Accumulation of the parkin substrate, FAF1, plays a key role in the dopaminergic neurodegeneration

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Received November 18, 2012; Revised December 25, 2012; Accepted January 4, 2013

This study reports the physical and functional interplay between Fas-associated factor 1 (FAF1), a death-promoting protein, and parkin, a key susceptibility protein for Parkinson’s disease (PD). We found that parkin acts as an E3 ubiquitin ligase to ubiquitinate FAF1 both in vitro and at cellular level, identifying FAF1 as a direct substrate of parkin. The loss of parkin function due to PD-linked mutations was found to disrupt the ubiquitination and degradation of FAF1, resulting in elevated FAF1 expression in SH-SY5Y cells. Moreover, FAF1-mediated cell death was abolished by wild-type parkin, but not by PD-linked parkin mutants, implying that parkin antagonizes the death potential of FAF1. This led us to investigate whether FAF1 participates in the pathogenesis of PD. To address this, we used a gene trap mutagenesis approach to generate mutant mice with diminished levels of FAF1 (Faftgt/gt). Using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mouse model of PD, we found that FAF1 accumulated in the substantia nigra pars compacta (SNc) of MPTP-treated PD mice, and that MPTP-induced dopaminergic cell loss in the SNc was significantly attenuated in Faftgt/gt mice versus Faft1+/− mice. MPTP-induced reduction of locomotor activity was also lessened in Faftgt/gt mice versus Faft1+/− mice. Furthermore, we found that FAF1 deficiency blocked PD-linked biochemical events, including caspase activation, ROS generation, JNK activation and cell death. Taken together, these results suggest a new role for FAF1: that of a positive modulator for PD.

INTRODUCTION

Parkinson’s disease (PD) is a common neurodegenerative disorder that is characterized by the progressive loss of dopaminergic neurons from the substantia nigra pars compacta (SNc) and the presence of Lewy bodies (LBs) (1,2). Familial PD cases have been associated with mutations in various susceptibility genes including parkin. Parkin serves as an important link between the dysregulation of proteosome-dependent degradation and protein aggregation in the pathogenesis of PD (3,4). Furthermore, parkin also participates in the LB-like inclusion formation via K63-linked chain in PD (5,6). In fact, several clinical parkin mutants have been shown to have lost their enzymatic activity, leading to the disruption of the ubiquitination process and abnormal accumulation of parkin substrates (3,7–20). In fact, the accumulation of parkin substrates has been shown in the brains of both PD patients and parkin-knockout mice (21,22). Therefore, the preservation of parkin activity seems to be essential for protecting against the pathogenesis of PD.

Fas-associated factor 1 (FAF1) participates in Fas-mediated apoptosis by serving as a member of the death-inducing signaling complex, and also suppresses NF-kB activity via an interaction with IKKB (23,24). Moreover, overexpression of FAF1 has been shown to sensitize cancer cells to various
chemotherapeutics (25), and FAF1 has been shown to negatively regulate the Aurora-A onco-protein and consequently induce the cell-cycle arrest in the G2/M phase and cell death (26). Consistent with these roles, decreased FAF1 expression has been observed in cases of gastric carcinoma (27). Collectively, these findings have led FAF1 to be classified as a tumor suppressor with death-promoting potential.

FAF1 has domains with high homology to ubiquitin (Ub), FAF1 interacts with the valosin-containing protein (VCP) via its N-terminal UBA domain (28). Considering that protein aggregation due to the dysregulation of ubiquitination has been found in neurodegenerative diseases, we investigated the possibility that FAF1 might be related to the pathogenesis of neurodegenerative diseases. Indeed, FAF1 has been reported to be selectively increased in the brains of PD patients (29). Interestingly, the human FAF1-encoding gene is located on chromosome 1p32 in a region that reportedly contains one or more of the susceptibility genes associated with the PARK 10 locus associated with late-onset PD (30,31).

In this study, we sought to elucidate the molecular mechanism that connects FAF1 to the pathogenesis of PD. Our finding that dopaminergic cells are protected from PD-linked stressors in FAF1 knock-down mice and that FAF1 accumulates in the dopaminergic cells containing PD-linked parkin mutants support our novel contention that FAF1 is clinically relevant to the pathogenesis of PD.

RESULTS
FAF1 interacts with parkin

Based on the increased expression of FAF1 in the brains of PD patients (29), we investigated whether FAF1 is involved in the pathogenesis of PD. Considering that FAF1 has Ub-homologous domains, we examined whether FAF1 interacts with the E3 Ub ligase, parkin. Cellular extracts of SH-SY5Y cells were prepared and separately subjected to immunoprecipitation with antibodies against parkin and FAF1, and then reciprocally immunoblotted with antibodies against FAF1 and parkin. An endogenous complex of FAF1 and parkin was clearly detected in both cases (Fig. 1A). Immunofluorescent staining demonstrated that FAF1 and parkin colocalized in the cytoplasm. And the colocalization was further confirmed by the Z-stack images (Fig. 1C). Taken together, these data indicate that cellular complexes are formed between FAF1 and parkin in SH-SY5Y cells.

To investigate the respective domain(s) responsible for mediating the putative interaction between FAF1 and parkin, we incubated GST-fusion truncates of FAF1 with in vitro-translated parkin proteins and performed GST pull-down assays. Our results showed that parkin interacts with the Ub homologous domain 1 (UB1)-containing fragment of FAF1 (amino acids 71–201) (Fig. 1D). Similarly, GST pull-down assays using various truncated mutants of GST-parkin and in vitro-translated FAF1 proteins showed that FAF1 interacts with multiple domains of parkin, including the Ub-like domain (UBL), the really interesting new gene 1 (RING-1) domain and the region between UBL and RING-1. In contrast, FAF1 did not bind to the in-between-ring (IBR) or the really interesting new gene (RING-2) domain of parkin (Fig. 1E).

