FBXO7 triggers caspase 8-mediated proteolysis of the transcription factor FOXO4 and exacerbates neuronal cytotoxicity

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initiators, caspase 8 plays a role in extrinsic apoptosis by combining with FAS-associated death domain (FADD) to form the death-inducing signaling complex (DISC). Moreover, active caspase 8 is released from the DISC, which sequentially activates the apoptotic effectors, such as caspases 7 and 3. Therefore, caspase 8 is able to promote apoptosis by either directly or indirectly activating downstream effector caspases (23).

FOXOs are involved in the aging process of nervous system, and their altered regulation or activity can be associated with age-related neurodegenerative diseases (NDDs), including Alzheimer’s disease (AD) and PD (19). However, little is known about this aspect of their activity. Furthermore, unlike FOXO1 and FOXO3, the neuronal function of FOXO4 is poorly understood. In the current study, we found that PD-associated FBXO7 interacted with FOXO4 in dopaminergic neuroblastoma MN9D cells, negatively regulating the stability of FOXO4 through the novel caspase 8-linked pathway. In addition, FOXO4 activity was reduced during 6-hydroxydopamine (6-OHDA)-induced apoptotic death in neuronal cells, which resulted from FBXO7-mediated proteolysis. Taken together, our findings suggest that the functional relationship between FBXO7 and FOXO4 may play a role in 6-OHDA-induced neuronal cell death, as well as PD pathogenesis.

**Results**

**FBXO7 interacted with FOXO4 in mammalian cells**

It has been recently reported that defective FOXO1 and FOXO3 activities are observed in both a PD mouse model and patients with PD (19). However, the putative role of FOXO4 in PD pathology and its underlying mechanism are poorly understood. In the current study, we investigated whether and how FOXO4 was biochemically and functionally linked to the PD-linked gene product FBXO7. We first examined whether FBXO7 binds to FOXO4 in mammalian cells. HEK293 cells were transfected with plasmids encoding Myc-tagged FBXO7, either alone or together with FLAG-FOXO4. Immunoprecipitation of cell lysates was then performed using an anti-FLAG antibody. The immunoblotting revealed that the half-life of FOXO4, HEK293 cells were transfected with plasmids encoding FLAG-FOXO4, either alone or in combination with Myc-FBXO7. Immunoblotting analysis of cell lysates using anti-FOXO4 antibodies revealed that, except the FBXO7-WT, the FOXO4 level was unaffected by all four deletion mutants (Fig. 2A). Similar to the patterns shown in Figure 2, A and B, endogenous FOXO4 levels were significantly increased in HEK293 cells by siRNA-mediated FBXO7 knockdown (Fig. 2C), suggesting that FBXO7 negatively regulated FOXO4 activity. This was further supported by the finding that endogenous FOXO4 levels were significantly increased in FBXO7-knockout HAP1 cells (Fig. 2D).

To investigate which protein domain of FBXO7 plays a role in the regulation of FOXO4 level, HEK293 cells were transfected with plasmids encoding either FLAG-FBXO7-WT or one of its deletion mutants. Immunoblotting analysis of cell lysates using anti-FOXO4 antibodies revealed that, except the FBXO7-WT, the FOXO4 level was unaffected by all four deletion mutants (Fig. 2E). These results suggested that the discrete domain within the FBXO7 protein but its whole structure appears to be required for the catalytic activity of FBXO7 and that the Ubl domain of FBXO7 is required for this interaction.

**FBXO7 decreased FOXO4 protein stability**

To gain further insight into the link between FBXO7 and FOXO4, we investigated whether FBXO7, as a component of ubiquitin E3 ligase, may affect the level of FOXO4. As shown in Figures 1A and 2, A and B, exogenous and endogenous levels of FOXO4 were greatly reduced in the presence of FBXO7 in a dose-dependent manner. In contrast, intracellular levels of FBXO7 were unaffected, irrespective of FOXO4 (Fig. 2A). Similar to the patterns shown in Figure 2, A and B, endogenous FOXO4 levels were significantly increased in HEK293 cells by siRNA-mediated FBXO7 knockdown (Fig. 2C), suggesting that FBXO7 negatively regulated FOXO4 activity. This was further supported by the finding that endogenous FOXO4 levels were significantly increased in FBXO7-knockout HAP1 cells (Fig. 2D).

To determine whether FBXO7 regulated the protein stability of FOXO4, HEK293 cells were transfected with plasmids encoding FLAG-tagged FOXO4, either alone or in combination with Myc-FBXO7. The cells were then treated with 25 μM cycloheximide for the indicated times, and immunoblot analysis of the cell lysates was performed using an anti-FLAG antibody. The immunoblotting revealed that the half-life of FOXO4 was markedly reduced by FBXO7 (Fig. 2F). However, this effect was not seen with FBXO7-ΔU (Fig. 2G). These results suggest that FBXO7-ΔU cannot mediate the proteolysis of FOXO4, possibly due to the lack of their binding.

Collectively, the overall results suggested that FBXO7 promoted the degradation of FOXO4.

