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The E163K DJ-1 Mutant Shows Specific Antioxidant Deficiency

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Abstract

Recent discoveries of genetic mutations linked to familial forms of Parkinson's disease (PD), including mutations in *DJ-1*, have provided insights into the pathogenesis of sporadic PD. Recently, a novel homozygous missense mutation in the gene encoding human DJ-1 protein resulting in the E163K amino acid substitution has been reported. This mutation is associated with early-onset and clinical presentations that include parkinsonism, cognitive decline, and amyotrophic lateral sclerosis. The specific effect of this mutation on the function of DJ-1 protein as it relates to disease pathogenesis is currently unknown. Herein we show that the E163K pathogenic mutant retains similar properties to wild-type DJ-1 protein as it relates to protein stability, solubility, and dimerization. However, we show that the E163K mutant loses the ability to protect against oxidative stress while demonstrating a reduced redistribution towards mitochondria, but retains the ability to mitigate toxicity due to mitochondrial stress and proteasomal impairment. These findings suggest that DJ-1 influences several neuroprotective pathways and that the E163K mutation impairs the mechanism that is more specific to oxidative stress.

Keywords

Parkinson's disease; DJ-1; oxidative stress; mitochondria

1. Introduction

Although the majority of Parkinson's disease (PD) cases are idiopathic, a growing number of mutations have been found to be associated with familial forms of the disease [12;24;38]. Mutations in genes at multiple loci, designated *PARK1* through *PARK13*, result in parkinsonian phenotypes with distinct features [6;11;24]. Various mutations in *PARK7* (DJ-1), including truncation, missense, splice-site, and large deletions have been discovered [1;9]. Mutations in *DJ-1* cause autosomal recessive PD with early to mid age of onset, and these may contribute to 1-2% of early onset PD cases [16;31].

DJ-1 encodes a 189 amino acid protein which is a member of the ThiJ/PfPI superfamily based on its structure [19;30;39;50]. It is expressed in both neurons and astrocytes in the brain [3;4;25;42;44], but it is also expressed in many other organs [13;41;54]. A number of studies have

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shown that DJ-1 can have protective functions against oxidative, proteasomal and mitochondrial stresses [10;21;23;33;46;52;53;55].

Interestingly, a novel missense E163K *DJ-1* mutation was reported for a family in southern Italy which results in a severe phenotype as early as 24 years. Subjects homozygous for this E163K mutation develop symptoms that include parkinsonism, dementia, and amyotrophic lateral sclerosis [2]. Characteristics include weakness and muscle atrophy in the upper and lower extremities, speech deficits, cognitive impairment, and parkinsonism. Because of the early onset and more extensive phenotype associated with this mutation, its analysis and an understanding of how it can lead to disease may provide new insights into the function of DJ-1. In this study, we explore the properties of human E163K mutant DJ-1 protein as it relates to its solubility, dimerization, stability, subcellular localization, and effects on cell viability as compared to wild-type (WT) protein.

2. Results

Neuro-2A (N2A) mouse neuroblastoma cell lines (Figure 1a) and Chinese Hamster Ovary (CHO) cell lines (data not shown) stably expressing WT and E163K DJ-1 were generated as described in “Material and Methods”. The studies on solubility, dimerization, and protein stability were reproduced in both types of cells, but toxicity studies were conducted exclusively on N2A cell lines (see below).

The E163K mutation does not lead to changes in solubility

Protein aggregation is known to be a key component in the disease pathogenesis of many neurodegenerative disorders [29] and reduced solubility of DJ-1 has been reported in some diseased brains [26;34;37;40;43]. To assess whether the E163K mutation may lead to changes in solubility, N2A cells stably expressing WT and E163K DJ-1 were sequentially extracted with buffers of increasing solubilization strengths. The WT and the E163K mutant form of human DJ-1 both were extracted most abundantly in the soluble Triton X-100 fractions (TX1 and TX2) (Figure 1b) and to a lesser extent in the RIPA fractions. Additionally, neither E163K mutant nor WT DJ-1 were detected in the SDS fractions, and there were no differences between the distribution of WT and E163K DJ-1 across any of the biochemical fractions.

Biochemical fractionation experiments also were performed in CHO cells stably expressing the human WT or E163K mutant DJ-1 protein and no differences in protein solubility between the WT and E163K variants were observed in these cells (data not shown).

