

Protein Factors Regulating Mitochondrial Respiratory Supercomplexes

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ABSTRACT

Mitochondrial ATP production is achieved using the electron transport chain (ETC), whereby the controlled oxidation of biomolecules is coupled to the activity of ATP synthase. ETC complexes organize into supramolecular structures called supercomplexes (ETC SCs). Protein factors regulating ETC SCs remain largely unknown despite their fundamental implications to mitochondrial respiratory function.

Recent knock-out studies have delineated external ETC proteins HIGD1A and HIGD2A as assembly factors of ETC complexes III and IV, and their incorporation into SCs. In order to clarify the primary functions of HIGD1A and HIGD2A, as well as other previously uncharacterized HIG1 protein family members, stable overexpression (OE) models of each HIG1 protein were generated in HEK293t cells to perform comparative studies. We uncover a general dichotomy in the effects observed from HIGD2A vs. HIGD1A/1B/1C OE. Furthermore, we demonstrate that the previously unstudied protein family member HIGD1C is a negative regulator of complex IV SCs.

A very limited number of protein factors specifically regulating the I₁III₂IV₁ “respirasome” ETC SC have been identified. We propose a new framework where select complex I accessory subunits regulate respirasome assembly through protein-protein interactions between ETC complexes. Through specific point mutations to one such subunit, we generate a novel cell model with selective disassembly of the respirasome but otherwise functional individual ETC complexes. We demonstrate that respirasome disassembly limits respiration and modifies electron transfer pathways within the ETC. These findings to respirasome assembly and function may represent just a portion of higher order regulation that we are beginning to describe within eukaryotic metabolism.

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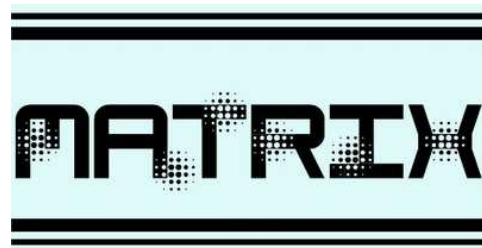


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LIST OF ABBREVIATIONS

A

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
APS	Ammonium persulfate
AA	Antimycotic-antibiotic
ATP5A	ATP synthase F1 subunit alpha
ATP5B	ATP synthase F1 subunit beta
ATP5C1	ATP synthase F1 subunit gamma
ANT1/2	Adenine nucleotide translocase 1 and 2
AKT	Protein kinase B
ANOVA	Analysis of variance
AOX	Alternative oxidase

B

BN-PAGE	Blue native polyacrylamide gel electrophoresis
BCA	Bicinchoninic acid
BSA	Bovine serum albumin

C

CI	Complex I (NADH:Ubiquinone Oxidoreductase)
CII	Complex II (Succinate Dehydrogenase)
CIII	Complex III (coenzyme Q:cytochrome <i>c</i> – oxidoreductase)
CIV	Complex IV (cytochrome <i>c</i> oxidase)
CV	Complex V (ATP synthase)
COX7A2	Cytochrome <i>c</i> oxidase subunit 7A2
COX7A2L	Cytochrome <i>c</i> oxidase subunit 7A2 Like
COX6C	Cytochrome <i>c</i> oxidase subunit 6C
cDNA	Complementary DNA
COX3	Cytochrome <i>c</i> oxidase subunit III regulatory module
CoQ/CoQH ₂	Coenzyme Q (ubiquinone)

D

DRP1	Dynamin-related protein 1
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
DMEM	Dulbecco's modified eagle medium

E

ETC	Electron transport chain
ETC SC	Electron transport chain supercomplexes
ETS	Electron transfer system
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
E _c	Elements per cluster

EV	Empty vector
F	
FA	Fatty acid
FADH ₂	Flavin adenine dinucleotide
FACS	Fluorescence activated cell sorting
FCCP	Trifluoromethoxy carbonylcyanide phenylhydrazone
FBS	Fetal bovine serum
G	
GTPase	Guanosine triphosphate hydrolase
GFP	Green fluorescent protein
H	
HIG1	Hypoxia inducible gene domain protein family
HIGD2A	Hypoxia inducible gene domain, family member 2A
HIGD1A	Hypoxia inducible gene domain, family member 1A
HIGD1B	Hypoxia inducible gene domain, family member 1B
HIGD1C	Hypoxia inducible gene domain, family member 1C
H ⁺	Hydrogen ion, proton
HEK293	Human embryonic kidney cells
HEK293t	Human embryonic kidney cells, SV40 T antigen producing
HIF-1 α	Hypoxia-inducible factor 1-alpha
HRR	High resolution respirometry
HRP	Horseradish peroxidase
I	
IRES	Internal ribosome entry site
ICC	Immunocytochemistry
J	
J/E	Junctions over ends
K	
KO	Knock out
L	
L-OPA1	Optic atrophy 1 long isoform
M	
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
MFN1/2	Mitofusin 1 and 2
mtDNA	Mitochondrial DNA
mtHSP70	Mitochondrial heat shock protein 70
MT-COI	Mitochondrially encoded cytochrome <i>c</i> oxidase subunit I

N

NADH	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
NDUFB4	NADH:ubiquinone oxidoreductase subunit B4
NDUFB6	NADH:ubiquinone oxidoreductase subunit B6
NDUFA3	NADH:ubiquinone oxidoreductase subunit A3
NDUFA9	NADH:ubiquinone oxidoreductase subunit A9

O

OE	Overexpression
OXPHOS	Oxidative phosphorylation
OPA1	Optic atrophy 1
OCR	Oxygen consumption rate
O ₂	Molecular oxygen

P

P/O	Phosphorylation to oxidation ratio
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
pH	Power of hydrogen
PCR	Polymerase chain reaction
Pink1	PTEN-induced kinase 1
PDB	Protein data bank

Q

qPCR	Quantitative polymerase chain reaction
------	--

R

ROS	Reactive oxygen species
Rcf1	Respiratory complex factor 1
Rcf2	Respiratory complex factor 2

S

SCAF1	Supercomplex assembly factor 1
SCs	Supercomplexes
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
s	Seconds
SNP	Single nucleotide polymorphism
siRNA	Small interfering ribonucleic acid
siKD	siRNA mediated knockdown
siCtrl	Negative control siRNA
SDHA	Succinate dehydrogenase complex flavoprotein subunit A

T

TCA	Tricarboxylic acid cycle
TBS	Tris buffered saline
TBS-T	Tris-buffered saline with 1% (v/v) Tween-20
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TMRM	Tetramethylrhodamine methyl ester perchlorate
Tom20	Translocase of outer mitochondrial membrane 20
T2D	Type 2 diabetes
U	
UQCRC1	Ubiquinol-cytochrome c reductase, core protein 1
UQCRC2	Ubiquinol-cytochrome c reductase, core protein 2
UQCR11	Ubiquinol-cytochrome c reductase, complex III subunit XI
W	
WT	Wildtype
X	
XF	Extracellular flux
#	
1BH	Higher expressing HIGD1B stable cell line
1BL	Lower expressing HIGD1B stable cell line
$\Delta\psi_{mt}$	Mitochondrial membrane potential
ΔG	Gibbs free energy

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1.0 INTRODUCTION

1.1.1.1 The electron transport chain and oxidative phosphorylation

Antoine Lavoisier, one of the forefathers of modern chemistry, demonstrated that living animals were consuming oxygen as early as 1789. In piloting a theory prior that describes the role of oxygen in the combustion process, Lavoisier speculated that physiological respiration was a slow process of combustion (1). Today we understand this combustion process as cellular respiration through action of the electron transport chain (ETC) found within the mitochondrial inner membrane (MIM).

The ETC is a series of protein complexes, named I-IV, which facilitate the movement of electrons in a sequence of small redox reactions. This process begins with the generation of reducing agent coenzymes NADH and FADH₂. In the mitochondrial matrix, the tricarboxylic acid (TCA) cycle and β -oxidation pathways generate these reducing agents by catabolising carbohydrates or fatty acids respectively, or other high energy biomolecules such as ketone bodies all initially present in the cytosol (2). NADH and FADH₂ possess destructively large amounts of free energy, and so electron transfer from NADH and FADH₂ to O₂ must occur in a relatively slow and controlled manner —just as Lavoisier predicted (1).

Two distinct pathways of electron transfer in the ETC are formed as a result of the two starting high energy electron carriers: the NADH-linked pathway (also known as N-linked or CI-linked pathway) and the FADH₂-linked pathway (also known as S-linked or CII-linked pathway) (**Figure 1**). NADH is first oxidized by NADH:ubiquinone oxidoreductase, also known as complex I (CI), while FADH₂ is oxidized by succinate dehydrogenase, also known as complex II (CII). The redox reaction catalysed by CI also mobilizes protons (H⁺) from the mitochondrial matrix to the

intermembrane space. In contrast, the enzymatic reaction of CII does not transfer any protons. Electrons from each reducing agent are passed onto the mobile electron carrier coenzyme Q (CoQ or Q), where they subsequently move to coenzyme Q:cytochrome *c* oxidoreductase (complex III or CIII), the mobile electron carrier cytochrome *c*, and finally to cytochrome *c* oxidase (Complex IV or CIV) in a series of redox reactions. Enzymatic reactions catalyzed by CIII and CIV also transfer protons across the MIM. Complex IV transfers two electron pairs to molecular oxygen, producing H₂O and thereby completing the process of respiration (3).

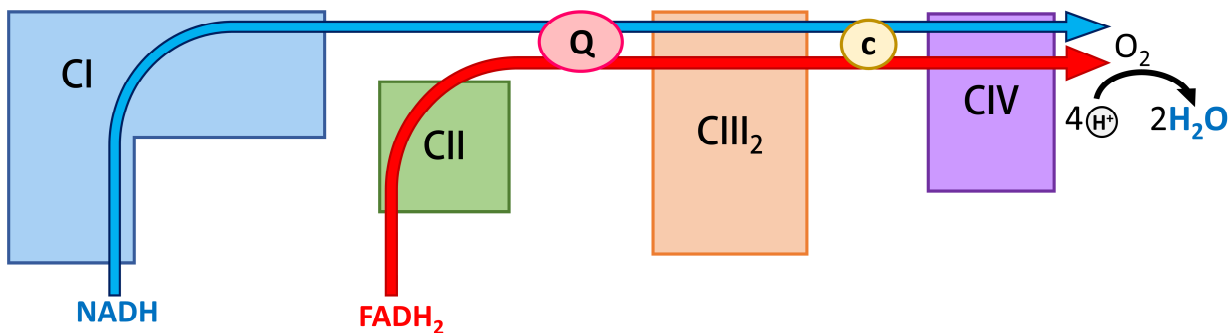


Figure 1. Major pathways of electron transfer in the electron transport chain. Complexes I-IV shown in order from left to right. Complexes I-IV facilitate the movement of electrons, initially received from NADH at CI (Blue) and FADH₂ at CII (Red). The actions of CI and CII transfer electron pairs to coenzyme Q (CoQ or Q), which subsequently move to Complex III, cytochrome *c*, and complex IV which catalyses the final transfer to molecular oxygen and produces H₂O.

The available free energy from the smaller redox reactions spread across the ETC complexes (excluding complex II) is utilized to transfer protons from the mitochondrial matrix to the intermembrane space. The imbalance of protons and other ions across the MIM contribute to a potential difference, and this source of energetic potential is referred to as the mitochondrial membrane potential (4).

Nobel laureate Peter Mitchell first proposed that the stored potential across the intermembrane space could be harnessed by a postulated “reversible ATPase” to phosphorylate ADP into ATP

(5). Following the discovery of ATP synthase (complex V or CV), a mechanism of returning protons back into the mitochondrial matrix was understood to complete his chemiosmotic theory (Figure 2).

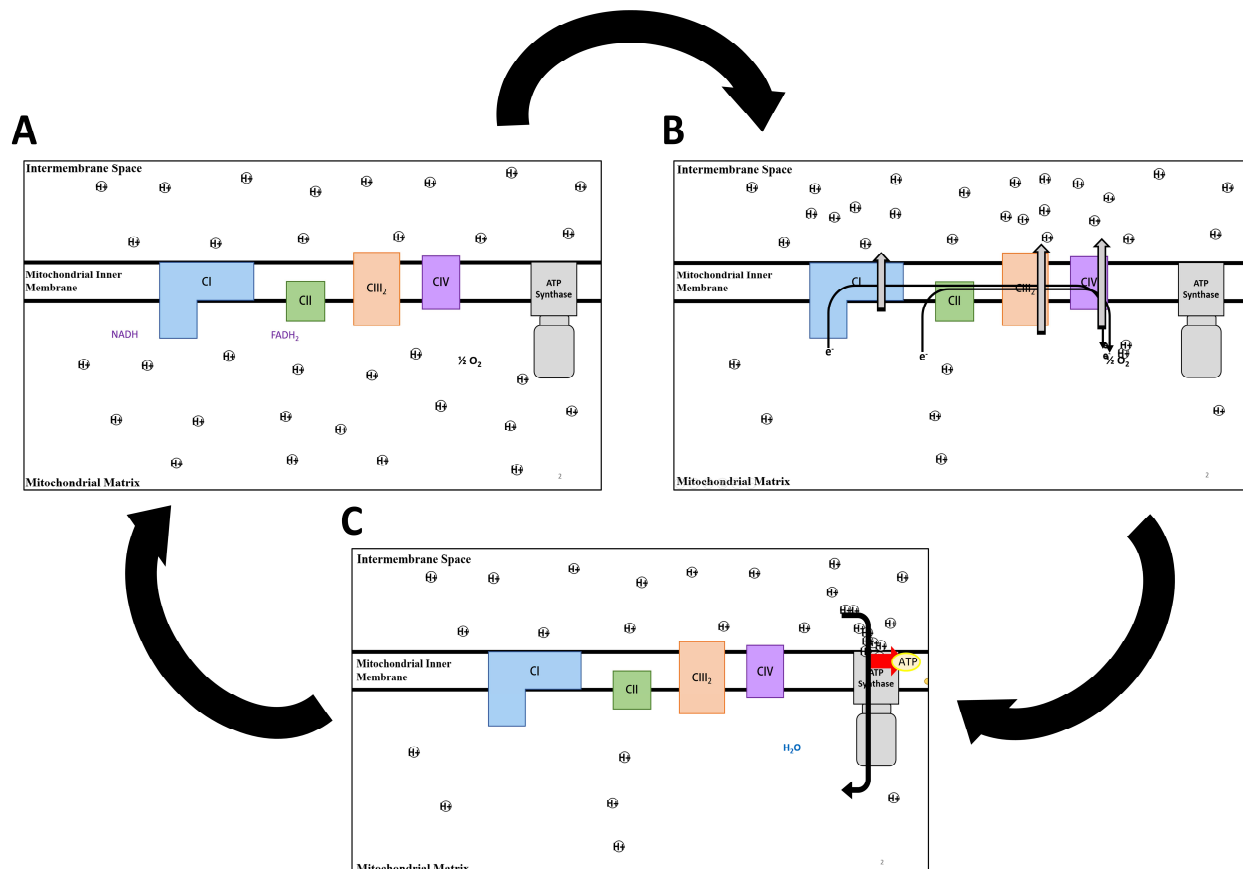


Figure 2. The oxidative phosphorylation system. (A) Prior to action of the electron transport chain, energy stored in the concentration of protons across the MIM cannot be harnessed for ATP synthesis. (B) Reducing agents NADH and FADH₂ supply electrons for a series of over 10 different redox reactions, which constitute the action of the ETC. Each redox reaction retains thermodynamic favourability (i.e., a $-\Delta G$), allowing for coupling of these reactions to the endergonic process of proton transfer from the mitochondrial matrix to the intermembrane space. Complexes I, III, and IV transfer protons as a result of their enzymatic reactions. (C) ATP synthase (CV) facilitates H⁺ movement back into the matrix, and couples the osmotic energy provided by the electrochemical gradient to phosphorylate ADP into ATP.

Mammalian ATP synthase harnesses energy from the movement of 2.7 protons to phosphorylate each ADP into ATP (6). The stoichiometry of phosphorylation to oxidation (known as the P/O

ratio) was initially listed as 3 for NADH and 2 for FADH₂ (7), though the modern theoretical maximums are estimated at 2.73 and 1.64 respectively (8). The amount of ATP produced per metabolic fuel source can be extrapolated using these values, however *in vivo* systems reveal that the *efficiency* of oxidative phosphorylation (OXPHOS) is variable. Proton leak across the MIM consumes a significant amount of oxygen —up to 1/3rd of resting oxygen consumption, and can vary depending on the state of the electron transfer system (ETS) (9). Moreover, redox reactions, primarily at complex I but also at complex III can lead to side reactions producing reactive oxygen species (ROS). The free energy available from these reactions is not used towards generating the electrochemical gradient and so is essentially lost (10).

1.1.1.2 The mitochondrial network

Mitochondria often exist as a dynamic and tubular interconnected network rather than individual entities in cells (11). The size and connectivity of a mitochondrial network has functional implications. A large size and degree of connectivity is referred to as a “fused” state, and is often associated with increased mitochondrial function. Conversely, a fragmented network (small size and lower connectivity) is often associated with mitochondrial dysfunction (12). Nonetheless, a fragmented network can be preferentially induced during stress-related situations to maintain mitochondrial quality. Dysfunctional mitochondria, often appearing as swollen, can be selectively removed by autophagic processes called mitophagy (13). Another recently described process is mitochondrial donut formation. Mitochondrial donuts form as result of a tubular branch fusing with itself. Donut formation could represent a protective method by healthy mitochondria to avoid mitophagy. A mitochondrial donut lacking ends is more inaccessible to the fission machinery and subsequent degradation (14).

Mitochondrial network size and connectivity is regulated by protein-based mechanisms of mitochondrial fusion and fission. Mitochondrial fission is regulated by the recruitment of the GTPase dynamin-related protein 1 (DRP1). In short, the mechanism involves DRP1 oligomerizing around the mitochondrial outer membrane (MOM), forming a ring-like structure which constricts and scissions a tubule into two (11). Conversely, fusion of the MOM is facilitated through the actions of mitofusins 1 and 2 (MFN1/2), which can oligomerize between outer mitochondrial ends in a GTPase dependant manner and lead to fusion. Fusion is then completed through the action of Optic atrophy 1 (OPA1) which oligomerizes to tether MIMs (13). OPA1 harbors a combination of splice variants and undergoes proteolytic processing in mammals, resulting in a multitude of OPA1 isoforms classified as either long or short. Although originally suggested that long isoforms exclusively mediated mitochondrial fusion (15), it is now known that both long and short isoforms must coexist to preform MIM fusion (16).

1.1.2 Electron transport chain supercomplexes (ETC SCs)

ETC complexes are also known to form active higher order structures titled supercomplexes (ETC SCs). ETC SCs have been detected across a variety of different organisms, including mammals, plants, yeast, and some bacteria (17). In 2000, Schägger first separated ETC SCs in yeast and mammals using blue-native polyacrylamide gel electrophoresis (BN-PAGE) when solubilizing the mitochondrial membrane with digitonin (18). Since then, intact ETC SCs have been verified to be functional through in-gel activity assays, and both enzymatic assays and O₂ consumption measurements on gel purified samples (19, 20). ETC SCs have also been viewed *in vivo* through fluorescence-lifetime imaging microscopy methods, confirming their endogenous presence (21). Moreover, high resolution crystal structures of ETC SCs have recently been solved (22, 23).

ETC supercomplexes have defined stoichiometries that dictate their function (**Figure 3**). Complex I, III and IV form the $I_1III_2IV_1$ “respirasome”, which contains all the required enzymes for electron transfer of NADH to O_2 (3). Complex II does not form any supercomplexes and acts as a link between the respiratory chain and the citric acid cycle. $CoQH_2$ produced by complex II must continue through the ETC however, and can be processed by the III_2IV_1 supercomplex. Intermediate supercomplexes such as I_1III_2 , and free form complexes such as III_2 and IV_1 also exist and are functional. The “plasticity” model proposes a dynamic system connecting both associated and disassociated states of ETC SCs, given the observation that the complexes retain functionality in non-associated states (24).

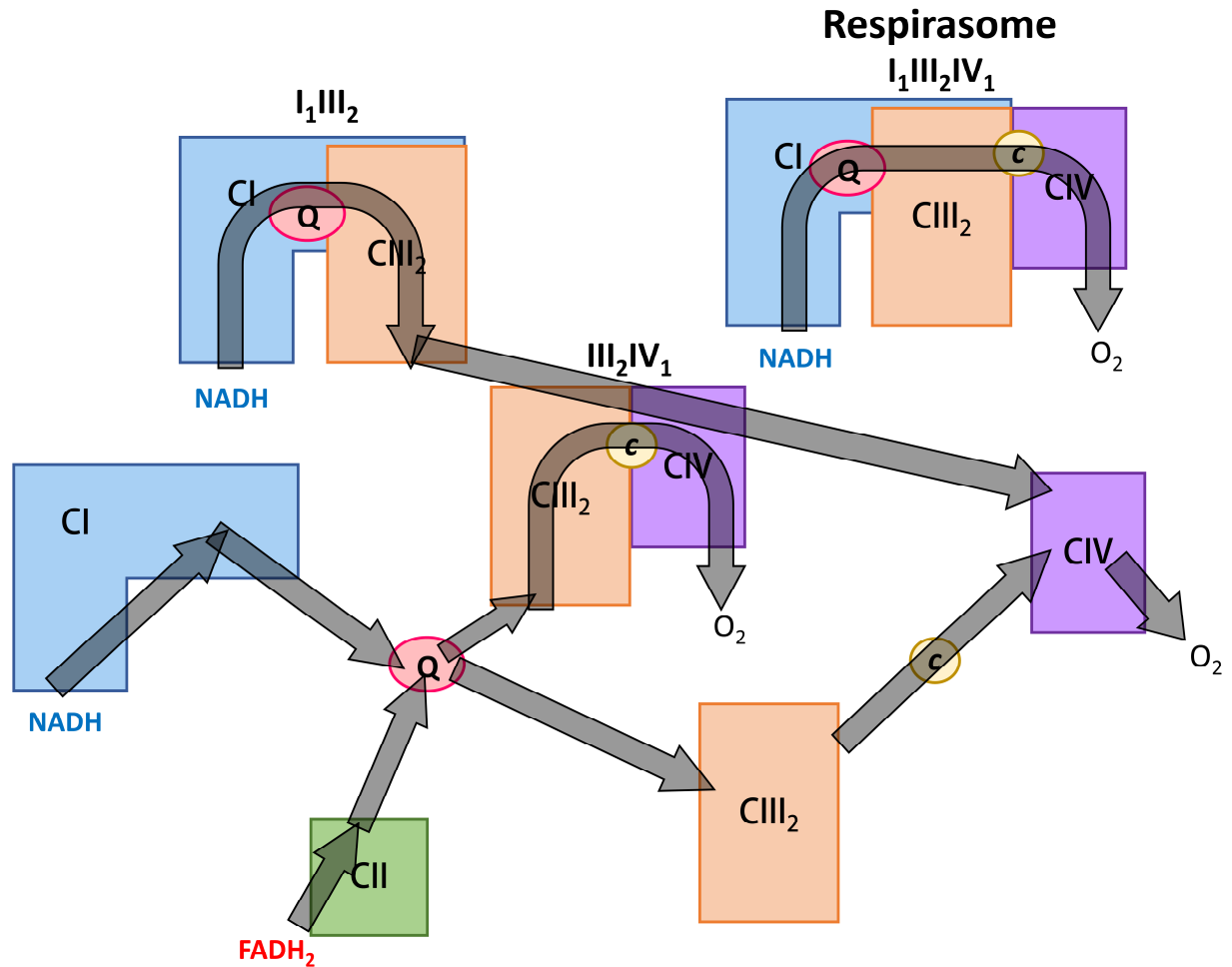


Figure 3. The plasticity model of ETC SC organization proposed by Moreno-Loshuertos et al. (2016). ETC SC assembly is “plastic” in that SCs can disassemble and reorganize, or complexes can remain active in their free form. Complexes I, III and IV form the I₁III₂IV₁ “respirasome” and process NADH exclusively. Complexes III and IV can also form the III₂IV₁ SC. Complex II, shown in the bottom left does not form any SCs. Higher order stoichiometries are also possible, building from the base forms depicted.

