

AWARD NUMBER: W81XWH-19-1-0213

TITLE: Investigation into the Mechanisms of Acute Myeloid Leukemia (AML)
Tumorigenesis and Chemoresistance via Systems Analysis of Mitochondrial
Form and Function

PRINCIPAL INVESTIGATOR: Kelsey Fisher-Wellman

CONTRACTING ORGANIZATION: East Carolina University, Greenville, NC

REPORT DATE: SEPTEMBER 2022

TYPE OF REPORT: FINAL

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE SEPTEMBER 2022		2. REPORT TYPE FINAL		3. DATES COVERED 01JUN2019 - 31MAY2022	
4. TITLE AND SUBTITLE Investigation into the Mechanisms of Acute Myeloid Leukemia (AML) Tumorigenesis and Chemoresistance via Systems Analysis of Mitochondrial Form and Function				5a. CONTRACT NUMBER W81XWH-19-1-0213	
				5b. GRANT NUMBER CA180334	
				5c. PROGRAM ELEMENT NUMBER PRCRP	
6. AUTHOR(S) Dr. Kelsey Fisher-Wellman E-Mail: fisherwellmank17@ecu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) East Carolina University / 1000 E 5th ST, Greenville, NC, 27858-2502				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Typified by oxidative phosphorylation (OXPHOS), mitochondria catalyze a wide variety of cellular processes seemingly critical for malignant growth. As such, there is considerable interest in targeting mitochondrial metabolism in cancer. However, notwithstanding the few drugs targeting mutant dehydrogenase activity, nearly all hopeful 'mito-therapeutics' cannot discriminate cancerous from non-cancerous OXPHOS and thus suffer from a limited therapeutic index. The present project was based on the premise that the development of efficacious mitochondrial-targeted anti-cancer compounds requires answering two fundamental questions: 1) is mitochondrial bioenergetics in fact different between cancer and non-cancer cells? and 2) If so, what are the underlying mechanisms? Such information is particularly critical for the subset of human cancers, including acute myeloid leukemia (AML), in which alterations in mitochondrial metabolism are implicated in various aspects of cancer biology (e.g., clonal expansion and chemoresistance). Herein, we leveraged an in-house diagnostic biochemical workflow to comprehensively evaluate mitochondrial bioenergetic efficiency and capacity in various hematological cell types, with a specific focus on OXPHOS dynamics in AML. Consistent with prior reports, clonal cell expansion, characteristic of leukemia, was universally associated with a hyper-metabolic phenotype which included increases in basal and maximal glycolytic and respiratory flux. However, despite having nearly 2-fold more mitochondria per cell, leukemic blasts, as well as chemoresistant AML were both consistently hallmarked by intrinsic limitations in oxidative ATP synthesis (i.e., OXPHOS). Remarkably, by performing experiments across a physiological span of ATP free energy (i.e., ΔG_{ATP}), we provide direct evidence that, rather than contributing to cellular ΔG_{ATP}, leukemic mitochondria are particularly poised to consume ATP. Relevant to AML biology, acute restoration of OXPHOS kinetics proved highly cytotoxic to leukemic blasts, suggesting that active OXPHOS repression supports aggressive disease dissemination in AML. Taken together, these findings argue against ATP being the primary output of mitochondria in leukemia and provide proof-of-principle that restoring, rather than disrupting, OXPHOS and/or cellular ΔG_{ATP} in cancer may represent an untapped therapeutic avenue for combatting hematological malignancy and chemoresistance.					
15. SUBJECT TERMS None listed.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	95	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	Page 2
2. Keywords	Page 2
3. Accomplishments	Pages 2-9
4. Impact	Page 11
5. Changes/Problems	Page 12
6. Products	Page 15
7. Participants & Other Collaborating Organizations	Page 17
8. Special Reporting Requirements	Page 20
9. Appendices	Page 20

1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The identification of discrete mitochondrial phenotypes across cancers is a prerequisite to successful implementation of mitochondrial precision medicine. At present, while several reports exist documenting differences in AML mitochondrial quantity and quality, the molecular details underpinning these observations remain largely unknown. Identifying the precise molecular cause of a given mitochondrial shift demarcates the difference between effective targeted drug delivery and catastrophic secondary toxicity. The natural sequitur to this is the central goal of this application; identify the unique bioenergetic signature(s) of AML tumorigenesis and chemoresistance. To accomplish this task, the current application proposes to employ a state-of-the-art assay platform which unites discovery-based molecular omics with comprehensive bioenergetic characterization. Given the unprecedented diagnostic utility of this methodological platform, we anticipate the discovery of distinct bioenergetic parameters and proteomic signatures which can subsequently be used to guide future AML-targeted therapies. Collectively, the data generated from the current proposal will represent the necessary first steps in the design and development of efficacious mitochondria-targeted chemotherapeutics.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Mitochondrial bioenergetics, respirometry, proteomics, acute myeloid leukemia, OXPHOS efficiency

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Aim 1: Determine the unique bioenergetic phenotypes that differentiate AML cells from non-cancerous hematopoietic controls, as well as those linked to AML chemoresistance, the major cause of treatment failure.

Major Task 1 – Seek IRB and HRPO approval for the use of PBMCs. Perform mitochondrial diagnostics workflow on specified AML cell lines, as well as human PBMCs. All experiments will be performed using isolated mitochondria.

Subtask 1 - Experiments will be carried out on 6 distinct AML cell lines (KG-1a, MV4-11, HL-60 control, HL-60/Ara-C, HL-60/DNR, HL-60/VCR), as well as PBMCs. To reach the target sample size per group of N=10 for AML cell lines and N=30 for human subjects will require ~90 full experimental work days (1 day is required to run a given mitochondrial preparation through the entire diagnostics workflow). Assuming mitochondrial preparations are performed 2-3 days/week, we anticipate all functional data being generated in the first 9 months of the project.

Expected Milestone(s) for Major Task 1 – At the conclusion of these experiments we will have generated a comprehensive bioenergetic profile of AML, as well as AML chemoresistance. We anticipate that these data will unveil several AML-specific bioenergetic signatures which can subsequently be utilized by the entire scientific community to determine the potential druggability of these novel mitochondrial targets.

Major Task 2 – Validate the diagnostic potential of a permeabilized cell model for bioenergetic characterization.

Subtask 1 - Experiments will be carried out on a subset of AML cell lines (MV4-11, HL-60 control) and PBMCs using a truncated version of our mitochondrial diagnostics workflow adapted for the utilization of a permeabilized cell model. For these experiments a sample size of N=10 will be achieved for all groups.

Expected Milestone(s) for Major Task 2 – The goal of these experiments is to determine which, if any, of the AML-specific bioenergetic phenotypes observed in isolated mitochondria can also be observed using a permeabilized cell model. While the use of permeabilized cells limits the diagnostic capabilities of the platform, such a system is more amenable to the translation of this work to the clinic where it may be difficult to obtain the necessary volume of blood needed for a full mitochondrial preparation. Validation of a permeabilized cell approach to blood cancer mitochondrial bioenergetic characterization could open the door for mitochondrial precision medicine in which unique bioenergetic profiles can be screened for on a patient to patient basis to determine whether a given “mito-therapeutic” should be prescribed.

Aim 2: Determine the phospho-proteomic landscape of mitochondria derived from hematopoietic controls and AML cells (sensitive and refractory to several chemotherapeutics) using bottom-up mass spectrometry.

Major Task 3 – Characterize the mitochondrial proteome and phosphoproteome of AML tumorigenesis and chemoresistance.

Expected Milestone(s) for Major Task 3: We anticipate the discovery of novel phospho-signatures associated with AML tumorigenesis and chemoresistance. These findings, while preliminary, are anticipated to serve as a powerful resource for future studies tasked with validating the druggability of implicated kinase families identified herein.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

This last year we published two major publications, which together describe the bulk of the discoveries made because of funding support by the DoD. The principal goal of our “Impact award” was to identify functional bioenergetic vulnerabilities entirely unique to AML mitochondria. Using in-house mitochondrial diagnostics, we were able to identify a novel mitochondrial dependency that effectively demarcates AML mitochondria from normal. Specifically, AML mitochondria were unexpectedly discovered to consume, rather than produce, ATP. Importantly, blocking mitochondrial ATP consumption to restore efficient mitochondrial ATP production proved highly cytotoxic, suggesting that mitochondrial ATP consumption underlies AML survival. In support of this, mitochondrial ATP consumption is most pronounced in AML cells made refractory to chemotherapy. Our findings detailing that AML mitochondria are functionally unique have important treatment implications, which we will describe below.

Publications during this period are detailed below. All articles are open access.

Article #1 - Intrinsic OXPHOS limitations underlie cellular bioenergetics in leukemia. [Intrinsic OXPHOS limitations underlie cellular bioenergetics in leukemia | eLife \(elifesciences.org\)](#). Full article can be found in Appendix 1.

Article #2 - Alterations in sphingolipid composition and mitochondrial bioenergetics represent synergistic therapeutic vulnerabilities linked to multidrug resistance in leukemia. [Alterations in sphingolipid composition and mitochondrial bioenergetics represent synergistic therapeutic vulnerabilities linked to multidrug resistance in leukemia - Fisher-Wellman - 2022 - The FASEB Journal - Wiley Online Library](#). Full article can be found in Appendix 2.

In addition to the original research articles above, this past reporting period, we co-authored a review in the Journal of Biological Chemistry on mitochondrial functional analysis. The intent of this review was to serve as a practical guide for the design and interpretation of both cellular and mitochondrial bioenergetic studies.

Article #3 - From OCR and ECAR to energy: Perspectives on the design and interpretation of bioenergetics studies. [From OCR and ECAR to energy: Perspectives on the design and interpretation of bioenergetics studies - ScienceDirect](#). Full article can be found in Appendix 3.

In addition to submitting our work for publication, this past funding period we also made several key discoveries related to Major Tasks 1-3. For clarity, such discoveries that relate to Major Task 3 are discussed first, as Major Task 3 was the focus of this past year. Additional discoveries related to Major Tasks 1-2 are also summarized.

There were 2 primary milestones set out for Major Tasks 3:

- 1) Characterize the mitochondrial proteome and phosphoproteome of AML, as well as chemoresistant AML.
- 2) Correlate changes in the AML mitochondrial phosphoproteome to AML respiratory control.

Accomplishments of stated milestones: This past year, we focused heavily on performing the phosphoproteomic experiments outlined in the SOW. We accomplished two major phosphoproteomic studies. In the first, we characterized the mitochondrial phosphoproteome in isolated mitochondria from AML cell lines (MV4;11 wildtype, venetoclax resistant MV4;11 and OCI-AML2), and then compared the data to isolated mitochondria from healthy peripheral blood cell mononuclear cells (PBMCs). Relative to PBMC, we observed a general increase in mitochondrial phosphopeptide abundance in AML cell line mitochondria. These data are consistent with our published findings that AML mitochondrial respiration is directly inhibited by ATP. Interestingly, among the 84 differentially expressed phosphopeptides, phosphorylation of serine 42 on SLC25A6 (ANT3) was a top hit (Figure 1). Phosphorylation of serine 42 on SLC25A6 has also been reported in other cancer phosphoproteomics screens and we recently published that SLC25A6 is differentially expressed in AML, relative to PBMC (PMID: 34132194).

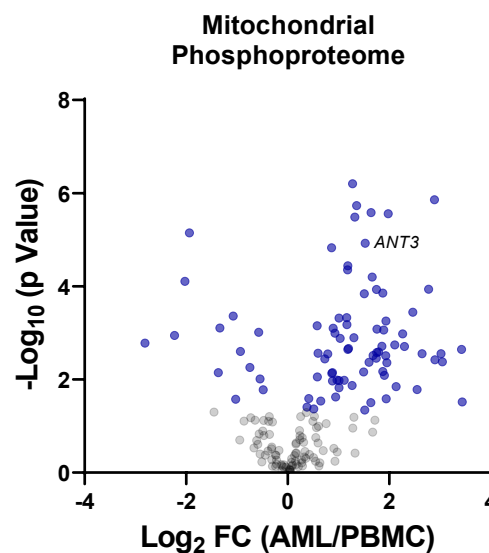


Figure 1. Volcano plot depicting phosphopeptide abundance from mitochondria isolated from AML cells, as well as PBMC. N=3/group

In our second phosphoproteomics study, we incubated isolated mitochondria from OCI-AML2 cells with different amounts of the ATP free energy and then assessed the phosphoproteome. As we have seen with out AML cell lines, in the presence of ATP free energy, maximal uncoupled respiration is severely blunted to < 10% of the no ATP condition in OCI-AML2 mitochondria (Figure 2A). Comparing high ATP free energy to the control group, we observed increased mitochondrial phosphopeptide abundance (Figure 2B). Together, these data demonstrate a direct relationship between inhibition of AML mitochondrial respiration by ATP and increased mitochondrial protein phosphorylation.

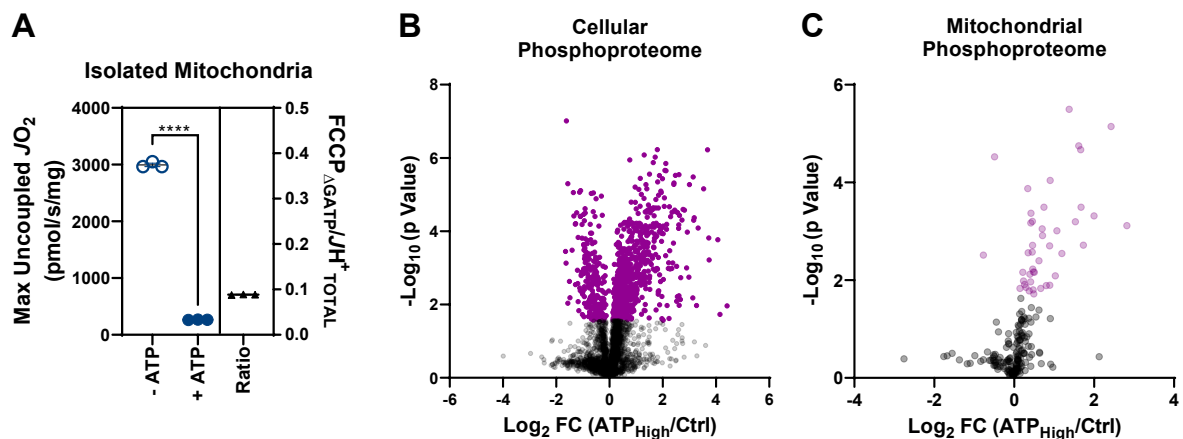


Figure 2. (A) Maximal uncoupled respiration in OCI-AML2 mitochondria in the absence and presence of ATP free energy. The right axis displays the ratio of respiration assessed in the presence versus the absence of ATP. (B) Volcano plot depicting phosphopeptide abundance in OCI-AML2 isolated mitochondria either incubated with high ATP free energy or vehicle control. Data in panel B includes both mitochondrial and non-mitochondrial phosphopeptides. (C) Volcano plot depicting phosphopeptide abundance in OCI-AML2 isolated mitochondria either incubated with high ATP free energy or vehicle control. Data in panel C includes mitochondrial phosphopeptides only. N=3/group, *P<0.05

Table 1 displays the mitochondrial phosphopeptides that were significantly (adjusted p value cutoff of 01.) increased in abundance by ATP. Although ATP free energy induced increased mitochondrial phosphorylation, this phosphorylation does not appear to be directed at the electron transport chain (ETC) respiratory complexes. The only ETC subunit that showed increased phosphorylation were ATP5F1A, which is the alpha subunit of ATP synthase, and COX4I1, a subunit of cytochrome c oxidase.

Aside from ETC complexes, many mitochondrial membrane bound proteins were hyperphosphorylated in response to ATP free energy. These included proteins implicated in apoptosis, as well as mitochondrial cristae morphology.

In accordance with the goals outlined in Aim 2, our results linking mitochondrial protein phosphorylation to ATP-dependent respiration inhibition in AML mitochondria are novel. Aside from ATP5F1A and COX4I1, most of the hyper-phosphorylated substrates in AML mitochondria did not correspond to the mitochondrial ETC. We interpret these data to mean that inhibition of AML respiration by ATP may not be due to direct inhibitory phosphorylation of the ETC complexes. An interesting hypothesis that sprang from our phosphoproteomics screen relates to the idea that hyper-phosphorylation of membrane bound proteins may alter mitochondrial fission/fusion or cristae architecture in a manner that results in respiratory inhibition. Whether any of these phosphosites are causally related to AML mitochondrial function is an exciting area of research for us as we move beyond this award funding period.

Table 1.			
Gene	Log2FC (ATP High/Control)	P Adjusted (q<0.1)	PhosphoSite
TFAM	1.377446675	0.000658172	1xPhospho [S124(100)]
PDHA1	2.424508217	0.000739396	
ACAD9	1.662567509	0.001103717	1xPhospho [S474(100)]
IMMT	1.614706831	0.001103717	1xPhospho [S387(100)]
LIG3	-0.493222602	0.001227496	1xPhospho [S210(100)]
AKAP1	0.897915527	0.003115582	1xPhospho [S251(99.5)]
BCL2L13	0.340377683	0.003935954	1xPhospho [S288(100)]
PDHA1	1.675286977	0.007375006	2xPhospho [S331(100)]
SPIRE1	0.746663869	0.007375006	1xPhospho [S523(100)]
BCL2L13	0.420700244	0.008760274	1xPhospho [S420(100)]
PDHA1	1.999282125	0.009024972	1xPhospho [S270(100)]
ARMC10	0.454764567	0.009917252	1xPhospho [S45(100)]
MFF	0.414975351	0.009917252	
TFAM	1.532516774	0.009917252	1xPhospho [S124(100)]
ETFA	2.821706737	0.010478547	1xPhospho [S15(100)]
COX4I1	0.701351627	0.011488208	1xPhospho [S26(100)]
PDHA1	1.070575298	0.011851047	1xPhospho [S331(100)]
BCKDHA	0.708516004	0.013975612	1xPhospho [S347(100)]
FKBP8	1.73203518	0.019405053	1xPhospho [T342(100)]
MTFR1L	0.458565806	0.019405053	1xPhospho [S103(100)]
PDHA1	0.88468733	0.019405053	1xPhospho [S331(100)]
BCL2L11	1.191186178	0.024460019	1xPhospho [S87(100)]
CHCHD3	0.346893866	0.024460019	1xPhospho [S50(100)]
FASTKD5	0.453239489	0.024460019	1xPhospho [S95(100)]
LIG3	-0.76972803	0.02533299	1xPhospho [S242(99.3)]
NRDC	0.618191353	0.031756287	1xPhospho [S94(100)]
C1QBP	0.464220392	0.045182903	
ATP5F1A	0.503740128	0.047513021	1xPhospho [S53(100)]
MFF	0.216582579	0.047513021	1xPhospho [S131(100)]
TIMM44	0.483115396	0.047513021	1xPhospho [S180(100)]
ARMC10	0.392163162	0.050286281	1xPhospho [S45(100)]
FKBP8	1.02806166	0.052329736	1xPhospho [S297(100)]
FUNDC2	0.232707993	0.065087142	1xPhospho [S151(100)]
MTFR1L	0.411880404	0.065569539	1xPhospho [S234(100)]
AIFM1	0.260365398	0.07195114	1xPhospho [S268(100)]
PLD6	0.892915111	0.07195114	1xPhospho [S239(100)]
TOMM20	0.804250548	0.07195114	1xPhospho [S138(99.7)]
AKAP1	0.293630736	0.075920064	1xPhospho [S592(100)]
BNIP3L	0.665682036	0.075971822	1xPhospho [S166(99.5)]
SHMT2	0.144730598	0.075971822	1xPhospho [T420(100)]
MFF	0.471699014	0.079185722	1xPhospho [S131(100)]
MRPL1	0.37617736	0.081389673	1xPhospho [S320(100)]
YRDC	0.490067186	0.091931117	1xPhospho [S37(100)]

Stated Goals not met – None

Additional Discoveries Related to Aim 1:

Mitochondria in AML consume cellular ATP to sustain mitochondrial polarization: Despite stark deficiencies in OXPHOS, AML cells have more mitochondria than normal cells and those mitochondria are more polarized. Mitochondrial membrane potential can be sustained in one of two ways; 1) via functional OXPHOS or 2) via ATP synthase reversal. Given that AML cells are highly OXPHOS deficient, we hypothesized that AML mitochondria may sustain polarization by hydrolyzing cellular ATP. To test this, we assessed mitochondrial polarization in AML cells in the absence and presence of ATP synthase inhibition. In wildtype MV411, administration of the ATP synthase inhibitor oligomycin triggered mitochondrial depolarization (**Fig. 3A-B**). Depolarization induced by oligomycin was indistinguishable from that induced by FCCP in venetoclax resistant MV411 (**Fig. 3B**; 'MV411_{vclax}'). Unlike that seen in AML, in healthy bone-marrow mononuclear cells, oligomycin induced mitochondrial hyperpolarization, consistent with functional OXPHOS in normal bone-marrow (**Fig. 3B**; 'Normal'). In agreement with our data using intact cells, in isolated mitochondria from healthy PBMCs, oligomycin again triggered hyperpolarization (**Fig. 3C**). In contrast, using mitochondria isolated from AML cell lines (HL-60, KG-1), acute blockade of mitochondrial ATP uptake with carboxyatractyloside induced stark depolarization (**Fig. 3D**). Taken together, these exciting findings indicate that mitochondria in AML are typified by a unique OXPHOS reversal phenotype whereby mitochondrial polarization is sustained by ATP hydrolysis. Moreover, non-canonical, OXPHOS-independent mitochondrial polarization appears most pronounced in refractory AML, suggesting that mitochondrial polarization is critical to the

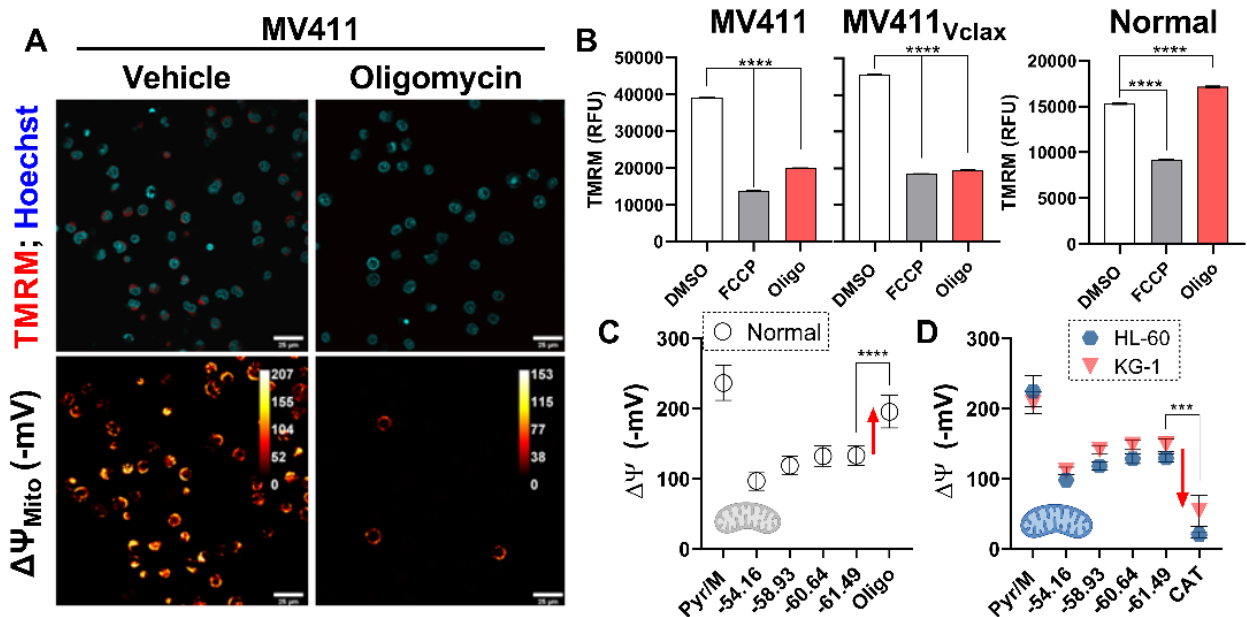


Fig. 3. (A-B) Mitochondrial polarization in intact cells assessed via TMRM fluorescence, using both (A) confocal microscopy and (B) flow cytometry. Prior to TMRM, cells were exposed for 15 minutes to vehicle, oligomycin (5 μ M), or FCCP (5 μ M). Normal indicates healthy bone marrow mononuclear cells. (C-D) Membrane potential ($\Delta\Psi$) in isolated mitochondria from healthy PBMCs, as well as AML cell lines. Red arrows indicate the impact of blocking ATP utilization on $\Delta\Psi$. (B, D) N=3/group; (C) N=8/group, *P<0.05.

Additional Discoveries Related to Aim 1:

Targeting mitochondrial membrane potential with organic cations restores venetoclax sensitivity in drug resistant AML: Using an in-house drug screen intended to identify novel disrupters of AML mitochondrial metabolism, we recently identified a family of small molecules uniquely capable of causing AML mitochondrial depolarization. The shared ability of these compounds to induce mitochondrial depolarization derives from the fact that they are all positively charged. At physiological pH, all 15 compounds carry a positive charge, and thus are functionally classified as organic cations (Fig. 4A). Driven by the mitochondrial voltage potential, organic cations accumulate in the negatively charged matrix where they directly interfere with mitochondrial polarization (Fig. 4B). In venetoclax resistant MV411, acute exposure to organic cations induced dose-dependent cytotoxicity (Fig. 4C). In addition to single-agent efficacy, organic cations synergized with venetoclax to completely eradicate the ability of AML cells to form colonies (Fig. 4D). In venetoclax resistant AML, co-administration of the organic cation dodecyl-triphenylphosphonium (dodecyl-TPP) restored venetoclax sensitivity (Fig. 4F). Taken together, these data provide pre-clinical evidence in support of leveraging organic cations to treat AML. Moreover, given that many of our identified organic cations are already FDA approved for various non-AML indications (Fig. 4A), such compounds can be reasonably repurposed for immediate testing in AML.

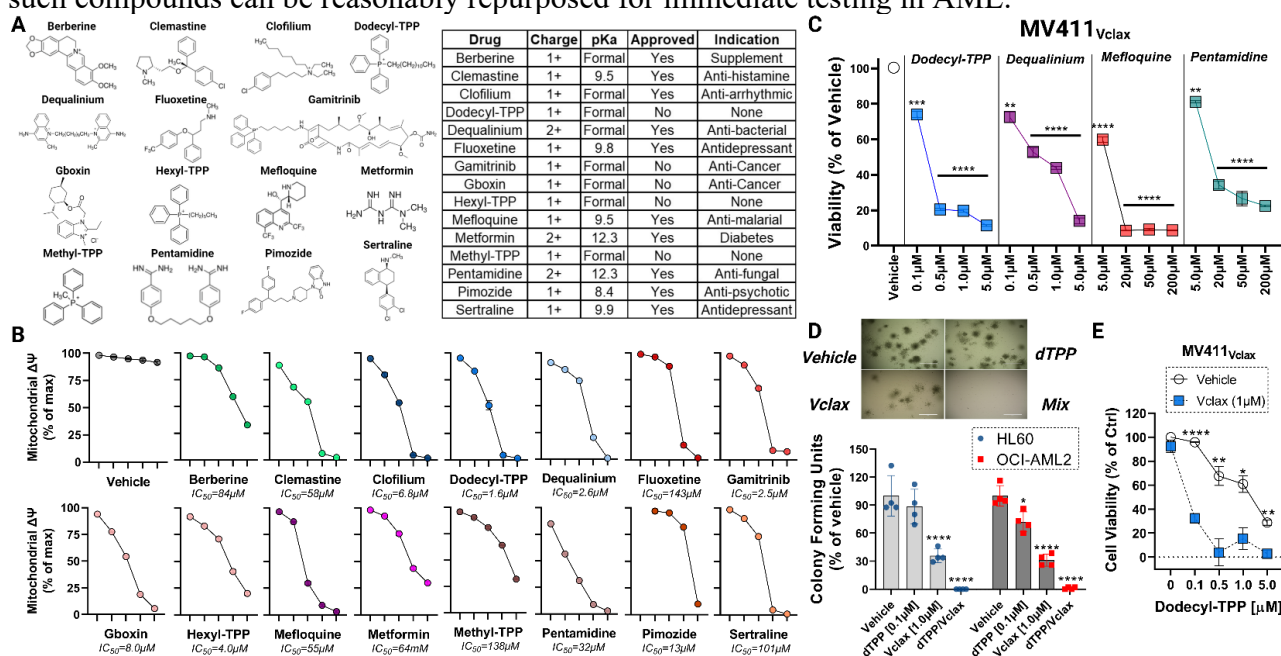


Fig. 4. (A) Organic cation structures, as well as a table detailing the charge state and pKa of each cation, as well as their FDA approval status and therapeutic implication. (B) Mitochondrial membrane potential in isolated mitochondria from OCI-AML2 cells in response to organic cation dose escalation. Mitochondria were energized with succinate (10mM). The IC₅₀ against membrane potential is listed below each cation. N=3/cation. (C) Cell viability in MV411_{Vclax} in response to 48hr exposure to organic cations. N=6/cation. (D) Quantification of colony forming units in HL60 and OCI-AML2 cells. Cells were continuously exposed to vehicle, dodecyl-TPP (0.1 μM), venetoclax (1 μM), or dodecyl-TPP + venetoclax. Representative images are from a CFU assay in HL60 cells. N=4/group. (C-D) *P<0.05 relative to vehicle. (E) Cell viability in MV411_{Vclax} cells in response to 48hr exposure to dodecyl-TPP. Exposures were performed in the absence and presence of venetoclax (1 μM). N=3/group, (E) *P<0.05 relative to the no venetoclax condition (open circles labeled as 'Vehicle').

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Results from our work were presented at the 2021 Virtual Keystone Symposia on Tumor Metabolism. Travel restrictions due to the COVID-19 pandemic have limited our ability to present our findings at scientific meetings.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Major findings have been published in peer-reviewed open-access journals.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

This award has ended. We plan to leverage the insights afforded by our Idea Award to thwart refractory AML by taking advantage of AML’s unique underlying mitochondrial bioenergetics.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Similar to a growing list of human solid organ malignancies, AML presents with alterations in mitochondrial metabolism. Pioneering studies over the past several years have repeatedly demonstrated efficacy in targeting mitochondria in AML. Although mitochondrial targeted therapies are beginning to enter clinical trials, the majority of these hopeful ‘mito-therapeutics’ suffer from a limited therapeutic index based on their inability to discriminate cancerous from non-cancerous OXPHOS. This represents a significant barrier to progress, as safe and effective mitochondrial therapies will undoubtedly require cancer-cell selectivity. Fortunately, evidence from our group demonstrates that all mitochondria are not alike but, in fact, are unique in composition and function within each cell type, including AML. For example, AML mitochondria are characterized by intrinsic OXPHOS deficiencies that force AML to sustain mitochondrial polarization exclusively by hydrolyzing cellular ATP. Informed by this discovery, AML mitochondria can be most effectively targeted using small molecules that either disrupt mitochondrial ATP exchange across the outer and inner membrane or specifically disrupt mitochondrial polarization. Because drug resistant AML prioritizes mitochondrial polarization over traditional ATP synthesis by OXPHOS, such therapies are also efficacious in otherwise refractory cells. Armed with our unique understanding of AML mitochondrial biology, moving forward we are excited to leverage this information to develop AML-specific mitochondrial targeted drugs.

To summarize our impact on the field, the goal of our Idea Award was to identify mitochondrial bioenergetic signatures that demarcate normal from AML. With respect to this goal, work funded through our Idea Award has resulted in the following seminal discoveries:

1. AML mitochondria present with intrinsic deficiencies in oxidative ATP synthesis (i.e., OXPHOS).
2. OXPHOS deficient AML sustains mitochondrial polarization by hydrolyzing cellular ATP.
3. Refractory AML is eliminated upon targeting mitochondrial membrane potential with organic cations.

While the first two discoveries have already been published (PMID: 34132194, PMID: 34888943), the exciting findings that organic cations may prove useful as an anti-leukemic therapy is an ongoing area of research in the lab. This line of investigation would not have been possible without the generous support of the Idea Award funding mechanism and the Department of Defense.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Aside from the implications of our work to leukemia, our results demonstrate that using intact respirometry alone to diagnose mitochondrial 'health' in cancer masks potential unique bioenergetic phenotypes of cancer that only become apparent when comprehensive approaches are leveraged to diagnose the mitochondrial network. The methodologies developed for this work thus have the potential to shift the paradigm with respect to mitochondrial 'function' in cancer and establish an entirely new field of research focused on understanding and leveraging the unique, intrinsic bioenergetic phenotypes of human malignancy.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

None to report.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

As detailed above, the methodologies developed for this work thus have the potential to shift the paradigm with respect to mitochondrial 'function' in cancer and open up an entirely new field of research focused on understanding and leveraging the unique, intrinsic bioenergetic phenotypes of human malignancy. While it will likely take some time for our work to translate to new therapies, our work represents a critical step towards new mitochondrial-localized therapy relative to leukemia and potentially other cancers.

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nelson MAM, **Fisher-Wellman KH**. Mitochondrial Diagnostics: A Discovery-Based Biochemical Platform for Phenotyping Human Peripheral Blood Cell Mitochondria. *Methods Mol Biol.* 2021;2277:371-389. doi: 10.1007/978-1-0716-1270-5_23. PubMed PMID: 34080163.

Fisher-Wellman KH, Hagen JT, Neufer PD, Kassai M, Cabot MC. On the nature of ceramide-mitochondria interactions - Dissection using comprehensive mitochondrial phenotyping. *Cell Signal.* 2021 Feb;78:109838. doi: 10.1016/j.cellsig.2020.109838. Epub 2020 Nov 17. Review. PubMed PMID: 33212155; PubMed Central PMCID: PMC7775270.

Nelson MA, McLaughlin KL, Hagen JT, Coalson HS, Schmidt C, Kew KA, McClung JM, Neufer PD, Brophy P, Vohra NA, Liles D, Cabot MC, **Fisher-Wellman KH**. Intrinsic oxidative phosphorylation limitations underlie cellular bioenergetics in leukemia. *Elife.* 2021 Jun 16;10:e63104. doi: 10.7554/eLife.63104, PMID: 34132194

Fisher-Wellman KH, Hagen JT, Kassai M, Kao LP, Nelson MAM, McLaughlin KL, Coalson HS, Fox TE, Tan SF, Feith DJ, Kester M, Loughran TP Jr, Claxton DF, Cabot MC. Alterations in sphingolipid composition and mitochondrial bioenergetics represent synergistic therapeutic vulnerabilities linked to multidrug resistance in leukemia. *FASEB J.* 2022 Jan;36(1):e22094. doi: 10.1096/fj.202101194RRR

Schmidt CA, **Fisher-Wellman KH**, Neufer PD. From OCR and ECAR to energy: Perspectives on the design and interpretation of bioenergetics studies. *J Biol Chem.* 2021 Oct;297(4):101140. doi: 10.1016/j.jbc.2021.101140. Epub 2021 Aug 28. PMID: 34461088

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Results from our work were presented at the 2021 Virtual Keystone Symposia on Tumor Metabolism.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

<i>Name:</i>	<i>Mary Smith</i>
<i>Project Role:</i>	<i>Graduate Student</i>
<i>Researcher Identifier (e.g. ORCID ID):</i>	<i>1234567</i>
<i>Nearest person month worked:</i>	<i>5</i>

<i>Contribution to Project:</i>	<i>Ms. Smith has performed work in the area of combined error-control and constrained coding.</i>
---------------------------------	---

<i>Funding Support:</i>	<i>The Ford Foundation (Complete only if the funding support is provided from other than this award.)</i>
-------------------------	---

Name: Kelsey Fisher-Wellman
Project Role: PI
Researcher Identifier (e.g. ORCID ID): ORCID ID: 0000-0002-0300-829X
Nearest person month worked: 4.2

Contribution to Project: Dr. Fisher-Wellman is involved with all aspects of the project.

Funding Support: Co-I on the following NIH R01 grants: R01DK125812, R01DK129455, R01AG069679, R01HL157659-01, P01CA171983. PI on the following DoD grant: W81XWH-22-1-0625

Name: Tyler Hagen
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2

Contribution to Project: Mr. Hagen assisted with data collection

Funding Support:

Name: Patricia Brophy
Project Role: Human Subjects Coordinator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.2

Contribution to Project: Ms. Brophy assisted with human subject recruitment

Funding Support:

Name: Myles Cabot
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.8

Contribution to Project: Dr. Cabot provided cell lines and participated in the chemoresistance arm of the project.

Funding Support: NIH P01 CA171983

Name: Hannah Coalson
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2

Contribution to Project: Ms. Coalson assisted with data collection

Funding Support:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ebrap.org/eBRAP/public/index.htm> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil/Pages/Resources.aspx>) should be updated and submitted with attachments.*

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Intrinsic OXPHOS limitations underlie cellular bioenergetics in leukemia

Margaret AM Nelson^{1,2†}, Kelsey L McLaughlin^{1,2†}, James T Hagen^{1,2}, Hannah S Coalson^{1,2}, Cameron Schmidt^{1,2}, Miki Kassai^{2,3}, Kimberly A Kew³, Joseph M McClung^{1,2,4}, P Darrell Neuffer², Patricia Brophy², Nasreen A Vohra⁵, Darla Liles⁶, Myles C Cabot^{2,3}, Kelsey H Fisher-Wellman^{1,2*}

¹Department of Physiology, Brody School of Medicine, East Carolina University, Greenville, United States; ²East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, United States; ³Department of Biochemistry and Molecular Biology, Brody School of Medicine, East Carolina University, Greenville, United States; ⁴Department of Cardiovascular Sciences, Brody School of Medicine, East Carolina University, Greenville, United States; ⁵Department of Surgery, Brody School of Medicine, East Carolina University, Greenville, United States; ⁶Department of Internal Medicine, Brody School of Medicine, East Carolina University, Greenville, United States

Abstract Currently there is great interest in targeting mitochondrial oxidative phosphorylation (OXPHOS) in cancer. However, notwithstanding the targeting of mutant dehydrogenases, nearly all hopeful ‘mito-therapeutics’ cannot discriminate cancerous from non-cancerous OXPHOS and thus suffer from a limited therapeutic index. Using acute myeloid leukemia (AML) as a model, herein, we leveraged an in-house diagnostic biochemical workflow to identify ‘actionable’ bioenergetic vulnerabilities intrinsic to cancerous mitochondria. Consistent with prior reports, AML growth and proliferation was associated with a hyper-metabolic phenotype which included increases in basal and maximal respiration. However, despite having nearly 2-fold more mitochondria per cell, clonally expanding hematopoietic stem cells, leukemic blasts, as well as chemoresistant AML were all consistently hallmarked by intrinsic OXPHOS limitations. Remarkably, by performing experiments across a physiological span of ATP free energy, we provide direct evidence that leukemic mitochondria are particularly poised to consume ATP. Relevant to AML biology, acute restoration of oxidative ATP synthesis proved highly cytotoxic to leukemic blasts, suggesting that active OXPHOS repression supports aggressive disease dissemination in AML. Together, these findings argue against ATP being the primary output of leukemic mitochondria and provide proof-of-principle that restoring, rather than disrupting, OXPHOS may represent an untapped therapeutic avenue for combatting hematological malignancy and chemoresistance.

***For correspondence:**
fisherwellmank17@ecu.edu

[†]These authors contributed equally to this work

Competing interests: The authors declare that no competing interests exist.

Funding: See page 26

Received: 15 September 2020

Accepted: 16 June 2021

Published: 16 June 2021

Reviewing editor: Ivan Topisirovic, Jewish General Hospital, Canada

© Copyright Nelson et al. This article is distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use and redistribution provided that the original author and source are credited.

Introduction

In comparison to normal hematopoietic cells, various human leukemias present with increases in mitochondrial mass and higher basal respiration rates (Farge et al., 2017; Goto et al., 2014b; Goto et al., 2014a; Sriskanthadevan et al., 2015; Suganuma et al., 2010), the latter of which appears to sensitize them to global disruptions in mitochondrial flux (Kuntz et al., 2017; Lagadinou et al., 2013; Lee et al., 2015; Mirali et al., 2020; Skrtić et al., 2011; Xiang et al., 2015). Although these studies have ignited interest in mitochondrial-targeted chemotherapeutics (Guièze et al., 2019; Panina et al., 2020), experimental rationale for targeting OXPHOS in leukemia is largely based on the assumption that heightened respiration is representative of the cancerous

mitochondrial network's attempt to accommodate an increased ATP demand (i.e. increased 'OXPHOS reliance'). However, identical increases in mitochondrial respiration can derive from any number of physiological stimuli, ranging from increased demand for ATP resynthesis to decreased OXPHOS efficiency. Distinguishing between these potential outcomes is critical, as such insight likely demarcates targeted drug efficacy from undesirable systemic toxicity. For example, it is currently unclear how targeting increased 'OXPHOS reliance' in leukemia can specifically disrupt leukemic oxidative metabolism without impacting OXPHOS in other highly metabolic organs (e.g. brain, heart, muscle).

Given the ubiquitous necessity of OXPHOS for healthy cellular metabolism, a major barrier to mitochondrial-targeted drugs in leukemia relates to the need for cancer-cell selectivity (Cohen, 2010; Dykens and Will, 2007). The current project was based on the premise that establishing cause and effect between mitochondrial bioenergetics and cancer is one of the keys to developing targeted and more effective therapies. As a first step, this will undoubtedly require advanced technical approaches capable of quantifying the interplay among the major mitochondrial thermodynamic free energy driving forces to distinguish between changes in bioenergetic demand versus efficiency. To this end, our group recently developed a diagnostic biochemical workflow that quantifies the changes in free energy driving forces over the entire range of respiratory demand, thus providing a comprehensive profile of mitochondrial bioenergetic efficiency and capacity, relative to the underlying proteome (Fisher-Wellman et al., 2019; Fisher-Wellman et al., 2018; McLaughlin et al., 2018).

Herein, we leveraged our mitochondrial diagnostics workflow across several hematological cell types, including AML cell lines, primary human leukemias, and AML cells made refractory to the chemotherapeutic venetoclax. Clonal cell expansion in leukemia, including chemoresistant AML, was universally associated with two primary phenotypes; (1) higher basal respiration driven by increased cellular mitochondrial content, and (2) intrinsic OXPHOS repression. Parallel assessment of the underlying mitochondrial proteome linked this unique ability to bolster cellular mitochondrial content while simultaneously constraining oxidative ATP synthesis to shifts in adenine nucleotide translocase (ANT) isoform expression. Specifically, decreased ANT1 and increased ANT2/ANT3 appears to prime leukemic mitochondria for ATP uptake, rather than export, that, in turn directly inhibits the ability of mitochondrial OXPHOS to contribute to the cellular ATP-free energy (ΔG_{ATP}). These findings are consistent with recent evidence demonstrating that mitochondrial OXPHOS is dispensable for tumor growth (Martínez-Reyes et al., 2020), and raise the intriguing possibility that the requirement for mitochondria in leukemia may have little to do with oxidative ATP production but instead reflect a requirement for unimpeded mitochondrial flux to support other aspects of anabolic growth [e.g., NAD^+ regeneration (Luengo et al., 2020), nucleotide synthesis (Martínez-Reyes et al., 2020)]. Critically however, accommodating such a flux demand, at least in leukemia, appears to require intrinsic mitochondrial remodeling that allows for forward electron transport/oxygen consumption to occur alongside ATP consumption. Given that acute restoration of OXPHOS kinetics in AML proved highly cytotoxic to leukemic blasts, the present findings provide proof-of-principle that interventions designed to restore, rather than disrupt, OXPHOS may impart therapeutic efficacy across various hematological malignancies.

Results

Mitochondrial bioenergetic profiling of acute leukemia reveals respiratory flux limitations

To begin to characterize the mitochondrial network in leukemia, we selected three commercially available acute leukemia cell lines – HL-60, KG-1, MV-4-11 – and comprehensively evaluated their bioenergetic profiles. These cells arise from unique precursors along the hematopoietic lineage, express a diverse array of cell surface markers, and have distinct underlying genetics (Inoue et al., 2014; Mrózek et al., 2003; Rücker et al., 2006). Results were compared to peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers. The decision to use PBMC as a control was based on the assumption that comprehensive bioenergetic phenotyping of non-proliferative PBMC compared to various AML cell lines would provide sufficient experimental design contrast to reveal fundamental mitochondrial bioenergetic phenotypes potentially required for clonal cell expansion.

Using intact PBMC and leukemia cell lines, respiratory flux (JO_2) was assessed under basal conditions, as well as in response to ATP synthase inhibition (oligomycin), and FCCP titration (i.e. mitochondrial uncoupler). All experiments were performed in bicarbonate-free IMDM growth media, supplemented with 10% FBS. Following FCCP titration, respiration was inhibited with a combination of rotenone (inhibits complex I) and antimycin A (inhibits complex III). Consistent with prior work in human leukemia (*Jitschin et al., 2014; Sriskanthadevan et al., 2015*), basal respiration normalized to cell count was elevated above PBMC across all leukemia lines and maximal respiratory flux was higher in KG-1 and MV-4-11 (**Figure 1A**). When normalized to basal respiration, oligomycin similarly inhibited respiration across groups and the fold change induced by FCCP was consistently blunted in leukemia (**Figure 1B**).

Given the large differences in cell size between PBMC and leukemia (**Figure 1—figure supplement 1A**), we reasoned that normalization to total protein would likely provide the most accurate index of absolute respiratory kinetics across groups. Interestingly, upon normalization to total protein, although basal respiration remained higher in KG-1 and MV-4-11, differences in maximal respiratory flux were eliminated, particularly at higher FCCP concentrations (**Figure 1C**). In fact, maximal FCCP-supported JO_2 was nearly two-fold lower in HL-60 compared to PBMC when normalized to total protein (**Figure 1C**). Relative to PBMC, maximal respiration induced by FCCP occurred at much lower concentrations in leukemia (i.e. lower K_m ; **Figure 1D**), with increasing FCCP concentrations leading to an overt bioenergetic collapse (i.e. diminishing respiration rates; **Figure 1C**; compare JO_2 at FC [2.0 μM] vs FC [5.0 μM]). Similar findings were observed using the mitochondrial uncoupler BAM15 (**Figure 1—figure supplement 1B–C**). Measurements of extracellular acidification (ECAR), an indirect readout of glycolytic flux, revealed higher extracellular acidification and a rightward shift in ECAR relative to oxygen consumption rate (OCR) in AML cell lines, consistent with prior reports detailing a hyper-metabolic phenotype in leukemia (**Figure 1—figure supplement 1E–F; Sukanuma et al., 2010**).

To determine if flux differences in leukemia could be explained by differences in mitochondrial content, nuclear and mitochondrial volumes were assessed independently by tetramethylrhodamine methyl ester (TMRM) or MitoTracker fluorescence and confocal microscopy (**Figure 1E–I, Figure 1—figure supplement 1D**). Absolute nuclear and mitochondrial volumes were higher in all leukemia lines (**Figure 1E–H**), consistent with leukemia's larger cell size (**Figure 1—figure supplement 1A**). However, when normalized to nuclear volume, mitochondrial content was elevated above PBMC only in HL-60 and MV-4-11 (**Figure 1I**). Interestingly, across all cell types, considerable discrepancies were apparent when protein-normalized maximal respiratory flux (**Figure 1C**) was compared to mitochondrial content (**Figure 1I**). This was particularly evident in HL-60 cells where mean maximal respiration, relative to the size of the underlying mitochondrial network, was ~5-fold lower compared to PBMC (**Figure 1J**). Together, these data suggested that at least a portion of the expansive mitochondrial network in AML may be biochemically constrained and thus unable to contribute to oxidative metabolism under basal conditions.

Intrinsic limitations to OXPHOS characterize the mitochondrial network in leukemia cell lines

To directly test OXPHOS kinetics in leukemia, two complementary assays were designed. Both assays used digitonin-permeabilized cells energized with identical carbon substrates, and respiratory flux was stimulated with either FCCP or ATP free energy. In the first assay, the maximal capacity of the electron transport system (ETS) was assessed by energizing permeabilized cells with saturating carbon substrates (P/M/G/S/O) and titrating in FCCP (**Figure 2A–B**). The use of multiple substrates was intended to saturate carbon substrate availability such that maximal ETS flux could be quantified. Using this approach, absolute respiration in substrate-replete permeabilized cells was comparable to that observed using intact cells treated with FCCP (**Figure 2C**), confirming maximal ETS flux in the permeabilized system. Note, maximum FCCP-supported flux under these conditions is indicated throughout as $\text{JH}^+_{\text{Total}}$ (**Figure 2A**). Relative to PBMC, $\text{JH}^+_{\text{Total}}$ was lower in HL-60, unchanged in KG-1, and higher in MV-4-11 (**Figure 2B**).

In mammalian cells, the vast majority of the adenylate pool is represented by ATP (i.e., ΔG_{ATP}), with typical values for ATP-free energy ranging from -56 to -64 kJ/mol (*Luptak et al., 2018; Roth and Weiner, 1991; Veech et al., 2002*). Thus, to evaluate OXPHOS kinetic efficiency in leukemia across a physiological range of ATP resynthesis demands, we utilized the creatine kinase (CK)

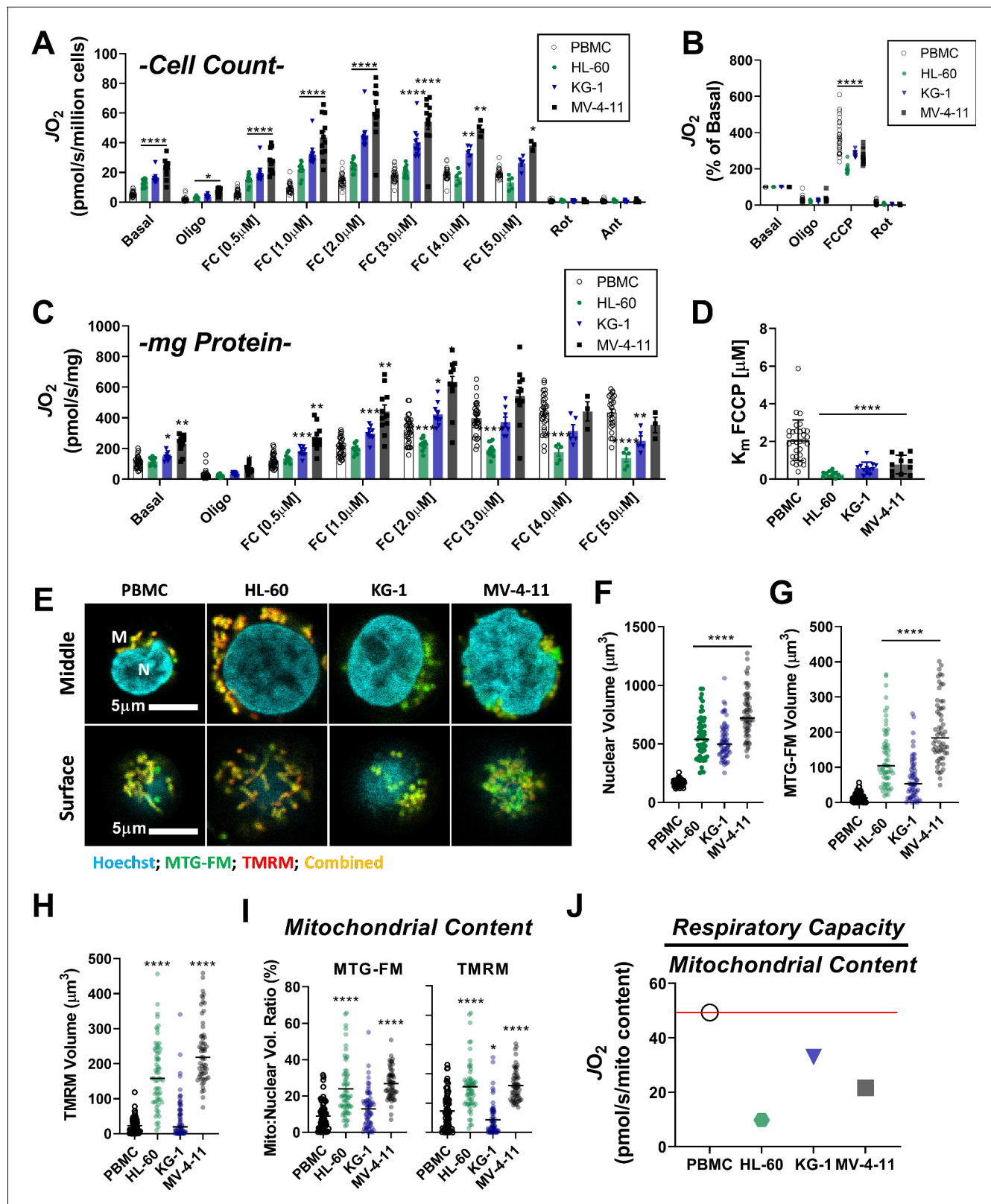


Figure 1. Leukemia exhibits impaired cellular respiratory capacity amid an increased mitochondrial network. All experiments were performed in intact cells. FCCP-stimulated flux normalized to cell count (A) and protein concentration (C) and represented as percentage of basal respiration (B). (D) K_m of FCCP calculated from FCCP titration (cell lines $n = 12$, PBMC $n = 31$). (E-I) Confocal microscopy was performed using two mitochondrial targeted cationic fluorescent dyes, MitoTracker Green FM (MTG-FM) and TMRM. (E) Representative fluorescent images of nuclear volume (F) and mitochondrial

Figure 1 continued on next page

Figure 1 continued

volume as measured by MTG-FM (G) and TMRM labeling (H) ($n = 65$ cells/cell type). (I) Ratios of mitochondrial to nuclear volumes assessed by MTG-FM and TMRM labeling. (J) Respiratory deficiency of cell type calculated by comparing respiratory capacity (the protein-normalized maximal respiration rate) to mitochondrial content (mitochondrial TMRM volume). Data are presented as mean $\pm$ SEM and analyzed by two-way ANOVA (A–D) and one-way ANOVA (F–I). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The online version of this article includes the following source data and figure supplement(s) for figure 1:

Source data 1. Raw values for 'Figure 1' and 'Figure 1—figure supplement 1'.

