Targeting the Mitochondrial Processing Peptidase as a therapeutic strategy for Acute Myeloid Leukemia

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Medical Biophysics
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2014

Abstract

Acute myeloid leukemia (AML) is a highly heterogeneous disease characterized by a wide variety of cytogenetic abnormalities. Treatment has been largely unchanged for the past two decades and prognosis is still poor; thus, new therapeutics is needed for this disease. Here, we explore the possibility of targeting the Mitochondrial Processing Peptidase (MPP) as a potential therapeutic target for AML. MPP is a heterodimeric zinc metallopeptidase that cleaves presequences following the import of nuclear encoded matrix space precursors. We demonstrate that MPP-α is overexpressed in a subset of AML patient blood and stem/progenitor populations. MPP-α is ubiquitously expressed in human tissues and strongly expressed in heart and brain. Furthermore, AML cell lines are differentially susceptible towards genetic knockdown of MPP-α with OCI-AML2 having the greatest decrease in cell viability and proliferation while K562 and TEX have moderate effects. These results warrant further investigation of MPP as a therapeutic target for AML.
Acknowledgments

I would like to offer my sincerest gratitude to my supervisor, Dr. Aaron Schimmer, for his invaluable guidance and continued support throughout the course of the project. I could not have imagined a better supervisor and mentor to guide me throughout the study. His patience and immense knowledge has been crucial to my learning and development during graduate studies. Additionally, I would also like to greatly thank the members of my advisory committee, Dr. Linda Penn and Dr. Rodger Tiedemann, for their thoughtful advice, insightful comments, and interesting questions. I thank all the members of the Schimmer Lab for the stimulating discussions and feedback to enhance my knowledge and critical thinking skills. In particular, I would like to thank Rose Hurren, Marcela Gronda, and Neil MacLean for their assistance with key experiments. Additionally, I thank Dr. Rebecca Laposa for providing the various human tissue mRNA samples. Last but not the least, I would like to thank my parents, Ramakrishnan and Sujatha, and other family members for always supporting and encouraging my endeavors in science.
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>ATFS-1</td>
<td>Activating Transcription Factor associated with Stress-1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CLPP</td>
<td>Caseinolytic protease proteolytic subunit</td>
</tr>
<tr>
<td>CRISPRi</td>
<td>CRISPR interference</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>HSP60</td>
<td>Heat Shock Protein member 60</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>LPC</td>
<td>Leukemia Progenitor Cell</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemia Stem Cell</td>
</tr>
<tr>
<td>MPP-α</td>
<td>Mitochondrial Processing Peptidase subunit α</td>
</tr>
<tr>
<td>MPP-β</td>
<td>Mitochondrial Processing Peptidase subunit β</td>
</tr>
<tr>
<td>MTS</td>
<td>Matrix Targeting Sequence</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>PAM</td>
<td>Presequence Associated Motor proteins</td>
</tr>
<tr>
<td>PBSC</td>
<td>Peripheral Blood Stem Cell</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN induced putative kinase 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Real Time Polymerase Chain reaction</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin RNA</td>
</tr>
<tr>
<td>TOM</td>
<td>The Outer Membrane complex</td>
</tr>
<tr>
<td>TIM</td>
<td>The Inner Membrane complex</td>
</tr>
<tr>
<td>UPR\textsuperscript{mt}</td>
<td>Mitochondrial Unfolded Protein Response</td>
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Chapter 1
Introduction

1.1 Acute Myeloid Leukemia

Acute Myeloid Leukemia (AML) is a malignancy of the myeloid group of blood cells in which immature myeloid precursors aggressively proliferate and fail to differentiate into mature functional cellular components. Eventually, this abnormal proliferation leads to an altered hematopoietic state whereby an accumulation of immature leukemic blasts override the abundance of mature granulocytes, monocytes, erythrocytes and platelets.\(^1\) AML comprises the largest group of hematological malignancies among acute adult leukemia and is highly heterogeneous in terms of cytogenetic abnormalities and mutations.\(^2\) Many AML cases arise abruptly and indiscriminant in whom it affects as a result of aberrant somatic mutations in hematopoietic progenitors.\(^3\) The highly heterogeneous nature of AML can be attributed to the diverse mutations such as chromosomal translocations leading to fusion products, chromosomal deletions of tumor suppressor genes, disruption of programmed cell death, single-gene mutations promoting leukemia, and perturbations of the telomere/telomerase system.\(^4\) It is generally thought that AML arises from the combination of two different kinds of mutations – proliferation stimulators (common targets include Ras, FLT3 and c-kit) and differentiation inhibitors (such as CBF, CEBPA and MLL).\(^5\) To promote and drive AML leukemogenesis, both types of mutations must occur in hematopoietic progenitors.

AML is similar to normal hematopoiesis in that it is maintained by leukemia stem cell (LSCs) which have a unique differentiation process distinct from normal hematopoiesis.\(^6\) A
major fraction of total AML cells do not possess the properties of hematopoietic stem cell that enable self-renewal and differentiation into various mature blood cells. LSCs which comprise a minority fraction of total AML cells possess these properties and proposed to be the candidates that maintain the leukemic state. Specifically, the CD34+38- population of total AML cells were first identified to possess the self-renewal and differentiation properties to maintain leukemic state. However, LSCs can also be found in the CD34+CD38+ and CD34- fractions in certain AML cases such as in patients with mutation in nucleophosmin (NPM1). The CD34+CD38-population in normal hematopoiesis is comprised of hematopoietic stem cells (HSC) and this suggests that LSC may originate due to mutations in HSCs. Intriguingly, Majeti et al. (2007) have recently identified a non-HSC multipotent progenitor with CD34+CD38-CD90-expression type in human cord blood which may contribute towards the origin of LSCs. LSCs differentiate into oligopotent leukemia progenitor cells (LPCs) with the surface markers expression CD34+CD38+ and LPCs, in turn, differentiate into the bulk leukemic blast population defined as simply CD34- due to loss of the CD34 marker expression. Figure 1 summarizes the sequence of AML differentiation process and marker phenotype expressed indicative of each fraction.

![Figure 1: Schematic representation of the self-renewal and differentiation properties of leukemia stem, progenitor, and blast cells along with their specific marker expression.](modified after McCellan and Majeti EMBO Mol Med (2013) 5, 7–89)
1.1.2 Prognosis and current therapy in AML

Survival of AML patients can be predicted by classifying AML patients into three broad risk group categories depending on cytogenetic abnormalities – favorable, intermediate, and adverse risk groups. Most common AML cytogenetic abnormalities are observed in chromosome 5, 7, and 11q23. Favorable risk group patients have the karyotype \( -t(15;17)(q22;q12-21), t(8;21)(q22;q22) \) or \( \text{inv}(16)(p13q22)/t(16;16)(p13;q22) \). Patients not classified into adverse or favorable risk groups fall into the intermediate category that includes a normal karyotype along with aberrations such as \(-Y, +11, +13, +21, \text{del6q, del9q, del11q, or del20q.}\) In addition, the type of mutations can also be indicative of AML prognosis in patients who have normal cytogenetics. Mutations of NPM1 and CEBPA have favorable prognosis and fusion products such as FLT3-ITD and MLL-PTD and overexpression of KIT, BAALC, and BAX all give rise to adverse prognosis. The variety of cytogenetic and mutation types having varying survival rates demonstrate the heterogeneity of AML and challenges in advancing therapeutics.

