DJ-1, a cancer- and Parkinson's disease-associated protein, stabilizes the antioxidant transcriptional master regulator Nrf2

Casey M. Clements*, Richard S. McNally*, Brian J. Conti*, Tak W. Mak†‡, and Jenny P.-Y. Ting*‡

*Department of Microbiology–Immunology, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599-7295; and †The Campbell Family Institute for Breast Cancer Research, University Health Network, 620 University Avenue, Suite 706, Toronto, ON, Canada M5G 2C1

Contributed by Tak W. Mak, August 22, 2006

DJ-1/PARK7, a cancer- and Parkinson's disease (PD)-associated protein, protects cells from toxic stresses. However, the functional basis of this protection has remained elusive. We found that loss of DJ-1 leads to deficits in NQO1 [NAD(P)H quinone oxidoreductase 1], a detoxification enzyme. This deficit is attributed to a loss of Nrf2 (nuclear factor erythroid 2-related factor), a master regulator of antioxidant transcriptional responses. DJ-1 stabilizes Nrf2 by preventing association with its inhibitor protein, Keap1, and Nrf2's subsequent ubiquitination. Without intact DJ-1, Nrf2 protein is unstable, and transcriptional responses are thereby decreased both basally and after induction. This effect of DJ-1 on Nrf2 is present in both transformed lines and primary cells across human and mouse species. DJ-1's effect on Nrf2 and subsequent effects on antioxidant responses may explain how DJ-1 affects the etiology of both cancer and PD, which are seemingly disparate disorders. Furthermore, this DJ-1/Nrf2 functional axis presents a therapeutic target in cancer treatment and justifies DJ-1 as a tumor biomarker.

oxidative stress | PARK7 | NQO1 | Keap1 | neurodegeneration

Oxidative stress has been implicated as a major contributing factor in a wide variety of ailments. Cancer, cardiovascular disease, neurodegenerative disorders, and aging all are associated with increased oxidative stress in tissues. Such stress results from the accumulation of oxidative species due to their metabolic generation and environmental exposures. These oxidative species are detoxified by a gambit of antioxidant enzymes and molecules. The balance between oxidative species generation and removal determines the oxidative stress on a given tissue. Not surprisingly, therefore, cellular responses to oxidative stress are major determinants of disease susceptibility, particularly in tissues that are sensitive to oxidative stress, such as in the central nervous system. Genetic defects in oxidative responses lead to neurodegenerative diseases. Examples include mutations in *SOD1* (superoxide dismutase 1) that lead to ALS (1) and loss of DJ-1, which leads to early onset Parkinson's disease (PD) with high penetrance (2).

DJ-1 was initially described as a putative oncogene that is able to transform cells weakly on its own and more strongly in combination with Ras (3). DJ-1 is expressed at high levels in primary lung and prostate cancer biopsies (4, 5), and its expression correlates negatively with clinical outcomes in nonsmall cell lung carcinoma patients (6). The DJ-1 protein affects cell survival, in part, by modulating cellular signaling cascades such as PTEN/phosphatidylinositol 3-kinase/Akt (6) and altering p53 activity (7). Additionally, we and others have previously shown that DJ-1 expression in cancer cell lines conveys protection against stresses, including chemotherapy, oxidative stress, endoplasmic reticulum stress, and proteosome inhibition (4, 8, 9). The mechanism by which DJ-1 imparts this protection remains unknown. We report here that DJ-1 is required for the activity of Nrf2 (nuclear factor erythroid 2-related factor), a master regulator of response to oxidative stress.

Nrf2 is a member of the cap 'n' collar family of basic leucine zipper transcription factors that regulate the expression of many antioxidant pathway genes (reviewed in ref. 10). Nrf2 is maintained at basal levels in cells by binding to its inhibitor protein, Keap1 (11, 12). Keap1 is a BTB (Broad complex, Tramtrack, Bric-a-Brac) domain-containing protein that targets Nrf2 for ubiquitination by Cul3/Roc-1, leading to its constitutive degradation (13–16). Upon exposure to oxidative stress, xenobiotics, or electrophilic compounds, Nrf2 protein is stabilized and translocates to the nucleus (17). There, it forms heterodimers with other transcription regulators, such as small Maf proteins, and induces the expression of antioxidant genes (18, 19). Nrf2 drives the expression of detoxification enzymes, such as NQO1 [NAD(P)H quinone oxidoreductase 1] and Hmox-1, and enzymes that generate antioxidant molecules, such as glutathione (20, 21). Nrf2 function and the expression of its regulated genes, including *NQO1*, have been implicated in the risk and/or prevention of both cancer and PD (22–27).

In this study, we find that DJ-1 is required for the expression of several genes, including the prototypic Nrf2-regulated antioxidant enzyme NQO1. We report here that DJ-1 is indispensable for Nrf2 stabilization by affecting Nrf2 association with Keap1, an inhibitor protein that promotes the ubiquitination and degradation of Nrf2. These findings implicate DJ-1's effects on Nrf2 in the development of Parkinson's disease and cancer and present potential therapeutic targets.

Results

siRNA-Mediated Knockdown of DJ-1 and Affymetrix GeneChip Analysis. To explore DJ-1's function, we examined its effect on global gene expression. DJ-1 expression was reduced by siRNA in H157 non-small-cell lung carcinoma cells (Fig. 1). The characterization of the antibody used to verify DJ-1 expression is shown in Fig. 6, which is published as supporting information on the PNAS web site. The first DJ-1 siRNA (referred to as siDJ-1#1) caused a modest decrease in DJ-1, whereas siDJ-1#2 caused a profound decrease. RNA samples from cells with siDJ-1#1, two control scrambled oligomers (siCTL), and one mock-transfected sample were subjected to GeneChip profiling (Affymetrix, Santa Clara, CA). To ensure that changes warranted further study, we

Author contributions: C.M.C., T.W.M., and J.P.-Y.T. designed research; C.M.C., R.S.M., and B.J.C. performed research; T.W.M. contributed new reagents/analytic tools; C.M.C., R.S.M., and B.J.C. analyzed data; and C.M.C. and J.P.-Y.T. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Abbreviations: ARE, antioxidant response element; tBHQ, *tert*-butylhydroquinone; MEF, mouse embryonic fibroblast; PD, Parkinson's disease.

Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE5519).

‡To whom correspondence may be addressed. E-mail: tmak@uhnresearch.ca or jenny_ting@med.unc.edu.

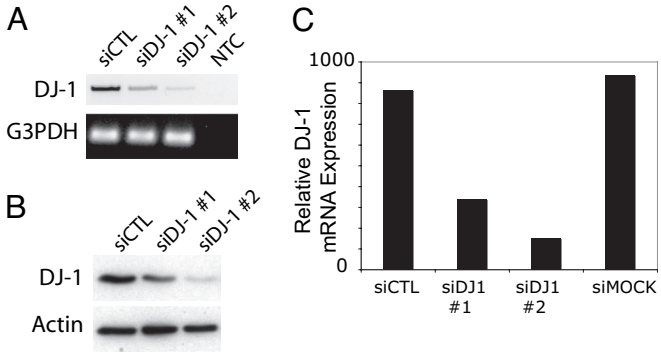
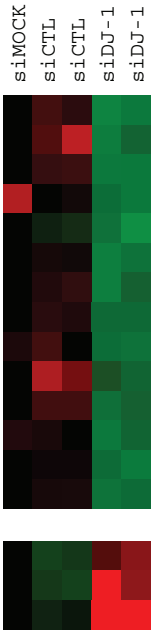


Fig. 1. siRNA-mediated knockdown of DJ-1 and GeneChip analysis. (*A*) End-point RT-PCR of H157 cells transfected with control siRNA (siCTL) or two different siRNA targeting DJ-1 (siDJ-1#1 and siDJ-1#2). The DJ-1 RT-PCR gel is presented as a negative image so bands can be more easily visualized. NTC is a nontemplate control. (*B*) Western blot analysis of siRNA-transfected H157 cells demonstrating DJ-1 knockdown at the protein level. (*C*) Quantitative real-time PCR of DJ-1 mRNA after siRNA transfection. Relative mRNA quantitation is normalized to 18S rRNA expression. siDJ-1#2 reduced DJ-1 expression to a greater degree than siDJ-1#1, whereas transfection with either a scrambled nonspecific oligomer siRNA or transfection reagent alone (siMOCK) did not affect DJ-1 expression.

stringently filtered expression to exclude differences <3-fold and any genes having spots with a raw signal intensity of <500 units in the samples where a gene was determined to be present. This stringent filtering produced a list of 3 genes that were increased and 14 genes that were decreased in cells with siDJ-1 (Fig. 2). As expected, siDJ-1 reduced *DJ-1* expression.

Among the genes whose expression decreased in the absence of DJ-1, one of particular interest was *NQO1*. NQO1 is a well described detoxification enzyme (28) that has been implicated in the risk and prevention of cancer and neurodegenerative diseases (29–32). NQO1 is regulated to a large degree by gene transcription by means of an antioxidant response element (ARE) in its promoter (33), which is a prototypic target of the antioxidant transcription factor Nrf2 (20). With this fact in mind, we used the tfsearch algorithm (as in ref. 34) to search for putative AREs within 1,000 bp upstream of the transcriptional start site of the genes identified in Fig. 2. Seven of 17 genes that were changed by >3-fold by siDJ-1 contained an ARE-like sequence (TMAnnRTGAYnnnGCRwwww) in their promoters (Fig. 2, rightmost column). We then reanalyzed our microarray data with respect to Nrf2 and found that several Nrf2-regulated genes were altered in the absence of DJ-1 (Fig. 7, which is published as supporting information on the PNAS web site). All array data have been deposited in the Gene Expression Omnibus online repository.

DJ-1 Is Required for Nrf2-Mediated Transcription. To verify the microarray data, we used *NQO1* as a prototypic target gene of DJ-1. Real-time PCR analysis shows that siDJ-1#2 reduced DJ-1 and NQO1 by >80%. However, Nrf2 mRNA expression was not changed (Fig. 3*A*), indicating that NQO1 expression differences are not due to a reduction of Nrf2 mRNA. To determine whether DJ-1 affects *NQO1* gene transcription by means of Nrf2 function, we used a reporter construct, pGL2-ARE, which contains the firefly luciferase gene under the control of an ARE from the human *NQO1* promoter (Fig. 3*B*). This construct was tested in the absence or presence of DJ-1. The liver cell line Huh7 was used because Nrf2 activity can be induced in these cells by the nontoxic food preservative *tert*-butylhydroquinone (tBHQ) (35). Cells were treated with either 50 μM tBHQ or DMSO vehicle control, and luciferase activity was measured (Fig. 3*B*). Flag-Nrf2 was transfected into cells as a positive control. Overexpressed Nrf2 robustly activated ARE-regulated luciferase (Fig. 3*B*, lanes 1 vs. lane 2). Cells with siCTL produced a basal level of luciferase, whereas tBHQ induced luciferase expression as expected (36) (Fig. 3*B*, lanes 3 and 4). In the presence of siDJ-1#1 or siDJ-1#2, luciferase activity was reduced (Fig. 3*B*, lanes 5 and 7), and it was no longer stimulated by tBHQ treatment (lanes 6 and 8). This effect is specific for the ARE element, as evidenced by the fact that siDJ-1 did not affect other promoter elements (Fig. 3 *C* and *D*).



