High expression levels of the D686N Parkinson's disease mutation in *VPS35* induces α-synuclein-dependent toxicity in yeast

YI HUANG[1*], XIANG CHEN[1*], XIAOFEI HE[1], CAIFENG GUO[2], XICUI SUN[2], FENGYIN LIANG[1], SIMEI LONG[1], XILIN LU[1], LUYANG FENG[1], WENYUAN GUO[1], YIXUAN ZENG[1] and ZHONG PEI[1]

[1]Department of Neurology, National Key Clinical Department and Key Discipline of Neurology, Guangdong Key Laboratory for Diagnosis and Treatment of Major Neurological Diseases, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510080; [2]Department of Neurology, Guangzhou Huiai Hospital, Guangzhou, Guangdong 510370, P.R. China

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Abstract. Parkinson's disease (PD) is a common neurodegenerative disorder that affects ~2% of the human population aged >65. α-synuclein serves a role in the pathogenesis of PD as it is a primary component of Lewy bodies, a pathological feature of PD. Endosomal-lysosomal dysfunction may be a key factor involved in the pathophysiology of PD, and may cause PD-associated neurodegeneration via α-synuclein-dependent and -independent mechanisms. The D620N mutation in the endosomal-lysosomal gene, vacuolar protein sorting-associated protein 35 (*VPS35*), has been linked to PD. To clarify the underlying cellular mechanism of the *VPS35* D620N mutation in PD, cell growth and endosomal-lysosomal functions were investigated in *Saccharomyces cerevisiae* (sc) yeast cells that exhibited various expression levels of sc*VPS35*, in the presence or absence of non-toxic expression levels of α-synuclein. Overexpression of the sc*VPS35* D686N mutation (the yeast equivalent of D620N) did not lead to toxicity in yeast. However, the co-expression of high copy numbers of sc*VPS35* D686N and low copy numbers of α-synuclein caused toxicity, whereas the co-expression of sc*VPS35* wild-type and α-synuclein did not. In addition, the sc*VPS35* D686N mutant enhanced α-synuclein aggregation. Fragmentation of vacuoles and subsequent inhibition of lysosome function was evident in yeast cells bearing the sc*VPS35* mutant. The results of the present study suggested that α-synuclein and sc*VPS35* were interlinked via the endosomal-lysosome pathway, which is important for the pathogenesis of PD.

Correspondence to: Professor Zhong Pei, Department of Neurology, National Key Clinical Department and Key Discipline of Neurology, Guangdong Key Laboratory for Diagnosis and Treatment of Major Neurological Diseases, The First Affiliated Hospital, Sun Yat-sen University, 58 Zhongshan 2nd Road, Guangzhou, Guangdong 510080, P.R. China
E-mail: peizhong@mail.sysu.edu.cn

*Contributed equally

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Introduction

Parkinson's disease (PD) is the most common neurodegenerative disorder that affects ~2% of the population aged >65. Clinically, PD is characterized by slow movement, rigidity and tremors (1). These classic symptoms are primarily caused by the loss of dopamine (DA) in neurons within the substantia nigra of the brain. The economic burden of PD exceeded $14.4 billion in 2010 in the United States, which equates to ~$22,800 per patient (2); the annual cost to Europe is ~€13.9 billion. At present, there are no effective treatments that prevent or reverse DA degeneration and current therapies primarily aim to alleviate the symptoms. When administered long-term, these treatments may cause serious side effects including dyskinesias, irritability and sleeplessness (3). Thus, extensive studies have investigated the aetiology and physiopathology of PD, to develop novel preventive or therapeutic strategies (4).

The aetiology of PD is complex, as PD may develop from a combination of genetic and environmental factors (5). Genetic PD is relatively rare and represents 10-15% of all PD cases. Determining the underlying genetic mechanisms involved in PD is key in identifying novel targets for the treatment of sporadic PD. Indeed, studies on genetic factors of PD aid understanding of its pathophysiology (6,7). For example, the identification of mutations in the gene encoding human α-synuclein, *SNCA*, led to the finding that α-synuclein aggregates are the primary components of Lewy bodies, a pathological feature of sporadic and genetic PD (8,9). Various causative genes have been identified in rare cases of familial PD that exhibit autosomal dominant or recessive patterns. Patients with recessive forms of PD present with a range of clinical signs and symptoms, including young-onset and onset with dystonia. However, the clinical symptoms of dominant forms of PD are indistinguishable from sporadic PD, suggesting that the underlying molecular signalling pathways are similar. Studies have suggested that the genes involved in the pathogenesis of dominant PD are associated with dysfunction of the endosomal-lysosomal system, which is responsible for synaptic vesicle and receptor recycling and protein degradation (10). Dysfunction of the endosomal-lysosomal system induces PD via the accumulation of α-synuclein (11); however, it may additionally cause PD via

an α-synuclein-independent pathway (12). Previously, it was reported that a point mutation (D620N) in vacuolar protein sorting-associated protein 35 (*VPS35*), a gene that serves a role in the endosomal-lysosomal system, is associated with autosomal dominant PD (13,14). *VPS35* is a subunit of the retromer complex, which serves a role in retrograde transport of cellular proteins from endosomes to the trans-Golgi network (TGN) (15). The *VPS35* D620 N mutation may disrupt cellular functions via endosomal-lysosomal dysfunction, autophagy impairment and mitochondrial dysfunction (16-19). In addition to PD, *VPS35* may be associated with Alzheimer's disease in humans and animals. For example, the expression levels of *VPS35* are reduced in the hippocampi of patients with Alzheimer's disease, and inhibition of *VPS35* resulted in elevated amyloid-β expression levels in animal models (20). Therefore, it remains uncertain how the D620 N mutation in *VPS35* is specifically associated with PD.

