

Drosophila parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress

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Summary

Mutations in the gene *parkin* in humans (*PARK2*) are responsible for a large number of familial cases of autosomal-recessive Parkinson disease. We have isolated a *Drosophila* homolog of human *PARK2* and characterized its expression and null phenotype. *parkin* null flies have 30% lower mass than wild-type controls which is in part accounted for by a reduced cell size and number. In addition, these flies are infertile, show significantly reduced longevity, and are unable to jump or fly. Rearing mutants on paraquat, which generates toxic free radicals *in vivo*, causes a further reduction in longevity. Furthermore, loss of *parkin* results in progressive degeneration of most indirect flight muscle (IFM) groups soon after eclosion, accompanied by apoptosis. However, *parkin* mutants have normal neuromuscular junction recordings during the

third larval instar stage, suggesting that larval musculature is intact and that *parkin* is required only in pupal and adult muscle. *parkin* flies do not show an age-dependent dopaminergic neuron loss in the brain, even after aging adults for 3 weeks. Nevertheless, degeneration of IFMs demonstrates the importance of *parkin* in maintaining specific cell groups, perhaps those with a high-energy demand and the concomitant production of high levels of free radicals. *parkin* mutants will be a valuable model for future analysis of the mechanisms of cell and tissue degeneration.

Key words: *parkin*, *Drosophila*, Parkinson disease, Paraquat, Oxygen radicals, Cell size, Infertility, Neurodegeneration, Dopaminergic, Brain, Apoptosis

Introduction

Parkinson disease (PD) is a progressive neurodegenerative disorder and individuals with PD present symptoms of resting tremors, rigidity and akinesia. Pathological examination of the brains of individuals with idiopathic PD show loss of dopaminergic (DA) neurons in the substantia nigra of the midbrain with the formation of cytoplasmic protein inclusions called Lewy bodies (LBs). The loss of these DA neurons might underlie the clinical symptoms of PD. Although the cause of idiopathic PD is unknown, various environmental toxins, such as pesticides and those structurally similar to MPTP, have been strongly implicated (Lockwood, 2000). MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), when converted to the active toxin MPP(+) by monoamine oxidase B, can induce parkinsonism in primates. MPP(+) interferes with mitochondrial complex I, resulting in an inhibition of ATP synthesis and the generation of free radicals, causing DA neuron damage (Dawson et al., 2002). In addition, endogenous toxins may also be a primary or an additional cause of DA neuron loss in a susceptible population. Specifically, dopamine readily oxidizes to produce free radicals, which can inflict damage on the cell. Mitochondria use several defense systems such as catalase,

superoxide dismutase and glutathione as protection against oxygen radicals. However, these systems can be overwhelmed, resulting in mitochondrial dysfunction and triggering apoptosis (Orth and Schapira, 2002).

Recent identification of a number of familial PD cases and the genes involved therein has provided new insights into the pathogenesis of PD. Missense mutations in α -Synuclein, identified in a small number of families, result in an autosomal dominant familial PD (Kruger et al., 1998; Polymeropoulos et al., 1997). α -Synuclein is a presynaptic protein and is a major component of LBs (Clayton and George, 1998). Misexpression of α -Synuclein in cultured cells results in aggregate formation, abnormal mitochondrial morphology, and increased levels of free radicals (Hsu et al., 2000). A number of transgenic models of PD have been generated using overexpression of α -Synuclein in both mice and flies (Feany and Bender, 2000; Giasson et al., 2002; Lee et al., 2002; Masliah et al., 2000).

Mutations in *PARK2* have been linked to Autosomal Recessive-Juvenile Parkinson's (AR-JP) (Kitada et al., 1998). The dopaminergic loss in AR-JP is generally not associated with LB formation (Mizuno et al., 1998). *PARK2* is highly expressed throughout the brain, is present in both the cytoplasm and the nucleus, and is also associated with

mitochondrial membranes (Darios et al., 2003; Kubo et al., 2001; Shimura et al., 1999; Stichel et al., 2000). The human PARK2 protein consists of four domains: an N-terminal ubiquitin-homology domain and two RING finger domains flanking a cysteine-rich in between RING fingers (IBR) domain (Fig. 1A) (Morett and Bork, 1999). Proteins containing RING finger domains frequently act as ubiquitin E3 ligases (Joazeiro and Weissman, 2000). E3 proteins function as part of the ubiquitin-proteasome system required to degrade proteins that are misfolded, damaged by oxidative stress, nitrated or ubiquitinated (Grune et al., 1998; Halliwell, 2001; Sitte et al., 2000; Stadtman and Berlett, 1998). Vertebrate parkin also acts as an E3 ligase, helping to ubiquitinate and degrade several proteins, including Pael receptor (Pael-R), α -Synuclein, CDCrel-1 and Synphilin 1. Familial mutations in PARK2 abolish its E3 activity (Chung et al., 2001; Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). This and other data (see below) suggest that loss of *PARK2* may result in increased amounts of damaged and misfolded proteins, leading to cellular dysfunction and apoptosis of DA neurons (Bence et al., 2001; Mezey et al., 1998). A familial mutation in another ubiquitin/proteasome system member, ubiquitin carboxy-terminal hydrolase I, also results in an early onset PD, underscoring the importance of this system in PD (Leroy et al., 1998).

Screens to identify *PARK2* targets and overexpression studies have provided evidence that *PARK2* plays an important role in protecting the cell from toxic insults. Misexpression of Pael-R, a substrate of human PARK2, causes it to become unfolded, insoluble and ubiquitinated, thereby resulting in cell death. An insoluble form of *Pael-R* was also found to accumulate in AR-JP brains. PARK2 binds and ubiquitinates Pael-R, leading to the degradation of its insoluble form, and protects cells from *Pael-R*-induced cell death (Imai et al., 2001). Furthermore, misexpression of Pael-R in *Drosophila* dopaminergic neurons results in DA cell loss that is suppressed by co-expression with *parkin*. By contrast, co-expression of Pael-R with RNAi knockdown of endogenous *Drosophila parkin* results in enhanced DA cell loss (Yang et al., 2003). α -Synuclein-induced DA cell loss is also suppressed by co-expression of *parkin* (Auluck et al., 2002; Yang et al., 2003). In addition, Parkin can promote the ubiquitination and degradation of an *O*-glycosylated form of α -Synuclein (Shimura et al., 2000). Overexpression of *PARK2* in tissue culture cells results in increased proteasomal activity and decreased levels of protein carbonyls and 3-nitrotyrosine-containing proteins (markers of proteins modified by reactive nitrogen species) (Hyun et al., 2002). Although the roles of two other Parkin-interacting proteins, CDCrel-1 and Synphilin 1, in AR-JP are still unclear, it is possible that Synphilin 1 may mediate interaction between Parkin and a more abundant non-glycosylated form of α -Synuclein. Finally, *PARK2* expression is sufficient to prevent mitochondrial swelling and cytochrome c release in ceramide-induced apoptosis and Parkin is associated with the outer mitochondrial membrane (Darios et al., 2003). Taken together, these results suggest that Parkin may be important in mitochondrial functions such as oxygen radical defense or the cell death pathway.

Previous work has shown that *Drosophila parkin* is required for longevity, male fertility and survival of flight muscle fibers (Greene et al., 2003). In this report, we confirm these

observations and extend the requirement for *parkin* to several other processes. Specifically, we show that *parkin* mutant flies have reduced mass, cell size and number; female infertility; and increased sensitivity to chemical and environmental stress. We also confirm that loss of *parkin* does not result in any significant DA neuron loss.