A few physiological functions of SC formation have since been demonstrated. Supercomplex formation enhances the stability of the individual complexes (25, 26), and minimizes the generation of damaging ROS (27, 28). Due to the relatively close distance between the complexes in ETC SCs, an increased *efficiency* in channeling substrate intermediates such as ubiquinone between the active sites of the complexes has been proposed. A recent study examining CIII-KO cells supplemented with alternative oxidase (AOX) suggested that CI-CIII₂ superassembly

partitions ubiquinone between CI- and CII- linked pathways and modifies their activities (20). Therefore, ETC SCs may serve as a partitioning agent, fine tuning the balance between the activity of CI-linked vs CII linked pathways. This has yet to be confirmed through functional models that can specifically disrupt the respirasome.

1.1.3 ETC SC assembly regulation and factors

The organization and proportion of ETC complexes in SCs varies between organisms, tissues, (20, 29, 30), and physiological states (31, 32), suggesting deliberate regulation of ETC function. Disassembly of SCs has been associated with diseases such as Parkinson's, heart failure, type 2 diabetes (T2D), and aging (33–36). Furthermore, ablation of SCs induces physiological changes within some model organisms. For example, deletion of SC assembly factor COX7A2L in zebrafish reduced their overall size, decreased fertility in females, and abnormally increased fat storage (37). These results suggesting impaired fatty acid (FA) metabolism coincide with previous results from Lapuente-Brun et al, who show that starved mice disassemble the respirasome to promote FAD route electron transfer (32). These studies together reiterate the suggestion that ETC SCs represent a higher order form of control to electron transfer pathways in order to optimize the catabolism of different metabolic fuels (37); however, more detailed studies confirming this idea are required.

Despite the molecular implications of ETC SCs for metabolic physiology and disease states, the supramolecular assembly of SCs remains largely uncharacterized. The discovery of COX7A2L and its now accepted role in III₂IV₁ SC assembly has prompted a search for additional protein factors regulating SC assembly. The hypothesis remains that additional protein factors control the formation and turnover of other SC species such as the respirasome, given that a regulatory factor specific to the III₂IV₁ SC has been found (38).

1.2.1 HIG1 protein family

Recent efforts in the search for regulatory protein factors of ETC SCs have focused on the HIG1 protein family. HIG1 proteins are mammalian homologs of yeast respiratory complex factor 1 (Rcf1). Deletion of Rcf1 and Rcf2 in yeast prevents III₂IV₁ SCs formation (39), though a more recent study shows that Rcf1 also interacts with pre-assembled CIV (40).

The Hig1 family in mammals includes hypoxia-inducible domain family member 2A (HIGD2A), HIGD1A, HIGD1B and HIGD1C, of which only HIGD2A and HIGD1A have been partially studied. All members are 10-12 kDa double pass transmembrane proteins generally localized in the MIM, with N and C termini that extend into the intermembrane space (41). The localization of HIGD1A and HIGD2A remain unclear (**Figure 4**). In addition to mitochondrial localization, these proteins have been either reported as partly nuclear localizing (42), or localizing to the nucleus under stress conditions (43).

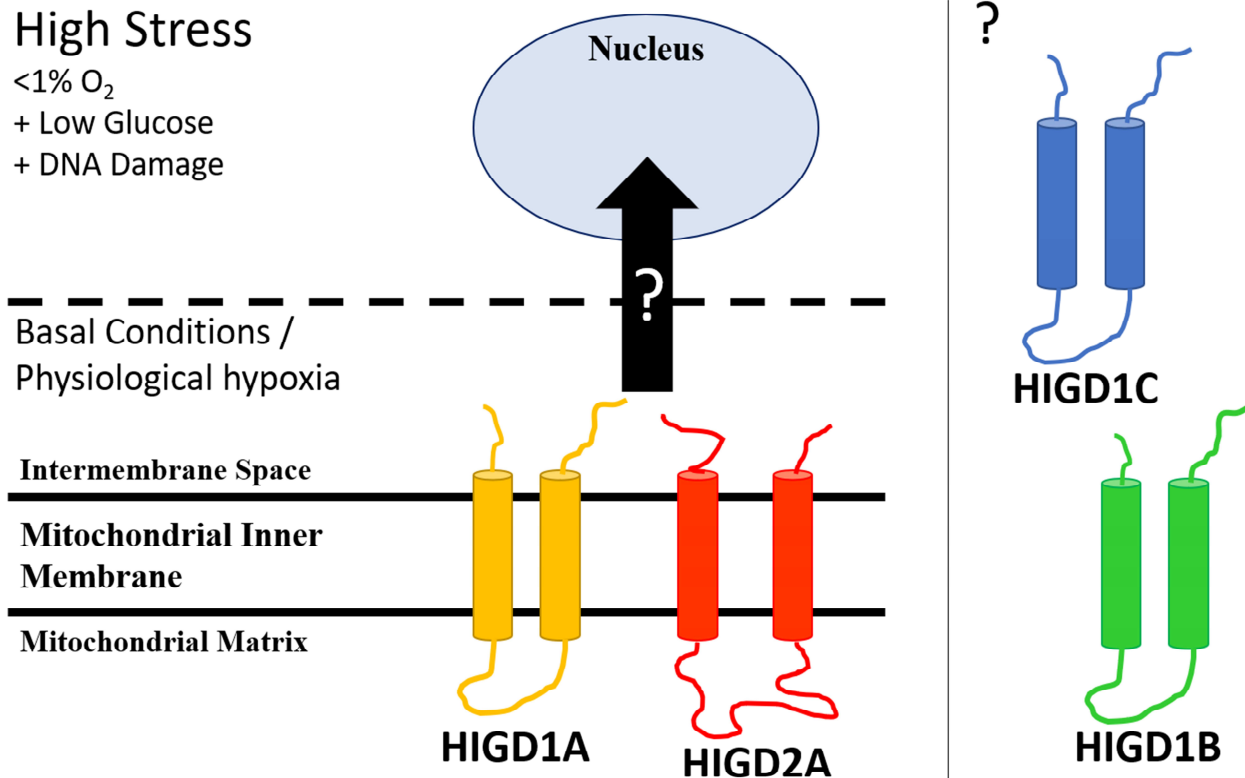


Figure 4. Subcellular localization of the HIG1 protein family. The HIG1 family proteins are small 10-12 kDa proteins with two transmembrane domains, and localize to the MIM under basal conditions. During conditions of high stress such as nutrient deprivation, and very low oxygen, HIGD1A may re-localize to the nucleus. Some studies report partial localization in both compartments. The subcellular localization of HIGD1C and HIGD1B are unknown.

HIGD1A and HIGD2A were originally characterized by their inducible expression under hypoglycaemic and hypoxic states (41). HIGD1A expression has consistently been associated with reduced cell death and anti-apoptotic signalling during hypoxia and low glucose treatment conditions (44–47). Moreover, similar effects from endogenous HIGD1A expression, including enhanced proliferation, were observed physiologically in tumor xenografts of glioma, breast cancer, and pancreatic cancer cells (48–50).

Both HIGD1A and HIGD2A were identified in early screening studies to be part of the HIF-1 α regulatory response (51), and although HIGD1A is canonically regulated by HIF-1 α (44, 50),

HIGD2A may or may not be regulated by the same mechanisms (50, 52). Altogether, the regulation and functional outcomes of HIG1 family protein expression appear to be specific to each model. The regulation and functions of HIGD1B and HIGD1C are unknown.

1.2.2 HIG1 protein family effects on cellular energetics and mitochondria

HIGD1A mediated cell survival during stress has been purportedly explained through effects measured in mitochondria. Stress conditions such as low glucose or hypoxia are thought to initially downregulate respiratory chain activity, and increase both non-mitochondrial and mitochondrial ROS production (53). Endogenous and exogenous HIGD1A expression during stress conditions can increase mitochondrial ATP production (46, 47), maintain mitochondrial membrane potential (54), and decrease mitochondrial ROS and general ROS production (50, 54, 55). One mechanism posits that HIGD1A binds CIV to increase its turnover rate, thereby lifting the supportive capacity of the ETC to promote cell survival (46); however, contrasting evidence shows interaction and function with CIII instead of CIV (50, 56). Alternatively, HIGD1A may modulate mitochondrial fusion dynamics through regulation of MIM fusion protein OPA1. In addition to pull-down experiments showing direct interaction between HIGD1A and the long isoform of OPA1, knockdown of HIGD1A induces mitochondrial fragmentation and a shift in OPA1 isoforms from long to short (57). Similar decreases in mitochondrial fusion and direct interactions with OPA1 were observed in another study exploring knockdown of HIGD2A, suggesting overlapping functions (42).

1.2.3 HIG1 protein family function in ETC biogenesis and SC assembly

The roles of HIGD1A and HIGD2A in CIII and CIV biogenesis and SC assembly have been a central focus of recent studies. HIGD2A incorporates the cytochrome *c* oxidase subunit III regulatory module (COX3) to complete CIV assembly, and knock-out of HIGD2A in leads to

decreased steady state levels of CIV and impairs biogenesis of CIV SCs (52, 56). Likewise, HIGD1A is a late assembly factor of CIII₂ and knock-out diminishes its steady state levels. The authors also note that HIGD1A-KO seems to impair CIV biogenesis and incorporation of both CIII₂ and CIV into SCs. Interestingly, overexpression of neither HIGD1A nor HIGD2A altered steady state levels of SCs under basal conditions; though the effects on assembly kinetics were not reported (56).

1.3.1 Current knowledge on respirasome assembly

While factors have been identified regulating III₂IV₁ assembly, there is a lack of direct protein factors to date have been identified in regulating respirasome (I₂III₂IV₁) formation. In models where COX7A2L is shown to be required for III₂IV₁ SC formation, respirasome levels remain unaffected by Δ COX7A2L (37, 58), suggesting that unknown protein factors can independently maintain interactions between CI and CIII₂ or CIV to form the respirasome (37). Furthermore, sub-respirasome SCs of CI-CIII₂ and CI-CIV suggest independent interactions are being maintained between CI and CIII₂ or CIV. One hypothesis that rectifies the dispensability yet functional aspect of COX7A2L is the observation that multiple configurations for the respirasome exist: one in which CIII₂ and CIV are tightly bound together using protein factors such as COX7A2L or possibly the HIG1 proteins, and a looser conformation in the absence of these factors (38). Regardless, the regulation of the interactions between CI and the other complexes in maintaining the respirasome is poorly understood.

1.3.2 Redefining ETC accessory subunits as regulatory protein factors of ETC SCs

Current research into SC assembly and regulation is limited to the known protein factors COX7A2L and the HIG1 family proteins. As such, research efforts into discovering new protein factors that regulate ETC SCs have assumed that these factors are external to the ETC subunits.

Within the last few years, crystal structures of the respirasome have been solved at atomic resolutions, which allows for detailed study of the interfaces shared between the complexes. Upon studying these structures, it is apparent that closely distanced protein-protein interactions between the complexes exist at particular interface locations (**Figure 5**). Potential protein-protein interactions between complexes have been highlighted by the authors of the crystal structures (22, 23, 59), and SC review articles following publication of the crystal structures (38, 60).

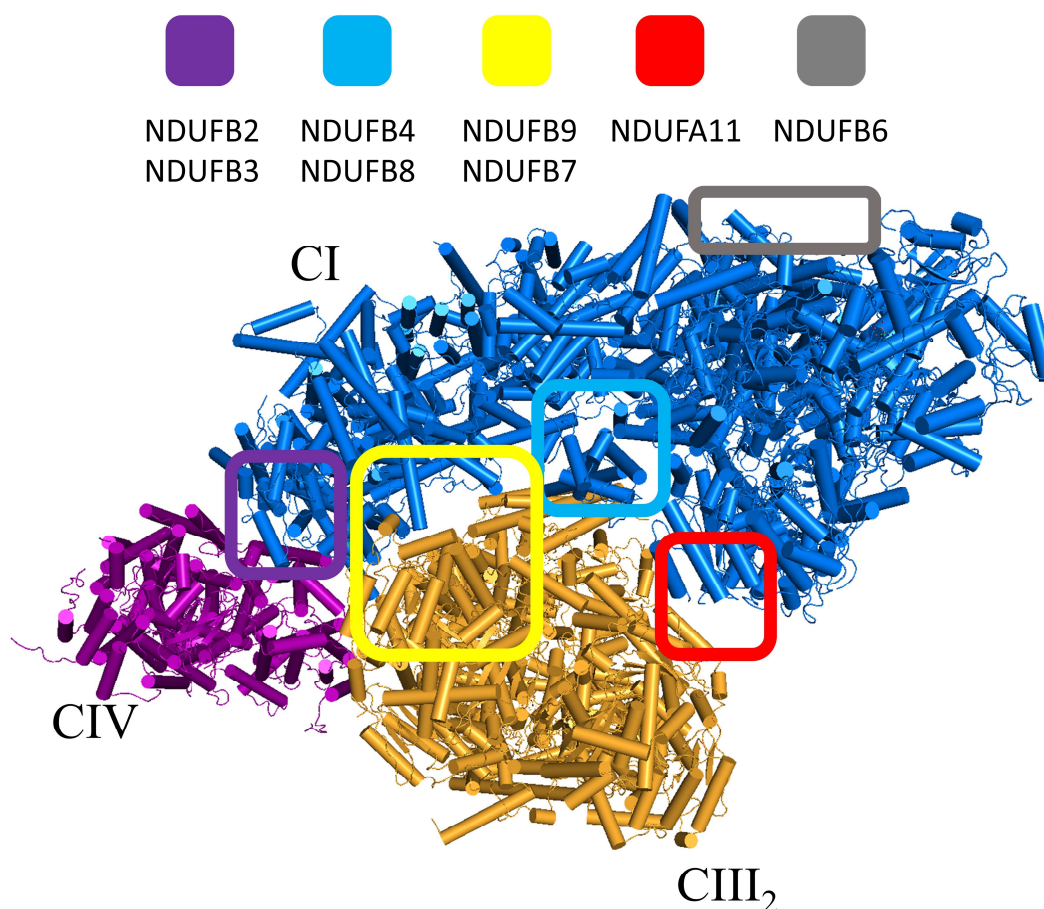


Figure 5. Candidate accessory subunits of complexes I with potential function in regulating respirasome assembly. Analyses of respirasome crystal structure (PDB: 5XTH) led to the identification of CI accessory subunits harboring potential interactions with CIII₂ or CIV. Subunits are labeled and color coded based on the interface they occupy (indicated on the respirasome diagram). NDUF6 was not identified through crystal structure datasets, but rather other mass-spectrometry datasets (see Figure 6).

Complex I is the largest ETC complex: in mammals it is comprised of 45 protein subunits total, of which 38 are nuclear DNA encoded and 7 are mitochondrial DNA encoded. Of the 45 subunits, just 14 are directly involved in catalytic activity of the complex (61). The remaining subunits are titled “accessory” and although they have no official function, they are likely involved in supporting the structure of the redox centers, in maintaining the integrity of the complex, or other regulatory functions.

A detailed study by Stroud et al. concluded that most CI accessory subunits were integral to the stability of the complex by generating knock-outs of every CI accessory subunit in HEK293 cells, and observing that the majority of the knockouts did not display CI-containing SCs at steady state (62). However, many CI-KO cell lines whose subunits are found at the interfaces between complexes display a faint band in CI-probed BN-PAGE immunoblots. This band could correspond to complete or N-module deficient complex I not in a respirasome, which is normally not detected in HEK293 cells at steady state. Accessory subunits NDUFA11 and NDUFAB1, which display this BN-PAGE pattern, have since been published in independent studies highlighting their importance to SC formation (63, 64).

Through analysis of a cryo-slicing mass spectrometry-BN-PAGE datasets developed by Muller et al. (65), a few of these same CI interface accessory subunits are enriched in BN-PAGE cryo-slices containing CI SCs (**Figure 6**).

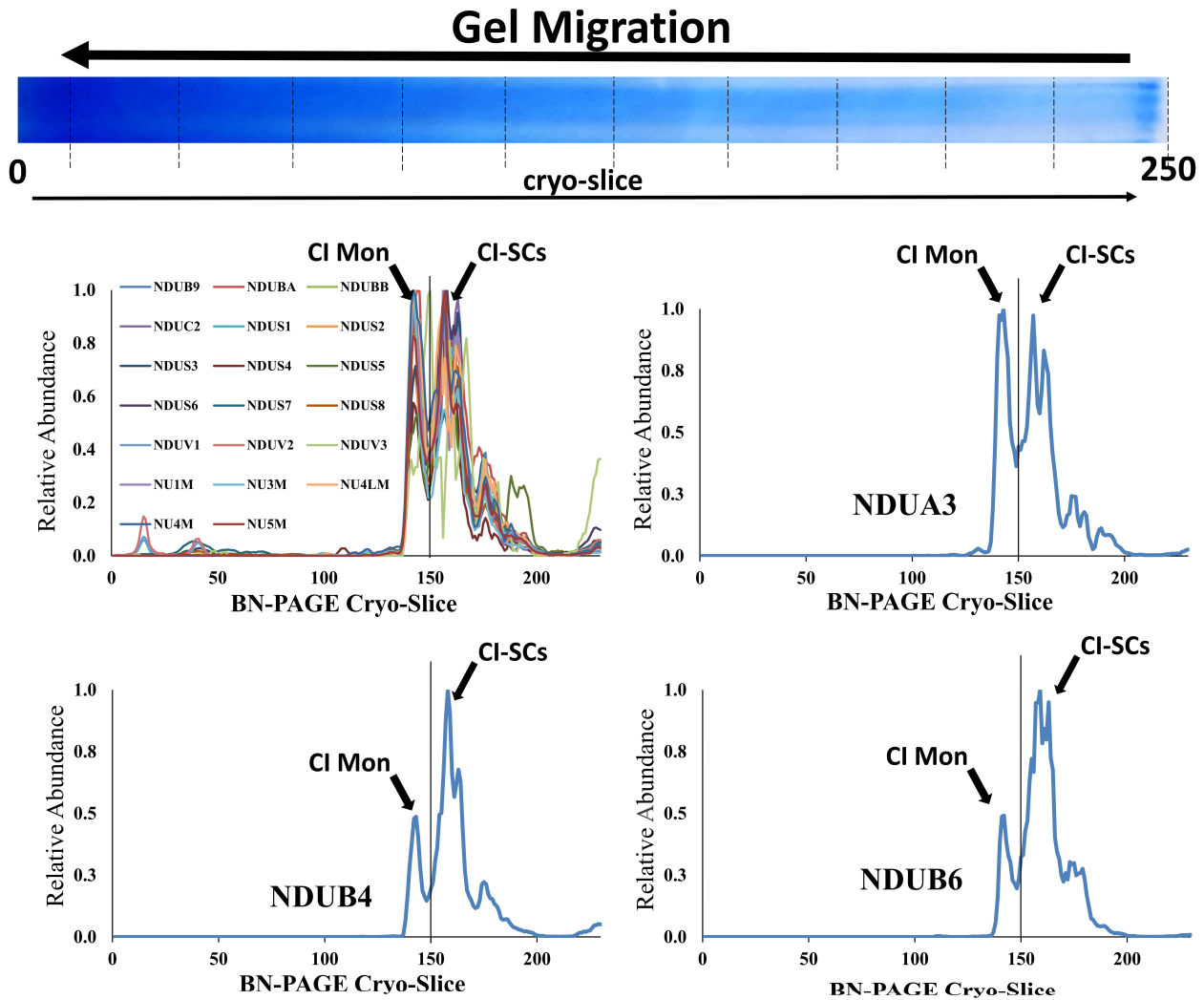


Figure 6. Cryo-slicing mass spectrometry-BN-PAGE datasets shows enrichment of select CI accessory subunits in CI SCs. Performed by Muller et al. (2016). BN-PAGE was performed on rat brain tissue to separate the respiratory chain complexes, followed by cryo-slicing of the gel into over 200 sections across its length (example shown on top). Mass Spectrometry analyses was performed on each fraction and the relative abundance of each identified protein was identified in each fraction. All CI subunits are shown in the top left graph. Unlike candidate subunits NDUB4 (bottom left) and NDUB6 (bottom right), NDUA3 (top right) is not enriched in respirasome fractions.

1.4 Purpose and Objectives

1.4.1 Characterization of the HIG1 protein family

The characterization of differences between the HIG1 proteins in ETC SC assembly and ETC function are incomplete. Furthermore, the functions of HIGD1C and HIGD1B have yet to be described in any capacity. We hypothesize that HIGD1C and HIGD1B may harbor both overlapping and distinct functionalities in cell survival under stress, cellular bioenergetics, and ETC SC formation. We additionally believe that HIGD1C and HIGD1B act as direct protein factors in ETC SC assembly. Through a stable overexpression model in HEK293t cells, we used comparative studies between the HIG1 protein family members to better identify the primary functionalities of each protein.

