

Glutathione S-Transferase Omega 1 Activity Is Sufficient to Suppress Neurodegeneration in a *Drosophila* Model of Parkinson Disease^{*[5]}

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Background: Glutathione S-transferase Omega has been shown to be associated with Parkinson disease.

Results: *Drosophila* GSTO1 regulates mitochondrial ATP synthase activity in *parkin* mutants.

Conclusion: *Drosophila* GSTO1 plays a protective role in a *Drosophila* model of Parkinson disease.

Significance: These findings may lead to a better understanding of the molecular mechanism of neuroprotection due to GSTO in Parkinson disease.

A loss-of-function mutation in the gene *parkin* causes a common neurodegenerative disease that may be caused by mitochondrial dysfunction. Glutathione S-transferase Omega (GSTO) is involved in cell defense mechanisms, but little is known about the role of GSTO in the progression of Parkinson disease. Here, we report that restoration of *Drosophila* GSTO1 (DmGSTO1), which is down-regulated in *parkin* mutants, alleviates some of the *parkin* pathogenic phenotypes and that the loss of DmGSTO1 function enhances *parkin* mutant phenotypes. We further identified the ATP synthase β subunit as a novel *in vivo* target of DmGSTO1. We found that glutathionylation of the ATP synthase β subunit is rescued by DmGSTO1 and that the expression of DmGSTO1 partially restores the activity and assembly of the mitochondrial F₁F₀-ATP synthase in *parkin* mutants. Our results suggest a novel mechanism for the protective role of DmGSTO1 in *parkin* mutants, through the regulation of ATP synthase activity, and provide insight into potential therapies for Parkinson disease neurodegeneration.

Parkinson disease (PD)³ is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic (DA) neurons. Previous reports suggested that mitochondrial dysfunction, oxidative stress, and ER stress induced by the aggregation of abnormal proteins can contribute to the pathogenesis of PD (1, 2). However, the molecular mechanisms of PD pathogenesis have not yet been fully elucidated. A loss-of-func-

tion mutation in the *parkin* gene is a major cause of autosomal recessive, early onset PD.

Glutathione S-transferases (GSTs) constitute a superfamily of enzymes that are grouped into at least 10 classes; some superfamily members are known to be involved in cell defense mechanisms (3, 4). The active sites of GST Omega (GSTOs) have a unique cysteine residue that can form a disulfide bond with GSH, whereas other eukaryotic GSTs have tyrosine or serine residues in their active sites (4). The biological functions of several GSTOs have been determined. Human GSTO1-1 modulates calcium channels (5), has a role in the activation of interleukin-1 β (6), and interacts with a serine protease inhibitor (7). Recently, our group reported that one of the four *GSTO* genes in *Drosophila*, *CG6781*, is the structural gene of the *Drosophila* eye color mutant, *sepia*, which encodes pyrimidodiazepine synthase, a key enzyme in the drosopterin biosynthetic pathway (8). In humans, GSTOs are mapped to the linkage region correlated with the age at onset of Alzheimer disease (9). Variations in human *GSTO1* genes that modify the age at onset of Alzheimer and Parkinson diseases have been reported (10). However, the *in vivo* function of GSTOs and their target proteins have not yet been fully identified.

Recently, Pallanck's group (11–13) reported that expression of *Drosophila melanogaster* GST Sigma 1 (DmGSTS1), which is another member of the GST family in *Drosophila*, suppresses the phenotypes of *parkin* mutants and α -synuclein-expressing mutants. To elucidate the protective role of GSTO in neurodegenerative diseases, we investigated the biological function of *D. melanogaster* GSTO (DmGSTO) in a *Drosophila* model of PD. We found that one of the DmGSTOs, DmGSTO1, is able to rescue some phenotypes of *parkin* mutants, including the degeneration of DA neurons and muscle. The ability of DmGSTO1 to rescue these phenotypes was dependent on the catalytic activity of DmGSTO1. Furthermore, tubulin accumulation and ER stress caused by the *parkin* mutation were also significantly reduced by ectopic expression of DmGSTO1. We discovered that the *Drosophila* ATP synthase β subunit (14) is a novel target of DmGSTO1, and up-regulation of DmGSTO1 restored glutathionylation levels of the ATP synthase β subunit in *parkin* mutants. The mitochondrial F₁F₀-ATP synthase is a

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[5] This article contains supplemental Figs. S1–S7.

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³ The abbreviations used are: PD, Parkinson disease; UAS, upstream activation sequence; IFM, indirect flight muscle; DA, dopaminergic; UPR, unfolded protein response; ANOVA, analysis of variance; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

membrane protein complex that couples the synthesis of ATP to the hydrogen ion gradient generated by the respiratory chain (15, 16). The up-regulation of DmGSTO1 also restored ATP synthase activity, complex assembly, and ATP levels in *parkin* mutants. Our findings suggest that DmGSTO1 plays a protective role in *parkin* mutants by regulating mitochondrial ATP synthase activity.

EXPERIMENTAL PROCEDURES

Drosophila Stocks—To generate *DmGSTO1* mutants, we obtained the *GE26508* *P*-element insertion line from the GenExel *Drosophila* library (KAIST, Daejeon, Korea). The *P*-element was mobilized using transposase, and *GE26508* was imprecisely excised to generate *DmGSTO1^{null}* (supplemental Fig. S1A). The UAS-*mitGFP* line was a gift from H. J. Bellen (Baylor College of Medicine, Houston, TX) (17). We generated four transgenic lines: *DmGSTO1A*, *DmGSTO1B*, *DmGSTO1A^{C31A}*, and *CG6662*. The coding sequences for these four genes were amplified by PCR. The four PCR products were ligated into the pUAST expression vector and introduced into the germ line by microinjection. All PCR products were confirmed by DNA sequencing. *Drosophila* stocks were maintained in standard food conditions at 25 °C. The *parkin* null mutant line, *park¹*, and *PINK1^{B9}* were gifts from J. Chung (Seoul National University) (18, 19). The *TH-Gal4* line was a gift from S. Birman (CNRS-Université de la Méditerranée) (20). The *Tub-Gal4* fly line and the *24B-Gal4* line were obtained from the Bloomington Stock Center. We obtained the ATP synthase β subunit RNAi lines, *CG11154R-1* and *R-3*, from the NIG-FLY stock center (National Institute of Genetics, Mishima, Japan).

Exposure to Paraquat—One- to two-day-old male flies were starved for 5 h and then kept in vials with 3M filter paper soaked with 20 mM paraquat (Methyl Viologen, Sigma-Aldrich) in 5% sucrose. Flies were kept in the dark at all times.

Immunoblot Analysis—Protein extracts for immunoblot analysis were prepared by homogenizing 10 fly thoraces from 3-day-old male flies in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% Nonidet P-40) containing a protease inhibitor mixture (1 \times ; Calbiochem-Merck4Biosciences). Total protein (20 μ g) was separated by 8 or 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked by Tris-buffered saline (TBS) with 4% nonfat dry milk or 4% BSA for 1 h. We used the following primary antibodies: rabbit anti-phospho-JNK (1:1,000; Promega), rabbit anti-JNK (1:1,000; Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), rabbit anti- β -actin (1:5,000; Sigma-Aldrich), mouse anti- α -tubulin (1:4,000; Sigma-Aldrich), mouse anti- β -tubulin (1:3,000; Sigma-Aldrich), rabbit anti-phospho-eIF2 α Ser-51 (1:1,000; Cell Signaling Technology), rabbit anti-eIF2 α (1:200; Abcam), rabbit anti-HSP60 (1:1,000; Stressgen Bioreagents, Kampenhout, Belgium), mouse anti-HSP/HSC70 (1:1,000; Stressgen Bioreagents), mouse anti-ATP synthase α subunit (1:20,000; Mitosciences), rabbit anti-prohibitin (1:500; Abcam), and rabbit anti-*Drosophila* ATP synthase β subunit (1:20,000; a kind gift from Rafael Garesse, Universidad Autonoma de Madrid). Detection of the primary antibodies was carried out with HRP-conjugated secondary antibodies and an ECL-Plus detection kit

(Amersham Biosciences). Polyclonal antibodies against *Drosophila* GSTO1A were produced by immunizing rabbits with a C-terminal synthetic peptide, ²³¹EFQKSKTLGNPQY²⁴³, as the antigen (Abfrontier).

Muscle Histology—Muscle section analysis was carried out as described previously (21) with some modifications. For muscle tissue analyses, whole thoraces from 3-day-old male flies were fixed with 4% formaldehyde overnight at 4 °C. After fixation, the samples were oxidized with 1% OsO₄ for 2 h at room temperature and then dehydrated in a series of acetone/water mixtures of increasing acetone concentration (50, 70, 80, 90, and 100% acetone). The samples were embedded in Spurr's resin. The thoraces were then trimmed and sectioned from the transverse side of the thorax. The sections were stained with a toluidine blue dye and observed by light microscopy (Carl Zeiss, Axio Imager A1).

