Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics, and extends lifespan

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Aberrant protein aggregation and mitochondrial dysfunction have each been linked to aging and a number of age-onset neurodegenerative disorders, including Parkinson disease. Loss-of-function mutations in *parkin*, an E3 ubiquitin ligase that functions to promote the ubiquitin–proteasome system of protein degradation and also in mitochondrial quality control, have been implicated in heritable forms of Parkinson disease. The question of whether *parkin* can modulate aging or positively impact longevity, however, has not been addressed. Here, we show that ubiquitous or neuron-specific up-regulation of Parkin, in adult *Drosophila melanogaster*, increases both mean and maximum lifespan without reducing reproductive output, physical activity, or food intake. Long-lived Parkin-overexpressing flies display an increase in K48-linked polyubiquitin and reduced levels of protein aggregation during aging. Recent evidence suggests that Parkin interacts with the mitochondrial fission/fusion machinery to mediate the turnover of dysfunctional mitochondria. However, the relationships between *parkin* gene activity, mitochondrial dynamics, and aging have not been explored. We show that the mitochondrial fusion-promoting factor *Drosophila* Mitofusin, a Parkin substrate, increases in abundance during aging. Parkin overexpression results in reduced *Drosophila* Mitofusin levels in aging flies, with concomitant changes in mitochondrial morphology and an increase in mitochondrial activity. Together, these findings reveal roles for Parkin in modulating organismal aging and provide insight into the molecular mechanisms linking aging to neurodegeneration.

energy metabolism | healthspan | mitophagy | neuronal aging | proteostasis

Advanced age is a major risk factor for many neurodegenerative disorders, including both Alzheimer's disease and Parkinson disease (PD), and yet the molecular mechanisms that link aging and neurodegeneration are poorly understood. To understand this connection, it is necessary to identify the molecular events and pathways that integrate aging and neurodegenerative disease. Several lines of evidence suggest that a decline in the ability to prevent and eliminate protein misfolding (1) and impaired mitochondrial function (2) are important factors in the increased risk of disease and death associated with aging. More specifically, although the correlation between neurodegenerative disease and protein aggregation in the brain has long been recognized (3), recent work in the nematode *Caenorhabditis elegans* (4–6) and the fruit fly *Drosophila melanogaster* (7) has shown that aging per se is associated with increased aggregation of a large number of proteins. At the same time, a decline in mitochondrial gene expression and/or energetic capacity is a hallmark of aging across diverse species (2, 8), whereas a failure to eliminate dysfunctional mitochondria has been implicated in the pathophysiology of a number of neurodegenerative diseases, including PD (9).

The ubiquitin–proteasome system (UPS) is a critical component of the cellular protein homeostasis (proteostasis) machinery and also acts in a regulatory capacity to mediate the controlled breakdown of specific proteins that impact biological processes (10). Proteins polyubiquitinated at specific lysine residues may undergo proteasomal degradation or have nondegradative functions; K48-linked polyubiquitin chains mainly target proteins for proteasomal degradation (11). Studies of genes involved in heritable forms of PD, including Parkin an E3 ubiquitin ligase (12), have greatly influenced our understanding of the molecular mechanisms underlying neurodegeneration in this disorder (13). Indeed, it has been postulated that *parkin* mutations impair the UPS of protein degradation, leading to the accumulation of toxic misfolded or aggregated proteins (14, 15). More recently, considerable attention has been focused on the role of Parkin in mitochondrial quality control (MQC) (16–18). Genetic studies in animal and cellular models, including *Drosophila* (19), have shown that loss of Parkin leads to the accumulation of degenerative mitochondria (20). The molecular basis for these findings was provided by the discovery that Parkin is recruited to dysfunctional mitochondria and targets these organelles for autophagic degradation (mitophagy) (16, 21). In this model, PTEN-induced putative kinase (PINK) 1, a mitochondrially targeted serine/threonine kinase, is selectively stabilized on the surface of dysfunctional mitochondria, promoting the recruitment of Parkin (22–24). Parkin then ubiquitinates the mitochondrial fusion-promoting factor Mitofusin (Mfn) (23, 25, 26) and other mitochondrial proteins (27, 28), promoting the segregation and autophagic turnover of the dysfunctional mitochondria (16, 17). Previous studies have reported that Parkin overexpression can ameliorate genetic and pharmacological models of both mitochondrial dysfunction (29–33) and also proteotoxicity (reviewed in ref. 34). However, the consequences of a long-term increase in Parkin activity, in a nondisease setting, on mitochondrial metabolism, proteostasis and organismal health are not known.

