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The ER protein Creld regulates ER-mitochondria contact dynamics and respiratory complex 1 activity

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Abstract

Dynamic contacts are formed between endoplasmic reticulum (ER) and mitochondria that enable the exchange of calcium and phospholipids. Disturbed contacts between ER and mitochondria impair mitochondrial dynamics and are a molecular hallmark of Parkinson's disease. Cystein-rich with EGF-like domain (Creld) are ER-proteins associated with atrioventricular septal defects, but human CRELD1 is also a poorly characterized risk gene for Parkinson's disease. Here we show that Creld is required for ER-mitochondria communication. Loss of Creld leads to mitochondrial hyperfusion and reduced ROS signaling in *Drosophila melanogaster*, *Xenopus tropicalis* and human cells. We found that reduced respiratory complex I activity lowers hydrogen peroxide levels, which disturbs neuronal activity and leads to impaired locomotion in Creld mutants. Our study presents a new paradigm of neuron dysfunction as a result of impaired ER-mitochondria communication and a new model for Parkinson's disease.

Introduction

The role of cellular reactive oxygen species (ROS) is ambiguous: according to the free radical theory of aging, oxidative stress caused by excessive ROS seems to be the major driver of aging and occurs due to a decline in mitochondrial function. This theory was challenged by findings that long-lived species have normal or elevated ROS levels, and increasing ROS genetically does not shorten the lifespan¹. Moreover, ROS are now acknowledged as important signaling molecules; for example, hydrogen peroxide (H₂O₂) is required for the excitability of neurons², and certain types of ROS have been implicated in increasing longevity³. Mitochondrial oxidative phosphorylation is the major cellular source of ROS. Superoxide anions (O₂⁻) are produced during small electron leaks mainly by the respiratory complexes I and III. They are cleared by superoxide dismutases, which convert them into H₂O₂. H₂O₂ can freely diffuse into the intermembrane space and into the cytosol, but is mostly converted to H₂O by mitochondrial catalase. If the equilibrium between ROS production and scavenging is disturbed, O₂⁻ and H₂O₂ accumulate⁴. Accumulation of ROS in the dopaminergic neurons of the substantia nigra as a result of dysfunctional mitochondria is considered as one of the main insults leading to degeneration of dopaminergic neurons in Parkinson's disease^{5,6}.

Mitochondria are highly dynamic and can form elongated tubes and networks or small, fragmented structures by undergoing cycles of fusion and fission. A key protein that coordinates mitochondrial quality control is PTEN Induced Putative Kinase 1 (PINK1), which is implicated in Parkinson's disease etiology⁷. PINK1 directly interacts with proteins that determine mitochondrial dynamics^{8,9}, among them the key player in mitochondrial fission, the large GTPase Dynamin-related-protein 1 (Drp1). PINK1 phosphorylates Drp1 at Ser616, which promotes its pro-fission activity. PINK1 further influences mitochondrial dynamics by inhibiting the pro-fusion factor Mitofusin 2⁹.

Mitochondrial fragmentation takes place at ER-mitochondria contact sites. Drp1 binds at these contact sites, and the ER structurally contributes to mitochondrial fission by wrapping itself around the fission site¹⁰. ER-mitochondria contacts are altered in PD patients and animal models, although reports on whether the contact increase or decrease are sometimes contradictory¹¹⁻¹⁴. Besides a role in mitochondrial fission, ER-mitochondria contacts also influence mitochondrial activity by allowing the exchange of calcium and phospholipids between both compartments, which also plays a role in Parkinson's disease pathology¹³.

Mitochondria produce energy by means of their electron transport chain: it contains five protein complexes that pump protons into the intermembrane space, thereby producing a proton gradient that drives an ATPase. The first and largest of the complexes is NADH:ubiquinone oxidoreductase or complex I, which oxidizes NADH and reduces ubiquinone and transports protons from the IMM to the intermembrane space¹⁵. In neurons of PD patients, complex I is functionally impaired and misassembled^{7,16}. Moreover, the complex I inhibitor rotenone, which has been used as a pesticide in agriculture, can induce PD in animal models, and PD is significantly more prevalent in people who were exposed to it in agriculture¹⁷. PINK1 is involved in the regulation of complex I: it mediates the phosphorylation of the complex I subunit NDUFA10 and thereby regulates its activity¹⁸. The phospholipids cardiolipin, phosphatidylethanolamine (PE) and phosphatidylcholine (PC), which are exchanged at ER-mitochondria contacts, support complex I function¹⁹; thus, ER-mitochondria communication might directly influence complex I activity. Strikingly, the functional relationship between ER-mitochondria contacts and complex I activity might be bidirectional: inhibition of complex I with rotenone increases the expression of tether proteins in neuroblastoma cells²⁰.

Genetically, PD is heterogenous: risk genes for familial forms of PD comprise *leucine rich repeat kinase 2* (LRRK), *PINK1* and *Parkin*. PD is characterized by aggregates in neurons, the Lewy bodies,

which contain α -synuclein folded into amyloid fibrils²¹. A recent study shows that Lewy bodies mostly contain organelles and membrane fragments, highlighting the importance of organelle quality control and neuronal organelle trafficking in PD insurgence²². CRELD1 is one of 10 genes that was identified as a PD risk gene in a combined proteomics and RNA-Sequencing approach in samples from the prefrontal cortex of PD²³.

Proteins of the Creld family (cysteine-rich with EGF-like domains) are ER proteins that have been reported to regulate ER stress response and untranslational protein response. CRELD1 is a risk gene for atrioventricular septal defects (AVSD) and murine CRELD1 is required during heart development²⁴. A recent study links human CRELD1 to T-cell survival²⁵. Mechanistic studies in *C. elegans* show that Creld1 is required for the maturation and assembly of acetylcholine receptors in the ER²⁶. The role of Creld proteins in PD pathology is unknown.

Here we show that *Drosophila* Creld is required for ER-mitochondria communication, mitochondrial dynamics and ROS signaling. Loss of Creld leads to failure of mitochondrial dynamics regulation and blocks mitochondrial fission. This results in increased mitochondrial abundance but decreased activity of the respiratory complex I. The subsequent lack of mitochondrial ROS inactivates dopaminergic neurons. We show that Creld is required for the transfer of phospholipids at ER-mitochondria contact sites and acts in parallel to the Pink1/Parkin pathway. In a study bridging from insect to vertebrate animal models to human cells, we describe the function of a novel PD risk factor whose mutation results in severe locomotion deficits due to decreased rather than increased ROS levels.

