

Parkin expression reverses mitochondrial dysfunction in fused in sarcoma-induced amyotrophic lateral sclerosis

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Abstract

Fused in sarcoma (FUS) is a DNA/RNA-binding protein associated with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration. The exact molecular mechanisms by which FUS results in neurotoxicity have not yet been fully elucidated. Here, we found that *parkin* is a genetic suppressor of defective phenotypes induced by exogenous human wild type FUS in *Drosophila*. Although *parkin* overexpression did not modulate the FUS protein expression level, the locomotive defects in FUS-expressing larvae and adult flies were rescued by *parkin* expression. We found that FUS expression in muscle tissues resulted in a reduction of the levels and assembly of mitochondrial complex I and III subunits, as well as decreased ATP. Remarkably, expression of *parkin* suppressed these mitochondrial dysfunctions. Our results indicate *parkin* as a neuroprotective regulator of FUS-induced proteinopathy by recovering the protein levels of mitochondrial complexes I and III. Our findings on *parkin*-mediated neuroprotection may expand our understanding of FUS-induced ALS pathogenesis.

Keywords: amyotrophic lateral sclerosis, FUS, *parkin*, mitochondrial dysfunction, *Drosophila*.

Introduction

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is characterized by the progressive degeneration of both upper and lower motor neurones, which leads to muscle weakness. Between 3 to 5 years after disease onset, muscle degradation can ultimately result in fatal muscle atrophy, paralysis, and death (Arai *et al.*, 2006; Neumann *et al.*, 2006). In approximately 90 to 95% of all ALS cases, the disease typically occurs sporadically with no clearly associated risk factors, while approximately 5 to 10% of ALS cases are familial. Mutations in several genes, including *superoxide dismutase 1 (SOD1)*, *chromosome 9 open reading frame 72 (C9orf72)*, *optineurin (OPTN)*, *p97/valosin-containing protein (VCP)*, *fused in sarcoma (FUS)*, *TAR DNA-binding protein (TDP-43)*, and *TATA box-binding protein-associated factor 15 (TAF15)*, have been linked to cases of familial ALS (Al-Chalabi *et al.*, 2012). Particularly, FUS is a DNA/RNA-binding protein that is implicated in RNA metabolism, including RNA processing, splicing, transport and translation, by regulating cellular localization and degradation (Lagier-Tourenne *et al.*, 2010; Ling *et al.*, 2013; Kapeli *et al.*, 2016). Recently, FUS has been identified as a component of cytoplasmic inclusions in patients with familial ALS (Mackenzie *et al.*, 2010). FUS is predominantly localized in the nucleus. ALS-linked mutations in FUS lead to the mislocalization of the protein to the cytoplasm from the nucleus (Munoz *et al.*, 2009; Neumann *et al.*, 2009; Dormann *et al.*, 2010). Rodents and flies expressing wild type or mutant FUS developed defective phenotypes, such as synapse loss in the neuromuscular junction, aggregate formation in the cytoplasm, imbalance of mitochondrial dynamics, and mitochondrial dysfunction (Lanson Jr. *et al.*, 2011; Altanbyek *et al.*, 2016; Nolan *et al.*, 2016). However, the molecular mechanisms underlying FUS-induced neuronal toxicity are not yet fully understood.

Parkin is a component of the cytosolic E3 ubiquitin ligase, which is associated with the ubiquitin/proteasome

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protein degradation pathway. Mutations in parkin are responsible for early-onset autosomal-recessive Parkinson's disease (Martin *et al.*, 2011). Various putative substrates for parkin have been described, and the dysregulation of parkin to ubiquitinate these substrates may be linked to neurodegeneration (Atkin and Paulson, 2014). Furthermore, parkin is recruited to depolarized mitochondria and promotes elimination by mitophagy in mammalian cells (Geisler *et al.*, 2010). Moreover, experimental evidence has revealed that parkin plays a critical role in mitochondrial quality control (Tanaka, 2010). In this study, we screened genetic modifiers, focusing on genes modified in a defective phenotype for retinal development and locomotive activity in exogenous human wild type FUS-expressing flies. We identified parkin as a novel regulator of the pathogenesis of FUS-induced ALS. We showed that overexpression of parkin under the control of the pan-neuronal Gal4 driver significantly restores impaired locomotive activity in FUS-expressing larvae and adult flies. However, parkin did not modulate the amount or the cytoplasmic distribution of FUS protein in the FUS-induced ALS model flies. Notably, we found that the reduced mitochondrial complex I and III expression and assembly can be rescued by the expression of parkin in *Drosophila* muscle. In addition, the decreased intracellular ATP level was restored by parkin expression. These results suggest that parkin is associated with the pathogenesis of FUS-induced ALS by regulating mitochondrial function. These findings on parkin-mediated neuroprotection may further our understanding of FUS-associated ALS pathogenesis and provide novel insights for the development of new therapeutic tools for FUS-induced proteinopathies.

