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L166P mutant DJ-1 promotes cell death by dissociating Bax from mitochondrial Bcl-X$_L$

Haigang Ren, Kai Fu, Chenchen Mu, Xuechu Zhen and Guanghui Wang[*]

Abstract

Background: Mutations or deletions in DJ-1/PARK7 gene are causative for recessive forms of early onset Parkinson's disease (PD). Wild-type DJ-1 has cytoprotective roles against cell death through multiple pathways. The most commonly studied mutant DJ-1(L166P) shifts its subcellular distribution to mitochondria and renders cells more susceptible to cell death under stress stimuli. We previously reported that wild-type DJ-1 binds to Bcl-X$_L$ and stabilizes it against ultraviolet B (UVB) irradiation-induced rapid degradation. However, the mechanisms by which mitochondrial DJ-1(L166P) promotes cell death under death stimuli are largely unknown.

Results: We show that DJ-1(L166P) is more prone to localize in mitochondria and it binds to Bcl-X$_L$ more strongly than wild-type DJ-1. In addition, UVB irradiation significantly promotes DJ-1(L166P) translocation to mitochondria and binding to Bcl-X$_L$. DJ-1(L166P) but not wild-type DJ-1 dissociates Bax from Bcl-X$_L$, thereby leading to Bax enrichment at outer mitochondrial membrane and promoting mitochondrial apoptosis pathway in response to UVB irradiation.

Conclusion: Our findings suggest that wild-type DJ-1 protects cells and DJ-1(L166P) impairs cells by differentially regulating mitochondrial Bax/Bcl-X$_L$ functions.

Keywords: Parkinson's disease, DJ-1, L166P, Mitochondria, Apoptosis, Bcl-X$_L$, Bax, UVB

Background

Parkinson's disease (PD), a common neurodegenerative movement disorder, is associated with progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) [1]. DJ-1, a product of DJ-1/PARK7 gene, was originally identified as an oncogene protein that protects cells against stress through multiple pathways including gene transcription regulation, protein stabilization, signal transduction and reactive oxygen species (ROS) elimination [2,3]. Recently, DJ-1 has attracted more attention due to its involvement in familial early onset PD as its deletion mutants or point mutations including L166P, A104T, M26I, D149A, E64D and L10P cause PD [1,4,5]. DJ-1(L166P) was the most commonly studied and traditionally considered as "loss of function by degradation" because of its instability and misfolded structure compared with wild-type DJ-1 [6-8]. In addition, DJ-1(L166P) exists as a

monomer, whereas wild-type DJ-1 exists as homodimers in cells [6,9]. However, many lines of recent evidence indicated that DJ-1(L166P) renders cells more susceptible to cell death under death stimuli [10-14].

Mitochondrial dysfunction is the key and common causative factor for pathogenesis of PD [1,15-19]. In PD patients and experimental PD models, dopaminergic neurodegeneration is caused at least partly by activation of mitochondria-dependent programmed cell death 2 (PCD) pathways [20,21]. For instance, positive Bax, caspase-3, caspase-9 have been observed in SNpc dopaminergic neurons in PD models [22-24]. In addition, PD-associated proteins such as PINK1, parkin and DJ-1 directly affect mitochondrial functions [25-29]. DJ-1 deficiency leads to impairments of mitochondrial connectivity, fusion rates, membrane potential ($\Delta\Psi$m), respiratory capacity and ROS scavenging [25,30-33]. Interestingly, wild-type DJ-1 partially localized in mitochondria and DJ-1 mutants including L166P are more prone to mitochondrial localization [5,8,12,34,35]. Moreover, both wild-type DJ-1 and DJ-1(L166P) are enriched in the mitochondrial fraction under death

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stimuli [34,36]. So, it is possible that DJ-1(L166P) impairs cells or neurons by "gain of function by translocation to mitochondria". In addition, the physiological roles of their translocation to mitochondria under oxidative stress are still unclear because wild-type DJ-1 translocation to mitochondria under oxidative stress is required for its oxidation of Cys106 [34], but DJ-1 (L166P) can not be oxidized [13], suggesting that these two proteins may differentially function in mitochondria.

Recently, we reported that wild-type DJ-1 translocates to mitochondria and binds to Bcl-X_L in response to UVB irradiation and inhibits Bcl-X_L rapid degradation and mitochondrial apoptosis pathway induced by UVB irradiation [37]. However, the roles of DJ-1(L166P) in mitochondria during oxidative stress are largely unknown. In this study, we further showed that DJ-1 (L166P) binds more tightly to Bcl-X_L than wild-type DJ-1. Under UVB irradiation, DJ-1(L166P) translocates to mitochondria to dissociate Bax from Bcl-X_L by its interaction with Bcl-X_L, resulting in an increased susceptibility of cells to UVB irradiation-induce cell death. Our results suggest that DJ-1 and DJ-1(L166P) differentially regulate Bcl-X_L functions in control of the mitochondrial apoptotic pathway.

Results
Subcellular distribution of wild-type DJ-1 and DJ-1(L166P)
Considering that DJ-1 and its pathogenic mutant DJ-1 (L166P) have potential functions in mitochondria, we first examined the subcellular localization of DJ-1 and DJ-1(L166P) in HEK293 cells. DJ-1-Myc was distributed diffusely in both the cytoplasm and nucleus, with a small portion co-localized with MitoTracker (green) (Figure 1A and 1B). However, DJ-1(L166P)-Myc was dominantly presented in the mitochondria with much less nuclear and cytosolic distribution (Figure 1A). Quantitative analysis showed that approximately 81.3% of cells transfected with DJ-1(L166P) displayed a mitochondrial localization, and approximately 18.7% of them displayed a cytosolic localization (Figure 1B). Consistent with the immunocytochemical results, subcellular fractionation assays also showed that both of distribution ratio and protein level of DJ-1(L166P) in the mitochondrial fraction were much higher than those of wild-type DJ-1, although the total protein level of DJ-1(L166P) was much less than that of wild-type DJ-1 (Figure 1C and 1D). The lower level of Flag-DJ-1(L166P) protein compared to Flag-DJ-1 should be caused by that L166P mutant is unstable and degraded rapidly through the ubiquitin proteasome system (UPS), when equal amounts of plasmids are used for transfection [6,7,38]. The mitochondrial localization of wild-type DJ-1 and its mutants increases under oxidative stresses such as

paraquat treatment,H_2O_2 and UV irradiation [34,36]. Consistent with those findings, we observed that UVB irradiation increased the mitochondrial localization of both endogenous DJ-1 and Flag-DJ-1(L166P), but did not change total protein levels of them (Figure 1E and 1F). These results indicated that DJ-1(L166P) is prone to mitochondrial localization and the mitochondrial distribution of wild-type DJ-1 and DJ-1(L166P) are increased in response to UVB irradiation.

