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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

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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.					
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1. INTRODUCTION:

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:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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.

Subtask 1 – Using the same mitochondrial preparations used for the diagnostics workflow, phosphoproteomics analysis will be performed using nLC-MS/MS. Initial experiments will be performed using aliquots from each mitochondrial prep which were either not subjected to the diagnostics workflow (i.e., flash-frozen immediately upon isolation) or collected at the completion of the respiration assays in which ATP free energy was maximal.

Subtask 2 – Additional experiments will be carried out in isolated mitochondria from AML cell lines in which mitochondrial aliquots are incubated with increasing ATP free energies. Phosphoproteomics analysis will then be performed on these samples to characterize phosphorylation dynamics as a function of ATP free energy.

Subtask 3 – Using phosphoproteomics data, prediction of kinase-substrate relationships will be performed using KinomeXplorer.

Subtask 4 – Integrate bioenergetic flux data with phosphoproteomics data into on-line resource which can be used by the entire scientific community to mine for potential mitochondrial-specific AML drug targets.

What was accomplished under these goals?

Although progress with respect to experimental data collection was impacted for this reporting period by the global COVID-19 pandemic, we were able to write up and submit all of our work pertaining to Aim 1 (Major Tasks #1 and #2) for peer review. Both Manuscripts are currently available on preprint servers.

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

Article #2 - Alterations in Sphingolipid Composition and Mitochondrial Complex I Deficiency Represent Synergistic Therapeutic Vulnerabilities Linked to Vincristine Resistance. [Alterations in Sphingolipid Composition and Mitochondrial Complex I Deficiency Represent Synergistic Therapeutic Vulnerabilities Linked to Vincristine Resistance | Research Square](#). Full article can be found in Appendix 2.

Article #1 is on its second round of peer-review at the journal E-Life and Article #2 is under review at Cancer & Metabolism.

In addition to submitting our work for publication, this past funding period we also made several key discoveries. For clarity, such discoveries that relate to Major Tasks 1 and 2 are briefly summarized below. Progress with respect to Aim 2 is discussed separately.

- Upon evaluation of our work via the peer-review process it was suggested that we include, in addition to PBMC, non-cancer control conditions that more accurately reflect the progenitor phenotype of leukemia. To do this, we performed our bioenergetic analyses on purified CD34+ hematopoietic stem cells, as well as mononuclear cells isolated from bone marrow of healthy subjects. These experiments were done using commercially available CD34+ and bone marrow cells, supplied by Hemacare ([HemaCare | Human Primary Blood Cells For Biomedical Research](#)). These experiments proved to be critical as they revealed two key findings: 1) CD34+ cells stimulated in culture to undergo clonal expansion increase the size of their mitochondrial network. 2) However, despite having nearly 2-fold more mitochondria per cell during clonal expansion, OXPHOS is actively repressed. These experiments indicated that intrinsic OXPHOS limitations in AML are not solely cancer specific. Rather, allosteric and/or post-translation regulation of mitochondrial respiration is likely a primary mode of OXPHOS regulation in hematopoietic progenitors that is maintained during leukemogenesis. This work is included in Article #1 above.

- Related to chemoresistance, although we originally planned to profile HL-60 cells made to be refractory to cytarabine (Ara-C) and daunorubicin, we made the decision to focus on vincristine resistance instead. This decision was based on the growing use of the compound to treat AML and the many reports detailing vincristine resistance in the clinic. We thus set out to comprehensively evaluate the mitochondrial network in vincristine resistant cells. Relative to chemosensitive controls, vincristine resistant cells had more mitochondria. However, despite having more mitochondria, their ability to synthesize ATP via OXPHOS was heavily depressed, suggesting that active OXPHOS repression supports resistant disease dissemination in AML. This work is detailed in Article #1.

There were 2 primary milestones set out for Major Tasks 1 and 2:

- 1) Generate a comprehensive bioenergetic profile of AML.
- 2) Determine which, if any, of the AML-specific bioenergetic phenotypes observed in isolated mitochondria can also be observed using a permeabilized cell model.

Accomplishments of stated milestones: With the addition of the control experiments using CD34+ and bone marrow mononuclear cells, as well as the vincristine resistant experiments, both milestones are now complete. In addition, the work has been written up and is under peer-review. Of note, as stated in the original grant, all raw data will be made available upon full publication.

Other Accomplishments – As outlined in Major Task 2, our protocol allowing for mitochondrial function to be assessed in permeabilized cells is now published as a methods article in *Methods in Molecular Biology – Mitochondrial Medicine* (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.).

In addition to this work, and using our permeabilized cell approach, we also identified a unique form of crosstalk between the mitochondrial network and the cellular sphingolipid profile in drug resistant AML. This work was also published during this past reporting period (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.).

Stated Goals not met – None

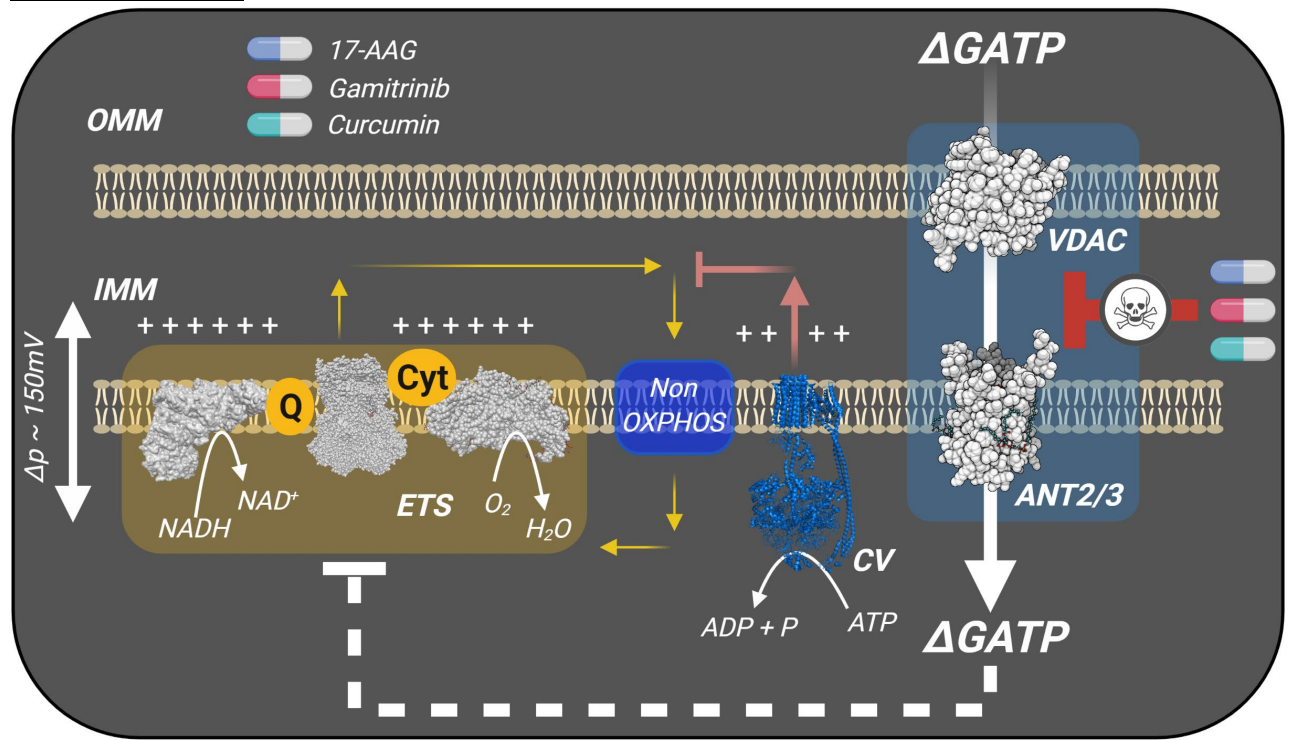
Stated Goals for Aim 2 – The Major Task associated with Aim 2 was to characterize the mitochondrial proteome and phosphoproteome of AML tumorigenesis and chemoresistance. In essence, this aim was designed to determine the molecular mechanism by which ATP leads to direct inhibition of the OXPHOS system in AML.

Major Accomplishments: Although analysis of the AML phosphoproteome remains still to be completed, we made substantial progress in identifying the mechanism of active OXPHOS repression AML. Based on our proteomics screen in AML vs PBMC we discovered that AML exclusively expressed the 2nd and 3rd isoform of adenine nucleotide translocase (ANT). Given that this gene is responsible for ADP/ADP exchange in the mitochondria, we reasoned that it may be the key mechanism by which ATP gains access to the matrix in AML to inhibit OXPHOS. Using specific inhibitors of ANT, we were able to confirm that indeed ANT is required for OXPHOS inhibition in AML.

Even more important, we uncovered a family of small molecules that are uniquely capable of selectivity blocking matrix ATP uptake, while still allowing for ADP to enter the matrix to support OXPHOS. The compounds are 17-AAG, gamitrinib and curcumin. It should be noted that the unique ability of these compounds to ‘restore’ OXPHOS in AML has not been described before. In fact, 17-AAG and gamitrinib are thought to exert anti-cancer effects by blocking the mitochondrial paralog of the HSP90 family -- TRAP1. Gamitrinib is currently be evaluated in a first in human phase I clinical trial (ClinicalTrials.gov Identifier: NCT04827810). To explore the role of TRAP1 in regulating OXPHOS in AML, we knocked down TRAP1 in MV-4-11 cells and remarkably, the ability of 17-AAG and gamitrinib to ‘restore’ OXPHOS was completely independent of TRAP1. Acute (24hrs) exposure of MV-4-11 cells to either gamitrinib or curcumin proved highly cytotoxic. Given that these compounds restore OXPHOS and induce cytotoxicity, these exciting results provide proof-of-principle that blocking mitochondrial ATP uptake may represent a novel treatment paradigm for combatting aggressive AML. Altogether, the work we have completed thus far informs the model depicted below:

Current Working Model of AML Bioenergetics: Currently, there is great interest in developing novel pharmacotherapies that target mitochondrial OXPHOS in leukemia. 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. 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 (**Summary Figure**). Although prior work has identified TRAP1 as a potential anti-cancer 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.

Summary Figure



Stated Goals not met – The only remaining goal to be completed is to investigate the AML phosphoproteome. We plan to perform these experiments, as well as explore the exciting results listed above for curcumin and gamitrinib in the next reporting period.

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

Results from our work were presented at the 2021 Virtual Keystone Symposia on Tumor Metabolism; Appendix 3 is a PDF of the abstract and poster that was presented.

How were the results disseminated to communities of interest?

Nothing to report.

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

The focus of the coming period will be on the phosphoproteomics experiments outlined in Aim 2. Related to this, we will also explore the mechanism of OXPHOS restoration and cytotoxicity by 17-AAG, curcumin and gamitrinib in AML. We will do this using gain and loss of function molecular techniques, as we have already performed for TRAP1 (see Article #1 for details).

4. IMPACT:

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

Our work thus far provides 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 as a component of this project 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).

What was the 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 open up 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?

None to report.

What was the impact on society beyond science and technology?

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 in years 1-2 represents a critical step towards new mitochondrial-localized therapy relative to leukemia and potentially other cancers.

5. CHANGES/PROBLEMS:

Nothing to report

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

Like most labs in the US, we did experience reduced experimentation due to the COVID-19 pandemic. This had an impact on all expenditures in the past reporting period.

Changes that had a significant impact on expenditures

Like most labs in the US, we did experience reduced experimentation due to the COVID-19 pandemic. This had an impact on all expenditures in the past reporting period.

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

Significant changes in use or care of human subjects

Continuing review by the institutional review board of East Carolina University was granted on 03.09.2021.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

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

Journal publications.

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, Neuffer 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, Neuffer PD, Brophy P, Vohra NA, Liles D, Cabot MC, **Fisher-Wellman KH**. Intrinsic OXPHOS limitations underlie cellular bioenergetics in leukemia. Research Square Pre-print, 2020, 10.21203/rs.3.rs-64203/v2.

Fisher-Wellman, K. H. *et al*. Alterations in Sphingolipid Composition and Mitochondrial Complex I Deficiency Represent Synergistic Therapeutic Vulnerabilities Linked to Vincristine Resistance. *Res. Sq.* (2021) doi:10.21203/rs.3.rs-506642/v1.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Results from our work were presented at the 2021 Virtual Keystone Symposia on Tumor Metabolism; Appendix 3 is a PDF of the abstract and poster that was presented.

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

Nothing to report.

- **Technologies or techniques**

Nothing to report.

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

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

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: DK110656, DK125812

Name: Margret Nelson
Project Role: Postdoc
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12

Contribution to Project: Dr. Nelson is involved with all aspects of the project.
Funding Support:

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

Contribution to Project: Ms. McLaughlin assisted with data collection
Funding Support:

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?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Intrinsic OXPHOS limitations underlie cellular bioenergetics in leukemia.

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Research Article

Keywords: leukemia, oxidative metabolism, oxidative phosphorylation

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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, clonally expanding hematopoietic stem cells, leukemic blasts, as well as chemoresistant AML were all 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.

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, 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; Škrtić 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, 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 (**Fig. 1A**). When normalized to basal respiration, oligomycin similarly inhibited respiration across groups and the fold change induced by FCCP was consistently blunted in leukemia (**Fig. 1B**).

Given the large differences in cell size between PBMC and leukemia (**Supp. Fig. 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 (**Fig. 1C**). In fact, maximal FCCP-supported JO_2 was nearly two-fold lower in HL-60 compared to PBMC when normalized to total protein (**Fig. 1C**). Relative to PBMC, maximal respiration induced by FCCP occurred at much lower concentrations in leukemia (i.e., lower K_m ; **Fig. 1D**), with increasing FCCP concentrations leading to an overt bioenergetic collapse (i.e., diminishing respiration rates; **Fig. 1C**; compare JO_2 at FC [2.0 μ M] vs FC [5.0 μ M]). Similar findings were observed using the mitochondrial uncoupler BAM15 (**Supp. Fig. 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 (**Supp. Fig. 1E-F**) (Suganuma 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 (**Fig. 1E-I, Supp. Fig 1D**). Absolute nuclear and mitochondrial volumes were higher in all leukemia lines (**Fig. 1E-H**), consistent with leukemia's larger

cell size (**Supp. Fig. 1A**). However, when normalized to nuclear volume, mitochondrial content was elevated above PBMC only in HL-60 and MV-4-11 (**Fig. 1I**). Interestingly, across all cell types, considerable discrepancies were apparent when protein-normalized maximal respiratory flux (**Fig. 1C**) was compared to mitochondrial content (**Fig. 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 (**Fig. 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 (**Fig. 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 (**Fig. 2C**), confirming maximal ETS flux in the permeabilized system. Note, maximum FCCP-supported flux under these conditions is indicated throughout as $\dot{J}H^+_{Total}$ (**Fig. 2A**). Relative to PBMC, $\dot{J}H^+_{Total}$ was lower in HL-60, unchanged in KG-1, and higher in MV-4-11 (**Fig. 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) 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 ‘ $\dot{J}H^+_{OXPHOS}$ ’ (**Fig. 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 $\dot{J}O_2$ between MV-4-11 and PBMC and revealed decreased respiratory kinetics in both HL-60 and KG-1 (**Fig. 2E**).

In both assays, the utilization of identical substrates (‘P/M/G/S/O’) allowed us to directly quantitate absolute OXPHOS kinetics (‘ $\dot{J}H^+_{OXPHOS}$ ’), relative to the maximal capacity of the electron transport system, (‘ $\dot{J}H^+_{Total}$ ’). Together, $\dot{J}H^+_{OXPHOS}$ and $\dot{J}H^+_{Total}$ 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 (**Fig. 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 (**Fig. 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 (**Fig. 2G**; 'BM_{Healthy}', **Supp. Fig. 2A-B**). As an additional control, identical experiments were carried out in primary human muscle precursor cells (human myoblasts – 'HMB' **Supp. Fig. 2C-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 (**Fig. 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 'FCCP $_{\Delta G_{\text{ATP}}}$ ' (**Fig. 2D**). In the intact cell assay (**Fig. 1C**), increased glycolytic flux is presumed to maintain cellular ΔG_{ATP} during FCCP titration. Thus, the continued presence of extra-mitochondrial ΔG_{ATP} in our permeabilized cell system was intended to model the adenylate constraints present in intact cells. By comparing maximal OXPHOS flux (' $J\text{H}^+_{\text{OXPHOS}}$ ') to maximum FCCP-stimulated respiration in the presence of ΔG_{ATP} ('FCCP $_{\Delta G_{\text{ATP}}}$ '), 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 ΔG_{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 ΔG_{ATP} titration restored respiration to levels obtained under low (-54.16 kJ/mol) ATP free energy (**Fig. 2E**, **Supp. Fig. 2D-E**), indicating minimal ETS flux inhibition by ΔG_{ATP} . Surprisingly, relative to $J\text{H}^+_{\text{OXPHOS}}$, FCCP-stimulated respiration was substantially blunted in the presence of high ATP free energy across all three leukemia cell lines (**Fig. 2E**), as well as healthy bone marrow cells (**Supp. Fig. 2C**), resulting in a near 2-fold difference in the

FCCP_{ΔG_{ATP}}/ JH^+ _{OXPHOS} ratio, termed 'FCCP Effect' throughout (**Fig. 2H**). Importantly, in the absence of ATP, the addition of CK and PCR up to 21mM did not impact FCCP-supported flux in permeabilized MV-4-11 cells (**Supp. Fig. 2E**). To determine the sensitivity of ETS inhibition by ΔG_{ATP}, FCCP-supported flux in MV-4-11 cells was assessed at defined ATP free energies. In these experiments, extramitochondrial ΔG_{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 ΔG_{ATP} (**Fig. 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 ΔG_{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 ΔG_{ATP} led to a more pronounced decrease in respiration in mitochondria of all three leukemia cell lines (**Fig. 3A**). The ability of FCCP to restore maximal respiratory flux was also once again blunted in leukemic mitochondria (**Fig. 3A-B**), consistent with lower fractional OXPHOS (**Fig 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 ΔG_{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 (**Fig. 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 ΔG_{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 of saturating malate in the CI substrate mix results in CII inhibition via malate-fumarate equilibration (**Fig. 3D**). Likewise, the addition of rotenone in the presence of succinate eliminates residual CI-supported flux by downstream products of succinate oxidation (**Fig. 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 (**Fig. 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 (**Supp. Table 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 (**Fig. 4A**). For pair-wise comparisons of mitochondrial protein expression between PBMC and each of the three leukemia cell lines, see **Supp. Table 1**. With respect to the shared 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) (**Fig. 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 (**Fig. 4B**). Although considerable heterogeneity was present across groups, comparing protein expression profiles of the individual subunits that comprise CI-CV and SLC25A3 (**Fig. 4C, Supp. Fig. 3A-D**) revealed that only 6 of the 110 subunits were similarly altered in leukemia (**Supp. Table 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 (**Fig. 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 (**Fig. 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 (**Fig. 5A-C**). Similar experiments performed in MV-4-11 isolated mitochondria (**Fig. 5D**), as well as with the ANT inhibitor bongkreikic acid (**Supp. Fig. 4A**) 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 (**Fig. 5E**), the addition of CAT or oligomycin caused a near complete elimination of mitochondrial membrane potential in HL-60 and KG-1 (**Fig. 5F-G, Supp. Fig. 4B**), indicative of matrix ATP uptake/consumption. Interestingly, in similar experiments with MV-4-11 mitochondria, the addition of oligomycin partially polarized membrane potential (**Fig. 5H**), presumably due to the higher ETS capacity of MV-4-11 compared to HL-60 or KG-1 (**Fig. 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 (**Fig. 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 (**Fig. 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.

