DJ-1 Transcriptionally Up-regulates the Human Tyrosine Hydroxylase by Inhibiting the Sumoylation of Pyrimidine Tract-binding Protein-associated Splicing Factor*[S]

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Loss-of-function mutations in DJ-1 cause a subset of familial Parkinson disease (PD). However, the mechanism underlying the selective vulnerability in dopaminergic pathway due to the inactivation of DJ-1 is unclear. Previously, we have reported that DJ-1 is a neuroprotective transcriptional co-activator interacting with the transcriptional co-repressor pyrimidine tract-binding protein-associated splicing factor (PSF). Here we show that DJ-1 and PSF bind and regulate the human tyrosine hydroxylase (TH) promoter. Inactivation of DJ-1 by small interference RNA (siRNA) results in decreased TH expression and L-DOPA production in human dopaminergic cell lines. Consistent with its role as a transcriptional regulator, DJ-1 specifically suppresses the global SUMO-1 modification. High molecular weight sumoylated protein species, including PSF, accumulate in the lymphoblast cells from the patients carrying pathogenic DJ-1 mutations. DJ-1 elevates the TH expression by inhibiting the sumoylation of PSF and preventing its sumoylation-dependent recruitment of histone deacetylase 1. Furthermore, siRNA silencing of DJ-1 decreases the acetylation of TH promoter-bound histones, and histone deacetylase inhibitors restore the DJ-1 siRNA-induced repression of TH. Therefore, our results suggest DJ-1 as a regulator of protein sumoylation and directly link the loss of DJ-1 expression and transcriptional dysfunction to impaired dopamine synthesis.

Parkinson disease (PD)[4] is a common progressive movement disease characterized by the selective loss of dopaminergic neurons and the decrease of striatal dopamine levels (1). Dopamine deficiency in PD patients contributes to the typical clinical features, which include tremor, bradykinesia, rigidity, and postural instability. These symptoms can be temporally controlled by administering medications targeting dopamine metabolism and function, such as the dopamine precursor L-DOPA and dopamine agonists. The rate-limiting enzyme for dopamine synthesis is tyrosine hydroxylase (TH).

Loss-of-function mutations in the *DJ-1* gene cause early-onset Parkinsonism (2), although the disease-causing mechanism remains to be fully resolved. The evolutionarily conserved DJ-1 has been shown to regulate oxidative stress, apoptosis, protein aggregation, and transcription in various subcellular compartments (3–8). *In vitro* experiments from several laboratories have clearly demonstrated the neuroprotective activity of DJ-1, although different molecular mechanisms have been proposed (4, 7–9). The role of DJ-1 in neuronal survival is strengthened by *in vivo* studies using *Drosophila* lacking DJ-1, which exhibit increased sensitivity to oxidative stress or environmental mitochondrial toxins (10, 11). In one study, inactivation of a *Drosophila* DJ-1 homolog by siRNA leads to the degeneration of the TH-positive dopaminergic neurons as in PD patients (10). On the other hand, DJ-1-deficient mice do not reproduce the typical neuropathology of PD patients, such as the loss of the dopaminergic neurons and the formation of intracellular inclusion Lewy bodies, although they exhibit subtle defects in the nigral-striatal pathway and motor functions (12–14). Patients carrying DJ-1 mutations demonstrate reduced dopamine

[4] The abbreviations used are: PD, Parkinson disease; TH, tyrosine hydroxylase; PSF, pyrimidine tract-binding protein-associated splicing factor; SUMO, small ubiquitin-like modifier; PIAS, protein inhibitors of activated STAT; STAT, signal transducers and activators of transcription; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation; siRNA, small interference RNA; DJBP, DJ-1 binding protein; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; CMV, cytomegalovirus; WT, wild type; IP, immunoprecipitation; Q-PCR, quantitative PCR; DEL, exon 1–5 deletion; HA, hemagglutinin; RNAi, RNA interference.

uptake indistinguishable to patients with sporadic PD (15). Due to the lack of pathological evidence from patients with DJ-1 mutations, it remains unclear whether the loss of DJ-1 function affects both dopaminergic neuronal survival and nigral-striatal pathways in humans.

Before mutations in the *DJ-1* gene were linked to familial PD, DJ-1 had been suggested to regulate transcription by modulating androgen receptor function in the testis (16, 17). DJ-1 restores the activities of androgen receptor by interacting with and antagonizing the transcriptional repressors PIASxα and DJBP (16, 17). Nevertheless, PIASxα and DJBP are mainly expressed in the testis (16, 17), and whether transcriptional regulation by DJ-1 is related to PD pathogenesis is yet to be fully characterized. We have recently demonstrated that DJ-1 forms complexes with nuclear proteins pyrimidine tract-binding protein-associated splicing factor (PSF) and p54nrb (7), two proteins that are highly expressed in the brain and affect RNA metabolism and transcription. DJ-1 serves as a transcriptional co-activator and prevents co-repressor PSF-mediated gene silencing and apoptosis.

Consistent with the role of DJ-1 in transcriptional regulation, several protein interaction studies suggest a potential functional link between DJ-1 and small ubiquitin-like modifiers (SUMO). Sumoylation is a reversible ATP-dependent process to covalently attach SUMO to target proteins, usually nuclear proteins (18). Similar to ubiquitination, this process requires the participation of E1 activating enzymes, E2 conjugating enzymes, and E3 ligases (18–20). Sumoylation has been shown to modulate the subcellular localization and transcriptional activity of the substrates (18, 20, 21). DJ-1 interacts with PIASxα and PIASy (17), which are SUMO E3 ligases belonging to the protein inhibitor of the activated STAT (PIAS) family. In addition, DJ-1 interacts with SUMO-1, SUMO-activating enzyme Uba2, and conjugating enzyme ubc-9 in the yeast two-hybrid systems (8, 22). However, the functional significance of the link between sumoylation and DJ-1 and the potential relevance to PD pathogenesis are unclear.

