Unexpected mitochondrial matrix localization of Parkinson's disease-related DJ-1 mutants but not wild-type DJ-1

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DJ-1 has been identified as a gene responsible for recessive familial Parkinson's disease (familial Parkinsonism), which is caused by a mutation in the PARK7 locus. Consistent with the inferred correlation between Parkinson's disease and mitochondrial impairment, mitochondrial localization of DJ-1 and its implied role in mitochondrial quality control have been reported. However, the mechanism by which DJ-1 affects mitochondrial function remains poorly defined, and the mitochondrial localization of DJ-1 is still controversial. Here, we show the mitochondrial matrix localization of various pathogenic and artificial DJ-1 mutants by multiple independent experimental approaches including cellular fractionation, proteinase K protection assays, and specific immunocytochemistry. Localization of various DJ-1 mutants to the matrix is dependent on the membrane potential and translocase activity in both the outer and the inner membranes. Nevertheless, DJ-1 possesses neither an amino-terminal alpha-helix nor a predictable matrix-targeting signal, and a post-translocation processing-derived molecular weight change is not observed. In fact, wild-type DJ-1 does not show any evidence of mitochondrial localization at all. Such a mode of matrix localization of DJ-1 is difficult to explain by conventional mechanisms and implies a unique matrix import mechanism for DJ-1 mutants.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disease affecting 1% of the population over the age of 65. PD is characterized by motor symptoms that are derived from a loss of dopaminergic neurons in the substantia nigra; however, neurons in brain regions associated with nonmotor symptoms are also affected in PD. Although PD commonly arises sporadically, in some cases, the disease is familial and inherited. PARKIN (PARK2), PINK1 (PARK6), and DJ-1 (PARK7) are causal genes for familial recessive Parkinsonism (Corti et al. 2011; Trempe et al. 2013). As pathogenic mutations in these genes lead to dysfunction of the corresponding proteins, we can deduce that PARKIN, PINK1, and DJ-1 typically function to counteract predisposition to PD. A better understanding of their roles will help decipher the molecular mechanisms underlying dopaminergic neuronal loss in PD.
In the 1990s, mitochondrial DNA mutations and reductions in the activity of the electron transport chain complex I were observed in sporadic PD (Schapira 2008; Corti et al. 2011). Furthermore, it has been reported that toxic parkinsonism is caused by various inhibitors of the mitochondrial respiratory chain, such as rotenone and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Thus, a correlation between PD and mitochondrial impairment is a well-accepted paradigm for disease pathogenesis. Recently, studies on PINK1 and Parkin have provided important insights into the pathophysiological mechanisms of familial PD and reinforced the aforementioned idea that a decrease in mitochondrial function causes PD. PINK1 and Parkin cooperate in the identification, labeling, and clearance of damaged mitochondria, and we now infer that dysfunction of PINK1 or Parkin causes an accumulation of low-quality mitochondria, which triggers familial Parkinsonism (Durcan & Fon 2015; Eiyama & Okamoto 2015; Herhaus & Dikic 2015; Koyano & Matsuda 2015; Pickrell & Youle 2015; Yamano et al. 2016).

The DJ-1 gene was first identified as an oncogene (Nagakubo et al. 1997), but was later re-identified as a gene responsible for recessive familial Parkinsonism, PARK7 (Bonifati et al. 2003). Since then, several hundred papers on DJ-1 function have been reported. Although the cumulative conclusions in those studies vary and frequently contradict one another, a number of reports suggest a correlation between mitochondrial integrity and DJ-1. It is generally well accepted that a decrease in DJ-1 activity or loss of the DJ-1 gene perturbs mitochondrial function (Kim et al. 2005; Meulener et al. 2005; Ved et al. 2005; Hao et al. 2010; Ircher et al. 2010; Krebiehl et al. 2010; Larsen et al. 2011; Shim et al. 2011; Giaine et al. 2012; Heo et al. 2012; Ren et al. 2012; Wang et al. 2012; Minakawa et al. 2013; Chang et al. 2014). In addition, genetic or functional interactions between DJ-1 and PINK1 or PARKIN have been reported (Ved et al. 2005; Hao et al. 2010; Kamp et al. 2010; Thomas et al. 2011; Joselin et al. 2012; Chang et al. 2014) as has mitochondrial localization of DJ-1 (Canet-Aviles et al. 2004; Zhang et al. 2005; Blackinton et al. 2009; Ren et al. 2011; Maita et al. 2013; Cali et al. 2015). Because DJ-1 is highly reactive to hydrogen peroxide through oxidation of Cys106, and functions as an important regulator of redox metabolism (Kinumi et al. 2004; Taira et al. 2004), we can infer that DJ-1 maintains mitochondrial integrity by protecting it from oxidative stress such as that generated by reactive oxygen species. However, the mechanism by which DJ-1 may affect the function and integrity of mitochondria is not yet fully understood, and the DJ-1 researcher community has yet to reach a consensus on DJ-1 function. Moreover, the mitochondrial localization of DJ-1 is controversial because other subcellular localizations of DJ-1, including the cytosol and nucleus, have been reported (Canet-Aviles et al. 2004; Xu et al. 2005; Zhang et al. 2005; Blackinton et al. 2009; Nural et al. 2009; Ren et al. 2011; Maita et al. 2013; Bjorkblom et al. 2014; Cali et al. 2015).

Here, we disentangle the conflicting reports regarding DJ-1 cellular localization and dissect mechanism driving mitochondrial import. We show that WT DJ-1 is cytosolic, whereas various PD-relevant mutations of DJ-1 cause translocation of DJ-1 to the mitochondrial matrix. Our results provide insights into understanding not only how DJ-1 accesses mitochondria but also how its function in maintenance of mitochondrial integrity contributes to the prevention of PD.

