

5-Aminolevulinic acid bypasses mitochondrial complex I deficiency and corrects physiological dysfunctions in *Drosophila*

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Abstract

Complex I (CI) deficiency in mitochondrial oxidative phosphorylation (OXPHOS) is the most common cause of mitochondrial diseases, and limited evidence-based treatment options exist. Although CI provides the most electrons to OXPHOS, complex II (CII) is another entry point of electrons. Enhancement of this pathway may compensate for a loss of CI; however, the effects of boosting CII activity on CI deficiency are unclear at the animal level. 5-Aminolevulinic acid (5-ALA) is a crucial precursor of heme, which is essential for CII, complex III, complex IV (CIV) and cytochrome c activities. Here, we show that feeding a combination of 5-ALA hydrochloride and sodium ferrous citrate (5-ALA-HCl + SFC) increases ATP production and suppresses defective phenotypes in *Drosophila* with CI deficiency. Knockdown of *sicily*, a *Drosophila* homolog of the critical CI assembly protein *NDUFAF6*, caused CI deficiency, accumulation of lactate and pyruvate and detrimental phenotypes such as abnormal neuromuscular junction development, locomotor dysfunctions and premature death. 5-ALA-HCl + SFC feeding increased ATP levels without recovery of CI activity. The activities of CII and CIV were upregulated, and accumulation of lactate and pyruvate was suppressed. 5-ALA-HCl + SFC feeding improved neuromuscular junction development and locomotor functions in *sicily*-knockdown flies. These results suggest that 5-ALA-HCl + SFC shifts metabolic programs to cope with CI deficiency.

Bullet outline

- 5-Aminolevulinic acid (5-ALA-HCl + SFC) increases ATP production in flies with complex I deficiency.
- 5-ALA-HCl + SFC increases the activities of complexes II and IV.
- 5-ALA-HCl + SFC corrects metabolic abnormalities and suppresses the detrimental phenotypes caused by complex I deficiency.

Introduction

Mitochondria generate ATP through oxidative phosphorylation (OXPHOS), which involves a series of electron transfer reactions mediated by the electron transfer chain (ETC) comprising complexes I–IV (CI–CIV) (1). An abnormality of the ETC leads to ATP deficiency and causes a group of multisystem disorders called mitochondrial diseases (2). CI, also called nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase, is the most common site for mitochondrial abnormalities, representing as many as one-third of respiratory chain deficiencies (1). CI deficiency causes various clinical symptoms in organs and tissues with high energy demands, such as the brain, the heart, the liver and skeletal muscle. Mitochondrial disorders associated with CI deficiency often lead to severe neurological presentations, including Leber's hereditary optic neuropathy, mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers

(MERRF) and Leigh syndrome. There is no cure for these disorders (2).

CII mediates electron input into the ETC and can function in parallel with CI (3). Although CI oxidizes NADH and provides most electrons that enter the respiratory chain, CII oxidizes succinate from the tricarboxylic acid (TCA) cycle and serves as another entry point of electrons into the ETC (3). CII does not have proton pump activity unlike CI, whereas CIII and CIV pump protons when electrons from CII pass through to create a proton gradient across the inner membrane that drives complex V (CV), the ATP synthase complex (4). It has been reported that cell membrane-permeable prodrugs of succinate increase ATP-linked mitochondrial respiration in CI-deficient cells (5), suggesting that enhancement of CII-linked respiration may rescue ATP deficits caused by CI deficiency. However, the effects of enhancement of CII activity on CI deficiency have not been tested at the animal level.

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CII, CIII, CIV and cytochrome c contain heme, which is essential for their activities (6–10). 5-Aminolevulinic acid (5-ALA) is a rate-limiting intermediate of heme synthesis (8,11,12). Treatment of cultured cells with 5-ALA in combination with sodium ferrous citrate (SFC) has been reported to increase the activity of cytochrome c oxidase, expression of CIII, CIV and CV, the oxygen consumption rate, and production of ATP (8–10).

In this study, we used *Drosophila* as a model system to investigate the effects of 5-ALA hydrochloride and SFC (5-ALA-HCl + SFC) treatment on CI deficiency. Loss of *sicily*, a *Drosophila* homolog of *NDUFA6*, has been reported to cause CI deficiency and lower ATP levels (13–15). We found that 5-ALA-HCl + SFC feeding increased ATP levels in *sicily*-knockdown flies. CII, CIII and CIV were upregulated, and lactate and pyruvate accumulation was improved. Detrimental phenotypes caused by CI deficiency, such as abnormal neuromuscular junction (NMJ) development and locomotor dysfunctions, were mitigated. These results suggest that 5-ALA-HCl + SFC enhances resilience against CI deficiency via shifting the metabolic mode.

Results

Sicily knockdown causes a developmental delay and neurological defects

We expressed *sicily* RNAi using the transgenic binary expression system Gal4/UAS (16). RNAi expression with the ubiquitous driver actin-GAL4 lowered mRNA levels of *sicily* in both males and females (Fig. 1A). Larvae expressing *sicily* RNAi developed without gross morphological defects, but muscles of third instar larvae with *sicily* knockdown were thinner, more fragile and less innervated than those of third instar larvae expressing control RNAi (Fig. 1B). Although control pupae were distributed all over the wall of the vial, pupae with *sicily* knockdown were found closer to the medium, suggesting that *sicily*-knockdown larvae could not climb far from the medium (Fig. 1C). Many males with *sicily* knockdown died before eclosion (Fig. 1D, Male). In contrast, females with *sicily* knockdown matured normally and survived to the adult stage (Fig. 1D, Female).

Adult males with *sicily* knockdown had severely impaired locomotor functions (Fig. 1E) and died prematurely (Fig. 1G). Adult females with *sicily* knockdown also exhibited locomotor defects (Fig. 1E) and premature death (Fig. 1G), and they developed bang sensitivity when they aged (30 days after eclosion, Fig. 1F). Similar results were obtained using another RNAi line (Supplementary Material, Fig. S1).

Sicily knockdown disrupts CI

We analyzed the expression and activities of mitochondrial respiratory complexes. *Sicily* knockdown did not affect the relative level of mitochondrial DNA (Fig. 2A), suggesting that overall mitochondrial numbers are similar. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) analyses revealed that *sicily* knockdown upregulated the CIII genes *mt:CytB* and *mt:COIII*, and ATP synthase B in CV (Fig. 2B). In contrast, CIV genes such as *COX4L*, *COX7AL* and *COX7A2I*, and genes encoding the uncoupling proteins *UCP4B*, *UCP4C* and *UCP5*, were downregulated (Fig. 2B).

We performed blue-native PAGE and an in-gel activity assay to analyze each complex. *Sicily* knockdown dramatically reduced CI levels in both males and females (Fig. 2C). A band was detected with mitochondria from *sicily*-knockdown flies but not with those from control flies (Fig. 2C, arrow). Considering that *sicily* encodes a CI assembly factor (17), this band may represent part of CI. The in-gel activity assay revealed that *sicily* knockdown reduced CI

activity in the portion of the gel corresponding to the size of the holoenzyme (Fig. 2C, CI activity). An additional band with a much smaller size was also detected (asterisk). This band may represent an intermediate of CI (17), and its intensity was not dramatically affected by *sicily* knockdown (Fig. 2C, CI activity). These results indicate that *sicily* knockdown causes a loss of CI activity.