The E163K mutation does not alter the ability of DJ-1 to dimerize

DJ-1 has been previously shown to form a homodimer in vitro which may be essential to its function [15;30;41]. Some mutations like L166P have previously been shown to affect protein folding such as to prevent dimer formation, resulting in rapid degradation [5;8;15;17;20;36;41]. Crystal structure analysis of DJ-1 protein shows that the L166P mutation occurs in helix 7, which is one of the helices in the dimer interface of DJ-1 [18;19;48;50]. Since the E163K mutation also occurs in helix 7, it is possible that a similar effect could occur. To assess whether E163K DJ-1 dimerizes under native conditions, size exclusion chromatography was performed on soluble extracts from N2A cells expressing endogenous DJ-1 alone or expressing WT or E163K human DJ-1. The elution of purified proteins with known molecular masses was used to standardize this assay. Both the WT DJ-1 and the E163K mutant DJ-1 exclusively eluted in fractions 24 through 26 (Figure 1c), which corresponds to a molecular mass of ~42 kDa, the proposed molecular mass for the DJ-1 dimer. Endogenous DJ-1 protein expressed in N2A cells also eluted in the same fractions.

Size exclusion chromatography was also performed in CHO cells stably expressing either the WT or the E163K mutant form of DJ-1 protein in order to confirm the protein dimerization results. In the CHO cells, both the WT and the E163K mutant DJ-1 protein eluted in fractions 24 and 25 (data not shown), corresponding to the mass of a dimer.

The DJ-1 E163K mutant shows comparable stability to WT

It has been previously shown that some pathogenic mutants of DJ-1 like M26I are structurally similar to WT DJ-1 in the ability to form a homodimer, but can demonstrate reduced stability compared to the WT protein [8;41;47;51]. Therefore, the effect of the E163K mutant on protein stability was assessed by pulse-chase experiments with ³⁵S-methionine and comparison to the WT protein. Both the E163K mutant and WT DJ-1 proteins showed similar turnover in N2A cells (Figure 1d), indicating that the E163K mutation does not result in reduced stability. These experiments were also performed in CHO cells (data not shown) and both protein variants also showed similar turnover rates in this alternate cell type.

The E163K mutant DJ-1 does not show altered subcellular localization by western blot

DJ-1 is normally diffusely expressed in cells [4], but WT DJ-1 as well as pathogenic mutants may localize to the mitochondria [54] and the latter localization may increase in response to mitochondrial stress [7;8]. However, the subcellular localization of the E163K pathogenic mutant form of DJ-1 has not been previously investigated. To determine a possible effect of this mutation on altering the localization of DJ-1, subcellular fractionation was performed by differential centrifugation as previously used by others [36]. Untransfected N2A cells and N2A cells stably expressing either human WT DJ-1 or human E163K DJ-1 were homogenized in triplicate in subfractionation buffer. Cytosolic and mitochondrial fractions were collected. Cell lysates were resolved by SDS-PAGE and analyzed by western blot analysis with DJ5 (human specific monoclonal DJ-1 antibody), 691 (polyclonal DJ-1 antibody that reacts with mouse and human protein), Tim23 (translocases of the inner mitochondrial membrane) as a mitochondrial marker, and ERK1/2 as a cytoplasmic marker. Endogenous DJ-1 protein in N2A cells and both human WT and E163K DJ-1 expressed in these cells demonstrated similar predominant cytoplasmic distributions (Figure 2).

The E163K mutant protein fails to rescue under conditions of oxidative stress

Many studies have reported that WT DJ-1 can serve in a protective capacity when overexpressed in various cells lines [10;45;49;53;55]. To investigate the ability of human E163K DJ-1 to protect N2A mouse neuroblastoma cells from mitochondrial stress, proteasomal stress, and oxidative stress, cells expressing WT or E163K human DJ-1 were challenged with various specific stressors.

Native N2A cells or N2A cells stably expressing WT DJ-1 or E163K mutant DJ-1 were treated with either fresh DMEM/FBS or DMEM/FBS containing MG-132 (a proteasome inhibitor) or MPP dihydrochloride (a mitochondrial complex I inhibitor) at a range of concentrations. N2A cells demonstrated reduced cell viability to MG-132 treatment at all concentrations used (10-30 uM). Additionally, N2A cells were vulnerable to MPP dihydrochloride concentrations that exceeded 10 uM and WT DJ-1 protein was able to protect against both of these stressors at all toxic concentrations tested (Figure 3A). Expression of E163K DJ-1 was also able to protect against MG-132 and MPP dihydrochloride toxicity.

To further assess the specific effect of the E163K mutation, other specific mitochondrial complex inhibitors were analyzed (Figure 3B). Antimycin, an inhibitor of mitochondrial complex III, resulted in a ~40% decrease in viability in N2A cells at the 100 nM concentration and a ~60% decrease in viability at the 200 nM concentration while expression of either WT or E163K DJ-1 had a protective effect at these concentrations (Figure 3B). The irreversible

inhibitor of mitochondrial complex II, 3-nitropropionic acid (3-NP), did not have a significant effect on the viability of any of the three cell lines at the 5 uM or 10 uM concentrations tested. However, N2A cells showed a slight vulnerability to 15 uM 3-NP and expression of either the E163K mutant DJ-1 or WT DJ-1 was protective.