The role of Parkin in the UPS of protein turnover and in MQC, together with its connection to age-related disease, led us to examine whether Parkin could modulate organismal aging. We find that ubiquitous or neuron-specific up-regulation of Parkin can increase both mean and maximum lifespan in *Drosophila*. This enhanced longevity can be observed upon adult-onset induction of Parkin, indicating that it is not attributable to developmental changes. Furthermore, long-lived Parkin flies do not display major physiological tradeoffs affecting reproduction, physical activity, or feeding behavior. Parkin-mediated life extension is associated with increased K48-linked polyubiquitination and reduced levels of protein aggregation in aged flies. With respect to mitochondrial biology, we show that the Parkin substrate *Drosophila* (*d*) Mfn increases in abundance during aging. Importantly, chronic up-regulation of Parkin leads to reduced levels of *d*Mfn, mitochondrial fragmentation, and an increase in multiple markers of mitochondrial

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activity in aging flies. Taken together, our findings demonstrate that an increase in Parkin expression can slow aging both at the level of biochemical and molecular markers of aging and also at the organismal level by extending healthy lifespan.

Results

Adult-Onset Induction of Parkin Increases Longevity. To determine whether Parkin can modulate organismal aging, we first examined the impact of Parkin overexpression on *Drosophila* lifespan. Studies of the genetics of aging and lifespan determination are prone to confounding effects because of uncontrolled differences in genetic background between test and control lines (35). To confront this issue, we have used the mifepristone (RU486) inducible-GAL4 system [annotated P[Switch] or Gene-Switch (36, 37)] throughout this study. This system eliminates genetic background effects because all flies share the same genetic background and only differ with respect to the presence of the inducing agent (RU486) or diluent (ethanol) in the food. Firstly, we used the ubiquitous *daughterless*–Gene-Switch (*daGS*) driver line to activate a *Upstream Activating Sequence (UAS)-parkin* transgene created by Greene et al. (19) during both development and adulthood. Western blot analysis showed that Parkin is significantly overexpressed in *daGS>UAS-parkin* flies upon RU486 treatment during development and the adult lifespan compared with uninduced flies (Fig. 1*A* and Fig. S1 *A* and *B*). RU486 treatment had no effect on Parkin expression levels in control flies (Fig. S1 *C* and *D*). Ubiquitous upregulation of Parkin with this driver line resulted in as much as a 28% increase in both the mean and also the maximum lifespan in female flies (Fig. 1*B* and Fig. S2*A*) and smaller positive effects in male flies (Fig. S2 *B* and *C*). Interestingly, lower levels of Parkin induction are sufficient to extend lifespan (Fig. S2 *D* and *E*), whereas increased doses of RU486 produced diminishing returns in terms of lifespan extension (Fig. S2 *F* and *G*). No major longevity effects were observed in female or male control flies exposed to RU486 (Fig. S2 *H–K*). Next, we took advantage of the inducible nature of the *daGS* driver to determine the temporal requirements for Parkin-mediated lifespan extension. Whereas induction of the *parkin* transgene exclusively during development had no major impact on adult longevity (Fig. S2*L*), *daGS>UAS-parkin* flies were significantly longer-lived when exposed to RU486 exclusively in the adult stage (Fig. 1*C*); again, no major effects on longevity were observed in control flies exposed to RU486 using these paradigms (Fig. S2 *M* and *N*). Together, our data demonstrate a role for Parkin in prolonging lifespan in adult *Drosophila*.

