Aconitase Causes Iron Toxicity in *Drosophila pink1* Mutants

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**Abstract**

The PTEN-induced kinase 1 (PINK1) is a mitochondrial kinase, and *pink1* mutations cause early onset Parkinson’s disease (PD) in humans. Loss of *pink1* in *Drosophila* leads to defects in mitochondrial function, and genetic data suggest that another PD-related gene product, Parkin, acts with *pink1* to regulate the clearance of dysfunctional mitochondria (mitophagy). Consequently, *pink1* mutants show an accumulation of morphologically abnormal mitochondria, but it is unclear if other factors are involved in *pink1* function in vivo and contribute to the mitochondrial morphological defects seen in specific cell types in *pink1* mutants. To explore the molecular mechanisms of *pink1* function, we performed a genetic modifier screen in *Drosophila* and identified aconitase (*acon*) as a dominant suppressor of *pink1*. Acon localizes to mitochondria and harbors a labile iron-sulfur [4Fe-4S] cluster that can scavenge superoxide to release hydrogen peroxide and iron that combine to produce hydroxyl radicals. Using Acon enzymatic mutants, and expression of mitoferritin that scavenges free iron, we show that [4Fe-4S] cluster inactivation, as a result of increased superoxide in *pink1* mutants, results in oxidative stress and mitochondrial swelling. We show that [4Fe-4S] inactivation acts downstream of *pink1* in a pathway that affects mitochondrial morphology, but acts independently of *parkin*. Thus our data indicate that superoxide-dependent [4Fe-4S] inactivation defines a potential pathogenic cascade that acts independent of mitophagy and links iron toxicity to mitochondrial failure in a PD–relevant model.

**Citation:** Esposito G, Vos M, Vilain S, Swerts J, De Sousa Valadas J, et al. (2013) Aconitase Causes Iron Toxicity in *Drosophila pink1* Mutants. PLoS Genet 9(4): e1003478. doi:10.1371/journal.pgen.1003478

**Editor:** Bingwei Lu, Stanford University School of Medicine, United States of America

**Received** August 23, 2012; **Accepted** March 12, 2013; **Published** April 25, 2013

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**Funding:** This work was supported by a Marie Curie Excellence Grant (MEXT-CT-2006-042267 [http://cordis.europa.eu/mariecurie-actions/ext/home.html]), the ERC Starting Grant (260678 [http://erc.europa.eu/starting-grants]), the Research Foundation Flanders (FWO grants G003913, G079013, G095511, and G074709 [http://www.fwo.be/]), the Methusalem grant of the Flemish Government ([http://www.belgium.be/en/about_belgium/government/communitys/flemish_community]), the Francqui Foundation ([http://www.francquifoundation.be/]), the Hercules Foundation (AKUL/09/037 [http://www.herculesstichting.be/en_English/index.php]), the Interuniversity Attraction Pole program by BELSPO (IAP P7/16 [http://www.belspo.be/iap/index_en.htm]), the research fund KU Leuven (OT Start, G003/10/7, [http://www.kuleuven.be/research/funding/bol/]), and VIB ([http://www.vib.be]). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

Parkinson’s disease (PD) is the most frequent neurodegenerative movement disorder, but the pathways that explain disease pathology remain poorly understood [1,2]. While the most recognized pathological feature of PD is the preferential loss of dopaminergic (DA) neurons, one of the earliest observations in post mortem PD brains was the accumulation of iron in the substantia nigra (SN) [3,4]. Iron-mediated toxicity may thus contribute to DA neuron dysfunction but the mechanism has not been established.

Mitochondrial dysfunction is thought to be an important aspect of PD progression. Mitochondrial toxins have been linked to sporadic forms of the disease and mitochondrial defects have been described in many cell types, also in SN of Parkinson’s disease patients [5,6]. Likewise some of the genetic factors linked to the disease also point to a role for mitochondria. PD-associated mutations in *pink1* and *parkin*, both affect mitochondrial function in genetic model organisms and in mammalian cells [7,8], but how mitochondrial dysfunction and iron toxicity are linked remains elusive.

Pink1 and Parkin have been implicated in the clearance of dysfunctional mitochondria, a process dubbed mitophagy. In support, loss of *parkin* or *pink1* in different cell types in flies, results in the accumulation of swollen and clumped mitochondria [9,10], believed to be the result of defective mitophagy [11]. Furthermore, expression of factors that promote mitochondrial fission and, as a consequence, also indirectly promote mitophagy (gain of *drp1* or loss of *opa1* or *mfn*) partially rescue defects seen in *pink1* and *parkin* mutants [12–14]. Further studies indicate that mitochondrial depolarization triggers the recruitment Parkin to mitochondria in a Pink1-dependent manner, facilitating mitophagy [15]. In line with this idea, over expression of Parkin in *pink1* mutants, alleviates *pink1*-associated defects [9–16]. Hence, Pink1 acts with Parkin to regulate mitophagy.

In parallel, *pink1* may also harbor supplementary roles. Expression of Parkin or Drp1, or loss of *opa1* or *mfn* only partially rescue *pink1*-associated defects, suggesting additional pathways are contributing to the phenotype. Furthermore, loss of *pink1* function causes defects in the electron transport chain in fly and mouse cells [17,18] that are not [19] or only partially [20] rescued by...
Author Summary

In this work we provide mechanistic insight linking together two of the earliest observations in Parkinson’s disease: the excessive build-up of iron in diseased substantia nigra neurons and mitochondrial dysfunction particularly increased reactive oxygen species production at the level of Complex I. We identify aconitase mutants as strong genetic suppressors of Parkinson-related pink1 mutant phenotypes, both at the organismal and at the cellular/mitochondrial level. We show that the mitochondrial dysfunction in pink1 mutants that includes Complex I dysfunction results in superoxide-dependent inactivation of the Aconitase iron-sulfur cluster, leading to the release of iron and peroxide that combine to produce hydroxyl radicals and mitochondrial failure. Consequently, scavenging free iron using expression of mitoferritin or decreasing the levels of aconitase both rescue pink1 mutants; while increased wild-type Aconitase, but not a mutant that does not harbor an iron-sulfur cluster, results in severe mitochondrial defects. Given that reduced electron transport chain activity, increased oxidative stress, and natural iron build-up in the substantia nigra are common factors in sporadic and familial forms of Parkinson’s disease, we believe that oxidative inactivation of Aconitase may represent an important pathogenic cascade underlying neuronal dysfunction in Parkinson’s disease.

expression of Drp1. Finally, bypassing Complex I dysfunction, by expressing a yeast Complex I equivalent protein Ndi1 partially rescues the defects in pink1 mutants, but not those seen in parkin mutants [19]. Hence, Pink1 may play multiple roles in mitochondria, but the relative contribution of these different pathways to the pink1-dependent phenotypes, including the accumulation of swollen, clumped mitochondria remains to be determined.