Expression of CII, CIII, CIV and CV was similar in control and *sicily*-knockdown flies (Fig. 2C). The activities of these complexes were also similar in control and *sicily*-knockdown males, whereas the activities of CII and CIV were elevated in females with *sicily* knockdown (Fig. 2C right). The higher activities of CII and CIV in females may contribute to their viability with CI deficiency.

Electron transport in respiratory complexes produces reactive oxygen species (ROS), which elevates expression of antioxidative stress response genes. Expression of the mitochondrial antioxidant gene *SOD2* was upregulated in males with *sicily* knockdown, whereas many cytoplasmic antioxidant genes (*cat*, *Prx3*, *Prx4*, *Prx5*, *SOD1*, *SOD3*, *Trxr-1* and *Trxr-2*) were downregulated (Fig. 2D), which may reflect lower mitochondrial ETC activity. The antioxidant genes *Prx2540-1* and *pxd* were upregulated in females with *sicily* knockdown (Fig. 2D), and the protein carbonyl assay indicated that the level of oxidative stress was higher in females than in males with *sicily* knockdown (Supplementary Material, Fig. S2). These results suggest that the detrimental phenotypes observed upon *sicily* knockdown are not caused by ROS.

Sicily knockdown causes lactate and pyruvate accumulation

Patients with CI deficiencies often suffer from lactic acidosis, which is characterized by the accumulation of lactate (18–21). *Sicily* knockdown caused lactate accumulation in males and females (Fig. 3A). Pyruvate also accumulated in males and females with *sicily* knockdown (Fig. 3B). Glycolytic genes such as *Pgi*, *Pfk*, *Pyk* and *HexC* were downregulated in flies with *sicily* knockdown, whereas lactate dehydrogenase (*LDH*) was upregulated (Fig. 3C), which may be because of a feedback response to accumulated pyruvate and lactate.

Genes encoding *ACOX1*, the first enzyme in the fatty acid β -oxidation pathway, and *Gdh*, which catalyzes the reversible conversion of glutamate to α -ketoglutarate using NAD(P)^+ , were downregulated in *sicily*-knockdown flies (Fig. 3C). These pathways provide acetyl CoA and α -ketoglutarate for the TCA cycle, and therefore their downregulation may also contribute to the lower metabolism in *sicily*-knockdown flies.

5-ALA-HCl + SFC increases the activities of CII and CIV and ATP levels in *sicily*-knockdown flies

To test the effect of 5-ALA-HCl + SFC treatment on mitochondria of *sicily*-knockdown flies, we raised these flies on media containing 5-ALA-HCl + SFC. 5-ALA-HCl + SFC feeding significantly increased the ATP levels in males and females with *sicily* knockdown (Fig. 4A). 5-ALA-HCl + SFC feeding did not affect mitochondrial DNA copy numbers (Fig. 4B), indicating that it does not increase the number of mitochondria. 5-ALA-HCl + SFC feeding upregulated the CIII genes *mt:CytB* and *mt:COIII* in both males and females with *sicily* knockdown (Fig. 4C). In males with *sicily* knockdown, CIV genes such as *COX4L* and *COX7A2I*, and genes encoding the uncoupling proteins *UCP4B*, *UCP4C* and *UCP5*, were also upregulated (Fig. 4C).

Blue-native PAGE and an in-gel activity assay revealed that the expression and activity of CI were still lacking in *sicily*-knockdown flies fed 5-ALA-HCl + SFC (Fig. 4D). Interestingly, 5-ALA-HCl + SFC feeding increased the activities of CII and CIV in males with

