Loss of Parkinson’s disease-associated protein CHCHD2 affects mitochondrial crista structure and destabilizes cytochrome c

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Mutations in CHCHD2 have been identified in some Parkinson’s disease (PD) cases. To understand the physiological and pathological roles of CHCHD2, we manipulated the expression of CHCHD2 in Drosophila and mammalian cells. The loss of CHCHD2 in Drosophila causes abnormal matrix structures and impaired oxygen respiration in mitochondria, leading to oxidative stress, dopaminergic neuron loss and motor dysfunction with age. These PD-associated phenotypes are rescued by the overexpression of the translation inhibitor 4E-BP and by the introduction of human CHCHD2 but not its PD-associated mutants. CHCHD2 is upregulated by various mitochondrial stresses, including the destabilization of mitochondrial genomes and unfolded protein stress, in Drosophila. CHCHD2 binds to cytochrome c along with a member of the Bax inhibitor-1 superfamily, MICS1, and modulated cell death signalling, suggesting that CHCHD2 dynamically regulates the functions of cytochrome c in both oxidative phosphorylation and cell death in response to mitochondrial stress.
Mutations in the CHCHD2 gene cause an autosomal dominant form of late-onset PD1. Several exonic variants, which may affect the protein levels or subcellular localization of CHCHD2, have been associated with Parkinson’s disease (PD) and dementia with Lewy bodies, albeit in a study with a limited number of cases2.

The gene product CHCHD2 contains a mitochondrial targeting sequence in the N-terminus and two cysteine-x-x-cysteine (twin CxxC) motifs at the C-terminus and has been localized to the intermembrane space of the mitochondria1,3. Although little is known regarding the physiological and pathological roles of CHCHD2, the close homologue CHCHD10 is believed to regulate cristae structure, maintaining the integrity of the mitochondrial respiratory complexes, at the crista junction of the intermembrane space4. A study of yeast CHCHD2 ortholog Mic17 indicated that the loss of Mic17 decreased oxygen consumption and altered activities in respiratory complexes III (ubiquinol-cytochrome c [Cyt c] reductase) and IV (Cyt c oxidase) in Saccharomyces cerevisiae6, whereas an oxidative phosphorylation (OXPHOS) computational expression screen using mouse and human microarray data revealed that CHCHD2 is required for the OXPHOS function in mammalian cells5. Further functional analysis has indicated that the loss of CHCHD2 destabilizes complex IV, which reduces oxygen consumption and complex IV activity but not complex I (NADH:ubiquinone oxidoreductase) activity6. Another study suggested that CHCHD2 positively regulates the function of complex IV through direct binding and transcriptional regulation of the complex IV subunit-4 isoform COX4i2 (ref. 3). It has also been suggested that CHCHD2 inhibits apoptosis through suppression of Cyt c release by binding to Bcl-xL7.

Dysregulation of the mitochondrial respiratory complexes generates reactive oxygen species (ROS), which leads to oxidative stress and affects the survival and function of neurons8,9. Insulin/IGF signalling is a key signalling pathway that maintains mitochondrial activity10, and the downstream components of insulin/IGF signalling, FoxO and the FoxO target elf4E-binding protein (4E-BP) regulate mitochondrial functions and aging in Drosophila11,12. Indeed, 4E-BP, which suppresses cap-dependent protein translation through binding to elf4E, has been shown to have neuroprotective roles against various stresses in Drosophila PD models13-17.

To understand the physiological and pathological roles of CHCHD2 in vivo, we established CHCHD2 mutant flies because fly models harbouring PD genes associated with mitochondrial functions exhibit pronounced mitochondrial phenotypes and have greatly contributed to the understanding of PD gene functions. Here, we report that the loss of CHCHD2 in flies leads to mitochondrial and neuronal phenotypes associated with PD pathology, including increased sensitivity to oxidative stress and loss of dopaminergic (DA) neurons with age. These phenotypes are rescued by 4E-BP and human CHCHD2 but not by CHCHD2 mutants found in PD cases. Our study suggests that mutations of CHCHD2 have a loss-of-function aspect in PD, exacerbating oxidative stress and cell death signalling.

Results

Generation of CHCHD2 loss-of-function flies. CHCHD2 orthologs are present in various species, including worm, yeast, and plants, and the affected amino acids found in PD cases are mildly conserved among these species (Supplementary Fig. 1a)6. We targeted Drosophila CHCHD2 (dCHCHD2) and generated hypomorphic dCHCHD2H43 and revertant alleles by imprecise and precise excision, respectively, using the artificial transposon p[EPgy2]EY05234 (Supplementary Fig. 1b,c). The expression of dCHCHD2 transcripts in dCHCHD2H43 was reduced to ~8.5% of the level associated with the revertant allele (Supplementary Fig. 1d). Western blot analysis revealed that expression of the dCHCHD2 protein was almost abrogated in dCHCHD2H43 homozygous flies (Fig. 1a and Supplementary Fig. 1e). We also generated a dCHCHD2 null allele (dCHCHD2+/−) mutant, which lacks the coding region of dCHCHD2, using CRISPR/Cas9 technology and confirmed that the protein expression disappeared (Fig. 1a and Supplementary Fig. 1e-g). Although dCHCHD2H43 and dCHCHD2+/− flies were grossly normal, emerging from pupae at the expected Mendelian ratio, the mitochondrial morphological in the indirect flight muscles were affected. The structure of mitochondrial cristae became disordered in 14-day-old dCHCHD2H43 homozygous flies, and a 'swirling' phenotype (white arrowheads) and dilatation of matrix spaces were observed (Fig. 1b,d,e). Similar results were obtained with 14-day-old dCHCHD2+/− flies (Supplementary Movies 1 and 2), and the disintegration of the mitochondrial cristae progressed in 40-day-old flies (Fig. 1c–e). ATP levels in the thorax muscles were mild decreased with age in dCHCHD2H43 flies, and the phenotype was exacerbated in dCHCHD2+/− flies because ATP reduction was detected at an earlier stage (Fig. 1f). Mild atrophy of muscles, irregular arrangement of nuclei and an increase in Tdt-mediated dUTP nick end labelling (TUNEL) -positive nuclei were observed in 30-day-old dCHCHD2+/− flies (Fig. 1g), whereas an increase in the number of TUNEL/tyrosine hydroxylase (TH)-positive neurons was not detected at a single point in time (Supplementary Fig. 1h). However, the loss of dCHCHD2 led to a reduction of TH signals, suggesting that the functions of dopaminergic neurons declined (Supplementary Fig. 1h). Our histochemical analyses of dCHCHD2 mutant flies indicate that loss of CHCHD2 affects the maintenance of the mitochondrial cristae structure, which is important for the activity of the mitochondrial respiratory complex and for muscle cell survival.

CHCHD2 loss leads to oxidative stress and neurodegeneration. Although dCHCHD2H43 adult flies emerged normally and showed normal survival at a younger stage, they exhibited shorter lifespans (Fig. 2a). Oxidative stress is considered a prominent causal factor of PD, and mutations in PD-associated genes that regulate mitochondrial functions have been reported to lead to oxidative stress18-20. dCHCHD2H43 adult flies were sensitive to oxidative stress from paraquat (Fig. 2b) and hydrogen peroxide (Fig. 2c), and they exhibited mild sensitivity to starvation (Fig. 2d). ROS production from mitochondria in dCHCHD2+/− embryonic cells was elevated by 1.77-fold (Fig. 2e,f). Consistent with the elevated ROS production from mitochondria, the protein translation regulator 4E-BP was upregulated at both the transcript and protein levels in the thorax of dCHCHD2H43 flies (Fig. 2g,h), and upregulation of 4E-BP protein was observed in dCHCHD2+/− flies (Fig. 2h). Increased protein oxidation and a decreased GSH/GSSG ratio was also detected in dCHCHD2+/− flies (Supplementary Fig. 2a,b). Conversely, the levels of the ROS scavengers SOD1 and DJ-1b were not changed in dCHCHD2+/− flies (Supplementary Fig. 2c). These results suggest that loss of dCHCHD2 causes chronic oxidative stress, overwhelming ROS scavenging activity in flies13-17.

