A Mitochondrial Membrane-Bridging Machinery Mediates Signal Transduction of Intramitochondrial Oxidation

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ABSTRACT

Mitochondria are the main site for generating reactive oxygen species, which are key players in diverse biological processes. However, the molecular pathways of redox signal transduction from the matrix to the cytosol are poorly defined. Here we report an inside-out redox signal of mitochondria. Cysteine oxidation of MIC60, an inner mitochondrial membrane protein, triggers the formation of disulfide bonds and the physical association of MIC60 with Miro, an outer mitochondrial membrane protein. The oxidative structural change of this membrane-crossing complex ultimately elicits cellular responses that delay mitophagy, impair cellular respiration, and cause oxidative stress. Blocking the MIC60-Miro interaction or reducing either protein, genetically or pharmacologically, extends lifespan and health-span of healthy fruit flies, and benefits multiple models of Parkinson's disease and Friedreich's Ataxia. Our discovery provides a molecular basis for common treatment strategies against oxidative stress.

Introduction

Mitochondria are vital organelles to support cellular functions and survival, and their activities decline with age and in diseases. A major byproduct from generating ATP in mitochondria are reactive oxygen species (ROS). Impairments of mitochondrial function cause elevation of ROS leading to oxidative stress, a common feature shared by various physiological and pathological conditions. While ROS are a well-known source of oxidative damage, they also play crucial roles in regulating tissue-specific physiology and signaling pathways, and certain ROS generated at unique locations in the electron transport chain (ETC) are beneficial for longevity [1,2]. However, the molecular pathways of redox signal transduction from the mitochondria to the cytosol remain elusive.

One key process through which ROS regulate biological processes is reversible covalent modification of protein cysteine residues [3]. Tissue-specific cysteine oxidation networks are remodeled by aging and age-related diseases in mice [2]. Oxidized cysteines can form disulfide bonds within the same protein or between two neighboring proteins, causing oxidative conformational changes of protein complexes. The redox-dependent switch of protein structures has been shown to allow for proper assembly, maturation, and stability of macromolecular complexes [4,5]. However, whether cysteine oxidation mediates redox signal transduction from the inside to the outside of the mitochondria is largely unexplored.

The ideal protein targets of intramitochondrial ROS modification for delivering redox signals to the cytosol are mitochondrial membrane proteins. The two membranes of mitochondria are closely apposed and separated by the narrow intermembrane space (IMS). Protein complexes located on the inner mitochondrial membranes (IMM) and outer mitochondrial membranes (OMM) can form super-complexes across the double membranes [6]. Interestingly, one component in the mitochondrial contact site and cristae organizing system (MICOS) complex, MIC19/CHCHD3, has been reported to regulate stability of the MICOS complex in a redox-dependent manner [4,7]. MICOS is localized on the IMM facing the IMS, and it is crucial for maintaining crista junctions and contact sites [7-12]. We have previously shown that another MICOS component, MIC60/Mitofilin, associates in the same complex with the OMM protein Miro [13]. Functioning as a motor-adaptor, Miro is best-known to recruit cytosolic motors to the mitochondrial surface and is regulated by signals at the cytosolic face of the mitochondria to mediate their motility [14-17]. In addition, Miro plays crucial roles in initiating mitophagy–a mitochondria-specific autophagy that involves recruitment of cytosolic autophagosomes and fusion with lysosomes [17-20], and likely in organizing mitochondria-ER contacts and balancing intracellular ion, metabolite, and nucleoid homeostasis [21-26]. This connection between the IMM protein MIC60 and the OMM protein Miro could

allow signal transduction of the intramitochondrial oxidation status to the cytosol. In this study, we tested this hypothesis by characterizing the nature and regulatory signals of the MIC60-Miro interaction.

Results

MIC60 Protein Undergoes an Oxidative Conformational Change

It is known that human MIC19 switches to an oxidative conformation by forming two disulfide bonds within the twin cysteine-x9-cysteine motifs in the CHCH domain [4]. We hypothesized that cysteine oxidation might also cause conformational changes of MIC60 protein. Fly MIC60 (dMIC60) has four cysteines, of which two (AAs 10 and 12) are in the putative mitochondrial targeting signal sequence (MTS) sticking into the matrix and are likely cleaved during maturation, and two (AAs 302 and 550) are present in the part of dMIC60 that remains in the IMS (Fig. 1a). A disulfide bond between cysteines 302 and 550 could cause folding of dMIC60 protein. To explore this possibility, we treated 15-day-old fly whole-body lysates with either the reducing agent, DTT, which opens disulfide bonds and linearizes proteins, or the non-reducing agent, PEG-MAL, which reacts to and tags only free cysteines leading to an upward mass shift of 5 kDa per cysteine but cannot access to oxidized cysteines [27]. dMIC60 protein migrated faster under the non-reducing condition (PEG-MAL) than the reducing condition (DTT) in SDS-PAGE (Fig. 1a), indicating that dMIC60 contains oxidized cysteines and undergoes oxidative conformational changes. Similarly, when we fed 2-day-old flies with an oxidizing agent, H_2O_2, the dMIC60 band ran faster in the non-reducing condition (PEG-MAL) than the reducing condition (DTT) (Fig. 1b), suggesting that dMIC60 is oxidized. To confirm that oxidized cysteines of dMIC60 formed disulfide bonds, we performed an indirect thiol trapping assay, which can detect disulfide bonds of native proteins [4]. We added TCEP to fly lysates, which reduced disulfide bonds, and then treated the lysates with PEG-MAL, which subsequently bond to cysteine residues that formed disulfide bonds in their

native form. Intriguingly, the dMIC60 band vanished in this condition (Fig. 1a; right lane), implying that the binding of PEG-MAL, which is a long-chained molecule, to cysteines might have blocked the detection by anti-dMIC60. Notably, anti-dMIC60 recognizes the regions close to both cysteines 302 and 550 [28]. To circumvent the problem that the detection of dMIC60 protein was masked by PEG-MAL, we replaced PEG-MAL with a smaller thiol-reacting agent, AMS, which adds 0.5 kDa to each cysteine, in the indirect thiol trapping assay [4]. This time, we observed a clear upward shift of the dMIC60 protein band in 15-day-old flies (Fig. 1c) or 2-day-old flies fed with H_2O_2 (Fig. 1d), compared to controls, confirming that dMIC60 forms disulfide bonds. Treating 2-day-old flies directly with AMS caused a dMIC60 band higher that treated with DTT (Fig. 1e), showing no disulfide bond formation. These data demonstrate that dMIC60 is oxidized with age.

To confirm that a disulfide bond formed between cysteines 302 and 550, we generated transgenic flies carrying either wild-type *dMIC60* ("dMIC60-WT"), or mutant *dMIC60* where both cysteines were converted to serines ("dMIC60-CS") which cannot form disulfide bonds [4]. Both transgenes were inserted into the same genomic region using the PhiC31 integrase-mediated transgenesis system [29]. We employed the UAS/GAL4 approach [30] to express *UAS-dMIC60* (WT or CS) transgenes ubiquitously by *Actin-GAL4*, and their expression levels were comparable (Extended Data Fig. 1a). We fed these flies with H_2O_2 to maximize oxidation and then examined the migration of dMIC60 protein under the non-reducing condition (PEG-MAL). As expected, oxidized dMIC60-WT migrated faster than dMIC60-CS (Fig. 1f). As a control, we treated flies harboring dMIC60-WT with TCEP and then AMS to trap cysteines that formed disulfide bonds, and this treatment consistently caused an upward shift of the dMIC60-WT band (Fig. 1f; right lane). Taken together, our results reveal that cysteines 302 and 550 of dMIC60 form a disulfide bond.

Oxidized MIC60 Promotes Its Physical Coupling with Miro

We have previously shown that endogenous human Miro1 binds to MIC60 in HEK293T cells [13]. Here, we confirmed that endogenous *Drosophila* Miro (DMiro) and dMIC60 resided in the same complex in vivo by co-immunoprecipitation (co-IP) from 10-day-old flies (Fig. 1g). We found that fly MIC19 (dMIC19) was also in the same complex (Fig. 1g), but not fly Mitofusin (Marf), VDAC, or ATP5β (Extended Data Fig. 1b). The association of Miro with MIC60 has been demonstrated in COS7 cells, Hela cells, and mouse brains as well [25,31]. We investigated whether the redox status of dMIC60 regulated its interaction with Miro. We discovered that in very young flies (day 2) when dMIC60 was not substantially oxidized (Fig. 1e), little dMIC60 co-IPed with DMiro (Fig. 1h; IP, left lane), indicating a weak interaction. By contrast, when we fed these flies with an oxidant (H_2O_2), significantly more native dMIC60 in fly lysates formed a complex with endogenous DMiro and with recombinant human full-length GST-Miro1 (Fig. 1h-i). Hence, the dMIC60 and Miro interaction is strengthened in an oxidizing environment. We corroborated this finding by a fluorescent resonance energy transfer (FRET)-based binding assay. Anti-dMIC60 and anti-T7, a tag fused to *DMiro* transgene [32], were validated for immunostaining using *dMIC60* null mutants and non-transgenic flies, respectively (Extended Data Fig. 1c-d) [28]. Feeding flies with H_2O_2 significantly increased the relative FRET between dMIC60 and T7-DMiro in fly brains (Fig. 1j), indicating enhanced association between these two proteins. We next determined whether blocking dMIC60 oxidation inhibited its interaction with Miro. Indeed, the reduced mutant, dMIC60-CS, lost the ability to bind to DMiro in young flies fed with H_2O_2 (Fig. 1k). In Vitro, recombinant wild-type dMIC60 [28] also associated with human GST-Miro1, and dMIC60-CS consistently eliminated this interaction (Extended Data Fig. 1e-g). Adding a strong reducing agent (2 mM β-mecaptoethanol) to the regular non-reducing buffer (Non) similarly abolished the binding between recombinant human Miro1 and dMIC60 in vitro, but not between human Miro1 and Parkin (Extended

Data Fig. 1h-i), a known Miro-interactor. These results suggest that the dMIC60 and Miro interaction is inhibited in a reducing environment. Given the known topology of DMiro (Fig. 1l), we predicted that the short segment of 11 amino acids at the C-terminus, which pokes into the IMS, was required for DMiro to directly interact with proteins in the IMS. As expected, we found that only full-length DMiro, but not DMiro1Δ11 (truncated DMiro lacking the last 11 amino acids), interacted with dMIC60 protein (Fig. 1l). Therefore, the C-terminal IMS section of DMiro is essential for binding to dMIC60. Collectively, our results demonstrate that dMIC60 couples with Miro in a redox-dependent manner (Fig. 1m).

From the in vivo experimental settings, we also found increased protein levels of endogenous DMiro and dMIC60 in whole-body lysates of flies fed with one of the two oxidants–paraquat and H_2O_2 (Extended Data Fig. 1j). This oxidation-induced increase in their protein levels was blocked when the oxidation-resistant mutant, dMIC60-CS, was introduced in flies (Fig. 2a). Together, our results support our model that the oxidation of dMIC60 facilitates its interaction with Miro and stabilizes the dMIC60-DMiro complex.