Interactions between Bcl-X_L and DJ-1(L166P)
In our previous study, we showed that wild-type DJ-1 translocates to mitochondria to bind to Bcl-X_L in response to UVB irradiation [37]. Considering that DJ-1 (L166P) is mainly distributed in mitochondria, and translocates more to mitochondria under oxidative stress, we wonder whether DJ-1(L166P) binds to Bcl-X_L. Although the interactions of wild-type DJ-1 and DJ-1 (L166P) with Bcl-X_L were not significant different in GST pulldown assays *in vitro* (Figure 2A), more DJ-1 (L166P) than wild-type DJ-1 bound to Bcl-X_L in cells (Figure 2B). Neither wild-type DJ-1 nor DJ-1(L166P) bound to Bcl$_2$ and Bax, another two typical Bcl-2 family proteins (data not shown). These data suggested that wild-type DJ-1 and DJ-1(L166P) specifically bind to Bcl-X_L. The monoclonal anti-Bcl-X_L antibody used in Figure 2B is suitable for immunoprecipitation assays as Flag-Bcl-X_L could be immunoprecipitated by anti-Bcl-X_L antibody but not by control mouse serum IgG (Additional file 1: Figure S1). Consistent with data from immunoprecipitation analyses, immunocytochemical studies showed that DJ-1(L166P)-Myc, but not DJ-1-Myc, was well co-localized with EGFP-Bcl-X_L in HEK293 cells (Figure 2C). We also examined the interactions between Bcl-X_L and another pathogenic DJ-1 mutant, DJ-1(M26I). Similar to DJ-1(L166P), DJ-1(M26I) interacted with Bcl-X_L and co-localized with Bcl-X_L (Additional file 1: Figure: S2). As DJ-1(L166P) increased in mitochondria under UVB irradiation (Figure 1E and 1F), we next performed immunoprecipitation assays to test if the interaction of Bcl-X_L with DJ-1(L166P) is affected by UVB irradiation. Interestingly, the binding affinity of Flag-DJ-1(L166P) for EGFP-Bcl-X_L significantly increased after UVB irradiation (Figure 2D). In addition, UVB irradiation led to larger punctate DJ-1(L166P)-RFP spots co-localizing with EGFP-Bcl-X_L (Figure 2E). Moreover, the mitochondria exhibited more severe abnormalities in cells harboring DJ-1(L166P) under UVB irradiation (Figure 2E).

Requirement of the C-terminal of Bcl-X_L for DJ-1(L166P) binding
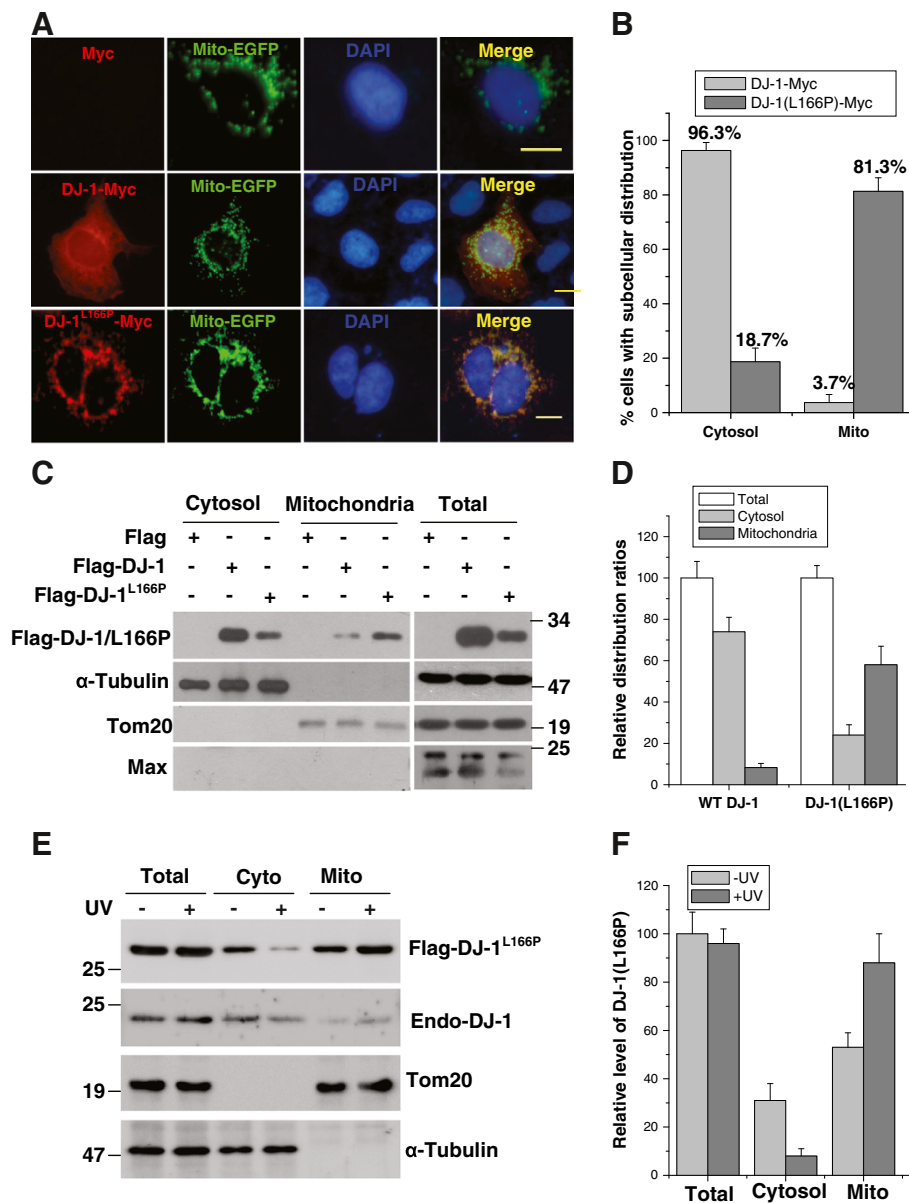We previously found that wild-type DJ-1 mainly binds to amino acids 86–195 of Bcl-X_L which contain BH1,