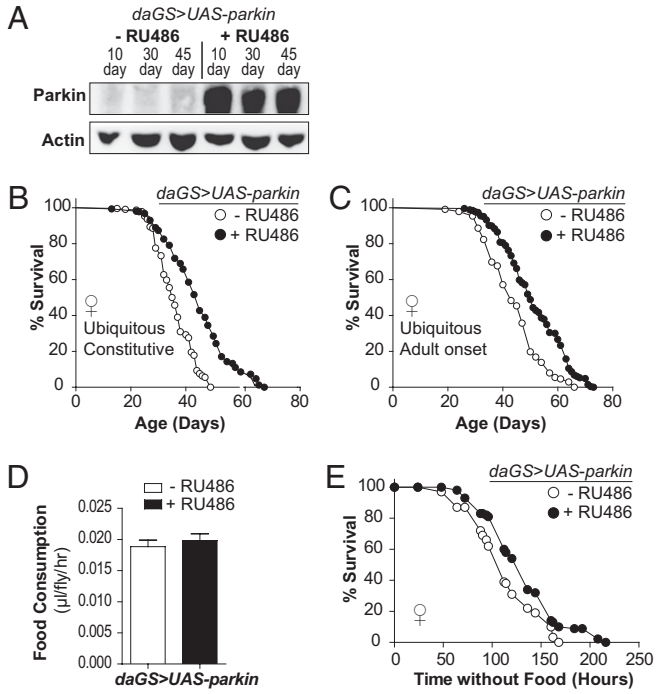

Fig. 1. Ubiquitous Parkin overexpression extends lifespan. (*A*) Western blot analysis of Parkin expression during aging. Parkin levels are increased in female flies by transgenic expression of a *parkin* cDNA under control of the ubiquitous *daGS* promoter. Transgenic expression is induced by exposure of flies to the drug RU486. See quantification in Fig. S1*A*. (*B* and *C*) Survival curves of *daGS>UAS-parkin* flies with or without RU486-mediated transgene induction. (*B*) Constitutive overexpression of Parkin increases fly lifespan ($P < 0.0001$; log-rank test; $n > 200$ female flies). (*C*) Adult-onset overexpression of Parkin increases fly lifespan ($P < 0.0001$; log-rank test; $n > 200$ female flies). (*D*) Capillary feeding assay (52). Constitutive overexpression of Parkin has no significant effect on feeding behavior ($n = 10$; 10 female flies per replicate). Data are represented as means ± SEM. (*E*) Survival curves under starvation. Constitutive Parkin overexpression confers increased survival under starvation ($P < 0.0001$; log-rank test; $n > 60$ female flies). RU486 was provided in the media at 0.2 µg/mL during development and 1 µg/mL during adulthood (*A*, *B*, *D*, and *E*) and 5 µg/mL during adulthood (*C*).

Toward an initial exploration of the mechanisms underlying Parkin-mediated lifespan extension, we examined a number of behavioral and physiological parameters in long-lived Parkin flies. Because overexpression of Parkin during both development and adulthood resulted in maximum extension of lifespan, this paradigm was used in all further experiments. Long-lived Parkin flies displayed normal food consumption (Fig. 1*D* and Fig. S3*A*) and an increase in spontaneous physical activity in young flies (Fig. S3 *B–D*). Moreover, although interventions that extend lifespan are often associated with reproductive tradeoffs (38), Parkin overexpression resulted in an increase in fecundity in young flies (Fig. S3*E*). An increased capacity to withstand extrinsic stress is a hallmark of many longevity interventions (39, 40). Interestingly, Parkin-mediated longevity was not associated with increased resistance to hyperoxia (85% oxygen) (Fig. S3*F*). However, long-lived Parkin flies showed improved survival when maintained on an agar-only diet to induce starvation (Fig. 1*E*). Feeding RU486 to control flies ($daGS>w^{1118}$) did not alter feeding behavior (Fig. S3*G*), physical activity (Fig. S3 *H–J*), fecundity (Fig. S3*K*), hyperoxia resistance (Fig. S3*L*), or starvation resistance (Fig. S3*M*). Taken together, our data indicate that Parkin-mediated lifespan extension is not associated with reduced feeding behavior, motor activity, or a reproductive tradeoff.

Parkin Overexpression Counteracts Age-Onset Proteotoxicity. Parkin functions as an E3 ubiquitin ligase to mediate attachment of ubiquitin monomers or chains to substrate proteins (12). To better understand Parkin-mediated lifespan extension, we set out to examine the relationships between *parkin* gene activity, polyubiquitination, and aging. K48-linked polyubiquitin predominantly, but not exclusively, targets proteins for proteasomal degradation, whereas K63-linked polyubiquitin regulates protein function, subcellular localization, or protein–protein interactions (11, 41). Firstly, we examined levels of K48-linked polyubiquitination in long-lived Parkin flies and controls during aging. Whereas no differences were observed in young flies, chronic overexpression of Parkin resulted in a significant increase in K48-linked polyubiquitination in aged flies (Fig. 2*A* and quantification in Fig. S4*A*). Feeding RU486 to control flies ($daGS>w^{1118}$) did not alter the levels of K48-linked polyubiquitination during aging (Fig. S4 *B* and *C*). Interestingly, however, no significant difference in the levels of K63-linked polyubiquitin chains were observed in long-lived Parkin flies (Fig. S4*D*).

To determine whether Parkin-mediated lifespan extension was associated with reduced proteotoxicity during aging, we characterized the age-related deposition of protein aggregates by immunofluorescence. In agreement with previous findings (7), we observed that *Drosophila* flight muscles accumulate aggregates of ubiquitinated proteins during aging (compare Fig. 2 *B* and *D*).

