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DJ-1, an oncogene and causative gene for familial Parkinson's disease, is essential for SV40 transformation in mouse fibroblasts through up-regulation of c-Myc

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ABSTRACT

Simian virus 40 (SV40) is a tumor virus and its early gene product large T-antigen (LT) is responsible for the transforming activity of SV40. Parkinson's disease causative gene *DJ-1* is also a *ras*-dependent oncogene, but the mechanism of its oncogene function is still not known. In this study, we found that there were no transformed foci when fibroblasts from DJ-1-knockout mice were transfected with LT. We also found that DJ-1 directly bound to LT and that the expression level of c-Myc in transformed cells was parallel to that of DJ-1. These findings indicate that DJ-1 is essential for SV40 transformation.

Keywords: SV40, DJ-1, transformation, p53

Abbreviations: SV40, simian virus 40; LT, large T-antigen.

1. Introduction

Simian virus 40 (SV40) is a tumor virus and its transformation mechanism in cells has been analyzed in detail. An early gene product of SV40, large T-antigen (LT), has been shown to be responsible for cell immortalization activity of SV40. Transformation of cells by LT depends on the cell type and on the presence or absence of other factors such as small t-antigen, another early gene product of SV40. LT binds to the DNA-binding region of p53, resulting in inactivation of p53, thereby activating the E2F-depenedent pathway [see review 1 and original references therein]. Recent findings have shown that the p53-LT complex promotes malignant cell growth through activation of the insulin-like growth factor-1-signaling pathway, indicating that binding of p53 (and pRB) by LT is a necessary, but not sufficient, condition for transformation [2]. Furthermore, Carbone et al. found that suppression of late viral products is an important step in SV40 transformation of human mesothelial cells [3]. The precise mechanism of SV40 transformation is, therefore, still not fully dissolved.

DJ-1 was first identified by our group as a novel oncogene that transformed mouse NIH3T3 cells in cooperation with activated *ras* [4] and was later found to be a causative gene for a familial form of Parkinson's disease [5]. DJ-1 has multiple functions, including transcriptional regulation [6-9], anti-oxidative stress function [10-12], mitochondrial regulation [11, 13-15], and functions as a chaperone [16] and protease [17-19]. DJ-1 binds to several signaling molecules such as Daxx, HIPK1 and Ask1 to repress the apoptosis signal. DJ-1 also binds to p53 and PTEN to repress their activities, thereby facilitating cell growth [20-23]. Although an elevated expression level of DJ-1 has been reported in many cancer cells and tissues [24-26], the molecular mechanism of the oncogene function of DJ-1 is still not known.

In this study, we found that whereas DJ-1 (+/+) fibroblasts transfected with SV40 LT produced many transformed foci, DJ-1 (-/-) fibroblasts transfected with SV40 LT produced no foci and that DJ-1 directly bound to both p53 and SV40 LT, suggesting that the transformation function of SV40 *in vitro* requires repression of p53 activity by DJ-1.

2. Materials and methods

2.1. Immortalization of cells with SV40 LT

DJ-1-heterozygous knockout mice [27] were kindly provided by J. Shen, and DJ-1-homozygous knockout mice (DJ-1(-/-)) and wild-type mice with the same background (DJ-1(+/+)) were obtained. Newborn mice with genotypes of DJ-1(-/-) and DJ-1(+/+) at 1 day after birth were cut with scissors, digested with trypsin, and seeded on a 10-cm dish in DMEM with 10% calf serum. Cells after two passages were then transfected with an expression vector for SV40 LT, pMTI [28]. About two weeks after transfection, immortalized cells appeared and were cloned. These cells were named T(D-/-) and T(D+/+) cells, respectively. Approximately 5% of the cells were immortalized.