Native N2A cells or stable clones expressing WT or E163K DJ-1 protein were challenged with H₂O₂ ranging from 10-30 uM in order to induce oxidative stress. We found that challenging the cell line expressing E163K mutant DJ-1 (E163K clone #16) that had been used in all of the aforementioned toxicity experiments not only demonstrated a lack of protection against H₂O₂ oxidative stress, but furthermore showed increased vulnerability to H₂O₂ exposure at all of the concentrations used (Figure 3C). To determine whether enhanced sensitivity to oxidative stress was due to expression of the E163K DJ-1 mutant, two additional N2A cell lines (clone #47 and clone #52) stably expressing the human E163K mutant DJ-1 were tested for H₂O₂ toxicity (Figure 3C). Both of these clones also revealed increased vulnerability to H₂O₂ oxidative stress when compared to native N2A cells. In contrast, two N2A cell lines stably expressing human WT DJ-1 (clones #11 and #12) demonstrated protection against H₂O₂ stress. These results indicate that the E163K mutation selectively compromises the protective role for DJ-1 on oxidative stress.

Human exogenous DJ-1 protein does not co-immunoprecipitate with endogenous DJ-1 in N2A cells

One plausible explanation for the selective vulnerability of N2A cell lines expressing E163K mutant DJ-1 to oxidative stress is that the mutant protein may behave as a dominant negative by forming a heterodimer with endogenous murine DJ-1 protein, inactivating the antioxidant capacity of the endogenous protein. Size exclusion chromatography analysis demonstrated that endogenous and human DJ-1 formed dimers, but these studies do not ascertain whether these proteins could form heterodimers (Figure 1). Coimmunoprecipitation experiments were performed to test the interaction between human WT and E163K DJ-1 with endogenous DJ-1. Since mouse and human DJ-1 migrate with the same mobility on SDS-PAGE, transfections with N-terminal flagged human DJ-1 constructs were used for these studies. Cell extracts from N2A cells or extracts from N2A cells transiently expressing either WT Flag-DJ-1 or E163K Flag-DJ-1 were immunoprecipitated with a mouse anti-flag monoclonal antibody. Immunoprecipitates were resolved by SDS-PAGE and analyzed by western blot with the polyclonal DJ-1 antibody, 691. Endogenous DJ-1 protein was not pulled down by immunoprecipitation with either WT-flag tagged or E163K-flag tagged DJ-1 (Figure 4), indicating that there is no detectable formation of heterodimers between the endogenous DJ-1 and exogenous human DJ-1 protein.

Immunofluorescence microscopy reveals an altered localization for E163K mutant DJ-1 protein in response to oxidative stress

To further understand the effect of the E163K mutation, quantitative immunofluorescence studies were conducted comparing untreated cells and cells challenged with oxidative stress. Under normal conditions, DJ-1 exhibited a diffuse staining pattern throughout the cell and co-immunofluorescence experiments with the mitochondrial marker, MitoTracker® Red CMXRos, revealed some overlap with both WT and E163K DJ-1 (Figure 5a). However, after treating with hydrogen peroxide, cells expressing WT DJ-1 showed a 5-fold increase in staining overlap of the human DJ-1 protein with the mitochondrial marker as compared to untreated cells. In striking contrast, under the same oxidative conditions, cells expressing the E163K mutant DJ-1 showed a 6-fold decrease in the overlap with mitochondrial marker (Figure 5b). To try to assess whether these alterations in DJ-1 distributions were due to changes in the presence of DJ-1 in the mitochondria, subcellular biochemical fractionation of cells treated with oxidative stress was performed. However, no biochemical mitochondrial localization was

observed under harsh conditions of oxidative stress (Figure 5c). These results suggest that under oxidative stress, WT human DJ-1 in N2A cells can relocate in close proximity to the mitochondria, but does not enter these organelles, while E163K DJ-1 is impaired in this property, which may play a role in its inability to protect against oxidative stress.

3. Discussion

DJ-1 mutations are associated with early-onset parkinsonism. The E163K mutation is also causal of early-onset disease, but clinical presentations are more extensive and diverse than for other DJ-1 disease-causing mutations [2]. In this study, the properties and effects of this mutant were assessed in cultured cells. Under normal conditions, the E163K mutant had properties similar to WT DJ-1 in terms of subcellular localization, dimerization abilities, stability, and solubility. These findings are consistent with recent findings by Lakshminarasimhan and colleagues that showed that in vitro E163K DJ-1 was able to form stable dimers [27].