We next examined the effects of dCHCHD2 loss on the central nervous system. Consistent with the above evidence that chronic oxidative stress occurs following loss of dCHCHD2, a lipid oxidation marker, 4-hydroxy-2-nonenal (4-HNE), was increased in both whole brain and central DA neurons of dCHCHD2H43 flies (Fig. 2i). Immunoreactivity of 8-hydroxy-2′-deoxyguanosine...
(8-OHdG), which is a product of oxidatively damaged DNA formed by hydroxyl radicals, was also increased in the whole brain and the PPL1 and PPM3 DA neuron clusters of dCHCHD2H43 flies (Supplementary Fig. 2d). The age-dependent loss of DA neurons was observed in the PPL1 cluster of dCHCHD2H43 adult flies even though no changes were observed in the TUNEL assay (Fig. 2j,k), and climbing ability, which is regulated by DA neurons, also declined with age (Fig. 2l)23. Taken together, these results indicate that loss of CHCHD2 produces chronic oxidative stress, which causes aging-dependent neurodegeneration in DA neurons.

**CHCHD2 is upregulated upon mitochondrial stresses.** During the stress assays using Drosophila, protein levels of dCHCHD2 were markedly changed following mitochondrial stresses (Fig. 3).
Mitochondrial genomic stress caused by mutations or overexpression of the mitochondrial DNA polymerase γ subunits and mitochondrial unfolded protein stress induced by truncated mitochondrial matrix protein ornithine transcarbamylase (ΔOTC) upregulated ΔCHCHD2 at both the transcript and protein levels (Fig. 3a,b and Supplementary Fig. 3a)24,25. Suppression of the mitochondrial respiratory complex subunits by gene knockdown and loss of PINK1, parkin and DJ-1b also increased or tended to increase ΔCHCHD2 protein levels (Supplementary Fig. 3b,c), suggesting that CHCHD2 is a mitochondrial stress-responsive protein.

Hyperoxia induces the mitochondrial swirl phenotype and defects in the Cyt c oxidase complex, which is likely to be accompanied by oxidative stress and/or dysregulation of the cristae junctions26,27. We thus examined the effects of CHCHD2 loss on hyperoxia. The mitochondrial cristae of the indirect flight muscle became disordered when normal flies were exposed to hyperoxia for 4 days (Fig. 3c,d versus Fig. 1e). The phenotype was further exacerbated in ΔCHCHD2 mutant flies. Consistent with the idea that hyperoxia produces oxidative stress, 4-E-BP was upregulated in both normal and mutant flies after hyperoxia exposure (Fig. 3e). Interestingly, ΔCHCHD2 expression was suppressed by hyperoxia (Fig. 3e). These results suggest that CHCHD2 expression is positively regulated by the reduction in the metabolic status.

CHCHD2 regulates mitochondrial electron transport. CHCHD2 has been suggested to be involved in the regulation of OXPHOS in human cultured cells and yeast6,28, which could be due to the positive regulation of complex IV activity by the binding of CHCHD2 (ref. 3). To determine respiratory ability in the presence or absence of ΔCHCHD2, we measured the oxygen consumption rate (OCR) of the mitochondria in combination with respiratory complex inhibitors. Although we initially used mitochondria isolated from the flight muscles of adult flies, they did not work well, consistent with a previous report29. Then, we employed embryonic cells prepared from ΔCHCHD2+/+ and ΔCHCHD2−/− flies. Consistent with the finding of lower OCR production in ΔCHCHD2 mutant flies, a steady OCR was lower in ΔCHCHD2−/− cells without detectable changes in the OXPHOS proteins (Fig. 4a,b and Supplementary Fig. 2c). Treatment with an inhibitor of ATP synthase, oligomycin, suppressed both OCRs to the same extent (Fig. 4a,b). Subsequent treatment with a proton ionophore, FCCP (carbonyl cyanide 4-[trifluoromethoxy] phenylhydrazone), stimulated maximal OCR, which was lower in ΔCHCHD2−/− cells (Fig. 4a,b). OCR was largely inhibited by rotenone and antimycin A, indicating that it was derived from mitochondrial matrix protein ornithine transcarbamylase (ΔOTC) upregulated ΔCHCHD2 at both the transcript and protein levels (Fig. 3a,b and Supplementary Fig. 3a)24,25. Suppression of the mitochondrial respiratory complex subunits by gene knockdown and loss of PINK1, parkin and DJ-1b also increased or tended to increase ΔCHCHD2 protein levels (Supplementary Fig. 3b,c), suggesting that CHCHD2 is a mitochondrial stress-responsive protein.

