Title
Dissecting Mitochondrial Redox-Regulated Protein Translocation Using Small Molecule Probes

Permalink
https://escholarship.org/uc/item/1n4516dd

Author
Maland, Matthew Dean

Publication Date
2016

Peer reviewed|Thesis/dissertation
Dissecting Mitochondrial Redox-Regulated Protein Translocation
Using Small Molecule Probes

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biochemistry and Molecular Biology

by

Matthew Dean Maland

2016
Mitochondria are responsible for a multitude of cellular functions that almost always require nuclear encoded proteins to be translocated. Defects in mitochondrial import pathways result in many devastating diseases including neurodegeneration, stroke, cardiac ischemia, and cancer. More specifically, mutations of the intermembrane space (IMS) import component DDP1/Tim8 lead to deafness-dystonia syndrome, mutations in the sulphhydryl-oxidase Erv1 result in inherited cardiomyopathy, and mutations in CHCHD10 leads to ALS. Therapies to treat such diseases are not readily available. The development of tools to characterize key components of mitochondrial import pathways is necessary to understand the mechanism of the defects, thus improving therapeutic development.

A collection of small molecules that inhibit Erv1 oxidase activity were identified in a high-throughput chemical screen. Leading candidates were characterized using a battery of
biochemical approaches in yeast, zebrafish, and mammalian systems. Moreover, these small molecules have been used to characterize the mechanism of the disulfide relay in mitochondria, dissect novel roles of Erv1/ALR and Mia40, and to investigate how defects in this redox pathway contribute to diseases.
This dissertation of Matthew Dean Maland is approved.

Catherine F. Clarke
Alexander M. Van der Bliek
Carla Marie Koehler, Chair

University of California, Los Angeles
2016
DEDICATION

To Luv and Miles
TABLE OF CONTENTS

Chapter 1. A high throughput screen to identify small molecule modulators for mitochondrial research .................................................................1
Chapter 2. A small molecule probe that selectively inhibits the import of a subset of Mia40/Erv1 substrates .........................................................39
Chapter 3. Import of the ALS protein CHCHD10 by small molecule modulators ..........88

LIST OF FIGURES

Chapter 1.
Figure 1.1 General mitochondrial protein translocation ........................................24
Figure 1.2 Mitochondrial intermembrane space import translocation ..........................25
Figure 1.3 Flowchart of fluorescent high throughput screening campaign ..................26
Figure 1.4 Scatterplot of potential modulators of Erv1 ............................................27
Figure 1.5 Mitochondrial membrane permeabilization assay .....................................28
Figure 1.6 Mitochondrial membrane potential fluorescent assay .........................29
Figure 1.7 Flowchart of small molecule modulator screen as performed by the Broad ......30

Chapter 2.
Figure 2.1 Mitoblock-7 structure, half maximal inhibitory concentration, and effect on Erv1/ALR homologs ..............................................62
Figure 2.2 MB-7 is not deleterious to general mitochondrial function ......................63
Figure 2.3 MB-7 inhibits the import of MIA substrates ........................................64
Figure 2.4 Structure activity relationship of MB-7 analogs ....................................65
Figure 2.5 Erv1 inhibitor MB-8 is not deleterious to general mitochondrial function ....66
Figure 2.6 MitoBloCK-8 inhibits the import of MIA substrates ..............................67
Figure 2.7 MB-7 inhibits import of Cmc1 and Cmc1 C64A ..................................68
Figure 2.8 Formation of Mia40-Cmc1 import intermediate is partially impaired by MB-7 and MB-8 .................................................................69
Figure 2.9 MB-7 attenuates the formation of MIA-substrate interaction ...............70
Figure 2.10 A high molecular complex that includes Cmc1 and Erv1 requires the cysteine 42 of Cmc1 for formation .................................................71
Figure 2.11 A high molecular complex that includes Cmc1 and Erv1 is inhibited by MB-6 and MB-8 ........................................................................72
Figure 2.12 Gel extractions submitted for mass spec analysis ..................................73
Figure S2.1 MB-7 is not toxic to yeast or HeLa cells .............................................74
Figure S2.2 MB-8 is not toxic to yeast but is toxic to HeLa cells ............................75
Figure S2.3 MB-7 treated zebrafish have defects in somatic and cardiac developments ....76
Figure S2.4 MB-8 treated fish have defects in somatic and cardiac development ........77
Figure S2.5 Structure modification of ALR by small molecule modulators ................78
Figure S2.6 Steady state levels of mitochondrial proteins in WT or 10xHis tagged Mia40 mitochondria .................................................................79
Table 2.1 Erv1 and Cmc1 pull-down hits identified by mass spectrometry ..............80-84
Chapter 3.

Figure 3.1 MB-6 causes the accumulation of Mia40-Cmc1 import intermediate ..................101
Figure 3.2 CHCHD10 accumulates into MB-6 and MB-8 treated mitochondria ..................102
Figure 3.3 MB-6 causes the accumulation of Mia40-CHCHD10 import intermediate ..........103
Figure 3.4 MB-6 and MB-8 causes the accumulation of Mia40-CHCHD10 import
     intermediate ........................................................................................................104
Figure 3.5 MitoBLOCK treatment results in a fragmented mitochondrial network ..........105
Figure 3.6 Radiolabeled CHCHD2 accumulates in MB-8 treated mitochondria ..............106
APPENDICES

Chapter 3.
Appendix A. Auranofin targets Mia40 .................................................................107
Appendix B. Recombinant Cmc1 import pulldown analysis by Mass spectrometry ..........109
ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my thesis advisor, Dr. Carla Koehler. Thank you for the opportunity to work in your lab, for your relentless guidance, and for keeping that proverbial torch lit under my proverbial ass. I feel confident that the little Carla voice in my head will follow me into my future endeavors. I would also like to thank my committee members, Dr. Alex van der Bliek, Dr. Michael Teitell, Dr. James Gober, and Dr. Cathy Clarke, for your support and guidance throughout the years. I am truly grateful for your motivation and encouragement.

I would like to thank my mentor Dr. Deepa Dabir. Graduate school is a roller coaster ride, sometimes with very deep troughs and your support has kept me on track. And to my colleagues Dr. Juwina Wijaya, Dr. Dan McNamara, Dr. Sonya Neal, Futures Dr.’s Sean Shen, Tanya Hioe, and Jisoo Han, thank you for the morning coffee breaks and sometimes evening non-coffee breaks. Thank you all for your mostly technical and sometimes not-so technical advice. I also would like to acknowledge the past members of the Koehler lab: Dr. Meghan Johnson, Dr. Colin Douglas, Dr. Yavuz Oktay (Go Turkiye!), Dr. Heather Tienson, Dr. Non Miyata, and Dr. Esther Nubel. Thank you for the great times we had in and out of the lab. And to the current Koehler Nation: Mike Puccio Conti, Eric Riggs Torres, Jenny Ngo, Jesmine Cheung, Vivian Pokemon Zhang, Dr. Janos Steffen, Kayla White, and Dr. Charles Bar. Thank you for the very necessary distractions.

They say there is a light at the end of tunnel but my path has been well lit. Finally, I would like to extend a very heartfelt thank you to my wife and family for being that guiding light. I would not be here if it weren’t for each and every one of you. THANK YOU ALL!
Chapter 1 introduces small molecule directed research, particularly in the mitochondrion, of essential proteins. Two high throughput screens are highlighted here resulting in the identification of small molecule modulators of Erv1. Special thanks to Samuel Hasson and Deepa Dabir who helped with the screen development. Dr. Hasson also wrote the macro for the data analysis and led the collaborative screen at the Broad Institute. Thank you to Robert Damoiseaux, Winnie Hong, and Jong Lee for their technical assistance at the screening center.

Chapter 2 describes the characterization and utilization of the lead hits. The Thorpe lab supplied the QSOX strain. Deepa Dabir made the Erv1 overexpressing strain and the Erv2 expression construct. The Barrientos lab supplied the Cmc1 cysteine mutant strains. Thanks to Sean Shen from the Loo Lab for mass spectrometry work and expertise. Meghan Johnson, Jisoo Han, Jenny Ngo performed the zebrafish and microscopy experiments. The Banci lab at the University of Florence performed the NMR experiments.

Chapter 3 highlights the capabilities of using these small molecules to dissect disease associated pathways, in this case involving Erv1 and Mia40. Thank you to Vivian Zhang for generating the CHCHD2, CHCHD10, and Gut2 clones. Thanks you Jenny Ngo for microscopy images. The Auranofin project was in collaboration with Shankar Thangamani, Tony R. Hazbun, and Mohamed N. Seleem. Ajay Vashisht and James Wohlschlaeger performed the Mass Spec analysis of the histidine tagged Cmc1, Mia40, and Erv1 pull downs.

This work was partially funded by the Ruth L. Kirschstein National Research Service Award GM007185.
VITA

2005-2007
College of the Canyons
Santa Clarita, CA

2010
B.S. Cellular and Molecular Biology
Department of Biological Sciences
California State University, Long Beach

2010-2016
Graduate Student Researcher
Department of Chemistry and Biochemistry
University of California, Los Angeles

2010-2016
Teaching Assistant
Department of Chemistry and Biochemistry
University of California, Los Angeles

PRESENTATIONS AND PUBLICATIONS


Matthew D. Maland, Deepa V. Dabir, Kiyoko Setoguchi, Megan E. Johnson, Robert Damoiseaux, Michael A. Teitell, and Carla M. Koehler A Chemical Screen to develop inhibitors for Redox-Regulated Protein Translocation into Mitochondria. Presented at the MBI Lake Arrowhead Retreat, Lake Arrowhead, CA

HONORS AND AWARDS
2007  J.A. Edmonson Math/Science Scholarship
       College of the Canyons
       Santa Clarita, CA

2008-2010  President’s Honor List
           College of the Canyons
           Santa Clarita, CA

2010  Summer Research Fellowship
       University of California, Los Angeles

2010  University Fellowship
       University of California, Los Angeles

2011-2014  Ruth L. Kirschstein National Research Service Award
           University of California, Los Angeles

2014  MBI Outstanding Poster Award
       University of California, Los Angeles

2015  UCLA Business of Science Center Venture Team Finalist
       University of California, Los Angeles
Chapter 1. A high throughput screen to identify small molecule modulators for mitochondrial research

Introduction

Basic research is a pursuit to understand biological events to strengthen our ability to identify and treat diseases. Human biology is a tremendously complicated machine with countless interactions, processes, and agents making direct and detailed investigations virtually impossible. To gain a conceptual grasp, a highly complex system must be simplified in a way to answer questions that would otherwise be insurmountable. But because of the many forces working in such a miniscule area, accurate conclusions are practically unachievable. Thus, researchers employ an ever growing collection of models and tools to analyze biological pathways. With these current technologies, systems can be easily modified or controlled to mimic human biology allowing us to gain insight into the proverbial black box that is molecular biology and develop and understanding of how dysfunction leads to disease.

Typically, lower order organisms with conserved and perhaps simpler pathways are used in experimental procedures and there is an ever growing body of options available to researchers. For example, bacteria have been used to study a vast array of molecular interactions and basic genetics, as well as exploited for their sturdy components to use as research tools\textsuperscript{1,2}. Saccharomyces cerevisiae has been used for decades to identify their human gene counterparts as
well as a myriad of other studies like ageing\textsuperscript{3,4}. \textit{Danio rerio}, or zebrafish, joined the pool of model organisms in the 1960s to help answer questions about human development previous invertebrates could not\textsuperscript{5,6}. These systems, among many others, have proved to be invaluable in biological research and the eventual development of therapeutics.

Nonetheless, no model is perfect. \textit{E. coli} are prokaryotes and humans are much more complex eukaryotes with features like organelles making many comparisons a large leap. Yeast, although eukaryotic, are single cell organisms and disease mechanisms, particularly tissue specific phenotypes are not existant\textsuperscript{7}. Zebrafish models are a more challenging to manipulate because of their larger genomes and less is known about the relatively new model creating hurdles when noting observations\textsuperscript{8}. Other organisms are available to researchers but limitations still exist. Thus, researchers make an exhausting effort to create multiple models that broaden the scope of research.

Using these models, a battery of assays is used to dissect biological processes. Traditional methods like knocking out a gene and observing a molecular or phenotypic outcome can aide in dissecting pathways or identifying function\textsuperscript{9}. However, a modified organism will be inviable if it is an essential component. Techniques that can remove proteins later in development such as RNA-interference can be an excellent tool, but abstracting a protein can stimulate compensatory responses and results are gradual over 72 hours\textsuperscript{10}. Another approach is to produce temperature sensitive mutants that perturb a function at a restrictive temperature. However, because most genes are pleiotropic, phenotype observations can be misleading. All things considered, modification of an experimental parameter or genetic code, or expelling a component, could lead to unforeseeable consequences that may misdirect our conclusions. Furthermore, because a collection of approaches are essential to eliminate artifact and non-specific effects, the
manufacturing of new systems to study a single pathway can be cumbersome. Taken together, it is advantageous to identify research tools that could be used ubiquitously and proficiently across all species to efficiently broaden the scope of the research. These challenges, among other impediments, created a need for scientific tools that can manipulate molecular mechanisms or pathways while limiting off-target consequences.

The advent of such tools became possible in recent years largely due to the integration of multiple scientific branches, specifically chemistry and biology\textsuperscript{11}. Born at the interface between these two disciplines, the conception of small molecule driven modulation of biological pathways has recently become a reality. Small molecules, as opposed to large molecules like proteins and DNA, are small organic compound typically used as therapeutic agents that regulate a biological pathway that countermeasures human disease. Alternatively, there has been a recent push to employ small molecules in basic research to perturb a single protein function or interaction to more accurately characterize biological processes where traditional genetic approaches have been limiting\textsuperscript{12,13}.

Small molecule tools have a variety of advantages. Small molecules can modulate activities quickly, and sometimes reversibly, enabling precise temporal control\textsuperscript{14}. This allows for regulation during various stages of the cell cycle. They can also be used in a range of concentrations resulting in a spectrum of phenotypes. Small molecules can also be used in large scale studies where traditional approaches like RNAi would be difficult to achieve\textsuperscript{10}. Lastly, small molecules have the potential to be used across species to speed up and simplify data collection. These are features previous approaches were lacking. Researchers can use these tools to dissect disease pathways and push our knowledge forward faster than ever before.
Small molecule tools are becoming more prevalent in this ‘post genomic era’. As huge data sets are revealed identifying genes and proteins, it is a race to determine how these newly discovered players function together in complex molecular systems. An assortment of approaches is being performed to find modulators for these countless biological mechanisms. Transcriptional activators, imaging tools, unnatural amino acids, and synthetic ligands are among the many types being used today. For instance, Tiechert et al. used neuronal receptor inhibitors to study the roles of fetal and adult neuromuscular nicotinic acetylcholine receptor subtypes during development, giving us insight into neuromuscular development and potential roles in associated pathologies. Also, Hong et al. identified ERK to be a specific marker of early arterial progenitors using small molecules identified in a chemical screen. Other tools have led to advances in the biological understanding of diverse areas such as cytoskeleton, mitosis, mTOR, PPARγ, and protein kinases, to name a few. Small molecules are becoming relevant in all areas of research.

Small molecule tools can also be used to recapitulate a disease in a model by perturbing a disease associated protein function. Current tools such as mouse models are expensive, take a long time for development, and have high variability with disease onset and progression. Furthermore, many current tools are not highly penetrant to diverse organisms, tissues, and cell types, requiring each system to use a different tool. Because many functional domains are conserved, a small molecule can have the ability to be used to model a dysfunction across a scope of tissues and organisms not only speeding up the discovery process but also improving the quality of the studies. Using small molecules to recapitulate disease mechanisms leads to superior models revealing better therapeutic targets vastly enhancing drug development pipelines.
It was understandably difficult to identify small molecule probes before advances in robotics and the stockpiling of chemical libraries. In 2005, the NIH launched the Molecular Libraries Program giving researchers broader access to small molecule science. Although the program is now defunct due to slashed funding, it illuminated the need to innovate and broaden our use of high throughput screening of small molecules that enable us to explore biological processes that were not otherwise testable. The Molecular Screening Shared Resource, MSSR, at UCLA’s California NanoSystems Institute collaborates with academic labs to design and execute a high throughput screening campaign to identify small molecule tools that can be used to study an otherwise difficult process. Combining the expertise of liquid handling and robotic automation, labs have the inexpensive ability to identify valuable research tools at a relatively quick pace.

There are two common ways to identify small molecule tools: phenotypic and target based screening. Phenotypic screening has been historically used to discover new pharmaceutical drugs. Compounds are screened in a cellular or animal disease model to identify biologically active small molecules that trigger a desired outcome. Although this type of screen has a larger breadth of new findings, it can be challenging to identify the target. Conversely, target based approaches have been used more recently as a result of the troves of targets revealed as a result of the advances in genome sequencing. Although phenotypic screening can be advantageous in therapeutic screening, target based approaches can be more effective in identifying small molecule tools that modulate a specific molecular pathway or enzymatic function.

Although tools are making headway in many areas of research, small molecule directed research in mitochondria is limited. Mitochondria are unique organelles with multiple
compartments, their own genome and protein expression machinery, and are responsible for a host of cellular functions. They are also responsible for a swarm of human maladies whose mechanisms, despite an army of researchers probing, remain a mystery\textsuperscript{31,32}. The objective of this research is to identify and employ small molecules to study mitochondrial biogenesis and the diseases that result from dysfunction.