**Results**

**Immunocytochemistry shows the mitochondrial localization of various pathogenic and artificial DJ-1 mutants**

To date, various subcellular localizations of DJ-1, including the cytosol and nucleus, have been reported (Canet-Aviles et al. 2004; Xu et al. 2005; Zhang et al. 2005; Nural et al. 2009). Although DJ-1 has no predictable mitochondria-targeting signal (MTS), WT DJ-1 has been reported to localize to mitochondria (Zhang et al. 2005) as have various pathogenic mutants (Maita et al. 2013; Björkblom et al. 2014). To confirm the mitochondrial localization of wild-type (WT) and the pathogenic DJ-1 mutants (M261I, A39S, E64D, E163K, L166P, and A179T), we generated constructs of each with a cysteine terminal HA-tag and expressed them in HeLa cells. In addition, because structural analyses suggest that E18, C106, and H126 of DJ-1 form a catalytic triad (Honbou et al. 2003; Huai et al. 2003; Tao & Tong 2003; Wilson et al. 2003), and C46, C53, and C106 have been reported to be functionally important (Canet-Aviles et al. 2004; Kim et al. 2013), we also similarly constructed a series of artificial DJ-1 mutants (E18A, C46S, C53A, and C106S) and expressed them in HeLa cells. For reference, a schematic diagram of the DJ-1 primary structure...
Figure 1 Several pathogenic and artificial mutations of DJ-1 induce mitochondrial translocation. Scale bars indicate 10 μm in all images. (A) DJ-1 constructs harboring the indicated mutation along with a carboxyl terminal HA-tag were expressed in WT HeLa cells and subjected to immunocytochemistry using anti-HA (green) and anti–Tom20 (red) antibodies. (B) DJ-1 knockout (KO) HeLa cells were transfected with the HA-tagged DJ-1 mutants and subjected to immunocytochemistry as in (A). (C) Carboxyl terminal GFP-tagged DJ-1 mutants were expressed in DJ-1 KO HeLa cells and GFP fluorescence observed. None of the tested mutants localized to mitochondria. (D) DJ-1 KO HeLa cells expressing nontagged DJ-1 mutants were subjected to immunocytochemistry using anti-DJ-1 (green) and anti–Tom20 (red) antibodies. Nontagged DJ-1 mutants showed the same mitochondrial localization pattern as HA-tagged mutants (B).

including location of the mutations used in this study is shown in Fig. S1 in Supporting Information. HA-tagged WT DJ-1 was diffusely localized throughout the cytosol and did not overlap with mitochondria in our experimental conditions (Fig. 1A). The A39S, C53A, E64D, C106S, and A179T DJ-1 mutants showed a similar cytosolic localization pattern. In contrast, localization of the E18A, M26I, C46S, E163K, and L166P mutants was clearly different from WT DJ-1 with mutant–derived signals predominantly localized to the mitochondria and to a lesser extent the cytosol (Fig. 1A).

Our finding that the C46S and C53A mutants localize to the mitochondria and cytosol, respectively (Fig. 1A), differs from previous reports. Canet-Aviles et al. (2004) reported that the C53A mutant localized to the mitochondria and the C46A mutant to the cytosol, whereas Maita et al. (2013) reported that both mutants localize to mitochondria. These differences might be attributable to variations in the experimental conditions. Our determination that the E18A, M26I, E163K, and L166P mutants exhibit mitochondrial localization is largely consistent with previous reports (Bonifati et al. 2003; Canet-Aviles et al. 2004; Maita et al. 2013; Bjorkblom et al. 2014).

Several reports suggest that DJ-1 forms a dimer (Miller et al. 2003; Wilson et al. 2003; Maita et al. 2013). To exclude the possibility that dimerization with endogenous DJ-1 drives localization of the exogenous DJ-1 mutants, we prepared DJ-1 knockout (KO) cells. Clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9-mediated genome editing (Cong et al. 2013) was used to generate DJ-1 KO HeLa cells. HeLa cells were transfected with CRISPR/Cas9 system plasmids targeting either exon3 or exon7 of DJ-1 and immunoblotting using an anti-DJ-1 antibody. Cells transfected with plasmid targeting exon3 showed a clear decrease in endogenous DJ-1 immunoblotting (Fig. S2A in Supporting Information) and thus were subjected to single clone selection. Among ten isolated single clones (#1–10), two clones (clone #2 and #7) showed very faint DJ-1 expression, whereas endogenous DJ-1 was undetectable in the other clones (clone #1, #3–6, and #8–10) (Fig. S2B in Supporting Information). Based on uniform cell shape and direct sequencing (not shown) confirmation of the DJ-1 genomic mutation, clone #5 was selected for all subsequent DJ-1 KO experiments. The mitochondrial localization patterns of the various DJ-1 mutants (E18A, M26I, C46S, E163K, and L166P) in the DJ-1 KO cell line (Fig. 1B) were the same as that in the normal HeLa cell line in Fig. 1A, indicating that endogenous DJ-1 did not influence mitochondrial localization of the exogenous DJ-1 mutants. In addition, we also confirmed that the expression levels of the exogenous WT and mutant DJ-1 constructs were comparable to endogenous DJ-1 (Fig. S2C in Supporting Information). In complementary reverse experiments, HA-tagged wild-type DJ-1 and untagged mitochondria-targeted DJ-1 mutants were co-expressed in DJ-1 knockout cells, and the subcellular localization of wild-type DJ-1 was examined. The cytosolic localization of wild-type DJ-1 was unchanged in the presence of the mitochondria-targeted DJ-1 mutants (Fig. S3A in Supporting Information) when they were similarly expressed (Fig. S3B in Supporting Information).

