

Short Communication

Zinc supplement greatly improves the condition of *parkin* mutant *Drosophila*

Nidhi Saini and Walter Schaffner*

Institute of Molecular Life Sciences, University of Zürich,
Winterthurerstrasse 190, CH-8051 Zürich, Switzerland

*Corresponding author
e-mail: walter.schaffner@imls.uzh.ch

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder in which oxidative stress is implicated as a major causative factor. Mutations in the gene encoding Parkin, a ubiquitin ligase, are responsible for a familial form of PD. In a *Drosophila* disease model lacking Parkin (*park*²⁵ null mutant), we tested the effect of zinc supplementation. Zinc is an essential trace metal and a component of many enzymes and transcriptional regulators. Unlike copper and iron, zinc is not redox-active and under most conditions serves as an antioxidant. We find that the condition of *parkin* mutants raised on zinc-supplemented food is greatly improved. At zinc concentrations where controls begin to show adverse effects as a result of the metal supplement, *parkin* mutants perform best, as manifested in a higher frequency of reaching adulthood, extended lifespan and improved motoric abilities.

Keywords: antioxidant; metal homeostasis; metallothioneins; MTF-1; Parkinson's disease; zinc transporters.

Parkinson's disease (PD), characterized by the loss of dopaminergic (DA) neurons in the substantia nigra, is a progressive neurodegenerative disorder with the second highest incidence rate and is the most common age-related movement disorder (Olanow and Tatton, 1999; Dawson and Dawson, 2003; Greene et al., 2003). Both genetic and environmental factors contribute to its pathogenesis. Oxidative stress is considered to be a major factor in the pathogenesis of PD, as evidenced by an elevated content of redox-active iron and lipid peroxides in the diseased brain, impaired mitochondrial function, and alterations in the antioxidant defense mechanisms (Dexter et al., 1989; Jenner and Olanow, 1996; Greene et al., 2003; Pesah et al., 2004). Mutations in six genes, including *parkin* which encodes an E3 ubiquitin ligase, have been associated with rare, early-onset, familial forms of PD (West and Maidment, 2004; Gasser, 2005; Sang et al., 2007). Interestingly, some alleles of these genes might

be susceptibility factors for environmental toxins (Choi et al., 2000; Warner and Schapira, 2003; Bueler, 2009).

In our study, we used a *Drosophila melanogaster* line in which the ortholog of the human *parkin* gene is disrupted by transposition of a P-element (Greene et al., 2003; Pesah et al., 2004). *Parkin* mutant flies present with male and female sterility (Riparbelli and Callaini, 2007), mitochondrial and muscle abnormalities, locomotor defects, an inability to fly owing to degeneration of indirect flight muscles, increased sensitivity to multiple stresses, including oxidative stress, and a severely reduced lifespan (Palacino et al., 2004; Greene et al., 2005; Whitworth et al., 2005). Some of these defects arise because *parkin* mutants have dysfunctional mitochondria with disturbances in the electron transport chain. In mice, in contrast to its pivotal role in humans (Choi et al., 2000), *Parkin* function does not seem to be critical for the survival of DA neurons (Goldberg et al., 2003; Itier et al., 2003; Palacino et al., 2004). Similarly, *Drosophila* that are null mutants for *parkin* do not generally display DA neuron loss (Greene et al., 2003; Pesah et al., 2004), although a partial loss of DA neurons in the PPL1 cluster of the brain has been reported (Whitworth et al., 2005).

In the late 1980s, the antioxidant function of the redox-inert metal zinc was recognized and proposed to be mediated by the protection of protein sulfhydryl groups and/or by competing against redox-active metals (Bray and Bettger, 1990). Additionally, zinc ions can upregulate the expression of metallothioneins, which owing to their high cysteine content can serve as antioxidants. Zinc has an established antiapoptotic function that minimizes ROS-induced cellular oxidative damage (Suzuki et al., 1991). This also occurs in the central nervous system, particularly in the brain (Kocaturk et al., 1996).

