The mutations of F-box protein 7 (FBXO7) gene (T22M, R378G and R498X) are associated with a severe form of autosomal recessive juvenile-onset Parkinson’s disease (PD) (PARK 15). Here we demonstrated that wild-type (WT) FBXO7 is a stress response protein and it can play both cytoprotective and neurotoxic roles. The WT FBXO7 protein is vital to cell mitophagy and can facilitate mitophagy to protect cells, whereas mutant FBXO7 inhibits mitophagy. Upon stress, the endogenous WT FBXO7 gets up-regulated, concentrates into mitochondria and forms FBXO7 aggregates in mitochondria. However, FBXO7 mutations aggravate deleterious FBXO7 aggregation in mitochondria. The FBXO7 aggregation and toxicity can be alleviated by Proline, glutathione (GSH) and coenzyme Q10, whereas deleterious FBXO7 aggregation in mitochondria can be aggravated by prohibitin 1 (PHB1), a mitochondrial protease inhibitor. The overexpression of WT FBXO7 could lead to FBXO7 protein aggregation and dopamine neuron degeneration in transgenic Drosophila heads. The elevated FBXO7 expression and aggregation were identified in human fibroblast cells from PD patients. FBXO7 can also form aggregates in brains of PD and Alzheimer’s disease. Our study provides novel pathophysiologic insights and suggests that FBXO7 may be a potential therapeutic target in FBXO7-linked neuron degeneration in PD.

Introduction

Parkinson’s disease (PD) is a chronic and progressive disease that is caused primarily by the loss of dopaminergic neurons in the substantia nigra (SN) (1–3). The mechanisms underlying the selective and progressive loss of nigral dopaminergic neurons in PD have not been fully elucidated. Although most individuals suffer from sporadic PD, about 10–15% of PD patients have inherited disease (familial form of PD) (4). Variants or mutations of at least six PD-related genes [such as α-synuclein (α-syn) and PAR-KIN] have been implicated in the pathogenesis of familial PD (5–11). Recently, mutations in the F-box protein 7 gene (FBXO7) have been associated with a severe form of autosomal recessive
juvenile-onset levodopa-responsive parkinsonism (PARK15) with rapid progressive parkinsonism and pyramidal signs (12,13). At present, at least 18 PARK15 patients from 5 families have been reported to carry FBXO7 mutations that include a homozygous nonsense mutation (R498X), a compound heterozygous mutation (T22M) (12) and a homozygous R378G mutation (14). Besides rigidity and bradykinnesia, other features include dementia or cognitive decline, abnormal eye movement, gait unsteadiness and early posture imbalance (13). The wide clinical spectrum associated with FBXO7 mutations suggests that FBXO7 mutation-induced pathological alterations are unlikely to be restricted only to dopaminergic neurons in SN.

FBXO7 is a member of F-box-containing protein family defined by the presence of an F-box domain, which binds with Skp1 protein (15,16). Through this interaction, F-box proteins are recruited as components of Skp-Cullin-F-box complex, an E3 ubiquitin ligase (17–19). FBXO7 is mainly expressed in the cerebral cortex, globus pallidum and SN, and to a lesser extent in the hippocampus and cerebellum (20). FBXO7 has been reported to catalyze the ubiquitination of HURP/DLG7, a regulator of mitotic spindle assembly, marking the protein for degradation by the proteasome (21). FBXO7 also promotes ubiquitination of clA1, an inhibitor of apoptosis family member that regulates canonical and non-canonical NF-κB signaling (22). Overexpression of FBXO7 in immortalized fibroblasts augments the levels of Cdk6 bound to D-type cyclins and led to transformation (23,24).

There is limited information on the pathophysiological mechanism of FBXO7-linked neuronal degeneration in PD. A recent study found alterations of subcellular localization and protein expression levels of endogenous FBXO7 proteins in lymphoblastoids and fibroblasts of PARK15 patients with FBXO7 mutations (20). Furthermore, mutant FBXO7 protein has decreased stability compared with wild-type (WT) FBXO7 (20). Another recent study reported that FBXO7 might be involved in mitochondrial quality maintenance via interaction with PINK1 and PARKIN (25). However, many questions regarding FBXO7 mutations in PD remain unanswered. In particular, it is still unclear if WT FBXO7 has neuroprotective functions and how FBXO7 mutations contribute to neuronal degeneration in PD. To address these knowledge gaps related to FBXO7, here we allude for the first time that FBXO7 is a stress response protein. The WT FBXO7 has paradoxical protective and toxic roles. However, PD-linked FBXO7 mutations lead to loss of protective functions and aggravation of toxic effects of WT FBXO7. The PD-related FBXO7 mutants can aggravate protein aggregates in mitochondria, impair mitochondrial function, promote reactive oxygen species (ROS) production and decrease cell viability. Our findings suggest that FBXO7 may be a potential therapeutic target in FBXO7-linked neuron degeneration in PD.

Results

WT FBXO7 have cytoprotective functions, while PD-linked FBXO7 mutants are toxic

Knocking down of endogenous WT FBXO7 in dopaminergic PC12 cells by FBXO7 shRNA transfection led to decreased PC12 cell viability even in the absence of stress (Fig. 1A and B). Similarly, knocking down of FBXO7 expression in HEK cells can impair HEK cell viability in the absence of stress and sensitized cells to H₂O₂-induced toxicity (Fig. 1C–E). Next, we transfected human HEK (Fig. 1F) and dopaminergic neuronal SK-N-SH cells (Fig. 1H) with GFP-tagged human isoform1 (WT1), isoform 2 (WT2) WT and 3 FBXO7 mutants (T22M, R378G and R498X). The overexpressed WT2 and T22M mutant FBXO7 in HEK cells showed decreased protein levels compared with WT1 and other FBXO7 mutants (Fig. 1F and G). WT1, WT2, R378G and R498X FBXO7 proteins localized mainly in nucleus (Fig. 1H and I). However, T22M FBXO7 localized primarily in cytoplasm (Fig. 1H and I). WT FBXO7 can protect against DA or H₂O₂ induced toxicity, whereas mutant FBXO7 protein aggravated stress-induced toxicity (Fig. 1J and K).

Mutations of FBXO7 promote deleterious aggregation of FBXO7 proteins

We found that overexpressed WT and mutant FBXO7 in human dopaminergic SK-N-SH cells could form FBXO7 protein aggregates (insoluble FBXO7 proteins) (Fig. 2A and B). Mutant FBXO7 formed more aggregates than WT FBXO7 (Fig. 2A–G). Most of FBXO7 aggregates were observed in cytoplasm (Fig. 2B). The FBXO7 protein aggregate formation was time- and stress-dependent (Fig. 2A, E–G). Stressing of cells significantly promoted FBXO7 aggregation (Fig. 2F). Furthermore, FBXO7 aggregation impaired cell viability, especially when transfected with mutant FBXO7 (Fig. 2H and I). However, FBXO7 aggregation- and FBXO7 overexpression-induced cell viability impairment can be alleviated by glutathione (GSH) and Proline treatments (Fig. 2G, H, L and M). The Proline is not a reductant but reported to have protein aggregation inhibiting capacity, which is different from GSH (Fig. S2) (30,31). On the contrary, the FBXO7 aggregation can be aggravated by MG132 proteasome inhibition, but not by 3-MA autophagy inhibition (Fig. 2L). The mono-ubiquitination of FBXO7 promotes FBXO7 aggregation significantly, which corroborated a recent report that mono-ubiquitination of α-syn promotes aggregate formation of α-syn (Fig. 2K) (32).

FBXO7 mutants promoted aggregation of WT FBXO7 and WT PARKIN (Fig. 2O and P). Cells were co-transfected with Myc-tagged WT1 FBXO7 together with GFP-tagged WT1 FBXO7 or mutant R378G FBXO7. We showed that GFP-tagged mutant R378G FBXO7 promoted aggregation of Myc-tagged WT1 FBXO7 (Fig. 2O). However, GFP-tagged WT1 FBXO7 was unable to affect aggregation of Myc-tagged WT1 FBXO7 (Fig. 2O). Similarly, GFP-tagged mutant R378G FBXO7 also promoted aggregate formation of WT PARKIN (Fig. 2P).