Results

Creld is conserved between fly and human

Cystein-rich with EGF-like domains (Creld) proteins belong to a family that is evolutionary conserved across species. Mammalian Creld proteins contain a signaling peptide at the N-terminus, a characteristic tryptophan and glutamic acid-rich (WE) domain, two epithelial-growth factor (EGF)-like and two calcium-binding domains (Fig. 1A, ²⁷). *Drosophila* Creld shows high conservation to both the human CRELD1 and CRELD2: protein sequence identity is 44,1% and 43%, respectively, and 89% and 78% identity in the WE domain (Fig. S1A). The *Drosophila* Creld protein contains a transmembrane domain like its human counterpart CRELD1, which according to protein domain prediction anchors it in the ER membrane, with the N-terminus facing the lumen. The *Drosophila* genome contains only one gene for Creld, which results in two peptides, Creld-PA and Creld-PB, by alternative splicing. We raised an antibody against the conserved WE-domain of Creld and found that it co-localized primarily with the ER-marker KDEL (Fig. S1B). We generated a Creld mutant (Creld^{Δ51}) by homologous recombination (Fig. S1C) and found that it is a transcript null for both *Creld* transcripts (Fig. S1D). Creld mutants are homozygous viable, but show slightly lower survival rates on a control diet, which is further reduced on a sugar-depleted diet (Fig. S1E). The lifespan of adult Creld mutants is shortened by ~ 50 % (Fig. S1F).

Figure 1: Impaired locomotion, increased diastole and mitochondrial accumulation in Creld mutants. A) Domain structure of human CRELD1 and 2 and Drosophila Creld. B) Single images from videos of w^{1118} and Creld mutants, showing their walking behavior. C) Startle-induced negative geotaxis (SING) assay of 2 days old w^{1118} and Creld mutants. D) M-mode tracing of pixel movement of the adult heart. E) Quantification of diastole, systole ($ns: p=0.43$) and heart period. F) Heart rate of w^{1118} and Creld mutant flies. G) Transmission electron

micrographs of abdominal tissue of *w¹¹¹⁸* and *Creld* mutant adults. Sections show myocardial cells. H)
Transmission electron micrographs of thorax tissue of *w¹¹¹⁸* and *Creld* mutant adults. Sections show indirect
flight muscles. Asterisks represent ****p*<0.001, ns: not significant

Loss of *Creld* leads to severe locomotion impairment

Adult *Creld* mutants show a strong locomotion deficit: they climb slowly, and are almost unable to fly. *Creld* mutants show a distinct walking behavior: while wildtypic flies show coordinated walking movements with an upright body position and a body axis of about 45° in relation to the surface, *Creld* mutants show uncoordinated walking movements and a horizontal body position, which causes them to fall on their heads frequently (Fig. 1B and video S1 and S2). We used the startle-induced negative geotaxis (SING) assay to measure adult locomotion and found that *Creld* mutants are hardly able to climb (Fig. 1C). Ubiquitous expression of RNAi directed against *Creld* also impairs the climbing performance (Fig. S2A). An independent allele generated by Crispr/Cas9-mediated k.o. shows the same phenotype (Fig. S2B, C).

Prolonged heart resting phase shows energy deficit in *Creld* mutants

Mutations in human CRELD1 are associated with heart development defects²⁸, and mouse *Creld1* mutants show defects in the proliferation of endocardial cells²⁹. The insect heart is morphologically different from the mammalian heart, but contains valves and cardiac cells. Flies do not have a closed, but an open circulatory system, and the heart pumps the hemolymph through the body to supply organs with nutrients and signaling molecules³⁰. We asked whether *Creld* mutants had heart defects and analyzed heart morphology and function. We found that heart development is normal, as is shown in 3rd instar larvae which show no morphological differences in the valve-like ostial cells (Fig. S2D). Next, we analyzed heart function in adult flies. M-mode recordings from semi-intact *Drosophila* heart preparation and digital high-speed movie analysis show that the heart rate is regular, but with a longer total heart period in *Creld* mutants compared to control flies (Fig. 1D). Upon quantification of the heart rate, we found that the total heart period is ~ 300 ms in wildtypic flies and ~ 400 ms in *Creld* mutants, and that this prolongation of the heart rate is due to a longer diastolic interval. The systole duration of the heart period is unaltered in *Creld* mutants, but the diastole duration increases from ~ 100 ms in wildtypes to ~ 250 ms in *Creld* mutants (Fig. 1E), and the heart rate is reduced from 3.5 to 2.5 Hz (Fig. 1F). We thus conclude that the contractility of the heart is not disturbed, but that the resting phase between the contraction is delayed. Prolonged heart rates and an increased diastolic interval is associated with a deficiency in energy supply³¹. We thus hypothesize that *Creld* mutants are not sufficiently supplied with ATP. Analysis of phosphorylated AMP-activated protein kinase (AMPK) confirmed that levels of phospho-AMPK are highly increased in protein extracts from adult *Creld* mutants compared to wildtypes (Fig. S2E, F). We conclude that heart development in *Drosophila* *Creld* mutants is normal, but that heart rate and diastolic interval are prolonged due to an energy deficit.

Mitochondria accumulate and elongate in *Creld* mutants

Mitochondria are a major source of energy in a cell. To investigate the energy crisis that provokes impaired locomotion and heart rate prolongation in *Creld* mutants, we analyzed mitochondrial morphology by transmission electron microscopy (TEM) for ultrastructural analysis. We found that in

myocardial cells, mitochondria are more abundant and elongated in Creld mutants compared to wildtype (Fig. 1G). We also used TEM for ultrastructural analysis of mitochondria in indirect flight muscles (IFM) and found that cristae structure is unchanged in mitochondria of Creld mutants, but that mitochondria are more abundant and enlarged (Fig. 1H). Both transversal and longitudinal sections of IFM show that in Creld mutants, mitochondria occupy all the space between the muscle fibers, whereas single mitochondria are well discernible in the wildtype. Our results indicate that in Creld mutants, there is a defect in mitochondrial network regulation and an imbalance towards mitochondrial fusion.

Mitochondrial hyperfusion upon Creld dysfunction is conserved

Members of the Creld protein family have not been associated with mitochondrial dynamics before. To assess whether mitochondrial fusion under Creld1 dysfunction can also be observed in vertebrates, we used the claw frog *Xenopus tropicalis* as a model. We injected *X. tropicalis* embryos with control or Creld1 morpholinos and a mito-GFP construct in the 2-cell stage and analyzed the mitochondrial morphology in bodywall muscles at stage 45. We found that muscles of animals injected with the control morpholino showed a balance between fused and fragmented mitochondria, while in animals injected with the Creld1 morpholino, mitochondria are mostly present as elongated and fused (Fig. 2A). We calculated the mitochondrial fragmentation index and found that it is significantly reduced in muscle cells of animals injected with the Creld1 morpholino (Fig. 2B). To test if a similar phenotype can be observed in mammalian cells, we used a double-stranded RNA construct against CRELD1 in HeLa cells. We stained control and dsRNA-treated cells with an antibody against Tom20, a receptor in the outer mitochondrial membrane³², and found that also in HeLa cells, mitochondria are mostly fragmented in controls, but are fused, elongated and network-like in cells that knock down CRELD1 (Fig. 2C). Our results indicate that loss of Creld leads to defects in mitochondrial network regulation and an imbalance towards mitochondrial fusion in various tissues and species.

Less association of the pro-fission factor Drp1 with mitochondria

Mitochondrial fission is regulated by Dynamin-related protein 1 (Drp1), which itself is regulated by several kinases and phosphatases. To assess whether Drp1 locates to mitochondria, we expressed a Drp1-HA fusion protein in *Crel*d mutants and compared the presence of Drp1-HA in the cytoplasmic and the mitochondrial fraction. After normalization to the protein content of the fractions, we found that 80% of Drp1-HA is found in the mitochondrial fraction in heterozygous controls. In *Crel*d mutants, the mitochondrial portion of Drp1 is reduced, and only ~60% associate with mitochondria, while ~40 % is found in the cytoplasmic fraction (Fig. S3 A-D). This shows that due to missing *Crel*d action, Drp1 locates less to mitochondria.