More than 70 different enzymes involved in the metabolism of biomolecules require zinc as a cofactor (Parkin, 2004). Zinc is an integral part of the hundreds of transcription factors that contain zinc finger domains (Berg and Shi, 1996), and it plays a role in cellular signal transduction and in modulation of synaptic neurotransmission. Zinc is critical for the growth and regulation of cells and alterations in zinc metabolism have been implicated in causing neurological dysfunctions on the one hand, and on the other hand providing neuroprotection. Maintenance of intracellular zinc homeostasis is thus an essential requirement in all living organisms (Valko et al., 2005).

The best characterized zinc-activated transcription factor is the metal response element-binding transcription factor-1,

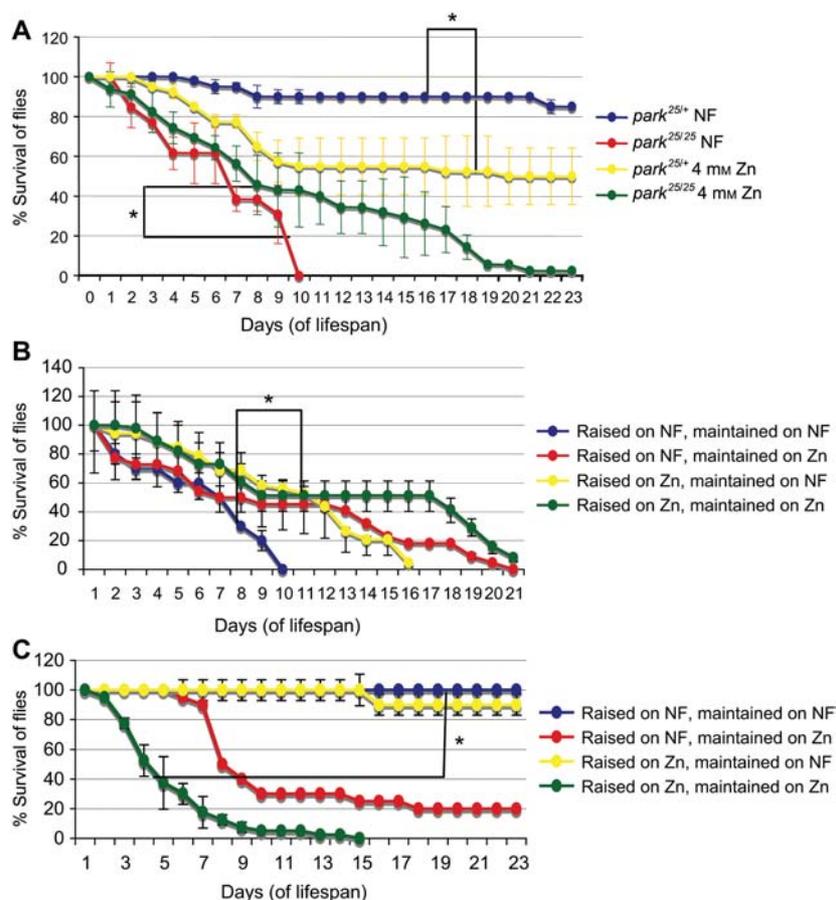


Figure 1 *Drosophila parkin* mutants show an enhanced lifespan on zinc-supplemented food.

(A) Zinc supplementation (4 mM $ZnCl_2$) increased the longevity of the *parkin* mutant flies (*park^{25/25}*). The maximum lifespan of 11 days on normal food (NF) increased to 23 days. Control flies (*park^{25/+}*) showed an opposite effect with a reduction of lifespan on zinc food. (B) Survival of *park^{25/25}* flies and (C) of *park^{25/+}* control flies raised and maintained on NF or zinc-supplemented food. Error bars indicate standard deviation. The significance between survival curves was analyzed using the Kaplan-Meier log-rank statistical test (* $p < 0.01$).

