Mnrr1: Understanding The Role Of A Novel Mitochondrial-Nuclear Regulator

Stephanie L. Gladyck
Wayne State University

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MNRR1: UNDERSTANDING THE ROLE OF A NOVEL MITOCHONDRIAL-NUCLEAR REGULATOR

by

STEPHANIE LYNN GLADYCK

DISSERTATION

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MAJOR: MOLECULAR GENETICS AND GENOMICS

Approved By:

________________________________________  Advisor

________________________________________  Date

________________________________________

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DEDICATION

For my parents, who have supported me through everything I’ve ever set my mind to accomplish, Eric Julien for being a supportive and loving partner, my family and friends for dealing with my absences and remaining steadfast, and the late James B. Cumming, for giving me a job when I needed one and threatening to fire me if I did not “get my butt back in school.”
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CHCHD</td>
<td>Coiled-coil-helix-coiled-helix domain</td>
</tr>
<tr>
<td>COX</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CytC</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DA</td>
<td>Dopaminergic neurons</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<td>EV</td>
<td>Empty vector</td>
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<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
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<tr>
<td>IMS</td>
<td>Intermembrane space</td>
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<tr>
<td>LONP1</td>
<td>Lon protease homolog 1</td>
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<td>MAM</td>
<td>Mitochondrial associated membrane</td>
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<td>MNRR1</td>
<td>Mitochondrial nuclear retrograde regulator 1</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>MTS</td>
<td>Mitochondrial targeting sequence</td>
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<tr>
<td>mtUPR</td>
<td>Mitochondrial unfolded protein response</td>
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<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
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<tr>
<td>ORE</td>
<td>Oxygen responsive element</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Ru-cytC</td>
<td>Ruthenium labeled cytochrome c</td>
</tr>
<tr>
<td>T61I</td>
<td>MNRR1 PD-associated mutation T61I</td>
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WT       Wild type
Y99E     Phosphomimetic MNRR1
Y99F     Unphosphorylated MNRR1
YME1L1   ATP-dependent zinc metalloprotease YME1L1
CHAPTER 1: INTRODUCTION

1.1 The Mitochondrion

Mitochondria are thread-like in appearance and contain an outer (OMM) and inner (IMM) mitochondrial membrane (Figure 1). The double membranes form compartments within the organelle structure: the intermembrane space (IMS) located between the membranes and the matrix, sequestered by the IMM. The IMM is responsible for the formation of the mitochondrial cristae, which are folds within the membrane, one of the hallmarks of mitochondrial morphology. They are dynamic organelles, which can combine to exchange contents and the separate through processes called fusion and fission (1). Mitochondria perform the critical function of aerobic respiration, generating most of the energy required to sustain life. As studies of the mitochondria progressed through out time, it was shown that they are also key regulators of the apoptosis, reactive oxygen species production, metabolic signaling, and calcium homeostasis (2).

Figure 1. Mitochondrial structure and landmarks. The mitochondrion organelle consists of two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). The IMM has a unique combination of lipids, including cardiolipin, that fold into cristae where the electron transport chain and MICOS complexes are located. The matrix (lumen) houses the mitochondrial genome, protein translational machinery, and metabolic pathways such as the tricarboxylic acid (TCA) cycle. This figure was obtained from Bornstein et. al., 2020 (3) with permission of the publisher (Elsevier).
Mitochondria are different from other cellular organelles, as they contain their own genetic material (mtDNA). The 16,569 bp genome was completely sequenced in 1987, revealing that mtDNA contains genes for the 12s and 16s ribosomal subunits, 22 tRNAs, 13 of the electron transport chain subunits for complexes III, IV and V (4), and more recently small peptides (5) and RNAs (6). All genes contained within the genome are critical for mitochondrial function and mutations or deletions have deleterious effects on cellular function which results in disease and aging pathologies (7, 8).

It was first proposed in 1967 that the mitochondrion may originate from an endosymbiotic relationship between bacterial and eukaryotic cells (9). Although very controversial at the time, this theory is now widely accepted by the scientific community, as subsequent analysis showed that the mitochondrial proteome and genome more closely resembles that of a prokaryotic lineage (10-12). In fact, with the development of high throughput technologies, the lineages of both the host eukaryote and the endosymbiotic proteobacterium were traced. These phylogenomic studies revealed that the host eukaryote is closely related to Asgard superphylum (archaea), specifically Lokiarchaeota (10, 11).

The debate in the scientific community is no longer if mitochondria originated from an ancestral endosymbiotic relationship but focuses on the timing of the event. The hypotheses within the field are divided into two extremes: “mitochondria-early” and “mitochondria-late.” Mitochondria-early postulations center around the idea that the endosymbiotic relationship is one of the first events, if not the event, leading to the evolution of modern eukaryotes (13). Mitochondria-late hypotheses suggest that the endosymbiotic relationship did not occur until ancestral cells began displaying eukaryotic characteristics (14-17). In either case, the fact remains that the endosymbiotic relationship between prokaryotes and pre-modern eukaryotes allowed modern eukaryotes to harness the capability of increased ATP production through aerobic respiration.
1.2 Mitochondrial Function

1.2.1 Oxidative phosphorylation

Mitochondria are well known as “the powerhouse of the cell”, a phrase that was coined by Peter Siekevitz in the 1950s. The organelles earned this nickname because they produce most of the energy required for cellular functions through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) (18). The process of OXPHOS occurs at the IMM via a series of four multi-subunit protein complexes called the electron transport chain (ETC) and ATP synthase (Figure 2). Subunits of these complexes are encoded by both the nuclear and the mitochondrial genomes.

![Figure 2. The electron transport chain and ATP synthase.](image)

The electron transport chain and ATP synthase are imbedded in the IMM. Substrates are fed from the TCA cycle to complexes I and II as electron donors. Electrons are transferred via the Q cycle to complex III and are then shuttled to complex IV via cytochrome c. Complex IV uses the electrons to ultimately convert oxygen to water. Hydrogen ions are pumped from the matrix into the IMS as the electrons move down the chain, creating a proton gradient that is utilized by ATP synthase to convert ADP to ATP. This figure was obtained from Nolfi-Donegan et. al., 2020 (19) with the permission of the publisher (Elsevier).

Substrates generated by the TCA cycle, NADH and FADH$_2$, donate a pair of electrons to complexes I (NADH dehydrogenase) and II (succinate dehydrogenase), respectively. The electron pair then enter the Q-cycle, where ubiquinone (CoQ) is reduced to ubiquinol (CoQH$_2$) which shuttles electrons to complex III (cytochrome c reductase or $bc_1$ complex). Ubiquinol is oxidized by complex III and one electron at a time is transferred from complex III by cytochrome
c (cytc). The reduced cytc shuttles an electron to complex IV (cytochrome c oxidase or COX), where molecular oxygen binds and is reduced to water.

As the electrons travel down the ETC, complexes I, III, and IV pump protons from the matrix across the IMM, allowing COX to reduce molecular oxygen to water while establishing an electrochemical proton gradient in the IMS, which generates a mitochondrial membrane potential (ΔΨ) (20). The ΔΨ, along with the proton concentration (pH), creates the proton motive force (Δp) that couples electron transfer/oxygen consumption and the activity of ATP synthase (19). ATP synthase moves protons from the IMS back to the matrix during the process of converting ADP to ATP, which cause ΔΨ to dissipate. In situations where proton leak across the IMM occurs, respiration becomes uncoupled, and uncoupling proteins shuttle protons back to the matrix, in an attempt to control ATP production (19).

1.2.2 Cellular metabolism, signaling, and mitophagy

In addition to the production of life sustaining amounts of cellular energy, mitochondria have been shown to participate in the synthesis of numerous biomolecules such as proteins, fatty acids, heme, iron-sulfur clusters, lipids, nucleotides, and amino acids via numerous pathways (Figure 3) (21). They also play key roles in the production of reactive oxygen species (ROS) and ROS signaling, calcium buffering and signaling, apoptosis, and mitophagy.
Figure 3. Metabolic pathways in the mitochondria. Yellow arrows indicate critical biosynthetic pathways, red arrows indicate redox-sensitive enzymatic reactions. This figure was obtained from Ahn et., al., 2015 (21) with permission from the publisher (Springer Nature).

The electron transport chain is a point of ROS generation in the cell, which is usually caused by electron leakage at complexes I and III (20). Free electrons can interact with oxygen, creating the free radicals known as superoxide, hydroxyl radical, singlet oxygen, and peroxide, which are highly reactive molecules (22-24). Under normal conditions a homeostatic amount of ROS is required for physiological signaling; however, too much ROS causes damage to proteins, lipids, and nucleic acids. An accumulation of ROS occurs when the ROS scavenging system is overwhelmed or dysfunctional resulting in disease pathologies including cancers, neurological diseases, diabetes, and organ failure (25).

Mitochondria also play a role in cytoplasmic calcium homeostasis. Mitochondria have two pathways for calcium uptake: The mitochondrial calcium uniporter (MCU) and cation transport and exchange channels (26, 27). Cytosolic calcium plays a role in apoptosis by inducing the release of cytc into the cytosol via the formation of the mitochondrial permeability transition pore through cristae remodeling (28, 29). Additionally, mitochondria can regulate calcium signaling through their close interaction with the endoplasmic reticulum. Mitochondria-associated membranes (MAMs) play numerous roles in metabolism and transport including cholesterol and
ceramide biosynthesis, phospholipids, proteins, ions, and many metabolites (30). The exchange of calcium between the ER and mitochondria affects OXPHOS and autophagy pathways as well. MAM dysfunction has been associated with various diseases including cancers and neurodegenerative diseases (31).

The mitochondrial membrane is made up of multiple lipids produced by the mitochondria itself or imported from the ER (32). As mentioned above, it has been shown that the close interaction with the ER at the MAMs allows for mitochondria and the ER to exchange lipids and thereby regulate lipid homeostasis (33). Dysregulation of mitochondrial lipid homeostasis results in stress signaling pathway activation of the mitochondrial and cytosolic unfolded protein responses (UPRs), discussed in detail in the next section (34).

Metabolic and stress signaling through the mitochondria is paramount in cellular homeostasis; however, there is a point at which mitochondrial damage and dysfunction is too great to repair. When mitochondria are damaged beyond repair, they are cleared via a mito-centric form of autophagy (the process of removing protein aggregates, organelles, and other contents of the cytoplasm), appropriately called mitophagy (35). During mitophagy, mitochondria are targeted by the autophagosome through a specific set of proteins activated by stresses such as an increase in ROS levels and disrupted membrane potential (36). One pathway by which mitophagy can be induced is ubiquitin mediated, involving the ubiquitination of mitochondria (37).

The classical signaling pathway for ubiquitin mediated mitophagy is the PINK1/Parkin pathway. Under normal physiological conditions, PINK1, a mitochondrial serine/threonine kinase, is continuously imported and cleaved by the matrix protein peptidases (MPP) and subsequently cleaved by presenilin-associated rhomboid like (PARL), a protease in the mitochondrial inner membrane. (38, 39). Upon mito-specific stress activation, PINK1 blocks mitochondrial import by accumulating on the OMM, which leads to autophosphorylation through dimerization of the protein (40-42). PINK1 then activates Parkin (an E3 ubiquitin ligase) through phosphorylation, which subsequently leads to OMM protein and cytosolic protein ubiquitination, fission, and recruitment.
of the autophagosome (42, 43). Defective mitophagy can be seen with aging, as well as cancers, heart and liver diseases, and neurodegenerative disorders (44).

1.2.2.1 Mitochondrial unfolded protein response

The mitochondrial unfolded protein response (mtUPR) is a stress signaling pathway that has recently emerged as important in protein homeostasis of the mitochondria (45, 46). The purpose of this pathway is to activate transcription of protein folding chaperones (heatshock proteins), proteases (YME1L1, LONP1, and CLPP), and other stress response proteins in the nucleus to protect the mitochondria from proteotoxic stress (Figure 4) (46, 47). Numerous stresses can trigger the mtUPR in addition to protein misfolding, including OXPHOS dysfunction and ROS, membrane depolarization, mtDNA damage, and immune response activation (48). The pathway has been well characterized in Caenorhabditis elegans (49, 50) and is now an emerging field in mammalian mitochondrial biology (46) and disease associations (51).

Figure 4. Mitochondrial matrix and IMS UPR overview. This figure was obtained from Arnould et. al., 2015 (52) with permission from the publisher (MDPI).
A main regulatory pathway of protein homeostasis is through the ubiquitin-proteasome system in the cytosol, both by degrading proteins on the OMM and by degrading proteins stuck in the cytosol due to slowed or blocked import machinery (53-56). An additional pathway has been identified to manage proteotoxic stress within the mitochondrion itself. In C. elegans, a transcription factor called ATFS-1 has been shown to be a key player in mtUPR. Under normal conditions, ATFS-1 is imported into the mitochondria; however, during stress, a portion of ATFS-1 is trafficked to the nucleus, where it activates transcription of genes involved in the mtUPR (50). In mammals, a transcription factor, ATF5, performs a similar role in mitochondrial-nuclear crosstalk and can rescue mtUPR in ATFS-1 deficient worms (57). ATF5 was recently shown to be transcriptionally activated by MNRR1 (58). More recently, compartment specific mtUPRs have emerged as important pathways in regulating proteotoxic stress within the mitochondria (Figure 4) (52).