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 (**Fig. 6A**). TRAP1 is the mitochondrial paralog of the heat shock protein 90 (HSP90) family and is widely recognized as a potential anti-cancer 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, 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 (**Supp. Fig. 5A**). Remarkably, the presence of 17-AAG increased $J\text{H}^+_{\text{OXPHOS}}$ and calculated fractional OXPHOS relative to vehicle control and completely restored FCCP-supported respiration in the presence of ATP free energy (**Fig. 6B-D**). Similar results were observed using permeabilized HL-60 cells (**Supp. Fig. 5B-D**), as well as using the mitochondrial-targeted TRAP1 inhibitor gamitrinib (**Supp. Fig. 4A**). 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 (**Supp. Fig. 5E-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 (**Supp. Fig. 5G**). 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 (**Supp. Fig. 5H**). In contrast, across all leukemia cell lines, restoration of respiratory flux by 17-AAG was exquisitely sensitive to ΔG_{ATP} (**Fig. 6E, Supp. Fig. 5I-J**).

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 24hr exposure to lentiviral particles and multiple rounds of puromycin selection, shRNA against TRAP1 led to a > 90% reduction in TRAP1 mRNA (**Fig. 6F**). Consistent with prior reports (Laquatra et al., 2021; Sciacovelli et al., 2013), TRAP1 knockdown increased basal respiration in intact cells (**Fig. 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} (**Supp. Fig. 5K**), TRAP1 knockdown decreased JH^+_{OXPHOS} and exacerbated the ability of ATP free energy to blunt ETS flux (**Fig. 6H-J**). This was surprising, given that acute administration of 17-AAG/gamitrinib consistently increased fractional OXPHOS across all AML lines (**Fig. 6C, Supp. Fig. 5C**). 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 (**Fig. 6I-J, Supp. Fig. 5L**). 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 anti-cancer 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 (**Fig. 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

(**Fig. 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 (**Fig. 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 hrs 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, 24hr exposure to either gamitrinib or curcumin decreased cell viability by as much as 60% (**Fig. 6N**). Note, identical cytotoxicity was observed in control and TRAP1 knockdown cells, and the additional presence of Ara-C had no further impact (**Fig. 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 ($\text{BM}_{\text{Healthy}}$), 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., quiescent) CD34^+ cells were also performed following chronic exposure to proliferative stimuli (e.g., TPO, SCF, FLT-3; ' $\text{CD34}^+_{\text{GFs}}$ '). Relative to PBMC, $\text{BM}_{\text{Healthy}}$, and quiescent CD34^+ , intact basal respiration was elevated in primary leukemia, as well as clonally expanding CD34^+ (**Fig. 7A**), reminiscent of that seen in AML cell lines and entirely consistent with their proliferative phenotypes. Relative to quiescent CD34^+ , $\text{JH}^+_{\text{Total}}$ in substrate-replete permeabilized cells was ~ 2-fold higher in PBMC, $\text{CD34}^+_{\text{GFs}}$ and primary leukemia (**Fig. 7B**), consistent with higher cellular mitochondrial density. Despite this apparent increase in mitochondrial content, $\text{JH}^+_{\text{OXPHOS}}$ remained elevated above quiescent CD34^+ only in PBMC, as OXPHOS kinetics were completely unaltered comparing clonally expanding CD34^+ or primary leukemia to quiescent CD34^+ (**Fig. 7C**). As seen in the AML cell lines, calculated fractional OXPHOS was decreased in clonally expanding CD34^+ and primary leukemia, entirely consistent with low fractional OXPHOS being required for hematopoietic clonal expansion (**Fig. 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 $\text{JH}^+_{\text{OXPHOS}}$ and restored FCCP-stimulated respiration in the presence of $\Delta\text{G}_{\text{ATP}}$ in primary AML and $\text{CD34}^+_{\text{GFs}}$ (**Fig. 7E-F**). Of note, acute 17-AAG had no impact on

OXPHOS kinetics in PBMC or BM_{Healthy} and 24hr exposure of PBMC to 17-AAG or gamitrinib did not impact cell viability (**Supp. Fig. 6A-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; Guièze 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 (**Fig. 8A-B**). In these experiments, $\mathcal{J}H^+_{\text{Total}}$ and $\mathcal{J}H^+_{\text{OXPHOS}}$ 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 ($\text{J}\cdot\text{s}^{-1}$). 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}) (**Fig. 8C**). Remarkably, despite a near ~2-fold increase in cellular respiratory capacity, OXPHOS kinetics were in fact decreased in HL60_{VR}, such that $\mathcal{J}H^+_{\text{OXPHOS}}$ failed to reach the respiratory rates observed under basal conditions prior to digitonin permeabilization (**Fig. 8D**). Of note, the addition of extramitochondrial cytochrome C did not impact $\mathcal{J}H^+_{\text{OXPHOS}}$ in either cell type (**Fig. 8E**). The combination of increased maximal respiratory capacity combined with decreased $\mathcal{J}H^+_{\text{OXPHOS}}$ translated to a striking decrease in both fractional OXPHOS (**Fig. 8F**), as well as OXPHOS power output in HL60_{VR} (**Fig. 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; Sukanuma 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 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, 2020), 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, 2014b; Sukanuma 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; Guièze 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 (**Fig. 6**). Although prior work has identified TRAP1 (Bryant et al., 2017; Ramkumar et al., 2020) as a potential anti-cancer 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).

Methods

All procedures involving human subjects were approved by the Institutional Review Board of the Brody School of Medicine.

Blood collection and isolation of PBMCs.

Venous blood from the brachial region of the upper arm was collected from healthy volunteers, ranging from 18-70 years. Whole blood was collected in sodium-heparinized Cell Preparation Tubes (CPT) (BD Biosciences, Franklin Lakes, NJ) and centrifuged at 1,800 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

Bone marrow aspirates were collected from healthy donors, ages 26-33, supplied by HemaCare (Northridge, CA). For primary leukemia samples, bone marrow aspirates were collected from patients undergoing confirmatory diagnosis for a range of hematological malignancies. Patients with confirmed leukemia were enrolled in the study. 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 32-78 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 1,800 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.

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 (25ng/mL), TPO (50ng/mL), and FLT-3 (50ng/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 1µM venetoclax in a manner previously described for other chemotherapy drugs (L. P. Kao et al., 2019). Upon reaching an average cell density of 1.5x10⁶cell/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.

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], 1 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 hours in individual wells of a 96-well plate in 0.1mL of IMDM growth media, supplemented with 4µg/mL polybrene (multiplicity of infection of approximately 20). At the end of the 24hrs, cells were spun down and resuspended in culture media devoid of polybrene. Cultures were then subjected to puromycin selection by continuous exposure to 2µg/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 200nM Mitotracker Green-FM dye (MTG-FM; Molecular Probes, Eugene, OR) at 37°C for 1hr. Cells were then centrifuged at 300 x g for 7min at ~25°C and resuspended in MTG-FM-free IMDM formulation media (Thermo Fisher) containing 50nM tetramethyl rhodamine methyl ester (TMRM) and 2 μ M 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 was 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 800x800 pixel with 2 μ s/pixel dwell time, sequential scan mode, resulting in a 4X digital zoom. Hoechst 33342 was excited using the 405nm line of a multiline argon laser; emission was filtered using a 560nm dichroic mirror and 420-460nm barrier filter. MTG-FM was excited using the 488nm line of a multiline argon laser; emission was filtered using a 560nm dichroic mirror and 505-540nm barrier filter. TMRM was excited using a 559nm laser diode; emission was filtered using a 575-675nm 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 μ m (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 $1-3 \times 10^6$ 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 1.0mL 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 $2,000 \times g$ 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 (17.7g/L Iscove's Modified Dulbecco's Medium (IMDM), 20mM HEPES, 1% Penicillin/Streptomycin, 10% FBS, pH 7.4). After basal respiration was established, oligomycin (Oligo; 0.02μM) was added followed by FCCP titration (FC; 0.5-5μM), rotenone (Rot; 0.5μM) and antimycin A (Ant; 0.5μM). For permeabilized cell measurements, cells were resuspended in Respiratory Buffer supplemented with creatine (105mM MES potassium salt, 30mM KCl, 8mM NaCl, 1mM EGTA, 10mM KH₂PO₄, 5mM MgCl₂, 0.25% BSA, 5mM creatine monohydrate, pH 7.2). Cells were permeabilized with digitonin (Digi; 0.02mg/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 (20U/mL), ATP (5mM), PCR (1mM, 6mM, 15mM, 21mM), pyruvate (Pyr or P; 5mM), malate (M; 1mM), glutamate (G; 5mM), octanoyl-carnitine (O; 0.2mM), succinate (Succ or S; 5mM) cytochrome C (Cyt C, 10μM), oligomycin (Oligo, 0.02μM), FCCP (FC, 0.5-2μM), rotenone (Rot, 0.5μM), antimycin A (Ant A, 0.5μM), carboxyatractyloside (CAT, 1μM), bongkreikic acid (20μM), 17-AAG (sigma, #100068, 1μM), Gamitrinib TPP hexafluorophosphate (MedChemExpress, #HY-102007A, 1μM).

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 2mM 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 50mM 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 (100mM KCl, 50mM MOPS, 1mM EGTA, 5mM 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 3 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 minutes 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, 21mM). Oligomycin (0.02 μ M) was added to inhibit ATP synthesis and cyanide (CN, 10mM) 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 2.5mL reaction volume in Respiratory Buffer supplemented with AP5A (0.2mM), and digitonin (0.02mg/mL). ATP synthesis and oxygen consumption were determined in permeabilized cells energized with P/M/G/S/O in the presence of 0.1mM ADP. The P/O ratio was then calculated using the ratio of the JATP and the steady state JO_2 , 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 μ g 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 5s 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 μ g, 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 μ g enzyme per 200 μ g 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 μ L 25% acetonitrile/0.1% TFA and 2 $\times$ 500 μ 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 μ L of 200 mM triethylammonium bicarbonate (TEAB), mixed with a unique 10-plex Tandem Mass Tag (TMT) reagent (0.8 mg re-suspended in 50 μ L 100% acetonitrile), and shaken for 4 hr at room temperature (Thermo Fisher). A total of 2 $\times$ 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 μ L 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 μ g) 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 8 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 μ g/ μ 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 μ L (1 μ g), the sample was first trapped on an Acclaim PepMap 100 20 mm $\times$ 0.075 mm trapping column (Thermo Fisher) 5 μ L/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 to 30% acetonitrile using a 2 μ m EASY-Spray PepMap RSLC C18 75 μ m $\times$ 250 mm column (Thermo Fisher) with a column temperature of 55 $^{\circ}$ 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 1 per MS1 scan, with dynamic exclusion enabled for 30s. 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 2 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 ten 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; Naugler and Lesack, 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.

Declarations

Proteomics data availability and software

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.

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AUTHOR CONTRIBUTIONS

M.A.N., K.L.M., J.T.H., H.S.C., C.S., M.K., J.M.M., M.C.C, and K.H.F.-W conceived and designed the study. M.A.N., K.L.M., J.T.H., H.S.C., C.S., M.K., K.A.K., and K.H.F.-W performed the experiments. N.A.V. and D.L. supplied clinical samples. M.A.N, K.L.M., and K.H.F.-W prepared the figures and wrote the manuscript. All authors reviewed and approved the manuscript.

ETHICS DECLARATION

The authors of this manuscript declare no competing interests.

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Figures

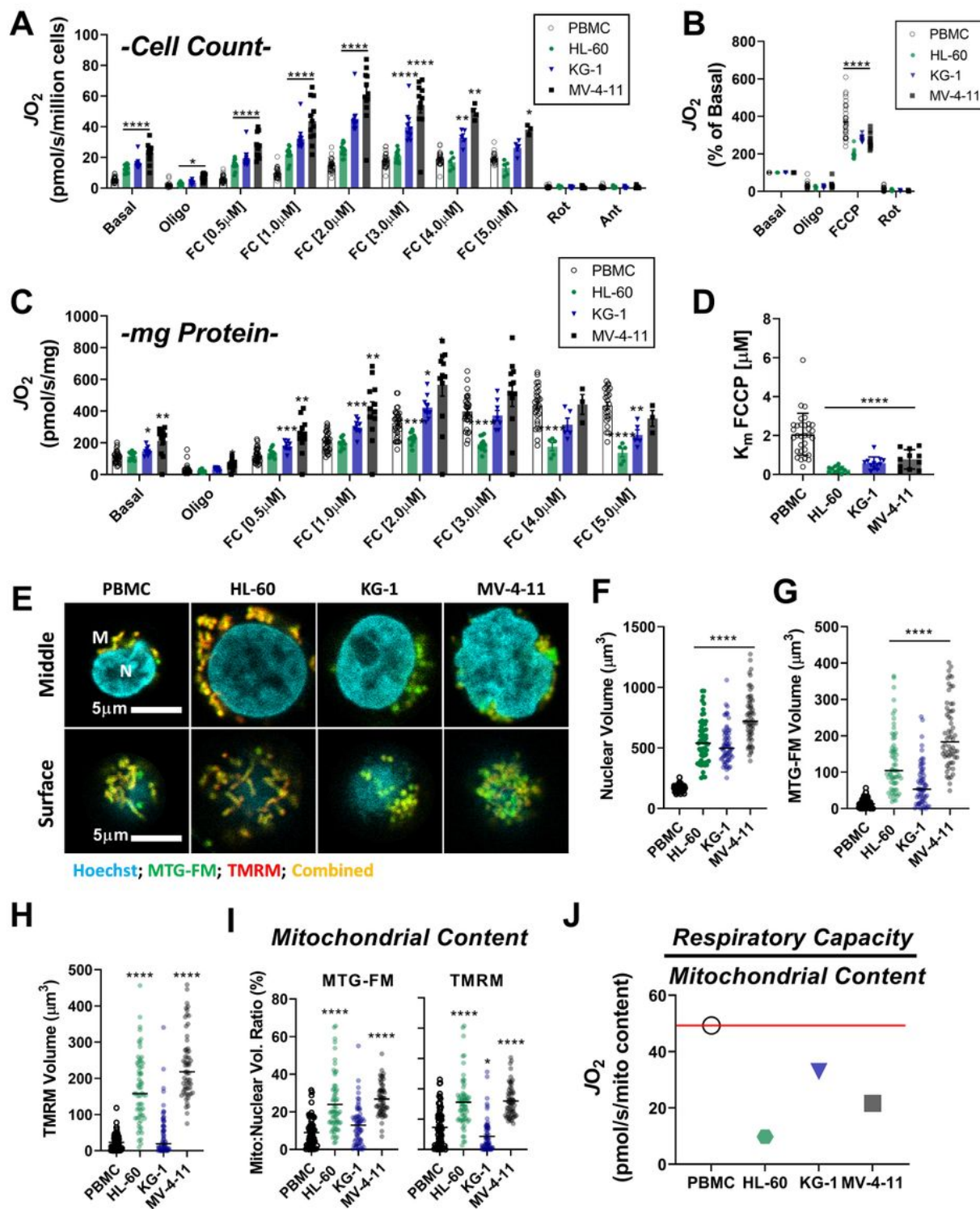


Figure 1

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 volume as measured by MTG-FM (G) and TMRM labelling (H) (n=65 cells/cell type). (I) Ratios of mitochondrial to nuclear volumes assessed by MTG-FM and TMRM labelling. (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). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