Methods: for survival assays, NF was supplemented with zinc chloride to a final concentration of 4 mM. One- to two-day-old flies (20 per vial) were maintained at 25°C on a 12:12 h light/dark cycle for each genotype in triplicate vials. Surviving flies were transferred to fresh food vials every 2 days and counted daily. As a control, flies of the same genotype were grown on food without metal supplement. In each lifespan assay testing different conditions, the controls of *park^{25/+}* and *park^{25/25}* flies raised on NF were the same. The variations in the median lifespan of control flies in different experiments can be attributed to subtle experimental variations.

also referred to as metal-responsive transcription factor-1 (MTF-1). In response to zinc, MTF-1 translocates from the cytoplasm to the nucleus where it regulates the expression of several genes, notably metallothioneins and some zinc

transporters (Andrews, 2000; Smirnova et al., 2000; Lichtlen and Schaffner, 2001; Saydam et al., 2001). MTF-1 requires elevated zinc concentration for DNA binding. This property was exploited for the activation of MTF-1-dependent pro-

Table 1 Zinc increases the frequency of *parkin* mutant *Drosophila* reaching adulthood.

Food condition	Genotype	<i>park^{25/25}</i> /total progeny	% of <i>park^{25/25}</i>
Normal food	Set 1 <i>park^{25/25}</i>	31/1116	2.8%
	Set 2 <i>park^{25/25}</i>	10/450	2.2%
4 mM Zn	Set 1 <i>park^{25/25}</i>	110/611	18%
	Set 2 <i>park^{25/25}</i>	186/956	19%

For the analysis of eclosure frequency, egg laying was allowed for 6 days and the parent population (10 males and 10 females each of *park^{25/+}*) was the same in all vials of normal food (NF) or zinc (Zn), and progeny flies were counted at the same time.

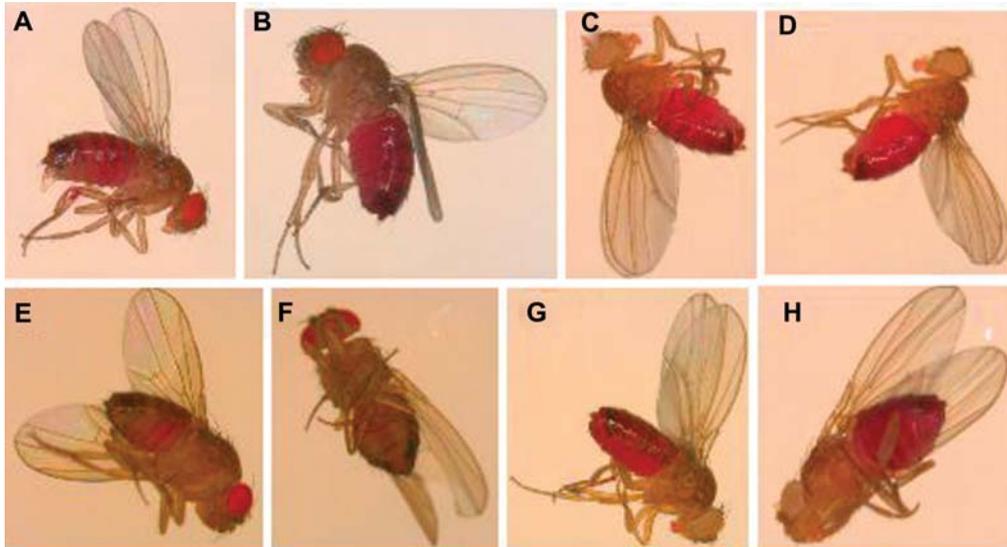


Figure 2 Gustatory assay with adult flies on normal or zinc-supplemented food with acid red dye as a marker.