**FBXO7 decreased FOXO4 stability through caspase activation**

Two major types of intracellular protein degradation machinery exist in eukaryotic cells, the ubiquitin-proteasome system (UPS) and lysosome-mediated autophagy pathway (24). Therefore, we next determined which pathway operates in FBXO7-mediated proteolysis of FOXO4. HEK293 cells were transfected for 24 h with plasmids encoding FLAG-FOXO4,
HA-FBXO7, or Myc-FBXO7, either alone or in combination, and treated the transfected cells with either proteasome inhibitor (MG132 or epoxomicin) or lysosomal autophagy inhibitor (NH4Cl or LY294002). As shown in Figure 3, pretreatment with proteasome inhibitors had no effect on FBXO7-mediated reduction of FOXO4 levels. These results indicated that the reduction of FOXO4 induced by FBXO7 occurred through SCF-independent and UPS-independent pathways. In addition, lysosomal inhibitors did not affect the proteolytic action of FBXO7 on FOXO4, suggesting that the proteolysis did not occur through the lysosomal autophagy pathway (Fig. 3, C and D).

We then examined whether FBXO7 promoted the reduction of FOXO4 through a caspase-dependent pathway. To investigate this possibility, HEK293 cells were transfected with plasmids encoding FLAG-FOXO4 and Myc-FBXO7, either alone or in combination, and the transfected cells were treated with the pan-caspase inhibitor Z-VAD-FMK. As shown in Figure 3E, immunoblotting analysis of cell lysates using anti-FLAG antibodies revealed that the presence of a caspase inhibitor markedly increased the reduction of FOXO4 levels. These results indicated that the FBXO7-mediated proteolysis of FOXO4 occurred through caspase activation. However, the pretreatment of caspase inhibitor could not recover the FOXO4 level completely by FBXO7, and so presumably, the caspase-independent mechanisms may also be involved in the reduction of FOXO4 level.

**FBXO7 promoted FOXO4 degradation via caspase 8 activation**

We further examined how the caspase pathway was linked downstream of FBXO7 and its underlying mechanism. For example, it was of interest which caspase(s) functionally interacted with FBXO7. Cells were transfected with plasmids encoding FLAG-tagged catalytically inactive caspases 1, 2, 3, and 7 or HA-tagged catalytically inactive caspase 8 and 10. Cell lysates were immunoprecipitated using anti-FLAG or anti-HA antibodies, accordingly. The immunoprecipitated samples were then immunoblotted using anti-FBXO7 antiserum, which revealed that exogenously added catalytic-inactive caspase
8 interacted with FBXO7 in HEK293 cells (Fig. 4A). However, this binding was not observed with caspases 1, 2, 3, 7, or 10 (Fig. 4A).

To determine which region of FBXO7 is responsible for its interaction with caspase 8, HEK293 cells were transfected with caspase 8-V5/His alone together with FBXO7-WT or one of its deletion mutants, and coimmunoprecipitation was performed. As shown in Figure S3B, immunoblot analysis of anti-FLAG immune complexes with anti-V5 IgG revealed that caspase 8 binds to FBXO7-ΔFa and FBXO7-ΔC, as well as FBXO7-WT. However, this interaction was not seen with FBXO7-ΔU or FBXO7-ΔN mutant. These results suggest that caspase 8 binds to the FBXO7 via its N-terminal Ubl domain.

To verify the biochemical interaction between FOXO4 and caspase 8, HEK293 cells were transfected with plasmid encoding the HA-tagged catalytically inactive caspase 8-C360S mutant and FLAG-FOXO4, either alone or in combination. Coimmunoprecipitation assays revealed that ectopically expressed FOXO4 is bound to caspase 8 (Fig. 4B).

To determine whether caspase 8 was involved in the FBXO7-induced degradation of FOXO4, we evaluated the effect of the caspase 8-specific inhibitor Z-IETD-FMK. Similar to the effects observed for Z-VAD-FMK, pretreatment with Z-IETD-FMK considerably inhibited the FBXO7 proteolysis of FOXO4 (Fig. 4C). Furthermore, siRNA-mediated knockdown of caspase 8 expression blocked the degradation of FOXO4 (Fig. 4D).
Conversely, the overexpression of caspase 8 plus FBXO7 enhanced FBXO7-mediated FOXO4 degradation compared with that of cells transfected with FBXO7 alone (Fig. 4E). However, this was not observed in cells transfected with catalytically inactive caspase 8-C360S (Fig. 4F). In addition, endogenous FOXO4 level was reversely increased by siRNA-mediated knockdown of caspase 8 expression (Fig. 4G). Taken together, these results indicated that the caspase 8 is involved in the FBXO7-mediated FOXO4 degradation. However, the FOXO4 level was not completely recovered by peptide inhibitor of caspase 8 or caspase 8-specific siRNA. So, we assume that the caspase 8-dependent pathway as well as caspase 8-independent novel mechanism may operate together in the downstream steps of FBXO7-induced FOXO4 proteolysis reaction.

Next, we examined whether FBXO7 activates the caspase 8. Upon activation, full-length caspase 8 is known to be processed into two auto-cleavage fragments having the sizes of 43/41 and 19 kDa (denoted as p43/p41 and p19, respectively) (25). Based on this report, cells were transfected with the plasmid encoding caspase 8-V5/His alone or together with one of FLAG-FBXO7-WT, FLAG-FBXO7-AU, or FLAG-FBXO7-ΔF, and we checked which cells could generate the cleavage intermediate of caspase 8, p43/p41. Immunoblotting analysis of cell lysates demonstrated that cells overexpressing exogenous FBXO7-WT produced a greater level of p43/p41 form by more than 18-fold than mock-transfected control cells (Fig. 4H). However, this effect was not seen with cells transfected with FBXO7-ΔU and FBXO7-ΔF mutant (Fig. 4H). Taken together, these results suggested that FBXO7 activates the caspase 8.

