Differential requirements for mitochondrial electron transport chain components in the adult murine liver.

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Summary

Mitochondrial electron transport chain (ETC) dysfunction is a common cause of metabolic disease in humans, but the mechanisms underlying tissue specific phenotypes are not understood. Complex I (cI) is classically considered the entry point for electrons into the ETC, and *in vitro* experiments indicate that cI is required for maintenance of the NAD⁺/NADH ratio, an indicator of cellular redox status. This finding has largely not been tested *in vivo*. Here, we report that mitochondrial complex I (cI) is dispensable for homeostasis of the adult mouse liver; animals with hepatocyte-specific loss of cI function display no overt phenotypes or signs of liver damage, and maintain liver function and redox status. Further analysis of cI-deficient livers did not reveal significant proteomic or metabolic changes, indicating little to no compensation is required in the setting of complex I loss. In contrast, complex IV (cIV) dysfunction in adult hepatocytes results in decreased liver function, steatosis, and liver damage, accompanied by significant metabolomic and proteomic perturbations. Correspondingly, we find that complex I-deficient livers are reliant on an alternative pathway, whereby electrons are donated to the ETC via dihydroorotate dehydrogenase, to maintain redox status. Our results support a model whereby complex I loss is tolerated in the mouse liver because hepatocytes make use of alternative electron donors to fuel the mitochondrial ETC.

Introduction

Mitochondrial ETC disease in humans exhibits a striking tissue specificity, whereby mutations in distinct ETC components are causative for specific and non-overlapping syndromes (1). As an example, genetic dysfunction of mitochondrial complex I (cI) is associated with neurological disease including Leigh syndrome (characterized by severe loss of motor abilities, necrotizing brain lesions and early death), and Leber Hereditary Optic Neuropathy (characterized by bilateral loss of vision in early adulthood due to degeneration of the optic nerve) (2-4). The mechanisms underlying sensitivity of the nervous system, with sparing of other organ systems, in the setting of complex I loss is not understood. In contrast, mutations in other mitochondrial ETC components have been associated with multi-organ diseases, and the basis for tissue specificity in those diseases is also unclear (5).

Liver involvement is a common component of mitochondrial diseases, present in approximately 20% of patients (6, 7), as well as being associated with specific syndromes caused by mutations in distinct ETC-related components (8-12). Common findings in affected patients include hepatomegaly, steatosis, cholestasis and additional metabolic abnormalities associated with liver failure (12-15). Genetic analysis of patients has revealed mutations in genes which influence multiple components of the ETC (e.g., *POLG*, *LARS*, *DGUOK*) (10). With respect to single-complex diseases, mutations in *SCO1*, an assembly protein for mitochondrial complex IV (cIV), and mutations in *BCS1L*, a chaperone protein for mitochondrial complex III, have been associated with liver failure (8, 16, 17). Interestingly, single-complex diseases associated with other components of

the ETC (complex I and complex II) do not typically present with liver failure, despite their placement directly upstream of cIV.

The ETC functions to transfer electrons (from the oxidation of nutrients) to O₂, as a final electron acceptor; this process is coupled to the formation of an electrochemical gradient across the mitochondrial inner membrane and the production of ATP. Mitochondrial complex I is the largest protein complex of the ETC, and is commonly considered the primary entry point for electrons into the ETC: In cultured cells, cI is required for maintenance of cellular redox status (assessed by the NAD⁺/NADH ratio) via its role in NADH oxidation, and subsequent electron transfer to ubiquinone (CoQ) (18). Complex I is composed of 14 core subunits, plus an additional 31 accessory subunits (19). Published mouse models of complex I deficiency have largely focused on accessory subunits, including deletion of *Ndufs4*, *Ndufs6*, and *Ndufa5*, which result in neurological and cardiac phenotypes associated with partial loss of complex I function (20-22); no liver phenotypes or metabolic perturbations were reported in these mice (23). A recent report conditionally deleted *Ndufs3*, a core subunit of cI, in skeletal muscle, resulting in progressive myopathy associated with significant reduction of cI activity (24). Thus, to date, mouse models have supported an essential role for cI *in vivo* in the central nervous system and muscle. Here, we report that deletion of the core subunit, *Ndufa9*, results in complete loss of cI function, but is well-tolerated in the adult mouse liver. Further analysis reveals intact liver function in *ndufa9*^{-/-} animals, which is not accompanied by significant metabolic alterations or changes in redox status. This is in contrast with hepatocyte-specific deletion of the complex IV component *cox10*, which results in altered redox status and impaired liver function. These results support a model in which cI is not an essential

upstream component of the ETC in adult mammalian liver, in contrast to the conventional
view of cl function.

RESULTS

Mitochondrial complex I function is dispensable for homeostatic function of adult murine hepatocytes

To understand how liver cells respond to loss of complex I, we made use of a conditional allele of the core subunit *Ndufa9*. *Ndufa9* deletion in mouse fibroblasts results in loss of mitochondrial respiratory function, as well as a significant drop in the NAD⁺/NADH ratio (Figure 1 – figure supplement 1A,B), indicating that *Ndufa9* is critical for redox status in these cultured cells. We depleted *Ndufa9* in livers of adult mice, making use of adeno-associated virus 8 (AAV8)-mediated Cre recombination driven by the liver specific TBG promoter in *ndufa9^{flox/flox}* animals (see Methods). *ndufa9^{flox/flox}* adult animals were injected with control AAV8-GFP virus (hereafter “*ndufa9^{f/f}*”) or AAV8-Cre virus (hereafter “*ndufa9^{-/-}*”). At 4 weeks post-AAV administration, AAV-Cre injected animals exhibited loss of *Ndufa9* mRNA and protein in hepatocytes, as well as loss of mitochondrial oxygen consumption (Figure 1A-C). At this time point, *ndufa9^{-/-}* livers displayed no differences in gross physiology (Figure 1D) or liver/body weight ratio (Figure 1F). Histological analysis of liver sections revealed no significant pathology, including no evidence of steatosis (Oil Red O staining) or glycogen loss (PAS staining) (Figure 1E). Liver function was maintained in *ndufa9^{-/-}* livers as indicated by unaltered levels of fasting glucose (Figure 1G) and circulating indicators of liver function and damage (Figure 1H,I). Similar results were observed in mice at eight weeks post-AAV injection (Figure 1 – figure supplement 1C-G). Thus, complex I loss is well-tolerated in adult mouse hepatocytes under homeostatic conditions, with no discernable effects on tissue function, or evidence of tissue damage.

Complex I inhibition is often associated with elevated reactive oxygen species (25, 26). In *ndufa9*^{-/-} livers, we observed a small decrease in the reduced to oxidized glutathione (GSH / GSSG) ratio, indicating increased oxidative stress in the setting of complex I deficiency (Figure 2A). Surprisingly, we did not observe altered NAD⁺/NADH or NADPH/NADP⁺ ratios in *ndufa9*^{-/-} liver (Figure 2B). We additionally examined compartment specific redox status by calculating pyruvate / lactate and acetoacetate / β -hydroxybutyrate ratios, which are indicative of cytosolic and mitochondrial redox status respectively; neither compartment display evidence of altered redox status (Figure 2C). A recent report suggests that circulating α -hydroxybutyrate levels serves as a marker of hepatic redox stress (27). *ndufa9*^{-/-} animals did not display alterations in tissue or plasma α -hydroxybutyrate levels (Figure 2D). Thus, complex I function is not required for maintenance of hepatic redox status, in contrast to its established role in cultured cells (Figure 1 – figure supplement 1A,B) (18).

To examine potential compensatory mechanisms that may be enabled in the setting of complex I inhibition, we assessed changes in the proteome and metabolome, comparing *ndufa9*^{fl/fl} and *ndufa9*^{-/-} whole livers. Our proteomics analysis detected 3353 proteins with high confidence; however, only 6 proteins were significantly upregulated ($\log_2(\text{fold change}) > 1$, Adjusted p-val < 0.05), and 20 proteins were significantly downregulated ($\log_2(\text{Fold Change}) < -1$; Adjusted p-val < 0.05) (Figure 2 – figure supplement 1). Of the down-regulated proteins, the majority were components of complex I (Figure 2E), consistent with destabilization of cI in the setting of Ndufa9 subunit loss. In addition, we did not detect significant changes in total mitochondrial content in *ndufa9*^{-/-} livers, as indicated by measurement of mitochondrial genome and protein content (Figure

2F,G). We assessed metabolism in livers of fasted mice, using LC/Q-TOF mass spectrometry based metabolomics. Steady-state targeted metabolomic analysis of >150 metabolites revealed only a single statistical metabolite difference (Figure 2H, Figure 2 – figure supplement 1), and no significant changes in mitochondrial TCA cycle metabolites (Figure 2I). Together, these results indicate that complex I is largely dispensable for homeostatic function in the adult mouse liver, without significant metabolic or proteomic compensation.