1.2.2.2 Apoptosis

Apoptosis is the result of a cell’s inability to overcome stress or damage while attempting to repair itself after an insult. There are two pathways by which cell fate can be committed to death: extrinsic apoptosis (relies on cell surface receptors) and intrinsic apoptosis (relies on the involvement of mitochondria), although it is possible for the extrinsic pathway to trigger the intrinsic pathway (59, 60). The first step in intrinsic apoptosis is the formation of mitochondrial outer membrane pores (MOMP) and the release of unphosphorylated cyt c into the cytosol, committing cell fate to death (Figure 5) (61). The interaction between cyt c and Apaf-1 forms the apoptosome, which in turn activates the caspase cascade (62). Caspase proteins, which are cysteine proteases, become active upon cleavage downstream of apoptosome formation, which begin to degrade cellular substrates, thereby playing the role of cell executioner (63).

The Bcl-2 family of proteins play a key role in regulating the first step of intrinsic apoptosis at the mitochondrial outer membrane. The Bcl-2 family of proteins is divided into two sections based on function: anti-apoptosis proteins (such as Bcl-xL) and pro-apoptosis proteins (Bax and
Bak) (64). Under normal conditions, Bax localizes to the cytosol and Bak is inserted into the OMM, where it interacts with the antiapoptotic protein Bcl-xL. At the induction of apoptosis, the interaction between Bcl-xL and Bak is interrupted by other proapoptotic proteins, allowing for the oligomerization of Bak and Bax at the OMM, an action which forms the MOMPs necessary for the release of cytc (60, 65, 66).

![Mitochondrial decisions in cell life and death](image)

**Figure 5. Mitochondrial decisions in cell life and death.** Under normal conditions, mitochondria actively respire by the process of OXPHOS. However, during stress, cytc is de-phosphorylated and Bcl-xL fails to prevent the oligomerization of Bak and Bax allowing the release of cytc from the mitochondrial IMS, committing cell fate to death. The apoptosome is formed through the interaction of Apaf-1 and dephosphorylated cytc and activates caspases. This figure was obtained from Kalpage et. al., 2020 (62) with the permission of the publisher (Elsevier).

### 1.3 Complex IV: Cytochrome c oxidase

Complex IV, or cytochrome c oxidase (COX), is one of the mitochondrial components that our lab studies. COX is the terminal enzyme of the ETC and is responsible for reducing oxygen to water. Physiologically, the mammalian complex is a dimer, with each monomer composed of
13 tightly bound subunits embedded in the IMM, an assembly supported by several crystal structures resolved from COX in bovine heart (Figure 6) (67, 68). However, more recently, monomeric crystal structures of COX were also published (69, 70) and monomeric COX was also reported in a supercomplex (71). It is therefore possible that an equilibrium exists between dimeric and monomeric COX, which could be subject to regulation. COX has been shown to be a tightly regulated complex via the presence of tissue-, developmental-, or species-specific subunit isoforms, small molecule interactions, reversible subunit phosphorylation, protein-protein interactions, and supercomplex formation (67, 72, 73).

Figure 6. Crystal structure of bovine heart cytochrome c oxidase at 2.8 Å. A. Transmembrane view of COX. B. View of COX from the intermembrane space. Each subunit and corresponding name are represented by its unique coloring within each COX monomer. This figure was obtained from Yoshikawa et. al., 2012 (74) with permission from the publisher (Elsevier).

Additionally, a 14th subunit has been proposed—NDUFA4—which was originally believed to be a subunit of complex I (75, 76). A structural study showed that NDUFA4 appears to be a subunit in the COX monomer, likely adding to the stability of the complex (76). NDUFA4 as part of the COX monomer is located at the interface of the dimeric complex, where it would prevent or interfere with dimer formation, which could be a reason that the protein was never detected in the dimeric crystal structure while being identified as part of monomeric COX structure (76). The validity of NDUFA4’s role as a true subunit has been questioned and it was argued that, because
NDUFA4 may bind to both complexes I and IV and is not consistently found in COX preparations, it may function as an assembly factor for the respirasome (77).

The three largest subunits are encoded by the mitochondrial genome whereas the other subunits are encoded by the nuclear genome. Among the mitochondrial-encoded subunits, subunits I and II contain the catalytic centers. The latter consist of metal centers that are involved in the electron acceptance from complex III via cytc and the pathway of the electron through the complex itself: electrons received from cytc first reach the CuA center in subunit II, are then transferred to heme a in subunit I, and finally reach the heme a3-CuB site of subunit I, where oxygen is reduced to water.

The biogenesis and maturation of COX is critical for its proper function. There are multiple steps in this tightly regulated process: the insertion of metal groups in COX I and COX II, the import and folding of nuclear encoded subunits, and the proper assembly of the subunits into the complex. Over 30 auxiliary proteins are involved in the biogenesis of the core enzyme composed of COX I, COX II, and COX III (78). The hypothesized assembly pathway favors a modular–linear assembly, where subunits are first assembled into module intermediates and then these modules are assembled into the COX monomer (Figure 7) (79, 80). The first step of monomer assembly is the synthesis of mitochondrially encoded COX I, including the insertion of heme a, which is followed by its association with the COX IV and COX Va module (79, 81). The COX II module, which requires the insertion of the CuA center into the subunit before assembly can continue (82-85), forms a complex intermediate with COX VIc, COX VIIb, COX VIIc, and COX VIIa. The COX III module consists of COX III, COX VIa, COX Vlb, and COX VIIa. These modules are then assembled in a linear fashion, upon which NDUFA4 interacts to assist in the stabilization of the COX monomer (79).

Over the years, many diseases have been studied that are associated with defective COX biogenesis, which often affect the brain, heart, and skeletal muscle (86-88). Most of these diseases involve assembly factors, chaperones, and other regulatory proteins because COX
biogenesis and activity are tightly regulated processes (89). Mutations in SCO1/SCO2 (83, 90-93), COX10 (94, 95), COX15 (96), and SURF1 (82, 92, 97, 98) have all been associated with COX dysfunction. Studies have also shown that mutations in members of the twin CX9C family, of which our lab also studies, can affect COX activity and are associated with various diseases including neurological disorders, cancers, and cardiomyopathy (see next section).

**Figure 7. Assembly of cytochrome c oxidase.** The assembly of COX is thought to be both modular and linear. Dark blue, mitochondrial encoded COX core subunits; light blue, nuclear encoded COX subunit; purple, assembly factors; gray, unknown function. *, Twin CX9C protein. Gray dashed lines represent assembly factor interactions. Black dashed lines represent subunit assembly. This figure was obtained from Gladyck et. al., 2021 (99) with the permission of the publisher (MDPI).

1.4 Twin CX9C Proteins

1.4.1 Unique structure for non-classical mitochondrial import

The twin CX9C family of proteins is characterized by its unique repeated motif of two cysteines separated by nine amino acid residues. This motif is found in the coiled-coil-helix-coiled-coil-helix (CHCH) domain, where pairs of cysteines form a helix turn helix fold by forming disulfide bonds with one another (Figure 8) (100-102). This domain provides structural stability for the twin alpha helices but also makes the proteins redox sensitive due to the reactive nature of cysteine residues (101-104). Another family of proteins, called the “small Tim” proteins, contain a similar
but shorter twin CX₃C motif, and act as chaperones in the TIM22 pathway for insertion of proteins into the IMS-facing side of the inner membrane (IM), an important role in mitochondrial function (105).

The CHCH domain is important for the import of the proteins into the intermembrane space (IMS) of the mitochondria. IMS import is facilitated through the Mia40/CHCHD4 redox mechanism based on cysteine residues (106-109). Unlike matrix or inner-membrane-bound proteins, proteins that use the Mia40 pathway do not require a mitochondrial-targeting sequence (MTS) precursor. Import via Mia40 works through a disulfide relay system wherein Mia40 is anchored to the inner mitochondrial membrane, facing into the IMS. In this system, CX₃C proteins are brought into the IMS from the cytosol via the translocase of the outer membrane (TOM) in a reduced, unfolded state. The oxidized cysteine residues of Mia40 then form disulfide bridges with the cysteine residues of the incoming twin CX₃C protein. After further modification to the disulfide bridges, the imported CX₃C protein is released into the IMS and Mia40 is re-oxidized by Erv1 (108).

It is interesting to note that some twin CX₃C proteins are predicted to have an MTS; these include CHCHD1, MNRR1/CHCHD2, and CHCHD10. Such observations suggest that these proteins can use the translocase of the inner membrane TIM/TOM import as an alternative route or that they may be able to also localize to the mitochondrial matrix (or the inner mitochondrial membrane). CHCHD1 has been identified as a mitochondrial ribosomal protein and therefore is able to use its MTS to localize to the mitochondrial matrix to participate in protein translation (110). A third possibility is that these pre-sequences are not functional since MNRR1/CHCHD2 has been shown to localize to the mitochondria even after the removal of its MTS (Aras and Grossman, unpublished data)(111).
1.4.2 The role of twin CX<sub>9</sub>C proteins in COX regulation

The first studies of this family of proteins took place in *Saccharomyces cerevisiae*, where a detailed study found that 13 of the 14 yeast family members were conserved across species (103). Subsequent research determined that family member proteins are important in COX complex assembly and function (Figure 6), as well as for direct regulation of the oxidase (Table 1) (112-115). Many of the protein family members are also associated with various diseases, although their mechanisms are not well understood in pathology (Table 2).

**Table 1. Human proteins, yeast nomenclature, and function of twin CX<sub>9</sub>C proteins (99).**

<table>
<thead>
<tr>
<th>Human Protein</th>
<th>Yeast Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX17</td>
<td>Cox17p</td>
<td>COX copper chaperone</td>
</tr>
<tr>
<td>COX19</td>
<td>Cox19p</td>
<td>COX assembly</td>
</tr>
<tr>
<td>CMC1</td>
<td>Cmc1p</td>
<td>COX assembly</td>
</tr>
<tr>
<td>CMC2</td>
<td>Cmc2p</td>
<td>COX assembly</td>
</tr>
<tr>
<td>COA5</td>
<td>Pet191p</td>
<td>COX assembly</td>
</tr>
<tr>
<td>COA6</td>
<td>Coa6p</td>
<td>COX assembly</td>
</tr>
<tr>
<td>CHCHD7</td>
<td>Cox23p</td>
<td>COX assembly</td>
</tr>
<tr>
<td>CHCHD8</td>
<td>Coa4p</td>
<td>COX/complex III assembly/function</td>
</tr>
<tr>
<td>MNRR1/CHCHD2</td>
<td>Mix17p</td>
<td>Activity regulation</td>
</tr>
<tr>
<td>CHCHD10</td>
<td>Mix17p</td>
<td>Activity regulation</td>
</tr>
<tr>
<td>CMC4</td>
<td>Cmc4p</td>
<td>Unknown</td>
</tr>
<tr>
<td>COX VIb1</td>
<td>Cox12p</td>
<td>Subunit</td>
</tr>
</tbody>
</table>
1.5 Mitochondrial Nuclear Retrograde Regulator 1 (MNRR1)

MNRR1 (also called CHCHD2) and its isoform CHCHD10 have a common ancestor in yeast called Mix17p (formerly Mic17p), which was initially shown to localize to the nucleus (143). However, as interest in IMS protein import increased, it was determined that Mix17p also localized to the IMS via the Mia40/Erv1 pathway (144). In a screen looking to determine which CX₃C proteins affect OXPHOS in yeast, the knockdown of Mix17p resulted in the reduction of oxygen consumption to approximately 50% of WT (103). The protein was later shown to be stress-sensitive using agents that induce DNA replication distress, a stimulus that also led to the characterization of changes in protein localization (145).
Mix17p and MNRR1 have 36% sequence identity, with a centrally located hydrophobic domain, which is largely conserved (Figure 9). This overlap suggests that the hydrophobic central domain plays an important role in the function of the protein. Additionally, the cysteines in the CHCH domain are conserved in the twin CX9C motif. MNRR1 and CHCHD10 have 56% sequence identity, which suggests they may perform similar functions in cells. As in yeast, mammalian MNRR1 was determined to affect OXPHOS in a computational screen coupled with functional assays in human cell lines (146). MNRR1 is a stress-sensitive protein and displays dual localization to the mitochondria and the nucleus (114, 147). During 20% oxygen tension growth in cell culture, most cellular MNRR1 is in the mitochondria; however, at more physiological 4% oxygen tension, MNRR1 levels rapidly turn over in the mitochondria and increase in the nucleus during stalled protein translation experiments (114). Our lab has recently shown that at 4% oxygen tension, import of MNRR1 in the mitochondria is blocked with accumulation of the protein in the nucleus at 8 hours (58). It is important to note that MNRR1 remains in the mitochondria during this time-point and a hypothesis will be presented in the results section as to why.