Many groups have shown that WT DJ-1 protein can act to mitigate the deleterious effects of various insults including oxidative stress both in cell culture and in animal models [21;28;33;35;46;53]. While the E163K mutant retains the ability to protect N2A cells against proteasome inhibition as well as mitochondrial stress through mitochondrial complex I and III inhibition, this mutation compromises the ability of DJ-1 to protect against oxidative stress and even increases sensitivity to oxidative insult. A number of studies demonstrated that the DJ-1 pathogenic mutant, L166P, insufficiently protects against oxidative stress [15;21;22;33;46;55], but in contrast to the E163K mutation the L166P mutation impairs the ability of DJ-1 to form stable dimers resulting in a dramatic instability of the mutant protein and loss of expression that can explain the loss of protective function [14;32;36;41]. The M26I mutant also displays reduced protection against oxidation stress [47] despite the ability to form stable dimers [8;37;47], but this mutation appears to reduce protein stability, albeit much less than the L166P mutation [8;37;47;51]. However, it is unclear if the reduced protection against oxidative stress by the M26I mutation is simply associated with reduced half-life or perhaps with an increased propensity for the M26I mutant protein to aggregate as recently reported [20].

In contrast to these other mutations in DJ-1, the effect of the E163K mutation on impairing DJ-1 function is specific to oxidative stress and it does not impair dimerization, protein stability or increase aggregation. E163 is in close proximity of L166, and both residues are located in α -helix G7 which is critical in forming stably folded protein [15;19]. The E163K mutation clearly does not cause the dramatic structural changes of the L166P mutation which introduces a helix-breaking residue, but the fact that E163 is highly conserved in DJ-1 across species underscores the importance of this residue [15]. The E163K mutation appears to result in subtle structural changes and recent biophysical studies by Lakshminarasimhan et al demonstrated that E163 can form a salt bridge with R145 and that the disruption of this interaction by the E163K mutation results in increased mobility of R145 [27].

Our findings that oxidative stress promoted the redistribution of DJ-1 towards mitochondria but without evidence of mitochondrial import are consistent with the studies of Canet-Aviles and colleagues that reported that oxidative stress can increase the amount of DJ-1 on the cytoplasmic side of this organelle without resulting in import [10]. It is shown in the present studies that under conditions of oxidative stress, the E163K mutant demonstrated a paucity of localization towards mitochondria. It is possible that simply the change from a negatively to positively charged residue and/or the subtle structural effects discussed above may prevent the interaction of DJ-1 with other proteins that may be involved in the redistribution of DJ-1 under conditions of oxidative stress. Identifying such mechanisms may be important to further

understand the function of DJ-1. The toxicity studies demonstrate that DJ-1 influences several neuroprotective pathways but that the E163K mutation specifically impairs the oxidative stress protective mechanism. The findings that this mutation appears to exacerbate the response to oxidation and diminish the redistribution of DJ-1 towards the mitochondria suggest that both processes may be related, but further studies will be needed to substantiate a direct association.

Our results reveal that the loss of DJ-1 protective function can occur without overt biochemical changes on the protein and demonstrate that alterations to specific residues in this protein can specifically affect individual functions indicating that DJ-1 is likely involved in multiple cellular pathways. A more detailed understanding of the mechanism by which DJ-1 can protect against stresses is still needed to better understand the activities of this protein and its role in neurodegenerative diseases.

4. Experimental Procedures

Antibodies

DJ-5 is a mouse monoclonal antibody specific for human DJ-1 protein [34]. 691 is a rabbit polyclonal antibody raised against recombinant human DJ-1 protein but that reacts with DJ-1 from various species [34].

Anti-Tim23 is a purified mouse monoclonal antibody to Tim23 (BD Transduction Laboratories, San Jose, CA). Anti-extracellular signal related kinase-1 (ERK-1) (C-16) is an affinity purified rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that reacts with both ERK-1 and -2. Anti-Flag-tag mouse monoclonal antibody (GenScript Corporation, Piscataway, NJ) is a purified antibody that reacts with proteins with the amino acid sequence, DYKDDDDK. Anti-Actin is a purified mouse monoclonal antibody (Millipore Corporation, Billerica, MA 01821) that reacts with all six isoforms of vertebrate Actin.

Cloning of human DJ-1 constructs

Human full-length WT DJ-1 cDNA was cloned into the mammalian expression vector pZeoSV2 (Invitrogen, Carlsbad, CA) at the Not I and Hind III restriction sites to create the plasmid pZeoWThDJ. Using the pZeoWThDJ construct, the QuickChange® Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used in order to generate the E163K mutant form of human DJ-1 in the pZeoSV2 vector. The sequences of the oligonucleotides used for mutagenesis were as follows: Forward- 5'-GGG CCT GGG ACC AGC TTC AAG TTT GCG CTT GCA ATT GTT-3' and Reverse- 5'- AAC AAT TGC AAG CGC AAA CTT GAA GCT GGT CCC AGG CCC-3'. The sequence of the plasmid with E163K mutant DJ-1 was verified by DNA sequencing as a service offered by the DNA Sequencing Facility of the University of Pennsylvania, and the construct was named pZeoEKhDJ.