Neurotoxins induced aggregate formation of endogenous WT FBXO7

Next, we studied the aggregation of endogenous WT FBXO7 in human dopaminergic neuronal SK-N-SH cells and mice primary neurons (Fig. 3). We found that challenging dopaminergic SK-N-SH cells with DA, Rotenone and H₂O₂ could induce dose- and time-dependent FBXO7 aggregation (Fig. 3A–H). Conversely, the amount of soluble WT FBXO7 in cells decreased accordingly (Fig. 3A–H). Stress-induced endogenous FBXO7 aggregate formation could also be observed in HEK cells (Supplementary Material, Fig. S1). However, quantitative real-time RT–PCR analysis demonstrated that expression of FBXO7 increased with time after stress challenges (Fig. 3I). In HEK and dopaminergic SHSY-5Y cells, the stress-induced up-regulation of FBXO7 expression levels was similar to those of FBXO7 transfection-induced alternations (Supplementary Material, Fig. S2). Both mice and human FBXO7 proteins formed aggregates after H₂O₂ challenge (Fig. 3I). The protein aggregates of mice and human FBXO7 co-localized with β-tubulin near nucleus (Fig. 3I). This indicated the localization of FBXO7 aggregate in aggresomes, suggesting the attempt by the cells to clear WT FBXO7 aggregate via aggresome-autophagy pathway.
Figure 1. FBXO7 proteins’ subcellular localization and impacts on cell viability. (A) Knock-down of FBXO7 expression impairs cell viability of mice dopaminergic PC12 cells. Cells were transfected with triple scramble shRNA or triple GIPZ mouse FBXO7 shRNA plasmids endowed with GFP expression marker [V3LMM-48860 (1), V3LMM-48863 (2), V3LMM-519091 (3) and V3LMM-519092 (4)] and cultured for 24 or 48 h, respectively. Cell viability was measured by MTT assay, * at least \( P < 0.01 \), compared with the cell viability of cells transfected with scramble shRNA. #, \( P < 0.05 \), compared with the cell viability of cells transfected with same set FBXO7 shRNA for 24 h. (B) Representative confocal image of cells with positive GFP fluorescence after PC12 cells has been transfected with GIPZ FBXO7 shRNA vectors (1 + 3 + 4) (magnification: 200×). (C) Knock-down of FBXO7 expression impairs HEK cell viability in the presence or absence of 20 µM H$_2$O$_2$. *, at least \( P < 0.01 \), compared with the cell viability of cells transfected with the same set of shRNA without H$_2$O$_2$ challenge. #, \( P < 0.05 \), compared with the cell viability of scramble shRNA transfected cells challenged with the same concentration of H$_2$O$_2$. (D) Two replicated western blot data of knocking down FBXO7 by human FBXO7 shRNA (triple scramble shRNA or human FBXO7 shRNA vectors [TRCN0000004338 (38), TRCN0000004339 (39), TRCN0000004340 (40), TRCN0000004341 (41)]). (E) Densitometric analysis of FBXO7 protein bands in western blot gels as in (D), *, \( P < 0.05 \), compared with FBXO7 protein bands of scramble shRNA transfected cells. (F) Representative western blot data of over-expressed GFP-tagged WT isoform 1 (WT1), WT isoform 2 (WT2), mutant R498X, R378G and T22M FBXO7 proteins in HEK cells after overnight transfection. (G) Fluorescent spectrophotometer analysis of expression levels of transfected GFP-tagged WT and mutant FBXO7 proteins in HEK cells, *, at least \( P < 0.01 \), compared with the fluorescent density of cells transfected with GFP. #, at least \( P < 0.05 \), compared with the fluorescent density of cells transfected with GFP-tagged WT1 FBXO7. (H) Human dopaminergic neuronal SK-N-SH cells were transfected with GFP-tagged FBXO7 WT and mutant FBXO7 proteins. Cells were stained with Hoescht 33342 and observed under confocal fluorescence microscopy (magnification: 600×). (I) Subcellular distribution of WT and mutant FBXO7 proteins in cells after transfection. N=C, indicates mainly nuclear localization of FBXO7 proteins. N>C, indicates almost equal distribution of FBXO7 proteins in nucleus and cytoplasm. N<C, indicates mainly cytoplasmic FBXO7 proteins localization. *, at least \( P < 0.05 \), compared with the percentage of cells transfected with WT1 FBXO7. (J) and (K) Influence on cell viability by overexpressed WT and mutant FBXO7 proteins under DA (J) or H$_2$O$_2$ (K) challenges. *, \( P < 0.05 \), compared with the cell viability of GFP-transfected cells under the same DA or H$_2$O$_2$ treatment conditions respectively.
Figure 2. Deleterious protein aggregate formation of GFP-tagged WT and mutant FBXO7 proteins in cells. (A) Confocal fluorescent microscopy images of GFP-tagged WT and mutant FBXO7 protein aggregates in human dopaminergic SKN-SH cells over time (1 or 3 days after transfection). (B) Subcellular distribution of GFP-tagged WT and mutant FBXO7 protein aggregates after overnight transfection, based on fluorescent microscopy observation. (C and D) Representative western blot data of soluble and insoluble (aggregate) fractions of GFP-tagged WT and mutant FBXO7 proteins (C) and GFP protein (D) after overnight transfection. ‘So’, refers to soluble parts; ‘In’, refers to insoluble parts. (E) Time-dependent aggregate formation of GFP-tagged FBXO7 proteins in HEK cells. * and #, at least P < 0.01, compared with the percentage of cells containing FBXO7 aggregates transfected with the same FBXO7 proteins (WT or mutant, respectively) and cultured for 24 h (*) or 48 h (#). (F) Aggregate formation of GFP-tagged WT and mutant FBXO7 proteins after overnight treatment with or without 300 µM DA or 50 µM H2O2, respectively. * and #, at least P < 0.05, compared with the percentage of WT1 FBXO7 transfected cells containing FBXO7 aggregates treated under the same conditions. * and #, at least P < 0.01, compared with the percentage of same FBXO7 transfected cells containing FBXO7 aggregates without challenges. (G and H) Alleviation of FBXO7 protein aggregate formation (G) and improvement of cell viability (H) of GFP-tagged FBXO7 transfected cells by GSH. GFP-tagged WT and mutant FBXO7 transfected cells were treated with or without 500 µM GSH and further cultured for 48 h. *, at least P < 0.05, compared with the percentage of WT1 FBXO7 transfected cells containing aggregates treated under the same conditions. #, at least P < 0.05, compared with the percentage of respective FBXO7 transfected cells containing aggregates (G) or the cell viability of respective FBXO7 transfected cells (H) in the absence of GSH. (I) Time-dependent influence on cell viability by overexpression of GFP or GFP-tagged WT and mutant FBXO7 proteins. Cell viabilities of cells transfected with GFP for 24 or 72 h were set as controls respectively, whereas the cell viabilities of other groups were shown as % control. *, at least P < 0.05, compared with the cell viability of cells transfected with GFP for 72 h. (J) Proteasome inhibitor (MG132) promotes GFP-tagged WT1 FBXO7 protein aggregate formation, whereas autophagy inhibitor (3-MA) does not. After transfection, cells were challenged with or without 3 µM MG132 or 15 µM 3-MA for 16 h respectively before harvested for western blot analysis. (K) Mono-ubiquitination of GFP-tagged WT1 FBXO7 promotes the most FBXO7 protein aggregate formation. Cells were co-transfected with GFP-tagged WT1 FBXO7 as well as ubiquitin WT (Ub-WT), ubiquitin KO (Ub-KO) or ubiquitin KO with G76V mutation respectively overnight before harvested for western blot analysis. (L) Representative western blot data to show the alleviation of WT-FBXO7 aggregation by Proline treatment. (M) Proline alleviate 3 days overexpression of WT and R498X FBXO7 proteins induced HEK cell viability impairment, visualized by quantified PI-Hoechst dual fluorescence staining. *, P < 0.05, compared with the PI fluorescent density of cells transfected with GFP for 3 days. (N) ABTS assay to determine reductive potency of Proline and GSH in solution. *, P < 0.001, compared with the ABTS value of control group in the absence of any reductants. (O) Mutant R378G FBXO7 protein promotes WT1 FBXO7 protein aggregate formation. Cells were co-transfected with Myc tagged WT1 FBXO7 as well as GFP-tagged WT1 FBXO7 or R378G FBXO7 overnight before harvested for western blot analysis of soluble and insoluble fractions of FBXO7 proteins. (P) Mutant R378G FBXO7 protein promotes WT PARKIN protein aggregate formation. Cells were co-transfected with PARKIN-HA as well as GFP-tagged WT1 or R378G FBXO7 overnight before harvested for western blot analysis.
We found that the level of ROS was elevated in our LRRK2 stable transfected dopaminergic SH-SY-5Y cells, especially in mutant G2019S LRRK2 cells (Fig. 3O). Furthermore, the expression level of FBXO7 protein was increased and the amount of insoluble FBXO7 was enhanced in LRRK2 stable cells, especially in G2019S mutant cells (Fig. 3K–N). However, the increased ROS levels, up-regulated FBXO7 expressions and increased FBXO7 aggregation in LRRK2 stable cells were improved by GSH and coenzyme Q10 (COQ10) treatment (Fig. 3K–O). The increased FBXO7 expression and FBXO7 aggregation were also found in LRRK2 transgenic mice brains (Fig. 3P and Q).