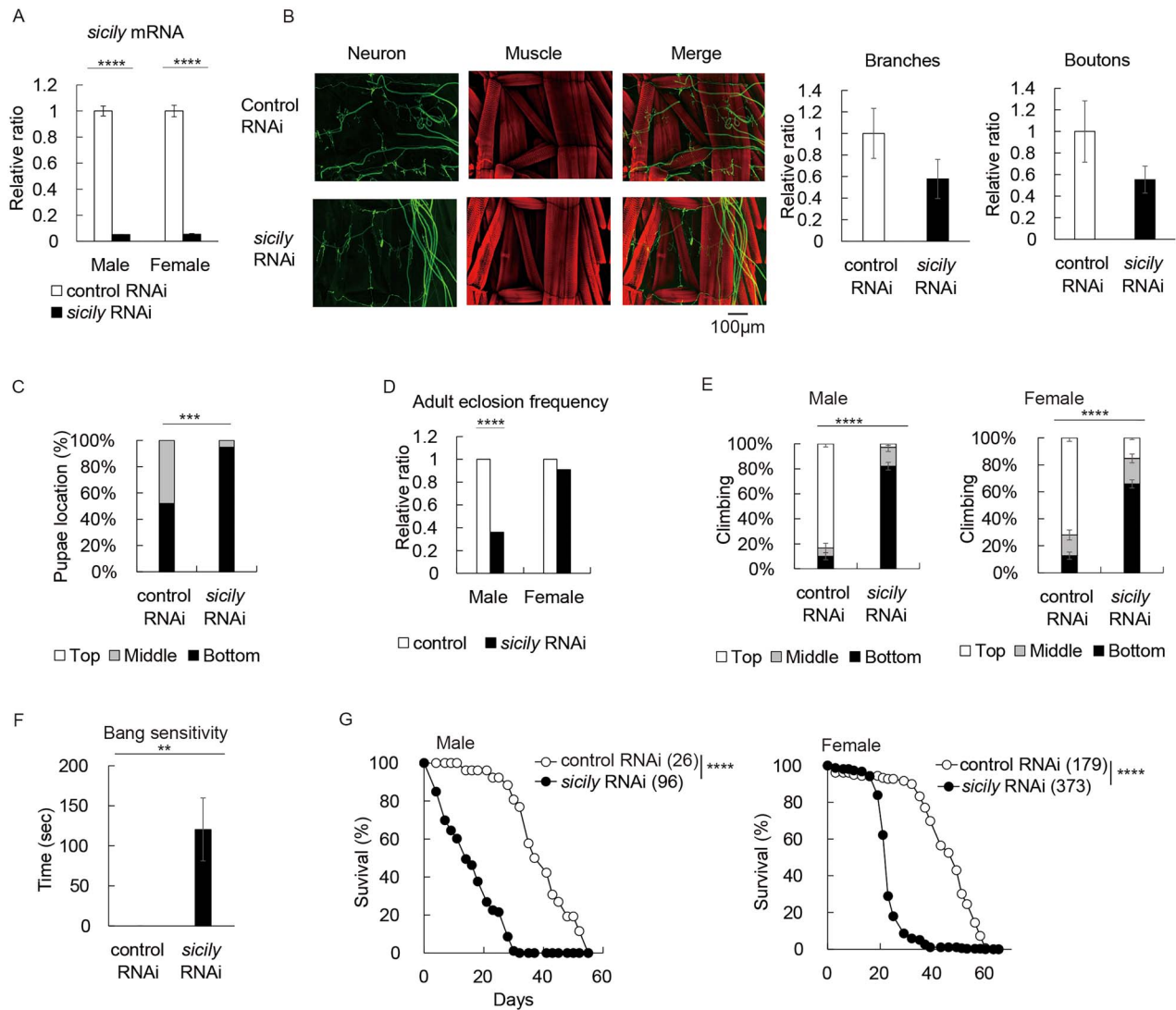


Figure 1. *sicily* knockdown causes a developmental delay and neurological defects. (A) Expression of *sicily* RNAi lowered *sicily* mRNA expression. Homogenates of adult flies with *sicily* RNAi expression driven by the ubiquitous driver *actin-GAL4* were subjected to RT-qPCR. Flies expressing mCherry RNAi were used as a control (mean \pm SE, $n = 3$; **** $P < 0.005$; unpaired t-test). (B) NMJs of third instar larvae were costained with phalloidin (red) and an anti-HRP antibody (green), which recognizes a neural membrane epitope. Genotypes or conditions as indicated. All scale bars, 100 μ m. NMJ growth was assessed by counting the number of branches and boutons at abdominal segment A4, muscle 5 (mean \pm SE, $n = 4$; $P = 0.05$; unpaired t-test). (C) The distance that larvae traveled to form pupae was assessed by counting the number of pupae formed in the top, middle and bottom areas of the vial wall (mean \pm SE, $n = 57$ or 100; *** $P = 0.005$; Chi-square test). (D) Egg-to-adult eclosion frequency for progeny flies generated by the cross *actin-GAL4/TM3Ser* \times *UAS-sicily RNAi/TM3Ser*. Eclosion frequency of *actin-GAL4*; *TM3Ser* (control) or *actin-GAL4*; *sicily RNAi* (*sicily RNAi*) expressed as relative ratios ($n = 873$ –1157; **** $P < 0.001$; Chi-square test). (E) *sicily* knockdown caused locomotor deficits. Flies were subjected to a climbing assay; they were tapped to the bottom of the vial, and the distance that they climbed in 10 s was measured. Flies were at 12 days after eclosion (mean \pm SE, $n = 6$ –29; **** $P < 0.001$; unpaired t-test). (F) Aged females with *sicily* knockdown developed shock-induced paralysis. The amount of time taken to recover from mechanical stress (bang sensitivity) is shown for flies of the indicated genotype at 24 days after eclosion (mean \pm SE, $n = 9$ or 11; ** $P < 0.01$; unpaired t-test). (G) Kaplan–Meier survival curves of *sicily*-knockdown males and females. Numbers in parentheses indicate sample size (number of flies). **** $P < 0.001$, log-rank test.

sicily knockdown (Fig. 4D). Considering that females were more resistant to loss of CI than males (Fig. 1), and that CII and CIV activities were higher in females than in males with *sicily* knockdown (Fig. 2C), these results suggest that elevated expression of CIII genes and CII and CIV activities mitigates the detrimental phenotypes of *sicily*-knockdown flies.

5-ALA-HCl + SFC suppresses lactate and pyruvate accumulation caused by *sicily* knockdown

5-ALA-HCl + SFC feeding reduced lactate and pyruvate accumulation in males and females with *sicily* knockdown (Fig. 5A and B).

It did not affect expression of the glycolytic genes *LDH*, *ACOX1* and *Gdh* in females with *sicily* knockdown, whereas *LDH* and *Gdh* were upregulated, and *ACOX1* was downregulated further, in males with *sicily* knockdown (Fig. 5C). Expression of several antioxidative stress genes was downregulated. The level of oxidative stress was similar in *sicily*-knockdown males fed 5-ALA-HCl + SFC and increased in *sicily*-knockdown females fed 5-ALA-HCl + SFC (Supplementary Material, Fig. S3), indicating that ROS levels do not correlate with improvement of the detrimental phenotypes. These results suggest that 5-ALA-HCl + SFC mitigates the metabolic abnormalities caused by CI deficiency.

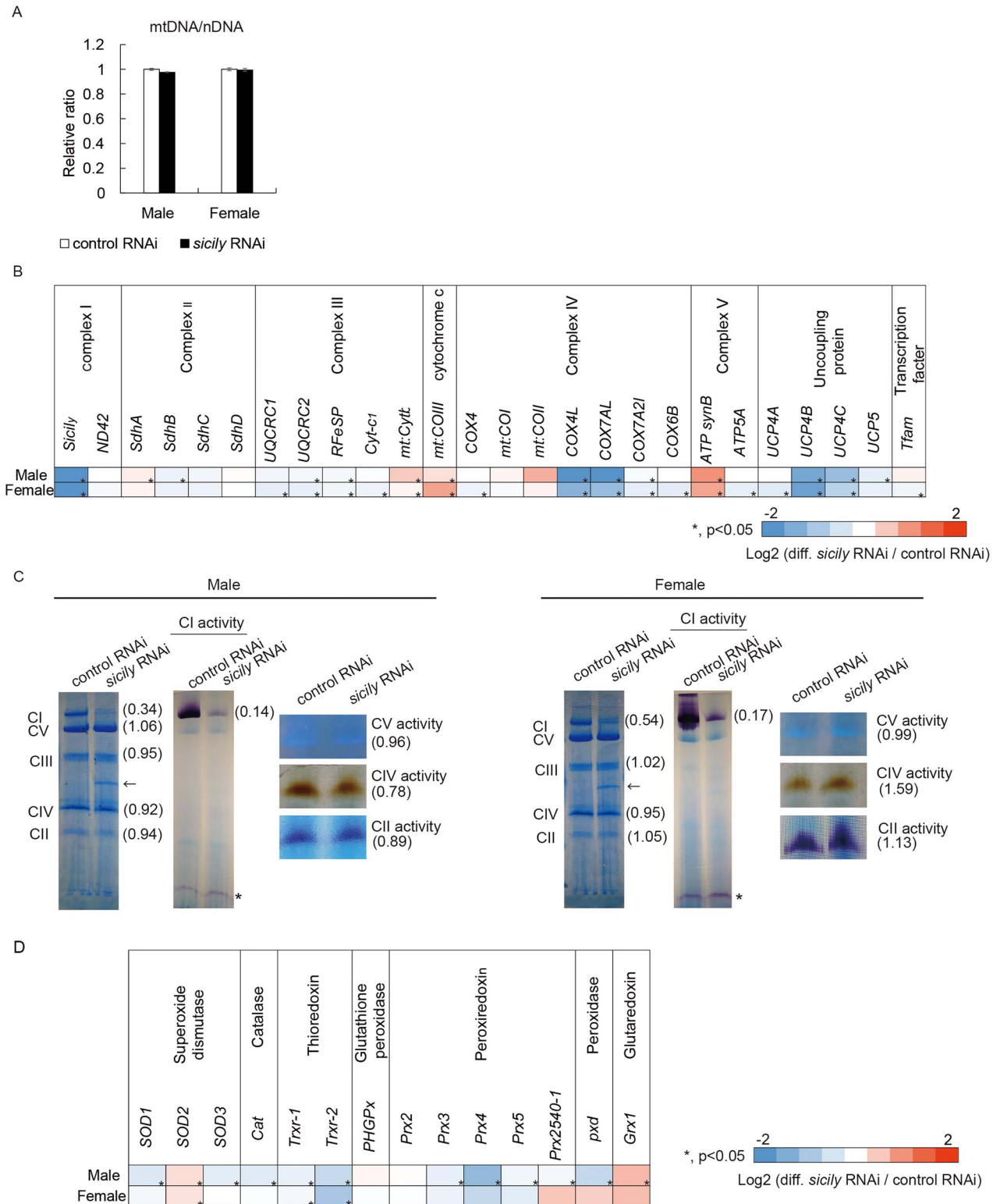


Figure 2. Effect of *sicily* knockdown on mitochondrial respiratory complexes. **(A)** The numbers of mitochondrial DNA copies in thoraxes dissected from 3-day-old flies expressing *sicily* RNAi and mCherry RNAi (control RNAi) were assessed by qRT-PCR and are shown as ratios relative to control RNAi (mean \pm SE, $n = 3$). No significant difference was detected ($P > 0.05$; unpaired t-test). **(B)** Heatmap showing fold changes in mRNA levels of the indicated genes ($n = 3$; $*P < 0.05$; unpaired t-test). **(C)** Blue-native PAGE and in-gel activity assay of mitochondria extracted from thoraxes of 3-day-old flies of the indicated genotype and sex. Arrows indicate bands specific to *sicily* knockdown. Asterisks indicate a possible CI fragment. Numbers in parentheses indicate the band intensities in flies expressing *sicily* RNAi relative to those in flies expressing control RNAi. **(D)** Heatmap showing fold changes in mRNA levels of the indicated antioxidant genes ($n = 3$; $*P < 0.05$; unpaired t-test).