FAF1 is a novel substrate of parkin

Based on our finding that FAF1 interacts with parkin, we next examined whether parkin ubiquititates FAF1. SH-SY5Y cells were transfected with the indicated combinations of FAF1, parkin WT, parkin mutant (T240R) and Ub, and cell lysates were immunoprecipitated with an anti-HA antibody, followed by western blotting with anti-HA and anti-Flag antibodies, respectively (Fig. 2A). Our results reveal that ubiquitination of FAF1 is significantly increased by overexpression of parkin WT, while T240R mutant parkin was incompetent to ubiquitinate FAF1. To confirm the ubiquitination of FAF1 by parkin, we incubated various combinations of 35S-labeled in vitro-translated FAF1, E1, E2 (UbcH7), Ub and parkin (Fig. 2B). Among the E2 enzymes, parkin is known to use UbcH7, UbcH8 and UbcH13 (3,4,32). Therefore, we investigated which type of E2 enzyme parkin uses to ubiquitinate FAF1, and found that UbcH7 was involved in the ubiquitination of FAF1 (Supplementary Material, Fig. S1).

Parkin promotes lysine 48 (K48)- and lysine 63 (K63)-linked ubiquitination (5). In order to determine the type of Ub chain used for the ubiquitination of FAF1, we constructed several substitution mutants of Ub [for detailed information, refer to Kim et al. (33)]: 7KR (all 7 lysine residues were substituted by arginines), K48R (only 48th lysine residue was substituted by arginine), K63R (only 63rd lysine residue was substituted by arginine), 48K (48th lysine residue was intact and the other 6 lysines were substituted by arginines) and 63K (63rd lysine residue was intact and the other 6 lysines were substituted by arginines). SH-SY5Y cells were co-transfected with FAF1, parkin and the indicated Ubs. FAF1 was strongly ubiquitinated in the presence of WT, K63R and 48K, but not in that of 7KR, K48R and 63K (Fig. 2C). Collectively, our data indicate that parkin mediates the K48-linked ubiquitination of FAF1, thereby targeting FAF1 to the proteasome for degradation.

Parkin promotes the degradation of FAF1

Since E3 Ub ligases regulate the proteasome-dependent degradation of their substrates, we examined whether parkin regulates FAF1 expression. Parkin+/+ and Parkin−/− mouse embryonic fibroblasts (MEFs) were transfected with Myc-parkin (Fig. 3A). FAF1 expression was elevated in Parkin−/− MEFs when compared with that in Parkin+/+. Moreover, reconstitution of parkin to Parkin−/− MEFs restored the potential to degrade FAF1. We also examined FAF1 expression in the ventral midbrain of Parkin−/− and Parkin+/+ mice. We observed a significant increase of FAF1 expression in the ventral midbrain of Parkin−/− mice when compared with that in the Parkin+/+ mice (Fig. 3B).

And, we generated a FAF1-overexpressing stable cell line and transfected with increasing amounts of Myc-parkin (Fig. 3C). FAF1 expression was dose-dependently decreased by parkin expression, and the de novo-synthesized FAF1 was degraded much faster in parkin-expressing cells versus MOCK-transfected cells (Fig. 3D).

We next examined whether the parkin-mediated degradation of FAF1 occurred in ts20-BALB cells, which lack E1
Ub-activating enzyme activity at the restrictive temperature (34). Our results showed that parkin-mediated degradation of FAF1 occurred in ts20-BALB cells held at the permissive temperature (32°C), but the level of FAF1 remained unchanged at the restrictive temperature (39°C) (Fig. 3E). When p53 was used in a comparable temperature-controlled system (34), we observed a similar degradation pattern, with p53 levels decreasing at 32°C but not at 39°C. Therefore, our data indicate that parkin promotes the degradation of FAF1 through the Ub-dependent degradation pathway. Next, we asked whether parkin reduces FAF1 expression via regulation of the proteasome-dependent degradation pathway, and found that the observed decrease of FAF1 expression in parkin-transfected cells was significantly inhibited by treatment with the proteasome
inhibitors, MG132 and lactacystin (Fig. 3F). The results show that overexpression of parkin was associated with decreased levels of FAF1, but only in the absence of MG132 or lactacystin; in the presence of MG132 or lactacystin, overexpression of parkin had little effect on the levels of FAF1. The decreased FAF1 protein level under parkin overexpression was not a result of FAF1 mRNA degradation, as real-time quantitative RT–PCR measurement indicated that parkin overexpression did not reduce FAF1 mRNA levels (Supplementary Material, Fig. S2). Similar results were obtained in the ventral midbrain of Parkin$^{−/−}$ mice: parkin deficiency does not affect FAF1 mRNA (Supplementary Material, Fig. S3). Taken together, our results demonstrate that parkin mediates the degradation of FAF1 via the Ub-dependent proteasome pathway.

Parkin mutations have been associated with autosomal recessive juvenile parkinsonism (35,36), and parkin mutants with compromised enzymatic potentials have been identified in PD patients (20). Therefore, we investigated whether the expression level of FAF1 could be affected by the clinically identified parkin mutants that have compromised enzymatic potentials (R42P, K161N, T240R, R275W, T415N, G430D, C431F and P437L) (3,7–19). SH-SY5Y cells were co-transfected with FAF1 and parkin WT or mutants, as indicated (Fig. 3G). We found that FAF1 expression was significantly decreased in cells expressing parkin WT, but not in cells expressing any of the parkin mutants. The result is more comparable with efficient degradation of p38 (37) (Supplementary Material, Fig. S4). Our data correlate well with those of a previous study that found accumulation of FAF1 in PD patients, although the prior paper did not examine the mutation status of parkin in the examined patients (29).

FAF1 phosphorylated by Aurora-A kinase, and phosphorylated FAF1 is capable of promoting cell death (26). Therefore, we examined whether the phosphorylation status of FAF1 affects its parkin-mediated degradation. SH-SY5Y cells were co-transfected with parkin and FAF1 WT, FAF1 AA (FAF1S289.291A, a phosphorylation-deficient mutant) or FAF1 DD (FAF1S289.291D, a phosphorylation-mimicking mutant). We found that FAF1 WT, FAF1 AA and FAF1 DD were degraded equally well in parkin-expressing cells, indicating that the phosphorylation status of FAF1 did not affect its parkin-mediated degradation (Supplementary Material, Fig. S5).

Since parkin participates in the protective mitophagy that occurs in response to PD-linked stressors (38), we examined whether FAF1 affects its parkin-mediated degradation. SH-SY5Y cells were co-transfected with parkin and FAF1 WT, FAF1 AA (FAF1S289-291A, a phosphorylation-deficient mutant) or FAF1 DD (FAF1S289-291D, a phosphorylation-mimicking mutant). We found that FAF1 WT, FAF1 AA and FAF1 DD were degraded equally well in parkin-expressing cells, indicating that the phosphorylation status of FAF1 did not affect its parkin-mediated degradation (Supplementary Material, Fig. S5).