In human skin fibroblasts from PD patients with or without mutations of PD-linked genes, we found that the FBXO7 protein levels were increased in the PD fibroblasts (Fig. 4A and B). The increased expression of FBXO7 in PD fibroblast cells was alleviated by GSH or COQ10 treatment (Fig. 4C–E). The amount of insoluble FBXO7 in PD fibroblast cells was also increased, which was reduced by GSH or COQ10 treatment (Fig. 4F and G). GSH is a reductant with strong reducing potency, whereas COQ10 (oxidized form) is not (Fig. 4H). However, they can both inhibit FBXO7...
Figure 4. Aggregation of endogenous WT FBXO7 in PD fibroblast cells and human postmortem brain samples from PD and AD patients. (A) Representative western blot data of soluble FBXO7 proteins in human fibroblast cells derived from PD patients with or without PD gene mutations. (B) Densitometric analysis of soluble FBXO7 protein bands in western blot gels from experiments in (A). *, at least $P < 0.05$, compared with the arbitrary value of FBXO7 protein bands in gels of control 1. (C) Quantitative real-time RT–PCR analysis of WT1 FBXO7 expression in human fibroblast cells from aged control or PD patients in the presence or absence of GSH or COQ10. *, at least $P < 0.05$, compared with the relative expression level of WT1 FBXO7 in aged control. (D) Representative western blot data of soluble FBXO7 proteins in human fibroblast cells derived from aged control or PD patients in the presence or absence of GSH or COQ10. (E) Densitometric analysis of soluble FBXO7 protein bands in western blot gels from experiments in (D). *, at least $P < 0.05$, compared with the arbitrary value of FBXO7 protein bands in gels of control 1. (F and G) Representative western blot data of insoluble FBXO7 proteins in human fibroblast cells derived from aged control or PD patients with PARKIN R275W mutation (F) or LRRK2 (G2019S) and Pink1 (I368N) mutations (G) in the presence or absence of COQ10. (H) ABTS assay to determine reductive force of GSH and COQ10 in solution. *, $P < 0.001$, compared with the ABTS value of control solution without any reductants. (I) Representative western blot analysis of soluble and insoluble FBXO7 proteins in cortex samples of two aged controls (Lane 1, A219/97 and lane 2, A283/96) and two PD patients (Lane 3, ST11/02 and lane 4, ST14/02). (J) Representative western blot analysis of soluble and insoluble FBXO7 proteins in cortex samples (gray and white matters) of two aged controls (Lane 1, 787 and lane 2, 963) and two AD patients (Lane 3, 1254 and lane 4, 1664).
protein aggregation, suggesting they inhibit FBXO7 aggregation via different non-oxidative mechanism.

FBXO7 aggregation in brains of PD and Alzheimer’s disease patients

We checked the FBXO7 aggregation in postmortem human brain samples of PD and Alzheimer’s disease (AD) patients (Fig. 4I and J). In the cortex of PD patients, the amounts of soluble and insoluble FBXO7 were up-regulated, compared with those of aged controls (Fig. 4I). In the cortex samples of AD patients, the levels of soluble FBXO7 did not alter much. However, the amount of insoluble FBXO7 increases significantly in gray matter and white matter of AD patients, compared with those of aged controls (Fig. 4I). These findings suggest that FBXO7 aggregation may be linked to neuron degeneration in both PD and AD.

Overexpression of WT FBXO7 leads to FBXO7 protein aggregation and dopaminergic neuron degeneration in Drosophila

We generated transgenic Drosophila models overexpressing human WT1 FBXO7 controlled by tissue-specific UAS-GAL4 system. Two transgenic fly lines were created: W;P{UAS-Fbxo7.WT}(II) and W;P{UAS-Fbxo7.WT}attp.ZH-51C(II). We found that overexpression of WT FBXO7 in dopaminergic neurons did not have any significant influence on fly life span (Fig. 5A). However, after 30–40 days culture, overexpression of WT1 FBXO7 in Drosophila dopaminergic neurons led to locomotor deficits in flight and climbing ability of flies (Fig. 5B). Overexpression of human WT FBXO7 in fly dopaminergic neurons for 40 days induced dopaminergic neuron loss, especially in PPM1/2 and PPM3 dopaminergic neuron clusters (Fig. 5D and F). Furthermore, overexpression of WT FBXO7 protein in fly heads for 40 days led to FBXO7 protein aggregation (Fig. 5C). The overexpression of WT1 FBXO7 for 40 days led to swelling of muscle mitochondria, broken mitochondria cristae and accumulated high density materials in the swollen mitochondria (Fig. 5E).

Stress-induced translocation of FBXO7 from nucleus into mitochondria and formation of deleterious FBXO7 aggregates

We found that endogenous FBXO7 protein could localize to mitochondria in the absence of stress (Fig. 6A). However DA, H2O2, or Rotenone challenges promoted translocation of endogenous FBXO7 from nucleus into mitochondria (Fig. 6A, Supplementary Material, Fig. S3B and C). Furthermore, stress of cells facilitated aggregation of endogenous FBXO7 in mitochondria (Supplementary Material, Fig. S3A). In HEK and SH-SY-5Y cells, the overexpressed WT and mutant FBXO7 proteins were localized to mitochondria (Fig. 6B–G and Supplementary Material, Fig. S3D). However, lesser amounts of mutant FBXO7 proteins were localized to mitochondria, compared with WT1 FBXO7 (Fig. 6B–G, J). Compared with (overexpressed or endogenous) WT FBXO7, H2O2 challenge led to reduced soluble FBXO7 mutant proteins (R378G and R498X) in both mitochondria and nucleus (Fig. 6C–F). The H2O2 challenge also significantly promoted aggregation of mutant FBXO7 in whole cell, nucleus as well as in mitochondria (Fig. 6D and E). Next, we investigated FBXO7 aggregation in mitochondria in the presence of overexpressed prohibitin 1 (PHB1). Co-transfection of PHB1 with WT1 or mutant R498X FBXO7 into HEK cells led to increased levels of soluble and insoluble FBXO7 in whole cell and mitochondria, especially the increased amount of insoluble fraction of FBXO7 proteins in mitochondria (Fig. 6H and I). However, PHB1 did not change the composition of soluble and insoluble FBXO7 in nucleus (Fig. 6J). Co-expression of PHB1 aggravated cell viability impairment, especially mutant R498X FBXO7 (Fig. 6K).