Results and Discussion

Parkin rescues the locomotive defect in neurone-specific FUS-expressing flies

ALS is characterized by the selective degeneration of motor neurones (Cleveland and Rothstein, 2001). Several studies have reported that ALS-induced models including mouse and zebrafish have many defective phenotypes, such as the loss of synapses at the neuromuscular junction, stress granule formation and shortened lifespan (Bosco *et al.*, 2010; Sharma *et al.*, 2016). FUS is a DNA/RNA-binding protein belonging to the FET family (Kwiatkowski Jr. *et al.*, 2009; Vance *et al.*, 2009). FUS was identified as a component of inclusions in neurones of patients with ALS and frontotemporal lobar degeneration (Broustal *et al.*, 2010; Deng *et al.*, 2010; Kuang *et al.*, 2017). Although FUS aggregation is thought to be a potential pathogenic signal for the initiation of selective neuronal death, the exact mechanisms underlying FUS-induced ALS remain unclear. Therefore, the identification of novel suppressors potentially linked to FUS neurotoxicity is key for the development of new therapeutic tools.

To identify novel regulators of the pathogenesis of FUS-induced ALS, we screened genetic modifiers, focusing on genes modified to give a defective phenotype for retinal development and locomotive activity in human wild type FUS-expressing flies (Stoica *et al.*, 2016; Bogaert *et al.*, 2018; Fig. 1). We crossed FUS-expressing flies with flies overexpressing various genes that regulate protein degradation and mitochondrial function. This screening process identified parkin as one of the genetic suppressors in FUS-expressing flies. As shown in Fig. 1A, the expression of FUS using the eye-specific Gal4 driver, glass multiple reporter (*GMR*)-Gal4, led to a retinal developmental defect with a disrupted pattern of ommatidia; however, overexpression of the *parkin* gene in the *Drosophila* eye suppressed the retinal defective phenotype induced by FUS (Fig. 1A, magnification view). Dysfunction of locomotive activity is one of the phenotypes for investigating ALS-induced *Drosophila* model. When ALS-causing genes, including *TDP-43*, *FUS* and *TAF15*, were overexpressed or mutated in a *Drosophila* model, their locomotive activities were reduced and their synapses were damaged (Lanson Jr. *et al.*, 2011; Shahidullah *et al.*, 2013). Therefore, we generated *Drosophila* models expressing FUS in neurones using the neurone-specific Gal4 driver, *elav*-Gal4. We tested the crawling behaviour activity of the larvae. Because of the organized architectural structure of the larval neuromuscular system, it can be used as a model for the study of many neurodegenerative diseases. We placed the larvae on a grape juice-agar plate for 90 s and measured their crawling distance. The distance crawled by the FUS-overexpressing larvae was shorter than that of the controls, indicating locomotive dysfunction. By contrast, the larvae that overexpressed both FUS and parkin exhibited a healthy phenotype (Fig. 1B). A recent study showed that a larval motor exon defect caused unbalanced locomotion including 'arching' and 'lifting' phenotypes (Kinold *et al.*, 2018). This finding might help to better understand the FUS-induced paralytic defect in *Drosophila*. Therefore, further studies are needed to investigate the exact mechanism and various phenotypes of FUS-induced movement disorder such as paralysis through the analysis of larval crawling activity.

We also examined the behaviour phenotypes of adults overexpressing both FUS and parkin. For this, we performed a climbing assay. We found that the climbing activity of FUS-expressing flies markedly degenerated in an age-dependent manner; almost all FUS-expressing flies exhibited a significant impairment in locomotive function 7 days after hatching. However, the overexpression of parkin suppressed the defective phenotype induced by FUS overexpression in flies (Fig. 1C). Furthermore, to confirm the effect of parkin knockdown in FUS-expressing flies, we coexpressed *UAS-FUS* and *UAS-parkin RNA interference* using *GMR-Gal4* and *elav-Gal4* drivers in

the eye and neurones and then examined the defective phenotypes induced by FUS (Fig. S1). Parkin knockdown did not significantly alter the rough eye phenotypes of FUS-expressing flies (Fig. S1A). Moreover, we found that flies with neuronal-specific knockdown of parkin, combined with overexpression of FUS, were not viable (Fig. S1B). Therefore, we could not perform further experiments to investigate the effect of parkin knockdown on neuronal specific FUS-expression. Thus, these data

indicate that parkin has a protective impact in a FUS-induced ALS model, based on the observed locomotive activity.

Total FUS expression is unaffected by parkin in fly head extracts

The observation that parkin suppresses FUS-induced behavioural defects in *Drosophila* prompted us to investigate the