Figure 2. Parkin catalyzes the ubiquitination of FAF1 via lysine 48. (A) SH-SY5Y cells were transfected with the indicated combinations of Myc-parkin WT, Myc-parkin T240R, Flag-ubiquitin (Flag-Ub) and HA-FAF1. Cells were treated with 10 μM MG132 for 12 h, and cell lysates were immunoprecipitated (IP) with an anti-HA antibody, followed by immunoblotting with anti-HA or anti-Flag antibodies. Cell lysates were analyzed by immunoblotting with anti-FAF1 and anti-parkin antibodies. Transfection efficiency was checked by immunoblotting with anti-HA, anti-Myc and anti-Flag antibodies. The equivalency of loading was confirmed by immunoblotting with an anti-β-actin antibody. (B) Each reaction contained 35S-labeled FAF1 in addition to the indicated ubiquitination components. After the in vitro ubiquitination reactions, samples were detected by autoradiography. (C) SH-SY5Y cells were transfected with Parkin, Flag-Ubs (WT, 7KR, K48R, K63R, 48K or 63K) and HA-FAF1. Cells were treated with 10 μM MG132 for 12 h, and lysates were subjected to IP with an anti-HA antibody, followed by immunoblotting with anti-HA and anti-Flag antibodies. Cell lysates were analyzed by immunoblotting with anti-FAF1 and anti-parkin antibodies. Transfection efficiency was analyzed by immunoblotting with anti-HA, anti-Myc and anti-Flag antibodies, and loading equivalency was confirmed using an anti-β-actin antibody.
FAF1 accumulates in the SNC of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice

We next sought evidence for a causal relationship between increased FAF1 expression and the pathogenesis of PD by testing whether FAF1 expression was elevated in the brain of the PD mouse model. It is known that parkin is incapacitated by S-nitrosylation in a mouse model of PD generated by treatment with the neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), as well as in a subset of PD patients (39–41). Mice were injected with MPTP (20 mg/kg body weight) once per day for 5 consecutive days (42), and FAF1 expression was analyzed in the ventral midbrain, cortex, striatum and cerebellum. There was a significant decrease of tyrosine hydroxylase (TH) expression upon MPTP treatment, implicating loss of dopaminergic cells. A significant increase of FAF1 expression was observed in the ventral midbrain of MPTP-treated mice versus saline-injected mice (Fig. 4A). Moreover, MPTP induced a time-dependent increase of FAF1 in the ventral midbrain until 7 days after MPTP treatment (Supplementary Material, Fig. S7). In contrast, FAF1 expression was not altered in the cortex (Fig. 4B), striatum (Fig. 4C) or cerebellum (Fig. 4D). In order to further confirm this increase of FAF1 in vivo, an immunohistochemical analysis was performed (Fig. 4E). Our results revealed that MPTP treatment enhanced FAF1 staining in the SNC which is immunoreactive for TH, a marker for the SNC. These findings indicate that FAF1 expression is elevated in the SNC of the MPTP-induced PD mouse model.

*Fafl*<sup>gt/gt</sup> mice are protected from MPTP-induced neuronal degeneration in the SNC

To further elucidate the function of FAF1, we generated FAF1-deficient mice harboring insertions at different positions of the *Fafl* locus. These mice were genotyped by Southern blot analysis. We found that although the disruption of *Fafl* by a gene-trap insertion in intron 2 resulted in embryonic lethality (BayGenomics cell line RRT033, data not shown), a gene-trap insertion in intron 8 generated a hypomorphic allele that allowed the mice to survive (BayGenomics cell line XK588); this mutant allele was designated *Fafl*<sup>gt/gt</sup> (Fig. 5A). Southern blotting showed that the WT allele generated an ~6.4 kb genomic DNA fragment that hybridized with the *Fafl*-specific probe, whereas the trapped allele yielded hybridizing fragments of 3.6 kb (Fig. 5B, left panel). Overall, FAF1 expression in MEts derived from *Fafl*<sup>gt/gt</sup> animals was markedly reduced compared with that in WT MEts at both the protein (Fig. 5B, right panel) and mRNA levels (Supplementary Material, Fig. S8). Western blot analysis of FAF1 protein expression levels in the brain regions of *Fafl*<sup>gt/gt</sup> versus *Fafl*<sup>+/+</sup> mice revealed that the mutant had significantly lower FAF1 expression levels in the ventral midbrain (Fig. 5C), striatum (Fig. 5E) and cerebellum (Fig. 5F) of *Fafl*<sup>gt/gt</sup> mice compared with those in *Fafl*<sup>+/+</sup> mice, but there was no apparent difference in the cortex (Fig. 5D).

Next, we examined whether FAF1 deficiency prevents dopaminergic neuronal loss in the MPTP-treated PD mouse model. First, we analyzed FAF1 expression in the *Fafl*<sup>gt/gt</sup> mice in response to MPTP. FAF1 level in the ventral midbrain of the MPTP-treated *Fafl*<sup>gt/gt</sup> mice was elevated when compared with that of the saline-treated mice. However, FAF1 expression level in the ventral midbrain of the MPTP-treated *Fafl*<sup>+/+</sup> mice was much higher when compared with that of the *Fafl*<sup>gt/gt</sup> mice (Fig. 5G). The extent of dopaminergic neuronal loss by MPTP treatment was significantly reduced in the ventral midbrain of *Fafl*<sup>gt/gt</sup> mice when examined immunohistochemically (Fig. 5H). Collectively, our results indicate that FAF1 deficiency prevents degeneration of dopaminergic neurons under MPTP treatment.

To address the immunohistochemical results showing dopaminergic neuronal loss is translated to the locomotor activity change, we evaluated the change of locomotor activity in MPTP-treated *Fafl*<sup>gt/gt</sup> mice by using rotarod analysis. Mice were trained on the rotarod and the locomotor activity was evaluated by the fall from the rotarod. Although the alternations of locomotor activity were significantly reduced in MPTP-treated *Fafl*<sup>+/+</sup> mice, *Fafl*<sup>gt/gt</sup> mice remained on the rotarod longer than *Fafl*<sup>+/+</sup> mice (Fig. 5I). Collectively, our data demonstrate that FAF1 deficiency blocks neuronal vulnerability to MPTP.

