**Introduction**

Oxidative stress, a state of imbalance between the generation and elimination of reactive oxygen and nitrogen species, has been implicated in a variety of neurodegenerative diseases [1–3]. The central nervous system is presumed to be particularly vulnerable to oxidative stress, as it consumes abundant quantities of oxygen and employs nitric oxide as a biological messenger, both of which can generate reactive species as by-products [1]. Oxidative stress provokes various cytotoxic processes, such as overstimulation of glutamate receptors (excitotoxicity), ER stress, and mitochondrial dysfunction, which lead to apoptosis, the predominant form of cell death in aging-related neurodegenerative diseases [1,2].

Parkinson’s disease (PD) is characterized by typical motor dysfunction and is thought to be caused by the loss of nigrostriatal dopaminergic (DA) neurons that connect the substantia nigra pars compacta (SNpc) to other brain regions [3–5]. The death of these neurons has been closely linked to oxidative stress [3–5]. Markers of oxidative damage to lipids, proteins and DNA, as well as mitochondrial DNA deletions, which can be caused by oxidative stress, are significantly elevated in postmortem samples of the SNpc of PD patients [4,5]. The nigrostriatal pathway is sensitive to 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP/MPP+), which destroy DA neurons via induction of oxidative stress [6]. Moreover, oxidative stress plays an important role in the function of the familial PD-related genes, α-synuclein and parkin [3]. Oxidative damaged α-synuclein is aggregated into Lewy bodies [7], and the function of Parkin is impaired by oxidative modifications [8,9]. Additionally, various animal models of familial PD show greater damage in response to oxidative stress [10–16]. Although these data demonstrate a correlation between PD and oxidative stress, the molecular mechanisms underlying oxidative stress-induced DA neuronal death in PD are not well understood.
Author Summary

DJ-1 is an antioxidant protein that has been implicated in autosomal recessive Parkinson’s disease (PD), although the mechanism by which DJ-1 deficiency causes PD remains elusive. Drosophila DJ-1 mutants are highly sensitive to oxidative stress and UV irradiation, and oxidative stress-induced cell death is significantly increased in dopaminergic neurons. In this study, we characterized a Drosophila homologue of death domain-associated protein (Daxx), Daxx-like protein (DLP), as a key player in the process of the oxidative stress-induced cell death in DJ-1 mutants. Upon oxidative stress, DLP expression was increased in the DJ-1 mutants, and locomotive defects and oxidative stress-induced phenotypes including apoptosis and lethality were dramatically rescued by DLP deficiency. More interestingly, we revealed that Drosophila forkhead box subgroup O was required for the increased DLP expression in DJ-1 mutants. Additionally, Drosophila DJ-1 suppressed DLP and Daxx translation from the nucleus to the cytosol in both fly brain and mammalian cells. Interestingly, targeted expression of Drosophila DJ-1 to mitochondria efficiently inhibited Daxx translocation. Our results show that Drosophila DJ-1 protects dopaminergic neurons from oxidative stresses by regulating the subcellular localization and gene expression of DLP, providing a clue to understanding the molecular mechanism underlying oxidative stress-induced neuronal death in PD.

Among the genes related to PD, DJ-1 is the most closely associated with oxidative stress [17]. DJ-1 was originally identified as an oncogene that transforms mouse NIH3T3 cells in cooperation with ras [18], and its gene expression is increased in various types of cancer [19–21]. Later, DJ-1 was linked to an autosomal-recessive early-onset type of familial PD [22,23]. The impairment of DJ-1 function sensitizes animal models to oxidative stress [11,12,14–24]. DJ-1 performs several critical functions in response to oxidative stress via diverse cellular mechanisms [5,17,23]. First, DJ-1 functions as an atypical peroxiredoxin-like peroxidase that scavenges peroxides by oxidizing Cys106 [14]. Second, DJ-1 regulates expression of several antioxidant genes [27–30] and stabilizes the antioxidant transcriptional master regulator, Nrf2 [29,31]. Third, DJ-1 inhibits UV- and oxidative stress-induced cell death by suppressing pro-apoptotic factors [32–34]. In Drosophila, there are 2 homologues of human DJ-1: DJ-1z and β [11,12,24]. DJ-1z is predominantly expressed in the testes, whereas DJ-1β is expressed in most tissues [11,24], similar to the expression pattern of mammalian DJ-1 [18]. Several previous studies have demonstrated that DJ-1β loss-of-function mutants are acutely sensitive to oxidative stress and prone to locomotive dysfunction, resembling the phenotypes seen in PD [11,12,14,24].

In this study, we identified a Drosophila homologue of death domain-associated protein (Daxx), Daxx-like protein (DLP), as a mediator of Drosophila DJ-1β mutant phenotypes. Daxx, originally identified as a binding partner of the pro-apoptotic receptor Fas (also called CD95) [35], performs a pivotal function in apoptosis [36,37]. Daxx activates apoptosis signal-regulating kinase 1 (ASK1), which in turn increases c-Jun N-terminal kinase (JNK) activity leading to apoptosis [38,39]. Daxx is increased in cells upon exposure to hydrogen peroxide and functions as a mediator of oxidative stress-induced apoptosis [33,40,41]. In neuronal cells, dominant negative-Daxx blocks Fas-induced cell death [42]. In addition, FADD/caspase-8 cascade-triggered cell death requires the transcriptional activation of Daxx in normal embryonic motor neurons [43]. Furthermore, Daxx has been identified as a potential component of the pathogenesis of neurodegenerative diseases, including PD [33,44]. For example, Daxx interacts with DJ-1 [33], and MPTP induces translocation of Daxx from the nucleus to the cytoplasm and activates the ASK1 signaling pathway in mouse SNpc [44].

Although previous studies have demonstrated that Drosophila DJ-1β mutants are acutely sensitive to oxidative stress [11,12,14,24], the cellular consequence of DJ-1β deficiency in the oxidative stress response remains unclear. Furthermore, the link between DJ-1 and PD, especially in the context of oxidative stress, has not been thoroughly explored. In this study, we used Drosophila and mammalian cells to investigate the functional interaction between DJ-1 and its downstream target, Daxx/DLP. We also characterized the relationship between loss of DJ-1 and PD-related phenotypes, such as DA neuronal degeneration and locomotive dysfunction, and examined the molecular mechanism of oxidative stress sensitivity in Drosophila DJ-1 mutants.

Results

Oxidative stress-sensitive neuronal death in DJ-1β mutants

Drosophila DJ-1β mutants do not have gross morphological defects or loss of DA neurons when raised under standard laboratory conditions (Figure S1) [24]. However, expression level of tyrosine hydroxylase and number of DA neurons were significantly reduced in DJ-1β mutant flies after hydrogen peroxide treatment compared to those in wild-type animals (Figure 1A). The numbers of DA neurons in 3 major clusters of the posterior brain, dorsomedial clusters, dorsolateral clusters 1, and posteriomedial clusters, were significantly decreased by oxidative stress (Figure 1B). Next, we tested the effect of similar oxidative stress conditions on the neurons of larvae. As shown in Figure 1C, oxidative stress-induced cell death was dramatically increased in the brains of DJ-1β mutant larvae compared to that in wild-type controls. This suggests that DJ-1β mediates developmentally universal protection of DA neurons from oxidative stress-induced cell death.

