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Chchd10, A Novel Bi-Organellar Regulator Of Cellular Metabolism: Implications In Neurodegeneration

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CHCHD10, A NOVEL BI-ORGANELLAR REGULATOR OF CELLULAR METABOLISM: IMPLICATIONS IN NEURODEGENERATION

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ABL2	Abelson murine leukemia viral oncogene homolog 2
ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
ARG	Abelson related gene
ATP	Adenosine triphosphate
cDNA	Complementary DNA
CHCHD10	Coiled-coil Helix Coiled-coil Helix Domain containing protein 10
CHCHD2	Coiled-coil Helix Coiled-coil Helix Domain containing protein 2
CM-H$_2$DCFDA	Chloromethyl- 2',7'-dichlorodihydrofluorescein diacetate
CMT	Charcot-Marie-Tooth
C$_T$	Threshold cycle
CO$_2$	Carbon dioxide
COX	Cytochrome *c* oxidase
CXXC5	CXXC finger protein 5
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ETC	Electron transport chain
EV	Empty vector
FBS	Fetal bovine serum
FTD	Frontotemporal dementia
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEK	Human embryonic kidney

HRP	Horseradish peroxidase
IB	Immunoblot
IgG	Immnoglobulin G
IMM	Inner mitochondrial membrane
IP	Immunoprecipitation
KD	Knockdown
kDa	kilo Dalton
KO	Knockout
MICOS	MItochondrial COntact Site
MND	Motor neuron disease
MNRR1	Mitochondrial Nuclear Retrograde Regulator 1
MS	Mass spectrometry
N_2	nitrogen
NSCLC	Non-small cell lung carcinoma
O_2	Oxygen
OCR	Oxygen consumption rate
OE	Ovexpressing
OMM	Outer mitochondrial membrane
ORE	Oxygen responsive element
OxPhos	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
pY	Phosphotyrosine

RBPJk	Recombination Signal Binding Protein For Immunoglobulin Kappa J Region
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulfate
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
TBS	Tris buffer saline
TCA	Tricarboxylic acid
TCGA	The Cancer Genome Atlas
TE	Tris EDTA
UPR	Unfolder protein response
UV	Ultraviolet
WT	Wild-type

CHAPTER I: INTRODUCTION

1. The structure and origin of mitochondria

Mitochondria (plural; singular-mitochondrion) are thread-like organelles found in the cytoplasm. The word mitochondrion comes from the Greek "mitos," "thread," and "chondrion," "granule," or "grain-like." Mitochondria have come a long way as signposts of cellular health under normal conditions, as well as key determinants of the cell's fate under stress (Duchen, 2004; McBride et al., 2006; Wallace, 2005). Mitochondria have an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) separated by an intermembrane space (IMS). The IMM is highly folded to form cristae, which surround the mitochondrial matrix (Figure 1).
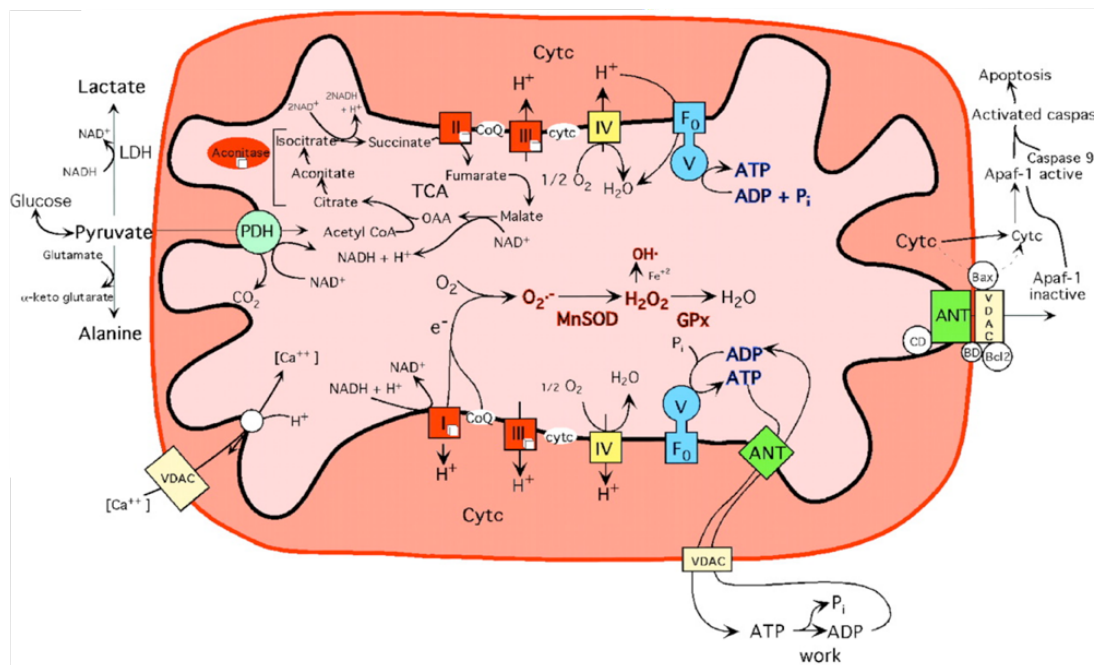
Figure 1. Diagrammatic representation of a single mitochondrion and the process of oxidative phosphorylation (Wallace, 2005)**.**

Besides a highly specialized IMS that distinguishes mitochondria from other organelles, mitochondria are unique because they harbor their own distinct genome in the mitochondrial matrix. The physical separation of the nuclear and mitochondria genomes allows discrete regulation of mitochondrial genomic DNA replication, transcription and translation, but with the

assistance of several proteins encoded in the nuclear genome. Human mitochondria genomic DNA is a 16,569 bp circular molecule (Figure 2). This encodes for 22 transfer RNAs, two subunits of mitochondrial ribosomal RNA (12S and 16S), and thirteen protein coding genes for oxidative phosphorylation (OxPhos, described in detail in section 2.1.) (Anderson et al., 1981). The coding region of the mitochondrial genome has two strands - a heavy strand (H-strand) and a light strand (L-strand) and the non-coding part consists of the D-loop region (Kasamatsu et al., 1971), which regulates transcription and replication of mtDNA (Fish et al., 2004). Mitochondrial transcription is initiated at the two promoters - the H-strand promoter (HSP) and the L-strand promoter (LSP). The H-strand encodes both the ribosomal RNAs, 12 messenger RNAs that code for OxPhos proteins, and 14 transfer RNAs whereas the L-strand encodes 1 messenger RNA and 8 transfer RNAs. Both strands generate long polycistronic RNA chains that are subsequently cleaved precisely into separate RNA species via an endonuclease. The L-strand also generates a primer for the replication of mtDNA. Using this primer and the H-strand as a template, replication is initiated at the O_H (on the L-strand) followed by replication initiation at the O_L (on the H-strand) (Clayton, 1991).
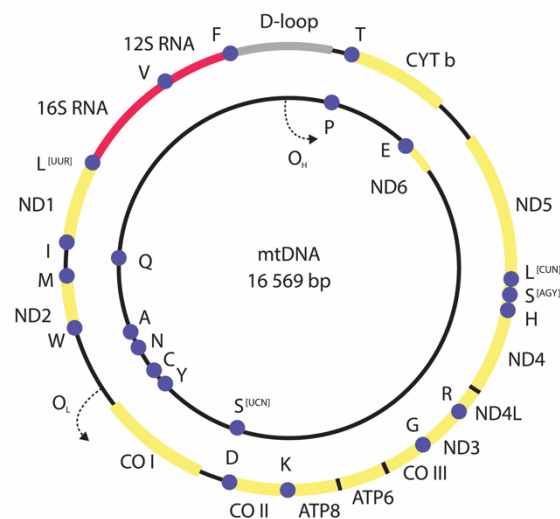
Figure 2. The human mitochondrial genome. The mitochondrial genome is 16569 bp and has two strands, the heavy strand (outer) and the light strand (inner). From the 37 genes (22 tRNAs, 2 rRNAs and 13 proteins), 28 genes (14 tRNAs, 2 rRNAs and 12 proteins) are encoded on the heavy strand and 9 (8 tRNAs and 1 protein) are encoded on the light strand. The non-coding D-loop regulates transcription and replication (Chinnery and Schon, 2003).

Due to their distinct structural features and the presence of their own genome, mitochondria are believed to originate from the endosymbiosis of small oxygen-utilizing and energy-generating cells within larger host cells. There are 2 competing theories to explain the origin of mitochondria and they have different considerations regarding the properties of the host, the endosymbiont and the ecological interactions that lead to the physical association of these (Embley and Martin, 2006). As per the first theory, a large, nucleus-bearing anaerobic eukaryote actively engulfed a small prokaryotic cell via phagocytosis that provided the anaerobe with the capacity to detoxify oxygen in the extracellular environment (ibid). Thus, mitochondria are obligate intracellular organelles. A second theory posits that the host was a archaebacterium or a larger prokaryotic cell that acquired a smaller prokaryotic facultative anaerobic cell that provided the host with energy, forming the first eukaryotic ancestor (ibid). In either case, mitochondria are now an essential part of the eukaryotic cell. Besides providing energy, mitochondria have now evolved to perform a diverse number of functions that are described below.

2. Mitochondria and their role in cellular physiology

2.1. Energy production

The basic energetic function of mitochondria is to couple electron flow to the generation of an electrochemical gradient that is ultimately used to generate energy in the form of ATP. In order to generate energy, mitochondria utilize reducing equivalents (NADH and $FADH_2$) generated from glycolysis and the tricarboxylic acid (TCA) cycle. These are oxidized, and the electrons are sequentially passed through a series of protein complexes embedded in the IMM. This process is initiated at either Complex I or Complex II that oxidize NADH and $FADH_2$, respectively, and the electrons generated during these steps are passed sequentially to complex III, and then to complex IV. Two mobile electron carriers are also part on this process. Ubiquinone transfers electrons from Complex I or II to Complex III and cytochrome c transfers these from Complex III to Complex IV. Ultimately, Complex IV uses these to reduce molecular oxygen to

water. These four protein complexes together form the electron transport chain (ETC). During this process, protons are pumped across the IMM to the IMS by all complexes except Complex II. The potential energy stored in this electrochemical gradient is utilized by Complex V (ATP synthase) to phosphorylate ADP, forming ATP, the main energy currency of the cell. During these steps, since oxygen is consumed and ADP is phosphorylated to ATP, this process is known as oxidative phosphorylation (OxPhos) (Figure 1).

2.2. Apoptosis

Mitochondria are important for producing energy required for cellular growth, differentiation, signaling, and protein folding and degradation under healthy conditions. Under conditions of cellular stress they regulate cell death through the process of apoptosis. The term apoptosis is derived from the Greek word for "falling off." Apoptosis is programmed cell death and is an essential part of physiological processes such as tissue remodeling during embryogenesis (Glucksmann, 1951) or may be initiated under cellular stress for removal of dead and damaged cells (Henson and Hume, 2006).

The first step that signals the apoptotic cascade is the release of cytochrome *c*, an important component of the ETC from the IMS into the cytoplasm. This cytoplasmic cytochrome *c* interacts with Apaf-1 forming the heptameric apoptosome (Liu et al., 1996; Zou et al., 1997), which activates the caspase cascade though cleavage of procaspase 9 (Acehan et al., 2002). Activation of the caspase cascade triggers programmed cell death through downstream "executioner" caspases such as caspase 3 (Li et al., 1997). Ultimately this gives rise to the classical apoptotic features of nuclear condensation and formation of apoptotic bodies (Kerr et al., 1972). Apoptosis is inhibited by anti-apoptotic proteins such as BCL-2 that prevent this release of cytochrome *c* (Yang et al., 1997). Excess or premature apoptosis is often associated with many neurodegenerative diseases, ischemic damage, and autoimmune disorders whereas inhibition or inactivation of apoptosis are linked to many types of cancer (Green and Reed, 1998; Wang and Youle, 2009).

2.3. Generation of reactive oxygen species

One of the major consequences of mitochondrial respiration is the generation of unpaired electrons. These electrons interact with oxygen to form highly reactive free radical species such as superoxide and peroxide known as reactive oxygen species (ROS). ROS generation in the mitochondria occurs at Complex I and III of the ETC. These ROS are released into the matrix by Complex I and into the matrix as well as the IMS by complex III (St-Pierre et al., 2002) Originally it was estimated that ~2% of the total oxygen consumed was diverted towards ROS generation. These estimates were made under non-physiological conditions; *in vivo* this number is actually much smaller, ~ 0.15% - 0.2% (Balaban et al., 2005; St-Pierre et al., 2002). ROS under normal physiological conditions are necessary for cellular signaling whereas under pathological conditions high levels cause damage to proteins (Imlay and Fridovich, 1992; Sablina et al., 2005), lipids (Montine et al., 2007; Vercellotti et al., 1991), and DNA (Jovanovic, 1997; Woo et al., 2012).

2.4. Calcium homeostasis

Mitochondria are essential to the maintenance of calcium levels in the cytoplasm. They import calcium via the mitochondrial calcium uniporter (MCU) (Baughman et al., 2011) as well as via calcium exchangers (Na^+/Ca^{2+} and H^+/Ca^{2+}) (Bernardi, 1999). Altered import of calcium may be a way to communicate changes and activate either OxPhos or apoptosis in the mitochondria under various pathologies (Giorgi et al., 2012). Excess mitochondrial calcium also activates the formation of a permeability transition pore (PTP), which releases the content of the IMS, particularly cytochrome *c*, into the cytoplasm to initiate apoptosis. The protein composition of PTP is still under debate, although there is evidence that several OMM, IMS, and IMM proteins are involved in its regulation, namely adenine nucleotide translocase, hexokinase, peripheral-type benzodiazepine receptor, voltage dependent anion channel, mitochondrial creatine kinase, and cyclophilin D (Cyp D) (Kroemer et al., 2007).

While the mitochondria play an important role in calcium signaling, the endoplasmic reticulum (ER) is the major organelle for storage of calcium (Somlyo et al., 1985). However,

mitochondria extensively associate with the ER, forming mitochondria-associated membranes (MAM). These can be identified using electron microscopy, which show the ER tubules closely apposed to mitochondria, forming the MAM. The MAM is now considered a distinct compartment with a unique proteome and biochemical properties and is an important regulator of calcium homeostasis (Patergnani et al., 2011) that has been associated with Alzheimer's disease (Area-Gomez and Schon, 2017).

2.5. Lipid homeostasis

Mitochondria constantly change their shape and size forming dynamic tubular networks in cells. This occurs via mitochondrial fusion and fission events to redistribute mitochondria-derived metabolites in response to extracellular cues. In order to provide the structural flexibility for these processes mitochondrial membranes (OMM and IMM) are enriched with several lipids. Some of these lipids such as phosphatidylglycerol and cardiolipin are autonomously synthesized in the mitochondria. Others are synthesized in part in mitochondria (phosphatidylethanolamine, phosphatidic acid, and CDP-diacylglycerol) or are imported (phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sterols, and sphingolipids). These lipids are distributed asymmetrically across the IMM and OMM and their distribution varies from species to species as well as different cell types (Horvath and Daum, 2013). These lipids also provide structural support for the components of OxPhos and import machinery in the mitochondria (Martensson et al., 2017). Mitochondrial membranes also exchange lipids with the ER and MAMs to facilitate cellular lipid homeostasis (Tatsuta et al., 2014). Due to these important effects, altered lipid metabolism in the mitochondria and MAMs has become an important mode of stress signaling (Kim et al., 2016) and is associated with disease (Aufschnaiter et al., 2017; Paradies et al., 1998; Porporato et al., 2016).

2.6. Iron homeostasis

Mitochondria are essential for the synthesis of heme and Fe-S clusters and hence are critical in iron homeostasis. Free iron is highly toxic due to its intrinsic ability to generate free

radicals through the Fenton reaction. This occurs under conditions of mitochondrial iron overload where the iron (II) reacts with hydrogen peroxide to form OH• radical and Fe (III). The Fe (III) may further react with hydrogen peroxide to form O_2^- and participates in other chain reactions (Halliwell and Gutteridge, 1984).The radicals promote DNA degradation and lipid peroxidation (Gutteridge, 1986). Defects in the assembly of mitochondrial Fe-S clusters are associated with mitochondrial iron overload and blood disorders (Chen and Paw, 2012).

3. Electron Transport Chain
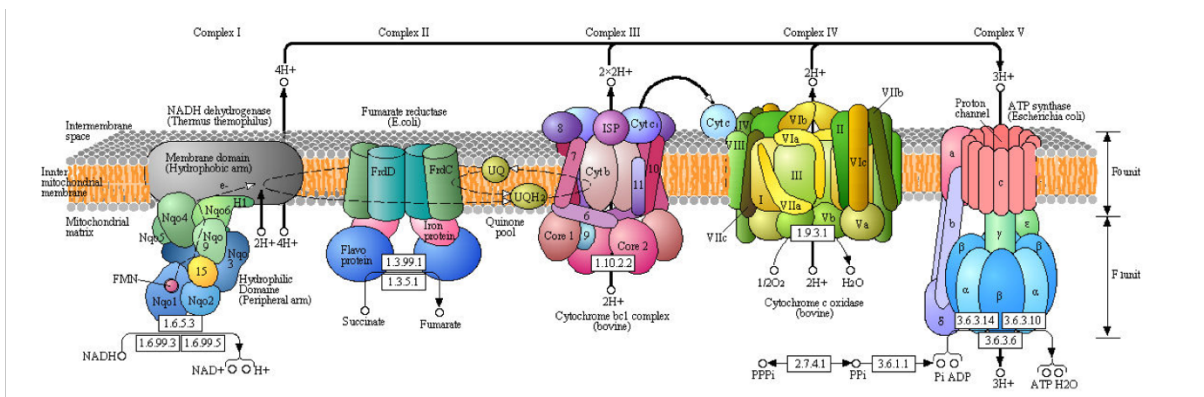
	Complex I	Complex II	Complex III	Complex IV	Complex V
Nuclear	37	4	10	10	14
Mitochondrial	7	0	1	3	2

Figure 3. **Components of the electron transport chain.** Diagrammatic representation of the components of the mitochondrial electron transport chain (above) with the number of subunits encoded by the mitochondrial and nuclear genomes (below the figure). Image source: KEGG pathway database.

Mitochondrial energy production occurs via the electron transport chain. Each of these complexes is composed of several subunits. The genes for these subunits are encoded in the nuclear as well as the mitochondrial genome as depicted in Figure 3.

3.1. Complex I

Complex I (NADH: ubiquinone oxidoreductase) is the largest of the ETC complexes and has 44 subunits. Of these 37 are nuclear encoded and 7 are encoded by the mitochondria genome. Two electrons are removed from NADH and ultimately transferred to the lipid-soluble carrier ubiquinone (UQ), forming ubiquinol (UQH$_2$). Complex I extrudes four protons (H$^+$) across the

membrane during this process thereby contributing to the proton gradient. Besides the "classical" Complex I found in higher organisms, lower organisms may have single subunit enzymes that carry out the typical NADH: quinone oxidoreductase function. The respiratory chain of the facultative aerobic yeast *Saccharomyces cerevisiae* does not contain a complex I. The oxidation of cytosolic and matrix NAD(P)H is performed by two external (NDE1 and NDE2) (Luttik et al., 1998; Small and McAlister-Henn, 1998) and one internal (NDI1) NAD(P)H dehydrogenases (Marres et al., 1991). These belong to the Type II category and other organisms such as bacteria may also have Type III (e.g., Nqr in *Vibro cholerae*) (Melo et al., 2004). Besides the reduced complexity of the enzyme, Type II differ from Type I NADH dehydrogenases because they are insensitive to the classical inhibitors of Complex I such as rotenone and Type III are Na^+-translocating NADH: quinone oxidoreductases (ibid). Complex I deficiency is the most frequently encountered enzyme deficiency in patients with a mitochondrial disorder and pathogenic mutations have been identified in 26 subunits thus far (Rodenburg, 2016).

3.2. Complex II

Complex II (succinate dehydrogenase) is the smallest of the ETC complexes and is composed of 4 nuclear encoded subunits. This complex is an important link between OxPhos and the TCA cycle because it is both part of the electron transport chain, and it catalyzes the conversion of succinate to fumarate in the TCA cycle. Two electrons are removed from $FADH_2$ and transferred to UQ by this complex without pumping of protons. Mutations in all 4 subunits have been associated with a number of cancers as well as with leukodystropy, Leigh syndrome and cardiomyopathy (Hoekstra and Bayley, 2013).

3.3. Complex III

Complex III (ubiquinol cytochrome *c* reductase) is composed of 11 subunits and only 1 is encoded by the mitochondrial genome. UQH_2 from Complex I and II is utilized by Complex III and the electrons are transferred to cytochrome *c*. During these steps, four H^+ are extruded by this complex thereby contributing to the proton gradient. Mutations in one mitochondrial encoded

subunit (for cytochrome *b*) and two nuclear encoded subunits of complex III have been associated with LHON (Leber's hereditary optic neuropathy), myopathy and encephalopathy (Benit et al., 2009).

Complex IV will be discussed in detail in Section 4.

3.4. Complex V (F$_O$F$_1$ ATP synthase)

Classically, the last member of the ETC is considered to be Complex IV since the electrons are passed to the terminal acceptor, molecular oxygen, which is reduced to water. However, ATP synthase is also described as Complex V of the electron transport chain since it performs the last functional step of OxPhos by utilizing the proton gradient to generate ATP from ADP. This complex is composed of 16 subunits - 2 mitochondrial encoded and the remaining ones encoded by the nuclear genome. From these, mutations in both mitochondrial-encoded subunits and one nuclear encoded subunit have been associated with LHON, FBSN (Familial Bilateral Striatal Necrosis), NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa), and encephalopathy (Kucharczyk et al., 2009).

4. Complex IV (Cytochrome *c* oxidase)

Complex IV, also known as cytochrome *c* oxidase (COX), is the terminal electron acceptor of the ETC. This enzyme accepts electrons from Complex III transported via cytochrome *c* and uses these to reduce molecular oxygen to water. During this process four H$^+$ are translocated across the IMM, contributing to the proton gradient. Complex IV is a dimeric enzyme, where each monomer of COX consists of 13 subunits (Figure 4). NDUFA4, originally a part of Complex I has been proposed as the 14th subunit, (Balsa et al., 2012). However, its identity as a bona fide subunit of COX has been recently contested (Kadenbach, 2017; Pitceathly and Taanman, 2018).
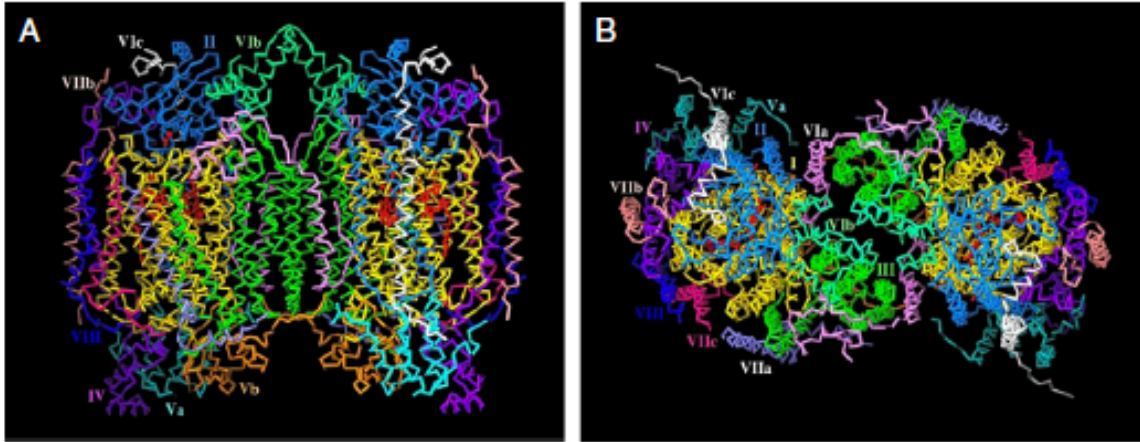
Figure 4. Ribbon diagram for subunits of Complex IV. The Cα backbone trace of the dimer of bovine heart COX at 2.8 Å resolution. Each subunit has been annotated with a distinct color. *A,* A view of the transmembrane surface and *B*, a view from the cytosolic side (Yoshikawa et al., 2012).

This complex is of interest to our lab because it is an important regulator of oxidative phosphorylation and is responsible for the utilization of >90% of the oxygen consumed by a cell (Babcock and Wikstrom, 1992) and is proposed to be the rate-limiting step for OxPhos (Villani et al., 1998).

COX function is tightly regulated both at the genetic as well as at the protein level. The three largest subunits are encoded *in situ* in the mitochondria (Anderson et al., 1981) and many of the nuclear encoded subunits have tissue-specific isoforms (Table 2) and there is highly controlled synthesis of the subunits aided by a host of assembly factors (Barrientos et al., 2009; Pecina et al., 2004; Shoubridge, 2001; Torraco et al., 2015). Some of the subunits such as COX I (Lee et al., 2005) and COX IV (Steenaart and Shore, 1997) are post-translationally modified, and the unassembled subunits are rapidly turned over (Nakai et al., 1995; Nijtmans et al., 1995; Weber et al., 1996).

Gene	Tissue enrichment	Reference
COX4I1	ubiquitous	(Huttemann, 2000)
COX4I2	lung (high); placenta, heart and brain (low)	(Hüttemann, 2000)
COXVIaL/COXVIa1	liver*	(Schmidt et al., 1997)

COXVIaH/COX6VIa2	heart and skeletal muscle	(Anthony et al., 1993; Chacinska et al., 2004; Schmidt et al., 1997)
COXVIb1	somatic (also in testes in humans)	(Tsukihara et al., 1996)
COXVIb2	testes (exclusively to testes in rats and mice)	(Weishaupt and Kadenbach, 1992)
COXVIIa1	heart and skeletal muscle	(Jaradat et al., 1998)
COXVIIa2	liver*	(Jaradat et al., 1998)
COXVIIaR/COXVIIa2L	ubiquitous, higher in kidney and liver	(Segade et al., 1996)
COXVIIIH/COXVIII1	ubiquitous	(Goldberg et al., 2003; Rizzuto et al., 1989)
COXVIIIL/COXVIII2	heart and skeletal muscle	(Goldberg et al., 2003; Rizzuto et al., 1989)
COXVIIIaC/COXVIII3	Testes, pancreas, placenta	(Huttemann et al., 2003)

Table 1. Tissue-specific isoform of various nuclear encoded COX subunits. List of various isoforms of COX subunits that have been identified, and the tissues in which they are found to be enriched. Liver* - during heart and skeletal muscle development, there is an isoform class switch from the liver (nonmuscle form) to the muscle isoform. The liver-type isoforms are thought to be ubiquitously expressed, while the heart-type subunits (VIaH, VIIaH, and VIIIH) are expressed in the heart and skeletal muscle (Kuhn-Nentwig and Kadenbach, 1985)

Additional regulation may also be exerted by the binding of small molecules. ATP allosterically inhibits COX (Arnold et al., 1998; Frank and Kadenbach, 1996) whereas 3,5-diiodothyronine abolishes this inhibition (Arnold and Kadenbach, 1999). Nitric oxide may competitively inhibit binding of oxygen to the catalytic site of COX (Brown, 2001).