**The Mitochondrion**

The mitochondrion, most widely known as the powerhouse of the cell, is a dual membrane organelle that is present in almost every eukaryotic cell. Their numbers range from just a few per cell to thousands, depending on the tissue or organism, and have a very distinct structure\textsuperscript{33}. The most well-known function of mitochondria is energy production in the form of adenosine triphosphate, or ATP\textsuperscript{34}. Lesser known but equally important functions include cell signaling, apoptosis, amino acid synthesis, heme and iron-sulfur biogenesis, and lipid metabolism\textsuperscript{35-40}. Mitochondria are fundamental organelles that are responsible for a number of essential cellular functions, many of which remain unresolved despite being heavily scrutinized for over a century.

Modern mitochondria are the result of the most important leap in eukaryotic evolution. Early mitochondrion ancestors were formerly free-living bacteria that were taken into another cell as an endosymbiont, although the specific sequence of events is still debated\textsuperscript{41}. As the endosymbiont evolved, the majority of genes were transferred to the host genome presumably as a result of several advantages that are still contested\textsuperscript{42}. Modern human mitochondrial DNA is 16,569 base pairs that encode 13 respiratory chain component proteins, a mere 1% of the required proteins, among other untranslated RNA transcripts\textsuperscript{43,44}. The other 99% of the mitochondrial proteome are nuclear encoded and translated in the cytosol, thus requiring a
transport mechanism that allows the mitochondria to amass the required proteins to function properly.

**Mitochondrial Protein Translocation**

Proteins translated in the cytosol have inherent properties to guide them to the appropriate location. Although some remain cryptic, most mitochondrial proteins have a known targeting sequence directing nascent peptides to the mitochondrial outer membrane and further ushering to the correct compartment. The most common targeting sequence is a cleavable N-terminal amphipathic α-helix, however other known sequences include non-cleavable signal anchors, internal, and cysteine motif signals\(^{45,46}\). These amino acid sequences interact with recognition proteins at the outer membrane of the mitochondrion.

The mitochondrial outer membrane is a phospholipid bilayer that separates the intermembrane space from the cytosol. The outer membrane not only mediates a variety of metabolic interactions, it also harbors proteins that mediate apoptosis as well as viral response elements\(^{47-49}\). Although the outer membrane contains porins, abundant channel proteins that allow almost any molecule less than 5 kD across, nearly all pre-proteins are translocated through the gateway protein, Tom40, or translocon of the outer membrane (Figure 1.1). Tom40 is an integral membrane beta barrel that interacts with a variety of receptors with pre-protein recognition sites to direct nascent proteins to translocate\(^ {46}\). The importing substrate continues across the lipid bilayer where it is sorted to the outer membrane, inner membrane, matrix, or folded and oxidized to remain the intermembrane space.

Importing pre-proteins destined for the outer membrane, such as beta barrels, will first reach the intermembrane space where they are met by the small Tim chaperone complexes, Tim9-Tim10 or Tim8-Tim13, that shield them from the hydrophilic intermembrane space and
assist them to the sorting and assembly machinery, or SAM, complex in the outer membrane\textsuperscript{50}. The proteins enter the lipid phase adjacent to the complex and assemble with the corresponding complex constituents.

The mitochondrial inner membrane is an exceedingly folded, cardiolipin rich, phospholipid bilayer that separates the matrix from the intermembrane space. The folds of the inner membrane, or cristae, greatly increase the workable surface area that houses important proteins of the electron transport chain and ATP synthase\textsuperscript{51, 52}. The mitochondrial inner membrane is permeable to O\textsubscript{2}, H\textsubscript{2}O, and CO\textsubscript{2}, but not permeable to ions and small molecules like the outer membrane. This provides a barrier to build a proton gradient that is used to drive ATP production.

There are three ways proteins are inserted in the mitochondrial inner membrane. Hydrophobic proteins are met by the small Tim chaperones which shield the protein as it is delivered to the translocon of the inner membrane, Tim22\textsuperscript{53-55}. The protein is then inserted into the lipid bilayer in a membrane potential dependent manner. Inner membrane proteins with only one transmembrane span are arrested at a stop transfer signal inserted laterally through the PAM lacking Tim23 complex\textsuperscript{46}. Finally, some inner membrane proteins enter into the matrix through the Tim23 complex where Oxa1 facilitates insertion\textsuperscript{56}.

The mitochondrial matrix houses nucleic acids, protein synthesizing machinery, metabolites, and numerous enzymes. Pathways in the matrix include the TCA and urea cycles, fatty acid oxidation, heme biosynthesis, as well as other components\textsuperscript{45, 57}. Proteins that need to reach the mitochondrial matrix are directed through the Tom40 complex in the outer membrane and pulled through the Tim23 complex, often by the PAM motor protein\textsuperscript{53, 58}. 
Contrary to early textbooks, the intermembrane space is more than just a narrow hydrophilic region that separates mitochondrial compartments. Because the majority of the proteins in the inner membrane are respiratory complexes, it is not a big surprise that the most abundant IMS protein is the electron carrier cytochrome c\(^59\). There are however, a variety of other essential processes ongoing in the IMS. One important role is mediating cellular signaling, for instance, employing reactive oxygen species (ROS) or pro-apoptotic factors\(^{35,36,59}\). Creatine kinase also functions in the IMS giving rise to phosphocreatine, a rapidly mobilizable form of energy\(^60\). Additional IMS proteins function in heme biosynthesis and the maturation of Fe/S cluster containing proteins\(^{61,62}\).

Most intermembrane space proteins are shuttled in through the Mitochondrial Intermembrane Space Import and Assembly (MIA) pathway after entering the Tom40 pore complex (Figure 1.2). The MIA pathway delivers proteins that require disulfides to reach their native confirmation to the intermembrane space - mostly small proteins with canonical C\(x(n)C\) motifs\(^63\). A major constituent of the MIA pathway is Mia40, Mitochondrial intermembrane space import and assembly 40, which interacts via a hydrophobic pocket while a CPC motif forms a transient disulfide bond with the importing substrate\(^{64,65}\). The resulting oxidation reaction is completed and the newly formed disulfide bond within the substrate prevents retro-translocation, leaving Mia40 in the reduced form. The next major constituent, Erv1, essential for respiration and viability, re-oxidizes Mia40 taking the electrons and passing them off to either cytochrome c to be used in the electron transport chain, or molecular oxygen\(^{64,66}\). Additional terminal electron acceptors have been postulated but yet to be identified.

ALR, the human homolog of Erv1, and Erv1 are members of the Erv1/ALR sulfhydryl oxidase family and share a great deal of homology\(^67\). Other homologous proteins include Erv2
found in the endoplasmic reticulum, QSOX in the Golgi and the extracellular environment, and E10R in the poxvirus family. ALR was originally found to promote growth in a regenerating or injured liver, however since has been found to have many more roles in mitochondrial biogenesis. ALR is ubiquitously expressed, yet the functions in other organs are not clear. It has been speculated that serum ALR levels can serve as a biomarker for liver injury or certain diseases, however, understanding ALR’s role in pathology would allow for better development of diagnostic tools. Furthermore, because the characterization of ALR has so far been lacking, it is not clear what the many roles of ALR are in mitochondrial biogenesis leaving important questions about ALR and MIA behaviors in disease pathology unanswered. New approaches are needed to elucidate disease mechanisms and dissect essential functions in mammalian cells.

It is clear that the mitochondria play many critical roles in cell, developmental, and organismal biology. Researchers are still trying to identify how all of these mitochondrial functions operate not only as independent pathways but also in concert with each other. Because of the vast machineries and mechanisms in the mitochondria, it is understandable that proper function is critical to human health and development. It is also understandable that even the slightest dysfunction can lead to a devastating disease. To this end, our goal is to dissect basic mitochondrial biogenesis to progress our understanding how dysfunction in these processes lead to disease.

**Mitochondrial Diseases**

Mitochondria are crucial to eukaryotic life and thus are intertwined with health, ageing, and disease. With so many essential functions, it isn’t a surprise that even the slightest dysfunctions can lead to devastating diseases. From cancers to myopathies to degenerative disorders, proper mitochondrial function is fundamental for proper development and well-being.
Many mitochondrial associated diseases are scrutinized yet it is not always clear how dysfunction leads to the pathological consequences.

Mitochondrial diseases range from the tissue specific, like Leber hereditary optic neuropathy, to more widespread symptoms associated with oxidative phosphorylation (OxPhos) defects\textsuperscript{71, 72}. These diseases affect every organ with a multitude of phenotypes. Dysfunction in the brain can cause cognitive defects and epilepsy as well as hepatic failure and cholestasis in the liver. Endocrine syndromes include diabetes and growth hormone defects. Muscles, peripheral nerves, sensory organs, and bone marrow are just a few additional tissues where known mitochondrial dysfunctions result in unrelenting manifestations\textsuperscript{73-75}.

Alzheimer’s and Parkinson’s diseases are well known and unfortunately common mitochondrial related disorders. Alzheimer’s disease is marked by a gradual onset of dementia where a growing body of evidence suggests mitochondrial defects, such as increased ROS production, decreased OxPhos, and network fragmentation contribute to the pathology\textsuperscript{76, 77}. Interestingly, patients with a polymorphism in the translocon TOM40 have increased susceptibility\textsuperscript{78}. Parkinson’s disease patients suffer from uncontrolled trembling resulting from dysfunctional mitochondrial turnover. PINK1 is a kinase that is recruited to the mitochondria to function in mitophagy with Parkin, an E3 ubiquitin ligase\textsuperscript{79-81}. Symptoms arise when PINK1 fails to translocate resulting in unsuccessful mitochondrial clearance.

Many other diseases are a consequence of mitochondrial biogenesis dysfunction, notably associated with proteins of the intermembrane space. Mia40 modulates tumor suppressor protein p53 localization and defects in expression or function can lead to cancers\textsuperscript{82}. Furthermore, Mia40 overexpression can be responsible for the hypoxic response that is required for the metabolic adaptation of tumors\textsuperscript{83}. Huntington’s disease patients have abnormally low levels of ALR but it
is not yet understood how this impacts pathology\textsuperscript{84}. Amyotrophic Lateral Sclerosis (ALS) results from neuronal cell death usually characterized by a SOD1, a superoxide scavenger, mutation\textsuperscript{85}. However, it was recently reported that mutations in the uncharacterized protein of the intermembrane space CHCHD10 also result in ALS\textsuperscript{86}. In a rare mutation in the sulfhydryl oxidase ALR, patients present with cataracts, hypotonia, and respiratory insufficiency and mutations to the ALR substrate Tim8 results in Deafness-dystonia-optic neuropathy\textsuperscript{87,88}. Although these disorders are uncommon, understanding rare syndromes also shed mechanistic insights into the more common disorders.

Defects in mitochondrial function impact human health greatly and result in a wide range of diseases from degenerative muscle and neural diseases to cancer. Dissecting many of these pathways using traditional techniques have proven to be difficult. Utilizing small molecule tools in mitochondrial research is a novel approach that will help further our understanding of mitochondrial biogenesis and how dysfunction leads to disease. Our current understanding of the mitochondrial inner membrane space is strong enough to illustrate general import but it is unclear how defects in redox regulated import lead to disease. Because traditional approaches are ineffective, small molecules would be an excellent tool in dissecting redox dependent import. The goal of this research is to design and execute a high throughput screen to identify small molecule modulators of Erv1/ALR to dissect the many functions, including import, allowing us to investigate how dysfunction leads to disease.

**Targeting small molecules to mitochondrial intermembrane space import machinery**

It has been established that disruption in the mitochondrial intermembrane space import machinery results in multiple tissue disease phenotypes\textsuperscript{87}. Although the import pathway is fairly well characterized, it remains unclear how these essential components relate to disease.
pathology. Although mitochondrial biogenesis has been studied extensively using classic genetic and biochemical analyses, many mitochondrial proteins, such the redox-regulated import machinery in the intermembrane space, are essential. As a consequence, results from classic approaches are unattainable. Some degree of success was made using temperature sensitive mutants, however pleiotropic results may misdirect our conclusions. Thus, novel approaches are necessary to elucidate the roles of essential proteins in the intermembrane space to shed light on how dysfunction leads to disease.

As eluded to earlier in the chapter, identifying small molecules that modulate a proteins function can be an invaluable asset in research studies. More specifically, using a small molecule tool will allow the quick modulation of the mitochondrial import pathway to investigate molecular functions and observe the results from disruption. Furthermore, because of the evolutionarily conserved nature of proteins, these small molecules can potentially be used across species widening the breadth of this research. The purpose of this research is to identify small molecule modulators of Erv1/ALR redox activity to dissect the molecular functions of this enigmatic protein and how dysfunction triggers disease.

Erv1 and ALR are sulfhydryl oxidases that oxidizes Mia40 and transfers electrons through 3 internal disulfides and a bound Flavin cofactor as proteins are imported into the intermembrane space. Erv1/ALR completes the linear pathway by reducing cytochrome c (to the electron transport chain) or molecular oxygen, forming hydrogen peroxide. Because Erv1/ALR has been implicated in a variety of pathways in addition to import, modulation of the enzymatic activity allows us to probe the many functions of Erv1/ALR. Mutations in ALR results in various clinical presentations and understanding the essential functions will give insight into how dysfunction impacts mitochondrial and cell functions.
Using a target based approach we have identified small molecule modulators of Erv1 sulfhydryl oxidase activity. Small molecule modulation of mitochondrial proteins is a novel approach to study mitochondrial biogenesis. Previous small molecule experiments have so far facilitated in expanding our working knowledge of the role and interactions of MIA constituents. However, more tools are needed to ensure quality and reproducibility as well as perturb enzymatic functions differently.

**A High-throughput Screen for Erv1 Modulators**

Using a simple in vitro assay, we can measure the amount of hydrogen peroxide produced by the Erv1 directed reduction of O$_2$ using the commercially available fluorogenic probe Amplex Red. Amplex Red, in conjunction with horseradish peroxidase (HRP), reacts with H$_2$O$_2$ at a 1:1 stoichiometry to produce resorufin, a fluorescent product. Reduced recombinant Erv1 enzymatically reduces O$_2$ to H$_2$O$_2$. The resulting amount of H$_2$O$_2$ directly correlates with the enzymatic activity of Erv1. This phenomenon will be the basis of a high throughput screen to identify small molecule modulators of Erv1 enzymatic activity. The goal of this study is to identify small molecule modulators of Erv1 enzymatic activity to use to identify unknown and corroborate previously identified functions of IMS import components.

Recombinant Erv1 with a 6xhis-tag is easily produced from bacterial expression and purified using Ni$^{2+}$ affinity resin. About 30-50 mg of active enzyme per liter can be produced. DTT, dithiothreitol, can be used as a non-physiological electron donor to the twin cysteines (C130 and C133) ultimately passing electrons to Erv1’s bound FAD molecule. The final step is the transfer of electrons to cytochrome c or molecular oxygen. Erv1 has been shown to reduce oxygen in the absence of cytochrome c previously studied in a spectrophotometer cuvette. A
simple assay of Erv1 catalytic activity can be achieved by coupling the DTT mediated production of peroxide from recombinant Erv1 to a fluorescent readout.

Amplex Red is a commercially available small molecule probe that is converted to the fluorescent compound resorufin by a reaction with hydrogen peroxide in the presence of HRP. Amplex Red has previously been used to measure activities of oxidases in high throughput format. Although Amplex Red is sensitive to temperature and light, precautions were taken into account during the assembly and execution of the screen.

After rounds of testing, the Erv1 assay was miniaturized to a volume of 55 μL. This allowed the screen to be performed in 384-well format. Each plate had a positive control lane of 1% DMSO. DMSO was shown not to cause significant changes in the assay behavior when less than 2%. Additionally, each plate has a negative control of a catalytically inactive mutant Erv1 C133S that is unable to produce peroxides by Erv1’s sulfhydryl oxidase activity.

The data is acquired using the Molecular Devices Flex Station plate reader and the SoftMax software package. All assay and screening data was processed though custom written data processing algorithms that computes well and plate statistics. The program calculates averages, standard deviations, Z score, Z’ factor, and percent inhibition for all wells and plates. Determination of Erv1 HTS assay quality signal to noise ratio was performed using Erv1 and the catalytically inactive C133S mutant. The assay read for 7 minutes and the signal to noise ratio could reach 10 fold. The Z’ factor was > 0.8 5 minutes after DTT addition. Taken together, this is a highly robust and reliable assay that facilitates clear hit determination.

The LS library of 40,000 compounds at the UCLA MSSR was screened in this fully automated assay with a liquid handling robotics and a plate scheduler. The library is a subset of hand selected small molecules to be drug like and filtered further to be chemically diverse as
well as unique to the UCLA collection. Each compound is >90% pure. The small molecules also fulfills Lipinski’s rule of 5 that evaluates a compounds pharmacological profile. These constraints insure the resulting hits will be of high quality.

Recombinant protein, Erv1 or the catalytically inactive C133S mutant, at 10 μM is incubated with either the vehicle control DMSO or small molecule (10 μM) pinned from a chemical library followed by the addition of Amplex Red and HRP. After equilibration, the reaction is initiated by the addition of 20 μM DTT. Endpoint fluorescence is measured at 10 minutes - within the linear range. It is important to note that the hit compounds must be rescreened to eliminate false positives. A low hit rate indicates that the Erv1 HTS assay is robust.