Immunohistochemistry and TUNEL Assay—Adult brains and thoraces of 1-, 3-, 5-, and 20-day-old male flies were fixed with 4% formaldehyde in a fixative buffer (100 mM PIPES, 1 mM EGTA, 1% Triton X-100, and 2 mM MgSO₄, pH 6.9) and blocked in a wash buffer (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, and 0.5 mg/ml BSA, pH 6.8) with 10 mg/ml BSA. The following antibodies were used: rabbit anti-TH (1:100; Pel-freeze), mouse anti-TH (1:100; Immunostar), rabbit anti-phospho-JNK (1:100; Promega), mouse anti-GFP (1:500; Roche Applied Science), and mouse anti- α -tubulin (1:500; Sigma-Aldrich). Alexa 488-conjugated streptavidin (1:100; Invitrogen) was used to identify mitochondria. Rhodamine phalloidin (Invitrogen) was used to visualize actin. All images were obtained on a Carl Zeiss confocal microscope (DE/LSM510 NLO). For the TUNEL assay, apoptosis in the indirect flight muscles (IFMs) of 3-day-old flies was detected using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science).

Quantitative RT-PCR and Real-time Quantitative RT-PCR—Total RNA was extracted with TRIzol reagent (Invitrogen), and cDNA was prepared from 2 μ g of total RNA using Moloney murine leukemia virus reverse transcriptase (Promega). The PCRs were performed using Ex Taq (Takara Bio, Otsu, Japan) and TaqDNA polymerase (Bioneer, Daejeon, Korea) with a PTC-100 programmable thermal controller (MJ Research). We used the previously reported *Drosophila* GSTO primers for *CG6776*, *CG6662*, *DmGSTO1A*, and *DmGSTO1B* (8). The following *dparkin* and *dPINK1* primers were used: *dparkin*-For (CAT ATG AGT TTT ATT TTT AAA TTT ATT GCC ACT TTT GTA C), *dparkin*-Rev (CTC GAG TTA GCC GAA CCA GTG GG), *dPINK1*-For (TTC TGC CAC CAC CGC CCC CAC ACT TC), and *dPINK1*-Rev (CCG CAG CAC ATT GGC AGC GGT GG).

The comparative cycle threshold (*Ct*) method was adapted to estimate transcript levels using an ABI7300 system (Applied Biosystems). The transcript levels were calculated as the relative -fold change over *rp49* mRNA. We used the following primers for α -tubulin, and β -tubulin: α -tubulin-For (ACA ACG AGG CTA TCT ACG ACA TCT), α -tubulin-Rev (TTT TCA GTG TTG CAG TGA ATT TTT) (22), β -tubulin-For (CAA GGC TTC CAA CTC ACA CAC TC), and β -tubulin-Rev (AGG TGG CGG ACA TCT TCA GAC) (23).

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Site-directed Mutagenesis and Expression of Mutant Proteins—The DmGSTO1A^{C31A} mutant was generated by site-directed mutagenesis (Cosmo Genetech, Seoul, Korea). Cysteine 31 at the active site was mutated to alanine by changing the TGC codon encoding cysteine 31 to GCC. Mutated DNA was sequenced to confirm the single codon change. The DmGSTO1A^{C31A} mutant was expressed in *Escherichia coli* strain BL21 after cloning the cDNA into a pET15b expression vector (Novagen-Merck4Biosciences).

In Vitro Glutathionylation Assay—Recombinant ATP synthase β subunit (5 μ g) was incubated at 37 °C in 50 mM potassium phosphate (pH 7.6), and 10 mM GSH in the presence of either recombinant DmGSTO1A or DmGSTO1B protein. After 30 min, the samples were placed on ice, and 5 \times non-reducing SDS loading buffer was added to the mixtures. Samples were separated by 12% SDS-PAGE, transferred, and probed with mouse anti-GSH (1:1,000; ViroGen Corp.) and rabbit anti-*Drosophila* ATP synthase β subunit (1:4,000) antibodies.

Immunoprecipitation and Glutathionylation Assay—Thoraxes from 3-day-old male flies were homogenized in lysis buffer containing 1 \times protease inhibitor mixture (Calbiochem-Merck4Biosciences). Thorax lysates were incubated with mouse anti-GSH antibodies (ViroGen) for 2 h at 4 °C and incubated overnight with 60 μ l of a solution of 50% protein G-Sepharose beads (Amersham Biosciences) at 4 °C. The resins were collected by centrifugation at 1,000 \times *g* for 20 s. Bound proteins, which were glutathionylated, were eluted by boiling in a 2 \times non-reducing SDS loading buffer for 5 min. Materials were subjected to SDS-PAGE and visualized with Coomassie Blue or silver staining.

After immunoprecipitation with mouse anti-GSH antibodies (ViroGen), proteins bound to resins were separated by 8% SDS-PAGE and transferred to PVDF membranes (Millipore) for the glutathionylation assay. Western blot analysis was carried out with rabbit anti-*Drosophila* ATP synthase β subunit antibodies (1:4,000). Signals were detected by HRP-conjugated secondary antibodies.

Mitochondrial ATP Synthase Activity Assay—To isolate mitochondria from fly thoraxes, a mitochondria isolation kit (Pierce) was used according to the manufacturer's protocol. Freshly prepared total mitochondrial protein was used for the ATP synthase activity assay.

ATP synthase activity was measured by ATP hydrolysis using a spectrophotometric method described previously (24). ATP synthase was assayed in 20 mM Hepes (pH 7.5), 5 mM KCl, 5 mM MgCl₂, 5 mM KCN, 2.5 mM phosphoenolpyruvate (Sigma-Aldrich), 15 units of pyruvate kinase (Sigma-Aldrich), 15 units of lactate dehydrogenase (Sigma-Aldrich), 300 μ M NADH (Sigma-Aldrich), and 20 μ g of total mitochondrial proteins. After 2 min of preincubation at 37 °C, the reaction was initiated by the addition of 2 mM ATP. The initial velocity of the reaction was followed for 2 min at 340 nm at 37 °C. The molar extinction coefficient of NADH is 6220 M⁻¹.

ATP Assay—Five thoraxes from 3-day-old male flies were homogenized in 100 μ l of 1 \times reporter lysis buffer (Promega) on ice. The homogenized samples were quickly frozen in liquid nitrogen to inhibit ATP synthase activity. The frozen samples

were boiled for 8 min to destroy endogenous ATP synthase and then centrifuged at 20,000 \times *g* for 15 min. ATP was quantified in the supernatant using the ATP bioluminescent assay kit (Sigma-Aldrich) according to the manufacturer's protocol.

Blue Native Electrophoresis—Mitochondria were isolated from 3-day-old male fly thoraxes using the mitochondrial isolation kit (Pierce). Mitochondrial proteins (10 μ g) were dissolved in NativePAGETM sample buffer (Invitrogen), 1% digitonin (Invitrogen), and 2% dodecylmaltoside (Invitrogen). Samples were incubated for 15 min on ice and centrifuged at 12,000 \times *g* for 25 min. Blue native electrophoresis was performed on NativePAGETM Novex 3–12% BisTris gels (Invitrogen).

RESULTS

DmGSTO1 Mutants Are Sensitive to Oxidative Stress—We previously reported that there are four different *GSTO* genes in *D. melanogaster*: *CG6781* (*sepia*), *CG6776*, *CG6673*, and *CG6662*. *CG6781* is the structural gene for *sepia*, which encodes pyrimidodiazepine synthase and is expressed exclusively in *Drosophila* heads (8). *CG6673* is also called *D. melanogaster GSTO1* (DmGSTO1) and has the highest thiol transferase and DHA reductase activity among the *GSTO* genes, reminiscent of thioredoxin and glutaredoxin (8). *CG6776* responds to heat stress in *Drosophila* (25). Although the function of *CG6662* is not known, *CG6662* transcripts are primarily expressed in the ovary and testes (26). Thus, we focused our study on *CG6673* (*DmGSTO1*) and excluded other *Drosophila GSTO* genes.