Parkin ameliorates FAF1-mediated cell death

We then examined whether parkin regulates PD-linked neuronal cell death. Death was assessed by the measurement of lactate dehydrogenase (LDH) release, and viability was assessed based on the detection of ATP levels in SH-SY5Y cells. We found that cells transfected with FAF1 showed increased cell death compared with MOCK-transfected cells (Fig. 6A and B). However, the co-expression of parkin attenuated the cell death seen among FAF1-expressing cells. Notably, FAF1 expression was found to sensitize these cells to the neuronal cell death caused by the oxidative-stress-inducing agent, 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>), whereas co-expression of parkin WT but not the parkin T240R mutant abrogated the FAF1-induced sensitization of neuronal cells to MPP<sup>+</sup>. Similar results were obtained in MEts: the MPP<sup>+</sup>-induced cell death of *Fafl*<sup>gt/gt</sup> MEts was significantly lower than that of *Fafl*<sup>+/+</sup> MEts, and parkin overexpression blocked this cell death in *Fafl*<sup>+/+</sup> MEts (Fig. 6C and D). Furthermore, *Parkin<sup>−/−</sup>* MEts were even a little more sensitive to MPP<sup>+</sup>-induced cell death than *Parkin<sup>+/−</sup>* MEts. Moreover, the FAF1 overexpression appeared to be largely sensitivity to cell death in *Parkin<sup>−/−</sup>* MEts (Fig. 6E and F). In contrast, MPP<sup>+</sup>-induced cell death in *Parkin<sup>−/−</sup>* MEts was significantly decreased when FAF1 was depleted by siRNA targeting FAF1 (Supplementary Material, Fig. S9). These observations clearly suggest that FAF1 is an important factor in MPP<sup>+</sup>-induced cell death. Collectively, our data indicate that parkin acts upstream of FAF1 in MPP<sup>+</sup>-induced cell death.

**FAF1 induces the generation of ROS and the activations of JNK and caspase-3 during PD-associated cell death**

A previous study showed that MPP<sup>+</sup> activates caspase-3 (Casp-3) and JNK and contributes to cell death in SH-SY5Y cells (43), and we previously reported that FAF1 overexpression enhances the activations of Casp-3 and JNK in response to Fas signaling and heat shock (44,45). Therefore, we herein examined whether FAF1 activates Casp-3 and JNK in
response to MPP\textsuperscript{+} treatment. Our results revealed that overexpression of FAF1 potentiated Casp-3 activation in MPP\textsuperscript{+}-treated SH-SY5Y cells, whereas co-expression of parkin WT, but not the parkin T240R mutant, attenuated this activation of Casp-3 (Fig. 7A). Similarly, JNK activation and Casp-3 cleavage were dose-dependently elevated in response to increasing concentrations of MPP\textsuperscript{+} in SH-SY5Y cells (Supplementary Material, Fig. S10). Casp-3 and JNK were activated in Faf1\textsuperscript{-/-} MEFs in response to MPP\textsuperscript{+}. However, activations of JNK and caspase upon MPP\textsuperscript{+} treatment were significantly attenuated in Faf1\textsuperscript{+/+} MEFs (Fig. 7B, upper panel). MPP\textsuperscript{+} increased FAF1 expression in Parkin\textsuperscript{-/-} MEFs, however to a much lesser extent than in Parkin\textsuperscript{+/+} implicating that parkin plays a suppressive role upon MPP\textsuperscript{+}-induced FAF1 expression (Fig. 7B, lower panel). Collectively, our data indicate that MPP\textsuperscript{+}-induced activations of Casp-3 and JNK correlate with the level of FAF1.

Overexpression of FAF1 reduced the cell viability of MPP\textsuperscript{+}-treated JNK\textsuperscript{I/II} MEFs more than that of JNK\textsuperscript{I/II} MEFs (Fig. 7C). Our data indicate that during MPP\textsuperscript{+}-induced cell death, FAF1 activates Casp-3 and JNK, and these activations are antagonized by parkin. Since changes in ROS homeostasis have also been implicated in MPP\textsuperscript{+}-mediated cell death (46), we investigated ROS generation over time in MPP\textsuperscript{+}-treated cells. Our results revealed that FAF1 overexpression enhanced ROS production in response to MPP\textsuperscript{+}, and this effect was reduced by co-expression of parkin WT (Fig. 7D). Treatment with the ROS scavenger, N-acetyl-l-cysteine (used as a control) decreased the level of MPP\textsuperscript{+}-induced ROS generation (Supplementary Material, Fig. S11). Collectively, these findings show that FAF1 acts upstream of Casp-3, JNK and ROS generation in the execution of MPP\textsuperscript{+}-induced cell death.

DISCUSSION

We herein show that parkin ubiquitinates death-promoting protein, FAF1, and induces its proteasome-dependent degradation, indicating that FAF1 is a novel substrate of parkin. We demonstrated that FAF1 was accumulated in SH-SY5Y cells expressing PD-linked mutant parkin (3,7–19) proteins having compromised Ub E3 ligase activity, and further report that elevated FAF1 expression enhances ROS generation and activates both Casp-3 and JNK in response to PD-linked stressor. This creates a death-prone milieu that contributes to the loss of dopaminergic neurons and decline in locomotor activity. Consistent with this, the dopaminergic neurons of FAF1\textsuperscript{-/-}\textsuperscript{-knockdown mice were resistant to PD-linked stressor, strongly suggesting that FAF1 is involved in PD pathogenesis. Collectively, our findings provide a molecular explanation for the contribution of FAF1 to PD.

FAF1\textsuperscript{gt/gt} embryos harboring a gene-trap vector insertion in intron 7 were found to die at the two-cell stage (47,48), whereas we observed survival among the Faf1\textsuperscript{gt/gt} mice generated in this study, which harbored a vector insertion in intron 8. This indicates that exon 8 of Faf1 is essential for embryonic development. Consistent with our data, FAF1 has been reported to promote β-catenin degradation and thereby affect Wnt signaling (49), which mediates anterior–posterior neuronal patterning during early embryogenesis (50). Therefore, it appears that FAF1 activity modulates the behavior of medially positioned neural plate cells.