Data analysis from the Cancer and Leukemia Group B study conducted by Clara Bloomfield and colleagues examined the 14-year survival rates of the 3 risk groups stratified according to cytogenetic abnormalities. Results revealed that most AML patients fall into the intermediate risk category which have 67% complete remission rate, 67% 5-year cumulative incidence of relapse, and 24% 5-year overall survival. In comparison, favorable risk group comprised the lowest proportion of AML patients and have 88% complete remission rate, 54% 5-year cumulative incidence of relapse, and 55% 5-year overall survival. Despite aggressive
chemotherapy, the adverse risk patients had even worse prognosis with 32% complete remission rate, 92% 5-year cumulative incidence of relapse, and 5% 5-year overall survival.

AML treatment protocol has been unchanged for at least 35 years. Standard therapy calls for induction chemotherapy with continuous infusion administration of cytarabine, which inhibits DNA synthesis, for seven days at 100 – 200 mg/m²/d along with intravenous administration of daunorubicin at 60 – 90 mg/m²/d for the first three days. After induction therapy, hematopoietic growth factors may be administered to promote neutrophil production. Success rate of induction chemotherapy is 50-70% of adult AML cases reaching complete remission and 20-30% of patients achieving long term disease free survival. A particular study by the Eastern Cooperative Oncology Group found that following induction chemotherapy of 3000 patients, 67% resulted in complete remission; however; 76% relapsed with more aggressive AML. Given the poor survival rates, low treatment success and the high occurrence of relapsed AML, improving therapeutic for this highly heterogeneous disease remains an unmet demand to treat AML.

1.2 Roles of the mitochondria and relevance to cancer

A prerequisite for the survival of any eukaryotic cell is energy generation, a task largely accomplished by the cell’s “powerhouse” double-membrane organelle, the mitochondria. Origins of the mitochondria date back about two billion years when a common eukaryotic precursor ingested a primitive α-proteobacterium as proposed by the endosymbiotic theory. Mitochondria are highly compartmentalized and, owing to their prokaryotic origin, possess an outer and inner membrane. The intermembrane space is sandwiched by outer and inner
membrane while the matrix space is defined by the inner membrane. The human mitochondrial genome is a circular chromosome in the matrix which encodes information for 13 proteins forming the core components of the respiratory complexes on the inner membrane and 22 transfer RNAs along with 2 ribosome-coding RNAs, which form the mitochondrial translation machinery distinct from its cellular counterparts.

There are approximately 1,000 proteins in the mitochondria albeit only 13 are encoded by mitochondrial DNA. The nuclear encoded mitochondrial proteins are translated on cytosolic ribosomes and are actively imported and sorted into mitochondrial sub-compartments by outer and inner membrane components. Regulation at the transcriptional, post-transcriptional and post-translational levels also occurs for these nuclear mitochondrial genes. The PGC-1 family of co-activators transcriptionally regulates nuclear mitochondrial genes in response to alterations in nutrient demand such as ATP and NADH. Extensive studies on yeast mitochondria have revealed that cytosolically synthesized mRNA encoding certain mitochondrial proteins are localized to mitochondrial outer membrane post transcriptionally and translated while localized. A common post-translational regulation includes the phosphorylation of genes encoding mitochondrial import pathway by cytosolic kinases in order to control the half-life in response to metabolic needs.

Mitochondrial also possess distinct quality-control pathways that sense and respond to both mitochondrial and cellular dysfunction. Molecular chaperones and quality control proteases act together to promote the assembly of protein complexes, both mitochondrial and nuclear encoded, to regulate and degrade unfolded proteins. When alterations in abundance of mitochondria and nuclear proteomes arise, the mitochondrial unfolded protein stress response pathway (mtUPR) is triggered by signals produced at the mitochondrial level that upregulate the
transcription of nuclear encoded mitochondrial chaperone genes, such as HAF1 and ATFS1, to restore balance and mitochondrial homeostasis.\textsuperscript{40} Studies of mtUPR activation in \textit{C. elegans} demonstrate increased cellular lifespan and the conservation of these mechanisms in mammalian cells could provide clues into longevity and cell survival.\textsuperscript{41}

Under physiological stress, damaged mitochondria can be targeted for degradation through autophagy. However, this process alone cannot account for rapid turnover of mitochondria proteins that was first described by Russell et al. (1980).\textsuperscript{42} This implied the existence of a unique proteolytic system within the mitochondria and was confirmed upon elucidation of the mitochondrial proteome. There are currently 20 different mitochondrial proteases that have been identified throughout all compartments of the human mitochondria. Mitochondrial proteases are encoded by nuclear genes, synthesized cytosolically and transported upon proteolytic maturation. Moreover, these proteases can be classified according to their main function as follows – processing peptidases, ATP-dependent proteases, and oligopeptidases.\textsuperscript{43}

Several studies have implicated the importance of mitochondria in tumor creation, progression, and maintenance. Mitochondria in tumor cells are unique in their structural features and have altered functions.\textsuperscript{44} Mutations in the any nuclear or mitochondrial encoded respiratory chain genes that cause aberrant oxidative phosphorylation and decreased ATP production can lead to accumulation of reactive oxygen species (ROS) and subsequent oxidative damage that can induce DNA damage, increasing likelihood of acquiring pro-oncogenic mutations.\textsuperscript{45} Canter et al. (2005) and Petros et al. (2005) have also observed specific mutations in mitochondrial DNA that are associated with a greater chance of developing malignancies such as breast, prostate, and thyroid cancers.\textsuperscript{46,47} For instance, mutations in cytochrome oxidase subunit I gene in certain prostate cancer patients leads to disruption of oxidative phosphorylation and thereby
increasing ROS and contributing to tumorgenicity. Altered mitochondrial functions have been addressed in key pathological hallmarks of cancer being high proliferative power, resistance to anti-proliferative signaling pathways, higher anabolic potential, and lower autophagy. Hence, therapeutic targeting of mitochondrial pathways in cancer represents a unique and feasible possibility.