Figure 1 Subcellular distribution of wild-type DJ-1 and DJ-1(L166P) in mitochondria. (A) HEK293 cells transiently transfected with Myc (control), DJ-1-Myc or DJ-1(L166P)-Myc along with Mito-EGFP were subjected to immunocytochemical staining with anti-Myc antibodies (red) and then labeled with the nuclear marker DAPI (blue). Bar, 10 μm. (**B**) The percentage of cells transfected with DJ-1-Myc or DJ-1(L166P)-Myc presenting mitochondrial or cytosolic staining in (A) was quantified (mean ± S.E.M., n = 3; by one-way ANOVA). (**C**) H1299 cells transiently transfected with Flag, Flag-DJ-1 or Flag-DJ-1(L166P) were subjected to subcellular fractionation. The total cell lysate and the cytosolic and mitochondrial fractions were subjected to immunoblot analysis. (**D**) The distribution ratio of Flag-DJ-1 or Flag-DJ-1(L166P) in cytosolic or mitochondrial fractions to their total level in (C) were analyzed using densitometric analysis (mean ± S.E.M., n = 3; by one-way ANOVA). (**E**) H1299 cells transiently transfected with Flag-DJ-1(L166P) were treated with or without 80 mJ/cm^2 UVB irradiation, and the cells were then subjected to the subcellular fractionation assay. The total cell lysate, cytosolic and mitochondrial fractions were subjected to immunoblot analysis. (**F**) The relative level of Flag-DJ-1(L166P) in (E) were analyzed using densitometric analysis (mean ± S.E.M., n = 3; by one-way ANOVA).

BH2 and BH3 domains [37]. We wonder whether DJ-1 (L166P) binds to the same amino acids of Bcl-X_L. Surprisingly, DJ-1(L166P) bound to the C-terminal fragment of Bcl-X_L at amino acids 196–233 (Figure 3A). We further examined the regions of Bcl-X_L binding to DJ-1(L166P) in H1299 cells. Similar to the results from

GST pulldown assays, EGFP-Bcl-$X_L^{196-233}$ but not EGFP-Bcl-X_L^{1-195} was found to interact with Flag-DJ-1 (L166P) (Figure 3B). These results suggest that DJ-1 (L166P) and wild-type DJ-1 bind to different domains of Bcl-X_L, indicating that they may regulate Bcl-X_L functions differently. Under UVB irradiation, Bcl-X_L is
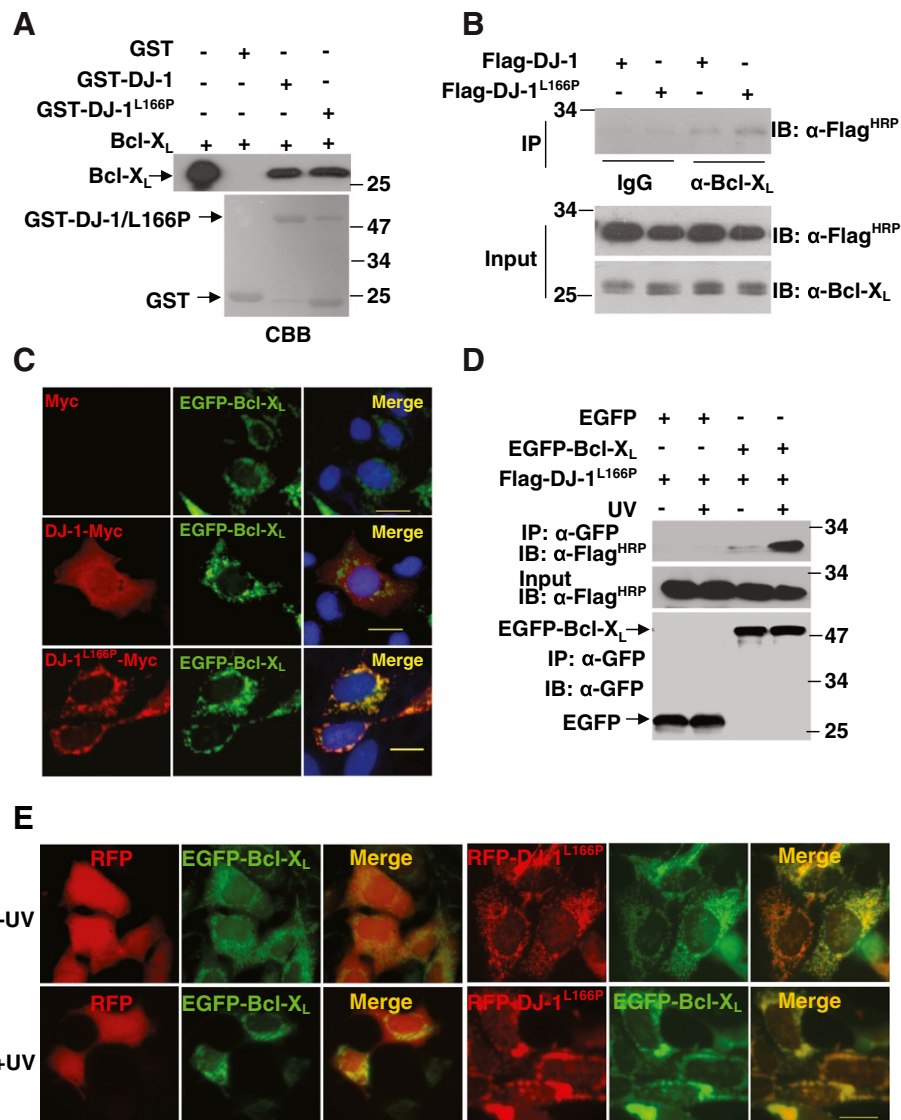
Figure 2 Interactions between Bcl-X$_L$ and DJ-1(L166P). (A) The supernatant of *E. coli* crude extract containing 50 μg of recombinant Bcl-X$_L$ was incubated with 20 μg of GST, GST-DJ-1 or GST-DJ-1(L166P) coupled to glutathione-agarose beads. The bound proteins were detected by immunoblot analysis. **(B)** H1299 cells transiently transfected with Flag-DJ-1 or Flag-DJ-1(L166P) were subjected to immunoprecipitation with normal mouse serum or anti-Bcl-X$_L$ antibody. **(C)** HEK293 cells transfected with Myc, DJ-1-Myc or DJ-1(L166P)-Myc along with EGFP-Bcl-X$_L$ were subjected to immunocytochemical staining with anti-Myc antibodies (red) and then labeled with DAPI (blue). Bar, 10 μm. **(D)** H1299 cells transfected with Flag-DJ-1(L166P) along with EGFP or EGFP-Bcl-X$_L$ were treated with or without 80 mJ/cm^2 UVB irradiation. Supernatants of the cell lysates were then subjected to immunoprecipitation with anti-GFP antibodies. **(E)** HEK293 cells transiently transfected with RFP or DJ-1 (L166P)-RFP along with EGFP-Bcl-X$_L$ were treated with or without 80 mJ/cm^2 UVB irradiation, and then the cells were observed with an inverted fluorescence microscope (Olympus, IX71). Bar, 10 μm.

degraded via the UPS [39,40]. In our previous study, we showed that wild-type DJ-1 stabilizes Bcl-X$_L$ by its inhibiting Bcl-X$_L$ under UVB irradiation. We therefore examined if DJ-1(L166P) also stabilize Bcl-X$_L$. Under UVB irradiation, knockdown of DJ-1 decreased Bcl-X$_L$ protein levels and re-overexpression of Flag-DJ-1(s), a synonymous mutant that is resistant to si-DJ-1, restored Bcl-X$_L$ protein levels, however, Flag-DJ-1 (L166P)(s) did not (Figure 3C and 3D). Meanwhile, the

ubiquitination of Bcl-X$_L$ was inhibited by DJ-1 but not DJ-1(L166P) (Figure 3E).