α-synuclein serves a role in the pathogenesis of genetic and sporadic PD. As α-synuclein is primarily degraded by the endosomal-lysosome system, its role in *VPS35*-associated PD has been extensively investigated; however, whether α-synuclein is a neuropathological feature of *VPS35*-associated PD remains unclear. Various studies have demonstrated an association between α-synuclein and *VPS35*-linked PD (21,22). For example, loss of *VPS35* led to an increase in α-synuclein accumulation and toxicity, whereas overexpression of *VPS35* reversed this effect (17). However, disruption of endosome-lysosomal function by mutations in PD-associated genes may additionally induce loss of DA and neurodegeneration via α-synuclein-independent mechanisms (22). In human brains from sporadic PD patients and in animal models, *VPS35* is not localised within Lewy bodies and is not associated with α-synuclein. Overexpression of *VPS35* harbouring the D620N mutation in the substantia nigra of rats may induce DA loss without formation of α-synuclein-positive Lewy pathology (21). Collectively, these findings suggested that α-synuclein-independent mechanisms may contribute to the pathogenesis of *VPS35*-associated PD; however, the association between α-synuclein and the D620N mutation within *VPS35* remains to be determined.

As numerous genes are conserved between species, simple model organisms have been utilized as tools for examining the underlying molecular mechanisms of neurodegenerative diseases. The budding yeast, *Saccharomyces cerevisiae* (sc), is a simple organism that has been widely used in the study of neurodegenerative diseases. In yeast models, high expression levels of α-synuclein are associated with key cellular defects implicated in the aetiology of PD, including α-synuclein aggregation, mitochondrial dysfunction and vesicle-trafficking dysfunction (23,24).

To determine the underlying cellular mechanism by which the *VPS35* D620N mutation causes PD, cell growth and endosomal-lysosomal function was examined in yeast cells with various expression levels of sc*VPS35* in the presence or absence of non-toxic levels of α-synuclein. The results of the present study revealed that high expression levels of sc*VPS35* D686N (the yeast equivalent of the human point mutation D620N) were associated with α-synuclein-dependent toxicity in yeast. In addition, sc*VPS35* D686N promoted α-synuclein aggregation whereas wild-type (WT) sc*VPS35* reduced α-synuclein aggregation. Fragmentation of vacuoles and dysfunction of the lysosome may be the potential mechanism underlying the synergistic effects of α-synuclein and sc*VPS35*.

Materials and methods

Yeast strains and plasmid construction. Saccharomyces cerevisiae strains BY4741, BY4743 and Δ*VPS35* were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The Q5® High-Fidelity PCR kit and restriction enzymes below were purchased from New England BioLabs, Inc. (Ipswich, MA, USA). Yeast vectors pRS414, pRS415 and pYES2nt were received as gifts from Dr. Lu Yongjun at the Sun Yat-sen University. The WT α-synuclein sequence (NM_001146055.1) was cloned into the pRS414 yeast vector in the C terminal region using *Spe*I and *Cla*I restriction enzymes. The plasmid contained the GAL1 promoter and an enhanced green fluorescent protein (eGFP) insert. To amplify the WT sc α-synuclein sequence, polymerase chain reaction (PCR) was performed with the following primer pair: Sense, 5'-GGACTAGTATGGATGTATTCATGAAAGG-3' and antisense, 5'-CCATCGATAGCTTCAGGTTCGTAGTCT TG-3'. PCR cycling conditions were as follows: an initial predenaturation step at 98˚C for 1 min, followed by 30 cycles of denaturation at 98˚C for 10 sec, annealing at 65˚C for 20 sec and extension at 72˚C for 30 sec. The sc*VPS35* sequence was amplified via PCR from yeast DNA and inserted into the pRS415 plasmid between *Xba*I and *Not*I restriction sites, and into the pYES2nt plasmid between *Sac*I and *Not*I restriction sites. The primer pair were as follows: sense, 5'-ATGCGG ACTCACC-3' and antisense, 5'-TACATATATGACTTTGAA AC-3'. PCR cycling conditions were as follows: an initial predenaturation step at 98˚C for 1 min, followed by 30 cycles of denaturation at 98˚C for 15 sec, annealing at 68˚C for 20 sec and extension at 72˚C for 1 min. The D686N mutation was generated as previously described (25). In addition, sc*VPS35* mutant sequences were inserted into the GAL1 promoter and fused with the mCherry tag in the C terminal of pRS415 and pYES2nt plasmids in the same manner as with sc*VPS35* WT.

Yeast cells were grown in non-selective medium at 30˚C and transformed as previously described (21). Selective synthetic complete (SC) medium (FunGenome Company, Beijing, China) contained 2% glucose or 2% galactose and lacked the nutrient corresponding to the marker (tryptophan for pRS414 and uracil for pYES2nt).

Spotting tests were conducted to assess growth differences. Yeast cells transfected with plasmids were grown overnight to achieve an optical density (OD) at a wavelength of 600 nm (OD600) of >2.0. Cells were subsequently diluted to mid-log phase (OD600, 0.5) and cultured for 4-6 h to achieve an OD600 of 1.0. Cells were normalized to equal densities, serially diluted 5-fold from an initial OD600 of 0.01, and spotted on plates containing 2% glucose or 2% galactose. Following 2 days incubation at 30˚C, plates were imaged.

Fluorescence microscopy. Yeast cell cultures were grown in SC medium with glucose to reach mid-log phase prior to replacement with medium supplemented with galactose. Cells were visualized using a Leica TCS SP5 II confocal

microscope (Leica Microsystems GmbH, Wetzlar, Germany) at x63 magnification following a specific incubation time.

Cells were stained with the FM® 1-43 lipophilic dye (T-35356; Thermo Fisher Scientific, Inc.) to observe yeast vacuoles, as previously described (11).

For quantification of aggregation, ≥300 cells were counted per strain in each experiment. For each strain, the number of cells that exhibited cytoplasmic foci were reported as the total number of cells with fluorescence and presented as a percentage. Cells exhibiting only bright peripheral halos around the plasma membrane, additional perivacuolar fluorescence or cytoplasmic distribution were not considered to be aggregated and were excluded from the count.

