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**Oxidized DJ-1 inhibits p53 by sequestering p53 from promoters in a
DNA-binding affinity-dependent manner**

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Running title: Oxidative Stress-dependent Repression of p53 by DJ-1

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ABSTRACT

DJ-1 is an oncogene and causative gene for familial Parkinson's disease. Although the oxidative status of DJ-1 at cysteine at 106 (C106) is thought to affect all of the activities of DJ-1 and excess oxidation leads to the onset of various diseases, the precise molecular mechanisms underlying the effects of oxidation of DJ-1 on protein-protein interaction of DJ-1 remain unclear. In this study, we found that DJ-1 bound to the DNA-binding region of p53 in an oxidation of C106-dependent manner. Of the p53-target genes, the expression level and promoter activity of the DUSP1 gene, but not those of the p21 gene, were increased in H₂O₂-treated DJ-1 (-/-) cells and were decreased in wild-type DJ-1- but not C106S DJ-1-transfected H1299 cells through sequestration of p53 from the DUSP1 promoter by DJ-1. DUSP1 down-regulated by oxidized DJ-1 activated ERK and decreased apoptosis. DUSP1 and p21 promoters harbor non-consensus and consensus p53-recognition sequences, respectively, which have low affinity and high affinity to p53. However, DJ-1 inhibited p21 promoter activity brought by p53 mutants harboring low DNA-binding affinity but not by wild-type p53. These results indicate that DJ-1 inhibits the expression of p53-target genes in p53 DNA-binding affinity- and oxidation of DJ-1 C106-dependent manners.

INTRODUCTION

DJ-1 was identified by us as a novel oncogene that induces anchorage-independent growth of fibroblasts cooperatively with activated *ras* (31) and was later found to be a causative gene for a familial form of Parkinson's disease, *Park7* (2). DJ-1 has 3 cysteines located at amino acid numbers 46, 53 and 106 (C46, C53 and C106, respectively). Of the 3 cysteines, C106 is first oxidized as SOH, SO₂H and SO₃H forms, and excessive oxidation then causes oxidation of C46 and C53 (21, 45). The C106S mutant of DJ-1, which is a substitution mutant of DJ-1 at amino acid number 106 from cysteine to serine, possesses no or little protective activity against neuronal cell death induced by oxidative stress (5, 19, 27, 44, 45), and abnormally oxidized forms of DJ-1 were observed in patients

with sporadic forms of Parkinsons disease (1). From these points, C106 is the most important cysteine to maintain DJ-1's function. Although oxidative status of DJ-1 affects DJ-1's activity toward cells and disease, the precise molecular mechanisms remain unclear.

DJ-1 binds to various factors, including transcriptional factors such as androgen receptor (32, 46), p53 (11, 41), polypyrimidine tract-binding protein-associated splicing factor (PSF) (51) and Keap1, an inhibitor for nuclear factor erythroid-2 related factor2 (Nrf2) (8). However, it is not known how DJ-1 chooses its suitable binding protein(s) during the course of oxidative stress.

p53 is a tumor suppressor protein that activates transcriptional programs under various types of cellular stress, including oxidative stress. It is, however, not clear how p53 determines a point leading to cell cycle arrest and to apoptosis. Recent reports suggest that activation of specific promoters by p53 is achieved through its interaction with heterologous transcription factors such as Hzf, human cellular apoptosis susceptibility (hCAS)/CSE1L and ankyrin-repeat, SH3-domain and proline-rich-region containing protein (ASPP) family proteins (9, 36, 47). DJ-1 directly binds to p53 to restore p53 transcriptional activity by inhibiting sumoylation of p53 through interaction of DJ-1 with Topors/p53BP3, a SUMO-1 ligase for p53 (50). Sumoylation of DJ-1 itself is necessary for DJ-1 to localize from the cytoplasm to nucleus (40) and DJ-1 is a negative regulator for sumoylation (10). Moreover, DJ-1 decreases Bax expression through repressing p53 transcriptional activity by an unknown mechanism (11). Although DJ-1 regulates p53 transcriptional activity through interaction with a SUMO-1 ligase of p53 and regulates its location, it is still unclear whether oxidative status of DJ-1 affects p53 activity. We hypothesized that oxidative status of DJ-1 contributes to its binding activity to various proteins to regulate their functions.

In this study, we found that DJ-1 bound to the DNA-binding region of p53 in an oxidative status of DJ-1-dependent manner and that the oxidation of C106 was essential for DJ-1 binding to p53, resulting in altering DNA-binding affinity of p53. Furthermore, DJ-1 repressed transcriptional activity of p53 in a p53 DNA-binding affinity-dependent manner.

69

70 MATERIALS AND METHODS

71 **Cell culture and mice.** HEK293T, A549, H1299 and mouse embryonic fibroblast cells were cultured
72 at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum.
73 DJ-1-heterozygous knockout mice were kindly provided by J. Shen (13), and DJ-1-homozygous
74 knockout mice (DJ-1(-/-)) and wild-type mice with the same background (DJ-1(+/+)) were obtained.
75 Newborn mice with genotypes of DJ-1(-/-) and DJ-1 (+/+) at 1 day after birth were cut with scissors,
76 digested with trypsin, and seeded on a 10-cm dish in DMEM with 10% calf serum. These cells were
77 used as mouse DJ-1 (-/-) and DJ-1 (+/+) cells. All animal experiments were carried out in accordance
78 with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the
79 protocols were approved by the Committee for Animal Research at Hokkaido University (the permit
80 number 08-0468).

81

82 **RT-PCR and real-time PCR.** Nucleotide sequences of primers used for RT-PCR were as follows:

83 β -actin sense: 5'-TCCTCCCTGGAGAAGAGCTA-3', β -actin as:
84 5'-CCAGACAGCACTGTGTTGGC-3', mouse p21 sense: 5'-CCGTGGACAGTGAGCAGTTG-3',
85 mouse p21 as2: 5'-GAAGACCAATCTGCGCTTGG-3', mouse NOXA sense:
86 5'-GAACGCGCCAGTGAACCCAA-3', mouse NOXA as:5'-CTTTGTCTCCAATCCTCCGG-3',
87 mouse PUMA sense: 5'-TCCTCAGCCCTCCCTGTCAC-3', mouse PUMA as:
88 5'-CCATTTCTGGGGCTCCAGGA-3', mouse DUSP1 sense2:
89 5'-CAGCTCCTGGTTCAACGAGG-3', and mouse DUSP1 as:
90 5'-GCAGCTTGGAGAGGTGGTGAT-3'. Nucleotide sequences of primers used for real-time PCR
91 were as follows: mACTB 192-211F: 5'-CCTAGGCACCAGGGTGTGAT-3', mACTB 734-753R:
92 5'-GCTCGAAGTCTAGAGCAACA-3', mACTB F-real-time:
93 5'-CCCTAAGGCCAACCGTGAAA-3', mACTB R-real-time:

94	5'-ACGACCAGAGGCATACAGGGA-3',	mDUSP1	F-real-time:
95	5'-CCTGGTTCAACGAGGCTATTG-3',	mDUSP1	R-real-time:
96	5'-CCAGCTTTACCCGGTTAGTCC-3',	human DUSP1	sense3:
97	5'-GTATCACGCTTCCCGCAAGG-3',	human DUSP1	as5:
98	5'-CAAACACCCTTCCTCCAGCATTC-3',	human Actin	sense3:
99	5'-CGGCTGAGGAGTGGCTGG-3', and human Actin as4: 5'-CCAGCCGAGACACGGCAT-3'.		

100 After mouse primary or H1299 cells had been treated with 300 μ M H₂O₂ for 0.25-4 hrs or 0.5 hrs,
101 total RNAs were prepared and subjected to semi-quantitative RT-PCR and quantitative RT-PCR
102 (real-time PCR) analyses. After reactions, PCR products were extracted, separated on 2% agarose
103 gels, and stained with ethidium bromide. PCR conditions for RT-PCR were as follows: 1 min at
104 94°C, 30 sec at 94°C, 30 sec at 58°C and 22 cycles of 1 min at 72°C for β -actin; 1 min at 94°C, 30
105 sec at 94°C, 30 sec at 58°C and 25 cycles of 1 min at 72°C for p21; 1 min at 94°C, 30 sec at 94°C, 30
106 sec at 58°C and 28 cycles of 1 min at 72°C for NOXA; 1 min at 94°C, 30 sec at 94°C, 30 sec at 58°C
107 and 34 cycles of 1 min at 72°C for PUMA; and 1 min at 94°C, 30 sec at 94°C, 30 sec at 58°C and 28
108 cycles of 1 min at 72°C for DUSP1. PCR conditions for real-time PCR were as follows: 10 sec at
109 95°C, 5 sec at 95°C and 44 cycles of 20 sec at 60°C for β -actin; and 10 sec at 95°C, 5 sec at 95°C
110 and 44 cycles of 20 sec at 60°C for DUSP1.

111

112 **Luciferase assay.** pGL4.10-hDUSP1 and pGL3-hp21 were digested with KpnI and HindIII and each
113 resultant fragment was inserted into KpnI and HindIII sites of pGL4.12[luc2CP] (Promega, Madison,
114 WI). H1299 cells in 6-well dishes were transfected with pGL4.12-hDUSP1 or pGL4.12-hp21
115 together with pcDNA3-FLAG-p53 and pEF-DJ-1-HA or pEF-DJ-1 C106S-HA by Lipofectamine
116 2000 (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. pActin- β -galactosidase
117 was also cotransfected with plasmids. At 24 hrs after transfection, cells were treated with or without
118 300 μ M H₂O₂ or with 10 μ g/mL cycloheximide for 30 min. Whole cell extracts were then prepared

by addition of a Triton X-100-containing solution from the Pica gene kit (Wako Pure Chemicals, Osaka, Japan) to cells. About a one-fifth volume of the extract was used for the β -galactosidase assay to normalize transfection efficiencies as described previously (14), and the luciferase activity due to the reporter plasmid was determined using a luminometer (Luminocounter Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany). Proteins in aliquots of the cell extract were analyzed by Western blotting with an anti-FLAG antibody (M2, Sigma, St. Louis, MO, USA) and anti-HA antibody (1:2000, MBL, Nagoya Japan) and visualized as described in the “Western blotting and antibody” section. The same experiments were repeated at least three times.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays using cultured A549 cells were performed according to the protocol of the ChIP assay kit (Millipore, Billerica, MA). Briefly, after proteins had been cross-linked with DNA, cell pellets were resuspended in an SDS-lysis buffer and sonicated on ice using a sonicator (UR-20P, Tomy, Tokyo, Japan) 4 times for 15 sec each time. Genomic DNA was sheared to 300 to 1200 base pairs in length. Chromatin solution was preincubated with salmon sperm DNA and protein A-agarose and incubated with species-matched IgG or with specific antibodies overnight at 4°C. DNA fragments immunoprecipitated were then used as templates for PCR with Ex taq (TaKaRa Bio, Kyoto, Japan) and reacted for 60 sec at 94°C, 60 sec at 94°C, 30 sec at 58°C and 35 cycles of 30 sec at 72°C. Nucleotide sequences of oligonucleotides used for PCR primers were as follows: hDUSP1 sense: 5'-AAGAGCAGGCCGGACAGC-3', hDUSP1 as: 5'-GAGCGCGTTTATATGCGGC-3', hDUSP1 sense 3: 5'-CCCAATCCCTCTCCCACTAG-3', hDUSP1 as3: 5'-CAGAGCCGCTAAAATGGGCA-3', hp21 sense: 5'-TGCTGCTCCACCGCACTC-3', hp21 as: 5'-GAAAACAGGCAGCCCAAGGAC-3', hp21 sense3: 5'-CTATCAGCTGCCTCGGGG-3' and hp21 as3: 5'-GGCGCCCCAAGTTCCTAAC. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Reverse images of black and white staining are shown.

144

145 **Western blotting and antibodies.** Proteins were extracted from cells with a buffer containing 150
146 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5) and 0.5% NP-40, loaded on 12% sodium dodecyl
147 sulfate (SDS)-polyacrylamide gels, and subjected to Western blotting. Antibodies used in this study
148 were as follows: anti-HA (1:2000, MBL, Nagoya Japan), anti-FLAG F7425 (1:1000, Sigma), anti-T7
149 (1:1000, Novagen, Madison, WI), anti-p53 (1:1000, DO-1, Santa Cruz Biotechnology, California,
150 CA), anti-phospho p53(serine6) (1:1000, Cell Signaling, Danvers, MA), anti-phospho p53(serine9)
151 (1:1000, Cell Signaling), anti-phospho p53(serine15) (1:1000, Cell Signaling), anti-phospho
152 p53(serine20) (1:1000, Cell Signaling), anti-phospho p53(serine37) (1:1000, Cell Signaling),
153 anti-phospho p53(serine46) (1:1000, Cell Signaling), anti-phospho p53(serine392) (1:1000, Cell
154 Signaling), anti-actin (1:4000, Chemicon, Temecula, CA), anti-phospho ERK1/2 (1:1000, Cell
155 Signaling), anti-ERK1/2 (1:1000, Santa Cruz Biotechnology, California, CA), anti-p53 (1:1000,
156 Pab240, Santa Cruz Biotechnology), anti-MKP1(1:500, C-19 , Santa Cruz Biotechnology), rat anti-
157 DJ-1 monoclonal (1:1000), anti-DJ-1 polyclonal (1:4000), mouse anti-DJ-1 monoclonal (1: 4000,
158 3E8, MBL) and anti-oxidized DJ-1 (1:1000) antibodies. Rabbit anti-DJ-1, rat anti-DJ-1 and
159 anti-oxidized DJ-1 antibodies were established by us as described previously (31, 35). After
160 membranes had been reacted with primary antibodies, they were reacted with Alexa Fluor
161 680-conjugated anti-mouse (Molecular Probes, Eugene, OR), Alexa Fluor 680-conjugated anti-rabbit
162 (Molecular Probes), IRDye 800-conjugated anti-mouse antibody (Rockland, Philadelphia, PA) or
163 IRDye 800-conjugated anti-rabbit antibody (Rockland) and visualized by using an infrared imaging
164 system (Odyssey, LI-COR, Lincoln, NE).

165

166 **Pull-down assay.** ³⁵S-labeled p53 was synthesized in vitro using reticulocyte lysate of the TNT
167 transcription-translation coupled system (Promega). Labeled proteins were reacted with GST or
168 GST-wild-type DJ-1 or GST-C106S DJ-1 expressed in and prepared from *Escherichia coli* in a

169 G-buffer containing 150 mM NaCl, 5 mM EDTA and 50 mM Tris (pH 7.5), 0.05% bovine serum
170 albumin and 0.1% Nonidet P-40 for 2 hrs at 4°C, mixed with glutathione sepharose beads, and
171 centrifuged. After washing pellets with the wash buffer containing 150 mM NaCl, 10 mM Tris-HCl
172 (pH 7.5), 0.1% Nonidet P-40, Laemmli buffer was added to pellets. Pellets were then heated at 97°C
173 for 5 min, separated on a 12% polyacrylamide gel containing SDS, and visualized by CBB staining
174 and by fluorography.

175 HEK293T or H1299 cells were transfected with expression vectors for wild-type DJ-1-HA or
176 C106S DJ-1-HA and One-STrEP-p53 and treated with H₂O₂. Proteins in cell extracts were then
177 subjected to pull-down assays with a Strep-Tactin sepharose beads according to the supplier's
178 protocol (IBA, Göttingen, Germany), and co-precipitated DJ-1 was detected by Western blotting
179 with anti-HA and anti-oxidized-DJ-1 antibodies.

180

181 **Co-immunoprecipitation assay.** Proteins were extracted from cultured cells with or without 300
182 μM H₂O₂ for 30 min by the procedure described previously (16). Proteins were immunoprecipitated
183 with a rabbit anti-DJ-1 antibody (1:500, MBL) or normal rabbit IgG, and precipitates were analyzed
184 by Western blotting with anti-p53 (1:1000, Santa Cruz Biotechnology) or mouse anti-DJ-1 antibody
185 (1:1000, 3E8, MBL). Proteins on membranes were visualized as described above.

186 HEK293T cells were transiently transfected with expression vectors for FLAG-p53 and DJ-1-HA
187 by the calcium phosphate method and were lysed by treatment of cells with or without 1 mM H₂O₂
188 for 30 min. Proteins were then immunoprecipitated by an anti-FLAG antibody and precipitates were
189 detected by anti-HA antibody or anti-DJ-1 antibody.

190

191 **Isoelectric focusing.** Cells were treated with 1 mM H₂O₂ for 30 min, and cell extracts were prepared
192 by the procedure described previously (16). Proteins in the extracts were then separated on pH 5–8
193 ranges of an isoelectric focusing gel or on 12.5% polyacrylamide gel containing SDS, transferred

194 onto nitrocellulose membranes, and reacted with an anti-HA antibody.