In the current study, we extend our prior findings and identify the human TH as a cellular target of transcriptional regulation by DJ-1 and PSF. DJ-1 promotes the expression of TH by inhibiting the SUMO-1 modification of PSF and preventing the recruitment of histone deacetylase (HDAC) 1 by PSF. Therefore, our results indicate that the transcriptional dysregulation caused by DJ-1 inactivation and the subsequent impairment of dopamine synthesis contribute to the development of PD.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Chemicals—Human CHP-212 cells were purchased from ATCC (Manassas, VA) and maintained in Eagle's minimum essential medium/F-12 (50%/50%) containing 10% fetal bovine serum and antibiotics. Human SH-SY5Y, HeLa, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. For immunofluorescence, cells were grown on coverslips. Wild-type and mutant DJ-1 constructs and SH-SY5Y cells stably expressing these constructs were described previously (7). The lymphoblasts from patients carrying pathogenic DJ-1 mutations (2) were described previ-

ously (23). Human HDAC1 construct (pCMV-FLAG-HDAC1) was kindly provided by Dr. Fang Liu at Rutgers University. Human SUMO-1 construct (pCMV-HA-SUMO-1) was a gift from Dr. Kim Orth at the University of Texas Southwestern Medical Center. Human PIASy (pCMV-PIASy) was purchased from ATCC. Rat TH-luc reporter plasmid was kindly provided by Dr. Kwang Soo Kim at McLean Hospital, Harvard Medical School, and the pTK-*Renilla* luciferase plasmid for transfection control was obtained from Promega (Madison, WI). A FLAG tag was fused in-frame to the N terminus of human wild-type PSF (7) to generate a tagged PSF construct (pCMV-FLAG-PSF). To introduce point mutations at the PSF sumoylation site (I337A and K338A), we used a two-step PCR mutagenesis approach to mutate and amplify the PSF fragments flanked by the EcoRI and BamHI sites. The resulting PCR products (979 bp) were then subcloned into an expression vector using pCMV-FLAG-PSF as backbone. The PSF plasmids were confirmed by sequencing. The flanking primers were: forward, 5′-gatgtcggttgtttgttg-3′; reverse, 5′-atctcccatgttcattgct-3′. Mutagenesis primers for PSF-I337A were: forward, 5′-ggattcg-gattt*gc*taagcttgaatctagagc-3′; reverse, 5′-gctctagattcaagcttag-caaatccgaatcc-3′. Mutagenesis primers for PSF-K338A were: forward, 5′-ggattcggatttattgcgcttgaatctagagc-3′; reverse, 5′-gc-tctagattcaagcgcaataaatccgaatcc-3′. Sodium butyrate and trichostatin A were from Sigma.

Transfection of siRNA and Plasmids—CHP-212 or SH-SY5Y cells plated in 6-well culture dishes were transfected with 100 nM siRNA constructs against the human DJ-1 (SMARTpool reagent, Dharmacon, Lafayette, CO) or nonspecific control siRNA constructs (siControl non-targeting pool, Dharmacon) using the Transfectin reagent (Bio-Rad, Hercules, CA) following the manufacturer's suggested protocol. Cells were harvested at 48 h post-transfection for RNA extraction or at days 1, 2, and 4 for Western blotting. To analyze the effects of HDAC inhibitors on the TH expression after DJ-1 inactivation, indicated sodium butyrate or trichostatin A was added to fresh medium 48 h after siRNA transfection, and the mixture was cultured for an additional 2 days. Lipofectamine 2000 (Invitrogen) was used to transfect various plasmids. To study the transcriptional repression of TH by PSF, CHP-212 cells plated in 10-cm dishes were co-transfected with 20 μg of control vector, WT, or mutant PSF plasmids and 2 μg of green fluorescent protein. Transfected cells were harvested 48 h later and enriched using a Cytomation Mo-Flo cell sorter (Core facility, Dana Farber Cancer Institute, Boston, MA) for subsequent total RNA extraction and mRNA analysis using quantitative PCR.

Western Blotting, Immunoprecipitation, and Antibodies—The procedures for Western blotting and immunoprecipitation were described previously (7). For SUMO-1-conjugated PSF IP, cells were lysed in denaturing radioimmune precipitation assay-deoxycholate buffer; for various co-immunoprecipitation experiments, cells were lysed in non-denaturing lysis buffer containing 1% Triton X-100. Antibodies used for IP included: a mouse monoclonal anti-PSF (Sigma), a rabbit polyclonal anti-DJ-1 (7), and a mouse monoclonal anti-HDAC1 (Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies for Western blotting included: a mouse monoclonal anti-TH (Sigma); monoclonal (Stressgen, San Diego, CA) and a poly-

clonal anti-DJ-1; a goat anti-β-actin and a mouse monoclonal anti-HDAC1 (Santa Cruz Biotechnology); a mouse monoclonal anti-SUMO-1 and a rabbit polyclonal anti-SUMO-2/3 (Invitrogen); and a rabbit polyclonal anti-FLAG tag and a rabbit polyclonal anti-acetylated histones (Histone sampler kit, Cell Signaling, Beverly, MA).

RNA Extraction and Real-time Quantitative PCR—RNA was extracted using TRIzol reagent (Invitrogen) and purified with an RNeasy Kit or an RNeasy Micro Kit (Qiagen). The quality of extracted RNA was determined by agarose gel electrophoresis. Reference RNA used for the calibration curve was made by pooling equal amounts of RNA from all samples. Q-PCRs were performed using a LightCycler (Roche Applied Science) and a One-Step QuantiTect™ SYBR Green RT-PCR kit (Qiagen). Experimental conditions and primer design parameter were set according to the Q-PCR kit instruction manual. Results were normalized to an internal control PCR amplified with glyceraldehyde-3-phosphate dehydrogenase or β-actin primers included in the same run of Q-PCR. The specificity of PCR amplifications was further confirmed by agarose gel electrophoresis. Primers for the human TH were: forward, 5′-cctcgcccatgcactc-3′; reverse, 5′-cctcgcccatgcactc-3′. Primers for PSF were: forward, 5′-accaccagcagcatcacc-3′; reverse, 5′-tcccaacaaacaaccgaca-3′.

Chromatin Immunoprecipitation Assays—ChIP assays using cultured cells were performed following the instructions of the EZ ChIP Kit (Upstate) with the following modifications. After protein-DNA cross-linking and harvesting, the cell pellets were resuspended in lysis buffer and sonicated on ice using a Branson Digital Sonifier (Branson Ultrasonics Corp., Danbury, CT) with 16 sets of 4-s pulse at 17% maximum power. The genomic DNA was sheared to 300–1200 bp of length. Aliquots of chromatin solution (each equivalent to 1×10^6 cells) were pre-cleared with Protein G-agarose and incubated with species-matched IgG or specific antibodies overnight at 4 °C with rotation. ChIP assays using brain tissues from normal patients were performed as described before (24). The substantia nigra tissues from a male (postmortem interval, 4.5 h) and a female (postmortem interval, 13 h) patient with no neurological or psychiatric conditions were acquired according to institutional regulations. Tissues surrounding the substantia nigra pars compacta were trimmed off to yield relatively pure nigral tissue. Samples were snap-frozen in liquid nitrogen after dissection and stored under −80 °C until usage. The specific antibodies applied in ChIP assays for DJ-1, PSF, HDAC1, and acetylated histones are described above. The final immunoprecipitated DNA fragments were used as templates for PCR with hot start Platinum Taq (Invitrogen) using the following conditions: 3 min at 94 °C; 32 cycles of 30 s denaturation at 95 °C, 30 s annealing at 57 °C, and 30 s elongation at 72 °C; with one final incubation for 2 min at 72 °C. 27–33 cycles were used for semiquantitative PCR in the TH-promoter-associated acetylated histones IP. Primer 3 software was used to design the PCR primers for amplifying the human TH promoter. The forward primer was 5′-gagccttcctggtgtttgtg-3′; the reverse primer was 5′-ctctccgattccagatggtg-3′. The PCR products correspond to the promoter sequences −2909 to −2707 upstream to the transcriptional initiation site of the human TH gene, and were analyzed by electrophoresis on 2% Tris-acetate-EDTA agarose gels. The primers used to amplify the control human glyceraldehyde-3-phosphate dehydrogenase promoter were: forward, 5′-tactagcggtttttacgggcg-3′; reverse, 5′-tcgaacaggaggagcagagagcga-3′.