Materials and methods

Drosophila strains

We obtained the *EP(3)3515* P-element insertion in the *orbit* gene from the Bloomington *Drosophila* Stock Center. This element, which causes recessive sterility, was mobilized using $\Delta 2$ -3 transposase and new insertions were identified using inverse PCR as described (Tavsanli et al., 2001). We identified a new insertion 1 kb 3' of *parkin* (named *dpk^{P30}*) that maintained an intact *EP(3)3515* element. Reversion of sterility was used to select for precise excision of the EP element and PCR was employed to identify those lines with an intact *dpk^{P30}* insertion. We then mobilized *dpk^{P30}* and screened for new insertions in or near *parkin*. We identified a new insertion 29 bp 5' of the *parkin* translational start site that also maintained an intact *dpk^{P30}* element. This line is called *dpk^{P21}*. In addition, we also identified two other lines, *dpk^{P23}* and *dpk^{P24}*, that truncate parkin 127 and 155 amino acids 3' of the N terminus and behave as genetic nulls. To generate *parkin* deletion chromosomes, the *dpk^{P30}-dpk^{P21}* double insertion was mobilized with $\Delta 2$ -3 transposase as described above. Progeny were screened for the absence of *w⁺* eyes. 66 *w⁻* males were identified and balanced, and their progeny were screened by Southern blot analysis. Five out of 66 lines were found to have *parkin* DNA deleted. These five deletion lines were screened by PCR and sequencing for the presence of intact genomic DNA flanking the two starting elements. We identified one *parkin* deletion line meeting these criteria, *dpk^{Δ21}* (Fig. 1B). The *c179-Gal4* that drives Gal4 expression in the adult muscle as well as other tissues, was obtained from the Bloomington collection (stock BL-6450) (Manseau et al., 1997).

Molecular characterization of *parkin*

Using a BLAST search of the *Drosophila* genome database, we identified an EST clone (SD01679) that encodes *Drosophila parkin*. We used this EST to probe a *Drosophila* genomic library (kindly provided by Ron Davis) and identified lambda phage clones that span the *parkin* locus. Phage integrity was verified by restriction digests as well as sequence analysis of the *parkin* locus. These phage were used to generate the *dpkR* (Rescue) genomic rescue construct that encompasses the following genomic coordinates: chromosome 3L, 21098849-21124696 (www.ensembl.org) (Fig. 2A). This rescue fragment was cloned into the pCasper 4.0 vector to generate transgenic flies. The *dpkSR* (Stop-Rescue) construct was created by introducing stop codons in all three reading frames of *parkin* using PCR mutagenesis but is otherwise identical to *dpkR*. These stop codons are located 66 base pairs 3' of the *parkin* AUG. Two and five independent transgenic lines of *dpkSR* and *dpkSR*, respectively, were obtained. In addition to the *parkin* mutant chromosome, *dpk^{Δ21}*, we also used an independent chromosome that had a deficiency uncovering the entire *parkin* gene, *Df(3L)pc-MK* (Bloomington, stock BL-3068). *UAS-HA::parkin* transgene was built using the *pUAST* vector with an engineered HA-tag sequence (courtesy of Beril Tavsanli) fused to the *parkin*-coding sequence from SD01679 EST clone. This EST clone contained an A→T difference at base pair 760 (of 1446) of the coding sequence compared to the genome database and our genomic phage clones. Therefore, we replaced a *NarI* fragment of SD01679 with the same fragment from EST clone AT25577 to correct for this change.

Developmental northern blot analysis

RNA was isolated from staged embryos, larvae, pupae, and adult flies

using 3 M LiCl, 6 M UREA and 0.2% SDS as previously described (Kelley, 1993). Poly (A)⁺ RNA was isolated using oligo(dT)-cellulose columns, subjected to electrophoresis in a 1% agarose, 6.25% formaldehyde gel and blotted. We hybridized blots with a *parkin* cDNA probe and subsequently with an RP49 cDNA probe using the RediPrime II Random Prime Labeling System (Amersham Pharmacia Biotech).

Immunohistochemistry

Whole-mount immunohistochemistry was performed as previously described (Davis et al., 2003). Brains from *parkin* mutant and heterozygous adults, aged 2 and 21 days post eclosion, were fixed in PLP, washed in PAXDG and stained with rabbit polyclonal anti-*Drosophila*-tyrosine hydroxylase antibody (courtesy of Wendi Neckameyer) (Mardon et al., 1994; Neckameyer et al., 2001). Subsequently, brains were stained with Alexa goat anti-rabbit antibodies in PAXDG. Brains were mounted in Vectashield and dorsomedial clusters identified and the number of cells counted using confocal microscopy (Zeiss LSM 510 microscope).

Light microscopy and TEM muscle fiber analysis

Two-day old adult flies were dissected by removing the head and abdomen in ice cold fixative (2% paraformaldehyde/3% glutaraldehyde/0.1 M sodium cacodylate/0.05% CaCl₂). Whole thoraces were fixed overnight at 4°C and then washed with 0.1 M cacodylate. Postfixation was performed with 2% OsO₄ for 2 hours at room temperature and the sample was washed with water before going through a series of progressive dehydration steps in ethanol:water mixtures. The sample was then embedded in Spurr's resin (Spurr, 1969). Thoraces were then trimmed to retain only the dorsal longitudinal muscles. Ultrathin sections of 50 nm were then made with a diamond knife and stained with Toluidine Blue dye, which labels nucleic acids, thereby marking both nuclei and cytoplasm (Balabanova et al., 2003). For TEM, sections were stained on the grid first with 4% uranyl acetate, then with 2.5% lead nitrate, five minutes each at room temperature. The sections were observed with a JEOL 1010 transmission electron microscope. Late pupae were removed from their pupal cases in the same fixative as above and processed as described above.

Courtship behavior and fertility assays

The courtship behavior assay was carried out on *parkin* mutant and heterozygous males that were aged individually for one week as previously described (Hall, 1994). Individual virgin w⁻ females, comparable in age to the males, were placed in a food vial with individual males, and the elements of the courtship behavioral sequence were noted for a total of 1 hour. Mating pairs were considered to be successful when the courtship behavior ended with copulation. Fertility was assayed by placing single *parkin* males with 3-4 virgin w⁻ females and by placing single virgin *parkin* females with two w⁻ males. Vials were then checked 3-7 days later for the presence of larvae.

Mass and cell size analysis

Mass analysis was performed as described (Bohni et al., 1999). Twenty individual males and females were weighed on a precision scale (range 0.01 mg to 50 g; Mettler Toledo AG245). In addition, groups of 20 and 30-100 flies of each genotype were weighed to confirm the accuracy of the individual fly weighing. Flies were reared under the same conditions and were age matched (48-72 hours post eclosion). Cell counts to determine the differences in cell size in the wing were assessed by counting the number of wing hairs on the dorsal wing surface in the same arbitrary area just posterior to the posterior cross vein for all genotypes ($n=20$ wings/genotype, $P<0.001$ one-way ANOVA test). Wing area was determined using NIH image 1.60 software (<http://rsb.info.nih.gov/nih-image/Default.html>) ($n=20$ wings/genotype, $P<0.001$ one-way ANOVA test).

Jump/flight analysis

Flies were anesthetized with CO₂ and individually placed in vials 24 hours before the assays were conducted to allow time for a full recovery from effects of CO₂. Each fly was dispelled into a plastic petri dish by gently tapping the vial. The dish was covered with a lid to prevent flies from escaping. The petri dish was then tapped lightly on the bench to initiate a jumping reaction. Flight tests were performed by removing the lid of the petri dish and inverting the dish at a height of 29 inches. The lid was lightly tapped to loosen the fly. Flies either fell in a straight line, fell by veering or flew. The number of flies exhibiting each behavior was recorded. Twenty flies of each genotype were assayed.