1.3 Mitochondrial therapeutic targets in cancer

Increased aerobic glycolysis in cancer, known as the Warburg effect, has been observed in various tumor cells to varying degrees. The Warburg effect stipulates that tumor cells are increasingly dependent on glycolysis rather than oxidative phosphorylation for their survival, even in the presence of oxygen. This metabolic alteration could be attributed to decreased mitochondrial respiration through mutations in mitochondrial DNA, mutations in nuclear encoded mitochondrial proteins, expression of oncogenes promoting glycolysis while decreasing mitochondrial respiration (Ras, Bcr-Abl, and Akt) and hypoxia in the tumor microenvironment decreasing the availability of oxygen. Intriguingly, AML stem/progenitor cells have higher mitochondrial biogenesis and, consequently, increased reliance on oxidative phosphorylation. Recent studies by Skrtic et al. (2011) have revealed the possibility of using the antimicrobial, tigecycline, to treat AML by inhibiting mitochondrial translation and selectively cytotoxic to leukemia cells over hematopoietic tissues. AML stem cell and progenitors were found to have an altered state of mitochondrial biogenesis where AML cells had higher mitochondrial mass and greater demand on oxidative phosphorylation.
Several studies have previously revealed the numerous possibilities of targeting the mitochondria with small molecule inhibitors. One common theme is targeting the mitochondrial metabolism, a highly attractive option given the altered metabolic demands of cancer cells. For instance, inhibiting glycolysis with 2-deoxy-D-glucose leads to increased oxidative stress (as a result of mitochondrial ROS production) when synergized with cisplatin.\textsuperscript{54} Currently, phase I/II trials are underway with 2-deoxy-D-glucose to treat solid tumors such as head and neck cancer and prostate cancer. Another strategy is targeting mitochondrial pyruvate dehydrogenase kinase (PDK) with dichloroacetate to promote glucose oxidation by stimulating activity of pyruvate dehydrogenase activity.\textsuperscript{55} This leads to higher ROS production selectively in malignant cells.

Furthermore, the targeting the apoptotic regulators is useful strategy to selectively induce cell death in cancerous cells. A commonly cited example is inhibition of Bcl-2, anti-apoptotic regulator on the outer mitochondrial membrane, which is overexpressed in a majority of n-Hodgkin’s lymphoma patients. G3139, a Bcl-2 specific inhibitor, has been demonstrated to be a promising candidate in phase I clinical trials for the treatment of hematological malignancies such as non-Hodgkin’s lymphoma, APL, and AML.\textsuperscript{56,57,58}

Dissipating the mitochondrial membrane potential is another popular strategy taking hold in mitochondrial cancer therapeutics to selectively inducing cell death.\textsuperscript{59} Permeability transition pore complexes (PTPC) are membrane potential dependent metabolite carries used to shuttle metabolites across the inner membrane. The adenine nucleotide translocase (ANT) is a PTPC that exchanges ATP in the matrix with ADP in the intermembrane space to complement oxidative phosphorylation. Inhibition of ANT specific agents such as clodronate or arsenite trioxide is rapidly becoming a strategy to treat more aggressive, relapsed tumors.\textsuperscript{60}
To add to the altered mitochondrial metabolic state in AML, Lagadinou et al (2013) showed that AML leukemic cell populations can be classified into ROS-high and ROS-low cell types. ROS-low cells comprised the majority of cell type and are characterized by quiescent state but greater dependence on oxidative phosphorylation. Moreover, ROS low cells seemed to much more susceptible to inhibition of oxidative phosphorylation. Hence, targeting mitochondrial genes to treat the highly heterogeneous nature of AML represents a unique and unexplored strategy.

1.4 Mitochondrial protein import pathway

Mitochondrial preproteins, or precursors, synthesized from the cytosol contain the mitochondria-targeting sequence (MTS) found within the N-terminus presequence that signals their localization. These presequences are highly varied in their lengths but most commonly are within 20 – 40 amino acid residues long while some precursors have smaller or larger lengths. Mutagenesis studies have shown that the presence of few positively charges amino acid residues such as arginine and lysine within the presequence are essential to elicit the import signal into the mitochondrial. The amino-terminal portion of the presequence, where the mitochondria-targeting sequences resides, forms an amphiphilic α-helical secondary structure in which the positively charged amino acid residues align on one side and the hydrophobic amino acid residues on the other side of the helix. The amphiphilicity of the amino acid sequence seems to be essential while the α-helix formation does not seem to be an important requirement for the function of MTS.
Mitochondrial membranes are highly lipophilic and translocation of precursors is achieved through multi-subunit aqueous channels. Mitochondrial protein precursors imported into the various compartments of the mitochondria must interact with two separate complexes mediating substrate translocation - the TOM complex (outer membrane) and the TIM complex (inner membrane).\textsuperscript{65} To date, a single TOM multi-subunit complex has been identified that mediates the translocation of almost all precursors across the outer membrane except the import of the cytochrome c precursor which carries out an import independent of the TOM complex.\textsuperscript{66} The TOM complex possesses unique characteristics to recognize both N-terminal and internal targeting presequences to strictly import in a unidirectional manner. Additionally, TOM complex is capable of differentiating hydrophobic membrane proteins for targeting and inserting into the outer membrane or the inner membrane and relays the precursors for further processing.

There are two separate TIM multi-subunit complexes with different functions that mediate protein import and share homology. The Tim23 complex is composed of the integral membrane proteins Tim23, Tim17, and Hsp70 that and is primarily responsible for the import of all precursors that are targeted to either the matrix or inner membrane insertion based on the N-terminal targeting presequence. On the other hand, Tim22 complex, which is formed by Tim54, Tim18, Tim12, Tim9 and Tim10, coordinates the insertion of specific inner membrane proteins such as metabolite channel and carriers that generally possess several membrane spanning domains and unique internal mitochondrial targeting signals at various locations.\textsuperscript{67} Figure 2 illustrates the various components of the import pathway discussed more specifically in the following sections.
Figure 2: Overview of the various mitochondrial import machinery components. OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane; PAM: presequence translocase-associated motor; MPP: mitochondrial processing peptidase; mtHSP70: mitochondrial heat shock protein member 70.
1.4.1 Translocation through outer mitochondrial membrane

The translocases of the outer membrane, TOM, complex consists of eight different proteins to form a multisubunit channel to recognize cytosolically translated preproteins and interact with cytosolic chaperones to permit entry into the intermembrane space. On the mitochondrial surface, receptor proteins Tom20, Tom22, and Tom70 function to recognize preprotein targeting signals and direct them from the cytosol to the mitochondria. A subcomplex of Tom20 and Tom22 specifically carries out recognition of precursors with cleavable, amino-terminal presequences. The interaction between a precursor and associated receptor subunit is mediated by the presence of acidic residues on the receptor cytosolic domains which form an electrostatic interaction between the receptor and the positively charged targeting presequence of the precursor. The other components of the TOM complex, present within the hydrophobic regions of outer membrane create the aqueous channel, general import pore (GIP). The core constituent is Tom40 and Tom5 (smallest TOM member) directly interacts with Tom40 to serve as a cross-linker between the receptor proteins and the GIP. Tom6 and Tom7 serve as regulators of the TOM import complex stability in response to metabolic demands.