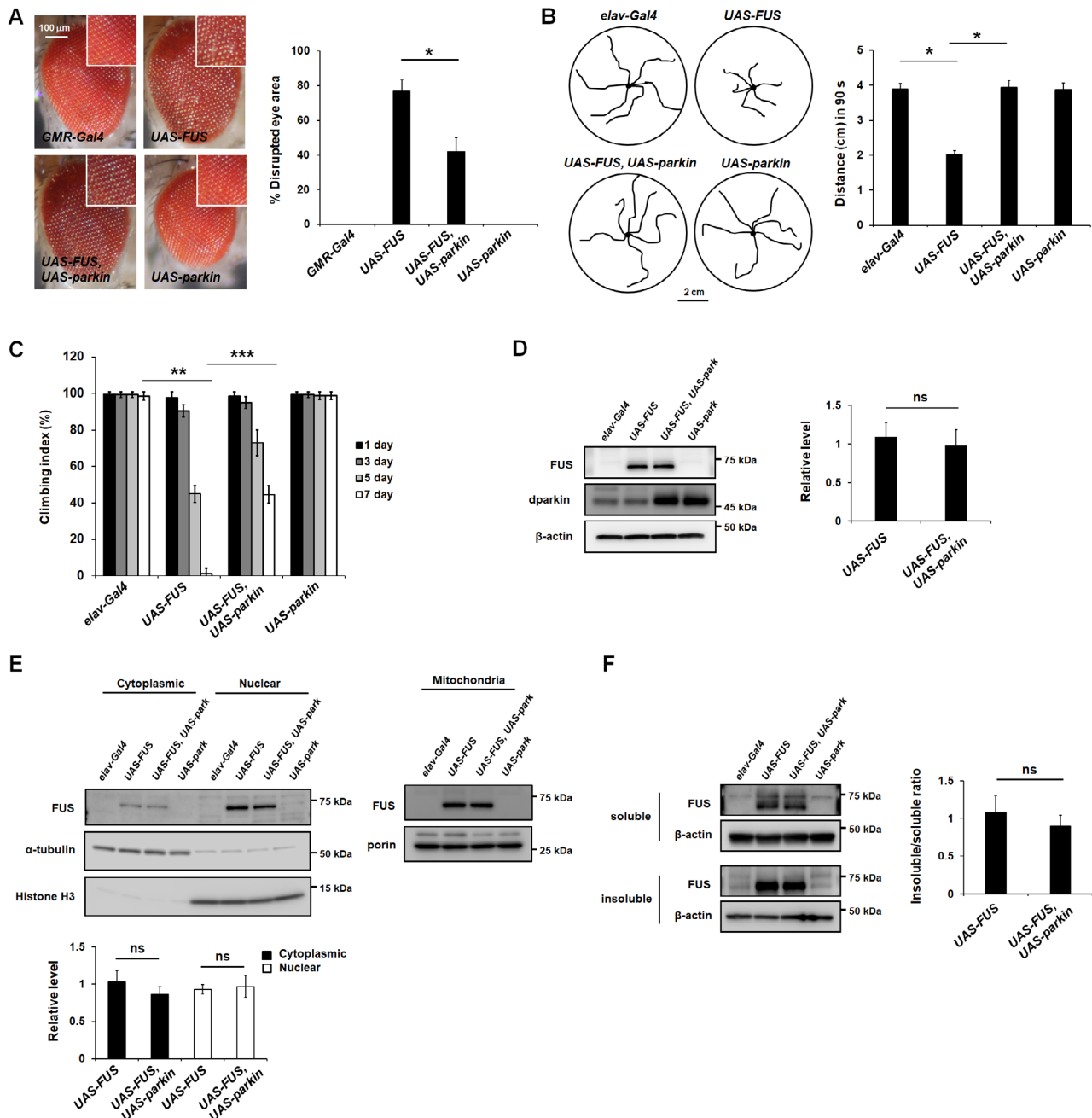


Figure 1. Legend on next page.

underlying molecular mechanisms. Parkin is an E3 ubiquitin ligase involved in the ubiquitin-proteasome system that protects the cell from numerous toxic molecules (Dawson and Dawson, 2003). Parkin is involved in mitochondrial quality control and in promoting the selective autophagy of depolarized mitochondria (mitophagy; Narendra *et al.*, 2012). Our recent work showed that the expression level of endogenous *Drosophila* parkin did not change in TDP-43-, FUS- and TAF-15-induced ALS fly models (Altanbyek *et al.*, 2016). To determine the effect of parkin on FUS protein stability in the FUS-induced ALS model, we generated a line of flies overexpressing both FUS and parkin driven by *elav-Gal4*. The overexpression of parkin in neurones did not affect the FUS protein level (Fig. 1D). Furthermore, to examine whether the expression of parkin leads to the mislocalization of FUS, we performed subcellular fractionation assays to measure FUS levels in the cytoplasm and nucleus. Parkin coexpression with FUS in neurones did not affect FUS protein levels in either the cytoplasmic or nuclear fractions (Fig. 1E). Moreover, mitochondrial FUS protein level was also not affected by parkin (Fig. 1E). To investigate the effects of parkin on FUS protein solubility, we separated the head extracts into detergent-soluble and -insoluble fractions. The solubility of FUS was not affected by parkin overexpression (Fig. 1F). Although the major role of parkin is in E3 ubiquitin ligase, parkin overexpression did not modulate FUS protein level in the FUS-induced ALS model. These data suggest that FUS may not be a direct physiological target of parkin in *Drosophila* and that the neuroprotective effect of parkin is not the result of a reduction in FUS protein levels.

FUS-induced mitochondrial dynamic imbalance is not related to parkin rescue effects

Mitochondria are important organelles involved in several cellular activities, including ATP generation, modulating the

biosynthesis of amino acids and steroids, and maintaining cytosolic calcium homeostasis (Davis and Williams, 2012). In addition, mitochondria are dynamic organelles and balance the morphology by undergoing fusion and fission. Mitochondrial dysfunction and an imbalanced mitochondrial morphology can result in an array of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS (Deng *et al.*, 2008; Oliveira, 2010; Johri and Beal, 2012).