		Fold Change	Upstream ARE-like sequence
TOMM20	TRANSLOCASE OF OUTER MITOCHONDRIAL MEMBRANE 20	-5.02	
RRN3	RNA POLYMERASE I TRANSCRIPTION FACTOR	-4.81	**GGA**GG**CTGAG**GCA**CGA**GAAT
P24A/TMED2	TRANSMEMBRANE EMP24 TRANSPORT DOMAIN-CONTAINING PROT.	-4.34	**CGA**GG**CTGAG**GCA**CGA**GAAT
LAMP1	LYSOSOME-ASSOCIATED MEMBRANE PROTEIN 1	-4.33	
ABCC3	ATP-BINDING CASSETTE, SUBFAMILY C, MEMBER 3	-3.88	**ACT**CA**ATGAC**TCA**TCG**GCCC
CLNS1A	CHLORIDE CHANNEL, NUCLEOTIDE SENSITIVE, 1A	-3.79	**TTG**CG**CTGAG**TCA**GTT**CCTG
DJ-1	ONCOGENE DJ-1/PARK7	-3.69	**CGG**AC**GTGAC**GCA**GCG**TGAG
PSIP1	PC4- AND SFRS1-INTERACTING PROTEIN 1	-3.57	
OB-RG/LEPR	LEPTIN-RELATED GENE/LEPTIN RECEPTOR	-3.49	
RAB14	RAS-ASSOCIATED PROTEIN	-3.38	
PIGB	PHOSPHATIDYLINOSITOL GLYCAN, CLASS B	-3.35	
NQO1	NAD(P)H DEHYDROGENASE, QUINONE 1	-3.32	**TCA**CA**GTGAC**TCA**GCA**GAAT
HRASLS3	HRAS-LIKE SUPPRESSOR 3	-3.31	
NOG1	NOGGIN HOMOLOG	-3.02	
GREM1	GREMLIN 1 HOMOLOG, CYSTEINE KNOT SUPERFAMILY	+4.00	
CALR	CALRETICULIN	+3.27	
CTGF	CONNECTIVE TISSUE GROWTH FACTOR	+3.10	**GCA**TC**CTGAG**TCA**CAC**GCGT

Fig. 2. Summary of Affymetrix GeneChip analysis. Genes shown represent changes of >3-fold between siCTL- and siDJ-1-transfected samples; fluorescence in the present (P) state is >500 in all samples. Green indicates decreased expression in normalized fluorescence; red indicates higher expression. Putative Nrf2-binding sequences within 1,000 bp upstream of the transcription start site are included to the right where present and were identified by using tfsearch and a score of >85.0.

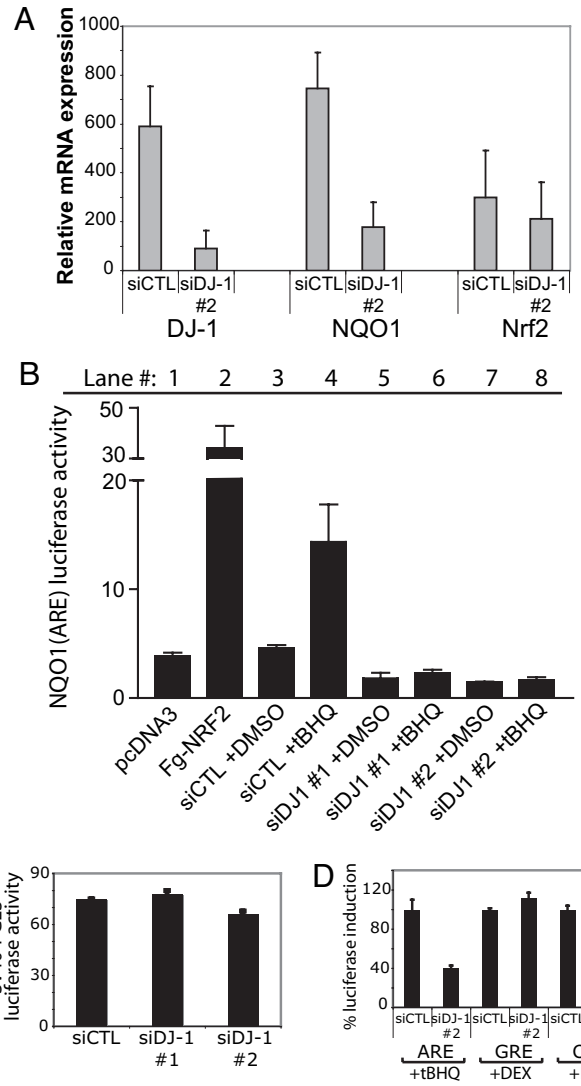


Fig. 3. DJ-1 is required for Nrf2-mediated transcription. (*A*) Real-time quantitative PCR analysis of mRNA expression verifies that siDJ-1#2 reduced DJ-1 mRNA expression, as well as NQO1 mRNA expression. However, the mRNA of Nrf2, a master regulator of NQO1 expression, is unaffected by the loss of DJ-1. All experiments were performed in triplicate, and error bars indicate SEM. (*B*) ARE-regulated luciferase reporter gene activity in Huh7 cells is reduced after siDJ-1 transfection. The firefly luciferase reporter construct is under the control of the NQO1 ARE (43), which is responsive to Nrf2. Cells were treated with 50 μM tBHQ or a DMSO vehicle control. Lysates were assayed for luciferase activity and normalized to crude protein present in the extract. Flag-Nrf2 was transfected as a positive control. Samples with lowered DJ-1 expression contained lower levels of the ARE-regulated luciferase activity and failed to increase luciferase activity after treatment with tBHQ. All experiments were performed in triplicate, and error bars indicate SEM. (*C*) Luciferase activity expressed from a construct under the control of the constitutively active viral SV40 promoter was not affected by siDJ-1. (*D*) Luciferase activity expressed from two mammalian promoters was not affected by siDJ-1. Huh7 cells with siDJ-1 were transfected with luciferase reporter constructs under control of the NQO1 ARE, glucocorticoid response element (GRE), or cAMP response element (CRE). Cultures were treated with the appropriate vehicle control, 50 μM tBHQ, 100 μM dexamethasone (DEX), or 10 μM forskolin (FOR) as indicated. Activation is presented as the percentage induction of control oligomer (siCTL)-transfected cells. All experiments were replicated at least three times.