MICOS Functions Upstream of Miro to Stabilize Miro

We discovered that ablating multiple MICOS components in flies, including *dMIC60* [13], *dMIC19*, or *dMIC26* (orthologue of *MIC26/27*) [33] using their null or RNAi mutants, lowered DMiro protein levels, but not *DMiro* mRNA expression detected by quantitative real-time PCR (qPCR) (Extended Data Fig. 2, 3a). This phenotype of DMiro downregulation was specific to *MICOS* mutants because we did not observe it in the null or RNAi mutants of *CHCHD2/10* [34] or *Sam50* (Extended Data Fig. 3a-c). The downregulation of DMiro protein by the lack of *dMIC60*, *dMIC26*, or *dMIC19* was not caused by a general mitochondrial destruction or mitophagy because other mitochondrial proteins such as Marf,

VDAC, and ATP5β were not affected (Extended Data Fig. 2a-c). Importantly, complete loss of *dMIC60* abolished dMIC19 and likewise, complete loss of *dMIC19* eliminated dMIC60 (Extended Data Fig. 2a), consistent with previous findings in yeast and human cells [4,7]. This demonstrates that both components are essential for MICOS complex stability. However, *DMiro* null mutant did not affect either dMIC60 or dMIC19 (Extended Data Fig. 3d). Hence, integrity of the MICOS complex functions upstream of stability of DMiro protein.

The Redox Sensor Is at Play during Biological Aging

Oxidative stress is a hallmark of normal aging [35]. Because we observed dMIC60 oxidation with age (Fig. 1a, c, e), we hypothesized that the dMIC60-DMiro complex functioned as a redox sensor during fly aging. We confirmed that mitochondrial ROS were elevated in old fly brains compared to young brains by MitoSox staining (Fig. 2b) [1]. Our proteomic evaluation of protein abundances in whole bodies of young (day 5) and old (day 50) flies, using tandem mass tag (TMT) isotopic labeling quantitative mass spectrometry, revealed molecular signatures of oxidative stress (upregulation of anti-oxidation enzymes and stress response proteins) with age (Fig. 2c, Supplementary Table 1). Furthermore, by performing co-IP experiments from fly lysates, we discovered that fly biological aging (day 50 compared with day 5) led to a significant increase in the amount of dMIC60 bound to DMiro (Fig. 2d; IP). This increase in their binding was eliminated when flies were fed with an antioxidant, AD4 (Fig. 2e; IP). The protein levels of dMIC60 and DMiro were also mildly upregulated in older flies, detected by both Western blotting (Fig. 2d; Input) and mass spectrometry (Fig. 2c, Supplementary Table 1), although the mRNA expression of *DMiro* did not change over age as detected by qPCR (Extended Data Fig. 3e); the protein levels of dMIC60 and DMiro were lowered by AD4 feeding (Fig. 2e; Input). These data demonstrate

that in older flies dMIC60 is more oxidized, resulting in more dMIC60 to interact with DMiro and stabilization of the dMIC60-DMiro complex.

Dissociating the Complex Extends Lifespan and Health-Span

We reasoned that the intramitochondrial redox status with age could be transmitted through the oxidation of dMIC60 and resultant stabilization of the dMIC60-DMiro interaction across the mitochondrial membranes, which would ultimately lead to cellular responses and subsequent changes in organismal performance and survival. To explore this possibility, we examined whether disrupting the dMIC60-DMiro interaction or reducing either protein extended lifespan. We first ubiquitously expressed either wild-type dMIC60 or the oxidation-resistant mutant, dMIC60-CS, which fails to bind to Miro (Fig. 1k, Extended Data Fig. 1f), in *dMIC60* null background. Loss of dMIC60 causes pupal lethality [13,28]. Expression of either dMIC60-WT or dMIC60-CS allowed *dMIC60* null flies to survive to adulthood; yet flies with dMIC60-CS lived significantly longer than those with dMIC60-WT (Fig. 3a). We next reduced DMiro levels by RNAi (Extended Data Fig. 3f), in a tissue-specific way by employing the GeneSwitch (GS) system–in pan-neurons driven by *Elav-GS-GAL4*, in intestine enterocytes by *5966-GS-GAL4*, in muscles by *MHC-GS-GAL4*, or ubiquitously by *Da-GS-GAL4*. We switched on *DMiro* RNAi either throughout adulthood or later in life from day 30 by feeding flies with RU486. All control flies (RU486 negative) were fed with the vehicle (ethanol). The GS system also enables RNAi manipulations to bypass the embryonic, larval, and pupal stages, thus preventing any impact by reducing DMiro on the development. For all lifespan experiments, we cultured female flies on the standard sugar-yeast-agar diet (1.0SYA) [36]. We confirmed that simply feeding wild-type flies with the highest dose of RU486 we used (200 μM) did not affect lifespan (Extended Data Fig. 4a), verifying the feasibility of our approach. We discovered that lowering DMiro levels by RNAi in neurons–but not in intestine enterocytes, muscles, or

ubiquitously–significantly extended lifespan (Fig. 3b, Extended Data Fig. 4b-g). Switching on *DMiro* RNAi either early or late in life was equally beneficial (Fig. 3b, Extended Data Fig. 4b). Knocking down *white*, the gene which determines the eye color, by RNAi using our GS system did not extend lifespan (Extended Data Fig. 4h), excluding the possibility of any artifact caused by our system. Furthermore, we reduced *dMIC60* or *dMIC19* levels which should lower the protein levels of dMIC60 and DMiro (Extended Data Fig. 2a-b). We used mild RNAi fly lines of *dMIC60* and *dMIC19*–which did not affect mitochondrial cristae confirmed by transmission electron microscopy (TEM) (Extended Data Fig. 5), but reduced DMiro levels (Extended Data Fig. 2b) [13,28]–and the GS system to bypass the development. Notably, adult-onset reduction of *dMIC19* or *dMIC60* in pan-neurons by *Elav-GS-GAL4*, but not in intestine enterocytes by *5966-GS-GAL4*, significantly extended lifespan (Fig. 3c-d, Extended Data Fig. 6a-d), like *DMiro* RNAi. Collectively, these results show that the dMIC60-DMiro complex can be targeted for extending lifespan in flies.

We then determined whether weakening the dMIC60-DMiro interaction extended fly health-span. The abilities to perform locomotor activity and to resist environmental stressors are distinctive features of an organism's health and decline with age [35]. We found that flies with dMIC60-CS had much better locomotor ability and resistance to the oxidative stressor, paraquat, than flies with dMIC60-WT (Fig. 3e-f, Supplementary Movie 1). Consistently, *DMiro* RNAi in neurons also significantly ameliorated the age-dependent locomotor decline (Fig. 3g) and improved the abilities of flies to withstand different environmental stressors including starvation and paraquat (Fig. 3h-i). A second independent *DMiro* RNAi line (*DMiro* RNAi 2) was similarly beneficial (Extended Data Fig. 6e). Therefore, our results demonstrate that dissociating the dMIC60-DMiro complex extends fly health-span and lifespan.

Targeting the Complex Improves Mitochondrial Function

Having demonstrated the significance of this redox-dependent regulation to fly physiology at the organismal level, we next sought to identify the underlying cellular mechanisms. If elevation of mitochondrial ROS with age damages a specific downstream cellular function via the dMIC60-DMiro complex, targeting the complex should restore this function. To this end, we examined several parameters of cellular metabolism. We first measured ATP levels to determine the energy homeostasis. We found that flies with the reduced mutant, dMIC60-CS, had higher total ATP levels than those with dMIC60-WT (Fig. 4a). Similarly, *DMiro* RNAi in pan-neurons from adulthood significantly elevated both total and head ATP levels (Fig. 4b). We then detected mitochondrial ROS levels in the brain by MitoSox staining. Again, flies with dMIC60-CS had lower ROS levels than those with dMIC60-WT (Fig. 4c), and *DMiro* RNAi in pan-neurons greatly lowered ROS levels in older brains (Fig. 4d), showing that oxidative stress is alleviated. We next determined the mitochondrial membrane potential, which is a driving force for mitochondrial ATP synthesis, in dopaminergic (DA) neurons of fly brains by TMRM staining. The intensity of TMRM was normalized to that of Mito-GFP in the same DA neurons driven by *TH-GAL4*. In line with our other observations, we found that the membrane potential was lowered in old brains, suggesting compromised mitochondrial function with age; *DMiro* RNAi considerably improved it (Fig. 4e). We also evaluated whether cellular respiration was affected by aging and whether *DMiro* RNAi could normalize it. We conducted the seahorse analysis on ex vivo intact fly brains and found that aging significantly decreased both the basal oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) (Fig. 5a), indicating both aerobic and anaerobic respiration is compromised with age. Interestingly, our mass spectrometry study revealed upregulation of multiple enzymes in the glycolysis pathway in old flies (Fig. 2c, Supplementary Table 1), which could be a feedback response to the impairments of glycolysis and oxidative phosphorylation. Remarkably, *DMiro* RNAi in pan-neurons by *Elav-GS-GAL4* fully restored both the OCR and ECAR to the levels of young

flies (Fig. 5a). Lastly, we assessed the key features of the initiation of mitophagy–the sequestration of the ubiquitin aggregates and the autophagy adaptor p62 from the cytosol to the mitochondria [37,38], because stabilization of the dMIC60-Miro complex may delay Miro removal from damaged mitochondria and consequently prolong the initiation of mitophagy [17-20]. We expressed Mito-GFP in DA neurons driven by *TH-GAL4*, and then immunostained ubiquitin and p62 [39]. Interestingly, we observed a decrease in the colocalization of Mito-GFP puncta with p62 in older brains compared to younger flies (Fig. 5b), indicating that the efficiency in recruiting the early autophagy machinery to the mitochondria is lowered with age. Consistent with this observation, our proteomic results showed a striking accumulation of mitochondrial matrix proteins in old flies compared to young flies (Fig. 2c, Supplementary Table 1), which could be a result of impaired mitophagy. Notably, *DMiro* RNAi significantly increased the colocalization of Mito-GFP puncta with both p62 and ubiquitin in old flies (Fig. 5b), suggesting an improvement in the mitophagy efficiency. We did not observe overall protein level changes of ubiquitin and p62 with age or *DMiro* RNAi by Western blotting (Extended Data Fig. 6f). Therefore, dissociating the dMIC60-DMiro complex promotes cellular respiration, improves mitochondrial function, and alleviates oxidative stress.