*Crel*d is required for the activation of the Drp1 phosphatase Calcineurin in mammals^{29,33}. To elucidate the *Crel*d-Calcineurin interaction in flies, we fed adult control flies and *Crel*d mutants with the Calcineurin inhibitor Cyclosporin (CsA). Treatment with CsA strongly reduces the climbing ability of wildtypes and thus mimics the phenotype of *Crel*d mutants (Fig. 2I). However, CsA treatment further reduces the climbing performance of *Crel*d mutants, suggesting that an additional function of *Crel*d, apart from activating Calcineurin, induces the locomotion phenotype. Drp1 can be activated by phosphorylation by PTEN-induced kinase 1 (PINK1,⁸). We overexpressed a constitutively active form of PINK1 (PINK1.C) under the control of the *tub*-Gal4 driver and found that it rescues the climbing performance of homozygous *Crel*d mutants (Fig. 2E). This PINK1-mediated rescue was maintained after treatment with CsA (Fig. 2E). This shows that *Crel*d interacts with PINK1, in addition to Calcineurin, to regulate mitochondrial dynamics.

*Crel*d rescues the locomotion deficit of PINK1 mutants

Human CRELD1 was identified as a Parkinson's disease (PD) risk gene²³, and the severe locomotion deficit is reminiscent of the PD phenotype in fly models. Moreover, expression of constitutively active PINK1 rescues the climbing performance of *Crel*d mutants. We asked if expression of *Crel*d would rescue the locomotion deficit of PINK1 mutants. We expressed the genetic rescue construct of *Crel*d under the control of the ubiquitous *actin*-Gal4 driver in PINK1 mutants and found that it significantly increases the climbing ability of PINK1 mutants (Fig. 2K). PINK1 recruits Parkin to mitochondria in the regulation of mitochondrial quality control. Heterozygous *Crel*d and Parkin mutants show similar climbing performance. Heterozygous double mutants for *Crel*d and Parkin show heterogenous, but not reduced climbing ability. Double mutants heterozygous for *Crel*d and homozygous for Parkin show no further reduction of the climbing ability compared to homozygous Parkin mutants. By contrast, double mutants homozygous for *Crel*d and heterozygous for Parkin show significantly worsened climbing behavior compared to *Crel*d mutants (Fig. 2L). We did not find flies homozygous for both mutations and assume that the combination is lethal. Our results suggest that *Crel*d modulates the PINK1-Parkin pathway of mitochondrial quality control.

*Crel*d is required in neurons for normal locomotion

To explore the connection between the defects in mitochondrial fission, locomotion and heart, we reintroduced *Crel*d function genetically in specific tissues. We generated a genetic rescue construct that allows for the expression of the full-length protein of *Crel*d under the control of the binary Gal4/UAS system. Homozygous *Crel*d mutants that only carry the rescue construct, but no Gal4 driver, show impaired climbing similar to homozygous *Crel*d mutants. Heterozygous *Crel*d mutants

show wildtypic climbing performance. We found that expression in the heart with the *handC*-Gal4 driver fails to rescue the climbing ability of *Credl* mutants. Expression in muscles with the *mef*-Gal4 driver partially rescues *Credl* mutants climbing. Similarly, ubiquitous expression of the genetic rescue construct with *tubulin*-Gal4 slightly but significantly rescues the climbing performance of homozygous *Credl* mutants (Fig. 3A). Intriguingly, expression of the genetic rescue construct in the nervous system by using the postmitotic neuron driver *elav*-Gal4 results in a strong rescue and markedly improves the climbing ability of homozygous *Credl* mutants (Fig. 3A). To further dissect the rescue in neurons, we used drivers specific for distinct neuron groups. Expression of the rescue construct restricted to the insulin-producing cells (*dilp2,3*-Gal4), a group of median neurosecretory cells organized in two clusters in the *pars intercerebralis* of the adult brain that secrete the insulin-like peptides 2, 3 and 5³⁴ fails to rescue the climbing deficit of *Credl* mutants (Fig. 3B). By contrast, use of the *D42*-Gal4 driver, which directs the expression of the rescue constructs to motor neurons³⁵, resulted in a complete rescue of the climbing deficit. Dopaminergic neurons are expressed in distinct clusters in the larval and adult brain³⁶ and integrate motor programs³⁷. In humans, dopaminergic neurons modulate locomotion by innervating the brain stem, and locomotion defects in Parkinson's disease (PD) are caused by degeneration of these neurons³⁸. This mechanism is strongly conserved, and also flies present impaired locomotion and other PD symptoms upon loss of dopaminergic neurons³⁹. We used tyrosine hydroxylase (*TH*)-Gal4 to direct the expression of the rescue construct to dopaminergic neurons and found that it rescues the locomotion impairment of homozygous *Credl* mutants (Fig. 3B). Our findings indicate that loss of *Credl* affects locomotion because its function is required in motor neurons and dopaminergic neurons, not the heart, and only to a minor degree in muscles.

Figure 3: *Credl* is required in neurons for normal locomotion. A) Startle-induced negative geotaxis (SING) assay of animals expressing UAS-*Credl*-HA in specific tissues. Climbed distance in mm was measured after 6 s. Genotypes are (from left to right) *Credl* -/-; UAS-*Credl*-HA/+, *Credl* +/- or -/- as indicated; *tubulin*-Gal4/UAS *Credl*-HA, *Credl* +/- or -/-; *mef*-Gal4; UAS-*Credl*-HA, *Credl* +/- or -/-; *handC*-Gal4; UAS-*Credl*-HA, *Credl* +/- or -/-; *elav*-Gal4; UAS-*Credl*-HA. ANOVA with $F=46.1$ and 195 degrees of freedom. B) SING assay of animals expressing UAS-*Credl*-HA in specific neurons. Climbed distance in mm was measured after 6 s. Genotypes are *Credl* -/-; UAS-*Credl*-HA/+, *Credl* +/- or -/- as indicated; *elav*-Gal4/UAS *Credl*-HA, *Credl* +/- or -/-; *dilp2,3*-Gal4; UAS-*Credl*-HA, *Credl* +/- or -/-; *D42*-Gal4; UAS-*Credl*-HA, *Credl* +/- or -/-; *TH*-Gal4; UAS-*Credl*-HA. ANOVA with $F=38.167$ and 168 degrees of freedom. Asterisks represent * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: not significant