In an unbiased genetic screen for heterozygous suppressors of Drosophila pink1 [21] we identified mitochondrial aconitase (acon) that encodes an enzyme catalyzing the first step of the Krebs Cycle [22]. Acon harbors an iron-sulfur [4Fe-4S] cluster [23] and we show that oxidative inactivation of this cluster in pink1 mutants is a major cause of iron toxicity that contributes to mitochondrial swelling and clumping in pink1 mutants. Our data are most consistent with acon acting downstream of pink1 and affecting mitochondrial morphology independently of parkin-mediated mitophagy. Thus oxidative inactivation of Aconitase is a source of iron toxicity that leads to mitochondrial defects in pink1 mutants and we propose a model where different pathways controlled by Pink1, including mitophagy and the maintenance of ETC activity can contribute to mitochondrial failure in specific cell types.

Results

Aconitase downregulation suppresses pink1 mutant phenotypes

Pink1 mutants show a severe defect to fly caused by mitochondrial dysfunction [19,21]. To identify genetic modifiers of pink1, we have tested a collection of 193 chemically induced (EMS) recessive lethal mutants that have been pre-selected for defects in mitochondrial function and neuronal communication [24–26], for their ability to modify the pink1 null mutant flight defect. At the 1% significance level we isolated 5 suppressors (p<0.01) [21] and to reveal mechanisms by which the modifiers affect Pink1, we mapped one of these recessive lethal suppressors to aconitase (acon) and named it acon1. This mutant fails to complement a deletion that uncovers acon as well as a lethal transposon insertion in acon that we named acon2 (Figure S1A). Sequence analysis of acon1 reveals a nonsense mutation in exon 2 (Figure S1A). In addition, semi-quantitative RT-PCR and Western blot analysis indicates severely reduced mRNA and protein levels in animals that are homozygous for either acon allele (Figure S1C and S1D), indicating that both are loss of function alleles. Moreover we can rescue the lethality and phenotypes associated with acon1/acon2 using a 20 kb genomic fragment encompassing the acon locus, yielding normal adult flies that do not show obvious behavioral abnormalities (Figure S1A, S1B). Likewise ubiquitous expression of acon CDN4 is also able to rescue acon1/acon2-associated lethality (Figure S1B). Thus, one of the suppressors of pink1 harbors a lethal lesion in acon and the lethality in the mutants is solely due to disruption of acon.

Heterozygosity of acon significantly suppresses the flight defect associated with pink1B90 mutants (Figure 1A, 1B). The extent of rescue we obtained by removing acon, is similar to previously reported conditions that suppress pink1 flight defects, including adding a copy of dep1 (dep1+) that facilitates mitochondrial fission, removing a copy of opa1 (opa185), reducing mitochondrial fusion (Figure S2A, S2B), expression of Parkin, expression of yeast NDI1 that bypasses Complex I of the electron transport chain (ETC), or feeding pink1 mutants ubiquinone or menaquinone that boost ETC function [9,10,13,19,21]. To test if the rescue that we observe is solely due to partial loss of acon (and not due to second site interactors on the chromosome), we determined flight but also ATP levels of pink1 mutants with one copy of a mutant acon allele. While heterozygous acon+ and acon− mutants alone do not show defects (Figure 1C), we find that one copy of either acon+ or acon− significantly rescue the reduced ATP levels in pink1 mutants (Figure 1D). This effect in pink1 mutants is specific to loss of acon as introduction of a genomic copy encompassing wild type acon in pink1B90/acon−/+ flies completely reverses both the flight and ATP level phenotypes to pink1B90 mutant levels (Figure 1B and 1D).

Thus, pink1 mutant phenotypes are specifically rescued by partial loss of acon expression.

To further quantify the effect of acon on pink1 mutant phenotypes we also analyzed mitochondrial morphology in adult indirect flight muscles using transmission electron microscopy. As previously described [9,10], the flight muscles of pink1 mutants exhibit swollen mitochondria with disorganized and fragmented cristae when compared to flight muscles from control flies or when compared to heterozygous acon mutants that do not show mitochondrial morphological defects (Figure 1E). Partial loss of acon in pink1 mutant results in a substantial rescue of the mitochondrial morphological defects in flight muscles, displaying substantially more intact cristae and less swollen mitochondria compared to pink1 mutants (Figure 1E). Hence, also at the ultrastructural level, partial loss of acon significantly alleviates mitochondrial morphological defects in pink1 mutant muscles.

Pink1 mutants also show swollen and clumped mitochondria in dopaminergic neurons in the adult brain [9,10]. To test if loss of acon can also rescue this defect, we expressed mitoGFP in pink1 mutant flies and in pink1 mutant animals heterozygous for acon. In line with the electron microscopy data of muscles, mitochondria in muscles labeled by mitoGFP (expressed using the ubiquitous da-GAL4) are spherical and aggregated in pink1 mutants and this defect is significantly rescued by partial loss of acon (Figure 1F and 1G). Next, we expressed mitoGFP in dopaminergic neurons using plet-Gal4 (also called TH-Gal4); While mitochondria are organized in a tubular network in wild type dopaminergic neurons, pink1 mutant mitochondria appear mostly as fragmented spherical aggregates in all dopaminergic neuron clusters analyzed (Figure
Aconitase Causes Iron Toxicity in pink1 Mutants

A

B

C

D

E

Thorax

control  pink1
pink1;acon
pink1;acon

F

Thorax

control  pink1
pink1

G

Average aggregates size (μm²)

H

Dopaminergic Neurons

control  acon
acon
acon
acon

I

Average aggregates size (μm²)
Figure 1. Partial loss of Acon suppresses pink<sup>1b9</sup> phenotypes. (A–D) Flight ability of 5-day-old adult flies (A, B) and ATP content in the head-thorax of 5-day-old flies (C, D). Data collected from at least 5 independent experiments. * Significantly different from pink<sup>1b9</sup>, One-way ANOVA, post hoc Dunnett p < 0.01, ns: not significantly different. (E) TEM analysis of thorax. Black arrows indicate swollen mitochondria. Scale bar: 5000 5 μm; 2000 2 μm. (F, H) GFP-labeled mitochondria in flight muscles (F; daGal4 UAS-mitoGFP) and in dopaminergic (H; pletGal4 UAS-mitoGFP) neurons that are double-labeled with anti-tyrosine hydroxylase (magenta). White arrows indicate mitochondrial aggregates. Scale bar: muscle 10 μm; DA neurons 2.5 μm. (G, I) Quantification of average mitochondrial aggregate size. 5 images from n=6 thoraces and about 5 neurons per brain from n=10 brains were analyzed. * Significantly different from pink<sup>1b9</sup>, One-way ANOVA, post hoc Dunnett p < 0.01, ns not significantly different. In all panel “control” is pink<sup>1b9</sup>, a precise P element excision; “genomic” indicates the insertion of a construct on the third chromosome that encompasses the wild type acon gene, data are shown as Mean ± SEM. doi:10.1371/journal.pgen.1003478.g001

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S1H) [9,10]. We quantified size and number of mitochondrial aggregates in the PPM3 cluster (Figure S1H and Methods). While heterozygous acon<sup>1</sup> and acon<sup>2</sup> mutants do not show defects compared to controls (Figure 1H, 1I), we find that both one copy of either acon<sup>1</sup> or acon<sup>2</sup> significantly rescue the increased size and number of mitochondrial aggregates in pink<sup>1</sup> mutants (Figure 1H, 1I and Figure S2A). This rescue in pink<sup>1</sup> mutants is specific to the partial loss of acon as introduction of a genomic copy encompassing wild type acon in pink<sup>1b9</sup>; acon<sup>1+/</sup> flies reverses these phenotypes back to pink<sup>1b9</sup> mutant levels (Figure 1F–1I). Furthermore, we confirm that protein levels are reduced by about 50% in pink<sup>1b9</sup>; acon<sup>1+/-</sup> compared to pink<sup>1b9</sup> mutants and are restored in flies expressing a genomic copy of wild type acon (Figure S1E). Thus, together our data indicate that morphological defects of mitochondria in pink<sup>1</sup> mutants are significantly rescued by partial loss of acon expression and the mitochondrial morphological defects in pink<sup>1</sup> mutants are dependent on acon expression.