GFP-fused DJ-1 mutants do not localize to mitochondria, whereas nontagged and HA-tagged mutants DJ-1 mutants do

To confirm the mitochondrial localization of the aforementioned DJ-1 mutants under nonfixed, live-cell conditions, we next added a GFP tag to the carboxyl terminus of the E18A, M26I, and L166P DJ-1 mutants. However, contrary to a previous study that reported localization of GFP-tagged DJ-1 mutants in mitochondria (Bjorkblom et al. 2014), none of the mutants we tested in the DJ-1 KO HeLa cells localized to mitochondria (Fig. 1C). This result suggests that the large, carboxyl terminal GFP tag inhibited localization of DJ-1 in mitochondria.
mitochondrial localization of the DJ-1 mutants. In Fig 1A,B, the HA-tag was fused to the carboxyl terminus of DJ-1. To exclude possible artificial effects of the HA-tag, we generated nontagged constructs of WT DJ-1 and the E18A, M26I, C46S, E163K, and L166P mutants. When the subcellular localization of the nontagged constructs was examined in the DJ-1 KO HeLa cells, we found nontagged DJ-1 mutants localized on mitochondria (Fig. 1D) similar to that observed for the HA-tagged mutants (Fig. 1A,B).

Taken together, the immunocytochemical analyses showed cytosolic localization of WT DJ-1, whereas various artificial and pathogenic DJ-1 mutants localized on mitochondria. In contrast with previously published results (Bjorkblom et al. 2014), we found that addition of GFP to the carboxyl terminus of the DJ-1 mutants inhibited their mitochondrial localization.

**Mutations to putative catalytic residues accelerate mitochondrial localization of DJ-1**

During construction of the various DJ-1 mutants, we serendipitously generated an exclusively mitochondria-localized mutant. Previous structural analyses suggested that E18, C106, and H126 form a catalytic triad in DJ-1 (Honbou et al. 2003; Huai et al. 2003; Tao & Tong 2003; Wilson et al. 2003). We found that the H126A mutation alone had no effect on the cytosolic localization of DJ-1, whereas the E18A/H126A double mutation clearly accelerated mitochondrial localization (Fig. 2A). This mutant showed almost exclusive mitochondrial localization with a barely detectable cytosolic signal, which differs from the other leaky mitochondria-localized mutants (E18A, M26I, C46S, E163K, and L166P).

Exclusive mitochondrial localization of the E18A/H126A mutant suggests a relationship between mitochondrial localization and enzyme activity. Although the biochemical function of DJ-1 is controversial, it has been reported that DJ-1 has protease activity that is dependent on the catalytic triad (E18, C106, and H126) and which is accelerated by the removal of 15 amino acids from the carboxyl terminus (Chen et al. 2010). We generated a deletion mutant lacking the carboxyl terminus 15 amino acids (ΔC15) and examined its cellular localization as before. The ΔC15 DJ-1 mutant showed leaky mitochondrial localization.
similar to that of the E18A, M26I, C46S, E163K, and L166P mutants (Fig. 2B). Because E18 and H126 comprise the catalytic center of DJ-1, clear mitochondrial localization of the DJ-1 E18A/H126A mutant seemingly suggests that catalytic inactivation accelerates mitochondrial localization. However, this hypothesis is incompatible with cytosolic localization of the C106S mutant, which disrupts the Cys106 catalytic center (Fig. 1), and mitochondrial localization of the ΔC15 mutant, which reportedly enhances catalytic activity (Fig. 2). Consequently, the specific interrelationships between the catalytic region, the enzymatic activity of DJ-1, and its cellular localization remain unclear.

The DJ-1 E18A/H126A mutant reaches the mitochondrial matrix

We next sought to confirm the mitochondrial localization of DJ-1 using a more quantitative, biochemical approach. DJ-1 KO HeLa cells were transiently transfected with WT DJ-1 or E18A/H126A DJ-1 mutants, and their total cell lysates (shown as Input) were separated into a cytosolic fraction (shown as Cyto) and a mitochondria-rich fraction (shown as Mito) (Fig. 3A). Lactate Dehydrogenase (LDH), Tom20 (also known as TOMM20) and Tom40 (also known as TOMM40), AIF (also known as AIFM1) and Tim23, and Hsp60 were used as cytosolic, outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), and soluble matrix markers, respectively. LDH was specifically detected in the cytosolic fraction (Fig. 3A, upper panel). Tom20, Tom40, AIF, and Tim23 were selectively recovered in the mitochondria-enriched fraction, whereas Hsp60 was present in both the mitochondrial and cytosolic fractions (Fig. 3A, middle panels). These findings indicate that the fractionation process disrupted the integrity of some mitochondria, and as a result soluble matrix proteins such as Hsp60 leak into the cytosol fraction. However, both intact and punctured mitochondria are present in the mitochondria-enriched fraction. WT DJ-1 was almost exclusively collected in the cytosolic fraction (Fig. 3A, lanes 1–3), whereas the E18A/H126A mutation caused a significant amount of DJ-1 to be distributed to the mitochondria-enriched fraction (Fig. 3A, lanes 4–6), confirming the immunocytochemical results.

To show the submitochondrial localization of the DJ-1 mutants, we carried out a proteinase K (Pro K) protection assay under various conditions. The aforementioned mitochondria-rich fraction containing the E18A/H126A mutant was incubated with Pro K before immunoblotting. In this instance, even if the mitochondria-enriched fraction is contaminated by a limited number of punctured mitochondria (Fig. 3A), the Hsp60 and DJ-1 (E18A/H126A) signals observed in this fraction are clearly derived from intact mitochondria because neither protein is retained by the punctured mitochondria. The OMM protein Tom20 was susceptible to Pro K treatment (Fig. 3B, lane 2), whereas the intermembrane space (IMS)-facing IMM protein AIF was resistant (lane 2). When mitochondria were pre-treated with a hypotonic solution, the osmotic swelling (SW) ruptured the OMM, which resulted in the formation of mitoplasts and exposed AIF to Pro K (lane 5). The matrix protein Hsp60 was resistant to Pro K in both mitochondria and mitoplasts (indicating that the IMM was intact; lane 5), but became susceptible to Pro K digestion after solubilization with Triton X-100 (TX-100) (lanes 3, 6). Surprisingly, the Pro K digestion pattern of the DJ-1 E18A/H126A mutant was the same as Hsp60 (compare the Pro K digested pattern with other panels in Fig. 3B), suggesting that the DJ-1 mutant reached the mitochondria matrix.