1.4.2 Understanding respirasome assembly through ETC accessory proteins

The lack of identified protein factors specific to respirasome formation suggest that the respirasome is regulated by alternative means. We hypothesize that specific CI accessory subunits function as the direct protein factors regulating respirasome assembly. In other words, the significant protein-protein interactions that mediate respirasome formation could be internal to the ETC and integral to control of electron transfer pathways (**Figure 7**).

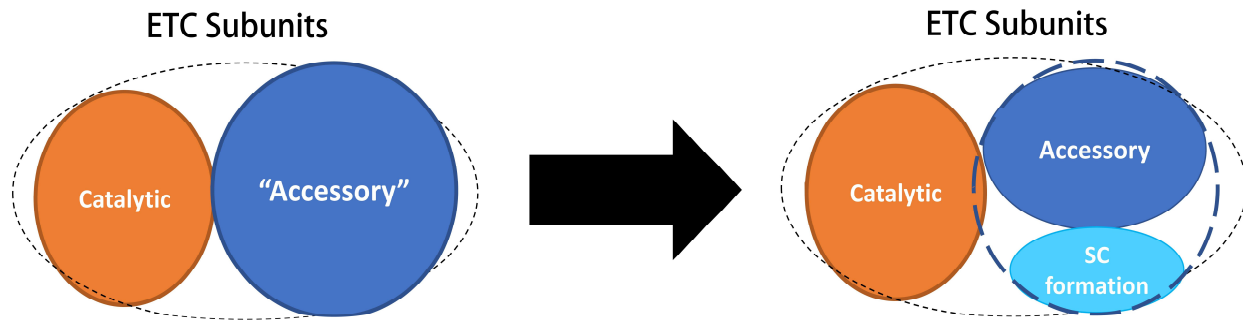


Figure 7. Proposed framework shift of ETC subunits and their categorical organization. We propose a shift in redefining subset of uncharacterized “accessory” subunits as respirasome assembly factors.

Due to the absence of CI-specific ETC SC factors, current interrogation into respirasome function is limited to the use of chemical agents which disrupt the entire MIM (28), or genetic ablation of important MIM proteins (20, 66, 67) which compromises more than just the respirasome itself: the individual complexes and the entire MIM structure. Adoption and characterization of this new framework could lead to an expansion in understanding the formation of CI-containing SCs, which would allow for more specific tools to study the function and importance of ETC SCs.

1.4.3 Research Objectives

The overall aim of this work is to delineate the role of regulatory protein factors in ETC SC assembly. This aim will be addressed through **two research objectives**:

1. Characterization of the complete HIG1 protein family, including previously uncharacterized members, through a stable expression cell line model
2. Delineating the function of CI accessory subunits in respirasome assembly, and the functional implications of redistributing SCs through selectively disassembling the respirasome

2.0 MATERIALS AND METHODS

2.1.1 HIGD protein family cell line culture:

Human Embryonic Kidney (HEK) 293T derived cells were maintained at 5% CO₂, 37°C and cultured in Dulbecco's modified Eagles' Medium (DMEM) (Gibco, 11995-065) supplemented with 10% Fetal Bovine Serum (FBS) (Wisent Bio Products) and 1% antimycotic-antibiotic (AA) (Gibco, 15240-062). Puromycin resistant stable cell lines were maintained with 5µg/mL puromycin dihydrochloride (Gibco, A11138-03). Cell lines were sub-cultured 1:20 using trypsin-EDTA dissociation reagent when reaching ~90% confluency. Passage 10-25 were used for experiments.

2.1.2 Internal SC cell line culture:

Human Embryonic Kidney (HEK) 293 unmodified (WT) and NDUFB4-KO cells were a kind gift from Dr. Mike Ryan (Monash University, Melbourne, Australia). Cell lines were maintained at 5% CO₂, 37°C and cultured in DMEM (Gibco, 11995-065) supplemented with 50µg/mL uridine (Sigma, U3003), 10% FBS (Wisent Bio Products), 1% Penicillin-Streptomycin (5,000 U/mL) (Gibco, 15070-063), and 1X GlutaMAX (Gibco, 35050-061). Puromycin resistant stable cell lines were maintained with 1µg/mL puromycin dihydrochloride (Gibco, A11138-03). Media on NDUFB4-KO cells were replaced daily due to rapid acidification. Cell lines were sub-cultured 1:5 using trypsin-EDTA dissociation reagent when reaching ~90% confluency. Passage 1-10 were used for experiments.

2.2 HIG1 protein family stable cell line generation

2.2.1 HIG1 protein family subcloning

Stable cell lines were generated by retroviral transduction through subcloning into the MSCV-PIG retroviral backbone vector, a kind gift from Dr. Ruth Slack (University of Ottawa, Ottawa,

Canada). C-terminal Myc-Flag tagged transient expression plasmids of human HIGD1A (NM_014056), HIGD2A (NM_138820), HIGD1B (NM_016438) and HIGD1C (NM_001109619) were purchased from Origene (CAT#: RC209997, RC201223, RC204663, RC225015). Encoding cDNA from each transient plasmid, including the upstream kozak sequence region, was amplified by PCR (FWD: 5'- GGAATTAGATCTCTCGAGGGTACCGAGGAGATCTGC-3', REV: 5'- CGGTAGAATTCGTTAACGGCCGGCCGTTTAAAC-3') to create complementary DNA (cDNA) constructs containing terminal base-pair overlaps with the MSCV-PIG retroviral vector. Gibson assembly (New England Biolabs, E5510S) was performed to ligate HIG1 protein family cDNA constructs to XhoI/HPAI restriction digested and agarose gel purified MSCV-PIG vector. Resulting bacterial clones were verified to contain the insert using sanger sequencing with the following primer: 5'-CCCTTGAASSTCCTCGTTCG-3'.

2.2.2 Retroviral Transduction

Packaging plasmids pCMV-VSVG and pCMV-Gag-Pol (Cell Biolabs, Inc.), and the backbone constructs were co-transfected into unmodified HEK293t cells using Lipofectamine LTX reagent (ThermoFisher) in a 1:2:3 ratio. Following 48 hours post transfection, viral supernatant was harvested and filtered through a 0.45µm filter. 1mL of viral supernatant was combined with 400,000 HEK293t cells and 8µg/mL hexadimethrine bromide (polybrene), and centrifuged at 800g for 1 hour. Cells were then incubated with viral supernatant for 48hrs, followed by replacement media supplemented with 10µg/mL puromycin for selection. Empty vector cells were generated using retroviral particles packaged with the unmodified MSCV-PIG vector. Retroviral transduction was repeated on HIGD2A stable cells using 1mL of frozen viral supernatant in order to achieve similar expression to the HIGD1 cell lines.

2.3 Internal SC stable cell line generation

Stable cells were generated by retroviral transduction through subcloning into the MSCV-PIG vector. Synthetic cDNA corresponding to human NDUFB4 (NM_004547.6), or modified with N24A and R30A substitutions were purchased from GenScript (Piscataway, New Jersey, United States). Amplification by PCR, Gibson assembly and retroviral transduction methods were performed as described in sections *2.2.1 HIG1 protein family subcloning/2.2.2 Retroviral Transduction* with the following modification: stable cells were selected with 2µg/mL puromycin. To increase expression, cell lines required an additional round of retroviral transduction.

2.4 Western blot analysis

2.4.1 Protein extraction and sample preparation

Samples were prepared at 4°C or on wet ice. Cells were pelleted in 1X phosphate buffered saline (PBS) and resuspended in RIPA Lysis Buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 0.25% deoxycholic acid, 1% NP-40, 10mM EDTA) supplemented with 1:500 Protease Inhibitor Cocktail (Sigma, P8340). Mechanical lysis was performed through 10 strokes of a 28-gauge needle, followed by sample clearing at 10,000g for 10 min, 4°C. Protein levels were quantified by the bicinchoninic acid (BCA) assay method using the Pierce™ BCA Protein Assay Kit (Thermofisher, 23225). Whole cell protein lysate was combined with 4X Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.005% Bromophenol Blue) and 10% β-mercaptoethanol. Samples were briefly boiled for 5 minutes at 95°C and stored at -20°C until use.

2.4.2 Immunoblotting

8%, 12% and 15% resolving acrylamide gels (30% acrylamide mix (Bio-Rad), 1.5M Tris pH 8.8, 10% SDS, 10% ammonium persulphate (APS), H₂O, N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) were casted with 5% stacking gels (30% acrylamide mix (Bio-Rad), 0.5M Tris pH 6.8,

10% SDS, 10% APS, H₂O, TEMED). 10-20µg of protein sample were run at 150V in running buffer (25mM Tris base, 192mM glycine, 0.1% SDS) for approximately 1 hour. Wet transfer to a nitrocellulose membrane (Bio-Rad) was performed at 100V in transfer buffer (25mM Tris Base, 192mM glycine, 20% methanol, H₂O) for 1 hour. Wet transfer quality was verified by ponceau-S staining, followed by blocking with 5% skim milk in tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 hour. Membranes were incubated with the indicated diluted primary antibodies in 1% BSA in TBS-T overnight at 4°C under gentle agitation: 1:1000 Anti-FLAG M2 (Sigma, F1804), 1:1000 ANTI-FLAG (Millipore, F7425), 1:10000 (CYG) FP Tag (Evrogen, AB121), 1:10000 Anti- α -Tubulin (Sigma-Aldrich, T6199), 1:2000 β -Actin (Cell Signaling Technology, #4967), 1:4000 Anti-Opa1 (Abcam, ab42364), 1:2000 Anti-NDUFB10 [EPR16230-47] (Abcam, ab196019), 1:2000 Anti-UQCRC2 [13G12AF12BB11] (Abcam, ab14745), 1:2000 MTCO1 [1D6E1A8] (ThermoFisher, 459600), 1:2000 Anti-Mitofusin 2 + Mitofusin 1 [3C9] (Abcam, ab57602), 1:2000 Anti-DLP1 (DRP1) (BD Transduction Laboratories, 611112), 1:20000 GAPDH (Santa Cruz, sc-47724), 1:5000 Vinculin [EPR8185] (Abcam, ab129002), 1:1000 p53 [1C12] (Cell Signaling Technology, #2524), 1:1000 Anti-Parkin (Santa Cruz, sc-32282), 1:1000 Akt (Cell Signaling Technology, #9272), 1:1000 Phospho-Akt (Ser473) (Cell Signaling Technology, #9271), 1:1000 ATP5B (Abcam, ab14730), 1:1000 ATP5C1 (Santa Cruz, sc-241858), 1:1000 ANT1/2 (N-19) (Santa Cruz, ac-9299), 1:2000 Tomm20 (Abcam, ab56783). 1:5000 anti-Mouse IgG-HRP (Promega), anti-Rabbit IgG-HRP (Promega), or anti-Goat IgG-HRP (Santa Cruz) secondary antibodies, prepared in 5% skim milk in TBS-T, were incubated for 1 hour at room temperature. Membranes were exposed with either Immobilon Classico Western HRP substrate (Millipore), Immobilon Crescendo Western HRP substrate (Millipore), or SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher), and captured by the ChemiDoc™ MP

Imaging System (Bio-Rad). Densitometry analysis was performed using Image J software and normalized to loading control targets.

2.5 Immunocytochemistry and mitochondrial network morphology analyses

2.5.1 Immunocytochemistry

Stable cell lines were plated onto #1.5 thickness, 12mm round glass coverslips (Fisher Scientific) coated with Matrigel at ~20% confluency and allowed to adhere overnight. For experiments investigating hypoxia, cells were plated onto coverslips at 10% confluency, allowed to adhere overnight, and then placed in an Xvivo System Model X3 Cytocentric Cell Incubation and Handling Platform (BioSpherix, Ltd., Parish, NY) at 1% O₂ for 48 hours. Coverslips were washed with PBS and subsequently fixed in 4% paraformaldehyde in PBS at room temperature for 15 minutes. Coverslips were then washed with PBS, followed by incubation with the following primary antibodies at the indicated dilutions in PBS and 1% BSA for 2.5 hours: 1:200 Anti-FLAG M2 (Sigma, F1804), 1:200 TOM20 (ProteinTech Group, 11802-1-AP), 1:100 mtHSP70 [JG1] (ThermoFisher, MA3-028). Coverslips were washed 3 times with PBS, and incubated with the following secondary antibodies at the indicated dilutions in PBS, 1% BSA, and 10µg/mL Hoechst 33342 counterstain (Invitrogen, H3570) for 2 hours: 1:200 Goat anti-Mouse IgG (H+L) Alexa Fluor 568 (Invitrogen, A-11004), 1:200 Donkey Anti-Rabbit IgG (H+L) Cy5 (Jackson ImmunoResearch, 711-175-152). Coverslips were mounted onto Fisherbrand™ Superfrost™ Plus Microscope Slides (Fisher Scientific) using Shandon™ Immu-Mount solution (Fisher Scientific). Images were acquired through a Zeiss LSM880 AxioObserver Z1 confocal microscope with AiryScan using a 63x 1.4 NA oil objective. Images were processed using ZenBlue 3.2 software (Zeiss).

2.5.2 Mitochondrial network morphology analyses

For network analyses experiments, a total of 10-12 images were acquired for each cell line across three independent experiments. Analysis was performed using Momito software (Dr. Marc Germain, University of Quebec at Trois-Rivieres, Canada) (68). Images were pre-processed using the “Basic setting” and the following parameters: enhance contrast selected, saturated pixel: 0.4, median filter radius: 2, local threshold radius: 15, variation to automatic threshold: 0, erode count: 4, dilate count: 7. Average morphology values of each 10-12 image set were plotted for statistical analyses.

2.6 Mitochondrial respiratory complex enzymatic assays

Enzymatic assays were performed on whole cell extracts prepared as described in *2.4.1 Protein extraction and sample preparation*. Activity of citrate synthase and OXPHOS complexes I, III, IV were determined similarly to Spinazzi et al. (69) with the following modification: Absorbance was quantified in ~0.2mL triplicate reactions using 96 well plate format and the BioTek Synergy Mx Microplate Reader (BioTek Instruments, Inc., Winooski, VT, United States). Enzyme activity is expressed per μg protein relative to empty vector cells in each set of biological replicate samples.

2.6.1 Citrate synthase activity

Protein lysate was combined with 100mM Tris (pH 8.0), 100 μM DTNB (5,5-dithio-bis-(2-nitrobenzoic acid), and 300 μM Acetyl-CoA. The reaction was started by the addition of 0.5mM oxaloacetic acid. Citrate synthase activity was determined by quantifying CoA-SH production, which further reacts with available 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce TNB. Production of TNB is measured by the increase in absorbance at 412nm for 5 minutes. Citrate synthase activity calculated using the extinction coefficient ϵ ($\text{mmol}^{-1} \text{cm}^{-1}$) = 13.6.

2.6.2 Complex I activity

Protein lysate was combined with 50mM potassium phosphate buffer (pH 7.5), 3mg/mL fatty acid-free BSA, 300 μ M KCN, and 100 μ M NADH. The reaction was started by the addition of 60 μ M ubiquinone. Complex I activity was determined by quantifying the oxidation of NADH through decrease in absorbance at 340nm for 10 minutes. Complex I activity calculated using the extinction coefficient ϵ ($\text{mmol}^{-1} \text{cm}^{-1}$) = 6.2.

2.6.3 Complex III activity

Protein lysate was combined with 25mM potassium phosphate buffer (pH 7.5), 75 μ M oxidized cytochrome C, 500 μ M KCN, and 100 μ M EDTA. The reaction was started by the addition of 100 μ M decylubiquinol. Complex III activity was determined by quantifying the reduction of cytochrome *c* through an increase in absorbance at 550nm for 10 minutes. Specific activity was determined subtracting absorbance rates upon the addition of 10 μ g/mL antimycin A. Complex III activity calculated using the extinction coefficient ϵ ($\text{mmol}^{-1} \text{cm}^{-1}$) = 18.5.

2.6.4 Complex IV activity

Protein lysate was combined with 50mM potassium phosphate buffer (pH 7.5). Cytochrome *c* was completely reduced by the addition of a few grains of sodium dithionite. The reaction was started by the addition of 60 μ M of reduced cytochrome *c*. Complex IV activity was determined by quantifying the oxidation of cytochrome *c* through a decrease in absorbance at 550nm for 10 minutes. Specific activity was determined subtracting absorbance rates upon the addition of 300 μ M KCN. Complex IV activity calculated using the extinction coefficient ϵ ($\text{mmol}^{-1} \text{cm}^{-1}$) = 18.5.

2.7 Mitochondrial to nuclear DNA ratio quantification

2.7.1 DNA extraction

Cells were pelleted in 1X PBS and were stored at -80°C until the day of DNA extraction. On the day of extraction, pelleted cells were allowed to thaw on ice, and resuspended in 0.5mL of DNA lysis buffer pH 8.0 (100 mM Tris, 5mM EDTA, 0.2% SDS, 200mM NaCl). Proteinase K solution (ThermoFisher, AM2546) was added to a final concentration of 0.4mg/mL, and samples were incubated at 55°C overnight. The next day, 0.5mL Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen, 15593031) was added to each sample followed by mixing by inversion for 5 minutes. Samples were centrifuged at 16000g for 10 minutes, and the top aqueous layer containing DNA was transferred to new microfuge tubes. DNA was precipitated by the addition of 0.6mL cold isopropanol to each sample, followed by incubation at -20°C overnight. The following day, precipitated DNA was centrifuged at 16000g for 10 minutes at 4°C, and DNA pellets were briefly washed and re-pelleted in 70% EtOH before final resuspension in 1mL of Tris-EDTA buffer pH 8.0 (10 mM Tris-HCl, 1 mM disodium EDTA). DNA concentration and purity were verified using the NanoDrop™ 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States).

2.7.2 Mitochondrial to nuclear DNA quantification

Mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) ratios were determined through quantitative PCR (qPCR) against mitochondrial DNA gene MT-ND1 (FWD: 5'- AACATACCCATGGCCAACCT -3', REV: 5'- AGCGAAGGGTTGTAGTAGCCC -3') and nuclear DNA gene 18SrRNA (FWD: 5'- TAGAGGGACAAGTGGCGTTC -3', REV: 5'- CGCTGAGCCAGTCAGTGT -3'). 10µL qPCR reactions were performed in triplicate using 1X SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 3.33µM forward and reverse

primers, and a final sample DNA amount of 1ng. qPCR was performed on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Following 3 minutes at 98°C for initial denaturation, thermal cycling consisted of 15s at 98°C for denaturation and 30s of annealing/extension/reading at 60°C. Cycling was continued for 40 cycles total, followed by melt curve analysis (65°C-95°C at 0.5°C increments per 5s) to verify product quality. qPCR data was compiled using the CFX Maestro software (Bio-Rad) and analyzed manually using the $2^{-\Delta\Delta CT}$ method in Microsoft Excel. mtDNA:nDNA ratios are expressed relative to empty vector cells in each set of biological replicate samples.

2.8 Immunoprecipitation and immunoblotting

Cells were pelleted in 1X PBS and resuspended in triton lysis buffer (10mM Tris-HCl (pH 7.4), 150mM NaCl, 0.5% v/v Triton X-100, and 1mM EDTA) with 1:500 Protease Inhibitor Cocktail (Sigma, P8340). Mechanical lysis was performed through 10 strokes of a 28-gauge needle, followed by sample clearing at 10,000g for 10 min, 4°C. Protein concentration was quantified by the Bradford assay method using Bio-Rad Protein Assay Dye Reagent (Bio-Rad) and measuring absorbance at 595nm using the BioTek Synergy Mx Microplate Reader (BioTek Instruments, Inc., Winooski, VT, United States). Three mg of whole cell protein extract was combined with 20µL of Anti-FLAG® M2 Magnetic Beads (Millipore, M8823) and was allowed to incubate overnight at 4°C with end over end rotation. Elution was carried through incubation with 50µL glycine-HCl (pH 3.0) for 15 minutes under constant agitation. Eluted protein samples neutralized with 10 µL of 0.5 M Tris, 1.5 M NaCl pH 8.0 solution, and were combined 3:1 with 4X Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.005% Bromophenol Blue) and 10% β-mercaptoethanol. Samples were briefly boiled for 5 minutes at 95°C. Immunoblotting was performed as described in section 2.4.2 *Immunoblotting*.

2.9 Long term hypoxia survival assay

2.9.1 Hypoxia survival experimental procedure

20000 cells were seeded into Matrigel coated 96 well clear cell cultured treated microplates, with a total of 6 technical replicates per cell line. The following day, cell culture media was replaced and supplemented with 1 drop/mL of NucBlue™ Live ReadyProbes™ Reagent (ThermoFisher, R37605) and 0.5µg/mL propidium iodide (PI) solution (Sigma, P4864). Cells were placed in an Xvivo System Model X3 Cytocentric Cell Incubation and Handling Platform (BioSpherix, Ltd., Parish, NY) at 1% O₂. At 0 hours, 48 hours, and 72 hours at 1% O₂, images capturing the number of Hoescht⁺ and PI⁺ cells were acquired using an EVOS® FL Auto Imaging System (ThermoFisher) placed in the hypoxia chamber. A 10X objective captured the center of each well, with a total of 300-600 cells per field of view.

2.9.2 Hypoxia survival imaging analysis

Acquired images were processed and cell count data was extracted using ImageJ software. Images showing total nuclei through the Hoescht⁺ channel were processed using the “make binary” and “watershed” functions. Nuclei were counted in the resulting images using the automated counting function. Images showing dead cells through the PI⁺ channel were thresholded manually prior to automated counting. The percentage of PI⁺ cells is expressed normalized to total nuclei (Hoescht⁺).