Mitochondrial complex IV is required in adult hepatocytes.

To further explore a role for the mitochondrial ETC, we examined the requirement for complex IV, making use of a *cox10^{fllox}* allele (28). Cox10 is a required assembly component of complex IV, and Cox10 deletion in skeletal muscle is associated with diminished cIV activity accompanied by myopathy (28). A previous report utilizing this allele in combination with the liver-specific Albumin-Cre transgenic reporter revealed partially penetrant phenotypes dependent on the transgene dosage (29), suggesting that the mitochondrial ETC is required for liver homeostasis. Thus, we made use of AAV-delivery of Cre recombinase to *cox10^{ff}* animals to investigate its role in the adult liver. Similar to above, we injected *cox10^{ff}* animals with AAV-GFP or AAV-Cre and characterized liver physiology at 4 and 8 weeks post-injection. At 4 weeks post-injection, AAV-Cre injected animals demonstrated significant reductions in Cox10 mRNA and protein (Figure 3A,B) and complex IV activity (Figure 3C). By 8 weeks post-AAV administration, *cox10^{-/-}* livers were grossly enlarged and pale in color, as compared with wild-type livers (Figure 3D,F). Histological analysis of liver sections revealed widespread

lipid accumulation indicating hepatic steatosis (Oil Red O staining), as well as decreased glycogen accumulation (PAS staining) (Figure 3E). Correspondingly, fasting glucose levels were decreased in animals with *cox10*^{-/-} livers (Figure 3G). To directly probe gluconeogenesis, fasted mice were challenged with a [U-¹³C]-lactate/pyruvate tolerance test; animals with *cox10*^{-/-} livers were unresponsive as compared with wild-type animals (Figure 3H,I, Figure 3 – figure supplement 1A). Additional plasma indicators of liver function were diminished in *cox10*^{-/-} animals, including decreased plasma total protein and increased plasma bilirubin (Figure 3J). We also observed significant elevations in ALT and AST, suggestive of liver damage (Figure 3K). Thus, in contrast to loss of complex I, the loss of complex IV in adult hepatocytes is associated with hepatic steatosis, diminished liver function and evidence of liver damage.

cox10^{-/-} livers exhibited increased oxidative stress (lowering of the GSH / GSSG ratio), consistent with mitochondrial ETC dysfunction (Figure 4A). In contrast to *cl*-deficient livers, *clV* deficiency significantly impacted the redox status of the cell, as measured by the NAD⁺ / NADH ratio (Figure 4B). We observed altered ratios of both pyruvate / lactate and acetoacetate / β -hydroxybutyrate, indicating impaired redox status in both the cytosol and mitochondrial compartments (Figure 4C). Interestingly, assessment of tissue and plasma α -hydroxybutyrate levels, revealed decreases in the setting of *clV* loss. Thus, impairment of mitochondrial complex IV has both biochemical and functional consequences in the adult liver, and is acutely required for maintenance of cellular redox status.

To probe the effects of complex IV loss in mammalian liver, we assessed proteome changes between *cox10*^{ff} and *cox10*^{-/-} livers (Figure 4E, Figure 4 – figure supplement 2).

A large number of proteins were significantly up-regulated, and this group was enriched for proteins that localize to mitochondria (Figure 4E). Consistent with this, we observed significant increases in total mitochondrial content in *cox10*^{-/-} livers, as measured by both mitochondrial genome and protein content (Figure 4F,G). We identified a number of complex IV components among the down-regulated proteins (Figure 4E), consistent with destabilization of intact complex IV. In addition, gene-ontology analysis of down-regulated proteins revealed enrichment of several metabolic pathways (Figure 4 – figure supplement 1A, Figure 4 – figure supplement 2), indicating extensive metabolic compensation in mutant livers.

Steady-state metabolomic analysis of *cox10*^{ff} and *cox10*^{-/-} livers revealed significant alterations in a subset of metabolites (Figure 4H, Figure 4 – figure supplement 2). Levels of mitochondrial TCA cycle metabolites were largely maintained, although we observed a significant increase in 2-hydroxyglutarate levels, which has been previously observed in the setting of mitochondrial dysfunction (Figure 4I) (30). We observed significant upregulation in cholate species, consistent with cholestasis and hepatomegaly in these animals (Figure 4H, Figure 4 – figure supplement 1C). Upregulated metabolites were also highly enriched for long-chain acylcarnitine fatty acid species, consistent with a fatty acid oxidation defect (Figure 4H, Figure 4 –figure supplement 1B). Concomitantly, we observed a significant decrease in β -hydroxybutyrate levels, a byproduct of fatty-acid oxidation (Figure 4J). Interestingly, downregulated metabolites were enriched for several electron carriers, including pyruvate, ascorbate and tetrahydrobiopterin (Figure 4H,J), suggesting that the mitochondrial ETC may be interacting with electron transfer from multiple sources.

DHODH activity maintains cellular redox status in the absence of mitochondrial complex I.

The phenotype of *cox10*^{-/-} livers noted above indicates that the mitochondrial ETC is required for liver homeostasis. However, the incongruence of phenotypes between *cl* and *clV* hepatic deficiency led us to consider alternative routes for electrons to enter the mitochondrial ETC, including complex II and DHODH (Figure 5A). Dihydroorotate dehydrogenase (DHODH) is highly expressed in the mammalian liver, localized to the mitochondrial inner membrane, and transfers electrons from dihydroorotate to coenzyme Q as part of the pyrimidine biosynthesis pathway (Figure 5A). Assessment of metabolites in this pathway revealed that levels of a subset of these metabolites (including aspartate, UDP-glucose and thymidine) were altered in the setting of *clV*-deficiency (Figure 4K). In contrast, we did not observe perturbations in succinate levels or TCA cycle metabolites in *cox10*^{-/-} livers (Figure 4I).

By donating electrons directly to coenzyme Q, DHODH activity offers a route to bypass *cl* and maintain ETC activity (Figure 5A). In complex I deficient livers, DHODH levels were not altered and metabolites in the pyrimidine synthesis pathway were not perturbed (Figure 5B,C). We therefore treated *ndufa9*^{ff} and *ndufa9*^{-/-} animals with brequinar, a DHODH inhibitor. DHODH inhibition is associated with hepatotoxicity in humans; however, we used a dosage which did not affect liver physiology (Figure 5 – figure supplement 1A-D), based on a previous protocol (31). Brequinar treatment increased dihydroorotate levels in both *ndufa9*^{ff} and *ndufa9*^{-/-} livers, indicating that DHODH activity was inhibited (Figure 5D). In vehicle-treated animals, NAD⁺ / NADH ratios

were not significantly altered by cl loss. However, in the setting of DHODH inhibition, complex I loss significantly lowered the NAD⁺ / NADH ratio (Figure 5E). This effect was not due to altered levels of pyrimidine pathway metabolites (Figure 5 – figure supplement 1E), suggesting a direct role for electron transfer into ETC by DHODH. These findings indicate that although complex I is largely dispensable in the adult liver, it can interact with alternative electron donor pathways to regulate cellular redox status.

Discussion:

The lack of a requirement for complex I in adult mammalian liver is not consistent with a key role for complex I in electron entry to the ETC in this tissue; in particular, we observed no alterations in mitochondrial of cytosol redox status in cl-deficient liver, or evidence of significant metabolic compensation. The liver is known to have relatively low flux through PDH, the major entry point of carbons to the TCA cycle which generates NADH for cl function (32, 33); thus, our data is consistent with a model in which the hepatic TCA cycle does not play a prominent role in nutrient oxidation via the generation of mitochondrial NADH. The impact of clV deficiency on cellular redox status indicates that the mitochondrial ETC is critical for removing electrons via transfer to oxygen, and thus, alternative electron donors are likely to fuel the ETC, including DHODH. We show that combined inhibition of DHODH and complex I is sufficient to impact redox status; however, there may still be additional important electron sources (such as complex II) which play a prominent role in the liver. A limitation of our study is that we have examined homeostatic functions of the mouse liver, and it is possible that complex I does have a required role in the setting of hepatic stressors (e.g., high fat diet, regeneration), and it will be important to assess these scenarios in future work.