We generated both loss-of-function and gain-of-function *DmGSTO1* mutant flies. Because *DmGSTO1* encodes two alternatively spliced transcripts, A and B, we created both DmGSTO1A- and DmGSTO1B-overexpressing fly lines. Loss-of-function mutant *DmGSTO1*^{null} was generated by imprecise *P*-element excision, which resulted in partial deletion of the *DmGSTO1* gene (supplemental Fig. S1A). The *DmGSTO1*^{null} mutant was viable and fertile, and it exhibited no obvious defects in adult morphology. In the *DmGSTO1* null mutant fly, there were no detectable levels of either *DmGSTO1A* or *DmGSTO1B* transcripts (supplemental Fig. S1B). *GSTO* enzymes exhibit higher glutathione-dependent thiol transferase and dehydroascorbate reductase (DHAR) activity than any other class of GSTs (27). We have shown that DmGSTO1B has much higher DHAR activity than DmGSTO1A *in vitro* (8). The DHAR activity in *DmGSTO1*^{null} flies was dramatically decreased to ~5% of the DHAR activity found in wild type flies (supplemental Fig. S1C). Furthermore, *DmGSTO1*^{null} mutants were sensitive to paraquat, an oxidative stress inducer (Fig. 1A). Using a ubiquitous driver, *Tub-Gal4*, we directed expression of DmGSTO1A or DmGSTO1B in the *DmGSTO1*^{null} mutant background. The paraquat sensitivity was rescued by expression of DmGSTO1B, which exhibited more oxidative stress protection than DmGSTO1A (Fig. 1A). These results suggest that the paraquat-sensitive phenotype in the *DmGSTO1*^{null} mutant is primarily due to the loss of DmGSTO1B function. Because DHAR catalyzes the conversion of dehydroascorbate (DHA) to ascorbate (AsA) using glutathione as a reducing agent, we investigated the level of DHA and AsA in the mutant

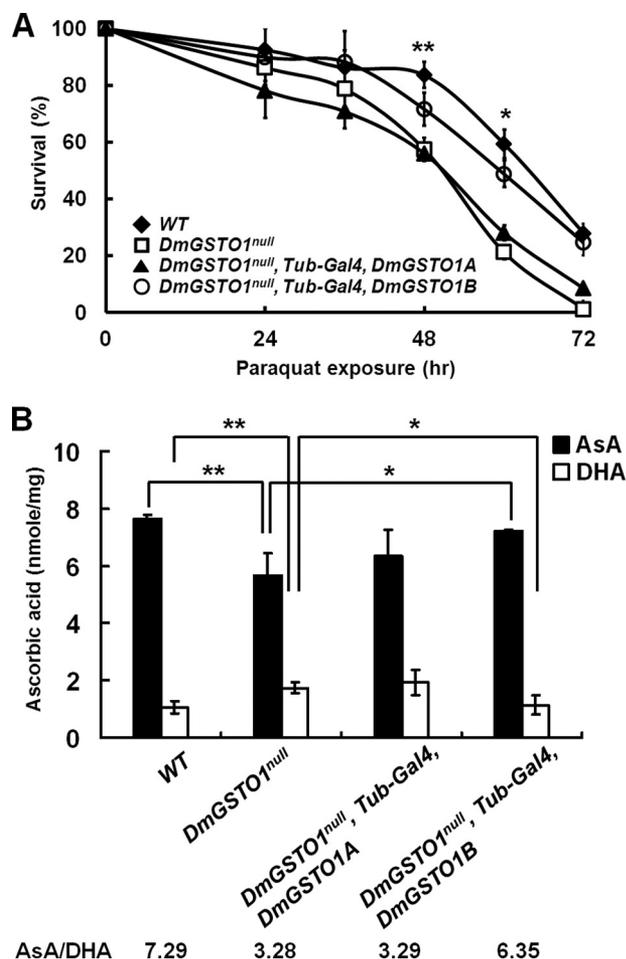


FIGURE 1. Sensitivity of *DmGSTO1* mutants to oxidative stress. A, survival rates of paraquat-treated (20 mM) flies overexpressing *DmGSTO1A*, and *DmGSTO1B* under the control of *Tubulin-Gal4*, a ubiquitously expressed driver ($n \geq 80$). *DmGSTO1*^{null} mutants were sensitive to oxidative stress. Overexpression of *DmGSTO1B* in a *DmGSTO1*^{null} mutant background reduced sensitivity to treatment with paraquat. Error bars, S.D. The significance was determined by one-way ANOVA (**, $p < 0.05$; *, $p < 0.01$). B, ascorbic acid, and dehydroascorbic acid content in fly extracts. The level of AsA was lower in *DmGSTO1*^{null} flies than in WT flies, whereas DHA was higher in *DmGSTO1*^{null} flies than in WT flies. The AsA/DHA ratio decreased from 7.29 in WT to 3.28 in *DmGSTO1*^{null}. Overexpression of *DmGSTO1B* in a *DmGSTO1*^{null} mutant background restored the ratio of AsA/DHA to 6.35. Error bars, S.D. Experimental significance was determined by one-way ANOVA (**, $p < 0.01$; *, $p < 0.05$). Experiments were performed in triplicate.

flies. As shown in Fig. 1B, only the overexpression of *DmGSTO1B* rescued the AsA/DHA ratio in *DmGSTO1*^{null} mutants. These results indicate that *DmGSTO1B* has a protective role in response to paraquat-induced stress and plays an important role in the *in vivo* conversion of DHA to AsA.

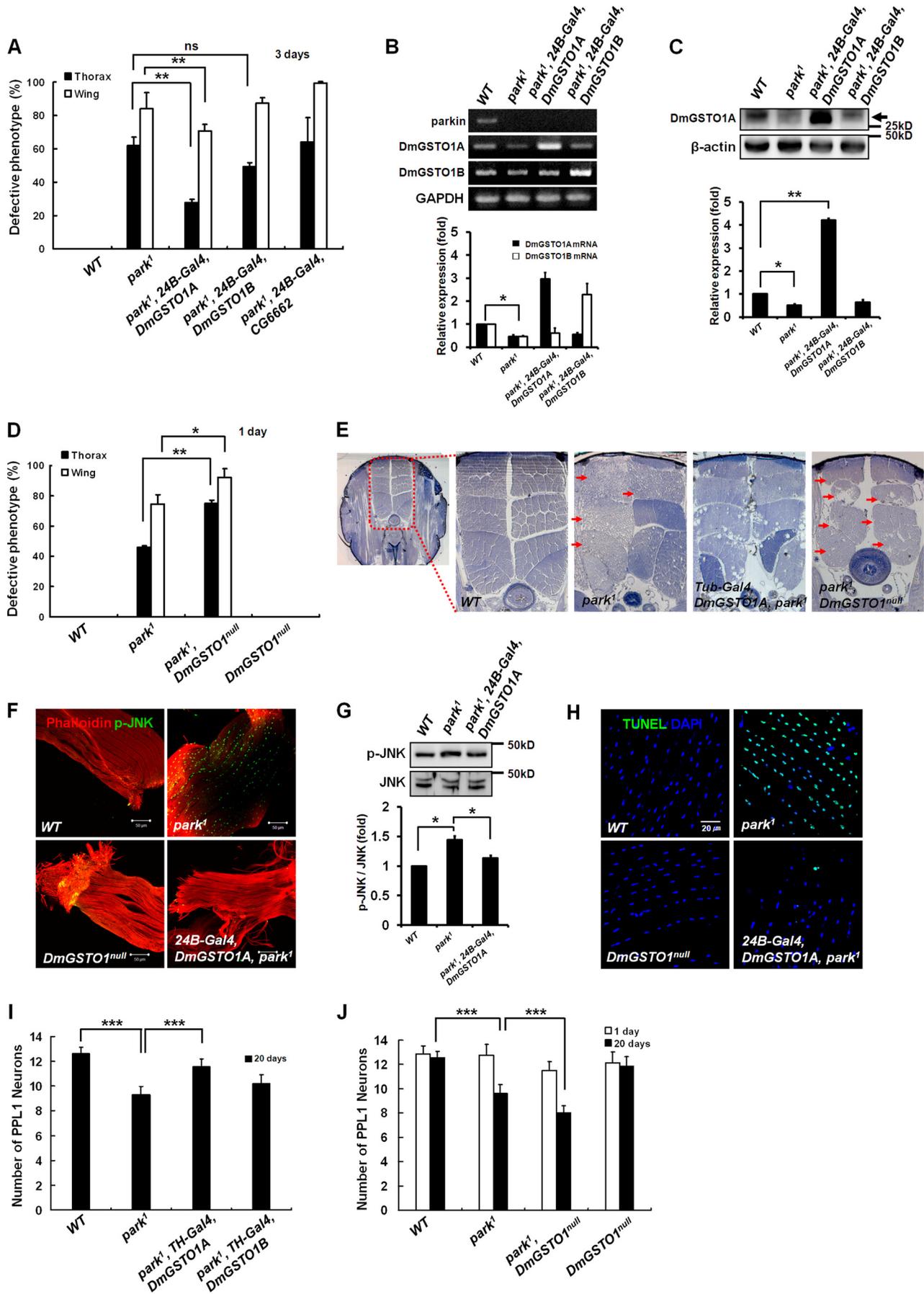
***DmGSTO1* Partially Rescues *park1* Mutant Phenotypes**—Although it has been reported that GSTs are involved in neurodegenerative disease (13, 28, 29), the molecular function of GSTOs remains unknown. To investigate the biological function of *DmGSTO1* in PD, we conducted genetic studies with *park1* mutants. As reported previously (18, 19), *park1* mutants showed collapsed thorax and downturned wing phenotypes. Surprisingly, increasing *DmGSTO1A* expression using the muscle-specific *24B-Gal4* driver significantly suppressed both thorax and wing phenotypes in *park1* mutants, whereas increased *DmGSTO1B* expression showed no effects (Fig. 2A

and supplemental Fig. S2, A and B). Of the *GSTO* genes, only the transcriptional expression of *DmGSTO1*, (*CG6673*) was decreased in *park1* mutants (Fig. 2B and supplemental Fig. S1D). Because the level of *DmGSTO1* mRNA decreased in *park1* mutants (Fig. 2B), we investigated whether the protein levels were also decreased in *park1* mutants. We generated a specific antibody against *DmGSTO1A*. As shown in Fig. 2C, the *DmGSTO1A* protein level was dramatically decreased in *park1* mutants compared with wild type flies. These results indicate that parkin regulates transcriptional expression of the *DmGSTO1* gene. We further examined the genetic interactions between parkin and *DmGSTO1* by introducing a *DmGSTO1* null mutation in the *park1* mutants. We found that the loss of function of *DmGSTO1* further enhanced the downturned wing and collapsed thorax phenotypes in the one-day-old *park1* mutants (Fig. 2D). In the *DmGSTO1*^{null} mutant, neither *DmGSTO1* nor *CG6662* transcripts were detected (supplemental Fig. S1B). We could eliminate the possibility that *CG6662* played a role in suppressing the *park1* mutant phenotype by demonstrating that *CG6662* overexpression in *park1* mutants had no effect on the *park1* mutant phenotype (Fig. 2A). These results indicate that *CG6662* is not involved in the suppression of *parkin* mutant phenotypes.

