Alternative assembly of respiratory complex II connects energy stress to metabolic checkpoints

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Cell growth and survival depend on a delicate balance between energy production and synthesis of metabolites. Here, we provide evidence that an alternative mitochondrial complex II (CII) assembly, designated as CIIlow, serves as a checkpoint for metabolite biosynthesis under bioenergetic stress, with cells suppressing their energy utilization by modulating DNA synthesis and cell cycle progression. Depletion of CIIlow leads to an imbalance in energy utilization and metabolite synthesis, as evidenced by recovery of the de novo pyrimidine pathway and unlocking cell cycle arrest from the S-phase. In vitro experiments are further corroborated by analysis of paraganglioma tissues from patients with sporadic, SDHA and SDHB mutations. These findings suggest that CIIlow is a core complex inside mitochondria that provides homeostatic control of cellular metabolism depending on the availability of energy.

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Mitochondria are semi-autonomous organelles found in the majority of eukaryotic cells. They have their own genome (mitochondrial DNA, mtDNA), which encodes subunits of respiratory complexes and RNA components for mitochondrial protein synthesis. Major roles of mitochondria include generation of energy and synthesis of metabolites.

Molecules that are a source of energy are oxidized in a series of biochemical reactions within the tricarboxylic acid (TCA) cycle. Intermediate products of the TCA cycle are used as signaling molecules and as building blocks for various macromolecules, while NADH and FADH₂ are metabolized via oxidative phosphorylation (OXPHOS) to yield ATP. OXPHOS comprises five complexes, CI–CV. CI (succinate dehydrogenase, SDH) contains nuclear-encoded SDHA, SDHB, SDHC, and SDHD subunits, which are recognized as tumor suppressors. CI converts succinate to fumarate in the TCA cycle, and is thus at the crossroad of the TCA cycle and OXPHOS.

Clinical data document the presence of somatic mutations in mtDNA in cancer in both the regulatory D-LOOP and the coding regions. Previous research has mainly concerned the effects of mtDNA mutations on CI, CII, CIII, CIV, and CV, with CII having been a relatively minor focus. This is explained by the fact that unlike all other OXPHOS complexes, CI does not contain mtDNA-encoded subunits, and therefore no direct effect of mtDNA defects on CII assembly and function has been expected. Further, individual respiratory complexes form supercomplexes (SCs), while CII acts as a stand-alone complex, with only one report indicating that it can be an SC component.

This study investigates whether CII subunits and mtDNA could have any form of interaction in energy production, and if so, whether there is a functional relationship that provides an advantage to cells with mtDNA mutations. We show that depletion of mtDNA has an unexpected effect on CII assembly, causing a shift from its tetrameric, fully processed, and assembled form to a slower migrating complex of ~100 kDa, referred to here as complex CHlow (CHlow). Our data suggest that CHlow links bioenergetic stress to negative regulation of de novo pyrimidine synthesis and cell cycle progression, which is supported by clinical data from paraganglioma patients with mutations in SDH subunits, indicating that CHlow plays an important role in homeostatic control of metabolite synthesis under bioenergetic stress.

Results

mtDNA-linked bioenergetics defects affect the assembly of CII.

Unlike other respiratory complexes, CII is encoded by nuclear DNA and is genetically independent of mtDNA. To understand if mtDNA dysfunction affects CII indirectly, we tested the effect of mtDNA perturbation on CII assembly, causing a shift from its tetrameric, fully processed, and assembled form to a slower migrating complex of ~100 kDa, referred to here as complex CHlow (CHlow). Our data suggest that CHlow links bioenergetic stress to negative regulation of de novo pyrimidine synthesis and cell cycle progression, which is supported by clinical data from paraganglioma patients with mutations in SDH subunits, indicating that CHlow plays an important role in homeostatic control of metabolite synthesis under bioenergetic stress.

SDHA is a stable constituent of CHlow. To better characterize the subunit composition of CHlow, SDHA, SDHB, and SDHC subunits were knocked down one at a time in 4T1 cells using two different siRNAs (siRNA1 and siRNA2), and CII assembly was assessed by Western blotting (WB) following SDS-PAGE for the SDH subunits, indicating that CHlow plays an important role in homeostatic control of metabolite synthesis under bioenergetic stress.

Characterization of the CHlow form of SDH. Based on crystal structure of CII22 (Supplementary Fig. 1a) and our data in Fig. 1e, we hypothesized that depletion of SDHB will result in cells containing only CHlow. We thus generated SDHB knockout (KO) MDA231 cells targeting exon 1 (Fig. 2a and b). With 4T1 cells (Fig. 1c and e), the steady-state level of SDHA was unchanged in SDHBKO MDA231 cells (Fig. 2c). In order to deplete CHlow, SDHBKO MDA231 cells were transfected with two different SDHA shRNAs (SDHBKO SDHBKOEV cells), and cells lacking both CII and SDHB were transfected with an empty vector (SDHBKOEV) as a control (Fig. 2c; Supplementary Fig. 1b). NBGE analysis showed high levels of CHlow in SDHBKO MDA231 cells and little CHlow in SDHBKO SDHBKOEV cells (Fig. 2d). We thus prepared models of cells with three variants of CII assembly: fully assembled CII and low levels of CHlow (parental cells), cells with only CHlow (SDHBKO SDHBKOEV cells), and cells lacking both CII and CHlow (SDHBKO SDHBKOEV cells). These models showed low proliferation for both SDHBKO and SDHBKO SDHBKOEV cells (Fig. 2e).

To specify its molecular composition, we transfected SDHBKO cells with SDHA-FLAG and immunoprecipitated the cell-free extract using anti-FLAG IgG. The immunoprecipitate was subjected to tryptic digest followed by MS analyses (Supplementary Fig. 2a), with results of the screen in Supplementary Fig. 2a and Supplementary Data 1. The analysis revealed the presence of the CII assembly factors SDHAF2 (16.7 kDa) and SDHAF4 (9.9 kDa). While SDHAF2 was readily detectable on NBGE membranes in CIIlow (Supplementary Fig. 2b), the available antibodies did not allow reproducible detection of SDHAF4 after NBGE, and its presence was verified by other means (see below).