195

196 **ELISA assay.** GST or GST-p53 purified from E coli was loaded on ELISA plates (BD) at 4°C
197 overnight. After plates had been blocked with 0.25 x block A and washed with 0.1% Tween 20-PBS,
198 wild-type or C106S DJ-1 was added to plates and incubated for 1 hr at 4°C. Plates were then reacted
199 with an anti-rat DJ-1 antibody (1:1000) for 1 hr at 37°C and reacted with an ABTS solution, and
200 absorbance of each well was measured using a plate reader (BIO-RAD).

201

202 **Immunofluorescence.** A549 cells were treated with or without 1 mM H₂O₂ for 30 min. Cells were
203 then fixed with acetone/methanol and reacted with an anti-p53 or anti-DJ-1 antibody, and
204 immunofluorescence images of proteins were detected by a fluorescein isothiocyanate- or
205 rhodamine-conjugated secondary antibody, respectively. Nuclei were stained with DAPI.

206

207 **Cell cycle FACS analysis.** Mouse DJ-1 (+/+) and DJ-1 (-/-) cells were transfected with 50 nM
208 AllStars negative control siRNA (QIAGEN), DUSP1 siRNA-1 (Scramble siRNA cocktail that
209 contains 4 sets of siRNA, Thermo scientific, 040753-01-0005, San Jose, CA) and DUSP1 siRNA-2
210 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Nucleotide sequences of DUSP1 siRNA-2 are
211 followed: DUSP1-2 sense: 5'-GGAUGCAGCUCCUGUAGUATT-3' and DUSP1-2 antisense:
212 5'-UACUACAGGAGCUGCAUCCTT-3'. At 48 hrs after transfection, the cells were starved for 6
213 hrs and then treated with or without H₂O₂. Cells were harvested at 40 hrs after the second H₂O₂
214 treatment, washed once with PBS, and fixed with 90% ethanol overnight. The cells were then treated
215 with 1 mg/ml RNase for 30 min at 37°C, washed once with PBS, and suspended in 300 µl of PBS
216 containing 50 µg/ml of propidium iodide (Sigma-Aldrich). After leaving for 1 hour at room
217 temperature, the cells were subjected to FACS analysis using a calibur flow cytometer
218 (Becton-Dickinson), and data obtained were analyzed using CellQuest software and ModFit

software.

Statistical analyses. Data are expressed as means $\pm$ S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test. For comparison of multiple samples, the Tukey-Kramer test was used.

RESULTS

Oxidative stress enhances DJ-1-binding to p53.

To address the effects of DJ-1 oxidation on interaction of DJ-1 with its target proteins, including p53, FLAG-tagged proteins were co-transfected with DJ-1-HA into HEK293T cells, and the cells were treated with 1 mM H₂O₂ for 30 min. Co-immunoprecipitation followed by Western blotting analyses showed that p53 and Daxx were strongly bound to DJ-1 in H₂O₂-treated cells (Fig. 1 and data not shown). We therefore focused on interaction of DJ-1 with p53 under the condition of oxidative stress in this study.

The same results as those shown in Fig. 1A were obtained when FLAG-DJ-1 was cotransfected with T7-p53 into HEK293T cells (Fig. 1B). In this case, both phosphorylated and unphosphorylated T7-p53 was bound to DJ-1. Proteins from H₂O₂-treated A549 cells and H1299 cells in which p53 is not expressed were immunoprecipitated with an anti-DJ-1 antibody and precipitates were analyzed by Western blotting with an anti-p53 antibody. The results showed that endogenous p53 bound to endogenous DJ-1 under normal culture conditions and that the amount of precipitated p53 was enhanced after treatment of A549 cells, but not H1299 cells, with H₂O₂ (Figs. 1C and D, respectively), suggesting that oxidative status of DJ-1 modulates p53-binding activity of DJ-1.

DJ-1 is localized in the cytoplasm, nucleus and mitochondria in cells and its localization is changed by oxidative stress (24). Oxidative stress might therefore alter localization of p53 and interaction of p53 with DJ-1. When A549 cells were treated with or without 300 μ M H₂O₂ for 30 min

and localization of proteins were analyzed by an immunofluorescence technique, DJ-1 and p53 were located both in the cytoplasm and nucleus and in the nucleus, respectively, and both proteins were co-localized in the nucleus in cells before and after H₂O₂ treatment (Figs.1E and 1F), indicating that oxidative stress enhances binding activity of DJ-1 to p53 without affecting localization of DJ-1.

Cysteine 106 of DJ-1 is essential for binding of DJ-1 to p53 under an oxidative stress condition.

To examine whether translational modification both of p53 and DJ-1 induced by oxidative stress contributes to H₂O₂-dependent increase of p53-DJ-1 complex formation, HEK293T cells were exposed to 1 mM H₂O₂ for 30 min, and phosphorylation of p53 was analyzed by Western blotting with several anti-phosphorylated p53 antibodies. As shown in Fig. 2A, enhanced phosphorylation of p53 at serine residues 15 and 20, but not at serine residues 6, 9, 37, 46 and 392, was observed. When HEK293T cells were transfected with serine mutants of p53 in which serine was changed to alanine and exposed to 1 mM H₂O₂ for 30 min, expected phosphorylation of serines 15 and 20 of FLAG-p53 mutants was not observed by single and double substitution (Fig. 2B). HEK293T cells were then co-transfected with wild-type and serine mutants of FLAG-p53 and DJ-1-HA and exposed to 1 mM H₂O₂ for 30 min at 48 hrs after transfection. Co-immunoprecipitation experiments showed that of the three serine mutants and wild-type p53, no or only a slight difference in binding activity of FLAG-p53 to DJ-1-HA was observed under the oxidative stress condition (Fig. 2C), indicating that phosphorylation of p53 does not contribute to H₂O₂-dependent enhancement of DJ-1-p53 interaction.

Oxidative status of cysteine106 (C106) of DJ-1 is critical for all of the functions of DJ-1. HEK293T cells were co-transfected with wild-type or C106S mutant of DJ-1-HA and FLAG-p53 and exposed to H₂O₂ under the same conditions as those described above. The oxidation levels of wild-type and C106S DJ-1-HA were first examined by using electron focusing gels. While C106S DJ-1-HA had no shifted band, approximately 50% of wild-type DJ-1-HA was shifted to a more acidic point in H₂O₂-treated cells (Fig. 3A). Furthermore, when HEK293T cells were treated with

H₂O₂ and with UV or doxorubicin, other stressors activating p53, and proteins were analyzed with anti-DJ-1 and anti-C106 oxidized-DJ-1 antibodies, H₂O₂ exposure clearly enhanced C106 oxidation of DJ-1 (Fig. 3B), suggesting that a shifted band observed in a sample of wild-type DJ-1-HA was derived from C106. The DJ-1-p53 complex was then analyzed by immunoprecipitation using an anti-FLAG antibody followed by Western blotting with an anti-DJ-1 antibody. The results showed that while wild-type DJ-1 was strongly bound to p53 in H₂O₂-treated cells, the C106S mutant of DJ-1 had lost p53-binding activity (Fig. 3C). Pull-down experiments were then carried out using GST-wild-type DJ-1, GST-C106S DJ-1 and GST with ³⁵S-labeled p53 in the presence or absence of H₂O₂ and reducing agent DTT. Recombinant DJ-1 purified from *E. coli* is comprised of a mixture containing reduced and oxidized forms of C106, and the amounts of SOH and SO₂H forms of C106 are about 10~20% of total forms of C106 (data not shown). Doublet bands of p53 in gels must be unphosphorylated and phosphorylated p53. As in the case of DJ-1 binding to p53 in 293T cells, H₂O₂-treated wild-type DJ-1 was bound to p53 more strongly than did H₂O₂-non-treated wild-type DJ-1, and C106S mutant of DJ-1 had lost the enhancement of p53-binding activity (Fig. 3D). The enhancement of binding activity of DJ-1 to p53 was diminished by addition of DTT, indicating that DJ-1 directly interacts with p53 and that oxidation of C106 is essential for this interaction.

To directly examine whether C106-oxidized DJ-1 binds to p53, two experiments were carried out. First, HEK293T cells were co-transfected with FLAG-wild-type or C106S mutant of DJ-1 and T7-p53 and exposed to H₂O₂. Proteins were subjected to co-immunoprecipitation and Western blotting analyses with an anti-C106-oxidized DJ-1 antibody (Fig. 3E). Second, HEK293T and H1299 cells were transfected with wild-type or C106S DJ-1-HA and One-Strep-p53 and treated with H₂O₂. Proteins were pulled-down using Strep-Tactin sepharose and precipitates were analyzed by Western blotting with the anti-oxidized DJ-1 antibody (Figs. 3F and 3G, respectively). The results showed that oxidized wild-type DJ-1, but not C106S DJ-1, bound to p53 under the oxidative stress condition.

DJ-1 down-regulates DUSP1 expression under an oxidative stress condition.

To address the role of increased formation of DJ-1-p53 complex after treatment of cells with H₂O₂, the expression levels of p53-target genes in mouse primary cells were examined by semi-quantitative RT-PCR. As shown in Fig. 4A, the expression level of DUSP1 mRNA was increased at a peak of 30 min after H₂O₂ treatment, while transcription of other p53-target genes such as p21, NOXA and PUMA was induced 1-2 hrs. The other stresses such as UV exposure and doxorubicin treatment, on the other hand, did not alter the DUSP1 gene expression level, while the expression levels of Noxa and p21 were increased at 0.5-6 hrs after UV exposure and doxorubicin treatment (Figs. 5A-5D), suggesting that the DUSP1 gene is a primary target of p53 against oxidative stress.

DUSP1, a mitogen-activated protein kinase phosphatase, regulates the apoptosis signaling pathway through dephosphorylating ERK and is known to be only the p53-target gene that specifically responds to H₂O₂ treatment (25, 43, and Figs. 4A, 5A and 5B). Since the amount of p53-DJ-1 complex was also increased at the peak of 30 min after H₂O₂ treatment in HEK293T cells transfected with DJ-1-HA and FLAG-p53 and in A549 cells (Figs. 4B and 4C) and p53-DJ-1 complex was not detected at 1-4 hrs after H₂O₂ treatment (Fig. 4B right), we focused on the effect of DJ-1 on p53-dependent DUSP1 expression.

The expression levels of DUSP1 and p21 mRNAs were examined using primary cells derived from DJ-1-knock out (DJ-1 (-/-)) and DJ-1 (+/+) mice. Semi-quantitative RT-PCR and real-time PCR analyses showed that the expression level of DUSP1 mRNA in DJ-1 (+/+) cells was increased at 30 min and then decreased at 2 hrs after H₂O₂ treatment and that the level at 30 min was further increased in DJ-1 (-/-) cells (Figs. 4D and 4F). The expression levels of p21 mRNA were, on the other hand, increased at 2 hrs and there was no difference between the expression levels in DJ-1 (-/-) and DJ-1 (+/+) cells (Figs. 4E and 4G). The expression levels of DUSP1 protein were increased at 2 hrs in DJ-1 (+/+) cells and further increased in DJ-1 (-/-) cells (Fig. 4H). On the other hand, there was little difference of expression levels of p21 protein at 2 hrs between DJ-1 (-/-) and DJ-1 (+/+) cells.

cells (Fig. 4H). When DJ-1 (-/-) and DJ-1 (+/+) cells were transfected with siRNA targeting p53, the expression levels of DUSP1 and p21 were decreased (Fig. 4I), suggesting that transcriptional activity of p53 is still active even in DJ-1 (-/-) cells. Furthermore, no significant differences in expression level and subcellular localization of p53 were observed in DJ-1 (-/-) and DJ-1 (+/+) cells (Figs. 4J and 4K), indicating that up-regulation of DUSP1 expression in DJ-1 (-/-) cells was not due to change of p53 localization.

Cysteine 106 of DJ-1 is essential for repression of p53-dependent DUSP1 transcription under an oxidative stress condition.

p53 binds to the promoter region of the DUSP1 gene (25). Since luciferase generally used in reporter assays (call conventional luciferase) has a relatively long half-life, it is not suitable for detecting prompt or immediate change of promoter activity. In the case of prompt decrease in promoter activity toward stress response, for instance, luciferase activity obtained is not parallel to promoter activity due to accumulation of already synthesized conventional luciferase. To overcome this problem, we set up an experimental condition using pGL4.12-Luciferase that harbors a modified version of luciferase with a short half-life of ~20 min and using cycloheximide (CHX), an inhibitor for translation. Even when pGL4.12-Luciferase was used, the short half-life of the modified version of luciferase was still not sufficient to decrease the background caused by accumulation of modified luciferase. Since CHX blocks further translation of luciferase, prompt promoter activity is parallel to promoter activity. Twenty-four hrs after cells had been transfected with reporter plasmids, the cells were divided to three sets. One set was a “control” set to measure the background signal and the other two sets were “test” sets either for an H₂O₂-treated or non-treated sample. The “control” set was first treated with CHX for 30 min just before addition of H₂O₂ to cells and then luciferase activity obtained in the “control” set was subtracted as the background signal from that in the “test” set. Furthermore, to measure transcriptional activity of transfected p53 without an effect of

344 endogenous p53, p53-nul H1299 cells were used.

345 Fig. 6A shows histograms of raw data of luciferase activities obtained in the three sets after
346 transfection of the luciferase reporter construct linked to the DUSP1 promoter: p53 strongly
347 activated DUSP1 promoter activity, and treatment of cells with H₂O₂ reduced its activation activity,
348 which is consistent with results reported previously (25). When wild-type or C106S DJ-1 was
349 co-transfected with p53, luciferase activities tended to increase compared to those without p53 both
350 in wild-type DJ-1- and C106S DJ-1-transfected cells, but luciferase activity was lower in wild-type
351 DJ-1-transfected cells than in C106S DJ-1-transfected cells in the presence of CHX (Fig. 6A).
352 Similar expression levels of introduced proteins were confirmed by Western blotting (Fig. 6C). To
353 compare accurate changes among these samples, ratios of luciferase activities in H₂O₂-treated cells to
354 those in non-treated cells were calculated by subtracting the background signal obtained as the
355 “control” set (Fig. 7A). The results showed that in H₂O₂-treated cells, p53 transcriptional activity
356 toward the DUSP1 promoter was significantly reduced by wild-type DJ-1 to 60% of that with p53
357 alone or with C106S DJ-1. No significant change of promoter-less luciferase activity was observed
358 both in wild-type and C106S DJ-1-transfected cells (Fig. 7C). Furthermore, to confirm that DUSP1
359 promoter activity detected by this assay is parallel with the expression level of DUSP1 mRNA,
360 H1299 cells were transfected with FLAG-p53 and wild-type DJ-1-HA or C106S DJ-1-HA.
361 Twenty-four hrs after transfection, cells were treated with H₂O₂ in the absence of CHX. The
362 expression levels of DUSP1 mRNA examined by quantitative RT-PCR (real-time PCR) were
363 decreased by wild-type DJ-1 but not C106S DJ-1 in H₂O₂-treated cells (Fig. 7D). These results
364 suggest that DJ-1 downregulates p53-dependent DUSP1 expression in a C106-dependent manner in
365 H₂O₂-treated cells and that oxidation of C106 is necessary for this activity. When the same assay
366 using the p21 promoter linked to the modified version of the luciferase gene was carried out, there
367 was no significant change in promoter activity after transfection of cells with wild-type and C106S
368 DJ-1 (Figs. 6B, 6D and Fig. 7B), indicating that the H₂O₂-dependent inhibitory effect of DJ-1 on p53

369 transactivation activity is specific to the DUSP1 gene.

370

371 **DJ-1 directly binds to the p53 DNA-binding region.**

372 p53 is comprised of five domains; an N-terminal transactivation domain (TAD) followed by a
373 proline-rich region (PRR), central DNA-binding domain (DBD), tetramerization domain (TET) and
374 extreme C-terminus (CT) (see recent review, 48). To determine DJ-1-binding region of p53,
375 GST-wild-type DJ-1 and GST were reacted with ³⁵S-labeled full-length p53 and three p53-deletion
376 mutants depicted in Fig. 8A. Pull-down experiments showed that DJ-1 bound to full-length p53,
377 p53ΔCT and p53DBD, but not to p53ΔDBD and that H₂O₂-treated DJ-1 more strongly bound to
378 full-length p53 and p53ΔCT than did H₂O₂-non-treated DJ-1 (Fig. 8B), indicating that DJ-1 binds to
379 p53-DBD (Fig. 8B). Furthermore, pull-down (Fig. 8C) and ELISA (Fig. 8D) assays showed that
380 DJ-1 bound to p53-DBD in a C106-dependent manner.