2.2. *Transformation and anchorage-independent growth assays*

For transforming assays, $4x10^5$ T(D-/-) and T(D+/+) cells in 10-cm dishes were cultured in a medium with 2% calf serum for two weeks and then stained with Giemsa (Merck). For anchorage-independent growth assays, transformed colonies were isolated and cultured in the medium containing 2% calf serum. After one week of cell culture, $4x10^3$ cells on 6-cm dishes were subjected to assays for anchorage-independent growth as described previously [23]. Experiments with duplicated cultures were carried out three times.

2. 3. Western blotting

Proteins were extracted from cells with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1% sodium deoxychloate and 0.1% SDS, loaded on 12% SDS-polyacrylamide gels, and subjected to Western blotting. Antibodies used in this study were as follows: anti-actin (Chemicon), anti-mouse DJ-1 [described previously [29]], anti-SV40 LT (Pab108, Lab Vision), anti-p53 (Pab240, Santa Cruz) and anti-c-Myc (N262, Santa Cruz), anti-HA (MBL) and anti-Cdk2 (Santa Cruz) antibodies. After membranes had been reacted with primary antibodies, they were reacted with Alexaflour 680-conjugated anti-mouse (Molecular Probes), Alexaflour 680-conjugated anti-rabbit (Molecular Probes) or IRDye800-conjugated anti-mouse (Rockland)

antibody, and proteins were visualized using an infrared imaging system (Odyssey, LI-COR).

2.4. Co-immunoprecipitation

Proteins were extracted from cells with a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, 0.1 mM Na3VO4, 20 mM NaF, 10 mM sodium β-glycerophosphate, 0.1 mM AEBSF, 1 μg leupeptin and 1 μg pepstatin. Proteins were immunoprecipitated with an agarose-conjugated anti-FLAG antibody (M2, Sigma) or with agarose-conjugated mouse IgG (Sigma) and precipitates were analyzed by Western blotting with anti-p53 (Pab240, Santa Cruz), anti-SV40 LT (Pab108, Lab Vision) and anti-FLAG (M2 Sigma) antibodies followed by visualization as described above.

2.5. Pull-down assay

Preparation of GST-DJ-1 and pull-down assays were described previously [6].

2.6. Cdk2 activity

Cdk2 activity was measured using proteins extracted from DJ-1(+/+) and DJ-1(-/-) primary cells as described previously [30].

2.7. Statistical analyses

Data are expressed as means ± S.E. Statistical analyses were performed using analysis of variance (one-way ANOVA) followed by unpaired Student's *t*-test.

3. Results

3.1. No SV40 large T-dependent transformed foci in DJ-1-knockout mice-derived cells

Whole newborn mice with DJ-1(+/+) and DJ-1(-/-) backgrounds at 1 day after birth were cut with scissors and digested with trypsin, and then primary cells were obtained after two passages of trypsinized cells. Almost all of the primary cells obtained were morphologically fibroblasts, and there were few or no cells such as mesothelial cells that were stained positive for calretinin. Expression levels of proteins related to the Erk-signaling pathway, Cdk2 and p21, were examined by Western blotting (Fig. 1A). The results showed that while expression levels of Raf and MEK and their phosphorylated forms were not different between DJ-1(+/+) and DJ-1(-/-)

primary cells, expression levels of phosphorylated Erk2 and c-Myc and expression level of p21 in DJ-1(-/-) primary cells were reduced and increased, respectively, compared with those in DJ-1(+/+) primary cells. Cdk2 activity was found to be reduced in DJ-1 (-/-) primary cells (Fig. 1B). DJ-1(+/+) and DJ-1(-/-) primary cells were then transfected with an expression vector for SV40 large T-antigen (LT). Two weeks after transfection, immortalized cell colonies appeared and were cloned. These immortalized cells were named T(D+/+) and T(D-/-) cells. Expression of SV40 LT and DJ-1 in these cells after more than 10 passages was confirmed by Western blotting (Fig. 2A), and there was little or no difference between the morphology of T(D+/+) cells and that of T(D-/-) cells (Fig. 2B). To examine the focus-forming ability of cells, T(D+/+) and T(D-/-) cells were cultured in a medium with 2% calf serum for two weeks and then stained with Giemsa (Fig. 2C). It was found that while T(D+/+) cells gave an average of 162 transformed foci in a 10-cm dish, T(D-/-) cells gave no foci. Morphology of cells containing a transformed focus is shown in Fig. 2C (lower panels). T(D+/+) and T(D-/-) cells were then cultured in a soft agar medium and their anchorage-independent growth activity was examined. NIH3T3 cells were used as a negative control. As shown in Fig. 2D, T(D+/+) cells grew well and made large colonies, but T(D-/-) cells did not grow in the soft agar medium. These results indicate that SV40 LT-induced immortalization of cells with anchorage-independent growth activity requires DJ-1.