We further confirmed the matrix localization of the DJ-1 E18A/H126A mutant by two different immunocytochemical approaches. Drp1 (dynamin-related protein 1) is a GTPase that regulates mitochondrial fission (Smirnova et al. 1998), and when removed results in fused, elongated mitochondria. Previously, Yamano et al. (2014) reported the presence of large bubble-like mitochondria in Drp1 KO cells following a decrease in the mitochondrial membrane potential (ΔΨm). We thus expected that matrix localization of DJ-1 would be more readily distinguishable in Drp1 KO HCT116 cells. Indeed, the signal for the OMM protein Tom20 was observed along the frame of the expanded mitochondria, whereas the DJ-1 E18A/H126A mutant signal was dispersed inside the bubble-like mitochondria in Drp1 KO HCT116 cells (Fig. 3C). Next, we carried out immunocytochemistry with distinct permeabilization methods. Triton X-100 (1%) permeabilizes both OMM and IMM and enables antibodies to access matrix proteins, whereas digitonin (50 μg/mL) does not sufficiently permeabilize the IMM (Okatsu et al. 2015). Thus, to allow detection of the matrix protein Hsp60, permeabilization with digitonin is insufficient; instead, permeabilization with Triton X-100 is required (Fig. 3D, left two panels). The precedent work by Maita et al. (2013) reported that the DJ-1
(A) DJ-1-HA

|       | WT  | E18A/H126A |
|-------|-----|------------|
| Input |   1 |  2         |
| Cito  |  3  |  4         |
| Mito  |  5  |  6         |
| LDH   | 38  | 14         |
| Tom20 | 14  | 64         |
| Tom40 | 38  | 64         |
| AIF   | 19  | 64         |
| Tim23 | 28  | 64         |
| Hsp60 | 19  | 64         |
| DJ-1  | 28  | 64         |

(kDa)

(B) DJ-1 E18A/H126A-HA

| SW | ProK | TX-100 |
|----|------|--------|
| +  | +    | +      |
| +  | +    | +      |
| +  | +    | +      |
| +  | +    | +      |
| +  | +    | +      |

Tom20

AIF

Hsp60

DJ-1

1 2 3 4 5 6 (kDa)

(C) DJ-1 E18A/H126A-HA, CCCP (+)

Merge

HA (DJ-1)

Tom20

(D) Permeabilize

|       | Digitonin | TX-100 |
|-------|------------|--------|
| Hsp60 |            |        |
|       |            |        |
| Tom20 |            |        |
|       |            |        |
| Merge |            |        |

(E) DJ-1-HA

|       | WT  | E18A/H126A |
|-------|-----|------------|
| SW    |   1 |  2         |
| ProK  |  3  |  4         |
| TX-100|  5  |  6         |
| Tom20 | 14  | 64         |
| AIF   | 38  | 64         |
| Hsp60 | 64  | 64         |
| DJ-1  | 28  | 64         |

(kDa)

(F) WT HeLa

|       | Input | Cito | Mito |
|-------|-------|------|------|
| LDH   | 38    | 1    |      |
| Tom20 | 14    | 2    |      |
| AIF   | 64    | 64   |      |
| Hsp60 | 64    | 64   |      |
| DJ-1  | 28    | 28   |      |

(kDa)
E18A and M26I mutants were weakly stained following digitonin treatment but strongly stained in mitochondria following Triton X-100 treatment. For the DJ-1 E18A/H126A mutant, anti-HA immunoreactivity was not observed in the mitochondria after permeabilization with digitonin, but was detectable following Triton X-100 permeabilization (Fig. 3D, right two panels). These immunocytochemical data support the Pro K protection assay results and indicate matrix localization of the DJ-1 E18A/H126A mutant.

In the fractionation experiments, WT DJ-1 was barely detectable in the mitochondria-enriched fraction (Fig. 3A, lane 3), which contrasts with previous studies that reported mitochondrial localization of WT DJ-1 using similar fractionation methods (Zhang et al. 2005). We thus scrutinized our WT DJ-1 data in detail. Treating the mitochondria-rich fraction of cells expressing WT DJ-1 with Pro K failed to degrade WT DJ-1 even in the presence of Triton (Fig. 3E, lanes 3,6), whereas in the parallel experiment with the DJ-1 E18A/H126A mutant degradation was observed (lanes 9,12) similar to that shown in Fig. 3B. The Pro K-resistant character of WT DJ-1 in the presence of Triton suggests aggregation. We also carried out fractionation and Pro K protection assays to examine the mitochondrial localization of endogenous DJ-1. When WT HeLa cells were fractionated, the endogenous DJ-1 signal was barely detectable in the mitochondria-enriched fraction (Fig. 3F, left panel). Moreover, even when an excessive amount of this fraction was subjected to the Pro K protection assay, the endogenous DJ-1 signal did not disappear despite the presence of Triton (Fig. 3F, lanes 3,6 in the right panel). Based on these data and the clear cytosolic localization of WT DJ-1 in the immunocytochemical data (Fig. 1), we concluded that WT DJ-1 does not localize in mitochondria. Thus, WT DJ-1 in the mitochondria-enriched fraction might be attributable to a small population of WT DJ-1 that forms a Pro K-resistant aggregate that precipitates under centrifugation.

In general, mitochondrial matrix proteins are synthesized in the cytosol as precursor proteins fused with a MTS at their amino terminus, and upon import into the matrix, the targeting signal is usually cleaved by mitochondrial processing peptidases. However, the molecular size of the DJ-1 mutant that localizes in the matrix is the same as cytosolic WT DJ-1 (Fig. 3A,E), suggesting that no proteolytic cleavage occurs.