Caspase 8 is indirectly involved in FOXO4 degradation

Next, we tried to examine whether caspase 8 directly cleaves FOXO4, and if it occurs, to identify the cleavage site(s) of caspase 8 on the FOXO4. Caspases cleave their substrates almost exclusively after Asp residues (26). Several studies have suggested the preferred cleavage sequences of caspase 8 (Fig. S4A) (27–30). Based on these sequences, nine point mutants of FOXO4 having the substitution at highly probable 9 Asp sites with Ala, respectively, out of total 23 Asp sites in human FOXO4 protein were generated (i.e., D21A, D53A, D143A, D251A, D373A, D473A, D482A, D492A, and D504A; Fig. S4B). We then examined whether FBXO7 could still digest each of these mutants. Immunoblotting analysis revealed that caspase 8 cleaved all those nine point mutants of FOXO4 to a similar extent of wild-type FOXO4 (Fig. S4C). To further determine whether caspase 8 cleaves non-conserved site of FOXO4, the remaining 13 Asp sites (i.e., D17 and 19A, D140A, D199A, D308A, D380A, D387A, D464A, D469 and 471A, D484A, D489A, D498A) that do not include the substrate cleavage sequence of caspase 8 were additionally mutated (27–30), excluding the D2 residue since...
it is close to the amino terminus. As shown in Figure S4D, all those 13 point mutants of FOXO4 were still cleaved by caspase 8. These results indicated that FBXO7-mediated caspase 8 activation appeared to be indirectly involved in FOXO4 degradation, although the detailed working mechanism is not clarified, which could occur through additional unknown target(s).

Figure 4. FBXO7 decreases FOXO4 stability through a caspase 8 activation. A, HEK293 cells were transfected for 24 h with the plasmids encoding FLAG-caspase 1-C284A, FLAG-caspase 2-C303A, FLAG-caspase 3-C163A, FLAG-caspase 7-C186A, HA-caspase 8-C360S, or HA-caspase 10-C401A. Cell lysates were immunoprecipitated using anti-FBXO7 antibody followed by immunoblotting with the indicated antibodies. B, HEK293 cells were transfected for 24 h with plasmids encoding HA-caspase 8-C360S and/or FLAG-FBXO4. Cell lysates were immunoprecipitated with anti-HA antibody. The precipitates were immunoblotted screened using the indicated antibodies. C, HEK293 cells were transfected for 24 h with plasmids encoding FLAG-FOXO4 and/or Myc-FBXO7. The cells were then treated for additional 3 h with vehicle (−) or Z-IETD-FMK (20 μM). Relative levels of FOXO4 were quantified and the results presented as the mean ± SD of three independent experiments (**p ≤ 0.001; *, p ≤ 0.05). D, HEK293 cells were transfected for 48 h with siRNA-control (CTL), siRNA-caspase 8, a plasmid encoding FLAG-FXO4, or a plasmid encoding Myc-FBXO7, either alone or in combination. Cell lysates were immunoprecipitated and screened using the indicated antibodies. Relative levels of FOXO4 were quantified and the results presented as the mean ± SD of three independent experiments (***p ≤ 0.001; **p ≤ 0.01; *, p ≤ 0.05). F, HEK293 cells were transfected for 24 h with a plasmid encoding caspase 8-V5/His, either alone or in combination. Cell lysates were immunoblotted and screened with anti-FOXO4 or anti-caspase 8 antibodies. H, HEK293 cells were transfected for 24 h with a plasmid encoding caspase 8-V5/His, Flag-FBXO7-WT, Flag-FBXO7-DU, or Flag-FBXO7-ΔF alone or in combination. Cell lysates were immunoblotted and screened using the indicated antibodies. Relative levels of p43/41 were quantified and the results presented as the mean ± SD of three independent experiments (***, p ≤ 0.001; ***, p ≤ 0.001; ***, p ≤ 0.001). Hsp90, β-actin, and Tubulin served as loading controls.

FBXO7 decreased FOXO4 transcriptional activity

The transcription factor FOXO4 plays a role in cell cycle arrest and apoptosis (31). For instance, FOXO4 regulates transcription of cyclin-dependent kinase inhibitor p27kip1 (17). Based on that report, we determined whether FBXO7, through proteolysis of FOXO4, controlled the transcription of p27kip1, which is a typical downstream target gene of FOXO4. To initially examine whether FBXO7-mediated degradation of FOXO4 regulated the endogenous levels of p27kip1 protein, HEK293 cells were transfected with plasmids encoding FLAG-FOXO4, either alone or in combination with Myc-FBXO7. Immunoblot analysis of cell lysates using an anti-p27kip1 antibody revealed that p27kip1 levels were significantly increased by the transfection of FOXO4 alone. In contrast, cotransfection of both FOXO4 and FBXO7 resulted in decreased p27kip1 levels via the reduced levels of FOXO4 (Fig. 5A). Meanwhile, knockdown of FBXO7 expression using
FBXO7-specific siRNA markedly increased p27kip1 levels (Fig. 5B).