We next re-expressed SDHB-FLAG in SDHBKO cells to see whether this would rescue the parental phenotype and whether CIIlow is reversible. SDH-reconstituted (SDHB⁶⁸⁶⁶ cells) were assessed by Western blotting (WB) following SDS-PAGE for the presence of SDHA, SDHB, SDHAF2, and SDHAF4. Supplementary Fig. 2c documents similar levels of SDHA in all three sublines and high levels of SDHB in parental and SDHB⁶⁶⁶ cells. In contrast, both SDHAF2 and SDHAF4 were low in parental and
SHDBrec cells, but accumulated in SDHBKO cells. We then tested the three sublines by NBGE followed by WB using anti-SDHA IgG and anti-SDHAF2 IgG. Supplementary Fig. 2d reveals that mature CII is re-assembled and the amount of CIIlow reverts to parental levels in SDHB rec cells, indicating SDHB-dependent reversibility of alternatively assembled CII. Furthermore, SDHAF2 and SDHAF4 were detectable only in SDHBKO cells that lack mature CII but feature CIIlow, supporting the presence of these assembly factors in CIIlow cells.

To verify the reversibility of CIIlow in response to stress, we treated parental cells with CAB for 48 h, followed by 24 h recovery. The amount of CIIlow was evaluated by NBGE using antibodies against SDHA and SDHAF2, two CIIlow components readily detectable in this assay (Supplementary Fig. 2e). Treatment with CAB increased the level of CIIlow, which reverted to baseline within 24 h of treatment cessation. Together with the SDHBrec data, this indicates reversibility of CIIlow assembly, providing flexibility in response to mitochondrial bioenergetic stress.

CIIlow does not modulate mitochondrial bioenergetics. We asked whether altered assembly of SDHA affects mitochondrial bioenergetics as well as expression and assembly of other mitochondrial complexes. We found that basal respiration, maximum respiratory capacity, and ATP production were decreased both in SDHBKOEV and SDHBKOSDHAlow cells (Fig. 3a–e). Unlike parental cells, absence of spare respiratory capacity and low ATP production were found in SDHBKOEV and SDHBKOSDHAlow cells (Fig. 3d, e), further indicating a state of bioenergetic stress.

We next assessed oxygen consumption in permeabilized MDA231 cells by high-resolution respirometry. This showed that alteration of the CII assembly status resulted not only in complete suppression of CII-dependent respiration, but also considerably altered CI-dependent respiration (Fig. 3f). Consistent with the above results, we found that assembly of SCs was reduced in SDHBKO and SDHBKOSDHAlow cells (Fig. 3g). This is further supported by lower steady-state levels of CI subunits but not CIII, CIV, and CV subunits (Fig. 3h). However, there was no significant difference between SDHBKO and SDHBKOSDHAlow cells.
for any of the assessed parameters. In contrast, re-expression of SDHB-FLAG (SDHBrec cells) substantially recovered the parental phenotype with respect to routine and CII-dependent respiration (Supplementary Fig. S2f).

Cells with severe defects in OXPHOS rely on glycolysis for ATP production and do not grow in the presence of galactose. We tested proliferation of MDA231 sublines in galactose- and glucose-containing media, and found that both SDHBKOEV and SDHBKOSDHAlow cells failed to proliferate in galactose-containing medium whereas parental cells proliferated efficiently (Fig. 3i). This is in agreement with previous reports on cells with defects in CI and CII assembly. Again, re-expressed SDHB rescued proliferation in galactose-containing media (Supplementary Fig. 2g).

Similarly, analysis of mitochondrial morphology using transmission electron microscopy (TEM) revealed that both SDHBKOEV and SDHBKOSDHAlow cells featured mitochondria with altered structure (Supplementary Fig. 3a), consistent with a previous report. These changes did not affect the steady-state level of several key proteins associated with mitochondrial maintenance and biogenesis (Supplementary Fig. 3b). Thus, while CII assembly status affected whole-cell bioenergetics and proliferation, CIIlow had little additional effect on mitochondrial bioenergetics and biogenesis.

Pyrimidine synthesis is attenuated in the presence of CIIlow. Having found no direct effect on bioenergetics, we reasoned that CIIlow could modulate adaptation to altered bioenergetic conditions. Thus, we first performed quantitative proteomic analysis in MDA231 sublines using SWATH-MS that resulted in identification and quantification of 1699 individual proteins (Supplementary Data 2). The most downregulated pathway in SDHBKOEV cells compared to parental cells was de novo pyrimidine synthesis (Fig. 4a). Quantification of the SWATH-MS data revealed that this downregulation is reversed in SDHBKOSDHAlow cells (Supplementary Fig. 4), indicating that SDHBKO cells may suppress anabolic processes to reduce the energy demand, and this is then relieved in SDHBKOSDHAlow cells. To further investigate this issue, we subjected parental, SDHBKO, and SDHBKOSDHAlow cells to RNAseq analysis (Supplementary Fig. 5a and b), which revealed significant clusters...
of genes either upregulated or downregulated in SDHB\textsuperscript{KO} cells with expression being reverted toward parental cell levels in SDHB\textsuperscript{KO}SDHAlow cells (Groups 1 and 2 in Supplementary Fig. 5b–f). Gene set enrichment analysis revealed the presence of catabolism-related processes in these clusters, including heterocycle catabolic process (GO:0046700), aromatic compound catabolic process (GO:0019439), organic cyclic compound catabolic process (GO:1901361), and cellular macromolecule catabolic process (GO:0044265) that were all downregulated in SDHBKOSDHAlow cells compared to SDHB KO cells (Supplementary Fig. 5e and f). However, the de novo pyrimidine synthesis pathway did not show significant enrichment, pointing...
to modest correlation of transcriptomic and proteomic data as previously reported\(^{29-33}\). Collectively, these data suggest that SDHB\(^{K^0}\) cells may upregulate catabolic and salvage pathways to compensate for defects in pyrimidine biosynthesis.

We next focused our studies on pyrimidine biosynthesis. Initially, we evaluated representative proteins of this pathway using WB, and found that the trifunctional polypeptide CAD (carbamoyl-phosphate synthase 2, aspartate transcarbamylase, glutamate dehydrogenase), DHODH, and CMPK1 may be altered in SDHB\(^{K^0}\) cells.
and dihydroorotase) and dihydroorotate dehydrogenase (DHODH) that catalyze the first four steps of de novo pyrimidine synthesis were attenuated in SDHBKO EV but recovered in SDHBKO SDHAlow cells (Fig. 4b–d). Re-expression of SDHB in SDHBKO cells also restored CAD and DHODH (Supplementary Fig. 2h).