Our previous study showed that the expression of TDP-43, FUS or TAF15 induced mitochondrial fragmentation in *Drosophila* muscle tissues (Altanbyek *et al.*, 2016). We used muscle tissue to study mitochondria, because muscle tissue is organized into muscle fibres with various mitochondrial sizes. First, we examined the crawling behaviour of larvae with muscle-specific overexpression of FUS and parkin and found that FUS-expressing flies displayed decreased crawling activity. However, the expression of parkin rescued the abnormal motor activity in FUS-expressing flies (Fig. 2A). In addition, to determine mitochondrial morphological changes, we crossed the fly lines overexpressing both FUS and parkin with myosin heavy chain (*mhc*)-*Gal4*. Due to mitochondria-targeted green fluorescent protein (mitoGFP) expression in the thoracic muscle, mitochondria were easily visualized using a confocal microscope. As controls, we detected changes in mitochondrial morphology after the up-regulation or down-regulation of the mitochondrial dynamics regulatory genes, *Drp1* and *Marf*. In wild type flies, mitochondrial morphology was found to be balanced, including both elongated and fragmented mitochondria (Fig. 2B). However, the mitochondria in flies overexpressing FUS exhibited increased mitochondrial fragmentation (ie smaller and rounder in size), similar to the phenotypes of the flies with *Drp1* overexpression or *Marf* knockdown (Fig. 2B). The overexpression of both FUS and parkin did not lead to any significant mitochondrial morphological changes compared to the flies

Figure 1. The transgenic expression of parkin rescues the fused in sarcoma (FUS)-induced defect phenotypes. (A) The genetic modulation of parkin altered FUS-induced retinal developmental defect. A normal eye phenotype was observed in *GMR-Gal4* control flies and flies expressing parkin alone in the eye. The overexpression of parkin by the *GMR-Gal4* eye-specific driver suppressed the rough eye phenotype in FUS-overexpressing flies (magnification view). The error bars represent the mean $\pm$ standard deviation. Statistical significance was determined using the Student's *t*-test (*, $P < 0.05$). (B) Locomotive traces from a 90-s trial are shown for four different genotypes. The locomotive activity of larvae overexpressing FUS was decreased. The overexpression of parkin by the *elav-Gal4* neurone-specific driver rescued the healthy phenotype from the defective locomotive phenotype of FUS-overexpressing flies. The movement of third-instar larvae on the agar plate was monitored for 90 s and the average distance of the larval trails was calculated ($n > 12$ for each genotype). The error bars represent the mean $\pm$ standard deviation. Statistical significance was determined using the Student's *t*-test (*, $P < 0.05$). (C) The percentage of locomotive activity in adult flies is shown. The FUS-induced locomotive defect in adult flies was suppressed by parkin expression. The error bars represent the mean $\pm$ standard deviation. Statistical significance was determined using one-way analysis of variance (**, $P < 0.01$; ***, $P < 0.001$). (D) The FUS protein level was not affected by parkin overexpression. The up-regulation of parkin by the *elav-Gal4* neurone-specific driver did not affect the FUS protein level. β -actin was used as a loading control. Quantification of the levels of FUS normalized to β -actin (ns, not significant). (E) Cytoplasmic, mitochondrial and nuclear FUS protein levels were not affected by parkin overexpression. The relative purity of the cytoplasmic, mitochondrial and nuclear fractions was confirmed by sequential probing for the cytoplasmic marker, α -tubulin, the mitochondrial marker, porin and the nuclear marker, histone H3. Quantification of FUS levels normalized to α -tubulin and histone H3. The error bars represent the standard deviation. Statistical significance was determined using the Student's *t*-test (ns, not significant). The experiments were performed in triplicate. (F) Soluble (supernatant) and insoluble (pellet) fractions isolated from adult heads of the indicated genotypes were analysed by western blotting. The solubility of FUS was not affected by parkin overexpression. β -actin was used as a loading control. Quantification of the insoluble/soluble FUS ratio of the indicated genotypes is shown. The error bars represent the standard deviation. Statistical significance was determined using the Student's *t*-test (ns, not significant). Experiments were performed in triplicate. [Colour figure can be viewed at wileyonlinelibrary.com].

