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**Studies on impairment of DJ-1, the product of a causative gene of
Parkinson's disease**

(パーキンソン病原因遺伝子産物 DJ-1 の機能破綻に関する研究)

A DISSERTATION

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Abbreviations

The abbreviations used in this thesis are follows:

ASK1, apoptosis signal-regulating kinase 1

CPR, NADPH cytochrome P450 reductase

DAT, dopamine transporter

DSS, disuccinimidyl suberate

DTT, dithiothreitol

ERK, extracellular signal-regulated kinase

GST, glutathione S-transferase

H₂O₂, hydrogen peroxide

IP, immunoprecipitation

KD, knockdown

kDa, kilo Dalton

KO, knockout

MAPK, mitogen-activated protein kinase

MEK, MAPK/ERK kinase

MPP⁺, 1-methyl-4-phenylpyridinium ion

M.W., molecular weight

Nrf2, nuclear factor erythroid 2-related factor 2

6-OHDA, 6-hydroxydopamine

PD, Parkinson's disease

PQ, paraquat

PTEN, phosphatase and tensin homolog

ROS, reactive oxygen species

Summary

Parkinson's disease (PD) is caused by dopaminergic neuronal cell death in the substantia nigra due to various triggers including oxidative stress and mitochondrial dysfunction. *DJ-1* is a causative gene for familial PD. Loss-of-function of DJ-1 protein is suggested to contribute to the onset of PD, but the causes of DJ-1 dysfunction remain insufficiently elucidated.

In this study, we found that the SDS-resistant irreversible dimer of DJ-1 protein was formed in human dopaminergic neuroblastoma SH-SY5Y cells, but scarcely in non-dopaminergic HEK293 cells, when the cells were exposed to paraquat and diquat, massive superoxide inducers. The dimer was also formed *in vitro* by superoxide in PQ redox cycling system and hydroxyl radical produced in fenton reaction. Moreover, the formation of the SDS-resistant dimer impaired anti-oxidative stress activity of DJ-1 both in H₂O₂-elimination assay *in vitro* and cell viability assay *in vivo* in cells. Similar SDS-resistant dimers were steadily formed with several mutants of DJ-1 found in familial PD patients.

These findings suggest that DJ-1 is impaired due to the formation of SDS-resistant dimer when the protein is directly attacked by ROS free radicals yielded by external and internal stresses and that the DJ-1 impairment is one of the causes of sporadic PD.

Introduction

Parkinson's disease

Parkinson's disease (PD) is the most common neurodegenerative disorder after Alzheimer's disease. PD occurs by dopaminergic neuronal cell death in the substantia nigra due to various causes such as oxidative stress, mitochondrial dysfunction, and accumulation of abnormal proteins. PD is a multifactorial disorder caused by various genetic and environmental factors and is caused in approximately 1/4000 between 50 and 65 years old and 1/100 above 65 years old people. There are two types of PD: more than 90% cases are sporadic PD whose onset risk increases with age, and 5-10% cases are familial PD. Mutations in various genes designated as *PARK* 1-18, including *parkin/PARK2*, *pink1/PARK6* and *DJ-1/PARK7* have been found to link to the onset of familial PD [1-3], and analyses of functions of these causative gene products contributed to elucidation of mechanisms of onsets and pathogenesis of both sporadic and familial PD. *DJ-1* is also known as *PARK7*, and various deletion and point mutations of DJ-1 have been found to be responsible for the onset of familial PD [2, 4-11].

DJ-1 as an oncogene

DJ-1 was first identified by our group as a novel oncogene that transformed mouse NIH3T3 cells in cooperation with activated H-*ras* [12]. It has been reported that regulatory mechanisms of oncogenic transformation by DJ-1 are related to inactivation of phosphatase and tensin homolog (PTEN) [13] and activation of c-Raf [14].

PTEN converts phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2) by dephosphorylation, and PIP3 activates

Akt which is serine/threonine protein kinase, also known as protein kinase B (PKB). In other words, PTEN leads to inactivation of Akt. When Akt is activated by PIP3, various cellular signals involved in physiological activities such as cell survival are transmitted. DJ-1 was reported to activate Akt through the suppression of PTEN followed by inhibition of cell cycle arrest on serum starvation in mouse fibroblast NIH-3T3 cells [13]. We reported later that DJ-1 directly associated with PTEN to suppress PTEN activity [15].

Ras superfamily is a protein superfamily of small GTPases. Receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) are activated by self-phosphorylation when growth factors such as epidermal growth factor (EGF) bind to RTKs. Activated RTKs lead to activation of Ras by the conversion from GDP-bound form to GTP-bound form. c-Raf protein, a serine/threonine kinase, binds to GTP-bound Ras and activates MEK by phosphorylation, and MEK activates ERK1/2 by phosphorylation. This Ras/Raf/MEK/ERK pathway is involved in various cellular functions such as cell survival and cell division. We recently reported that DJ-1 directly bound to c-Raf and stimulated c-Raf kinase activity [14]. The paper described that EGF-induced activation of c-Raf, MEK and ERK1/2 was reduced in DJ-1 knockdown (KD) and knockout (KO) cells, and that DJ-1 promoted c-Raf kinase activity *in vitro*.

Moreover, a recent study showed the levels of DJ-1 protein in sera of breast cancer patients were about 2-fold higher than those of healthy people [16]. Although the mechanism and significance of DJ-1 secretion remain unclear, it is possible that a high level of DJ-1 protein in serum can be a good marker of breast cancer.

These results indicate that *DJ-1* is an oncogene and that acceleration of DJ-1 leads to oncogenesis.

Transcriptional regulation of DJ-1

DJ-1 is suggested to regulate transcription through binding to various proteins such as protein inhibitor of activated STAT α (PIAS α), DJBP, pyrimidine tract-binding protein-associated splicing factor (PSF), and p53.

PIAS α , a member of transcriptional co-regulators PIAS family, interacts with androgen receptor (AR) and inhibits the transcriptional activity. AR is one of nuclear receptors and binds to androgen in cytoplasm, followed by translocation into nucleus and binding to the specific DNA sequence to regulate gene expression. We reported that DJ-1 directly bound to the AR-binding region of PIAS α and antagonized the interaction between PIAS α and AR, resulting in restoring the suppression of AR transcriptional activity of by PIAS α [17]. We also reported that DJBP, a novel DJ-1-binding protein cloned by our group, repressed the AR transcriptional activity by binding to androgen-bound AR and then recruiting histone deacetylase (HDAC) complex and that DJ-1 bound to DJBP to abrogate the DJBP-HDAC complex [18]. In other words, DJ-1 restored the AR transcriptional activity suppressed by DJBP. The results suggest that DJ-1 is a positive regulator of AR.

PSF is another nuclear protein and regulates splicing and transcription. PSF was reported to bind to the *tyrosine hydroxylase (TH)* promoter. Being sumoylated, PSF repressed TH transcription by recruiting HDAC1. DJ-1 bound to PSF and inhibited the sumoylation of PSF [19]. DJ-1 thus promotes TH expression by inhibiting sumoylation of PSF and recruitment of HDAC1 to the *TH* promoter. Since TH is one of the enzymes involved in dopamine biosynthesis, it is suggested that DJ-1 is a positive regulator of dopamine biosynthesis and therefore the dysfunction of DJ-1 leads to the onset of PD.

Nuclear factor erythroid 2-related factor (Nrf2), a transcriptional factor, is kept in cytoplasm by kelch-like ECH-associated protein 1 (Keap1) and is degraded in ubiquitin-proteasome system. Keap1 interacts with Nrf2 and functions as an adaptor between Nrf2 and E3 ubiquitin ligase. When cells are exposed to oxidative stresses, Nrf2 translocates into nucleus by cancelling the interaction with Keap1 and activates expression of various genes involved in anti-oxidative stress responses. It was reported that DJ-1 stabilized Nrf2 protein by inhibiting the ubiquitination of Nrf2 through preventing the Nrf2-Keap1 interaction [20]. Either the Nrf2 protein expression or transcription of the target genes of Nrf2 such as *NAD(P)H quinone oxidoreductase 1 (NQO1)* was decreased in DJ-1 KD and KO cells. The regulatory mechanism of Nrf2-Keap1 system by DJ-1 was not clarified in the report, but we have found that DJ-1 regulated Nrf2-Keap1 system through activation of the upstream phosphatidylinositol-3 kinase (PI3K)/Akt pathway by the association with PTEN [Niki *et al.* in preparation].

The product of a tumor suppressor gene *p53* functions as a transcriptional factor activating expression of many genes involved in anti-cancer phenomena such as apoptosis, cell cycle arrest, cell senescence and DNA repair. In the induction of apoptosis, *p53* activates several genes including *Bax*, *p53 upregulated modulator of apoptosis (PUMA)* and *Noxa*. DJ-1 was reported to directly interact with *p53* and to inhibit the transcriptional activity of *p53*, followed by the repression of *Bax* expression [21]. The cell death induced by ultraviolet (UV) exposure was decreased by DJ-1 overexpression but was increased by DJ-1 KD. While the *Bax* expression was increased by DJ-1 KD in A549 cells which express intact *p53*, but not in *p53*-null H1299 cells, and the enhancement of *Bax* expression by DJ-1 KD was inhibited by a *p53* inhibitor.

The transcriptional regulations in the Nrf2-Keap1 system and the *p53*-*Bax* pathway

are of a variety of anti-oxidative stress mechanisms of DJ-1.

Protease activity of DJ-1

The structure of DJ-1 is very similar to that of protease I, a cysteine protease from *Pyrococcus horikoshii*, but DJ-1 has an additional α -helix (the ninth α -helix, α -helix 9) at the C-terminal region compared to protease I [22]. Although another group reported the protease activity of the wild-type (WT) DJ-1 using a casein derivative as a substrate [23], DJ-1 WT showed no or little protease activity in our experiments. A DJ-1 mutant designated as DJ-1 Δ H9 lacking the α -helix 9, the structure of which was more similar to that of protease I, showed protease activity stronger than DJ-1 WT [24]. Similar observation was also reported by another group [25]. Among various amino acids sequences examined for the target of digestion by DJ-1 Δ H9, valine–lysine–valine–alanine (VKVA) was most susceptible and Abelson murine leukemia viral oncogene homolog 1 (ABL1) and kinesin family member 1B (KIF1B) were identified as possible substrates of DJ-1 Δ H9. The optimum pH of the digestion was pH 5.5, and Cu²⁺ inhibited the protease activity of DJ-1 Δ H9. [26].

Several structural analyses suggested that E18, C106 and H126 comprised catalytic triad in DJ-1 and that DJ-1 was a cysteine protease requiring C106 as an active center [22, 27-29]. Although it remains unclear whether DJ-1 has protease activity *in vivo*, it is possible that α -helix 9 of DJ-1 may be released by certain cellular protease to activate DJ-1 as a protease.

Mitochondrial regulation by DJ-1

The mitochondrial regulation by DJ-1 has been reported by many groups. DJ-1 KD

and KO leads to mitochondrial dysfunction such as decreased mitochondrial respiration, increased mitochondrial reactive oxygen species (ROS), decreased mitochondrial membrane potential and mitochondrial fragmentation [30, 31]. It was reported that mitochondrial fragmentation and decreased mitochondrial membrane potential was due to increased mitochondrial ROS caused by loss of DJ-1, resulting in the induction of autophagy [31]. Increased mitochondrial ROS is supposed to be caused indirectly by loss of DJ-1 anti-oxidative stress activity, described below, and/or directly by impaired interaction between DJ-1 and mitochondrial regulating proteins. We reported that DJ-1 directly bound to NDUFA4 and ND1, subunits of mitochondrial complex I, and maintained mitochondrial complex I activity [32]. The mitochondrial complex I activity was decreased in DJ-1 KD cells and the decrease was restored by the expression of DJ-1 WT but not of DJ-1 L166P mutant found in PD patients.

A majority of DJ-1 localizes in cytoplasm as well as nucleus, but also in mitochondria despite of no atypical mitochondrial targeting sequence in DJ-1. Regarding the mitochondrial localization of DJ-1, various controversial observations have been reported and there is thus room for argument. It was reported that DJ-1 translocated to mitochondria by the oxidation at C106 on cell exposure to oxidative stress [33]. We previously reported that DJ-1 bound to mitochondrial 70kDa heat shock protein (mtHsp70), one of chaperones, and the binding was promoted by oxidative stress [34]. It is then suggested that translocation of DJ-1 to mitochondria by oxidative stress is executed by chaperones, however the mechanism remains unclear.

We and others reported that several DJ-1 mutants including DJ-1-L166P, -M26I and -E163K found in PD patients mainly localized to mitochondria in a steady state [35-37]. Neither the reason nor the mechanism of the localization of the DJ-1 mutants is clarified,

we reported that mitochondrial membrane potential and N-terminal 12 amino acids were necessary for mitochondrial localization of the DJ-1 mutants including DJ-1-L166P and –M26I [36]. It was also reported that the DJ-1 mutants localized to mitochondrial matrix without molecular weight change by processing of a mitochondrial targeting sequence and the localization was dependent on TOM40 and TIM23, channel components of translocases on the outer membrane and the inner membrane, respectively, which play key roles in the import into mitochondria in general [37]. The localization of endogenous DJ-1 to mitochondrial matrix was also reported [38]. Further analyses of mitochondrial localization of DJ-1 are required.

