EXPLORATION OF THE MECHANISTIC ROLE OF CYCLOPHILIN D IN THE MITOCHONDRIA

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Doctor in Philosophy

by

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Abstract

Cyclophilin D (CypD) is an approximately 18KDa protein, and it is the only reported cyclophilin family of the peptidyl prolyl cis trans isomerase that is present in the mitochondrial matrix. It functions mainly as a regulator of the open and closed state of the mitochondrial permeability transition pore (MPTP); however, the mechanism by which this occur is still largely undetermined. Being a peptidyl prolyl isomerase, CypD is believed to also function alongside other mitochondrial quality control proteins to execute the important task of folding and refolding of nascent and unfolded matrix proteins respectively.

Indeed, many studies have investigated the MPTP, using CypD as ideal modulator; unfortunately, most of these studies were carried out under pathological and/or in vitro artificial experimental conditions. It was hypothesized that exploring the basal physiological role of CypD in the mitochondria, in addition to signalling changes that accompany its modulation using both knock down and knock out cell-based systems would enhance the understanding of CypD and this may prove useful in the delineation and characterisation of the MPTP.

The work herein quantified the amount of CypD present in the mitochondria of both mouse tissues and HAP1 cells. It was observed that CypD was more than 5-fold higher in cancer cells, depending on the cell type than normal, non-transformed cells/tissues. Furthermore, the Cys203 on CypD directly regulates the amount of mitochondrial NAD(P)H by forming stable sulfenic acid which may constantly require the mitochondrial NADPH-dependent thioredoxin or glutaredoxin systems for its reduction. Moreover, CypD elevated basal mitochondrial ROS, membrane potential (ΔΨ_m) /proton motive force and mitochondrial ATP while decreasing the level of forward flux TCA cycle enzymes such as inhibiting PDH activity while decreasing the level of OGDH and IDH3 when compared to CypD deficient cells. Furthermore, extracellular signalling pathways such as AKT, mTORC1, ERK, AMPK and HIF-1α were
differentially altered between CypD WT and deficient cells. More importantly, it was observed that CypD deficient cells were susceptible to all forms of cellular stress most likely via inhibition of autophagy.

This result emphasized the physiological importance of CypD in the regulation of basal cellular ROS signalling and ATP synthesis / energy homeostasis, and this effect may be used by cancer cells to enhance the amount of circulating ROS. The findings herein suggest that inhibition of the MPTP during CypD deficiency may be associated with decreased inner membrane proton gradient and subsequent acidification of the matrix. However, CypD ablation may not be an overall useful technique against cell death, as it confers stress-like phenotype on the cells.
Acknowledgements

I would first like to thank my supervisory team; Prof. Lu-Yun Lian and Dr. David Criddle, chaired by Prof. Lu-Yun Lian for the mentorship and guidance they have shown during the past four years. It has been absolute pleasure working with them. I appreciate your commitments and passion, and this has helped me immensely to develop myself in critical thinking and independent research. The discussions and *uncountable* meetings we had were very useful, encouraging and stimulating. I would also like to thank my assessors, Prof. Caroline Dart and Prof. Alexey Tepikin for your interest in my project, and your advice during my annual IPAP meetings.

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Thanks be to God who has given me the breath of life to will and to work during these years of study through His Son Jesus Christ.

‘For it is God who works in me both to will and to do, for His good pleasure’

*Philippians 2:13*
# Table of Contents

Abstract .................................................................................................................................i
Acknowledgement ................................................................................................................iii
Table of Contents ..................................................................................................................v
List of Figures .......................................................................................................................ix
List of Tables ........................................................................................................................xi
List of Abbreviations .............................................................................................................xii

1 Introduction .........................................................................................................................5
1.1 Overview of the mitochondria .........................................................................................5
  1.1.1 Different perspectives of the mitochondria ..............................................................5
  1.1.2 Morphological Features of the Mitochondria ..........................................................5
  1.1.3 Mitochondrial Compartmentalisation and Function ...............................................7
1.2 Mitochondrial ROS Production and Control .................................................................23
  1.2.1 Mitochondrial Superoxide ......................................................................................23
  1.2.2 Measurement of Mitochondrial Superoxide ............................................................27
  1.2.3 ROS induced Protein Modification .....................................................................28
  1.2.4 Mitochondrial Antioxidant Defence Systems .......................................................29
  1.2.5 Mitochondrial Inner Membrane Uncoupling or Proton leak ................................31
  1.2.6 Mitochondria Retrograde Signalling ..................................................................35
1.3 Mitochondrial Cyclophilin D .........................................................................................44
  1.3.1 Inhibitors of CypD ...............................................................................................47
  1.3.2 CypD and Protein Folding ...................................................................................48
  1.3.3 CypD and the Mitochondrial Permeability Transition Pore (MPTP) ..................51
  1.3.4 Post translational modification of CypD ...............................................................61
  1.3.5 Role of CypD in Disease Pathology .....................................................................62
1.4 Aim and Objectives ........................................................................................................64
  1.4.1 Purification of Recombinant and Quantitation of mitochondrial Cyclophilin D ....65
1.4.2 Assessment of the Role of Cyclophilin D in the Regulation of Mitochondrial Redox and Energy Homeostasis ........................................ 65
1.4.3 Evaluation of Metabolic Reprogramming in CypD Deficient Cells 65

2 General Materials and Methods ........................................................................................................ 70
2.1 Materials ........................................................................................................................................ 70
2.1.1 Plasmids ....................................................................................................................................... 70
2.1.2 Cell lines and lysates .................................................................................................................... 70
2.1.3 Culture media ............................................................................................................................... 71
2.1.4 siRNA Oligonucleotides .............................................................................................................. 71
2.1.5 Tissue sources ............................................................................................................................. 71
2.2 Methods ......................................................................................................................................... 71
2.2.1 Bacteria Culture .......................................................................................................................... 71
2.2.2 Protein purification ....................................................................................................................... 73
2.2.3 Protein Activity and Modulation assay ....................................................................................... 74
2.2.4 Human Tissue culture ................................................................................................................ 76
2.2.5 Mitochondria extraction and activity ......................................................................................... 85
2.3 Statistical Analysis ........................................................................................................................ 88

3 Purification of Recombinant and Quantitation of Mitochondrial Cyclophilin D ...................... 91
3.1 Introduction ....................................................................................................................................... 91
3.2 Results ............................................................................................................................................ 94
3.2.1 Purification and Characterisation of Recombinant Cyclophilin D ............................................. 94
3.2.2 Mitochondria extraction and verification .................................................................................. 95
3.2.3 Quantitation of CypD in the mitochondria .............................................................................. 97
3.2.4 Quantification of PTPMT1 in HAP1 cell mitochondria ............................................................. 102
3.3 Discussion ...................................................................................................................................... 105
3.3.1 Purified Recombinant CypD is Structurally intact ................................................................. 105
3.3.2 Peptidyl-prolyl cis-trans isomerisation ................................................................................... 105
3.3.3 Mitochondria extraction and verification ................................................................................ 106
3.3.4 Quantification of CypD in the mitochondria .......................................................................... 108
5.2.5 CypD Regulates HIF-1α in HAP1 and HeLa Cells .......... 166
5.2.6 CypD Deficiency Prevents Lipid Droplet Formation in HAP1 and HeLa Cells ................................................................. 168
5.2.7 CypD Deficiency Differentially Regulate the PI3K/AKT Pathway in Different Cell Types .................................................... 169
5.2.8 CypD Deficiency Differentially Regulates mTORC1 Pathway in HAP HeLa and HT1080 Cells ..................................................... 170
5.2.9 CypD Deficiency Differentially Alters the NF-κB Pathway in HAP1 and HeLa Cells ................................................................. 172
5.2.10 CypD Regulates Autophagy in HAP1 Cells ..................... 174

5.3 Discussion ........................................................................... 178
5.3.1 CypD Deficiency Enhances Mitochondrial TCA Cycle forward flux while Repressing ATP Synthesis in HAP1 Cells ................. 178
5.3.2 Linking CypD to the MPTP through its role in coupling TCA with oxidative phosphorylation ................................................. 181
5.3.3 CypD Modulates Cellular ROS in HAP1 Cells ................. 182
5.3.4 AMPK is Activated in CypD Knock Downs ....................... 183
5.3.5 CypD Deficiency Suppresses HIF-1α Activity in HAP1 and HeLa Cells 185
5.3.6 CypD is Required for Lipid Droplet Formation in HAP1 and HeLa Cells 187
5.3.7 CypD Deficiency Differentially Regulates PI3K/AKT and MEK/ERK Pathways in Different Cell Lines ................................. 189
5.3.8 CypD Deficiency Activates mTORC1 Pathway in HAP1 and HeLa Cells ........................................................................ 191
5.3.9 CypD Maintains Survival via Enhanced Autophagy and mTORC2 Stabilisation in HAP1 Cells .................................................. 193
5.3.10 CypD Deficiency Differentially Regulates NF-κB in HAP1 and HeLa Cells ........................................................................ 195

5.4 Conclusion ........................................................................... 196
6 Overall Discussion and Conclusion ......................................... 199
7 Future work ............................................................................. 205
References ........................................................................................................ 209
Appendices ........................................................................................................ 270

List of Figures

Figure 1.1: Variety of Mitochondrial Morphology ............................................. 6
Figure 1.2: Cellular Mitochondrial (Intrinsic) and Extrinsic Apoptotic Pathway ........................................................................................................... 11
Figure 1.3: Mitochondrial Electron Transport Chain and its Associated Chemiosmosis ........................................................................................... 15
Figure 1.4: Overview of ROS-Induced Protein Thiol Modification ................. 29
Figure 1.5: Thioredoxin and Glutaredoxin Systems .......................................... 30
Figure 1.6: Mitochondrial Uncoupling Proteins and Cardioprotection .......... 34
Figure 1.7: The Role of Mitochondria on some Signalling Pathways ............. 46
Figure 1.8: Crystal Structure of CypD in Complex with CsA ......................... 49
Figure 1.9: Cyclosporin A and its Structural Analogues ................................ 50
Figure 1.10: VDAC-ANT-CypD Model of the MPTP ..................................... 54
Figure 1.11: CypD-PiC-ANT Model of the MPTP ....................................... 55
Figure 1.12: ATP Synthase Dimer as a Model of the MPTP ......................... 57
Figure 3.1: Purification of Recombinant CypD .............................................. 95
Figure 3.2: Verification of Recombinant CypD ............................................... 96
Figure 3.3: Verification of Mitochondria Extraction in Mouse Tissues and HAP1 Cells ................................................................................................. 97
Figure 3.4: Quantitation of CypD in Mouse Liver Mitochondria ................. 98
Figure 3.5: CypD is More Abundant in Mouse Heart than Liver Tissue Mitochondria .............................................................................................. 99
Figure 3.6: Quantitation of CypD in Mouse Liver Tissue Lysates .......... 100
Figure 3.7: Quantitation of CypD in HAP1 Mitochondria ........................... 101
Figure 3.8: CypD is Abundant in Cancer than Non-Cancer Cells ............... 103
Figure 3.9: Quantitation of PTPMT1 in HAP1 Mitochondria .................... 104
Figure 4.1: Modulation of Ca^{2+}-Induced Swelling in CypD WT and KO Isolated Mitochondria .............................................................................. 121
Figure 4.2: Phosphatases and Prolyl Hydroxylation does not Affect Mitochondrial Swelling. .......................................................... 122
Figure 4.3: NAD(P)H in Isolated CypD WT and KO HAP1 Mitochondria. .. 123
Figure 4.4: Thapsigargin Induced Mitochondrial NAD(P)H in CypD KO HAP1 Cells. .......................................................................................................................... 124
Figure 4.5: CypD Deficiency Promotes Protein Lysine Acetylation. ........ 125
Figure 4.6: CypD does not Affect the Activities of Respiratory Chain Complexes I and II. ................................................................................. 126
Figure 4.7: CypD Deficiency Decreases Basal Mitochondrial Membrane Potential ......................................................................................... 127
Figure 4.8: Respiratory Chain Inhibition Dissipates Membrane Potential in HAP1 Cells.................................................................................. 128
Figure 4.9: CypD Deficiency Decreases Mitochondrial ROS................ 130
Figure 4.10: Cellular and Mitochondrial ATP are Lower in CypD KO HAP1 Cells. ............................................................................................ 131
Figure 4.11: CypD Modulates the Activity of Mitochondrial Antioxidants. .. 132
Figure 4.12: H₂O₂ Accelerates NAD(P)H Depletion in WT HAP1 Cells..... 133
Figure 4.13: H₂O₂ Induces Cell Death in CypD KO than WT HAP1 Cells.. 134
Figure 4.14: CypD Undergoes Stable Protein Thiol Sulfenylation at Cys203 Residue......................................................................................... 135
Figure 4.15: Recombinant CypD is Resistant to H₂O₂ Induced Oxidative Damage. ......................................................................................... 136
Figure 4.16: PTPMT1 is Inactivated by both Mutation and H₂O₂ Treatment. ................................................................................................. 137
Figure 4.17: Schematic Diagram of CypD Sulfenylation and Endogenous Reduction by Mitochondrial Glutathione and Thioredoxin Systems. .... 153
Figure 5.1: CypD Deficiency Upregulates Forward TCA Cycle Metabolising Enzymes. ......................................................................................... 163
Figure 5.2: CypD KO are Susceptible to ATP Synthase Inhibition. .......... 164
Figure 5.3: CypD Modulates AMPK Activity. ......................................... 165
Figure 5.4: CypD Regulates Cellular ROS level in HAP1 Cells.............. 166
Figure 5.5: CypD Modulates HIF-1α in HAP1 Cells. .............................. 167
Figure 5.6: CypD Modulates HIF-1α in HeLa Cells. .............................. 168
Figure 5.7: CypD Regulates Cellular Lipid Droplets in HAP and HeLa Cells. ................................................................. 169
Figure 5.8: CypD Modulates PI3K/AKT and ERK Pathway. ......................... 171
Figure 5.9: CypD Deficiency Modulates mTORC1 Activity. ........................ 172
Figure 5.10: CypD Deficiency is Associated with mTOR-Lysosomal Localisation in HeLa Cells ................................................................. 173
Figure 5.11: CypD Deficiency Modulates NF-κB Pathway. ........................ 173
Figure 5.12: CypD Regulates Autophagy in HAP1 Cells ............................... 174
Figure 5.13: Modulation of Mitochondrial TCA cycle in CypD KD and KO cells. ......................................................................................... 173
Figure 5.14: Regulation of AMPK by ATP and Glycolysis. ............................ 187
Figure 6.1: Speculative Effect of CypD on Mitochondrial Metabolism ...... 199
Figure 6.2: Overall Summary of CypD Deficiency ................................................................. 200
Figure 9.1: Modulation of Ca$^{2+}$-induced mitochondrial swelling by TCA cycle metabolites ......................................................................................... 270
Figure 9.2: Succinyl-CoA represses CypD-CsA interaction. ............................ 271
Figure 9.3: PTPMT1 activity is not modulated by CypD ................................. 272
Figure 9.4: Amyloid beta proteins in CypD WT and KO isolated mitochondria. ......................................................................................... 273
Figure 9.5: Nuclear localisation of mitochondrial aconitase in HeLa cells . 274
Figure 9.6: Nuclear localisation of mitochondrial OGDH and Aconitase in HT1080 cells ......................................................................................... 275

List of Tables

Table 1.1: Regulation of BCl 2 family on the outer mitochondrial membrane ................................................................................................. 10
Table 1.2: Source of mitochondrial ROS production ....................................... 26
Table 1.3: ppif protein and gene homology across other mammalian species relative to H. sapiens ................................................................. 47
Table 2.1: Antibodies for Immunofluorescence .............................................. 80
Table 2.2: QPCR primers for gene expression ................................................. 81
Table 2.3: Resolving gel ................................................................................ 82
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>2-OGDD</td>
<td>2-Oxoglutarate-Dependent Dioxygenase</td>
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<td>ALT</td>
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<td>BCL-XL</td>
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<td>Carbonyl Cyanide M-Chlorophenyl Hydrazine</td>
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<td>ClpP</td>
<td>Caseinolytic protease proteolytic subunit</td>
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<td>ERK</td>
<td>Extracellular Signal-Regulated Kinases</td>
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ERR  Estrogen-Related Receptor
ETC  Electron Transport Chain
ETF-QOR  Electron Transfer Flavoprotein-Ubiquinone Oxidoreductase
F6 (ATP5J)  ATP synthase-coupling factor 6
FADH2  Flavin Adenine Dinucleotide (FAD) + Dihydrogen (H2)
FCCP  Carbonyl Cyanide-4-(Trifluoromethoxy) Phenylhydrazone
Fe-S  Iron–sulfur
FIH  Factor Inhibiting HIF-1
FK506  FK506-binding protein 15
FKBPS  Peptidyl-prolyl cis-trans isomerase FKBP12
Flavin Binding Site Of BCOADHC
FMN  Flavin Mononucleotide
G3P  Glycerol-3-phosphate
G3PDH  Glyceraldehyde-3-phosphate dehydrogenase
GDP  Guanosine Diphosphate
GPCR  G Protein-Coupled Receptor
GPX  Glutathione Peroxidase
GSH  Reduced Glutathione
GSK3β  Glycogen Synthase Kinase-3β
GSSG  Oxidised Glutathione
GST  Glutathione S-Transferase
GTP  Guanosine triphosphate
H₂O₂  Hydrogen Peroxide
HAP1  Near Haploid cells 1
HIF  Hypoxia-Inducible Factors
HRK  Activator of Apoptosis Harakiri
HSP  Heat Shock Proteins
IAP  Inhibitor of Apoptosis Protein
IDH  Isocitrate Dehydrogenase
IKK  Inhibitor of nuclear factor kappa-B kinase
IL-1  Interleukin-1 Family (IL-1 Family)
ILK  Integrin-Linked Kinase
IPC          Ischemic Preconditioning
IR           Ischemia reperfusion
IkB (NFKB1A) NF-kappa-B inhibitor alpha
JC-1         Fluorescent Cationic Carbocyanine Dye
LC3          1B-Light Chain 3
LC8 (DYL)    Dynein light chain
LKB1 (STK11) Liver kinase B1
Lonp         Lon Protease Homolog
M-AAA        Multifunctional mitochondrial AAA
MAPK         Mitogen-Activated Protein Kinase
MCL-1        Induced Myeloid Leukemia Cell Proliferation Protein
MCU          Mitochondrial Calcium Uniporter
MDM10        Mitochondrial Distribution and Morphology Protein 10
MFN          Mitofusin
MGL          Monoacylglycerol Lipase
Mitosox      Mitochondrial superoxide indicator
MLST8        Mammalian Lethal with Sec13 Protein 8
MOMP         Mitochondrial Outer Membrane Permeabilization
MPTP         Mitochondrial Permeability Transition Pore
MSIN1        Mammalian Stress-Activated Protein Kinase Interacting Protein 1
MTORC1/2     Mammalian Target of Rapamycin Complex 1/2
NAD          Nicotinamide Adenine Dinucleotide
NAD(P)H      Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NADH         Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
NEM          N-Ethylmaleimide
NF- KB       Nuclear factor kappa B
NFAT         Nuclear Factor of Activated T-Cells
NF-KB        Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NIM811       N-Methionine-4-Isolecine-Cyclosporin A
NNT          Nicotinamide Nucleotide Transhydrogenase
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</tr>
<tr>
<td>Opa1</td>
<td>Dynamin-Like 120 KDa Protein</td>
</tr>
<tr>
<td>OSCP</td>
<td>Oligomycin Sensitivity-Conferring Protein</td>
</tr>
<tr>
<td>OXA</td>
<td>Oxidase Assembly (OXA) Translocase</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative Phosphorylation</td>
</tr>
<tr>
<td>P50 (NFκB1)</td>
<td>Nuclear factor NF-kappa-B p105 subunit</td>
</tr>
<tr>
<td>P52 (NFκB2)</td>
<td>Nuclear factor NF-kappa-B p100 subunit</td>
</tr>
<tr>
<td>P53</td>
<td>Cellular tumour antigen p53</td>
</tr>
<tr>
<td>P62</td>
<td>Ubiquitin-Binding protein, sequestosome</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate Dehydrogenase</td>
</tr>
<tr>
<td>PDHE1α</td>
<td>Pyruvate Dehydrogenase E1 subunit alpha</td>
</tr>
<tr>
<td>PDHK1</td>
<td>Pyruvate Dehydrogenase Kinase 1</td>
</tr>
<tr>
<td>PGC-1alpha</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin Homology</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl Hydroxylases</td>
</tr>
<tr>
<td>PHLPP1</td>
<td>Pleckstrin Homology Domain Leucine Rich Repeat Containing Protein Phosphatases</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-Kinases</td>
</tr>
<tr>
<td>PiC</td>
<td>Phosphate Carrier</td>
</tr>
<tr>
<td>PINK1</td>
<td>PTEN-Induced Kinase 1</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)-Trisphosphate (PtdIns (3,4,5) P3)</td>
</tr>
<tr>
<td>PLIN</td>
<td>Perilipins</td>
</tr>
<tr>
<td>PMF</td>
<td>Protein Motive Force</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein Phosphatase 2A</td>
</tr>
<tr>
<td>PPIase</td>
<td>Peptidyl Prolyl <em>cis-trans</em> Isomerase</td>
</tr>
<tr>
<td>PPID</td>
<td>Peptidyl-Prolyl Cis-Trans Isomerase D</td>
</tr>
<tr>
<td>PPIF</td>
<td>Peptidyl-Prolyl Cis-Trans Isomerase, Mitochondrial</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-Rich AKT Substrate 40kDa</td>
</tr>
<tr>
<td>PROTOR-1</td>
<td>Protein Observed with Rictor-1</td>
</tr>
<tr>
<td>PRX</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>PUMA</td>
<td>P53 Upregulated Modulator of Apoptosis</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von-Hippel Lindau Protein</td>
</tr>
<tr>
<td>RAPTOR</td>
<td>Regulatory-Associated Protein of mTOR</td>
</tr>
<tr>
<td>REL</td>
<td>Rel-Like Domain-Containing Proteins</td>
</tr>
<tr>
<td>RET</td>
<td>Reverse Electron Transport</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel Homology Domain</td>
</tr>
<tr>
<td>RHEB</td>
<td>Ras Homolog Enriched in Brain</td>
</tr>
<tr>
<td>RICTOR</td>
<td>Rapamycin-Insensitive Companion of mTOR</td>
</tr>
<tr>
<td>RISK</td>
<td>Reperfusion Injury Salvage Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SAFE</td>
<td>Survival Activation Factor Enhancement</td>
</tr>
<tr>
<td>SAM50</td>
<td>Sorting Assembly Machinery 50 KDa Subunit</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate Dehydrogenase</td>
</tr>
<tr>
<td>SGK1</td>
<td>Serum/Glucocorticoid Regulated Kinase 1</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second Mitochondria Derived Activator of Caspases</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second Mitochondrial Derived Activator of Caspases</td>
</tr>
<tr>
<td>SNO</td>
<td>S-Nitrosylation</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SPG7</td>
<td>Spastic Paraplegia 7</td>
</tr>
<tr>
<td>TNF-Alpha</td>
<td>Tumor Necrosis Factor Alpha</td>
</tr>
<tr>
<td>TOM40</td>
<td>Translocase of Outer Mitochondrial Membrane 40</td>
</tr>
<tr>
<td>TRAP1</td>
<td>TNF Receptor-Associated Protein 1</td>
</tr>
<tr>
<td>TRX</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Proteins.</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling Protein</td>
</tr>
<tr>
<td><strong>VDAC</strong></td>
<td>Voltage Dependent Anion Channel</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td><strong>YME1L</strong></td>
<td>ATP-dependent zinc metalloprotease</td>
</tr>
</tbody>
</table>
Chapter One

Introduction
1 Introduction ........................................................................................................................................ 5
1.1 Overview of the mitochondria ........................................................................................................ 5

1.1.1 Different perspectives of the mitochondria .................................................................................. 5

1.1.2 Morphological Features of the Mitochondria .............................................................................. 5

1.1.3 Mitochondrial Compartmentalisation and Function ..................................................................... 7

1.1.3.1 Mitochondrial Outer Membrane .............................................................................................. 8

1.1.3.1.1 Solute and Protein Transport .............................................................................................. 8

1.1.3.1.2 Mitochondrial Apoptosis .................................................................................................. 9

1.1.3.1.3 Mitophagy .......................................................................................................................... 12

1.1.3.2 Mitochondrial Inner Membrane ............................................................................................. 13

1.1.3.2.1 Respiratory Chain ............................................................................................................. 14

1.1.3.2.2 Experimental States of Respiration .................................................................................. 16

1.1.3.2.3 Mitochondria Proton Motive Force .................................................................................. 16

1.1.3.2.4 Mitochondrial ATP Production ......................................................................................... 17

1.1.3.3 Mitochondrial Matrix .............................................................................................................. 19

1.1.3.3.1 Mitochondrial DNA .......................................................................................................... 20

1.1.3.3.2 Mitochondrial TCA cycle .................................................................................................. 20

1.1.3.3.3 Mitochondrial Matrix Quality Control ................................................................................ 22

1.2 Mitochondrial ROS Production and Control .................................................................................. 23

1.2.1 Mitochondrial Superoxide ............................................................................................................ 23

1.2.1.1 Superoxide at Complex I ...................................................................................................... 25

1.2.1.2 Superoxide at Complex III .................................................................................................. 26

1.2.2 Measurement of Mitochondrial Superoxide ................................................................................ 27

1.2.3 ROS induced Protein Modification ............................................................................................ 28

1.2.4 Mitochondrial Antioxidant Defence Systems ............................................................................ 29

1.2.5 Mitochondrial Inner Membrane Uncoupling or Proton leak .................................................... 31

1.2.5.1 Small Molecule Uncouplers .................................................................................................. 32

1.2.5.2 Endogenous Protein Uncouplers ............................................................................................ 32

1.2.5.2.1 Basal Proton Leak ............................................................................................................. 32

1.2.5.2.2 Inducible Proton Leak ....................................................................................................... 33
1.2.5.3 Cation Flux and Membrane Potential Dissipation .......... 34

1.2.6 Mitochondria Retrograde Signalling .................................. 35

1.2.6.1 Mitochondrial ROS Signalling Pathways ............................ 36
    1.2.6.1.1 Hypoxia Inducible Factor 1 (HIF-1) Pathway ............... 36
    1.2.6.1.2 Cellular Lipid Droplets Formation ........................... 37
    1.2.6.1.3 NF-KB Signalling .............................................. 39
    1.2.6.1.4 PI3K/AKT Signalling ......................................... 40

1.2.6.2 Mitochondrial Metabolism and Cell Signalling .................. 42
    1.2.6.2.1 mTOR Pathway .................................................. 42
    1.2.6.2.2 AMPK Signalling ............................................... 44

1.3 Mitochondrial Cyclophilin D .............................................. 44

    1.3.1 Inhibitors of CypD .................................................. 47
    1.3.2 CypD and Protein Folding ......................................... 48

    1.3.3 CypD and the Mitochondrial Permeability Transition Pore
           (MPTP)51
        1.3.3.1 Components of the MPTP ..................................... 52
           1.3.3.1.1 CypD as an MPTP regulator ............................... 52
           1.3.3.1.2 Previous Hypothesized Molecular Interactors of CypD. 52
                1.3.3.1.2.1 VDAC-ANT-CypD interaction .................................. 52
                1.3.3.1.2.2 CypD-PiC-ANT interaction .................................... 54
                1.3.3.1.2.3 Misfolded protein Aggregates ............................... 55
           1.3.3.1.3 Currently Debated Molecular Interactors of CypD ...... 56
                1.3.3.1.3.1 ATP Synthase Dimer Hypothesis ............................. 56
                1.3.3.1.3.2 ATP Synthase C Ring Hypothesis ............................. 58
                1.3.3.1.4 Other Possible CypD-Mediated MPTP Models ............ 59

    1.3.3.2 Mitochondrial matrix proteins and CypD-mediated MPTP . 60
        1.3.3.2.1 Mitochondrial Chaperones Stress response and MPTP60

1.3.4 Post translational modification of CypD ............................. 61

    1.3.4.1 S-Nitrosylation .................................................. 61
1.3.4.2 Acetylation ................................................................. 61
1.3.4.3 Phosphorylation .......................................................... 62

1.3.5 Role of CypD in Disease Pathology ................................. 62
   1.3.5.1 Role of CypD in Amyloid Beta-Induced Toxicity ........... 62
   1.3.5.2 Role of CypD in Ischemia-Reperfusion Injury ............... 63

1.4 Aim and Objectives ........................................................... 64
   1.4.1 Purification of Recombinant and Quantitation of mitochondrial Cyclophilin D .............................................................. 65
   1.4.2 Assessment of the Role of Cyclophilin D in the Regulation of Mitochondrial Redox and Energy Homeostasis ......................... 65
   1.4.3 Evaluation of Metabolic Reprogramming in CypD Deficient Cells 65
1 Introduction

1.1 Overview of the mitochondria

1.1.1 Different perspectives of the mitochondria

Mitochondria are dynamic, pleomorphic organelles generally regarded as the power house of the cell (Logan, 2006). The name mitochondrion was coined from the Greek words, ‘mitos’ (thread) and ‘chondros’ (granule) by Benda in 1898 (Ernster and Schatz, 1981; Westermann, 2012). Mitochondria have become the focus of intensive research and scrutiny for the past six decades. Indeed, multiple fields of biological science have viewed this important organelle differently, and have form varying opinion on its biological importance.

For the biochemist, mitochondria are the hub of cellular respiration-linked phosphorylation, governed by a complex set of molecular processes involved in the coupling of matrix oxidation of carbon skeleton to the phosphorylation of ADP in the inner mitochondrial membrane. For the cytologist, mitochondria presented the experimental samples for the study of membrane structures and tissue and organellar fractionation. The physiologists have a somewhat different view; they perceived the mitochondria as tools for functional studies, particularly, those involved in membrane transport and metabolic regulation (Ermster and Schatz, 1981).

1.1.2 Morphological Features of the Mitochondria

Mitochondria are characterised with different morphological features dictated by either the cell type or physiological condition. They usually present as dynamic interconnected networks; however, occasionally, they exist as distinct spherical small organelles (Kuznetsov et al., 2009). Notably, physiological conditions that regulate mitochondrial morphology are mainly cristae transition (Logan et al., 2004) and fission-fusion events. Fluorescence confocal and electron microscopy have contributed immensely to the study and analysis of their morphology, behaviour and distribution of the mitochondria in time and
space (Ong et al., 2010; Westermann, 2012). Mitochondria morphology can vary from the following features: regular patterns, dense networks, elongated threads, small spheres, short rod-like shapes and long filaments. Figure 1.1 describes the various mitochondrial morphological features acquired by live cell imaging. In humans, mitochondria shaping proteins include Opa1, Mfn1, Mfn2, Drp1, Fis1 and Mtp18. These proteins are involved in mitochondria fission-fusion dynamics. These proteins are either permanent or temporary residents of the mitochondria.

Figure 1.1: Variety of Mitochondrial Morphology (Kuznetsov et al., 2009). (A) Arrangement of mitochondria in adult rat heart tissues. (B) Mitochondria from rat skeletal muscles. (C) Human pancreatic cells thread-like mitochondria. (D) Mitochondrial networks in HL-1 cells with cardiac phenotype. (E) Isolated rat liver mitochondria. (F) Mitochondria of small promyeloid cells. Mitochondrial images were obtained by confocal microscopy using autofluorescence of mitochondrial flavoproteins (E) or fluorescent signals from either TMRM or MitoTracker (A, B, C, D and F). Image was obtained with permission from Elsevier.
Mfn1 and 2 were discovered in 2001 as outer mitochondrial membrane proteins involved in mitochondrial fusion. They are G-proteins containing a GTPase domain and two coiled coil domains (Heptad repeat 1 and 2; HR 1 and 2). Both GTPase and HR2 domain are essential for mitochondrial fusion, as they are both exposed to the cytosol. Mitochondria undergoing fusion are tethered by either homotypic or heterotypic adjacent HR2 domains of Mfn (Schrepfer and Scorrano, 2016).

DRP1 was first identified in 1998 as mainly a cytosolic G-protein that translocate to the outer mitochondrial membrane to activate mitochondrial fission at scission sites (Yoon et al., 1998). Notably, Drp1 does not possess a transmembrane domain and so exerts its effect on the mitochondria through oligomerisation and constriction at scission sites or directly interacting with resident outer mitochondrial membrane proteins such as Fis1 (Yoon et al., 2003). The activity of Drp1 is regulated post-translationally by s-nitrosylation, ubiquitination, phosphorylation and sumoylation. All these modifications have been shown to activate Drp1, hence increasing mitochondrial fission (Ong et al., 2010).

Fis1 was also discovered in 2001 as a promoter of mitochondrial fission (Ong et al., 2010). Unlike Drp1, it is localised as a permanent resident of the outer mitochondrial membrane. It is reported to bind and inhibit the GTPase activities of fusion proteins such as Mfn1, Mfn2 and OPA1, hence repressing mitochondria fusion. Conversely, inhibition of Fis1 resulted in mitochondrial elongation (Yu et al., 2019).

1.1.3 Mitochondrial Compartmentalisation and Function
Like every other organelle, mitochondria are membrane bound; however, unlike every organelle, mitochondria are divided into three compartments: a smooth outer membrane, folded inner membranous cristae and a protein-rich matrix (Logan, 2006). These compartments play distinct roles in the
mitochondria and the status of their integrity contributes immensely to cellular health and disease.

1.1.3.1 Mitochondrial Outer Membrane

The mitochondrial outer membrane is the entry point into the mitochondria. They are porous membrane structures that accommodate the passage of small and uncharged molecules through the abundant pore-forming proteins present on it, while preventing the passage of larger molecules such as peptides in and out of the mitochondria. Because of their permeability to small molecules and ions, the outer membrane is devoid of $\Delta \Psi_m$ (Kühlbrandt, 2015). Several mitochondrial functions are executed on the outer membrane. Some of these functions include solute and protein transport, fission and fusion processes, apoptosis and mitophagy. Notably, the role of the outer membrane in fission-fusion events have already been discussed in the previous section (Section 1.1.2).

1.1.3.1.1 Solute and Protein Transport

Not much channel-forming proteins have been associated with the outer mitochondrial membrane. Indeed, all the channel-forming proteins of the outer mitochondrial proteins are beta-barrel proteins. A typical and most prominent outer mitochondrial membrane protein is the voltage dependent anion channel (VDAC), otherwise known as porin (Naghdi and Hajnoczky, 2016). VDAC/Porins are regulated channels that mediates the flux of ions and metabolites across the outer mitochondrial membrane (Kruger et al., 2017). They translocate anions at high conductance states while showing preferences for cations at low conductance states (Campo et al., 2017).

Other known channels of the outer membrane are those involved in protein transport; Tom40, a member of the translocase of outer mitochondrial membrane complex and Sam50, a member of the sorting and assembly machinery (Bredemeier et al., 2007). As their names implied, these two proteins play separate roles on the outer membrane. Tom40 translocate
precursor proteins into the mitochondria while Sam50 enables the insertion of beta barrel precursors into the outer membrane (Kruger et al., 2017). A fourth well characterised channel of the outer membrane is mitochondrial distribution and morphology protein Mdm10 which promotes the biogenesis of TOM by associating with the SAM machinery. It is also well known for providing enabling environment for mitochondria-ER tethering (Ellenrieder et al., 2016). Apart from VDAC, these other channels and protein transporters of the outer membrane preferentially translocate cations in and out of the mitochondria (Poynor et al., 2008; Kruger et al., 2017).

1.1.3.1.2 Mitochondrial Apoptosis
At the time of its introduction in 1972, ‘Apoptosis’ was defined as programmed cell death (Kerr et al., 1972; Luna-Vargas et al., 2016). Indeed, other forms of programmed cell death now exist, and these include; necroptosis and ferroptosis (Dhuriya and Sharma, 2018; Mou et al., 2019). Apoptotic cell death is characterised by the activation of caspases that facilitates the proteolysis of vital cellular components that are involved in DNA repair and cytoskeletal functions. This event signals the detection and clearance of compromised cells by phagocytes accompanied by reduced damage to the cell microenvironment. The mitochondrial outer membrane plays significant role in mediating apoptosis via the intrinsic pathway (Reidl and Salvesen, 2007). Stress stimuli converge on the outer mitochondrial membrane thereby resulting in mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c into the cytosol. Cytosolic cytochrome c binds apoptotic protease activating factor 1 (APAF 1) and pro-caspase 9 to form the apoptosome (Riedl and Salvesen, 2007), resulting in the downstream activation of executioner caspases -3, -6 and -7 (Yuan and Akey, 2013; Luna-Vargas et al., 2016)).

The outer mitochondria membrane is home to some members of the B cell lymphoma 2 (BCL-2) family of proteins that regulates the mitochondrial pathway of apoptosis. Interaction between members of this family of proteins
is crucial for MOMP. Members of the BCL-2 family of proteins are divided into pro-apoptotic and anti-apoptotic proteins and this is based on the composition of their BCL-2 homology 1-4 (BH1-4) domains (Westphal et al., 2011; Luna-Vargas et al., 2016). The Anti-apoptotic proteins contain BH1-4 domains while pro-apoptotic proteins contain only BH3 except for apoptosis effector proteins; BAK and BAX which possess BH1-4 domains (Kuwana et al., 2005; Chipuk and Green, 2008; Westphal et al., 2011) while BOK possess BH1-3 (Hsu et al., 1997; Harris and Thompson, 2000). Members of the pro- and anti-apoptotic subfamily are represented in Table 1.1.