Oxidative inactivation of [4Fe-4S] clusters results in increased H<sub>2</sub>O<sub>2</sub> and Fe<sup>2+</sup> levels in pink<sup>1</sup> mutants

acon is predicted to encode mitochondrial Aconitase (Acon), an iron sulfur cluster containing protein, that catalyzes the formation of isocitrate in the first step of the Krebs cycle [22]. To assess whether Acon localizes to mitochondria we fractionated fly tissue in cytoplasmic and mitochondrially enriched fraction and performed Western blotting using anti-Acon antibodies. Acon is enriched in the mitochondrial fraction (Figure S1G).

Acon harbors a single unligated iron atom in its [4Fe-4S]<sup>2+</sup> cluster, and the enzyme is in this respect unique in mitochondria. Such an unligated iron atom is particularly sensitive to superoxide (O<sub>2</sub><sup>-</sup>)-dependent oxidation [27–29] that results in cluster instability. Oxidation is followed by the release of Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> that may contribute to oxidative damage and mitochondrial morphological defects through the formation of the potent hydroxyl radical (OH) by the Fenton reaction [30]. Thus, the specific configuration of the Acon [4Fe-4S]<sup>2+</sup> cluster in combination with its proximity to mitochondrialy generated superoxide place Acon as a major mediator of oxidative stress in mitochondria. We therefore wondered if O<sub>2</sub><sup>-</sup> leaking from defective pink<sup>1</sup> mutant mitochondria could be a source of Acon inactivation resulting in morphological defects. To test if also in fruit flies the loss of pink<sup>1</sup> function results in increased O<sub>2</sub><sup>-</sup> production, we incubated mitochondrial preparations from pink<sup>1</sup> mutant flies and controls with Complex I substrates (pyruvate/malate) and used the fluorescent probe dihydroethidium (DHE) to measure O<sub>2</sub><sup>-</sup> production [31,32]. Similar to wild type mitochondria in the presence of AntimycinA, known to induce O<sub>2</sub><sup>-</sup> production (Figure 2A), pink<sup>1b9</sup> mitochondria show a significant increase in DHE fluorescence compared to controls (Figure 2A). These data indicate that pink<sup>1</sup> loss leads to increased O<sub>2</sub><sup>-</sup> production.

If the increased O<sub>2</sub><sup>-</sup> in pink<sup>1</sup> mutants can act via the Acon [4Fe-4S] cluster to cause mitochondrial swelling, we expect (1) that partial loss of acon does not rescue the increased O<sub>2</sub><sup>-</sup> production in pink<sup>1</sup> mutants; (2) that Acon enzymatic activity normalized to total Acon protein is reduced in pink<sup>1</sup> mutants; (3) that H<sub>2</sub>O<sub>2</sub> and Fe<sup>2+</sup> levels are increased in pink<sup>1</sup> mutants as a result of Acon inactivation, and (4) that this defect is rescued by partial loss of acon. First we assessed O<sub>2</sub><sup>-</sup> in pink<sup>1</sup> mutants heterozygous for acon<sup>1</sup> or acon<sup>2</sup> that we showed rescues morphological defects in pink<sup>1b9</sup>. However, in line with our model, heterozygosity for acon does not reduce pink<sup>1b9</sup>-induced O<sub>2</sub><sup>-</sup> production (Figure 2A), indicating that increased O<sub>2</sub><sup>-</sup> production per se does not induce mitochondrial morphological defects. Next we measured Acon activity in pink<sup>1</sup> mutant mitochondria and we find that Acon activity normalized to total Acon protein levels is significantly reduced compared to the controls. These data are in line with increased Acon inactivation in pink<sup>1</sup> mutants (Figure 2B), likely as a result of the increased O<sub>2</sub><sup>-</sup>.

Further testing our model, we also measured H<sub>2</sub>O<sub>2</sub> and Fe<sup>2+</sup> content. To measure H<sub>2</sub>O<sub>2</sub> and its radical derivatives we incubated fly lysates with the fluorescent probe dichlorofluorescein diacetate (DHCF-DA) [33]. We find a 50% increase in fluorescence in pink<sup>1</sup> mutant lysates compared to the control (Figure 2C). Thus, pink<sup>1</sup> mutants accumulate H<sub>2</sub>O<sub>2</sub> and/or its derivatives thereof. We also measured mitochondrial Fe<sup>2+</sup> content by incubating mitochondrial enriched fractions with Rhodamine B-{[1,10-phenanthroline-5-yl]aminocarboxyl}benzyl ester (RPA) [34]. In the presence of Fe<sup>2+</sup>, RPA fluorescence quenches and in pink<sup>1b9</sup> mitochondria, we observe a significant increase in RPA quenching compared to controls (Figure 2D). These data indicate increased mitochondrial Fe<sup>2+</sup> levels in pink<sup>1b9</sup> mutants. This effect is specific, as incubating mitochondria of controls and mutants in Rhodamine B-{[Phenanthren-9-yl]Aminocarbonyl}benzyl ester (RPAC) that consists of the same fluorophore as RPA but without ironchelating properties, does not show quenching in pink<sup>1b9</sup> or in controls (Figure 2D). Thus, our data indicate that pink<sup>1b9</sup> mutants harbor increased levels of Fe<sup>2+</sup> and of H<sub>2</sub>O<sub>2</sub> and/or its radical derivatives.

Next we tested if increased mitochondrial Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> accumulation in pink<sup>1</sup> mutants is a consequence of Acon-[4Fe-4S] inactivation by O<sub>2</sub><sup>-</sup>. We therefore measured Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> and its derivatives levels in mitochondria of pink<sup>1</sup> mutants heterozygous for acon<sup>1</sup> or acon<sup>2</sup>. While the increased O<sub>2</sub><sup>-</sup> production in pink<sup>1b9</sup> mutants was not reduced by heterogeneous acon, as shown above (Figure 2A), we find that compared to pink<sup>1b9</sup>, mitochondrial Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> levels are significantly lower in pink<sup>1b9</sup> heterozygous for acon<sup>1</sup> or acon<sup>2</sup> (Figure 2C and 2D). Thus, these data are consistent with the possibility that mitochondrial Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> and/or its radical derivatives-accumulation in pink<sup>1</sup> mutants is caused by oxidative inactivation of Acon.