Promoter shut-off studies and drug treatment. Yeast cells were cultured overnight in SC medium with glucose and without tryptophan and uracil, and were subsequently cultured in SC medium containing galactose for 12 h to induce α-synuclein expression. Following pre-incubation in galactose medium, cells were cultured in SC medium with glucose to switch off the promoter for 2 h and observed under a fluorescence microscope. A total of 1 mM phenylmethanesulfonyl fluoride (PMSF) or ethanol (control; the solvent utilized to reconstitute PMSF) were applied to yeast cells when the glucose medium was added.

Statistical analysis. Data are expressed as the mean ± standard error, of three independent experiments. GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analyses. A Brown-Forsythe test was used to confirm that the group variances were statistically equal. A one-way analysis of variance was performed to determine significant differences between groups, followed by a Dunnett's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Overexpression of scVPS35 D686N does not lead to toxicity in yeast. *VPS35* is a highly conserved protein among various species. Mouse, chicken, and zebrafish *VPS35* share ≥90% similarity with humans. In addition, protein sequence alignment revealed that the human *VPS35* sequence is 52% homologous to that of the budding yeast *Saccharomyces cerevisiae*. Notably, the human PD-associated D620N mutation site in *VPS35* is conserved in yeast and corresponds to D686N (Fig. 1A). To avoid possible artificial dominant-negative impact by direct expression of human genes in yeast, the human D620N mutation was mimicked by introducing the analogous mutation D686N to examine the effects on the biological functions in yeast. WT sc*VPS35* was directly cloned from yeast DNA and site 686 was subsequently mutated from aspartic acid (D) to asparagine (N; Fig. 1B).

Cell growth is a key biological function of yeast. To examine the potential role of *VPS35* in cell growth, a spotting assay was performed in yeast strains with or without sc*VPS35* expression. A spotting assay is a simple and effective way to determine the effect of targeted genes on growth. Under the control of the GAL1 inducible promoter, medium with galactose significantly increases target gene expression, whereas medium containing glucose represses gene expression. Therefore, the effect of targeted genes on growth may be evaluated by assessing the density of yeast in galactose medium. Under this system, yeast strains with different expression levels of sc*VPS35*, including a *VPS35* deleted strain (Δ*VPS35*), in addition to BY4741 and BY4743 strains, were utilized to determine whether sc*VPS35* is essential for cell growth. Δ*VPS35*, BY4741 and BY4743 strains contained zero, one or two copies of sc*VPS35*, respectively. The cell density was similar in these three strains (data not shown). Therefore, endogenous sc*VPS35* may not be essential for cell growth. To examine the effect of the sc*VPS35* D686N mutation on cell growth, sc*VPS35* WT and sc*VPS35* containing the D686N mutant were cloned into the low-copy plasmid pRS415 and transfected into the yeast strains. Yeast with low expression levels of the sc*VPS35* WT or D686N mutant exhibited similar growth to the pRS415 control vector, following incubation with repression and induction medium in BY4741 and BY4743 strains (Fig. 1C and D). A high-copy plasmid, pYES2nt, was utilized to address the potential threshold expression levels of the sc*VPS35* D686N mutant. As observed with the low-copy plasmid, high expression levels of the sc*VPS35* WT and D686N mutant were equally as toxic in yeast strains as the control (Fig. 1E, F and G). Therefore, this data suggested that *VPS35* D686N alone is not sufficient for inhibition of growth, even at relatively high expression levels.

High levels of scVPS35 D686N mutant induce toxicity in an α-synuclein-dependent manner. α-synuclein has a central role in the pathogenesis of PD. Clinical and experimental studies have suggested that overexpression of WT α-synuclein may lead to cell toxicity in a dose-dependent manner (26). As high expression of α-synuclein alone may lead to cell toxicity, α-synuclein was expressed in yeast at non-toxic levels to determine whether the interaction of *VPS35* with physiological levels of α-synuclein induces toxicity. Consistent with previous studies (17,27), low expression levels of WT α-synuclein in yeast were not toxic compared with the pRS415 vector control (Fig. 2A). Co-expression of sc*VPS35* WT or D686N at low copy numbers with α-synuclein did not affect cell growth compared with the vector control (Fig. 2A). sc*VPS35* WT and D686N were subsequently cloned into the pYES2nt plasmid to enhance their expression levels. High copy numbers of sc*VPS35* D686N or sc*VPS35* WT were co-expressed with low copy numbers of α-synuclein. Under induction, yeast cells showed relatively lower growth rate due to carrying more protein compared with the control. However, the interaction of sc*VPS35* D686N with α-synuclein suppressed cell growth significantly, compared with sc*VPS35* WT (Fig. 2B). Collectively, this data suggested that the negative effect of the *VPS35* mutant on yeast cell growth is dose- and α-synuclein-dependent.

scVPS35 D686N promotes fragmentation of vacuoles in yeast. The yeast vacuole is the equivalent of the mammalian lysosome and serves numerous crucial functions in maintaining cellular homeostasis, including protein degradation, removal of harmful molecules and storage of organic molecules. To determine whether the *VPS35* mutant impairs vacuolar function, the distribution of sc*VPS35* in a pYES2nt plasmid tagged with the mCherry fluorescent protein, was investigated in BY4741 cells.