Results

Utilizing a high throughput platform, a total of 40,000 compounds on 125 384-well plates was screened with a hit rate of 0.32% (126 compounds with a greater than 50% apparent enzyme inhibition). Every plate had a Z score above 0.5 and in all averaging 0.8 indicating the screen was consistent and robust. These results are comparable to similar screens performed in previous pilot screens. In total, 126 small molecules displayed an apparent enzyme inhibition rate of greater than 50% and were designated as active candidates. To eliminate false positives, the compounds were subjected to a battery of counter screens and secondary assays.

It is noteworthy that although there were potential enhancers identified, further testing revealed that the top candidates either were auto-fluorescent or influenced the Amplex Red-HRP reaction and no further testing followed.

Counter screen and secondary assays

To eliminate false positives, counter screens were conducted to test whether the small molecule directly inhibited the Amplex Red assay or DTT reaction. Small volumes of cherry-
picked small molecules were individually pinned onto separate plates and subjected to additional assays. First, the assay was repeated in the presence of small molecule, DTT, HRP, Amplex Red, and 800nM H₂O₂ (experimentally determined physiological concentration) but in the absence of Erv1 to eliminate false positives that inhibit HRP, reacts with the conversion of Amplex Red to resorufin, or directly reduces molecular oxygen to peroxide. Second, the original reaction is repeated however in the absence of DTT and Erv1 and in the presence of H₂O₂ to eliminate false positives that inhibit Amplex Red directly. Third, a physiological substrate, Tim13, is used to reduce Erv1 to eliminate false positives that interact with DTT or prevent DTT reduction of Erv1. After performing each of these assays, 94 small molecules were subjected to further analysis.

Previous screening campaigns revealed small molecules that non-specifically permeabilize the mitochondrial membranes can present themselves as hits. To eliminate false positives, candidate compounds were incubated with respiring purified mitochondria and supernatant and pellet fractions analyzed for mitochondrial proteins. Compounds whose supernatant lane contains mitochondrial proteins were removed from the pool of active candidates.

Because Erv1 ablation does not affect mitochondrial membrane potential or NADH induced respiration, compounds that uncouple mitochondria are likely false positives. By measuring respiration using a Clark-type oxygen electrode, we were able to determine whether compounds inhibit respiration or uncouple mitochondria. Compounds that displayed oxygen consumption rates that were greater than basal NADH respiration and closer to known uncouplers, like carbonyl cyanide m-chlorophenyl hydrazine (CCCP), likely had deleterious non-specific activities. These results were complemented by a fluorescence based assay in which a
potentiometric dye, 3,3’-Dipropylthiadicarbocyanide Iodide (DiSC₃(5)), was used to measure the presence of a membrane potential. DiSC₃(5) accumulates in a hyperpolarized membrane where it translocates into the lipid phase where fluorescence is quenched, i.e. a low relative fluorescence. On the contrary, DiSC₃(5) in the presence of depolarized mitochondria, such as in the presence of CCCP, is released into the supernatant resulting in a high relative fluorescence. Candidate compounds were incubated with mitochondria and DiSC₃(5) and fluorescence was observed. It should be noted that small molecules can be absorb or emit fluorescence at the same wavelengths as DiSC₃(5) and proper controls were maintained. Candidate compounds that uncoupled mitochondria were removed from the active list.

The characterization of hits from the first round of screening suggests that most Erv1 modulators do not permeabilize the mitochondrial membrane, impair respiration, or uncouple mitochondria. The active candidate compounds were noted as Erv1 inhibitors and designated mitochondrial protein import blockers from the Carla Koehler lab (MitoBloCK). Of these potential lead inhibitors, MitoBloCK-7, MB-7, was chosen for further analyses and is discussed in the following chapters.

A collaborative luminescence screen for additional Erv1 modulators

The previous screens that identified MB-6 and the current screen that identified MB-7 were both completed using in-house libraries. Although the MSSR screening facility is an excellent resource, the identification and use of small molecules in studying mitochondrial biogenesis would greatly benefit by expanding our screening to include a collaboration with the NIH Molecular Libraries Probe Production Centers Network (MLPCN). This will be important for providing an opportunity to screen the larger library or different types of libraries (i.e., natural products), to consult with chemists and determine if a library of compounds similar to our
hit can be obtained from the large libraries available through the MLPCN, and to collaborate with chemists for optimization chemistry to identify the specific targets or design modified probes that can be used in vivo, etc.

An additional screen was performed at the Broad Institute in collaboration with the Koehler Lab. The screen was performed in a similar manner to the fluorescent *in vitro* assay previously described. In short, recombinantly purified Erv1, reduced by the non-physiological substrate DTT, reduced molecular oxygen to hydrogen peroxide. The levels of H$_2$O$_2$ were measured by Luminol which delivers light in the presence of HRP and peroxide. Molecules were also further evaluated for their mode of action and their potential for modulation of Erv1’s human homolog, ALR.

The Broad Institute collaborators screened 288,806 compounds which had a hit rate of 3.7%. After applying cheminformatic filters, 4678 compounds were active. Of those, the top 2500 were selected for re-screen. The resulting 1447 compounds were subjected to secondary assays for further assessment.

A counter screen repeated the luminescent assay with ALR and DTT but with exogenous H$_2$O$_2$ to eliminate false positives that inhibited off target elements of the *in vitro* reaction. Secondly, an assay similar to the primary screen, however with physiological substrate Tim13, was performed in order to identify specificity for ALR. Moreover, a Proteostat PDI Assay Kit (Enzo Life Sciences) was used to eliminate small molecules that were non-specific inhibitors of all oxidases. PDI is a non-homologous oxidase that reduces insulin. The reduction of insulin causes formation of aggregates, which bind a fluorogenic detection reagent. Recombinant Erv1 and Erv2 dose response studies as well as DiSC$_3$(5) membrane potential studies followed suit. Following secondary assays, 1038 compounds remained in the pool of active hits.
A small collection of these hits were sent to the Koehler lab for further analyses. Following preliminary experiments, small molecules that were designated MB-8, MB-9, and MB-13 were selected for further characterization and utilized for dissecting mitochondrial intermembrane space biogenesis and will be discussed further in the following chapters.

Concluding Remarks

In summary, the goals of these screening campaigns were to identify and validate a collection of small molecule modulators of Erv1. Between the screens performed at UCLA’s MSSR and the Broad Institute, we have discovered small molecules that have great potential to be used to investigate the roles of Erv1 in mitochondrial biogenesis. These small molecules allow for specific and rapid manipulations that will allow us to investigate mechanisms that were previously too difficult. Furthermore, these versatile tools can be used cooperatively with additional genetic or biochemical approaches in diverse models and will expand our understanding of cellular functions.
Materials and Methods

Protein expression and purification.
Recombinant Erv1 and Erv1 C133S were expressed and purified under native conditions as described previously\textsuperscript{89}.

High-throughput screen for Erv1 modulators.
Freshly purified recombinant Erv1 (in buffer Screening Buffer, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH7.4, 100 mM NaCl, 1 mM EDTA) at a concentration of 10 µM, which was expressed and purified as described previously. A Titertek multidrop (Thermofisher) was used to dispense 25 µl Erv1 or 25 µl of catalytically inactive enzyme Erv1C133S into clear bottom 384-well plate (GreinerBio). A Biomek FX (Beckman Coulter) was used to pin transfer 0.5 µl compound from 1mM stock (from the diverse smart 40,000 compound LS library) or DMSO (1% vehicle) to respective wells. Approximate screening concentration was 12.5 µM.
After completed compound transfer, all plates were incubated at 25°C in a humidified incubator for 1 hr. A Titertek multidrop was used to dispense 50 µl of Amplex Red-HRP (Sigma) mix into all wells of the 384-well plate. The final concentration of Amplex Red and HRP were 46 µM and 0.092 U/ml, respectively. The Amplex Red-HRP solution was shielded from light during the entire experiment. The plates were incubated for an additional 10 minutes, and then 15 µl of the substrate DTT (20 µM) was added to initiate the reduction of O₂ to H₂O₂ by Erv1. The plates were incubated for 12 minutes to achieve a maximal signal-to-noise ratio in the kinetic linear range. Plates were then read at an endpoint using an excitation wavelength of 545 nM and an emission wavelength of 590 nM. All operations were performed by an automated plate scheduler to ensure consistency across the screening run. Assay quality and reproducibility of each plate was monitored using the statistical parameter, \( Z' \textsuperscript{92} \). All plates had a \( Z' \) value greater than 0.8.
than 0.5. Compounds that inhibited activity by 50% were marked as potential hits. Hits were then rescreened against Erv1 in the presence of H$_2$O$_2$ to ensure compounds did not inhibit HRP. Commercially available compounds were purchased for further analysis.

**Oxygen consumption and membrane potential measurements.**

Mitochondria were purified from yeast cells grown on YPEG as described previously $^{93}$. Oxygen consumption measurements with isolated mitochondria were performed using an oxygen electrode (Hansatec) as described previously $^{90}$. Membrane potential measurements of purified mitochondria were performed with fluorescent 3,3'-dipropylthiadicarbocyanine iodide dye [DISC3(5)]. 1% DMSO, carbonyl cyanide $m$-chlorophenyl hydrazone (CCCP), MB-7, or MB-8 was added to mitochondria in import buffer (0.6 M sorbitol, 2 mM KH$_2$PO$_4$, 60 mM KCl, 50 mM HEPES-KOH, 5 mM MgCl$_2$, 2.5 mM EDTA, 5 mM L-methionine, pH 7.1) for 10 min. Subsequently 0.2 μM DISC3(5) in import buffer was added, incubated for 5 min, and fluorescence was measured at excitation and emission length of 620 nm and 670 nm, respectively.

**Mitochondria integrity assays.**

25 μg of isolated yeast mitochondria were incubated in import buffer and varying concentrations of small molecules at 25°C for 30 min. Mitochondria were then pelleted by centrifugation at 8,000 x g for 10 min at 4°C. The supernatant containing released proteins was TCA precipitated on ice for 30 min. Precipitated proteins were recovered by centrifugation at 15,000 x g for 15 min at 4°C. The mitochondrial pellet and the precipitated supernatant were resuspended in SDS sample buffer and analyzed on SDS-PAGE. Resulting gel were stained with Coomassie blue for 30 min or transferred to nitrocellulose membrane and blotted against key mitochondrial proteins.
Purification of mitochondria

Mitochondria were purified from yeast cells grown in YPEG as described previously. Yeast cultures were kept at 25°C with vigorous shaking during growth. Mitochondria concentration was measured by BCA assay and stored in 25 mg/ml at -80°C.
**Figures**

Figure 1.1 – General Mitochondrial Protein Translocation – Proteins are translocated through the gate protein Tom40, or translocon of the outer membrane. Importing proteins destined for the outer membrane enter the lipid phase via the SAM complex. Proteins destined for the inner membrane enter through the Tim22 complex. The Tim23 import pathway usually directs proteins to the matrix, but there are also situations where proteins can be inserted into the inner membrane. The MIA pathway is responsible for the oxidative folded proteins of the intermembrane space.
Figure 1.2 – MIA import pathway – Usually small, cysteine rich substrates, such as small Tim chaperones, are imported through the Translocon of the Outer Membrane (TOM40) complex and form a mixed disulfide bond with Mia40. The substrate is oxidized, locking it in the intermembrane space. Mia40 is subsequently re-oxidized by the sulfhydryl oxidase, Erv1. Erv1 passes the acquired electrons to cytochrome c (and eventually the electron transport chain), or molecular oxygen.
Figure 1.3 – Flowchart of high-throughput screening campaign. A high throughput screening assay was designed and executed to identify modulators of the sulfhydryl oxidase Erv1. Following the initial screen secondary assays followed to qualify lead hits.
Figure 1.4 – Potential modulators of Erv1 – The LS compound library of 40,000 small molecules was screened. Inhibitors with apparent inhibition of greater than 50% (126) were further subjected to secondary screens. Top potential enhancers were also re-screened.
Figure 1.5 – Mitochondrial membrane permeabilization assay – Biologically active compounds identified in the primary screen were assessed for the non-specific permeabilization of mitochondrial membranes. Shown is a select group of candidate small molecules that were incubated with purified mitochondria. Supernatant (S) and pellet (P) fractions were separated by SDS-PAGE and analyzed by Coomassie staining. Membrane permeabilizing compound 5447 was included as a positive control.
Figure 1.6 – Membrane potential assay – Mitochondria were incubated with DMSO, chemical uncoupler CCCP, or small molecule in the presence of a fluorescent dye, DiSC3(5), that is taken up by the mitochondria and quenched in the presence of a membrane potential.
2 Assays - Critical Path Flow chart

**Primary Assay**
HTS-Luminescence assay for inhibitors of ALR by detection of hydrogen peroxide production.

Stop if there are no hits

- **Confirmatory Assay**
  Repeat of Primary Assay, at dose.
  BIPDeC

  < 10 µM IC₅₀
  Stop if no compounds show repeat activity

- **Secondary Assay 1**
  Luminescence assay for the detection of hydrogen peroxide activity in the absence of ALR and DTT, at dose.
  BIPDeC

  < 10% inhibition
  Stop if compounds are luminal/HRP inhibitors

- **Secondary Assay 2**
  Turbidity counter screen for activity with non-homologous sulfhydryl oxidase PDI, at dose.
  BIPDeC

  >10-fold specificity
  Stop if compounds inhibit PDI

- **Secondary Assay 3 & 4**
  Luminescence assay for inhibitors of ALR with:
  3. CₓₓC
  4. CₓₓC
  BIPDeC

  >10 µM IC₅₀
  Stop if compounds show no activity

- **Secondary Assay 5**
  Mitotoxicity assay with a coupled fluorescence/luminescence read-out in HUVEC cells, at dose.
  BIPDeC

  >5-fold activity
  Stop if compounds do not show mitotoxicity

  Stop after 3 rounds of SAR optimization if no probe

- **Secondary Assay 6**
  High content mitochondrial health assay in HUVEC cells, at dose.
  BIPDeC

  >5-fold activity
  Stop if compounds do not show activity in cells

**Legend**

- Primary protocol
- Secondary assay
- Assay cut-off
- Decision point

Figure 1.7 – Flowchart of small molecule modulator screen as performed by the collaborators at the Broad Institute.
References


34. Maldonado, E. N.; Lemasters, J. J., ATP/ADP ratio, the missed connection between mitochondria and the Warburg effect. Mitochondrion 2014, 19 Pt A, 78-84.


Chapter 2. A small molecule probe that selectively inhibits the import of a subset of Mia40/Erv1 substrates

Abstract

The mitochondrial disulfide relay system of Mia40 and Erv1/ALR facilitates the import of cysteine rich proteins, including the small Tim proteins. Our lab has previously characterized mitochondrial biogenesis and import using classical genetics and biochemical assays with purified mitochondria. However, new strategies are needed to elucidate the detailed mechanisms of mitochondrial protein translocation and their role in mammalian development and human disease. To this end, we developed a chemical biology approach to identify small molecules that inhibit Erv1 oxidase activity to enable dissection of the disulfide relay system in yeast and mammalian mitochondria. We have recently completed a new screen of 40,000 small molecules and have identified potential inhibitors. Several molecules, including MB-6, MB-7, and MB-8, have been characterized in detail. Our collection of small molecules differentially affects the import of various Erv1 substrates, which suggests that each probe may have alternative mechanisms of inhibiting Erv1. Thus, the approach of using a target based chemical screen validates the generation of new tools to understand mammalian mitochondrial import into the IMS and how dysfunction is linked to disease.
Introduction

Mitochondria are dynamic organelles that are responsible for many cellular processes including energy, heme, iron, and lipid metabolism, as well as signaling and apoptosis\textsuperscript{1-5}. The mitochondrial genome encodes only a small fraction of the organelle’s proteome requiring most proteins to be imported from the cytosol via a complicated translocation system\textsuperscript{6}. Defects in mitochondrial import leads to a host of degenerative diseases in the muscular and neural systems\textsuperscript{7}. Most studies on mitochondrial biogenesis have been done in yeast because yeast is amenable to biochemistry and genetics. As a result, our understanding of the protein import in \textit{S. cerevisiae} is advanced and serves as a model for understanding mitochondrial assembly in vertebrates\textsuperscript{8,9}. However, differences do exist because many homologs are absent in yeast. For example, it is difficult to study apoptosis and signaling pathways in yeast mitochondria because these pathways are at best rudimentary. Expanding our research of mechanisms in a variety of models will broaden the scope of our understanding thus facilitating in identifying how dysfunction leads to disease\textsuperscript{10}. But adequate tools are not widely available\textsuperscript{11}. There has been a recent push to employ small molecules in basic research to perturb a single protein function or interaction to more accurately characterize biological processes where traditional genetic approaches have been limiting\textsuperscript{12,13}. The objective of this research is to identify and employ small molecules to study mitochondrial biogenesis to aide in the determination of how dysfunction results in disease.

The mitochondrial intermembrane space is crucial for a variety cell processes like apoptosis and house important and sometimes disease linked proteins like the Parkinson’s disease associated kinase Pink1\textsuperscript{14}. The redox regulated intermembrane space import components, Mia40 and Erv1, have been implicated in a variety of essential cellular processes like iron-sulfur
cluster maturation and heme biogenesis\textsuperscript{15,16}. Mutations in ALR result in a rare autosomal recessive myopathy and it remains unclear how these defects lead to disease\textsuperscript{17}. Previous attempts to study these processes have less than favorable outcomes because many of the components are essential\textsuperscript{11}.