2.10. Seahorse extracellular flux (XF) analyses of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR)

2.10.1 XF assessment of HIG1 protein family stable cells

OCR and ECAR were measured using the XF96^e Analyzer (Seahorse Biosciences). 30,000 cells (experimentally optimized) were seeded onto 96-well XF96 culture plates coated with Matrigel. The following day, cell culture media was replaced with phenol red free, sodium bicarbonate free

Seahorse XF Media pH 7.4 (DMEM, 25mM D-glucose) supplemented with 4mM L-glutamine and 1mM sodium pyruvate on the day of the experiment. The XF96 culture plate was allowed to equilibrate at 37°C in a non-CO₂ incubator for 30 minutes prior to the start of the experiment. Following the acquisition of resting (basal) OCR and ECAR, cells were treated with the following compounds in order: 1.5µM oligomycin to measure non-phosphorylating OCR, 1µM Trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) to measure uncoupled (maximal) OCR, 1.5µM Antimycin A and 1.5µM Rotenone to measure non-mitochondrial OCR, and 6.5µM Monensin to measure maximal glycolytic capacity. Three measurement cycles were performed for resting OCR, non-phosphorylating OCR, uncoupled OCR, non-mitochondrial OCR and monensin induced ECAR, which included 3-minute intervals of mixing and measurement (6 minutes total per cycle). Following completion of the assay, Seahorse XF Media was removed and cells were washed briefly with PBS followed by Lysis with RIPA Lysis Buffer (0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA). The amount of protein per well was determined by the BCA assay method using the Pierce™ BCA Protein Assay Kit (Thermofisher, 23225). Results from each independent experiment are expressed as the average value for the 3 measurements of each treatment condition. Data was analyzed using the XF software (Seahorse Biosciences) and Microsoft Excel.

2.10.2 XF assessment of HIG1 protein family stable cells following hypoxia pre-treatment

Experiments were performed as described in *2.10.1 XF assessment of HIG1 protein family stable cells*, with the following modification: 20,000 cells (experimentally optimized) were seeded onto a 96-well XF96 culture plate coated with Matrigel. The following day, the XF96 culture plate was placed into an Xvivo System Model X3 Cytocentric Cell Incubation and Handling Platform

(BioSpherix, Ltd., Parish, NY) at 1% O₂ for 24 hours. After 24 hours the cells were removed from the hypoxia chamber and XF analyses were continued as previously described.

2.10.3 XF Assessment of internal SC stable cell lines

Experiments were performed as described in *2.10.1 XF assessment of HIG1 protein family stable cells*, with the following modification: 40,000 cells (experimentally optimized) were seeded onto a 96-well XF96 culture plate coated with Matrigel.

2.11. High resolution respirometry (HRR) in permeabilized cells

2.11.1 HRR assessment of HIG1 protein family stable cells

HRR of digitonin permeabilized cells was performed on the Oxygraph-2k system (OROBOROS Instruments, Innsbruck, Austria). Cell lines were dissociated from culture flasks using Trypsin-EDTA and resuspended in mitochondrial respiration (MiR05) buffer (0.5mM EGTA, 3mM MgCl₂-6H₂O, 20mM taurine, 10mM K₂H₂PO₄, 20mM HEPES, 110mM sucrose, and 1g/L BSA; pH 7.1). 2.5 million cells were added to each measurement chamber, suspended in MiR05 and held at 37°C. To the chamber, the following additions were performed sequentially: 16.2μM digitonin, 2mM malate, 5mM pyruvate, 10mM glutamate (CI Leak respiration), 5mM ADP (CI-linked respiration), 10mM succinate (CI+CII linked respiration), 2.5μM oligomycin (CI+CII leak respiration), 0.5μM titrations of FCCP (uncoupled or maximal respiration), and 2.5μM of antimycin A (non-mitochondrial respiration). Respiration rates are corrected by subtracting non-mitochondrial respiration rates. Data is expressed per million cells.

2.11.2 Simultaneous mitochondrial membrane potential measurement during HRR in HIG1 protein family stable cells

Mitochondrial membrane potential ($\Delta\psi_{mt}$) was determined fluorometrically during HRR respiratory states using tetramethylrhodamine methyl ester perchlorate (TMRM). TMRM was

added after cells were resuspended in the measurement chamber, through stepwise titrations of 0.5 μ M to a final concentration of 2 μ M. The HRR protocol was then followed similarly as described in 2.11.1 *HRR assessment of HIG1 protein family stable cells*. Raw fluorescence signal was calibrated to μ M TMRM, which represents the proportion of unaccumulated fluorescence signal in the mitochondrial matrix (inversely proportional to $\Delta\psi_{mt}$). μ M TMRM values were converted to $\Delta\psi_{mt}$ (expressed in mV) using the following equation adapted from a template provided by OROBOROS Instruments (Innsbruck, Austria), which expresses $\Delta\psi_{mt}$ as a function of μ M TMRM:

$$\Delta\psi_{mt} (\mu\text{M TMRM}) = 61.5 \times \log_{10} \left(\frac{\frac{(\text{inital } C_{TMRM}) \times (V_{total})}{\mu\text{M TMRM}} \times 10^{-6} - (V_{total} - (V_{mt} \times P_{mt}) - (K_o' \times P_c))}{(V_{mt} \times P_{mt}) + (K_i' \times P_{mt})} \right)$$

Where *inital* C_{TMRM} is the initial concentration of TMRM, equal to 2 $\cdot 10^{-6}$ M.

V_{total} is the total volume of the measurement chamber, equal to 2000 μ L.

V_{mt} is the physical volume mitochondrial occupy per mg weight, assumed to be 1 μ L/mg.

P_c is the protein content in the measurement chamber, estimated to be 0.6 mg total based on experimental determinations.

P_{mt} is the mitochondrial protein content in the measurement chamber, assumed to be 1/5th total protein content, equal to 0.12mg.

K_o' is the partition coefficient between the external medium and the inter-membrane surface of the mitochondrial inner membrane, assumed to be 88 μ L/mg.

K_i' is the partition coefficient between the mitochondrial matrix volume and the matrix facing surface of the mitochondrial inner membrane, assumed to be 36 μ L/mg

2.11.3 HRR of internal SC stable cell lines

Experiments were performed by Dr. Chantal Pileggi from the University of Ottawa. HRR of digitonin permeabilized internal SC stable cell lines were performed using the Oxygraph-2k system (OROBOROS Instruments, Innsbruck, Austria). Cell lines were dissociated from culture flasks using Trypsin-EDTA and resuspended in MiR05 buffer (0.5mM EGTA, 3mM MgCl₂·6H₂O, 20mM taurine, 10mM K₂HPO₄, 20mM HEPES, 110mM sucrose, and 1g/L BSA; pH 7.1). 2-3 million cells were added to each measurement chamber, suspended in MiR05 and held at 37°C. A total of 3 separate protocols were performed simultaneously in separate measurement chambers. The first protocol determining CI-specific respiration featured the following additions performed sequentially: 8.1µM digitonin, 5mM malonate, 2mM malate, 5mM pyruvate, 10mM glutamate (CI leak respiration), 5mM ADP (CI linked respiration), 0.5µM rotenone (non-CI respiration), and 2.5µM antimycin A (non-mitochondrial respiration). All respiration values were corrected by subtracting non-mitochondrial respiration values. CI-specific respiration was calculated by subtracting respiration values in the presence of rotenone. The second protocol determining CII-specific respiration featured the following additions performed sequentially: 8.1µM digitonin, 0.5µM rotenone, 0.2mM succinate (CII leak respiration), 5mM ADP (CII linked respiration), 0.5µM Malonate (non-CII respiration), 2.5µM antimycin A (non-mitochondrial respiration). All respiration values were corrected by subtracting non-mitochondrial respiration values. CII-specific respiration was calculated by subtracting respiration values in the presence of malonate. The third protocol determining CI+CII linked respiration featured the following additions performed sequentially: 8.1µM digitonin, 5mM malonate, 2mM malate, 5mM pyruvate, 10mM glutamate (CI leak respiration), 5mM ADP (CI linked respiration), 10mM succinate (CI+CII linked respiration), 2.5µM oligomycin (CI+CII leak respiration), 0.5µM titrations of FCCP (uncoupled or maximal

respiration), and 2.5 μ M of antimycin A (non-mitochondrial respiration). Data is expressed per million cells.

2.12. siRNA mediated knockdown of mitochondrial respiratory complex subunits

500,000 unmodified HEK293t cells were plated 24 hours prior to transfection in 60mm cell culture plates, achieving ~60% seeding density at the time of transfection. 1 hour prior to transfection, the cell culture media was replaced to 10% FBS supplemented DMEM high glucose media without antibiotics. Transfection was performed with siLentFect Lipid Reagent (Bio-Rad) according to the manufacturers protocol, with optimizations as indicated. The following siRNA were individually transfected per cell plate to a final concentration of 25nM: NDUFB4 Silencer Select siRNA (ThermoFisher, 4390824, siRNA ID: s224102), NDUFB6 Silencer Select siRNA (ThermoFisher, 4392420, siRNA ID: s9366), COX6C Silencer Select siRNA (ThermoFisher, 4390824, siRNA ID: s223298), UQCRI1 Silencer Select siRNA (ThermoFisher, 4390824, siRNA ID: s132), NDUFA3 Silencer Select siRNA (ThermoFisher, 4392420, siRNA ID: s9327) and Silencer Select Negative Control 1 siRNA (ThermoFisher, 4390843). 10 μ L of siLentFect Lipid Reagent was used per 60mm cell plate. Reagents were prepared separately in Opti-MEM reduced serum medium (Invitrogen) before being combined and added dropwise onto the cells. 48 hours post transfection, cells were harvested for downstream applications.

2.13. Blue-Native PAGE (BN-PAGE) analysis of mitochondrial respiratory supercomplexes

12.13.1 Sample preparation for BN-PAGE

Samples were prepared on ice or at 4°C. Cells were pelleted in 1X PBS and were resuspended in either digitonin based solubilization buffer (50mM imidazole pH 7.0, 50mM NaCl, 5mM 6-aminocaproic acid, 1mM EDTA and 1.5% w/v digitonin) or Triton X-100 based solubilization

buffer (50mM imidazole pH 7.0, 50mM NaCl, 5mM 6-aminocaproic acid, 1mM EDTA and 1% v/v Triton X-100) in a ratio of 0.7mL per 100mg wet weight of cells. Membranous proteins were gently extracted for 30 mins with occasional pipetting. Samples were cleared at 10,000g for 30 minutes, and supernatants were combined with glycerol (5% v/v final) and 5% w/v Coomassie dye G250 solution (Bio-Rad) to achieve a final 1:4 dye:digitonin/triton ratio. Samples were held on ice until loading, or stored at -80°C and allowed to thaw on ice before immediately loading.

2.13.2 BN-PAGE immunoblotting

Two 3-13% acrylamide gradient gels (acrylamide mix: 48g acrylamide, 1.5g bis-acrylamide in 100mL H₂O, 25mM imidazole pH 7.0, 0.5M 6-aminocaproic acid, 10% APS, TEMED, H₂O, glycerol) were casted by a gradient mixer. Equal volumes of each sample were loaded and run at 4°C with anode buffer (25mM imidazole pH 7.0) and dark cathode buffer (50mM tricine, 7.5mM imidazole pH 7.0, 0.02% Coomassie Blue G250) at 150V for approximately 2.5 hours. The dark cathode buffer replaced with light cathode buffer (50mM tricine, 7.5mM imidazole pH 7.0, 0.002% Coomassie Blue G250) and the gels were continued overnight at 200V. The next day, gels were incubated in 0.1% SDS in transfer buffer for 30 minutes. Wet transfer to nitrocellulose membranes (Bio-Rad) were completed at a constant 400 milliamps for 2.5 hours. Membranes were blocked for 1 hour with 5% skim milk in TBST. Membranes were incubated with 1:2000 complex I NDUFA9 [20C11B11B11] (Invitrogen, 459100) and 1:2000 complex III Anti-UQCRC2 [13G12AF12BB11] (Abcam, ab14745) primary antibodies with 1% BSA in TBS-T overnight at 4°C with gentle agitation. 1:5000 Anti-mouse IgG HRP secondary antibody (Promega) in 5% skim milk in TBS-T was added for 1 hour at room temperature with shaking. Membranes were exposed with the SuperSignal West Pico Chemiluminescent Substrate Kit (ThermoFisher), and chemiluminescence signal was captured by the ChemiDoc™ MP Imaging System (Bio-Rad).

Membranes were then incubated with 1:2000 MTCO1 [1D6E1A8] (ThermoFisher, 459600) overnight at 4°C or 1:20000 complex II SDHA [2E3GC12FB2AE2] (Invitrogen, 459200) and 1:50000 complex V ATP5A (Abcam, ab14748) primary antibodies for 1 hour. Similar secondary antibody and exposure procedures were completed. Membranes incubated with SDHA or ATP5A were exposed with Immobilon Classico Western HRP substrate (Millipore). Densitometry analysis was performed using Image J software.

2.14. Statistical Analyses

All statistical tests were performed using GraphPad Prism 9 software. Unless stated otherwise, statistical significance between EV and HIG1 cell lines was determined using one-way ANOVA, followed by Dunnett's *post hoc* method for comparison to EV. Statistical significance for siRNA mediated knockdown experiments was performed using one-way ANOVA, followed by Dunnett's *post hoc* method for comparison to siCtrl. Statistical significance between HEK293 WT, NDUFB4-KO, B4-rescue and N24A, R30A cell lines was determined using one-way ANOVA, followed by Holm-Šidák *post hoc* method for pairwise comparisons. Data are shown as means $\pm$ SEM. Statistical significance was accepted at $p < 0.05$, with p values listed or represented with the following denominations: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. Graphs were generated using either GraphPad Prism 9 or Microsoft Excel software.

3.0 RESULTS

3.1 Chapter 1: HIG1 Protein Family Regulation of Mitochondrial Respiratory Supercomplex Formation and Function

3.1.1 HIG1 protein family stable cell line generation

The primary functions and differences between the HIG1 family proteins are not completely defined. Furthermore, the cellular functions of protein family members HIGD1B and HIGD1C have yet to be described in any capacity. In order to characterize the distinct functions of each HIG1 protein in the context of SC formation and mitochondrial function, HEK293t cell lines stably expressing each HIG1 protein were generated through retroviral transduction (**Figure 8A**). A cell line harboring the empty retroviral backbone was generated as a control. Polyclonal HIG1 family overexpression (OE) HEK293t populations with relatively similar expression were kept and further characterized (**Figure 8B**). Both lower expressing and higher expressing HIGD1B cells (1BH and 1BL) were kept for further characterization.

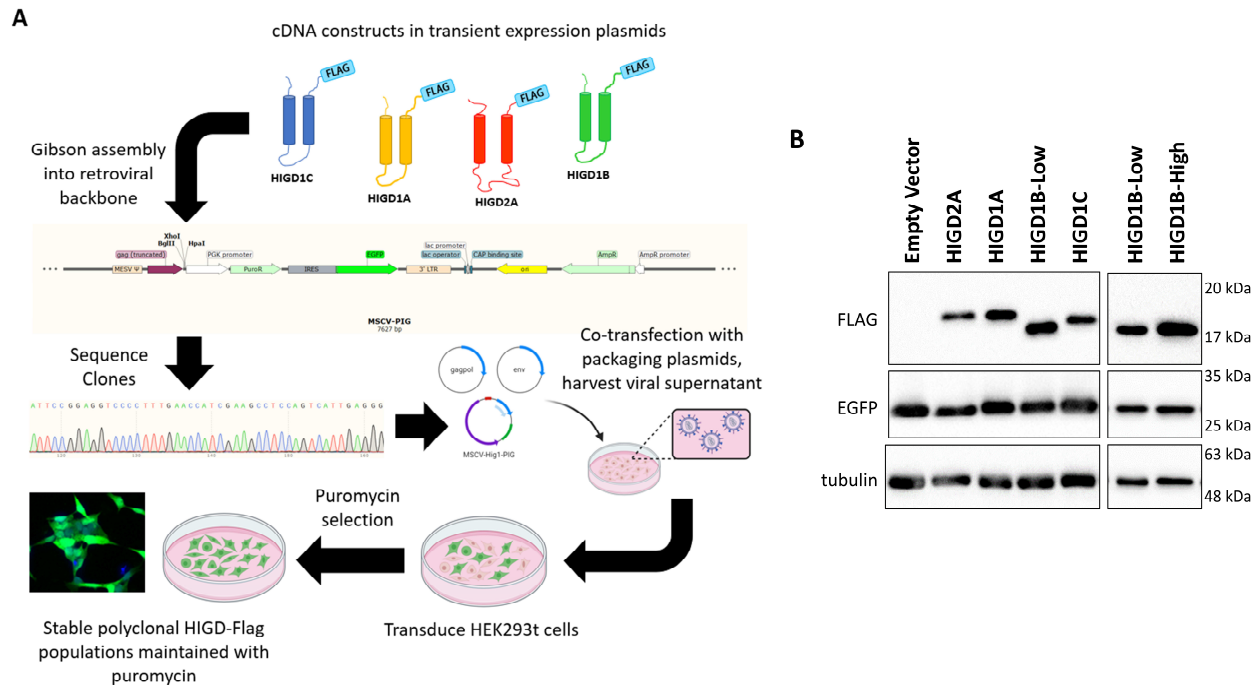


Figure 8. Generation of cell lines stably expressing each HIG1 protein family member. (A) DNA constructs in transient expression plasmids were subcloned into a retroviral vector backbone through high fidelity PCR and Gibson assembly approaches. Resulting clones were screened by sequencing, and harvested plasmid was co-transfected with retroviral packaging plasmids to generate encoding retrovirus. Transduction was performed on HEK293t cells with varying amounts of viral supernatant, followed by selection with 10 μ g/mL puromycin. Resulting cell populations were maintained in 5 μ g/mL puromycin for further analyses. **(B)** Relative expression of each FLAG-tagged HIG1 protein family cell line probed by immunoblotting.

3.1.2 HIG1 proteins co-localize with mitochondria under normoxia and hypoxia conditions

We confirmed the localization of each HIG1 protein under both a basal and stress-related condition to settle discrepancies in subcellular localization for HIGD1A and HIGD2A. To promote cellular stress, cells were incubated for 48 hours in 1% O₂ to induce hypoxic conditions and activate HIG1 protein expression and function (44). Subcellular localization of the HIG1 proteins was investigated by immunocytochemistry (ICC) using the FLAG tag, and mitochondria were visualized through ICC of the MOM protein TOM20. All HIG1 family proteins co-localize exclusively with mitochondria under both basal and hypoxia conditions (**Figure 9A-B**).

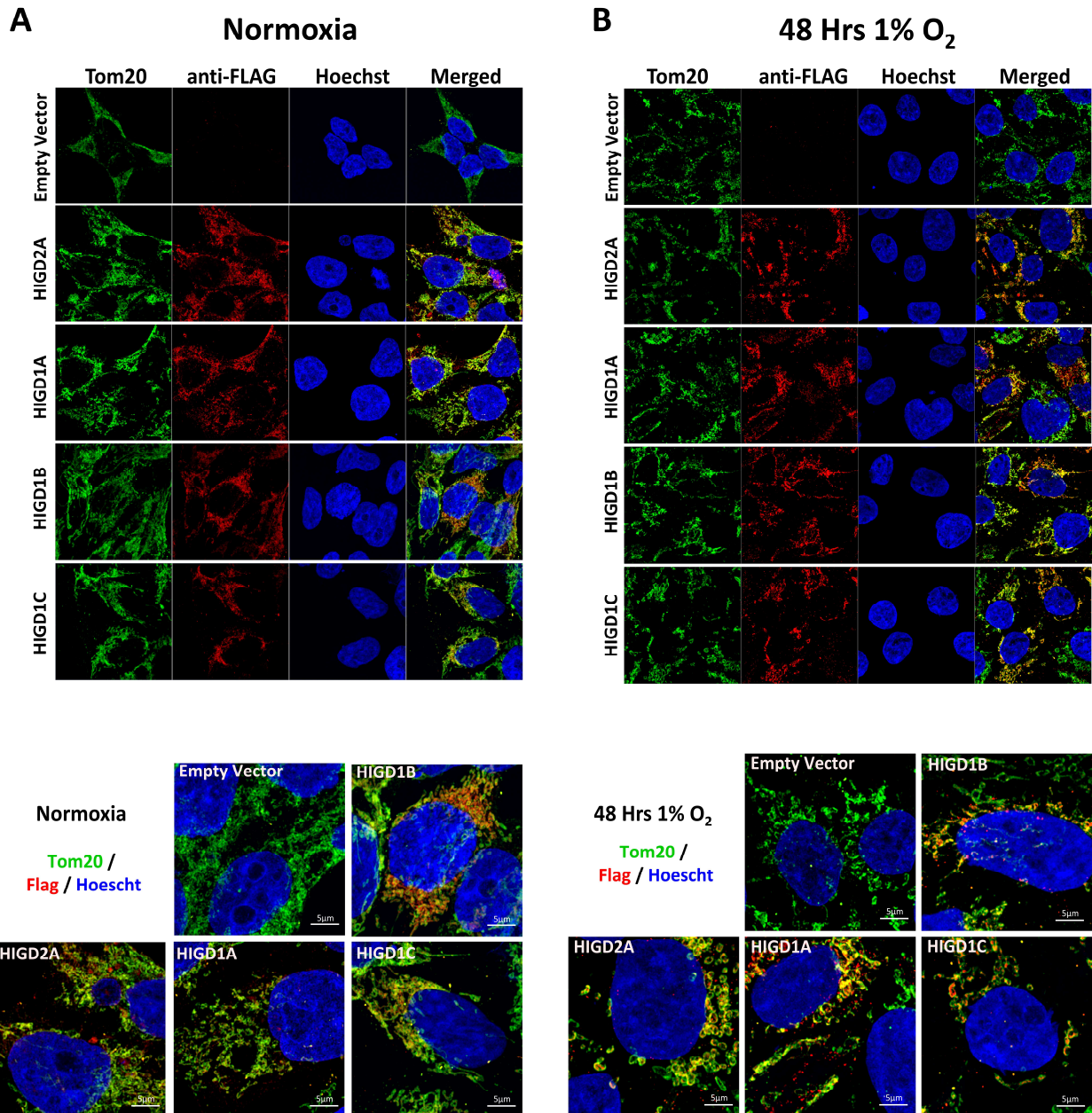


Figure 9. HIG1 family proteins co-localize with mitochondria under (A) basal and (B) long term hypoxia. HIG1 family OE HEK293t cells were plated onto coverslips prior to paraformaldehyde fixation and immunofluorescence. For hypoxia experiments, cells were placed under 1% O₂ for 48 hours prior to fixation. Flag-tagged HIG1 family proteins are shown in red, mitochondria visualized by Tom20 are shown in green, and counterstained nuclei are shown in blue. Images were acquired through a Zeiss LSM880 AxioObserver Z1 confocal microscope with AiryScan using a 63x 1.4 NA oil objective. Scale bars shown in zoomed images depict 5µm.