Chaperone activity of DJ-1

Although DJ-1 has neither ATPase activity nor ATP binding sites, chaperone activity has been suggested for DJ-1. It was reported that the structure of DJ-1 is quite similar to that of *E. coli*. heat shock protein 31 (Hsp31), another molecular chaperone, and that DJ-1 had chaperone activity to prevent heat-induced aggregation of citrate synthase (CS) and luciferase, classical chaperone substrates [39]. It was also reported that DJ-1 prevented aggregation/fibrillization of α -synuclein, one of the products of causative genes of PD, dependently on the oxidation state of DJ-1 [40, 41]. Zhou *et al.* reported that the chaperone activity of DJ-1 toward α -synuclein was promoted by the moderate oxidation at C106 of DJ-1, with hydrogen peroxide (H_2O_2) at 1-20 fold molar ratio, and was impaired by the excess oxidation with overdose of H_2O_2 [41]. It is thus suggested that DJ-1 is activated by the moderate oxidation at C106 while excess oxidation of the protein causes dysfunction. The redox dependent chaperone activity was also indicated for YajL, an *E. coli*. homolog of DJ-1 [42]. YajL formed mixed disulfides with various

cellular proteins identified as partners. When bacteria were exposed to H₂O₂, those complexes due to mixed disulfide were detected with YajL, but not with YajL C106A mutant, in immunoblot analyses following SDS-PAGE under non-reducing conditions, while the complexes were not detected by dithiothreitol (DTT) treatment before SDS-PAGE. The results indicate that DJ-1 may be a redox-dependent molecular chaperone.

DJ-1 is also suggested to be a copper chaperone [43, 44]. It was reported that AtDJ-1a, one of *Arabidopsis thaliana* homologs, protected cells against oxidative stress by the activation of superoxide dismutase (SOD). Multiple lesions in leaves induced by H₂O₂ treatment were increased in AtDJ-1a-null *Arabidopsis* mutant plants and were decreased in *Arabidopsis* mutant plants overexpressing AtDJ-1a compared with WT *Arabidopsis* plants [43]. AtDJ-1a could bind to either *Arabidopsis* copper SOD (CSD1) or copper and copper-binding AtDJ-1a promoted CSD1 activity [43]. It is thus suggested that AtDJ-1a transfer copper to CSD1 to stimulate its enzymatic activity. It was also reported that human DJ-1 could bind to copper and activated SOD1 (human copper SOD) by the transfer of copper to SOD1 *in vitro* [44], however the copper chaperone activity of DJ-1 in cells was not examined. It is thus suggested that DJ-1 is a copper chaperone which transfers copper to copper proteins such as SOD1.

Anti-oxidative stress activity of DJ-1

DJ-1 has multiple functions including transcriptional regulation, protease, mitochondrial regulation and chaperone as described above. The major function of DJ-1 is, however, suggested to be anti-oxidative stress activity [33, 45-48]. The cell death induced by oxidative stress was increased in DJ-1 deficient dopaminergic neurons [46],

and PD phenotypes such as cell death in dopaminergic neurons, decrease of dopamine and motor abnormality were improved by the injection of recombinant DJ-1 protein into brain of PD model rats prepared by injection of 6-hydroxydopamine (6-OHDA) [47]. DJ-1 pathogenic mutants have lost or decreased protective activity against oxidative stress-induced cell death [49].

It was reported that DJ-1 isoforms with excessive oxidation was observed in the brains of familial PD patients with DJ-1 R98Q polymorphisms [50]. Moreover, not only the excess-oxidation forms of DJ-1 but also an SDS-resistant dimer of DJ-1, which was detected after denaturing by SDS in immunoblotting, appeared in the brains of sporadic PD patients as reported in a previous study [51]. The SDS-resistant DJ-1 dimer is a major topic to discuss in this thesis. These isoforms of DJ-1 are suggested to be loss-of-function forms, but the causes of their formation remain unclear.

Two mechanisms have been suggested for DJ-1 anti-oxidative stress activity: one is that DJ-1 itself scavenges reactive oxygen species (ROS) [45], while the other is that activation of anti-oxidative stress pathways through the association with other proteins such as Nrf2 [20], apoptosis signal-regulating kinase 1 (ASK1) and its regulatory proteins [52-54] and PTEN [15].

DJ-1 scavenges H_2O_2 through self-oxidation of its three cysteine residues at positions 46, 53 and 106 (C46, C53 and C106, respectively) to sulfenic acid (SOH), sulfinic acid (SO_2H) and sulfonic acid (SO_3H), however it was reported that the second-order rate constant for the reaction of DJ-1 with H_2O_2 were $0.56 \pm 0.05 \text{ M}^{-1}\cdot\text{s}^{-1}$ which was 10^6 - 10^8 lower than that of peroxiredoxins [55]. C106 is the most sensitive cysteine residue to ROS [56], and DJ-1 loses the chaperone activity by excess oxidation of C106 to sulfonic acid as described above. It was reported that C106 comprised catalytic triad

with E18 and H126 in DJ-1 protease activity [57] and that C106 also comprised a copper binding site with E18 in DJ-1 copper chaperone activity [58]. The steric positions of C46, C53, C106 and E18 in DJ-1 dimer are shown in Fig. 1. We previously reported that a substitution mutant of DJ-1 at C106 from cysteine to serine (C106S) has extremely reduced anti-oxidative stress activity [45, 49], indicating that, among the three cysteine residues, C106 is the most important cysteine residue for DJ-1 function. Furthermore, the gene silencing of the *DJ-1* gene in cultured cells decreased its anti-oxidative stress activity [33, 45, 46], and DJ-1-knockout mice increased dopaminergic neuronal cell death induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [59].

In regards to anti-oxidative stress activity of DJ-1 by the association with other proteins, the regulation by DJ-1 of Nrf2-Keap1 system and PI3K/Akt pathway through the association with PTEN was described above. ASK1 is one of mitogen-activated protein kinase kinase kinases (MAPKKK) and is activated by various stresses such as oxidative stress to induce cell death through the activation of MAP kinase cascade. It was reported that DJ-1 interacted with Daxx which is a positive regulator of ASK1 [52]. DJ-1 inhibited the translocation of Daxx to cytoplasm by keeping Daxx in nucleus to prevent ASK1 activation and cell death induced by oxidative stress, while DJ-1 L166P mutant found in PD patients did not bind to Daxx, resulting in the translocation of Daxx to cytoplasm, ASK1 activation and increase of cell death [52]. Another report tells that DJ-1 interacted with ASK1 and inhibited ASK1 kinase activity [54]. DJ-1 bound to ASK1 by forming mixed disulfides with C106 when cells were exposed to H₂O₂ and suppressed ASK1 activity to protect cells against oxidative stress. The results suggest that DJ-1 is one of negative regulators of ASK1 and that DJ-1 plays a role as a redox

sensor which requires C106.

We reported that DJ-1 interacted with pyrroline-5-carboxylate reductase 1 (PYCR1), a mitochondrial enzyme of proline biosynthesis [48]. PYCR1 catalyzes the conversion of pyrroline-5-carboxylate (P5C) to proline using NAD(P)H as the cofactor, regulates intracellular redox potential and has anti-oxidative stress activity. DJ-1 directly bound to PYCR1 and promoted the enzyme activity [48]. DJ-1 and PYCR1 cooperated to protect cells from oxidative stress [48]. The interaction with PYCR1 is thus suggested to be a part of the anti-oxidative stress activity of DJ-1.

Although oxidative stress is considered as one of the major causes of the onset of PD, the precise mechanism underlying how oxidative stress affects the onset of PD remains unsolved. Moreover, it is insufficiently elucidated how the anti-oxidative stress activity of DJ-1 is impaired.

In this study, we found a novel inactive form of DJ-1 in the anti-oxidative stress activity. We show that the anti-oxidative stress activity of DJ-1 was impaired by free radicals induced in cells by exposure to PQ and diquat, free radical-inducers, through the formation of SDS-resistant irreversible dimer of DJ-1 (SDS-resistant DJ-1 dimer), different from DJ-1 functional dimers which are SDS sensitive and usually formed to explore various functions [60]. These results suggest that the anti-oxidative stress activity of DJ-1 is abrogated by SDS-resistant dimer formation, which causes to render dopaminergic cells vulnerable to oxidative stress and thereby leads to the onset of PD.

Materials and Methods

Cell culture and transfection

SH-SY5Y and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and maintained in an atmosphere with 5% CO₂ at 37°C. DJ-1-knockout (KO) SH-SY5Y cells were established using methods the same as those for establishment of DJ-1-KO 293T cells as described previously [61]. Transfection of plasmids into SH-SY5Y cells and HEK293 cells was carried out using Xfect transfection reagent (TaKaRa, Kyoto, Japan) and Polyethylenimine Max (Polysciences, Warminster, PA), respectively.

Expression plasmids and mutagenesis

pEF-DJ-1-HA harboring WT and various mutants of DJ-1 with C-terminal HA-tag, pGEX-6P-1-DJ-1-WT and pGEX-6P-1-DJ-1-C106S, and pGEX-6P-1-FLAG-DJ-1 were described previously [17, 18, 26, 36, 62]. pEF-L166P-, M26-, D149- and R98Q-HA were constructed by insertion of respective DJ-1 fragments from DJ-1 fragment-containing pcDNA3-FLAG or pGEX-6P-1. pcDNA3-FLAG-DAT was constructed by insertion of human DAT cDNA into EcoRI and XhoI sites of pcDNA3-FLAG. Mutagenesis into DJ-1 was carried out using PrimeSTAR mutagenesis basal kit (TaKaRa) and PCR. Nucleotide sequences used for PCR primers and PCR conditions are described in Table 1.

Expression and purification of recombinant proteins

E. coli (BL21) was transfected with pGEX-6P-1-DJ-1, pGEX-6P-1-C106S DJ-1 and pGEX-6P-1-FLAG-DJ-1 and proteins with an N-terminal GST-tag were expressed by

addition of isopropyl β -D-thiogalactopyranoside for 4 hours at 30°C. Cell pellets were suspended in PBS and lysed with 0.5 mg/ml lysozyme (Wako Pure Chemicals, Osaka, Japan) for 20 min and with 1% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO) for 10 min at 4°C. After sonication of mixtures, lysates were centrifuged at 12,000 x g for 20 min at 4°C. Proteins in supernatants were then applied onto Glutathione Sepharose 4B (GE Healthcare, Buckinghamshire, UK), washed with PBS containing 0.1% IGEPAL CA-630 and dissolved in PBS. GST-tag in proteins was cleaved off by reaction with PreScission Protease (GE Healthcare) for 16 hours at 4°C.

Immunoblotting and CBB staining

Cells were reacted a mixture containing with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS and 1% TritonX-100 (RIPA buffer) for 20 min at 4°C and centrifuged at 12,000 x g for 10 min. To detect dopamine transporter (DAT), cells were lysed with an SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M DTT, 10% glycerol and 0.02% bromophenol blue. Protein samples were then mixed with the SDS sample buffer, heated at 100°C for 5 min, separated on a 12% polyacrylamide gel or 8% polyacrylamide gel for DAT detection and applied to nitrocellulose membranes (Amersham Protran Premium NC 0.2, GE Healthcare). Proteins on membranes were reacted with an Alexa Fluor 680-conjugated secondary antibody (1:10000, Thermo Fisher Scientific, Waltham, MA) and visualized using an infrared imaging system (Odyssey, LI-COR, Lincoln, NE). Antibodies used were anti-DJ-1 (3E8, 1:1000, MBL, Nagoya, Japan), anti-GAPDH (6C5, 1:4000, Merck Millipore, Billerica, MA), anti-DAT (6-8D6, 1:1000, Santa Cruz Biotechnology, Dallas, TX), anti-FLAG (M2, 1:1000, Sigma-Aldrich) and anti-HA (HA-7, 1:10000,

Sigma-Aldrich) antibodies. For the detection of SDS-resistant DJ-1 dimers formed in vitro, samples were separated on a 12% polyacrylamide gel and stained with coomassie brilliant blue (CBB) using Rapid CBB KANTO 3S (Kanto Chemical, Tokyo, Japan) according to manufacturer's instructions.

Cross-linking with disuccinimidyl suberate (DSS)

Cells were washed three times with PBS and lysed with a solution containing PBS, 1% TritonX-100 and 1 mM DSS (Tokyo Chemical Industry, Tokyo, Japan) for 30 min at 25 °C. Cross-linking reactions were stopped by addition of final 50 mM Tris-HCl (pH 7.5) for 15 min at 25 °C. Lysates were cleared by centrifugation at 12,000 x g at 4 °C for 10 min and their supernatants were used for analyses.

***In vitro* NADPH cytochrome P450 reductase (CPR) system**

A reaction mixture containing 0.05 µg/µl recombinant DJ-1, 1 mM NADPH, 5 ng/µl CPR (Thermo Fisher Scientific) and 1 mM PQ in PBS was incubated for 30 min at 37°C. The reaction mixture was added with NADPH and then with an SDS sample buffer to start and stop the reaction, respectively. Twenty µl aliquot of the mixture containing 1 µg DJ-1 was used for SDS-PAGE.