The Redox Sensor Responds to α-Synuclein Toxicity

We explored whether this redox signal transduction pathway was associated with pathological conditions afflicted by ROS. We have previously shown that upregulation of either wild-type α-synuclein (α-syn) or pathogenic α-syn with the A53T mutation causes an elevation of Miro protein in multiple model systems including flies (Extended Data Fig. 7a-d), HEK293T cells, and human neurons, which delays Miro removal and slows mitophagy [20]. This increase in Miro protein is not a result of increased mRNA synthesis [20]. α-Syn is the main constituent of Lewy bodies [40], a pathological feature of Parkinson's

disease (PD) which is characterized by selective loss of DA neurons in the substantia nigra. It has been well-documented that α-syn accumulation enhances mitochondrial ROS production and impairs mitochondrial function [41-43]. Indeed, we observed increased ROS levels detected by MitoSox staining in fly brains expressing human α-syn-A53T (Fig. 6a) [44]. Therefore, it is likely that the dMIC60-DMiro-mediated redox signal pathway is activated by α-syn toxicity. To explore this possibility, we performed co-IP experiments from fly lysates and discovered that ubiquitous expression of α-syn-A53T [20] in young flies caused significantly more dMIC60 to interact with DMiro (Fig. 6b; IP), just as in normal aging flies (Fig. 2d), suggesting that the dMIC60-DMiro redox sensor responds to α-syn toxicity in flies.

Importantly, we found a temporal relation of these phenotypes in α-syn-A53T-expressing flies. The increase in the binding of DMiro and dMIC60 occurred first (day 5) (Fig. 6b), followed by DMiro protein upregulation (day 15) (Fig. 6c) and subsequent DA neuron loss (day 40) (Fig. 6d). The elevation of DMiro protein levels could be a result of increased stability of the complex. Because the phenotypes of the DMiro-dMIC60 complex preceded DA neurodegeneration, it suggests that the molecular changes may have predisposed these neurons to cell death. We have previously shown that knocking down *DMiro* by RNAi alleviates age-dependent locomotor decline and protects DA neurons in fly α-syn models of PD [20], supporting our hypothesis. Similarly, when we knocked down *dMIC60* using the mild *dMIC60* RNAi fly strain that did not affect crista structure (Extended Data Fig. 5) but reduced DMiro levels (Extended Data Fig. 2b), the behavioral deficits in climbing and flying and DA neurodegeneration were fully rescued in α-syn-A53T-expressing flies (Fig. 6d, Extended Data Fig. 7e-f, Supplementary Movie 2-4). Consistently, expression of the reduced mutant, dMIC60-CS, in flies with α-syn-A53T restored the DA neuron number and climbing ability (Fig. 6e-f). Taken together, these results demonstrate that α-syn elevation causes PD-relevant phenotypes at least in part via the dMIC60-DMiro pathway.

We substantiated the discovery we made in flies in the human HEK293T cell line. We knocked down endogenous *MIC60* by RNAi [28]. This significantly reduced endogenous Miro1 protein levels (Extended Data Fig. 7g), consistent with the observation in flies (Extended Data Fig. 2a-b) [13], suggesting that the regulation of Miro by MIC60 is conserved between flies and human cell lines. EGFP-α-syn expression upregulated Miro1 levels (Extended Data Fig. 7g) [20], just as in flies (Fig. 6c, Extended Data Fig. 7d); however, EGFP-α-syn no longer caused Miro1 elevation in the presence of *MIC60* RNAi (Extended Data Fig. 7g), indicating that MIC60 is required for α-syn to upregulate Miro1. We next expressed dMIC60-WT and oxidation-resistant dMIC60-CS in these cells and found dMIC60-CS similarly eliminated the phenotype of Miro1 upregulation caused by EGFP-α-syn expression (Extended Data Fig. 7h). These results corroborate our conclusion that dMIC60 oxidation underlies the impact that α-syn has on Miro in both flies and human cells.

The Redox Sensor Acts in Flies with Frataxin Deficiency

Now that we have demonstrated how the dMIC60-DMiro pathway is activated during both normal aging and α-syn toxicity in flies, we wondered whether this mechanism also underlay childhood-onset mitochondrial diseases, many of which are characterized by oxidative stress due to mitochondrial dysfunction. Friedreich's Ataxia (FA) is an early-onset autosomal recessive genetic disease, caused by the extension of GAA repeats in the *FTX* gene, which encodes a mitochondria-localized protein called frataxin. Disease mutations reduce frataxin and disrupt mitochondrial function. Therefore, oxidative stress might play a part in pathogenesis and anti-oxidation strategies have been beneficial in multiple FA models [45-47]. Flies deficient in the frataxin ortholog (Fh) have been established as useful models for understanding FA mechanisms [45,47,48]. We found that in one such fly model (RNAi against *Fh*) [47], mitochondrial ROS levels in fly brains were elevated (Fig. 6g). In addition, more dMIC60 bound to

DMiro and both proteins were mildly upregulated in whole-body lysates of these *Fh* RNAi flies (Fig. 6h). These results demonstrate that the redox-dependent dMIC60-DMiro mechanism is at play during frataxin deficiency. Furthermore, we discovered that expressing *dMIC60-WT* transgene, but not *dMIC60-CS*, in *Fh* RNAi flies ubiquitously caused adult lethality (0% survival rate, compared to ~100% of *dMIC60-CS*), showing a genetic interaction between dMIC60 oxidation and frataxin deficiency. Together, these results reveal a third oxidative condition associated with the activation of this redox pathway.

Miro Reducers Benefit Fly Models of Oxidative Stress

Because the dMIC60-DMiro pathway underlies multiple oxidative conditions including biological aging, PD, and FA in flies (Fig. 2-6), pharmacological intervention in this pathway might provide a common therapeutic strategy. We have previously discovered small molecules that are predicted to bind to one of the two GTPase domains of human Miro1 through an in-silico screen [18]. Feeding wild-type flies with each of the top 4 hits (Miro1 Reducer 3, 4, 5, 6 or MR3, 4, 5, 6) partially lowers DMiro protein levels [18]. Importantly, 3 out of these 4 hits, MR3, 4, 5, share structural similarity (Fig. 7a), suggesting that these drugs belong to a compound series. To confirm these small molecules were true Miro1-binders, we performed a thermal shift assay using human Miro1 protein and MR3 in vitro. We found that MR3 caused a dose-dependent decrease in the melting temperature of Miro1 protein (Extended Data Fig. 8a). This result demonstrates that MR3 directly binds to Miro1 and this binding triggers Miro1 protein unfolding and destabilization, consistent with the observation in flies in vivo [18]. Therefore, this compound series may provide a pharmacological approach to deactivate this redox pathway.

We first examined this series in PD models. We have previously shown that MR3 rescues Parkinson's relevant phenotypes in human neuron and fly models of PD, without affecting Miro1's

overall GTPase activity or other mitochondrial proteins including Miro2, Mitofusin, OPA1, VDAC, and ATP5β [18]. If MR4 and MR5 function similarly as MR3, those compounds should also exhibit protection against PD. To explore this possibility, we fed MR5 to a fly model of PD with α-syn-A53T expression driven by *Elav-GAL4*. We confirmed that adult-onset administration of MR5 in those PD flies for 15 days partially reduced DMiro protein levels (Extended Data Fig. 8b), similar to MR3 [18]. We then examined the DA neuron number in the brain and the climbing ability. MR5 treatment significantly rescued the age-dependent DA neuron loss and locomotor decline of α-syn-A53T-expressing flies (Fig. 7b-c). We next tested MR5 in a human neuron model of PD. We differentiated induced pluripotent stem cells (iPSCs) from one familial patient with the A53T mutation in *SNCA* (encodes α-syn) and the corresponding isogenic wild-type control [18], to neurons expressing tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine synthesis as previously described (Extended Data Fig. 8c-d) [18-20]. We identified DA neurons by TH-immunostaining [20] and cell death by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). iPSC-derived DA neurons from PD patients are more vulnerable to stress than those from healthy controls [18-20,49]. The treatment with Antimycin A, the complex III inhibitor, for 6 hrs, caused acute neuronal cell death leading to the loss of TH and the increase of TUNEL signals in PD patient-derived neurons, but not in healthy controls (Fig. 7d, Extended Data Fig. 8c) [18,20]. Notably, MR5 treatment at 5 μM for 30 hrs completely rescued the stress-induced degeneration of DA neurons derived from the PD patient (Fig. 7d, Extended Data Fig. 8c), just like MR3 [18]. These data support that MR3 and MR5 belong to the same compound series.

We hypothesized that Miro1 Reducers were also beneficial for normal aging and frataxin deficiency. Similar to PD flies, *Fh* RNAi flies exhibit age-dependent locomotor decline [47]. Notably, both MR3 and MR5 improved the locomotor ability of *Fh* RNAi flies (Fig. 7e), reminiscent of the effect in PD flies (Fig. 7c) [18], indicating the possibility of a common treatment method for both PD and FA. We next fed

MR3 or MR5 to wild-type flies, and found that administration of either compound from adulthood significantly extended their lifespan (Fig. 7f). MR5 also improved the overall ATP production of wild-type flies (Fig. 7g). Taken together, we have discovered 2 pilot molecules that alleviate multiple oxidative stressed conditions.

Discussion

In this study, we have discovered a novel signal transduction pathway of intramitochondrial oxidation (Fig. 8). The redox status of the matrix is transmitted through the oxidative structural change of a protein complex bridging the mitochondrial double membranes, which ultimately elicits adverse cellular responses. Our work also presents a new means whereby Miro receives cellular instructions: from the inside of the mitochondria rather than from the outside. This mechanism places mitochondrial membrane complexes at the central intersection to relay commands from both internal and external mitochondria to guide mitochondrial behaviors.

Our work sheds light on a new biological process regulated by reversible covalent modification of cysteine residues [3]. ROS released from the ETC oxidize the IMM protein dMIC60 at cysteines 302 and 550 leading to the formation of a disulfide bond, which consequently causes folding of dMIC60 protein in the IMS. This oxidative structure of dMIC60 stabilizes its physical coupling with the OMM protein Miro. Interestingly, a recent proteomic study reports that among the 7 cysteine residues in mouse MIC60, the 3 cysteines localized in the IMS are all oxidized [2], similar to our finding of dMIC60, suggesting that the redox-dependent regulation of MIC60 may be conserved across species. There are other examples where cysteine oxidation switches protein structures to allow for stability of macromolecular complexes [4,5]. MIC19, another component of the MICOS complex, stabilizes the MICOS assembly via the oxidation

of the cysteines in its CHCH domain [4,7]. It will be of importance to determine whether MIC19 oxidation regulates the interaction between MIC60 and Miro. In addition, MIC60 interacts with a myriad of proteins in the IMS and on the OMM. MIC60 oxidation may affect those additional MIC60-associated protein complexes and consequently regulate their respective functions. A systemic understanding of how MIC60 oxidation affects a network of mitochondrial proteins will reveal further mechanistic details and molecular pathways of MIC60 oxidation beyond Miro, and help us understand MIC60's larger role in dictating multiple mitochondrial activities.