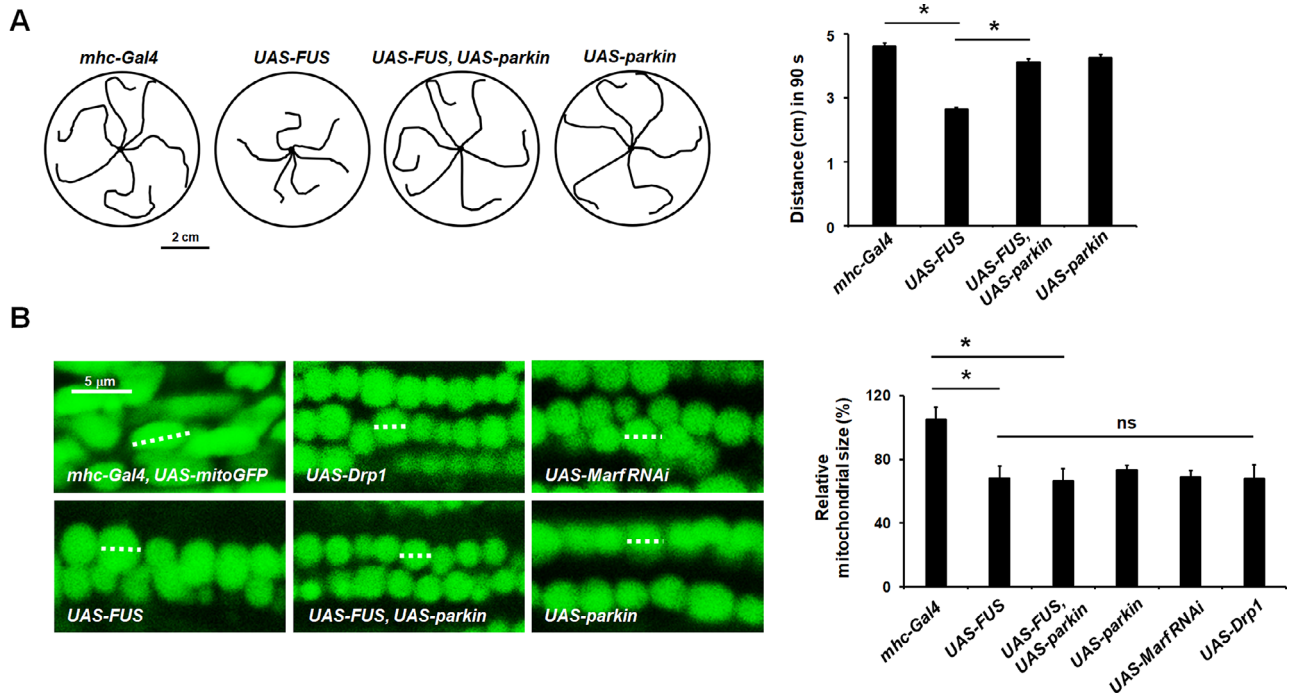


Figure 2. Parkin rescues fused in sarcoma (FUS)-induced motility deficits in muscle. (A) Locomotive traces from a 90-s trial are shown for four different genotypes. Lower locomotive activity was observed in larvae overexpressing FUS compared to the control larvae (*mhc-Gal4*). The overexpression of parkin by the *mhc-Gal4* muscle-specific driver rescued the flies from the defective locomotive phenotype of FUS-overexpressing flies. The movement of third-instar larvae on the agar plate was monitored for 90 s and the average distance of the larval trails was calculated ($n > 12$ for each genotype). The error bars represent the standard deviation. Statistical significance was determined using the Student's *t*-test (*, $P < 0.05$). (B) The mitochondria were visualized using mitochondria-targeted green fluorescent protein (mitoGFP). The overexpression of *Drp1* or knockdown of *Marf* leads to mitochondrial fragmentation. Mitochondrial morphology was highly fragmented in flies overexpressing either FUS or parkin alone. Fragmented mitochondrial morphology in FUS-overexpressing flies was not affected by the co-overexpression of parkin. The error bars represent the standard deviation ($n = 5$ for each genotype). Statistical significance was determined using the Student's *t*-test (*, $P < 0.05$; ns, not significant). [Colour figure can be viewed at wileyonlinelibrary.com].

only overexpressing FUS (Fig. 2B). By contrast, when we overexpressed parkin alone, the flies also exhibited fragmented mitochondria (Fig. 2B). These results indicate that, although parkin can suppress the locomotive defect in FUS-expressing flies, its protective effect is not related to modulating mitochondrial dynamics in *Drosophila*.

Down-regulation of complex I and III subunits is compensated by parkin

Mitochondrial function is an important indicator of neurodegenerative disease (Lin and Beal, 2006). Patients with ALS and SOD1, as well as TDP-43 mutant transgenic mice, show mitochondrial dysfunction (Shi *et al.*, 2010; Xu *et al.*, 2010; Cozzolino and Carri, 2012). Mitochondria consist of five distinct multi-subunit complexes (complexes I–V). In the spinal cords of ALS patients, the activities of complexes I, II and III are reduced (Sasaki and Iwata, 2007; Johri and Beal, 2012). Here, we investigated the protein expression level of the five complex subunits in *Drosophila* thoracic extracts. The total proteins were extracted from fly thoraces and western blot analysis was performed

to measure the protein levels of the complex subunits in flies overexpressing both FUS and parkin compared to those in flies overexpressing FUS alone. Owing to the availability of ideal antibodies for the *Drosophila* model, we could only detect complexes I, III and V. Surprisingly, we found that the expression levels of the subunits of complexes I (NDUFS3) and III (UQCRC2) were significantly decreased in FUS-induced ALS flies compared to those in the control flies (Fig. 3A). The flies that overexpressed both FUS and parkin partially recovered the level of the complex I subunit (NDUFS3). Moreover, the expression level of the complex III subunit (UQCRC2) was fully rescued in flies overexpressing both FUS and parkin compared to that in flies overexpressing FUS alone (Fig. 3A). By contrast, the expression level of the complex V subunit (ATP5A) did not vary across any of the fly lines. The expression levels of all complex subunits did not vary in flies that overexpressed parkin alone (Fig. 3A). We studied the assembly of the oxidative phosphorylation complex using blue native polyacrylamide gel electrophoresis (BN-PAGE). In FUS-induced ALS flies, disassembly was observed for complexes I and III. The cluster of complexes I and III was recovered in flies overexpressing