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CHCHD2 maintains mitochondrial cristal integrity. A close homologue of CHCHD2, CHCHD10 (identity 58% by ClustalW), is reported to be enriched at the cristal junction3. We thus searched for the binding partners of CHCHD2 that are involved in the OXPHOS pathway by mass spectrometry analysis. Among them, we focused on an inner mitochondrial membrane protein, MICS1, which has been reported to maintain cista structure and to negatively regulate Cyt c release by direct binding during apoptosis (Supplementary Fig. 5a)30. Because MICS1 contains several transmembrane domains, it can be solubilized only in detergent-containing solutions. However, because it was difficult to detect stable binding between human CHCHD2 (hCHCHD2) or Cyt c and MICS1 in detergent-containing solutions, probably due to the unfolding of MICS1 by the removal of the inner mitochondrial memnbrane, we used a thiol-cleavable crosslinker (dithiobis (succinimidyl) propionate) (DSP) to detect the binding in their native conformations. Human embryonic kidney (HEK) 293T cells were transiently transfected with plasmids encoding MICS1, hCHCHD2 or Cyt c. We first confirmed the interaction between MICS1 and hCHCHD2 in mitochondria (Supplementary Fig. 5b,c). We then examined whether these three proteins form a tertiary complex in HEK293T cells (Fig. 5a). Cyt c with a FLAG tag was immunoprecipitated with anti-FLAG beads, and co-precipitated MICS1-HA and hCHCHD2-Myc were detected in western blots. Under this condition, an unrelated inner mitochondrial membrane protein, Tim23, was not present in this complex, indicating that DSP specifically links protein complexes.
Figure 2 | dCHCHD2 mutant flies exhibit phenotypes associated with PD. (a) dCHCHD2H43 adult flies showed shortened lifespans compared with dCHCHD2Rev. P < 0.00016 by log-rank test; n = 122 (H43) and 127 (Rev) male flies. (b-d) dCHCHD2H43 flies are sensitive to 2 mM paraquat treatment (b, P < 0.0030 by log-rank test; n = 96 (H43) and 99 (Rev) males), 0.5% hydrogen peroxide treatment (c, P < 0.000087; n = 98 (H43) and 81 (Rev) males) and starvation (d, P < 0.023; n = 117 (H43) and 111 (Rev) males). (e-f) MitoSOX indicates chronic ROS production from mitochondria in dCHCHD2−/− cells. FACS plots were gated on Hoechst/MitoSOX-positive cells (e), and relative MitoSOX intensities are shown as histograms (f). Rotenone (Rot, 1 µM for 90 min) served as a positive control. The values indicate the average fold increase ± s.e.m. from three independent experiments. *P < 0.027. (g-h) 4E-BP is upregulated at both the transcript (g) and protein (h) levels (mean ± s.e.m., n = 3) in dCHCHD2 mutant flies. *P = 0.050, ***P = 0.00019. (i) Lipid oxidation is exacerbated in dCHCHD2H43 flies. The whole brain tissues of 30-day-old flies were stained with anti-4-HNE and anti-dTH antibodies. Scale bar, 75 µm. Intensity of anti-4-HNE immunoreactive signals in whole brain (upper graph, n = 5) and TH-positive regions (lower graph, n = 39) were measured and graphed (mean ± s.e.m.). *P = 0.048, **P < 0.009 versus Rev. (j-k) The number of PPL1 DA neurons decreases in dCHCHD2H43 flies with age. (j) The number of the indicated cluster of DA neurons (mean ± s.e.m.) was counted. ***P < 0.0001 versus Rev. (k) The number of PPL1 DA neurons at 3, 14, 30 and 50 days of age (mean ± s.e.m.). (l) The motor defect of dCHCHD2H43 flies with age. Mean ± s.e.m. from 20 trials with 48-85 male flies from three independent experiments. ***P < 0.0001 versus age-matched Rev. (g-l) two-tailed student’s t-test. FACS, fluorescence-activated cell sorting.
**Figure 3 | CHCHD2 responds to mitochondrial stresses.** (a, b) CHCHD2 responds to mitochondrial DNA damage and mitochondrial unfolded protein stress. (a) Thorax muscle tissues of flies harbouring mutations in the α- or β-subunit of the mitochondrial polymerase γ were subjected to western blot analysis with the indicated antibodies. (b) Overexpression of ΔOTC, but not OTC, produced unfolded protein stress in mitochondria, whereas overexpression of the polymerase γ, α subunit destabilized the mitochondrial genome24. The indicated UAS-transgenes were driven by MHC-GAL4. NDUFS3 (Complex I), ATP5a (Complex V) and actin served as loading controls. w- and GFP were used as control lines. The graph represents relative values (mean ± s.e.m.). *P < 0.05, **P < 0.01 by one-way ANOVA with Tukey–Kramer test. (c) The loss of CHCHD2 results in sensitization to hyperoxia. TEM images of the indirect flight muscles of adult flies with the indicated genotypes incubated under 90% O₂ for 4 days are shown (upper). Higher-magnification images of boxed regions in upper panels (lower). Normal mitochondria of 14-day-old flies are also shown as a control (dCHCHD2+/+, Normoxia). Scale bars, 1 μm (upper); and 500 nm (lower), respectively. (d) Mitochondria with abnormal cristae or degenerating mitochondria in (c) were counted as shown in Fig. 1e (mean ± s.e.m.). n = 265–335 from three independent samples. *P = 0.045, **P = 0.0002, ***P < 0.0001 versus the same class of dCHCHD2+/+. (e) CHCHD2 is downregulated under hyperoxia. The indicated proteins were analysed using thorax muscle tissues by western blot, and dCHCHD2 levels were graphed (mean ± s.e.m.). *P = 0.0054. n = 5 from two independent experiments. (d, e) two-tailed student’s t-test. TEM, transmission electron microscopy. ANOVA, analysis of variance.
Importantly, the amounts of co-precipitated MICS1 with Cyt c-FLAG were largely unchanged in the presence or absence of hCHCHD2-Myc and vice versa, suggesting that MICS1 and CHCHD2 form a tertiary complex with Cyt c rather than compete for binding of Cyt c (Fig. 5a). Endogenous interactions of three proteins were also detected (Fig. 5b). CHCHD2 T61I mutation found in PD cases affected the interactions of three proteins were also detected (Fig. 5b). CHCHD2 T61I mutation found in PD cases affected the interactions of three proteins were also detected (Fig. 5b). CHCHD2 T61I mutation found in PD cases affected the interactions of three proteins were also detected (Fig. 5b).

Figure 4 | CHCHD2 maintains the electron flow in OXPHOS. (a-d) The function of the mitochondrial electron respiratory complex in Drosophila embryonic cells was assessed as the OCR and the ECAR via serial injection of 5 μM oligomycin (Oligo), 1 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and 5 μM rotenone plus 5 μM antimycin A (Rot + AA). The mitochondrial content was comparable between the two genotypes, as estimated based on mitochondrial proteins (Supplementary Fig. 2c). The OCR and ECAR values (mean of the three points ± s.e.m.) were graphed in b and d, respectively. (e) Proton leak from the intermembrane space, ATP production, and spare respiratory capacity (mean ± s.e.m.) were calculated using basal OCR and its alterations with the inhibitors against each respiratory complex. (a-e) n = 17 (dCHCHD2+/−) and 19 (dCHCHD2−/−) from three independent experiments. *P = 0.028, **P < 0.01, ***P < 0.001; N.S., not significant (P = 0.66) by two-tailed student’s t-test.

Knockdown of the Drosophila MICS1 ortholog dMICS1, the efficiency of which was ~55%, had little effect on mitochondrial morphology in the dCHCHD2Rev genetic background, whereas it dramatically degenerated the mitochondrial matrices in the dCHCHD2H43 background (Fig. 5g,i versus Fig. 1b,e and Supplementary Fig. 7a). Ubiquitous overexpression of dMICS1 by Da-GAL4 at 25 °C results in death at the 1st instar larval stage. Reduced expression levels (Da-GAL4 at 22 or 18 °C) caused reduced hatching efficiency (~10%), slowed growth and death at the second instar larval stage, whereas the loss of dCHCHD2 did not affect its developmental phenotype and hatching efficiency of dMICS1-overexpressing larvae (Supplementary Fig. 7b). Muscle-specific overexpression of dMICS1 was viable and promoted mitochondrial fragmentation in both genetic backgrounds, showing mild mitochondrial defects to the same extent (Fig. 5h,i versus Fig. 1b,e). Consistent with the morphological defects, knockdown of dMICS1 largely reduced ATP production in dCHCHD2H43 flies (Fig. 5j). Importantly, overexpression of dMICS1 rescued the ATP reduction and motor defects observed in dCHCHD2H43 flies, which strongly suggested that MICS1 maintains OXPHOS function in cooperation with CHCHD2 (Fig. 5j and Supplementary Fig. 7c).
Rescue of fly phenotypes by expression of human CHCHD2. We next examined whether hCHCHD2 rescues the mitochondrial phenotypes observed in dCHCHD2H43 flies. We introduced LacZ, dCHCHD2 or hCHCHD2, including WT and two mutants, into dCHCHD2H43 flies; hCHCHD2 WT and mutant genes were expressed at similar levels (Fig. 6a). Reintroduction of dCHCHD2 and hCHCHD2 WT almost completely rescued the morphological defects of mitochondria in the indirect flight muscles,
whereas the dilation of matrix space, disordered cristae, and ‘swirl’ phenotype were still observed with a control LacZ, hCHCHD2 T61I or R145Q (Fig. 6b,c and Supplementary Fig. 8). Importantly, the age-dependent loss of PPL1 DA neurons observed in dCHCHD2-deficient flies was rescued by hCHCHD2 WT but not by the mutants T61I or R145Q (Fig. 6d). Consistent with the rescue of the mitochondrial morphology and the DA neuron loss, impairment of ATP production, locomotor defects, short lifespan, and sensitivity to oxidative stress observed in dCHCHD2-deficient flies were suppressed by dCHCHD2 or hCHCHD2 WT, whereas rescue effects were generally weaker in hCHCHD2 mutants, raising the possibility that these two missense mutations are loss-of-function mutations (Fig. 6e–g). To test this idea, hCHCHD2 T61I and R145Q were expressed in dCHCHD2 heterozygous flies. dCHCHD2 heterozygous flies expressing LacZ exhibited almost normal mitochondrial morphology and showed slightly decreasing ATP levels compared to a normal control (Fig. 7a–c). Overexpression of PD-associated mutants did not produce morphological defects, and ATP levels were comparable to the heterozygous mutant with LacZ (Fig. 7a–c). We found that the T61I mutation, which occurs in a central conserved region (Supplementary Fig. 1a), destabilized the CHCHD2 protein, leading to its appearance in the insoluble fraction when expressed in bacteria (Fig. 7d). CHCHD2 formed a homodimer (Fig. 7e). R145Q, which is located adjacent to the second Cys-C motif (Supplementary Fig. 1a), affected the dimer formation (Fig. 7e). These genetic and molecular analyses suggest that the two mutations affect the normal functions of CHCHD2 showing loss-of-function properties.