Because Miro anchors mitochondria to microtubule tracks for movement [14,15], stabilization of Miro on the OMM could enhance mitochondrial motility. However, long-range mitochondrial transport also relies on motor activities and coupling efficiencies with microtubules. Recent reports have revealed a decline in motor activities with age in flies [50,51]. Our proteomic study consistently showed downregulation of both kinesin heavy chain (KHC) and dynein heavy chain (DHC) in old flies (Fig. 2c, Supplementary Table 1). These data explain the observation of decreased mitochondrial motility with age in an adult-wing-neuron system [50]. Therefore, stabilization of the dMIC60-DMiro interaction with age does not seem to enhance mitochondrial motility. Nevertheless, the redox-dependent regulation of this complex does elicit other cellular responses, including impairments in mitophagy and cellular respiration. Our results highlight the importance of mitochondrial quality control in biological aging and neurodegenerative diseases, supporting the existing compelling evidence across model systems and diseases [18-20,52-54].

METHODS

Fly Stocks

The following fly stocks were used: w^{1118}, *Actin-GAL4* (Bloomington Drosophila Stock Center–BDSC), *Elav-GAL4* (BDSC), *TH-GAL4* (BDSC), *UAS-mito-GFP* (BDSC), *UAS-SNCA*^{A53T} [43], *dMIC60*^{LL02849} (Drosophila Genomics Resource Center) [28,55], *DMiro*^{null} [56], *dMIC19*^{null} [33], *CHCHD2*^{null} [34], *Fh* RNAi (#24620, BDSC), *MHC-GeneSwitch-GAL4* (#43641, BDSC), *Elav-GeneSwitch-GAL4* [30], *5966-GeneSwitch-GAL4* (a gift from Benjamin Ohlstein), *Daughterless (Da)-GeneSwitch-GAL4* [57], *DMiro*^{RNAi} (#106683, Vienna *Drosophila* Resource Center–VDRC), *DMiro*^{RNAi} (RNAi 2; #330334, VDRC), *dMIC60*^{RNAi} (#47615, VDRC), *dMIC19*^{RNAi} (#52251, VDRC), *Sam50*^{RNAi} (#33642, VDRC), *dMIC26*^{RNAi} (#31098, VDRC), *white*^{RNAi} (#30033, VDRC), and *UAS-T7-DMiro* [32]. *UAS-dMIC60*^{WT}*-Myc* and *UAS-dMIC60*^{CS}*-Myc* were generated using PhiC31 integrase-mediated transgenesis, with an insertion at an estimated position of 25C6 at the attP40 site (BestGene, Inc.) [29]. Similarly, *UAS-SNCA* flies were generated by injecting pJFRC8-40×UAS-SNCA into 25C6 at the attP40 site at the Fly Facility at Department of Genetics of University of Cambridge. All fly stocks and experiments were kept at 25°C with a 12:12 hrs light:dark cycle and constant humidity (65%) on standard sugar-yeast-agar (SYA) medium (15 g/l agar, 50 g/l sugar, 100 g/l autolyzed yeast, 6 g/l nipagin, and 3 ml/l propionic acid) [58]. Flies were raised at standard density in 200 ml bottles unless otherwise stated. All fly lines were backcrossed 6 generations into a w^{1118} background to ensure a homogeneous genetic background. The ages and developmental stages of flies for each experiment were stated in Figure Legends and Method Details. All behavioral and imaging experiments were carried out on mated females [36], except Fig. 6d and Extended Data Fig. 7e-f which used male and female flies (close to equal number).

Constructs

pJFRC8-40×UAS-SNCA was generated by PCR amplifying the human *SNCA* cDNA from the pCMV6-XL5 vector (#SC119919, OriGene), engineered with the unique *Xhol*/*Xbal* restriction sites at either side,

and cloning it into a pJFRC8-40×UAS-IVS-mCD8::GFP plasmid (a gift from Gerald Rubin, #26221, Addgene) [59]. PCR primers were: Forward- "GCGCCTCGAGATGGATGTATTCA"; Reverse- "GCGCTCTAGATTAGGCTTCAGGT". pcDNA3.1-dMIC60-Flag was purchased from Genescript (OFa05646), and pcDNA3.1-dMIC60-Flag with the C302S and C550S mutations was custom-made by Genescript. pUASTAttB-dMIC60-CS (C302S, C550S), pET101-TOPO-dMIC60-CS (AAs 92-739; C302S, C550S)-His-V5, and pA1-T7-DMiroΔ11 were custom-made by Synbio. pUASTAttB-dMIC60-WT [28], pCMV-EGFP [20], pEGFP-SNCA (a gift from David Rubinsztein, #40822, Addgene) (Furlong, 2000), pET101-TOPO-dMIC60 (AAs 92-739)-His-V5 [28], pA1-T7-DMiro [14], and pRK5-Myc-Miro1 [17] were used.

qPCR

Total RNA was extracted from at least 10 whole flies each experiment using TRIzol® (GIBCO) according to the manufacturer's instructions. Concentrations of total RNA were measured using a Nano Drop. Total RNA (5 μg) was then subjected to DNA digestion using DNase I (Ambion), immediately followed by reverse transcription using the iScript Reverse Transcription Supermix (1708841, BIO-RAD). qPCR was performed using the StepOnePlus™ instrument (Thermo Fisher Scientific) and SYBR® Green Supermix (172-5270, BIO-RAD) by following the manufacturer's instructions. qPCR was analyzed by the Step One™ Software (Version 2.2.2), and relative expression level was presented by the ratio of target gene to the internal standard gene, *RP49*. Each sample was analyzed in duplicate. Four independent biological repeats were obtained. The following primers were used:

DMiro forward: 5'-GCGGAGAGCAAGATTCCAGT -3'

DMiro reverse: 5'-GCCTCAGGTGAGGAAACGC -3'

dMIC19 forward: 5'-CGACGATGTGGTCAAGCGACT -3'

dMIC19 reverse: 5'-ACTTTCGGAGCAGGAGAAGC -3'

dMIC26 forward: 5'-CAGCTCCTGACCACTTCGAG -3'

dMIC26 reverse: 5'-TGGCTTGGGTTCTGTCTTGC -3'

Sam50 forward: 5'-GGTTGGAGTGGATTTGACGC -3'

Sam50 reverse: 5'-CAAAGAGGCCAATTTGGGGC -3'

RP49 forward: 5'-GCTAAGCTGTCGCACAAA -3'

RP49 reverse: 5'-TCCGGTGGGCAGCATGTG -3'

Western Blotting

Five to 10 whole flies, 15 fly heads, 5 pupae, or 5 Larvae were homogenized in 2×Laemmli loading buffer (100 mM Tris 6.8, 20% glycerol, 4% SDS) containing 5% β-mercaptoethanol, and then boiled for 5 min prior to being loaded into SDS-PAGE. Pre-cast polyacrylamide gels (10%) and Tris-Glycine-SDS buffer (24.8 mM Tris, 192 mM glycine, 0.1% SDS) or manually made gels were used for electrophoresis. After electrophoresis, nitrocellulose membranes (1620115, Bio-Rad) were used in semi-dry transfer with Bjerrum Schafer-Nielsen buffer (48 mM Tris, 39 mM glycine, 20% Methanol (v/v), pH 9.2). Transferred membranes were first blocked in TBST (TBS with 0.05% Tween-20) with 5% milk for 1 hr at room temperature, and then immunoblotted with the following primary antibodies in TBST with 5% milk at 4°C overnight: guinea pig anti-DMiro (GP5) [32] at 1:20,000, rabbit anti-dMIC60 [28] at 1:3,000, rabbit anti-dMIC19 [33] at 1:3,000, mouse anti-ATP5β (ab14730, Abcam) at 1:5,000, mouse anti-α-tubulin (T6199, T5168, Sigma; 62204, Invitrogen) at 1:3,000, mouse anti-β-actin (ab8224, Abcam) at 1:5,000, mouse anti-VDAC (ab14734, Abcam) at 1:3,000, rabbit anti-marf [60] at 1:1,000, mouse anti-α-synuclein (ab27766, Abcam) at 1:3,000, mouse anti-Miro1 (WH0055288M1, Sigma) at 1:1,000, rabbit anti-Miro1 (HPA010687, Sigma) at 1:1,000, rabbit anti-GAPDH (5174S, Cell Signaling Technology) at 1:3,000,

mouse anti-MIC60 (ab110329, Abcam) at 1:750, mouse anti-Parkin (sc-32282, Santa Cruz) at 1:3,000, mouse anti-GST (8-326, Thermo Fisher) at 1:3,000, rabbit anti-GFP (A11122, Invitrogen) at 1:1,000-3,000, mouse anti-Myc (sc-40, Santa Cruz) at 1:200, mouse anti-T7 (69522, Sigma) at 1:1,000, rabbit anti-p62/ref(2)P (ab178440, Abcam) at 1:1,000, mouse anti FK2 (mono- and polyubiquitinated conjugates, BML-PW8810, Enzo) at 1:1,000, or rabbit anti-Flag (F7425, Sigma) at 1:1,000. Secondary antibodies used were HRP-conjugated goat anti-guinea pig, mouse, or rabbit IgG (Jackson ImmunoResearch Laboratories) at 1:3-10,000 for 1 hr at room temperature. In IP when detecting the dMIC19 band, VeriBlot for IP Detection Reagent (HRP) (ab131366, Abcam) was used at 1:1000. Luminata Crescendo (Millipore) and West Dura ECL Reagents (34075, GE Healthcare) were used for chemiluminescence. Membranes were exposed by a Konica Minolta SRX-101A developer or scanned using a Biorad ChemiDoc XRS system. Quantification was carried out with ImageJ. Experiments were repeated for more than 3 times.

IP and Cell Culture

Ten whole flies were homogenized in 300 µl lysis buffer (50 mM Tris pH7.5, 1% Triton, 300 mM NaCl, 5 mM EDTA, 1:1000 Protease Inhibitor Cocktail III–#539134, Millipore), followed by centrifugation at 16,200 ×g for 10 min at 4°C. Supernatant (30 µl) was reserved as "Input". The remaining 270 µl was incubated with 2 µl anti-DMiro or normal guinea pig IgG (#5051291, Thermo Fisher) for 2 hrs on a nutator at 4°C, and then combined with 60 µl washed protein A-Sepharose beads (Amersham) for 1 hr at 4°C. Or lysates were mixed with 18 µl glutathione beads (GE Healthcare) and 1 µg GST-Miro1 (H00055288-P01, Abnova). Beads were then washed 5 times with lysis buffer. Residual buffer was removed from the last wash and the beads were mixed with 50 µl 2×laemmli buffer and loaded into SDS-PAGE. For each gel running, approximate 10% of total lysates (Input) and 40% of total IPed proteins

were loaded. For HEK293T cell transfection, cells (100,000 cells/well in 6-well plate) were plated, and when 30-50% confluent co-transfected with (1) a combination of *dMIC60-Flag* (WT or CS), *Myc-Miro1*, *EGFP-SNCA*, and *EGFP*; (2) a combination of *MIC60* dsRNA [28], control dsRNA [28], *EGFP-SNCA*, and *EGFP*; or (3) *T7-DMiro* (FL or Δ11) and *dMIC60-Flag*, using the calcium phosphate transfection protocol [16]. After 48 hrs of transfection, cells were lysed with 250 µl of Triton X-100 lysis buffer (300 mM NaCl, 50 mM Tris PH 7.5, 1% Triton X-100, 5 mM EDTA, 0.2 mM PMSF, Protease Inhibitor Cocktail III). The following Western blotting was described as above. For IP in HEK293T cells, 10 mM H_2O_2 was applied to cells for 10 min right before cells were lysed. For each preparation, cells from 2 wells were pooled and lysed in 200 µl Triton X-100 lysis buffer (300 mM NaCl, 50 mM Tris PH 7.5, 1% Triton X-100, 5 mM EDTA, 0.2 mM PMSF, Protease Inhibitor Cocktail III). Cell debris was removed by centrifugation at 16,200 ×g for 10 min at 4°C. 50 µl cell supernatant was collected as "Input". The rest of supernatant was first incubated with 3 µl mouse anti-T7 (69522, Sigma) for 2 hrs at 4°C, and then was mixed with 60 µl washed protein A-Sepharose beads (Amersham) for 1 hr at 4°C. Beads were then washed four times with lysis buffer. After washes, beads were resuspended in 60 µl 2×SDS loading buffer and boiled for 10 min prior to being loaded into an SDS-PAGE.