Besides small molecules, small proteins such as BCL2 and HIGD1A can also bind and regulate COX activity. BCL2 binds to COXVa, stabilizing its levels under oxidative stress (Chen and Pervaiz, 2010). HIGD1A (hypoxia inducible domain family, member 1A) is induced by 1% O_2, binds to the active center of COX, and enhances its activity. Overexpression of HIGD1A increases oxygen consumption and ATP production (Hayashi et al., 2015).

In our lab we study two novel regulators of Complex IV, MNRR1 (Mitochondrial Nuclear Retrograde Regulator 1, also known as CHCHD2 (Coiled-coil Helix Coiled-coil Helix Domain containing protein 2) and CHCHD10. These have come to light in recent years due to the expanding spectrum of diseases that both proteins have been associated with, including

neurodegeneration and cancer (Grossman et al., 2017; Modjtahedi et al., 2016). MNRR1 was first identified in our lab as a regulator of mitochondrial function by acting in the mitochondria as well as the nucleus (Aras et al., 2017; Aras et al., 2015; Aras et al., 2013) and I have characterized the role of CHCHD10 in these two compartments for my graduate work.

5. Twin CX$_9$C Proteins

MNRR1 and CHCHD10 belong to the twin CX$_9$C family of proteins. As the name suggests, this family is composed of proteins that contain two pairs of cysteine residues separated by nine amino acids, necessary for proper import and retention within the mitochondrial IMS (Muller et al., 2008). CHCHD4 (Mia40 in yeast) and ALR (Augmenter of Liver Regeneration; Erv1 in yeast) form a redox relay system (ibid) that oxidizes the two pairs of cysteine residues, thereby forming the prototypical Coiled-coil Helix Coiled-coil Helix Domain (Figure 5). This oxidation prevents escape of MNRR1, CHCHD10, and other twin CX$_9$C proteins to the cytoplasm, where they would be rapidly degraded by the proteasomal machinery (Bragoszewski et al., 2013).

Besides its role in protein import, CHCHD4 is also necessary for HIF1α stabilization under hypoxia and high expression is correlated with high tumor grade and low patient survival (Yang et al., 2012). Two other well characterized members of this family are CHCHD3 and CHCHD6. Both were identified as proteins necessary for maintenance of mitochondrial structure (An et al., 2012; Darshi et al., 2011) and hence are a part of the MICOS (MItochondrial COntact Site) complex (Pfanner et al., 2014). Other twin CX$_9$C proteins that are less studied include CHCHD1, which is necessary for mitochondrial translation (Koc et al., 2013), and CHCHD5, which is required for cellular growth and as a marker overexpressed in breast and colon cancer (Babbar et al., 2018).

Structurally, CHCHD5 is unique due to the presence of two sets of twin CX$_9$C motifs forming 2 CHCH domains. The crystal structure of only one twin CX$_9$C protein, CHCHD4 (as Mia40), has been determined (Kawano et al., 2009) at a resolution of 3 Å and the NMR (Nuclear

Magnetic Resonance) structures of human CHCHD5 and CHCHD7 have been solved (Banci et al., 2012).
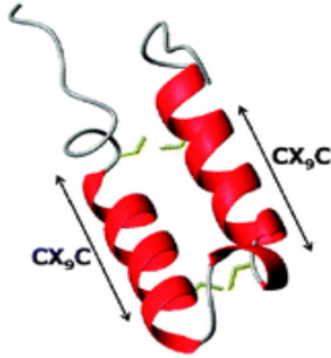
Figure 5. Diagrammatic representation of the Coiled-coil Helix Coiled-coil Helix Domain. Representative image of a CHCHD domain from CHCHD4 (Cavarallo 2010).

MNRR1 and CHCHD10, are also members of the twin CX_9C family originally identified by bioinformatics approaches as modulators of OxPhos. MNRR1 was picked up on a screen of genes necessary to regulate OxPhos (Baughman et al., 2009) and CHCHD10 as a heart and skeletal muscle enriched mitochondrial gene that is required for optimal COX activity (Martherus et al., 2010). MNRR1 and CHCHD10 are 58% identical and arose due to a gene duplication event that predates the human-mouse speciation (Figure 6). There are several common orthologs and these have been described in detail in section 5 of this chapter.

The depletion of either MNRR1 or CHCHD10 results in decreased oxygen consumption (Aras et al., 2015; Baughman et al., 2009; Burstein et al., 2017; Martherus et al., 2010; Straub et al., 2017). This is similar to the functioning of other twin CX_9C family members such as CHCHD3 and CHCHD4, which are also found in the mitochondria and regulate oxygen consumption (Darshi et al., 2011; Yang et al., 2012). It is clear from several studies that MNRR1 and CHCHD10 are both necessary for maintenance of mitochondrial structure (Straub et al., 2017; Woo et al., 2017) and function (Meng et al., 2017; Straub et al., 2017; Woo et al., 2017); our unpublished data), regulation of apoptosis (Genin et al., 2016; Liu et al., 2015), and optimal electron transport chain

function (Aras et al., 2015; Bannwarth et al., 2014; Martherus et al., 2010; Meng et al., 2017; Straub et al., 2017), as are several other members of this family (Modjtahedi et al., 2016; Zhou et al., 2017b).

What distinguishes MNRR1 and CHCHD10 from the other twin CX_9C family members is the causal association of several mutations in both proteins with a myriad of neurodegenerative disorders, in particular ALS for CHCHD10 (Bannwarth et al., 2014; Chaussenot et al., 2014; Chio et al., 2015; Dols-Icardo et al., 2015; Johnson et al., 2014; Jokela et al., 2016) and Parkinson's disease for MNRR1 (Funayama et al., 2015; Ikeda et al., 2017; Koschmidder et al., 2016; Meng et al., 2017). These have led to several different studies focusing on these mutations in animal models (Burstein et al., 2017; Meng et al., 2017; Woo et al., 2017) and in cell culture using patient fibroblasts (Bannwarth et al., 2014; Brockmann et al., 2018; Genin et al., 2016; Straub et al., 2017). Moreover, altered protein or transcript levels for both MNRR1 and CHCHD10 have been identified in several cancers including breast cancer (Lamb et al., 2014), non-small cell lung carcinoma (Wei et al., 2015), hepatocellular carcinoma (Song et al., 2015), thyroid follicular carcinoma (Lai et al., 2017) for MNRR1 and prostate (Chen et al., 2012) and ovarian cancer (Cheng et al., 2010) for CHCHD10. Besides these effects, both MNRR1 and CHCHD10 have also been associated with pleiotropic effects. Depletion of MNRR1 and CHCHD10 decreased cellular growth (Aras et al., 2013; Straub et al., 2017) and enhanced ROS levels (Aras et al., 2015; Meng et al., 2017; Woo et al., 2017). MNRR1 is also required for differentiation to a neuroectordermal lineage (Wei et al., 2015; Zhu et al., 2016) and cell migration (Seo et al., 2010; Wei et al., 2015).

Another feature that makes the MNRR1-CHCHD10 pair novel, is that a fraction of each protein is also localized to the nucleus, where each regulates the transcription of genes controlling OxPhos (Aras et al., 2015; Aras et al., 2013; Woo et al., 2017). None of the other twin CX_9C family
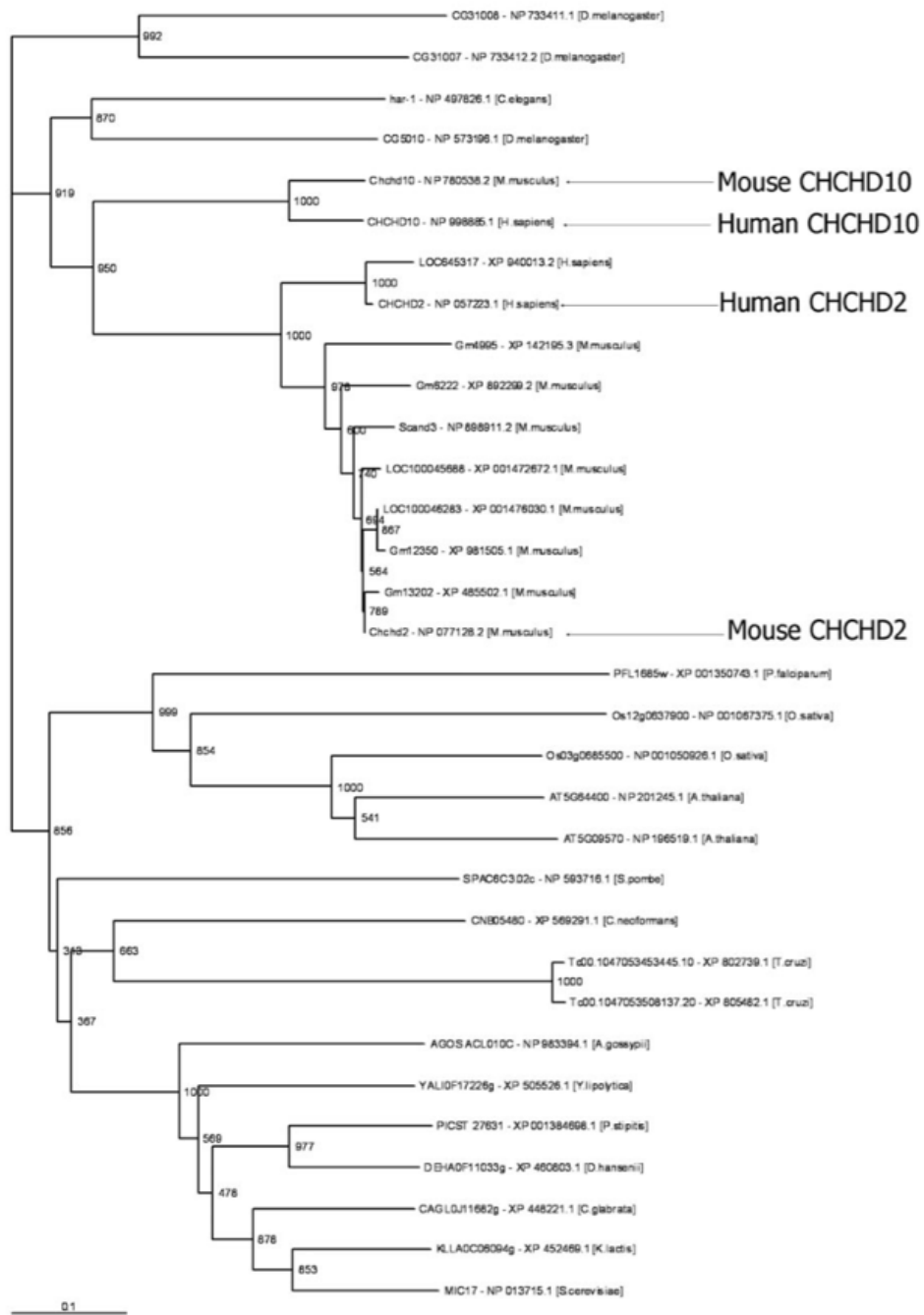
 Figure 6. Neighbor-joining tree built from the multiple alignment of protein homologs for MNRR1 and CHCHD10. The *MNRR1* and *CHCHD10* genes present in *H. sapiens* and *M. musculus* appear to have originated from a duplication predating their speciation (Cavarallo 2010).

members have been characterized to also be present the nucleus and affect transcription with

the exception of CHCHD3 (Liu et al., 2012). A notable feature that sets these two proteins apart

from the other twin CX_9C members is the fact they are 58% identical and yet associated with distinct pathologies.

This observation and the bi-organellar localization of both proteins indicates that they may be involved in regulation of signaling from the mitochondria to the nucleus under different conditions. Hence, understanding MNRR1 and CHCHD10 function both independently, and as a regulatory pair, makes for an intriguing model for retrograde mito-nuclear signaling in cell physiology. Understanding how the proteins work to regulate the same process under different conditions or how the differences between the two proteins may give rise to different effects under the same stress, are key to learning how mutations in either cause so many different pathologies.

6. Orthologs for CHCHD10 and MNRR1

MNRR1 and CHCHD10 are 58% identical and arose from a gene duplication that predates the human-mouse speciation (Cavallaro, 2010). Table 1 summarizes various orthologs for both MNRR1 and CHCHD10. Both proteins have a common homolog in yeast (Mix17, formerly known as Mic17), worms (*har1*), and flies (CG5010). Knockdown of Mix17 decreases oxygen consumption (Longen et al., 2009) is predicted to regulate cell division (Cavallaro, 2010). Originally, however, Mix17p was characterized as a nuclear (Huh et al., 2003) stress responsive protein (Tkach et al., 2012). Another study (Gabriel et al., 2007) considered the possibility that the C-terminal GFP-tag on Mix17, since it is a twin CX_9C protein, prevents its entry into the mitochondria, allowing it to localize to the nucleus. They showed that untagged Mix17p does localize to the mitochondrial IMS.

Another common ortholog for CHCHD10 and MNRR1, CG5010, found in *Drosophila melanogaster,* has been characterized and shown to also regulate ATP levels, mitochondrial morphology, and apoptosis (Meng et al., 2017). In the case of worms (*Caenorhabditis elegans*), *har1* is necessary to maintain ATP levels (Zubovych et al., 2010), mitochondrial networks formation (ibid), reduced ROS levels (Woo et al., 2017), and longevity (ibid). Disease-associated mutations in MNRR1 and CHCHD10 have been studied in a fly model (Meng et al., 2017) and

worm (Woo et al., 2017), respectively. However, in both studies the authors did not consider the fact that CHCHD10 and MNRR1have a common ortholog. It is also important to note that most of the worm and fly orthologs more closely resemble MNRR1, an issue when human orthologs are overexpressed in these models. Specifically, in the Woo study that analyzed *har1*-KO worms, overexpressing WT human *CHCHD10* resulted in only a complementation of the KO phenotype (increased ROS, shortened life span). If human CHCHD10 was indeed entirely sufficient to replace har1, one would predict that overexpression would enhance lifespan as compared to WT worms and decrease ROS production to levels below that of WT worms. The higher similarity of har1 to MNRR1 may explain why these effects were not seen when only human CHCHD10 was overexpressed without MNRR1 co-overexpression in the same system. Further, it highlights that, despite the high similarity, MNRR1 and CHCHD10 have some functions that are distinct, and the model system must be chosen with care in regard to the orthologs of one's protein of interest.

Organism	MNRR1	CHCHD10
Saccharomyces cerevisiae	Mic17 (42% identity)	Mic17 (42% identity)
Caenorhabditis elegans	har1 (52% identity) (Zubovych et al., 2010)	har1 (41% identity) (Woo et al., 2017)
Drosophila melanogaster	CG5010 (61% identity) (Meng et al., 2017)	CG5010 (41% identity)
Danio rerio	CHCHD2 (60% identity)	CHCHD10 (72% identity) (Brockmann et al., 2018)
Mus musculus	Chchd2 (86% identity)	Ndg2 (87% identity) (Burstein et al., 2017)

Table 2. **Orthologs for MNRR1 and CHCHD10 in model organisms**. List of orthologous genes for MNRR1 and CHCHD10 used for studying protein function or disease models. The percent identity for each organism is with respect to the corresponding human ortholog followed by any studies using this ortholog in a model system.

There are other systems where *MNRR1* and *CHCHD10* are independently conserved such as zebrafish and mouse. In both these organisms only *CHCHD10* knockout/knockdown have been analyzed so far. For the zebrafish system, a knockdown of *CHCHD10* affected motor neuron

function by decreasing axon length and altered muscle myofibrillar structure, giving rise to motility defects (Brockmann et al., 2018). However, in the *CHCHD10* knockout mouse model, no distinct phenotype was observed (Burstein et al., 2017). The absence of a phenotype in the mouse system indicates that *CHCHD10* may be regulated differently in mice as compared to humans.

7. Similarities and differences between CHCHD10 and MNRR1

Some similarities and differences between CHCHD10 and MNRR1 have been highlighted in Table 2. Both proteins have clear roles in maintenance of mitochondrial function (Aras et al., 2017; Aras et al., 2015; Aras et al., 2013; Bannwarth et al., 2014; Baughman et al., 2009; Martherus et al., 2010; Straub et al., 2017; Woo et al., 2017). The nuclear presence of both has been confirmed in yeast (Mic17p; Huh 2003) and humans (Aras et al., 2017; Aras et al., 2015; Aras et al., 2013; Woo et al., 2017). MNRR1 functions as a transcriptional activator at the promoter of genes harboring an oxygen responsive element (ORE) (Aras et al., 2013) and is upregulated at distinct oxygen tensions – 4% O_2 cell culture system (Described in detail in section 8 of this chapter). CHCHD10's role in the nucleus is yet to be clarified. Woo et al. were the first to show that a fraction of CHCHD10 is in the nucleus and it is imported via a complex formed with TDP-43. Knockdown of CHCHD10 decreases transcript levels for *COX4I2, NDUFS3* and *NDUFB6*. They also show that the reduced transcript levels for *NDUFS3* and *NDUFB6* are rescued with WT-CHCHD10 overexpression, but do not show these results for *COX4I2*. Further, they show that CHCHD10 mutants that do not localize to the the nucleus as well as WT due to which confounds the interpretation of their data.

	MNRR1	CHCHD10
Protein length	151	142
CHCH domain	114-144	102-132
Expression (Human Protein Atlas)	Expressed in all tissues at medium to high levels	Muscle, heart, liver (high), brain (medium) and low levels for other tissues

Mitochondrial function	Regulation of COX activity, ROS level (Aras et al., 2015), apoptosis (Meng et al., 2017) (Liu et al., 2015) 2014)	Regulation of COX activity and ATP levels (Martherus et al., 2010), cristae morphology ((Bannwarth et al., 2014) Genin (Genin et al., 2016) 2016), apoptosis (Genin et al., 2016), (Woo et al., 2017)
Nuclear function	Transcriptional activator for *COX4i2* (Aras et al., 2013) and itself (Aras et al., 2015)	Transcriptional activator* for *COX4i2*, NDUFB6 and NDUFS3 (Woo et al., 2017)
Hypoxia sensitivity	Upregulated at 4% oxygen (Aras et al., 2013)	Unknown
Interactions identified using mass spectrometry (Bio Grid database)	102 total unique interactors (Common interactors for both: C1QBP, CHCHD2, CHCHD10, NDUFS3, NDUFA8, COX5A, COX6A1, COX6C, ATP5C1, ATP5F1, ATP5H, ECH1, CLPX, PITRM1, POLDIP2, RNASEH1, TIMM44, TIMM50, USMG5, GHITM)	70 total unique interactors (Common interactors for both: C1QBP, CHCHD2, CHCHD10, NDUFS3, NDUFA8, COX5A, COX6A1, COX6C, ATP5C1, ATP5F1, ATP5H, ECH1, CLPX, PITRM, POLDIP2, RNASEH1, TIMM44, TIMM50, USMG5, GHITM)
Post-translational regulation	Phosphorylated at Y99 by ARG which activates mitochondrial function (Aras et al., 2017)	Unknown
Disease association (altered protein/transcript levels)	Huntington's disease (Feyeux et al., 2012), Lissenchephaly (Shimojima et al., 2015), Hepatocellular carcinoma (Song et al., 2015), breast cancer (Lamb et al., 2014), non-small cell lung carcinoma (Wei et al., 2015), follicular carcinoma (Lai et al., 2017)	Ovarian cancer (Cheng et al., 2010), Gastric cancer (Chen et al., 2012)
Mutation in protein associated with disease	Parkinson's disease, Lewy body disorder, Charcot-Marie-Tooth disease type 1A Multiple system atrophy (specific references and mutations summarized in Table 5)	Mitochondrial myopathy, Amyotrophic Lateral Sclerosis, Alzheimer's disease, Frontotemporal dementia, Cerebellar ataxia, Spinomuscular atrophy, Charcot-Marie-Tooth disease type 2A, motor neuron disease (specific references and mutations summarized in Table 6)
Functionally characterized mutations	Q112H (Aras et al., 2017), 300+5G>A (Funayama et al., 2015),T61I and R145Q (Meng et al., 2017)	S59L and P34S (Bannwarth et al., 2014; Genin et al., 2016), R15L/G58R (Ajroud-Driss et al., 2015) , R15L (Burstein et al., 2017) (Straub et al., 2017) G66V (Brockmann et al., 2018)

Table 3. Similarities and differences between MNRR1 and CHCHD10. Table for comparison of various identified functions, effects, and properties of MNRR1 and CHCHD10*, see Section 6, paragraph 1.

What are the conditions that cause such distinctive properties for each protein *in vivo* despite such a high degree of conservation with each other? Current studies have been focused

on identification of several disease-associated mutations in both proteins and altered protein levels in several different cancers (Table 3) without a clear understanding of the basic function of these proteins. The distinct properties of these proteins in two different compartments suggests that these proteins are necessary to respond to different conditions. Their similarities with respect to regulation of mitochondrial function suggest that as organisms evolved from single cells to multicellular organisms a single version of this protein could no longer satisfy its requirements. What did not change was the need to regulate energy production in the cells but perhaps it needed a more diverse panel of factors that would signal back to the nucleus to respond to different stresses. As compared to MNRR1, which is ubiquitously expressed, CHCHD10 has a more tissue dependent profile wherein expression in enhanced in the liver and heart (Bannwarth et al., 2014). This, along with the observation that MNRR1 protein levels are altered in several cancers, and the observation that more mutations have been identified in CHCHD10 with regard to neurodegeneration, all indicate that CHCHD10 and MNRR1 may be necessary to respond to different stresses. Their dual and compartment-specific function requires detailed study. In summary, we may speculate that a gene duplication event was exploited by the evolutionary process to generate two slightly different proteins that respond to discrete conditions, in particular to altered oxygen levels, and respond by signaling to two essential compartments – the nucleus to regulate transcription of genes and the mitochondria to modulate energy production needed for cellular homeostasis.

Several studies have assessed binding partners for both CHCHD10 and MNRR1 using mass spectrometry (MS) (Table 3). Both proteins have several novel interactors that are found in the nucleus as well as the mitochondria that may yield clues to resolve the mechanism by which both proteins regulate mito-nuclear crosstalk and may help understand the role of these proteins in distinct processes. They also have several common interactors, which reflect the two proteins' localization profiles – most interactors for both are mainly mitochondrial and a smaller proportion are found in the nucleus.

Some of these common interactors have been linked to key processes such as cell growth, migration, and apoptosis and hence may be important mediators for executing CHCHD10's and MNRR1's roles in both compartments. CHCHD10 and MNRR1 interact with each other (Floyd et al., 2016; Wei et al., 2015) and C1QBP (Complement c1q binding protein) (Hein et al., 2015; Huttlin et al., 2017; Wei et al., 2015; Yu et al., 2011) as seen by several studies. These interactions have also been validated in a cell culture model (Burstein et al., 2017; Straub et al., 2017; Wei et al., 2015) but the exact effects of these three together are yet to be assessed. C1QBP was identified as a secreted protein that binds to C1q complement protein (Ghebrehiwet et al., 1994) and was later termed HABP1 (Hyaluronan Binding Protein-1) due to its role in modulating the sperm-oocyte interaction (Ghosh et al., 2004; Gupta et al., 1991). However, further characterization revealed that this protein is localized to the mitochondria, where it affects RNA splicing (Deb and Datta, 1996). C1QBP is upregulated in a number of cancer cell lines – breast, lung, and colon cancer, and overexpression protects from staurosporine induced apoptosis in fibroblasts and increases cell migration and proliferation in breast cancer cells (McGee et al., 2011). This is highly similar to MNRR1's effects and the interaction of MNRR1 and C1QBP has been studied in the context of non-small cell lung carcinoma. Using MS and interactome analysis of the data from BioGrid the network of C1QBP-associated proteins including MNRR1 has been predicted to affect cell proliferation, (Wei et al., 2015). The same paper also shows experimental evidence that CHCHD2 is required for cell migration, and respiration in cancer cells (ibid). Another study, however (Seo et al., 2010), reports that MNRR1 and C1QBP have opposing effects on cell migration in NIH3T3 cells where MNRR1 enhances and C1QBP decreases cell proliferation. These differences in the effects of C1QBP and MNRR1 may be attributed to the differences in human (Wei et al., 2015) versus mouse (Seo et al., 2010) cells or due to cancer (Wei et al., 2015) versus transformed cells (Seo et al., 2010) used in the two studies. These differences need to be resolved by further characterization, of how CHCHD10 affects MNRR1 and C1QBP, and the formation of this ternary complex (MNRR1-CHCHD10-C1QBP). Another important binding

partner for both proteins is GHITM or MICS1. The interaction of MNRR1 and MICS1 was assessed in a recent study (Meng et al., 2017) along with a third binding partner – cytochrome *c* – in a cell culture system. This complex was proposed as a regulator of apoptosis and may play a protective role. The MNRR1-MICS1 interaction has been validated by a second MS study (Floyd et al., 2016). The MNRR1-cytochrome *c* interaction, however, was seen only by the one study (Meng et al., 2017) and only in the presence of a cross-linker, suggesting it may be a transient interaction.

Other common interactors include proteins associated with mitochondrial function such as ETC proteins NDUFS3, NDUFA8 (subunits of Complex I), COX5A, COX6A1, COX6C (subunits of Complex IV), and ATP5C1, ATP5F1, ATP5H (subunits of Complex V). These interactions suggest that MNRR1 and CHCHD10 may play a role in supercomplex formation.

Other mitochondrial interactors include TIMM44 and TIMM50 (membrane transport machinery); ECH1 (beta oxidation pathway) (FitzPatrick et al., 1995); CLPX, a component of the in ATP-dependent Clp protease (Kang et al., 2005), and PITRM1, a non-ATP dependent metalloproteinase that is involved in degradation of amyloid β-protein (Aβ) in human brain mitochondria (Falkevall et al., 2006), which has been linked to Alzheimer's disease (Alikhani et al., 2011). Another interactor, GLRX, is a redox regulatory enzyme linked with Alzheimer's disease (Akterin et al., 2006). A mutation in CHCHD10 has also been identified in a patient with late onset Alzheimer's disease in a Chinese population (Xiao et al., 2017) but no mutations have been identified in MNRR1. Another interesting common interactor is USMG5 (Up-Regulated During Skeletal Muscle Growth Protein 5), also known as DAPIT (Diabetes-Associated Protein In Insulin-Sensitive Tissues), which is involved in maintaining ATP synthase (Complex V) subunit levels in mitochondria (Ohsakaya et al., 2011). Although there are no known studies linking MNRR1 with diabetes, a study identified a CHCHD10 mutation (G66V) in one family to be associated with adult onset type 2 diabetes (Pasanen et al., 2016). However, the authors

themselves conclude that data from one family are insufficient to establish a concrete link and, hence, more studies will be needed to confirm an actual disease association.