DLP is a downstream gene of DJ-1β

To characterize the protective mechanism of DJ-1β, we used microarrays to compare the gene expression profiles of DJ-1β mutants and wild-type controls under oxidative stress conditions. We identified 143 upregulated and 134 downregulated genes (>1.5-fold changes in expression DJ-1β mutants) in the mRNA extracted from fly heads (Table S1). The role of DLP in the DJ-1-dependent oxidative stress response was first examined among the upregulated genes because Daxx, the mammalian homologue of DLP, has been implicated in oxidative stress-induced apoptosis [40] and identified as a potential component of PD pathogenesis [33,44]. DLP is a 183.9-kDa protein with approximately 46% similarity to human Daxx in the Daxx-homology region; it is the only Daxx homologue in the Drosophila genome [45]. Interestingly, DLP protein levels were significantly higher in the fly head than in the body (Figure 2A and Figure S2), suggesting that it performs an important function in the brain. Furthermore, DLP mRNA and protein levels were increased by both UV irradiation and oxidative stress (Figure 2B and 2C), implying that DLP is involved in these stress responses, similar to its mammalian counterpart.

To confirm our microarray data, we evaluated DLP expression in the heads of wild-type and DJ-1β mutant flies following treatment with H2O2 using real-time quantitative PCR and western blot analysis. The levels of DLP mRNA and protein did not differ between the DJ-1β mutants and wild-type controls.
Figure 1. Decreased DA neurons and increased apoptosis in DJ-1β mutant under oxidative stress conditions. (A) DA neurons visualized by immunohistochemical analysis with anti-tyrosine hydroxylase antibody in the brains of wild-type (WT) and DJ-1β mutant (DJ-1βex54) flies fed with...
under standard laboratory conditions (Figure 2D and 2E). However, as anticipated, the levels of DLP mRNA and protein were significantly elevated in DLP knockdown to confirm the DLP mutant phenotypes.

DLP functions as a pro-apoptotic gene by activating the JNK/dFOXO signaling pathway

Daxx was originally identified as a pro-apoptotic gene that induced cell death [35]. However, the role of Daxx and DLP in apoptosis is somewhat controversial [38,45]. In order to confirm the function of DLP in non-stress-induced apoptosis, we utilized the UAS-GALA system to evaluate the effects of DLP overexpression on developing tissues. As EY09290 harbors a P-element in the 5’ region of DLP and the direction of the P-element is oriented to induce DLP gene expression (Figure 3A), the mutant was employed to study DLP overexpression. The induction of DLP expression was confirmed by RNA in situ hybridization in the wing imaginal discs of the EY09290 line harboring MS1096-GALA driver (Figure S6A). Interestingly, DLP overexpression in the developing wing under the control of MS1096-GALA reduced organ size in a dose-dependent manner (Figure 4A). When DLP was overexpressed in neurons, reduced survival and defects in locomotive behavior were observed (Figure S6B and S6C), suggesting that increased DLP expression has an adverse effect on neuronal development and function in Drosophila. Indeed, DLP overexpression strongly induced cell death in the imaginal disc (Figure 4B; compared with MS1096/Y and MS1096>DLP0/2), without affecting the cell cycle or differentiation (Figure S6D and S6E). Furthermore, the DLP-induced reduction in wing size was suppressed almost completely by co-expression of Drosophila inhibitor of apoptosis protein 1 (DLP1), a caspase inhibitor. These results demonstrate that DLP activity induces apoptosis through caspase activation and reduces overall survival, as demonstrated by the reduced survival of DLP-overexpressing flies (Figure S6B).

In mammals, Daxx induces apoptosis by activating the JNK signaling pathway [33,39]. We attempted to determine whether...
Figure 2. Gene expression of DLP by oxidative stress, UV, and DJ-1β

(A) DLP protein levels in the wild-type (WT) fly body and head (Student’s t-test, n = 5, * p < 0.05). (B–C) DLP mRNA (B) and protein (C) levels in WT embryos exposed to UV (50 mJ/cm²) and the heads of WT flies fed with 1% H₂O₂ (B, Student’s t-test: UV, n = 5, ** p < 0.01; H₂O₂, n = 5, *** p < 0.001; C, Student’s t-test: UV, n = 5, * p < 0.05; H₂O₂, n = 4, ** p < 0.01). (D–G) DLP mRNA (D, F) and protein (E, G) levels in the heads of WT and DJ-1βex54 flies grown with cornmeal-soybean standard fly food (D–E) and fed with 1% H₂O₂ for 3 days (F–G). (D, Student’s t-test, n = 8; E, Student’s t-test, n = 7; F, Student’s t-test, n = 6, * p < 0.05; G, Student’s t-test, n = 7, * p < 0.05). (H–I) DLP mRNA (H) and protein (I) levels in control (elav/Y) and pan-neuronally DJ-1β-overexpressing (elav>HA-DJ-1β) fly head (H, Student’s t-test, n = 5, *** p < 0.001; I, Student’s t-test, n = 4, *** p < 0.001). (J) Daxx mRNA level in the WT and DJ-1 null SN4741 cells treated with 1 mM H₂O₂ (Student’s t-test, n = 3, ** p < 0.01). (K–L) Western blot (K) and statistical (L) analysis of cytosolic (c) and nuclear (n) fractions of WT and DJ-1βex54 fly head extracts showed that increased translocation of DLP to the cytosol in DJ-1βex54 compared to WT controls (Student’s t-test, n = 4, ** p < 0.01). Relative cytosolic DLP levels were calculated by dividing the normalized cytosolic DLP level by the normalized nuclear DLP level. Lamin and β-tubulin were used as loading controls for nuclear and cytosolic fractions, respectively. (M) Confocal images of DLP immunohistochemistry in the larval brains of WT and DJ-1βex54. Hoechst-stained regions represent nuclei. Magnification, 8,000×. (N) Confocal images showing subcellular localization of Daxx in WT and DJ-1 null SN4741 cells. MitoTracker-stained spots represent mitochondria. The cells were treated with 0.4 mM H₂O₂ for 1 h. (O) The ratio of the cells with cytosolic localized Daxx in DJ-1 null SN4741 cells transfected with wild-type DJ-1β or nucleus (Nuc), cytoplasmic membrane (CM), Golgi (Golgi), or mitochondria (Mito)-targeted DJ-1β. The cells were treated with 0.4 mM H₂O₂ for 1 h. More than 70 cells per each samples were counted to calculate the ratio of the cells with cytosolic Daxx (Student’s t-test, n = 4, * p < 0.05, *** p < 0.001). All data are expressed as means ± s.e. values. Actin was employed as an internal control of total extract.