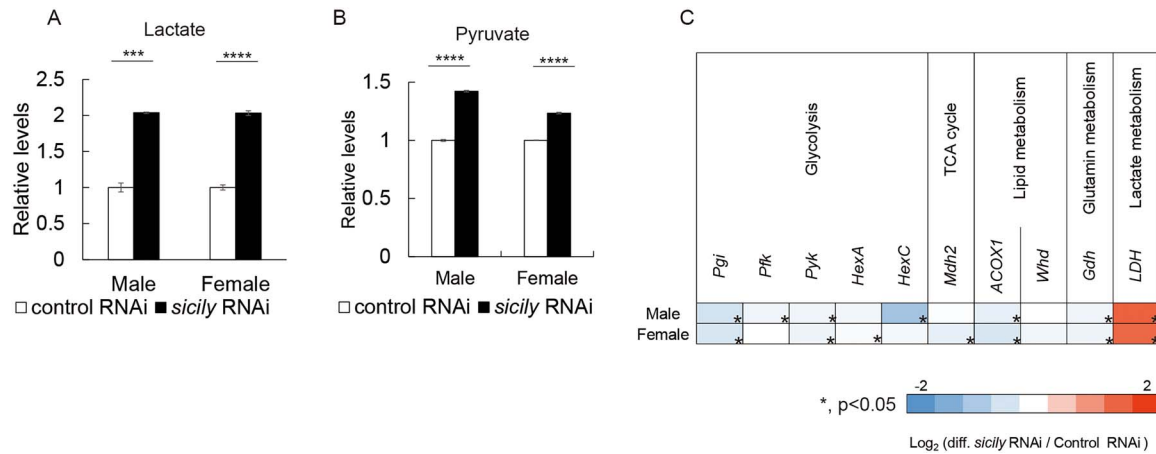


Figure 3. sically knockdown causes lactate and pyruvate accumulation. (A–B) Relative abundance of lactate (A) and pyruvate (B) in flies of the indicated genotype and sex (mean \pm SE, $n = 3–4$; *** $P < 0.005$, **** $P < 0.0001$; unpaired t-test). (C) Heatmap showing fold changes in mRNA levels of metabolic genes ($n = 3$; * $P < 0.05$; unpaired t-test).

5-ALA-HCl + SFC rescues premature death and neurological dysfunction caused by sically knockdown

We tested the effect of 5-ALA-HCl + SFC treatment on the detrimental phenotypes of sically-knockdown flies. 5-ALA-HCl + SFC feeding increased the numbers of branches and boutons in the muscles of third instar larvae with sically knockdown (Fig. 6A). Sically-knockdown larvae climbed higher to form pupae in 5-ALA-HCl + SFC-containing vials than in 5-ALA-HCl + SFC-lacking vials, suggesting that their locomotor functions are improved (Supplementary Material, Fig. S4). 5-ALA-HCl + SFC feeding improved the eclosion rate in males with sically knockdown (Fig. 6B) and significantly suppressed locomotor defects in males and females (Fig. 6C) and bang sensitivity in females (Fig. 6D). In addition, 5-ALA-HCl + SFC feeding extended the lifespan of females with sically knockdown (Fig. 6E).

We tested whether 5-ALA-HCl + SFC feeding at the adult stage has protective effects in sically-knockdown flies. Sically-knockdown flies were raised in regular food, and eclosed flies were transferred to food containing 5-ALA-HCl + SFC. Sically-knockdown flies fed 5-ALA-HCl + SFC after eclosion exhibited improved locomotor functions (Fig. 6F). In addition, 5-ALA-HCl + SFC feeding at the adult stage rescued bang sensitivity of females (Fig. 6G) and extended the lifespan of males (Fig. 6H). These results suggest that some of the defective phenotypes caused by CI deficiency can be mitigated after the developmental period.

Discussion

In this study, we found that 5-ALA-HCl + SFC mitigated detrimental phenotypes caused by CI deficiency in flies. Knockdown of *sically*, the *Drosophila* homolog of *NDUFA6*, caused abnormal NMJ development, locomotor defects and premature death, with accumulation of pyruvate and lactate (Figs 1–3). 5-ALA-HCl + SFC feeding improved NMJ development and locomotor functioning in sically-knockdown flies (Fig. 6). Although CI activity was still lacking, ATP levels were increased, and metabolic abnormalities such as accumulation of pyruvate and lactate were suppressed, in sically-knockdown flies fed 5-ALA-HCl + SFC (Fig. 4A and B). Detrimental phenotypes were negatively correlated with the mRNA levels of genes encoding CIII and activities of CII and CIV (Figs 2, 4, and 5).

Metabolic bypassing of mitochondrial CI has been reported in cultured tissues (5,22) but has not been reported at the animal level. This study, for the first time, showed that 5-ALA-HCl + SFC promotes bypassing of CI.

sically knockdown caused more severe phenotypes in males than in females (Fig. 1). Although the knockdown efficiency was similar in males and females (Fig. 1A, sically RNAi), and CI activity was lost in both males and females with sically RNAi expression (Fig. 2C), sically knockdown was partially lethal in males but not in females (Fig. 1). A similar result was obtained with another RNAi line (Fig. S1), indicating that the sex-specific lethality is not attributable to an off-target effect or transgene insertion. Interestingly, sically knockdown upregulated CIII genes and increased CII and CIV activities only in females (Fig. 2B and C). 5-ALA-HCl + SFC feeding, which suppressed abnormal NMJ development and locomotor defects, also upregulated CIII genes and increased CII and CIV activities in males (Fig. 4C and D). These results suggest that enhancement of CII, CIII and CIV mediates resilience against CI deficiency, and females may be more resilient to sically knockdown because of their ability to enhance CII, CIII and CIV (Fig. 7).

In addition to CII, CIII and CIV, our results suggest that 5-ALA-HCl + SFC affects multiple ATP-synthesizing reactions, including glycolysis, the TCA cycle, OXPHOS, β -oxidation and other routes for oxidation of NADH such as the glutamate dehydrogenase pathway (Figs 4 and 5). 5-ALA stimulates the synthesis of porphyrins and heme (8,11,12). Heme regulates various cellular processes associated with oxygen by controlling the activities of enzymes, signal transducers and transcriptional regulators (23). 5-ALA is also metabolized to protoporphyrin IX, which affects gene expression by interacting with DNA or RNA sequences that fold into non-canonical four-stranded nucleic acid structures known as G-quadruplexes (24–26). 5-ALA-HCl + SFC may shift the cellular metabolic mode to cope with the lack of CI via orchestrated changes in multiple pathways (Fig. 7).

In summary, our results revealed that 5-ALA-HCl + SFC bypasses CI deficiency and promotes ATP production, and thus corrects metabolic abnormalities and mitigates detrimental phenotypes in *Drosophila*. Further investigation would enhance our understanding of metabolic regulation and the pathogenesis of mitochondrial diseases.