The few common nuclear interactors include proteins involved in DNA repair. These are POLDIP2 (DNA polymerase delta p50 subunit interacting protein), required for repair of DNA lesions (Maga et al., 2013), and RNASEH1, which encodes a ribonuclease that specifically degrades the RNA of RNA-DNA hybrids and plays a key role in DNA replication and repair (Parajuli et al., 2017). A fraction of MNRR1 (Aras et al., 2017; Aras et al., 2015) and CHCHD10 (Woo et al., 2017) are found in the nucleus and their interaction with proteins that are a part of the DNA repair system suggests that both are part of the nuclear stress response machinery. This repair machinery may be activated due to conditions that affect mitochondrial function such as oxygen levels altered to 4% O_2 where MNRR1 is upregulated (Aras et al., 2013).

8. MNRR1

8.1. MNRR1 and its role in the nucleus and mitochondria

MNRR1 was originally identified on a screen of genes that regulate oxidative phosphorylation (Baughman et al., 2009). In this study, a transient of knockdown MNRR1 decreased complex I and Complex IV protein levels, and also decreased activities of complex I by ~20% (non-significant) and complex IV activity by ~50% (significant). While knockdown of MNRR1 has clear effects on mitochondrial function, such as decreased in oxygen consumption (Aras et al., 2015; Wei et al., 2015) and mitochondrial membrane potential (Aras et al., 2015), it also has several pleiotropic effects on cellular function, including cell migration (Lamb et al., 2014; Seo et al., 2010; Wei et al., 2015), growth (Aras et al., 2015) , and ROS levels (Aras et al., 2015; Meng et al., 2017). MNRR1 was identified as a factor that promotes cell migration on an unbiased functional genetic screen of a 3-dimensional migration assay (Seo et al., 2010). This study was also the first to identify that MNRR1 interacts with C1QBP (aka HABP1 or p32), a protein that inhibits cell migration. Next, they examined the pathway that may be involved and found that the Akt/Rho/ROCK/JNK signaling pathway is activated during cell migration. Other pleiotropic effects

of MNRR1 knockdown include decreased growth and enhanced ROS levels (Aras et al., 2015). These pleiotropic effects may be attributed to the functioning of MNRR1 in two compartments, and hence more mechanistic studies are necessary to identify how this regulation occurs under normal and pathological conditions.

In the nucleus, MNRR1 functions as a transcription activator of genes harboring a 13-bp ORE in their gene promoters (Aras et al., 2013). These include genes that regulate oxidative phosphorylation such as *COX4I2* (ibid) and *MNRR1* itself (Aras et al., 2013). MNRR1 is a part of regulatory system at the ORE that includes two other proteins, Recombination Signal Binding Protein For Immunoglobulin Kappa J Region (RBPJK), which functions as a scaffold to recruit MNRR1 at the ORE, and CXXC Finger Protein 5 (CXXC5), which functions as an inhibitor of transcription. At 20% O_2, CXXC5 binds RBPJK at the ORE, preventing transcription of *COX4I2*. MNRR1 is hypoxia-sensitive, and upregulated at 4% oxygen, which displaces the inhibitory CXXC5 from the ORE in order to maximally upregulate transcription (Figure 7).

systematic *in silico* analysis of human genes containing the ORE identified 28 genes containing the ORE derived from *COX4I2* or *MNRR1* upstream of the first exon. These are listed in Table 4. Many of the genes in the list are yet to be characterized (LOC105370119, RBBP8NL, KIAA1614, ADPRHL1, NOL9, C18ORF8, C2CD2, RNF150), or are microRNA genes (MIR36481, MIR661), long non-coding RNA genes (LINC00403), and pseudogenes (EEF1DP3), and hence cannot be classified into any major category for cell function.

The genes on the list whose function has been characterized to some extent have interesting implications. The target list includes genes that control mitochondrial function such as SDHAF1 (Succinate Dehydrogenase Assembly Factor 1), a complex II assembly factor (Maio et al., 2016), and FBP1 (Fructose Bisphosphatase 1), an enzyme that regulates gluconeogenesis in mice (Lamont et al., 2006). Other target genes include proteins such as MADCAM1 (Mucosal Vascular Addressin Cell Adhesion Molecule 1) (Xu et al., 2010), MARCKSL1 (Macrophage Myristoylated Alanine-Rich C Kinase Substrate Like 1) (Bjorkblom et al., 2012), and CDH4

(Cadherin 4) (Schmitz et al., 2008), which are associated with cell adhesion and migration, a process known to be regulated by MNRR1 (Seo et al., 2010; Wei et al., 2015), and LACTB (Lactamase Beta); the latter forms filaments in the mitochondrial IMS and is part of a network of
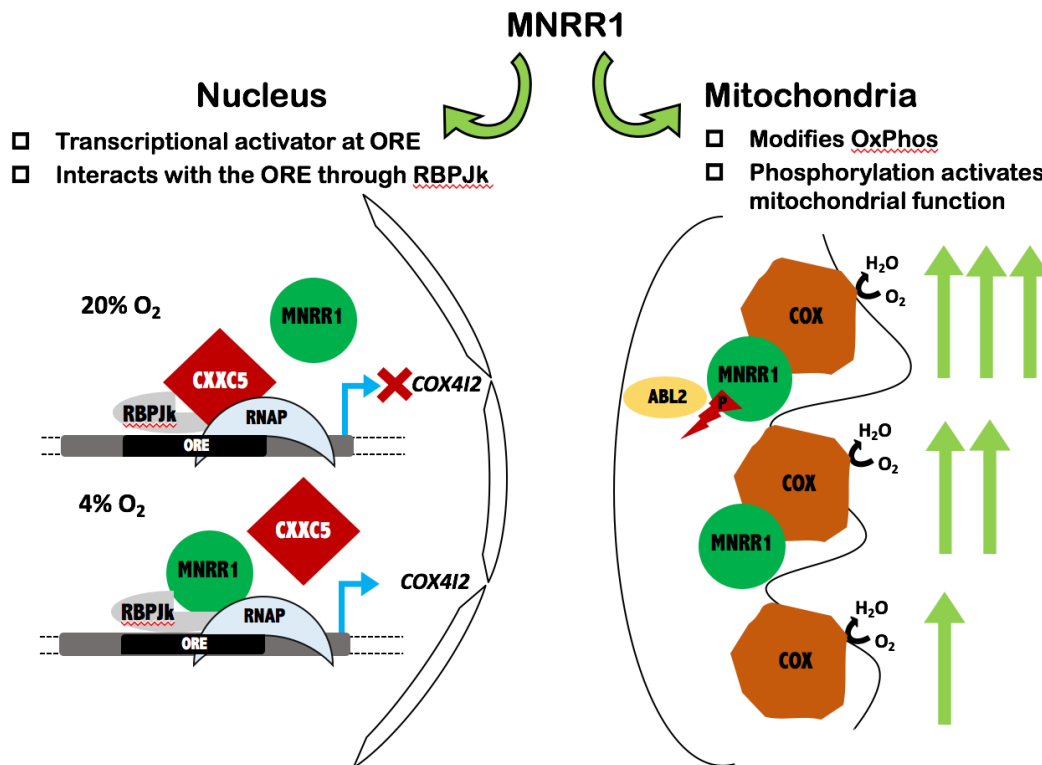
Figure 7. Model for MNRR1's role in the nucleus and mitochondria. MNRR1 functions as a transcriptional activator at the ORE in the nucleus. In the mitochondria, MNRR1 activates of OxPhos in the and the phosphorylation at Tyrosine-99 is necessary for optimal activity of MNRR1.

genes that were validated to have a causal association with obesity traits (Chen et al., 2008). Another putative MNRR1 target gene is USP28 (Ubiquitin Specific Peptidase 28), which encodes a deubiquitinating enzyme that contributes to DNA damage-induced activation of apoptosis (Zhang et al., 2006), another key pathway with which MNRR1 is associated (Liu et al., 2015).

Other ORE harboring genes include some that may affect neuronal and CNS function but require detailed characterization. WWC1 plays a role in Hippo/SWH signaling (Yu et al., 2010) and variants of this protein have been associated with memory performance and lipid binding (Duning et al., 2013). CNPY4 is a transcriptional inhibitor that modulates FGF signaling in the midbrain-hindbrain region in a zebrafish model system (Hirate and Okamoto, 2006). ADRA2A is

a protein belonging to the GPCR family and is involved in the regulation of neurotransmitter release from adrenergic neurons in the CNS (Charpentier et al., 2013).

ORE	Genes containing ORE upto 1000 bp upstream of the gene
MNRR1 (5'TGTCCCACGTCCGGA-3')	*LOC105370119, MIR661, MNRR1, ST18, MADCAM1, RBBP8NL*
COX4i2 (5'TTCCCACGCTGGGG-3')	*ADPRHL1, ADRA2A, C18orf8, C2CD2, CASZ1, CDH4, CNPY4, COX4I2, EEF1DP3, ESYT1, FBP1, KIAA1614, LACTB, LINC00403, MAP2K5, MARCKSL1, MIR36481, NOL9, RNF150, SDHAF1, USP28, WWC1*

Table 4. List of genes containing the Oxygen Responsive Element (ORE). The genes were identified using Geneious (www.geneious.com). ORE sequences for *MNRR1* and *COX4I2* in the table were used as reference sequences and searched against the human genome (GRCH38/hg38). Matches of 83.5% or above within 1000 bp 5' to the start of translation were listed.

MNRR1 interacts with COX to maintain optimal ETC function (Figure 7). This interaction requires phosphorylation of MNRR1 on Tyrosine-99 by ABL2 kinase/ARG (Aras et al., 2017). This residue is found in the COX-binding domain of MNRR1 (unpublished data) and this phosphorylation of MNRR1 occurs exclusively in the mitochondria (phosphorylation not observed in the nucleus). ARG and ABL1 kinase are both important members of the ABL kinase family and promote formation of filopodia and lamellipodia (Courtemanche et al., 2015). These actin-based protrusive structures are essential for axon formation and axonal growth cones are enriched for mitochondria, which provide energy for the migrating and extending cells (Smith and Gallo, 2018).

From the ABL family, only ARG, not ABL1, localizes to the mitochondria (Aras et al., 2017), indicating that under normal conditions, ARG specifically phosphorylates MNRR1 in the mitochondria. This is confirmed by the observations that MNRR1 fails to activate oxygen consumption in ARG knockout cells and ARG overexpression cannot enhance oxygen consumption in MNRR1 knockout cells. To further analyze the effect of this phosphorylation, they overexpressed phosphomimetic MNRR1 and non-phosphorylatable MNRR1 in cells with a depletion of endogenous MNRR1. Only the phosphomimetic MNRR1 enhanced the defective oxygen consumption (ibid) indicating that this phosphorylation is necessary for the mitochondrial

function of MNRR1. They also tested a missense mutation in MNRR1 (Q112H), from CMT1A patients, in a cell culture model that is described in the next section.

As of now, phosphorylation is the only post-translational modification for MNRR1 that has been identified and analyzed. However, more work needs to be done in order to characterize other potential modifications on MNRR1 and their compartment-specific effects on its function. Since MNRR1 has differential phosphorylation in the nucleus versus the mitochondria (ibid), this may serve as a target to modulate function in a niche-specific manner. MNRR1 also has been associated with regulation of apoptosis, where it binds to Bcl-xL, an anti-apoptotic protein that prevents Bax-mediated pore formation in the OMM and induction of apoptosis (Liu et al., 2015). When cells are subjected to UV stress mitochondrial MNRR1 levels are reduced, which allows Bax to oligomerize and form pores in the OMM, thereby releasing cytochrome *c* from the IMS to induce apoptosis*.* Another recent study used a *Drosophila* model to analyze MNRR1's mitochondrial function (Meng et al., 2017). The investigators generated MNRR1-KO flies that had fragmented and deformed mitochondria, decreased oxygen consumption, and increased ROS, leading to oxidative stress, dopaminergic neuron loss, and motor dysfunction with age. These flies also had higher levels of eIF4E-binding protein (4EBP), which functions as a translational inhibitor and is upregulated due to cellular stress that may be caused by lower MNRR1 levels. Further, they tested MNRR1 sensitivity to a number of stresses. These included activators of mitochondrial UPR (ΔOTC, Pol γ-α) that increased MNRR1 levels, and hyperoxia, which decreases MNRR1 levels. They also identified two important proteins that interact with MNRR1: MICS1 and cytochrome *c*. MICS1 (Mitochondrial Morphology and Cristae Structure 1) is a poorly characterized protein that was also identified as a binding partner for MNRR1 by a previous study (Floyd et al., 2016). Depletion of the *Drosophila* ortholog of MICS1 had no effect by itself; however, the depletion of MICS1 in a MNRR1 hypomorphic background exacerbated the defective mitochondrial morphology and reduced ATP levels caused by loss of MNRR1. This indicates that MNRR1 is necessary for mediating MICS1's effects on mitochondrial function. A more detailed

analysis is necessary to determine the actual function of MNRR1 and MICS1 for maintaining mitochondrial structure and function in other organisms as well as in human cells.

8.2. MNRR1 and disease

Altered MNRR1 transcript and protein levels have been associated with neurodegeneration as well as cancer. One of the first studies to associate MNRR1 with a disease was by Feyeux et al, linking a change in MNRR1 transcript levels to Huntington's disease. They found that MNRR1 is upregulated in human embryonic stem cells (ESCs) bearing a mutant copy of *HTT* (encodes Huntingtin protein), as well as during differentiation of normal WT stem cells into a neuronal lineage (Feyeux et al., 2012). This study did not assess a mechanism for this process. However, a potential mechanism was later identified by Zhu and co-workers wherein they showed that inhibition of the TGFβ signaling pathway mediated by MNRR1 is necessary for differentiation of both human ESCs and induced pluripotent stem cells (iPSCs) to a neuroectodermal lineage. MNRR1 interacts with SMAD4, an activator of TGFβ signaling. This interaction sequesters SMAD4 to the mitochondria and prevents it from entering the nucleus to activate this pathway, thereby allowing differentiation of these cells into a neuroectodermal lineage (Zhu et al., 2016). MNRR1 levels are also lower in patients with lissencephaly (literally means "smooth brain"), a rare, gene-linked brain malformation characterized by the absence of normal convolutions (folds) in the cerebral cortex and an abnormally small head (microcephaly). These lower levels may prevent proper differentiation of iPSCs from these patients into neuronal cells (Shimojima et al., 2015). Besides these effects MNRR1 also affects cell migration (Seo 2010, Wei 2015, Lamb 2015) and growth signaling (Lamb et al., 2014; Meng et al., 2017; Seo et al., 2010) and hence is increasingly associated with regulation neuronal differentiation (Keller et al., 2018; Zhu et al., 2016).

Mutation	Disease	Reference
Pro2→Leu	Autosomal dominant Parkinson's disease	(Ogaki et al., 2015; Shi et al., 2016)

Gly4→Arg	Parkinson's disease (PD), Lewy Body Disorder (LBD)	(Ogaki et al., 2015)
Arg8→His	Sporadic PD	(Ikeda et al., 2017)
Pro14→Ser	PD	(Ogaki et al., 2015)
Pro34→Leu	PD, LBD	(Ogaki et al., 2015)
Ala37→Val	PD, LBD	(Ogaki et al., 2015)
Ala49→Val	PD, LBD	(Ogaki et al., 2015)
Thr61→Ile	PD	(Funayama et al., 2015; Meng et al., 2017)
GLy66→Met	Multiple System Atrophy	(Nicoletti et al., 2018)
Ala93→Val	PD, LBD	(Ogaki et al., 2015)
Gln112→His	CMT1A	(Aras et al., 2017)
Arg145→Gln	PD	(Funayama et al., 2015; Meng et al., 2017)
Gln126→X	PD	(Koschmidder et al., 2016)
300+5G>A*	PD	(Funayama et al., 2015)

Table 5. List of MNRR1 mutations identified in association with neurodegenerative diseases. Mutations in MNRR1 are mostly found in the coding region and associated by multiple studies with Parkinson's and other neurodegenerative diseases (*- found in non-coding region).

Besides altered transcript and protein levels, there have also been several mutations in MNRR1 linking it mainly to Parkinson's disease and Lewy Body disorders, summarized in Table 5. A few of these have also been functionally characterized. The first study linking MNRR1 to Parkinson's disease identified three mutations – T61I and R145Q in the coding region and 300+5G>A in a non-coding region. A splicing assay also revealed that the 300+5G>A mutation caused skipping of exon 2. The other two had no effect on splicing. However, the group assessed the other two mutations in a *Drosophila* model with a hypomorphic expression of *Drosophila* MNRR1 ortholog (CG5010) and reintroduced either human WT or mutant MNRR1 (T61I and R145Q). Both mutations failed to rescue defects in mitochondrial morphology and ATP levels seen in the hypomorphic MNRR1-harboring flies whereas both could significantly improve climbing defects. With respect to lifespan studies, neither mutation could improve the shortened

lifespan in MNRR1 hypomorphic flies. Furthermore, the difference in the lifespan of hypomorphic files overexpressing WT and mutant forms of human MNRR1 was higher under peroxide stress. Another mutation of MNRR1, Q112H, has been identified in CMT1A patients with an exaggerated

The mitochondrial effects as well as effects on cell migration have led to the association of MNRR1 with cancer, specifically, non-small cell lung carcinoma (NSCLC) (Wei et al., 2015). MNRR1 is co-amplified with EGFR (Epidermal Growth Factor Receptor) in patients with NSCLC as well as in tumor xenografts derived from NSCLC patients in comparison with normal lungs. They further assessed these effects in context of MNRR1's role in cell migration and mitochondrial respiration, where a knockdown of MNRR1 decreases cell migration and oxygen consumption in the NSCLC cell line HCC827 and the xenograft derived lung primary cell line LPC43. To explore whether other proteins are involved, Wei et al. also performed a high-throughput MS analysis for interactors and found MNRR1 to associate with two "hub" proteins (centers of highly connected subclusters of other proteins) – C1QBP found in the mitochondria, and YBX1, an oncogenic transcription factor. Another new study examines MNRR1 and EGFR in a different cancer model, glioblastoma multiforme, where the investigators found that the constitutively active EGFR[viii] mutant enhances the stability of MNRR1 and its nuclear translocation under hypoxia (Lumibao 2017– meeting abstract not published yet). Other cancers with which MNRR1 is associated are breast cancer, thyroid follicular carcinoma, and hepatocellular carcinoma. In a study by Lamb and co-workers, MNRR1 was found to be highly upregulated in mammospheres derived from MCF7 and T47D cells. Using patients samples, they also determined that MNRR1 was the highest transcriptionally upregulated protein common to the set of genes upregulated in mammospheres and in 28 breast cancer patients' tumor stroma vs adjacent epithelial cells from the same individuals (Lamb et al., 2014). Another study assessed protein levels using a high throughput quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach from frozen samples of patients with follicular carcinoma (metastatic), follicular adenoma (localized), and normal thyroid cells. MNRR1 was upregulated in the follicular carcinoma set and was

considered as a potential biomarker and therapeutic candidate for the disease (Lai et al., 2017). MNRR1 is also linked to hepatocellular carcinoma via its upregulation in livers of patients. The mechanism for this upregulation is proposed to be via CREB, a transcription factor that binds to and activates transcription from the *MNRR1* promoter in hepatocellular carcinoma cells (Song et al., 2015). Data from The Cancer Genome Atlas (TCGA) (human protein atlas) also suggest high MNRR1 levels as an unfavorable marker for liver cancer survival and also for head and neck cancer survival (Figure 8).
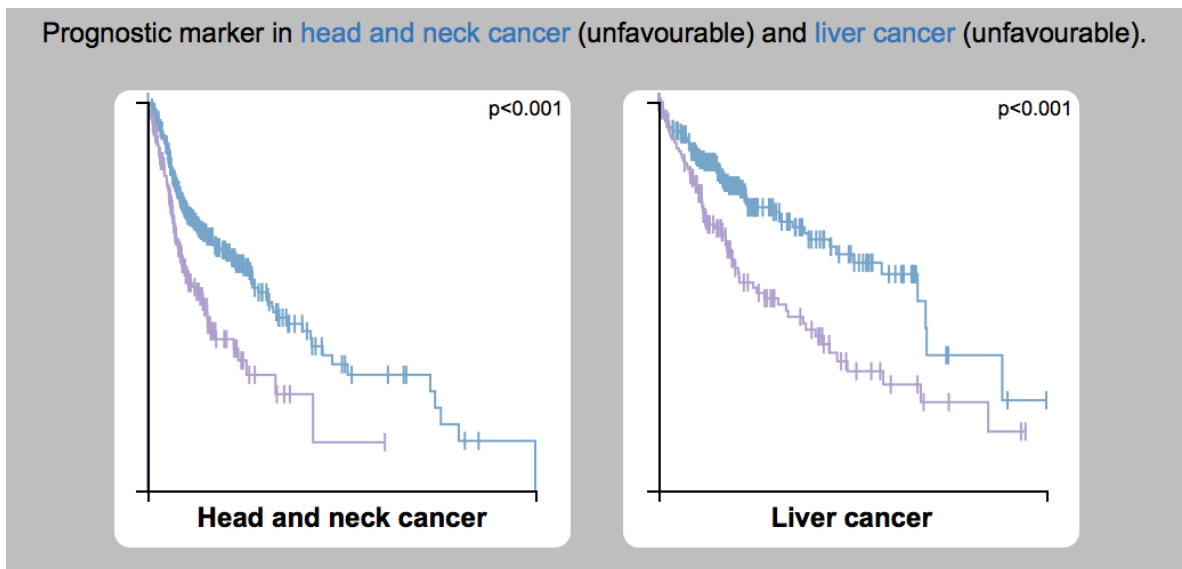
Figure 8. MNRR1 is an unfavorable prognostic marker for head and neck and for liver cancer. Kaplan-Meier survival data from TCGA consortium showing that head and neck cancer patients (left) and liver cancer patients (right) with high MNRR1 expression (purple line) have poorer survival as compared to those with low expression (blue line) (Images obtained from Human Protein Expression Atlas)

9. CHCHD10

9.1. CHCHD10's role in the nucleus and mitochondria

CHCHD10 is a 142 amino acid protein originally discovered as a heart and skeletal muscle enriched gene using a bioinformatics approach (Martherus et al., 2010). This study also confirmed that CHCHD10 is a mitochondrial protein and a transient knockdown decreases both COX activity and ATP levels to ~50% of wild-type cells.

Preliminary characterization of CHCHD10 indicates that a majority of the protein is located in the mitochondria, specifically in the IMS (Bannwarth et al., 2014) and is required for maintenance of mitochondrial cristae (ibid, (Genin et al., 2016)) by interacting with CHCHD3 and Mitofilin, components of the MICOS complex. This association has recently been questioned based on the demonstration that mitofilin, a member of the MICOS complex, may be detected in an immunoprecipitation experiment due to non-specific binding of mitofilin with IgG (Burstein et al., 2017). The same group also showed that mitofilin and CHCHD10 do not co-sediment on a sucrose gradient, and another study showed that CHCHD10 and CHCHD3 do not co-migrate on a native gel (Straub et al., 2017). Straub and co-workers suggest that CHCHD10 functions in a complex with MNRR1 and regulates oxygen consumption by affecting Complex I stability. They also showed that CHCHD10 and MNRR1 co-migrate with a 220kDa complex containing COX I (at the expected size for Complex IV) but did not study the effects of these proteins on Complex IV in detail. Other work has indicated that CHCHD10 plays a role in regulating apoptosis (Genin et al., 2016).

With respect to nuclear effects, Woo *et al* showed that the fraction of CHCHD10 localized to the nucleus is necessary for import of TDP-43 (Woo et al., 2017) and as a transcriptional activator* (See section 6 paragraph 1) for *COX4I2*, *NDUFB6* and *NDUFS3*. Besides these effects, very little is known regarding CHCHD10's physiological role. Most work is focused on CHCHD10 mutations described in the next section.

10. CHCHD10 and its role in disease

CHCHD10 has stimulated the interest of the research community due to its association with a myriad of neurodegenerative disorders such as amyotrophic lateral sclerosis, spinomuscular atrophy, spinocerebellar ataxia, and mitochondrial myopathy. Two initial studies identified CHCHD10 to be causally associated with these diseases. The first group found that mutation of Serine-59 to Leucine (S59L) is associated with a frontotemporal dementia-amyotrophic lateral sclerosis phenotype (Bannwarth et al., 2014) in a family of French origin. The

other mutation was R15S in *cis* with a G58R mutation of CHCHD10, identified in a family of Puerto Rican origin by Ajroud-Driss *et al*, which caused a mitochondrial myopathy. They investigated its effects in cells overexpressing this mutant protein and found that it led to a loss of mitochondrial networks, forming smaller, more punctate mitochondria. On comparing this double mutant with individual mutants, that is only R15S or G58R, they found that the G58R mutation is sufficient to cause the altered cell phenotype. Hence, they concluded that the R15S mutation may not be pathogenic and the effects seen may be only due to the G58R mutation in CHCHD10.

Table 6 summarizes the mutations identified so far; the potential clinical implications of these mutations have been well summarized in a recent review (Ait-El-Mkadem et al., 2015). While most studies identified these mutations in large-scale screenings, a few have characterized them *in vivo* using patient fibroblasts. The original Bannwarth study analyzed the S59L mutation in fibroblasts from two patients and found that there was decreased mitochondrial length and altered cristae. A follow up study by the same group also looked at patient fibroblasts with another mutation, P34S, and found it also affected mitochondrial cristae formation due to defects in MICOS assembly and nucleoid formation and that patient fibroblasts harboring mutant CHCHD10 are protected from actinomycin D-induced apoptosis (Genin et al., 2016). This is surprising finding since CHCHD10 is necessary for maintenance of optimal COX activity (Burstein et al., 2017; Martherus et al., 2010; Straub et al., 2017) and mitochondrial structure. One potential explanation which was not assessed by the authors in the case of these mutations is that there may be a

Mutation	Disease	Reference
Pro12→Ser*	ALS	(Dols-Icardo et al., 2015)
Arg15→Leu	ALS, Motor neuron disease	(Johnson et al., 2014; Kurzwelly et al., 2015; Muller et al., 2014; Zhang et al., 2015)
His22→Tyr	Behavioral variant FTD	Jiao et. al 2016

Pro23→Thr/Ser/Leu	FTLD (T), Behavioral variant FTD(S), semantic dementia (L)	(Zhang et al., 2015) (T), (Dols-Icardo et al., 2015) (S), (Jiao et al., 2016) (L)
Ser30→Leu	Early onset Parkinson's Disease	(Zhou et al., 2017a)
Pro34→Ser	FTD-ALS, ALS	(Chaussenot et al., 2014; Dols-Icardo et al., 2015)
Ala35→Asp	FTLD, Alzheimer's disease	(Jiao et al., 2016; Zhang et al., 2015)
G58→Arg (in cis with Arg15→Ser)	Mitochondrial myopathy	(Ajroud-Driss et al., 2015)
Ser59→Leu	FTD-ALS, cerebellar ataxia	(Bannwarth et al., 2014; Chaussenot et al., 2014)
Gly66→Val	ALS, LOSMoN/SMAJ, Motor neuron disease,CMT2A	(Auranen et al., 2015; Johnson et al., 2014; Muller et al., 2014; Penttila et al., 2016)
Pro80→Leu	ALS	(Dols-Icardo et al., 2015; Ronchi et al., 2015; Zhang et al., 2015)
Gln82→X	Atypical FTD with Parkinsonism	(Dols-Icardo et al., 2015)
Tyr92→Cys**	ALS	(Zhou et al., 2017a)
Pro96→Thr*	ALS	(Dols-Icardo et al., 2015; Teyssou et al., 2016)
Gln102→His**	ALS	(Zhou et al., 2017a)
Gln108→X/P*	Atypical FTD and Parkinson's disease (X), Early onset ALS (P)	(Lehmer et al., 2018; Perrone et al., 2016)

Table 6. List of CHCHD10 mutations associated with neurodegenerative diseases. Mutations in CHCHD10 are mostly found in exon 2 and associated by multiple studies with a plethora of neurodegenerative disorders (*-found outside exon 2, **-incorrectly assigned mutations)

compensatory upregulation of MNRR1. MNRR1 has a well-characterized role in regulation of apoptosis (Liu 2014), which may protect the patient fibroblasts.