Indeed, the relevant and high flux modes of electron entry into the ETC may be different among distinct tissues, and this provides a plausible hypothesis for the non-overlapping syndromes present in mitochondrial ETC diseases affecting different components. As an upstream component of the ETC, cl mutations may manifest in a narrow set of tissues systems which rely on mitochondrial NADH to fuel respiration. In contrast, complex IV, the terminal component of the ETC which feeds electrons to oxygen

as a final electron acceptor, might be predicted to have effects in a wider variety of tissues, particularly those which do not significantly generate and export lactate as a means to remove electrons. Understanding the key ETC fuel sources in both normal and stressed tissue systems will therefore provide insight into the pathophysiological processes which occur downstream of specific mitochondrial mutations.

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References:

1. G. S. Gorman, P. F. Chinnery, S. DiMauro, M. Hirano, Y. Koga, R. McFarland, A. Suomalainen, D. R. Thorburn, M. Zeviani, D. M. Turnbull, Mitochondrial diseases. *Nature reviews. Disease primers* **2**, 16080 (2016); published online EpubOct 20 (10.1038/nrdp.2016.80).
2. R. J. Rodenburg, Mitochondrial complex I-linked disease. *Biochimica et biophysica acta* **1857**, 938-945 (2016); published online EpubJul (10.1016/j.bbabo.2016.02.012).
3. M. A. Martin, A. Blazquez, L. G. Gutierrez-Solana, D. Fernandez-Moreira, P. Briones, A. L. Andreu, R. Garesse, Y. Campos, J. Arenas, Leigh syndrome associated with mitochondrial complex I deficiency due to a novel mutation in the NDUFS1 gene. *Archives of neurology* **62**, 659-661 (2005); published online EpubApr (10.1001/archneur.62.4.659).
4. F. Distelmaier, W. J. Koopman, L. P. van den Heuvel, R. J. Rodenburg, E. Mayatepek, P. H. Willems, J. A. Smeitink, Mitochondrial complex I deficiency: from organelle dysfunction to clinical disease. *Brain : a journal of neurology* **132**, 833-842 (2009); published online EpubApr (10.1093/brain/awp058).
5. S. T. Ahmed, L. Craven, O. M. Russell, D. M. Turnbull, A. E. Vincent, Diagnosis and Treatment of Mitochondrial Myopathies. *Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics* **15**, 943-953 (2018); published online EpubOct (10.1007/s13311-018-00674-4).
6. W. S. Lee, R. J. Sokol, Mitochondrial hepatopathies: advances in genetics and pathogenesis. *Hepatology* **45**, 1555-1565 (2007); published online EpubJun (10.1002/hep.21710).

7. S. Rahman, Gastrointestinal and hepatic manifestations of mitochondrial disorders. *Journal of inherited metabolic disease* **36**, 659-673 (2013); published online EpubJul (10.1007/s10545-013-9614-2).
8. I. Valnot, S. Osmond, N. Gigarel, B. Mehaye, J. Amiel, V. Cormier-Daire, A. Munnich, J. P. Bonnefont, P. Rustin, A. Rotig, Mutations of the SCO1 gene in mitochondrial cytochrome c oxidase deficiency with neonatal-onset hepatic failure and encephalopathy. *American journal of human genetics* **67**, 1104-1109 (2000); published online EpubNov (10.1016/S0002-9297(07)62940-1).
9. J. P. Casey, P. McGettigan, N. Lynam-Lennon, M. McDermott, R. Regan, J. Conroy, B. Bourke, J. O'Sullivan, E. Crushell, S. Lynch, S. Ennis, Identification of a mutation in LARS as a novel cause of infantile hepatopathy. *Molecular genetics and metabolism* **106**, 351-358 (2012); published online EpubJul (10.1016/j.ymgme.2012.04.017).
10. W. S. Lee, R. J. Sokol, Liver disease in mitochondrial disorders. *Seminars in liver disease* **27**, 259-273 (2007); published online EpubAug (10.1055/s-2007-985071).
11. A. A. Morris, J. W. Taanman, J. Blake, J. M. Cooper, B. D. Lake, M. Malone, S. Love, P. T. Clayton, J. V. Leonard, A. H. Schapira, Liver failure associated with mitochondrial DNA depletion. *Journal of hepatology* **28**, 556-563 (1998); published online EpubApr (10.1016/s0168-8278(98)80278-x).
12. H. Koh, G. S. Park, S. M. Shin, C. E. Park, S. Kim, S. J. Han, H. Q. Pham, J. H. Shin, D. W. Lee, Mitochondrial Mutations in Cholestatic Liver Disease with Biliary Atresia. *Scientific reports* **8**, 905 (2018); published online EpubJan 17 (10.1038/s41598-017-18958-8).
13. J. P. Molleston, R. J. Sokol, W. Karnsakul, A. Miethke, S. Horslen, J. C. Magee, R. Romero, R. H. Squires, J. L. Van Hove, N. Childhood Liver Disease Research Education, Evaluation of the child with suspected mitochondrial liver disease. *Journal of pediatric gastroenterology and nutrition* **57**, 269-276 (2013); published online EpubSep (10.1097/MPG.0b013e31829ef67a).
14. K. Cloots, J. Verbeek, H. Orlent, W. Meersseman, D. Cassiman, Mitochondrial hepatopathy in adults: a case series and review of the literature. *European*

- journal of gastroenterology & hepatology* **25**, 892-898 (2013); published online EpubAug (10.1097/MEG.0b013e32835ee629).
15. P. F. Chinnery, S. DiMauro, Mitochondrial hepatopathies. *Journal of hepatology* **43**, 207-209 (2005); published online EpubAug (10.1016/j.jhep.2005.05.012).
16. L. De Meirleir, S. Seneca, E. Damis, B. Sepulchre, A. Hoorens, E. Gerlo, M. T. Garcia Silva, E. M. Hernandez, W. Lissens, R. Van Coster, Clinical and diagnostic characteristics of complex III deficiency due to mutations in the BCS1L gene. *American journal of medical genetics. Part A* **121A**, 126-131 (2003); published online EpubAug 30 (10.1002/ajmg.a.20171).
17. P. de Lonlay, I. Valnot, A. Barrientos, M. Gorbatyuk, A. Tzagoloff, J. W. Taanman, E. Benayoun, D. Chretien, N. Kadhon, A. Lombes, H. O. de Baulny, P. Niaudet, A. Munnich, P. Rustin, A. Rotig, A mutant mitochondrial respiratory chain assembly protein causes complex III deficiency in patients with tubulopathy, encephalopathy and liver failure. *Nature genetics* **29**, 57-60 (2001); published online EpubSep (10.1038/ng706).
18. L. B. Sullivan, D. Y. Gui, A. M. Hosios, L. N. Bush, E. Freinkman, M. G. Vander Heiden, Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. *Cell* **162**, 552-563 (2015); published online EpubJul 30 (10.1016/j.cell.2015.07.017).
19. L. K. Sharma, J. Lu, Y. Bai, Mitochondrial respiratory complex I: structure, function and implication in human diseases. *Current medicinal chemistry* **16**, 1266-1277 (2009)10.2174/092986709787846578).
20. S. E. Kruse, W. C. Watt, D. J. Marcinek, R. P. Kapur, K. A. Schenkman, R. D. Palmiter, Mice with mitochondrial complex I deficiency develop a fatal encephalomyopathy. *Cell metabolism* **7**, 312-320 (2008); published online EpubApr (10.1016/j.cmet.2008.02.004).
21. S. Peralta, A. Torraco, T. Wenz, S. Garcia, F. Diaz, C. T. Moraes, Partial complex I deficiency due to the CNS conditional ablation of Ndufa5 results in a mild chronic encephalopathy but no increase in oxidative damage. *Human molecular genetics* **23**, 1399-1412 (2014); published online EpubMar 15 (10.1093/hmg/ddt526).