4E-BP rescues neurodegeneration by loss of CHCHD2. Our data suggest that loss or mutation of CHCHD2 produces ROS due to the incomplete OXPHOS activity, leading to oxidative stress and subsequent neurodegeneration. Supporting this idea, a compensatory upregulation of 4E-BP, which has a protective role against oxidative stress maintaining mitochondrial integrity, was observed in dCHCHD2-deficient flies (Fig. 2g,h)\textsuperscript{12,13,31}. Overexpressing CHCHD2 and LacZ with the GAL4-UAS system caused 4E-BP expression to increase slightly, probably due to GAL4, LacZ and/or CHCHD2 overexpression. However, reintroduction of dCHCHD2 at a level similar to endogenous levels suppressed 4E-BP induction in dCHCHD2-deficient flies (Fig. 8a and Supplementary Fig. 9a). Under the same expression conditions as the dCHCHD2 introduction, WT hCHCHD2 successfully suppressed 4E-BP induction in dCHCHD2-deficient flies, whereas PD mutants failed to do so, suggesting that mitochondrial stress was alleviated by intact CHCHD2 (Fig. 8a). We then introduced 4E-BP into dCHCHD2-deficient flies because 4E-BP has multiple beneficial roles in mitochondria. Ubiquitous expression of 4E-BP lessened the ATP decline in dCHCHD2-deficient flies (Fig. 8b). A similar effect was obtained by treating with rapamycin, which activates 4E-BP through inhibition of a 4E-BP kinase target of rapamycin (TOR) (Fig. 8b)\textsuperscript{17}. Consistent with the effect on ATP, mitochondrial morphology and protein oxidation were also improved by 4E-BP overexpression (Fig. 8c–e). Dopaminergic and serotoninergic expression of 4E-BP in dCHCHD2-deficient flies extended the lifespan and improved the motor activity and DA neuron loss to the extent of Rev expressing LacZ or 4E-BP (Fig. 8f–h).

Although DJ-1 and SOD1 have anti-oxidative activity, we could not detect an obvious genetic interaction between CHCHD2 and DJ-1 or between CHCHD2 and SOD1 in Drosophila\textsuperscript{18,22}. Loss of DJ-1 did not exacerbate mitochondrial morphology in dCHCHD2-deficient flies (Supplementary Fig. 9b). DJ-1 or SOD1 overexpression did not extend the lifespan of dCHCHD2-deficient flies (Supplementary Fig. 9c,d). In addition, SOD1 failed to improve the climbing defect. TH neuron loss of dCHCHD2-deficient flies (Supplementary Fig. 9e), suggesting that simple redox control is not sufficient for mitochondrial protection. We also tested the genetic association of CHCHD2 with PARK1 and parkin, both of which are involved in mitochondrial quality control in the same signalling pathway\textsuperscript{32}. Although dCHCHD2 is upregulated in PARK1- and parkin-deficient flies, there was no evidence that loss of CHCHD2 exacerbates the mitochondrial phenotypes caused by the loss of PARK1 or parkin (Fig. 9a) or that CHCHD2 regulates mitochondrial size or dynamics, as observed in PARK1 and Parkin signalling (Fig. 9b). However, overexpression of PARK1 or Parkin led to much sharper reductions in ATP and stronger changes in mitochondrial morphology caused by the loss of dCHCHD2, whereas PARK1 or Parkin overexpression alone promoted the fragmentation of mitochondria with normal cristae integrity (Fig. 9c,d).

**Discussion**

Mitochondrial dysfunction is a prominent feature of PD. The newly identified PD-related gene CHCHD2, which encodes a mitochondrial protein, could provide new insight into the aetiology of PD. In this study, we characterized the physiological and pathological roles of CHCHD2 using Drosophila and mouse cultured cells. Although CHCHD2-deficient flies and mice develop normally, age-dependent mitochondrial defects, increased oxidative stress, and DA neuron loss were detected in Drosophila. Previous studies suggest that CHCHD2 has a role in OXPHOS function. During OXPHOS, electrons are extracted

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**Figure 5 | CHCHD2 interacts with MICS1 and Cyt c. (a) CHCHD2 interacts with MICS1 and Cyt c in HEK293T cells. Co-precipitated proteins with Cyt c-FLAG were detected after DSP treatment. An inner mitochondrial membrane protein, Tim23 served as a control. (b) Endogenous interaction of MICS1 with CHCHD2 and Cyt c. Lysates from cells treated with or without DSP were immunoprecipitated with anti-MICS1 or a control antibody. Co-precipitated proteins were analysed in the presence or absence of DTT. (c) CHCHD2 negatively regulates Cyt c release. An empty vector (KO), hCHCHD2 WT, T61I or R145Q was virally introduced into dCHCHD2-deficient MEFs. Cells were treated with 500 nM actinomycin D for the indicated periods of time. The amounts of cytosolic Cyt c (mean ± s.e.m.) were determined by western blot and were normalized to lactate dehydrogenase (LDH) levels. *P < 0.047, **P < 0.0013, ***P < 0.001 versus the same period of WT from eight independent experiments (one-way ANOVA with Tukey–Kramer test). (d) A representative western blot of the Cyt c release is shown. LDH and Tim23 serve as cytosolic and mitochondrial loading controls, respectively. (e) CHCHD2 suppresses caspase activation by actinomycin D. (e) Caspase-3/7 activity (mean ± s.e.m.) was measured by a chemiluminescence-based assay kit. RLU, relative light unit. ***P < 0.0001 versus 6 h of all other groups. (f) Cleaved caspase-3 was detected by western blot. (g,h) TEM images of the indirect flight muscles of 7-day-old adult flies with the indicated genotypes are shown. (upper) Mitochondrion, mito; myofibril, myo. (Lower) Higher-magnification images of boxed regions in upper panels. Scale bars, 2 μm (upper in g) and 1 μm (lower in g and h), respectively. (i) Mitochondrial defects were counted as shown in Fig. 1e (mean ± s.e.m.). n = 9 from two independent experiments (one-way ANOVA with Tukey–Kramer test). (j) ATP contents of 30-day-old flies were measured as in Fig. 1f. Mean ± s.e.m., n = 7–10. *P < 0.001 versus the same class of dCHCHD2Rev (two-tailed student’s t-test). (k) ATP contents of 30-day-old flies were measured as in Fig. 1f. Mean ± s.e.m., n = 7–10. *P < 0.046, **P < 0.001 (one-way ANOVA with Dunnett’s test). Transgenes were driven by the MHC-GAL4 (g–j). ANOVA, analysis of variance; LDH, lactate dehydrogenase, TEM, transmission electron microscopy.**
from substrates and are then transferred to molecular oxygen through a chain of enzymatic complexes, I–IV. In the final step, complex IV (Cyt c oxidase) ensures the reduction of molecular oxygen to water, without formation of oxygen radicals. CHCHD2 binds to Cyt c in conjunction with MICS1. Cyt c, which transfers electrons from complex III (ubiquinol-Cyt c reductase) to complex IV, is localized in the mitochondrial intermembrane space. When apoptotic signalling is activated by proapoptotic Bcl-2 family members, such as Bax or tBid, Cyt c is released through the permeabilized mitochondrial outer membrane. Knockdown of MICS1 and CHCHD2 facilitates Cyt c release upon treatment with apoptosis-inducing reagents and UV irradiation7,30. Considering our findings together with the above studies, we hypothesize that CHCHD2 and MICS1 stabilize Cyt c to maintain normal electron flow during OXPHOS (Fig. 10). When Cyt c function is compromised, electron flow from complex III to complex IV is impaired, and excess electrons from Cyt c may react with molecular oxygen,
resulting in the production of superoxide and hydrogen peroxide, a major cause of oxidative stress. Supporting this idea, dCHCHD2-deficient flies exhibit various phenotypes associated with oxidative stress, including increased 4-HNE, 8-OHdG and protein/GSH oxidation and upregulation of 4E-BP. In these flies, aging is a key factor in mitochondrial degeneration and DA neuron loss. An age-related decline in the activity of complex IV, but not other respiratory complexes, has been reported without detectable alteration of complex IV protein levels in Drosophila. This observation raises the possibility that the function of complex IV, in addition to that of Cyt c, is compromised in dCHCHD2-deficient flies as they aged, resulting in a high incidence of ROS.