Dissociation of Bax from Bcl-X$_L$ by DJ-1(L166P)

Bcl-2 family proteins mediate apoptosis in a manner dependent on their homo- or hetero-dimerization [41]. Bcl-X$_L$ interacts with Bax to block its oligomerization in the mitochondrial membrane, thereby protecting cells from Bax-induced mitochondrial membrane
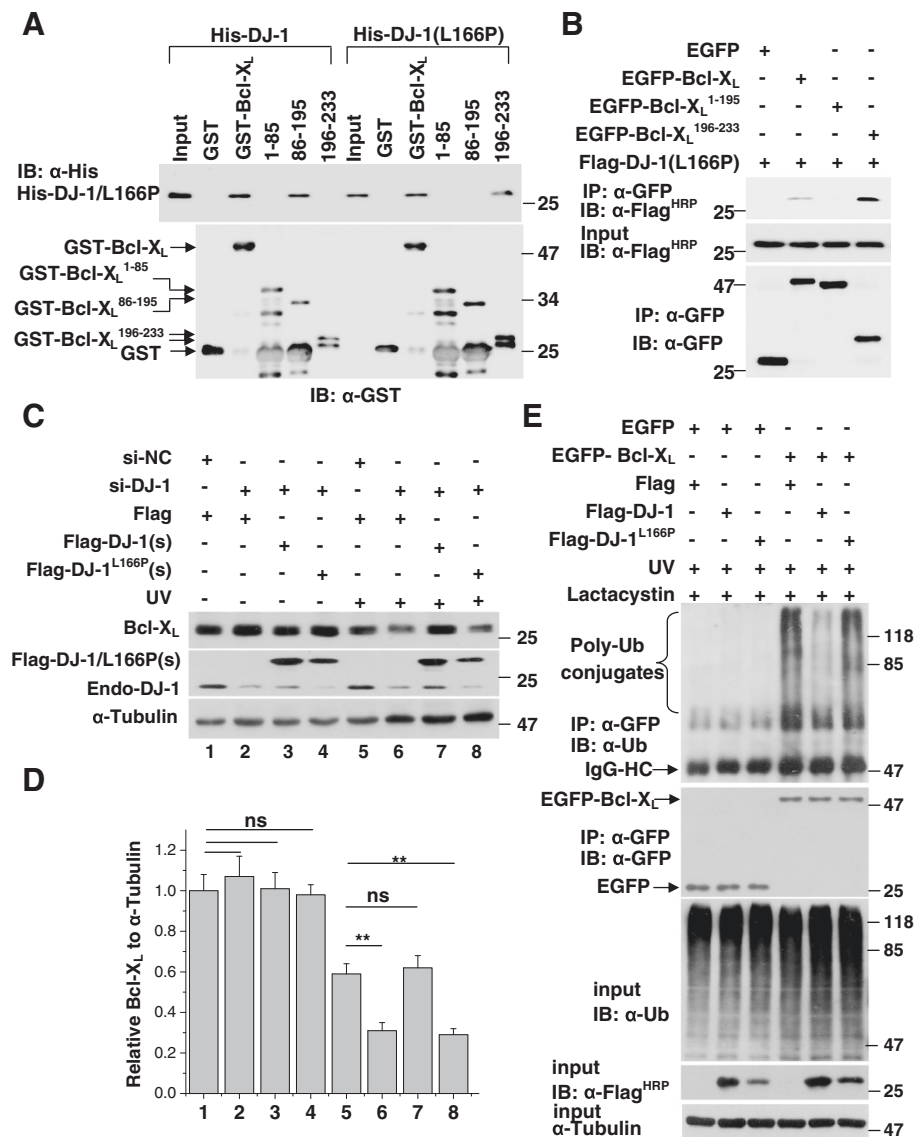
Figure 3 Requirement of the C-terminus of Bcl-X$_L$ for DJ-1(L166P) binding. (**A**) The supernatant of *E. coli* crude extract containing recombinant His-DJ-1 or His-DJ-1(L166P) was incubated with GST, GST-Bcl-X$_L$, GST-Bcl-X$_L$(1–85), GST-Bcl-X$_L$(86–195) or GST-Bcl-X$_L$(196–233) coupled to glutathione-agarose beads. The bound proteins were subjected to immunoblot analysis. (**B**) The supernatants of H1299 cells transfected with EGFP, EGFP-Bcl-X$_L$, EGFP-Bcl-X$_L$(1–195) or EGFP-Bcl-X$_L$(196–233) along with Flag-DJ-1(L166P) were subjected to immunoprecipitation with anti-GFP antibodies. (**C**) H1299 cells transfected with si-NC or si-DJ-1 along with Flag, Flag-DJ-1(s) or Flag-DJ-1(L166P)(s) were treated with or without 80 mJ/cm^2 UVB irradiation. Total cell lysates were subjected to immunoblot analysis. (**D**) The relative ratios of Bcl-X$_L$ to α-Tubulin in (C) were analyzed using densitometry (mean ± S.E.M., n = 3; **, p <0.01; ns, no statistical significance by one-way ANOVA). (**E**) H1299 cells transfected with EGFP or EGFP-Bcl-X$_L$ along with Flag, Flag-DJ-1 or Flag-DJ-1(L166P) were treated with 80 mJ/cm^2 UVB and then with 5 μM Lactacystin for 16 hours. The supernatants of the cell lysates were subjected to immunoprecipitation with anti-GFP antibodies.