**Table 1.1: Regulation of BCL 2 family on the outer mitochondrial membrane**

<table>
<thead>
<tr>
<th>Apoptotic regulator</th>
<th>BH domain</th>
<th>Interaction with effector BAK/BAX</th>
<th>Apoptosis orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>1-4</td>
<td>Yes</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>1-4</td>
<td>Yes</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>Bcl-w</td>
<td>1-4</td>
<td>Yes</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>1-4</td>
<td>Yes</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>A1</td>
<td>1-4</td>
<td>Yes</td>
<td>Anti-apoptotic</td>
</tr>
<tr>
<td>Bid</td>
<td>3</td>
<td>Yes</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Bim</td>
<td>3</td>
<td>Yes</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Puma</td>
<td>3</td>
<td>Yes</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Noxa</td>
<td>3</td>
<td>No</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Bad</td>
<td>3</td>
<td>No</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Hrk</td>
<td>3</td>
<td>No</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Bmf</td>
<td>3</td>
<td>No</td>
<td>Pro-apoptotic</td>
</tr>
<tr>
<td>Bik</td>
<td>3</td>
<td>No</td>
<td>Pro-apoptotic</td>
</tr>
</tbody>
</table>

Data obtained from Westphal et al., 2011.
BH3 only pro-apoptotic proteins antagonise the effect of mitochondrial outer membrane anti-apoptotic proteins while activating effector pro-apoptotic effector proteins on the outer mitochondrial membrane, Bax/Bak. Extrinsic pathways activate the truncation and translocation of Bid through caspase 8. Truncated Bid also inhibits anti-apoptotic proteins present on the outer membrane while activating effector Bax/Bak proteins. Following these events, cytochrome C is released from the intermembrane space and pro-caspases 3 and 9 are activated to mediate apoptosis. Image was adapted from Fox and MacFarlane, 2016.

Activation of MOMP is mediated by apoptotic effector proteins BAK, BAX and BOK following their interaction with certain members of the pro-apoptotic subfamilies as shown in Table 1.1. This interaction results in their oligomerisation at the outer membrane, hence resulting in pore formation (Lindsten et al., 2000). However, between the two widely studied pore-forming apoptotic effector proteins BAK and BAX, only BAK is a constitutive resident of the outer membrane, as the presence and interaction with the anti-apoptotic member BCL-XL results in retro-translocation of BAX to and from the outer membrane which is abrogated upon apoptotic stimuli (Schellenberg et al., 2013). Such stimuli include the truncation of BID by caspase 8 resulting in BID-BAX interaction and activation of membrane localisation (Luna-Vargas et al., 2000).
2016). Indeed, the presence of anti-apoptotic subfamilies on the outer membrane prevents their oligomerisation and the subsequent release of cytochrome c and Smac/DIABLO from the intermembrane space into the cytosol (Westphal et al., 2011). However, the mechanism of the pore-forming oligomerised BAK/BAX remains elusive.

1.1.3.1.3 Mitophagy
Mitophagy is a form of macro-autophagic catabolic process coined by Lemasters and colleagues (Kim et al., 2007). It is directed at eliminating dysfunctional or superfluous mitochondria preventing them from adversely affecting the surrounding mitochondrial network. In addition, mitophagy occurs beyond quality control to adjust the mitochondria to the dynamic cellular metabolic requirement (Kissová et al., 2004; Youle and Narendra, 2011). The outer mitochondrial membrane is the base for mitophagy signals, usually mediated by specific proteins such as BNIP3L and PINK1/parkin.

PINK1 is a ubiquitously expressed outer mitochondrial membrane protein that is rapidly degraded by proteolysis in healthy mitochondria (Matsuda et al., 2010, Narendra et al., 2010; Youle and Narendra, 2011). However, upon mitochondrial damage mostly due to the loss of its $\Delta \Psi_m$, PINK1 rapidly accumulates on the outer membrane, facilitating the recruitment of the E3 ubiquitin ligase parkin to the outer membrane (Shiba et al., 2009; Matsuda et al., 2010). The ubiquitylation of mitochondrial substrates at Lys 63 results in the sequestration and aggregation of p62 on the mitochondrial membrane, thereby leading to autophagosome binding, hence mitophagy. Following PINK1 mitochondrial accumulation, parkin has also been shown to ubiquitylate a number of other outer membrane proteins, such as VDAC (Ordureau et al., 2018), Mfn1, Mfn2 (Tanaka et al., 2010; Basso et al., 2018). Mitofusin ubiquitylation by parkin results in their degradation, thus, promoting mitochondrial fragmentation while preventing the fusion of damaged mitochondria with the remaining mitochondrial network (Youle and Narendra, 2011; Basso et al., 2018).
BNIP3L also known as NIX is upregulated on the outer mitochondria membrane during hypoxia via HIF-induced transcriptional activity (Esteban-Martinez et al., 2018; Qiu et al., 2019). It is believed to be responsible for mitophagy-mediated mitochondria clearance in red blood cells, as mature red blood cells lack mitochondria. BNIP3L directly interacts with LC3, an autophagosome ubiquitin like protein, via its cytosolic facing WXXL-like motif. Recruitment of LC3 to the mitochondria results in autophagosome engulfment and mitophagy (Novak et al., 2010; Pickles et al., 2018). NIX functions independently of the PINK1/parkin pathway, as mitophagy is still activated during BNIP3L deficiency following $\Delta \Psi_m$ depolarisation (Youle and Narendra, 2011; Yuan et al., 2017).

1.1.3.2 Mitochondrial Inner Membrane
Mitochondria inner membrane encompasses the matrix and it is uniquely folded into cristae structures that confers it with large surface area (Mannella, 2006; Kim, 2014), particularly for the diffusion of oxygen during respiration. It is highly abundant in proteins, as its phospholipid content is about 30% (Milenkovic et al., 2017). The inner membrane is home to various solute carrier proteins (Gutiérrez-Aguilar and Baines, 2013), and other numerous enzymes such as the respiratory chain complexes which are directly linked to the matrix, making it the limiting factor for several matrix enzyme activities. It is also closely associated with the mitochondrial DNA (Friedman and Nunnari, 2014). The matrix side is also widely recognised for the $\Delta \Psi_m$ component of its proton motive force, which is coupled to ATP production (Martin, 1991; Walker, 2013), solute/protein transport (Geissler et al., 2000; Maldonado et al., 2016), ROS quenching (Zorov et al., 2014), $\text{Ca}^{2+}$ buffering (Friedman and Nunnari, 2014) and thermogenesis (Porter, 2017). It plays direct roles in regulating energy metabolism, mitochondria morphology (Opa1), mitophagy (PINK1/parkin pathway), apoptosis/necrosis (Mitochondrial permeability transition pore) and cellular ROS signalling. Mitochondrial proteins are translated by oxidase assembly (OXA), whereby the translating ribosomes are coupled to insertase
present in the inner membrane and are directly translated into the membrane (Pfanner et al., 2019).

1.1.3.2.1 Respiratory Chain

The respiratory chain involves the transfer of electrons from reducing equivalent to molecular oxygen in the inner mitochondrial membrane. It is facilitated by respiratory complexes I-IV, which are about 1.4nm apart (Murphy, 2009). Electrons are transferred in step-wise manner aided by the diffusion of lipophilic ubiquinone and membrane-anchored cytochrome c which respectively embed within the membrane and the external surface of the inner membrane (Lobo-Jarne and Ugalde, 2018). The initial electron acceptors of the respiratory chain are complex I (NADH dehydrogenase:ubiquinone oxidoreductase) and complex II (succinate:ubiquinone oxidoreductase, otherwise referred to as succinate dehydrogenase complex). These respectively accepts electrons from NADH and FADH$_2$ and transfer to ubiquinone, reducing it to ubiquinol. The reduced ubiquinol rapidly transfers the electrons to complex III (ubiquinol:cytochrome c oxidoreductase) and then to cytochrome c, and finally to complex IV (cytochrome c oxidase). Complex IV reduces molecular oxygen to water molecules within the inner membrane.

The electrogenic proton transfer from the matrix to the intermembrane through complexes I, III and IV creates an electrochemical potential and free energy or proton motive force which is coupled to ATP synthesis via the ATP synthase (Nath, 2019). This concept popularly regarded as the chemiosmotic theory was proposed by Peter Mitchell in 1961 (Mitchell, 1961) which earned him a Nobel Prize in 1978. There are no evidences of proton pump at complex II, while complex I, III and IV pumps 4H$^+$ each from the matrix to the intermembrane space. The coupling of electron transfer to ATP synthesis in the mitochondrial inner membrane is termed oxidative phosphorylation.
The transfer of electrons from NADH and oxidizable substrates to mobile carriers in the inner mitochondrial membrane are accompanied by proton transfer across the membrane into the intermembrane space creating both chemical and electrical gradient otherwise known as an electrochemical gradient. Since the inner membrane is impermeable to protons, a proton motive force is generated, and this drives protons back into the matrix through protons specific channels, the FO component of the F1FO ATP synthase. The energy generated from the downward flow of protons through the electrochemical gradient is used to synthesize ATP from ADP and Pi at the F1 component of the ATP synthase. OMM, outer mitochondrial membrane, IMM, inner mitochondrial membrane.

In the last twenty years, the respiratory chain complexes have emerged to exist largely as supercomplexes, defying the originally accepted fluid state model where individual respiratory components diffuse feely in the membrane while electrons are carried through coenzyme Q and cytochrome C through undefined paths to these complexes (Milenkovic et al., 2017; Lobo-Jarne and Ugalde, 2018). Complex II is not involved in supercomplex formation. However, supercomplexes of CI/CIII2/CIV1-4, CI/CII2, and CIII2/CIV1-2 have been shown to exist using blue native PAGE. Notably 85%-100% of CI, 55%-65% of CIII and 15%-25% of CIV are estimated to exist as supercomplexes (Milenkovic et al., 2017). Supercomplexes consisting of complexes I, III and IV are referred to as respirasomes. Indeed, cardiolipin, a highly abundant phospholipid in the inner mitochondria membrane is suggested to stabilise supercomplexes in the mitochondria (Mileykovskaya and Dowhan, 2014). It has been proposed that supercomplex formation decreases ROS production, regulates respiratory chain activities, prevents protein aggregation in the inner mitochondrial membrane.

### 1.1.3.2.2 Experimental States of Respiration

There are four experimentally determined respiratory states in the mitochondria. These are measured by the rate of oxygen consumption in an airtight temperature-controlled oxygen tension measuring chamber. State 1 respiration is defined as the basal oxygen consumption upon the addition of isolated mitochondria to the chamber while state 2 respiration is measured when one or more respiratory substrates are added to the mitochondrial suspension. State 3 and 4 respiration are different as they involve the alteration of the respiration rate by the dissipation of the proton motive force. Thus, state 3 respiration is the maximal oxygen consumption rate, following the addition of molecules that dissipates the proton motive force such as ADP (dissipation via the ATP synthase) or protonophores (FCCP). Upon inhibition of the ATP synthase with oligomycin, provided that state 3 respiration is induced by ADP, the respiratory rate reverts to state 2. However, oxygen consumption during basal proton leak when the ATP synthase is excluded is referred as state 4 (Hoeks *et al.*, 2012).

### 1.1.3.2.3 Mitochondria Proton Motive Force

The mitochondrial proton motive force (PMF) is created by the proton gradient across the inner mitochondrial membrane. It consists of both pH and $\Delta \Psi_m$ components; with strong contribution from the $\Delta \Psi_m$ component. Physiologically, the $\Delta \Psi_m$ component is estimated to be about -180mv, and it is determined the inward transport of cations and the export of anions (Feng *et al.*, 2019). Examples include the mitochondrial importation of $\text{ADP}^3$ in exchange for $\text{ATP}^4$ by the electrogenic ANT present in the inner mitochondria membrane (Maldonado *et al.*, 2016). Other notable cation influxes are $\text{Fe}^{2+}$ transport for Fe-S cluster formation (Zorov *et al.*, 2007), and $\text{Ca}^{2+}$ transport by
the mitochondrial Ca\(^{2+}\) uniporter (De Stefani et al., 2016). Indeed, significantly low \(\Delta \Psi_m\) can also put brakes on mitochondrial protein transport.

As much as high PMF is important for bioenergetics, it should also be known that its elevation is a significant contributor to mitochondrial ROS, especially the pH component which is determined by the proton gradient across the membrane. Therefore, excessive PMF can be toxic to the mitochondria, as it creates stronger electrochemical force for proton translocation across the membrane, thus, slowing down the ETC. However, excessively low PMF is also detrimental to cell survival, as dissipation of the \(\Delta \Psi_m\) would proceed into loss of mitochondrial function. Interestingly, moderate decline in the PMF can alleviate mitochondrial ROS when protons leak from the intermembrane space into the matrix, creating both an acidic matrix environment and lower electrochemical barrier, enabling forward electron transfer in the ETC and proton translocation to the intermembrane space (Zorova et al., 2018). This paves the way for harnessing potential benefits of mild mitochondrial uncouplers in pathological conditions that are underlined by mitochondrial ROS.

Due to the rather small size of the mitochondria, fluorescent probes have been employed to measure \(\Delta \Psi_m\) component of the PMF across the inner mitochondrial membrane. Examples of these probes include; Tetramethylrhodamine (TMRM; 580nm), Mitotracker green (MTG; 510) and the dual colour probe JC-1 which detects high and low \(\Delta \Psi_m\) at 595nm and 525nm, respectively (Feng et al., 2019).

### 1.1.3.2.4 Mitochondrial ATP Production

Re-emphasizing the popular saying, ‘ATP is the energy currency of the biological world’ (Walker, 2013), it is indeed remarkable to understand that the mitochondria generate more than 95% of ATP in cardiac tissues during normoxic conditions while the remainder of the ATP results from a combination of glycolysis and other forms of substrate level phosphorylation (Doenst et al.,
This confers immense and significant importance on the mitochondrial ATP synthase otherwise known as the F1FO ATP synthase.

The ATP synthase is divided into two domains; the FO regulatory and the F1 (fraction 1) catalytic domain. The FO domain consists of three main a, b, c subunits and other associated subunits, d, e, f, g, A6L, DAPIT, 6.8KDa, F6, and OSCP. The F1 domain is made up of α, β, γ, δ, ε subunits with a stoichiometry of 3:3:1:1:1 respectively (Walker, 2013). The proton motive force generated by the electron transport chain is indirectly coupled to the catalytic phosphorylation of ADP by the ATP synthase. A rotary and step-wise transfer of protons through c subunit at the upper half of the a-c interphase mediates a clockwise rotation and eventual proton release into the matrix via the second half of the a-c interphase, this results in a 360° rotation of the γ subunit, hence providing energy to produce three molecules of ATP at the β subunit in the F1 domain. This mechanism is called ‘the binding change mechanism’ (Boyer, 1993; Walker, 2013). Solving both the structure and the mechanism of the F1 component of this splendid molecular machine greeted John Walker and Paul Boyer with a Nobel Prize in chemistry in 1997.

ATP synthase can exist in more complex structures, more likely as an adaptation to more robust ATP synthesis. Dimers, oligomers and supramolecular complexes with ANT and PiC referred as the ATP synthasome revealed the need for an efficient energy production both in higher animals and single-celled organisms (Ko et al., 2003; Clémençon, 2012; Guo et al., 2017). Indeed, the ATP synthase and ANT are the most abundant proteins of the inner mitochondrial membrane (Liu and Chen, 2013; Pfanner et al., 2019). Of note, electron microscopy has revealed an array of ATP synthase dimers at bends or curvature of the inner mitochondria membrane, suggesting that they contribute to cristae curvature, hence mitochondrial morphology (Blum et al., 2019).
1.1.3.3 Mitochondrial Matrix

The mitochondrial matrix is the centre of oxidative metabolism enclosed by the inner mitochondrial membrane. It contains the machineries for the TCA cycle and fatty acid beta oxidation to metabolise carbon fuels such as pyruvate, ketoacids, amino acids and fatty acids to yield reducing equivalents (NADH and FADH$_2$) which is used to produce ATP and NADPH (Paul et al., 2017). Cataplerotic reactions of the mitochondrial TCA lead to the biosynthesis of cholesterol, haem, pyrimidine, amino acids, lipids and other metabolites (Cheng and Ristow, 2013; Paul et al., 2017).

The matrix mediates cellular Ca$^{2+}$ homeostasis by sequestering excessive cytosolic Ca$^{2+}$ for storage, signalling and regulation of cell death (Paupe and Prudent, 2018). It is also involved in iron homeostasis, sequestering cytosolic iron for the synthesis of haem and Rieske Fe-S clusters which are incorporated as prosthetic groups cytochrome bc1, cytochrome c, respiratory chain complexes, and TCA cycle enzyme, aconitase (Farhan et al., 2014; Paulos, 2014).

The matrix chaperones (mtHSPs; 70, 60 and 10) and PPIase (CypD) mediates protein homeostasis and ensures the proper folding of mitochondrial proteins (Porter and Beutner, 2018). It also houses all the proteins involved in the maintenance of the mitochondrial genome (Ott et al., 2016). Furthermore, quality control activities of the matrix include the sequestration of the integrated stress factor, ATF5 (ATFS-1) into the matrix by the inner $\Delta\Psi_m$ dependent mitochondrial import machinery and degradation by the matrix Lon protease (LonP) (Nargund et al., 2012). Upon disturbance of protein import, ATF5 is translocated into the nucleus where it induces the transcription of stress response proteins, hence promoting mitochondrial unfolded protein response (Melber and Haynes, 2018; Pfanner et al., 2019).
1.1.3.3.1 Mitochondrial DNA
Mitochondrial genome of all existing organisms has sizes ranging from 15-60kbp (Burger et al., 2003; Ingman and Gyllensten, 2006). The human mitochondrial genome is a circular supercoiled DNA of 16,298bp with no introns, encoding 13 polypeptides, 2 rRNA and 22 tRNAs. Remarkably, all 13 polypeptides in the human mitochondria are constituents of the electron transport chain, hence indicating that most of the approximately 1500 proteins that make up the mitochondria proteome are nuclear encoded (Friedman and Nunnari, 2014; El-Hattab et al., 2017). Transcriptional regulation of these mitochondrial proteins is widely coordinated by the nucleus via PGC-1alpha, NRF1, NRF2 and ERR (Friedman and Nunnari, 2014). However, other forms of transcriptional regulation may exist within the mitochondria.

In addition, all the battery of enzymes, replisomes, necessary for mitochondrial DNA replication are nuclear encoded (El-Hattab et al., 2017). Because of the multiple copy numbers of mitochondrial DNA in a single mitochondrion, in addition to the numerous and different mitochondria filaments that could exist in a cell, variable amount of mutations may exist on the mitochondria DNA, which is referred as heteroplasmy. Indeed, the severity of the mutation on organismal health is highly dependent on the mutant load (Stefano et al., 2017). Notably, mutations in a gene have been reported to present with separate outcomes in adults and infants (Sonam et al., 2015). For example complex I mutation results in optic atrophy in adults while infants’ manifests encephalopathy (Friedman and Nunnari, 2014).

1.1.3.3.2 Mitochondrial TCA cycle
The TCA cycle is the central hub of mitochondrial metabolism due to its anaplerotic and cataplerotic characteristics. It was discovered in 1937 by Hans Krebs which got him his Nobel Prize in Physiology in 1953. The TCA cycle begins with the condensation of two-carbon acetyl-CoA with four-carbon oxaloacetate to generate six-carbon citrate. Other intermediates of the TCA include; isocitrate, 2-oxoglutarate, succinyl-CoA, succinate, fumarate, and
malate. Indeed, 3 mols of NADH, 1 mol of FADH₂, 2 mols of CO₂ and 1 mol of GTP/ATP are produced from a single cyclic run. NADH and FADH₂ of the TCA are coupled to the inner membrane oxidative phosphorylation. The major anaplerotic amino acid of the TCA cycle is glutamine which produces 2-oxoglutarate when citrate is exported for lipid synthesis, while 2-oxoglutarate is oxidised to maintain the forward flux of the TCA cycle.

The reductive equivalent, NADH, and the ultimate product of OXPHOS, ATP are inhibitors of the TCA cycle. NADH represses the activity of all regulatory enzymes of the TCA, most predominantly IDH and OGDH, while ATP inhibits IDH and PDH. Indeed, elevated acetyl-CoA inhibits PDH but activates PC to produce oxaloacetate which drives the TCA forward flux. The TCA intermediate succinyl-CoA inhibits OGDH and CS while oxaloacetate inhibits SDH to slow down the cycle as the accumulating intermediates are used up (Martinez-Reyes et al., 2020).

The TCA cycle was initially thought to only produce metabolites for the sole intention of anabolic processes such as biosynthesis of proteins, lipids and nucleotides. However recently, other functions of the mitochondrial TCA cycle have emerged, and these include, regulation of chromatin modification, post translational modification of proteins and DNA methylation.

Cytosolic acetyl-coA is mainly derived from the mitochondrial exported cytosolic citrate, catalysed by cytosolic acetyl-coA lyase. Acetyl-coA regulates chromatin modification by enhancing histone acetylation which is an epigenetic control of gene transcription and protein expression (Shi and Tu, 2015). Acetyl-coA has also been associated with elevated cytosolic Ca²⁺, subsequently resulting in the positive regulation of NFAT transcription factor which aids cell adhesion and migration (Lee et al., 2018). Other pro-inflammatory transcription factors and molecules such as production of interferon-γ, NO and other ROS have been reported to be upregulated during elevated level of acetyl-coA (Infantino et al., 2013; Peng et al., 2016; Martinez-Reyes et al., 2020).
2-oxoglutarate / succinate/ fumarate are implicated in the activities of various 2-oxoglutarate dependent dioxygenase (2-OGDD) which are involved in the hydroxylation of varying protein prolyl substrates (Martinez-Reyes et al., 2020). The intracellular ratio of 2-oxoglutarate to either succinate or fumarate determines the activity of the enzymes, as 2-oxoglutarate is an activator while succinate and fumarate are inhibitors of these enzymes (Park et al., 2016b). Currently, succinate and fumarate are referred as oncometabolites because of their role in cancer cell progression. Pathways implicated by 2-oxoglutarate, succinate and fumarate include, HIF 1alpha (Patten et al., 2010; Semenza, 2012), Histone and DNA methylation (Batie et al., 2019; Chakraborty et al., 2019), mTORC1 (Dando et al., 2019), and NF-KB (Liu et al., 2017) pathways. In addition, succinate has been implicated in the activation of the G-protein coupled receptor, SUCNR1 which has been reported to activate MAPK, ERK, NF-kB pathways (Dando et al., 2019).

1.1.3.3.3 Mitochondrial Matrix Quality Control

Misfolding and misassembling of mitochondrial proteins may result in protein aggregation and malfunctioning of important mitochondrial metabolic pathways. These phenomena occur quite often especially during protein import or at increased metabolic rates. The mitochondria possess an array of quality control enzymes to combat this stress factors and these are located at both the inner mitochondrial membrane and the matrix. The import machinery which consist of protein transporters and cleavage peptidases are found in the inner membrane while chaperones and two important proteases are in the matrix (Moehle et al., 2019).

Mitochondrial molecular chaperones are proteins dedicated to correctly aid the folding of nascent proteins following their importation, or the re-folding of misfolded or unfolded proteins, essentially to repress the proteotoxic effects of misfolded proteins in the mitochondria (Ruan et al., 2020). Mitochondrial chaperons include mtHSP70, mtHSP 90 also known as TRAP1 and the large
chaperonin HSP60/10 complex. Other proteins that contributes to mitochondrial folding are CypD, a peptidyl-prolyl \textit{cis-trans} isomerase (Porter and Beutner, 2018) and SOD2 which detoxifies toxic superoxide from effecting damage on proteins.

In addition to chaperones and associated proteins, mitochondrial proteases play important role in preventing the accumulation of toxic proteins. It has been reported that mitochondria degrade 6-12\% of its proteome per generation (Moehle \textit{et al}., 2019). The two main proteases existing in the matrix are LonP and ClpP (Yang \textit{et al}., 2018; Rolland \textit{et al}., 2019). The inner membrane also consists of two main proteases, each facing opposite direction of the membrane; the m-AAA protease (AFG3L1, AFG3L2 and SPG7) faces the matrix while i-AAA protease (YME1L) faces the intermembrane space of the mitochondria (Patron \textit{et al}., 2018). While, matrix proteases are important for degradation of unfolded matrix proteins, the inner membrane proteases control the inner membrane proteins, especially, the OXPHOS machinery (Moehle \textit{et al}., 2019).

1.2 Mitochondrial ROS Production and Control
This is a wide subject area; the various aspects below are described as these have direct relevance to the work undertaken in this thesis.

1.2.1 Mitochondrial Superoxide
Oxidative phosphorylation results in mitochondrial ROS production and this was first reported in 1966 (Jensen, 1966). Mitochondrial ROS starts with the production of superoxide, \( \text{O}_2^\bullet^- \), which results from the thermodynamically favourable one electron reduction of molecular oxygen (Roca-Agujetas \textit{et al}., 2019). Oxygen have increased capacity to produce ROS due to its high reduction potential of -160mV at 1M concentration at pH 7 (Murphy, 2009). Indeed, mitochondrial oxygen concentration ranges from 3-30\( \mu \text{M} \) in vivo while mitochondrial \( \text{O}_2^\bullet^- \) is estimated to be around 10-200pM (Turrens, 2003; Mailloux, 2018). The source of electron leakage for superoxide production is usually from either flavin prosthetic groups of mitochondrial enzymes or at the
interphase of CoQH\(_2\)-protein interaction situated in the inner mitochondrial membrane. Small electron carriers such as reduced glutathione (GSH), reduced coenzyme Q (CoQH\(_2\)), NADH and NADPH do not produce O\(_2\)•− (Murphy, 2009). In addition, superoxide dismutation results in H\(_2\)O\(_2\) formation which is further reduced to H\(_2\)O by mitochondrial antioxidant enzymes (Sharma et al., 2012).

The production of mitochondrial O\(_2\)•− is regulated by the concentration of the protein carrying electron carriers and the proportion of the partially reduced prosthetic group available for interaction with molecular oxygen. Indeed, eleven sites of ROS production exists in the mitochondria (Wong et al., 2017) and these are represented in Table 1.2. These sites are either exposed to the membrane or to the matrix and may serve as accessible oxygen reactive centres. Notably, different tissues possess varying amounts of the ROS generating proteins (Tahara et al., 2009). In vitro, the concentration of molecular oxygen is also positively correlated with ROS production. It has been reported in previous studies that superoxide production at isolated complex I decreases with dissipating level of molecular oxygen in vitro. Cytochrome c oxidase (CIV) of the electron transport chain have a high propensity to consume molecular oxygen with Km less than 1\(\mu\)M (Li et al., 2006; Murphy, 2009). Intriguingly, oxygen dissolves more than 3-fold in biological membranes than in solution. Therefore, enhancing the rate of oxygen consumption leads to decrease in mitochondrial ROS production, as the propensity for one electron transfer to oxygen rapidly becomes insignificant. Moreover, a significant part of the respiratory complex I (especially the matrix arm) serves as shields to the Fe-S clusters, thus, preventing oxygen from interacting with the electron carrying groups (Hirst et al., 2003; Larosa and Remacle, 2018). However, protein post-translational modification and interaction with other proteins may modify their structures and enable exposure of electron carriers to oxygen, hence, resulting in superoxide production (Murphy, 2009; Chen and Zweier, 2014).
1.2.1.1 Superoxide at Complex I

Mitochondrial $O_2^-$ is mainly produced at both the flavin and ubiquinone binding sites of complex I and III respectively (Wong et al., 2017). The complex I of the ETC is a 1MDa flavo-protein complex divided into a matrix hydrophilic and membrane incorporated lipophilic arm (Vinothkumar et al., 2014; Zhu et al., 2016). The mega-protein consists of 45 polypeptides with prosthetic groups; one FMN electron acceptor and seven Fe-S clusters embedded in the matrix hydrophilic arm, which are well shielded from $O_2$ except for the FMN which accepts electron from NADH (Efremov et al., 2010). FMN results in superoxide production only when fully reduced, and at that redox state, electrons leak to molecular oxygen. Conditions resulting in the full reduction of FMN are those that increase the mitochondrial NADH/NAD ratio or the inhibition of complex I with rotenone at the CoQ-binding site. Rotenone binding prevents the transfer of electrons to CoQ, thus leading to the build-up of electrons downwards through the Fe-S clusters to the FMN site, subsequently leading to superoxide production (Kussmaul and Hirst, 2006). Therefore, pathological conditions that limits complex I activity such as, mutation, therapeutic inhibition, and ischemia can result in superoxide production at complex I (Prag et al., 2020).

The production of superoxide at complex I can also occur via reverse electron transport (RET), whereby electrons are transferred from fully reduced CoQH$_2$ to the complex I of the ETC during elevated proton gradient / PMF across the inner mitochondrial membrane (Scialò et al., 2017). The source of ROS during this process is unknown but it is believed to originate at either the FMN site in the matrix or CoQ binding site of complex I (Murphy, 2009). This is proposed because the FMN inhibitor diphenyleneiodonium (DPI) and rotenone both significantly diminished the production of superoxide. Of note, DPI, being an FMN inhibitor might have repressed any of the ETC respiratory complexes containing FMN prosthetic groups (Andreyev et al., 2015; Dubouchaud et al., 2018). Indeed, mitochondria respiring on succinate in the absence of ADP (for ATP production) were characterised with elevated level of superoxide production which was repressed when ADP was supplied or during mild
dissipation of the proton gradient of the inner mitochondria membrane via uncouplers (Barbeau et al., 2018).

**Table 1.2: Source of mitochondrial ROS production**

<table>
<thead>
<tr>
<th>Mitochondrial compartment</th>
<th>Source of ROS Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial matrix</td>
<td>Flavin binding site of OGDH</td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>Flavin binding site of BCOADHC</td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>Flavin binding sites of OADHC</td>
</tr>
<tr>
<td>Mitochondrial matrix</td>
<td>Flavin binding site of PDH</td>
</tr>
<tr>
<td>Matrix surface of the inner membrane</td>
<td>Flavin binding site of complex I</td>
</tr>
<tr>
<td>Mitochondrial inner membrane</td>
<td>Ubiquinone binding sites of complex I</td>
</tr>
<tr>
<td>Matrix surface of the inner membrane</td>
<td>Flavin binding sites of complex II</td>
</tr>
<tr>
<td>Mitochondrial inner membrane</td>
<td>Ubiquinone binding site of CIII (Q&lt;sub&gt;i&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Mitochondrial inner membrane</td>
<td>Ubiquinone binding site of G3PDH</td>
</tr>
<tr>
<td>Matrix inner membrane</td>
<td>Flavin binding site of ETF:QOR</td>
</tr>
<tr>
<td>Mitochondrial inner membrane</td>
<td>Ubiquinone binding site of DHODH</td>
</tr>
</tbody>
</table>

Data obtained from (Wong et al., 2017).

### 1.2.1.2 Superoxide at Complex III

The respiratory complex III exists as a functional dimer with monomeric size of 240KDa consisting of 11 polypeptides, three haems and a single Fe-S cluster. It mediates the Q-cycle and transiently binds CoQ at both the Q<sub>o</sub> and Q<sub>i</sub> sites (Wenz et al., 2009). It is proposed that during basal electron transfer, complex III contribution to the mitochondrial O<sub>2</sub><sup>•−</sup> pool is negligible when compared to ROS production at complex I. However, inhibition of complex III at the Q<sub>i</sub> site by antimycin results in the superoxide burst as a result of the interaction of the stably formed ubisemiquinone with O<sub>2</sub> at the Q<sub>o</sub> site (Dröse and Brandt, 2012; Bleier and Dröse, 2013; Larosa and Remacle, 2018). It is suggested that ubisemiquinone stabilisation at the Q<sub>o</sub> may be promoted by elevated proton gradient across the inner membrane, or the loss of cytochrome c (Murphy, 2009; Bleier and Dröse, 2013).

However, recent evidence suggested that superoxide production at complex III was significantly higher than any other site in rat skeletal muscle isolated
mitochondria and that superoxide production at complex I was confounded by other flavin binding superoxide producing sites (Brand, 2016). Notably, the produced superoxide at complex III is released to both the matrix and the intermembrane space (Muller et al., 2004), thus, having the capacity to exert oxidation on both the outer and inner mitochondrial membrane simultaneously (Wong et al., 2017).

Conditions leading to the formation of mitochondrial O$_2^{-}$ include elevated matrix NADH, elevated inner membrane CoQH$_2$ pool, and elevated proton gradient across the inner membrane (Murphy and Hartley, 2018). Therefore, elevated NADH and CoQH$_2$ with concomitant decrease in the PMF will not result in the production of superoxide within the mitochondrial membrane. Most probably because the ETC is forced to proceed in the forward reaction and also because oxygen will be less available for ROS production. It should be noted that the accumulation of NADH may also exert superoxide production at other sites in the matrix such as OGDH (Murphy, 2009).

1.2.2 Measurement of Mitochondrial Superoxide

Mitochondrial O$_2^{-}$ is difficult to measure due to its rapid dismutation into H$_2$O$_2$ by the matrix MnSOD which possesses an approximate rate constant, K, of 2 x 10$^9$ M$^{-1}$. s$^{-1}$ (Murphy, 2009). Therefore, superoxide is being measured indirectly by H$_2$O$_2$. The limitation of this method is that mitochondrial O$_2^{-}$ can react very rapidly as well with other electron acceptors such as NO•, forming the reactive nitrogen species, free radical, peroxynitrites (ONOO$^-$) with an approximate rate constant K, of 10$^{10}$ M$^{-1}$. s$^{-1}$ (Murphy, 2009). Other confounding factors are the production of H$_2$O$_2$ by other compartments of the mitochondria such as the outer membrane and the intermembrane space, and the rapid quenching of H$_2$O$_2$ by mitochondrial antioxidant systems; catalase, peroxiredoxins 3, and 5 and glutathione peroxidase 1 and 4. Therefore, following these observations, numerous research groups have provided useful information on the production of mitochondrial O$_2^{-}$ by using various techniques ranging from fluorescent dye measurements such as MitoSOX™
and hydroethidine to enzyme activity assays, such as superoxide sensitive aconitase activity assay (Gardner, 2002; St-Pierre et al., 2002; Muller et al., 2004; Murphy, 2009, Miwa and Brand, 2005; Treberg et al., 2010; Brand, 2016).

1.2.3 ROS induced Protein Modification

ROS are products of aerobic metabolism which can affect biological processes at both the protein and transcriptional level. ROS can regulate enzyme activities, protein structure and interactions as well as regulate the binding and activities of transcription factors (Corcoran and Cotter, 2013; Klotz et al., 2015; Flohé, 2016). ROS, most especially superoxide, also targets unshielded Fe-S clusters on proteins, oxidising the Fe$^{2+}$ to Fe$^{3+}$. However, labile Fe$^{2+}$ forms a toxic association with ROS, as it can react with H$_2$O$_2$ to produce toxic hydroxyl radicals, thus, resulting in cell and tissue damage (Choudhury et al., 2017).

Protein are usually oxidised on amino acid side chains that imparts their structure and function. Tryptophan, histidine, proline, methionine and cysteine are all redox sensitive amino acids. Usually, enzyme activity is regulated by reversible thiol modifications such as sulfenylation (RS-OH), glutathionylation (RS-SG) and nitosation (RS-NO) which are reduced by thioredoxin and glutaredoxin (Mieyal and Chock, 2012; Hanschmann et al., 2013). Many a times, the thiol group undergoes irreversible oxidation if not rescued by the antioxidant systems available in the mitochondria. Sulfinylation (RS-O$_2$H) of protein thiols may be reversed by sulfiredoxins (SRXN), however, thiol sulfonylation (RS-O$_3$H) is irreversible (Yoube et al., 2018). Following these modifications, the proteins completely lose their enzymatic activities and may be degraded by the mitochondrial chaperones and proteases such as the Lon protease. Figure 1.4 discusses protein thiol modification in details.
Cysteine thiol sulfenylation (SOH) is induced by H₂O₂ which usually is followed by additional ROS-induced modifications which are sulfinylation (SO₂H) and sulfonylation (SO₃H). Protein thiol sulfinylation may be reversibly catalysed by sulfiredoxins. Other cysteine modifications include, sulfhydration, nitrosylation and intra or intermolecular disulphide bonds. Image was adapted and modified from Yoboue et al., 2018.

1.2.4 Mitochondrial Antioxidant Defence Systems

The first line of mitochondrial ROS defence systems is intermembrane space SOD1 and matrix SOD2, which converts superoxide into H₂O₂ (Kowaltowski et al., 2009). The produced H₂O₂ is then immediately exported out of the mitochondria by mitochondrial aquaporin-8 which also transports ammonia (Chauvigné et al., 2015). However, matrix H₂O₂ can be immediately reduced to H₂O molecules by GPX 1 and 4 or PRX 3 and 5 and this is dependent on the GSH/GSSG or TRX₂red/TRX₂ox ratios respectively (Cox et al., 2010; Brigelius-Flohe and Maiorino, 2013). Furthermore, in the case of protein oxidation, during oxidative stress, glutaredoxin or thioredoxin reduces the oxidised proteins to their native state (Chalker et al., 2018; Holzerova et al., 2016).
Both the glutaredoxin and thioredoxin systems are abundant in the mitochondria where they are utilised for protein thiol reduction consequently to the oxidation of protein cysteine residues. They are also involved in the conversion of oxidised peroxiredoxins and glutathione peroxidase back to their reduced states following their loss of electron during the reduction of peroxides to H2O. Both glutaredoxin and thioredoxin losses electrons and are oxidised during their reductive activity. While thioredoxin is reduced by thioredoxin reductase, glutaredoxin is reduced by GSH which is maintained by glutathione reductase. Both thioredoxin reductase and glutathione reductase are maintained by the available NADPH. Thioredoxin, TRX, thioredoxin reductase TrxR, peroxiredoxin, PRX, glutathione peroxidase, GPX, glutaredoxin, GRX, Reduced glutathione, GSH, oxidised glutathione, GSSG, glutathione reductase, GR. Image was obtained with permission from Mary Ann Liebert, Inc.