22. B. X. Ke, S. Pepe, D. R. Grubb, J. C. Komen, A. Laskowski, F. A. Rodda, B. M. Hardman, J. J. Pitt, M. T. Ryan, M. Lazarou, J. Koleff, M. M. Cheung, J. J. Smolich, D. R. Thorburn, Tissue-specific splicing of an Ndufs6 gene-trap insertion generates a mitochondrial complex I deficiency-specific cardiomyopathy. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 6165-6170 (2012); published online EpubApr 17 (10.1073/pnas.1113987109).
23. L. Yang, J. C. Garcia Canaveras, Z. Chen, L. Wang, L. Liang, C. Jang, J. A. Mayr, Z. Zhang, J. M. Ghergurovich, L. Zhan, S. Joshi, Z. Hu, M. R. McReynolds, X. Su, E. White, R. J. Morscher, J. D. Rabinowitz, Serine Catabolism Feeds NADH when Respiration Is Impaired. *Cell metabolism* **31**, 809-821 e806 (2020); published online EpubApr 7 (10.1016/j.cmet.2020.02.017).
24. C. V. Pereira, S. Peralta, T. Arguello, S. R. Bacman, F. Diaz, C. T. Moraes, Myopathy reversion in mice after restauration of mitochondrial complex I. *EMBO molecular medicine* **12**, e10674 (2020); published online EpubFeb 7 (10.15252/emmm.201910674).
25. N. Li, K. Ragheb, G. Lawler, J. Sturgis, B. Rajwa, J. A. Melendez, J. P. Robinson, Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *The Journal of biological chemistry* **278**, 8516-8525 (2003); published online EpubMar 7 (10.1074/jbc.M210432200).
26. R. Fato, C. Bergamini, M. Bortolus, A. L. Maniero, S. Leoni, T. Ohnishi, G. Lenaz, Differential effects of mitochondrial Complex I inhibitors on production of reactive oxygen species. *Biochimica et biophysica acta* **1787**, 384-392 (2009); published online EpubMay (10.1016/j.bbabo.2008.11.003).
27. R. P. Goodman, A. L. Markhard, H. Shah, R. Sharma, O. S. Skinner, C. B. Clish, A. Deik, A. Patgiri, Y. H. Hsu, R. Masia, H. L. Noh, S. Suk, O. Goldberger, J. N. Hirschhorn, G. Yellen, J. K. Kim, V. K. Mootha, Hepatic NADH reductive stress underlies common variation in metabolic traits. *Nature* **583**, 122-126 (2020); published online EpubJul (10.1038/s41586-020-2337-2).

28. F. Diaz, C. K. Thomas, S. Garcia, D. Hernandez, C. T. Moraes, Mice lacking COX10 in skeletal muscle recapitulate the phenotype of progressive mitochondrial myopathies associated with cytochrome c oxidase deficiency. *Human molecular genetics* **14**, 2737-2748 (2005); published online EpubSep 15 (10.1093/hmg/ddi307).
29. F. Diaz, S. Garcia, D. Hernandez, A. Regev, A. Rebelo, J. Oca-Cossio, C. T. Moraes, Pathophysiology and fate of hepatocytes in a mouse model of mitochondrial hepatopathies. *Gut* **57**, 232-242 (2008); published online EpubFeb (10.1136/gut.2006.119180).
30. R. J. Hunt, L. Granat, G. S. McElroy, R. Ranganathan, N. S. Chandel, J. M. Bateman, Mitochondrial stress causes neuronal dysfunction via an ATF4-dependent increase in L-2-hydroxyglutarate. *The Journal of cell biology* **218**, 4007-4016 (2019); published online EpubDec 2 (10.1083/jcb.201904148).
31. D. B. Sykes, Y. S. Kfoury, F. E. Mercier, M. J. Wawer, J. M. Law, M. K. Haynes, T. A. Lewis, A. Schajnovitz, E. Jain, D. Lee, H. Meyer, K. A. Pierce, N. J. Tolliday, A. Waller, S. J. Ferrara, A. L. Eheim, D. Stoeckigt, K. L. Maxcy, J. M. Cobert, J. Bachand, B. A. Szekely, S. Mukherjee, L. A. Sklar, J. D. Kotz, C. B. Clish, R. I. Sadreyev, P. A. Clemons, A. Janzer, S. L. Schreiber, D. T. Scadden, Inhibition of Dihydroorotate Dehydrogenase Overcomes Differentiation Blockade in Acute Myeloid Leukemia. *Cell* **167**, 171-186 e115 (2016); published online EpubSep 22 (10.1016/j.cell.2016.08.057).
32. M. E. Merritt, C. Harrison, A. D. Sherry, C. R. Malloy, S. C. Burgess, Flux through hepatic pyruvate carboxylase and phosphoenolpyruvate carboxykinase detected by hyperpolarized ¹³C magnetic resonance. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 19084-19089 (2011); published online EpubNov 22 (10.1073/pnas.1111247108).
33. D. A. Cappel, S. Deja, J. A. G. Duarte, B. Kucejova, M. Inigo, J. A. Fletcher, X. Fu, E. D. Berglund, T. Liu, J. K. Elmquist, S. Hammer, P. Mishra, J. D. Browning, S. C. Burgess, Pyruvate-Carboxylase-Mediated Anaplerosis Promotes Antioxidant Capacity by Sustaining TCA Cycle and Redox Metabolism in Liver.

479 *Cell metabolism* **29**, 1291-1305 e1298 (2019); published online EpubJun 4
 480 (10.1016/j.cmet.2019.03.014).

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Figure 1

Figure 1. Mitochondrial complex I is dispensable in adult murine hepatocytes. (A)

Ndufa9 mRNA levels (relative to β 2-microglobulin; normalized) in isolated hepatocytes of the indicated genotype, assessed by qRT-PCR. $n=3$ animals per group. The same color scheme is used throughout this figure. **(B)** Ndufa9 protein levels in isolated hepatocytes of the indicated genotype, assessed by Western blot. β 2-microglobulin levels are shown as a loading control. **(C)** Representative mitochondrial oxygen consumption rates (Mito-

OCR) per 2500 cells in isolated hepatocytes of the indicated genotype. n=8 wells per group. **(D)** Representative images of gross liver anatomy in animals of the indicated genotype. Scale bar: 0.5cm. **(E)** Representative histology (H&E, Oil Red O, PAS staining) images of liver cross sections from animals of the indicated genotype. Scale bar: 50µm. **(F)** Liver weight (normalized to body weight) from mice of the indicated genotype. n=4-5 animals per group. **(G)** Fasting plasma glucose levels in mice of the indicated genotype. n=12-14 animals per group. **(H)** Circulating plasma markers (total protein, albumin, and total bilirubin) of liver function in mice of the indicated genotype. n=3-5 animals per group. **(I)** Plasma markers (ALT and AST) of liver damage in mice of the indicated genotype. n=4-5 mice per group. Statistical significance was assessed using t-test (A,C,G,H,I) or Mann-Whitney (F,H) tests with adjustments for multiple comparisons. All data represent mean +/- standard deviation from biological replicates. Source data is available in Figure 1 – source data file 1. Uncropped western blots are available in Figure 1 – source data file 2.

Figure 1 - figure supplement 1

Figure 1 – figure supplement 1. Analysis of *ndufa9^{ff}* and *ndufa9^{-/-}* livers at 8 weeks post-AAV administration. (A) Oxygen consumption rates in *ndufa9^{ff}* and *ndufa9^{-/-}* mouse embryonic fibroblasts (MEFs). n = 22-24 wells per group. The same color scheme is used throughout this figure. (B) Normalized NAD⁺ / NADH ratios in MEFs of the indicated genotype. n=3 wells per group. (C) Representative images of gross liver anatomy in animals of the indicated genotype. Scale bar: 0.5cm. (D) Representative

histology (H&E, Oil Red O, PAS staining) images of liver cross sections from animals of the indicated genotype. Scale bar: 50µm. **(E)** Fasting plasma glucose levels in mice of the indicated genotype. n=4 mice per group. **(F)** Circulating plasma markers (total protein, albumin, and total bilirubin) of liver function in mice of the indicated genotype. n=4 animals per group. **(G)** Plasma markers (ALT and AST levels) of liver damage in mice of the indicated genotype. n=4-5 mice per group. Statistical significance was assessed using t-test (B,E,F,G) or Mann-Whitney (A,F,G) tests with adjustments for multiple comparisons. All data represent mean +/- standard deviation from biological replicates, unless noted otherwise. Source data is available in Figure 1 – figure supplement 1 - source data file 1.