Since a previous study reported that FAF1 expression was enhanced in a dopaminergic neurons containing region of the SNc of PD patients, the FAF1 activity might be tightly linked to PD pathogenesis (29). Consistent with this, we herein show that PD-linked stressor elevates FAF1 expression in the SNc of the mice. It is known that oxidative stress induces misfolding and aggregation of parkin, leading to loss of function (51–53). Moreover, the exogenous expressions of various clinically identified ligase-activity-deficient parkin mutants were found to compromise the degradation of parkin substrates, leading to their accumulation (21,22). This is consistent with reports that the brains of PD patients can show increased expression levels of various parkin substrates, including the death-promoting proteins, p38 and Pael-R, in which all contribute to neurodegeneration when their expression and stability are misregulated (21,54). Our data indicate that FAF1 should be added to this list of death-promoting parkin substrates.

FAF1 might participate in the pathogenesis of PD via another mechanism in addition to the one shown in this

Figure 3. Parkin-induced degradation of FAF1 occurs through the ubiquitin–proteasome system. (A) Left panel: Parkin\textsuperscript{+/+} and Parkin\textsuperscript{-/-} MEFS were transfected with Myc-parkin. Cell lysates were analyzed by immunoblotting with anti-parkin and anti-FAF1 antibodies and transfection efficiency was confirmed with an anti-Myc antibody. The equivalency of loading was confirmed using an anti-β-actin antibody. Right panel: densitometric analyses of band intensities normalized with respect to β-actin levels are presented as mean ± SEM; * * * P < 0.001. (B) Left panel: brain lysates were prepared from the ventral midbrain of 8-month Parkin\textsuperscript{+/+} and Parkin\textsuperscript{-/-} mice (n = 5), and anti-FAF1, anti-parkin and anti-β-actin antibodies were used for immunoblotting. Right panel: densitometric analyses of band intensities normalized with respect to β-actin levels are presented as mean ± SEM; * * * P < 0.001. (C) Upper panel: FAF1 levels were detected in the stable cell line. Numbers below the bands indicate relative levels of FAF1 normalized with respect to β-actin; lower panel: stable cells were transfected with increasing concentrations of Myc-parkin and cell lysates were analyzed by immunoblotting with anti-FAF1 and anti-parkin antibodies. Transfection efficiency was confirmed with an anti-Myc antibody, and an anti-β-actin antibody was used as the loading control. (D) SH-SY5Y cells transfected with or without Myc-parkin and Flag-FAF1. Twenty-four hours post-transfection, cells were washed and incubated with methionine-free medium for 1 h. The cells were pulsed with 100 μCi of \textsuperscript{35}S-methionine for 3 h. Next, the cells were harvested at the indicated times and immunoprecipitated with an anti-Flag antibody, followed by autoradiography and quantification of the Flag-FAF1 levels. Data are expressed as percentages of the control ± SEM; * P < 0.05 and ** P < 0.01. (E) ts20-BALB cells were transfected with or without Myc-parkin, Flag-ubiquitin (Flag-Ub) and HA-FAF1. Twenty-four hours post-transfection, cells were divided into two groups, one set was maintained at 32°C, and the other, at 39°C for 16 h, and then cell lysates were analyzed by immunoblotting with anti-HA and anti-p53 antibodies. The transfection efficiency was assessed with an anti-Myc antibody, and the equivalency of loading was confirmed using an anti-β-actin antibody. (F) SH-SY5Y cells were transfected with or without Myc-parkin and Flag-FAF1, and treated with MG132 (10 μM) or lactacystin (10 μM) for 12 h. Cell lysates were analyzed by western blot (WB) using anti-Flag, anti-FAF1 and anti-parkin antibodies. Transfection efficiency was determined by immunoblotting with an anti-Myc antibody, whereas the equivalency of loading was confirmed using an anti-β-actin antibody. (G) SH-SY5Y cells were transfected with Flag-FAF1 and Myc-parkin (WT and mutants), and cell lysates were analyzed by immunoblotting with an anti-Flag antibody. The transfection efficiency was confirmed using an anti-Myc antibody, and an anti-β-actin antibody was used as a loading control.
We previously reported that FAF1 interacts with VCP, a chaperon protein required for the proteasome-dependent protein destruction of the ubiquitinated substrates (55). FAF1 inhibited the chaperon function of VCP via direct interaction, and resulted in cellular accumulation of ubiquitinated proteins (28). This leads us to speculate that FAF1 accumulation might contribute to the formation of LBs (the pathological hallmark of PD), which are composed of α-synuclein plus various ubiquitinated proteins (56). Therefore, in addition, activating the death effectors, Casp-3 and JNK, we propose that FAF1 might also contribute to PD through the FAF1-dependent failure of Ub-mediated protein degradation.

To date, 15 loci have been reported to contain susceptibility genes for PD (57). The genes responsible for PD susceptibility have been identified for 12 loci, whereas those for three loci (PARK3, PARK10 and PARK12) have not yet been identified (57). Notably, the Faf1 gene maps to 1p32, where PARK10 locus is located (30,31). Several prior studies have investigated candidate susceptibility gene(s) in the PARK10 region, and mutations or deletions have been found in the genes encoding human immunodeficiency virus type 1 enhancer-binding protein 3, eukaryotic translation initiation factor 2B, subunit 3 gamma, Ub-specific peptidase 24, embryonic lethal, abnormal vision-like 4, CUB domain-containing protein 2 and ring finger protein 11 (58–62). Although a mutation analysis of Faf1 has not yet been performed, it is not highly likely that Faf1 is a candidate gene for PD susceptibility in the PARK10 locus because the pathogenesis of PD would be associated with a gain of function (not a loss of function) in FAF1.