Fenton reaction

A reaction mixture containing 0.05 µg/µl recombinant DJ-1, 100 µM H₂O₂ and 10µM FeCl₂ in PBS was incubated for 60 min at 37°C. The reaction was added with FeCl₂ and then with an SDS sample buffer to start and stop the reaction, respectively. Twenty µl aliquot of the mixture containing 1 µg DJ-1 was used for SDS-PAGE.

H₂O₂ scavenging activity of DJ-1

A reaction mixture containing 0.2 µg/µl recombinant FLAG-DJ-1, 1 mM NADPH, 5 ng/µl CPR and 0-500 µM PQ in 20 µl PBS was incubated for 30 min at 37°C. The mixture was then diluted with cold PBS to the volume of 500 µl, added with an anti-FLAG M2 agarose affinity gel (Sigma-Aldrich) and incubated for 1.5 hours at 4°C with gentle mixing. Precipitates were washed four times with cold PBS, eluted with 100 µg/ml FLAG peptide (Sigma-Aldrich) in 25µl PBS on ice for 30 min and mixed every 10 min using a vortex mixture. Twenty µl of aliquot in a 96-well black microplate (Corning, Corning, NY) was then reacted with 300 nM H₂O₂ in 50 µl of total volume of PBS-containing mixture for 30 min at 25°C. Ten µl of aliquot from the mixture was separated on SDS-PAGE and proteins were stained with CBB. The amount of H₂O₂ was measured using an Amplex Red hydrogen peroxide/peroxidase assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Briefly, test samples in microplates were added with 50 µl of Amplex Red reagent (100 µM)/horseradish peroxidase (0.2 U/mL) and incubated for 30 min at 25°C under no light condition, and their fluorescence intensity was measured at Ex/Em = 525 nm/580-640 nm using a fluorometer (GloMax-Multi+ Detection System, Promega, Fitchburg, WI).

Cell viability assay

SH-SY5Y and DJ-1-KO SH-SY5Y cells at a density of 2.5 x10⁴ cells/well in a 96-well microplate were treated with 2 mM PQ for 8 hours and then with various concentrations of H₂O₂ for 1.5 hours, or with various concentrations of PQ for 8 hours and then with 0.2 mM H₂O₂ for 1.5 hours. Their viability was then measured at the absorbance of 450

nm using Cell Counting Kit-8 (DOJINDO, Kumamoto, Japan).

Two-dimensional (2D) gel electrophoresis

For isoelectric focusing (IEF), proteins were extracted from SH-SY5Y cells treated with or without 2 mM PQ using an IEF lysis buffer containing 0.1 M Tris-HCl (pH 8.8), 8M urea, 2% IGEPAL CA-630 and 0.1 M DTT. After sonication, the cell lysates were centrifuged at 12,000 x g for 5 min at 4°C. Recombinant DJ-1 was reacted in PQ redox system as described above and precipitated with acetone. The precipitates were dissolved by an IEF lysis buffer. Proteins were then separated in the 5% polyacrylamide gel containing 9 M urea, 2% IGEPAL CA-630 and 2.5% Pharmalyte, broad range pH 3.0-10.0 (GE Healthcare). The sample lanes were cut and equilibrated in a buffer containing 20 mM Tris-HCl (pH 6.8), 2% SDS and 0.01% BPB for 15 min, and subjected to SDS-PAGE.

For native-PAGE, SH-SY5Y cells treated with or without PQ were incubated in a mixture containing 50 mM Tris-HCl (pH 7.4), 50 mM NaCl and 0.5 % TritonX-100 for 20 min at 4°C and centrifuged at 12,000 x g for 10 min at 4°C. Recombinant DJ-1 was reacted in PQ redox system as described above and diluted by 4-fold using 200 mM Tris-HCl (pH 7.4). Proteins were then separated in a 5 - 13% gradient polyacrylamide gel with a buffer containing 25 mM Tris and 192 mM glycine. The sample lanes were cut and equilibrated in a buffer containing 20 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M DTT and 0.01% BPB for 15 min, and subjected to SDS-PAGE.

Proteinase K digestion

Cell extracts were prepared from SH-SY5Y cells treated with or without 2 mM PQ.

After incubation in PBS containing 0.5% TritonX-100 at 4°C for 20 min followed by centrifugation at 12,000 x g at 4°C for 10 min, the supernatants were reacted with 100 µg/ml Proteinase K at 37°C for 30 min. Recombinant DJ-1 or GST reacted in PQ redox system as described above was treated with 5 µg/ml Proteinase K and 0.5% SDS at 37°C for 30 min.

Other reagents

Paraquat (PQ) were purchased from Tokyo Chemical Industry. Ascorbate, FeCl₂ and H₂O₂ were purchased from Wako Pure Chemicals. NADPH and diphenyleneiodonium chloride (DPI) were purchased from Oriental Yeast (Tokyo, Japan) and Sigma-Aldrich, respectively.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical analyses were performed using ANOVA followed by the Tukey-Kramer test for multiple comparisons.

Results

DJ-1 forms an SDS-resistant dimer in SH-SY5Y cells by ROS free radicals

PQ, one of a widely used herbicide, is reduced to PQ radicals by intracellular flavoenzymes, including NADPH cytochrome P450 reductase (CPR) [63], xanthine oxidase [64] and NADPH oxidase [65], all of which depend on electron donors such as NADPH. PQ radicals react with oxygen in turn and are recycled back to PQ. In this redox cycling, massive superoxide ($O_2^{\bullet-}$) is produced (see Fig. 2A), resulting in increasing oxidative stress and cellular injury [66]. To investigate the effect of PQ-induced $O_2^{\bullet-}$ on DJ-1, we treated human neuroblastoma SH-SY5Y cells, the most widely used cells as a model system of PD research, with various amounts of PQ, and the cell lysates were separated on an SDS-containing polyacrylamide gel and analyzed by immunoblotting with an anti-DJ-1 antibody. Molecular weights of monomer and dimer of DJ-1 are 21-kDa and 42-kDa, respectively. Although the cell lysates were boiled in the presence of SDS and dithiothreitol (DTT), a band around ~40 kDa, a similar molecular weight to that of DJ-1 dimer, appeared in both time- and dose-dependent manners of PQ treatment (Fig. 2B). In detail, the dimer-like band was clearly detected in certain conditions of exposure to PQ, such as in 1.0 mM for 3 hrs, in 0.5 mM for 6 hrs and 0.1 mM for 12 hrs. To confirm that the protein band induced by PQ treatment is due to an SDS-resistant DJ-1 dimer, cell lysates from SH-SY5Y cells exposed to PQ were treated with 1 mM disuccinimidyl suberate (DSS), a protein cross-linking agent that should render monomers into dimers. As shown in Fig. 2C, the band observed in cells treated with vehicle DMSO and PQ had the same mobility of the bands observed in cells treated with DSS alone or DSS and PQ. In addition, a substitution mutant of glutamine, E18A DJ-1-HA, the level of which homodimer

formation is reduced as described previously [36], and WT DJ-1-HA were expressed in DJ-1-knockout (KO) SH-SY5Y cells to avoid the formation of a heterodimer between endogenous DJ-1 and expressed mutant DJ-1 (Fig. 2D). No band was detected for E18A DJ-1-HA at the mobility of the dimer observed with WT DJ-1-HA in PQ-exposed cells. These results indicated that the band induced by PQ treatment is due to DJ-1 dimer. Three cysteine residues in DJ-1, reported as oxidation hot spots involved in anti-oxidative functions were examined for possible roles in PQ-triggered formation of an SDS-resistant DJ-1 dimer. Three substitution mutants of the cysteines, C46S DJ-1-HA, C53A DJ-1-HA and C106S DJ-1-HA were expressed as E18A DJ-1-HA and WT DJ-1-HA described above (Fig. 2D). The results showed that, while WT DJ-1 formed the SDS-resistant dimer in PQ-exposed cells, the dimer was not detected in C106S DJ-1-HA and C46S DJ-1-HA-expressing cells, except for in C53A DJ-1-HA-expressing cells. Since the expression levels of C46S DJ-1-HA, C53A DJ-1-HA and E18A DJ-1-HA were lower than that of WT DJ-1-HA, it was indicated that these mutants became unstable by the introduction of the mutation. Since the SDS-resistant dimer of C53A DJ-1-HA was clearly observed although the expression level of C53A DJ-1-HA was lower than that of WT DJ-1-HA, it was suggested that the SDS-resistant dimer of C53A DJ-1-HA was formed more than that of WT DJ-1-HA. Among the three cysteine substitution mutants of DJ-1, a mutant of C46 has been reported for low ability to form a homodimer [33]. DJ-1-KO SH-SY5Y cells were transfected with the DJ-1 mutants as in Fig. 2D, and the lysates from the cells were treated with DSS. As shown in Fig. 2E, DSS-crosslinked dimers were detected significantly in the lysates prepared from the cells expressing WT and C106S, moderately in those with C53A, but only faintly for C46S and E18A. The results

indicate that the band observed in PQ-treated cells is SDS-resistant DJ-1 dimer and that the cysteine 106, a preferential oxidation spot [56], is required for the SDS-resistant dimer formation upon PQ exposure of cells. Since the SDS-resistant dimer was detected in the presence of DTT, an association form of the dimer is unlikely due to disulfide bond. We also confirmed that SDS-resistant DJ-1 was not due to a dityrosine bond (data not shown).

To clarify whether the formation of SDS-resistant DJ-1 dimer depended on free radicals produced by PQ, SH-SY5Y cells were treated with PQ in the presence of various amount of ascorbate, i.e. vitamin C, absorbing free radicals. As shown in Fig. 3A, the SDS-resistant DJ-1 dimer induced by PQ exposure was significantly decreased in an ascorbate-dose-dependent manner, suggesting that the formation of SDS-resistant DJ-1 dimer was dependent on free radicals. The $O_2^{\bullet-}$ production by PQ has been reported to be catalyzed by flavoenzymes [66]. SH-SY5Y cells were therefore co-treated with PQ and diphenyleneiodonium (DPI), an inhibitor of flavoenzymes. Co-treatment with DPI significantly attenuated the PQ-induced formation of SDS-resistant DJ-1 dimer (Fig. 3B), suggesting that the SDS-resistant DJ-1 dimer is formed by $O_2^{\bullet-}$ yielded through PQ reduction by flavoenzymes. We further treated SH-SY5Y cells with other oxidative stress agents, including diquat, H_2O_2 , 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium ion (MPP^+), at the respective maximal doses which allowed the cells to survive. Among the agents tested, only diquat, another massive free radical-inducing agent similar to PQ structure (Fig. 3C), yielded SDS-resistant DJ-1 dimer in a dose-dependent manner as in the case of PQ (Fig. 3D). To confirm whether the formation of SDS-resistant DJ-1 dimer by diquat was also dependent on free radicals, SH-SY5Y cells were treated with diquat in the presence of various amount of

ascorbate. The SDS-resistant DJ-1 dimer induced by diquat was similarly attenuated by ascorbate as that induced by PQ (Fig. 3E). The results indicated that the formation of free radical-induced SDS-resistant DJ-1 dimer is not specific for PQ treatment, but also observed with another free radical producer, diquat. For further analyses described below, PQ was used as a free radical inducer.

We found an SDS-resistant dimer was induced by PQ treatment in human neuroblastoma SH-SY5Y cells. We further examined whether such an SDS-resistant dimer was also formed in non-dopaminergic cells. Human embryonic kidney cells HEK293 were exposed to PQ, and DJ-1 in the cells was analyzed as described above. Interestingly, similar SDS-resistant DJ-1 dimer was formed in HEK293 cells, but less clearly than in SH-SY5Y cells (Fig. 4A). PQ has been reported to be transported into cells via dopamine transporter (DAT) into dopaminergic cells [67]. Since the expression of DAT was detected in SH-SY5Y cells, but not in HEK293 cells (Fig. 4A), lack or little of PQ transportation into HEK293 cells may possibly result in a faint band of DJ-1 dimer in the cells, compared to that in SH-SY5Y cells. To examine the possibility, HEK293 cells were transfected with FLAG-DAT and then exposed to PQ. As shown in Fig. 4B, the increase of the band due to SDS-resistant DJ-1 dimer by PQ exposure was more significant in the FLAG-DAT-transfected cells than in the vector-transfected cells. The results indicated that the increase of PQ transportation via DAT into HEK293 cells enhances the formation of SDS-resistant DJ-1 dimer in the cells.

SDS-resistant DJ-1 dimer is formed by free radicals *in vitro*

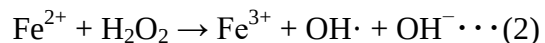
To clarify whether $O_2^{\bullet -}$ produced in the PQ redox cycling system directly turns DJ-1 to the SDS-resistant dimer, the PQ redox cycling system was reconstituted *in vitro*.

Recombinant DJ-1 expressed in and prepared from *E. coli* was reacted with various combinations of NADPH cytochrome P450 reductase (CPR), NADPH and PQ. As observed *in vivo* in cells, the SDS-resistant DJ-1 dimer was clearly formed in the reaction mixture containing CPR, NADPH and PQ (Fig. 5A). A faint band of the SDS-resistant DJ-1 dimer was also detected in the reaction containing CPR and NADPH but lacking PQ, which is probably due to the production of $O_2^{\bullet-}$ from a CPR-catalyzed reaction (1).