Peroxiredoxins have been reported to undergo protein sulfinylation and inactivation upon exposure to excessive H2O2. This modification accounts for the redox signalling that emanates from the mitochondria, as it allows the export of H2O2 from the mitochondria into the cytosol (Veal et al., 2018). However, under such conditions, sulfiredoxins is imported into the mitochondria for the reduction and activation of peroxiredoxin (Okabe et al., 2019). The activities of mitochondrial antioxidant enzymes are ultimately regulated by the matrix NADPH/NADP+ ratio (Figure 1.5).

Indeed, NADPH is produced from NADH by the inner membrane transhydrogenase (Nickel et al., 2015) and also the TCA cycle enzyme, NADP+ dependent IDH2. Therefore, the TCA cycle and the ETC contributes immensely to the level of mitochondrial NADPH, as elevated PMF is necessary.
for transhydrogenase activity. Notably, elevated matrix NADH, inner membrane coenzyme Q pool and PMF with no concomitant production of ATP or uncoupling activities results in the elevation of mitochondrial ROS production.

1.2.5 Mitochondrial Inner Membrane Uncoupling or Proton leak

Mitochondrial inner membrane uncoupling is the dissociation of the oxidative activities of the electron transport chain from ADP phosphorylation by the ATP synthase. Effectively, the ETC activities is futile, as the generated inner membrane proton motive force during this process is dissipated by other proteins of the inner membrane (Cadenas, 2018).

The inner mitochondrial membrane proton gradient / PMF is important for mitochondrial \( \text{O}_2^- \) production, as it modulates the direction of electron carriers in the inner mitochondrial membrane. The PMF can be uncoupled from ATP synthesis physiologically via a *wasteful proton leak back into the matrix*, independently of the ATP synthase, hence generating heat because the energy created via leakage down their electrochemical gradient is not being used for any metabolic process (Cheng *et al.*, 2017).

Interestingly, proton leak accounts for about 20-50% of the basal metabolic rate in mammals depending on the tissue type and it results in alteration of both \( \Delta \Psi_m \) and pH component of the PMF (Brand *et al.*, 2005; Cadenas, 2018; Nanayakkara *et al.*, 2019). Since the mitochondrial inner membrane consists of numerous ion transporters and channels, therefore, uncompensated cationic influx into the matrix also results in the dissipation of the \( \Delta \Psi_m \) component of the PMF without altering the pH component, hence reduced ETC. Therefore, it is believed that the pH component of PMF is important in uncoupler-induced ROS scavenging, as alkalinized matrix enhances both RET and ubisemiquinone stabilisation, thus potentiating ROS formation. Indeed, it has been established that ROS production is low at matrix acidic pH and high ETC (Brookes, 2005).
1.2.5.1 Small Molecule Uncouplers
Mitochondrial uncoupling is monitored by measuring both inner $\Delta \Psi_m$ and oxygen consumption rate. This is because dissipation of the PMF lowers the electrochemical gradient, which favours respiration in the forward direction. Indeed, state 3 respiration is favoured by the treatment of the mitochondria with ADP, as it activates the ATP synthase to dissipate the proton gradient across the inner membrane (see section 1.1.3.2.3 for respiratory states). Chemical uncouplers also referred to as protonophores have been used to treat metabolic diseases in experimental animal models. Such small molecules include; 2,4-dinitrophenol (DNP), carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Minners et al., 2000; Brennan et al., 2006; Modriansky and Gabrielova, 2009). Unfortunately, therapeutically, it is difficult to regulate the pharmacokinetics of uncouplers. However, recent findings have demonstrated the regulation of modified DNP, termed MitoPhotoDNP, which accumulates in the mitochondria matrix via the PMF and released into the inter membrane space upon exposure to light (Ost et al., 2017).

1.2.5.2 Endogenous Protein Uncouplers
Endogenous uncoupling proteins are present in the inner mitochondrial membrane and they mediate both basal and inducible proton leak down their chemical gradient into the matrix (Cheng et al., 2017).

1.2.5.2.1 Basal Proton Leak
Adenine nucleotide translocators account for most of the proton leak in the inner membrane at mitochondrial basal metabolic states. Their primary function is to exchange matrix ATP for inter membrane space ADP at the expense of PMF (Cheng et al., 2017). Inhibition of their activities with carboxyatractyloside did not significantly alter ANT mediated proton leak or PMF dissipation. Indeed, it is reported that their abundance positively correlates with proton conductance across the inner membrane. In vitro, the
capacity of ANT for proton leak is regulated by the presence of fatty acids, AMP or alkenals (Brand et al., 2005).

1.2.5.2.2 Inducible Proton Leak

Inducible proton leak is mediated by proteins of the inner mitochondrial membrane that dissipate the proton motive force of the inner mitochondrial membrane upon induction. Two mostly studied inducible proton translocators of the inner membrane are uncoupling proteins (UCPs) and nicotinamide nucleotide transhydrogenase (NNT) (Brown et al., 2010).

UCPs are members of the mitochondrial anion carrier proteins that mediate the reflux of protons back into the matrix (Zhao et al., 2019). Five isomers of UCPs exist in nature (UCP1-5). They are basically inactive, inhibited by nucleotides and nucleosides (ATP, ADP and GDP). The induction of UCPs require free fatty acid but the mechanism is still being investigated (Cannon and Nedergaard, 2017). UCPs have been implicated in oxidative stress, type 2 diabetes, immune response and cardiovascular diseases. They have also been associated with tolerance to ischemia reperfusion injury. UCPs are activated by ROS, exhibiting a protective feedback loop against oxidative stress (Nishikawa et al., 2000; Koziel et al., 2015; Cadenas, 2018).

Nicotinamide nucleotide transhydrogenases (NNT) are mitochondrial inner membrane proteins that regulates the conversion of NADP$^+$ to NADPH utilising electrons from NADH. They utilise the PMF of the membrane to perform this role, thus, function as endogenous uncouplers (Kampjut and Sazanov, 2019). They contribute immensely to mitochondrial NADPH pool with an estimate of nearly 50% in mouse liver mitochondria (Ward et al., 2019). Knock down of NNT has been associated with increased mitochondria ROS (Chortis et al., 2018) and reduced oxygen consumption when fed at both complex I and complex III (Ward et al., 2019). Other PMF dissipating carriers of the inner mitochondrial membrane are phosphate carrier and glutamate/aspartate exchanger.
1.2.5.3 Cation Flux and Membrane Potential Dissipation

Cations such as K\(^{+}\) and Ca\(^{2+}\) are abundant intracellularly. The mitochondria have a large driving force for the accumulation of cations because of its high electrochemical gradient / PMF despite their non-permeability through biological membranes. Therefore, different proteins located in the inner membrane facilitates the influx of cations into the matrix. Cations dissipate the \(\Delta \Psi_m\) without affecting the pH composition of the PMF, thereby enhancing the propensity for ROS formation in the mitochondria (Heinen et al., 2007).

Less than 1nM of K\(^{+}\) ionophore, valinomycin, resulted in the upregulation of ROS production, however, K\(^{+}\) influx is usually accompanied by the electroneutral Pi and H\(^{+}\) matrix uptake, thus, dissipating the proton gradient.
while inducing an acidic matrix which downregulates ROS production (Berry et al., 2018). In addition, the K⁺/H⁺ exchanger mediates the exchange of matrix potassium for the import of intermembrane space H⁺, thus, lowering the matrix pH. The production of ROS is dependent on the matrix pH status which is usually determined by the nature of the influx and the associated compensatory mechanism (Berry et al., 2018).

Ca²⁺ is imported into the mitochondria through the mitochondrial Ca²⁺ uniporter (MCU), which is also driven by the inner mitochondrial ΔΨₘ. Indeed, the MCU has a low affinity for Ca²⁺ (Kd = 10µM), therefore it requires Ca²⁺ at very high concentration to transmit it into the mitochondria (De Stefani et al., 2016). Numerous reports have associated Ca²⁺ influx with ROS production either directly or indirectly (Huang et al., 2014; Xu and Chisholm, 2014; Tosatto et al., 2016; Paupe and Prudent, 2018).

1.2.6 Mitochondria Retrograde Signalling
Mitochondrial retrograde signalling pathway is the communication between the mitochondria and the nucleus which culminates in the activation and repression of different cellular pathways under both physiological and pathological conditions (Butow and Avadhani, 2004). In other words, retrograde signalling is broadly referred to as cellular responses resulting from mitochondrial activity changes, and these are mostly associated with the mitochondrial regulation of ROS, intracellular Ca²⁺ and TCA cycle metabolites (oxaloacetate, succinate, fumarate, 2-oxoglutarate, succinylCoA and acetyl-CoA). ROS and Ca²⁺ signalling are somewhat inter-related, as they control the stabilisation and phosphorylation of various transcription factors such as HIF-1α and calcineurin/calmodulin dependent pathways. On the other hand, the TCA cycle intermediates highlighted here play important role in HIF-1α stabilisation, DNA and histone methylation, histone acetylation and succinylation (Hunt and Bateman, 2018; Janssen et al., 2019).
Recently, studies suggest the possibility of mitochondria to nucleus direct communication as opposed to the use of the above named second messengers (Desai et al., 2019a, b). Indeed, we also observed the presence of certain mitochondrial proteins (aconitase-2 and 2-oxoglutarate dehydrogenase enzymes) in the nuclear compartment of HT1080 cells, and interestingly, CypD deficiency increased the level of these proteins in the nucleus (Figure 9.6).

### 1.2.6.1 Mitochondrial ROS Signalling Pathways

Metabolic pathways in the mitochondria results in variable level of numerous metabolites with specific roles in global cell metabolism. Some of the pathways affected by mitochondria metabolites are already discussed in the previous section. However, other signalling pathways that mirror mitochondrial health which are relevant to the results discussed in this thesis will be elaborated in this section.

#### 1.2.6.1.1 Hypoxia Inducible Factor 1 (HIF-1) Pathway

HIF-1 was first observed in 1991 as a human erythropoietin gene inducer in ischemic kidney and liver tissues by Semenza and colleagues (Semenza et al., 1991). HIF-1 is a heterodimeric transcription factor consisting of two subunits, the oxygen sensitive HIF-1alpha (or analogues, HIF-2alpha and HIF-3alpha) and the constitutively expressed oxygen insensitive HIF-1beta, also referred to as aryl hydrocarbon nuclear translocator (ARNT). Degradation of HIF-1alpha is largely dependent on the availability of molecular oxygen mediated by the ubiquitin proteasome system (Rocha, 2007). However, other oxygen independent modifications contribute to its stability (Masoud and Li, 2015).

The hydroxylation of HIF-1alpha by PHDs is regulated by the mitochondrial TCA cycle metabolite, 2-oxoglutarate. PHDs catalyses the decarboxylation of 2-oxoglutarate to succinate and carbon IV oxide in the presence of molecular oxygen and ferrous ($\text{Fe}^{2+}$) ion while leading to the hydroxylation of HIF-1alpha
at P406 and P562 of its oxygen dependent degradation domain (ODDD). Following hydroxylation at the ODDD, HIF-1alpha is recognised by the E3 ubiquitin ligase, von-Hippel Lindau protein (pVHL) complex which targets HIF-1alpha for degradation by the 26s proteasome (Rocha, 2007). Conversely, oxygen-dependent hydroxylation at another site, N803, by Factor Inhibiting HIF-1 (FIH) present in the C-terminal domain does not affect protein stability but prevents its transcriptional activities (Azimi, 2018; Lee et al., 2019). Transcriptional targets of HIF-1alpha adapts the cell to survival and metabolic changes. Due to its stabilisation by hypoxia, HIF-1alpha has been observed to be upregulated at the core of tumour tissues (Schofield and Ratcliffe, 2004).

Mitochondrial derived ROS has been reported to stabilise HIF-1alpha accumulation during normoxia and hypoxia, and the complex III of the ETC have been implicated as the major ROS producer during hypoxia. The decreased activity of complex IV during hypoxia may result to the harmful transfer of electrons to O₂ thereby resulting in superoxide production (Guzy et al., 2005). The mechanism of HIF-1alpha stabilisation by ROS is associated with the redox state of the ferrous cofactor present at the active site of PHDs. The oxidation of ferrous, Fe²⁺ to ferric Fe³⁺ ion has debilitating effect on the activity of PHDs, hence decreasing the hydroxylation and subsequent degradation of HIF-1alpha and its homologues. Indeed, under normoxia, the treatment of cells with H₂O₂ also resulted in the accumulation of HIF-1alpha (Metzen et al., 2003; Jung et al., 2008). Previous reports have also shown that sustained oxidative stress may result in the inactivation of the proteasome, thus, resulting in reduced degradation of HIF-1alpha (Wu et al., 2009). As much as the role of ROS during hypoxia is controversial, there are increasing evidences for ROS induced HIF-stabilisation during normoxia (Movafagh et al., 2015; Thomas and Ashcroft, 2019).

### 1.2.6.1.2 Cellular Lipid Droplets Formation

Lipid droplets have been reported to be associated with mitochondrial metabolism and can be used as markers for mitochondrial health (Yu et al.,
Lipid droplets otherwise known as adiposomes or lipid bodies are specialised intracellular organelles composed of neutral lipids surrounded by phospholipid monolayer that contains numerous coat proteins which are the various isoforms of perilipins (PLIN1-5). Lipid droplets exist in most cells and undergo dynamic formation and degradation via specific mechanisms (Walther and Farese, 2012; Thiam et al., 2013). Lipid droplets sequesters toxic proteins such as histones (Cermelli et al., 2006) and viral proteins (Herker et al., 2010). It also stores fat soluble vitamins such as Vitamins A, D, E and K (Thiam et al., 2013). ROS has been reported to have positive relationship with lipid droplet accumulation (Tirinato et al., 2019).

Lipid droplets are formed from the ER (Wilfling et al., 2013). Excessive intracellular toxic lipids are esterified into more inert neutral triacylglycerol and sterol esters. These newly formed oily lipids leave intracellular membranes into the cytosol where they form emulsion of lipid droplets. The surface phospholipid monolayer of lipid droplets is derived from the ER membrane. However, surface phospholipids on droplets differ from the ER, as they possess more phosphatidylcholine content (Tauchi-Sato et al., 2002).

Lipid droplets breakdown their triacylglycerol and sterol esters via lipolysis to generate energy for metabolism and lipids for the formation of intracellular and plasma membranes. In adipose tissues, adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase and monoacylglycerol lipase (MGL) are the three lipases that sequentially hydrolyses fatty acids from triacylglycerol present in lipid droplets yielding glycerol and fatty acids. Lipophagy has also been shown to degrade lipid droplets by the engulfment of autophagosomes (Ouimet and Marcel, 2012; Thiam et al., 2013).

As mentioned above, ROS appears to have positive relationship with lipid droplet accumulation (Tirinato et al., 2019). Other experiments where cellular antioxidative capacity were knocked down also resulted in the accumulation of lipid droplets (Liu et al., 2015). Recently, it was reported that ROS generated
from mitochondrial dysfunction is associated with lipid droplet formation (Lee et al., 2013; Zheng et al., 2017). Interestingly, elevated lipid droplet formation during oxidative stress has been proposed as an antioxidant measure utilised by cells to prevent the peroxidation of free fatty acids. This supports why oxidative stress in cancer cell does not drive apoptosis but fuel growth and survival (Sabharwal and Schumacker, 2014; Jin et al., 2018; Shyu et al., 2018).

Indeed, lipid droplet dissipation or shrinking is associated with mitochondrial UCPs, as their activities are activated by the presence of free fatty acids (Yu et al., 2015; Lomax et al., 2019; Velickovic et al., 2019). Indeed, it was also reported that mild mitochondrial inner membrane uncoupling with either FCCP or DNP resulted in ATGL/HSL-independent lipid droplet depletion (Demine et al., 2018; Demine et al., 2019). These evidences reveal the role of ROS signalling on cellular lipid droplets in the cell and suggests that droplet formation aids cell survival by conferring protection from oxidative and proteotoxic stress.

1.2.6.1.3 NF-κB Signalling

Nuclear factor-κB (NF-κB) is a transcription factor that regulate the expression of genes involved in a variety of cellular processes which includes, cell proliferation, apoptosis, innate immune response, embryogenesis and organ development, and stress response to stimuli (Wan and Leonardo, 2010). NF-κB exists as both homo- and heterodimers of five different proteins p65/RELA, RELB, c-REL, p50 and p52. They possess a Rel homology domain (RHD), which is responsible for their dimerization, DNA recognition and binding, and interaction with endogenous inhibitor, IκB proteins. Binding of IκB to the RHD prevents dimerization and DNA binding, this consequently results in their nuclear export and cytosolic sequestration (Mitchell et al., 2016). NF-κB is activated by inflammatory signals such as TNF-alpha, lipopolysaccharide and IL-1 and ligand binding. This results in the phosphorylation of IκB by IKK and
subsequent ubiquitination and degradation of IkB which in turn leads to NF-kB dimerization, nuclear translocation and DNA binding (De Simone et al., 2015).

ROS have both activating and inhibitory effects on the NF-kB pathway and this depends on the cellular context (Morgan and Liu, 2011; Lingappan, 2018). The activation of NF-kB by mitochondrial ROS have been reported in fibroblast cells (Hamblin, 2018). However, on a general note, ROS modulates the activity of NF-kB either directly or indirectly. Thioredoxin has been shown to affect NF-kB activity, and this modulation depends on the cellular compartment; in the cytosol, it prevents dimerization by stabilising IkB while in the nucleus, DNA binding was enhanced (Baratz-Goldstein et al., 2016). Another protein, LC8 prevents the degradation of IkB by binding and inhibiting IKK phosphorylation; however, ROS exposure results in the oxidation and dissociation of LC8 from IkB (Jung et al., 2008). Oxidation of Cys62 in the RHD domain of p50 prevents DNA binding of the NF-kB heterodimer (Krithika, 2018). In addition, ROS has been reported to decrease TNF-induced IKK activation, hence downregulating the NF-kB pathway. Furthermore, ROS have been shown to enhance the PI3K/AKT which also activates the NF-KB pathway (Mantamadiotis, 2017; Krithika, 2018). These evidences suggest dual effect on the NF-kB signalling pathway following ROS exposure.

1.2.6.1.4 PI3K/AKT Signalling

PI3k are a family of lipid kinase that phosphorylate the 3'-OH group in the inositol ring of phosphatidylinositol 4, 5 bisphosphates present on the plasma membrane. There are three classes of the intracellular PI3K which is based on their structure and substrate specificity. The class I PI3Ks (α, β, δ, γ) are the most studied set of PI3Ks. The PI3Kα, β, δ are regulated by the receptor tyrosine kinase while PI3Kγ is regulated by G-protein coupled receptor (GPCR) and the Ras family of GTPases (Dobbin and Landen, 2013; Nitulescu et al., 2018). Activated PIP3 results in the downstream activation of PIP3 dependent kinase (PDK1), which further results in the phosphorylation and
activation of AKT. PIP3 is hydrolysed and downregulated by the lipid phosphatase, PTEN which converts PIP3 back to PIP2.

AKT is a serine/threonine kinase previously referred to as protein kinase B. It consists of three isoforms AKT1, AKT2 and AKT3 (Koseoglu et al., 2007). AKT2 has been implicated in numerous forms of human cancer. AKT has a pleckstrin homology (PH) domain for binding and interacting with 3-phosphoinositide components of the plasma membrane. It is activated when sequestered to the membrane and phosphorylated at Thr308, although full activation requires phosphorylation at Ser473 by either PDK1, AKT, DNA-PK, ILK and mTORC2 (Jiang et al., 2000; Sabatini, 2017). The dephosphorylation of Thr308 of AKT is mediated by PP2A while pleckstrin homology domain leucine rich repeat containing protein phosphatases (PHLPP1 or 2) preferentially dephosphorylates AKT at Ser437 (Nitsche et al., 2012).

The PI3K/AKT signalling pathway controls cellular processes which includes, proliferation, motility, growth, and metabolism. It is found to be highly dysregulated in human cancer cells. This pathway is activated by numerous growth factor receptor tyrosine kinases or G protein-coupled receptors and also modulated by ROS. Activation of the PI3K/AKT ultimately culminate in the enhancement of cell survival while suppressing autophagy and apoptosis (Janku et al., 2018).

AKT has been reported to be sensitive to cellular ROS signals. Indeed, the addition of oxidants to cells have activated AKT phosphorylation at both Thr308 and Ser437. However, several mechanisms may account for this observation and these include the following: the inactivation of PTEN by ROS (Leslie et al., 2003), oxidative inactivation of other protein tyrosine phosphatases, ligand-independent dimerization of receptor tyrosine kinases, inactivation of the feedback inhibition by MAPKs and inactivation of serine phosphatase PHLPP1 (Genestra, 2007; Wani et al., 2011; Nitsche et al., 2012). Inhibition of mitochondrial complex I by rotenone also resulted in the activation of AKT2 (Wani et al., 2011). Indeed, mitochondrial complex I
inhibition results in NADH accumulation which predisposes the mitochondria to ROS production at various FMN sites. However, it was also reported that oxidative stress induced by catalase inhibitor, 3-AT, resulted in the inactivation of AKT2 without affecting the phosphosites. This was attributed to sulfenylation of Cys124 residue present in the protein, thus, inactivating downstream AKT effectors (Wani et al., 2011).

### 1.2.6.2 Mitochondrial Metabolism and Cell Signalling

Mitochondrial metabolism can also directly affect cytosolic cell signalling. Metabolic products and intermediates such as ATP, amino acids and TCA cycle intermediates can act as direct signalling molecules for an array of pathways localised to the lysosome and other organelles. However, due to the complexity of cell signalling, other cues that determines the direction of the pathways can also originate extracellularly.

#### 1.2.6.2.1 mTOR Pathway

The mTOR kinase, a 289KDa and associated with the PI3K related kinase family exist in a multi-protein complex consisting of two distinct mTOR nucleation multi-protein complexes. The two mTOR complexes are mTORC1 and mTORC2, comprising of separate protein constituents, hence, they are involved in different physiological roles (Laplante and Sabatini, 2009).

mTORC1 is made up of five protein components consisting of regulatory associated protein of mTOR (RAPTOR), DEP-domain-containing mTOR-interacting protein (DEPTOR), mammalian lethal with Sec13 protein 8 (mLST8) and proline-rich AKT substrate 40KDa (PRAS40). mTORC1 is negatively regulated by rapamycin-FKBP12 complex. However, mTORC1 complex consists of endogenous inhibitors, PRAS40 and DEPTOR which negatively regulates the binding of mTORC1 to target substrates. This was proposed due to the physical reduction in these protein components upon mTORC1 activation. Notably, RAPTOR is associated with the activation of the mTORC1 complex by ensuring the lysosomal translocation of mTORC1 via
interaction with GTP-bound Rag GTPases (Laplante and Sabatini, 2012; Laplante and Sabatini, 2013).

Signalling input that converge on the mTORC1 includes, growth factors, oxygen, genotoxic stress, energy and amino acid status. Except for amino acid sensing carried out by the Rag GTPases, all the other signalling pathways are regulated by RHEB GTPase via TSC1-TSC2 kinases. Hypoxia, energy deficit via AMPK and genotoxic stress all activates TSC1-TSC2 and promotes its translocation to the lysosome where it inhibits RHEB, while amino acids promotes the translocation of mTORC1 to the lysosome for RHEB binding and activation (Laplante and Sabatini, 2013; Sabatini, 2017). The major role of mTORC1 in cells are growth and metabolic regulation (Liu and Sabatini, 2020).

mTORC2 is made up of six protein complexes, mammalian stress-activated protein kinase interacting protein (mSIN1), rapamycin-insensitive companion of mTOR (RICTOR), protein observed with Rictor-1 (PROTOR-1), DEPTOR and mLST8. Unlike mTORC1, the function of mTORC2 is still largely undetermined. Furthermore, acute rapamycin treatment does not affect its activity. However, it is associated with cell survival and cytoskeletal organisation via the phosphorylation of some AGC kinases such as, AKT, PKC and SGK1 (Zoncu et al., 2011; Liu and Sabatini, 2020).

Mitochondria regulates the mTOR by regulating both energy and amino acid homeostasis via TCA anaplerosis and cataplerosis. Mitochondrial energy output through the ATP synthase modulates the phosphorylation of cytoplasmic AMPK status via cellular AMP/ATP ratio. Elevated mitochondrial ATP inhibits phosphorylation of AMPK, hence activating the mTORC1. However, reduced ATP inhibits the mTORC1 through the TSC1-TSC2 axis. Likewise, transamination in the mitochondria may enhance the level of cytoplasmic amino acids. This induces the lysosomal translocation of the mTORC1 for further activation by RHEB. These two major mitochondrial processes are major contributors to mTOR regulation (Saxton and Sabatini, 2017; Lieu et al., 2020; Liu and Sabatini, 2020).
1.2.6.2.2 AMPK Signalling

AMPK is a heterotrimeric protein complex consisting of a catalytic alpha subunit and two regulatory beta and gamma subunits. There are two isoforms of the alpha and beta subunits while the gamma subunit has three isoforms in mammals. However, these isoforms are tissue specific (Hardie et al., 2011). Phosphorylation of Thr172 in the catalytic alpha subunit by either LKB1 (Hawley et al., 2003) and CaMKK2 (Fogarty et al., 2010) results in the activation of AMPK. In addition, the beta subunits undergo myristoylation and membrane incorporation (Oakhill et al., 2010).

Activated AMPK is a metabolic switch that redirects cellular metabolism from anabolism to catabolism (Carling et al., 2011; Hardie, 2011). During energy deficiency, the ratio of AMP in relation to ATP significantly increases and binds AMPK, creating a conformation on that both enhances and stabilises its phosphorylation (Carling, 2017).

While both ADP and AMP prevents the dephosphorylation. However, during re-energisation, ATP binding to the gamma subunit results in the dephosphorylation of the Thr172 of the alpha subunit (Mihaylova and Shaw, 2011). Indeed, ADP was observed to protect the phosphorylation status of AMPK (Oakhill, 2010). Recent findings have also showed the activation of AMPK on the lysosome via the axin-LKB1-AMPK axis (Zhang et al., 2013; Steinberg and Carling, 2019). Regulation of AMPK are elaborated in Fig 5.11. However, it was observed that AMPK may directly regulate mTORC1 by phosphorylating RAPTOR and inhibiting its activity. Indirect inhibition of the mTORC1 occurs via AMPK mediated activation of the TSC1-TSC2 complex which inhibits mTORC1 (Steinberg and Carling, 2019).

1.3 Mitochondrial Cyclophilin D

Following the advent of their discovery in 1984, seventeen (17) cyclophilins are present in the human proteome and majority of them binds the
immunosuppressant drug, CsA (Fisher et al., 1984). The nascent CypD protein is a nuclear-encoded 207 amino acid protein of molecular mass 22KDa (Uniprot P30405) which possesses both an N-terminal 43 amino acid mitochondrial targeting sequence and a 164 amino acid cyclophilin domain which confers on it the PPIase activity (Elrod and Molkentin, 2013). Full-length CypD is transported from the nucleus to the mitochondria where the MTS is cleaved to yield the mature 18kD mitochondrial isoform, Cyclophilin D (CypD) which resides in the mitochondrial matrix protein. CypD is a member of the cyclophilin family of protein with characteristics peptidyl-prolyl-cis-trans isomerase (PPIase) (Elrod and Molkentin, 2013, Baines et al., 2005) activity due to their ability to catalyse an $180^\circ$ rotation about the C-N linkage of an X-P peptide bond (Shore et al., 2016). Structurally, CypD consists of two alpha helices, eight beta strands, and a $3_{10}$ helix (Göthel and Marahiel, 1999), and structurally similar to Cyclophilins A, B, C and E with 75%, 61%, 58% and 67% amino acid sequence similarity respectively. The amino acid residues around the CsA binding sites, residues, Ser59, Ser81, Arg82, Ile117, Lys148 and Ser149 of CypD (mature protein sequence numbering) are not conserved among the other cyclophilins, suggesting their difference in protein specificity (Kajitani et al., 2008). The gene encoding CypD, ppif is located on human chromosome 10 and consists of 6 exons. It was incorrectly named as PPIF instead of PPID by the HUGO gene nomenclature committee (Elrod and Molkentin, 2013). The homology of CypD across mammalian species is represented in Table 1.4 below. CypD is suggested to play two important roles in the mitochondria these include; protein folding and intracellular chaperone binding, and the regulation of the mitochondrial permeability transition pore (MPTP).
Mitochondrial ROS can activate the PI3K/AKT pathway positively by inhibiting the phosphatase and tensin homologue (PTEN). This enhances protein synthesis via mTORC1, translation and stabilisation of HIF-1α activation and cell survival via mTORC2. Mitochondrial succinate can exit the mitochondria to activate its plasma membrane receptor GPR91, this results in the activation of the ERK pathway and phospholipase C (PLC). Inositol-3,4,5 trisphosphate, a product of PLC activity can potentiate Ca$^{2+}$ release from the ER. The subsequent elevated cytosolic Ca$^{2+}$ can also activate the AKT pathway and mitochondrial activities when sequestered by the mitochondria. The mitochondrial urea cycle and other mitochondrial transaminase activities (not shown) can release arginine and other amino acids, these activates the mTORC1 pathway, further leading to protein synthesis. During mitochondrial energy stress or hypoxia, activated AMP dependent protein kinase (AMPK) and REDD1 (regulated in development and DNA damage responses 1) respectively activates tuberous sclerosis protein complex (TSC) which results in the inhibition of mTORC1. Adapted and modified from Roux and Topisirovic, 2018. Detailed abbreviations are in the list of abbreviations.

Figure 1.7: The Role of Mitochondria on some Signalling Pathways

Mitochondrial ROS can activate the PI3K/AKT pathway positively by inhibiting the phosphatase and tensin homologue (PTEN). This enhances protein synthesis via mTORC1, translation and stabilisation of HIF-1α activation and cell survival via mTORC2. Mitochondrial succinate can exit the mitochondria to activate its plasma membrane receptor GPR91, this results in the activation of the ERK pathway and phospholipase C (PLC). Inositol-3,4,5 trisphosphate, a product of PLC activity can potentiate Ca$^{2+}$ release from the ER. The subsequent elevated cytosolic Ca$^{2+}$ can also activate the AKT pathway and mitochondrial activities when sequestered by the mitochondria. The mitochondrial urea cycle and other mitochondrial transaminase activities (not shown) can release arginine and other amino acids, these activates the mTORC1 pathway, further leading to protein synthesis. During mitochondrial energy stress or hypoxia, activated AMP dependent protein kinase (AMPK) and REDD1 (regulated in development and DNA damage responses 1) respectively activates tuberous sclerosis protein complex (TSC) which results in the inhibition of mTORC1. Adapted and modified from Roux and Topisirovic, 2018. Detailed abbreviations are in the list of abbreviations.
Table 1.3: ppif protein and gene homology across other mammalian species relative to H. sapiens

<table>
<thead>
<tr>
<th>Species</th>
<th>Symbol</th>
<th>HomoloGene Pairwise Alignment Scores</th>
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<tr>
<td></td>
<td></td>
<td>Identity (%)</td>
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<tr>
<td></td>
<td></td>
<td>DNA</td>
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<td>R. norvegeicus</td>
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<td>88.70</td>
</tr>
</tbody>
</table>

Adapted from Elrod and Molkentin, 2013.

1.3.1 Inhibitors of CypD

CsA is a classical inhibitor of various cyclophilins which has immunosuppressive activities by simultaneously binding with calcium-calmodulin dependent protein phosphatase, calcineurin (CN) to form a ternary CypA-CsA-CN complex. CsA has been modified to analogues that are much more specific to CypD only. Some of these analogues include, D-Me-Ala-3-Et-Val-4-cyclosporin (Debio 025) (Gomez et al., 2007), N-Me-4-Ile-cyclosporin A (NIM811) (Argaud et al., 2005; Shore et al., 2016), N-Me-Val-4-cyclosporin A and N-Me-Ala-6-cyclosporin A (6-MeAla-CsA) (Griffiths and Halestrap, 1995; Khaspekov et al., 1999; Kalani et al., 2018; Oliveira, 2018).

These derivatives blocked MPTP activation through the inhibition of CypD. Indeed, Debio-025, NIM811 and 6-MeAla-CsA also reduced cardiac dysfunction and infarct size induced by ischemia reperfusion injury (Gomez et al., 2007; Panel et al., 2019). Sanglifehrin A (SfA) is also a CypD inhibitor; however, its structure is unrelated to CsA (Lim et al., 2007). It does not bind calcineurin and it has also been shown to provide cardioprotection against ischemia reperfusion injury (Hauserloy et al., 2002; Oliveira, 2018). Recently, compound 19 (1-(4-Aminobenzyl)-3-((s)-4-(methylthio)-1-((s)-2-(2-
(methylthio) phenyl)pyrrolidine-1-yl)-1-oxobutan-2-yl)urea) is a urea based novel inhibitor of CypD and it also prevented the loss of $\Delta \Psi_m$ in murine pancreatic acinar cells (Shore et al., 2016).

1.3.2 CypD and Protein Folding

Protein folding involves the transformation of nascent linear primary polypeptide sequence into a well-defined three-dimensional structure. Folding of polypeptides are usually chaperone guided spontaneous processes, and all the information necessary for folding are present in the polypeptide sequence (Altieri, 2011). However, the rate limiting step during protein folding is the formation of the trans conformation in a peptidyl-prolyl peptide bond, as this is the only peptide bond that usually exist as mix of both the cis and trans conformation. Notably, most of the other peptide bonds in nascent polypeptides are usually synthesized in the trans conformation on the ribosome. Therefore, the rate limiting step in protein folding processes was suggested as the peptidyl-prolyl cis-trans isomerisation (Göthel and Marahiel, 1999). Since folding intermediates were prone to aggregation and degradation, it was suspected that certain enzymes are dedicated to the rapid isomerisation of prolyl peptide bonds (Göthel and Marahiel, 1999).
Figure 1.8: Crystal Structure of CypD in Complex with CsA Surface representation of human CypD in complex with cyclosporin A (represented as magenta sticks) (Red patches represent acidic functional groups while blue patches represent basic functional groups (PDB Accession 2Z6W)(Kajitani et al., 2008).
Three classes of peptidyl-prolyl *cis-trans* isomerase were discovered to occur in eukaryotes, with presence in prokaryotes as well. These include; Cyclophilins, FKBPs and Parvulins. They differ significantly in their amino acid sequence and structure although they are generally referred as immunophilins due to their ability to bind immunosuppressant drugs. Cyclophilins are inhibited by CsA, FKBPs are inhibited by FK506 and rapamycin, and Parvulins are inhibited by juglone. Members of these immunophilins are localised to different subcellular locations where they are most likely involved in protein folding activities.

CypD, the mitochondrial cyclophilin, although not the only immunophilin in the mitochondria, has been reported to interact with two mitochondrial molecular chaperones. These include, TRAP-1, a mitochondrial HSP90 and HSP60, a chaperonin that exist in complex with HSP10 (Kang *et al*., 2007; Altieri, 2011). These interactions with CypD do not form stable complex (Ghosh *et al*., 2010).
Furthermore, it is thought that the interactions between CypD and the mitochondrial chaperones are more related to MPTP modulation rather than protein folding (Kang et al., 2007; Ghosh et al., 2010). Therefore, detailed work is needed on the exact role of CypD during mitochondrial protein folding.

1.3.3 CypD and the Mitochondrial Permeability Transition Pore (MPTP)

Haworth and Hunter were the first to characterise the MPTP in 1979. They referred to it as the abrupt non-selective permeability of the inner mitochondrial membrane to molecules below 1.5KDa in size under specific conditions such as mitochondrial Ca$^{2+}$ overload or oxidative stress (Haworth and Hunter, 1979). However, other divalent cations such as Mg$^{2+}$ and adenine nucleotides such as ADP inhibited pore opening. MPTP opening result in mitochondrial matrix swelling, drastic collapse of the inner $\Delta\psi_m$, uncoupling of OXPHOS and the rapid depletion of ATP. Transient MPTP opening, also referred as the low conductance state is beneficial to the mitochondrial health, as excessive matrix Ca$^{2+}$ and ROS are extruded from the mitochondria. However, the persistent opening of the MPTP could result in outer membrane rupture and necrotic cell death, because of both the dysregulation of ion homeostasis and ATP depletion (Crompton, 1999; Javadov and Karmazyn, 2007).

The role of MPTP in apoptosis was initially argued by some research groups at the onset of MPTP discovery. They showed that the rupture of the outer membrane that results from matrix swelling resulted in the release of intermembrane space pro-apoptotic proteins such as cytochrome C, endonuclease G, second mitochondria derived activator of caspases (SMAC) / direct IAP-binding protein with low PI (DIABLO) and apoptosis inducing factor (AIF). Evidences revealed that MPTP inhibition prevented apoptotic cell death and also that BAX-induced apoptosis was sensitive to CsA treatment (Marchetti et al., 1996; Ellerby et al., 1997; Marzo et al., 1998; Narita et al., 1998). However, compelling evidences supports MPTP-induced necrotic cell
death to apoptotic cell death (Kinnally et al., 2011; Miura and Tanno, 2012; Karch and Molkentin, 2015).

1.3.3.1 Components of the MPTP

1.3.3.1.1 CypD as an MPTP regulator

The use of CsA as a therapeutic target for cardioprotection during ischemia reperfusion in 1988 by Crompton and colleagues revealed that CsA inhibited MPTP activation (Crompton et al., 1988). This experiment was done as a result of observations from previous report that CsA treatment resulted in mitochondrial matrix Ca\(^{2+}\) accumulation (Halestrap et al., 1997; Hausenloy et al., 2012). Further studies by Halestrap and colleagues in 1990 showed that a matrix localised PPIase was the target of CsA and that it possibly interacts with the inner membrane ANT to mediate the MPTP. Afterwards, Crompton and colleagues identified the PPIase as CypD following the use of photoactive CsA (Tanveer et al., 1996). Many reports have thereafter validated the role of CypD in MPTP activation (Basso et al., 2005).