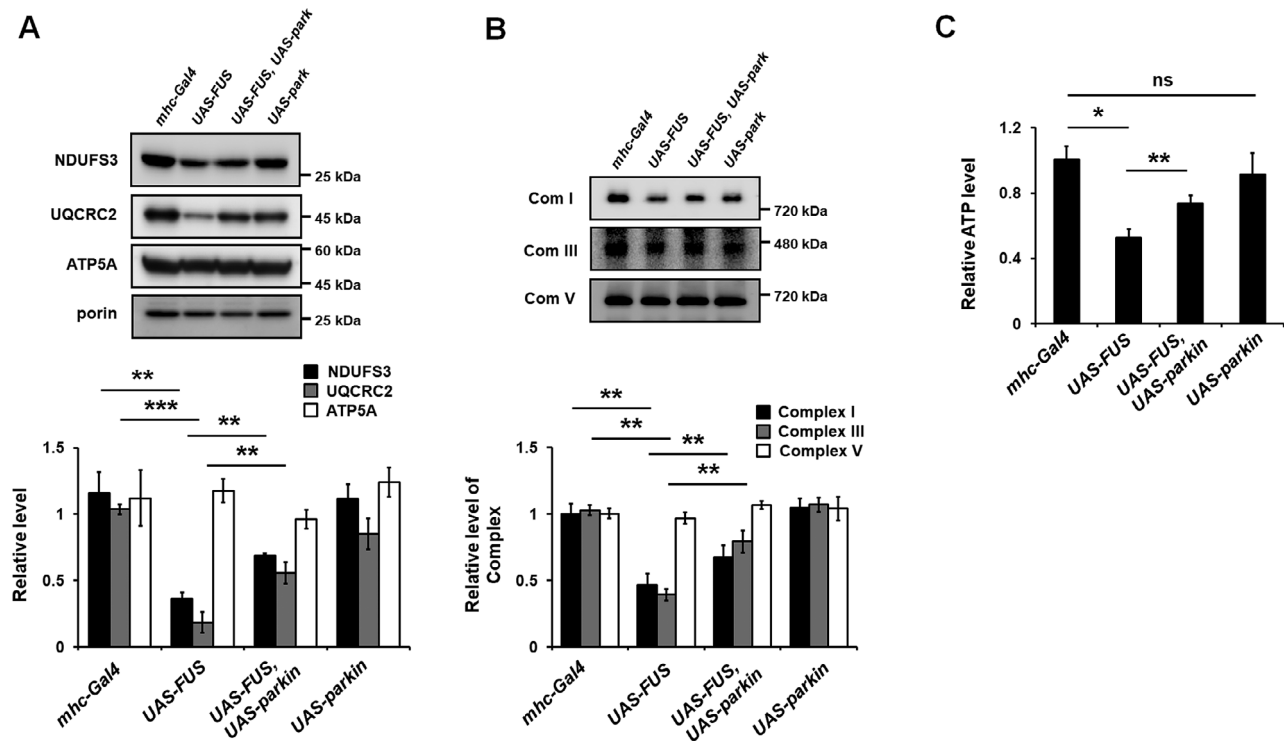


Figure 3. Parkin can restore the defects of the mitochondrial oxidative phosphorylation (OXPHOS) system in fused in sarcoma (FUS)-overexpressing flies. (A) NDUF3, UQCRC2 and ATP5A protein levels in thorax extracts from the different fly lines are shown. NDUF3 and UQCRC2 levels in FUS-overexpressing flies were partially restored by parkin overexpression. The levels of ATP5A in the thorax extracts did not change in any of the fly lines. The relative contents of mitochondrial complexes were normalized to porin. The error bars represent the standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA; **, $P < 0.01$; ***, $P < 0.001$). The experiments were performed in triplicate. (B) The assembled complexes I and III were significantly decreased in FUS-overexpressing flies. The level of assembled complexes I and III was rescued by parkin overexpression in FUS-overexpressing flies. The relative protein levels were analysed quantitatively by densitometry. The error bars represent the standard deviation. Statistical significance was determined using one-way ANOVA (**, $P < 0.01$). The experiments were performed in quadruplicate. (C) FUS-overexpressing flies had reduced ATP levels. Parkin overexpression partially recovered ATP levels in FUS-overexpressing flies. The relative ATP content in the thorax was normalized to the total protein. The error bars represent the standard deviation ($n = 5$ for each genotype). Statistical significance was determined using the Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

both FUS and parkin. In line with its protein expression levels, complex V did not affect complex assembly in flies overexpressing either both FUS and parkin or FUS alone (Fig. 3B). One of the major functions of mitochondria is the synthesis of ATP, which is necessary for cellular activities. We measured the ATP level in *Drosophila* thoraces with the muscle-specific Gal4 driver. The ATP level was significantly decreased in FUS-induced ALS flies compared to that in the controls. By contrast, flies overexpressing both FUS and parkin had partially restored ATP levels (Fig. 3C). Taken together, these data suggest that parkin ameliorated FUS-induced mitochondrial dysfunction through a mechanism not related to modulating mitochondrial morphology.