Figure 2

Figure 2. Complex I deficiency in adult liver does not impact redox status. (A)

Reduced glutathione to oxidized glutathione ratio (GSH:GSSG) ratios in livers of the indicated genotype, as a proxy of oxidative stress. n=8-9 mice per group. The same color scheme is used throughout this figure. **(B)** NAD⁺/NADH and NADPH/NADP⁺ ratios in livers of the indicated genotype. n=6 mice per group. **(C)** Normalized cytosolic (pyruvate / lactate) and mitochondrial (acetoacetate / β-hydroxybutyrate) redox ratios in livers of the indicated genotype. n=6 mice per group. **(D)** Tissue and plasma α-hydroxybutyrate

levels in animals of the indicated genotype. n=4-6 mice per group. **(E)** Volcano plot of protein abundances changes in *ndufa9^{-/-}* vs. *ndufa9^{fl/fl}* livers, based on proteomics analysis. Log₂(Fold change) is plotted against the -log₁₀(adjusted p-value) for each protein. Complex I components are labeled. Significantly changing proteins (log₂(Fold change) >1 or < -1; Adjusted p-val<0.05) are colored black, and mitochondrial proteins are colored red. **(F)** Mitochondrial genome (mtDNA) to nuclear genome (nDNA) ratios in livers of the indicated genotype. n=8-9 mice per group. **(G)** Levels of mitochondrial proteins (PC, pyruvate carboxylase, and Tomm20) in livers of the indicated genotype, as assessed by Western blot. Actin levels are shown as a loading control. **(H)** Volcano plot of metabolite abundance changes in *ndufa9^{-/-}* vs. *ndufa9^{fl/fl}* livers, based on metabolomics analysis. Log₂(Fold change) is potted against the -log₁₀(adjusted p-value) for each metabolite. Significantly changing metabolites (log₂(Fold change) >1 or < -1; Adjusted p-val<0.05) are colored black. TMP, Thiamine monophosphate. **(I)** Relative abundances of mitochondrial TCA cycle metabolites from livers of the indicated genotype. n=6 mice per group. Statistical significance was assessed using t-test (B,C,D,F,I) or Mann-Whitney (A) with adjustments for multiple comparisons. All data represent mean +/- standard deviation from biological replicates. Source data is available in Figure 2 – source data file 1, including proteomics and metabolomics data sets. Uncropped western blots are available in Figure 2 – source data file 2.

Figure 2 – figure supplement 1

Excel spreadsheet containing statistical analysis of proteomics and metabolomics data for *ndufa9*^{-/-} vs. *ndufa9*^{ff/ff} livers.

Figure 3

Figure 3. Complex IV deficiency in adult hepatocytes induces liver dysfunction. (A)

Cox10 mRNA levels (relative to β 2-microglobulin; normalized) in livers of the indicated genotype, assessed by qRT-PCR. n=4-5 animals per group. The same color scheme is used throughout this figure. **(B)** Cox10 protein levels in livers of the indicated genotype, assessed by Western blot. β 2-microglobulin levels are shown as a loading control. **(C)** Complex IV activity (pmol O₂ consumed / min) in isolated mitochondria supplemented

with ascorbate/TMPD substrates from livers of the indicated genotype. n=5-6 animals per group. **(D)** Representative images of gross liver anatomy in animals of the indicated genotype. Scale bar: 0.5cm. **(E)** Representative histology (H&E, Oil Red O, PAS staining) images of liver cross sections from animals of the indicated genotype. Scale bar: 50µm. **(F)** Liver weight (normalized to body weight) from mice of the indicated genotype. n=7 animals per group. **(G)** Fasting plasma glucose levels in mice of the indicated genotype. n=6-7 animals per group. **(H)** Relative changes in plasma glucose following a lactate/pyruvate tolerance test in mice of the indicated genotype. n=4-5 mice per group. **(I)** Fractional gluconeogenesis (plasma) in animals of the indicated genotype, measured via pyruvate/lactate tolerance test. n=4-5 animals per group. **(J)** Circulating plasma markers (total protein, albumin, and total bilirubin) of liver function in mice of the indicated genotype. n=5 animals per group. **(K)** Plasma markers (ALT and AST) of liver damage in mice of the indicated genotype. n=5 mice per group. Statistical significance was assessed using two-way ANOVA (H), t-test (C,F,G,I,J,K) or Mann-Whitney (A,J) tests with adjustments for multiple comparisons. All data represent mean +/- standard deviation from biological replicates. Source data is available in Figure 3 – source data file 1. Uncropped western blots are available in Figure 3 – source data file 2.

Figure 3 - figure supplement 1

Figure 3 – figure supplement 1. Enrichment data for ^{13}C -lactate/pyruvate challenge.

(A) Mass isotopomer distributions related to lactate/pyruvate tolerance test (Fig. 3H,I), for plasma lactate and glucose. n=4-5 mice per group. Source data is available in Figure 3 – figure supplement 1 - source data file 1.

Figure 4

Figure 4. Complex IV deficiency alters metabolism and cellular redox status in adult liver. (A) Reduced glutathionine to oxidized glutathionine ratio (GSH:GSSG) ratios in

livers of the indicated genotype, as a proxy of oxidative stress. n=5-6 mice per group. The same color scheme is used throughout this figure. **(B)** NAD⁺/NADH and NADPH/NADP⁺ ratios in livers of the indicated genotype. n=5-6 mice per group. **(C)** Normalized cytosolic (pyruvate / lactate) and mitochondrial (acetoacetate / β-hydroxybutyrate) redox ratios in livers of the indicated genotype. n=5-6 mice per group. **(D)** Normalized tissue and plasma α-hydroxybutyrate levels in animals of the indicated genotype. n=4-5 mice per group. **(E)** Volcano plot of protein abundances changes in *cox10*^{-/-} vs. *cox10*^{ff} livers, based on proteomics analysis. Log₂(Fold change) is plotted against the -log₁₀(adjusted p-value) for each protein. Complex IV components are labeled. Significantly changing proteins (log₂(Fold change) >1 or < -1; Adjusted p-val<0.05) are colored black; of these, mitochondria-localized proteins are colored red. **(F)** Mitochondrial genome (mtDNA) to nuclear genome (nDNA) ratios in livers of the indicated genotype. n=9 mice per group. **(G)** Levels of mitochondrial proteins (PC, pyruvate carboxylase, and Tomm20) in livers of the indicated genotype, as assessed by Western blot. Actin levels are shown as a loading control. **(H)** Volcano plot of metabolite abundance changes in *cox10*^{-/-} vs. *cox10*^{ff} livers, based on metabolomics analysis. Log₂(Fold change) is plotted against the -log₁₀(adjusted p-value) for each metabolite. Significantly changing metabolites (log₂(Fold change) >1 or < -1; Adjusted p-val<0.05) are colored black. **(I)** Relative abundances of mitochondrial TCA cycle metabolites from livers of the indicated genotype. n=5-6 mice per group. **(J)** Relative abundance of electron acceptors/donors from livers of the indicated genotype. n=4-6 mice per group. BH₄, tetrahydrobiopterin. **(K)** Relative abundance of pyrimidine synthesis pathway metabolites in livers of the indicated genotype. n=4-6 mice per group. Statistical significance was assessed using t-test (A,B,C,D,F,I,J,K) or Mann-Whitney

(B,D) tests with adjustments for multiple comparisons. All data represent mean +/- standard deviation from biological replicates. Source data is available in Figure 4 – source data file 1, including proteomics and metabolomics data sets. Uncropped western blots are available in Figure 4 – source data file 2.

Figure 4 - figure supplement 1

Figure 4 – figure supplement 1. Altered metabolism in *cox10*^{-/-} livers. (A) The top enriched biological pathways from gene ontology analysis of down-regulated proteins ($\log_2(\text{Fold change}) < -1$; Adjusted p -value < 0.05) in *cox10*^{-/-} livers. **(B)** Relative abundance of acylcarnitine metabolites in livers of the indicated genotype. $n=5-6$ mice

per group. **(C)** Relative abundance of cholate species in livers of the indicated genotype.
n=5-6 mice per group. Statistical significance was assessed using t-tests (B,C) with
adjustments for multiple comparisons. All data represent mean +/- standard deviation
from biological replicates. Source data is available in Figure 4 – figure supplement 1 -
source data file 1.

Figure 4 – figure supplement 2

Excel spreadsheet containing statistical analysis of proteomics and metabolomics data for *cox10^{-/-}* vs. *cox10^{ff}* livers.

Figure 5

Figure 5. DHODH inhibition impairs redox status in the background of complex I deficiency. (A) Overview of the pyrimidine synthesis pathway and its interaction with the mitochondrial electron transport chain. DHODH resides in the inner mitochondrial membrane and transfers electrons from dihydroorotate directly to coenzyme Q (CoQ), bypassing complex I. **(B)** Levels of DHODH in livers of the indicated genotype, assessed by Western blot. β2-microglobulin levels are shown as a loading control. **(C)** Relative abundance of pyrimidine synthesis pathway metabolites in livers of the indicated genotype. n=6 mice per group. The same color scheme is used throughout this figure. **(D)** Relative abundance of dihydroorotate and the orotate / dihydroorotate ratio in livers of the indicated genotype, following treatment with vehicle or brequinar (BRQ). n=3-4 mice per group. **(E)** NAD⁺ / NADH ratios in livers of the indicated genotype, following treatment with vehicle or brequinar (BRQ). n=3-4 mice per group. Statistical significance was assessed using two-way ANOVA (C,D,E) with adjustments for multiple comparisons.