The downturned wing and thorax disruption phenotypes of *parkin* mutant flies are caused by the degeneration of the IFMs (21, 30). Therefore, we investigated whether *DmGSTO1* prevented muscle degeneration in *park1* mutants. As determined by histological analysis of thoracic IFMs, the integrity of IFMs in dorsal longitudinal muscles, which regulate adult wing posture, was clearly disrupted in *park1* mutants (Fig. 2E). Although *DmGSTO1*^{null} mutants showed a normal muscle phenotype, *park1*/*DmGSTO1*^{null} double mutants showed dramatically enhanced degeneration of IFMs compared with *park1* single mutants (Fig. 2E). Furthermore, overexpression of *DmGSTO1A* using a ubiquitous driver, *Tub-Gal4*, suppressed the degeneration of IFMs in *park1* mutants. Overexpression of *DmGSTO1A* in *park1* mutants resulted in regular and compact muscle tissues in the dorsal longitudinal IFMs, which were similar to those of wild type flies except for occasional vacuoles (Fig. 2E). These data suggest that *DmGSTO1* expression partially rescues the morphological defects and muscle degeneration in *park1* mutants.

Phospho-JNK Signal and Apoptosis Are Suppressed by *DmGSTO1* in *park1* Mutants—Many studies have suggested that neuronal cell death in various neurodegenerative diseases is closely related to JNK activation. Cha *et al.* (18) reported that parkin inhibits the JNK pathway. As shown in Fig. 2F, phospho-JNK was dramatically increased in IFMs of the *park1* mutant, whereas there was no change in phospho-JNK in the *DmGSTO1*^{null} mutant compared with wild type. The expression of *DmGSTO1A* suppressed activation of phospho-JNK in *park1* mutants (Fig. 2F), and the degree of suppression was confirmed by Western blot analysis (Fig. 2G). Degeneration of IFMs in *parkin* mutants occurs through an apoptotic mechanism (30). The IFMs in *park1* mutants were examined by a terminal deoxynucleotidyltransferase-mediated dUTP end labeling (TUNEL) assay. As shown in Fig. 2H, the increased TUNEL signal in *park1* mutants was suppressed by

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DmGSTO1A expression, similar to the suppression in the phospho-JNK signal. These data suggest that DmGSTO1 prevents degeneration of IFMs by blocking the activation of JNK and apoptosis in *park¹* mutants.

DmGSTO1 Suppresses Dopaminergic Neuronal Degeneration in *park¹* Mutants—*park¹* mutants show an age-dependent degeneration of DA neurons, especially in the protocerebral posterior lateral 1 (PPL1) cluster (18, 31). To further clarify the effect of DmGSTO1 on *park¹* mutants, we examined the degeneration of DA neurons that is characteristic of PD. As shown in Fig. 2I, DmGSTO1A expression under the control of a DA neuron-specific tyrosine hydroxylase (*TH*-*Gal4* driver resulted in significant restoration of the lost DA neurons in 20-day-old *park¹* mutant flies. In addition, 20-day-old *park¹/DmGSTO1^{null}* double mutants showed significantly increased DA neuron degeneration in the PPL1 cluster compared with the *park¹* single mutants of the same age, whereas there was no difference in neuronal degeneration between *park¹/DmGSTO1^{null}* double mutants and *park¹* single mutants in 1-day-old flies (Fig. 2J). These results indicate that DmGSTO1 protects the DA neurons from age-dependent degeneration in *park¹* mutants.

DmGSTO1 Restores Accumulation of Tubulin in IFMs in *park¹* Mutants—Parkin functions as an E3 ubiquitin ligase and has important roles in the degradation of many substrates (32, 33). Recent *in vitro* studies demonstrated that parkin binds to microtubule and tubulin proteins with high affinity and that parkin ubiquitinates and promotes the degradation of α/β -tubulin (34). It remains controversial whether putative *in vitro* substrates are relevant *in vivo*. Therefore, we investigated the α/β -tubulin protein levels in *park¹* mutants *in vivo*. Levels of α -tubulin were increased in *park¹* mutant muscles (Fig. 3A). Interestingly, the accumulation of α -tubulin in *park¹* mutant muscles was dramatically reduced by DmGSTO1A expression, and it was enhanced in *park¹/DmGSTO1^{null}* double mutant muscles (Fig. 3A). The levels of actin filaments in IFMs were unchanged in all mutants (Fig. 3B). These changes were confirmed by Western blot analysis (Fig. 3, C and D). We found that tubulin was slightly increased in *DmGSTO1^{null}* mutants by Western blot analysis (Fig. 3D). We also detected tubulin accumulation in DA neurons in *park¹* mutants and *park¹/DmGSTO1^{null}* double mutants (supplemental Fig. S3). These data indicate that parkin does not directly regulate the level of tubulin in *Drosophila in vivo*. In contrast to the change in total

protein levels (Fig. 3C), there were no detectable changes in the α/β -tubulin transcript levels in *park¹* mutants regardless of DmGSTO1A expression level (Fig. 3E). Thus, the increased α/β -tubulin levels were not caused by increased transcription but by protein accumulation. These results indicate that parkin is required for the regulation of tubulin levels and that DmGSTO1 suppresses the accumulation of tubulin in *park¹* mutants.

DmGSTO1 Suppresses Activation of Unfolded Protein Response (UPR) in *park¹* Mutant Muscles—Many studies have claimed that ER stress is involved in the progression of neurodegenerative diseases, such as PD. The UPR is a signaling pathway that is activated in response to ER stress. UPR activation has been observed in DA neurons of PD patients and is exemplified by an increase in phospho-PERK and eIF2 α (35). To determine whether the *parkin* mutation induces UPR activation, we examined eIF2 α , which is one of the components of the UPR signaling pathway and is activated by the upstream kinase PERK (36). The level of active phospho-eIF2 α was highly increased in *park¹* mutants and dramatically restored by DmGSTO1A expression (Fig. 3F). The ER and mitochondrial UPR share similar pathways that increase chaperone levels to promote protein homeostasis in the cytoplasm and mitochondria (37). We also examined heat shock proteins (HSPs), including Hsp60, and Hsp/Hsc70. As shown in Fig. 3G, the levels of HSPs were increased in *park¹* mutants and reduced by DmGSTO1A expression. These data indicate that DmGSTO1 suppresses UPR activation in *park¹* mutants.

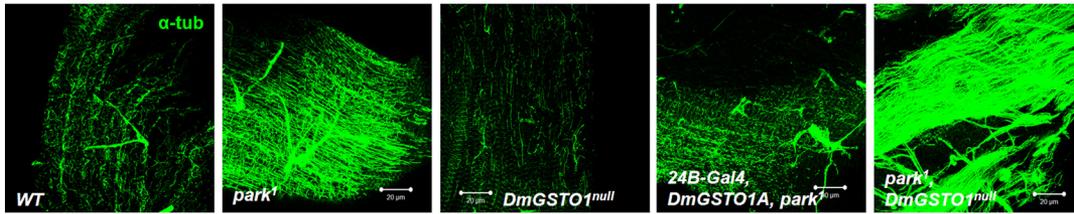
DmGSTO1 Restores Mitochondrial ATP Synthase Activity in *park¹* Mutants—We next examined whether the catalytic activity of DmGSTO1A was critical for the rescue the *park¹* mutant phenotypes. GSTOs have a unique cysteine at their active site that binds to GSH (27). We constructed a catalytically inactive form of DmGSTO1A, DmGSTO1A^{C31A}, in which cysteine 31 was mutated to alanine. In contrast to wild type DmGSTO1A, expression of DmGSTO1A^{C31A} in muscle did not rescue the collapsed thorax and downturned wing phenotypes in *park¹* mutants (Fig. 4A). Moreover, tubulin accumulation was not suppressed by DmGSTO1A^{C31A} expression in *park¹* mutants (Fig. 4B). Our data demonstrate that DmGSTO1 catalytic activity is required to rescue the defective phenotypes of *park¹* mutants.