![Figure 9. MNRR1/CHCHD2, CHCHD10, and Mix17. Alignment of human MNRR1/CHCHD2 and CHCHD10 (D10) and yeast Mix17 protein sequences. Black boxes indicate conserved residues. Human sequence annotation is as follows: Blue box indicates the CHCH domain. Solid black lines indicate the twin CX9C motif. Dashed lines indicate the cysteine pairs that form structural disulfide bonds. The arrow indicates the Y99 residue discussed elsewhere. This figure was obtained from Gladyck et al., 2021 (99) with the permission of the publisher (MDPI).](image)

In the mitochondria, MNRR1 has been shown to interact with COX and regulate its activity (113, 114). MNRR1 knockdown in cells affected multiple mitochondrial processes including a ~50% reduction of oxygen consumption rate, an increase in reactive oxygen species (ROS), a reduction in mitochondrial membrane potential, slower growth (114), increased sensitivity to apoptosis (61), and fragmented mitochondria, which increasingly form during oxidative stress...
A large-scale protein–protein interaction study showed that MNRR1 interacts with two COX subunits on the IMS-facing side of the IM (COX Vlc and COX VIa1), as well as cytochrome c, further suggesting that MNRR1 is a regulator of COX activity (151). However, further work is needed as the interaction study, which was carried out with total cell lysate, also identified an interaction with wholly matrix-localized COX subunits yet MNRR1 has not been detected in the matrix (58). These identifications may not be physiological, as the matrix subunits would not interact with MNRR1 in the IMS.

The interaction between MNRR1 and COX is promoted when MNRR1 is phosphorylated at tyrosine residue 99 (Figure 9, arrow) by Abl2/Arg kinase (113). In order for this site-specific phosphorylation to take place, CHCHD10 must be present. It has been shown that MNRR1 and CHCHD10 are able to form homo- and hetero-dimers, potentially through the GXXXGXXG motif located near the center of the protein sequence in a conserved structural helix (152-154). The glycine residues are of critical importance in protein-protein interaction, as illustrated through residue mutational studies (155). Glycine has the smallest sidechain of the amino acids, and therefore it is likely that these residues provide a flat interaction surface, allowing proteins to interact closely with one another at this site (154). When Y99 is mutated to glutamate to mimic phosphorylation, an increase in oxygen consumption is detected compared to WT. MNRR1 has been shown to have another phosphorylation site, in the retained (non-cleaved due to IMS localization) putative mitochondrial targeting sequence of the protein.

In the nucleus, MNRR1 acts as a transcriptional activator by binding to RBPJκ at the highly conserved oxygen responsive element (ORE) in the COX4I2 promoter, stimulating the expression of the COX IV-2 subunit isoform, which is highly expressed in the lung, trachea, and carotid body (73, 114, 147, 156, 157). Expression of COX IV-2 is maximal at 4% oxygen tension (73, 114, 147, 156). When COX IV-2 is present in the complex isolated from lung, COX activity was twofold higher than the activity of COX isolated from liver with only COX IV-1 present (158). Genes that harbor the ORE include mitochondrial proteases, ETC complex components (including subunits),
and molecular chaperones, all of which are critical in maintaining mitochondrial homeostasis (99, 147). Based on this information, MNRR1 regulates COX activity and mitochondrial function with a bi-organellar approach: both through physical interaction with the complex in the mitochondria and through stress-induced transcription in the nucleus.

1.5.1 MNRR1 and Disease

Presently, alterations of MNRR1 have been associated with a handful of diseases, including Parkinson’s disease (123), Huntington’s disease (124), lissencephaly (a neuronal migration disorder) (125), Charcot-Marie Tooth disease (159), EGFR-positive non-small cell lung cancer (128), hepatitis B or C virus-associated hepatocellular carcinoma (129), breast cancer (126, 127) and very recently MELAS (58) (Table 2, Figure 10); Interestingly, CHCHD10 has also been associated with several neurodegenerative diseases (130, 131, 134, 135, 160, 161).

MNRR1 has been shown to regulate cell migration (162), suggesting that it could be important for metastasis in cancer and affecting migration in lissencephaly. Serine residue 41 has been shown to be phosphorylated in breast cancer and levels of MNRR1 are also increased with tumor grade, seemingly aiding the aggressiveness of breast cancer cells (126, 127).

A family with Charcot-Marie-Tooth disease type 1A has a Q112H mutation in MNRR1. This mutation results in defective respiration and an exaggerated phenotype. We have shown that the Q112H mutation in MNRR1 hinders its ability to interact with Abl2 kinase, resulting in defective tyrosine phosphorylation and therefore defective mitochondria (113). Many of the disease associated alterations of MNRR1 are mostly correlated with a change in protein levels, suggesting that the MNRR1 mediated mitochondria-nuclear crosstalk could be dysfunctional in these diseases. However, in most cases, the mechanistic involvement of MNRR1 in the disease is unknown.
Figure 10. The cellular functions and disease associations of MNRR1. Cellular functions are colored in orange, neuro-type disease are colored in dark green, and cancers are colored in light green.

1.6 Parkinson’s Disease

Parkinson’s Disease (PD) is a complex, multifactorial neurodegenerative disorder that affects more than 10 million people world-wide, according to the Parkinson’s Foundation, making it the second most common neurodegenerative disorder. PD patients generally present with movement symptoms that include resting tremor, rigidity, bradykinesia, and postural instability (163). Patients also have non-motor symptoms as well, including depression, cognitive impairment, autonomic nervous system dysfunction, sleep disturbance, pain, and gastrointestinal issues, all of which are likely due to widespread degeneration within the nervous system (164, 165). PD can either be sporadic (majority of PD cases) or familial (autosomal dominant).

On a molecular level, PD is caused by death of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) of the midbrain, which controls movement, and the presence of alpha-synuclein aggregates, called Lewy Bodies, in the brain tissue due to a
dysfunctional proteasomal pathway (166, 167). Excessive production in ROS, alterations to catecholamine metabolism, decreased inflammatory response markers, dysregulation metal cation homeostasis, overactive mtUPR, and impaired mitochondrial fission and fusion, and dysfunctional autophagy/mitophagy have also been associated with PD (168-171). Unsurprisingly, mitochondrial dysfunction has been shown in both early-onset familial and late-onset sporadic forms of PD (38, 164, 172). This is likely due to neuronal physiology, as these cell types require an increased number of mitochondria due to high energy demands, which make them sensitive to oxidative stress (173). Only a handful of genes have been identified as causing pathogenicity within the etiology of the heritable form of the disease (and sometimes the sporadic form) when they are altered (Table 3).

Table 3. Known genetic mutations in Parkinson’s Disease and effects on the mitochondria (172).

<table>
<thead>
<tr>
<th>Gene Name/Protein</th>
<th>Healthy Gene Function</th>
<th>Gene Dysfunction</th>
<th>Type of PD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SNCA/alpha-Synuclein</strong></td>
<td>May modulate synaptic transmissions (174) Can localize to the mitochondria (and MAM) and affect their function (175-178)</td>
<td>Forms aggregates called Lewy bodies (179) Induce mitochondrial fragmentation and increase ROS (180, 181)</td>
<td>Autosomal Dominant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lewy bodies</td>
<td>Sporadic</td>
</tr>
<tr>
<td><strong>LRRK2/Leucine repeat kinase 2</strong></td>
<td>Serine/threonine kinase Neuronal plasticity (182) Autophagy (183) Neuronal vesicle trafficking (184)</td>
<td>Most common cause of AD PD Mutations lead to increased kinase activity Affects mitochondrial fission and fusion (185) Causes protein leak and uncoupled respiration (186) Impairs mitophagy (187)</td>
<td>Autosomal Dominant</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Affects mitochondrial fission and fusion (188)</td>
<td>Sporadic</td>
</tr>
<tr>
<td><strong>VPS35/Vacuolar protein sorting-associated protein 35</strong></td>
<td>Mediates retrograde delivery of cargo from endosomes to Golgi (189) Recycles endosomal cargo to the cell surface Regulates mitochondrial fission and fusion (190, 191)</td>
<td>Impairs complex I assembly and activity (192) Impairs mitochondrial fission and fusion</td>
<td>Autosomal Dominant</td>
</tr>
<tr>
<td><strong>PRKN/E3 ubiquitin-protein ligase parkin</strong></td>
<td>E3 ubiquitin ligase involved in proteasomal degradation and cell signaling by targeting mitochondrial proteins (193) Mitochondrial biogenesis and mitophagy (38)</td>
<td>Most frequent cause of AR PD (194) Dysfunctional mitophagy and biogenesis pathway (195) Loss of mitochondrial mass and respiration (196)</td>
<td>Autosomal Recessive</td>
</tr>
<tr>
<td><strong>PINK1/Serine-threonine protein kinase pink1</strong></td>
<td>Serine/threonine kinase Key regulator of mitophagy Enhances fission in damaged mitochondria (197) Mitochondrial biogenesis (198)</td>
<td>Reduced mitophagy (43) Mitochondrial calcium overload (199) Reduction of complexes I and III (200)</td>
<td>Autosomal Recessive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sporadic</td>
</tr>
</tbody>
</table>
By the time patients are diagnosed with the disease, there is already substantial death of DA neurons, and the degeneration has spread to other parts of the central nervous system (165).

There is no cure for PD but there are treatments such as dopamine replacement to manage symptoms. This progressive disorder has a high socioeconomic burden to patients, as therapeutics are quite costly (especially for the uninsured), not to mention the on-going care required as those affected by the disease become less independent. Therefore, research into the mechanism of the disease and the expansion of therapeutic targets to halt or slow the progression of the disease is of incredible importance for patient care.

### 1.6.1 Parkinson's Disease associated mutations in MNRR1

Genome-wide association studies have identified rare mutations in MNRR1 that are associated with Parkinson's Disease and other neurodegenerative disorders (Figure 10). The mutations associated with neurodegeneration are interesting, as they span across the protein sequence (Figure 11) and differ based on ancestral ethnicity. Meaning, MNRR1 mutations associated with PD in Chinese and Japanese patients are not the same in Western European/Caucasian patients (Table 4). All individuals who possess PD-associated variants are heterozygous for the mutation, apart from one variant, A71P, which caused mitochondrial dysfunction in patient fibroblasts (208). It is also worth mentioning that not all ethnic populations
(or cohorts) studied to date contain rare MNRR1 variants (209-215), suggesting that PD-associated mutations in MNRR1 may exacerbate an already complicated and heterogenic disease.

The first rare MNRR1 PD-associated mutations were identified in a genome wide-linkage study in a Japanese family with autosomal dominant PD as well as the Comprehensive Genetic Study on Parkinson’s Disease and Related Disorders databank (123). Patients who were selected for the study met the neurological exam criteria to submit DNA samples to the databank and had no known PD-associated mutations. The novel variants T61I and R145Q were detected in 3 out of the 340 Japanese families with autosomal dominant PD, while not detected in the control population (n=559) or the sporadic PD population (n=517). The T61I mutation was also found in a Chinese pedigree study in autosomal dominant PD patients (216). The T61I mutation will be discussed further as it is of interest to this research.

Table 4. Rare mutations in MNRR1 that are associated with Parkinson’s Disease.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Genotype</th>
<th>Associated Disease</th>
<th>Ethnicity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro2Leu</td>
<td>+/-</td>
<td>Sporadic</td>
<td>Japanese/Chinese</td>
<td>(123, 216-219)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Early-onset</td>
<td>Chinese</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Risk factor</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg8His</td>
<td>+/-</td>
<td>Sporadic</td>
<td>Japanese</td>
<td>(220)</td>
</tr>
<tr>
<td>Ala32Thr</td>
<td>+/-</td>
<td>PD</td>
<td>Japanese</td>
<td>(221)</td>
</tr>
<tr>
<td>Pro34Leu</td>
<td>+/-</td>
<td>PD</td>
<td>Western European</td>
<td>(221)</td>
</tr>
<tr>
<td>Ala49Val</td>
<td>+/-</td>
<td>PD</td>
<td>Caucasian</td>
<td>(222)</td>
</tr>
<tr>
<td>Thr61Ile</td>
<td>+/-</td>
<td>Late-onset autosomal dominant</td>
<td>Japanese</td>
<td>(216, 223)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autosomal dominant</td>
<td>Chinese</td>
<td></td>
</tr>
<tr>
<td>Ala71Pro</td>
<td>+/-</td>
<td>Early onset PD with TOPIMT mutation</td>
<td>Caucasian</td>
<td>(208)</td>
</tr>
<tr>
<td>Ala79Ser</td>
<td>+/-</td>
<td>Sporadic</td>
<td>Chinese</td>
<td>(224)</td>
</tr>
<tr>
<td>lle80Val</td>
<td>+/-</td>
<td>PD</td>
<td>Western European</td>
<td>(221)</td>
</tr>
<tr>
<td>Gln126X</td>
<td>+/-</td>
<td>Early-onset</td>
<td>German</td>
<td>(225)</td>
</tr>
<tr>
<td>Arg145Gln</td>
<td>+/-</td>
<td>Late-onset autosomal dominant</td>
<td>Japanese</td>
<td>(123, 226)</td>
</tr>
</tbody>
</table>
Figure 11. Alignment of MNRR1 (hCHCHD2) with neurodegenerative disorder mutations. This alignment includes human CHCHD10 (isoform of MNRR1, hCHCHD10), and MNRR1 orthologs in C. elegans (Har-1) and D. melanogaster (CG5010). Mutations are denoted with boxes with degree of conservation: red (identical), purple (conserved), dashes (semi-conserved), and blue (not conserved).