Oxidative stress toxicity

Paraquat (methyl viologen) toxicity was performed as previously described (Clancy et al., 2001). Two-day old animals were placed in vials (10 per vial) with food media made of 0.8% agar containing 5% sucrose and 2 mM paraquat (Sigma). Control flies for each genotype were placed under the same conditions minus paraquat. All experiments were performed in the dark. Animals were scored daily and the media was replaced every 3-4 days.

Cold stress

Resistance to cold stress of 2-day old flies was performed as previously described (Mockett et al., 2003). Flies were placed ($n=10$) in a vial and kept at 4°C, except for a 1 hour recovery period (25°C) daily prior to scoring mortality.

Longevity

Flies were maintained on standard media at 25°C, ten per vial, and transferred to new food medium daily. Mortality was scored daily.

Results

Drosophila parkin is a highly conserved homolog of human Parkin

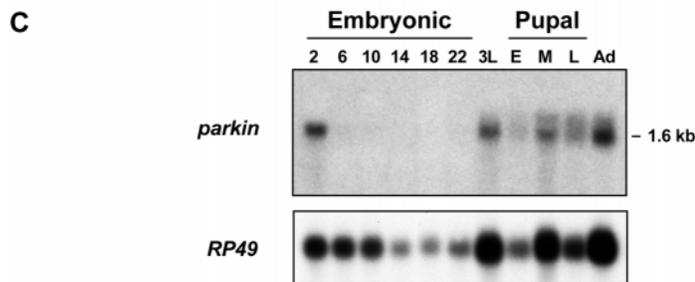
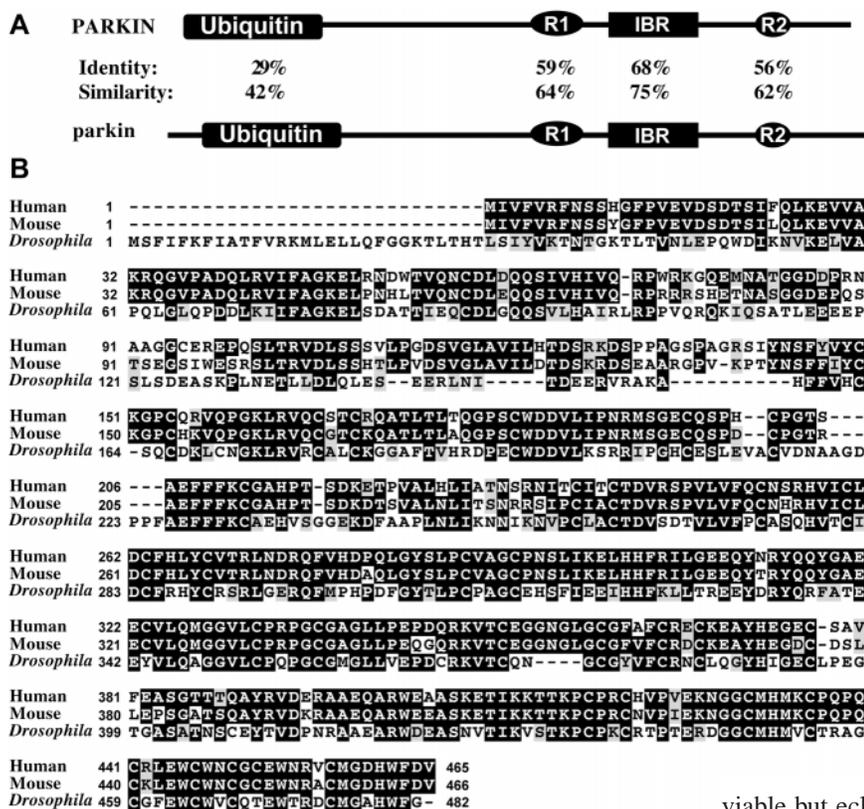
Analysis of EST clone sequences and genomic data indicates that *parkin* consists of six exons (CG10523, www.bdgp.org). The predicted open reading frame encodes a 482 amino acid protein with four domains that follow the same order as in human Parkin: an N-terminal ubiquitin-homology domain, two ring finger domains and an in between ring (IBR) domain which are all highly conserved (Fig. 1A). The human and *Drosophila* proteins have an overall identity of 46% and an E-value of $3.1E^{-102}$ (Fig. 1B). No other obvious human Parkin homolog is present in the *Drosophila* genome.

parkin is expressed at multiple stages of development

To determine the developmental expression of *parkin* we performed northern blot analysis of embryonic, larval, pupal and adult RNAs. This reveals a 1.6 kb transcript, which is similar in length to the longest *parkin* EST clone identified (SD01679) (Fig. 1C). *parkin* transcript is first detected early (0- to 2-hour-old embryos) and is probably due to a maternal contribution. Although *parkin* transcript is not detected at high levels during later embryonic stages, it is present in third instar larvae, as well as throughout pupal development and into adulthood (Fig. 1C).

parkin mutants have reduced body mass, cell size, and cell number

We used P-element mutagenesis to generate null mutations in



parkin (Fig. 2A,B) (Kaiser and Goodwin, 1990). A P-element located 26 kb 3' of *parkin* [*EP(3)3515*] was used to conduct a local hop screen for new insertions in or near the *parkin* locus (see Materials and methods) (Fig. 2A). We obtained a new insertion 1 kb 3' of *parkin*, termed *dpk^{P30}*. After precisely excising the starting element [*EP(3)3515*], the new element (*dpk^{P30}*) was mobilized to generate new insertions in and flanking *parkin* (*dpk^{P21}*, *dpk^{P23}*, and *dpk^{P24}*) (Fig. 2B and data not shown). Subsequent simultaneous mobilization of *dpk^{P30}* and *dpk^{P21}* resulted in deletion of the entire *parkin*-coding region. PCR and Southern analyses were used to confirm deletion of the gene (data not shown). We also obtained two insertions directly in the *parkin*-coding region, *dpk^{P23}* and *dpk^{P24}*, which behave genetically as null alleles (data not shown). To confirm that observed phenotypes are specific to *parkin* we generated two rescue transgenes. The first transgene contains *parkin* and 10-12 kb flanking it on either side. The second transgene is identical to the first with the exception that stop codons were introduced in the coding sequence of *parkin* shortly after the translational start site. Rescue by the first

Fig. 1. Analysis of Parkin amino acid sequence conservation and developmental expression. (A) Schematic of human and *Drosophila* Parkin and PARK2 proteins. The ubiquitin-homology domain is an N-terminal domain, followed by the first RING finger domain (R1), an in between ring (IBR) domain, and a second RING finger domain (R2). Similarity and identity values for each domain are shown. (B) Multiple sequence alignment of human, mouse and *Drosophila* Parkin proteins shows a high degree of conservation throughout the lengths of the proteins. Solid boxes indicate identities; shaded boxes represent similarities. (C) Developmental northern analysis of *parkin* expression. A 1.6 kb *parkin* transcript is first detected in 0-2 hour embryos but is undetected in subsequent embryonic stages. *parkin* is again expressed during third instar larval (3L), early (E), mid (M) and late (L) pupal stages, as well as in adults. *RP49* was used as a loading control on the same filter.

transgene, *dpkR* (*Rescue*), but not the second, *dpkSR* (*Stop Rescue*), indicates that observed phenotypes are specific to loss of *parkin* function.

parkin mutant animals are homozygous viable but eclose one day later than heterozygous controls. In addition, loss of *parkin* results in a significant pupal lethality (Fig. 2C). Although there is little or no lethality prior to pupation, 68% of *parkin* mutant animals fail to eclose from their pupal cases, suggesting that *parkin* plays an important role during pupal development (Fig. 2C). *parkin* null animals that do eclose exhibit a significant reduction in body size (Fig. 3A). Mutant flies present a mass reduction of 34% in females and 30% in males when compared with controls of the same age and sex. This phenotype is partially but significantly rescued with *dpkR* but not *dpkSR* transgenes, confirming that the mass reduction is due to *parkin* and not any of the surrounding genes that could have been affected by the *parkin* deletion (Fig. 3B).