The death effector domain-interacting domain (DEDID) of FAF1, which confers its death-effector potential in apoptosis, interacts with caspase-8 (23). However, the DEDID is a novel structural entity that does not share homology with the structural domains of any mammalian protein registered in the Protein Data Bank (www.rcsb.org). In the context of developing new therapeutic strategies, this uniqueness might give FAF1 a selective advantage over other death proteins containing more widely shared death effector domains. The development of small-molecule inhibitors that target DEDID would...
Figure 5. Faf1<sup>gt/gt</sup> mice are protected against MPTP-induced dopaminergic neurotoxicity. (A) A genomic map of the mouse Faf1 gene, which comprises 19 exons and spans ~10 kb on chromosome 4. Our gene-trap insertion into intron 8 is depicted in the mouse Faf1 locus, and the probe is indicated. B, BamHI restriction site; H, HindIII restriction site. (B) Southern blot analysis of BamHI-digested genomic DNA from the progeny from Faf1<sup>+/+</sup> mouse intercrosses. The Faf1<sup>gt/gt</sup> and trapped alleles yielded hybridizing fragments of 6.4 and 3.6 kb, respectively (left panel). FAF1 expression levels were analyzed by immunoblot analysis of immortalized Faf1<sup>+/+</sup> and Faf1<sup>gt/gt</sup> MEFs (right panel). An anti-β-actin antibody was used as the loading control. (C–F) Brain lysates were prepared from the ventral midbrain (C), cortex (D), striatum (E) and cerebellum (F) of Faf1<sup>gt/gt</sup> and Faf1<sup>+/+</sup> mice (<i>n</i> = 5), and anti-FAF1 and anti-β-actin antibodies were used for immunoblotting. Right panel: densitometric analyses of band intensities normalized with respect to β-actin levels are presented as mean ± SEM. Statistically significant differences (compared with the control saline group) were determined by ANOVA; **<i>P</i> < 0.01 and ***<i>P</i> < 0.001. (G) Left panel: brain lysates were prepared from the ventral midbrain of Faf1<sup>gt/gt</sup> and Faf1<sup>+/+</sup> mice treated with saline or MPTP, and anti-FAF1 and anti-β-actin antibodies were used for immunoblotting. Right panel: densitometric analyses of band intensities normalized with respect to β-actin levels presented as mean ± SEM; *<i>P</i> < 0.05 and ***<i>P</i> < 0.001. (H) The SNC of Faf1<sup>gt/gt</sup> mice versus Faf1<sup>+/+</sup> mice following MPTP treatment. Left panel: representative TH-stained sections of the SNC of mice that were injected with saline (a, c, e and g) and MPTP (b, d, f and h). The panel was acquired using a ×10 lens (a, b, c and d) and ×40 lens (e, f, g and h). Right panel: stereological cell counts of TH-positive dopaminergic neurons were performed on coronal sections isolated from the SNC of Faf1<sup>gt/gt</sup> versus Faf1<sup>+/+</sup> mice injected with either saline and MPTP. Statistically significant differences (compared with the control saline group) were determined by ANOVA; ***<i>P</i> < 0.001. Scale bar: 200 μm. Details of the boxed regions of the upper panel are shown in the lower panel. (I) Locomotor activity was measured in Faf1<sup>gt/gt</sup> and Faf1<sup>+/+</sup> mice, using rotarod (<i>n</i> = 10). Mice were injected with saline or MPTP (20 mg/kg body weight) once per day for 5 consecutive days. Average latency of mice to fall from rotarod was measured for 6 consecutive days after last injection. Statistically significant differences (compared with MPTP-treated Faf1<sup>++</sup> mice group) were determined by ANOVA; ***<i>P</i> < 0.001.
thus seem to represent a promising approach for the future establishment of new PD therapeutics.

MATERIALS AND METHODS

Cell culture, treatments and antibodies

Human neuroblastoma SH-SY5Y cells and MEFs were grown in DMEM (WelGENE, Daegu, South Korea) with 10% fetal bovine serum (Invitrogen, Carlsbad, OR, USA) and penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere. The ts20-BALB cells were kindly provided by Dr Harvey Ozer (International Center for Public Health, Newark, NJ, USA). The parkin-knockout MEFs were kindly provided by Dr Valina L. Dawson (The Johns Hopkins University School of Medicine, Baltimore, MD, USA). The JNK1-knockout MEFs were kindly provided by Dr Gang Min Hur (Chungnam National University, Daejeon, South Korea). MG132, lactacystin, MPTP, MPP⁺ and CCCP were purchased from Sigma (St Louis, MO, USA). To block proteasome-mediated protein degradation, cells were treated with 10 μM MG132 for 12 h or 10 μM lactacystin for 12 h. The antibodies were obtained as follows: mouse anti-FAF1 (provided by Dr Jong-Seok Lim; Sookmyung Women’s University, Seoul, South Korea); rabbit anti-FAF1 (Proteintech, Chicago, IL, USA); mouse anti-parkin, anti-PARP1, anti-SOD2, anti-Myc and anti-p53 (Santa Cruz Biotechnology, San Diego, CA, USA); rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AbFrontier, Seoul, South Korea); mouse anti-Flag and anti-TRITC and rabbit anti-HA, anti-TH, anti-β-actin (Sigma); mouse anti-JNK (BD Biosciences, San Jose, CA, USA); rabbit anti-Phospho-JNK (P-JNK) and rabbit anti-Casp-3 (Cell Signaling Technology, Danvers, MA, USA); rabbit anti-FITC (Invitrogen). The horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce (Rockford, IL, USA).

Plasmid constructs and site-directed mutagenesis

The constructs FAF1 and its deletion mutants have been described previously (44). The parkin deletion constructs were generated by PCR and cloned into the pGEX 4T-1 vector. Specific Ub substitution mutants (7KR, K48R, K63R, 48K and 63K) were generated using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The PD-linked parkin substitution mutants (R42P, K161N, T240R, R275W, T415N, G430D, C431F and P437L) were also generated using a QuikChange kit (Stratagene).

Immunoprecipitation

SH-SY5Y cells were treated with MG132, lysed in mammalian lysis buffer (24), sonicated and then centrifuged at 14,926g for 10 min at 4°C. The resulting whole-cell lysates (WCLs) were incubated for 6 h at 4°C with anti-parkin or anti-FAF1 antibodies, and then with protein A/G-Sepharose beads (Santa Cruz Biotechnology). The WCLs were then subjected to SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies.