3.1.3 HIG1 family proteins interact with OXHPOS complexes but do not modify their activity

We next sought to further characterize the relationship between the HIG1 proteins and mitochondria. We first aimed to determine whether stable expression of the HIG1 proteins influenced the steady state levels of mitochondria. Total mitochondrial content was unaltered relative to empty vector cells, assessed using two independent measures: citrate synthase activity and the mtDNA:nDNA ratio (**Figure 10A**). We subsequently confirmed and extended upon protein-protein interactions within mitochondria that had been observed in earlier studies. HIGD2A has been previously shown to interact with CIV, while HIGD1A has been shown to interact with CIII and CIV (56). Furthermore, HIGD1A was reported to interact with the long isoform of OPA1 (L-OPA1) (57). In order to individually characterize the associations between the HIG1 proteins and these proposed targets, co-immunoprecipitation (co-IP) experiments were performed by pulling down the FLAG-tagged HIG1 proteins, followed by immunoblotting. Interestingly, OPA1 and the OXPHOS complexes I, III and IV were pulled down with all the HIG1 family members, though to varying extents (**Figure 10B**). More specifically, HIGD2A appeared to have a much weaker interaction with OPA1 than the other HIG1 family members. Furthermore, all HIG1 proteins preferentially interacted with the long isoform of OPA1. Conversely, HIGD2A strongly associated with the OXPHOS complexes CI, CIII and CIV whereas HIGD1A/1B/1C proteins did to a lesser degree.

After establishing that each HIG1 protein interacted with complexes I, III and IV by varying amounts, we assessed the effects of HIG1 overexpression on the activity of the ETC complexes. Despite interactions with the HIG1 proteins, the individual activity of each OXPHOS complex remained unmodified relative to the EV control (**Figure 10C**).

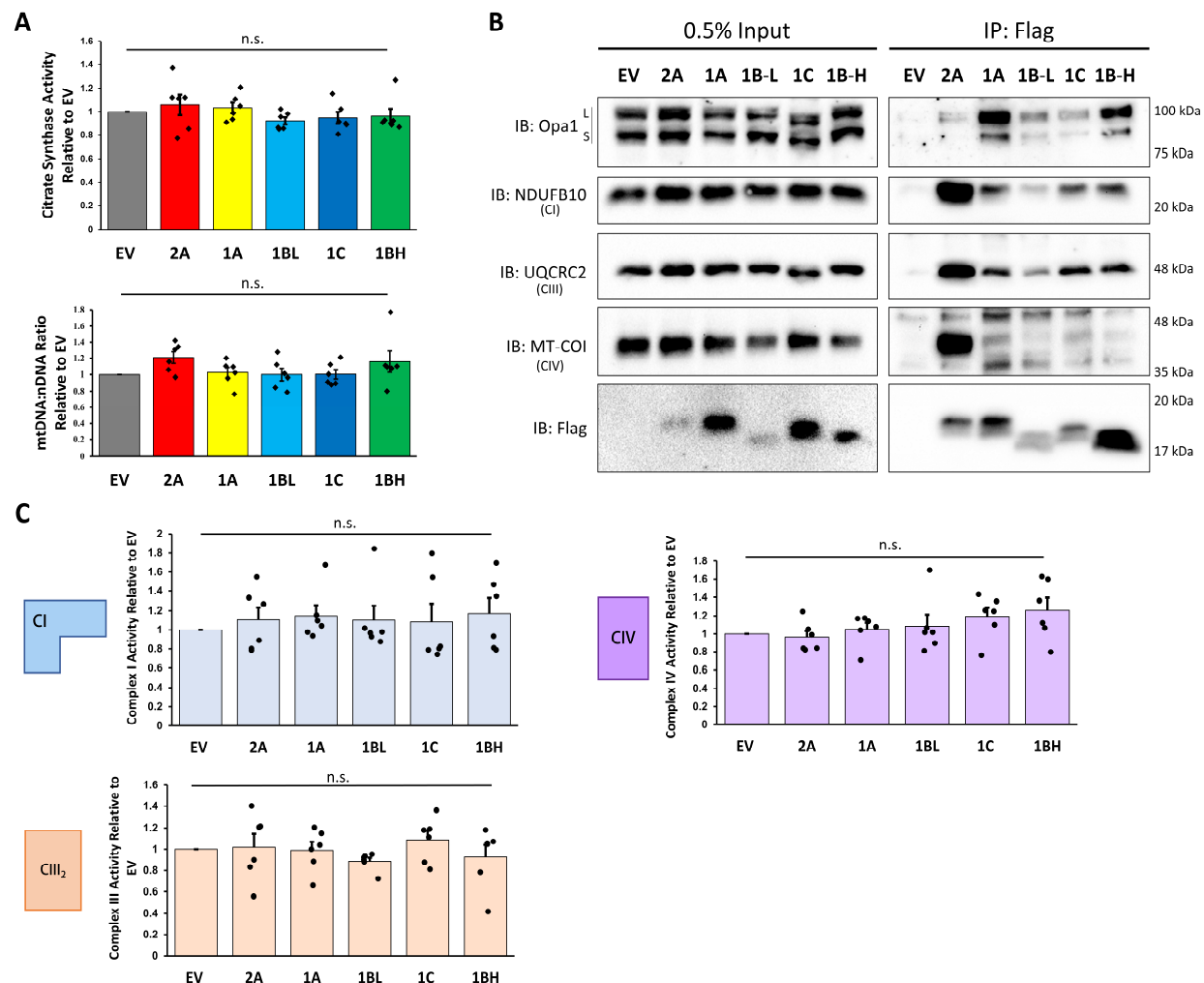


Figure 10. Interactions of HIG1 family proteins in mitochondria. (A) HIG1 family proteins do not influence total mitochondrial content. Citrate synthase activity was measured using an *in vitro* spectrophotometric assay on whole cell lysates. Activity was normalized to protein content and expressed relative to empty vector control. mtDNA levels were quantified and normalized to nDNA levels through quantitative PCR on DNA isolated by liquid-liquid extraction. Primers against MT-ND1 were used for mtDNA quantification and 18SrRNA for nDNA reference. Ratios expressed relative to empty vector control cells. Means $\pm$ SEM, n=6 biological replicates measured in triplicate for both experiments. One-way ANOVA with Dunnett's post-hoc test vs. EV. (B) HIG1 proteins pull down L-Opa1 and OXPHOS complexes I, III and IV to varying degrees. FLAG-tagged HIG1 proteins were pulled down by Anti-FLAG conjugated magnetic beads and interacting proteins were probed by immunoblotting. (C) HIG1 proteins do not modify the activity of OXPHOS complexes I, III and IV. *In vitro* spectrophotometric assays were performed on whole cell lysates. Activity was normalized to protein content and expressed relative to empty vector control. Means $\pm$ SEM, n=6 biological replicates measured in triplicate. One-way ANOVA with Dunnett's post-hoc test vs. EV.

3.1.4 Proteins regulating mitochondrial dynamics are altered in higher expressing HIGD1B OE cells during hypoxia.

Given the observed interactions between L-OPA1 and the HIG1 proteins, we sought to determine whether stable expression of the HIG1 proteins influenced mitochondrial dynamics under basal and hypoxia conditions. First, mitochondrial network morphology was characterized quantitatively through automated analyses using the program Momito (68). Long term hypoxic conditions depress mitochondrial function and evoke fragmentation of the mitochondrial network, which can be visually observed (**Figure 11A-B**). Distributions of mitochondrial length are unaltered under basal conditions in the stable cells, however during hypoxia conditions the higher expressing HIGD1B cells (1BH) displayed trending increases to mitochondrial length ($p=0.0505$ for $<1.0\mu\text{m}$, $p=0.1037$ for $<1.0\mu\text{m}$, $p=0.0549$ for $>3.0\mu\text{m}$) (**Figure 11C-D**). Parameters of mitochondrial network connectivity, the number of elements per cluster (E_c) and the number of junctions over ends (J/E), demonstrate that overall connectivity is repressed under hypoxic conditions, though no differences to EV are observed (**Figure 11E-F**).

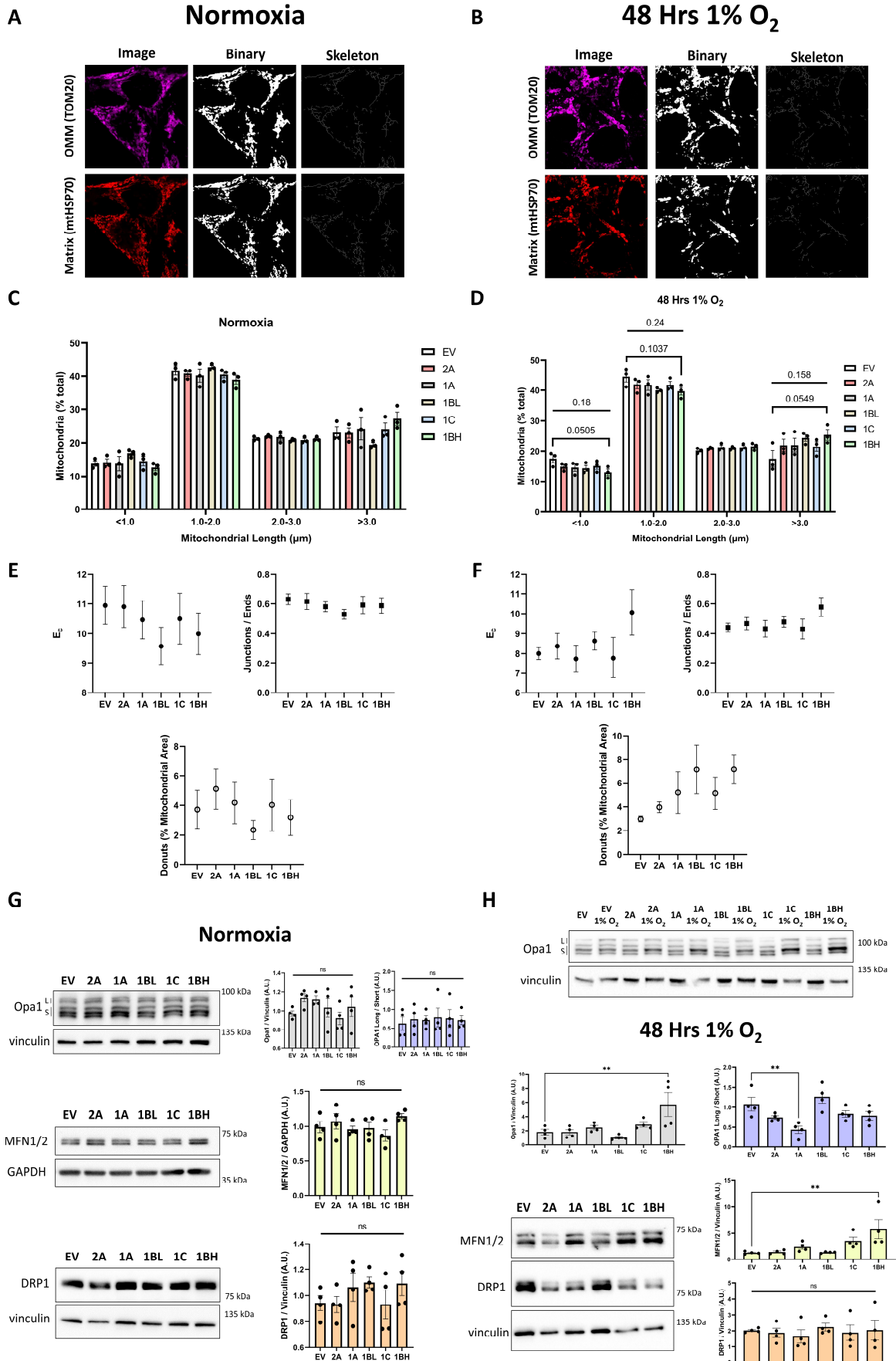


Figure 11. HIG1 stable cell mitochondrial network morphology and dynamics regulating proteins under basal and long-term hypoxia. (A and B) HEK293t cells stably expressing each HIG1 family protein were plated onto coverslips and fixed by paraformaldehyde under (A) normoxia or (B) 1% O₂ for 48 hours. Images were processed and analysed using Momito software. (C and D) Average mitochondrial length distributions under (C) normoxia or (D) 48 hours 1% O₂ conditions. Means±SEM for 3 independent experiments under each condition. One-way ANOVA with Dunnett's post-hoc test vs. EV. (E and F) Network morphology parameters under (E) normoxia or (F) 48 hours 1% O₂ conditions. Elements per cluster (E_c) and junctions over ends (J/E) are measurements of connectivity, while donuts represent the proportion of mitochondria fused with itself. Means±SEM for 3 independent experiments under each condition. (G and H) Immunoblot analyses of mitochondrial dynamics regulating proteins under (G) normoxia or (H) 48 hours 1% O₂. Means±SEM, n=4 biological replicates. One-way ANOVA with Dunnett's post-hoc test vs. EV. ** = p<0.01.

Cellular stress can evoke mitochondrial donut formation. Donut formation is a result of a mitochondrial network branch fusing with itself, and could represent a protective measure from stress-induced mitophagy (14). While under basal conditions there were no differences in mitochondrial donut formation between the HIG1 and empty vector cells, 1BH cells displayed increased donut formation relative to EV cells under long-term hypoxia, though only by pairwise comparison (p=0.02, unpaired 2-tailed student's t-test) (**Figure 11E-F**).

Lastly, the expression of proteins regulating mitochondrial dynamics were examined under basal and long-term hypoxia conditions. Though levels of proteins regulating mitochondrial dynamics appear unchanged under basal conditions, total levels of fusion proteins OPA1 and MFN1/2 are increased in the 1BH cell line during hypoxia (**Figure 11G-H**). Interestingly, the balance of OPA1 isoforms shifted towards the short form exclusively in HIGD1A stable cells during hypoxia.

3.1.5 Stable expression of some HIG1 proteins may alter the p53-parkin axis during hypoxia

HIGD1A expression during hypoxia is associated with decreased cell death and apoptotic signalling (44–47). Moreover, endogenous HIGD1A expression is also observed physiologically in tumor xenografts of glioma, breast cancer, and pancreatic cancer cells (48–50). In order to

investigate whether stable expression of the HIG1 proteins altered cell death signalling and enhanced survival in our cell model, we first performed a long-term hypoxia survival assay. Interestingly, none of the HIG1 stable cells demonstrated enhanced survival during the 72-hour hypoxia challenge (**Figure 12A**). we next questioned whether any hypoxia related cell death/survival signalling could be altered, as we suspected that perhaps the combinatory effects of both enhanced cell survival and anti-apoptotic signalling could have been unique to the previous models in literature. Immunoblotting of the canonical tumor suppressor gene p53 revealed a trending reduction in p53 levels during hypoxia in some HIGD1A/1B/1C subfamily cell lines ($p=0.0616$ for HIGD1C, $p=0.125$ for HIGD1A, one-way ANOVA vs. EV) (**Figure 12B**).

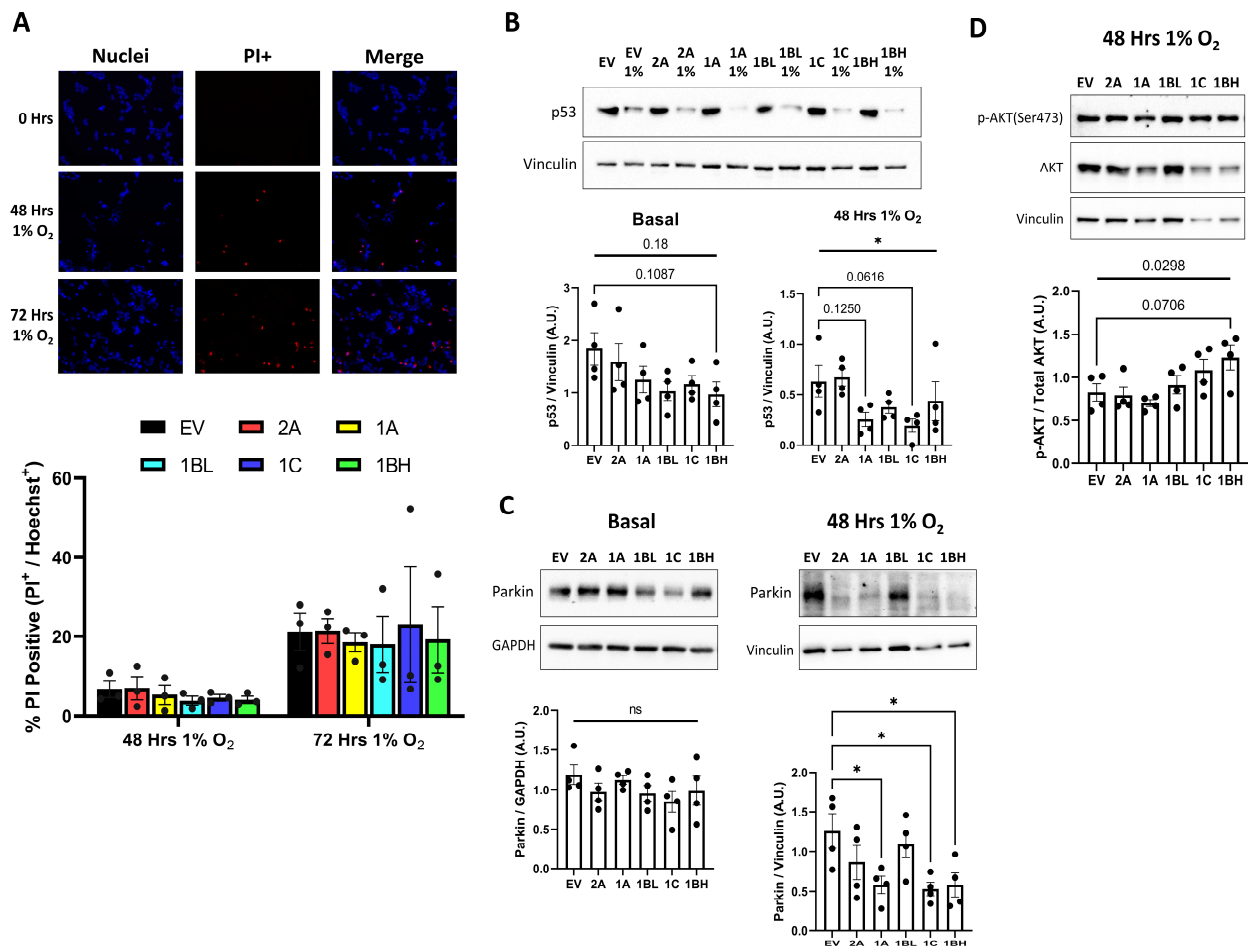


Figure 12. HIG1 protein family involvement in cell death/survival and related signalling during hypoxia. (A) Long-term hypoxia survival assay. HIG1 stably expressing and empty vector

cells were stained with Hoechst and PI dyes and placed in 1% O₂ for up to 72 hours. The percentage of dead cells is reported by the number of dead cells (PI⁺) normalized to the number of total cells (Hoechst⁺) in view. Images were acquired using EVOS FL auto placed in the hypoxia chamber, using a 10X objective. Means±SEM for 3 independent experiments. **(B-D)** Immunoblotting of **(B)** p53 during normoxia and 48 hours 1% O₂, **(C)** parkin during normoxia and 48 hours 1% O₂, and **(D)** phospho-AKT (Ser473) and total AKT during 48 hours 1% O₂. Means±SEM, n=4 biological replicates for each condition and target. One-way ANOVA with Dunnett's post-hoc test vs. EV. * = p < 0.05.

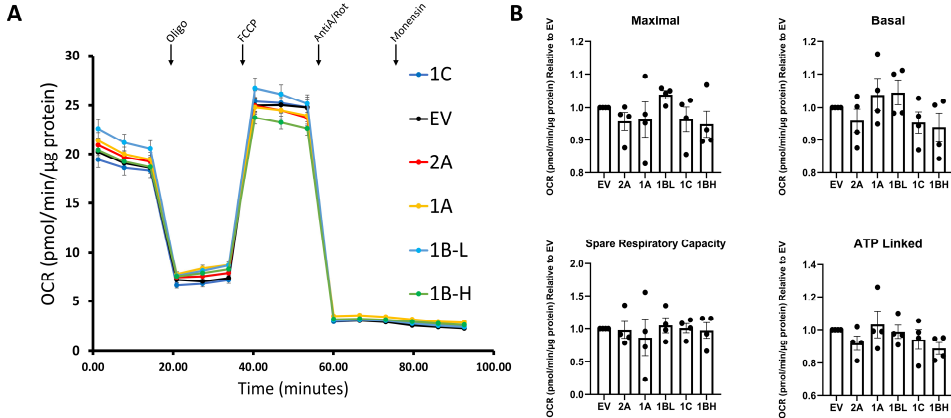
Next, we sought to find the altered signalling axis involving p53 repression in the HIGD1A/1B/1C subfamily cells. The parkin gene is a well-established transcriptional target of p53 (70), which promotes autophagic clearance of dysfunctional mitochondria (13). Remarkably, parkin levels were decreased in the HIGD1A/1B/1C subfamily cell lines during hypoxia (**Figure 12C**). We suspected the action of actors upstream of p53, such as AKT, were leading to its repression (71, 72). Immunoblotting of total and phosphorylated AKT forms revealed a trending increase in its activation, though only in the higher expressing HIGD1B cell line (p=0.0706) (**Figure 12D**).