The reduction in body size could be accounted for by a reduction in cell number and/or a reduction in the size of individual cells. We used the *Drosophila* wing to determine whether cell number, cell size or both is reduced in *parkin* mutants. Each wing cell normally secretes cuticle and a single hair. Therefore, cell number and cell size in the wing can be determined by counting wing hairs and determining their density over an area (Bohni et al., 1999). Compared with heterozygous controls, cell size in *parkin* mutant female wings is reduced by 10% and the wing area itself is reduced by 23% ($n=20$, $P<0.001$ one-way ANOVA test) (Fig. 3C; Table 1). This suggests that *parkin* may play a role in cell growth, proliferation and/or survival.

Loss of *parkin* results in reduced longevity and resistance to oxidative and cold stress

Similar to previously published work, we observe that loss of *parkin* function also reduces longevity (Greene et al., 2003). We analyzed lifespans of *w; dpk^{A21}* and *w; dpk^{A21}/Df(3)pc-MK* flies. *Df(3)pc-MK* is an independent deficiency that

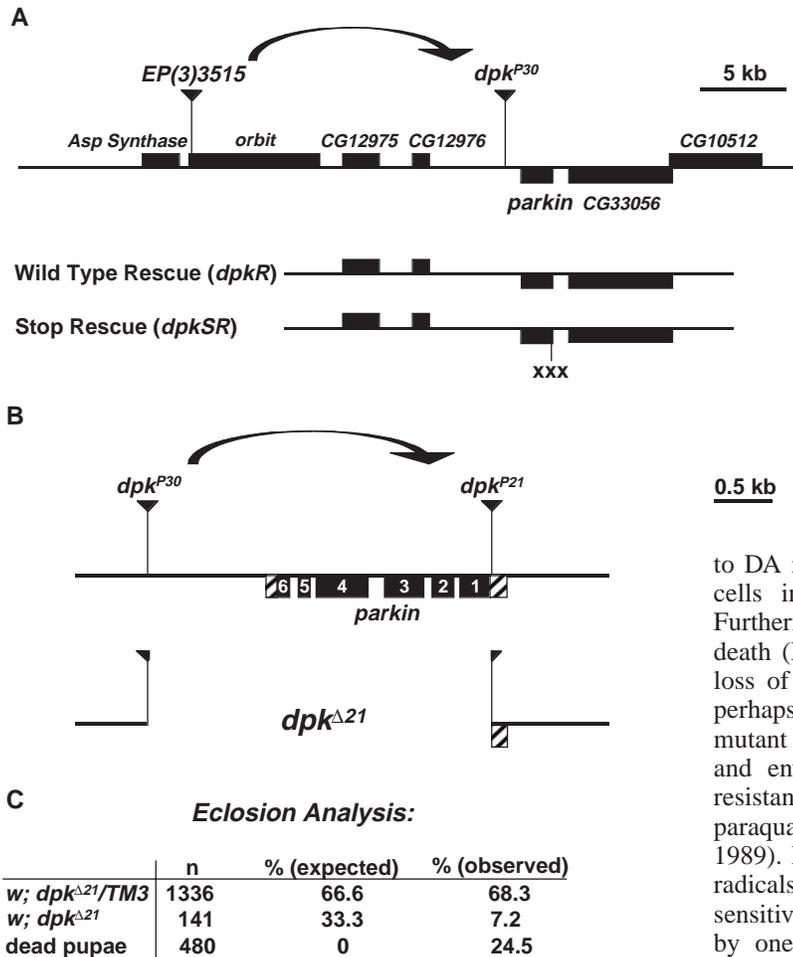


Fig. 2. Genomic map of *parkin* locus, P-element mutagenesis and eclosion analysis of *parkin* mutants. (A) Locations of predicted genes and P-elements surrounding the *parkin* locus are shown. Solid boxes represent *Drosophila* genes and putative ORFs. The *parkin* rescue (*dpkR*) and stop-rescue (*dpkSR*) transgenes are identical except that *dpkSR* contains stop codons in all three reading frames of *parkin*. xxx indicates the position of the stop codons. (B) Generation of a null *parkin* allele. The *dpk^{P30}* element was mobilized to generate the *dpk^{P21}* insertion. The resulting double insertion chromosome was then used to delete the entire *parkin*-coding region while the surrounding genomic DNA remained intact. (C) Eclosion analysis. Progeny of w; *dpk^{Δ21}/TM3* self cross was analyzed. Although little or no pre-pupal lethality was observed, 68% of *parkin* mutant pupae fail to eclose (92% of the dead pupae are *parkin* mutants).

to DA neurons is thought to be related to the death of these cells in individuals with PD (Braunwald et al., 2001). Furthermore, overexpression of *PARK2* protects against cell death (Darios et al., 2003). Therefore, we hypothesized that loss of *parkin* may lead to a reduction in stress resistance, perhaps contributing to reduced longevity. We subjected *parkin* mutant flies to two types of stress: chemical (paraquat) and environmental (cold stress). To assay oxygen radical resistance, *parkin* mutant animals were placed on 2 mM paraquat (methyl viologen)-supplemented food (Phillips et al., 1989). Paraquat is an MPTP-like toxin that produces oxygen radicals. Mutant animals, compared with controls, show high sensitivity to this low dose of paraquat as most mutants died by one week of age while heterozygous controls showed minimal lethality (Fig. 4B). We used cold to further test the stress resistance of *parkin*-null animals and discovered that *parkin* mutant flies show a significant reduction in resistance to cold temperature. The mean lifespan of *parkin* mutant flies maintained at 4°C is 5-7 days compared with 50 days for heterozygous controls (Fig. 4C) (Mockett et al., 2003). Taken together, these results suggest that *parkin* may be required for stress resistance and protection against oxygen radical damage.

Loss of *parkin* results in infertility, apoptotic indirect flight muscle degeneration, and mitochondrial dysfunction

Previous work has shown that *parkin* is required for male fertility and in particular for proper sperm maturation (Greene et al.,

completely uncovers the *parkin* locus. The mean life spans for w; *dpk^{Δ21}* and for w; *dpk^{Δ21}/Df(3)pc-MK* flies are 13 and 32 days, respectively, while heterozygous controls have a mean life span greater than 60 days (Fig. 4A). This phenotype is rescued by *dpkR* but not *dpkSR* transgenes and w; *dpk^{Δ21}* and w; *dpk^{Δ21}/Df(3)pc-MK* animals are rescued to the same degree by *dpkR*. These results suggest that the less severe phenotype of w; *dpk^{Δ21}/Df(3)pc-MK* compared with w; *dpk^{Δ21}* is due to a second site suppressor on the *Df(3)pc-MK* chromosome (Fig. 4A).