Figure supplement 1. Morphology of leukemia and respiratory flux stimulated by BAM15, a mitochondrial uncoupler.

energetic clamp (Fisher-Wellman et al., 2018; Glancy et al., 2013; Messer et al., 2004). This technique leverages the enzymatic activity of CK, which couples the interconversion of ATP and ADP to that of phosphocreatine (PCR) and free creatine (CR) such that extramitochondrial ATP-free energy (i.e. ΔG_{ATP}) can be empirically titrated using PCR. Using this approach, permeabilized cells were energized with the same carbon substrate mix used for the ETS capacity assay and respiration was stimulated at minimal ATP-free energy. Note, ΔG_{ATP} equal to -54.16 kJ/mol reflects an ATP/ADP ratio in vivo that would be expected to induce 'maximal' OXPHOS flux and is thus referred to throughout as ' $\text{JH}^+_{\text{OXPHOS}}$ ' (Figure 2D). Cytochrome C (Cyt C) was added to assess the integrity of the mitochondrial outer-membrane, and ΔG_{ATP} was then titrated via sequential additions of PCR. With respect to the ETS capacity assay, respiration stimulated by ΔG_{ATP} partially normalized JO_2 between MV-4–11 and PBMC and revealed decreased respiratory kinetics in both HL-60 and KG-1 (Figure 2E).

In both assays, the utilization of identical substrates ('P/M/G/S/O') allowed us to directly quantify absolute OXPHOS kinetics (' $\text{JH}^+_{\text{OXPHOS}}$ '), relative to the maximal capacity of the electron transport system (' $\text{JH}^+_{\text{Total}}$ '). Together, $\text{JH}^+_{\text{OXPHOS}}$ and $\text{JH}^+_{\text{Total}}$ provide a quantitative index of fractional OXPHOS capacity as the ratio of the two reflects the proportion of the entire respiratory system that can be used for OXPHOS (Figure 2F). A ratio of '1' reflects maximal OXPHOS reliance, whereas a ratio of '0' indicates that the mitochondrial proton current cannot be utilized for ATP synthesis. Strikingly, calculated fractional OXPHOS in leukemic mitochondria was consistently decreased compared to PBMC, corresponding to a factor of ~ 0.5 (Figure 2G), indicating that only half of the available ETS capacity in leukemia can be dedicated to OXPHOS under physiological ATP free energy constraints. Given that the OXPHOS network is responsible for driving ATP/ADP disequilibrium to establish cellular ΔG_{ATP} , low fractional OXPHOS was interpreted to reflect reduced bioenergetic efficiency in leukemia. Moreover, such findings indicate that traditional measurements of 'OXPHOS' capacity using intact cells (e.g. extracellular flux analysis) woefully underestimate true OXPHOS kinetics.

To differentiate between bioenergetic signatures inherent to proliferating cells and those which are unique to leukemia, experiments were repeated in mononuclear cells isolated from bone marrow aspirates collected from healthy volunteers. In these experiments, fractional OXPHOS in healthy bone marrow cells was comparable to PBMC and once again elevated above all leukemia cell lines (Figure 2G; ' $\text{BM}_{\text{Healthy}}$ ', Figure 2—figure supplement 1A–B). As an additional control, identical experiments were carried out in primary human muscle precursor cells (human myoblasts – 'HMB' Figure 2—figure supplement 1C–D). These cells were cultured from muscle biopsies uniformly collected from the gastrocnemius muscle (10 cm distal to the tibial tuberosity) of healthy human subjects and were intended to serve as a non-cancerous, proliferative human progenitor control. Importantly, fractional OXPHOS was elevated above leukemia in human muscle progenitor cells (Figure 2G: 'HMB'), indicating that decreased bioenergetic efficiency is not an absolute requirement of cellular proliferation, but rather a unique bioenergetic feature of leukemic mitochondria.

Exposure to physiological ΔG_{ATP} reveals direct inhibition of ETS flux by ATP in leukemic mitochondria

To gain insight into the mechanism of OXPHOS limitations in leukemia, at the end of the ΔG_{ATP} titration, oligomycin was added to inhibit ATP synthesis and maximal uncoupled respiration was stimulated with FCCP titration. Maximal FCCP-supported flux under these conditions is denoted as ' $\text{FCCP}_{\Delta G_{\text{ATP}}}$ ' (Figure 2D). In the intact cell assay (Figure 1C), increased glycolytic flux is presumed to

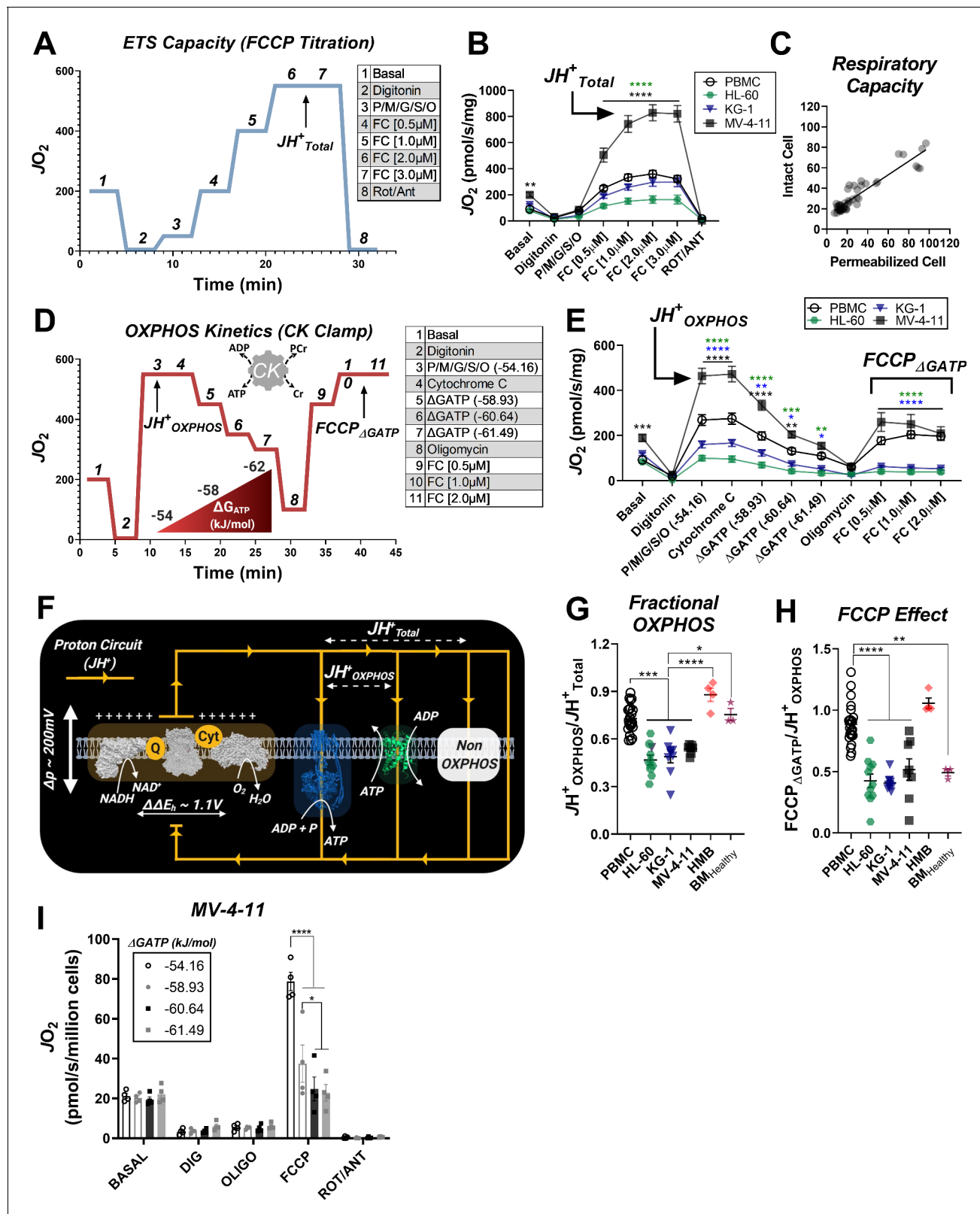


Figure 2. Impaired OXPHOS kinetics and ATP-dependent inhibition of ETS flux are unique phenotypes of leukemic mitochondria. All experiments were performed using digitonin-permeabilized cells. (A) Schematic depicting changes in oxygen consumption (JO_2) during an ETS capacity protocol (FCCP titration) where points 6–7 represent the maximum proton conductance of the respiratory system (JH^+ Total). (B) ETS capacity protocol measured in leukemia cell lines and PBMC. (C) Comparison of respiratory capacity between intact and permeabilized experimental conditions across cell types. (D) Figure 2 continued on next page

Figure 2 continued

Schematic depicting JO_2 during an OXPHOS kinetics protocol ($\Delta\text{G}_{\text{ATP}}$ titration) where point three represents maximum proton conductance by the OXPHOS system ($\text{JH}^+_{\text{OXPHOS}}$) and point 10–11 represents maximum proton conductance of the respiratory system in the presence of $\Delta\text{G}_{\text{ATP}}$ (FCCP $\Delta\text{G}_{\text{ATP}}$). (E) OXPHOS capacity protocol measured in leukemia cell lines and PBMC. (F) Illustration detailing maximal proton current generated by the electron transport system ($\text{JH}^+_{\text{Total}}$) and proportion of current harnessed by the phosphorylation system ($\text{JH}^+_{\text{OXPHOS}}$). (G) Comparison of fractional OXPHOS calculated as the ratio of $\text{JH}^+_{\text{OXPHOS}}$ to $\text{JH}^+_{\text{Total}}$. (H) Comparison of FCCP Effect calculated as the ratio of FCCP $\Delta\text{G}_{\text{ATP}}$ to $\text{JH}^+_{\text{OXPHOS}}$. For all experiments, $n = 10$ for leukemia cell lines, $n = 22$ for PBMC, $n = 4$ for HMB, and $n = 3$ for BM_{Healthy}. (I) FCCP-stimulated flux was measured under four ATP-free energy ($\Delta\text{G}_{\text{ATP}}$) conditions in permeabilized MV-4–11 cells; $n = 4$ independent experiments. Data are presented as mean $\pm$ SEM and analyzed by two-way ANOVA in (B, E, I) and one-way ANOVA in (G–H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The online version of this article includes the following source data and figure supplement(s) for figure 2:

Source data 1. Raw values for 'Figure 2' and 'Figure 2—figure supplement 1'.

Figure supplement 1. Respiratory profile of healthy bone marrow mononuclear cells and primary human myoblasts.

maintain cellular $\Delta\text{G}_{\text{ATP}}$ during FCCP titration. Thus, the continued presence of extra-mitochondrial $\Delta\text{G}_{\text{ATP}}$ in our permeabilized cell system was intended to model the adenylate constraints present in intact cells. By comparing maximal OXPHOS flux (' $\text{JH}^+_{\text{OXPHOS}}$ ') to maximum FCCP-stimulated respiration in the presence of $\Delta\text{G}_{\text{ATP}}$ ('FCCP $\Delta\text{G}_{\text{ATP}}$ '), it becomes possible to quantitate any flux limitations imposed by physiological ATP/ADP. Importantly, ATP synthase is not functional during the assay, thus any flux limitations imposed by $\Delta\text{G}_{\text{ATP}}$ would be interpreted to reflect direct ETS regulation (e.g. allostery). In PBMC and human muscle progenitor cells, the addition of FCCP at the end of the $\Delta\text{G}_{\text{ATP}}$ titration restored respiration to levels obtained under low (–54.16 kJ/mol) ATP free energy (Figure 2—figure supplement 1D–E), indicating minimal ETS flux inhibition by $\Delta\text{G}_{\text{ATP}}$. Surprisingly, relative to $\text{JH}^+_{\text{OXPHOS}}$, FCCP-stimulated respiration was substantially blunted in the presence of high ATP-free energy across all three leukemia cell lines (Figure 2E), as well as healthy bone marrow cells (Figure 2—figure supplement 1C), resulting in a near twofold difference in the FCCP $\Delta\text{G}_{\text{ATP}}$ / $\text{JH}^+_{\text{OXPHOS}}$ ratio, termed 'FCCP Effect' throughout (Figure 2H). Importantly, in the absence of ATP, the addition of CK and PCR up to 21 mM did not impact FCCP-supported flux in permeabilized MV-4–11 cells (Figure 2—figure supplement 1E). To determine the sensitivity of ETS inhibition by $\Delta\text{G}_{\text{ATP}}$, FCCP-supported flux in MV-4–11 cells was assessed at defined ATP-free energies. In these experiments, extramitochondrial $\Delta\text{G}_{\text{ATP}}$ was administered after CV inhibition with oligomycin, followed by FCCP titration. Results revealed a dose-dependent decrease in uncoupled respiration in response to increasing $\Delta\text{G}_{\text{ATP}}$ (Figure 2I), confirming that ATP-free energy is both necessary and sufficient to induce direct ETS flux inhibition in leukemia.

Inhibition of respiratory flux mediated by $\Delta\text{G}_{\text{ATP}}$ could reflect a number of potential mechanisms ranging from cytoskeletal alterations, direct inhibition of the matrix dehydrogenase network (e.g. inhibitory phosphorylation of pyruvate dehydrogenase), and/or ETS inhibition (Fisher-Wellman et al., 2018). To differentiate between these potential outcomes, mitochondria were isolated from PBMC and each of the three leukemia cell lines and similarly assessed for OXPHOS kinetics. In mitochondria energized with saturating carbon, increasing $\Delta\text{G}_{\text{ATP}}$ led to a more pronounced decrease in respiration in mitochondria of all three leukemia cell lines (Figure 3A). The ability of FCCP to restore maximal respiratory flux was also once again blunted in leukemic mitochondria (Figure 3A–B), consistent with lower fractional OXPHOS (Figure 2G). Evidence of ATP-dependent inhibition of ETS flux using both isolated mitochondria and permeabilized cells was used as criteria to rule out any involvement of the cytoskeleton. To differentiate between respiratory flux inhibition localized to the matrix dehydrogenases or the ETS, NADH/NAD⁺ redox poise was measured in substrate-replete isolated mitochondria exposed to an identical $\Delta\text{G}_{\text{ATP}}$ span. Results are depicted as a percentage of complete reduction, where 0% reduction reflects isolated mitochondria at 37°C without added substrates and 100% reduction is recorded at the end of the assay with the addition of the CIV inhibitor cyanide. Except for a slight hyper-reduction in HL-60 mitochondria, NADH/NAD⁺ redox was similar across groups (Figure 3C), indicating that ATP-mediated respiratory flux inhibition in leukemia is not due to a generalized impairment in carbon substrate uptake and/or dehydrogenase flux. Having eliminated the cytoskeleton and the dehydrogenase network as potential sites of inhibition, we next turned our attention to the ETS. To determine if flux inhibition induced by $\Delta\text{G}_{\text{ATP}}$ was specific to a given respiratory complex, OXPHOS kinetics were assessed in isolated mitochondria energized with either complex I (CI)- or CII-linked substrate combinations. Note, the presence

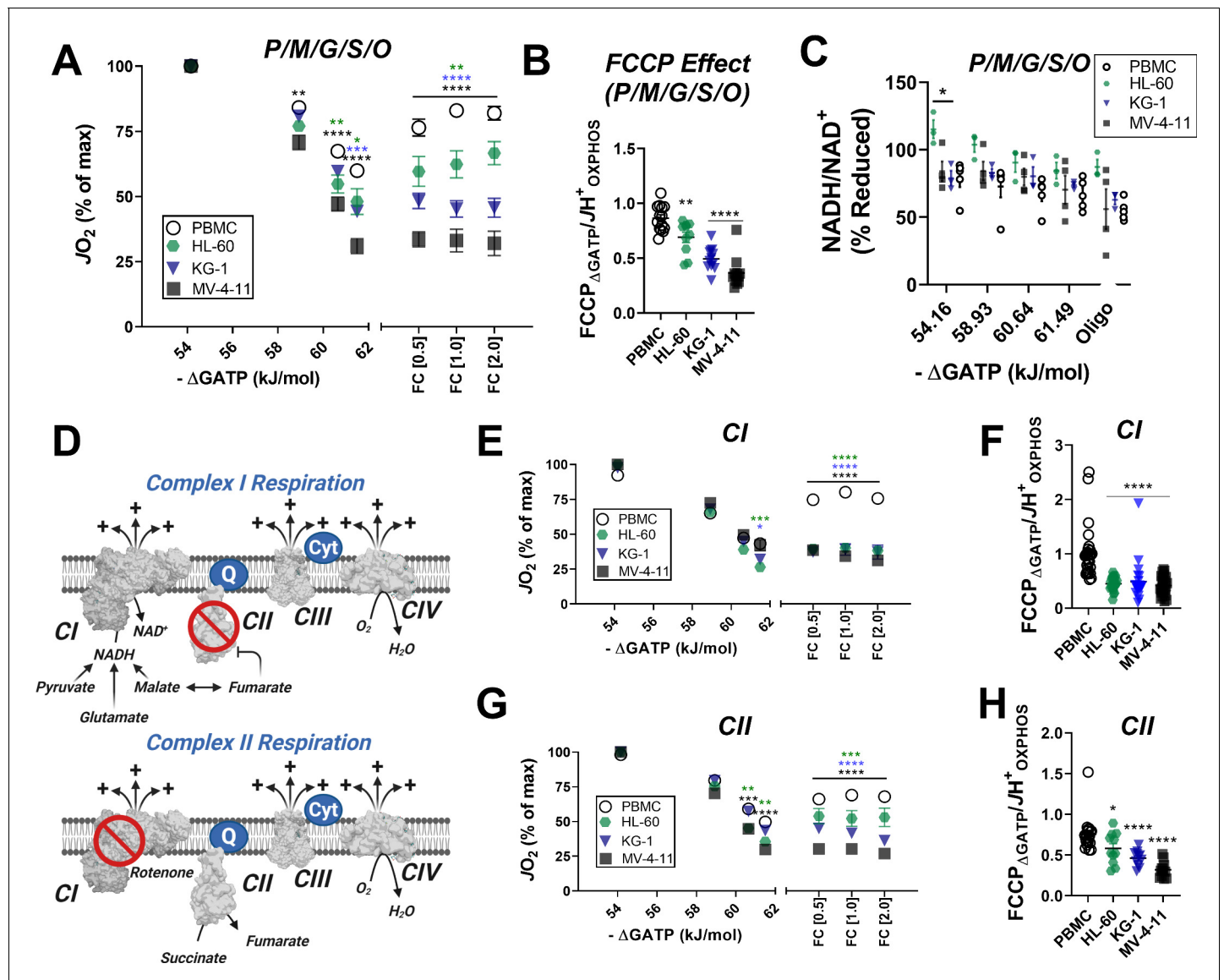


Figure 3. In leukemic mitochondria ΔG_{ATP} regulates ETS flux independent of substrate condition. (A) OXPHOS kinetics supported by P/M/G/S/O in mitochondria isolated from PBMC and leukemia cells. (B) Comparison of FCCP Effect calculated as the ratio of $FCCP \Delta G_{ATP}$ to $J_{H^+ OXPHOS}$ from B. (C) Relationship between ΔG_{ATP} and NADH/NAD⁺ redox supported by P/M/G/S/O in mitochondria isolated from leukemia cell lines and PBMC. (D) Schematic depicting CI supported respiration driven by pyruvate/glutamate/malate and inhibition of CII by equilibration of malate/fumarate (top) and CII supported respiration driven by succinate and inhibition of CI by rotenone (bottom). OXPHOS kinetics of mitochondria isolated from PBMC and leukemia cells and supported by Complex I substrates (E) and Complex II substrates (G). FCCP Effect of complex I (F) and complex II (H) supported respiration. (A, B, E–H) $n = 7$ – 10 for leukemia cell lines and $n = 22$ for PBMC. (C) $n = 3$ – 5 independent experiments. Data are mean $\pm$ SEM and analyzed by one-way ANOVA in (A) and two-way ANOVA in (B–C, E–H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The online version of this article includes the following source data for figure 3:

Source data 1. Raw values for 'Figure 3'.

of saturating malate in the CI substrate mix results in CII inhibition via malate-fumarate equilibration (Figure 3D). Likewise, the addition of rotenone in the presence of succinate eliminates residual CI-supported flux by downstream products of succinate oxidation (Figure 3D). Using either CI- or CII-linked substrates, we once again observed a more pronounced decrease in respiration in response to ΔG_{ATP} titration in leukemic mitochondria, as well as a relative inability of FCCP to restore maximal respiratory flux (Figure 3E–H). Taken together these findings demonstrate that leukemic

mitochondria are characterized by a unique form of OXPHOS regulation involving ETS inhibition by ATP free energy.

Subcellular proteomics reveals unique isoform expression of the adenine nucleotide translocase (ANT) in leukemia

To identify potential protein mediators responsible for OXPHOS insufficiency in leukemia, we conducted a proteomics screen using TMT-labeled peptides prepared from the same isolated mitochondria samples used for functional characterization. To control for group differences in percent mitochondrial enrichment, nLC-MS/MS raw data were searched using the MitoCarta 2.0 database, as previously described (McLaughlin et al., 2020). Using this approach, total mitochondrial protein abundance was similar between groups (Supplementary file 1), thus allowing for intrinsic mitochondrial signatures to be identified across leukemia. In total, 135 differentially expressed mitochondrial proteins (adjusted p value < 0.01) were identified comparing PBMC to each of the three leukemia lines (Figure 4A). For pair-wise comparisons of mitochondrial protein expression between PBMC and each of the three leukemia cell lines, see Supplementary file 1. With respect to the shared

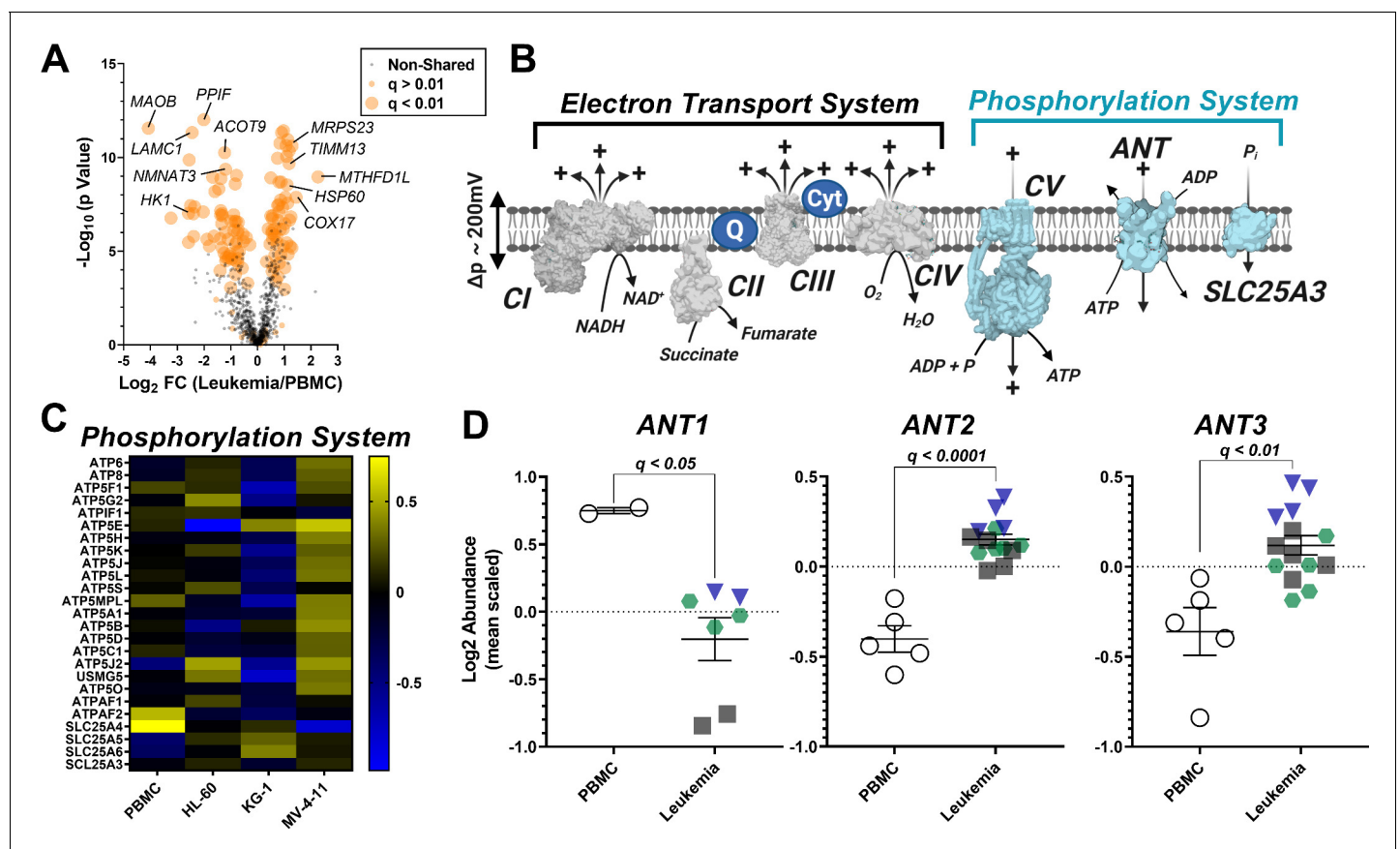


Figure 4. Analysis of mitochondrial proteome reveal disparate expression of ANT isoforms in leukemia. TMT-labeled nLC-MS/MS was performed on mitochondrial lysates from each cell type. (A) Volcano plot depicting changes in proteome between leukemia cell lines and PBMC with mitochondrial proteins shown in orange. Significance is indicated by size of each circle with changes in significance ($p < 0.01$) represented by larger circles. (B) Schematic depicting the OXPHOS system with enzymes integral to the ETS shown in gray and the phosphorylation system shown in blue. (C) Heat map displaying the common differentially expressed proteins across the phosphorylation system of leukemia and PBMC. Data are displayed as Log₂ protein intensity of all quantified master proteins. (D) Comparison of log₂ abundance of ANT isoforms in leukemia and PBMC; $n = 4-6$ mitochondrial preparations per cell lines. Data are presented as mean $\pm$ SEM and analyzed by unpaired t-tests with multiple hypothesis correction ($P_{adjusted}$, Benjamini Hochberg FDR correction, significance cutoff of $q < 0.1$).

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Raw values for 'Figure 4' and 'Figure 4—figure supplement 1'.

Figure supplement 1. Heatmap analysis depicting abundance of subunits and assembly factors that comprise the ETS complexes.

differentially expressed proteins, several of these proteins have previously been implicated in cancer biology, such as decreased MAOB (Ryu *et al.*, 2018) and HK1 (Rai *et al.*, 2019), and increased MTHFD1L (Lee *et al.*, 2017), and COX17 (Singh *et al.*, 2020; Figure 4A).

Focusing on the OXPHOS proteome, we assessed the abundance of the individual protein subunits that comprise CI, CII, CIII, and CIV, as well as the protein components of the phosphorylation system which include ATP synthase (CV), the phosphate carrier (SLC25A3), and ANT (Figure 4B). Although considerable heterogeneity was present across groups, comparing protein expression profiles of the individual subunits that comprise CI-CV and SLC25A3 (Figure 4C, Figure 4—figure supplement 1A–D) revealed that only 6 of the 110 subunits were similarly altered in leukemia (Supplementary file 1). With the exception of COX6A1, all protein subunits were involved in the assembly of CI (NDUFB10), CIV (COA4, COA7, COX17) or CV (ATPAF2). In stark contrast, the expression profiles of the three main ANT isoforms were entirely distinct between PBMC and leukemia mitochondria, highlighted by reduced ANT1 (SLC25A4) and increased ANT2 (SLC25A5) and ANT3 (SLC25A6) in leukemia (Figure 4D).

Inhibition of ETS flux by ΔG_{ATP} is a result of matrix ATP consumption in leukemia

Given that ATP-free energy was required to induce ETS flux inhibition, we hypothesized that this effect may be mediated by ATP transport into the matrix, facilitated by dominant ANT2/3 expression in leukemia (Chevrollier *et al.*, 2011). To test this hypothesis, FCCP-supported respiration was assessed in permeabilized MV-4-11 and HL-60 cells exposed to ΔG_{ATP} of -61.49 kJ/mol in the absence and presence of the ANT inhibitor carboxyatractyloside (CAT) (Maldonado *et al.*, 2016). Consistent with our prior findings, the addition of FCCP in the presence of ΔG_{ATP} was incapable of restoring flux to levels obtained with minimal ATP-free energy in leukemia (Figure 5A–B; ' ΔG_{ATP} (-61.49 kJ/mol)'). However, relative to no adenylates, as well as minimal ΔG_{ATP} (e.g. -54.16 kJ/mol), the addition of CAT restored maximal FCCP-supported ETS flux in the presence of maximal ATP free energy (Figure 5A–C). Similar experiments performed in MV-4-11 isolated mitochondria (Figure 5D), as well as with the ANT inhibitor bongkreikic acid (Figure 5—figure supplement 1A) revealed nearly identical results, indicating that ETS flux inhibition by ΔG_{ATP} requires matrix ATP uptake via ANT.

As a component of OXPHOS, ANT functions to exchange matrix ATP for cytosolic ADP in a process that consumes (i.e. depolarizes) the electrochemical proton gradient across the inner membrane. It is critical to point out that the directionality of transport by ANT is dependent on inner membrane polarization. Based on this, if indeed ANT2/3 were favoring matrix ATP uptake in leukemia then chemical inhibition of the phosphorylation system should depolarize, rather than hyperpolarize, membrane potential in leukemic isolated mitochondria. To test this, we quantified mitochondrial membrane potential in substrate-replete isolated mitochondria from HL-60 and KG-1, as well as PBMC, across a ΔG_{ATP} span and then assessed the impact of ANT inhibition with CAT or CV inhibition with oligomycin. Contrary to that seen in PBMC (Figure 5E), the addition of CAT or oligomycin caused a near complete elimination of mitochondrial membrane potential in HL-60 and KG-1 (Figure 5F–G, Figure 5—figure supplement 1B), indicative of matrix ATP uptake/consumption. Interestingly, in similar experiments with MV-4-11 mitochondria, the addition of oligomycin partially polarized membrane potential (Figure 5H), presumably due to the higher ETS capacity of MV-4-11 compared to HL-60 or KG-1 (Figure 2B). However, in the absence of any carbon substrates (i.e. no forward electron transport), membrane potential generated exclusively by ΔG_{ATP} was ~ 2 fold more polarized in all AML lines compared to PBMC (Figure 5I). In fact, for each AML line, membrane potential generated by ATP consumption was comparable to that generated during forward electron transport with saturating carbon substrates (Figure 5J). Together, these data indicate that leukemic mitochondria are particularly poised to consume, rather than produce, ATP. Such a mechanism appears mediated by the dominant expression of ANT2/3 in AML and once inside the matrix, ATP exerts a direct inhibitory effect on ETS flux.

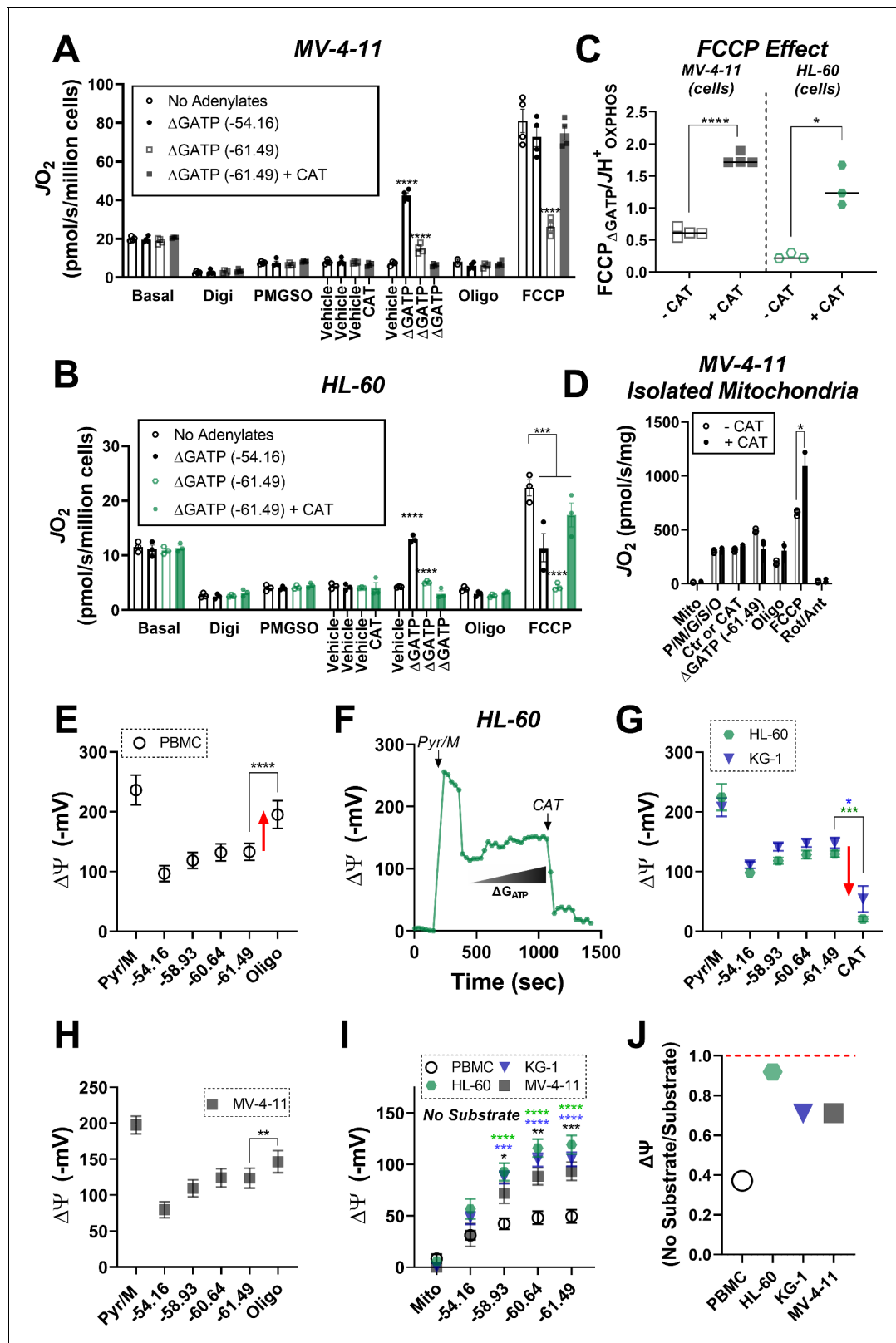


Figure 5. ETS flux inhibition by ΔG_{ATP} links to matrix ATP consumption in leukemia. (A–B) OXPHOS kinetics (via CK clamp) were assessed in the absence of adenylates or in the presence of minimal ΔG_{ATP} (–54.16), maximal ΔG_{ATP} (–61.49), or maximal ΔG_{ATP} + CAT (Carboxyatractylide; ANT inhibitor). Comparison of OXPHOS kinetics in MV-4-11 (A) and HL-60 cells (B). (C) Ratio of FCCP ΔG_{ATP} to JH⁺ OXPHOS with and without CAT in MV-4-11 and HL-60 cells. (D) OXPHOS kinetics measured in the presence of CAT in mitochondria isolated from MV-4-11. (A–D) n = 4 independent experiments

Figure 5 continued on next page

Figure 5 continued

per cell type. (E) Mitochondrial membrane potential ($\Delta\Psi$) in PBMC isolated mitochondria across a ΔG_{ATP} span, followed by CV inhibition with oligomycin; $n = 8$ independent experiments. (F) Representative trace of $\Delta\Psi$ in HL-60 isolated mitochondria across a ΔG_{ATP} span, followed by ANT inhibition with CAT. (G) Quantification of the experiment depicted in panel F in HL-60 and KG-1 isolated mitochondria; $n = 3$ independent experiments. (H) Mitochondrial $\Delta\Psi$ in MV411 isolated mitochondria across a ΔG_{ATP} span, followed by CV inhibition with oligomycin; $n = 9$ independent experiments. (I) Mitochondrial $\Delta\Psi$ in isolated mitochondria across a ΔG_{ATP} span in the absence versus presence of carbon substrates (pyruvate, malate); $n = 4$ independent experiments. (J) Ratio of group mean $\Delta\Psi$ generated at $\Delta G_{ATP} = -61.49$ kJ/mol in the absence versus presence of carbon substrates (pyruvate, malate). Data are presented as mean $\pm$ SEM and analyzed by two-way ANOVA (A–B, I) or paired t-tests (C–E, G–H). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. Raw values for 'Figure 5' and 'Figure 5—figure supplement 1'.

Figure supplement 1. ETS flux in the presence of ΔG_{ATP} is restored by ANT inhibition ANT.

Low fractional OXPHOS in AML is reversed by small-molecule inhibitors of TRAP1

Having established that extramitochondrial ΔG_{ATP} must gain access to the matrix space to inhibit ETS flux in leukemia, we next set out to elucidate the potential protein mediator(s) of this effect. To do this, we searched our proteomics dataset for mitochondrial proteins with known kinase and/or ATPase function that were substantially upregulated across all three leukemia lines and identified mitochondrial TRAP1 (Figure 6A). TRAP1 is the mitochondrial paralog of the heat shock protein 90 (HSP90) family and is widely recognized as a potential anticancer drug target across multiple human malignancies, including leukemia (Bryant et al., 2017; Kim et al., 2020; Li et al., 2020; Sanchez-Martin et al., 2020a; Sanchez-Martin et al., 2020b; Sciacovelli et al., 2013; Yoshida et al., 2013). Given that ATPase activity is required for TRAP1 function (Ramkumar et al., 2020), we hypothesized that ΔG_{ATP} -mediated ETS inhibition may be driven by acute activation of TRAP1. To test this hypothesis, OXPHOS kinetics were assessed in permeabilized MV-4-11 cells in the absence and presence of the purported TRAP1 inhibitor 17-AAG (Kamal et al., 2003). In substrate-replete permeabilized MV-4-11 cells, acute exposure to 17-AAG had no impact on maximal FCCP-supported respiration in the absence of adenylates (Figure 6—figure supplement 1A). Remarkably, the presence of 17-AAG increased JH^+_{OXPHOS} and calculated fractional OXPHOS relative to vehicle control and completely restored FCCP-supported respiration in the presence of ATP-free energy (Figure 6B–D). Similar results were observed using permeabilized HL-60 cells (Figure 6—figure supplement 1B–D), as well as using the mitochondrial-targeted TRAP1 inhibitor gamitrinib (Figure 5—figure supplement 1A). Using both MV-4-11 and KG-1 permeabilized cells, the ability of 17-AAG to restore OXPHOS flux was similar in the presence of CI or CII-based carbon substrates (Figure 6—figure supplement 1E–F).

Although ATP is widely understood to be the universal energy currency in cells, it is critical to consider that ATP alone has minimal bio-synthetic power; rather, its utilization as a common energy currency is solely a function of the remarkable displacement of the molecule from equilibrium (~10 orders of magnitude) (Willis et al., 2016). This means that biological processes driven by ATP hydrolysis are presumably fueled by ATP-free energy, rather than ATP levels per se. The primary advantage of the CK clamp technique is that it allows for mitochondrial bioenergetics to be evaluated across a physiological ΔG_{ATP} span without appreciable changes in ATP concentration (Figure 6—figure supplement 1G). Thus, we reasoned that the CK clamp could be utilized to assess 17-AAG effectiveness across a ΔG_{ATP} span under conditions in which free [ATP] is not rate-limiting. For contrast, we compared the respiratory impact of 17-AAG to that of the commonly used ETC inhibitor antimycin A. Although antimycin A decreased respiratory flux in permeabilized MV-4-11 cells, percent inhibition by the compound was largely insensitive to ΔG_{ATP} , consistent with its known mechanism of action at CIII (Figure 6—figure supplement 1H). In contrast, across all leukemia cell lines, restoration of respiratory flux by 17-AAG was exquisitely sensitive to ΔG_{ATP} (Figure 6E, Figure 6—figure supplement 1I–J).

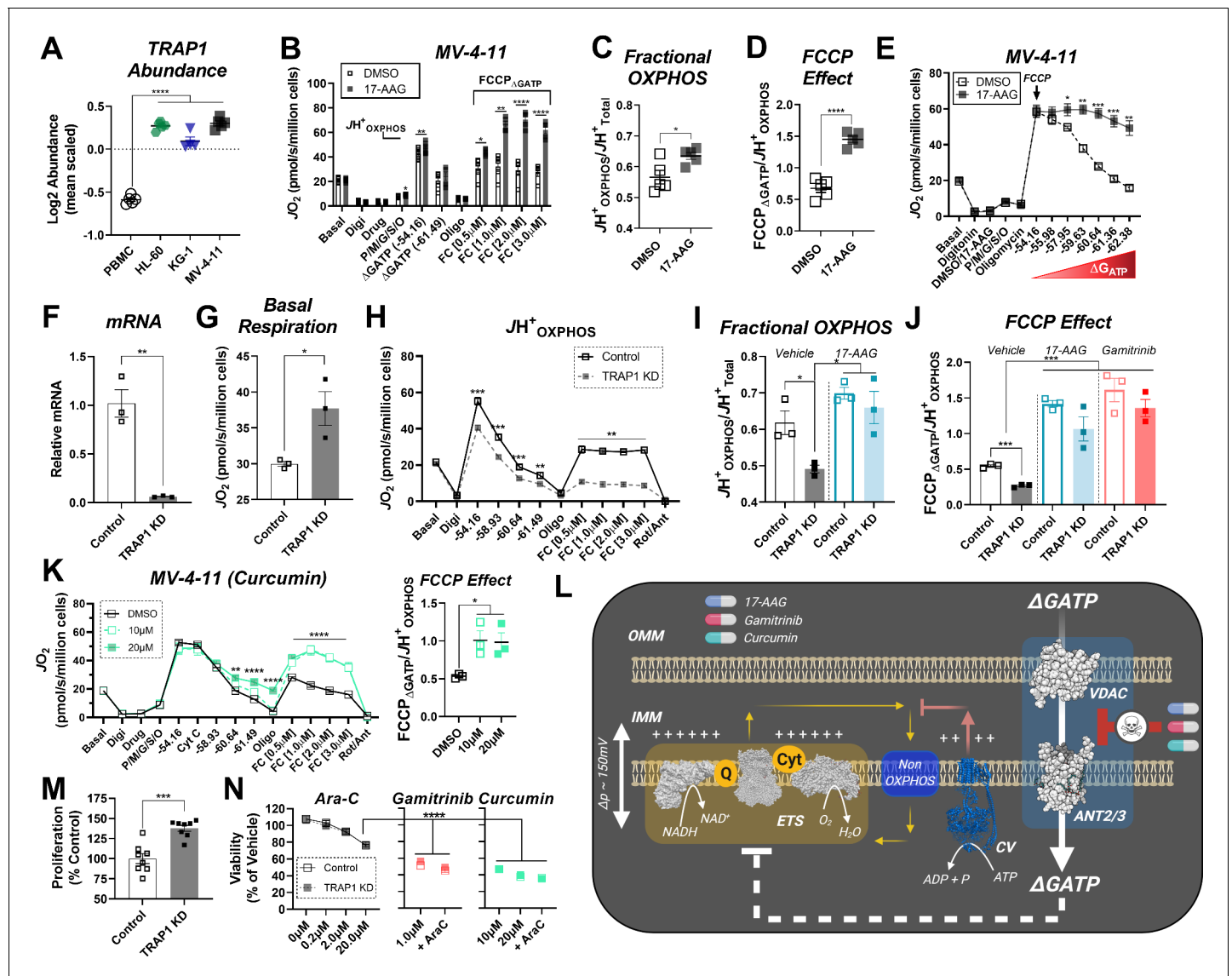


Figure 6. 17-AAG and gamitrinib increase fractional OXPHOS and restore ETS flux in the presence of ΔGATP . (A) Log2 Abundance of TRAP1 in PBMC and leukemia cells. (B) Comparison of OXPHOS kinetics in presence of the TRAP1 inhibitor, 17-AAG (15 μM); $n = 4$ independent cell experiments. Comparison of (C) Fractional OXPHOS and (D) FCCP Effect in MV-4-11 cells treated with DMSO or 17-AAG (15 μM). (E) Comparison of respiratory flux inhibition within MV-4-11 cells across a range of ΔGATP in the absence and presence of 17-AAG (15 μM). Respiration was stimulated by the addition of FCCP (1 μM), followed by PCR titration to manipulate ΔGATP . (F) Relative abundance of TRAP1 mRNA following shRNA knockdown of TRAP1 in MV-4-11 cells. (G) Basal respiration in intact cells. (H) OXPHOS kinetics in permeabilized MV-4-11 cells infected with lentivirus encoding shRNA targeted to TRAP1 (TRAP1 KD) or scrambled shRNA (Control). (I–J) Fractional OXPHOS and FCCP Effect measured in the presence of vehicle (DMSO), 17-AAG (15 μM), or gamitrinib (1 μM) in control and TRAP1 KD cells. (K) OXPHOS kinetics in permeabilized MV-4-11 cells in the presence of DMSO or curcumin (10–20 μM). FCCP Effect is graphed to the right. (L) Schematic depicting the presumed mechanism of action of 17-AAG, gamitrinib and curcumin in which the compounds selectively block ATP uptake via the VDAC-ANT axis to restore OXPHOS kinetics. (M) Cell proliferation expressed as a percentage of Control. (N) Cell viability in MV-4-11 cells infected with lentivirus encoding shRNA targeted to TRAP1 or scrambled shRNA and treated for 24 hr with increasing concentrations of Ara-C. Additional treatments included gamitrinib (1 μM), 17-AAG (15 μM) or curcumin (10–20 μM) either alone or plus Ara-C (20 μM). Data depicted as viability based on the percentage of vehicle using the propidium iodide assay. (A) $n = 4/6/\text{group}$; (B–D) $n = 4$ independent experiments; (E–K) $n = 3$ independent experiments; (M) $n = 8$ independent experiments; (N) $n = 4$ –6 independent experiments. Data are presented as mean $\pm$ SEM analyzed by unpaired t-tests (F–H, M) two-way ANOVA (B, K), one-way ANOVA (A, I, J, N), paired t-tests (B–E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The online version of this article includes the following source data and figure supplement(s) for figure 6:

Source data 1. Raw values for 'Figure 6' and 'Figure 6—figure supplement 1'.

Figure supplement 1. Effects of 17-AAG on OXPHOS kinetics.

OXPHOS restoration by 17-AAG and gamitrinib is independent of TRAP1

To control for potential off-target effects mediated by 17-AAG or gamitrinib, MV-4-11 cells were infected with lentivirus containing pooled short hairpin RNA (shRNA) against TRAP1. Control cells were infected with lentivirus containing scrambled shRNA. All constructs encoded GFP, as well as a puromycin selection gene to allow for stable selection. Following 24 hr exposure to lentiviral particles and multiple rounds of puromycin selection, shRNA against TRAP1 led to a > 90% reduction in TRAP1 mRNA (**Figure 6F**). Consistent with prior reports (*Laquatra et al., 2021*; *Sciacovelli et al., 2013*), TRAP1 knockdown increased basal respiration in intact cells (**Figure 6G**). To determine the impact of TRAP1 knockdown on OXPHOS kinetics, JH^{+}_{Total} and JH^{+}_{OXPHOS} were assessed in substrate-replete permeabilized cells. Despite no change in JH^{+}_{Total} (**Figure 6—figure supplement 1K**), TRAP1 knockdown decreased JH^{+}_{OXPHOS} and exacerbated the ability of ATP-free energy to blunt ETS flux (**Figure 6H–J**). This was surprising, given that acute administration of 17-AAG/gamitrinib consistently increased fractional OXPHOS across all AML lines (**Figure 6C**, **Figure 6—figure supplement 1C**). Based on this, we hypothesized that the ability of 17-AAG and/or gamitrinib to bolster OXPHOS kinetics may be independent of TRAP1. To test this, we repeated the JH^{+}_{OXPHOS} experiments in control and TRAP1 knockdown cells in the presence of either 17-AAG or gamitrinib. In control cells, acute administration of 17-AAG and gamitrinib once again increased fractional OXPHOS and restored the FCCP effect and near identical results were also apparent in TRAP1 knockdown cells (**Figure 6I–J**, **Figure 6—figure supplement 1L**). Such findings indicated that OXPHOS restoration by 17-AAG/gamitrinib occurs independent of TRAP1 and is thus attributable to an ‘off-target’ mechanism. One such off-target effect documented for 17-AAG relates to the ability of the compound to bind voltage-dependent anion transporter (VDAC) (*Xie et al., 2011*), the principal gatekeeper to adenylate transport across the outer mitochondrial membrane. A similar mechanism has also been described for the anticancer compound curcumin (*Tewari et al., 2015*); however, the implications of these interactions on cellular bioenergetics remain incompletely understood. Interestingly, nearly identical to the effect observed in the presence of 17-AAG/gamitrinib, acute exposure to curcumin also reversed ΔG_{ATP} -mediated ETS inhibition in MV-4-11 cells (**Figure 6K**). Based on the striking functional similarities between 17-AAG, gamitrinib and curcumin, such findings suggest a potential novel mechanism whereby these small molecules are uniquely capable of selectively blocking matrix ATP uptake, while remaining permissive to ADP uptake, presumably via interaction with the VDAC-ANT axis (**Figure 6L**). In support of this, curcumin-VDAC interaction is known to lock VDAC in the ‘closed’ confirmation, in turn making it partially selective for cation (i.e. ADP^{3+}), rather than anion (i.e. ATP^{4-}), transport (*Tewari et al., 2015*).

To investigate the relationship between mitochondrial fractional OXPHOS and AML biology, we assessed cellular proliferation and viability in control and TRAP1 knockdown cells exposed to gamitrinib, curcumin, or vehicle control. In the absence of any small-molecule intervention, cellular proliferation was higher in TRAP1 knockdown cells (**Figure 6M**), consistent with low fractional OXPHOS being advantageous to AML growth. To determine the therapeutic efficacy of restoring fractional OXPHOS in AML, cells were exposed for 24 hr to either gamitrinib or curcumin, either alone or in combination with the front-line AML chemotherapeutic cytarabine (Ara-C). Compared to marginal cytotoxicity in response to Ara-C dose escalation, 24 hr exposure to either gamitrinib or curcumin decreased cell viability by as much as 60% (**Figure 6N**). Note, identical cytotoxicity was observed in control and TRAP1 knockdown cells, and the additional presence of Ara-C had no further impact (**Figure 6N**).

Intrinsic limitations in OXPHOS characterize human primary leukemia

To determine if the bioenergetic phenotypes present in leukemia cell lines translated to the clinic, we recruited patients diagnosed with leukemia and comprehensively evaluated mitochondrial bioenergetic function in mononuclear cells isolated from bone marrow aspirates. All patients had confirmed leukemia at the time of sample acquisition. Biochemical results were compared to PBMC isolated from age-matched participants without a prior history of leukemia. As additional controls, OXPHOS kinetics were assessed in mononuclear cells isolated from bone marrow aspirates of healthy subjects ($BM_{Healthy}$), as well as purified CD34 +hematopoietic stem cells. To ascertain the impact of clonal cell expansion on the underlying mitochondrial network, experiments in unstimulated (i.e.