Since the initial studies in patient populations were done to screen for rare PD-associated variants, some functional studies have been done for some of the mutants, but the most information to date is regarding the T61I variant. The first studies were performed in D. melanogaster, which has ortholog of MNRR1 called CG5010. Flies over-expressing various MNRR1 PD-associated mutations, including T61I, displayed decreased climbing ability over time, which could be exacerbated by exposure to rotenone (complex I inhibitor) (207). The transgenic flies also had a reduced lifespan, number of DA neurons, and mitochondrial dysfunction. Knockout of CG5010 in flies results in an increase in ROS, disruption of mitochondrial structure, inhibition of OXPHOS, resulting in the loss of DA neurons and motor dysfunction with aging (227). The introduction of human WT MNRR1 was able to rescue the PD-associated phenotype while the introduction of human T61I MNRR1 did not.
In HEK 293 cells, overexpression of the T61I mutation results in a greater binding affinity for endogenous CHCHD10 and endogenous MNRR1 interaction (152, 228). Interestingly, the T61I mutation occurs just outside of a GXXXGXXXG motif (Figure 11). It is important to note that the presence of increased CHCHD10 in the pulldown performed could be due to the protein binding to the endogenous WT MNRR1 in the lysate, and not necessarily due to interaction with the overexpressed mutant. This would suggest an alteration in MNRR1 function in the mitochondria, including COX regulation, as we have shown the interaction of CHCHD10 and MNRR1 is necessary for finetuning COX activity (113).

1.7 Project Summary

MNRR1 has been shown to play a role in numerous pathways within cellular physiology (Figure 10). Our lab is interested in studying the mitochondrial- and nuclear-specific functions of the protein. The long-term goal of this project is to better understand the mechanisms by which MNRR1 and its post-translational profile help to regulate mitochondrial respiration, apoptosis, and nuclear transcriptional activation in basal and Parkinson’s disease backgrounds. A more complete understanding of the impact of MNRR1 function in the mitochondria and nucleus is critical, as a clearer functional picture may describe underlying functional mechanisms linked to disease and cellular dysfunction, as well as provide a novel therapy target for diseases with a mitochondrial dysfunction component.

One limitation to understanding the mitochondrion’s role in disease, stress signaling, and metabolic pathways is the lack of functional characterization of much of the organelle’s proteome as well as regulatory post-translational modifications. Therefore, the first aim of this project is to understand how MNRR1 is turned over in the mitochondria during cellular stress. The second aim is to determine where MNRR1 is binding on COX, how cytochrome c function is affected when interacting with COX, and how phosphorylation of MNRR1 may be a regulatory switch between cellular life and death. Finally, rare mutations in MNRR1 associated with Parkinson’s disease are becoming a popular niche for research among the small group of researchers that study this
protein. It an interesting topic, as patients in the flagship study had no known PD-associated variants but had autosomal dominant PD in at least two generations of the family. The third aim of this project is to add information to the emerging functional annotation of the PD-associated T61I mutation of MNRR1.
CHAPTER II: RESULTS

My thesis project would not have been possible without my many collaborators. Collaborator experiments have been included in my thesis because they help to paint the mechanistic picture of MNRR1. The experimental contributions are as follows: multiple experiments in sections 2.1 and 2.3 were performed in part by Dr. Siddhesh Aras (Wayne State University, Detroit, MI) as a collaborative effort to determine the mechanism of the stress response activities of MNRR1. The spectral shift assay in section 2.2 was done by Dr. Sachiko Yanagisawa (Hyogo University, Kakogawa, Japan) with materials prepared by me. The ruthenium-labeled cytochrome c assay in section 2.2 was performed by Marti Scharlau in the laboratory of Dr. Francis Millett (University of Arkansas, Fayetteville, AR, USA) with materials prepared by me. All contributions are denoted in figure legends and specifically acknowledged in Chapter IV: Materials and Methods.

2.1 MNRR1 turnover and implications in a mitochondrial stress response

2.1.1 MNRR1 is not targeted by the ubiquitin-proteasomal system during moderate hypoxia

Previous studies in our lab show that MNRR1 is upregulated at 4% oxygen tension and that the nuclear levels of the protein increase, while mitochondrial levels decrease (114, 147). Others have used a quantitative proteomics approach which showed that MNRR1 is one of the most rapidly turned over proteins in the cell (229). During 20% oxygen tension, total cellular MNRR1 is degraded after 3 hours of stalled translation; however, at 4% oxygen, MNRR1 is completely lost by 3 hours suggesting that MNRR1 is more rapidly turned over at 4% oxygen tension (58). Our lab has also found that MNRR1 was more rapidly turned over in the mitochondria, where turnover happens within an hour of stalled translation. The nuclear fraction of MNRR1 is mostly turned over by hour two, suggesting that during moderate hypoxic stress, the levels of MNRR1 are more stable in the nucleus compared to the mitochondria (58).

It has been shown that during cellular stress the mitochondrial proteome can be ubiquitinated and degraded by the cytosolic proteasomal system to regulate function and stress
response (230, 231). Larance et. al. (229) showed that MNRR1 was not a target for the proteasomal system because turnover of MNRR1 still occurred with proteasome inhibition using MG132. We confirmed this finding by looking at the ubiquitination profile of MNRR1 in cells maintained at 20% and 4% oxygen tension using immunoprecipitation. The resulting pulldown revealed that there was no difference in the ubiquitination profile of MNRR1 between basal and stress conditions, ruling out the ubiquitin proteasomal system as responsible for mitochondrial MNRR1 turnover (Figure 12).

**Figure 12. MNRR1 is not targeted by the ubiquitin-proteasomal system.** HEK 293 cells were co-transfected with MNRR1-FLAG and UB-HA expression plasmids; 24 hours post-transfection, one plate was moved to 4% oxygen for 12 hours. Cells were then harvested and lysed. The lysate was immunoprecipitated with FLAG beads and probed with HA to look at the ubiquitination profile of MNRR1 in 20% vs 4% oxygen. Published in PNAS (58).

### 2.1.2 YME1L1 is a candidate protease for MNRR1 turnover in the mitochondria

Identifying the protease(s) responsible for turnover of MNRR1 in the mitochondria during moderate hypoxic stress became an important mechanistic question. We knew the ubiquitin proteasomal system was not responsible, so it must be a protease localized within the mitochondria during basal or stress conditions. We chose to focus on two AAA-proteases: YME1L1 and LONP1. These proteases play important roles in mitochondrial protein quality control systems (232, 233). Although both proteases reside within the mitochondria, it has been predicted that LONP1 resides solely in the matrix (233) and that YME1L1 is bound to the IMS side of the IMM (234). MNRR1 is a soluble IMS protein and, although it retains its N-terminal MTS,
we have yet to observe its localization to the matrix. To ensure that our localization model was correct, we checked the protein compartmentalization through mitochondrial fractionation. Indeed, MNRR1 and YME1L1 were found to localize to the IMS, while LONP1 localizes to the matrix (Figure 13A).

We have shown that MNRR1 levels increase at 4% oxygen tension in cells and that during this moderate stress, MNRR1 predominately localizes to the nucleus where it acts as a transcriptional activator at the ORE. We were interested to know if YME1L1 protein levels increase at this same oxygen tension, where we see a decrease in mitochondrial MNRR1. Cells were maintained at 20% and 4% oxygen tension and, it was determined that YME1L1 protein levels increase at 4% oxygen (Figure 13B, top). Given our model of MNRR1 transcriptional activation in the nucleus, our hypothesis was that if YME1L1 gene contains an ORE, the expression could be regulated by MNRR1 during stress. As expected, a promoter scan resulted in the identification of the COX4i2 ORE in the YME1L1 promoter (Figure 13B, bottom), suggesting that MNRR1 is a transcriptional regulator of its target protease.

To test if YME1L1 was able to directly degrade MNRR1, an in vitro protease assay was performed with purified YME1L1 (catalytic domain), MNRR1, and bovine liver COX. In the reaction containing YME1L1, it was observed that the levels of MNRR1 decreased slightly compared to the no-protease control (Figure 13C). The MNRR1 degradation by YME1L1 was specific, in that COXIV levels did not differ between the presence or absence of the protease (Figure 13C). It should be noted that this experiment needs to be repeated using a known YME1L1 target substrate as a positive control to ensure that the catalytic domain of the enzyme is functionally active.

To determine if the turnover of MNRR1 by YME1L1 was true in cell culture models, YME1L1 was knocked down in HEK 293 cells using siRNA. The knock down of YME1L1 resulted in an increase in MNRR1 protein levels (Figure 13D, left), suggesting that the protein is stabilized at the depletion of YME1L1. In YME1L1Δ MEFS, we observed the same result (Figure 13D, right).
These experiments collectively support that YME1L1 is a protease responsible for targeting MNRR1 in the mitochondria for turnover during stress.

Figure 13. MNRR1 is targeted for degradation by IMS protease YME1L1. A. IMS and matrix compartments were isolated from HEK 293 mitochondria. Compartment lysates were assessed for the presence of the indicated proteins. B. Top, total YME1L1 was detected in HEK 293 cells that were incubated at 20% or 4% oxygen tension for 24 hours. Tubulin served as a loading control. Bottom, YME1L1 promoter ORE aligned to classical COX4I2 ORE. C. In vitro protease degradation assay using purified YME1L1, MNRR1, and COX. D. MNRR1 levels were analyzed in YME1L1 knockdown HEK 293 cells using siRNA (left) or YME1L1 knockout MEFs (right). GAPDH served as a loading control. This experiment was done by Dr. Siddhesh Aras, Wayne State University, Detroit, MI and are published in PNAS (58).

2.1.3 MNRR1 is involved in the mitochondrial unfolded protein response

YME1L1 has been shown to be a key player in the mitochondrial unfolded protein response (mtUPR) (47). When protein misfolding occurs in the mitochondria, a stress response, similar to that of the endoplasmic reticulum UPR (erUPR) (235) is set in motion (45, 46). Stress signaling from mitochondria to the nucleus kicks off transcriptional activation of proteins required to restore protein homeostasis in the organelle.
We have shown that under moderate hypoxic stress MNRR1 levels increase in the nucleus and decrease in the mitochondria (114) and that MNRR1 may serve as a transcriptional activator for YME1L1, we were interested in determining if MNRR1 plays a role in the mtUPR. Therefore, we looked at protein levels of key mtUPR regulators in MNRR1\(^{-/-}\) cells (Figure 14A). Depletion of YME1L1, LONP1, NRF-1, and Hsp60 protein levels were observed in the MNRR1\(^{-/-}\) cells as compared to the WT cells, suggesting that MNRR1 may transcriptionally regulate other mtUPR proteins besides YME1L1.

Our lab has shown that mtUPR induction at the protein level of Hsp60 did not occur in MNRR1\(^{-/-}\) cells treated with doxycycline (an inducer of mtUPR) (58). To confirm that MNRR1 was involved in the transcriptional regulation of mtUPR response, our dual luciferase COX4I2 ORE and Mutant ORE (no RBPJk binding sequence) was transfected into cells which were treated with doxycycline for 48 hours (Figure 14B). The WT ORE reporter a statistically significant increase in activity compared to its control, whereas the mutant ORE response was blunted with no significant change compared to control. The increase in seen in WT ORE transcriptional activity was also significant from that seen in the mutant. This data is supported by the observation of similar effects with our MNRR1 ORE luciferase reporter and other mtUPR inducers shown elsewhere (58).
Figure 14. MNRR1 is involved in mtUPR signaling in the nucleus. A. Protein levels of key mtUPR markers in MNRR1 knockout HEK 293 (R1<sup>+</sup>) cells compared with control cells (WT). GAPDH and actin served as loading controls. B. Dual luciferase assay in HEK 293 cells expressing the WT or Mutant COX4I2-luciferase reporter treated with mtUPR inducer doxycycline (50 μg/mL) for 48 h. Data are presented relative to cells treated with vehicle. ** p<0.01.