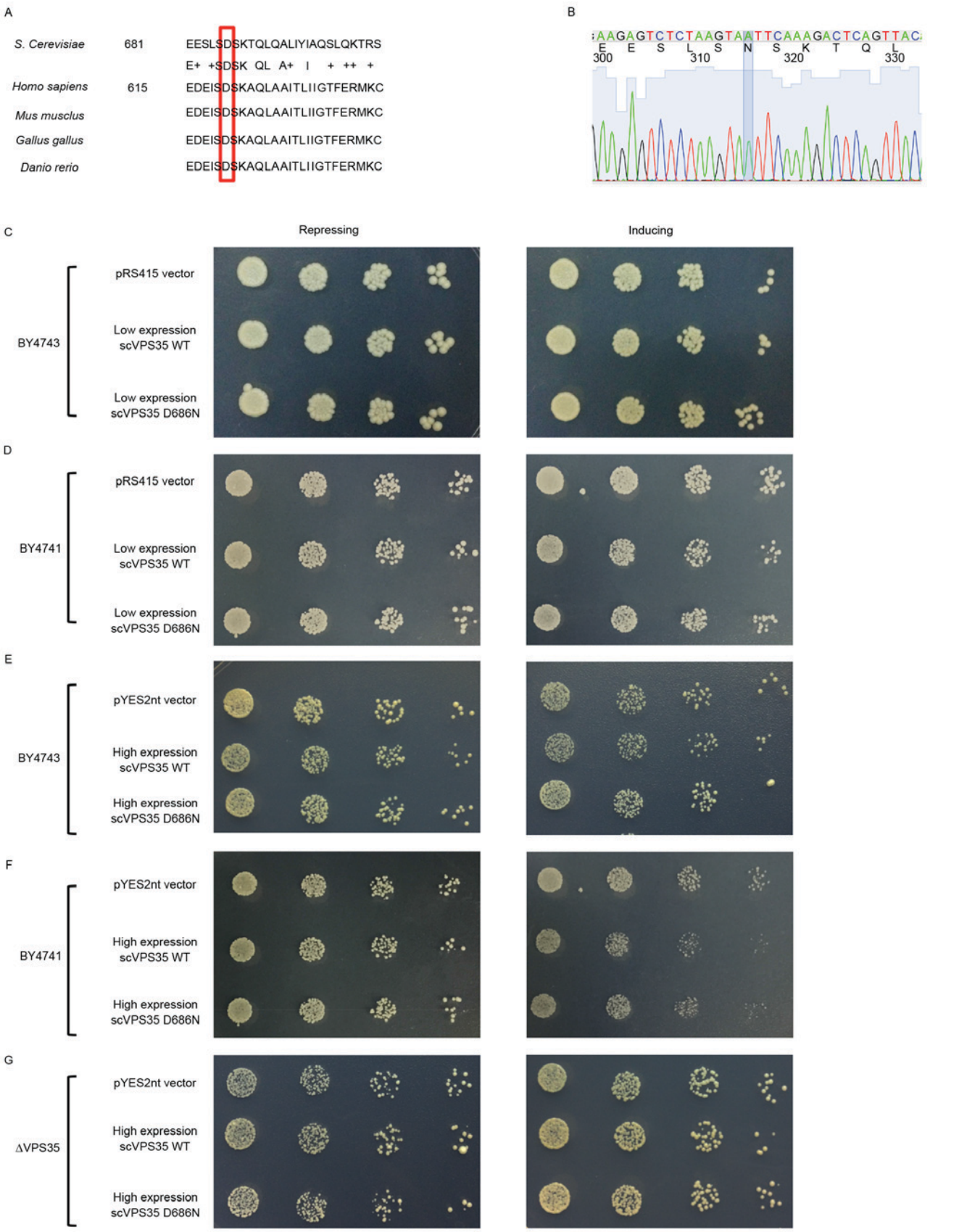

Figure 1. Overexpression of wild type or mutant sc*VPS35* does not inhibit yeast growth. (A) Schematic representation of the conserved region of the *VPS35* sequence in various species. (B) Sequencing results of the sc*VPS35* mutation. The blue frame demonstrated the base pair that had been successfully replaced, resulting in an amino acid mutation from aspartic acid to asparagine. The growth of (C) BY4743 (diploid) and (D) BY4741 (haploid) yeast strains that contained low copy numbers of sc*VPS35* WT or sc*VPS35* D686N were not significantly different compared with cells expressing the pRS415 vector alone, as determined by spotting assays. The growth of (E) BY4743, (F) BY4741 and (G) Δ*VPS35* yeast strains were assessed by spotting assays following transfection with high copy numbers of the pYES2nt plasmid vector that contained sc*VPS35* WT or sc*VPS35* D686N. Five-fold serial dilutions starting with equal numbers of cells (optical density 600=0.01) were spotted in medium containing glucose (repressing) or galactose (inducing). sc*VPS35*, *Saccharomyces cerevisiae* vacuolar protein sorting-associated protein 35; WT, wild-type.