1.4.2 Translocation through inner mitochondrial membrane

The translocase of inner membrane, TIM, complex is composed of the members Tim17, Tim23, and Tim44. Tim17 and Tim23 are integral membrane proteins that span through the inner membrane and have both matrix and intermembrane space associated domains in order to form the aqueous channel to shuttle precursors designated into the matrix. Tim23 interacts with presequence translocase-associated motor (PAM) proteins 16, 17 and 18. The inner
membrane translocation process is energy and membrane potential dependent. Uncoupling inhibitors (ex. Valinomycin) decrease matrix specific import and suggests initial translocation is promoted by maintenance of membrane potential gradient. An electrophoretic effect is generated due to the membrane potential which draws precursor candidates into the matrix since the presequence has positively charges residues. The energy dependent phase of the translocation process occurs in conjunction with matrix HSP70 via hydrolysis of ATP. Although the precise mechanism is still unclear, HSP70 is thought to interact with Tim44 to form a motor complex that drives the movement of precursors into the matrix. The phospholipid cardiolipin in the inner mitochondrial membrane is also imperative for protein translocation. Furthermore, it is proposed that cardiolipin, a phospholipid in the inner membrane, facilitates in interaction between the precursor and Tim44-HSP70 sub-complex by properly orienting the precursor in the appropriate position to optimize its interaction with Tim44-HSP70. Following translocation through the inner membrane, the precursors are cleaved at the N-terminus to remove the presequence in order to properly fold and function optimally. This step is mediated by the mitochondrial processing peptidase (MPP) which results in a lower molecular weight matured product which is folds into its native conformation by interacting with HSP60. The MPP mediated cleavage and HSP60 assisted folding occurs proximal to the TIM complex so as to efficiently coordinate import and maturation.

1.5 Mitochondrial Processing Peptidase

MPP is a metalloendopeptidase that specifically cleaves off presequences from several mitochondrial matrix precursor proteins. Specifically, MPP is categorized into the pitrilysin
family of proteases due to the presence of an inverted Zn-binding motif in the catalytic site.\textsuperscript{82} MPP is a heterodimer of two structurally similar subunits that coordinate its activity, MPP-\(\alpha\) and MPP-\(\beta\) (about 55 kDa per subunit). MPP-\(\beta\) contains the active site and performs the cleavage while MPP-\(\alpha\) plays a role in recognition of presequence. The MPP complex is localized to the matrix in in yeast and mammals. The matrix targeting presequences cleaved by MPP vary greatly in size and are highly varied in their sequences.

The two subunits forming MPP are structurally related; MPP-\(\alpha\) is between 53-57 kDa while MPP-\(\beta\) ranges from 48 and 52 kDa.\textsuperscript{83} Both MPP subunits are synthesized as larger precursor proteins with matrix-targeting presequences and therefore require the presence of active MPP already in the mitochondria in order to be processed to the mature form. There is 43\% identity between yeast and neurospora MPP-\(\alpha\) and 36\% identity between each of these proteins and the rat MPP-\(\alpha\).\textsuperscript{84} The human MPP identity is 45–52\% among rat, yeast, and neurospora. The zinc cation within MPP-\(\beta\) active site is bound by two glutamates (proton acceptors) and two histidines (proton donors). Albeit no knockout mice have been reported for MPP-\(\alpha\) in literature, the Mouse Genome Informatics database reveals that complete knockout of MPP-\(\beta\) is embryonically lethal at day 8 of development.\textsuperscript{85}

\subsection{1.5.1 MPP mediated cleavage of matrix targeting signal}

Mitochondrial presequences vary in length from 10 – 50 residues. The sequence analyses of mitochondrial precursor proteins showed that three common features in most presequences cleaved by MPP – 1) series of positively charges residues, 2) formation of an \(\alpha\)-helix, and 3) an arginine residue at the -2 position from the cleavage site.\textsuperscript{86,87,88} There is a relatively high degree
of amino acid sequence degeneracy at MPP cleavage sites and this variation makes it difficult to predict whether a given presequence is amendable to MPP cleavage.\textsuperscript{89} Mutational studies revealed that arginine residue at -2 or -3 position does play a role but does not necessarily direct cleavage.\textsuperscript{90} The role of other positively charged residues in the presequence is still unclear. Structural element rather than sequence identity appears to play a larger role in directing cleavage.\textsuperscript{91} A conserved HXXEH motif is present in the MPP-β while absent in the MPP-α polypeptide.\textsuperscript{92} The MPP-α has a conserved region enriched in negatively charged residues to facilitate electrostatic interactions with positively charged residues of the presequence. MPP-α also has a unique glycine-rich domain that further facilitates in substrate binding and processing of the MPP complex.\textsuperscript{93}

1.5.2 Implication in mitochondrial diseases

Recent studies have implicated MPP in the Friedreich ataxia (FRDA), an autosomal recessive neurodegenerative disease characterized by defect processing of the mitochondria frataxin.\textsuperscript{94} Frataxin, thought to play a role in mitochondrial iron homeostasis, is nuclear encoded and synthesized in the cytoplasm with an N-terminal matrix targeting presequence.\textsuperscript{95} The frataxin presequence is cleaved by MPP between residues 41–42 and 55–56.\textsuperscript{96} The initial cleavage of the precursor is rapid and produces the intermediate form which in turn is cleaved much slower into the mature product.\textsuperscript{97} In FRDA, point mutations at I154F and G130V lead to disruption in interaction of precursor frataxin with MPP and thereby inhibiting the initial cleavage.\textsuperscript{98} Reduction in mature frataxin leads to dysfunctional mitochondria and subsequent neurodegeneration.
Chapter 2
Rationale and Hypothesis

One key finding by Skrtic et al. (2011) was the demonstration that some but not all regulators of mitochondrial translation are essential for maintaining mitochondria integrity and cell viability. Due to the differential cytotoxic effects of IF-3 and EF-Tu on leukemic cell viability, a genetic screen was performed in order to identify mitochondrial proteins whose genetic suppression reduces the viability of leukemia cells. From this screen, the top hits in the mitochondrial proteome consisted of several mitochondrial proteases including ClpP, AFG3L2, PMPCA (MPP-α).

The implications of mitochondrial proteases in the context of cancer are unclear. However, preliminary results show that ClpP, a top hit from above mentioned RNAi screen, chemical and genetic inhibition in AML cells is selectively cytotoxic compared to normal hematopoietic cells. In vivo studies are currently underway in examining therapeutic window of the ClpP inhibitor, A2-32-01. These promising results potentially imply a unique role of mitochondrial proteases in developing therapeutics for AML.

While the precise role of MPP in AML is not well characterized, it remains an unexplored pathway for which the therapeutic window has not been elucidated. Furthermore, no specific MPP inhibitors have been identified to date which further signifies a unique therapeutic opportunity worth exploring. As a result of higher mitochondrial biogenesis in AML cells, it may be necessary to meet this demand by upregulation of mitochondrial protein import so as to maintain equilibrium between nuclear and mitochondrial proteome. Hence, it is expected that
MPP subunits would be overexpressed in AML cells to coordinate the increased mitochondrial precursor import. Moreover, inhibition of MPP activity may lead to accumulation of misfolded polypeptide precursors whose accumulation could elicit the mtUPR response leading to subsequent cell death selectively in AML cells. Henceforth, the aims are two fold – 1) determine the expression of MPP-α in AML cells including primary patient blood and stem cell/progenitor populations and normal human tissues 2) determine the impact of MPP-α inhibition, consequently MPP complex activity suppression, on viability of AML cell lines.
Chapter 3
Methods

NanoString

Six adult AML samples were isolated from peripheral blood samples from consenting AML patients. Six normal peripheral blood stem cell (PBSC) samples were obtained from healthy volunteers who donated peripheral blood after being treated with granulocyte colony-stimulating factor (G-CSF) to mobilize normal hematopoietic stem cells from the bone marrow. The use of total RNA extract from a panel of 19 different human tissues (ClonTech) was approved by University Health Network Research Ethics Board. Primary AML patient samples were sorted for CD34+ and CD38+ cell surface markers by fluorescence-activated cell sorting in order to determine expression levels in AML stem cell and progenitor fractions.