3.1.6 Stable expression of the HIG1 proteins do not alter mitochondrial respiratory function

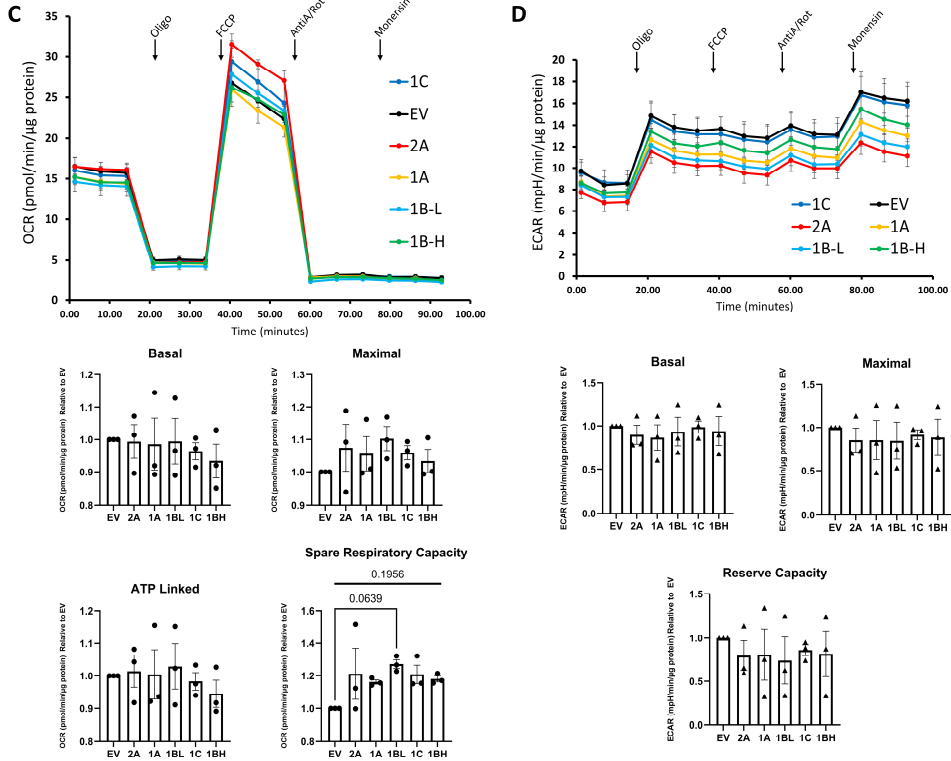
The HIG1 family proteins are thought to confer survival under stress conditions by maintaining or enhancing mitochondrial function. Studies involving HIGD1A/2A knockout report defects in mitochondrial respiration (52, 56), while conversely ectopic HIGD1A expression in CI-damaged cells can partially recover respiration (47). To determine whether the HIG1 family proteins directly modify mitochondrial respiratory function in a non-challenged setting, we performed Seahorse extracellular flux (XF) analyses to measure changes in respiratory rate and capacity (**Figure 13A**). Surprisingly, no changes in resting or maximal respiration were observed (**Figure 13B**). Next, we assessed whether mitochondrial respiration and capacity was modified under a stress condition. Pre-treatment of 24 hours at 1% O₂ was able to decrease resting mitochondrial respiration

compared to non-challenging conditions, however respiratory parameters relative to EV were largely unchanged (**Figure 13C**). The glycolytic capacity of the HIG1-transduced cells was also similarly unaffected compared to EV cells (**Figure 13D**). As differences in mitochondrial function may be more subtle than the overall respiratory parameters obtained by Seahorse, we performed high resolution respirometry (HRR) on permeabilized cells to directly stimulate mitochondria. HRR and simultaneous measurements of mitochondrial membrane potential revealed no changes to ETC pathways (**Figure 13E-F**).

Normoxia



24 Hrs 1% O₂



Normoxia

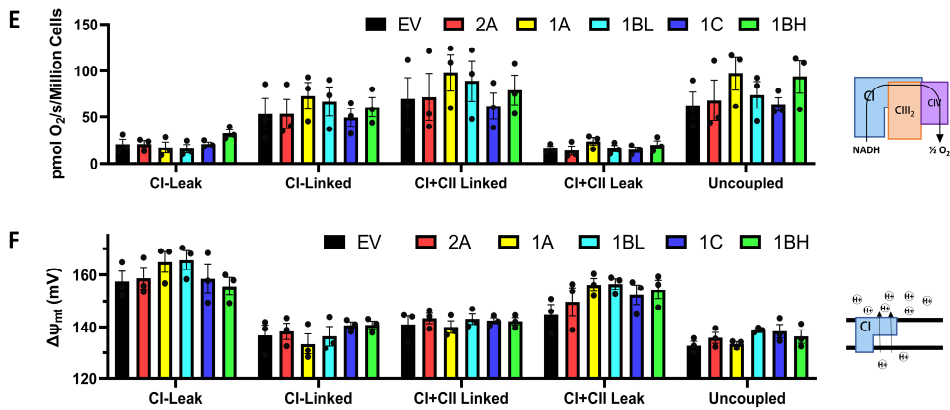


Figure 13. Stable expression of the HIG1 proteins do not alter mitochondrial respiratory function. (A) Approximately 30,000 cells were plated per well 24 hours prior to Seahorse XFe96 assay. A mito stress test protocol with monensin was followed: 1.5 μ M oligomycin, 1 μ M FCCP, 1.5 μ M antimycin-A + rotenone, 6.5 μ M monensin. Data normalized to μ g total protein per well using the BCA method following the assay. One representative experiment shown. (B) Basal respiration, maximal respiration, ATP-linked respiration and spare respiratory capacity of each HIG1 stable cell line following standard culturing conditions. Data presented relative to empty vector control. Means $\pm$ SEM, n =4 independent experiments. One-way analysis of variance with Dunnett's post-hoc test. (C) OCR and (D) ECAR parameters of HIG1 stable cells following 24 hours 1% O₂ pre-treatment. One representative experiment shown Means $\pm$ SEM, n =3 independent experiments. One-way analysis of variance with Dunnett's post-hoc test. (E) High resolution respirometry of digitonin-permeabilized HIG1 stable cells. Data presented normalized per million cells. (F) Simultaneous membrane potential ($\Delta\psi_{mt}$) acquired during HRR through TMRM fluorescence. TMRM fluorescence signal intensity is converted to $\Delta\psi_{mt}$ expressed in mV. HRR and $\Delta\psi_{mt}$ presented as means $\pm$ SEM, n =3 independent experiments. One-way analysis of variance with Dunnett's post-hoc test.

3.1.7 Stable expression of the HIGD1C alters complex IV supercomplex formation at steady state

The roles of HIGD1A and HIGD2A in CIII and CIV biogenesis and SC assembly have recently been elucidated. Knockout studies have confirmed HIGD1A as a late assembly factor of CIII₂, while HIGD2A is an assembly factor of CIV. Furthermore, these studies show the importance of HIGD1A and HIGD2A in mediating incorporation of CIII₂ and CIV into SCs (52, 56). To further explore whether other HIG1 family proteins regulated CIII or CIV related SC assembly, we probed the steady state levels of the ETC complexes and SCs in the stable HIG1 cell lines through BN-PAGE and immunoblotting (**Figure 14A**). Surprisingly, CIV related SCs corresponding to the respirasome were decreased in the HIGD1C cell line, with a trending decrease in CI SCs (**Figure 14B**). Analysis of CIV SCs in ratio to CIV monomer confirmed that CIV SC assembly is impaired following HIGD1C OE (**Figure 14C**). Immunoblotting of the FLAG-tagged HIG1 proteins revealed accumulation into a mid-sized complex, slightly larger than monomeric CIV but well below the III₂IV₁ SC. (**Figure 14D**). Interestingly, the HIGD1C and HIGD1B cell lines displayed greater immunoreactive signal compared to HIGD2A, while HIGD1A showed almost none.

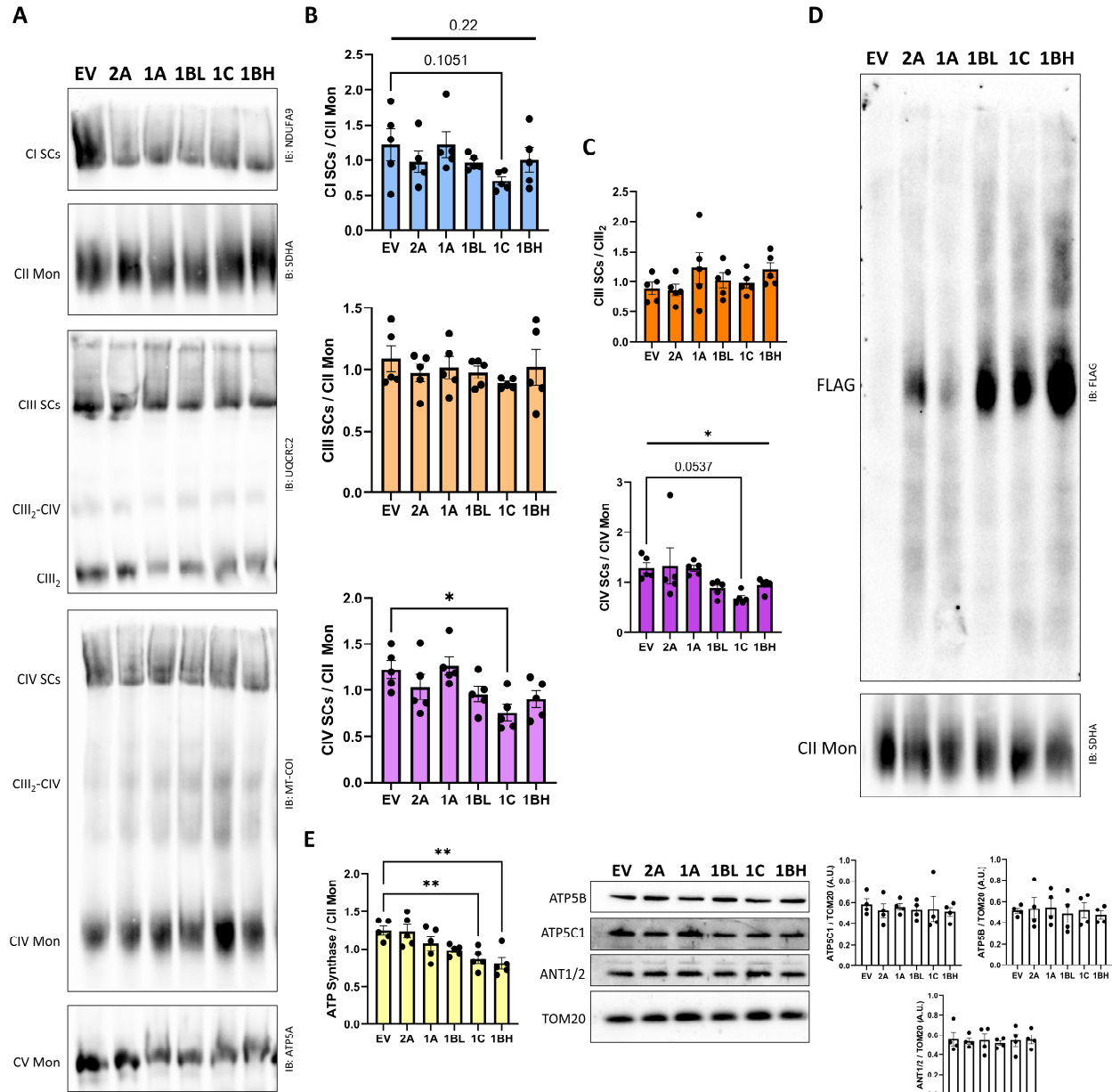


Figure 14. Steady state levels of mitochondrial respiratory supercomplexes in cells stably expressing HIG1 proteins. (A) HIG1 family OE HEK293t cells were solubilized with 1.5% vol/wt digitonin, followed by BN-PAGE and immunoblotting with the indicated primary antibodies. (B) Relative intensities of complex I, III and IV related SCs presented relative to complex II monomer. (C) Complex III and IV SCs as a ratio to dimer / monomer forms. Means $\pm$ SEM, n=5. One-way ANOVA with Dunnett's post-hoc test vs. EV. * = $p < 0.05$. (D) FLAG-tagged HIG1 family proteins resolve by BN-PAGE into a mid-sized complex slightly larger than CIV. (E) ATP synthase levels are decreased in the HIGD1C and higher expressing HIGD1B cell lines, but are not attributable to ATP5B/ATP5C subunit levels. ANT1/2 levels are not compensating as a result of CV decreases. Analysis of immunoblotting presented as means $\pm$ SEM, n=4 biological replicates. One-way ANOVA with Dunnett's post-hoc test vs. EV. * = $p < 0.05$, ** = $p < 0.01$.

ATP synthase levels were decreased in the HIGD1C and higher expressing HIGD1B cell lines (**Figure 14E**). Immunoblotting of two ATP synthase subunit levels were probed to investigate whether decreases in the complex could be attributed to the comprising subunits, however no changes to these subunits were found. Moreover, protein levels of the adenine nucleotide translocator (ANT1/2), which mediates ADP and ATP exchange across the MIM (73), remain unaltered.

3.2 Chapter 2: A New Role for ETC Accessory Subunits in Regulating Respirasome Assembly

3.2.1 Redistribution of respiratory supercomplexes following silencing of ETC accessory subunits

The outstanding lack of novel protein factors regulating respirasome assembly prompted us to speculate about protein factors within the ETC. The individual complexes comprising the respirasome must harbor key protein-protein interactions to maintain its structure. We hypothesized that respirasome formation was mediated by select accessory subunits of CI, and that these CI subunits harbored key protein-protein interactions with subunits of CIII and CIV. Analysis of recent $I_1III_2IV_1$ crystal structures (23) and proteomic datasets (65) led us to speculate that CI accessory subunits NDUF B4 and NDUF B6 were involved in respirasome formation. An important issue encountered when considering CI subunits as assembly factors is that although they are not directly involved in catalysis, they may assist in the stabilization and assembly of the individual complexes (62). As an internal control, we also identified accessory subunit NDUF A3—an accessory CI subunit which we hypothesized to not be directly involved in respirasome assembly. NDUF A3-KO cells do not contain signatures of monomeric CI unlike NDUF B4 and NDUF B6 (62). Crystal structure analysis shows that NDUF A3 is not positioned near interfaces with the other complexes (23), and cryo-slicing-BN-PAGE-mass spectrometry datasets show that

NDUFA3 is not enriched in ETC SCs (65). CIII subunit UQCRI1 and CIV subunit COX6C were also identified in potentially mediating respirasome formation using crystal structure data (23) and cryo-slicing-BN-PAGE-mass spectrometry datasets (65). To investigate the extent to which each subunit plays a role in individual complex stability vs respirasome formation, we performed siRNA mediated knockdown (siKD) of select candidate subunits in HEK293t cells to determine the transient effects of individual subunit removal (**Figure 15A**).

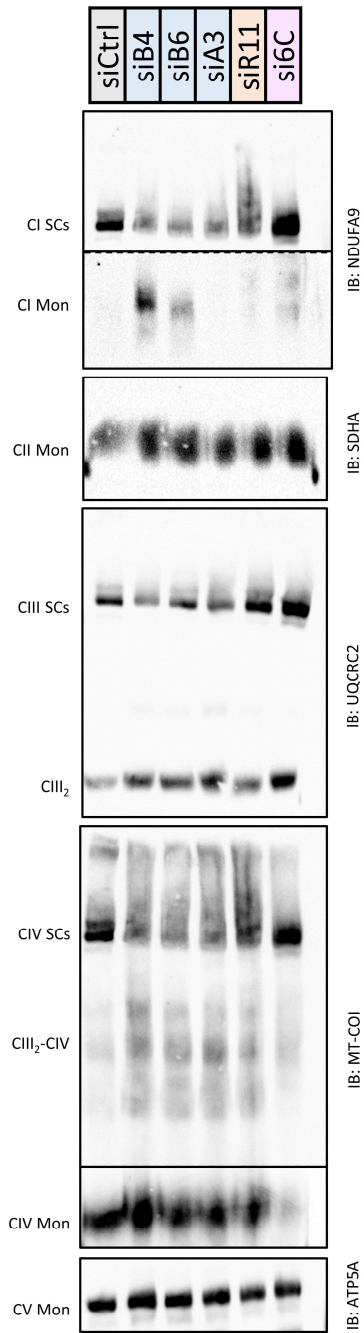
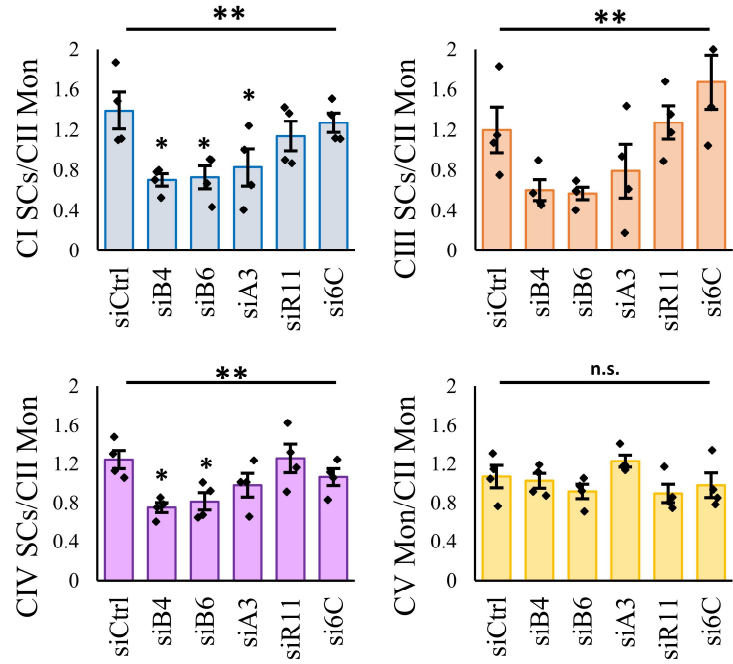
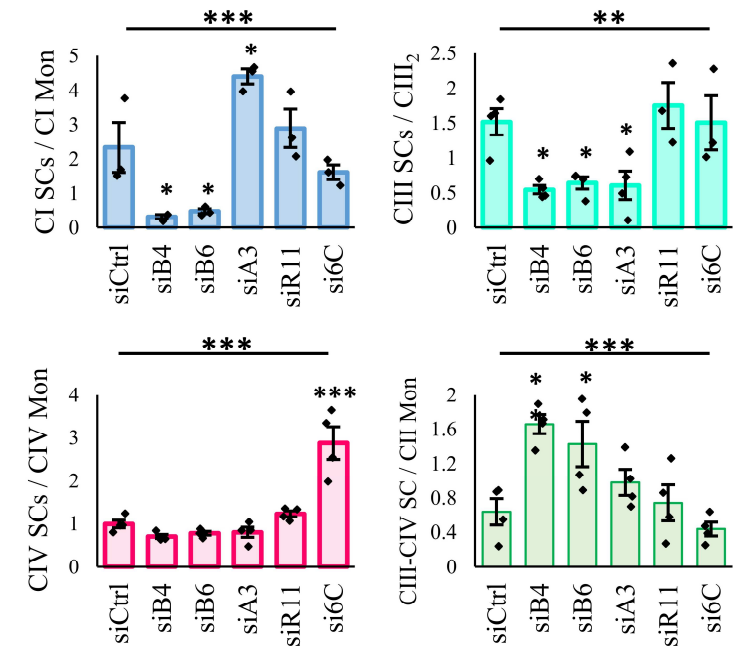
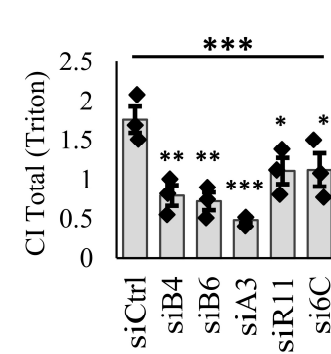
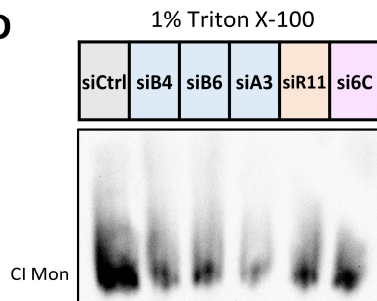
A**B****C****D**

Figure 15. Redistribution of ETC SCs following transient siRNA mediated knockdown of ETC accessory subunits. (A) HEK293t cells were transfected siRNA for 48 hours prior to solubilization with 1.5% vol/wt digitonin, separation by BN-PAGE and immunoblotting with the indicated primary antibodies. siCtrl = negative control siRNA, siB4 = siRNA against CI subunit NDUFB4, siB6 = siRNA against CI subunit NDUFB6, siA3 = siRNA against CI subunit NDUF3, siR11 = siRNA against CIII subunit UQCRC1, si6C = siRNA against CIV subunit COX6C. (B) Intensities of complex V and complex I, III and IV related SC relative to complex II. (C) Complex I, III, and IV SCs as a ratio to monomeric forms, and CIII-CIV SC levels relative to CII. Means $\pm$ SEM from 4 independent experiments. One-way ANOVA with Dunnett's post-hoc test, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ vs. siCtrl. (D) HEK293t cells were transfected siRNA for 48 hours with prior to solubilization with 1% vol/wt triton X-100 detergent to disrupt all interactions between ETC complexes. Immunoblotting of whole CI complex following BN-PAGE from three independent experiments. Relative normalized intensities presented as Means $\pm$ SEM, $n = 3$. One-way ANOVA with Dunnett's post-hoc test, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ vs. siCtrl.

Knockdown of CI subunits NDUFB4 and NDUFB6 decreased both CI and CIV SCs levels, while only CI SCs were decreased when silencing NDUF3 (Figure 15B). In order to account for the effects of CI degradation, the ratio of SCs to their monomeric forms can be analyzed since disassembly of SCs should theoretically follow with redistribution into the individual complexes. siKD of NDUFB4 and NDUFB6 show disassembly of the respirasome and redistribution into the monomeric complexes or other SCs (Figure 15C). Knockdown of NDUFB4 and NDUFB6 cause the enhanced appearance of a lower CI immunopositive band likely corresponding to a monomeric or N-module deficient CI form, while siKD of NDUF3 further diminishes this pre-SC form. Decreased CI monomer increases the CI SCs/monomer ratio for NDUF3, which is in stark contrast to NDUFB4/B6 (Figure 15C). Moreover, the intermediate III₂IV₁ SC is upregulated during siKD of NDUFB4 and NDUFB6, but not NDUF3 (Figure 15C). Surprisingly, knockdown of COX6C increased the ratio of CIV SCs to its monomer form. In other words, monomeric CIV is likely specifically destabilized upon knockdown of COX6C.

The stability of CI following siKD of each subunit was determined by probing total CI levels. Knockdown of each subunit decreased total CI levels to varying extents (**Figure 15D**). Interestingly, siKD of the two non-CI subunits, UQCR11 and COX6C, also decreased total CI levels.

3.2.2 N24A and R30A mutations of CI accessory subunit NDUFB4 modify mitochondrial bioenergetics

In order to better separate the effects of CI disassembly from ETC SC formation, an alternate approach from transient subunit knockdown was required. In-depth analyses of respirasome crystal structures show that certain residues of the CI accessory subunit NDUFB4, Asn24 and Arg30, may hydrogen bond with residues of subunit UQCRC1 of CIII (**Figure 16A**). Modification at these residues may alter CI-CIII₂ superassembly specifically while maintaining CI integrity. HEK293 CRISPR/Cas9 NDUFB4-KO cells were rescued with stable expression of either WT NDUFB4 or NDUFB4 with N24A and R30A substitutions (**Figure 16B-C**). As a control, NDUFB4-KO cells and HEK293 WT cells were supplied with empty vector.