permeabilization [41-43]. It has been reported that the BH1-2 domains and the C-terminus of Bcl-X$_L$ are required for Bcl-X$_L$/Bax heterodimer formation [43,44]. To investigate if DJ-1(L166P) affects the interactions between Bcl-X$_L$ and Bax or Bcl-2, we performed competitive binding assays. With less amount of His-DJ-1 (L166P), more Bax bound to Bcl-X$_L$ (Figure 4A). However, the binding ability of Bcl-2 to Bcl-X$_L$ was

not affected by His-DJ-1(L166P) (Figure 4B). To further identify if DJ-1(L166P) influences the interactions between Bcl-X$_L$ and Bax in mammalian cells, we transfected various amounts of Flag-DJ-1(L166P) into H1299 cells stably expressing EGFP-Bcl-X$_L$ or EGFP-Bcl-X$_L^{1-195}$ and performed immunoprecipitation assays. Under UVB irradiation, the amount of endogenous Bax that interacted with EGFP-Bcl-X$_L$ was decreased
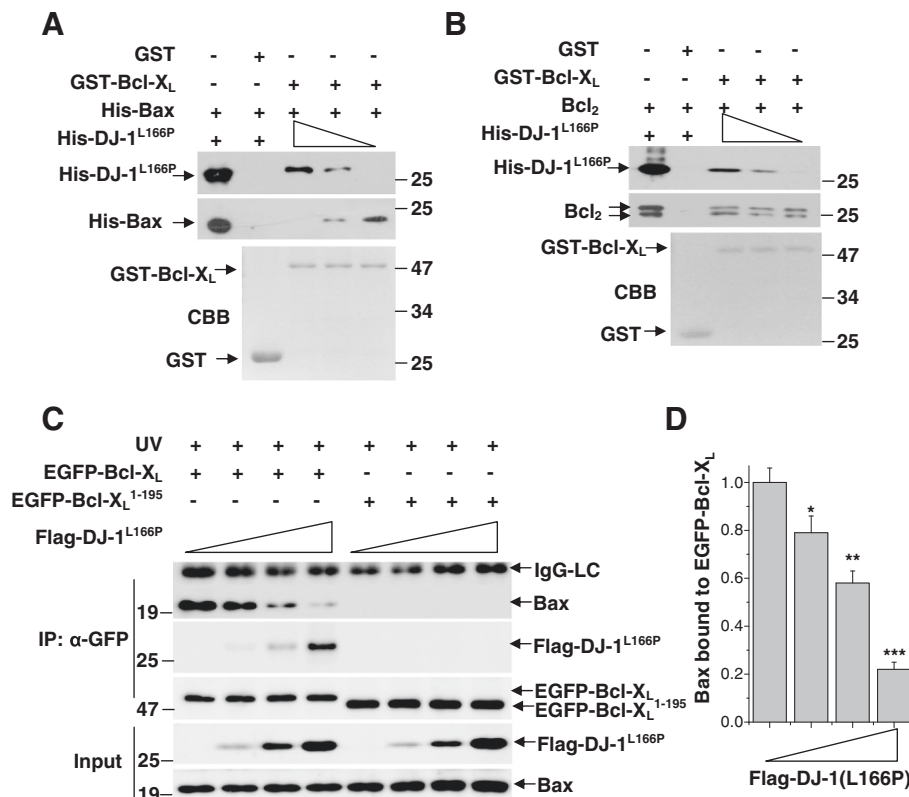
Figure 4 Dissociation of Bax from Bcl-X$_L$ by DJ-1(L166P). (**A**-**B**) Supernatant containing 50 μg of recombinant His-Bax (A) or Bcl$_2$ (B) was incubated with 20 μg of GST or GST-Bcl-X$_L$ coupled to glutathione-agarose beads. After being washed to remove unbound proteins, the glutathione-agarose beads were incubated with various amounts of His-DJ-1(L166P) supernatant. The bound proteins were subjected to immunoblot analysis. (**C**) H1299 cells stably expressing EGFP-Bcl-X$_L$ or EGFP-Bcl-X$_L$(1–195) were transiently transfected with various amounts of Flag-DJ-1(L166P) and treated with 80 mJ/cm^2 UVB irradiation. The supernatants of cell lysates were subjected to immunoprecipitation with anti-GFP antibodies. (**D**) The relative levels of Bax bound to EGFP-Bcl-X$_L$ in (D) were analyzed using densitometry (mean ± S.E.M., n = 3; *, p <0.05; **, p <0.01; ***, p <0.001; by one-way ANOVA).

when more Flag-DJ-1(L166P) was inputted (Figure 4C and 4D). However, EGFP-Bcl-X$_L^{1-195}$, which does not interact with Bax [44], was unable to interact with DJ-1(L166P) (Figure 4C).

DJ-1(L166P) promotes cell death by interfering with Bcl-X$_L$/Bax heterodimerization

The mitochondrial localization of Bax is important for its ability to induce cell death [41]. Because DJ-1 and DJ-1(L166P) re-distribute to mitochondria upon UVB irradiation but differentially influence Bcl-X$_L$, we performed cytosolic and mitochondrial fractionation assays and MTT assays to examine the effects of DJ-1 and DJ-1 (L166P) on mitochondrial Bax translocation and cell viability. We performed experiments in H1299 cells, a p53 null cell line to exclude the possibility that DJ-1 inhibits Bax transcription by binding to p53 [45-47]. Because endogenous DJ-1 expression is abundant [48], we constructed a H1299 cell line stably transfected with

sh-DJ-1 to silence endogenous DJ-1 to examine the effects of exogenous wild-type DJ-1 and DJ-1(L166P).