(A, B) *park*^{25/4+} control flies and (C, D) *park*^{25/25} flies show the same feeding behavior on normal food, exemplified by the fully red abdomens. A food supplement of 4 mM Zn (E, F) altered feeding behavior of *park*^{25/4+} (only partially red abdomens) but no change in the behavior of *park*^{25/25} flies was observed (G, H) (fully red abdomens). The mutants are recognized by white eyes because of the loss of *w*⁺ (red eyes) which are present in control flies.

Methods: the adult gustatory assay was essentially carried out as described by A. Hilliker and colleagues (Bahadorani, 2008). Briefly, newly eclosed flies were reared on normal food for 2–3 days, then starved for 18 h on Whatman paper soaked with distilled water. After this treatment, starved flies (20 per vial) were transferred onto zinc-supplemented food with 0.2% sulforhodamine B sodium salt (acid red) for 2 h. For control flies, culture medium was supplemented with 0.2% acid red, without metal supplement. After 2 h of feeding at optimum temperature (25°C) and relative humidity, flies were anesthetized and the degree of abdomen redness was visually inspected. Abdomen redness was used as an indicator of the amount of food taken up.

motors by zinc and other metals in a cell-free transcription system (Zhang et al., 2003).

Zinc is not redox-active, but nevertheless toxic when in excess (Beyersmann and Haase, 2001). Acute zinc toxicity is rare but has been reported (Duncan et al., 1992; Whittaker, 1998; Prasad et al., 1999). If the extracellular concentration of zinc exceeds the capacity of zinc homeostasis mechanisms, it becomes cytotoxic and an excess of free intracellular zinc can trigger apoptosis (Choi et al., 1988; Duncan et al., 1992; Kim et al., 1999; Beyersmann and Haase, 2001; Wilhelm et al., 2001; Beyersmann, 2002; Walther et al., 2003). Zinc transport in *Drosophila*, as in vertebrates, is mediated by two families of solute linked carrier proteins: zinc importers (ZIPs), which function in the uptake of zinc to the cytoplasm, and zinc exporters (ZnTs), which reduce cytoplasmic zinc concentrations by promoting zinc efflux (Liuzzi and Cousins, 2004; Yepiskoposyan et al., 2006). More than 10 zinc transporter genes are annotated in *Drosophila melanogaster* based on their sequence similarities to vertebrate zinc transporters. The ZIP family gene *foi* (*fear of intimacy*) was characterized in *Drosophila* and shown to be a zinc importer that is critical during development (Moore et al., 1998; Mathews et al., 2005). Transcriptional responses to zinc in *Drosophila* larvae were analyzed in our laboratory (Yepiskoposyan et al., 2006). Apart from the expected upregulation of metallothioneins and the zinc exporters *ZnT35C*

and *ZnT63C*, there was also an induction of neurotransmitters, detoxification enzymes (such as glutathione S-transferase), ferritin and chaperone encoding genes.

We found that whereas *parkin* mutant flies readily feed on high-zinc food, their wild type counterparts avoid zinc-loaded food. The mutants also had an increased survival rate on zinc-supplemented food, which prompted us to investigate their response to zinc in more detail.

With standard ‘normal food’ (NF), *parkin* mutant flies have a median lifespan of 6 days with a maximum of 11 days (Figure 1A). Zinc supplementation in the form of zinc chloride increased the lifespan of *parkin* mutants. When maintained on supplements of 4 mM Zn, the mutant flies survived up to 23 days with a median lifespan of 8 days (Figure 1). This increase was as a result of zinc and not chloride ions, as similar survival assays on NaCl-supplemented medium did not extend lifespan (data not shown). Zinc supplementation also increases the eclosion frequency of *parkin* mutants, from 2.5% to 19%, i.e., close to 25% which is the expected frequency of *parkin* mutants in the progeny of *parkin* heterozygous parents (Table 1). In contrast, heterozygous control flies did not draw any benefit from zinc-supplemented food: if kept on food with 4 mM ZnCl₂, the median lifespan reduced significantly to 17 days, whereas on NF, 80% were still alive at the end of the experiment (23 days) (Figure 1A). Similar adverse effects of zinc load were