Dopaminergic neurons are inactive in *Credl* mutants

Locomotion deficits in PD patients as well as PD fly models are elicited by degeneration of dopaminergic neurons. However, we found no loss of dopaminergic neurons in *Crel*d mutants (Fig. S4A, B), nor any sign of neurodegeneration in histological sections (Fig. S4C). The number of apoptotic cells in the brain is similar in wildtypic flies and *Crel*d mutants (Fig. S4D). To evaluate mitochondrial morphology in dopaminergic neurons, we expressed a construct that carries the transmembrane domain of the mitochondrial protein Miro tagged with mCherry, and thus labels the outer mitochondrial membrane (mCherry-mitoOMM), under the control of *TH*-Gal4. We found that mitochondria are round and fragmented in heterozygous *Crel*d mutants, but that the mCherry signal accumulates and shows mitochondria fused and elongated in homozygous *Crel*d mutants (Figure 4A). Next, we asked if dopaminergic neurons are less active in *Crel*d mutants. We used the Calcium-modulated photoactivatable ratiometric integrator CaMPARI2⁴⁰ for calcium imaging of active dopaminergic neurons. We found that upon stimulation with UV light for 30 s and 4 min after stimulation, dopaminergic neuron somata show conversion of green to red in wildtypic controls, but less conversion in *Crel*d mutants. This indicates that dopaminergic neurons are less active, rather than degenerated, in *Crel*d mutants (Fig. 4B, C). Dopaminergic neuron loss in PD fly models is induced by oxidative stress, elicited by old and dysfunctional mitochondria. We thus asked if reactive oxygen species (ROS) are altered in *Crel*d mutants. Mitochondrial ROS form during mitochondrial respiration and are a byproduct of mitochondrial metabolism, but also serve as important signaling molecules. ROS are indispensable for cell differentiation and neuronal activity, including motor neurons⁴¹. We tested if the observed mitochondrial phenotype led to reduced ROS levels. We used the superoxide indicator DHE and the mitochondria-specific superoxide dye MitoSOX to assess superoxide levels in *Crel*d mutants. We found that overall superoxide was reduced. By contrast, mitochondrial superoxide levels as stained by MitoSOX were increased (Figure 4D). Superoxide anions are relatively stable and remain in the mitochondrial matrix and intermembrane space, while hydrogen peroxide can diffuse freely into the cytoplasm⁴. Thus, we next measured hydrogen peroxide, a product of superoxide dismutation by Sod 1 and 2, with AmplexRed, and found that hydrogen peroxide is drastically reduced in *Crel*d mutants (Figure 4E). We thus concluded that in mitochondria of *Crel*d mutants, superoxide accumulates but is not degraded to hydrogen peroxide. Also in extracts of *X. tropicalis* embryos that knock down *Crel*d1, hydrogen peroxide is reduced (Figure S5A). We measured the transcript levels of ROS-scavenging enzymes and found that catalase and NOS are expressed at wildtypic levels, while superoxide dismutase 1 and 2 are significantly downregulated (Figure 4F). To induce ROS formation in *Crel*d mutants, we expressed Sod1 under the control of the ubiquitous *arm*-Gal4 driver and found that it rescues the climbing performance of *Crel*d mutants (Figure 4G), and ubiquitous expression of PINK1 and Sod1 restores hydrogen peroxide to control levels (Figure 4H). We tested if modulation of hydrogen peroxide in dopaminergic neurons was sufficient to restore the climbing of *Crel*d mutants. Upon induction of hydrogen peroxide formation by expression PINK1, Sod1 or downregulation of catalase, the locomotion deficit was abolished. When hydrogen peroxide formation was repressed by downregulation of Duox, climbing was not restored (Figure 4I). Our results show that aberrant ROS signaling underlies the locomotion deficit in *Crel*d mutants, and suggest that missing ROS lead to inactivity of dopaminergic neurons.

Figure 4: Low H₂O₂ impairs dopaminergic neuron function. A) PPL1 cluster of dopaminergic neurons labeled by mCherry-mitoOMM under the control of TH-Gal4. Genotypes are Cred^{+/+}; TH Gal4/UAS mCherry-mitoOMM and Cred^{-/-}; TH Gal4/UAS mCherry-mitoOMM. mCherry was detected with a DsRed antibody. B, C) Calcium imaging in PPL2 clusters of dopaminergic neurons with UAS CaMPARI2. Images were taken after 30 s UV light induction and 4 min photoconversion. Genotypes are Cred^{+/+} or ^{-/-}; TH Gal4/UAS CaMPARI2. D) Adult gut tissue was stained with dihydroethidium (DHE) to label superoxide anions. Adult gut was stained with mitoSOX to

label mitochondrial superoxide anions. E) Hydrogen peroxide was measured in extracts of whole adults with AmplexRed (n=8). F) Transcript levels of ROS-scavenging enzymes in *Credl* mutants, fold change normalized to *w¹¹¹⁸* (n=8). G) SING assay of *Credl* mutants ubiquitously expressing UAS *Sod1*. Genotypes are *Credl*^{-/-}; *armadillo Gal4/+*, *Credl/+* or *-/-*; *armadillo Gal4/UAS Sod1*. H) Hydrogen peroxide was measured in extracts of whole adults with AmplexRed. Genotypes are *Credl*^{-/-}; *armadillo Gal4/+*, *Credl/+* or *-/-*; *armadillo Gal4/UAS PINK1.C* and *Credl/+* or *-/-*; *armadillo Gal4/UAS Sod1* (n=12). I) SING assay of *Credl* mutants expressing *PINK1*, *Sod1*, *catalase-RNAi* or *Duox-RNAi* in dopaminergic neurons. Genotypes are *Credl*^{-/-}; *TH-Gal4/+*; *Credl/+* or *-/-*; *TH-Gal4/UAS PINK1.C*, *Credl/+* or *-/-*; *TH Gal4/UAS Sod1*, *Credl/+* or *-/-*; *TH-Gal4/UAS catalase-RNAi*, *Credl/+* or *-/-*; *TH-Gal4/UAS Duox-RNAi*. ANOVA with F=33.627 and 145 degrees of freedom. J) Oxygen consumption of larval bodywall preparations of *w¹¹¹⁸* and *Credl* mutants treated with pyruvate and malate and rotenone (n=9). K) SING assay of *w¹¹¹⁸* and *Credl* mutants treated with vehicle (DMSO) and rotenone. ANOVA with F=56.2 and 160 degrees of freedom. Asterisks represent ***p<0.001, ns: not significant

Mitochondrial activity is impaired at the level of respiratory complex I

A major source of superoxide and hydrogen peroxide in mitochondria is the respiratory complex I, which is regulated by *PINK1*⁴². We analyzed mitochondrial respiration in larval body wall muscles using a Seahorse analyzer (Agilent). We found that both wildtypic and *Credl* mutant tissue react to injection of pyruvate and malate as substrates for complex I by increasing mitochondrial respiration, but that only oxygen consumption in wildtype tissue can be blocked with rotenone. Oxygen consumption in *Credl* mutant tissue remains at high levels upon rotenone treatment, which indicates that complex I is inactive (Fig. 4J). Feeding wildtypic adults with sub-lethal levels of rotenone (100 μ M) decreases their climbing performance, but not to the extent of *Credl* loss-of-function (Figure 4K). By contrast, the poor climbing performance of *Credl* mutants is not further affected by rotenone, and even ameliorates slightly. Rotenone treatment lowers the levels of hydrogen peroxide in wildtypes, but not in *Credl* mutants (Fig. S5B). This shows that the climbing deficit of *Credl* mutants can be attributed to low complex I activity. Our results show that in *Credl* mutants, ROS production by complex 1 is inhibited.