Figure 3 The E18A/H126A DJ-1 mutant reaches the mitochondrial matrix. (A) DJ-1 E18A/H126A is detectable in the mitochondria-rich fraction (lane 6) following cellular fractionation. DJ-1 knockout (KO) HeLa cells expressing HA-tagged DJ-1 WT or E18A/H126A were subjected to fractionation and detected using anti-DJ-1, anti-Lactate Dehydrogenase (LDH) anti-Tom20, anti-Tom40, anti-AIF, anti-Tim23, and anti-Hsp60 antibodies. LDH, Tom20 and Tom40, AIF and Tim23, and Hsp60 were used as cytosolic, outer mitochondrial membrane (OMM), inner mitochondrial membrane (IMM), and soluble matrix markers, respectively. Cyto and Mito indicate the cytosolic and mitochondria-rich fractions, respectively. (B) The Pro K digestion pattern of the E18A/H126A DJ-1 mutant is the same as Hsp60, indicating matrix localization of DJ-1 E18A/H126A. The mitochondria-rich fraction collected from DJ-1 KO HeLa cells expressing DJ-1 E18A/H126A was subjected to a Pro K protection assay. Tom20, AIF, and Hsp60 are the OMM, intermembrane space (IMS), and matrix markers, respectively. (C) The DJ-1 E18A/H126A signal localizes to the mitochondrial interior, whereas the Tom20 signal localizes to the mitochondria periphery. Drp1 KO HCT116 cells expressing DJ-1 E18A/H126A were treated with CCCP and then subjected to immunocytochemistry using anti-DJ-1 and anti-Tom20 antibodies. The graph indicates the fluorescence intensity of the white line in the merged picture. Note that cells were transiently treated with CCCP after the DJ-1 E18A/H126A mutant had reached the mitochondria, and thus, mitochondrial translocation of DJ-1 E18A/H126A was not inhibited, unlike Fig. 5D. (D) Permeabilization of the IMM is required to detect the DJ-1 E18A/H126A signal. Nontransfected and DJ-1 E18A/H126A transfected DJ-1 KO HeLa cells were subjected to immunocytochemistry using anti-Hsp60, anti-HA, and anti-Tom20 antibodies. Digitonin (50 μg/mL) or 0.2% Triton X-100 were used for permeabilization. Similar to the matrix marker Hsp60, the mitochondrial localization of DJ-1 E18A/H126A was not observed in cells permeabilized with digitonin but was observed in cells treated with Triton X-100. (E) The WT DJ-1 signal did not disappear following Pro K treatment even in the presence of Triton X-100, unlike the E18A/H126A mutant. The mitochondria-rich fraction collected from DJ-1 KO HeLa cells expressing HA-tagged DJ-1 WT or E18A/H126A was subjected to a Pro K protection assay as (B). (F) Endogenous DJ-1 does not localize in mitochondria. The endogenous DJ-1 signal was rarely observed in the mitochondria-enriched fraction (left panel) and was resistant to Pro K digestion in the presence of Triton X-100 (right panel, prolonged exposure of DJ-1).
Various mitochondria-localized DJ-1 mutants reach the matrix. We carried out additional fractionation experiments for the other mitochondria-localized DJ-1 mutants to investigate whether they also reach the matrix. The E18A (Fig. 4A, lane 6), M26I (lane 15), C46S (lane 18), E163K (lane 9), L166P (lane 12), and ΔC15 (lane 21) mutants were distributed in the mitochondria-rich fraction, albeit not to the same extent as the E18A/H126A mutant (lane 3). We carried out a Pro K digestion pattern indicates matrix localization of the E18A, E163K, L166P, C46S, and ΔC15 mutants. The mitochondria-rich fractions collected in (A) were subjected to a Pro K protection assay as in Fig. 3B. E18A (lanes 1–6), E163K (lanes 7–12), L166P (lanes 13–18), C46S (lanes 25–30), and ΔC15 (lanes 31–36) had the same Pro K digestion pattern as the matrix marker Hsp60.

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Figure 5 Mitochondrial translocases (Tom40 and Tim23) and membrane potential (ΔΨm) are required for the localization of DJ-1 mutants. (A) DJ-1 knockout (KO) HeLa cells pre-treated with control, TIM23, or TOM40 siRNA were transfected with Su9-GFP or DJ-1 E18A/H126A, and then subjected to immunocytochemistry using anti-GFP, anti-HA, and anti-Tom20 antibodies. (B) Statistical analysis of (A). The graphs show the percentage of cells exhibiting clear mitochondrial localization of Su9-GFP or DJ-1 E18A/H126A (merge level high, representative figure is shown in Fig. S4 in Supporting Information) or no mitochondrial localization (merge level low, Fig. S4 in Supporting Information). Data represent the mean ± SD of 100 cells from three independent experiments. Black, gray, and white bars indicate control siRNA-, TIM23 siRNA-, and TOM40 siRNA-treated cells, respectively. *P < 0.01. (C) Decreased expression of endogenous Tim23 and Tom40 following siRNA treatment. DJ-1 KO HeLa cells treated with control, TIM23, or TOM40 siRNAs used in (A) and (B) were immunoblotted using the indicated antibodies. (D) DJ-1 KO HeLa cells were pre-treated with 10 μM valinomycin or 15 μM CCCP and then transfected with Su9-GFP or DJ-1 E18A/H126A. Subcellular localization was observed by immunocytochemistry using anti-GFP, anti-HA, and anti-Tom20 antibodies. The mitochondrial localization of Su9-GFP and DJ-1 E18A/H126A was hampered by valinomycin and CCCP treatment.
K protection assay as in Fig. 3B to confirm the matrix localization for these DJ-1 mutants. The Pro K sensitivity pattern of the E18A (Fig. 4B, lanes 1–6), E163K (lanes 7–12), L166P (lanes 13–18), C46S (lanes 25–30), and ΔC15 (lanes 31–36) mutants was the same as Hsp60, indicating that they also reach the mitochondrial matrix similar to E18A/H126A. For the M26I mutant, degradation was apparent after the initial Pro K treatment (lanes 20) and was accelerated following both hypotonic and Triton X-100 pre-treatment (lanes 21, 23, 24), suggesting localization of not only the matrix but also the OMM and IMS. Moreover, when compared to the other mitochondria-localized mutants, the Pro K digestion pattern of the M26I mutant was clearly different (lanes 19–24), suggesting that the M26I mutation had a significant effect on the overall structure of DJ-1 or promoted aggregation of the protein.