2.1.4. The mechanism of MNRR1 in mtUPR

Taken together, these data support that MNRR1 plays an active role in mtUPR signaling via its nuclear function. The proposed mechanism based on the above data is that during proteotoxic stress due to misfolded proteins in the mitochondria, MNRR1 rapidly accumulates in the nucleus due to the blockage of import (Figure 15). At the same time, the mitochondrial pool of MNRR1 that is interacting with COX is rapidly turned over by YME1L1 to attenuate mitochondrial metabolism. In the nucleus, ORE containing genes involved in the mtUPR are actively expressed and allow for the rescue of proteotoxicity in the mitochondria, restoring organelle homeostasis.
Figure 15. The role of MNRR1 in mtUPR. Left, under non-stress conditions most of the cellular MNRR1 is bound to COX in the mitochondrial IMS, with a fraction localized to the nucleus. Right, mitochondrial stress due to proteotoxicity initiates the mtUPR. MNRR1 is rapidly turned over by YME1L1, while blocked IMS import results in MNRR1 accumulating in the nucleus. The increase in MNRR1 levels results in the displacement of ORE repressors and transcriptional activation of mtUPR genes occurs in attempt to restore mitochondrial homeostasis.

2.2 MNRR1 modulates COX activity through direct interaction with the complex

2.2.1 Purification of HIS-MNRR1 from *Escherichia coli*

Our lab has previously shown that MNRR1 regulates COX activity and that phosphorylation of tyrosine residue 99 (Y99) plays an important role in fine-tuning oxygen consumption (113, 114). One unanswered question was if MNRR1 directly regulates COX activity through interaction or if this was an indirect effect. To answer this question, large quantities of MNRR1 would need to be purified from bacteria.

Given the unique properties of MNRR1 (numerous redox sensitive cysteines, disulfide bonded folding, and highly disordered regions within the protein), every step of the purification process needed to be optimized. The original protocol used in the lab for 6xHIS-tagged MNRR1
(HIS-MNRR1) expression in BL21(DE3) *E. coli* did not result in a high yield (Figure 16A) or a clean preparation of purified target protein (Figure 16B). Thus, the protein expressed and purified using this protocol was not suitable for *in vitro* assays, where protein purity plays an important role in the accuracy of measurements.

Therefore, a highly specialized strain of *E. coli*, SHuffle® T7 Express *lysY* (NEB), was used to ensure proper protein folding in the bacterial cytoplasm. DsbC is a soluble, enzymatic chaperone that catalyzes the formation of disulfide bonds in the oxidizing environment of the periplasm of bacterial cells (236, 237). This strain of *E. coli* expresses DsbC in the cytoplasm. The co-expression of DsbC has been shown to increase expression of the target protein and to help increase purity of preparations (238-240). This strain also contains mutant lysozyme, allowing competent bacteria to survive toxic protein expression, which is visible as the highly degraded product seen in BL21(DE3) (Figure 16B). The implementation of the SHuffle® T7 Express *lysY* resulted in higher protein expression (Figure 16C) as well as a highly pure protein purification yield (Figure 16D).

A secondary issue was also addressed in post-purification, after protein concentration. Recombinant MNRR1 left in the high-salt purification buffer would result in the protein irreversibly precipitating out of solution. To address this issue, different storage buffer formulations were used, including high glycerol content and low salt concentrations. Glycerol was used to prevent the protein aliquots from freezing; however, high glycerol content can affect protein-protein interactions. Taking the downstream applications for which the recombinant protein would be used in to account, it was determined that a low salt, glycerol free buffer allowed the protein to stay in solution during storage. To prevent freeze-thaw of the protein, small volume aliquots were made and stored at -80°C. For more information on other modifications made during the purification process please see Materials and Methods Section 4.5.
Figure 16. Purification optimization for HIS-MNRR1. A. Expression test of transformed BL21(DE3) *E. coli* transformed with 6xHIS-MNRR1 expression plasmid. Whole bacterial lysate is shown on Coomassie blue stained gel. B. Pooled elution fractions from nickel column purification showing massive degradation and co-purifiers when 6xHIS-MNRR1 is purified from BL21(DE3) *E. coli*. C. Expression test of SHuffle® T7 Express lysY *E. coli*. D. Comparison of end product between 6xHIS-MNRR1 expressed in BL21(DE3) (Old Protocol) and SHuffle® T7 Express lysY (New Protocol). -IPTG denotes the uninduced control culture, +IPTG denotes the induced culture, * indicates the intact 6xHIS-MNRR1 protein.

2.2.2 MNRR1 changes COX activity through direct interaction near the cyt c binding pocket

To test if the addition of MNRR1 to an *in vitro* system affects COX activity, oxygen consumption was measured using a Clark-type oxygen sensing electrode. The addition of unphosphorylated (WT) MNRR1 resulted in an ~20% increase in COX activity (Figure 17A). As expected, the phosphomimetic (Y99E) MNRR1 maximally increased COX activity, superior to that of WT. This *in vitro* data supports our cell culture model and shows that, in both cases, Y99 phosphorylation of MNRR1 is a critical PTM in fine-tuning the activity of the tightly regulated complex IV.

Another important question in this study was where MNRR1 binds to COX to regulate the activity of the complex. A high throughput interaction study performed using affinity capture-mass spectrometry (AP-MS) revealed a handful of COX subunits as potential MNRR1 interactors: COX Va, COX Vb, COX Vla, and COX Vlc (151). However, the COX V subunit isoforms localize to the
mitochondrial matrix side of the membrane (Figure 17B), suggesting that their capture is an artifact of using whole cell lysate in the experiment. COX VIa is almost exclusively inserted into the inner mitochondrial membrane, although ~20 amino acid residues extend into the IMS, as is true with of the COX VIc subunit (Figure 17B).

Figure 17. MNRR1 regulates COX activity through direct interaction near the cyt c binding pocket. A. In vitro COX activity in the absence and presence of WT MNRR1 and Y99E MNRR1. *p<0.05, **p<0.01, ***p<0.001 B. COX structure (67). COXVa (pink), COXVb (purple), COXVIa (red), and COXVIc (light green). Hemes (dark blue) and copper (orange circles). C. Oxidized spectrum of bovine liver COX in the absence and presence of recombinant WT MNRR1. D. Cross-linking-MS peptide identification of COX6c and MNRR1. E. COX6c interacts with the holoenzyme, circled in purple. This experiment was performed by Dr. Sachiko Yanagisawa (Hyogo University, Kakogawa, Japan) with materials prepared by me. Figures 1B and modified 1E were obtained from Kadenbach and Hüttemann, 2015 (67) with permission from the publisher (Elsevier).
To detect the physical interaction of MNRR1 and COX, the UV-VIS absorbance spectrum of COX was measured in the absence and presence of WT MNRR1. A shift in the absorbance spectrum of COX in the presence of WT MNRR1 indicates a physical interaction and a potential location of the interaction between the protein and the COX complex. The measurements showed a slight shift in the absorbance spectrum of COX in the presence of WT MNRR1, from 412 nm to 436 nm (Figure 17C, green line). The shift in the spectra between these wavelengths suggests that a structural change is occurring in the heme vicinity of COX because the heme absorption spectrum is observed from 410-460 nm (241). The hemes are located in the holoenzyme of the complex in COX subunit I and are involved in electron transfer.

To determine where MNRR1 may be binding and inducing structural changes in COX, \textit{in vitro} crosslinking was performed followed by mass spectrometry. The results showed only one band containing peptide from both WT MNRR1 and a COX subunit, which was identified as COX VIc (Figure 17D). COX VIc binds to subunits I (location of the heme groups) and II on the outside of the complex (Figure 17E). This subunit also protrudes into the IMS and is located near the cytC binding pocket, suggesting that MNRR1 regulates the interaction between the electron shuttle and COX.

**2.2.3 Phosphorylated MNRR1 influences the binding between cytC and COX \textit{in vitro}**

Given the evidence that MNRR1 likely binds near the cytC binding pocket and induces structural changes within the complex itself, we assessed cytC-COX interaction by measuring \textit{in vitro} electron transfer and binding affinity. Measurements were taken spectroscopically using a ruthenium labeled derivative of cytC (Ru-cytC). Ruthenium is a rare transition metal that can act as an electron donor through metal to ligand transfer. The ruthenium moiety is added to cytC at residue C39 (mutated from lysine), which was shown to have similar kinetics as wildtype cytC (242). With laser excitation, ruthenium transfers an electron to cytC, which is then subsequently accepted by COX via the CuA site (Figure 18A) (243). In the assay, the rate constant of electron transfer from Ru-cytC to the CuA site of bovine liver COX (k<sub>a</sub>) was unaffected by the presence of
recombinant WT or Y99E MNRR1. However, in the presence of increasing ionic strength, a difference in the rate constant for the formation of 1:1 cyt$c$:COX complex ($k_f$) is observed (Figure 18B). The $k_f$ is lower for COX control and in the presence of recombinant WT MNRR1 as compared to when recombinant Y99E MNRR1 is present. Phosphorylation at Y99 appears to stabilize the cyt$c$:COX complex when exposed to increasing NaCl concentrations.

![Figure 18](image.png)

**Figure 18. Phosphorylated MNRR1 affects the binding kinetics between COX and cyt$c$.** A. Electron flow (blue arrows) from Ru-39-cyt$c$ to COX (243). B. Electron transfer measurements (brown square) ($k_o$) and cyt$c$:COX binding in increasing amounts of NaCl in the absence (red circles) and presence of recombinant MNRR1($k_f$) (WT, blue diamond and Y99E, green triangle). This experiment was performed by Marti Scharlau in the laboratory of Dr. Francis Millett (University of Arkansas, Fayetteville, AR, USA) with materials sent by me. Figure 18A was obtained from Scharlau et. al., 2019 (243) with permission for use from the publisher (ACS Publications).

### 2.3 Unphosphorylated MNRR1 preferentially interacts with Bcl-xL

A previous study had suggested that MNRR1 plays a role in apoptosis in the mitochondria. In that study, it was shown that MNRR1 knockout cells are sensitized to apoptosis because Bcl-xL is unable to inhibit Bax activation and that MNRR1 interacts with Bcl-xL at the IMS facing side of the OMM (61). Our hypothesis was that the unphosphorylated pool of MNRR1 in the mitochondria mostly interacts with Bcl-xL, while the phosphorylated pool of MNRR1 is reserved for COX regulation.
To test this hypothesis, flag-tagged phosphomimetic (Y99E) or unphosphorylatable (Y99F) MNRR1 was overexpressed in HEK 293 cells. Immunoprecipitation was performed with FLAG beads and the presence Bcl-xL was assessed in the pulldown. An increase in Bcl-xL protein was observed in the Y99F MNRR1 pulldown as compared to the Y99E MNRR1 pulldown (Figure 19A). This finding supports the hypothesis of unphosphorylated MNRR1 playing a role in the anti-apoptotic function of the protein.

The proposed complete model for MNRR1 function in the mitochondria is presented in Figure 19B. In this model, MNRR1 interacts with COX, where Y99 phosphorylation plays a role in the fine-tuning of COX activity through cytc interaction. Due to the location of COX in the IMM, YME1L1, another membrane bound protein, can turn over the phosphorylated pool of MNRR1 as needed for adaptation to stress. This allows for the unphosphorylated pool of MNRR1 to remain bound to Bcl-xL on the IMS side of the OMM, giving the cell time to return to homeostasis after stress insult, by inhibiting apoptosis.

Figure 19. Unphosphorylated MNRR1 preferentially interacts with Bcl-xL. A. HEK 293 cells were transfected with FLAG tagged phosphomimetic (Y99E) or unphosphorylated (Y99F) MNRR1. MNRR1 was immunoprecipitated using anti-FLAG antibody and the precipitate was probed for Bcl-xL and FLAG. B. The working model of MNRR1 function in the mitochondria. Phosphorylated MNRR1 (purple oval with grey dot) preferentially interacts with COX to attenuate COX activity. Due to the proximity COX-bound MNRR1 to the IMM, YME1L1 turns over the phosphorylated MNRR1 pool during stress, leaving the unphosphorylated MNRR1 to inhibit apoptosis through Bcl-xL interaction. *This experiment was done by Dr. Siddhesh Aras, Wayne State University, Detroit, MI and included to complete the model of MNRR1 in the mitochondria.