Mitochondrial Isolation

Mitochondria fractions were isolated as described previously [16] with minor modifications. Briefly, forty 20-day-old whole flies were mechanically homogenized with a glass Dounce homogenizer in 1000 µl cold mitochondrial isolation buffer (70 mM sucrose, 210mM Mannitol, 50mM Tris-HCl pH7.5, 10 mM EDTA/TRIS pH 7.4, 1:1000 Protease Inhibitor Cocktail III). After first centrifugation at 600 g for 10 min at 4°C to remove debris, crude supernatant was spun at 7,000 ×g for 10 min at 4°C to pellet intact mitochondria. After this step, supernatant was referred to "cytosolic fraction (Cyto)". Mitochondrial

pellet referred to “mitochondrial fraction (Mito)” was washed 3 times with mitochondrial isolation buffer and resuspended in 100 µl 2×laemmli buffer. “Cyto” fraction was mixed 1:1 with 2×laemmli buffer and boiled for 5 min prior to being loaded together with “Mito” (Mito:Cyto=10:1) into SDS-PAGE.

ATP Assay

ATP concentrations were determined using the Roche ATP Bioluminescence Assay Kit HS II (#11699709001, Sigma). Briefly, 1 whole fly or 8 fly heads were homogenized in 150 µl ice-cold lysis buffer using a Kontes pellet pestle. Lysate was then boiled for 5 min and centrifuged at 20,000 g at 4°C for 1 min. Cleared lysate was diluted 1:200 in dilution buffer and loaded with 10 µl luciferase. Luminescence was immediately measured using a FlexStation 3 (Molecular Devices). Total protein amount was measured using the bicinchoninic acid protein (BCA) assay (Thermo Fisher). The ATP level in each sample was normalized to the total protein amount.

Live ROS Detection

Fly brains were dissected in HBSS buffer (156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$ 10 mM glucose and 10 mM HEPES at pH 7.35), incubated with 30 µM MitoSox (M36008, Thermo Fisher) for 10 min, washed 3 times with HBSS, and then imaged immediately in a chamber on a slide with a coverslip. Images were acquired using a Leica SPE laser scanning confocal microscope equipped with a 10×/N.A.0.40 objective or 20×/N.A.0.60 oil Plan-Apochromat objective as Z stacks, with identical imaging parameters among different genotypes in a blinded fashion. The total intensity of each individual brain was measured with ImageJ (Ver. 1.48, NIH), and normalized to the background intensity.

Live Membrane Potential Imaging

Fly brains were dissected in HBSS buffer and stained with 25 nM tetramethylrhodamine methylester (TMRM) (T668, Molecular Probes) in HBSS for 40 min at room temperature and imaged immediately in a chamber on a slide with a coverslip. TMRM was present in the imaging media (25 nM TMRM in HBSS). TMRM fluorescence was excited at 561 nm and Mito-GFP at 488 nm using a Leica SPE laser scanning confocal microscope. Confocal images were obtained with a 63×/N.A.1.30 oil Plan-Apochromat objective. Images were collected from the same neuron cluster. The mean intensity of TMRM is normalized to that of matrix-localized Mito-GFP within the same neuron cell body.

Immunocytochemistry and Confocal Microscopy

Adult fly brains and larval muscles were dissected in PBST (0.3% Triton X-100 in PBS), and incubated with fixative solution (4% formaldehyde in PBST) for 15 min. Fixed samples were immunostained with rabbit anti-TH (AB-152, EMD Millipore) at 1:200, mouse anti-FK2 (mono- and polyubiquitinylated conjugates, BML-PW8810, Enzo) at 1:200, rabbit anti-p62/Ref(2)P (ab178440, Abcam) at 1:200, rabbit anti-dMIC60 at 1:500, or mouse anti-ATP5β (ab14730; AbCam) at 1:100. Colocalization coefficient was calculated using the Coloc 2 function of ImageJ for individual neuron cell bodies from the same neuron cluster.

For sensitized emission FRET, brain dissection and fixation were as described above. Fixed samples were immunostained with rabbit anti-dMIC60 at 1:200 and Alexa Fluor 488 donkey anti-rabbit (ab150073, Abcam) at 1:250, and mouse anti-T7 (69522, Sigma) at 1:200 and Cy3 donkey anti-mouse (NC9606617, Fisher scientific) at 1:250. All samples were prepared at the same time with the same handling and all images were obtained from a single acquisition session with identical imaging settings. Donor (dMIC60) was excited at 488 nm with 18% laser power and emitted at 510-540 nm. Acceptor

(T7-DMiro) was excited at 561 nm with 19% laser power and emitted at 580-700 nm. FRET was excited at 488 nm and emitted at 580-700 nm. Images were taken from the same brain area for all brains. The donor/acceptor expression ratio was fixed at close to 1 (0.90-1.18). Colocalized FRET index was calculated for neuron cell bodies using the FRET analyzer (colocalization FRET index) plugin of ImageJ, with the correction of donor/acceptor bleed through, and normalized to the DMiro intensity from the same neurons.

For immunostaining on iPSC-derived neurons, neurons were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min, then washed twice in PBS (5 min each), and rinsed in deionized water. Alexa Fluor 594 picolyl azide based TUNEL assays were performed according to the manufacturer's instruction (C10618, Invitrogen). Coverslips were incubated with TdT reaction buffer at 37°C for 10 min, followed by TdT reaction mixture at 37°C for 60 min in a humidified chamber. Coverslips were then rinsed with deionized water, washed with 3% BSA and 0.1% Triton X-100 in PBS for 5 min, followed by incubation with Click-iT plus TUNEL reaction cocktail at 37°C for 30 min, and then washed with 3% BSA and 0.1% Triton X-100 in PBS for 5 min. The following procedures were performed in the dark. Neurons were blocked in PBS with 3% BSA for 60 min, and then immunostained with rabbit anti-TH (AB-152, EMD Millipore) at 1:500 at 4°C overnight, followed by Alexa Fluor 488 fluorochrome conjugated goat anti-rabbit IgG (A11008, Invitrogen) at 1:500 at room temperature for 1 hr. Next, 4', 6-Diamidino-2-phenylindole (Dapi; D9542, Sigma) at 0.5 µg/ml was applied at room temperature for 10 min. Coverslips were washed with PBS, and then mounted on slides with Fluoromount-G mounting medium (SouthernBiotech). Samples were imaged at room temperature with a 20×/N.A.0.60 oil Plan-Apochromat objective on a Leica SPE laser scanning confocal microscope, with identical imaging parameters among different genotypes in a blinded fashion. Images were processed with ImageJ (Ver. 1.48, NIH) using only linear adjustments of contrast and color.

Lifespan

Flies were raised at a standard density in 200 ml bottles. After eclosion, flies were allowed to mate for 24-48 hrs in the bottles. Mated females were then split into vials containing SYA medium with or without compounds, with 15 flies in each vial. MR3/5 was dissolved in DMSO at 100 mM and supplemented into SYA medium when applicable. Control flies were fed with the same volume of the solvent of MR3/5, DMSO. Adult-onset, tissue–specific RNAi was achieved as previously described [36]. Briefly, 24-28 hrs after eclosion, female flies carrying a heterozygous copy of a *GeneSwitch* driver and at least one UAS-RNAi were fed on SYA medium supplemented with ethanol or with 200 μM mifepristone (RU486) to induce UAS expression from adulthood. Alternatively, flies were initially fed on normal SYA medium for 30 days and then switched to 200 μM RU486-supplemented food. For Fig. 3d, RU concentration supplemented in SYA medium was 25 μM. For all un-induced control food, the same volume of the solvent, ethanol, was added [61]. Flies were tipped onto fresh food and fly deaths were scored 3 times a week. The final n was the total number of dead flies. Lifespan experiments were performed at 25ºC unless otherwise stated. Data are presented as cumulative survival curves, and survival rates are compared using Log-Rank Test.

Stress and Antioxidant Assays

Paraquat (#856177, Sigma) was dissolved in water and then added into SYA medium at a final concentration of 20 mM. For starvation, female flies were maintained on pure 1.5% agar medium. 30% H_2O_2 (#H1009, Sigma) was added into SYA medium at a final concentration of 5%. AD4 (HY-110256, MedChemExpress) was added into SYA medium at a final concentration of 40 μg/ml.

Fly Behavior Assays

We performed two assays to measure fly locomotor abilities. The average Performance Index (PI) (negative geotaxis) was evaluated as previously described [36,62,63]. Briefly, adult flies were gently tapped to the base of a modified 25 ml climbing tube and their climbing progress was recorded after 45 sec. Three to six populations of flies were assessed, and for each population, flies were examined 3 times per experiment. The recorded values were used to calculate the average PI. The second measurement, the climbing time, was described in [32]. Briefly, the climbing ability was quantified by time it took adult flies to climb 8 cm.

To assess flying ability, flies were tapped one at a time out of a vial held upside down 1 foot above a benchtop. If the fly flew away, it was recorded as "1", and if it was unable to fly, it was recorded as "0".