381

382 **DJ-1-p53 complex stably binds to the p21 promoter but not to the DUSP1 promoter.**

383 A previous study showed that p53 binds to a 10-bp perfect palindromic site in the DUSP1 promoter
384 to activate its transcription, though the p53-binding sequence in the DUSP1 promoter is not a
385 sequence similar to the consensus sequence found in the p21 promoter (25). Chromatin
386 immunoprecipitation (ChIP) assays were carried out using DUSP1 and p21 genes. First, H1299 cells
387 were co-transfected with FLAG-p53 and wild-type or C106S DJ-1-HA, treated with H₂O₂ for 30 min
388 and subjected to ChIP assays with an anti-p53 antibody targeting two regions containing the
389 promoter and other regions (downstream region) were amplified by PCR. As shown in Fig. 9A, the
390 anti-p53 antibody precipitated the DUSP1 promoter region but not the downstream region under a
391 non-oxidative stress condition. In H₂O₂ treated-cells, precipitation of the DUSP1 promoter region by
392 the anti-p53 antibody was cancelled by transfection of wild-type DJ-1-HA but not by that of C106S
393 DJ-1-HA. Although the p21 promoter region contains the consensus p53-binding sequence,

precipitated p21 promoter region was not cancelled by transfection of wild-type DJ-1-HA and C106S DJ-1-HA both under oxidative and non-oxidative stress conditions (Fig. 9B). The p53 levels precipitated in reactions were at the similar range by Western blotting (Fig. 9C). Furthermore, ChIP assays were carried out using A549 cells. While the anti-p53 antibody precipitated the DUSP1 promoter region but not the downstream region in H₂O₂-treated cells, an anti-DJ-1 antibody did not precipitate regions even after more than 50 cycles of amplification by PCR (Fig. 9D). Both anti-p53 and anti-DJ-1 antibodies precipitated the p21 promoter region before and after H₂O₂ treatment and the precipitated levels were increased after H₂O₂ treatment (Fig. 9E). Equal levels of precipitated p53 and DJ-1 were confirmed (Fig. 9F). These results indicate that binding activity of p53 to the DUSP1 promoter is weaker than that to the p21 promoter and that the p53-DJ-1 complex endogenously binds to the p21 promoter but not to the palindromic site in the DUSP1 promoter. It is thought that DJ-1-free p53 binds to the DUSP1 promoter and that DJ-1-p53 complex binds to the p21 promoter (Fig. 9D). Time course analysis of ChIP assays were then carried out using A549 cells. p53 bound to the DUSP1 promoter in H₂O₂-treated cells at peak of 15 min exposure to H₂O₂ and then the level of promoter-bound p53 decreased (Fig. 10A). No binding of DJ-1 to the DUSP1 promoter was observed. The amount of promoter-bound p53 in DJ-1(-/-) cells was larger than that in DJ-1(+/+) (Fig. 10C). The precipitated levels of p53 and DJ-1 were at the similar levels during the course of H₂O₂ exposure (Figs. 10B and 10D). Taken together, these results suggest that p53 binding to the DUSP1 promoter is sequestered by DJ-1 through its binding to p53DBD in a C106-dependent manner under an oxidative stress condition, resulting in suppression of transactivation activity of p53 toward the DUSP1 gene.

DJ-1 increases phosphorylation of ERK and reduces apoptosis under an oxidative stress condition.

Since DUSP1 dephosphorylates ERK as MAPK phosphatase, we examined whether the

dephosphorylation level of ERK is up-regulated in DJ-1 (-/-) cells by the increased level of DUSP1. DJ-1 (-/-) and DJ-1 (+/+) cells were exposed to H₂O₂ twice and the phosphorylation level of ERK1/2 was analyzed by Western blotting. Since culture medium was changed before second H₂O₂ treatment, cells were treated with the same concentration of H₂O₂ at respective stimulation. As shown in Figs. 11A and 11C, the expression level of DUSP1 was increased 2 hrs after the first H₂O₂ treatment in DJ-1 (-/-) cells but not in DJ-1 (+/+) cells, and the phosphorylation level of ERK1/2 was lower in DJ-1 (-/-) cells than that in DJ-1 (+/+) cells after the second H₂O₂ treatment. The similar decreased curve of ERK1/2 phosphorylation level after second H₂O₂ treatment was obtained both in DJ-1 (-/-) and DJ-1 (+/+) cells, indicating that DJ-1 did not prolong the ERK activation. There were no or little difference in the expression levels of p53 and total ERK1/2 in DJ-1 (-/-) and DJ-1 (+/+) cells regardless of H₂O₂ treatment (Fig. 11C). Furthermore, knockdown of DUSP1 using two different siRNA (siDUSP1-1 and siDUSP1-2) increased ERK phosphorylation in DJ-1 (-/-) cells but not in DJ-1 (+/+) cells (Figs. 11B and 11D), indicating a reverse correlation between the expression levels of DUSP1 and phosphorylated ERK in DJ-1 (-/-) cells under an oxidative stress condition. Since DUSP1 activates the apoptosis pathway in cells after oxidative stress, DJ-1 (-/-) and DJ-1 (+/+) cells were treated twice with H₂O₂ and then treated with propidium iodide, and their apoptosis levels were analyzed by using FACS in which apoptotic cells are observed in the sub-G1 phase. As shown in Figs. 12A and 12B, H₂O₂ treatment induced apoptosis in both DJ-1 (-/-) and DJ-1 (+/+) cells, but the level of apoptosis in DJ-1 (-/-) cells was about 10-times higher than that in DJ-1 (+/+) cells. Furthermore, knockdown of DUSP1 expression in DJ-1 (-/-) cells reduced apoptosis to 60% of that in control siRNA-treated cells (Fig. 12B). There was no difference between the expression levels of p53 in DJ-1 (-/-) and DJ-1 (+/+) cells (Fig. 11D). These results suggest that DJ-1 decreases apoptosis in cells under intermittent oxidative stress condition through downregulation of DUSP1 expression.

DJ-1 inhibits p53 activity in a DNA-binding affinity-dependent manner.

444 We showed that DJ-1 inhibited transcriptional activity of p53 targeting the DUSP1 gene, but not that
445 of p53 targeting the p21 gene, by inhibiting DNA-binding activity of p53 in the DUSP1 promoter but
446 not in the p21 promoter. Since DUSP1 and p21 promoters contain non-consensus and consensus
447 p53-binding sequences and since non-consensus and consensus p53-binding sequences possess low
448 and high binding affinities to p53, respectively, we considered two possible mechanisms by which
449 DJ-1 inhibited p53-binding activity only to the DUSP1 promoter: One is the difference in the
450 p53-binding region between the consensus and non-consensus sequences and the other is the
451 difference in binding affinity of p53 to DNA. To address this point, we used substitution mutants of
452 p53 at amino acid number 181 from arginine to leucine (R181L), to cysteine (R181C) and to proline
453 (R181P), which are linked to tumor development in families with the hereditary Li-Fraumeni or
454 Li-Fraumeni-like cancer susceptibility syndrome (37). DNA-binding activity of these p53 mutants
455 changed in the order of wild-type (WT) > R181L > R181C > R181P (38). Pull-down assays using
456 GST-wild-type DJ-1, GST and ³⁵S-labeled p53 mutants showed that DJ-1 bound to R181L, R181C,
457 R181P and WT p53 with the same or similar affinity (Fig. 13A). Assays using the luciferase reporter
458 gene conjugated to the p21 promoter showed that transcriptional activity of p53 mutants toward the
459 p21 promoter was reduced compared to that of WT p53 in a DNA-binding activity-dependent
460 manner and that the R181P mutant had no transcriptional activity (Fig. 13B). We therefore used
461 R181L and R181C mutants of p53 for further study. Pull-down assays showed that H₂O₂ treatment
462 enhanced DJ-1-binding activity of R181L and R181C p53 (Fig. 13C). H1299 cells were then
463 co-transfected with wild-type DJ-1 or C106S DJ-1 together with R181L or R181C p53 and treated
464 with 300 μM H₂O₂ or with 10 μg/mL cycloheximide for 30 min at 20 hrs after transfection, and their
465 luciferase activities toward the p21 promoter were examined. Luciferase activities and similar
466 expression levels of introduced proteins in transfected cells are shown in Figs.14B-14E, and ratios of
467 luciferase activity in cells treated with or without H₂O₂ were calculated. The results showed that
468 wild-type DJ-1 significantly inhibited transcriptional activities of R181L p53 and R181C p53 toward

the p21 promoter in H₂O₂-treated cells to 50% and 40%, respectively, of that in cells without DJ-1 or in cells transfected with C106S DJ-1 (Fig. 14A). Wild-type DJ-1 suppressed p21 promoter activity brought by R181C p53 more effectively than that by R181L p53, suggesting that DJ-1 inhibits p53 transcriptional activity in a p53 DNA-binding affinity-depending manner. Furthermore, H1299 cells were co-transfected with FLAG-R181L p53 or -R181C p53 together with wild-type DJ-1-HA or C106S DJ-1-HA, then treated with or without H₂O₂ and subjected to ChIP assays targeting the p21 promoter. As shown in Figs. 15A and 15B, the anti-p53 antibody precipitated the p21 promoter region but not the downstream region under a non-oxidative stress condition, and precipitated p21 promoter region was inhibited by transfection of wild-type DJ-1-HA but not by that of C106S DJ-1-HA under an oxidative stress condition. The precipitated p53 levels were at the similar range by Western blotting (Figs. 15C and 15D). These results suggest that DJ-1 inhibits p53 activity in a DNA-binding affinity-dependent manner.

DISCUSSION

In this study, we showed that oxidized DJ-1 at C106 induced by oxidative stress strongly binds to p53 and that the enhanced interaction of DJ-1 with p53 under the oxidative stress condition is required to suppress p53-dependent transcriptional activation of the DUSP1 gene through preventing promoter recognition of p53 (Fig. 16A). C106 of DJ-1 is essential for increase of complex formation with p53 (Fig. 3C). Although several studies, including studies by us, have shown direct interaction of DJ-1 with p53 (11, 41), this is the first evidence that oxidative status of DJ-1 regulates its interaction.

C106 of DJ-1 is oxidized from forms of SH (reduced), SOH, SO₂H and SO₃H, and excess oxidation of C106, probably as an SO₃H form, has been found in brains of patients with Parkinsons disease and Alzheimer's disease (1, 6). What type of oxidation form of C106 is the active form is still under debate. Zhou et al. reported that DJ-1 at C106 with SO₂H is an active form in terms of

494 chaperone activity toward α -synuclein (53). We have, on the other hand, reported that stimulating
495 activity of DJ-1 toward tyrosine hydroxylase and L-DOPA decarboxylase requires the presence of
496 reduced and SOH forms of C106, which account for more than 50% of total forms (16). In this study,
497 cells were treated with 1 mM H₂O₂ for 30 min and pI of DJ-1 spots was shifted from pI 6.2 to 5.8.
498 (Fig. 3B). Since a spot of pI 5.8 contained more than 50% of the SO₂H form of C106 to total forms
499 of C106 (data not shown), it is possible that DJ-1 with SO₂H of C106 preferentially binds to p53 to
500 inhibit p53-dependent DUSP1 expression. Since other forms of C106 were also involved in the pI
501 5.8 spot, however, we cannot rule out the possibility that C106 forms other than SO₂H much more
502 effectively work toward p53. Establishment of methods to purify DJ-1 possessing respective forms
503 of C106 is necessary for analysis of DJ-1.

504 p53 bound to the DUSP1 promoter at 15 min after exposure of cells to H₂O₂ (Fig. 10A) and the
505 expression level of DUSP1 mRNA then peaked at 30 min (Fig. 4A). The DJ-1-binding level with p53
506 was also peaked at 30 min after H₂O₂ exposure and then decreased (Fig. 4B) concomitant with
507 decreased binding of p53 to the DUSP1 promoter (Fig. 10A). When the same experiments were
508 carried out using DJ-1(-/-) cells, the expression level of DUSP1 mRNA was increased at 30 min
509 after H₂O₂ exposure (Figs. 4D and 4E). If the p53/DJ-1 interaction is a negative feedback that shuts
510 down DUSP1 after its initial activation, binding of DJ-1 to p53 must be correlated with repression of
511 DUSP1 expression at later time points after H₂O₂. As described above, this is not a case. As shown in
512 Figs. 11A and 11C, DJ-1 increased the phosphorylation level of ERK through down-regulation of
513 DUSP1 expression at 15 min after second exposure of H₂O₂, but thereafter there was no difference of
514 decreasing curve of ERK phosphorylation between DJ-1(+/+) and DJ-1(-/-) cells, suggesting that
515 DJ-1 determines the maximal level of ERK phosphorylation. From these results, the mechanism of
516 DJ-1 action toward DUSP1 expression may be followings: DJ-1 receives oxidative stress as an
517 oxidative stress sensor and its C106 is oxidized. Oxidized DJ-1 binds to p53 at the limited time point,
518 resulting in suppression of DUSP1 expression, thereby regulating the activation level of ERK.

519 p53 is post-translationally modified by various stresses, and tetramerization of p53 occurs to
520 function. We did not find any evidence that phosphorylation of p53 affects its interaction with DJ-1.
521 p53, however, receives other post-translational modifications such as lysine-acetylation. Acetylation
522 of p53 enhances DNA-binding activity of p53 to specific DNA regions (15) and it is therefore
523 possible that acetylation of p53 affects DUSP1 expression. Furthermore, it has been reported that p53
524 induces phosphorylation of DJ-1 (34). Thus, it is thought that in addition to oxidation, DJ-1 chooses
525 its binding proteins dependently on various modifications of DJ-1.

526 Thirty-min exposure of cells to H₂O₂ induced phosphorylation of serines 15 and 20, but not
527 serine 46, of p53 and the p53-DJ-1 complex level was increased at the peak of 30 min after H₂O₂
528 treatment (Figs. 2 and 4B). Since severe DNA damage leads to phosphorylation of p53 serine 46 (33),
529 it is thought that weak DNA damage occurred under the oxidative stress condition used in this study
530 and that DJ-1 contributes to suppression of p53 under the condition of such weak damage.

531 Of the p53 DNA-binding sequences, p21 and DUSP1 promoters contain consensus and
532 non-consensus p53-binding sequences, respectively, which have high affinity and low affinity to p53
533 (12, 25). The protein level of Bax, the promoter of which contains the consensus p53-binding
534 sequence, is down-regulated by DJ-1 before and after H₂O₂ treatment of cells (11, 29), suggesting
535 that suppressive activity of DJ-1 toward Bax does not depend on the oxidative status of DJ-1. It is of
536 interest that DJ-1 inhibited p53 transcriptional activity toward the DUSP1 promoter but not that
537 toward the p21 promoter (Fig. 7) and that these phenomena were obtained through interference of the
538 DNA-binding activity of p53 toward the DUSP1 promoter by DJ-1 (Fig.9). When p53 mutants with
539 low DNA-binding affinity to the p21 promoter were used, however, DJ-1 inhibited their
540 transcriptional activity even to the p21 promoter (Fig. 14A). Binding activity of DJ-1 to p53 mutants
541 was the same as that to wild-type p53 (Fig. 13A). It is therefore thought that DJ-1 interferes with
542 p53-binding activity to the DNA region that possesses weak association with p53 and that DJ-1 does
543 not affect p53-binding activity to the DNA region that strongly interacts with p53 under an oxidative

stress condition. In other words, the binding affinity of p53 to the respective DNA-binding region determines whether or not DJ-1 inhibits DNA-binding and transcriptional activities of p53 (Fig. 16B). The present findings suggest that DJ-1 plays a novel role in suppressing the expression of p53-target genes through preventing promoter recognition of p53 in a DNA-binding affinity-dependent manner. Furthermore, DJ-1 and p53 were co-localized in the nucleus before and after H₂O₂ treatment (Figs. 1E and F) and p53 was localized to the nucleus both in DJ-1 (-/-) and DJ-1 (+/+) cells with or without H₂O₂ treatment (Figs. 4J and 4K), suggesting that DJ-1 inhibits binding of p53 to specific DNA regions without affecting localization of p53.

Exposure of cells to H₂O₂ quickly induces phosphorylation of p53 and ERK. Activated p53 then up-regulates the expression of pro-apoptotic genes (4, 42). Activation of ERK via the Ras/Raf/MEK pathway, on the other hand, supports cell survival (3, 49). It has been reported that inhibition of p53 activates ERK after H₂O₂ treatment of cells, suggesting the existence of a cross-talk of the negative signaling pathway between p53 and ERK (22). Oxidized DJ-1 inhibited p53-dependent DUSP1 expression under an oxidative stress condition (Figs. 4D, 4F and 7A), and it increased ERK phosphorylation and cell survival (Figs. 11 and 12). Suppression of DUSP1 expression also results in a decrease of oxidative stress-induced cell death in SH-SY5Y cells (18), and activation of ERK regulates tyrosine hydroxylase transcription through activating the orphan nuclear receptor Nurr1 (17, 26). In addition to ERK, DJ-1 inhibits p38, MKK3 and MKK6 activity through inactivating ASK1 under an oxidative stress condition (30). It is therefore thought that DJ-1 contributes to activation of survival pathways by suppressing the apoptosis pathway through regulating expression of MAPK phosphatase under an oxidative condition.