We then assessed changes in p27kip1 mRNA expression in the absence and presence of FBXO7. Real-time PCR analysis revealed that cells overexpressing exogenous FOXO4, which was used as a positive control, displayed 4.8-fold greater induction of p27kip1 mRNA levels. There was no considerably change in p27kip1 mRNA levels compared with that with the mock-transfected cells, regardless of FBXO7 (Fig. 5C). Moreover, cells coexpressing exogenous FOXO4 and FBXO7 exhibited significantly reduced p27kip1 mRNA levels compared with that of cells overexpressing FOXO4 alone (Fig. 5C). We further measured the effect of FBXO7 on p27kip1-specific activity using a dual luciferase reporter assay. Consistent with the pattern of p27kip1 mRNA expression determined by the PCR analysis, cells overexpressing exogenous FOXO4 showed 1.6-fold higher p27kip1-specific luciferase activity compared with that of mock-transfected control cells. In addition, cells coexpressing exogenous FOXO4 and FBXO7 exhibited greatly reduced p27kip1 luciferase activity compared with that of cells transfected with FOXO4 alone (Fig. 5D). Taken together, these results suggested that FBXO7 negatively regulated the transcriptional activity of FOXO4 via its protein degradation.

FBXO7 exacerbated 6-OHDA-induced neuronal cell death via negative regulation of FOXO4 levels

Treatment of neuronal cells with 6-OHDA produced oxidative stress through the generation of reactive oxygen species (ROS). The toxic effect of 6-OHDA in dopaminergic neurons reflects pathological circumstances and cell death in PD (32). In addition, several reports have revealed that 6-OHDA treatment triggers dysregulated autophagy in neuronal cells through caspase-dependent cell death (33). Based on these reports, the current finding of caspase-8 activation being involved in FBXO7-induced proteolysis of FOXO4 raises the possibility that this novel pathway may be activated or/and contribute to neuronal cell death in response to 6-OHDA. To evaluate the validity of this possibility, we tested the effect of 6-OHDA treatment on the proteolysis of FOXO4 in mouse dopaminergic MN9D cells. When the cells...
were exposed to 6-OHDA, endogenous FOXO4 levels were greatly reduced in a dose-dependent manner (Fig. 6A). Interestingly, FBXO7 levels were also reduced in a dose-dependent but in a less sensitive manner than that of FOXO4. To determine whether FBXO7 affected 6-OHDA-induced cell death and to identify the underlying mechanism, MN9D cells were transfected with plasmids encoding FLAG-FBXO7-ΔF, which lacked the F-box domain at the C-terminus. Cells treated with 6-OHDA and expressing the dominant-negative FLAG-FBXO7-ΔF mutant showed 3-fold higher levels of FOXO4 compared with cells treated with 6-OHDA alone (Fig. 6B). Based on a previous finding that 6-OHDA treatment mediates caspase-8 activation in MN9D cells (34), we examined whether Z-IETD-FMK could rescue 6-OHDA-mediated reduction of FOXO4 levels. Immunoblot analysis revealed that FOXO4 levels increased 2.6-fold in MN9D cells cotreated with 6-OHDA and Z-IETD-FMK compared with that in MN9D cells treated with 6-OHDA alone (Fig. 6C). These results suggested that 6-OHDA treatment caused FOXO4 proteolysis in MN9D cells through the action of both FBXO7 and caspase 8.

Many reports have demonstrated that the cytoprotective roles of FOXOs are mainly attributable to antioxidative function in response to oxidative stress (35). Therefore, we examined whether 6-OHDA-induced FOXO4 degradation affected cell viability and whether FBXO7 played a role during the process. Firstly, we checked the transfection efficiency of FOXO4-siRNA and FOXO4-siRNA in MN9D cells. Immunoblotting analysis revealed that the expression of endogenous FOXO4 was efficiently downregulated by its siRNA. In addition, endogenous FOXO4 level was correspondingly increased by FOXO4-siRNA (Fig. 7A and B).

After MN9D cells were treated with various concentrations of 6-OHDA, cell viability was measured using LDH cytotoxicity assays. As shown in Figure 7C, MN9D cell cytotoxicity increased in the presence of 6-OHDA in a dose-dependent manner. MN9D cells transfected for 48 h with control siRNA, siRNA-FOXO4, or siRNA-FBXO7 were treated with 100 μM 6-OHDA or vehicle. Cells that underwent siRNA-mediated FOXO4-knockdown alone exhibited a greater than 1.7-fold increase in 6-OHDA-induced cytotoxicity compared with that of cells transfected with control siRNA (Fig. 7D). Meanwhile, cells that underwent siRNA-mediated FBXO7-knockdown alone showed approximately 7% lower levels of 6-OHDA-induced cytotoxicity compared with that of cells transfected with control siRNA (Fig. 7D). When cells were cotransfected with both FOXO4-siRNA and FBXO7-siRNA and then treated with 6-OHDA, cellular cytotoxicity decreased by more than 2-fold compared with that of cells that underwent FOXO4-knockdown alone (Fig. 7D). Taken together, these findings suggested FBXO7 potentiated the cytotoxicity of 6-OHDA in neuronal cells and that this occurred through the degradation FOXO4 and consequent blockade of the cytoprotective effect of FOXO4.