observed with wild type *yw* and *OregonR* flies (data not shown). To determine if zinc has a stronger effect during development from eggs to adults or during the adult feeding stage, we followed the lifespan of both *parkin* mutant and *parkin* heterozygous flies under four conditions: (i) development and adult maintenance in zinc supplement, (ii) raising the flies on NF until adult stage and then maintaining them on zinc-supplemented food post-eclosion, (iii) raising the flies on zinc food but maintaining them on NF after eclosion, and (iv) raising and maintaining the flies on NF. We observed that the strongest positive effect of zinc on *park*^{25/25} flies was when they were both raised and maintained on 4 mM Zn-supplemented food (Figure 1B). The strongest negative effect of zinc on *park*^{25/+} control flies was also observed under these conditions (Figure 1C). Development of *park*^{25/+} and *OregonR* wild type flies was delayed but generally less affected by zinc than the survival of adults; egg transfer (200 each) from NF to vials containing increasing concentrations of zinc resulted in an equal percentage of eclosing adults in NF, 4 and 6 mM Zn but none developed in 10 mM Zn (data not shown; see also Egli et al., 2003).

parkin mutants might sense a deficiency in and/or a rescuing effect of zinc, and in response to this eat normal amounts of zinc-supplemented food unlike heterozygous or wild type flies. This was indicated by a visual inspection of adult *Drosophila* in a gustatory test and was quantitatively confirmed by the measurement of total zinc uptake by flies (Figures 2 and 3). On NF, the zinc content in heads of *parkin* mutant flies was much lower than in controls, consistent with a zinc deficiency in the mutants. Zinc supplementation indeed resulted in an, albeit minor, increase of zinc content in mutant heads (Figure 3A), whereas in thoraces the zinc content was similar in mutant and control flies (Figure 3B). Interestingly, the abdomens of *park*^{25/25} flies fed on zinc food showed a 10-fold increase in zinc levels compared with flies fed on NF (Figure 3C). Such an effect was, however, not observed in the control flies which only showed a minor increase in zinc levels upon zinc feeding, which is in line with their tendency to avoid zinc-loaded food.

We also determined the transcript levels of *parkin*, MTF-1 and some other genes involved in zinc import/export (Figure 4). As expected, *parkin* mutants have no detectable *parkin* transcripts. In comparison to control flies, transcript levels of the metallothionein *MtnB* are higher in *parkin* mutants (Figure 4A,B). Zinc supplementation induced *MtnB* transcripts 24-fold in *parkin* mutants; in control flies the increase was 14-fold (Figure 4B). Boosting expression of metallothioneins, which act as ROS scavengers, is one way zinc could play an antioxidant role. The elevated metallothionein levels could also explain at least in part why an increased zinc concentration is not toxic to the *parkin* mutants. We also determined the transcript levels of three zinc transporters, the exporters *ZnT35C* and *ZnT63C* and the importer *foi* (Mathews et al., 2005; Yepiskoposyan et al., 2006) both in *parkin* mutants and in the heterozygous controls. The most conspicuous difference was observed with the exporter gene *ZnT35C*, a known target of MTF-1. In *parkin* mutants, expression was dramatically reduced com-