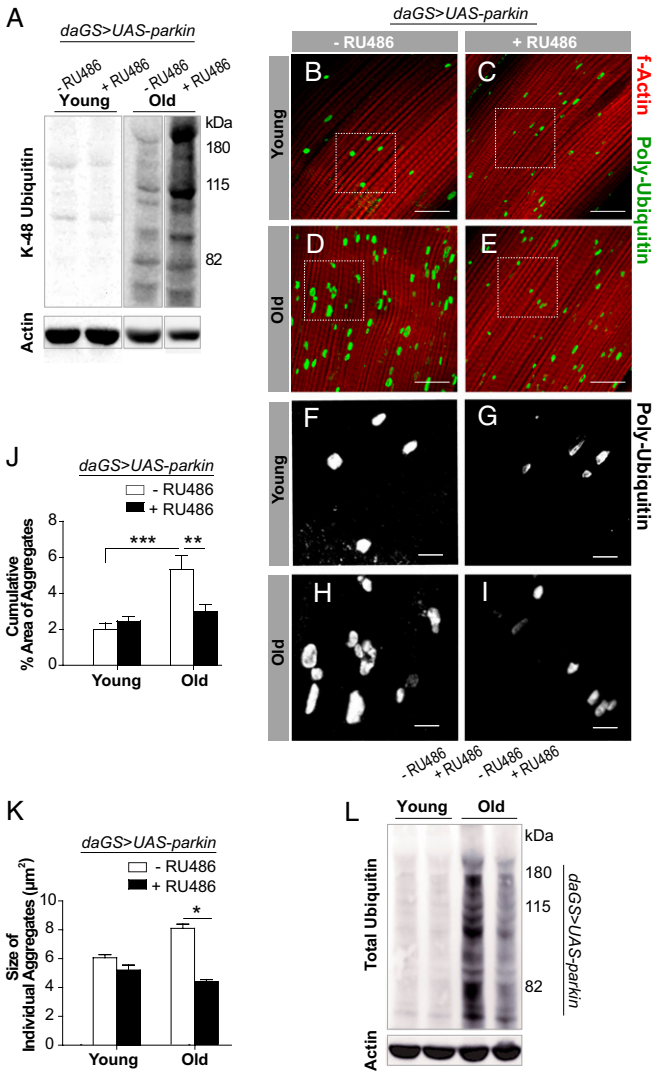

Fig. 2. Ubiquitous Parkin overexpression counteracts age-onset proteotoxicity. (*A*) Western blot detection of K48-polyubiquitinated proteins in whole-body detergent-insoluble extracts from young and aged *daGS>UAS-parkin* female flies with or without RU486-mediated transgene induction. Parkin overexpression leads to increased K48-polyubiquitinated proteins in aged flies (see quantification in Fig. S4*A*). (*B–I*) Immunostaining of indirect flight muscles from young and aged flies. Polyubiquitin (green) immunoreactivity reveals an age-related increase in the deposition of aggregates containing polyubiquitin proteins in uninduced control flies (*B* and *D*) and, to a lesser extent, in Parkin-overexpressing flies (*C* and *E*). *F–I* represent insets of *B–E*, respectively. Parkin overexpression counteracts the age-related increase in the cumulative area and size of protein aggregates (see quantification in *J* and *K*). $^{**}P < 0.01$; $^{*}P < 0.05$ (Student *t* test; $n > 6$; one fly per replicate). Phalloidin staining (red) shows F-actin, which is a component of muscle myofibrils. Data are represented as means ± SEM. (Scale bar: *B–E*, 20 μm; *F–I*, 5 μm.) (*L*) Western blot detection of total ubiquitin-conjugated proteins in whole-body detergent-insoluble extracts from young and aged female flies. Parkin overexpression leads to a reduced amount of detergent-insoluble ubiquitin-conjugated proteins in aged flies (see quantification in Fig. S4*H*). RU486 was provided in the media at 0.2 μg/mL during development and 1 μg/mL during adulthood. Young flies were 10 d of age, and old flies were 30 d of age.

The cumulative area of these aggregates increases substantially with age (compare Fig. 2 *F* and *H* and see quantification in Fig. 2 *J* and *K*), consistent with a loss of proteostasis. Importantly, long-lived Parkin flies displayed reduced levels of protein aggregates during aging (compare Fig. 2 *D* and *E*) and also a decrease in the size of individual aggregates (Fig. 2 *H–K*). Feeding RU486 to control flies (*daGS>w*^{1118}) did not alter the accumulation of protein aggregates during aging (Fig. S4 *E–G*). In a complementary approach, we examined the levels of insoluble ubiquitinated proteins in whole bodies of long-lived Parkin flies and controls during aging. Consistent with the immunofluorescence data, we observed that long-lived Parkin flies display reduced levels of insoluble ubiquitinated proteins during aging (Fig. 2*L* and quantification in Fig. S4*H*). Feeding RU486 to control flies (*daGS>w*^{1118}) did not alter the levels of insoluble ubiquitinated proteins during aging (Fig. S4 *I* and *J*). Taken together, our findings indicate that up-regulation of Parkin preserves protein homeostasis during aging, which is associated with reduced levels of proteotoxicity in aged flies.

Parkin Overexpression Modulates Mitochondrial Dynamics and Activity During Aging. In recent years, considerable attention has been focused on the role of Parkin in mediating the elimination of dysfunctional mitochondria via mitophagy (16–18). Although the details of how this happens remain uncertain, a growing body of data supports the idea that the Parkin-mediated polyubiquitination of mitochondrial outer-membrane (OM) proteins is important for mitophagy (17). Indeed, proteomic studies have shown that the translocation of Parkin to dysfunctional mitochondria is associated with a broad activation of the UPS, resulting in the K48-linked polyubiquitylation and degradation of many OM proteins (27). Studies in *Drosophila* and mammalian cells have shown that Mfn represents an evolutionarily conserved mitochondrial substrate of Parkin (23, 25, 42). These findings have led to the proposal that the ubiquitin-mediated turnover of Mfns would then inhibit mitochondrial fusion and, thus, promote the segregation of dysfunctional mitochondria and facilitate their clearance by mitophagy (16–18, 23, 25, 43).