All data represent mean +/- standard deviation from biological replicates. Source data is available in Figure 5 – source data file 1. Uncropped western blots are available in Figure 5 – source data file 2.

Figure 5 - figure supplement 1

Figure 5 – figure supplement 1. Liver function in brequinar-treated mice. (A) Liver weight (normalized to body weight) from mice of the indicated genotype and treatment condition. n=3-4 mice per group. BRQ, brequinar. The same color scheme is used throughout this figure. **(B)** Fasting plasma glucose levels in mice of the indicated genotype and treatment condition. n=3-4 animals per group. **(C)** Circulating plasma markers (total protein, albumin, and total bilirubin) of liver function in mice of the indicated genotype and treatment condition. n=3-4 mice per group. **(D)** Plasma markers (ALT and AST levels) of liver damage in mice of the indicated genotype and treatment condition. n=3-4 mice per group. **(E)** Relative abundance of pyrimidine synthesis pathway metabolites in livers of the indicated genotype and treatment condition. n=3-4 mice per group. Statistical significance was assessed using two-way ANOVA (A,B,C,D,E) with adjustments for

multiple comparisons. All data represent mean +/- standard deviation from biological replicates. Source data is available in Figure 4 – figure supplement 1 - source data file 1.

Figure 1 – source data file 1: Excel file with source data for Figure 1A,C,F,G,H,I.

Figure 1 – source data file 2: Uncropped western blots for Figure 1B.

Figure 1 – figure supplement 1 - source data file 1: Excel file with source data for Figure 1 – figure supplement 1A,B,E,F,G.

Figure 2 – source data file 1: Excel file with source data for Figure 2A,B,C,D,E,F,H,I, including proteomics and metabolomics data sets.

Figure 2 – source data file 2: Uncropped western blots for Figure 2G.

Figure 3 – source data file 1: Excel file with source data for Figure 3A,C,F,G,H,I,J,K.

Figure 3 – source data file 2: Uncropped western blots for Figure 3B.

Figure 3 – figure supplement 1 - source data file 1: Excel file with source data for Figure 3 – figure supplement 1A.

Figure 4 – source data file 1: Excel file with source data for Figure 4A,B,C,D,E,F,H,I,J,K, including proteomics and metabolomics data sets.

Figure 4 – source data file 2: Uncropped western blots for Figure 4G.

Figure 4 – figure supplement 1 - source data file 1: Excel file with source data for Figure 4 – figure supplement 1A,B,C.

Figure 5 – source data file 1: Excel file with source data for Figure 5C,D,E.

Figure 5 – source data file 2: Uncropped western blots for Figure 5B.

Figure 5 – figure supplement 1 - source data file 1: Excel file with source data for Figure 5 – figure supplement 1A,B,C,D,E.

Supplementary File 1: Zip folder with raw image files for all western blots.

MATERIALS AND METHODS:

Reagents:

Most chemicals and reagents including antimycin A (A8674), carbonyl cyanide 3-chlorophenylhydrazone [CCCP (C2759)], EGTA (E3889), glutamate (49621), glycerol (G5516), hematoxylin (GHS132), HEPES (H4034), magnesium chloride (208337), malate (240176), D-mannitol (M4125), NAD⁺ (N8285), NADP⁺ (N8160), NADH (N6785), NADPH (N9960), ¹⁵N₅-AMP (900382), Orotic acid (O2750), L-Dihydroorotic acid (D7128), Oil Red O (O0625), periodic acid (P7875), PEG 400 (91893), potassium chloride (P9541), rotenone (R8875), sodium chloride (S9888), sodium lactate (L7022), sodium phosphate monobasic (RDD007), sodium pyruvate (P2256), sucrose (S0389), N,N,N',N'-Tetramethyl-p-phenylenediamine [TMPD (T7394)] were purchased from Sigma-Aldrich. HPLC-grade acetonitrile (A955), formic acid (A117), methanol (A456), and water (W6) were purchased from Fisher Scientific. Eosin Y (SE23) and Tris base (BP301) was purchased from Fisher Scientific. GSH (G597951), GSSG (G597970), ¹³C₂; ¹⁵N GSH (G597952), and ¹³C₄; ¹⁵N₂ GSSG (G597972) were purchased from Toronto Research Chemicals. [U-¹³C] lactate (CLM-1579) and [U-¹³C] pyruvate (CLM-2440) were purchased from Cambridge Isotope Laboratories. N-carbamoyl-DL-aspartic acid (B7906) was purchased from ApexBio. Brequinar sodium was purchased from Tocris (6196). Schiff's Reagent was purchased from Electron Microscopy Sciences (26052-06). Liver perfusion medium (17701038), liver digest medium (17703034), and hepatocyte wash medium (17704024) were purchased from Thermo Scientific. pAAV.TBG.PI.eGFP.WPRE.bGH (AAV-GFP) and pAAV.TBG.PI.Cre.rBG (AAV-Cre) were a gift from James M. Wilson (Addgene viral prep #105535-AAV8 and #107787-AAV8). Antibodies used: Tom20

(Proteintech, 11802), Pyruvate carboxylase (Proteintech, 16588), DHODH (Proteintech, 14877), Actin (Proteintech, 66009), Ndufa9 (ThermoScientific, 459100), β 2-microglobulin (ThermoScientific, 701250).

Mice

The *cox10^{ff}* mice (strain 024697) were purchased from The Jackson Laboratory and were maintained on a C57BL6 background. The *ndufa9^{ff}* mice were obtained from David McFadden (UT Southwestern) and were on a C57BL6 background; generation of these mice are described here (1). All mice were housed in the Animal Resource Center at University of Texas Southwestern Medical Center under a 12-hour light-dark cycle and fed ad libitum. Prior to all experiments, mice were fasted for 16-18 hours over night (unless otherwise indicated). All mouse experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas Southwestern Medical Center. Both male and female mice were used in all experiments; if sex differences were not present, both male and female mice were analyzed together. For genotyping, the following primers were used:

Cox10 Forward: GAGAGGAGTCAAGGGGACCT

Cox10 Reverse: GGCCTGCAGCTCAAAGTGTA

Ndufa9 Forward: TTGATTGCCTGTGAGCTTTG

Ndufa9 Reverse: TGCTAGGAAAGAGGCAGGTC

For AAV injections, virus was prepared in dilution buffer [NaCl (136mM), KCl (5mM), MgCl₂ (1mM), Tris (10mM, pH 7.4), and glycerol (10%)] and injected into the retroorbital vein at 5x10¹⁰ GC per mouse. For brequinar treatments, brequinar sodium was dissolved

in a 70:30 (PBS:PEG 400) solution, and was administered via intraperitoneal injection 2x/week into mice at a dose of 25 mg/kg, based on a previous protocol (2).

Mitochondrial Respiration Measurements

Oxygen consumption rates (OCR) were measured using a Seahorse XFe96 Analyzer (Agilent Technologies) based on a previously published protocol (3). Liver tissue was homogenized with 40 strokes of a dounce homogenizer in mitochondrial isolation buffer [HEPES (5mM), sucrose (70mM), mannitol (220mM), MgCl₂ (5mM), KH₂PO₄ (10mM), and EGTA (1mM), pH 7.2]. Mitochondria were isolated via differential centrifugation. 5μg of mitochondria were plated in an XFe96 well plate on ice, spun at 4700 RPM (Beckman Coulter S6096) for 2 minutes at 4°C. Mitochondria were then supplemented with media containing isolation buffer with complex I, II, or IV substrates to probe respiration. At the times indicated, ADP (final concentration 4mM), oligomycin (2uM), CCCP (2uM), and either antimycin A (4uM) or sodium azide (40mM) was injected.