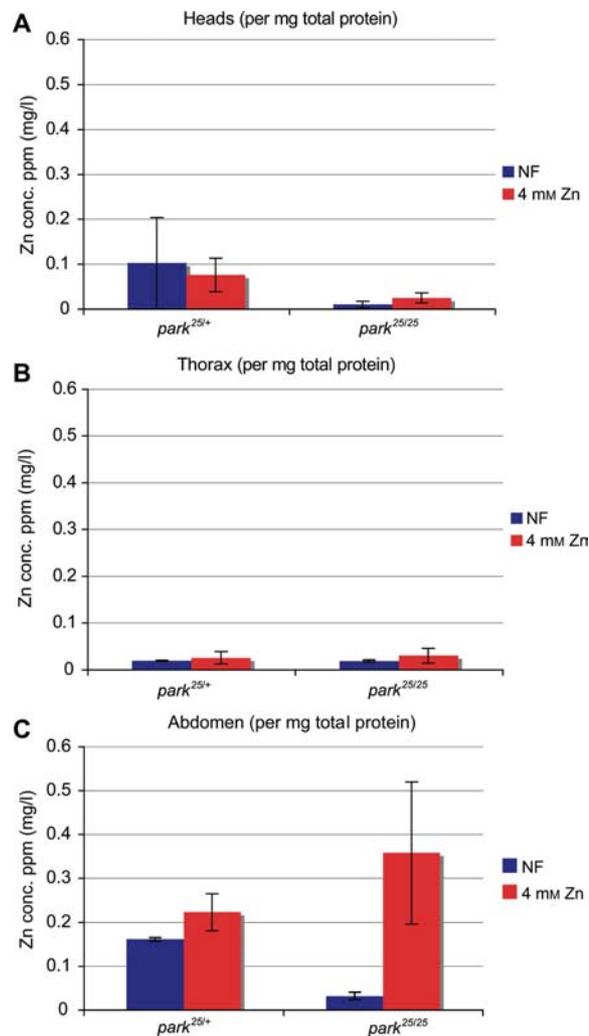
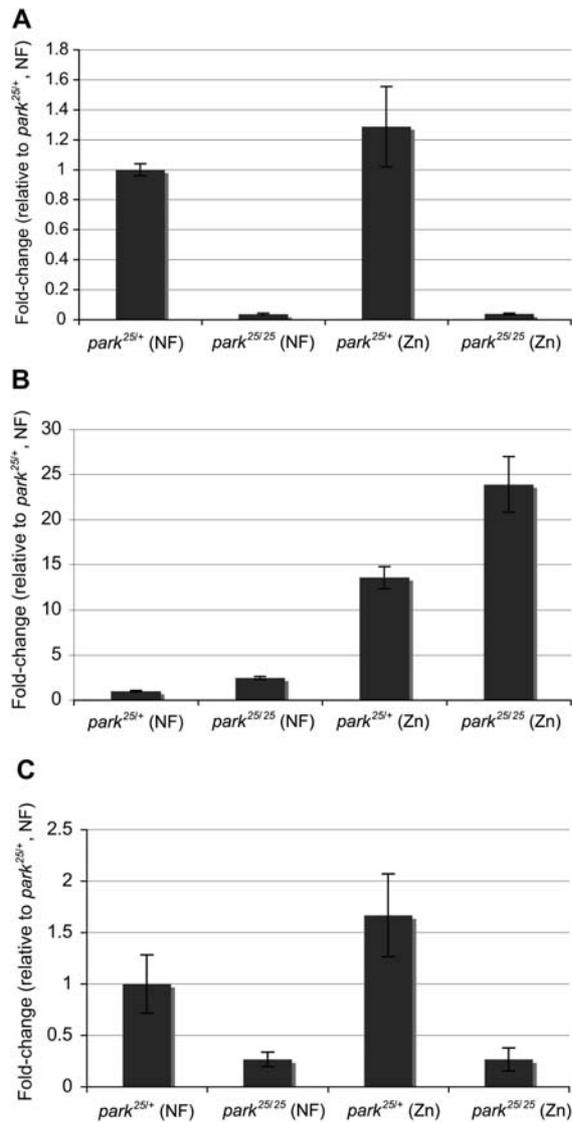


Figure 3 Quantification of Zn content in the different body parts of *park*^{25/+} control flies and *park*^{25/25} flies.

Flies were raised on normal food (NF) or zinc-supplemented food (4 mM Zn). (A) Heads, (B) thoraces, (C) abdomens.