Glutathione S-transferase pull-down assay

To identify the FAF1 domain responsible for its putative direct interaction with parkin, GST-tagged full-length FAF1 and truncated mutant proteins were purified from bacteria, using...
Figure 6. Parkin ameliorates FAF1-mediated cell death. (A and B) SH-SY5Y cells were transfected with or without Flag-FAF1 and Myc-parkin (WT or T240R) and treated with 5 mM MPP\textsuperscript{+} for 24 h. Upper panel: death was determined by the assessment of LDH release; lower panel: an immunoblot of the input with anti-Flag, anti-FAF1, anti-Myc, anti-parkin and anti-\(\beta\)-actin antibodies (A). Upper panel: viability was determined by the measurement of ATP levels; lower panel: an immunoblot of the input with anti-Flag, anti-FAF1, anti-Myc, anti-parkin and anti-\(\beta\)-actin antibodies (B). (C and D) Faf1\textsuperscript{+/+} and Faf1\textsuperscript{gt/gt} MEFs were transfected with or without Myc-parkin and treated with 5 mM MPP\textsuperscript{+} for 24 h. Upper panel: death was determined by the assessment of LDH release; lower panel: cell lysates were analyzed by immunoblotting with the reciprocal antibody, as indicated (C). Upper panel: viability was determined by the measurement of ATP levels; lower panel: cell lysates were analyzed by immunoblotting with the reciprocal antibody, as indicated (D). (E and F) Parkin\textsuperscript{+/+} and Parkin\textsuperscript{−/−} MEFs were transfected with or without Flag-FAF1 and treated with 5 mM MPP\textsuperscript{+} for 24 h. Upper panel: death was determined by the assessment of LDH release; lower panel: cell lysates were analyzed by immunoblotting with the reciprocal antibody, as indicated (E). Upper panel: viability was determined by the measurement of ATP levels; lower panel: cell lysates were analyzed by immunoblotting with the reciprocal antibody, as indicated (F). Data are expressed as percentages of the control ± SEM; *\(P<0.05\); **\(P<0.01\); ***\(P<0.001\).
Figure 7. FAF1 activates cell death pathways. (A) SH-SY5Y cells were MOCK-transfected or transfected with Flag-FAF1 with or without co-transfection of Myc-parkin (WT or T240R). The transfected cells were subjected to 5 mM MPP+ treatment for 24 h and then assayed for Casp-3 activity. Cell lysates were analyzed by immunoblotting with the reciprocal antibody, as indicated. Data are expressed as folds of the control ± SEM; **P < 0.01. (B) Fafl (upper panel) and Parkin (lower panel) MEFs were treated with or without 5 mM MPP+ for 24 h, and cell lysates were analyzed by western blot (WB) using anti-FAF1, anti-parkin, anti-JNK, anti-P-JNK, anti-Casp-3 and anti-β-actin antibodies. Numbers below the bands indicate relative levels of FAF1 normalized with respect to β-actin. (C) JNK1+/− and JNK1−/− MEFs were transfected with or without Flag-FAF1 and treated with 5 mM MPP+ for 24 h. Viability was determined by the measurement of ATP levels. Data are expressed as percentages of the control ± SEM; ***P < 0.001. Cell lysates were analyzed by immunoblotting with the reciprocal antibody, as indicated. (D) SH-SY5Y cells were MOCK-transfected or transfected with Flag-FAF1 with or without co-transfection of Myc-parkin (WT or T240R). The cells were then incubated with 5 mM MPP+ for the indicated time periods, washed with PBS and incubated with DCFA for 15 min. Finally, the fluorescence intensity of dichlorofluorescein (DCF) was measured using a VICTOR microplate reader.
glutathione-sepharose beads. 35S-labeled parkin proteins were transcribed and translated in vitro using the TNT T7-coupled reticulocyte lysate system (Promega, Madison, WI, USA). The GST-fusion proteins were incubated with 35S-labeled in vitro-translated parkin for 12 h at 4°C, the samples were washed with binding buffer (50 mM HEPES, pH 7.6, 50 mM NaCl, 5 mM EDTA, 0.1% NP-40 and 10% glycerol) and the bound proteins were resolved by SDS–PAGE and detected by autoradiography. To determine the parkin domain responsible for interacting with FAF1, GST-tagged full-length parkin and truncated mutant proteins were purified from bacteria, using glutathione-sepharose beads. The GST-fusion proteins were incubated with 35S-labeled in vitro-translated FAF1 for 12 h at 4°C, the samples were washed with binding buffer and the bound proteins were resolved by SDS–PAGE and detected by autoradiography.

Immunofluorescence staining

Cells were grown on poly-L-lysine (Sigma)-coated coverslips, washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% bovine serum albumin (BSA) in PBS. The cells were then incubated for 12 h at 4°C with primary antibodies diluted in 0.1% Triton X-100 and 1% BSA in PBS, followed by incubation for 1 h with secondary antibodies diluted in 0.1% BSA in PBS. Finally, nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR, USA), the coverslips were mounted onto microscope slides using fluorescence-mounting medium (Dako, Carpinteria, CA, USA) and the samples were analyzed using a Zeiss LSM 510 Laser Scanning Confocal Microscope (Carl Zeiss, Jena, Germany).

Generation of stable cell lines

SH-SY5Y cells (5 × 10^5) were seeded in six-well plate per well. And cells were transfected with pcDNA3.1-FAF1. After 24 h after transfection, the culture medium was changed. Following another 24 h, cells were transferred to 10 cm dishes in selective medium containing G418 (600 μg/ml).

In vivo ubiquitination assay

SH-SY5Y cells were transfected with Myc-parkin, Flag-Ub and HA-FAF1, either alone or in combination, and treated with 10 μM MG132 for 12 h. Cells were harvested 48 h after transfection, lysed in modified RIPA buffer (2 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.025% SDS and 1 mM PMSF) and immunoprecipitation was performed with an anti-HA antibody and protein A/G-Sepharose beads. The samples were then resolved by SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies.

In vitro ubiquitination assay

For the FAF1 ubiquitination assays, 35S-labeled FAF1 proteins were transcribed and translated in vitro using the TNT translation system (Promega). The in vitro-translated FAF1 proteins (10 μl) were combined with recombinant parkin (5 μg, Calbiochem, Darmstadt, Germany) and incubated on ice for 1 h. The resulting complexes were then mixed with ubiquitination buffer (50 mM Tris–HCl, pH 7.4, 2 mM ATP, 5 mM MgCl₂, 2 mM DTT, 30 mM creatine phosphate and 0.05 mg/ml creatine phosphokinase, rabbit E1 (200 ng, Calbiochem), UbCH7 (100 ng, Calbiochem) and Ub (5 μg, Sigma). The reactions were incubated at 30°C for 2 h and stopped with SDS loading buffer, and the proteins were separated by SDS–PAGE and detected by autoradiography.

35S-methionine pulse-chase analysis

Twenty-four hours after transfection with the indicated constructs, the cells were washed and starved in methionine-free medium for 1 h. The cells were then pulsed with 100 μCi of 35S-methionine for 3 h, washed and chased in normal medium for the indicated time periods. Subsequently, the cells were harvested for immunoprecipitation with an anti-Flag antibody. The immunoprecipitates were separated by SDS–PAGE, detected by autoradiography and quantified using the image analysis software obtained from the NIH.