Although multiple lines of evidence indicate that chronic oxidative stress occurs by CHCHD2 loss, overexpression of SOD1 and DJ-1 or removal of DJ-1 did not affect dCHCHD2 phenotypes, which strongly suggests that oxidative stress is only part of the pathogenesis of PD that is linked to CHCHD2. The functional difference between SOD1/DJ-1 and 4E-BP is that the latter regulates mitochondrial functions and proteostasis in addition to upregulation of anti-oxidative proteins such as anti-oxidant GST-S1 (refs 11,12,17,31). Another study has proposed that 4E-BP suppresses OXPHOS proteins and mitochondrial respiration31. We favour the latter idea because hypoxia improves the mitochondrial defects of dCHCHD2 flies (Supplementary Fig. 10a,b).

In sharp contrast to 4E-BP overexpression, PINK1 or Parkin overexpression decreased the mitochondrial integrity of dCHCHD2-deficient flies. According to the hypothesis that PINK1-Parkin signalling is responsible for mitochondrial quality control, PINK1 or Parkin overexpression might activate mitophagy. This effect would cause most of the mitochondria in dCHCHD2-deficient flies to be recognized as damaged mitochondria, leading to lysosomal degradation. Nevertheless, given that CHCHD2 responds to a variety of mitochondrial stresses, including PINK1, parkin and DJ-1 mutations, CHCHD2 could be a potential biomarker of PD associated with mitochondrial dysfunction.

Because the mitochondrial respiratory chains are localized at the cristae, cristal structure is closely associated with OXPHOS activity. CHCHD10 has been reported to be enriched at the cristal junctions, and expression of a CHCHD10 mutant, which causes frontotemporal dementia-amyotrophic lateral sclerosis, led to fragmentation of the mitochondrial network and defects in cristal structures4. Although our electron microscopic study using CHCHD2-deficient MEFs did not detect obvious abnormalities in cristal organization due to a variety of mitochondrial morphologies in the cytosol (Supplementary Fig. 11), the lack of dCHCHD2 led to moderate cristal deformation in the indirect flight muscles of Drosophila. Because MICS1 has also been reported to regulate cristal organization49, CHCHD2 along with MICS1 might maintain cristal integrity to accommodate Cyt c properly.

PD caused by missense mutations in CHCHD2 is inherited as a dominant trait1. This suggests that neurodegeneration may be caused by gain-of-function mutations. Our study indicates that human pathogenic T61I and possible R145Q mutants do not fully rescue the mitochondrial defects caused by the lack of dCHCHD2, suggesting that these mutations compromise its physiological functions. Supporting this idea, we found that CHCHD2 harbouring PD mutations exhibits reduced solubility or impaired homodimerization, although it should be determined whether dimerization is important for its physiological function in future research. Importantly, the expression of T61I and R145Q in dCHCHD2 heterozygous flies did not produce mitochondrial defects, which strongly suggests that the aetiology of PD linked to CHCHD2 includes a loss-of-function aspect. However, further studies should determine whether gain-of-function events are also involved in neurodegeneration by CHCHD2 mutations.

CHCHD2 levels were altered by various mitochondrial stresses. A previous study showed that CHCHD2 transactivates COX4I2 and CHCHD2 itself in the nucleus3,34. Thus, the upregulation of CHCHD2 due to mitochondrial stress might contribute to the transcriptional regulation of these genes, resulting in a more complex pathogenesis than we hypothesize here. Hyperoxia suppressed dCHCHD2 levels in Drosophila, which suggests that hypoxia-inducible factor (HIF) is involved in its regulation. However, we could not find the consensus sequence of the hypoxia-responsive element upstream of the dCHCHD2 gene. In addition, hypoxia or knockdown of HIF-1α/Sima or HIF-1β/Tango did not change dCHCHD2 levels (Supplementary Fig. 12). Cyclic adenosine monophosphate response element and a novel oxygen-responsive element have been proposed as regulation sequences for CHCHD2 gene expression in mammals3,35. Although we could not find putative sequences for these elements in the 5'-untranslated region of the dCHCHD2 gene, further analysis is needed to clarify the involvement of these elements in Drosophila.

In summary, we propose that CHCHD2 controls OXPHOS through regulation of Cyt c and cristal integrity. Loss or mutation of CHCHD2 leads to oxidative stress probably through dysregulation of electron flow in OXPHOS. Our study sheds light on the aetiology of PD linked to CHCHD2 and potential therapeutic targets in PD caused by mitochondrial dysfunction.

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**Figure 6** | PD-associated mutations of hCHCHD2 affect mitochondrial functions and DA neuron survival. (a) Expression of hCHCHD2 in dCHCHD2H43 flies. LacZ, dCHCHD2, and hCHCHD2 (WT, T61I and R145Q), were expressed in dCHCHD2H43 flies, and thorax muscle tissues were subjected to western blot. Actin, a loading control. (b) TEM images of the indirect flight muscles of 14-day-old flies with the indicated genotypes. (Lower) Higher-magnification images of boxed regions in upper panels. Scale bars, 2 μm (upper) and 500 nm (lower), respectively. (c) Mitochondria with abnormal cristae or showing degeneration were quantified as shown in Fig. 1e (mean ± s.e.m.). n = 134–172 from three independent samples. *P < 0.0001 versus class 1 of LacZ; # P < 0.0001 versus total defects (class 1 + class 2) of LacZ (one-way ANOVA with Tukey-Kramer test). (d) Expression of hCHCHD2 WT but not PD mutants rescues the loss of PPL1 DA neurons in dCHCHD2H43 flies. Fifty-day-old flies were analysed (mean ± s.e.m., n = 12–18), and representative images of the PPL1 DA neurons visualized with anti-DH are shown. Scale bar, 10 μm. *P < 0.026 versus PPL1 of hCHCHD2 WT (one-way ANOVA with Tukey-Kramer test). (e) ATP reduction and climbing defects in dCHCHD2H43 flies were rescued by dCHCHD2 and hCHCHD2 WT, whereas hCHCHD2 T61I and R145Q did not fully suppress these phenotypes. Thirty-day-old flies were analysed (mean ± s.e.m.). n = 15 for ATP assay; 20 trials with 114–128 flies from five independent experiments for the climbing assay. *P < 0.05, **P < 0.01, ***P < 0.001 versus LacZ (one-way ANOVA with Tukey-Kramer test). (f) dCHCHD2 (P < 0.001 by log-rank test) and hCHCHD2 WT (P < 1 × 10^−6) by log-rank test), but not PD mutants, extend the lifespan of dCHCHD2H43 flies. No differences with LacZ, T61I, and R145Q. (g) hCHCHD2 WT (P < 0.05 by log-rank test), but not PD mutants, improved the sensitivity to oxidative stress by peroxide. No differences with LacZ, T61I and R145Q. All transgenes were driven by Da-GAL4, and LacZ served as a negative control. ANOVA, analysis of variance; TEM, transmission electron microscopy.
Figure 7 | Biochemical properties of hCHCHD2 are altered in PD mutants. (a) Mitochondrial defects in 14-day-old adult flies, defined as classes 1 and 2 defects, were counted as shown in Fig. 1e (mean ± s.e.m.). Transgenes (LacZ, T61I and R145Q) were driven in dCHCHD2 heterozygous flies by Da-GAL4. n = 335–428 from three independent samples. N.S., not significant by one-way ANOVA with Tukey–Kramer test. (b) Representative TEM images of the mitochondria in the indirect flight muscles counted in a. Scale bar, 2 μm. (c) ATP content in the thorax muscle tissues of 14-day-old adult flies with the indicated genotypes. Mean ± s.e.m. from seven independent samples. N.S., not significant by one-way ANOVA with Tukey–Kramer test. (d) Bacterially expressed hCHCHD2 T61I has difficulty in solubilizing. hCHCHD2 WT and PD mutants were expressed in SHuffle T7 Express Escherichia coli (New England Biolabs), which promotes disulfide bond formation in the cytoplasm. Total lysate (T), phosphate-buffered saline-soluble (S) and insoluble (I) protein fractions solubilized in SDS sample buffer containing 2% SDS were separated on SDS–PAGE and were visualized by Coomassie brilliant blue staining. An asterisk indicates insoluble hCHCHD2 T61I, whereas there were no insoluble hCHCHD2 bands in WT and R145Q. (e) hCHCHD2 forms a dimer. hCHCHD2-expressing MEFs treated with or without 0.5 μg ml⁻¹ DSP in PBS for 20 min at RT were lysed and subjected to western blot analysis with anti-CHCHD2. The ratio of monomer signals to total signals was graphed (mean ± s.e.m., n = 6 in each group). A monomer form of hCHCHD2 WT and T61I accounts for less than 50% of total CHCHD2 signals under this experimental condition, whereas the R145Q monomer form was higher (~60%, *P = 0.027 by one-way ANOVA with Tukey–Kramer test). ANOVA, analysis of variance; TEM, transmission electron microscopy.
Methods