Discussion

In this study, we demonstrated that FBXO7 protein is a stress response protein and that stress-induced up-regulation of transient WT FBXO7 expression can be protective to cells. However, mutant FBXO7 proteins aggravate neurotoxin-induced cell demise. Cellular stress can promote FBXO7 proteins’ deleterious protein aggregate formation, especially in mitochondria. The PD-linked FBXO7 mutations facilitate FBXO7 aggregates formation in mitochondria, impair mitochondria function, promote ROS generation and decrease cell viability. WT FBXO7 facilitates cytoprotective mitophagy, whereas PD-linked FBXO7 mutations inhibit mitophagy process. The PD-linked mutations of FBXO7 lead to loss of protective functions and aggravation of toxic effects of WT FBXO7.

A role for mitochondria dysfunction in the pathogenesis of PD is well recognized (30). The mitochondria are recognized as dynamic and mobile organelles that constantly undergo membrane

FBXO7 aggregation in mitochondria impairs mitochondria integrity and enhances ROS production

We found that FBXO7 aggregates co-localized with ROS indicator RedoxSensor RCC-1 (Fig. 7A) and WT1 or mutant R498X FBXO7 aggregates co-localized well with TOMM20 (a mitochondria marker) and 4-HNE (a marker for lipid peroxidation) (Fig. 7B and C). The overexpression of mutant FBXO7 decreased mitochondrial membrane potential in a time-dependent manner (Fig. 7D). The overexpression of WT FBXO7 for 1 day increased the mitochondrial membrane potential (Fig. 7D). However, the increased mitochondrial membrane potential of cells transfected with WT FBXO7 was reversed after 3 days’ culture (Fig. 7D). These observations suggest that WT FBXO7 can be protective to cells, but longer time exposure to high level of WT FBXO7 may lead to FBXO7 aggregation in mitochondria. Next, we quantitatively detected the ROS levels in cells with our newly developed procedure using RedoxSensor RCC-1 dye (Fig. 7E). We found that overexpressing mutant FBXO7 could enhance ROS levels (Fig. 7F and G). Overexpression of WT FBXO7 for 2 and 3 days (longer time exposure) also promoted ROS generation (Fig. 7G).

FBXO7 mutants inhibit mitophagy

Knocking down of endogenous FBXO7 expression impaired FCCP-induced mitophagy (Fig. 8A and B). We used NAO-Hoechst dual dyes staining protocol and western blot analysis of the content of cytochrome c to quantitatively monitor mitophagy process (29). We found that overexpression of WT PARKIN promoted FCCP (20 µm)-induced mitophagy (Fig. 8E). The mutant PARKIN inhibited FCCP-induced mitophagy (Fig. 8E). However, overexpression of WT FBXO7 facilitated mitophagy either in the presence or in the absence of FCCP (Fig. 8F, G and I). Overexpression of mutant FBXO7 proteins inhibited FCCP-induced mitophagy (Fig. 8F, H and J). Recent findings reported that WT FBXO7 can rescue PARKIN mutation-induced mitophagy defects (25). Our data confirmed that WT FBXO7 promotes mitophagy, whereas mutations of FBXO7 lead to inhibition of mitophagy process.

We found that overexpression of PARKIN promoted FCCP-induced mitophagy (Fig. 8E). The mutant PARKIN inhibited FCCP-induced mitophagy (Fig. 8E). However, overexpression of WT FBXO7 facilitated mitophagy either in the presence or in the absence of FCCP (Fig. 8F, G and I). Overexpression of mutant FBXO7 proteins inhibited FCCP-induced mitophagy (Fig. 8F, H and J). Recent findings reported that WT FBXO7 can rescue PARKIN mutation-induced mitophagy defects (25). Our data confirmed that WT FBXO7 promotes mitophagy, whereas mutations of FBXO7 lead to inhibition of mitophagy process.
Figure 5. Overexpression of human WT FBXO7 induced FBXO7 aggregation and dopaminergic neuron degeneration in Drosophila heads. (A) Influence of overexpression of human WT FBXO7 in dopaminergic neurons in fly heads cannot influence life span of flies. FBXO7 transgenic flies were crossed with ddc-GAL4 lines and life span of flies was monitored for 40 days. (B) Overexpression of human WT FBXO7 in dopaminergic neurons in fly heads leads to locomotor deficits in climbing ability of these flies. FBXO7 transgenic flies were crossed with ddc-GAL4 lines and climbing assay was performed after different time culture. *, P < 0.05, compared with the percentage of flies on top of yellow white flies crossed with ddc-GAL4 line. (C) Western blot analysis of soluble and insoluble FBXO7 in fly heads. Yellow white control flies or WT FBXO7 transgenic flies were crossed with elva-GAL4 line and cultured for 40 days. FBXO7 in fly heads was analyzed by western blot analysis. (D) Representative confocal image of TH neurons in fly heads of yellow white control and FBXO7 transgenic flies crossed with ddc-GAL4 lines. After control yellow white and FBXO7 transgenic flies crossed with 24B-Gal4 lines and cultured for 40 days, TH-positive neurons in fly heads were counted and calculated. *, P < 0.05, compared with the number of TH-positive neurons in heads of yellow white flies crossed with ddc-GAL4 line.
remodeling through repeated cycles of fusion and fission as well as mitophagy (31). These processes help to maintain a steady pool of healthy mitochondrial essential for energy production and beyond (31). Mitophagy is a process where mitochondria are targeted for degradation via autophagy pathway (32). The impaired mitophagy process and subsequent delayed clearance of dysfunctional mitochondria in cell can contribute to decreased ATP production, ROS generation and finally dopaminergic neuron degeneration in PD (30). Mitophagy is regulated by PINK1 and PARKIN pathway (33). A recent report demonstrates that FBXO7 binds with PARKIN and may be involved in the translocation of PARKIN to damaged mitochondria (25). Here, we demonstrated that FBXO7 may interact with key FBXO7 targets in mitochondria to modulate mitophagy process. Further studies to identify FBXO7 mitochondrial targets will further unravel the mechanism of FBXO7-modulated mitophagy.

The WT FBXO7 can be protective but FBXO7 can form deleterious protein aggregates. Recently, the FBXO7 immunoreactivity was reported to be detected in large proportions of insoluble α-syn-positive inclusions (20). The similar scenario has been reported in α-syn and SOD1. Both WT α-syn and SOD1 have protective functions (34,35). However, WT and mutant α-syn can both form protein aggregate, which predispose to dopaminergic neuron degeneration in PD (36). The aggregation of mutant SOD1 is relevant to familial form of amyotrophic lateral sclerosis (ALS) (37,38). However, oxidative stress-induced misfolding and aggregation of WT SOD1 is implicated in the pathogenesis of sporadic ALS (39).

Figure 6. Stress-induced FBXO7 proteins translocation from nucleus into mitochondria and formation of PHB1 aggravated FBXO7 aggregation in mitochondria. (A) Confocal fluorescent microscopy images of mice primary neurons challenged with different neurotoxins (DA, H2O2, and Rotenone). Green arrowheads point to FBXO7 aggregates formed outside of mitochondria. Yellow arrowheads point to FBXO7 aggregates co-localized with mitochondria. (B) Confocal fluorescent microscopy images of localization of GFP-tagged WT and mutant FBXO7 in human dopaminergic SK-N-SH cell lines. Yellow arrowheads point to co-localization of FBXO7 with mitochondria. (C-G) Representative western blot gels of soluble and insoluble GFP-tagged WT and mutant FBXO7 or endogenous FBXO7 proteins in whole HEK cell, mitochondria and nucleus, respectively. (C) WT1 FBXO7, (D) R498X FBXO7, (E) R378G FBXO7, (F) Endogenous FBXO7 protein only, (G) Cell fractionation markers. (H-J) Representative western blot data of soluble and insoluble WT and mutant FBXO7 in whole cell (H), mitochondria (I) and nucleus (J) when co-transfected into HEK cells with ATP5B or PHB1, respectively. Cell organelle markers PARP and cytochrome c (Cyto C) are used respectively for quality monitoring of achieved nucleus and mitochondria fractions. (K) HEK cell viability detected by PI-Hoechst dual fluorescent dye staining protocol *. P < 0.001, compared with the relative PI fluorescent intensity of control cells transfected with GFP or ATP5B.
We showed that FBXO7 can concentrate into mitochondria under stress and form deleterious protein aggregate in mitochondria, leading to cell viability impairment. Maintaining protein homeostasis involves an elaborate protein quality control system, consisting of chaperones and proteases in the mitochondria (40–42). The highly conserved mitochondrial proteases can clear and remove non-assembled, misfolded and aggregated proteins to maintain protein homeostasis in mitochondria (40). Impairment of m-ATP mitochondria proteases can contribute to neuron degeneration (43,44). PHB1 is an endogenous m-ATP protease inhibitor, located in the inner membrane of mitochondria (45). We found for the first time that PHB1 promoted deleterious FBXO7 aggregation in mitochondria. Thus, PHB1 may become a therapeutic target to alleviate FBXO7-linked neuron degeneration in PD. Our observations were corroborated by recent studies that showed that IMD-019064 and FL40, new small molecular PHB1 inhibitors, can protect dopaminergic neurons in both in vitro and in vivo PD models (MPP+-induced neurotoxicity) and traumatic brain injury (38).