The knockdown efficiency of sh-DJ-1 is shown in Figure 5A and 5B. In the absence of UVB irradiation, neither Flag-DJ-1(s) nor Flag-DJ-1(L166P)(s) had significant effects on the translocation of Bax from the cytosol to the mitochondria in sh-DJ-1 cells (Figure 5C and 5D). However, under UVB irradiation, less Bax was presented in the mitochondrial fraction in cells transfected with Flag-DJ-1(s), but more Bax was present in the mitochondrial fraction in cells transfected with Flag-DJ-1(L166P) (s) (Figure 5C and 5D). Overexpression of Flag-DJ-1(s) but not Flag-DJ-1(L166P)(s) significantly increased mitochondrial Bcl-X$_L$ in response to UVB irradiation (Figure 5C and 5E). In addition, in the absence of death stimulus, overexpression of Flag-DJ-1(s) or Flag-DJ-1 (L166P)(s) had no significant effects on Bcl-X$_L$ levels, caspase-3 and PARP cleavage (Figure 5F) or cell viability (Figure 5G). However, with UVB irradiation, Flag-DJ-1 (s) partially restored Bcl-X$_L$ levels and accordingly
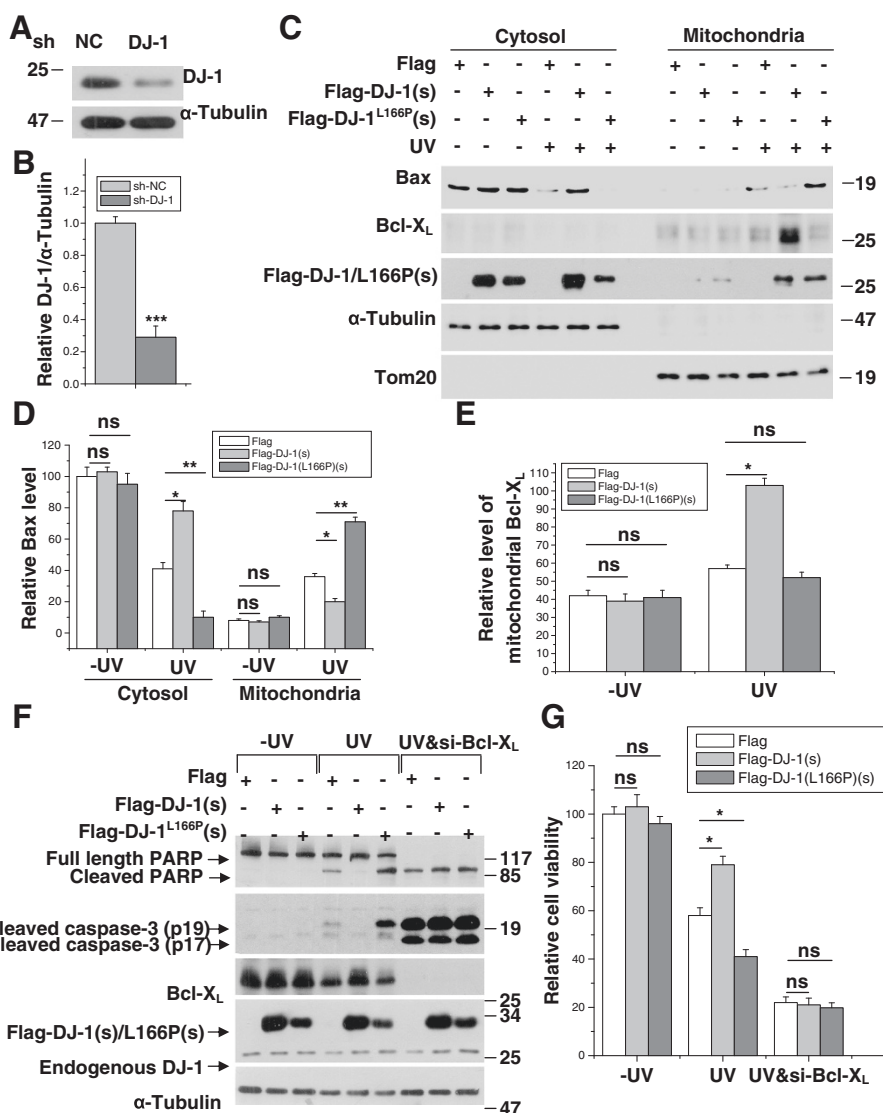
Figure 5 Promotion of cell death by DJ-1(L166P)-influenced Bcl-X$_L$/Bax heterodimerization. (A) Cell lysates of H1299 cells stably expressing sh-NC or sh-DJ-1 were subjected to immunoblot analysis. **(B)** The relative ratios of DJ-1 to α-Tubulin in (A) were analyzed using densitometry (mean ± S.E.M., n = 3; ***, p <0.001; by one-way ANOVA). **(C)** H1299 cells stably expressing sh-DJ-1 were transfected with Flag, Flag-DJ-1(s) or Flag-DJ-1(L166P)(s) and treated with or without 80 mJ/cm^2 UVB irradiation. The cells were then subjected to subcellular fractionation. **(D)** The relative level of Bax in (C) were analyzed using densitometry (mean ± S.E.M., n = 3; *, p <0.05; **, p <0.01; by one-way ANOVA). **(E)** The relative level of Bcl-X$_L$ in mitochondria in (C) were analyzed using densitometry (mean ± S.E.M., n = 3; *, p <0.05; by one-way ANOVA). **(F-G)** H1299 cells stably expressing sh-DJ-1 transfected with si-NC or si-Bcl-X$_L$ along with Flag, Flag-DJ-1(s) or Flag-DJ-1(L166P)(s) were treated with or without 80 mJ/cm^2 UVB irradiation. Cell lysates were then subjected to immunoblot analysis (F) or the cells were analyzed with the MTT assay (G) (mean ± S.E.M., n = 3; *, p <0.05; ns, no statistical significance by one-way ANOVA).

inhibited the cleavage of caspase-3 and PARP (Figure 5F) and increased cell viability (Figure 5G). In contrast to Flag-DJ-1(s), Flag-DJ-1(L166P)(s) greatly increased the cleavage of both caspase-3 and PARP (Figure 5F) and decreased cell viability (Figure 5G). Moreover, the effects of DJ-1 and DJ-1(L166P) on cell death under UVB irradiation were abrogated by Bcl-X$_L$ knockdown (Figure 5F and 5G). These results suggest that wild-type DJ-1 protects cells against UVB

irradiation by inhibiting Bcl-X$_L$ degradation, but DJ-1 (L166P) promotes cell death by dissociating Bcl-X$_L$/Bax heterodimerization.

Discussion

Many studies have shown that wild-type DJ-1 and DJ-1 (L166P) are partially localized in mitochondria [5,8,12,34,35], and that their mitochondrial distribution is enhanced under death stimuli [34,36]. Several lines of

evidence indicate that wild-type DJ-1 exhibits cytoprotective roles by maintaining mitochondrial integrity, fusion rates, membrane potential, respiratory capacity and ROS elimination [25,30-33].

In our previous study, we identified that wild-type DJ-1 is a novel partner of Bcl-X$_L$ in mitochondria, to stabilize Bcl-X$_L$ [37]. Here, we found that DJ-1(L166P) binds to Bcl-X$_L$ as well. However, DJ-1(L166P) does not stabilize Bcl-X$_L$ but dissociates Bax from Bcl-X$_L$. The binding ability of DJ-1(L166P) to Bcl-X$_L$ is stronger than that of wild-type DJ-1 (Figure 2B). In contrast to DJ-1 that interacts with Bcl-X$_L$ dependent on its oxidation [37], DJ-1(L166P) interacts with Bcl-X$_L$ independent on its oxidation as DJ-1(L166P) is a loss of oxidized form [13]. In addition, DJ-1(L166P) binds to the C-terminus of Bcl-X$_L$ (Figure 3A and 3B) which is required for Bcl-X$_L$/Bax heterodimer formation [44], but wild-type DJ-1 mainly binds to middle regions (Figure 3A) containing BH1, BH2 and BH3 domains which are essential for Bcl-X$_L$ stability [49,50]. Wild-type DJ-1 and DJ-1(L166P) that bind to different domains of Bcl-X$_L$ may be due to the fact that L166P mutant interrupts the normal folding and exposes new domains or amino acid sites [38,51,52]. Taken together, our study suggests that the different roles of DJ-1 and DJ-1(L166P) in mitochondria may result from the different oxidative status of these two proteins and from their functioning differentially in mitochondria.