Mitochondrial morphological defects are critically dependent on the Acon dose

Our biochemical data support a model in which oxidative inactivation of Acon and ensuing Fe<sup>2+</sup> and H<sub>2</sub>O<sub>2</sub> accumulation contributes to the mitochondrial morphology defects in pink<sup>1</sup> mutants. We reasoned that if partial loss of acon protects against mitochondrial stress in pink<sup>1</sup> mutants, increased levels of Acon...
expression may predispose cells to develop mitochondrial morphological defects, provided sufficient O$_2$ is around. We therefore created transgenic animals that overexpress wild type Acon (Figure 3A) resulting in increased Acon activity (Figure 3B). We then determined mitochondrial morphology using mito-GFP and the plea-GAL-4 driver upon expression of Acon in DA neurons. While mitochondria in DA neurons of control flies organize in a long tubular network, mitochondria in DA neurons that overexpress Acon form fragmented spherical aggregates (Figure 3C, 3D and Figure S2A). Hence, in contrast to partial loss of acon that rescues mitochondrial defects in pink1 mutants, overexpression of Acon causes mitochondrial morphological defects and swelling of mitochondria in DA neurons.

Based on the finding that increased expression of Acon causes mitochondrial morphological defects we tested if pink1 mutant flies upregulate Acon expression. We measured acon mRNA and protein levels in pink1 mutants, but in contrast to our expectation, we find a significant downregulation of both acon mRNA and Acon protein levels in pink1 flies (Figure S2C, S2D) suggesting that an adaptive mechanism already acts in pink1 mutants to down regulate Acon expression. Thus, the pink1-induced stress response results in lower Acon levels and, as shown above, further reducing Acon expression (using heterozygous acon mutant) is protective against mitochondrial defects in pink1 mutants. Taken together, the data are consistent with Acon being a dosage sensitive modifier of morphological defects in mitochondria.

Mitochondrial morphological defects as a consequence of Acon inactivation depend on its [4Fe-4S] cluster

To test if the mitochondrial morphological defects in DA neurons following Acon overexpression are induced by increased Acon catalytic activity or by the presence of an [4Fe-4S] cluster we generated transgenic flies that either overexpress a catalytic inactive Acon (Acon$^{S677A}$) that still harbors its [4Fe-4S] cluster, or flies that overexpress an Acon without its [4Fe-4S] cluster...
and is thus also catalytically inactive [22,35]. Western blotting indeed indicates overexpression of the mutant Acon proteins (Figure 3A), and as expected, Acon enzymatic activity measured in fly head lysates is only increased when wild type Acon is expressed, and not when AconS677A or AconC459S are expressed (Figure 3B). While overexpression AconS677A in DA neurons results in obvious mitochondrial morphological defects similar to the overexpression of wild type Acon, overexpression of AconC459S.

Figure 3. Acon[4Fe-4S] cluster induces mitochondrial defect in DA neurons that is not rescued by increased mitophagy. (A) Quantification of Western blots of fly heads expressing UAS-acon (see text) in DA neurons probed with anti-Acon normalized to tubulin, relative to control. Data from 3 independent experiments. (B) Mitochondrial Aconitase activity relative to the control. Data collected from 5 independent mitochondrial preparations. (C,E) GFP-labeled mitochondria in dopaminergic (DA) neurons DA neurons labeled with anti-tyrosine hydroxylase (magenta). White arrows indicate mitochondrial aggregates. Scale bar: 2.5 μm. (D,F) Quantification of average mitochondrial aggregate size. 5 neurons per brain from n=10 brains were analyzed. The genotype of control is: w1118; pleGal4 UAS-mitoGFP/+; and of mutants that express wild type or mutant Acon is: w1118; UAS-acon*/+; pleGal4 UAS-mitoGFP/+ . Significantly different * from control, $ from ple-acon+; $ from ple-aconS677A One-way ANOVA, post hoc Dunnett p<0.01, ns: not significantly different. Data are shown as Mean ± SEM. doi:10.1371/journal.pgen.1003478.g003
is inert (Figure 3C, 3D and Figure S2A). Hence, the Acon [4Fe-4S] cluster predisposes DA neurons to mitochondrial morphological defects.

Our data are in line with a model where oxidative inactivation of the Acon [4Fe-4S] cluster by O$_2^-$ contributes to mitochondrial morphological defects. To find further evidence for this idea we expressed Drosophila mitochondrial Ferritin (Fer3HCH) [36] in DA neurons of pink1$^{B9}$, using the ple-GAL4 driver and assessed mitochondrial morphology using mito-GFP. We find that expression of Fer3HCH significantly rescues defects in mitochondrial morphology in pink1$^{B9}$ mutants (Figure 4E, Figure 2F, and Figure S2A), suggesting that iron toxicity causes mitochondrial defects in pink1 mutants. Consistent with this model, expression of Fer3HCH in flies that over express Acon also results in a significant rescue of the mitochondrial morphological defects in the DA neurons (Figure S2E, S2F). Hence, the mitochondrial swelling as a result of Acon overexpression is at least in part mediated by iron. Together these data indicate that Acon is a critical source of Fe$^{2+}$-mediated mitochondrial toxicity.

Mitochondrial morphological defects upon Acon overexpression are not rescued by Drp1 or Parkin

Mitochondrial dynamics and mitophagy are critical processes in maintaining a healthy population of mitochondria. Pink1 has been implicated to regulate mitochondrial homeostasis via several mechanisms. Deregulation of these pathways may be a source of O$_2^-$, responsible for Acon inactivation. While Pink1 has been found to maintain the activity of Complex I in the ETC [17–20], the protein has also been linked to mitophagy in a pathway involving Drp1 and Parkin [11–13,20,37–40]. Dysfunctional mitochondrial parts may be segregated by the fission factor Drp1 [41,42]. Pink1 stabilized on depolarized mitochondria then mediates Parkin recruitment causing the ubiquitination of mitochondrial proteins and activation of the autophagic machinery [41,42]. To test if enlarged and swollen mitochondria upon Acon over expression are a consequence of defective remodeling or mitophagy we co-expressed Parkin, a protein that ubiquitinates mitochondrial targets, or Drp1, a mitochondrial fusion factor, two conditions thought to facilitate mitophagy. While over expression of Parkin or Drp1 -as expected- result in fragmentation of mitochondria, these conditions do not rescue the defect in mitochondrial swelling and clumping induced by expression of Acon or Acon$^{S677A}$ (Figure 3E, 3F and Figure S2A). Hence, our data suggest that the defects in mitochondrial morphology induced by Acon expression are at least in part caused independently from defects in remodeling and mitophagy.