Indeed, CypD is the only genetically proven regulator or component of the MPTP, as other proposed components have failed rigorous experimental scrutiny. Single channel recordings of the MPTP using patch-clamping techniques revealed that the MPTP conductance was not different between CypD WT and KO mitoplasts, only that KO mitoplasts were insensitive to CsA treatment, thus, further confirming the role of CypD as a regulator and not a structural component of the MPTP (De Marchi et al., 2006).

1.3.3.1.2 Previous Hypothesized Molecular Interactors of CypD

1.3.3.1.2.1 VDAC-ANT-CypD interaction

The contact site hypothesis was attributed to the research by Crompton, Le-Quoc and Halestrap, and this proposed that the MPTP is a CypD regulated interaction between outer mitochondrial membrane VDAC and the inner membrane ANT (Elrod and Molkentin, 2013). Le-Quoc and colleague
suggested that VDAC is a structural component of the MPTP in an experiment where mitoplasts, mitochondria devoid of the outer membrane did not respond effectively to MPTP stimulation (Le-Quoc and Le-Quoc, 1985). It was suspected that the assembly of the pore may require VDAC. Subsequent pull-down assay carried out by Crompton and colleagues further revealed that GST tagged CypD pulled out VDAC and ANT. Moreover, reconstitution of purified VDAC and ANT in phosphatidylcholine liposomes showed pore-like characteristics (Crompton et al., 1998). In addition, inactivating phosphorylation of VDAC was reported to attenuate MPTP activation (Elrod and Molkentin, 2013).

This proposition gained more acceptance when it was observed that ANT inhibition with bongkrekic acid, which locks the ANT in the matrix ‘m’ conformation, resulted in delayed Ca\(^{2+}\)-induced permeability transition while carboxyatractyloside which inhibits ANT by locking it in the cytosolic ‘c’ conformation sensitized the mitochondria to Ca\(^{2+}\)-induced MPTP activation (Haworth and Hunter, 2000; Bernardi, 2013). In addition, it was observed by Brdiczka and colleagues that carboxyatractyloside activated contact between the inner and outer mitochondrial membrane (Bücheler et al., 1991; Ruck et al., 1998). Indeed, reconstitution of recombinant CypD, ANT and VDAC in liposomes showed PTP-like characteristics with conductance of about 1.3 nano-siemens (nS) (Brustovetsky and Klingenberg, 1996; Bauer and Murphy, 2020). Further studies revealed that overexpression of BAX was able to induce cell death only in the presence of ANT and that BAX co-precipitated with ANT (Marzo et al., 1998).

Despite the detailed and promising work that has been carried out on the structure of the MPTP, genetic ablation studies revealed that both ANT and VDAC were dispensable for pore formation. Indeed, double knock out of ANT1 and 2 still showed CsA dependent MPTP activation. Notably, elevated Ca\(^{2+}\) concentration was required to trigger pore opening (Kokoszka et al., 2004). More importantly, inhibition of the MPTP by ADP was abolished in ANT KO models. This was also observed for VDAC, as there was no effect on the
MPTP (Baines, 2007). Following these observations, the structural components of the MPTP still remain elusive, however, ANT may play some regulatory role during the activation of the MPTP.

Figure 1.10: VDAC-ANT-CypD Model of the MPTP. Mitochondrial Ca2+ overload was proposed to enhance the interaction between outer membrane VDAC and inner membrane ANT in a CypD dependent manner. The interaction was between the two different membrane protein components was believed to make the up the active permeability transition pore. Voltage dependent anion channel, VDAC, Adenine nucleotide translocator, ANT.

1.3.3.1.2.2 CypD-PiC-ANT interaction

Experiments carried out by Halestrap and colleagues in 2008, revealed that phosphate carrier (PiC), which is the primary matrix translocator of Pi, interacted with CypD in a CsA-dependent manner. In addition, ANT interaction with PiC further resulted in a conformational change that enhanced CypD interaction (Leung et al., 2008). It is noteworthy, that numerous groups have reported that MPTP opening is dependent on the level of matrix Pi (Crompton et al., 1988) which was consistent with the hypothesis that PiC plays significant role in MPTP activation. Furthermore, inhibition of PiC by N-ethylmaleimide (NEM) was also reported to inhibit the MPTP and the overexpression of PiC induced apoptosis in HeLa cells (Leung et al., 2008).

Few years later, additional studies by Baines and colleagues revealed that Pi did not affect or enhance MPTP opening in CypD KO or CsA treated mitochondria (McGee and Baines, 2012) and that the genetic ablation of PiC did not affect the MPTP (Varanyuwatana and Halestrap, 2012; Gutiérrez-Aguilar et al., 2014; Kwong et al., 2014). It was concluded that PiC may be an
important modulator of the MPTP. However, downregulation of PiC will alter Pi level in the matrix, thus having a negative effect on the mitochondrial ATP/ADP ratio which is an established modulator of the MPTP (Bauer and Murphy, 2020).

Mitochondrial Ca\textsuperscript{2+} overload was hypothesised to facilitate the interaction between two inner mitochondrial membrane proteins, ANT and PiC in a CypD dependent manner. This complex formation was believed to make up the permeability transition pore. Adenine nucleotide translocator, ANT, inorganic phosphate carrier, PiC.

**Figure 1.11: CypD-PiC-ANT Model of the MPTP.** Mitochondrial Ca\textsuperscript{2+} overload was hypothesised to facilitate the interaction between two inner mitochondrial membrane proteins, ANT and PiC in a CypD dependent manner. This complex formation was believed to make up the permeability transition pore. Adenine nucleotide translocator, ANT, inorganic phosphate carrier, PiC.

**1.3.3.1.2.3 Misfolded protein Aggregates**

The MPTP was proposed by He and Lemasters to consist of an aggregation of misfolded chaperone-regulated amphipathic proteins during the high conductance state when triggered by stronger inducers (He and Lemasters, 2002). This was shown to be unregulated and insensitive to CsA or Mg\textsuperscript{2+}. Exposure to oxidative stress induced the translocation of pore-forming lipophilic regions of the protein into the membrane. Following this event, chaperones, especially CypD would bind in an attempt to initiate proper folding, thus, creating a CsA-regulated pore. However, persistent oxidative stress increases the number of misfolded proteins, thereby outnumbering the chaperones (He and Lemasters, 2002). This would therefore result in an unregulated pore which is insensitive to MPTP inhibitors. It was acknowledged that ANT may play some unidentified role, being the most abundant inner membrane protein. Although, the hypothesis has not been scrutinised (He and Lemasters, 2002), Bauer and Murphy suggested that the hypothesis may be
irreconcilable with the features of the MPTP, which is regulated by adenine nucleotides, matrix pH and the $\Delta \Psi_m$ (Bauer and Murphy, 2020).

### 1.3.3.1.3 Currently Debated Molecular Interactors of CypD

The current hypothesis of the MPTP centres around the F1FO ATP synthase. Two separate hypotheses have been raised about the MPTP and the ATP synthase. These are the dimer hypothesis and the C ring hypothesis. However, the validity of these two hypotheses have faced immense scrutiny and criticism in the last couple of years, and direct experimental evidences have suggested the fallibility of these hypotheses.

#### 1.3.3.1.3.1 ATP Synthase Dimer Hypothesis

The attention on the ATP synthase started when Bernardi and colleagues reported that CypD binds in a CsA and Pi dependent manner to the lateral stalk (b and OSCP subunits) of the ATP synthase to inhibit the hydrolysis of ATP. This was confirmed in another experiment by Chinopoulos and colleagues (Giorgio et al., 2009; Chinopoulos et al., 2011). Indeed, already established regulators of the MPTP such as Pi, Mg$^{2+}$, adenine nucleotides, and $\Delta \Psi_m$ also modulated activities of the ATP synthase. In 2013, Bernardi and colleagues also showed that CypD binding to OSCP or OSCP depletion sensitised the MPTP to Ca$^{2+}$ via enhanced ATP hydrolysis without proton translocation to the intermembrane space (Giorgio et al., 2013). In addition, reconstituted dimers of the ATP synthase in liposomes were induced by Ca$^{2+}$ to form channels with conductance similar to the MPTP and that these were not observed with monomeric ATP synthase (Giorgio et al., 2013; Bernardi et al., 2015).

Furthermore, experiments by the Bernardi lab reported that Thr163 of the beta subunit of the ATP synthase were necessary for Ca$^{2+}$ binding, which prevents the generation of proton gradient by ATP hydrolysis (Giorgio et al., 2017) and the sensitisation to the collapse of the $\Delta \Psi_m$ during Ca$^{2+}$ overload (Nathanson
and Gromet-Elhanan, 1998). Mutation of this residue T159S (Bovine equivalent which can still bind Mg-ATP but not Ca-ATP) resulted in decreased sensitivity to Ca^{2+}-induced MPTP activation due to the attempt rescue to maintain the $\Delta\Psi_m$. One other claim in favour of the ATP synthase include the loss of the MPTP sensitivity to low pH inhibition following mutation of H112Q/Y of the OSCP subunit (Antoniel et al., 2018).

![Figure 1.12: ATP Synthase Dimer as a Model of the MPTP.](image)

Dimers of the ATP synthase form mostly at membrane curvature of the inner mitochondrial membrane. ATP synthase dimers were believed to form the MPTP during Ca2+ overload in a CypD dependent manner. CypD binding to OSCP and b subunit is believed to facilitate the opening of the MPTP. Oligomycin sensitivity conferring protein, OSCP.

However, in 2017, deletion of both OSCP and b subunit in HAP1-A11 cells by the Walker lab still showed CsA dependent Ca^{2+} activated MPTP. This experiment challenged the possibility of OSCP and b subunit being the binding site for CypD mediated MPTP (He et al., 2017a). However, this argument was refuted by Bernardi, as he suggested the plausibility of the cells developing
vestigial ATP synthase and these are incapable of dimerization following the deletion of multiple subunits. The case for the size of the MPTP channel observed by Walker and colleagues was also raised in Bernardi’s defence (Bernardi, 2018). However, in 2019, the Walker lab published another set of data showing the persistence of a CsA-dependent MPTP in the absence of ATP synthase dimerization (Carroll et al., 2019). They inhibited dimerization by deleting five other subunits (e, f, g, DAPIT and 6.8KDa proteolipid) of the ATP synthase, and this has indeed challenged the plausibility of the MPTP consisting of the ATP synthase dimers (Bauer and Murphy, 2020).

1.3.3.1.3.2 ATP Synthase C Ring Hypothesis

Porter and colleagues reported that the reconstitution of recombinant C subunit into liposomes formed voltage-sensitive channel that was sensitive to recombinant F1 beta subunit, adenine nucleotides and anti-c subunit antibody (Jonas et al., 2015). They also showed that Ca\(^{2+}\) and CsA modulated the opening and closing of the C subunit in cells and that cell death promoting factors increased the single channel conductance of the C subunit. In addition, elevated mitochondrial matrix Ca\(^{2+}\) resulted in the dissociation of the F1 from the C subunit. Conversely, genetic depletion of cellular C subunit resulted in the lack of Ca\(^{2+}\) induced inner mitochondrial membrane depolarisation (Alavian et al., 2014; Jonas et al., 2015).

In response to this hypothesis, Walker and colleagues showed that CsA sensitive MPTP persisted in the absence of the C subunit of the ATP synthase (He et al., 2017b). Gomez and colleagues also reported that atomistic simulations showed that the C subunit cannot be the MPTP (Zhou et al., 2017). In fact, this model is seemingly implausible because of the lipophilicity of the internal surface of the C subunit. The lipid plug occupying the internal surface is impermeable to solutes and will have to be dislodged for MPTP activation to occur (Gerle, 2016, Bauer and Murphy, 2020).
In the defence of the C subunit, another group argued that the pore conductance observed in C subunit deficiency of HAP1 cells was lower (300pS) than the characteristic conductance of the MPTP which is about 1.3nS. They indicated that the MPTP was not formed in this KO cells and that the conductance observed may have been contributed by ANT because of its sensitivity to Bongkrekic acid. Therefore, the loss of the C subunit may enhance the formation of the MPTP via other proteins which may include the ANT (Neginskaya et al., 2019). However, another concern that needs to be addressed about the involvement of the C subunit is centred around the reversibility of the MPTP under physiological conditions. Not until these concerns are addressed, the hypothesis may remain invalid.

1.3.3.1.4 Other Possible CypD-Mediated MPTP Models

Two other groups have suggested that that the MPTP is centred around the ATP synthase and the accessory proteins necessary for the synthesis of ATP such as, ANT and PiC. To support this hypothesis, one of the groups suggested that the supernumerary complex of the ATP synthase may form the pore in a CypD-dependent manner. It was suggested that dimers of the ATP synthase, ANT and PiC are assembled into a synthasome and CypD activation and interaction dislodges this complex interaction, thus resulting in the increased permeability of the inner mitochondrial membrane, hence MPTP (Beutner et al., 2017; Bauer and Murphy, 2020).

The other group suggested that the MPTP comprises of the ANTs and a yet elusive protein component that requires CypD. Their opinion was based on the observation that triple KO of ANT1, 2 and 4 in mouse model had an inner membrane characteristic that was resistant to pore opening, although which was still sensitive to CsA. However, when CypD was KO, there was a complete abrogation of the MPTP (Karch et al., 2019; Bauer and Murphy, 2020).
1.3.3.2 Mitochondrial matrix proteins and CypD-mediated MPTP

1.3.3.2.1 Mitochondrial Chaperones Stress response and MPTP

The MPTP has been reported to be negatively regulated by the activity of both TRAP-1 and HSP60. It is proposed that they antagonise CypD-mediated MPTP via the refolding of unfolded mitochondrial proteins during MPTP potentiating stress signals (Kang et al., 2007). Furthermore, some studies have reported direct interaction between CypD and HSP60, and that these interactions impeded the formation of MPTP (Ghosh et al., 2010; Tang et al., 2016). It has been proposed that cancer cells accumulate these chaperones in their mitochondria to prevent MPTP mediated necrotic cell death. In support of this, treatment of cells with Gamitrinibs, a potent inhibitor of TRAP-1 abolished protection against CypD mediated MPTP, activated unfolded protein response and potentiated tumour shrinkage (Kang and Altieri, 2009).

Another mitochondrial protein involved in mitochondrial protein transport, which is associated with HSP70, is the DnaJ homolog, subfamily C, member 15 (DnaJC15); this was reported to enhance cisplatin-induced MPTP via direct interaction with CypD (Sinha and D’Silva, 2014). Notably, resistance to chemotherapy in both ovarian and breast cancer were associated with hypermethylation of the CpG island in the gene encoding DnaJC15, hence, resulting in their reduced protein expression (Bonora and Pinton, 2014).

P53, has also been dubbed 'the guardian angel of the mitochondrial genome' (Park et al., 2016a), as it is implicated in triggering the MPTP via direct interaction with CypD during oxidative stress. It is proposed that oxidative stress is the main driver for p53-mitochondrial translocation, where unfolded p53 displaces CypD-TRAP1 interaction and binding to CypD trigger MPTP opening (Lebedev et al., 2016). Mitochondrial matrix p53-CypD interaction resulted in necrotic damage during ischemia reperfusion injury (Vaseva et al., 2012; Javadov et al., 2013; Morciano et al., 2015).
1.3.4 Post translational modification of CypD

1.3.4.1 S-Nitrosylation

Protein thiol S-nitrosylation (SNO) of cysteine residues is an important post translational modification in cell signalling whereby a NO moiety is covalently attached to thiol groups of protein cysteine residues (Elrod and Molkentin, 2013). CypD is reported to undergo SNO modification at specific Cys203, and this modification has been shown to negatively regulate the activation of the MPTP similarly to CypD deletion (Nguyen et al., 2011). It was suggested that endogenous SNO modification of CypD may prevent MPTP formation by masking the oxidation of Cys203/161 (full length/mature protein sequence) to a highly reactive protein species. It should however be noted that *Bos taurus* still displays CsA dependent MPTP, even though it lacks this residue (Porter and Beutner, 2018).

1.3.4.2 Acetylation

Most of the published work supports the notion that acetylation activates CypD activity, hence, enhancing pore opening (Elrod and Molkentin, 2013; Porter and Beutner, 2018). Indeed, it was reported that the activation of sirt3 by mitochondrial NAD treatment repressed pathologies associated with the MPTP (Teodoro et al., 2018). Lys166 (full-length sequence), a residue near the PPIase catalytic site is attributed as the acetylation site on CypD (Hafner et al., 2010). Another proposed acetylation site on CypD is Lys145 (full length sequence), and it was reported that deacetylation at this site resulted in the dissociation of CypD from ANT (Shulga et al., 2010). The acetylation of CypD can occur spontaneously in the matrix, as favourable conditions such as abundant acetyl-coA and alkaline environment, are readily available (Wagner and Payne, 2013). However, setback to this hypothesis is only one mitochondrial transacetylase has been reported (Scott et al., 2012; Porter and Beutner, 2018).
1.3.4.3 Phosphorylation

Phosphorylation of CypD has been reported to increase its activity, thereby enhancing MPTP activation. However, the mechanism and compositions of kinases and phosphatases in the mitochondrial matrix are relatively unknown. GSK 3β has been reported to phosphorylate recombinant CypD \textit{in vitro} (Elrod and Molkentin, 2013). However, other groups have reported the GSK 3β mediated phosphorylation of CypD and the subsequent activation of the MPTP \textit{in vivo} (Teodoro et al., 2018). The Bernardi lab reported that matrix ERK represses MPTP activation by phosphorylating and inhibiting GSK 3β (Rasola et al., 2010). AKT2 has also been shown to mediate CypD phosphorylation, thus, activating the MPTP (Ghosh et al., 2015). In addition, deletion of the mitochondrial Ca\textsuperscript{2+} uniporter also resulted in CypD phosphorylation and enhanced MPTP sensitivity to Ca\textsuperscript{2+} (Parks et al., 2019). The mechanism of kinase translocation to the mitochondria is unknown because of the lack of mitochondrial targeting sequence in their composition. Two serine sites have been reported so far for CypD phosphorylation, Ser31 and Ser42 (mature protein sequence) (Porter and Beutner, 2018).

1.3.5 Role of CypD in Disease Pathology

1.3.5.1 Role of CypD in Amyloid Beta-Induced Toxicity

Amyloid beta has been implicated in ROS production and mitochondrial toxicity which may be associated with MPTP activation (Lustbader et al., 2004; Reddy and Beal, 2008; Du et al., 2014; Rao et al., 2014). CypD deficiency has been used to alleviate mitochondrial and synaptic function, in addition to significantly enhanced learning and memory in mice. This might have resulted from reduction in MPTP formation (Du et al., 2008; Du et al., 2011; Du et al., 2014; Swerdlow, 2018). Further studies by the same group using surface plasmon resonance showed that amyloid beta directly interacted with CypD (Du et al., 2008), thus potentiating ROS production and subsequent MPTP formation (Pagani and Eckert, 2011).
1.3.5.2 Role of CypD in Ischemia-Reperfusion Injury

The MPTP has been associated with ischemia reperfusion injury because of the sudden burst of respiration via complex II during reperfusion which occurs immediately after a period of ischemia or hypoxia. During ischemia, CoQH$_2$ and PMF accumulates in the inner mitochondrial membrane because of the unavailability of oxygen (Chouchani et al., 2014). This result in the activation of fumarate reductase system which involves the transfer of electron to fumarate via complex II leading, thus leading to the accumulation of succinate. However, PMF is still being generated across the inner membrane through proton translocation via the oxidation of NADH by complex I to replenish CoQH$_2$ for more succinate production. In the process, NADH producing enzymes such as OGDH oxidises 2-oxoglutarate, thus, making more succinate. Overall, succinate becomes the metabolic terminal point of the TCA cycle (Chouchani et al., 2014; Pell et al., 2016b).

Another group has also recently suggested that glycolysis and glycogenolysis during ischemia drives the production of succinate. Glycolytic pyruvate accumulation during ischemia is aminated by alanine amino transferase to alanine and by-product 2-oxoglutarate. Consequently, 2-oxoglutarate then drives succinate production in the mitochondria (Zhang et al., 2018).

During reperfusion, the increase in oxygen results in the abrupt oxidation of succinate and this return of succinate back to the baseline within five minutes of reperfusion. Elevated CoQH$_2$ and inner membrane PMF favours the electron leakage to molecular oxygen resulting in superoxide production. In addition, superoxide production by reverse electron transfer is favoured at complex I. Indeed, the accumulation of NADH due to succinate oxidation also results in superoxide production at OGDH and other free FMN sites. These processes culminate in mitochondrial oxidative stress and damage (Chouchani et al., 2014; Pell et al., 2016a).

Indeed, the protection against ischemia reperfusion injury in different experimental models involving either the therapeutic inhibition or the genetic
ablation of CypD has no doubt confirmed the role of CypD and the MPTP in ischemia reperfusion injury (Shiga et al., 1992; Halestrap et al., 2004; Ikeda et al., 2016). More importantly, Hausenloy and colleagues revealed that CsA treatment prior to or within five minutes of reperfusion rescued IR injury whereas treatment outside this time frame had no protective effect on IR injury (Ong et al., 2015). This is consistent with previous findings that showed the depletion of ischemic succinate within five minutes of reperfusion (Zhang et al., 2018).

Interestingly, pore opening through CypD activity have also been suggested to significantly contribute to ischemic preconditioning (IPC) signalling. IPC is cytoprotective and it involves the transient, brief episodes of ischemia reperfusion before a major ischemic event. Interestingly, inhibition of CypD during ischemic preconditioning has been shown to prevent IPC induced protection during a prolonged ischemic insult (Elrod and Molkentin, 2013). It has been suggested that IPC results in the activation of mitochondrially terminating signalling factors that help protect from MPTP opening during a major ischemic episode. The contributing pathways are, Reperfusion Injury Salvage Kinase (RISK) and Survival Activation Factor Enhancement (SAFE) pathways (Ong et al., 2015).

1.4 Aim and Objectives

The role of CypD in MPTP activation has been the subject of research for the past three decades; however, a conclusive consensus has not been reached as to how CypD mediates Ca\(^{2+}\) and ROS-dependent pore opening. It is, therefore, expedient to examine CypD from a different view from the MPTP and focus on its role in the modulation of basic mitochondrial and cytoplasmic metabolic and signalling pathways, as the data obtained may provide insight into the regulation of the MPTP and possibly decipher some of its structural protein players. This project is divided into three main objectives and they are listed below.
1.4.1 Purification of Recombinant and Quantitation of mitochondrial Cyclophilin D
The hypothesis is that the quantification of CypD in the mitochondria relative to the total mitochondrial proteome will enable a more complete understanding of its importance in the mitochondria. This is because protein abundance can be associated with its metabolic function. This section will also differentiate between the level of CypD between non-cancer and cancer cells.

1.4.2 Assessment of the Role of Cyclophilin D in the Regulation of Mitochondrial Redox and Energy Homeostasis
The hypothesis is that CypD regulates mitochondrial Redox and energy homeostasis in a dynamic way. It directly modulates both the level of ROS and reductive equivalents via both redox sensing by its Cysteine 203 residue and the modulation of the electron transport chain.

1.4.3 Evaluation of Metabolic Reprogramming in CypD Deficient Cells
The hypothesis is that the bioenergetic and redox dysregulation that arise from CypD deficiency may result in metabolic changes, alteration of signalling pathways and global metabolic reprogramming. Moreover, tracing the adaptive response signalling pathways may give insight into the specific role of CypD in the mitochondria.
Chapter Two

General Materials and Methods
2 General Materials and Methods ................................................................. 70
2.1 Materials ......................................................................................... 70
  2.1.1 Plasmids ...................................................................................... 70
  2.1.2 Cell lines and lysates ................................................................. 70
    2.1.2.1 Bacteria cells ........................................................................ 70
    2.1.2.2 Mammalian cell lines ......................................................... 70
    2.1.2.3 Cell Lysates ......................................................................... 70
  2.1.3 Culture media ................................................................................ 71
    2.1.3.1 Bacteria culture media ....................................................... 71
    2.1.3.2 Tissue culture media .......................................................... 71
  2.1.4 siRNA Oligonucleotides ............................................................ 71
  2.1.5 Tissue sources ............................................................................. 71
2.2 Methods .......................................................................................... 71
  2.2.1 Bacteria Culture ............................................................................ 71
    2.2.1.1 Transformation .................................................................... 71
    2.2.1.2 Plasmid extraction miniprep .............................................. 72
    2.2.1.3 Plasmid concentration and purity ....................................... 72
    2.2.1.4 Protein overexpression .......................................................... 72
    2.2.1.5 Cell harvest ......................................................................... 72
    2.2.1.6 Cell lysis ............................................................................ 73
  2.2.2 Protein purification ....................................................................... 73
    2.2.2.1 Affinity chromatography ...................................................... 73
    2.2.2.2 His-tag cleavage and reverse his-trap .................................. 73
    2.2.2.3 Size exclusion chromatography .......................................... 74
  2.2.3 Protein Activity and Modulation assay ....................................... 74
    2.2.3.1 Peptidyl prolyl cis-trans isomerase (PPIase) activity ......... 74
    2.2.3.2 Redox modulation of CypD and PTPMT1 ........................... 74
      2.2.3.2.1 Protein Sulfenylation assay Oxidation assay ............... 75
      2.2.3.2.2 Differential scanning fluorimetry .................................. 75
      2.2.3.2.3 Phosphatase activity assay ......................................... 75
2.2.4 Human Tissue culture ................................................................. 76
  2.2.4.1 Culture and passaging ........................................................... 76
    2.2.4.1.1 Preparation of Complete Media ........................................... 76
    2.2.4.1.2 Cell defrosting and culturing ............................................ 76
    2.2.4.1.3 Cell splitting and passaging ............................................. 76
  2.2.4.2 siRNA Transfection and knock down ....................................... 77
    2.2.4.2.1 2.2.4.2.1 siRNA preparation ............................................ 77
    2.2.4.2.2 Transfection of 35mm (6 well) plates ................................ 77
  2.2.4.3 RIPA lysis ............................................................................. 77
  2.2.4.4 Drug Treatments .................................................................... 78
  2.2.4.5 Colony forming (Clonogenic) assay ......................................... 78
  2.2.4.6 Luciferase assay ..................................................................... 78
  2.2.4.7 Immunofluorescence ............................................................... 79
    2.2.4.7.1 Cell plating and transfection ............................................... 79
    2.2.4.7.2 Mitochondrial membrane potential Superoxide Cellular ROS or Lipid droplet dye staining experiments ......................... 79
    2.2.4.7.3 Antibody staining ............................................................... 79
    2.2.4.7.4 Cell mounting and Imaging .................................................. 80
  2.2.4.8 RNA extraction and quantitative PCR ....................................... 80
  2.2.4.9 Electrophoresis and Western Blots ........................................... 81
    2.2.4.9.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) .......................................................... 81
    2.2.4.9.2 Coomassie brilliant blue staining and de-staining ............... 82
    2.2.4.9.3 Western and Dot blot ......................................................... 83
      2.2.4.9.3.1 Western blot transfer ...................................................... 83
      2.2.4.9.3.2 Dot blot transfer ............................................................ 83
      2.2.4.9.3.3 Antibody Incubation and Imaging .................................. 83
    2.2.4.9.4 Measurement of Mitochondrial Membrane potential in Intact Cell 85
    2.2.4.9.5 Measurement of NAD(P)H in Intact Cell or Mitochondria 85
2.2.5 Mitochondria extraction and activity .............................................. 85
  2.2.5.1 Mitochondria extraction ......................................................... 85
    2.2.5.1.1 Mammalian tissue mitochondria extraction ..................... 85
    2.2.5.1.2 HAP1 cell mitochondria extraction .................................. 86
  2.2.5.2 Mitochondria activity assays ............................................... 86
    2.2.5.2.1 Mitochondrial swelling assay ....................................... 86
    2.2.5.2.2 Measurement of NAD(P)H in isolated mitochondria ......... 87
    2.2.5.2.3 Measurement of Cellular or Mitochondrial ATP .............. 87
    2.2.5.2.4 Respiratory Chain complex activity ............................... 88
    2.2.5.2.5 Mitochondrial antioxidants activities ............................ 88
  2.3 Statistical Analysis ........................................................................ 88
2 General Materials and Methods

2.1 Materials

2.1.1 Plasmids

pET-M11a plasmids were obtained from EMBL. Genes for the mitochondrial mature form of ppm1 (uniport accession number: P30405) and full length ptpmt1 (uniport accession number: Q8WUK0) were synthesized by UoL Gene Mill and cloned in pETM11a vector.

2.1.2 Cell lines and lysates

2.1.2.1 Bacteria cells

E. coli; XL blue and BL21 star DE3 strains (Invitrogen, USA).

2.1.2.2 Mammalian cell lines

Near Haploid HAP1 WT and CRISPR/Cas9 ppm1 KO cells (Horizon Discovery, UK). Other cell lines; Human cervical carcinoma HeLa cells and Human fibrosarcoma cell line HT1080 were generous gifts from Professor Sonia Rocha, Department of Biochemistry, University of Liverpool. HeLa cells used were Hypoxia response element (HRE) luciferase stable cell lines and were generated via co-transfection of a puromycin resistant construct with an HRE luciferase reporter vector.

2.1.2.3 Cell Lysates

Cell lysates from Human osteosarcoma U2OS, Human embryonic kidney HEK293, Human renal cell adenocarcinoma 786-O, Human umbilical vein endothelial cell HUVEC, Human clear cell renal cell carcinoma RCC4, Human foreskin fibroblasts HFF, Human breast adenocarcinoma MB-MDA-231 were obtained from Dr. Michael Batie, Department of Biochemistry, University of Liverpool.
2.1.3 Culture media

2.1.3.1 Bacteria culture media
Luria agar culture was made following the manufacturer’s instruction (Merck, East Yorkshire, UK).
Luria Broth (LB) culture was made following the manufacturer’s instruction (Merck, East Yorkshire, UK).

2.1.3.2 Tissue culture media
Iscove’s Modified Dulbecco’s Medium IMDM (ThermoFisher Scientific, UK) and Dulbecco’s Modified Eagle Medium DMEM (ThermoFisher Scientific, UK).

2.1.4 siRNA Oligonucleotides
siRNAs were obtained from Eurofins Genomics, Germany.

<table>
<thead>
<tr>
<th>siRNAs</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CAG UCG GGU UUG GGA CUG G</td>
</tr>
<tr>
<td>CypD</td>
<td>AGA UUG ACU AGA AGU GAA A</td>
</tr>
</tbody>
</table>

2.1.5 Tissue sources
Mouse (*Mus Musculus*; C57BL/6)

2.2 Methods

2.2.1 Bacteria Culture

2.2.1.1 Transformation
Competent cells (*E. coli*; 50µL) was incubated with 100ng vector (pET-M11a-ppi* or pET-M11a-PTPMT1) for 30 min on ice. This was then heat shocked for 45s at 42°C. 200µL SOC media (Merck, East Yorkshire, UK) was added and the mixture incubated at 37°C for 1h in a shaker at 210 rpm. The cells were collected by centrifugation and re-suspended into 50µL SOC media. These were plated onto a sterile LB Agar plate containing 50µg/mL Kanamycin for cell selection. The culture was incubated overnight at 37°C in a static incubator.
2.2.1.2 Plasmid extraction miniprep
A transformed XL blue colony from the plate was sub-cultured to 10 mL LB broth which was grown overnight at 37°C, 210 rpm. The overnight culture was centrifuged for 10 min at 4000rpm to pellet the cells. Plasmids were extracted using a Qiagen miniprep kit; this was done according to the manufacturer’s instructions (Qiagen, UK).

2.2.1.3 Plasmid concentration and purity
Plasmid concentration and purity was determined by Nano-drop spectrophotometer (Thermo scientific, UK). Ratio of absorbance at 260nm and 280nm was used to assess plasmid purity. Values around 1.9 suggests pure DNA.

2.2.1.4 Protein overexpression
A colony of transformed BL21 star DE3 colony was inoculated into 20mL of LB broth containing 50μg/mL kanamycin. This was incubated at 37°C overnight with shaking at 210rpm. Overnight culture was inoculated into 500 mL LB media containing 50μg/mL kanamycin in a ratio of 1:50. The starting absorbance at OD600 was 0.1. Cultures were grown at 37°C (210 rpm) until an absorbance of 0.7 was reached. Overexpression of protein was induced with 1mM IPTG (Bioline UK, London, UK) and incubated overnight at 18°C in a shaker at 180 rpm.

2.2.1.5 Cell harvest
The cultures were transferred to pre-chilled, sterile 100 mL tubes and centrifuged (Sorvall RC 5B refrigerated super speed centrifuge) for 30 min, at 5000rpm at 4°C. Cell pellets were re-suspended in pre-chilled sterile buffer containing 50mM Tris-HCl, pH 8.0, 500mM NaCl, 3mM DTT supplemented with lysozyme and stored on ice in a cold 250mL beaker for lysis.
2.2.1.6 Cell lysis
Cells were passed through a pressure cell homogenizer (Stansted, UK) 5 times for a 30s period with intermittent resting time on ice of 1 min. Samples were centrifuged (Sorvall RC 5C super speed refrigerated centrifuge, UK) in pre-chilled sterile 50 mL centrifuge tubes at 18,000 ×g for 40 min, 4°C.

2.2.2 Protein purification

2.2.2.1 Affinity chromatography
Affinity Nickel-NTA resin column (5mL Fast flow, GE, Sweden) was connected to a purifying system (AKTA, GE, Sweden). The column was equilibrated with the loading and elution buffers at a flow rate of 4 mL/min. Cell lysate were loaded on to the column at a flow rate of 2.5mL/min and washed with 3 column volume of loading buffer (50mM Tris-HCl, pH 8.0, 500mM NaCl, 3mM DTT) at the same flow rate. Histidine-tagged protein was eluted with a gradient of elution buffer (50mM Tris-HCl, pH 8.0, 500mM NaCl, 3mM DTT, 1M Imidazole). Protein absorbance on the AKTA Purifier were monitored at 280 nm.

2.2.2.2 His-tag cleavage and reverse his-trap
Eluate was immediately buffer exchanged into non-imidazole buffer with desalting column (Hi prep 26/10 desalting, GE, Sweden) at flow rate of 10mL/min. CypD desalting buffer: 25mM Tris-HCl, pH 8.0, 150mM NaCl, 5% glycerol. Histidine tag of eluted proteins were then cleaved by TEV (Tobacco Etch Virus) protease on their TEV cleavage site overnight at 25°C. These were immediately loaded back unto the Affinity Nickel-NTA resin column (5mL Fast flow, GE, Sweden) and flow-through were collected as the non-tagged protein. Cleaved tag was eluted using imidazole gradient and the column was washed and stored in 20% (v/v) ethanol.
2.2.2.3 Size exclusion chromatography
Protein (CypD) was further purified by size exclusion chromatography using a gel filtration column (Hiload 26/600, GE, Sweden). The column was equilibrated overnight with gel filtration buffer (50mM NaH₂PO₄·H₂O, pH 6.8, 100mM NaCl, 5mM DTT, 2mM EDTA) at flow rate of 1mL/min. Protein were loaded unto the column at 1mL/min and elution under a single peak were analysed further on polyacrylamide. The column was washed with 1 column volume of MilliQ water and stored in 1 column volume of 20% (v/v) ethanol.

2.2.3 Protein Activity and Modulation assay
2.2.3.1 Peptidyl prolyl cis-trans isomerase (PPIase) activity
Recombinant and mitochondrial CypD activity were monitored spectrophotometrically in a chymotrypsin-coupled reaction. The assay was performed in 50mM HEPES pH7.5, 25mM NaCl in total reaction volume of 250µL at 10°C. Freshly prepared 0.5mM alpha-chymotrypsin in 1mM HCl was diluted 10-fold following addition into the reaction mixture containing either 30µg/mL or 80µg/mL of recombinant CypD or mitochondrial lysate respectively. Recombinant protein or mitochondrial lysate were incubated with 2-10µM inhibitor for 15 min prior addition to buffer medium where necessary. The peptide substrate, succinyl-alanine-alanine-proline-phenylalanine-p-nitroanilide (Suc-AAPF-pNA; Sigma, UK) was immediately added to a final concentration of 60µM from a 10mM stock dissolved in 0.47M LiCl/TFE and quickly mixed to homogeneity. The reaction was monitored by kinetic measurement of the absorbance at 390nm for 2.5-5mins on a Varian Cary 300 Bio UV-Vis spectrophotometer.

2.2.3.2 Redox modulation of CypD and PTPMT1
Protein samples were used at final concentration of 5µM. PTPMT1 and its mutant C132S alongside CypD mutant C203S were expressed and purified by Dr. Amy Wood, Department of Biochemistry, University of Liverpool.
2.2.3.2.1 Protein Sulfenylation assay Oxidation assay
Recombinant CypD or PTPMT1 were incubated at a final concentration of 5µM in 10µM H$_2$O$_2$ [Buffer: 20mM NaCl, 20mM Na$_2$PO$_4$ (pH 7.2)] at varying time points at 37°C. Sulfenic acid was stabilised by Dimedone (Sigma, UK) at a final concentration of 1mM. Samples were immediately mixed with non-reducing SDS PAGE sample buffer and loaded onto SDS gels. Electrophoresis was run at 80V for 30min, then 120V through to the end. Western blot was immediately carried out and membranes were incubated in primary antibody specific for sulfenic acid. Dimedone and sulfenic acid antibody were generously provided by Dr. Dominic Byrne, Department of Biochemistry, University of Liverpool.