On the other hand, it is reported that small molecules such as the anti-oxidant pyrroloquinoline quinone (PQQ) prevents the toxic amyloid fibril formation of α-syn, Aβ1–42 and mouse prion protein in vitro (46,47). Furthermore, PQQ-modified α-syn prevents the amyloid formation of intact α-syn (46). In our current

Figure 7. FBXO7 aggregation in mitochondria promotes ROS production in mitochondria and impairment of mitochondrial integrity. (A) FBXO7 aggregation enhances ROS production. HEK cells were transfected with GFP-tagged WT and mutant FBXO7 and cultured for 2 days. Cells were stained with 1 µM RedoxSensor Red CC-1 before observed and photographed under fluorescent microscopy. White arrowheads point to co-localization of FBXO7 aggregates with RedoxSensor Red CC-1 dyes. (B and C) FBXO7 proteins induced mitochondria ROS production. After transfection of HEK cells with GFP or GFP-tagged WT and mutant FBXO7 proteins for different days, mitochondria membrane potential was analyzed using TRAM dye. Cells transfected with GFP were set as control and the mitochondrial potential of GFP-transfected cells was set as 100%. *, at least P < 0.05, compared with the mitochondrial potential of cells transfected with GFP for 1, 2 or 3 days respectively. #, P < 0.05, compared with the mitochondrial potential of cells transfected with the same FBXO7 protein for 1 day, respectively. (D) Influence on mitochondria membrane potential by WT and mutant FBXO7 proteins. After transfection of HEK cells with GFP or GFP-tagged WT and mutant FBXO7 proteins for different days, ROS levels were measured using RedoxSensor Red CC-1 dye. Cells transfected with GFP were set as control and the ROS level of control cells was set as 100%. *, at least P < 0.01, compared with the ROS level of cells without challenge. (E) Influence on cell ROS levels by overexpression of GFP-tagged WT and mutant FBXO7 proteins. After transfection of HEK cells with GFP or GFP-tagged WT and mutant FBXO7 proteins for 1, 2 and 3 days respectively, ROS levels were measured using RedoxSensor Red CC-1 dye. Cells transfected with GFP were set as control and the ROS level of control cells was set as 100%. *, at least P < 0.05, compared with the ROS level of GFP-transfected cells. (F) Time-dependent influence on cell ROS levels by overexpression of GFP-tagged WT and mutant FBXO7 proteins. After transfection of HEK cells with GFP or GFP-tagged WT and mutant FBXO7 proteins for 1, 2 and 3 days respectively, ROS levels were measured using RedoxSensor Red CC-1 dye. Cells transfected with GFP were set as control and the ROS level of control cells for 1 day was set as 100%. *, at least P < 0.05, compared with the ROS level of cells transfected with GFP for the same time duration. #, at least P < 0.05, compared with the ROS level of cells transfected with the same plasmid for 1 day.
Figure 8. FBXO7 proteins regulate mitophagy process. (A and B) Knock-down of FBXO7 in mice dopaminergic PC12 cells inhibits mitophagy process. (A) Representative western blot data of cytochrome c with or without FCCP challenge for 2 h. (B) Densitometric analysis of cytochrome c protein bands in western blot gels from experiments in (A). *, $P < 0.05$, compared with the arbitrary value of cytochrome c bands in gels of scramble shRNA transfected cells. (C) Fluorescent images of NAO stained mitochondria in HEK cells. Cells were transfected with Mito-RFP, stained by NAO dye and photographed under fluorescence microscopy. (D) Monitoring FCCP-induced mitophagy using NAO fluorescence dye. HEK cells were challenged with FCCP for 2 or 3 h before NAO and Hoechst dyes staining and analyzed by fluorescent spectrometer. Cells without FCCP challenge were set as control and the value of NAO fluorescence of control cells was set as 100%. *, at least $P < 0.01$, compared with the value of NAO fluorescence of cells without FCCP challenge. #, at least $P < 0.01$, compared with the value of NAO fluorescence of cells challenged with the same concentration of FCCP for 2 h. (E and F) Monitoring FCCP-induced mitophagy in cells overexpressing WT or mutant PARKIN or FBXO7 proteins. HEK cells were transfected with WT or mutant PARKIN or FBXO7 proteins overnight before challenged with FCCP for 2 h and stained with NAO and Hoechst dyes. Cells transfected with RFP without FCCP challenge were set as controls and the value of NAO fluorescence of control cells was set as 100%. *, $P < 0.05$, compared with the NAO fluorescence density of cells challenged with the same concentration of FCCP. (G–J) Monitoring mitophagy in cells overexpressing GFP-tagged WT1 or mutant FBXO7 proteins by western blot analysis of cytochrome c content in human dopaminergic SH-SY-5Y cells. Cells were transfected with GFP or GFP-tagged WT1 or mutant FBXO7 proteins overnight and then challenged with FCCP for 2 h before western blot analysis of cytochrome c content in cells. (G) Representative western blot gel of cytochrome c in the presence or absence of overexpression of GFP-tagged WT1 FBXO7. (H) Representative western blot gel of cytochrome c in the presence or absence of overexpression of GFP-tagged WT1 FBXO7 proteins. (I) Densitometric analysis of cytochrome c protein bands in western blot gels from experiments in (G). *, $P < 0.01$, compared with the arbitrary value of cytochrome c bands in gels of GFP-transfected cells without FCCP challenge. #, $P < 0.05$, compared with the arbitrary value of cytochrome c bands in gels of GFP-transfected cells without FCCP challenge.
study, we showed that Proline, GSH and COQ10 could alleviate FBXO7 mutation and stress-induced accumulation of deleterious FBXO7 aggregation. These small molecules may inhibit FBXO7 aggregation and benefit FBXO7-linked neuron degeneration in PD. We hypothesize that FBXO7 may interact with its various binding partners in cytosol, mitochondria and nucleus. In cytosol and mitochondria, FBXO7 may interact with chaperones to prevent the self-aggregation of FBXO7 proteins. FBXO7 mutations may alter FBXO7 structure, leading to impaired binding capacity with chaperone proteins and aggregation formation. Under stress, the up-regulated FBXO7 level may disturb the interaction between FBXO7 and chaperone. This may contribute to stress-induced FBXO7 aggregation. Thus, searching for key FBXO7 binding partners, especially chaperones in mitochondria, will have therapeutic significance. We find that T22M mutant FBXO7 is mainly localized to cytoplasm, which is a different primary subcellular location compared with WT, R378G and R498X FBXO7 proteins. It is possible that mutations in different domains of FBXO7 cause FBXO7 proteins aggregation via different pathways, or the different FBXO7 domains interact and regulate each other. These possibilities warrant further investigations. The potential pathological links among FBXO7 aggregation, mitochondria impairment, ROS generation as well as mitophagy modulation are illustrated in Scheme 1.