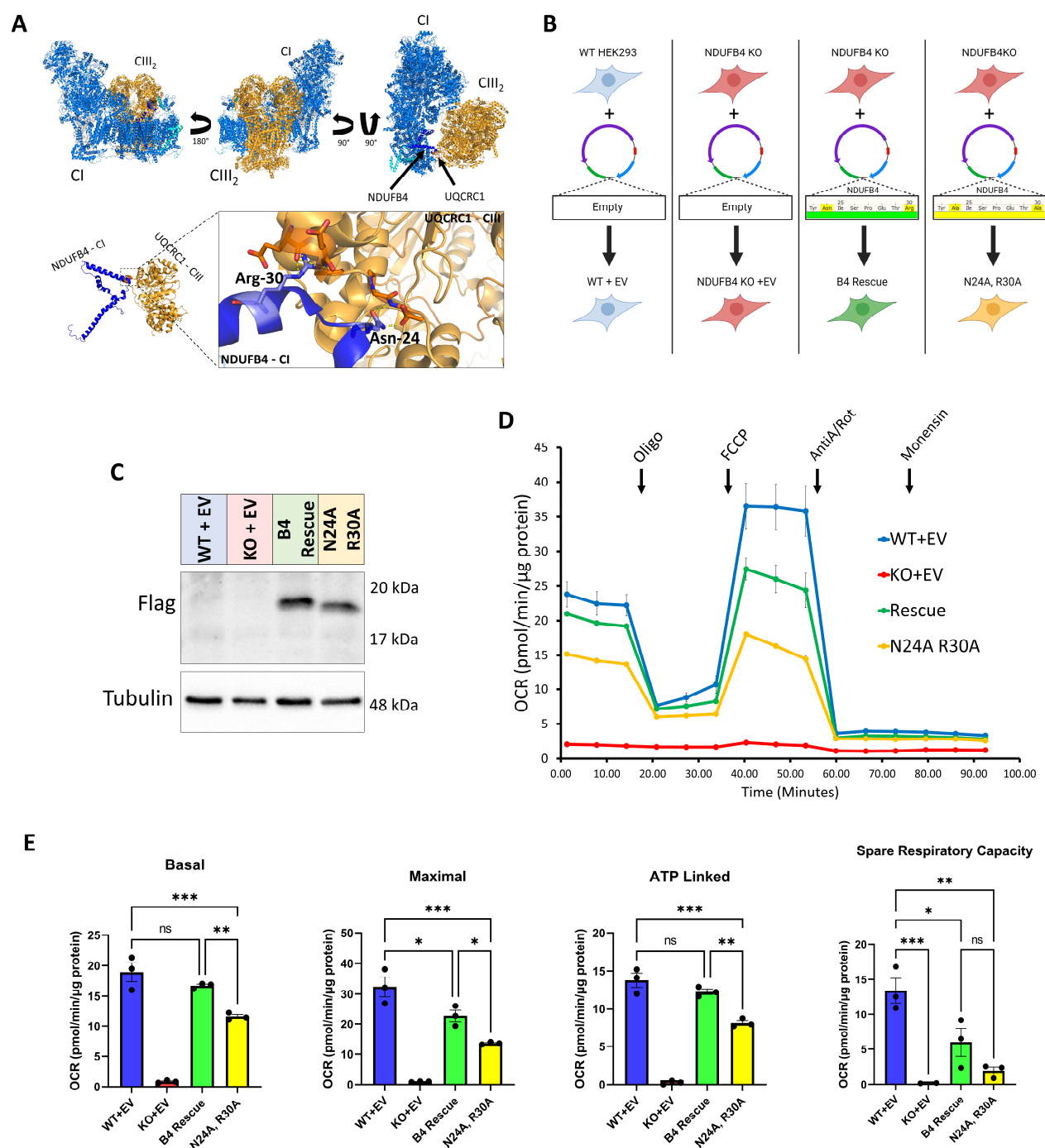


Figure 16. Generation of stable NDUFB4 rescue and N24A, R30A mutated cell lines to modulate respirasome formation. (A) In-depth analyses of respirasome crystal structure (PDB: 5XTH) identifies NDUFB4 residues N24A and R30A potentially conferring CI-CIII₂ super assembly. (B) Schematic of stable cell lines generated through retroviral transduction, restoring NDUFB4 or a mutated copy with N24A and R30A substitutions. (C) Immunoblot of generated cell lines. Similar expression of WT NDUFB4 or N24A, R30A subunits are detected in the rescue cell lines. (D) Approximately 40,000 cells were plated per well 24 hours prior to a Seahorse XFe96

assay. A Mito stress test protocol with monensin was followed: 1.5 μ M oligomycin, 1 μ M FCCP, 1.5 μ M antimycin-A + rotenone, 6.5 μ M monensin. Data normalized to μ g total protein per well using the BCA method following the assay. Mean values $\pm$ SEM presented for 3 independent experiments. (C) Basal respiration, maximal respiration, ATP-linked respiration and spare respiratory capacity values from Seahorse assays. Means $\pm$ SEM, n =3 independent experiments. One-way ANOVA with Holm-Šídák post-hoc test, *** = p<0.001, ** = p <0.01, * = p <0.05.

To verify the rescue of respiration, NDUFB4-KO cells stably expressing WT NDUFB4 (titled B4 rescue) or NDUFB4 with N24A and R30A substitutions (titled N24A, R30A) were probed through Seahorse XF analyses. B4 rescue cells displayed basal (resting) and leak respiration similar to WT+EV cells, indicating a restoration of OXPHOS function (**Figure 16D-E**). Maximal (uncoupled) respiration was reduced in the rescue cells compared to WT+EV, though this may be attributable to the differences in cell origins: previous long-term genetic ablation of NDUFB4, and previous long-term loss of mitochondrial respiratory function in B4 rescue cells.

Remarkably, the N24A, R30A cells display markedly decreased resting and uncoupled respiration compared to the B4 rescue (**Figure 16D-E**). Due to similarities in leak respiration, ATP-linked respiration was decreased in N24A, R30A compared to B4 rescue. In contrast, spare respiratory capacity (maximal – basal) was not significantly altered.

3.2.3 N24A and R30A mutations of CI accessory subunit NDUFB4 modify respirasome formation

Next, changes to respirasome formation were determined through BN-PAGE, followed by immunoblotting. Compared to NDUFB4-KO cells, B4 rescue cells display appreciable reassembly of the respirasome, apparent through the restoration of CI, CIII and CIV containing SCs (**Figure 17A-B**). In contrast, N24A, R30A cells displayed impaired respirasome formation as evidenced by the distinct decreases in CI, CIII and CIV SCs. Moreover, N24A, R30A cells feature a unique, high molecular weight CI-immunopositive band just below CI SCs. This complex is likely a

functional, monomeric form of CI, indicating that the lower molecular weight CI complexes that are present in KO, B4 rescue and N24A, R30A cells correspond to non-functional precursor CI forms.

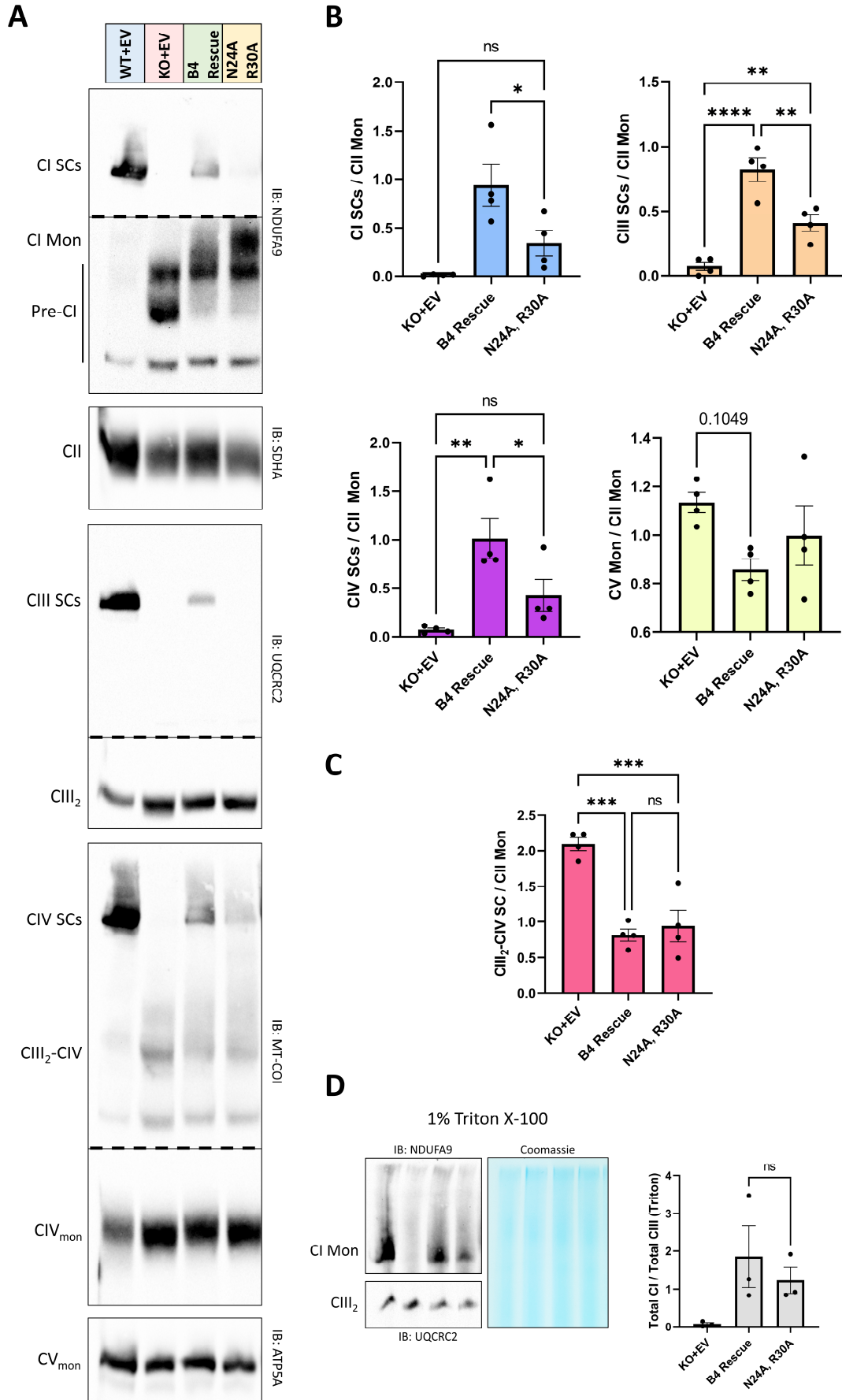


Figure 17. N24A and R30A substitutions to CI accessory subunit NDUFB4 impair assembly of the respirasome. (A) WT or NDUFB4-KO HEK293 cells stably expressing empty vector, NDUFB4 WT, or NDUFB4 with N24A and R30A substitutions were solubilized with 1.5% vol/wt digitonin, separated by BN-PAGE and immunoblotted with the indicated primary antibodies. Representative blots shown. (B) Intensities of complex V and complex I, III and IV related SC relative to complex II and (C) III₂IV₁ SC levels relative to CII. Means $\pm$ SEM, n=4. One-way ANOVA with Holm-Šídák post-hoc test, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. (D) Solubilization with 1% vol/vol triton X-100 followed by BN-PAGE and immunoblotting with the indicated antibodies. Coomassie stain shown to verify loading. Intensities presented as Means $\pm$ SEM, n = 3. One-way ANOVA with Holm-Šídák post-hoc test, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

Disassembly of the respirasome by the removal of steady state CI resulted in a redistribution of SCs into monomeric forms, and increased III₂IV₁, which was visually apparent in NDUFB4-KO cells. Interestingly, rescue of either WT NDUFB4 or the N24A R30A mutant decreases III₂IV₁ SC levels (**Figure 17C**).

The stability of CI was determined in these rescue models by probing total CI levels through solubilization using Triton X-100. Both B4 rescue and N24A R30A show appreciable recovery of CI compared to NDUFB4-KO cells (**Figure 17D**). No difference in total CI levels is observed between the B4 rescue and N24A R30A cell lines.

3.2.4 N24A and R30A mutations of CI accessory subunit NDUFB4 alter CI vs CII linked electron transfer pathways during mitochondrial respiration

OXPHOS is classically driven by two major electron pathways: CI-linked or N-linked respiration which begins with NADH oxidation at CI, and CII-linked or S-linked respiration which begins with succinate oxidation at CII. An emerging but unconfirmed theory is that mitochondrial respiratory supercomplexes fine tune activities of these electron transfer pathways. Assembly of the respirasome would likely favor the activity of the CI-linked pathway, whereas respirasome disassembly would free CIII and CIV complexes and allow for their participation in the CII-linked respiration pathway. CI and CII linked pathways were independently stimulated through HRR on

B4 rescue cells and N24A, R30A cells (**Figure 18A**). The ratio of CII to CI linked OXPHOS is increased in N24A, R30A cells compared to B4 rescue cells (**Figure 18B**). In contrast to mitochondrial respiration analyses by Seahorse, CI + CII linked stimulation showed no differences in uncoupled respiration between B4 rescue and N24A, R30A cells (**Figure 18C**).

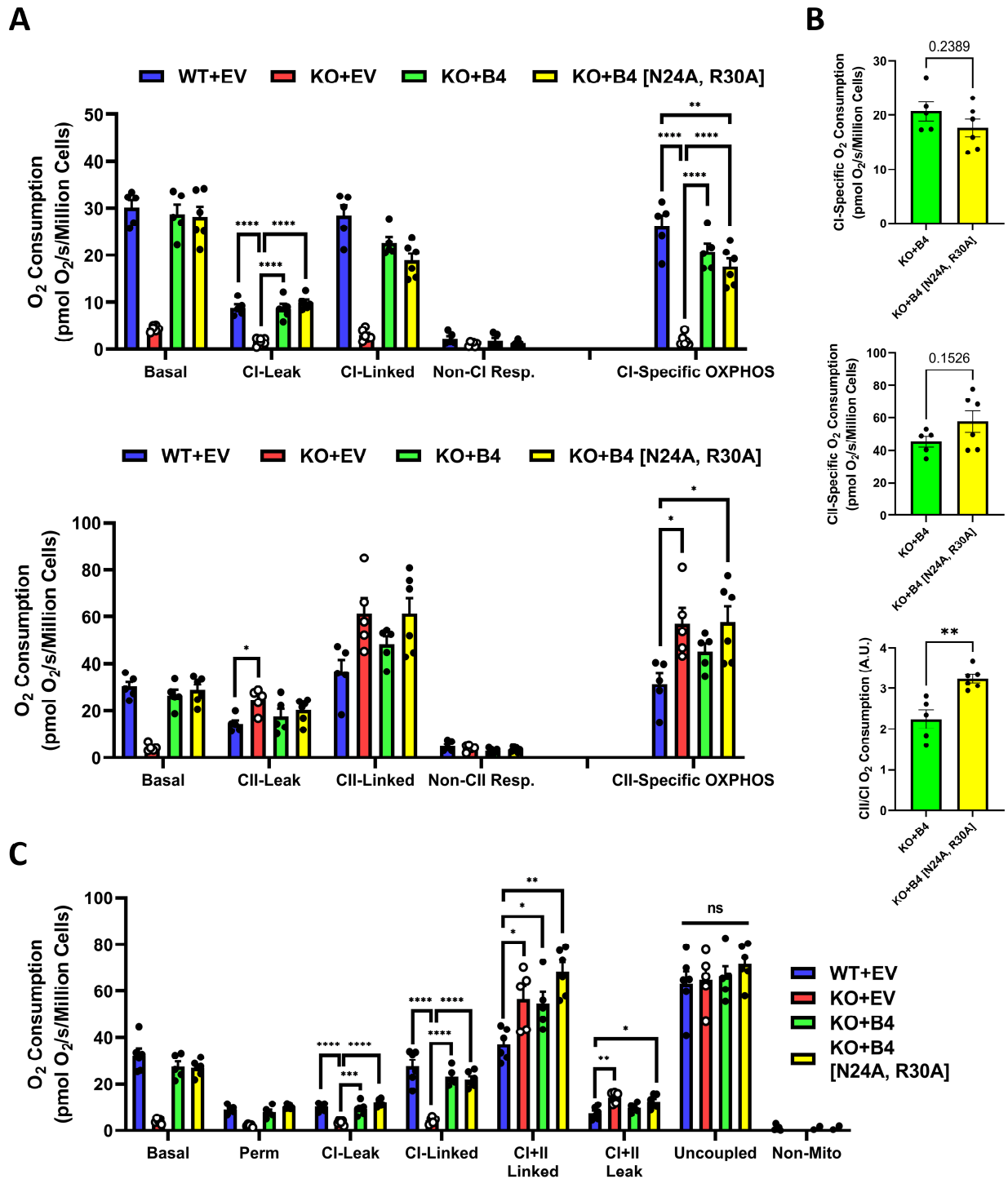


Figure 18. N24A and R30A mutations of CI accessory subunit NDUFB4 alter CI vs CII linked electron transfer pathways. High resolution respirometry of digitonin-permeabilized HEK293 WT or NDUFB4-KO cells stably expressing empty vector, NDUFB4 WT, or NDUFB4 with N24A and R30A substitutions. (A) CI-specific and CII-specific respiration pathway. Data presented normalized per million cells. Means $\pm$ SEM, $n = 5-6$. One-way ANOVA with Holm-Šídák post-hoc test, $** = p < 0.01$, $* = p < 0.05$. (B) Comparison of CI vs CII linked OXPHOS in

NDUFB4-KO cells stably expressing NDUFB4 WT, or NDUFB4 with N24A and R30A substitutions. Unpaired, 2 tailed student's T-test. ** = $p < 0.01$. (C) High resolution respirometry with CI + CII linked respiration. Means $\pm$ SEM, $n = 5-6$. One-way ANOVA with Holm-Šídák post-hoc test, ** = $p < 0.01$, * = $p < 0.05$.

4.0 DISCUSSION

4.1 HIG1 Protein Family

HIGD1A and HIGD2A have been described as both mitochondrial and nuclear localized (41–43), as regulators of cell death during hypoxia (44–47), as regulators of mitochondrial dynamics (57), as regulators of cellular bioenergetics (46, 47), and as OXPHOS complex assembly factors (52, 56). Taken together, the functional mechanisms of the HIG1 proteins are unclear. We hypothesized that the different HIG1 family proteins harbor different primary mechanisms to achieve their regulatory functions. To help explain the previously reported functions, we employed a stable overexpression model to evaluate the effects of OE for each HIG1 family protein. As opposed to knock-out based studies which can induce downstream system-wide dysfunctions, stable OE allows for interrogation of roles within a complete and functional system. In this context the primary effects of each HIG1 family protein can be better identified.

4.1.1 The HIG1 family proteins are mitochondrial

The localization of the HIG1 family proteins had not been definitively shown despite previously reported functions in mitochondria. Observed anti-apoptotic effects could lend to the plausibility that the HIG1 proteins could be partly nuclear localizing to act as transcription factors (42, 43). However, these studies used anti-HIGD1A/HIGD2A primary antibodies to establish their findings, which should warrant caution to interpretations due to the possibilities of off-target labeling. In this study, we probed the localization of FLAG-tagged HIG1 proteins using anti-FLAG labeling. With the specificity of tagged labeling, we definitively show that all the HIG1 proteins localize to mitochondria in normoxia and following 48 hours of hypoxia. We did not find evidence of nuclear localization under the conditions investigated, suggesting that the previously described anti-apoptotic effects of HIGD1A expression may be linked to functions within mitochondria.

An et al. demonstrated interactions between HIGD1A and L-OPA1 (57), which we confirmed along with HIGD1B and HIGD1C in our work. We suspect that previously reported enhanced cell survival under stress conditions could be linked to L-OPA1 interaction. OPA1 mediated regulation of cristae prevents the release of cytochrome *c* and other apoptotic cofactors (74). Increased cell survival under stress conditions could follow as a downstream effect of primary HIG1 protein family function through regulating mitochondrial dynamics. This possibility is partly supported by our findings that show 1) enhanced expression of mitochondrial fusion proteins OPA1 and MFN1/2 in the higher expressing HIGD1B cell line under hypoxic stress, and 2) no increase in cell survival during hypoxia following stable expression of the HIG1 proteins.

Secondary effects resulting from changes in mitochondrial dynamics could be specific to the cell model, tissue, or stress conditions being investigated. In the context of our cell model, longer exposure to 1% O₂ conditions may be necessary to observe enhanced survival resulting from changes to mitochondrial dynamics. Although mitochondrial connectivity was decreased in all cell lines with hypoxia compared to normoxia, increased mitochondrial length was only trending in the higher expressing HIGD1B cell line.

Trending increases in mitochondrial length suggest that 48 hours of hypoxia may be too early to observe clear differences in mitochondrial dynamics, and enhanced cell survival. Depending on the cell model, and the severity/duration of hypoxia, p53 proteins levels can either increase or decrease. Under hypoxia conditions as those utilized in this study, p53 protein levels can decrease from degradation, promoting cell survival and adaptation (75). A decrease in p53 levels was observed in all cells including EV, suggesting all cell lines were in an adaptive phase during the hypoxia condition tested. Following prolonged hypoxia, cells generally forego their adaptive

response and become apoptotic (75). Maintenance of mitochondrial length and cell survival following HIG1 OE may become more pronounced with longer hypoxia conditions.

Previous studies have established HIGD1A as a transcriptional target of HIF-1 α (44, 50). As such, the HIG1 proteins could represent part of the cellular adaptation response to hypoxia. Low oxygen and glucose conditions mimic both ischemia/reperfusion injury and tumor growth environments, explaining HIGD1A upregulation in tumor xenografts (48–50) and challenged cardiomyocyte models (46). In this study, we introduce additional functionality of the HIGD1A/1B/1C subfamily in modifying the p53-parkin signalling axis.