T(D+/+), T(D-/-) and primary cells from wild-type and DJ-1-knockout mice were injected subcutaneously into 6-week-old nude mice and their longevity was examined. All of the cells injected were fibroblasts. All of the five mice injected with T(D+/+) cells died within 58 days after injection, while other mice injected with T(D-/-) and primary cells from wild-type and DJ-1-knockout mice still survived (data not shown). Although typical tumors such as sarcoma were not observed in mice injected with T(D+/+) cells, some tumor cells, which might to be too small to be identified under this condition, are thought to have injured mice.

3.2. Binding of DJ-1 to SV40 LT

To examine the interaction of DJ-1 with SV40 LT, proteins extracted from T(D+/+) and

T(D-/-) cells were immunoprecipitated with an anti-LT antibody or with non-specific IgG, and precipitates were then analyzed by Western blotting with anti-mouse DJ-1, anti-LT and anti-p53 antibodies. As shown in Fig. 3A, p53 used as a positive control and DJ-1 in T(D+/+) cells but not in T(D-/-) cells were co-immunoprecipitated with SV40 LT. Pull-down experiments were then carried out using GST-DJ-1, which was expressed in and purified from *E. coli*, and ^{35}S-labeled SV40 LT and p53, which were synthesized in reticulocyte lysates *in vitro*. The results showed that DJ-1 bound to both SV40 LT and p53 (Fig. 3B), indicating direct binding of DJ-1 to SV40 LT and p53.

3.3. Enhanced expression of c-Myc by DJ-1 in SV40 LT-transformed cells

T(D-/-) cells were re-transfected with an expression vector for HA-tagged DJ-1, and T(D-/-) cells expressing DJ-1-HA, T(D-/-)D cells, were established. The expression levels of c-Myc, DJ-1, SV40 LT, p53, Cdk2 and p21 in T(D+/+), T(D-/-) and T(D-/-)D cells were examined by Western blotting and intensities of bands were quantified (Figs. 4A and 4B, respectively). The results showed that while expression levels of SV40 LT, p53 and Cdk2 were not changed, the expression level of c-Myc was parallel to that of DJ-1 in T(D+/+) and T(D-/-)D cells, and the order of expression levels of c-Myc and DJ-1 was T(D-/-)D, T(D+/+) and T(D-/-) cells. The reduced expression level of c-Myc in T(D-/-) cells is thought to be affected by that in primary DJ-1(-/-) cells shown in Fig. 1. A high expression level of p21 in T(D-/-) cells compared with that in T(D+/+) and T(D-/-)D cells was also observed. Colony size of these cells that had been cultured in a soft agar medium was also found to be parallel to expression levels of c-Myc and DJ-1 (Fig. 4C). These results suggest that DJ-1 up-regulates a pathway(s) that leads to activation of c-Myc.