In summary, our study demonstrated that FBXO7 is a stress response protein and upon stress, the endogenous WT FBXO7 gets up-regulated, concentrates into mitochondria and forms FBXO7 aggregates in mitochondria. However, FBXO7 mutations

Scheme 1. Potential mechanisms for FBXO7-linked neurodegeneration. (A) FBXO7-linked pathogenesis involving FBXO7 aggregation, mitochondrial impairment, ROS production and deregulation of mitophagy process. FBXO7 mutation can lead to increased aggregation of FBXO7, especially in mitochondria. The FBXO7 aggregate that formed in mitochondria will impair mitochondrial integrity, leading to decreased ATP production and increased ROS production. The enhanced ROS production will facilitate FBXO7 aggregation. Therefore, an adverse amplification loop was proposed. Besides ROS production, mutant FBXO7 proteins can inhibit mitophagy process and hence impair the clearance of damaged mitochondria via autophagy process, aggravating the positive feedback loop. The positive feedback loop can be exacerbated by pathological factors, such as factors that inhibit UPS, aggravate oxidative stress or inhibit mitochondria. However, ROS scavengers, protein aggregate inhibitors and mitophagy inducers may break the debilitating cycle and have potential therapeutic significance against FBXO7-linked dopaminergic neuron degeneration in PD. (B) The potential mechanism of ROS facilitated FBXO7 aggregation. The increased ROS may directly oxidize FBXO7, thus promoting misfold and FBXO7 aggregation. Furthermore, ROS challenge can up-regulate FBXO7 expressions. ROS can also inhibit proteasome activities. Thus, the increased FBXO7 expression and impaired FBXO7 clearance capacity under stress will favor FBXO7 aggregation. Subsequently, the accumulated FBXO7 aggregation and increased ROS levels in cells will impair mitochondrial integrity. Thus, will lead to more ROS production and decreased cell ATP production. The decreased ATP production can inhibit UPS function and impair clearance of FBXO7 aggregate in cells. Finally, these complicated pathological events may interact and converge to contribute to FBXO7 aggregation. (C) The balance between protective roles and deleterious impacts of FBXO7 proteins. Soluble WT FBXO7 protein can have protective effects. Mild and transient stress may promote up-regulation of FBXO7 expression and help cells to survival. However, strong and persistent stress of cells may lead to FBXO7 aggregation and disturb the balance, contributing to FBXO7-linked neuron degeneration.
aggravate deleterious FBXO7 aggregation in mitochondria. The elevated FBXO7 expression and aggregation were identified in human fibroblast cells from PD patients. FBXO7 can also form aggregate in patient brains of PD and AD. Our study provides novel pathophysiological insights and suggests that FBXO7 may be a potential therapeutic target in FBXO7-linked neuron degeneration in PD.

Materials and Methods

Plasmids and vectors

Human FBXO7-dsred vector (a gift from Prof Laman, Department of Pathology, Cambridge) was used as a template to sub-clone WT and mutant FBXO7 constructs (23). The In-Fusion™ Advantage PCR Cloning Kit (Clontech) was used to clone isoform 1 and isoform 2 WT FBXO7 into pcDNA3-myc/His vector or pcDNA3-GFP vector (Addgene plasmid 13031) which was developed by Dr Doug Golenbock. The pCMV6-XL5-FBXO7 (Plasmid ID SC12556) was purchased from OriGene Technologies, Inc., USA. The WT human PARKIN expression vector pRK5-HA-PARKIN used in this study was purchased from Addgene (Plasmid 17613, deposited by Ted Dawson) (48). FBXO7 mutants (R498X, R378G and T22M) and PARKIN R275W mutant were created based on the template of pcDNA3-FBXO7-GFP and pRK5-HA-PARKIN plasmids respectively by PCR-based technique using QuickChange site-directed mutagenesis kit (Stratagene, USA). Primers used for cloning of WT FBXO7 and creation of mutant FBXO7 were summarized in Supplementary Material, Table S1. The ubiquitin plasmids [pRK5-HA-Ubiquitin-KO (17603, all Lysine residues were mutated to Alanine, so substrates can only be mono-ubiquitinated instead of being poly-ubiquitinated), pRK5-HA-Ubiquitin- WT (17608)] were purchased from Addgene deposited by Ted Dawson (49). The pRK5-HA-Ubiquitin-KO plus G76V vector was created based on the template pRK5-HA-Ubiquitin-KO using QuickChange site-directed mutagenesis kit (Stratagene, USA). The mitochondria targeting Mito-RFP plasmid was constructed by cloning of N terminal mitochondria targeting sequence (MTS) and transmembrane domain (TM) of human PINK1 (1–150 amino acid) into pcDNA3-mRFP plasmid (Addgene plasmid, 13032, developed by Dr Doug Golenbock) (50). Scramble shRNA was purchased from Addgene (Plasmid 1864) deposited by David Sabatini (50). The FBXO7 shRNA plasmids for human (TRCN vectors) or mice (GIPZ vectors) FBXO7 knock down were obtained from Sigma (TRCN vectors) or Open biosystem. The pLX304-PHB plasmid (plasmid ID: HsCD00441072) was purchased from DNASU, USA. pRK5-HA-PARKIN expression vector pRK5-HA-PARKIN used in this study was purchased from Addgene (Plasmid 17613, deposited by Ted Dawson) (48). The FBXO7 mutants (R498X, R378G and T22M) and PARKIN R275W mutant were created based on the template of pcDNA3-FBXO7-GFP and pRK5-HA-PARKIN plasmids respectively by PCR-based technique using QuickChange site-directed mutagenesis kit (Stratagene, USA). The mitochondria targeting Mito-RFP plasmid was constructed by cloning of N terminal mitochondria targeting sequence (MTS) and transmembrane domain (TM) of human PINK1 (1–150 amino acid) into pcDNA3-mRFP plasmid (Addgene plasmid, 13032, developed by Dr Doug Golenbock) (50). Scramble shRNA was purchased from Addgene (Plasmid 1864) deposited by David Sabatini (50). The FBXO7 shRNA plasmids for human (TRCN vectors) or mice (GIPZ vectors) FBXO7 knock down were obtained from Sigma (TRCN vectors) or Open biosystem. The pLX304-PHB plasmid (plasmid ID: HsCD00441072) was purchased from DNASU, USA. All cloned or created mutant constructs were sequenced using BigDye® Terminator v3.1 cycle sequencing kit (Life Technologies) before transfection into cells.

Cell lines

The protocol to create human dopaminergic SH-SY-5Y LRRK2 stable cell lines used in the current study had been reported previously (5). Briefly, SH-SY-5Y cells were transfected with pcDNA3.1 vector, pcDNA3.1-LRRK2-WT or pcDNA3.1-LRRK2-G2019S vector respectively using the lipofectamine reagents. After transfection, cells were treated with selection medium containing 0.6 g/1 w/v G418 antibiotics (Promega) for 2–3 weeks before resistant cells were separated by serial dilution and allowed to grow from single cells. The stably transformed clones were verified by real-time RT-PCR and western blot analysis. Cells were maintained at 37°C in 5% CO2 incubator with 0.4 g/1 w/v G418. Human skin fibroblast cells from PD patients were purchased from the Coriell Institute for Medical Research, USA. Fibroblast cells were cultured and maintained in DMEM with 20% FBS at 37°C in 5% CO2 incubator before experiments.

Transgenic animals

Transgenic mutant LRRK2 G2019S mice were provided by The Jackson Laboratory (#009604, B6.Cg-Tg (LRRK2*G2019S)2YuejaJ). The transgenic and non-transgenic mice were maintained in accordance with institutional guidelines, and all protocols were approved through the Institutional Animal Care and Use Committee (IACUC) of the National Neuroscience Institute, Tan Tock Seng Hospital. The mice were maintained in a pathogen-free facility and exposed to a 12-h light/dark cycle with food and water. The hippocampus, cortex, striatum and midbrain samples were isolated from 12-month-old transgenic and non-transgenic control mice for further experimental analysis.