Mitochondrial defects caused by Complex I dysfunction are rescued by partial loss of Acon and by mitoferritin

Given that pink1 mutants display reduced Complex I activity [17–20] and this feature may also be a source of increased O$_2^-$ we tested if mitochondrial swelling and clumping seen in animals where we downregulated an evolutionary conserved Complex I component, NDUFA8, can be rescued by partial loss of acon. First, we confirm increased O$_2^-$ production and find a concomitant inactivation of Acon activity upon RNAi-mediated downregulation of NDUFA8 (Figure 4A and 4B). Second, we believe that this O$_2^-$ is produced at least partly independently from defects in mitochondrial remodeling because expression of Drp1 in DA neurons with reduced NDUFA8 function does not fully rescue the mitochondrial swelling and clumping phenotypes in PPM3 DA neurons (Figure 4C, 4D and Figure S2A). Next, we tested the ability of heterozygous acon to modulate the mitochondrial

Figure 4. Mitochondrial morphological defects in DA neurons of Complex I-deficient and pink1$^{B9}$ flies involve iron-mediated toxicity. (A) Superoxide production measured as fluorescence change of DHE, a superoxide sensitive dye, in isolated mitochondria from control (w$^{1118}$; daGal4/+) and from Complex I RNAi expressing flies (w$^{1118}$; UAS-NDUFA8RNAi/+; daGal4/+). (B) Relative mitochondrial Aconitase activity in Complex I RNAi expressing flies normalized to Acon protein levels. Data collected from 4 independent mitochondrial preparations. * Significantly different from control, Student’s t test p<0.01. (C,E) GFP-MitoGFP/anti-TH in wild type flies used as a control. (D,F) Average aggregates size from control (w$^{1118}$; daGal4/+; ple>NDUFA8$^{RNAi}$/ +; pleGal4 UAS-NDUFA8$^{RNAi}$/+) and from Complex I RNAi expressing flies (w$^{1118}$; UAS-NDUFA8$^{RNAi}$/+; pleGal4 UAS-mito-GFP/+). Notably, while overexpression of the mitophagy gene Fer3HCH rescues mitochondrial morphology in pink1 mutants, the mitochondrial swelling and clumping phenotypes in PPM3 DA neurons with reduced NDUFA8 function does not fully rescue the mitochondrial remodeling because expression of Drp1 in DA neurons with reduced NDUFA8 function does not fully rescue the mitochondrial swelling and clumping phenotypes in PPM3 DA neurons (Figure 4C, 4D and Figure S2A). Next, we tested the ability of heterozygous acon to modulate the mitochondrial...
labeled mitochondria in DA neurons pink1B9, pink1 mutants that express mitoferritin (pink1RNAi, pleGal4 UAS-mito-GFP/UAS-mitoFerIII, Complex I RNAi: expressing flies (w; UAS-NDUFA8 Pink8/NNA/4; pleGal4 UAS-mito-GFP/FerIII), and Complex I RNAi, mitoferritin co-expressing flies (w; UAS-NDUFA8 Pink8/NNA/4; pleGal4 UAS-mito-GFP/UIA-mitoFerIII). White arrows indicate mitochondrial aggregates. Scale bar 2.5 μm. (DF) Quantification of average mitochondrial aggregate size. Significantly different * from control. ** significantly different from ple–NDUFA8 RNAi. One-way ANOVA, post hoc Dunnett p < 0.01. Data are shown as Mean ± SEM.

doi:10.1371/journal.pgen.1003478.g004

morphological defect induced by NDUFA8 RNAi and find that heterozygous acon is more effective than expression of Drp1 in rescuing the mitochondrial deficits in DA neurons (Figure 4C, 4D and Figure S2A). Likewise, and in line with our model, expression of mitoferritin (Fer3HCH) also alleviates mitochondrial defects in animals that express RNAi to NDUFA8 in DA neurons (Figure 4E, 4F and Figure S2A). Hence, our data suggest that Acon is inactivated by ETC-derived O2− causing oxidative stress.

Our work suggests that mitochondrial morphological defects in pink1 mutant DA cells can be of different origin: both O2− dependent Acon inactivation or loss of Parkin-dependent mitophagy yield swollen and clumped mitochondria. Alleviating the defects induced by either pathway using heterozygous acon or expressing Drp1 or Parkin both rescue the mitochondrial morphological defects in pink1 mutants (this work; [12–14,16]). To further support this notion, we first assessed if mitochondrial defects in parkin mutants can be rescued by partially removing acon function. parkin mutants display enlarged and swollen mitochondria in muscles and DA neurons, many of the flies also fail to fly and animals harbor lower ATP levels. In contrast to removing acon function in pink1 mutants, heterozygosity for acon fails to rescue the inability of parkin mutants to fly, their reduced ATP levels and their defects in mitochondrial morphology (Figure 5A–5D). Hence, our data suggest that acon acts independently from defects in Parkin-dependent mitophagy. Finally if our model is correct, we reasoned that the combination of Drp1 expression and acon heterozygosity in pink1 mutants should yield additive ‘super rescue’. We therefore tested the ability of these flies to fly and find that they fly significantly better than pink1 mutants or than pink1 mutants partially rescued by either Drp1 expression or by heterozygous acon (Figure 5E). Hence, these data are in line with Pink1 controlling different mitochondrial pathways that can be targeted largely independently. We speculate that increased O2− derived from a defective Complex I in pink1 mutants is an important contributor to Acon inactivation, but other sources of O2− may contribute to mitochondrial failing as well.

Discussion

Iron accumulation in the substantia nigra, systemic mitochondrial dysfunction and oxidative stress have all been implicated in PD pathology; however, a link between these factors remains elusive. Here we show that oxidative inactivation of Acon generates iron-mediated oxidative stress that contributes to mitochondrial swelling in Drosophila pink1 mutants (Figure 6). Inactivation of Acon[4Fe-4S] clusters could contribute to mediating O2− toxicity by simultaneous release of Fe2+ and H2O2 [43] that combine in the Fenton reaction to generate highly toxic hydroxyl radicals [30,44] (Figure 6). Hydroxyl radicals can induce mitochondrial permeability transition and swelling [45–47], in line with electron microscopic analyses of pink1 mutants where mitochondria appear swollen and show disorganized cristae [9,10] (Figure 1). Four major findings support that this iron-mediated toxic mechanism is an additional important aspect of mitochondrial dysfunction in pink1 mutants. First, we find increased O2− production, increased Acon inactivation and more Fe2+ and H2O2 accumulation in pink1

Figure 5. Acon inactivation and Parkin-mediated mitophagy act in parallel in pink1 mutants. Analysis of parkin mutants (parkin+/A2) and parkin flies heterozygous for acon1 or 2 (A) Flight ability of 5-day-old adult flies and (B) ATP content in the head-thorax of 5-day-old flies. Data collected from at least 5 independent experiments. (C) GFP-labeled mitochondria in flight muscles (daGal4 UAS-mitoGFP), Scale bar: muscle 10 μm. (D) Quantification of average mitochondrial aggregate size. 5 images from n=5 thoraxes. (E) Flight ability of control flies, pink1B9, pink1 mutants heterozygous for acon1 or 2, heterogeneous for acon1 or 2 overexpressing drp1 and pink1 mutants with a combination of acon1 or 2 and heterozygosity and drp1 overexpression. * Significantly different from pink1B9, One-way ANOVA, post hoc Dunnett p < 0.01; ns: not significantly different.

doi:10.1371/journal.pgen.1003478.g005
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Figure 6. Model of oxidative Acon inactivation in pink1 mutants. Pink1 loss induces increased superoxide production that inactivates the Acon[4Fe-4S] resulting in the generation of Fe²⁺ and H₂O₂. These combine to form hydroxyl radicals that lead to mitochondrial failure. Mitoferritin chelates Fe²⁺ and is thus able to rescue mitochondrial failure in pink1 mutants. Acon inactivation act in parallel to Parkin-mediated mitophagy in controlling mitochondrial integrity. doi:10.1371/journal.pgen.1003478.g006

mutants (Figure 2). Second, partial loss of Acon reduces Fe²⁺ and H₂O₂ accumulation and alleviates pink1-associated phenotypes including mitochondrial morphological defects in muscle and DA neurons (Figure 1). Third, overexpression of wild type Acon in dopaminergic neurons produces a mitochondrial morphological defect and this effect is completely dependent on the presence of the [4Fe-4S] cluster in Acon (Figure 3). These data also indicate mitochondrial integrity is sensitive to Acon [4Fe-4S] cluster dosage. Finally, chelating iron by expressing mitochondrial Ferritin is sufficient to rescue pink1 mitochondrial morphological defects (Figure 4). Thus, our data suggest that inactivation of Acon and iron accumulation might be a pathogenic mechanism triggered by loss of pink1 and increased superoxide, linking iron accumulation and mitochondrial failure.