A high rate of oxygen radical production (as part of dopamine oxidation) and corresponding mitochondrial damage

Table 1. Cell size and cell number are reduced in wings of *parkin* mutant animals

Genotype*	Cell number (±s.e.m.)†	Cell size (μm ²)‡	Relative cell size (%)	Wing size (±s.e.m.)§	Relative wing size
w; <i>dpk^{Δ21}</i>	90.5±0.8	259	90.2	8.4±0.1	77.3
w; <i>dpkR/+; dpk^{Δ21}</i>	78.4±0.8	298	103.8	10.8±0.1	98.4
w; <i>dpkRS/+; dpk^{Δ21}</i>	87.1±1.0	269	93.7	8.6±0.2	78.9
w; <i>dpk^{Δ21}/+</i>	81.4±0.6	287	100.0	10.9±0.2	100.0

*Twenty animals of each genotype were assayed.

†Number of wing hairs on the dorsal wing surface in the same arbitrary area just posterior to the posterior cross vein.

‡Determined by dividing the area in b by the number of hairs.

§Wing size was determined using NIH image 1.60 software after importing the image from Adobe Photoshop.

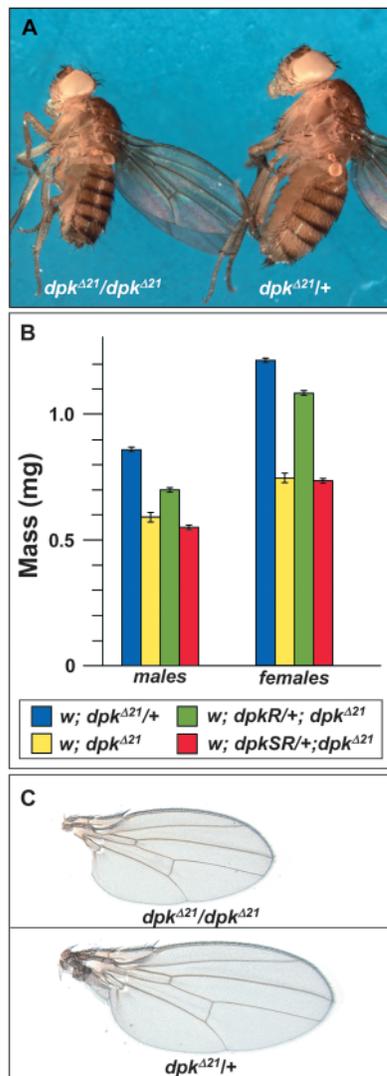


Fig. 3. Body size of *parkin* mutants is significantly reduced at eclosion. (A) *parkin* mutant animals are visibly smaller than controls 2 days after eclosion. (B) Body mass measurements of 20 individual males and females were performed. In *parkin* mutants, mass was reduced by 30% in males and 34% in females compared with heterozygous controls. This phenotype shows significant but partial rescue with *dpkR* but not with *dpkSR*, confirming the specificity of phenotype to *parkin* ($P < 0.001$, one-way ANOVA test). (C) *parkin* mutant wing size is reduced by 23% in females ($n = 20$, $P < 0.001$, one-way ANOVA test).

2003). We confirmed that *parkin* mutant males are sterile and have found that females are infertile as well. Neither sex yields progeny when crossed with wild-type animals of the opposite sex. Furthermore, only three out of 20 mutant males exhibited one of seven tested courtship behaviors (data not shown, see Materials and methods) (Hall, 1994). This suggests that *parkin* is important in either sexual behavior, function or both.

We also confirmed that *parkin* null animals are unable to fly (Table 2) and exhibit a drooped wing phenotype: mutant wings are positioned at an approximately 45° angle to the ground, while wild-type flies hold their wings parallel to the ground (Greene et al., 2003). To investigate the flight and drooped

Table 2. Jumping/flying ability of flies (1-day-old flies)

Genotype*	Jump (%)	Fly (%)	Veer (%)	Fall (%)
w; <i>dpk</i> ^{Δ21}	15	0	5	95
w; <i>dpkR</i> /+; <i>dpk</i> ^{Δ21}	100	100	0	0
w; <i>dpkRS</i> /+; <i>dpk</i> ^{Δ21}	10	0	10	90
w; <i>dpk</i> ^{Δ21} /+	100	100	0	0

*Ten males and ten females of each genotype were assayed.

wing phenotypes, we analyzed thoracic indirect flight muscles (IFMs) using light microscopic analysis of thin resin sections and transmission electron microscopy (TEM) at both pupal and adult stages (Figs 5, 6). At a late pupal stage (94 hours after pupation) all IFMs appear normal (Fig. 5A-D). By contrast, 2 days post eclosion *parkin* mutant animals have significant IFM degeneration (Fig. 5F,H, asterisks). By 11 days, most of the IFMs show signs of degeneration (Fig. 5J,L, asterisks). Heterozygous controls and mutant animals containing the rescue transgene, *dpkR*, did not show any signs of a muscle loss, while *parkin* mutants carrying the *dpkSR* transgene were not rescued (Fig. 5E,G,I,K).

We used TEM to further investigate the nature of this IFM defect. Wild-type muscle fibers have a highly regular and uniform structure (Fig. 6A). At the late pupal stage, *parkin* mutant IFM fibers have normal morphology that is indistinguishable from control animals (data not shown). However, at 2 days post eclosion, *parkin* mutants IFMs have roughened edges, increased spacing between fibril groups, and a reduced number of fibrils (Fig. 6B,D). There is also a reduced number of mitochondria which are of a generally smaller and variable size with a significant loss of cristae (Fig. 6B, asterisk). In addition, signs of apoptosis, including chromatin condensation and nuclear envelope breakdown, are readily apparent in *parkin* mutant muscle tissues (Fig. 6F) (Cotran, 1999). The *dpkR*, but not *dpkSR*, transgene rescues these phenotypes (Table 2; Fig. 6). In addition, we were able to rescue the flight defect with a *UAS-HA-parkin* transgene using both ubiquitous (*Ubi-Gal4*) and muscle (*C179-Gal4*) drivers (data not shown). These data further confirms that loss of *parkin* is responsible for the IFM degeneration phenotype. At 2 days post-eclosion not all IFM groups within each animal exhibit this phenotype, suggesting that muscle degeneration is progressive. *parkin* mutant flies can survive for several weeks and are able to walk, but not jump or fly, suggesting that only specific muscle groups are affected. As these flies are able to feed, smooth muscle is also unlikely to be significantly affected. We conducted neuromuscular junction (NMJ) electrophysiological studies to determine if there is a neural and/or muscular defect at the NMJ during the third larval stage (L3). Our tests show that *parkin* mutants have normal L3 recordings (data not shown). As third instar larval neuromuscular junction recordings in *parkin* null animals are normal, the primary role for *parkin* in muscle development and maintenance probably occurs during pupal stages.