Because mitochondrial dysfunction is a hallmark of aging (2), we set out to examine the relationships between *parkin* gene activity, aging, and mitochondrial dynamics. In the first place, we sought to explore the effects of aging on the steady-state abundance of the *d*Mfn. Using antibodies against *d*Mfn (23), we examined *d*Mfn levels in control and long-lived Parkin flies during aging. In doing so, we observed a significant increase in *d*Mfn levels in aged control flies relative to young control flies (Fig. 3*A* and quantification in Fig. S5*A*). Interestingly, long-lived Parkin flies displayed significantly reduced levels of *d*Mfn at both time points. Next, we examined whether Parkin-mediated changes in *d*Mfn levels were associated with altered mitochondrial morphology during aging. *Drosophila* adult flight muscle is an ideal system to study alterations in mitochondrial structure and function (44–46). In this tissue, we observed mitochondrial fragmentation (i.e., smaller and rounder mitochondria), as visualized by electron microscopy (EM), in young and aged Parkin-overexpressing flies (compare Fig. 3 *B, b* and *D, d* with Fig. 3 *C, c* and *E, e*, respectively, and quantification in Fig. 3*F*). These findings are consistent with a model whereby Parkin-mediated turnover of *d*Mfn inhibits mitochondrial fusion and, thereby, results in an increased segregation of individual mitochondria from the mitochondrial network. Feeding RU486 to control flies (*daGS>w*^{1118}) did not alter *d*Mfn levels during aging (Fig. S5*B*) or mitochondrial morphology (Fig. S5 *C–G*).

Next, we sought to determine whether Parkin-mediated changes in mitochondrial dynamics were functionally significant. To do so, we assayed a number of markers of mitochondrial activity in long-lived Parkin flies and controls during aging. Firstly, we assayed the enzymatic activity of citrate synthase, a key enzyme in the Krebs cycle and a widely used mitochondrial marker. Indeed, Parkin overexpression conferred a significant increase in citrate synthase activity both in young and aged flies (Fig. 3*G*). Furthermore, long-lived Parkin flies showed an increase in the activity of mitochondrial respiratory complexes I (Fig. 3*H*) and complex II (Fig. 3*I*) in young flies. The peroxisome proliferator-activated receptor-γ