4. Discussion

In this study, we found that transformation of mouse fibroblasts by SV40 *in vitro* requires the presence of DJ-1 and that DJ-1 directly binds to SV40 large LT, which is responsible for SV40

immortalization and/or transformation dependent on cell type. Although the transformation mechanism by SV40 has long been studied, this is the first report showing that DJ-1 is an essential protein for SV40 transformation. In the process of SV40 transformation, it is thought that SV40 LT binds to the DNA-binding region of p53 to repress its transcriptional activity, leading to repression of p21 expression followed by activation of Cdk and then inhibition of Rb phosphorylation, thereby activating E2F that leads to up-regulation of c-*myc* expression [1]. SV40 LT also binds to Rb to inhibit its activity, resulting in activation of E2F [1]. Since DJ-1 binds both to p53 and LT and LT-dependent transformation *in vitro* does not occur in DJ-1-knockout cells, it is thought that repression of p53 activity by DJ-1 is at least necessary for SV40 transformation and alternatively that binding of LT to p53 requires DJ-1. Indeed, the expression level of p21 was increased both in primary and LT-immortalized DJ-1(-/-) cells, and Cdk2 activity was decreased in primary DJ-1(-/-) cells. Furthermore, the expression level of c-Myc in LT-transformed DJ-1(+/+) cells and DJ-1(-/-) cells re-introduced with DJ-1 was found to be parallel to that of DJ-1, affecting the colony size of transformed cells in a soft agar medium. Activated Erk phosphorylates c-Myc, resulting in prevention of its ubiquitination and subsequent proteasomal degradation. Since the expression levels of c-Myc and phosphorylated/activated Erk2 were decreased in primary DJ-1(-/-) cells compared to those in DJ-1(+/+) cells (Fig. 1), immortalized DJ-1(+/+) and DJ-1(-/-) cells are thought to have these characters. It has been reported that DJ-1 activated the Erk signaling pathway through increased phosphorylation of Erk1/2 [31], suggesting that DJ-1 is a positive regulator upstream of c-Myc. Since DJ-1 does not directly bind to c-Myc [4], the activity levels of SV40 LT and p53, which are activated and repressed by DJ-1, respectively, may affect the expression level of c-Myc. The present findings suggest that DJ-1 activates c-Myc by several pathways, including the SV40 LT/p53-dependent Cdk/p21 pathway and DJ-1-dependent Erk pathway, and will contribute to the understanding of oncogenic activity of DJ-1.

Acknowledgements

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Legends of figures

Fig. 1. Changes in expression of activated Erk, p21 and c-Myc in primary DJ-1(+/+) and DJ-1(-/-) cells.

A. Proteins were extracted from primary cells from wild-type and DJ-1-knockout mouse and expression levels of DJ-1, Erk pathway-related proteins, p21, Cdk2 and actin were analyzed by Western blotting as described in Materials and methods. The results showed reduced expression levels of phosphorylated Erk2 and c-Myc and increased expression level of p21 in

DJ-1(-/-)-derived cells.

B. Cdk2 activity was measured using proteins extracted from primary cells with DJ-1(+/+) and DJ-1(-/-) backgrounds. The results showed reduced Cdk2 activity in DJ-1(-/-)-derived cells.

Fig. 2. Requirement of DJ-1 in SV40 transformation.

A. Proteins were extracted from T(D+/+) and T(D-/-) cells and expression levels of SV40 LT, DJ-1 and actin were analyzed by Western blotting as described in Materials and methods.

B. Morphology of T(D+/+) and T(D-/-) cells is shown. The results of A and B showed that the expression levels of LT in LT-immortalized (D+/+) and (D-/-) cells were not changed and that there was little difference in morphology between T(D+/+) and T(D-/-) cells.

C. T(D+/+) and T(D-/-) cells were cultured in a medium with 2% calf serum for two weeks and then stained with Giemsa (upper panel). Numbers beside figures indicate the average number of transformed foci in a 10-cm dish from six experiments. Morphology of cells containing foci is shown (lower panel). The results showed that about 0.4% of the cells were transformed.