In addition, the formation of SDS-resistant DJ-1 dimer in the presence of NADPH and CPR was augmented by PQ in a dose dependent manner (Fig. 5B), and the formation augmented by PQ was inhibited by ascorbate dose-dependently (Fig. 5C). Furthermore, the results of similar *in vitro* experiments using a C106 mutant of DJ-1, which did not give rise to the band of SDS-resistant dimer, indicated that intact C106 of DJ-1 was required for the SDS-resistant dimer formation (Fig. 5D).

Hydroxyl radical ($\cdot OH$), another free radical produced by Fenton reaction between H_2O_2 and Fe^{2+} (2), was also subjected to the *in vitro* reactions for the SDS-resistant DJ-1 dimer formation.



The results showed that SDS-resistant dimer was formed by WT DJ-1, but not by the C106 mutant, in the presence of hydroxyl radical produced by H_2O_2 and Fe^{2+} (Fig. 5E), and that the formation was inhibited by ascorbate (Fig. 5F). These results indicate that free radicals, either superoxide ($O_2^{\bullet-}$) or hydroxyl radical ($\cdot OH$), directly attack DJ-1, resulting in the formation of SDS-resistant DJ-1 dimer.

Anti-oxidative stress activity of DJ-1 is attenuated by SDS-resistant dimer formation

We examined the effect of the SDS-resistant dimer formation on the anti-oxidative stress activity of the protein. H₂O₂ scavenging activity of DJ-1 was examined in an *in vitro* system. FLAG-DJ-1 prepared from *E. coli* was reacted with CPR, NADPH and PQ, and immunoprecipitated with an anti-FLAG antibody to separate from unreacted O₂^{•-} produced by the CPR system. FLAG-DJ-1 was eluted from the precipitate using FLAG peptides and then reacted with H₂O₂, and the amount of quenched H₂O₂ was measured. As shown in Fig. 6A, the recombinant DJ-1 strongly quenched H₂O₂, even after treated with CPR and NADPH, but the amount of H₂O₂ quenched by DJ-1 was reduced by addition of PQ in a dose-dependent manner and in parallel to the increased level of the SDS-resistant DJ-1 dimer formation. The results indicate that the SDS-resistant dimer formation leads to dysfunction of H₂O₂ scavenging activity of DJ-1.

DJ-1 activity toward cell viability against oxidative stress was then examined. DJ-1-KO SH-SY5Y cells as well as the parental SH-SY5Y cells were treated with 2 mM PQ for 8 hours followed by another 1.5 hours treatment with 0 - 0.35 mM H₂O₂, and the cell viability was measured by Water Soluble Tetrazolium (WST) assay using Cell Counting Kit-8. WST-8 contained in the kit is reduced by cellular dehydrogenases in living cells and turns into WST-8 formazan. The cell viability can be assayed by measurement of the absorbance of formazan. As reported by several groups including ours [45, 52, 68], cell viability of DJ-1-KO SH-SY5Y cells was decreased against H₂O₂ treatment without PQ exposure more quickly than that of parental SH-SY5Y cells (Fig. 6B). When the cells were pre-treated with PQ, however, difference of cell viability between DJ-1-KO SH-SY5Y and parental SH-SY5Y cells became statistically

insignificant (Fig. 6B). The results suggest that PQ-treatment abolished the DJ-1 activity of retaining cell viability against H₂O₂ treatment. Next, DJ-1-KO SH-SY5Y cells and parental SH-SY5Y cells were treated with 0 - 2 mM PQ for 8 hours before incubation with 0.2 mM H₂O₂ for 1.5 hours. As shown in Fig. 6C, viability of SH-SY5Y cells against H₂O₂ was significantly higher than that of DJ-1-KO SH-SY5Y cells, and the difference of viability between the two cell lines was decreased dependently on PQ concentration and no significant difference was detected as for 1.0 and 2.0 mM PQ, the concentrations where the SDS-resistant DJ-1 dimer was clearly observed (Fig. 2B). The decrease in cell viability difference was thus suggested to be due to the formation of SDS-resistant DJ-1 dimer. These results suggest that DJ-1 in SH-SY5Y cells was induced by PQ to form the SDS-resistant dimer that has no, or low, protection activity against H₂O₂-induced cell death, and that SH-SY5Y cells thereby behaved similarly to DJ-1-KO SH-SY5Y cells.

Characterization of SDS-resistant DJ-1 dimer

To characterize the SDS-resistant DJ-1 dimer, we performed three experiments. First, we performed two-dimensional (2D) gel electrophoresis of the combination of isoelectric focusing and SDS-PAGE for the cell extracts from SH-SY5Y cells treated with or without 2 mM PQ (Fig. 7A). DJ-1 showed multiple isoforms in both samples and the isoforms with more acidic isoelectric point (pI) values were increased by PQ treatment. The isoforms with more acidic pI were suggested to be oxidized forms of DJ-1 at cysteine residues as previously reported [45, 50]. Several signals corresponding to the SDS-resistant DJ-1 dimers were detected rather at more acidic pI region, i.e. of more oxidized forms of untreated DJ-1 monomer, than at the most neutral pI, probably

reflecting reduced form. In the experiment using in vitro PQ redox system and recombinant DJ-1, several isoform-like signals of SDS-resistant DJ-1 dimer were also detected at more acidic pI values than DJ-1 monomer showed (Fig. 7B). The results indicated that the isoform-like variants of SDS-resistant DJ-1 dimer in SH-SY5Y cells as well as DJ-1 monomer isoforms were probably due to oxidation, by free radicals, but not due to various possible modifications by other cellular factors than free radicals.

Secondly, to examine the behavior of the SDS-resistant DJ-1 dimer under non-denaturing conditions, we performed native-PAGE for recombinant DJ-1 reacted with or without PQ redox system in vitro. While the SDS-resistant DJ-1 dimer appeared in SDS-PAGE in a dose-dependent manner of PQ, no significant change was observed in native-PAGE: a thick hazy band was similarly detected in all lanes (Fig. 7C). The proteins separated in lanes '0' and '500' μ M PQ of Fig 7C, left panel, were subjected to SDS-PAGE (Fig. 7D). As for the proteins treated with 500 μ M PQ, the SDS-resistant DJ-1 dimer was detected together with DJ-1 monomer in the same thick band on native-PAGE (Fig. 7D, lower panel). The results implied that there was no dramatic difference in the structure as a bulk between healthy DJ-1 dimer and SDS-resistant DJ-1 dimer. We further performed native-PAGE for the extracts from SH-SY5Y cells treated with or without PQ (Fig. 7E). Comparing with the results using the recombinant protein, DJ-1 was detected in much broader smear band probably due to complex formation with various cellular proteins as suggested [69]. Between the cell lysates prepared with and without PQ treatment, no significant difference was observed in electrophoretic patterns of the first native-PAGE, but the second SDS-PAGE revealed that the SDS-resistant DJ-1 dimer was detected only in the lysate of the PQ-treated cells, while most of the DJ-1 molecules were similarly observed as monomers in both the lysates with or

without PQ treatment (Fig. 7F), where the strongest signals were observed at the mobility on native-PAGE corresponding to the mobility in the analysis of recombinant DJ-1 (Fig. 7D). The signals corresponding to the SDS-resistant DJ-1 dimer were much weaker than those for monomer, but smaller amounts of the SDS-resistant DJ-1 dimer were also recovered from the lower mobility zone in native-PAGE (Fig. 7F). The results suggested that the SDS-resistant DJ-1 dimer was similarly involved in various protein complexes as healthy, SDS-sensitive dimer or monomer, in a similar proportion, though in much lower amount. The SDS-resistant DJ-1 dimer is thus suggested to have a bulk conformation similar to that of healthy DJ-1 dimer, and the conformation may allow the partner proteins to recognize the SDS-resistant dimer as well as healthy dimer.

To examine whether SDS-resistant DJ-1 was sensitive to ProK digestion, the extracts were prepared from SH-SY5Y cells treated with or without PQ and were reacted with ProK (Fig. 7G). Both of healthy (SDS-sensitive) and SDS-resistant DJ-1 dimers became undetectable by immunoblotting after ProK digestion. Since we could not exclude the possibility that we might miss partial digests of either healthy or SDS-resistant DJ-1 dimer because of nonreactivity with the anti-DJ-1 antibody used in the experiment, recombinant DJ-1 proteins were reacted with ProK before and after reacted in PQ redox system and detected by CBB staining (Fig. 7H). The results show SDS-resistant DJ-1 dimer was digested by ProK same as DJ-1 in the extracts from PQ-exposed SH-SY5Y cells. SDS-resistant DJ-1 dimer was then indicated to be ProK sensitive and was suggested to be not highly aggregated form.

Several DJ-1 mutants found in familial PD patients form SDS-resistant dimers in a steady state

Various mutants of DJ-1 found in familial PD patients were then examined for the formation of the SDS-resistant dimers. DJ-1-KO SH-SY5Y cells were transfected with expression vectors for various HA-tagged DJ-1 mutants and treated with or without PQ. As shown in Fig. 8A, the SDS-resistant dimer was induced in the cells expressing M26I and A179T as in the case of WT DJ-1, but such dimer was not induced in the cells expressing L10P, A107P and L166P. No dimer was detected for these three mutants when the cell lysates were reacted with DSS (Fig. 8B), which suggests L10P, A107P and L166P DJ-1 mutants have lost their intrinsic dimerization ability. Interestingly, the rest of the examined mutants of DJ-1, including E64D, R98Q, A104T, D149A and E163K, formed SDS-resistant dimers in cells even without PQ treatment. These mutants are categorized into two groups in terms of response to PQ: SDS-resistant dimers of E64D and R98Q were of similar amounts with or without PQ treatment, while A104T, D149A and E163K showed increase of the SDS-resistant dimers upon PQ treatment. It is therefore considered that these DJ-1 mutants, especially E64D and R98Q, spontaneously formed SDS-resistant dimers, in transfected cells, similar to those induced by PQ. To examine whether the SDS-resistant dimers of DJ-1 mutants steadily formed without stress inducers were the same as those induced by PQ treatment, additional C106S mutation was introduced into the mutants and the resulting double mutants were expressed in DJ-1-KO SH-SY5Y cells (Fig. 8C). The results showed that all of the C106S-containing mutants, except for a single C106S mutant, formed SDS-resistant dimers under the condition without PQ treatment, suggesting that several DJ-1 mutants found in familial PD patients spontaneously form SDS-resistant dimers in a C106-independent manner probably due to some structural changes or unknown mechanism.

Discussion

In this study, we demonstrated that an SDS-resistant dimer of DJ-1 is induced by $O_2^{\bullet -}$ in PQ-treated human dopaminergic cells and in an *in vitro* reconstituted system for PQ redox cycling (Figs. 2-5). Diquat, also inducing free radical in cells, as well as $\cdot OH$ produced by Fenton reaction *in vitro* yield the SDS-resistant dimer (Figs. 3D and 5E, respectively). It is therefore suggested that the SDS-resistant DJ-1 dimer is formed by free radicals regardless of PQ. Since the SDS-resistant DJ-1 dimer was also formed in a reconstituted system *in vitro*, it was indicated that the dimer was directly formed by free radicals independently of other cellular factors, which is a phenomenon of a novel finding.

C46S, C53A and E18A DJ-1 were unstable compared to WT DJ-1 (Fig. 2D). Since the abilities of C46S and E18A mutants to form healthy dimers were decreased, compared to WT-DJ-1 (Fig. 2E), the two mutants were suggested to become unstable due to loss of the ability of forming healthy dimers. Since the SDS-resistant dimer of C53A DJ-1-HA was clearly observed although the expression level of C53A DJ-1-HA was lower than that of WT DJ-1-HA, it was thus suggested that C53A DJ-1 was triggered to form SDS-resistant dimer by PQ more efficiently than WT-DJ-1. C53A DJ-1, on the other hand, showed instability although the mutant had the ability of forming healthy dimer similarly as WT DJ-1 (Fig. 2D and 2E, respectively), it is therefore suggested that the C53 residue of DJ-1, structurally involved in the intra-DJ-1 dimer interaction spots (see Fig. 1), is required for the stabilization of DJ-1 as dimer so as not to impair the protein.

The SDS-resistant DJ-1 dimer was incompetent for anti-oxidative stress functions, which suggests that free radicals render DJ-1 inactive in anti-oxidative stress functions (Fig. 6). It has been reported that functions of DJ-1, including chaperone activity [40,

41] and stimulation of TH and DDC activities [62], are abrogated by excessive oxidation at SH of C106 to SO₃H. In addition to the aberrant oxidation at C106 of DJ-1, we found a novel mechanism eliciting DJ-1 dysfunction through the formation of SDS-resistant dimer. Moreover, dopamine is oxidized during its metabolism, thereby producing free radicals in dopaminergic cells. Sporadic PD is a multifactorial disorder and considered to be caused by various genetic and environmental factors, including pesticides such as paraquat (1,1-dimethyl-4,4'-bipyridinium dichloride, PQ) [70]. SDS-resistant DJ-1 dimers therefore can be formed in dopaminergic cells when a large amount of free radicals is produced under pathological conditions, leading to inactivation of the DJ-1 function.