Although the ability of DmGSTO1B to respond to oxidative stress was higher than that of DmGSTO1A, only up-regulation of DmGSTO1A suppressed the defective phenotypes in *parkin*

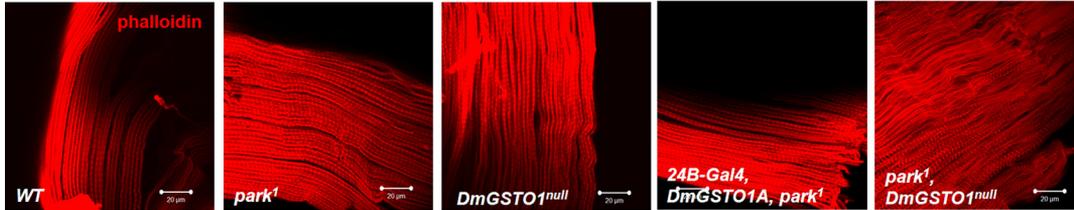
FIGURE 2. Up-regulation of DmGSTO1 suppresses phenotypes caused by *parkin* loss of function. A, statistical analysis of the percentage of collapsed thorax ($n > 120$) and downturned wing ($n > 90$) phenotypes in 3-day-old flies. DmGSTO1A overexpression by the *24B-Gal4* muscle-specific driver suppresses the thorax and wing defects of *parkin* mutant flies. Experimental significance was determined by one-way ANOVA (**, $p < 0.05$; ns, not significant). B, DmGSTO1 mRNA levels determined by RT-PCR were also reduced in *park¹* mutants. Error bars, S.D. Significance was determined by one-way ANOVA (*, $p < 0.01$). Experiments were performed in triplicate. C, endogenous DmGSTO1A levels (black arrow) were dramatically reduced in *park¹* mutants. Error bars, S.D. The significance was determined by one-way ANOVA (**, $p < 0.0001$; *, $p < 0.01$). D, percentage of collapsed thorax ($n > 300$) and downturned wing ($n > 110$) phenotypes in 1-day-old *park¹/DmGSTO1^{null}* double mutants. The DmGSTO1 mutation enhanced the thorax and wing defects exhibited by *parkin* mutants. Error bars, S.D. Statistical analysis was carried out with one-way ANOVA (**, $p < 0.001$; *, $p < 0.05$). E, light microscopy was used to examine IFM morphology (red arrow, muscle degeneration). Shown are magnified views of the dorsal longitudinal muscle ($\times 200$). Tubulin-*Gal4*-driven DmGSTO1A expression rescues muscle degeneration of *park¹* mutant flies. The *park¹/DmGSTO1^{null}* double mutants showed enhanced degeneration of muscles compared with *park¹* single mutants. F, muscle-specific expression of DmGSTO1A in *park¹* mutants reduced phosphorylated JNK. Activated JNK (phospho-JNK; p-JNK) is visualized in green, and phalloidin-labeled muscle tissues are shown in red. G, Western blot analysis of phospho-JNK. Error bars, S.D. Experimental significance was determined by one-way ANOVA (*, $p < 0.05$). Experiments were performed in triplicate. H, merged images of apoptotic cells (TUNEL, green) and nuclei (DAPI, blue). Up-regulation of DmGSTO1A resulted in fewer apoptotic cells in the muscle. I and J, quantification of TH-positive neurons in PPL1 clusters in 1- and 20-day-old flies ($n > 10$). Up-regulation of DmGSTO1A by the *TH-Gal4* DA neuron-specific driver can rescue DA neuron loss in *parkin* mutants. Error bars, S.D. Experimental significance was determined by one-way ANOVA (***, $p < 0.0001$).

Function of Glutathione S-Transferase Omega 1

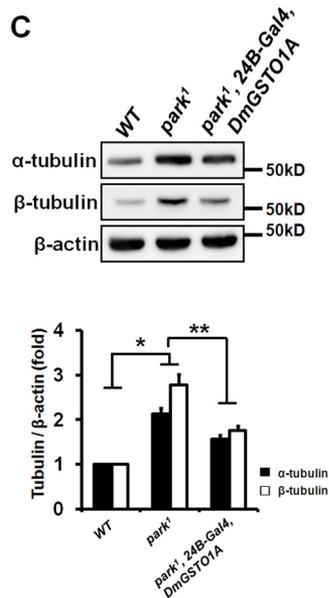
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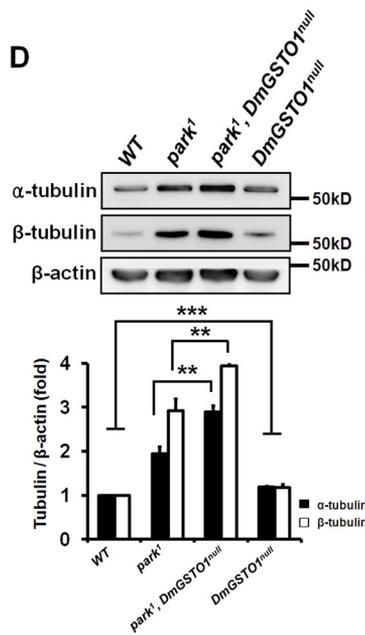
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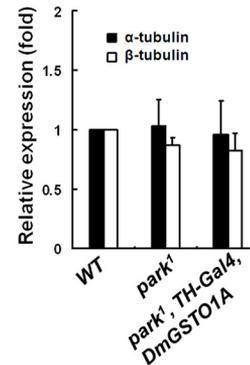
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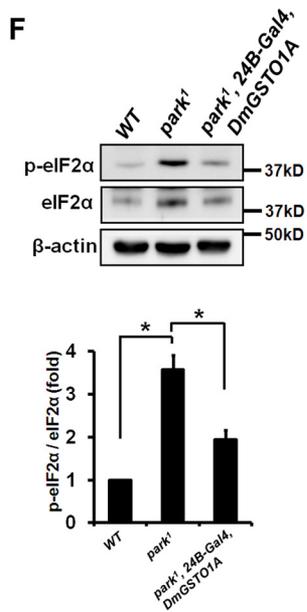
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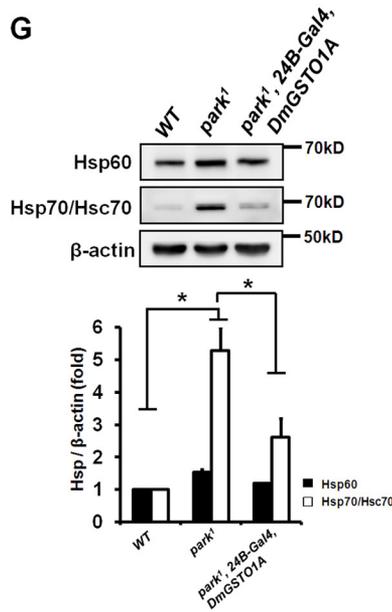
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mutants (Figs. 1A and 2A). Therefore, DmGSTO1A may suppress *parkin* mutant phenotypes by other mechanisms. The catalytic detoxification functions of the GST family have been studied by several research groups. However, some members of the GST family have physiological functions unrelated to detoxification (38). Previous reports revealed that the rate of protein glutathionylation, a post-translational modification that regulates the function of proteins, is enhanced by the presence of active GSTP (GST Pi family) (39). Based on the result that DmGSTO1 enzyme activity is required to suppress the *parkin* mutant phenotypes, we screened *in vivo* targets of DmGSTO1 by immunoprecipitation with an anti-GSH antibody in adult thorax extracts. Only one glutathionylated protein showed altered abundance in *park¹* mutants. DmGSTO1A expression increased the level of glutathionylation of this protein, whereas DmGSTO1A^{C31A} did not. The protein was identified by MALDI-TOF mass spectrometric analysis as the mitochondrial F₁F₀-ATP synthase β subunit. We also confirmed that DmGSTO1A can directly glutathionylate the ATP synthase β subunit in a dose-dependent manner in the presence of GSH (Fig. 5A). Furthermore, the endogenous levels of the glutathionylated form of the ATP synthase β subunit in thorax extracts were decreased in *park¹* mutants and decreased even more in *park¹/DmGSTO1^{null}* double mutants, whereas the total expression levels of the ATP synthase β subunit were unchanged in all fly lines. DmGSTO1A expression in *park¹* mutants restored the levels of glutathionylated ATP synthase β subunit to wild type levels, whereas the expression of DmGSTO1A^{C31A} or DmGSTO1B in *park¹* mutants had no effect on the level of ATP synthase β subunit glutathionylation (Fig. 5B). However, in the *DmGSTO1^{null}* mutant, the level of glutathionylation of the ATP synthase β subunit was only slightly decreased compared with control. This finding suggests that a compensatory mechanism related to glutathionylation of the ATP synthase β subunit exists. Another DmGSTO, CG6662, did not affect glutathionylation of the ATP synthase β subunit (supplemental Fig. S4). These data indicate that the ATP synthase β subunit is a novel and specific target of DmGSTO1A in *Drosophila* and that only DmGSTO1A expression in *park¹* mutants is sufficient to partially restore glutathionylation of the ATP synthase β subunit.