Mitochondrial localization of DJ-1 mutants is dependent on mitochondrial translocase activity and the mitochondrial membrane potential

In general, matrix proteins are imported into the mitochondria by a sequential passage through TOM40 (also known as TOMM40) and TIM23 (also known as TIMM23), channel components of the translocases on the outer membrane complex (TOM complex), and the inner membrane complex (TIM23 complex), respectively. TOM40 and TIM23 are essential genes and difficult to knock out. We thus tried to knockdown TOM40 and TIM23 and examined whether mitochondrial localization of the E18A/H126A DJ-1 mutant was inhibited. The amino-terminal region of subunit 9 of the FoATPase (Su9) functions as a typical MTS that directs GFP to the matrix through the TOM and TIM23 complexes (Ishihara et al. 2006). We thus used Su9–GFP as a positive control. DJ-1 knockout HeLa cells were transfected with small interfering (si)RNA for TOM40 or TIM23, followed by transfection with DJ-1 E18A/H126A or Su9–GFP, and their cellular localization was examined. The Su9–GFP mitochondrial signals decreased in both siRNA conditions, confirming that knockdown of TOM40 and TIM23 indeed hampers matrix targeting (Fig. 5A, left panels). The mitochondrial localization of DJ-1 E18A/H126A was also blocked by TOM40 and TIM23 siRNAs (Fig. 5A, right panels). For quantitative analysis, HeLa cells under all siRNA conditions were categorized into four groups: those in which the GFP or DJ-1 signal was completely co-localized with mitochondria (90–100%), modestly co-localized (50–90%), poorly co-localized (10–50%), and not co-localized (0–10%). The quantity of cells in each category was determined for 100 cells across three independent experiments (Figs 5B and S4 in Supporting Information). Whereas TOM40 siRNA almost completely abolished the mitochondrial translocation of Su9–GFP and the DJ-1 E18A/H126A mutant, TIM23 siRNA did not completely inhibit their translocation. This difference is likely derived from leaky suppression of endogenous TIM23 expression by siRNAs (Fig. 5C). It is important to note that mitochondrial localization of the DJ-1 E18A/H126A mutant was inhibited by TOM40 and TIM23 siRNAs in a manner similar to Su9–GFP (Fig. 5B), suggesting the DJ-1 E18A/H126A mutant and MTS-containing Su9–GFP are targeted to the matrix by the same mechanism.

The ΔΨm is essential for MTS-containing proteins to pass the IMM and reach the matrix (Hartl et al. 1989). Treatment with uncouplers such as valinomycin and carbonyl cyanide m-chlorophenyl hydrazine (CCCP) eliminates ΔΨm in cells. As we have reported previously (Okatsu et al. 2015), incubation with valinomycin or CCCP inhibited import of the Su9–GFP matrix marker, whereas mitochondrial localization of the OMM protein Tom20 was not affected (Fig. 5D, left panel). Consequently, we examined the mitochondrial localization of the DJ-1 E18A/H126A mutant following uncoupler treatment (note that Fig. 3C does not show ΔΨm-independent mitochondrial localization of the DJ-1 E18A/H126A because it had reached the mitochondria before CCCP treatment). In Fig. 5D, cells were pre-treated with the uncoupler and then transfected with the DJ-1 E18A/H126A mutant. Similar to the previous result, CCCP treatment changed localization of the DJ-1 E18A mutant from the mitochondria to the cytosol (Maita et al. 2013); the mitochondrial import of DJ-1 E18A/H126A was inhibited by both valinomycin and CCCP treatment (Fig. 5D, right panel). Taken together with the results shown in Fig. 5, we determined that matrix translocation of the DJ-1 E18A/H126A mutant is dependent on translocase (TOM40 and TIM23) function and an intact ΔΨm.

Discussion

Approximately 1500 different proteins are contained in the human mitochondria (Pagliarini et al. 2008). Of these, 13 proteins (approximately 1%) are encoded in mtDNA (Anderson et al. 1981; Andrews et al. 1999) and are synthesized in the matrix by
mitochondrial ribosomes. The remainder are synthesized on cytosolic ribosomes and imported across the mitochondrial membranes. To pass through the mitochondrial membranes and reach the matrix, precursor proteins use general entry gates (i.e., the TOM and TIM23 complexes). Furthermore, protein import into the matrix is driven by $\Delta \Psi_m$. To be destined for the mitochondrial matrix, positively charged amphipathic alpha-helices (presequences) are usually located at the amino terminus of matrix-localizing proteins. Upon import into the matrix, most presequences are then proteolytically removed by the mitochondrial processing peptidase (Chacinska et al. 2009).

DJ-1 has a unique pattern of mitochondrial localization. Several DJ-1 mutants are transported to the mitochondrial matrix (Figs 3,4), a process that is dependent on the TOM and TIM23 complexes and $\Delta \Psi_m$ (Fig. 5). These results seemingly suggest a conventional mode of matrix localization for DJ-1; however, based on our findings, we conclude that DJ-1 mitochondrial localization proceeds via a noncanonical pathway. As stated previously, the MTS consists of a positively charged, amino-terminal amphiphilic alpha-helix. However, structural analysis of DJ-1 showed that its amino terminus does not form an alpha-helix, but rather forms a beta-sheet (Honbou et al. 2003; Huai et al. 2003; Tao & Tong 2003; Wilson et al. 2003). In addition, two mitochondrial localization prediction databases, TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) and PSORT II (http://psort.hgc.jp/form2.html), suggested WT DJ-1 lacks a predictable MTS (data not shown). Because