Straub and co-workers characterized patient fibroblasts with another mutation, R15L. WT CHCHD10 forms a complex with MNRR1 necessary for mitochondrial respiration (Straub et al., 2017) whereas the mutant protein fails to form this complex, which may explain the pathogenic effects. CHCHD10 levels are reduced in patient fibroblasts, suggesting lower levels of mutant

CHCHD10 as the mechanism for these effects. Another key observation made in this study showed that lower CHCHD10 levels in patient fibroblasts, or silencing of CHCHD10 in normal fibroblasts, increased the levels of MNRR1. However, when MNRR1 is knocked out in cells, CHCHD10 levels are decreased. This, and the observation that MNRR1 and CHCHD10 have several common ancestors, lead to the conclusion that, though MNRR1 and CHCHD10 play a common role in a highly conserved process (OxPhos), the gene duplication event gave rise to two forms that may contribute in distinct ways. Hence, studying these proteins in lower organisms with common homologs for MNRR1 and CHCHD10 such as yeast, worms and flies may lead to observations that cannot be replicated in human cells. Studies in other organisms such as zebrafish, where MNRR1 and CHCHD10 have distinct orthologs, show that when CHCHD10 is depleted the embryos have decreased axon length, defective myofibrillar structure, and a poor touch-induced flight response as compared to the control embryos. The authors suggest that this may be a good model for testing the effect of various treatments in an *in vivo* system, at least in the case where the mutations affect CHCHD10 protein levels in patient fibroblasts harboring the R15L and G66V mutations (Brockmann et al., 2018). They also show that CHCHD10 levels are reduced in patient fibroblasts but show no defects in mitochondrial structure (ibid). Although there is a distinct phenotype seen in the zebrafish knockdown model, the mouse knockout model for CHCHD10 does not show any distinct effects (Burstein et al., 2017). This may partially be explained by the observation made by Calvo and co-workers that the N-terminal 80 amino acids from CHCHD10 are deleted in the murine system (Calvo et al., 2017) and indicates that, even when the organism has distinct orthologs, the mechanism by which the protein is regulated may be species-specific.

Another important study to assess CHCHD10 function was done in a *C. elegans* model by Woo and co-workers, in which they analyzed two mutations, R15L and S59L. The knockout of CHCHD10 in *C. elegans* caused a decrease in longevity, increased mitochondrial ROS, and produced a motility defect in the worms termed "curling morphology" by the authors. Moreover,

they also tested the mutant human proteins in the worm system and showed that, unlike WT human CHCHD10, the mutant proteins fail to rescue the phenotypic defects. The study further showed that in human cells CHCHD10 protein is also localized to the nucleus and is necessary for the import of TDP-43 into this compartment. In the presence of mutant CHCHD10, TDP-43 fails to be imported to the nucleus and mislocalizes to the mitochondria. The authors suggest that, like MNRR1, CHCHD10 affects transcription of mitochondrial genes. However, the authors made these observations in multiple systems, some in human cells (TDP-43 import to nucleus), some in rat neurons (transcription), and some in mouse hippocampal neurons (TDP-43 mislocalization). Moreover, their initial studies are in *C. elegans,* where CHCHD10 and MNRR1 are represented by same gene, *har1*. This confounds their analyses since it is becoming increasingly clear that even the most used model to generate disease phenotype, the mouse system (Ericsson 2013), may have a different mechanism for the function of CHCHD10 (Burstein 2018, Calvo 2016) that may not be applicable to study human mutations.

Since the original discovery of mutations in CHCHD10 being linked to ALS, many screens were conducted across different populations to detect other harmful mutations in CHCHD10. Despite the seemingly large number of mutations identified, it is slowly coming to light that all the variants do not necessarily contribute to the disease. There have been cases where the same mutation is found both in disease patients and in control patients, suggesting the mutation is a benign polymorphism in the protein. The P34S mutation is associated with ALS and FTD by several studies (Chaussenot et al., 2014; Chio et al., 2015; Kurzwelly et al., 2015) but the same mutation has been identified in healthy control individuals and hence is considered non-pathogenic (Dols-Icardo et al., 2015; Marroquin et al., 2016; Wong et al., 2015; Zhou et al., 2017a). One possibility, as suggested by Marroquin *et al* is that some of these studies that found the mutation to be associated with ALS did not consider a sufficient number of control individuals.

There has been one case where the identified mutation is due to improper annotation of the gene. The canonical CHCHD10 protein sequence (UniProtKB) has 4 cysteines at positions

102, 112,122, and 132 that are connected by 2 disulfide linkages to form the CHCH domain. Any mutation in one of these critical cysteines would lead to misfolding of the protein. One study has identified a mutation of glutamine 102 to histidine whereas in the actual protein position 102 is a cysteine, a part of the twin CX_9C motif. The same group also found a mutation at Tyrosine-92 whereas the original residue in the CHCHD10 sequence is Alanine (Zhou et al., 2017a). Hence, all the data must be analyzed meticulously and compared with the known studies before asserting that the mutation is associated with disease.

Another important consideration is the age of onset and the phenotype. However, a problem with the interpretation of the disease association data is that the age of onset for neurodegenerative disorders in many cases is later in life. In individuals where the mutation has been identified as non-pathogenic it may be that the disease has not manifested yet, thereby leading to erroneous conclusions. Until recently, most mutations of CHCHD10 seen were late onset, but a new mutation, Q108P, was recently identified in a patient of 29 years with rapidly progressing ALS. The investigators propose that this mutation in the CHCH domain of the protein causes defective import and, hence, upregulating import to the mitochondria would be a potential therapy for mutations that affect import (Lehmer et al., 2018). A nonsense mutation of the same residue (Q108X) was associated with another patient presenting with atypical FTD and a Parkinson's-like phenotype. This disease was relatively late onset wherein the symptoms began to appear at 48 years of age. If the missense mutation at residue Q108 reduced import, a nonsense mutation would probably prevent entry of the protein into mitochondria altogether. One would predict that the phenotype with the protein of reduced import would be less severe and probably have effect later in life. However, the nonsense mutation presents much later than the import defective one, and both have different effects, adding to the paradox of disease variability seen in individuals harboring CHCHD10 mutations. Therefore, it is essential to assess the available data with caution and also to consider follow-up studies for both cases and controls after data collection.

The observation for multiple distinct presentations of the same mutation are also seen in the case of the G66V, which has been associated with a diverse spectrum of disorders including ALS (Muller et al., 2014), SMAJ (Penttila et al., 2016), motor neuron disease (Johnson et al., 2014), and CMT2A (Auranen et al., 2015). In one study it was seen that even within a single family, in which all the affected individuals carried the G66V variant, members displayed many different phenotypes ranging from CMT2-type axonal neuropathy to spinal muscular atrophy that presented as an ALS-like disease with ages ranging from 30 to 55 years (Pasanen et al., 2016). Another mutation, P80L, is almost exclusively present in patients with ALS (Dols-Icardo et al., 2015; Ronchi et al., 2015). The P80L mutation recently was seen in one control patient, but the authors state that this patient was 57 years of age at the time of the study and may develop symptoms at a later age. They concluded that the P80L mutation may be a pathogenic one with reduced penetrance (Perrone et al., 2016). Thus, it is essential to carefully consider the data regarding age of presentation, phenotype, and the presence in control individuals before establishment of any mutation in CHCHD10 as pathogenic.

Although most of the studies have focuses on CHCHD10's role in neurodegeneration, there is some data linking CHCHD10 to cancer. Two studies link CHCHD10 to gastric cancer, one where lower promoter methylation was identified to be a prognostic marker (Chen et al., 2012) and another where loss of the CHCHD10 locus was found in higher frequency in patient samples (Kawauchi et al., 2010). Another study also found lower CHCHD10 levels in a cisplatin-resistant line of ovarian cancer (Cheng et al., 2010); data from the TCGA consortium suggest high CHCHD10 expression to be a favorable prognostic maker for renal cancer (Figure 9). This is a surprising finding and will be addressed in the discussion section, which may provide a possible explanation for this observation.
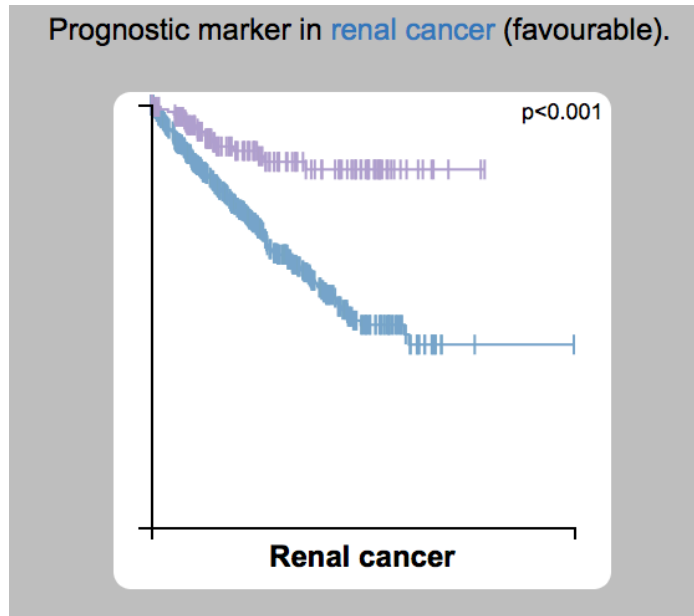
Figure 9. CHCHD10 is a favorable prognostic marker for renal cancer. Kaplan-Meier survival data from the TCGA consortium showing that renal cancer patients with high CHCHD10 expression (purple line) have better survival as compared to those with low expression (blue line) (Images obtained from Human Protein Expression Atlas).

With such variability with respect to disease presentation, age of onset, and potential population specificity, CHCHD10 has become a key protein for neurodegenerative research and a candidate gene in the pathogenicity of gastric and ovarian cancers. However, there is still not much data regarding the physiological role of this protein and no functional data regarding the protein's role in cancer. Most current studies have focused on its neurodegeneration-associated mutations, and contrasting reports have led to confounding data that question the relevance of this protein under pathological conditions. Part of the perplexity, at least with respect to model organisms, may be explained by the presence of common orthologs for MNRR1 and CHCHD10 in lower organisms such as fly and the worm. Even in the mouse model, where there are distinct orthologs, there is data regarding altered import of CHCHD10 to mitochondria (Calvo et al., 2017). One essential aspect of this protein's function that requires in depth analysis is its presence in the nucleus as identified by Woo and co-workers (Woo et al., 2017).

Besides these effects, more studies need to focus on the physiological role of CHCHD10 as well as its effects in conjunction with MNRR1 in order to elucidate the mechanism of the

disease. There are several reasons to study these proteins as a pair such as their conservation (58%), the presence of common orthologs, and common interacting partners. Despite these similarities, both genes have evolved separately after a gene duplication event (Cavallaro, 2010). The persistence of these two distinct yet highly conserved genes and the altered tissue specificity for only one of them (Table 3) indicate that each protein may play a different role that affects a common process (ibid). With respect to mitochondrial function, there are several studies linking the proteins to the optimal maintenance of ETC function (Burstein et al., 2017; Martherus et al., 2010; Straub et al., 2017). A fraction of each protein is also found in the nucleus (Aras et al., 2017; Aras et al., 2015; Aras et al., 2013; Woo et al., 2017) and little is known regarding CHCHD10's function in that compartment, either by itself or in conjunction with MNRR1. We also know another important property of MNRR1– it's hypoxia-mediated activation at 4% O_2 in a cell culture model (Aras et al., 2013) whereas it is not clear if CHCHD10 responds to altered oxygen levels. Moreover, this pair of proteins and other twin CX_9C family members may be part of a comprehensive mito-nuclear communication system that regulates energy production as well as transcription under distinct physiological or pathological conditions.

CHAPTER II: RESULTS

1. Preliminary characterization of CHCHD10 localization and function

1.1. CHCHD10 is localized to the nucleus and the mitochondria

CHCHD10 has been characterized as a mitochondrial protein in previous studies (Martherus 2010, Bannwarth 2014). Due to its high degree of conservation with MNRR1 (58%), a bi-organellar protein that localizes to both the mitochondria and the nucleus, we examined the subcellular localization of CHCHD10. Like MNRR1, CHCHD10 is also present in both the mitochondria (primarily) and the nucleus as analyzed by Western blot (Figure 10A) and by confocal microscopy (Figure 10B). In the nucleus CHCHD10 (red) co-localizes with DAPI (blue), with a small but positive correlation coefficient. This suggests that CHCHD10, like MNRR1, is found in both organelles and may have a role in both compartments. In order to confirm the nuclear signal, we used CHCHD10-KD cells, where we see that the correlation coefficient of CHCHD10 (red) signal with DAPI (blue) is negative, indicating that the small positive correlation signal is not due to non-specific staining with the antibody (Figure 10C).

B

C
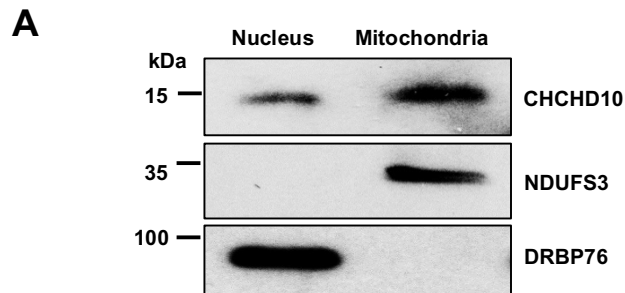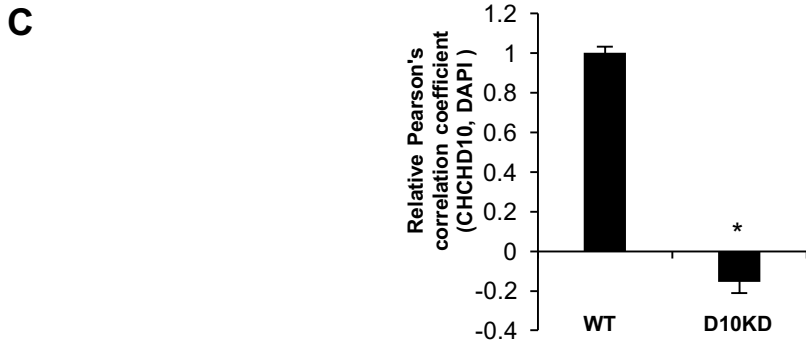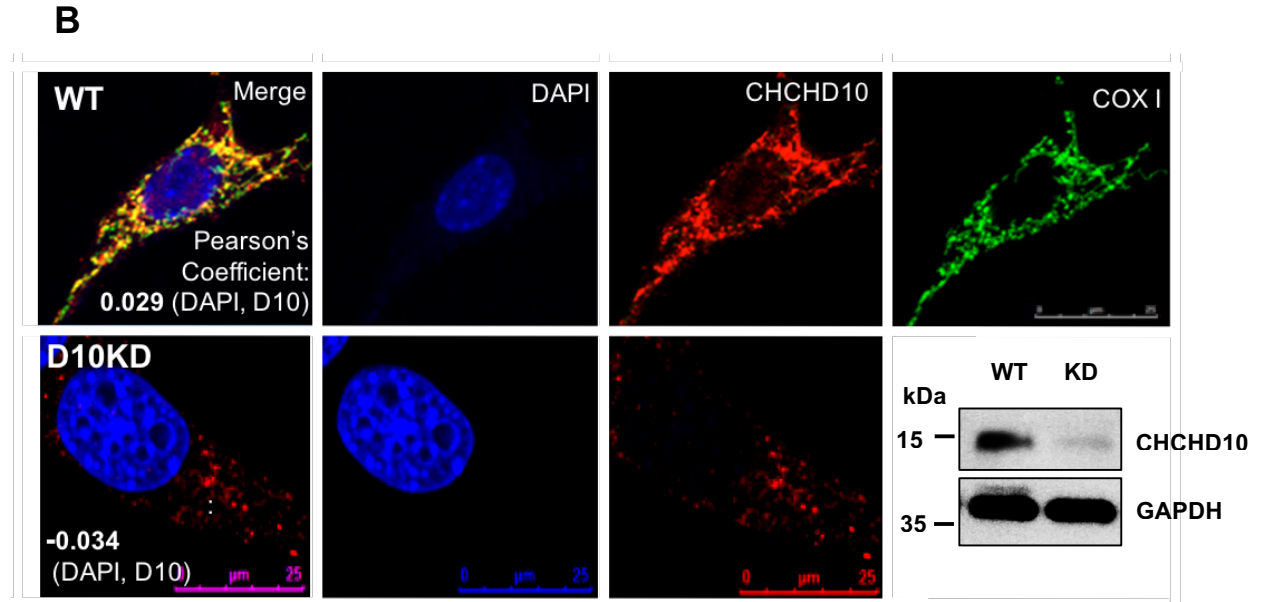
Figure 10. **CHCHD10 is found in the nucleus and the mitochondria.** *A,* nuclear and mitochondrial fractions (HEK293 cells) were analyzed for levels of CHCHD10. Effectiveness of fractionation was shown with DRBP76 as a nuclear marker and NDUFS3 as a mitochondrial marker. *B,* Representative images of CHCHD10 (red) co-localization with the nucleus (DAPI: blue; merge: purple) in HEK293 WT and CHCHD10-KD cells. To show effectiveness of CHCHD10 knockdown, equal amounts of lysate from HEK293 WT and CHCHD10-KD cells were probed for CHCHD10 levels. GAPDH was probed as loading control. (bottom panel, right). *C,* Relative Pearson correlation coefficient values (negative in the KD cells) indicate the specificity of nuclear signal of CHCHD10 (n=4, *, $p < 0.05$). (graph below).

1.2. CHCHD10 is a hypoxia-sensitive gene

Another important feature of MNRR1 is its hypoxia-sensitivity. MNRR1 is transcriptionally

upregulated at 4% oxygen in a cell culture model (Aras et al., 2013). Hence, we tested the hypoxia

sensitivity of CHCHD10 at a range of physiologically encountered oxygen tensions between 1 and 13% (Carreau et al., 2011). CHCHD10 is upregulated at 8% oxygen and downregulated at 4% oxygen in a manner opposite to MNRR1, which is maximally induced at 4% oxygen and downregulated at 8% (Figure 11A). This up-regulation of CHCHD10 at 8% is transcriptionally mediated (Figure 11B). In order to confirm the specificity of these effects, we analyzed CHCHD10 levels at 7,8, and 9% oxygen and found that CHCHD10 is maximally upregulated at 8% oxygen in a surprisingly narrow window (Figure 11C). We also confirmed the upregulation at 8% using microscopy and saw this upregulation in both compartments (Figure 11D).
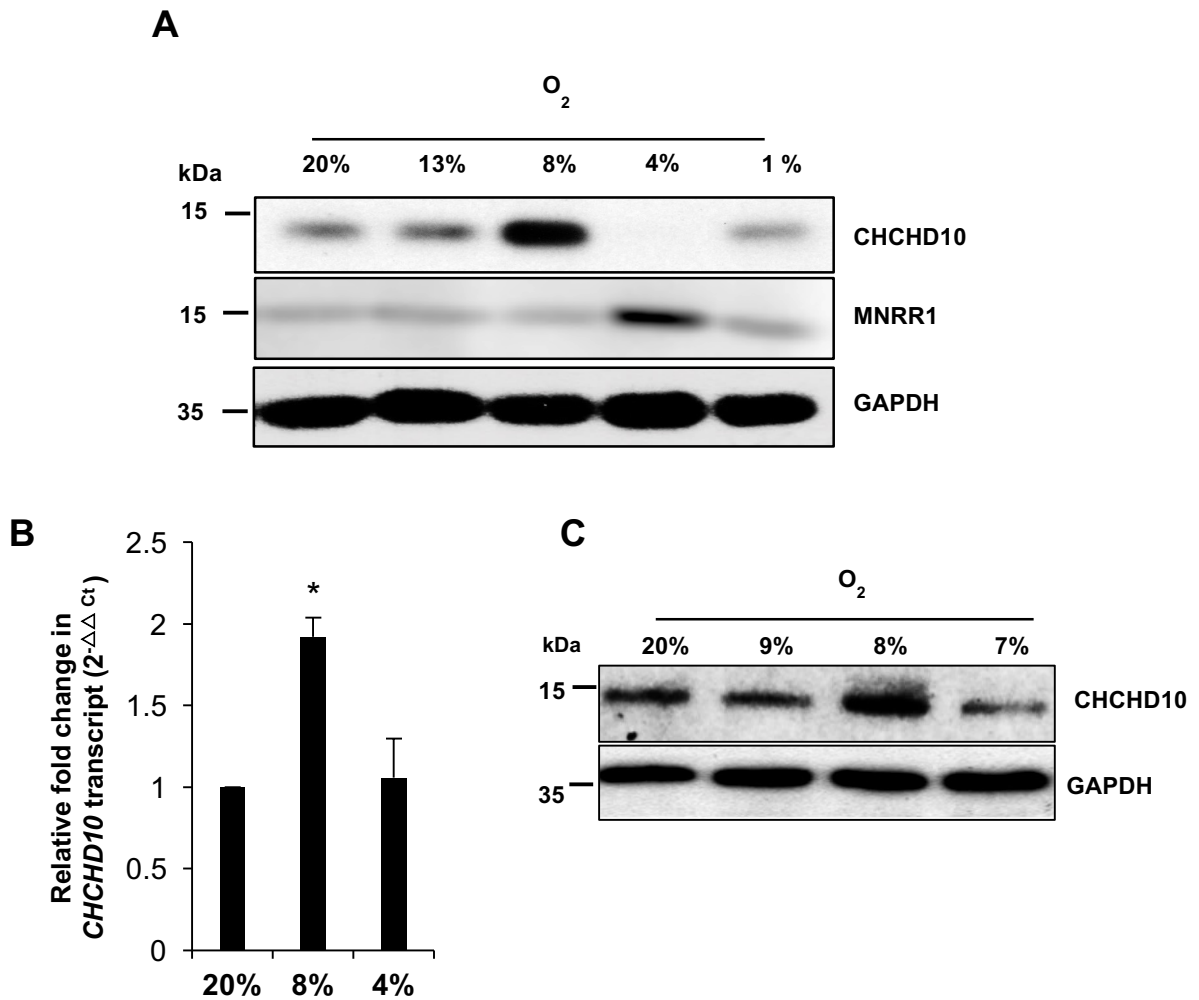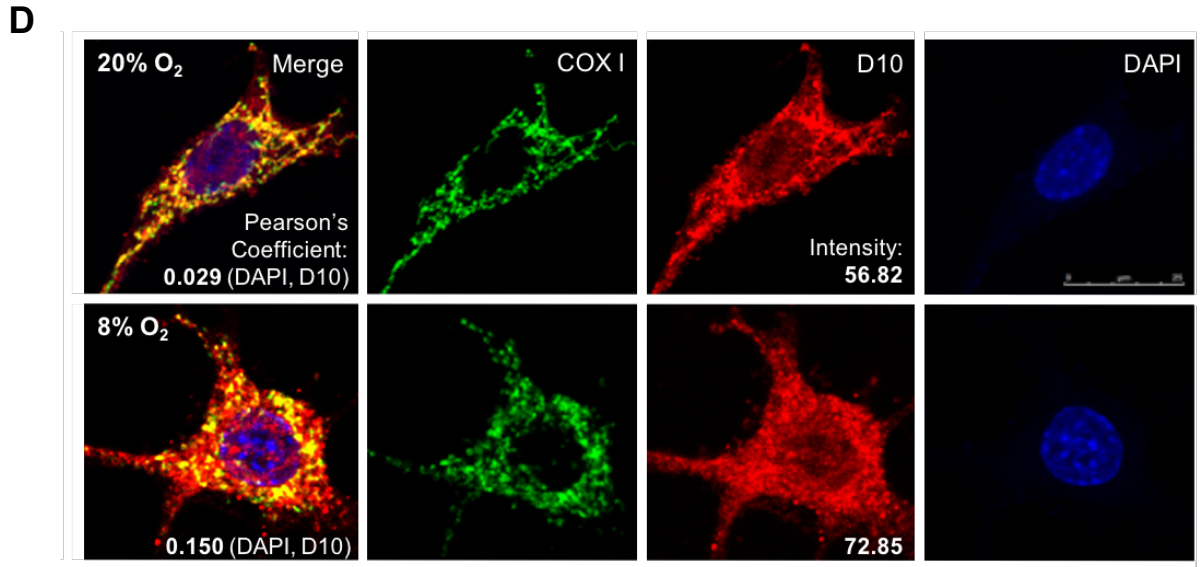
Figure 11. CHCHD10 is a hypoxia-sensitive gene. *A,* HeLa cells were incubated for 48 h at the stated O₂ levels. Whole cell lysates were separated on an SDS-PAGE gel and analyzed for CHCHD10 and MNRR1 levels. GAPDH was probed as a loading control. *B,* Real-time PCR analysis of transcript levels of endogenous CHCHD10 at 20%, 8%, and 4% oxygen (n=4, *, p<0.05). *C,* as in *A* but not probed for MNRR1. *D*, representative images of CHCHD10 (red) localization in HEK293 cells at normoxia (20% O₂) (middle panel) and hypoxia (8% O₂) (bottom panel). DAPI (blue) was used as nuclear marker (merge-purple) and COX I (green) was used as mitochondrial marker (merge-yellow).