TH Activity Assay—The TH activity in CHP-212 cells transfected with control or DJ-1-specific siRNA was determined by measuring L-DOPA formation using methods as described (25). In brief, 4 days after the siRNA transfection, CHP-212 cells plated in 6-well dishes were rinsed twice with 1 ml of the physiological medium and then incubated for 12 h at 37 °C with 100 mM L-tyrosine and 500 μM n-hydroxybenzylhydrozine (Sigma), a selective DOPA decarboxylase inhibitor. After the incubation, medium was discarded and the cells were collected, resuspended with 0.1 ml of 0.1 N perchloric acid, and sonicated. An aliquot (10 μl) was used to determine protein concentration, and the remainder was centrifuged at $15,000 \times g$ at 4 °C for 10 min. A 20-μl aliquot of the supernatant was injected onto an ODS Hypersil column (150 $\times$ 4.6 mm, 5-μm particle size, Keystone Scientific Inc., Bellefonte, PA). Mobile phase consisted of (in mM): 50 potassium phosphate, 0.1 EDTA, 0.2 heptane sulfonic acid, and 10% methanol, pH 2.6. L-DOPA peaks were detected with an Antec Leyden Intro amperometric high-performance liquid chromatography system (GBC Analytical Systems). Peaks from samples were compared against a daily 200-pictrogram L-DOPA external standard. Results were normalized per microgram of protein and analyzed through one-way analysis of variance.

RESULTS

DJ-1 and PSF Transcriptionally Regulate the Human TH Promoter—Since we have shown that DJ-1 is a transcriptional co-activator, we examined whether DJ-1 regulated the expression of genes involved in dopaminergic neurotransmission, such as tyrosine hydroxylase, the rate-limiting enzyme that converts tyrosine to the dopamine precursor L-DOPA. To mimic the loss-of-function effects as seen in PD patients with DJ-1 mutations, we used DJ-1-specific siRNA constructs to inhibit the synthesis of endogenous DJ-1 in two human dopaminergic neuroblastoma cell lines, CHP-212 and SH-SY5Y cells. The protein levels of TH and DJ-1 showed time-dependent decreases in CHP-212 cells transfected with DJ-1-specific siRNA (Fig. 1*A*). 4 days after DJ-1 siRNA transfection, the TH protein expression was reduced by 90% (Fig. 1*A*). Quantitative real-time PCR results indicated that DJ-1 inactivation by siRNA significantly decreased the TH mRNA levels in both CHP-212 and SH-SY5Y cells as determined by quantitative real-time PCR (Fig. 1*B*). In addition, the reduction in the TH expression following siRNA knockdown of DJ-1 decreased the TH activity by almost 40% in CHP-212 cells, as determined by the production of L-DOPA using high-performance liquid chromatography (Fig. 1*C*). Consistent with these observations, the TH mRNA expression was increased by >100% in SH-SY5Y cells stably expressing the human wild-type DJ-1 (Fig. 1*D*).

Previously, we have shown that DJ-1 interacts with and blocks the functions of a transcriptional repressor PSF in human dopaminergic cells (7). As in SH-SY5Y cells, PSF specifically interacted with DJ-1 in untransfected CHP-212 cells (data not shown). Therefore, we tested whether PSF repressed TH transcription. Transient expression of the wild-type PSF inhib-
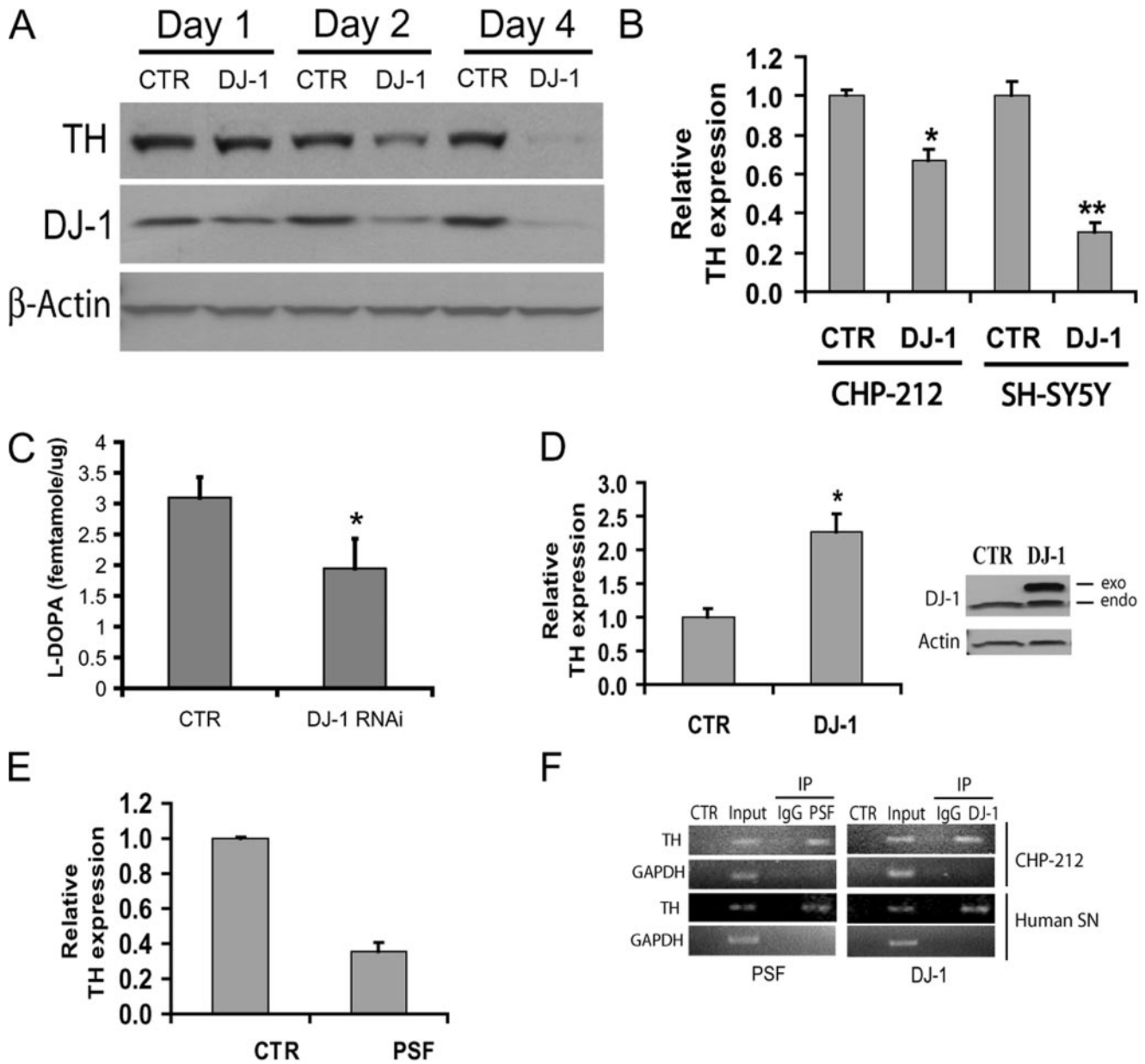
FIGURE 1. **DJ-1 and PSF transcriptionally regulate the human tyrosine hydroxylase.** *A*, Western blotting showing the expression of TH, DJ-1, and β-actin at various time points in CHP-212 cells transfected with nonspecific control or DJ-1 RNAi constructs. *B*, the relative TH mRNA levels determined by quantitative real-time PCR (Q-PCR) in CHP-212 and SH-SY5Y cells at 48 h after the transfection of control (*CTR*) or DJ-1 RNAi (*DJ-1*) constructs. DJ-1 inactivation (>60%) by DJ-1 RNAi was confirmed by Western blotting and Q-PCR (data not shown). The TH mRNA levels from each sample were normalized to β-actin mRNA. Values represent the mean ± S.E. *n* = 3 experiments (*, *p* < 0.01; **, *p* < 0.01) relative to the control by unpaired *t* test. *C*, L-DOPA production determined by the high-performance liquid chromatography analysis in CHP-212 cells transfected with control or DJ-1-specific siRNA. Values are the mean ± S.E.; femtamoles of L-DOPA/μg of protein lysate; *n* = 12 per condition. *, *p* < 0.01 relative to the control by one-way analysis of variance. *D*, the relative TH mRNA levels in SH-SY5Y cells stably expressing a vector control (*CTR*) or the human *myc*-his tagged wild-type DJ-1 (*DJ-1*). Values are the mean ± S.E. *n* = 3 experiments; *, *p* < 0.05 relative to the control by unpaired *t* test. The *side panels* are Western blots indicating the expression levels of DJ-1 and β-actin in stable cells. *E*, the relative TH mRNA levels in CHP-212 cells at 48 h after transient transfection of a vector control (*CTR*) or the human wild-type PSF. Values represent the mean ± S.E., *n* = 2. *F*, ChIP assays showing the binding of the endogenous PSF (*left*) and DJ-1 (*right*) to the human TH promoter in CHP-212 cells and in the human substantia nigra pars compacta (*SN*) tissue. *CTR*, no input DNA; *Input*, 0.5% of the total DNA before IP; *IgG*, species-matched pre-immune control antibodies for IP; *PSF* or *DJ-1*, antibodies specifically recognizing PSF or DJ-1. The results were confirmed using three different pairs of primers specifically amplifying the human TH promoter sequences. Primers specifically for the human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoter were used in negative control experiments.