Sequential restriction digestions with Not I and Apa I were performed on both pZeoWThDJ and pZeoEKhDJ. The DNA fragments containing the full-length DJ-1 sequences were ligated into the pcDNA3.1 (Invitrogen, Carlsbad, CA) mammalian expression vector in order to generate the human WT and E163K mutant DJ-1 constructs named pc3.1WThDJ1 and pc3.1EKhDJ1, respectively.

An N-terminal flag-tagged WT DJ-1 construct was generated by PCR, using the pZeoWThDJ-1 construct as a template. The sequences for the oligonucleotides used were as follows: Forward-5'- GAT CGC GGC CGC CAC CAT GGA TTA CAA GGA TGA CGA CGA TAA GGC TTC CAA AAG AGC TCT GGT CAT CCT -3' and Reverse-5'-GAT CAA GCT TCT AGT CTT TAA GAA CAA GTG GAG CCT TC-3'. The tagged insert was cloned into the pCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA) and subsequently cloned into the pcDNA3.1 (-) vector at the Hind III and Not I restriction sites. The construct was named pc3.1WThDJ1-

Nflag. The sequence of the plasmid was verified by DNA sequencing as described above. The E163K N-terminal flag-tagged DJ-1 construct was generated by performing site directed mutagenesis using the pc3.1WThDJ1-Nflag construct as a template. The same oligonucleotides were used as described above for mutagenesis. The plasmid was named pc3.1E163KhDJ1-NFlag.

Cell culture

N2A and CHO cells were cultured in Dulbecco-modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St.Louis, MO), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C and 95% air/5% CO₂ atmosphere.

Generation of stable cell lines expressing WT and E163K DJ-1

The pc3.1WThDJ and pc3.1EKhDJ constructs were used to transfect N2A cells using FuGENE reagent following the manufacturer's protocol. Stably expressing clones were isolated and selected with G418 (Invitrogen, Carlsbad, CA) at 200-500 µg/mL and screened by Western blotting for the expression of DJ-1 using the antibody DJ5 that is specific for human DJ-1. Stably expressing clones expressing WT (clone #11 and clone #12) or E163K (clone #16, #47 and #52) human DJ-1 were used in the studies (See Figure 1A).

The pZeoWThDJ and pZeoEKhDJ constructs were used to transfect CHO cells using FuGENE (Roche, Basel, Switzerland) transfection reagent according to the manufacturer's protocol. Stably expressing clones were isolated following selection with Zeocin (Invitrogen, Carlsbad, CA) at 50 µg/mL.

Biochemical fractionation

Native N2A and CHO cells or stable clonal lines expressing WT or E163K human DJ-1 were cultured in 10 cm dishes as described above. Cells were grown to confluency, rinsed and scraped in PBS, and harvested by centrifugation at 13,000 × g. Cells were vortexed vigorously in 2 pellet volumes of PBS/0.1% Triton, sedimented, and the supernatant was collected as the TX1 fraction. This was repeated on the remaining pellet and the supernatant was collected as the TX2 fraction. The pellet was resuspended in 2 volumes of RIPA buffer, vortexed vigorously, sedimented, and the supernatant was collected as the RIPA fraction. The remaining pellet was solubilized in 2%SDS/17mM Tris, pH 8.0 and kept as the SDS fraction.

Gel filtration chromatography

Gel filtration chromatography was performed by calibrating a Superose 6 column (Amersham Biosciences) attached to a fast performance liquid chromatography (FPLC) system with standards of known molecular mass using 10 mM Tris, pH 7.5, 100 mM NaCl as the mobile phase. Molecular mass standards [BSA (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12 kDa)] were resolved separately to calibrate the column. The elution of the standards was monitored by protein absorbance at 280 nm. N2A or CHO cells and stable cell lines thereof were cultured in 10 cm dishes and grown to confluency. Cells were rinsed and scraped in phosphate buffered saline, pH 7.4. After recovery by centrifugation, cells were lysed in PBS/0.1% Triton and the cell debris was sedimented at 13,000 × g for 5 min. The extracts were filtered through a 0.45 µm filter and loaded onto the column. Fractions were analyzed by immunoblotting with anti-DJ-1 antibodies, DJ5 (1:1000) and 691 (1:1000).