1.3. Knockdown of CHCHD10 has pleiotropic effects in cells

To gain insight into CHCHD10's role *in vivo* we tested cell growth of WT versus CHCHD10-KD (knockdown) cells in both glucose and galactose media. Growth of cells with glucose as the primary carbon source yielded indistinguishable growth rates independent of CHCHD10 level (Figure 12A blue solid and dotted lines representing WT and CHCHD10-KD, respectively). However, when galactose was used as the primary carbon source, requiring cells to make ATP primarily through mitochondria (Robinson et al., 1992), CHCHD10-KD cells grew more slowly than WT cells (Figure 12A red solid and dotted lines representing WT and CHCHD10-KD, respectively). The decrease in growth in galactose media was also seen by counting live cell numbers (Figure 12B). We also tested total cellular ROS levels using CM-H₂DCFDA and found that CHCHD10-KD cells have higher ROS levels (Figure 12C). Increased ROS levels and slower

growth specifically in galactose indicate defective oxidative phosphorylation (Robinson et al., 1992).
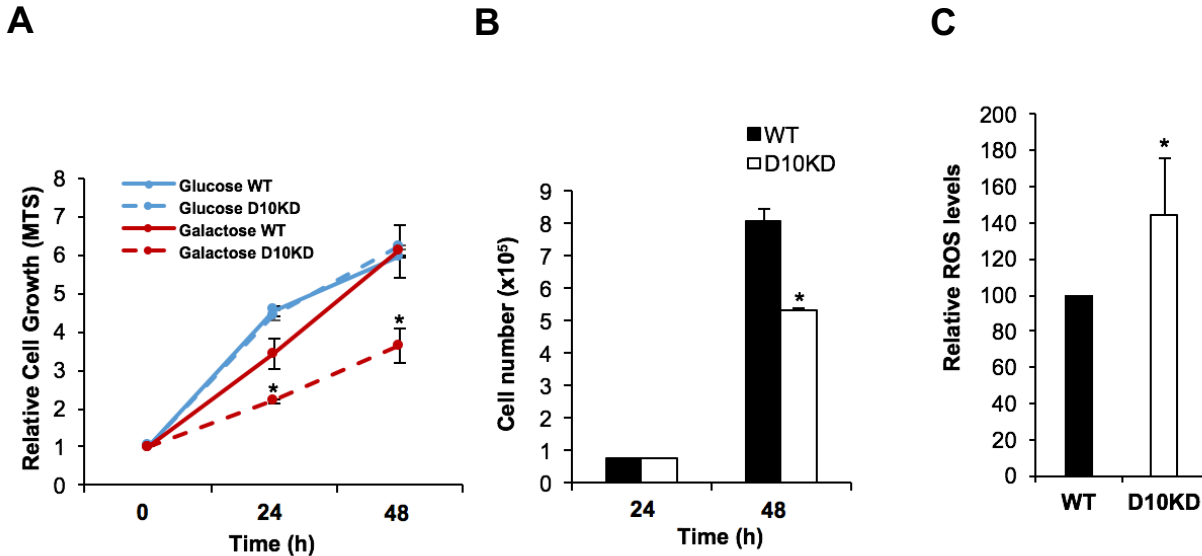
Figure 12. Knockdown of CHCHD10 has pleiotropic effects on cells. *A,* equal numbers of HEK293 WT and CHCHD10-KD (D10KD) cells were plated and grown in medium containing either glucose or galactose as the primary carbon source. Growth was assessed in real time using CellTiter 96 AQ$_{ueous}$ One Solution Cell Proliferation Assay (MTS) (n=4, *, p<0.05 for CHCHD10-KD as compared to WT grown in galactose, NS for glucose). *B,* equal numbers of HEK293 WT and CHCHD10-KD cells were plated and grown in galactose medium (n=4 each in quadruplicate, *, p<0.05). *C,* total cellular ROS was measured from WT and CHCHD10-KD HEK293 cells using CM-H$_2$DCFDA (n=4, *, p<0.05).

2. CHCHD10 regulates transcription in the nucleus

2.1. CHCHD10 functions as a repressor at the oxygen responsive element (ORE) in the nucleus

In order to characterize the effects of CHCHD10 in the nucleus, we used a luciferase reporter carrying the *COX4I2* promoter, which harbors the 13-bp ORE. Overexpression of CHCHD10 inhibits transcription at the *COX4I2* ORE (Figure 13A). To confirm that these effects occur exclusively at the ORE we used a mutant *COX4I2* ORE that has three point mutations in the 13-bp sequence (Aras et al., 2013). Overexpression of CHCHD10 had no effect on the mutant ORE, indicating that CHCHD10 functions as a repressor at the ORE. We confirmed this role using

an *MNRR1* and *COX4I2* ORE-harboring reporters in CHCHD10-KD cells. In these cells, both

MNRR1 and *COX4I2* are transcriptionally upregulated (Figures 13B and C).
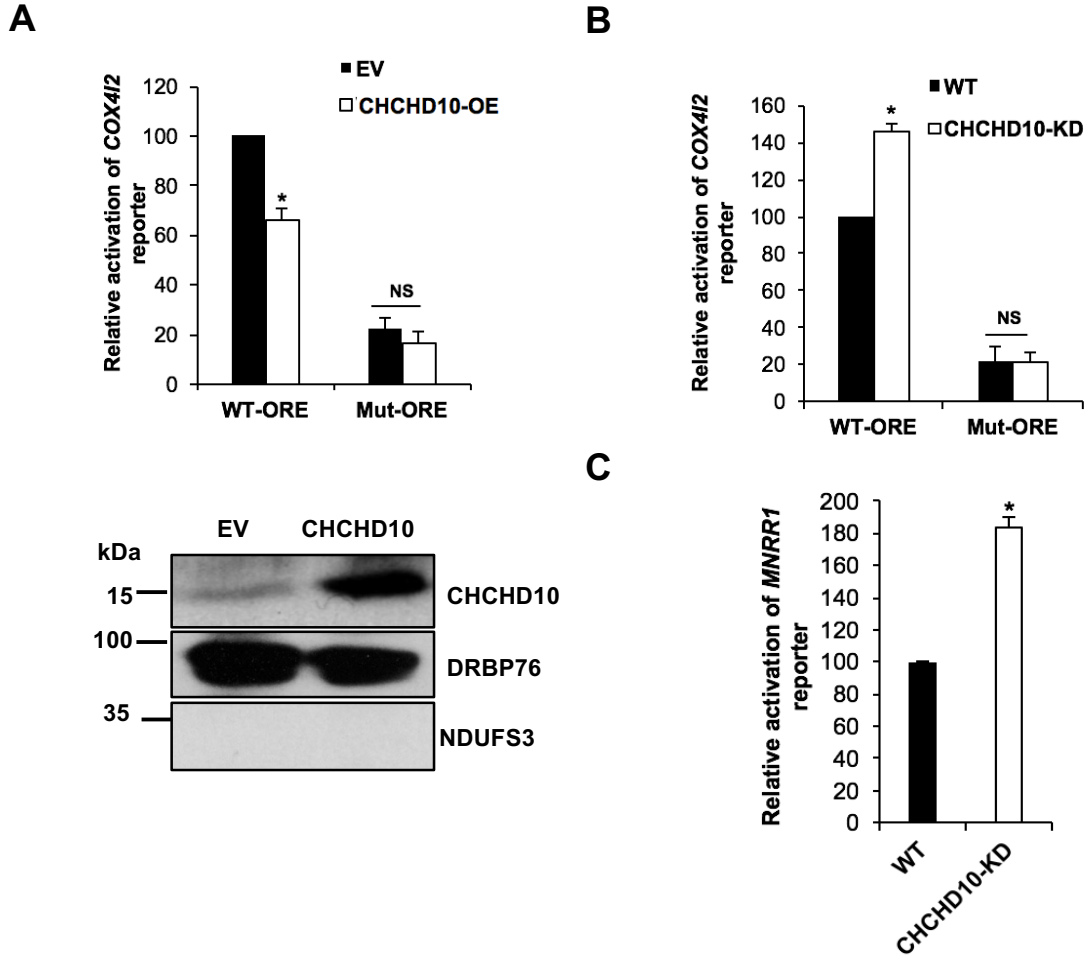
Figure 13. CHCHD10 functions as a repressor at the oxygen responsive element (ORE) in the nucleus. *A,* (Upper) HEK293 cells were co-transfected with 100 ng *COX4I2*-luciferase WT or mutant reporter plasmid and either 200 ng of an empty vector (EV) or a WT-CHCHD10 expression plasmid and a dual luciferase assay was performed 48 h post-transfection (n=4, *, p<0.05). (Lower) Equal amounts of nuclear lysate overexpressing EV or WT-CHCHD10 were loaded on an SDS-PAGE gel and probed with CHCHD10. DRBP76 was probed a loading control and mitochondrial protein NDUFS3 monitored fractionation. *B*, HEK293 or CHCHD10-KD cells were transfected with 100 ng *COX4I2*-luciferase WT or mutant reporter plasmid and a dual luciferase assay was performed 48 h after transfection (n=4, *, p<0.05). *C*, HEK293 or CHCHD10-KD cells were transfected with 100 ng *MNRR1*-luciferase reporter plasmid and assessed as in *B* (n=4, *, p<0.05).

2.2. CHCHD10 functions as a repressor by interacting with the inhibitory CXXC5 at the ORE

Transcription at the ORE is governed by three proteins: RBPJK, CXXC5, and MNRR1. At

20% oxygen, CXXC5 interacts with RBPJK at the ORE to repress transcription, whereas at 4%

MNRR1 displaces the inhibitory CXXC5 and activates RBPJκ-mediated transcription at the ORE (Aras et al., 2013). Since CHCHD10 functions as a repressor at the ORE, we hypothesized that CHCHD10 interacts with CXXC5 to strengthen repression and tested the interaction of CHCHD10 with CXXC5. CHCHD10 co-immunoprecipitates with CXXC5 (Figure 14A) whereas a transient knockdown of CXXC5 prevents the interaction of CHCHD10 with the *COX4I2* ORE (Figure 14B). These results suggest that CXXC5 is necessary for CHCHD10's effects at the ORE. Furthermore, CHCHD10 does not interact with MNRR1 in the nucleus (Figure 14C), consistent with opposed functions of CHCHD10 and MNRR1 in this cellular compartment.
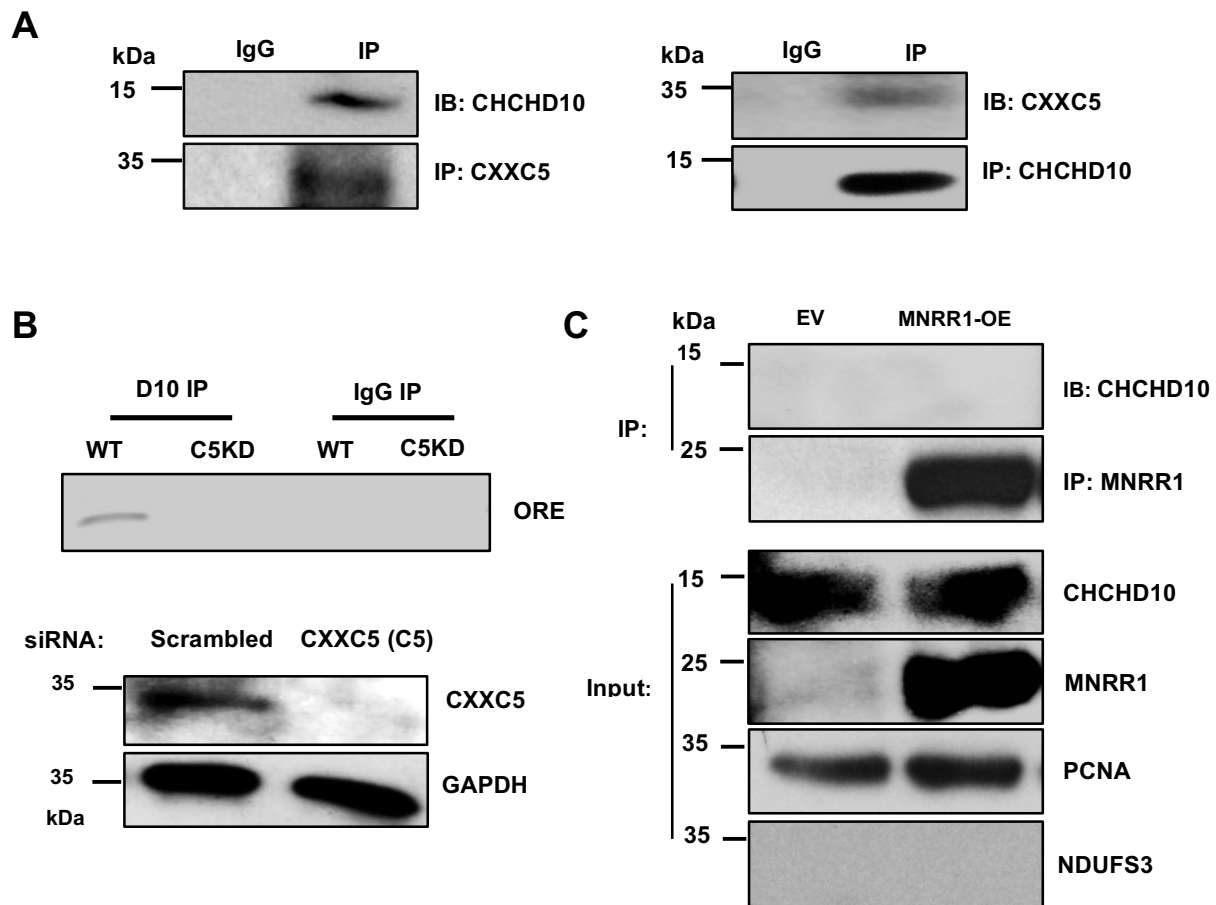
Figure 14. CHCHD10 functions as a repressor by interacting with the inhibitory CXXC5 at the ORE. *A,* HEK293 cells were transfected with a CXXC5 expression plasmid; 48 h post-transfection nuclear lysate was immunoprecipitated with CXXC5 beads and probed for endogenous CHCHD10 (left). HEK293 cells were transfected with a CHCHD10 expression plasmid; 48 h post-transfection nuclear lysate was immunoprecipitated with CHCHD10 beads and probed for endogenous CXXC5 (right). *B,* HEK293 cells (WT or CXXC5 knockdown) transfected with a CHCHD10 expression plasmid were used for a

DNA binding assay. Anti-CHCHD10 immunoprecipitate was used to detect bound ORE-specific DNA by PCR amplification using endogenous *COX4I2* ORE-specific primers. Knockdown of CXXC5 was confirmed by Western blot; GAPDH was probed as a loading control. *C,* HEK293 cells were transfected with EV or FLAG-tagged MNRR1 expression plasmids. After 48 h, equal amounts of a nuclear enriched lysate were immunoprecipitated with FLAG-beads and probed for CHCHD10. Equal amounts of input lysate were also separated on an SDS-PAGE gel and probed for MNRR1 (FLAG) and CHCHD10 levels. Nuclear protein PCNA was used as a loading control and mitochondrial protein NDUFS3 was used to monitor fractionation.

3. CHCHD10's regulates oxygen consumption in the mitochondria

3.1. CHCHD10 interacts with COX

CHCHD10 is a soluble protein in the IMS (Figure 15A and Bannwarth et al., 2014) and is

a substrate of the the CHCHD4-ALR pathway (Figure 15B and Lehmer et al., 2018). A transient

knockdown of CHCHD10 decreased COX activity and ATP levels (Martherus et al., 2010).

Complementing these observations, we find that CHCHD10 co-purifies with COX and interacts

with the intact complex *in vivo* (Figure 15C left)*.* Moreover, CHCHD10 is defective for its

interaction with COX in the absence of MNRR1 (Figure 15C, right). When we separated purified

bovine heart COX on a high percentage urea-acrylamide gel (Kadenbach et al., 1983) that

separates COX into its component subunits, we detected CHCHD10 immunologically (Figure

15C). However, the amount of CHCHD10 appears to be submolar, similar to MNRR1 (Figure 15C

and (Aras et al., 2015).
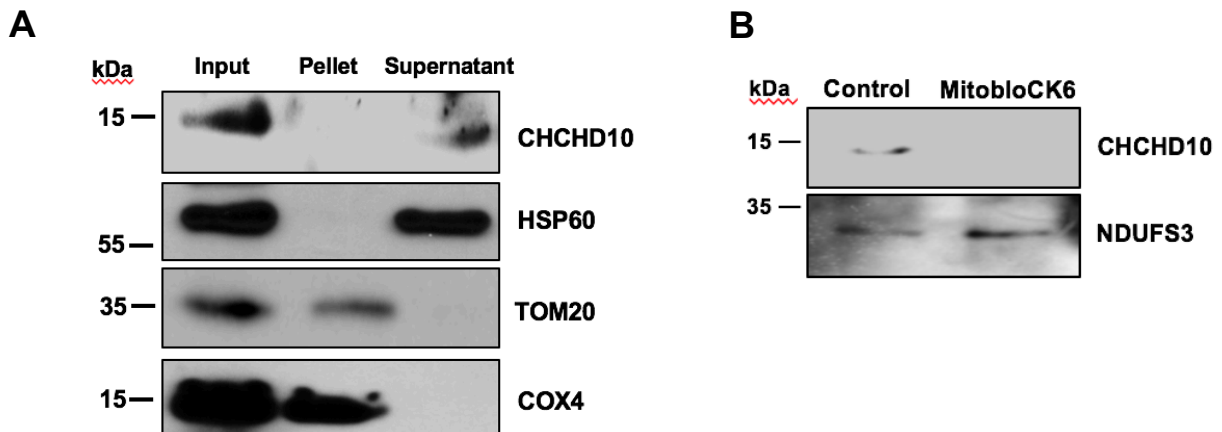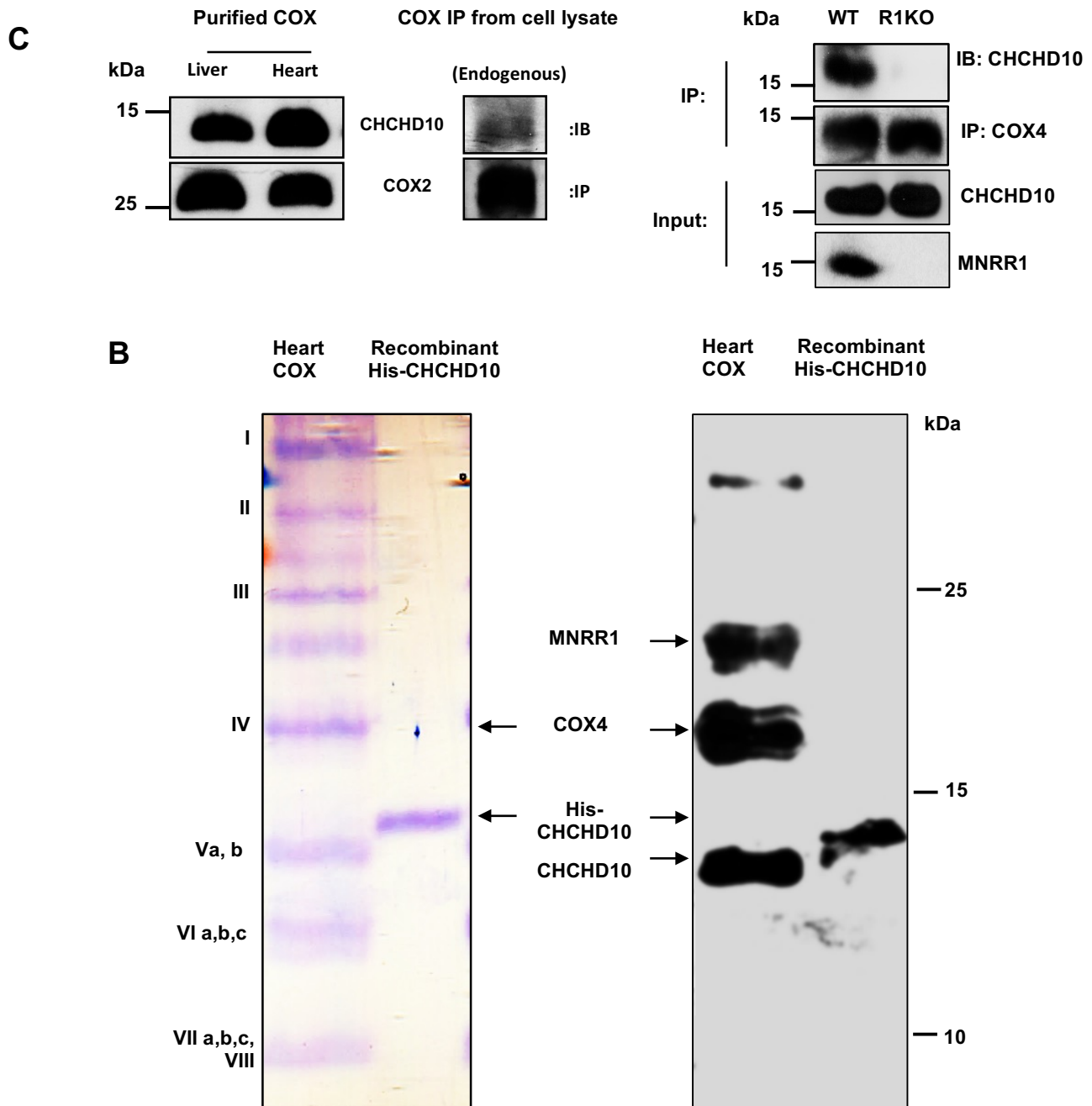
Figure 15. CHCHD10 interacts with COX. *A,* Mitochondria were isolated from HEK293 cells and treated with 100mM Na_2CO_3 (pH 11.5) for 30 mins on ice. Half the volume was saved as input fraction and the remaining was centrifuged at 14000rpm for 1.5 hrs. Equal volumes of input, pellet, supernatant fraction were separated on a 15 % SDS PAGE gel and probed for HSP60 (soluble fraction marker), TOM20 (integral membrane fraction marker for OMM), COX4 (integral membrane fraction marker for IMM) and CHCHD10. *B,* HEK293 cells were treated with DMSO (control) or 10µM MitobloCK overnight and mitochondria were isolated. Equal amounts of mitochondrial lysates were separated using SDS-PAGE and were probed for CHCHD10 and NDUFS3. *C,* (Upper) purified COX from cow liver and heart and mitochondrial fractions

from HEK293 cells were probed for CHCHD10. COX2 is probed as a loading control for the tissue COX and COX2-beads were used to immunoprecipitate endogenous COX from HEK293 cells. All samples were probed for CHCHD10. (Lower) To show lack of COX binding of CHCHD10 in MNRR1-KO cells, both WT and HEK293 MNRR1-KO cells were transfected with an expression plasmid for WT-CHCHD10 (FLAG-tagged). After 48 h, COX was immunoprecipitated from mitochondrial lysates with COX4-conjugated beads and probed for CHCHD10 (FLAG). Input fractions were also probed for CHCHD10 (FLAG) and MNRR1 levels. *B,* purified bovine heart COX (lane 1) and recombinant His-tagged CHCHD10 (lane 2) were separated on a 9-inch-long 50% acrylamide gel containing 7.2 M urea (Kadenbach 1983). Samples were separated in parallel and one set was stained with Coomassie Brilliant Blue and the other was transferred to a membrane and probed for CHCHD10, MNRR1, and COX4.

3.2. CHCHD10 stimulates oxygen consumption in the mitochondria

The stimulation by CHCHD10 of COX activity could be visualized in a cell-free assay by adding increasing amounts of recombinant CHCHD10 to purified COX *in vitro* (Figure 16A). We then tested the effect of CHCHD10 on COX activity *in vivo*, by measuring intact cellular oxygen consumption. In WT cells, overexpression of CHCHD10 increases oxygen consumption by ~ 22% (Figure 16B, bars 1 and 2) whereas in CHCHD10-KD cells oxygen consumption is decreased to ~60% that in WT cells (Figure 16B, bars 1 and 3). Overexpression of CHCHD10 in KD cells restores oxygen consumption (Figure 16B, bars 3 and 4). Note that the restoration is equivalent to wild-type cells overexpressing CHCHD10 (Figure 16B, bars 2 and 4). We then asked whether overexpression of WT-CHCHD10 can increase oxygen consumption in MNRR1-KO cells. We found that it could not (Figure 16C), consistent with the requirement of MNRR1 for CHCHD10 to interact with COX (Figure 15C, right). Taken together, the data show that CHCHD10 functions to stimulate COX activity in the mitochondria and that MNRR1 is necessary for this stimulation.

3.3. Defective mitochondrial oxygen consumption in CHCHD10-KD cells arises *via* defective phosphorylation of MNRR1

To investigate the mechanism of decreased oxygen consumption in CHCHD10-KD cells, we assessed whether MNRR1, which is 58% identical to CHCHD10, can replace and rescue defective oxygen consumption in CHCHD10-KD cells. We found that transfecting increasing amount of MNRR1 cannot increase oxygen consumption in CHCHD10-KD cells (Figure 17A). Since MNRR1's mitochondrial stimulation of respiration requires phosphorylation on Tyrosine-99 by ABL2 kinase/ARG, we evaluated the phosphorylation level of endogenous MNRR1. Despite

elevated levels of MNRR1 in the mitochondria, there is less phosphorylation of MNRR1 in CHCHD10-KD cells (Figure 17B). We also found that phosphorylation of overexpressed mitochondrial MNRR1 was reduced (Figure 17C).
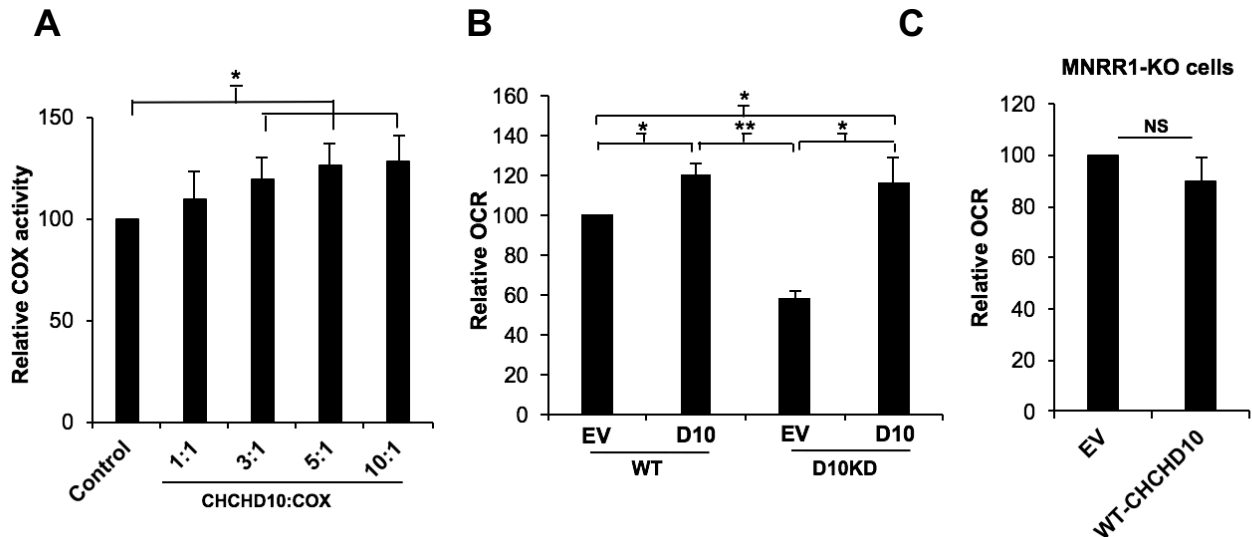
Figure 16. CHCHD10 stimulates oxygen consumption in the mitochondria. *A, in vitro* COX activity was assessed using a kit (Sigma) that measures change in cytochrome *c* absorbance. Dialyzed purified COX and cytochrome *c* from bovine heart were used for the assay (n=4, *, $p<0.05$). COX (10 nM) activity was measured after supplementation with the stated amounts of CHCHD10. *B,* HEK293 WT and CHCHD10-KD cells were transfected with either empty vector (EV) or WT-CHCHD10. After 24 h, 40,000 cells per well were plated and used for measuring oxygen consumption using the Seahorse Bioanalyzer (n=4, *, $p<0.05$; **, $p<0.01$). *C,* HEK293 MNRR1-KO cells were transfected with either empty vector (EV) or WT-CHCHD10. After 24 h, 40,000 cells per well were plated and used for measuring oxygen consumption using the Seahorse Bioanalyzer (n=3, NS).