Though we observed decreased p53 expression in all cell lines during hypoxia, we demonstrated a trending further decrease of p53 compared to EV cells in some HIGD1A/1B/1C subfamily cell lines. Moreover expression of parkin, a transcriptional target of p53 (70), was decreased in all HIGD1A/1B/1C subfamily cell lines. Given that HIF-1 α overexpression represses p53 transcription (76), the HIGD1A/1B/1C subfamily proteins may be involved in the mechanism by which HIF-1 α promotes cell survival and adaptation (**Figure 19**). Transcriptional repression of parkin may lead to decreased canonical pink1-parkin mitophagy and preserve the mitochondrial network (13), though this was not clearly observed under the hypoxia conditions in this study.

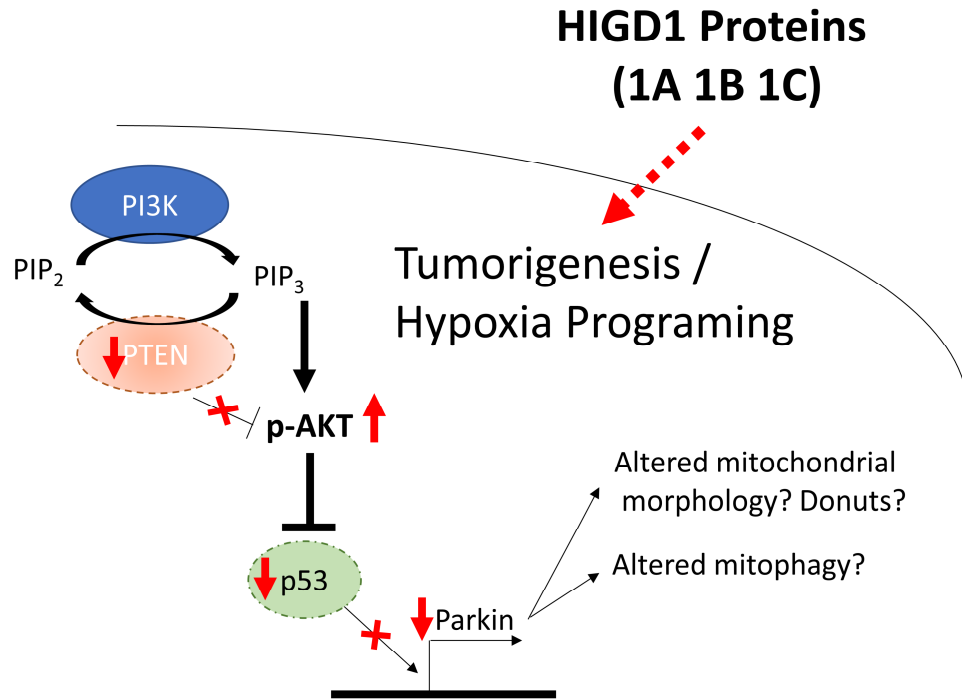


Figure 19. Proposed model of HIGD1A/1B/1C subfamily involvement during tumorigenesis and hypoxia cell survival and adaptation. Stable expression of HIGD1A/1B/1C may amplify cell survival by further silencing the p53-parkin axis.

4.1.2 Differing effects of HIGD2A vs. HIGD1A/1B/1C OE

Previous studies have suggested HIGD2A is an assembly factor of CIV (52, 56), though the extent of HIGD2A functional overlap with HIGD1A was unclear. In the present study, we establish a general dichotomy between HIGD2A and HIGD1A/1B/1C in specific protein-protein interactions as well as in ETC SC assembly. Immunoprecipitation of the FLAG-tagged HIG1 proteins verify that HIGD2A associates with the OXPHOS complexes I, III and IV to a greater extent than HIGD1A/1B/1C. Conversely, HIGD1A/1B/1C proteins interact with L-OPA1 to a greater extent than HIGD2A. Moreover, stable expression of HIGD2A does not appear to directly influence mitochondrial dynamics under hypoxic conditions, nor does it directly influence the p53-parkin

signalling axis. These findings suggest that the primary effects of HIGD2A OE may be limited to interactions with the OXPHOS complexes.

Loss of HIGD2A has been shown to fragment mitochondria and alter mitochondrial dynamics (42). We did not observe regulation of mitochondrial dynamics in our HIGD2A OE model, suggesting that the effects on mitochondrial dynamics may be a by-product of CIV disassembly and OXPHOS dysfunction, an effect which was also observed during Δ HIGD2A (52). Dysfunction of the OXPHOS system can lead to depolarization of mitochondria, resulting in fragmentation and mitophagy (13).

4.1.3 Proposing HIGD1C as a novel dominant negative homolog of HIGD2A in ETC SC assembly

The dichotomy between HIGD2A and HIGD1A/1B/1C does not rule out the possibilities of overlapping function. Overexpression of HIGD1A or HIGD2A did not alter steady state levels of SCs, as previously described (56). Instead, we demonstrate a novel function of HIGD1C in regulating CIV SC assembly. Stable expression of HIGD1C decreases CIV SC levels, and shifts CIV into a monomeric form. HIGD1C's interaction with CIV may prevent the latter from incorporating into SCs, and so CIV is trapped in monomeric form. Moreover, FLAG-tagged immunoblotting of the HIG1 proteins suggests that HIGD1C, HIGD1B, and HIGD2A are localizing into a mid-sized complex slightly larger than CIV. Absence of HIGD2A was previously shown to decrease CIV steady state levels and CIV incorporation into SCs. (56). Our findings suggest that HIGD1C could have a dominant negative role in regulating HIGD2A's association and stabilization of CIV-containing ETC SCs (**Figure 20**). In other words, HIGD1C may displace HIGD2A during late assembly of CIV, and prevent incorporation of CIV into ETC SCs. Attempts to confirm this hypothesis are currently ongoing. Studies confirming that HIGD1C binds the

COX3 module of CIV, similar to HIGD2A (52), are needed to confirm the dominant negative model of HIGD1C function.

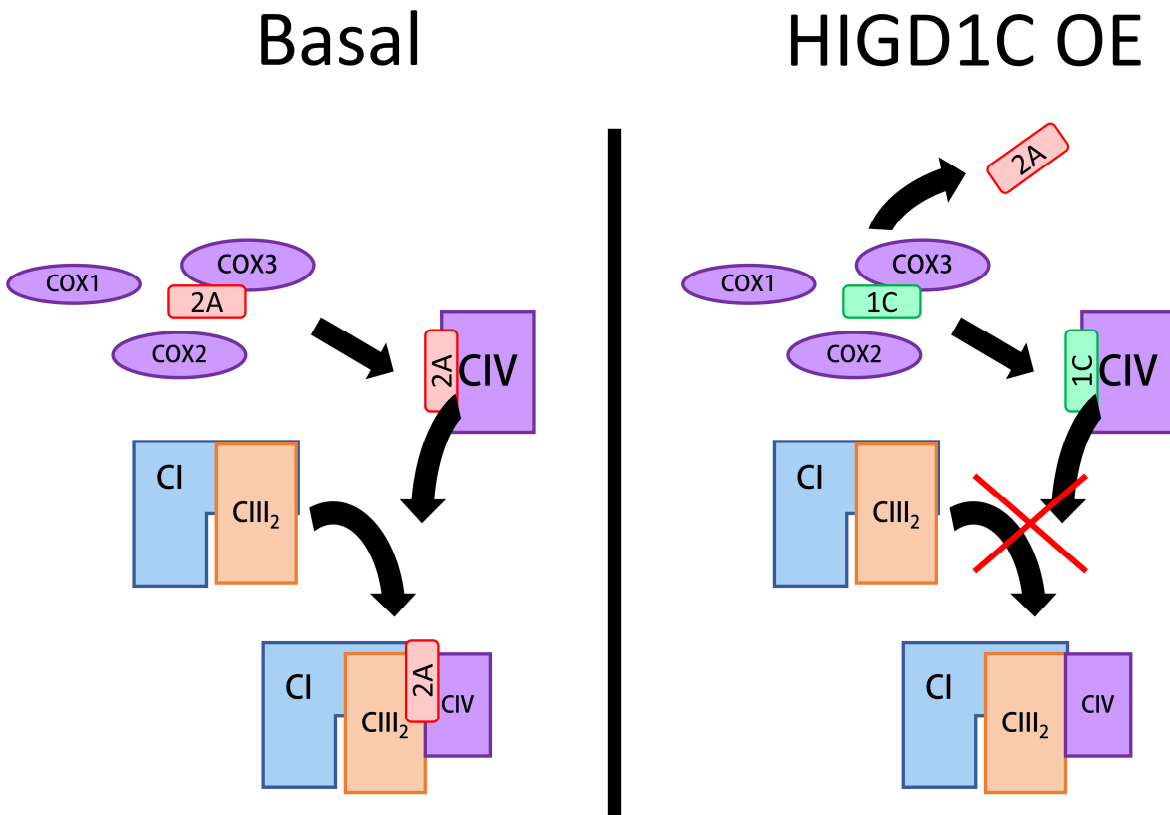


Figure 20. Proposed model of dominant negative HIGD1C function. Under basal conditions, endogenous HIGD2A is completing CIV assembly by incorporating the COX3 module, and subsequently aiding in CIV incorporation into SCs. During HIGD1C OE, HIGD1C displaces HIGD2A but is non-functional towards assembly in SCs. As a result, CIV is trapped in monomer form, leading to decreased CIV SCs at steady state.

Though no published studies to date have addressed HIGD1C protein, a single nucleotide polymorphism (SNP) upstream of the *HIGD1C* gene was previously identified in genome wide association studies against diabetes (77). Disassembly of ETC SCs has been previously reported by our group in abdominal muscle of diabetic obese individuals (33). Thus, an exciting hypothesis, to be formally tested, has emerged where altered HIGD1C expression levels in a subset of T2D patients may explain their ETC SC disassembly and resulting mitochondrial dysfunction.

4.2 ETC Accessory Subunits Regulate Respirasome Formation

4.2.1 ETC accessory subunits regulate ETC SCs and represent a new model to understand the functional significance of respirasome assembly

Functional characterization of ETC SCs have been largely driven by manipulation to the only recognized ETC SC regulatory protein factor known to date: COX7A2L (20, 32, 37, 58). COX7A2L is a direct assembly factor of the III₂IV₁ SC. As an isoform of the COX7A2 subunit, COX7A2L replaces COX7A2 during CIV assembly and harbors a modified N-terminus to form protein-protein interactions with CIII₂ (20). During studies interrogating III₂IV₁ function through its removal by Δ COX7A2L, the I₁III₂IV₁ respirasome is still present (37, 58).

The respirasome is a major ETC SC form and is the focus of assembly in studies that link ETC SC formation to physiological states such as exercise (31), and disease states such as Parkinson's, heart failure, type 2 diabetes, and aging (33–36). Unfortunately, there is a lack of known protein factors that directly regulate respirasome assembly. As a result, the functionality of the respirasome and thus all connections to physiology and disease remain largely unknown. In this study, we demonstrate a new framework to understand respirasome assembly: protein-protein interactions between OXPHOS complexes are mediated through previously uncharacterized accessory subunits of CI.

The siRNA mediated knockdown of candidate subunits revealed respirasome disassembly and degradation of complex I. As such, NDUFB4 and NDUFB6 were found to regulate both complex I monomer assembly and ETC SC assembly. Selective point mutations N24A and R30A in NDUFB4 allowed for exclusive respirasome disassembly without impacting CI stability. No previous model has disrupted respirasome formation while maintaining the OXPHOS system and

the MIM. Furthermore, no model had previously defined the residues conferring CI-CIII₂ super-assembly.

It is still debated whether respirasome assembly involves either fully assembled CI or partially assembled CI lacking its NADH substrate binding N-module (38). Here we demonstrate a case for fully assembled CI being incorporated into the respirasome. Upon disrupting CI-CIII₂ super-assembly through NDUF_B4 point mutations, a distinct high molecular weight band just below the respirasome is formed, which was not observed in either NDUF_B4-KO cells or NDUF_B4 rescue cells. NDUF_B4 point mutation cells retain CI-specific activity as measured through direct stimulation in HRR, suggesting that fully assembled and functional CI can exist and that CI does not need to be incorporated into a respirasome to assemble its catalytic N-module.

Another question in respirasome assembly is whether CIII₂ and CIV must complete the respirasome by interacting with CI as a III₂IV₁ SC (38). In the model examining transient knockdown of CI accessory subunits, a clear redistribution of SCs from the respirasome into the III₂IV₁ SC was observed. Upregulation of III₂IV₁ can also be observed in NDUF_B4-KO cells. Interestingly, our NDUF_B4 point mutation model displays downregulation of III₂IV₁ similar to cells rescued with WT NDUF_B4. Downregulation of III₂IV₁ despite impaired respirasome formation is a novel and unexpected finding, as it suggests that selective disassembly of the respirasome does not necessarily lead to upregulation of the III₂IV₁ SC. Upregulation of III₂IV₁ observed in NDUF_B4 knockdown and NDUF_B4 knockout could be a result of CI degradation. In our point mutation NDUF_B4 model, we demonstrate that I₁III₂IV₁ and III₂IV₁ formation are independently regulated, and that CIII and CIV need not form the III₂IV₁ SC in the absence of the respirasome, or to incorporate into the respirasome.

4.2.2 Respirasome formation has a global effect on respiration, and fine tunes the ETC

We describe for the first time the functional consequences of disrupting respirasome assembly while maintaining individual OXPHOS complexes, and MIM integrity. Disruption of CI-CIII₂ super-assembly decreased both resting and uncoupled respiration by a third, while leak respiration was generally unaltered in intact cells, suggesting a global dysfunction of the OXPHOS system. Moreover, ATP-linked respiration (resting – leak) was decreased supporting an overall suppression in the OXPHOS system. However, interrogation into the ETC through HRR suggests that when directly stimulating OXPHOS with supplied substrates, the ETC in respirasome deficient cells is similar to rescued cells. Thus, general function of the ETC does not explain the dramatic 1/3rd decrease to bioenergetic activity in respirasome deficient cells. Instead, other metabolic pathways feeding into the OXPHOS system may be limiting its activity in intact cells. One potential reason to limit OXPHOS activity is to limit the production of ROS. CI is a large source of mitochondrial ROS production (10), and CI ROS production is greatly increased when CI is dissociated from SCs (28). Due to respirasome disassembly, our NDUFB4 point mutation cell line features monomeric, functional CI. Therefore, in our respirasome deficient model metabolic pathways could be limiting the activity of monomeric CI and the ETC as a protective measure to decrease ROS production in intact cells; however, this limiting metabolic pathway would then be bypassed during permeabilization and HRR to agree with our findings that HRR-probed ETC activity is generally unaltered. Metabolomic studies could confirm this hypothesis by revealing the altered metabolic pathway(s) feeding into the ETC, causing the respiratory dysfunction observed in intact, respirasome deficient cells.

During selective disassembly of the respirasome, modification to the ETC is subtle. The ratio of CII-linked to CI-linked OXPHOS is greater in our NDUFB4 point mutation model, indicating a

shift towards CII-linked respiration. This observation is similar to the pioneering study by Lapuente-Brun et al, who showed decreases to respirasome levels in mice starved for 18 hours, coupled with decreased CI-linked respiration and increased CII-linked respiration. Interestingly, increases to CII-linked respiration were not observed in starved mice lacking functional COX7A2L (32). Increases to CII/CI OXPHOS in our model, which features a disassembled respirasome and unaltered COX7A2L, suggest that modification to electron transfer pathways can occur specifically through the respirasome and independent of COX7A2L.

Multiple mechanisms could explain how the SCs fine tune the ETC. First, optimal CI-linked respiration could be dependant on respirasome formation, and any decrease to respirasome levels results in a shift from CI-linked to CII-linked respiration to compensate. Moreover, ETC SCs could sequester ubiquinone for CI-linked respiration, thereby limiting the pool of available ubiquinone for CII linked respiration. This idea is supported by a very recent study where CIII was genetically removed to disassemble the respirasome and cells were supplied with an alternative oxidase (AOX) to complete respiration. In this model, CIII-KO+AOX cells had increased CI-linked respiration compared to CIV-KO+AOX cells, suggesting that CI-CIII₂ super-assembly is channeling its own ubiquinone, and is therefore insensitive to AOX (20). In our study, we use a model specifically disrupting respirasome formation to show that ETC SCs modify electron transfer. Since the ratio of NADH/FADH₂ generated is dependant upon the carbon source used to fuel the OXPHOS system (37), ETC SCs may represent a method to optimize CI-linked vs CII-linked respiration to fit the incoming NADH/FADH₂ supply.

4.3 Conclusion

In this work, we broadened the characterization of HIG1 family proteins: human orthologs of ETC SC factors found in yeast. Through comparative studies between partially characterised family members HIGD2A/HIGD1A and previously unstudied members HIGD1B/HIGD1C, we revealed a general dichotomy between the effects observed with HIGD2A vs. HIGD1A/1B/1C OE. Furthermore, we identified HIGD1C as a negative regulator of CIV SCs. Future studies will determine the mechanism of HIGD1C-mediated ETC SC disassembly, and whether modification of *HIGD1C* expression is linked to a subset of T2D patients with observed ETC SC disassembly and mitochondrial dysfunction.

To address the current gaps in understanding respirasome assembly, we proposed a new model of ETC SC regulation through non-catalytic subunits of OXPHOS complex I. By introducing point mutations to one such subunit NDUFB4, we developed a new cell model with respirasome disassembly but otherwise intact and functional OXPHOS complexes. Through this model, we demonstrate that the respirasome regulates both respiration and ETC pathway activities. Further study of the NDUFB4 point mutation model could yield additional insight into functional causes of diseases implicated with disassembly of ETC SCs.

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6.0 CONTRIBUTIONS OF COLLABORATORS

High resolution respirometry experiments of HEK293 WT or NDUFB4-KO cells stably expressing empty vector, NDUFB4 WT, or NDUFB4 with N24A and R30A substitutions (**Figure 18**) were performed by Dr. Chantal Pileggi (University of Ottawa, Ottawa, Canada).

I performed all other experiments and data analysis featured, including generation of all stable cell line models.

7.0 CURRICULUM VITAE

Gaganvir Singh Parmar

Education

2019-Present Master's of Science (MSc.) in Biochemistry

University of Ottawa, Ottawa, ON

Currently completing 2nd year of studies

2015-2019 Honours Bachelor of Science (B.Sc.) with specialization in Biochemistry

University of Ottawa, Ottawa, ON

Degree conferred May 2019 *Summa Cum Laude*, holding 9.88 cGPA (on 10)

2011-2015 Ontario Secondary School Diploma

Awards and Honors

- NSERC CREATE MATRIX Graduate Student Scholarship (2020)
- University of Ottawa Admission's Scholarship – Master's (2020)
- Ontario Graduate Scholarship (2019)
- Maxime Claveau Excellence Scholarship (2018)
- National Science and Engineering Research Council Undergraduate Student Research Award (NSERC-USRA) (2018)
- National Science and Engineering Research Council Undergraduate Student Research Award (NSERC-USRA) (2017)
- Undergraduate Research Opportunity Program Award (2016-2017)
- Dean's Honor List (2016-2019)
- University of Ottawa Admission's Scholarship (2015-2019)
- Ontario Scholar (2015)

Certificates

- B1 DELF French certification, Ministère de l'Éducation Nationale (France)
- 3rd Degree Black Belt (N° C-3-665), granted by the International Taekwondo Federation

Work Experience

Harper lab (Dr. Mary-Ellen Harper) Ottawa, ON

May 2019 – Present Master's Student in Biochemistry

Sep 2019 – Apr 2019 Biochemistry Honour's Research Project

May 2018 - Aug 2018 NSERC-USRA Summer Student

Science Preparatory Workshop (Faculty of Science, University of Ottawa) Ottawa, ON

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May 2017 - Sep 2017 NSERC-USRA Summer Student

Nov 2016 - Mar 2017 Undergraduate Research Opportunity Program (UROP) Student

May 2016 - Sep 2016 Research Assistant Internship (volunteer)

Peer-reviewed Publications

1. Pileggi CA, **Parmar G**, Harper M-E. 2021. The lifecycle of skeletal muscle mitochondria in obesity. *Obes Rev* 22:e13164.
2. Liaghati A, Pileggi CA, **Parmar G**, Patten DA, Hadzimustafic N, Cuillerier A, Menzies KJ, Burelle Y, Harper M-E. 2021. Grx2 Regulates Skeletal Muscle Mitochondrial Structure and Autophagy. *Front Physiol* 12:604210.
3. Boardman NT, Migally B, Pileggi C, **Parmar GS**, Xuan JY, Menzies K, Harper M-E. 2021. Glutaredoxin-2 and Sirtuin-3 deficiencies impair cardiac mitochondrial energetics but their effects are not additive. *Biochim Biophys Acta Mol Basis Dis* 1867:165982.
4. Patten DA, McGuirk S, Anilkumar U, Antoun G, Gandhi K, **Parmar G**, Iqbal MA, Wong J, Richardson RB, St-Pierre J, Slack RS, Harper M-E. 2021. Altered mitochondrial fusion drives defensive glutathione synthesis in cells able to switch to glycolytic ATP production. *Biochim Biophys Acta Mol Cell Res* 1868:118854.

Abstracts and Presentations at Scientific Meetings

1. **Parmar G**, Patten DA, Harper M-E. 2021. Novel Factors Regulating Electron Transport Chain Supercomplexes. 5th Ottawa International Conference on Neuromuscular Disease and Biology. October 17-19th 2019

Non-Academic Activities

Taekwondo Ottawa, ON

2011 – Present Practicing

Jun 2019 – Mar 2020 Assistant Instructor

BMI Graduate Student Association Ottawa, ON

Sep 2020 – Present VP Academic

- Organizing info sessions, seminars, workshops and career day for graduate students in the department
- Participating in student council meetings

MATRIX Metabolomics

Ottawa, ON

January 2020 – Present

Journal club seminar coordinator

- Working with program directors and collaborating lab researchers to organize a weekly journal club seminar in mitochondrial biology and metabolomics
- Handling weekly communication of presentation information and upcoming events

Let's Talk Science Ottawa

Ottawa, ON

Oct 2017 – Mar 2020

Community outreach volunteer

- Visiting classrooms to provide STEM based learning experiences through organized presentations and activities

The Ottawa Hospital

Ottawa, ON

May 2017 – Mar 2020

Patient ambassador, info guide, in-patient care volunteer

Canadian Blood Services

Ottawa, ON

May 2016 – Nov 2017

In-community volunteer

- Participating in local community events, providing information on various donation programs and assisting with donor recruitment

References

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