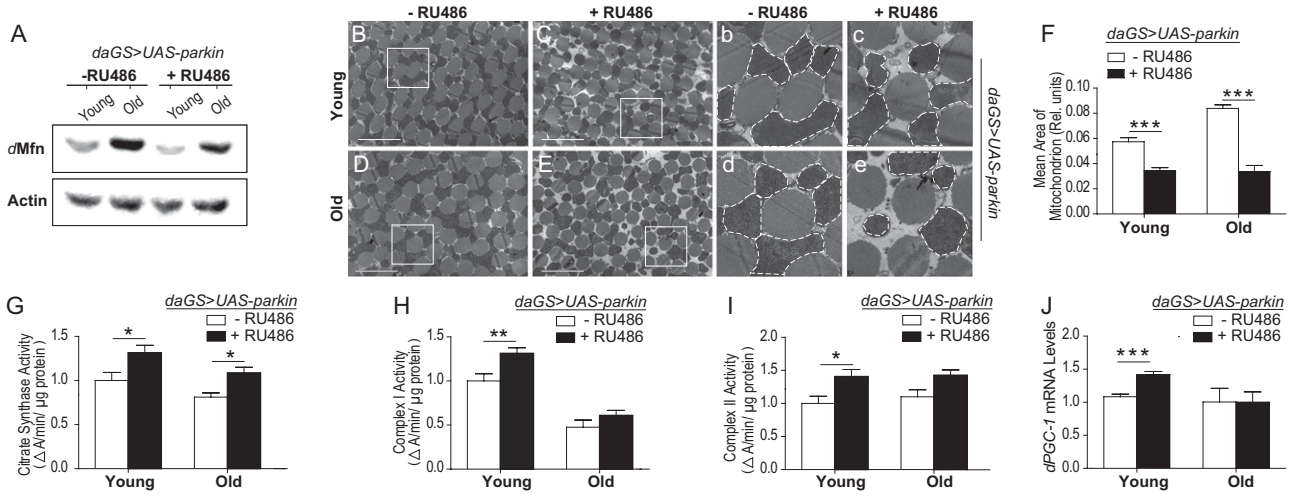

Fig. 3. Ubiquitous Parkin overexpression alters mitochondrial dynamics and activity during aging. (*A*) Western blot detection of *d*Mfn in young and aged *daGS>UAS-parkin* female flies, with or without RU486-mediated transgene induction. *d*Mfn levels are increased in aged flies compared with young flies. Parkin overexpression reduces *d*Mfn levels in young and aged flies (see quantification in Fig. S5*A*). (*B–E*) Electron micrographs of indirect flight muscles from young and aged flies. Parkin overexpression leads to a more fragmented mitochondrial network. *b–e* are insets of *B–E*. (Scale bars: 5 μm.) (*F*) Quantification of mitochondrial area in indirect flight muscles from young and aged flies. Parkin overexpression leads to smaller mitochondria in young and aged flies ($n > 81$ mitochondria per sample). (*G–I*) Quantification of markers of mitochondrial activity in young and aged flies. Parkin overexpression leads to an increase in citrate synthase activity (*G*), complex I activity (*H*), and complex II activity (*I*) in young flies and an increase in citrate synthase activity in aged flies (*G*) ($n \geq 3$; 10 flies per replicate). (*J*) Quantification of *d*PGC-1 mRNA levels in young and aged flies. Parkin overexpression leads to an increase in *dPGC-1* gene expression in young flies ($n = 3$; five flies per replicate). Data are represented as means ± SEM. RU486 was provided in the media at 0.2 μg/mL during development and 1 μg/mL during adulthood. Young flies were 10 d of age, and old flies were 42 d of age. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (Student *t* test).

coactivator (PGC)-1 family of transcriptional coactivators are key regulators of mitochondrial activity in mammals (47) and *Drosophila* (48, 49). Hence, we examined the expression of *dPGC-1* in long-lived Parkin flies and controls. Interestingly, we observed that Parkin-mediated changes in mitochondrial activity were associated with increased *dPGC-1* gene expression in young flies (Fig. 3*J*). Feeding RU486 to control flies (*daGS>w^{1118}*) did not alter markers of mitochondrial activity during aging (Fig. S5 *H–K*). Taken together, our findings show that Parkin-mediated longevity is associated with alterations in mitochondrial dynamics and activity during aging.

Parkin Overexpression in Adult Neurons Extends Longevity. To better understand the relationships between *parkin* gene activity, chronic maintenance of the nervous system and organismal aging, we examined the impact of neuron-specific overexpression of Parkin on *Drosophila* lifespan. To do so, we used the pan-neuronal *Elav*–Gene-Switch (*ElavGS*) driver line to increase *parkin* gene expression specifically in neurons. Firstly, we validated that there was an increase in *parkin* gene activity in heads of *ElavGS>UAS-parkin* flies upon RU486 treatment (Fig. S6 *A* and *B*). Our previous results with the ubiquitous *daGS* driver line showed that increasing Parkin levels exclusively in adult tissues can extend lifespan. Therefore, we tested the effects of both constitutive and adult-onset neuronal overexpression of Parkin to refine our understanding of the spatiotemporal requirements of Parkin-mediated lifespan extension. Induced neuronal up-regulation of Parkin, both constitutively (Fig. 4*A*) and in adult neurons (Fig. 4*B*), increased both mean and maximum lifespan in female flies and produced no major effect in male flies (Fig. S6*C*). Other doses of RU486 gave diminishing returns in lifespan extension and higher doses shortened lifespan (Fig. S6*D*). Again, no positive effects on longevity were observed in control flies exposed to RU486 using this paradigm (Fig. S6 *E–G*). Similar to our findings with ubiquitous overexpression, we observed that neuron-specific overexpression of Parkin did not reduce food consumption (Fig. 4*C* and Fig. S7*A*) or physical activity (Fig. S7 *B–D*) and increased fecundity in young flies (Fig. S7*E*). Interestingly, neuron-specific Parkin overexpression was sufficient to improve survival under starvation conditions (Fig. 4*D*). Feeding RU486 to control flies did not alter feeding (Fig. S7*F*), starvation resistance (Fig. S7*G*), physical activity (Fig. S7 *H–J*), or fecundity (Fig. S7*K*).

Neuronal Overexpression of Parkin Reduces Proteotoxicity and Enhances Mitochondrial Activity in the Aging Brain. To better understand neuronal Parkin-mediated lifespan extension, we examined a number of markers of Parkin activity in heads, which are greatly enriched for neuronal tissue. Firstly, we examined levels of K48-linked polyubiquitination in heads of long-lived neuronal Parkin flies and controls during aging. Whereas no differences were observed in young flies, neuronal overexpression of Parkin resulted in a significant increase in K48-linked polyubiquitination in heads of aged flies (Fig. S8*A*). To determine whether targeted activation of Parkin impacts protein homeostasis in the aging brain, we examined the levels of protein aggregates in aged brains of neuronal Parkin-overexpressing flies and controls. Long-lived flies with neuronal Parkin overexpression displayed reduced levels of protein aggregation in the aged brain (Fig. 4*E* and Fig. S8*C*). Feeding RU486 to control flies did not alter levels of protein aggregation in the aged brain (Fig. S8 *B* and *B′*). Together, these data indicate that neuronal Parkin overexpression can protect against proteotoxicity in the aging brain.