The PD-linked FBXO7-T22M mutant reduced FOXO4 stability much greater than wild-type FBXO7

We then investigated the effect of familial PD-linked pathogenic mutations of FBXO7 on FOXO4 degradation. Four missense or nonsense point mutations have been reported to date in FBXO7 that are linked to familial PD (36). We selected three of the mutations (T22M, R378G, and R498X) to examine their effect on FOXO4 degradation. The T22M mutation is located in the N-terminal Ubl domain, the R378G mutation is located at the end of the F-box domain, and the R498X mutation is in the C-terminal proline-rich

**Figure 6. Treatment with 6-OHDA reduces FOXO4 levels via FBXO7-mediated proteolysis.** A, MN9D cells were treated for 6 h with vehicle (−) or 6-OHDA. Cell lysates were then immunoblotted with anti-FOXO4 or anti-FBXO7 antibodies. B, MN9D cells were transfected for 24 h with a plasmid encoding FLAG-FBXO7-ΔF mutant and then treated for additional 6 h with vehicle (−) or 6-OHDA (50 μM). Cell lysates were immunoblotted and screened using anti-FOXO4 or anti-FLAG antibodies. Relative levels of FOXO4 were quantified and the results presented as the mean ± SD of three independent experiments (**p ≤ 0.0001; *p ≤ 0.05**). C, MN9D cells were left untreated or treated for 3 h with 6-OHDA (50 μM), either alone or in combination with Z-IETD-FMK (20 μM). Cell lysates were immunoblotted and screened using anti-FOXO4 antibody. Relative levels of FOXO4 were quantified and the results presented as the mean ± SD of three independent experiments (**p ≤ 0.0001; **p ≤ 0.001**). Tubulin, GAPDH, and Hsp90 were served as loading controls.
region (PRR) (Fig. 8A). Cells were transfected with FOXO4, either alone or in combination with wild-type FBXO7 or one of the FBXO7 mutants. All three point mutants of FBXO7 reduced the levels of FOXO4, similar to the pattern observed with the transfection of wild-type FBXO7. In particular, the FBXO7-T22M mutant facilitated the degradation of FOXO4 approximately 3.9-fold greater than that of wild-type FBXO7 (Fig. 8B). These results indicated that the FBXO7 T22M mutation within the Ubl domain may affect the proteolytic activity of FBXO7, possibly by increasing FBXO7-binding to FOXO4 or by further stimulation of the novel caspase 8-linked pathway. This could consequently contribute to

Figure 7. FBXO7 reduces cytoprotective effects of FOXO4 through 6-OHDA-induced cell death. A, MN9D cells were transfected for 48 h with control siRNA (CTL) or FBXO7-siRNA, and the cell lysates were immunoblotted and screened using anti-FOXO4 or anti-FBXO7 antibodies. B, MN9D cells were transfected for 48 h with control siRNA (CTL), FOXO4-siRNA #1, or FOXO4-siRNA #2. Cell lysates were immunoblotted and screened using the indicated antibodies. Hsp90 and Tubulin served as loading controls. C, MN9D cells were treated for 4 h with DMSO (vehicle) or the indicated concentrations of 6-OHDA. Cell toxicity was measured using LDH assays. Data are presented as the mean ± SD of three independent experiments (***p ≤ 0.0001). D, MN9D cells were transfected for 48 h with siRNA-control (CTL), siRNA-FOXO4, or siRNA-FBXO7 and then treated for additional 4 h with DMSO or 6-OHDA (100 μM). Data are presented as the mean ± SD of six independent experiments (***p ≤ 0.0001).

Figure 8. The PD-linked FBXO7-T22M mutant reduces FOXO4 stability much greater than wild-type FBXO7. A, Schematic of PD-linked mutations in FBXO7. B, HEK293 cells were transfected for 24 h with plasmids encoding FLAG-FOXO4, wild-type FLAG-FBXO7, mutant FLAG-FBXO7-T22M, mutant FLAG-FBXO7-R378G, or mutant FLAG-FBXO7-R498X, either alone or in combination. Cell lysates were immunblotted and screened using the indicated antibodies. Relative levels of FOXO4 were quantified and the results presented as the mean ± SD of three independent experiments (***p ≤ 0.0001; **p ≤ 0.001). Hsp90 served as a loading control.
more severe dysfunction of FOXO4 in variant FBXO7-linked familial PD.

**Discussion**

Age-related changes in a number of cellular processes, such as excessive oxidative stress and mitochondrial dysfunction, can promote cell death, including neuronal cell death (37–40). Accordingly, aging is a major risk factor for the development of NDDs. FOXO transcription factors are important determinants in extending the human life span, and their functional defects are consequently involved in age-related diseases, such as cancer, diabetes, and NDD. Similar to other FOXOs, FOXO4 is associated with longevity through the insulin/IGF-1 signaling pathway (15, 41). As many FOXOs are essential for neuronal survival or apoptosis (19), FOXO4 may be linked to neuronal cell death and PD pathogenesis; however, its molecular mechanism has not been elucidated. Here we found that PD-associated FBXO7 promoted the degradation of FOXO4 in mammalian cells. Moreover, the cytoprotective levels of FOXO4 were greatly reduced in dopaminergic MN9D cells treated with 6-OHDA through the action of FBXO7 and caspase 8.