To investigate the mechanism, we examined levels of ARG in CHCHD10-KD mitochondria and found them to be lower than WT levels (Figure 17C). We therefore conclude that reduced ARG in mitochondria caused reduced MNRR1 phosphorylation, and thereby reduced interaction with COX and decreased oxygen consumption.

We hypothesized from the above observations that reduced ARG in mitochondria caused reduced MNRR1 phosphorylation, and thus reduced interaction with COX (Figure 17D) and decreased oxygen consumption, thereby rationalizing the inability of overexpressed MNRR1 to suppress the oxygen consumption defect in CHCHD10-KD cells (Figure 17A). To test this hypothesis, we overexpressed phosphomimetic MNRR1 (Y99E), which is expected to circumvent the reduced MNRR1 phosphorylation seen in CHCHD10-KD cells. We indeed observed rescue

of oxygen consumption in CHCHD10-KD cells (Fig.17E) whereas we did not see this rescue when we overexpressed WT or non-phosphorylatable (Y99F) MNRR1 at the same level. We can also see this effect in vitro using purified components: maximum COX stimulation is seen with a combination of phosphomimetic-MNRR1 and CHCHD10 as compared to each by itself (Figure 17F).

A

B
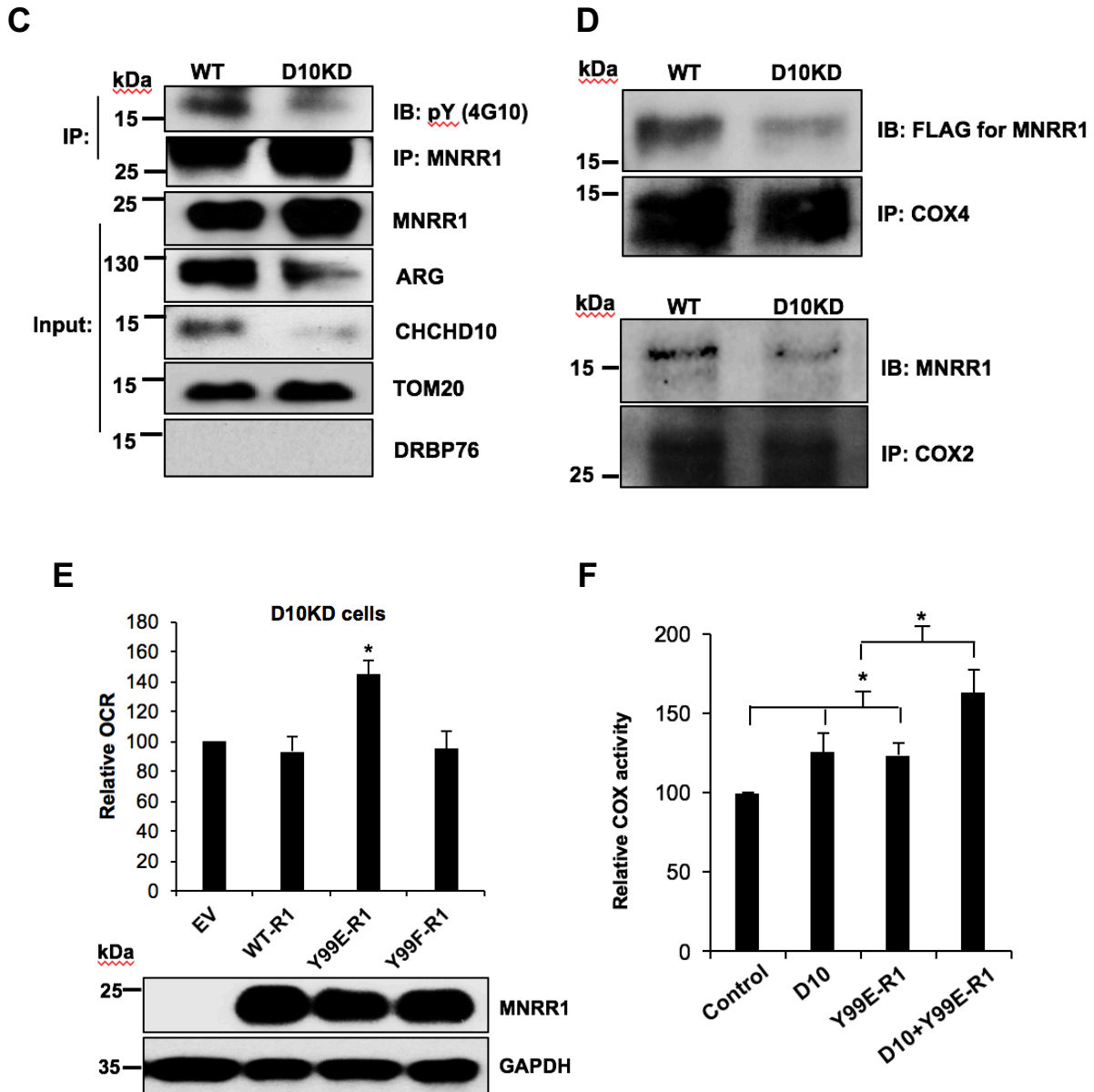
Figure 17. Overexpression of WT-MNRR1 fails to suppress the oxygen consumption defect of CHCHD10-KD. *A,* (Upper) HEK293 WT and CHCHD10-KD cells (bars 1 & 2) are each overexpressing an empty vector (EV). CHCHD10-KD cells (D10KD) are overexpressing increasing amounts of MNRR1 (bars 3-5) or WT-CHCHD10 (bar 6). In each case, 40,000 cells per well were taken and oxygen consumption was measured 48 h after transfection (n=4, *, p<0.05; **, p<0.01). (Lower) Equal amounts of lysate were probed for MNRR1 (shown at 2 exposure levels) and CHCHD10 levels. GAPDH was probed as a loading control. *B,* purified mitochondrial fractions from WT or CHCHD10-KD cells were immunoprecipitated with phosphotyrosine (pY)-beads. Equal volumes of immunoprecipitate were separated on an SDS-PAGE gel and probed for MNRR1 and CHCHD10 levels. NDUFS3 was probed as a loading control and nuclear protein DRBP76 monitored fractionation. *C,* WT or CHCHD10-KD cells were transfected with FLAG-tagged MNRR1 plasmid. After 48 h, equal amounts of purified mitochondrial lysate were used for immunoprecipitation (IP) with FLAG beads. Equal IP volumes were separated by SDS-PAGE and probed with anti-pY and -FLAG (for MNRR1). Input fractions were probed for ARG and CHCHD10 levels; TOM20 was a loading control and nuclear protein DRBP76 monitored fractionation. *D,* (Above) purified mitochondrial fractions from WT or CHCHD10-KD cells were immunoprecipitated with FLAG-beads. Equal

volumes of immunoprecipitate were separated on an SDS-PAGE gel and probed for MNRR1(FLAG) and COX4 levels. (Below) purified mitochondrial fractions from WT or CHCHD10-KD cells were immunoprecipitated with COX2 (MTCO2) beads. Equal volumes of immunoprecipitate were separated on an SDS-PAGE gel and probed for MNRR1(endogenous) and COX2 levels. *E*, CHCHD10-KD cells were transfected with either EV or one of the following FLAG-tagged constructs: WT-MNRR1 (WT-R1), Y99E-MNRR1 (Y99E-R1) or Y99F-MNRR1 (Y99F-R1). Y99E and Y99F represent glutamic acid (phosphomimetic) and phenylalanine (non-phosphorylatable) replacements, respectively, for WT tyrosine at position 99. After transfection (48 h), 40,000 cells per well were utilized for oxygen consumption measurement using the Seahorse Bioanalyzer (n=4, *, p<0.05). (Below) equal amounts of lysate were probed for FLAG (MNRR1 expression) and the GAPDH loading control. *F, in vitro* COX activity was assessed using an assay kit (Sigma). Dialyzed purified COX and cytochrome *c* from bovine heart were used for the assay of COX with the addition of CHCHD10, MNRR1 (R1) bearing a phosphomimetic replacement (Y99E), and CHCHD10 plus MNRR1-Y99E (n=4, *, p<0.05).

4. CHCHD10's effects in the nucleus and mitochondria under stress

4.1. CHCHD10 function is enhanced at 8% hypoxia

CHCHD10 levels are upregulated under hypoxia (Figure 11A) and this upregulation is seen in both compartments (Figure 11D). Hence, we tested for the functional effects in both compartments under hypoxia. In the nucleus, we tested these effects using the dual luciferase reporter assay and found that the transcription of the *COX4I2* reporter is repressed at 8% oxygen to ~60% of transcriptional activity seen at 20% oxygen (Figure 18A - comparing bars 1 and 2). This is comparable to effect seen by overexpressing WT-CHCHD10 under normoxia (Figure 13A). Protein levels of RBPJκ and CXXC5 are not affected by 8% oxygen (Figure 18B left and middle). MNRR1, which itself harbors an ORE is repressed (Figure 18B right). Taken together, CHCHD10 upregulation at 8% oxygen represses transcription at the ORE as seen at both transcript (Figure 18A - comparing bars 1 and 2) and protein level (Figure 18B right).

To assess mitochondrial effects, we also tested oxygen consumption under hypoxia in WT and CHCHD10-KD cells. Under hypoxia, there is ~89% increase in WT-cells and ~40% increase in CHCHD10-KD cells (Figure 18B). We reasoned that the increase in CHCHD10-KD cells may be due to upregulation of CHCHD10 under hypoxia and tested protein levels in CHCHD10-KD cells at 8% oxygen. Using Western blot analysis, we see that CHCHD10 levels are upregulated in the CHCHD10-KD under hypoxia comparable to WT levels, similar to the effect seen on oxygen consumption where, under hypoxia, oxygen consumption in CHCHD10-KD cells increases to

levels comparable to WT (Figure 18C). Hence, at 8% hypoxia, the upregulated CHCHD10 activates oxygen consumption.

During regular growth and maintenance, cells in the incubator are exposed to ~20% oxygen, which corresponds to atmospheric levels. Eight percent oxygen may represent physiological hypoxic "stress" in a cell culture system since cells this is lower than the normal growth conditons; 8% is a part of physiological oxygen levels that range from 1-13% (Carreau et al., 2011). Under these conditions, CHCHD10 function is enhanced in both compartments.

A

B
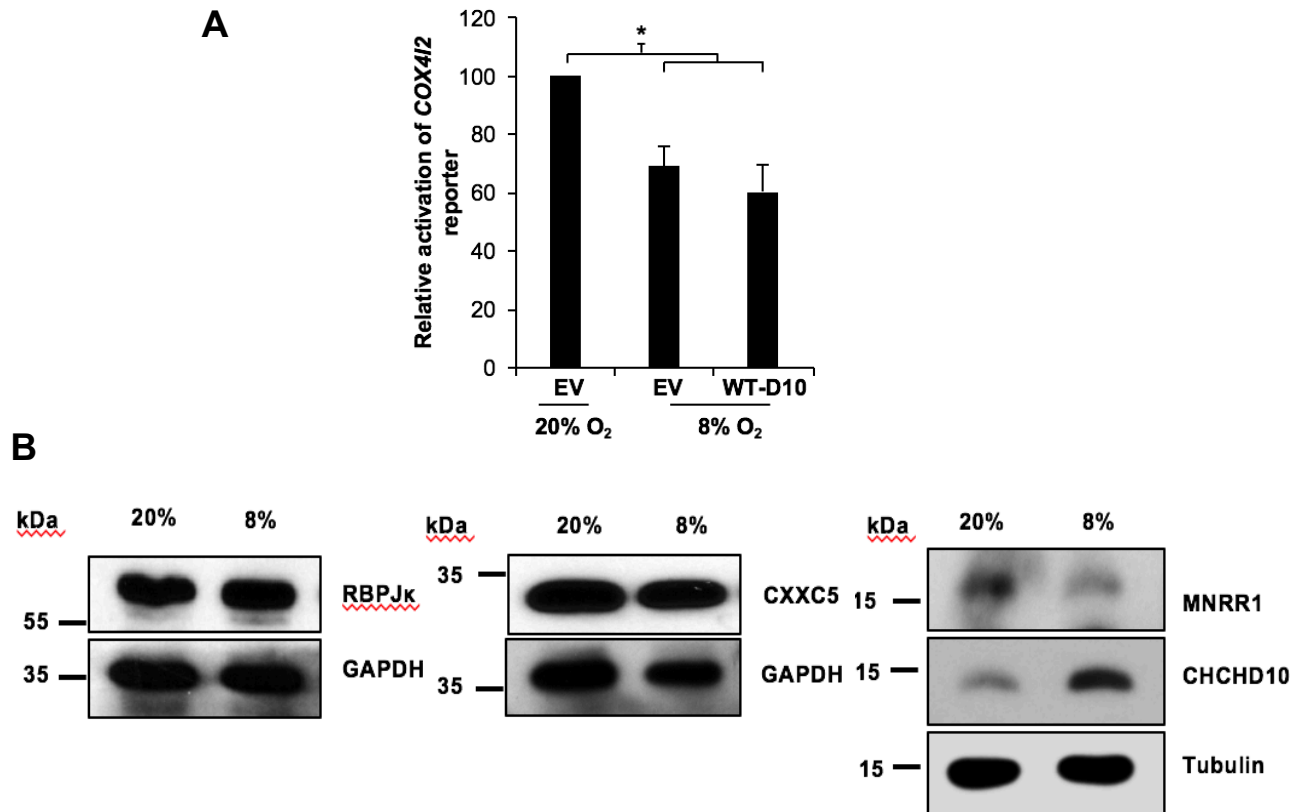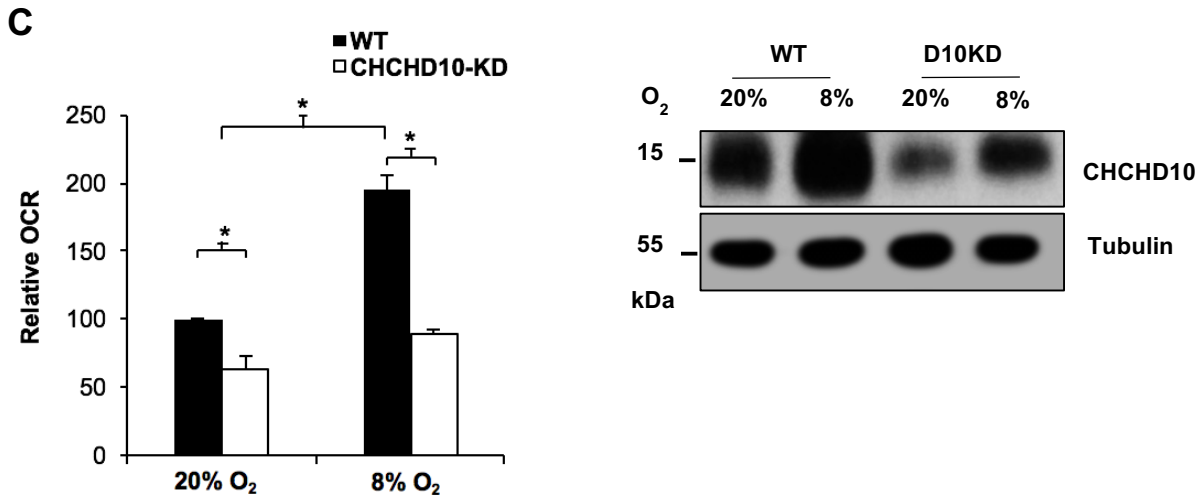
Figure 18. CHCHD10 function is enhanced at 8% hypoxia. *A*, HEK293 cells were co-transfected with 100 ng *COX4I2*-luciferase WT reporter plasmid and either 200 ng empty vector (EV) or WT-CHCHD10 plasmid and incubated at 20% O_2 or 8% O_2. After 48 h, a dual luciferase assay was performed to measure reporter activity (n=4, *, p<0.05). *B,* HEK293 cells were incubated for 48 h at the 20% O_2 or 8% O_2. Whole cell lysates were separated on an SDS-PAGE gel and analyzed for RBPJκ (left) and CXXC5 (middle). GAPDH was probed as a loading control. Lysates were also probed for CHCHD10 and MNRR1 levels (right). Tubulin was probed as a loading control. *C*, (Left) WT-HEK293 or CHCHD10KD-HEK293 (30,000 cells/well) were plated in a 96 well plate and incubated at 20% O_2 or 8% O_2 for 24 h. Oxygen consumption was analyzed using the Oxygen Consumption Rate Assay Kit (Cayman) per the manufacturer's instructions (n=4, each in triplicate). *C*, equal amounts of the cell lysates from either WT or CHCHD10-KD incubated at 20% O_2 and 8% O_2 were loaded on an SDS-PAGE gel and probed using anti-CHCHD10 antibody. Tubulin was probed as loading control.

4.2. Point mutations in CHCHD10 abrogate CHCHD10's function in the nucleus and mitochondria

More than 10 different mutations in CHCHD10 have been linked with neurodegenerative diseases, and most lie within the region encoded by exon 2 (amino acids 15-87). Two functionally uncharacterized mutations found in this region, G66V and P80L, were chosen for study because they have been associated with several different phenotypes including ALS (Dols-Icardo et al., 2015; Johnson et al., 2014; Muller et al., 2014; Ronchi et al., 2015; Zhang et al., 2015), CMT2A (G66V- (Auranen et al., 2015)), and spinomuscular atrophy (Penttila et al., 2016). In fact, the G66V mutation has been associated with a spectrum of phenotypes within the same family ranging from proximal sensorimotor neuropathy to spinal muscular atrophy and, in one case, resembled motor neuron disease (Pasanen et al., 2016). Importantly, unlike some mutations such

as P34S, these have not been identified in any control populations in several different studies, strengthening their disease association. Since CHCHD10 localizes to the nucleus and the mitochondria, we first asked whether the mutations affect localization of the protein. Both mutations show a similar localization profile as WT-CHCHD10 (Figure 19) and hence we assessed compartment-specific effects.
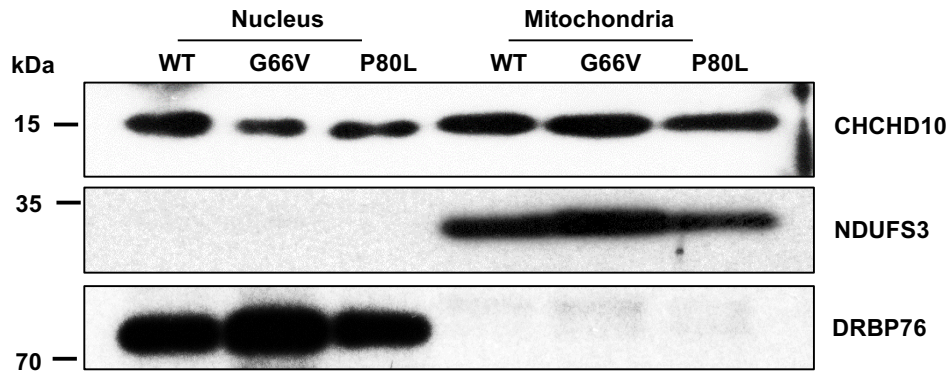
Figure 19. CHCHD10 point mutants are localized to the nucleus and mitochondria. *A,* Nuclei and mitochondria from HEK293 transfected with WT or CHCHD10 point mutants were fractionated and analyzed for levels of CHCHD10. Purity of fractionation was confirmed using DRBP76 as a nuclear marker and NDUFS3 as mitochondrial marker.

4.2.1. Point mutations in CHCHD10 fail to repress ORE-mediated transcription in the nucleus

To test for nuclear effects, we overexpressed WT-CHCHD10 or the point mutants in HEK293 cells and assessed their effects on the *COX4I2*-ORE driven luciferase reporter. Unlike WT-CHCHD10, the mutants failed to repress transcription (Figure 20A bars 1-4). These effects are abolished when we used the mutant ORE (Figure 20A, bars 5-8) indicating that they are ORE-mediated. We also examined these effects at the translational level by analyzing levels of COX4I2 and MNRR1, two ORE-regulated proteins. Both are repressed by WT-CHCHD10 but not by the mutants (Figure 19B). To identify the mechanism for the failure to repress, we compared the interaction of WT-CHCHD10 and the point mutants with CXXC5 and found that both mutants are defective for this interaction (Figure 20C).
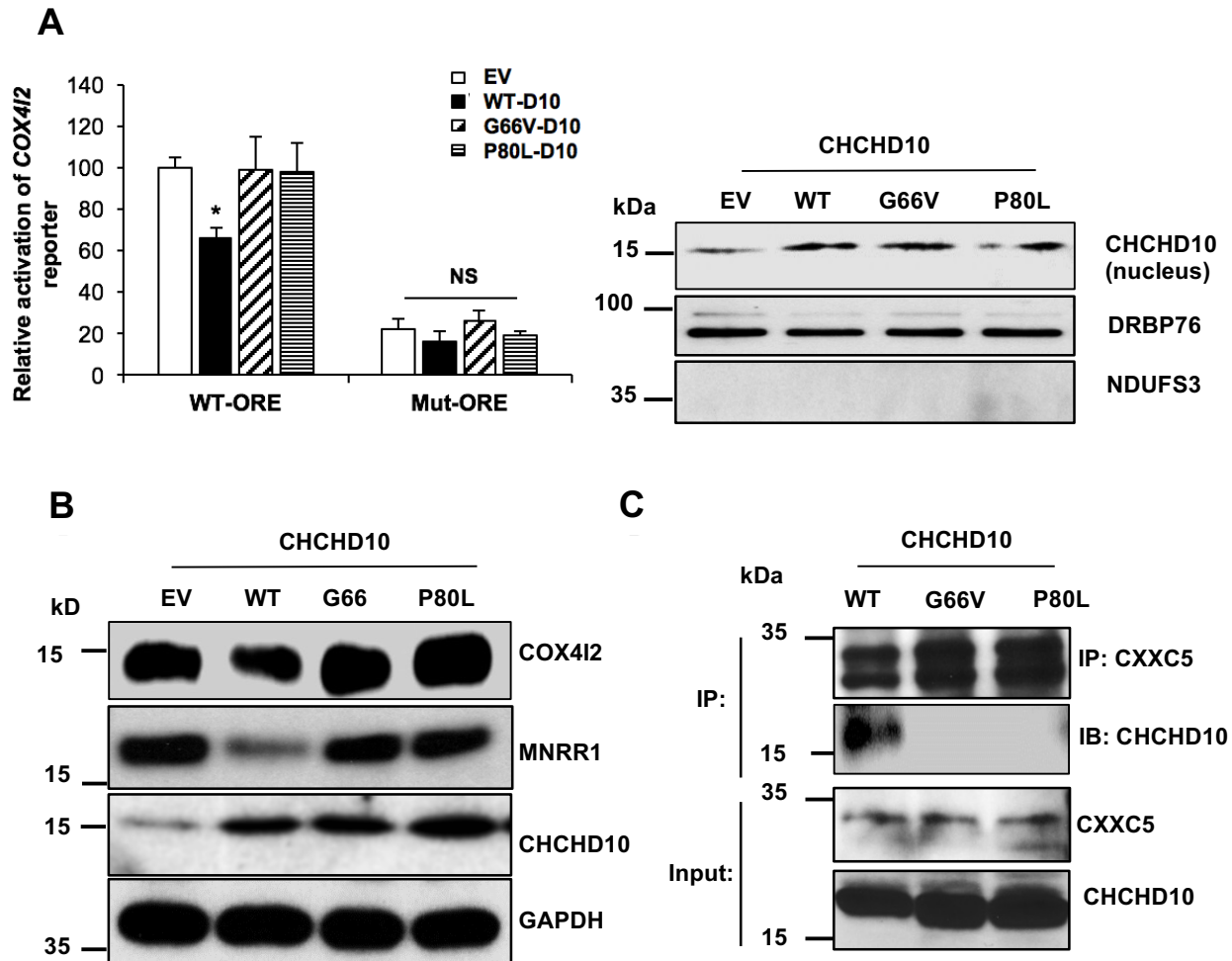
Figure 20. Point mutations in CHCHD10 fail to repress ORE-mediated transcription in the nucleus
A, (Left) HEK293 cells were co-transfected with 100 ng *COX4I2*-luciferase WT or mutant reporter plasmid and either 200 ng empty vector (EV) or the different CHCHD10 expression plasmids. After 48 h, a dual luciferase assay was performed to measure reporter activity (n=4, *, p<0.05). (Right) Equal amounts of nuclear fractions transfected with EV, WT-CHCHD10, or point mutants were separated on an SDS-PAGE gel and probed with CHCHD10. DRBP76 was probed as a loading control and mitochondrial protein NDUFS3 monitored fractionation. *B,* equal amounts of whole cell lysates transfected with EV, or with WT or point mutants of CHCHD10, were separated on an SDS-PAGE gel and probed for MNRR1 and CHCHD10. GAPDH was used as a loading control. *C,* HEK293 cells were co-transfected with CXXC5 and with WT or CHCHD10 (FLAG-tagged) point mutants. After 48 h nuclear lysates were immunoprecipitated with CXXC5-conjugated beads and probed for FLAG-tagged CHCHD10. Equal amounts of input fractions were also probed for FLAG and CXXC5.

4.2.2. Point mutations in CHCHD10 are defective in maintaining optimal ETC function in the mitochondria

Since CHCHD10 interacts with COX in the mitochondria and promotes its activity, we examined whether either of the two mutations could rescue the oxygen consumption defect seen in

CHCHD10-KD cells. We found that, unlike WT-CHCHD10, neither point mutant was able to rescue these defects, being no better than an overexpressed empty vector (Figure 21A). We also tested the mitochondrial membrane potential in CHCHD10-KD cells and found it to be ~50% of WT cells. Overexpression of WT-CHCHD10 can rescue this defect whereas neither point mutant was able to rescue the defective membrane potential (Figure 21B). Overexpression of WT and CHCHD10 mutants was confirmed via Western blot (Figure 21C). Since the mutants were defective for regulating oxygen consumption and membrane potential we also tested mitochondrial ROS levels. Overexpression of both mutants enhanced ROS levels, in contrast to WT-CHCHD10, which decreased ROS levels (Figure 21D).