To determine whether Parkin overexpression can impact mitochondrial metabolism in aging neurons, we examined a number of markers of mitochondrial activity in heads. Interestingly, neuronal overexpression of Parkin was associated with increased citrate synthase activity (Fig. 4*F*) and *dPGC-1* gene expression (Fig. S8*D*) in heads of young flies and an increase in respiratory complex I activity in heads of aged flies (Fig. 4*G*). Feeding RU486 to control flies (*ElavGS>w^{1118}*) did not alter markers of mitochondrial activity in heads during aging (Fig. S8 *E–I*). Taken together, these findings indicate that an increase in neuronal Parkin expression protects against proteotoxicity and enhances mitochondrial activity in the aging brain. Moreover, these Parkin-mediated changes in the aging brain result in an increase in health span at the organismal level.

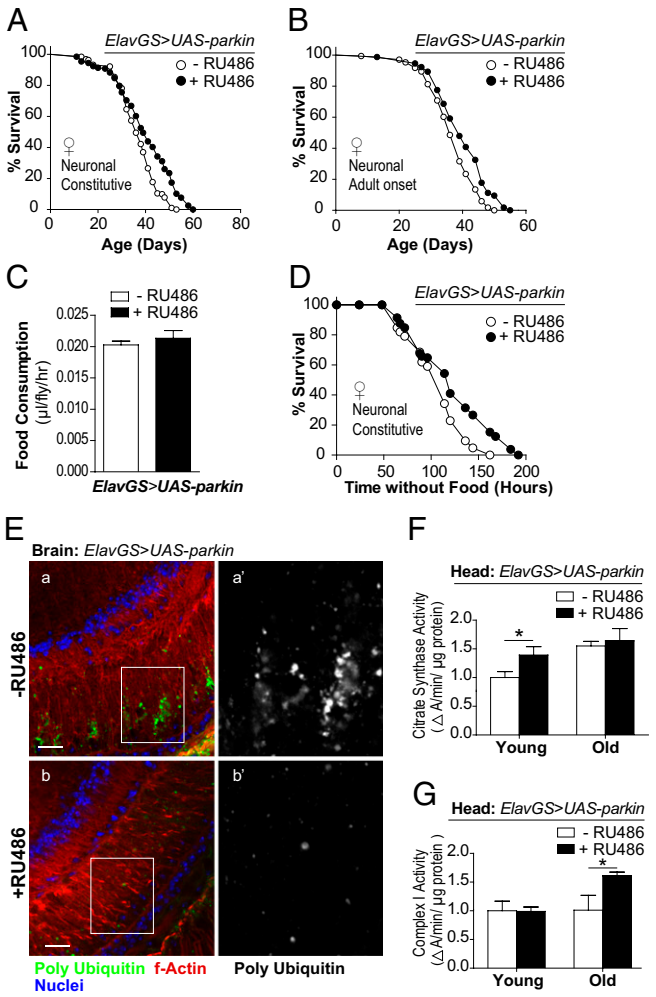

Fig. 4. Neuron-specific Parkin overexpression extends lifespan. (*A* and *B*) Survival curves of *ElavGS>UAS-parkin* flies with or without RU486-mediated transgene induction. (*A*) Constitutive neuron-specific overexpression of Parkin increases fly lifespan ($P < 0.0001$; log-rank test; $n > 180$ female flies). (*B*) Adult-onset neuron-specific overexpression of Parkin increases fly lifespan ($P < 0.0001$; log-rank test; $n > 180$ female flies). (*C*) Capillary feeding assay (52). Neuronal overexpression of Parkin has no significant effect on feeding behavior (n= 10; 10 female flies per replicate). (*D*) Survival curves under starvation. Constitutive neuron-specific overexpression of Parkin confers increased survival under starvation ($P < 0.0001$; log-rank test; $n > 60$ female flies). (*E*) Immunostaining of adult brains from aged (30 d) flies. Polyubiquitin (green) immunoreactivity revealed the deposition of aggregates containing polyubiquitin proteins (*Ea* and inset *Ea′* showing only aggregates). Neuronal Parkin overexpression significantly reduces the number of protein aggregates in the aged brain (*Eb* and inset *Eb′* showing only aggregates). See quantification in Fig. S8C. Phalloidin staining (red) outlines F-actin, and TO-PRO-3 stains nuclei (blue). (Scale bar: 10 µm.) (*F* and *G*) Quantification of markers of mitochondrial activity in heads of young and aged flies. Constitutive neuron-specific overexpression of Parkin overexpression leads to an increase in citrate synthase activity in heads of young flies (*F*) and complex I activity in heads of aged flies (*G*) (*$P < 0.05$; Student *t* test; $n = 3$; 10 heads from female flies per replicate). Young flies were 10 d of age, and old flies were 42 d of age. RU486 was provided in the media at 0.2 µg/mL during development and 1 µg/mL during adulthood (*A*, *C*, and *D*–*G*) and 0.5 µg/mL during adulthood (*B*). Data are represented as means ± SEM.