2.4 MNRR1 in Parkinson’s Disease

2.4.1 MNRR1 cannot rescue OXPHOS defects seen with MPP+ treatment
It has been suggested that PD-associated mutations in MNRR1 may lead to mitochondrial dysfunction and oxidative stress (207). To test this hypothesis in vitro, purified proteins were used to measure COX activity. The T61I PD-associated mutant was included in this study because it is the most studied of the rare mutants, but more information is needed to fully understand the pathogenic alterations to the protein’s function. Oxygen consumption was measured using a Clark-type oxygen sensing electrode. The Y99E control produced an ~20% increase in COX activity (Figure 20A). The T61I mutant did not produce a change in COX activity upon addition to the electrode chamber, indicating that in the in vitro system this PD-associated mutant does not alter COX activity. However, PD patients harboring the T61I mutant are heterozygous at the CHCHD2 locus, meaning they have one WT allele present, suggesting the mutant allele has a dominant-negative effect. The in vitro finding may not necessarily reflect the physiology of the heterozygous phenotype because only one type of MNRR1 was added to the chamber for COX activity measurement, which is why it was important to test the mutant in a WT cell culture model.

To find an appropriate system to induce a PD phenotype in WT SY5Y cells, MPP+ was used. MPP+ was discovered to induce rapid PD phenotypes in drug users when the precursor metabolite was found to be an ingredient in synthetic heroine (244, 245). MPP+ uses the dopamine transporter to enter DA neurons, after which it enters the mitochondria and inhibits complex I; it has thus become a common PD phenotype inducer in mammalian systems.

To test whether the T61I mutant was defective for oxygen consumption rate (OCR) in WT SY5Y cells, intact cellular respiration was measured in both vehicle and MPP+ transfected cells. Both WT and T61I cells showed a significant decrease in OCR with MPP+ treatment compared to the vehicle control (Figure 20D).

Since MNRR1 is a biorganellar protein, it was important to determine if the T61I mutant's effect was mediated in the nucleus or in the mitochondria. To do so, the mitochondrial import deficient mutant (C-S) of MNRR1, which stimulates transcriptional activation at the ORE in the nucleus, and our Y99E mutant, which stimulates respiration in the mitochondria were utilized. To
answer this question WT SY5Y cells were transfected with WT, C-S, or Y99E MNRR1 and either treated with vehicle or MPP+ before intact respiration was measured. Neither the overexpression of C-S or Y99E MNRR1 could rescue the defects observed in OCR in the presence of MPP+ treatment, indicating that MNRR1’s nuclear activity or post-translational status cannot overcome MPP+ inhibition (Figure 20B).

Figure 20. T61I mutant is defective for oxygen consumption and MPP+ phenotype cannot be rescued by MNRR1 overexpression. A. In vitro COX activity in the absence and presence of Y99E and T61I MNRR1. **p<0.005, ***p<0.001 B. Relative oxygen consumption rate (OCR) of intact WT SY5Y cells expressing WT, T61I, C-S, and Y99E MNRR1 pre-treated with 250 µM MPP+ of DMSO vehicle for 7 hours. Measured in Seahorse bioanalyzer. MPP+ treatment is normalized to respective vehicle controls for comparison. Flag expression is shown below (loaded by volume). *p<0.05, **p<0.005.

2.4.2. The expression of T61I in WT SH-SY5Y cells affects the nuclear function of MNRR1

MNRR1 has been shown to activate transcription of stress response genes in cells. MNRR1-mediated transcription has not yet been studied in PD. To determine whether MNRR1 over-expression can alter transcription at the ORE, WT, C-S, T61I, and Y99E MNRR1 variants were co-transfected into SH-SY5Y cells with our MNRR1 ORE reporter plasmid. Cells were subsequently treated with DMSO vehicle or MPP+ for 24 hours and relative luminescence from the reporter plasmid was read. The C-S mutant, which is defective for mitochondrial import but can perform its nuclear function (114), behaved as expected by increasing transcripts despite the presence of MPP+. Others in the lab have shown that Y99E MNRR1 is defective for transcriptional activation using our reporter (data unpublished) and this was reproduced in the assay.
The T61I mutant behaved in an interesting manner, where under basal conditions, an increase in transcriptional activation was observed mirroring that which is observed with the C-S MPP+ treated cells. However, with MPP+ treatment there is a decrease in the reporter activity in the T61I cells. Taken together, the data suggests that the nuclear behavior of MNRR1 is affected by the T61I mutation.

**Figure 21. T61I MNRR1 is defective for transcriptional activation at the MNRR1 ORE.** Activity measurements were taken in WT SH-SY5Y cells co-transfected with the MNRR1 ORE reporter, internal control luciferase, and the indicated MNRR1 expression plasmid. Cells were then treated with DMSO vehicle or 500 µM MPP+ for 24 hours. Relative luminescence was measured and normalized to the internal control luciferase. Experiment must be repeated once more for statistics.

### 2.4.3. T61I mutant may cause an imbalance in compartment-specific mtUPRs

Due to the ORE transcriptional changes observed with the T61I mutant, protein levels were investigated for CHCHD10 (transcriptional repression at the ORE) and mtUPR markers (Hsp60 and YME1L1) in WT or T61I transfected WT SH-SY5Y cells treated with either vehicle or 500 µM MPP+ for 24 hours. In the T61I cells, we see an increase in CHCHD10 levels compared to untreated and WT treated cells (Figure 22A). The reduction in reporter activity may be due to the rise in CHCHD10 levels, as our lab has previously shown CHCHD10 to act as a transcriptional repressor of the ORE (115). Confirmation experiments will need to be performed to determine if CHCHD10 levels increase in the nucleus in the presence of T61I with MPP+ stress.
When assessing the levels of endogenous and Flag-tagged transfected MNRR1, it was observed that T61I (top band) and endogenous (WT) MNRR1 (bottom band) levels were lower in vehicle treated cells compared to WT cells, with the most noticeable decrease observed with the T61I protein (Figure 22B). Both endogenous and T61I MNRR1 increased with MPP+ treatment (Figure 22B). This was unexpected, as we see repression of the MNRR1 ORE in the T61I cells with MPP+ treatment, leading to a hypothesis that this type of stress may stabilize MNRR1 in cells.

As previously discussed in Results Section 2.1, YME1L1 is a protease that targets MNRR1 in the mitochondria. While no change in YME1L1 protein levels was observed in WT expressing cells, there were differences in the T61I cells (Figure 22C). In vehicle T61I cells, the levels of YME1L1 are higher compared to WT controls. This finding could be an explanation for the reduction in T61I and endogenous MNRR1 levels we see in the same cells. In the MPP+ treated T61I cells, the levels of YME1L1 are depleted compared to the vehicle control, supporting the hypothesis that T61I and endogenous MNRR1 proteins are stabilized with MPP+ treatment. The levels of Hsp60 were also altered in the T61I expressing cells, but not WT expressing cells. Compared to the vehicle control, the level of Hsp60 drastically increased in MPP+ treated T61I cells (Figure 22D).

When the data is analyzed cumulatively, a soft hypothesis can be made, with the caveat that many more experiments need to be performed to solidify a mechanism of T61I functional alterations that contribute to PD. This data suggests that under basal conditions, T61I stimulates MNRR1 nuclear activity (potentially through a stress response or mis-localization), where we see an increase in YME1L1 protein levels. The localization of YME1L1 coupled with an increase in protein level may result in a mtUPR response in the IMS, turning over the T61I mutant, while not inducing the matrix mtUPR; this is reflected by uninduced Hsp60 levels in the T61I cells compared to WT. When the T61I cells undergo oxidative stress due to MPP+ treatment, ORE activity is inhibited in the nucleus due to the rise in CHCHD10 protein levels. T61I and endogenous MNRR1
are stabilized during this stress due to a decrease in the levels of YME1L1 protein, which could be indicative of a dysfunctional IMS mtUPR. Hsp60 levels increase in these conditions, suggesting that the matrix mtUPR is activated by an unknown mechanism.

Figure 22. T61I MNRR1 may cause an imbalance in compartment mtUPR activation. A. Western blot showing protein levels in lysate from WT SH-SY5Y cells transfected with indicated MNRR1 expression plasmid. Transfected cells were treated with DMSO vehicle (-) or 500 µM MPP+ (+) for 24 hours before harvest. Gapdh was used as a loading control for soluble proteins and Complex II (SDH) was used as a loading control for membrane protein.
CHAPTER III: DISCUSSION

Mitochondrial-nuclear crosstalk is a crucial molecular mechanism that allows the mitochondria to relay a message to the nucleus to maintain or restore cellular homeostasis (246, 247). Our lab has shown that MNRR1 is a multi-functional protein, performing specific duties in the mitochondria and the nucleus dependent upon stress crosstalk. In the mitochondria, we have previously shown that MNRR1 regulates COX activity, and that the regulatory activity can be fine-tuned through phosphorylation of residue Y99 (113, 114). We have also identified MNRR1 as a transcriptional activator in the nucleus during inducible stress (58, 147). Others have shown that MNRR1 plays an anti-apoptotic role in the mitochondria by demonstrating the inability of Bcl-xL to prevent the release of cytC during cellular stress in MNRR1 knockout cells (61).

As mentioned above, during times of stress, such as moderate hypoxia, MNRR1 activates transcription in the nucleus. Our lab showed that bi-organellar levels of MNRR1 switch upon cellular stress so that MNRR1 accumulates in the nucleus due to inhibited mitochondrial import, resulting in decreased mitochondrial MNRR1 protein levels. These results suggest that there is a delicate balance required by the remaining mitochondrial MNRR1 to deal with stress attenuation while inhibiting apoptosis, giving the cell time to repair itself through retrograde signaling.

The research presented here suggests that phosphorylation of residue Y99 is a key regulator of this balance between life and death decisions. Under basal conditions, mitochondrial MNRR1 binds to COX, whereby unphosphorylated MNRR1 can increase activity of the enzyme complex and Y99 phosphorylation results in maximal activity (Figure 17A). Depending on the energetic need of the cell, it is possible that MNRR1 is preferentially phosphorylated or dephosphorylated, which acts as a mechanistic switch. During cellular stress, we observe the rapid reduction of MNRR1 protein levels in the mitochondria (Figure 12B) and an accumulation of MNRR1 in the nucleus for transcriptional activation to take place; however, some MNRR1 remains in the mitochondria (58, 114). A hypothesis of this project was that unphosphorylated MNRR1 may preferentially interact with Bcl-xL, allowing it to remain in the mitochondria, inhibiting
apoptosis and giving the nucleus time to facilitate a cellular rescue response to deleterious stimuli. An immunoprecipitation assay showed that the unphosphorylated mutant of MNRR1 preferentially interacts with Bcl-xL compared to the phosphomimetic MNRR1 (Figure 19A).

Further studies should be done to complement our localization microscopy shown elsewhere (58). The data shows that MNRR1 remains in the mitochondria of cells incubated at 4% oxygen tension for 8 hours, accompanied by nuclear accumulation due to blocked IMS import. To understand the full picture of the above proposed mechanism, microscopy should be done at 4% oxygen tension for longer time points, including 12 hours (where reporter activation was observed), 24 hours (used in other experiments), etc. The time points will give an accurate depiction of bi-organellar MNRR1 localization during acute and chronic stress and determine the timeframes of the functions of MNRR1 in transcription, OXPHOS, and apoptosis. The addition of functional studies at each time point would also add to the study, such as assessing ORE transcriptional activation, COX activity, and apoptosis.

Another important question addressed in this project was regarding how MNRR1 was being turned over in the mitochondria during stress. We have observed MNRR1 localizing to the mitochondrial IMS via the Mia40 import machinery (114) and have yet to observe conditions in which MNRR1 uses its retained MTS to enter the mitochondrial matrix. A classic pathway in mitochondrial protein turnover is the ubiquitin-proteasomal pathway (53); however, the ubiquitination profile of MNRR1 did not change between basal and stressed conditions (Figure 1C). The next logical step in identifying a candidate protease that targets MNRR1 as a substrate was to test proteases with access to the IMS. YME1L1 is a membrane bound protease that protrudes into the IMS (Figure 13A). YME1L1 levels also increase at 4% oxygen tension (Figure 13B) and contains an ORE in the promoter of its gene, which suggests that MNRR1 is a transcriptional activator of the protease during stress. In both YME1L1 knockdown and knockout cells, the reduction in the protease results in the stabilization of MNRR1 protein levels (Figure 13D-E).
YME1L1 is a key regulator of proteotoxic stress in the mitochondria and participates in the mtUPR (47, 232). The fact that MNRR1 is a transcriptional activator in conditions that cause mitochondrial proteotoxic stress further implicates its role in crosstalk signaling. It was determined that MNRR1 does indeed play a transcriptional activation role in the mtUPR via the upregulation of YME1L1 and HSP60 (a protein folding chaperone) (Figures 13B and 14B). Additionally, MNRR1 deficient cells were unable to induce a mtUPR response as apparent by reduction in key players in the pathway (Figures 14A-B).

The identification of YME1L1 as a protease that facilitates mitochondrial MNRR1 turnover further supports the notion that unphosphorylated MNRR1 is left in the mitochondria to regulate apoptosis. This hypothesis is based upon proximity of the protease to the OMM. As discussed above, YME1L1 is an IMM bound protein with the catalytic domain protruding into the IMS. Additionally, COX is a transmembrane complex with MNRR1 binding on the IMS-facing side. This would place the pool of MNRR1 directing COX activity within reach of YME1L1 and thereby attenuating COX activity through turnover during stress. The Bcl-xL bound pool of mitochondrial MNRR1 would be out of reach of the protease, allowing for its antiapoptotic function to remain intact (Figure 19B).