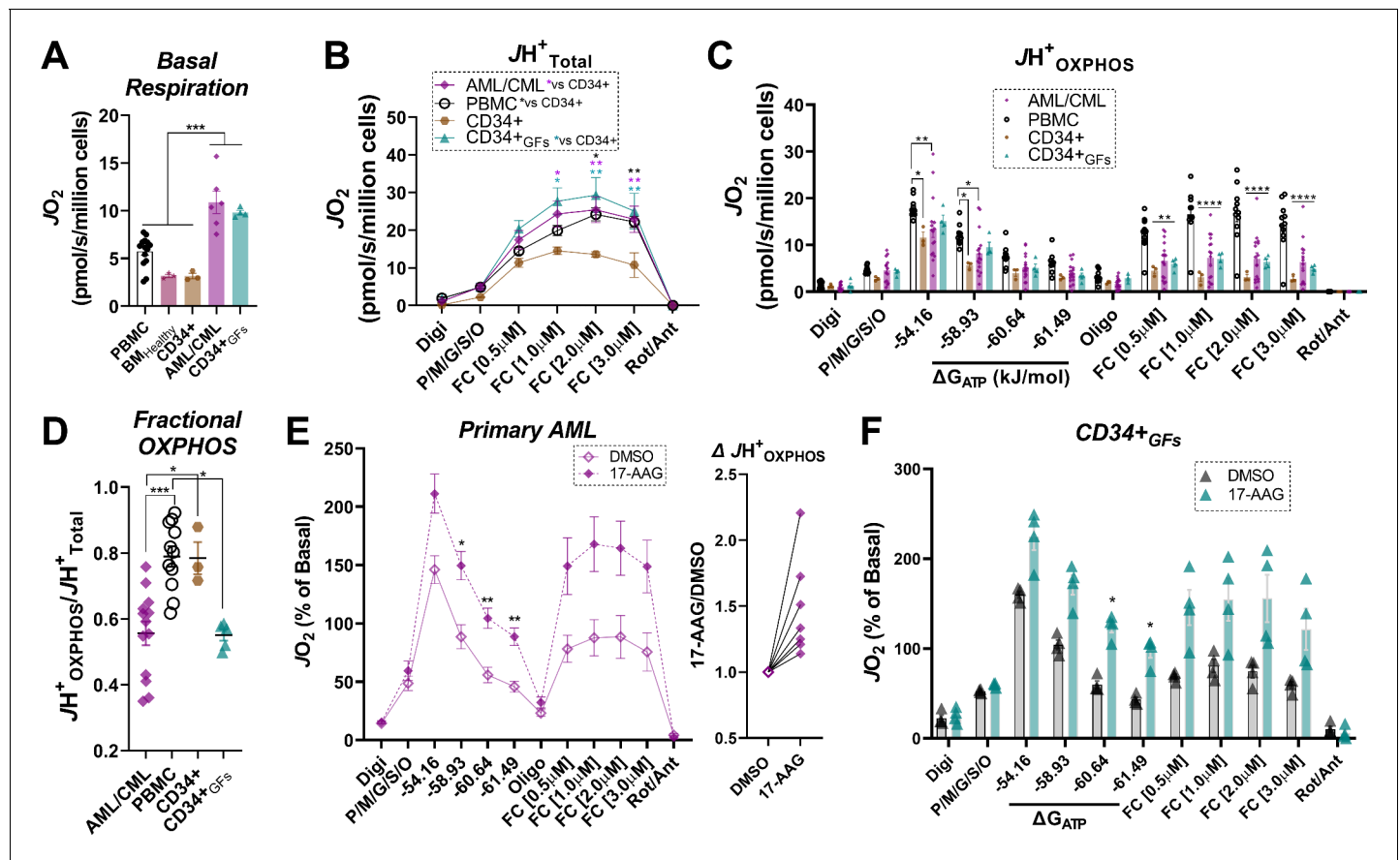


Figure 7. Human primary leukemia is characterized by low Fractional OXPHOS. (A) Basal respiration in intact cells – ‘PBMC’ (age-matched to the primary leukemia samples); ‘BM_{Healthy}’ (bone marrow mononuclear cells); ‘CD34⁺’ (pure CD34⁺ cells not exposed to growth factors); ‘P. Leukemia’ (mononuclear cells isolated from bone marrow of patients with confirmed leukemia); ‘CD34⁺G_Fs’ (pure CD34⁺ cells stimulated to undergo clonal expansion in culture). (B) ETS capacity assay in permeabilized cells. (C) OXPHOS kinetics in permeabilized cells. (D) Fractional OXPHOS. (A–D) $n = 3$ –15 independent experiments. (E) Comparison of OXPHOS kinetics in the presence of DMSO or 17-AAG (15 μ M) in a cohort of patients with confirmed AML. Graph to the right depicts JH⁺ OXPHOS depicted as a fold change from DMSO; $n = 7$ independent cell experiments. (F) Comparison of OXPHOS kinetics in the presence of DMSO or 17-AAG (15 μ M) in CD34⁺G_Fs; $n = 4$ independent cell experiments. (E–F) Data depicted as a percentage of basal respiration based on oxygen consumption rates obtained in intact cells. Data are presented as mean $\pm$ SEM analyzed by one-way ANOVA (A, D) two-way ANOVA (B–C), or paired t-tests (E–F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The online version of this article includes the following source data and figure supplement(s) for figure 7:

Source data 1. Raw values for ‘Figure 7’ and ‘Figure 7—figure supplement 1’.

Figure supplement 1. Effects of 17-AAG on OXPHOS kinetics and cell viability in PBMC and healthy bone marrow mononuclear cells.

quiescent) CD34⁺ cells were also performed following chronic exposure to proliferative stimuli (e.g. TPO, SCF, FLT-3; ‘CD34 + G_Fs’). Relative to PBMC, BM_{Healthy}, and quiescent CD34⁺, intact basal respiration was elevated in primary leukemia, as well as CD34⁺G_Fs (Figure 7A), reminiscent of that seen in AML cell lines and entirely consistent with their proliferative phenotypes. Relative to quiescent CD34⁺, JH⁺ Total in substrate-replete permeabilized cells was ~2 fold higher in PBMC, CD34⁺G_Fs and primary leukemia (Figure 7B), consistent with higher cellular mitochondrial density. Despite this apparent increase in mitochondrial content, JH⁺ OXPHOS remained elevated above quiescent CD34⁺ only in PBMC, as OXPHOS kinetics were completely unaltered comparing CD34⁺G_Fs or primary leukemia to quiescent CD34⁺ (Figure 7C). As seen in the AML cell lines, calculated fractional OXPHOS was decreased in CD34⁺G_Fs and primary leukemia, entirely consistent with low fractional

OXPHOS being required for hematopoietic clonal expansion (**Figure 7D**). To determine the impact of 17-AAG on OXPHOS kinetics, CK clamp experiments were performed in a separate cohort of primary AML samples, as well as clonally expanding CD34⁺ cells. Relative to vehicle control, 17-AAG increased JH^{+}_{OXPHOS} and restored FCCP-stimulated respiration in the presence of ΔG_{ATP} in primary AML and CD34⁺GFs (**Figure 7E–F**). Of note, acute 17-AAG had no impact on OXPHOS kinetics in PBMC or BM_{Healthy} and 24 hr exposure of PBMC to 17-AAG or gamitrinib did not impact cell viability (**Figure 7—figure supplement 1A–C**).

Intrinsic limitations in OXPHOS power output characterize venetoclax resistance in AML

The preponderance of evidence related to mitochondrial bioenergetics in AML indicates that active repression of OXPHOS is advantageous to the leukemia phenotype. Related to this, although several reports have linked AML chemoresistance to apparent increases in ‘OXPHOS reliance’ (*Farge et al., 2017; Gui  ze et al., 2019; Liu et al., 2020; Roca-Portoles et al., 2020*), such conclusions are based largely on intact cellular respirometry in which OXPHOS kinetics are not directly quantified. To address the specific role for OXPHOS in AML chemoresistance, we conducted a series of experiments in chemosensitive HL-60 cells and compared the results to HL-60 cells made to be refractory to venetoclax (**Figure 8A–B**). In these experiments, JH^{+}_{Total} and JH^{+}_{OXPHOS} were determined as described above. In addition, OXPHOS kinetic data generated using the CK clamp was integrated with parallel analysis of OXPHOS efficiency (mitochondrial ATP synthesis relative to oxygen consumption; quantified as the P/O ratio). By incorporating empirically derived P/O, respiratory flux at each ΔG_{ATP} can be converted to ATP production rate. Assuming extra-mitochondrial force applied via the CK clamp is fixed at each titration step, ATP production rate can be used to quantitate OXPHOS power output in Watts ($J \cdot s^{-1}$). Power is an extremely useful parameter because it encompasses thermodynamic, kinetic, as well as stoichiometric descriptions that effectively report on the actual quantities of OXPHOS work performed across the demand range. Looking first at intact cellular respirometry, both basal and maximal respiratory capacity were elevated in venetoclax-resistant HL-60 (HL60_{VR}) (**Figure 8C**). Remarkably, despite a near ~2 fold increase in cellular respiratory capacity, OXPHOS kinetics were in fact decreased in HL60_{VR}, such that JH^{+}_{OXPHOS} failed to reach the respiratory rates observed under basal conditions prior to digitonin permeabilization (**Figure 8D**). Of note, the addition of extramitochondrial cytochrome C did not impact JH^{+}_{OXPHOS} in either cell type (**Figure 8E**). The combination of increased maximal respiratory capacity combined with decreased JH^{+}_{OXPHOS} translated to a striking decrease in both fractional OXPHOS (**Figure 8F**), as well as OXPHOS power output in HL60_{VR} (**Figure 8G–I**), indicating that intrinsic OXPHOS insufficiency also underlies cellular bioenergetics in chemoresistant AML.

Discussion

Increased mitochondrial oxidative metabolism, an established metabolic hallmark of leukemia (*Byrd et al., 2013; Kuntz et al., 2017; Lee et al., 2015; Sriskanthadevan et al., 2015; Suganuma et al., 2010*), has been historically interpreted to reflect an increased reliance on mitochondrial ATP production. However, fractional OXPHOS kinetics had not been empirically evaluated in leukemia at the onset of this project. Thus, it remained to be determined whether higher basal respiration in leukemia reflected accelerated demand for ATP regeneration or intrinsic OXPHOS insufficiency. Both conditions would be expected to similarly restrict cellular ATP/ADP equilibrium displacement (i.e. ΔG_{ATP} charge) and thus could potentially result in identical respiratory profiles in intact cells. For example, a small network of mitochondria each respiring near maximal capacity could in theory produce an identical ‘basal’ oxygen consumption rate to that of a comparatively larger mitochondrial network in which forward respiratory flux was constrained across each mitochondrial unit. Our findings provide definitive support for the latter scenario in AML, as application of our diagnostic biochemical workflow revealed that intrinsic limitations in fractional OXPHOS characterize an expansive mitochondrial network in human leukemia. In fact, a substantial portion of the AML mitochondrial network is incapable of contributing to oxidative ATP production, as leukemic mitochondria primarily consume, rather than produce, ATP across a physiological ΔG_{ATP} span. Intrinsic OXPHOS limitations in AML appear to derive from a unique biochemical mechanism whereby extra-mitochondrial ATP gains access to the matrix space, where it then directly inhibits electron

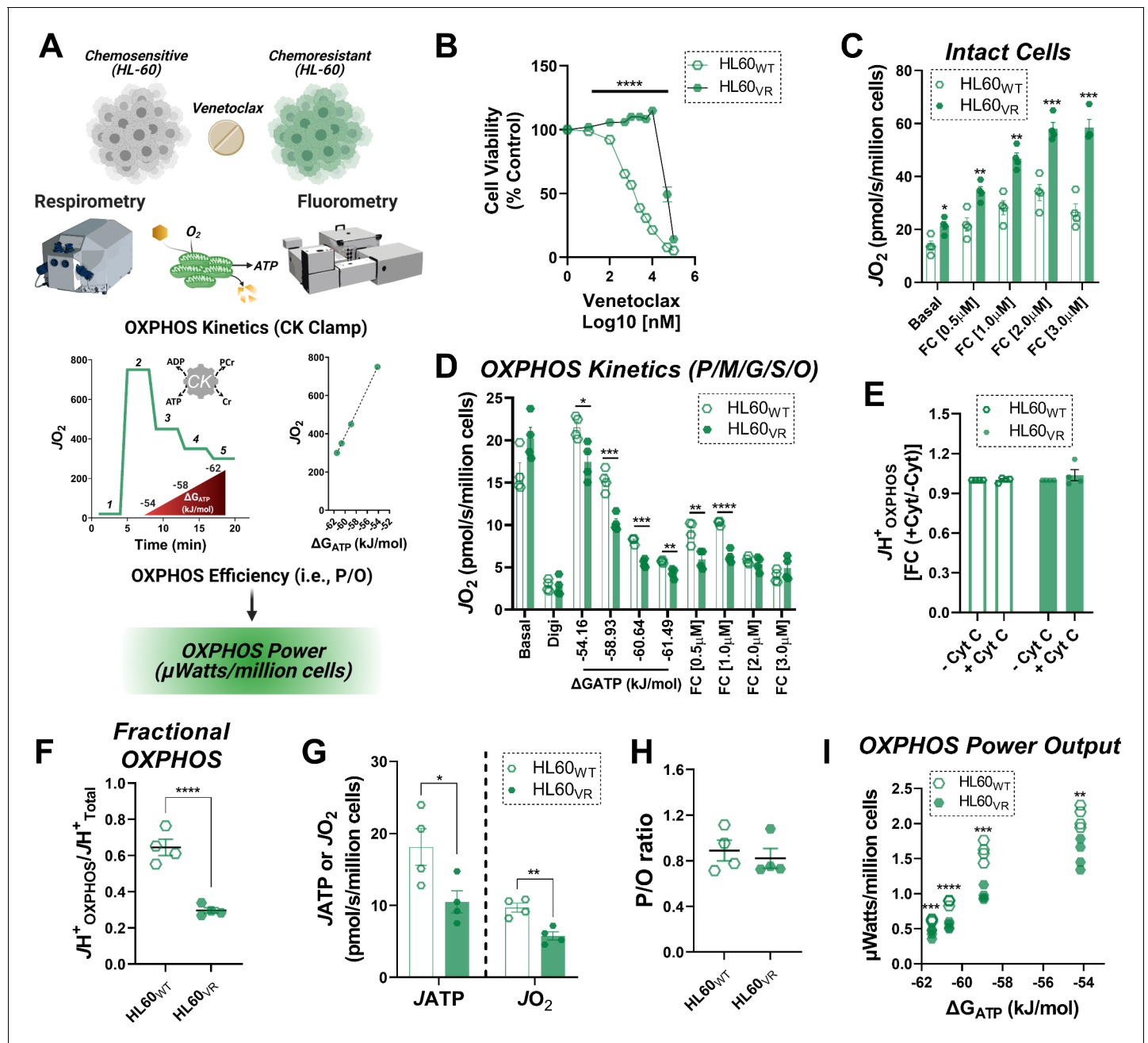


Figure 8. OXPHOS power output is reduced in the setting of venetoclax resistance. (A) Study schematic depicting bioenergetic characterization of OXPHOS power output in HL-60 cells either sensitive (HL60_{WT}) or made resistant to venetoclax by continuous exposure to 1 μ M venetoclax (HL60_{VR}). (B) Cell viability, expressed as a percentage of control, following 48 hr exposure to increasing concentrations of venetoclax; n = 6 independent experiments. (C) Intact cellular respiration under basal conditions, as well as in response to FCCP titration in bicarbonate free RPMI media. (D) OXPHOS kinetics in permeabilized cells. (E) Effect of cytochrome C addition on JH^+_{OXPHOS} . Data expresses as fold change from rates obtained prior to cytochrome C addition. (F) Fractional OXPHOS - note, JH^+_{Total} was calculated from the intact cell assay in C using the maximal rate stimulated by FCCP. (G) Rate of ATP synthesis (JATP) and oxygen consumption (JO_2) in permeabilized cells energized with P/M/G/S/O. (H) Calculated P/O ratio. (I) Calculated OXPHOS power output. (C–I) n = 4 independent experiments. Data are presented as mean $\pm$ SEM analyzed by unpaired t-tests. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

The online version of this article includes the following source data for figure 8:

Source data 1. Raw values for 'Figure 8'.

transport flux in a ΔG_{ATP} -dependent manner. Such inhibition is independent of ATP synthase (i.e. CV) and presumably localized to the respiratory complexes downstream of the ubiquinone pool (i.e. CIII, Cyt C, CIV). Given that evidence for this effect was also observed in bone marrow-derived CD34+ stem cells, allosteric and/or post-translation regulation of ETS flux is likely a primary mode of OXPHOS regulation in hematopoietic progenitors that is maintained during leukemogenesis. Importantly, reversal of this effect was strongly cytotoxic to AML, indicating that direct OXPHOS regulation by ΔG_{ATP} confers a survival advantage during hematopoietic clonal cell expansion. Although additional work will be required to fully elucidate the mechanism(s) by which ATP uptake directly inhibits OXPHOS flux in AML, our preliminary findings leveraging gamitrinib and curcumin provide proof-of-principle that such regulation can indeed be targeted therapeutically.

While prior work has implicated ANT2 (Chevrollier et al., 2011; Lee et al., 2019; Maldonado et al., 2016) in cancer biology, we present for the first time here a potential mechanism whereby ANT2/3 regulate leukemic cell metabolism via ΔG_{ATP} . Specifically, our findings inform a model of leukemia bioenergetics in which decreased ANT1, and increased ANT2/3 favor the uptake of extra-mitochondrial ATP into the matrix space. The transfer of ΔG_{ATP} from the cytosol to the matrix in turn constrains OXPHOS via direct inhibition of the ETS. Direct ETS inhibition by ΔG_{ATP} could derive from multiple sources, such as adenylate-mediated allosteric regulation (Kadenbach, 2021), inhibitory phosphorylation of the respiratory complexes (Helling et al., 2012), or chaperone mediated protein-protein interactions (Sciacovelli et al., 2013). In either case, the exquisite sensitivity of the ETS to ΔG_{ATP} likely prevents the mitochondrial network from contributing to the cellular ΔG_{ATP} charge in the leukemic blasts. Consequentially, maintenance of a low cellular ΔG_{ATP} would be predicted to maximize both glycolytic and mitochondrial metabolism, entirely consistent with the known hyper-metabolic phenotype of human leukemia (Goto et al., 2014a; Goto et al., 2014b; Suganuma et al., 2010). Such conditions are likely advantageous to proliferating leukemic blasts, as insufficient ATPase activity has been demonstrated to be rate-limiting for cellular proliferation (Luengo et al., 2020).

Currently, there is great interest in developing novel pharmacotherapies that target mitochondrial OXPHOS in leukemia (Farge et al., 2017; Gui  ze et al., 2019; Kuntz et al., 2017; Lee et al., 2015; Panina et al., 2020). Yet, a large caveat of targeting mitochondria is the ubiquitous necessity of OXPHOS for healthy cellular metabolism. If indeed the proliferative potential of leukemia depends upon mitochondrial ATP consumption, rather than production, it is tempting to speculate that a pharmaceutical intervention designed to restore OXPHOS kinetics and/or ΔG_{ATP} could effectively halt cell proliferation, in turn allowing for proliferating blasts to succumb to apoptosis. Such a targeted approach would be expected to minimize secondary toxicity as increased OXPHOS efficiency is likely advantageous across non-cancerous, highly metabolic tissues (e.g. brain, heart, muscle), as well as in the context of adaptive cellular immunity (Buck et al., 2016; Vardhana et al., 2020). Based upon our proposed model, targeting the acute OXPHOS regulating capabilities of ΔG_{ATP} through the VDAC-ANT axis provides an appealing, leukemia-specific target for pharmaceutical intervention, as small-molecule interference of this pathway restored OXPHOS kinetics in leukemic mitochondria and induced cytotoxicity (Figure 6). Although prior work has identified TRAP1 (Bryant et al., 2017; Ramkumar et al., 2020) as a potential anticancer target in leukemia, genetic knockdown in MV-4-11 cells increased, rather than decreased, cellular proliferation in the present study. Of note, the mitochondrial-localized TRAP1 inhibitor gamitrinib is currently being evaluated in a first-in-human phase I clinical trial for treatment of advanced cancers (ClinicalTrials.gov Identifier: NCT04827810). It is currently unclear how much of the cytotoxicity induced by 17-AAG and/or gamitrinib is attributable to on-target TRAP1 inhibition vs off-target interference with ATP exchange via VDAC-ANT. Moreover, the mechanism of cytotoxicity induced by 17-AAG/gamitrinib/curcumin requires additional experimentation. Although speculative, it is possible that the ability of these compounds to selectively block matrix ATP uptake acutely restores full respiratory competence of the expansive AML mitochondrial network, in turn increasing redox pressure across the ETS and boosting ROS production above a cytotoxic threshold.

Taken together, the present findings provide novel insight into the hyper-metabolic phenotype characteristic of human leukemia. With respect to the mitochondria, it bears repeating that heightened basal respiration in AML is almost exclusively assumed to reflect an increased reliance on oxidative ATP synthesis (i.e. OXPHOS). Although this assumption largely underscores the growing interest in targeting mitochondrial oxidative metabolism in cancer, direct interrogation of the

mitochondrial network herein consistently revealed active OXPHOS repression in AML. Representative of this phenotype, TRAP1 knockdown and venetoclax resistance both resulted in an increase in basal respiration that was subsequently linked to decreased, not increased, OXPHOS kinetics. Such findings highlight the diagnostic limitations of metabolic measurements made in intact cells and suggest that too much reliance on binary readouts of respiration versus glycolysis may in fact be masking ‘actionable’ cancer-specific mitochondrial biology. By leveraging comprehensive mitochondrial diagnostics, our collective findings inform a model whereby intrinsic OXPHOS limitations support aggressive disease dissemination in leukemia and raise the intriguing possibility that pharmaceutical interventions aimed at blocking mitochondrial ATP uptake/consumption warrants further exploration. Given that increased OXPHOS efficiency is advantageous across non-cancerous tissues, a potential benefit to this novel treatment paradigm is the minimization of secondary toxicity (i.e. a wide therapeutic window).

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Biological sample (<i>Homo sapiens</i>)	Peripheral Blood Mononuclear Cells; PBMC	Venous puncture		Freshly isolated from <i>Homo sapiens</i> , male and female, 18–75 years
Biological sample (<i>Homo sapiens</i>)	Bone Marrow Aspirate; Primary Leukemia	Posterior Iliac Crest		Freshly isolated from <i>Homo sapiens</i>
Biological sample (<i>Homo sapiens</i>)	Human Myoblast; HMB	Muscle biopsies from gastrocnemius muscle		Freshly isolated from <i>Homo sapiens</i>
Biological sample (<i>Homo sapiens</i>)	CD34+ Cells	HemaCare	CAT#: BM34C	Isolated freshly from bone marrow (<i>Homo sapiens</i> , M)
Biological sample (<i>Homo sapiens</i>)	CD34+ Cells	HemaCare	CAT#: BM34C	Isolated freshly from bone marrow (<i>Homo sapiens</i> , M)
Biological sample (<i>Homo sapiens</i>)	CD34+ Cells	HemaCare	CAT#: BM34C	Isolated freshly from bone marrow (<i>Homo sapiens</i> , F)
Biological sample (<i>Homo sapiens</i>)	Bone Marrow Aspirate; BM Healthy	HemaCare	CAT#: BM008F	Isolated freshly from bone marrow (<i>Homo sapiens</i> , F)
Biological sample (<i>Homo sapiens</i>)	Bone Marrow Aspirate; BM Healthy	HemaCare	CAT#: BM008F	Isolated fresh (<i>Homo sapiens</i> , M)
Biological sample (<i>Homo sapiens</i>)	Bone Marrow Aspirate; BM Healthy	HemaCare	CAT#: BM008F	Isolated fresh (<i>Homo sapiens</i> , M)
Cell line (<i>Homo sapiens</i>)	HL-60	ATCC	CAT#: CCL-240	
Cell line (<i>Homo sapiens</i>)	MV-4–11	ATCC	CAT#: CRL-9591	
Cell line (<i>Homo sapiens</i>)	KG-1	ATCC	CAT#: CCL-246	
Chemical compound, drug	Oligomycin; Oligo	Tocris	CAT#: 4110	
Chemical compound, drug	FCCP	Millipore Sigma	CAT#: C2920	
Chemical compound, drug	Rotenone; Rot	Millipore Sigma	CAT#: R8875	
Chemical compound, drug	Antimycin; Ant	Millipore Sigma	CAT#: A8674	
Chemical compound, drug	Digitonin; Digi	Millipore Sigma	CAT#: D1410599	
Chemical compound, drug	Potassium Pyruvate; P; Pyr	Combi-Blocks	CAT#: QA-1116	

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Chemical compound, drug	L-Malic Acid; Malate; M	Alpha Aesar	CAT#: A13702	
Chemical compound, drug	L-Glutamic Acid; Glutamate; G	RPI	CAT#: G25200	
Chemical compound, drug	Succinic Acid; Succinate; S; Succ	Fisher	CAT#: BP336	
Chemical compound, drug	Octanoyl-L-Carnitine; O	Millipore Sigma	CAT#: 50892	
Chemical compound, drug	Cytochrome C	Millipore Sigma	CAT#: C2506	
Chemical compound, drug	ATP	Ark Pharma	CAT#: AK54737	
Chemical compound, drug	Creatine Kinase	Millipore Sigma	CAT#: C2506	
Chemical compound, drug	Phosphocreatine; PCr	Millipore Sigma	CAT#: 237911	
Chemical compound, drug	Carboxy Atractyloside; CAT	Millipore Sigma	CAT#: C4992	
Chemical compound, drug	17-AAG	Millipore Sigma	CAT#:100068	
Chemical compound, drug	Gamitrinib TPP hexafluorophosphate; Gamitrinib	MedChem Express	CAT#: HY-102007A	
Chemical compound, drug	Curcumin	Millipore Sigma	CAT#: 239802	
Chemical compound, drug	Venetoclax	Selleckchem	CAT#: S8048	
Chemical compound, drug	Bongkreic acid	Cayman Chemical	CAT#: 19079	
Chemical compound, drug	ACK Lysis Buffer	Lonza	CAT#: BP10-548E	
Chemical compound, drug	Mitotracker Green-FM Dye; MTG-FM	Thermo Fisher	CAT#: M7514	
Chemical compound, drug	Tetramethyl rhodamine methyl ester (TMRM)	Invitrogen	CAT#: 1924079	
Chemical compound, drug	Pierce Lys-C Protease	Thermo Fisher	CAT#: 90307	
Transfected construct (<i>Homo sapien</i>)	shRNA to TRAP1	Origene	CAT#: TL300868V	shRNA lentiviral particles packaged from pGFP-C-shLenti vector
Sequence-based reagent	Primer for TRAP1	Thermo Fisher	CAT#: 4331182	
Sequence-based reagent	Primer for 18 s rRNA	Thermo Fisher	CAT#: 4319413E	
Peptide, recombinant protein	Seq Grade Trypsin	Millipore Sigma	CAT#: V5113	
Peptide, recombinant protein	Human Stem Cell Factor; SCF	Gibco	CAT#: PHC2115	
Peptide, recombinant protein	Human Thrombopoietin	Gibco	CAT#: PHC9514	
Peptide, recombinant protein	FLT-3 Ligand	Sigma Aldrich	CAT#: SRP3044	
Commercial assay or kit	RNeasy Midi kit	Qiagen	CAT#: 74124	
Commercial assay or kit	Superscript IV reverse transcriptase	Invitrogen	CAT#: 18090010	

Continued on next page

Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Commercial assay or kit	TMT 10-plex	Thermo Fisher	CAT#: A34808	
Software, algorithm	Prism 8.4	GraphPad	RRID:SCR_002798	
Software, algorithm	Proteome Discoverer 2.2	Thermo Fisher		
Software, algorithm	Mito Carta 2.0		RRID:SCR_018165	

Ethical approval of human subject research

All procedures involving human subjects were approved by the Institutional Review Board of the Brody School of Medicine at East Carolina University (study ID: UMCIRB 18–001328, UMCIRB 19–002331).

Blood collection and isolation of PBMCs

For PBMC samples, healthy subjects (ages 18–70 years), without a prior history of hematological malignancy, were recruited from the surrounding area. Following informed consent (study ID: UMCIRB 18–001328), venous blood from the brachial region of the upper arm was collected. Whole blood was collected in sodium-heparinized Cell Preparation Tubes (CPT) (BD Biosciences, Franklin Lakes, NJ) and centrifuged at 1800 x g for 15 min. Prior to experimentation, mononuclear cells were washed in ammonium-chloride-potassium (ACK) lysis buffer to remove red blood cells and either used immediately for experiments or cultured overnight in IMDM (Thermo Fisher Scientific, Waltham, MA) supplemented with glutamine, 10% FBS, and 1% penicillin/streptomycin.

Mononuclear cell isolation from bone marrow aspirates

For primary leukemia samples, bone marrow aspirates were collected from patients undergoing confirmatory diagnosis for a range of hematological malignancies as a component of an already scheduled procedure. All patients provided informed consent prior to study enrollment (study ID: UMCIRB 19–002331). Type of leukemia ranged from acute myeloid leukemia (AML, N = 14), chronic myeloid leukemia (CML, N = 2), and granular lymphocytic leukemia (N = 1). Patient age ranged from 32 to 78 years (male/female, 8/9). Bone marrow aspirates were collected in sodium-heparinized Cell Preparation Tubes (CPT) (BD Biosciences, Franklin Lakes, NJ) and centrifuged at 1800 x g for 15 min. Mononuclear cells were isolated and then washed in ACK lysis buffer to remove red blood cells and used immediately for experiments. In addition to primary leukemia samples, bone marrow aspirates were collected from healthy donors, ages 26–33, supplied by HemaCare (Northridge, CA).

CD34+ stem/progenitor cell collection and culture

CD34+ cells were purchased from HemaCare (Northridge, CA). These cells were isolated and purified from bone marrow aspirates of healthy donors, ages 20–39. A portion from each vial was cultured in IMDM supplemented with 15% HyClone FBS, recombinant human SCF (25 ng/mL), TPO (50 ng/mL), and FLT-3 (50 ng/mL).

AML cell line culture

HL-60, KG-1, and MV-4–11 (ATCC, Manassas, VA) human leukemia cells were cultured in IMDM (Thermo Fisher Scientific, Waltham, MA) supplemented with glutamax, 10% FBS, and 1% penicillin/streptomycin and incubated at 37°C in 5% CO₂. All cell lines were obtained from ATCC (Manassas, VA). Cell lines were not tested or authenticated over and above documentation provided by ATCC, which included antigen expression, DNA profile, short tandem repeat profiling, and cytogenetic analysis. For experiments involving venetoclax resistance, HL-60 cells were cultured in RPMI-1640, supplemented with glutamax, 10% FBS, and 1% penicillin/streptomycin. To model venetoclax resistance, HL-60 cells were conditionally adapted over time to 1 μM venetoclax in a manner previously described for other chemotherapy drugs (Kao et al., 2019). Upon reaching an average cell density

of 1.5×10^6 cell/mL the cells were harvested and used for whole cell, permeabilized cell, and isolated mitochondria experiments. Primary human muscle progenitor cells (human myoblasts, 'HMB') were derived from fresh muscle biopsy samples, as described previously (Ryan *et al.*, 2018). Cells were cultured on collagen-coated flasks using HMB growth medium (GM: Ham's F10, supplemented with 20% FBS and 1% penicillin/streptomycin, and supplemented immediately prior to use with 5 ng/ml basic FGF).

Cell viability assays

Cell viability was determined by viable cell count using Trypan Blue (0.4%). Where indicated, viability was determined by fluorescence measurement as previously described (L.-P. Kao *et al.*, 2019). Briefly, cells were seeded in black-wall, 96-well plates, in growth medium. After addition of agents (0.1 ml final well volume), cells were incubated at 37° C, 5% CO₂, for the times indicated. Viability was determined using propidium iodide (PI) as follows. Positive control cells were permeabilized by addition of 10 µl of 1.0 mg/ml digitonin and incubated at 37° C, 5% CO₂, for 20 min. Plates were then centrifuged at 1200 x g for 20 min, and after dumping the media, 0.1 ml of a 5.0 µM PI solution in PBS was added. The plate was again incubated for 20 min, and viability was calculated as the mean fluorescence (minus permeabilized vehicle control) at 530 nm excitation and 620 nm emission. For venetoclax-induced cell death assays, cell viability was determined using a standard MTT assay and absorption was read at 570 nm. For all viability assays, each biological replicate was derived from the mean of three technical replicates.

TRAP1 knockdown in MV-4-11 cells

MV-4-11 cells were cultured in IMDM (Thermo Fisher Scientific, Waltham, MA) supplemented with glutamax, 10% FBS, and 1% penicillin/streptomycin and incubated at 37°C in 5% CO₂. Human shRNA lentiviral particles packaged from pGFP-C-shLenti vector (4 unique 29mer TRAP1-specific shRNA [ACAGCCGCAAAGTCCTCATCCAGACCAAG; ATGGTGGCTGACAGAGTGGAGGTCTATTC; GGAGACGGACATAGTCGTGGATCACTACA; TGGCTTTCAGATGGTTCTGGAGTGTGTTGA], one scramble control; 0.5 ml each, >10⁷ TU/ml) were purchased from Origene (CAT#: TL300868V). To facilitate infection, MV-4-11 cells and lentiviral particles were co-cultured for 24 hr in individual wells of a 96-well plate in 0.1 mL of IMDM growth media, supplemented with 4 µg/mL polybrene (multiplicity of infection of approximately 20). At the end of the 24 hr, cells were spun down and resuspended in culture media devoid of polybrene. Cultures were then subjected to puromycin selection by continuous exposure to 2 µg/mL puromycin in the culture media. Confirmation of TRAP1 knockdown was performed via real-time PCR. To do this, total RNA was extracted from cell pellets using Qiagen RNeasy Midi kits per manufacturer instructions. RNA was reverse transcribed using Superscript IV reverse transcriptase according to manufacturer instructions (Invitrogen). Real-time PCR on TRAP1 was performed using a Quantstudio 3 Real-Time PCR system (Applied Biosystems). Relative quantification of mRNA levels was determined using the comparative threshold cycle ($\Delta\Delta CT$) method using FAM-labeled Taqman gene expression assays (Applied Biosystems) specific to TRAP1 run in multiplex with a VIC-labeled 18S control primer.

Confocal microscopy

Cells were pre-loaded with 200 nM Mitotracker Green-FM dye (MTG-FM; Molecular Probes, Eugene, OR) at 37°C for 1 hr. Cells were then centrifuged at 300 x g for 7 min at ~25°C and resuspended in MTG-FM-free IMDM formulation media (Thermo Fisher) containing 50 nM tetramethyl rhodamine methyl ester (TMRM) and 2 µM Hoechst 33342. Cells were plated on glass-bottom dishes (MatTek, Ashland, MA) for imaging. Cells were held in place with a thin 1% agarose pad that was applied immediately prior to imaging in order to minimize rapid motion interference during imaging of live non-adherent cells (Ivanusic *et al.*, 2017).

All imaging were performed using an Olympus FV1000 laser scanning confocal microscope (LSCM) with an onstage incubator at 37°C. Acquisition software was Olympus FluoView FSW (V4.2). The objective used was 60X oil immersion (NA = 1.35, Olympus Plan Apochromat UPLSAPO60X(F)). Images were 800 × 800 pixel with 2µs/pixel dwell time, sequential scan mode, resulting in a 4X digital zoom. Hoechst 33342 was excited using the 405 nm line of a multiline argon laser; emission was filtered using a 560 nm dichroic mirror and 420–460 nm barrier filter. MTG-FM was excited using the

488 nm line of a multiline argon laser; emission was filtered using a 560 nm dichroic mirror and 505–540 nm barrier filter. TMRM was excited using a 559 nm laser diode; emission was filtered using a 575–675 nm barrier filter. Zero detector offset was used for all images and gain at the detectors was kept the same for all imaging. The pinhole aperture diameter was set to 105 μm (1 Airy disc).

Images were analyzed using Fiji (*Schindelin et al., 2012*). Spatial resolution was measured using sub-resolution fluorescent beads (Thermo Fisher) and curve fitting was performed using the MetroloJ plugin in Fiji. 16-bit images were made into a composite. Circular ROIs were manually selected using the ROI manager plugin. Images were then decomposed into separate 16-bit image stacks leaving the ROI positions intact. A Huang auto-threshold was used for automated selection of signal for all three channels. Following threshold application, each signal was measured using the multi-measure feature. Only whole cells were analyzed (i.e. cells on edges of the FOV were excluded). Slices containing cells above the lowest monolayer were removed from stacks to avoid oversampling. The following calculations were performed to determine the relevant signal volumes.

$$\text{Signal Volume } (\mu\text{m}^3) = [A \cdot Z] / N$$

where A is the signal-positive area selected using a Huang auto-threshold (μm^2), Z is the optical section thickness (axial resolution; μm), and N is the number of steps within each optical section (i.e. axial resolution divided by the step size). The latter operation is necessary to correct for oversampling of the signal volumes.

Respiratory flux in intact and permeabilized cells

Approximately $1\text{--}3 \times 10^6$ cells were used for each intact and permeabilized cell experiment. High-resolution respirometry measurements were performed using the Oroboros Oxgraph-2k (O2k; Oroboros Instruments, Innsbruck, Austria) in a 0.5 or 1.0 mL reaction volume at 37°C. For normalization to total protein, at the conclusion of each experiment the cell suspension was collected from each chamber and centrifuged at 2000 $\times$ g for 10 min at 4°C. Cells were lysed using low-percentage detergent buffer (CellLytic M) followed by a freeze-thaw cycle, and protein concentration was determined using a BCA protein assay.

Respiratory flux was measured using previously described methods (*Fisher-Wellman et al., 2018*). For intact cell measurements, cells were resuspended in Intact Cell Respiratory Media (17.7 g/L Iscove's Modified Dulbecco's Medium (IMDM), 20 mM HEPES, 1% Penicillin/Streptomycin, 10% FBS, pH 7.4). After basal respiration was established, oligomycin (Oligo; 0.02 μM) was added followed by FCCP titration (FC; 0.5–5 μM), rotenone (Rot; 0.5 μM) and antimycin A (Ant; 0.5 μM). For permeabilized cell measurements, cells were resuspended in Respiratory Buffer supplemented with creatine (105 mM MES potassium salt, 30 mM KCl, 8 mM NaCl, 1 mM EGTA, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 0.25% BSA, 5 mM creatine monohydrate, pH 7.2). Cells were permeabilized with digitonin (Digi; 0.02 mg/mL), and respiratory flux was measured using the creatine kinase (CK) clamp and FCCP titration assays. Within the CK clamp assay, the free energy of ATP hydrolysis (ΔG_{ATP}) is calculated using the equilibrium constant for the CK reaction (K'_{CK}) and is based upon the addition of known concentrations of creatine (CR), phosphocreatine (PCR), and ATP in the presence of excess amounts of CK (*Fisher-Wellman et al., 2018*). Calculation of ΔG_{ATP} at defined PCR concentrations was done using the online resource (https://dmpio.github.io/bioenergetic-calculators/ck_clamp/) previously described (*Fisher-Wellman et al., 2018*).

For all assays, various combinations of carbon substrates and inhibitors were employed. Substrates and inhibitors utilized are indicated in the figure legends: CK (20 U/mL), ATP (5 mM), PCR (1 mM, 6 mM, 15 mM, 21 mM), pyruvate (Pyr or P; 5 mM), malate (M; 1 mM), glutamate (G; 5 mM), octanoyl-carnitine (O; 0.2 mM), succinate (Succ or S; 5 mM) cytochrome C (Cyt C, 10 μM), oligomycin (Oligo, 0.02 μM), FCCP (FC, 0.5–2 μM), rotenone (Rot, 0.5 μM), antimycin A (Ant A, 0.5 μM), carboxyatractyloside (CAT, 1 μM), bongkreic acid (20 μM), 17-AAG (sigma, #100068, 1 μM), Gamitrinib TPP hexafluorophosphate (MedChemExpress, #HY-102007A, 1 μM).

Extracellular flux analysis

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a Seahorse XF24e flux analyzer (Agilent Technologies, Santa Clara, CA). Prior to analysis, wells were coated with Cell-Tak (Corning, Cat# 354240). Cells were then seeded at 3×10^5 cells/well. The assay

was performed in bicarbonate free IMDM, supplemented with 2 mM HEPES, in the absence of FBS, pH 7.4. The flux analysis protocol was as follows: Basal OCR and ECAR were measured followed by the addition of 5 μ M Oligomycin, and 50 mM 2-deoxyglucose (2-DG). All data were normalized to viable cell count.

Isolation of mitochondria from PBMCs and leukemia cells

In order to pellet cells, PBMC fractions were washed with PBS and centrifuged at 300 x g for 10 min at 4°C and leukemia cells were centrifuged at 300 x g for 10 min followed by a PBS wash. Cell pellets were resuspended in Mitochondrial Isolation Buffer with BSA (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄, 0.2% BSA, pH 7.1) and homogenized using a borosilicate glass mortar and Teflon pestle. Homogenates were centrifuged at 800 x g for 10 min at 4°C. The supernatant was collected, and the remaining pellet was resuspended in Mitochondrial Isolation Buffer with BSA, then homogenized and centrifuged again. This process was repeated a total of three times. The collected supernatant was centrifuged at 10,000 x g for 10 min at 4°C to pellet the mitochondrial fraction. The fraction was resuspended in Mitochondrial Isolation Buffer without BSA, transferred to a microcentrifuge tube and subjected to another spin at 10,000 x g. The mitochondrial pellet was resuspended in ~100 μ L of Mitochondria Isolation Buffer and protein concentration was calculated using the Pierce BCA assay. Respiration assays using isolated mitochondria were similar to that described for permeabilized cells.

Mitochondrial NADH/NAD⁺ redox in isolated mitochondria

Fluorescent determination of NADH/NAD⁺ was performed using a QuantaMaster Spectrofluorometer (QM-400, Horiba Scientific, Kyoto, Japan). The NADH/NAD⁺ was detected at Ex/Em: 350/450. NADH/NAD⁺ was measured in mitochondria isolated from PBMC and leukemia cell lines using the CK clamp assay. Experiments were performed at 37°C in a 200 μ L reaction volume. To start, Respiratory Buffer supplemented with creatine (200 μ L), Cyt C (10 μ M), mitochondrial lysate (100 μ g) were added into a glass cuvette. Mitochondria were incubated at 37°C for ~5 min in the absence of substrate to induce 0% reduction of the NADH pool. Saturating carbon substrates were added (P/M/G/S/O), and respiration was stimulated with the CK clamp. Titration of ΔG_{ATP} was performed via PCR titration (6, 15, 21 mM). Oligomycin (0.02 μ M) was added to inhibit ATP synthesis and cyanide (CN, 10 mM) was added to induce 100% reduction of the matrix NADH pool. The NADH/NAD⁺ was expressed as a percentage reduction of the CN value (i.e. 100% reduction) based upon the formula % Reduction = $(F - F_{0\%}) / (F_{100\%} - F_{0\%}) * 100$.

Mitochondrial membrane potential ($\Delta\Psi$) in isolated mitochondria

Fluorescent determination of $\Delta\Psi$ was carried out via a QuantaMaster Spectrofluorometer (QM-400; Horiba Scientific). Determination of $\Delta\Psi$ via TMRM was done as described previously (Fisher-Wellman et al., 2018), taking the fluorescence ratio of the following excitation/emission parameters [Ex/Em, 576/590 and 551/590]. The 576/551 ratio was then converted to millivolts via a KCl standard curve performed in the presence of valinomycin as described (Fisher-Wellman et al., 2018).

Mitochondrial JATP synthesis, P/O ratio and OXPHOS power output in permeabilized cells

Parallel respiration and fluorometric experiments were carried out in order to generate an ATP/O (P/O) ratio, as previously described (Lark et al., 2016). Experiments were performed at 37°C in a 2.5 mL reaction volume in Respiratory Buffer supplemented with AP5A (0.2 mM), and digitonin (0.02 mg/mL). ATP synthesis and oxygen consumption were determined in permeabilized cells energized with P/M/G/S/O in the presence of 0.1 mM ADP. The P/O ratio was then calculated using the ratio of the JATP and the steady state JO₂, divided by 2. Empirically derived P/O ratios were then used to convert oxygen consumption rates recorded during the CK clamp assay (using identical substrates) to ATP production rate [(oxygen consumption rate) x (P/O x 2)]. A fixed extra-mitochondrial force was assumed to be applied via the CK clamp at each PCR titration. These forces corresponded to -54.16, -58.93, -60.64, and -61.49 kJ/mol. These forces were used along with ATP production rate to quantitate OXPHOS power output in Watts (J/s) according to the following formula [(pmol ATP/s/million cells) x (5.416 or 5.893 or 6.064 or 6.149 * 10⁻⁸ J/pmol ATP) = μ Watts/million cells].

Mitochondrial lysis, protein digestion, and peptide labeling for TMT quantitative proteomics

Mitochondrial pellets from leukemia cells and PBMC (approximately 250 μg of protein) were lysed in ice-cold 8 M Urea Lysis Buffer (8 M urea in 50 mM Tris, pH 8.0, 40 mM NaCl, 2 mM CaCl_2 , 1x cOmplete ULTRA mini EDTA-free protease inhibitor tablet), as described previously (McLaughlin *et al.*, 2020). The samples were frozen on dry ice and thawed for three freeze-thaw cycles and further disrupted by sonication with a probe sonicator in three 5 s bursts set at an amplitude of 30 (Q Sonica, Newtown, CT). Samples were centrifuged at $10,000 \times g$ for 10 min at 4°C to pellet insoluble material. Protein concentration was determined by BCA, and equal amounts of protein (200 μg , adjusted to 2.5 mg/mL with Urea Lysis Buffer) were reduced with 5 mM DTT at 32°C for 30 min, cooled to room temperature, and then alkylated with 15 mM iodoacetamide for 30 min in the dark. Unreacted iodoacetamide was quenched by the addition of DTT up to 15 mM. Initial digestion was performed with Lys C (Thermo Fisher) 1:100 w-w; 2 μg enzyme per 200 μg protein for 4 hr at 32°C . Following dilution to 1.5 M urea with 50 mM Tris (pH 8.0), 30 mM NaCl, 5 mM CaCl_2 , the samples were digested overnight with trypsin (Promega, Madison, WI) 50:1 w/w, protein:enzyme at 32°C . Samples were acidified to 0.5% TFA and centrifuged at $10,000 \times g$ for 10 min at 4°C to pellet insoluble material. Supernatant containing soluble peptides was desalted on a 50 mg tC18 SEP-PAK solid phase extraction column (Waters, Milford, MA) and eluted (500 μL 25% acetonitrile/0.1% TFA and $2 \times 500 \mu\text{L}$ 50% acetonitrile/0.1% TFA). The 1.5 mL eluate was frozen and lyophilized.

TMT labeling

TMT labeling was performed as previously described (McLaughlin *et al.*, 2020). The samples from isolated mitochondria were re-suspended in 100 μL of 200 mM triethylammonium bicarbonate (TEAB), mixed with a unique 10-plex Tandem Mass Tag (TMT) reagent (0.8 mg re-suspended in 50 μL 100% acetonitrile), and shaken for 4 hr at room temperature (Thermo Fisher). A total of 2×10 plex kits were used and one sample was TMT-labeled in both kits to control for quantification differences across multiplex preparations. Following quenching with 0.8 μL 50% hydroxylamine samples were frozen, and lyophilized. Samples were re-suspended in ~ 1 mL of 0.5% TFA and again subjected to solid phase extraction, but with a 100 mg tC18 SEP-PAK SPE column (Waters). The multiplexed peptide sample was subjected to high pH reversed phase fractionation according to the manufacturer's instructions (Thermo Fisher). In this protocol, peptides (100 μg) are loaded onto a pH-resistant resin and then desalted with water washing combined with low-speed centrifugation. A step-gradient of increasing acetonitrile concentration in a high-pH elution solution is then applied to columns to elute bound peptides into eight fractions. Following elution, fractions were frozen and lyophilized.

nLC-MS/MS for TMT proteomics nLC-MS/MS was performed as described previously (McLaughlin *et al.*, 2020). Peptide fractions were suspended in 0.1% formic acid at a concentration of 0.25 $\mu\text{g}/\mu\text{L}$, following peptide quantification (ThermoFisher). All samples were subjected to nanoLC-MS/MS analysis using an UltiMate 3000 RSLCnano system (Thermo Fisher) coupled to a Q Exactive PlusHybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher) via nanoelectrospray ionization source. For each injection of 4 μL (1 μg), the sample was first trapped on an Acclaim Pep-Map 100 20 mm $\times$ 0.075 mm trapping column (Thermo Fisher) 5 $\mu\text{L}/\text{min}$ at 98/2 v/v water/acetonitrile with 0.1% formic acid, after which the analytical separation was performed over a 90-min gradient (flow rate of 300 nanoliters/min) of 3–30% acetonitrile using a 2 μm EASY-Spray PepMap RSLC C18 75 $\mu\text{m} \times 250$ mm column (Thermo Fisher) with a column temperature of 55°C . MS1 was performed at 70,000 resolution, with an AGC target of 1×10^6 ions and a maximum IT of 60 ms. MS2 spectra were collected by data-dependent acquisition (DDA) of the top 20 most abundant precursor ions with a charge greater than one per MS1 scan, with dynamic exclusion enabled for 30 s. Precursor ions were filtered with a 1.0 m/z isolation window and fragmented with a normalized collision energy of 30. MS2 scans were performed at 17,500 resolution, AGC target of 1×10^5 ions, and a maximum IT of 60 ms.

Data analysis for TMT proteomics

Proteome Discoverer 2.2 (PDv2.2) was used for raw data analysis, with default search parameters including oxidation (15.995 Da on M) as a variable modification and carbamidomethyl (57.021 Da on

C) and TMT6plex (229.163 Da on peptide N-term and K) as fixed modifications, and two missed cleavages (full trypsin specificity). Data were searched against human Mito Carta 2.0 database (Calvo et al., 2016). PSMs were filtered to a 1% FDR. PSMs were grouped to unique peptides while maintaining a 1% FDR at the peptide level. Peptides were grouped to proteins using the rules of strict parsimony and proteins were filtered to 1% FDR using the Protein FDR Validator node of PD2.2. MS2 reporter ion intensities for all PSMs having co-isolation interference below 0.5 (50% of the ion current in the isolation window) and an average S/N > 10 for reporter ions were summed together at the peptide and protein level. Imputation was performed via low abundance resampling.

Statistical analysis for TMT proteomic

The protein group tab in the PDv2.2 results was exported as tab delimited.txt. files, and analyzed based on a previously described workflow (McLaughlin et al., 2020). First, M2 reporter (TMT) intensities were summed together for each TMT channel, each channel's sum was divided by the average of all channels' sums, resulting in channel-specific loading control normalization factors to correct for any deviation from equal protein input in the 10-plex experiments. Reporter intensities for proteins were divided by the loading control normalization factors for each respective TMT channel. All loading control-normalized reporter intensities were converted to log₂ space and the average value from the 10 samples per kit was subtracted from each sample-specific measurement to normalize the relative measurements to the mean of each kit. Data from each kit were then combined for statistical comparisons. For comparison of PBMC to leukemia cell lines, condition average, standard deviation, p-value (p, two-tailed student's t-test, assuming equal variance), and adjusted p-value ($P_{adjusted}$, Benjamini Hochberg FDR correction) were calculated (Benjamini and Hochberg, 1995; Lesack and Naugler, 2011). For protein-level quantification, only Master Proteins—or the most statistically significant protein representing a group of parsimonious proteins containing common peptides identified at 1% FDR—were used for quantitative comparison.

Data availability

All data from the manuscript are available upon request. In addition, all data are available in the source data files provided with this paper. All raw data for proteomics experiments is available online using accession number 'PXD020715' for Proteome Xchange (Deutsch et al., 2017) and accession number 'JPST000934' for jPOST Repository (Okuda et al., 2017).

Statistical analysis and software

Statistical analysis was performed using GraphPad Prism 8.4. All data are represented as mean ± SEM and analysis were conducted with a significance level set at p<0.05. Details of statistical analysis are included within figure legends. Figures were generated using Biorender and GraphPad Prism 8.4.

Acknowledgements

The work was supported by DOD-W81XWH-19-1-0213 (K.H.F.-W.) and NIH P01 CA171983 (M.C.C). This project used the North Carolina Tissue Consortium (NCTC) shared resource which is supported in part by the University Cancer Research Fund (UCRF).

Additional information

Funding

Funder	Grant reference number	Author
U.S. Army Medical Research and Materiel Command	W81XWH-19-1-0213	Kelsey H Fisher-Wellman
National Cancer Institute	P01 CA171983	Myles C Cabot

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Author contributions

Margaret AM Nelson, Kelsey L McLaughlin, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing; James T Hagen, Conceptualization, Data curation, Investigation, Methodology, Writing - review and editing; Hannah S Coalson, Data curation, Investigation, Writing - review and editing; Cameron Schmidt, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing; Miki Kassai, Investigation; Kimberly A Kew, Data curation, Methodology, Writing - review and editing; Joseph M McClung, Conceptualization, Resources, Methodology, Writing - review and editing; P Darrell Neuffer, Conceptualization, Supervision, Writing - review and editing; Patricia Brophy, Resources, Project administration, Writing - review and editing; Nasreen A Vohra, Darla Liles, Conceptualization, Resources, Project administration, Writing - review and editing; Myles C Cabot, Conceptualization, Resources, Funding acquisition, Project administration, Writing - review and editing; Kelsey H Fisher-Wellman, Conceptualization, Resources, Data curation, Software, Formal analysis, Supervision, Funding acquisition, Validation, Investigation, Visualization, Methodology, Writing - original draft, Project administration, Writing - review and editing

Author ORCIDs

Kelsey H Fisher-Wellman  <https://orcid.org/0000-0002-0300-829X>

Ethics

Human subjects: All procedures involving human subjects were approved by the Institutional Review Board of the Brody School of Medicine at East Carolina University (study ID: UMCIRB 18-001328, UMCIRB 19-002331). For PBMC samples, healthy subjects (ages 18-70 years), without a prior history of hematological malignancy, were recruited from the surrounding area. Following informed consent (study ID: UMCIRB 18-001328), venous blood from the brachial region of the upper arm was collected. For primary leukemia samples, bone marrow aspirates were collected from patients undergoing confirmatory diagnosis for a range of hematological malignancies as a component of an already scheduled procedure. All patients provided informed consent prior to study enrollment (study ID: UMCIRB 19-002331).