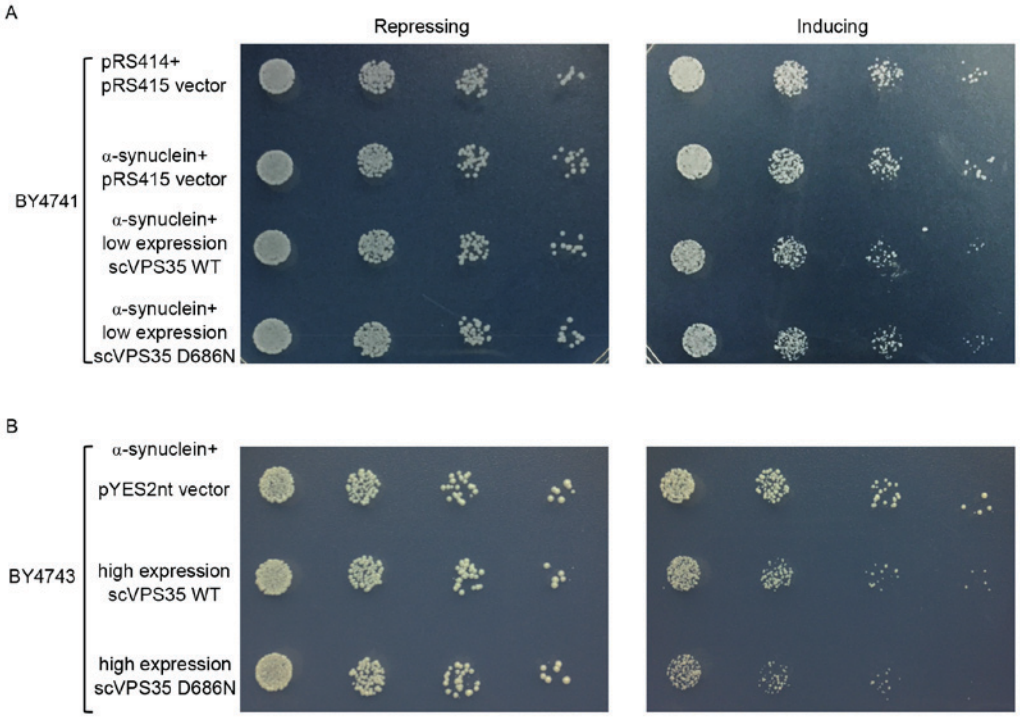

Figure 2. Co-expression of α-synuclein and sc*VPS35* in yeast cells. (A) Spotting assays were performed to compare the effect on growth following overexpression of α-synuclein and sc*VPS35* WT or sc*VPS35* D686N. Growth in BY4741 cells transfected with low copy numbers of α-synuclein and sc*VPS35* WT or sc*VPS35* D686N, was not significantly different from the growth in cells treated with the pRS415 vector alone. (B) Toxicity was greater in BY4743 cells transfected with low copies of α-synuclein and high copies of sc*VPS35* D686N, compared with the pYES2nt vector alone and cells transfected with α-synuclein and sc*VPS35* WT. Five-fold serial dilutions starting with equal numbers of cells (optical density 600=0.01) were spotted in medium containing glucose (repressing) or galactose (inducing). sc*VPS35*, *Saccharomyces cerevisiae* vacuolar protein sorting-associated protein 35; WT, wild-type.

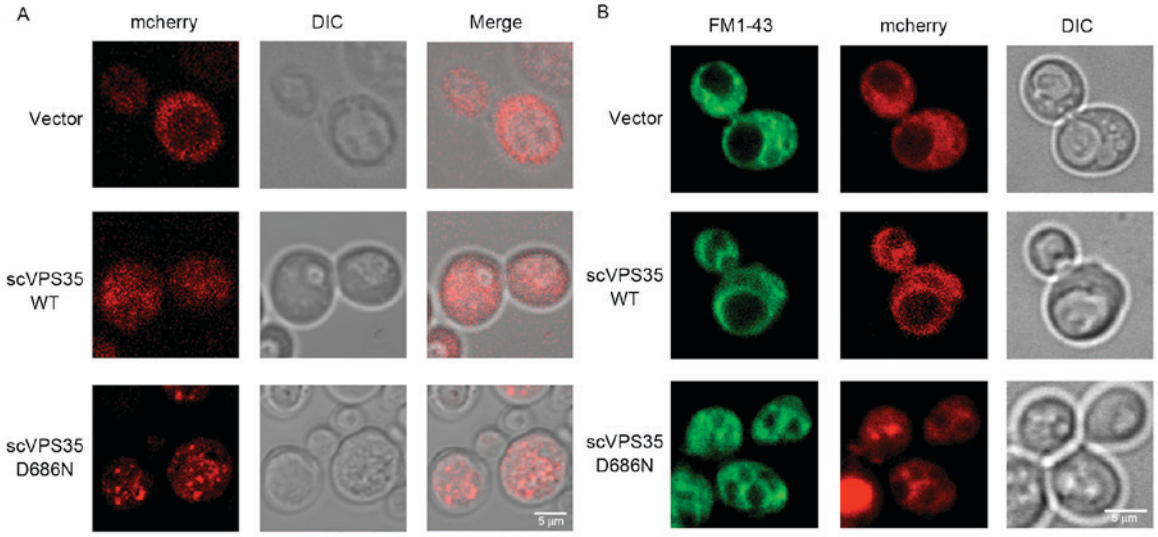

Figure 3. Morphology of vacuoles in yeast cells overexpressing sc*VPS35* WT or sc*VPS35* D686N. (A) BY4741 yeast cells were seeded in medium containing glucose and lacking uracil to mid-log phase and subsequently induced in galactose-containing medium. Cells were examined under a fluorescence microscope with a x63 objective lens following 4 h of induction. mCherry-expressing red fluorescent cells were utilized as a control. pYES2nt plasmids containing N-terminally mCherry-tagged sc*VPS35* WT or sc*VPS35* D686N were transfected into cells. Cells expressing sc*VPS35* D686N demonstrated small puncta throughout cytoplasm, whereas cells containing the vector alone or sc*VPS35* WT did not. (B) Fluorescence microscopy of yeast cells expressing the mCherry control, pYES2nt-mCherry-sc*VPS35* WT and pYES2nt-mCherry-sc*VPS35* D686N. Following induction in galactose-containing medium for 12 h, yeast vacuoles were stained with green fluorescent FM® 1-43 and morphology was examined. Vacuoles fragmented into multiple compartments in cells transfected with sc*VPS35* D686N. sc*VPS35*, *Saccharomyces cerevisiae* vacuolar protein sorting-associated protein 35; WT, wild type.