***parkin* mutants do not show an age dependent dopaminergic neuronal loss**

The pathological presentation of familial cases of Parkinson disease is an age-dependent degeneration of dopaminergic

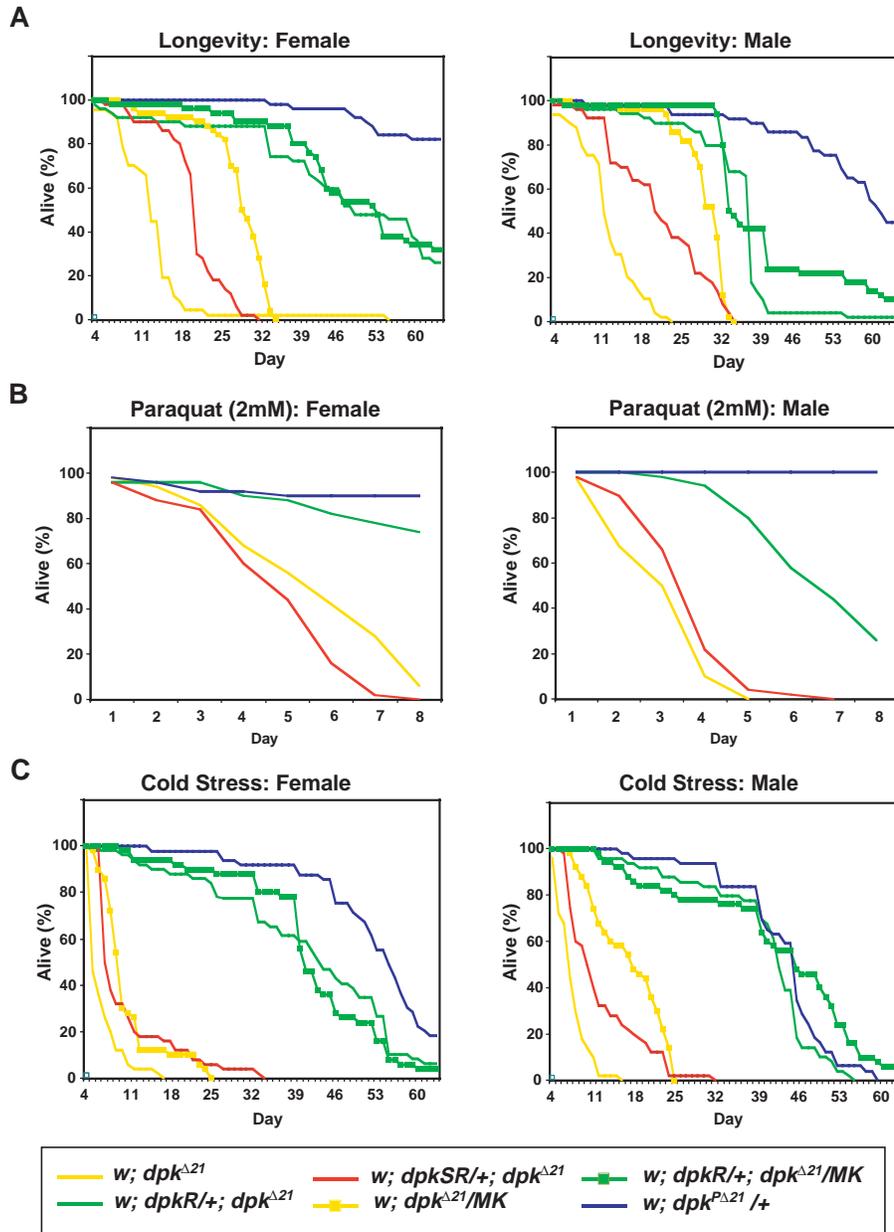


Fig. 4. *parkin* null flies have reduced longevity and stress resistance. We analyzed life span, and oxidative and cold stress resistance of *parkin* mutant animals. (A) Longevity profile of $w; dpk^{\Delta 21}$ as well as $w; dpk^{\Delta 21}/Df(3L)pc-MK$ animals. The *Df(3L)pc-MK* deficiency uncovers the entire *parkin* locus. Mean life span for male $w; dpk^{\Delta 21}$ flies is 13 days and for $w; dpk^{\Delta 21}/Df(3L)pc-MK$ flies is 32 days, compared with more than 60 days for heterozygous controls. Similar results were observed with females. (B) Paraquat sensitivity of *parkin* mutants. Loss of *parkin* results in rapid mortality when flies are maintained on 2 mM paraquat-containing media. (C) Cold stress resistance test. *parkin* mutants exhibit poor survival at 4°C. Fifty flies per genotype per sex were analyzed for each assay. Similar results were obtained with $w; dpk^{P23}$ and $w; dpk^{P24}$ mutants.

cells (Fig. 7C). Furthermore, we did not observe any defects in DA cell body appearance. These findings are consistent with previously published results in flies and mice, and suggest that *parkin* is not essential for DA neuron survival at least during the 21 days post eclosion (Greene et al., 2003; Itier et al., 2003). In addition, *parkin* mutants do not have an abnormal electroretinogram (ERG) shortly after eclosion or at 2 weeks of age (data not shown). This suggests that photoreceptor neurons are also intact.

Discussion

Human *PARK2* is involved in several processes that may provide important insights concerning the pathogenesis of Parkinson disease. Parkin is an E3 ubiquitin ligase that is thought to target several proteins for degradation, including α -Synuclein and Pael receptor (Shimura et al., 2001; Yang et al., 2003).

Misexpression of α -Synuclein results in mitochondrial damage and increased levels of free radicals while misexpression of *Pael-R* results in cell death (Hsu et al., 2000). This suggests that human Parkin may play a role in protecting the cell from cytotoxic insults. In addition, Parkin interacts with cyclin E as part of the SCF^{hSel-10} complex (Staropoli et al., 2003). This may be important in PD as accumulation of cyclin E is associated with apoptotic death in postmitotic neurons that are exposed to the glutamatergic excitotoxin kainate. Glutamatergic toxicity has been previously implicated in the pathogenesis of PD (Olanow and Tatton, 1999). Overexpression of *PARK2* suppresses both cyclin E accumulation and excitotoxin-induced apoptosis of granular neurons. In addition, *PARK2* deficiency potentiates and *PARK2* overexpression protects against kainate-mediated toxicity in cultured dopaminergic neurons (Staropoli et al., 2003).

neurons in the substantia nigra of the midbrain (Cotran, 1999). Therefore, we were interested to determine if there was a corresponding age-dependent loss of dopaminergic neurons in *parkin* mutant animals. We specifically focused on the dorsomedial clusters (DMC) of the *Drosophila* brain dopaminergic system. DMC neurons were previously reported to be affected in transgenic *Drosophila* misexpressing α -Synuclein and *Pael* receptor. These clusters contain 14–18 tyrosine hydroxylase (TH)-positive cells that were shown to degenerate in an age-dependent manner in the previously reported transgenic models (Auluck and Bonini, 2002; Auluck et al., 2002; Feany and Bender, 2000; Yang et al., 2003). To assay DA neurons in these clusters, we used whole-mount brain staining and confocal microscopy (Fig. 7A,B). We analyzed 2- to 3-day-old and 21-day-old animals, and did not observe any significant difference in the number of TH-positive