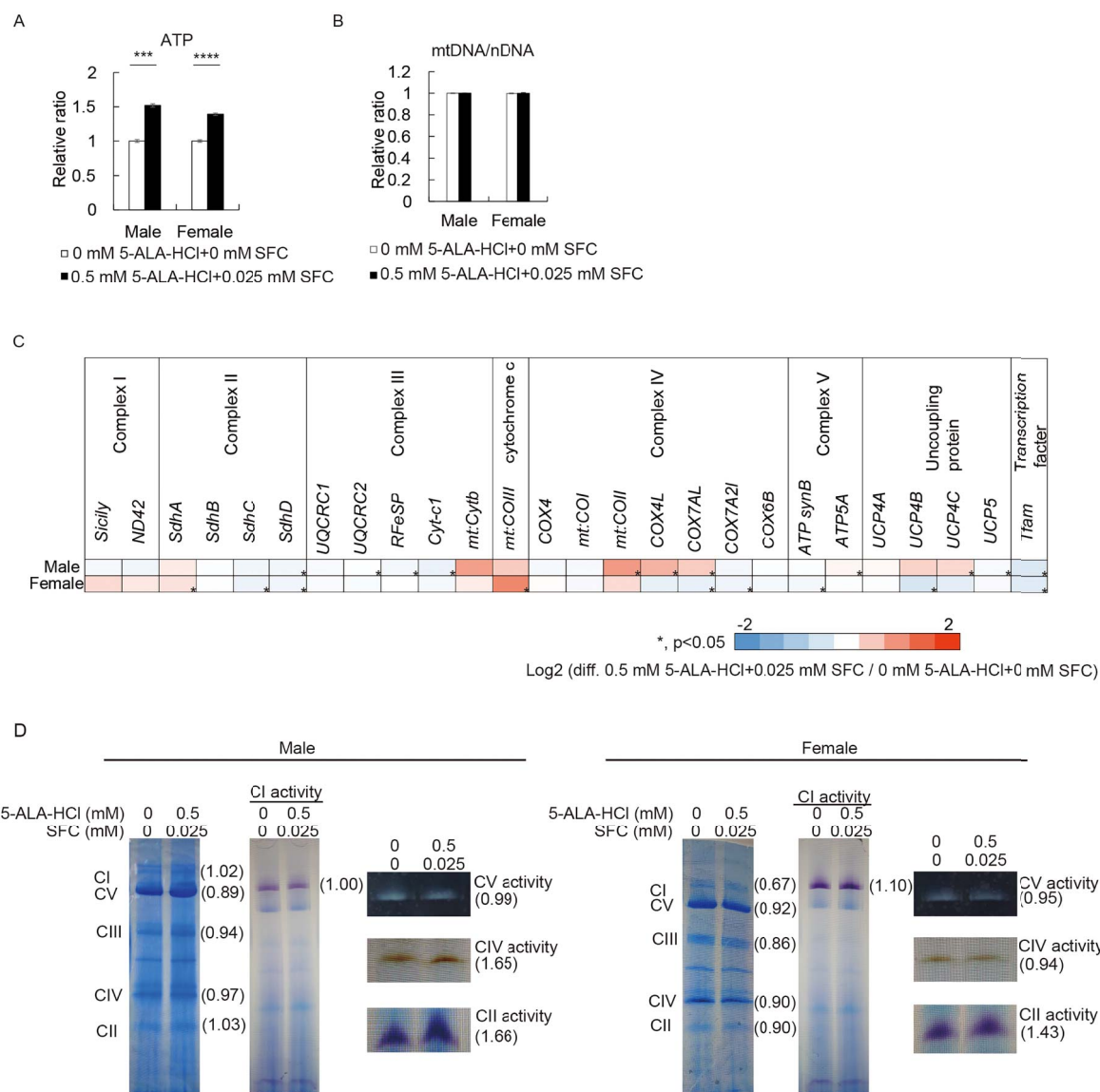


Figure 4. 5-ALA-HCl + SFC feeding increases ATP levels without CI. **(A)** Quantitation of the ATP levels in thoraxes of sicily-knockdown flies fed food containing the indicated concentrations of 5-ALA-HCl + SFC. Flies were 3 days old. ATP levels are normalized to protein levels and shown as ratios relative to the control (mean \pm SD, $n = 3$; *** $P < 0.005$, **** $P = 0.001$; unpaired t-test). **(B)** The number of mitochondrial copies was determined by qRT-PCR. The numbers of mitochondrial copies are shown as ratios relative to the control (mean \pm SE, $n = 3$). No significant difference was detected ($P > 0.05$, unpaired t-test). **(C)** Heatmap showing fold changes in mRNA levels of the indicated genes ($n = 3$; * $P < 0.05$, unpaired t-test). **(D)** Blue-native PAGE and an in-gel activity assay of mitochondria extracted from thoraxes of flies fed food containing the indicated concentrations of 5-ALA-HCl + SFC. Flies were at 3 days after eclosion. Numbers in parentheses indicate the band intensities in flies expressing sicily RNAi relative to those in flies expressing control RNAi.

Materials and Methods

Chemicals

5-ALA hydrochloride (neo-ALA Co. Ltd, Tokyo, Japan) and SFC (Komatsuya Corporation, Osaka, Japan) were provided by SBI Pharmaceuticals Co., Ltd. Alexa 488 Goat Anti-Horseradish Peroxidase (Jackson Immuno Research), Rhodamin-Phalloidin (Merck/Sigma-Aldrich, Germany), NativePAGE™ Running Buffer and NativePAGE™ Cathode Buffer Additive (Thermo Fisher Scientific, USA) were purchased.

Fly stocks and husbandry

Flies were maintained in standard cornmeal media (10% glucose, 0.7% agar, 9% cornmeal, 4% yeast extract, 0.3% propionic acid and 0.1% n-butyl p-hydroxybenzoate) at 25°C under light-dark

cycles of 12:12 h. The flies were transferred to fresh food vials for every 2–3 days. Actin-GAL4 (Act5C-GAL4, #3954), UAS-mCherry RNAi (#35785) and UAS-sicily RNAi (#55332) are obtained from Bloomington Drosophila Stock Center. UAS-sicily RNAi (#103029) is from VDRC.

5-ALA-HCl + SFC feeding

Flies were raised on regular cornmeal. After eclosion, male flies were maintained on regular cornmeal food mixed with 5-ALA-HCl + SFC at the indicated concentration. Food vials were changed every 2–3 days.

Muscle and NMJs staining

The muscles of third instar larvae were dissected in HL3.1 buffer (70 mM NaCl, 5 mM KCl, 0.2 mM CaCl₂, 20 mM MgCl₂,