Two sequence-specific probes were constructed for each gene of interest and were complementary to a 100-base region of the target mRNA. One probe was covalently linked to an oligonucleotide containing biotin (the capture probe), and the other was linked to a uniquely color-coded fluorescent tag that produced the signal (the reporter probe). The nCounter CodeSet contained probes for 40 genes 12 control genes for a standard curve. Sample RNA (200 ng) was loaded onto nCounter Gene Expression CodeSets chip and subsequently hybridized with complementary probes and analyzed for raw mRNA counts from each sample. Following hybridization, the tripartite structures are purified and robotically on custom liquid handling robot (Prep Station, NanoString Technologies). Purified complexes are attached to a streptavidin coated slide through biotinylated capture probes. Voltage (160 V/cm) is applied to elongate and
align complexes. Stretched complexes are subsequently immobilized and voltage is turned off to prepare for imaging and counting. Raw mRNA counts were normalized with respect to endogenous β2 microglobin mRNA counts for data analysis.

Cell Culture

Human leukemia OCI-AML2 and K562 leukemia cell lines were maintained in IMDM (Iscove’s modified Dulbecco’s medium) and 10% FCS (Fetal Calf Serum). TEX leukemia cells were maintained in IMDM, 20% FCS, 2mM L-glutamine, 20 ng/mL SCF (stem cell factor), 2 ng/mL IL-3 (interleukin-3). All cells were incubated at 37°C in a humidified air atmosphere supplemented with 5% CO2.

shRNA knockdown

Lentivirus was constructed using hairpin-pLKO.1 vector (containing the puromycin antibiotic resistance gene PAC) with shRNA insert according to standard protocol for transient virus production in HEK293T cells. The coding sequences of the shRNAs targeting MPP-α (Accession No. NM_015160) are as follows: shRNA 174 – 5’-GCTACAGTTGATGGACAGGAA-3’, shRNA 309 5’- GCGAAATACCTTAGGGAATT-3’, shRNA 366 – 5’-GCTCGATTTGACAGCAAAGAT-3’, shRNA 1295 – 5’-GCTGACATCAATGCTCATGAT-3’, and shRNA 1413 – 5’-CGCAACGTGAAGCCGGAAGAT-3’. Lentiviral infections in suspension cell lines were performed according as described previously in Skrtic et. al (2011). Each treatment group
contained 5 x 10⁶ suspension cells in culture that were centrifuged and re-suspended in 10 ml media containing protamine sulfate at 5 ug/ml, appropriate quantity of virus cocktail (1 ml for K562, 2 ml for OCI-AML2, TEX, and HL-60), followed by overnight incubation (37 °C, 5% CO2) with virus. The following day, cells were centrifuged and resuspended in fresh media with appropriate puromycin concentration (1 g/mL for OCI-AML2 and 2 g/ml for K562, TEX) was added. Following a three day selection period, equal numbers of live cells in each shRNA treatment were plated for growth assays and counted with trypan blue exclusion staining to measure total viable cells for a period of 8 days. To confirm knockdown, 2 x 10⁶ cells were collected at Day 3 post puromycin selection for q-RTPCR to confirm mRNA knockdown and cells were collected for immunoblot analysis on Days 4, 7, and 10 post puromycin selection to confirm knockdown at the protein level.

Quantitative reverse transcriptase polymerase chain reaction

MPP-α cDNA was amplified using the following primer pair: Forward 5’-AGCCGGAAGATGTGAAGAGA-3’ and Reverse 5’-AAACTAACACGCACGGAAC-3’. Equal quantities of cDNA for each sample treatment were added to a prepared master mix (SYBR Green PCR Master mix; Applied Biosystems). Quantitative reverse-transcriptase polymerase chain reaction reactions were performed on an ABI Prism 7900 sequence detection system (Applied Biosystems). The relative abundance of a transcript was indicated by the threshold cycle of amplification that is inversely proportional to the amount of mRNA. In order to normalize mRNA quantity from each sample, 18s mRNA was used as an endogenous loading
control. MPP-α mRNA expression level was then calculated with respect to threshold cycle of endogenous 18s mRNA level and normalized to untreated sample.

**Immunoblot Analysis**

To prepare cell lysates, cells were washed twice with phosphate buffered saline pH 7.4 and suspended in lysis buffer 1.5% dodecyl β-maltoside (Sigma Aldrich) containing protease inhibitor tablets (Roche). Protein concentrations were measured by the DC Protein assay (Bio Rad). Equal amounts of protein were subjected to sodium dodecyl sulphate (SDS)-polyacrylamide gels followed by transfer to polyvinylidene fluoride (PVDF) membranes. Membranes were probed with anti-MPP-α 1:1000 (Abcam), anti-β-actin 1:10000 (Santa Cruz Biotechnology) and secondary antibodies from GE Health (IgG peroxidase linked species-specific whole antibody). Detection was performed by the enhanced chemical luminescence method (Pierce Chemical).

**Rescue of OCI-AML2 cell viability upon MPP-α knockdown**

To determine the on-target nature of MPP-α shRNAs on OCI-AML2 cell viability, mouse MPP-α cDNA in a pCMV6 Kanamycin/Neomycin construct (OriGene) was amplified by a two-stage PCR using the following primer pairs to add attB1 and attB2 recombination sites at either end of PCR product – Forward 5’-AAAGCAGGCACCATGGCGACGGCAGTGGG-3’, Reverse 5’-ACAAGAAAGCTGGTGCCCTACCGGAACAGCCGTAT-3’ and Forward 5’-GGGGACAAGTTTTGTAACAAAAAAGCAGGCACCATG-3’, Reverse 5’-GGGGACCACTTTT
GTACAAGAAAGCTGGGTC-3’. The amplified product was subsequently inserted into pLX304-Blast-V5 destination lentiviral overexpression vector by gateway cloning at sequence specific recombinant sites. An independent construct containing Renilla luciferase cDNA insert was constructed to serve as a transduction control. Lentivirus was constructed using pLX304 with respective insert according to standard protocol for transient virus production in HEK293T cells.

Lentiviral infections were performed with each construct, mouse MPP-α and Renilla luciferase cDNA inserts, in 5 x 10^6 OCI-AML2 cells that were centrifuged and resuspended in 5 ml containing protamine sulfate at 5 ug/ml and 2 ml of virus cocktail followed by overnight incubation (37°C, 5% CO2) with virus. The following day, cells were centrifuged and resuspended in fresh media with blasticidin concentration at 10 ug/ml. Following a four day selection period, cells from each treatment group were counted by trypan blue exclusion staining and resuspended in fresh media with blasticidin. On the sixth day of selection, independently selected populations overexpressing mouse MPP-α and Renilla luciferase were prepared for shRNA transduction using shRNA clones 174 and 1295 in hairpin-pLKO.1 vector and selected with puromycin.