To examine the mechanism by which this could occur, we tested for the interaction of WT or mutant CHCHD10 with MNRR1 and COX. Each mutant protein was defective for binding to MNRR1 and thereby COX (Figure 21E). A failure to bind MNRR1 leads to reduced ARG-mediated phosphorylation of MNRR1 (Figure 21F), which then cannot bind to and stimulate COX activity. WT as well as both mutant CHCHD10 proteins bind ARG (Figure 21F). Hence, the point mutations G66V and P80L in CHCHD10 allow it to bind ARG, but not MNRR1. This leads to a failure to rec ruit ARG to phosphorylate MNRR1.Defective phosphorylation impairs MNRR1's binding to COX

C

D

E

F

CHCHD10-KD

G
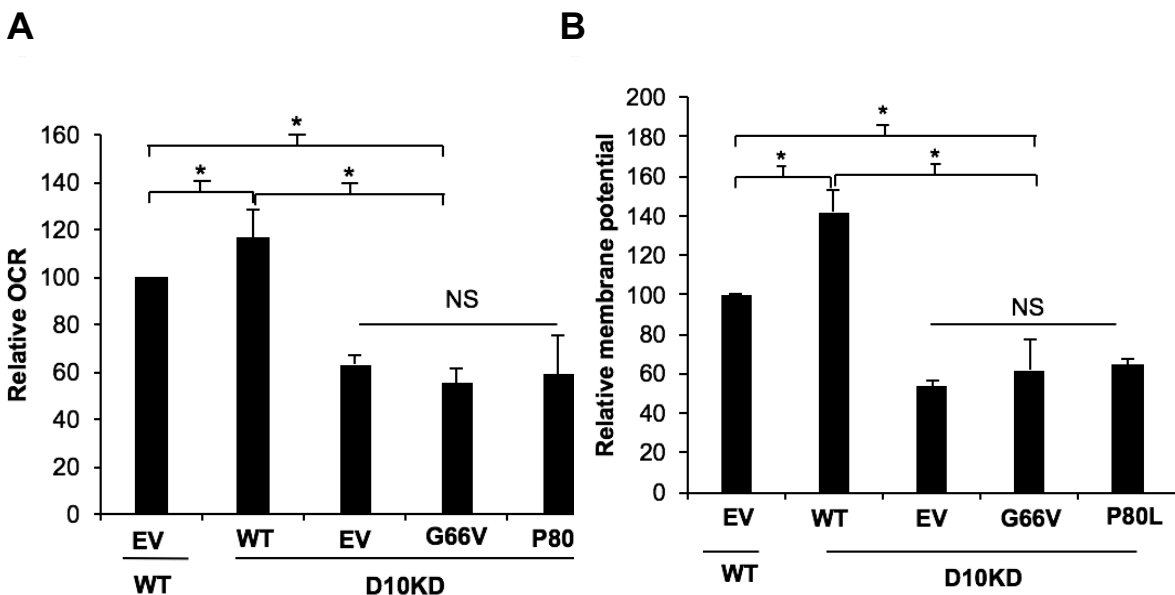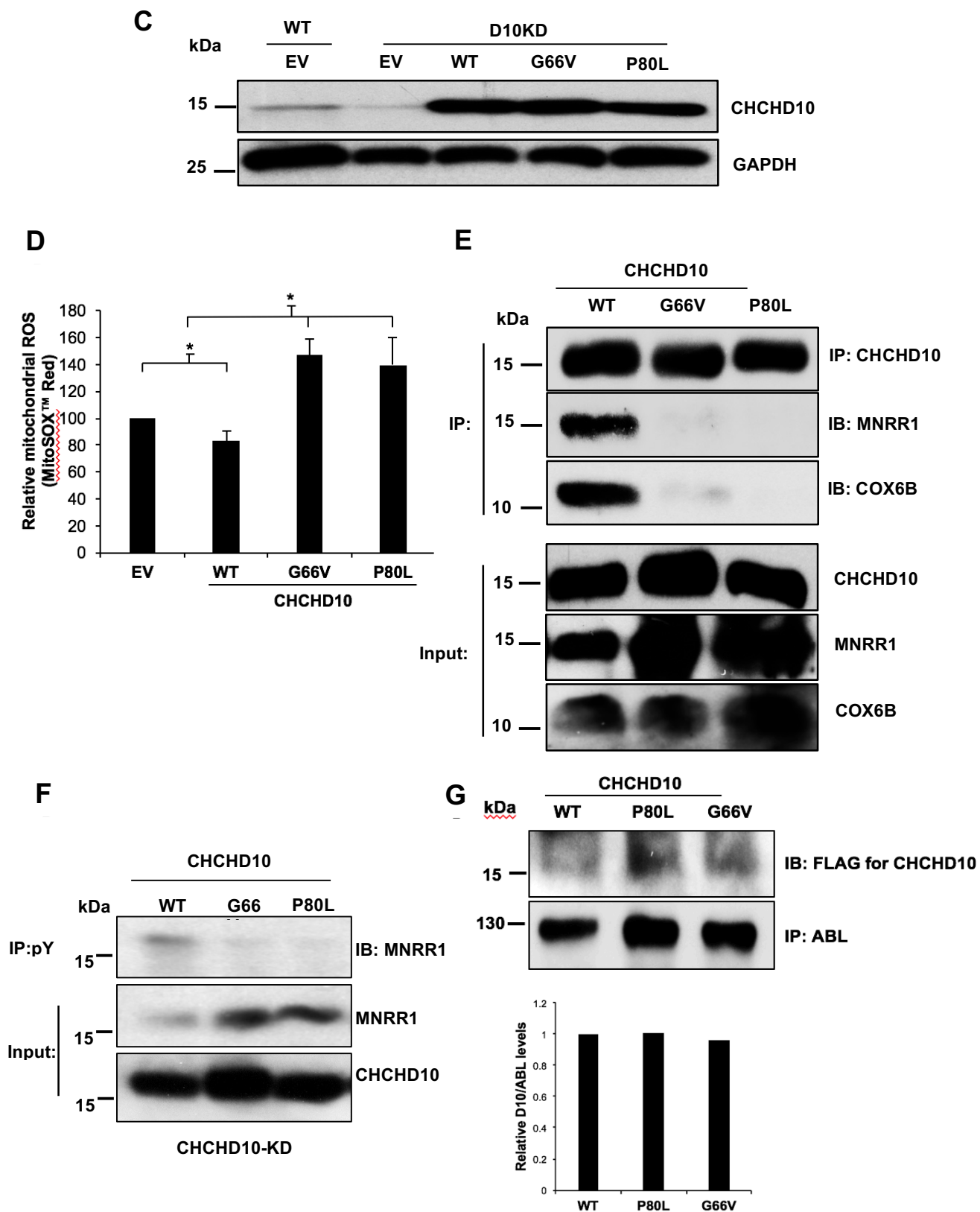
Figure 21. Point mutations in CHCHD10 are defective in maintaining optimal ETC function in the mitochondria. *A,* (Above) WT cells were transfected with an empty vector as a control and CHCHD10-KD cells were transfected with either EV, WT, or point mutants for CHCHD10. Oxygen consumption was measured after 24 h (n=4, *, p<0.05). *B,* WT cells were transfected with an empty vector as a control and CHCHD10-KD cells were transfected with either EV, WT, or point mutants for CHCHD10. After 24 h, 40,000 cell/well were plated on a 96-well plate and mitochondrial membrane potential was measured using TMRM (Invitrogen) per the manufacturer's protocol (n=4, each in quadruplicate). *C,* Equal amounts of whole cell lysates of WT cells transfected with an empty vector and CHCHD10-KD cells transfected with EV, WT-CHCHD10, or point mutants were separated on an SDS-PAGE gel and probed with CHCHD10. GAPDH served as a loading control. *D,* HEK293 cells were transfected with an empty vector (EV), or with WT or mutant CHCHD10 expression plasmids. After 48 h, 1.5×10^5 cells were plated on a 24-well plate and mitochondrial ROS was measured with MitoSOX Red (n=4, *, p<0.05). Results are shown as fluorescence relative to the EV sample. *E,* HEK293 cells were co-transfected with WT- or (FLAG-tagged) CHCHD10 point mutants. After 48 h mitochondria-enriched lysates were used for immunoprecipition (IP) of FLAG-tagged CHCHD10 and probed (IB) for MNRR1 and COX6B levels. Equal amounts of input fractions were also probed for FLAG (for CHCHD10), MNRR1, and COX6B. *F,* CHCHD10-KD HEK293 cells were transfected with WT-CHCHD10 or point mutants (FLAG-tagged). Equal amounts of mitochondrial enriched fractions were used for immunoprespicitation using anti-phosphotyrosine (pY) antibody. Equal volumes of the IP was separated on an SDS-PAGE gel and and probed using anti-MNRR1 antibody and anti-FLAG (for CHCHD10) to confirm expression. *G,* (Above) HEK293 cells were co-transfected with ABL2 overxpression plasmid and either WT or mutant CHCHD10 (FLAG tagged). ABL was immunoprecipated from equal amount of mitochondria-enriched fractions and probed for FLAG-CHCHD10. (Below) Quantification of bands from Western blot above.

preventing it from activating mitochondrial oxygen consumption. This presumably leads to higher

ROS levels.

CHAPTER III: DISCUSSION

The regulation of mitochondrial energy production in response to extracellular changes is a key feature of cellular homeostastic mechanisms that require, at least in part, mito-nuclear signaling to alter gene transcription (Kotiadis et al., 2014; Quiros et al., 2016). These changes in the extracellular environment need to be communicated in order to alter energy production for modulating protein levels by synthesis or degradation in the short-term, as well as to alter transcription of genes in the long-term. This is essential for the cell to adapt to environmental changes (Cagin and Enriquez, 2015). There are several mechanisms that regulate energy production in the mitochondria and one important mechanism is via signaling to the nucleus (Liu and Butow, 2006; Quiros et al., 2016). In order to signal changes in the short term, mitochondria produce ROS and may release other metabolites such as acetyl CoA, succinate, and lactate (Haas et al., 2016; Pietrocola et al., 2015). In the long-term, various stresses may activate transcriptional pathways. Key examples are activation of HIF1α under as low oxygen levels (Wang et al., 1995) and SIRT1 (Canto et al., 2009) and AMPK (Shao et al., 2014) activated by low energy and starvation. Newer studies also indicate that TCA cycle intermediates may localize to the nucleus during activation of the zygotic genome (Nagaraj et al., 2017). All these signals arise from and affect one key process, mitochondrial OxPhos.

We previously showed that MNRR1 is a component of a mito-nuclear pathway that has a distinct function in each compartment. MNRR1 regulates oxygen consumption in the mitochondria and transcription in the nucleus (Aras et al., 2017; Aras et al., 2015; Aras et al., 2013). CHCHD10 is a protein that is 58% identical with MNRR1 and both work in conjunction to regulate OxPhos in the mitochondria (Burstein et al., 2017; Straub et al., 2017). They belong to the twin CX_9C family that primarily form scaffolds for the formation and the organization of the large macromolecular complexes found in mitochondria (Cavallaro, 2010). COX is one such complex, composed of 13 or 14 subunits (Balsa et al., 2012; Kadenbach, 2017; Pitceathly and Taanman, 2018) and MNRR1 and CHCHD10 interact with and regulate its activity.

MNRR1 and CHCHD10 have a common homolog in yeast, Mix17, and appear to be the product of a gene duplication event prior to the mammalian radiation. Moreover, the presence of disulfide linkages to stabilize the CHCH domain imparts potential redox sensitivity to these proteins, which may be essential to their ability to regulate the stability (Cavallaro, 2010) and hence activity of COX under altered oxygen tensions. The fact that MNRR1 and CHCHD10 function as a complex was also recognized in a recent analysis of an ALS-associated mutation, R15L, in CHCHD10 (Straub et al., 2017).

1. CHCHD10 and MNRR1's effects in the mitochondria

CHCHD10 and MNRR1 in the mitochondria have a cooperative role to bind COX and modulate its function. MNRR1's mitochondrial role has been characterized previously (Aras et al., 2015), including the key step of its phosphorylation by Abl2 kinase/ARG (Aras et al., 2017). In the present study, we identified the mitochondrial role of CHCHD10 to recruit ARG in order to phosphorylate MNRR1 (Figure 22 right). This phosphorylation enhances the binding of MNRR1 to COX and increases COX activity. The above role for CHCHD10 is supported by the observations of reduced phosphorylation of MNRR1 and decreased oxygen consumption in CHCHD10-KD cells.

In CHCHD10-KD cells there is lower phosphorylation of both endogenous (Figure 17C), and overexpressed MNRR1 (Figure 17B). One other effect of this modification is that the phosphorylated form is turned over faster as compared to the non-phosphorylatable version of MNRR1 (unpublished data). In CHCHD10-KD cells, there is reduced MNRR1 phosphorylation, and the observation that the non-phosphorylatable form is more stable is bolstered by the finding that MNRR1 levels are elevated in CHCHD10-KD cells. Moreover, the non-phosphorylatable form of MNRR1 is defective in activating oxygen consumption as compared to the phosphomimetic version (ibid). The elevated MNRR1 levels in CHCHD10-KD as compared to WT cells are not active due to the lower phosphorylation of MNRR1 in the knockdown cells. Hence this protein cannot rescue the defective oxygen consumption. This failure to rescue defective OxPhos was

confirmed with the overexpression of WT-MNRR1, which has defective phosphorylation (Figure 17B) and cannot enhance oxygen consumption in CHCHD10-KD cells (Figure 17A). Only the overexpression of the phosphomimetic form can rescue this defect (Figure 17E). This finding indicates that CHCHD10 is necessary for optimal MNRR1 function. However, CHCHD10 cannot function in the absence of MNRR1. CHCHD10 fails to bind COX (Figure 15C) and activate OxPhos (Figure 16C) in MNRR1-KO cells. These observations indicate that each is necessary for the optimal role of the other in mitochondria. Thus, maximal activation would occur when both CHCHD10 and MNRR1 are added together as seen in the *in vitro* COX activity assay (Figure 17F).

CHCHD10 and MNRR1 have common orthologs in many lower organisms including yeast, worms and flies. Their similar role in regulating mitochondrial function is clearly conserved throughout evolution. The knockdown of their common orthologs (Mix17 in yeast, har1 in worms, and CG5010 in flies) all decrease oxygen consumption (Longen et al., 2009; Meng et al., 2017; Woo et al., 2017) and enhance ROS levels (Meng et al., 2017; Woo et al., 2017; Zubovych et al., 2010). The common form in lower organisms that was duplicated and maintained as distinct genes in higher organisms (Cavallaro, 2010) may indicate that this was necessary in order to provide an additional layer of regulation for fine-tuning COX activity in the mitochondria. This additional layer is reflected in the observation that CHCHD10 binds COX via MNRR1 (Figure 15C) and MNRR1 is activated fully only in the presence of CHCHD10 that brings ARG to phosphorylated MNRR1 (Figure 17).
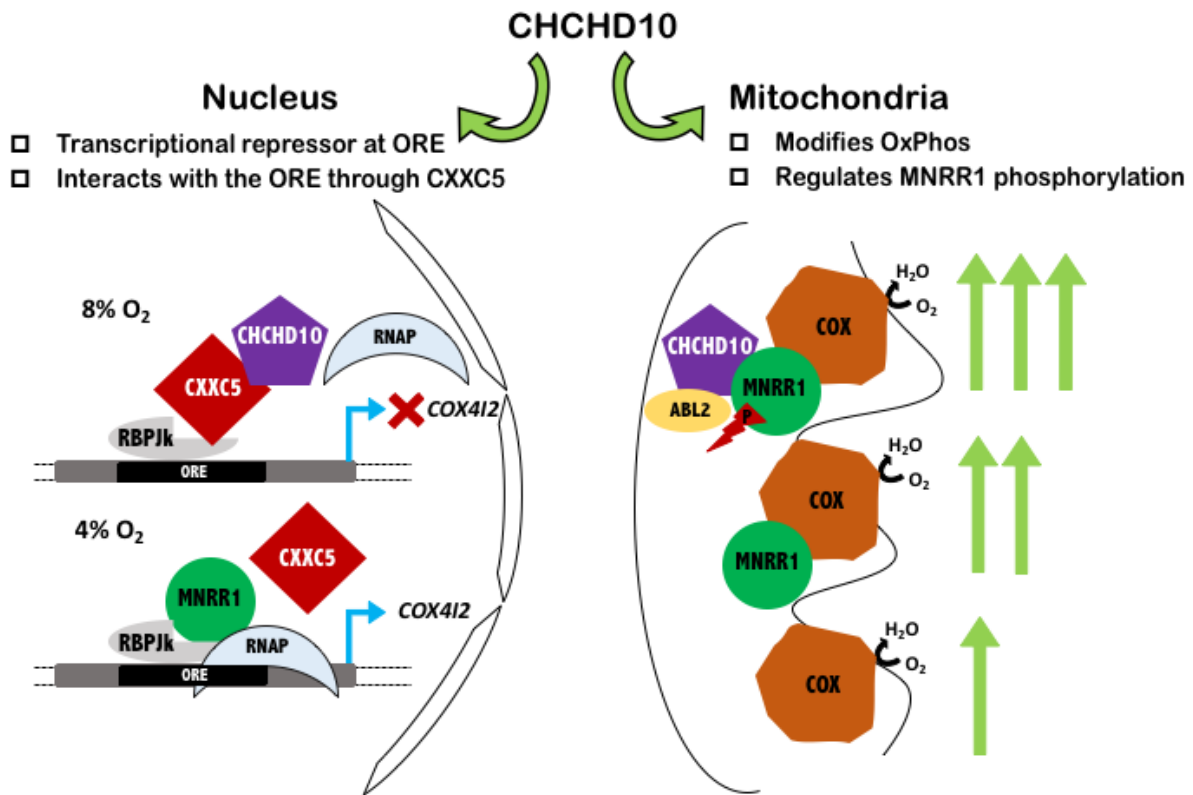
Figure 22. CHCHD10's effects in the nucleus and mitochondria. Left, nuclear CHCHD10 functions as a transcriptional repressor at the ORE. CHCHD10 is upregulated at 8% and interacts with the ORE via CXX5 to enhance its function as an inhibitor at the ORE. Right, in the nucleus and as an activator of OxPhos in the mitochondria.

2. CHCHD10 and MNRR1's effects in the nucleus

A fraction of both MNRR1 and CHCHD10 is found in the nucleus. But in this compartment, despite the high conservation, both proteins have an opposite effect on transcription at the ORE. MNRR1 functions as an activator at the ORE and whereas CHCHD10 functions as an inhibitor. In the nucleus, MNRR1 is not phosphorylated (Aras et al., 2017), and the phosphomimetic MNRR1 is defective for transcription as compared to WT-MNRR1 (unpublished data). Hence phosphorylated MNRR1 may have a different three-dimensional configuration, i.e., folding that is necessary for MNRR1's role of binding COX in the mitochondria but prevents MNRR1 from

interacting with RBPJk in the nucleus. This is supported by the finding that MNRR1 and CHCHD10 do not interact in the nucleus (Figure 13C).

Another important observation is that CHCHD10 interacts with CXXC5, an inhibitor at the ORE (Figure 13A), where it may enhance its repressive function. Moreover, CXXC5 is necessary for CHCHD10 to bind the ORE (Figures 14B, 22 left). Two conclusions can be drawn from these observations. The first, at a structural level, is that MNRR1 and CHCHD10, despite high similarity, may be differently folded due to different post-translational modifications in the nucleus. We already have some preliminary clues regarding the functional effects of MNRR1's differential phosphorylation in the two compartments. However, nothing yet is known about CHCHD10's post-translational profile and this may be an important next step to understand CHCHD10's functioning in the nucleus. The second, more important conclusion, can be drawn functional level. CHCHD10 and MNRR1's opposing effects may represent that the fraction for each in the nucleus is maintained because this is required under only under certain conditions which specifically activate either MNRR1 or CHCHD10.s

Another potential explanation for CHCHD10's repressive effects may be that, when the protein is upregulated by overexpression or under hypoxia, it would recruit ARG (also found in the nucleus) and phosphorylate MNRR1, altering its structure such that the protein cannot bind the ORE. However, MNRR1 and CHCHD10 do not interact in the nucleus (Figure 14C) and there are other known factors at play. MNRR1 interacts with SIRT1 (Law et al., 2009) and is deacetylated in the nucleus. This deacetylated form of MNRR1 is a superior transcription factor compared to WT-MNRR1 (unpublished data). CHCHD10 interacts with CXXC5 to enhance its repressive effects. Hence, in the nucleus, MNRR1 and CHCHD10 simply may be differently modified and have a different structure that allows them to bind other partners rather than each other. Another way to look at this problem is to assess the binding domains for each. If the binding of CHCHD10 to CXXC5 requires a specific domain, then would the addition of this domain on MNRR1 allow it to bind CXXC5? Or would the addition of the deacetylated domain of MNRR1 on

CHCHD10 make it function as an activator in the nucleus? However, addition of a small domain that may change the role of the protein may be a rather simplistic way of studying this pair because there are other factors at play, as well as other potential binding partners. Hypoxia adds a second layer of regulation that will be discussed in the next section.

One more factor with these proteins is the formation of the CHCH domain. In the IMS, the cysteines form disulfide linkages to stabilize this domain. This is necessary to ensure that the proteins are retained in the IMS and this may also protect it from the slightly acidic conditions in the IMS (Porcelli et al., 2005). In the nucleus the environment differs from the IMS, and how, or if the proteins are the same configuration, is unknown. An interesting question to ask would be if it is the post-translational modification changes structure and that hence the binding partners or the binding partners (such as SIRT1 for MNRR1) change the post-translational modification, and hence structure for each protein. But all these observations, point to a difference in the folding of both proteins in the nucleus and analyzing the post-translational profile for MNRR1 and CHCHD10 would yield important clues to understand their function.

An important point to note is all the observations made in CHCHD10 - or MNRR1-overexpressing or deficient cells represent the ends of the expression range. The situation *in vivo* may be more graded as represented by different oxygen levels in different tissues (Carreau et al., 2011; McKeown, 2014) and the known tissue-specificity for CHCHD10 (Bannwarth et al., 2014). Further, the fraction of MNRR1 and CHCHD10 in the nucleus is lower as compared to the mitochondria and enhanced only under specific stresses. The mitochondrial levels for both are higher . This means when there is increase in the levels of these proteins under altered stress conditions, casues a major change in the nuclear levels relative to normal conditions. With respect to mitochondria, the normal levels are quite high and hence not affected significantly under stress. This is key to understanding the mechanism for their opposing effects in the nucleus.

Learning about the contrasting effects of MNRR1 and CHCHD10 is necessary because they seem to have important consequences in the context of cancer. While high MNRR1 levels

are unfavorable for progression of liver cancer and progression of head and neck cancer (Figure 8), high CHCHD10 levels are favorable in the case of renal cancer (Figure 9). Were both favorable, one would predict that their common effect, i.e., their role in the mitochondria is important. However, they have clear opposite effects, and this supports their opposing effects in the nucleus may have a role to play in different cancers. MNRR1 is highly upregulated in breast cancer (Wei et al., 2015), thyroid follicular carcinoma (Lai et al., 2017) and hepatocellular carcinoma (Song et al., 2015). High CHCHD10 levels on the other hand seem to provide some protection against ovarian cancer, conferring sensitivity to cisplatin (Cheng et al., 2010). Hence, an important future direction is to identify the other ORE-harboring genes that are regulated by MNRR1 and CHCHD10 using transcriptomic analysis which may help elucidate the effects of these genes in such a diverse number of cancers.

MNRR1 has established effects in activating cell migration (Seo et al., 2010; Wei et al., 2015) and proliferation (Aras et al., 2015; Wei et al., 2015). There are some clues for MNRR1's role in pathways that affect these processes, especially Akt (Meng et al., 2017; Seo et al., 2010) and EGFR signaling (Li et al., 2014; Li et al., 2016; Wei et al., 2015). CHCHD10 represses MNRR1 expression at both transcript (Figure 20A) and protein levels (Figure 21B). Hence, high CHCHD10 may repress MNRR1-mediated cell growth and migration. However, identifying the common genes regulated by both MNRR1 and CHCHD10 would provide mechanistic insight into the global consequences of altered protein levels for MNRR1 and CHCHD10. One important point to note, is that in all these cases, the analysis has been done in the context of either MNRR1 and CHCHD10 by itself, but not both. All these data need to be reassessed with a focus on both genes as a system for ORE-regulation in order to make real conclusions regarding these effects.

3. Hypoxia sensitivity of MNRR1 and CHCHD10

One of the most important observations regarding MNRR1 and CHCHD10 is their hypoxia responsiveness in a cell culture system. Both proteins are regulated by oxygen levels and induced maximally at two distinct oxygen tensions – 4% for MNRR1 (Aras et al., 2013) and 8% for

CHCHD10 (Figure 10). With regard to their role in the mitochondria this makes sense intuitively – they interact with and regulate an enzyme that is responsible for >90% of cellular oxygen consumption (Babcock and Wikstrom, 1992) and may play a role in modulating its effects under different oxygen levels. This specificity for each level suggests that, though oxygen sensitivity may be common to both, the formation of two genes may have conferred specificity to distinct oxygen tensions and this allowed the duplication to be maintained throughout evolution. Besides fine-tuning mitochondrial function, the relative effect of each in the nucleus on transcription of ORE-containing genes may change continuously as oxygen levels decline. Experimental oxygen tension is 20% although this is high compared to physiological levels *in vivo*, which range from ~3% to 8% in most tissues and ~13% in lung (McKeown, 2014). One of the most critical periods of oxygen sensing is during placental development where oxygen levels switch from 2-3% prior to 12 weeks of gestation to 8% afterwards (Rodesch et al., 1992). Besides MNRR1 and CHCHD10, two other proteins have been described to bind and regulate COX – HIGD1A, which is also activated at 1% oxygen (Hayashi et al., 2015), and BCL2, which modulates the COXVa/COXVb ratio at 0.5% hypoxia (Chen and Pervaiz, 2010). Different oxygen levels likely require the regulation of factors that respond appropriately and alter transcription.

Both MNRR1 and CHCHD10 are transcriptionally upregulated at their selective oxygen levels and in the case of MNRR1, the ORE mediates its upregulation. An important observation also is that at 4% CHCHD10 levels are greatly depleted (Figure 11A). This again supports the observation that MNRR1 is upregulated in CHCHD10-KD cells and it is the absence of CHCHD10 at 4% oxygen that enables the upregulation of MNRR1 via transcription at the ORE. While CHCHD10 transcription is not affected at 4% (Figure 11B), the DNA element in the CHCHD10 promoter that responds maximally at 8% oxygen is yet to be characterized. Furthermore, other cellular factors besides MNRR1 and CHCHD10 and other oxygen responsive elements may be responsible for regulating mitochondrial function at different oxygen tensions. The twin CX_9C

protein CHCHD3 is also known to regulate transcription, at the BAG1 promoter (Liu et al., 2012), although its hypoxia-sensitivity is yet to be assessed.