Complex I inhibition enhances ER-mitochondria contacts

ER-mitochondria contacts regulate mitochondrial dynamics and are sites of material exchange. Drp1 binds at ER-mitochondria contacts to induce fission, and *PINK1* and *Parkin* accumulate at contact sites in the regulation of mitophagy. Transfer of phospholipids and calcium ions (Ca²⁺) between ER and mitochondria takes place at their contact sites and is required for mitochondrial function: for example, phospholipids support the function of respiratory complex I¹⁹. Human *CRELD1* locates primarily in the ER²⁵, and *Drosophila Credl* also localizes mostly to the ER (Fig. S1B). We thus asked how *Credl* affects the function of respiratory complex I from its ER position. To analyze mito-ER contacts by imaging, we expressed a mCherry-mitoOMM construct in dopaminergic neurons and co-stained with an antibody against the ER-marker KDEL and performed colocalization analysis on single confocal sections of single somata of dopaminergic neurons. In wildtypes, KDEL signal intensity is low and colocalization of ER and mitochondria markers is rare (Fig. 5A, B). To inhibit complex I activity, we treated the flies for 48 h with sublethal concentrations of rotenone. Upon rotenone treatment, KDEL signal intensity increases especially in the somata of dopaminergic neurons, resulting in enhanced colocalization of ER and mitochondria markers (Fig. 5C, D, I). We asked if *Credl* is required under conditions of low complex I activity and measured the transcript levels of *Credl* under different conditions in adult wildtype flies. We found that while under reduction of dietary sugar or upon starvation, *Credl* transcript levels remain stable, they increase upon feeding with rotenone

(Figure S5C). We expressed a UAS-Creld construct tagged with HA in dopaminergic neurons and co-stained with an antibody against endogenous Creld. We found that upon rotenone feeding, the signal intensity of both the ectopically expressed Creld and endogenous Creld dramatically increases (Figure S5D). Our results show that Creld is required under conditions of low complex I activity, and that complex I activity modulates ER-mitochondria contacts.

Figure 5: Creld and complex I activity modulate ER-mitochondria contact. A) PPL1 cluster of dopaminergic neurons expressing mCherry-mitoOMM, stained with anti-DsRed and anti-KDEL in +/+; TH-Gal4/UAS mitoOMM. B, D, F, H) Regions of interest (ROI) of single somata of dopaminergic neurons after colocalization analysis. C) PPL1 cluster of dopaminergic neurons expressing mCherry-mitoOMM, stained with anti-DsRed and anti-KDEL in +/+; TH-Gal4/UAS mitoOMM after treatment with rotenone. E, G) PPL1 cluster of dopaminergic neurons expressing mCherry-mitoOMM, stained with anti-DsRed and anti-KDEL in Creld/+; TH-Gal4/UAS mitoOMM (A) and Creld-/-; TH-Gal4/UAS mitoOMM. Arrowheads show colocalization of ER and mitochondria. I) Pearson's correlation coefficient of KDEL/mitoOMM colocalization of +/+; TH-Gal4/UAS mitoOMM (n=9). J) Pearson's correlation coefficient of KDEL/mitoOMM colocalization in Creld/+ or -/-; TH-Gal4/UAS mitoOMM (n=12/15). K) PPL1 cluster of dopaminergic neurons expressing an artificial tether construct (mitoER-tether-RFP) stained with α -Creld. Asterisks represent * $p < 0.05$, *** $p < 0.001$

Creld localizes to ER-mitochondria contacts

Complex I and mitochondrial dynamics are inhibited in Creld mutants, and complex I inhibition enhances ER-mitochondria contacts. To investigate ER-mitochondria contacts in Creld mutants, we used the mCherry-mitoOMM construct in dopaminergic neurons and co-stained with an antibody against the ER-marker KDEL. We found that in heterozygous controls, ER and mitochondrial markers show some few points of colocalization (Fig. 5 E, F). In homozygous Creld mutants, the KDEL signal intensifies specifically in the somata of the dopaminergic neurons and colocalization between ER and mitochondrial markers increases, similar to rotenone-treated wildtypes (Fig. 5G, H, J). To address if endogenous Creld is located at ER-mitochondria contact sites, we expressed a synthetic mito-ER tether¹¹ in dopaminergic neurons. Tethering sites are marked with RFP in this line, and we found large spots of the RFP signal in the somata of dopaminergic neurons. The endogenous Creld signal colocalizes with these spots (Figure 5K). Our results show that contacts between ER and mitochondria increase upon loss of Creld and upon complex I inhibition, and that Creld localizes to ER-mitochondria contact sites in wildtypes.

Phospholipids accumulate at mitochondria-associated membranes (MAMs) in Creld mutants

ER-mitochondria contacts have been identified as the so-called mitochondria-associated membranes (MAMs): crude mitochondrial fractions contain fragments of ER-membranes. Phospholipids are transferred at ER-mitochondria contacts, and the phospholipids phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are required for the function and stability of the respiratory complex I¹⁹. We thus asked if phospholipids can be transferred to mitochondria in Creld mutants. We isolated the cytoplasmic fraction, containing also plasma membrane, ER and lysosomes, the crude mitochondrial fraction comprising mitochondria and MAMs, and the MAM fraction by ultracentrifugation and subjected the fractions to lipidomics analysis. Importantly, we measured a technical sample containing only the buffers for each fraction and normalized the obtained data to the corresponding technical sample. We found that the total lipid content is reduced in all fractions in Creld mutants, but that phospholipids are at similar levels in the cytoplasm-ER fraction. Phospholipids are reduced in the mitochondrial fraction, but accumulate in the MAM fraction. PE also increases in the MAM fraction of Creld mutants. PC is strongly reduced in mitochondria, but unchanged in MAMs (Figure 6 A-C). Together, the data from our lipidomics analysis suggest that transfer of phospholipids from the ER to mitochondria is impaired, and that PE and PC, which are required for complex I activity, get stuck at ER-mitochondria contacts. This also suggests that while ER-mitochondria contacts increase

in *Credl* mutants, they might be less functional (Fig. 6D-F). We propose that *Credl* functions under conditions of low complex I activity to facilitate phospholipid flux at ER-mitochondria contacts.

Figure 6: Phospholipid transfer at ER-mitochondria contacts is impaired in *Credl* mutants. A) Total lipid, phospholipid, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) content of fractions containing the cytoplasm, ER, plasma membrane and lysosomes. Fold change of extracts of *Credl* mutant flies normalized to

w¹¹¹. B) Lipids as indicated in fractions containing mitochondria and MAM (crude mitochondria). C) Lipids as indicated in fractions containing MAM. n=200, n=2 per fraction. D-E) Model of Creld action under D) wildtypic, E) complex I-inhibited, F) Creld loss-of-function conditions.