2.2.3.2.2 Differential scanning fluorimetry
Differential scanning fluorimetry (DSF) also known as thermal shift assay was used to assess the interaction of recombinant CypD and PTPMT1 with potential modulators. CypD or PTPMT1 (5µM) were incubated with varying concentrations of H$_2$O$_2$ or CsA (10µM) for 10min at 37°C in buffer containing 20mM NaH$_2$PO$_4$ pH 7.2 and 20mM NaCl. Afterwards, Sypro orange was added to the mixture at a final concentration of 1X and was immediately transferred into PCR plates (25µL final reaction volume), centrifuged for 10 seconds at 1000xg and then placed into QPCR machine for measurement of fluorescent intensities with changes in temperature (ThermoFisher, UK). At the end of the run, a Boltzmann curve was plotted and melting temperatures were calculated.

2.2.3.2.3 Phosphatase activity assay
PTPMT1 was incubated at a final concentration of 5µM with 10µM H$_2$O$_2$ in buffer containing 20mM NaCl and 20mM Na$_2$PO$_4$ pH 7.2 in a 96 well plate. Treatment groups were incubated in 1mM DTT for 10min at 37°C prior to H$_2$O$_2$ treatment. 10µL of 4-nitrophenyl phosphate (pNPP; NEB, USA) was added and air bubbles were immediately removed by gentle aeration. Kinetic absorbance measurements were immediately taken at 405nm.
2.2.4 Human Tissue culture

2.2.4.1 Culture and passaging

2.2.4.1.1 Preparation of Complete Media

Human Cell lines were maintained at 5% v/v CO\textsubscript{2}, 37°C in complete media. Iscove’s modified Dulbecco’s medium (IMDM) or Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2mM glutamine, 50 units/mL Penicillin, 50µg/mL Streptomycin and 10% FBS (Gibco, ThermoFisher, UK).

2.2.4.1.2 Cell defrosting and culturing

Adherent cells were plated onto cells according to prescribed instructions. Briefly, IMDM (only for HAP1 cells) or DMEM media (Gibco, ThermoFisher, UK) supplemented with 2mM glutamine, 50 units/mL Penicillin, 50µg/mL Streptomycin and 10% FBS (Gibco, ThermoFisher, UK) to make rich media. Media were warmed up to 37°C in water bath and 10mL of media were transferred into 60mm plates. Cryovials containing cells were removed from storage (Liquid nitrogen or -80°C freezer) and immediately placed in 37°C water bath to thaw until a tiny fraction of iced cells remains. Cells were immediately transferred into the pre-warmed growth media and incubated at 5% v/v CO\textsubscript{2}, 37°C for 24hr. Following incubation, cells were split into 150mm plates containing pre-warmed media at 1:15000 dilution.

2.2.4.1.3 Cell splitting and passaging

Complete IMDM or DMEM Media, PBS and Trypsin- EDTA (0.05%) were warmed at 37°C in water bath. Prior to splitting, 15mL of pre-warmed media were transferred into 150mm plates. Cultured adherent cells were washed thrice with warm 1x PBS and trypsinized with 5mL of trypsin in an incubator at 5% v/v CO\textsubscript{2}, 37°C for maximum of 5 mins. Trypsinisation was quenched with 5mL of rich media and detached cells were resuspended in 15mL tube. Cell suspension was re-plated in pre-warmed complete media and incubated at 5% v/v CO\textsubscript{2}, 37°C till 90% confluence is achieved.
2.2.4.2 siRNA Transfection and knock down

2.2.4.2.1 siRNA preparation

siRNA oligonucleotides were purchased from MWG/Eurofins, UK and diluted to 20µM by adding 1x siRNA buffer. Once diluted siRNAs were aliquoted out into Eppendorf tubes and stored at -20. Samples were thawed on ice before use.

2.2.4.2.2 Transfection of 35mm (6 well) plates

Cells were plated onto 35mm plates for 24hr before transfection at a density of 1.5x10^5 cells in a total volume of 2mL media. In 1.5mL Eppendorf tubes, 200µL of Opti-MEM® I Reduced Serum Media (Life technologies), 3µLs of interferin (Polyplus Transfection, Illkirch, France) and 3µL of 20µM siRNA were mixed by scraping Eppendorf across TC hood grill. Reaction mixture was incubated at room temperature for 15-20min. Prior to transfection, the cell media was replaced with fresh media and 200µL of the transfection complex was added to the cells by dropwise pipetting. The culture was gently mixed by cross-moving the plates thrice and cells were incubated at 5% v/v CO₂, 37°C for 48hr prior to collecting lysates.

2.2.4.3 RIPA lysis

For collecting cell lysates, cell media was removed, cells were washed with Dulbecco’s Phosphate-Buffered Saline (DPBS) (Life Technologies) and then harvested in Radio-Immunoprecipitation Assay (RIPA) lysis buffer (50mM Tris-HCl pH 8.0 (VWR chemicals), 150mM sodium chloride(VWR chemicals), 1.0% v/v NP-40 (Calbiochem), 0.5% Sodium deoxycholate (Sigma), 0.1% w/v SDS (VWR chemicals), 10mM sodium fluoride (Sigma), 2mM sodium orthovanadate (sigma) and 1 protease inhibitor cocktail tablet (Roche) per 10mL of lysis buffer). 100µL for 35mm plates, 200µL for 100mm plates and 400µL for 150mm plates of RIPA buffer was used. Cells were scraped into 1.5mL Eppendorf tubes, the suspension was then incubated on ice for 10min and centrifuged for 10min at 13000rpm, 4°C. The supernatant was collected
and stored at -80°C. Cells treated with hypoxia were harvested in the hypoxia chamber to prevent reoxygenation. Protein concentrations of lysates were determined by BCA assay (BioRad, USA).

2.2.4.4 Drug Treatments
Rotenone, Antimycin A, Oligomycin A, CsA, Pifithrin µ, Thapsigargin and MG132 (Sigma, UK) were dissolved in DMSO and used at a final concentration of 10µM. MitoSox, TMRM, FCCP, BODIPY were obtained from ThermoFisher and were dissolved in DMSO and used at a final concentration of 5µM, 0.8µM, 5µM, and 2µM respectively. Hypoxia treatments were performed in an In Vivo 300 hypoxia workstation (Ruskin, UK) at 1% O₂ 5% CO₂, and 37°C.

2.2.4.5 Colony forming (Clonogenic) assay
Cells were seeded onto 35mm plates at a density of 200 cells in 2mL media. Cultured cells were incubated at 5% v/v CO₂, 37°C for 14 days before treatment with 10µM inhibitors of the electron transport chain (Rotenone, Antimycin A, Oligomycin A; Sigma, UK) for varying time points 24hr. Control groups were treated with DMSO. Following treatments, media was gently removed by pipetting and wells were washed once with 1x PBS. Immediately, 2mL of staining solution (0.2% Coomassie brilliant blue R250 in 50% Methanol /7% Acetic acid) was added onto wells for 10mins. Solution was removed by pipetting and washed once with 1x PBS and allowed to airdry. Colonies were imaged with Chemi Doc XRS system (BioRad, USA).

2.2.4.6 Luciferase assay
Cells stably transfected with an HRE luciferase reporter construct (3 copies of a 21bp sequence from the iNOS promoter containing the HRE sequence (5'-AGTGACTACGTGCTGCCTAGG-3') (Lia et al., 2013) cloned into the KpnI and MluI restriction sites of the pGL3 promoter vector (Promega, UK)) were plated onto 35mm plates at a density of 1.5 x10⁵ cells in a total volume of 2mL media. 48hr following cell seeding, cells were stimulated with hypoxia for 24hr.
Cells were then harvested using 400μL Passive lysis buffer (Promega, UK). Luciferase activity was measured according to manufacturer’s instructions using a luminometer (Lumat LB 9507, EG&G Berthold). Briefly, 5μL of the supernatant was pipetted into a Sarstedt round-bottom polystyrene tube (Thermo Scientific; 50-809-212) that is compatible with Berthold Lumat LB 9507 Luminometer. The tube containing supernatant was placed in the Luminometer that had been set to add 50μL of luciferase assay reagent (Promega) into the tube. Luciferase activity was measured 10sec after the addition of reagent by the Luminometer. Results were normalised to the protein concentration, which was determined by BCA Assay (BioRad).

2.2.4.7 Immunofluorescence

2.2.4.7.1 Cell plating and transfection
Cells were plated onto sterilised glass coverslips (VWR 19mm) in 35mm plates 24hr before transfection at a density of 1.2x10^5 cells in a total volume of 2mL media.

2.2.4.7.2 Mitochondrial membrane potential Superoxide Cellular ROS or Lipid droplet dye staining experiments
48hr following transfection cells were treated with either MitoSox, TMRM, CellRox or BODIPY (ThermoFisher, UK) for 1 hour and then fixed on coverslips in 1mL 3.7% formaldehyde in PBS (pH 6.8) for 15min at 37°C inside cell incubator, washed with PBS and incubated with either Hoechst (1μg/mL) or Topro-3 (1:1000) for 2 or 30mins respectively.

2.2.4.7.3 Antibody staining
48hr following transfection, cells were fixed to coverslips in 1mL 3.7% formaldehyde in PBS (pH 6.8) for 15min at 37°C inside cell incubator, washed with PBS permeabilised in 1mL PBS-0.1% Triton X-100 for 10min at room temperature. Cells were then washed with PBS and blocked with 120μL 1% donkey serum in PBS 0.05% Tween for 30min at room temperature. Cells
were placed in a humidified chamber incubated with primary antibody diluted in 1% donkey serum in PBS 0.05% Tween (120μL) (Table 2.1) overnight at 4°C, washed 3 times 5min in PBS and incubated for 1hr with secondary antibody 1% donkey serum in PBS 0.05% Tween (120μL) (Table 2.1). Slides were washed 3 times for 5min in PBS and nucleus staining was performed by incubation with 120μL 33.3μM Hoechst (Sigma, UK) for 2min.

### 2.2.4.7.4 Cell mounting and Imaging

Coverslips were then washed in water and mounted onto VWR slides (VWR) using mounting medium (H-1000; Vector labs) and sealed with nail varnish. Laser line of 488nm, 568nm and 633nm were used to observe green, red and far red fluorescence on a Zeiss LSM710 confocal microscope while RFP, GFP and DAPI filters were used to obtain red, green and blue images respectively on a Zeiss Epifluorecent microscope (Centre for Cell Imaging, University of Liverpool). Images were analysed with Image J open source analysis software [NIH, Maryland, USA (Abràmoff et al., 2004)] and OMERO client software [Open Microscopy Environment (Allan et al., 2012)]

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR</td>
<td>Cell Signalling Technology, 7C10</td>
<td>R</td>
<td>1:300</td>
</tr>
<tr>
<td>Lamp2</td>
<td>Proteintech, 27823-1-AP</td>
<td>M</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-rabbit Alexa fluor 488</td>
<td>ThermoFisher, A11070</td>
<td>G</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti-mouse Alexa fluor 555</td>
<td>ThermoFisher, A11020</td>
<td>G</td>
<td>1:500</td>
</tr>
</tbody>
</table>

(R= rabbit, M= mouse, G=Goat)

### 2.2.4.8 RNA extraction and quantitative PCR

Total RNA from cells was extracted using peqGOLD total RNA kit (Peqlab) and converted to cDNA using Quantitect Reverse Transcription Kit (Qiagen). Brilliant II Sybr green kit (Stratagene/Agilent), 96 well plates (Starlab) and the
Mx3005P qPCR platform (Stratagene/Agilent) were used to analyse cDNA by qPCR. 18S ribosomal RNA was used as a normalising gene for cDNA analysis. Primers used for qPCR analysis of cDNA are shown in (Table 2.2).

**Table 2.2: QPCR primers for gene expression**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD2</td>
<td><strong>F</strong> ACCTGATACGCCACTGTAACG <strong>R</strong> CCCGGATAACAAGCAACCAT</td>
</tr>
<tr>
<td>PDHK1</td>
<td><strong>F</strong> ATTTTCCTCAAAGGAACGCC <strong>R</strong> CAACAGAGGTGTTACCCCC</td>
</tr>
<tr>
<td>CypD</td>
<td><strong>F</strong> CTTCTCCACGACCTCATTTTC <strong>R</strong> GTCCAAGGCACAGATGAAT</td>
</tr>
<tr>
<td>18S Ribosomal RNA (control)</td>
<td><strong>F</strong> AAACGGCTACCACATCCAAG <strong>R</strong> CGCTCCCAAGATCCAACTAC</td>
</tr>
</tbody>
</table>

(F=Forward, R=Reverse)

### 2.2.4.9 Electrophoresis and Western Blots

#### 2.2.4.9.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (12.5 % (w/v) acrylamide resolving gels and 4 % (w/v) acrylamide stacking gels) were made according to an established method stated table 2.3 below; gels were poured in a Bio-Rad (Bio-Rad, USA) mini protein gel system apparatus with 0.75mm spacers and left to solidify. Other reagents used for the SDS PAGE were sample buffer (4×: 240mM Tris HCl, pH 6.8, 8% (w/v) SDS, 0.04% (w/v) bromophenol blue, 5% (v/v) beta-mercaptoethanol, 40% (v/v) glycerol), running buffer (50mM Tris, 192mM glycine and 0.1 % (w/v) SDS), pre-stained protein ladder (ThermoFisher Scientific, UK), and Coomassie brilliant blue (CBB R250, G250, Bio-Rad, UK).

Clear protein lysates were mixed with 4× sample buffer in the ratio 3:1 (v/v). The samples were heated to 95°C for 5min and 20-100µg of protein was
loaded into the gel. Electrophoresis was carried out at 100V, 55min for each gel.

**Table 2.3: Resolving gel**

| Reagent                                                      | Volume  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis-acrylamide stock (30% w/v)</td>
<td>4.20mL</td>
</tr>
<tr>
<td>Tris-HCl (1.5M), pH 8.8</td>
<td>2.50mL</td>
</tr>
<tr>
<td>Water</td>
<td>3.20mL</td>
</tr>
<tr>
<td>10% sodium dodecyl sulphate (SDS) w/v</td>
<td>100µL</td>
</tr>
<tr>
<td>TEMED (N, N, N, N, Tetramethylethylene diamine)</td>
<td>10µL</td>
</tr>
<tr>
<td>Ammonium persulphate 10% (w/v)</td>
<td>100µL</td>
</tr>
</tbody>
</table>

**Table 2.4: Stacking gel**

| Reagent                                                      | Volume  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis-acrylamide stock (30%, w/v)</td>
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</tr>
<tr>
<td>Tris-HCl (0.5M), pH 6.8</td>
<td>2.50mL</td>
</tr>
<tr>
<td>Water</td>
<td>6.10mL</td>
</tr>
<tr>
<td>10% sodium dodecyl sulphate (SDS) w/v</td>
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</tr>
<tr>
<td>TEMED</td>
<td>10µL</td>
</tr>
<tr>
<td>Ammonium persulphate 10% (w/v)</td>
<td>100µL</td>
</tr>
</tbody>
</table>

**2.2.4.9.2 Coomassie brilliant blue staining and de-staining**

The gels were soaked in Coomassie brilliant blue (CBB) stain (CBB R250, water, methanol, acetic acid in percentage proportion of 0.1:5:4:1 respectively) for up to 1hr, then de-stained in CBB de-stain solution (water, methanol, acetic acid in percentage proportion of 5:4:1 respectively) until the background became clear.
2.2.4.9.3 Western and Dot blot

2.2.4.9.3.1 Western blot transfer
Following SDS-PAGE, proteins in gels were then transferred onto 100% methanol pre-soaked PVDF membranes (Millipore) via wet transfer method (transfer buffer; 25mM Tris, 190mM glycine and 20% methanol) at 4°C for 1hr at 100V.

2.2.4.9.3.2 Dot blot transfer
Protein lysate (20µL) were slowly spotted onto 100% methanol pre-soaked PVDF membranes (Millipore) in a humidified chamber and spotted samples were left to dry on the membrane for 1hr in the humidified chamber.

2.2.4.9.3.3 Antibody Incubation and Imaging
The membrane was then blocked with 10% milk in TBS tween buffer (50mM Tris pH7.6, 150mM sodium chloride and 1% Tween 20) for 10-30min. After three 10min washes with TBS tween buffer, the membranes were incubated with primary antibody (Table 2.5) diluted in antibody dilution buffer (5% BSA and 0.5% sodium azide in TBS tween buffer) for between 1hr and overnight at 4°C. After three 5min washes with TBS tween buffer the membranes were incubated with the appropriate secondary HRP antibody (ab131368 or ab6721; Abcam) at 1:15000 dilutions in 5% milk in TBS tween buffer for 1hr. After three 5min washes with TBS tween buffer the membranes were developed using ECL solution (Pierce). Chemiluminescent substrate was applied to the blot according to the manufacturer’s instruction. Signals were captured with a Chemi Doc XRS system (BioRad, USA).
Table 2.5: Primary antibodies for western blot.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Species</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CypD</td>
<td>Abcam, ab231156</td>
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<td>1:5000</td>
</tr>
<tr>
<td>ATP5B</td>
<td>Santa Cruz, sc-135903</td>
<td>Mouse</td>
<td>1:1000</td>
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<tr>
<td>HSP60</td>
<td>Proteintech, 66041-1-lg</td>
<td>Mouse</td>
<td>1:5000</td>
</tr>
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<td>HSP75</td>
<td>Santa Cruz, sc-13557</td>
<td>Mouse</td>
<td>1:1000</td>
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2.2.4.9.4 Measurement of Mitochondrial Membrane potential in Intact Cell

Cells were seeded at 50,000 density in 100µL media in black fluorescent 96 well plates for 18-24hr at 37°C, 5% CO₂. Media was gently aspirated and pre-warmed 100µL culture media containing 800nM of TMRM (ThermoFisher, UK). Respiratory complex inhibitors were added to a final concentration of 10µM and samples were immediately incubated in the dark for 1hr at 37°C, 5% CO₂. The media was then removed, and cells washed 2x with prewarmed PBS (Gently aspirate). Immediately 200µL of 1% Triton X 100 in PBS was added and incubated for 5min at 37°C, 5% CO₂. Bubbles were removed from the surface of any well by gentle aeration. TMRM fluorescence was measured at 549/575 (EX/EM).

2.2.4.9.5 Measurement of NAD(P)H in Intact Cell or Mitochondria

Cells were seeded at 50,000 density in 100µL media in 96 well plates for 18-24hr at 37°C, 5% CO₂. Varying concentrations of thapsigargin was added and samples were incubated in the dark for 1hr at 37°C, 5% CO₂. Immediately, 20µL of WST-1 solution was added and incubated for another 1hr. Bubbles were removed from the surface of any well by gentle aeration. Absorbance of the samples were measured at 430nm and 630nm using a plate reader (Spectramax plus, Molecular Devices, USA).

2.2.5 Mitochondria extraction and activity

2.2.5.1 Mitochondria extraction

2.2.5.1.1 Mammalian tissue mitochondria extraction

Bovine and murine heart or liver tissue mitochondria were extracted by differential centrifugation at 4°C. Organs were excised and quickly rinsed in
pre-chilled homogenizing buffer (210mM mannitol, 70mM sucrose, 1mM EDTA, 5mM HEPES, pH 7.4). Organs were macerated and mixed with same buffer in ratio 1:9 to make a 10% homogenate. The suspension was homogenized using a potter-elvehjem glass tube (Sigma, UK) before centrifugation in a high speed refrigerated centrifuge (Sorvall, UK) at 2300rpm. Pellet were discarded, and supernatant were then centrifuged at 13000rpm, the pellet being the mitochondria were washed with buffer containing 210mM mannitol, 70mM sucrose, 5mM HEPES-KOH, pH 7.4 and 0.5% (w/v) BSA. Mitochondria were immediately re-suspended in swelling buffer (210mM mannitol, 70mM sucrose, and 5mM HEPES-KOH, pH 7.4).

2.2.5.1.2 HAP1 cell mitochondria extraction
HAP1 cell mitochondria were extracted by differential centrifugation. Briefly, 10x 150mm plates were allowed to grow to 90% confluence. Cells were washed 3x in warm 1X DPBS and trypsinized for 4 mins at 37°C. Trypsinisation was stopped by diluting culture 2-fold with media. Cells were collected in 50mL tubes on ice and centrifuged at 1000Xg (Allegra X-30R Beckman Coulter, USA) at 4°C for 5min. Cell pellet was resuspended in 6mL of mitochondria extraction buffer (210mM mannitol, 70mM sucrose, 1mM EDTA, 5mM HEPES pH 7.4) in the presence of 1x protease inhibitor (Proteoloc, Expedeon, UK). Resuspended cells were homogenised with 100 strokes in a dounce homogenizer. Samples were immediately centrifuged at 1500xg for 10min at 4°C and the supernatant was centrifuged at 8000xg for 10min at 4°C. The mitochondrial pellet was resuspended in 6mL of extraction buffer and centrifuged at 10000xg for 10min at 4°C. Mitochondrial pellet was resuspended in 100µL of extraction buffer and stored on ice.

2.2.5.2 Mitochondria activity assays
2.2.5.2.1 Mitochondrial swelling assay
Basal mitochondrial swelling was assessed by a microplate reader (Spectramax 340, USA). The assay was performed with either 1mg/mL or
0.25mg/mL of rat liver or HAP1 cells mitochondria respectively in 100μL of assay buffer (Mitochondria challenge buffer: 250mM sucrose, 10mM MOPS, 10mM Tris-Pi, pH 7.4). Samples were incubated in the presence of 2μM inhibitor for 3 mins at 25°C where necessary. Mitochondrial swelling was quantified as progressive change in absorbance for 5-15min at 540nm following energisation with 10mM sodium succinate. Induced permeability transition pore opening was triggered by incubating the mitochondria with of 200μM CaCl₂ for 3min prior to succinate energisation.

### 2.2.5.2.2 Measurement of NAD(P)H in isolated mitochondria

Isolated mitochondria were prepared at 0.75mg/mL in 100μL mitochondria challenge buffer in the presence of 2μM rotenone and 10mM sodium succinate in 96 well plate. CsA was added as an inhibitor at a final concentration of 2μM and incubated at room temperature for 5min. Then 20μL of WST-1 (Sigma, UK) was added and samples were immediately incubated in the dark for 1hr at 37°C, 5% CO₂. Bubbles were removed from the surface of any well by gentle aeration. Absorbance of the samples were measured at 430nm and 630nm using a plate reader (Spectramax plus, Molecular Devices, USA).

### 2.2.5.2.3 Measurement of Cellular or Mitochondrial ATP

Total ATP was determined in seeded HAP1 cells or isolated mitochondria from HAP1 cells in white opaque 96 well plate. Cells were seeded at density of 50,000 per well and incubated for 18-24hr at 37°C, 5% CO₂. Isolated mitochondria were prepared at 0.75mg/mL in 100μL mitochondria challenge buffer. Then 20μL of Cell titre Glo reagent (Promega, UK) was added and Bubbles were immediately removed from the surface of any well by gentle aeration. Kinetic luminescence measurement was taken at 37°C with vigorous shaking before every read for 10-20min at 1min interval using a luminescence plate reader (Flex station 3, Molecular Devices, USA). Total mitochondrial ATP level was determined as the maximum peak.
2.2.5.2.4 Respiratory Chain complex activity

Respiratory chain complex I or II were measured in isolated mitochondrial using Dichlorophenolindophenol (DCPIP) as the terminal electron acceptor. Mitochondria were energised with 10mM pyruvate or succinate for either complex I or II activity, respectively. 0.75mg/mL mitochondria was incubated in mitochondrial challenge buffer. Prior to substrate energisation, the reaction was incubated in the presence of the appropriate incubator 10µM rotenone or CsA and 5mM malonate for 5min at room temperature. Immediately after substrate addition, kinetic measurements were recorded using a plate reader at 600nm at 1min interval. CsA, Rotenone and malonate were used to inhibit CypD, complex I and complex II respectively. All chemicals were product of Sigma, UK.

2.2.5.2.5 Mitochondrial antioxidants activities

Superoxide dismutase and Glutathione peroxidase activity of isolated murine liver mitochondria were measured colourimetrically with SOD (ab65354) and GPX (ab102530) activity assay kit and were performed according to the manufacturer’s instruction (Abcam, UK).

2.3 Statistical Analysis

Images were analysed with Image J open source analysis software [NIH, Maryland, USA (Abràmoff et al., 2004)] and OMERO client software [Open Microscopy Environment (Alan et al., 2012)]. Student’s T-test and Analysis of Variance (ANOVA) with Tukey’s HSD post hoc test were used for data analysis, and level of significance determined using both R programming (Vienna, Austria) and SPSS 2015 (IBM Inc, USA). Minimum of 2 replicates were used in the experiments. Results were represented as mean ± SEM. * represents p<0.05.
Chapter Three

Purification of Recombinant and Quantitation of Mitochondrial Protein
3.1 Introduction ........................................................................................................ 91
3.2 Results ............................................................................................................... 94

3.2.1 Purification and Characterisation of Recombinant Cyclophilin D .... 94

3.2.1.1 CypD overexpression and purification ........................................... 94
3.2.1.2 Verification Ligand binding and PPIase activity of CypD ... 94

3.2.2 Mitochondria extraction and verification ................................................. 95

3.2.3 Quantitation of CypD in the mitochondria ............................................. 97

3.2.3.1 Mouse liver mitochondria .............................................................. 97
3.2.3.2 Mouse heart mitochondria ............................................................ 98
3.2.3.3 CypD in various rat tissue lysate .................................................... 98
3.2.3.4 HAP1 cell mitochondria ............................................................... 100
3.2.3.5 CypD Variation between non-cancer and cancer mammalian tissues 102

3.2.4 Quantification of PTPMT1 in HAP1 cell mitochondria .............. 102

3.3 Discussion ....................................................................................................... 105

3.3.1 Purified Recombinant CypD is Structurally intact ....................... 105
3.3.2 Peptidyl-prolyl \( cis\text{-}trans \) isomerisation ........................................ 105
3.3.3 Mitochondria extraction and verification ......................................... 106
3.3.4 Quantification of CypD in the mitochondria ................................. 108
3.3.5 CypD accumulates in cancer cells ............................................... 110

3.4 Conclusion .................................................................................................. 112
3 Purification of Recombinant and Quantitation of Mitochondrial Cyclophilin D

3.1 Introduction

Cyclophilin D is a major player in the regulation of the MPTP. However, its specific role in the mitochondria and the MPTP remains contentious. As pointed earlier, CypD has been reported to interact with certain mitochondrial protein candidates which play important roles in effecting efficient mitochondrial functions. Some of the proteins suspected to interact with CypD are important in: the assembly and functioning of the electron transport chain by enhancing respiratory chain super-complex formation (Beutner et al., 2014; Etzler et al., 2017), the synthesis and export of mitochondrial ATP by interacting with the ATP synthase - the peripheral stalk of the ATP synthase (Giorgio et al., 2009; Giorgio et al., 2012; Gauba et al., 2017), folding and stabilisation of mitochondrial proteins (Kang et al., 2007; Ghosh et al., 2010; Sinha et al., 2014) and regulation of signalling events involved in mitochondrial apoptosis, gene expression and ROS generation within the mitochondria (Matouschek et al., 1995; Eliseev et al., 2009; Boengler et al, 2010; Rasola et al., 2010; McGee and Baines, 2011; Chen et al., 2015; Youcef et al., 2015; Meier et al., 2017). Other protein interactors include the Adenine Nucleotide Translocator (Halestrap and Davidson, 1990; Crompton et al., 1998; Woodfield et al., 1998), phosphate carrier (Leung et al., 2008), and mitochondrial creatine kinase (Beutner et al., 1998).

The interaction of CypD with various classical and non-classical mitochondrial proteins have suggested its possible role in their improved or repressed activities, and this has revealed plausible role as a central regulator of mitochondrial activity. Proteins are usually expressed based on their cellular importance; thus, cellular processes and molecular phenotypes are influenced by the levels of individual proteins (Wu et al., 2013; Ho et al., 2017a). Studies in yeast showed that proteins involved in cellular polarity, morphogenesis and Ribosome synthesis are highly abundant while less abundant proteins played roles in DNA replication and repair, and least abundant proteins are associated
with DNA recombination and protein ubiquitination (Ho et al., 2017a). In the same vein, the abundance of mitochondrial proteins indicated by enrichment analysis has been associated with their physiological functions.

The most abundant mitochondrial protein is Adenine nucleotide translocator (ANT), accounting for approximately 10% of the entire mitochondrial proteome (Liu and Chen, 2013). It plays vital role in the electrogenic translocation of adenine nucleotide across the inner mitochondrial membrane for cellular energy homeostasis. Another protein of high abundance is the translocator of the outer membrane (TOM). A recent study carried out in yeast mitochondria revealed high level of TOM complexes, this is consistent with its role as the main protein entry point into the mitochondria (Morgenstern et al., 2017). Other mitochondrial proteins that have been established to be highly abundant are Porin 1 or VDAC, respiratory complexes (II-IV), ATP synthase, Phosphate carrier (PiC), and components of the TCA cycle (Ohlmeier et al., 2004; Zahedi et al., 2006; Murphy et al., 2015). This is consistent with their role in energy metabolism since this is the most important function of the mitochondria. However, proteins associated with iron homeostasis were lowly but stably abundant since they are paramount for steady mitochondrial function (Morgenstern et al., 2017).

It is expedient to quantify the level of CypD within the mitochondria for a complete understanding of its functional activities as this has not been previously done. This chapter is aimed at establishing the proportion of mitochondrial cyclophilin D in relation to the total mitochondrial proteome of mouse Liver and heart tissues and in different cancer cell lines including HAP1 cells. It also aims at differentiating the abundance of CypD between cancer and non-cancer cells.

Results discussed in this chapter encompasses recombinant protein purification, mitochondria extraction and quantitation of mitochondrial CypD using recombinant protein as the standard of measurement. Techniques used in this chapter are chromatography (affinity and size exclusion), differential
centrifugation, Western blot, and differential scanning fluorimetry (DSF) with quantitative PCR.
3.2 Results

3.2.1 Purification and Characterisation of Recombinant Cyclophilin D

3.2.1.1 CypD overexpression and purification
Recombinant CypD was produced as the mature form corresponding to residues 43-207 of the fully translated gene sequence (Uniprot acc. P30405) using the protocol described in Shore et.al (2016). The gene was cloned in pETM11 vector and protein expressed in BL21 (DE3) E. coli cells grown in LB. The protein was successfully purified using a His-TRAP affinity column followed by size-exclusion chromatography. The recombinant protein was verified to be CypD by MALDI mass spectrometry (Figure 3.1).

3.2.1.2 Verification Ligand binding and PPIase activity of CypD
Following overexpression and purification, the protein was identified as CypD by Western blot and the protein was selected while the band corresponded to about 18kDa (Figure 3.11A). Differential scanning fluorimetry (DSF) was used to analyse both the folding and ligand binding property of CypD. In this assay 5µM of CypD was mixed with 10µM of CsA. It was observed that the Boltzmann melting temperature of CypD before and after CsA introduction was 50.9°C and 59.8°C, respectively (Figure 3.2C, D).

Further to this, PPIase activity showed enhanced rate of trans isomerisation of the substrate Suc-AAPF-pNA following incubation with 30µg/mL rec CypD. The cis-trans isomerisation presented with the rate constant K of 1.8±0.12min⁻¹ (Figure 3.2B).
3.2.2 Mitochondria extraction and verification

Differential centrifugation was optimised and used to extract mitochondrial enriched fractions from liver tissues and HAP1 cells. Mouse liver mitochondria extracts were resolved on 12.5% polyacrylamide gel and Coomassie staining revealed numerous protein bands with no significant difference between bands characteristics of the WT and KO mitochondria (Figure 3.3A). Total tissue/cell lysate and mitochondrial fractions were immunoblotted for mitochondrial markers; CypD (both murine tissues and HAP1 cells), ATP

Figure 3.1: Purification of Recombinant CypD. SDS PAGE representing the purification steps of recombinant CypD cloned into pETM11 vector and expressed in E. coli (BL21DE3 star). The chromatograph was used to monitor protein lysate loaded onto Ni2+ column and the elution of Histidine tagged CypD by imidazole gradient. Flow rate for loading and elution was 2mL/min. (B) SDS PAGE representing the purification steps of CypD from TEV protease mediated His tag cleavage. Chromatograph shows the purification process, as cleaved CypD was collected as the flow through. (C) SDS PAGE representing gel filtration of recombinant CypD. Chromatograph shows the purified protein eluted from the gel filtration column. Blue curve represents the protein conductance at 280nm while brown curve represents buffer conductivity in mS/cm.
synthase b subunit and HSP75 (HAP1 cells only), nuclear marker, H2A (mouse liver tissues) and cytosolic markers; beta Actin (Both murine tissues and HAP1 cells) and HSP 90 (HAP1 cells only). The mitochondrial markers were elevated in the mitochondrial fraction, while the nuclear marker, H2A was not found in the mitochondrial fraction. Level of cytosolic markers; HSP90 and beta actin were lower in the mitochondrial fraction than the total tissue or cell lysate (Figure 3.3B).

Figure 3.2: Verification of Recombinant CypD. (A) Western blot showing recombinant CypD in expression bacterial lysate and purified protein. CypD in the bacterial lysate were his-tagged which were of higher molecular weight than the non-tagged purified protein. (B) Plots represent the rate constants for the PPIase assay of both his-tagged and non-tagged CypD. PPIase activity was significantly higher in both tagged and untagged protein with rate constants of 1.3min⁻¹ and 1.8min⁻¹ respectively when compared with experimental control which had no enzyme in the reaction. (C) Fluorimetric trace showing the thermal shift of recombinant CypD in combination with CsA. (D) Boltzmann melting temperature of both CypD and CypD-CsA interaction. The melting temperature of CypD alone was 50.9°C which significantly increased to 59.8°C following CsA binding. N=3, * represents p<0.05 when compared to the control.
3.2.3 Quantitation of CypD in the mitochondria

3.2.3.1 Mouse liver mitochondria

Mouse liver mitochondrial CypD was quantified by interpolation from standard curve obtained by densitometry analysis of recombinant CypD with Image J software. Results reveal that mitochondrial CypD consist of 0.1% of the entire mitochondrial protein (Figure 3.4).

Figure 3.3: Verification of Mitochondria Extraction in Mouse Tissues and HAP1 Cells

(A) SDS PAGE showing resolved isolated mitochondria from CypD WT and KO mouse liver mitochondria. Purified recombinant CypD (5µg) was loaded into lane 2 while 100µg of mitochondria lysates were loaded into lanes 3 and 4. (B) Western blot showing the various cellular compartments following mitochondria extraction from murine tissues and HAP1 cells. CypD, ATP b subunit (ATP5F1) and HSP75 were used for mitochondrial selection. Beta actin and HSP90 were used for cytosolic selection while H2A was used for the nuclear selection. The mitochondrial proteins were enriched in the mitochondrial fraction.
3.2.3.2 Mouse heart mitochondria

The amount of CypD in heart tissue mitochondria was not directly measured as in the liver tissues. However, its amount in both liver and heart tissues were compared using Western blot. Densitometric analysis showed that heart tissue mitochondria contain 2.5-fold higher CypD content than their liver tissues counterpart (Figure 3.5; Table 3.1, 3.2). CypD in heart tissues mitochondria is 0.25% of the total mitochondrial proteome.

3.2.3.3 CypD in various rat tissue lysate

Following tissue preparation, protein quantification and Western blot, densitometric analysis of rat liver, heart, brain, pancreas, and lung tissues protein revealed CypD was 2-fold and 1.3-fold higher in heart and pancreas tissues respectively than liver tissues, however, liver tissues CypD was 4- and 12-fold higher than brain and lungs tissue lysate, respectively (Figure 3.6).

Figure 3.4: Quantitation of CypD in Mouse Liver Mitochondria. (A) Western blot of purified recombinant CypD concentration gradient alongside 3 replicates of liver mitochondrial CypD. (B) Standard curve of recombinant CypD showing the plot of image J percentage intensities vs recombinant protein concentration loaded into the electrophoresis gel.
Table 3.1: Percentage Intensity of Recombinant and Mouse Liver Mitochondrial CypD

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<tr>
<td>16.07267</td>
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Table 3.2: Proportion of Mouse Liver Mitochondrial CypD

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<td>Proportion of CypD to total mitochondrial lysate</td>
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Figure 3.5: CypD is More Abundant in Mouse Heart than Liver Tissue Mitochondria.

(A) Western blot of mouse liver and heart mitochondria shows more CypD protein intensity in the heart than liver mitochondria. (B) Plot shows significantly 2.5-fold higher amount of CypD in heart than liver tissues mitochondria. Band intensity was quantified with image J. N=3, * represents p<0.05 when compared with liver mitochondria.
3.2.3.4 HAP1 cell mitochondria

Following mitochondrial extraction from HAP 1 cells, proteins were quickly quantified using BCA assay and 5µg of mitochondrial protein alongside varying amount of recombinant CypD were loaded on 12.5% gel for Western blot. Densitometric analysis of both recombinant CypD and mitochondrial CypD revealed that the percentage proportion of CypD in the mitochondria was 0.6% (Figure 3.7; Tables 3.3, 3.4).

Figure 3.6: Quantitation of CypD in Mouse Liver Tissue Lysates. (A) Western blot and ponceau stain of mouse liver heart and pancreas tissue lysate. CypD intensity was higher in heart and pancreas than liver tissues. Ponceau stain of total protein was used as the internal control, as it shows the same amount of protein intensity around 100KDa. (B) Western blot of mouse liver brain and lungs tissue lysate. Higher CypD band intensity was observed in liver than brain and lungs tissue lysate. Ponceau stain was also used as the internal control. (C) Plots reveal that CypD was significantly higher in heart tissue lysate than liver while brain and lungs possessed significantly lower level of CypD than the liver. However, there was no significant difference between the liver and pancreas. N=3, * represents p<0.05 when compared with the liver tissues.
Figure 3.7: Quantitation of CypD in HAP1 Mitochondria. (A) Western blot of purified recombinant and mitochondrial CypD. 4 replicates of 5µg of mitochondrial protein was loaded into the gel. (B) Standard curve of recombinant CypD. Protein band intensity was plotted against the amount of recombinant protein loaded into the gel. $R^2$ score is 0.9929 which was higher than the $R^2$ value initially observed in a linear graph.