We then analyzed pyrimidine nucleotide synthesis directly using stable isotope labeling and LC-MS/MS. The data in Fig. 4e and f, and Supplementary Fig. 7, show that biosynthesis of CTP, CDP, UTP, and UDP from major nutrients glucose and glutamine was low in SDHBKO EV cells but reversed variably in SDHBKO SDHAlow cells. These results document that compared to the baseline situation (fully assembled CII), formation of CIILow is linked to depressed de novo pyrimidine synthesis, which is reversed with its depletion. The data suggest that under low-energy conditions, CIILow may activate cellular processes reducing ATP-consuming pathways such as DNA synthesis to maintain energy balance, which is deregulated in SDHBKO SDHAlow cells.

**CIILow plays a role in cell cycle progression.** De novo pyrimidine synthesis is essential for maintaining the nucleotide pool for replication of DNA, thereby controlling cell cycle progression34–37. We thus analyzed cell cycle distribution in MDA231 sublines, which showed that SDHBKO and SDHBKO EV cells were arrested in the S-phase. However, SDHBKO SDHAlow cells partially recovered from S-phase arrest with cell cycle distribution similar to that of parental cells (Fig. 5a and b). Cell cycle is regulated by cyclins and cyclin-dependent kinases (CDKs). WB confirmed a marked decrease in the level of CDK6 as well as p16, phosphorylated histone H3 (H3H3), and cMYC in SDHBKO and SDHBKO SDHAlow cells compared to parental cells (Fig. 5c).

Interestingly, p18 was downregulated in SDHBKO and SDHBKO EV cells and recovered in SDHBKO SDHAlow cells (Fig. 5c), similar to that seen for steady-state levels of CAD and DHODH and for nucleotide synthesis (Fig. 4). Further, we observed near parental levels of pH3 in SDHBKO cells (Supplementary Fig. 2h). This result suggests that alteration in SDHA assembly status affects de novo pyrimidine synthesis, and consequently cell cycle progression. Our data suggest that under low-energy conditions, the shift of CII to CIILow is associated with a switch to bioenergetically less-demanding processes.

**CIILow supports growth of SDHB-deficient tumors.** Figure 2e reveals no difference in proliferation between SDHBKO EV and SDHBKO SDHAlow cells, suggesting that CIILow is not critical for proliferation under nutrient-rich conditions. However, imbalance of energy production and anabolic metabolism such as that linked to DNA synthesis could be critical for biomass production required for growth and survival under sub-optimal conditions38. To see whether CIILow is important for the tumorigenic potential of MDA231 sublines, migration of parental, SDHBKO EV, and SDHBKO SDHAlow cells was tested. Contrary to the cell proliferation assay (Fig. 2e), SDHBKO SDHAlow cells showed a lower rate of migration from serum-free media compared to both parental and SDHBKO EV cells (Fig. 5d). To extend these results to a pathologically relevant situation, we tested the capacity of the sublines to form tumors. Parental, SDHBKO EV, and SDHBKO SDHAlow cells were grafted subcutaneously into Balb-c nu/nu mice, and tumor progression was analyzed by ultrasound imaging (USI). Compared to parental cells, SDHBKO EV cells formed tumors with a delay of about 15 days and at lower rate, while SDHBKO SDHAlow cells failed to form tumors 60 days post grafting (Fig. 5e and f). To understand whether the inability of SDHBKO SDHAlow cells to form tumors is linked to their higher vulnerability under nutrient-poor conditions, we evaluated the level of cell death in individual sublines grown in galactose media. Supplementary Fig. 3c, d shows that all sublines are mostly viable in glucose-containing media, while SDHBKO SDHAlow cells are more vulnerable to cell death than SDHBKO cells in non-permissive, galactose-containing media. These data point to a link between CIILow and energy balance regulation to maintain cellular fitness under nutrient-poor conditions.

**CIILow modulates the metabolome.** In order to better understand metabolic consequences of alternative SDHA assembly, we examined the metabolic profiles of MDA231 sublines. Metabolomic data obtained by one-dimensional (1D) nuclear magnetic resonance (NMR) were combined with additional 16 metabolites, measured using targeted LC-MS/MS that are important in central energy metabolism but are not readily quantifiable by NMR (Supplementary Data 3). Results of this analysis, represented by the heat map in Fig. 6a, indicate an effect of CIILow on the differential metabolite profile in MDA231 sublines. The data show that the metabolic profile of SDHBKO EV cells has very little overlap with that of the parental cells, whereas SDHBKO SDHAlow cells have significantly more overlap. This is consistent with partial least square discriminant analysis (PLS-DA) of the metabolomic data showing the metabolic differences between sublines and the closer position of the SDHBKO SDHAlow cells to parental cells along the PLS1 axis (Fig. 6b). Taken together with the results in Fig. 1 which demonstrated CIILow formation during OXPHOS dysfunction, these data suggest that CIILow is linked to metabolic modulation when bioenergetics is compromised.

**Depletion of CIILow reverses accumulation of succinate.** Given the role of SDHA in the TCA cycle, we expect that CIILow could affect succinate metabolism and TCA cycle activity. We therefore subjected parental, SDHBKO EV, and SDHBKO SDHAlow cells to two-dimensional (2D) in-cell NMR analysis, which allows monitoring of metabolites in live cells in real time39. The approach detects metabolite levels as a function of time, as opposed to steady-state levels at a single time-point as reported above and in most metabolomics studies. This facilitates estimation of actual activities of metabolic pathways leading to particular metabolites in live cells. Thus, cells were incubated with [U-13C]glucose, and production of 13C isotope-containing pyruvate, lactate, alanine, and the TCA cycle intermediate succinate was monitored. As expected, parental cells differed in all assessed metabolites from the two sublines. SDHBKO EV cells showed increased intracellular levels of lactate and alanine compared to SDHBKO SDHAlow cells.
while fatty acid synthesis was not altered (Fig. 6c). Therefore, reduction of CII_{low} in SDHBKO/SDHAlow cells seems to induce shunting of pyruvate to pathways other than glycolysis without affecting fatty acid metabolism. Importantly, SDHBKO/SDHAlow cells had a higher level of succinate than parental cells, in agreement with previous reports for other SDHBKO models^{40-44}. However, SDHBKOSDHAlow cells showed reduced succinate levels compared to SDHBKO/SDHAlow cells (Fig. 6c), suggesting a CII_{low}-dependent change in succinate metabolism, which may be due to activation of an alternative metabolic route of succinate utilization.