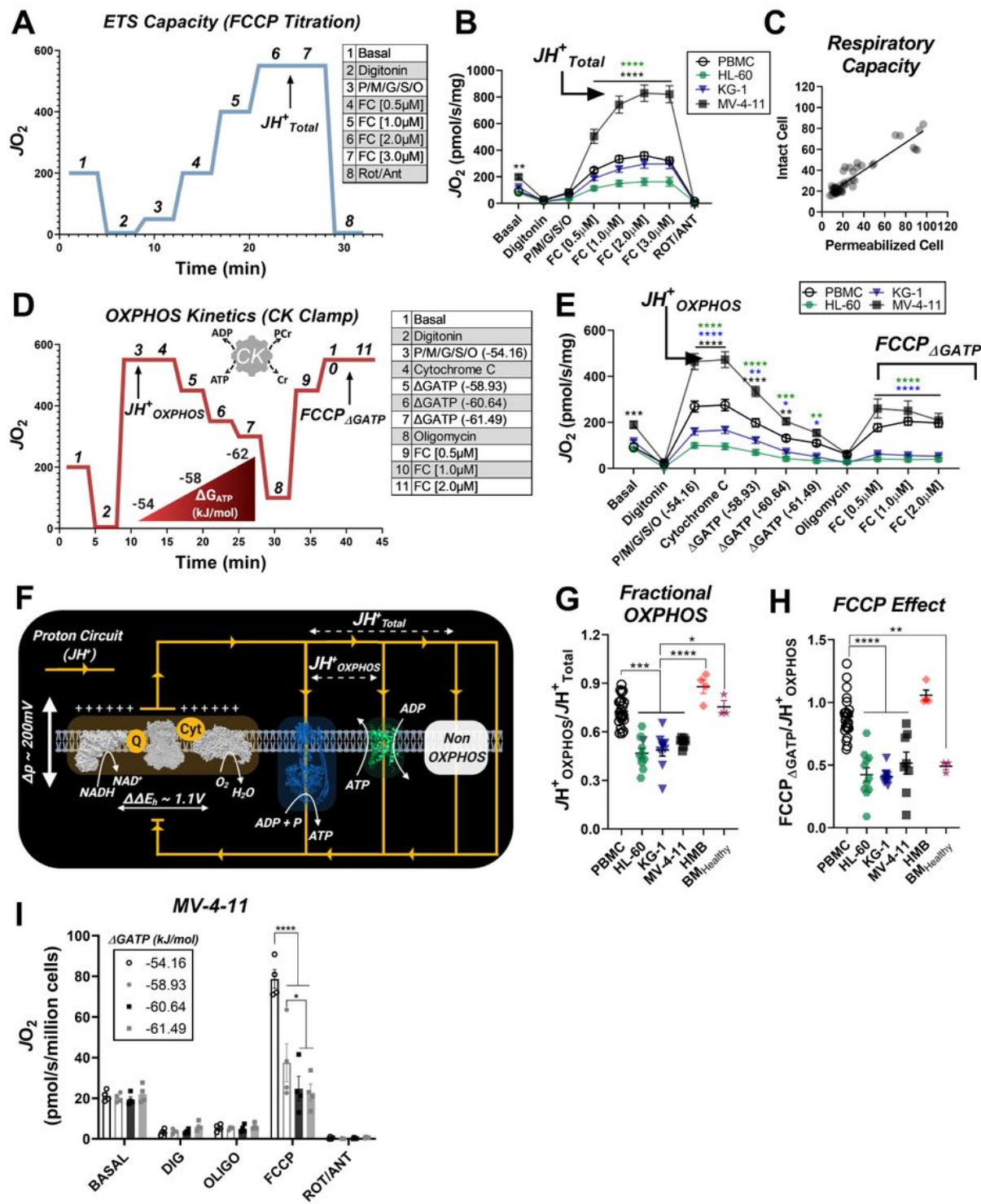


Figure 2
52

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 ($\text{JH}+\text{Total}$). (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) Schematic depicting JO_2 during an OXPHOS kinetics protocol (ΔGATP titration) where point 3 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 ΔGATP (FCCP ΔGATP). (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 ΔGATP 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 BMHealthy. (I) FCCP-stimulated flux was measured under four ATP-free energy (ΔGATP) 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) and one-way ANOVA in (G-I). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

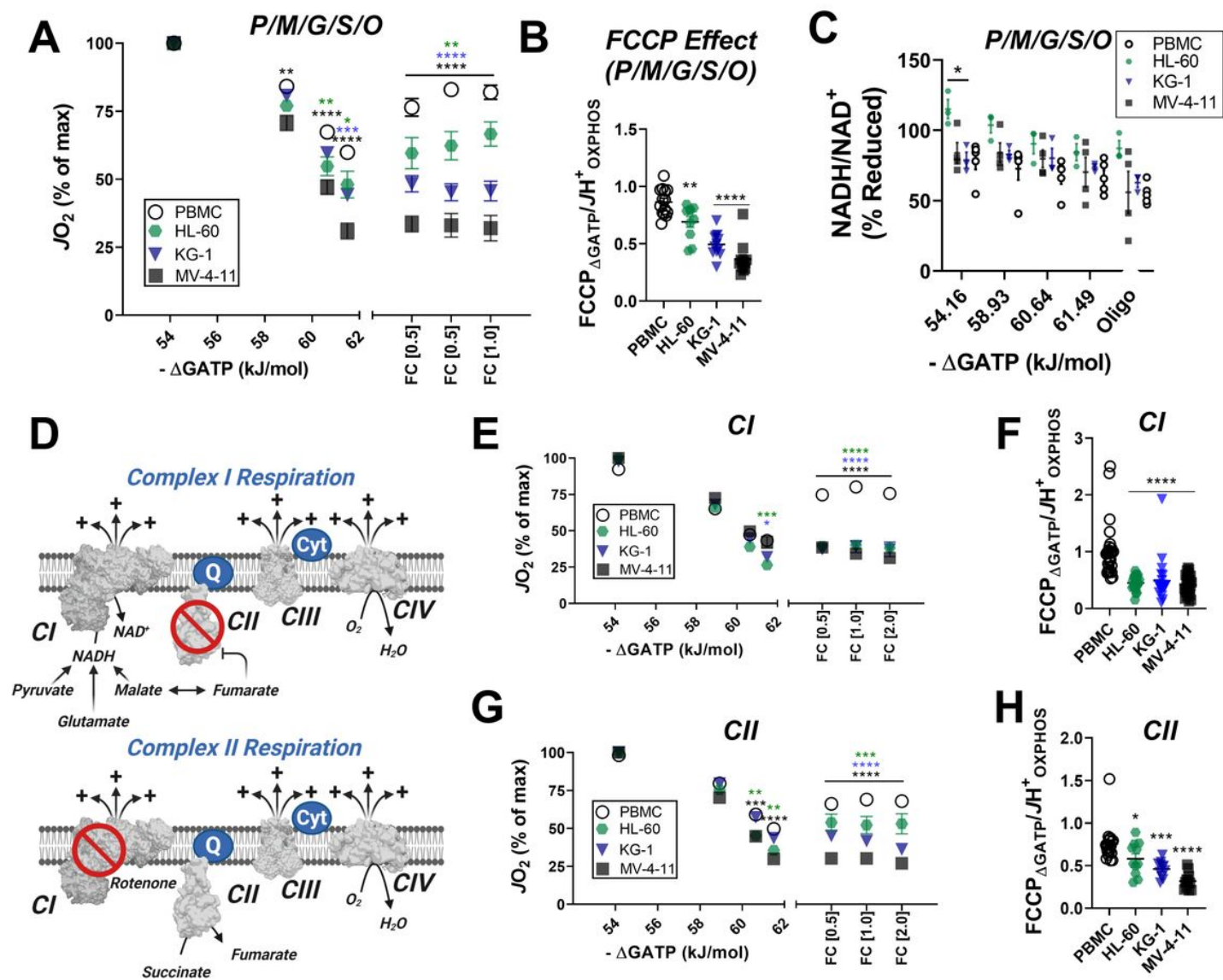


Figure 3

In leukemic mitochondria Δ GATP 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 Δ GATP to JH+OXPHOS from B. (C) Relationship between Δ GATP 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

±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.

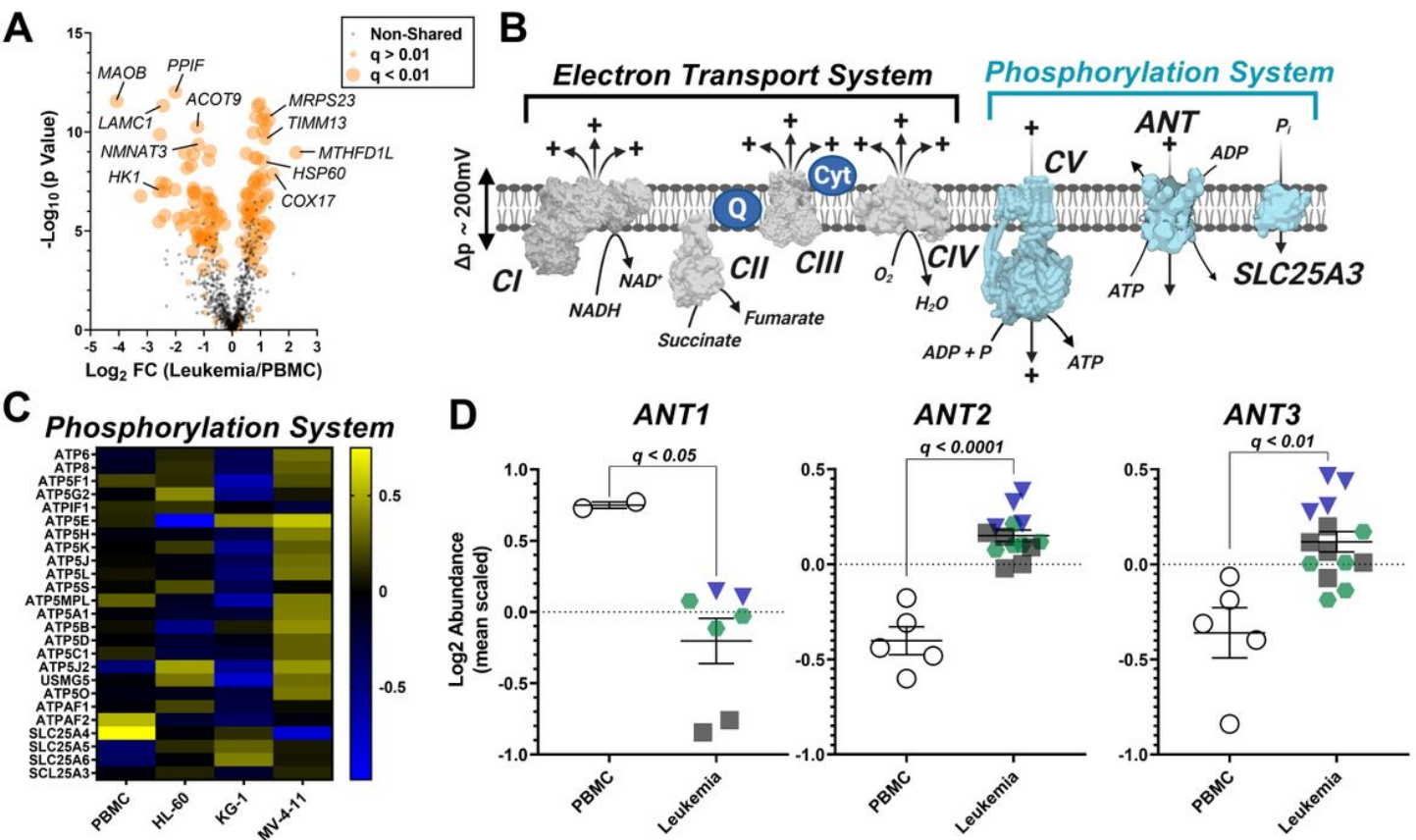


Figure 4

Analysis of mitochondrial proteome reveal disparate expression of ANT isoforms in leukemia. TMT-labelled 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 ±SEM and analyzed by unpaired t-tests with multiple hypothesis correction (Padjusted, Benjamini Hochberg FDR correction, significance cutoff of q < 0.1).

mitochondria isolated from MV-4-11. (A-D) n=4 independent experiments per cell type. (E) Mitochondrial membrane potential ($\Delta\Psi$) in PBMC isolated mitochondria across a Δ GATP span, followed by CV inhibition with oligomycin; n=8 independent experiments. (F) Representative trace of $\Delta\Psi$ in HL-60 isolated mitochondria across a Δ GATP 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 Δ GATP span, followed by CV inhibition with oligomycin; n=9 independent experiments. (I) Mitochondrial $\Delta\Psi$ in isolated mitochondria across a Δ GATP span in the absence of any carbon substrates; n=9 independent experiments. (J) Ratio of group mean $\Delta\Psi$ generated at Δ GATP = -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, E, G, H, I) or paired t-tests (C-D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

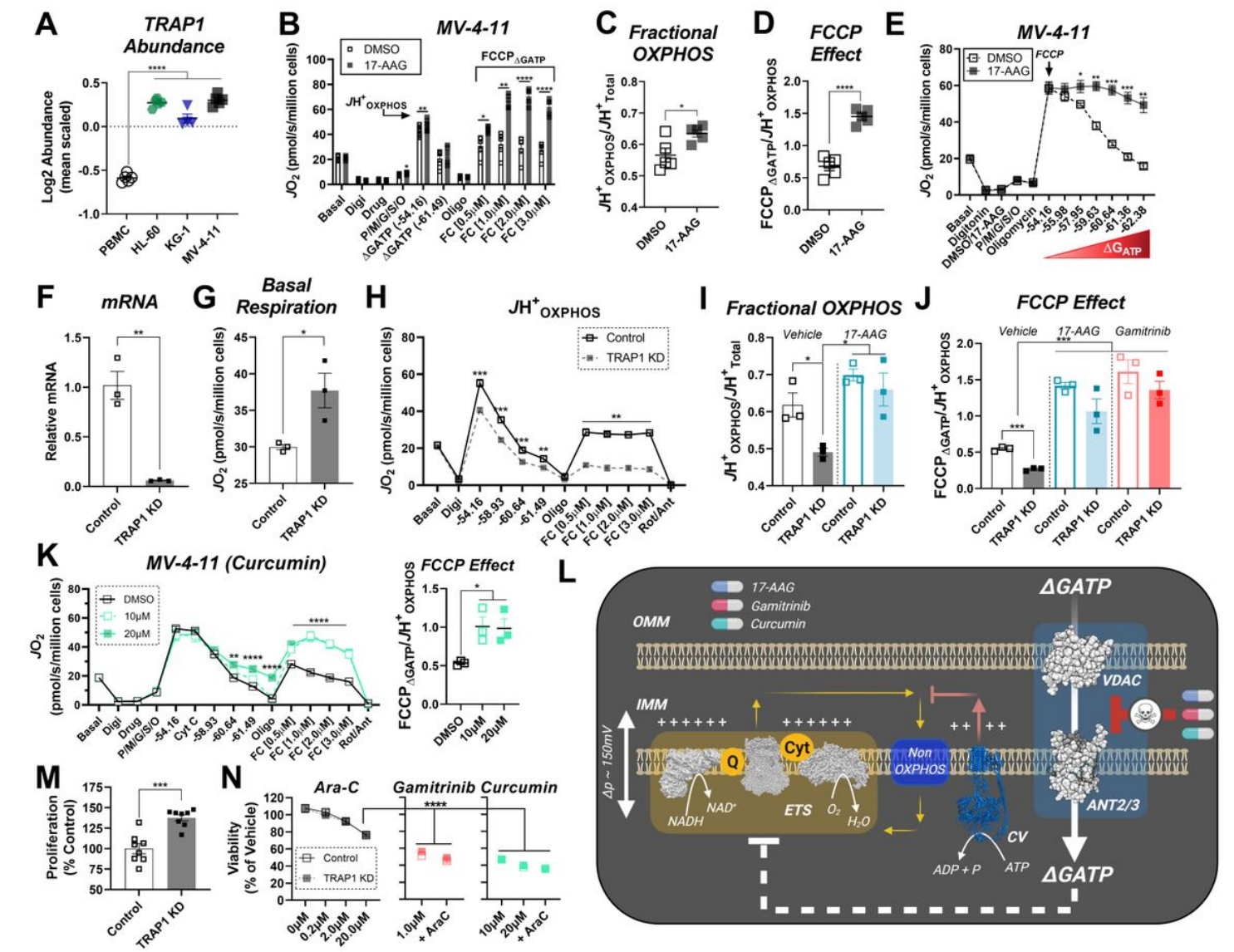


Figure 6

17-AAG and gamitrinib increase fractional OXPHOS and restore ETS flux in the presence of Δ GATP. (A) Log2 Abundance of TRAP1 in PBMC and leukemia cells. (B) Comparison of OXPHOS kinetics in presence of the TRAP1 inhibitor, 17-AAG (15 μ M); 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 μ M). (E) Comparison of respiratory flux inhibition within MV-4-11 cells across a range of Δ GATP in the absence and presence of 17-AAG (15 μ M). Respiration was stimulated by the addition of FCCP (1 μ M), followed by PCR titration to manipulate Δ GATP. (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 μ M), or gamitrinib (1 μ M) in control and TRAP1 KD cells. (K) OXPHOS kinetics in permeabilized MV-4-11 cells in the presence of DMSO or curcumin (10-20 μ M). 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 hours with increasing concentrations of Ara-C. Additional treatments included gamitrinib (1 μ M), 17-AAG (15 μ M) or curcumin (10-20 μ M) either alone or plus Ara-C (20 μ M). Data depicted as viability based on the percentage of vehicle using the propidium iodide assay. (A) n=4/6/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 (A, F-H, M) two-way ANOVA (B, K), one-way ANOVA (I, J, N), paired t-tests (C-D). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