Since SDS-resistant DJ-1 dimer was formed in a dose-dependent manner of PQ both in SH-SY5Y cells and in the *in vitro* system (Figs. 2B and 5B, respectively), the level of formation of SDS-resistant DJ-1 dimer is determined by the amount of free radicals in cells. Since the SDS-resistant dimer was detected in the presence of DTT (see 'Materials and methods'), an association form of the dimer was unlikely to be disulfide bond. The SDS-resistant DJ-1 dimer was detected as a band by immunoblotting, not as atypical aggregation bands or smear of high molecular weight. The results suggested that the dimer-like band was due to tightly conjunct dimers, possibly structurally intertwined or locked together or yielding additional covalent bonds, but not due to disordered aggregates. Free radicals may create intermolecular covalent bonds other than disulfide bond and dityrosine bond, or induce strong intermolecular entanglement within DJ-1 dimer, yielding a tight coupling of the dimer, which is not dissociated even by boiling in the presence of SDS, but the dimer structure as a bulk is suggested to be retained (Figs. 7C and 7E).

Prions (Pr), unique infectious proteins involved in neurodegenerative diseases such as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cows and Creutzfeldt-Jakob disease (CJD) in humans, turn infectious altering steric structure. PrP^{Sc}, infectious prion molecules, are resistant to ProK digestion and grow into huge aggregates in neuronal cells. In analogy of prions, DJ-1 may alter higher-order structure, form SDS-resistant dimers and possibly become ProK-resistant. We thus investigated the susceptibility of SDS-resistant DJ-1 dimer to Proteinase K (ProK). The dimer-like band was SDS-resistant but sensitive to Proteinase K digestion (Figs. 7G and 7H). Since the SDS-resistant dimer was not induced by PQ for the mutants that cannot or can hardly form dimers (Fig. 2D), it is hypothesized that formation of the SDS-resistant dimer is based on formation of SDS-sensitive, reversible dimer. Free radicals may attack certain structures or vicinal atoms at closely interacting spots within the dimer, probably around C106 (Figs. 2D, 5D and 5E), and yield intra-dimer entanglement or covalent bonds, resulting in a tight irreversible, SDS-resistant coupling of the dimer. While the increase of SDS-resistant DJ-1 dimer in PQ-exposed SH-SY5Y cells was not in a large part of the total DJ-1 (Fig. 2B), the decrease of anti-oxidative stress activity of DJ-1 was more significant (Figs. 6B and 6C). It is therefore implied that the SDS-resistant DJ-1 dimer is dominant-negative to interfere other DJ-1 molecules, or a certain amount of DJ-1 dimers may be entangled by free radicals to some intermediate degree where the association is cancelled by SDS but is inflexible enough to lose functions.

The formation of SDS-resistant DJ-1 dimer was induced by PQ strongly in SH-SY5Y cells and weakly in HEK293 cells, in which dopamine transporter (DAT) is strongly and scarcely expressed, respectively (Fig. 4A). PQ has been reported to be transported into

cells via organic cation transporter 3 (OCT3) and also via DAT into dopaminergic cells [67]. Probably because of DAT, which is lacking in HEK293 cells, the more PQ transportation and consequently the stronger induction of the SDS-resistant DJ-1 dimer were observed in SH-SY5Y cells than in HEK293 cells. The induction of the SDS-resistant DJ-1 dimer was thus suggested to be dependent on the level of PQ transportation into cells. The suggestion was strongly supported by the results that the introduction of DAT expression in HEK293 cells increased the SDS-resistant DJ-1 dimer induced by PQ treatment (Fig. 4B). Dopaminergic cells expressing DAT are thus suggested to be vulnerable to PQ and other similar herbicides and chemicals, resulting in PD.

We previously reported that DJ-1 eliminates H_2O_2 by self-oxidation [45]. The H_2O_2 -quenching activity was attenuated inversely with the increase of the SDS-resistant DJ-1 dimer formation promoted by increasing concentrations of PQ (Fig. 6A), indicating that the H_2O_2 -quenching activity of DJ-1 was abolished in the SDS-resistant dimer form, or weaker than that in the intact form. When cells were treated with H_2O_2 , viability of DJ-1(-/-) SH-SY5Y cells was decreased more quickly than that of DJ-1(+/+) SH-SY5Y cells as described previously [45, 52, 68]. When cells were pre-treated with PQ and then with H_2O_2 , viability of DJ-1(+/+) SH-SY5Y cells became close to that of DJ-1(-/-) SH-SY5Y cells (Figs. 6B and 6C). These results suggest that the H_2O_2 -quenching activity of DJ-1 in DJ-1(+/+) cells was decreased by PQ-treatment through formation of the SDS-resistant dimer and that the DJ-1(+/+) cells thus became vulnerable against oxidative stresses such as H_2O_2 exposure similarly as DJ-1(-/-) cells.

DJ-1 normally functions as a dimer [60], and monomer DJ-1 observed in pathogenic mutants such as L166P and M26I is considered to be inactive [2, 36, 71]. The present

study indicates that there are two types of DJ-1 dimer, SDS-sensitive and SDS-resistant forms, and showed that the SDS-resistant DJ-1 dimer is incompetent, or less competent, as for the anti-oxidative stress activity.

Two-D gel electrophoresis combining isoelectric focusing with SDS-PAGE revealed that the DJ-1 molecules were shifted to lower (more acidic) pI, in other words, more oxidized states. Strong oxidation of DJ-1 by free radicals may attack certain points of DJ-1 molecules and affect local conformation yielding intra-dimer entanglement and thereby SDS-resistant DJ-1 dimer. As the isoforms having more acidic pI was observed for monomer DJ-1 when the cells or recombinant DJ-1 were exposed to PQ, the increase of several isoform-like SDS-resistant DJ-1 dimers with different pI values were detected at more acidic region than PQ-untreated DJ-1 monomer (Figs. 7A and 7B). Different combination of monomer DJ-1 oxidized to different levels by PQ-derived free radicals may result in isoform-like variants of the SDS-resistant DJ-1 dimer with different pI values. Furthermore, the results of native gel electrophoresis indicated that the SDS-resistant dimer was involved in complexes with other cellular proteins similarly as healthy, SDS-sensitive dimer is (Fig. 7F). It is therefore suggested that the SDS-resistant DJ-1 dimer shares a bulk conformation with healthy, sensitive dimer at least as for the recognition by partner proteins to form complexes. The SDS-resistant DJ-1 dimer, however, caused a significant decrease in the anti-oxidative cell protection activity, suggesting the SDS-resistant dimer formation alters DJ-1 into loss-of-function.

We also examined various DJ-1 mutants found in familial PD patients for SDS-resistant dimer formation at PQ-exposure. SDS-resistant dimers were observed with five of the ten point mutants of DJ-1 (E64D, R98Q, A104T, D149A and E163K) even without PQ-exposure (Fig. 8), suggesting that many pathological mutants of DJ-1

form SDS-resistant dimers and so that SDS-resistant DJ-1 dimers are either causes or effects of the disease. In Fig. 9, the mutation sites in the DJ-1 mutants used in Fig. 8 are shown in the healthy dimer of wild type DJ-1. Since the locations of mutation sites are not adjacent but rather scattered for the five mutants E64D, R98Q, A104T, D149A and E163K, we cannot define domains required for the formation of SDS-resistant dimer. As for M26I and A179T DJ-1 mutants, SDS-resistant dimers were induced by PQ similarly as for WT DJ-1. M26I is very unstable, while A179T was as stable as WT DJ-1 (Fig. 8A). M26I DJ-1 is probably an inactive form due to protein instability. On the other hand, A179T DJ-1 may fall into another inactive form via unknown mechanism. Since the formation of SDS-resistant dimers of the five mutants occurs independently of intact C106, these mutants may have transformed structures allowing the molecules to form the SDS-resistant dimers independently of C106, while the wild-type DJ-1 are inclined to form the SDS-resistant dimer only after attack of free radicals at C106. We do not exclude at present other possibilities of mechanisms in terms of SDS-resistant dimer formation for either the wild-type or mutants of DJ-1.

Although increased levels of SDS-resistant DJ-1 dimers have been reported in brains of sporadic PD patients [51] and suggested to be associated with pathogenesis of PD, no article dealing with the SDS resistant DJ-1 dimer has been so far published and the details have not been clarified yet. In addition to the report that DJ-1 functions as a dimer [60], various proteins have been reported to associate with DJ-1 to express different functions [69]. DJ-1 may be competent in flexible, reversible dimer forms to be ready to associate with various partner proteins to play various roles. We showed the data suggesting the SDS-resistant dimer is bound by cellular proteins similarly as the healthy dimer is. Inflexibility in dimer association or mal-form of the SDS-resistant

DJ-1 dimer may deprive the protein of competency to collaborate with partner proteins. Further analyses using animal models of PD are necessary to ascertain that the formation of SDS-resistant dimers of DJ-1 is a cause or a progress of PD.

Although SDS-resistant DJ-1 dimer was Proteinase K sensitive in contrast to Prion, it is still possible that SDS-resistant DJ-1 dimer may convert healthy DJ-1 dimer to SDS-resistant *in vivo* because DJ-1 is suggested to be a molecular chaperone as described in 'Introduction'. It is interesting whether SDS-resistant DJ-1 dimer can be formed in mouse by PQ administration and whether SDS-resistant DJ-1 dimer is spontaneously increased by its injection to mouse brain. Longer period-observation of the experiments based on cultured cells may also be useful for further discussion. It is interesting whether SDS-resistant DJ-1 is degraded same as healthy DJ-1 in cells. Stability including a half-life of SDS-resistant DJ-1 dimer is under examination compared to that of healthy DJ-1 using cycloheximide chase analysis in cultured cells.

In conclusion, the present study demonstrates that the formation of SDS-resistant dimers of DJ-1 gives rise to dysfunction of the protein that possibly leads to the onset of PD. It is therefore promising that SDS-resistant DJ-1 dimers will be used as a target for remedy of PD. Positron emission tomography (PET) imaging using a monoclonal antibody was reported [72]. Trastuzumab is an anti-human epidermal growth factor receptor 2 (HER2) monoclonal antibody widely used as a drug against HER2-positive breast cancer. Trastuzumab labeled with ⁶⁴Cu using DOTA, a chelating agent, could be used for the identification of primary and metastatic lesions of HER2-positive breast cancer in patients by PET imaging [72]. The SDS-resistant DJ-1 dimers dealt in this thesis can be also used as a biomarker for diagnosis of PD, when anti-SDS-resistant DJ-1 dimer antibodies are successfully prepared.

Other proteins may also form oligomers which are ready to be converted to SDS-resistant by some stimulation, but yet unidentified. The present study may trigger elucidation of the significance of such SDS-resistant oligomer formation in physiological regulation and pathogenesis due to protein dysfunction.

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Figure Legends

Figure 1. Diagram of the structure of DJ-1 dimer and the positions of three cysteines and glutamate 18 residue

The positions of C46, C53, C106 and E18 are shown in the diagram of the structure of DJ-1 dimer, where the amino acid residues are displayed as red sticks. C106, the most important cysteine residue for DJ-1 function, are indicated in blue. The diagram was prepared using PyMOL software.

Figure 2. Formation of SDS-resistant DJ-1 dimer by PQ and requirement of cysteine residues

Proteins were extracted from cells and analyzed by immunoblotting with anti-DJ-1, anti-HA and anti-GAPDH antibodies in Figs. 2B to 2E as described in Materials and methods. GAPDH was used as a loading control. MW: molecular weight.

(A) Schematic drawing of the reduction/oxidation cycle of PQ. PQ is reduced to PQ radical by intracellular flavoenzymes such as NADPH cytochrome P450 reductase dependently on an electron donor of NADPH. PQ radical in turn reacts with oxygen and is recycled back to PQ. During the redox cycling, superoxide ($O_2^{\bullet -}$) is produced, leading to the increase of oxidative stress and cell injury.

(B) SH-SY5Y cells were treated with various concentrations of PQ for 3-12 hours.

(C) SH-SY5Y cells were treated with 2 mM PQ for 12 hours and reacted with a lysis buffer containing 1 mM DSS or DMSO as a vehicle control for 30 min.

(D) DJ-1-KO SH-SY5Y cells were transfected with expression vectors for WT-HA DJ-1, C46S-HA DJ-1, C53A-HA DJ-1, C106S-HA DJ-1 and E18A-HA DJ-1 with vector alone. At 24 hours after transfection, cells were treated with 0-2 mM PQ for 12 hours.

(E) DJ-1-KO SH-SY5Y cells were transfected and reacted with a lysis buffer as described in legends for Figs. 2D and 2C, respectively. Ratios of DJ-1 dimer / DJ-1 monomer were quantified by measuring intensity of bands using an Odyssey infrared imaging system software (bottom graph).

Figure 3. Requirement of free radicals and flavoenzymes for the formation of SDS-resistant DJ-1 dimer

Proteins were extracted from cells and analyzed by immunoblotting with anti-DJ-1 and anti-GAPDH antibodies. GAPDH was used as a loading control. MW: molecular weight.

(A and B) SHSY5Y cells were pre-treated with various concentrations of ascorbate (A) or DPI (B). At 1 hour after the reaction, cells were treated with or without 2 mM PQ for 12 hours.

(C) The structural formula of paraquat and diquat are shown..