Neuronal Derivation from iPSCs

The iPSC work was approved by Stanford Stem Cell Oversight Committee. iPSCs were purchased under an MTA from the NINDS human and cell repository, which is in a partnership with multiple institutions that deposited iPSCs, approved study protocols, ensured consent from donors, and explained the conditions for donating materials for research. All iPSC lines in this study are summarized below and have been fully characterized by our previous study [18] and the NINDS human and cell repository.

iPSC line	Source	Etiology	PD mutation	Age	Sex
PD	NINDS (ND50050); Episomal reprogramming	Genetic	*SNCA (A53T)*	51	Female
Wild-type	NINDS (ND50085)	Isogenic control for *PD*	*SNCA (A53T) corrected to wild-type*	51	Female

iPSCs were derived to midbrain dopaminergic neurons as previously described with minor modifications [19,49,64-66]. Briefly, neurons were generated using an adaptation of the dual-smad inhibition

method with the use of smad inhibitors dorsormorphin (P5499, Sigma-Aldrich) and SB431542 (1614, Tokris), and the addition of GSK3β inhibitor CHIR99021 (04-0004, Stemgent) and smoothened agonist SAG (566661, CalBioChem). To gain a higher purity of neural precursor cells, 12 days after neural induction, rosette-forming neuroectodermal cells were manually lifted and detached en bloc, and then cultured in suspension in a low-attachment dish (430589, Corning Inc.) with N2 medium with 20 ng/ml BDNF (450-02, Peprotech), 200 µM Ascorbic Acid (A5960, Sigma-Aldrich), 500 nM SAG, and 100 ng/ml FGF8a (4745-F8-050, R&D systems). On day 17, neurons were transferred onto poly-ornithine and laminin-coated glass coverslips in a 24-well plate. On day 18, medium was switched to N2 medium supplemented with 20 ng/ml BDNF, 200 µM Ascorbic Acid, 20 ng/ml GDNF (450-10, Peprotech), 1 ng/ml TGFβ3 (AF-100-36E, Peprotech), and 500 µM Dibutyryl-cAMP (D0627, Sigma-Aldrich) for maturation of dopaminergic neurons. Neurons were used at day 21-22 after neuronal induction, when about 80-90% of total cells expressed the neuronal marker TUJ-1, and 17.08%-19.25% of total cells expressed TH and markers consistent with ventral midbrain neuronal subtypes [20,67]. Antimycin A (A8674, Sigma-Aldrich) was applied to neurons at 10 µM.

In Vitro Binding Assay

For expression and purification of dMIC60 protein (containing AAs 92-739), pET101-TOPO-dMIC60 (WT or CS)-His-V5 [28] was transformed in BL21 DE3 non-pathogenic *E. Coli* by standard transformation methods. A 3 ml culture suspended in a culture tube was incubated overnight at 37ºC and shook at 250 rpm in a shaking incubator. The following day, the culture was added to 200 ml of growth media (YT media/1% glucose/Amp+), and then incubated at 37ºC, 250 rpm in a shaking incubator for at least 2 hrs or until it reached an O.D. of 0.6. Next, IPTG (367-93-1, Millipore Sigma) was added at a concentration of 0.1 mM, and the culture was allowed to incubate for an additional 2.5-3 hrs at 25°C, 180 rpm for

optimum expression. Bacteria were then pelleted by centrifugation at 2,400 ×g for 15 min. After discarding the supernatant and resuspending the pellet in 1×PBS with Protease Inhibitor Cocktail III (1:1000), bacteria were sonicated on ice with a sonicator at 50% power, 32% amplitude for 6-7 min or until the solution changed color (generally going from a darker consistency to a lighter one). Alternatively, pellets were lysed by Bugbuster (70922-3, 10 ml per 200 ml culture, Sigma) and Lysozyme (L3790, 2.5 ku per 1 ml Bugbuster, Sigma) with Protease Inhibitor Cocktail III (1:1000). Cellular debris was removed by centrifugation at 13,800 ×g for 20 min. All subsequent steps were performed at 4ºC or on ice. Ni-NTA beads were washed and resuspended 3-4 times in 1×PBS with Protease Inhibitor Cocktail III (1:1000) to a final solution of 50% Ni-NTA beads. Generally, 500 µl of beads was used for every 200 ml of bacteria. Beads were added to the supernatant of the post-sonication step and were incubated in a cold room at 4ºC for 2 hrs. Next, Ni-NTA beads and protein supernatant were added to a Gravitrap column. After the flow-through was discarded, 2 column volumes (CVs) of a weak wash buffer (1×PBS, 30 mM Imidazole, Protease Inhibitor Cocktail III at 1:1000) was added to the beads and allowed to incubate for 5 min. The aqueous phase was drained and collected for further analysis. This step was repeated along an imidazole concentration gradient with wash buffers consisting of 100 mM imidazole, 200 mM imidazole, and finally with an elution buffer containing 300 mM imidazole. Elution buffer was run over beads at least twice until no more protein of interest was eluted. Protein was concentrated to 2-4× and exchanged into NETN buffer (0.2 mM PMSF, 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5% NP-40, Protease Inhibitor Cocktail III) using a 2-6 ml Pierce™ protein concentrator with a molecular weight cutoff (MWCO) of 30 KDa.

GST-tagged human full-length Miro1 was purchased from Abnova (H00055288-P01). Untagged Parkin was purchased from Boston Biochem (E3-160).

For in vitro GST pull-down, glutathione beads (GE Healthcare) were washed and transferred to NETN buffer. Glutathione beads (18 μl/3 μg) were mixed with a GST-tagged protein and a non-GST-tagged protein suspended in NETN buffer to a final volume of 150 μl. For specific pull-down experiments, 1-3 μg of full-length GST-Miro1 (H00055288-P01, Abnova) and 5-10 μg of dMIC60 (as described above), or 1 μg of full-length GST-Miro1 and 3 μg of Parkin (E3-160, Boston Biochem) were used. For all assays, 0.25-3 μg of GST (ab81793, Abcam) was used as a negative control. All reactions were incubated at 4ºC for 3.5 hrs. Beads were washed 3-4 times with reaction buffer to eliminate all unbound proteins, and then mixed with 4×Laemmli buffer and run in 10% SDS-PAGE. Reducing buffers consisted of NETN buffer and 2 mM β-mercaptoethanol (Sigma). Western blotting was described as above.

Indirect Thiol Trapping Assay

Inspired by [4]. Flies were lysed in lysis buffer (50 mM Tris pH7.5, 1% Triton, 300 mM NaCl, 5 mM EDTA, 1:1000 Protease Inhibitor Cocktail III–#539134, Millipore), and debris was removed by centrifugation at 16,200 ×g. Laemmli buffer and 50 mM DTT were added to supernatant and the sample was boiled at 95ºC for 5 min. Or flies were lysed in lysis buffer containing 1 mg/ml methoxypolyethylene glycol maleimide 5000 (PEG-MAL; 63187, Sigma), followed by centrifugation, and Laemmli buffer was added to supernatant at 37ºC for 30 min. Or flies were lysed in lysis buffer containing 10 mM tris(2-carboxyethyl)phosphine (TCEP; 51805-45-9, Sigma), followed by centrifugation. Supernatant was incubated at 37ºC for 30 min, then Laemmli buffer together with 15 mM 4-Acetamido-4'-Maleimidylstilbene-2,2'-Disulfonic Acid, Disodium Salt (AMS; A485, Thermo Fisher) or 1 mg/ml PEG-MAL was added, and the sample was incubated at 37ºC for 10 min. Alternatively, mitochondrial pellet isolated from flies was suspended in mitochondrial isolation buffer, and treated with 50 mM DTT or 15

mM AMS at 37ºC for 10 min. Or mitochondrial fraction was treated with 10 mM TCEP at 37ºC for 30 min, pelleted via centrifugation and resuspended again, then Laemmli buffer containing 15 mM AMS was added, and the sample was incubated at 37ºC for 10 min.

Thermal Shift Assay

Human Miro1ΔTM (AAs 1-592) was prepared from Sf9 cell pellets collected three days post-infection. Frozen cell pellets were resuspended in lysis buffer (30 mM HEPES pH 7.4, 150 mM KAc, 2 mM MgAc, 3 mM $CaCl_2$, 20 μM GTP, 0.1% Triton X-100, 5% glycerol, 1×protease inhibitor cocktail–Sigma) and lysed by a dounce homogenizer. Supernatant was batch-bound to IgG sepharose (GE Healthcare, Chicago, IL, USA) and washed with lysis buffer and then with wash buffer (30 mM HEPES pH 7.4, 250 mM KAc, 2 mM MgAc, 3 mM $CaCl_2$, 20 μM GTP, 5% glycerol). Protein was eluted from sepharose by cleavage with Tobacco Etch Virus (TEV) protease in elution buffer (30 mM HEPES pH 7.4, 150 mM KAc, 2 mM MgAc, 3 mM $CaCl_2$, 20 μM GTP, 5% glycerol) at 16ºC for 2 hrs. The eluate was concentrated in 10 KDa-MWCO spin filters (Millipore, Burlington, MA, USA). TEV and residual affinity tag was removed by passage of the protein through a Superdex 200 Increase 10/300 GL (GE Healthcare) in SEC buffer (30 mM HEPES pH 7.4, 150 mM KAc, 2 mM MgAc, 3 mM $CaCl_2$, 20 μM GTP). Miro1 protein (0.4 mg/ml starting concentration) was combined with Sypro Orange protein stain (Sigma, St. Louis, MO, USA) at a final concentration of 2×(manufacturer's assignment) in PBS. Protein was diluted in a 2-fold series in a final volume of 10 μl. MR3 was diluted to 150 μM in PBS and then diluted in a 2-fold series. Diluted MR3 was added to wells of a 384-well plate, followed by protein and dye in equal proportion to yield the peak well-concentration of MR3 at 100 μM. Plate was sealed with optical grade plastic, spun for 1 min at 500×g to mix, and incubated at room temperature for 10 min prior to initiation of the melt program. Dilution values were measured in quadruplicate with PBS as the

control. A separate row of MR3 plus dye alone confirmed there was no fluorescence in the absence of Miro1 protein. The plate was read in a QuantFlex RT-PCR and a thermal melt curve from 25-95°C was applied at 0.03°C/s. Dye fluorescence was read with QuantStudio and analyzed with Thermal Shift Analyzer software.