It has been reported that DJ-1 is overexpressed in many types of cancers, especially in cancer cells with poor prognosis, and that almost half of the cancer cells possess p53 mutations (7, 23, 28, 52, 54). In this study, DJ-1 inhibited transcriptional activity of p53 mutants more effectively than that of wild-type p53 after cells had been exposed to H₂O₂ (Fig. 14A): p21 promoter activity brought by

R181L p53 was decreased to 82% of that brought by wild-type p53 and was inhibited by wild-type DJ-1 to 57.2% of that without DJ-1, indicating that the final promoter activity by R181L p53 became 47% of that by wild-type p53. In the case of R181C p53, the final activity was reduced only to 8% of that obtained by wild-type p53 (Figs. 13B and 14A). DJ-1 activates ERK (Fig. 11A) and Akt pathways by binding to PTEN (20) and has a cooperative transforming activity with H-Ras, which is located upstream of both pathways (31). These results suggest that in addition to stimulation of cell proliferation pathways, overexpressed DJ-1 contributes to the poor prognosis in cancer cells by suppressing cell death pathways.

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LEGEND OF FIGURES

FIG. 1. Oxidative stress enhances DJ-1-binding to p53.

A. HEK293T cells were transfected with FLAG-p53 and DJ-1-HA and treated with 1 mM H₂O₂ for

745 30 min at 48 hrs after transfection. Proteins were analyzed by immunoprecipitation followed by
746 Western blotting.

747 **B.** HEK293T cells were transfected with FLAG-DJ-1 and T7-p53 and analyzed as described in a
748 legend for Fig. 1A.

749 **C, D.** A549 cells (p53+/+) (C) and H1299 cells (p53-/-) (D) were treated with 300 μ M H₂O₂ for 30
750 min, and proteins analyzed by immunoprecipitation followed by Western blotting. The intensity of
751 precipitated p53 bands in lanes 5 and 6 was quantified and their relative level is shown under the
752 figure.

753 **E, F.** A549 cells were treated with (F) or without (E) 300 μ M H₂O₂ for 30 min. Immunofluorescence
754 analyses were carried out using anti-p53 and anti-DJ-1 antibodies as described in Materials and
755 methods.

756

757 **FIG. 2.** Phosphorylation levels of serines in p53 under an oxidative stress condition.

758 **A.** HEK293T cells with or without 1 mM H₂O₂ for 30 min, and proteins were analyzed by Western
759 blotting with anti-p53 and respective anti-p-p53 antibodies.

760 **B.** HEK293T cells transfected with FLAG-wild-type and -mutants of p53 were treated with 1 mM
761 H₂O₂ for 30 min at 48 hrs after transfection. Proteins were detected by Western blotting.

762 **C.** HEK293T cells transfected with DJ-1-HA, FLAG-wild-type and mutants of p53 were treated with
763 1 mM H₂O₂ for 30 min at 48 hrs after transfection. Proteins were analyzed by immunoprecipitation
764 followed by Western blotting. A precipitated p53 band in lane 2 is overflow from a band in lane 3.

765

766 **FIG. 3.** Cysteine 106 of DJ-1 is essential for binding to p53 under an oxidative stress condition.

767 **A.** HEK293T cells were transfected with wild-type or C106S DJ-1-HA and FLAG-p53. Forty-eight
768 hrs after transfection, cells were treated with 1 mM H₂O₂ for 30 min, and proteins were analyzed by
769 an isoelectric focusing gel and subjected to Western blotting with an anti-HA antibody.

770 B. HEK293T cells were treated with 20 J/m² UV, 1 μM doxorubicin or 1 mM H₂O₂ for 0.5-4 hrs and
771 proteins were analyzed by Western blotting.

772 C. HEK293T cells were transfected with wild-type DJ-1-HA or C106S DJ-1-HA and FLAG-p53 and
773 treated with H₂O₂ as described in the legend of Fig. 2A. Proteins were analyzed by
774 immunoprecipitation followed by Western blotting.

775 D. GST, GST-wild-type DJ-1 and GST-C106S DJ-1 were incubated with ³⁵S-labeled p53 in the
776 presence or absence of H₂O₂ and DTT, and subjected to pull-down assays. CBB: Coomassie brilliant
777 blue.

778 E. HEK293T cells were transfected with FLAG-wild-type DJ-1 or FLAG -C106S DJ-1 and T7-p53
779 and treated with H₂O₂. Proteins were then analyzed by immunoprecipitation followed by Western
780 blotting.

781 F, G. HEK293T cells (F) or H1299 cells (G) were transfected with wild-type or C106S DJ-1-HA and
782 One-STrEP-p53 and treated with H₂O₂. Proteins were then subjected to pull-down assays using
783 Strep-Tactin sepharose beads.

784

785 **FIG. 4.** DJ-1 down-regulates DUSP1 expression under an oxidative stress condition.

786 A. Mouse primary cells were treated with H₂O₂ for 0.25-6 hrs, and the expression levels of
787 respective mRNA were examined by semi-quantitative RT-PCR and their relative expression to that
788 of β-actin is shown.

789 B. HEK293T cells were transfected with FLAG-p53 and DJ-1-HA and treated with 1 mM H₂O₂ for
790 15-45 min (left panel) and for 0.5-4 hrs (right panel) at 48 hrs after transfection. Proteins were
791 analyzed by immunoprecipitation followed by Western blotting.

792 C. A549 cells were treated with 300 μM H₂O₂ for 15-120 min. Proteins were analyzed by
793 immunoprecipitation with an anti-DJ-1 antibody followed by Western blotting.

794 D, E. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were treated with H₂O₂ for 0.5 hrs (C) or 2 hrs (D). The

expression levels of DUSP1 (C) and p21 mRNA (D) were examined by semi-quantitative RT-PCR and their relative expression to that of β -actin is shown.

F, G. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were treated with H₂O₂ for 0.5 and 2 hrs. The expression levels of DUSP1 (F) and p21 mRNA (G) were examined by quantitative RT-PCR.

H. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were treated with H₂O₂ for 0.5 and 2 hrs. The expression levels of respective protein were analyzed by Western blotting.

I. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were transfected with control siRNA or p53 siRNA and treated with 300 μ M H₂O₂ for 30 min or 2 hrs at 72 hrs after transfection. The expression levels of DUSP1 and p21 mRNAs were examined by semi-quantitative RT-PCR. Nucleotide sequences of p53 siRNA are as follows: mp53 sense: 5'- CCAGAAGAUAUCCUGCCAUTT-3' and mp53 antisense: 5'- AUGGCAGGAUAUCUUCUGGTT-3'.

J, K. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were treated with H₂O₂ for 0.5 and 2 hrs. p53 was visualized as described in Materials and methods.

Values in Figures D-G are means $\pm$ S.E. n=3 experiments. Significance: ** p < 0.01 and *** p < 0.001. N.S. represents no significance.

FIG. 5. Expression of DUSP1, p21 and NOXA in UV- and doxorubicin-treated cells.

Mouse DJ-1 (+/+) cells were treated with 20 J/m² UV (A, C) or 1 μ M doxorubicin (B, D) for 0.5-6 hrs. The expression levels of mRNA and protein of DUSP1, p21 and NOXA were examined by semi-quantitative RT-PCR (A, B) and by Western blotting (C, D), respectively.

FIG. 6. Raw data in three sets of luciferase assays.

A, B. H1299 cells were co-transfected with pGL4.12-DUSP1-luciferase (A) or pGL4.12-p21-luciferase (B) and expression vectors for FLAG-p53 and wild-type or C106S DJ-1-HA. Twenty-four hrs after transfection, cells were treated with 300 μ M H₂O₂ or with 10 μ g/mL

cycloheximide (CHX) for 30 min. Luciferase activities were then calculated.

C, D. Proteins were prepared from H1299 cells transfected with FLAG-p53 and wild-type or C106S DJ-1-HA and analyzed by Western blotting.

FIG. 7. Cysteine 106 of DJ-1 is essential for repression of p53-dependent DUSP1 transcription under an oxidative stress condition.

A, B, C. H1299 cells were co-transfected with pGL4.12-DUSP1-luciferase (A), pGL4.12-p21-luciferase (B) or pGL4.12-luciferase (C) and FLAG-p53 and wild-type or C106S DJ-1-HA. Cells were treated as described in the legend for Fig. 6. Fold repression of luciferase activity was calculated as described in the text.

D. H1299 cells were transfected with FLAG-p53 and DJ-1-HA and treated with H₂O₂ for 30 min in the absence of cycloheximide as described in the legend for Fig. 6. The expression levels of DUSP1 mRNA were examined by quantitative RT-PCR.

Values are means $\pm$ S.E. n=3 experiments. Significance: ** $p < 0.01$, *** $p < 0.001$. N.S. represents no significance.

FIG. 8. DJ-1 directly binds to the p53 DNA-binding region.

A. Schematic diagram of p53 deletion mutants. FL: full-length; CT: C-terminus; DBD: DNA-binding domain.

B. GST and GST-DJ-1 were incubated with ³⁵S-labeled p53 in the presence or absence of H₂O₂, and then subjected to pull-down assays. I: Input; G: GST; D: GST-DJ-1; CBB: Coomassie brilliant blue.

C. GST, GST-DJ-1 and GST-C106S DJ-1 were incubated with ³⁵S-labeled p53DBD and subjected to pull-down assays. CBB: Coomassie brilliant blue.

D. ELISA assays were carried out using GST-p53, GST, DJ-1 and C106S DJ-1.

845

846 **FIG. 9.** DJ-1-p53 complex stably binds to the p21 promoter but not to the DUSP1 promoter.

847 **A, B.** H1299 cells were transfected with FLAG-p53 and wild-type or C106S DJ-1-HA. Forty-eight
848 hrs after transfection, cells were treated with 300 μ M H₂O₂ for 30 min, and ChIP assays were carried
849 out targeting DUSP1 (A) and p21 (B) genes.

850 **C.** Proteins prepared from transfected H1299 cells were analyzed by Western blotting.

851 **D, E.** A549 cells were treated with 300 μ M H₂O₂ for 30 min, and ChIP assays were carried out
852 targeting DUSP1 (D) and p21 (E) genes. The intensity of precipitated DNA bands in lanes 4 and 6 of
853 Figure E was quantified and their relative level is shown.

854 **F.** Proteins prepared from H₂O₂-treated A549 cells were analyzed by Western blotting.

855

856 **FIG. 10.** A time course of endogenous DJ-1/p53 interactions and endogenous p53 ChIP on the DUSP
857 promoter in cells exposed to oxidative stress.

858 **A.** A549 cells were treated with 300 μ M H₂O₂ for 0.25-2 hrs, and ChIP assays targeting the DUSP1
859 gene were carried out.

860 **B.** Proteins prepared from H₂O₂-treated A549 cells were analyzed by Western blotting.

861 **C.** DJ-1 (+/+) and DJ-1 (-/-) mouse cells were treated with 300 μ M H₂O₂ for 0.5 hrs, and ChIP assays
862 targeting the DUSP1 gene were carried out.

863 **D.** Proteins prepared from H₂O₂-treated mouse DJ-1 (+/+) and DJ-1 (-/-) cells were analyzed by
864 Western blotting.

865

866 **FIG. 11.** Phosphorylation levels of ERK in cells under an oxidative stress condition.

867 **A, C.** DJ-1 (+/+) and DJ-1 (-/-) mouse cells were starved for 6 hrs and then treated with or without
868 two pulses of H₂O₂. Proteins were analyzed by Western blotting (C) and relative phosphorylation
869 levels of ERK were quantified (A).

B, D. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were transfected with control siRNA or DUSP1 siRNA-1 or DUSP1 siRNA-2, starved for 6 hrs at 48 hrs after transfection, and then treated with two pulses of H₂O₂. Proteins were analyzed by Western blotting (B) and relative phosphorylation levels of ERK were quantified (D). Values are means $\pm$ S.E. n=3 experiments. Significance: * p < 0.05.

FIG. 12. DJ-1 regulates cell survival under an oxidative stress condition.

A. DJ-1 (+/+) and DJ-1 (-/-) mouse cells were transfected with control siRNA or DUSP1 siRNA-1 and treated as described in the legend for Fig. 11. Forty hrs after H₂O₂ addition, cell-cycle profiles were examined by using flow cytometry.

B. The sub-G1 phase of the cell cycle obtained in FIG. 12A was quantified, and relative number of cells in the sub-G1 fraction compared to that in DJ-1 (+/+) cells with control siRNA and H₂O₂ was calculated. Values are means $\pm$ S.E. n=3 experiments. Significance: *** p < 0.001.

FIG. 13. Binding activity of p53 mutants to DJ-1

A and C. GST and GST-DJ-1 were incubated with ³⁵S-labeled wild-type and mutant p53 in the presence or absence of H₂O₂, and subjected to pull-down assays. I: Input; G: GST; D: GST-wild-type DJ-1; C: GST-C106S DJ-1; CBB: Coomassie brilliant blue.

B. H1299 cells were co-transfected with pGL4.12-p21-luciferase and expression vectors for wild-type, R181L, R181C and R181P of FLAG-p53. Twenty-four hrs after transfection, luciferase assays were carried out. Values are means $\pm$ S.E. n=3 experiments. Significance: ** p < 0.01 . N.S. represents no significance.

FIG. 14. DJ-1 inhibits transcriptional activity of p53 to the p21 promoter under an oxidative stress condition in a DNA-affinity-dependent manner.

A. H1299 cells were co-transfected with pGL4.12-p21-luciferase and expression vectors for FLAG-p53 mutants and for wild-type or C106S DJ-1-HA. Twenty-four hrs after transfection, their

luciferase activities were examined. Values are means $\pm$ S.E. n=3 experiments. Significance: * p < 0.05 and ** p < 0.01 .

B, C. H1299 cells were co-transfected with pGL4.12-p21-luciferase and an expression vector for wild-type or C106S DJ-1-HA together with a vector for FLAG-p53 R181L (B) or FLAG-p53 R181C (C). Twenty-four hrs after transfection, the cells were treated with H₂O₂ and cycloheximide (CHX) as described in the legend for Fig. 6 and their luciferase activities were examined.

D, E. Proteins were prepared from H1299 cells transfected with FLAG-p53 mutants and wild-type or C106S DJ-1-HA and analyzed by Western blotting.

Fig. 15. DJ-1 inhibits DNA-binding activity of p53 mutants.

A, B. H1299 cells were transfected with FLAG-p53 R181L (A) or R181C (B) and wild-type or C106S DJ-1-HA. Forty-eight hrs after transfection, cells were treated with 300 μ M H₂O₂ for 30 min, and ChIP assays were carried out targeting the p21 gene.

C, D. Proteins were prepared from H1299 cells transfected with FLAG-p53 R181L (C) or R181C (D) and wild-type or C106S DJ-1-HA and analyzed by Western blotting.

FIG. 16. Schematic models of the role of DJ-1 in inhibition of p53 transactivation

A. DJ-1 activates cell survival pathways under an oxidative stress condition through down-regulation of DUSP1 expression.