Acon inactivation is dependent on O₂⁻ that, amongst other sources (see below), may be produced in defective mitochondria. While various mitochondrial insults can result in increased O₂⁻ production, our work is most consistent with Parkin-dependent mitophagy being not the major source of Acon inactivation in pink1 mutants. The mitochondrial morphological defects induced by Acon overexpression were not strongly rescued by expressing Drp1, a condition that indirectly promotes mitophagy and parkin mutants were not majorly rescued by partial loss of acon (Figure 3 and Figure 5). In contrast, mitochondrial morphological defects in DA neurons of flies with reduced Complex I activity are significantly rescued when acon is heterozygous (Figure 4). Hence, Acon seems to act in a Pink1-dependent pathway that can operate largely independently of mitophagy (Figure 6).

Defects at the level of Complex I are often associated with increased leaking of the toxic O₂⁻ [48,49], and likewise, systemic inhibition of Complex I mimics features of PD in animal models [50–53]. Previous work in flies or mice has indicated reduced ETC inhibition of Complex I mimics features of PD in animal models [54]. Given that both genetic forms of PD as well as sporadic cases of PD show ETC defects [5,6,17–19], our work may be relevant for idiopathic cases that suffer from mitochondrial dysfunction as well. Acon inactivation and iron-mediated toxicity might thus have a more general role in the pathogenesis of PD.

While pink1 loss affects numerous cell types, our data also start to provide insight as to why DA neurons in the substantia nigra are more vulnerable in PD. While overexpression of Acon or downregulation of Complex I produces mitochondrial morphological defects in DA neurons, in Drosophila flight muscles mitochondria appear morphologically largely normal (data not shown). These data suggest a tissue-specific response in that Acon inactivation has a stronger impact in DA neurons than in muscle cells. Each cell type is exposed to various sources of O₂⁻, but DA neurons in particular are exposed to dopamine-induced oxidative stress that is a source of O₂⁻ [55–57]. Furthermore, the substantia nigra in humans is naturally rich in iron [58] and this feature may lower the threshold for hydroxyl radical production in the Fenton reaction that is facilitated by Acon inactivation. Pink1 mutations or environmental factors in some sporadic cases of PD already result in increased levels of O₂⁻, but we hypothesize that in DA neurons, additional dopamine-induced oxidative stress may facilitate Acon inactivation and hydroxyl radical production providing insight into one of the pathways underlying mitochondrial failure in pink1 mutants.

Methods

Drosophila stocks and maintenance

Flies were raised on standard cornmeal and molasses medium at 25°C. w¹¹¹⁸; UAS-mitoGFP, w¹¹¹⁸; daGal4, w¹¹¹⁸; pleGal4, w¹¹¹⁸; UAS-4EBP and w¹¹¹⁸; M(tET1) Acn[M495T]/S06a (acon⁻) and were obtained from Bloomington stock center (Indiana, USA). w¹¹¹⁸; pink¹⁰⁶ and w¹¹¹⁸; pink¹⁰⁶, parkin¹⁰⁶ and parkin¹⁰⁸ [59] were provided by Jongkyeong Chung (Advanced Institute of Science and Technology, Korea) [10]. parkin⁻¹⁰⁷ mutant flies were a gift from Graeme Mardon (Baylor College of Medicine) [60] and dpt¹⁰⁷ genomic rescue constructs were provided by Hugo Bellen (Baylor College of Medicine) [60] and dpt¹⁰⁷ genomic rescue constructs were provided by Hugo Bellen (Baylor College of Medicine) [60].

Indeed, our data indicate that pink1 mutants heterozygous for acon show increased levels of O₂⁻ but normal mitochondrial morphology. Our data also indicate that upstream events in pink1 mutants that result in increased O₂⁻ production contribute mitochondrial morphological defects because of oxidative inactivation of Acon. In line with this, overexpression of the mitochondrial superoxide dismutase 2 (SOD2) that scavenges O₂⁻, successfully rescues mitochondrial swelling phenotype of pink1 in DA neurons [54]. Given that both genetic forms of PD as well as sporadic cases of PD show ETC defects [5,6,17–19], our work may be relevant for idiopathic cases that suffer from mitochondrial dysfunction as well. Acon inactivation and iron-mediated toxicity might thus have a more general role in the pathogenesis of PD.

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UAS-CG3683RNAi info.nih.gov/ij/). software from the US National Institute of Health (http://rsb.info.nih.gov/ij/).

Quantification was performed using gel analyzer tool in ImageJ software. Blots were developed in 1:1000 anti-Tubulin (B5–12, Sigma) and 1:1000 HRP coupled secondary antibodies (Jackson ImmunoResearch). Blots were developed in Ethanol 10% SDS loading buffer and boiled for 5 min and 15 seconds.

Mitochondria isolation

Fifty flies were gently crushed in 1 ml chilled mitochondrial isolation medium (Mitosciences) by using a porcelain mortar and pestle, then spun twice at 1,000 g for 5 min at 4°C to remove debris. The supernatant was then spun at 12,000 g, for 15 min at 4°C. The pellet, containing the mitochondria, was washed with 1 ml of isolation medium and resuspended in 40 μl of isolation medium supplemented with complete protease inhibitor mixture without EDTA (Roche).

Superoxide production

Mitochondrial Superoxide production was measured as described [32]. 10 μg of mitochondria were incubated in experimental buffer (EB: 125 mM KCl, 10 mM Tris-MOPS, 1 mM KPi, 10 μM EGTA-Tris, pH 7.4, 25°C) supplemented with 1.25 mM Pyruvate/1.25 mM malate and 5 μM DHE (Molecular probe) in a 96-well plate format for 10 min. The fluorescence was measured (485exc/590em) using Wallac Victor2 1420 (Perkin Elmer). Fluorescence intensity was normalized to the initial value and expressed as relative to the control. 10 μM antimycin A was used to induce superoxide production in control mitochondria.

Fe2+ measurements

For mitochondrial ferrous iron level measurements, 10 μg of mitochondria were resuspended in isolation buffer (Mitosciences) and incubated with 20 μM of RPA or RPAC (Squarix Biotechnology) in a 96-well plate format at room temperature for 10 min. RPA/RPAC fluorescence (560 exc/600 em) was measured using Wallac Victor2 1420 (Perkin Elmer). Quenching was calculated as percent of initial fluorescence.