According to multiple studies, the cytoprotective effect of FOXO4 is attributable to its antioxidant activity. For example, FOXO4 promotes the detoxification reaction through transcriptional activation of antioxidant enzymes, such as superoxide dismutase 2 and catalase (42). In contrast to FOXO4, FBXO7 is able to exert either cytoprotective or cytotoxic action in response to stress, depending on the differential cellular context. Regarding the neurotoxic effect, FBXO7 promotes PINK1 ubiquitination and degradation. The FBXO7 small-molecule inhibitor increases PINK1 stability by reducing the interaction between FBXO7 and PINK1. As PINK1 has cytoprotective effects, the treatment of FBXO7 inhibitors improves cell viability and reduces dendrite injury against 1-methyl-4-phenylpyridium ion (MPP+) toxicity in human SH-SY5Y cells and primary neurons (43). On the other hand, soluble wild-type FBXO7 exhibits cytoprotective activity. For example, mild and transient stress can induce FBXO7 expression to protect cells, which facilitates neuroprotective mitophagy (44).

The current study provided additional evidence that FBXO7 displays neurotoxic action, which may be produced in part via FOXO4 degradation. Furthermore, it was found that FBXO7 exaggerated MN9D cell death in response to 6-OHDA. Interestingly, all three PD-linked FBXO7 mutants reduced FOXO4 degradation, wherein the FBXO7-T22M mutant’s effect was most pronounced.

In regard to the SCF-independent noncanonical function of FBXO7, FBXO7 affects the biochemical and functional activity of its multiple binding partners. For example, binding of FBXO7 to Cdk6 does not change its expression levels, but does enhance Cdk6 activity, suggesting that FBXO7 acts as an assembly scaffold for the formation of the cyclin D/Cdk6 complex (12). The current study suggested that FBXO7 also interacted with caspase 8, triggering its activation and leading to FOXO4 degradation.

In eukaryotic cells, intracellular protein degradation is mediated by two major pathways, the ubiquitin-proteasome pathway and lysosomal autophagy pathway (24). In the current study, we attempted to clarify which of these systems was involved in FOXO4 degradation. We showed that a caspase inhibitor markedly blocked the FBXO7-mediated proteolysis of FOXO4, but this effect was not observed with proteasome or lysosome inhibitors. These results demonstrated that FBXO7 reduced the protein stability of FOXO4 through an SCF-independent caspase-linked pathway. Consistent with our findings, a previous study reported that treatment with a proteasome inhibitor fails to rescue Mdm2-mediated FOXO4 degradation (45). In support of our finding, FOXO4 is reported to be a target of degradation via both the non-UPS and UPS pathways. Concerning FOXO4 degradation through non-UPS pathway, Mdm2 acts as an E3 ligase for FOXO4 degradation and mediates its mono-ubiquitination. Mdm2-mediated reduction of FOXO4 levels is not rescued by MG132 treatment (45). In contrast, the viral oncoprotein Tax induces FOXO4 degradation and generates its cleavage products via the proteosome pathway in human T-cell leukemia virus type 1 (HTLV-1)-infected cells (46). Moreover, in RAS-mutant cancer cells, casein kinase 1α phosphorylates FOXO4 at Ser265 and Ser268, leading to its proteolysis by nuclear proteasomes (47).

We also demonstrated that caspase 8 binds to both FBXO7 and FOXO4. Although protease-dependent protein degradation is a minor pathway, several proteins have been identified as targets of the caspase-dependent pathway (48, 49). No known target has been reported as substrate(s) of caspase 8, except for cIAP-1. As a substrate for caspase 8, cIAP-1 is likely to have multiple cleavage sites. TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of liver cancer cells is associated with the degradation of cIAP-1 and X-linked IAP (XIAP) (50). Pan-caspase inhibitor Q-VD-OPH stabilizes cIAP-1 protein levels during TRAIL treatment, but lysosomal inhibitors (Bafilomycin A1, CRA025850) and proteasome inhibitor (MG132) do not. Specifically, caspase-8 knockdown stabilizes both cIAP-1 and XIAP, while caspase-9 knockdown prevents XIAP degradation, but not cIAP-1 degradation (50). These results suggest that TRAIL-induced cIAP-1 degradation requires caspase-8 activity. While our current data suggested that FBXO7-mediated caspase 8 activation appeared to be indirectly involved in the proteolysis of FOXO4, further study is necessary to clarify the entire pathway and to characterize the unknown target(s) participating in the degradation of FOXO4 after caspase 8 activation.

Mutation of FBXO7 has been found to cause autosomal recessive early-onset PD (4). For example, the R378G mutation of FBXO7 has been shown to reduce the affinity of the SCF complex for Skp1, whereas the R498X mutation results in the removal of 24-amino acids from the substrate-binding domain, reducing SCF-dependent E3 ligase activity. In addition, the T22M mutation in the Ubl domain interferes with its interaction with parkin, preventing its translocation to depolarized mitochondria and consequently suppressing mitophagy (51–53). Interestingly, all three pathogenic PD-linked point
mutants of FBXO7 reduced the degradation of FOXO4 in our current study with the strongest effect being observed for the FBXO7-T22M mutant.

Both 6-OHDA and MPP+ are neurotoxins commonly used to induce PD symptoms in mice. A previous study revealed that cytochrome c is released into the cytosol in dopaminergic neurons and MN9D dopaminergic cells after 6-OHDA and MPP+ treatment, respectively. Meanwhile, treatment with a caspase inhibitor blocks 6-OHDA-mediated neurotoxicity, but not MPP+ cytotoxicity (54, 55). These results indicate that 6-OHDA treatment induces caspase-dependent neuronal cell death. This hypothesis was further supported by our current finding that 6-OHDA-induced degradation of FOXO4 in MN9D cells occurred through the FBXO7-mediated novel caspase-linked pathway, specifically, via caspase 8.