Decision letter and Author response

Decision letter <https://doi.org/10.7554/eLife.63104.sa1>

Author response <https://doi.org/10.7554/eLife.63104.sa2>

Additional files

Supplementary files

- Supplementary file 1. Mitochondrial proteome of AML cell lines, relative to PBMC. (A) Exported results from PDv2.2. (B) Analyzed master protein expression by group.
- Transparent reporting form

Data availability

All data from the manuscript are available upon request. In addition, all data are available in the source data files provided with this paper. Raw data for proteomics experiments are available online using accession number "PXD020715" for Proteome Xchange and accession number "JPST000934" for jPOST Repository <http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD020715>.

The following datasets were generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Fisher-Wellman KH	2020	Mitochondrial proteome of human leukemia.	http://proteomecentral.proteomexchange.org/	Proteome Xchange , PXD020715

cgi/GetDataset?ID=
PXD020715

Fisher-Wellman KH	2020	Mitochondrial proteome of human leukemia	https://repository.jpostdb.org/entry/JPST000934	jPOST Repository , JPST000934
-------------------	------	--	---	-------------------------------

References

- Benjamini Y**, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B* **57**:289–300. DOI: <https://doi.org/10.1111/j.2517-6161.1995.tb02031.x>
- Bryant KG**, Chae YC, Martinez RL, Gordon JC, Elokely KM, Kossenkov AV, Grant S, Childers WE, Abou-Gharbia M, Altieri DC. 2017. A Mitochondrial-targeted purine-based HSP90 antagonist for leukemia therapy. *Oncotarget* **8**:112184–112198. DOI: <https://doi.org/10.18632/oncotarget.23097>, PMID: 29348817
- Buck MD**, O’Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O’Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. 2016. Mitochondrial dynamics controls T cell fate through metabolic programming. *Cell* **166**:63–76. DOI: <https://doi.org/10.1016/j.cell.2016.05.035>, PMID: 27293185
- Byrd JC**, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, Grant B, Sharman JP, Coleman M, Wierda WG, Jones JA, Zhao W, Heerema NA, Johnson AJ, Sukbuntherng J, Chang BY, Clow F, Hedrick E, Buggy JJ, James DF, et al. 2013. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *New England Journal of Medicine* **369**:32–42. DOI: <https://doi.org/10.1056/NEJMoa1215637>, PMID: 23782158
- Calvo SE**, Clauser KR, Mootha VK. 2016. MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Research* **44**:D1251–D1257. DOI: <https://doi.org/10.1093/nar/gkv1003>, PMID: 26450961
- Chevrollier A**, Loiseau D, Reynier P, Stepien G. 2011. Adenine nucleotide translocase 2 is a key mitochondrial protein in Cancer metabolism. *Biochimica Et Biophysica Acta (BBA) - Bioenergetics* **1807**:562–567. DOI: <https://doi.org/10.1016/j.bbabi.2010.10.008>
- Cohen BH**. 2010. Pharmacologic effects on mitochondrial function. *Developmental Disabilities Research Reviews* **16**:189–199. DOI: <https://doi.org/10.1002/ddrr.106>, PMID: 20818734
- Deutsch EW**, Csordas A, Sun Z, Jarnuczak A, Perez-Riverol Y, Ternent T, Campbell DS, Bernal-Llinares M, Okuda S, Kawano S, Moritz RL, Carver JJ, Wang M, Ishihama Y, Bandeira N, Hermjakob H, Vizcaino JA. 2017. The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Research* **45**:D1100–D1106. DOI: <https://doi.org/10.1093/nar/gkw936>
- Dykens JA**, Will Y. 2007. The significance of mitochondrial toxicity testing in drug development. *Drug Discovery Today* **12**:777–785. DOI: <https://doi.org/10.1016/j.drudis.2007.07.013>, PMID: 17826691
- Farge T**, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, Bosc C, Sugita M, Stuanil L, Fraisse M, Scotland S, Larrue C, Boutzen H, Féliu V, Nicolau-Travers ML, Cassant-Sourdy S, Broin N, David M, Serhan N, Sarry A, et al. 2017. Chemotherapy-Resistant human acute myeloid leukemia cells are not enriched for leukemic stem cells but require oxidative metabolism. *Cancer Discovery* **7**:716–735. DOI: <https://doi.org/10.1158/2159-8290.CD-16-0441>, PMID: 28416471
- Fisher-Wellman KH**, Davidson MT, Narowski TM, Lin CT, Koves TR, Muoio DM. 2018. Mitochondrial diagnostics: a multiplexed assay platform for comprehensive assessment of mitochondrial energy fluxes. *Cell Reports* **24**:3593–3606. DOI: <https://doi.org/10.1016/j.celrep.2018.08.091>, PMID: 30257218
- Fisher-Wellman KH**, Draper JA, Davidson MT, Williams AS, Narowski TM, Slentz DH, Ilkayeva OR, Stevens RD, Wagner GR, Najjar R, Hirschey MD, Thompson JW, Olson DP, Kelly DP, Koves TR, Grimsrud PA, Muoio DM. 2019. Respiratory phenomics across multiple models of protein hyperacetylation in cardiac mitochondria reveals a marginal impact on bioenergetics. *Cell Reports* **26**:1557–1572. DOI: <https://doi.org/10.1016/j.celrep.2019.01.057>, PMID: 30726738
- Glancy B**, Willis WT, Chess DJ, Balaban RS. 2013. Effect of calcium on the oxidative phosphorylation cascade in skeletal muscle mitochondria. *Biochemistry* **52**:2793–2809. DOI: <https://doi.org/10.1021/bi3015983>, PMID: 23547908
- Goto M**, Miwa H, Shikami M, Tsunekawa-Imai N, Suganuma K, Mizuno S, Takahashi M, Mizutani M, Hanamura I, Nitta M. 2014a. Importance of glutamine metabolism in leukemia cells by energy production through TCA cycle and by redox homeostasis. *Cancer Investigation* **32**:241–247. DOI: <https://doi.org/10.3109/07357907.2014.907419>, PMID: 24762082
- Goto M**, Miwa H, Suganuma K, Tsunekawa-Imai N, Shikami M, Mizutani M, Mizuno S, Hanamura I, Nitta M. 2014b. Adaptation of leukemia cells to hypoxic condition through switching the energy metabolism or avoiding the oxidative stress. *BMC Cancer* **14**:1–9. DOI: <https://doi.org/10.1186/1471-2407-14-76>, PMID: 24506813
- Guièze R**, Liu VM, Rosebrock D, Jourdain AA, Hernández-Sánchez M, Martínez Zurita A, Sun J, Ten Hacken E, Baranowski K, Thompson PA, Heo JM, Cartun Z, Aygün O, Iorgulescu JB, Zhang W, Notarangelo G, Livitz D, Li S, Davids MS, Biran A, et al. 2019. Mitochondrial reprogramming underlies resistance to BCL-2 inhibition in lymphoid malignancies. *Cancer Cell* **36**:369–384. DOI: <https://doi.org/10.1016/j.ccell.2019.08.005>, PMID: 31543463

- Helling S**, Hüttemann M, Ramzan R, Kim SH, Lee I, Müller T, Langenfeld E, Meyer HE, Kadenbach B, Vogt S, Marcus K. 2012. Multiple phosphorylations of cytochrome c oxidase and their functions. *Proteomics* **12**:950–959. DOI: <https://doi.org/10.1002/pmic.201100618>, PMID: 22522801
- Inoue T**, Swain A, Nakanishi Y, Sugiyama D. 2014. Multicolor analysis of cell surface marker of human leukemia cell lines using flow cytometry. *Anticancer Research* **34**:4539–4550. PMID: 25075097
- Ivanusic D**, Madela K, Denner J. 2017. Easy and cost-effective stable positioning of suspension cells during live-cell imaging. *Journal of Biological Methods* **4**:80. DOI: <https://doi.org/10.14440/jbm.2017.203>
- Jitschin R**, Hofmann AD, Bruns H, Giessl A, Bricks J, Berger J, Saul D, Eckart MJ, Mackensen A, Mougiakakos D. 2014. Mitochondrial metabolism contributes to oxidative stress and reveals therapeutic targets in chronic lymphocytic leukemia. *Blood* **123**:2663–2672. DOI: <https://doi.org/10.1182/blood-2013-10-532200>, PMID: 24553174
- Kadenbach B**. 2021. Complex IV - The regulatory center of mitochondrial oxidative phosphorylation. *Mitochondrion* **58**:296–302. DOI: <https://doi.org/10.1016/j.mito.2020.10.004>, PMID: 33069909
- Kamal A**, Thao L, Sensintaffar J, Zhang L, Boehm MF, Fritz LC, Burrows FJ. 2003. A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* **425**:407–410. DOI: <https://doi.org/10.1038/nature01913>, PMID: 14508491
- Kao LP**, Morad SAF, Davis TS, MacDougall MR, Kassai M, Abdelmageed N, Fox TE, Kester M, Loughran TP, Abad JL, Fabrias G, Tan SF, Feith DJ, Claxton DF, Spiegel S, Fisher-Wellman KH, Cabot MC. 2019. Chemotherapy selection pressure alters sphingolipid composition and mitochondrial bioenergetics in resistant HL-60 cells. *Journal of Lipid Research* **60**:1590–1602. DOI: <https://doi.org/10.1194/jlr.RA119000251>, PMID: 31363040
- Kim D**, Kim SY, Kim D, Yoon NG, Yun J, Hong KB, Lee C, Lee JH, Kang BH, Kang S. 2020. Development of pyrazolo[3,4-d]pyrimidine-6-amine-based TRAP1 inhibitors that demonstrate in vivo anticancer activity in mouse xenograft models. *Bioorganic Chemistry* **101**:103901. DOI: <https://doi.org/10.1016/j.bioorg.2020.103901>, PMID: 32590225
- Kuntz EM**, Baquero P, Michie AM, Dunn K, Tardito S, Holyoake TL, Helgason GV, Gottlieb E. 2017. Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. *Nature Medicine* **23**:1234–1240. DOI: <https://doi.org/10.1038/nm.4399>, PMID: 28920959
- Lagadinou ED**, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, Ashton JM, Pei S, Grose V, O'Dwyer KM, Liesveld JL, Brookes PS, Becker MW, Jordan CT. 2013. BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. *Cell Stem Cell* **12**:329–341. DOI: <https://doi.org/10.1016/j.stem.2012.12.013>, PMID: 23333149
- Laquatra C**, Sanchez-Martin C, Dinarello A, Cannino G, Minervini G, Moroni E, Schiavone M, Tosatto S, Argenton F, Colombo G, Bernardi P, Masgras I, Rasola A. 2021. HIF1 α -dependent induction of the mitochondrial chaperone TRAP1 regulates bioenergetic adaptations to hypoxia. *Cell Death & Disease* **12**:434. DOI: <https://doi.org/10.1038/s41419-021-03716-6>, PMID: 33934112
- Lark DS**, Torres MJ, Lin CT, Ryan TE, Anderson EJ, Neuffer PD. 2016. Direct real-time quantification of mitochondrial oxidative phosphorylation efficiency in permeabilized skeletal muscle myofibers. *American Journal of Physiology-Cell Physiology* **311**:C239–C245. DOI: <https://doi.org/10.1152/ajpcell.00124.2016>, PMID: 27335172
- Lee EA**, Angka L, Rota SG, Hanlon T, Mitchell A, Hurren R, Wang XM, Gronda M, Boyaci E, Bojko B, Minden M, Sriskanthadevan S, Datti A, Wrana JL, Edginton A, Pawliszyn J, Joseph JW, Quadriatero J, Schimmer AD, Spagnuolo PA. 2015. Targeting mitochondria with avocatin B induces selective leukemia cell death. *Cancer Research* **75**:2478–2488. DOI: <https://doi.org/10.1158/0008-5472.CAN-14-2676>, PMID: 26077472
- Lee D**, Xu IM, Chiu DK, Lai RK, Tse AP, Lan Li L, Law CT, Tsang FH, Wei LL, Chan CY, Wong CM, Ng IO, Wong CC. 2017. Folate cycle enzyme MTHFD1L confers metabolic advantages in hepatocellular carcinoma. *Journal of Clinical Investigation* **127**:1856–1872. DOI: <https://doi.org/10.1172/JCI90253>, PMID: 28394261
- Lee CH**, Kim MJ, Lee HH, Paeng JC, Park YJ, Oh SW, Chai YJ, Kim YA, Cheon GJ, Kang KW, Youn H, Chung JK. 2019. Adenine nucleotide translocase 2 as an enzyme related to [¹⁸F] FDG Accumulation in Various Cancers. *Molecular Imaging and Biology* **21**:722–730. DOI: <https://doi.org/10.1007/s11307-018-1268-x>, PMID: 30225759
- Lesack K**, Naugler C. 2011. An open-source software program for performing bonferroni and related corrections for multiple comparisons. *Journal of Pathology Informatics* **2**:52. DOI: <https://doi.org/10.4103/2153-3539.91130>, PMID: 22276243
- Li XT**, Li YS, Shi ZY, Guo XL. 2020. New insights into molecular chaperone TRAP1 as a feasible target for future Cancer treatments. *Life Sciences* **254**:117737. DOI: <https://doi.org/10.1016/j.lfs.2020.117737>, PMID: 32376268
- Liu F**, Kalpage HA, Wang D, Edwards H, Hüttemann M, Ma J, Su Y, Carter J, Li X, Polin L, Kushner J, Dzinic SH, White K, Wang G, Taub JW, Ge Y. 2020. Cotargeting of mitochondrial complex I and Bcl-2 shows antileukemic activity against acute myeloid leukemia cells reliant on oxidative phosphorylation. *Cancers* **12**:2400–2419. DOI: <https://doi.org/10.3390/cancers12092400>
- Luengo A**, Li Z, Gui D, Sullivan L, Zagorulya M, Do B, Ferreira R, Naamati A, Ali A, Lewis C, Thomas C, Spranger S, Matheson N, Vander Heiden M. 2020. Increased demand for NAD⁺ relative to ATP drives aerobic glycolysis. *Molecular Cell* **17**:691–707. DOI: <https://doi.org/10.1016/j.molcel.2020.12.012>
- Luptak I**, Sverdlov AL, Panagia M, Qin F, Pimentel DR, Croteau D, Siwik DA, Ingwall JS, Bachschmid MM, Balschi JA, Colucci WS. 2018. Decreased ATP production and myocardial contractile reserve in metabolic heart disease. *Journal of Molecular and Cellular Cardiology* **116**:106–114. DOI: <https://doi.org/10.1016/j.yjmcc.2018.01.017>, PMID: 29409987

- Maldonado EN**, DeHart DN, Patnaik J, Klatt SC, Gooz MB, Lemasters JJ. 2016. ATP/ADP turnover and import of glycolytic ATP into mitochondria in Cancer cells is independent of the Adenine nucleotide translocator. *Journal of Biological Chemistry* **291**:19642–19650. DOI: <https://doi.org/10.1074/jbc.M116.734814>, PMID: 27458020
- Martínez-Reyes I**, Cardona LR, Kong H, Vasan K, McElroy GS, Werner M, Kihshen H, Reczek CR, Weinberg SE, Gao P, Steinert EM, Piseaux R, Budinger GRS, Chandel NS. 2020. Mitochondrial ubiquinol oxidation is necessary for tumour growth. *Nature* **585**:288–292. DOI: <https://doi.org/10.1038/s41586-020-2475-6>, PMID: 32641834
- McLaughlin KL**, McClung JM, Fisher-Wellman KH. 2018. Bioenergetic consequences of compromised mitochondrial DNA repair in the mouse heart. *Biochemical and Biophysical Research Communications* **504**:742–748. DOI: <https://doi.org/10.1016/j.bbrc.2018.09.022>
- McLaughlin KL**, Kew KA, McClung JM, Fisher-Wellman KH. 2020. Subcellular proteomics combined with bioenergetic phenotyping reveals protein biomarkers of respiratory insufficiency in the setting of proofreading-deficient mitochondrial polymerase. *Scientific Reports* **10**:3603. DOI: <https://doi.org/10.1038/s41598-020-60536-y>, PMID: 32107436
- Messer JI**, Jackman MR, Willis WT. 2004. Pyruvate and citric acid cycle carbon requirements in isolated skeletal muscle mitochondria. *American Journal of Physiology-Cell Physiology* **286**:C565–C572. DOI: <https://doi.org/10.1152/ajpcell.00146.2003>
- Mirali S**, Botham A, Voisin V, Xu C, St-Germain J, Sharon D, Hoff FW, Qiu Y, Hurren R, Gronda M, Jitkova Y, Nachmias B, MacLean N, Wang X, Arruda A, Minden MD, Horton TM, Kornblau SM, Chan SM, Bader GD, et al. 2020. The mitochondrial peptidase, neurolysin, regulates respiratory chain supercomplex formation and is necessary for AML viability. *Science Translational Medicine* **12**:eaaz8264–17. DOI: <https://doi.org/10.1126/scitranslmed.aaz8264>
- Mrózek K**, Tanner SM, Heinonen K, Bloomfield CD. 2003. Molecular cytogenetic characterization of the KG-1 and KG-1a acute myeloid leukemia cell lines by use of spectral karyotyping and fluorescence in situ hybridization. *Genes, Chromosomes and Cancer* **38**:249–252. DOI: <https://doi.org/10.1002/gcc.10274>, PMID: 14506699
- Okuda S**, Watanabe Y, Moriya Y, Kawano S, Yamamoto T, Matsumoto M, Takami T, Kobayashi D, Araki N, Yoshizawa AC, Tabata T, Sugiyama N, Goto S, Ishihama Y. 2017. jPOSTrepo: an international standard data repository for proteomes. *Nucleic Acids Research* **45**:D1107–D1111. DOI: <https://doi.org/10.1093/nar/gkw1080>
- Panina SB**, Pei J, Baran N, Konopleva M, Kiriienko NV. 2020. Utilizing synergistic potential of Mitochondria-Targeting drugs for leukemia therapy. *Frontiers in Oncology* **10**:435. DOI: <https://doi.org/10.3389/fonc.2020.00435>, PMID: 32318340
- Rai Y**, Yadav P, Kumari N, Kalra N, Bhatt AN. 2019. Hexokinase II inhibition by 3-bromopyruvate sensitizes myeloid leukemic cells K-562 to anti-leukemic drug, daunorubicin. *Bioscience Reports* **39**:BSR20190880. DOI: <https://doi.org/10.1042/BSR20190880>, PMID: 31506393
- Ramkumar B**, Dharaskar SP, Mounika G, Paithankar K, Sreedhar AS. 2020. Mitochondrial chaperone, TRAP1 as a potential pharmacological target to combat Cancer metabolism. *Mitochondrion* **50**:42–50. DOI: <https://doi.org/10.1016/j.mito.2019.09.011>, PMID: 31669620
- Roca-Portoles A**, Rodríguez-Blanco G, Sumpton D, Cloix C, Mullin M, Mackay GM, O'Neill K, Lemgruber L, Luo X, Tait SWG. 2020. Venetoclax causes metabolic reprogramming independent of BCL-2 inhibition. *Cell Death & Disease* **11**:616. DOI: <https://doi.org/10.1038/s41419-020-02867-2>, PMID: 32792521
- Roth K**, Weiner MW. 1991. Determination of cytosolic ADP and AMP concentrations and the free energy of ATP hydrolysis in human muscle and brain tissues with ³¹P NMR spectroscopy. *Magnetic Resonance in Medicine* **22**:505–511. DOI: <https://doi.org/10.1002/mrm.1910220258>, PMID: 1812384
- Rückert FG**, Sander S, Döhner K, Döhner H, Pollack JR, Bullinger L. 2006. Molecular profiling reveals myeloid leukemia cell lines to be faithful model systems characterized by distinct genomic aberrations. *Leukemia* **20**:994–1001. DOI: <https://doi.org/10.1038/sj.leu.2404235>, PMID: 16721385
- Ryan TE**, Yamaguchi DJ, Schmidt CA, Zeczycki TN, Shaikh SR, Brophy P, Green TD, Tarpey MD, Karnekar R, Goldberg EJ, Sparagna GC, Torres MJ, Annex BH, Neuffer PD, Spangenburg EE, McClung JM. 2018. Extensive skeletal muscle cell mitochondriopathy distinguishes critical limb ischemia patients from claudicants. *JCI Insight* **3**:e123235. DOI: <https://doi.org/10.1172/jci.insight.123235>
- Ryu I**, Ryu MJ, Han J, Kim SJ, Lee MJ, Ju X, Yoo BH, Lee YL, Jang Y, Song IC, Chung W, Oh E, Heo JY, Kweon GR. 2018. Ldeprenyl exerts cytotoxicity towards acute myeloid leukemia through inhibition of mitochondrial respiration. *Oncology Reports* **40**:3869–3878. DOI: <https://doi.org/10.3892/or.2018.6753>, PMID: 30272370
- Sanchez-Martin C**, Menon D, Moroni E, Ferraro M, Masgras I, Elsey J, Arbiser JL, Colombo G, Rasola A. 2020a. Honokiol Bis-Dichloroacetate is a selective allosteric inhibitor of the mitochondrial chaperone TRAP1. *Antioxidants & Redox Signaling* **00**:1–13.
- Sanchez-Martin C**, Moroni E, Ferraro M, Laquatra C, Cannino G, Masgras I, Negro A, Quadrelli P, Rasola A, Colombo G. 2020b. Rational design of allosteric and selective inhibitors of the molecular chaperone TRAP1. *Cell Reports* **31**:107531. DOI: <https://doi.org/10.1016/j.celrep.2020.107531>, PMID: 32320652
- Schindelin J**, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. *Nature Methods* **9**:676–682. DOI: <https://doi.org/10.1038/nmeth.2019>, PMID: 22743772
- Sciacovelli M**, Guzzo G, Morello V, Frezza C, Zheng L, Nannini N, Calabrese F, Laudiero G, Esposito F, Landriscina M, Defilippi P, Bernardi P, Rasola A. 2013. The mitochondrial chaperone TRAP1 promotes

- neoplastic growth by inhibiting succinate dehydrogenase. *Cell Metabolism* **17**:988–999. DOI: <https://doi.org/10.1016/j.cmet.2013.04.019>, PMID: 23747254
- Singh RP**, Jeyaraju DV, Voisin V, Hurren R, Xu C, Hawley JR, Barghout SH, Khan DH, Gronda M, Wang X, Jitkova Y, Sharon D, Liyanagae S, MacLean N, Seneviratne AK, Mirali S, Borenstein A, Thomas GE, Soriano J, Orouji E, et al. 2020. Disrupting mitochondrial copper distribution inhibits leukemic stem cell Self-Renewal. *Cell Stem Cell* **26**:926–937. DOI: <https://doi.org/10.1016/j.stem.2020.04.010>, PMID: 32416059
- Skrčić M**, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, Hurren R, Jitkova Y, Gronda M, Maclean N, Lai CK, Eberhard Y, Bartoszko J, Spagnuolo P, Rutledge AC, Datti A, Ketela T, Moffat J, Robinson BH, Cameron JH, et al. 2011. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* **20**:674–688. DOI: <https://doi.org/10.1016/j.ccr.2011.10.015>, PMID: 22094260
- Sriskanthadevan S**, Jeyaraju DV, Chung TE, Prabha S, Xu W, Skrtic M, Jhas B, Hurren R, Gronda M, Wang X, Jitkova Y, Sukhai MA, Lin FH, Maclean N, Laister R, Goard CA, Mullen PJ, Xie S, Penn LZ, Rogers IM, et al. 2015. AML cells have low spare reserve capacity in their respiratory chain that renders them susceptible to oxidative metabolic stress. *Blood* **125**:2120–2130. DOI: <https://doi.org/10.1182/blood-2014-08-594408>, PMID: 25631767
- Suganuma K**, Miwa H, Imai N, Shikami M, Gotou M, Goto M, Mizuno S, Takahashi M, Yamamoto H, Hiramatsu A, Wakabayashi M, Watarai M, Hanamura I, Imamura A, Mihara H, Nitta M. 2010. Energy metabolism of leukemia cells: glycolysis versus oxidative phosphorylation. *Leukemia & Lymphoma* **51**:2112–2119. DOI: <https://doi.org/10.3109/10428194.2010.512966>, PMID: 20860495
- Tewari D**, Ahmed T, Chirasani VR, Singh PK, Maji SK, Senapati S, Bera AK. 2015. Modulation of the mitochondrial voltage dependent anion channel (VDAC) by curcumin. *Biochimica Et Biophysica Acta (BBA) - Biomembranes* **1848**:151–158. DOI: <https://doi.org/10.1016/j.bbamem.2014.10.014>
- Vardhana SA**, Hwee MA, Berisa M, Wells DK, Yost KE, King B, Smith M, Herrera PS, Chang HY, Satpathy AT, van den Brink MRM, Cross JR, Thompson CB. 2020. Impaired mitochondrial oxidative phosphorylation limits the self-renewal of T cells exposed to persistent antigen. *Nature Immunology* **21**:1022–1033. DOI: <https://doi.org/10.1038/s41590-020-0725-2>, PMID: 32661364
- Veech RL**, Kashiwaya Y, Gates DN, King MT, Clarke K. 2002. The energetics of ion distribution: the origin of the resting electric potential of cells. *IUBMB Life* **54**:241–252. DOI: <https://doi.org/10.1080/15216540215678>, PMID: 12587974
- Willis WT**, Jackman MR, Messer JI, Kuzmiak-Glancy S, Glancy B. 2016. A simple hydraulic analog model of oxidative phosphorylation. *Medicine & Science in Sports & Exercise* **48**:990–1000. DOI: <https://doi.org/10.1249/MSS.0000000000000884>, PMID: 26807634
- Xiang W**, Cheong JK, Ang SH, Teo B, Xu P, Asari K, Sun WT, Than H, Bunte RM, Virshup DM, Chuah C. 2015. Pyrvinium selectively targets blast phase-chronic myeloid leukemia through inhibition of mitochondrial respiration. *Oncotarget* **6**:33769–33780. DOI: <https://doi.org/10.18632/oncotarget.5615>, PMID: 26378050
- Xie Q**, Wondergem R, Shen Y, Cavey G, Ke J, Thompson R, Bradley R, Daugherty-Holtrop J, Daugherty-Holtrop J, Xu Y, Chen E, Omar H, Rosen N, Wenkert D, Xu HE, Vande Woude GF. 2011. Benzoquinone ansamycin 17aag binds to mitochondrial voltage-dependent anion channel and inhibits cell invasion. *PNAS* **108**:4105–4110. DOI: <https://doi.org/10.1073/pnas.1015181108>, PMID: 21368131
- Yoshida S**, Tsutsumi S, Muhlebach G, Sourbier C, Lee M-J, Lee S, Vartholomaiou E, Tatokoro M, Beebe K, Miyajima N, Mohny RP, Chen Y, Hasumi H, Xu W, Fukushima H, Nakamura K, Koga F, Kihara K, Trepel J, Picard D, et al. 2013. Molecular chaperone TRAP1 regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis. *PNAS* **110**:E1604–E1612. DOI: <https://doi.org/10.1073/pnas.1220659110>

RESEARCH ARTICLE

Alterations in sphingolipid composition and mitochondrial bioenergetics represent synergistic therapeutic vulnerabilities linked to multidrug resistance in leukemia

Kelsey H. Fisher-Wellman¹ | James T. Hagen¹ | Miki Kassai² | Li-Pin Kao² | Margaret A. M. Nelson¹ | Kelsey L. McLaughlin¹ | Hannah S. Coalson¹ | Todd E. Fox³ | Su-Fern Tan⁴ | David J. Feith^{4,5} | Mark Kester^{4,5} | Thomas P. Loughran Jr^{4,5} | David F. Claxton^{6,7} | Myles C. Cabot²

¹Department of Physiology, Brody School of Medicine, and the East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, North Carolina, USA

²Department of Biochemistry & Molecular Biology, Brody School of Medicine, and the East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, North Carolina, USA

³Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia, USA

⁴Department of Medicine, Hematology/Oncology, University of Virginia School of Medicine, Charlottesville, Virginia, USA

⁵University of Virginia Cancer Center, Charlottesville, Virginia, USA

⁶Department of Medicine, Division of Hematology and Oncology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania, USA

⁷Penn state Cancer Institute, Hershey, Pennsylvania, USA

Correspondence

Kelsey H. Fisher-Wellman, Department of Physiology, Brody School of Medicine, and the East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC 27834, USA.
Email: fisherwellmank17@ecu.edu

Myles C. Cabot, Department of Biochemistry & Molecular Biology, Brody School of Medicine, and the East Carolina Diabetes and Obesity Institute, East Carolina University, Greenville, NC 27834, USA.
Email: cabotm@ecu.edu

Funding information

The work was supported by DOD-W81XWH-19-1-0213 (K.H.F.-W.) and

Abstract

Modifications in sphingolipid (SL) metabolism and mitochondrial bioenergetics are key factors implicated in cancer cell response to chemotherapy, including chemotherapy resistance. In the present work, we utilized acute myeloid leukemia (AML) cell lines, selected to be refractory to various chemotherapeutics, to explore the interplay between SL metabolism and mitochondrial biology supportive of multidrug resistance (MDR). In agreement with previous findings in cytarabine or daunorubicin resistant AML cells, relative to chemosensitive wildtype controls, HL-60 cells refractory to vincristine (HL60/VCR) presented with alterations in SL enzyme expression and lipidome composition. Such changes were typified by upregulated expression of various ceramide detoxifying enzymes, as well as corresponding shifts in ceramide, glucosylceramide, and sphingomyelin (SM) molecular species. With respect to mitochondria, despite consistent increases

Abbreviations: AC, acid ceramidase; AML, acute myeloid leukemia; Ant A, antimycin; CI, complex I; CK, creatine kinase; CN, cyanide; CNL, ceramide nanoliposome; Cyt C, cytochrome c; DHO, dihydroorotate; DNR, daunorubicin; ETS, electron transport system; G, glutamate; GC, glucosylceramides; GCS, glucosylceramide synthase; M, malate; MDR, multidrug resistance; MEF, mitochondrial enrichment factor; Multi, pyruvate/malate/glutamate/succinate/octanoyl-carnitine; O or Oct, octanoyl-carnitine; Oligo, oligomycin; P or Pyr, pyruvate; PBMC, peripheral blood mononuclear cell; PCr, phosphocreatine; Rot, rotenone; S or Succ, succinate; S1P, sphingosine 1-phosphate; SL, sphingolipids; SM, sphingomyelin; SPHK1, sphingosine kinase; TF, teriflunomide; VBL, vinblastine; VCR, vincristine; Vlcax, venetoclax; ΔG_{ATP} , ATP-free energy of hydrolysis.

NIH P01 CA171983 (M.C.C.), as well as a grant from the Brody Brothers Foundation, Kinston, NC

in both basal respiration and maximal respiratory capacity, direct interrogation of the oxidative phosphorylation (OXPHOS) system revealed intrinsic deficiencies in HL60/VCR, as well as across multiple MDR model systems. Based on the apparent requirement for augmented SL and mitochondrial flux to support the MDR phenotype, we explored a combinatorial therapeutic paradigm designed to target each pathway. Remarkably, despite minimal cytotoxicity in peripheral blood mononuclear cells (PBMC), co-targeting SL metabolism, and respiratory complex I (CI) induced synergistic cytotoxicity consistently across multiple MDR leukemia models. Together, these data underscore the intimate connection between cellular sphingolipids and mitochondrial metabolism and suggest that pharmacological intervention across both pathways may represent a novel treatment strategy against MDR.

KEYWORDS

acute myeloid leukemia, chemotherapy resistance, HL-60 cells, mitochondrial bioenergetics, sphingolipids, vincristine

1 | BACKGROUND

A major element underlying treatment failure in cancer is chemotherapy resistance. Factors contributing to resistance include drug efflux driven by multidrug resistance (MDR) membrane transport proteins (e.g., P-glycoprotein; P-gp), drug activation/inactivation missteps, DNA damage repair, cell death inhibition (i.e., resistance to apoptosis), mutation of the target to reduce inhibitor binding, and alterations in mitochondrial bioenergetics.^{1–3} These current research paradigms by and large drive the study of MDR in cancer. *Vinca* alkaloids, such as vincristine (VCR) and vinblastine (VBL), part of the World Health Organization's List of Essential Medicines, are no exception; their benefits are supplanted by upregulated expression of MDR proteins, dysfunctional apoptosis, and alterations in β -tubulin that thwart *Vinca*-associated microtubule targeting benefits. Despite these limitations, *Vinca* alkaloids are used widely in the treatment of both liquid and solid tumors, and VCR, as well as VBL, are frequent agents in combination regimens. VCR is a component in CVP (cyclophosphamide, VCR, prednisone) used in follicular B-cell lymphoma, CHOP (cyclophosphamide, doxorubicin, VCR, prednisone) used in large B-cell lymphoma, and was recently demonstrated to exert anti-leukemic effects in CD44+ acute myeloid leukemia (AML) when administered as A6 peptide-functionalized polymersomal vincristine sulfate.⁴ Although VCR resistance is the main model in the present work, this study also encompasses resistance to daunorubicin and venetoclax, drugs frequently used in AML therapy.

In addition to the hallmark factors underlying chemotherapy resistance, the interplay of SL's and mitochondrial

bioenergetics merits consideration. Many anticancer agents raise intracellular levels of ceramides, SL's that exhibit strong tumor-suppressing activities.⁵ With respect to VCR, Olshefski and Ladisch⁶ have shown that VCR exposure increases ceramide levels in leukemia cells, resulting in apoptotic responses that were intensified by blocking ceramide glycosylation. Interestingly, inhibitors of SL enzymes were recently found to interfere with energy transduction across the AML mitochondrial network, suggestive of a direct link between SL metabolism and mitochondrial bioenergetics.⁷ Together, these findings raise the intriguing possibility that SL-mitochondria crosstalk may play a fundamental role in chemotherapy response and thus represent an opportunity for novel therapeutic interventions against MDR.

Herein, we utilized AML cell lines, selected to be refractory to various chemotherapeutics, to evaluate the interplay between SL metabolism and mitochondrial bioenergetics and explore the therapeutic potential of co-targeting these pathways in MDR. Consistent with prior work focused on cytarabine and daunorubicin,⁸ in HL-60/VCR cells as opposed to wild-type (wt) HL-60 cells, we detail striking alterations in SL composition, highlighted by specific increases in S1P, ceramides, as well GC, and changes in levels of specific SM molecular species. These changes were accompanied by intrinsic mitochondrial remodeling that allowed for higher basal respiration and overall respiratory capacity, alongside substantial deficiencies in oxidative phosphorylation (OXPHOS). Apparent increases in respiratory capacity combined with intrinsic OXPHOS limitations were previously observed in the context of venetoclax resistance⁹ and were once again observed herein for multiple MDR AML models. Based on consistent observations linking

both SL and mitochondrial remodeling, therapeutic synergy was explored across both pathways. Importantly, across multiple MDR leukemia and despite minimal cytotoxicity as single agents, the combination of forced ceramide accumulation, via the addition of C6-ceramide nanoliposomes (CNL), with CI inhibition, induced synergistic cell death.

2 | MATERIALS AND METHODS

2.1 | Materials

C6-ceramide (*N*-hexanoyl-*D*-erythro-sphingosine) was from Avanti Polar Lipids, Alabaster, AL. Ceramide nanoliposomes (CNL) were a generous gift from Keystone Nano, Inc. Control (ghost) formulations included all liposomal ingredients except C6-ceramide. Propidium iodide (PI) was obtained from Life Technologies/Thermo Fisher, Carlsbad, CA. SK1-i, a SPHK1 inhibitor, was from Enzo, Farmingdale, NY. Vincristine sulfate was purchased from LC Laboratories, Woburn, MA. Daunorubicin (DNR) and venetoclax (Vclax) were from R&D Systems, Minneapolis, MN. Metformin and phenformin were purchased from Sigma. IACS-010759 was purchased from SelleckChem.com (Cat # S8731). All chemicals for mitochondrial analysis were purchased from Sigma. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) was purchased from Cayman, Ann Arbor, MI.

2.2 | Peripheral blood mononuclear cells (PBMC)

For Peripheral blood mononuclear cells (PBMC) samples, healthy subjects, without a prior history of hematological malignancy, were recruited. Following informed consent (study ID: UMCIRB 18-001328), venous blood from the brachial region of the upper arm was collected. Collection of whole blood was performed in sodium-heparinized Cell Preparation Tubes (CPT) (BD Biosciences, Franklin Lakes, NJ) and tubes were centrifuged at 1800×g for 15 min. Mononuclear cells were washed in ammonium-chloride-potassium (ACK) lysis buffer to remove red blood cells and cultured for 48 h in IMDM (Thermo Fisher Scientific, Waltham, MA) supplemented with glutamine, 10% FBS, and 1% penicillin/streptomycin.

2.3 | Cell culture

The human VCR resistant (multidrug-resistant) cell line, HL-60/VCR, was developed by Safa and colleagues

from HL-60/Vinc cells, originally developed by Melvin S. Center, Division of Biology, Kansas State University, Manhattan, KS, by step-wise selection to increasing concentrations of VCR.¹⁰ Cells were cultured in either IMDM (MV411 only) or RPMI-1640 medium, supplemented with 10% FBS (Peak Serum, Wellington, CO), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Science Technologies), and 1.0 µg/ml (1.2 µM) vincristine sulfate (dissolved in water). For experiments testing the impact of VCR withdrawal, HL-60/VCR cells were cultured in RPMI-1640 growth medium devoid of vincristine sulfate for 7 days. HL-60 cells were obtained from the ATCC, Manassas, VA. HL-60 daunorubicin resistant cells (HL-60/DNR), grown to tolerate 1.0 µg/ml DNR, were established as previously described⁸; HL-60 and MV411 venetoclax resistant cells (HL-60/VclaxR), established to tolerate 1.0 µM Vclax, were established in a similar fashion.⁹ All other cell lines were obtained from ATCC (Manassas, VA). Cell lines were not tested or authenticated over and above documentation provided by ATCC, which included antigen expression, DNA profile, short tandem repeat profiling, and cytogenetic analysis.

2.4 | Cell viability assays

Cell viability was determined by fluorescence measurement as previously described.¹¹ Briefly, cells were seeded in Corning black wall, 96-well plates (VWR, Suwanee, GA), treated with indicated agents in a final volume of 0.2 ml RPMI-1640 complete medium, and incubated at 37°C, 5% CO₂, for the times indicated. Viability was determined using PI as follows. Cells (positive controls corresponding to 100% cell death) were permeabilized by addition of 10 µl of 1 mg/ml digitonin and incubated at 37°C, 5% CO₂ for 20 min, followed by addition of PI dissolved in PBS, at a final concentration of 5 µM. The plate was incubated for 20 min, and viability was calculated as the mean ($n = 6$) fluorescence (minus non-permeabilized control) at 530 nm excitation and 620 nm emission, using a BIO-TEK Synergy H1 microplate reader (Winooski, VT). Viability was also determined using the MTS Cell Proliferation Assay (Promega), as well as trypan blue (0.4%) exclusion and cell counting on a Countess II apparatus, Life Science Technologies, using disposable hemocytometers, from Invitrogen, Thermo/Fisher Scientific; cell growth rates were assessed by the same method.

2.5 | Caspase activation

Cells (25 000/well) were seeded into Corning white wall, 96-well plates, treated with indicated agents in the final

volume of 0.1 ml RPMI-1640 complete medium, and incubated at 37°C, 5% CO₂, for 24 h, after which the cells were exposed to Promega Caspase-Glo® 3/7 Assay mixture (Madison, WI). The plate was incubated at 37°C for 1 h, and caspase activation was calculated as the mean ($n = 8$) luminescence using a BIO-TEK Synergy H1 microplate reader (Winooski, VT).

2.6 | Lipidomics

Lipidomic analysis was conducted using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as previously described.¹¹ Briefly, total lipids were extracted from cells after the addition of internal standards using ethyl acetate/isopropanol/water (60:30:10, v/v) without phase partitioning. Separation of lipid extracts was achieved using a Waters I-class Acquity LC with a 2.1 × 100 mm C18 CSH column and detected with a Waters Xevo TQ-S MS system.

2.7 | Immunoblotting

Immunoblotting was conducted as previously described^{12,13} with modifications. SPHK1 (47 kDa, catalog # 12071), used at 1:1000 dilution, and β-actin (45 kDa, catalog # 3700), used at 1:3000 dilution, were from Cell Signaling. P-gp (180 kDa, catalog # NB60D-1036), used at 1:1000 dilution, was from Novus (Imgenex). GCS (40 kDa, catalog # PA5-42111), used at a 1:1000 dilution, was from Invitrogen. AC (13 kDa, catalog # 612302), used at a 1:1000 dilution, was from Labome (BD Bioscience). Briefly, cells were lysed in 1× RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing phosphatase inhibitor cocktail 2, protease inhibitor, and PMSF (1.0 mM; Sigma). The bicinchoninic acid (BCA) method was employed for determining protein concentrations. Lysates were centrifuged to remove debris and then heated in 1× NuPage LDS sample buffer at 95°C for 5 min (GCS lysate was heated at 55°C for 5 min). Equal amounts of total protein were separated by SDS–polyacrylamide gel electrophoresis and subsequently transferred onto PVDF or nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in PBS for 1 h at room temperature and incubated with primary antibodies according to the dilutions above, at 4°C overnight. The membranes were washed with PBST, followed by incubation with the corresponding HRP-conjugated secondary antibodies, for 1 h at room temperature. The membranes were washed with PBST before exposure. Bound antibodies were measured using

an enhanced chemiluminescence detection kit (Thermo Fisher Scientific) and surveyed using a ChemiDoc imaging system (Bio-Rad, Hercules, CA). The relative intensity of protein expression was calculated using ImageJ software (National Institutes of Health).

2.8 | Quantitative reverse transcription PCR (RT-PCR)

An RNA extraction kit (Qiagen Pty., Ltd., Waltham, MA) was used to extract total RNA, which was then reverse-transcribed into cDNA using a ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). The synthesized cDNAs were amplified with SYBR Premix (Bimake, Houston, TX) using the ABI VII7 Real-Time PCR System (Invitrogen). The PCR cycling parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative mRNA levels were calculated using the comparative Ct method (Applied Biosystems instruction manual) and presented as ratios to their biological controls. The fold change in expression of each target mRNA relative to β-actin was calculated as $2^{\Delta(\Delta Ct)}$, where $\Delta Ct = \Delta Ct_{\beta\text{-actin}} - Ct_{\text{genes}}$. β-actin transcript levels were confirmed to correlate well with total RNA levels and therefore were used for normalization throughout the experiments.^{14,15} The primers used for real-time PCR were designed by primerbank (http://pga.mgh.harvard.edu/prime_rbank/): (ABCB1: F:TTGCTGCTTACATTTCAGGTTTCA, R:AGCCTATCTCCTGTCGCATTA; ABCC1: F:CTCTATCTCTCCCGACATGACC, R:AGCAGACGATCCACAGCAAAA; ABCG2: F:ACGAACGGATTAACAGGGTCA, R:CTCCAGACACACCACGGAT; β-actin: F:GCTGTGCTACGTCGCCCTG, R:GGAGGAGCTGGAAGCAGCC).

2.9 | Cellular respirometry (intact and permeabilized)

High-resolution O₂ consumption measurements were conducted using the Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) in intact and digitonin permeabilized cells. For each intact cell experiment, cells were centrifuged at 1000 rpm for 7 min at room temperature, washed in PBS, centrifuged once more, and then suspended in assay media at a cell concentration of 2–3 × 10⁶ viable cells/ml. Assay media was RPMI 1640, without bicarbonate, supplemented with 20 mM HEPES (pH 7.4), 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. All experiments were carried out at 37°C in a 1-ml reaction volume. Following the assessment of

basal respiration, the following sequential additions were made (oligomycin (5.0 μ M), FCCP (0.5, 1, 2, 3 μ M), rotenone (Rot; 5 μ M), antimycin A (Ant A; 5 μ M)). For experiments designed to assess the impact of P-gp, verapamil (10 μ M) was added prior to oligomycin to intact cells. For permeabilized cell experiments, cells were centrifuged at 1000 rpm for 7 min at room temperature, washed in respiration buffer, centrifuged once more, and then suspended in respiration buffer at a cell concentration of $2\text{--}3 \times 10^6$ viable cells/ml. Respiration buffer consisted of potassium-MES (105 mM; pH 7.2), KCl (30 mM), KH_2PO_4 (10 mM), MgCl_2 (5 mM), EGTA (1 mM), and BSA (2.5 g/L). After recording basal respiration, cells were permeabilized with digitonin (20 μ g/ml), energized with various carbon substrates (pyruvate, malate, glutamate, succinate, octanoyl-l-carnitine; P, M, G, S, O; 5, 2, 5, 5, 0.2 mM) and flux was stimulated across a physiological ATP free energy demand using the creatine kinase (CK) clamp, see below for details. Additional respiratory stimulation was carried out via respiratory uncoupler (FCCP) titration (0.5–3.0 μ M). Cytochrome C (10 μ M) was added to check the integrity of the outer mitochondrial membrane. Note, the absence of an increase in respiration, relative to the pre cytochrome C rate, was used as a quality control assessment for outer membrane integrity. Non-mitochondrial respiration was controlled by adding rotenone (Rot; 5 μ M) and/or antimycin A (Ant A; 5 μ M). Data were normalized to viable cell count and expressed as pmol/s/million cells. All additions were made directly to the O2K chamber during the period of each assay. Typical assay length was 20–40 min.

2.10 | Mitochondrial isolation

As described previously^{16,17} and with some modifications, cells were centrifuged at $300\times g$ for 10 min followed by a PBS wash. Cell pellets were resuspended in mitochondrial isolation buffer (MIB) with BSA (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO_4 , 0.2% BSA, pH 7.1) and homogenized using a borosilicate glass mortar and Teflon pestle. Homogenates were centrifuged at $800\times g$ for 10 min at 4°C. The supernatant was collected, and this process was repeated for a total of 3 times. The collected supernatant was centrifuged at $10\,000\times g$ for 10 min at 4°C to pellet the mitochondrial fraction. The fraction was resuspended in MIB without BSA, transferred to a microcentrifuge tube, and spun down at $10\,000\times g$. Final resuspension of the mitochondrial pellet was done in Mitochondria Isolation Buffer, without BSA, and protein concentration was calculated using the Pierce BCA assay. Respiration assays using isolated mitochondria were like that described for permeabilized cells.

2.11 | Mitochondrial functional assessment (isolated mitochondria)

High-resolution O_2 consumption rate (JO_2) measurements were conducted as above. The base assay buffer was identical to that used in permeabilized cells (105 mM potassium-MES, 30 mM KCl, 10 mM KH_2PO_4 , 5 mM MgCl_2 , 1 mM EGTA, 2.5 g/L BSA, and 5 mM creatine monohydrate; pH = 7.2). As previously described,^{16,18} a modified version of the CK clamp technique was used to determine steady-state JO_2 across a physiological ΔG_{ATP} span. For complete details regarding the calculation of ΔG_{ATP} at each titration point see.¹⁸ To begin, mitochondria were added to the respiration buffer, followed by the addition of respiratory substrates to stimulate State 4 (i.e., non-phosphorylating) respiration. The CK clamp was then initiated by the addition of ATP (5 mM), PCr (1 mM), and CK (20 U/ml), simulating a “maximal” demand for ATP re-synthesis. Sequential additions of PCr to 6, 15, and 21 mM were then performed to gradually lower the ATP demand state and assess OXPHOS conductance. Four substrate conditions were used: Pyr/M (5/1 mM); glutamate/M (G/M; 10/1 mM); S/Rot (10 mM/0.5 μ M); P/M/G/S/O (5/1/5/5/0.2 mM; Multi). For experiments involving dihydroorotate (DHO), DHO (2 mM) was added to isolated mitochondria in the presence of saturating ADP (2 mM). DHO-supported respiration was then inhibited using a specific inhibitor of dihydroorotate dehydrogenase (DHODH), teriflunomide (20 μ M). Data were normalized to protein loaded per experiment and then corrected for the mitochondrial enrichment factor (MEF; *) calculated for that sample, with the final values expressed as pmol/s/mg protein* (see proteomics methodology below regarding MEF).

2.12 | Mitochondrial NADH/NAD⁺ redox in isolated mitochondria

Fluorescent determination of NADH/NAD⁺ was performed using a QuantaMaster Spectrofluorometer (QM-400, Horiba Scientific, Kyoto, Japan). The NADH/NAD⁺ was detected at Ex/Em: 350/450. NADH/NAD⁺ was measured in mitochondria using the CK clamp assay. Experiments were performed at 37°C in a 200 μ l reaction volume. Mitochondria were incubated at 37°C for ~ 5 min without a substrate to induce a 0% reduction of the NADH pool. Saturating carbon substrates were added (Multi), and respiration was stimulated with the CK clamp. Titration of ΔG_{ATP} was performed via PCr titration (6, 15, 21 mM). Oligomycin (0.02 μ M) was added to inhibit ATP synthesis and cyanide (CN, 10 mM) was added to induce a 100% reduction of the matrix NADH pool. The NADH/

NAD⁺ was expressed as a percentage reduction of the CN value (i.e., 100% reduction) based upon the formula % Reduction = $(F - F_{0\%}) / (F_{100\%} - F_{0\%}) \times 100$.

2.13 | Mitochondrial H₂O₂ emission

Mitochondrial peroxide emission was assessed in Respiration buffer, supplemented with amplex ultra red (5 μ M), horse radish peroxidase (1 U/ml), and superoxide dismutase (25 U/ml), as described previously.¹⁹ Experiments were run in the presence of mitochondria alone, Pyr/M (5/2 mM), and the CK clamp. Relative fluorescence was converted to pmol of H₂O₂ using an H₂O₂ standard curve, as previously described.¹⁹

2.14 | Mitochondrial lysis and sample prep for label-free proteomics

Isolated mitochondria were lysed in urea lysis buffer (8 M urea in 40 mM Tris, 30 mM NaCl, 1 mM CaCl₂, 1 $\times$ cOmplete ULTRA mini EDTA-free protease inhibitor tablet; pH = 8.0), as described previously.^{16,20} The samples were subjected to three freeze-thaw cycles and sonicated with a probe sonicator in three 5s bursts (Q Sonica #CL-188; the amplitude of 30). Samples were centrifuged at 10 000 $\times$ g for 10 min at 4°C. Protein concentration was determined by BCA. Equal amounts of protein were reduced with 5 mM DTT at 37°C for 30 min and then alkylated with 15 mM iodoacetamide for 30 min in the dark at room temperature. Unreacted iodoacetamide was quenched with DTT (15 mM). Initial digestion was performed with Lys C (ThermoFisher Cat# 90307; 1:100 w:w) for 4 h at 37°C. Following dilution to 1.5 M urea with 40 mM Tris (pH = 8.0), 30 mM NaCl, 1 mM CaCl₂, samples were digested overnight with trypsin (Promega; Cat# V5113; 50:1 w/w) at 37°C. Samples were acidified to 0.5% TFA and then centrifuged at 4000 $\times$ g for 10 min at 4°C. The supernatant containing soluble peptides was desalted, as described previously²⁰ and then eluate was frozen and subjected to speedvac vacuum concentration.

2.15 | nLC-MS/MS for label-free proteomics

Final peptides were resuspended in 0.1% formic acid, quantified (ThermoFisher Cat# 23275), and then diluted to a final concentration of 0.25 μ g/ μ l. Samples were subjected to nanoLC-MS/MS analysis using an UltiMate 3000 RSLCnano system (ThermoFisher) coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap mass spectrometer (ThermoFisher)

via a nanoelectrospray ionization source. For each injection, 4 μ L (1 μ g) of the sample was first trapped on an Acclaim PepMap 100 20 mm $\times$ 0.075 mm trapping column (ThermoFisher Cat# 164535; 5 μ l/min at 98/2 v/v water/acetonitrile with 0.1% formic acid). Analytical separation was performed over a 95 min gradient (flow rate of 250 nl/min) of 4%–25% acetonitrile using a 2 μ m EASY-Spray PepMap RSLC C18 75 μ m $\times$ 250 mm column (ThermoFisher Cat# ES802A) with a column temperature of 45°C. MS1 was performed at 70 000 resolution, with an AGC target of 3×10^6 ions and a maximum injection time (IT) of 100 ms. MS2 spectra were collected by data-dependent acquisition (DDA) of the top 15 most abundant precursor ions with a charge greater than 1 per MS1 scan, with dynamic exclusion enabled for 20 s. Precursor ions isolation window was 1.5 m/z and the normalized collision energy was 27. MS2 scans were performed at 17 500 resolution, maximum IT of 50 ms, and AGC target of 1×10^5 ions.