Another important question addressed in this study is regarding where MNRR1 is binding on COX. Of the potential subunits identified by Floyd et. al (151) in their high throughput AP-MS study, it seemed that the IMS protruding subunits COX VIa and COX VIc were the most likely candidates due to the mitochondrial compartment localization of MNRR1. The use of tissue-purified COX and recombinant MNRR1 for irreversible chemical cross-linking seemed like the most straightforward approach. However, it should be noted, that tissue-purified COX has hundreds of co-purifying proteins (Hüttemann Lab, unpublished MS data); it is, after all, how our lab was able to determine that MNRR1 interacts with the complex (114). Immunoblotting allowed us to track MNRR1 binding in the crosslinking reaction to COX and unconventional mass spec was performed from membrane bands. Despite low peptide count (likely due to the high sensitivity
of the FLAG antibody used), peptides from MNRR1 and COX VIc were found in the same band, suggesting interaction (Figure 17D).

The function of COX VIc is not well understood; however, mutations of the *D. melanogaster* COX VIc homolog *cyclope* causes lethality in the germline and inhibition of cell growth in somatic cells (248). The growth defect was observed in HEK293 cells overexpressing COX VIc as well (249). These findings suggest that the functionality and the levels of COXVIc protein play an important role in mitochondrial energetics. Follow up studies need to be done, but the findings presented here suggest that COX VIc plays a role in regulating COX activity. Further, the addition of MNRR1 to COX causes the spectra of COX to shift from 412 nm to 436 nm, indicating a structural change in the heme vicinity of the core subunit. It would be interesting to see if the COX VIc subunit is flexible upon MNRR1 binding through *in silico* structure prediction of MNRR1 and molecular dynamics and docking simulations with COX and MNRR1.

The protrusion of COX VIc into the IMS is near the cyt c binding pocket (Figure 17E). We assessed the effect of MNRR1 phosphorylation on the electron transfer and binding between cyt c and COX using ruthenium labeled cyt c and tissue-purified COX in an *in vitro* system. We observed that the presence of neither WT or Y99E MNRR1 changed the electron transfer from cyt c to COX from basal conditions (Figure 18A) but the addition of Y99E did increase the stability of the cyt c-COX complex at increasing ionic strength compared to WT and basal measurements. This finding is supported by a study that shows the knockout of MNRR1 destabilizes cyt c (227).

Research assessing the function of MNRR1 in PD is relatively new in the field. Upon literature review for this portion of the project, I noted that research involving MNRR1 and PD, or just PD itself, is fairly incohesive between researchers. One issue is that obtaining primary dopaminergic neurons from patients is not practical and primary neurons are notoriously difficult to culture, so the gold standard cell line has become neuroblastoma SH-SY5Y cells, which are immortalized, have DA neuronal features, and are much easier to culture. Meta-analysis of literature done by others suggests that SH-SY5Y differentiation does not significantly affect the
Another issue that I noted is that the induction of PD using MPP+ varies in a broad range of concentrations and incubation times of treatment. Yet another issue comes from the use of peripheral tissue cells instead of a neuronal cell line. The metabolism between the brain and peripheral tissues is quite different, and it should be expected that not all findings in commonly used cells such as HEK293 and HeLa cells will recapitulate neuronal physiology. Standardization of models in PD research using human tissues will greatly help reproducibility in the field and allow for more streamlined research of the disease.

A good portion of MNRR1 PD research has been done in D. melanogaster. It should be noted that this protein shares considerable sequence overlap with both human MNRR1 and CHCHD10. This may have caused some of the observations noted in CG5010 KO flies over-expressing human MNRR1 but not co-expressing human CHCHD10. Tio et al., 2017 (207), saw an increase in H$_2$O$_2$ production, death of DA neurons, and reduced lifespan in flies over-expressing human WT MNRR1 compared to the EV control.

Despite these difficulties, we tested both WT and PD-associated T61I MNRR1 in both basal and MPP+ treated conditions in WT SH-SY5Y cells. Our data show that overexpression of T61I MNRR1 causes mitochondrial OCR to increase in basal conditions compared to WT (data not shown). This finding conflicts with another study, which showed no difference after overexpression of the T61I mutant in the same cell line (228). However, it should be noted that this publication does not list the cell culture conditions or details of their Seahorse Bioanalyzer protocol. The number of cells plated for the assay, as well as the type of medium used, can have effects that could explain the discrepancy in results. One common example of variation would be in the media in which the OCR reading was performed. In many studies, intact respiration is performed in galactose medium, which forces the cells into aerobic respiration. However, neuronal cells perform oxidative metabolism of glucose and glucose is a main sugar source of ATP used by the brain (251), which would explain the difference in results. In any case, there was no significant difference shown in OCR when the cells were treated with MPP+. OCR in WT SH-
SY5Y cells could not be rescued by the overexpression of the C-S (nuclear localization) mutant or the expression of Y99E MNRR1 (Figure 20B). These data suggest that the overexpression of MNRR1 is not sufficient to rescue the oxygen defect with complex I inhibition by MPP+ in neuronal cells.

In WT SH-SY5Y cells expressing our MNRR1 ORE luciferase reporter with the T61I mutation shows a difference in transcriptional activation from WT MNRR1 cells in vehicle and MPP+ treated conditions. In vehicle treated T61I cells, the MNRR1 ORE reporter is activated on the level of the positive control C-S transcriptionally active mutant of MNRR1. When the cells are treated with MPP+, a decrease in transcriptional activity occurs, returning to WT levels. These data suggest that the T61I mutant is transcriptionally defective during stress and may contribute to a PD phenotype through its nuclear function. It would be interesting to test the interaction between RBPJκ to determine if the mechanism of the reduced capacity of T61I MNRR1 to activate transcription is based on an inability to bind RBPJκ.

Protein level of MNRR1’s isoform, CHCHD10, is affected by the presence of the T61I mutant in WT SH-SY5Y cells. In the same MPP+ treated T61I cells where transcriptional repression is observed, the levels of CHCHD10 increase. CHCHD10 has been shown to be a transcriptional repressor of the ORE (115). One hypothesis is that the increase in CHCHD10 levels observed in these cells is nuclear specific. This can be tested by isolating mitochondria and nuclei from cells in the same experimental conditions as described Figure 22. If the increase in CHCHD10 is localized to the nucleus, it is likely that this increase is resultant in ORE transcriptional repression.

The difference in FLAG-tagged and endogenous MNRR1 protein levels between the vehicle and MPP+ treated T61I cells could be due to alternating levels of YME1L1 between treatments. In vehicle treated T61I cells, where the T61I mutant protein level is low compared to WT controls, there is a marked increase in YME1L1 levels. Because YME1L1 targets MNRR1 in
the mitochondria, an increase in the protease could lead to turnover of the PD-associated mutant and endogenous MNRR1. The reduced levels of MNRR1 in the presence of T61I may have to do with the ability of the mutant to oligomerize with WT MNRR1, causing the protein to precipitate in the mitochondrial (252). This would likely turn on the mtUPR, at least in the IMS, causing the increase in YME1L1 to respond to proteotoxic stress in the presence of the T61I mutant. When the cells are challenged with MPP+, the levels of YME1L1 decrease, which matches the repression of the ORE in the nucleus and the stabilization of both endogenous and T61I MNRR1. Given what our lab has shown regarding the involvement of MNRR1 in the mtUPR response, a second marker was assessed for mtUPR induction in the presence of the T61I mutant. The increase in Hsp60 protein in the presence of the T61I mutant and stress challenge suggests that the mtUPR is induced.

The difference between the IMS and matrix mtUPR markers is an interesting observation. It is possible that the T61I mutant causes a compartment specific induction of the mtUPR without further stress challenge. However, upon further stress induction, we note an induction of the matrix mtUPR. It has been suggested in literature that over activation of mtUPR may be a contributing factor to disease pathogenesis and mitochondrial dysfunction accompanying ageing (253). In fact, the over-activation of mtUPR contributes to neurodegenerative diseases, including PD (170), however this has yet to be studied in a compartment-specific manner. The T61I mutant may induce mtUPR in the IMS which in turn causes the pathway to be chronically activated in the heterozygous cells. This hypothesis warrants further investigation to fully determine the dysfunction occurring in T61I expressing cells.

Overall, this project was able to add knowledge of the function of MNRR1 in the mitochondria and the nucleus. MNRR1 has been shown to participate in mitochondria-nuclear crosstalk by participating in the mtUPR. The findings added to the working model of the Grossman lab, showing that MNRR1 regulates mitochondrial function based on its phosphorylation status (Figure 17-19). The preferential binding between phosphorylated and unphosphorylated MNRR1
dictates the ability for YME1L1 to preferentially degrade the phosphorylated pool of mitochondrial MNRR1, allowing the pool of Bcl-xL associated MNRR1 to inhibit apoptosis. When MNRR1 is interacting with COX, it may be doing so through a subunit of unknown function, COX VIc. This finding provides molecular insights to a potential function of the subunit in the regulation of the complex activity. The role of MNRR1 in PD is still unclear and much more work needs to be done as this project goes forward. This thesis project does show that MNRR1 is involved mtUPR, fine-tuning COX function, and an unbalanced or chronic mtUPR response, which can be further studied in the context of PD.
CHAPTER IV: MATERIALS AND METHODS

4.1 Reagents

*Antibodies.* Actin (catalog no. 12748), GAPDH (3683), tubulin (9099), HSP60 (46611), NDUFS3 (15066-1-AP), DRBP76 (19887-1-AP), COX IV (11242-1-AP), and LONP1 (56266) antibodies were purchased from Cell Signaling Technologies. MNRR1 (19424-1-AP), p62 (18420-1-AP), and YME1L1 (11510-1-AP) antibodies from Proteintech. FLAG (A8592) antibody was purchased from Sigma.

*Chemicals.* Chemicals used in this study include doxycycline (sc-337961) and cycloheximide (sc-3508) purchased from Santa Cruz Biotechnology. MPP+ (ab144783) was purchased from Abcam. Dual luciferase assay kit (E1910) was purchased from Promega. Thermo Fisher Mitochondrial Isolation Kit for Cultured Cells (Thermo Fisher Scientific, 89874).

4.2 Cell lines

HEK 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (HyClone, SH30243), with 10% FBS (Sigma Aldrich, F2442), 1% penicillin-streptomycin (P/S) (Gibco, 15140-122). To generate an MNRR1 KO, HEK 293 cells were transfected with a human MNRR1 CRISPR/Cas9 KO plasmid pair (Santa Cruz Biotechnology, sc-412127 and sc-412127-HDR), followed by selection for puromycin-resistant colonies and clones. Knockout was confirmed by immunoblotting. YME1L1 WT and KO MEF cells were a gift from Dr. Thomas Langer, Institute of Genetics, University of Cologne. The medium for MEFs has been described previously (254). To generate YME1L1 KD in HEK 293, cells were transfected with YME1L1 siRNA (and scramble for control) and then grown in DMEM with 10% FBS and 1% P/S for 48 hours. SH-SY5Y cells were maintained in DMEM/F-12 with L-glutamine (Corning, 10-090-CV) with 10% FBS (Sigma Aldrich, F2442), 1% P/S (Gibco, 15140-122).

All cells were cultured in a humidified incubator at 37 °C. The normoxia-hypoxia experiments were performed as described previously (147).
4.3 Plasmids

All MNRR1 mammalian expression plasmids were cloned into pCI-Neo vector with a c-terminal 3× Flag tag. The WT, Y99E, Y99F, and C-S MNRR1 expression plasmids have been described previously (113, 147). The T61I MNRR1 expression vector was constructed by Genscript. The Ubiquitin-HA expression vector was a gift from Dr. Kezhong Zhang, Wayne State University, Detroit, MI. The MNRR1 ORE luciferase reporter plasmid and the COX4I2 luciferase reporter plasmids have been described previously (114, 147).

The c-terminal 6xhistidine tagged WT and Y99E MNRR1 were cloned into pET15b vector by Dr. Siddhesh Aras, Wayne State University, Detroit, MI. The catalytic domain of YME1L1 in pET15b was purchased from DNASU (HsCD00598203). The T61I MNRR1 expression plasmid in pCold2 vector was a gift from Dr. Manabu Funayama, Juntendo University, Tokyo, Japan (227).

4.4 Transient transfection of cells

Cells were transfected with the indicated plasmids using TransFast transfection reagent (Promega, Madison, WI) according to the manufacturer's protocol. A TransFast:DNA ratio of 2:1 in serum and antibiotic free medium was used. Following incubation at room temperature for ~15 min, the mixture was added to the cells. The plates were incubated overnight at 37 °C followed by replacement with complete medium and further incubation for 48 hours.