ited the human TH mRNA expression in CHP-212 cells (Fig. 1*E*). To further confirm the transcriptional regulation of the human TH promoter by both DJ-1 and PSF, we performed ChIP assays to assess the physical interactions between these two transcriptional regulators and the TH promoter *in vitro* and *in vivo*. The DNA co-immunoprecipitated with either a

monoclonal anti-PSF or a polyclonal anti-DJ-1 antibody using the lysates from CHP-212 cells or human substantia nigra pars compacta tissues were amplified by primers specifically recognizing the human TH promoter but not by primers recognizing the human glyceraldehyde-3-phosphate dehydrogenase promoter (Fig. 1*F*). Taken together, our results strongly demon-
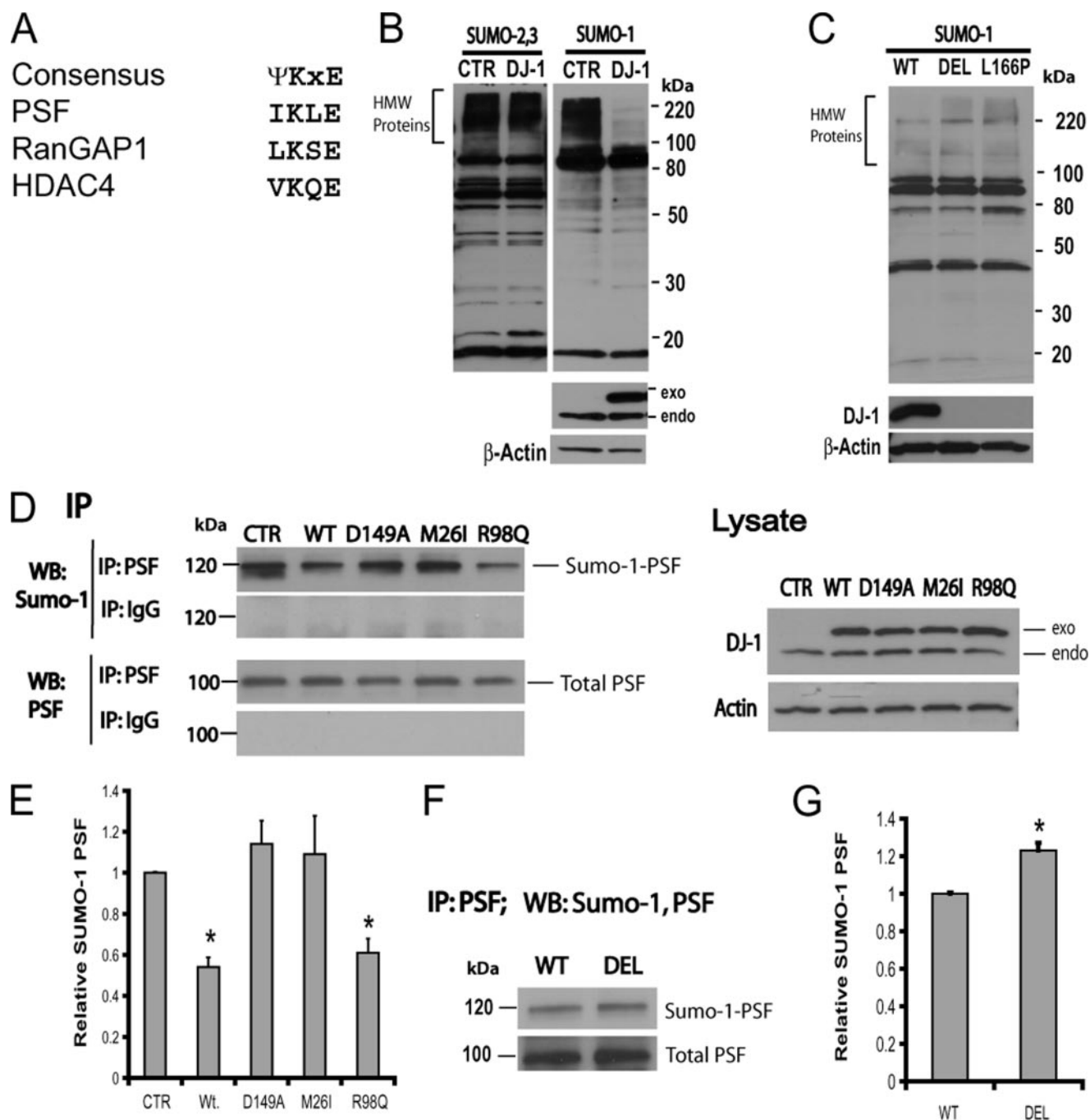
FIGURE 2. **Wild-type DJ-1 inhibits the sumoylation of PSF.** *A*, the alignment of the putative sumoylation site in PSF with the consensus site. Ψ is typically a hydrophobic residue, and *X* can be any amino acid. The PSF site is located between residues 337 and 340. The sumoylation sites from two well characterized proteins RanGAP1 and HDAC4 are listed for comparison (42). *B*, Western blots of proteins modified by endogenous SUMO-2 or 3 (*left*), or SUMO-1 (*right*), in stable SH-SY5Y cells expressing an empty vector (*CTR*) or wild-type DJ-1 (*DJ-1*). Identical samples were separated in duplicate gels and probed with a rabbit polyclonal anti-SUMO-2/3 or a mouse monoclonal anti-SUMO-1. Note the reduced abundance of high molecular weight SUMO-1-conjugated proteins in cells overexpressing DJ-1. The membranes were reprobed for β-actin and DJ-1 to confirm equal loading and the overexpression of DJ-1. *C*, Western blotting showing the amount of SUMO-1-conjugated proteins in the lymphoblast cells from a normal control individual (*WT*) and PD patients carrying the exon 1–5 deletion (*DEL*) or the L166P mutation in the *DJ-1* gene. The loss of DJ-1 expression in the PD patients was confirmed by reprobing the membrane for DJ-1. The abundance of high molecular mass proteins ($>$100 kDa) from each sample was quantified with the NIH Image J software and was normalized to that of the WT. (WT = 1; DEL = 1.47 ± 0.09; L166P = 3.07 ± 0.61; $p < 0.05$ by one-way analysis of variance, $n = 3$ experiments). *D*, Western blots of sumoylated and total PSF. Total endogenous PSF was immunoprecipitated using 1 mg of denaturing lysates from the SH-SY5Y cells stably expressing equivalent amount (confirmed in the *right panels*, *exo*: exogenous *myc*-His tagged DJ-1; *endo*: endogenous DJ-1) of indicated *myc*-his tagged DJ-1 or a vector control (*CTR*), and then immunoblotted with an anti-SUMO-1 (*top left panels*) or anti-PSF (*bottom left panels*). Mouse pre-immune IgG was used as control antibodies for IP. *E*, quantitative analysis of sumoylated PSF in SH-SY5Y cells stably expressing the wild-type or mutant DJ-1. The levels of SUMO-1-conjugated PSF were normalized to those of total PSF and are represented as ratios to the control (*CTR*). $n = 5$ experiments for CTR and WT. *, $p < 0.001$ relative to control by one-way analysis of variance with post-hoc test. $n = 3$ for samples from D149A, M26I, and R98Q. *F*, Western blots of sumoylated and total PSF in lymphoblast cells from a control (WT) and PD patient carrying the exons 1–5 deletion mutation of DJ-1 (*DEL*). PSF was immunoprecipitated from 300 μg of cell lysate. The L166P sample was not analyzed due to insufficient materials for immunoprecipitation assays. *G*, quantitative analysis of the relative SUMO-1-modified PSF in the lymphoblast cells from the patients. Values represent normalized mean $\pm$ S.E., *, $p < 0.01$ by unpaired *t* test; $n = 4$.