doi:10.1371/journal.pgen.1003412.g002
**Figure 3. Generation and characterization of DLP mutants.** (A) Genomic structure of the DLP gene. Exons of the DLP gene are shown in black (coding region) and white (non-coding region) boxes. The inverted triangles indicate the P-elements, EY09290 and KG01694. The deletion sites of DLP1, DLP2, DLP3, and DLP4 are illustrated under the genomic structures. (B) Determination of the deleted size in DLP mutants by genomic DNA PCR. (C) Western blotting of DLP in wild type (WT), DLP loss-of-function (DLP1 and DLP2) and gain-of-function (elav>DLP) mutants. Intact DLP protein is not detected in the DLP mutants. (D) Comparison of DLP gene expression in the third-instar larval brains of WT and a DLP mutant via RNA in situ hybridization. (E–F) Survival rates of DLP loss-of-function (DLP1, DLP2, and elav>DLP) and gain-of-function (elav>DLP) mutants under oxidative stress conditions. (G) WT and DLP1/DLP2 were used as controls (log-rank test: WT, n = 300; DLP1, n = 250; DLP2, n = 250; DLP1/DLP1, n = 300, p<0.01, groups with the same letter do not differ significantly). (F) elav/Y was used as a control (log-rank test: elav/Y, n = 350; elav>DLP, n = 300; elav>DLP, n = 300, p<0.01, groups with the different letter differ significantly). The genotypes of the samples were elav/Y (elav-GAL4/Y), elav>DLP (elav-GAL4/Y; EY09290+), and elav>DLP (elav-GAL4/Y; UAS-DLP-RNAi/+). (G) Acridine orange staining of larval brains of DLP1 and WT treated with 0.1% H2O2 for 24 h. (H) Survival rates of WT and DLP mutant (DLP1 and DLP2) pupae after exposure to UV irradiation (10 mJ/cm²; black bars) as described in the
Materials and Methods (Kruskal-Wallis test: CTL, n=6, p<0.1; UV, n = 6, p<0.01, groups with the same letter do not differ significantly). CTL, UV-untreated control pupae; UV, UV-treated pupae. All data are expressed as means ± s.e. values. (i) TUNEL-stained images of UV-exposed 0–3 h embryos of WT and DLP. The lower panels are higher-magnification images of the boxes indicated with dotted lines in the upper panels. CTL, control; rv, revertant; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.
doi:10.1371/journal.pgen.1003412.g003

the JNK signaling pathway is activated by DLP in Drosophila. We initially examined the genetic interaction of DLP with basket (bsk), a Drosophila JNK, and hemipterous (hep), a Drosophila JNK kinase (JNKK). Although overexpression of DLP, bsk, or hep in the wing produced only a slight reduction in wing size, overexpression of either bsk or hep in conjunction with DLP resulted in severely rumpled and shrunked wings (Figure 4C). Consistently, co-expression of hep with DLP strongly induced cell death in the wing imaginal disc (Figure 4B). On the other hand, bsk or hep loss-of-function mutation or co-expression of puckered (puc), a negative regulator of JNK (a JNKK phosphatase), suppressed the cell death and wing deformities induced by DLP overexpression (Figure 4B and 4C). Furthermore, co-expression of bsk or hep with DLP resulted in a strong increase of JNK phosphorylation (Figure 4D). These results demonstrate that DLP activates the JNK signaling pathway, similar to mammalian Daxx.

As FOXO is a target of JNK in mammalian systems [47] and acts downstream of JNK signaling in the control of apoptosis [48], we investigated the role of FOXO in DLP-induced apoptosis. We examined the effect of FOXO deficiency on the DLP-induced gain-of-function phenotype and apoptosis. FOXO deficiency (dFOXO2−/−) suppressed the DLP-induced wing (Figure 4E) and cell death (Figure 4B) phenotypes, suggesting that the JNK/dFOXO pathway is downstream of DLP.

It has been shown that JNK activates FOXO4 activity through phosphorylation at Thr447 and Thr451 [47]. Therefore, we assessed whether dFOXO is also phosphorylated by JNK. Amino acid sequence analysis between mammalian FOXOs and dFOXO implies that JNK regulates dFOXO activity by increasing its protein level or stability in Drosophila. Interestingly, expression of constitutively active JNK (JNKK) induced cell death and wing deformities (Figure 5B) phenotypes, suggesting that the JNK/dFOXO pathway is downstream of DLP.

DLP contributes to the oxidative stress-related phenotypes of DJ-1β mutants

Because DLP expression is regulated by DJ-1β under oxidative stress conditions (Figure 2) and DLP is important for oxidative stress-induced apoptosis and lethality (Figure 3 and Figure S5), we assessed the role of DLP in the oxidative stress-related phenotypes of DJ-1β mutants, specifically their acute sensitivity to oxidative stress and locomotive dysfunction. To accomplish this, we initially examined the genetic interaction of DJ-1 with DJ-1β double mutants, specifically their acute sensitivity to oxidative stress-induced apoptosis and lethality (Figure 3 and Figure S5). Consistently, oxidative stress-induced DA neuronal death in DJ-1β mutants was almost completely inhibited by DLP deficiency (Figure 5C and Figure S7). These findings indicate that DLP is a key mediator in the oxidative stress-induced neuronal cell death of DJ-1β mutants. Furthermore, DLP deficiency rescued the UV sensitivity (Figure 5D) and locomotive dysfunction (Figure 5E) of DJ-1β mutants. These results strongly suggest that DLP mediates the H2O2-induced oxidative stress responses, UV sensitivity, and locomotive dysfunction of DJ-1β mutants.

DJ-1β negatively regulates DLP expression through dFOXO

Next, we investigated the mechanism by which DJ-1β regulates DLP expression. Previous studies with mammalian DJ-1 suggest that DJ-1 affects oxidative stress-related gene expression by stabilizing Nrf2 [29] or by suppressing p53 transcriptional activity [34]. Alternatively, DJ-1 regulates phosphatidylinositol 3-kinase (PI3K)/Akt signaling [19,49,50], which inhibits FOXO. Therefore, we tested whether cap’n’collar C (cncC, Drosophila Nrf2), p33, or dFOXO is involved in the regulation of DLP gene expression by DJ-1β under oxidative stress conditions. As demonstrated in Figure 6A and Figure S8, neither cncC nor p33 affected the DLP levels in wild-type or DJ-1β mutant flies. However, the increased DLP mRNA (Figure 6B) and protein (Figure 6C) levels in DJ-1β mutants were restored to normal levels by dFOXO deficiency, suggesting dFOXO is required for elevation of DLP expression in DJ-1β mutants. Thus, when dFOXO was overexpressed in neurons, DLP expression was elevated more than 2-fold for both mRNA (Figure 6D) and protein (Figure 6E). Since a putative FOXO recognition element (ERE, AAAAAAAC) is located at 1,041 bp upstream of the transcription start site of the DLP gene (Figure 6F) [51], to examine the positive effect of dFOXO on the DLP gene expression at the transcriptional level, we cloned two different sizes of the DLP promoter region into a firefly reporter plasmid; 0.5 kb and 1.3 kb, respectively. Upon transient co-transfection with a construct expressing the constitutively active form of dFOXO (pMT-dFOXO A3), the construct containing the 1.3-kb fragment of the promoter region, but not the 0.5-kb fragment, exhibited the dFOXO-dependent promoter activity in a dose dependent manner (Figure 6F). To confirm whether this putative CRE site is critical, a mutation that disrupts the dFOXO binding was introduced into the 1.3-kb promoter construct by site-directed mutagenesis. Upon transfection, the construct containing the mutant 1.3-kb promoter region no longer showed dFOXO-dependent promoter activity (Figure 6F), indicating that dFOXO regulates DLP expression through this CRE site.