Antibodies. Recombinant 6xHis-GST-tagged dCHCHD2 was purified from bacteria and used for antibody production (Eveto Science, Japan). Antibodies used in western blot analysis were as follows: anti-dCHCHD2 (1:1,000 dilution; in-house), anti-hCHCHD2 (1:1,000; Proteintech, 19424-1-AP), anti-FLAG (1:1,000; Sigma-Aldrich, clone M2), anti-Myc (1:1,000; Millipore, clone 4A6), anti-HA (1:1,000; Roche, clone 3F10), anti-d4E-BP (1:1,000; in-house16), anti-MICs1 (1:1,000; Everest Biotech, EB09815), anti-cleaved caspase-3 (1:1,000; Cell Signaling Technology, #9664), anti-Cyt c (1:1,000; Abcam, 37BA11), anti-LDH (1:1,000; Santa Cruz, H-160), anti-Tim23 (1:2,000; BD, clone 32/Tim23).

Figure 8 | 4E-BP rescues neurodegeneration by loss of CHCHD2. (a) hCHCHD2 WT, but not PD mutants, suppresses 4E-BP expression. dCHCHD2 and hCHCHD2 were induced in adult flies with 2.0 μg ml−1 RU486 for 3 days (Supplementary Fig. 9a). UAS-LacZ crossed with Da-G5 served as a negative control. 4E-BP levels (mean ± s.e.m., n = 6) were quantified and normalized to actin levels. *P = 0.038; LacZ versus dCHCHD2, *P = 0.029; hCHCHD2 WT versus T61l, *P = 0.013; hCHCHD2 WT versus R145Q. (b-h) 4E-BP rescues dCHCHD2 phenotypes. (b) ATP content in thorax muscle tissues from 14-day-old flies. 4E-BP or LacZ was ubiquitously expressed by using the Da-GAL4 driver. Rapamycin treatment (50 μM) for 14 days, but not DMSO treatment, reduced the mildly (a white dot) and highly (a black dot) phosphorylated 4E-BP levels in western blots. Mean ± s.e.m. from seven independent samples. *P = 0.014, **P = 0.003. (c) TEM images of the indirect flight muscle of 40-day-old adult flies with the indicated genotypes and higher-magnification images of the boxed regions are shown. Scale bar = 1 μm. (d) Mitochondria with abnormal cristae or degenerating mitochondria in c were counted as shown in Fig. 1e (mean ± s.e.m.). n = 280–411 from three independent samples. *P = 0.0021, **P < 0.0001 versus dCHCHD2−/−; LacZ. (e) Detection of protein oxidation. Thoraxes of 45-day-old flies were used for the Oxyblot assay. Protein oxidation of dCHCHD2-deficient flies was suppressed by 4E-BP overexpression (n = 5, *P = 0.02). (f-h) 4E-BP or LacZ was expressed using the Ddc-GAL4 driver in dCHCHD2−/− and revertant flies, and flies were analysed for the number of remaining DA neurons (f, *P = 0.047 versus PPL1 of H43; 4E-BP, n = 10–11), climbing ability (g, ***P < 0.001; n = 46–50) and lifespan (h, P < 0.04, H43; LacZ versus any other groups by log-rank test). (a,b,d,g) Data represent the mean ± s.e.m. and were analysed by one-way ANOVA with Tukey-Kramer test. ANOVA, analysis of variance; TEM, transmission electron microscopy.
Figure 9 | Effects of PINK1-Parkin signalling on the dCHCHD2 mitochondrial phenotypes. (a) Mitochondrial morphology of PINK1 RNAi (upper) and parkin-deficient (lower) flies with or without dCHCHD2 activity. Fluorescent images of the indirect flight muscles of 7-day-old adult male flies with the indicated genotypes are shown. To visualize the mitochondria, the mitoGFP (green) transgene was co-expressed, and the muscle tissue was counterstained with phalloidin (magenta). Scale bar, 10 μm. (b) Loss of CHCHD2 and expression of PD mutants do not affect mitochondrial size. Fluorescent images of the indirect flight muscles of 14-day-old adult male flies with the indicated genotypes are shown, as in (a). Scale bar, 5 μm. A graph shows the length of the long axis of the muscle mitochondria (mean ± s.e.m.). n = 9 from three independent flies in each group. N.S., not significant (P = 0.0629) versus the LacZ group by Dunnett’s test. (c) TEM images of the indirect flight muscle in the indicated genotypes of 14-day-old adult flies. Scale bars, 1 μm (upper) and 2 μm (lower), respectively. (d) ATP levels in the thorax muscle tissues in the indicated genotypes. n = 8. *P = 0.045, ***P < 0.0001 (one-way ANOVA with Tukey-Kramer test). The genotypes used were as follows: (a) dCHCHD2Rev/Y; UAS-mitoGFP/+; MHC-GAL4, UAS-PINK1 RNAi/+ (PINK1RNAi; dCHCHD2Rev), dCHCHD2H43/Y; UAS-mitoGFP/+; MHC-GAL4, UAS-PINK1 RNAi/+ (PINK1RNAi; dCHCHD2H43), dCHCHD2Rev/Y; UAS-mitoGFP/+; Da-GAL4, Parkin+/D21/Parkin1 (Parkin+/C0/C0; dCHCHD2Rev), dCHCHD2H43/Y; UAS-mitoGFP/+; Da-GAL4, Parkin+/D21/Parkin1 (Parkin+/C0/C0; dCHCHD2H43). (b) dCHCHD2null/Y; UAS-mitoGFP/+; MHC-GAL4/UAS-LacZ (LacZ), dCHCHD2null/Y; UAS-mitoGFP/+; MHC-GAL4/UAS-dCHCHD2 (dCHCHD2), dCHCHD2null/Y; UAS-mitoGFP/+; MHC-GAL4/UAS-hCHCHD2 WT (WT), dCHCHD2null/Y; UAS-mitoGFP/+; MHC-GAL4/UAS-hCHCHD2 T61I (T61I), dCHCHD2null/Y; UAS-mitoGFP/+; MHC-GAL4/UAS-hCHCHD2 R145Q (R145Q). (c.d) +/Y; MHC-GAL4/UAS-LacZ (LacZ), dCHCHD2+/−, +/Y; UAS-dPINK1; MHC-GAL4/UAS-dPINK1 (dPINK1; dCHCHD2+/−), +/Y; UAS-dParkin+/+; MHC-GAL4/+ (dParkin; dCHCHD2+/−), dCHCHD2null/Y; MHC-GAL4/UAS-LacZ (LacZ; dCHCHD2+/−), dCHCHD2null/Y; UAS-dPINK1; MHC-GAL4/UAS-dPINK1 (dPINK1; dCHCHD2+/−), +/Y; UAS-dParkin+/+; MHC-GAL4/+ (dParkin; dCHCHD2+/−), dCHCHD2null/Y; UAS-dParkin+/+; MHC-GAL4/+ (dParkin; dCHCHD2+/−). ANOVA, analysis of variance; TEM, transmission electron microscopy.
tandem guide RNA plasmids (pCFD4) and a fly line expressing Cas9 during