Pulse-chase protein turnover experiments

N2A or CHO cells stably expressing WT or E163K DJ-1 were cultured in 6-well dishes. Cells were methionine-deprived for 15 minutes by incubation in methionine-free DMEM

(Invitrogen, Carlsbad, CA)/ 10% dialyzed FBS before adding 100 μ Ci [^{35} S]-methionine (Invitrogen, Carlsbad, CA) per ml of methionine free DMEM/10% dialyzed FBS for 30 min. Chase experiments were conducted in quadruplicates with normal DMEM/FBS for 0, 3, 6, 12, and 25 hours. Cells were then rinsed with PBS and harvested in CSK buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 1 % Triton X-100) containing 1 % SDS and boiled at 100 °C for 5 minutes. CSK buffer was added to the lysates in order to bring the final concentration of SDS to 0.25%. Lysates were frozen on dry ice and kept frozen at -20 °C until the last time point was harvested. The radiolabelled protein extracts were pre-cleared with a rabbit serum pre-incubated with protein A-agarose (Santa Cruz Biotechnologies, Santa Cruz, CA) for 3 hours at 4°C and radiolabeled extracts were then immunoprecipitated overnight at 4°C with anti-DJ-1 polyclonal antibody 691 pre-incubated with protein A-agarose (Santa Cruz Biotechnologies, Santa Cruz, CA). The antibody-protein complexes were washed 3 times with 10 volumes of CSK buffer, resuspended in 2 volumes of 2X SDS sample buffer and boiled at 100 °C for 5 minutes. The beads were removed by centrifugation and the samples were loaded on 12 % polyacrylamide gels. Following electrophoresis, gels were fixed with 50% methanol/ 5% glycerol, dried and exposed to a PhosphorImager plate and the signal was quantified using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Subcellular fractionation

The subcellular fractionation procedures used were similar to those previously described [36] with some changes. Native N2A, or N2A cells stably expressing WT or E163K DJ-1 were cultured in 10 cm plates. Cells were rinsed and scraped in phosphate-buffered saline, pH 7.4 (Invitrogen, Carlsbad, CA) and pelleted at 13,000 $\times$ g. Cells were resuspended in 3 pellet volumes of subfractionation buffer (0.25 M sucrose, 10 mM HEPES/NaOH, pH 7.5, 1 mM DTT, and protease inhibitors). Cells were homogenized with 20 strokes of a Dounce homogenizer (Kontes Glass Co; Vineland, NJ). The nuclei and unlysed cells were pelleted by sedimentation at 489 $\times$ g for 10 minutes at room temperature (RT). The supernatants were further cleared at 1585 $\times$ g for 10 minutes. The supernatants (S1) were removed to fresh tubes and sedimented for 10 minutes at 1585 $\times$ g at RT. The supernatants (S2) were removed to fresh tubes and pelleted for 17 minutes at 22000 $\times$ g at 4° C. The supernatants (S3) were removed as the crude cytoplasmic fraction and incubated on ice. In the mean time, the pellet (mitochondrial enriched fraction) was rinsed with 3 pellet volumes of subfractionation buffer and centrifuged again at 22000 $\times$ g for 17 minutes at 4°C. The supernatant from this rinse was discarded and the mitochondrial pellet was solubilized in 3 pellet volumes of 2% SDS/17mM Tris and boiled at 100 °C for 5 minutes. The tube containing the crude cytoplasmic supernatant (S3) was then sedimented at 103,000 $\times$ g for 25 minutes at 4 °C in order to pellet small organelles and cell debris, and the resulting supernatant (S4) was collected as the cytosolic fraction. The protein concentrations were determined in each fraction using the bicinchoninic acid protein (BCA) Assay (Pierce, Rockford, IL) with and bovine serum albumin as a standard. Fractions were analyzed by immunoblotting with antibodies DJ5 (1:1000), 691 (1:1000), Tim23 (0.5 μ g/mL), and ERK1 (0.2 μ g/mL).

For subcellular fractionation experiments that followed oxidative stress, N2A cells or cells stably expressing human WT or E163K mutant DJ-1 were cultured in 10 cm plates and were treated for 1.5 hours with DMEM/FBS or DMEM/FBS containing 350 μ M H₂O₂. Cells were treated with H₂O₂ in duplicate. Cells were rinsed and scraped in PBS. Subcellular fractionation by differential centrifugation was performed as described above.