D. T(D+/+) and T(D-/-) cells were cultured in a soft agar medium for two weeks and morphology of cell is shown.

Since no transformed foci appeared in cultured T(D-/-) cells and since transformed T(D+/+) cells grew in an anchorage-independent manner, DJ-1 is necessary for LT-induced transformation *in vitro*.

Fig. 3. Binding of DJ-1 to SV40 LT.

A. Proteins were extracted from T(D+/+) and T(D-/-) cells and immunoprecipitated with an anti-LT antigen antibody or with IgG. Precipitates were then analyzed by Western blotting with anti-DJ-1, anti-LT and anti-p53 antibodies as described in Materials and methods.

B. GST-DJ-1 and ^{35}S-labeled SV40 LT or p53 were mixed and pull-down assays were carried out as described in Materials and methods. An autoradiograph of the results is shown in the upper part of the figures, and GST and GST-DJ-1 used were stained with Coomassie Brilliant

Blue (CBB) and shown in the lower part of the figures.

The results showed that DJ-1 directly binds to LT.

Fig. 4. Enhanced expression of c-Myc in SV40-transformed cells expressing a high level of DJ-1.

A. Proteins were extracted from T(D+/+), T(D-/-) and T(D-/-)D cells and expression levels of SV40 LT, DJ-1, p53, Cdk2, p21 and actin were analyzed by Western blotting as described in Materials and methods.

B. Intensity of bands in Fig. 4A was quantified and that corresponding to c-Myc and DJ-1 is shown. Experiments were carried out three times.

C. Morphology of colonies of T(D+/+), T(D-/-) and T(D-/-)D cells cultured in a soft agar medium is shown.

Since T(D-/-) cells re-introduced with DJ-1 grew in an anchorage-independent manner concomitant with increased expression of c-Myc, DJ-1 is a factor necessary for LT-induced transformation *in vitro*.