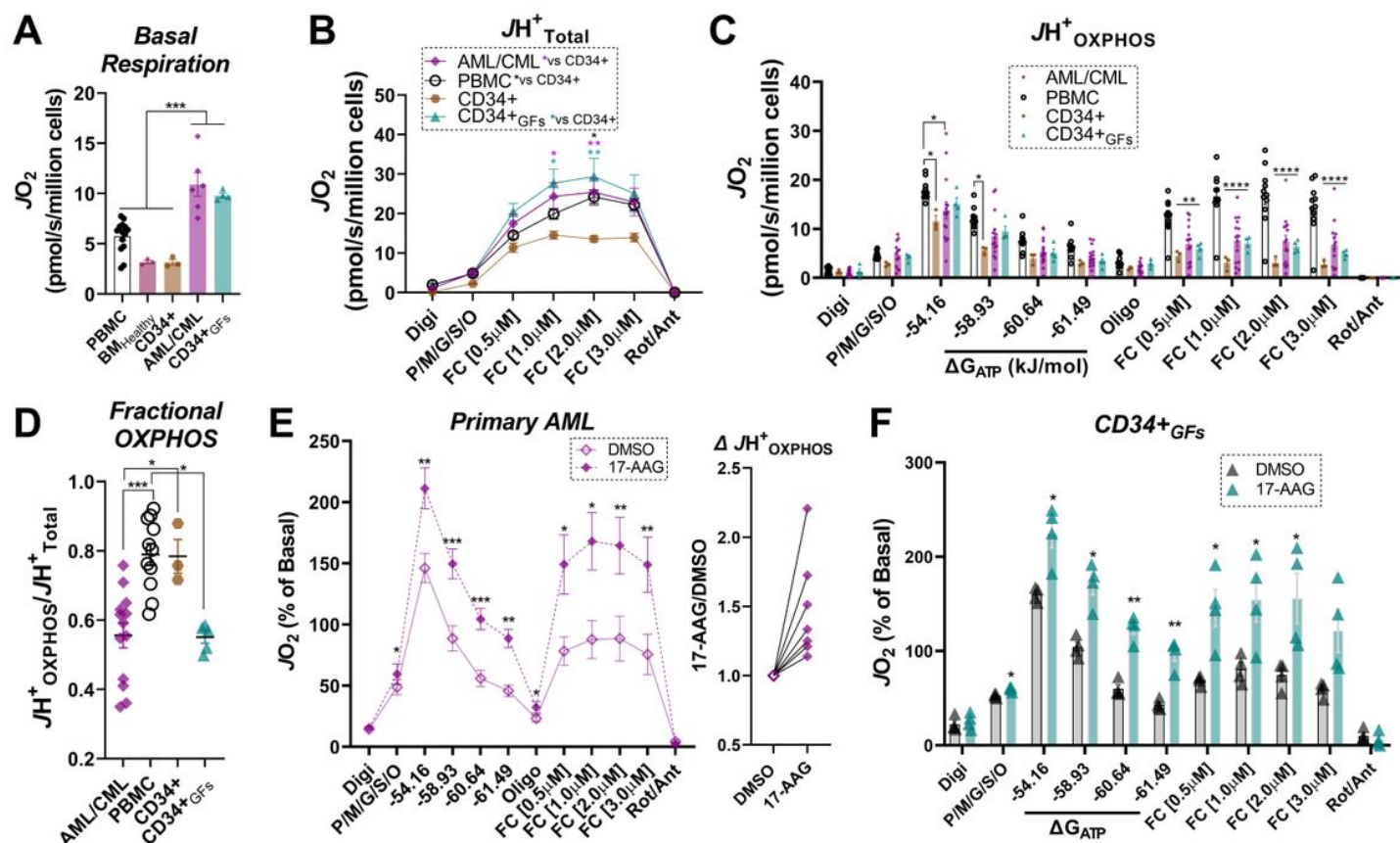


Figure 7

Human primary leukemia is characterized by low Fractional OXPHOS. (A) Basal respiration in intact cells – ‘PBMC’ (age-matched to the primary leukemia samples); ‘BMHealthy’ (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+GFs’ (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+GFs; $n=4$ 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). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

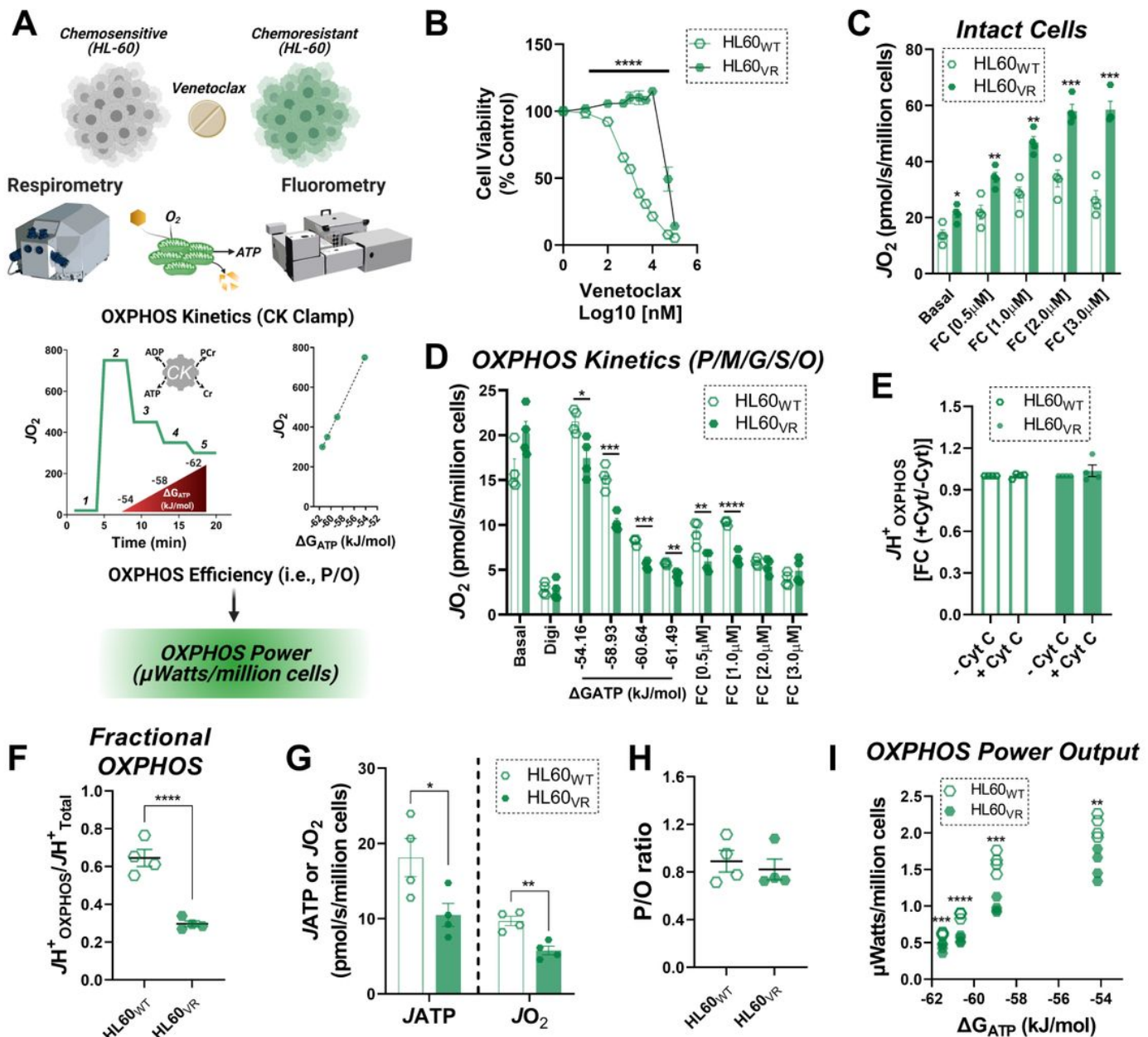


Figure 8

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 48hr 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.

Supplementary Files

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Alterations in Sphingolipid Composition and Mitochondrial Complex I Deficiency Represent Synergistic Therapeutic Vulnerabilities Linked to Vincristine Resistance

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Research

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

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Alterations in sphingolipid composition and mitochondrial complex I deficiency represent synergistic therapeutic vulnerabilities linked to vincristine resistance

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Keywords (6): acute myeloid leukemia, sphingolipids, vincristine, chemotherapy resistance, mitochondrial bioenergetics, HL-60 cells

Abstract

Background: Modifications in sphingolipid (SL) metabolism and mitochondrial bioenergetics are key factors implicated in cancer cell response to chemotherapy, including chemotherapy resistance. *Vinca* alkaloids such as vincristine (VCR), widely used in cancer treatment, are no exception, as their beneficial actions are often supplanted by resistance. In the present work we utilized HL-60 human leukemia cells and a VCR-resistant counterpart, HL-60/VCR cells, to determine potential interplay between SL metabolism and mitochondrial bioenergetics supportive of VCR resistance.

Methods: Cellular SL alterations were assessed via a combination of enzyme immunoblot analysis and lipidomics. Mitochondrial function was evaluated in intact cells, permeabilized cells and isolated mitochondria using a comprehensive diagnostic assay system designed to directly interrogate oxidative phosphorylation (OXPHOS) kinetics. Informed by SL and bioenergetic readouts, experiments were designed to assess the therapeutic efficacy of co-targeting SL metabolism and mitochondrial flux to combat VCR resistance.

Results: Relative to wild-type cells, HL-60/VCR presented with global alterations in SL composition, typified by upregulated expression of sphingosine kinase (SPHK1), which catalyzes formation of sphingosine 1-phosphate (S1P), glucosylceramide synthase (GCS), which catalyzes formation of glucosylceramides (GC), and acid ceramidase, responsible for ceramide hydrolysis. In support of these changes, VCR resistance was also characterized by increases in S1P, several molecular species of ceramide and GC, and changes in sphingomyelin (SM) molecular species. With respect to mitochondria, despite increased basal respiration in HL-60/VCR cells, direct interrogation of the mitochondrial network revealed intrinsic respiratory complex insufficiency, largely localized to complex I (CI). Importantly, forced ceramide accumulation in wild-type cells phenocopied the respiratory insufficiency observed in HL-60/VCR, and co-targeting SL metabolism and CI induced synergistic cytotoxicity in HL-60/VCR.

Conclusions: 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 VCR resistance.

Background

Chemotherapy resistance is a major element underlying treatment failure in cancer. 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 drug resistance 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 treatment of both liquid and solid tumors, and VCR as well as VBL are frequent agents in combination regimens. VCR, the focus of the present study, 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+ AML when administered as A6 peptide functionalized polymersomal vincristine sulfate [4].

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 Ladisch [6] 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 drug resistance.

Herein, we utilized VCR-resistant human leukemia cells (HL-60/VCR) to comprehensively evaluate the interplay between SL metabolism and mitochondrial bioenergetics and explore the therapeutic potential of co-targeting these pathways in drug resistance. 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 maintenance of cellular respiratory kinetics, despite intrinsic limitations in respiratory CI. Interestingly, despite minimal cytotoxicity as single agents, the combination of various inhibitors of SL metabolism with CI inhibition induced synergistic cell death in HL-60/VCR.

Methods

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. All chemicals for mitochondrial analysis were purchased from Sigma.

Cell culture

The human, VCR resistant (multidrug resistant) cell line, HL-60/VCR was provided by A.R. Safa, Indiana University School of Medicine, Indianapolis, IN. Cells were cultured in RPMI-1640 medium, (Life Science Technologies, Carlsbad, CA), 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. This cell line was not tested and authenticated over, and above documentation provided by the ATCC, which includes antigen expression, DNA profile, short tandem repeat profiling, and cytogenic analysis.

Cell viability assays

Cell viability was determined by fluorescence measurement as previously described [8]. 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 minutes, 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 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.

Lipidomics

Lipidomic analysis was conducted using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) as previously described [8]. Briefly, total lipids were extracted from cells after 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 x 100mm C18 CSH column and detected with a Waters Xevo TQ-S MS system.

Immunoblotting

Immunoblotting was conducted as previously described [9,10] 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 1x RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing phosphatase inhibitor cocktail 2, protease inhibitor, and PMSF (1.0 mM), according to the manufacturer's protocol (Sigma Chemical Company). The bicinchoninic acid (BCA) method was employed for determining protein concentrations. Lysates were centrifuged to remove debris and then heated in 1x 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 hr at room temperature and incubated with primary antibodies according to the manufacturer's instructions, at 4°C overnight. The membranes were washed with PBST, followed by incubation with the corresponding HRP-conjugated secondary antibodies according to the manufacturer's instructions, for 1 hr 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).

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 [11,12]. The primers used for real-time PCR were designed by primerbank (<http://pga.mgh.harvard.edu/primerbank/>): (ABCB1: F:TTGCTGCTTACATTACAGGTTTCA, R:AGCCTATCTCCTGTCGCATTA; ABCC1: F:CTCTATCTCTCCCGACATGACC, R: AGCAGACGATCCACAGCAAAA; ABCG2: F:ACGAACGGATTAACAGGGTCA, R: CTCCAGACACACCACGGAT; β -actin: F:GCTGTGCTACGTGCGCCCTG, R:GGAGGAGCTGGAAGCAGCC).

Cellular respirometry (intact and permeabilized)

High-resolution O_2 consumption measurements were conducted using the Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria) in intact and digitonin permeabilized HL-60 and HL-60/VCR cells. For each intact cell experiment, cells were centrifuged at 1,000 rpm for 7 min at room-temperature, washed in PBS, centrifuged once more and then suspended in assay media at a cell concentration of $2-3 \times 10^6$ 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 μ g/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 1,000 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 $2-3 \times 10^6$ 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 saturating carbon (pyruvate/malate/glutamate/succinate/octanoyl-L-carnitine; P/M/G/S/O; 5 mM/2 mM/5 mM/5 mM/0.2 mM) and flux was stimulated with FCCP titration (0.5-3.0 μ M). Cytochrome C (10 μ M) was added to check the integrity of the outer mitochondrial membrane and then respiration was inhibited with rotenone (Rot; 5 μ M) and antimycin A (Ant A; 5 μ M). Data were normalized to viable cell count and expressed as pmol/s/million cells.

Mitochondrial isolation

As described previously [13] and with some modifications, 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 (100mM KCl, 50mM MOPS, 1mM EGTA, 5mM $MgSO_4$, 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 this process was repeated for a total of 3 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 spun down at 10,000 x g. Final resuspension of the mitochondrial pellet was done in 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 functional assessment (isolated mitochondria)

High-resolution O_2 consumption rate (JO_2) measurements were conducted using the Oroboros Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria). The base assay buffer was identical to that used in permeabilized cells (105mM potassium-MES, 30mM KCl, 10mM KH_2PO_4 , 5mM $MgCl_2$, 1mM EGTA, 2.5g/L BSA and 5mM creatine monohydrate; pH=7.2). As previously described [13,14], 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 [14]. To begin, mitochondria were added to 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 (5mM), PCr (1mM), and CK (20U/ml), simulating a 'maximal' demand for ATP re-synthesis. Sequential additions of PCr to 6, 15, and 21mM were then performed to gradually lower the ATP demand state and assess OXPHOS conductance. Four substrate conditions were used: Pyr/M (5mM/1mM); glutamate/M (G/M; 10mM/1mM); S/Rot (10mM/0.5 μ M); P/M/G/S/O (5mM/1mM/5mM/5mM/0.2mM; Multi). For experiments involving dihydroorotate (DHO), DHO (2mM) was added to isolated mitochondria in the presence of saturating ADP (2mM). 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 (*) calculated for that sample, with the final values expressed as pmol/s/mg protein* (see proteomics methodology below).

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 minutes without substrate to induce 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, 21mM). Oligomycin (0.02 μ M) was added to inhibit ATP synthesis and cyanide (CN, 10mM) 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 H₂O₂ emission

Mitochondrial peroxide emission was assessed in Respiration buffer, supplemented with amplex ultra red (5 μ M), horse radish peroxidase (HRP; 1U/mL), and superoxide dismutase (25U/mL), as described previously [15]. Experiments were run in the presence of mitochondria alone, Pyr/M (5mM/2mM), and the CK clamp. Relative fluorescence was converted to pmol of H₂O₂ using an H₂O₂ standard curve, as previously described [15].

Mitochondrial lysis and sample prep for label-free proteomics

Isolated mitochondria were lysed in urea lysis buffer (8M urea in 40mM Tris, 30mM NaCl, 1mM CaCl₂, 1 x cComplete ULTRA mini EDTA-free protease inhibitor tablet; pH=8.0), as described previously [13,16]. The samples were subjected to three freeze-thaw cycles, and sonication with a probe sonicator in three 5s bursts (Q Sonica #CL-188; amplitude of 30). Samples were centrifuged at 10,000 x g for 10min at 4°C. Protein concentration was determined by BCA. Equal amounts of protein were reduced with 5mM DTT at 37°C for 30min,

and then alkylated with 15mM iodoacetamide for 30min in the dark at room temperature. Unreacted iodoacetamide was quenched with DTT (15mM). Initial digestion was performed with Lys C (ThermoFisher Cat# 90307; 1:100 w:w) for 4hr at 37°C. Following dilution to 1.5M urea with 40mM Tris (pH=8.0), 30mM NaCl, 1mM 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 4,000 x g for 10min at 4°C. Supernatant containing soluble peptides was desalted, as described previously [16] and then eluate was frozen and subjected to speedvac vacuum concentration.

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 0.25µg/µL. 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, 4µL (1µg) of sample was first trapped on an Acclaim PepMap 100 20mm × 0.075mm trapping column (ThermoFisher Cat# 164535; 5µL/min at 98/2 v/v water/acetonitrile with 0.1% formic acid). Analytical separation was performed over a 95min gradient (flow rate of 250nL/min) of 4-25% acetonitrile using a 2µm EASY-Spray PepMap RSLC C18 75µm × 250mm column (ThermoFisher Cat# ES802A) with a column temperature of 45°C. MS1 was performed at 70,000 resolution, with an AGC target of 3x10⁶ ions and a maximum injection time (IT) of 100ms. 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 20s. Precursor ions isolation window was 1.5m/z and normalized collision energy was 27. MS2 scans were performed at 17,500 resolution, maximum IT of 50ms, and AGC target of 1x10⁵ ions.

Data analysis for label-free proteomics

As described previously [16], 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 [17]. 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 described [13] 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.

Statistical evaluation

All mass spectrometry 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 [18,19].

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 [13]. 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: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Unless otherwise stated, figures were generated using GraphPad Prism 8 software (Version 8.4.2) or Biorender.

Results

HL-60/VCR cell characteristics

The cell line used in the present work, 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 [20]. 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 more resistant to VCR compared to the VCR-naïve, wt counterpart (**Fig. 1A**), with IC₅₀ values of 10.6 (12.8 μ M) and 0.001 μ g/ml (1.2 nM), respectively, translating to a >10,000-fold difference in sensitivity. 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). 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 (**Fig. 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 (multidrug resistance protein, MRP), and (25-fold) ABCG2 (breast cancer resistant protein, BCRP) compared to wt cells (**Fig. 1C**).

Alterations in SL enzyme expression accompany VCR resistance

Because of the key role that SL's play in cancer growth [21,22] and response to chemotherapy [23], we were interested in assessing the impact of VCR resistance on expression of several enzymes involved in SL metabolism. Firstly, levels of total SPHK1 were elevated in HL-60/VCR cells compared to the wt counterpart (**Fig. 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 (**Fig. 1D-E**). This change in glycosphingolipid metabolism has been shown to accompany MDR [24]. Acid

ceramidase (AC) expression was also enhanced in HL-60/VCR compared to wt cells (**Fig. 1D-E**), as previously demonstrated in HL-60/VCR cells [25]. Lastly, although expected, HL-60/VCR cells exhibited markedly elevated levels of P-gp (**Fig. 1D-E**).