Primary cortical neuron culture

Embryonic day 16.5 female mice were euthanized with ketamine. Uteri were removed and individual fetuses were extracted and placed in sterile petri dish filled with sterile dissection medium [DM, HEPES-buffered Hanks’ balanced salt solution (HBSS) without calcium or magnesium]. Cortex tissue was removed; trypsin (1 mg/mL) was added before cells were seeded on dishes or coverslips at the following density: 1 x 105 cells per well for 4-well-plate and 1 x 106 cells per well for 6-well-plate. Medium was changed 4 h after seeding cells to feeding medium (Neurobasal containing Glutamax, B27, and antibiotic) and maintained in feeding medium for 7 days before further experiments.

Drosophila stocks, preparation and behavioral assays

Promoter lines containing elav-GALA4, ddc-GALA4 and 24B-GALA4 were obtained from Bloomington Stock Center (Bloomington, IN, USA). WT FBXO7 transgenic lines (W;P{UAS-Fbxo7.WT}(II)) and W;P{UAS-Fbxo7.WT}attP.ZH-51C(II)) were gifts from Prof Alexander J Whitworth (MRC Centre for Developmental and Biomedical Genetics, Sheffield S10 2TN, UK). Flies were routinely raised at 25°C on cornmeal media that were replaced every 3 days. For longevity experiments, day-old flies were transferred to fresh media every 3 days. The lifespan was assessed by monitoring survival of 50 flies in five cohorts for each genotype. The triplicate cohorts of 50 flies per genotype were monitored for survival daily. For the climbing assay, motor ability was assessed at 15 min interval using the negative geotaxis assay. Briefly, 3 cohorts of 20 female and age-matched control flies were anesthetized and placed in a vertical plastic column (length, 25 cm; diameter, 1.5 cm). After a 2-h recovery period, flies were tapped to the bottom and the percentage of flies that climb to or above the top column line in 1 min was calculated. Triplicate trials were performed in each experiment at 15 min interval.

Human postmortem brain samples from AD and PD patients

Human postmortem frozen tissue Frontal Cortex brain samples of AD patients (sample ID: 1254 and 1664) and aged controls...
cells and 500 µl of solubilizing solution [0.04 M Tris–HCl, pH 7.4, 3% glutaraldehyde in 0.1 M phosphate buffer, dehydrated in ethanol–acetone series then embedded in araldite. Ultrathin sections were observed under Jeol Jem1010. Ultrathin sections were picked up onto copper slot grids, stained with uranyl acetate and lead citrate then observed under Jeol jem1010 and photographed (Peabody, MA, USA).

**Drug administration**

After cells were transfected with WT or mutant FBXO7 or FBXO7 shRNA for 6 h, cells were treated with different concentration of H2O2 or DA and further incubated for 24 h at 37°C prior to analysis of cell viability or FBXO7 aggregation. To study the protective effects of GSH against aggregate-formation and cell viability decline, cells were transfected with WT or mutant FBXO7 and 500 µM GSH was added. Cells were further cultured for 2 days before protease aggregation and cell viability were analyzed. To study aggregation formation of endogenous FBXO7 induced by stressors, human or mice cells were treated with different concentrations of DA, H2O2, FCCP or Rotenone for different periods of time before FBXO7 protein aggregation or cell fractionation was performed. To study the inhibition of endogenous FBXO7 aggregation in human LRRK2 stable SH-SY-5Y dopaminergic cells or fibroblast cells from PD patients, cells were cultured in the presence or absence of 500 µM GSH or 300 µM COQ10 for 3 days before harvested for western blot analysis. To study the influence of FCCP-induced mitophagy by overexpression of PARKIN or FBXO7, cells were transfected with WT or mutant FBXO7 or PARKIN and treated with different concentration of FCCP for 2 h before mitophagy was analyzed. Unless specifically stated, all chemicals were purchased from Sigma-Aldrich.

**MTT cell viability assay**

Cell viability was monitored by MTT cell viability assay as previously described (52). Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was prepared as 2 mg/ml stock solution in 1 × phosphate-buffered saline (1 × PBS) and stored at 4°C. The MTT/DMEM solution (15% v/v of 2 mg/ml MTT stock solution mixed with 85% v/v DMEM) was prepared freshly. Cells in 24-well dishes were washed with PBS and incubated in 500 µl of MTT/DMEM solution in the dark at 37°C for 3 h. The solution was then aspirated without disrupting the cells and 500 µl of solubilizing solution [0.04 M hydrochloric acid/isopropanol plus 3% w/v sodium dodecyl sulfate (SDS)] was added and mixed well. The plates were incubated at room temperature for 1 h in the dark. The solution optical density (OD) was then measured at 595 nm using a spectrophotometer (Elisa Reader Spectra Max 340) in a 96-well plate (Iwaki).

**PI-Hoechst dual fluorescent dyes staining of cell viability**

The PI-Hoechst dual fluorescent dyes staining protocol was derived and modified from calcein-PI dual fluorescent cell viability detection protocol. The aim to introduce Hoechst dye into the assay is to prevent any adverse influence on final PI fluorescent intensity due to variance of cell numbers among respective groups. In brief, 3.5 × 10^5 cells were plated into each well of 96-well μClear tissue culture-treated black plate (Greiner). After transfection and drugs administration, 15 µl of Opti-MEM containing PI (2.5 µg/ml) and Hoechst (2 µg/ml) were added to each well. After incubation of cells at 37°C for 30 min in the dark, the fluorescence intensity PI and Hoechst was measured by Tecan Infinite M200 microplate reader at different wavelengths: 530 nm excitation and 620 nm emission for PI and 335 nm excitation and 460 nm emission for Hoechst. The relative fluorescent intensity of PI was acquired via division of PI readings with Hoechst readings of each well, respectively. Cells without any treatment were set as control group. The cell viability of other groups was expressed as the relative PI fluorescent intensity (% control).

**Western blot analysis**

Cells were collected in lysis buffer [100 mM HEPES pH 7.5, 5 mM magnesium chloride, 150 mM sodium chloride, 1 mM EDTA, 1% v/v Triton and 1% v/v protease inhibitor cocktail (Calbiochem)] with the help of a cell scraper and centrifuged at 12 000g at 4°C for 20 min. From the supernatant, 30 µg proteins were resolved by 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane using an electric blotting apparatus (Invitrogen), blocked with washing buffer A (150 mM sodium chloride plus 13 mM Tris-hydrochloric acid pH 7.5 and 0.1% w/v Tween 20) containing 5% w/v skim milk at room temperature before incubation with primary antibody in washing buffer A with 2% w/v skim milk (anti-FBXO7, Abnova, 1:1000; anti-PARP, Abcam 1:1000; anti-cytochrome-c, Abcam, 1:600, mouse anti-GFP antibody, Roche, 1:1000) for overnight at 4°C. The membrane was washed 5 × 5 min each with washing buffer A and subsequently incubated with the secondary antibody (goat-anti-mouse or goat-anti-rabbit antibody, Santa Cruz Biotechnology, 1:1000) for 1 h at room temperature. Following subsequent washes, the blots were developed with the enhanced chemiluminescent kit (Pierce) on Kodak CL-Xposure™ films.

**Quantitative analysis of western blot data**

Quantitative analysis of the densities of protein bands in western blot gels was carried out by densitometric analysis using the image software Bandscan 4.30. The ratio of the densities of respective protein bands in control lanes in western blot gels was set as 50%. The relative densities of protein bands of other lanes were expressed as the ratio of densities, after automatic comparison with the ratio of densities of control lanes by the software.

**Analysis of FBXO7 protein aggregates by western blot analysis**

After transfection of cells or stress of cells with different stressors, cells were lysed in buffer [25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% (w/v) SDS] containing protease inhibitors (Roche Applied Science) and phenylmethylsulfonyl fluoride and incubated...
on ice for 30 min. The lysate was then sonicated before centrifugation at 13 200 rpm for 30 min to obtain the soluble (supernatant) and insoluble (pellet) fractions. The pellet was solubilized in 30 µl of 4× SDS loading buffer (161-0737, BIO-RAD) with continuous shaking at 4°C overnight, then boiled and loaded for gel electrophoresis, SDS-PAGE and western blot analysis.