Methods: female flies were allowed to lay eggs on zinc-supplemented (4 mM ZnCl₂) fly food for 6 days and removed afterwards. The resulting progeny was collected at regular intervals and the percentage of eclosing *parkin* homozygous mutants and heterozygous controls were calculated each time. These progeny were then frozen and stored until the required number of 200 flies was obtained in triplicate for each genotype. Frozen flies were then dissected to separate their heads, thoraces and abdomens which were collected separately. Each sample set was then subjected to homogenization using cold protein homogenization buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM MgCl₂, 0.5 mM PMSF protease inhibitor at pH 7.4) and the samples were normalized for protein content. A highly sensitivity flame atomic absorption spectrophotometer (FAAS; GTA-120/PSD-120, Varian Australia Pty Ltd, Mulgrave, VIC, Australia) was used to detect the zinc content in each body part of each genotype assessed.

pared to controls and not responsive to zinc. In heterozygous controls, zinc supplement resulted in a 1.5-fold upregulation, which indicates a response to zinc overload (Figure 4C). An increased expression of zinc exporters in control flies is in



agreement with their avoidance of zinc-supplemented food. Transcripts of *ZnT63C* and, somewhat unexpectedly of *foi*, were also lower in *parkin* mutants compared to *parkin* heterozygous controls (data not shown). Taken together, these data suggest that zinc homeostasis is distorted in *parkin* mutants, with the effect that zinc supplement in food produces a strong phenotypic rescue effect. It remains to be seen whether zinc supplementation also has a positive effect in a mouse model of familial PD and ultimately in Parkinson's patients.

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Figure 4 Real-time analysis of *parkin* transcript levels. (A) In *park*^{25/25} flies no *parkin* transcripts are detectable. In *park*^{25+/+} *parkin* transcripts are, if anything, elevated in flies raised on zinc food. (B) *park*^{25/25} flies have increased levels of metallothionein *MtnB* transcripts in NF compared to control flies. Zinc induces *MtnB* in both genotypes, but to a higher level in *park*^{25/25}. (C) The zinc exporter, *ZnT35C*, shows increased transcript levels in *park*^{25+/+} on zinc food, which is not observed in *park*^{25/25} flies.

Methods: total RNA was purified from the adult *Drosophila* tissue using the nucleospin RNA II protocol and eluted in 60 μ l RNase-free water. cDNA was prepared using the transcriptor high fidelity cDNA synthesis kit from Roche (Mannheim, Germany). The cDNA obtained was further purified using the AM 1906 DNA-free kit (Ambion, Rotkreuz, Switzerland). This was then used for analysis by real-time PCR on the Tecan Genesis 200/8 robot (Tecan, Männedorf, Switzerland) using the Mesa Green qPCR Mastermix Plus (Eurogentec, Seraing, Belgium) for SYBR assays. The qPCR run was performed on an Applied Biosystems machine (ABI Prism SDS 7900 HAT; Seraing, Belgium) in a 384-well format with a reaction volume of 10 μ l. All fold-change values are normalized to corresponding *park*^{25+/+} values on normal food (NF). Housekeeping genes used for reference were *actin5C*, *sec24* and *GAPDH*. The primer sequences used for the transcripts quantified were: for *parkin*, 5'-AAG ATC ATA TTT GCC GGT AAG GAA-3' and 5'-CGC TTT GCT GAC CCA AGT C-3' amplify a 73-bp fragment only from the *parkin* heterozygous control flies; for *MtnB*, 5'-TTG CAA GGG TTG TGG AAC AAA-3' and 5'-TGC AGG CGC AGT TGT CC-3' amplify a 65-bp fragment, and for *ZnT35C*, 5'-GCT CTC GCC GAT CTG AAA GT-3' and 5'-CGC ACC GAC AAG TGT TTC TTA TA-3' amplify a 75-bp fragment.

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