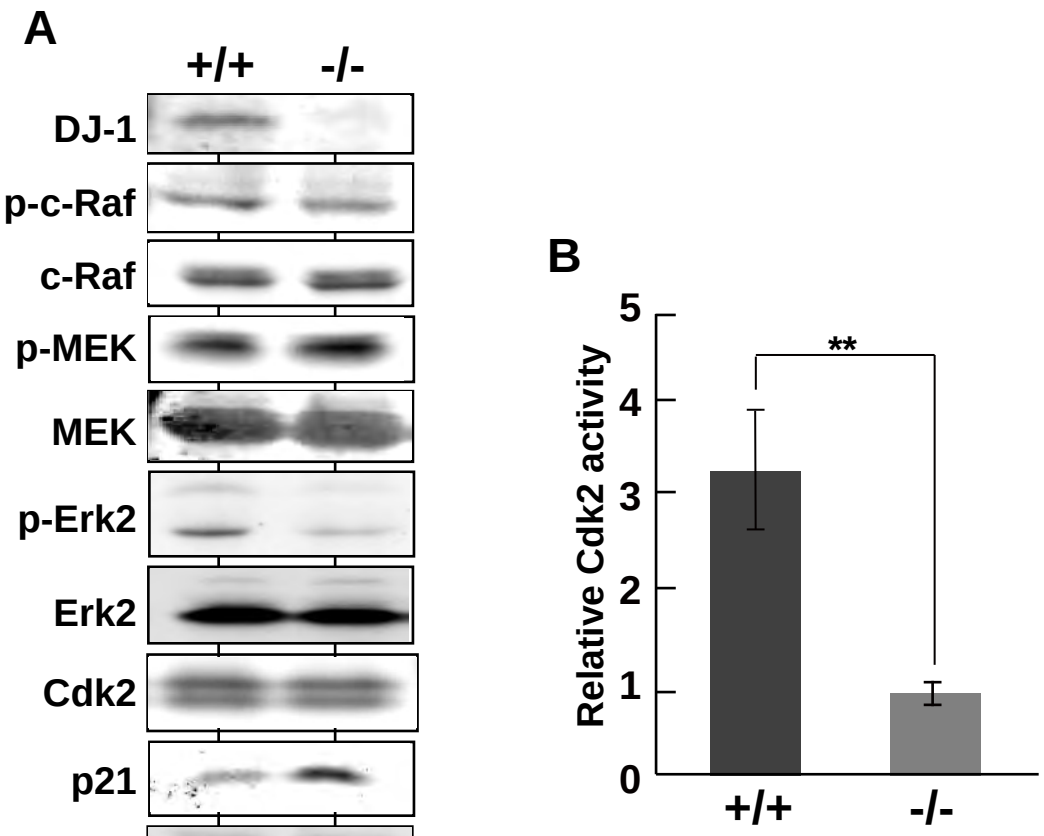
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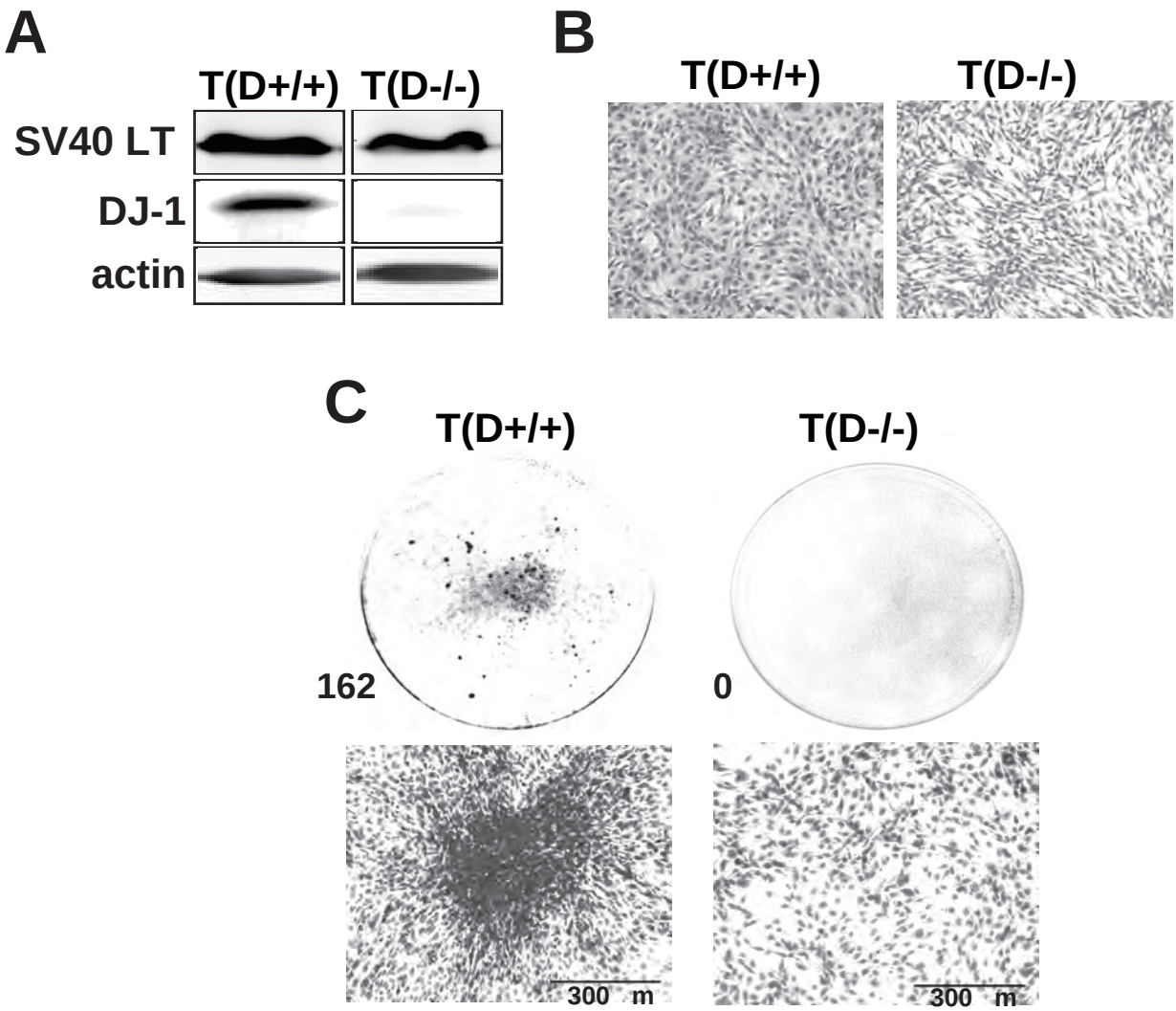
Fig. 1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T(D+/+)
T(D-/-)