B. DJ-1 suppresses transactivation activity of p53 depending on p53-DNA binding-affinity.

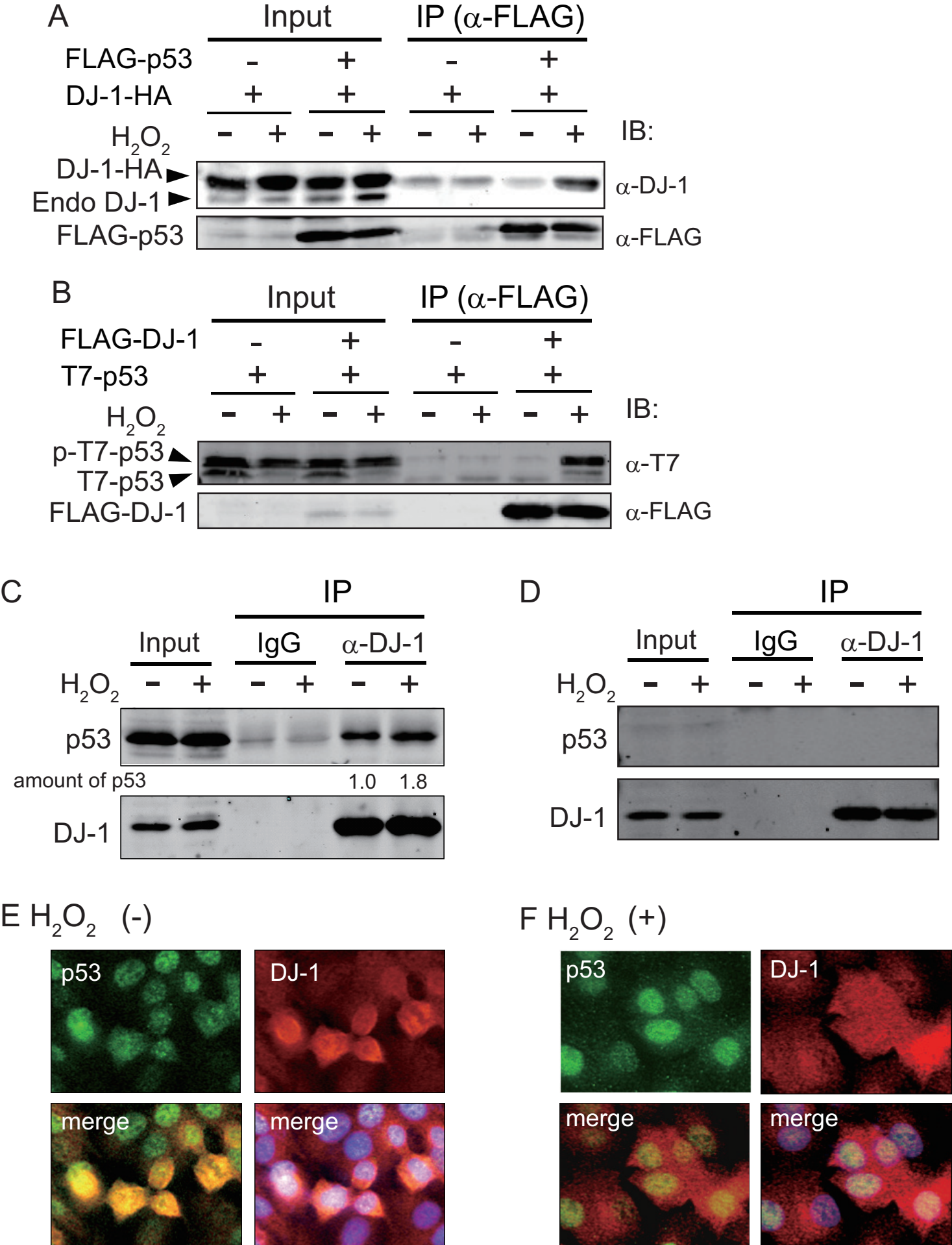
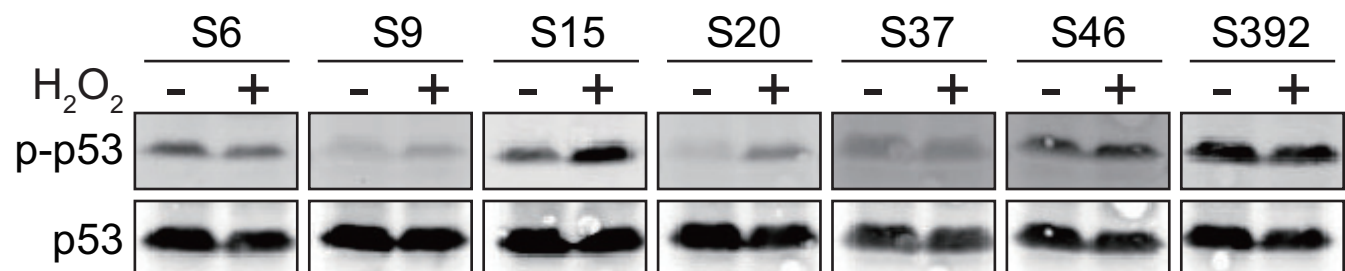
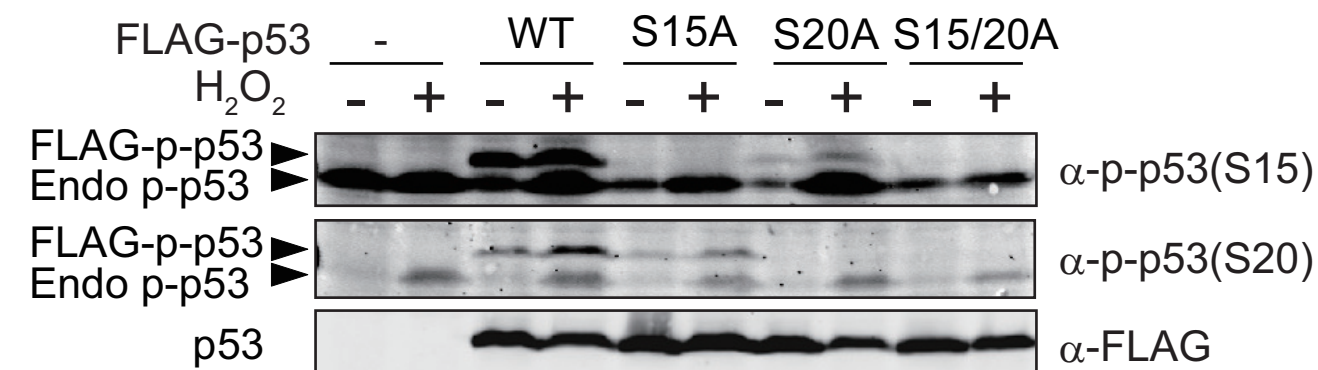


Fig. 1

A



B



C

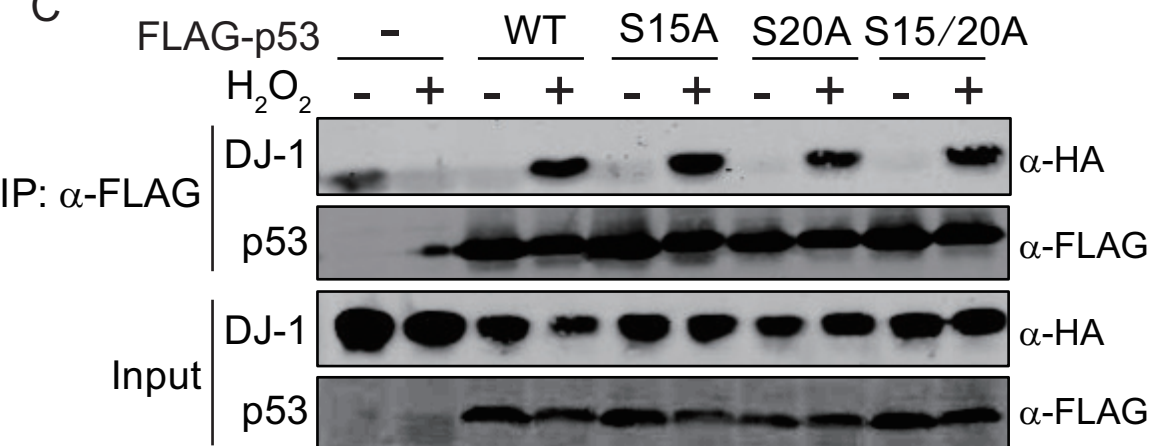


Fig. 2

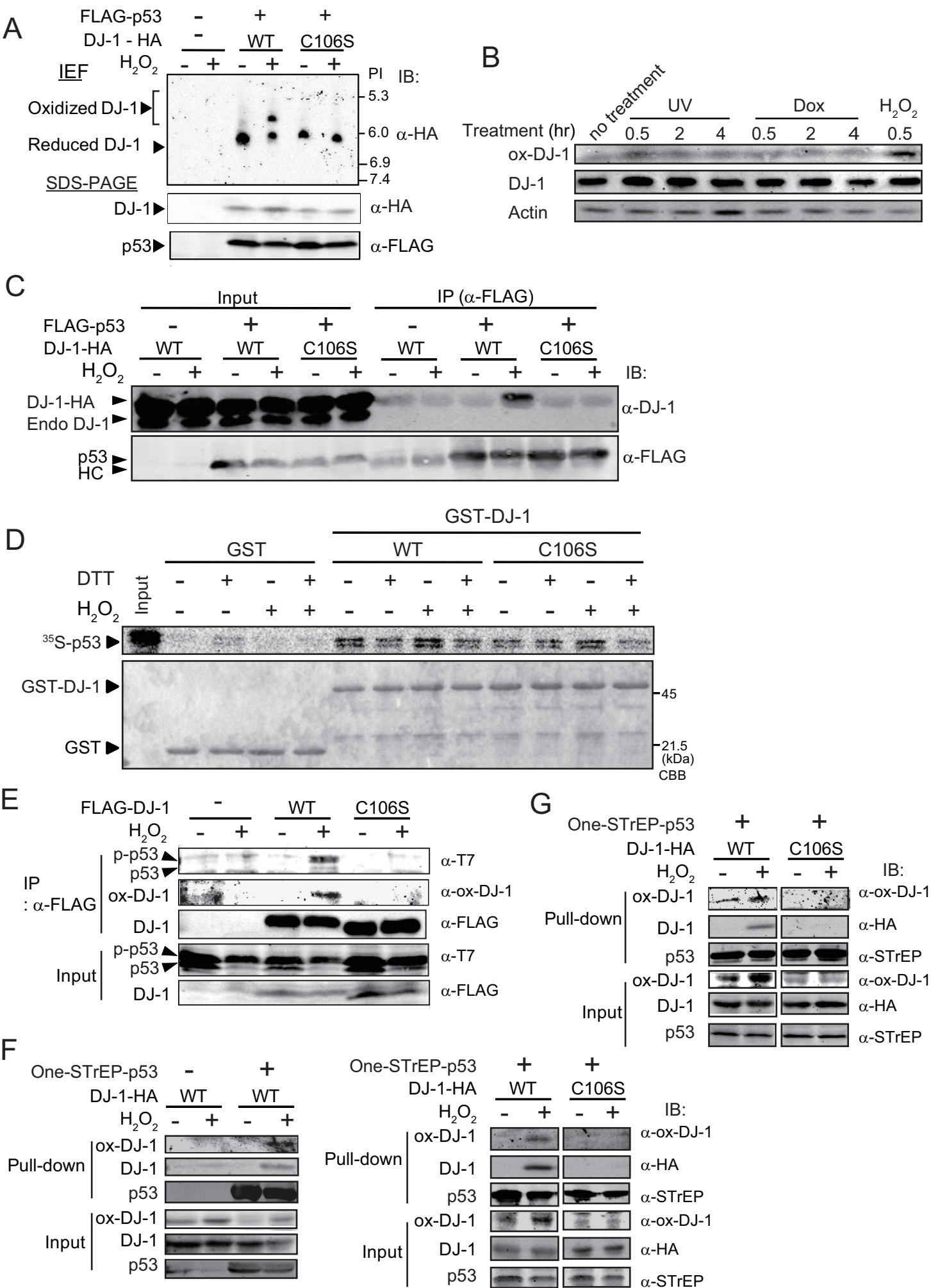


Fig. 3

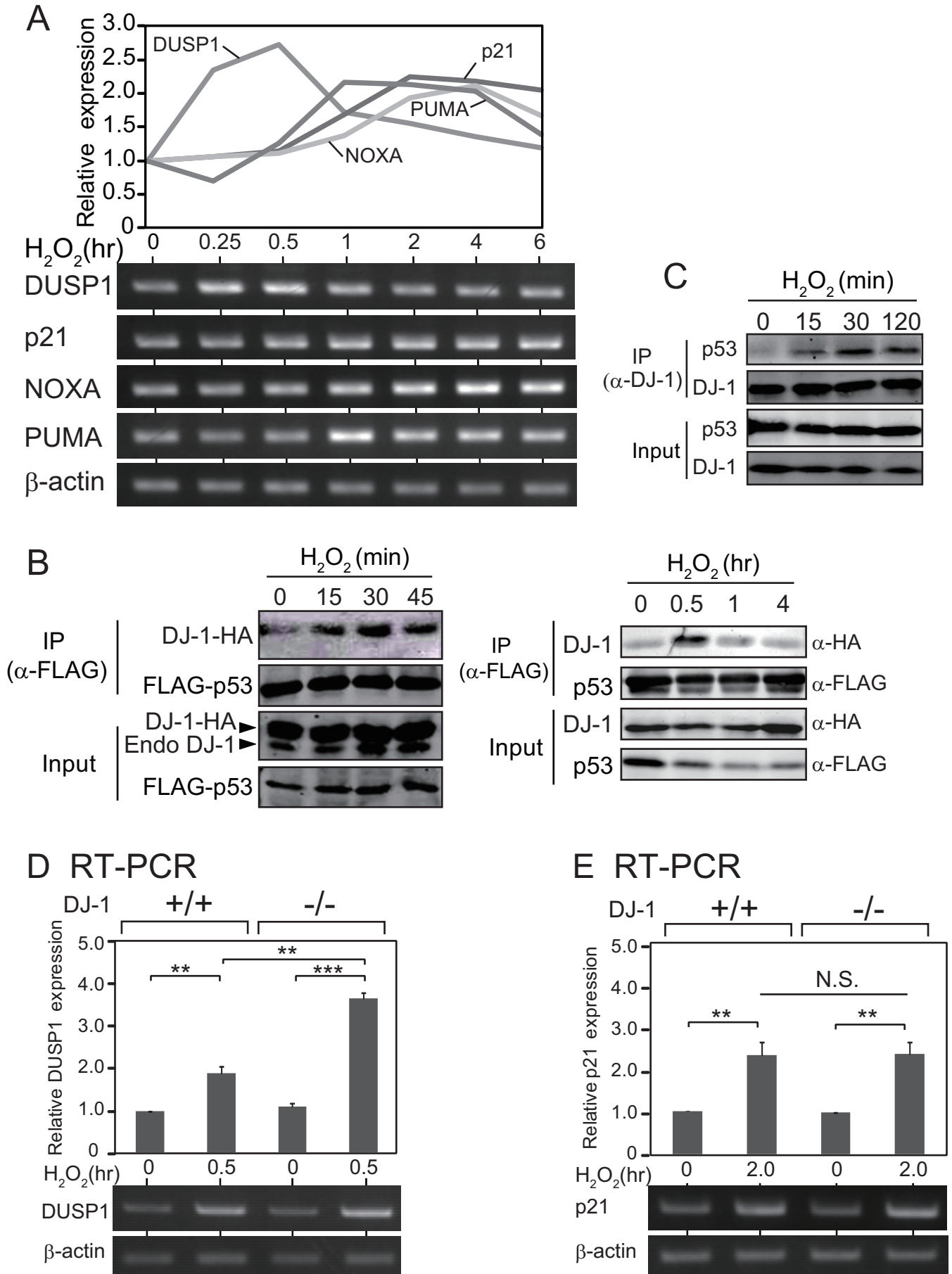
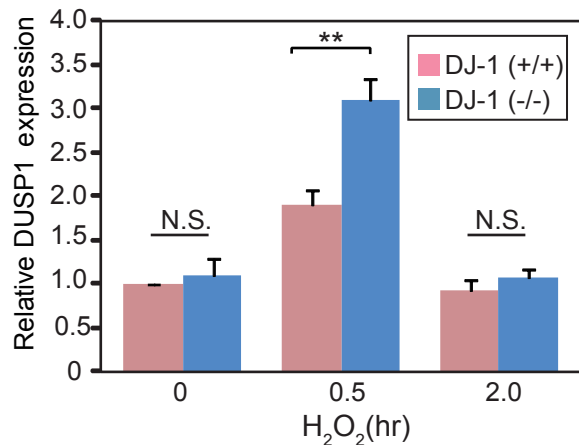
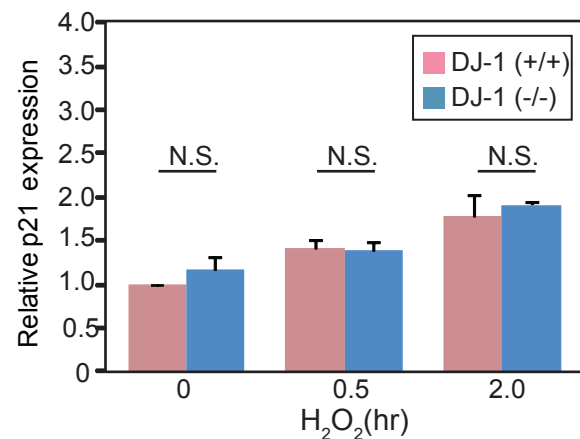