Additionally, we revealed that phospho-Akt (an activated form of Drosophila Akt (dAkt), a negative regulator of dFOXO) was significantly reduced in DJ-1β mutants (Figure 6G). We also found that gene expression of Drosophila 4E-BP, a target of dFOXO, increased in DJ-1β mutants, although dFOXO gene expression remained unaltered (Figure 6H). Moreover, overexpression of PTEN, a negative regulator of the Akt signaling pathway and, therefore, an activator of dFOXO, elevated the DLP level.
Figure 4. DLP activates apoptosis and the JNK/dFOXO signaling pathway. (A) Comparison of tissue sizes of control (MS1096/Y), DLP-overexpressing (MS1096>DLp and MS1096>DLp^{x2}), and DLP- and DIAP1-coexpressing (MS1096>DLp^{x2}+DIAP1) fly wings. Two copies of the DLP gene were overexpressed in MS1096>DLp^{x2}. (B) Acridine orange-stained images of control (MS1096/Y), DLP-overexpressing (MS1096>DLp and MS1096>DLp^{x2}), DLP- and hep-coexpressing (MS1096>DLp+hep), DLP-overexpressing and hep deficient (MS1096>DLp, hep^{1}), DLP-overexpressing
and bsk deficient (MS1096->DLP, bsk^1), DLP- and puc-coexpressing (MS1096->DLP^2+puc), and DLP-overexpressing and dFOXO deficient (MS1096->DLP^2, dFOXO^1) wing imaginal discs. (C) Genetic interactions of DLP with bsk, hep, and puc in the developing wing. The reduced wing phenotype induced by DLP overexpression (MS1096->DLP->bsk or hep (MS1096->DLP->hep) overexpression, and suppressed by bsk (MS1096->DLP->bsk) or hep (MS1096->DLP->hep) deficiency or co-expression of puc (MS1096->DLP^2+puc). (D) Comparison of JNK activity in the DLP- and coexpressing olfactory sensory neurons (MCl) and DLP- and hep-coexpressing wing imaginal discs (MS1096->bsk or MS1096->DLP->hep) with DLP, bsk- or hep-overexpressing wings (MS1096->bsk or MS1096->hep) by anti-phospho-JNK antibody staining. (E) Genetic interactions of DLP with dFOXO in the developing wing. The wing phenotype of DLP overexpression (MS1096->DLP^2) was strongly suppressed by dFOXO deficiency (MS1096->DLP^2, dFOXO^1). MS1096 with dFOXO deficiency (MS1096, dFOXO^1) was used as controls. (F) DLP protein levels in the control (sev-GAL4) and constitutive active hep-overexpressing (sev->hep^1) flies heads (Student's t-test, n = 9, **p < 0.001). sev, sevenless-GAL4. The data are expressed as means ± s.e. values. The genotypes of the samples were MS1096/Y, MS1096->bsk (MS1096->GAL4/Y, UAS-bsk^1), MS1096->hep (MS1096-GAL4/Y, UAS-hep^1), MS1096->hep (MS1096-GAL4/Y, EY09290/+), MS1096->DLP+bsk (MS1096-GAL4/Y, EY9290/bsk), MS1096->DLP+hep (MS1096-GAL4/Y, EY09290/UAS-hep), MS1096->DLP, bsk^1 (MS1096-GAL4/Y, EY9290/bsk), MS1096->DLP, hep^1 (MS1096-GAL4/hb; EY09290/+), MS1096->DLP^2 (MS1096-GAL4/Y, EY09290/+), MS1096->DLP^2, dFOXO^1 (MS1096-GAL4/Y, EY9290/bsk, MS1096->DLP^2, dFOXO^1, EY09290/UAS-hep), MS1096->DLP^2, dFOXO^1, sev-GAL4 (MS1096-GAL4/Y, sev-GAL4/+), and sev->hep^1 (sev-GAL4/+; UAS-hep^1). bsk, basket; DIAPI1, Drosophila inhibitor of apoptosis protein 1; hep, hemipterous; pJNK, phospho-JNK; puc, pucked.

doi:10.1371/journal.pgen.1003412.g004

(Figure 6f). These results consistently indicate that DJ-1β regulates DLP gene expression through the PI3K-Akt-dFOXO pathway.

Finally, we investigated whether DJ-1β could suppress dFOXO-induced apoptosis. As shown in Figure 6j, DJ-1β overexpression strongly suppressed dFOXO-induced eye degeneration and apoptosis (compare e17>dFOXO with e17>dFOXO+DJ-1β).

Discussion

Recently, several Drosophila PD models, each of which represents a different PD-associated gene mutant, have been developed and characterized [10–13,16,24,49,52–54]. Although each exhibits a distinct phenotype, a common feature of all these models is sensitization to oxidative stress. This, along with pathology data from PD patients [55–57], strongly indicates a significant role for oxidative stress in the development and progression of PD. Since the DJ-1 mutations have been linked to familial PD and hypersensitivity to toxins that induce oxidative stress [23], we examined a signaling pathway that controls oxidative stress responses by DJ-1 in Drosophila. We demonstrated that DJ-1β inhibits oxidative stress-induced neuronal apoptosis by regulating DLP gene expression and protein subcellular localization, suggesting a causal relationship between DJ-1β mutation and oxidative stress-induced DA neuronal loss in PD.

Our genetic and cellular analyses indicate DLP functions as a pro-apoptotic gene and as a JNK activator in Drosophila, like its mammalian homologue Daxx. Previous studies have shown that Daxx is upregulated in response to oxidative stress and UV irradiation; it also mediates apoptosis in these contexts [40,41]. Consistent with these reports, our results demonstrate that DLP expression is elevated by H2O2 and UV exposure. Moreover, the apoptosis induced by these insults is reduced dramatically in DLP mutants. In contrast to oxidative stress or UV irradiation, γ-ray irradiation [40] gray-induced apoptosis was unaffected by DLP deficiency (data not shown). This is consistent with a previous report, which demonstrated that DLP is not associated with radioresistance [45]. These findings suggest DLP does not function as a general pro-apoptotic factor, but rather exerts a pro-apoptotic function in response to specific insults, including oxidative stress and UV irradiation. Moreover, the level of DLP in neurons was associated with fly survival rates under oxidative stress conditions. The pan-neuronal overexpression of DLP rendered flies more sensitive to oxidative stress than controls, while knockdown or loss of DLP resulted in resistance to oxidative stress. Therefore, we believe DLP functions as a stress response mediator that generates appropriate cellular responses to oxidative stress. The similarities between the functions of DLP and Daxx suggest that this oxidative stress response pathway is highly conserved from insects to mammals.

Due to the pronounced increase in oxidative damage within DJ-1β mutants [52], we hypothesized that DLP functions as an important mediator of hypersensitivity to oxidative stress in DJ-1β mutants. Indeed, DLP expression and translocation from the nucleus to the cytoplasm increased in DJ-1β mutants, and DLP deficiency almost completely rescued the phenotypes of DJ-1β mutants, including oxidative stress-induced DA neuronal loss. Moreover, overexpression of DJ-1β reduced the level of endogenous DLP. These findings suggest DLP plays an important function in the oxidative stress-related phenotypes of DJ-1β mutant flies and that DJ-1β protects flies against oxidative stress, at least in part, by suppression of DLP expression and cytosolic localization.

These observations raised the question of how DJ-1β negatively regulates DLP expression at the transcriptional level under oxidative stress conditions. Our data indicate that DJ-1β controls DLP gene expression by regulating the activity of dFOXO. Furthermore, DLP harbors a consensus FRE in its promoter region and dFOXO overexpression increased DLP expression in neurons. Previous studies as well as this work identified DJ-1 as a positive regulator of the PI3K/Akt pathway [19,49,50], which suppresses the activity of FOXO by phosphorylation [58]. Therefore, it was not surprising to see that loss of DJ-1β function reduced dAkt activity and increased the transcriptional activity of dFOXO (Figure 6G–6H). FOXO activity may be crucial for setting the sensitivity threshold for oxidative stress and determining the appropriate level of stress responses, which ultimately determines whether the cells live or die. From this perspective, the elevated level of dFOXO activity in DJ-1β mutants may render their neurons more sensitive to stress, and thus neurons in mutant animals die more readily than their wild-type counterparts. We also found that dFOXO performs a dual role in DJ-1β mutants with DJ-1β function associated with the upregulation of DLP and as an effector of DLP. Both of these roles increase the DLP-mediated apoptosis in response to oxidative stress in DJ-1β mutants. This suggests that dFOXO is involved in the loss of neurons due to oxidative stress, and possibly, DJ-1 mutation-associated familial PD cases.