Analysis of FBXO7 protein aggregates in fluorescent image

Cells transfected with GFP-tagged WT or mutant FBXO7, were seeded on cover slips, fixed with 4% (w/v) p-formaldehyde at room temperature for 20 min, washed with 1× PBS and mounted on slides using 10 µl of mounting medium containing 0.2% (w/v) Hoechst dye (Invitrogen). Subsequent analysis was done with Axio Imager.M1 fluorescence microscope (Carl Zeiss). For cells without transfection but challenged with different stressors, the endogenous FBXO7 protein was visualized by immunocytochemistry procedure. Cells on cover slides were observed and photographed in a random and clockwise manner under the fluorescent microscope. Cells with uniformly distributed FBXO7 were recognized as cells without aggregates, whereas cells with obvious condensed and brightly colored FBXO7 particles were recognized as cells with FBXO7 aggregates. Percentage of cells with aggregates was obtained based on the observation of at least 400 cells.

Quantitative real-time RT–PCR

Expression levels of FBXO7 in human dopaminergic SH-SY-5Y and human fibroblast cells were analyzed using real-time quantitative RT–PCR. Total RNA was isolated using NucleoSpin RNA II (Macherey-Nagel). A two-step quantitative RT–PCR was carried out. Reverse transcription was performed with Maxima First Strand cDNA synthesis kit for RT-qPCR (Fermentas); 1 µg RNA sample was used for each reverse transcription. RT-qPCR primers used in this study are as follows: forward primer (GAGGCTGCGGG TGGCGCT) and reverse primer (TCAAGTCCCCAGAAACAA). All real-time PCR reactions were performed using the CFX96 real-time PCR detection system (Bio-Rad Laboratories) and the amplifications were done using Maxima SYBR Green/Fluorescein qPCR master mix (2X) (Fermentas). Thermal cycling conditions comprised incubation at 50°C for 2 min followed by an initial denaturation step at 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 45 s. Expression data were normalized to the geometric mean of housekeeping gene β-actin to control the variability in expression levels. The relative quantification in gene expression was determined using the 2−ΔΔCT method (53). The expression levels of WT1 FBXO7 protein in control cells without any stresses were set as 100% and FBXO7 expressions of other groups were expressed as % of control cells.

Monitoring GFP-tagged FBXO7 overexpression by fluorescent spectrophotometry

After transfection of cells with GFP-tagged WT or mutant FBXO7 overnight, cells were harvested and lysed in M-PER mammalian extraction buffer (Thermo Scientific). After determination of protein concentration, 400 µg protein lysates in 150 µl M-PER buffer were transferred into black flat bottom 96-well plates (Nunc). Samples were analyzed with Infinite M200 Microplate Reader (Tecan) at excitation and emission wavelength at 488 and 509 nm respectively for EGFP fluorescence.

Immunocytochemistry

After cells on cover slips were stressed with neurotoxins, they were fixed with 4% p-formaldehyde at room temperature for 20 min, washed with 1× PBS containing 0.3% (v/v) Triton-X for three times 5 min each, blocked with 5% (w/v) bovine serum albumin (BSA) at room temperature for 1 h. Cells were then incubated with primary anti-FBXO7 (1:100, Abnovas); anti-TOM20 (1:100, Sigma) or anti-4-HNE (1:100, antibodies online) antibody for 1 h at room temperature, followed by incubation of secondary Alexa Fluor® 488 chicken anti-rabbit IgG (1:500, Molecular Probes) or Alexa Fluor® 594 chicken anti-rabbit IgG antibodies at room temperature for 1 h. After washing, cover slips were mounted on slides using 10 µl of mounting medium containing 0.2% (w/v) Hoechst dye (Invitrogen). Slides were dried for 24 h before observation and photographed under Axio Imager.M1 fluorescence microscope (Carl Zeiss) or LSM710 Carl Zeiss upright confocal microscope (Zeiss).

Quantitative detection of ROS levels in living cells

In this study, we have developed a novel protocol that can detect the ROS levels in living cells using the RedoxSensor Red CC-1 dye (R14060, Invitrogen). Briefly, cells were seeded onto 6-well plates 1 day before experiments. After transfection or challenge with neurotoxin stressors, cells were incubated with 1 µM RedoxSensor Red CC-1 for 10 min at 37°C. Cells were then washed with 1× PBS three times before cells were harvested and resuspended in PBS buffer. About 2 × 10^6 cells were transferred into each well of black color flat bottom 96-well plates (Nunc), and samples were measured by the Infinite M200 Microplate Reader (Tecan) at excitation and emission wavelength at 540 and 600 nm respectively for RedoxSensor Red CC-1 fluorescence.

Mitochondria potential assay

After transfected cells were incubated in Opti-MEM (Invitrogen) containing 1 µM tetrathymethylrhodamine ethyl ester perchlorate (TMRE) for 30 min at 37°C in a CO2 incubator, cells were collected and resuspended. A total of 100 µl aliquots were transferred into black flat bottom 96-well plates (Nunc). Samples were analyzed with Infinite M200 Microplate Reader (Tecan) at excitation and emission wavelength at 549 and 575 nm respectively for TMRE fluorescence. The mitochondrial potential of control cells transfected with GFP was set as 100% and mitochondrial potential of cells transfected with respective FBXO7 vectors was expressed as % of the mitochondrial potential of control cells.

Cell fractionation

Cell fractionation experiments were performed using the Cell Fractionation Kit from Mitosciences in accordance with the manufacturer’s protocol. After H2O2 or FCCP challenge, the cells were harvested. Mitochondrial and nuclear components were separated and harvested. The purity of each fraction was tested by western blot analysis using anti-PARP (1:1000, Abcam) or anti-cytochrome-c (1:1000, Abcam) antibodies. The content of endogenous FBXO7 in nucleus or mitochondria in the presence or absence of stresses was analyzed by western blot analysis, respectively.

NAO-based quantitative mitophagy analysis

An improved Hoechst and NAO double staining protocol based on a recent published NAO single staining method was used to quantitatively analyze mitophagy process (29). Briefly, cells were
challenged with 1, 10 or 20 μM FCCP for 2, 3 or 24 h and incubated at 37°C with 5% CO2 before cells were stained with 10 μg/ml (w/v) Hoechst 33342 and 0.5 μM 10-N-nonyl acridine orange (NAO, Invitrogen) dyes. Cells were washed with 1 x PBS for 5 min. Subsequently, cells were harvested and resuspended in 1 x PBS. 1 x 10⁴ cells were transferred into each well of Nunc® flat bottom black 96-well plates before analysis by Infinite M200 Microplate Reader (Tecan) at excitation wavelength 488 nm and emission wavelength 535 nm for NAO fluorescence, and excitation wavelength 355 nm and emission wavelength 460 nm for Hoechst. NAO fluorescence values were normalized using Hoechst fluorescence values, thus increasing the accuracy of mitophagy analysis.

ABTS cation decolorization assay

The monitoring of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity of chemicals was measured by the ABTS cation decolorization assay as described by Re et al. (54) with some modifications. The ABTS radical cation (ABTS⁺) was produced by reaction of 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in dark at room temperature for 12 h before use. The ABTS⁺ solution was diluted with methanol to give an absorbance of 0.7 ± 0.01 at 734 nm. Chemicals in solutions (1 ml) were allowed to react with 2 ml of the ABTS⁺ solution and the absorbance was measured at 734 nm after 1 min. The group in the absence of any reductants was set as control group. The reductants were added to the ABTS solution and the absorbance was measured at 734 nm after 1 min.

Statistical analyses were conducted using one-way or two-way ANOVA followed by post hoc Student’s t-test using software MiniTab 14. Graphs were constructed with Microsoft Excel 2007 or SigmaPlot 2001.

Supplementary Material

Supplementary Material is available at HMG online.

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References