strate that DJ-1 activates the human TH expression and regulates dopamine synthesis.

DJ-1 Inhibits the Sumoylation of PSF and Its Repression of the TH Promoter—To elucidate the molecular mechanism of this transcriptional regulation, we assessed the possibility that DJ-1 might regulate the sumoylation of PSF. DJ-1 has been shown to interact with SUMO-1, SUMO-activating enzyme Uba2, and conjugating enzyme ubc-9 (8, 22) using the yeast two-hybrid system. In addition, DJ-1 interacts with SUMO ligases PIASxa and PIASy and potentially regulates their functions (17). Meanwhile, PSF harbors a potential sumoylation site (IKLE) that completely matches the consensus sumoylation motif ΨKXE, where the conserved lysine (K) and glutamic acid (E) are preceded by a hydrophobic amino acid (Ψ) and any amino acid (*X*), respectively (Fig. 2*A*). Recently, a proteomic study suggested that PSF is sumoylated, although the site of modification has not been mapped (26). We first examined the effect of DJ-1 on global sumoylation in SH-SY5Y cells stably expressing the human wild-type DJ-1. Overexpression of the wild-type DJ-1 resulted in an overall decrease in the amount of high molecular weight proteins modified by SUMO-1 but not by SUMO-2 or SUMO-3 (Fig. 2*B*). To examine whether DJ-1 similarly regulates sumoylation *in vivo*, we analyzed the lymphoblast cells harvested from normal or PD patients carrying pathogenic mutations in the *DJ-1* gene. Consistent with the results from Fig. 2*B*, DJ-1 inactivation caused by the homozygous deletion of exons 1 to 5 (DEL) or the L166P point mutation resulted in a slight but reproducible increase in the amount of SUMO-1-modified high molecular weight proteins (Fig. 2*C*, -fold change relative to WT, mean $\pm$ S.E., DEL: 1.47 ± 0.09; L166P: 3.07 ± 0.61; $p < 0.05$, $n = 3$ for both conditions).

Next, we examined whether PSF was sumoylated and whether DJ-1 affected this process. Immunoprecipitation of PSF and Western blotting of SUMO species and PSF using lysates from SH-SY5Y cells stably expressing various DJ-1 constructs indicated that PSF was modified by SUMO-1 (Fig. 2*D*) but not by SUMO-2 or SUMO-3 (data not shown). Further, the wild-type and the non-pathogenic R98Q DJ-1, but not the pathogenic DJ-1 mutants expressed at similar levels, specifically reduced the abundance of SUMO-1-conjugated PSF (Fig. 2, *D* and *E*). The inability of the pathogenic DJ-1 mutants to suppress PSF sumoylation suggests a functional link between the regulation of sumoylation and PD pathogenesis. To examine the effect of DJ-1 inactivation on the sumoylation of PSF *in vivo*, we evaluated the abundance of sumoylated PSF in the lymphoblast cells from a normal and a PD patient (DEL) using immunoprecipitation and Western blotting. As shown in Fig. 2 (*F* and *G*), the loss of DJ-1 resulted in minor but statistically significant accumulation of the SUMO-1-modified PSF. Therefore, our results indicate that DJ-1 suppresses the modification of PSF by SUMO-1, and DJ-1-inactivating mutations may lead to the accumulation of sumoylated proteins.