CII_{low} negatively regulates anabolic activity of TCA cycle. To obtain further insight into succinate metabolism as well as metabolism of other TCA cycle intermediates, we monitored the fate of [U-13C] glucose and [U-13C] glutamine in MDA231 sublines using LC-MS. The first cycle of TCA metabolism of [U-13C] glucose via acetyl-CoA will generate four-carbon metabolites with two 13C nuclei (m+2 isotopomer) through oxidative decarboxylation (Fig. 7a-f). Alternatively, an m+3 isotopomer can be formed if glucose enters the TCA cycle via the pyruvate carboxylase (PC) pathway (Fig. 7a). In agreement with the above NMR data (Fig. 6c), we observed increased m+2 isotopomers of succinate in SDHBICO/SDHAlow cells, and this was reduced in SDHBKO/SDHAlow cells (Fig. 7d). Concurrently, the levels of m+2 isotopomers of aspartate, malate, and fumarate increased in SDHBKO/SDHAlow cells compared to SDHBKO/SDHAlow cells (cf. Fig. 7e-g), suggesting more efficient consumption of succinate in cells with lower levels of CII_{low}. The increase in m+3...
Fig. 6 CII<sub>low</sub> modulates metabolome of MDA231 sublines. a The heat map was constructed with the Z-score of peak intensities of each metabolite identified by both 1D NMR and LC-MS/MS from parental, SDHB<sup>KO</sup>EV, and SDHB<sup>KO</sup>SDHA<sup>low</sup> cells. The Z-score was obtained by dividing the difference between actual peak intensities and mean value by the standard deviation. b Score plot from partial least squares discriminant analysis (PLS-DA) on MDA231 sublines with the entire 1D NMR spectral data. PLS1 axis accounts for the major metabolic discrimination (63.5%), and the PLS2 16.8%. The symbols represent the mean score values from the multivariate analysis and the whiskers represent one standard deviation. Transitions are shown by arrows. c Real-time flux comparison in live parental, SDHB<sup>KO</sup>EV, and SDHB<sup>KO</sup>SDHA<sup>low</sup> cells. Time-dependent metabolic changes were obtained using 2D in-cell NMR metabolomics approach. The absolute quantification is based on the level of 13C carbon on a particular atom of metabolites detectable by NMR and was performed as described previously. Although it is not possible to differentiate pre-existing natural abundance metabolites with those derived from 13C-glucose with real-time NMR, the natural abundance of 13C is 1% and pre-existing metabolites should not make significant contributions to the quantitation. Fatty acid represents the aggregate level of the 13C-labeled CH<sub>2</sub> peak from free fatty acids at 1.36 and 32.0 ppm on the HSQC spectrum. Parental, black; SDHB<sup>KO</sup>EV, blue; SDHB<sup>KO</sup>SDHA<sup>low</sup>, red.
isotomer of aspartate and malate in SDHB^KOSDHAlow cells, but not that of fumarate (Fig. 7e–g), suggests that the PC pathway may also contribute to the four-carbon metabolites in these cells. [U-13C] glutamine feeds carbons into the TCA cycle via α-ketoglutarate. A subsequent oxidative decarboxylation in the TCA cycle generates the m+4 isotopomer of succinate that can lead to m+4 aspartate (Fig. 7h, j–n). Succinate and aspartate m+2 isotopomers can also be formed through the TCA cycle if the m+4 succinate condenses with acetyl-CoA, followed by oxidative decarboxylation in subsequent TCA cycles (Fig. 7h, green). [U-13C] glutamine can also contribute to formation of the m+3 isotopomer of aspartate via reductive carboxylation (Fig. 7h, red). In parental cell lines, we observed simultaneous oxidative decarboxylation and reductive carboxylation of glutamine as

![Diagram showing metabolic pathways](image-url)
shown by high levels of incorporation of [U-13C] glutamine into m+2, m+3, and m+4 aspartate isotoformers (Fig. 7n), consistent with reports on other cancer cells43–48. Significant formation of m+5 citrate isotoformomer also suggests reductive carboxylation in parental cells (Fig. 7i). We observed very high absolute incorporation of [U-13C] glutamine into m+4 succinate in SDHBKOEV and a significant decrease to about half in SDHBKO/SDHAlow cells (Fig. 7k). These were accompanied by higher incorporation of [U-13C] glutamine into m+4 aspartate and fumarate in SDHBKO/SDHAlow cells than SDHBKOEV cells (Fig. 7l and n). Similar patterns were observed for the m+2 succinate isotoformomer (Fig. 7k), although the absolute incorporation was much lower, probably due to a second round of the TCA cycle. These glutamine incorporation data clearly indicate that glutamine-derived succinate accumulates in SDHBKOEV cells, whereas it is converted to fumarate and ultimately to aspartate more efficiently in SDHBKO/SDHAlow cells.

Overall, both glucose and glutamine isotope incorporation experiments indicate a block in succinate metabolism in SDHBKOEV cells that is at least partially lifted when the CIIlow is depleted on the SDHBKO background (Supplementary Fig. 5a and c). This phenomenon is also consistent with the reverse trend of fumarate, whose level increased in SDHBKOSDHAlow cells (Supplementary Fig. 5b and d). This re-flow of succinate is suggested to induce redistribution of carbon atoms of glucose and glutamine to four-carbon metabolites leading to the recovery of synthesis of aspartate, a precursor for pyrimidine synthesis.

Previous studies showed that CII dysfunction caused by SDHB–SDHD mutations/deficiency results in accumulation of succinate24,40,42–44,49,50. Here we demonstrate that SDHA maintains high levels of succinate following CII dysfunction. Our data also implicate a switch of carbon metabolism in a CIIlow-dependent manner, mainly from succinate accumulation to anabolic reactions, including synthesis of aspartate to produce pyrimidines. Further investigations are needed to elucidate the enzymatic activity of CIIlow, especially in the context of succinate accumulation due to reductive carboxylation of glutamine (Fig. 7h).