Small molecules identified in a high throughput screen by the Koehler lab and in collaboration with the Broad Institute have been used to dissect mitochondrial intermembrane space biogenesis and facilitate in determining how dysfunction leads to disease. Furthermore, these small molecules will expedite the development of therapeutics. Currently, we have used these small molecules to dissect key features of intermembrane space import mechanisms and identified import complexes that are affected by the various Erv1 modulators.

\textbf{Results}

\textbf{MB-7 inhibits Erv1 activity in vitro.}

From a similar small molecule screen with recombinant Erv1 and the non-physiologic substrate DTT that resulted in the identification of MB-6, we identified an additional modulator designated MitoBloCK-7 (MB-7) that inhibited Erv1 activity by greater than 50\% in the screen. The assay was based on the reduction of oxidized DTT by Erv1 with the production of H\textsubscript{2}O\textsubscript{2}. A standard fluorometric assay with Amplex Red and horseradish peroxidase (HRP) measured H\textsubscript{2}O\textsubscript{2} production. To eliminate false-positives that inhibited the Amplex Red reaction, a counter screen was used to test whether the small molecule compounds directly inhibited the Amplex Red-HRP assay. MB-7 did not inhibit the HRP reaction. Thus, MB-7 was deemed a potential Erv1 modulator.

MB-7 is a sulfonamide, N-(4-(2-(cyclopropylamino)-6H-1,3,4-thidiazin-5yl) phenyl)-N-methylmethanesulfonamide from the LS library (\textbf{Figure 2.1A}). Upon reordering from the
vendor, MB-7 showed the same Erv1 inhibitory activity as the original aliquot from the LS library. The IC50 for MB-7 that inhibited Erv1 oxidase activity in the in vitro Amplex Red-HRP assay was 18 μM (Figure 2.1B). To determine if MB-7 inhibited similar proteins in the sulfhydryl oxidase family, we also tested MB-7 (50 μM) with ALR, the yeast paralog in the endoplasmic reticulum Erv2, and QSOX at 3 μM using the in vitro Amplex Red-HRP assay (Figure 2.1C). MB-7 inhibited sulfhydryl oxidase activity for Erv1 and ALR, but not Erv2 and QSOX. Thus, MB-7 seems to show more specificity at inhibiting Erv1 and ALR than closely related Erv2 and QSOX.

**Mitochondria are not damaged by MB-7**

A potential mechanism by which MB-7 could alter protein translocation is to nonspecifically permeabilize membranes, resulting in the release of mitochondrial proteins, particularly from the intermembrane space. We have previously shown that MB-2, an inhibitor of the TIM22 import pathway, nonspecifically permeabilizes mitochondrial membranes. We incubated energized mitochondria with 1% DMSO or MB-7 for 30 min on ice followed by centrifugation. Released proteins were recovered in the supernatant (S) fraction and analyzed by Coomassie staining for the collective release of proteins (Figure 2.2A) and by immunoblot assay for key proteins (Figure 2.2B). The results from Coomassie staining indicated that MB-7 did not alter mitochondrial membrane integrity, because proteins were not released into the supernatant fraction (Figure 2.2A). Similarly, immunoblot analysis showed that marker proteins aconitase (matrix), AAC (inner membrane), and Ccp1 (intermembrane space) were not released with MB-7 or DMSO treatment (Figure 2.2B).

Another potential mechanism by which MitoBloCK-6 may disrupt protein translocation is indirect, by dissipation of the membrane potential (ΔΨ) or disruption of oxidative
phosphorylation, both of which can be measured with a Clark-type oxygen sensing electrode (Figure 2.2C)\textsuperscript{11}. Isolated mitochondria were incubated in a 1 ml chamber at 25°C with an oxygen electrode and respiration was initiated with NADH. The measured oxygen consumption rate was indicative of well-coupled mitochondria. The addition of the vehicle (1% DMSO) does not alter respiration (data not shown and \textsuperscript{20}). The subsequent addition of 100 μM MB-7 did not alter the oxygen consumption rate (Figure\textsuperscript{2.2C,D}). As a control, mitochondria were treated with CCCP and the respiration rate increased drastically, indicative of uncoupled mitochondria (Figure\textsuperscript{2.2C,D}). The ΔΨ was also measured with the fluorescent dye DiSC\textsubscript{3}(5), which is taken up by mitochondria and then released when the ΔΨ is dissipated with an uncoupler such as CCCP (Figure\textsuperscript{2.2E})\textsuperscript{21}. The relative change of fluorescence between dye uptake and release is a relative measure of the ΔΨ. The membrane potential measurements indicated that mitochondria had a robust ΔΨ in the presence of DMSO or 100 μM MB-7. In contrast, CCCP uncoupled the mitochondria as the dye was released. Taken together, MB-7 does not alter mitochondrial function or disrupt mitochondrial integrity.

Erv1 is an essential protein and thus it is expected that dysfunction would result in cytotoxicity. We tested the toxicity of MB-7 in drug pump mutant yeast GA74ΔSNQ2ΔPDR5 (Figure S2.1A). Cultures were grown for 72 hrs at 30°C in YPEG and varying concentrations of MB-7 and OD\textsubscript{600} was measured. MB-7 treatment is not toxic to yeast. It is not clear if the Erv1 function modified by MB-7 treatment is not essential for survival or if MB-7 is being degraded by an alternative pathway. Moreover, we tested MB-7 toxicity in mammalian cells. Using a commercially available MTT colorimetric toxicity assay, we measured the viability of HeLa cells in varying concentrations of MB-7 (Figure S2.1B).

MB-7 inhibits Erv1-dependent import into mitochondria
The import of Erv1 substrates was tested with an \textit{in organello} import assay. Substrates included twin CX\textsubscript{9}C proteins (Cmc1, and Mia40), twin CX\textsubscript{3}C protein Tim13, Erv1, and Tim22 (\textbf{Figure 2.3}). Additional controls included Su9-DHFR that uses the TIM23 translocon and AAC that uses the TIM22 translocon. Energized mitochondria were pre-incubated with varying concentrations of MB-7 or vehicle (1% DMSO) for 15 min, followed by the addition of the radiolabeled substrate. First, time course assays were performed with a fixed amount of MB-7 and precursors Cmc1, Tim13, Mia40, Erv1, and AAC were tested. The import of Cmc1 and Tim13 was strongly decreased by 85% (\textbf{Figure 2.3A,B}). Erv1 and Mia40 depend on themselves for import; MB-7 did not markedly inhibit import of these substrates as import was decreased by 65% (Erv1) and 35% (Mia40) (\textbf{Figure 2.3C,D}). Tim22 and AAC import has been shown to partially depend on Erv1\textsuperscript{11,22,23}. Tim22 translocation was decreased by 40% and AAC import was unaffected in the presence of MB-7 (\textbf{Figure 2.3E,F}).

To identify the minimal concentration of MB-7 that was needed to abrogate import, we performed titrations with varying concentrations of MB-7 at a single endpoint that was set in which the maximal amount of precursor was expected to accumulate. Cmc1 and Su9-DHFR were tested (\textbf{Figure 2.3G,H}). 10 μM MB-7 strongly inhibited Cmc1 import, whereas MB-7 at 100 μM did not block Su9-DHFR import. Thus, this analysis supports that MB-7 seems to be a specific inhibitor for typical CX\textsubscript{9}C and CX\textsubscript{3}C substrates, but the import of other substrates (Mia40, Erv1, AAC, and Tim22) in which Erv1 has an auxiliary role were not strongly inhibited by MB-7.

To verify that mitochondrial Erv1 is the target of MB-7, an increased abundance of Erv1 should require an increased MB-7 concentration to inhibit protein import. Previously, we designed a yeast strain in which Erv1 with a C-terminal hexahistidine tag (designated \textsuperscript{\textdagger}Erv1)
was expressed from a high copy plasmid. This strain contained an approximate 5-fold increase in Erv1 with no aberrant phenotypes detected. The import of Cmc1 was tested in isolated WT and ΔErv1 mitochondria (Figure 2.3I). The concentration of MB-7 that was required to inhibit import increased from 10 μM to 50 μM (Figure 3I). Therefore, Erv1 is the likely target of MB-7 in isolated mitochondria.

To further confirm that MB-7 inhibition of Erv1 is specific, we tested additional small molecules that shared a similar structure with MB-7 in structure-activity relationship (SAR) studies (Figure 2.4). Small molecules with similar structures were purchased from the vendor and tested in the in vitro import assay with Cmc1 as in Figure 3. Of the SAR compounds, the compound designated 7.1 partially inhibited Cmc1 import, but the other four compounds did not alter import. Based on structure, the sulfone may be important for MB-7 interactions with Erv1 and MB-7 inhibition of Erv1 seems very specific.

**MitoBloCK-8 is an additional Erv1 inhibitor that is not deleterious to general mitochondrial function**

From a chemical screen performed by the Broad Institute (previously described in Chapter 1), several possible Erv1 inhibitors were sent to the Koehler lab for further analysis. One promising hit, MitoBloCK-8 (MB-8), inhibited Erv1 activity by more than 50%. The screen was similar to previous screens using DTT as a substrate of Erv1 producing a measurable amount of H₂O₂. Luminol reacts with HRP and H₂O₂ producing light. To eliminate false positives, that inhibited the in vitro reactions, a counter screen was performed and MB-8 did not inhibit the reduction of DTT or the HRP reaction. MB-8 was deemed an Erv1 modulator.

MB-8, N-(3-(2-((5-methylpyridin-2-yl)amino)thiazol-4-yl)phenyl)acetamide (Figure 2.5A), was ordered from a vendor and displayed similar inhibitory activity as the original screen.
Attempts at repeating the efficacy of MB-8 were unsuccessful because MB-8 inhibited the Amplex Red reaction. Additional modulators were identified in the screen and currently being further characterized.

**MB-8 does not affect general mitochondrial function**

Because Erv1 inhibition should not affect general mitochondrial functions, we assessed for deleterious effects on mitochondrial respiration, membrane stability, and membrane potential. Isolated mitochondria were incubated in a 1 mL chamber at 25°C with a Clarke-type oxygen electrode and respiration was initiated with NADH. Upon addition of 100 μM MB-8, respiration rate was unchanged indicating that mitochondria remain well coupled in the presence of MB-8 (Figure 2.5B). As a control CCCP was added and the respiration rate increased drastically. The membrane potential was also measured with the fluorescent dye DiSC₃(5), which is taken up by the mitochondria and then released when the membrane potential is dissipated with an uncoupler such as CCCP (Figure 2.5C). The relative change fluorescence between dye uptake and release is a relative measure of the membrane potential. The low fluorescence of the MB-8 treated samples are indicative that the membrane potential is preserved (Figure 2.5C). To show that MB-8 does not non-specifically permeabilize the mitochondrial membrane, energized mitochondria were incubated either 1% DMSO (data not shown), a compound known to permeabilize the membrane, 5447, or 100μM MB-8 for 30 min on ice. Released proteins were recovered in the supernatant (S) fraction and analyzed by Coomassie staining for the collective release of proteins. The results from Coomassie staining indicated that MB-7 did not alter mitochondrial membrane integrity, because proteins were not released into the supernatant fraction (Figure 2.5D). Taken together, MB-8 does not alter mitochondrial function or disrupt mitochondrial integrity.
Erv1 is an essential protein and thus it is expected that dysfunction would result in cytotoxicity. We tested the toxicity of MB-8 in drug pump mutant yeast GA74ΔSNQ2ΔPDR5 (Figure S2.2A). Cultures were grown for 72 hrs at 30°C in YPEG and varying concentrations of MB-8 and OD$_{600}$ was measured. Unlike MB-7, MB-8 treatment is toxic to yeast, particularly above 50 μM. Moreover, we tested MB-8 toxicity in mammalian cells. Using a commercially available MTT colorimetric toxicity assay, we measured the viability of HeLa cells in varying concentrations of MB-8 (Figure S2.2B). MB-8 was toxic to mammalian cells at concentrations greater than 25 μM.

**MB-8 inhibits Erv1-dependent import into mitochondria**

The import of Erv1 substrates is a classic way to demonstrate the effectiveness of Erv1 modulation$^{11}$. As shown previously with MB-7, canonical Mia40 substrates with twin Cx(n)C motifs (Cmc1, Erv1 Mia40, and Tim10) were tested in an in organello import assay (Figure 2.6). Additional controls include Su9-DHFR which uses the Tim23 translocon and AAC that uses the Tim22 translocon. Energized mitochondria were incubated with varying concentrations of MB-8 or vehicle control (1% DMSO) for 15 min, followed by the addition of radiolabeled substrate. Non-imported precursor was removed by protease treatment. The previously described MB-7 as well as uncharacterized MB-9 and MB-13 were also analyzed. Single time-point assays were performed to assess inhibition of import. The import of Cmc1 and Erv1 were strongly decreased by 75% and 90%, respectively (Figure 2.6A,C). Mia40 and Su9-DHFR were only slightly inhibited (Figure 2.6B,E) and AAC and Tim10 did not show any inhibition (Figure 2.6D,F). To identify the minimal concentration of MB-8 that is needed to abrogate import, a titration of varying concentrations of MB-8 treated mitochondria were incubated with radiolabeled Cmc1 and import was measured at a single time-point. It is evident that like MB-7, MB-8 is an
inhibitor of Erv1. However, it is not clear what auxiliary roles Erv1 plays that may contribute to the mild and inconsistent inhibition of non MIA substrates. In collaboration with the Banci lab, we analyzed the residue peaks shifts in the presence of various MitoBloCKs and the each treatment (MB-5,6,7,8,13) showed similar shifts in residue demonstrating a consistent structural change among MitoBloCKs (Figure S2.5). It remains unclear if the small molecules remain bound to Erv1.

**ALR structure analysis by NMR in the presence of Erv1 modulators**

To identify how small molecules modulate Erv1 activity, we investigated structural rearrangements of ALR in collaboration with the Banci lab. Because the N-terminal region of Erv1 is mobile, a full structure by X-ray crystallography or NMR has been difficult to achieve. The Banci lab has purified ALR and assigned ΔHN peaks. ALR was incubated with our Erv1 inhibitors and overlaid with the DMSO control. Residue shift distances were measured and compared (Figure S2.5). Briefly MB-6 and MB-8 had similar profiles with shifts at residues 10-20, 20-30, 40, 70, and 120-130. MB-7 had a similar profile, however lacking the shifts at 10-12. It is not yet clear how these shifts contribute to the observed phenotypes. The interacting regions involve solvent exposed residues and a pocket where the compounds can sit. The shifting residues were mapped on PDB 3O55 (Figure S2.5).

**The abundance of the Mia40-substrate import intermediate is decreased in the presence MB-7.**

During import, Mia40 forms a disulfide bonded intermediate with the reduced substrate\(^{25}\). The substrate is subsequently released in an oxidized and folded state and Mia40 is oxidized by Erv1. We investigated whether MB-7 or MB-8 treatment blocked the import pathway at this intermediate stage by extending the aforementioned import studies (Figure 2.7). We used Cmc1
as a substrate as well as two mutants Cmc1\textsuperscript{C42A} and Cmc1\textsuperscript{C64A} to investigate how MB treatment might affect interactions with the different cysteine residues (Figure 2.7). The import of Cmc1, Cmc1\textsuperscript{C42A}, and Cmc1\textsuperscript{C64A} was first tested into mitochondria. As expected, Cmc1\textsuperscript{C42A} import was impaired, because complete translocation into mitochondria requires this cysteine residue\textsuperscript{26}. In contrast, Cmc1\textsuperscript{C64A} import was similar to WT Cmc1. The addition of MB-7 and, to a lesser extent, MB-8, inhibited the import of both Cmc1 and Cmc1\textsuperscript{C64A}.

We also investigated the formation of the Mia40-Cmc1 import intermediate that is detected in non-reducing gels, which marks the initial interaction in translocation (Figure 2.8A). An intermediate between Mia40 and Cmc1 was present. The intermediate for Cmc1\textsuperscript{C42A} and Cmc1\textsuperscript{C64A} was increased in abundance (Figure 2.8A), which is expected because the translocation pathway is less efficient and the intermediate accumulates bound to Mia40. The addition of MB-7 and MB-8 resulted in decreased abundance of the Mia40-Cmc1 intermediate (Figure 2.8 B,C). For Cmc1\textsuperscript{C42A}, this intermediate was also decreased (Figure 2.8D), whereas the abundance of the intermediate with Cmc1\textsuperscript{C64A} was slightly decreased (Figure 2.8C).

To investigate the abundance of the intermediate, we performed pulldowns in a yeast strain in which Mia40 was tagged at the C-terminus. This strain was generated by integrating a His-tag into the genome that corresponded to the C-terminus of Mia40 using homologous recombination. The his-tag did not interfere with growth and the steady-state levels of mitochondrial proteins (Mia40, Erv1, Tim13, Ccp1, and Cym1) in the Mia40-His tagged strain were similar to WT (Figure S1). Radiolabeled Cmc1 as well as Tim10, Erv1, and Mia40 were imported into WT and Mia40-His mitochondria in the presence of MB-7 and vehicle control (1% DMSO) (Figure 2.9). The mitochondria were solubilized with 8M Urea, pH 8.0 and 20 mM IAA and pulldowns were performed with Ni\textsuperscript{2+}-NTA affinity resin. The columns were washed
with 8M Urea, pH 6.3, and bound Mia40 (M) was eluted at pH 4.5 and boiled with SDS sample buffer (Eluate). 5% of the total lysate (Load) and the eluate were separated by non-reducing SDS-PAGE. The imported substrate was detected with autoradiography. The abundance of the Cmc1-Mia40 intermediate was markedly decreased in the presence of MB-7 (Figure 2.9A). In addition, MB-7 treatment slightly decreased the abundance of the Mia40-Tim10 intermediate (Figure 2.9B), whereas the Mia40-Erv1 and Mia40-Mia40 intermediate were strongly decreased in the presence of MB-7 (Figure 2.9C,D). As a control, untagged Mia40 was not purified in WT mitochondria. Thus, MB-7 inhibition of Erv1 activity blocks the import of MIA substrates at an early stage, supporting the idea that Erv1 is necessary at the initial steps of translocation.

