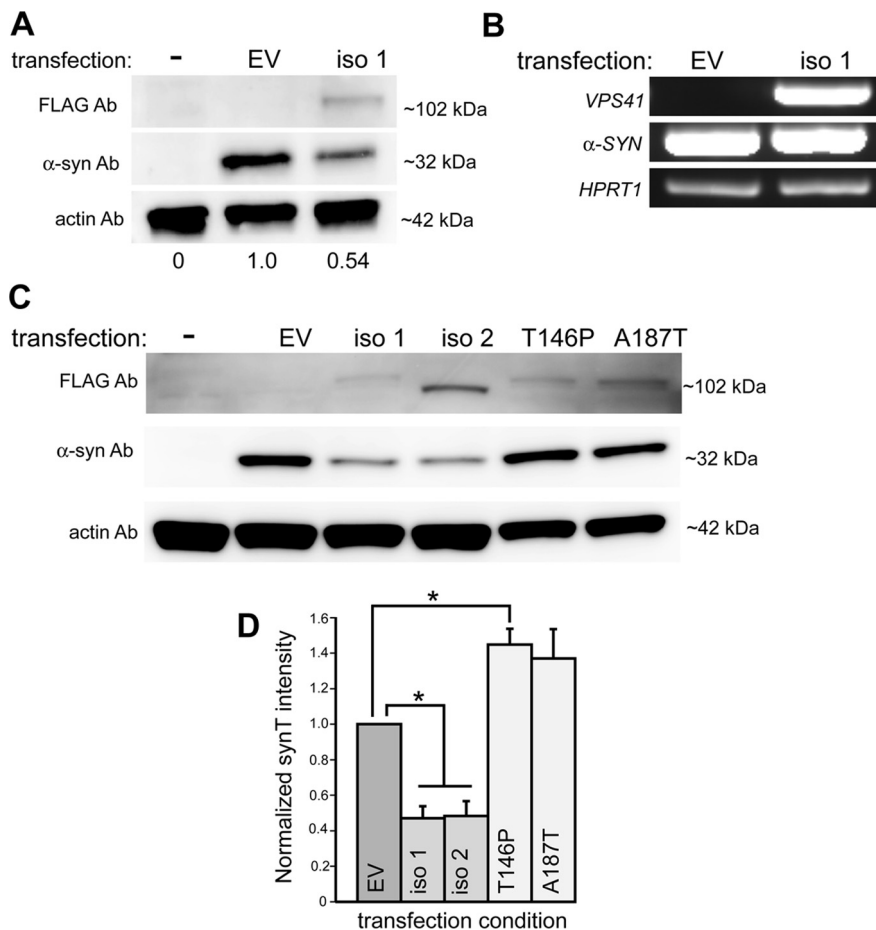
Figure 7. Overexpression of hVPS41 in H4 cells reduces the level of overexpressed α-syn/truncated GFP. **A**, Representative Western blot of protein levels in H4 cells transfected with synT, synphilin, and either empty vector (EV) control or hVPS41 isoform 1. A total of 10 μg of total cell protein was resolved using SDS-PAGE, transferred to PVDF membrane, and immunoblotted using primary antibodies against FLAG (for hVPS41), α-syn, or actin (loading control). The normalized intensity values of synT (α-syn) are reported below the blot image. Data analysis using Multigauge software revealed ~45% decrease in synT (~32 kDa) levels when hVPS41 (~102 kDa) was being coexpressed. **B**, Semiquantitative RT-PCR of transfected H4 cells reveals that the transcriptional level of α-syn is not being altered between the empty vector control and hVPS41-expressing cells. HPRT-1 was used as an internal loading control. **C**, Overexpression of hVPS41 SNPs in H4 cells fails to reduce the level of overexpressed synT protein. Representative Western blot of protein levels in transfected H4 cells was performed as described above (**A**). Reduced synT (~32 kDa) levels compared with EV were observed when hVPS41 isoform 1 (~102 kDa) or hVPS41 isoform 2 (~100 kDa) are coexpressed. Expression of either hVPS41 SNP (~102 kDa) failed to reduce the level of synT (~32 kDa) compared with EV, supporting functional defects associated with these SNPs. **D**, The normalized intensity values of synT (α-syn) were compared between the various hVPS41 constructs and the EV control. Data analysis using Multigauge software revealed a ~53% and ~52% decrease in synT levels compared with EV when hVPS41 isoform 1 or hVPS41 isoform 2 are coexpressed, respectively. When either SNP is present in hVPS41, an increase in synT level is observed when compared with EV. Results represent data collected from three independent experiments. Data are reported as the mean ± SD. *$p < 0.05$, Student's t test.