Mutations That Abolish the Sumoylation of PSF Relieve the Transcriptional Repression of the TH Promoter by PSF—To further evaluate the regulation of PSF sumoylation by DJ-1 and to study its functional consequence, we mutated the conserved lysine (K338A) or the preceding hydrophobic isoleucine (I337A). Western blotting of various immunoprecipitated PSF
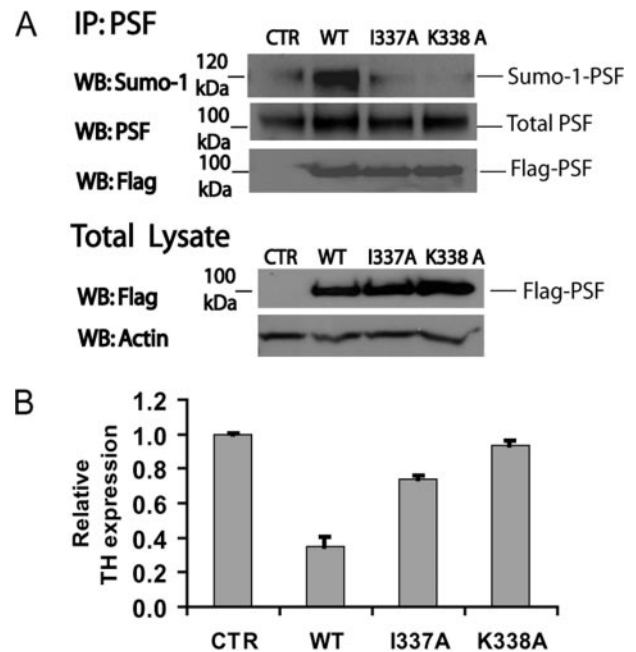

FIGURE 3. **Mutations at the putative PSF sumoylation site abolish its sumoylation and relieve PSF-mediated transcriptional repression of the human TH promoter.** *A*, the sumoylation status of the WT, I337A, or K338A PSF. SH-SY5Y cells grown in 10-cm culture plates were co-transfected with vector plasmid (*CTR*), WT or mutant FLAG-tagged PSF constructs (20 µg), SUMO-1 (10 µg), and the E3 ligase PIASy (10 µg). Total PSF was immunoprecipitated, and the sumoylation status of each PSF construct was determined by the SUMO-1 Western blotting. The amount of total (*top middle panel*) and FLAG-tagged (*top bottom panel*) PSF immunoprecipitated from each sample was examined in parallel to confirm that equal amounts of transfected PSF were precipitated. This approach was used because anti-FLAG antibodies failed to pull down FLAG-tagged PSF. In addition, the equivalent expression of transfected WT or mutant PSF was determined by direct Western blotting (*bottom panels*). *B*, the relative TH mRNA levels in CHP-212 cells at 48 h after transient transfection of a vector control (*CTR*) or indicated PSF constructs. Values represent the mean $\pm$ S.E., $n = 2$. The equivalent amount of the transfected WT and mutant PSF was confirmed by Q-PCR (data not shown).

constructs for SUMO-1 indicated that mutation at either site decreased the abundance of sumoylated PSF and confirmed that Lys-338 was the sumoylation site (Fig. 3*A*). In addition, sumoylation-deficient PSF mutants exhibited decreased colocalization with co-transfected HA-tagged SUMO-1 in SH-SY5Y cells (Supplemental Fig. S1). To evaluate whether sumoylation is required for PSF to repress TH expression, we transiently transfected the wild-type or sumoylation-deficient PSF constructs in CHP-212 cells and analyzed the TH mRNA expression using quantitative real-time PCR. Mutations that abolished the consensus sumoylation site relieved the repression of the human TH promoter by PSF (Fig. 3*B*). Taken together, these observations strongly indicate that DJ-1 prevents PSF-mediated repression of the TH promoter by inhibiting the sumoylation of PSF.

DJ-1 Prevents the Sumoylation-dependent Recruitment of HDAC1 by PSF—In most cases, sumoylation of transcriptional regulators leads to increased transcriptional repression (20, 21). One possible mechanism is the increased recruitment of HDAC complexes to the promoter (20, 21, 27). HDAC complexes remove acetyl groups from histones and transcriptional factors and repress transcription. In addition, PSF is known to repress nuclear hormone receptor-mediated transcription by
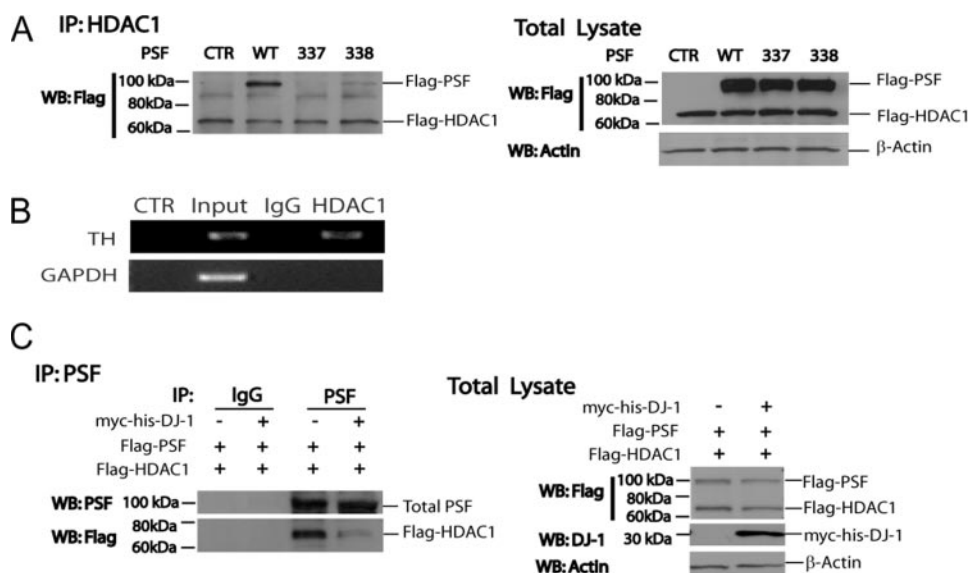
FIGURE 4. **DJ-1 prevents the sumoylation-dependent recruitment of HDAC1 by PSF.** *A*, mutations that abolish the sumoylation of PSF disrupt the recruitment of HDAC1. After lysis, total HDAC1 was immunoprecipitated and the amount of HDAC1-associated transfected PSF was determined by Western blotting using an anti-FLAG antibody (*left panel*). Note the equivalent amount of immunoprecipitated HDAC1 from each sample (*left panel*). Note the equivalent amount of immunoprecipitated HDAC1 from each sample (*top panel*). Equivalent expression of transfected PSF and HDAC1 was confirmed by Western blot (anti-FLAG) of the total lysates (*right panel*). *WT*, wild-type PSF; *337*, I337A PSF; *338*, I338A PSF. *B*, ChIP assays showing that HDAC1 was recruited to the human TH promoter. HEK293 cells were transfected with PSF and HDAC1 as in *A*, total HDAC1 was immunoprecipitated, and the immunocomplex was processed for ChIP assays using primers specifically for the human TH and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) promoters. *C*, DJ-1 disrupts the binding between the WT PSF and HDAC1. Non-denaturing cell lysates from the HeLa cells co-transfected with a vector or wild-type DJ-1 (20 μg), FLAG-tagged WT PSF (10 μg), and FLAG-tagged HDAC1(10 μg) were used to immunoprecipitate PSF and PSF-associated HDAC1 was determined by immunoblotting with an anti-PSF and anti-FLAG antibody, respectively (*left panels*). The expression of transfected plasmids was confirmed by Western blots (anti-FLAG-tag and anti-DJ-1) of the total lysates (*right panels*). Similar results were observed in HEK293 cells with identical experimental conditions. HeLa and HEK293 cells were used in co-immunoprecipitation experiments due to the high transfection efficiency in these cells.