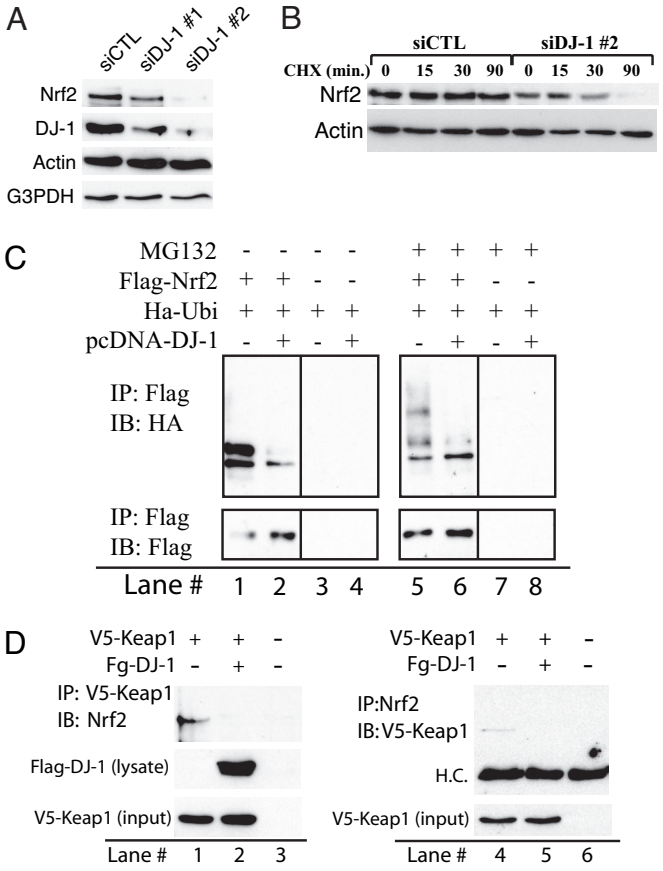


Fig. 4. DJ-1 is required for Nrf2 protein stability. (*A*) Western blot analysis of Nrf-2, DJ-1, and control proteins in Huh7 cell lysates after siRNA knockdown of DJ-1. (*B*) Time course of protein expression after cyclohexamide (CHX) treatment. Western blot analysis confirms the presence of Nrf2 at times after CHX treatment in control samples. Actin is used as an unaffected control. (*C*) *In cellulo* assay of Nrf2 ubiquitinylation. Nrf2 and covalently bound ubiquitin were immunoprecipitated from Huh7 extracts and analyzed by SDS/PAGE and Western blot analysis. (*D*) Nrf2/Keap1 coimmunoprecipitation in the presence of DJ-1. V5 epitope-tagged Keap1 was expressed in Huh7 cells with and without overexpressed Flag-DJ-1. Immunoprecipitation using anti-V5 antibody coimmunoprecipitated endogenous Nrf2 protein, and, conversely, immunoprecipitation of endogenous Nrf2 coisolated V5-Keap1. ''H.C.'' denotes a cross-reacting band of IgG heavy chain that was present from the immunoprecipitating antibody. Data are representative of at least three independent experiments.

DJ-1 Is Required for Nrf2 Protein Stability. Given that DJ-1 was required for both basal and induced ARE-driven transcription, we explored some possible mechanisms. DJ-1 was not associated with the *NQO1* promoter as assessed by chromatin immunoprecipitation assay, suggesting that DJ-1 is not likely tethered on the *NQO1* promoter with Nrf2 (Fig. 8, which is published as supporting information on the PNAS web site). Furthermore, RNA expression of Nrf2 (see Fig. 2*A*) or its inhibitor, Keap1, was not changed by siDJ-1 (Fig. 9, which is published as supporting information on the PNAS web site). However, Western blot analysis revealed that Nrf2 protein expression was drastically reduced in the absence of DJ-1, with siDJ-1#1 causing a more modest decrease and siDJ-1#2 causing a dramatic decrease (Fig. 4*A*), consistent with the level of DJ-1 reduction achieved with these two siRNA (see Fig. 1*A*).

To determine whether DJ-1 reduced Nrf2 stability, DJ-1 was decreased by siDJ-1 in Huh7 cells, and the cells were treated with the translation inhibitor cyclohexamide to prevent new protein synthesis. Cells were lysed at various time points, and the degradation kinetics of Nrf2 and actin (as a control) was analyzed by Western blot. Nrf2 protein was decreased by siDJ-1 compared with siCTL or transfection reagents alone, and, by 90 min, Nrf2 disappeared in cells with siDJ-1 (Fig. 4*B*), indicating that DJ-1 stabilizes Nrf-2 protein.

Nrf2 protein stability is an important regulatory event that is tightly controlled by its association with a cytosolic inhibitor protein, Keap1 (11, 12). Under unstimulated conditions, Nrf2 associates with Keap1, which targets Nrf2 for ubiquitination by a Cullin-3-dependent mechanism (13–16), leading to proteosome-dependent degradation (37). Given our data implicating DJ-1 in Nrf2 stability, we tested DJ-1's effect on Nrf2 ubiquitination (Fig. 4*C*). Huh7 cells expressing HA-tagged ubiquitin and Nrf2 were transfected with DJ-1 or pcDNA. Nrf2 was immunoprecipitated from denatured lysates, isolating only molecules covalently linked to Nrf2. Ubiquitin-Nrf2 conjugates were visualized by immunoblotting for the ubiquitin epitope HA. Nrf2 was ubiquitinated to a much lesser degree when DJ-1 was overexpressed (Fig. 4*C*, upper blot, lanes 1 and 2), correlating with an increase of Nrf-2 protein in the presence of DJ-1 (lower blot). The addition of the proteosome inhibitor MG132 prevented degradation of ubiquitinated Nrf2 (Fig. 4*C*, lanes 5 and 6).

Given that the association of Keap1 with Nrf2 is known to trigger Nrf2 ubiquitination/degradation (38) and that DJ-1 reduces Nrf2 ubiquitination, we determined whether DJ-1 affects the association of Nrf2 and Keap1. Huh7 cells were transfected with V5-tagged Keap1 (the tagged epitope is required because of the lack of a sufficient and commercially available Keap1 antibody). The anti-V5 antibody recognized V5-Keap1 and coimmunoprecipitated Nrf2 (Fig. 4*D*, lane 1); the inclusion of Flag-DJ-1 eliminated this coimmunoprecipitation (Fig. 4*D*, lane 2). Reverse immunoprecipitation shows that antibody to endogenous Nrf2 coprecipitated V5-Keap1 (Fig. 4*D*, lane 4), which was also decreased by Flag-DJ-1 (lane 5). These data suggest that DJ-1 stabilizes Nrf2 by preventing its association with Keap1.