4.5 Recombinant protein purification from E. coli

The catalytic domain of YME1L1 in pET15b (DNASU, HsCD00598203) was expressed in BL21 (DE3) competent cells (NEB). Colonies were cultured in LB broth with 100 µg/ml ampicillin (Sigma) at 37°C until an OD₆₀₀ between 0.5 and 0.6. Cells were induced with 1mM IPTG and incubated at 37°C for 16 hours with 240 rpm agitation. Cells were harvested by centrifugation at 3.5k rpm for 10 minutes. Bacterial pellets were suspended in 500 mM KCl, 20 mM Tris pH 7.9, 0.2% NP-40, 10% glycerol, 10 mM Imidazole with protease cocktail inhibitor (PIC) and 5 mM 2-mercaptoethanol. Suspension was frozen at -20°C overnight. Next day, thawed lysate was applied to HIS-Select® iLap® Column (Sigma, H9913) and purified per manufacturer protocol.
MNRR1 bacterial expression vectors (WT and Y99E in pET15b), and T61I in pCold2) were expressed in SHuffle® T7 Express lysY competent cells (NEB). Colonies were cultured in terrific broth with 100 µg/ml ampicillin (Sigma) at 37°C until an OD_{600} between 0.5 and 0.6. Cells were induced with 100 µM IPTG and incubated at 16°C overnight for 16 hours with 240 rpm agitation. Cells were harvested by centrifugation at 4.5k rpm for 45 minutes. Bacterial pellets were suspended in 500 mM NaCl, 40 mM NaH_{2}PO_{4}, and 5% glycerol with PIC and lysozyme. Cell suspension was lysed using a French pressure cell press (AMINCO, American Instrument Co., Silver Spring, MD, USA). Lysate debris was pelleted at 12k RPM for one hour at 4°C. Supernatant was used to purify the HIS-tagged recombinant proteins using HIS-Select® Nickel Affinity gel (Sigma) per manufacturer’s protocol with some modifications. The buffer wash step was repeated 2x to ensure all unbound proteins were exiting the column. A 25 mM and 50 mM imidazole wash steps were included because this showed further removal of non-specific binding of contaminants. The final elution was performed in 150 mM imidazole. Eluted protein was concentrated using Amicron® Ultra 15 ml centrifugal filters (Millipore) and buffer exchanged to 20 mM Tris, pH 7.5, 100 mM NaCl. Protein aliquots were stored at -80°C for long term storage.

4.6 In vitro protease assay

The assay to assess in vitro protease activity has been described elsewhere (255). Briefly, recombinant HIS-tagged YME1L1 and MNRR1 (1 µg) each were combined in a reaction buffer with final conditions of 50 mM Trish-HCl pH 8, 10 mM MgCl_{2}, 25 mM NaCl, 25 mM imidazole, 0.001% NP40, and 0.5% glycerol in a 25 µl total volume. An ATP regeneration system was added to the reactions as well with final concentrations of 50 mM pyruvate kinase, 4 mM ATP, and 80 µg/ml phosphoenolpyruvate. Reactions were incubated at 37°C for one hour and reactions were stopped via the addition of 4xSample Loading Buffer. Reactions were run on SDS-PAGE and the presence of proteins was detected through immunoblotting. Control reactions included a MNRR1 only reaction, as well as tissue purified COX reactions, to show the specificity of the protease.
4.7 Knockdown of YME1L1 in HEK 293 cells

YME1L1 siRNA was purchased from Santa Cruz Biotechnology, Inc. (sc-90696) as was the scramble control. The siRNA was transfected according to manufacturer’s protocol.

4.8 Mitochondrial isolation and sub-fractionation

Cellular mitochondrial and nuclear fractions were isolated from cells with the Thermo Fisher Mitochondrial Isolation Kit for Cultured Cells (Thermo Fisher Scientific, 89874) according to the manufacturer’s protocol. Purity of fractions were determined using organelle specific markers. Submitochondrial fractionation was performed by Dr. Siddhesh Aras (Wayne State University, Detroit, MI) as described previously (256). These experiments are shown in Figure 12A and 12B and were included in the thesis as part of the MNRR1 turnover story to fully illustrate the mechanism of MNRR1 activity during stress.

4.9 Reporter assays

Luciferase reporter assays were performed using the dual luciferase reporter assay kit (E1910, Promega) as described previously (114, 147). Transfection efficiency was normalized with the co-transfected pRLSV40 Renilla luciferase expression plasmid.

4.10 In vitro COX activity

Purified bovine liver COX was a gift from the lab of Dr. Maik Hüttemann, Wayne State University, Detroit, MI. The purified complex was diluted to 3 µM and cardiolipin (Sigma, St. Louis, MO, USA) was added at a 40:1 cardiolipin:COX molar ratio. COX was dialyzed using a 12K-14K molecular weight cut-off membrane in measurement buffer containing 10 mM K-HEPES pH 7.4, 40 mM KCl, 1% Tween-20, and 0.2 mM ATP overnight at 4˚C overnight to remove bound collate, an artifact from purification, and replenish cardiolipin, as described in Lee et. al. (241).

Oxygen consumption was measured using a Clark-type oxygen electrode (Oxygraph System, Hasentech, Pentney, UK) at a final concentration of 30 nM COX in 220 µl measurement buffer. Ascorbate (20 mM) was added to donate electrons to cyt c. Basal oxygen consumption was measured and then purified cyt c (30 nM) (Sigma, C3131) was injected into chamber and oxygen
consumption was measured. Next, buffer, WT, Y99E, or T61I recombinant MNRR1 was injected for final oxygen consumption measurement. Measurements were performed at 25°C. Oxygen consumption was recorded and analyzed using Hansatech Oxygraph software. COX activity was determined as oxygen consumed per minute (min⁻¹).

4.11 Chemical cross-linking and mass spectrometry

Purified and dialyzed bovine liver COX and recombinant WT MNRR1 were used in the cross-linking experiment. A 5:1 molar ratio of MNRR1:COX was used in the presence of 0.25 mM DSS in a buffer containing 100 mM Na₂PO₄, 0.15 M NaCl, pH 7.5. Reactions were incubated on ice for 2 hours. Reactions were quenched using 45 mM Tris-HCl, pH 7.5 for 20 minutes on ice. Identification of cross-linking of recombinant WT MNRR1 to COX was performed with immunoblotting and membrane bands were submitted for mass spectrometry at the Wayne State University Proteomics Core (Detroit, MI, USA) for peptide identification.

4.12 Absorbance spectra of COX

The absorbance spectrum of bovine liver COX in the absence and presence of recombinant WT MNRR1 (my preparation) was determined by Dr. Sachiko Yanagisawa (Hyogo University, Kakogawa, Japan). Oxidized COX spectra was measured from 350-700 nm using UV-VIS absorbance spectroscopy. The molar ratio of recombinant WT MNRR1:COX was 2:1.

4.13 In vitro electron transfer and cytc:COX binding measurements

Assays were performed by Marti Scharlau in the laboratory of Dr. Francis Millett (University of Arkansas, Fayetteville, AR, USA) with materials sent by me as described elsewhere (243). Briefly, flash photolysis and transient absorbance spectroscopy were performed to determine electron transfer rate and binding between ruthenium labeled cytc and bovine heart COX. The dissociation rate constant k_d and the equilibrium dissociation constant K_D = k_d/k_f were measured from 20 to 60 mM NaCl, and the formation rate constant k_f was measured from 40 to 110 mM NaCl. See Scheme 1 below (243).
4.14 **Co-immunoprecipitation and immunoblotting**

Co-immunoprecipitation and immunoblotting were performed as described previously using specific antibodies (113, 114, 147). Primary antibodies were used at 1:1000 dilution and secondary antibodies were used at 1:5000 dilution, unless otherwise specified. The pulldown with the Y99E and Y99F MNRR1 mutants for Bcl-xL interaction was performed by Dr. Siddhesh Aras, Wayne State University, Detroit, MI.

4.15 **Intact cellular oxygen consumption**

Cellular oxygen consumption was measured with a Seahorse XFe24 Bioanalyzer. Cells were plated at a concentration of 30,000 per well for transfection 72 hrs before assay (see Material and Methods section 4.4 for transfection methods). On the day of assay, plates were pre-treated with 250 µM MPP+ or DMSO (vehicle) 7 hours before assay (Pilot assay for optimal MPP+ concentration was done by Dr. Siddhesh Aras, Wayne State University, Detroit, MI). Basal oxygen consumption reads were performed according to the manufacturer's instructions in SH-SY5Y cell culture media.

4.16 **Statistical analysis**

Statistical analyses were performed with MSTAT version 6.1.1 (N. Drinkwater, University of Wisconsin, Madison, WI). The two-sided Wilcoxon rank-sum test was applied to determine statistical significance for p-values. Data were considered statistically significant with p <0.05.

4.17 **Publications**

Chapter I contains materials published in the following reviews:


Chapters II – IV contains materials published in the following research article:

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ABSTRACT

MNRR1: UNDERSTANDING THE ROLE OF A NOVEL MITOCHONDRIAL-NUCLEAR REGULATOR

by

STEPHANIE LYNN GLADYCK

August 2021

Advisor: Dr. Lawrence I. Grossman

Major: Molecular Genetics and Genomics

Degree: Doctor of Philosophy

Mitochondria are complex organelles that generate most of the energy required to sustain life and function in metabolic and signaling pathways required to maintain cellular homeostasis. MNRR1 (mitochondrial nuclear retrograde regulator 1 or CHCHD2) is a small, bi-organelar twin CX2C protein that is emerging as an important regulator of mitochondrial function, apoptosis, and cellular stress by participating in mitochondrial-nuclear crosstalk. Our lab has previously shown that in the mitochondria, MNRR1 regulates complex IV (Cytochrome c oxidase or COX) and is able to finetune the oxidase function through phosphorylation status. We have also shown that during stress, mitochondrial MNRR1 levels deplete, while nuclear levels increase. MNRR1 in the nucleus activates transcription of genes containing an oxygen responsive element (ORE) in their promoter. Additionally, others have shown that MNRR1 acts as an anti-apoptotic molecule through interaction with Bcl-xL. Here we report that a protease responsible for MNRR1 degradation in the mitochondria is YME1L1, a key protease for mitochondrial quality control and for the mitochondrial unfolded protein response (mtUPR). Through this association, the role of MNRR1 in mtUPR was investigated and determined to be a transcriptional activator during mtUPR through its nuclear function. We also report that MNRR1 likely binds near the cytochrome c (cytc) binding pocket located on COX via interaction with COX Vic and that this interaction increases the stability of the cytc:COX when MNRR1 is phosphorylated. We also show that
unphosphorylated MNRR1 preferentially binds to Bcl-xL compared to phosphorylated. Taken together, the findings presented here suggest that phosphorylation of MNRR1 plays a role in finetuning its mitochondrial function. The proposed model is that at the inner mitochondrial membrane, YME1L1 targets the MNRR1 bound to COX during stress to attenuate the production of energy, while the Bcl-xL bound MNRR1 pool remains to prevent apoptosis. At the same time, blocked intermembrane space import results in the accumulation of MNRR1 in the nucleus, to activate a transcriptional response in the ORE. Mutations in MNRR1 are associated with Parkinson’s disease (PD), yet the pathogenic mechanism is not clear. Here, we report that induction of PD in a cell culture model shows that protein levels of MNRR1 and other mtUPR proteins are affected in the presence of the T61I mutant. It was also determined that the PD-associated mutation T61I is defective for nuclear function as a transcription factor in MPP+ treated cells.
AUTOBIOGRAPHICAL STATEMENT

STEPHANIE LYNN GLADYCK

Education
Wayne State University School of Medicine, Detroit, MI
2015-2021  PhD in Molecular Genetics and Genomics, Graduate Research Assistant, Center for Molecular Medicine and Genetics

Madonna University, Livonia, MI
2014-2015  Graduate Certificate in Forensic Science Research, Research Assistant, Madonna University Forensic Science Research Facility

Syracuse University, Syracuse, NY
2013-2015  MS in Forensic Science (Advanced Track), Research Assistant, FNSSI Bioforensics Lab

Madonna University, Livonia, MI
2010-2013  BS in Forensic Science (Minor in Chemistry), Research Assistant, Madonna University Forensic Science Research Facility

Academic Honors and Awards
2019  Thomas Rumble Graduate Fellowship, Wayne State University
2018  First Place, Graduate and Postdoctoral Research Symposium, Wayne State University
2017  Thomas Rumble Graduate Fellowship, Wayne State University
2014  Maxwell School Summer Project Assistantship Grant, Syracuse University
2013  First Place, Research Day, Madonna University
2012-2013  Title III: Strengthening Institutions Program Faculty-Student Collaborative Research, Madonna University
2012  Earhart Emerging Scholars Scholarship, Michigan College Fund
2010-2013  Transfer Student Scholarship, Madonna University

Publications