In conclusion, based on findings of the present study, we propose a new regulatory pathway for the action of FOXO4. The findings also suggest that FBXO7-mediated proteolysis of FOXO4 may contribute to the pathogenesis of PD through the cytotoxic effect of FBXO7.

**Experimental procedures**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Lipofectamine 2000, polyethylenimine (PEI) reagent, and anti-rabbit and anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from PerkinElmer Life and Analytical Sciences. Protein A Sepharose beads were purchased from GE Healthcare Bio-Sciences. Proteasome inhibitor MG132 was purchased from Sigma-Aldrich. PI3K inhibitor LY294002 and caspase inhibitor Z-VAD-FMK were purchased from Calbiochem, and caspase-8 inhibitor Z-IETD-FMK was purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence (ECL) reagent was purchased from Abclon and Advansta. All other chemicals used in the study were analytical-grade commercial products and purchased from Sigma-Aldrich. Primary antibodies for western blotting included mouse monoclonal anti-Myc (1:1000, sc-40, Santa Cruz Biotechnology), anti-FLAG (1:1000, F3165-1MG, Sigma-Aldrich), anti-HA (1:1000, sc-7392, Santa Cruz Biotechnology), anti-V5 (1:10,000, R960-25, Invitrogen), anti-FBXO7 (1:1000, sc-271763, Santa Cruz Biotechnology), anti-caspase 8 (1:1000, 9746S, Cell Signaling Technology), anti-tubulin (1:10,000, sc-8017, Santa Cruz Biotechnology), anti-Hsp90 (1:10,000, sc-13119, Santa Cruz Biotechnology), anti-actin (1:10,000, sc-47778, Santa Cruz Biotechnology), and rabbit monoclonal anti-GAPDH (1:10,000, sc-22323, Santa Cruz Biotechnology) and rabbit monoclonal anti-FOXO4 (1:5000, ab128908, Abcam), anti-FBXO7 (1:1000, sc-86450, Santa Cruz Biotechnology), and anti-p27kip1 (1:500, #2552, Cell Signaling Technology). Secondary antibodies were HRP-conjugated anti-mouse (AP124P) and anti-rabbit (AP132P) purchased from EMD Millipore.

**DNA constructs and RNA interference**

Mammalian expression vectors for FLAG-tagged human wild-type FBXO7 isoform-1 and its deletion mutants were provided by Dr H. J. Kuiken (The Netherlands Cancer Institute, Amsterdam, Netherlands). Mammalian expression vectors for all caspase constructs were provided by Dr Y. K. Jung (Seoul National University, Seoul, Republic of Korea). The mammalian expression vector for FLAG-tagged human FOXO4 (plasmid #17549) and pGL3-p27Luc vector (plasmid #23047) were purchased from Addgene Korea. All plasmid sequences were verified by DNA sequencing (COSMO Genetics). Small interfering RNAs (siRNAs) for FBXO7 and FOXO4 and control scrambled siRNA (cat. # 51-01-14-04) were designed and synthesized by Integrated DNA Technologies (Hanam-si). The FBXO7-specific siRNA duplex sense and antisense sequences were 5’-UUGGUUCCUCUCUAGAGUGAAGAGUdTdT-3’ and 5’-ACUUCAACUCAGAGGAGAACCAdTdT-3’, respectively. The FOXO4-specific siRNA duplex sense and antisense sequences were 5’-UAGAUAGUGACUAAGUAAAAACACdTdT-3’ and 5’-GUGUUUAGUACUGUCACUAUCdTdT-3’, respectively. The caspase 8-specific siRNA was designed and synthesized by Bioneer. The caspase 8-specific siRNA duplex sense and antisense sequences were 5’-GCUGCUCCUCGGAUUUAUdTdT-3’ and 5’-AUUAAUUCGGAGACGACdTdT-3’, respectively.

**Cell culture and DNA transfection**

Human embryonic kidney 293 (HEK293) cells and human breast cancer MCF7 cells were maintained in DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. FBXO7- knockout HAP1 cells (#HZGHC 002684c003; Horizon Discovery) were maintained in Iscove’s Modified Dulbecco’s Medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Mouse neuroblastoma MN9D cells were cultured on dishes coated with 25 μg/ml Poly-D-lysine (Sigma-Aldrich) in high-glucose DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin in an atmosphere of 90% air and 10% CO2. Human neuroblastoma SH-SY5Y cells were maintained in DMEM/F12 supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. All cells were grown at 37 °C with 5% CO2 unless indicated otherwise. All DNA transfections were carried out using Lipofectamine 2000 or PEI reagent according to the manufacturer’s instructions.