Discussion

In this study, we use the fruit fly *D. melanogaster* as a model system to address the question of whether the E3 ubiquitin ligase Parkin, previously implicated in PD, can modulate organismal aging. Using an inducible gene expression system, we show that overexpression of Parkin leads to a significant increase in longevity without any obvious physiological tradeoffs. These findings identify Parkin as a molecular link between the underlying aging process and age-onset neurodegeneration. Regarding the spatiotemporal requirements of Parkin-mediated lifespan extension, we show that adult-onset and/or neuron-specific overexpression of Parkin is sufficient to enhance longevity. However, the extent of lifespan extension was considerably greater upon ubiquitous Parkin overexpression compared with neuron-specific Parkin overexpression. Therefore, it would appear that an increase in Parkin activity beyond the nervous system is important to maximize the antiaging capabilities of Parkin.

There are several apparent candidate mechanisms that could explain Parkin-mediated lifespan extension. In recent years, the idea that the accumulation of damaged or misfolded proteins (proteotoxicity) during aging is a key determinant of lifespan has gained considerable empirical support (1). Using two independent measures, we observed that Parkin overexpression reduces proteotoxicity during aging. Firstly, using immunofluorescence, we observed a reduction in the number of protein aggregates in aged flight muscle and brains of long-lived Parkin-overexpressing flies. Secondly, Western blot analysis showed reduced levels of insoluble ubiquitinated proteins in whole bodies of aged Parkin-overexpressing flies compared with uninduced controls. In *C. elegans*, several proteins have been identified that become aggregation-prone as a function of age (4). An interesting area of future research will be to determine the identity of the substrates that become targeted for proteasomal degradation in response to Parkin overexpression. A related challenge will be to better understand how age-onset proteotoxicity affects the physiology of individual organ systems and how this, in turn, influences the health and viability of the organism.

A loss of mitochondrial homeostasis has been proposed as an underlying cause of aging (2). Given the role of Parkin in mediating the clearance of dysfunctional mitochondria via mitophagy and a recent report claiming that Pink1 overexpression in dopaminergic neurons can enhance lifespan (50), it is appropriate to speculate on the role of mitochondria in Parkin-mediated lifespan extension. The process of mitophagy appears to be intimately linked to the Parkin-mediated turnover of mitochondrial OM proteins, including the mitochondrial fusion-promoting factor Mfn (17). Our results demonstrate that *d*Mfn shows a significant increase in abundance during aging. A previous *Drosophila* study reported that acute overexpression of Parkin leads to reduced *d*Mfn levels (25). Consistent with this, we observed that chronic overexpression of Parkin resulted in reduced *d*Mfn levels in both young and aged flies. Moreover, we observed that the Parkin-mediated turnover of *d*Mfn in aging flies was associated with reduced mitochondrial fusion/increased mitochondrial fission and an increase in multiple markers of mitochondrial activity. One interpretation of these findings is that Parkin overexpression leads to a more segregated mitochondrial network, thereby facilitating the clearance of damaged mitochondria via mitophagy. However, it is also possible that the observed increase in markers of mitochondrial activity in long-lived Parkin flies is attributable to an increase in mitochondrial biogenesis. Indeed, we detected a significant increase in *dPGC-1* gene activity in long-lived Parkin flies. Interestingly, Parkin Interacting Substrate, PARIS (ZNF746), a repressor of mammalian PGC-1α, was recently identified as a Parkin substrate (51). Future work could focus on elucidating the relationships between Parkin, mitochondrial dynamics, mitophagy, and aging.

We have shown that long-lived Parkin flies display improved proteostasis and altered mitochondrial dynamics during aging. However, the relative contribution of each of these processes to lifespan extension remains unclear. Further study will be required to determine the cause and effects relationships between

proteotoxicity, mitochondrial homeostasis, and Parkin-mediated lifespan extension. Regardless of the underlying mechanism, however, our work strongly suggests that treatments designed to augment Parkin expression during aging may delay the onset and progression of a number of age-onset diseases including, but not limited to, PD and other neurodegenerative disorders.

Materials and Methods

Below we provide a brief overview of the methods used for experiments presented in this article. For further details, please see *SI Materials and Methods*.

Lifespan Analysis and Physiological Assays. Lifespan analysis and other physiological assays were carried out as described previously (48). See *SI Materials and Methods* for details.

Immunostaining and EM. Hemithoraces or brains were immunostained and analyzed as described in ref. 7. Standard procedure for EM analysis was carried out as described previously (44, 45). See *SI Materials and Methods* for details.

Molecular Biology. Protocols for Western blot, analysis of detergent-insoluble fractions, mitochondria purifications, citrate synthase assay, and complex I and II activity assays used in this study can be found in *SI Materials and Methods*.

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