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 (**Fig. 1F**). 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 (**Fig. 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 (**Fig. 1G**). The increases in ceramide molecular species were mirrored by concomitant increases in corresponding GC species, as shown for example in C14:0, C16:0, C22:1, C24:1, and C26 molecular species (**Fig. 1G**). Several molecular species of SM were decreased in HL-60/VCR cells versus wt, as demonstrated in C18, C20, C22, and C24 (**Fig. 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 (**Fig. 1G**). In summary, the data in **Fig. 1** demonstrate widespread alterations in SL composition occur with VCR resistance, and interestingly, C22:1 ceramide appears to be sequestered in GC and SM.

Intact cellular respirometry in VCR resistance reveals increased mitochondrial content.

Mitochondria play a key role in cancer cell biology and are increasingly recognized as potential targets in cancer therapy [26]. Mitochondria are also important downstream targets of ceramide [27,28]. Considering the dynamic changes in SL profiles and enzyme expression patterns that accompanied VCR resistance, 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 2-fold higher in HL-60/VCR compared to wt cells (**Fig. 2A**, basal abbreviated by 'Basal'). Interestingly, the ability of oligomycin to lower respiration was blunted in HL-60/VCR (**Fig. 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 (**Fig. 2B**), confirming P-gp mediated efflux of oligomycin. Maximal uncoupled respiration was nearly 2-fold 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 (**Fig. 2A**, 'Rot/Ant'). Correcting for non-mitochondrial JO_2 , maximal uncoupled respiration was higher in HL-60/VCR (**Fig. 2C**), consistent with previous findings linking increased mitochondrial content to AML chemoresistance [2,29]. In our model system, HL-60/VCR were continuously cultured in the presence of 1.0 μ g/mL (1.2 μ M) VCR. To determine 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 (**Fig. 2A**, 'VCR^{+VCR}' vs 'VCR^{-VCR}').

Comprehensive mitochondrial phenotyping in VCR resistance reveals functional limitations in CI

Increased basal respiration and higher mitochondrial mass are commonly observed bioenergetic phenotypes associated with drug-resistance [2,30]. Although such phenotypes are presumed to reflect an increased reliance on oxidative ATP production, how the mitochondrial network is intrinsically remodeled to support the drug resistant phenotype remains poorly understood. To begin to address this gap, we first sought to validate our intact cell respirometry data by directly measuring mitochondrial respiratory capacity in substrate replete permeabilized cells. Surprisingly, despite higher basal respiration (**Fig. 2D 'Cells'**), maximal respiratory capacity induced via FCCP titration was decreased, rather than increased, in HL-60/VCR cells (**Fig. 2D 'FCCP'**), suggesting intrinsic dysregulation across the VCR mitochondrial network. Similar results were observed in HL-60/VCR cultured for seven days in VCR-free culture media (**Fig. 2D**). Importantly, the addition of cytochrome C did not increase respiratory flux in any group (**Fig. 2D**), indicating that the respiratory limitations in VCR were not attributable to outer membrane permeabilization. To probe deeper, we isolated mitochondria from wt and VCR resistant HL-60 cells and comprehensively evaluated mitochondrial bioenergetic function. Using this workflow, we can model the impact of a physiological ATP free energy (i.e., ΔG_{ATP}) span on respiratory flux [14,31]. Experimental manipulation of ΔG_{ATP} is made possible via a modified version of the creatine kinase (CK) energetic clamp [32]. 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.

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 (**Fig. 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 (**Fig. 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., 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 (**Fig. 3D**), despite no

differences in the presence of S/Rot (**Fig. 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 (**Fig. 3F**). Under these conditions, G/M supported JO_2 was again reduced in HL-60/VCR (**Fig. 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 (**Fig. 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 (**Fig 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 (**Fig. 3H-I**). Results revealed an over 10-fold increase in H_2O_2 emission in HL-60/VCR under all conditions (**Fig. 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 (**Fig. 3H-I**). Such results are consistent with elevated NAD(P)H oxidase activity in HL-60/VCR mitochondria and provide a potential explanation for the increased rotenone/antimycin insensitive respiration observed in intact cells (**Fig. 2A**).

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 < 0.01) comparing wt to HL-60/VCR (**Supplementary Table 1**). Focusing in on the respiratory complexes exclusively, the vast majority of CI subunits were specifically downregulated in HL-60/VCR (**Fig. 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 (**Fig. 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 (**Fig. 4B-C**). Consistent with partial CI loss-of-function, HL-60/VCR cells were found to be highly sensitized to rotenone-induced cytotoxicity (**Fig 3C**). Taken together, these findings reveal CI deficiency as a previously unidentified bioenergetic vulnerability that accompanies VCR resistance.

Forced ceramide accumulation in wt cells phenocopies respiratory complex insufficiency observed with VCR resistance.

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 present

study, VCR resistance was associated with substantial increases in a variety of ceramide molecular species (**Fig. 1G**). Based on this, we hypothesized that CI deficiency may be consequential to ceramide accumulation in HL-60/VCR. To determine whether elevated ceramide levels seen in HL-60/VCR cells infringe on mitochondrial function, we grew HL-60 wild-type cells for extended periods (7-days) in 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 respiration in the presence of Pyr/M in the absence of adenylate stimulation (**Fig. 5A**; 'Pyr/M'), stimulation of near-maximal OXPHOS flux with the CK clamp revealed a nearly 2-fold decrease in respiratory flux in cells exposed to both C6 and CNL (**Fig. 5A**; ' Δ GATP'). The addition of cytochrome C did not impact respiration, thus confirming functional integrity of the outer mitochondrial membrane across all groups (**Fig. 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 (**Fig. 5A**; 'Succ'). Similar differences in respiration regardless of the carbon substrate strongly suggests that the respiratory impairments induced by C6/CNL are most likely attributable to ETS dysregulation. Such findings are highly reminiscent to those observed in HL-60/VCR and thus support a model whereby mitochondrial dysregulation, primarily localized to the ETS (e.g., CI), may be secondary to altered SL metabolism.

Therapeutic synergy induced by co-targeting SL metabolism and respiratory CI in HL-60/VCR.

Given that sweeping alterations in SL metabolism were observed alongside bioenergetic remodeling in HL-60/VCR, we sought to explore the synergistic potential of targeting respiratory complex I in conjunction with pharmacological ceramide delivery (e.g., CNL) and/or inhibition of SL metabolism. In these experiments, CI was inhibited using the well characterized biguanides metformin or phenformin [35]. Induction of cellular ceramides was achieved by adding CNL or by blocking AC, SPHK1, or GCS with SACLAC (5 μ M), SK1-i (20 μ M), or PDMP (20 μ M), respectively [36–41]. In HL60/VCR, 48hr exposure to CNL (20 μ M), nanoliposome devoid of ceramide (ghost; 20 μ M), phenformin (0.5mM), or phenformin plus ghost had no impact on cell viability (**Fig. 5B**). Remarkably, despite no change in viability for any of the single agents, 48hr exposure to both CNL and phenformin led to a dramatic (>80% of control) decrease in cell viability (**Fig. 5B**). To further the therapeutic efficacy of targeting mitochondrial CI alongside SL metabolism, experiments were designed to determine the ability of metformin or phenformin to boost cytotoxicity of other agents that can act to increase ceramide levels (inhibitors of SL metabolism). 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 (**Fig. 5C-E**). However, the combination of each SL enzyme inhibitor with either metformin or phenformin boosted cytotoxicity, relative to vehicle or single agent conditions (**Fig. 5C-E**). This relationship was particularly evident for the AC inhibitor SACLAC, 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 hours (**Fig. 5C**). 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 VCR resistance.

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 [21,42,43]. Gouaze-Andersson and Cabot [44] 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 levels of S1P and C16 ceramide. In agreement with recent observations in daunorubicin and cytarabine resistant AML [29], VCR resistance herein was accompanied by substantial alterations in SL composition. The novelty of the present findings stems from our focus on VCR resistance and its impact on both SL's and mitochondrial function. Moreover, given our recent findings linking SL metabolism to mitochondrial bioenergetics [7,29], 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 [46], specifically designed to comprehensively evaluate OXPHOS efficiency and capacity, relative to the underlying mitochondrial proteome. Using this platform, we made the exciting discovery that VCR resistance is accompanied by intrinsic mitochondrial remodeling, typified by specific limitations in respiratory CI. These changes were subsequently found to sensitize VCR resistant cells 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 VCR resistance 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 [10]. 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 recycling of sphingosine via the salvage pathway of ceramide synthesis [47]. Interestingly, 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 [24,48–50]. 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 work of Tan et al [51] and Morad et al [52] 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, their growth rate was considerably lower than HL-60 cells. This biology could be related to S1P receptor status in HL-60/VCR cells, 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 [24]. Thus, the association of GCS and GC levels with drug resistance [53–55] has solid footing. Of further relevance, Shabbits and Mayer [56] have shown that ceramide sensitivity in breast cancer cells is modulated by P-gp, and using vincristine as a ceramide generator, Olshefski and Ladisch [6] have shown that GCS inhibition boosts vincristine efficacy.

In addition to changes in SL metabolism, VCR resistance in the present study was accompanied by extensive mitochondrial remodeling. In comparison to wt cells, HL-60/VCR 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 **Fig. 5** 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 **Fig. 1G**), the inclusion of either SACLAC or PDMP would be expected to drive ceramide to cytotoxic levels [29,36,57,58]. 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 (see **Fig. 4B-E**) 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 HL-60/VCR cells, by cell supplementation with either C6 or CNL.

Conclusions

In conclusion, increases in SPHK1, GCS, and AC expression that accompany VCR resistance 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 induction of autophagy for survival [59]. These factors, in conjunction with the intrinsic mitochondrial remodeling allied with VCR resistance, promote a resilient cancer phenotype, but also reveal(s) opportunities for design of novel, targeted therapeutics [21,60–62]. Our

approach of leveraging comprehensive bioenergetic phenotyping to unveil actionable cancer-specific mitochondria targets holds great potential as a novel drug-discovery platform centered upon mitochondrial precision medicine in drug resistance that can easily be adapted across cancer types.

DECLARATIONS

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Competing interest: Authors declare that they have no conflicts of interest with the contents of this article. MK is Chief Medical Officer and cofounder 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.

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Figure Legends

Fig. 1. Alterations in SL metabolism underlie 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 hr. Viability was determined by PI staining. (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 $\pm$ SEM, (A-B) N=6/group, (C-F) N=3/group, (G) N=4-7/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Fig. 2. Increased basal and maximal respiration accompanies VCR resistance. (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 μ M), rotenone (Rot), and antimycin A (Ant). (B) FCCP stimulated respiration corrected for oxygen consumption remaining following rotenone/antimycin. (C) Percent decrease in basal respiration induced by oligomycin in wt cells, as well as vehicle or verapamil (10 μ M) treated HL-60/VCR cells. (D) Oxygen consumption in digitonin (0.02mg/mL) permeabilized cells in the absence of substrates (Digi), as well as following the addition of saturating substrate (Multi; pyruvate/malate/glutamate/octanoyl-L-carnitine/succinate), FCCP titration, cytochrome C (Cyt), and Rot/Ant. All experiments were conducted in VCR-free medium. Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean $\pm$ SEM, (A, B, D) N=4-10/group, (C) N=3-5/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

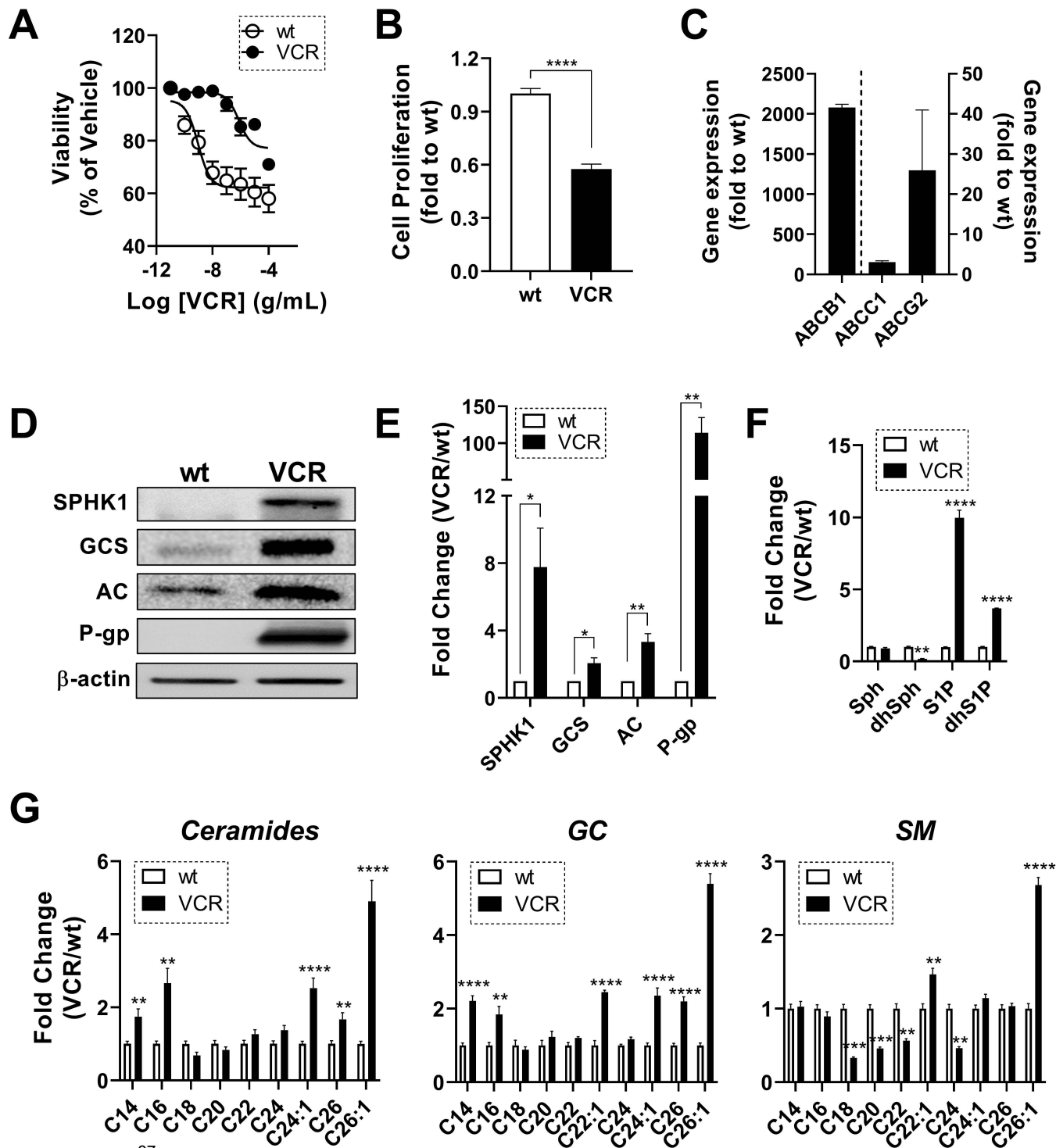
Fig. 3. Comprehensive mitochondrial phenotyping reveals specific CI deficiency in HL-60/VCR. All experiments were performed in isolated mitochondria. (A) Relationship between JO_2 and ATP free energy (ΔG_{ATP}) clamped with the CK clamp in mitochondria energized with Multi. (B) Respiratory conductance – 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 depicts 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<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

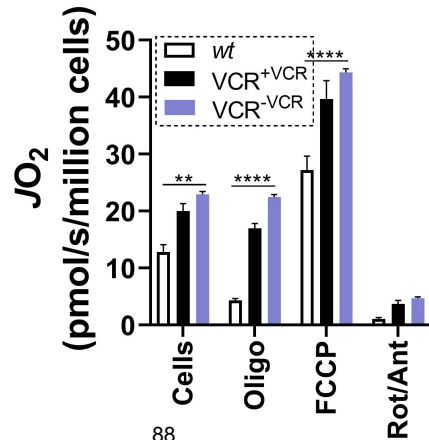
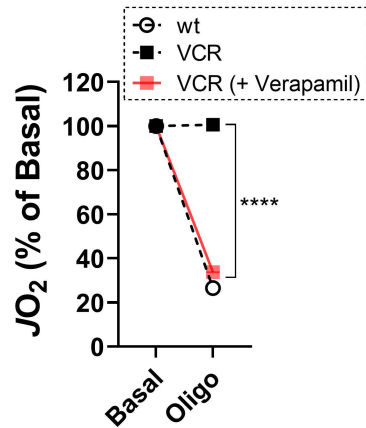
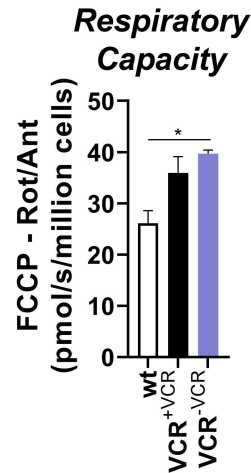
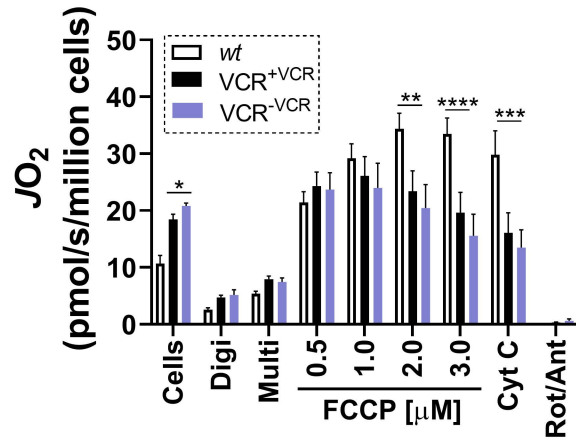
Fig. 4 Decreased CI expression underlies VCR resistance. (A) Label-free nLC-MS/MS was performed on mitochondrial lysates from each sample. Volcano plot depicting changes in the mitochondrial proteome between HL-60/VCR and wt. Protein subunits of each respiratory complex (CI, CII, CIII, CIV) are indicated by color. (B) Cartoon depicting CI inhibition by rotenone. (C) Cell viability in response to 24 hours 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/group, (C) N=6/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