(D) SH-SY5Y cells were treated with 0-2 mM PQ, 0-0.6 mM diquat, 0.1 mM H₂O₂, 50 µM 6-OHDA or 2 mM MPP⁺ for 12 hours.

(E) SH-SY5Y cells were pre-treated with various concentrations of ascorbate. At 1 hour after the reaction, cells were treated with 2 mM PQ or 0.5 mM diquat for 12 hours.

Figure 4. Inefficient formation of SDS-resistant DJ-1 dimer by PQ in non-dopaminergic HEK293 cells

Proteins were extracted from cells and analyzed by immunoblotting with anti-DJ-1, anti-DAT, anti-FLAG and anti-GAPDH antibodies. GAPDH was used as a loading

control. MW: molecular weight.

(A) SH-SY5Y and HEK293 cells were treated with 0-2 mM PQ.

(B) HEK293 cells were transfected with an expression vector for FLAG-DAT or with vector alone, and treated with 0-2 mM PQ.

Figure 5. Formation of SDS-resistant DJ-1 dimer by free radicals *in vitro*

Reactions were carried out as described below, and proteins in the reaction mixture were then separated on SDS-PAGE and stained with CBB.

(A) DJ-1 (0.05 µg/µl) was reacted with various combinations of 5 ng/µl CPR, 1 mM NADPH and 1 mM PQ for 30 min at 37°C.

(B) DJ-1 (0.05 µg/µl) was reacted with 5 ng/µl CPR, 1 mM NADPH and 0-200 µM PQ for 30 min at 37°C.

(C) DJ-1 (0.05 µg/µl) was reacted with 5 ng/µl CPR, 1 mM NADPH, 1 mM PQ and 0-500 µM ascorbate for 30 min at 37°C.

(D) WT DJ-1 or C106S DJ-1 (0.05 µg/µl) was reacted with various combinations of 5 ng/µl CPR, 1 mM NADPH and 1 mM PQ for indicated for 30 min at 37°C .

(E) WT DJ-1 or C106S DJ-1 (0.05 µg/µl) was reacted with 100 µM H₂O₂ and 10µM FeCl₂ for 60 min at 37°C.

(F) DJ-1 (0.05 µg/µl) was reacted with 100 µM H₂O₂, 10µM FeCl₂ and 0-250 µM ascorbate for 60 min at 37°C.

Figure 6. Effect of the formation of an SDS-resistant dimer on anti-oxidative stress activity of DJ-1.

(A) FLAG-DJ-1 purified from *E. coli* (0.2 µg/µl) was reacted with various combinations

of 1 mM NADPH, 5 ng/μl CPR and 0-500 μM PQ for 30 min at 37°C, immunoprecipitated with an anti-FLAG M2 agarose affinity gel and eluted with FLAG peptide. Eluted FLAG-DJ-1 was then reacted with 300 nM H₂O₂ for 30 min at 25°C, and the amount of H₂O₂ was measured using an Amplex Red hydrogen peroxide/peroxidase assay kit and indicated as relative amounts standardized by the value without any additives. Immunoprecipitated FLAG-DJ-1 was separated on SDS-PAGE and stained with CBB. n=6. Significance: ** $p < 0.01$.

(B) SH-SY5Y and DJ-1-KO SH-SY5Y cells were treated with 2 mM PQ for 8 hours and then with 0-0.3 mM H₂O₂ for 1.5 hours. Cell viability was measured at the absorbance of 450 nm using Cell Counting Kit-8 and indicated as relative value to that for respective cell line in the absence of PQ and H₂O₂. n=5. NS: not significant. Significance: ** $p < 0.01$.

(C) SH-SY5Y and DJ-1-KO SH-SY5Y cells were treated with 0-2 mM PQ for 8 hours and then with 0.2 mM H₂O₂ for 1.5 hours, and their cell viability was measured as described in the legend for Fig. 6B. Relative viability was standardized by that for respective cell line in the absence of PQ and H₂O₂. The expression levels of DJ-1 in SH-SY5Y and DJ-1-KO SH-SY5Y cells were analyzed by immunoblotting with anti-DJ-1 and anti-GAPDH antibodies. n=5. NS: not significant. Significance: * $p < 0.05$, ** $p < 0.01$.

Figure 7. Characterization of SDS-resistant DJ-1 dimer.

(A) Proteins were extracted from SH-SY5Y cells treated with or without 2 mM PQ and analyzed by 2D gel electrophoresis combining isoelectric focusing with SDS-PAGE, followed by immunoblotting with an anti-DJ-1 antibody.

(B) DJ-1 was reacted with or without 5 ng/μl CPR, 1 mM NADPH and 1 mM PQ for 30 min at 37°C and analyzed by 2D gel electrophoresis combining isoelectric focusing with SDS-PAGE, followed by CBB staining.

(C) DJ-1 was reacted with or without 5 ng/μl CPR, 1 mM NADPH and indicated concentrations of PQ for 30 min at 37°C and analyzed by SDS-PAGE or native-PAGE followed by CBB staining.

(D) DJ-1 reacted with 0 or 2 mM PQ and separated by 2D gel electrophoresis combining native-PAGE (as in Fig. 7C) with SDS-PAGE, followed by CBB staining.

(E) Proteins were extracted from SH-SY5Y cells treated with indicated concentrations of PQ, separated by native-PAGE or SDS-PAGE and immunoblotted with an anti-DJ-1 antibody.

(F) Proteins were extracted from SH-SY5Y cells treated with or without 2 mM PQ and analyzed by 2D gel electrophoresis combining native-PAGE with SDS-PAGE, followed by immunoblotting with an anti-DJ-1 antibody.

(G) Proteins extracted from SH-SY5Y cells treated with or without 2 mM PQ were reacted with 100 μg/ml Proteinase K (ProK) for 30 min at 37°C and analyzed by immunoblotting with an anti-DJ-1 antibody.

(H) GST or DJ-1 (0.05 μg/μl) was reacted with or without 5 ng/μl CPR, 1 mM NADPH, 1 mM PQ for 30 min at 37°C, followed by the reaction with 5 μg/ml ProK for 30 min at 37°C. Proteins in the reaction mixture were then separated by SDS-PAGE and stained with CBB.

Figure 8. Formation of SDS-resistant DJ-1 dimer in DJ-1 mutants found in PD patients.

(A) SH-SY5Y-DJ-1-KO cells were transfected with expression vectors for WT and various mutants of DJ-1 tagged with HA at their C-terminus. At 24 hours after transfection, cells were treated of 2 mM PQ for 12 hours. Proteins extracted from cells were then analyzed by immunoblotting with anti-DJ-1 and anti-GAPDH antibodies.

(B) DJ-1-KO SH-SY5Y cells were transfected with expression vectors for WT and three pathogenic mutants of DJ-1 tagged with HA at their C-terminus. At 24 hours after transfection, cells were reacted with a lysis buffer containing 1 mM DSS or DMSO as a vehicle control for 30 min, and proteins in cells were analyzed by immunoblotting with an anti-HA antibody.

(C) DJ-1-KO SH-SY5Y cells were transfected with expression vectors for WT and various mutants of DJ-1, and proteins in cells were analyzed as described in the legend for Fig. 8A.

Figure 9. Diagram of the structure of DJ-1 dimer and the positions of mutations found in PD patients

The structural diagram of DJ-1 dimer shows the positions of mutations in the mutants derived from PD patients used in Fig. 8. The amino acid residues are displayed as red sticks. The mutation sites, E64, R98, A104, D149 and E163, in the mutants which steadily formed the SDS-resistant DJ-1 dimer are indicated in blue. The diagram was prepared using PyMOL software.

Tables and Figures

Table 1. Nucleotide sequences of the upper and lower strands

Plasmid name	Sense/anti-sense	Nucleotide sequence	Template
pEF-DJ-1-L10P-HA	sense anti-sense	5'-GTCATCCCAGCTAAAGGAGCAGAGGAA-3' 5'-TTTAGCTGGGATGACCAGAGCTCTTTT-3'	pEF-DJ-1-WT-HA
pEF-DJ-1-E64D-HA	sense anti-sense	5'-AAAAAGACGGACCATATGATGTGGTGG-3' 5'-ATGGTCCGTCTTTTTTTGCATCTTCAA-3'	pEF-DJ-1-WT-HA
pEF-DJ-1-A104T-HA	sense anti-sense	5'-GATAGCCACCATCTGTGCAGGTCCTAC-3' 5'-CAGATGGTGGCTATCAGGCCCTTCCGG-3'	pEF-DJ-1-WT-HA
pEF-DJ-1-A107P-HA	sense anti-sense	5'-ATCTGTCCAGGTCCTACTGCTCTGTTG-3' 5'-AGGACCTGGACAGATGGCGGCTATCAG-3'	pEF-DJ-1-WT-HA
pEF-DJ-1-E163K-HA	sense anti-sense	5'-CAGCTTCAAGTTTGCGCTTGCAATTGT-3' 5'-GCAAACCTTGAAGCTGGTCCCAGGCCCC-3'	pEF-DJ-1-WT-HA
pEF-DJ-1-A179T-HA	sense anti-sense	5'-GTGGCGACTCAAGTGAAGGCTCCACTT-3' 5'-CACTTGAGTCGCCACCTCCTTGCCATT-3'	pEF-DJ-1-WT-HA
pEF-DJ-1-E64D/C106S-HA			pEF-DJ-1-E64D-HA
pEF-DJ-1-R98Q/C106S-HA	sense	5'-CGCCATCAGTGCAGGTCCTACTGCTCT-3'	pEF-DJ-1-R98Q-HA
pEF-DJ-1-D149A/C106S-HA	anti-sense	5'-CCTGCACTGATGGCGGCTATCAGGCCCC-3'	pEF-DJ-1-D149A-HA
pEF-DJ-1-E163K/C106S-HA			pEF-DJ-1-E163KHA
pEF-DJ-1-A104T/C106S-HA	sense anti-sense	5'-CACCATCAGTGCAGGTCCTACTGCTCT-3' 5'-CCTGCACTGATGGTGGCTATCAGGCCCC-3'	pEF-DJ-1-A104T-HA