In addition to transcriptional regulation, DJ-1 controls DLP translocation from the nucleus to the cytosol. Daxx is translocated to the cytosol under oxidative stress conditions [59] and this translocation is important for its pro-apoptotic function [60]. These studies and our results showed that both mammalian and Drosophila DJ-1 strongly suppress the cytosolic translocation of Daxx/DLP. The molecular mechanism by which DJ-1 suppresses the DLP translocation is elusive. It has been proposed that mammalian DJ-1 directly binds to Daxx and inhibits its
Figure 5. DLP deficiency reduces acute sensitivity to oxidative stress and UV, and improves locomotive dysfunction in DJ-1β mutant.
(A) Comparison of the survival rates of DLP and DJ-1β double mutants (DLP1; DJ-1βex54 and DLP2; DJ-1βex54) with wild-type (WT) and DJ-1βex54 flies under oxidative stress conditions (log-rank test: WT, n = 250; DJ-1βex54, n = 250; DLP1; DJ-1βex54, n = 250; DLP2; DJ-1βex54, n = 250, p < 0.01, groups with the same letter do not differ significantly). (B) Reduced oxidative stress-induced apoptosis was noted in the larval brain of the DLP and DJ-1β double mutant (DLP1; DJ-1βex54) compared to DJ-1βex54. The larval brains were treated with 0.1% H2O2 for 24 h and cell death was detected via acridine orange staining. (C) Sensitized DA neuronal death of DJ-1βex54 under oxidative stress conditions was rescued by DLP deficiency. The flies were fed with 1% H2O2 for 3 days. (n = 10, Student’s t-test: DM, ** p < 0.01, *** p < 0.001; DL1, ** p < 0.01; PM, ** p < 0.01). (D) Survival rates of WT, DJ-1βex54, and
Drosophila DJ-1 Regulates DLP through dFOXO

Figure 6. The role of dFOXO in the regulation of DLP by DJ-1.

(A) DLP protein levels in the heads of control (tub-GAL4), DJ-1 mutant (tub-GAL4, DJ-1<sup>pex4</sup>) and the double mutant of cncc and DJ-1 (tub>cncCI, DJ-1<sup>pex4</sup>) flies fed with 1% H<sub>2</sub>O<sub>2</sub> (Student’s t-test, n = 4, * p < 0.05). NS, not significant. (B–C) DLP mRNA (B) and protein (C) levels in the head of WT, DJ-1<sup>pex4</sup>, and DFOXO and DJ-1 double mutant (DFOXO<sup>31</sup>, DJ-1<sup>pex4</sup>) flies fed with 1% H<sub>2</sub>O<sub>2</sub> (B, Student’s t-test, n = 5, ** p < 0.01; C, Student’s t-test, n = 4, * p < 0.05). (D–E) DLP mRNA (D) and protein (E) levels in control (elav/Y) and pan-neuronally DLP-overexpressing (elav>UAS-dFOXO) fly heads (D, Student’s t-test, n = 6, *** p < 0.001; E, Student’s t-test, n = 5, * p < 0.05). (F) Luciferase assays showed activation of DLP promoters in S2 cells after cotransfection with dFOXO-A3. (Open circle) Empty vector. (Open triangle) 0.5-kb fragment of DLP promoter. (Filled triangle) 1.3-kb fragment of DLP promoter with mutation in the putative FRE site; pGL3–1.3 kb (mut). Bold characters in the putative FRE site represent the mutated nucleotides. (Student’s t-test, n = 3, * p < 0.05; ** p < 0.01; *** p < 0.001). (G) The levels of phospho-Akt in the head of WT and DJ-1<sup>pex4</sup> flies fed with 1% H<sub>2</sub>O<sub>2</sub> (Student’s t-test, n = 3, * p < 0.05). dAkt was used as an internal control. (H) dAkt, a target of dFOXO, and dFOXO mRNA levels in the head of WT and DJ-1<sup>pex4</sup> flies fed with 1% H<sub>2</sub>O<sub>2</sub> (Student’s t-test: dAkt, n = 7, *** p < 0.001; dFOXO, n = 7). (I) DLP protein levels in the control (elav/Y) and pan-neuronally PTEN-overexpressing (elav>UAS-PTEN) fly heads (Student’s t-test, n = 4, * p < 0.05). (J) Genetic interactions of dFOXO with DJ-1 in the developing eye. The upper pictures are scanning electron micrographs of the eye flies. The lower pictures are acridine orange-stained images of the eye imaginal discs. The genotypes of the samples were tub-GAL4 (tub-GAL4/+), tub-GAL4, DJ-1<sup>pex4</sup> (tub-GAL4, DJ-1<sup>pex4</sup>), tub>cncCI, DJ-1<sup>pex4</sup> (UAS-cncC-RNAi/+; tub-GAL4, DJ-1<sup>pex4</sup>), DJ-1<sup>pex4</sup> (DFOXO<sup>31</sup>, DJ-1<sup>pex4</sup> (DFOXO<sup>31</sup>, DJ-1<sup>pex4</sup>)), elav/Y (elav-GAL4/Y), elav>dFOXO (elav-GAL4/Y, UAS-dFOXO/+), and elav>PTEN (elav-GAL4/Y, UAS-PTEN/+), ey-GAL4 (ey-GAL4/+), ey>dFOXO (UAS-dFOXO/+; ey-GAL4/+), and ey>dFOXO+DJ-1 (UAS-dFOXO/UAS-HA-DJ-1; ey-GAL4/+). pAkt, phospho-Akt; ey, eyesless. All data are expressed as means ± s.e. values. Actin was used as an internal control.

doi:10.1371/journal.pgen.1003412.g006
translocation. However, we did not observe prominent binding between Drosophila DJ-1 and DLP (data not shown), suggesting Drosophila DJ-1 may regulate the DLP translocation by an alternative mechanism. Interestingly, the mitochondrial targeted DJ-1β efficiently inhibited the Daxx translocation under oxidative stress conditions, suggesting the function of DJ-1 in mitochondria is important for the translocation. Further studies are necessary to understand how DJ-1 inhibits the cytosolic localization of Daxx/DLP under oxidative stress.

Our work with DJ-1β and its downstream effector, DLP, has led us to propose the models illustrated in Figure 7. In a wild-type animal, DJ-1 protects the cells from oxidative stress-induced apoptosis. This protection is a result of activation of the PI3K/Akt pathway that inhibits dFOXO. DLP, among its many functions, induces DLP transcription. DLP expression levels have a direct positive correlation with the likelihood of a cell to undergo apoptosis in response to oxidative stress. It is important to note that not only does DJ-1β suppress DLP expression, but DJ-1β also prevents DLP translocation to the cytosol, which may be critical for the pro-apoptotic function of DLP. However, once cells are damaged by oxidative stress and UV irradiation, the DLP protein acts through the JNK pathway to initiate apoptosis. Since the JNK pathway can increase dFOXO activity, DLP expression can be further increased by a hypothetical feed-forward loop of DLP-JNK-dFOXO. In wild-type animals, this pro-apoptotic loop can be negatively regulated by DJ-1β, while in DJ-1β mutant animals, the inability to control this process leads to increased DLP levels and apoptosis. This increased chance of apoptosis may be an important factor in the development of PD.