Although the above experiments demonstrate a strong functional link between DJ-1 and Nrf2, we have so far been unable to determine where DJ-1 physically exerts this effect. Coimmunoprecipitation experiments have failed to find DJ-1 in physical association with Nrf2, Keap1, or Cullin-3 (Fig. 10, which is published as supporting information on the PNAS web site). Therefore, it remains to be determined whether DJ-1's profound effect on Nrf2 is the result of direct or indirect molecular mechanisms.

DJ-1 Is Required for Nrf2 Function in Primary Mouse Embryonic Fibroblasts (MEFs). To determine whether DJ-1 is required for Nrf2 expression in primary untransformed cells, we isolated day-13.5 MEFs from DJ-$1^{-/-}$ mice (39) and induced Nrf2 protein expression by using tBHQ treatment. tBHQ induced murine Nrf2 (mNrf2) protein expression in WT littermates (n = 4; two are shown in Fig. 5*A*), whereas DJ-$1^{-/-}$ mice failed to show induced mNrf2 expression (n = 4; two are shown in Fig. 5*A*). Restoration of DJ-1 with a Flag-DJ-1 expression plasmid also restored Nrf2 protein expression with tBHQ treatment (Fig. 5*B*), which indicates that the loss of Nrf2 protein in DJ-$1^{-/-}$ fibroblasts is a specific consequence of the loss of DJ-1.

To examine the necessity of DJ-1 for Nrf2 function, we resorted to the Nrf2-activated reporter plasmid pGL2-ARE. DJ-$1^{+/+}$ and DJ-$1^{-/-}$ MEFs from four mice (two representatives are shown in Fig. 5*C*) were separately transfected with pGL2-ARE and then induced with 50 μM tBHQ. WT DJ-$1^{+/+}$ cells showed increased luciferase expression upon tBHQ treatment, whereas DJ-$1^{-/-}$ cells did not (Fig. 5*C Left*). SV40 promoter activity was independent of DJ-1 (Fig. 5*C Right*).

To use a more physiologic measurement, we tested the effect of DJ-1 on the expression of Nrf2-regulated detoxification enzymes: NQO1 and GCLM (glutathione cysteine ligase modifier subunit) (Fig. 5*D*). Based on the microarray analysis (Fig. 7), siDJ-1 reduced GCLM expression by 1.478-fold; hence, we selected it in addition to NQO1 for further analysis. Induction of MEF cultures with 25 μM tBHQ led to a substantial increase of

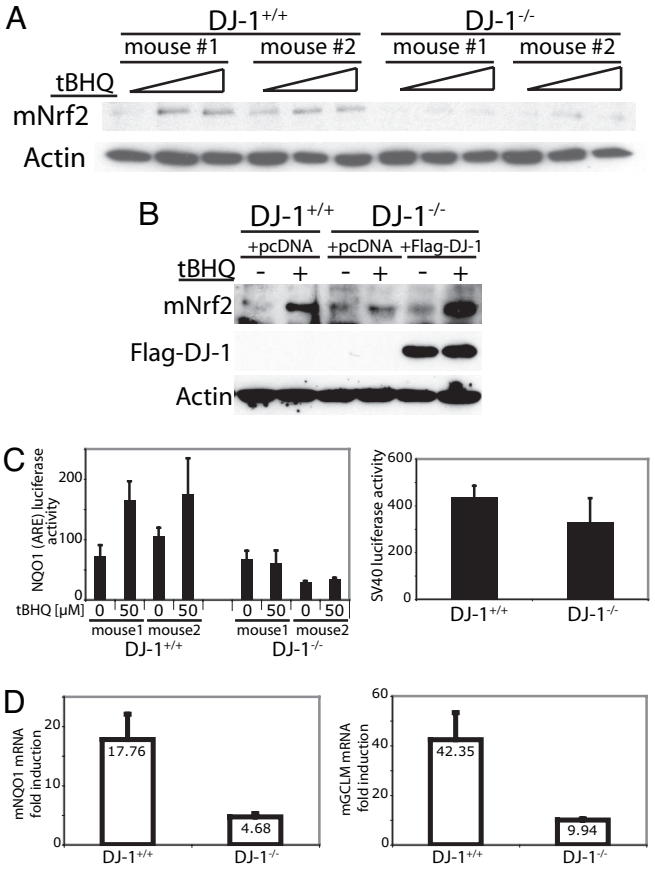


Fig. 5. DJ-1 is required for Nrf2 function in MEFs. (*A*) Western blot analysis of mNrf2 protein expression in primary MEFs derived from DJ-1 gene deletion mice and WT littermates. Cultures were treated with tBHQ at 0, 50, and 100 μM. (*B*) Western blot analysis of mNrf2 cultures transfected with either pcDNA or Flag-DJ-1 and treated with 50 μM tBHQ or vehicle control. (*C*) ARE-luciferase activity in DJ-$1^{+/+}$ and DJ-$1^{-/-}$ MEF cultures. Luciferase is under control of the ARE from the human NQO1 gene promoter (*Left*); SV40-luciferase is under control of the constitutively active viral SV40 promoter (*Right*). Luciferase activity is normalized to protein concentration in the extract. (*D*) Real-time quantitative PCR of Nrf2-mediated target gene expression in primary MEFs. NQO1, NAD(P)H quinone oxidoreductase I; GCLM, glutathione cysteine ligase modifier subunit. Data are presented as fold induction after treatment with tBHQ compared with vehicle control. All mRNA measurements are normalized to mouse G3PDH expression. All experiments were performed in triplicate, and error bars indicate SEM. All data are representative of at least three independent experiments.

mNQO1 in DJ-$1^{+/+}$ MEFs, but this increase was drastically reduced in DJ-$1^{-/-}$ cells. This pattern is also found for murine GCLM. However, at higher (100 μM) dosage, even though differences in mNrf2 protein expression persisted (Fig. 5*A*), induction of detoxification enzymes was only slightly reduced in DJ-$1^{-/-}$ compared with DJ-$1^{+/+}$ MEFs (Fig. 11, which is published as supporting information on the PNAS web site), indicating that a high concentration of tBHQ can activate a DJ-1-independent pathway to cause NQO1 and GCLM expression.