2.16 | Data analysis for label-free proteomics

As described previously,²⁰ with some modification, Proteome Discoverer 2.2 (PDv2.2) was used for raw data analysis, with default search parameters including oxidation (15.995 Da on M) as a variable modification and carbamidomethyl (57.021 Da on C) as a fixed modification. Data were searched against the Uniprot Homo Sapiens reference proteome (Proteome ID: UP000005640), as well as the Human Mito Carta 2.0 database.²¹ PSMs were filtered to a 1% FDR and grouped to unique peptides while maintaining a 1% FDR at the peptide level. Peptides were grouped to proteins using the rules of strict parsimony and proteins were filtered to 1% FDR. Peptide quantification was done using the MS1 precursor intensity. Imputation was performed via low abundance resampling. Using only high confidence master proteins, mitochondrial enrichment factor (MEF) was determined as previously described¹⁶ by comparing mitochondrial protein abundance (i.e., proteins identified to be mitochondrial by cross-reference with the MitoCarta 2.0 database) to total protein abundance.

2.17 | Statistical evaluation

All proteomics samples were normalized to total protein abundance, and the protein tab in the PDv2.2 results was exported as a tab delimited .txt. file and analyzed. Protein abundance was converted to the Log₂ space. For pairwise comparisons, tissue mean, standard deviation, p value (p ; two-tailed Student's t -test, assuming equal variance),

and adjusted p value (Benjamini Hochberg FDR correction) were calculated.²²

Mitochondrial assay results are expressed as the mean $\pm$ SEM (error bars). Data were normalized to protein loaded per experiment and then corrected for the mitochondrial enrichment factor (*) calculated for that sample, or the group mean, with the final values expressed as pmol/s/mg protein*. For a detailed description of this method, see previous publication.¹⁶ Throughout the paper, differences between groups were assessed by t -test, or one-way ANOVA, followed by Tukey's test where appropriate using GraphPad Prism 8 software (Version 8.4.2). Other statistical tests used are described in the figure legends. Statistical significance in the figures is indicated as follows: * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$. Unless otherwise stated, figures were generated using GraphPad Prism 8 software (Version 8.4.2) or Biorender.

3 | RESULTS

3.1 | HL-60/VCR cell characteristics

To confirm the VCR resistance status in our model, we determined sensitivity to VCR in wt and in HL-60/VCR cells. As shown, HL-60/VCR cells were near completely refractory to VCR compared to the VCR-naïve, wt counterpart (Figure 1A). From a morphological perspective, wt and VCR-resistant cells were similar in size and appearance; however, HL-60/VCR cells tend to clump in suspension (photos not shown). The cell growth rate was, however, slower in drug-resistant cells compared to wt, with HL-60/VCR growing at 57% the rate of wt cells (Figure 1B). Lastly, and as expected, ABCB1 transcription (P-glycoprotein, P-gp) was markedly elevated (2000-fold) in HL-60/VCR cells along with a slight but significant increase (3-fold) in ABCC1 (MDR protein, MRP), and (25-fold) ABCG2 (breast cancer resistant protein, BCRP) compared to wt cells (Figure 1C).

3.2 | Alterations in SL enzyme expression accompany VCR resistance

Because of the key role that SL's play in cancer growth^{23,24} and response to chemotherapy,²⁵ we were interested in assessing the impact of VCR resistance on the expression of several enzymes involved in SL metabolism. First, the levels of total SPHK1 were elevated in HL-60/VCR cells compared to the wt counterpart (Figure 1D,E). Regarding SPHK1, it is important to note that the product of this enzyme, S1P, is mitogenic, and thus favors neoplastic growth. GCS, which catalyzes glycosylation of ceramide

to GC, and in effect dulls ceramide's tumor-suppressor activity by reducing ceramide levels, was overexpressed in VCR-resistant cells (Figure 1D,E). This change in glycosphingolipid metabolism has been shown to accompany MDR.²⁶ Acid ceramidase (AC) expression was also enhanced in HL-60/VCR compared to wt cells (Figure 1D,E), as previously demonstrated in HL-60/VCR cells.²⁷ Lastly, although expected, HL-60/VCR cells exhibited markedly elevated levels of P-gp (Figure 1D,E).

3.3 | Lipidomic analysis

Complementary with immunoblot data, HL-60/VCR cells presented with multi-fold increases in S1P and dh-S1P levels, compared to wt cells (Figure 1F); this could be the result of elevated AC activity or reduced S1P lyase expression. Sphingosine levels were similar in both cell lines, whereas the levels of dihydrosphingosine (dhSph), also termed sphinganine, were diminished by approximately 75% in HL-60/VCR cells (Figure 1F). The levels of several ceramide molecular species were increased in HL-60/VCR cells, including C14:0, C16:0, C24:1, as well as the C26 species, C26 and C26:1 (Figure 1G). Although ceramide is proapoptotic, VCR resistance could result in ceramide tolerance that is assisted by enhanced ceramide hydrolysis and glycosylation. Thus, it appears that the increases in ceramide molecular species were mirrored and kept in check by concomitant increases in corresponding GC species, as shown, for example, in C14:0, C16:0, C22:1, C24:1, and C26 molecular species (Figure 1G). Several molecular species of SM were decreased in HL-60/VCR cells versus wt, as demonstrated in C18, C20, C22, and C24 (Figure 1G). On the opposing side, C22:1 SM levels were elevated and a marked increase in C26:1 SM was clearly shown in VCR-resistant cells (Figure 1G). In summary, the data in Figure 1 demonstrate widespread alterations in SL composition occur with VCR resistance, and interestingly, C22:1 ceramide appears to be sequestered in GC and SM. Consistent with a central role for SL metabolism in MDR, SL alterations apparent in HL60/VCR are highly reminiscent to those previously observed in other MDR AML models.⁸

3.4 | Intact cellular respirometry in MDR AML reveals increased mitochondrial content

Mitochondria play a key role in cancer cell biology and are increasingly recognized as potential targets in cancer therapy.²⁸ Mitochondria are also important downstream targets of ceramide.^{29,30} Considering the dynamic

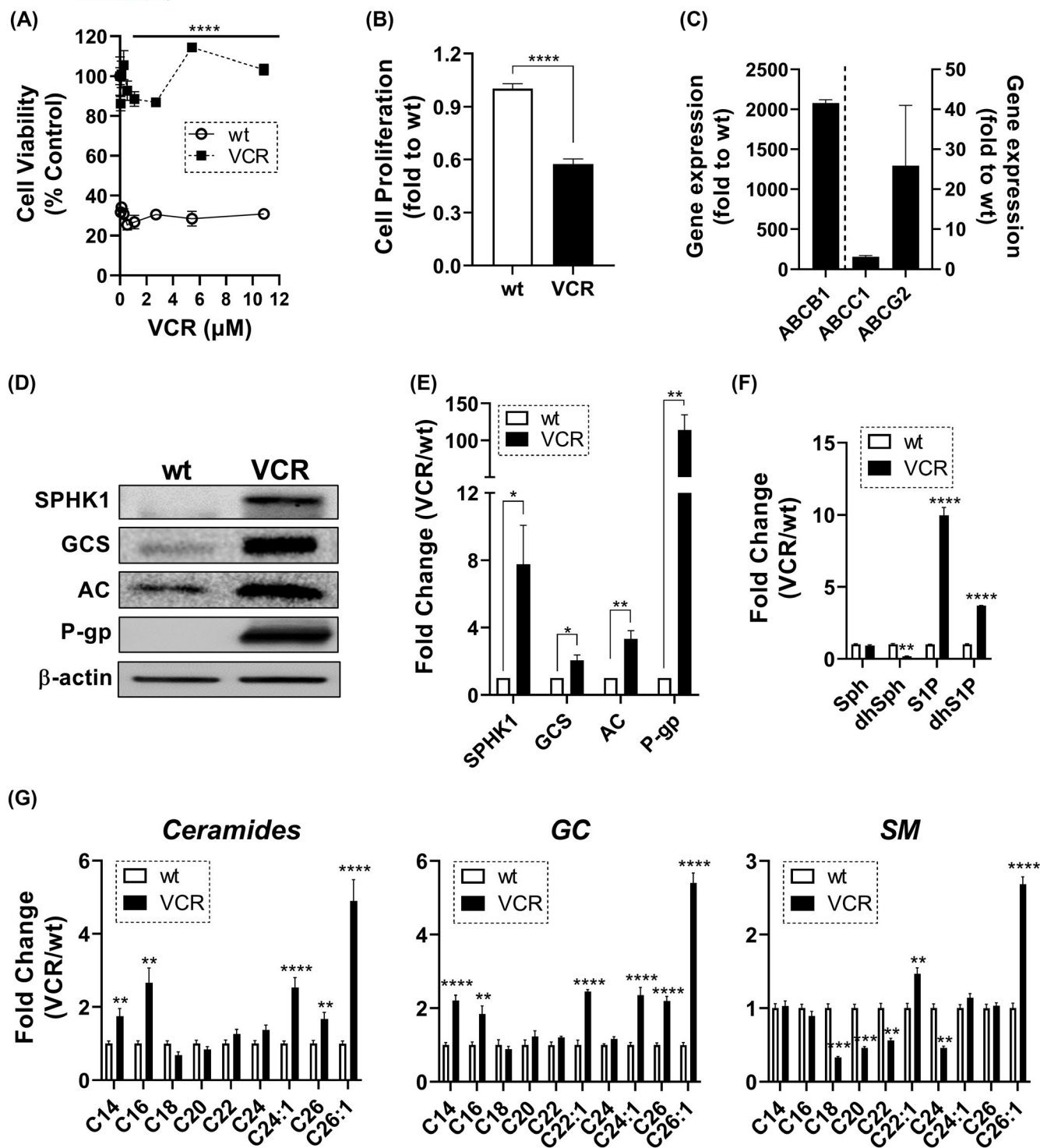


FIGURE 1 Alterations in sphingolipid metabolism underlie vincristine (VCR) resistance in HL-60 cells. (A) VCR cytotoxicity. HL-60 and HL-60/VCR cells were seeded in 96-well plates (50 000 cells/well) and exposed to VCR concentrations indicated for 48 h. Viability was determined by MTS assay. (B) Cell growth rates determined by viable cell counts. VCR was present in HL-60/VCR growth medium. (C) ABC transporter gene expression profile. Changes in gene expression in HL-60/VCR cells are represented as fold-increase over levels measured in wt cells. (D) Representative immunoblots for SPHK1, GCS, AC, P-gp, and β-actin. (E) Immunoblot quantification represented by fold-increases in blot density over wt. (F) Long-chain bases and S1P profiles. (G) Ceramide, GC, and SM molecular species profiled in wt and HL-60/VCR cells. Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean ± SEM, (A,B) $N = 6$ /group, (C–F) $N = 3$ /group, (G) $N = 4$ –7/group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$

changes in SL profiles and enzyme expression patterns that accompanied prior observations in MDR AML and VCR resistance herein,⁸ we sought to determine whether mitochondrial metabolism was impacted in the process. To begin, initial respirometry experiments were performed in intact cells under basal conditions, as well as in response to ATP synthase inhibition (i.e., oligomycin), chemical uncoupling (i.e., the addition of the protonophore FCCP), and electron transport inhibition (i.e., rotenone and antimycin A). Respiration under basal conditions was twofold higher in HL-60/VCR compared to wt cells (Figure 2A, basal abbreviated by “Basal”). Interestingly, the ability of oligomycin to lower respiration was blunted in HL-60/VCR (Figure 2A, “Oligo”). Although inhibition of JO_2 by oligomycin is typically interpreted to reflect OXPHOS “coupling efficiency,” we hypothesized that high P-gp expression in HL-60/

VCR could be driving oligomycin efflux. To test this, oligomycin-mediated inhibition of basal respiration was assessed in HL-60/VCR cells in the presence of the P-gp inhibitor verapamil. Under these conditions, oligomycin inhibited basal respiration similar to that seen in wt cells (Figure 2B), confirming P-gp mediated efflux of oligomycin. Maximal uncoupled respiration was nearly twofold higher in HL-60/VCR and respiration remained higher in HL-60/VCR following the addition of both rotenone and antimycin A, suggesting increased non-mitochondrial oxidase activity in HL-60/VCR (Figure 2A, “Rot/Ant”). Correcting for non-mitochondrial JO_2 , maximal uncoupled respiration was higher in HL-60/VCR (Figure 2C), consistent with previous findings linking increased mitochondrial content to AML chemoresistance.^{2,8} In our model system, HL-60/VCR were continuously cultured in the presence of 1.0 $\mu\text{g/ml}$ (1.2 μM) VCR. To determine

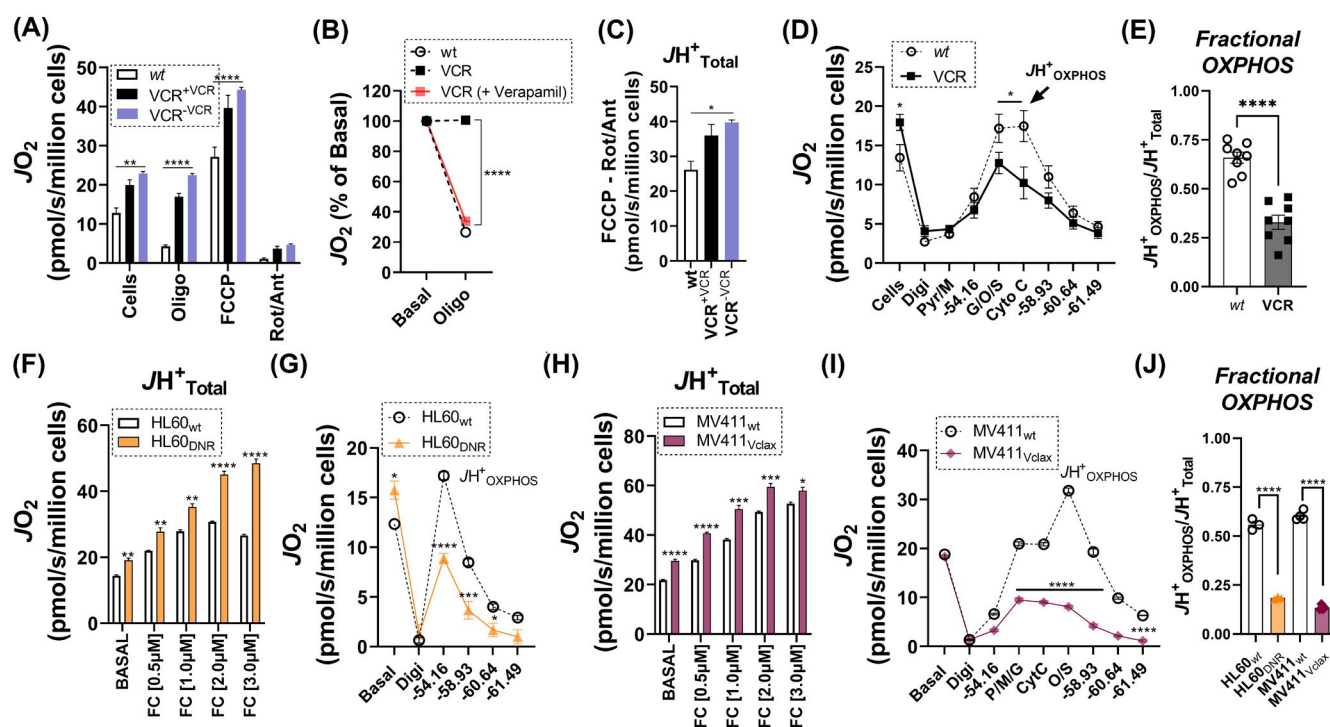
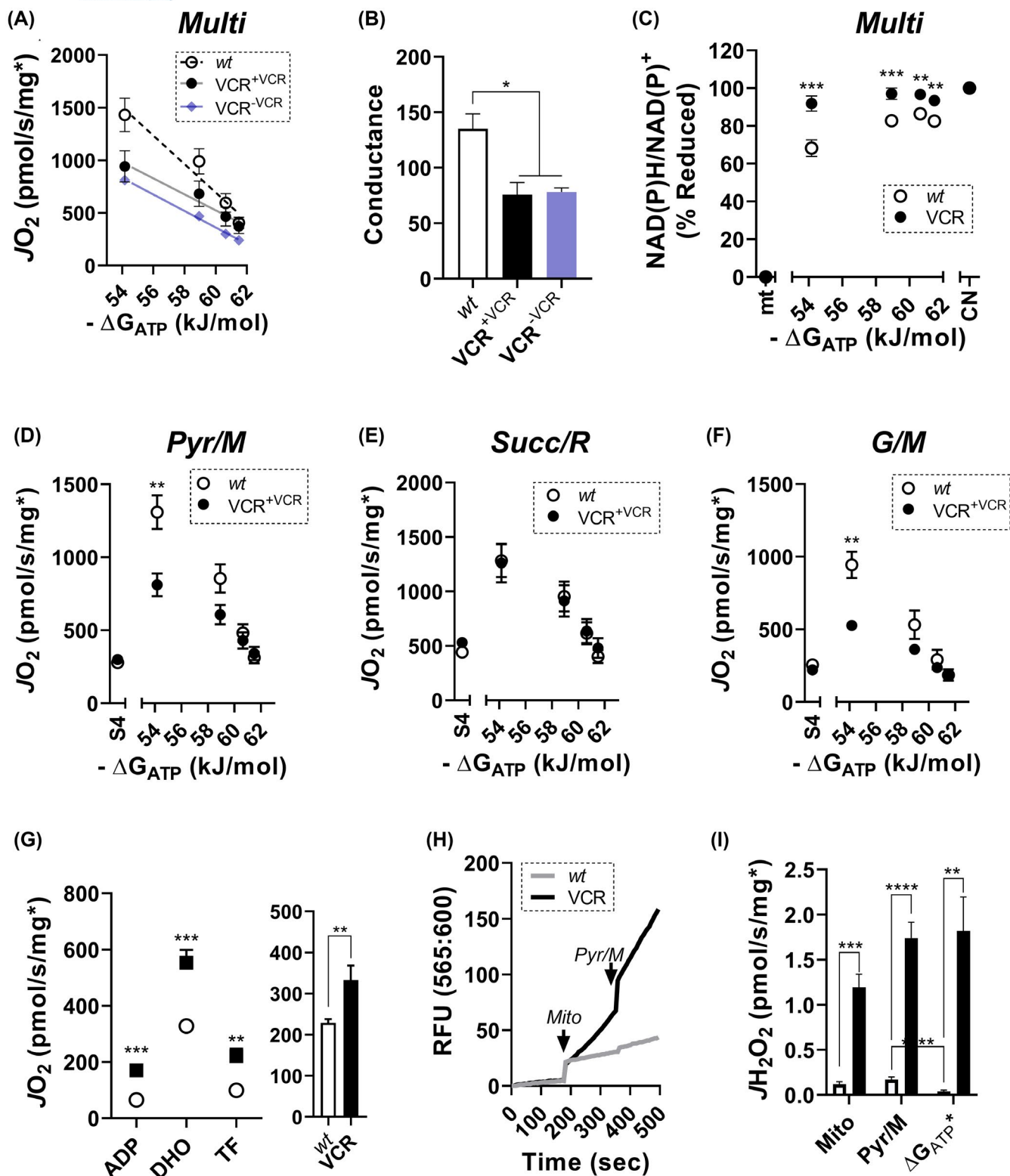


FIGURE 2 Increased basal and maximal respiration along with low fractional oxidative phosphorylation (OXPHOS) characterizes multidrug resistance leukemia. (A) Oxygen consumption in intact HL-60 wt and HL-60/VCR cells under basal conditions, as well as following the addition of oligomycin (Oligo), FCCP (max rate depicted based on titrations of 0.5, 1, 2, and 3 μM), rotenone (Rot), and antimycin A (Ant). (B) Percent decrease in basal respiration induced by oligomycin in wt cells, as well as vehicle or verapamil (10 μM)-treated HL-60/VCR cells. (C) FCCP stimulated respiration corrected for oxygen consumption remaining following rotenone/antimycin. Indicated throughout as total respiratory capacity— JH^+_{Total} . (D) Oxygen consumption in digitonin (0.02 mg/ml) permeabilized cells in the absence of substrates (Digi), as well as following the addition of P/M (5 mM/1 mM), ATP-free energy (−54.16 kJ/mol), additional substrate G/O/S (5 mM/0.2 mM/5 mM), cytochrome C (Cyt), phosphocreatine titration to manipulate ATP-free energy. Data displayed were corrected for respiration remaining following Rot/Ant. Maximal respiration in the presence of saturating substrate and minimal ATP-free energy was denoted as maximal OXPHOS flux (i.e., JH^+_{OXPHOS}). (E) Ratio of JH^+_{OXPHOS} to JH^+_{Total} —Fractional OXPHOS. (F) Intact cellular respiration to assess basal and JH^+_{Total} in wildtype HL-60 and HL60_{DNR}. (G) OXPHOS kinetics assay, like panel D. (H) Intact cellular respiration to assess basal and JH^+_{Total} in wildtype MV411 and MV411_{Vclax}. (I) OXPHOS kinetics assay, like panel D. (J) Calculated Fractional OXPHOS. Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean $\pm$ SEM, (A, B, D, E) $N = 4$ –10/group, (C, F–J) $N = 3$ –5/group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$



if the bioenergetic phenotype observed in VCR resistant cells was attributable to maintaining our stock cultures in VCR medium, we repeated our respirometry experiments in HL-60/VCR cultured in growth media devoid of VCR for seven days. Results revealed nearly identical results to that observed in cells continuously exposed to VCR (Figure 2A, “VCR⁺VCR” vs “VCR⁻VCR”).

3.5 | Mitochondrial phenotyping in MDR AML reveals intrinsic OXPHOS deficiencies linked to partial CI loss-of-function

Increased basal respiration and higher mitochondrial mass are commonly observed bioenergetic phenotypes

FIGURE 3 Mitochondrial phenotyping links low fractional oxidative phosphorylation to CI partial loss-of-function. All experiments were performed in isolated mitochondria. (A) Relationship between JO_2 and ATP-free energy (ΔG_{ATP}) clamped with the CK clamp in mitochondria energized with Multi. (B) Respiratory conductance—the slope of the relationship between JO_2 and ΔG_{ATP} . (C) Relationship between NAD(P)H/NAD(P)⁺ redox and ATP-free energy (ΔG_{ATP}) clamped with the CK clamp in mitochondria energized with Multi. (D–F) Relationship between JO_2 and ATP-free energy (ΔG_{ATP}) clamped with the CK clamp in mitochondria energized with Pyr/M, Succ/R, and G/M. (G) Respiration experiments in mitochondria exposed to saturating ADP, followed by the addition of DHO, and teriflunomide. Bar graph inset displays DHO-specific flux calculated from comparing respiration in the presence of DHO minus that observed in the presence of teriflunomide. (H) Representative trace of a mitochondrial H_2O_2 emission experiment in wt and HL-60/VCR mitochondria. Data depict fluorescence in response to mitochondrial addition, as well as Pyr/M. (I) Quantified mitochondrial H_2O_2 emission. (A–I) Data normalized to protein corrected for each sample's mitochondrial enrichment factor (MEF), assessed via nLC-MS/MS (see methods). Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean $\pm$ SEM, (A–C) $N = 3$ –11/group, (D–E) $N = 9$ –10/group, (F) $N = 4$ /group, (G) $N = 7$ –8/group, (I) $N = 5$ /group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$

associated with MDR.^{2,31} Although such phenotypes are presumed to reflect an increased reliance on oxidative ATP production, how the mitochondrial network is intrinsically remodeled to support the MDR phenotype remains poorly understood. To begin to address this gap, we first sought to validate our intact cell respirometry data by directly measuring OXPHOS kinetics in substrate replete permeabilized cells. Surprisingly, despite higher basal respiration (Figure 2D “Cells”), maximal OXPHOS capacity (indicated throughout as JH^+_{OXPHOS}) was decreased, rather than increased, in HL-60/VCR cells (Figure 2D). Consistent with this, the ratio of OXPHOS capacity (JH^+_{OXPHOS}), to total respiratory capacity (JH^+_{Total})—indicated as Fractional OXPHOS—was reduced nearly twofold in HL60/VCR (Figure 2E). Given that we recently observed low fractional OXPHOS in the setting of venetoclax resistance,⁹ we next sought to evaluate both OXPHOS and total respiratory kinetics across multiple MDR AML models. Consistent with data in HL60/VCR, HL60 cells refractory to daunorubicin, as well as MV411 cells refractory to venetoclax all presented with near-identical bioenergetic phenotypes; higher basal respiration and respiratory capacity (Figure 2F,H), lower OXPHOS capacity (Figure 2G,I), and reduced fractional OXPHOS (Figure 2J). Taken together, these data corroborate recent findings⁹ and suggest that the combination of increased mitochondrial volume, sufficient to boost both basal and maximal respiratory capacity, along with intrinsic OXPHOS deficiencies are hallmark bioenergetic features of MDR leukemia.

To probe deeper into MDR OXPHOS, we isolated mitochondria from wt and VCR resistant HL-60 cells and evaluated mitochondrial bioenergetic function. Using this workflow, we can further model the impact of a physiological ATP free energy (i.e., ΔG_{ATP}) span on respiratory flux.^{18,32} As in the permeabilized cell assay, experimental manipulation of ΔG_{ATP} is made possible via a modified version of the creatine kinase (CK) energetic clamp. This technique leverages the enzymatic activity of CK, which couples the interconversion of ATP and ADP to that of phosphocreatine (PCr) and free creatine (Cr) to titrate the

extra-mitochondrial ATP/ADP ratio and allow direct interrogation of OXPHOS kinetics in the presence of various substrate/inhibitor combinations. In isolated mitochondria, respiration assays can then be coupled with parallel measurements of mitochondrial membrane potential and electron reducing the potential to localize OXPHOS deficiencies across distinct bioenergetic nodes.

To begin, isolated mitochondria from wt and VCR resistant HL-60 cells, either continuously exposed to VCR in culture or following 7-day withdrawal, were energized with saturating carbon (pyruvate, malate, glutamate, octanoyl-carnitine, succinate; indicated by “Multi”) and respiration was stimulated with the CK clamp at minimal ATP free energy to mimic maximal OXPHOS demand. Subsequent titrations in PCr were then performed to gradually lower ATP resynthesis. Both maximal respiratory flux and OXPHOS conductance (i.e., the slope of the JO_2 vs ΔG_{ATP} relationship) were decreased in HL-60/VCR (Figure 3A,B). To identify the source of decreased OXPHOS conductance in HL-60/VCR, NADH/NAD⁺ redox poise was measured in parallel in substrate-replete isolated mitochondria exposed to an identical ΔG_{ATP} span. Results revealed NADH/NAD⁺ hyper-reduction in HL-60/VCR mitochondria (Figure 3C), indicating that OXPHOS impairments in HL-60/VCR are not due to a generalized impairment in dehydrogenase flux, but likely attributable to respiratory complex limitations (i.e., lesions in the electron transport system; ETS).

To investigate complex specific OXPHOS kinetics, mitochondria were energized with either a CI (Pyr/M) or CII (Succ/R) specific substrate combination and respiration was again stimulated with the CK clamp. At minimal ΔG_{ATP} , respiration support by Pyr/M was nearly 2-fold lower in HL-60/VCR (Figure 3D), despite no differences in the presence of S/Rot (Figure 3E). These findings suggested that the respiratory impairments observed with Pyr/M were either caused by limitations in pyruvate uptake/metabolism or due to a generalized impairment in NADH-linked flux (i.e., CI limitations). To distinguish between these possible phenotypes, we performed an

additional respirometry assay in which CI-supported flux was fueled with G/M to provide NADH that circumvents pyruvate metabolism (Figure 3F). Under these conditions, G/M supported JO_2 was again reduced in HL-60/VCR (Figure 3F), suggesting a specific bioenergetic limitation localized at CI. In support of this, a dihydroorotate (DHO) assay was performed to provide electrons directly to the ubiquinone pool, thus bypassing CI in a manner comparable to succinate. Respiration supported by DHO was higher in HL-60/VCR (Figure 3G), further corroborating a CI-specific limitation in HL-60/VCR. Interestingly, even in the absence of carbon substrate, mitochondrial respiration was elevated in HL-60/VCR, suggesting the involvement of increased mitochondrial oxidase activity (Figure 3G, “ADP”). To address this, we assessed mitochondrial H_2O_2 emission in deenergized mitochondria, as well as in response to Pyr/M and the CK clamp (Figure 3H,I). Results revealed an over 10-fold increase in H_2O_2 emission in HL-60/VCR under all conditions (Figure 3H,I). Interestingly, while the addition of the CK clamp decreased peroxide emission in wt mitochondria, presumably as a result of decreased redox pressure, H_2O_2 emission was unaffected by ATP-free energy in HL-60/VCR (Figure 3H,I). Such

results are consistent with elevated NAD(P)H oxidase activity in HL-60/VCR mitochondria.

To identify the mechanism of CI deficiency in HL-60/VCR, we conducted a label-free quantitative proteomics screen using peptides prepared from the same isolated mitochondria samples used for functional characterization. In total, we identified 385 differentially expressed mitochondrial proteins (adjusted p value < .01) comparing wt to HL-60/VCR (Table S1). Focusing on the respiratory complexes exclusively, many CI subunits were specifically downregulated in HL-60/VCR (Figure 4A). Interestingly, the core subunits of succinate dehydrogenase (i.e., CII) were increased in HL-60/VCR, presumably to compensate for CI partial loss-of-function (Figure 4A). To explore the biological consequences of CI deficiency in HL-60/VCR, we assessed cell viability in the absence and presence of increasing concentrations of the CI inhibitor, rotenone (Figure 4B,C). Consistent with partial CI loss-of-function, HL-60/VCR cells were found to be highly sensitized to rotenone-induced cytotoxicity (Figure 4C). Taken together, these findings reveal CI deficiency as a previously unidentified bioenergetic vulnerability that accompanies VCR resistance.

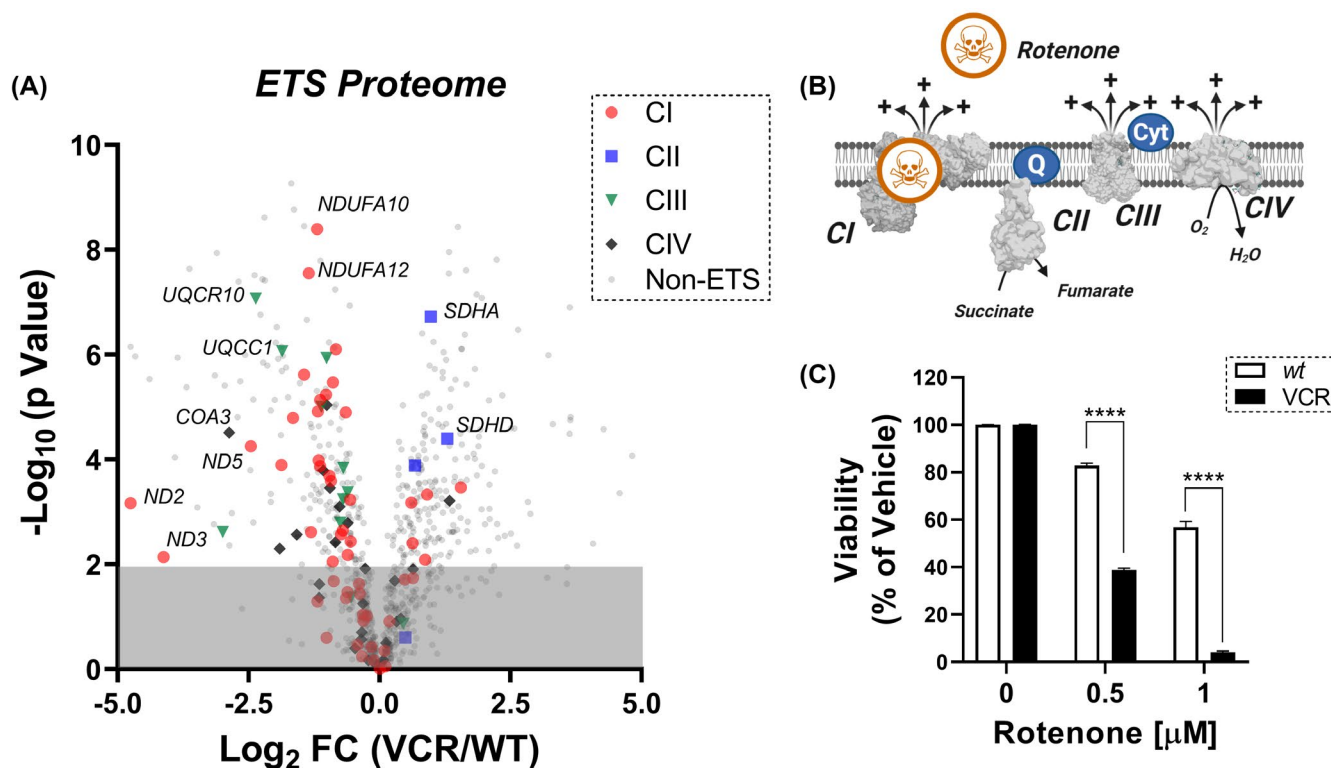


FIGURE 4 Decreased CI expression underlies vincristine (VCR) resistance. (A) Label-free nLC-MS/MS was performed on mitochondrial lysates from each sample. Volcano plot depicting changes in the mitochondrial proteome between HL-60/VCR and wt. Protein subunits of each respiratory complex (CI, CII, CIII, CIV) are indicated by color. (B) Cartoon depicting CI inhibition by rotenone. (C) Cell viability in response to 24 h of exposure to increasing concentrations of rotenone in wt and HL-60/VCR cells. Viability assessed via counting in the presence of trypan blue. Figures generated using GraphPad Prism 8 software (Version 8.4.2) and Biorender. Data are Mean $\pm$ SEM, (A) $N = 5/\text{group}$, (C) $N = 6/\text{group}$. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$

3.6 | Forced ceramide accumulation in wt cells phenocopies respiratory complex insufficiency observed with MDR

Pharmacological inhibition of AC, sufficient to increase cellular ceramide molecular species, was recently demonstrated to decrease mitochondrial respiratory competence in drug-resistant AML.⁷ Interestingly, respiratory limitations were independent of both carbon substrate and respiratory stimulus and were observed despite no evidence of mitochondrial outer membrane permeabilization. Such findings suggested the intriguing possibility that cellular ceramides may be capable of directly regulating mitochondrial ETS function. In the prior work,⁸ as well as herein, AML MDR has been observed to associate with substantial alterations in SL metabolism that center around ceramide detoxification (Figure 1G). Based on this, we hypothesized that OXPHOS deficiency may be consequential to ceramide accumulation in MDR. To determine whether elevated ceramide levels can infringe on mitochondrial function, we grew HL-60 wild-type cells for extended periods (7-days) in a medium containing either C6-ceramide (C6; 10 μ M) or ceramide delivered via nanoliposome (CNL; 10 μ M), both sources for the production of long-chain ceramides.^{33,34} Following 7-days of exposure, mitochondrial OXPHOS kinetics in permeabilized cells was assessed. Despite no change in respiration in the presence of Pyr/M in the absence of adenylate stimulation (Figure 5A; “Pyr/M”), stimulation of near-maximal OXPHOS flux with the CK clamp revealed a near twofold decrease in respiratory flux in cells exposed to both C6 and CNL (Figure 5A; “ Δ GATP”). The addition of cytochrome C did not impact respiration, thus confirming functional integrity of the outer mitochondrial membrane across all groups (Figure 5A; “Cyt C”). Relative to vehicle control, respiration remained lower in C6/CNL exposed cells upon addition of glutamate, as well as succinate, following rotenone (Figure 5A; “Succ”). Similar differences in respiration regardless of the carbon substrate strongly suggest that the respiratory impairments induced by C6/CNL are most likely attributable to ETS dysregulation. Such findings are highly reminiscent of those observed in HL-60/VCR, HL60/DNR, as well as MV411/Vclax (see Figure 2), and thus support a model whereby mitochondrial OXPHOS dysregulation (i.e., low Fractional OXPHOS) may be secondary to altered SL metabolism.

3.7 | Therapeutic synergy induced by co-targeting SL metabolism and respiratory CI in MDR leukemia

Given that sweeping alterations in SL metabolism were observed alongside bioenergetic remodeling in MDR

AML, we first sought to explore the synergistic potential of targeting respiratory complex I in conjunction with pharmacological ceramide delivery (e.g., CNL). In these experiments, CI was inhibited using the well-characterized biguanides metformin or phenformin,³⁵ as well as with the small-molecule IACS-010759.³⁶ Induction of cellular ceramides was achieved by adding CNL. In HL60/VCR, 48 h exposure to CNL (20 μ M), nanoliposome devoid of ceramide (ghost; 20 μ M), phenformin (0.5 mM), or phenformin plus ghost had no impact on cell viability (Figure 5B). Remarkably, despite no change in viability for any of the single agents, 48 h exposure to both CNL and phenformin led to a dramatic (>80% of control) decrease in cell viability (Figure 5B). Consistent with this effect, caspase activation was apparent 24 h into CNL/phenformin co-treatment, indicative of apoptotic cell death (Figure 5C). To further the therapeutic efficacy of targeting mitochondrial CI alongside SL metabolism, CNL exposure experiments were repeated in the absence and presence of IACS-010759. Once, again, despite minimal single-agent efficacy, the combination of CNL with IACS-010759 was highly cytotoxic (Figure 5D). Given that alterations in both SL metabolism and mitochondrial bioenergetics were observed herein to be common metabolic features of MRD leukemia, we next sought to investigate the broader therapeutic applications of CNL/phenformin. To do this, we repeated our cell viability assays in the presence of CNL or phenformin, both as single agents and in combination, in HL-60 cells made to be refractory to frontline AML therapies daunorubicin (HL-60/DNR) and venetoclax (HL-60/Vclax), as well as in various other AML cell lines (Kasumi-1, KG-1, THP-1, U937). Of note, both PBMCs from healthy donors, as well as parental HL-60 cells were also included in these experiments (HL-60 wt). Relative to PBMC and across all AML cell lines, despite minimal cytotoxicity as single agents, the combination of CNL and phenformin proved to be remarkably cytotoxic (Figure 5E).

As an alternative strategy to boost cellular ceramides, cell viability experiments, plus and minus metformin/phenformin, were repeated using a variety of SL metabolic inhibitors. These included SK1-i to block SPHK1, SACLAC to block acid ceramidase, and D-threo-PDMP to inhibit GCS.^{8,37–41} Except for SK1-i, minimal cytotoxicity was observed in HL-60/VCR cells in the presence of the indicated doses of SACLAC, PDMP, metformin, or phenformin alone (Figure 6A–C). However, the combination of each SL enzyme inhibitor with either metformin or phenformin boosted cytotoxicity, relative to vehicle or single-agent conditions (Figure 6A–C). This relationship was particularly evident for the acid ceramidase inhibitor SACLAC,

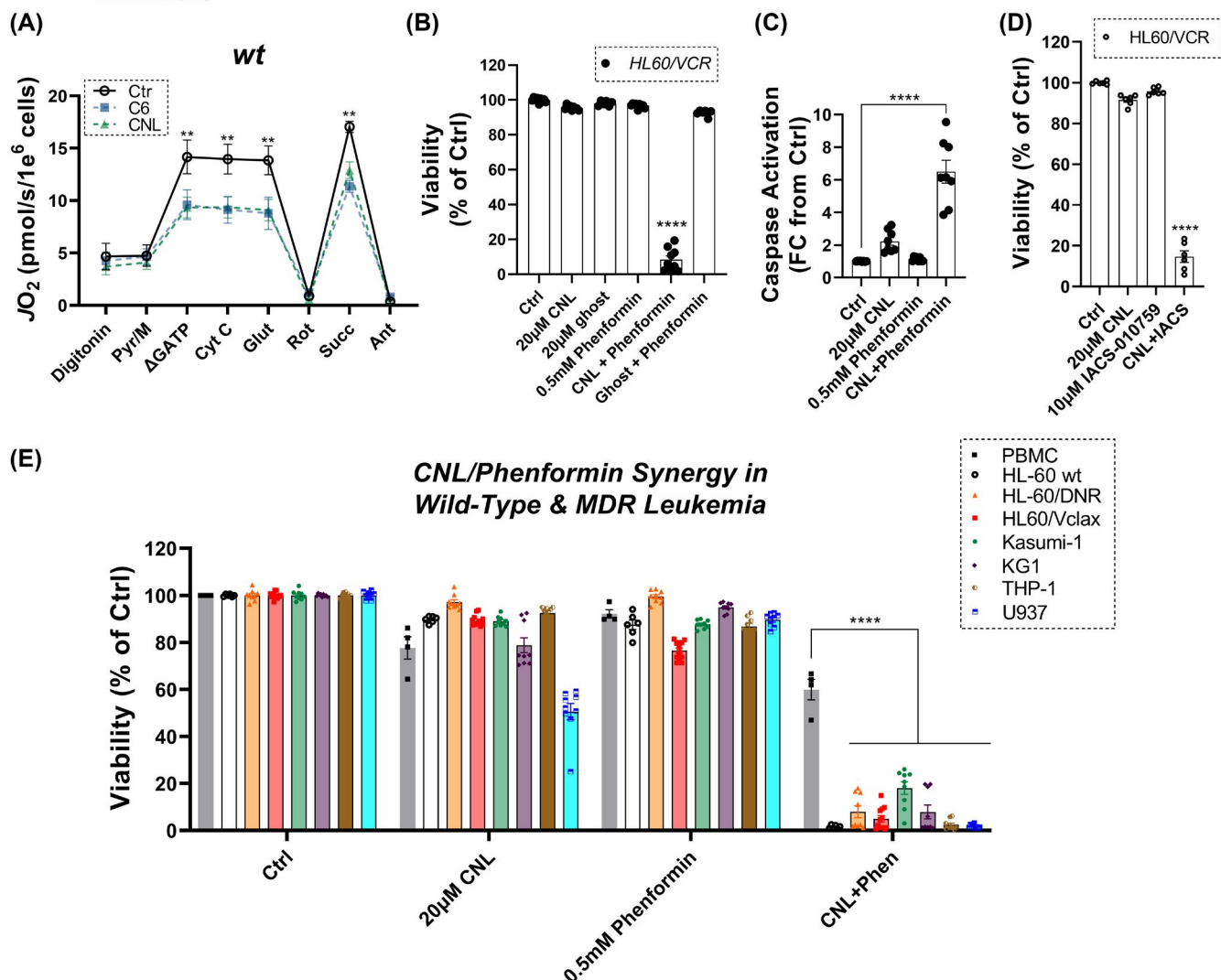


FIGURE 5 Mitochondrial CI inhibition in conjunction with administration of short-chain nanoliposomal ceramide results in synergistic cytotoxicity. (A) Respiration in permeabilized HL-60 wt cells following exposure to vehicle C6 (10 μ M) or CNL (10 μ M) for 7-days. Cells were permeabilized with digitonin (0.02 mg/ml) and respiration was assessed in response to the indicated additions. Data normalized to viable cell count. (B–D) All experiments were done in HL-60/VCR cells. (B) Cell viability, determined by viable cell count, in HL-60/VCR cells exposed for 48 h to CNL (20 μ M), ghost (20 μ M), phenformin (0.5 mM), CNL (20 μ M) + phenformin (0.5 mM), or ghost (20 μ M) + phenformin (0.5 mM). (C) Caspase activation in HL-60/VCR cells exposed for 24 h to vehicle (Ctrl), CNL (20 μ M), phenformin (0.5 mM), or CNL (20 μ M) + phenformin (0.5 mM). (D) Cell viability in response to 48 h of exposure to either CNL (20 μ M), IACS-010759 (10 μ M), or CNL (20 μ M) + IACS-010759 (10 μ M). (E) Cell viability, determined by viable cell count, in PBMC, HL-60 wt, HL-60/DNR, HL-60/Vclax, Kasumi-1, KG-1, THP-1, and U937 cells exposed for 48 h to vehicle (Ctrl), CNL (20 μ M), phenformin (0.5 mM), or CNL (20 μ M) + phenformin (0.5 mM). Viability assessed via counting in the presence of trypan blue. Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean $\pm$ SEM, (A) $N = 3$ /group, (B–E) $N = 6$ –12/group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$

where relative to the single-agent conditions, the combination of SACLAC and phenformin led to a near 70% decrease in cell viability after only 24 h in both HL60/VCR, as well as wild-type THP-1 (Figure 6C,D). Together, these data raise the intriguing possibility that co-targeting of cellular sphingolipid metabolism and mitochondrial metabolism, specifically at CI, may represent a novel treatment strategy in difficult to treat MDR leukemia, as well as in wild-type chemotherapy-sensitive phenotypes.

4 | DISCUSSION

A number of studies have been conducted on the relationship of SL's and SL metabolism to chemotherapy resistance, and results have been captioned in excellent works, including reviews.^{23,42,43} Gouaze-Andersson and Cabot⁴⁴ have reviewed the subject with specific regard to hematological malignancies, and recent work by Snider et al. discloses multiple actions of doxorubicin on SL metabolism in MCF-7 breast cancer cells,⁴⁵ including increased

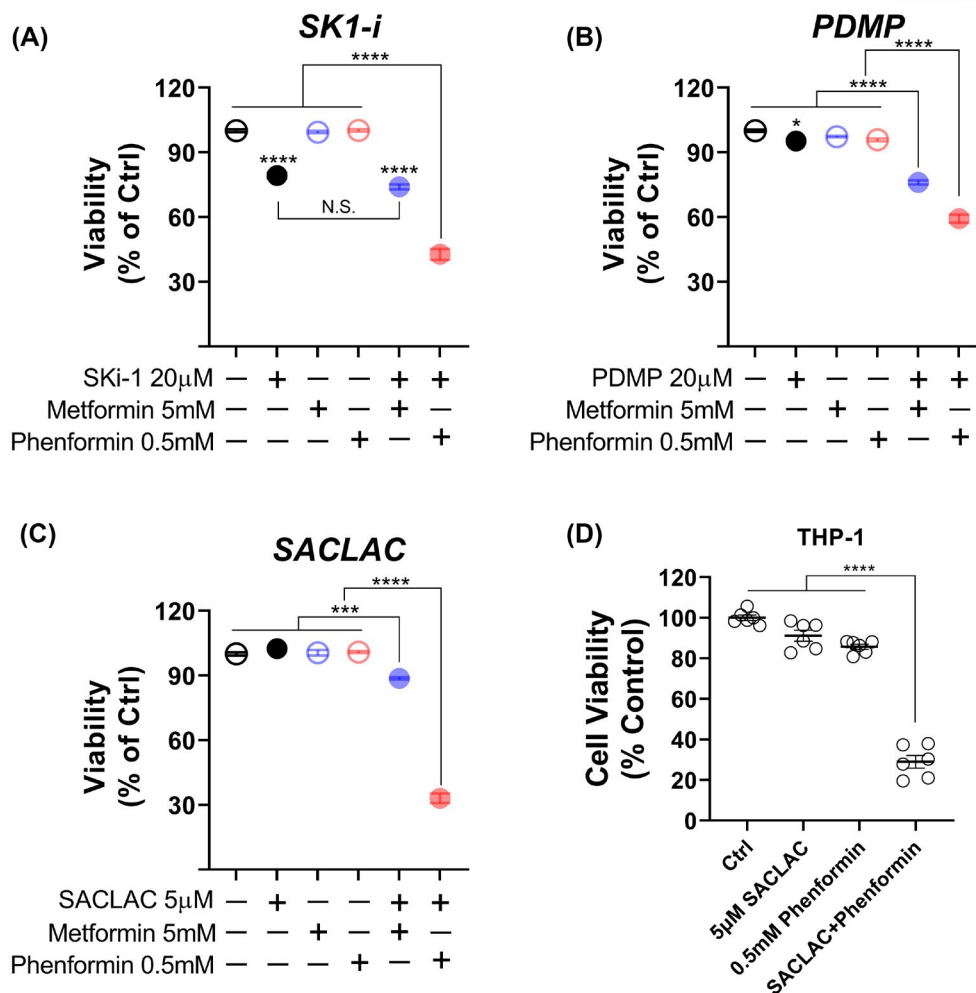


FIGURE 6 Co-targeting of respiratory CI and enzymes of sphingolipid metabolism induces synergistic cytotoxicity. (A–C) Experiments performed in HL60/VCR. Cell viability in response to 24 h of exposure to Ski-1 (20 μM), 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (20 μM), SACLAC (5 μM), metformin (5 mM) or phenformin (0.5 mM). Metformin and phenformin were administered alone or in combination with Ski-1, PDMP, or SACLAC. Viability assessed via counting in the presence of trypan blue. (D) Cell viability in THP-1 cells treated with vehicle, SACLAC (5 μM), phenformin (0.5 mM), or SACLAC + phenformin. Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean ± SEM, (A–D) $N = 3$ –6/group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$

levels of S1P and C16 ceramide. In agreement with recent observations in daunorubicin and cytarabine resistant AML,⁸ VCR resistance herein was accompanied by substantial alterations in SL composition. The novelty of the present findings stems from our focus on MDR leukemia and its impact on both SL's and mitochondrial function. Moreover, given our recent findings linking SL metabolism to mitochondrial bioenergetics,^{7,8} we hypothesized that developing a detailed understanding of the mitochondrial perturbations that accompany the drug-resistant phenotype may reveal new opportunities for targeted therapies that regulate cancer growth. To this end, the present study leveraged a recently described mitochondrial phenotyping platform,¹⁶ specifically designed to evaluate OXPHOS efficiency and capacity, relative to the underlying mitochondrial proteome. Using this platform, we made the exciting discovery that MDR leukemia is accompanied by intrinsic

mitochondrial remodeling, typified by increases in a cellular mitochondrial volume sufficient to raise both basal and maximal respiratory capacity. However, contrary to the common assumption associating high basal respiration with enhanced OXPHOS reliance, direct interrogation of the OXPHOS system in MDR leukemia revealed striking OXPHOS limitations. Such limitations are reminiscent of those recently reported in primary AML samples and suggest that the combination of high mitochondrial volume with intrinsic OXPHOS deficiency is advantageous to AML survival.⁹ Herein, these changes were subsequently found to sensitize MDR leukemia, as well as chemosensitive AML cell lines, to combinatorial treatment directed at increasing cellular ceramides and further decreasing CI flux within the mitochondria, thus offering an exciting, potential treatment strategy for combatting MDR in the clinic.

With respect to SL metabolism, VCR resistance was accompanied by upregulated expression of SPHK1, GCS, and AC, in addition to the concomitant and expected upregulation of P-gp, a predominant player in MDR.¹³ Interestingly, the changes observed in enzyme expression were reflected in the results from lipidomic analyses. For example, increased expression of SPHK1 in HL-60/VCR cells was mirrored by elevated levels of S1P. Overexpression of AC, as noted in HL-60/VCR cells, can also contribute to elevated S1P, as AC liberates sphingosine that is converted to S1P by SPHK1. With regard to ceramide, it is possible that elevated ceramide levels in HL-60/VCR cells resulted from the recycling of sphingosine via the salvage pathway of ceramide synthesis.⁴⁶ However, increases in cellular ceramides were countered in part by conversion to GC, a reflection of elevated levels of GCS. High GCS expression and elevated levels of GC have previously been linked to MDR.^{26,47–49} As ceramides induce apoptosis, our data suggest that VCR-resistant cells keep levels of ceramide “in check” via the combined activities of GCS and AC. This is supported by the work of Tan et al⁵⁰ and Morad et al⁵¹ who showed that the employ of AC and GCS inhibitors, respectively, in HL-60/VCR cells, increased ceramide levels. Interestingly, even though HL-60/VCR cells contained high levels of S1P, possibly a consequence of S1P lyase deficiency, their growth rate was considerably lower than HL-60 cells. This biology could be related to S1P receptor status in HL-60/VCR cells, as related to work by Van Brocklyn et al on the regulation of proliferation and survival,⁵² although this has not been evaluated. We also detected reductions in C18, C20, C22, and C24 molecular species of SM in HL-60/VCR cells, indicative of high sphingomyelinase activity that would contribute to increased ceramide levels. However, only some minor increases in C18, C20, C22, and C24:0 species were detected in the ceramides or in GC. This discrepancy may be a product of altered SL flux.

With regard to P-gp, the bastion of MDR, Gouaze et al., have shown coincidental overexpression of P-gp and GCS in breast cancer and epidermoid carcinoma cells selected for resistance to natural product chemotherapy drugs.²⁶ Thus, the association of GCS and GC levels with drug resistance^{53–55} has a solid footing. Of further relevance, Shabbits and Mayer⁵⁶ have shown that ceramide sensitivity in breast cancer cells is modulated by P-gp, and using vincristine as a ceramide generator, Olshefski and Ladisch⁶ have shown that GCS inhibition boosts vincristine efficacy.