Materials and Methods

**Drosophila strains**

DJ-1βAc and p3EBd were previously described [24,61], and UAS-HA-DJ-1β was generated via microinjection of the corresponding plasmid into w1118 embryos. EY0290, KG01694, basket (bok)1, UAS-DLP1, UAS-dFOXO, UAS-PTEN, elav-GAL4, Glass multimer reporter (GMR)-GAL4, tubulin (tub)-GAL4, eyeless (ey)-GAL4, sevenless (sev)-GAL4, wingless (wg)-lacZ, and engrailed (en)-lacZ were acquired from the Bloomington Drosophila Stock Center (Bloomington, IN, USA). UAS-DLP-RNAi was obtained from the Vienna Drosophila RNAi Center (Vienna, Austria). UAS-basket (bok) and UAS-hemipterous (hep) were gifts from Dr. M. Mlodzik (EMBL, Germany). UAS-pucked (puc) and MS1096-GAL4 were generously provided by Dr. M. Peifer (University of North Carolina, Pembroke, NC) and Dr. M. Freeman (MRC Laboratory of Molecular Biology, Cambridge, UK), respectively. UAS-cncC and UAS-cncC-RNAi were gifts from Dr. D. Bohmann (University of Rochester Medical Center). DLP1/2 and dFOXO2/3 were gifts from Dr. I. M. Boros (University of Szeged, Hungary) and Dr. E. Hafen (University of Zurich, Switzerland), respectively. hemipterousCA (hepCA) was gift from Dr. S. Noselli (CNRS, France). UAS-hemipterousCA (hepCA), the constitutively active form of Drosophila JNK, was gift from Dr. K. Mastsumoto (Nagoya University, Japan). All fly strains were maintained at 25°C.

**Microarray**

Total RNA was extracted from the heads of hydrogen peroxide-treated wild-type and DJ-1β mutant flies using an RNeasy Mini kit (Qiagen) in accordance with the manufacturer’s instructions. Total RNA was used as a probe for microarray analyses. GeneChip Drosophila Genome 2.0 Arrays for Drosophila melanogaster were probed, hybridized, stained, and washed in accordance with the manufacturer’s recommendations. Hybridized arrays were scanned using an Affymetrix Command Console, and normalization was conducted using an Affymetrix Expression Console 1.1 (MAS5). These experiments were repeated three times for each sample. We required that the fold change difference between the average of three independent wild-type samples and the average of three independent DJ-1β mutant samples exceed 1.5 (p<0.005).

**Figure 7. Schematic representation of the role of Drosophila DJ-1 in the cellular response to oxidative stress or UV.** dFOXO, DLP, and JNK form a circuit that controls cellular responses to stress, and DJ-1 sets neural sensitivity to stress by regulating this circuit.

doi:10.1371/journal.pgen.1003412.g007
Generation of DLP mutants
To generate DLP mutant flies, we used the EY09290 and KG01694 lines (Bloomington, USA) containing the UAS in the first exon and first intron of the DLP gene, respectively. Two DLP mutants, DLP\(_1^0\) and DLP\(_3^0\), were generated via the imprecise excision of a P-element in the EY09290 line, and DLP\(_1^0\) and DLP\(_4^0\) were obtained from the KG01694 line. Additionally, the revertant line, DLP\(_1^1\), was also generated via precise excision of the P-element in the EY09290 line. Among the excision lines, the deletion lines were selected via genomic DNA PCR. Genomic DNA was prepared from each independent line, and PCR was conducted with primer pairs to amplify sequences lying upstream of the deletion lines were selected via genomic DNA PCR. Genomic DNA was prepared from each independent line, and PCR was conducted with primer pairs to amplify sequences lying upstream of the deletion.

Immunoblotting
A polyclonal antibody against the C-terminus of Drosophila DLP (amino acids 1300–1559) was synthesized in rabbits via injection of pGEX-fused DLP. The specificity of the DLP antibody was verified by immunoblotting and immunohistochemistry using wild-type and DLP mutant fly tissues (Figure 3C and Figure S2). Cell and fly lysates were prepared in lysis buffer A (150 mM NaCl, 25 mM Tris, 10% Glycerol, 0.1% NP-40, and 1 mM EDTA). For western blotting, the membranes were probed with anti-DLP (1:1,000 in Tris-buffed saline with Tween 20 (TBST)), anti-lamin (1:2,000 in TBST; Developmental Studies Hybridoma Bank (DSHB)), anti-β-tubulin (1:2,000 in TBST; DSHB), anti-phospho-Drosophila Akt (Ser505) (1:1,000 in TBST; Cell Signaling Technology), anti-Actin (1:2,000 in TBST; DSHB at the University of Iowa) antibodies. Western blot analyses were conducted with standard procedures using horseradish peroxidase-conjugated secondary antibodies (1:2,000 in TBST; Cell Signaling Technology).

Nuclear/cytosolic fractionation
To separate nuclear and cytosolic fractions, 15 fly heads were collected and homogenized. Nuclear and cytosolic fractions from the fly heads were isolated with Nuclear Extract Kit (Active Motif) in accordance with the manufacturer’s instructions.

RNA in situ hybridization
In situ hybridization experiments were conducted using a digoxigenin-labeled RNA probe (Roche Applied Science) in accordance with the manufacturer’s instructions. The probe was prepared using the PCR product of DLP. Hybridization was conducted at 55°C, and the RNA hybrids were detected with alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody followed by nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) staining.

Analysis of Drosophila development
One hundred embryos of each genotype were placed on grape juice agar plates. After incubation for 2 days at 25°C, the number of larvae hatched was counted to determine embryonic lethality. These larvae were then transferred to standard media (cornmeal, yeast, molasses, agar) and aged at 25°C in upright standard plastic shell vials. Larvae were maintained under non-crowded conditions with 20 individuals per vial. The numbers of pupae and enclosed adult flies were counted. Experiments were repeated 3 times with 100 flies per genotype.

UV irradiation
UV irradiation experiments were conducted as previously described with some modifications [48]. In brief, 10 mid-aged pupae were collected, and the pupal shells surrounding the heads were surgically removed. The samples were UV irradiated at 10 mJ/cm\(^2\) using a UV crosslinker (CL-1000, UV Products). Following irradiation, the pupae were kept in darkness until processing, and the survival rate was determined. Each experiment was repeated more than 5 times. To analyze the effects of UV irradiation on cell death or DLP gene expression in the embryos, 0–3 h embryos were UV-irradiated at a dose of 50 mJ/cm\(^2\).

Oxidative stress test
The effects of oxidative stress on the survival of the indicated lines were evaluated by feeding with hydrogen peroxide. Fifty 3-day-old male flies of the indicated lines were starved for 6 h and then transferred to vials containing 1% hydrogen peroxide in 5% sucrose solution. Surviving flies were counted semi-diurnally. We carried out each survival experiment at least 5 times with 50 flies per genotype (n=250). In order to evaluate gene expression and protein levels under oxidative stress conditions, the flies were fed with 1% hydrogen peroxide for 3 days.