Fig.4-1

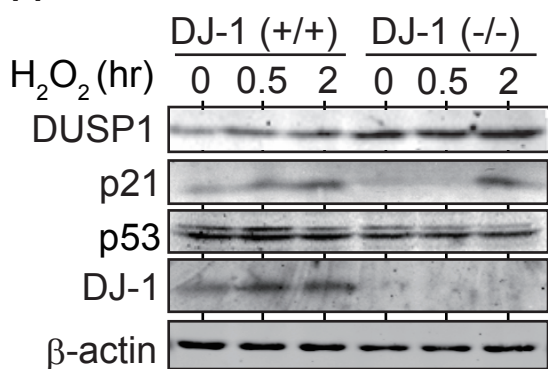
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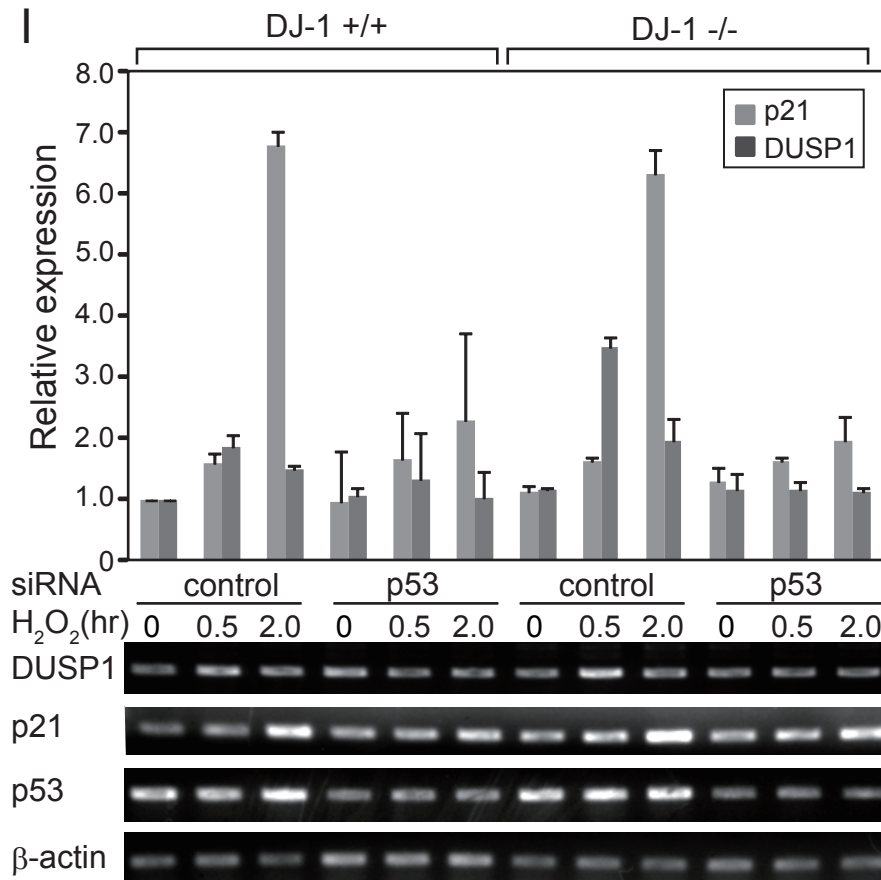
G Real-time PCR



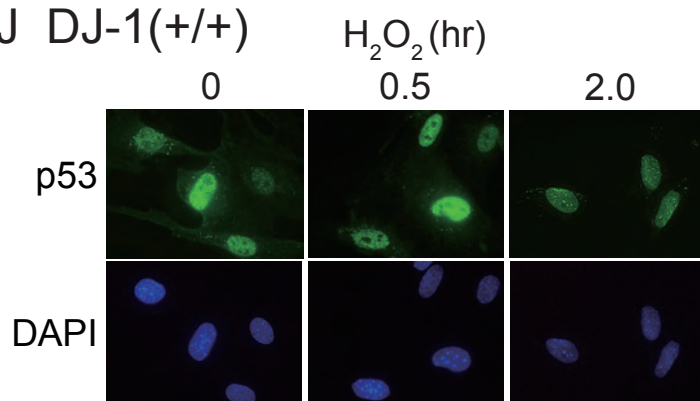
H



I



J DJ-1(+/+)



K DJ-1 (-/-)

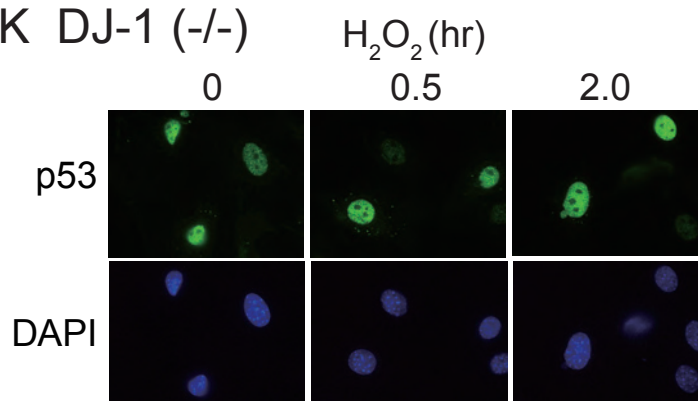


Fig. 4-2

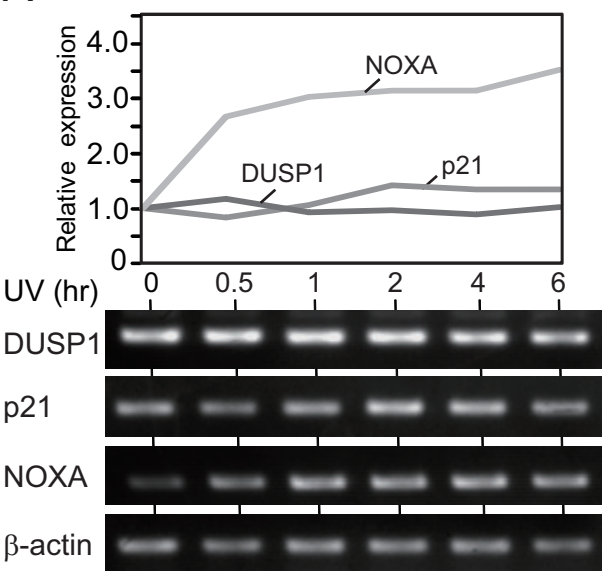
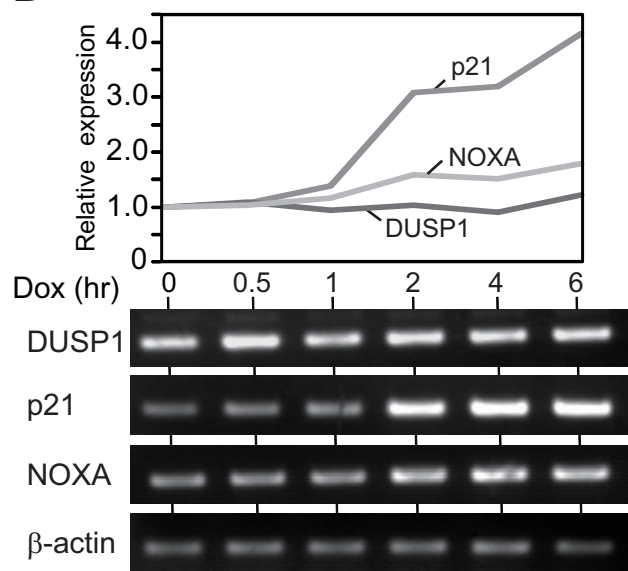
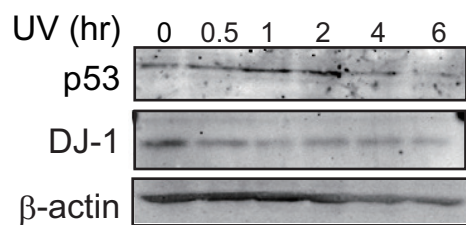
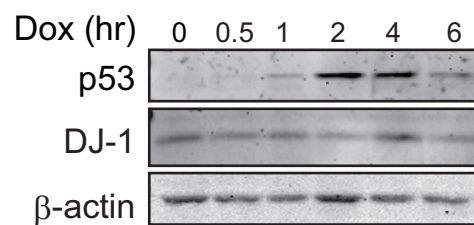
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Fig. 5