We expanded the pulldown experiments with mitochondria that contained Erv1 with a C-terminal His-tag and substrate Cmc1\textsuperscript{11}. As described in Fig. 2.9, Cmc1 was imported followed by pulldowns with Mia40 (M) and Erv1 (E) (Figure 2.10). A new translocation intermediate was detected at a mass of \(~130\) kDa that contained Erv1 and Cmc1, but not Mia40; this intermediate may also contain additional protein(s). This \(~130\) kDa intermediate was also detected with Cmc1 C64A but not Cmc1 C42A. The addition of MB-6 and MB-8 decreased the abundance of this translocation intermediate (Figure 2.11). An additional translocation intermediate of \(~180\) kDa containing Mia40 and Cmc1 was also detected (marked with an asterisk); this may be a dimer of Cmc1-Mia40. Note that the conditions to visualize the \(~130\) kDa Erv1-Cmc1 intermediate result in overexposure for the Cmc1-Mia40 intermediates, which have been examined closely in Figure 2.8 and 2.9.

**Identification of MIA import complexes by mass spectrometry**

The import complexes identified in in the radiolabeled pull down experiments revealed previously undescribed import complexes. The high molecular weight complex was identified by
pulling down his-tagged Erv1 and autoradiography revealed a band at 130 kDa. The band was not present with his-tagged Mia40. To identify substrates that were in complexes, recombinantly purified Cmc1 was imported into either Wt or his-tagged Erv1 mitochondria. His-tagged proteins were separated by Ni$^{2+}$-NTA affinity resin in the presence of 8M urea and iodoacetamide to preserve disulfide bonds and prevent aberrant ones from forming. The columns were eluted with 250 mM imidazole and samples were separates by non-reducing SDS-PAGE. Gels were imaged by UV fluorescence and gel slices were extracted (Figure 2.12). The gel slices were subjected to in-gel trypsin digestion and transferred to the Loo lab for Mass spectrometry analysis. Results revealed that many bands contained Cmc1 and Erv1 suggesting the assay was successful. A promising hit returned was mitochondrial glycerol-3-phosphate dehydrogenase (GUT2). GUT2 is in localized in the intermembrane space and uses an FAD cofactor, similar to Erv1. Further confirmatory analyses are currently underway.

**MB-7 impairs cardiac development in zebrafish**

To test specificity of the small molecules, we have incubated zebrafish embryos with small molecules starting at 4 hours post fertilization and investigated development. MB-6 treatment impaired general development as well as cardiac development; cardiac mitochondria marked with DsRed were also not detectable, supporting an important role for mitochondria in cardiac development. MB-7 was less potent than MB-6, but shared similar developmental defects. At concentrations of 50-100 μM, the larvae showed slight body curvature, cardiac edema, and failure of the heart to loop (Figure S2.3). MB-8, on the other hand, was more potent than MB-6. No fish survived when treated with over 10 μM MB-8. At low concentrations, the larvae showed slight body curvature, cardiac edema, and failure of the heart to loop, a similar common phenotype for Erv1 inhibition (Figure S2.4). Moreover, the mitochondria marked with
DsRed were not detectable in cardiac tissue in both MB-7 and MB-8 treated fish. Thus, MB-7 and MB-8 seems to interfere with Erv1 function in a manner similar to MB-6.

**Discussion**

From two high throughput chemical screens, we identified multiple modulators of the sulfhydryl oxidase Erv1. Among those identified, we labeled MB-7 and MB-8 as selective inhibitors of the redox-mediated import pathway. Because small molecules may non-specifically alter mitochondrial function, we investigated the effects of MB-7 and MB-8 on membrane potential, respiration, and mitochondrial integrity. MB-7 and MB-8 does not generally damage mitochondria. In organello import assays demonstrated that MB-7 and MB-8 selectively inhibit Erv1/Mia40 precursors. These experiments, among others, strongly suggest that MB-7 and MB-8 are specific inhibitors of Erv1/ALR enzymatic activity.

MB-7 inhibited almost all MIA intermediates from importing as well as seemed to have secondary effects on non-canonical substrates at higher concentrations, particularly of the Tim22 pathway. AAC is imported via the Tim22 complex and yet showed mild inhibition because import of AAC requires the small Tim chaperones for protection while traversing the hydrophilic intermembrane space. It was previously suggested Erv1 plays a role in maintaining the small Tim disulfides. MB-8 appeared to be more selective of precursor inhibition in that it did not inhibit AAC or Mia40 strongly. These results suggest each small molecule may have a different mode of inhibition.

Yeast, mammalian, and zebrafish toxicity studies revealed additional differences between the small molecules. MB-7 does not appear to be toxic to yeast and HeLa cells, but is toxic to zebrafish at concentrations above 50μM. MB-8 is toxic to yeast and HeLa cells at higher concentrations, and is toxic to zebrafish as low as 5μM. It is not clear if MB-7 is more sensitive
to degradative pathways in the cell, but nonetheless raises questions about how these small molecules are interacting differently.

Because of the variability during in organello import inhibition as well as differences in toxicity profiles, we asked whether these small molecules are modulating Erv1/ALR differently. In collaboration with the Banci lab, we investigated the effects of our small molecules on the structure of ALR. Because the long form of ALR is quite unstable, the short form was incubated with the small molecules or DMSO and analyzed by Heteronuclear Single Quantum Correlation NMR spectroscopy to identify spectra shifts that represent protein structure modification. The protein bound with compound is in slow exchange with the free one, on the NMR time scale. Indeed we detect in the NMR spectra of the protein the formation of a new species, while the signals of the unbound species disappeared. The regions of the protein interacting with MB-6, MB-7, MB-8 are the same for all compounds, with the exception of some residues at N-terminal of the SF-ALR which are not interacting with MB-7 is not interacting as indeed shown by chemical shifts changes. The interacting regions involve solvent exposed residues and a pocket where the compounds can sit. Interacting residues are 15, 16, 18, 22, 25-27, 40, 68, 69, 119-124 in the case of MB-8, while MB-7 interact only with residues 40, 68, 69, 119-124. Interacting residues are mapped on the protein surface on the ALR PDB 3O55.

Because it is not clear how defects in Erv1/ALR defects lead to disease, we investigated the mechanisms of redox-regulated import into the IMS. It was previously shown the Mia40 forms disulfides with importing precursor. These interactions are inhibited in the presence of MB-7. It is not clear if this is a result of a disruption in the Erv1-Mia40 interaction, if the flow of electrons, or if Erv1 is getting locked in with one of the interacting partners, i.e. cytochrome c. Furthermore, we have identified an import complex around 130 kDa. A ternary complex has
previously been suggested but the species has yet to be seen in physiological conditions. The complex migrates at a much higher complex than what we would expect a 1:1:1 stoichiometric complex to run. To identify this larger complex, we isolated the bands and subjected to mass spectrometry. Although the peaks weren’t abundant, an interesting hit was mitochondrial glycerol-3-phosphate dehydrogenase. Yeast Gut2 may be an additional terminal electron acceptor and play a role in import during non-oxidative growth. Further studies are being carried out to confirm this, among other hits.

Defects in mitochondrial function impact human health greatly and result in a wide range of diseases from degenerative muscle and neural diseases to cancer. Dissecting many of these pathways using traditional techniques have proven to be difficult. Utilizing small molecule tools in mitochondrial research is a novel approach that will help further our understanding of mitochondrial biogenesis and how dysfunction leads to disease.
Materials and Methods

Protein expression and purification.

Recombinant Erv1, ALR, and Erv2 were expressed and purified under native conditions as described previously\textsuperscript{11}. QSOX was cloned into BL21 Gold E. coli as previously described\textsuperscript{19}. Enzymatic activity was confirmed by H\textsubscript{2}O\textsubscript{2} production measured by Amplex Red (Sigma) fluorescence assay.

High-throughput screen for Erv1 modulators.

Freshly purified recombinant Erv1 (in buffer Screening Buffer, 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH7.4, 100 mM NaCl, 1 mM EDTA) at a concentration of 10 µM, which was expressed and purified as described previously\textsuperscript{29}. A Titertek multidrop (Thermofisher) was used to dispense 25 µl Erv1 or 25 µl of catalytically inactive enzyme Erv1C133S into clear bottom 384-well plate (GreinerBio). A Biomek FX (Beckman Coulter) was used to pin transfer 0.5 µl compound from 1mM stock (from the diverse smart 40,000 compound LS library) or DMSO (1% vehicle) to respective wells. Approximate screening concentration was 12.5 µM. After completed compound transfer, all plates were incubated at 25°C in a humidified incubator for 1 hr. A Titertek multidrop was used to dispense 50 µl of Amplex Red-HRP (Sigma) mix into all wells of the 384-well plate. The final concentration of Amplex Red and HRP were 46 µM and 0.092 U/ml, respectively. The Amplex Red-HRP solution was shielded from light during the entire experiment. The plates were incubated for an additional 10 minutes, and then 15 µl of the substrate DTT (20 µM) was added to initiate the reduction of O\textsubscript{2} to H\textsubscript{2}O\textsubscript{2} by Erv1. The plates were incubated for 12 minutes to achieve a maximal signal-to-noise ratio in the kinetic linear range\textsuperscript{30}. Plates were then read at an endpoint using an excitation wavelength of 545 nM and an emission wavelength of 590 nM. All operations were
performed by an automated plate scheduler to ensure consistency across the screening run. Assay quality and reproducibility of each plate was monitored using the statistical parameter, $Z'$. All plates had a $Z'$ value greater than 0.5. Compounds that inhibited activity by 50% were marked as potential hits. Hits were then rescreened against Erv1 in the presence of $\text{H}_2\text{O}_2$ to ensure compounds did not inhibit HRP. Commercially available compounds were purchased for further analysis.

**In vitro activity assays for Erv1, Erv2, ALR, and QSOX**

For IC$_{50}$ assays, 25 µl of freshly purified 3 µM (final) of Erv1 (previously described) was incubated in 1% DMSO or varying concentrations of small molecule in screening buffer for 1 hr prior to addition of 25 µl Amplex Red-HRP (Sigma) followed by 10 min incubation at 25°C. The reaction was initiated by the addition of 18.33 µM (final) DTT and incubated at for 12 minutes and plates were then read at an endpoint using an excitation wavelength of 545 nM and an emission wavelength of 590 nM. Other recombinant enzymes of the sulfhydryl oxidase family, ALR, Erv2, and QSOX were treated with 50 µM MB-7 and fluorescence was measured as above at a 12 minute endpoint.

**Oxygen consumption and membrane potential measurements.**

Mitochondria were purified from yeast cells grown on YPEG as described previously$^{20}$. Oxygen consumption measurements with isolated mitochondria were performed using an oxygen electrode (Hansatec) as described previously$^{11}$. Membrane potential measurements of purified mitochondria were performed with fluorescent 3,3'-dipropylthiadicarbocyanine iodide dye DISC3(5). 1% DMSO, carbonyl cyanide $m$-chlorophenyl hydrazone (CCCP), MB-7, or MB-8 was added to mitochondria in import buffer (0.6 M sorbitol, 2 mM KH$_2$PO$_4$, 60 mM KCl, 50 mM HEPES-KOH, 5 mM MgCl$_2$, 2.5 mM EDTA, 5 mM L-methionine, pH 7.1) for 10 min.
Subsequently 0.2 μM DISC3(5) in import buffer was added, incubated for 5 min, and fluorescence was measured at excitation and emission length of 620 nm and 670 nm, respectively.

**Mitochondria integrity assays.**

25 μg of isolated yeast mitochondria were incubated in import buffer and varying concentrations of small molecules at 25°C for 30 min. Mitochondria were then pelleted by centrifugation at 8,000 x g for 10 min at 4°C. The supernatant containing released proteins was TCA precipitated on ice for 30 min. Precipitated proteins were recovered by centrifugation at 15,000 x g for 15 min at 4°C. The mitochondrial pellet and the precipitated supernatant were resuspended in SDS sample buffer and analyzed on SDS-PAGE. Resulting gel were stained with Coomassie blue for 30 min or transferred to nitrocellulose membrane and blotted against key mitochondrial proteins.

**Purification of mitochondria**

Mitochondria were purified from yeast cells grown in YPEG as described previously.^32^ Yeast cultures were kept at 25°C with vigorous shaking during growth. Mitochondria concentration was measured by BCA assay and stored in 25 mg/ml at -80°C.

**Import of radiolabeled proteins into yeast mitochondria.**

Prior to import into purified mitochondria,^35^S-methionine and cysteine labeled proteins were generated with TNT Quick Coupled Transcription/Translation kits (Promega) and plasmids carrying the genes of interest. Transcription of genes was driven by either a T7 or SP6 promoter. Import reaction were conducted as previously described.^11,20^ After frozen mitochondria aliquots were thawed and added to the import buffer at a final concentration of 100 μg/ml, 1% DMSO or small molecule was added as indicated. A final concentration of 1 % DMSO was used in all experiments. Following incubation at 25°C for 15 min, import reactions were initiated by the
addition of 5-10 µl of translation mix. Aliquots were removed at intervals during the reaction
time course and import was terminated with addition either of cold buffer or 25 µg/ml trypsin, or
the combination. If trypsin was added to digest non-imported precursor protein, soybean trypsin
inhibitor was subsequently added in excess after 15 min incubation on ice. After a final recovery
of by centrifugation (8,000 x g, 6 min), mitochondria were disrupted in Laemmli sample buffer.
Imports of membrane proteins included a carbonate extraction step to remove proteins that had
not inserted into the membrane\textsuperscript{33}. Samples from import reaction time points were resolved by
SDS-PAGE and visualized by autoradiography. For non-reducing experiments, reactions were
stopped in the presence of 20 mM iodoacetamide and disrupted in Laemmli sample buffer
lacking β-mercaptoethanol.

**Pulldown of radiolabeled imported precursor**

Radiolabeled precursors were imported as described above. After 8 min incubation, reactions
were stopped by diluting with cold buffer, 0.6 M sorbitol, 20 mM K\textsuperscript+HEPES, pH 7.4, 40 mM
iodoacetamide (IAA). Mitochondria were pelleted by centrifugation (8,000 x g, 6 min) and
pellets were washed in 20 mM IAA and recollected. Mitochondria were disrupted with 8 M urea
buffer, pH 8.0 for 30 min. Samples were then centrifuged at 14,000 RPM for 15 min at room
temp. The supernatant was incubated with Ni\textsuperscript{2+}-NTA affinity resin at room temp while rotating
for 2 hours (5% was kept for the load lane). Samples were washed with 8M urea, pH 6.3 twice
for 10 minutes and aspirated with a Hamilton syringe. The bound protein was then eluted for 10
min in Urea buffer, pH 4.5. Laemmli sample buffer (without β-me) was added and heated at
60°C for 10 min. Samples from were resolved by SDS-PAGE and visualized by autoradiography
or transferred to nitrocellulose for Western analysis.
Gel extraction and Mass Spec analysis

Protein isolates eluted from Ni$^{2+}$-NTA affinity resin were separated by non-reducing SDS-PAGE. Gels were stained with Sypro Ruby (ThermoFisher) and illuminating bands were excised. A random gel slice containing no bands and a sample without a gel slice were included for controls. Samples were washed several times with 100mM NH$_4$HCO$_3$ and 100mM NH$_4$HCO$_3$:CH$_3$CN. Samples were reduced with 10mM DTT and blocked with 20 mM iodoacetamide. Proteins were digested with 300 ng modified sequencing grade trypsin (Thermo-Pierce) overnight at 37°C. Proteins were extracted with 50% CH$_3$CN/5% Formic acid. Samples were analyzed on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer and data was analyzed by Proteome Discover 1.4 configured with Mascot that searches the SwissProt Saccharomyces cerevisiae database.

Yeast toxicity studies

GA74ΔPDR5ΔSNQ2 yeast cultures were diluted to 0.002 OD$_{600}$ in 24 well plates in YPEG with 1% DMSO or varying concentrations of MB-7 or MB-8. OD$_{600}$ was measured at 72 hours.

HeLa Cell toxicity assay

Measurements for cell viability were made with a MTT based toxicology assay kit (Sigma) as described previously$^{20}$. Briefly, HeLa cells were grown in 24-well tissue culture dishes to 80% confluency. Cells were then treated with DMSO or MB-7 or MB-8 in complete medium for 24 h and reacted with MTT solution supplement for additional 4 hr as described in manufacturer’s protocols. Percentage viability of each cell sample was calculated as: (Butterfield and Shahabuddin, 1982) X 100.
Mitochondrial Network Microscopy

HeLa cells were grown to 40% confluency in the presence of DMSO, CCCP, or varying concentrations of MB-7 or MB-8. Mitochondria were labeled with Mitotracker red CMXRos (Invitrogen), fixed with 3.7% formaldehyde, and visualized on a Leica TCS SP8 confocal microscope and processed with the Leica AF software package.