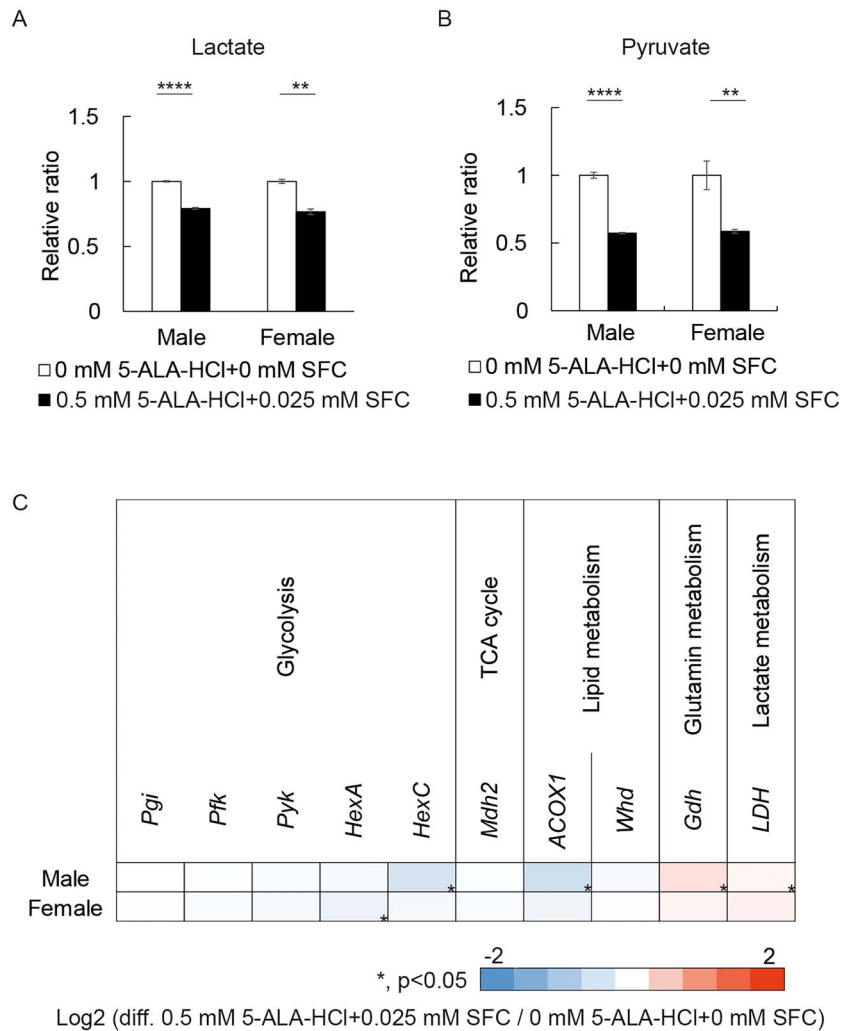


Figure 5. 5-ALA-HCl + SFC suppresses lactate and pyruvate accumulation caused by sicily knockdown. (A-B) Relative abundance of lactate (A) and pyruvate (B) in sicily-knockdown flies fed food containing the indicated concentrations of 5-ALA-HCl + SFC (mean \pm SE, $n = 3-4$; ** $P < 0.01$, **** $P < 0.0001$; unpaired t-test). (C) Heatmap showing fold changes in mRNA levels of metabolic genes ($n = 3$; * $P < 0.05$; unpaired t-test).

10 mM NaHCO₃, 5 mM trehalose and 5 mM HEPES) and fixed in 4% PFA for 10 min. Samples were incubated in PBS with 0.05% Triton X-100 (PBST) containing 5% normal goat serum (Jackson Immuno Research) for 30 min, then incubated with PBST containing 2 $\mu\text{g}\cdot\text{ml}^{-1}$ phalloidin/tetramethylrhodamine B isothiocyanate peptide (Sigma-Aldrich) and 3 $\mu\text{g}\cdot\text{ml}^{-1}$ Alexa 488 goat anti-horseradish peroxidase (Jackson Immuno Research) at 4°C for overnight. The samples were washed three times in 0.1% PBST and mounted in VectaShield mounting medium (Vector Laboratories). Images were captured using a fluorescence microscope (Keyence, BZ-X710).

Climbing assay

The climbing assay was performed as previously described (27). Flies were placed in an empty plastic vial (2.5 cm in diameter \times 10 cm in length). The vial was gently tapped to knock the flies to the bottom, and the number of flies reached to the top, middle and bottom areas of the vials in 20 s was counted. Experiments were repeated 10 times, and the mean percentage of flies in each area and standard deviations was calculated. Experiments were repeated with independent cohorts more than three times, and a representative result was shown.

Bang-sensitivity

About 9–30 flies were placed in an empty plastic vial (2.5 cm in diameter \times 10 cm in length). Flies were tapped down to the bottom, and the mean recovery time and standard deviations were calculated.

Lifespan analysis

Flies were placed in the food vials on their sides at 25°C under conditions of a 12:12-h light and dark cycle, and transferred to fresh food vials every 2–3 days, and the number of dead flies was counted each time.

Blue-native PAGE

Blue-native PAGE to analyze mitochondrial respiratory chain protein was carried out as described with a modification (28,29). Thorax from 54–100 flies was homogenized in 1 ml of chilled mitochondrial isolation medium [250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 0.15 mM MgCl₂]. The samples were centrifuged twice for 15 min at 600g at 4°C to remove debris. The supernatant was centrifuged again for 5 min at 13000g at 4°C to collect mitochondrial fraction as the pellet. Mitochondrial