Statistical Analysis

All data are presented as mean and standard deviation (SD) to represent data variability. Statistical analyses were performed by unpaired student’s t-test, one-way ANOVA and Tukey’s test. Differences were considered statistically significant at p < 0.05.
Chapter 4
Results

4.1 Expression of MPP-α in AML cells and human tissues

4.1.1 MPP-α mRNA is overexpressed in AML patient blood, cell lines, and a subset of patient stem and progenitor cells.

To investigate expression of MPP-α, peripheral blood was collected from six consenting adult AML patients and healthy volunteers who donated peripheral blood after being treated with G-CSF to mobilize normal hematopoietic stem cells from the bone marrow. Primary AML patient samples were sorted for CD34+ and CD38+ cell surface markers by fluorescence-activated cell sorting in order to determine expression levels in bulk and stem/progenitor fractions with immunotypes of CD34+38+ and CD34+38- respectively. As Figure 3A demonstrates, we observe that MPP-α mRNA is higher in each AML patient blood compared to peripheral blood stem cells (PBSCs) from healthy volunteers. Furthermore within AML patients, levels of MPP-α mRNA is variable albeit consistently higher than PBSCs. When examining the expression in stem and progenitor cells (Fig 3B), MPP-α mRNA is higher in a subset of these samples. When PBSCs are sorted for CD34+ marker expression, MPP-α mRNA is higher compared to their unsorted counterparts. Among the five AML patient samples examined in Fig 3B, AML311 has highest MPP-α mRNA expression in both bulk and stem/progenitor populations. AML102, AML307, and AML35 are indifferent in their expression of MPP-α compared to PBSCs sorted for CD34+ expression. AML27 has higher MPP-α mRNA expression in stem/progenitor population only. However, only single replicated were performed and sample
size of both AML and normal groups are small and larger sample size is required to relate to clinical characteristics. To further investigate the relevance of higher MPP-α in some AML patients, we examined the mRNA expression in AML cell lines by q-RTPCR (Figure 4). MPP-α mRNA is higher in the three AML cell lines used for genetic knockdown studies – OCI-AML2, K562, and TEX leukemia cells. K562 cells have the highest mRNA expression followed by OCI-AML2 and TEX cells.
Figure 3 – MPP-α mRNA expression in AML patient blood and stem/progenitor populations. Relative mRNA expression in peripheral blood stem cells (PBSCs) from healthy volunteers and AML patient blood (3a). Expression of mRNA in AML stem/progenitor cell populations was compared with CD34+ PBSCs (3b) sorted by fluorescent activated cell sorting. Unsorted PBSC and patient blood expression levels are also provided in 1B. Data are represented as normalized counts with respect to endogenous B2-microglobulin mRNA counts as measured by a custom NanoString chip.
Figure 4 – MPP-α mRNA expression in AML cell lines. Relative mRNA expression in AML cell lines OCI-AML2, K562, and TEX leukemia cells evaluated by quantitative real time polymerase chain reaction (qRT-PCR). Expression in AML cell lines are compared to PBSC from four healthy volunteers. Abundance of MPP-α mRNA was normalized with respect to abundance of 18s rRNA as an endogenous control. Data represented are mean (n=3) with standard deviation.
4.1.2 MPP-α mRNA is ubiquitously expressed in various human tissues and strongly expressed in some mitochondrial dependent tissues.

To understand potential toxicities upon inhibiting MPP activity in in vivo models, we examined MPP-α mRNA expression in 19 human tissues by NanoString. As evident in Figure 5, some mitochondrial enriched tissues such as the skeletal muscle and brain have very high levels of MPP-α mRNA while other energy dependent tissues such as the heart, kidney, and liver have intermediate expression of MPP-α mRNA. Lung, spleen, trachea, and small intestine had the lowest relative abundance of MPP-α mRNA. However, only single replicates were performed and validation by immunoblotting is needed for useful interpretation in the various tissues.

![Figure 5 – MPP-α mRNA expression across human tissues.](image)

**Figure 5 – MPP-α mRNA expression across human tissues.** Relative mRNA expression in RNA extracts from a panel of 19 human tissues derived from healthy volunteers examined by a custom NanoString chip. MPP-α mRNA counts were normalized with respect to endogenous B2-microglobulin mRNA counts.
4.2 Impact of genetic inhibition of MPP-α on viability of AML cell lines

We also examined the impact of knocking down MPP-α on the growth and cell viability of AML cell lines. Five different shRNAs targeting various regions of MPP-α mRNA in OCI-AML2 were used and 2 shRNAs tested for MPP-α knockdown were evaluated in K562 and TEX leukemia cells. A single independent shRNA targeting ClpP mRNA was used as a positive control in all three cell lines. As previously shown, ClpP is overexpressed in TEX, K562, and OCI-AML2 and knockdown of ClpP is cytotoxic to these cell lines. Target knockdown was confirmed by q-RTPCR and immunoblotting at day 4 and 7 post infection (Fig 6, 7, and 8). Target knockdown was quantified by densitometry using ImageJ software for all cell lines using β-actin as a loading control (Table 1). In OCI-AML2, knockdown was demonstrated to be >95% and >94% for clones 309, 366, 1295, and 1413 on Days 4 and 7 respectively (Table 1). Clone 174 had 77.9% and 71.9% knockdown on Days 4 and 7 respectively in OCI-AML2 (Table 1). The q-RTPCR data also demonstrates similar levels of mRNA knockdown on Day 3 (Figure 6). The knockdown of MPP-α reduced the growth and cell viability of OCI-AML2 and is consistent with all five shRNA clones (Figure 6). We also evaluated effects of MPP-α knockdown in K562 and TEX cells using shRNA clones 174 and 1295. In K562, clones 174 and 1295 had knockdown levels of 41.6% and 81.6% on Day 4 and 10.3% and 33.1% on Day 7. While in TEX cells, 174 and 1295 were demonstrated to have knockdown levels of 69.8% and 63.7% on Day 4 and 74.4% and 88.6% on Day 7 (Table 1). However, K562 and TEX cells are only weakly sensitive towards MPP-α knockdown (Figure 7 and 8) since MPP-α knockdown has a lower effect on growth and viability of these cell lines. The ClpP shRNA (knockdown not shown) is still cytotoxic to all three cell lines.
Figure 6 – OCI-AML2 MPP-α genetic knockdown. Impact of MPP-α shRNA knockdown on OCI-AML2 cell viability and proliferation (6a) over a 5 day period counted by Trypan blue exclusion staining. Knockdown of MPP-α mRNA was confirmed using qRT-PCR (18s mRNA endogenous control) on Day 3 (6b) and immunoblotting (β-actin loading control) with a rabbit anti-PMPCA antibody on Days 4 and 7 (6c). Data are represented as means (n=3) with standard deviation.
**Figure 7 – MPP-α genetic knockdown in K562 cells.** Impact of MPP-α shRNA knockdown on K562 cell viability and proliferation (7a) over a 5 day period counted by Trypan blue exclusion staining. Knockdown of MPP-α mRNA was confirmed using immunoblotting (β-actin loading control) with a rabbit anti-PMPCA antibody on Days 4 and 7 (7b). Data are represented as means (n=3) with standard deviation.
**Figure 8 – MPP-α genetic knockdown in TEX cells.** Impact of MPP-α genetic knockdown on TEX leukemia cell viability and proliferation over a 5 day period (8a) counted by Trypan blue exclusion staining. Knockdown of MPP-α mRNA was confirmed using immunoblotting (β-actin loading control) with a rabbit anti-PMPCA antibody on Days 4 and 7 (8b). Data are represented as means (n=3) with standard deviation.
Table 1: Quantification of MPP-α knockdown in AML cell lines