Animals and MPTP injections

All mice were maintained in the animal facility of Chungnam National University (Daejeon, South Korea), and all animal studies were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Parkin /− mice and their wild-type (WT) control C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To generate the MPTP-induced model of PD, 8-week-old male C57BL/6 mice (Japan SLC, Hamamatsu, Japan) received intraperitoneal injections of MPTP in saline (20 mg/kg body weight) or saline alone once per day for 5 consecutive days. Animals were sacrificed by decapitation 7 days after the last injection, and samples were processed for analysis.

Immunohistochemistry

Animals were anesthetized and then perfused with PBS followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were promptly removed and post-fixed overnight in 4% paraformaldehyde at room temperature, and paraffin-embedded sections were deparaffinized, treated with H₂O₂ and blocked with 3% serum in PBS. The sections were incubated overnight at 4°C with antibodies against TH and FAF1, followed by incubation with avidin–biotin–horseradish peroxidase complexes. Finally, the sections were stained using a DBA kit (Dako).

Generation of Faf1 wt mice and MEFs

FAF1 gene-trapped embryonic stem cells (XK588) were obtained from BayGenomics (part of the International Gene Trap Consortium, San Francisco, CA, USA), and mice heterozygous for the trapped Faf1 allele were generated according to the provided protocol. All the mouse lines were backcrossed onto the C57BL/6 background (backcrossed more than 10
generations) and were maintained under specific pathogen-free conditions at the Seoul National University Animal Facility. The insertion site of the gene-trap vector was confirmed by Southern blot analysis and sequencing. Genotypes were determined by denaturing gel electrophoresis followed by hybridization with the radioactively labeled FAF1 PCR fragments generated with primers for the trapped allele (forward, 5′-GACGTCTCGTGGCTGACAA-3′; reverse, 5′-CAGCAGCATCATTTCAGA-3′) and the WT allele (forward, 5′-TC CATCATCAGCAGGACAGATA-3′; reverse, 5′-GATGTCGTGAA GATGGTCAA-3′). MEFs were isolated from embryos (embryonic day 13.5) that had been digested with trypsin-EDTA, and the obtained primary MEFs were immortalized by transfection with a vector encoding the large-T antigen of simian virus 40. FAF1 expression in the immortalized MEFs was examined by reverse transcription and real-time PCR analysis, and also by immunoblot analysis.

Stereological cell counting
The stereological estimation was used to analyze of dopaminergic neuronal loss in the ventral midbrain after MPTP injection. Dopaminergic neurons were outlined on the basis of TH-positive immunostaining. Optical fractionator sampling was carried out on an Olympus BX51 microscope equipped with a motorized stage. TH-positive neuron cell counts were performed at ×40 magnification using a 100 × 75 μm² counting frame.

Behavioral analysis
Six days after MPTP or saline injections, the mice were subjected to behavioral testing. We first modified the standard rotarod test to emphasize the learning aspect of the test and minimize other factors. The rotarod consisted of a rotating spindle (diameter 3 cm), automatic timers and falling sensors, where mice were challenged for speeds (increased from 5 to 30 r.p.m.) with an intermission of 10 min between two speeds. Mice were trained twice daily (2 h intervals) for 6 days, with increasing speed of rotation ranging from 5 to 30 r.p.m.

Measurement of cell death and cell viability
For the assessment of cell death, we measured the LDH activity in the extracellular medium, using a Cytotoxicity Detection Kit (Roche, Mannheim, Germany). Cell viability was determined with the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Casp-3 activity
SH-SY5Y cells were MOCK-transfected or transfected with Flag-FAF1 with or without co-transfection of Myc-parkin (WT or T240R). Cells were harvested 48 h after transfection, lysed in ice-cold lysis buffer (Peptron, Daejeon, South Korea). The lysates were clarified by centrifugation and the supernatants were used in Casp-3 assays. Reaction buffer containing 10 mM dithiothreitol and 2.5 mM DEVD-AMC as substrates (Peptron) were added to each sample and incubated at 37°C for 30 min. Casp-3 activity was detected by measuring the relative fluorescence intensity at 460 nm following excitation at 360 nm, using a VICTOR microplate reader (Perkin-Elmer, Boston, MA, USA).

ROS assay
SH-SY5Y cells were MOCK-transfected or transfected with Flag-FAF1 with or without co-transfection of Myc-parkin (WT or T240R). The cultures were then centrifuged, and the cell pellets were resuspended with DMEM. For each sample, 100 μl of cell suspension was then transferred into a well of a 96-well plate (black background) and treated with 5 mM MPP⁺ for the indicated time periods. Five microliters (2.5 mg/ml) of dichlorofluorescin acetate (DCFA) solution was added into each well, and the plate was incubated at 37°C for 15 min. The fluorescence intensity was then assayed with a VICTOR microplate reader (Perkin-Elmer).

RNA extraction and RT reaction
Total RNA was extracted from cells using the easy-BLUE™ Total RNA Extraction Kit (iNtRON, Seongnam, South Korea). The total RNA was reverse-transcribed into cDNAs using the Rever Tra Ace Kit (Toyobo, Osaka, Japan).

Real-time PCR analysis of FAF1 mRNA
Relative gene expression for FAF1 was measured using the Quantitative Green real-time PCR master mix kit (iNtRON), with GAPDH as an internal reference gene. The primers used for hFAF1 were forward, 5′-CTGCAGGAGTCAT CATCTAGCATAAC-3′ and reverse, 5′-GGTGACTGCCATCCTGTATTTT-3′. The primers used for mFAF1 were forward, 5′-GGTGACACTGCCATCCTGTATTTT-3′ and reverse, 5′-TGCTCTGTGTTGGCGTCTTTT-3′.

Data analysis
All data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was evaluated by one-way or two-way analysis of variance (ANOVA) for repeated measures within a sample.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank Dr Valina L. Dawson for providing the parkin-knockout MEF cell line and Dr Harvey Ozer for providing the ts20-BALB cell line and Dr Gang Min Hur for providing the JNK1-knockout MEF cell line.

Conflict of Interest statement. None declared.
FUNDING
This work was supported by the National Research Foundation of Korea grant funded by the Korean government (Ministry of Education, Science, and Technology in South Korea) (2012M3A9A90055088).

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