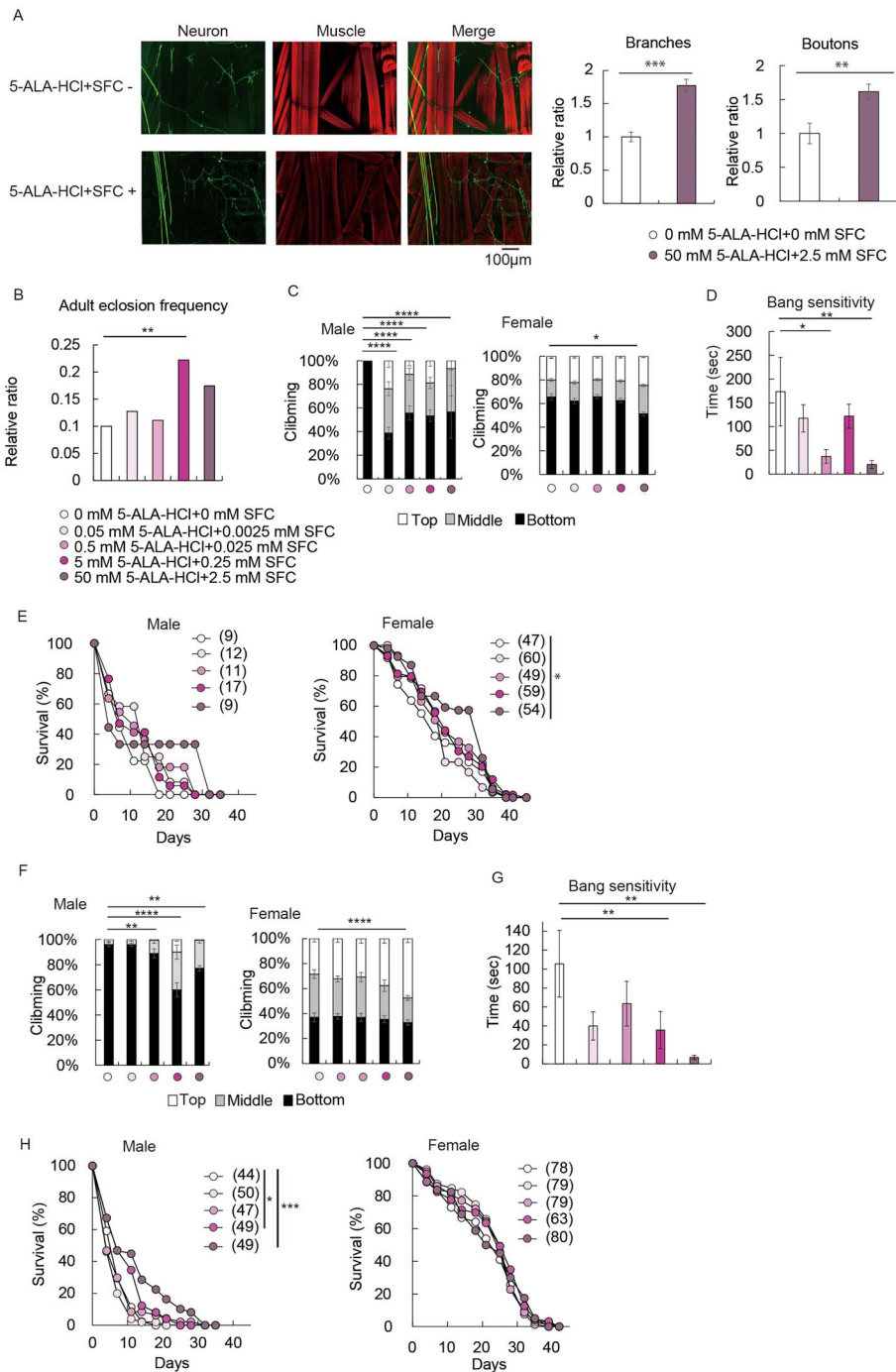


Figure 6. 5-ALA-HCl + SFC feeding rescues developmental delays, premature death and neurological dysfunction caused by sicily knockdown. (A) Third instar larvae were costained with phalloidin (red) and an anti-HRP antibody (green), which recognizes a neural membrane epitope. Concentrations of 5-ALA-HCl + SFC in food are as indicated. NMJ growth was assessed by counting the numbers of branches and boutons at abdominal segment A4, muscle 6/7 (mean \pm SE, $n = 4$; $**P = 0.01$, $***P = 0.005$; unpaired t-test). (B) Egg-to-adult eclosion frequency of flies with the indicated concentrations of 5-ALA-HCl + SFC in food ($n = 74$ –115; $**P < 0.01$; Chi-square test and Holm–Sidak post hoc test). (C) Locomotor functions of flies fed food containing the indicated concentrations of 5-ALA-HCl + SFC were assessed by a climbing assay. Flies were tapped to the bottom of the vial, and the number of flies that reached the top, middle and bottom third of the vial in 20 s was counted. Flies were at 9 days after eclosion (mean \pm SE, $n = 4$ –29; $*P < 0.05$, $****P < 0.0001$; one-way ANOVA followed by Dunnett’s test). (D) Amount of time taken to recover from mechanical stress (bang sensitivity) by females fed food containing the indicated concentrations of 5-ALA-HCl + SFC. Flies were at 21 days after eclosion (mean \pm SE, $n = 11$ –26; $*P < 0.05$, $**P < 0.01$; one-way ANOVA followed by Dunnett’s test). (E) Survival curves of sicily-knockdown males and females raised on food containing the indicated concentrations of 5-ALA-HCl + SFC. Numbers in parentheses indicate sample size (number of flies); $*P < 0.05$; log-rank test and Holm–Sidak post hoc test. (F–H) 5-ALA-HCl + SFC feeding after eclosion suppressed detrimental phenotypes caused by sicily knockdown. Flies were raised on food lacking 5-ALA-HCl + SFC until eclosion and then transferred to food containing the indicated concentrations of 5-ALA-HCl + SFC. (F) Climbing assay with flies fed food containing the indicated concentrations of 5-ALA-HCl + SFC. Flies were at 12 days after eclosion (mean \pm SE, $n = 10$ –22; $**P < 0.01$, $****P < 0.0001$; one-way ANOVA followed by Dunnett’s test). (G) Amount of time taken to recover from mechanical stress by females fed food containing the indicated concentrations of 5-ALA-HCl + SFC. Flies were at 30 days after eclosion (mean \pm SE, $n = 13$ –30; $**P < 0.01$; one-way ANOVA followed by Dunnett’s test). (H) Survival curves of sicily-knockdown males and females maintained on food containing the indicated concentrations of 5-ALA-HCl + SFC after eclosion. Numbers in parentheses indicate sample size (number of flies); $*P < 0.05$, $***P < 0.005$; log-rank test and Holm–Sidak post hoc test.

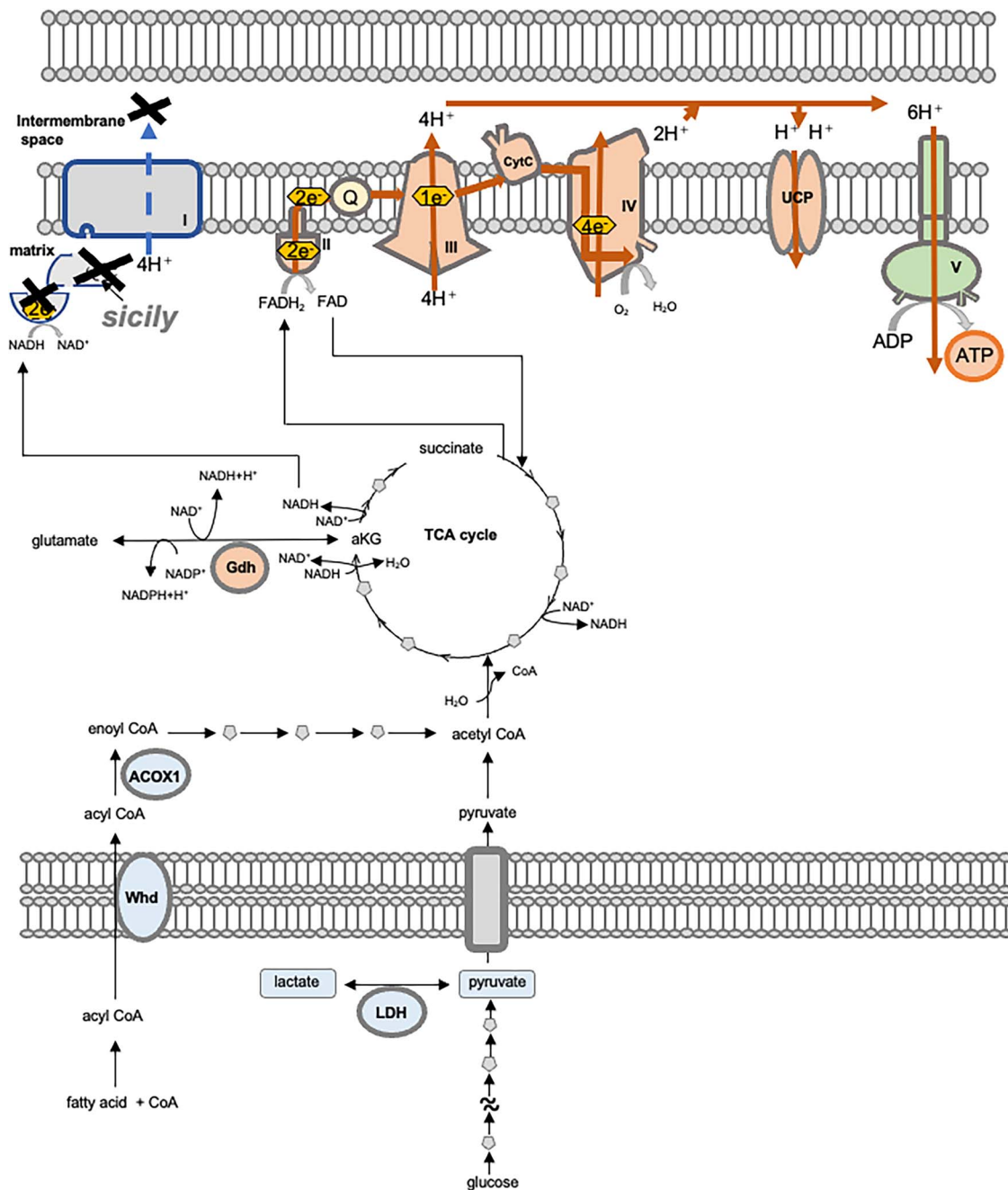


Figure 7. Possible mechanisms by which 5-ALA-HCl + SFC increases ATP production without CI. The components upregulated and downregulated by 5-ALA-HCl + SFC are colored orange and blue, respectively. Sicily knockdown causes loss of CI, whereas upregulation of CII compensates for the lack of electron transfer in CI. Upregulation of CIII and CIV increases the number of protons pumped into the IMS. 5-ALA-HCl + SFC may also affect other ATP-synthesizing reactions, including glycolysis, the TCA cycle and β -oxidation, which in combination may counteract the accumulation of lactate and pyruvate and increase ATP synthesis.