OCI-AML2

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4.3 Rescue of cell viability in OCI-AML2 upon MPP-α genetic knockdown

To confirm whether the observed reduction in cell viability and proliferation were on-target effects of the shRNAs targeting MPP-α, we attempted a rescue experiment using MPP-α mouse cDNA in OCI-AML2, the most susceptible cell line to MPP-α knockdown. The mouse MPP-α cDNA was inserted into a lentiviral overexpression construct (pLX304) which had a V5 tag at the C-terminus. The mouse cDNA has 86% mRNA and 91% protein sequence identity compared to its human counterpart. Mouse cDNA is resistant to two human shRNA clones - 174 and 1295. As a transduction control for the overexpression construct, a Renilla luciferase cDNA was also created. OCI-AML2 cells were then transduced with mouse MPP-α or Renilla luciferase cDNA separately. Next, we tested whether overexpression of mouse MPP-α could rescue the shRNA effects on growth and cell viability. Knockdown of human MPP-α was carried out with two human shRNA clones 174 and 1295. We confirmed the overexpression of mouse MPP-α by q-RTPCR (Figure 9a). Overexpression of mouse MPP-α did not rescue the effects of MPP-α shRNA. MPP-α shRNAs continued to reduce growth and viability of OCI-AML2 similar to control (b). Moreover, immunoblots for anti-V5 revealed no bands for either Renilla luciferase (40 kDa) or mouse MPP-α (55 kDa) and failing to detect the protein (Figure 10). Hence, we could not confirm overexpression of MPP-α mouse protein or determine whether MPP-α mouse protein is active in human cells. Thus, the on-target specificity of MPP-α shRNA remains to be determined.
Figure 9 – Rescue of OCI-AML2 cell viability and proliferation. Expression of mouse MPP-α mRNA was confirmed by qRT-PCR on Day 3 (9a). MPP-α knockdown rescue in OCI-AML2 cells with overexpression of mouse MPP-α cDNA as counted by trypan blue exclusion staining (9b). Concomitantly, Renilla Luciferase was overexpressed in a separate population OCI-AML2 cells to serve as a negative control. Data are presented as means (n=3) with standard deviation.
Figure 10 – Anti-V5 immunoblot of transduced OCI-AML2 overexpressing mouse MPP-α and Renilla luciferase (A). Lysates were collected on Day 3 post puromycin selection. V5 lysate from HEK293 cells was used as a positive control (65 kDa). Equal protein loading was confirmed with staining PVDF membrane with amido black (B).
Chapter 5
Discussion

5.1 Expression of MPP-α in AML cells and human tissues

As shown, MPP-α is overexpressed in a subset of primary AML bulk and stem/progenitor cells. Previous studies have already revealed that a subset of AML patients have increased mitochondrial biogenesis.\textsuperscript{100,101} Hence, it may be possible that mitochondrial import is upregulated to keep up with the increased mitochondrial biogenesis. Therefore as a next step, it would be useful to examine mitochondrial mass in the same patients to confirm their dysregulated mitochondrial biogenesis status and determine if expression relates to patient characteristics. The need for greater rate of import correlates with the expression of MPP-α in some mitochondrial dependent tissues such as skeletal muscle and brain. Furthermore, this effect may not be specific to MPP and it remains to be identified whether other regulators of mitochondrial import are also overexpressed such as components of the TOM and TIM23 complexes. Takahashi et al. (1998) have shown that TOM20 expression levels increase in response to higher skeletal muscle contractile activity over 7 day period.\textsuperscript{102} Thus, examining expression of other import pathway components and mitochondrial mass would support the need for greater mitochondrial import in the subset of these AML patients.

Examining expression of MPP-β in these same samples would complement current expression results. Although knockout models of either subunit of MPP have not been reported in literature, the Mouse Genome Informatics database reports that MPP-β knockout in homozygous mice are embryonically lethal at Day 8 of development. This may suggest a very
narrow therapeutic window. However, while MPP subunits are essential for coordinating embryonic development, their role in adult tissues remains to be examined, and is potentially less important when inhibiting in adult tissues. The embryonic lethality at 8 dpc indicates failure to form somites, sections of mesoderm aligned along the anterior-posterior axis to give rise to skeletal muscle, cartilage, tendons, endothelial cells, and dermis. Development of hematopoietic progenitors initiates in the visceral yolk sac at 8 dpc but do not enter circulation occurs until 8.5 – 9.5 dpc which possibly indicates the requirement of MPP-β during embryonic hematopoiesis. In addition, transient inhibition of MPP subunits may be less toxic. To further explore the effect of transient knockdown, one may use previously developed siRNA-containing stabilized nucleic acid lipid nanoparticles (SNALPs) to transfet suspension primary blood and leukemia cells in vivo to transiently knockdown target mRNA specifically in hematopoietic tissues. An alternative to the mouse knockout could be the use of tissue specific conditional knockout of MPP subunits in mice using Cre-Lox recombination (with tissue specific promoter) in order to examine its impact in adult hematopoietic tissues.

A limitation of the expression data is the small sample size of AML patients examined and single replicate performed. To validate and further explore the clinical implications as a next step, one may use high-throughput techniques such as reverse phase protein arrays to simultaneously validate overexpression of MPP-α protein in primary AML samples and CD34+ sorted normal HSCs as well as examine a significantly higher number of samples within each group to generate clinically useful data.
5.2 Differential sensitivity of AML cell lines towards MPP-α genetic knockdown

We assessed the effect of knocking down MPP-α on the growth and viability of OCI-AML2, K562 and TEX cells. OCI-AML2 cells were more sensitive to MPP-α knockdown compared to K562 and TEX. We propose a few explanations for the difference in sensitivity between these cell lines. First, differences in the level of target knockdown where OCI-AML2 had >95% target knockdown while K562 and TEX cells had 40-80% and 60-70% target knockdown respectively as quantified by densitometry. However, a potential downfall of the densitometry analysis is that signal strength of the bands being out of linear range (thus skewing calculated knockdown levels) and single replicate performed for each immunoblot. As such a greater level of knockdown in K562 and TEX cells may be needed to observe impacts on cell viability and proliferation similar to OCI-AML2. To achieve this, one may need to increase the multiplicity of infection in K562 and TEX cells by using a higher titer of virus cocktail so as to optimally increase the abundance of shRNAs needed to knockdown MPP-α at high levels. Another alternative to achieve higher knockdown efficiencies is the use of CRISPR interference. CRISPR (clustered regularly interspaced short palindromic repeats) and associated Cas family of genes are prokaryotic in origin and have been repurposed for targeted knockdown in mammalian cells by disrupting transcription initiation and elongation of target gene. Briefly, CRISPRi requires coexpression of mutant Cas9 and subgenomic RNA (sgRNA) that contains Cas9 bound hairpin loop and base pairing region (~20 nucleotides) complementary to target gene sequence. Following formation of protein-RNA complex assembly, the sgRNA base pairing region binds to target sequence and can result in either a Cas9 mediated double strand break or hindering elongation by RNA polymerase. Another explanation accounting for the differential sensitivity could be due to differential rates of protein import within these cell lines.
Due to lower target knockdown levels in K562 and TEX cells, the residual MPP-α remaining in the matrix may be sufficient to mediate basal rates of mitochondrial protein processing in these cell lines.