Zebrafish analysis

Zebrafish displaying fluorescent hearts were derived from transgenic TL fish expressing a fusion of the CoxIV targeting sequence with DsRed regulated by a cmlc2 (cardiac myocyte light chain-2) promoter (Shu et al., 2007). Zebrafish used for o-dianisidine staining of red blood cells in DMSO and MB-7 or MB-8 treated fish were albino lines generated from crosses of TL and Tü fish lines were maintained in a 14-hr light/10-hr dark cycle and mated for one hour to obtain synchronized embryonic development. Embryos were grown for 3 hpf in E3 buffer (5 mM sodium chloride, 0.17 mM potassium chloride, 0.33 mM calcium chloride, 0.33 mM magnesium sulfate) and then incubated with E3 buffer supplemented with 1% DMSO or MB-7 or MB-8 for 3 days at 28.5 °C. Following treatment, embryos were imaged using a Leica MZ16F fluorescent stereoscope (TexasRed filter set) at 5X magnification. Alternatively, 3-day embryos were stained with o-dianisidine [40% (v/v) ethanol, 0.01 M sodium acetate, 0.65% hydrogen peroxide, 0.6 mg/ml o-dianisidine] and incubated for 15 min in complete darkness. Embryos were then washed with E3 buffer to remove residual stain and stereoscopically imaged under white light using a Leica S8AP0 at 1.575X magnification. Images were resized to 300dpi without resampling using Adobe Photoshop software.
NMR spectra shifts of ALR

NMR studies were performed by the Banci Lab. Briefly, up to 2 mM short form ALR (amino acid 81-205, PDB 3O55) and up to 4 mM compound or DMSO. Spectra were monitored and chemical shifts were recorded.
Figures

Figure 2.1 – Mitoblock-7 structure, half maximal inhibitory concentration, and effect on Erv1/ALR homologs. (A) The structure of MB-7. (B) IC50 analysis of MB-7 in the in vitro assay. 10 µM Erv1 was incubated with varying concentrations of MB-7 as described for the chemical screen. (C) 10 µM Erv1, ALR, Erv2, or QSOX was incubated with 50 µM MB-7 or DMSO and fluorescence was measured as described in the chemical screen.
Figure 2.2 – MB-7 is not deleterious to general mitochondrial function. (A) Purified mitochondria were incubated with MB-7 and the supernatant (S) and pellet (P) fractions were assessed for the presence of mitochondrial proteins by Coomassie stain or (B) western blot. (C) Using a Clarke-type oxygen electrode, oxygen consumed by mitochondria was measured after adding the electron donor NADH and DMSO or (D) MB-7. The chemical uncoupler CCCP was added as an internal control. (E) A fluorescent dye, Disc3(5), that is inserted into lipid bilayers and quenched in the presence of a membrane potential was added to DMSO, CCCP, or MB-7 treated mitochondria.
Figure 2.3 – MitoBloCK-7 inhibits the import of MIA substrates. (A-F) Radiolabeled precursors of the MIA pathway were imported into WT mitochondria in the presence of DMSO or MB-7. Non-imported precursor was removed by protease treatment. A 10% standard (Std) from the translation was included. (G) Mitochondria were treated with varying concentrations of MB-7 and MIA substrate Cmc1 or Tim23 substrate (H) Su9-DHFR was imported as described above. (I) MIA substrate Cmc1 was imported (as described above) into WT or Erv1 overexpressed mitochondria treated with varying concentrations of MB-7.
Figure 2.4 – Structure activity relationship of MB-7 analogs. (A) Structures of MB-7 analogs. (B) Import radiolabeled Cmc1 into DMSO, MB-7, or analog treated mitochondria as previously described.
Figure 2.5 – Erv1 inhibitor Mb-8 is not deleterious to general mitochondrial function

The structure of MB-8. (B) Using a Clarke-type oxygen electrode, oxygen consumed by mitochondria was measured after adding the electron donor NADH followed by MB-8. The chemical uncoupler was added as an internal control. (C) A fluorescent dye, Disc3(5), that is inserted into lipid bilayers and quenched in the presence of a membrane potential was added to DMSO, CCCP, or MB-7 treated mitochondria. (D) Purified mitochondria were incubated with MB-7 and the supernatant (S) and pellet (P) fractions were assessed for the presence of mitochondrial proteins by Coomassie stain.
Figure 2.6 – MitoblocK-8 inhibits the import of MIA substrates. (A-G) Radiolabeled precursors of the MIA pathway were imported into WT mitochondria in the presence of DMSO or MB-7. Non-imported precursor was removed by protease treatment. A 10% standard (Std) from the translation was included. (G) Mitochondria were treated with varying concentrations of MB-7 and MIA substrate Cmc1 was imported as described above.
Figure 2.7 – MB-7 inhibits import of Cmc1 and Cmc1 C64A. Import of radiolabeled Cmc1, Cmc1 C42A, Cmc1 C64A are imported into WT mitochondria treated with DMSO, MB-7, or MB8 as described previously.
Figure 2.8 – Formation of Mia40-Cmc1 import intermediate is partially impaired by MB-7 and MB-8. (A) Radiolabeled Cmc1 or cysteine mutants were imported into WT mitochondria and separated by non-reducing gel electrophoresis. (B-E) Import of radiolabeled Cmc1, Cmc1 C42A, Cmc1 C64A are imported into WT mitochondria treated with DMSO, MB-7, or MB8 and separated by non-reducing gel electrophoresis.
Figure 2.9 – MB-7 attenuates the formation of MIA-substrate interaction. Radiolabeled precursors Cmc1 (A), Tim10 (B), Erv1 (C), and Mia40 (D) were incubated with DMSO or MB-7 treated Wt or 10xHis tagged Mia40 mitochondria. Mitochondria were lysed with 8M urea and isolated with Ni\(^2+\)-NTA affinity resin. Bound complexes were eluted at pH 4.5 and separated by non-reducing gel electrophoresis. A 10% total (Load) was also included.
Figure 2.10 – A high molecular complex that includes Cmc1 and Erv1 requires the cysteine 42 of Cmc1 for formation. Radiolabeled WT, C42A, or C64A Cmc1 was imported into WT, 10xHis-tagged Mia40, or 10xHis-tagged Erv1 mitochondria. Mitochondria were lysed with 8M urea and isolated with Ni\textsuperscript{2+}-NTA affinity resin. Bound complexes were eluted at pH 4.5 and separated by non-reducing gel electrophoresis. A 10\% total (Load) was also included.
Figure 2.11 – A high molecular complex that includes Cmc1 and Erv1 is inhibited by MB-6 and MB-8. Radiolabeled Cmc1 was imported into WT, 10xHis-tagged Mia40 (M), or 10xHis-tagged Erv1(E) mitochondria that were treated with MB-6 (A) or MB-8(B). Mitochondria were lysed with 8M urea and isolated with Ni²⁺-NTA affinity resin. Bound complexes were eluted at pH 4.5 and separated by non-reducing gel electrophoresis. A 10% total (Load) was also included.
Figure 2.12 – Gel extractions submitted for mass spec analysis – Purified Wt or 10xHis-tagged Erv1 mitochondria were either incubated with recombinantly purified his tagged Cmc1 or mock control. Protein complexes were isolated by Ni\(^{2+}\)-NTA affinity resin in the presence of 8M urea. Isolates were separated by non-reducing gel electrophoresis and gel slices were separated, subjected to in gel digestion, followed by mass spec analysis. The above gel is one of 3 replicated and the numbers (1-25) indicate the area of the gel isolated.
**Supplemental**

Figure S2.1- MitoBloCK-7 is not toxic to yeast or HeLa cells. (A) Drug pump knockout yeast, ΔPdr5 and ΔSnq2, were grown in YPEG and varying concentrations of MB-7 for 72 hours. (B) HeLa cells that were grown in DMEM and varying concentrations of MB-7 for 24 hours were assessed for survival using a MTT colorimetric assay.
Figure S2.2 MitoBloCK-8 is not toxic to yeast but is toxic to HeLa cells. (A) Drug pump knockout yeast, ΔPdr5 and ΔSnq2, were grown in YPEG and varying concentrations of MB-8 for 72 hours. (B) HeLa cells that were grown in DMEM and varying concentrations of MB-8 for 24 hours were assessed for survival using a MTT colorimetric assay.
Figure S2.3 – MB-7 treated zebrafish have defects in somatic and cardiac developments.

Zebrafish were treated with varying concentrations of MB-7 starting at 4 hours post fertilization and visualized by microscopy at 72 hours post fertilization. Corresponding zebrafish hearts (right) containing cardiac mitochondria DsRed were visualized by fluorescent microscopy.
Figure S2.4 – MB-8 treated fish have defects in somatic and cardiac development. Zebrafish were treated with varying concentrations of MB-8 starting at 4 hours post fertilization and visualized by microscopy at 72 hours post fertilization. Corresponding zebrafish hearts (right) containing cardiac mitochondria DsRed were visualized by fluorescent microscopy.
Figure S2.5 – Structure modification of ALR by small molecule modulators. Recombinantly purified ALR treated with small molecules or DMSO was analyzed by NMR and $\Delta_HN$ peaks were observed. A space filled diagram (A) of ALR shows the residues affected in red. An example of shifted peaks (B) shows the shifts of Glu68 and Leu26. Bar graphs (C) treated samples reveal the residues shift patterns for each MB compared to the DMSO control.
Figure S2.6 – Steady state levels of mitochondrial proteins in WT or 10xHis tagged Mia40 mitochondria. Mitochondrial lysates were separated by SDS-PAGE and probed for mitochondrial protein concentration by western analysis.
<table>
<thead>
<tr>
<th>Band 1</th>
<th>Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q07653</td>
<td>Score 1302.75 Coverage 53.15% #proteins 1 #uniquepeptide 44 #peptides 44 PSM 58 AA 1046 MW (kDa) 113.5 pl 6.38</td>
</tr>
<tr>
<td>P18239</td>
<td>ADP-ATP carrier protein 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=PE19 PE=1 SV=2 - [ADT2_YEAST]</td>
</tr>
<tr>
<td></td>
<td>Score 48.61 Coverage 5.66% #proteins 1 #uniquepeptide 2 #peptides 2 PSM 318 AA 34.4 MW (kDa) 9.79</td>
</tr>
<tr>
<td>Band 2</td>
<td>Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
</tr>
<tr>
<td>Q07653</td>
<td>Score 351.21 Coverage 9.08% #proteins 1 #uniquepeptide 9 #peptides 9 PSM 10 AA 1046 MW (kDa) 113.5 pl 6.38</td>
</tr>
<tr>
<td>Band 3</td>
<td>Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
</tr>
<tr>
<td>Q07653</td>
<td>Score 92.03 Coverage 3.73% #proteins 1 #uniquepeptide 4 #peptides 4 PSM 10 AA 1046 MW (kDa) 113.5 pl 6.38</td>
</tr>
<tr>
<td>Band 4</td>
<td>Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ERV1 PE=1 SV=2 - [ERV1_YEAST]</td>
</tr>
<tr>
<td>P27882</td>
<td>Score 76.25 Coverage 11.11% #proteins 1 #uniquepeptide 2 #peptides 2 PSM 189 AA 21.6 MW (kDa) 8.21</td>
</tr>
<tr>
<td>P32191</td>
<td>Glycerol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GT2 PE=1 SV=2 - [GPDM_YEAST]</td>
</tr>
<tr>
<td>Q07653</td>
<td>Score 29.15 Coverage 2.62% #proteins 1 #uniquepeptide 2 #peptides 2 PSM 649 AA 72.3 MW (kDa) 7.9</td>
</tr>
<tr>
<td>Band 5</td>
<td>Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
</tr>
<tr>
<td>P36046</td>
<td>Score 26.73 Coverage 2.10% #proteins 1 #uniquepeptide 2 #peptides 2 PSM 104 AA 1046 MW (kDa) 113.5 pl 6.38</td>
</tr>
<tr>
<td>Band 6</td>
<td>Mitochondrial intermembrane space import and assembly protein 40 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
</tr>
<tr>
<td>P27882</td>
<td>Score 95.33 Coverage 10.42% #proteins 1 #uniquepeptide 4 #peptides 4 PSM 403 AA 44.5 MW (kDa) 4.56</td>
</tr>
<tr>
<td>P27882</td>
<td>Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ERV1 PE=1 SV=2 - [ERV1_YEAST]</td>
</tr>
<tr>
<td>Q07653</td>
<td>Score 79.54 Coverage 11.11% #proteins 1 #uniquepeptide 2 #peptides 2 PSM 189 AA 21.6 MW (kDa) 8.21</td>
</tr>
<tr>
<td>P32191</td>
<td>Glycerol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GT2 PE=1 SV=2 - [GPDM_YEAST]</td>
</tr>
<tr>
<td>P32191</td>
<td>Score 53.06 Coverage 2.68% #proteins 1 #uniquepeptide 2 #peptides 2 PSM 104 AA 113.5 MW (kDa) 6.38</td>
</tr>
<tr>
<td>P32563</td>
<td>V-type proton ATPase subunit a, vacuolar isomor OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=VPH1 PE=1 SV=3 - [VPH1_YEAST]</td>
</tr>
<tr>
<td></td>
<td>Score 51.67 Coverage 7.09% #proteins 1 #uniquepeptide 5 #peptides 5 PSM 649 AA 72.3 MW (kDa) 7.9</td>
</tr>
<tr>
<td>Band 7</td>
<td>Mitochondrial intermembrane space import and assembly protein 40 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
</tr>
<tr>
<td>P36046</td>
<td>Score 44.81 Coverage 2.86% #proteins 1 #uniquepeptide 3 #peptides 3 PSM 840 AA 95.5 MW (kDa) 5.48</td>
</tr>
<tr>
<td>P36046</td>
<td>Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ERV1 PE=1 SV=2 - [ERV1_YEAST]</td>
</tr>
<tr>
<td>P36046</td>
<td>Score 358.34 Coverage 20.10% #proteins 1 #uniquepeptide 10 #peptides 10 PSM 403 AA 44.5 MW (kDa) 4.56</td>
</tr>
<tr>
<td>P36046</td>
<td>Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ERV1 PE=1 SV=2 - [ERV1_YEAST]</td>
</tr>
<tr>
<td>P36046</td>
<td>Score 101.31 Coverage 19.05% #proteins 1 #uniquepeptide 5 #peptides 5 PSM 189 AA 21.6 MW (kDa) 8.21</td>
</tr>
<tr>
<td>P36046</td>
<td>Glycerol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GT2 PE=1 SV=2 - [GPDM_YEAST]</td>
</tr>
<tr>
<td>A62N18</td>
<td>Score 85.39 Coverage 4.01% #proteins 1 #uniquepeptide 3 #peptides 3 PSM 649 AA 72.3 MW (kDa) 7.9</td>
</tr>
<tr>
<td>A62N18</td>
<td>Mitochondrial escape protein 2 OS=Saccharomyces cerevisiae (strain YJM789) GN=YM2 PE=1 SV=3 - [YM2_YEAST]</td>
</tr>
<tr>
<td></td>
<td>Score 50.54 Coverage 2.59% #proteins 2 #uniquepeptide 2 #peptides 2 PSM 850 AA 96.6 MW (kDa) 8.81</td>
</tr>
<tr>
<td>Band 7</td>
<td>Mitochondrial intermembrane space import and assembly protein 40 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
</tr>
<tr>
<td>P36046</td>
<td>Score 225.79 Coverage 18.86% #proteins 1 #uniquepeptide 8 #peptides 8 PSM 403 AA 44.5 MW (kDa) 4.56</td>
</tr>
<tr>
<td>Protein</td>
<td>Score</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>P32191 Glycol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GUT2 PE=1 SV=2 - [GPDM_YEAST]</td>
<td>114.8</td>
</tr>
<tr>
<td>Q08179 Mitochondrial distribution and morphology protein 38 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MMD38 PE=1 SV=1 - [MMD38_YEAST]</td>
<td>52.82</td>
</tr>
<tr>
<td>Band 8</td>
<td></td>
</tr>
<tr>
<td>P36046 Mitochondrial intermembrane space import and assembly protein 40 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
<td>258.03</td>
</tr>
<tr>
<td>P32191 Glycol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GUT2 PE=1 SV=2 - [GPDM_YEAST]</td>
<td>124.53</td>
</tr>
<tr>
<td>P39925 Mitochondrial respiratory chain complexes assembly protein AFG3 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=AFG3 PE=1 SV=1 - [AFG3_YEAST]</td>
<td>55.87</td>
</tr>
<tr>
<td>P39987 Heat shock protein SSCP3, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ECM10 PE=1 SV=1 - [HSP70_YEAST]</td>
<td>30.5</td>
</tr>
<tr>
<td>Band 9 Compare to Band 1</td>
<td></td>
</tr>
<tr>
<td>Q07653 Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
<td>430.78</td>
</tr>
<tr>
<td>Band 10 Compare to 2</td>
<td></td>
</tr>
<tr>
<td>Q07653 Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
<td>358.34</td>
</tr>
<tr>
<td>Band 11 Compare to 3</td>
<td></td>
</tr>
<tr>
<td>Q07653 Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
<td>37.55</td>
</tr>
<tr>
<td>Band 12 Compare to 5</td>
<td></td>
</tr>
<tr>
<td>P32191 Glycol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GUT2 PE=1 SV=2 - [GPDM_YEAST]</td>
<td>71.67</td>
</tr>
<tr>
<td>P36046 Mitochondrial intermembrane space import and assembly protein 40 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
<td>43.96</td>
</tr>
<tr>
<td>P32563 V-type proton ATPase subunit a, vacuolar isoform OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=VPH1 PE=1 SV=3 - [VPH1_YEAST]</td>
<td>33.36</td>
</tr>
<tr>
<td>Band 13 Compare to 6</td>
<td></td>
</tr>
<tr>
<td>P36046 Mitochondrial intermembrane space import and assembly protein 40 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
<td>186.11</td>
</tr>
<tr>
<td>P32191 Glycol-3-phosphate dehydrogenase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GUT2 PE=1 SV=2 - [GPDM_YEAST]</td>
<td>81.21</td>
</tr>
<tr>
<td>P23782 Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ERV1 PE=1 SV=2 - [ERV1_YEAST]</td>
<td>78.17</td>
</tr>
<tr>
<td>A6Z2N8 Mitochondrial escape protein 2 OS=Saccharomyces cerevisiae (strain YJM789) GN=YM2 PE=3 SV=1 - [YM2_YEAST]</td>
<td>73.31</td>
</tr>
<tr>
<td>Band 14 Compare to 1</td>
<td></td>
</tr>
<tr>
<td>Q07653 Protein HBT1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=HBT1 PE=1 SV=1 - [HBT1_YEAST]</td>
<td>109.35</td>
</tr>
<tr>
<td>Protein</td>
<td>Score</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Q07580 External NADH-ubiquinone oxidoreductase 2, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--NDE2 PE=1 SV=1 - [NDH2_YEAST]</td>
<td>104.24</td>
</tr>
<tr>
<td>Q06493 LETM1 domain-containing protein YLH47, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--YLH47 PE=1 SV=1 - [YLH47_YEAST]</td>
<td>102.43</td>
</tr>
<tr>
<td>P07251 ATP synthase subunit alpha, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--ATP1 PE=1 SV=5 - [ATPA_YEAST]</td>
<td>82.71</td>
</tr>
<tr>
<td>Q12428 Probable 2-methylcitrate dehydratase OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--PDH1 PE=1 SV=1 - [PRPD_YEAST]</td>
<td>70.45</td>
</tr>
<tr>
<td>P46367 Potassium-activated aldehyde dehydrogenase, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--ALD4 PE=1 SV=2 - [ALDH4_YEAST]</td>
<td>60.47</td>
</tr>
<tr>
<td>P40215 External NADH-ubiquinone oxidoreductase 1, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--NDE1 PE=1 SV=1 - [NDH1_YEAST]</td>
<td>53.22</td>
</tr>
<tr>
<td>P32346 Rotenone-insensitive NADH-ubiquinone oxidoreductase, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--NDE1 PE=1 SV=1 - [NDH1_YEAST]</td>
<td>45.92</td>
</tr>
<tr>
<td>P54783 D-arabino-1,4-lactone oxidase OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--ALO1 PE=1 SV=1 - [ALO_YEAST]</td>
<td>24.98</td>
</tr>
</tbody>
</table>