L166P mutant prevents normal folding of wild-type DJ-1 and itself is instable with a rapid degradation via UPS [6,7,51,52]. However, DJ-1(L166P) appears not only to be "loss of function" of wild-type DJ-1. It also forms larger complexes with other proteins but not wild-type DJ-1 [8]. Although DJ-1(L166P) loses the ability to bind to proteins that wild-type DJ-1 does, such as Daxx [53], DJ-1(L166P) existing as a monomer in cells may allow it to gain an ability to bind to proteins that wild-type DJ-1 does not. For instance, DJ-1(L166P) and DJ-1(M26I) bind more TTRAP than wild-type DJ-1 does, and they block the protective activity of TTRAP, leading to cell death [10]. Wild-type DJ-1 represses UV-induced JNK activation to protects cells, but DJ-1(L166P) significantly activates JNK pathway to promote cell death in response to UV irradiation [11]. As much more DJ-1(L166P) is translocated to mitochondria than wild-type DJ-1 under UVB stimulation, and DJ-1(L166P), but not wild-type DJ-1, dissociates Bax from mitochondrial Bcl-X$_L$, it is therefore possible that DJ-1(L166P) may gain functions by translocation to mitochondria to affect mitochondrial pathway. We also found that another PD-associated mutant DJ-1(M26I) mainly distributes in mitochondria and binds to Bcl-X$_L$, similar to DJ-1(166P) (Additional file 1: Figure S2). These results suggest that the mitochondrial Bcl-X$_L$/Bax pathway influenced by mutant DJ-1 might be

a common mechanism involved in mutant DJ-1-associated PD pathogenesis.

Mitochondrial dysfunction is a key feature involved in both sporadic and genetic forms of PD [1,15-19]. Although familial PD is rare, to understand the mechanisms and functions of familial PD-associated proteins in mitochondria may shed light on the pathogenesis of PD. Our findings suggest that wild-type DJ-1 and DJ-1(L166P) differentially mediate Bcl-X$_L$ functions providing us to further understand the pathogenesis of PD.

Conclusion

We found that a small portion of wild-type DJ-1 and most of DJ-1(L166P) is presented in mitochondria and wild-type DJ-1 and DJ-1(L166P) increased in mitochondria in response to UVB irradiation. DJ-1(L166P) binds to mitochondrial Bcl-X$_L$ more tightly than wild-type DJ-1 and UVB irradiation further promotes their binding affinity. Unlike wild-type DJ-1, DJ-1(L166P) fails to stabilize Bcl-X$_L$, but it dissociates Bax from Bcl-X$_L$ that leading Bax enrichment in outer mitochondrial membrane and subsequently triggers cell death in response to UV irradiation. Our findings suggest that wild-type DJ-1 protects cells and DJ-1(L166P) impairs cells by differentially regulating Bcl-X$_L$ functions. Our study provides a novel insight into the underlying mechanisms of PD pathogenesis.

Materials and methods

Cell culture and plasmid transfection

Human HEK293 cells, a human kidney cell line, and H1299 cells, a human lung cancer cell line, were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum (Hyclone, USA). Plasmid transfections were performed using Lipofectamine2000 reagent (Invitrogen, USA).

UVB irradiation treatment

HEK293 or H1299 cells were irradiated with UVB using a UV crosslinker. Briefly, the cultured cells covered with a thin layer of phosphate buffer solution (PBS, pH 7.4), were exposed to UVB irradiation (312 nm) with 80 mJ/cm2 with a UV crosslinker (SCIENTZ03-II, Ningbo, China). After UVB irradiation, the cells were cultured for 16 hours and subsequently subjected to additional experiments.

siRNA or shRNA knockdown

si-DJ-1^{328} was described previously [47]. siRNA against human Bcl-X$_L$ mRNA was purchased from GenePharma (GenePharma, Shanghai, China) with the following sequences: sense: 5′-GAGAUGCAGGUAUUGGUGATT-3′, anti-sense: 5′-UCACCAAUACCUGCAUCUCTT-3′.

The oligonucleotides were transfected with Oligofectamine reagent (Invitrogen, USA). Briefly, cultured cells were washed with Opti-MEM medium (Invitrogen) and then transfected with siRNA using Oligofectamine reagent in Opti-MEM medium without serum. Six hours after transfection, the culture medium was replaced with fresh complete medium. The cells were subjected to further experiments 72 hours after transfection. pGPU6/GFP/Neo-sh-DJ-1 encoding a short hairpin RNA (shRNA) against nucleotide 328 to 346 of human DJ-1 mRNA (sh-DJ-1) or a negative control short hairpin (sh-NC) was constructed by GenePharma (GenePharma, Shanghai, China). H1299 cells stably expressing sh-NC or sh-DJ-1 were obtained by selection with 200 μg/ml Geneticin (Invitrogen, USA) after transfection.

Plasmid constructs

Full-length DJ-1 in p3 × Flag-myc-cmv-24, pET-15b, pDsRed-N1, pmyc-cmv-24 and pGEX-5x-1, and pET-21a-Bcl$_2$, pET-21a-Bax, pET-21a-Bcl-X$_L$, pEGFP-C2-Bcl-X$_L$, p3 × Flag-myc-cmv-24-Bcl-X$_L$, pGEX-5x-1-Bcl-X$_L$(1–85), pGEX-5x-1-Bcl-X$_L$(86–195) and pGEX-5x-1-Bcl-X$_L$(196–233) were described previously [37]. pEGFP-C2-Bcl-X$_L$(1–195) and pEGFP-C2-Bcl-X$_L$(196–233) were created by subcloning PCR products into pEGFP-C2 at its *Eco*RI/*Sal*I sites. The PCR products were amplified with the following primers: 5′-CGGAATTCATGTCTCAGAGCAAC-3′ and 5′-GCGTCGACTCAATAGAGTTCCACAAA-3′ for 1-195aa; 5′-CGGAATTCGGGAACAATGCAGCA-3′ and 5′-GCGTCGACTCATTTCCGACTGAAG-3′ for 196-233aa. DJ-1(L166P) and DJ-1(M26I) mutants were obtained by site-directed mutagenesis using wild-type DJ-1 plasmids as template with the following primers: 5′-CCTGCAATTGTTGAAGCCCTGGAATG-3′ and 5′-CGCAAACTCGAAGCTGGTCCCAG-3′, for DJ-1(L166P); 5′-GTAGATGTCATTAGGCGA-3′ and 5′-GGTGACCTTAATCCCAGC-3′, for DJ-1(M26I); respectively. Two synonymous mutants, p3 × Flag-myc-cmv-24-DJ-1(s) and p3 × Flag-myc-cmv-24-DJ-1(L166P)(s), that are resistant to si-DJ-1[328] and sh-DJ-1 were described previously [54].