Preparation of RNA and real-time quantitative PCR
For real-time quantitative PCR, total RNA from the 20 fly heads was isolated with an RNasey Protect Mini kit (Qiagen). Then, cDNA was synthesized with a Maxime kit (iNtRON Biotechnology), and real-time quantitative PCR was undertaken using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer’s recommended protocols. Real-time quantitative PCR was performed using StepOne Real-time PCR system (Applied Biosystems). Quantification was performed using the ‘delta-delta Ct’ method to normalize to Actin transcript levels and to control. Each experiment was repeated at least 5 times (n=5). The relative level of DLP, dHE-BP or dFOXO mRNA to Actin mRNA was statistically analyzed by Student’s t-test.

Primers
To determine the deletion sites of the DLP mutant lines, PCR was conducted using the following primer pairs: 5’-ACTGAACAA-TAGTTGAATTAAGGCAAC-3’ and 5’-TGCAACATGG-GGAAGTCTCTG-3’ for DLP\(_1^0\), 5’-TGCCAAATTAGTCTGTCC-3’ and 5’-GGCGTTGTTGCTGAAGA-GA-3’ for DLP\(_3^0\), 5’-TCACCGG-TATCGTTCTGGACT-3’ and 5’-GCCTGGAGGTGCTCAATAAC-3’ for DLP\(_4^0\), 5’-GGGAACACCCGACGGTTGAAAGATC-3’ and 5’-GCCCTGGAGTTGCTCAATAAC-3’ for DLP\(_1^1\), and 5’-GGGGAACACCCGACGGTTGAAAGATC-3’ and 5’-GCCCTGGAGTTGCTCAATAAC-3’ for DLP\(_2^0\).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay
For the TUNEL assay, embryos treated with UV were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. The samples were then washed with PBS and permeabilized by a 2-min incubation in PBS containing proteinase K (10 µg/mL) and 0.1% Triton X-100 on ice. After extensive washing, the samples were incubated for an additional 3 h in TUNEL reaction solution (Roche Applied Science) at 37°C in accordance with the manufacturer’s recommendations. After three rinses with PBS, the embryos were incubated with anti-digoxigenin-AP antibody and stained with NBT/BCIP.
Construction of subcellular organelle-targeted DJ-1β

Full-length DJ-1β cDNA was cloned into diRed2-Mito vector (Clontech) to target DJ-1β to mitochondria. diRed2-Mito vector contains the mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase at the N-terminus. For the nucleus-targeted DJ-1β, pEF/3×NL5/Mtv vector (Invitrogen) was used. To construct the cytoplasmic membrane-targeted DJ-1β, 20-amino acid farnesylation signal from c-Ha-Ras was fused to the C-terminus of DJ-1β resulting in pcDNA3 3×HA DJ-1β-F. For Golgi-targeted DJ-1β, Golgi targeting sequence from β 1,4-galactosyltransferase was fused to the N-terminus of DJ-1β resulting in pcDNA3 Golgi DJ-1β.

Mammalian cell culture and DNA transfection

HeLa cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. WT and DJ-1 null SN4741 cells were established from the substantia nigra region of E13.5 wild-type and DJ-1 knockout mouse embryos, respectively [62]. SN4741 cells were grown in RF medium containing DMEM supplemented with 10% fetal bovine serum, 1% glucose, and L-glutamine (2 mM) at 33°C with 5% CO₂. The transfection of expression plasmids was performed using Lipofectamine plus reagent according to the manufacturer's instruction.

Immunocytochemistry

For immunocytochemistry, SN4741 or HeLa cells were sub-cultured on 12-well culture plates coated with poly-L-lysine (Sigma). Appropriately treated cells were washed once with PBS and fixed in 2% paraformaldehyde for 15 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 5 min. Then, the cells were washed with 0.1% Triton X-100 in PBS (PBS-T) and incubated in blocking solution (4% BSA and 1% normal goat serum in PBS-T) for 1 h. Primary antibodies were added to the blocking solution and the cells were incubated overnight at 4°C. After washing with PBS-T 3 times, the cells were incubated with appropriate secondary antibodies in blocking solution for 45 min at room temperature. The antibody-labeled cells were washed with PBS-T 6 times and mounted with mounting solution [100 mg/mL 1,4-diazabicyclo[2.2.2]octane (DABCO) in 90% glycerol]. The slides were observed with LSM710 laser-scanning confocal microscope (Carl Zeiss). All immunostaining experiments with HeLa cells were conducted at least 3 times (n = 300). Anti-mouse DJ-1β [24] and anti-rabbit Daxx (Cell Signaling Technology) were used as primary antibodies. MitoTracker Red CMXRos (Invitrogen) was used to visualize mitochondria.

Immunohistochemistry

For immunohistochemistry, the wing or eye imaginal discs or adult brains were fixed in 4% paraformaldehyde in PBS at room temperature. The tissues were then washed in PBST (PBS+0.5% Triton X-100) and blocked in PBST with 2% normal goat serum (NGS). The samples were incubated first with rabbit anti-phospho-JNK antibody (1:200 in PBST containing 2% NGS; Promega) or rabbit anti-phospho-histone H3 antibody (1:200 in PBST containing 2% NGS; Upstate Biotechnology) or rabbit anti-DLG antibody (1:200 in PBST containing 2% NGS) or rabbit anti-tyrosine hydroxylase antibody (1:50 in PBST containing 2% NGS; Pel-Freeze Biologicals) and then subsequently incubated with rhodamine-labeled goat anti-rabbit immunoglobulin G secondary antibody (1:200 in PBST; Sigma-Aldrich). The tissues were then washed in PBST (PBS+0.5% Triton X-100) with 0.1% Triton X-100 and blocked in PBST with 2% normal goat serum (NGS; Upstate Biotechnology) or rabbit anti-tyrosine hydroxylase antibody (1:50 in PBST containing 2% NGS; Pel-Freeze Biologicals) and then subsequently incubated with rhodamine-labeled goat anti-rabbit immunoglobulin G secondary antibody (1:200 in PBST; Sigma-Aldrich).

Ectopic gene expression with the UAS-GAL4 system

The UAS-GAL4 system was used to evaluate the phenotypes induced by the overexpression of several target genes, including DLP. The GAL4 gene was placed near a tissue-specific enhancer, allowing for the ectopic expression of the target gene in the desired tissue. GMR-GAL4, MS1096-GAL4, elav-GAL4, tub-GAL4, and ey-GAL4 were used to induce target gene expression in the eye, the whole wing, the nervous system, the whole body, and the eye, respectively.

Acridine orange staining

Acridine orange staining was conducted as previously described [63] with some modifications. The wing or eye imaginal discs of stage L3 larvae were dissected in PBS. In order to characterize the effects of oxidative stress on cell death, we incubated the larval brains for 24 h in Schneider’s Drosophila media with 0.1% hydrogen peroxide. The discs or brains were then incubated for 5 min in 1.6×10⁻⁵ M acridine orange (Sigma-Aldrich) and briefly rinsed in PBS. The samples were subsequently observed under an Axiophot2 fluorescence microscope (Carl Zeiss).

5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining

For X-gal staining, the wing discs were fixed for 4 min in 4% formaldehyde in PBS, washed, and incubated in standard X-gal staining solution (4.9 mM X-gal, 3.1 mM K₃Fe(CN)₆, 3.1 mM K₄Fe(CN)₆, 1 mM MgCl₂, 150 mM NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 0.3% Triton X-100) for 30 min at 37°C before observation.