Table 3.3: Percentage Intensity of Recombinant and Mitochondrial CypD

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Table 3.4: Proportion of Hap1 Mitochondrial CypD

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</table>

### 3.2.3.5 CypD Variation between non-cancer and cancer mammalian tissues

CypD was quantified between rat liver, heart and brain tissues and HAP1, HeLa and HT1080 cancer cell lines. ATP synthase beta subunit was used as control. Western blot analysis of 20µg protein of each sample showed that the cancer cell lines accumulated more CypD than the normal tissues (Figure 3.8A). The level of ATP synthase, 2-oxoglutarate dehydrogenase and HSP60 were used to account for both inter-species and neoplastic variation. Immediately, CypD was quantified between cultured non-cancer (Huvec), immortalised (HFF) and cancer (RCC4, MDA, U2OS and 786-O) cells. It was observed that CypD was more than 5-fold lower in the non-cancer cell than immortalised and cancer cells (Figure 3.8, B, C).

### 3.2.4 Quantification of PTPMT1 in HAP1 cell mitochondria

PTPMT1 was quantified using 50µg of isolated HAP1 mitochondria and recombinant PTPMT1. Densitometric analysis and standard curve revealed that the percentage proportion of PTPMT1 in HAP1 cell mitochondria is 0.004% of the mitochondrial proteome (Figure 3.9). This makes it 160-fold lower than CypD in the mitochondria.
Figure 3.8: CypD is Abundant in Cancer than Non-Cancer Cells. (A) Western blot of mitochondrial proteins alongside HSP90 in mouse tissue lysates and cancer cells. CypD was observed to be highly expressed in the cancer cells than the tissue lysates. (B) Western blot of mitochondrial proteins in non-cancer cell (HUVEC) and immortalised (HFF) / cancer (RCC4, MB-MDA-231, U2OS and 786-O) cells. (C) Plots of figure B revealed that CypD was highly abundant by more than 10-fold in both immortalised and cancer cells, except for MB-MDA-231 cells where it was 5-fold higher than the non-cancer HUVEC cells. OGDH, HSP60 and ATP synthase were used as mitochondrial controls. 20µg of cell lysates were loaded into the gel.
Figure 3.9: Quantitation of PTPMT1 in HAP1 Mitochondria. (A) Western blot of purified recombinant PTPMT1 alongside 50µg of mitochondrial protein. (B) Standard curve of band intensities of recombinant PTPMT1 (%) vs amount of protein in ng. R² score is 0.9858 which is higher than that initially observed in a linear graph.

Table 3.5: Percentage intensities of recombinant and mitochondrial PTPMT1

<table>
<thead>
<tr>
<th>Amount of recombinant PTPMT1 (ng)</th>
<th>Percentage Band intensity (%)</th>
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<tr>
<td>2</td>
<td>4.08</td>
</tr>
<tr>
<td>4</td>
<td>10.04</td>
</tr>
<tr>
<td>8</td>
<td>20.91</td>
</tr>
<tr>
<td>16</td>
<td>25.31</td>
</tr>
<tr>
<td>32</td>
<td>32.65</td>
</tr>
<tr>
<td>Mitochondria 1</td>
<td>2.38</td>
</tr>
<tr>
<td>Mitochondria 2</td>
<td>4.63</td>
</tr>
</tbody>
</table>

Table 3.6: Percentage Proportion of PTPMT1 in Mitochondrial Protein

<table>
<thead>
<tr>
<th>Mitochondrial sample</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mit1</td>
<td>0.003%</td>
</tr>
<tr>
<td>mit2</td>
<td>0.004%</td>
</tr>
<tr>
<td>Average</td>
<td>0.004%</td>
</tr>
</tbody>
</table>
3.3 Discussion

3.3.1 Purified Recombinant CypD is Structurally intact

Following the expression and purification of rec CypD, it was indeed important to verify its native conformation, as it is common for some proteins to lose their intactness ex vivo. A notable property of CypD is its affinity for CsA, a cyclic peptide of 11 amino acids discovered in 1971 as an immunosuppressant (Borel, 2002). Numerous studies have further established CsA as a potent ligand to some peptidyl prolyl isomerase proteins such as CypD and CypA as they both bind readily (Kajitani et al., 2008; Elrod et al., 2010; Lim et al., 2011; Kim et al., 2014; Shore et al., 2016; Neginskaya et al., 2019).

DSF was used to explore the folding and binding property of the purified recombinant CypD. It employed the fluorescence characteristics of Sypro orange dye when bound to hydrophobic parts of proteins. The dye possesses low fluorescing characteristics in aqueous environment which is different from its high fluorescent ability when attached to hydrophobic surfaces of the protein (Ericsson et al., 2006; Niesen et al., 2007). This result in the detection of the thermostability of CypD because hydrophobic parts of the protein are only exposed when unfolded. The increase in thermal stability of CypD by CsA binding (melting temperature increased by 8.6°C) suggests strong interaction with CsA (Figure 3.2C, D). This is consistent with the Kd value for CsA binding of 30nM (Shore et al., 2016). The modulatory increase in the melting temperature of CypD (51°C) by 8.6°C after ligand binding suggests that the purified recombinant CypD was structurally intact.

3.3.2 Peptidyl-prolyl cis-trans isomerisation

To verify the enzymatic function of the recombinant protein, PPIase activity assay was carried out on the purified recombinant CypD. The chymotrypsin coupled assay measured the hydrolysis of phenylalanine-p-nitroanilide when the adjacent alanine-proline peptide bond in the standard tetrapeptide Suc-AAPF-p-NA is in the trans conformation (Shore et al., 2016). Since 4-nitroanilide has an absorbance maximum at 390nm, a steady increase in
absorbance was measured over time. Addition of high concentration of Li\(^+\) ions (2-5mM) in anhydrous solvents such as trifluoroethanol favours the cis conformation (about 60%) of A-P peptide bond (Kofron et al., 1991). In this assay, the further addition of recombinant CypD shifted the peptide bond conformation from the cis towards the trans isoform, hence, enhancing the hydrolysis of the standard tetrapeptide, thereby generating a colorimetric signal measured at 390nm. Kinetic curves of the change in absorbance at 390nm is coupled to the rate of cis-trans isomerisation per second of the peptide and indeed, the purified recombinant CypD enhanced this isomerisation with rate constant greater than the control (Figure 3.2B). The change in absorbance (390nm) observed in the control group accounted for the residual trans isoform of the peptide present in the reaction mixture. Apart from the structural intactness of the protein which is already verified, this results further indicates the functional intactness of the purified recombinant CypD.

### 3.3.3 Mitochondria extraction and verification

CypD was quantified in extracted mitochondrial fractions of murine hepatic and cardiac tissues, and HAP1 cells. Differential centrifugation has been established as a reliable method for cellular compartmentalisation. To ensure that an enriched mitochondrial fraction was obtained following differential centrifugation, Actin and HSP90\(\alpha\) were used to probe for the level of cytosolic contamination while CypD (both murine tissues and HAP1 cells) alongside other resident mitochondrial proteins (HAP1 cells) were chosen as markers for mitochondrial enrichment. Both murine tissues and HAP1 cell mitochondrial fractions showed high levels of mitochondrial proteins suggesting an enrichment of the mitochondria in these fractions. In addition to mitochondrial selection by CypD, HAP1 mitochondria were probed for ATP5F1 and HSP75. The subunits of the F1FO ATP synthase were selected in HAP1 cells because they are solely mitochondrial (He et al., 2018), hence, useful markers for mitochondrial selection.
Interestingly, both cytosolic markers used were observed to be very low when compared with the total tissue lysate in the hepatic mitochondrial fractions (Figure 3.3B). However, this was different in HAP1 cell mitochondrial fractions as actin was only slightly lower in the mitochondrial fraction relative to the entire cell lysate. This high level of actin in the mitochondria was initially thought to have resulted from increased cytosolic contamination of the mitochondrial fraction; however, comparing this with the level of HSP90, another cytosolic protein showed convincing evidences of very little cytosolic contamination.

Recent evidences revealed interactions between actin filaments and the mitochondria. Cytoskeletal proteins bind to and are required for various mitochondrial activities, ranging from respiration to motility (Ligon and Steward, 2000; Boldogh and Pon, 2006; Liu et al., 2011; Korobova et al., 2013; Moore et al., 2016). The effect of actin on mitochondria may be tissue or cell specific because lower actin was observed in the heart tissues when compared with the liver. Cardiac tissues are highly muscular and contain lots of actively respiring mitochondria. Therefore, lower actin filaments observed seemed to be inconsistent with the physiological function of the heart tissues.

It can be assumed that elevated actin is observed in the mitochondria of actively dividing cells as opposed to animal tissues. Indeed, it has been shown in previous studies that actin polymerisation and cytosolic interactions positively correlates with stemness and cell division, while terminally differentiated cells, like heart tissues possesses monomeric actin filaments which are localised to the plasma membrane, hence, enriched in membrane fractions during tissue processing (Romih et al., 1999; Boraas et al., 2018). This therefore account for the reduced level of actin observed in cardiac tissues non-membranous soluble fractions. Therefore, beta actin may not be an ideal marker for cytosolic contamination when dealing with cardiac tissues.

HSP 90α on the other hand, being a well characterised cytosolic protein (Wang et al., 2009; Li et al., 2015) was enriched in the total cell lysate than the
mitochondrial enriched fraction and has proved to be a reliable cytosolic marker (Figure 3.3B).

3.3.4 Quantification of CypD in the mitochondria

Protein expression generally varies in tissues and many a times, tissue function is dependent on specific protein level. Heart tissues are typically abundant in cellular mitochondria because of their highly muscular and energy demanding characteristics (Boengler et al., 2017). Moreover, at the protein level, they also possess high amount of different proteins such as TCA cycle enzymes and respiratory chain complexes; 2-oxoglutarate and the mitochondrial ATPase (Figure 3.8A) so as to meet up with its high energy demand. Since the abundance of these mitochondrial proteins corelates with their function, understanding the proportion of CypD in various organs and cell types would be informative, as this may also be associated with tissue specific functions.

Quantification of mitochondrial CypD by standard curve using concentration gradient of recombinant CypD revealed that the total mitochondrial protein constitution of CypD are; 0.1% in mouse liver mitochondria (Figure 3.4, Tables 3.1, 3.2), 0.2% in mouse heart mitochondria (Figure 3.5) and 0.6% in HAP1 isolated mitochondria (Figure 3.7, Tables 3.3, 3.4). The natural abundance of CypD observed in this study is novel. In HAP1 cells, when compared to another mitochondrial protein, PTPMT1 which has a mitochondrial abundance of 0.004% (Figure 3.9; Tables 3.5, 3.6), and is involved in the biosynthesis of cardiolipins (Zhang et al., 2011), CypD (0.6%) seems to be 160-fold higher. However, when compared with ANT, which has an established abundance of 10% (Liu and Chen, 2013), then the level of CypD is severely low in the mitochondria. Depending on which way it is viewed, CypD in the mitochondria has a very significant effect on the mitochondrial health via regulation of the MPTP. More so, it would be interesting to quantify other mitochondrial proteins to know how CypD fairs among the rest in the mitochondria.
Amongst the two studied mice tissues, liver mitochondrial samples had the lowest proportion of CypD to total protein as opposed to the 2-fold higher level of CypD observed in heart mitochondria. The same pattern was observed in whole tissue lysates when the liver and heart were probed for CypD (Figure 3.6). Following the observation that the fold difference between the liver and heart mitochondrial samples were the same as their total tissue lysate, the differential expression between the tissue lysates of the brain, pancreas and lungs was also determined. This was done by calculating the fold differences of CypD in these tissues in relation to the liver using densitometric analysis. It was observed that CypD level in the pancreas, brain and lungs were respectively, 1.3-, 0.25- and 0.07-fold the level of CypD in the liver (Figure 3.6C). Recall that the percentage proportion of CypD is 0.1% in the liver (Table 3.2), therefore, this suggests that the percentage proportion of CypD in the pancreas, brain and lungs are 0.13%, 0.025% and 0.007% respectively.

According to the database of the human proteome atlas (Uhlén et al., 2015), the level of the ppif gene expression of the liver, brain, pancreas and lungs translate properly into their observed protein level, however, this was not observed in heart tissues as there was no difference in the RNA transcripts between the heart and liver tissues whereas the observed protein was 2-fold higher in the heart than liver tissues (Figure 3.6C). This observation may be attributed to unknown effects from post transcriptional modifications or enhanced mitochondrial CypD stabilisation in heart tissues.

Following the quantification of CypD in various tissues it would appear that CypD is necessary for mitochondrial energy production because of its positive relationship with mitochondrially active tissues (heart>liver) and rapidly dividing cells (HAP1 cells). However, quantities found in brain tissues contrast this proposition as brain cells are also constantly in very high energy demand. Indeed, it is still not clear which is the preferred route of energy production in the brain, whether aerobic glycolysis or oxidative phosphorylation (Dienel, 2012).
Recent evidences suggest enhanced aerobic glycolysis as opposed to oxidative phosphorylation during stimulated neuronal activities. This is because of high level of lactate observed to exit neurons during physical activities, however, during resting state, oxidative phosphorylation is enhanced. Therefore, some brain tissues, may be characterised with reduced mitochondrial oxidative phosphorylation as the bulk of utilised glucose are not oxidised but rather converted into lactate either for neuronal export or channelled towards other signalling pathways (Dienel, 2012; Zhang et al., 2014; Díaz-García et al., 2017; Yellen, 2018). However, more findings reported in this thesis may suggest plausible reasons for the downregulation of CypD in brain and lungs tissues.

3.3.5 CypD accumulates in cancer cells

Many independent studies have associated cancer tissues with reduced mitochondrial activities. However, it has been suggested that the parameters used to measure mitochondrial activity may be far from accurate in these studies as lactate production from aerobic glycolysis is consistently being used as marker for mitochondrial activity (Gaude and Frezza, 2014). Rather, this phenomenon may suggest other preferred route of metabolites entry into the mitochondria from the cytosol. In fact, recent studies have suggested that mitochondria play important roles in oncogenesis (Weinberg and Chandel, 2009; Porporato et al., 2018). Since CypD abundance correlated positively with mitochondrially active heart tissues and even more in HAP1 cancer cell lines, its level in other cancer cells was worth investigating.

Indeed, the level of proteins may vary between species, although differences between phylogenetically related species may be negligible. Interestingly, CypD in HAP1 cells mitochondria was 6-fold more abundant than mouse tissues mitochondria, as it accounts for 0.6% of the mitochondrial protein content (Table 3.4). Consistent with this finding, the amounts of CypD observed in tissue lysates from Hela and HT1080 cells were also very
comparable to that found in HAP1 cells; both HeLa and HT1080 are cancer cell lines (Figure 3.8A).

To show that the observed accumulation of CypD was not a phenomenon of general protein accumulation in cancer cells, the levels of some mitochondrial proteins were compared between normal tissues and cancer cells. Variation in the amount of ATP synthase beta subunit between the different tissues and cell lines was negligible except for the heart tissue which had more than 2-fold the amount of the ATP synthase which is consistent with its high dependency on ATP (Figure 3.8). Interestingly, 2-oxoglutarate dehydrogenase was elevated in normal tissues than in cancer cells suggesting that these tissues were adapted to increasing the flux of the TCA cycle in the forward direction.

The level of CypD was compared between an array of other cancer cells and HUVEC – a non-immortalised endothelial cell. Interestingly, cancer cells alongside the non-cancer but immortalised HFF cells were characterised with more than 10-fold higher level of CypD compared to the level in the HUVEC cells except for MDA breast cancer cells which was 5-fold higher (Figure 3.8B, C). This CypD elevation was consistent with what was observed between the murine tissues and HAP1, HeLa and HT1080 cells. These findings paradoxically indicate the importance of CypD in cancer cell survival – perhaps proliferation and invasion, or more importantly enhancing mitochondrial bioenergetics (ATP synthesis).

It is not clear what role CypD performs in the mitochondria apart from activation of the MPTP. However, its elevation in cancer cells suggests other possible roles in the mitochondria such as playing important role in cancer cell survival. Since it is upregulated alongside another mitochondrial chaperones, HSP60 in cancer cells, it is plausible to assume that CypD may also be involved in the mitochondrial unfolded protein response (Pellegrino et al., 2013). Although HSP60, which has been reported to antagonise CypD-mediated MPTP activation (Ghosh et al., 2010; Tang et al., 2016) was also observed to be higher in most of the cancer cells (Ciocca et al., 2005; Rappa
et al., 2012; Meng et al., 2018), nevertheless, it is difficult to reconcile the elevation of a supposed pro-apoptotic protein such as CypD in immortalised and cancer cells.

3.4 Conclusion
In conclusion, the level of CypD varies among tissue mitochondria; Liver - 0.1%, heart - 0.2% and HAP1 - 0.6% of total mitochondrial proteins. Indeed, there seems to be more than 10-fold higher CypD in most cancer cells when compared with non-cancer cells (HUVEC). Different murine tissues possess varying level of abundance, which increases in the order of lungs, brain, liver, pancreas, and heart tissues while HAP1, HeLa, and HT1080 cells possess more CypD than the physiologically normal tissues. Indeed, HUVEC cells which are non-malignant endothelial cells (Bagley et al., 2003) possessed significantly lower level of CypD than other immortalised cells such as HFF cells. Although HFF cells are regarded as non-cancer cells, nevertheless they are immortalised cells (Thellman et al., 2017) which also were characterised with higher level of CypD. These findings suggest the possible role of CypD in mediating cell survival under pathological conditions.

Besides the elevation of ATP synthase and downregulation of HSP60 in HUVEC cells, the level of all other proteins investigated were relatively the same amongst all cell lines (except MB-MDA-231 cells). Moreover, it was interesting to observe that MB-MDA-231 – a highly metastatic breast cancer cell line was characterised with downregulated mitochondrial proteins when compared with other cancer cells (Figure 3.8B, C), suggesting that not all, but some cancer cells evade mitochondrial metabolism. Notably in the tissue samples, there were higher level of OGDH and HSP90 than in HAP, HeLa and HT1080 cells.

More importantly, it is important to investigate other physiological roles of CypD outside of the MPTP activation, by studying changes in mitochondrial metabolism / bioenergetics and extra-mitochondrial signalling pathways that
accompanies CypD modulation. Findings from this may shed light into why CypD accumulates in cancer cells while also solving the mystery surrounding the regulation of the MPTP.
Chapter Four

CypD Regulates Mitochondrial Redox Environment
4.1 Introduction ........................................................................................................ 117
4.2 Results .............................................................................................................. 120

4.2.1 CypD Regulates Mitochondrial NAD(P)H ................................................. 120
  4.2.1.1 Modulation of Mitochondrial Swelling in both HAP1 cells and Murine tissue mitochondria ................................................................. 120
  4.2.1.2 Effect of CypD on Mitochondrial NAD(P)H ........................................... 120
  4.2.1.3 Mitochondrial calcium sequestration increases NADH in HAP 1 KO 122
  4.2.1.4 CypD Deficiency Enhances Total Protein Lysine Acetylation 123

4.2.2 Effect of CypD on Mitochondrial Respiratory Chain Activity ... 124
  4.2.2.1 Mitochondrial Respiratory Complex I and II Activity ........... 124
  4.2.2.2 Basal Mitochondrial Membrane Potential ................................. 126
  4.2.2.3 Respiratory Chain Complexes Inhibition and Membrane potential 128
  4.2.2.4 Mitochondrial Superoxide Production ...................................... 129
  4.2.2.5 Measurement of Mitochondrial ATP ....................................... 129

4.2.3 Mitochondrial Antioxidant Activity Assay ............................................. 129

4.2.4 CypD and Cellular ROS Signalling ....................................................... 129
  4.2.4.1 CypD Promotes H$_2$O$_2$ Induced NADPH Depletion .......... 129
  4.2.4.2 CypD Deficiency Enhances H$_2$O$_2$-Induced Cell Death in HAP1 Cells .............................................................................................................. 130
  4.2.4.3 Protein Thiol Sulfenylation of WT and Mutant Recombinant CypD and PTPMT1 ........................................................................................................ 132
  4.2.4.4 Structural Modulation of rec CypD by H$_2$O$_2$ Using Differential Scanning Fluorimetry ................................................................. 133
  4.2.4.5 PTPMT1 Phosphatase Activity is Impaired by Both C132S Mutation and H$_2$O$_2$ ........................................................................................................ 137

4.3 Discussion ........................................................................................................ 140
  4.3.1 CypD Deficiency Represses Mitochondrial Swelling ...................... 140
  4.3.2 CypD deficiency stabilises mitochondrial NADPH ...................... 141
4.3.3 Mitochondrial NADH is shuttled to the Cytosol .......................... 144
4.3.4 CypD Enhances ROS -induced NADPH depletion ....................... 144
4.3.5 CypD Deficiency Alters Membrane Potential Without Affecting Complex I and II Respiratory Chain Components .................................. 146
4.3.6 CypD Deficiency Downregulates Mitochondrial Superoxide... 148
4.3.7 Reduced Mitochondrial ATP is Associated with CypD Deficiency 150
4.3.8 Recombinant CypD Enhances Redox Cycling by Forming Stable Sulfenic Acid .............................................................................................. 151
4.4 Conclusion .......................................................................................... 155
4 Chapter Four CypD Regulates Mitochondrial Redox Environment

4.1 Introduction

Mitochondrial redox environment is critical for the maintenance, functioning and efficient turnover of macromolecules—proteins, lipids and DNA—in the mitochondria. Redox imbalance can interfere with many signalling pathways, thereby deciding the viability of both the mitochondria and the entire cell. Maintenance of mitochondrial redox environment is dependent on the efficient coupling of NADPH to the two primary redox regulatory systems: the glutathione and thioredoxin systems (Holmgren et al., 2005; Jones, 2006).

Mitochondrial-generated ROS has been established to result from respiration when electrons leak from respiratory complexes I and III of the electron transport chain (Koopman et al., 2010; Rigoulet et al., 2011). Following ROS production, mitochondrial antioxidant enzymes kick in to prevent their debilitating effects (Handy and Loscalzo, 2012). However, when antioxidant enzymes are overwhelmed and evaded by ROS, proteins and other macromolecules are oxidised, resulting in the formation of protein disulphides, protein glutathionylation and lipid peroxides. It becomes expedient for redox regulatory systems which are dependent on reduced glutathione and thioredoxin to become activated, thus, correcting this damaging effect.

Glutathione peroxidase (GPX), glutaredoxin (GRX) and peroxiredoxin (PRX) are mobilised to sites of peroxides and thiol disulphides during oxidative stress resulting in oxidised glutathione and thioredoxins. In the mitochondria, NADPH is coupled to either glutathione reductase or thioredoxin reductase to regenerate their reduced forms. Indeed, this result in the depletion of NADPH which must also be regenerated for the mitochondria to regain its viability. GSH is highly abundant in the mitochondria with estimated concentration varying between 1 and 14 mM (Mari et al., 2009). It possesses very low redox potential (-330 to -300 mv) with ratios of 100:1 (GSH: GSSG) in the
mitochondria, thus may require glutathione S-transferase (GST) for glutathionylation (Schafer and Beuttner, 2001).

Deficiency of mitochondrial GPX (isoforms 1 and 4) has been implicated in ischemia-reperfusion injury (Dabkowski et al., 2008; Wong et al., 2008; Lim et al., 2009) while overexpression decreases mitochondrial $\Delta\psi_m$, ROS generation and ATP synthesis (Handy et al., 2009). In the same vein, mitochondrial GRX and PRX protects against ROS mediated apoptosis (Lillig et al., 2004; Enoksson et al., 2005; Song et al., 2011).

Under oxidative stress, protein thiols can be modified into sulfenic ($\text{S-OH}$), sulfinic ($\text{S-O}_2\text{H}$), sulfonic ($\text{S-O}_3\text{H}$) acids and disulphides ($\text{R-s-s-R}$). Sulphenylated thiol is electrophilic and unstable; it is prone to nucleophilic attack by thiols or thiolate anions, consequently resulting in the formation of protein disulphides which can be intramolecular or intermolecular, S-glutathionylation or S-homocysteinylation (Rehder and Borges, 2010; Handy and Loscalzo, 2012). The disulphide bonds formed are reversible and are reduced by GRX and TRX at the overall expense of NAD(P)H (Manevich and Fisher, 2005).

CypD has been implicated to play roles in the MPTP through mitochondrial unfolded protein response (as suggested in the previous chapter) and redox sensing (as will be studied in this chapter). Its function in the MPTP has been validated across many research groups (Connem and Halestrap, 1994; Baines et al., 2005; Tsujimoto et al., 2006; Nakagawa et al., 2005). However, its role in redox sensing is still considered a grey area. Indeed, C203S mutation was shown to attenuate MPTP opening resulting from ischemia/reperfusion injury (Nguyen et al., 2011) and CypD interaction with TRX2 and PRX3 was also recently published (Folda et al., 2016). Other notable evidences provided were CsA prevented ROS-induced MPTP associated with increased CypD level and that oxidation of CypD resulted in its conformational change which potentiated MPTP opening (Linard et al., 2009).
This chapter is based on the hypothesis that the roles of CypD may be involved in more than ROS sensing, but actually potentiating the mitochondria to ROS-induced damage and subsequently MPTP opening. CypD directly modulates both the level of ROS and reductive equivalents via both redox sensing by its Cysteine 203 residue and the modulation of the electron transport chain. In addition, CypD keeps the level of mitochondrial reductive equivalents (GSH and NAD(P)H) at the lowest minimum for efficient functioning of the mitochondrial bioenergetic processes such as the TCA cycle and the electron transport chain. Therefore, tipping this balance by either overexpressing or deleting/ mutating CypD determines the fate of the mitochondria, respectively experiencing either oxidative or reductive stress.
4.2 Results

The results of this chapter are summarised in Table 4.1.

4.2.1 CypD Regulates Mitochondrial NAD(P)H

4.2.1.1 Modulation of Mitochondrial Swelling in both HAP1 cells and Murine tissue mitochondria

Incubation with Ca\(^{2+}\) induced mitochondrial swelling in both 10mM succinate or glutamate energised mitochondria in WT more than KO mitochondria of both HAP1 cells and mice tissues. CsA reversed Ca\(^{2+}\)-induced mitochondria swelling in WT mitochondria. In isolated WT mouse liver mitochondria, Ca\(^{2+}\) (100µM) resulted in mitochondrial swelling by 7-fold relative to both WT-CsA and KO mitochondria while in HAP1 mitochondria, the increase is 3-fold higher in WT relative to the KO mitochondria. CsA treatment reversed mitochondrial swelling in both mouse and HAP1 mitochondria. Indeed, swelling was minimal in KO mitochondria (Figure 4.1). Since cytosolic Cyclophilin A is associated with calcineurin, a Ca\(^{2+}\)-dependent phosphatase, it was hypothesized that phosphatase inhibition may modulate the MPTP. However, treatment with okadaic acid (phosphatase inhibitor) did not affect Ca\(^{2+}\) induced mitochondrial swelling in mice tissues mitochondria (Figure 4.2). Moreover, it was also hypothesized that certain protein prolyl hydroxylation may proceed after CypD mediated peptidyl prolyl isomerisation and mitochondrial prolyl hydroxylation may alter the MPTP. However, oxalylglycine (an inhibitor of prolyl hydroxylases) did not modulate Ca\(^{2+}\)-induced mitochondrial swelling.

4.2.1.2 Effect of CypD on Mitochondrial NAD(P)H

The level of mitochondria NAD(P)H was assessed by the extent of reduction of WST-1: the greater the reduction, the higher the level of NAD(P)H. Following the incubation of isolated mitochondria with WST-1 in the presence and absence of succinate the level of NAD(P)H was not significantly different in the latter conditions between the WT, WT-CsA and KO samples. However, treatment with 10mM succinate resulted in the elevation of NAD(P)H only in the KO by 1.5-fold when compared with the WT control. CsA treatment (2µM)
resulted in the depletion of NAD(P)H significantly by 1.25-fold when compared with the control (Figure 4.3).

Figure 4.1: Modulation of Ca²⁺-Induced Swelling in CypD WT and KO Isolated Mitochondria.

(A) Absorbance trace at 540nm of isolated mouse liver CypD WT and KO succinate energised mitochondria. Measurement was taken at both basal and 200µM Ca²⁺ treatment. 10µM CsA was used to inhibit mitochondrial swelling. (B) Induction fold of Ca²⁺-induced mitochondrial swelling at 540nm of CypD WT, WT-CsA and KO isolated mouse liver mitochondria. (C) Absorbance trace at 540nm of isolated WT and KO HAP1 succinate energised mitochondria at both basal and 100µM Ca²⁺ incubation. 2µM CsA was used to inhibit mitochondrial swelling. (D) Absorbance trace at 340nm of isolated HAP1 CypD WT and KO glutamate energised mitochondria. Samples were treated with 100µM Ca²⁺ and inhibited with 2µM CsA. (E) Induction fold at 340nm of Ca²⁺ induced mitochondrial swelling of CypD WT and KO HAP1 mitochondria. Murine and HAP1 mitochondria were used at 1mg/mL and 0.25-0.5mg/mL concentration, respectively. Minimum of 3 replicates were used and * represents p<0.05 when compared with the WT.
Figure 4.2: Phosphatases and Prolyl Hydroxylation does not Affect Mitochondrial Swelling.

(A) Absorbance trace of mouse liver succinate energised mitochondrial swelling in the presence of oxalylglycine (PHDs inhibitor). CsA completely reversed Ca\(^{2+}\)-induced swelling while oxalylglycine (10µM) had no effect on swelling. (B) Ca\(^{2+}\) in the absence and presence of oxalylglycine induced mitochondrial swelling by more than 10-fold of the untreated control. (C) Absorbance trace of mouse liver succinate energised mitochondrial swelling in the presence of okadaic acid (phosphatases inhibitor). Okadaic acid (5µM) had no effect on Ca\(^{2+}\)-induced swelling. (D) Ca\(^{2+}\) induced mitochondrial swelling by more than 10-fold in the absence or presence of okadaic acid whereas, CsA inhibited Ca\(^{2+}\)-induced swelling. Mitochondria were used at 1mg/mL. Minimum of 3 replicates were used and * represents p<0.05 relative to the Ca\(^{2+}\) only.

4.2.1.3 Mitochondrial calcium sequestration increases NADH in HAP 1 KO

Incubation of WT and KO cells in differential concentration of thapsigargin (an inducer of cytosolic Ca\(^{2+}\) overload, thus, mitochondrial Ca\(^{2+}\) sequestration) for 1hr induced NADH in KO HAP1 cells. This was measured by the reduction of WST-1 dye added to the cells. Thapsigargin significantly induced the reduction of WST-1 at 5, 10 and 20µM concentrations in KO cells by 1.3, 1.4 and 1.6 folds respectively, while WT cells remained relatively at the same level when
compared with the control. There was significant reduction in both WT and KO cells at 40µM concentration (Figure 4.4).

![Graph A](image1)

![Graph B](image2)

**Figure 4.3: NAD(P)H in Isolated CypD WT and KO HAP1 Mitochondria.** (A) WST-1 absorbance of isolated mitochondria following 45mins of incubation at 37°C, 5% CO₂. Unenergized mitochondria were suspended in buffer. Although there seems to be higher reduction by NAD(P)H in both 2µM CsA and KO mitochondria, however, this was not significant (P>0.05). (B) Fold change of NAD(P)H induced WST-1 reduction in 10mM succinate energised mitochondria. Relative to WT mitochondria, CsA decreased mitochondrial NAD(P)H – induced reduction of WST-1 by 1.25-fold while KO mitochondria increased NAD(P)H-induced reduction of WST-1 by 1.5-fold. Minimum of 3 replicates were used and * represents p<0.05 relative to the WT.

### 4.2.1.4 CypD Deficiency Enhances Total Protein Lysine Acetylation

Total cell lysate of both HAP and HeLa CypD deficient cells were probed for protein lysine acetylation. Western blot revealed that overall acetylated proteins were higher in KD than WT in HeLa cells. In HAP 1 cells, lysine acetylated proteins were also higher in KO than their WT counterparts (Figure 4.5).
Increasing thapsigargin concentrations from 5µM to 20µM elevated NAD(P)H-induced WST-1 reduction significantly in KO cells and this was not observed in the WT cells. However, 40µM thapsigargin resulted in the depletion of NAD(P)H in both WT and KO cells. 50,000 cells were seeded in 96 well plate and a minimum of 4 biological replicates was used. Treatment groups were normalised to their controls. * represents p<0.05 relative to the untreated controls.

**4.2.2 Effect of CypD on Mitochondrial Respiratory Chain Activity**

DCIP was used as the final electron acceptor for both complex I and II activity measurement. Measurement of colour change by progressive depletion of absorbance at 600nm following substrate addition was used as either complex I or II activity assay.

**4.2.2.1 Mitochondrial Respiratory Complex I and II Activity**

Respiratory chain complex I was measured in pyruvate energised mitochondria. There was no difference in the progressive reduction of DCPIP at 600nm between the WT and KO mitochondria. In addition, treatment with 100µM Ca^{2+} did not result in any significant change between the untreated groups (Figure 4.6A, B).
Figure 4.5: CypD Deficiency Promotes Protein Lysine Acetylation. (A) Western blot showing acetylated proteins in CypD WT and KD HeLa cells. KD cells are characterised with more band intensity than the WT. GAPDH was used as the loading control. (B) Acetylated proteins are significantly higher in KD than WT HeLa cell lysates. (C) Western blot showing acetylated proteins in CypD WT and KO HAP1 cells. Higher protein band intensity was observed in the KO than WT. Most of the protein bands are observed above 70KDa. HSP75 was used as the loading control. (D) Acetylated proteins are significantly higher in KO than WT HAP1 cell lysate. 3 biological replicates of samples were used, and image J was used to quantify bands intensity. * represents p<0.05 relative to the untreated controls.

Respiratory chain complex II activity assay was measured in succinate energised HAP WT and KO mitochondria. There was no difference in the reduction of DCPIP between HAP1 WT, KO and 10µM CsA treated WT mitochondria. However, treatment with 10mM Malonate repressed the reduction of DCPIP by 3-fold in both WT and KO mitochondria (Figure 4.7C, D).
Depletion of absorbance indicates the reduction of DCPIP by electrons supplied via the ETC. 10mM pyruvate and succinate were used to stimulate electron transfer via complex I and complex II respectively. (A) Absorbance trace at 600nm of DCPIP reduction by pyruvate energised CypD WT and KO isolated HAP1 mitochondria. NADH produced from pyruvate metabolism transfers electron to DCPIP via complex I. 100µM Ca²⁺ and 10µM rotenone had no effect on complex I activity. (B) Bar plots represents fold change of complex I activities of the treatment groups relative to the WT. There are no significant differences in the various treatment groups. (C) Absorbance trace at 600nm of succinate energised CypD WT and KO isolated mitochondria. The absence of succinate represents basal complex II activity. 5mM Malonate repressed absorbance depletion in both WT and KO mitochondria while 10µM CsA further enhanced DCPIP reduction in both WT and KO mitochondria. (D) Bar plots represents fold induction relative to the unstimulated WT mitochondria (No succinate). Malonate significantly repressed DCPIP reduction by electrons supplied at complex II in both WT and KO mitochondria. CsA further enhanced DCPIP reduction, with KO treatment being significantly enhanced in relation to the WT. plots represent a total of 3 biological replicates. * represents p<0.05 relative to the WT.

4.2.2.2 Basal Mitochondrial Membrane Potential

The basal level of mitochondrial $\Delta \Psi_m$ was quantified in HAP and HeLa cells using the cell permeant TMRM dye at 548/574nm (Excitation/Emission) with confocal microscope. Following 1hr treatment of both WT, KO (HAP 1 cells) and WT, KD (HeLa cells) with 800nM TMRM, CypD KO and KD cells presented with significantly lower TMRM fluorescence.
HAP1 and HeLa cells were treated with 800nM TMRM for 1hr at 37°C, 5% CO₂. Cells were fixed in 3.7% PFA and imaged with Zeiss confocal microscope LSM 710. (A) Panel shows the depletion of TMRM fluorescence in HAP1 KO, CsA and rotenone treated cells. Rotenone completely depleted TMRM fluorescence in these cells while CsA resulted in unquantifiable punctate structures. (B) Panel shows depletion of TMRM fluorescence in HeLa KD, CsA and rotenone treated cells. CsA treatment was also characterised with punctate mitochondrial structures. (C) Box plot shows significant reduction in TMRM fluorescence when compared with the WT HAP1 cells. (D) Box plot of HeLa cells shows significant reduction in TMRM fluorescence in KD when compared WT and CsA treatment group. However, there is no significant difference between rotenone treatment and CypD KD. Cells were treated with 10µM of CsA and rotenone for 4hr. N=3, n>50. P values are represented in the plots. Image was quantified with Image J.

Figure 4.7: CypD Deficiency Decreases Basal Mitochondrial Membrane Potential.
Complex I-IV respiratory chain inhibition by rotenone, malonate, antimycin and sodium azide respectively dissipated mitochondrial $\Delta \Psi_m$. All treatment groups were characterised with reduced TMRM fluorescence when compared with the control. Antimycin had the lowest TMRM fluorescence. However, there was no difference between treatment counterparts. FCCP was used as control. Cells were seeded in 96 well plates at density of 50,000 for 18hrs. Prior to 800nM TMRM treatment for 1hr, cells were incubated in inhibitors for 4hrs. TMRM fluorescence was measured at 549/575 (Ex/Em). Plots represent a total of 5 biological replicates. * represents p<0.05 relative to the control.

Treatment of HAP1 and HeLa cells with 10µM rotenone resulted in the significant loss of TMRM fluorescence. Moreover, treatment with 10µM CsA resulted in mitochondrial fragmentation and loss of $\Delta \Psi_m$ in both HAP1 and HeLa cells (Figure 4.7).