Discussion

In summary, this work describes functional effects of the DJ-1 protein by means of Nrf2, a master regulator of antioxidant gene responses. Cancer and PD lie at opposite ends of a spectrum defined by dysfunctions in cell death. Our finding may explain how DJ-1 plays an important role in both diseases. One of the hallmarks of PD is the loss of substantia nigra dopaminergic neurons, leading to motor deficits (40). DJ-$1^{-/-}$ mice did not

exhibit widespread neuronal loss in a PD disease model (39, 41), but these neurons were more susceptible to death after toxic insults (39). Likewise, human neuronal cell lines with DJ-1 knockdown are more sensitive to toxic compounds (8, 9). The loss of antioxidant gene transcription could account for these phenotypes that are only evident after environmental harm.

It is noteworthy that we initially identified DJ-1's effect on Nrf2 in lung tumor cells. Studies of Nrf2 knockout mice show that Nrf2 plays a significant role in lung biology (reviewed in ref. 10). In our studies, we found that the H157 lung tumor cells did not consistently induce Nrf2 activity after tBHQ treatment; instead, they had a very high basal level of activity that was not inducible by treatment (data not shown). High basal NQO1 expression allowed us to confidently quantify changes in NQO1 expression and implicated the broader effect of DJ-1 on Nrf2. To study gene induction, we then used liver cell line models, which are highly inducible. These models allowed us to identify the effects of DJ-1 on Nrf2, which heretofore remained unrecognized.

Enhanced expression of DJ-1 in cancer cells, leading to increased detoxification enzymes, is likely to provide a survival advantage. These enzymes may be exploited as treatment targets in tumors. For example, NQO1, an obligate two-electron reductase, can reduce antitumor quinones, leading to their bioactivation. Mitomycin C (MMC) and the antitumor compound 2,5-diaziridinyl-3- (hydroxymethyl)-6-methyl-1,4-benzoquinone are activated by NQO1 activity, and NQO1 is shown to increase the efficacy of MMC *in vivo* (42). It is possible that tumors with high DJ-1 levels might be more susceptible to therapies that rely on enzymes such as NQO1, underscoring the potential of DJ-1 as a biomarker to define specific antitumor therapies.

Materials and Methods

Cell Cultures, Treatments, and Plasmid Constructs. Huh7 cells were grown in DMEM (Sigma, St. Louis, MO) with 7% FCS. H157 cells were grown in RPMI medium 1640 (Gibco, Carlsbad, CA) plus 10% FCS. All mammalian cell cultures were grown in the presence of penicillin and streptomycin to minimize contamination effects.

tBHQ (Fluka, St. Louis, MO) was dissolved in DMSO (final concentration on cells was 0.0001%), and cells were treated for 18–24 h. Dexamethasone and forskolin (MP Biochemicals, Irvine, CA) were dissolved in DMSO and ethanol, respectively. Dexamethasone was used at a final concentration of 100 μM, and forskolin was used at 10 μM. In experiments determining Nrf2 protein stability, cells were treated with cyclohexamide (Sigma) in DMSO at a concentration of 75 μg/ml for up to 2 h. The peptide proteosome inhibitor MG132 (Calbiochem, San Diego, CA) was used at 25 μM for 4–6 h for ubiquitination studies.

Other investigators generously provided Flag-DJ-1 (5), Flag-Nrf2 (16), and hNQO1-ARE-pGL2 (43) plasmids. SV40-luciferase (pGL3-control), GRE-luciferase (pGRE-Luc, Clontech, Mountain View, CA), and CRE-luciferase (pCRE-Luc; Clontech) were all purchased from commercial sources. We directionally cloned human Keap1 into the V5/His-containing pcDNA3.1D-Topo plasmid (Invitrogen, Carlsbad, CA) by amplifying the Keap1 ORF with the primers 5′-CACCATGCAGCCAGATCCCAGGCCTAGC-3′ and 5′-ACAGGTACAGTTCTGCTGGTCAATCT-3′ by using platinum-*pfx* polymerase (Invitrogen). Clone directionality and expression was verified by sequencing and Western blot analysis. Human cell lines were transfected with DNA by using FuGENE 6 (Roche, Basel, Switzerland), and MEF cultures were transfected with Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions.

siRNA Knockdown of DJ-1. Cell lines were transfected with siDJ-1-1 5′-NNGACCCAGUACAGUGUAGCC-3′, siDJ-1-2 5′-NNUGGAGACGGUCAUCCCUGU-3′, scrambled control oligomer (Xeragon, Huntsville, AL), siCONTROL (siCTL) nontargeting siRNA no. 1 (Dharmacon, Lafayette, CO), or transfection reagent alone by using Oligofectamine (Invitrogen) for H157 cells and Lipofectamine 2000 (Invitrogen) for Huh7 cells per the manufacturer's protocols. Cells were transfected on consecutive days for 2–3 days in a row, and lysates were taken for RNA and protein analysis 96 h after the first transfection.

Generation of Anti-DJ-1 Antibody. DJ-1 was cloned into 6× histidine-tagged *Escherichia coli* overexpression vector QE82L (Qiagen, Valencia, CA) by standard methodology. Expression of DJ-1 was induced with 1 mM isopropyl-β-D-thiogalactopyranoside in the *E. coli* strain BL21 (DE3). Cells were lysed in PBS plus EDTA-free protease inhibitor mixture (Roche), and DJ-1 was purified to >95% homogeneity with Ni-nitrilotriacetic acid (Qiagen) according to the manufacturer's instructions. Recombinant DJ-1 was sent to Proteintech Group (Chicago, IL) for the production of the anti-DJ-1 rabbit polyclonal serum.