protein levels were determined using a bicinchoninic acid (BCA) assay. For blue-native PAGE, The NativePAGE Novex Bis-Tris Gel System (Life Technologies) was used according to the manufacturer's protocol. About 18–96 μ g mitochondrial protein was solubilized in sample buffer (50 mM NaCl, 20 mM Tris-HCl (pH 7.4), 1% Triton X-100). After centrifugation for 5 min at 14000 rpm at 4°C, the supernatants were collected and mixed with 10 \times loading dye solution (5% Coomassie Blue G, 1 M aminohexanoic acid, 100 mM Bis-Tris). The separated on 3–12% NativePAGE gels.

In-gel activity assay

In-gel activity assay to analyze mitochondrial respiratory chain activity was carried out as described previously with a modification (30). Mitochondrial fractions were subjected to blue-native PAGE. For CI activity, the gel was incubated in complex I activity substrate [2 mM Tris-HCl (pH 7.4), 0.01% (w/v) NADH, 0.025% (w/v) Nitroretorazolium Bule chloride (NTB)] for 15 min at room temperature. The gel was then transferred to 10% acetic acid to stop reaction. For CII activity, the gel was incubated in complex II activity substrate [5 mM Tris-HCl (pH 7.4), 20 mM sodium

succinate, 0.025% (w/v) NTB, 200 μ M phenazine methosulphate] for 40 min at 37°C. The gel was then transferred to 10% acetic acid to stop reaction. For CIV, the gel was incubated with complex IV activity substrate [0.05% (w/v) diaminobenzidine, 0.01% (w/v) cytochrome c, 45 mM phosphate buffer (pH 7.4)] for overnight at room temperature. For CV activity, the gel was incubated with complex V activity substrate [35 mM Tris, 270 mM glycine, 14 mM MgSO₄, 10 mM ATP, 0.2% (w/v) Pb(NO₃)₂] for overnight at room temperature. The gel was transferred to 50% of methanol to stop the reaction.

High-resolution clear native-PAGE

High-resolution Clear Native-PAGE to analyze mitochondrial respiratory chain protein was carried out as described with a modification (31). Thorax from 59 to 100 flies was homogenized in 1 ml of chilled mitochondrial isolation medium [250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 0.15 mM MgCl₂]. The samples were centrifuged twice for 15 min at 600g at 4°C to remove debris. The supernatant was centrifuged again for 5 min at 13 000g at 4°C to collect mitochondria as pellet. Mitochondrial protein levels were determined using a BCA assay. Mitochondrial fractions were incubated in sample buffer [50 mM NaCl, 50 mM imidazole/HCl, 2 mM 6-aminohexanoic acid, 1 mM EDTA (pH 7.0), DDM (2.5 g/g protein) and digitonin (4 g/g protein)] for 10 min on ice. After centrifugation for 15 min at 10 000g at 4°C, the supernatants were mixed with 10 \times loading dye solution (50% glycerol, 0.1% Ponceau S) and separated on 3–12% NativePAGE Novex Bis-Tris Gel System (Life Technologies). The gels were stained with colloidal blue staining kit (Thermo Fisher Scientific).

Pyruvate assay

Thoraxes from the 35 flies were homogenized in pyruvate assay buffer (Pyruvate Assay kit, Abcam), and pyruvate concentration was measured by using the Pyruvate Assay kit (Abcam) according to the manufacturer's manual.

Lactate assay

Thoraxes from the 40 flies were homogenized in lactate assay buffer [L-Lactate Assay kit (Abcam)], and lactate concentration was measured by using the L-Lactate Assay kit (Abcam) according to the manufacturer's manual.

ATP assay

Thoraxes from the 20 flies were homogenized in CellTiter-Glo[®] buffer [Celltiter-Glo[®] Luminescent Cell Viability Assay (Promega)], and ATP was measured by using the Celltiter-Glo[®] Luminescent Cell Viability Assay (Promega) according to the manufacturer's manual. Protein levels were measured with the BCA Protein Assay kit (Thermo Fisher Scientific).

Oxyblot (protein carbonyl assay kit)

Thoraxes from the 20 flies were homogenized in PBS (pH 7.2), and oxidative stress levels were measured by using the Protein Carbonyl Assay kit (Abcam) according to the manufacturer's manual. Protein levels were measured with the BCA Protein Assay kit (Thermo Fisher Scientific). A total of 85 μ g of mitochondrial protein was spotted to polyvinylidene difluoride membranes, blocked with 5% skim milk in PBS-T and incubated with DNP antibody followed by HRP goat anti-rabbit secondary antibody. Immunolabeled proteins were analyzed by using a chemiluminescence kit (ImmunoStar LD) and a lumino-image analyzer (ChemiDoc MP System, Bio-Rad Laboratories).

mtDNA/nDNA ratio

The total DNA of the 20 thoraxes was purified according to the protocol provided with the QIAamp DNA Mini kit (QIAGEN). The expression level of each gene was measured using the PowerUp SYBR Green Master Mix (Life Technologies) and the StepOnePlus Real-Time PCR System (Life Technologies). The average value of levels of *mito:COI* and *mito:COIII* was used as mitochondrial DNA, and the average value of levels of *sicily* and *Act5C* was used to quantify nuclear DNA. Primers were designed using DRSC Fly-PrimerBank (32). Primer sequences are shown in [Supplementary Material, Table S1](#).

qRT-PCR

The total RNA of the 20 thoraxes was purified according to the protocol provided with the RNAeasy Plus Universal Mini kit (QIAGEN). Copy DNA (cDNA) was synthesized from 2.0 μ g of total RNA using the High Capacity RNA-to-cDNA kit (Life Technologies). The expression level of each gene was measured using the PowerUp SYBR Green Master Mix (Life Technologies) and the StepOnePlus Real-Time PCR System (Life Technologies). The expression of genes of interest was standardized relative to *Act57B*. Primers were designed using DRSC FlyPrimerBank (33). Primer sequences are listed in [Supplementary Material, Table S1](#).

Statistics

Statistics were done with GraphPad Prism9 (GraphPad Prism ver.9.10 for Windows, GraphPad Software, San Diego California USA). Differences were assessed using unpaired t-test, one-way ANOVA followed by Dunnett's test or log-rank test and Holm-Sidak post hoc test. P-values < 0.05 were considered statistically significant.

Supplementary material

[Supplementary Material](#) is available at HMG online.

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Authors' contributions

N.N. and K.A. designed the experiments. N.N., M.N., K. S., A.A., T.S. and K.A. performed the experiments. N.N., K.S. and K.A. analyzed the data. N.N., M.N., T.I, K.T, M.I. and K.A. wrote the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article or available from the lead contact upon request.

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