In addition to changes in SL metabolism, MDR leukemia was accompanied by extensive mitochondrial remodeling. In comparison to wt cells, HL-60/VCR, HL60/DNR, and MV411/Vclax all presented with increases in basal respiration, as well as higher maximal uncoupled oxygen

consumption, the latter suggestive of higher mitochondrial content. Increased reliance on mitochondrial metabolism is a commonly emerging theme in cancer drug resistance.^{2,3} Although such findings are often interpreted to reflect an increased reliance on oxidative ATP synthesis, it is critical to point out that a large collection of these findings are based solely on measurements of intact cellular respirometry. As demonstrated herein, such methodologies are largely incapable of identifying nuanced bioenergetic inefficiencies across the mitochondrial network in cancer. As such, too much reliance on macro-level changes in cellular bioenergetics (i.e., binary assessments of mitochondrial versus glycolytic “reliance”) may in fact be masking a “goldmine” of cancer-specific mitochondrial biology that if known could potentially be leveraged to develop highly efficacious targeted therapies with a favorable therapeutic index.

Results from experiments in Figures 5 and 6 highlight the integration between SL's and mitochondrial function and suggest that this link can be exploited therapeutically. For example, with ceramide levels already high in HL-60/VCR cells compared to wild-type (see Figure 1G), the inclusion of either SACLAC or PDMP would be expected to drive ceramide to cytotoxic levels.^{8,37,57,58} SK1-i could also backup ceramide levels while limiting S1-P's mitogenic effects. Interestingly, consequent to baseline disruptions in CI expression, the addition of CI inhibitors metformin or phenformin to the mix likely add “insult to injury,” as amply demonstrated by the synergistic, cytotoxic outcome, most strikingly regarding the CNL-phenformin combination and the SACLAC-phenformin regimen. Moreover, it is noteworthy that we were able to mimic infringement of mitochondrial function typified in MDR leukemia, by cell supplementation with either C6 or CNL. Given the growing interest in targeting mitochondrial metabolism in cancer,²⁸ C6/CNL impingement on OXPHOS warrants further investigation.

5 | CONCLUSIONS

In conclusion, increases in SPHK1, GCS, and AC expression that accompany MDR leukemia serve to support cancer cell growth, as the coordinated action of these enzymes may limit the full cancer suppressor actions of ceramide while boosting mitogenicity and leukemic dissemination. Although levels of several ceramide molecular species increased with VCR resistance, a change perhaps favoring apoptosis, it is clear from the lipidomic profiles that VCR-resistant cells endeavor to keep ceramide levels in-check by increasing several GC and SM molecular species. Ceramides may also be of benefit in

the induction of autophagy for survival.⁵⁹ These factors, in conjunction with the intrinsic mitochondrial remodeling allied with MDR leukemia, promote a resilient cancer phenotype, but also reveal(s) opportunities for the design of novel, targeted therapeutics.^{23,60–62} Our approach of leveraging bioenergetic phenotyping to unveil actionable cancer-specific mitochondrial targets holds great potential as a novel drug-discovery platform centered upon mitochondrial precision medicine in MDR that can easily be adapted across cancer types.

DISCLOSURES

Authors declare that they have no conflicts of interest with the contents of this article. MK is Chief Medical Officer and co-founder of Keystone Nano, Inc., and TPL is a member of the scientific advisory board. TPL is on the Scientific Advisory Board and has stock options for Keystone Nano, Bioniz Therapeutics, and Dren Bio. TPL and DF received honoraria from Kymera Therapeutics.

AUTHORS' CONTRIBUTIONS

Kelsey H. Fisher-Wellman, James T. Hagen, Miki Kassai, Li-Pin Kao, Margaret A.M. Nelson, Kelsey L. McLaughlin, Hannah S. Coalson, Todd E. Fox, Su-Fern Tan, David J. Feith, Mark Kester, Thomas P. Loughran Jr, David F. Claxton, and Myles C. Cabot took part in the data collection and analysis. Kelsey H. Fisher-Wellman and Myles C. Cabot did the study design. Kelsey H. Fisher-Wellman and Myles C. Cabot participated in drafting and editing the manuscript. Kelsey H. Fisher-Wellman and Myles C. Cabot were responsible for the funding support. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available in the Open Science Framework (OSF) repository (DOI available on acceptance for publication). For proteomics data, all raw data are available online using accession number “JPST001142” for jPOST Repository^{63,64}.

REFERENCES

- Gottesman MM, Lavi O, Hall MD, Gillet J-P. Toward a better understanding of the complexity of cancer drug resistance. *Ann Rev Pharmacol Toxicol*. 2016;56:85-102.
- Farge T, Saland E, de Toni F, et al. Chemotherapy-resistant human acute myeloid leukemia cells are not enriched for leukemic stem cells but require oxidative metabolism. *Cancer Discov*. 2017;7:716-735.
- Guièze R, Liu VM, Rosebrock D, et al. Mitochondrial reprogramming underlies resistance to BCL-2 inhibition in lymphoid malignancies. *Cancer Cell*. 2019;36:369-384.e13.
- Gu W, Liu T, Fan D, et al. A6 peptide-tagged, ultra-small and reduction-sensitive polymersomal vincristine sulfate as a smart and specific treatment for CD44+ acute myeloid leukemia. *J Control Release*. 2020;329:706-716.
- Senchenkov A, Litvak DA, Cabot MC. Targeting ceramide metabolism—a strategy for overcoming drug resistance. *JNCI J Natl Cancer Inst*. 2001;93:347-357.
- Olshefski RS, Ladisch S. Glucosylceramide synthase inhibition enhances vincristine-induced cytotoxicity. *Int J Cancer*. 2001;93:131-138.
- Fisher-Wellman KH, Hagen JT, Neufer PD, Kassai M, Cabot MC. On the nature of ceramide-mitochondria interactions – dissection using comprehensive mitochondrial phenotyping. *Cell Signal*. 2021;78:e109838.
- Kao L-P, Morad SAFF, Davis TS, et al. Chemotherapy selection pressure alters sphingolipid composition and mitochondrial bioenergetics in resistant HL-60 cells. *J Lipid Res*. 2019;60:1590-1602.
- Nelson MA, McLaughlin KL, Hagen JT, et al. Intrinsic OXPHOS limitations underlie cellular bioenergetics in leukemia. *eLife*. 2021;10:1-31.
- Ogretmen B, Safa AR. Identification and characterization of the MDR1 promoter-enhancing factor 1 (MEF1) in the multidrug resistant HL60/VCR human acute myeloid leukemia cell line. *Biochemistry*. 2000;39:194-204.
- Morad SAF, Ryan TE, Neufer PD, et al. Ceramide-tamoxifen regimen targets bioenergetic elements in acute myelogenous leukemia. *J Lipid Res*. 2016;57:1231-1242.
- Bartholomae S, Gruhn B, Debatin K-M, et al. Coexpression of multiple ABC-transporters is strongly associated with treatment response in childhood acute myeloid leukemia. *Pediatr Blood Cancer*. 2016;63:242-247.
- Shaffer BC, Gillet J-P, Patel C, Baer MR, Bates SE, Gottesman MM. Drug resistance: still a daunting challenge to the successful treatment of AML. *Drug Resist Updates*. 2012;15:62-69.
- Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*. 2008;3:1101-1108.
- Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat Protoc*. 2006;1:1559-1582.
- McLaughlin KL, Hagen JT, Coalson HS, et al. Novel approach to quantify mitochondrial content and intrinsic bioenergetic efficiency across organs. *Sci Rep*. 2020;10:17599.
- Nelson MAM, Fisher-Wellman KH. Mitochondrial diagnostics: a discovery-based biochemical platform for phenotyping human peripheral blood cell mitochondria. *Methods Mol Biol*. 2021;2277:371-389.
- Fisher-Wellman KH, Davidson MT, Narowski TM, Lin C-T, Koves TR, Muoio DM. Mitochondrial diagnostics: a multiplexed assay platform for comprehensive assessment of mitochondrial energy fluxes. *Cell Rep*. 2018;24:3593-3606.e10.
- Fisher-Wellman KH, Gilliam LAA, Lin C-T, Cathey BL, Lark DS, Darrell Neufer P. Mitochondrial glutathione depletion reveals a novel role for the pyruvate dehydrogenase complex as a key H₂O₂-emitting source under conditions of nutrient overload. *Free Radic Biol Med*. 2013;65:1201-1208.
- McLaughlin KL, Kew KA, McClung JM, Fisher-Wellman KH. Subcellular proteomics combined with bioenergetic phenotyping reveals protein biomarkers of respiratory insufficiency in the setting of proofreading-deficient mitochondrial polymerase. *Sci Rep*. 2020;10:3603.

21. Calvo SE, Clauser KR, Mootha VK. MitoCarta2.0: an updated inventory of mammalian mitochondrial proteins. *Nucleic Acids Res.* 2016;44:D1251-D1257.
22. Lesack K, Naugler C. An open-source software program for performing Bonferroni and related corrections for multiple comparisons. *J Pathol Inform.* 2011;2:52.
23. Morad SAF, Cabot MC. Ceramide-orchestrated signalling in cancer cells. *Nat Rev Cancer.* 2013;13:51-65.
24. Truman J-P, Garcia-Barros M, Obeid LM, Hannun YA. Evolving concepts in cancer therapy through targeting sphingolipid metabolism. *Biochim Biophys Acta.* 2014;1841:1174-1188.
25. Morad SAF, Cabot MC. The onus of sphingolipid enzymes in cancer drug resistance. *Adv Cancer Res.* 2018;140:235-263.
26. Gouazé V, Yu JY, Bleicher RJ, et al. Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy. *Mol Cancer Ther.* 2004;3:633-639.
27. Tan S-F, Dunton W, Liu X, et al. Acid ceramidase promotes drug resistance in acute myeloid leukemia through NF- κ B-dependent P-glycoprotein upregulation. *J Lipid Res.* 2019;60:1078-1086.
28. Vasan K, Werner M, Chandel NS. Mitochondrial metabolism as a target for cancer therapy. *Cell Metab.* 2020;32:341-352.
29. Colombini M. Ceramide channels and their role in mitochondria-mediated apoptosis. *Biochim Biophys Acta.* 2010;1797:1239-1244.
30. Neuzil J, Dong L-F, Rohlena J, Truksa J, Ralph SJ. Classification of mitocans, anti-cancer drugs acting on mitochondria. *Mitochondrion.* 2013;13:199-208.
31. Chen J, Kao YR, Sun D, et al. Myelodysplastic syndrome progression to acute myeloid leukemia at the stem cell level. *Nat Med.* 2019;25:103-110.
32. McLaughlin KL, McClung JM, Fisher-Wellman KH. Bioenergetic consequences of compromised mitochondrial DNA repair in the mouse heart. *Biochem Biophys Res Commun.* 2018;504:742-748.
33. Song MS, Posse De Chaves EI. Inhibition of rat sympathetic neuron apoptosis by ceramide. Role of p75NTR in ceramide generation. *Neuropharmacology.* 2003;45:1130-1150.
34. Barth BM, Wang W, Toran PT, et al. Sphingolipid metabolism determines the therapeutic efficacy of nanoliposomal ceramide in acute myeloid leukemia. *Blood Adv.* 2019;3:2598-2603.
35. Bridges HR, Sirviö VA, Agip A-NA, Hirst J. Molecular features of biguanides required for targeting of mitochondrial respiratory complex I and activation of AMP-kinase. *BMC Biol.* 2016;14:65.
36. Molina JR, Sun Y, Protopopova M, et al. An inhibitor of oxidative phosphorylation exploits cancer vulnerability. *Nat Med.* 2018;24:1036-1046.
37. Pearson JM, Tan SF, Sharma A, et al. Ceramide analogue SACLAC modulates sphingolipid levels and MCL-1 splicing to induce apoptosis in acute myeloid leukemia. *Mol Cancer Res.* 2020;18:352-363.
38. Chatzakos V, Rundlöf AK, Ahmed D, De Verdier PJ, Flygare J. Inhibition of sphingosine kinase 1 enhances cytotoxicity, ceramide levels and ROS formation in liver cancer cells treated with selenite. *Biochem Pharmacol.* 2012;84:712-721.
39. Maurer BJ, Melton L, Billups C, Cabot MC, Reynolds CP. Synergistic cytotoxicity in solid tumor cell lines between N-(4-hydroxyphenyl)retinamide and modulators of ceramide metabolism. *J Natl Cancer Inst.* 2000;92:1897-1909.
40. Yang YL, Ji C, Cheng L, et al. Sphingosine kinase-1 inhibition sensitizes curcumin-induced growth inhibition and apoptosis in ovarian cancer cells. *Cancer Sci.* 2012;103:1538-1545.
41. Huang W, Tsai C, Chen C, et al. Glucosylceramide synthase inhibitor PDMP sensitizes chronic myeloid leukemia T315I mutant to Bcr-Abl inhibitor and cooperatively induces glycogen synthase kinase-3-regulated apoptosis. *FASEB J.* 2011;25:3661-3673.
42. Giussani P, Tringali C, Riboni L, Viani P, Venerando B. Sphingolipids: key regulators of apoptosis and pivotal players in cancer drug resistance. *Int J Mol Sci.* 2014;15:4356-4392.
43. Lee W-K, Kolesnick RN. Sphingolipid abnormalities in cancer multidrug resistance: chicken or egg? *Cell Signal.* 2017;38:134-145.
44. Gouaze-Andersson V, Cabot MC. Sphingolipid metabolism and drug resistance in hematological malignancies. *Anticancer Agents Med Chem.* 2011;11:891-903.
45. Snider JM, Trayssac M, Clarke CJ, et al. Multiple actions of doxorubicin on the sphingolipid network revealed by flux analysis. *J Lipid Res.* 2019;60:819-831.
46. Kitatani K, Idkowiak-Baldys J, Hannun YA. The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell Signal.* 2008;20:1010-1018.
47. Gouazé-Andersson V, Yu JY, Kreitenberg AJ, Bielawska A, Giuliano AE, Cabot MC. Ceramide and glucosylceramide up-regulate expression of the multidrug resistance gene MDR1 in cancer cells. *Biochim Biophys Acta.* 2007;1771:1407-1417.
48. Messner MC, Cabot MC. Glucosylceramide in humans. *Adv Exp Med Biol.* 2010;688:156-164.
49. Liu YY, Patwardhan GA, Xie P, Gu X, Giuliano AE, Cabot MC. Glucosylceramide synthase, a factor in modulating drug resistance, is overexpressed in metastatic breast carcinoma. *Int J Oncol.* 2011;39:425-431.
50. Tan S-F, Liu X, Fox TE, et al. Acid ceramidase is upregulated in AML and represents a novel therapeutic target. *Oncotarget.* 2016;7:83208-83222.
51. Morad SAFF, Tan S-F, Feith DJ, et al. Modification of sphingolipid metabolism by tamoxifen and N-desmethyltamoxifen in acute myelogenous leukemia—impact on enzyme activity and response to cytotoxics. *Biochim Biophys Acta.* 2015;1851:919-928.
52. Van Brocklyn JR, Lee M-J, Menzeleev R, et al. Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J Cell Biol.* 1998;142:229-240.
53. Lavie Y, Cao H, Bursten SL, Giuliano AE, Cabot MC. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J Biol Chem.* 1996;271:19530-19536.
54. Lucci A, Cho WI, Han TY, Giuliano AE, Morton DL, Cabot MC. Glucosylceramide: a marker for multiple-drug resistant cancers. *Anticancer Res.* 1998;18:475-480.
55. Liu YY, Han TY, Giuliano AE, Cabot MC. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J.* 2001;15:719-730.
56. Shabbits JA, Mayer LD. P-glycoprotein modulates ceramide-mediated sensitivity of human breast cancer cells to tubulin-binding anticancer drugs. *Mol Cancer Ther.* 2002;1:205-213.
57. Baran Y, Bielawski J, Gunduz U, Ogretmen B. Targeting glucosylceramide synthase sensitizes imatinib-resistant chronic

- myeloid leukemia cells via endogenous ceramide accumulation. *J Cancer Res Clin Oncol*. 2011;137:1535-1544.
58. Yamane M, Miyazawa K, Moriya S, Abe A, Yamane S. D, L-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (DL-PDMP) increases endoplasmic reticulum stress, autophagy and apoptosis accompanying ceramide accumulation via ceramide synthase 5 protein expression in A549 cells. *Biochimie*. 2011;93:1446-1459.
59. Jiang W, Ogretmen B. Autophagy paradox and ceramide. *Biochim Biophys Acta—Mol Cell Biol Lipids*. 2014;1841:783-792.
60. Bonhoure E, Pchejetski D, Aouali N, et al. Overcoming MDR-associated chemoresistance in HL-60 acute myeloid leukemia cells by targeting sphingosine kinase-1. *Leukemia*. 2006;20:95-102.
61. Paugh SW, Paugh BS, Rahmani M, et al. A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. *Blood*. 2008;112:1382-1391.
62. Ogretmen B. Sphingolipid metabolism in cancer signalling and therapy. *Nat Rev Cancer*. 2018;18:33-50.
63. Okuda S, Watanabe Y, Moriya Y, et al. jPOSTrepo: an international standard data repository for proteomes. *Nucleic Acids Res*. 2017;45:D1107-D1111.
64. Deutsch EW, Csordas A, Sun Z, et al. The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. *Nucleic Acids Res*. 2017;45:D1100-D1106.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Fisher-Wellman KH, Hagen JT, Kassai M, et al. Alterations in sphingolipid composition and mitochondrial bioenergetics represent synergistic therapeutic vulnerabilities linked to multidrug resistance in leukemia. *FASEB J*. 2022;36:e22094. doi:[10.1096/fj.202101194RRR](https://doi.org/10.1096/fj.202101194RRR)

From OCR and ECAR to energy: Perspectives on the design and interpretation of bioenergetics studies

Received for publication, January 11, 2021, and in revised form, August 25, 2021. Published, Papers in Press, August 28, 2021, <https://doi.org/10.1016/j.jbc.2021.101140>

Cameron A. Schmidt^{1,2}, Kelsey H. Fisher-Wellman^{1,2,*}, and P. Darrell Neuffer^{1,2,3,*} 

From the ¹East Carolina Diabetes and Obesity Institute, ²Department of Physiology, ³Department of Biochemistry and Molecular Biology, Brody School of Medicine, East Carolina University, Greenville, North Carolina, USA

Edited by Qi-Qun Tang

Biological energy transduction underlies all physiological phenomena in cells. The metabolic systems that support energy transduction have been of great interest due to their association with numerous pathologies including diabetes, cancer, rare genetic diseases, and aberrant cell death. Commercially available bioenergetics technologies (e.g., extracellular flux analysis, high-resolution respirometry, fluorescent dye kits, etc.) have made practical assessment of metabolic parameters widely accessible. This has facilitated an explosion in the number of studies exploring, in particular, the biological implications of oxygen consumption rate (OCR) and substrate level phosphorylation *via* glycolysis (i.e., *via* extracellular acidification rate (ECAR)). Though these technologies have demonstrated substantial utility and broad applicability to cell biology research, they are also susceptible to historical assumptions, experimental limitations, and other caveats that have led to premature and/or erroneous interpretations. This review enumerates various important considerations for designing and interpreting cellular and mitochondrial bioenergetics experiments, some common challenges and pitfalls in data interpretation, and some potential “next steps” to be taken that can address these highlighted challenges.

In recent decades, a multitude of genetically modified cells and organisms have been developed with the aim of elucidating etiological and/or pathophysiological mechanisms of disease (1–3). These model systems often demonstrate features of modified or compromised energy metabolism (4–6). Recent technological advances and widespread commercial availability of instrumentation and reagents have facilitated an explosion of investigations into the consequences of gene variance in metabolic systems, with emphasis placed on processes related to cellular energy transduction through mitochondrial oxidative phosphorylation (OxPhos) and substrate-level phosphorylation in glycolysis (7–10). These bioenergetics assays, exemplified by the widely popular phosphorescent probe-based extracellular flux analysis (EFA) (8, 9), have also found broad use in other areas of biomedical research. For example, EFA studies have contributed to significant advances in

identification of novel metabolic liabilities of common drugs (11, 12), high-throughput comparison of metabolic features of cancer-derived cell lines (13–15), and elucidation of the links between cell energy transduction and programmed death (16).

Though there has been great utility in the widespread application of bioenergetics assays to biomedical science, some assumptions regarding cellular metabolic organization persist that may ultimately impede scientific progress. These assumptions can be nuanced and likely persist because they can be made to be observable by experimental conditions. As examples, the “Warburg effect” in cancerous and noncancerous cells and mitochondrial “dysfunction” in metabolic diseases (e.g., type II diabetes mellitus) can both be detected depending on conditions, but both have physiological implications that remain disputed (17, 18). Additionally, common bioenergetics measurements have significant caveats that can dramatically impact the quality of their interpretations. For example, the ECAR to OCR ratios that are often used to “diagnose” the Warburg effect are subject to several modifiers that can impact their proportionality to the metabolic processes they are meant to represent (9, 10).

The purpose of this article is to provide an overview of cellular bioenergetics assay design, emphasizing key conceptual and practical considerations, as well as perspectives on some common pitfalls in data interpretation. Some key considerations in flexible bioenergetics assay design are covered in the first section, followed by additional discussion of three topic areas of general importance: 1) cellular energy and ATP, 2) glycolysis, ECAR, and energy partitioning, and 3) mitochondrial dysfunction.

Designing bioenergetics experiments

Experimental platforms

Nearly all bioenergetics studies take advantage of the principle of steady-state mass balance to determine metabolic fluxes. In this scheme, external and/or internal fluxes are measured, then the net flux is fit to a model of the metabolic system(s) under study, and the unknown fluxes are then estimated. The main discriminating factor among different approaches lies in the degree of molecular “resolution,” or the ability to distinguish accurately between pathways or individual reactions. The current experimental modality with the highest molecular resolution is metabolic flux analysis (MFA)

* For correspondence: P. Darrell Neuffer, neufferp@ecu.edu; Kelsey H. Fisher-Wellman, fisherwellmank17@ecu.edu.

(19). In this method, internal fluxes are measured by fitting isotopomer labeling patterns to a mass balance simulation of fluxes through a network model to interpolate individual reaction fluxes (20). Two alternative methods include respirometry and extracellular flux analysis, which involve time-resolved measurement of external fluxes only, paired with metabolic substrate and inhibitor protocols to reductively interpolate fluxes through specific reactions/pathways. These methods are low molecular resolution compared with MFA but are capable of measurements over short timescales, which is a limitation of MFA studies due to the typically long incubation times required to achieve an isotopic steady state (20). Because of technical challenges, MFA studies also tend to be performed by researchers with extensive experience in biochemistry/metabolism. Respirometry and extracellular flux analysis will be the continued focus of this review because of their broad use by researchers that do not necessarily specialize in bioenergetics research.

Measuring mitochondrial metabolic fluxes *via* oxygen consumption rate (*i.e.*, respirometry) has been used for decades and has been essential in the development of the modern understanding of mitochondrial physiology (21). In these experiments, cells/tissues/organelles in an enclosed chamber are exposed to defined conditions and oxygen tension in the media is measured over time. The structure and coupling stoichiometries of the electron transfer system (ETS) and energetically coupled transport processes are well characterized in a variety of cell types (22, 23). This allows the interpolation of metabolic flux through mitochondrial metabolic pathways (*e.g.*, OxPhos) from OCR data. There are two common methods currently employed to measure OCR: 1) a potentiometric method that uses a platinum hydrogen (Clark) electrode, and 2) quenching of the fluorescence lifetime of metalloporphyrin complexes (MPCs) by the presence of elemental oxygen (24, 25). Clark electrodes are widely used in commercial systems (*e.g.*, Oxygraph-2K; Oroboros) as well as in "homemade" apparatuses, and principles of their design and function are well described (26, 27). Solid-state MPCs are the most likely technology used in the extremely popular Seahorse Extracellular Flux Analyzer (Agilent technologies) (8), although that information has not been made explicitly available to the scientific community. MPCs can also be prepared/purchased in soluble form and their molecular design and function have been described (24, 25, 28, 29). ECAR is another external parameter that can be measured and interpolated to

metabolic flux. Like the methods employed in respirometry, this can be measured using fluorescence lifetime quenching of lanthanide-based small-molecule probes (30).

Several additional measurements are commonly used in conjunction with measured external fluxes. For example, measurement of the electrochemical potential across the inner mitochondrial membrane (IMM) is proportional to the energetic state of the ETS and can be measured potentiometrically or with fluorescent organic cations (*e.g.*, tetramethyl rhodamine methyl ester) (31, 32). The redox potential of the reduced pyridine nucleotide pool (NAD(P)H) within specific subcellular compartments can be proportional to the energetic state of specific pathways and can be monitored *via* autofluorescence (ideally fluorescence lifetime) (33, 34). Additionally, enzyme activity assays are cost-effective and relatively simple to perform and can be used in conjunction with other assays to provide information regarding specific reactions and/or to reveal allosteric interactions (34). Many other fluorometric probes are available for measuring pH and ion gradients in a variety of model systems (35, 36). There has also been significant advancement in design of genetically encoded probes that can report additional changes in internal conditions, for example, intracellular pH, GSH/GSSG ratio, NAD(P)H redox state, etc. (37–39). Finally, some platforms/instrumentation is best suited for specific types of preparations. Some of these considerations are highlighted in Table 1.

Buffer/media chemistry

A major consideration in the design of bioenergetics assays is the buffer (or media) chemistry. The media/buffer composition for intact cell *versus* permeabilized cells/isolated mitochondria is essentially opposite in ionic composition (*i.e.*, intact high sodium and calcium/low potassium; permeabilized-high potassium/low sodium and calcium). Intact cells placed in buffers designed for permeabilized cells/isolated mitochondria will likely be lysed due to the hypotonic nature of the buffer (and vice versa). Both ionic strength and osmolarity should be considered to avoid significant effects on the kinetics and energetics of metabolic pathways (40–42). Additionally, certain ions may modulate metabolic fluxes on their own, which could necessitate the use of salts with alternative (inert) ions. For example, sodium alters isolated mitochondrial respiration rates, which can be circumvented by using tris-based salts (34).

Table 1
Considerations for choosing instrumentation for extracellular flux analysis studies

Considerations	Potentiometric (electrode)	Solid-state phosphorescent probe	Soluble phosphorescent or fluorescent probe
Manufacturer	Oroboros O2K	Agilent Seahorse XF Flux Analyzer	Agilent Mito-Xpress and pH-Xtra
Format	Sealed Chamber(s)	Specialized Microplate	Standard (culture) microplate
Variables measured	OCR, Membrane Potential	OCR, ECAR	OCR, ECAR
Suitable for adherent cells?	No	Yes	Yes
Suitable for suspension cells/isolated mitochondria?	Yes	No	Yes
Suitable for permeabilized cells?	Yes	Yes	Yes
Measurement type	Kinetic	Kinetic (>4 Injections)	Serial

To further emphasize the impact of assay media conditions on tonicity and osmolarity, consider that many metabolic intermediates are multivalent ions, which contribute substantially to both parameters. For example, a 5 mmol/l addition of sodium citrate, imparts 20 mOsmol/l to the osmolarity and 30 mmol/l to the ionic strength of the buffer. Additionally, sodium citrate is a salt of a weak acid that may raise the pH and buffering capacity of the media, particularly in low or unbuffered media that is often used for ECAR measurements. To address this type of issue, reported buffer/assay media descriptions should include information regarding changes in these parameters over the course of the assay. Additionally, controls should be included to account for the relative contributions of these changes, for example: 1) metabolically inert sugar alcohols (*e.g.*, mannitol) to account for osmotic effects, 2) salts with alternative counterions in accordance with the type of preparation, or 3) inert weak acids with a pK_a similar to the metabolite under investigation to account for pH effects.

Substrate/inhibitor titration

Because respirometry and EFA are limited by low molecular resolution, experiments are typically designed to compensate by reductively separating out components of individual reactions or pathways and by measuring multiple related parameters to reduce ambiguity (34, 43, 44). The most common general strategy is known as substrate-inhibitor titration (SUIT). In this strategy, external flux is measured while "input" metabolite (substrate) for a given pathway is provided in increasing concentration until the saturating substrate concentration is reached. To obtain additional resolution, specific inhibitors of steps within the pathway are titrated to determine the degree of control exerted by those steps under that specific set of experimental conditions (45). This strategy is often necessarily implemented through multiple parallel experiments to obtain a more complete working picture of the metabolic system (34, 44, 46, 47).

Importantly, there is no single way to approach SUIT design, and flexible protocol design can be generalized to a few key steps: 1) define the pathway (metabolic network model), 2) identify experimentally measurable fluxes, 3) identify candidate substrate/inhibitor combinations to isolate flux through specific pathway components, and 4) determine the most appropriate statistical design for the specific comparisons of interest. Notably, each of these steps must be very carefully considered to avoid ambiguity in the resulting interpretations. Several articles and books have been published that serve as excellent starting points for SUIT design in isolated mitochondria, permeabilized cells/tissue, and intact cells (9, 10, 44, 46–50).

Energy substrate choice

Choice of substrates (*i.e.*, metabolic intermediates that support catabolic pathway flux) is significantly influenced by the model of the metabolic network under study. In permeabilized cells/isolated mitochondrial preparations, the metabolic systems are separated from their normal physiological context. This necessitates careful consideration of substrate choices because multiple

metabolites may need to be present to fully support the metabolic network or prevent feedback inhibition. As an example, fatty acid oxidation (FAO) in isolated skeletal muscle mitochondria cannot proceed in the absence of low (~ 0.1 mM) concentrations of malate, but can in liver mitochondrial preparations due to a different mechanism of relieving feedback inhibition by acetyl-CoA (44). Additionally, substrate transport may be influenced by several other factors including energy dependency of the transport process, substrate ionization state, substrate concentration, or cofactors required for symport/antiport mechanisms (51–54). For intact cells, transport of metabolites across the plasma membrane also adds an additional layer of complexity and some metabolites may require specific cofactors or growth factor stimulation for uptake (55). Ultimately the network model chosen by the experimenter must account for these factors, which will manifest differently depending on preparation type, as well as subculture conditions or cell type/tissue of origin.

Inhibitor choice

There are a large number of possible inhibitors that can be used to target specific reactions within metabolic pathways and reports/reviews that may aid in identifying appropriate compounds are readily available in the literature (56–58). There are a few considerations to keep in mind when choosing inhibitors for bioenergetics experiments: 1) not all inhibitors are permeable to all preparation types (*e.g.*, intact cells *versus* permeabilized cells/isolated mitochondria), 2) some inhibitors may be metabolized or transported out of cells, which can result in unstable kinetic effects, and 3) minimal effective inhibitor concentrations should be determined using titrations and curve fitting to determine the appropriate experimental concentration from the dose–response kinetics. Importantly, the details involved in choosing the inhibitor concentration should be reported when possible.

Normalization factor(s)

Another critical factor in the design of bioenergetics experiments is the choice of normalization factor(s). Metabolic flux through a given pathway will depend on the amount/activity of the enzymes and metabolic intermediates present in the sample, neither of which necessarily scales with other variables (*e.g.*, cell volume). Cell counts are sometimes used for normalization in intact cells. However, some experimental designs may require comparisons among cells that are physically different sizes and would thus have a different total cell mass for the same number of cells. A popular way to circumvent this challenge is to normalize flux data to protein concentration, which can be measured using a bicinchoninic acid assay (59). This strategy may present challenges in situations where enzymes participating in the metabolic pathway under study are outnumbered by other soluble proteins, which act as background noise in the assay. The same considerations apply to isolated mitochondria studies, with the additional challenge that cellular proteins may contaminate the mitochondrial preparation, which may skew normalization when comparing different tissue/cell types or conditions (60). Ultimately, there is no single optimal normalizing factor. Instead,

normalization must be carefully considered early in the experimental design and several options should be tested to determine the best approach.

Standardization and quality control

All bioenergetics experiments are subject to variability due to the mode of preparation. For intact cells, this may include growth conditions of the cells (*e.g.*, media formulation, incubation conditions), cell viability (especially in drug studies), and cell type/tissue of origin. For permeabilized cells and tissues, significant variability can be introduced by the permeabilization agent and procedure during tissue isolation/separation. Additionally, isolated mitochondria preparations are very sensitive to the isolation conditions, and obtaining undamaged mitochondria is not trivial. Finally, variation may be introduced due to drift in the instrumentation being used. To account for potential sources of variation, standardization practices should be incorporated into all bioenergetics designs (and reported when possible). The simplest way to standardize the effects of preparation on bioenergetics experiments is to develop abbreviated assay protocols that are not anticipated to vary so long as underlying conditions remain the same. Several previously described assay designs can be adapted for use as standard assays, including the popular "stress tests" often implemented in the xF analyzer platforms or the respiratory control ratios described for isolated mitochondria studies (9, 47). These can be performed repeatedly throughout the course of experiments to detect drift in preparation or instrumentation. To standardize drift in instrumentation, positive and negative control conditions can be created that test the dynamic ranges of the measurement. For example, glucose/glucose oxidase can be used to induce a high noncellular rate of oxygen consumption and cyanide can be used to completely inhibit most biological respiratory processes (29).

Additionally, due to the variability that preparation quality can introduce, it is important to include quality control assessments in assay design whenever possible. For example, damaged mitochondria may still respire, but at very low coupling efficiency with OxPhos, which can be assessed by using respiratory control ratios that determine the degree of kinetic control of OxPhos over total respiration (47). To account for mitochondrial damage, a cytochrome *c* titration is often performed to test the integrity of the outer mitochondrial membrane. Cytochrome *c* may also be limiting under certain assay conditions or physiological circumstances, necessitating its addition in the assay buffer (49). For intact cells, several cost-effective cell viability assay strategies are available and should be used in conjunction with qualitative morphological analysis to ensure that cells are healthy and viable. Note that some viability assays such as luciferase-based ATP assays or diaphorase activity-dependent assays (*e.g.*, resazurin or tetrazolium dyes) are sensitive to changes in cellular metabolism and should not be used as indicators of viability. Necrosis (permeability)-based assays such as ethidium homodimer-2 or propidium iodide are recommended.

Interpreting bioenergetics experiments

The preceding section highlighted key considerations regarding general design of bioenergetics studies. The following section addresses a few issues that commonly impact the interpretation of cellular bioenergetics studies. Experimental interpretations are not just sensitive to the caveats intrinsic to the measurements being made, but also to some of the assumptions that underlie the questions being asked. The following section will focus on four areas in which both issues have and continue to influence data interpretation: 1) cellular energy and the meaning of ATP measurements, 2) energy partitioning between glycolysis and OxPhos, 3) challenges underlying interpretation of ECAR measurements, and 3) whether differences in mitochondrial functional parameters are indicative of pathological "dysfunction," as well as strategies to discern when (or if) that is the case.

Cellular energy and ATP measurements

Though there are many energy transducing reactions within cells, adenosine triphosphate (ATP) is generally considered to be the premier energy intermediate (61). A very common strategy for approximating cellular energetic state is to associate a change in metabolic flux (*e.g.*, OCR and/or ECAR) with a reduction in total ATP concentration (often qualitatively denoted ATP "levels"), which is commonly measured using luciferase-based enzyme coupled reaction schemes. This association is often interpreted as a failure of metabolic flux to support a given physiological ATP concentration (62–64). However, there are several problems with this interpretation. First, ATP levels reflect the steady-state balance between the rates of ATP synthesis and ATP hydrolysis, and thus both must be considered. Second, there is a common misconception regarding how ATP acts as an energy intermediate in cells; specifically, that the energy "stored" by ATP is held within the gamma phosphate bond. The gamma phosphate bond is sometimes referred to as "high-energy," which is physically correct but colloquially misleading. The term "high-energy" refers to both charge repulsion and the fact that inorganic phosphate is resonance stabilized in aqueous solution (61). However, it is not the case that the bond itself "contains" energy, and it is certainly not the case that energy is released by breaking of the bond (a process that *requires* energy).

From a thermodynamic perspective, energy is transduced in proportion to the direction and extent of displacement of the ATP hydrolysis reaction rather than through the ATP molecule itself. Quantifying this relationship requires measuring concentrations of both the product (ATP) and reactants (ADP+P_i). The free energy of ATP hydrolysis can be expressed as a phosphate group transfer potential and quantitated by the Gibbs free energy of the hydrolysis reaction (ΔG_{ATP} , kJ/mol) (65, 66).

$$\Delta G_{ATP} = \Delta G^{\circ'}_{ATP} + RT \ln \Gamma = RT \ln \left(\frac{\Gamma}{K_{eq}} \right) \quad (1)$$

ΔG_{ATP} is empirically derived from the equilibrium (K_{eq}) and observed (Γ) mass action ratios of the reaction. $\Delta G_{\text{ATP}}^{\circ}$ is the Gibbs energy at physiological pH and temperature and R and T are the gas constant and absolute temperature, respectively. This equation demonstrates that the energy available from ATP hydrolysis is quantitated from its relative *displacement* from thermodynamic equilibrium, which in biological systems is held at a steady-state value dependent on the energetic and kinetic states of *both* catabolic (supply) and anabolic (demand) reactions (67–69).

In extreme situations, total ATP may be an indicator of cell health. In fact, the most popular luciferase-based assay, the cell-titer glo assay (Promega), is billed by the manufacturer as a viability assay, not as an energetic assay. A reduction in cellular ATP may indicate a reduction in the total adenylate pool due to purine degradation during energetic crisis or other physiologically relevant scenarios (70). However, if the energy status of the cell is what is being investigated, then a more comprehensive approach is required. The theoretical ATP hydrolysis mass action ratio is challenging to measure due to the many ionic forms of reactants and products involved in the gross reaction (69). However, in the early 1970s, Atkinson and Chapman proposed a practical "shorthand" mass action ratio involving free ATP, ATP + ADP, and total adenylates measured using enzyme coupled reactions (71, 72). This (rudimentary) approach offers an improvement over measuring ATP levels alone, as it leverages the same concept as commercial luciferase kits. It also accounts for the ATP hydrolysis mass action ratio and is not significantly more difficult to perform. Alternatively, the ATP hydrolysis mass action ratio can be determined using high (or ultra) performance liquid chromatography (73). Note that in this case, free ADP is difficult to measure because it is present in very low concentrations, and extraction methods often capture ADP that was not actually free within the cell, but rather, bound to cellular proteins. Approaches that leverage the known equilibrium constant of the creatine kinase reaction, along with HPLC measurement of other phosphagens, may be used to calculate free ADP concentrations in some tissues (73).

The rate of ATP turnover (J_{ATP}) is another representation of the cell's energetic phenotype that compliments static measurements of the ATP mass action ratio. It is directly proportional to the power transferred through the phosphate potential (Box 1), which if measured over the duration of a cellular process is proportional to the total work required by the process itself (74). Directly measuring J_{ATP} is complex and limited by technical challenges (75), but *estimating* J_{ATP} from EFA data is straightforward and has been recently described in detail (9, 10, 31). Importantly, there is a major caveat to this approach: *i.e.*, the quality of the estimation is only as good as the assumed reaction coupling stoichiometries for a given metabolic pathway, and the model of the pathway is ultimately up to the experimenter. It can be very difficult to determine the coupling stoichiometries explicitly in intact cells because it is not always possible to isolate specific substrate oxidation patterns. For example, a sample of intact cells may oxidize stored carbohydrates and fatty acids simultaneously,

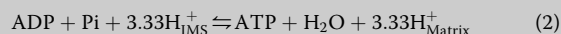
Box 1. Mitochondrial energy transduction and power output

In the standard model of mitochondrial energy transduction (43, 210, 211): 1) organic molecules (*i.e.*, fuels) transfer electrons through a series of coupled redox reactions that terminate on the reduction of O_2 to H_2O ; 2) this series of redox reactions is coupled with proton translocation to the intermembrane space, resulting in a rapid equilibration of other permeant ions and counter ions that constitutes an electrochemical potential; 3) the electrochemical potential (or proton motive force, pmf) is dissipated through membrane-bound enzyme complexes (*e.g.*, ATP synthase) in order to perform useful work. For additional visualization, this concept has also been described through several didactic analog models (47, 212–214).

The potential energy of each step is constrained by the maximum transfer potential of the previous step, which depends on both the magnitude of the "driving" force as well as the stoichiometry of the process (212).

$$\Delta G_{\text{Redox} \rightarrow \text{pmf}} = \Delta G_{\text{Driving}} + \Delta G_{\text{Driven}} = -2F(E_{\text{h}}^{\text{O}} - E_{\text{h}}^{\text{NADH}}) + 10\Delta G_{\text{pmf}} \quad (1)$$

This equation demonstrates the maximal work transfer from the combined redox reactions of the ETS to the proton motive force (pmf) for a two-electron transfer from NADH to oxygen. O indicates $\frac{1}{2}$ mole O_2 , F is Faraday's constant (converts from Volts to J/mol). The stoichiometric coefficient 10 comes from assuming a proton pumping stoichiometry of 4, 4, and 2 for respiratory complexes I, III, and IV, respectively (27, 215–217). The pmf derived from the entire redox span ($\text{NADH}:\text{O}_2$) is approximately -185 mV or 17.8 kJ mol^{-1} , assuming that the steady-state redox potential of the ETS is -925 mV (43, 168). ADP phosphorylation by the mammalian F_0F_1 ATP synthase enzyme complex is thought to phosphorylate ~ 3 ADP from ~ 10 protons transferred from the intermembrane space (IMS) toward the matrix, resulting in a final stoichiometry of $\sim 3.33 \text{ mol H}^+ \cdot \text{mol}^{-1} \text{ ATP}$ (22, 218):



The Gibbs energy differential from pmf to ADP phosphorylation can be determined by:

$$\Delta G_{\text{pmf} \rightarrow \text{ATP}} = 3.33\Delta G_{\text{pmf}} + \Delta G_{\text{ATP}} \quad (3)$$

Thus, the estimated *maximum* work that the full redox span from NADH/NAD^+ to $\text{O}_2/\text{H}_2\text{O}$ can transfer to the ATP phosphate group transfer potential (for this specific value of ΔG_{redox}) has a magnitude of $3.33 \cdot 17.8 \text{ kJ mol}^{-1} = 59.2 \text{ kJ mol}^{-1}$. If the magnitude of driven force drops due to net ATP hydrolysis (demand), the driving force will tend toward this value, so long as the ETS redox potential (supply) remains unchanged.

Thermodynamics describe the direction and extent of biochemical reactions, but these expressions do not contain any kinetic information. Steady-state kinetics of mitochondrial energy transduction are similarly constrained by flux through preceding and succeeding steps as well as the coupling stoichiometries of the individual reactions that comprise the system (34). Work (or energy) transferred per unit time is denoted as power and has SI units of Watts ($\text{J} \cdot \text{s}^{-1}$). Power is an extremely useful parameter because it encompasses both kinetic and energetic information and is capable of supplying information regarding the actual quantities of work required to perform cellular processes (*i.e.*, by integrating the power over the time course of the process) (74). If a sample of a known quantity of isolated mitochondria or permeabilized cells respire on pyruvate/malate, then electron transfer to the CoQ/CoQH_2 redox couple can be attributed to only NADH linked redox transfer through respiratory complex I (34). The ATP/O stoichiometry is approximately:

$$\frac{10 \left(\frac{\text{H}^+}{\text{O}} \right)}{3.33 \left(\frac{\text{H}^+}{\text{ATP}} \right)} = 3.0 \left(\frac{\text{ATP}}{\text{O}} \right) \text{ or } 6.0 \left(\frac{\text{ATP}}{\text{O}_2} \right) \quad (4)$$

If ΔG_{ATP} is $-59.2 \text{ kJ mol}^{-1}$, which represents a moderate to high cellular energetic demand state (34), then an equivalent $+59.2 \text{ kJ mol}^{-1}$ work transfer is required to maintain it. If mitochondria respiring against a $\Delta G_{\text{ATP}} = -59.2 \text{ kJ} \cdot \text{mol}^{-1}$ have a measured steady-state respiration rate of $J_{\text{O}_2} = 4.5 \text{ nmol O}_2 \cdot \text{s}^{-1} \text{ mg}^{-1}$ mitochondrial protein (34), the ATP phosphorylation rate can be calculated:

$$6.0 \left(\frac{ATP}{O_2} \right) * 4.5 \text{ nmol } O_2 \cdot s^{-1} \cdot \text{mg}^{-1} = 27.0 \text{ nmol } ATP \cdot s^{-1} \cdot \text{mg}^{-1} \quad (5)$$

The steady-state work transfer rate to the phosphate potential is:

$$27.0 \text{ nmol } ATP \cdot s^{-1} \cdot \text{mg}^{-1} * 5.92 * 10^{-5} \text{ J} \cdot \text{nmol } ATP^{-1} \\ = 1.6 * 10^{-3} \text{ J} \cdot s^{-1} \cdot \text{mg}^{-1} = 1.6 \text{ mWatts} \cdot \text{mg}^{-1} \quad (6)$$

In the case of an alternative fuel source with a different ATP/O ratio, such as isolated mitochondria respiring in the presence of succinate/rotenone (34), the electron transfer to the CoQ/CoQH₂ redox couple can be attributed to only FADH₂ linked redox transfer through respiratory complex II (34). This results in only 6 H⁺/O for the entire redox span, and the ATP/O₂ ratio changes to:

$$\frac{6 \left(\frac{H^+}{O} \right)}{3.33 \left(\frac{H^+}{ATP} \right)} = 1.8 \left(\frac{ATP}{O} \right) \text{ or } 3.6 \left(\frac{ATP}{O_2} \right) \quad (7)$$

To maintain the same rate of ATP phosphorylation (*i.e.*, the same power output), the estimated respiration rate would need to be approximately 7.5 nmol O₂·s⁻¹·mg⁻¹. This is congruent with experimentally determined values using isolated mitochondria from mouse hearts (Fig. 7) (34). Together these examples illustrate a critical bioenergetic concept: two different catabolic substrates exhibit different rates of respiration but support the same power output. Even though it may be tempting to assign "dysfunction" to any condition that results in slower respiration rates, these observations may simply represent a common metabolic strategy that ensures consistent power output (108).

which are predicted to have different ATP/O stoichiometries in OxPhos (10). In this case, SUITs could be designed to isolate oxidation patterns of specific substrates with known stoichiometries in mitochondria isolated from the intact cells, and the ATP/O stoichiometry could be measured directly under the same substrate conditions (34, 41, 76, 77). Parallel experiments could then be performed to measure oxidation patterns in the intact cells using nutrient-restricted media or inhibitors designed to reduce ambiguity in the substrates being oxidized (31).

To the best of our knowledge, no similar approach is available for isolating substrate level phosphorylation stoichiometries in glycolysis, which would likely necessitate the use of isotopomer-based metabolic flux analysis (78, 79). This is important because: 1) not all hexose equivalents will enter the ADP phosphorylating steps of lower glycolysis in intact cells (80, 81), and 2) lactate is likely oxidized in the mitochondria of many cell/tissues, which means that the ECAR may not be directly proportional to substrate level phosphorylation of ADP (82). For these reasons, ECAR measurements must be very carefully interpreted, which is discussed in greater detail in the next section.

Cellular energy partitioning between glycolysis and OxPhos

Despite the importance of the ATP hydrolysis reaction in the function of many cells, the number of biochemical pathways that result in net phosphorylation of ADP are surprisingly sparse. The two most well-understood energy transducing reaction schemes are OxPhos and substrate level phosphorylation in glycolysis. There is a well-known difference in molar efficiency between OxPhos and substrate-level

phosphorylation (*i.e.*, ~38 mol of ATP/mol glucose *via* OxPhos, 2 mol ATP/mol glucose in substrate level phosphorylation in glycolysis), which is often used as a basis for classifying the energetic economy of cells. EFA technologies have facilitated an explosion in studies that compare the relative contributions of these two pathways to cellular energetic states. The comparisons made often include multi-parametric "stress" tests that use SUITs to assess several flux parameters (64, 83, 84). Additionally, the ratio of OCR/ECAR is popularly used as an index of cellular bioenergetic function (85, 86). However, the interpretation of some of these parameters, particularly OCR/ECAR, can be quite problematic. The problems arise from two important issues: 1) OCR/ECAR is not quantitatively related to any metabolic fluxes, and 2) the physiological meaning of a shift in flux between these two pathways is complicated and can be artifactually created by the assay conditions.

Challenges underlying interpretation of ECAR measurements

Assumptions about the meaning of OCR/ECAR stem from the so-called "Warburg Effect" but may not be meaningful

In the 1920s, Otto Warburg famously observed that Ehrlich ascites tumors produced venous blood lactate under normoxic conditions (87). He later hypothesized that this observation could be caused by intrinsic damage to cellular respiration, citing the observation that other common causes of respiratory damage result in a similar metabolic phenotype (*i.e.*, ischemia/reperfusion and exposure to respiratory poisons) (88). At the time, it was a very reasonable hypothesis, because fermentation was being heavily studied in yeast and skeletal muscle, neither of which performs extensive fermentation under aerobic conditions. Warburg's hypothesis has persisted (inconclusively) for nearly 70 years and has continued to be broadly associated with cancer despite the lack of consensus model, mechanism, or consistent evidence of therapeutic potential (17, 80, 89–91). The acceptance of the Warburg hypothesis as an "effect," seems to have also inadvertently led to persistent generalizations regarding the relationship between central carbon metabolism and cellular energy partitioning even in noncancerous cells (17). More specifically, it has reinforced the notion that some cells may "prefer" either aerobic or anaerobic glycolysis in support of their energy metabolism, and that this "preference" indicates metabolic inefficiency if the "anaerobic" pathway prevails under normoxic conditions (13).

The biochemical "logic" underlying glycolytic energy transduction has been very nicely reviewed by Bar-Even *et al.* (92). In summary, a hexose (typically glucose) is phosphorylated, isomerized, and enzymatically cleaved, ultimately resulting in 2 M equivalents of triose phosphate. In "lower" glycolysis, these triose phosphate equivalents undergo two major electron rearrangements, which provide the net driving force for substrate-level phosphorylation of ADP. Mass action ratios of glycolytic intermediates and calculations of pathway energetics have been assessed under resting conditions in erythrocytes (which notably lack mitochondria) and other subcultured cell lines (79, 93, 94). In those studies, substrate-

level phosphorylation supported a steady-state ΔG_{ATP} between approximately $-50 \text{ kJ} \cdot \text{mol}^{-1}$ and $-59 \text{ kJ} \cdot \text{mol}^{-1}$, which are similar (albeit lower on average) compared with ΔG_{ATP} supported by OxPhos (34, 41, 66, 77). This supports the notion that, under certain conditions (e.g., hypoxia or ETS inhibition), homolactic fermentation may support the energy metabolism of some cell types. For example, this phenomenon has been well described in ρ^0 cells that have been depleted of mitochondria *via* chronic inhibition of mtDNA replication (95).

Sole reliance on fermentative energy metabolism would certainly impose significant kinetic constraints on the required flux through carbohydrate metabolism, but such an effect may only be meaningful when high-power output is required over sustained periods of time. Indeed, this likely explains why genetic diseases that affect mitochondria disproportionately impact tissue types that undergo sustained increases in power output as part of their physiological function (e.g., striated muscle and neurons) (96). Thus, the limitations on cellular function imposed by energy homeostasis depend in large part on the physiological activities of the cell type, as well as the specific metabolic constraints determined by the cell's microenvironment (e.g., nutrient availability and/or regulation of nutrient uptake) (60, 97–99). The extent to which this limitation applies to cell/tissue types that do not depend on high-power output is less understood. Unfortunately, it is not uncommon for assumptions about the energetic requirements of cellular processes to be implied with very little (if any) supporting evidence. The energetic requirement of cell proliferation in cancer is one prominent example and is often assumed to be highly dependent on ATP demand (84, 100, 101). However, findings from several recent investigations indicate that cancer energetics are much more nuanced, and that other energy intermediates such as reduced pyridine nucleotides may be more limiting than ATP in essential macromolecular synthesis reactions that limit proliferation rate (102–104).

A more modern view of the balance in energy partitioning between OxPhos and substrate-level phosphorylation hinges on several important observations. First, both OxPhos and glycolysis transduce chemical energy while simultaneously providing molecular "backbones" and reducing equivalents for anabolic reactions (termed amphibolism) (103–107). This is important because either of these requirements may constrain flux depending on the context/conditions. For example, the "warburg effect" in cancer has been proposed to be a by-product of maintaining high flux through the pentose phosphate and 1-carbon metabolism pathways, which may be more important for mitosis than supporting a high rate of ATP turnover (80, 102). Second, cells likely balance the energetic and kinetic trade-offs between the two pathways to ensure consistent power output and may use subcellular localization of mitochondria or glycolytic "metabolons" to support specific processes such as maintenance of transmembrane ion gradients (108, 109). Finally, flux through glycolysis and OxPhos are integrated through electron "shuttling" pathways such as the malate-aspartate shuttle (MAS). Though the MAS is often depicted as a way to deliver "excess" electron equivalents to the ETS, this process is actually *energy-dependent* due to

electrogenic transport of neutral glutamic acid for anionic aspartate (54, 110, 111). Manipulating MAS has been shown to induce complex effects in both OxPhos and glycolysis in several cell types, further highlighting that electron shuttling reaction schemes serve as points of integration between the two pathways (112, 113).