**Coimmunoprecipitation and immunoblot analysis**

Cell lysates were washed with ice-cold phosphate-buffered saline (PBS), scraped, and mixed with 1% Nonidet P-40 lysis buffer (50 mM Tris, pH 7.5; 1% Nonidet P-40; 150 mM NaCl; 10% glycerol; 1 mM sodium orthovanadate; 1 mM EDTA; 1 mM sodium fluoride; 2 mM phenylmethylsulfonyl fluoride). The cells were sonicated and the supernatants then collected by centrifugation at 13,000g for 15 min at 4 °C. For immunoprecipitation, 500–1000 μg of cell lysates was incubated with 1 μg of appropriate antibodies overnight at 4 °C with gentle rotation. Protein A Sepharose
beads were then added and incubated for 2 h at 4 °C with
gentle rotation. The beads were washed with 1% Nonidet P-40
lysis buffer and the immunocomplexes dissociated by boiling
in 2× sample buffer. The samples were resolved by SDS-PAGE
and transferred to a nitrocellulose membrane (Whatman, GE
Healthcare Life Sciences). The membranes were blocked in
TBST buffer (20 mM Tris, pH 7.5; 137 mM NaCl; and 0.1%
Tween 20) plus 5% nonfat dry milk for 1 h at room temper-
ature. The membranes were then incubated with the appro-
priate primary antibody at 4 °C overnight. Membranes were
washed in TBST and incubated with the HPR-conjugated
secondary antibody for 2 h at room temperature. The mem-
branes were washed with TBST and the protein bands visual-
ized using ECL reagents, following the manufacturer’s
instructions.

**Preparation of mouse whole-brain lysates**

Whole brains were obtained from 6-week-old male C57BL/
6 mice (Orient). The brain tissues were homogenized and
sonicated in lysis buffer containing 50 mM Tris (pH 7.4),
150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate,
0.1% SDS, and a protease inhibitor cocktail (Sigma-Aldrich).
The samples were centrifuged at 13,000 g for 20 min at 4 °C
and the supernatants collected.

**Immunocytochemistry analysis**

Human SH-SY5Y cells were seeded onto poly-D-lysine-
coated cover glasses. The adherent cells were washed twice
with PBS and immediately fixed in 3.7% formaldehyde for
10 min at room temperature. After fixation, the cells were
permeabilized with 0.1% Triton X-100 for 10 min and blocked
with 1% BSA in TBST for 1 h at room temperature. The cells
were immunostained using rabbit polyclonal anti-FOXO4
and/or mouse monoclonal anti-FBXO7 antibodies, washed,
and incubated with Alexa Fluor 488- or Alexa Fluor 594-
coujugated anti-IgG antibodies. Images were captured using
an LSM 880 confocal microscope (Carl Zeiss) and processed
using a Zeiss LSM Image Browser (Carl Zeiss).

**RNA extraction and real-time analysis**

Total RNA was isolated from cells and target mRNAs were
amplified by real-time PCR using complementary DNA (cDNA)
as the template. The primer sequences for the p27kip1 and
GAPDH genes were as follows: p27kip1-forward, 5′-AACCGG
CCACAGAAACAGA-3′; p27kip1-reverse, 5′-CTGGTGGATTCACTGAAGGAC-3′,
GAPDH-forward, 5′-TGGACACC
AATCATGACT-3′, and GAPDH-reverse, 5′-GCATTCCCC
GTTACCAGAC-3′.

**Luciferase reporter assays**

MCF7 cells were transfected with the luciferase reporter
plasmid pGL3-p27-Luc. The cells were cotransfected with
pRL-Luc vector, which was used to normalize the transfection
efficiency. After 24 h of DNA transfection, cell lysates were
collected and firefly and Renilla luciferase activities were
measured using the Dual-Luciferase Reporter Assay System
(Promega), following the manufacturer’s instructions. All
firefly luciferase measurements were normalized to the Renilla
luciferase measurements from the same samples.

**Lactate dehydrogenase (LDH) cytotoxicity assays**

Cytotoxicity was evaluated using an LDH Cytotoxicity
Detection Kit (Takara). After siRNA transfection for 48 h,
MCF7 cells were treated with 100 μM 6-OHDA for an addi-
tional 6 h. Cell-free culture media was then collected and used
in the LDH assay, according to the manufacturer’s
instructions. The maximum LDH release (referred to as “high
control”) was determined by solubilizing cells in 1% Triton
X-100; the spontaneous LDH release (referred as “low
control”) was determined by incubating cells in medium alone.
Absorbance was measured at 490 nm using a microplate
reader. The percentage of cytotoxicity was determined using
the formula: Cytotoxicity = [(experimental value – low con-
trol)/(high control – low control)] × 100%.

**Statistical analysis**

Statistical analysis of data from different groups was per-
formed using an unpaired Student’s t test. All graphs were
generated using GraphPad Prism 5.0 program (GraphPad
Software, Inc.). All values are reported as mean ± Standard
Deviation (SD). The intensities of the western blot bands were
measured using GelQuant.NET software (version 1.8.2).

**Data availability**

All data that support the findings of this study are contained
within the manuscript and are available from the corre-
sponding author upon reasonable request.

**Supporting information**—This article contains supporting
information.

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**Abbreviations**—The abbreviations used are: AD, Alzheimer’s dis-
ease; DISC, death-inducing signaling complex; DMEM, Dulbecco’s
modified Eagle’s medium; ECL, enhanced chemiluminescence;
FADD, FAS-associated death domain; FBS, fetal bovine serum;
FBXO7, F-box only protein 7; HEK293, human embryonic kidney 293; HRP, horseradish peroxidase; MPP+, 1-methyl-4-phenylpyridium ion; NDDs, neurodegenerative diseases; 6-OHDA, 6-hydroxydopamine; PD, Parkinson’s disease; PEI, poly-ethylenimine; PRR, proline-rich region; ROS, reactive oxygen species; SCF, SKP1-Cullin1-F-box; TRAIL, TNF-related apoptosis-inducing ligand; Ubl, ubiquitin-like; UPS, ubiquitin-proteasome system; X-IAP, X-linked IAP.

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