Cell viability assay

Native N2A or N2A cell lines expressing WT or E163K DJ-1 were cultured separately into 48 well plates. Each cell type was treated for 20 hours in sextuplicates with DMEM/FBS containing either 10, 20 or 30 μ M H₂O₂, 7.5, 10, or 20 μ M MPP dihydrochloride (Sigma,

St. Louis, MO), 10, 20, or 30 μ M MG-132 (Sigma, St. Louis, MO), or fresh DMEM/FBS. In separate experiments, these cell lines were treated for 96 hours in sextuplicates with either 50, 100, or 200 nM Antimycin A (Sigma, St. Louis, MO) or 5, 10, or 15 μ M 3-nitropropionic acid (Sigma, St. Louis, MO). After treatment, the media from each well was collected into separate 1.5 mL microfuge tubes. The cells remaining in the wells were trypsinized, and harvested in corresponding microfuge tubes. Cells were pelleted and resuspended in fresh DMEM/FBS, and then 3-fold volumes of Trypan Blue solution (Sigma, St. Louis, MO) was added. Live and dead cells were counted manually using a hemacytometer and an Olympus CKX41 microscope. The percentage of live cells relative to the total number in each well was calculated.

Co-immunoprecipitation and heterodimer studies

N2A cells were cultured in 10 cm plates. Cells were mock transfected or transiently transfected with pc3.1E163KhDJ1-NFlag, or pc3.1WThDJ1-NFlag constructs using Lipofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 24 hours, cells were rinsed and scraped in PBS and lysed into 500 μ L of 1X CSK buffer by vortexing. Cell debris was sedimented at $13,000 \times g$ for 5 minutes and supernatants were removed to fresh tubes. 30 μ L of each supernatant was saved as the "Start" fraction. The remainders of the supernatants were incubated at 4 $^{\circ}$ C with a mouse anti-flag antibody preabsorbed to protein A/G PLUS Agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The beads were sedimented, repeatedly rinsed with 1X CSK buffer, resuspended in sample buffer, heated to 100 $^{\circ}$ C and saved as the "IP" fractions. The supernatants remaining after the immunoprecipitation were saved as the "Unbound" fractions. Sample buffer was added to the "Start" and "Unbound" fractions and then they were heated to 100 $^{\circ}$ C for 5 minutes.

Immunofluorescence and confocal microscopy

Native N2A, or N2A cells stably expressing WT or E163K DJ-1 were treated with DMEM/FBS or DMEM/FBS containing 20 μ M H_2O_2 for 3 hours. The media was removed and replaced with warmed DMEM/FBS containing 100 nM Mitotracker $\text{\textcircled{R}}$ Red CMXRos (Invitrogen, Carlsbad, CA). The cells were allowed to respire for 20 minutes and then were fixed with neutral buffered formalin according to the manufacturer's protocol. The cells were rinsed with PBS for 5 minutes and then blocked for 30 minutes at room temperature in PBS/2% FBS/0.1% Triton. Cells were then incubated at 4 $^{\circ}$ C with DJ5 antibody diluted into PBS/2% FBS at a concentration of 1:500 overnight, washed 3 times with PBS at 10 minutes each, and then incubated at room temperature with a goat anti-mouse secondary antibody conjugated to Alexa Fluor $\text{\textcircled{R}}$ 488 (Invitrogen) diluted into PBS/2% FBS at a concentration of 1:500 for 2 hours. Cells were rinsed for 10 minutes with PBS, incubated at room temperature with DAPI (Pierce, Rockford, IL) diluted into PBS at a concentration of 1 μ g/mL for 5 minutes, and then rinsed 3 times with PBS at 10 minutes each. Cells were coverslipped with Cytoseal $^{\text{TM}}$ 60 mounting media (Richard-Allen Scientific, Kalamazoo, MI). The images were visualized with a Zeiss LSM-510 Meta confocal microscope. For each sample, five $143 \times 143 \mu$ m images were taken from a single plane using the 63 $\times$ /1.4 oil objective. The images were then quantified using MetaMorph 6.0 software (Molecular Devices, Sunnyvale, CA). The relative integrated staining for DJ-1 per mitochondrial area was calculated by measuring the integrated pixel intensity for DJ5 signal that overlapped with pixels positive for Mitotracker Red CMXRos divided by total mitochondrial area. Integrated pixel intensity is defined as pixel intensity times the area of pixels positive for the signal. The average values for the replicate images were calculated.

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Figure 1. Assessment of the solubility, dimerization and stability of E163K human DJ-1 in N2A cells