CIIlow is a feature of SDHB-deficient paraganglioma. To assess the clinical relevance of our findings, we inspected tumor tissue from paraganglioma patients with SDHA and SDHB mutations, as well as sporadic paraganglioma patients. We assessed tumors from six patients for the presence of SDHA and SDHB using immunohistochemistry. Fig. 8a shows that samples from patients with mutated SDHB exhibit high levels of SDHA, while tumor tissue derived from a patient with SDHB mutation showed very low levels of both SDHA and SDHB proteins. Next, tumors of these patients were evaluated for CII assembly and for the level of de novo pyrimidine synthesis. The rate-limiting trifunctional CAD protein as well as cell cycle regulatory proteins were assessed. NBGE showed high levels of CIIlow and low levels of fully assembled CII in tumors with SDHB mutations, while tumors with SDHA mutations showed low levels of fully assembled CII and the absence of CIIlow (Fig. 8b). Further, SDHA-mutated paragangliomas lacked FAD, a cofactor of CII associated with the catalytic subunit SDHA, while it was detected in sporadic paragangliomas or those with SDHB mutations. Paragangliomas with high levels of CIIlow were found to contain low levels of CAD and p18 (Fig. 8c and d). These data are in agreement with results found for sublines of MDA231 cells with different CII assembly status (Fig. 2d; Fig. 4c; Fig. 5c). The data imply that CIIlow may vary under different (patho)physiological conditions, giving our findings clinical relevance.

Discussion

In the present study, we show that alternative assembly of CII fine-tunes cellular metabolic homeostasis to compensate for chronic bioenergetic stress. SDHA is known to exist in a complex with other subunits of CII, with an overall molar mass of 124 kDa, migrating at ~140 kDa22,23. We found an assembly form of SDHA migrating at ~100 kDa (designated here as CIIlow; Figs. 1 and 2), lacking SDHB and SDHC. This is consistent with previous studies using an experimental model of yeast, mammalian cell culture, and human pathological conditions showing that SDHA is stable in the absence of SDHB40–44,52, although its biological function has never been investigated. In addition, CIIlow is more prevalent when mtDNA is depleted or its expression is compromised (Fig. 1). This suggests biological relevance of CIIlow with mtDNA mutations and under conditions that limit mitochondrial energy production. We propose that CII/ CIIlow has a role in mitochondria similar to that of pyruvate kinase 2 (PKM2), which changes its assembly from a highly active tetrameric to low-active dimeric form in response to different cellular signaling pathways. A switch from the tetrameric to dimeric form of PKM2 has profound consequences for cellular metabolism as well as proliferation in the context of diabetic nephropathy53 and tumorigenic capacity of cancer cells54–56.

The molecular weight of SDHA is about 70 kDa and thus there are likely to be other SDH-interacting proteins in CIIlow of approximately 100 kDa. To identify SDHA-interacting proteins, we used immunoprecipitation followed by MS analysis. This analysis, supported by WB, identified the assembly factors SDHAF2 and SDHAF4 as proteins that together with SDHA constitute CIIlow accounting for its ~100 kDa mass (Supplementary Fig. 2). We have recently reported that SDHAF2 has a redundant function in relation to CII activity in MDA231 cells57. While we do not know the function of SDHAF2 and SDHAF4 in CIIlow, it is possible that these factors form a functional association with SDHA within CIIlow and in the absence of the other three subunits (SDHB–D). The role of SDHAF2 and SDHAF4 in CIIlow is the subject of current investigation.

To study the cellular function of CIIlow, we established stable cellular models with three different assembly forms of SDHA (Fig. 2) and initially analyzed bioenergetics difference of these sublines. We found that SDHBKO cells with prominent presence of CIIlow switched to low basal respiration with no spare respiratory capacity, indicating an energy stress situation. SDHBKO/SDHAlow cells with depleted CIIlow showed a bioenergetic pattern similar to that of SDHBKO cells (Fig. 3), indicating...
that CII\textsubscript{low} plays only a minimal role in mitochondrial bioenergetics.

Label-free proteomic and pathway analysis allowed for identification of differences in the de novo pyrimidine pathway in MDA231 sublines. We found that CII\textsubscript{low} abundance is inversely correlated with de novo pyrimidine pathway activity (Fig. 4), as exemplified by the steady-state level of CAD, a trifunctional and rate-limiting polypeptide in the pathway. The level of the CAD protein was reduced by approximately 60% in SDHB\textsubscript{KO} cells resulting in cell cycle arrest in the S-phase, and its re-expression in SDHB\textsubscript{KO}SDHA\textsubscript{low} cells with depleted CII\textsubscript{low} normalized cell cycle progression (Fig. 5). Recently, the de novo pyrimidine synthesis pathway has been shown to be essential in metabolic rewiring that modulates chemotherapy resistance of triple-negative breast cancer\textsuperscript{58} and other neoplastic pathologies\textsuperscript{59,60}, supporting the notion that de novo pyrimidine synthesis improves plasticity of cancer cells at the expense of increased energy consumption. Further investigations will be needed to provide unequivocal evidence for a functional linkage between CII\textsubscript{low} and pyrimidine biosynthesis. However, our data suggest that CII\textsubscript{low} exerts its protective function not by modulating mitochondrial bioenergetics (i.e., OXPHOS), but through other mitochondria-related mechanisms, which are not yet fully defined but seem to involve energy conservation strategies such as a reduction of the de novo pyrimidine synthesis pathway. Therefore, we propose that restriction of this pathway and possibly of other energy-demanding processes by CII\textsubscript{low} may provide a selective advantage under suboptimal nutrient conditions when bioenergetics is compromised, exemplified by increased cell death in galactose media and a failure to form tumors in a mouse xenograft model (Fig. 5) when SDHB-deficient cells lose CII\textsubscript{low}. In line with this, CII\textsubscript{low} is less abundant in optimal physiological conditions and becomes the dominant SDHA form of CII during sub-optimal energy production (Fig. 1).

Our data suggest that deficiency of CII assembly and the shift of SDHA to CII\textsubscript{low} modulates metabolism in response to compromised mitochondrial bioenergetics. It introduces checkpoints of cellular functions with high demands for energy, for example reducing formation of aspartate that is essential for pyrimidine nucleotide synthesis. This indicates that CII\textsubscript{low} might be a sensor of bioenergetic stress, providing a feedback from OXPHOS to central carbon metabolism. Similarly, a number of proteins

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**Fig. 8** CII assembly and de novo pyrimidine pathway in paragangliomas. a Histological examination of SDHA and SDHB expression from tumor tissue of paraganglioma patients with SDHA or SDHB mutations or with sporadic paragangliomas. Tissues were processed for H&E staining and immunohistochemistry using SDHA and SDHB IgGs. Representative images are collected using a 40× objective. Scale bars represent 100 μm. b–d Paraganglioma tissues, as shown, were assessed by WB following protein separation by NBGE for CII assembly using anti-SDHA IgG (b), and by SDS-PAGE for CAD and FAD (c) and for markers of cell cycle (d). Images are representative of three independent experiments.
capable of sensing mitochondrial energy production or metabolite levels that reflect energy status have been identified. AMP-activated protein kinase (AMPK) has been shown to sense the ratio of ATP to ADP and AMP level in order to initiate an energy compensating stress response. Previous studies have also demonstrated that certain sirtuins are sensors of organellar energy production in relation to the NADH/NAD++ ratio. Recently, the ubiquinol/ubiquinone ratio has been reported to sense mitochondrial energy production linked to the respiratory status. Possible links between CII assembly forms and other sensors of mitochondrial bioenergetic dysfunction need to be further investigated. Merging these various individual components into a highly integrated regulatory system presents the next challenge.