Challenges of interpreting ECAR and ECAR/OCR ratio data and some suggested strategies for overcoming these limitations

Homolactic fermentation produces two net lactate anion equivalents from a hexose equivalent. Monocarboxylate transporters on the plasma membrane may then transport lactate out of the cell *via* an electroneutral lactate/ H^+ symport mechanism, which results in net acidification of the external microenvironment (51). Thus, in minimally buffered media, ECAR is *proportional* to the rate of lactate efflux resulting from homolactic fermentation (Fig. 1A). This measurement is typically included in EFA studies, but is very often misinterpreted as being a direct measure of "glycolytic flux" (85, 86). Importantly, the proportionality of ECAR with glycolytic flux can be confounded by other sources of extracellular acidification. Additionally, typical ECAR units (mpH/min) are not stoichiometrically related to any metabolic fluxes (*i.e.*, how many ATP/mpH/min are expected to result from glycolysis?) (114).

It is generally assumed that all lactate produced in glycolysis will be effluxed (Fig. 1B). However, a recent radioisotope labeling study in mice determined that circulating lactate turnover was the highest of all blood metabolites, resulting in labeling of a variety of TCA cycle intermediates in all tissues tested (82, 115). Additionally, mitochondria isolated from many cells and tissues have been shown to oxidize lactate at rates that are similar to (or greater than) pyruvate (111, 116–118). Lactate dehydrogenase reactions tend to favor lactate

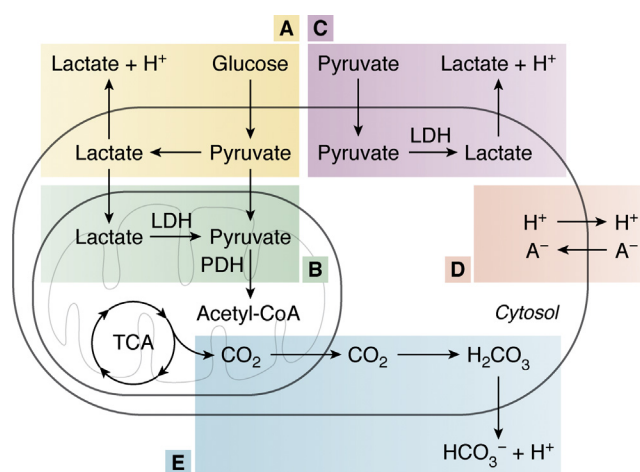


Figure 1. Potential sources of error in ECAR measurements. A, homolactic fermentation may result in symport of lactate/ H^+ , making ECAR proportional to substrate-level phosphorylation in glycolysis. B, oxidation of lactate in mitochondria may result in glycolytic flux that is not detectable through ECAR. C, exogenous pyruvate may be reduced to lactate without being directly coupled to glycolytic flux. D, ECAR may be unrelated to lactate/ H^+ symport (e.g., alternative anion/ H^+ exchange mechanisms). E, CO_2 derived from respiration may induce acidification due to accumulation through bicarbonate and carbonic acid equilibrium.

formation from pyruvate at equilibrium under physiological conditions ($\Delta G'^{\circ}_{LDH} = -23.4 \text{ kJ mol}^{-1}$) and LDH isoforms generally exhibit high activity (111). At relevant concentrations of reaction intermediates *in vivo*, the LDH reaction operates close to thermodynamic equilibrium, implying that the direction and extent of the reaction are sensitive to modulation by changing lactate, pyruvate, or NADH/NAD⁺ concentrations. This is particularly important for studies involving subcultured cells because it suggests that substrate conditions will likely affect the oxidation rate of lactate and thus skew the interpretation of EFA studies.

ECAR units can be made to be more stoichiometrically useful by conversion to a proton efflux rate (JH^+ ; a.k.a proton production rate- PPR) by empirically determining the buffer capacity of the media *via* titration of known amounts of strong acid (31, 114). This conversion is useful because JH^+ can be linked to the substrate-level ATP phosphorylation rate (APR_{Glyc}) *via* the following expression (10).

$$APR_{Glyc} = JH^+ \cdot \left(\frac{ATP}{H^+} \right) \cdot Q \quad (2)$$

where $\left(\frac{ATP}{H^+} \right)$ is generally considered to equal 1, and Q is a unitless proportionality constant that must account for the apparent coupling efficiency of JH^+ with substrate-level phosphorylation (9). In other words, Q represents the percentage of JH^+ that is linked to substrate level phosphorylation. This can be difficult to determine because it can be influenced by several factors (discussed above). However, a good place to start would be to begin the assay in the absence of exogenous lactate and measure the effluxed lactate at the end of the assay using an enzyme coupled reaction (*e.g.*, the hydrazine sink method) (119). Assuming that lactate is effluxed at a constant rate, the correction factor can be applied to JH^+ (9). This does, however, leave two remaining sources of error. First, in some cell types (*e.g.*, mammalian spermatozoa), the LDH reaction produces lactate from exogenous pyruvate independent of flux through lower glycolysis (Fig. 1C) (120). The extent to which this occurs in other cell types is not known. Thus, control experiments may need to be performed in the presence of an inhibitor that targets reactions of glycolysis (*e.g.*, α -chlorohydrin or 3-bromopyruvic acid; 2-deoxyglucose is not recommended due to confounding osmotic stress) or in the absence of exogenous pyruvate. Second, as mentioned above, lactate produced from homolactic fermentation may be oxidized in mitochondria, which would have the effect of "uncoupling" JH^+ from glycolytic flux. Whether or not a given cell type retains the ability to oxidize lactate in support of cellular respiration is testable using isolated mitochondria or permeabilized cell preparations (111, 116). Unfortunately, controlling for the magnitude of this effect in the intact cell to correct the ECAR measurement does not appear to be possible without altering the integration of OxPhos and glycolysis, because inhibiting lactate oxidation would likely alter glycolytic flux. Thus, further study would be helpful in this area.

Another caveat to ECAR measurements is that Lactate/ H^+ symport may not be the only source of H^+ exchange (Fig. 1D). In a closed system near physiological pH, 1 mol of glucose will produce 6 mol of CO_2 if completely oxidized. Each mole of CO_2 generated will produce a molar equivalent of H^+ , therefore the CO_2 produced by oxidation of substrates in the TCA cycle can contribute substantially to ECAR through reaction equilibrium with bicarbonate and carbonic acid (Fig. 1E) (9, 114). The CO_2 contribution to ECAR is far from trivial and reported increases in ECAR are very often accompanied by increases in OCR that may confound the ECAR interpretation. This effect may be accounted for by leveraging a mathematical correction factor, described by Mookerjee *et al.* (9), which estimates CO_2 contribution to ECAR. Assays can also be performed in the presence/absence of purified carbonic anhydrase, which speeds the rate of CO_2 to carbonic acid equilibration (121). This will ensure that the rate of CO_2 hydration is not limiting, which could result in overestimation of the CO_2 contribution to the acidification rate (9). In a final word of caution, some cell types (*e.g.*, adipocytes, polarized-epithelial cells, osteoclasts, etc) acidify their environments as part of their normal physiological function, and many cells exchange free acid for cations (*e.g.*, Na^+/H^+ antiport), which may necessitate the use of additional controls (121).

Nonphysiological assay conditions may create artifactual glycolytic flux states

A major challenge in bioenergetics studies is that assay conditions themselves often create artifactual conditions that demonstrate questionable physiological relevance. Glycolysis has been extensively studied in a variety of cells and tissues, and nearly every enzyme in the pathway has been identified in one study or another as either energetically or kinetically limiting to overall pathway flux (122). Importantly, under normal physiological conditions, glycolysis does not appear to be regulated by cellular energetic demand. A recent study by Tanner *et al.* systematically overexpressed each catalyzing step in the glycolytic pathway and combined metabolic flux analysis with metabolic control analysis in two mouse cell lines (45, 78). It was determined that glycolytic flux control under basal conditions was exerted at only four reactions: glucose transport, the hexokinase reaction, the phosphofructokinase reaction, and lactate transport (*i.e.*, *via* the monocarboxylate transporter). Interestingly the ADP phosphorylating reactions of "lower" glycolysis did not exert control under resting cellular conditions. This is similar to another metabolic control study performed in primary rat hepatocytes that determined the majority of glycolytic flux control was exerted by glycolytic enzymes (*i.e.*, ~66% by enzymes, ~47% by ATP hydrolysis rate) (123).

However, control of flux in energy transducing systems is highly sensitive to conditions, and inhibition of a step within a given pathway can result in redistribution of control to the inhibited step (45). Under extreme conditions, such as exposure to the F-Type ATP synthase inhibitor Oligomycin A,

glycolytic flux exhibits an increase in net driving force as well as flux through the pathway, indicating sensitivity to increased energetic demand similar to kinetic regulation by adenylates observed in the OxPhos system (79, 124). Increased ECAR is commonly observed response to OxPhos inhibitors (e.g., oligomycin, antimycin A, Rotenone, etc.), which is interpreted to represent compensatory flux through aerobic fermentation in support of the phosphate potential. However, this condition does not strictly represent any physiologically meaningful steady state and instead creates artifactual changes in flux that do not necessarily provide information about physiological energy partitioning. Additionally, in cells with active electron-shuttling systems (e.g., malate aspartate shuttle), inhibiting the ETS may affect glycolytic flux through the cytosolic NAD⁺/NADH redox potential, which may change glycolytic flux kinetics independent of energetic demand (110, 112, 125).

With these considerations in mind, how can assumptions about energy partitioning among OxPhos and glycolytic substrate-level phosphorylation be addressed moving forward? Abandoning the simplified view that cellular energy transducing pathways are independent is a recommended start. Instead, research should focus on metabolic system integration and adaptation under experimental conditions conducted in the most physiologically relevant micronutrient environment that can be reasonably achieved (10, 31, 126). Non-physiological metabolic states may inform the limitations of the assay, aid in standardizing among replicates, or aid in interpolating a model of rate limiting steps in the pathway (45). However, buzzwords such as "max glycolytic capacity" and OCR/ECAR index simply are not meaningful and should not be used to compare experimental treatments or group effects unless this practice can be clearly justified under the circumstances.

Mitochondrial function and "dysfunction"

The details regarding the measurement of specific parameters related to mitochondrial function and OxPhos are quite numerous/complex and have been the subject of several books and reviews (21, 43, 44, 46, 47, 127, 128). Typical assays for assessing OxPhos in isolated mitochondria were developed in the 1950s and have remained surprisingly similar to the manner in which they were first described (21, 23). Standardized respiratory states are commonly achieved *via* saturating substrate conditions in the absence of ADP (State 4; Low respiration rate- JO_2), in the presence of ADP (State 3; High JO_2), or *via* addition of ATP synthase inhibitors and protonophore uncouplers such as Oligomycin and Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; state u; High JO_2). Additional resolution is often achieved by using combinations of substrates and inhibitors to isolate activities of specific ETS complexes (44).

Intact cell measurements became fairly standardized with the advent of EFA and typically involve basal respiration as well as "max" or "uncoupled" respiration induced by a cell permeable protonophore (e.g., FCCP), which are often reported with calculated "ATP-linked" respiration (*i.e.*, basal –

oligomycin) and "respiratory reserve capacity" (*i.e.*, "max" – basal) (129, 130). Mitochondrial membrane potential measurements are also often included with both experimental preparation types (130). Methods typically involve use of a cationic indicator molecule that equilibrates with the steady-state voltage potential across the IMM (131–135). As was the case for OCR/ECAR, interpretation of mitochondrial functional parameters hinges on several important assumptions about the organization of the system as well experimental caveats that should be carefully considered. This is perhaps most relevant when attributing physiological effects to mitochondrial pathology or the more nebulous (and arguably less useful) term "dysfunction."

Assumptions about mitochondrial dysfunction stem from observed changes/differences in functional parameters that may not be meaningful

Bioenergetics studies often identify functional changes in mitochondrial metabolism (e.g., reduced respiration, membrane potential, etc) and then interpret these changes through a lens of mitochondrial pathology, leading to the conclusion that many diseases are mediated by some degree of mitochondrial dysfunction (96, 136–138). However, there are a few problems with this practice. First, mitochondrial dysfunction is not really a testable condition because it lacks a consensus definition. Second, and perhaps more importantly, changes in mitochondrial functional parameters often simply reflect the underlying energetic and kinetic flexibility of the system rather than a *bona fide* mitochondrial pathology (43).

A good way to illustrate this point is through brief discussion of the debate regarding the role of mitochondrial dysfunction in the etiology of skeletal muscle insulin resistance (IR) and glucose intolerance that arise as a consequence of obesity and type II diabetes mellitus (18). A popular hypothesis regarding the etiology of IR in skeletal muscle emerged in the early 2000s stemming from the work of Shulman and others (139). This hypothesis stipulated that ectopic accumulation of proinflammatory lipids (e.g., acyl-CoAs, diacylglycerol, and ceramides) occurs because of dysfunctional mitochondrial β -oxidation, which ultimately results in stress-dependent serine kinase activity that antagonizes insulin signaling (140). The proposed mitochondrial dysfunction was suggested to be the result of age- or environment-related decline based on observations of reduced rates of TCA cycle flux and ADP phosphorylation at rest in elderly IR patients *versus* young weight-matched controls (141). However, from simple bioenergetic principles, a decrease in mitochondrial function at rest should have minimal impact on the ability to oxidize fatty acids because the capacity of mitochondria to oxidize substrates is far in excess of what is needed at rest. Experimentally, several transgenic models in rodents have recapitulated loss-of-function in muscle mitochondrial β -oxidation, which results in ectopic lipid accumulation but not IR (142, 143). Additionally, reductions in state 3/state u respiration were not detected at the onset of IR in humans, and observations in rodent models have demonstrated that *in vitro* reductions in

respiratory kinetics and changes in mitochondrial ultrastructure/content follow (rather than precede) development of IR, indicating that these changes are simply adaptive in nature (144–146).

The connection between overnutrition and the development of IR is more likely mediated by substrate competition among fatty acid and glucose oxidation pathways, which is a manifestation of straightforward metabolic flux balance and allosteric feedback regulation of reaction kinetics by metabolic intermediates (147–150). In other words, these effects can be reasonably explained by the core principles of bioenergetics, rather than the presence of underlying mitochondrial pathology. More specifically, overnutrition results in an "oversupply" of catabolites, and mitochondrial energy transduction systems are energetically and kinetically limited by demand when supply is not limiting (18, 43, 151). Reduced rates of respiration can be normal (or even beneficial), rather than pathological, but to appreciate why requires an understanding of a few fundamental bioenergetics concepts (Box 1).

Overnutrition stimulus has been shown to be followed by adaptive reorganization of mitochondrial enzyme activity and/or content that manifests as a reduction in intrinsic mitochondrial functional parameters (*i.e.*, in isolated mitochondria that are removed from nutritional conditions of the cell) (152, 153). This effect has even been shown to occur in well-trained noninsulin-resistant individuals placed on a high-fat diet for just 5 days (154). But why would an *in vivo* shift in substrate oxidation patterns cause this effect? Though the ATP/O ratios for carbohydrate oxidation and β -oxidation are similar (~ 2.78 versus ~ 2.45 respectively), the molar ATP equivalents produced from complete β -oxidation are nearly fourfold higher (10). This also means that the number of electrons donated to the ETS per mole of substrate is also about fourfold higher. Additionally, β -oxidation donates ~ 2.4 times more electron equivalents to the CoQ/CoQH₂ redox couple, which is not directly linked to the thermodynamic "backpressure" of proton pumping through complex I. These consequences of metabolic organization result in the production of superoxide because the demand remains unchanged, but the input "pressure" from supply is increased (34, 155). Mitochondrial H₂O₂ production/emission (derived from superoxide) in turn is directly linked to the development of insulin resistance (156–159). Reactive oxygen species (ROS) have been demonstrated to induce rapid changes in various ETS enzyme activities through post-translation modifications, and Guaras *et al.* showed that complex I is degraded under conditions that result in a hyperreduced CoQ/CoQH₂ redox couple (160, 161). Both effects would be expected to manifest in conditional reductions in respiratory kinetics in isolated mitochondria or intact cell preparations, but these changes should be considered homeostatic rather than pathological.

There is no doubt that mitochondria play a critical role in the physiology of many cell types. Mitochondria-derived pathologies have been experimentally induced by genetic disruption of mitochondrial ultrastructure (162, 163) or toxic effects of small molecules on mitochondrial metabolism (164, 165). Though similar associations have been inserted into the etiology of

several diseases (*e.g.*, cardiovascular disease, neurodegeneration, and cancer), the interpretation of those data generally suffer from the same ill-defined causality as that promoted for the association of mitochondrial dysfunction with insulin resistance (136, 166, 167). To confidently conclude pathology, experiments must be designed to test the hypothesis that changes in mitochondrial functional parameters or cellular energy status *precede* the development of the disease phenotype.

Challenges involved with interpreting mitochondrial functional parameters and some suggested strategies for overcoming these limitations

Different types of preparations (*i.e.*, isolated mitochondria/permeabilized cells (IM/PCs) *versus* intact cells) have major differences in both experimental design and interpretation. IM/PCs offer more control over the specific biochemical conditions that regulate mitochondrial metabolism, but lack physiological context (47). IM/PC assay conditions are typically designed to saturate OxPhos kinetics (48), which can be useful for determining rate limiting steps when combined with inhibitor titrations but may also be susceptible to misinterpretations because the conditions are not reasonably recapitulated in live cells. Intact cell conditions are more physiologically relevant but limit unambiguous interpretation due to the complex and integrated nature of whole cell metabolic systems. Thus, interpreting data from either preparation type alone involves trade-offs between physiological relevance and experimental tractability (Fig. 2).

Nonphysiological metabolic states and artifacts

As mentioned earlier (Box 1), mitochondrial energy transduction can be modeled as an energetic and kinetic balance among four network control nodes (or modules) (34, 47). These include: 1) substrate oxidation by matrix dehydrogenases (energy supply), 2) coupling of the ETS redox reactions ETS with proton "pumping," 3) the electrochemical potential ($\Delta\Psi_m$; energy intermediate), and 4) $\Delta\Psi_m$ dissipating processes (energy demand). Importantly, these nodes are kinetically and energetically linked, and a change in a parameter that reports on only one node cannot be interpreted unambiguously without having additional information about

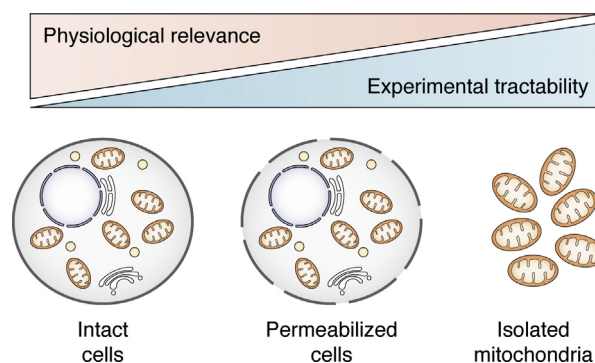


Figure 2. Experimental versus physiological tradeoffs made when using intact cell, permeabilized cell, or isolated mitochondria preparations to study bioenergetics.

the other nodes. Thus, the ideal approach to defining mitochondrial function under a given set of conditions requires a multidimensional analysis that can account for the net behavior of the entire system. In fact, this concept is not new. In their widely read article on assessing mitochondrial function in cells, Nicholls and Brand highlighted the use of the respiratory control ratio (RCR) as a practical single parameter indicator of mitochondrial function, but also cautioned that a more detailed modular analysis should be performed to avoid ambiguity in the interpretation of RCRs (47).

One of the most prevalent limitations in the interpretation of bioenergetics studies is that mitochondrial functional parameters are measured under extreme assay conditions that (by design) elicit large effects. For example, maximal state 3 or state 4 respiration is often used in conjunction with SUIIT strategies to identify rate or energetically limiting steps in OxPhos. These measurements are then interpreted as a diagnosis of respiratory complex deficiency or more broad mitochondrial limitation and/or dysfunction. However, this logic is flawed both conceptually and experimentally. Conceptually, a limitation measured at capacity is only relevant if the physiological system reaches that capacity, which is rarely (if ever) demonstrated. Additionally, measurements made under extreme assay conditions do not inform the potential limitations of a system functioning below maximal capacity, which more closely represents the physiological state of cells. From an experimental perspective, the conditions in which typical SUIIT strategies are performed include: 1) saturated substrate transport (potentially superseding carrier mediated transport), 2) collapsed membrane potential induced by saturating ADP or uncoupling agents, and 3) adenylate mass action ratios that are opposite to those that occur in live cells, which likely understates the importance of thermodynamic and allosteric control mechanisms (53, 69, 168). These SUIIT strategies have been time tested and are useful but were explicitly developed to interrogate the mechanisms that underlie OxPhos, not to investigate the complex roles that OxPhos and other energy-linked processes play in cellular physiology. To accomplish the latter, new iterative testable models derived from multiplexed measurements in both intact cells and IM/PCs must be developed.

Intact cells

The most common EFA assay applied to intact cells is sometimes referred to as a "mito stress test" and is comprised of oxygen consumption rate measurement combined with a brief SUIIT protocol from which several parameters are often calculated including: basal, ATP linked, non-ATP linked (leak), uncoupled (reserve), and nonmitochondrial respiration (47). This design evolved from several decades of work in isolated mitochondria aimed at determining the net coupling stoichiometry between respiration in the ETS and ADP phosphorylation (8, 22, 23, 47). There are, however, several important considerations that can influence the interpretation of this assay and its individual parameters (Fig. 3). First, different net substrate oxidation patterns can exhibit different rates of respiration in support of the same power output (Box 1) and

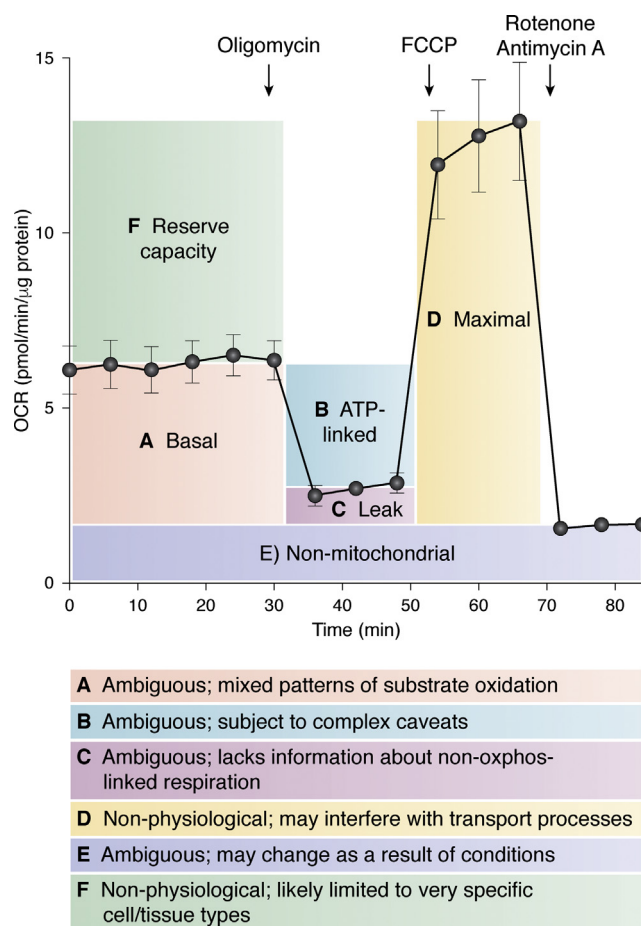


Figure 3. Ambiguities and other considerations involved in interpretation of the popular "mito stress test" respirometry assay and its related parameters.

are difficult to control in intact cells. Second, ATP-linked respiration is determined from additions of Oligomycin, but there are several complex caveats that impact the quality of this assumption (169). Third, the difference between uncoupled and basal respiration rates is often interpreted to be a measure of respiratory "reserve capacity" (83, 170). Though this may seem a reasonable interpretation, empirical correlation between uncoupled respiration rates and intact tissue respiratory reserve has not been demonstrated outside of very specific circumstances (*e.g.*, in resting *versus* exercising skeletal muscle) (171, 172). The concept likely holds in skeletal muscle because myofibers undergo large changes in energy demand over short timescales during exercise. However, the extent to which this concept applies to other cell types or cells growing in subculture is not known. Finally, chemical uncoupling agents disrupt membrane transport processes that depend on voltage potential, which may result in a biased interpretation of substrate utilization due to confounding effects of these compounds alone (48). This assay design is indeed a good "starting point" and does provide some information regarding respiratory control by OxPhos. However, some of the measured and subsequently calculated parameters are limited by ambiguities or lack a clear physiological meaning (Fig. 3).

Force-flow analysis

Many of the limitations discussed above can be explored further using IM/PC preparations. For example: 1) Kinetic parameters involving specific substrate oxidation pathways can be more precisely assessed by isolating specific ETS-linked reactions (46), 2) OxPhos coupling can be targeted with other inhibitors such as carboxyatractyloside or bongkreikic acid (ATP/ADP carrier inhibitors) to provide additional resolution on the degree of coupling with OxPhos (43, 58), and 3) removing the plasma membrane barrier relieves some of the transport related ambiguity that occurs in intact cells. Despite these considerations, the lack of physiologically relevant adenylate ratios presents a major limitation to interpretation of these measurements due to the absence of normal energetic and allosteric regulatory mechanisms exerted by physiological ΔG_{ATP} . Additionally, $\Delta \Psi_m$ measurements can only be made under extreme states involving depolarization or hyperpolarization (*i.e.*, state 4 or state 3/u respectively), where this measurement has limited physiological relevance.

Fortunately, a collection of pioneering studies over the last few decades has led to the development of an elegant *in vitro* IM/PC-based system that utilizes purified creatine kinase enzyme to "clamp" the free ATP/ADP concentration ratio (and thus, ΔG_{ATP}) at fixed values by taking advantage of the known equilibrium constant of the reaction (K'_{CK}) (Fig. 4A) (41, 77, 173).

$$\Delta G_{\text{ATP}} = \Delta G'^{\circ}_{\text{ATP}} + RT \ln \left(\frac{[\text{Creatine}][\text{Phosphate}]}{[\text{Phosphocreatine}]K'_{\text{CK}}} \right) \quad (3)$$

This can be used to assess respiratory kinetics over a physiologically relevant range of phosphate potential values and allows unprecedented examination of the energetic-kinetic relationships that support OxPhos (34, 41). Another advantage of this system is that the phosphate potential can be increased (*i.e.*, more negative ΔG_{ATP} ; analogous to reduced cellular energetic demand) through the creatine kinase reaction equilibrium by titration of known amounts of phosphocreatine (Fig. 4, B and C) (34, 41, 77). This more closely models the *in vivo* control over respiration rate that is exerted over a physiological demand range (34, 41, 77, 174), resulting in concomitant reduction in steady-state respiratory flux described by the following relationship:

$$J_{\text{O}_2} = \sigma \Delta G_{\text{ATP}} \quad (4)$$

where σ is a phenomenological coefficient denoted "conductance" that is equal to the rate of change in flux (J_{O_2}) with respect to unit change in ΔG_{ATP} (34, 168). Conductance is the inverse of resistance, and as such, an observed change/difference in conductance among experimental groups implies that one or more source(s) of resistance in the mitochondrial energy transduction system (*i.e.*, the four control nodes) are responsible (Fig. 4D). It is important to note that the empirical relationship becomes nonlinear at extreme values of ΔG_{ATP} and the interpretation of this assay becomes complex. Thus, the conductance is best interpreted in the "linear" range of the curve and must be optimized for different tissue/cell types to

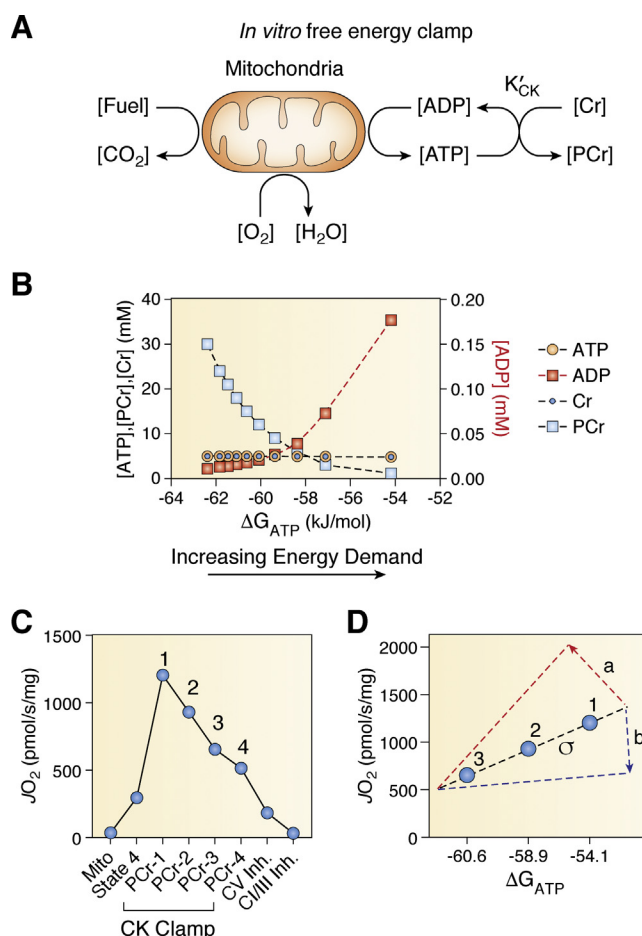


Figure 4. Creatine kinase (free energy) clamp system for assessing mitochondrial function in IM/PCs under a physiological range of ATP hydrolysis free energy (ΔG_{ATP}). A, diagram summarizing clamp concept. B, changes in ADP, ATP, Creatine, and Phosphocreatine concentrations versus resulting values of ΔG_{ATP} . More negative ΔG_{ATP} represents a lower energetic demand state. C, hypothetical example of a free energy clamp experiment using isolated mitochondria. PCr- Phosphocreatine; CV- Respiratory complex V (ATP synthase); Cl/III- Complexes I and III respectively. D, representative conductance (σ) over the linear portion of the curve in subpanel C. An increase in slope (conductance) indicates greater control of flux by OxPhos (a), a decrease indicates lower control by OxPhos (b).

ensure that this is the case. Additionally, the free energy values are calculated from the assay conditions, which is not trivial. To aid researchers in designing and interpreting experiments, an open-source online calculator is available (Bioenergetic Calculators (dmpio.github.io)) (34).

The goal of force-flow analysis is to simultaneously account for the driving and driven energetic forces that influence the kinetics of mitochondrial respiration under conditions that more closely recapitulate the living cell. A change/difference in conductance implies alteration in the apparent coupling efficiency of OxPhos, which is also related to the ATP/O stoichiometry. The stoichiometries of proton translocation by the ETS and ATP synthase are generally considered to be fixed (though may vary by taxonomy) (22). Thus, the two most likely sources of variation in conductance in IM/PC preparations are derived from substrate oxidation stoichiometries (Box 1) or non-OxPhos coupled respiration. Though a change/difference in conductance does

not indicate the specific source of altered "efficiency," this can be further investigated by including additional measurements (34).

The $\Delta\Psi_m$ (energy intermediate) node is proportional to the mitochondrial proton motive force. Importantly, $\Delta\Psi_m$ has complex properties that should be considered when measuring this variable (highlighted in Box 2). $\Delta\Psi_m$ is typically measured using cationic indicators (fluorescent or potentiometric), which have many uses/interpretations depending on the preparation type and context (47, 134, 175). Quantitatively monitoring $\Delta\Psi_m$ can be a complicated business (133, 176). However, it can be made slightly less complicated (albeit at a cost to accuracy) by making a few simplifying assumptions and fluorescence can be calibrated using described methods (34, 177). Note that cationic indicators often affect respiration rates and should be included uniformly across replicates/conditions

Box 2. Mitochondrial membrane potential and proton motive force

The principal function of the mitochondrial pmf is to facilitate the energetically favorable transfer of molecules across the selectively permeable inner membrane. As discussed in Box 1, pmf is a form of energy intermediate derived from the ΔG of the redox spans from the various electron carriers to oxygen. The highly directional suite of transport processes that depend on pmf are linked to critical mitochondrial and greater cellular functions including protein import (219), ADP phosphorylation (220), control of cytosolic and matrix redox states (110, 112, 221), and metabolite interconversion (54). The pmf is derived from the proton pumping activities of NADH:ubiquinone oxidoreductase (Complex I), CoQ:cytochrome c oxidoreductase (Complex III), and cytochrome c oxidase (Complex IV) (128, 222). The pmf can be stated compactly as:

$$\text{pmf} = F\Delta\Psi_m + 2.3RT\Delta\text{pH} \quad (1)$$

where $\Delta\Psi_m = \Psi_{\text{matrix}} - \Psi_{\text{cytosol}}$ and $\Delta\text{pH} = \text{pH}_{\text{matrix}} - \text{pH}_{\text{cytosol}}$. The constant (2.3) converts the natural logarithm that is classically used in this expression to base 10, which is the scale on which pH is represented. F is Faraday's constant, and R and T are the ideal gas constant and absolute temperature, respectively. F is Faraday's constant, and R and T are the ideal gas constant and absolute temperature, respectively. The pmf is an expression of an electrochemical potential and is comprised of both the voltage potential difference ($\Delta\Psi_m$) and the proton concentration gradient (ΔpH). Importantly, the $\Delta\Psi_m$ component is a manifestation of the balance of all charged species that equilibrate in response to changes in ΔpH (e.g., K^+ , Na^+ , Ca^{2+} , Mg^{2+} , HPO_4^{2-} , organic acids, etc) (202). In animal cells, $\Delta\Psi_m$ generally makes a larger energetic contribution to the steady-state pmf value compared with ΔpH (approx. -150 mV versus -50 mV respectively) (132, 176). Changes in each term can relate to distinct physical consequences. For example, transport of cations into the matrix (or anions out) is energetically dependent on the magnitude of the $\Delta\Psi_m$ term (54, 110, 223), while processes that directly transfer protons into the matrix such as ADP phosphorylation by ATP synthase are kinetically and energetically dependent on the magnitude of ΔpH (224). Additionally, many carbon substrates of the TCA cycle and other pathways are weak acids (i.e., ionize at different pH values), and their distribution is dependent on magnitude of both $\Delta\Psi_m$ and ΔpH (54). Notably, $\Delta\Psi_m$ is framed in reference to only one ionic species (H^+), because it is actively produced by proton pumping, and all other permeant ions are considered to equilibrate with the diffusive and electrical forces through passive or secondary-active transport processes (54, 128, 202). For this reason, it is important that the net ion current can be reasonably assumed to be zero during experimental determination of $\Delta\Psi_m$ (i.e., no change in net ion concentrations across the membrane over time). During steady-state respiration, this is taken to be the case. However, if such a steady state cannot be achieved, then permeabilities of other ions may contribute significantly to the $\Delta\Psi_m$ requiring use of the Goldman-Hodgkin-Katz relation (225). Though changes in mitochondrial membrane potential are often reported in conjunction with pathological states, such results must be interpreted cautiously. The membrane potential is a function of both energy "supply" and "demand" and small changes may reflect large kinetic responses. For these reasons, measurements of membrane potential alone are insufficient to define mitochondrial function.

at the lowest concentrations that achieve the desired effects (with additional attention paid the importance of dye quenching) (177, 178). Additionally, cationic indicators should not be used without accompanying respiration experiments to account for potential inhibitory or uncoupling effects that are quite common among these molecules. When appropriately measured, $\Delta\Psi_m$ is useful because it provides information about the balance between energy supply and demand. When the third node (demand) is fixed under free energy clamped conditions (Fig. 5A), $\Delta\Psi_m$ becomes proportional to the input force and the relationships among energetics and kinetics (i.e., force flow) can be compared among experimental groups/conditions. A leftward shift (more negative/polarized state) in $\Delta\Psi_m$ at the same conductance implies increased coupling efficiency of OxPhos (Fig. 5B). A rightward shift in $\Delta\Psi_m$ (less negative/depolarized state) at the same conductance implies reduced coupling efficiency of OxPhos (i.e., physiological or nonphysiological uncoupling) or may result from rate limitations in the ETS or substrate oxidation nodes (Fig. 5B).

Changes in the input forces (i.e., the substrate oxidation node) can be difficult to quantify. However, under controlled conditions, the fractional reduction of the pyridine nucleotide pool (NAD(P)H) can provide some additional qualitative information regarding the dehydrogenase reactions that support the ETS and can be measured using the autofluorescence of these molecules that is excited by long-wavelength UV light (34). This can be readily measured using a fluorometer or microtiter plate reader, and experimental conditions can be designed to determine the maximal/minimal reduced states via SUT assays (Fig. 5C). Notably, the NAD(P)H redox state may not change significantly over the clamped ΔG_{ATP} range in isolated mitochondria preparations due to the necessity of including saturating substrates, which by design causes the substrate oxidation node to be fully reduced. This effect may vary with the biological system/conditions under study, and a hyper-reduced state (Fig. 5D) may indicate specific respiratory complex deficiencies as the primary mode of resistance becomes the ETS. This effect has been demonstrated in heart mitochondria of mice with compromised DNA repair (162). A more oxidized state (Fig. 5D) may indicate a limitation imposed by specific dehydrogenase expression or activities, or uncoupling mechanisms that can each be investigated with other methods (34).

Finally, ADP phosphorylation rate is often interpolated from respiration rates due the presumed tightly coupled nature or these processes, but the degree of OxPhos coupling may change under different conditions. Thus, an additional method that empirically measures the ADP phosphorylation rate can be useful for further investigating observed changes/differences in conductance. This can be accomplished in IM/PC preparations using hexokinase clamped ATP hydrolysis with glucose-6-phosphate dehydrogenase linked NADPH autofluorescence in the presence of respiring mitochondria to empirically determine OxPhos rates in real time (76). When measured in parallel with JO_2 under matched conditions, this method can be used to empirically determine ATP/O stoichiometry, and the causes of apparent discrepancy between

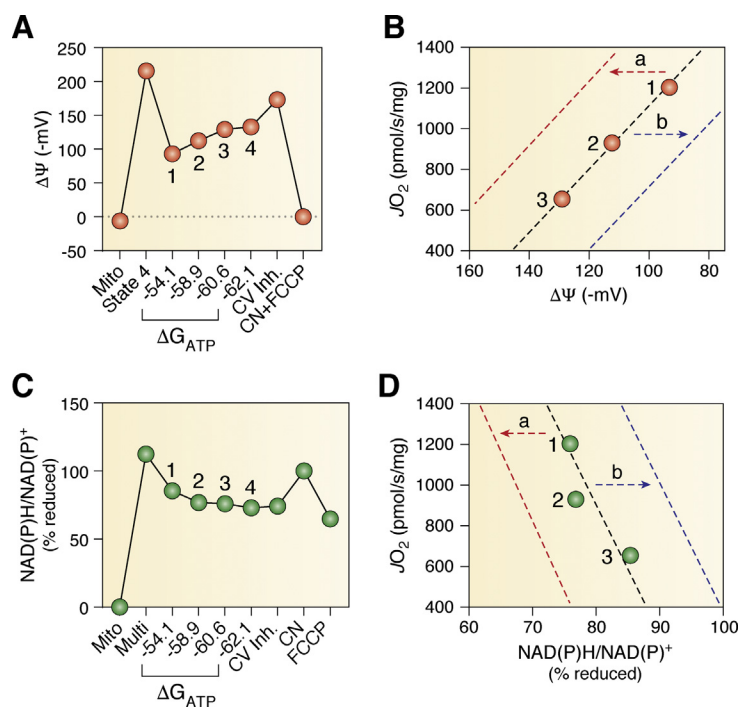


Figure 5. Force-flow analysis for additional investigation of mitochondrial energy transduction. A, hypothetical experiment demonstrating inner mitochondrial membrane potential ($\Delta\Psi_m$) via quench mode TMRM measured under CK clamp conditions using isolated mitochondria. CN- Potassium Cyanide. Numbers indicate chronological order of PCR addition. B, a decrease in $\Delta\Psi_m$ with unchanged conductance indicates reduced OxPhos coupling efficiency or limitations in the energy supply node (a). An increase in $\Delta\Psi_m$ with unchanged conductance indicates increased OxPhos coupling efficiency or rate limitation by respiratory complex V (b). C, hypothetical experiment for qualitative assessment of the redox state of the substrate oxidation node under CK clamp conditions using isolated mitochondria. D, a decrease in % reduced with unchanged conductance indicates reduced OxPhos coupling efficiency or limitations in the energy supply node (a). An increase in % reduced with unchanged conductance indicates increased OxPhos coupling efficiency or rate limitation by proton pumping complexes in the ETS (b).

measured and theoretical ATP/O ratios for the substrate conditions can be further explored using other methods (22, 34). Importantly, the hexokinase enzymatic clamp system does not support physiological adenylate ratios (*i.e.*, ADP is much higher than ATP), but this is the best approach currently available. When this assay is performed in parallel with force-flow analysis, and ADP concentrations are carefully matched among the two experiments, all the parameters necessary to calculate power output to the phosphate potential are determined (as described in Box 1).

Respiration versus OxPhos and the efficiency of oxidative phosphorylation

As support for the chemiosmotic theory grew in the mid-20th century, many early studies were performed with the aim of determining mechanistic ATP/O ratios and were designed to minimize implications of ETS flux that was not linked to the phosphorylation of ADP (*i.e.*, uncoupled respiration) (22). Many of these early studies formed the basis of contemporary practices for assaying mitochondrial function, which have ultimately retained some of those design elements: *e.g.*, use of oligomycin, Ca²⁺ chelators, albumin, and buffer compositions optimized for high RCRs (23, 179). Due to the popularity of the "mito stress test" assay (Fig. 3), the apparent coupling efficiency of OxPhos has now been determined for a wide variety of cultured cell types under various substrate supported conditions. A brief survey of representative data for

several distinct cell types indicates that astonishing variation (40–90%) in coupling efficiency is quite common (180–184). This demonstrates that, in some cell types/conditions, most of the respiration may not be committed to supporting OxPhos. Though such conclusions could be met with skepticism due to the fact that media conditions may facilitate artifactual adaptations in central carbon metabolism (185, 186), observations of age-dependent reduction in ADP phosphorylation rate in the absence of change in JO_2 in mouse skeletal muscle *in vivo* provide further support for the complexity and physiologic relevance of OxPhos independent respiration (187–189).

In addition to the observations discussed above, several other recent studies have documented that mitochondrial flux may be more important than OxPhos under certain conditions implying that it is time to rethink the universality of the "powerhouse of the cell" anachronism (103, 104, 106, 190, 191). The putative role of physiological uncoupling of respiration from OxPhos is not well understood. It has been shown that less than 5% of basal proton leak is mediated by passive diffusion of protons through the lipid bilayer, suggesting that most of the uncoupling is mediated by inner membrane proteins and is probably tightly regulated (192). The expression of uncoupling proteins (UCPs) was first noted in brown adipose tissue and ascribed to a nonshivering thermogenic function (193, 194). The discovery of widespread UCP isoform expression in other cells/tissues and putative proton translocating activities of mitochondrial anion transporters of the SLC25 family suggests that processes other than

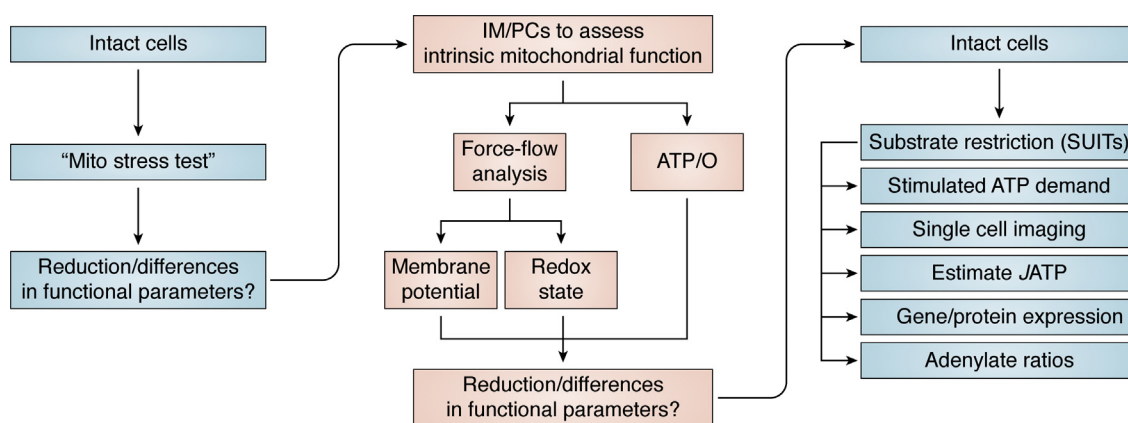


Figure 6. Representative workflow for reducing some of the ambiguity inherent in the "mito stress test" assay design by incorporating isolated mitochondria/permeabilized cells (IM/PC) data followed by further iterative testing in intact cells.

thermogenesis are also implicated in energy uncoupling (195–199). Another role of physiological uncoupling may be to facilitate increased TCA cycle flux in support of metabolite synthesis and exchange. For example, TCA cycle intermediates provide carbon backbones for nonessential amino acids and other anabolic reactions through cataplerosis (200, 201). Additionally, several well-characterized transport processes are electrogenic and are energetically dependent on $\Delta\Psi_m$ (54, 202, 203), and others depend on compensated proton symport (or as electroneutral protonated acids) and are thus dependent on the pH gradient component of $\Delta\Psi_m$ (54). A final mechanism of uncoupling worth mentioning is the prevention of oxidant stress *via* nicotinamide nucleotide transhydrogenase—an IMM protein that activates proton conductance to catalyze the resynthesis of NADPH needed to support flux through redox buffering circuits (32, 204).

Importantly, all of these processes are capable of contributing substantially to basal energy expenditure *in vivo*, but have been considered to make only a small contribution to respiration rate in isolated organelle experiments that have been classically focused on determining mechanistic ATP/O ratios (22). This is important because this view shades the way in which the physiological role of mitochondria within cells is framed, which is then echoed in the design of

bioenergetics experiments that typically focus on OxPhos. These factors must be considered as the field evolves to understand how mitochondria fit into the greater functionality of the cell, and it is clear that current assays must be expanded to include strategies that look beyond respiratory control exerted by OxPhos. Moving this idea forward will require approaches that can practically account for the coupling stoichiometry of OxPhos, and its relationship with non-OxPhos dependent mitochondrial respiration as it varies among cell types/conditions. The methods described in the previous section can be used to identify circumstances in which non-OxPhos coupled flux is occurring, but other approaches must be undertaken to identify the underlying causes.

Integration of intrinsic functional parameters with intact cell measurements

To reiterate, the IM/PC-based assays described in previous sections are intended to provide additional resolution on observations made in intact cells, with the ultimate goal of returning to the intact cells to test the new hypotheses (Fig. 6). To a limited degree, substrate oxidation patterns can be isolated in intact cells using strategic substrate deprivation/refeeding and appropriate inhibitors (31, 186). Under conditions in which specific pathways can be reasonably isolated, JATP can be estimated using theoretical ATP/O ratios, which can then be compared with JATP calculated from empirically derived ATP/O ratios. Gene/protein expression strategies can be undertaken to investigate underlying causes/consequences of specific metabolic patterns identified in the analysis and cell/tissue fractionation may aid in identifying intrinsic mitochondrial expression patterns (162, 205). Controlled increases in physiological demand state can be induced by using ionophores that selectively permeabilize plasma membranes to ions in the media (206–209). In many cells, ATP-dependent ion exchange in response to depolarization results in increased energy expenditure, which can be selectively investigated using SUITs (31, 108, 114). However, these experiments must be very carefully controlled (and optimized) using titration of ion transporter inhibitors to ensure

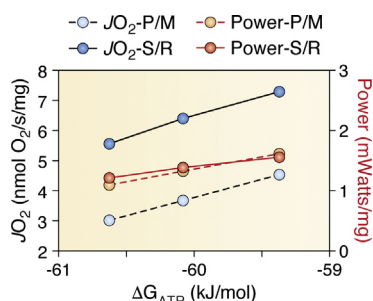


Figure 7. Coupling stoichiometry affects respiration rate required to maintain fixed power output. Creatine kinase (free energy) clamped respiration and power output to the phosphate potential calculated as described in Box 1 using previously published data obtained in isolated cardiac mitochondria from mouse heart (34). P/M, Pyruvate/Malate; S/R, Succinate/Rotenone.

specificity of the effect, and ionophore concentrations must be minimized because they are generally not membrane specific and may result in depolarization of the IMM in addition to the plasma membrane (176). Finally, quantitative imaging of potentiometric dyes can be extremely useful for investigating the importance of mitochondrial structure, content, and spatial distribution within cells and can also be performed under conditions that parallel other functional parameter measurements (31). This approach can also be used to assess whether mitochondrial structure/content is retained under permeabilizing conditions.

Conclusion

Bioenergetics assay design must account for minute changes in assay conditions as well as emphasize normalization and standardization due to the many factors that may independently introduce variability. Additionally, it may be time to rethink some of the historical assumptions that have shaded the view of bioenergetic organization, particularly with regard to the universality of the "textbook" model of glycolysis and oxidative phosphorylation and the ill-defined notion of mitochondrial "dysfunction." Bioenergetics research has been challenged historically by the reliance on extreme assay conditions that fail to recapitulate the balance of free energies (ΔG_{redox} , $\Delta G_{\Delta\psi}$, ΔG_{ATP} , etc.) and the demand-driven nature under which the system operates *in vivo* to draw conclusions that are at best tenuously related to physiology. Improvements can be made on these approaches by leveraging recent advances in multiplexed assay design that unify information obtained in both intact cells as well as isolated mitochondria/permeabilized cells that ultimately culminate in iterative development of testable models in intact cells.

Finally, looking to the future, much of bioenergetics research has been focused on "metabolically active tissues and cell types" (*i.e.*, striated muscle, liver, pancreas, cancerous cells, etc). Perhaps the most promising progressive aim of bioenergetics research is simply the expansion of the collective understanding of the organization/regulation of cellular metabolism in cell types and biological contexts that have not yet been adequately studied. To achieve this, some of the assumptions regarding the canonical roles of metabolic systems must be cast off and assay design/interpretation must be afforded a measure of flexibility to accommodate unexpected observations. It is our hope that the concepts/approaches discussed in this review provide a modest starting point for such endeavors.

Author contributions—C. A. S., K. H. F.-W., and P. D. N., conceptualization; C. A. S., writing—original draft; C. A. S., K. H. F.-W., and P. D. N., writing—review and editing.

Funding and additional information—This work was supported by NIH NIDDK Award number F32DK127632 (C. A. S.), DoD-W81XWH-19-1-0213 (K. H. F.-W.), and NIH R01 AR071263, DK110656 and AG069679 (P. D. N.). The content is solely the

responsibility of the authors and does not necessarily represent the official views of the DoD or NIH.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ECAR, extracellular acidification rate; EFA, extracellular flux analysis; ETS, electron transfer system; FAO, fatty acid oxidation; MAS, malate-aspartate shuttle; MFA, metabolic flux analysis; MPC, metal-porphyrin complex; OCR, oxygen consumption rate; OxPhos, oxidative phosphorylation; pmf, proton motive force; RCR, respiratory control ratio; ROS, reactive oxygen species; SUIT, substrate-inhibitor titration.