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**Figure 6** Model for DJ-1 translocation to the mitochondrial matrix. (A) DJ-1 possesses dual subcellular localization signals, that is, a cytosolic retention signal (CRS) and a mitochondria-targeting signal (MTS). Under normal conditions, the CRS is dominant and maintains DJ-1 in the cytosol. However, when the CRS is inhibited by mutations, the MTS directs DJ-1 localization to mitochondria. (B) DJ-1 possesses an intrinsic MTS, but the signal is suppressed under steady-state conditions by some inhibitory molecule or auto-inhibition, as a result DJ-1 is cytosolic. However, when various mutations abolish the inhibitor interaction or auto-inhibition, DJ-1 is transported to the matrix by its intrinsic MTS. (C) WT DJ-1 (blue) is imported to the matrix but promptly recycled back to the cytosol under steady-state conditions. Various mutations hamper mitochondrial export of DJ-1 to the cytosol and thus DJ-1 mutants (red) are retained in the mitochondria.
pathogenic or artificial mutations are required for matrix localization of DJ-1, whereas intact WT DJ-1 is cytosolic (Figs 1,3), the simplest explanation is that the introduced mutations generate an MTS or MTS-like sequence in DJ-1. However, this is unlikely for the following reasons. First, the translocating mutations are widely distributed throughout the DJ-1 sequence and are not concentrated in the amino terminus. Second, the nature of the amino acid substitutions varies among the mutants with no rule governing amino acid replacement. Third, TargetP 1.1 and PSORT II analysis of mitochondria-localized DJ-1 mutants (e.g., E18A/H126A) likewise failed to detect an MTS-like sequence. Moreover, DJ-1 mutants localized in the matrix do not exhibit the change in molecular weight expected with removal of a presequence (Fig. 3).

Having rejected the hypothesis that the mutations generate an MTS-like sequence, we propose an alternative model to explain mitochondrial localization of the DJ-1 mutants. Interestingly, the L166P mutation that triggers transport of DJ-1 to mitochondria (Figs 1,4) has been reported to promote protein unfolding and destabilization (Miller et al. 2003; Moore et al. 2003; Olzmann et al. 2004). This linkage between mitochondrial transport of DJ-1 and the loss-of-function mutation suggests that transport occurs by a recessive (nonpositive) mechanism. Based on this premise, we propose two models to fit the aforementioned conditions. First, DJ-1 might possess dual subcellular localization signals, that is, a cytosolic retention signal and an atypical (unpredictable) matrix-targeting signal. Under normal conditions, the cytosolic retention signal is dominant and maintains DJ-1 in the cytosol. However, when the cytosolic retention signal is inhibited by mutations, the atypical matrix-targeting signal directs DJ-1 to the mitochondria (Fig. 6A). Similarly, we can speculate that DJ-1 possesses a potential MTS, but the signal is latent under steady-state conditions by some inhibitory molecule or auto-inhibition. However, when mutations used in this study impair interactions with the inhibitory factor or liberate the auto-inhibition, DJ-1 is transported to the mitochondrial matrix (Fig. 6B). In either model, the putative cytosolic retention signal or inhibitory signal for matrix localization is likely structurally coded because the L166P translocation-associated mutation also promotes protein unfolding and destabilization of DJ-1 (Miller et al. 2003; Moore et al. 2003; Olzmann et al. 2004). A similar type of regulation using two distinct localization signals has been reported for ATFS-1 (activating transcription factor associated with stress-1). ATFS-1 possesses a recessive nuclear localization sequence and a dominant amino-terminal MTS. Under normal conditions, ATFS-1 is transported to mitochondria, however, when the MTS function is inhibited, such as when damaged proteins accumulate on mitochondria, the subcellular localization of ATFS-1 changes to the nucleus (Nargund et al. 2012).

Alternatively, DJ-1 might be imported to the matrix but promptly recycled back to the cytosol under steady-state conditions. In this case, the various mutations that we used in this study may hamper the mitochondrial export of DJ-1; as a consequence, the DJ-1 mutants are retained in the mitochondria (Fig. 6C). This model seems less plausible because this type of retrograde transport system from the mitochondrial matrix to the cytosol has not yet been reported. Although, in yeast cells, some proteins relocate from the mitochondrial IMS back to the cytosol with Tom40 functioning as an escape gate in this retrograde transport (Bragoszewski et al. 2015). We thus cannot completely rule out this possibility.

In conclusion, the major implication of our work is that DJ-1 is transported to the mitochondrial matrix by an atypical mechanism in which matrix localization of DJ-1 mutants (Figs 3,4) is dependent on both translocase activity and ΔΨm (Fig. 5), despite the absence of a predictable MTS or cleavage of a presequence (Fig. 3). Although further analyses are required, we hope that the data presented here will contribute to not only elucidation of a novel mitochondrial localization mechanism for DJ-1, but to increased understanding of how DJ-1 maintenance of mitochondrial integrity contributes to the prevention of PD.

**Experimental procedures**

**Cells**

HeLa cells were cultured at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) containing 1× nonessential amino acids (Gibco), 1× sodium pyruvate (Gibco), 1× Penicillin–Streptomycin–Glutamine (Gibco), and 10% fetal bovine serum (Gibco). Dpn1 KO HCT116 cells were provided by Dr. Richard Youle (NIH) and were cultured at 37 °C with 5% CO₂ in McCoy’s 5A medium (Gibco) containing 1× nonessential amino acids (Gibco), 1× Penicillin–Streptomycin–Glutamine (Gibco), and 10% fetal bovine serum (Gibco).

DJ-1 KO HeLa cells were established using the CRISPR/Cas9 system (GeneArt CRISPR Nuclease System; Life Technologies). The vector in this kit encodes a Cas9 nuclease expression cassette and a guide RNA cloning cassette. Two
types of DJ-1 guide RNAs targeting 5′-AGTACAGTG-TAGCCTGATG-3′ in exon 3 and 5′-TGCAACGC-CAAACTCGAAGC-3′ in exon 7 were selected using the CRISPR Design web site (crispr.mit.edu) and ligated into the GeneArt CRISPR Nuclease vector (Life Technologies). HeLa cells were transfected with the obtained GeneArt CRISPR Nuclease vector and a pSilencer5.1-H1 Retro empty vector (for puromycin selection) for 24 h. The cells were treated with 5 μg/mL puromycin for 48 h and then maintained in fresh puromycin-free medium. Polyclonal cells were immunoblotted using an anti-DJ-1 antibody, and cells transfected with the GeneArt CRISPR Nuclease vector harboring the DJ-1 guide RNA targeting 5′-AGTACAGTG-TAGCCTGATG-3′ were chosen. Monoclonal cell lines were generated through limiting dilutions of the aforementioned cells and were validated by immunoblotting with an anti-DJ-1 antibody and genomic DNA sequencing.