Discussion

Parkinson's disease is characterized by the loss of dopaminergic neurons. It is believed that one of the main pathology driving factors is oxidative stress induced by failure of mitochondrial quality control, leading to neuron loss. In accordance with this theory, PINK1, one of the commonest risk genes for PD, induces clearance of old mitochondria, but also directly regulates the machinery of mitochondrial fusion and fission. However, more recent data implicates PINK1 also in a role as a direct regulator of mitochondrial function via phosphorylation of the respiratory complex I subunit NDUFA10^{18,42}. Here we present data on an unexplored PD risk gene, Creld, and show that Creld mutants exhibit a strong, PD-like locomotion deficit and mitochondrial hyperfusion, as well as genetic interaction with PINK1. In contrast to canonical PD models, dopaminergic neurons do not degenerate in Creld mutants, and oxidative stress in the form of hydrogen peroxide is absent. Consistently, mitochondrial ultrastructure appeared undamaged. Instead, dopaminergic neurons are inactive, and the locomotion phenotype can be rescued by reintroducing Creld function specifically in dopaminergic neurons, and similarly by increasing hydrogen peroxide production via overexpression of Sod1. ROS, especially mitochondrial hydrogen peroxide, have been recognized as important signaling molecules in recent years: hydrogen peroxide is produced as a consequence of dopaminergic neuron activity, but is also required for their activity^{43,44}. We argue that mitochondrial ROS signaling, especially hydrogen peroxide resulting from complex I activity, is required in dopaminergic neurons to support their function. This notion is supported by a study showing that reverse electron transport (RET) at complex I is not detrimental, but instead delays aging³. Furthermore, the PD causing pesticide rotenone is a specific and well-studied complex I inhibitor which specifically blocks RET at complex I and reduces the formation of hydrogen peroxide. Of note, we found that rotenone treatment provokes increased expression of Creld. We therefore conclude that cells require Creld under conditions of low complex I activity and/or low levels of hydrogen peroxide signaling from mitochondria. As Creld mutants show differences in the abundance and lipid composition of ER-mitochondria contact sites, we propose that it is needed to facilitate ER-mitochondria communication and phospholipid exchange, which fits to a known role of phospholipids in regulating complex I activity¹⁹. We show that Creld accumulates after rotenone treatment, and that rotenone inhibition of complex I induces the formation of ER-mitochondria contacts. When contacts are forced by use of a synthetic tether, Creld is enriched at the contact sites. In Creld mutants, contact sites can be formed, but phospholipid exchange is impaired; thus, low complex I activity cannot be alleviated, and the resulting drop in hydrogen peroxide levels impairs neuron function. Therefore, Creld mutants, despite their marked locomotion defect, do neither show loss of dopaminergic neurons nor other signs of neurodegeneration. However, we cannot rule out that dopaminergic neurons might degenerate eventually as a result of their inactivity in old Creld mutants.

Creld is an ER protein that in *C. elegans* has been shown to act as a chaperone for the assembly of acetylcholine receptors²⁶. Misassembly of acetylcholine receptors might account for a muscle-induced climbing deficit also in fly Creld mutants, but does not sufficiently explain mitochondrial hyperfusion and low complex I activity. It is conceivable that Creld might be required for the assembly of subunits of the respiratory complex I, a large protein complex comprising several subunits, or proteins that form the tether between ER and mitochondria.

To date, it is not entirely clear if loose or tight contacts between ER and mitochondria better support the function of the cell: the formation of the contacts is important for mitochondrial dynamics quality control, but increased contacts have been associated with depletion of phospholipids from the ER¹³. Furthermore, reduced contacts in flies as a result of knock-down of the tether protein Pdzd8 promotes mitochondrial turnover in neurons and decelerates age-related locomotion deficits⁴⁵. Our data suggest that Creld is required for the formation of functional contacts between ER and mitochondria to provide phospholipids for the support of complex I function. Complex I turns to its inactive state when oxygen is limiting¹⁵, but phases of low complex I activity might also occur during development and in dependence of nutrition⁴⁶. We suggest that Creld is required for dynamic ER-mitochondria contacts that enable the transfer of phospholipids to support complex I function and thus allows the formation of hydrogen peroxide as a signaling molecule for the activity of dopaminergic neurons.

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Author contribution

Conceptualization: JS, MHB; Methodology: MHB, JS, MP, NK, CT, AP; Investigation: MHB, MP, NK, GEF, SJMP, BS, CM, JK, IJ; Writing – Original Draft: MHB; Resources – MHB, RB; Writing – Review & Editing: MHB; Visualization: MHB; Funding Acquisition: MHB; Supervision: MHB, JS, AP, CT, RB.

Abbreviations

arm: armadillo; DHE: dihydroethidium; ER: endoplasmic reticulum; OMM: outer mitochondrial membrane; PD: Parkinson's disease; ROS: reactive oxygen species; TH: tyrosine hydroxylase; tub: tubulin

Material and Methods

Fly husbandry

Flies were reared on standard cornmeal food (130g yeast agar, 248g Baker's yeast, 1223g Cornmeal and 1.5 l sugar beet syrup in 20 l distilled water) and kept in a 25°C incubator with light-dark-cycle. Fly stocks were obtained from the Bloomington Drosophila Stock Center and the Vienna Drosophila Resource Center. For stainings and SING assays, 2-5 day old female flies were kept on control diet or on filter paper soaked with 10 % sucrose solution and 50 or 100 µM rotenone, or 20 µM CsA. We obtained the UAS-CaMPARI2 and the TH-Gal4 line from Andreas Schoofs and the UAS mito-ER tether

lines from Elena Ziviani. Other fly lines used in this study were *w*¹¹¹⁸ (Bloomington Drosophila Stock center (BDSC) # 6326), *elav-Gal4* (BDSC # 8760), *tubulin-Gal4* (BDSC # 5138), *mef-Gal4* (BDSC # 27390), *handC-Gal4*⁴⁷, *actin-Gal4* (BDSC # 4414), *armadillo-Gal4* (BDSC # 1561) UAS-Creld-HA (this study), UAS-mCherry-mitoOMM (BDSC # 66533), UAS-Drp1-HA (BDSC # 42208), UAS-sra-RNAi (BDSC # 27260), UAS-PKA-C3-RNAi (BDSC # 27569), UAS PINK1.C (BDSC # 51648), UAS-Sod1 (BDSC # 24750), PINK1⁵ (BDSC # 51649) and *park*^{25 48}.

Creld mutant generation

The Creld^{Δ51} mutant was generated by homologous recombination. 5' and 3' homology arms (HA) were cloned into the pGX-attP vector⁴⁹ and injected into *w*¹¹¹⁸ embryos. Primers were 5' HA fwd: *GCGGCCGCCTTATTCGTATAGAACTTTGCCCG*, 5' HA rev: *GCGGCCGCTTTAACCGGCAAACCTATCTGAG* (both including NotI restriction sites), 3' HA fwd: *TGGCGCGCCAAATGATACGGCAACTCTCC*, 3' HA rev: *TGGCGCGCCAGTCCGCAGTGATTCAAACCTC* (both including AsclI restriction sites). Creld was deleted by homologous recombination and replaced by the mini-white ORF and an attP site. The white gene was removed by Cre-recombinase. The resulting mutants are transcript-null. Expression of the neighboring genes *zir* and *nhe1* was tested and found to be at wildtype levels. We generated a Crispr/Cas9-mediated mutant allele (Creld 12-2) by non-homologous end-joining. Primers were *Creld_fwd*: *CTTCGCCTGTTCGCCGCCCTGCA* and *Creld_rev*: *AAACTGCAGGGCGCGGAACAGGC*. Annealed oligos were cloned into the *pU6-BbsI* vector and sent to BestGene Inc. (USA) for injection into *vas-Cas9* (BDSC # 55821). Mutants were identified by high-resolution melting analysis and sequencing. For the rescue construct, we designed a gblock containing a UAS coding sequence, the open reading frame for the Creld gene and an HA tag (Integrated DNA Technologies, IDT). Cloning of the gblock construction was done by using EcoRI and NotI, followed by ligation into the pGE-attBGMR vector⁴⁹. Clones were sequenced to confirm successful integration and the plasmid was sent to BestGene Inc. (USA) for injection.