| Band 15 | | | |
|---------| | | |
| P29704  Squalene synthase OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--ERG9 PE=1 SV=2 - [FDFT_YEAST] | 100.92 | 6.08%    | 1         | 3              | 3         | 3   | 444 | 51.7     | 5.9  |
| Q08970  Mitochondrial metal transporter 2 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--MMT2 PE=3 SV=2 - [MMT2_YEAST] | 97.34  | 11.36%   | 1         | 5              | 5         | 5   | 484 | 52.4     | 7.64 |
| P00989  Citrate synthase, mitochondrial OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--CIT1 PE=1 SV=2 - [CISV1_YEAST] | 79.48  | 5.01%    | 1         | 3              | 3         | 3   | 479 | 53.3     | 8.29 |
| P27882  Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--ERV1 PE=1 SV=2 - [ERV1_YEAST] | 65.27  | 10.58%   | 1         | 2              | 2         | 2   | 189 | 21.6     | 8.21 |

| Band 16 | | | |
|---------| | | |
| P27382  Mitochondrial FAD-linked sulfhydryl oxidase ERV1 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--ERV1 PE=1 SV=2 - [ERV1_YEAST] | 90.82  | 12.17%   | 1         | 3              | 3         | 3   | 189 | 21.6     | 8.21 |
| A6Z35   COX assembly mitochondrial protein OS--Saccharomyces cerevisiae (strain YJM789) GN--CMC1 PE=3 SV=2 - [COXM1_YEAST] | 50.56  | 22.52%   | 2         | 3              | 3         | 3   | 111 | 13       | 8.91 |
| P18239  ADP,ATP carrier protein 2 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--PET9 PE=1 SV=2 - [ADT2_YEAST] | 49.35  | 5.35%    | 1         | 2              | 2         | 2   | 318 | 34.4     | 9.79 |

| Band 17 | | | |
|---------| | | |
| Q07653  Protein HBT1 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--HBT1 PE=1 SV=1 - [HBT1_YEAST] | 341.59 | 18.36%   | 1         | 16             | 16        | 17  | 1046 | 113.5    | 6.38 |

| Band 18 | | | |
|---------| | | |
| Q07653  Protein HBT1 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--HBT1 PE=1 SV=1 - [HBT1_YEAST] | 54.96  | 4.68%    | 1         | 4              | 4         | 4   | 1046 | 113.5    | 6.38 |

| Band 19 | | | |
|---------| | | |
| Q08372  Serine/threonine-protein kinase HRK1 OS--Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN--HRK1 PE=1 SV=1 - [HRK1_YEAST] | 58.87  | 2.11%    | 1         | 2              | 2         | 2   | 759  | 85.6     | 7.31 |

<p>| Band 20 | | | |
|---------| | | |
| Too faint | | | |</p>
<table>
<thead>
<tr>
<th>Band 21</th>
<th>Protein</th>
<th>Score</th>
<th>Coverage</th>
<th>#proteins</th>
<th>#uniquepeptide</th>
<th>#peptides</th>
<th>#PSM</th>
<th>#AA</th>
<th>MW (kDa)</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>P36046</td>
<td>Mitochondrial intermembrane space import and assembly protein 40 OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
<td>387.76</td>
<td>22.83%</td>
<td>1</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>403</td>
<td>44.5</td>
<td>4.56</td>
</tr>
<tr>
<td>P32191</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GUT2 PE=1 SV=2 - [GPD_M_YEAST]</td>
<td>115.52</td>
<td>6.47%</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>649</td>
<td>72.3</td>
<td>7.9</td>
</tr>
<tr>
<td>A62Z15</td>
<td>COX assembly mitochondrial protein OS-Saccharomyces cerevisiae (strain YJM789) GN=CMC1 PE=3 SV=2 - [COX1_M_YEAST]</td>
<td>59.34</td>
<td>34.23%</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>111</td>
<td>13</td>
<td>8.91</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Band 22</th>
<th>Protein</th>
<th>Score</th>
<th>Coverage</th>
<th>#proteins</th>
<th>#uniquepeptide</th>
<th>#peptides</th>
<th>#PSM</th>
<th>#AA</th>
<th>MW (kDa)</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>P36046</td>
<td>Mitochondrial intermembrane space import and assembly protein 40 OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MIA40 PE=1 SV=2 - [MIA40_YEAST]</td>
<td>411.27</td>
<td>29.03%</td>
<td>1</td>
<td>11</td>
<td>11</td>
<td>13</td>
<td>403</td>
<td>44.5</td>
<td>4.56</td>
</tr>
<tr>
<td>P32191</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GUT2 PE=1 SV=2 - [GPD_M_YEAST]</td>
<td>105.75</td>
<td>7.09%</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>649</td>
<td>72.3</td>
<td>7.9</td>
</tr>
<tr>
<td>P06782</td>
<td>Carbon catabolite-activator protein kinase OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SNF1 PE=1 SV=1 - [SNF1_YEAST]</td>
<td>77.14</td>
<td>5.37%</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>633</td>
<td>72</td>
<td>6.65</td>
</tr>
<tr>
<td>P39987</td>
<td>Heat shock protein SSC3, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ECM10 PE=1 SV=1 - [HSP70_YEAST]</td>
<td>60.5</td>
<td>2.95%</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>644</td>
<td>70</td>
<td>6.27</td>
</tr>
<tr>
<td>Q08179</td>
<td>Mitochondrial distribution and morphology protein 38 OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MMD38 PE=1 SV=1 - [MMD38_YEAST]</td>
<td>49.03</td>
<td>5.24%</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>573</td>
<td>65</td>
<td>7.77</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Band 23</th>
<th>Protein</th>
<th>Score</th>
<th>Coverage</th>
<th>#proteins</th>
<th>#uniquepeptide</th>
<th>#peptides</th>
<th>#PSM</th>
<th>#AA</th>
<th>MW (kDa)</th>
<th>pl</th>
</tr>
</thead>
<tbody>
<tr>
<td>P07251</td>
<td>ATP synthase subunit alpha, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=ATPA PE=1 SV=5 - [ATPA_YEAST]</td>
<td>122.07</td>
<td>7.71%</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>545</td>
<td>58.6</td>
<td>9.04</td>
</tr>
<tr>
<td>P40215</td>
<td>External NADH-ubiquinone oxidoreductase 1, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=NDE1 PE=1 SV=1 - [NDH1_YEAST]</td>
<td>102.69</td>
<td>5.36%</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>560</td>
<td>62.7</td>
<td>9.28</td>
</tr>
<tr>
<td>P27929</td>
<td>ATP synthase subunit beta, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=NAM9 PE=1 SV=2 - [NAM9_YEAST]</td>
<td>101.57</td>
<td>5.36%</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>486</td>
<td>56.3</td>
<td>9.64</td>
</tr>
<tr>
<td>P00830</td>
<td>ATP synthase subunit beta, mitochondrial OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=NAM9 PE=1 SV=2 - [NAM9_YEAST]</td>
<td>101.27</td>
<td>6.40%</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>511</td>
<td>54.8</td>
<td>5.71</td>
</tr>
<tr>
<td>Q12428</td>
<td>Probable 2-methylcitrate dehydratase OS-Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=PDP1 PE=1 SV=1 - [PDP1_YEAST]</td>
<td>75.83</td>
<td>5.04%</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>516</td>
<td>57.6</td>
<td>9.07</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Band 24</th>
<th>No hits (gel control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 25</td>
<td>No hits (buffer control)</td>
</tr>
</tbody>
</table>
Table 2.1 Erv1 and Cmc1 pull-down hits identified by mass spectrometry

Purified Wt or 10xHis-tagged Erv1 mitochondria were either incubated with purified 10xHis-tagged recombinant Cmc1 or mock control. Protein complexes were isolated by Ni\textsuperscript{2+}-NTA affinity resin in the presence of 8M urea. Isolates were separated by non-reducing gel electrophoresis and gel slices were separated, subjected to in gel digestion, followed by mass spec analysis. Proteins were extracted with 50% CH\textsubscript{3}CN/5% Formic acid. Samples were analyzed on a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer and data was analyzed by Proteome Discover 1.4 configured with Mascot that searches the SwissProt Saccharomyces cerevisiae database
References


Chapter 3. Studying the import of the ALS protein CHCHD10 by small molecule modulators

Abstract

Mitochondrial dysfunction leads to a host of devastating diseases. ALS is a disease of motor neurons in which mitochondria have been strongly associated. It has been recently reported that a mutation in the intermembrane space protein CHCHD10 results in familial ALS. Studies revealed ALS mitochondria have a sundry of defects, notably a marked redox abnormality, protein import dysfunction, and have dysregulation in fission fusion dynamics resulting in a fragmented network. However, results are becoming more challenging to interpret because of the pleiotropic nature of disease. Thus, new strategies are necessary to elucidate how dysfunction of CHCHD10 leads to ALS. Although the function of CHCHD10 is unknown, it has been suggested that the Mia40/Erv1 import pathway is required for translocation. Our lab has recently identified and characterized small molecule tools, MB-6, MB-7, and MB-8 that modulate the enzymatic activity of the MIA import protein Erv1/ALR. We have employed these small molecules to dissect CHCHD10 import. The success of these studies suggests that small molecules can be powerful tools in studying mitochondrial protein biogenesis, determining how dysfunction leads to disease, and ultimately facilitating in the development of superior therapeutics.
Introduction

Defects in mitochondrial protein import have been linked to neurodegeneration, cardiac ischemia, and other brutal disorders.\textsuperscript{1-3} Mutations of the intermembrane space import component DDP1/Tim8 lead to deafness-dystonia syndrome, a disease marked by hearing loss, vision and movement impairment, and behavioral problems.\textsuperscript{4} Dysfunctions in the import component Erv1/ALR result in autosomal recessive cardiomyopathy and neuropathy.\textsuperscript{5, 6} These diseases, albeit sometimes rare, do not have readily available therapies partly due to the slow progression of research discovery. New approaches are crucial to not only understand disease mechanisms, but also to reveal optimal therapeutic targets. Moreover, understanding rare syndromes will also shed mechanistic insights into the more common disorders.

For that purpose, small molecule directed research is gaining momentum in a wide variety of fields.\textsuperscript{7-10} Small organic compounds are being used to modulate pathways that were otherwise too challenging to accomplish. Although they are making advancements in many areas of research, small molecule directed research in mitochondria is sparse. To this end, we have recently identified small molecule tools from high throughput chemical screening campaigns to use to dissect mitochondrial biogenesis. The collection of tools, or MitoBloCKs (MB), has been used to dissect a number of import mechanisms that would be otherwise insurmountable using traditional approaches. Of particular interest are MB-6, recently published, in addition to MB-7 and MB-8, described in chapter 2.\textsuperscript{9} These small molecules, identified from a chemical screen against Erv1, have helped elucidate a variety of functions and mechanisms of redox regulated import as well as additional roles of both yeast Erv1 and the mammalian homolog ALR. Interestingly, MB-6 was used to elucidate a pro survival role for ALR in stem cells as well as have key roles in neuronal development. MB-7 and MB-8 were used to dissect high molecular
weight import complexes and identify potential previously unknown electron acceptors. These tools have proven to be very useful in dissecting basic mitochondrial function and have the potential to help identify how mitochondrial dysfunctions lead to disease.

Recently, Bannwarth et al. reported that a mutation in the mitochondrial protein CHCHD10 results in Amyotrophic Lateral Sclerosis (ALS)\textsuperscript{11}. ALS, also known as Lou Gehrig’s disease, is a motor neuron disease that causes involuntary muscle movements resulting from the demise of neurons, eventually becoming so bad that patients have extreme difficulty swallowing and breathing\textsuperscript{12,13}. Mitochondria have been implicated in ALS for many years but it is still hotly debated whether mitochondria play a causative role. ALS is a multi-systemic disorder that presents primarily in the highly metabolic motor neurons and muscle tissue\textsuperscript{14}. Although it is not clear if mitochondria play a primary role in ALS pathology, patient mitochondria have a marked redox abnormality, protein import dysfunction, and have dysregulation in fission fusion dynamics resulting in a fragmented network\textsuperscript{11,15,16}. CHCHD10 has a twin cysteine motif that suggests it is imported into the intermembrane space by Mia40/Erv1 pathway. Although the function is unknown, CHCHD10 localizes to the mitochondrial intermembrane space, is enriched at the cristae junctions, and plays a role in the MICOS complex\textsuperscript{11,17}. Dissecting the biogenesis and function(s) of CHCHD10 will advance our understanding of ALS pathophysiology and facilitate in the development of superior therapeutics.

The mitochondrial network is governed by a tightly regulated series of proteins\textsuperscript{18}. The balance between these proteins determines the overall shape of the mitochondrial network and is important for all tissues. It is reported that the mitochondrial network is clearly fragmented when the mutant form of CHCHD10 is overexpressed\textsuperscript{11}. Genin et al. reported that CHCHD10 is in a complex with CHCHD3, CHCHD6, and Mic60 (mitofilin) to form key, and well conserved,
components of the MICOS complex\textsuperscript{19}. RNAi experiments that knockdown Mic60 or CHCHD3 both reportedly result in fragmented mitochondria\textsuperscript{20,21}. It is evident that the mitochondrial intermembrane space proteome consists of players that are critical for mitochondrial morphology but it remains unclear how these defects fit in ALS pathology and cause tissue specific cell death.

The redox status is a critical feature of mitochondria and is defective in ALS patient mitochondria\textsuperscript{22}. Thus, it is not surprising that many of the general mitochondrial functions are affected by the redox melee in the mitochondria of ALS patients, notably respiratory chain deficiency and mitochondrial protein translocation\textsuperscript{23,24}. The electron transport chain and the Mia40/Erv1 import pathway both rely heavily on redox reactions and thus a shift in redox balance will undoubtedly complicate general function. These attributes of ALS, among many others, make characterization of ALS pathology a challenging task. Thus, additional tools are necessary to aide in the dissection of the mechanisms of ALS.

Here we investigate the import of CHCHD10 into the mitochondrial intermembrane space and demonstrate that that our small molecules can be useful in the dissection of CHCHD10 and other disease-associated proteins of the mitochondrial intermembrane space.