(A) Western blot analysis with DJ5 antibody, specific for human DJ-1, showing the expression of WT human DJ-1 in two stable N2A cell lines (clones 11 and 12) and E163K human DJ-1 in three stable N2A cell lines (clones 16, 47 and 52). An immunoblot with an anti-Actin antibody is shown as a loading control. (B) Biochemical fractionation was performed on N2A cells and stable cell clones of N2A cells expressing WT (clone 11) or E163K (clone 16) human DJ-1. Cellular proteins were extracted with buffers of increasing solubilization strengths as described in “Experimental Procedures”. Each fraction was analyzed by western blotting with the anti-human DJ-1 antibody, DJ5, and an anti-ERK 1/2, ERK1, antibody to verify equal protein loading. (C) Size-exclusion chromatography was performed to analyze the native size of E163K human DJ-1 compared to WT human DJ-1 and endogenous murine DJ-1. Soluble extracts from N2A cells and stable cell clones of N2A cells expressing WT (clone 11) or E163K (clone 16) human DJ-1 were loaded on a precalibrated Superose 6 column as described in “Experimental Procedures”. The total cell lysates (“Start”) and fractions were collected and analyzed by western blot analysis with DJ5 antibody to detect human DJ-1 or 691 antibody to detect endogenous murine DJ-1. Fractions 20-30 are shown. The elution profile of known molecular mass standards [BSA (66 kDa), ovalbumin (44 kDa), carbonic anhydrase (29kDa) and cytochrome C (12kDa)] are indicated above. (D) Pulse-chase turnover assay of E163K human DJ-1 compared to WT human DJ-1. N2A stable cell clones expressing WT (clone 11) or E163K (clone 16) human DJ-1 were pulsed with ^{35}S -methionine for 30 minutes and chased for 0, 3, 6, 12, or 25 hours. Experiments were conducted in quadruplicates. The results are plotted as percentage of protein over time standardized to the 0 hrs time point. The error bars show standard deviation.

Figure 2. The E163K mutant does not show altered subcellular localization

(A) N2A cells and stable cell clones of N2A cells expressing WT (clone 11) or E163K (clone 16) human DJ-1 were subjected to subcellular fractionation by differential centrifugation as described in "Experimental Procedures". Cellular fractions were analyzed by western blot with the monoclonal anti-human DJ-1 antibody, DJ5, the polyclonal anti-DJ-1 antibody, 691, anti-Tim 23 antibody (used as a mitochondrial marker), and anti-ERK1/2 antibody (used as a cytoplasmic marker). Experiments are shown in triplicates.

Figure 3. The E163K mutant fails to rescue under conditions of oxidative stress

(A) N2A cells and stable cell clones of N2A cells expressing WT (clone 11) or E163K (clone 16) human DJ-1 were treated with various chemical stressors and the Trypan Blue Exclusion Assay was used to assess viability. Results were plotted as the percentage of viable cells over time of chemical treatment. The error bars show standard deviation. In A, cells were treated for 20 hrs with either fresh DMEM/FBS or DMEM/FBS containing 10-30 uM MG-132, or 7.5-20 uM MPP dihydrochloride. In B, cells were treated for 96 hours with either fresh DMEM/FBS or DMEM/FBS containing 50-200 nM Antimycin or 5-15 uM 3-nitropropionic acid. (C) Several N2A stable clones expressing either WT human (clones 11 and 12) or E163K

human mutant DJ-1 (clones 16, 47 and 52) were treated for 20 hours with either fresh DMEM/FBS or DMEM/FBS containing 10-30uM H₂O₂.

Figure 4. Human exogenous DJ-1 protein does not co-immunoprecipitate with N2A endogenous DJ-1
 N2A cells were mock transfected or transiently transfected with either flag-tagged human WT or E163K mutant DJ-1 constructs. Soluble protein extracts were immunoprecipitated with anti-flag antibody. Fractions were resolved by SDS-PAGE and immunoblotted with the polyclonal DJ-1 antibody, 691. The “Start” fraction represents the total soluble extracts before IP and the “Unbound” fraction represents the protein remaining in the protein lysates after the IP.

Figure 5. Immunofluorescence microscopy reveals an altered mitochondrial localization for E163K mutant DJ-1 protein in response to oxidative stress

(A) N2A cells stably expressing WT (clone 11) or E163K (clone 52) human DJ-1 were treated either DMEM/FBS containing 20uM H₂O₂ or with DMEM/FBS. Cells were stained with Mitotracker (red), DJ5 (green), and DAPI (blue) and visualized by confocal microscopy. Representative images are shown. Bar = 13.5μm (B) The confocal microscopy images were quantified using MetaMorph 6.0. The graph represents the average of the relative pixel intensity for DJ-1 per mitochondrial area. The error bars show the standard deviation between replicate images. (C) N2A cells and N2A cells stably expressing WT (clone 11) or E163K (clone 52) human DJ-1 were either untreated (lanes labeled with “U”) or treated with 350uM H₂O₂ (lanes

labeled with “T”) for 1.5 hrs and then fractions enriched for mitochondria were isolated by differential centrifugation as described in “Material and Methods”. Cellular fractions were analyzed by western blot with the monoclonal anti- human DJ-1 antibody, DJ5, the polyclonal anti-DJ-1 antibody, 691, anti-Tim 23 antibody (used as a mitochondrial marker), and anti-ERK1/2 antibody. Experiments are shown in duplicates.