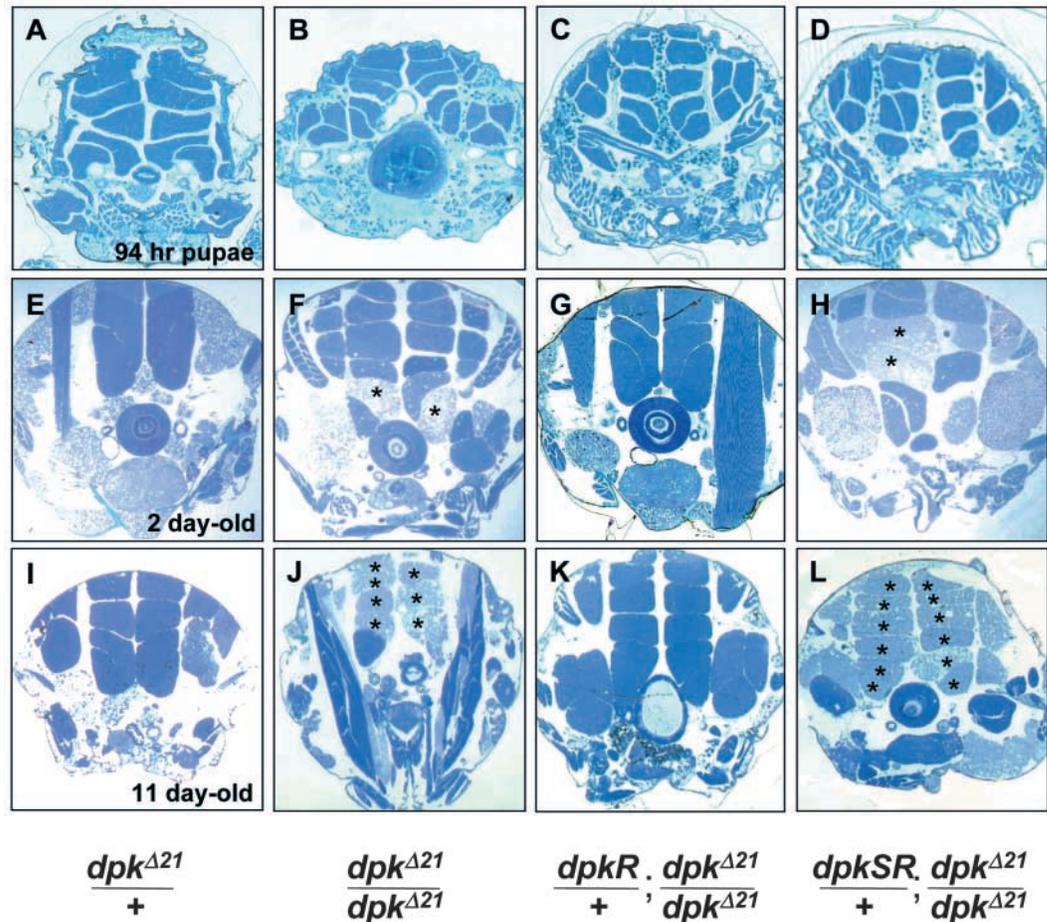


Fig. 5. Loss of *parkin* results in indirect flight muscle degeneration. Light microscopy was used to examine IFM architecture. (A-D) Transverse sections through resin-embedded thoraces at 94 hours of pupation and (E-H) at 2 days after eclosion stained with Toluidine Blue to visualize tissue morphology. (A,E) Heterozygous controls show normal IFM architecture. At 2 days post eclosion (F), but not at the late pupal stage (B), *parkin* mutants show loss of muscle tissue (asterisks). At 11 days (I-L) the muscle loss becomes much more pronounced (compare J with F and L with H). This phenotype is rescued by the *dpkR* (C,G,K) but not *dpkSR* (D,H,L).

Furthermore, overexpression of *PARK2* in tissue culture protects cells from mitochondrial swelling and cytochrome c release during apoptosis (Darios et al., 2003). Therefore, *PARK2* may be involved in the regulation of cell death. Furthermore, interaction of human Parkin with cyclin E suggests that it may also be involved in regulation of the cell cycle. Nevertheless, the molecular mechanisms of Parkin function and its precise role in PD are poorly understood. In this report, we describe a *Drosophila parkin* model of PD that will help our understanding of the molecular mechanisms of *parkin* function and may thereby provide insights into the pathogenesis of PD. To this end, we generated loss of function mutations in *Drosophila parkin*. Many of the phenotypes of *parkin* mutant animals parallel its inferred roles based on in vitro experiments outlined above. First, *parkin* mutant animals have reduced body size and cell size at eclosion, consistent with a possible role for *parkin* in regulating the cell cycle via interaction with cyclin E. Second, *parkin* mutants are highly sensitive to oxidative and environmental stress, suggesting possible defects in oxygen radical defense. Finally, *parkin* null animals show apoptotic flight muscle degeneration supporting other data that *parkin* may be involved in the regulation of cell death (Greene et al., 2003).

parkin mutant animals have reduced body and cell size at eclosion, suggesting possible defects in cell growth, proliferation and/or cell survival. This phenotype is particularly interesting given the interaction of human Parkin with cyclin

E, an important regulator of cell cycle progression (Staropoli et al., 2003). The reduced body size of *parkin* mutants is similar to the phenotypes of *insulin growth factor (IGF) receptor* mutant flies and mice (Bruning et al., 2000). Physiological effects of insulin in the brain are not limited to regulation of food intake and control of glucose uptake, but are also important in trophic actions on neurons and glial cells. Administration of the N-terminal tripeptide of IGF1 prevents loss of DA neurons after chemically (6-hydroxydopamine)-induced DA cell lesion in rats (Guan et al., 2000). In addition, IGF1 also protects against DA-induced neurotoxicity in vitro (Offen et al., 2001). This, together with our data, suggests *parkin* may play a role in the insulin signaling pathway during development or in adults.

Our results show that *parkin* mutant flies have increased sensitivity to paraquat toxicity. Paraquat, with its two N-methyl pyridinium moieties, is structurally similar to MPP(+), a toxic metabolite of the MPTP. In humans, toxins such as MPTP cause DA neuron-specific death in the substantia nigra because production of oxygen radicals, resulting in Parkinsonian symptoms. Glutathione and superoxide dismutase (SOD) inactivate H₂O₂ and superoxide radicals, respectively, thereby reducing MPTP neurotoxicity in mice (Przedborski et al., 2001). Paraquat also causes high oxygen radical production and interferes with mitochondrial respiration, resulting in cell death. The effects of paraquat are not specific to DA cells and sensitivity to its toxicity reflects a general impairment of

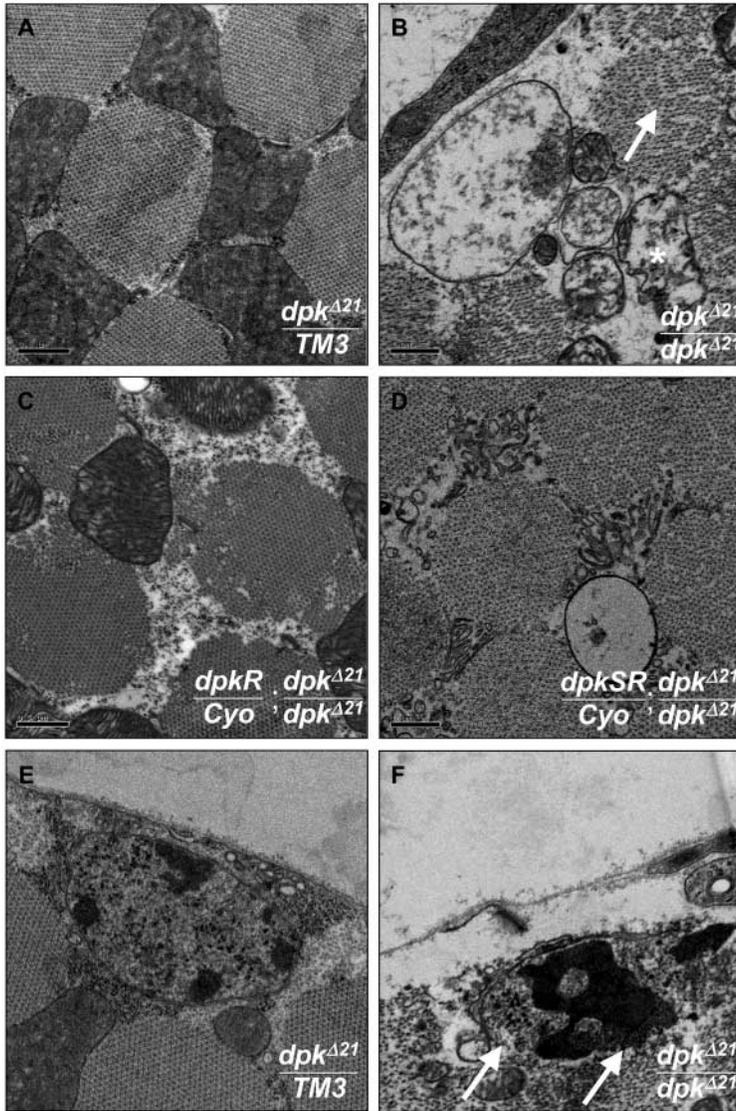


Fig. 6. TEM analysis of IFM ultrastructural morphology reveals muscle fiber degeneration, mitochondrial defects and apoptosis in *parkin* mutants 2 days after eclosion. (A) Control animals have well organized uniform muscle architecture and electron dense mitochondria. (B) *parkin* mutant muscle fibers are fragmented (arrow) and mitochondria show loss of cristae (asterisk). These phenotypes are rescued with *dpkR* (C), but not with *dpkSR* transgenes (D). (E) Control animals do not show any signs of muscle death; however, *parkin* mutant IFMs (F) show clear signs of apoptosis, including chromatin condensation and nuclear envelope breakdown (arrows).