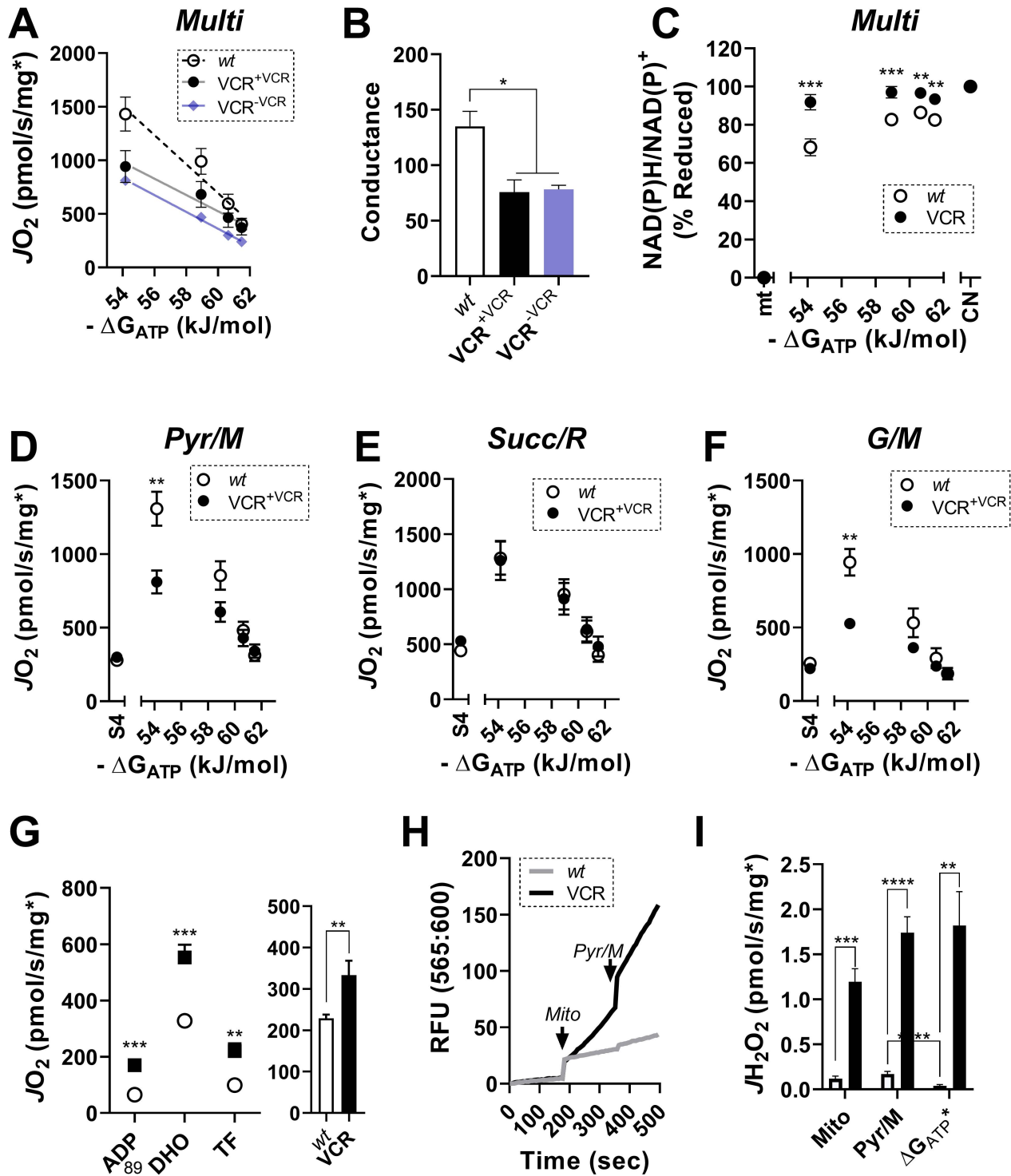
Fig. 5. Mitochondrial CI inhibition combined with various ceramide analogues results in synergistic cytotoxicity. (A) Respiration in permeabilized HL-60 wt cells following exposure to vehicle C6 (10 μ M) or CNL (10 μ M) for 7-days. Cells were permeabilized with digitonin (0.02mg/mL) and respiration was assessed in response to the indicated additions. Data normalized to viable cell count. (B-E) All experiments were done in HL-60/VCR cells. (B) Cell viability, determined by viable cell count, in HL-60/VCR cells exposed for 48hr to CNL (20 μ M), ghost (20 μ M), phenformin (0.5mM), CNL (20 μ M) + phenformin (0.5mM), or ghost (20 μ M) + phenformin (0.5mM). (C-E) Cell viability in response to 24 hours of exposure to SACLAC (5 μ M), Ski-1 (20 μ M), PDMP (20 μ M), metformin (5mM) or phenformin (0.5mM). Metformin and phenformin were administered alone or in combination with SACLAC, Ski-1, or PDMP. 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, C-E) N=3/group, (B) N=6-9/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

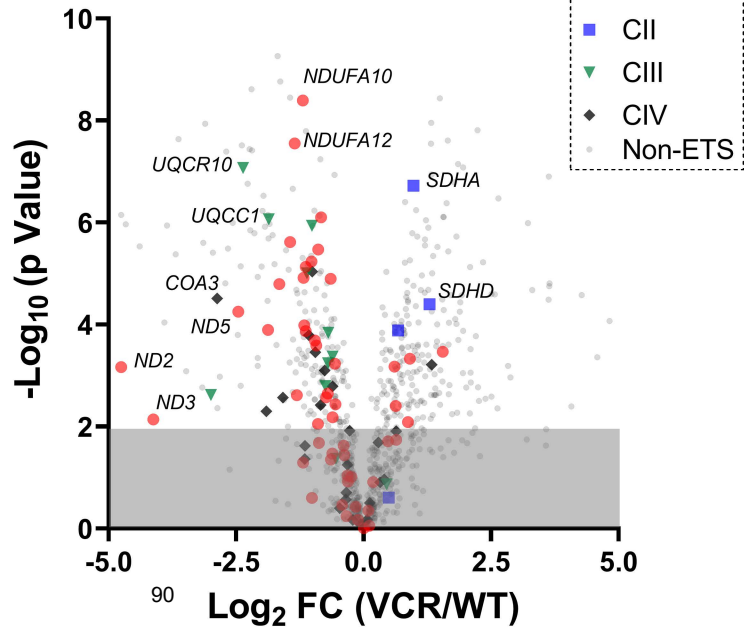
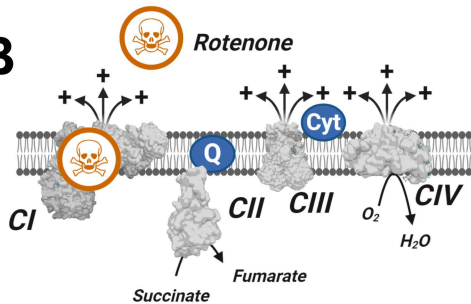
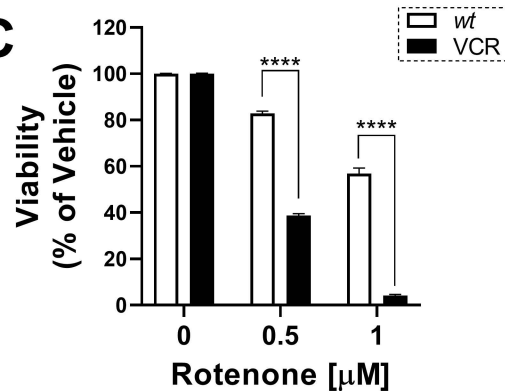
SUPPLEMENTAL TABLES

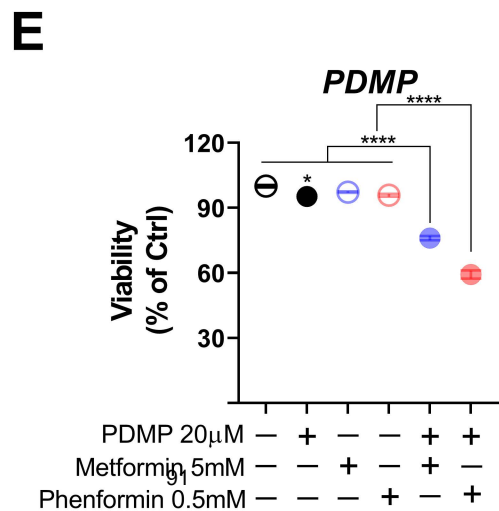
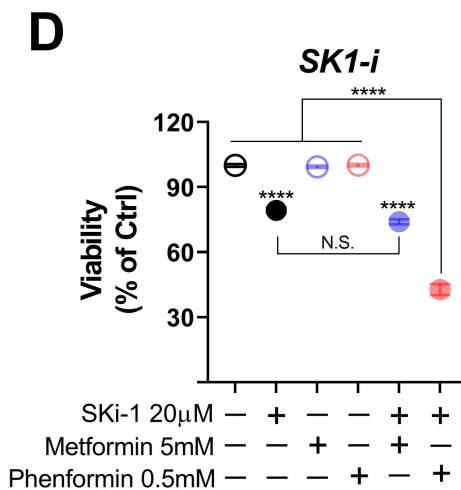
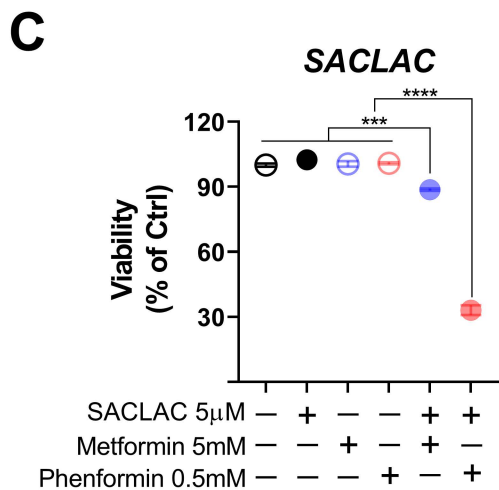
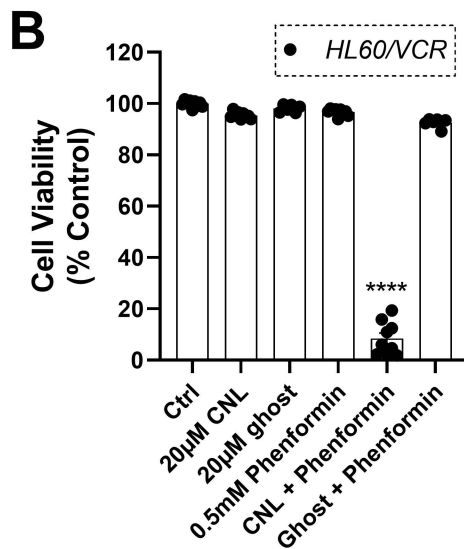
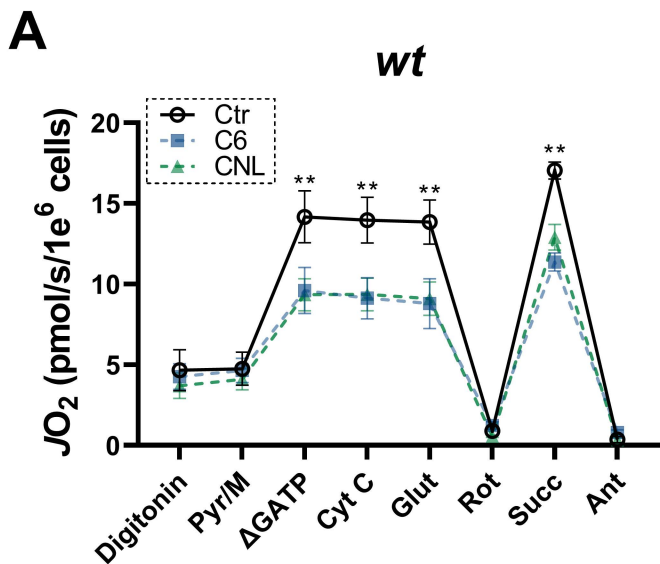
Supplement Table 1 – (A) Exported results from PDv2.2 (Proteome ID: UP000005640). (B) Analyzed master protein expression and calculation of sample-specific MEF (data were searched against the Uniprot Homo Sapiens reference proteome). (C) Exported results from PDv2.2 (MitoCarta 2.0 Database). (D) Analyzed mitochondrial master protein expression.



A**Intact Cells****B****C****D****Permeabilized Cells**



A**ETS Proteome****B****C**



Figures

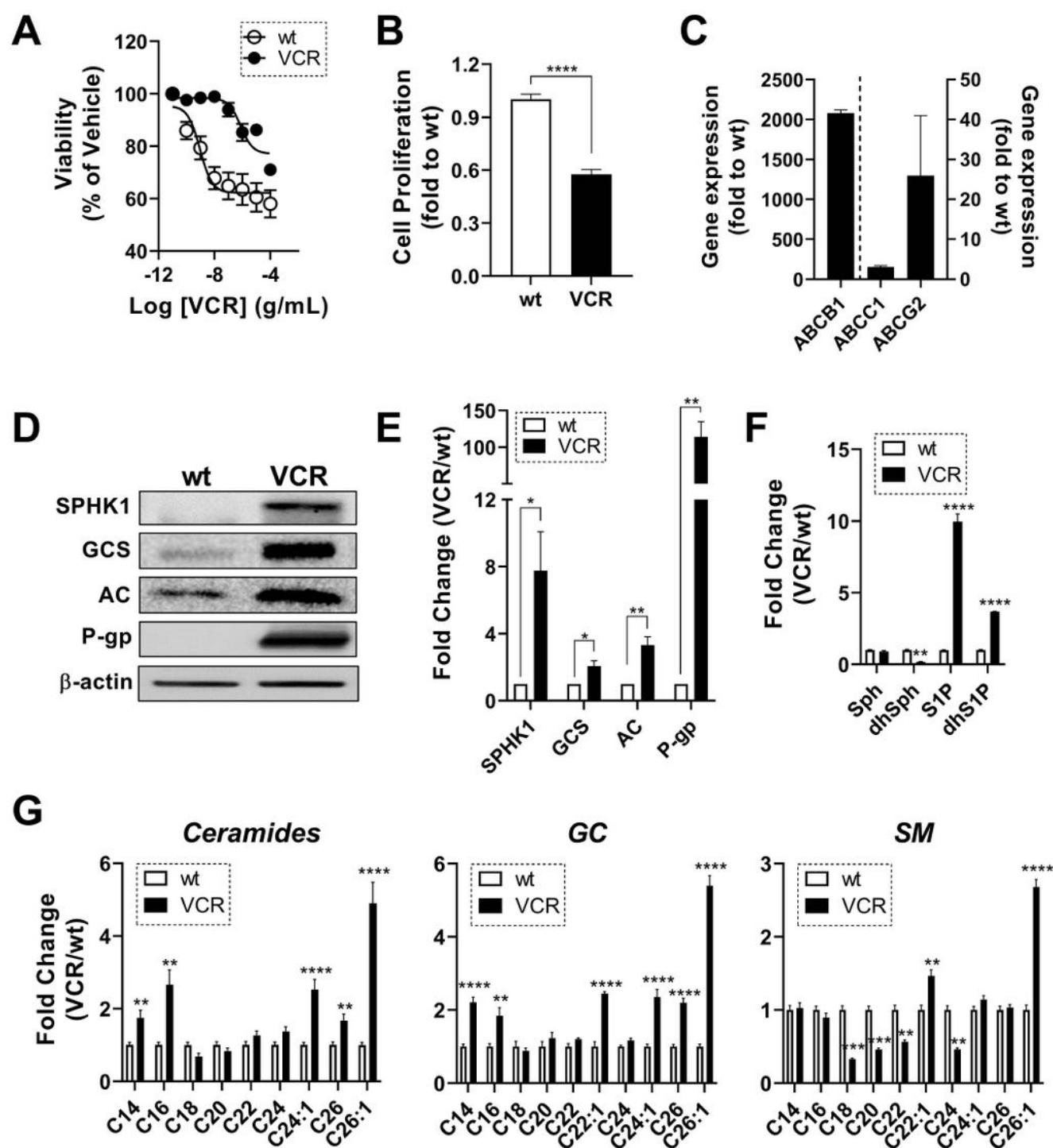


Figure 1

Alterations in SL metabolism underlie 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 hr. Viability was determined by PI staining. (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 $\pm$ SEM, (A-B) N=6/group, (C-F) N=3/group, (G) N=4-7/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

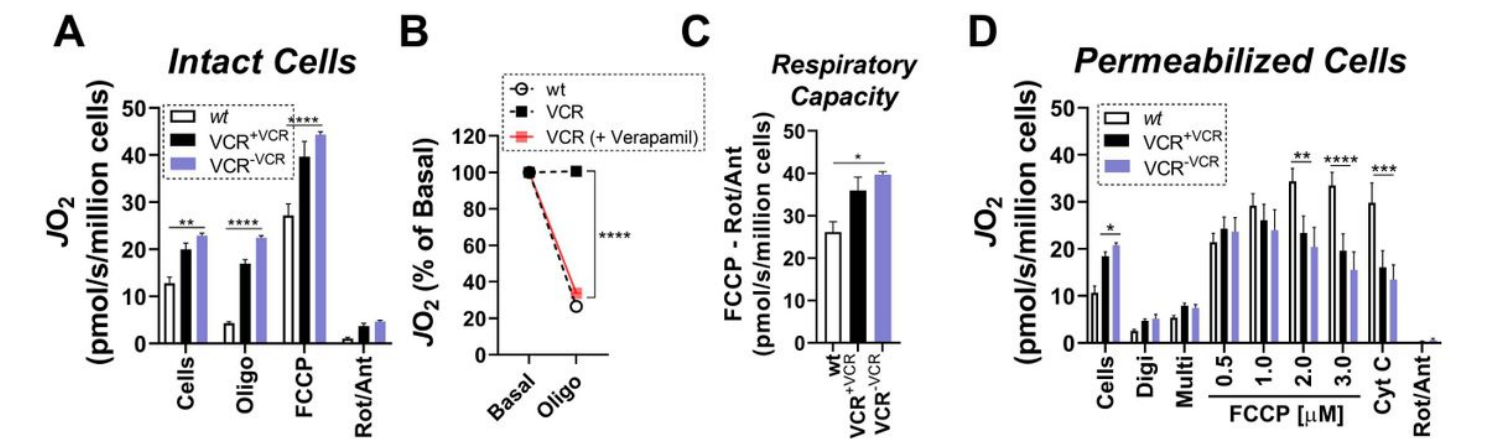


Figure 2

Increased basal and maximal respiration accompanies VCR resistance. (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 μ M), rotenone (Rot), and antimycin A (Ant). (B) FCCP stimulated respiration corrected for oxygen consumption remaining following rotenone/antimycin. (C) Percent decrease in basal respiration induced by oligomycin in wt cells, as well as vehicle or verapamil (10 μ M) treated HL-60/VCR cells. (D) Oxygen consumption in digitonin (0.02mg/mL) permeabilized cells in the absence of substrates (Digi), as well as following the addition of saturating substrate (Multi; pyruvate/malate/glutamate/octanoyl-l-carnitine/succinate), FCCP titration, cytochrome C (Cyt), and Rot/Ant. All experiments were conducted in VCR-free medium. Figures generated using GraphPad Prism 8 software (Version 8.4.2). Data are Mean $\pm$ SEM, (A, B, D) N=4-10/group, (C) N=3-5/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