No substrate	Isolation buffer (IB)
Complex I – Pyruvate	IB with pyruvate (10mM) + malate (1mM)
Complex I – Glutamate	IB with glutamate (10mM) + malate (1mM)
Complex II	IB with succinate (5mM) + rotenone (2uM)
Complex IV	IB with ascorbate (10mM) + TMPD (100uM) + antimycin A (2uM)

For primary hepatocyte experiments, cells were first isolated from fresh liver by a 2-step collagenase perfusion as previously described (4). Hepatocytes were plated (2500 cells per well), allowed to settle for 20 minutes, and centrifuged. Seahorse assay buffer [Sigma D5030, glucose (10mM), L-glutamine (1mM), and penicillin/streptomycin (1%)] was

added to the cells and measurements were started. For mouse embryonic fibroblasts, cells were plated overnight and exchanged into seahorse assay buffer and measurements started. Mitochondrial oxygen consumption rates (mito-OCR) were calculated by subtracting non-mitochondrial respiration from baseline respiration.

qRT-PCR and qPCR measurements.

Genomic DNA was isolated by ethanol precipitation and RNA was isolated from tissue using RNeasy Mini kits (Qiagen). Samples were run using the Luna Universal One-Step RT-qPCR Kit (New England Biolabs) on a CFX384 (Bio-Rad).

Target	Primer Sequence
<i>cox10</i>	F: AGGGTCAGCATCACCAATAC
	R: GGAGACACTTACCAGCATCAA
<i>ndufa9</i>	F: GGAGACACTTACCAGCATCAA
	R: CCTCCTTTCCCGTGAGGTA
mtDNA (<i>ND2</i> mtDNA)	F: CCTATCACCTTGCCATCAT
	R: GAGGCTGTTGCTTGTGTGAC
nDNA (<i>pecam1</i>)	F: ATGGAAAGCCTGCCATCATG
	R: TCCTTGTTGTTTCAGCATCAC

Histology of liver tissue

Fresh tissue was fixed overnight in 10% neutral buffered formalin, rinsed with 30% sucrose in PBS, and paraffin embedded. 10 micron sections were cut and placed on slides at -20°C until staining. Sections were stained by either haemotoxylin and eosin, Oil

Red O), or periodic acid Schiff base (PAS) and visualized under 20X magnification on an Olympus IX83 microscope and processed with ImageJ (NIH).

Plasma Analysis

Blood was collected on ice with heparin and centrifuged at 5000xg for 10 minutes to obtain plasma. Plasma levels of alanine aminotransferase, albumin, aspartate aminotransferase, glucose, lactate, total bilirubin, and total protein were measured using a VITROS 250 Microslide at the UT Southwestern Metabolic Phenotyping Core.

GSH/GSSG and NAD(P)(H) Quantitation

For glutathione quantitation, 20mg of frozen tissue was treated with 300uL of 80% methanol with 0.1% formic acid and homogenized on ice. The samples were freeze-thawed, centrifuged to remove protein, and the supernatant was dried. Dried samples were reconstituted in 1mL water with 0.1% formic acid, diluted, spiked with internal labeled standards, and analyzed as previously described (5, 6).

NAD(P)⁺/NAD(P)H was measured as previously described (7). Briefly, ~20 mg of tissue was homogenized on ice in cold 40:40:20 (acetonitrile: methanol: water) with 0.1M formic acid buffer. Tissue was vortexed, chilled, quenched with 15% ammonium bicarbonate, and centrifuged to pellet protein. Supernatant was diluted in 10mM ammonium bicarbonate and ¹⁵N₅-AMP was added as an internal standard and injected onto a Sciex 6500 Qtrap using a reverse-phase ion pairing method. Pelleted protein was lysed and quantified using the DC protein assay (Bio-Rad).

For MEFs, cells were plated in 6-well dishes and allowed to adhere overnight; the following day cells were at ~80% confluence. Media was removed, cells were rinsed with ice cold saline, and metabolites were collected by scraping cells in dry ice-cold 80% methanol / 20% water with an internal standard (series of ¹³C, ¹⁵N-labeled amino acids). Metabolite extracts were stored at -80°C overnight and centrifuged the following day to remove debris. Raw AUC values were obtained using MultiQuant and TIC values for normalization were obtained using Analyst (SCIEX software for MS analysis).

Proteomics analysis of liver tissue

50 µg of liver protein homogenate was loaded onto a 4-20% Mini-PROTEAN TGX precast protein gel (BioRad), stained with Coomassie Blue, and destained. Samples were analyzed at the UT Southwestern Proteomics Core. Gel slices were digested overnight with trypsin (Pierce) following reduction and alkylation with DTT and iodoacetamide (Sigma–Aldrich). The samples then underwent solid-phase extraction cleanup with an Oasis HLB plate (Waters) and the resulting samples were injected onto an Orbitrap Fusion Lumos mass spectrometer coupled to an Ultimate 3000 RSLC-Nano liquid chromatography system. Samples were injected onto a 75 µm i.d., 75-cm long EasySpray column (Thermo) and eluted with a gradient from 0-28% buffer B over 90 min. Buffer A contained 2% (v/v) ACN and 0.1% formic acid in water, and buffer B contained 80% (v/v) ACN, 10% (v/v) trifluoroethanol, and 0.1% formic acid in water. The mass spectrometer operated in positive ion mode with a source voltage of 1.8 kV and an ion transfer tube temperature of 275 °C. MS scans were acquired at 120,000 resolution in the Orbitrap and up to 10 MS/MS spectra were obtained in the ion trap for each full spectrum acquired

using higher-energy collisional dissociation (HCD) for ions with charges 2-7. Dynamic exclusion was set for 25 s after an ion was selected for fragmentation. Raw MS data files were analyzed using Proteome Discoverer v2.4 SP1 (Thermo), with peptide identification performed using Sequest HT searching against the mouse protein database from UniProt. Fragment and precursor tolerances of 10 ppm and 0.6 Da were specified, and three missed cleavages were allowed. Carbamidomethylation of Cys was set as a fixed modification, with oxidation of Met set as a variable modification. The false-discovery rate (FDR) cutoff was 1% for all peptides. Mitochondrial-localized proteins were classified based on their presence in MitoCarta2.0 (8). Gene ontology analysis was performed using DAVID (<https://david-d.ncifcrf.gov/home.jsp>).

Metabolomics analysis

Flash frozen liver tissue (20 mg) was pulverized with a mortar and pestle on liquid nitrogen (H37260-0100, Bel-Art Products), 80% methanol was added, and samples subjected to three freeze-thaw cycles. For plasma, 20 μ L was extracted with 0.2 mL cold acetone. Protein was removed by centrifugation and supernatant dried (SpeedVac, ThermoFisher).

Data acquisition was performed by reverse-phase chromatography on a 1290 UHPLC liquid chromatography (LC) system interfaced to a high-resolution mass spectrometry (HRMS) 6550 iFunnel Q-TOF mass spectrometer (MS) (Agilent Technologies, CA). The MS was operated in both positive and negative (ESI+ and ESI-) modes. Analytes were separated on an Acquity UPLC® HSS T3 column (1.8 μ m, 2.1 x 150 mm, Waters, MA). The column was kept at room temperature. Mobile phase A composition was 0.1% formic

992 acid in water and mobile phase B composition was 0.1% formic acid in 100% ACN. The
 993 LC gradient was 0 min: 1% B; 5 min: 5% B; 15 min: 99% B; 23 min: 99% B; 24 min: 1%
 994 B; 25 min: 1% B. The flow rate was 250 μ L min⁻¹. The sample injection volume was 5 μ L.
 995 ESI source conditions were set as follows: dry gas temperature 225 °C and flow 18 L min⁻¹,
 996 1, fragmentor voltage 175 V, sheath gas temperature 350 °C and flow 12 L min⁻¹, nozzle
 997 voltage 500 V, and capillary voltage +3500 V in positive mode and -3500 V in negative.
 998 The instrument was set to acquire over the full m/z range of 40–1700 in both modes, with
 999 the MS acquisition rate of 1 spectrum s⁻¹ in profile format. Raw data files were processed
 1000 using Profinder B.08.00 SP3 software (Agilent Technologies, CA) with an in-house
 1001 database containing retention time and accurate mass information on 600 standards from
 1002 Mass Spectrometry Metabolite Library (IROA Technologies, MA) which was created
 1003 under the same analysis conditions. The in-house database matching parameters were
 1004 mass tolerance 10 ppm; retention time tolerance 0.5 min. Peak integration result was
 1005 manually curated in Profinder for improved consistency
 1006 HILIC chromatographic separation of metabolites related to *de novo* pyrimidine
 1007 metabolism were extracted with 80% acetonitrile, normalized to 0.1mg/mL and analyzed
 1008 as previously described (6).
 1009 GC-MS analysis was used to quantitate α -hydroxybutyrate and α -ketobutyrate, as well
 1010 as measure mass isotopomer distributions for the lactate/pyruvate tolerance test. Dried
 1011 metabolites were derivatized to form methoxime-TBDMS adducts by incubating with 1%
 1012 methoxyamine hydrochloride (Sigma-Aldrich) in pyridine at 70°C for 15 min followed by
 1013 addition of *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA, Sigma-
 1014 Aldrich) for 1 hour. Derivatized samples were analyzed using an Agilent Technologies

7890B gas chromatographer with a HP-5MS 5% phenyl methyl Silox column (Agilent) coupled to an Agilent Technologies 5977A mass spectrometer.