radical defense (Kirby et al., 2002; Phillips et al., 1989). Our results suggest that antioxidant defenses in *parkin* mutant flies are also impaired and that *parkin* may play a role in the oxidative stress response. One likely mechanism of Parkin function is to help rid the cell of specific proteins that are misfolded because of oxygen radical damage. This is consistent with the identification of human Parkin as an E3 ligase (Imai et al., 2000; Shimura et al., 2000; Staropoli et al., 2003; Zhang et al., 2000).

parkin mutant flies show a progressive apoptotic degeneration of indirect flight muscles (IFMs). Furthermore, this degeneration is accompanied by mitochondrial disintegration and loss of cristae (Greene et al., 2003). *Drosophila* IFMs are groups of specialized muscle that are in a constant state of vibration. They require a high oxygen supply to sustain their respiratory activity, making this tissue especially susceptible to mitochondrial dysfunction. It is possible that the mitochondrial degeneration we observe in *parkin* mutants results in increased susceptibility to oxygen radical damage because of the impairment of antioxidant defenses by mitochondria, culminating in cell death. The data from animal and tissue culture models of PD outlined above suggest that mitochondrial dysfunction and oxygen radical damage are two crucial factors in the development of PD pathology. In addition, they also underscore the importance of the apoptotic pathway in

oxygen radical defense (Ding and Keller, 2001; Phillips et al., 1989). Loss of *Drosophila* SOD also results in sensitivity to low doses of paraquat, reflecting a similar defect in oxygen

DA cell loss. Oxidative stress such as that produced by MPTP can trigger apoptosis (Kruman et al., 1998). Transgenic mice overexpressing the anti-apoptotic gene *Bcl2* and mice null for the proapoptotic gene *Bax* are resistant to MPTP (Offen et al., 1998; Vila et al., 2001). Loss of *parkin* results in similar phenotypes in flies: increased sensitivity to oxygen radical stress and

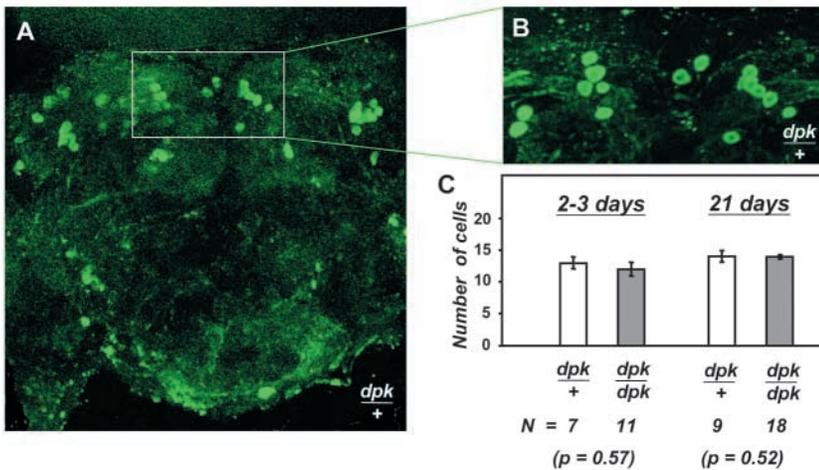


Fig. 7. *parkin* mutants do not show dopaminergic neuron loss on whole-mount analysis of dopaminergic dorsomedial cluster (DMC) neurons. (A) Example of a morphologically normal *Drosophila* brain stained with anti-tyrosine hydroxylase antibodies with a higher magnification of DMC cluster neurons shown in B. (C) Dopaminergic cell counts of *parkin* mutant animals (*w; dpk^{Δ21}*) at 2 and 21 days after eclosion do not show any reduction in numbers of tyrosine hydroxylase-positive cells in dorsomedial clusters compared with heterozygous controls (*w; dpk^{Δ21/+}*).

IFM apoptosis, suggesting that underlying mechanisms of cellular dysfunction maybe be similar between flies and humans.

Individuals with AR-JP exhibit a loss of DA neurons in the substantia nigra similar to that of idiopathic PD. Therefore, we expected to see a similar phenotype in *parkin* mutant animals. We focused on the dorsomedial cluster (DMC) neurons because they were shown to be affected in other *Drosophila* models of PD (Auluck et al., 2002; Feany and Bender, 2000). However, we do not observe any significant loss of DA neurons at three weeks of age in *parkin* mutant animals compared with controls. In addition, in contrast to a previously published report, we do not see any changes in DA cell morphology in *parkin* mutants (Greene et al., 2003). Loss of mouse *PARK2* also does not result in DA cell loss. However, increased extracellular DA concentration, abnormal neurophysiology and motor and cognitive behavioral deficits are observed in *PARK2* mutant mice (Goldberg et al., 2003; Itier et al., 2003). By contrast, only motor deficits have been observed thus far in *Drosophila* (Greene et al., 2003). In another model of PD, overexpression of α -synuclein in mice does not result in DA cell loss but does cause DA neuron dysfunction (Giasson et al., 2002; Masliah et al., 2000; van Der Putten et al., 2000). Similarly, in Alzheimer's disease mouse models employing overexpression of amyloid precursor protein, cognitive defects are present in the absence of cortical neuronal loss (Hock and Lamb, 2001). The absence of cell loss in multiple models of neurodegenerative disease may reflect the shorter life span of flies and mice compared with humans. Although the mean onset of PD symptoms in AR-JP is the earliest of all genetically defined forms of Parkinson's disease, it is not clear how to compare fly, mouse and human life spans in this regard (Gasser, 1998).

Even though we do not observe DA loss in *parkin* mutant animals, the mechanism of cell loss may be similar between humans and *Drosophila*. Neurons and muscle are two of the most energy-dependent tissues, and therefore have high numbers of mitochondria and are highly sensitive to mitochondrial insults. Although the mechanisms of cell death in IFMs and in DA neurons might be similar, it is possible that *Drosophila* IFMs are more sensitive than DA neurons to mitochondrial defects. The nature of this defect is still unclear; however, three mechanisms are likely. First, it is possible that *parkin* functions in a trophic factor pathway that promotes cell survival, and in its absence cells become more susceptible to insults such as oxygen radicals. Second, *parkin* might be important in the stress response pathway, and in its absence the cell becomes more susceptible to various stimuli such as oxygen radical damage that trigger apoptosis. Finally, it is possible that *parkin* is part of the cell death pathway, and its absence results in susceptibility to proapoptotic insults. *Drosophila parkin* mutants will serve as an invaluable model for understanding the biological role of *parkin* and may provide important clues concerning the molecular mechanisms of PD.

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