Mass Spectrometry

Ten flies (day 5 and 50) in each group were lysed in RIPA buffer. The protein samples were mixed with 4×volume of ice cold (-80ºC) acetone and incubated at -80ºC overnight. Samples were centrifuged at 9,600 ×g for 10 min to separate the supernatant acetone from the precipitated protein pellet. Air-dried protein pellets were reconstituted in 100 mM triethylammonium bicarbonate buffer, sonicated, and vortexed to solubilize proteins. The samples were then reduced with 10 mM DTT at 55ºC for 30 min followed by alkylation with 30 mM acrylamide for 30 min at room temperature. 1 µg of Trypsin/LysC protease (Promega) was added to each sample for digestion and the samples were incubated overnight at 37ºC. 10 µl aliquots of each sample were used for a peptide quantification with the Pierce Quantitative Fluorometric Pepitde Assay kit (Thermo Fisher Scientific). Following peptide quantification, 10 µg aliquots of each sample of digested peptides were used for TMT labelling. Eight channels (126-130N) of a TMT 10plex Isobaric Label Reagent set (#90110, Thermo Fisher) were used to label individual samples. The 130C channel was left as an unused channel, and the 131 channel was used as a pool channel for all eight samples. Post-labelling, the samples were quenched with 5% hydroxylamine, combined into one series, and de-salted with C18 Monospin Reverse Phase Columns (GL Sciences). The combined sample was dried by speed vac before dissolution into 200 µl of reconstitution buffer (2% acetonitrile with 0.1% formic acid). 2 µl of the sample was injected into the instrument. Mass spectrometry was performed using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific,

San Jose, CA) attached to an Acquity M-Class UPLC system (Waters Corporation, Milford, MA) by an analytical column. The column was prepared in-house with an I.D. of 100 microns pulled to a nanospray emitter using a P2000 laser puller (Sutter Instrument, Novato, CA). The column was packed using C18 reprosil Pur 1.8 micron stationary phase (Dr. Maisch) to a length of ~25 cm. The UPLC system was set to a flow rate of 450 nL/min, where mobile phase A was 0.2% formic acid in water and mobile phase B was 0.2% formic acid in acetonitrile. Labelled peptides were directly injected into the column with a gradient of 2-45% mobile phase B, followed by a high-B wash over a total 180 minutes. The mass spectrometer was operated in a data-dependent mode using MS3 HCD and CID fragmentation for spectral generation. The collected mass spectra were analyzed using Proteome Discoverer 2.2 (Thermo Scientific) against the Uniprot *Drosophila Melanogaster* database. Data were searched using Byonic v3.7.13 (Protein Metrics) as the peptide identification node. The precursor ion tolerance was set to 12 ppm. The fragment ion tolerance was set to 0.4 Da and 12 ppm for MS2 and MS3 scans, respectively. Trypsin was set as the enzyme, and up to two missed cleavages were allowed. N-termini and lysine residues modified by TMT10plex and cysteine modified with propionamide were set as fixed modifications in the search. Oxidation on methionine and deamidation on asparagine and glutamine were set as variable modifications. Data were validated using the standard reverse-decoy technique at a 1% false discovery rate.

TEM

Third instar male larvae were filleted in 1×Ca^{2+} free saline (0.128 M NaCl, 2 mM KCl, 5 mM EGTA, 4 mM $MgCl_2$, 5 mM HEPES, 0.0355 M sucrose) and fixed in Karnovsky's fixative (0.1 M sodium cacodylate buffer pH 7.4, 2% glutaraldehyde, and 4% paraformaldehyde) at room temperature (22°C) for 30 min and then kept at 4°C overnight. Specimens were post-fixed in cold/aqueous 1% osmium

tetroxide for 1 hr, warmed to room temperature for 2 hrs, and then rinsed 3 times in ultra-filtered H_2O. Specimens were subsequently stained en bloc with 1% uranyl acetate at room temperature for 2 hrs, dehydrated in a graded ethanol series, immersed in propylene oxide (PO) for 15 min, infiltrated with a graded series of PO and EMbed-812 resin, and then embedded in EMbed-812 resin at 65°C overnight. Sections were cut to a thickness of 75-90 nm and laid on formvar/carbon-coated slot Cu grids. Sections were stained for 40 sec with 3.5% uranyl acetate in 50% acetone followed by Sato's Lead Citrate for 2 min. Sections were observed using a JEM-1400 120kV (Joel, Japan) and images were taken using an Orius 832 4k X 2.6k digital camera with 9 μm pixel (Gatan, CA). Muscles 6/7 were selected for imaging. Images were processed with Photoshop CS6.

Seahorse Analysis

Fly brains were dissected in Agilent seahorse basal media (Agilent Technologies, Santa Clara, CA, USA) supplemented with glucose (10 mM), pyruvate (1 mM), and glutamine (2 mM) pH 7.4, and each brain was placed at the bottom of a well of a XF24 cell culture plate containing 500 μl Agilent basal media. Fly brains were then placed into a Seahorse XFe24 Analyzer (Agilent Technologies) with temperature set at 25ºC. For OCR and ECAR experiments, samples were treated with 10 μM oligomycin (complex V inhibitor) and 5 μM rotenone/antimycin (complex I & III inhibitor). Black arrows in the Figure represent the times of oligomycin and rotenone/antimycin injections. A total of at least three OCR and pH measurements were taken after each compound was administered. Basal respiration was calculated as follows: (average(initial three measurements))-(average(last three measurements)) from the O_2 pmol/min readings. ECAR was calculated as the average of the initial three measurements from the mpH/min readings.

QUANTIFICATION AND STATISTICAL ANALYSIS

Throughout the paper, the distribution of data points is expressed as box-whisker, dot-plot with Mean±SEM, or survival plot, except otherwise stated. Box center line is median and box limits are upper and lower quartiles. One-Way or Two-Way ANOVA Post-Hoc Tukey or Fisher LSD Test was performed for comparing multiple groups. Mann-Whitney *U* or T Test was performed for comparing two groups. Chi-Square Test was performed for flying ability analysis because the data was categorical. Statistical analyses were performed using the Prism software (ver. 8.01, GraphPad) or Excel (ver. 16.51). For all experiments, between 3 and 157 animals or independent experiments were used or performed. The number of animals and experimental replications (n) can be found in Figure Legends and Method Details. No statistical methods were used to predetermine sample sizes, but the number of flies, experiments, and biological replicates were chosen based on the nature of the experiments (it is usually difficult to assess an outcome that follows a normal distribution in our experiments), degree of variations, and published papers describing similar experiments. We did not exclude any data. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, for all Figures.

Data availability

Further information and reagents are available from the corresponding author upon reasonable request. Source Data are provided with this paper. Uniprot Drosophila Melanogaster database was used.

Acknowledgements

We thank Drs. W. Ge, A. Whitworth, and K. Zinsmaier for antibodies, L. Pallanck, W. Ge, H. Tricoire, B. Ohlstein, V. Monnier, B. Lu, and Y. Imai for flies, G. Rubin and D. Rubinsztein for plasmids, Z. Tan for Miro1 protein, A. Gonzalez, R. Vanhauwaert, K. Kinghorn, J. Castillo-Quan, Y. Zhao, N. Vavouraki,

J. Navarro, E. Chouchani, H. Xiao, W. Weis, and Steinberg lab for advice and assistance, J. Perrino and Stanford Cell Science Imaging EM Facility for support with TEM, Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University (RRID:SCR_017801) for generating proteomic data, and the Fly Facility at Department of Genetics of University of Cambridge for fly injection. This work was supported by the Department of Defense (W81XWH-16-1-0282, X.W.), NIA (R21AG06131501, X.W.), Stanford Gabilan and McCormick Fellowship (X.W.), Stanford Parkinson's Disease Seed Grant (X.W.), Stanford MCHRI Pilot Grant (X.W.), Glenn Foundation Postdoctoral Fellowship (L.L.), Parkinson's UK (L.L. and L.P), and in part National Center for Research Resources (ARRA, 1S10RR026780-01) and NIH (P30CA124435 and S10RR027425).

Author contributions

Fly work: L.L., M.J.K., A.M.P. Biochemistry: D.M.C, L.L., V.B. Mammalian work: C.H., V.B. Protein purification and thermal shift assay: A.L., M.A.C. Seahorse: P.S.M., A.S.D., K.I.A. Reagent generation and discussion: L.P. Conception and supervision: X.W. All authors designed the experiments and wrote the paper.

Competing interests

The authors declare the following competing interests: X.W. is a co-founder, adviser, and shareholder of AcureX Therapeutics Inc, and a shareholder of Mitokinin Inc. L. L. and C.H. are shareholders of AcureX Therapeutics Inc. A patent on Miro1 Reducers was filed by Stanford University with X.W., C.H., and L.L. as inventors. The remaining authors declare no competing interests.

Figure Legends

Fig. 1. dMIC60 Is Oxidized and Binds to Miro in a Redox-Dependent Manner. (a-f) Wild-type flies (w^{1118}) (a-e), or flies with indicated genotypes (f), were treated with the reducing agent DTT, the thiol-reacting agent PEG-MAL (PEG) or AMS, or by the indirect thiol trapping assay, and blotted with anti-dMIC60. Inspired by [4]. H_2O_2 was fed at day 1 for 24 hrs. (g-h) Whole-body lysates of wild-type flies (w^{1118}) were IPed with anti-DMiro or IgG, and immunoblotted (IB) as indicated. (h) Flies were fed with or without H_2O_2 at day 1 for 24 hrs. (i) GST pull-down using recombinant human full-length GST-Miro1, and whole-body lysates of flies expressing *UAS-dMIC60-WT* driven by *Actin-GAL4*, fed with or without H_2O_2 at day 7 for 24 hrs. (j) Confocal single-section images overlaying dMIC60 (green) and T7-DMiro (magenta) of fly brains of *Elav-GAL4>T7-DMiro*, fed with or without H_2O_2 at day 7 for 24 hrs. The FRET images are color-coded colocalized FRET. From FRET images, colocalized FRET index is calculated. n=68 and 88 neurons from 17 and 22 brain regions from 5 and 6 brains for H_2O and H_2O_2, respectively. Two-sided Mann-Whitney Test. p=0.049. Boxes show $25^{th}/75^{th}$ percentiles, whiskers are the minimum and maximum, and middle line is median. Scale bars: 20 μm. (k) Flies with indicated genotypes were fed with H_2O_2 at day 1 for 24 hrs, IPed with anti-DMiro, and blotted. (l) Left: The topology of DMiro and dMIC60. Right: HEK cells expressing indicated constructs were treated with H_2O_2 for 10 min, IPed, and blotted as indicated. FL: full length. (a-i, k, l) Same results were reproduced more than 3 times. (m) Schematic summarization of our results showing that Miro binds to dMIC60 in a redox-dependent manner.

Fig. 2. Mitochondrial Changes with Age. (a) Flies with indicated genotypes were fed with or without H_2O_2 at day 1 for 24 hrs, lysed, and blotted. The band intensity is normalized to β-actin on the same blot. n.s.: not significant. n=4 independent experiments. p=0.0228, 0.0354, 0.0013 for DMiro, 0.0231, 0.0278, 0.0003 for dMIC60. The rest of the precise p values are in Source Data. (b) Representative confocal

stack images of MitoSox staining of fly brains. The total MitoSox intensity is measured for each fly brain and normalized to the background intensity. Genotype: *Elav-GS>UAS-DMiro*^{RNAi} uninduced (RU-). n=5 (young) and 7 (old) fly brains. Scale bar: 200 μm. p=0.0025. (c) The heatmap shows relative protein abundances (normalized mean values from Supplementary Table 1) in selective clusters of protein groups. (d) Upper: Whole-body fly lysates of young (day 5) and old (day 50) wild-type flies (w^{1118}) were IPed with anti-DMiro and blotted as indicated. The band intensity of dMIC60 in IP is normalized to that of DMiro in IP. Lower: The band intensity of each protein in lysate is normalized to that of β-actin from the same blot. n=4 independent experiments. p=0.0286. (e) Similar to (d), old (day 50) wild-type flies, fed with or without AD4 from day 30-50, were IPed. n=5 for DMiro Input and 4 for the rest. p=0.0286 and 0.0159. (a) Two-Way ANOVA Post Hoc Fisher LSD Test. (b, d-e) Two-sided Mann-Whitney Test. Boxes show 25th/75th percentiles, whiskers are the minimum and maximum, and middle line is median.