Determination of lifespan

For determination of the lifespan of *w*- and Creld mutant flies a longevity assay was performed. Flies were separated by sex and transferred to Longevity (LG) food (37.5 g Baker's yeast, 10 g Agar Kobe I, 300 mL dH₂O; 3ml of a 10 % Nipagin (in 70% EtOH) and 37.5 g glucose were added after autoclavation) with 20 flies per tube. Number of viable and dead flies was controlled every day and LG food was changed every two days.

Semi-intact Drosophila heart preparation and digital high-speed movie analysis

All dissection steps were done in artificial hemolymph (108 mM NaCl₂, 5 mM KCl₂, 2 mM CaCl₂ 2xH₂O, 8 mM MgCl₂ 6xH₂O, 1 mM NaH₂PO₄, 4 mM NaHCO₃, 15 mM HEPES, 1 mM sucrose, 0.5 mM Trehalose, pH 7.1). The flies were anesthetized with fly-nap, followed by transfer to a Petri dish coated with Vaseline for dissection. After dissection, the submerged hearts were kept in oxygenated hemolymph for 15 min at room temperature for equilibration. The heart movements were recorded with a digital high-speed camera (connected to a Leica DM-LB microscope with a 10x water immersion lens) and Fire capture Red 1.2 software. Movie analysis of the heart activity is then carried using the MATLAB R2010a software. Statistical analysis was done using Microsoft Excel. Images of morphology of semi-dissected fly hearts were taken by screenshots of the videos described above.

Startle-induced negative geotaxis (SING) assay

For the analysis of the adult climbing performance, we used the startle-induced negative geotaxis assay. Flies show an innate escape response after being tapped to the bottom of a cylinder (negative geotaxis), and by connecting several tubes that allow simultaneous recording of several genotypes or conditions, the climbing performance can be monitored reproducibly (Gargano et al., 2005). 5-10 female flies were collected and allowed to recover from CO₂ anesthesia for 24 h. On the day of the experiment, flies were transferred into glass tubes for climbing. After 5 min of conditioning, flies were tapped to the bottom of the tubes and their climbing was recorded. The climbing apparatus was millimetrically marked to measure the climbing of the flies. Mp4 files were converted to JPEG using VideoProc – video converter or VirtualDub. The height climbed by each individual fly was measured after 3, 6 and 10 s with ImageJ.

Transmission electron microscopy (TEM)

Adult female flies were aged to 10 days on standard food. Heart tubes were dissected with the semi-dissection method described in section 2.2.9. For the imaging of indirect flight muscles, the thorax of the flies was cut open. Dissection was carried on in artificial hemolymph on ice. The tissue was fixed in TEM fixation buffer (2.0 % paraformaldehyde and 2.5% glutaraldehyde (both electron microscopy grade) in 0.1 M Na-Cacodylate buffer, pH 7.4) for 4h. Fixed specimens were further processed as previously described^{50,51}. In brief, samples were postfixed in 1% osmium tetroxide in PBS for 1h, dehydrated in a graded ethanol series up to 100% ethanol and transferred into mixture of 100% ethanol and propylenoxide (1:1) for 10 min. Afterwards samples were rinsed in mixture of propylenoxide and Epon 812 and finally embedded in Epon 812. Polymerization was carried out at 60 °C for 72h. For transmission electron microscopy ultra-thin sections of 70 nm were prepared with a diamond knife on a Leica UC 6 microtome. Mounted sections were contrasted with 2% uranyl acetate and lead citrate and finally investigated with a Zeiss 902 transmission electron microscope.

***Xenopus tropicalis* husbandry and mitochondria labeling**

2 cell stage embryos of *X. tropicalis* were obtained by natural mating using wild-type mature male and female frogs. Embryos were raised in 1/9x Modified Frog Ringers (1/9x MR) at 25°C. The stage of the embryos was defined according to Nieuwkoop and Faber (1994) (NF). To label mitochondria in the somatic muscle of *X. tropicalis*, 2 cell stage embryos (NF stage 2) were injected with 4 nl of 100 ng/μl mito-eGFP reporter construct were injected into 1 blastomere. The embryo was raised in 1/9x MR buffer, and kept at 25°C. Screening of eGFP+ injected embryos was performed under an epifluorescence microscope after 24 hrs. of the injection. Half body fluorescent animals were selected and kept in PTU buffer (0,03 g/L phenylthiourea in 1/9x MR buffer) at 25°C. Larvae at NF stage 40 was anesthetized and fixed in PFA 4% overnight at 4°C. After PBS washes, the nuclei were stained with DAPI (1:5000). Mitochondria fragmentation index (MFI) was calculated blind using ImageJ software. Briefly, images were first thresholded and converted to binary images, eGFP+ muscle cells were delimited, and then the particle number was quantified. The MFI was calculated by dividing the number of particles by the total area of mitochondria.

Cell culture

HeLa cells were grown in T-75cm² flasks using DMEM medium (Thermo Fisher Scientific), and Penicillin-Streptomycin 1x (Thermo Fisher Scientific), supplemented with 10% FBS, in an atmosphere of 37°C and 5% CO₂. HeLa cells were subcultured 3 times a week at a ratio of 1:3 and 1:5 depending on the confluence. Cell density was determined using a hemocytometer. Cells were detached using 2.5mL of Trypsin-EDTA solution (Thermo Fisher Scientific). Experiments were conducted between passages 5 to 20. DsRNA were resuspended as described by the manufacturer. On the day of the

experiment, 10nM of dsRNA were reverse transfected into 10.000 HeLa cells per well of an 8 well tissue culture chamber (SARSTEDT) using Lipofectamine RNAiMAX as transfection reagent. Cells used for transfection were grown in media without antibiotics and imaged after 48 hours.

Oxygen consumption assay

We used an Agilent Seahorse XFe96 to measure the oxygen consumption rate (OCR) of larval bodywall tissue. Cartridges were watered and equilibrated according to the manufacturer's instruction. Plates were coated with poly-L-lysine. Bodywall muscles of 3rd instar larvae were dissected in HL3A buffer (115 mM sucrose, 70 mM NaCl, 20 mM MgCl₂, 10 mM NaHCO₃, 5 mM KCl, 5 mM HEPES, 5 mM trehalose) by inside-out preparation and attached to the bottom of the well (n = 6). Tissue was covered with 180 µl HL3A buffer. Assay was performed at 25 °C. For the assay, three measurements without injection (baseline), three with pyruvate and malate (end concentration 10 and 1 mM, respectively) and three with rotenone (end concentration 5 µM) were taken.