Figure 10 | A model for roles of CHCHD2 in mitochondria. (a) CHCHD2 in conjunction with MICS1 stabilizes Cyt c in the respiratory chains, allowing for proper electron flow from complex III to complex IV and consequent ATP production. (b) Mutation or absence of CHCHD2 destabilizes Cyt c in the respiratory chain, which causes electron leakage and ROS generation.

anti-Tom20 (1:500; Santa Cruz, FL-145), anti-complex I (1:10,000; Abcam, 17D955), anti-complex IV (1:1,000; Abcam, 208C12), anti-complex V (1:10,000; Abcam, 15H4C4), anti-SOD1 (1:1,000; Abcam, ab13498), anti-OXPHOS cocktail (1:250; Abcam, ab110143), anti-DJ-1 (1:1,000; in-house), anti-oxidative stress markers (1:10,000; Abcam, 20E8C12), anti-complex V (1:10,000; Abcam, 20E8C12), anti-Myc (1:1,000; Millipore, clone M2), anti-Myc (1:1,000; Millipore, clone 4A6), anti-Myc (1:1,000; Cell Signaling Technology), anti-Hsp60 (1:500; BD, clone 24/HSP60) and anti-dTH (1:250; in-house).

Drosophila genetics. Fly culture and crosses were performed on standard fly food containing yeast, cornmeal, and molasses, and the flies were raised at 25°C. The w[+]/w[–] or revertant line was used as a WT genetic background. Complementary DNA (cDNA) for dCHCHD2 or hCHCHD2 was subcloned into the pUAST vector, and transgenic lines were generated on the w[–]/w[–] background (BestGene). For gene targeting of dCHCHD2, we used conventional mutagenesis by transposon mobilization of p[EPgy2]EY05234 (Bloomington stock 15790) and generated a hypomorphic CHCHD2 allele and its revertant allele as a WT control. We also employed CRISPR/Cas9 technology to generate a dCHCHD2 null allele using tandem guide RNA plasmids (pCFD4) and a fly line expressing Cas9 during oogenesis (Bloomington stock 35491[4]). Two transposers corresponding to sequences of dCHCHD2 were cloned into pCFD4 digested with BbsI using an In-fusion HD cloning kit (Takara Bio) with the following primer pairs: 5’TATATAGGAAAGATATCCGGGACTGGCTGACAGTCCAGGTTTTAGAGCTAGAAATAGCAAG-3’, 5’TATATAGGAAAGATATCCGGGACTGGCTGACAGTCCAGGTTTTAGAGCTAGAAATAGCAAG-3’. The indirect Western blot analysis, an OxyBlot Protein Oxidation Detection Kit (Merck Millipore) was used according to the manufacturer’s instructions. The band intensity was analysed using ImageJ software. GSH/GSSG ratios from twenty male flies were measured by using a glutathione assay kit (Cayman Chemical), following a protocol provided by the manufacturer.

Plasmids and cell culture. Human CHCHD2 cDNAs were inserted in the pCDNA3 vector containing FLAG or Myc-His tag and in the pMXs-puro vector, followed by sequencing of the entire gene. Expression vectors for MICS1-HA and Cyt c-FLAG were a kind gift from Dr T. Oka. MEFs were retrovirally transfected with pMXs-puro harbouring hCHCHD2, and the transfected cells were then selected with 1 μg ml–1 puromycin. The source of HEK293T cells was described elsewhere[43] and HeLa cells were purchased from RIKEN cell bank. They were maintained at 37°C with a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich) supplemented with 10% FCS (Gibco), GlutaMax (Gibco), non-essential amino acids (Gibco) and 1% penicillin-streptomycin. The plasmids were transfected using Lipofectamine 2000 reagent (ThermoFisher Scientific) according to the manufacturer’s instructions. The total amount of transfected RNA was adjusted with vector DNA in every transfection experiment. All the cells used here were tested and negative for mycoplasma contamination.

Identification of CHCHD2-binding proteins and binding assay. For identification of CHCHD2-binding proteins, HEK293T cells expressing C-terminally FLAG-tagged human CHCHD2 were transiently transfected with a cell lysis buffer (20 μM HEPES pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 0.5% digitonin, 1 mM PMSE, 5 μg ml–1 leupeptin, 5 μg ml–1 aprotinin and 3 μg ml–1 pepstatin A). CHCHD2-FLAG was purified from the cell lysate with anti-FLAG M2 affinity gel (Sigma-Aldrich) and eluted with FLAG peptide (Sigma-Aldrich). The elution fraction was subjected to trichloroacetic acid precipitation and subsequent digestion with lysyl endopeptidase (Lys-C, Wako Chemicals USA). Samples were analysed on a liquid chromatography system coupled to a time-of-flight mass spectrometer (Q-STAR XL, AB Sciex).

For co-immunoprecipitation assay using chemical crosslinker, cells were harvested and washed twice with PBS and then incubated with 0.5 mM maleimide-1-DSP (ThermoFisher Scientific) at 25°C for 15–20 min. After washing with TBS, the cells were solubilized at 4°C for 30 min in cell lysis buffer (50 mM Tris-HCl pH 8.0, 130 mM NaCl, 1% Triton X-100 and 0.1% SDS) and then centrifuged to remove insoluble precipitates. The resultant supernatant was incubated with anti-FLAG or anti-Myc beads (Medical & Biological Laboratories) for 3 h at 4°C, washed twice with PBS containing 1% Triton X-100, and then washed once with PBS alone. The precipitates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent western blot analysis. For preparation of MEFs from CHCHD2-deficient mice. CHCHD2–/– mice were generated by Unitech in Japan. In brief, the targeting vector was constructed to replace exons 2 and 3 with the neomycin resistance gene. The targeting vector was integrated into C57BL/6 embryonic stem (ES) cells. Following selection with G418, ES clones were subject to PCR screening and Southern blotting to identify homologous recombinant clones. Recombinant ES cells were
microinjected into CS7BL/6 blastocysts to obtain chimeric mice, which were then mated to generate heterozygous and homozygous mice. Animal protocols were performed in accordance with the Juntingo University Animal Experiment Committee. CHCHD2−/− MEFs were prepared from embryos at 13.5–15.5 d.p.c. and were cultured at 37 °C with a 5% CO2 atmosphere in DMEM containing with GlutaMax, supplemented with 10% fetal bovine serum, gentamicin (50 μg/ml−1; Gibco), and 1% non-essential amino acids.

Cyt c release and caspase assay. MEFs treated with 500 mM actinomycin D or 30 mM peroxide were homogenized in mitochondrial isolation buffer mannitol, 70 mM sucrose, 20 mM HEPES-KOH pH 7.4, 1 mM EDTA and protease inhibitor cocktail (Roche), then homogenized by 20 passages through a 26-G syringe (Terumo) on ice23. Homogenates were centrifuged at 700 g for 10 min at 4 °C to obtain a post-nuclear supernatant. The post-nuclear supernatant was further centrifuged at 12,000 g for 15 min. Pellets were washed several times with the mitochondrial isolation buffer. Caspase activity was estimated with a Caspase-Glo 3/7 assay kit (Promega) or western blot with anti-cleaved caspase-3.