S2 cell culture and luciferase assay

Drosophila S2 cells were transiently transfected using the Effectene transfection reagent (Qiagen Inc., Valencia, CA) using a standard protocol. After 24 h, CuSO₄ (Sigma) was added to a final concentration of 0.6 mM for the optimal expression of the dFOXO A3 construct (a gift from Dr. Oscar Puig, Roche). Dual luciferase assays were performed using the dual luciferase assay system (Promega Corp., Madison, WI). Harvested cells were lysed with luciferase cell lysis buffer. Cell lysates (20 μl out of 30 μl total lysate per sample) were analyzed for firefly luciferase activity by adding 20 μl of firefly reaction buffer. Furthermore, Renilla luciferase activity was measured by adding 20 μl of Renilla reaction buffer. Luminescence was measured from a 96-well plate by using VictorX5 multilabel plate reader (Perkin-Elmer). The data are presented as fold changes relative to the negative control (transfection with the pMT empty vector as an effecter plasmid) normalized to 1.

Climbing assay

The climbing assay was conducted as previously described [64,65] with some modifications. Ten male flies of the indicated lines were transferred into the climbing ability test vial and incubated for 1 h at room temperature for environmental acclimation. After tapping the flies down to the bottom, we counted the number of flies that climbed to the top of the vial within 4 sec. Ten trials were conducted for each group. The experiment was repeated at least 10 times with independently derived transgenic lines. Climbing scores (ratio of the number of flies that climbed to the top to the total number of flies, expressed as a percentage) were obtained for each test group, and the mean climbing score for 10 repeated tests was compared to the scores of the wild-type flies. All climbing assay experiments were conducted at 25°C.
Statistics
Western blotting data were measured using the Multi gauge V3.1 (Fuji, Japan) software program and converted into ratios of band intensity relative to the controls. Using the non-parametric Wilcoxon signed-rank test or the Kruskal-Wallis test, the data were analyzed to detect any statistical differences between treatments. In particular, when the data analyzed with the Kruskal-Wallis test revealed a statistical difference, the data were arcsine-transformed and subsequently analyzed by ANOVA followed by Tukey’s HSD post-hoc analysis. The climbing assay data were arcsine-transformed, and then ANOVA with Tukey’s HSD post hoc analyses were conducted to detect any differences in climbing ability between treatments. The Kaplan-Meier estimator and the log-rank test were conducted on the pooled cumulative survival data to determine whether each treatment had any effect on the longevity of individuals using Online Application Survival Analysis Lifespan Assays (http://sbi.postech.ac.kr/oasis) [66].

Supporting Information
Figure S1 DA neurons in the brains of wild-type and DJ-1β mutant flies grown under standard laboratory condition. (A) DA neurons visualized by immunohistochemical analysis with anti-tyrosine hydroxylase antibody in the brains of wild-type (WT) and DJ-1β mutant (DJ-1βex54) flies fed cornmeal-soybean standard fly food. The lower pictures, including DM, DL1, DL2, and PM, are the magnified areas of the upper pictures. Magnification of the upper pictures, 100×; Magnification of the lower pictures, 400×. (B) Graphs showing the number of DA neurons in each cluster of upper pictures, 100×. Graphs showing subcellular localization of Daxx in HeLa cells transfected with wild-type DJ-1β or mitochondrial targeted DJ-1β (Mito-DJ-1β). The cells were treated with 1 mM H2O2 for 2 h. MitoTracker-stained spots represent mitochondria. (C) Confocal images showing subcellular localization of Daxx in HeLa cells transfected with wild-type DJ-1β or mitochondrial targeted DJ-1β (Mito-DJ-1β). The cells were treated with 1 mM H2O2 for 2 h. MitoTracker-stained spots represent mitochondria. Daxx was expressed in blue in merged images.

Figure S5 Survival rates of DLP mutants under oxidative stress conditions. (A–B) DLP loss-of-function mutants (A, DLP1 and DLP6) and DLP trans-heterozygous mutants (B, DLP1/DLP6) were resistant to H2O2 treatment relative to the wild-type (WT) strain (log-rank test: n=250, p<0.01).

Figure S6 The effects of DLP overexpression on various biological processes. (A) Confirmation of ectopic DLP gene expression in wing imaginal discs via RNA in situ hybridization. DLP-overexpressing wing (MS1096>DLP), but not the control (MS1096/Y), evidenced a strong DLP mRNA signal. (B–C) Survival rates (B) and climbing ability (C) of flies pan-neuronally overexpressing DLP. Pan-neuronal overexpression of DLP (elav>DLP) reduced both the survival rate of the embryos (ANOVA: n=10, p<0.01) and climbing ability (Wilcoxon rank sum test: n=30, p<0.01) relative to the controls (elav/Y). All data are expressed as means ± s.e. values. (D–E) Immuno-staining using anti-phospho-histone H3 antibody (D) and X-gal staining (E) of control (MS1096/Y, wg-lacZ/+), en-lacZ/+ and DLP-overexpressing (MS1096>MS1096; MS1096>MS1096, wg-lacZ or MS1096>MS1096, en-lacZ) wing imaginal discs. wg-lacZ and en-lacZ are transgenes expressing lacZ under the control of the wings (wg) and engrafted (en) gene promoters, respectively. The genotypes of the samples were elav/Y (elav-GAL4/Y), elav>DLP (elav-GAL4/Y; EY09290/+), MS1096/Y (MS1096-GAL4/Y, MS1096>MS1096, wg-lacZ or MS1096>MS1096, en-lacZ), MS1096>DLP, en-lacZ (MS1096-GAL4/Y; EY09290/wg-lacZ, en-lacZ/+), MS1096>DLP, en-lacZ (MS1096-GAL4/Y; EY09290/en-lacZ), ph3, phospho-histone H3.

Figure S7 DLP deficiency reduces the loss of DA neurons in DJ-1β mutants under conditions of oxidative stress. DA neurons visualized by immunohistochemical analysis with anti-tyrosine hydroxylase antibody in the brains of wild-type (A, WT), DJ-1β mutant (B, DJ-1βex54), and double mutant of DLP and DJ-1β (C, DLP6; DJ-1βex54) flies fed with 1% H2O2 for 3 days. The lower pictures, including DM, DL1, DL2, and PM, are the magnified areas of the upper pictures. Magnification of the upper pictures, 100×; Magnification of the lower pictures, 400×. D, dorsal; DL, dorsal lateral clusters; PM, posteromedial clusters.

Figure S8 Effect of cncC or p53 level on the regulation of DLP protein level. (A) DLP protein levels in the control (elav/Y), cncC-overexpressing (elav>cnC), and cncC knock-down (elav>cncC) fly heads (n=3). (B) DLP protein levels in the heads of the control (WT), DJ-1β mutant (DJ-1βex54) and double mutant of DJ-1β and p53 (DJ-1βex54; p53ex54) flies fed with 1% H2O2 for 3 days. Actin was used as an internal control. The genotypes of the samples were elav/Y (elav-GAL4/Y), elav>cnC (elav-GAL4/Y; UAS-cncC/+), elav>cncC (elav-GAL4/Y; UAS-cncC-RNAi/+), DJ-1βex54 (DJ-1βex54; DJ-1βex54; DJ-1βex54), DJ-1βex54 (DLP1/DLP6), and DJ-1βex54, p53ex54 (DLP1/DLP6, p53ex54).
Table S1 The list of genes for which expression is up- and downregulated in DJ-1/β mutant fly heads compared to wild type under the 1% hydrogen peroxide insult condition. (DOCX)

Acknowledgments
The authors thank Drs. Elizabeth M. H. Tank (University of California at San Francisco) and James Clegg (University of California at San Francisco) for critical review of this manuscript.

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