Fig. 2

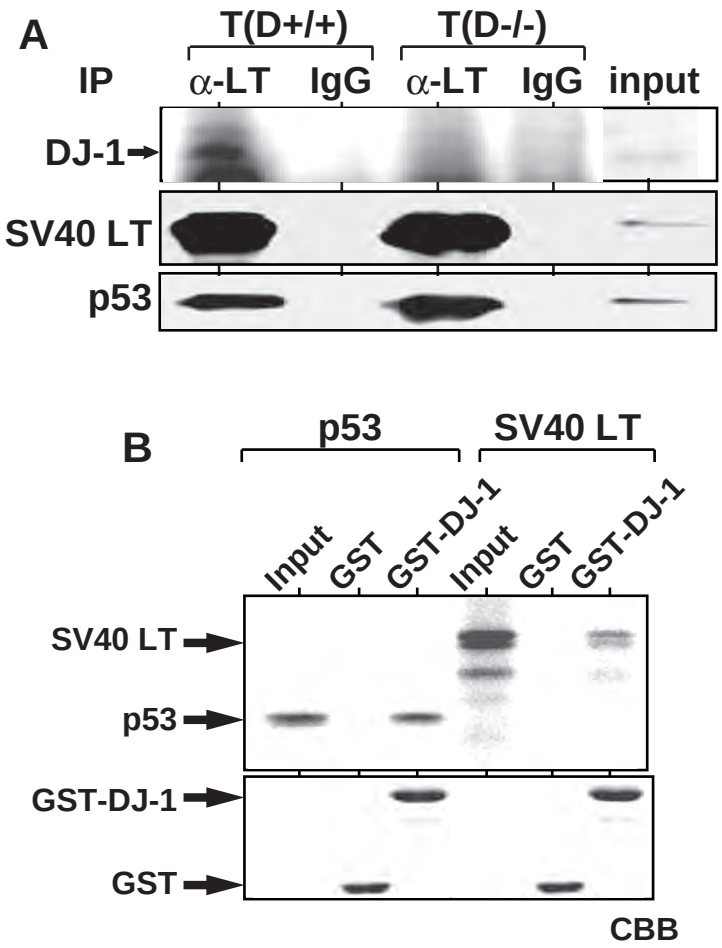
A
T(D+/+)
T(D-/-)
IP
α-LT
IgG
α-LT
IgG
input
DJ-1
SV40 LT
p53
B
p53
SV40 LT
Input
GST
GST-DJ-1
Input
GST
GST-DJ-1
SV40 LT
p53
GST-DJ-1
GST
CBB