Affymetrix GeneChip Analysis. Total RNA isolated from H157 cells was DNase I-treated and column-purified (Promega, Madison, WI). The quality of the RNA was determined by formamide-agarose electrophoresis and comparison of expression of housekeeping genes. Seven micrograms of total RNA was used to synthesize cDNA. A custom cDNA kit from Life Technologies (Carlsbad, CA) was used with a T7-$(dT)_{24}$ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction by using the BioArray High Yield RNA transcript kit (Ento Life Sciences, Farmingdale, NY). The cRNA was fragmented in fragmentation buffer (5× fragmentation buffer: 200 mM Tris-acetate, pH 8.1/500 mM KOAc/150 mM MgOAc) at 94°C for 35 min before the chip hybridization. Fragmented cRNA (15 μg) was then added to the hybridization mixture (0.05 μg/μl fragmented cRNA/50 pM control oligonucleotide B2, *BioB*, *BioC*, *BioD*, and *cre* hybridization controls/0.1 mg/ml herring sperm DNA/0.5 mg/ml acetylated BSA/100 mM Mes/1 M $[Na^+]$/20 mM EDTA/0.01% Tween 20). Ten micrograms of cRNA was used for hybridization. Arrays were hybridized for 16 h at 45°C in the GeneChip Hybridization Oven 640. The arrays were washed and stained with R-phycoerythrin streptavidin in the GeneChip Fluidics Station 400. After this, the arrays were scanned with a GeneArray scanner (Hewlett–Packard, Palo Alto, CA). Affymetrix GeneChip Microarray Suite 5.0 software was used for washing, scanning, and basic analysis. Sample quality was assessed by examination of 3′ to 5′ intensity ratios of certain genes.

These data were then further analyzed, filtered, and compared by using GeneSpring software (Silicon Genetics, Redwood City, CA). Genes defined as "changed" were filtered to include those differing >3-fold between both siCTL chips and siDJ-1 chips, with a raw fluorescence intensity of at least 500 in both of the highly expressed (present) arrays. Both siDJ-1 arrays were transfected with siDJ-1#1 and then verified by real-time PCR using both siDJ-1#1 and siDJ-1#2.

Real-Time Quantitative PCR. Reactions were carried out in an ABI 7900HT PCR system (Applied Biosystems, Foster City, CA) using a 15-μl, 384-well format and master mixes from ABGene (Rochester, NY). TaqMan PCR primer/probe sets were designed for human DJ-1: primer 1, 5′-CCATATGATGTGGTGGTTCTAC-3′; primer 2, 5′-ACTTCCACAACCTATTTCATGAG-3′; probe, 5′-[6-FAM]ACCTGCACAGATGGCGGCTATCA[Tamra-Q]-3′. Primer/probe sets for human NQO1 were as follows: primer 1, 5′-CCGTGGATCCCTTGCAGAGA-3′; primer 2, 5′-AGGACCCTTCCGGAGTAAGA-3′; probe, 5′-[6-FAM]ACATGGAGCCACTGCCACCA[Tamra-Q]-3′. SYBR green real-time PCR primers were designed for human Nrf2: primer 1, 5′-AGTGGATCTGCCAACTACTC-3′; primer 2, 5′-CATCTACAAA-

CGGGAATGTCTG-3′. We used previously published mouse G3PDH primers that were designed to be used with SYBR green quantitation (44). Predesigned TaqMan PCR primer and probe sets were purchased from Applied Biosystems for mouse NQO1 and GCLM.

Luciferase Reporter Gene Assays. Cells were grown and transfected as described above in six-well plates (Falcon, San Jose, CA). Cultures were lysed in reporter lysis buffer (Promega) by using a single round of freeze–thaw at −80°C. Luciferase assays were then performed as described in ref. 45.

Western Blot Analysis and Immunoprecipitation. For all Western blot analyses, cells were lysed in RIPA buffer (10 mM NaPO4, pH 7.4/300 mM NaCl/0.1% SDS/1% Nonidet P-40/1% deoxycholic acid/2 mM EDTA) with protease inhibitors (Roche), diluted with SDS loading buffer, and boiled in the presence of the reducing agent DTT. Proteins were then separated by molecular weight by SDS/PAGE through polyacrylamide gels ranging from 6% to 12%. Proteins were electrophoretically transferred to nitrocellulose membranes and blocked by using 5% nonfat dry milk in TBS with 0.1% Tween 20. Antibodies used for blotting were anti-Nrf2 H-300 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-DJ-1, anti-actin-HRP (Santa Cruz Biotechnology), anti-G3PDH, anti-HA-HRP (Roche), and anti-Flag (M2)-HRP (Sigma).

Protein complexes were isolated from cell lysates by immunoprecipitation using antibodies specific for Nrf2 (H-300, Santa Cruz Biotechnology) and anti-V5 (Invitrogen), followed by incubation with protein A/G agarose (Pierce Biotechnologies, Rockford, IL). Protein A/G antibody–protein complexes were washed extensively and eluted by boiling in loading buffer with reducing equivalents. Eluates and input lysate controls were then Western blotted to assay for protein expression and isolation.

Ubiquitination assays were performed in Huh7 cells transfected with epitope-tagged Nrf2 and ubiquitin grown in 100-mm^2 plates. The cells were lysed in 200 μl of SDS lysis buffer (50 mM Tris·HCl, pH 7.5/0.5 mM EDTA/1% SDS/1 mM DTT) and boiled for 10 min. Cellular debris was pelleted, and SDS concentrations were diluted by the addition of 1,200 μl of 0.5% Nonidet P-40 lysis buffer with added protease inhibitors. Anti-Flag (M2) agarose was then added and incubated for 14–16 h. The agarose matrix was washed extensively with 0.5% Nonidet P-40 lysis buffer, and the proteins were eluted by boiling in 2× loading buffer with DTT. The eluates were then analyzed by Western blot analysis for the expression of the epitope tags.

DJ-1 Knockout Mice and Embryonic Fibroblast Culture. DJ-1 knockout mice and WT littermates (39), backcrossed six generations onto the C57BL6 strain, were housed according to the guidelines of the National Institutes of Health under an approved Institutional Animal Care and Use Committee protocol at the University of North Carolina. Primary MEFs were isolated from day-13.5 embryos and grown in DMEM supplemented with 10% FCS. All MEF experiments were performed on cells within two cell passages of isolation from the mice.

We thank Dr. Yue Xiong (University of North Carolina) for his valuable guidance and generous gift of several plasmid expression constructs, Dr. Yacov Hod (Stony Brook University, Stony Brook, NY) for the gift of the Flag-DJ-1 construct, Dr. Anil K. Jaiswal (Baylor College of Medicine, Houston, TX) for generously providing the pGL2-ARE (NQO1) expression plasmid, and Drs. Daniel T. Bergstralh and Willie June Brickey for assistance with microarray data analysis. This work was supported by National Cancer Institute Grants P50CA58223 and AI067798 and Training Grant 5T32AR007416-24 (to C.M.C.).