References

- Jucker, M. (2010) The benefits and limitations of animal models for translational research in neurodegenerative diseases. *Nat. Med.* **16**, 1210–1214
- Ruggeri, B. A., Camp, F., and Miknyoczki, S. (2014) Animal models of disease: Pre-clinical animal models of cancer and their applications and utility in drug discovery. *Biochem. Pharmacol.* **87**, 150–161
- King, A. J. F. (2012) The use of animal models in diabetes research. *Br. J. Pharmacol.* **166**, 877–894
- Cheng, Z., Tseng, Y., and White, M. F. (2010) Insulin signaling meets mitochondria in metabolism. *Trends Endocrinol. Metab.* **21**, 589–598
- Nagarajan, A., Malvi, P., and Wajapeyee, N. (2016) Oncogene-directed alterations in cancer cell metabolism. *Trends Cancer* **2**, 365–377
- Gao, C., Wang, Q., Chung, S. K., and Shen, J. (2017) Crosstalk of metabolic factors and neurogenic signaling in adult neurogenesis: Implication of metabolic regulation for mental and neurological diseases. *Neurochem. Int.* **106**, 24–36
- Reily, C., Mitchell, T., Chacko, B. K., Benavides, G. A., Murphy, M. P., and Darley-Usmar, V. M. (2013) Mitochondrially targeted compounds and their impact on cellular bioenergetics. *Redox Biol.* **1**, 86–93
- Gerencser, A. A., Neilson, A., Choi, S. W., Edman, U., Yadava, N., Oh, R. J., Ferrick, D. A., Nicholls, D. G., and Brand, M. D. (2009) Quantitative microplate-based respirometry with correction for oxygen diffusion. *Anal. Chem.* **81**, 6868–6878
- Mookerjee, S. A., Goncalves, R. L. S., Gerencser, A. A., Nicholls, D. G., and Brand, M. D. (2015) The contributions of respiration and glycolysis to extracellular acid production. *Biochim. Biophys. Acta* **1847**, 171–181
- Mookerjee, S. A., Gerencser, A. A., Nicholls, D. G., and Brand, M. D. (2017) Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. *J. Biol. Chem.* **292**, 7189–7207
- Rana, P., Aleo, M. D., Gosink, M., and Will, Y. (2019) Evaluation of *in vitro* mitochondrial toxicity assays and physicochemical properties for prediction of organ toxicity using 228 pharmaceutical drugs. *Chem. Res. Toxicol.* **32**, 156–167
- Gohil, V. M., Sheth, S. A., Nilsson, R., Wojtovich, A. P., Lee, J. H., Perocchi, F., Chen, W., Clish, C. B., Ayata, C., Brookes, P. S., and Mootha, V. K. (2010) Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondrial respiration to glycolysis. *Nat. Biotechnol.* **28**, 249–255
- Wu, M., Neilson, A., Swift, A. L., Moran, R., Tamagnine, J., Parslow, D., Armistead, S., Lemire, K., Orrell, J., Teich, J., Chomicz, S., and Ferrick, D. A. (2007) Multiparameter metabolic analysis reveals a close link between attenuated mitochondrial bioenergetic function and enhanced glycolysis dependency in human tumor cells. *Am. J. Physiol. Cell Physiol.* **292**, 125–136
- Martin, S. D., and McGee, S. L. (2019) A systematic flux analysis approach to identify metabolic vulnerabilities in human breast cancer cell lines. *Cancer Metab.* **7**, 12

15. Dier, U., Shin, D. H., Madhubhani, L. P., Hemachandra, P., Uusitalo, L. M., and Hempel, N. (2014) Bioenergetic analysis of ovarian cancer cell lines: Profiling of histological subtypes and identification of a mitochondria-defective cell line. *PLoS One* **9**, e98479
16. Robinson, G. L., Dinsdale, D., MacFarlane, M., and Cain, K. (2012) Switching from aerobic glycolysis to oxidative phosphorylation modulates the sensitivity of mantle cell lymphoma cells to TRAIL. *Oncogene* **31**, 4996–5006
17. DeBerardinis, R. J., and Chandel, N. S. (2020) We need to talk about the Warburg effect. *Nat. Metab.* **2**, 127–129
18. Muoio, D. M., and Neufer, P. D. (2012) Lipid-induced mitochondrial stress and insulin action in muscle. *Cell Metab.* **15**, 595–605
19. Wiechert, W. (2001) ¹³C metabolic flux analysis. *Metab. Eng.* **3**, 195–206
20. Antoniewicz, M. R. (2018) A guide to ¹³C metabolic flux analysis for the cancer biologist. *Exp. Mol. Med.* **50**, 1–13
21. Chance, B., and Williams, G. R. (1955) A simple and rapid assay of oxidative phosphorylation. *Nature* **175**, 1120–1121
22. Hinkle, P. C. (2005) P/O ratios of mitochondrial oxidative phosphorylation. *Biochim. Biophys. Acta* **1706**, 1–11
23. Chance, B., and Williams, G. R. (1956) The respiratory chain and oxidative phosphorylation. *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134
24. O'Donovan, C., Hynes, J., Yashunski, D., and Papkovsky, D. B. (2005) Phosphorescent oxygen-sensitive materials for biological applications. *J. Mater. Chem.* **15**, 2946–2951
25. Zhdanov, A. V., Ogurtsov, V. I., Taylor, C. T., and Papkovsky, D. B. (2010) Monitoring of cell oxygenation and responses to metabolic stimulation by intracellular oxygen sensing technique. *Integr. Biol. (Camb.)* **2**, 443–451
26. Haller, T., Ortner, M., and Gnaiger, E. (1994) A respirometer for investigating oxidative cell metabolism: Toward optimization of respiratory studies. *Anal. Biochem.* **218**, 338–342
27. Ripple, M. O., Kim, N., and Springett, R. (2013) Mammalian complex I pumps 4 protons per 2 electrons at high and physiological proton motive force in living cells. *J. Biol. Chem.* **288**, 5374–5380
28. Zhdanov, A. V., Favre, C., O'Flaherty, L., Adam, J., O'Connor, R., Pollard, P. J., and Papkovsky, D. B. (2011) Comparative bioenergetic assessment of transformed cells using a cell energy budget platform. *Integr. Biol. (Camb.)* **3**, 1135–1142
29. O'Riordan, T. C., Zhdanov, A. V., Ponomarev, G. V., and Papkovsky, D. B. (2007) Analysis of intracellular oxygen and metabolic responses of mammalian cells by time-resolved fluorometry. *Anal. Chem.* **79**, 9414–9419
30. Hynes, J., O'Riordan, T. C., Zhdanov, A. V., Uray, G., Will, Y., and Papkovsky, D. B. (2009) *In vitro* analysis of cell metabolism using a long-decay pH-sensitive lanthanide probe and extracellular acidification assay. *Anal. Biochem.* **390**, 21–28
31. Schmidt, C. A., McLaughlin, K. L., Boykov, I. N., Mojalagbe, R., Ranganathan, A., Buddo, K. A., Lin, C.-T., Fisher-Wellman, K. H., and Neufer, P. D. (2021) Aglycemic growth enhances carbohydrate metabolism and induces sensitivity to menadione in cultured tumor-derived cells. *Cancer Metab.* **9**, 1–21
32. Fisher-Wellman, K. H., Lin, C. T., Ryan, T. E., Reese, L. R., Gilliam, L. A. A., Cathey, B. L., Lark, D. S., Smith, C. D., Muoio, D. M., and Neufer, P. D. (2015) Pyruvate dehydrogenase complex and nicotinamide nucleotide transhydrogenase constitute an energy-consuming redox circuit. *Biochem. J.* **467**, 271–280
33. Blacker, T. S., and Duch, M. R. (2016) Investigating mitochondrial redox state using NADH and NADPH autofluorescence. *Free Radic. Biol. Med.* **100**, 53–65
34. Fisher-Wellman, K. H., Davidson, M. T., Narowski, T. M., Lin, C. T., Koves, T. R., and Muoio, D. M. (2018) Mitochondrial diagnostics: A multiplexed assay platform for comprehensive assessment of mitochondrial energy fluxes. *Cell Rep.* **24**, 3593–3606.e10
35. Takahashi, A., Zhang, Y., Centonze, V. E., and Herman, B. (2001) Measurement of mitochondrial pH *in situ*. *Biotechniques* **30**, 804–815
36. Roe, M. W., Lemasters, J. J., and Herman, B. (1990) Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium* **11**, 63–73
37. Shirmanova, M. V., Druzhkova, I. N., Lukina, M. M., Matlashov, M. E., Belousov, V. V., Snopova, L. B., Prodanetz, N. N., Dudenkova, V. V., Lukyanov, S. A., and Zagaynova, E. V. (2015) Intracellular pH imaging in cancer cells *in vitro* and tumors *in vivo* using the new genetically encoded sensor SypHer2. *Biochim. Biophys. Acta* **1850**, 1905–1911
38. Fan, Y., Makar, M., Wang, M. X., and Ai, H. (2017) Monitoring thio-redoxin redox with a genetically encoded red fluorescent biosensor. *Nat. Chem. Biol.* **13**, 1045–1052
39. Tao, R., Zhao, Y., Chu, H., Wang, A., Zhu, J., Chen, X., Zou, Y., Shi, M., Liu, R., Su, N., Du, J., Zhou, H.-M., Zhu, L., Qian, X., Liu, H., *et al.* (2017) Genetically encoded fluorescent sensors reveal dynamic regulation of NADPH metabolism. *Nat. Methods* **14**, 720–728
40. Bradshaw, P. C., and Pfeiffer, D. R. (2006) Release of Ca²⁺ and Mg²⁺ from yeast mitochondria is stimulated by increased ionic strength. *BMC Biochem.* **7**, 1–12
41. Glancy, B., Willis, W. T., Chess, D. J., and Balaban, R. S. (2013) Effect of calcium on the oxidative phosphorylation cascade in skeletal muscle mitochondria. *Biochemistry* **52**, 2793–2809
42. Golding, E. M., Teague, W. E., and Dobson, G. P. (1995) Adjustment of K⁺ to varying pH and pMg for the creatine kinase, adenylate kinase and ATP hydrolysis equilibria permitting quantitative bioenergetic assessment. *J. Exp. Biol.* **198**, 1775–1782
43. Nicholls, D., and Ferguson, S. (2013) *Bioenergetics*, 4th Ed, Academic Press, London, UK
44. Gnaiger, E. (2020) *Mitochondrial Pathways and Respiratory Control: An Introduction to OXPHOS Analysis*, 5th Ed., Bioenergetics Communications, Innsbruck, Austria
45. Kacser, H., Burns, J. A., and Fell, D. A. (1995) The control of flux: 21 years on. *Biochem. Soc. Trans.* **23**, 341–366
46. Perry, C. G. R., Kane, D. A., Lanza, I. R., and Neufer, P. D. (2013) Methods for assessing mitochondrial function in diabetes. *Diabetes* **62**, 1041–1053
47. Brand, M. D., and Nicholls, D. G. (2011) Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435**, 297–312
48. Wollenman, L. C., Vander Ploeg, M. R., Miller, M. L., Zhang, Y., and Bazil, J. N. (2017) The effect of respiration buffer composition on mitochondrial metabolism and function. *PLoS One* **12**, e0187523
49. Jaber, S. M., Yadava, N., and Polster, B. M. (2020) Mapping mitochondrial respiratory chain deficiencies by respirometry: Beyond the Mito Stress Test. *Exp. Neurol.* **328**, 113282
50. Makrecka-Kuka, M., Krumschnabel, G., and Gnaiger, E. (2015) High-resolution respirometry for simultaneous measurement of oxygen and hydrogen peroxide fluxes in permeabilized cells, tissue homogenate and isolated mitochondria. *Biomolecules* **5**, 1319–1338
51. Halestrap, A. P., and Wilson, M. C. (2012) The monocarboxylate transporter family-role and regulation. *IUBMB Life* **64**, 109–119
52. McCommis, K., and Finck, B. (2015) Mitochondrial pyruvate transport: A historical perspective and future research directions. *Biochem. J.* **466**, 443–454
53. Edlund, G. L., and Halestrap, A. P. (1988) The kinetics of transport of lactate and pyruvate into rat hepatocytes. Evidence for the presence of a specific carrier similar to that in erythrocytes. *Biochem. J.* **249**, 117–126
54. LaNoue, K. F., and Schoolwerth, A. C. (1979) Metabolite transport in mitochondria. *Annu. Rev. Biochem.* **48**, 871–922
55. Vander Heiden, M. G., Plas, D. R., Rathmell, J. C., Fox, C. J., Harris, M. H., and Thompson, C. B. (2001) Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol. Cell. Biol.* **21**, 5899–5912
56. Xintaropoulou, C., Ward, C., Wise, A., Marston, H., Turnbull, A., and Langdon, S. P. (2015) A comparative analysis of inhibitors of the glycolysis pathway in breast and ovarian cancer cell line models. *Oncotarget* **6**, 25677–25695
57. Miyoshi, H. (1998) Structure-activity relationships of some complex I inhibitors. *Biochim. Biophys. Acta* **1364**, 236–244
58. Hong, S., and Pedersen, P. L. (2008) ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. *Microbiol. Mol. Biol. Rev.* **72**, 590–641

59. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85
60. McLaughlin, K. L., Hagen, J. T., Coalson, H. S., Nelson, M. A. M., Kew, K. A., Wooten, A. R., and Fisher-Wellman, K. H. (2020) Novel approach to quantify mitochondrial content and intrinsic bioenergetic efficiency across organs. *Sci. Rep.* **10**, 17599
61. Westheimer, F. H. (1987) Why nature chose phosphates. *Science* **235**, 1173–1178
62. Chen, Z., Zuo, X., Zhang, Y., Han, G., Zhang, L., Wu, J., and Wang, X. (2018) MiR-3662 suppresses hepatocellular carcinoma growth through inhibition of HIF-1 α -mediated Warburg effect. *Cell Death Dis.* **9**, 549
63. Kovalenko, I., Glasauer, A., Schöckel, L., Sauter, D. R. P., Ehrmann, A., Sohler, F., Hägebarth, A., Novak, I., and Christian, S. (2016) Identification of KCa 3.1 channel as a novel regulator of oxidative phosphorylation in a subset of pancreatic carcinoma cell lines. *PLoS One* **11**, e0160658
64. Wang, L., Yang, Q., Peng, S., and Liu, X. (2019) The combination of the glycolysis inhibitor 2-DG and sorafenib can be effective against sorafenib-tolerant persister cancer cells. *Onco. Targets Ther.* **12**, 5359–5373
65. Calderwood, S. K., Bump, E. A., Stevenson, M. A., Van Kersen, I., and Hahn, G. M. (1985) Investigation of adenylate energy charge, phosphorylation potential, and ATP concentration in cells stressed with starvation and heat. *J. Cell. Physiol.* **124**, 261–268
66. Veech, R. L., Lawson, J. W., Cornell, N. W., and Krebs, H. A. (1979) Cytosolic phosphorylation potential. *J. Biol. Chem.* **254**, 6538–6547
67. Gnaiger, E. (1993) Nonequilibrium thermodynamics of energy transformations. *Pure Appl. Chem.* **65**, 1983–2002
68. Welch, G. R. (1985) Some problems in the usage of Gibbs free energy in biochemistry. *J. Theor. Biol.* **114**, 433–446
69. Alberty, R. A. (1968) Effect of pH and metal ion concentration on the equilibrium hydrolysis of adenosine triphosphate to adenosine diphosphate. *J. Biol. Chem.* **243**, 1337–1343
70. Furuhashi, M. (2020) New insights into purine metabolism in metabolic diseases: Role of xanthine oxidoreductase activity. *Am. J. Physiol. Endocrinol. Metab.* **319**, E827–E834
71. Atkinson, D. E., and Walton, G. M. (1967) Adenosine triphosphate conservation in metabolic regulation rat liver citrate cleavage enzyme. *J. Biol. Chem.* **242**, 3239–3241
72. Chapman, A. G., Fall, L., and Atkinson, D. E. (1971) Adenylate energy charge in *Escherichia coli* during growth and starvation. *J. Bacteriol.* **108**, 1072–1086
73. Brault, J. J., Pizzimenti, N. M., Dentel, J. N., and Wiseman, R. W. (2013) Selective inhibition of ATPase activity during contraction alters the activation of p38 MAP kinase isoforms in skeletal muscle. *J. Cell. Biochem.* **114**, 1445–1455
74. Glancy, B., and Balaban, R. S. (2021) Energy metabolism design of the striated muscle cell. *Physiol. Rev.* **101**, 1561–1607
75. Wiseman, R. W., and Kushmerick, M. J. (1995) Creatine kinase equilibration follows solution thermodynamics in skeletal muscle: ³¹P NMR studies using creatine analogs. *J. Biol. Chem.* **270**, 12428–12438
76. Lark, D. S., Torres, M. J., Lin, C. T., Ryan, T. E., Anderson, E. J., and Neuffer, P. D. (2016) Direct real-time quantification of mitochondrial oxidative phosphorylation efficiency in permeabilized skeletal muscle myofibers. *Am. J. Physiol. Cell Physiol.* **311**, C239–C245
77. Messer, J. I., Jackman, M. R., and Willis, W. T. (2004) Pyruvate and citric acid cycle carbon requirements in isolated skeletal muscle mitochondria. *Am. J. Physiol. Cell Physiol.* **286**, C565–C572
78. Tanner, L. B., Goglia, A. G., Wei, M. H., Sehgal, T., Parsons, L. R., Park, J. O., White, E., Toettcher, J. E., and Rabinowitz, J. D. (2018) Four key steps control glycolytic flux in mammalian cells. *Cell Syst.* **7**, 49–62.e8
79. Park, J. O., Tanner, L. B., Wei, M. H., Khana, D. B., Jacobson, T. B., Zhang, Z., Rubin, S. A., Li, S. H. J., Higgins, M. B., Stevenson, D. M., Amador-Noguez, D., and Rabinowitz, J. D. (2019) Near-equilibrium glycolysis supports metabolic homeostasis and energy yield. *Nat. Chem. Biol.* **15**, 1001–1008
80. Xiao, Z., Dai, Z., and Locasale, J. W. (2019) Metabolic landscape of the tumor microenvironment at single cell resolution. *Nat. Commun.* **10**, 3763
81. Reitner, L. J., Wice, B. M., and Kennell, D. (1979) Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J. Biol. Chem.* **254**, 2669–2676
82. Hui, S., Ghergurovich, J. M., Morscher, R. J., Jang, C., Teng, X., Lu, W., Esparza, L. A., Reya, T., Zhan, L., Yanxiang Guo, J., White, E., and Rabinowitz, J. D. (2017) Glucose feeds the TCA cycle via circulating lactate. *Nature* **551**, 115–118
83. Perron, N. R., Beeson, C., and Rohrer, B. (2013) Early alterations in mitochondrial reserve capacity; a means to predict subsequent photo-receptor cell death. *J. Bioenerg. Biomembr.* **45**, 101–109
84. Hardie, R.-A., van Dam, E., Cowley, M., Han, T.-L., Balaban, S., Pajic, M., Pinese, M., Iconomou, M., Shearer, R. F., McKenna, J., Miller, D., Waddell, N., Pearson, J. V., Grimmond, S. M., Sazanov, L., et al. (2017) Mitochondrial mutations and metabolic adaptation in pancreatic cancer. *Cancer Metab.* **5**, 1–15
85. Keuper, M., Berti, L., Raedle, B., Sachs, S., Böhm, A., Fritsche, L., Fritsche, A., Häring, H. U., Hrabě de Angelis, M., Jastroch, M., Hofmann, S. M., and Staiger, H. (2019) Preadipocytes of obese humans display gender-specific bioenergetic responses to glucose and insulin. *Mol. Metab.* **20**, 28–37
86. Vijayan, V., Pradhan, P., Braud, L., Fuchs, H. R., Gueler, F., Motterlini, R., Foresti, R., and Immenschuh, S. (2019) Human and murine macrophages exhibit differential metabolic responses to lipopolysaccharide - a divergent role for glycolysis. *Redox Biol.* **22**, 101147
87. Warburg, O., Franz, W., and Negelein, E. (1927) The metabolism of tumours in the body. *J. Gen. Physiol.* **8**, 519–530
88. Warburg, O. (1956) On the origin of cancer cells. *Science* **123**, 309–314
89. Cassim, S., Vučetić, M., Ždravčević, M., and Pouyssegur, J. (2020) Warburg and beyond: The power of mitochondrial metabolism to collaborate or replace fermentative glycolysis in cancer. *Cancers (Basel)* **12**, 13–15
90. Morandi, A., and Indraccolo, S. (2017) Linking metabolic reprogramming to therapy resistance in cancer. *Biochim. Biophys. Acta Rev. Cancer* **1868**, 1–6
91. Handel, M. E., Brand, M. D., and Mookerjee, S. A. (2019) The whys and hows of calculating total cellular ATP production rate. *Trends Endocrinol. Metab.* **30**, 412–416
92. Bar-Even, A., Flamholz, A., Noor, E., and Milo, R. (2012) Rethinking glycolysis: On the biochemical logic of metabolic pathways. *Nat. Chem. Biol.* **8**, 509–517
93. Minakami, S., and Yoshikawa, H. (1966) Studies on erythrocyte glycolysis II. Free energy changes and rate limiting steps in erythrocyte glycolysis. *J. Biochem.* **59**, 139–144
94. Xie, J., Dai, C., and Hu, X. (2016) Evidence that does not support pyruvate kinase M2 (PKM2) - catalyzed reaction as a rate-limiting step in cancer cell glycolysis. *J. Biol. Chem.* **291**, 8987–8999
95. King, M. P., and Attardi, G. (1989) Human cells lacking mtDNA: Repopulation with exogenous mitochondria by complementation. *Science* **246**, 500–503
96. Alston, C. L., Rocha, M. C., Lax, N. Z., Turnbull, D. M., and Taylor, R. W. (2017) The genetics and pathology of mitochondrial disease. *J. Pathol.* **241**, 236–250
97. Johnson, D. T., Harris, R. A., Blair, P. V., and Balaban, R. S. (2007) Functional consequences of mitochondrial proteome heterogeneity. *Am. J. Physiol. Cell Physiol.* **292**, 698–707
98. Johnson, D. T., Harris, R. A., French, S., Blair, P. V., You, J., Bemis, K. G., Wang, M., and Balaban, R. S. (2007) Tissue heterogeneity of the mammalian mitochondrial proteome. *Am. J. Physiol. Cell Physiol.* **292**, 689–697
99. Konarzewski, M., and Książek, A. (2013) Determinants of intra-specific variation in basal metabolic rate. *J. Comp. Physiol. B* **183**, 27–41
100. Zhu, Z., Wang, Z., Zhang, C., Wang, Y., Zhang, H., Gan, Z., Guo, Z., and Wang, X. (2019) Mitochondrion-targeted platinum complexes suppressing lung cancer through multiple pathways involving energy metabolism. *Chem. Sci.* **10**, 3089–3095

101. O'Neill, S., Porter, R. K., McNamee, N., Martinez, V. G., and O'Driscoll, L. (2019) 2-Deoxy-D-glucose inhibits aggressive triple-negative breast cancer cells by targeting glycolysis and the cancer stem cell phenotype. *Sci. Rep.* **9**, 3788
102. Luengo, A., Li, Z., Gui, D. Y., Sullivan, L. B., Zagorulya, M., Do, B. T., Ferreira, R., Naamati, A., Ali, A., Lewis, C. A., Thomas, C. J., Spranger, S., Matheson, N. J., and Vander Heiden, M. G. (2021) Increased demand for NAD⁺ relative to ATP drives aerobic glycolysis. *Mol. Cell* **81**, 691–707.e6
103. Martínez-Reyes, I., Cardona, L. R., Kong, H., Vasan, K., McElroy, G. S., Werner, M., Kihshen, H., Reczek, C. R., Weinberg, S. E., Gao, P., Steinert, E. M., Piseaux, R., Budinger, G. R. S., and Chandel, N. S. (2020) Mitochondrial ubiquinol oxidation is necessary for tumour growth. *Nature* **585**, 288–292
104. Cardaci, S., Zheng, L., Mackay, G., Van Den Broek, N. J. F., Mackenzie, E. D., Nixon, C., Stevenson, D., Tumanov, S., Bulusu, V., Kamphorst, J. J., Vazquez, A., Fleming, S., Schiavi, F., Kalna, G., Blyth, K., *et al.* (2015) Pyruvate carboxylation enables growth of SDH-deficient cells by supporting aspartate biosynthesis. *Nat. Cell Biol.* **17**, 1317–1326
105. Fan, J., Kamphorst, J. J., Mathew, R., Chung, M. K., White, E., Shlomi, T., and Rabinowitz, J. D. (2013) Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia. *Mol. Syst. Biol.* **9**, 1–11
106. Mullen, A. R., Wheaton, W. W., Jin, E. S., Chen, P. H., Sullivan, L. B., Cheng, T., Yang, Y., Linehan, W. M., Chandel, N. S., and DeBerardinis, R. J. (2012) Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature* **481**, 385–388
107. Yang, C., Ko, B., Hensley, C. T., Jiang, L., Wasti, A. T., Kim, J., Sudderth, J., Calvaruso, M. A., Lumata, L., Mitsche, M., Rutter, J., Merritt, M. E., and DeBerardinis, R. J. (2014) Glutamine oxidation maintains the TCA cycle and cell survival during impaired mitochondrial pyruvate transport. *Mol. Cell* **56**, 414–424
108. Epstein, T., Xu, L., Gillies, R. J., and Gatenby, R. A. (2014) Separation of metabolic supply and demand: Aerobic glycolysis as a normal physiological response to fluctuating energetic demands in the membrane. *Cancer Metab.* **2**, 7
109. Jang, S. R., Nelson, J. C., Bend, E. G., Rodríguez-Laureano, L., Tueros, F. G., Cartagena, L., Underwood, K., Jorgensen, E. M., and Colón-Ramos, D. A. (2016) Glycolytic enzymes localize to synapses under energy stress to support synaptic function. *Neuron* **90**, 278–291
110. Davis, E. J., Bremer, J., and Akerman, K. E. (1980) Thermodynamic aspects of translocation of reducing equivalents by mitochondria. *J. Biol. Chem.* **255**, 2277–2283
111. Glancy, B., Kane, D. A., Kavazis, A. N., Goodwin, M. L., Willis, W. T., and Gladden, L. B. (2021) Mitochondrial lactate metabolism: History and implications for exercise and disease. *J. Physiol.* **599**, 863–888
112. Barron, J. T., Gu, L., and Parrillo, J. E. (1998) Malate-aspartate shuttle, cytoplasmic NADH redox potential, and energetics in vascular smooth muscle. *J. Mol. Cell. Cardiol.* **30**, 1571–1579
113. Birsoy, K., Wang, T., Chen, W. W., Freinkman, E., Abu-Remaileh, M., and Sabatini, D. M. (2015) An essential role of the mitochondrial electron transport chain in cell proliferation is to enable aspartate synthesis. *Cell* **162**, 540–551
114. Mookerjee, S. A., Nicholls, D. G., and Brand, M. D. (2016) Determining maximum glycolytic capacity using extracellular flux measurements. *PLoS One* **11**, e0152016
115. Psychogios, N., Hau, D. D., Peng, J., Guo, A. C., Mandal, R., Bouatra, S., Sinelnikov, I., Krishnamurthy, R., Eisner, R., Gautam, B., Young, N., Xia, J., Knox, C., Dong, E., Huang, P., *et al.* (2011) The human serum metabolome. *PLoS One* **6**, e16957
116. Herbst, E. A. F., George, M. A. J., Brebner, K., Holloway, G. P., and Kane, D. A. (2018) Lactate is oxidized outside of the mitochondrial matrix in rodent brain. *Appl. Physiol. Nutr. Metab.* **43**, 467–474
117. Faubert, B., Li, K. Y., Cai, L., Hensley, C. T., Kim, J., Zacharias, L. G., Yang, C., Do, Q. N., Doucette, S., Burguete, D., Li, H., Huet, G., Yuan, Q., Wigal, T., Butt, Y., *et al.* (2017) Lactate metabolism in human lung tumors. *Cell* **171**, 358–371.e9
118. Young, A., Oldford, C., and Mailloux, R. J. (2020) Lactate dehydrogenase supports lactate oxidation in mitochondria isolated from different mouse tissues. *Redox Biol.* **28**, 101339
119. Prabhu, A. V., Krycer, J. R., and Brown, A. J. (2013) Overexpression of a key regulator of lipid homeostasis, Scap, promotes respiration in prostate cancer cells. *FEBS Lett.* **587**, 983–988
120. Hereng, T. H., Elgstøen, K. B. P., Cederkvist, F. H., Eide, L., Jahnsen, T., Sklhegg, B. S., and Rosendal, K. R. (2011) Exogenous pyruvate accelerates glycolysis and promotes capacitation in human spermatozoa. *Hum. Reprod.* **26**, 3249–3263
121. Owicki, J. C., and Wallace Parce, J. (1992) Biosensors based on the energy metabolism of living cells: The physical chemistry and cell biology of extracellular acidification. *Biosens. Bioelectron.* **7**, 255–272
122. Orlenko, A., Hermansen, R. A., and Liberles, D. A. (2016) Flux control in glycolysis varies across the tree of life. *J. Mol. Evol.* **82**, 146–161
123. Ainscow, E. K., and Brand, M. D. (1999) Internal regulation of ATP turnover, glycolysis and oxidative phosphorylation in rat hepatocytes. *Eur. J. Biochem.* **266**, 737–749
124. Shestov, A. A., Liu, X., Ser, Z., Cluntun, A. A., Hung, Y. P., Huang, L., Kim, D., Le, A., Yellen, G., Albeck, J. G., and Locasale, J. W. (2014) Quantitative determinants of aerobic glycolysis identify flux through the enzyme GAPDH as a limiting step. *Elife* **3**, e03342
125. Altinok, O., Poggio, J. L., Stein, D. E., Bowne, W. B., Shieh, A. C., Snyder, N. W., and Orynbayeva, Z. (2020) Malate–aspartate shuttle promotes l-lactate oxidation in mitochondria. *J. Cell. Physiol.* **235**, 2569–2581
126. Louie, M. C., Ton, J., Brady, M. L., Le, D. T., Mar, J. N., Lerner, C. A., Gerencser, A. A., and Mookerjee, S. A. (2020) Total cellular ATP production changes with primary substrate in MCF7 breast cancer cells. *Front. Oncol.* **10**, 1–12
127. Hill, B. G., Benavides, G. A., Lancaster, J. J. R., Ballinger, S., Dell'Italia, L., Zhang, J., and Darley-Usmar, V. M. (2012) Integration of cellular bioenergetics with mitochondrial quality control and autophagy. *Biol. Chem.* **393**, 1485–1512
128. Zorova, L. D., Popkov, V. A., Plotnikov, E. Y., Silachev, D. N., Pevzner, I. B., Jankauskas, S. S., Babenko, V. A., Zorov, S. D., Balakireva, A. V., Juhaszova, M., Sollott, S. J., and Zorov, D. B. (2018) Mitochondrial membrane potential. *Anal. Biochem.* **552**, 50–59
129. Lefranc, C., Friederich-Persson, M., Braud, L., Palacios-Ramirez, R., Karlsson, S., Boujardine, N., Motterlini, R., Jaisser, F., and Nguyen Dinh Cat, A. (2019) MR (mineralocorticoid receptor) induces adipose tissue senescence and mitochondrial dysfunction leading to vascular dysfunction in obesity. *Hypertension* **73**, 458–468
130. Rojas-Morales, P., León-Contreras, J. C., Aparicio-Trejo, O. E., Reyes-Ocampo, J. G., Medina-Campos, O. N., Jiménez-Osorio, A. S., González-Reyes, S., Marquina-Castillo, B., Hernández-Pando, R., Barrera-Oviedo, D., Sánchez-Lozada, L. G., Pedraza-Chaverri, J., and Tapia, E. (2019) Fasting reduces oxidative stress, mitochondrial dysfunction and fibrosis induced by renal ischemia-reperfusion injury. *Free Radic. Biol. Med.* **135**, 60–67
131. Ehrenberg, B., Montana, V., Wei, M. D., Wuskell, J. P., and Loew, L. M. (1988) Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. *Biophys. J.* **53**, 785–794
132. Hoek, J. B., Nicholls, D. G., and Williamson, J. R. (1980) Determination of the mitochondrial protonmotive force in isolated hepatocytes. *J. Biol. Chem.* **255**, 1458–1464
133. Ward, M. W., Rego, A. C., Frenguelli, B. G., and Nicholls, D. G. (2000) Mitochondrial membrane potential and glutamate excitotoxicity in cultured cerebellar granule cells. *J. Neurosci.* **20**, 7208–7219
134. Scaduto, R. C., and Grotyohann, L. W. (1999) Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J.* **76**, 469–477
135. Lemasters, J. J., and Ramshesh, V. K. (2007) Imaging of mitochondrial polarization and depolarization with cationic fluorophores. *Methods Cell Biol.* **80**, 283–295
136. Park, J. S., Davis, R. L., and Sue, C. M. (2018) Mitochondrial dysfunction in Parkinson's disease: New mechanistic insights and therapeutic perspectives. *Curr. Neurol. Neurosci. Rep.* **18**, 21
137. Kelley, D. E., He, J., Menshikova, E. V., and Ritov, V. B. (2002) Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* **51**, 2944–2950

138. Romanello, V., Guadagnin, E., Gomes, L., Roder, I., Sandri, C., Petersen, Y., Milan, G., Masiero, E., Del Piccolo, P., Foretz, M., Scorrano, L., Rudolf, R., and Sandri, M. (2010) Mitochondrial fission and remodelling contributes to muscle atrophy. *EMBO J.* **29**, 1774–1785
139. Roden, M., Price, T. B., Perseghin, G., Petersen, K. F., Rothman, D. L., Cline, G. W., and Shulman, G. I. (1996) Mechanism of free fatty acid-induced insulin resistance in humans. *J. Clin. Invest.* **97**, 2859–2865
140. Lowell, B. B., and Shulman, G. I. (2005) Mitochondrial dysfunction and type 2 diabetes. *Curr. Diab. Rep.* **307**, 384–387
141. Petersen, K. F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D. L., DiPietro, L., Cline, G. W., and Shulman, G. I. (2003) Mitochondrial dysfunction in the elderly: Possible role in insulin resistance. *Science* **300**, 1140–1142
142. Muoio, D. M. (2010) Intramuscular triacylglycerol and insulin resistance: Guilty as charged or wrongly accused? *Biochim. Biophys. Acta* **1801**, 281–288
143. Ghosh, S., Wicks, S. E., Vandanmagsar, B., Mendoza, T. M., Bayless, D. S., Salbaum, J. M., Dearth, S. P., Campagna, S. R., Mynatt, R. L., and Noland, R. C. (2019) Extensive metabolic remodeling after limiting mitochondrial lipid burden is consistent with an improved metabolic health profile. *J. Biol. Chem.* **294**, 12313–12327
144. Bonnard, C., Durand, A., Peyrol, S., Chanseume, E., Chauvin, M. A., Morio, B., Vidal, H., and Rieusset, J. (2008) Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J. Clin. Invest.* **118**, 789–800
145. Fisher-Wellman, K. H., Weber, T. M., Cathey, B. L., Brophy, P. M., Gilliam, L. A. A., Kane, C. L., Maples, J. M., Gavin, T. P., Houmard, J. A., and Neuffer, P. D. (2014) Mitochondrial respiratory capacity and content are normal in young insulin-resistant obese humans. *Diabetes* **63**, 132–141
146. Phielix, E., Schrauwen-Hinderling, V. B., Mensink, M., Lenaers, E., Meex, R., Hoeks, J., Kooi, M. E., Moonen-Kornips, E., Sels, J. P., Hesselink, M. K. C., and Schrauwen, P. (2008) Lower intrinsic ADP-stimulated mitochondrial respiration underlies *in vivo* mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes* **57**, 2943–2949
147. Randle, P. J., Garland, P. B., Hales, C. N., and Newsholme, E. A. (1963) The glucose fatty-acid cycle its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* **281**, 785–789
148. Denton, R. M., and Randle, P. J. (1966) Citrate and the regulation of adipose-tissue phosphofructokinase. *Biochem. J.* **100**, 420–423
149. Ramakrishna, R., Edwards, J. S., McCulloch, A., and Palsson, B. O. (2001) Flux-balance analysis of mitochondrial energy metabolism: Consequences of systemic stoichiometric constraints. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **280**, 695–704
150. Savinell, J. M., and Palsson, B. O. (1992) Network analysis of intermediary metabolism using linear optimization. I. Development of mathematical formalism. *J. Theor. Biol.* **154**, 421–454
151. Neuffer, P. D. (2019) Cutting fuel offers new clues in diabetic mystery. *J. Biol. Chem.* **294**, 12328–12329
152. Hancock, C. R., Han, D. H., Chen, M., Terada, S., Yasuda, T., Wright, D. C., and Holloszy, J. O. (2008) High-fat diets cause insulin resistance despite an increase in muscle mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 7815–7820
153. Holloszy, J. O. (2013) "Deficiency" of mitochondria in muscle does not cause insulin resistance. *Diabetes* **62**, 1036–1040
154. Leckey, J. J., Hoffman, N. J., Parr, E. B., Devlin, B. L., Trewin, A. J., Stepto, N. K., Morton, J. P., Burke, L. M., and Hawley, J. A. (2018) High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans. *FASEB J.* **32**, 2979–2991
155. Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1–13
156. Anderson, E. J., Lustig, M. E., Boyle, K. E., Woodlief, T. L., Kane, D. A., Lin, C.-T., Price, J. W., Kang, L., Rabinovitch, P. S., Szeto, H. H., Houmard, J. A., Cortright, R. N., Wasserman, D. H., and Neuffer, P. D. (2009) Mitochondrial H₂O₂ emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. *J. Clin. Invest.* **119**, 573–581
157. Houstis, N., Rosen, E. D., and Lander, E. S. (2006) Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* **440**, 944–948
158. Fazakerley, D. J., Minard, A. Y., Krycer, J. R., Thomas, K. C., Stöckli, J., Harney, D. J., Burchfield, J. G., Maghzal, G. J., Caldwell, S. T., Hartley, R. C., Stocker, R., Murphy, M. P., and James, D. E. (2018) Mitochondrial oxidative stress causes insulin resistance without disrupting oxidative phosphorylation. *J. Biol. Chem.* **293**, 7315–7328
159. Fazakerley, D. J., Chaudhuri, R., Yang, P., Maghzal, G. J., Thomas, K. C., Krycer, J. R., Humphrey, S. J., Parker, B. L., Fisher-Wellman, K. H., Meoli, C. C., Hoffman, N. J., Diskin, C., Burchfield, J. G., Cowley, M. J., Kaplan, W., *et al.* (2018) Mitochondrial CoQ deficiency is a common driver of mitochondrial oxidants and insulin resistance. *Elife* **7**, e32111
160. Hüttemann, M., Lee, I., Pecinova, A., Pecina, P., Przyklenk, K., and Doan, J. W. (2008) Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease. *J. Bioenerg. Biomembr.* **40**, 445–456
161. Guarás, A., Perales-Clemente, E., Calvo, E., Acín-Pérez, R., Loureiro-Lopez, M., Pujol, C., Martínez-Carrascoso, I., Nuñez, E., García-Marqués, F., Rodríguez-Hernández, M. A., Cortés, A., Diaz, F., Pérez-Martos, A., Moraes, C. T., Fernández-Silva, P., *et al.* (2016) The CoQH₂/CoQ ratio serves as a sensor of respiratory chain efficiency. *Cell Rep.* **15**, 197–209
162. McLaughlin, K. L., McClung, J. M., and Fisher-Wellman, K. H. (2018) Bioenergetic consequences of compromised mitochondrial DNA repair in the mouse heart. *Biochem. Biophys. Res. Commun.* **504**, 742–748
163. Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J. N., Rovio, A. T., Bruder, C. E., Bohlooly-Y, M., Gidlöf, S., Oldfors, A., Wibom, R., Törnelli, J., Jacobs, H. T., and Larsson, N. G. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417–423
164. Dykens, J. A., Jamieson, J., Marroquin, L., Nadanaciva, S., Billis, P. A., and Will, Y. (2008) Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes *in vitro*. *Toxicol. Appl. Pharmacol.* **233**, 203–210
165. Pereira, C. V., Nadanaciva, S., Oliveira, P. J., and Will, Y. (2012) The contribution of oxidative stress to drug-induced organ toxicity and its detection *in vitro* and *in vivo*. *Expert Opin. Drug Metab. Toxicol.* **8**, 219–237
166. Ježek, J., Cooper, K. F., and Strich, R. (2018) Reactive oxygen species and mitochondrial dynamics: The yin and yang of mitochondrial dysfunction and cancer progression. *Antioxidants (Basel)* **7**, 13
167. Peoples, J. N., Saraf, A., Ghazal, N., Pham, T. T., and Kwong, J. Q. (2019) Mitochondrial dysfunction and oxidative stress in heart disease. *Exp. Mol. Med.* **51**, 1–13
168. Rocha, M., and Springett, R. (2019) Measuring the functionality of the mitochondrial pumping complexes with multi-wavelength spectroscopy. *Biochim. Biophys. Acta Bioenerg.* **1860**, 89–101
169. Ruas, J. S., Siqueira-Santos, E. S., Amigo, I., Rodrigues-Silva, E., Kowaltowski, A. J., and Castilho, R. F. (2016) Underestimation of the maximal capacity of the mitochondrial electron transport system in oligomycin-treated cells. *PLoS One* **11**, e0150967
170. Dranka, B. P., Hill, B. G., and Darley-Usmar, V. M. (2010) Mitochondrial reserve capacity in endothelial cells: The impact of nitric oxide and reactive oxygen species. *Free Radic. Biol. Med.* **48**, 905–914
171. Tonkonogi, M., and Sahlin, K. (1997) Rate of oxidative phosphorylation in isolated mitochondria from human skeletal muscle: Effect of training status. *Acta Physiol. Scand.* **161**, 345–353
172. Suarez, R. K., Staples, J. F., Lighton, J. R. B., and West, T. G. (1997) Relationships between enzymatic flux capacities and metabolic flux rates: Nonequilibrium reactions in muscle glycolysis. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7065–7069
173. Connett, R. J. (1988) Analysis of metabolic control: New insights using scaled creatine kinase model. *Am. J. Physiol.* **254**, R949–R959
174. Cairns, C. B., Walther, J., Harken, A. H., and Banerjee, A. (1998) Mitochondrial oxidative phosphorylation thermodynamic efficiencies reflect physiological organ roles. *Am. J. Physiol.* **274**, R1376–R1383

175. Waggoner, A. S. (1979) Dye indicators of membrane potential. *Annu. Rev. Biophys. Bioeng.* **8**, 47–68
176. Nicholls, D. G. (2006) Simultaneous monitoring of ionophore- and inhibitor-mediated plasma and mitochondrial membrane potential changes in cultured neurons. *J. Biol. Chem.* **281**, 14864–14874
177. Krumschnabel, G., Eigentler, A., Fasching, M., and Gnaiger, E. (2014) *Use of Safranin for the Assessment of Mitochondrial Membrane Potential by High-Resolution Respirometry and Fluorometry*, 1st Ed, Elsevier Inc, Amsterdam, The Netherlands
178. Perry, S. W., Norman, J. P., Barbieri, J., Brown, E. B., and Gelbard, H. A. (2011) Mitochondrial membrane potential probes and the proton gradient: A practical usage guide. *Biotechniques* **50**, 98–115
179. Chance, B. (1952) Spectra and reaction kinetics of respiratory pigments of homogenized and intact cells. *Nature* **169**, 215–221
180. Nisr, R. B., and Affouitit, C. (2014) Insulin acutely improves mitochondrial function of rat and human skeletal muscle by increasing coupling efficiency of oxidative phosphorylation. *Biochim. Biophys. Acta* **1837**, 270–276
181. Choi, S. W., Gerencser, A. A., and Nicholls, D. G. (2009) Bioenergetic analysis of isolated cerebrocortical nerve terminals on a microgram scale: Spare respiratory capacity and stochastic mitochondrial failure. *J. Neurochem.* **109**, 1179–1191
182. Nickens, K. P., Wikstrom, J. D., Shirihi, O. S., Patierno, S. R., and Ceryak, S. (2013) A bioenergetic profile of non-transformed fibroblasts uncovers a link between death-resistance and enhanced spare respiratory capacity. *Mitochondrion* **13**, 662–667
183. Malinska, D., Szymański, J., Patalas-Krawczyk, P., Michalska, B., Wojtala, A., Prill, M., Partyka, M., Drabik, K., Walczak, J., Sewer, A., John, S., Luettich, K., Peitsch, M. C., Hoeng, J., Duszyński, J., *et al.* (2018) Assessment of mitochondrial function following short- and long-term exposure of human bronchial epithelial cells to total particulate matter from a candidate modified-risk tobacco product and reference cigarettes. *Food Chem. Toxicol.* **115**, 1–12
184. Abe, Y., Sakairi, T., Kajiyama, H., Shrivastav, S., Beeson, C., and Kopp, J. B. (2010) Bioenergetic characterization of mouse podocytes. *Am. J. Physiol. Cell Physiol.* **299**, 464–476
185. Selenius, L. A., Lundgren, M. W., Jawad, R., Danielsson, O., and Björnstedt, M. (2019) The cell culture medium affects growth, phenotype expression and the response to selenium cytotoxicity in A549 and HepG2 cells. *Antioxidants* **8**, 130
186. Ježek, J., Plečičá-Hlavatá, L., and Ježek, P. (2018) Aglycemic HepG2 cells switch from aminotransferase glutaminolytic pathway of pyruvate utilization to complete Krebs cycle at hypoxia. *Front. Endocrinol. (Lausanne)* **9**, 1–14
187. Marcinek, D. J., Schenkman, K. A., Ciesielski, W. A., and Conley, K. E. (2004) Mitochondrial coupling *in vivo* in mouse skeletal muscle. *Am. J. Physiol. Cell Physiol.* **286**, 457–463
188. Marcinek, D. J., Schenkman, K. A., Ciesielski, W. A., Lee, D., and Conley, K. E. (2005) Reduced mitochondrial coupling *in vivo* alters cellular energetics in aged mouse skeletal muscle. *J. Physiol.* **569**, 467–473
189. Siegel, M. P., Kruse, S. E., Knowels, G., Salmon, A., Beyer, R., Xie, H., van Remmen, H., Smith, S. R., and Marcinek, D. J. (2011) Reduced coupling of oxidative phosphorylation *in vivo* precedes electron transport chain defects due to mild oxidative stress in mice. *PLoS One* **6**, e26963
190. To, T. L., Cuadros, A. M., Shah, H., Hung, W. H. W., Li, Y., Kim, S. H., Rubin, D. H. F., Boe, R. H., Rath, S., Eaton, J. K., Piccioni, F., Goodale, A., Kalani, Z., Doench, J. G., Root, D. E., *et al.* (2019) A compendium of genetic modifiers of mitochondrial dysfunction reveals intra-organelle buffering. *Cell* **179**, 1222–1238.e17
191. Sullivan, L. B., Gui, D. Y., Hosios, A. M., Bush, L. N., Freinkman, E., and Vander Heiden, M. G. (2015) Supporting aspartate biosynthesis is an essential function of respiration in proliferating cells. *Cell* **162**, 552–563
192. Brookes, P. S., Rolfe, D. F. S., and Brand, M. D. (1997) The proton permeability of liposomes made from mitochondrial inner membrane phospholipids: Comparison with isolated mitochondria. *J. Membr. Biol.* **155**, 167–174
193. Ježek, P., Jabárek, M., and Porter, R. K. (2019) Uncoupling mechanism and redox regulation of mitochondrial uncoupling protein 1 (UCP1). *Biochim. Biophys. Acta Bioenerg.* **1860**, 259–269
194. Ricquier, D., and Kader, J. C. (1976) Mitochondrial protein alteration in active brown fat: A sodium dodecyl sulfate-polyacrylamide gel electrophoretic study. *Biochem. Biophys. Res. Commun.* **73**, 577–583
195. Bertholet, A. M., Chouchani, E. T., Kazak, L., Angelin, A., Fedorenko, A., Long, J. Z., Vidoni, S., Garrity, R., Cho, J., Terada, N., Wallace, D. C., Spiegelman, B. M., and Kirichok, Y. (2019) H⁺ transport is an integral function of the mitochondrial ADP/ATP carrier. *Nature* **571**, 515–520
196. Samartsev, V. N., Smirnov, A. V., Zeldi, I. P., Markova, O. V., Mokhova, E. N., and Skulachev, V. P. (1997) Involvement of aspartate/glutamate antiporter in fatty acid-induced uncoupling of liver mitochondria. *Biochim. Biophys. Acta* **1319**, 251–257
197. Voza, A., Parisi, G., De Leonardi, F., Lasorsa, F. M., Castegna, A., Amorese, D., Marmo, R., Calcagnile, V. M., Palmieri, L., Ricquier, D., Paradies, E., Scarcia, P., Palmieri, F., Bouillaud, F., and Fiermonte, G. (2014) UCP2 transports C4 metabolites out of mitochondria, regulating glucose and glutamine oxidation. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 960–965
198. Bouillaud, F., Alves-Guerra, M. C., and Ricquier, D. (2016) UCPs, at the interface between bioenergetics and metabolism. *Biochim. Biophys. Acta* **1863**, 2443–2456
199. Starkov, A. A., Dedukhova, V. I., and Skulachev, V. P. (1994) 6-Ketocholestanol abolishes the effect of the most potent uncouplers of oxidative phosphorylation in mitochondria. *FEBS Lett.* **355**, 305–308
200. Owen, O. E., Kalhan, S. C., and Hanson, R. W. (2002) The key role of anaplerosis and cataplerosis for citric acid cycle function. *J. Biol. Chem.* **277**, 30409–30412
201. Hakimi, P., Johnson, M. T., Yang, J., Lepage, D. F., Conlon, R. A., Kalhan, S. C., Reshef, L., Tilghman, S. M., and Hanson, R. W. (2005) Phosphoenolpyruvate carboxykinase and the critical role of cataplerosis in the control of hepatic metabolism. *Nutr. Metab.* **2**, 1–12
202. Bernardi, P. (1999) Mitochondrial transport of cations: Channels, exchangers, and permeability transition. *Physiol. Rev.* **79**, 1127–1155
203. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009) Importing mitochondrial proteins: Machineries and mechanisms. *Cell* **138**, 628–644
204. Smith, C. D., Schmidt, C. A., Lin, C.-T., Fisher-Wellman, K. H., and Neuffer, P. D. (2020) Flux through mitochondrial redox circuits linked to nicotinamide nucleotide transhydrogenase generates counterbalance changes in energy expenditure. *J. Biol. Chem.* **295**, 16207–16216
205. McLaughlin, K. L., Kew, K. A., McClung, J. M., and Fisher-Wellman, K. H. (2020) Subcellular proteomics combined with bioenergetic phenotyping reveals protein biomarkers of respiratory insufficiency in the setting of proofreading-deficient mitochondrial polymerase. *Sci. Rep.* **10**, 1–10
206. Maxfield, F. R. (1982) Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* **95**, 676–681
207. Al-Shaikhal, M. H. M., Nedergaard, J., and Cannon, B. (1979) Sodium-induced calcium release from mitochondria in brown adipose tissue. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 2350–2353
208. Souza, A. C., Machado, F. S., Celes, M. R. N., Faria, G., Rocha, L. B., Silva, J. S., and Rossi, M. A. (2005) Mitochondrial damage as an early event of monensin-induced cell injury in cultured fibroblasts L929. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* **52**, 230–237
209. Erecinska, M., Dagani, F., Nelson, D., Deas, J., and Silver, I. A. (1991) Relations between intracellular ions and energy metabolism: A study with monensin in synaptosomes, neurons, and C6 glioma cells. *J. Neurosci.* **11**, 2410–2421
210. Mitchell, P. (1977) Vectorial chemiosmotic processes. *Annu. Rev. Biochem.* **46**, 996–1005
211. Mitchell, P., and Moyle, J. (1969) Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria. *Eur. J. Biochem.* **7**, 471–484
212. Willis, W. T., Jackman, M. R., Messer, J. I., Kuzmiak-Glancy, S., and Glancy, B. (2016) A simple hydraulic analog model of oxidative phosphorylation. *Med. Sci. Sports Exerc.* **48**, 990–1000
213. Fisher-Wellman, K. H., and Neuffer, P. D. (2012) Linking mitochondrial bioenergetics to insulin resistance via redox biology. *Trends Endocrinol. Metab.* **23**, 142–153

214. Neuffer, P. D. (2018) The bioenergetics of exercise. *Cold Spring Harb. Perspect. Med.* **8**, 1–12
215. Hirst, J. (2013) Mitochondrial complex I. *Annu. Rev. Biochem.* **82**, 551–575
216. Brand, M. (2005) The efficiency and plasticity of mitochondrial energy transduction. *Biochem. Soc. Trans.* **33**, 897–904
217. Hollis, V. S., Palacios-Callender, M., Springett, R. J., Delpy, D. T., and Moncada, S. (2003) Monitoring cytochrome redox changes in the mitochondria of intact cells using multi-wavelength visible light spectroscopy. *Biochim. Biophys. Acta* **1607**, 191–202
218. Soga, N., Kimura, K., Kinoshita, K., Yoshida, M., and Suzuki, T. (2017) Perfect chemomechanical coupling of FoF1-ATP synthase. *Proc. Natl. Acad. Sci. U. S. A.* **114**, 4960–4965
219. Schendzielorz, A. B., Schulz, C., Lytovchenko, O., Clancy, A., Guiard, B., Ieva, R., van der Laan, M., and Rehling, P. (2017) Two distinct membrane potential-dependent steps drive mitochondrial matrix protein translocation. *J. Cell Biol.* **216**, 83–92
220. Jonckheere, A. I., Smeitink, J. A. M., and Rodenburg, R. J. T. (2012) Mitochondrial ATP synthase: Architecture, function and pathology. *J. Inherit. Metab. Dis.* **35**, 211–225
221. Hoek, J. B., and Rydstrom, J. (1988) Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem. J.* **254**, 1–10
222. Berry, B. J., Trewin, A. J., Amitrano, A. M., Kim, M., and Wojtovich, A. P. (2018) Use the protonmotive force: Mitochondrial uncoupling and reactive oxygen species. *J. Mol. Biol.* **430**, 3873–3891
223. Klingenberg, M. (2008) The ADP and ATP transport in mitochondria and its carrier. *Biochim. Biophys. Acta* **1778**, 1978–2021
224. Anandakrishnan, R., Zhang, Z., Donovan-Maiye, R., and Zuckerman, D. M. (2016) Biophysical comparison of ATP synthesis mechanisms shows a kinetic advantage for the rotary process. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 11220–11225
225. Hafner, R. P., and Brand, M. D. (1991) Effect of protonmotive force on the relative proton stoichiometries of the mitochondrial proton pumps. *Biochem. J.* **275**, 75–80