Coupling assay of mitohondria in Drosophila embryonic cells. Drosophila embryos (~800 in each) at the blastoderm stage collected 2–3 h post laying at 25 °C were dechorionated with 40% bleach for 2 min. Embryos were then homogenized in Schneider’s insect medium containing 5% FCS using a Dounce tissue homogenizer by 10–12 up and down strokes with a loose pestle. Embryonic cells filtered with a 40-μm cell strainer were plated onto 24-well cell culture microplates (Seahorse Bioscience) pre-coated with Cell-Tak (Corning) at 80,000 cells per well and were subjected to a MitoStress test using the XF24 analyser (Seahorse Bioscience) at 29 °C.

MitoSOX analysis in Drosophila embryonic cells. Drosophila embryonic cells prepared as described above were stained with 20 μM MitoSOX (Thermo Fisher Scientific) and 1 μM HaloGreen (Hoechst) for 20 min at RT. Fluorescence intensities of MitoSOX in cells (1 × 103) were measured using LSRFortessa (BD Biosciences), and Hoechst 33342-positive cells were analysed using FlowJo version 9 (TreeStar).

Mitochondrial respiratory chain complex activity assay. Mitochondria from the thoracic muscles of 14-day-old adult flies were isolated by a reported protocol with some modifications24. Briefly, isolated thoraces from 20 flies were gently crushed in 200 μl of mitochondrial isolation buffer (250 mM sucrose, 25 mM Tris-HCl pH 7.3) using a plastic pestle homogenizer and then spun twice at 500 g for 5 min at 4 °C to remove debris. After centrifugation for 10 min at 2,200 g, the pellet was resuspended in 100 μl of isolation buffer. The activities of complexes I–IV were measured following a previously reported protocol, which was adapted to the 96-well plate scale16. Briefly, for the measurement of the Complex I activity, NADH dehydrogenase assay buffer (50 mM potassium phosphate buffer pH 7.5, 2.5 mg/ml−1 bovine serum albumin [BSA], 240 μM KCN, 70 μM decylubiquinone [dUb], 25 μM antimycin A) containing 2 μM rotenone or ethanol was divided into 100 μl aliquots in a microplate and left at RT for 5 min. Complex I activity was monitored by adding 0.4 μg of mitochondrial preparations and 200 μM NADH (2 mM NADH stock) to the reaction mix and following a decrease in absorbance at 340 nm (with reference wavelength at 425 nm). The measurement of the Complex II activity, succinate dehydrogenase assay buffer (25 mM potassium phosphate buffer pH 7.5, 2 mM NADH [BSA], 20 mM potassium succinate, 2.6-dichlorophenolindophenol, 25 μM antimycin A, 2 μM KCN, 2 μM rotenone) with or without or 10 mM malonate was divided into 100 μl aliquots in a microplate and left at RT for 5 min. Complex II activity was monitored by adding 0.4 μg of mitochondrial preparations and 100 μM dUb to the reaction mix and following a decrease in absorbance at 600 nm. For the measurement of the Complex III activity, cytochrome c reductase assay buffer (50 mM potassium phosphate buffer pH 7.5, 1 mM n-dodecyl β-D-maltoside [DDM], 1 mM KCN, 2 μM rotenone, 250 μM EDTA, and 1 mg/ml−1 BSA, 50 μM oxidized cytochrome c) with or without or 25 μM antimycin A was divided into 100 μl aliquots in a microplate and left at RT for 5 min. The cytochrome c reductase activity was monitored by adding 200 μM decylubiquinol, and 0.075 μg/ml−1 of mitochondrial samples to the reaction mix and following an increase in absorbance at 550 nm. For the measurement of the Complex IV activity, cytochrome c oxidase assay buffer (25 mM potassium phosphate pH 7.0, 0.45 mM DDM and 100 μM reduced cytochrome c) with or without or 2 mM KCN was divided into 100 μl aliquots in a microplate. The cytochrome c oxidase activity was monitored by adding 0.1 μg of mitochondrial samples to the reaction mix and following a decrease in absorbance at 550 nm. Each activity was evaluated by subtracting the activity in the presence of specific complex inhibitors, including rotenone, malonic acid, antimycin A and KCN.

Binding assay of CHCHD2 to complex IV. Exp293F cells (Thermo Fisher Scientific) were transfected with an expression plasmid for full-length human CHCHD2 cDNA by a PA plus protocol according to the manufacturer’s protocol. The cells from a 50 ml culture were lysed via sonication in lysis buffer containing 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% glycerol, 0.1% Triton-X and complete Ultra protease inhibitor cocktail (Roche), followed by centrifugation at 27,216 g for 15 min. The supernatant (10 ml in total) was incubated with 100 μl of anti-PA (B9-1-Sepharose for 1 h at 4 °C), and the beads were sequentially washed with 1 ml of wash buffer containing 50 mM Tris-HCl pH 7.5, 300 mM NaCl, 10% glycerol, followed by 1 ml of interaction buffer containing 50 mM Tris-HCl pH 7.5, 300 mM NaCl 10% glycerol and 0.2% n-decyl-β-D-maltopyranoside (DM) (Anatrace, D322). The CHCHD2-bound beads were incubated in the presence or absence of purified bovine heart complex IV (provided by Dr Tsukihara) for 1.5 h at 4 °C and washed twice with 1 ml of the interaction buffer. Co-precipitated proteins were eluted with SDS-containing buffer and analysed via SDS–PAGE and subsequent Coomassie Brilliant Blue (CBB) staining or western blotting with anti-complex IV subunit IV (Abcam, 2868C12).

Quantitative PCR. Analysis of mitochondrial DNA content was performed by quantitative PCR (qPCR) on a 7500 Fast real-time cycler (Applied Biosystems, Thermo Fisher Scientific) using the EXPRESS SYBR GreenER qPCR Supermix kit (ThermoFisher Scientific). Specific primers for mitochondrial DNA (mtDNA; Fw primer: 5′-CAACAATCCATCTGACAGTTG-3′; Rev primer: 5′-GAAAATTTT-TAAATGCGGCA-3′) were designed. The relative levels of mitochondrial DNA were normalized to nuclear DNA using primers for actin (Dm_Ac79B, Quant iT Primer Test Assay, Qiagen). For reverse transcription (RT)-qPCR analyses, the isolation of total RNA was performed using Sepasol RNA (CBB) staining or western blotting with anti-complex IV subunit IV (Abcam, 2868C12).

Survival assay and climbing assay. For the lifespan studies, ~20 adult flies per vial (25 mm diameter × 95 mm height) were maintained at 25 °C, transferred to fresh fly food, and scored for survival every 4 days. To control for isogeny, we used dCHCHD2−/− and its revertant for comparison or we crossed dCHCHD2−/− and the revertant with driver and transgenic lines that were backcrossed to the w background for six generations or generated in the w background. For the oxidative stress assays, the survival rate of 3-day-old male adult flies kept in a vial containing tissue paper soaked with 0.5% H2O2 or 2 mM paraquat prepared in Schneider’s insect medium was measured as described16. Starvation assay was performed with distilled water instead of toxins. For a climbing assay, vials (25 mm diameter × 180 mm height) containing 20–25 flies were tapped gently on the table and left standing for 18 seconds. The number of flies that climbed at least 60 mm was recorded.

Exposure to hyperoxia. Adult males (1–2-day-old) in plastic vials (20–25 flies per vial) containing laboratory-standard food were placed in an atmosphere chamber at 20% 21% oxygen (O2) balanced with nitrogen. The experiment was conducted on at least 50 flies of a given genotype. Flies were removed after 4 days for EM studies and western blot analysis.

Statistical analysis. Two-tailed student’s t-test or a one-way repeated measures analysis of variance was used to determine significant differences between two or among multiple groups, respectively, unless otherwise indicated. If a significant result was determined using analysis of variance (P < 0.05), the mean values of the control and the specific test group were analysed using a Tukey–Kramer test. Dunnett’s test were used to determine significant differences between two specific groups or among multiple groups of interests. Data distribution was assumed to be normal, but this was not formally tested. With the exception of collecting data for Drosophila mitochondrial phenotypes, data collection and analysis were not performed blind to the conditions of the experiments and no randomization was used.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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