Data shown here reveal that patients with SDHB and SDHA mutations have different CII assembly status. We found that CIIlow is the remaining unit of CII present in patients with mutations that have different CII assembly status. We demonstrated that certain sirtuins are sensors of organelle energy homeostasis. Thus, our data provide more detailed insights into rare, possibly due to an imbalance in energy and metabolic marker.

siRNA transfections. Mouse Scleron-select pre-designed SDHA, SDHB, and SDHC-targeting siRNAs and non-silencing controls were obtained from Life Technologies. The sequences are as follows: SDHA target sequence-1 (s414416): GGA ACA CUC AAA CAA CAG ATT; SDHA target sequence-2 (s211850): CCA GGU AUU UGG AGG AUA ATT; SDHB target sequence-1 (s205969): GCC UUA AUC AAG AUC AAG ATT; SDHB target sequence-2 (s205970): CCC UCC UCC ACA UAU GUA UTT; SDHC target sequence-1 (s24767): GAU CUA CUG GCC UAA GGU UU; SDHC target sequence-2 (s20571): GAC CAG CAC GUA UAC CGG UTT. 4T1 cells were transiently transfected with 20 nM siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer’s instructions and harvested for analysis 72 h after transfection.

Cell cycle analysis. Cells were harvested with trypsin, rinsed with PBS, fixed by drop-wise addition into 70% (v/v) ethanol and kept at −20 °C for at least 4 h. They were then centrifuged, rinsed in PBS, re-suspended in PBS containing 80 μg/ml RNase and 30 μg/ml of propidium iodide plus 100 μg/ml RNase A. After incubating for 30 min at room temperature, DNA content was assessed using the FACScalibur flow cytometer (Becton Dickinson). Cell cycle distribution was estimated using the Flowjo software and the Watson distribution model.

Western blotting. Cells were lysed in 20 mM Tris (pH 8), 200 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP-40, and 10% glycerol supplemented with protease inhibitors (Roche). After addition of the Laemmli solution to the samples, the proteins were separated by SDS-PAGE (8–15% gel) and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% non-fat milk, the blots were incubated with the primary antibodies overnight at 4 °C. The membranes were then treated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature, followed by visualization (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The following antibodies were used in the study: SDHA (Abcam, ab14715; 1:2000); SDHB (Abcam, ab14714; 1:1000); SDHC (Abcam, ab15599; 1:1000); SDHB (Abcam, ab137692; 1:200); and Cell Signaling (Dox) (Abcam, ab110262, 1:2000); NDUF9 (Life Technologies/Thermo Fisher, 49100; 1:2000); Core I/UQCRC1 (Life Technologies/Thermo Fisher, 45910; 1:2000); NDUFV (Origab, ab5535; 1:1000); NDUFAS (Abcam, ab17936; 1:1000); SDHAF2 (Cell Signaling, 58489; 1:1000); SDHAF4 (Thermo Fisher, PAS-73014; 1:1000); or Sigma, HPA018284; 1:500); FAD (Rainin/BioSource, MBS2185813; 1:500); HRP-conjugated tubulin (Thermo Scientific, MA1-63086-HRP; 1:2000); HRP-conjugated heparin (Abcam, ab9990; 1:5000); and actin (Abcam, ab14715 or 3700, or Cell Signaling, 4970 or 3700; all 1:1000); DHODH (Proteintech, 14877-1-AP; 1:500); CAD (Cell Signaling, 93925; 1:1000); phospho-Histone H3 (Ser10) (Cell Signaling, 3642; 1:1000); GADD45 (Cell Signaling, 8337; 1:1000). Uncropped western blots are in Supplementary Fig. 8.

Isolation of mitochondria and NBGE. Mitochondria were isolated using Dounce or Balch homogenizers, followed by standard differential centrifugation. Experimental procedure and antibodies for NBGE were used as described previously.

Methods

Cells and transfection. MDA231 and MCF7 cells were obtained from the ATCC and cultured in DMEM (Life Technology) supplemented with 10% FCS and antibiotics plus antymycotics (Gibco). To generate SDHBKO cells, and cultured in DMEM (Life Technology) supplemented with 10% FCS and antibiotics plus antymycotics (Gibco). To generate SDHBKO cells, we transfected the pMDM2-neo plasmid. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions; transfectants were selected with ganciclovir. Two shRNAs targeting the human SDHA gene and empty vector (EV) were purchased from OriGene. SDHBKO cells were transfected with the shRNA plasmids. After 36 h, cells were treated for 5 days with 0.8 µg/ml puromycin. Preparation of mdDNA-depleted cells (4T1 sp and MCF7 sp) was accomplished by their long-term incubation with 50 µM EthB. Human SDHB was re-expressed in SDHBKO cells from the pLYS5-SDHB-Flag plasmid (Addgene # 50055, a kind gift of Vamsi Mootha) using lentiviral transduction. Lentivirus particles were produced from 293FT cells using second generation packaging plasmids and Lipofectamine 2000 plasmids and lipofection (Lipofectamine 3000, Invitrogen). Virus-containing media were collected after 48 h, centrifuged at 3000× g for 15 min and stored at −80 °C.