Aconitase activity

Aconitase enzyme activity microplate kit (Mitosciences) was used according to the manufacturer’s protocol to measure Aconitase activity. 20 μg of mitochondria were incubated with assay buffer and the activity was measured by following conversion to Aconitase Causes Iron Toxicity in pink1 Mutants
of isocitrate to cis-aconitate as in increased in 240 nm UV absorbance. Measurements were recorded over 30 min. at 1 min intervals and aconitase activity were calculated from the linear increase in absorbance and normalized to the amount of aconitase, determined by western blot, in the same mitochondrial preparation. Values were reported as relative activity to the control.

Mitochondrial morphology in DA neurons

Brain dissection and whole-mount immunohistochemistry for tyrosine hydroxylase (TH) was performed as described [65]. Primary 1:300 antibody against TH (Chemicon) and secondary alexa 555 (Invitrogen) were used. Brains were imaged on a Zeiss LSM 510 META confocal microscope using a 63xoil NA 1.4 lens. Mitochondrial tagged GFP (mito-GFP) was visualized using 488 nm laser and 500–530 band pass emission filter. Because mitochondrial morphology is sensitive to environmental conditions, variations did occur from batch to batch. We only compared flies of different genotypes if normal mitochondrial morphology was observed in the control samples (Figure S1H–S1H) in the same batch. For quantification of mitochondrial aggregates size and numbers, DA neurons of PPM3 cluster (Figure S1H–S1H) were scored. Quantification of aggregate size was done using “analyzing particles” plugin in Imagej [http://rsb.info.nih.gov/ij/] : rounded particles were automatically detected and the average surface area of aggregates in each neuron was determined as total area occupied by aggregates/number of aggregates.

Mitochondrial morphology in flight muscles

Adult flies were fixed in PBS with 5% formaldehyde and 0.4% Triton for 3 hours. Thoraces were dissected in PBS and mounted in vectashield (Vector Laboratories) and were imaged on a Zeiss LSM 510 META confocal microscope using a 63xoil NA 1.4 lens. Mitochondrial tagged GFP (mito-GFP) was visualized using 488 nm laser and 500–530 band pass emission filter. For muscle section with same area were scored and quantification of mitochondrial aggregates was performed as described above.

Supporting Information

Figure S1  (A) Schematic representation of the acon gene. C→A in acon is cytosine adenine transition that results in a STOP codon; The insertion site of Mi[ET1]Acon BM095176 is indicated. (B) complementation test table of different heteroallelic combinations, “genomic” indicates a genomic fragment containing the wild type acon locus and da>nasAcon indicates flies with ubiquitous expression of qcon cDNA; “−/−” means fail to complement, and “+” means adult fertile flies emerge. (C) Quantification of acon mRNA by semi-quantitative RT-PCR in embryos. (D) Quantification of Acon protein levels in embryos normalized for tubulin levels using Western blotting. The presence of remaining Acon protein in acon+/−; acon−/− mutants may indicate maternal component.

References

1. Dawson TM, Dawson VL (2003) Molecular pathways of neurodegeneration in Parkinson’s disease. Science 302: 819–822.
2. Thomas B, Beal MF (2005) Parkinson’s disease. Hum Mol Genet 16 Spec No. 2: R183–194.
3. Horowitz MP, Greenamyre JT (2010) Mitochondrial iron metabolism and its role in neurodegeneration. J Alzheimers Dis 20 Suppl 2: S551-560.
4. Sofic E, Papas W, Jellinger K, Riederer P, Youdim MB (1991) Selective increase of iron in substantia nigra zona compacta of parkinsonian brains. J Neurochem 56: 978–982.
5. Lesteinne P, Nelson J, Riederer P, Jellinger K, Reichmann H (1990) Normal mitochondrial genome in brain from patients with Parkinson’s disease and complex I defect. J Neurochem 55: 1810–1812.
6. Schapira AH, Cooper JM, Dexter D, Jenner P, Clark JB, et al. (1989) Mitochondrial complex I deficiency in Parkinson’s disease. Lancet 1: 1269.
7. Dawson TM, Ko HS, Dawson VL (2010) Genetic animal models of Parkinson’s disease. Neuroen 66: 646–661.
8. Jones R (2010) The roles of PINK1 and Parkin in Parkinson’s disease. PLoS Biol 8: e1000299. doi:10.1371/journal.pbio.1000299
9. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, et al. (2006) Drosophila pink1 mutants overexpressing dplp1 (pink1P0; dplp1) or with reduced dplp1 gene dosage (pink1P0; dplp1−/−). (E) Quantification of Acon protein levels by Western blot in 3-day-old adult flies, anti-Acon normalized to tubulin, relative to control. Data collected from at least 4 independent experiments. * Significantly different from pink1B9; Student’s t test p<0.01. (F) TEM analysis of thorax. Black arrows indicate swollen mitochondria. Scale bar: x5000 μm; x2000 2 μm. (G) Western blot analysis on mitochondrial and cytoplasmic fractions using antibodies against Acon and Complex V. (H) DA neuron clusters in the protocerebrum of the Drosophila brain with identified clusters indicated. (H) Magnification of the PPM3 cluster and (H0) of a single PPM3 neuron. Scale bar: 50 μm (H) 5 μm (H0) 2.5 μm (H0). (TIF)

Figure S2 (A) Quantification of the mitochondrial aggregate number per DA neurons in PPM3 cluster. Data collected from 5 neurons per brain in at least 10 brains. Significantly different * from control, ** from pink1B9, *** ple> NDUFA8 Δ/−, ns not significantly different. One-way ANOVA, post hoc Dunnett p<0.01. Data are shown as Mean ± SEM. (B) Flight ability of pink1P0; pink1 mutants overexpressing dplp1 (pink1P0; dplp1+) or with reduced dplp1 gene dosage (pink1P0; dplp1−/−). * Significantly different from pink1P0. (C) Quantification of acon mRNA by semi-quantitative RT-PCR in 5-day-old controls (pink1P0) and in pink1P0 flies. (D) Quantification of Acon protein levels in 5-day-old control and pink1P0 mutant flies using Western blotting with anti-Acon and normalized for tubulin levels, relative to control. Data were collected from 5 independent experiments. * Significantly different from control, Student’s t test p<0.01. (E, F) Quantification of the mean number of mitochondrial aggregates per DA neuron and of average mitochondrial aggregate size of GFP-labeled mitochondria in controls (pink1P0; pleGal4 UAS-mitoGFP/+); in flies over expressing wild type Acon in DA neurons (pink1P0; UAS-acon−/+; pleGal4 UAS-mitoGFP/+); and in flies overexpressing wild type Acon and mitoferritin in DA neurons (pink1P0; UAS-acon−/+; pleGal4 UAS-mitoGFP/ UAS-mitoFerrIII). Significantly different * from ple>acon−/−; t-test: p<0.01. In all panels data are shown as Mean ± SEM. (TIF)

Acknowledgments

We thank the Bloomington and Harvard Drosophila stock centers and the Developmental Studies Hybridoma bank as well as Dr Fanis Missirlis and Jongkyeung Chung for reagents, and Wim Vandenberghe and members of the Verstreken and De Strooper labs for comments.