4.2.2.3 Respiratory Chain Complexes Inhibition and Membrane potential

Following incubation with inhibitors of mitochondrial respiratory chain components for 2hr, the $\Delta \Psi_m$ was measured with TMRM between WT and KO
HAP1 cells. There was no significant difference between WT and KO cells in the reduction of $\Delta \Psi_m$ by respiratory chain complexes inhibitors. However, when compared with the control, all treatments reduced mitochondrial $\Delta \Psi_m$ significantly. FCCP (5µM) was used as the positive control (Figure 4.8).

4.2.2.4 Mitochondrial Superoxide Production
Since superoxide production are mainly by-products of the respiratory chain, mitochondrial $O_2^{-•}$ was measured by MitoSox red in both HAP1 and HeLa cells. Following the treatment of both cell types with 100nM of MitoSox red for 1hr, there was a significant reduction in MitoSox fluorescence in KO HAP1 cells and KD HeLa when compared with their scrambled controls (Figure 4.9).

4.2.2.5 Measurement of Mitochondrial ATP
Quantities of ATP was measured in HAP WT and KO extracted mitochondria using Cell Titre Glo Luminescence Assay Kit. Kinetic measurements of mitochondrial ATP at 37°C revealed significantly higher level of mitochondrial ATP by 2-fold in the WT than KO. Total cellular ATP was significantly higher in WT than in KO cells (Figure 4.10).

4.2.3 Mitochondrial Antioxidant Activity Assay
Mitochondrial SOD and GPX were measured in WT and KO mouse liver isolated mitochondria. SOD activity was significantly lower (P<0.05) in KO than WT mitochondria by more than 2-fold. (Figure 4.11). There was no significant difference in basal GPX activity between WT and KO mitochondria although KO activity level was lower by 1.3-fold (Figure 4.11).

4.2.4 CypD and Cellular ROS Signalling
4.2.4.1 CypD Promotes $H_2O_2$ Induced NADPH Depletion
HAP1 WT and KO cells were treated with varying concentrations of $H_2O_2$ for 2hr. WST- 1 based assay revealed no difference between WT and KO at 5
and 10µM treatment. However, concentrations at 20, 40, 80 and 100µM revealed reduction in about 40% loss of NADPH only in the WT cells while no significant difference was observed in the KO cells. Higher concentrations; 200, 400 and 800µM resulted in 80% loss of NAD(P)H in both cell types (Figure 4.12).

**Figure 4.9: CypD Deficiency Decreases Mitochondrial ROS.** Mitochondrial ROS was determined by incubating cells in 5µM MitoSox red for 1hr. Cells were fixed in 3.7% PFA and imaged with Zeiss confocal microscope LSM 710. (A) CypD KO HAP1 cells were characterised with significantly lower MitoSox fluorescence than the WT. (B) CypD KD HeLa cells also showed significantly lower MitoSox red fluorescence than the WT. Nucleus (Blue) was stained with Topro-3 at 1:1000 dilution. N=3, n>50. Respective p values are represented on the plots. Image was quantified with Image J.

### 4.2.4.2 CypD Deficiency Enhances H$_2$O$_2$-Induced Cell Death in HAP1 Cells

Clonogenic assay was carried out on both WT and KO HAP1 cells treated with varying concentrations of H$_2$O$_2$ for 2hr. No difference was observed in the number of colonies at 40µM of H$_2$O$_2$ treatment. However, at 0.8mM to 6.4mM treatment, more colony loss was observed in the KO cells (Figure 4.13).
Figure 4.10: Cellular and Mitochondrial ATP are Lower in CypD KO HAP1 Cells.

(A) Luminescence trace of ATP measuring cell titre Glo of HAP1 cell isolated mitochondria. (B) CypD KO mitochondria have significantly lower ATP signals than their WT counterpart. (C) Luminescence trace of ATP measuring cell titre Glo of HAP1 cells, seeded at 50,000 per well. Cells were treated with 10µM oligomycin as the control. (D) Luminescence signal (ATP level) was significantly lower in KO cells than WT cells and oligomycin treatment groups were characterised with significantly lower ATP signals than their untreated counterpart. N=5, * represents p<0.05 between WT and KO, ‘a’ represents p<0.05 between control and oligomycin treatments.
SOD activity is significantly lower in CypD KO isolated mouse liver mitochondria than WT. (B) GPX activity is lower but not significantly different between WT and KO isolated mitochondria. Antioxidant activity were measured spectrometrically at absorbance 430nm using the manufacturers instruction. N=3, * represents p value<0.05.

4.2.4.3 Protein Thiol Sulfenylation of WT and Mutant Recombinant CypD and PTPMT1
Recombinant CypD and PTPMT1 were treated with 10μM H₂O₂ and incubated at different times ranging from 30sec to 32min. PTPMT1 was used for comparison; this is a mitochondrial protein whose phosphatase activity is redox regulated. In recombinant CypD, sulfenic acid formation determined by western blot was observed to be relatively stabilised in all treatment groups and were not different from the untreated control group. However, H₂O₂ treatment of recombinant PTPMT1 resulted in the formation of thiol sulfenic acid by 20-fold at 30sec incubation which declined with increasing incubation time. Mutations of cysteine to serine amino acid at positions 203 in CypD and 132 of PTPMT1 resulted in significant loss of thiol sulfenic acid when incubated in H₂O₂ for 2min (Figure 4.14).
133

2hr treatment with 20, 40, 80 and 100µM of \( \text{H}_2\text{O}_2 \) resulted in the depletion of WST-1 based NAD(P)H in HAP1 WT cells when compared with the untreated control group. KO cells at this concentration were not affected adversely but were characterised with steady non-significant increase in NAD(P)H when compared with the untreated control group. Moreover NAD(P)H in KO cells remained significantly higher than their WT counterpart at 20, 40, 80 and 100µM of \( \text{H}_2\text{O}_2 \). Higher concentration of \( \text{H}_2\text{O}_2 \) rapidly depreciated NAD(P)H in both WT and KO cells when compared with their control groups. FCCP was used as standard. N=5, * represents p<0.05 when cells are compared between counterparts (WT and KO). ‘a’ represents p<0.05 when compared with untreated control.

Figure 4.12: \( \text{H}_2\text{O}_2 \) Accelerates NAD(P)H Depletion in WT HAP1 Cells. 2hr treatment with 20, 40, 80 and 100µM of \( \text{H}_2\text{O}_2 \) resulted in the depletion of WST-1 based NAD(P)H in HAP1 WT cells when compared with the untreated control group. KO cells at this concentration were not affected adversely but were characterised with steady non-significant increase in NAD(P)H when compared with the untreated control group. Moreover NAD(P)H in KO cells remained significantly higher than their WT counterpart at 20, 40, 80 and 100µM of \( \text{H}_2\text{O}_2 \). Higher concentration of \( \text{H}_2\text{O}_2 \) rapidly depreciated NAD(P)H in both WT and KO cells when compared with their control groups. FCCP was used as standard. N=5, * represents p<0.05 when cells are compared between counterparts (WT and KO). ‘a’ represents p<0.05 when compared with untreated control.

4.2.4.4 Structural Modulation of rec CypD by \( \text{H}_2\text{O}_2 \) Using Differential Scanning Fluorimetry

Thermal shifts of \( \text{H}_2\text{O}_2 \)-treated recombinant CypD was determined using differential scanning fluorimetry (DSF). There was no difference in the melting temperature of CypD following treatment in varying concentration of \( \text{H}_2\text{O}_2 \) ranging from 5µM to 160µM. The control protein, recombinant PTPMT1 resulted in loss of protein stability in all concentrations of \( \text{H}_2\text{O}_2 \) used. The melting temperature decreased by more than 5°C; decreasing with increasing \( \text{H}_2\text{O}_2 \) concentration starting from 10µM to 160µM. Treatment of mutant rec CypD (C203S) and PTPMT1 (C132S) with \( \text{H}_2\text{O}_2 \) did not destabilise the protein structure. Mutant CypD formed stable interaction with CsA with 8°C increase in melting temperature (Figure 4.15).
Figure 4.13: H$_2$O$_2$ Induces Cell Death in CypD KO than WT HAP1 Cells.

(A) clonogenic assay showing HAP1 cell colonies following treatment with varying concentrations of H$_2$O$_2$ for 2hr. (B) Plot of clonogenic assay shows that treatment with 0.8mM, 3.2mM and 6.4mM of H$_2$O$_2$ results in significantly lower number of cell colonies in the KO than WT. N=3, * represents p<0.05 when compared with WT counterparts. ‘a’ represents p<0.05 when compared with untreated controls.
**Figure 4.14: CypD Undergoes Stable Protein Thiol Sulfenylation at Cys203 Residue.**

(A) Western blots show protein thiol sulfenic acid. Recombinant protein was incubated in 10µM H₂O₂ at varying time points and cysteine residue thiol sulfenylation was stabilised with dimedone at a final concentration of 1mM. (B) CypD possessed sulfenylated Cys even when untreated with H₂O₂ while control protein, PTPMT1 was characterised with fluctuating cysteine sulfenic acid with 20-fold sulfenic acid formation at 30sec which was depleted at subsequent time points. (C) Western blot of sulfenic acid following CsA or DTT incubation with CypD prior to H₂O₂ treatment. (D) CsA did not affect CypD sulfenylation while DTT reduced sulfenylation by 1.8-fold. (E) Western blot indicates sulfenic acid formation between CypD WT and C203S mutant following 10µM H₂O₂ incubation for 1min. PTPMT1 was also used as the control and the mutant form was C132S. Mutant PTPMT1 resolved at lower molecular weight than the WT. (D) Plots indicates that CypD and PTPMT1 mutation depleted sulfenic acid formation. Image was quantified with image J. N=4, * represents p<0.05 when compared with the WT.
Figure 4.15: Recombinant CypD is Resistant to H₂O₂ Induced Oxidative Damage.

(A) Fluorimetric trace of recombinant CypD when incubated with varying concentrations of H₂O₂. (B) Plot showed no change in the melting temperature of CypD after incubation with H₂O₂. (C) Fluorimetric trace of Recombinant WT PTPMT1 when incubated with varying concentrations of H₂O₂. (D) Plot revealed that the melting temperature of PTPMT1 was significantly reduced following incubation with H₂O₂. (E) Fluorimetric trace of mutant CypD (C203S) following incubation with either 50µM H₂O₂ or 10µM CsA. (F) Fluorimetric trace of mutant PTPMT1 (C132S) following incubation with 50µM H₂O₂. (G) Plots reveal that there is no difference in melting temperature between H₂O₂ untreated and treated mutant CypD. In addition, CsA was able to bind mutant CypD, as it increases its melting temperature. Mutant PTPMT1 was also stabilised after treatment with H₂O₂. 5µM recombinant protein was used. N=3.
4.2.4.5 PTPMT1 Phosphatase Activity is Impaired by Both C132S Mutation and H$_2$O$_2$

PTPMT1 activity assay was determined using 4-nitrophenyl phosphate (pNPP) as the colorimetric substrate. WT PTPMT1 activity was significantly reduced by 10µM H$_2$O$_2$. Activity was restored when treated with 1mM DTT. Mutant PTPMT1 (C132S) did not show any PTPMT1 activity and H$_2$O$_2$ treatment had no effect on it (Figure 4.16).

Figure 4.16: PTPMT1 is Inactivated by both Mutation and H$_2$O$_2$ Treatment.

(A) spectrophotometric trace of PTPMT1 activity in the presence of H$_2$O$_2$. Mutant PTPMT1 showed no activity trace. (B) Activity was repressed by 10µM H$_2$O$_2$ and reversed by 1mM DTT while there is no activity observed for the C132S mutant. 5µM of recombinant protein was used. N=3, * represents p<0.05 in relation to untreated WT PTPMT1.
Table 4.1: Summary of Results for Chapter Four.

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<th>CypD and Ca$^{2+}$-Induced swelling</th>
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| CypD and the effect of ROS |
| CypD modulates mitochondrial antioxidant activities |
| Ppif cell type relative to WT | SOD | GPX |
| KO | Lower | Not different |

| CypD modulates ROS-induced NAD(P)H depletion |
| Ppif cell type | Level of NAD(P)H after H_2O_2 treatment (relative to WT) |
| KO | Higher |

| CypD modulates H_2O_2 induced cell death |
| Ppif cell type | Number of cells after H_2O_2 treatment (relative to WT) |
| KO | Lower |

| Recombinant CypD forms stable sulfenic acid on Cysteine 203 after H_2O_2 treatment |
| Protein cysteine modification | CypD | PTPMT1 |
| Cysteine sulfenic acid | Not different | Higher |
| Protein activity | Not different | Inactivity |
4.3 Discussion

4.3.1 CypD Deficiency Represses Mitochondrial Swelling

Mitochondrial swelling is the hallmark of Ca\(^{2+}\)-activated permeability transition pore, as persistent opening of the pore results in the unregulated exchange of solutes less than 1.5KDa between the matrix and the inter membrane space. Osmotic pressure drives matrix swelling and the eventual rupture of the outer mitochondrial membrane (Haworth and Hunter, 1979). Indeed, Ca\(^{2+}\) was able to activate mitochondrial swelling in the WT mitochondria while the KO and CsA treatment were characterised with reduced matrix swelling in both mouse liver and HAP1 isolated mitochondria (Figure 4.1). This result confirms that the isolated mitochondria was active and could respond to Ca\(^{2+}\) overload. More importantly, the influence of CypD on Ca\(^{2+}\) induced MPTP was also highlighted in the experiment.

Since CsA, an inhibitor of the MPTP has also been reported to inhibit calcineurin, a calcium-calmodulin dependent protein phosphatase, the possibility of phosphatase involvement in the MPTP was speculated. To verify this hypothesis, isolated mouse liver mitochondria were incubated with okadaic acid, a generic phosphatase inhibitor, which did not reverse Ca\(^{2+}\) induced mitochondrial swelling (Figure 4.2). This is consistent with findings that MPTP opening was inhibited with CsA analogues that do not interact with calcineurin.

Following this observation, it was also reasoned that mitochondrial protein prolyl hydroxylation may be important in MPTP activation, as CypD being a peptidyl prolyl isomerase may mediate the MPTP activation by priming prolyl peptide bonds for prolyl hydroxylation. Indeed, CypB has been reported to associate with Proline 3-hydroxylase during the formation of collagen (Vranka et al., 2004; Weis et al., 2010; Ishikawa et al., 2012). However, incubation of isolated mitochondria with oxalylglycine, an inhibitor of prolyl hydroxylases did not affect Ca\(^{2+}\) induced mitochondrial swelling (Figure 4.2). These results confirmed that neither mitochondrial phosphatases nor protein prolyl hydroxylases are important in MPTP activation, and that CsA binds CypD to
modulate the MPTP activation which is consistent with hypothesis that the permeability transition pore is PPIase regulated.

The mechanism by which CypD activates the MPTP still remains elusive. Indeed, previous reports have implicated acidosis associated with ischemia as an inhibitor of the MPTP even in the presence of CypD while reperfusion in the presence of CypD reactivates mitochondrial activities and produces elevated ROS as a side effect of the metabolic overload (Baines, 2009). This suggests that the MPTP is mainly regulated by metabolism, hence, placing CypD at the centre stage of both mitochondrial metabolic and ROS regulation.

### 4.3.2 CypD deficiency stabilises mitochondrial NADPH

CypD KO was associated with elevated mitochondrial NAD(P)H level in succinate energised mitochondria (Figure 4.3). Interestingly, unenergized mitochondria reflected very low NAD(P)H measurement and did not show any difference between WT, KO and CsA inhibition. This suggests that mitochondrial energisation is indeed important for the measurement of the matrix bioenergetics status. However, it is also plausible to assume that succinate may play a rather direct role on the generation of mitochondrial NAD(P)H. A possibility is the production of substrate for malate dehydrogenase, an NADH generating enzyme located downstream of succinate dehydrogenase in the TCA cycle. However, succinate-induced increase in $\Delta\Psi_m$ without ADP administration results in state 2 respiration, hence, grounding the TCA forward reaction, therefore preventing NADH accumulation.

Interestingly, previous reports have shown that succinate mediates NAD(P)H depletion rather than NAD(P)H production. Succinate depletes NADPH via the dissipation of mitochondrial GSH (Zheng et al., 2015). High level of GSH has been reported to bind fumarate (product from succinate), hence producing succinic-GSH which immediately depletes mitochondrial GSH. This increases
the NADPH requirement for GSH biosynthesis by glutathione reductase (Zheng et al., 2015; Janssen et al., 2019).

Most likely, succinate energisation of the mitochondria may have aided the transfer of the cell permeable WST-1 reagent into the mitochondria for measurement of the mitochondrial reductive equivalents. This supports the low and indifferent level of NAD(P)H when the isolated mitochondria were not treated with succinate. Therefore, the measurements observed in the result is a direct measure of the NAD(P)H. The direct effect of CypD on NAD(P)H was attempted via CsA treatment of the WT mitochondria. Surprisingly, the level of NAD(P)H was observed to be lower than the untreated control. This suggests that CsA directly and negatively modulates mitochondrial NAD(P)H. This result was supported by previous reports which demonstrated that CsA directly elevates mitochondrial ROS production independent of CypD (Lopez-Ongil et al., 1998; Chen et al., 2002; Seidlmayer et al., 2015; Esterberg et al., 2016; Folda et al., 2016). Unfortunately, the effect of CsA on CypD KO NAD(P)H status was not tested to verify its independence of CypD.

The results here suggest that the depletion of NAD(P)H by CsA (Figure 4.3) is mediated via the NADPH antioxidant systems involving either glutathione or thioredoxins (Figure 1.5). This also suggests that the reductive equivalent measured by WST-1 is most likely NADPH. The ROS produced from CsA is minimal and may pose no immediate threat to the mitochondria, and, therefore, CypD inhibition by CsA efficiently inhibits the MPTP. Previous studies have also shown that CsA failed to reverse MPTP opening following severe mitochondrial insult (Armstrong et al., 2018). Mitochondrial compromise in this event may be ascribed to the additive ROS producing activity of CsA.

In this result, the elevated level of NADPH in the KO when compared to the WT is hypothesized to be associated with either increased NADH derived TCA cycle activity of the intact cells and/or reduced mitochondrial ROS-generating activities. Elevated TCA cycle of the intact KO cells has been reported to be
closely associated with their enhanced Ca\textsuperscript{2+} buffering capacity during endogenous cellular activities. This was also tested in this study by activating mitochondrial Ca\textsuperscript{2+} sequestration via stimulating the release of Ca\textsuperscript{2+} from the ER with thapsigargin, as Ca\textsuperscript{2+} store in the ER are immediately released upon ER stress (Lytton et al., 1991; Garcia-Casas et al., 2018).

Treatment of HAP1 cells with 5, 10, and 20µM thapsigargin was accompanied by 1.3, 1.5 and 1.6-fold increase in cellular NAD(P)H in the KO as shown by WST-1 reduction-based assay (Figure 4.4). Surprisingly, WT cells showed no difference in the level of NAD(P)H. except at 40µM thapsigargin where there was a significant reduction in cellular NAD(P)H of both WT and KO. Indeed, higher Ca\textsuperscript{2+} buffering and accumulation must have triggered the elevation of NAD(P)H in the KO cells. Since the ER Ca\textsuperscript{2+} depletion takes place within 5min of thapsigargin treatment, and the incubation in this experiment was done for 2hrs, the unchanged level of NAD(P)H observed in WT must have resulted from the extrusion of mitochondrial Ca\textsuperscript{2+} either through transient MPTP or the sodium-calcium exchanger.

Ca\textsuperscript{2+} accumulation in the KO results in enhanced dehydrogenase activity, hence NADH production (Denton and McCormack, 1986; Hopper et al., 2006; Marcu et al., 2015; Warne et al., 2016). However, accumulation of NADH is reported to stall the TCA cycle (Martinez-Reyes and Chandel, 2020), therefore, oxidation via the electron transport chain and conversion into NADPH is immediately required in order for the cycle to proceed (Bradshaw, 2019). Moreover, other experiments are required to validate the elevation of the TCA cycle. More importantly, CypD may play direct role in the regulation of mitochondrial ROS and NADPH status. Indeed, elevated NADPH in the KO cells may be associated with downregulated oxidative stress in the KO HAP1 cells.

It is noteworthy that the NAD(P)H assay used in these experiments cannot differentiate between NADPH and NADH, and there are chances that both reducing equivalents are both high in KO mitochondria since they are highly
interrelated (Blacker and Duchen, 2016). Hence, both contribute individually to the characteristics associated with their abundance.

4.3.3 Mitochondrial NADH is shuttled to the Cytosol
To confirm that NADH accumulation in the mitochondria is shuttled to the cytosol in CypD deficient HAP1 and HeLa cells, global cellular lysine acetylation was determined, as lysine acetylation and deacetylation is dependent on the activity of, respectively, acetyl transferases and NAD-dependent deacetylases. Cell lysates from the CypD WT and KO cells were probed for lysine acetylation. Interestingly, the level of acetylated proteins was upregulated in both HAP1 and HeLa CypD deficient cells (Figure 4.5). Indeed, the build-up of NADH facilitated the transmission of NADH from the matrix to the cytosol through redox shuttles, such as the malate aspartate shuttle (Marcu et al., 2014). This is further evidence that CypD deficiency leads to the accumulation of NADH in these cells and indeed the mitochondria.

4.3.4 CypD Enhances ROS-induced NADPH depletion
The effects of exogenously added H$_2$O$_2$ on cellular NAD(P)H status was determined in WT and KO HAP1 cells. Treatment of cells with non-lethal doses of 20, 40, 80 and 100µM H$_2$O$_2$ resulted in the loss of NAD(P)H level of WT cells (Figure 4.12). H$_2$O$_2$ is a potential free radical-producing reactive oxygen species if it is not immediately reduced to water by antioxidant enzymes such as glutathione peroxidase, catalase and peroxiredoxins (Fukai and Ushio-Fukai, 2011). Since cell death was not observed at low concentration of H$_2$O$_2$, the H$_2$O$_2$ is most likely reduced through coupled reactions utilising GSH and NAD(P)H (Figure 1.5).

The depletion of NAD(P)H observed here in the WT cells (as opposed to KO cells) could potentially be explained by the WT cells utilising large quantities of NAD(P)H to make reducing small molecule thiols molecules such as GSH in response to oxidative stress (Martinez-Reyes and Chandel, 2020), hence explaining why the cells survived at low H$_2$O$_2$ levels. However, NAD(P)H in KO
cells remained remarkably unchanged (Figure 4.12). Results in this chapter indicates that CypD is implicated in enhancing ROS production (Figure 4.9), therefore, exogenous ROS may sensitise WT cells to rapid NADPH depletion in order to repress oxidative stress damage. Notably, the assay used in measuring NADPH level is often used for cell viability assay and this finding may be misleading, i.e. suggesting that KO cells are more viable than their WT counterpart. It is therefore important to combine this assay with other assays such as clonogenic and mitochondrial $\Delta \Psi_m$ to have a clearer view of the cellular NADPH dynamics. Surprisingly, clonogenic assay showed that KO cells were more susceptible to cell death induced by 2hr incubation in 0.8, 3.2 and 6.4mM H$_2$O$_2$ (Figure 4.13) even though the level of NAD(P)H was depleted alike under these conditions in both WT and KO cells. This could be explained by changes in response to death signals which may be underlined by numerous metabolic changes associated with the KO.

Elevated NAD(P)H in the KO may also be responsible for H$_2$O$_2$ induced cell death, as reductive equivalents keep chelated iron in the reduced Fe$^{2+}$ state which can result in dysfunctional mitochondrial TCA and the ETC. In addition to these, Fe$^{2+}$ can potentiate the formation of dangerous radicals such as hydroxyl radical during oxidative burst (Gores et al., 1989). The results here clearly demonstrate that radical-induced cell death is more prominent in CypD deficient cells than their WT indicating the usefulness of CypD in maintaining the balance between oxidative and reductive stress. Furthermore, this result emphasizes the possibility of CypD playing direct role in the modulation of mitochondrial NAD(P)H.

It appears that the NAD(P)H dynamics is rather being controlled in a CypD dependent manner, as HAP1 KO cells were unable to utilise their elevated NAD(P)H to combat ROS-induced cell stress, whereas, WT cells were characterised with deflating level of NAD(P)H while simultaneously maintaining their survival at non-lethal dose of H$_2$O$_2$. It was indeed pertinent to investigate further into the endogenous mitochondrial ROS production and
the modulation of bioenergetics associated with CypD and its deficiency, and to know if CypD plays direct role in mediating mitochondrial ROS production.

4.3.5 CypD Deficiency Alters Membrane Potential Without Affecting Complex I and II Respiratory Chain Components

Activity of complex I of the ETC was assessed by pyruvate-induced reduction of DCPIP and was not found to be different between WT and KO isolated mitochondria. Treatment with Ca\(^{2+}\) did not alter this effect – it was done to observe if Ca\(^{2+}\) overload alters complex I activity. Surprisingly, rotenone, an inhibitor of complex I slightly enhanced the reduction of DCIP by about 1.3-fold rather than inhibiting it in both WT and KO isolated mitochondria (Figure 4.6). This may have resulted from superoxide-induced reduction of DCPIP arising from rotenone treatment (Forman and Kennedy, 1975). Findings from this result suggests that the rate of electron transfer through the respiratory complex I remains the same between WT and KO isolated mitochondria, however, other methods should be explored (Figure 4.6).

For complex II activity, there was no significant difference in SDH between WT and KO isolated mitochondria. Malonate, a competitive inhibitor of SDH significantly inhibited the activity of SDH by 3-fold in both WT and KO mitochondria while CsA sustained the activity of SDH for another extra 2.5min before reaching a plateau in the kinetic curve (Figure 4.6). The effect of CsA was independent of CypD because WT and KO mitochondria presented similar phenomenon. Sustaining the reduction of DCPIP may be justified by the ROS-producing effect of CsA as it was also shown in the NAD(P)H depletion (Figure 4.3). Indeed, ROS generated by CsA can reduce DCPIP, giving a false positive result of complex II activity. This observed effect is the same with rotenone, as it also enhances the reduction of DCPIP in complex I mediated reaction via ROS production. Moreover, it is also possible that these mitochondrial protein inhibitors can inhibit the activities of other protein electron acceptors present in the mitochondria, thereby sustaining the number of electrons available for DCPIP reduction.
To evaluate the ETC activity in more comprehensive way, inhibition of respiratory chain complexes and its effect on the inner mitochondrial $\Delta \Psi_m$ was adopted. It was hypothesized that the differential respiratory chain activities would result in variable response of the inner membrane to ETC inhibition, as the $\Delta \Psi_m$ of cells growing independent of / reduced dependence on the ETC are likely to be less affected by respiratory chain activity suppression when compared with active users of the ETC. Respiratory chain inhibition resulted in the depression of $\Delta \Psi_m$ by the same order of magnitude in HAP1 WT and KO cells (Figure 4.8). This suggests that there are no differences in the respiratory chain activities between the WT and KO HAP1 cells.

Interestingly, basal mitochondrial $\Delta \Psi_m$ assessed in both HAP1 and HeLa cells was significantly lower in CypD deficient cells and CsA-treated HeLa cells. Treatment with rotenone was used as experimental control and this also resulted in loss of $\Delta \Psi_m$ in both HAP1 and HeLa cells (Figure 4.7). Low $\Delta \Psi_m$, or generally PMF is often associated with impairment of the ETC or disassembly of the respirasome. However, since no difference in respiratory chain complex activity was observed (Figures 4.6; 4.8), and Ca$^{2+}$ actively induced NAD(P)H production in the KO via thapsigargin (Figure 4.4), the hypothesis of impaired ETC in the KO may be rejected.

However, mild inner mitochondrial membrane uncoupling may depress the inner mitochondrial $\Delta \Psi_m$ (Cheng et al., 2017), as observed in the CypD deficient cells when compared with their WT counterpart. This is supported by evidences which suggests the forward flow of the TCA cycle being elevated during CypD deficiency (Chapter five). It is, however, important to verify the mitochondrial protein level of known uncouplers such as the ANT, UCP and the nicotinamide nucleotide transhydrogenases (NNT). Indeed, elevation of this uncouplers especially the NNT play important roles in both TCA cycle forward flux and NADPH accumulation (Rousset et al., 2004; Gameiro et al., 2013; Ho et al., 2017b).
It is plausible to assume that CypD has direct effect on the $\Delta\psi_m$ as CsA inhibition significantly reduced the $\Delta\psi_m$ (Figure 4.7) while also altering the mitochondrial morphology. Indeed, inhibition of calcineurin by CsA has been reported to enhance DRP-1 induced mitochondrial fragmentation following depolarisation of the $\Delta\psi_m$ (Cereghetti et al., 2008). In addition, free radicals can also activate mitochondrial uncoupling protein 2 (UCP-2) which can quickly dissipate the inner $\Delta\psi_m$ of the mitochondria (Echtay et al., 2002). However, the level of mitochondrial ROS is determined in subsequent section.

Complex I and II activities were not affected in CypD deficient cells, which is consistent with other recently published reports (Menazza et al., 2013; Tavecchio et al., 2015; Etzler et al., 2017). On the contrary, it has been reported that overexpression of CypD promoted cell survival by enhancing ETC via the complex III axis (Etzler et al., 2017). In light of this, a detailed understanding of the importance of CypD on the ETC is required as it is possible for any of the other downstream respiratory complexes or electron carriers such as cytochrome c or ubiquinone may be affected adversely either individually or collectively. Therefore, future work will be directed at understanding the effect of CypD on the ETC in a more detailed way. Indeed, the hypothesis of this chapter places CypD at the pivotal position of bioenergetics. Although its exact mechanism of regulating the $\Delta\psi_m$ is unknown, nevertheless, it may be involved in low level ROS signalling that drives the mitochondrial energetics in the forward path.

4.3.6 CypD Deficiency Downregulates Mitochondrial Superoxide

To verify if the depressed $\Delta\psi_m$ observed is associated with mitochondrial respiratory chain, the production of superoxide was compared between CypD deficient cells and their WT counterpart. Since most of the produced mitochondrial $O_2^•−$ is linked to the respiratory chain, therefore in this context, the persistence of mitochondrial $O_2^•−$ is determined by the balance between respiration and clearance by mitochondrial superoxide dismutase (MnSOD)
(St-Pierre et al., 2002; Kudin et al., 2004; Murphy, 2009). Interestingly, the level of superoxide was significantly reduced in the CypD deficient mitochondria (Figure 4.9). Since there were no observed difference in the activity of complex I and II between WT and KO mitochondria, coupled with undetectable differences during respiratory complexes inhibition (Figure 4.8), it is plausible to assume that low level of superoxide in the KO cells may have originated from either reduced ROS production at other mitochondrial sites (Wong et al., 2017), elevated superoxide dismutation (Van Raamsdonk et al., 2012), or activation of state 3 respiration via mild inner mitochondrial membrane uncoupling (Echtay et al., 2002).

Mitochondrial uncoupling may decrease ROS production by increasing the respiratory rate, hence, resulting in reduced oxygen tension in the mitochondrial microenvironment (Balaban et al., 2005; Caldeira da Silva et al., 2008). It also results in the maintenance of complex I and II intermediate at more oxidised state which prevents them from electron leakage. Also, ROS production via the 2-oxoglutarate and pyruvate dehydrogenase axis is repressed as a result of the TCA forward flux, thus, suppressing the reverse electron transfer to complex I (Turrens et al., 2003; Caldeira da Silva et al., 2008).

To verify antioxidant activities, MnSOD activity was observed to be lower in murine liver KO mitochondria than WT (Figure 4.11). Since the activities of enzymes are usually determined by the availability of substrate (Robinson, 2015), lower activity of SOD in the KO is therefore unsurprising as this correlates with the level of superoxide in CypD deficient cells (Figure 4.9). However, as SOD protein levels were not measured, it cannot be ignored that the results could reflect a difference in SOD protein levels in the WT and KO samples rather than a difference in enzymatic activity as influenced by superoxide concentrations.

Recall in the previous chapter that CypD may be implicated in mitochondrial unfolded protein response, the absence of CypD in KO mitochondria may alter
the structure and function of important mitochondrial enzymes such as MnSOD, thus, exhibiting reduced enzymatic activity in vitro. Likewise, metabolic changes associated with KO arising from the accumulation of NAD(P)H may result in increased acetylation of MnSOD, as acetylation has been reported to reduce its activity (Ozden et al., 2011; Zou et al., 2016). Although KO cells exhibited increased protein lysine acetylation, likewise, the level of total and acetylated Mn-SOD is worth checking in both WT and KO mitochondria.

On the contrary, there was no difference in the activity of glutathione peroxidase between WT and KO murine mitochondria (Figure 4.11). In this case, GPX activity is downstream of mitochondrial ROS and RNS production as it helps to mop up peroxides and peroxynitrites using reduced glutathione (Benhar, 2018).

4.3.7 Reduced Mitochondrial ATP is Associated with CypD Deficiency

Following observations suggesting both reduced $\Delta \Psi_m$ and electron flux through the ETC, levels of ATP was then measured in isolated mitochondria of WT and KO HAP1 cells. Unsurprisingly, the level of ATP was significantly (P<0.05) lower in KO than their WT isolated mitochondria (Figure 4.10). This result is consistent with the implications of low $\Delta \Psi_m$ or PMF. Indeed, the proton motive force is required for ATP production via the F1FO ATP synthase (Boyer, 1997; Boyer, 1999; Walker, 1998). Moreover, other authors have shown that mitochondrial matrix Ca$^{2+}$ accumulation also observed in CypD KO mitochondria had previously been associated with reduced $\Delta \Psi_m$ and ATP synthesis (Santulli et al., 2015).

Notably, reduced level of ATP observed in CypD-deficient cells may have predisposed them to necrotic death during H$_2$O$_2$-incubation (Figure 4.13). This is consistent with previous reports (Nikoletopoulou et al., 2013). On the other hand, apoptotic cell death requires energy. Other studies have also shown that
ROS-induced necrotic cell death can occur in the absence of CypD (Armstrong et al., 2018).

To determine whether the reduction of mitochondrial ATP corresponds with reduction in cellular ATP, the level of ATP was measured between WT and KO HAP1 cells. Notably, there was a 2-fold reduction (p<0.05) of ATP in HAP1 KO cells (Figure 4.10C, D). This observed reduced cellular energy in the KO cells most likely reflects the low ATP synthesis in the mitochondria since this is the organelle where 98% of cellular ATP is made: glycolysis generates a net of 2 ATP whereas oxidative phosphorylation by active F1/Fo ATP synthase in the mitochondria generates 38 ATP molecules. (Bonora et al., 2012; Walker, 2013). Similar trends of reduced mitochondrial ATP synthesis, ΔΨm and superoxide production were also observed in the CypD KD Hela cells. This leads to the conclusion that CypD has a role in regulating these important mitochondrial bioenergetics characteristics.

### 4.3.8 Recombinant CypD Enhances Redox Cycling by Forming Stable Sulfenic Acid

Treatment of recombinant CypD with H₂O₂ over a 32-minute time course revealed the modulation of sulfenic acid (R-SOH) formation (Figure 4.14). Two-electron oxidants such as H₂O₂ can lead to oxidation of protein thiols (R-SH) to R-SOH (Mieval and Chock, 2012). Thiol sulfenylation is not damaging to proteins and is reversible. It is usually dependent on the protein microenvironment, thiol nucleophilicity and proximity of the target thiol to the source of the ROS. However, when not reversed, sulfenylation may proceed irreversibly to formation sulfonic acids which is damaging to the proteins (Conte and Carroll, 2013).

We explored the sulfenylation behaviour of CypD and compared it to that of another mitochondrial protein PTPMT1 whose phosphatase activity is redox regulated. CypD sulfenylation was relatively stable in the presence and absence of H₂O₂. However, this was not the case when PTPMT1 where H₂O₂
treatment resulted in huge increase in R-SOH which immediately declined afterwards, until sulfenylation was almost completely dissipated (Figure 4.14).

Of note, CypD possessed naturally existing R-SOH as there was no difference in its level following H₂O₂ incubation whereas the level in PTPMT1 was 23-fold higher following 30sec incubation which later disappeared as incubation time increased (Figure 4.14A, B). R-SOH stabilisation is determined by the availability of nearby hydrogen bond acceptors, limited solvent access and the absence of neighbouring cysteine residues (Conte and Carroll, 2013). As expected, cysteine to serine mutations of both CypD and PTPMT1 resulted in drastic loss of sulfenic acid (Figure 4.14E, F). The data from CypD C203S mutant is particularly interesting; this mutant shows a dramatic loss of sulfenylation of CypD as a whole, suggesting that the majority of the sulfenic acid detected following incubation with 10µM H₂O₂ is from the sulfenylation of Cys203 (Figure 4.14E). CsA did not prevent CypD sulfenylation and this implies that formation of R-SOH is independent of its PPlase activity (Figure 4.14C, D).

Following endogenous protein sulfenylation, low molecular weight thiol groups such as GSH and thioredoxin are nucleophiles utilised in the reduction and reversal of the protein sulfenic acid (Conte and Carroll, 2013). Therefore, the presence of stable R-SOH in mitochondrial CypD makes it a potential distractor of NADPH for other mitochondrial or cellular reductive activities, as CypD cycles between sulfenylation and spontaneous intermolecular disulphide bond formation with either glutathione, homocysteine or thioredoxin, and reduction of these oxidised small molecule thiols will require NADPH (Figure 4.17).

Some studies have reported CypD to be involved in mitochondrial redox processes, in which suggested interactions include: hydrophobic interaction and formation of salt bridges with thioredoxin 2 and peroxiredoxin 3 respectively (Folda et al., 2016), cysteine nitrosylation (Nguyen et al., 2011), and cysteine glutathionylation (Itani et al., 2016). Interestingly, blockade of
CypD C203 by artificial S-nitrosylation and glutathionylation was reported to prevent both MPTP opening and ischemia reperfusion injury (Nguyen et al., 2011; Ong et al., 2015; Itani et al., 2016; Porter and Beutner, 2018). Our results confirm that redox cycling, and consequently the depletion of reductive equivalent (NADPH) at the Cysteine 203 of CypD plays important role in sensitising the mitochondria to modulation by exogenous ROS triggers.