Proliferation, migration, and cell death assays. Parental, SDHBKO, and SDHBKO-Flag cells were plated in a 24-well dish in DMEM. At indicated times, cell proliferation was assessed by crystal violet assay using a standard protocol. xCELLigence Real-Time Cellular Analysis system was used to evaluate cell proliferation in glucose- or galactose-containing medium and using dialyzed FBS (Gibco). For this, cells were seeded in 16-well E-plates (4 replications per cell type) as recommended by the manufacturer. For migration assays, cells were transfected to the CIM-16 plates and allowed to migrate toward FBS placed in the other compartment. Cell proliferation was measured using the xCELLigence instrument for 50 h, and the cell index was recorded every 5 min. Cell death was assessed using the standard annexin V/propidium iodide (PI) method.
Xenograft experiments. All procedures with animals were performed according to the Institutional guidelines and ethical authorization by the Griffith University Animal Ethics Committee. MDA231 sublines were injected into the mammary fat pad of Balb-c nu/nu mice at 10^6 cells per animal. Tumor volume was quantified using the Vevo3100 USI system using a 30-μm resolution scan-head. To generate a sample specific spectral library for SWATH analysis, the fractionated sample was run on an LC-MS-MS system at a 150 m/z range. Peptides were identified using ProteinPilot (v4.2) (Sciex) to search the uniproT Human protein database (20,198 entries, downloaded June 2015) against the Paragon group of functional and protein expression (protein expression - 1) using PeakView software 2.1. The SWATH MicroApp 2.0 was used to generate a sample specific spectral library.

To extract SWATH peak areas with PeakView software 2.1, we carried out retention time calibration with endogenous peptides and data processing following the following settings: 100 maximal peptides per protein, 6 transitions per peptide, peptide confidence threshold of 99%, transition false discovery rate < 0.1%, 10 min per transition window, and fragment extraction tolerance of 75 ppm, exclusion of shared peptides. The protein peak areas were normalized to the total peak area and log-transformed peak areas and subjected to Student’s t-test to compare relative protein peak area between samples. Proteins were considered to be differentially expressed with p < 0.05 and protein fold change ±1.5 fold. DAVIP was used to check the Benjamini method (adjusted p value) was used to control the family-wise false discovery rate for enrichment analysis.

Identification of SDHA-interacting proteins. Mitochondria isolated from SDHBKO cell stably expressing SDHA-FLAG and empty vector were lysed using membrane solubilization buffer (1% n-dodecyl β-D-maltoside, 20 mM Tris-HCl, pH 7.4, 1 mM NaCl, 10 mM NaH2PO4, 5 mM Na2HPO4, 10% glycerol) supplemented with protease inhibitors (Roche). Protein concentrations were determined using the BCA protein assay kit. Equal amounts of protein were incubated with 20 μl anti-FLAG M2 agarose beads (Sigma) overnight at 4°C. Beads were washed three times with the membrane solubilization buffer. The proteins bound to the beads were then eluted with 2x SDS lysis buffer and separated by 10% SDS-PAGE followed by silver staining (Mass spectrometry compatible, Life Technologies). Protein bands from control and treatment lanes were subjected to tryptic digest and analyzed by mass spectrometry.

Sample preparation for NMR spectroscopy and LC-MS. Metabolites were extracted from parental, SDHBKO-ΔV, and SDHBKO-ΔV-HA cells (3 x 10^6 cells) with 400 μl of mixture composed of methanol, acetonitrile, and distilled water (5:3:2). The samples were centrifuged at 15,000g for 20 min at 4°C. The supernatant was collected, divided into two portions at the ratio of 1:5 for LC-MS and NMR analysis, and dried with a vacuum centrifuge (V). The pellets for LC-MS were dissolved with 30 μl mixture of HPLC-grade acetonitrile and water (1:1); and those for NMR analysis were suspended in 500 μl D2O (pH 7.0). The samples were centrifuged at 12,000g for 20 min at 4°C. The supernatant was collected, divided into two portions at the ratio of 1:5 for LC-MS and NMR analysis, and dried with a vacuum centrifuge (V).

Measurement and analysis for NMR spectroscopy and LC-MS. Untargeted metabonomic profiling was performed using NMR, and targeted profiling by LC-MS multiple reaction monitoring for metabolites that are not readily discernible by NMR. Metabolites detected by both methods, such as succinate and glutamate, exhibited consistent results. 1D NMR spectra were obtained using a 500-MHz Bruker Avance spectrometer equipped with a cryogenic triple resonance probe (KBSI). For the pulse program, 'noesygrpd' was used with 64 scans, and the final spectra were reconstructed to 16K points. The metabolites were identified with Chenomx spectral database (Edmonton, Alberta, Canada) and comparison with standard compounds. The data obtained were processed using an LC-MS high-performance linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization source. The operating conditions of the mass spectrometer were as follows: 5 kV of ion spray voltage, heated capillary temperature of 275°C, and sheath gas (nitrogen), auxiliary gas (nitrogen), and sweep gas (nitrogen) pressures of 35arb, 5arb, and 2arb, respectively. MS/MS scan- ing analyses were performed in the range of m/z 85–1000, and a 35-nM-precursor ion selection was used for MS/MS. Mobile phases were 10 mM ammonium carbonate (pH 9.1) in distilled water (A) and acetonitrile (B), and the flow rate was 0.5 ml/min. The gradient scheme was as follows: 80% A at 0 min, 80% A at 10 min, 5% A at 12 min, 5% A at 25 min, 80% A at 25.1 min, and 80% B at 35 min. The Zic-Phlic Polymeric Peaks Column (150 mm x 2.1 mm, 5 μm; Merck) was used at 35°C, and the autosampler temperature was set at 4°C. The peak areas of parental ion and its isotopologues were measured using Xcalibur (Thermo Fisher Scientific). The metabolites were identified by m/z values and MS/MS fragmentation patterns from Human Metabolome Database (HMDB) and METLIN databases and also by comparison with standard compounds. For multivariate analysis, the NMR data were Fourier-transformed, phase-corrected, and baseline-corrected manually using MestReNova (Mestrelab Research, Santiago de Compostela, Spain). The signal intensities were normalized against the intensity of the 0.025% 1H TSP signal at 0 ppm and total area values. The processed NMR data were imported into SIMCA-P software (Umetrics) for PLS-DA. The heat maps were constructed with a relative Z-score of peak area of each metabolite identified by NMR and LC-MS.

RNA sequence analysis. RNA was isolated using RNAzol (Molecular Research Center) according to the manufacturer’s instructions. RNA was dissolved in the TE buffer (20 mM Tris-Cl, pH 8) and the RNA contamination was removed using DNase I (Sigma-Aldrich) for 30 min. To clean RNA, the same volume of 8 M LiCl (Sigma-Aldrich) was added and incubated overnight at −20°C. Samples were centrifuged for 30 min at 16,100g at 2°C, the supernatant was removed and the pellet washed twice in 80% ethanol, followed by centrifugation as above. The remaining ethanol was evaporated at 65°C for 10 min, and RNA was dissolved in 20 μl of TE buffer. Total RNA concentration was measured by Nanodrop 2000, and its quality was assessed by the Fragment Analyzer (Advanced Analytical) using Standard Sensitivity RNA Analysis Kit (Advanced Analytical). The libraries were prepared from 2 μg of total RNA using QuantSeq 2’RNA-Seq Library Prep Kit for Illumina (Lexogen), pooled and sequenced on the MiSeq v3 kit in the 150 bp SE mode.