Previous studies have reported contradictory results for parkin activity in FUS-expressing fly models (Chen *et al.*, 2016). It has been shown that parkin knockdown partially rescues FUS-induced rough eye and larval movement phenotypes in *Drosophila*. However, emerging evidence suggests that parkin plays a protective role in various neurodegenerative diseases. Parkin overexpression in an Alzheimer's disease model was found to reduce β -amyloid

deposition (Hong *et al.*, 2014). Moussa's group recently demonstrated that parkin expression reverses TDP-43-mediated cell death (Hebron *et al.*, 2014). Parkin has also been found to protect against expanded polyglutamine-induced toxicity (Tsai *et al.*, 2003). Furthermore, we recently reported that overexpression of parkin suppresses neurotoxicity in TAF15-induced proteinopathy (Kim *et al.*, 2018). In particular, increasing evidence points to a mitochondrial defect as an important factor in the pathogenesis of ALS (Wang *et al.*, 2016; Nakaya and Maragkakis, 2018). Various studies have suggested that parkin regulates mitochondrial biogenesis and mitophagy (Jin and Youle, 2012; McWilliams and Muqit, 2017). Interestingly, parkin overexpression in *Drosophila* during aging has been shown to reduce proteotoxicity and increase mitochondrial activity (Rana *et al.*, 2013). In this study, we found that parkin overexpression ameliorated FUS-induced defective phenotypes by suppressing mitochondrial dysfunction in *Drosophila*. Thus, our results suggest that parkin contributes to counteracting FUS-induced neurotoxicity by decreasing mitochondrial damage.

In conclusion, our results demonstrate that parkin was able to rescue the healthy phenotype from the FUS-induced defective phenotype, including impaired locomotive ability and mitochondrial dysfunction. We found that the FUS-induced *Drosophila* models had defective locomotive activities resulting from mitochondrial impairment and dysfunction. Importantly, we provided *in vivo* evidence that the locomotive disability of FUS-overexpressing flies was alleviated by parkin, which we screened as a novel genetic modifier. Furthermore, we showed that parkin is a neuroprotective regulator for FUS-induced proteinopathy, which was able to recover the protein levels of mitochondrial complexes I and III. Therefore, our findings on parkin-mediated neuroprotection may further our understanding of ALS pathogenesis and provide novel insights for the development of new therapeutic tools for FUS-induced proteinopathies.

Experimental procedures

Drosophila stocks

All *Drosophila* stocks were kept under standard food conditions, normal temperature conditions (25 °C), and normal humidity conditions (60%). Crossing of the flies was performed according to the standard procedure. The *UAS-mitoGFP* line was a gift from H. J. Bellen (Baylor College of Medicine; Sandoval *et al.*, 2014). The *UAS-FUS* line was obtained from Dr N. Bonini (University of Pennsylvania; Kim *et al.*, 2014). The *UAS-parkin* line was from Dr J. Chung (Cha *et al.*, 2005). The *GMR-Gal4*, *elav-Gal4*, *mhc-Gal4* and *UAS-parkin RNAi* (BL31259) fly lines were obtained from the Bloomington *Drosophila* Stock Center.

Locomotive activity assay

For the larva crawling assay, the third-instar larvae were washed with phosphate-buffered saline to remove the remaining food medium. Larvae were briefly dried on clean filter paper and put on a 2% grape juice-agar Petri plate. The larvae of each genetic line were allowed to crawl for 90 s. To quantify the crawling behaviour of the larvae, we drew lines to track the larvae and measured the distance using IMAGEJ software (National Institute of Health, Bethesda, MD, USA). Results for at least 12 larvae were averaged for each transgenic line.

For the adult climbing assay, the flies were collected based on age (1 day old, 3 day old, 5 day old and 7 day old) and then placed into cohorts of 20 male flies per vial. The flies were transferred to an empty vial and incubated for 1 h at room temperature to acclimatize to the environment. We tapped the vial and the flies dropped to the bottom. We counted the number of the flies that were able to climb to the top of the vial within 10 s. The experiment was repeated five times independently for each transgenic line. All climbing experiments were performed at room temperature.

Western blot analysis

Several 5-day-old male fly heads or 7-day-old male fly thoraxes were homogenized in the protein extracts in preparation for western blot analyses. The total protein extracts (10 µg) were

separated in a 4–12% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were blocked in Tris-buffered saline with 4% non-fat dry milk or 4% bovine serum albumin (BSA) for 1 h. We treated the membranes with the following primary antibodies: rabbit anti-FUS (1:1000; A300-302A; BETHYL Laboratories, Montgomery, TX, USA), rabbit anti-dparkin (1:2000; a gift from Alexander Whitworth, University of Cambridge), rabbit anti- α -tubulin (1:1000; 9099S, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-histone H3 (1:5000; ab1791, Abcam, Cambridge, UK), mouse anti-VDAC1/porin (1:2000; ab14734, Abcam), mouse anti-NDUFS3 (1:5000; ab14711; Abcam), mouse anti-UQCRC2 (1:1000; ab14745, Abcam), mouse anti-ATP5A (1:10 000; ab14748, Abcam) and rabbit anti- β -actin (1:5000; 4967L; Cell Signaling Technology). Detection of the primary antibodies was performed with the following horseradish peroxidase (HRP)-conjugated secondary antibodies: goat anti-rabbit IgG HRP conjugate and goat anti-mouse IgG HRP conjugate (1:2000; AP308P, Millipore). Detection was performed using an ECL-Plus Kit (Amersham, Little Chalfont, UK). Each protein band was analysed and quantified by performing densitometry of the blots using IMAGEJ software.