Immunocytochemistry

HEK293 cells were washed with PBS and fixed with 4% paraformaldehyde. After being blocked with 4% fetal bovine serum containing 0.25% Triton X-100 in PBS, the cells incubated with rabbit anti-myc polyclonal antibodies (Santa Cruz Biotechnology, USA) followed by an incubation with rhodamine-conjugated donkey anti-rabbit IgG (Santa Cruz biotech, Inc). After staining with DAPI (4′,6-diamidino-2-phenylindole), the labeled cells were observed using an inverted fluorescent microscope (Olympus, IX71).

GST pulldown assay

Equal amounts of GST or GST-fused proteins (20 μg) expressed by Escherichia coli strain JM109 were incubated with 20 μl of glutathione agarose beads (Pharmacia, USA) for 30 min at 4°C. After washing three times with ice-cold PBS, the beads were incubated with 50 μg of His-fused protein expressed by Escherichia coli strain BL21 for 2 hours at 4°C. After incubation, the beads were washed five times with ice-cold HNTG buffer (20 mM Hepes-KOH, pH 7.5, 100 mM NaCl, 0.1% Triton X-100 and 10% glycerol). Bound proteins were eluted from the beads and subjected to immunoblot analysis with specific antibodies. The input represents 10% of the protein that was incubated with GST or a GST-fused protein. The inputs of purified GST and GST fusion proteins are stained with Coomassie Brilliant Blue (CBB) or anti-GST antibody.

Immunoprecipitation assay

The cells were lysed in 1 ml cell lysis buffer (50 mM Tris–HCl pH 7.5 buffer containing 150 mM NaCl, 1% NP40 and 0.5% deoxycholate) supplemented with the protease inhibitor cocktail (Roche, USA) for 30 min at 4°C. After centrifugation at 12,000 g for 15 min at 4°C, the supernatants were incubated with appropriate antibodies coupled to protein G Sepharose (Roche, USA). The immunoprecipitants were then washed five times with cell lysis buffer. Bound proteins and cell lysates were subjected to immunoblot analysis. The input represents 10% of the supernatant used in the co-immunoprecipitation experiment.

Immunoblot analysis and antibodies

Proteins were separated by 12% or 15% SDS-PAGE and subjected to immunoblot analysis with specific antibodies. The following primary antibodies were used: Monoclonal anti-Bcl2, anti-Bcl-XL, anti-GFP, anti-GST, anti-Tom20, anti-Ub and polyclonal anti-Myc, anti-Bax, anti-Max antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-Bcl-XL, anti-cleaved caspase-3 and anti-PARP antibodies were from Cell Signaling. Polyclonal anti-DJ-1 antibodies were purchased from Chemicon. Monoclonal anti-Flag-HRP and anti-α-Tubulin antibodies were purchased from Sigma. Monoclonal anti-GAPDH antibody was from Millipore. The secondary antibodies, sheep anti-mouse IgG-HRP and anti-rabbit IgG-HRP were purchased from Amersham Pharmacia Biotech. The proteins were visualized using an ECL detection kit (Amersham Pharmacia Biotech). Immunoblot densitometric analysis of data from three independent experiments was performed using Photoshop 7.0 (Adobe, USA).

Subcellular fractionation assay

The cytosolic and mitochondrial fractions were isolated using Mitochondria Isolation Kit for Cultured Cells (Beyotime, China). The total cell lysates and isolated fractions were subjected to immunoblot analysis with specific antibodies. Tom20, α-Tubulin and Max served as the mitochondrial, cytosolic and nuclear maker, respectively.

Cell viability assay

The cell viability was measured by MTT (methylthiazo-letetrazolium) assay. Briefly, the cells were washed with DMEM without phenol red (Gibco, USA) and incubated with 0.5 mg/ml MTT (Sigma) for three hours. The medium was removed and the formazan crystals were dissolved in DMSO (dimethyl sulfoxide). Cell viability was measured by spectrometry at OD570. The data were normalized to a control and the ratios are presented as means ± S.E.M from three independent experiments.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) using origin 6.0 software (Originlab, USA). Values are shown as mean ± S.E.M.

Additional file

Additional file 1: Figure S1. Anti-Bcl-X$_L$ antibody was suitable for immunoprecipitation. The supernatants of HEK293 cells that were transiently transfected with Flag-Bcl-X$_L$ were subjected to immunoprecipitation analysis using normal mouse serum or anti-Bcl-X$_L$ antibody. **Figure S2.** DJ-1(M26I) also interacted and co-localized with Bcl-X$_L$ in cells. (A) HEK293 cells were co-transfected with EGFP or EGFP-Bcl-X$_L$ along with Flag-DJ-1, Flag-DJ-1(L166P) or Flag-DJ-1(M26I) as indicated, the supernatants of cell lysates were subjected to immunoprecipitation analysis using anti-GFP antibodies. (B) HEK293 cells transiently transfected with DJ-1(M26I)-Myc with EGFP-Bcl-X$_L$ were subjected to immunocytochemical staining with anti-Myc antibodies (red), Bar, 10 μm.

Abbreviations

ANOVA: Analysis of variance; CBB: Coomassie brilliant blue; DAPI: 4′,6-diamidino-2-phenylindole; EGFP: Enhanced green fluorescent proteins; HRP: Horseradish peroxidase; PD: Parkinson's disease; PBS: Phosphate buffer solution; ROS: Reactive oxygen species; SNpc: Substantia nigra pars compacta; UPS: Ubiquitin proteasome system; UVB: Ultraviolet B.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RHG designed and carried out all the experiments, and drafted the manuscript. FK and MCC participated in experiments and manuscript. ZXC edited the manuscript. WGH conceived and designed the study, and edited the manuscript. All authors read and approved the final manuscript.

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