Knockdown of MPP-α reduced growth and cell viability, ultimately leading to cell death. However, the mechanism by which MPP-α knockdown reduced growth and cell viability is unknown. Potentially, reducing MPP-α activity impairs the ability of MPP-β to cleave matrix presequences and leads to increases levels of immature uncleaved proteins in the mitochondrial matrix. The accumulation of immature precursors in the matrix could lead to dysfunctional mitochondrial that could elicit the mitochondrial unfolded protein response (UPR\textsuperscript{mt}) to restore function. A key regulator of UPR\textsuperscript{mt} is the transcription factor ATFS-1 that contains both a nuclear and mitochondrial localization sequence.\textsuperscript{112} Under basal conditions, ATFS-1 is imported into the mitochondria through TOM/TIM complexes but is proteolytically inactive following processing by Lon protease.\textsuperscript{113,114} During UPR\textsuperscript{mt}, ATFS-1 import into the mitochondrial is inhibited due to alteration in mitochondrial membrane potential and remains in nucleus to activate expression of mitochondrial chaperones.\textsuperscript{115,116} Hence, measuring the changes in mitochondrial membrane potential upon decreased MPP activity using potential dependent dye such as JC-1 optimized for flow cytometry can serve as an indicator of mitochondrial health. Another mechanism to ameliorate dysfunctional mitochondria is targeted degradation by mitophagy mediated by the PINK1/PARKIN system. PINK1 is serine/threonine kinase localized on inner mitochondrial membrane and due to alteration in mitochondrial membrane potential under mitochondrial stress, PINK1 is translocated to the outer mitochondrial membrane where it interacts with PARKIN to be targeted for mitophagy.\textsuperscript{117,118} Interestingly, Greene et al. (2012) have demonstrated that activity of MPP-β is essential to import and process PINK1 and under
MPP-β knockdown, mitophagy is induced.\textsuperscript{119} Cell death could arise as a result of increased mitophagy and dysfunctional mitochondria upon MPP-β knockdown.

To directly test the effect of MPP-α knockdown on mitochondrial respiration, one may assess basal oxygen consumption rate using the Seahorse XF96 analyzer by measuring levels of O\textsubscript{2} in extracellular media and the extracellular acidification rate (based on H\textsuperscript{+} levels in extracellular media) to determine glycolytic compensation. Concomitantly, mitochondrial enzymatic assays such as citrate synthase and complex II, or succinate dehydrogenase, activity assays can be formed to characterize effect of uncleaved presequences on nuclear encoded genes involved in mitochondrial respiration.

Additionally, one may also examine the expression of mature and immature forms of MPP substrates such as N-acetylglutamate synthase and N-acetyl-glutamyl-phosphate reductase upon MPP-α knockdown to identify impact on import and processing of precursors. Activity assays of MPP substrates, such as ClpP activity assay, may also be performed to mechanistically probe the effect of impaired MPP activity.

Albeit the activity of MPP-β and MPP complex as a whole are only indirectly disrupted upon knockdown of MPP-α, genetic knockdown of MPP-β will have to be performed to validate these results. No specific inhibitor of MPP-β have yet been developed and designing inhibitors specific to MPP-β could serve as the next step in further developing MPP-β as a therapeutic target. Intriguingly, Mukhopadhyay et al. (2002) have recently shown inhibition of MPP activity by multiple HIV-1 protease inhibitors including indinavir, amprenavir, ritonavir, and saquinavir.\textsuperscript{120} Although not mechanistically proven in their inhibitory effects, these may serve as
drugs repurposed for targeting MPP and/or serve as lead compounds in developing specific inhibitors.

5.3 Failure to rescue cell viability in OCI-AML2

As evident in Figure 7, overexpression of mouse MPP-α failed to rescue OCI-AML2 cell viability upon knockdown of human MPP-α. Several reasons can account for this result. First, mouse cDNA may not be translated. Differences in 5’UTR and 3’UTR could account for its failure to be translated in OCI-AML2. Anti-V5 immunoblots seem to indicate failure to translate the mRNA as no bands were detected for mouse MPP-α (55 kDa). However, the same blots revealed no bands for Renilla luciferase suggesting more fundamental problems with this technique. Although the immunogenic sequence for human anti-MPP-α antibody is 90% identical to the mouse, using the human anti-MPP-α antibody to detect expression of mouse MPP-α could serve as a first step. Alternatively, using a mouse anti-MPP-α antibody could further confirm presence of protein in transduced cells.

If mouse MPP-α protein is expressed in transduced OCI-AML2, another possibility accounting for the failure to rescue cell viability is the inability to import the mouse MPP-α subunit. Residual MPP subunits in matrix coordinate import of its own subunits and differences in presequences of mouse and human could account for the inability of mouse MPP-α to be imported and thereby leading to accumulation in the cytosol and targeted for degradation. Even if mouse MPP-α is imported into the matrix, it is possible that mouse MPP-α is unable to dimerize with human MPP-β, thereby failing to form a functional complex. Finally, the effects observed in OCI-AML2 may possibly be due to the off-target nature of the shRNAs.
Future directions to troubleshoot the rescue experiment can include the following – using a different construct (with a different tag), rescuing cell viability in different cell lines, and performing rescue using human cDNA with site directed mutagenesis at sites specifically targeted by the shRNAs.
In summary, our results show that targeting MPP remains a potential therapeutic option for AML. MPP-α is overexpressed in AML cell lines and a subset of AML patient bulk (CD34+38+) and stem/progenitor (CD34+38-) cells. MPP-α is abundant in mitochondrial dependent tissues including the brain and skeletal muscle while relatively low in lung, spleen, trachea, and small intestine. Knockdown of MPP-α using shRNAs reduced the growth and viability of OCI-AML2 cells. Future work should focus on validating MPP as a therapeutic target in AML. Specifically, the following experiments are proposed for future work –

1) Expression of MPP-α and MPP-β by reverse phase protein array
2) Examine MPP-α and MPP-β expression in bulk and leukemia stem cells data sets
3) Validate on-target effect of MPP-α shRNAs in OCI-AML2
4) Use CRISPRi to achieve greater level of knockdown in K562 and TEX cells
5) Generate a conditional mouse knockout models of MPP-α and MPP-β using the Cre-Lox recombination to knockout specifically in hematopoietic tissues
6) Mechanistically probe the effect of decreased MPP activity on mitophagy and UPR\textsuperscript{mt}
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