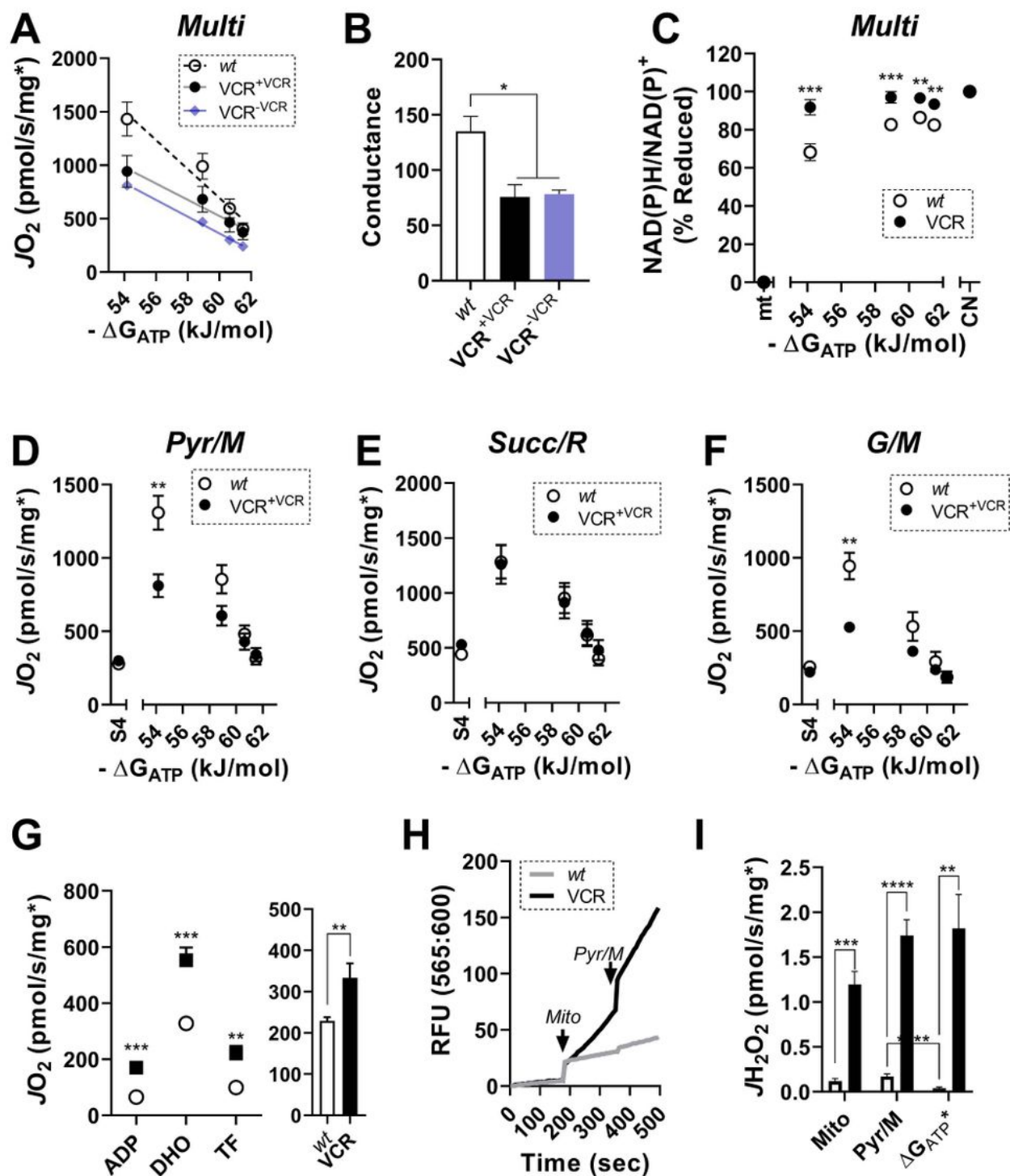


Figure 3

Comprehensive mitochondrial phenotyping reveals specific CI deficiency in HL-60/VCR. 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 – 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 JO2 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 H₂O₂ emission experiment in wt and HL-60/VCR mitochondria. Data depicts fluorescence in response to mitochondrial addition, as well as Pyr/M. (I) Quantified mitochondrial H₂O₂ 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<0.05, **P<0.01, ***P<0.001, ****P<0.0001

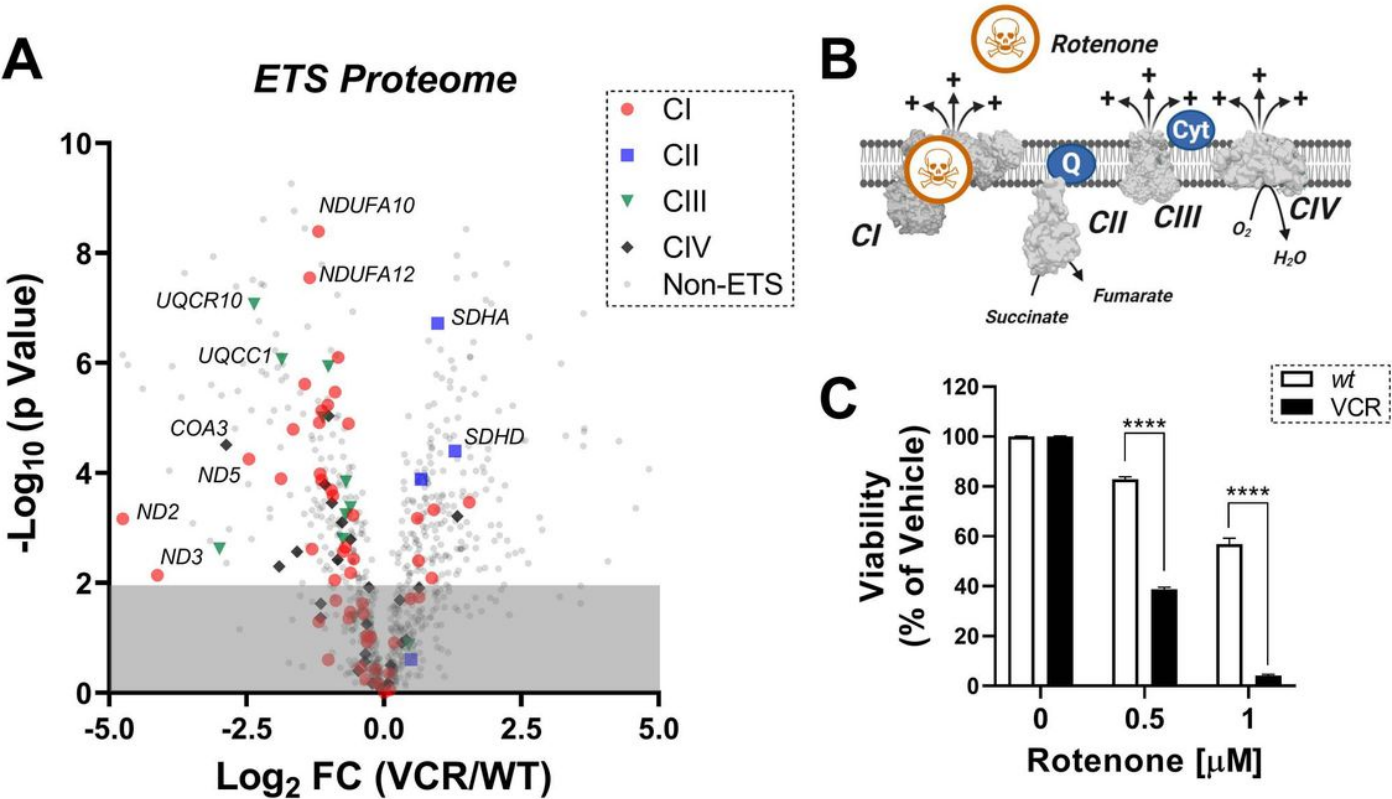


Figure 4

Decreased CI expression underlies 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 hours 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/group, (C) N=6/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

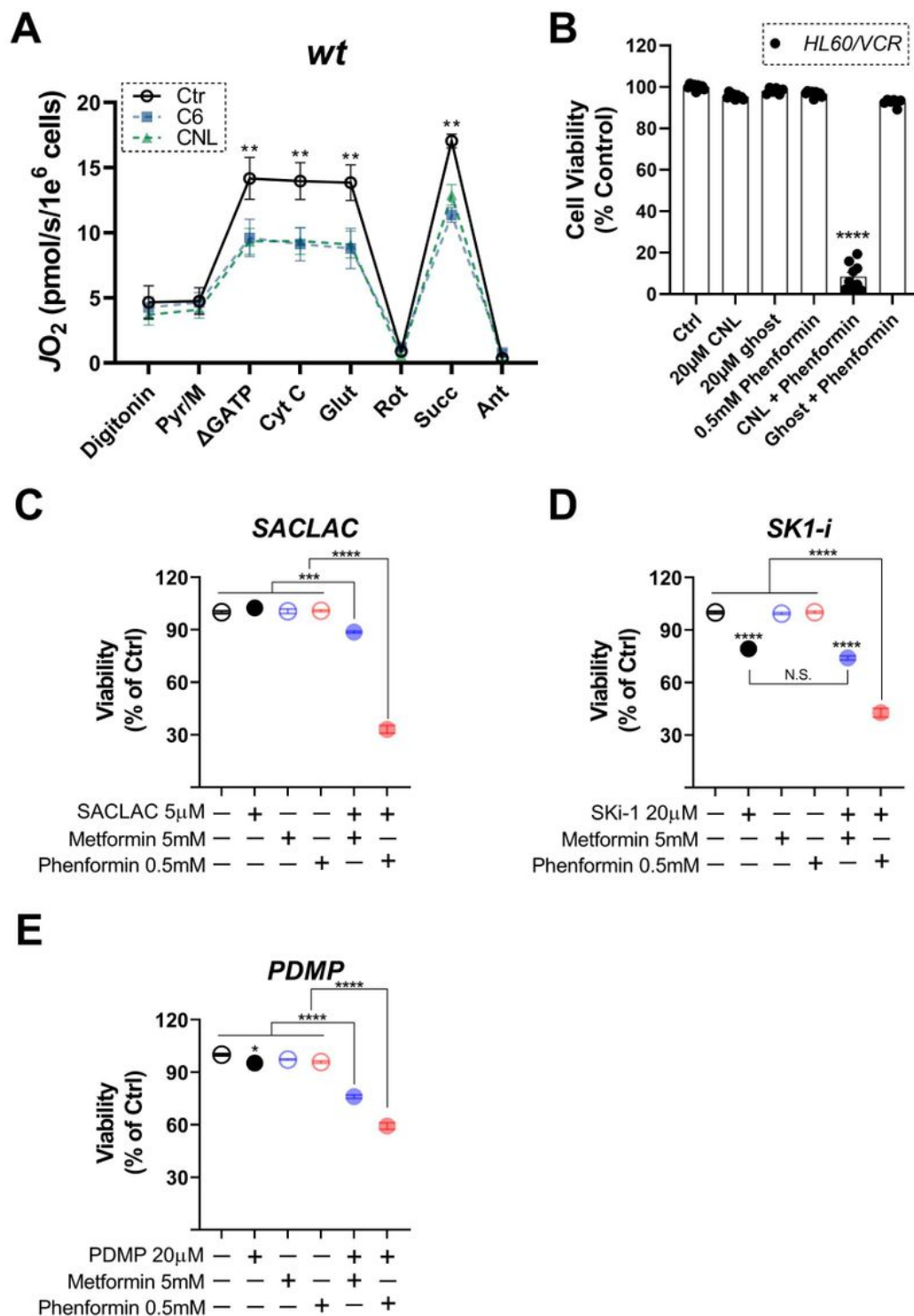


Figure 5

Mitochondrial CI inhibition combined with various ceramide analogues 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 (0.02mg/mL) and respiration was assessed in response to the indicated additions. Data normalized to viable cell count. (B-E) All experiments were done in HL-60/VCR cells. (B) Cell viability, determined by viable cell count, in HL-60/VCR cells exposed for 48hr

to CNL (20 μ M), ghost (20 μ M), phenformin (0.5mM), CNL (20 μ M) + phenformin (0.5mM), or ghost (20 μ M) + phenformin (0.5mM). (C-E) Cell viability in response to 24 hours of exposure to SACLAC (5 μ M), Ski-1 (20 μ M), PDMP (20 μ M), metformin (5mM) or phenformin (0.5mM). Metformin and phenformin were administered alone or in combination with SACLAC, Ski-1, or PDMP. 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, C-E) N=3/group, (B) N=6-9/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.xlsx](#)

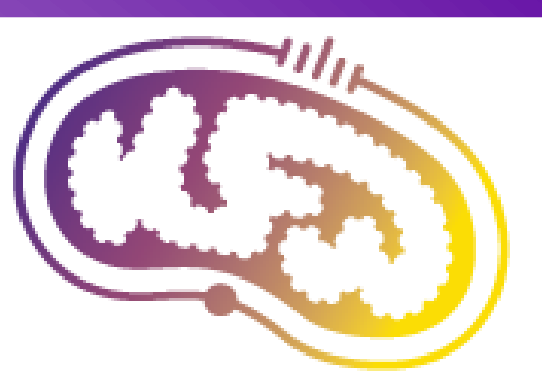
Intrinsic oxidative phosphorylation limitations underlie cellular bioenergetics in leukemia

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In comparison to normal hematopoietic cells, various human leukemias present with increased mitochondrial mass and higher basal respiration rates, the latter of which appears to sensitize them to respiratory inhibition. Although these studies have ignited interest in mitochondrial-targeted chemotherapeutics, it is currently unclear whether heightened respiration in leukemia derives from increased demand for ATP resynthesis or decreased oxidative phosphorylation (OXPHOS) efficiency. 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. To do this requires 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 OXPHOS efficiency and capacity, relative to the underlying proteome. Herein, we leveraged this workflow across several acute leukemia cell lines and primary human leukemias. Compared to healthy controls, intrinsic limitations to mitochondrial OXPHOS were found to characterize the mitochondrial network in leukemia. Parallel assessment of the underlying mitochondrial proteome linked leukemia-specific OXPHOS deficiency to shifts in adenine nucleotide translocase (ANT) isoform expression and increased abundance of TNF receptor associated protein 1 (TRAP1). Specifically, decreased ANT1 and increased ANT2 in leukemia facilitated matrix ATP uptake, rather than export, that, in the presence of functional TRAP1, directly constrained the ability of mitochondrial OXPHOS to contribute to the cellular ATP free energy (ΔG_{ATP}) charge. These findings are consistent with recent evidence demonstrating that mitochondrial OXPHOS is dispensable for tumor growth, 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 universal requirement for continuous mitochondrial flux to support other cellular functions (e.g., metabolite export, nucleotide synthesis).

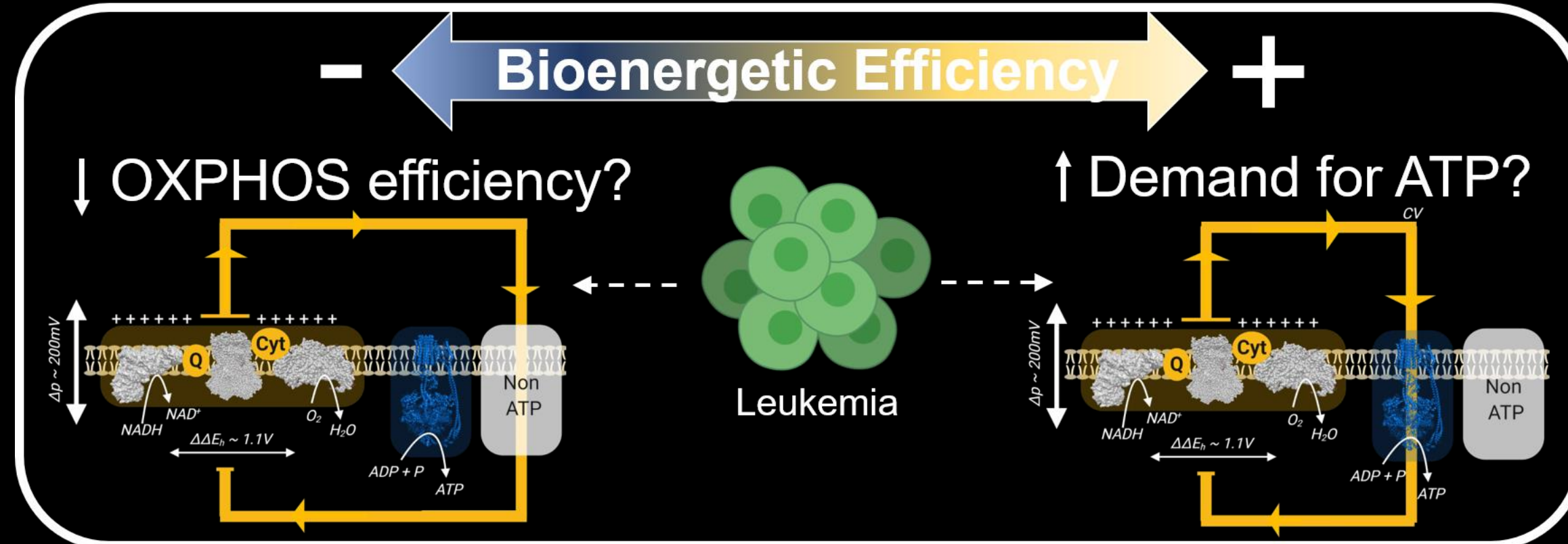
Funding: DOD-W81XWH1910213



Introduction

- Current cancer pharmacotherapies are aimed at disruption of mitochondrial energy, specifically inhibiting oxidative phosphorylation (OXPHOS).
- Human leukemias present with increased basal respiration and mitochondrial mass
- Whether increased respiration in leukemia is caused by a decrease in OXPHOS deficiency or an increase demand for ATP remains unknown.