Figure 1

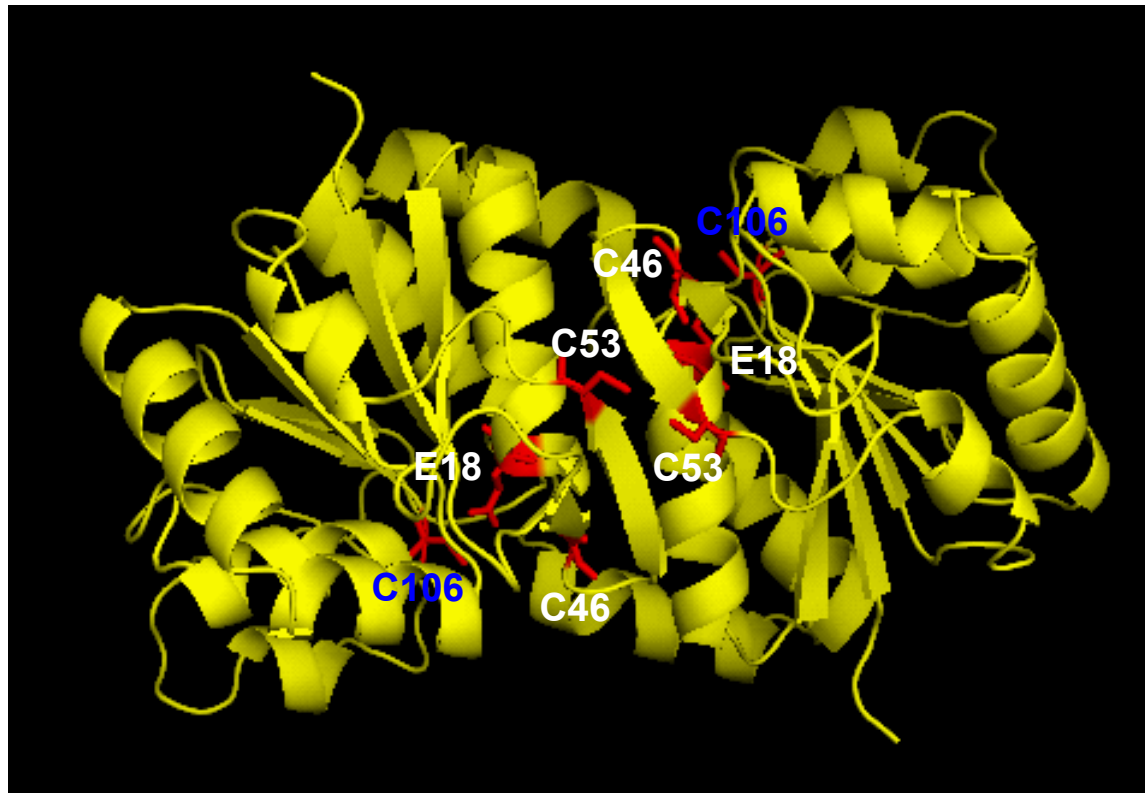


Figure 2

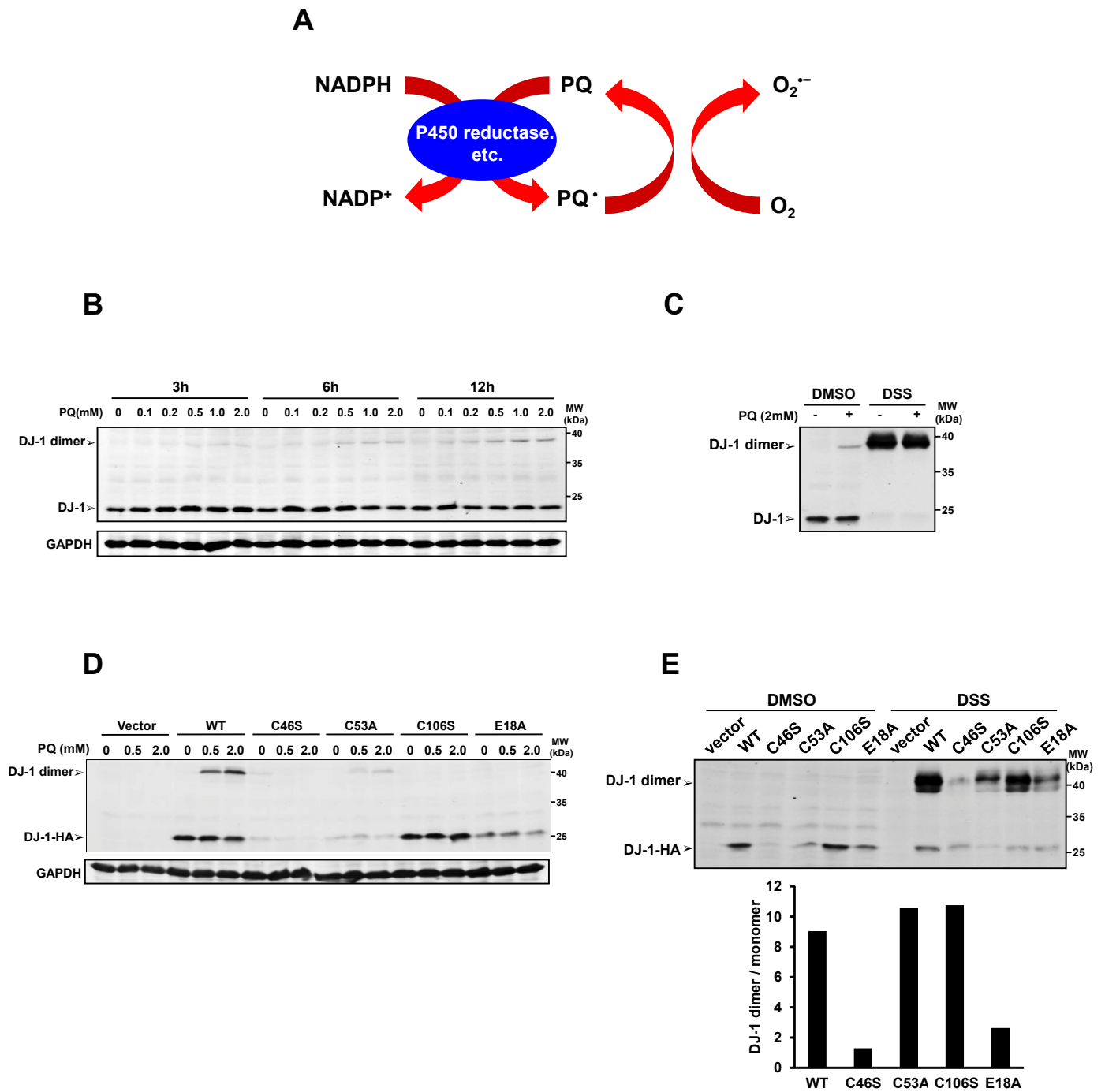
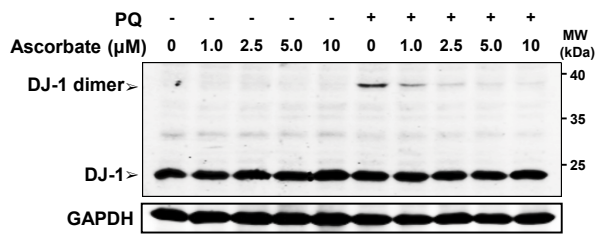
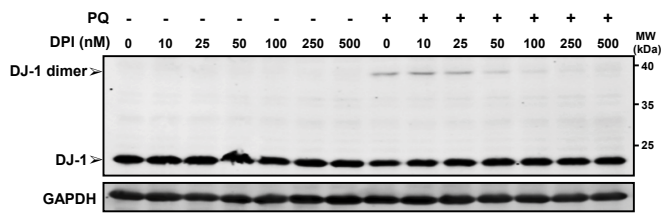


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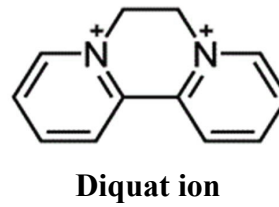
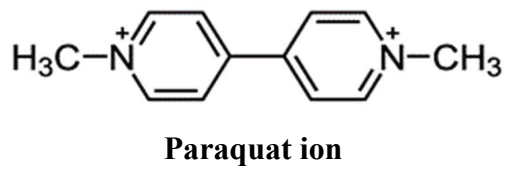
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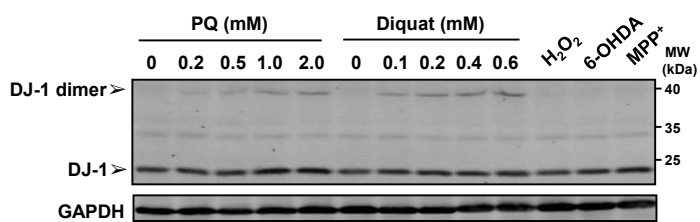
B



C



D



E

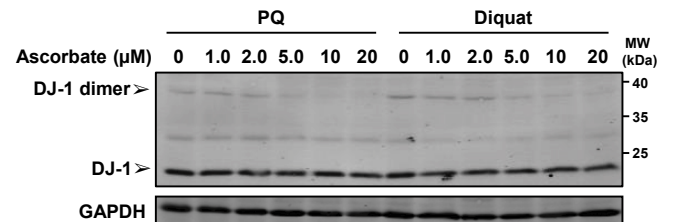
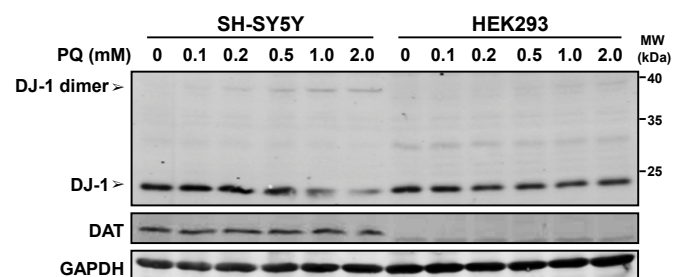


Figure 4

A



B

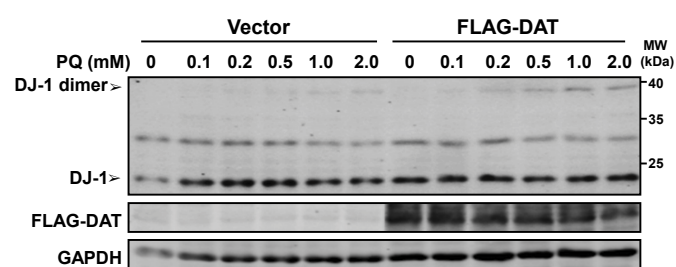


Figure 5

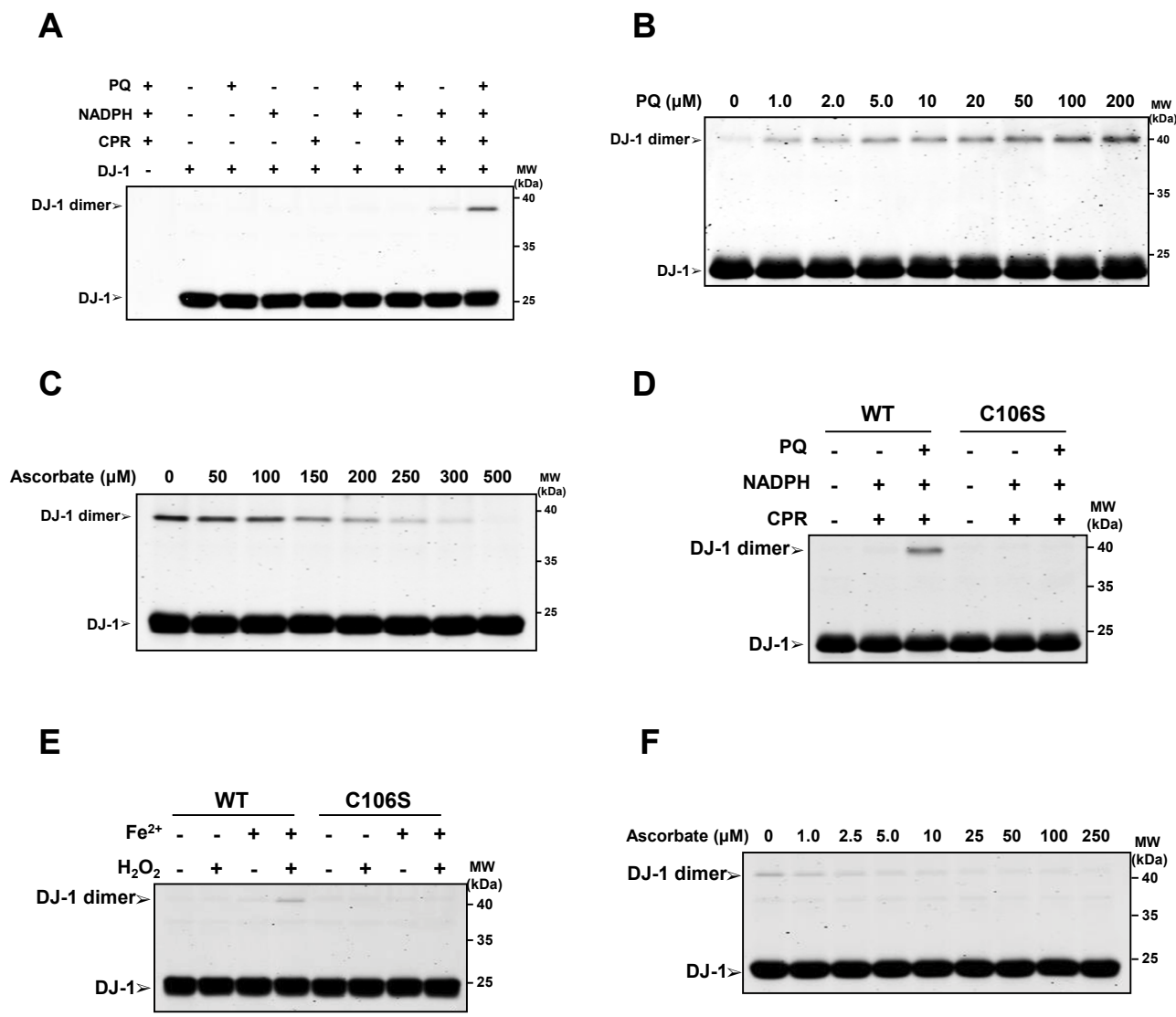


Figure 6

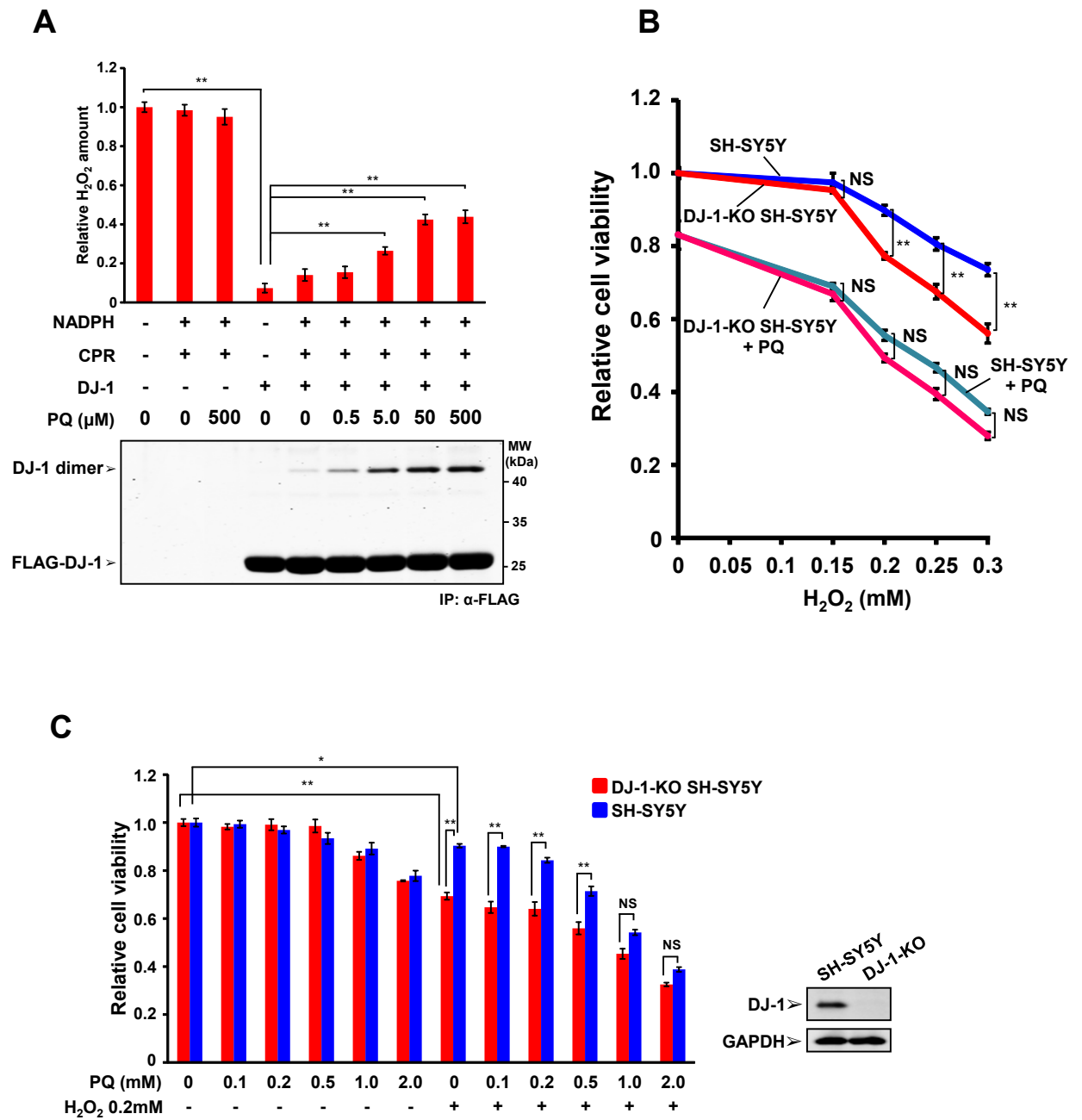


Figure 7 (1/3)