FIGURE 5. **DJ-1 inactivation leads to decreased acetylation of the human TH promoter-bound histones.** *A*, ChIP assays of acetylated histones bound to the human TH promoter. Various acetylated histone species from CHP-212 cells transfected with control or DJ-1 RNAi for 4 days were immunoprecipitated with specific antibodies and amplified with primers specifically for the human TH promoter using semiquantitative PCR. Reactions were stopped at indicated PCR cycles for gel analysis. *Input*: 0.5% of input DNA before IP. The inhibition of endogenous TH and DJ-1 by the DJ-1-specific siRNA for the ChIP assays was confirmed by Western blots (data not shown). *B*, the restoration of TH expression by the HDAC inhibitor sodium butyrate (*NaButy*) in cells transfected with DJ-1 RNAi. 8 h after the transfection of control (*CTR*) or DJ-1 RNAi constructs, CHP-212 cells were treated with increasing amounts of sodium butyrate for an additional 88 h before harvesting, with two changes of fresh medium containing sodium butyrate during the course of the experiment. The optimal dosage of sodium butyrate was determined empirically to achieve minimal cellular toxicity and was comparable to the tolerable dosages tested *in vivo* (43). The increased histone acetylation caused by sodium butyrate was confirmed by Western blotting (data not shown). The protein levels of TH, DJ-1, and β-actin were determined by Western blotting.

lation-deficient PSF constructs and then co-immunoprecipitated PSF with an anti-HDAC1 antibody. Mutations that abolish the sumoylation of PSF completely disrupted the interactions between PSF and HDAC1 (Fig. 4*A*). This result agrees entirely with our observation showing that sumoylation-deficient PSF mutants failed to repress the expression of TH (Fig. 3*B*). To confirm the recruitment of HDAC1 to the human TH promoter, we co-expressed HDAC1 and the wild-type PSF in HEK293 cells, and performed ChIP assays using an anti-HDAC1 antibody. Primers recognizing the human TH promoter specifically amplified the DNA from the HDAC1 immunocomplex (Fig. 4*B*).

Because DJ-1 inhibited PSF sumoylation, we assessed whether DJ-1 overexpression might prevent the recruitment of HDAC1 by PSF. FLAG-tagged wt PSF and HDAC1 were co-transfected with an empty vector or the wild-type DJ-1 in HeLa or HEK293 cells, and HDAC1 was co-immunoprecipitated with an anti-PSF antibody. Overexpression of DJ-1 disrupted the binding between PSF and HDAC1 in both HeLa (Fig. 4*C*) and HEK293 cells (data not shown). Taken together, our data suggest that by suppressing the sumoylation of PSF, DJ-1 prevents the recruitment of the HDAC1 repressor complex to the human TH promoter and maintains an active transcription of TH.

HDAC Inhibitors Reverse the Inhibition of the TH Promoter by DJ-1 Inactivation—To validate the role of histone acetylation in DJ-1-mediated regulation of the human TH promoter, we transfected CHP-212 cells with control or DJ-1-specific siRNA, performed ChIP assays with antibodies specifically recognizing acetylated histones, and amplified the human TH promoter sequences using semi-quantitative PCR. Consistent with the concurrent inhibition of the TH protein expression (data not shown), DJ-1 inactivation caused a decrease in
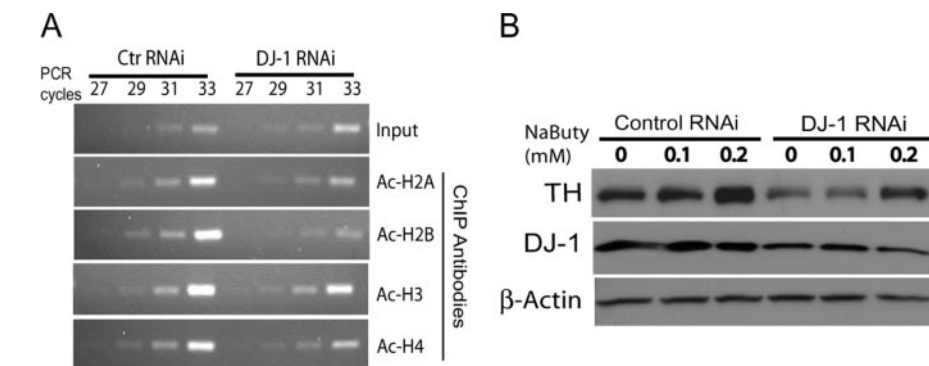
recruiting Sin3A and the HDAC1 (28). To examine the role of PSF sumoylation in the recruitment of HDAC1, we co-transfected HEK293 cells with HDAC1 and the wild-type or sumoy-

the amount of TH promoter-associated acetylated histones, indicating transcriptional silencing (Fig. 5*A*). Because DJ-1 acted as a transcriptional co-activator eventually promoting histone acetyla-

tion, we reasoned that chemical inhibitors of HDAC would reverse the effects of DJ-1 inactivation. Therefore, we pre-transfected CHP-212 cells with control or DJ-1-specific siRNA and treated cells with increasing amount of the HDAC inhibitor sodium butyrate. Sodium butyrate almost completely restored the TH expression (to 88% of the levels in the control at 0.2 mM) even in the presence of DJ-1 siRNA (Fig. 5*B*). In separate experiments, another HDAC inhibitor trichostatin A similarly reversed the TH inhibition caused by DJ-1 inactivation (data not shown).