pression and identify the cellular pathway in which hVPS41 is acting to protect neurons against α-syn-induced neurodegeneration. Moreover, the development of transgenic nematodes enabling neuron-specific RNAi allows for postembryonic phenotypic evaluation of essential gene products, such as VPS-41, for which mutant animals are not viable (Lackner et al., 2005).

With the rapid advancement in genome sequencing technologies, numerous SNPs have been identified within the general human population, yet limited reports have emerged on the functional implications of such variation or their mechanistic implications for neurodegenerative, and other, diseases. In this context, the two SNPs, T146P and A187T, that have been identified within the WD40 domain of hVPS41 represent such an uncharacterized example. Here we report that when either SNP is introduced into the WD40 domain of hVPS41, the modified protein failed to protect cells against α-syn accumulation (in H4 cells) and toxicity (in *C. elegans* dopaminergic neurons). These results correlate to our structure/function analysis, wherein we also showed that the WD40 domain was required to maintain neuroprotection against α-syn-induced dopaminergic neurodegeneration. Both of these SNPs have a minor allelic frequency of $f = 0.02$, indicating that only a small percentage of the general population contains either polymorphism. With only 5–10% of PD cases having a known genetic mutation, these SNPs may indicate susceptibility factors that possibly sensitize individuals to environmental or genetic cues promoting the onset or progression of PD. The molecular effect of these SNPs on hVPS41 may prevent hVPS41 from interacting with the AP-3 complex, recruiting of the HOPS complex, or activating the vacuolar RAB7 to promote lysosomal fusion. Further protein interaction studies will need to be conducted to clarify the molecular mechanism underlying the functional effect of these variants. Likewise, as with the recent *PARK16*/RAB7 finding, it will be interesting to determine whether additional modifying polymorphisms are uncovered within this pathway as disease-associated genomic datasets expand among patient populations.

In this study, we exploited the strengths of both invertebrate and cellular models of PD to functionally analyze the neuroprotective property of the lysosomal trafficking protein hVPS41. Together, these data reveal the cellular mechanism by which hVPS41 is acting to protect neurons from α-syn accumulation and toxicity, and to identify two SNPs in hVPS41 that may enhance the susceptibility of PD pathology within certain populations. Overall, our data support hVPS41 as a potential therapeutic target for treating the progression of PD. This

genetic background of RNAi-resistant *sid-1* mutant animals. The use of neuron-specific RNAi in animals resistant to RNAi in other cell types enables cell-specific depletion of genes that are otherwise lethal to the nematode when knocked down in all cell types. Genetic knockdown of the AP-3 δ-subunit or HOPS complex (VPS-39 and VPS-41) either pan-neuronally or selectively in the dopaminergic neurons significantly enhanced degeneration in worms expressing α-syn. In this manner, the pan-neuronal RNAi strain served to validate the results obtained using the dopaminergic neuron-specific RNAi, and also confirm that overexpression of an additional gene in the dopaminergic neurons does not simply alter the level of α-syn-induced neurodegeneration through decreased transgene expression. These data reveal the importance of Golgi–lysosomal trafficking in regulating cellular toxicity elicited by α-syn overex-

strategy serves as an example by which the experimental paradigm of model systems can be expanded to evaluate functional consequences of human genomic variation and prospective disease-modifying factors.

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