About 2 million reads per sample were obtained, and low-quality reads were filtered out using Trimmomatic (v. 0.36) with parameters "trimmomatic/TRIM_MD_A4_1_sequence.fastq CROP:165 HEADCROP:12 ILLUMINACLIP: ~30:" and "trimmomatic/SLIDINGWINDOW:4:15 MINLEN:36." The reads were then normalized across cell lines by dividing the number of clusters set to ten83. Clusters that showed similar expression profiles were chosen as DEGs. The function "plotPCA" from DESeq2 package with default parameters was used for PCA. The heat map was generated using the heatmap.3 (v 0.8.20). Full-size images were used for functional enrichment analysis. The Benjamini method (adjusted p value) was used to control the family-wise false discovery rate for enrichment analysis.

For gene set enrichment analysis, DEGs were filtered and used for clustering and ontology analysis. The normalized counts of these genes for all biological replicates and cell lines (parental–MDA231, SDHBKO–SHB, and SDHBKO-SDHA–SHB) were used to analyze patterns of expression across the three cell lines. The data were first normalized across all genes by dividing the mean of the replicates by the sum of the mean of the replicates. The R package optCluster (R ver. 3.4.3) was then used to determine the best clustering algorithm among agnes, clara, diana, hierarchical, kmeans, pam, and sota clustering methods using the validation methods connectivity, Dunn index and silhouette width, and the number of clusters set to 10. The resulting clusters were then merged.

Gene ontology was performed on the genes from each unique cluster, using the R package qgoefr, to identify significantly (p-value < 0.05) enriched GO terms. The genes were analyzed with no specified ranking while the gscs (Set Counts and Statistical锦) were used to sign the enrichment. The annotated enriched genes from the RNASEq were used as background. GO annotations inferred by in silico methods were included in the analysis.
After the genes were linked to their appropriate GO terms, REVIGO was used to cluster similar GO terms together while using the p-value output from goploter to create a ranked list. A similarity of 0.7 using the SimRel semantic similarity method was used to group similar GO terms and the UniProt database (15 March 2017) was used to find the relevant GO term sizes. Output from REVIGO was visualized as treemaps.

2D in-cell NMR analysis. Six plates of 70% confluent cultured cells were harvested with centrifugation. After re-suspending the cells with 5 ml DPBS, the cells were counted and 3 x 10^7 cells were transferred into a fresh tube. After centrifugation, the harvested cells were re-suspended in 500 μl glucose-free DMEM media (Gibco) supplemented with 10% dialyzed FBS, 25 mM U-13C6-labeled glucose, and 10% D2O. The cells were spun in an NMR (30 g, 100 s) to allow sedimentation, enough to cover the active region of the NMR detection coil. H1–13C Heteronuclear Single Quantum Coherence (HSQC) NMR spectra were measured using the 800-MHz Bruker Avance spectrometer equipped with a cryogenic triple resonance probe (Seoul National University). The dataset comprised 1024 x 128 points for both the indirect and direct dimensions, respectively. The time course spectral measurement was obtained at 310 K for 24 time points, with each experiment lasting for 288 s (13 points). Each metabolite was identified by spiking the standard compound.

Transmission electron microscopy. TEM was accomplished as follows. Cells were grown on cover-slips, fixed with 2% glutaraldehyde (Agar Scientific) and post-fixed with 1% OsO4, made up in Sorensen’s phosphate buffer (0.1 M, pH 7.2–7.4), dehydrated, and embedded in Epon-Durcupan (Sigma-Aldrich). Ultrathin sections (~70–90 nm) were cut, contrasted with uranyl acetate (Agar Scientific), and examined in the Morgagni 268 transmission electron microscope (FEI) at 80 kV and in the Tecnai G2 20 LaB6 electron microscope (FEI) at 200 kV. Images were captured with Mega View III CCD camera (Olympus Soft Imaging Solutions).

Paraganglioma patients. Tumor tissue from 6 patients (3 with SDHB mutations, 1 with SDHA mutation, 2 sporadic) was used in this study. Tumor tissue was obtained during surgery at the National Institutes of Health (NIH). Genetic testing was performed by NIH in collaboration with the Mayo Clinic (Rochester, MN, USA) or the results were sent from external facilities to the NIH. Expression of SDHB and SDHA proteins was also evaluated by immunohistochemistry as previously described. Signed written consent was obtained from all patients prior to any experimental work. The use of patient samples was allowed by the Eunice Kennedy Shriver NICHD IRB (Ethics Approval #00-CH-0093). The tumor samples examined were as follows (Sample code, affected subunit, DNA base pair change, nonviability of cells with oxidative defects in galactose medium: a screening test for affected patient fibroblasts). Each metabolite was identified by spiking the standard compound.

Statistical evaluation. Unless stated otherwise, data are mean values ± SD of at least three independent experiments. In mouse experiments, groups of 6 animals were used, unless stated otherwise. The two-tailed t-test was used to assess statistical significance with p < 0.05 being regarded as significant. Images are representative of three independent experiments.

Data availability. RNA sequence data are filed in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE108938. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009656. All other data that support findings of this study are available from the corresponding authors upon request.

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Author contributions
A.B.-G. and J.N. conceived the project and designed the study. A.B.-G. performed the majority of the experiments. H.W. and S.P. designed and performed metabolomics study and analyzed the data. V.C., Y.P., and K.P. provided patient data and samples, and they performed H&E and ICH analysis. I.K., K.V., R.Z., S.M.N., and S.B. performed the respiration studies, WB, and SDHB re-expression. M.S. and P.H. performed TEM analysis. T.Z. and M.P.M. performed SWATH-MS and bioinformatics analysis. L.D., B.Y., J.V., J.R., S.Z., and M.V.B. contributed reagents, materials, analytical tools, technical support, and ideas. P.A., R.N., R.S., S.M.N., and M.K. performed RNA sequence analysis and interpreted the data. A.B.-G., J.R., and J.N. wrote the manuscript. All authors discussed the results and edited the manuscript.

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