Glutathionylation is a reversible post-translational modification that can lead to alteration of protein or enzyme function, such as the Ca²⁺ uptake activity of the sarco-/endoplasmic reticulum calcium ATPase (40). To test the hypothesis that glutathionylation of the ATP synthase β subunit by DmGSTO1A in *park¹* mutants regulates ATP synthase activity, we isolated mitochondria from the thorax and measured F₁F₀-ATP synthase activity. As shown in Fig. 5C, the level of mitochondrial

F₁F₀-ATP synthase activity was partially rescued by DmGSTO1A expression in *park¹* mutants and was decreased in *park¹/DmGSTO1^{null}* double mutants. Indeed, the change in mitochondrial F₁F₀-ATP synthase activity was correlated to the change in glutathionylation of the ATP synthase β subunit. Consistent with the ATP synthase activity results, we observed a change in the total ATP levels in all of the mutant lines (Fig. 5C). Impaired mitochondrial respiration caused by decreased ATP production has been reported in PD, and agents that improve mitochondrial respiration can exert beneficial effects in animal models of PD (2). These reports are consistent with our findings; ATP synthase activity and ATP levels are significantly decreased in *park¹* mutants and can be modulated by DmGSTO1 expression.

Decreased ATP Synthase β Subunit in *Drosophila* Muscle Leads to *park¹* Mutant-like Phenotype—Mutations of ATP synthase subunits are known to exhibit mitochondrial dysfunction and neuromuscular impairment (41, 42). To determine whether decreased ATP synthase activity was directly linked to the phenotypes found in *parkin* mutants, we down-regulated the ATP synthase β subunit in *Drosophila* thorax muscle using a UAS-RNAi line (obtained from the NIG-FLY stock center) together with the muscle-specific *24B-Gal4* driver. Because the ATP synthase β subunit knockdown flies reared at 25 °C displayed pupal lethality, we performed all knockdown experiments at 18 °C to increase adult viability. When ATP synthase β subunit knockdown was induced at 18 °C in muscle using *24B-Gal4*, fewer than 20% of the flies were able to eclose. We observed abnormal muscle structure in the knockdown mutant flies, similar to the phenotypes found in *parkin* mutants (Fig. 5D). Moreover, the ATP synthase β subunit RNAi flies showed accumulation of total α/β -tubulin (Fig. 5E). These results demonstrate that loss of the ATP synthase β subunit induces some of the *parkin* mutant phenotypes, including muscle degeneration and accumulation of tubulin.

Next, to examine whether the ATP synthase β subunit RNAi enhances the *parkin* mutant phenotype, the ATP synthase β subunit was knocked down in *park¹* mutant muscles. As shown in Fig. 5F, we observed an increase in α/β -tubulin accumulation. Moreover, the levels of Hsp70/Hsc70 and phosphorylated eIF2 α in the *park¹* mutant were further increased by RNAi knockdown of the ATP synthase β subunit (Fig. 5G). These data indicate that down-regulation of ATP synthase activity in the *park¹* mutant results in a more severe phenotype compared with that of *park¹* single mutants.

DmGSTO1 Rescues Mitochondrial ATP Synthase Assembly in *park¹* Mutants—To investigate how expression of DmGSTO1A affects ATP synthase activity, we examined the assembly level of the F₁F₀-ATP synthase complex (Complex V)

FIGURE 3. Up-regulation of DmGSTO1 in muscle decreases tubulin accumulation and UPR activation in *park¹* mutants. Shown are representative images of flight muscle stained with anti- α -tubulin antibody and phalloidin. *A*, α -tubulin accumulates in *park¹* mutant muscle. The accumulation of tubulin in the *park¹* mutant was suppressed by the muscle-specific up-regulation of DmGSTO1A. *B*, the levels of actin filaments in muscle did not change in any mutants as visualized with phalloidin. *C*, the increased levels of α/β -tubulin in *park¹* mutants were rescued by DmGSTO1A expression. Error bars, S.D. The experimental significance was determined by one-way ANOVA (**, $p < 0.05$; *, $p < 0.01$). *D*, tubulin levels were higher in the double mutants than in the *park¹* single mutants. Error bars, S.D. The significance was determined by one-way ANOVA (***, $p < 0.01$; **, $p < 0.05$). *E*, mRNA levels of male thoraces were determined by real-time PCR analysis. Relative amounts of tubulin mRNA were unchanged in all mutants. *F*, Western blot analysis of phosphorylated and total eIF2 α . *G*, increased HSPs in *park¹* mutants were decreased with the up-regulation of DmGSTO1A. *F* and *G*, error bars, S.D. The significance was determined by one-way ANOVA (*, $p < 0.05$). β -Actin was used as a loading control. Experiments were performed in triplicate.

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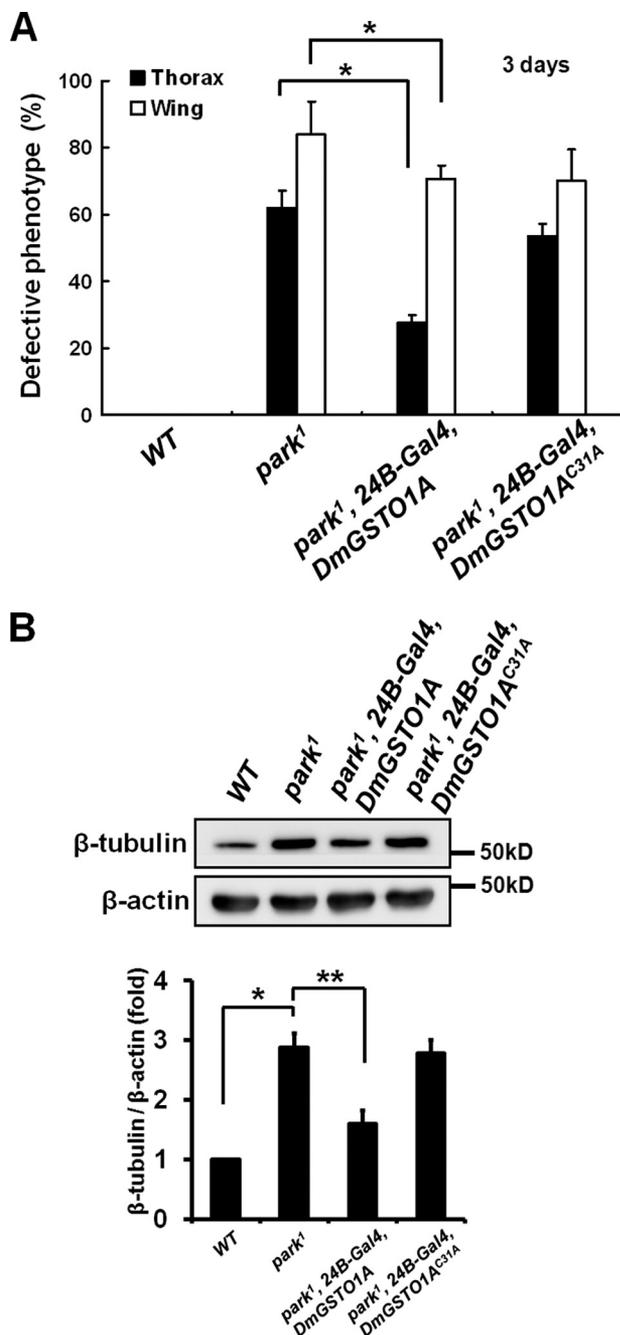


FIGURE 4. The catalytic activity of DmGSTO1 is critical for rescue of the defective phenotypes of the *park¹* mutant. *A*, expression of DmGSTO1A^{C31A} in the *park¹* mutant background did not suppress the collapsed thorax ($n > 200$) and downturned wing ($n > 160$) phenotypes of *parkin* mutants. Error bars, S.D. The experimental significance was determined by one-way ANOVA (*, $p < 0.05$). *B*, Western blot analysis of adult thorax extracts using anti- β -tubulin antibodies. DmGSTO1A^{C31A} expressed in the *park¹* mutants did not rescue the tubulin accumulation phenotypes. Error bars, S.D. The significance was determined by one-way ANOVA (**, $p < 0.05$; *, $p < 0.01$). β -Actin was used as a loading control. Experiments were performed in triplicate.

by blue native PAGE and Western blot analysis with an anti-ATP synthase α subunit antibody. Three bands (supercomplex, >800 kDa; assembled ATP synthase, Complex V, >600 kDa; F1 subcomplex, >400 kDa) were detected (Fig. 6). All three bands associated with the mitochondrial F₁F₀-ATP synthase complex were dramatically decreased by RNAi knockdown of the ATP

synthase β subunit (supplemental Fig. S5). The amount of assembled ATP synthase was normalized using mitochondrial prohibitin. In comparison with the wild type, the amount of assembled ATP synthase complex was decreased in *park¹* mutants. Expression of DmGSTO1A in *park¹* mutants increased the amount of assembled ATP synthase complex (Fig. 6A, Complex V). Interestingly, *park¹/DmGSTO1^{null}* double mutants tend to exhibit lower amounts of assembled ATP synthase than those in *park¹* mutants, although the effect was not statistically significant (Fig. 6B). Blue native electrophoresis analysis is not sensitive to small changes; nevertheless, the change in the amount of assembled ATP synthase complex correlated to the change in ATP synthase activity. These results indicate that DmGSTO1A affects mitochondrial ATP synthase activity by regulating the assembly efficiency of ATP synthase in *park¹* mutants.