Plasmids and DNA transfections
The plasmids used in this study are summarized in supplementary Table S1 in Supporting Information. DJ-1 was amplified from a cDNA library by PCR and inserted into pcDNA3 (Invitrogen) with or without a carboxyl terminal HA-tag or pEGFP-N1 (Clontech). Plasmids for expressing various DJ-1 mutants were constructed by conventional site-directed mutagenesis. DJ-1 or DJ-1 mutants were suspended in HKSS buffer and disrupted by 30 passages through a 25-gauge needle as described above. The lysates were divided into half, debris was removed by centrifugation at 800 g for 10 min, and supernatants were further centrifuged at 10 000 g for 25 min at 4°C to obtain the cytosolic fraction (supernatant) and the mitochondrial transport depen-
tency of Su9-GFP or the DJ-1 E18A/H126A mutant on an

dependency of Su9-GFP or the DJ-1 E18A/H126A mutant on an

Mitochondria-enriched fractionation and proteinase K protection assay
For proteinase K protection assays, cells were re-suspended in HKSS buffer and disrupted by 30 passages through a 25-gauge needle as described above. The lysates were divided into half, debris was removed by centrifugation at 800 g for 10 min, and supernatants were further centrifuged at 10 000 g for 25 min at 4°C to obtain the mitochondria-enriched fraction. One mitochondria-enriched fraction was re-suspended in HKSS buffer, and another was re-suspended in HK buffer [10 mM CaCl2, 20 mM HEPES-KOH pH 7.5] for hypotonic treatment. Each sample was then divided into three and incubated on ice for 2 h with 12.5 μg/mL Pro K (Wako chemicals) or with Pro K and 0.1% Triton X-100. The reaction was stopped by the addition of 5 mM phenylmethylsulfonyl fluoride before boiling for electrophoresis. All fractions were then immunoblotted.

siRNA analyses
For siRNA analysis, the siGENOME siRNA SMART pool TIMM23 (M-190121-00, Thermo Fisher Scientific) and siGENOME siRNA SMART pool TOMM40 (M-012732-00, Thermo Fisher Scientific) were used for knockdown of TIMM23 and TOMM40, respectively. As controls, cells treated with the siGENOME Control siRNA pool (D-001206-13-20, Thermo Fisher Scientific) were also used. Finally, 10 nM TIMM23, TOMM40, and control siRNAs were introduced into HeLa cells using Lipofectamine RNAiMAX (Life Technologies). Cells were incubated for 48 h, re-seeded on 35-mm glass bottom dishes (MatTek Corporation), and then either Su9-GFP or DJ-1 E18A/H126A plasmids were transfected using Fugene 6 (Promega). The resulting cells were incubated for another 24 h and then subjected to immunocytochemical analysis. For statistical analyses, cells were categorized based on
the degree of DJ-1 E18A/H126A or Su9-GFP mitochondrial co-localization: robust co-localization (90–100%), modest co-localization (50–90%), poor co-localization (10–50%), or no co-localization (0–10%). These analyses were carried out using 100 cells per siRNA condition. Error bars represent the mean ± SD values of three independent experiments. Statistical significance was calculated using Student’s t-test.

SDS-PAGE and immunoblotting

For SDS-PAGE, 12% acrylamide gels (NuPAGE Gels; Thermo Fisher Scientific) and MES or MOPS buffers were used. After electrophoresis, gels were washed using transfer buffer for 10 min and then transferred to PVDF membranes according to the manufacturer’s protocol. PVDF membranes with transferred proteins were blocked with gentle shaking in 5% skim milk and detected using antibodies by standard immunoblotting procedures. The following primary antibodies were used for immunoblotting: anti-HA (TANA2, MBL, 1:2000), anti-DJ-1 (3E8, MBL, 1:750), anti-LDH (Abcam, 1:500), anti-AIF (E-1, Santa Cruz, 1:700), anti-Hsp60 (N-20, Santa Cruz, 1:600), anti–Tom20 (FL-145, Santa Cruz, 1:400), anti-Actin (clone C4, 1:500), anti-Tim23 (BD Biosciences, 1:500), anti-AIF (E-1, Santa Cruz, 1:700), anti-Hsp60 (N-20, Santa Cruz, 1:600), anti–Tom20 (FL-145, Santa Cruz, 1:400), anti-Actin (clone C4, 1:500), anti-Tim23 (BD Biosciences, 1:500), and anti-Tom40 (provided by Dr. Katsuyoshi Mihara, Kyushu University, Fukuoka, Japan; 1:1000). We also used the following secondary antibodies for immunoblotting: anti-mouse-IgG, anti-rabbit-IgG, or anti-goat-IgG antibody conjugated to alkaline phosphatase (Santa Cruz, 1:5000).

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Schematic diagram of the DJ-1 primary structure including mutation sites used in this study.

Figure S2 Establishment of DJ-1 knockout (KO) HeLa cells by CRISPR/Cas9-mediated genome editing (S2A, S2B), and comparison of the expression levels between endogenous DJ-1 and exogenous WT or M26I DJ-1 (S2C).

Figure S3 (A) Cytoplasmic localization of HA-tagged wild-type DJ-1 did not change in the presence of mitochondria-targeted DJ-1 mutants. (B) Comparison of the expression levels between exogenous HA-tagged WT DJ-1 and untagged DJ-1 mutants using an anti-DJ-1 antibody.

Figure S4 (A) Representative figures used in the statistical analysis examining the degree of mitochondrial localization for DJ-1 E18A/H126A and Su9-GFP. (B) The graphs show the ratio of cells exhibiting clear, modest, poor or no mitochondrial localization of Su9-GFP or E18A/H126A.

Table S1 List of plasmids used in this study