4. The mechanism of mitochondrial dysfunction for CHCHD10 mutations

Mutations in CHCHD10 have been associated with a number of neurodegenerative diseases. Several studies have characterized the effects of CHCHD10 by analyzing these mutations in skin fibroblasts from patients (Bannwarth et al., 2014; Brockmann et al., 2018; Genin et al., 2016; Straub et al., 2017) or by overexpression of these mutations in WT cells (Ajroud-Driss et al., 2015; Burstein et al., 2017). Here we show that CHCHD10 plays a key role in the regulation of electron transport chain function in the mitochondria as well as a novel role in the nucleus as a transcriptional repressor at the ORE. We selected two CHCHD10 mutations – P80L and G66V – for detailed study. These mutations are associated with several phenotypes across different populations and are absent in control populations. In the mitochondria, each mutant decreased mitochondrial oxygen consumption and increased mitochondrial ROS. High ROS levels have been extensively associated with neurodegeneration and specifically are consistent with neurodegenerative phenotypes such as ALS and motor neuron disease (Lin and Beal, 2006), where ROS may affect crosstalk between neurons and muscles (Jackson, 2016), and Parkinson's (Zuo and Motherwell, 2013). Unlike WT-CHCHD10, which is necessary to recruit ARG to phosphorylate MNRR1 (Figures 16 B, C), the mutants do not interact with MNRR1 (Figure 21G) and they may not recruit ARG to phosphorylate MNRR1. MNRR1 phosphorylation by ARG is essential to its role in regulation of COX activity and a mutant version of MNRR1 that has defective phosphorylation has been linked to another neurodegenerative disorder, Charcot-Marie-Tooth disease type 1A (Aras et al., 2017). Thus, even in the presence of elevated levels of MNRR1 the two mutant versions of CHCHD10 prevent phosphorylation of MNRR1, giving rise to the mitochondrial phenotype seen (Figure 23). A recent study also showed that CHCHD10 is localized to both the mitochondria and the nucleus and that its normal function prevents the neurotoxic mitochondrial localization of the protein TDP-43 whereas two other disease associated mutations,

R15L and S59L, promote its cytoplasmic mislocalization (Woo et al., 2017). These mutations have also been shown to accumulate excessive mitochondrial iron (Burstein et al., 2017).
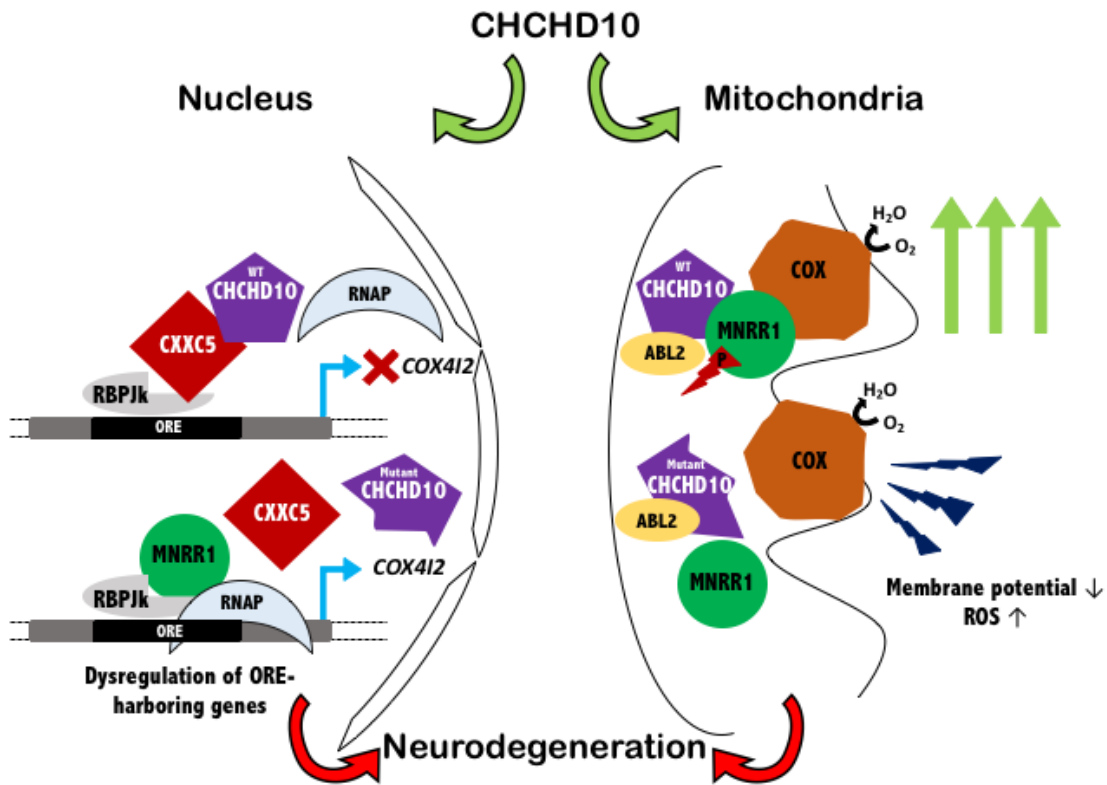
Figure 23. Effects of point mutations (G66V and P80L) on the bi-organellar role of CHCHD10. Point mutations (G66V and P80L) in CHCHD10 abrogate its function in the nucleus and mitochondria, which may give rise to the neurodegenerative phenotype seen in patients.

A feature of the pathogenic mutations not well explained thus far is their generally heterozygous appearance, suggesting they are dominant mutations, whereas in cultured cells the two mutations examined do not appear dominant: despite being overexpressed, endogenous expression needs to be reduced to see a clear phenotype. An important caveat, however, is that the neurodegenerative diseases to which these mutations have been linked show late onset in most cases. Consequently, these experiments do not preclude a small degree of haploinsufficiency that, over time, leads to the observed pathophysiologies. Since there is evidence of population-specific effects of some of the mutations (Marroquin et al., 2016), it is also

possible that other variants must be present to elicit a full phenotype, a possibility also suggested by the diversity of neurodegenerative diseases in which mutations in CHCHD10 have been found.

5. Summary

In summary, we have characterized the role of CHCHD10 as a key modulator of mitochondrial function via inter-organellar signaling (Figure 22). The failure of several pathogenic mutants, which no longer interact with COX, to rescue the mitochondrial effects of CHCHD10 depletion provides a potential mechanism for CHCHD10 dysfunction during the pathogenesis of neurodegenerative diseases. In addition, the altered transcriptional regulation in the nucleus of these mutants at ORE-harboring promoters provides an additional pathogenic pathway (Figure 23). Lastly, the hypoxia sensitivity of both CHCHD10 and MNRR1 transcription places the CHCHD10-MNRR1 pair in a regulatory network that can respond to altered oxygen levels. Such altered levels may be present in different tissues, during different developmental stages, or as a result of disease, and whose lack may therefore contribute to neurodegenerative disease phenotypes with which CHCHD10 mutations have been associated.

CHCHD10 and MNRR1 are both important proteins that regulate cell growth and metabolism. The functional studies regarding MNRR1's role as biorganellar regulator of oxidative phosphorylation (Aras et al., 2015; Aras et al., 2013) and the characterization of a post-translational modification (Aras 2017), provide clues to identify the role of this protein in cellular function and pathways that can be targeted in order to regulate its levels under hypoxic stress conditions. Similar studies regarding CHCHD10's post-translational effects are also necessary.

Since hypoxia is associated with such a large proportion of diseases, it is not surprising that disease associated variants are coming to light. One important corollary that can be drawn from the high similarity between the two proteins and the fact that both have a common ancestor in yeast is that both proteins would be part of a similar process (Cavallaro, 2010). It would be tempting to speculate that during the course of evolution, when the ancestral gene was duplicated, both copies underwent distinct changes, giving rise to two separate genes, perhaps in order to

respond to different conditions but to regulate one critical process – oxidative phosphorylation – which is vital for cell survival. Hence, more mechanistic studies in the case of CHCHD10 and MNRR1 would provide a platform for identifying the effects of both these proteins individually, and as part of a system of regulation in response to different stress conditions including but not limited to hypoxia.

6. Future Directions

In this work, we have identified a novel signaling mechanism for a synchronized response to cellular stress in two distinct compartments, the mitochondria for regulating oxygen consumption and in the nucleus to modulate transcription. Though MNRR1 and CHCHD10 began as a single gene, the evolution of multicelluarity required refinement of protein structure and function to meet the cells' energetic and signaling demands. MNRR1 and CHCHD10, which regulate COX, an enzyme that consumes majority of cellular oxygen, and govern transcription of genes in the nucleus, are ideal candidates to communicate these changes. As members of the twin CX_9C family, the presence of their cysteine residues may impart partial redox sensitivity (Khalimonchuk and Winge 2007) to these proteins and one of the most important next steps must be to assess if other members share this sensitivity to altered oxygen levels. However, hypoxia is just one form of stress; there may be other conditions that elicit a signaling response from MNRR1, CHCHD10, or other twin CX_9C proteins. Identifying those stresses, and the twin CX_9C proteins that respond, would serve as useful to modulate the cell's response. CHCHD3 has been associated with transcription (Liu et al., 2012), CHCHD1 with mitochondria translation (Koc et al., 2013), and most recently CHCHD5 was shown to respond to metabolic stress to affect PGC1a and CREB signaling (Babbar et al., 2018). Characterization of these responses would be useful to understand the role of these proteins within and outside the mitochondria.

The MNRR1 and CHCHD10 pair, however, continues to dominate research on twin CX_9C family proteins because of their association with a plethora of neurodegenerative disorders. The point mutations in both have been associated with defects in mitochondrial function and it would

be useful to further assess how these proteins regulate energy production, particularly important to neuronal function. The mechanism we have identified may just explain a part of this intriguing pair's effects in the mitochondria. In particular, C1QBP, a protein that is required for oligodendrocyte differentiation and axon formation (Yagi et al., 2017), is strongly associated with both proteins, forming a trimeric complex (Burstein et al., 2017; Straub et al., 2017). This association presents a puzzle about why only mutations in MNRR1 and CHCHD10 are associated with neurodegeneration.

With respect to the nuclear function, the pair is important due to their opposite effects on transcription of ORE harboring genes. Identifying a list of genes via transcriptomics and ChIP sequencing would help understand the downstream effects of both on cellular function. We have preliminary data using RNA transcriptomics analysis to identify genes regulated by MNRR1 and CHCHD10. Direct confirmation of this may be done using ChIP sequencing. One important result from this work is the identification of CHCHD10 as a transcriptional repressor for MNRR1. Finding a selective activator for CHCHD10 or for its interaction with CXXC5 interaction would provide a novel method to inhibit cancer progression in patients with high MNRR1 levels. Another potential way to target MNRR1 would be to identify the domain on CHCHD10 that is necessary to recruit ARG to phosphorylate MNRR1 and to generate a small peptide. This peptide may be targeted to the nucleus to enhance MNRR1 phosphorylation and repress its function. Even without selective targeting to the nucleus, this peptide would enhance mitochondrial MNRR1 function and repress nuclear function. This is because the phosphorylation at Tyr-99 regulates MNRR1 activity in a compartment-specific manner. Hence, it becomes even more important to identify other potential modifications that can be used to target protein function in a compartment-specific manner.

The MNRR1 and CHCHD10 pair represent an elegant example of customization the protein structure and function to meet the cell's energetic demands. Identifying how this pair regulates mito-nuclear function and dysfunction will help to provide novel targets to ameliorate

defects in neurodegeneration, restrict growth and metastasis in cancer, and other pathologies affected by mito-nuclear signaling.

CHAPTER IV: MATERIALS AND METHODS

1. Cell culture

Experiments were performed with human embryonic kidney 293 (HEK293) cells and HeLa (human cervical cancer) cells. The HEK293 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT) with 10% fetal bovine serum (FBS) (Sigma Aldrich, St. Louis, MO) plus antibiotic and antimycotic (HyClone); CHCHD10 knockdown (sc-72754 from Santa Cruz Biotechnology (SCBT) Inc, Dallas, TX) cells were grown under puromycin selection. HEK293 cells stably overexpressing wild-type (WT) or mutant CHCHD10 were selected using G418. Inc, Dallas, TX).

The CHCHD10 shRNA is a pool of 2 different shRNA plasmids:

Hairpin sequence:

GATCCGTGCAAGTACTACCATGGTTTCAAGAGAACCATGGTAGTACTTGCACTTTTT

Corresponding siRNA sequences (sc-72754A):

Sense: GUGCAAGUACUACCAUGGUTT

Antisense: ACCAUGGUAGUACUUGCACTT

sc-72754-B: Hairpin sequence:

GATCCCAGACCACAACACCAGATTTTCAAGAGAAATCTGGTGTTGTGGTCTGTTTTT

Corresponding siRNA sequences (sc-72754B):

Sense: CAGACCACAACACCAGAUUTT

Antisense: AAUCUGGUGUUGUGGUCUGTT

Note that all sequences are provided in 5′ → 3′ orientation.

2. Effector and reporter plasmids

The WT and mutant 579-bp *COX4I2* promoter luciferase reporter plasmids (Aras et al., 2013), and MNRR1 reporter plasmid (5), have been described previously. The WT MNRR1 and CHCHD10 expression plasmid was cloned in the pCI-Neo vector with a C-terminal 3xFLAG epitope. The G66V and P80L mutants were cloned in the pCI-Neo vector using overlap extension

PCR. The mutation was confirmed by sequencing. All the expression plasmids were purified using the EndoFree plasmid purification kit (Qiagen, Valencia, CA). CXXC5 was expressed from a pCI-Neo vector.

3. Transient transfection of HEK293 cells

HEK293 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 3:1 in serum and antibiotic free medium was used. Following incubation at room temperature for ~15 min, the cells were overlaid with the mixture. The plates were incubated for 1 h at 37 °C followed by replacement with complete medium and further incubation for the indicated time interval.

4. Real-time polymerase chain reaction

Total cellular RNA was extracted using an RNeasy Plus Mini Kit (Qiagen) per the manufacturer's instructions. Complementary DNA (cDNA) was generated by reverse transcriptase polymerase chain reaction (PCR) using the ProtoScript® II First Strand cDNA Synthesis Kit (NEB, Ipswich, MA, USA). Transcript levels were measured by real time PCR using SYBR green on an ABI7500 system. Real-time analysis was performed by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The primer sequences used were as follows (F, forward; R, reverse): *CHCHD10* (F-CGCCTACGAGATCAGGCAGT, R-GGAGCTCAGACCATGGTAGTACTTG), and 1*8SrRNA* (F-AGTCCCTGCCCTTTGTACACA, R-GATCCGAGGGCCTCACTAAAC).

5. Hypoxia assays

Cells were maintained either at 20% oxygen (normoxia) or at 13, 9, 8, 7, 4, or 1% oxygen in a chamber infused with N_2 and CO_2 at 37 °C. The gas flow was controlled with PRO-OX 110 and PRO-CO_2 controllers (BioSpherix, Redfield, NY) to achieve desired oxygen levels and 5% CO_2. Oxygen equilibration time (3–4 h) was accounted for in the hypoxia experiments.

6. Luciferase reporter assays

Luciferase assays were performed using the dual-luciferase reporter assay kit (Promega) per the manufacturer's instructions. Transfection efficiency was normalized with the co-transfected pRL-SV40 *Renilla* luciferase expression plasmid.

7. DNA binding assays

DNA binding assays were performed as described (Aras et al., 2013) with a few modifications. HEK293 WT (scrambled) or CXXC5 KD (siRNA- sc-91677 from SCBT) were transfected with a CHCHD10 expression plasmid.

sc-91677: CXXC5 siRNA is a pool of 3 different siRNA duplexes:

sc-91677A:

Sense: CUCUCCCACUACUCUUCUUTT

Antisense: AAGAAGAGUAGUGGGAGAGTT

sc-91677B:

Sense: CCAGGCCUCUUCAUUAUGATT Antisense: UCAUAAUGAAGAGGCCUGGTT

sc-91677C:

Sense: GCAGUUGUAGGAAUCGAAATT

Antisense: UUUCGAUUCCUACAACUGCTTA

All sequences are provided in 5′ → 3′ orientation.

After 48 h proteins were crosslinked and nuclei were separated. DNA was fragmented by incubation with Sau3AI and nuclei were lysed. The antibody–matrix complex was prepared as described under co-immunoprecipitation (IP) using anti-CHCHD10 antibody and shaken overnight with nuclear lysate at 4°C. Cross-linking was reversed, and the precipitated DNA was purified with the QIAquick purification kit (Qiagen) and amplified with primers encompassing the segment harboring the endogenous ORE for both the CHCHD10 and IgG IP samples.

8. Cell proliferation assay

Cell proliferation was assessed with the CellTiter96 AQueous One colorimetric solution kit (Promega) in a 96-well plate according to the manufacturer's instructions. Equal numbers of cells were plated and analyzed for proliferation at 24 h (~1.5 doublings) and 48 h (~3 doublings) by treating with CellTiter96 cell aqueous solution and recording absorbance at 490 nm.

9. Cell counting assay

Live cell counts were performed by trypan blue staining on a Countess™ automated cell counter (Invitrogen). Live cells (7.5×10^4) were plated, allowed to grow for 48 h (~3 doublings), and the number of live cells counted again.

10. Intact cellular oxygen consumption

Cellular oxygen consumption was measured with a Seahorse XFe^{24} Bioanalyzer. Cells were plated at a concentration of 40,000 per well a day prior to basal oxygen consumption measurements, performed according to the manufacturer's instructions.

For the hypoxia experiments, oxygen consumption was analyzed using the MitoXpress® Xtra HS Oxygen Consumption Rate Assay Kit (Cayman, Ann Arbor, MI). Cells were plated at a concentration of 30,000 per well a day prior to measurements, incubated at 20% or 8% oxygen and measurements were performed according to the manufacturer's instructions.

11. Cytochrome *c* oxidase activity measurement

In vitro COX activity was measured using the Cytochrome *c* Oxidase Assay Kit from Sigma Aldrich as per manufacturer's instructions using bovine heart cytochrome *c*. The cytochrome *c* oxidase used was purified from bovine heart and dialyzed before being used for the assay.

12. ROS measurements

Total cellular ROS measurements were performed with CM-H_2DCFDA (Life Technologies, Grand Island, NY). Cells were distributed into 12-well plates at 10^5 cells per well and incubated for 24 h. Cells were then treated with 10 μM CM-H_2DCFDA in serum- and antibiotic-free medium for 1 h.

Cells were washed twice in phosphate buffered saline and analyzed for fluorescence on a Gen5 Microplate Reader (BioTek Inc, Winooski, VT). For mitochondrial ROS measurements, the cells were treated as above but with 5 μM Mitosox Red (Life Technologies) for 30 min.

13. Confocal microscopy

Confocal microscopy was performed as described (Aras et al., 2017). Primary antibodies were CHCHD10 (1:50, Sigma) and COXI (1:100, Life Technologies). Secondary antibodies were goat anti-rabbit IgG Alexa 488 and goat anti-mouse IgG Alexa 596 (1:300, Jackson Labs, Bar Harbor, ME). Cells were imaged with a Leica TCS S5P microscope and images were combined in Adobe Photoshop. Co-localization (overlap of the two fluorophores) and intensity (number of pixels per unit area) were quantitated using Volocity image analysis software (Perkin Elmer, Waltham, MA). The Pearson correlation coefficient determined for the images is indicated on the figures.

14. Immunoblotting and co-immunoprecipitation

Immunoblotting on a PVDF membrane was performed as described previously (Aras et al., 2015; Aras et al., 2013). The high percentage urea-acrylamide gel was run as described previously (Kadenbach et al., 1983).

Unless specified otherwise, primary antibodies (Table 7) were used at a concentration of 1:500 and secondary antibodies at a concentration of 1:5000.

Antibody	Host	Source	Catalog #
α-ARG	Mouse	Dr. Koleske	–
α-CHCHD10	Rabbit	Sigma	HPA003440
α-COX I	Mouse	Invitrogen	459600
α-COX4	Rabbit	Proteintech	11242-1-AP
α-COX4i2	Mouse	Abnova	H00084701-M01
α-COX6B1	Rabbit	Proteintech	11425-1-AP
α-CXXC5	Mouse	SCBT	sc-376348
α-DRBP76	Rabbit	Proteintech	19887-1-AP
α-FLAG	Mouse (HRP)	Sigma	A8592
α-GAPDH	Mouse (HRP)	Proteintech	HRP-60004
α-MNRR1	Rabbit	Proteintech	19424-1-AP
α-NDUFS3	Rabbit	Proteintech	15066-1-AP

α-Phosphotyrosine (4G10)	Mouse	Millipore	05-321
α-TOM20	Rabbit (HRP)	Cell Signaling	72610
α-Tubulin	Rabbit	Proteintech	1HRP-66031

Table 7. List of antibodies used in assays. List of antibodies and the source from where they were acquired.

15. Mitochondria isolation

Mitochondria were isolated from cells with a Mitochondrial Isolation Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. The nuclear fraction was obtained by low-speed centrifugation and the mitochondrial fraction was obtained after high-speed centrifugation of the nuclear supernatant. Cross-contamination between the fractions was analyzed with compartment-specific antibodies.

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 $p < 0.05$.

17. Publications

Some sections of Chapter I have been published as a part of Grossman et al. 2017.

Some sections of Chapters II-IV have been published as a part of Purandare et al. 2018.

18. Author contributions

Confocal imaging and analysis was performed by Dr. Mallika Somayajulu-Nitu.

All other experiments were performed by Neeraja Purandare.

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ABSTRACT

CHCHD10, A NOVEL BI-ORGANELLAR REGULATOR OF CELLULAR METABOLISM: IMPLICATIONS IN NEURODEGENERATION

by

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CHCHD10 (Coiled-coil Helix Coiled-coil Helix Domain containing protein 10) and MNRR1 (Mitochondrial Nuclear Retrograde Regulator 1, also known as CHCHD2), have been shown by us to be stress regulators of mitochondrial function that act both in the mitochondria and in the nucleus. Both are members of the twin CX_9C family, but CHCHD10 in particular, has been found in mutant form to be linked to a myriad of neurodegenerative conditions. In mitochondria, both activate cytochrome *c* oxidase (COX) whereas in the nucleus, both act as transcription regulators of a subset of genes that contain a 13-bp sequence termed as the oxygen responsive element (ORE). We previously modeled events at the ORE to consist of a complex of RBPJκ with either the transcriptional repressor CXXC5 or the transcriptional activator MNRR1. We show that CHCHD10 co-purifies with COX and up-regulates its activity by serving as a scaffolding protein required for MNRR1 phosphorylation, mediated by ARG (ABL2 kinase). Surprisingly, in the nucleus CHCHD10 protein down-regulates the expression of ORE-harboring genes by interacting with and augmenting the activity of CXXC5. The CHCHD10 gene is maximally transcribed in cultured cells at 8% oxygen, unlike MNRR1, which is maximally expressed at 4%, suggesting a fine-tuned oxygen-sensing system that adapts to the varying oxygen concentrations in the human body under physiological conditions. The nuclear inhibitory role at the ORE is bolstered by our observation that at oxygen tensions of 4 and 8%, there is a reciprocal downregulation of MNRR1

and CHCHD10 respectively. Furthermore, we show that cells predominantly harboring two CHCHD10 disease mutants (c.197G>T p.G66V and c.239C>T p.P80L) are defective for cellular oxidative phosphorylation, have lower membrane potential and higher reactive oxygen species (ROS) levels as compared to WT-CHCHD10. The mutant proteins are also defective in the nucleus as they fail to interact with CXXC5 and repress transcription at the ORE. In summary, CHCHD10 and MNRR1 have similar functions in the mitochondria where both regulate cellular oxygen consumption. In the nucleus however, they have opposing effects at the ORE. We discuss these findings to generate a generalized model for cellular responses to moderate levels of hypoxia and a possible mechanism for the observed phenotype in patients with mutations in this gene.

AUTOBIOGRAPHICAL STATEMENT

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PUBLICATIONS:
1) **Purandare N,** Somayajulu M, Hüttemann M, Grossman LI, Aras S. The cellular stress proteins CHCHD10 and MNRR1 (CHCHD2): Partners in mitochondrial and nuclear function and dysfunction. *J Biol Chem*. 2018 Mar 14. pii: jbc.RA117.001073. doi: 10.1074/jbc.RA117.001073.
2) Lawrence Grossman, **Neeraja Purandare**, Rooshan Arshad, Stephanie Gladyck, Mallika Somayajulu, Maik Hüttemann and Siddhesh Aras. MNRR1, a bi-organellar regulator of mitochondria. Invited review; *Oxidative Medicine and Cellular Longevity*. 2017:6739236. doi:10.1155/2017/6739236.Epub 2017 Jun 8.
3) Siddhesh Aras, Hassan Arrabi, **Neeraja Purandare**, Maik Huttemann, John Kamholz, Stephan Zuchner, Lawrence Grossman. Abl2 Kinase Phosphorylates Bi-organellar Regulator MNRR1 in Mitochondria, Stimulating Respiration. *BBA Mol Cell Res*. 2016 doi: 10.1016/j.bbamcr.2016.11.029.

ORAL PRESENTATIONS:
1) Targeting MNRR1 protein acetylation to regulate mitochondrial oxidative phosphorylation. **Neeraja Purandare**, Siddhesh Aras, Lawrence Grossman
   Graduate Student Research Day, Wayne State University, September 2015, Detroit, Michigan
2) CHCHD10 and MNRR1: It takes two to tango but one to cause disease**Neeraja Purandare**, Siddhesh Aras, Maik Hüttemann, Lawrence I. Grossman. United Mitochondrial Disease Foundation (UMDF) Meeting, June 2017, Washington D.C.

ABSTRACTS PRESENTED:
1) Lawrence Grossman, **Neeraja Purandare**, Mallika Somayajulu, Maik Huttemann, Siddhesh Aras. CHCHD10 and MNRR1 (CHCHD2): Partners in mitochondrial and nuclear function and dysfunction. Cell Symposia; Multifaceted Mitochondria, June 4-6th 2018, San Diego, CA.
2) **Neeraja Purandare,** Mallika Somayajulu, Siddhesh Aras, Maik Huttemann, Lawrence I. Grossman. CHCHD10 and MNRR1 (CHCHD2): It takes two to tango but one to cause disease. United Mitochondrial Disease Foundation (UMDF) mitochondrial medicine 2017 conference, Washington DC.
3) Siddhesh Aras, Arrabi Hassan, **Purandare Neeraja**, Zuchner Stephan, Kamholz John, Huttemann Maik, Grossman Lawrence. MNRR1 ABLed to activate oxidative phosphorylation. NHLBI Symposium, 2016, National Institutes of Health, Bethesda, MD.
4) Aras Siddhesh, Arrabi Hassan, **Purandare Neeraja**, Zuchner Stephan, Kamholz John, Huttemann Maik, Grossman Lawrence. *CX9C proteins as new stress responsive bi-organellar regulators and disease modifiers*. 6th World Congress on Targeting Mitochondria, Oct 28-30th 2015, Berlin, Germany.
5) **N.A. Purandare**, S Aras, L.I Grossman. *Acetylation profile of MNRR1, a critical bi-organellar OxPhos regulator, dictates its functioning*. Cell symposia: Multifaceted Mitochondria, July 19-21 2015, Chicago, IL.

AWARDS:
1) **First Place** for Poster Presentation at Center for Molecular Medicine and Genetics Annual Retreat 2015, Detroit, Michigan
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