DISCUSSION

In this study, we suggest that DmGSTO1 is a novel genetic suppressor of parkin dysfunction and has a protective role in a model of PD. Moreover, we showed that the ATP synthase β subunit is a novel target of DmGSTO1 in *Drosophila*. The ATP synthase β subunit is an essential catalytic core component of the F₁F₀-ATP synthase complex in mitochondria. We found that levels of glutathionylation of the ATP synthase β subunit were significantly decreased in *park¹* mutants, whereas its total protein level remained unchanged. Glutathionylation is a mechanism of post-translational regulation of several proteins, including protein-tyrosine phosphatase 1B (PTP1B), and MEK1. The glutathionylation of a calcium channel, ryanodine receptor 1 (RyR1), activates it and enhances calcium release (43). Our results suggest that expression of DmGSTO1A in *park¹* mutants increases the level of glutathionylated ATP synthase β subunit and restores mitochondrial F₁F₀-ATP synthase activity, thereby partially rescuing *park¹* mutant phenotypes, including the degeneration of DA neurons.

The increase in F₁F₀-ATP synthase activity and assembly by DmGSTO1A in the *park¹* mutant led to a recovery of ATP depletion. Interestingly, because mitochondrial F₁F₀-ATP synthase has a role in maintaining inner membrane morphology, and mitochondrial membrane potential (41, 44) and mutation of F₁F₀-ATP synthase ϵ subunit can cause mitochondrial dysfunction (42), the restoration of F₁F₀-ATP synthase activity in the *park¹* mutant is important for rescuing the *parkin* mutant phenotypes. Moreover, RNAi mutants of the ATP synthase β subunit exhibit phenotypes similar to *park¹* mutants, including α/β -tubulin accumulation, locomotor dysfunction, UPR activation, and muscle degeneration.

Although the change in mitochondrial F₁F₀-ATP synthase activity and ATP levels correlated with the degree of glutathionylation of the ATP synthase β subunit, the exact regulatory mechanism between glutathionylation of the ATP synthase β subunit and ATP synthase activity is not known. It is technically difficult to show a direct relationship between glutathionylation of the ATP synthase β subunit and ATP synthase activity because in *Drosophila*, mitochondrial ATP synthase is a large multiprotein complex composed of eight different subunits. Further studies will be required to determine how glutathiony-

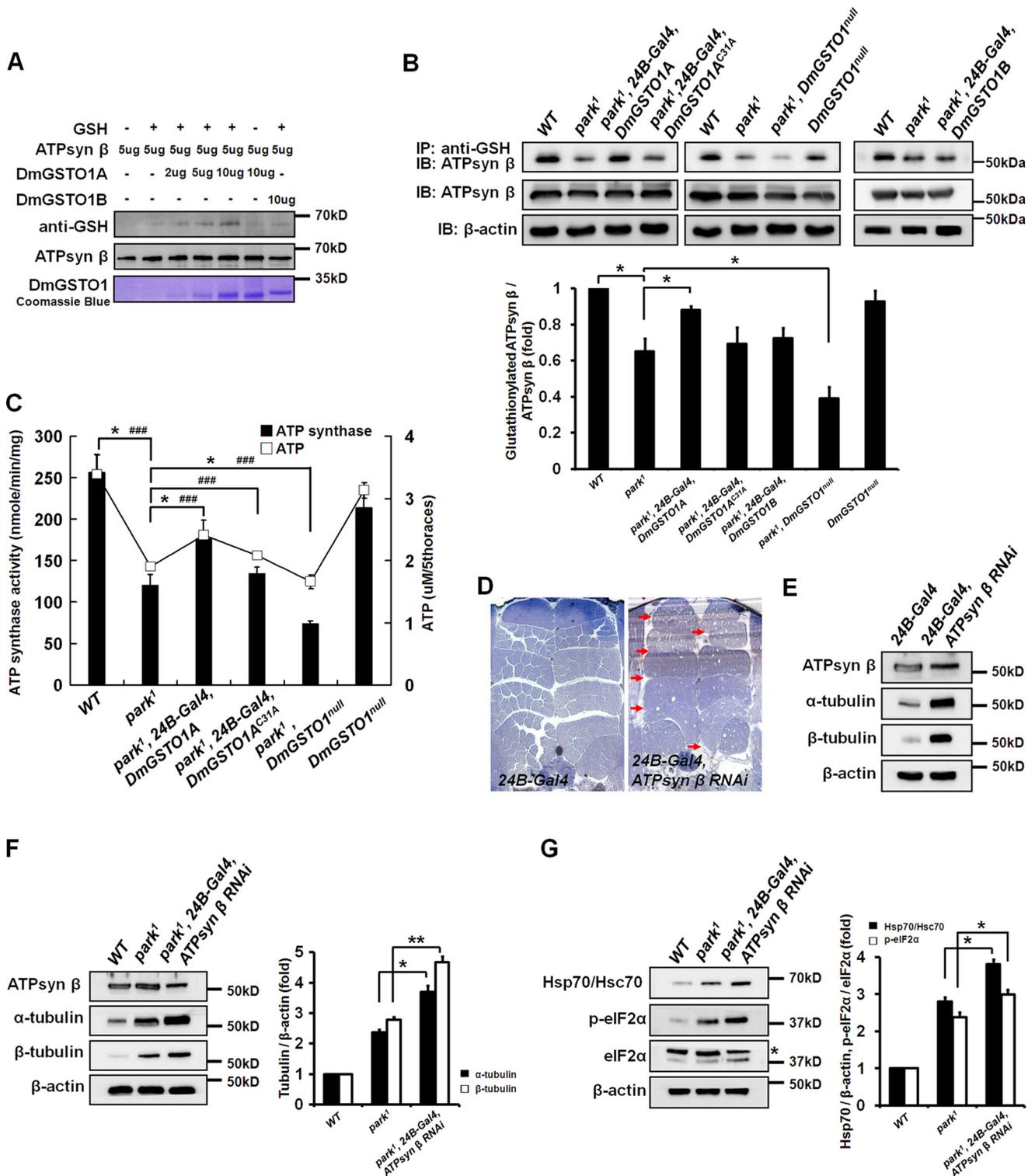


FIGURE 5. DmGSTO1 partially restored mitochondrial F_1F_0 -ATP synthase activity in *park1* mutants. *A*, in the presence of GSH, recombinant ATP synthase β subunit was glutathionylated by DmGSTO1A in a dose-dependent manner. *B*, glutathionylated proteins were immunoprecipitated from thorax extracts with an anti-GSH antibody and were immunoblotted with an anti-ATP synthase β antibody. Glutathionylation of endogenous ATP synthase β subunit in *park1* mutants was regulated by the GSH-conjugating catalytic activity of DmGSTO1A but not by DmGSTO1B. The endogenous levels of the glutathionylated form of the ATP synthase β subunit were decreased even more in *park1*/DmGSTO1^{null} double mutants. *Error bars*, S.D. The experimental significance was determined by one-way ANOVA (*, $p < 0.05$). *C*, mitochondrial F_1F_0 -ATP synthase activity and ATP levels in *Drosophila* thoraces. DmGSTO1A expression enhances ATP synthase activity and ATP level of *park1* mutants. *Error bars*, S.D. One-way ANOVA was used for statistical analysis (ATP synthase activity: *, $p < 0.05$; ATP level: ###, $p < 0.001$). *D*, muscle morphology of wild type and ATP synthase β subunit RNAi mutant flies (arrow, muscle degeneration). Muscle-specific ATP synthase β subunit RNAi resulted in degeneration of the flight muscles. *E*, Western blot analysis of adult thorax extracts from the ATP synthase β subunit RNAi mutant flies. α - and β -tubulin showed significant accumulation in the RNAi mutant muscle. *F*, Western blot analysis of adult thorax extracts from the ATP synthase β subunit RNAi in a *park1* mutant background. Accumulation of tubulin was increased more in the RNAi mutants than in the *park1* single mutants. *Error bars*, S.D. The significance was determined by one-way ANOVA (**, $p < 0.01$; *, $p < 0.05$). *G*, Western blot analysis of Hsp70/Hsc70, phosphorylated eIF2 α , and total eIF2 α (*, nonspecific band). *Error bars*, S.D. The significance was determined by one-way ANOVA (*, $p < 0.01$). Experiments were performed in triplicate.