Fig. 3

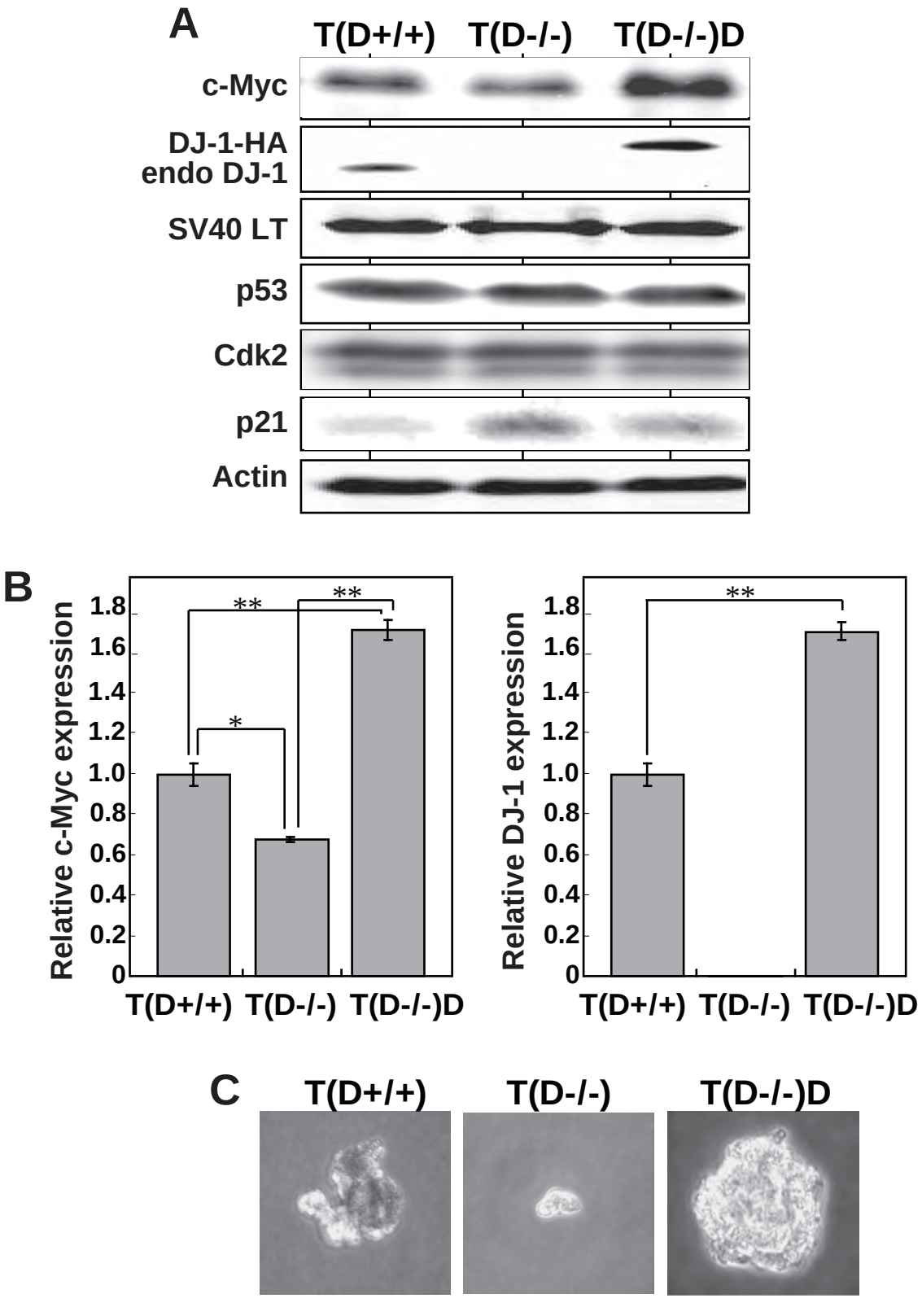
A
T(D+/+)
T(D-/-)
T(D-/-)D
c-Myc
DJ-1-HA
endo DJ-1
SV40 LT
p53
Cdk2
p21
Actin
B
Relative c-Myc expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
**
*
T(D+/+)
T(D-/-)
T(D-/-)D
Relative DJ-1 expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
T(D+/+)
T(D-/-)
T(D-/-)D
C
T(D+/+)
T(D-/-)
T(D-/-)D

Fig. 4