Bioenergetics of leukemia



Aim: To comprehensively profile mitochondrial bioenergetics of leukemia

Bioenergetic profile of leukemia cells reveal respiratory flux limitations

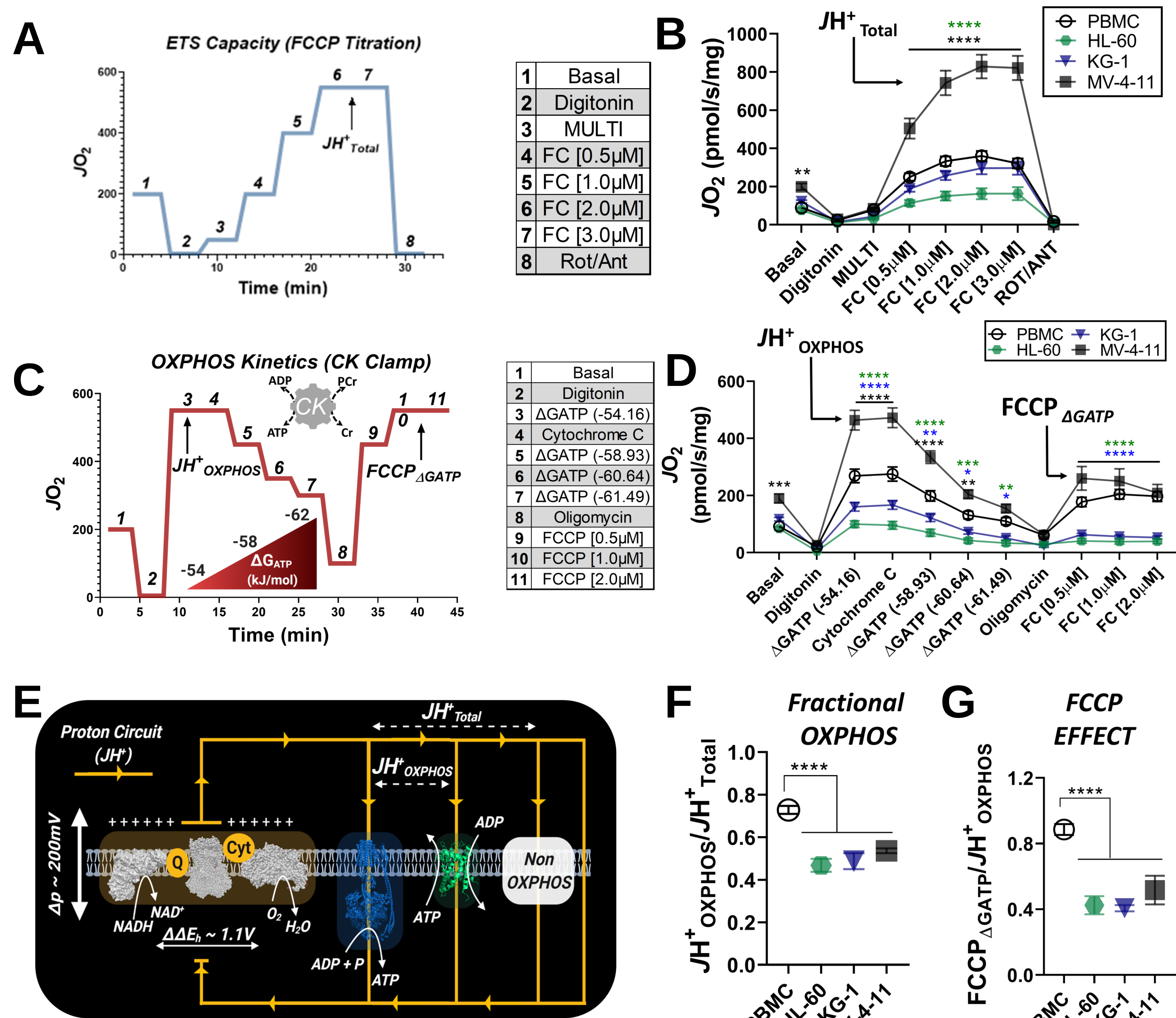


Fig 1. All experiments were performed using digitonin-permeabilized cells. (A) Schematic depicting changes in oxygen consumption (JO_2) during an ETS capacity protocol (FCCP titration) where points 6-7 represent the maximum proton conductance of the respiratory system (JH^+_{Total}). (B) ETS capacity protocol measured in leukemia cell lines and PBMC. (C) Schematic depicting JO_2 during an OXPHOS kinetics protocol (ΔG_{ATP} titration) where point 3 represents maximum proton conductance by the OXPHOS system (JH^+_{OXPHOS}) and point 10-11 represents maximum proton conductance of the respiratory system in the presence of ΔG_{ATP} (FCCP ΔG_{ATP}). (D) OXPHOS capacity protocol measured in leukemia cell lines and PBMC. (E) Illustration detailing maximal proton current generated by the electron transport system (JH^+_{Total}) and proportion of current harnessed by the phosphorylation system (JH^+_{OXPHOS}). (F) Comparison of fractional OXPHOS calculated as the ratio of JH^+_{OXPHOS} to JH^+_{Total} . (G) Comparison of FCCP effect calculated as the ratio of FCCP ΔG_{ATP} to JH^+_{OXPHOS} . For all experiments, n=10 for leukemia cell lines and n=22 for PBMC. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001.

OXPHOS impairments are a hallmark of primary leukemia

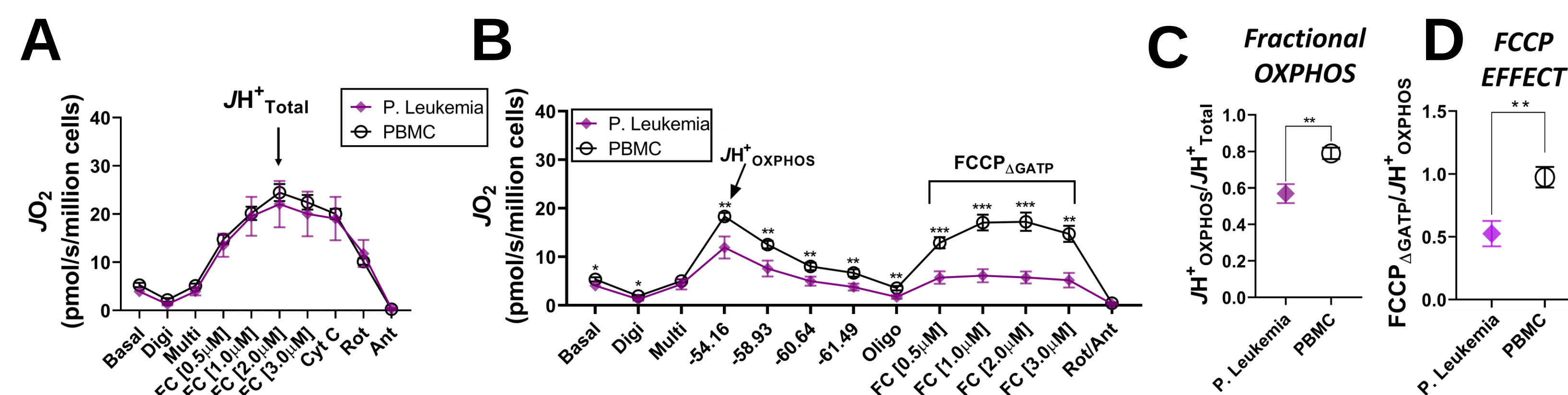


Fig 2. Permeabilized cells were used for all experiments. JO_2 of primary leukemia (AML/CML) and PBMC were measured by ETS capacity (A) and OXPHOS kinetic (B) protocols. Comparison of fractional OXPHOS (C) and FCCP effect (D) ratios in primary leukemia and PBMC. Primary leukemia n=8, PBMC n=11 *p<0.05, **p<0.005, ***p<0.0005

Increased ANT2/3 facilitates matrix ATP uptake and mediates OXPHOS inhibition

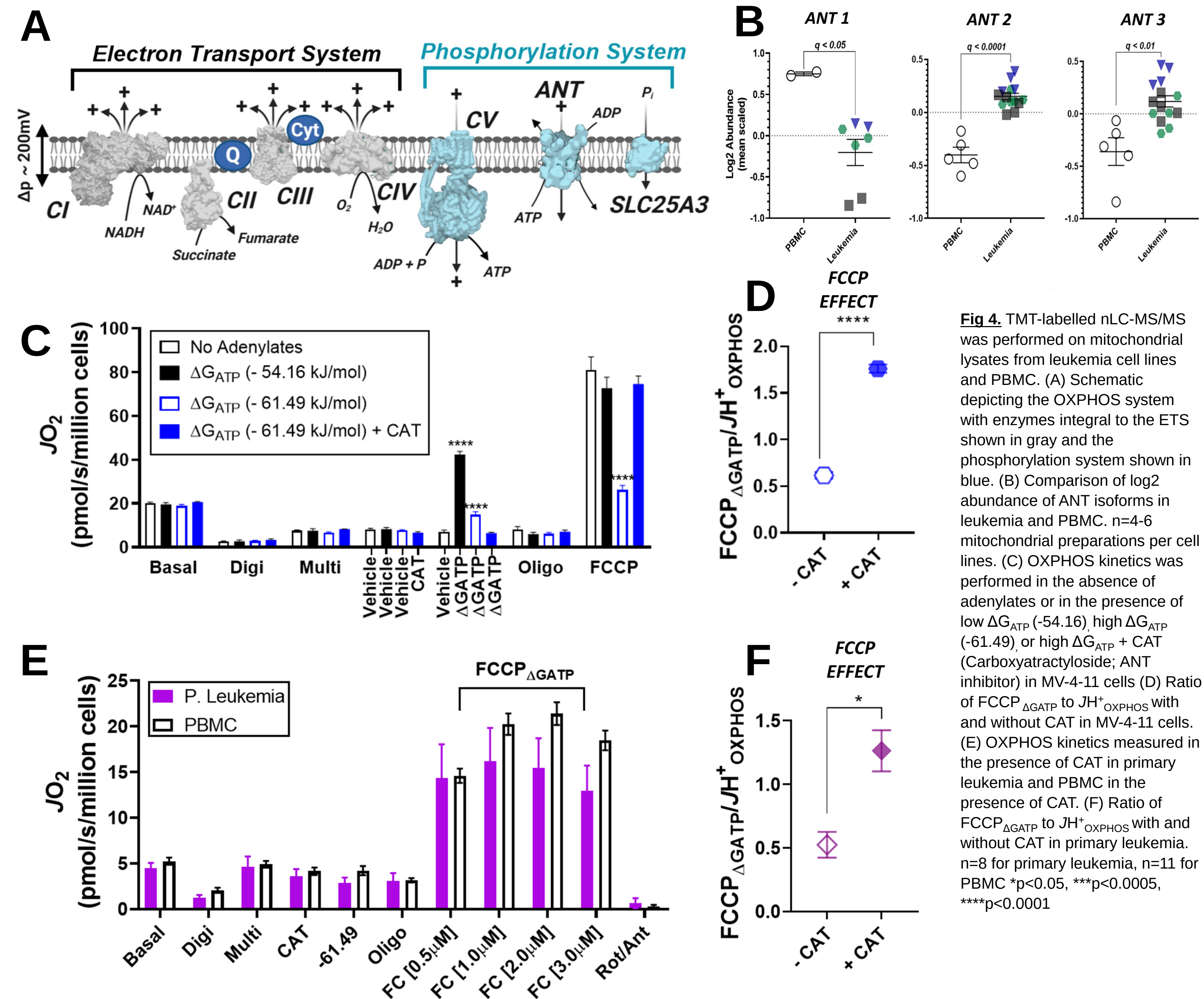


Fig 4. TMT-labelled nLC-MS/MS was performed on mitochondrial lysates from leukemia cell lines and PBMC. (A) Schematic depicting the OXPHOS system with enzymes integral to the ETS shown in gray and the phosphorylation system shown in blue. (B) Comparison of log2 abundance of ANT isoforms in leukemia and PBMC. n=4-6 mitochondrial preparations per cell lines. (C) OXPHOS kinetics was performed in the absence of adenylates or in the presence of low ΔG_{ATP} (-54.16) high ΔG_{ATP} (-61.49) or high ΔG_{ATP} + CAT (Carboxyatractylolide; ANT inhibitor) in MV-4-11 cells (D) Ratio of FCCP ΔG_{ATP} to JH^+_{OXPHOS} with and without CAT in MV-4-11 cells. (E) OXPHOS kinetics measured in the presence of CAT in primary leukemia and PBMC in the presence of CAT. (F) Ratio of FCCP ΔG_{ATP} to JH^+_{OXPHOS} with and without CAT in primary leukemia. n=8 for primary leukemia, n=11 for PBMC *p<0.05, ***p<0.0005, ****p<0.0001

Inhibition of ETS flux by ΔG_{ATP} requires functional TRAP1

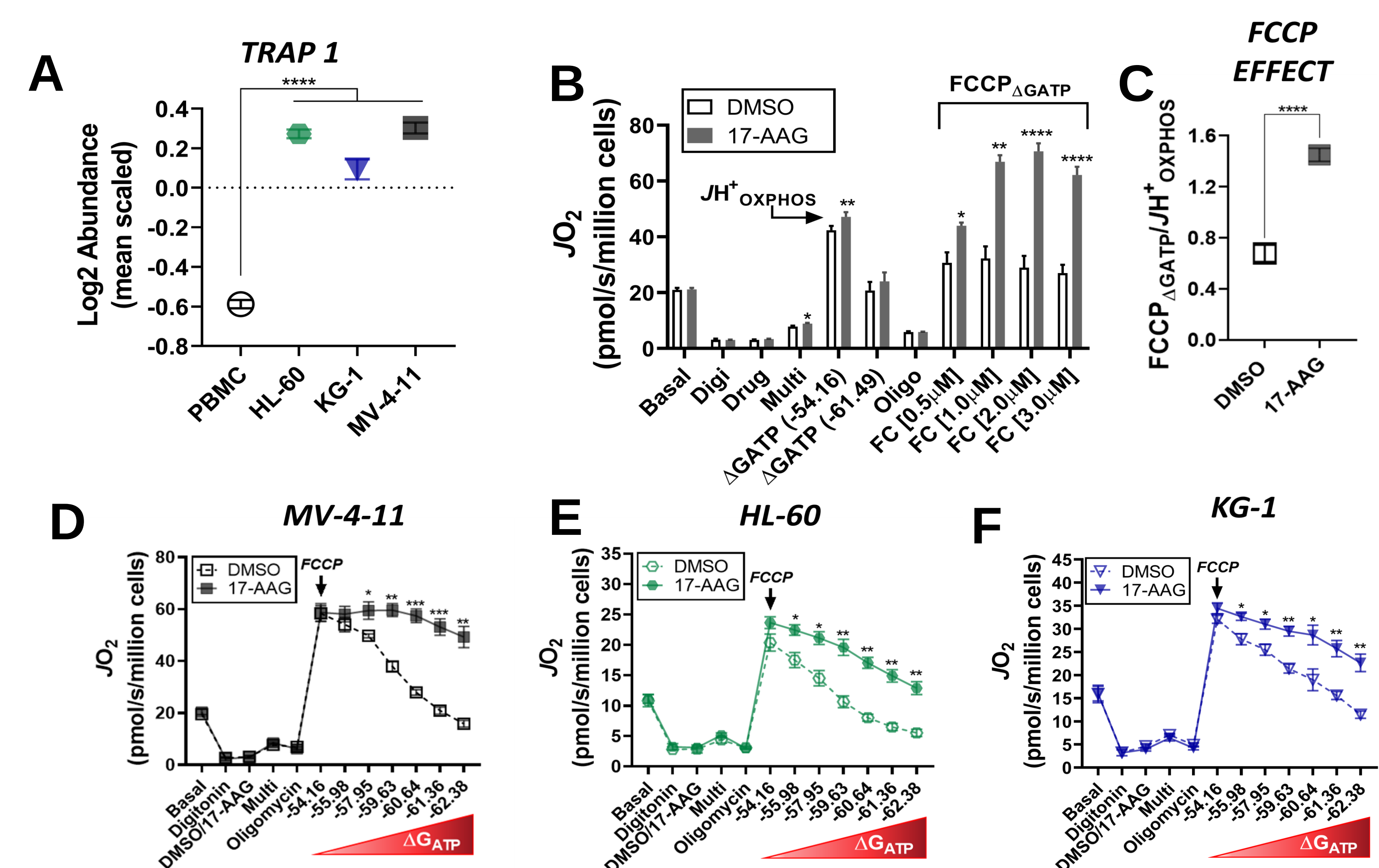


Fig 5. (A) Log2 Abundance of TRAP1 in PBMC and leukemia cells, n=6 per cell line. (B) Comparison of OXPHOS kinetics in presence of the TRAP1 inhibitor, 17-AAG in MV-4-11 cells. (C) Comparison of FCCP effect in MV-4-11 cells in presence of 17-AAG. Comparison of respiratory flux inhibition within (D) MV-4-11 (E) HL-60 and (F) KG-1 cells across a range of ΔG_{ATP} induced by functional TRAP1. Respiration was stimulated by the additional FCCP (1μM) n=7. *p<0.05, **p<0.005, ***p<0.0005,

Key Findings

- Only 50% of respiratory flux contributes to ADP phosphorylation in leukemia
- Activity of ANT and TRAP1 limit the ability of OXPHOS to contribute to cellular ΔG_{ATP}
- OXPHOS limitations may reflect a universal requirement for continuous mitochondrial flux to support other cellular functions
- Restoring OXPHOS capacity may provide therapeutic efficacy in leukemia