VPS35 is involved in vesicle trafficking. Following a short induction time of 4 h, sc*VPS35* WT was distributed uniformly throughout the cytoplasm with very few small puncta, which was comparable to the mCherry vector control. By contrast, sc*VPS35* D686N exhibited large puncta, which is a characteristic of a deficit in vesicle formation (Fig. 3A). This was

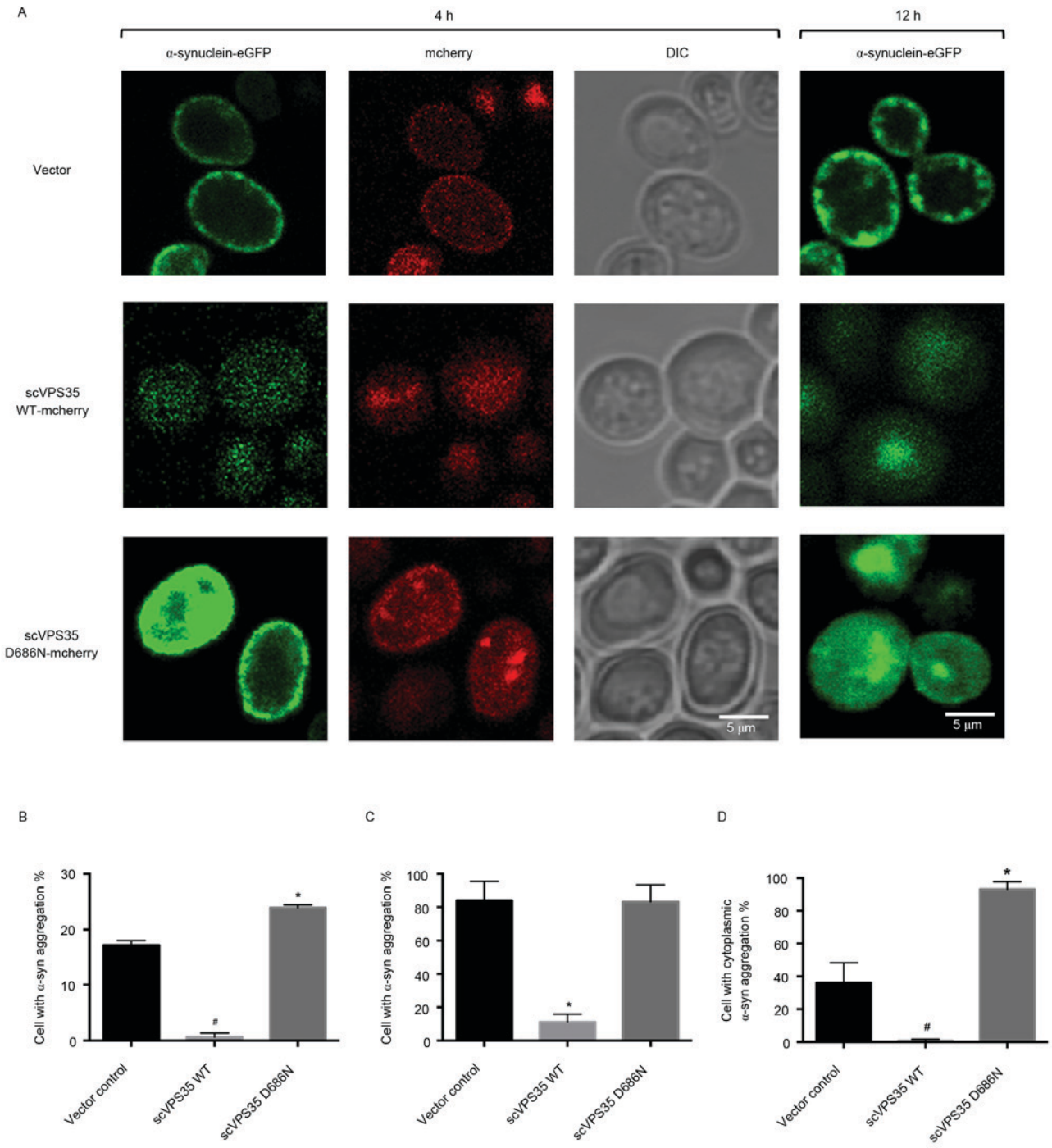

Figure 4. Fluorescence microscopy of yeast cells co-expressing α-synuclein and sc*VPS35*. (A) At low copy numbers, α-synuclein fused with eGFP co-expressed with high copy numbers of sc*VPS35* fused with mCherry in BY4741. Images were captured following 4 or 12 h induction with galactose, with a x63 objective lens under a live-cell fluorescence microscope. Expression levels of α-synuclein were reduced in cells expressing sc*VPS35* WT and enhanced in cells expressing sc*VPS35* D686N, compared with the vector control. (B) Following 4 h of induction, the number of cells exhibiting aggregates were normalised to the total number of cells with fluorescence and calculated as a percentage. (C) Following 12 h induction, α-synuclein aggregates were counted in yeast cells. Cells transfected with α-synuclein and the vector or sc*VPS35* D686N exhibited a similar percentage of cells bearing aggregates following 12 h induction. (D) Following 12 h of induction, the number of cells exhibiting cytoplasmic foci was normalised to the total number of cells and calculated as a percentage. Expression of sc*VPS35* D686N enhanced the percentage of cytoplasmic α-synuclein compared with the vector control. The total number of cells counted was >300 and values represented mean ± standard error. *P<0.05 and $^{\#}$P<0.05 vs. vector control. eGFP, enhanced green fluorescence protein; sc*VPS35*, *Saccharomyces cerevisiae* vacuolar protein sorting-associated protein 35; WT, wild-type.

consistent with the characteristics of *VPS35* in mammalian cells. As vesicle formation is the first step of vesicle trafficking, the results of the present study suggested that overexpression of sc*VPS35* D686N disrupts vesicle trafficking.