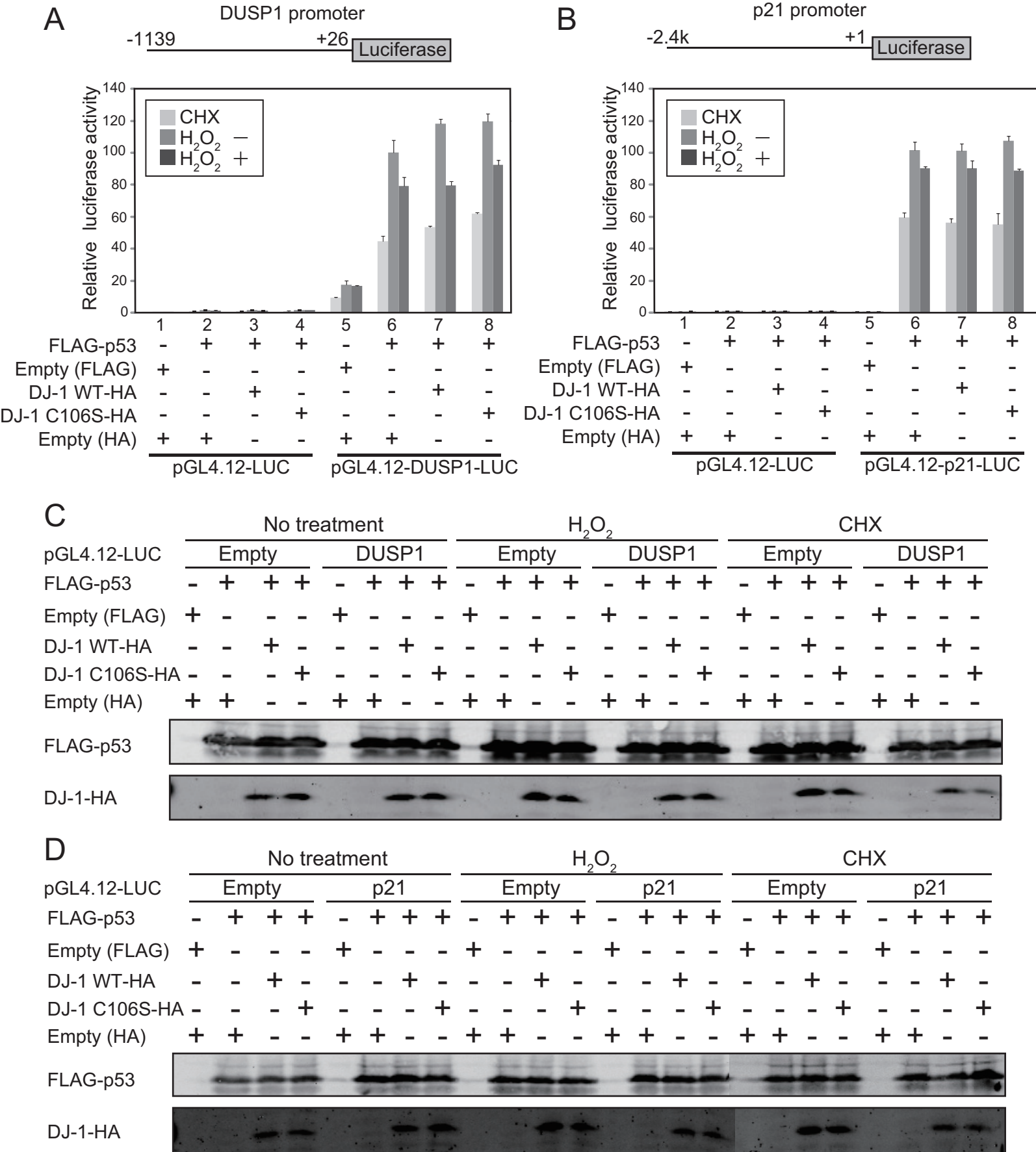


Fig. 6

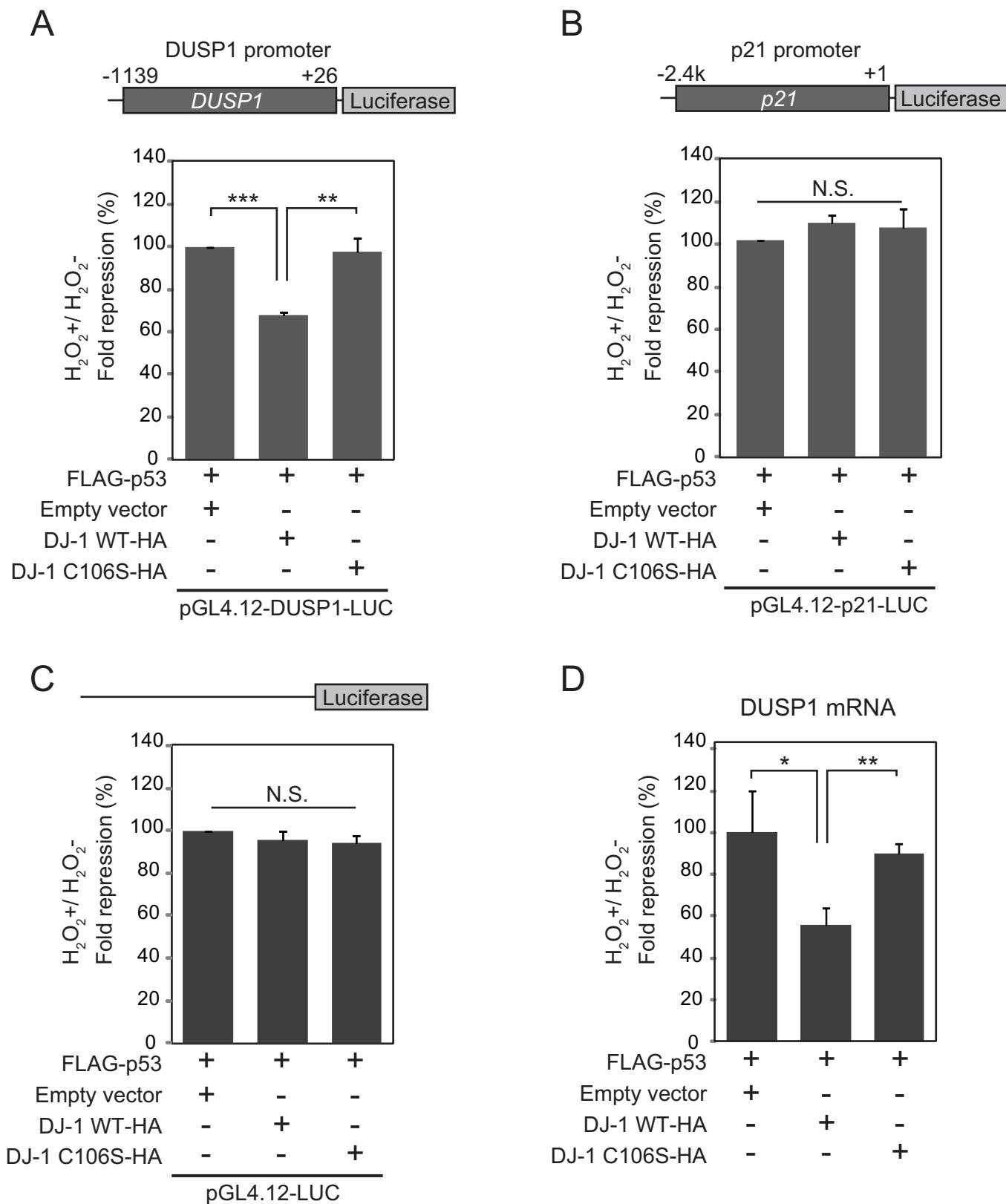


Fig. 7

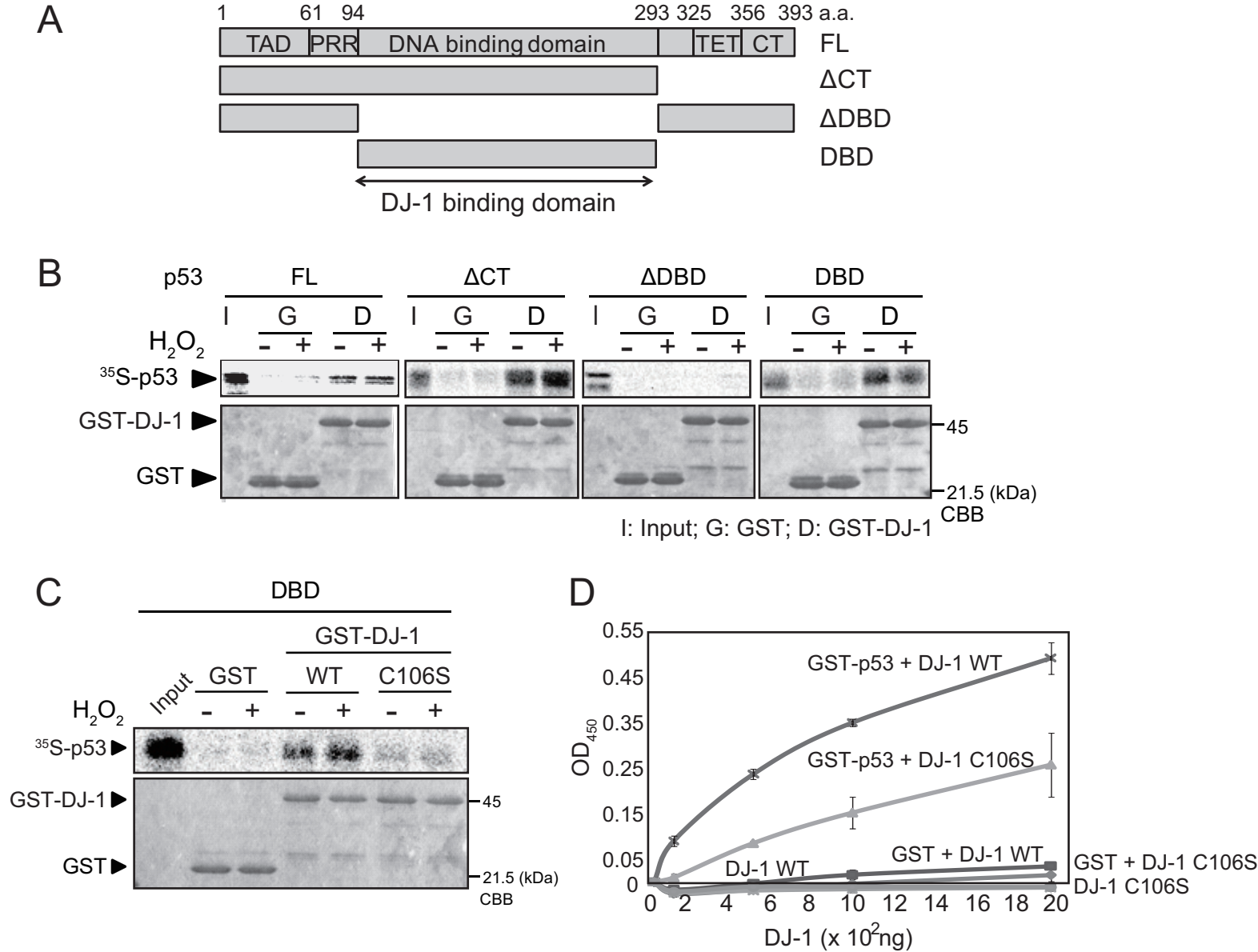


Fig. 8

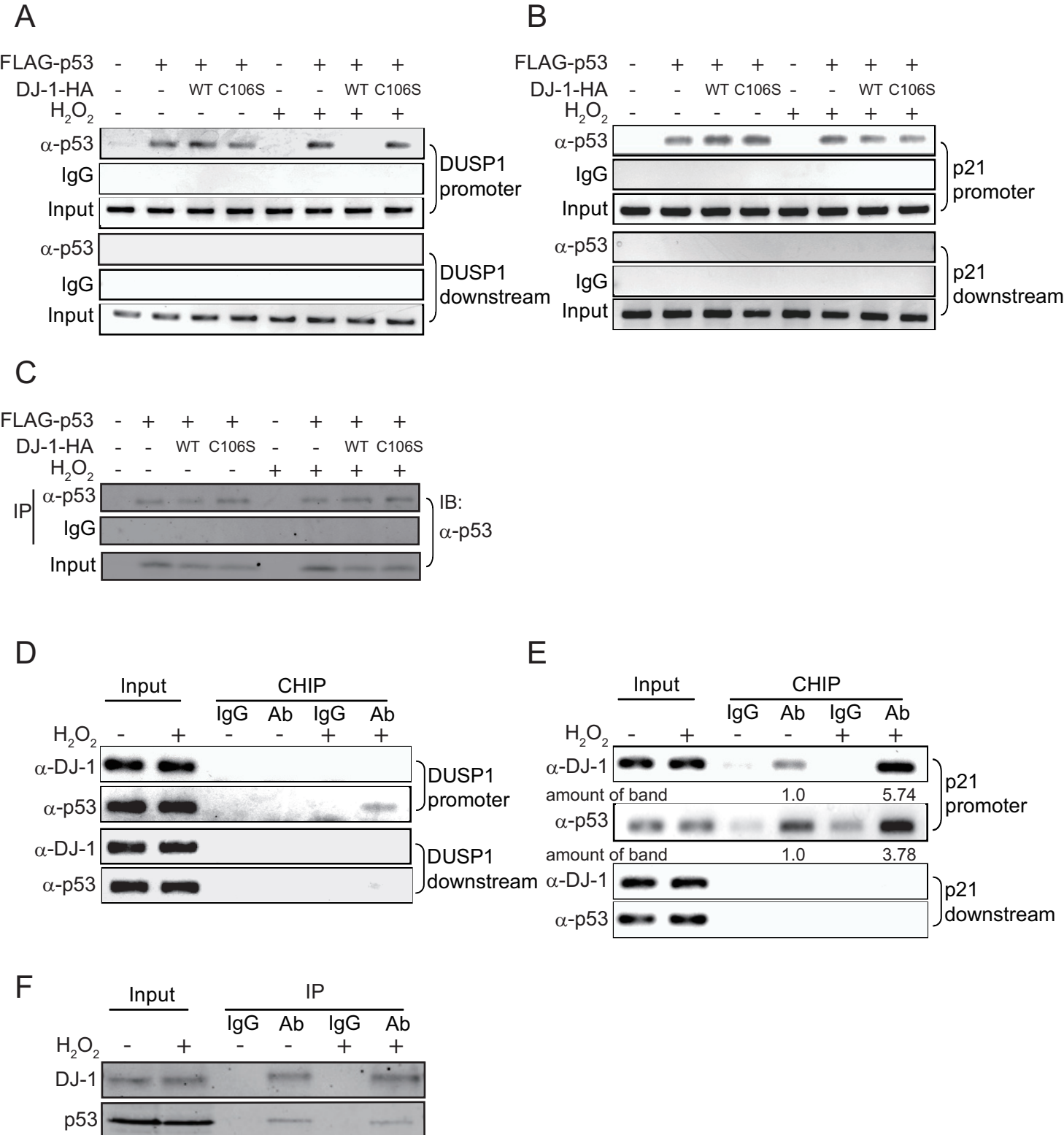


Fig. 9

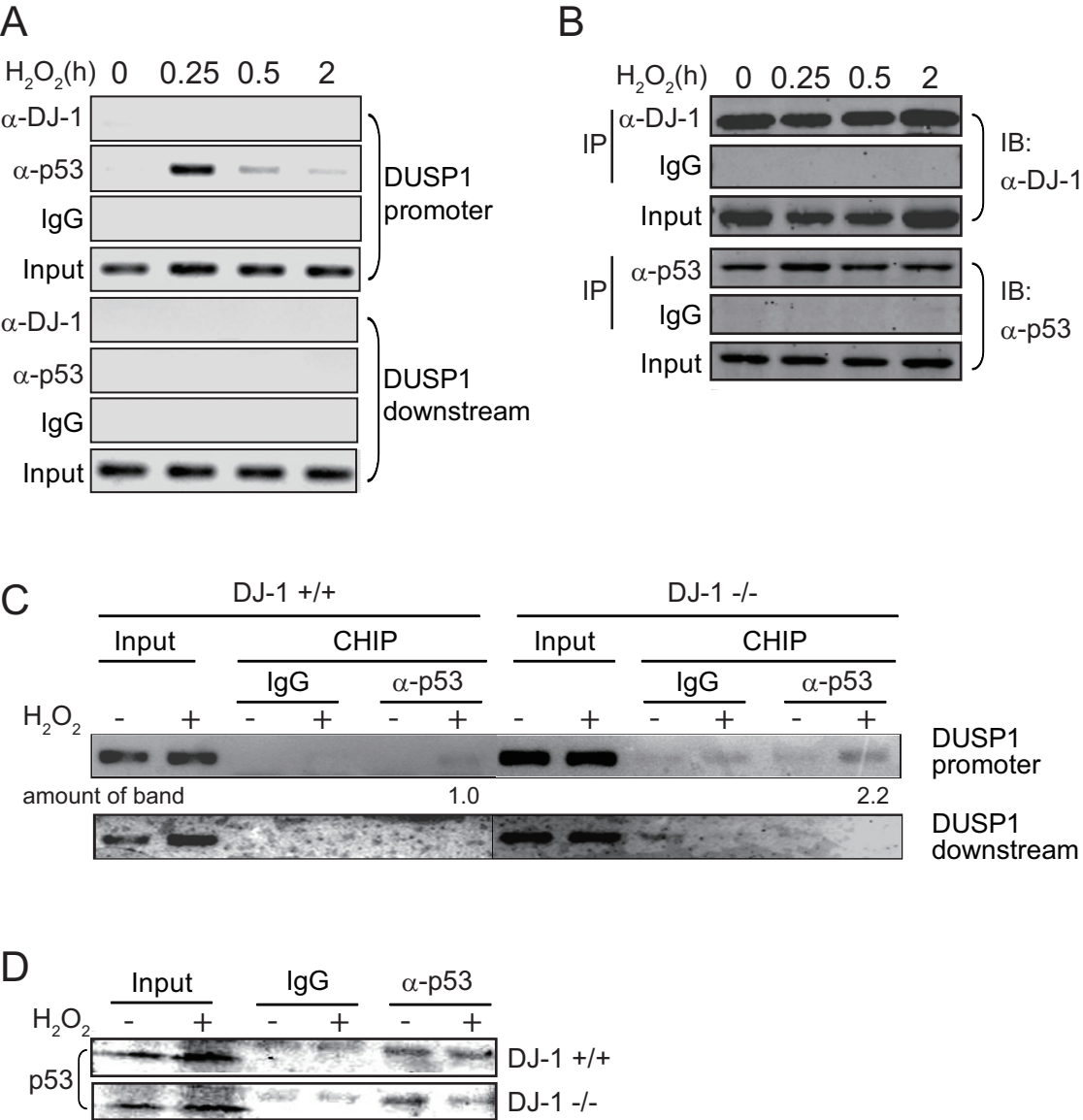


Fig. 10

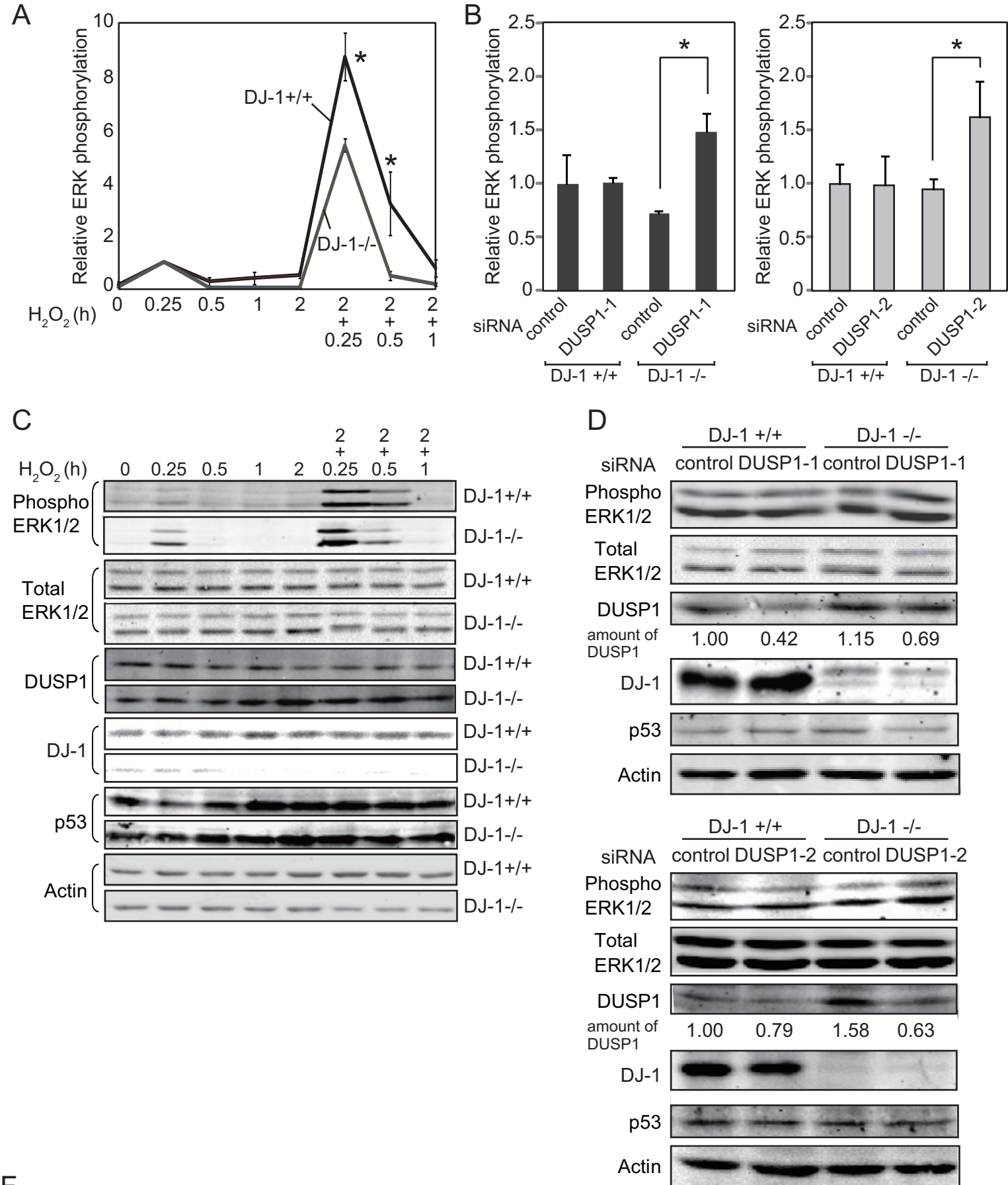


Fig. 11

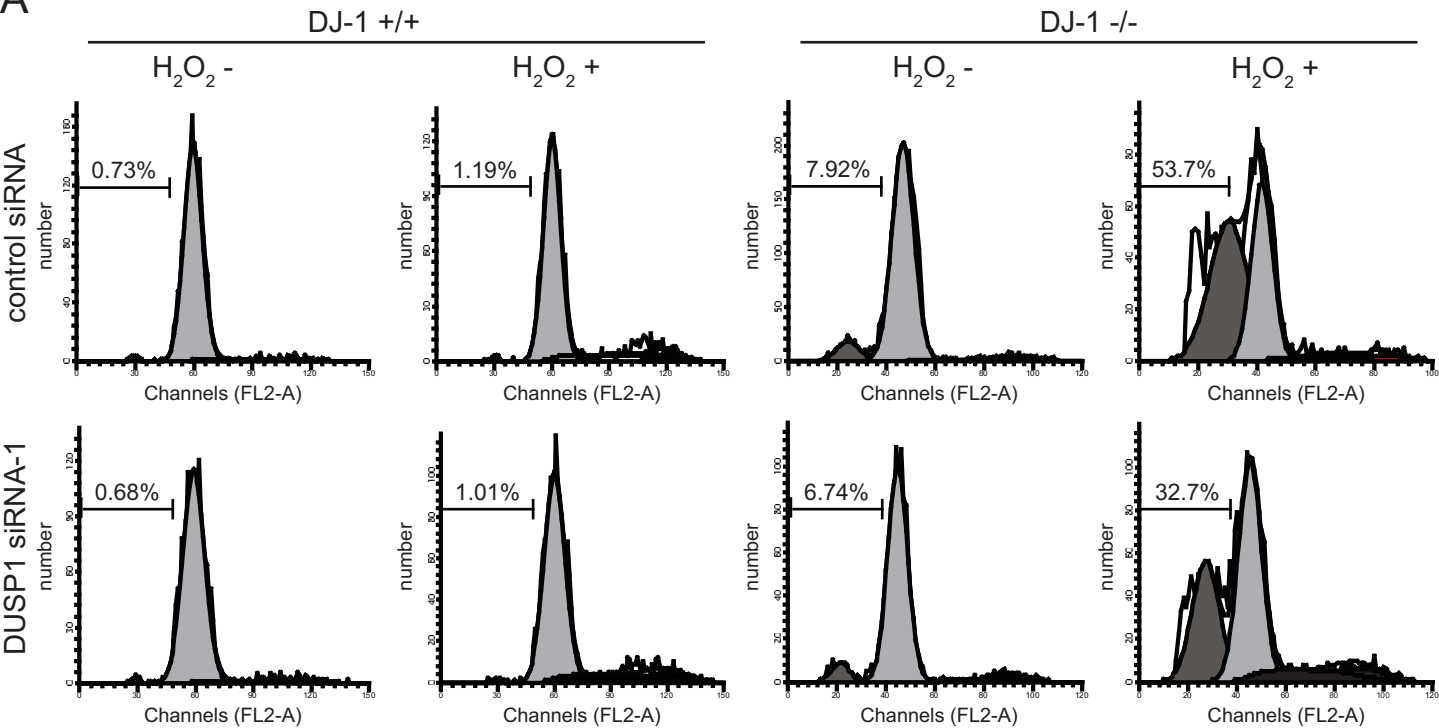
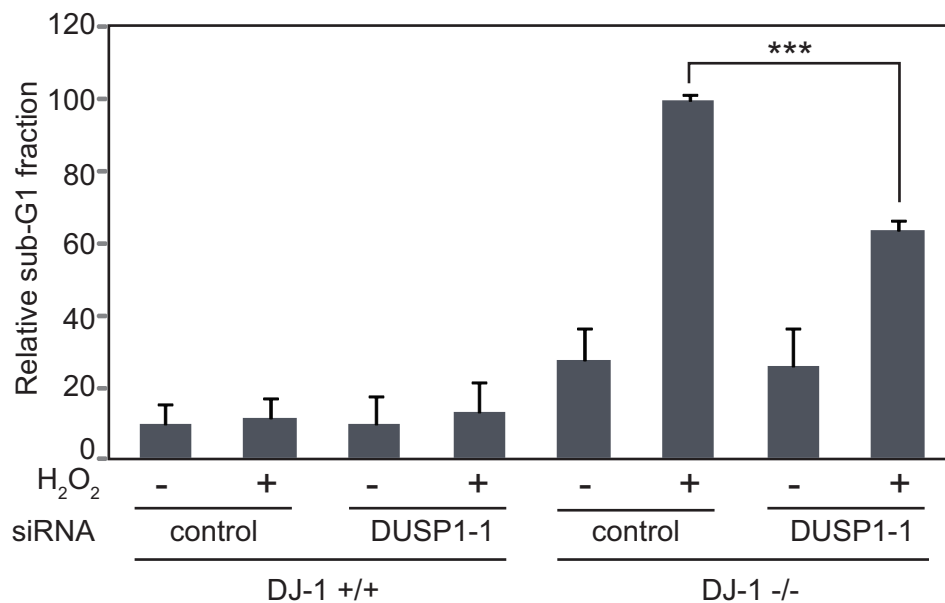
A**B**