Fig. 3. Dissociating the DMiro-dMIC60 Interaction Benefits Fly Lifespan and Health-Span. For all panels, "RU-" flies were fed with the same volume of the vehicle, ethanol. All flies were female. (a-d) Survival plots of flies with genotypes indicated. (a) n=81 (WT) and 89 (CS). (b-d) Blue lines (RU+): Adult-onset neuronal downregulation using *Elav-GS-GAL4*. Black lines (RU-): Un-induced controls. (b) n=144 (RU+) and 145 (RU-) flies. (c) n=122 (RU+) and 142 (RU-) flies. (d) n=67 (RU+) and 74 (RU-) flies. (e) The climbing ability shown as Performance Index, or (f) the survival ability in response to 20 mM paraquat (starting from day 4), of flies with ubiquitous expression of *UAS-dMIC60-WT* or *UAS-dMIC60-CS* by *Actin-GAL4* in *dMIC60* null background. p=0.0152. (e) n=19 (WT) and 30 (CS), 6 independent experiments. p=0.0152. (f) n=29 (WT) and 34 (CS). p=1.4 ×10^{-12}. (g) The climbing ability shown as Performance Index of flies with indicated genotypes. n=59 (RU-) and 60 (RU+) flies, 3

independent experiments. p=0.0037. (h-i) Survival plots. (h) Starvation ($p=1.17\times10^{-22}$) and (i) 20 mM Paraquat ($p=5.12\times10^{-9}$) started after 14 days of adult-onset neuronal knockdown of *DMiro*. (h) n=148 (RU-) and 151 (RU+). (i) 149 (RU-) and 150 (RU+). (a-d, f, h, i) Log-Rank Test. (e) Two-sided Mann Whitney Test. (g) Two-Way ANOVA Post Hoc Tukey Test. (e, g) Data are presented as Mean±S.E.M. with dots.

Fig. 4. Dissociating the DMiro-dMIC60 Interaction Improves Mitochondrial Function. (a-b) ATP levels were measured in flies with indicated genotypes. (a) Day 2. n= 8. p=0.0019. (b) Blue bar (RU+): Adult-onset neuronal knockdown of *DMiro* (*Elav-GS>UAS-DMiro*^{RNAi}) for 20 days. Gray bar (RU-): Un-induced control flies. n=6 (heads) and 7 (whole flies) independent experiments. p=0.0022 (heads), 0.0111 (whole body). (c-d) Representative confocal stack images of MitoSox staining of fly brains with indicated genotypes. The total MitoSox intensity is measured for each fly brain and normalized to the background intensity. (c) Day 12. n=5 (WT) and 6 (CS) fly brains. p=0.0303. (d) n=5, 5, 7, 6 fly brains (from left to right). Uninduced data (RU-) are the same as in Fig. 2b. p=0.0046, 0.015. The rest of the precise p values are in Source Data. (e) Representative confocal images of TMRM staining (magenta) of fly brains expressing Mito-GFP (green) in DA neurons driven by *TH-GAL4*, with or without *DMiro* RNAi. The fluorescence intensity of TMRM is normalized to that of Mito-GFP within the same neuron cell body. n=20 (old, RU-), 28 (old, RU+), 29 (young, RU-), 38 (young, RU+) neurons from 3 (young) and 4 (old) brains. p<0.0001. Scale bars: (c) 100 μm; (d) 200 μm; (e) 25 μm. (a-c) Two-sided Mann-Whitney Test. (d-e) One-Way ANOVA Post Hoc Tukey Test. (a, c, d, e) Boxes show $25^{th}/75^{th}$ percentiles, whiskers are the minimum and maximum, and middle line is median. (b) Data are presented as Mean±S.E.M. with dots.

Fig. 5. *DMiro* RNAi Restores Cellular Respiration and Mitophagy. (a) Seahorse analysis on ex vivo fly brains (*Elav-GS>UAS-DMiro*^{RNAi}) of different ages, with or without RU induction, as indicated. "RU-" flies were fed with the same volume of the vehicle, ethanol. n=7 brains. Black arrows show the times of oligomycin and rotenone/antimycin injections. $p<0.0001$. (b) Representative confocal single-section images, overlaying Mito-GFP (green), ubiquitin (red), and p62 (blue), of fly brains expressing Mito-GFP in DA neurons driven by *TH-GAL4*, with or without *DMiro* RNAi. Yellow arrow heads show Mito-GFP puncta that colocalize with p62, ubiquitin, or both. The colocalization coefficient is quantified for Mito-GFP versus ubiquitin, and Mito-GFP versus p62, respectively. n=31, 24, 19, 25 neurons for ubiquitin, and 32, 24, 19, 23 for p62 (from left to right) from 6 brains. Scale bar: 10 μm. $p<0.0001$, $=0.0415$. The rest of the precise p values are in Source Data. (a) Two-Way ANOVA Post Hoc Tukey Test. (b) One-Way ANOVA Post Hoc Tukey Test. (a) Data are presented as Mean±S.E.M. with dots. (b) Boxes show $25^{th}/75^{th}$ percentiles, whiskers are the minimum and maximum, and middle line is median.

Fig. 6. α-Syn Expression or Frataxin Deficiency Impacts the DMiro-dMIC60 Complex. (a, g) Representative confocal stack images of MitoSox staining of fly brains as indicated. The total MitoSox intensity is measured for each fly brain and normalized to the background intensity. (a) n=4 (*Elav-GAL4*) and 5 (*Elav>SNCA-A53T*) fly brains. p=0.0317. (g) n=5 (*Actin>Fh RNAi*) and 7 (*Actin-GAL4*) fly brains. p=0.0313. (b) Whole-body lysates of 5-day-old α-syn-A53T-expressing flies (*Actin>UAS-SNCA*^{A53T}) and controls (*Actin-GAL4*) were IPed with anti-DMiro and blotted as indicated. The band intensity of dMIC60 in IP is normalized to that of DMiro. n=4 independent experiments. p=0.0286. (c) Whole-body lysates of 15-day-old flies were immunoblotted. The DMiro band intensity is normalized to that of β-actin from the same blot. n=4. p=0.0286. (d) Confocal stack images show DA neurons immunostained

with anti-TH in the PPL1 clusters of 40-day-old flies. The DA neuron number in the PPM1/2 and PPL1 clusters is quantified. n=14, 12, 13, 15 brains for PPL1, and 19, 12, 12, 20 for PPM1/2 (from left to right). p<0.0001, =0.0027, 0.0018. Details of comparisons and p values are in Source Data. (e) The DA neuron number in the PPL1 clusters is quantified. n=6 (*TH>dMIC60-WT*), 8 (*TH>dMIC60-CS*), 10 (*TH<SNCA^{A53T}, dMIC60-WT*), and 7 (*TH>SNCA^{A53T}, dMIC60-CS*) brains. p<0.0001. (f) Locomotor ability shown as Performance Index of 42-day-old flies. n=25 (*TH>dMIC60-WT*), 40 (*TH>dMIC60-CS*), 45 (*TH<SNCA^{A53T}, dMIC60-WT*), and 33 (*TH>SNCA^{A53T}, dMIC60-CS*); 3 independent experiments. p=0.009, 0.0007, 0.003. The rest of the precise p values are in Source Data. (h) Upper: Whole-body lysates of 30-day-old flies were IPed with anti-DMiro and blotted as indicated. The band intensity of dMIC60 in IP is normalized to that of DMiro. Lower: The DMiro or dMIC60 band intensity in Input is normalized to that of β-actin from the same blot. n=4. p=0.0286. Scale bars: (a, g) 100 μm; (d) 27.5 μm. (a-c, g-h) Two-sided Mann-Whitney Test. (d-f) One-Way ANOVA Post Hoc Tukey Test. Boxes show 25th/75th percentiles, whiskers are the minimum and maximum, and middle line is median. Data in (f) are presented as Mean±S.E.M. with dots.

Fig. 7. MR3 and MR5 Benefit Normal Flies, and Fly Models of PD and FA. (a) Chemical properties of MR3-5. (b) Confocal stack images show DA neurons in the PPL1 clusters of 40-day-old brains immunostained with anti-TH. Quantification of the DA neuron number in the PPL1 clusters is shown. n=7, 10, 9, 11 brains (from left to right). p<0.0001. (c) Locomotor ability shown as Performance Index at indicated dates. n=40, 42, 40, 46 flies (from left to right), 3 independent experiments. p=0.0072, 0.0482, 0.0018. The rest of the precise p values are in Source Data. (d) Neurons were pretreated with MR5 24 hrs before Antimycin A for another 6 hrs. The same volume of ethanol was applied at the same time in negative controls. The density of TH-positive neurons is calculated in each condition under 20×.

n=19 (PD, MR5) or 20 fields (the rest) from 4 experiments. $p<0.0001$. The neuron density without Antimycin A is not significantly different among all conditions ($p=0.4543$). Comparisons with "*Wild-type*, no treatment" except otherwise indicated. (e) Locomotor ability shown as Performance Index was measured in flies as indicated at different days. n=43, 41, 44, 44, 44, 42 flies (from left to right), 3 independent experiments. Data is compared at the same age. p=0.0249, 0.0092, 0.0076, 0.0281, 0.0038. The rest of the precise p values are in Source Data. (f) Survival plots of flies orally administered with MR3 or MR5 from adulthood (colored lines) and controls fed with DMSO (black line). n=126 (1 μM MR3), 131 (2.5 μM MR5), 132 (2.5 μM MR3), 157 (DMSO) flies. (g) ATP levels of wild-type flies fed with either 2.5 or 10 μM MR5 from adulthood for 20 days. n=8 independent experiments. p=0.0499, 0.0207. (b, d) One-Way ANOVA Post Hoc Tukey Test. (c) One-Way ANOVA Post Hoc Fisher LSD Test. (e) Two-Way ANOVA Post Hoc Tukey Test. (f) Log-Rank Test. (g) Two-sided Mann-Whitney Test. Scale bars: (b) 50 μm; (d) 100 μm. (b, d) Boxes show $25^{th}/75^{th}$ percentiles, whiskers are the minimum and maximum, and middle line is median. (c, e, g) Data are presented as Mean$\pm$S.E.M. with dots.

Fig. 8. Schematic Representation of a Redox-Dependent Regulation of dMIC60-DMiro. (a) In young flies (low ROS), dMIC60 shows a weak interaction with DMiro. MT: microtubules. (b) ROS upregulation (aging, PD, FA) causes an increase in the amount of dMIC60 bound to DMiro, leading to stabilization of the complex, which may consequently exacerbate ROS accumulation by slowing mitophagy and disrupting cellular respiration. (c) Genetically or pharmacologically targeting the DMiro-dMIC60 complex extends lifespan and health-span, promotes mitophagy, restores cellular respiration, and rescues phenotypes of PD and FA models.

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