**Results**

**MB-6 and MB-8 destabilize a high molecular weight Erv1-substrate complex**

From a similar small molecule screen with recombinant Erv1 and the non-physiologic substrate DTT that resulted in the identification of MB-6, we identified additional inhibitors MB-7 and MB8\textsuperscript{9}. These small molecules attenuated the import of IMS substrates and differentially regulated the Mia40-substrate interaction. To determine if mitochondrial precursors are indeed imported in a disulfide dependent manner that can be modulated by are different small
molecules, we incubated a radiolabeled substrate of Mia40, Cmc1, an incubated with purified yeast mitochondria. Non-imported precursor was removed by protease. Solubilized mitochondria were separated by non-reducing SDS-PAGE and subjected to autoradiography. The Mia40 substrate forms a disulfide interaction that is abolished in the presence of reductant (Figure 3.1). MB-8 causes the Mia40- Cmc1 interaction to be stabilized whereas MB-7 inhibits the interaction. This suggests that by modulating Erv1 with different small molecules, we are able to attenuate translocation at various import stages.

**ALS protein CHCHD10 uses the Mia40/Erv1 translocation pathway**

CHCHD10 is a member of the coiled helix coiled helix family with a classic cysteine motif suggesting it is imported via the Mia40 pathway. To test if CHCHD10 is indeed imported into the mitochondria, energized mitochondria were incubated with a TNT (Promega) generated radiolabeled CHCHD10 precursor. Non-imported precursor was removed by protease. Solubilized mitochondria were separated by reducing SDS-PAGE and subjected to autoradiography (data not shown). We were able to show that CHCHD10 is indeed imported into mitochondria. To test whether our small molecules affected import, we pre-incubated energized mitochondria with DMSO or varying concentrations of MBs and executed import analysis as described above (Figure 3.2). Interestingly, MB-5 and MB-8 seemed to slightly accumulate precursor in the mitochondria, but MB-6 treated mitochondria overwhelmingly accumulates imported CHCHD10. These results are surprising because we would expect import to be attenuated when we inhibit Erv1 enzymatic activity.

To investigate how the precursor is accumulating, energized mitochondria were incubated with a radiolabeled CHCHD10 precursor as before and separated by non-reducing SDS-PAGE and subjected to autoradiography (Figure 3.3). Because redox regulated import can
be increased in mitochondria in the absence of a membrane potential (ΔΨ), both untreated and
CCCP treated analysis were included. Interestingly, a high molecular weight complex
accumulates in MB-6 treated mitochondria. We speculate that this interaction is with Mia40 and,
as with the case of Cmc1, the interaction to be stabilized.

To confirm that this interaction is with Mia40, radiolabeled CHCHD10 was imported into
WT, 10xHis-tagged Mia40, or 10xHis-tagged Erv1 mitochondria in the presence of small
molecule or vehicle control (1% DMSO). The mitochondria were solubilized with 8M Urea, pH
8.0 and 20 mM IAA and pulldowns were performed with Ni$^{2+}$-NTA affinity resin. The columns
were washed with 8M Urea, pH 6.3, and bound Mia40 (M) was eluted at pH 4.5 and boiled with
SDS sample buffer (Eluate). 5% of the total lysate (Load) and the eluate were separated by non-
reducing SDS-PAGE. The imported substrate was detected with autoradiography. A high
molecular weight band appears much darker in the MB-6 and MB-8 compared to DMSO or MB-
7 treated lanes in the Mia40-His gel suggesting that MB-6 and MB-8 accumulates a stable form
of CHCHD10 and Mia40 (Figure 3.4). The high molecular weight precursor is not in the eluate
lanes of in the Erv1-His pull down gel confirming that the interaction is with Mia40. Taken
together, these results confirm that CHCHD10 is indeed imported into mitochondria via a
disulfide interaction with Mia40.

**ALR plays a role in cristae maintenance via the MICOS complex**

It is widely acknowledged that ALS patients have a defect in mitochondrial networks.
The MICOS constituents Mic60, CHCHD3, and CHCHD6 are all speculated precursors of the
Mia40/Erv1 pathway. By inhibiting the import of CHCHD10 with small molecules, we
hypothesized that the mitochondrial morphology would have a similar phenotype to disease
models. HeLa cells were grown to 40% confluency in the presence of DMSO, CCCP, or varying
concentrations of small molecules for 6 hours. Mitochondria were labeled with Mitotracker red CMXRos (Invitrogen), fixed with 3.7% formaldehyde, and visualized on a Leica TCS SP8 confocal microscope and processed with the Leica AF software package. DMSO treated cells had an elongated and interconnected mitochondrial network (Figure 3.5). CCCP treatment showed cells diffuse and light staining as expected because Mitotracker Red requires a membrane potential. Interestingly, all MB treated HeLa cells demonstrate a fragmented mitochondrial network. Although, we can speculate that this is due to a defect in MICOS stability, further testing is necessary to determine if this is the case.

**CHCHD2 accumulates in MB-8 treated mitochondria**

To determine if our collection of small molecules can be used against other disease associated proteins of the intermembrane space, we repeated our *in organello* import analyses with CHCHD2, a Parkinson’s disease associated protein. Briefly, energized mitochondria were incubated with a radiolabeled CHCHD2 precursor as before and separated by reducing SDS-PAGE and subjected to autoradiography (Figure 3.6). As expected, CHCHD2 accumulates in MB-8 treated mitochondria. It has also been shown that deleting a speculative N-terminal targeting sequence abolishes import. Indeed, this hydrophobic region is necessary for import and the precursor does not accumulate even in MB-8 treated mitochondria. Further testing is necessary to determine if CHCHD2 accumulation under MB-8 treatment is a result being trapped with Mia40, similar to CHCHD10.

**Discussion**

In this study, we first demonstrated that the collection of small molecules called MitoBloCKs modulate the import and import complexes of precursors destined for the mitochondrial intermembrane space. Interestingly, these small molecules seem to modulate Erv1
differently, exemplified by the stabilization of the Mia-40-substrate complex in MB-6 treated mitochondria and the destabilization under MB-7 treatment. Additional studies are currently underway to dissect the unknown import complexes that involve Erv1 and substrates and appear to lack Mia40.

As we dissect these mechanisms, we hypothesized that our small molecules can be used to facilitate in investigating the functions and pathways of disease associated proteins of the mitochondrial IMS that rely on Mia40/Erv1 import. Of particular interest, CHCHD10 is a recently identified protein that causes late-onset ALS. Although the function remains elusive, it is thought to enter the mitochondria through the Mia40/Erv1 import pathway. Using a radiolabeled import assay, we show that CHCHD10 is indeed imported into the mitochondria. We also demonstrate that, unlike other precursors, CHCHD10 accumulates in MB-6 and MB-8 treated mitochondria. Upon further investigation in non-reducing conditions, we show that CHCHD10 forms a complex with Mia40 which is stabilized in MB-6, and to a lesser degree MB-8, treated mitochondria. In order to show that the interaction is specific to Mia40, we imported radiolabeled CHCHD10 precursor into histidine tagged Mia-40 or his-tagged Erv1 mitochondria and purified the mitochondria lysates on Ni$^{2+}$-NTA affinity resin. To preserve disulfides, mitochondria were denatured in 8 M urea and separated by non-reducing SDS-PAGE. The Mia40-CHCHD10 interaction was evident in the vehicle control DMSO treated samples and enriched in the MB-6 and MB-8 treated lanes. This complex did not show up in Erv1 purified lanes. Thus, we conclude that CHCHD10 is indeed a substrate of the MIA pathway in mitochondria and that our small molecules modulate its translocation.

Interestingly, SOD1 mutant forms of ALS have defects in mitochondrial import. However, these studies are commonly done in models overexpressing mutant SOD1 that form...
toxic aggregates causing a plethora of other problems. Thus, results from these studies can be challenging to interpret. It is interesting to speculate that MB treatment can be used to study defects arising from import inhibition without additional consequences from SOD1 aggregates.

It has been well established that ALS mitochondria have similar morphological defects. Bannwarth et al. reported that CHCHD10 is involved in mitochondrial network maintenance by playing a role in the MICOS complex with Mic60, CHCHD3, and CHCHD6\textsuperscript{19}. Disruption of this complex disrupts cristae formation and results in fragmented mitochondria. Bannwarth et al. overexpressed mutant CHCHD10 and observed fragmented mitochondria\textsuperscript{11}. It is interesting to speculate that the defects in import reported in SOD1 mutants prevent MICOS complex components from importing and assembling resulting in a fragmented network. To this end, we hypothesized that Erv1/ALR may play a critical role in mitochondrial morphology. We observed MB or DMSO treated HeLa mitochondrial networks by Mitotracker Red staining and visualizing by confocal microscopy. DMSO treated HeLa cells exhibit elongated and interconnected mitochondrial network. In all MB treated HeLa cells, mitochondria were fragmented and terse. Although these results correspond with those reported by Bannwarth et al., making it tempting to conclude that the network disruption is a direct result of ALR inhibition, this disruption can ultimately be caused by a number of direct or indirect consequences.

Additional proteins of similar composition and characteristics have been associated with other common neuronal diseases. To test if other disease associated proteins are affected by our small molecules, we tested import of a Parkinson’s disease associated protein CHCHD2, a paralog of CHCHD10 with 60% homology\textsuperscript{25}. Interestingly, our small molecules again show the accumulation of precursor in MB-8 treated mitochondria. Moreover, it has been speculated that
CHCHD2 has a small N-terminal mitochondrial targeting sequence (MTS) and MB-8 augmented import is attenuated when deleted.

These results suggest that our collection of small molecules differentially modulate the import of mitochondrial proteins of the intermembrane space. Because the identification of these proteins is relatively new and not well understood, we present the case that these small molecules will be excellent tools to facilitate in the dissection of protein function and disease pathology. Moreover, these small molecules can be used in a variety of models and have already been extensively studied in yeast, multiple cell lines, and zebrafish.
Materials and Methods

Purification of mitochondria

Mitochondria were purified from yeast cells grown in YPEG as described previously. Yeast cultures were kept at 25°C with vigorous shaking during growth. Mitochondria concentration was measured by BCA assay and stored in 25 mg/ml at -80°C.

Import of radiolabeled proteins into yeast mitochondria.

Prior to import into purified mitochondria, 35S-methionine and cysteine labeled proteins were generated with TNT Quick Coupled Transcription/Translation kits (Promega) and plasmids carrying the genes of interest. Transcription of genes was driven by either a T7 or SP6 promoter. Import reaction were conducted as previously described. After frozen mitochondria aliquots were thawed and added to the import buffer at a final concentration of 100 µg/ml, 1% DMSO or small molecule was added as indicated. A final concentration of 1 % DMSO was used in all experiments. Following incubation at 25°C for 15 min, import reactions were initiated by the addition of 5-10 µl of translation mix. Aliquots were removed at intervals during the reaction time course and import was terminated with addition either of cold buffer or 25 µg/ml trypsin, or the combination. If trypsin was added to digest non-imported precursor protein, soybean trypsin inhibitor was subsequently added in excess after 15 min incubation on ice. After a final recovery of by centrifugation (8,000 x g, 6 min), mitochondria were disrupted in Laemmli sample buffer. Imports of membrane proteins included a carbonate extraction step to remove proteins that had not inserted into the membrane. Samples from import reaction time points were resolved by SDS-PAGE and visualized by autoradiography. For non-reducing experiments, reactions were stopped in the presence of 20 mM iodoacetamide and disrupted in Laemmli sample buffer lacking β-mercaptoethanol.
**Pulldown of radiolabeled imported precursor**

Radiolabeled precursors were imported as described above. After 8 min incubation, reactions were stopped by diluting with cold buffer, 0.6 M sorbitol, 20 mM K⁺HEPES, pH 7.4, 40 mM iodoacetamide (IAA). Mitochondria were pelleted by centrifugation (8,000 x g, 6 min) and pellets were washed in 20 mM IAA and recollected. Mitochondria were disrupted with 8 M urea buffer, pH 8.0 for 30 min. Samples were then centrifuged at 14,000 RPM for 15 min at room temp. The supernatant was incubated with Ni²⁺-NTA affinity resin at room temp while rotating for 2 hours (5% was kept for the load lane). Samples were washed with 8M urea, pH 6.3 twice for 10 minutes and aspirated with a Hamilton syringe. The bound protein was then eluted for 10 min in Urea buffer, pH 4.5. Laemmli sample buffer (without β-me) was added and heated at 60°C for 10 min. Samples from were resolved by SDS-PAGE and visualized by autoradiography.

**Oxygen consumption and membrane potential measurements.**

Mitochondria were purified from yeast cells grown on YPEG as described previously. Oxygen consumption measurements with isolated mitochondria were performed using an oxygen electrode (Hansatec) as described previously. Membrane potential measurements of purified mitochondria were performed with fluorescent 3,3'-dipropylthiadicarbocyanine iodide dye DISC3(5). 1% DMSO, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), MB-7, or MB-8 was added to mitochondria in import buffer (0.6 M sorbitol, 2 mM KH₂PO₄, 60 mM KCl, 50 mM HEPES-KOH, 5 mM MgCl₂, 2.5 mM EDTA, 5 mM L-methionine, pH 7.1) for 10 min. Subsequently 0.2 μM DISC₃(5) in import buffer was added, incubated for 5 min, and fluorescence was measured at excitation and emission length of 620 nm and 670 nm, respectively.
Mitochondrial Network Microscopy

HeLa cells were grown to 40% confluency in the presence of DMSO, CCCP, or varying concentrations of MB-7 or MB-8. Mitochondria were labeled with Mitotracker red CMXRos (Invitrogen), fixed with 3.7% formaldehyde, and visualized on a Leica TCS SP8 confocal microscope and processed with the Leica AF software package.
Figures

Figure 3.1 – MB-6 causes the accumulation of Mia40-Cmc1 import intermediate.

Radiolabeled Cmc1 was imported into purified Wt yeast mitochondria in the presence of MB-6 or MB-7 and separated by non-reducing (left) or reducing (right) SDS-PAGE and visualized by autoradiography.
Figure 3.2 – Radiolabeled CHCHD10 accumulates into MB-6 or MB-8 treated mitochondria. Radiolabeled CHCHD10 was imported into purified Wt yeast mitochondria in the presence of DMSO, or varying concentrations of MB-5, MB-6, MB-7, or MB-8 and separated by SDS-PAGE and visualized by autoradiography.
Figure 3.3 – MB-6 causes the accumulation of Mia40-CHCHD10 import intermediate.

Radiolabeled CHCHD10 was imported into purified Wt yeast mitochondria in the presence of 1% DMSO, or 50µM MB-6, MB-7, or MB-8 and separated by non-reducing SDS-PAGE and visualized by autoradiography. Both coupled and uncoupled (+CCCP) mitochondria were analyzed.
Figure 3.4 – MB-6 and MB-8 causes the accumulation of Mia40-CHCHD10 import

**Intermediate.** Radiolabeled Cmc1 was imported into purified Wt, 10xHis-tagged Mia40, or 10xHis-tagged Erv1 yeast mitochondria in the presence of DMSO, or 50µM MB-6, MB-7, or MB-8. Mitochondria were solubilized in 8M urea and separated on Ni$^{2+}$-NTA affinity resin. Proteins were eluted (E) with imidazole and separated by non-reducing SDS-PAGE and visualized by autoradiography. 10% Load (L) and Flow through (Ft) were included.
Figure 3.5 – MitoBloCK treatment results in a fragmented mitochondrial network.

Mitochondrial networks from Hela cells grown in the presence of DMSO, various MBs, or CCCP were visualized by confocal microscopy using MitoTracker Red (scale 10 μm).
Figure 3.6 – Radiolabeled CHCHD2 accumulates in MB-8 treated mitochondria.

Radiolabeled CHCHD2 or truncated CHCHD2ΔMTS was imported into purified Wt yeast mitochondria in the presence of DMSO or MB-8 and separated by SDS-PAGE and visualized by autoradiography.
Appendices

Appendix A. Rheumatoid arthritis drug Auranofin targets Mia40. The Seleem group demonstrated that auranofin, an FDA-approved drug for rheumatoid arthritis patients, has potent...
antifungal activity against a wide range of *Candida* and *Cryptococcus* sp. and identified Mia40 as a potential target from a chemogenic screen. Our biochemical analysis confirmed Auranofin does not uncouple the mitochondria (A) or strongly affect respiration (B). Auranofin appears to target the Mia40-Erv1 pathway as the drug inhibited Mia40 from interacting with its substrate, Cmc1 (C), in a dose-dependent manner similar to the control, MB-7. Furthermore, yeast mitochondria overexpressing Erv1 (D) were shown to exhibit resistance to Auranofin as an increase in Cmc1 import was observed compared to wild-type yeast.
Proteins enriched Erv1 pulldowns

Proteins enriched Erv1+CMC1 pulldowns

Fold increase over background
Appendix B. Mass Spec analysis of disulfided partners with Mia40, Erv1, or Cmc1.

Wildtype, his-tagged Mia40, or his-tagged Erv1 were either mock treated or incubated with purified recombinant Cmc1. Disulfide interactions were trapped, mitochondria were lysed with 8M urea, and the complexes were isolated with Ni$^{2+}$-NTA affinity resin. The samples were digested with sequencing grade trypsin while still bound to the beads and extracts were subjected to mass spec analysis in collaboration with the Wohlschlegal lab. Bar graphs represent proteins that were enriched in each sample and reported by the fold change over the background. Red lanes represent proteins that had no detectable background so caution must be taken into account when comparing levels.
References