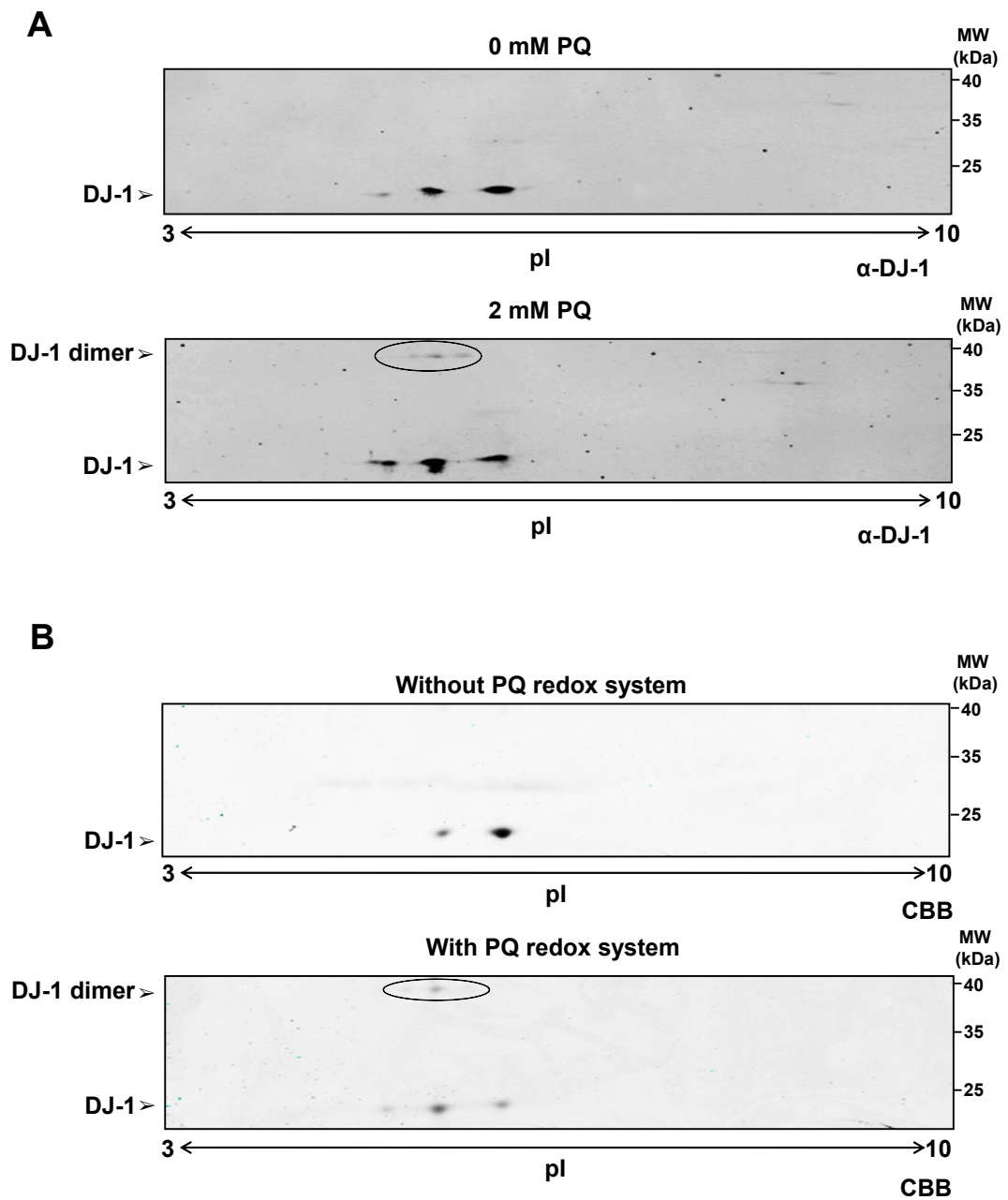


Figure 7 (2/3)

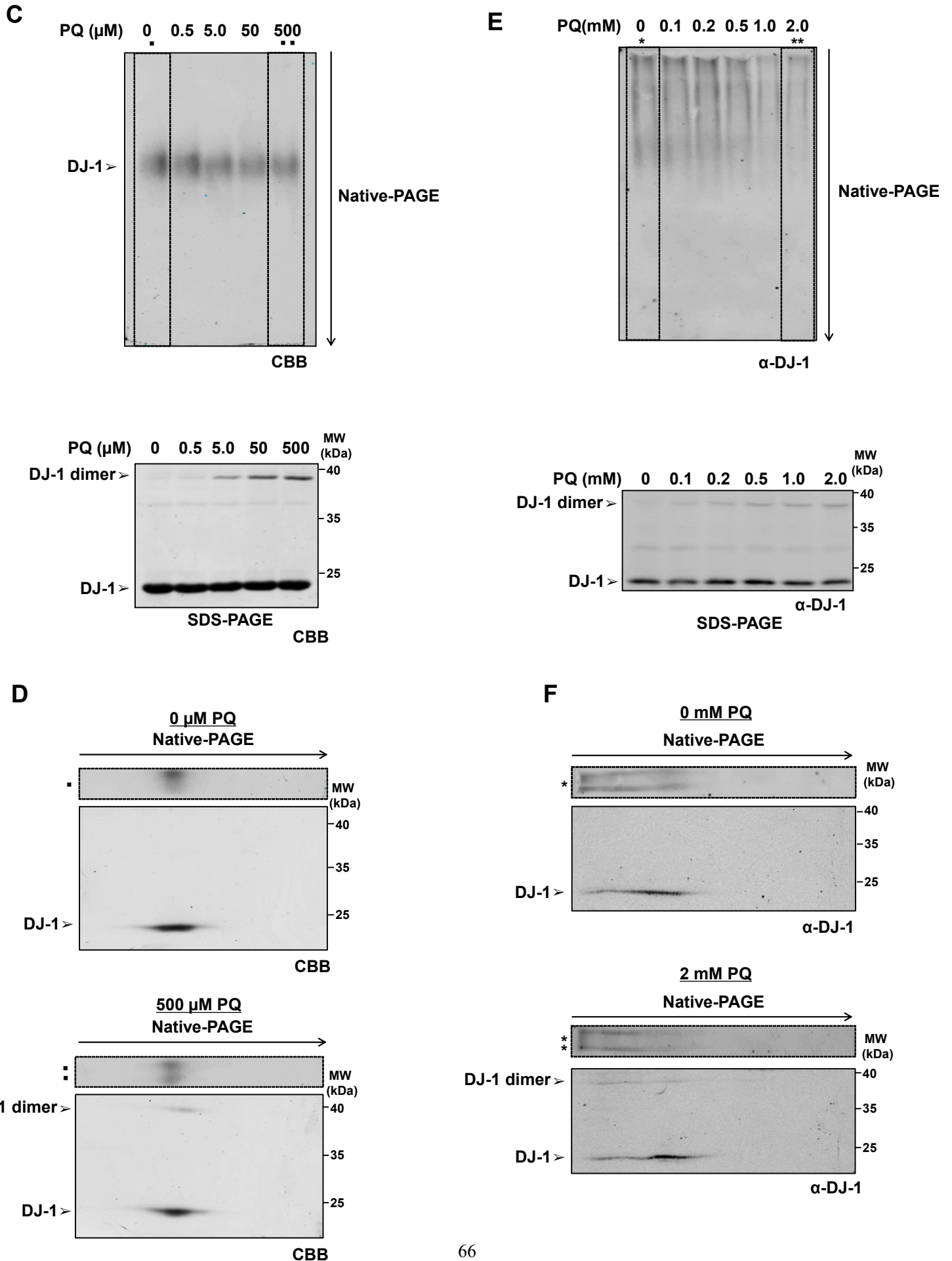
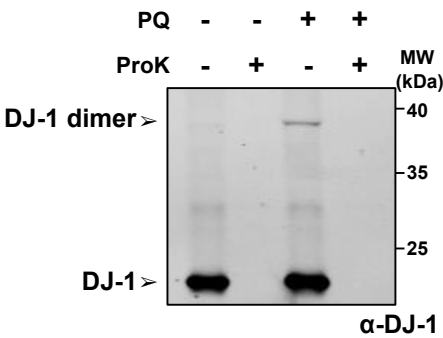


Figure 7 (3/3)

G



H

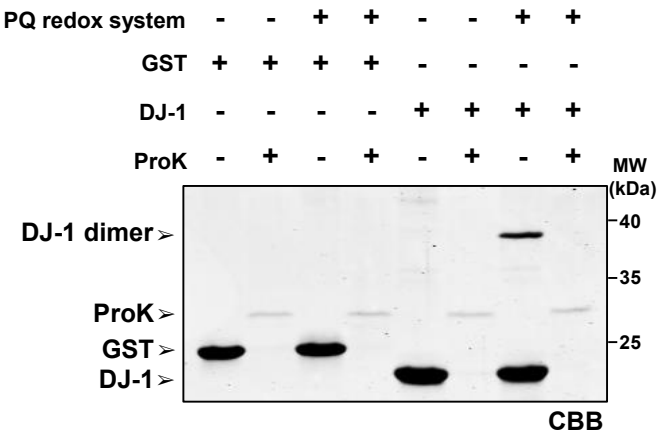


Figure 8

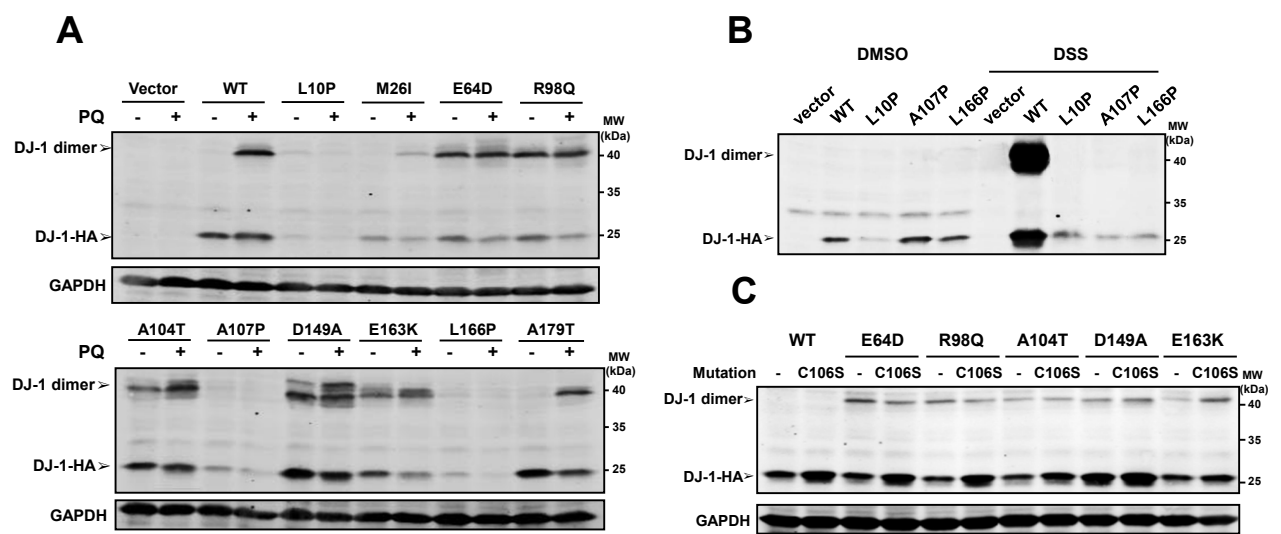


Figure 9