1. Beckman JS, Carson M, Smith CD, Koppenol WH (1993) *Nature* 364:584.
2. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, *et al.* (2003) *Science* 299:256–259.
3. Nagakubo D, Taira T, Kitaura H, Ikeda M, Tamai K, Iguchi-Ariga SM, Ariga H (1997) *Biochem Biophys Res Commun* 231:509–513.
4. MacKeigan JP, Clements CM, Lich JD, Pope RM, Hod Y, Ting JP (2003) *Cancer Res* 63:6928–6934.
5. Hod Y (2004) *J Cell Biochem* 92:1221–1233.
6. Kim RH, Peters M, Jang Y, Shi W, Pintilie M, Fletcher GC, DeLuca C, Liepa J, Zhou L, Snow B, *et al.* (2005) *Cancer Cell* 7:263–273.
7. Shinbo Y, Taira T, Niki T, Iguchi-Ariga SM, Ariga H (2005) *Int J Oncol* 26:641–648.
8. Taira T, Saito Y, Niki T, Iguchi-Ariga SM, Takahashi K, Ariga H (2004) *EMBO Rep* 5:213–218.
9. Yokota T, Sugawara K, Ito K, Takahashi R, Ariga H, Mizusawa H (2003) *Biochem Biophys Res Commun* 312:1342–1348.
10. Cho HY, Reddy SP, Kleeberger SR (2006) *Antioxid Redox Signal* 8:76–87.
11. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, Yamamoto M (1999) *Genes Dev* 13:76–86.
12. Dhakshinamoorthy S, Jaiswal AK (2001) *Oncogene* 20:3906–3917.
13. Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA (2004) *Mol Cell Biol* 24:8477–8486.
14. Kobayashi A, Kang MI, Okawa H, Ohtsuji M, Zenke Y, Chiba T, Igarashi K, Yamamoto M (2004) *Mol Cell Biol* 24:7130–7139.
15. Zhang DD, Lo SC, Cross JV, Templeton DJ, Hannink M (2004) *Mol Cell Biol* 24:10941–10953.
16. Furukawa M, Xiong Y (2005) *Mol Cell Biol* 25:162–171.
17. Chen C, Kong AN (2004) *Free Radic Biol Med* 36:1505–1516.
18. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) *Biochem Biophys Res Commun* 236:313–322.
19. Wild AC, Moinova HR, Mulcahy RT (1999) *J Biol Chem* 274:33627–33636.
20. Venugopal R, Jaiswal AK (1996) *Proc Natl Acad Sci USA* 93:14960–14965.
21. Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL (1999) *J Biol Chem* 274:26071–26078.
22. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, Kensler TW (2001) *Proc Natl Acad Sci USA* 98:3410–3415.
23. Kwak MK, Wakabayashi N, Itoh K, Motohashi H, Yamamoto M, Kensler TW (2003) *J Biol Chem* 278:8135–8145.
24. Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002) *Proc Natl Acad Sci USA* 99:7610–7615.
25. Cao TT, Ma L, Kandpal G, Warren L, Hess JF, Seabrook GR (2005) *J Neurochem* 95:406–417.
26. Nakaso K, Nakamura C, Sato H, Imamura K, Takeshima T, Nakashima K (2006) *Biochem Biophys Res Commun* 339:915–922.
27. Satoh T, Okamoto SI, Cui J, Watanabe Y, Furuta K, Suzuki M, Tohyama K, Lipton SA (2006) *Proc Natl Acad Sci USA* 103:768–773.
28. Iyanagi T, Yamazaki I (1970) *Biochim Biophys Acta* 216:282–294.
29. Cresteil T, Jaiswal AK (1991) *Biochem Pharmacol* 42:1021–1027.
30. Kolesar JM, Kuhn JG, Burris HA, III (1995) *J Natl Cancer Inst* 87:1022–1024.
31. Hara H, Ohta M, Ohta K, Kuno S, Adachi T (2003) *Brain Res Mol Brain Res* 119:125–131.
32. van Muiswinkel FL, de Vos RA, Bol JG, Andringa G, Jansen Steur EN, Ross D, Siegel D, Drukarch B (2004) *Neurobiol Aging* 25:1253–1262.
33. Jaiswal AK (2000) *Free Radic Biol Med* 29:254–262.
34. Kast C, Wang M, Whiteway M (2003) *J Biol Chem* 278:6787–6794.
35. Huang HC, Nguyen T, Pickett CB (2000) *Proc Natl Acad Sci USA* 97:12475–12480.
36. Dhakshinamoorthy S, Jaiswal AK (2002) *Oncogene* 21:5301–5312.
37. Stewart D, Killeen E, Naquin R, Alam S, Alam J (2003) *J Biol Chem* 278:2396–2402.
38. McMahon M, Itoh K, Yamamoto M, Hayes JD (2003) *J Biol Chem* 278:21592–21600.
39. Kim RH, Smith PD, Aleyasin H, Hayley S, Mount MP, Pownall S, Wakeham A, You-Ten AJ, Kalia SK, Horne P, *et al.* (2005) *Proc Natl Acad Sci USA* 102:5215–5220.
40. Kish SJ, Shannak K, Hornykiewicz O (1988) *N Engl J Med* 318:876–880.
41. Goldberg MS, Pisani A, Haburcak M, Vortherms TA, Kitada T, Costa C, Tong Y, Martella G, Tscherter A, Martins A, *et al.* (2005) *Neuron* 45:489–496.
42. Begleiter A, Leith MK, Thliveris JA, Digby T (2004) *Br J Cancer* 91:1624–1631.
43. Dhakshinamoorthy S, Jaiswal AK (2000) *J Biol Chem* 275:40134–40141.
44. Engelbrecht Y, de Wet H, Horsch K, Langeveldt CR, Hough FS, Hulley PA (2003) *Endocrinology* 144:412–422.
45. Piskurich JF, Linhoff MW, Wang Y, Ting JP (1999) *Mol Cell Biol* 19:431–440.