To investigate the impact of sc*VPS35* D686N on the lysosome, the expression of *VPS35* was extended to 12 h and FM 1-43 lipophilic dye was utilized to visualize the morphology of yeast vacuoles. The vacuole is a dynamic organelle whose

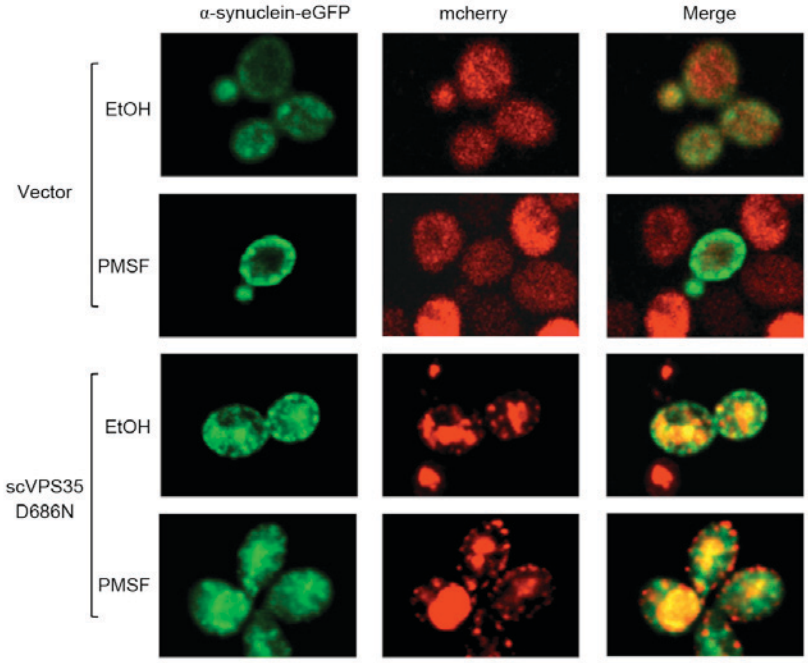

Figure 5. Effect of sc*VPS35* D686N expression on clearance of α-synuclein aggregation following promoter shut-off with PMSF. Following 4 h pre-incubation with galactose, cells expressing α-synuclein or co-expressing α-synuclein and sc*VPS35* D686N were cultured in glucose medium supplemented with 1 mM PMSF dissolved in EtOH, or medium containing EtOH as a control. Following promoter shut off with or without PMSF treatment for 2 h, the expression of α-synuclein in yeast cells was measured under a fluorescent microscope. sc*VPS35*, *Saccharomyces cerevisiae* vacuolar protein sorting-associated protein 35; PMSF, phenylmethanesulfonyl fluoride; eGFP, enhanced green fluorescence protein; EtOH, ethanol.

morphology is responsive to varying intracellular conditions. Normal cells contain one to two vacuoles with medium-sized lobes. The morphology of vacuoles in cells treated with the sc*VPS35* WT plasmid was comparable to that of the vector control. However, in yeast cells expressing sc*VPS35* D686N, vacuoles fragmented into multiple compartments, suggesting that the cells were undergoing osmotic stress (Fig. 3B). The alteration in size and compartment number corresponded to dysfunction of vacuoles and an imbalance of the intracellular environment, which suggested that sc*VPS35* D68N overexpression causes defects in the lysosome system.

α-synuclein aggregation is enhanced by expression of scVPS35 WT and is reduced by expression of scVPS35 WT. Tagging sc*VPS35* and α-synuclein with the fluorescent proteins mCherry and eGFP allowed visualization of their interaction via fluorescent microscopy. Following 4 h of induction, α-synuclein was localised primarily to the plasma membrane in vector control cells with limited aggregation. Transfection of cells with WT sc*VPS35* resulted in reduced expression levels of α-synuclein, which was uniformly distributed. By contrast, expression of sc*VPS35* D686N resulted in enhanced expression levels of α-synuclein, which was localised to the plasma membrane (Fig. 4A). In addition, the percentage of α-synuclein aggregation was greater in cells expressing sc*VPS35* D686N compared with the vector control (23.88±0.52 vs. 17.14±0.89%; P<0.05; Fig. 4B). Induction for 12 h resulted in formation of α-synuclein aggregates at the membrane in control cells, and larger α-synuclein aggregates in the cytoplasm of yeast cells expressing sc*VPS35* D686N. However, cells transfected with sc*VPS35* WT had reduced expression levels of α-synuclein compared with the control. Quantification analysis revealed that the percentage of control cells bearing aggregates was not significantly different to cells expressing sc*VPS35* D686N (84.03±11.47 vs. 83.30±10.01%; Fig. 4C). However, compared with the control, the percentage of cells containing cytoplasmic aggregates was significantly enhanced following expression of sc*VPS35* D686N (35.98±12.45 vs. 93.30±4.40%; P<0.05; Fig. 4D). These results suggested that sc*VPS35* WT reduces α-synuclein aggregation and the sc*VPS35* D686N mutant enhances α-synuclein aggregation.

scVPS35 D686N mutant increases α-synuclein aggregation in a lysosome-dependent manner. As the lysosome serves a key role in the clearance of α-synuclein aggregation, it was hypothesized that the sc*VPS35* mutant may interfere with the degradation of α-synuclein via the lysosome system. The GAL1 promoter shut-off system is an effective tool to evaluate the efficiency of protein clearance, and enables investigation of α-synuclein clearance and the effect of sc*VPS35* on cytoplasmic fluorescent foci. Promoter shut-off was achieved by culturing cells in glucose-containing medium. Following 12 h of pre-incubation in galactose-containing medium, cells harbouring α-synuclein-eGFP exhibited aggregates. Promoter shut-off for 2 h resulted in a significant reduction in aggregate formation. The vacuolar protease inhibitor PMSF was utilized to investigate α-synuclein aggregation in yeast cells expressing the WT or mutant *VPS35*. PMSF blocked the lysosome system and clearance of α-synuclein aggregation was prevented following promoter shut-off. Aggregation was primarily localized to the plasma membrane; however, aggregates were additionally localized to cytoplasmic vesicular inclusions. The fluorescence intensity of α-synuclein was maintained;

however, there were fewer aggregated and co-localized vacuoles in the cytoplasm of yeast cells expressing sc*VPS35* D686N following PMSF treatment compared with cells treated with ethanol (Fig. 5). The alteration in the distribution of aggregated α-synuclein in the cell suggested that scVSP35 D686N may affect clearance of α-synuclein prior to the lysosome, and direct inhibition of lysosome function by PMSF may lead to accumulation of α-synuclein in the cytoplasm. Therefore, the data of the present study suggested that impaired endosome-to-Golgi retrieval of proteins may contribute to VSP35 D686N mutant-mediated α-synuclein aggregation.