DISCUSSION

These experiments describe a novel transcriptional regulation of the human TH promoter by DJ-1. Consistent with its role as a transcriptional co-activator (7), DJ-1 stimulates the acetylation of TH-promoter-bound histones by blocking the sumoylation of a transcriptional co-repressor PSF and the subsequent recruitment of HDAC1. Inactivation of DJ-1 by siRNA results in the loss of TH expression and L-DOPA production. Therefore, our study provides a molecular mechanism whereby the loss of DJ-1 leads to impaired nigral-striatal functions, thus predisposing patients to PD.

Despite multiple important functions attributed to DJ-1 (3–5, 7–9), and the fact that the loss of DJ-1 functions in patients causes early-onset PD (2, 29), surprisingly, DJ-1-deficient mice appear normal without histological abnormalities, although exhibiting very minor motor deficits (12–14). In contrast to our results showing the regulation of the human TH promoter by DJ-1, mice lacking DJ-1 do not exhibit reduced TH expression or decreased dopamine production. Although genetically engineered mice are invaluable tools for understanding neurodegenerative diseases, they often do not reproduce all the symptoms and pathological hallmarks of the human diseases, likely due to the sum of multiple factors, including compensatory response, short life span, and difference in biology. For instance, *parkin* is the most frequently mutated gene in autosomal recessive juvenile parkinsonism (29), but *parkin*-deficient mice do not exhibit clear differences from control littermates in behavioral and histological analyses (30). A better animal model of DJ-1 deficiency is needed to fully understand the function of DJ-1.

Several groups have established *Drosophila* models of DJ-1 deficiency (10, 11, 31, 32). However, different strategies to inactivate DJ-1 have led to distinct phenotypes (33). Interestingly, only the study using siRNA to inactivate *Drosophila* DJ-1 demonstrates the age-dependent decrease in the number of TH+ neurons and total brain dopamine content that resembles the neuropathology in PD patients (10). Although DJ-1 siRNA-induced apoptosis certainly contributes to this observation, it will be of interest to determine whether DJ-1 inactivation leads to transcriptional down-regulation of the TH expression in *Drosophila* as well. It is conceivable that the loss of DJ-1 function not only promotes apoptosis in dopaminergic neurons but also inhibits normal dopamine synthesis. Due to the lack of neuropathological evidence from the patients carrying DJ-1 mutations, it is difficult to determine presently whether DJ-1 inactivation similarly causes a gradual decrease in the TH expression in the human brain, and whether the reduced TH expression and activity precede dopaminergic neuronal loss. Our data

indicate that the regulation of the human TH promoter in neuroblastoma cell lines by DJ-1 is mediated by its role as a suppressor of sumoylation. Consistent with this mechanism, in lymphoblasts harvested from the PD patients carrying homozygous DJ-1 mutations, the loss of DJ-1 contributes to the accumulation of SUMO-1-conjugated proteins, including PSF. Although overexpression of DJ-1 in neuroblastoma cell lines results in a much more robust change in the abundance of proteins modified by SUMO-1, the subtle increase in the sumoylated proteins *in vivo* may reflect aging-dependent gene silencing (24) and the chronic progression of neurodegenerative diseases.

Our experiments demonstrate that DJ-1 is a cellular inhibitor of sumoylation. A previous study has suggested DJ-1 may alter the sumoylation of the tumor suppressor p53 (34). Nevertheless, the exact mechanism by which DJ-1 modulates SUMO-1 modification is not clear. DJ-1 interacts with key components of the sumoylation machinery (8, 22) and may block their access to SUMO substrates, such as PSF. Alternatively, DJ-1 may serve as a substrate to compete for sumoylation (22). However, DJ-1 does not harbor a consensus SUMO acceptor site and we were unable to convincingly detect endogenous sumoylated DJ-1 (data not shown), even though DJ-1 significantly reduced the abundance of SUMO-1-modified proteins (Fig. 2*B*). In addition, the presence of cysteine protease activity of DJ-1 (35) raises an intriguing possibility that DJ-1 could be a SUMO protease. The evolutionarily conserved catalytic cysteine residue (Cys-106) is highly sensitive to oxidation (36), and the mutation at this site abolishes its neuroprotective activity (9, 37). Although DJ-1 is not highly homologous to other known SUMO proteases, it will be of interest to clarify this possibility.

Our findings also support the theory that sumoylation and acetylation are coupled molecular switches controlling gene expression (20, 27, 38). The suppression of PSF sumoylation by DJ-1 prevents the recruitment of HDAC1 and leads to increased acetylation of the TH promoter-bound histones. Surprisingly, our co-immunoprecipitation experiments examining the interactions between HDAC1 and PSF suggested that sumoylation of PSF may facilitate, rather than directly mediate, the binding to HDAC1, because it was the 100-kDa PSF corresponding to unmodified PSF that was associated with HDAC1 (Fig. 4). Sumoylation is a dynamic process; at any given time, <1% of substrate proteins are sumoylated (18). We have found that <5% of total PSF are sumoylated in human neuroblastoma cell lines (Supplemental Fig. S2), and these species may be beyond the detection limit in our co-immunoprecipitation assays. More importantly, our results show that the mutations abolishing PSF sumoylation also prevent the association between PSF and HDAC1. These observations are consistent with the model (18, 39) suggesting that transient sumoylation may cause conformational change of the substrate and lead to increased interaction with transcriptional repressors, such as HDAC1. It is possible that, once PSF and HDAC1 form a stable protein complex, the SUMO molecules are cleaved from PSF and recycled. This idea agrees with the observations that a number of transcriptional regulators, like PSF, exhibit strong sumoylation-dependent transcriptional repression activity, yet only a very small fraction of these proteins are sumoylated (18). With the identification of more SUMO substrates, we will gain

additional insight into the mechanism and functional consequences of sumoylation.

In addition to regulating TH expression, DJ-1 likely affects multiple cellular pathways, such as oxidative stress and apoptosis (4, 5, 8, 40), but it is unclear whether these activities require the nuclear functions of DJ-1. However, the ability of DJ-1 to modulate gene expression as a transcriptional co-activator agrees with the observations indicating that DJ-1 affects multiple cellular activities. For instance, a recent study confirms our previous finding showing that transcriptional regulation by DJ-1 may be involved in its protection against apoptosis mediated by oxidative stress and α-synuclein (7, 41). In that report, Zhou and Freed (41) demonstrate that DJ-1 protects against dopaminergic neuronal apoptosis at least partially by inducing the expression of glutamate cysteine ligase and HSP70. Another study has indicated that DJ-1 interacts with Daxx and prevents its translocation to the cytoplasm and the initiation of apoptotic signaling (8). Interestingly, like PSF, Daxx is a known transcriptional repressor modified by SUMO-1 (39, 42). Whether the regulation of Daxx and the inhibition of apoptosis by DJ-1 require blocking the sumoylation of Daxx deserves further investigation. Therefore, mutations that inactivate DJ-1 may result in concurrent impairment in multiple cellular pathways linked to PD pathogenesis. If transcriptional regulation by DJ-1 is involved in several of these pathways, we may be able to develop potential therapeutic approaches for PD by targeting sumoylation and acetylation to mimic the functions of DJ-1.

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