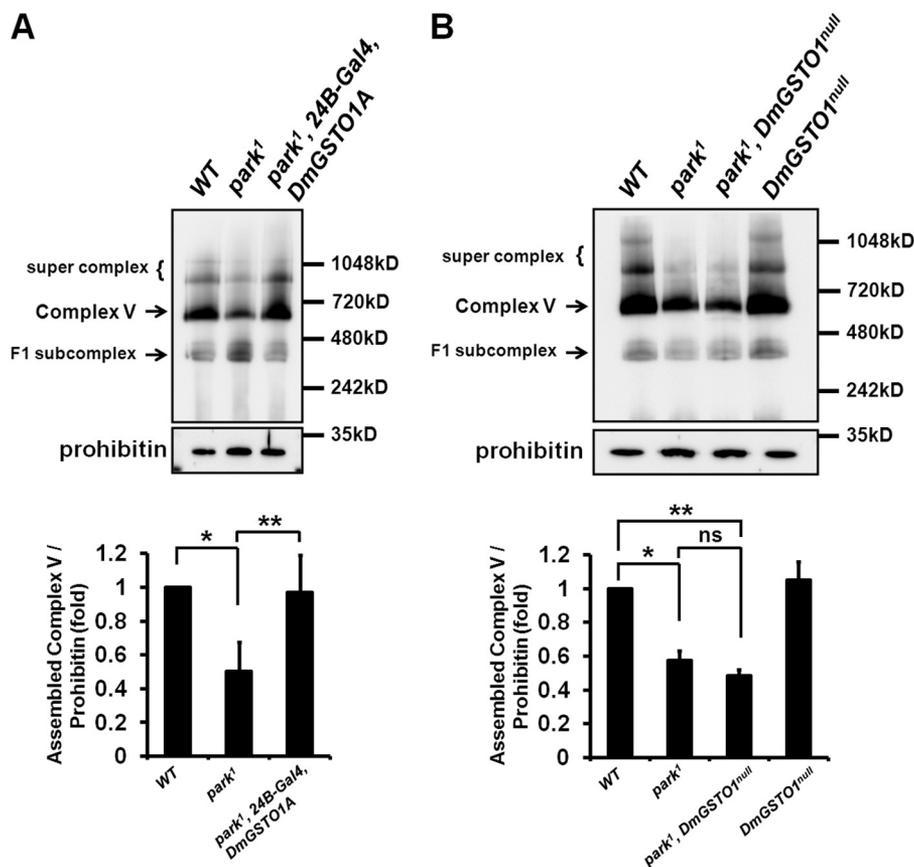


FIGURE 6. **Mitochondrial F₁F₀-ATP synthase (Complex V) assembly is affected by DmGSTO1 expression in *park¹* mutants.** *A* and *B*, mitochondrial protein extracts from the thorax of mutant fly lines were subjected to blue native PAGE, followed by Western blot analysis with anti-ATP synthase α subunit antibody. Three bands were detected: supercomplex (>800 kDa), assembled ATP synthase (Complex V, >600 kDa), and F1 subcomplex (>400 kDa). Prohibitin was used as a mitochondrial loading control. *A*, the assembly of ATP synthase (Complex V) was significantly decreased in *park¹* mutants. The amount of assembled ATP synthase was restored by DmGSTO1A up-regulation in *park¹* mutants. Error bars, S.D. The experimental significance was determined by one-way ANOVA (**, $p < 0.01$; *, $p < 0.0001$). Experiments were performed in quintuplicate. *B*, The amount of assembled ATP synthase tended to decrease more in *park¹/DmGSTO1^{null}* double mutants than *park¹* mutants; however, the effect was not statistically significant. Error bars, S.D. The experimental significance was determined by one-way ANOVA (**, $p < 0.0001$; *, $p < 0.001$; ns, not significant). Experiments were performed in quadruplicate.

lation of the ATP synthase β subunit regulates mitochondrial ATP synthase activity and assembly at the molecular level.

However, the loss-of-function mutant *DmGSTO1^{null}* exhibited no obvious morphological defects and slightly reduced glutathionylation of the ATP synthase β subunit and ATP synthase activity. It is not clear why *DmGSTO1^{null}* single mutants show a weak effect on the glutathionylation and activity of ATP synthase. One possible explanation is that compensatory mechanisms related to glutathionylation of the ATP synthase β subunit exist *in vivo*.

Although our current study focused on the specific target of GSTO related to neurodegeneration, previous studies have shown that GSTs have protective functions against oxidative stress in neurodegenerative diseases. Loss of function of the yeast *GSTS1* homolog, *gtt-1*, enhances α -synuclein toxicity (45), and mouse GST Pi contributes to the sensitivity to xenobiotics in idiopathic PD (29). Additionally, increased GST Pi expression protected cells from rotenone-induced neurotoxicity (46). However, the various roles of GSTs remain controversial because of the diversity and complexity of these proteins. Because the expression levels of several GSTs increase due to oxidative stress, we measured endogenous DmGSTO1A expression in *park¹* mutants and found that the protein and

mRNA levels of DmGSTO1A were significantly lowered in *park¹* mutants. Transcriptional profiling of *parkin* mutants showed that the oxidative stress response genes are up-regulated, and overexpression of *GSTS1* in DA neurons suppressed neurodegeneration in *parkin* mutants (11, 12). DmGSTS1 was also up-regulated by the 4E-BP1-mediated stress response (47). Therefore, DmGSTS1 in *park¹* mutants may be increased in an effort to decrease the stress induced by the *parkin* mutation, but its increased level is not sufficient to rescue the *park¹* phenotype. Both DmGSTO1 mRNA and protein levels were decreased in *park¹* mutants, suggesting that the normal function of parkin is critical for the regulation of DmGSTO1 expression. Little is known about the factors regulating *DmGSTO1* gene expression, but we can make the following speculation. Because the *DmGSTO1* gene contains a potential NF- κ B-like transcription factor binding motif (48), and parkin stimulates NF- κ B-dependent transcription (49), DmGSTO1 may be down-regulated in the *park¹* mutant. Thus, the mechanism underscoring the protective role of DmGSTO1 in *park¹* mutants is distinct from the mechanisms used by general antioxidants and the detoxifying enzyme DmGSTS1. Furthermore, GSTO1 has an active site cysteine residue, which is distinct from the tyrosine residue found in GSTS1. This cys-

teine residue could enable GSTO1 to modulate the disulfide status of cysteine residues on substrate proteins (27). Although the specific substrates of individual GSTs remain unknown, the functional diversity of GSTs might explain why they have different substrates against various stresses *in vivo*.

DmGSTO1B more strongly protects against oxidative stress than DmGSTO1A, but only the up-regulation of DmGSTO1A improved the defective phenotypes in *parkin* mutants. Although DHAR activity and the reduced form of ascorbate have protective roles under oxidative stress conditions (50), they are not sufficient to rescue *parkin* mutant phenotypes. As shown in Fig. 5, A and B, DmGSTO1B was not able to glutathionylate the ATP synthase β subunit. Thus, we propose that these two isoforms, DmGSTO1A and DmGSTO1B, have different substrates and act differently on the stress response pathway *in vivo*.

Mitochondrial defects have been detected in PD cases with *parkin* mutations. Recent studies suggest that *parkin* promotes mitochondrial fission and/or inhibits mitochondrial fusion in muscle tissues and DA neurons (51–53). Staining mitochondria with streptavidin revealed severe defects in mitochondrial integrity in the IFMs of *park¹* mutants. Interestingly, these defects were not restored by DmGSTO1A expression, and *park¹/DmGSTO1^{null}* double mutants were not distinguishable from *park¹* single mutants (supplemental Fig. S6). These findings were further confirmed by expressing *mito-GFP* in thorax muscles (supplemental Fig. S6). In contrast to the *park¹* mutants, the ATP synthase β subunit RNAi mutants did not show disrupted mitochondrial morphology (supplemental Fig. S6). These results suggest that DmGSTO1 is not important for restoring mitochondrial morphology in *park¹* mutants and might act downstream or in parallel to the mitochondrial dynamics pathway.

PTEN-induced putative kinase 1 (PINK1) is a Ser/Thr kinase containing a mitochondrial targeting motif (54). Previous studies reported that mutations of *PINK1* lead to muscle degeneration, DA neuron loss, and mitochondrial dysfunction. PINK1 and *parkin* are linked in the same pathway, and PINK1 acts upstream of *parkin* (19, 51–53, 55). We therefore hypothesized that DmGSTO1A would genetically interact with the *PINK1* null mutant *PINK1^{B9}*. Interestingly, consistent with *park¹* mutants, we found that *DmGSTO1* and *parkin* mRNA were decreased in *PINK1^{B9}* mutants (supplemental Fig. S7A). Glutathionylation of the mitochondrial ATP synthase β subunit was significantly decreased in *PINK1^{B9}* mutants (supplemental Fig. S7B). Therefore, it seems possible that DmGSTO1A up-regulation may also contribute to the prevention of degeneration in *PINK1^{B9}* mutants. A detailed study of *PINK1^{B9}* mutants is currently under way in our group.

Our results support the hypothesis that DmGSTO1 is linked to the pathogenic phenotypes displayed in *parkin* mutants. We found that DmGSTO1 is a novel genetic suppressor of *parkin* dysfunction. The two isoforms of DmGSTO1 have different functions. DmGSTO1B in *Drosophila* is required to protect the flies against oxidative stress. Although the exact molecular mechanism is not clear, glutathionylation of the ATP synthase β subunit by DmGSTO1A regulates mitochondrial F_1F_0 -ATP synthase activity, and the restoration of ATP synthase activity

by DmGSTO1A expression is critically important for partial rescue of the mitochondrial function in *park¹* mutants. These findings present a novel mechanism of regulation of ATP synthase by DmGSTO1 in *parkin* mutants. Our results strongly suggest that promoting DmGSTO1 activity could alleviate neurodegeneration in *parkin* mutants. These findings will lead us to a better understanding of the molecular mechanism of neuroprotection due to GSTO in PD and could help in developing new therapeutic approaches for PD.

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