Discussion

In the present study, the role of the *VPS35* D686N mutant was examined in yeast. It was demonstrated that deletion of *VPS35,* and expression of WT or mutant *VPS35,* did not suppress cell growth. Therefore, *VPS35* may not be necessary to maintain yeast homeostasis. However, when high expression levels of *VPS35* D686N were combined with non-toxic levels of α-synuclein, cell growth was inhibited, suggesting that α-synuclein is involved in *VPS35*-associated PD. In addition, vacuoles, which are the equivalents of lysosomes in mammalian cells, were compromised in cells expressing *VPS35* D686N, and α-synuclein aggregation was evident in yeast cells expressing high levels of *VPS35* D686N and low levels of α-synuclein. Together with previous studies (17,28), the data of the present study supported the hypothesis that impairment of lysosomal degradation of α-synuclein is responsible for the phenotypes in *VPS35*-associated PD.

The endosome is a component of the endocytic membrane transport pathway that primarily mediates cellular transport of endocytic proteins. Once proteins enter into endocytic system, they are transferred to lysosomes for degradation or recycled back to the plasma membrane for reuse. There are two primary pathways for the reuse of endocytic proteins, including the recycling endosome-mediated endosome-to-plasma membrane pathway and the endosome-to-TGN retrograde pathway. In the TGN pathway, the retromer serves a critical role in shifting proteins from endosomes back to the TGN, resulting in escape of lysosomal degradation. *VPS35* is a subunit of the retromer complex and functions to sort cellular cargo within the retromer complex (29). Although mutations in *VPS35* have been associated with a late-onset, autosomal dominant form of PD, the underlying mechanism by which *VPS35* mutants contribute to PD is not fully understood. Loss and gain of function have been proposed to be involved in the pathogenesis of *VPS35*-associated PD. As genetic manipulation in yeast is relatively simple, the present study generated yeast cells with varying expression levels of *VPS35*, and the subsequent effect on cell growth was investigated. In agreement with previous studies, deletion of sc*VPS35* and varying expression levels of WT sc*VPS35* did not inhibit cell growth (21,27). This suggested that *VPS35* may not be involved in the maintenance of yeast homeostasis. Similarly, varying the expression levels of the *VPS35* mutant did not affect cell growth. However, large puncta, a sign of a compromised lysosome, was detected in cells transfected with the mutant *VPS35*, indicating that the *VPS35* mutant may induce lysosome dysfunction. However, the non-toxic effect of the *VPS35* mutant observed in the present study was not in agreement with previous reports, which demonstrated that the *VPS35* mutant induced toxicity (30,31). This discrepancy may be due to the yeast model expressing insufficient levels of mutant *VPS35* to induce toxicity. An alternative explanation may be the lack of endogenous α-synuclein in yeast cells. In addition, as α-synuclein is degraded by the endosome-lysosome pathway, accumulation of α-synuclein may impair this pathway. Therefore, the interaction with α-synuclein may be necessary for other cellular pathways, which may synergistically lead to neurodegeneration.

Endogenous expression levels of the α-synuclein gene and protein are associated with the disease phenotype, and have been investigated in yeast (11). Consistent with clinical observations (17,32), the present study demonstrated that at low to moderate expression levels, α-synuclein was localized to the membrane and did not induce toxicity, whereas at high levels, α-synuclein formed protein aggregation and caused toxicity. Previous studies have demonstrated that *VPS35* deficiency induces toxicity, whereas overexpression of WT *VPS35* reduces α-synuclein toxicity (18), suggesting that α-synuclein is involved in *VPS35*-associated PD. However, the majority of studies were conducted in animals with high expression levels of α-synuclein, and it is important to assess the impact of the *VPS35* mutant in models that express α-synuclein at low levels, which corresponds with physiological endogenous expression in sporadic PD. As the majority of sporadic PD patients do not exhibit point mutations in genes that are associated with high expression of α-synuclein, yeast strains expressing low levels of α-synuclein were utilized to investigate the effect of the interaction between *VPS35* and a physiological level of α-synuclein. The results of the present study revealed that the interaction between low expression levels of α-synuclein and high expression levels of the *VPS35* mutant impaired cell growth. Additionally, α-synuclein aggregation was evident. This suggested that high expression levels of the *VPS35* mutant may be required to trigger α-synuclein-dependent toxicity. However, whether this is the case in PD patients requires further investigation. Notably, α-synuclein was less aggregated in cells transfected with WT *VPS35*, and high expression levels of WT *VPS35* did not affect the morphology of the lysosome. These results suggested that overexpression of WT *VPS35* may have a protective function in α-synuclein pathology.

As high expression levels of the sc*VPS35* mutant caused morphological alteration of lysosomes, the effect of the sc*VPS35* mutant on clearance of α-synuclein was investigated via a GAL1 promoter shut-off system. Under control of the shut-off system, α-synuclein accumulation was evident in sc*VPS35* mutant yeast cells; however, this was not observed in sc*VPS35* WT yeast cells, suggesting that the *VPS35* mutant impaired the degradation of α-synuclein via the endosome-lysosome pathway.

In conclusion, the present data suggested that a high expression level of the analogous PD mutation D686N in *VPS35* disrupted α-synuclein homeostasis in yeast. These results, in addition to previous studies (21,27), suggested that a mutual mechanism may be responsible for the interaction between α-synuclein and mutant *VPS35*. However, the *VPS35* mutant may negatively modulate the clearance of α-synuclein and trigger toxicity; accumulation of α-synuclein may impair

vacuolar function and vesicular trafficking to exacerbate *VPS35* mutant-mediated cell sensitivity. Collectively, the data suggested that α-synuclein and scVPS35 is interlinked via the endosomal-lysosome pathway. The present study thus implicates endosome-lysosome as a promising therapeutic target for PD.

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