Fig. 12

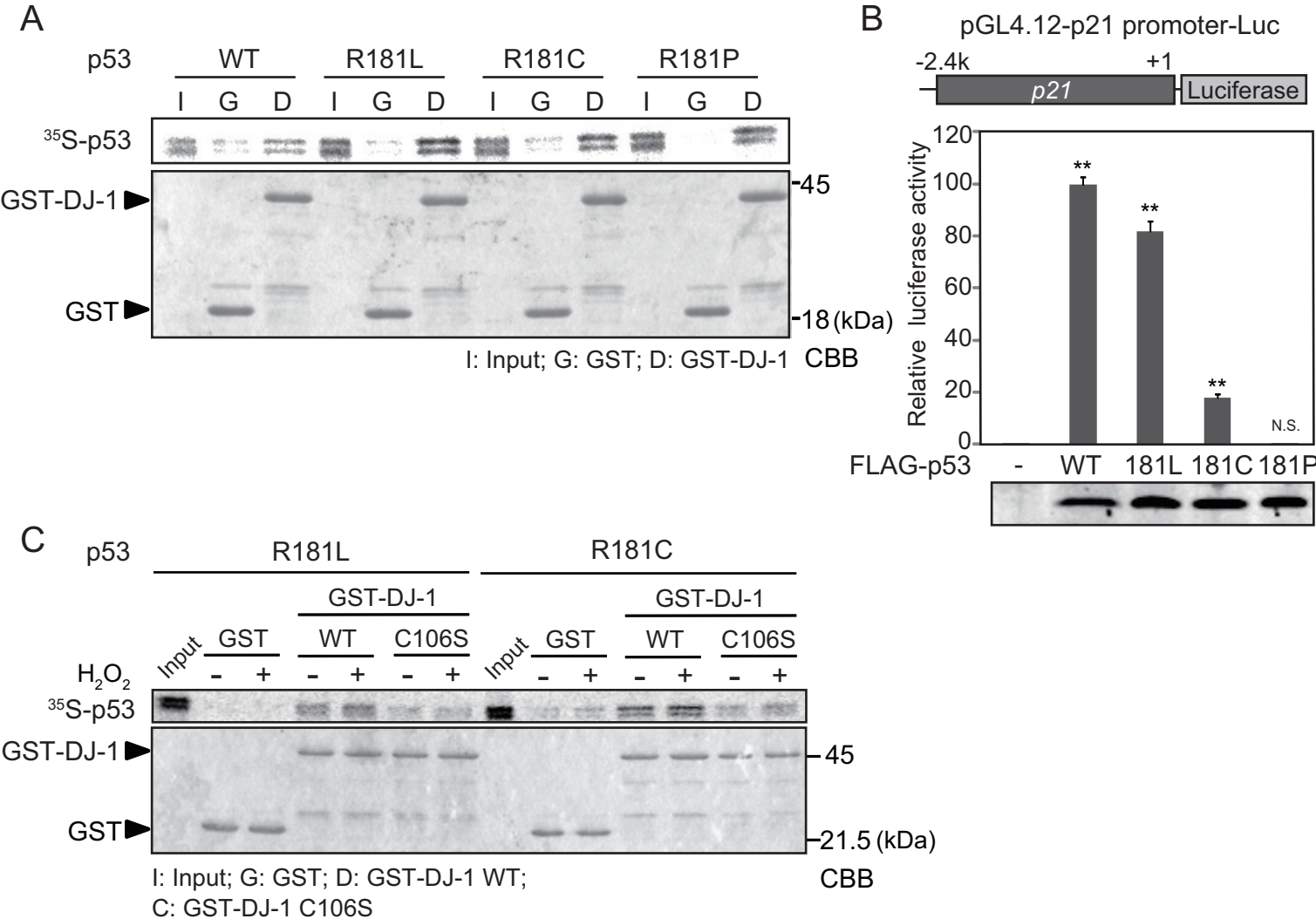


Fig. 13

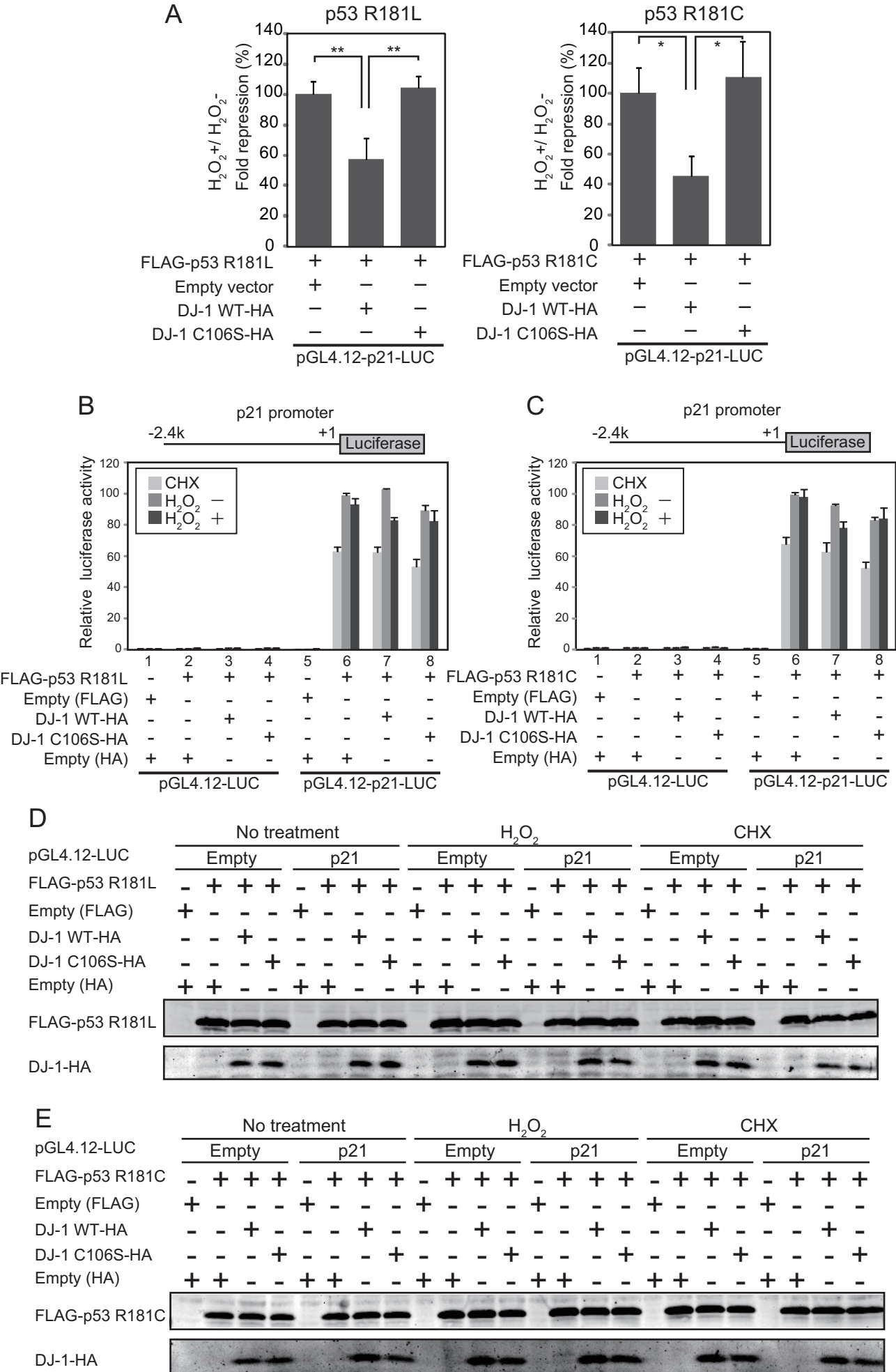


Fig. 14

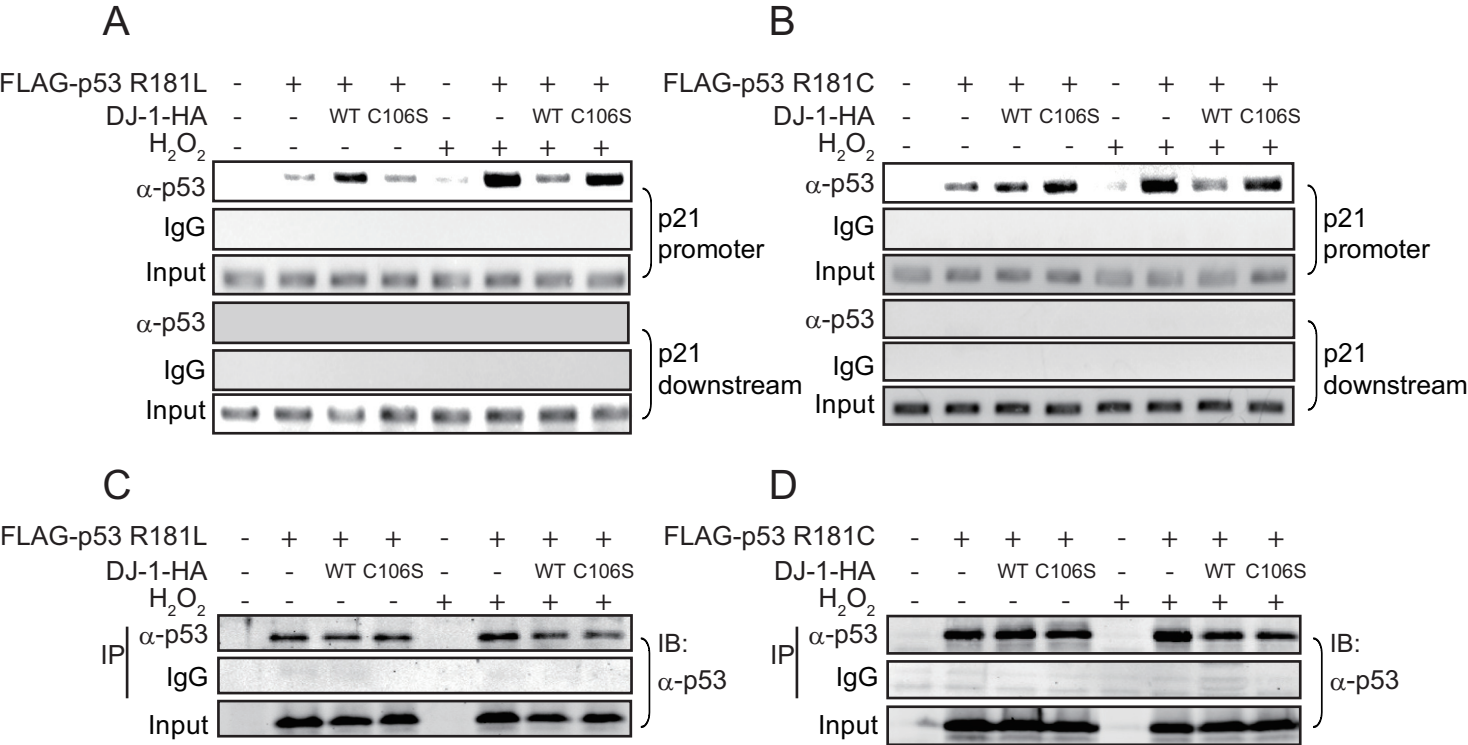
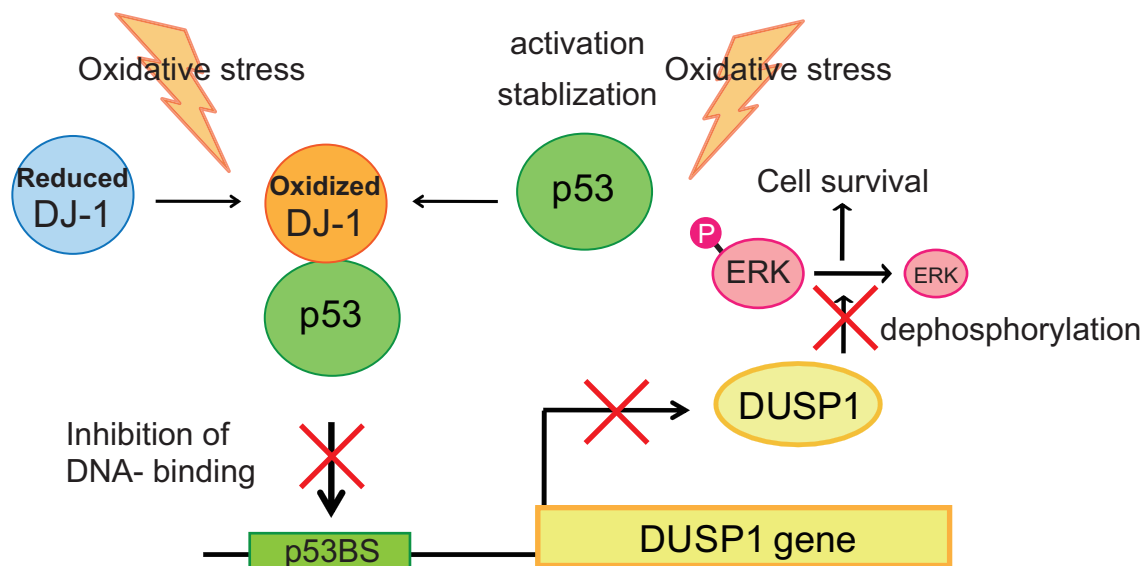


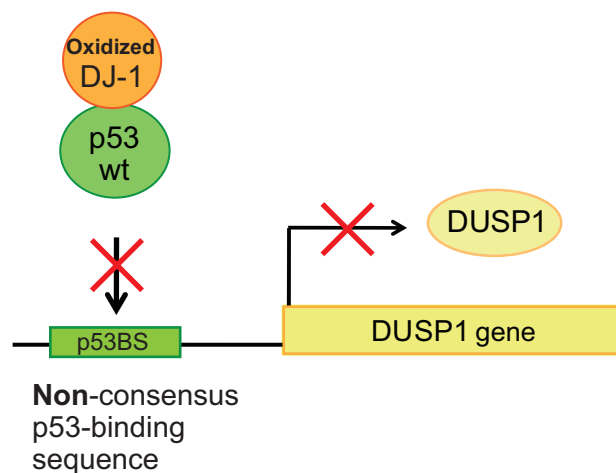
Fig. 15

A

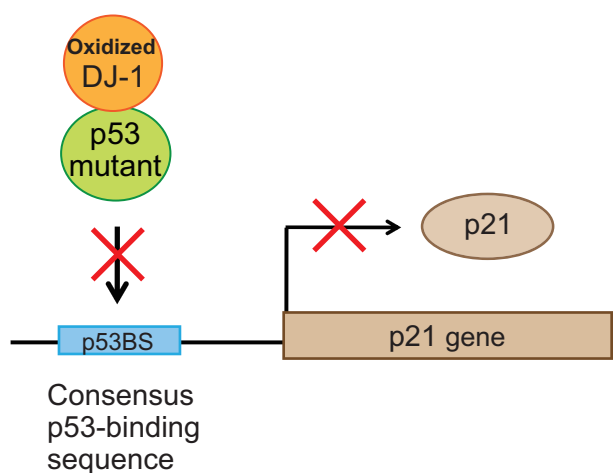


B

Low DNA-binding affinity



Low DNA-binding affinity



High DNA-binding affinity

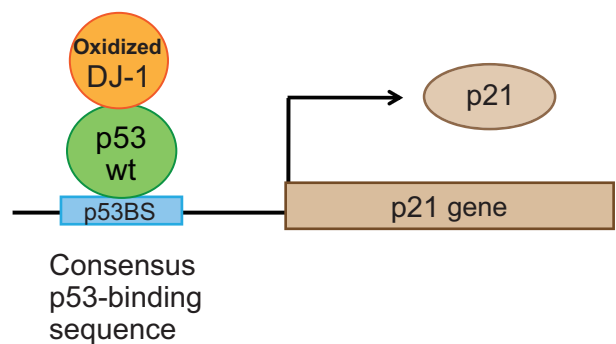


Fig. 16