Lactate/Pyruvate Tolerance Test

Lactate/Pyruvate tolerance tests were performed as previously described (9). Briefly, mice were fasted for 16 hours overnight and given an IP injection of a 1.5mg/g of a 10:1 sodium lactate: sodium pyruvate solution. 40% of the sodium lactate and sodium pyruvate were [U-¹³C] sodium lactate and [U-¹³C] sodium pyruvate. Blood glucose was measured 0, 10, 20, and 30 minutes post injection using a blood glucometer (Contour Next) and lactate was measured using a lactate meter (Nova Biomedical Lactate Plus). Fractional gluconeogenesis (GNG) was determined as previously described (10), using the following formula:

$$\text{GNG} = (\text{M1}_{\text{glucose}} + \text{M2}_{\text{glucose}} + \text{M3}_{\text{glucose}}) / (2 * \text{M0}_{\text{lactate}} * (\text{M1}_{\text{lactate}} + \text{M2}_{\text{lactate}} + \text{M3}_{\text{lactate}}))$$

where Mx represents the fractional enrichment of the m+x species for the indicated metabolite.

Statistical analyses

All data represent mean and standard deviations from biological replicates. No statistical tests were used to predetermine sample size. Animals were randomly allocated into experimental groups. No blinding or masking of samples was performed. Data sets for each group of measurement was tested for normality using the Shapiro-Wilk test. If the data was not normally distributed, the data was log-transformed and retested for normality. For normally-distributed data, groups were compared using the two-tailed

Student's t-test (for 2 groups), or one-way ANOVA or two-way ANOVA (> 2 groups), followed by Tukey's or Dunnett's test for multiple comparisons. For data that was not normally distributed, we used non-parametric testing (Mann-Whitney or Kolmogorov-Smirnov tests for two groups and Kruskal-Wallis test for multiple groups), followed by Dunn's multiple comparisons adjustment. For metabolomics and proteomics data sets, abundances were compared by multiple t-tests, followed by Bonferroni correction of p-values. Proteomics and metabolomics analysis were performed once using biological replicates (individual mice); for other experiments, multiple (two to four) independent experiments with biological replicates (individual mice) were performed for reported data, and the number of biological replicates are indicated in the figures. No data were excluded.

References:

1. X. S. Wang, SD; Bordieanu, B; Frank, A; Yi, Y; Venigalla, SSK; Gu, Z; Glogauer, M; Chandel, NS; Zhao, H; Zhao, Z; McFadden, DG; Mishra, P, Scinderin promotes removal of electron transport chain dysfunctional muscle stem cells via induction of stem cell-myofiber fusion. *in revision*, (2021).
2. D. B. Sykes, Y. S. Kfoury, F. E. Mercier, M. J. Wawer, J. M. Law, M. K. Haynes, T. A. Lewis, A. Schajnovitz, E. Jain, D. Lee, H. Meyer, K. A. Pierce, N. J. Tolliday, A. Waller, S. J. Ferrara, A. L. Eheim, D. Stoeckigt, K. L. Maxcy, J. M. Cobert, J. Bachand, B. A. Szekely, S. Mukherjee, L. A. Sklar, J. D. Kotz, C. B. Clish, R. I. Sadreyev, P. A. Clemons, A. Janzer, S. L. Schreiber, D. T. Scadden, Inhibition of Dihydroorotate Dehydrogenase Overcomes Differentiation Blockade in Acute Myeloid Leukemia. *Cell* **167**, 171-186 e115 (2016); published online EpubSep 22 (10.1016/j.cell.2016.08.057).
3. G. W. Rogers, M. D. Brand, S. Petrosyan, D. Ashok, A. A. Elorza, D. A. Ferrick, A. N. Murphy, High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. *PloS one* **6**, e21746 (2011)10.1371/journal.pone.0021746).
4. Y. H. Lin, S. Zhang, M. Zhu, T. Lu, K. Chen, Z. Wen, S. Wang, G. Xiao, D. Luo, Y. Jia, L. Li, M. MacConmara, Y. Hoshida, A. G. Singal, A. Yopp, T. Wang, H. Zhu, Mice With Increased Numbers of Polyploid Hepatocytes Maintain Regenerative Capacity But Develop Fewer Hepatocellular Carcinomas Following Chronic Liver Injury. *Gastroenterology* **158**, 1698-1712 e1614 (2020); published online EpubMay (10.1053/j.gastro.2020.01.026).
5. J. M. Ubellacker, A. Tasdogan, V. Ramesh, B. Shen, E. C. Mitchell, M. S. Martin-Sandoval, Z. Gu, M. L. McCormick, A. B. Durham, D. R. Spitz, Z. Zhao, T. P. Mathews, S. J. Morrison, Lymph protects metastasizing melanoma cells from ferroptosis. *Nature* **585**, 113-118 (2020); published online EpubSep (10.1038/s41586-020-2623-z).
6. A. Tasdogan, B. Faubert, V. Ramesh, J. M. Ubellacker, B. Shen, A. Solmonson, M. M. Murphy, Z. Gu, W. Gu, M. Martin, S. Y. Kasitinon, T. Vandergriff, T. P. Mathews, Z. Zhao, D. Schadendorf, R. J. DeBerardinis, S. J. Morrison, Metabolic

- heterogeneity confers differences in melanoma metastatic potential. *Nature* **577**, 115-120 (2020); published online EpubJan (10.1038/s41586-019-1847-2).
7. W. Lu, L. Wang, L. Chen, S. Hui, J. D. Rabinowitz, Extraction and Quantitation of Nicotinamide Adenine Dinucleotide Redox Cofactors. *Antioxidants & redox signaling* **28**, 167-179 (2018); published online EpubJan 20 (10.1089/ars.2017.7014).
8. S. E. Calvo, K. R. Clauser, V. K. Mootha, MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. *Nucleic acids research* **44**, D1251-1257 (2016); published online EpubJan 4 (10.1093/nar/gkv1003).
9. D. A. Cappel, S. Deja, J. A. G. Duarte, B. Kucejova, M. Inigo, J. A. Fletcher, X. Fu, E. D. Berglund, T. Liu, J. K. Elmquist, S. Hammer, P. Mishra, J. D. Browning, S. C. Burgess, Pyruvate-Carboxylase-Mediated Anaplerosis Promotes Antioxidant Capacity by Sustaining TCA Cycle and Redox Metabolism in Liver. *Cell metabolism* **29**, 1291-1305 e1298 (2019); published online EpubJun 4 (10.1016/j.cmet.2019.03.014).
10. J. K. Kelleher, Estimating gluconeogenesis with [U-13C]glucose: molecular condensation requires a molecular approach. *The American journal of physiology* **277**, E395-400 (1999); published online EpubSep (10.1152/ajpendo.1999.277.3.E395).

Figure 2 - figure supplement 1: Proteomic and metabolomic analysis of ndufa9^{-/-} vs. ndufa9

Tables

<i>Proteomics Ndufa9</i>	Statistics of differential abundance from proteomics anal
<i>Metabolomics Ndufa9</i>	Statistics of differential abundance from metabolomics a

^{f/f} liver.

ysis of ndufa9^{-/-} vs. ndufa9^{f/f} liver.

nalys of ndufa9^{-/-} vs. ndufa9^{f/f} liver.

Figure 4 - figure supplement 2 - Proteomics and Metabolomics Analysis of cox10^{-/-} vs. cox1

Tables

Proteomics Cox10

Statistics of differential abundance from proteomics anal

GO - Cox10 downregulated

Gene ontology statistics for down-regulated proteins in c

Metabolomics Cox10

Statistics of differential abundance from metabolomics a

0^{ff} liver.

ysis of $\text{cox10}^{-/-}$ vs. cox10^{ff} liver.

$\text{cox10}^{-/-}$ liver.

nalysis of $\text{cox10}^{-/-}$ vs. cox10^{ff} liver.