Author Contributions

Conceived and designed the experiments: GE MV SV PV. Performed the experiments: GE MV SVM SV JS. Analyzed the data: GE MV SV. Contributed reagents/materials/analysis tools: GE MV SVM PV JDSV OS. Wrote the paper: GE PV.
38. Geisler S, Holmstrom KM, Treis A, Skujat D, Weber SS, et al. (2010) The
36. Missirlis F, Holmberg S, Georgieva T, Dunkov BC, Rouault TA, et al. (2006)
34. Petrat F, Weisheit D, Lensen M, de Groot H, Sustmann R, et al. (2002) Selective
32. Horak P, Crawford AR, Vadysirisack DD, Nash ZM, DeYoung MP, et al.
30. Vasquez-Vivar J, Kalyanaraman B, Kennedy MC (2000) Mitochondrial
29. Gardner PR, Fridovich I (1991) Superoxide sensitivity of the Escherichia coli
26. Verstreken P, Ohyama T, Haueter C, Habets RL, Lin YQ, et al. (2009) Tweek,
23. Lauble H, Kennedy MC, Beinert H, Stout CD (1992) Crystal structures of
22. Beinert H, Kennedy MC (1993) Aconitase, a two-faced protein: enzyme and
20. Liu W, Acin-Perez R, Greggman KD, Manfredi G, Lu B, et al. (2011) Pink1
18. Morais VA, Verstreken P, Roethig A, Smet J, Snellinx A, et al. (2009)
17. Gautier CA, Kitada T, Shon J (2008) Loss of PINK1 causes mitochondrial
functions altered and increased sensitivity to oxidative stress. Proc Natl Acad
Sci U S A 105: 11361–11366.
15. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, et al. (2010) PINK1 is
selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8:
e1000289. doi:10.1371/journal.pbiol.1000289
14. Yang Y, Gehrke S, Imai Y, Huang Z, Oyung Y, et al. (2006) Mitochondrial
pathology and muscle and dopaminergic neuron degeneration caused by
inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci U S A
103: 10795–10797.
13. Gautier CA, Kitada T, Shen J (2008) Loss of PINK1 causes mitochondrial
Toxicity. Proc Natl Acad Sci U S A 105: 1638–1643.
12. Poole AC, Thomas RE, Andrews LA, McBride HM, Whitworth AJ, et al. (2008)
The PINK1/Parkin pathway regulates mitochondrial morphology. Proc Natl
Acad Sci U S A 105: 1638–1643.
11. Yang Y, Ouyang Y, Yang L, Beal MF, McQuibban A, et al. (2008) Pink1 regulates
mitochondrial dynamics through interaction with the fusion/fission
machinery. Proc Natl Acad Sci U S A 105: 7070–7075.
10. Narendra DF, Jin SM, Tanaka A, Suen DF, Gautier CA, et al. (2010) PINK1 is
selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol 8:
e1000289. doi:10.1371/journal.pbiol.1000289
9. Yang Y, Gehrke S, Imai Y, Huang Z, Oyung Y, et al. (2006) Mitochondrial
pathology and muscle and dopaminergic neuron degeneration caused by
inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci U S A
103: 10795–10797.
8. Geisler S, Holmstrom KM, Skujat D, Weber SS, et al. (2010) The
PINK1/Parkin pathway regulates mitochondrial dynamics and function in
mammalian hippocampal and dopaminergic neurons. J Neurobiol 2009; 79:
3297–3304.
7. Imai Y, Lu B (2012) Mitochondrial dynamics and mitophagy in Parkinson’s
disease: disordered cellular power plant becomes a big deal in a major
movement disorder. Curr Opin Neurobiol 21: 935–941.
6. Twig G, Shiraih OS (2011) The interplay between mitochondrial dynamics and
mitophagy. Antioxid Redox Signal 14: 1939–1951.
5. Cantu D, Schaeck J, Patel M (2009) Oxidative inactivation of mitochondrial
aconitase results in iron and H2O2-mediated neurotoxicity in rat primary
ependymal cultures. PLoS ONE 4: e7093. doi:10.1371/journal.pone.0007095
4. Lochev SI, Fridovich I (1994) The role of O2.- in the production of HO.: in
vitro and in vivo. Free Radic Biol Med 16: 29–33.
3. Sakurai K, Stocanovsky DA, Fujimoto Y, Cederbaum AI (2000) Mitochondrial
permeability transition induced by 1-hydroxyethyl radical. Free Radic Biol Med
28: 273–280.
2. Srivastava S, Chan C (2007) Hydrogen peroxide and hydroxyl radicals mediate
palmitate-induced cytotoxicity to hepatoma cells: relation to mitochondrial
permeability transition. Free Radic Res 41: 38–49.
1. Vercesi AE, Kossowski AJ, Grijalba MF, Meinicke AR, Castillo RF (1997)
The role of reactive oxygen species in mitochondrial permeability transition.
Biosci Rep 17: 43–52.
0. Pitkans T, Robinson BH (1996) Mitochondrial complex I deficiency leads to
increased production of superoxide radicals and induction of superoxide
dismutase. J Clin Invest 97: 345–351.
9. Turrens JF (1997) Superoxide production by the mitochondrial respiratory
chain. Biosci Rep 17: 5–8.
8. Betarbet R, Sherry TB, MacKenzie G, Garcia-Osma M, Panov AV, et al.
(2000) Chronic systemic pesticide exposure reproduces features of Parkinson’s
disease. Nat Neurosci 3: 1301–1306.
7. Cannon JR, Tapias V, Na HM, Honick AS, Droet RE, et al. (2009) A highly
reproducible retoine model of Parkinson’s disease. Neurobiol Dis 34: 279–290.
6. Coulom H, Birman S (2004) Chronic exposure to rotenone models sporadic
Parkinson’s disease in Drosophila melanogaster. J Neurosci 24: 1306–1310.
5. Dauer W, Przedborski S (2003) Parkinson’s disease: mechanisms and models.
Neuron 39: 889–899.
4. Koh H, Kim H, Kim MJ, Park J, Lee HJ, et al. (2012) Silent information
regulator 2 (Sir2) and Forkhead box a (FOXO) complement mitochondrial
respiration and deficient synaptic function. EMBO Mol Med 1: 99–111.
3. Yang Y, Gehrke S, Imai Y, Huang Z, Oyung Y, et al. (2006) Mitochondrial
pathology and muscle and dopaminergic neuron degeneration caused by
inactivation of Drosophila Pink1 is rescued by Parkin. Proc Natl Acad Sci U S A
103: 10795–10797.
2. Liu W, Acin-Perez R, Greggman KD, Manfredi G, Lu B, et al. (2011) Pink1
regulates the oxidative phosphorylation machinery via mitochondrial fusion.
Proc Natl Acad Sci U S A 108: 12980–12984.
1. Vasquez-Vivar J, Kalyanaraman B, Kennedy MC (2000) Mitochondrial
aconitase is a source of hydroxyl radical. An electron spin resonance
investigation. J Biol Chem 275: 14646–14654.
Aconitase Causes Iron Toxicity in pink1 Mutants

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