Cellular fractionation assay

For subcellular fractionation, 30 male fly heads were lysed in the reagent of a nuclear extract kit (Active Motif, Carlsbad, CA, USA) and mitochondrial isolation kit (Pierce, Rockford, IL, USA) according to the manufacturers' protocols. Protein extracts in different subcellular fractions were mixed with SDS loading buffer, boiled and subjected to SDS-PAGE for immunoblot analysis.

Solubility assay

Twenty heads of male flies were homogenized in lysis buffer without SDS [50 mM Tris-HCl, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1% NP-40 and 10% glycerol, pH 7.5]. The homogenate samples were centrifuged at 100 000 g for 30 min at 4 °C. The supernatant from this step was collected as the soluble fraction. The resting pellets were further extracted with 2× SDS loading buffer including 2% SDS, sonicated and boiled for 10 min at 95 °C. The supernatant was used as the insoluble fraction.

Immunohistochemistry

To analyse the mitochondria in muscle tissue, adult fly thoraces dissected from 7-day-old male flies were fixed with 4% formaldehyde in fixation buffer (100 mM PIPES, 1 mM EGTA, 1% Triton X-100 and 2 mM MgSO₄, pH 6.9) and blocked with wash buffer (50 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 0.5 mg/ml BSA, pH 6.8) with 10 mg/ml BSA. All images were obtained under an LSM710 Carl Zeiss confocal microscope (Carl Zeiss, Oberkochen, Germany).

Morphological analysis of mitochondria

Confocal microscopy images (1500× magnification) were analysed with IMAGEJ software. Non-overlapping mitochondria from

images were selected for length measurement. After setting the scale, each mitochondrion on the image was selected and its length calculated. The mitochondrial length was analysed as average length from 90 mitochondria per image. At least three images were analysed for each thorax and at least five thoraxes of each genotype were examined.

BN-PAGE

Mitochondria were isolated from 7-day-old male fly thoraces using a mitochondrial isolation kit (Pierce) following the manufacturer's protocol. The purified mitochondria extracts were resuspended in 60 µl of 1× Native PAGE Sample Buffer (Invitrogen, Carlsbad, CA, USA) containing 2% n-dodecyl-β-D-maltoside, 1% digitonin, and protease inhibitor (Thermo Fisher Scientific, Waltham, MA, USA). The sample was incubated on ice for 15 min and centrifuged at 12 000 *g* for 30 min. We measured the mitochondrial protein concentration in the supernatant. The supernatant (20 µg) was mixed with 0.5% G-250 sample additive (Invitrogen). We performed blue native electrophoresis using 3–12% Native PAGE Bis-Tris gel (Invitrogen) at 4 °C as previously described (Kim *et al.*, 2012). Anode running buffer was used outside the gel and cathode running buffer was used inside the gel. After PAGE, western blot analysis was performed. For each protein, the bands were analysed and quantified by performing densitometry of the blots using IMAGEJ software.

ATP assay

Five thoraxes from 27-day-old flies were homogenized in 100 µl extraction buffer (6 M Guanidine-HCl, 100 mM Tris, 4 mM EDTA, pH 7.8) to inhibit ATPase. The extracts were immediately boiled for 5 min to denature the endogenous ATP synthase. The samples were centrifuged at 20 000 *g* for 15 min and the supernatant was transferred to a new tube. The supernatant was diluted (1/100) with the extraction buffer. On a 96-well plate, the sample was placed in each well and mixed with the luminescent solution of an Enliten ATP assay kit (Promega, Madison, WI, USA). We measured the luminescence with an interval of 10 s using a GloMax microplate reader (Promega). The supernatant was diluted (1/5) with the extraction buffer to measure the protein concentration using a BCA protein assay kit (Pierce). We calculated the relative ATP level compared to the standard by dividing by the total protein concentration.

Acknowledgements

This work was supported by the Basic Science Research Program, and the Korea Brain Research Institute (KBRI) Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Science and ICT (MSIT) (2017R1C1B1008825, 18-BR-02-06), Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare (MOHW) (HI14C1135) and by the Soonchunhyang University Research Fund.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Knockdown of parkin by the *GMR-Gal4* eye-specific driver did not affect the rough eye phenotype of fused in sarcoma (FUS)-overexpressing flies. (A) Down-regulation of parkin did not alter FUS-induced retinal toxicity (see magnified views). (B) Scheme of the genetic cross used to obtain parkin down-regulation in an FUS-expression background. Knockdown of parkin by *elav-Gal4* in FUS-expressing flies was lethal.