Western Blot

For the detection of p-AMPK, 15 µL 1X PBS were added and flies were homogenized. 1x Lämmli buffer was added and the lysate was boiled at 99°C for 3 minutes to destroy all proteases. For the detection of Drp1-HA, 1X Laemmli buffer was added to the cytoplasmic and mitochondrial fraction, and the fractions were boiled at 99°C for 3 min. Electrophoresis was performed at 150 V in 1X SDS running buffer. Semi-dry blotting was performed to transfer the proteins onto a PVDF (polyvinylidene difluoride) membrane. Blotting was done at 100 V for 1.5 hours on a prior in methanol activated membrane. The membrane was blocked in 5% milk in TBS-T buffer (1M) for 20 minutes followed by incubation with anti-p-AMPK (Cell Signaling, 1:1000) or anti-HA (Roche, 1:400) antibody in 5% milk/TBS-T buffer at 4°C o/n. For immunodetection of the antibody membrane was incubated with HRP-coupled secondary antibody (Santa Cruz Biotechnology, 1:7500) in 5% milk/TBS-T buffer in the dark at RT for 2 hours. Detection of antibodies was done with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermofisher Scientific) incubated for 1 minute and exposed for 30 seconds. Signal acquisition was performed with Curix 60 (AGFA).

Histology

For semi-thin sections, heads of Creld and w- adult *D. melanogaster* were fixed in 4% formaldehyde in 1X PBS over night at 4°C. Following dehydration of tissue in a graded ethanol series (70%, 80%, 90%, 96%, and 100%) for half an hour per step, the tissue was embedded in JB-4 solution (Polysciences, Inc.). Embedded tissue was cut into 5 µm sections with an ultramicrotome by a glass knife and transferred to a coated microscope slide (superfrost® plus, thermofisher scientific). Furthermore, cut tissue was stained with Hematoxylin for 3 minutes, then washed in ddH₂O for 3 minutes and stained with Eosin for 3 minutes followed by washing in ddH₂O for 30 seconds. Sections were mounted in Entellan® New (Merck).

Hydrogen peroxide detection

H₂O₂ was measured in extracts of 3 adult female flies. 3 flies were homogenized in ice-cold PBS and centrifuged for 15 min at 4°C at 12000 g. 1 ml working reagent contained 970 µl PBS, 20 µl horse-reddish peroxidase (HRP) and 10 µl Amplex Red. 50 µl supernatant of each sample was measured in triplicates. Fluorescence was measured at 544/590 excitation/emission using a TECAN plate reader. A 3 µM dilution of H₂O₂ was used for the standard curve.

Imaging

Antibodies used in this study were α -GFP, α -KDEL (both Santa Cruz Biotechnology), α -DsRed (Takara), α -HA (Invitrogen), α -TOMM20 (Sigma-Aldrich). α -Creld was generated by immunization with a peptide targeting the conserved WE domain (TAWEEEKLRSYKNSE, Davids Biotechnologie). For immunohistochemistry, brains from adult female flies were dissected in PBS and fixed for 1 h in 0.5 % PBS-Tween20 and 4 % formaldehyde. Tissue was washed with 0.5 % PBS-Tween20 and blocked with donkey serum before incubation with the primary antibody (over night at 4 °C). Tissue was washed in 0.1 % PBS-Tween20 before incubation with the secondary antibody at room temperature in the dark for 1 h. Secondary antibodies coupled to Alexa or Cyanine dyes were from Molecular probes. Tissue was washed and incubated for 5 min with DAPI (4',6-diamidino-2-phenylindole). For colocalization analysis of KDEL and mitoOMM-mcherry, adult brains were stained with α -KDEL-mouse and α -DsRed-rabbit. Secondary antibodies were donkey- α -mouse-Alexa488 and donkey- α -rabbit-Cy3. Single confocal sections of dopaminergic neuron PPL1, PPL2 and PAL clusters were analyzed with the ImageJ colocalization threshold tool. For live staining with MitoSOX (ThermoFisher), guts and Malpighian tubes were dissected in cold PBS and incubated with 5 μ M MitoSOX for 10 min. Tissue was washed in PBS, mounted in Fluoromount (ThermoFisher) and analyzed immediately. For staining of superoxide with dihydroethidium (DHE, Sigma Aldrich), tissue was dissected as described above and incubated for 15 min in x μ M DHE and fixed for 15 min in 4 % formaldehyde. Tissue was washed and incubated with DAPI, mounted in Fluoromount and analyzed immediately. For imaging, we used a Zeiss LSM 710 with a 25x water lens (Plan-Neofluar, Zeiss), 40x water lens (C-Apochromat, Zeiss) and 63 x water lens (Plan-Apochromat, Zeiss).

Subcellular fractionation

For the analysis of cytoplasmic and mitochondrial protein, the mitochondrial fraction was enriched from extracts of 10 female flies by homogenization in 200 μ l mitochondria isolation buffer (250 mM sucrose, 10 mM Tris, pH 7.4, 0.15 mM $MgCl_2$). Homogenates were filtered with cotton by centrifugation at 4000 g for 15 min, and the cleared homogenate was transferred to a fresh cup. Mitochondria and cytoplasm were fractionated by centrifugation at 16000 g for 30 min at 4 °C. The cytoplasmic fraction in the supernatant was transferred to fresh cups, and the mitochondrial pellet was dissolved in 200 μ l mitochondria isolation buffer. For isolation of MAMs, we followed the protocol by Wieckowski et al., 2009. In brief, 200 flies (100 male, 100 female) were homogenized in isolation buffer 1, 10 μ l per fly, using a Precellys (peqlab) homogenizer. The homogenate was centrifuged twice at 1000 g to remove chitin and other bulk material. The cleared homogenate was centrifuged at 7000 g for 10 min and contained cytosol, ER, plasma membrane and lysosomes in the supernatant and crude mitochondria in the pellet. The pellet was resuspended in 200 μ l isolation buffer 2 and pelleted again at 10000 g. The pellet was resuspended in 2 ml mitochondria resuspension buffer (MRB) and ultracentrifuged in a Beckman Optima L-60 Ultracentrifuge on Percoll medium to isolate mitochondria from MAMs. Lipidomics analysis of cytoplasmic, crude mitochondria and MAM was performed. For each isolation step, we included a technical sample containing only buffer. Lipidomics analysis was performed in a Thermo Scientific Q Exactive Plus Hybrid quadrupole-orbitrap mass spectrometer as described previously (Thiele et al., 2019). Reads were normalized to protein content of the fraction, and reads from the technical samples were subtracted.

Statistics

Bar charts represent mean and standard deviation. Boxes in box plots represent the interquartile range and median, whiskers represent minimum and maximum. Green squares in box plots represent single data points. We used Microsoft Excel for bar charts and Origin Pro 8G for box plots. We used the software GraphPad InStat for our statistical analyses. Two-sided Student's t-test was applied for normally distributed data in single comparisons, assuming heteroschedasticity. One-way ANOVA with Tukey-Kramer post-test was used for multiple comparisons. The Kolmogorov-Smirnow test was applied to test normality, and Bartlett's method was used to test for equal standard deviations within groups. Asterisks represent * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. A minimum of 3 biological replicates was used for each analysis.

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