Figure 4.17: Schematic Diagram of CypD Sulfenylation and Endogenous Reduction by Mitochondrial Glutathione and Thioredoxin Systems.

At basal mitochondrial respiratory rates, the spontaneous sulfenylation of CypD at the exposed Cys203 amino acid residue may occur consequently to ROS production at different sites in the mitochondria. The electrophilic nature of sulfenic acid may result in the spontaneous formation of intermolecular disulphide bonds with either other proteins or small molecule thiols such as glutathione or thioredoxins. The reduction of the formed disulphide bond is enhanced by the abundant NADPH-dependent mitochondrial glutaredoxin and thioredoxin systems. Because of the position of Cys203, it becomes easily oxidised following its reduction, hence, a wasteful depletion of mitochondrial NADPH.
Sulfenylation was first reported in 1976 (Allison, 1976; Salsbury et al., 2008). Indeed, few proteins have been identified to undergo sulfenylation and notable protein family of such are protein tyrosine phosphatases like PTPMT1 (Tanner et al., 2011). R-SOH enhances the formation of intramolecular disulphide bond which modifies the structure and inactivates PTPMT1. This study observed phosphatase inactivation when PTPMT1 was incubated with H₂O₂ (Figure 4.16). In this study, the sulfenylation status of cysteine 203 present in recombinant CypD in this study is novel. Unlike PTPMT1, sulfenylation at this residue does not lead to disulphide bond formation since there is no evidence of this disulphide bond being present from the many crystal structures of Cyclophilin D (Shore et al., 2016) although it is possible this sulfenylation plays a role in the folding of CypD itself. In the light of this result, further experiments must be carried out to confirm the effect of glutathionylation and nitrosylation on CypD sulfenylation in vitro and to identify the possibility of other cysteine molecules involved in it sulfenylation.

Next, to know if CypD remained structurally intact during oxidative stress, the effect of H₂O₂ on rec CypD stability was analysed using differential scanning fluorimetry (DSF). Because it has been established in this result that stable R-SOH occurs in CypD, it was not surprising to observe that CypD was resistant to H₂O₂ induced damage, even at a very high 32-fold concentration of H₂O₂. Interestingly, mutant CypD was also stable when incubated with H₂O₂. Moreover, the mutant specie (C203S) also formed stable interaction with CsA like the WT protein (Figure 4.15E).

However, PTPMT1 was characterised with structural changes and unfolding by more than 5°C reduction melting temperature as the concentration of H₂O₂ increased (Figure 4.15C, D). Phosphatase activity was impaired in the presence of H₂O₂. Interestingly, its mutant form was resistant to H₂O₂ destabilisation, but it possessed no phosphatase activity. This proposes that the decline in R-SOH in recombinant PTPMT1 (Figure 4.14A, B) might have resulted from intramolecular disulphide bond formation or perhaps the irreversible conversion of sulfenic acid to sulfinic and sulfonic acid which are
detrimental to the protein structure. On the other hand, recombinant CypD was able to prevent its thiol R-SOH from proceeding to these dangerous modifications. Supporting this view, R-SH groups on CypD possess very high reducing potential, and this posits them as distractors for the abundant mitochondrial matrix low molecular weight thiols, hence, NADPH depletion over time; in the case when CypD is not present, the NADPH depletion is much reduced, creating an overall mitochondrial reductive environment.

4.4 Conclusion

In conclusion, CypD is implicated in mitochondrial metabolism which determines the ROS status. CypD deficiency have shown reduction in mitochondrial O$_2$$^•$ and the inner $\Delta \Psi_m$ with concurrent low SOD activities. Additionally, KO cells are characterised with elevated NAD(P)H and while being susceptible to H$_2$O$_2$ induced cell death, it contrasts numerous experiments from different groups that have reported that CypD deficiency provides protection against ROS induced MPTP opening (Lee et al., 2011; Briston, et al., 2017; Teixeira et al., 2018).

Although NAD(P)H was depleted in the WT, following H$_2$O$_2$ treatment, yet clonogenic assay revealed excessive cell death in the KO, hence, indicating susceptibility to death in the KO than WT. It is clearly evident that though CypD deficiency may protect the mitochondria against MPTP induced cell death, the overall health of CypD deficient cells may be impaired if the stress is from outside the mitochondria, such as exogenous ROS-induced cell death observed in our data. It may be plausible to assume that the cellular reductive environment observed in the KO may prime the conditions for the formation of more dangerous radicals, such as hydroxyl radical in the presence of H$_2$O$_2$. Moreover, the depletion of ATP observed in the KO can activate necrotic cell death upon stimuli (Armstrong et al., 2018).

In addition, $\Delta \Psi_m$ or PMF was observed to be lower in CypD deficient HAP1 and HeLa cells and this may have indirectly resulted in both NAD(P)H
accumulation and Ca\(^{2+}\) retention. It has been reported that lower \(\Delta \Psi_m\) inhibits the mitochondrial Na\(^+\)/H\(^+\) exchanger which also inhibits the Na\(^+\)/Ca\(^{2+}\) exchanger, hence, resulting in matrix Ca\(^{2+}\) accumulation and activation of mitochondrial dehydrogenases (Numata et al., 1998). However, the underlying cause of the \(\Delta \Psi_m\) depletion would be further investigated.

Notably the activities of the respiratory chain complexes I and II were not different between WT and KO HAP1 cells while mitochondrial O\(_2^-\) and ATP were significantly lower in the deficient cells. It is suggested that mild mitochondrial uncoupling may be responsible for these effects including reduction of the inner mitochondrial \(\Delta \Psi_m\) (Caldeira da Silva et al., 2008). However, respiratory rate should be monitored to verify these assumptions.

Intriguingly, the redox status of Cysteine 203 present in CypD posits it as potential wasteful site for mitochondrial reductive equivalents, hence, plunging the mitochondria into oxidative stress. The role of CypD in redox sensing by stabilising cysteine sulfenic acid as potential distractor for mitochondrial reductive equivalent NAD(P)H here is novel and it provides probable mechanism for MPTP modulation. Further verification and validation of this hypothesis will now be required.
Chapter Five

CypD Regulates Metabolic Reprogramming via ROS and Energy Modulation
5.1 Introduction .............................................................................................................. 160
5.2 Results ................................................................................................................... 162

5.2.1 CypD Deficient Cells Possess Higher Level of Forward Flux Mitochondrial TCA Cycle Metabolising Enzymes .................................................. 162
5.2.2 ATP Synthase Inhibition Enhanced Cell Death in CypD Deficient Cells 162
5.2.3 AMPK is Differentially Regulated in KD and KO Cells........... 164
5.2.4 CypD Regulates Cellular ROS in HAP1 Cells ......................... 165
5.2.5 CypD Regulates HIF-1α in HAP1 and HeLa Cells ................. 166

5.2.5.1 Proteasome Inhibition Upregulates HIF-1α in CypD Deficient Cells More Than WT ................................................................. 166
5.2.5.2 HIF 1 Target Proteins are Downregulated in CypD Deficient Cells 166
5.2.5.3 CypD Deficiency Differentially Express HIF-1α and its Target Genes in HAP1 Cells .............................................................................. 167
5.2.5.4 CypD Deficiency Negatively Regulates HIF-1α Activity in HeLa Cells .................................................................................... 167
5.2.6 CypD Deficiency Prevents Lipid Droplet Formation in HAP1 and HeLa Cells ................................................................................. 168
5.2.7 CypD Deficiency Differentially Regulate the PI3K/AKT Pathway in Different Cell Types ................................................................. 169
5.2.8 CypD Deficiency Differentially Regulates mTORC1 Pathway in HAP HeLa and HT1080 Cells ................................................................. 170
5.2.9 CypD Deficiency Differentially Alters the NF-κB Pathway in HAP1 and HeLa Cells ............................................................................. 172
5.2.10 CypD Regulates Autophagy in HAP1 Cells .................................. 174

5.3 Discussion .............................................................................................................. 178

5.3.1 CypD Deficiency Enhances Mitochondrial TCA Cycle forward flux while Repressing ATP Synthesis in HAP1 Cells ..................... 178
5.3.2 Linking CypD to the MPTP through its role in coupling TCA with oxidative phosphorylation ............................................................. 181
5.3.3 CypD Modulates Cellular ROS in HAP1 Cells ...................... 182
5.3.4 AMPK is Activated in CypD Knock Downs ......................... 183
5.3.5 CypD Deficiency Suppresses HIF-1α Activity in HAP1 and HeLa Cells 185
5.3.6 CypD is Required for Lipid Droplet Formation in HAP1 and HeLa Cells 187
5.3.7 CypD Deficiency Differentially Regulates PI3K/AKT and MEK/ERK Pathways in Different Cell Lines .......................... 189
5.3.8 CypD Deficiency Activates mTORC1 Pathway in HAP1 and HeLa Cells ................................................................. 191
5.3.9 CypD Maintains Survival via Enhanced Autophagy and mTORC2 Stabilisation in HAP1 Cells ................................. 193
5.3.10 CypD Deficiency Differentially Regulates NF-κB in HAP1 and HeLa Cells .............................................................. 195
5.4 Conclusion ........................................................................... 196
5 CypD Regulates Metabolic Reprogramming Via ROS and Energy Modulation

5.1 Introduction

The aims of this chapter are to investigate how CypD deficiency affects two aspects of mitochondria function – the TCA cycle and oxidative phosphorylation. The strategy taken to achieve these aims is to measure the cellular effects brought about by changes in metabolite levels, such as changes in the levels of phosphorylation of extramitochondrial proteins.

The previous chapter revealed changes in mitochondrial bioenergetics that were associated with the loss of CypD. These spanned physiological changes in the mitochondrial membrane activities and the matrix NAD(P)H status. Indeed, these changes are powerful enough to evoke cell-wide metabolic reprogramming, hence redefining the entire cellular signalling pattern. Moreover, tracking the changes in cellular metabolic preferences may give relevant information on finding the underlying perturbation that has given rise to the observed changes (Ward and Thompson, 2012; Phan et al., 2014).

Metabolic reprogramming or rewiring is an adaptive response by which cells or tissues maintain their survival or self-renewing potential by either upregulating or downregulating cellular signalling pathways in response to drastic changes in internal or external microenvironment (Yoshida, 2015). Following the genetic ablation of certain genes, pathway analysis may give insight into the metabolic function of the gene of interest. However, ablation of some genes could be fatal and lethal, revealing how indispensable such gene products are to the survival of the cell (Mikula et al., 2001; Orlacchio et al., 2018).

This chapter focusses on the modulation of various cell signalling protein molecules, such as PI3k/AKT, mTORC1, HIF-1α and NF-κB and their association with partial and total mitochondrial CypD absence, i.e. KD and KO
HAP1 and HeLa cells. The differences in phosphorylated protein targets, neutral lipids, and level of expressed proteins were used to determine the direction and preference of cell signalling associated with CypD deficiency. Moreover, metabolomics data obtained by another member of our lab are referred to occasionally in support of the claims presented in this chapter as they are pertinent to the discussions.

Previous work has demonstrated the effect of CypD on various signalling pathways such as the AKT, mTORC1, JAK/STAT pathways and elevation of these pathways in CypD deficient cells had previously been reported although these were carried out in several separate studies (Klawitter et al., 2017a, Klawitter et al., 2017b). However, by studying all the pathways using one system, an important finding in this chapter suggests that CypD may exert varying effects on these pathways and demonstrate that these can be dependent on the metabolic background of the cell in question. Since mitochondrial metabolism intermediates such as 2-oxoglutarate and succinate contribute immensely to the activation of all of the signalling pathways being studied here, observations of perturbations of these metabolite levels are of particular interest.
5.2 Results
The results of this chapter are summarised in Table 5.1

5.2.1 CypD Deficient Cells Possess Higher Level of Forward Flux Mitochondrial TCA Cycle Metabolising Enzymes
Western blot revealed significantly higher level of OGDH in CypD KO than WT of HAP1 cells. However, other mitochondrial proteins such as ATP synthase b subunit and IDH3 were not significantly different between WT and KO cells. Interestingly, IDH2 was not detected in both HAP1 cell experimental models, and the level of cytosolic IDH1 was significantly lower in HAP1 KO than WT cells. However, in HeLa cells, CypD KD resulted in higher level of IDH3 and IDH1 coupled with lower level of IDH2 whereas OGDH was not significantly different between the WT and KD cells. HSP75 was used as the control (Figure 5.1).

5.2.2 ATP Synthase Inhibition Enhanced Cell Death in CypD Deficient Cells
Respiratory chain complexes I, III and V (ATP synthase) were inhibited respectively with rotenone, antimycin, and oligomycin for 24hr. Rotenone treatment resulted in about 100% cell death of both WT and KO cells while antimycin treatment did not negatively affect the number of colonies. However, following oligomycin treatment, 60% of the colonies survived in the KO cells while WT were also not adversely affected. Colony sizes were significantly reduced in all treatment groups when compared with the control. Oligomycin treatment resulted in more decrease in colony size in KO than WT cells (Figure 5.2).
(A) TCA forward flux enzyme, OGDH was upregulated in HAP1 CypD KO cells by more than 2-fold. IDH3 was also higher but not significantly different from the WT. Cytosolic IDH1 was significantly lower in KO cells when compared with WT cells. Other mitochondrial enzymes such as SDHC and ATP synthase b subunit were not found to be different between WT and KO HAP1 cells. Blot intensities were normalised to HSP75. (B) In HeLa cells, OGDH was not found to be significantly different between CypD WT and KD cells. However, IDH3 was observed to be significantly upregulated in the KD. IDH2 was detected and was downregulated in KD cells. Cytosolic IDH1 was upregulated in KD cells. Other mitochondrial enzymes such as SDHC and ATP synthase b subunit were not observed to be significantly different. (C) in HAP1 cells, phosphorylated (ser 239) pyruvate dehydrogenase (PDHE1α) was significantly reduced in KO cells when compared with the WT. Blot intensities were normalised to HSP75. N=3, * represents p<0.05 when compared with the WT.

Figure 5.1: CypD Deficiency Upregulates Forward TCA Cycle Metabolising Enzymes.
5.2.3 AMPK is Differentially Regulated in KD and KO Cells

Western blot analysis revealed that CypD KD in HAP1 cells resulted in the elevation of p-AMPK at Thr172 than both WT and KO cells although this elevation was not statistically significant (p>0.05). However, in the KO cells, p-AMPK was significantly lower than the WT (p<0.05). HeLa cells also presented with elevated p-AMPK in the KD than WT, although again this was not statistically significant (P>0.05; Figure 5.3).

Figure 5.2: CypD KO are Susceptible to ATP Synthase Inhibition. (A) Clonogenic assay shows the colony differences between WT and KO cells and the effect of ETC inhibition. KO cells are characterised with bigger colonies than WT. (B) The percentage survival of both WT and KO cells were 10% following the inhibition of complex I with rotenone. There were slightly higher colonies in both WT and KO which were not significantly different from the control after antimycin treatment. ATP synthase inhibition with oligomycin resulted in the depletion of cell colonies in the KO while WT cells maintained an elevated number of colonies. (C) The percentage colony size was significantly lower in all treated groups when compared with their control. However, oligomycin treatment resulted in smaller sizes in the KO cells than WT cells. Image J was used to analyse the images. N=3, * represents p<0.05.
Figure 5.3: CypD Modulates AMPK Activity. (A) P-AMPK is differentially activated in CypD KD and KO HAP 1 cells. KD cells were characterised with the upregulation of P-AMPK, although this difference was not significant while in KO cells. P-AMPK was significantly lower. Band intensity were normalised to HSP75. (B) P-AMPK was elevated in CypD KD HeLa cells. The observed elevation was not statistically significant. Band intensities were normalised to GAPDH. Image J was used for western blot image analysis. N=3, * represents p<0.05 when compared to the WT.

5.2.4 CypD Regulates Cellular ROS in HAP1 Cells

Immunofluorescence studies revealed that cellular ROS was significantly higher in CypD KD cells, and significantly lower in KO cells when compared with the WT group in HAP1 cells (Figure 5.4).
Cellular ROS level were significantly higher in KD cells while in KO cells, ROS level were significantly lower when compared to the WT in HAP1 cells. ROS level were stained with 2µM Cell ROX in IMDM media for 40mins. Cells were fixed in 3.7% PFA. Image was acquired using the laser channel 488nm of Zeiss LSM 710 confocal microscope. N=3, n>30 for HAP1 cells. P values are represented on the box plot.

5.2.5 CypD Regulates HIF-1α in HAP1 and HeLa Cells
5.2.5.1 Proteasome Inhibition Upregulates HIF-1α in CypD Deficient Cells More Than WT
Western blot analysis revealed that treatment of cells with MG132, a proteasome inhibitor, for 4hr resulted in the stabilisation of HIF-1α along with elevated ubiquitinated HIF resolving at higher molecular weight. However, accumulation of both HIF-1α and its ubiquitinated form was significantly higher in CypD deficient HAP1 and HeLa cells. Indeed, untreated HAP1 cells revealed enhanced degradation of HIF-1α for the KD and KO cells than WT (Figure 5.5).

5.2.5.2 HIF 1 Target Proteins are Downregulated in CypD Deficient Cells
Western blot analysis revealed that HIF-1α target protein, PHD2 was significantly higher in KD and lower in KO cells when compared with the WT cells (Figure 5.5).
5.2.5.3 CypD Deficiency Differentially Express HIF-1α and its Target Genes in HAP1 Cells

Quantitative PCR analysis revealed that CypD KD significantly (p<0.05) upregulated the expression of PHD2 and PDHK1, while their expression was significantly reduced in KO cells (Figure 5.5D).

Figure 5.5: CypD Modulates HIF-1α in HAP1 Cells. (A) Normoxic HIF-1α was elevated in KD cells and downregulated in the KO cells. HIF target protein, PHD2 was elevated in KD and downregulated in the KO cells. Protein blots were normalised to HSP75. (B) Proteasome inhibition by MG132 resulted in elevated HIF-1α in both WT and KO HAP1 cells. HIF-1α in KO were significantly higher than WT cells. Untreated groups were undetected in both WT and KO cells. Band intensity were normalised to HSP75. (C) MG132 treatment resulted in elevated hydroxylated HIF-1α in the KO cells than their WT counterpart. (D) QPCR expression analysis of PHD2 and PDHK1 expression in CypD WT and KO HAP1 cell. PHD2 and PDHK1 expression were significantly lower in KO cells. N=3, * represents p<0.05 when compared with WT, ‘a’ represents p<0.05 when compared with untreated groups.

5.2.5.4 CypD Deficiency Negatively Regulates HIF-1α Activity in HeLa Cells

HIF-1α activity was measured by the activity of luciferase which is expressed by a plasmid that possessed a HIF Response Element (HRE) that is upstream of the luciferase gene. During normoxia (21% O₂), the activity of luciferase was significantly lower in KD cells than their WT counterpart. However, in hypoxia (1% O₂) where the HIF-1α activity was expected to be significantly higher than
normoxia, the difference between the CypD WT and KD was not statistically significant. However, CA9, which is an established HIF-1alpha target protein was observed to be significantly lower in hypoxic CypD KD cells when compared with the WT (Figure 5.6).

**Figure 5.6: CypD Modulates HIF-1α in HeLa Cells.** (A) Proteasome inhibition with MG132 resulted in the stabilisation of HIF-1alpha in both CypD WT and KD cells. However, KD cells were characterised with elevated HIF-1alpha when compared with their WT counterpart. (B) HIF-1alpha activity measured by HRE coupled luciferase activity revealed that hypoxia increased HIF-1alpha activity with no difference in activity between WT and KD cells. However, normoxic activity was significantly downregulated in CypD KD cells. (C) Hypoxic CA9, a HIF-1α target protein, was significantly reduced in CypD KD cells when compared to the WT. Image J was used for western blot band intensity quantification. N=3, ‘a’ represents p<0.05 when compared with the untreated or normoxic groups, * represents p<0.05 when compared with the WT.

**5.2.6 CypD Deficiency Prevents Lipid Droplet Formation in HAP1 and HeLa Cells**

The formation of neutral lipid droplets was assessed in both HAP1 and HeLa cells following CypD deficiency. Indeed, both HAP1 KO and HeLa KD cells showed significantly reduced lipid droplet sizes than their respective WT
counterpart. However, the size of lipid droplets in CypD KD in HAP1 cells were not significantly different from the WT cells (Figure 5.7).

![Figure 5.7: CypD Regulates Cellular Lipid Droplets in HAP and HeLa Cells.](image)

(A) Lipid droplets size in HAP1 cells. HAP1 CypD KO cells have reduced level of lipid droplet size and overall amount of lipid droplets when compared with WT and KD cells. (B) Lipid droplet size in HeLa cells. HeLa KD cells are characterised with reduced droplet size than WT cells. Lipid droplets were stained with 2µM BODIPY green in HBSS media for 15mins. Cells were fixed in 3.7% PFA and nucleus stained with TOPRO-3 (1:1000) for 30mins. Image was acquired using the laser channel 488nm and 633nm of Zeiss LSM 710 confocal microscope. N=3, n>30 for HAP1 cells and N=10, n>20 for HeLa cells. P values are represented on the box plot.

5.2.7 CypD Deficiency Differentially Regulate the PI3K/AKT Pathway in Different Cell Types

Western blot analysis revealed that CypD deficiency upregulated the level of phosphorylated AKT at Thr308 more than the WT in HAP1 cells. Interestingly, AKT phosphorylation at Thr308 decreased significantly with decreasing level of CypD in hypoxia. The downstream target of the AKT, p-GSK3 β at Ser9 was significantly (P<0.05) higher in CypD deficient HAP1 cells than the WT, this was also reduced during hypoxia, however, not significant in the KO (Figure
5.8A). Furthermore, phosphorylated ERK at Thr202/Tyr204 was higher in the KO than WT in HAP1 cells (Figure 5.8B).

In Hela cells, phosphorylated AKT at Thr308 was not detected in both WT and KO, however, hypoxia elevated the level of p-AKT in the WT while in KD cells, it remained undetected. Phosphorylation of the downstream GSK 3β at Ser9 was observed to higher in KD than WT cells. However, CypD KD in HT1080 cells presented with reduced level of p-AKT (Ser473) and p-GSK3 β (Ser9) than the WT (Figure 5.8D).

5.2.8 CypD Deficiency Differentially Regulates mTORC1 Pathway in HAP HeLa and HT1080 Cells

Western blot and immunofluorescence were used to study the activity of mTORC1. The level of phosphorylated ribosomal protein S6 and p70 S6 kinase at Ser235/236 and Thr389 were respectively upregulated in HAP1 KO and HeLa KD cells. In HAP1 KD, the two phosphorylated mTORC1 markers were not significantly different from the WT cells. However, in HT1080 cells, the phosphorylation status of these proteins was downregulated in CypD KD cells when compared with their WT counterpart (Figure 5.9).

Immunofluorescence was used to determine the co-localisation of mTORC1 and the lysosome in HeLa cells. CypD KD resulted in the upregulation of mTOR-lysosome co-localisation than WT HeLa cells (Figure 5.10).
The phosphorylation of AKT at both Thr308 and Ser473 were modulated by CypD deficiency. Decreasing mitochondrial CypD resulted in increasing phosphorylation of AKT at the Thr308 site at normoxic conditions while the phosphorylation at Ser473 was not different between WT KD and KO HAP1 cells. However, during hypoxia, the phosphorylation at both Thr308 and Ser473 decreased with decreasing CypD. Phosphorylation of GSK3 β at Ser9, a downstream effector of AKT, was increased significantly only in the KO during normoxia and declined however, insignificantly during hypoxia. (B) CypD KO cells were characterised with upregulated total and p-ERK at Thr202/Tyr204. (C) CypD KO in HeLa cells resulted in the downregulation of p-AKT (Thr308) only during hypoxia, as normoxic levels were undetected for AKT. Phosphorylation of GSK 3β at Ser9 was observed in normoxic KD cells, while hypoxia maintained higher level of P-GSK3 β. (D) In HT1080 cells, during normoxia, CypD KD resulted in the downregulation of the AKT pathway, as observed by the reduced level of phosphorylated GSK 3β. N ≥ 2. * represents p<0.05 when compared with the WT. ‘a’ represents p<0.05 when compared with their normoxic counterpart.
172

5.2.9 CypD Deficiency Differentially Alters the NF-κB Pathway in HAP1 and HeLa Cells

Western blot was used to determine differences in the regulation of NF-κB. Phosphorylated IκB-α at Ser32/36 was higher in HAP1 KO cells than WT resulting in the degradation of IκB-α. This correlates with the observed lower level of IκB-α total protein. Conversely, in HeLa cells, no significant difference was observed in phosphorylated IκB-α, however, the protein level was higher in the KD than the WT (Figure 5.11).
mTOR (green) were observed in both WT and KD HeLa cells. However, KD cells showed mTOR condensation at specific cellular locations when compared with the WT and this correlated with lysosomal (LAMP2 stained in red). Nucleus (blue) was stained with Hoechst (1µg/mL). Yellow arrows point at mTOR clusters in the cell cytoplasm. Brown arrows shows dispersed mTOR around the cytoplasm. Red arrows show mTOR-lysosomal co-localisation in the cytosol. Cells were fixed in 3.7% PFA. Primary antibodies (1:300), Secondary antibodies (Alexa Fluor 555 and Alexa Fluor 488; 1:500). N=3; Image was acquired using RFP, GFP and DAPI filters on a Zeiss Epifluorescent microscope. Image analysis was done with Image J.

Figure 5.10: CypD Deficiency is Associated with mTOR-Lysosomal Localisation in HeLa Cells.

mTOR (green) were observed in both WT and KD HeLa cells. However, KD cells showed mTOR condensation at specific cellular locations when compared with the WT and this correlated with lysosomal (LAMP2 stained in red). Nucleus (blue) was stained with Hoechst (1µg/mL). Yellow arrows point at mTOR clusters in the cell cytoplasm. Brown arrows shows dispersed mTOR around the cytoplasm. Red arrows show mTOR-lysosomal co-localisation in the cytosol. Cells were fixed in 3.7% PFA. Primary antibodies (1:300), Secondary antibodies (Alexa Fluor 555 and Alexa Fluor 488; 1:500). N=3; Image was acquired using RFP, GFP and DAPI filters on a Zeiss Epifluorescent microscope. Image analysis was done with Image J.

Figure 5.11: CypD Deficiency Modulates NF-κB Pathway. (A) NF-κB activation is upregulated in CypD KO HAP1 cells. KO cells were characterised with increased level of phosphorylated IκB alpha, an endogenous inhibitor of the NF-κB pathway at Ser32/36, thereby resulting in its degradation. (B) CypD deficiency did not result the overall phosphorylation of IκB alpha in HeLa cells, however, there was an accumulation of IκB alpha.
5.2.10 CypD Regulates Autophagy in HAP1 Cells

Autophagy marker LC3B was used to verify the extent of autophagy in HAP1 cells. KD were characterised with elevated autophagy by their characteristic reduced level of LC3B-1 whereas, KO cells showed elevated LC3-1B which is characterised with reduced autophagy (Figure 5.12).

![Autophagy marker LC3B-1 is differently expressed in KD and KO HAP1 cells. LC3B-1 was lower in KD cells but was higher in KO cells when compared with the WT. N=3, * represents p<0.05 when compared with the WT, 'a' represents p<0.05 when compared with the KD.]

Figure 5.12: CypD Regulates Autophagy in HAP1 Cells. Autophagy marker LC3B-1 is differently expressed in KD and KO HAP1 cells. LC3B-1 was lower in KD cells but was higher in KO cells when compared with the WT. N=3, * represents p<0.05 when compared with the WT, ‘a’ represents p<0.05 when compared with the KD.
Table 5.1: Summary of Result for Chapter 5. NA means that the data is not available.

| CypD Deficient Cells Possess Higher Level of Forward Flux Mitochondrial TCA Cycle Metabolising Enzymes |
|-------------------------------------------------|----------------|----------------|----------------|----------------|----------------|
| **ppif** cell types compared to WT              | OGDH           | IDH1           | IDH2           | IDH3           | ATP Synthase Subunit b |
| Hap1 KO                                         | 2-fold higher  | lower          | undetect ed    | Not significantly different | Not significantly different | Lower |
| Hap1 KD                                         | NA             | NA             | NA             | NA             | NA             | NA              |
| Hela KD                                         | Not significantly different | higher         | Lower          | Higher          | Not significantly different | NA              |

| Membrane Potential Level and Cell Death |
|------------------------------------------|----------------|----------------|----------------|----------------|----------------|
| Mitochondrial membrane potential and cell death |
| Antimycin A (CIII inhibitor)              | Rotenone       | Malonate       | Sodium Azide   | Oligomycin     |
| Residual Level of Membrane potential after 2hr incubation relative to WT |
| Higher in KO than WT                      | No difference between WT and KO | No difference between WT and KO | No difference between WT and KO |
| Cell death after 24hr incubation relative to WT |
| Higher in WT than KO                      | Complete cell death in both WT and KO | NA             | NA             | Higher in KO than WT |

| HAP1 Cellular ROS |
|-------------------|----------------|
| **ppif** cell type | ROS level compared to WT |
| KD                | Higher          |
| KO                | Lower           |

| AMPK is Differentially Regulated in KD and KO Cells |
|---------------------------------------------------|----------------|
| **ppif** cell types compared to WT                | pAMPK T172     |
| Hap1 KO                                          | Lower than WT  |
| Hap1 KD                                          | Higher than WT |
| HeLa KD                                          | Not different  |

| HIF-1α is Both Highly Expressed and Degraded in CypD Deficient Cells |
|---------------------------------------------------------------------|----------------|
| **ppif** cell types compared to WT                                  | HIF-1α         |


<table>
<thead>
<tr>
<th>MG132 treated cells</th>
<th>Elevated HIF-1α accumulation in <em>ppif</em> KO HAP1 and KD HeLa cells respectively. Lower level of HIF-1α in <em>ppif</em> KD HAP1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG132 untreated</td>
<td>Increased degradation of HIF-1α in HAP1 KO cells</td>
</tr>
<tr>
<td>Hydroxylated HIF-1α</td>
<td>Elevated hydroxylated HIF-1α in HAP KO</td>
</tr>
</tbody>
</table>

### Levels of HIF-1α target genes

<table>
<thead>
<tr>
<th><em>ppif</em> cell types compared to WT</th>
<th>PHD2</th>
<th>Hypoxic CA9</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP1 KO</td>
<td>Lower than WT</td>
<td>NA</td>
</tr>
<tr>
<td>HAP1 KD</td>
<td>Higher than WT</td>
<td>NA</td>
</tr>
<tr>
<td>HeLa KD</td>
<td>NA</td>
<td>Lower than WT</td>
</tr>
</tbody>
</table>

### Level of HIF-1α activity relative to WT

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Hypoxia</th>
<th>Normoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP1 KO</td>
<td>Not significantly different</td>
<td>Lower</td>
</tr>
<tr>
<td>HAP1 KD</td>
<td>Not significantly different</td>
<td>Higher</td>
</tr>
<tr>
<td>HeLa KD</td>
<td>Not significantly different</td>
<td>Lower</td>
</tr>
</tbody>
</table>

### Cellular Lipid Droplets relative to WT

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Hap 1 KO</th>
<th>HAP1 KD</th>
<th>HeLa KD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower</td>
<td>Not different</td>
<td>Lower</td>
</tr>
</tbody>
</table>

### AKT Pathway

<table>
<thead>
<tr>
<th><em>ppif</em> cell types compared to WT</th>
<th>AKT T308 phosphorylation (by PDK1)</th>
<th>AKT S473 phosphorylation (by mTORC2)</th>
<th>GSK 3β S9 phosphorylation</th>
<th>ERK T202/Y204</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAP1 KO (normoxia)</td>
<td>Higher</td>
<td>Not significantly different</td>
<td>Higher</td>
<td>Higher</td>
</tr>
<tr>
<td>HAP1 KD (normoxia)</td>
<td>Higher</td>
<td>Not significantly different</td>
<td>Higher</td>
<td>NA</td>
</tr>
<tr>
<td>HAP1 KO (hypoxia)</td>
<td>Lower</td>
<td>Lower</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HeLa KD (normoxia)</td>
<td>Not detected in WT and KD</td>
<td>NA</td>
<td>Higher</td>
<td>NA</td>
</tr>
<tr>
<td>HeLa KD (hypoxia)</td>
<td>Not detected in KD but present in WT</td>
<td>NA</td>
<td>Lower</td>
<td>NA</td>
</tr>
<tr>
<td>HT1080 KO (normoxia)</td>
<td>NA</td>
<td>Lower</td>
<td>Lower</td>
<td>NA</td>
</tr>
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</table>

### mTORC1 Pathway (levels compared with WT)

<table>
<thead>
<tr>
<th>Hap 1 KO</th>
<th>Hap 1 KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-S6 S235/236</td>
<td>p-70 S6K T389</td>
</tr>
<tr>
<td>Higher</td>
<td>Higher</td>
</tr>
<tr>
<td>Not different</td>
<td>Not different</td>
</tr>
<tr>
<td></td>
<td>HeLa KD</td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
</tr>
<tr>
<td>H1080 KD</td>
<td>Lower</td>
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**NF-κB Pathway (levels compare with WT)**

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<tbody>
<tr>
<td>Hap1 KO</td>
<td></td>
<td></td>
<td></td>
<td>Phosphorylated IκB-α at Ser32/36 higher in KO, lower level of IκB-α total protein.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein degradation in KO.</td>
</tr>
<tr>
<td>Hela KD</td>
<td></td>
<td></td>
<td></td>
<td>No difference was observed in phosphorylated IκB-α, higher level of protein in the KD than the WT</td>
</tr>
</tbody>
</table>

**Autophagy (Levels compare with WT)**

<p>| | | | | |</p>
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<th></th>
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<tbody>
<tr>
<td>HAP 1 KD</td>
<td></td>
<td></td>
<td></td>
<td>Significantly higher</td>
</tr>
<tr>
<td>HAP 1 KO</td>
<td></td>
<td></td>
<td></td>
<td>Significantly lower</td>
</tr>
</tbody>
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5.3 Discussion

5.3.1 CypD Deficiency Enhances Mitochondrial TCA Cycle forward flux while Repressing ATP Synthesis in HAP1 Cells

Previous reports have shown that dysfunctional oxidative phosphorylation caused by inner mitochondrial uncoupling can increase the flux through the TCA cycle to compensate for reduced ATP synthesis (Smith et al., 2004; Befroy et al., 2008). Likewise, it has been shown in the previous chapter that CypD deficiency increased mitochondrial NAD(P)H; following this observation, an indirect measure of the TCA cycle flux was determined by the abundance of vital TCA cycle metabolising enzymes in both HAP WT and KO cells. The magnitude of abundance of TCA cycle enzymes depicts the adaptive response to the mitochondrial metabolic need and flow (Ilic et al., 2017).

As shown in the summary of results in Table 5.1 and Figure 5.13, the mitochondrial NADP+ dependent isocitrate dehydrogenase, IDH2 was not detected in both WT and KO HAP1 cells while its NAD+-dependent isoform, IDH3 was detected and observed to be elevated in the KO cells, however, the difference was not significant. Moreover pyruvate dehydrogenase activity, an enzyme that commits glycolysis into the TCA cycle was significantly elevated in HAP KO cells when compared with the WT. Interestingly, the level of 2-oxoglutarate dehydrogenase, OGDH was 2-fold higher in the KO than WT; this elevated level suggests that in the KO, the TCA cycle is driven by the forward reaction pathway (Figure 5.1A). This result also emphasizes the need to metabolise 2-oxoglutarate - the substrate for OGDH; this is an important intermediate of the TCA cycle which is positioned at the junction of pyruvate, glutamine metabolism and amino acid transamination reactions (Vatrinet et al., 2017).

It is also plausible that the accumulation of OGDH might have resulted from its reduced activity due to improper protein folding (Yoon et al., 2017). If this was the case, reverse TCA may be activated via IDH2, however the very low or undetectable levels of IDH2 in both WT and KO cells makes this an unlikely
process in HAP1 cells. Interestingly, cytosolic IDH1, an enzyme which is essential for cytosolic reductive carboxylation was also downregulated in the KO HAP1 cells (Figure 5.1) suggesting that reductive carboxylation both within and outside the mitochondria may not be favoured in CypD deficient HAP1 cells.

In addition, lipid accumulation is usually the product of reductive carboxylation, and the fact that the level of lipid droplets was significantly lower in the KO cells than WT also supported the notion that reductive carboxylation was not prevalent in the KO cells (Figure 5.7). In addition, unpublished metabolomics data in our lab revealed elevated level of glutamine, 2-oxoglutarate and succinate in the HAP1 KO cells when compared to the WT, again suggesting a forward flux of the TCA cycle (Data not shown).

Interestingly, HeLa KD cells presented with significantly higher IDH3, coupled with lower level of IDH2 (Figure 5.1B), hence metabolising more isocitrate in the forward TCA flux, as IDH3 irreversibly oxidises isocitrate to 2-oxoglutarate while the reversible reaction pathway is mediated by IDH2. This suggests that CypD deficient cells favour the forward TCA flux, providing more electron supply to the ETC via NADH while also producing ATP.

Taking all these observations together (both in the previous and present chapter), it is evident that HAP1 KO cells have the following characteristics: elevated TCA forward flux, coupled with glutamine anaplerotic supply of 2-oxoglutarate and increased NADH and surprisingly presenting with reduced levels of both the inner mitochondrial ΔΨm / PMF and ATP (Figure 6.1). This suggests that depletion of CypD in the HAP1 and HeLa cells might have triggered mitochondrial ATP depletion via various mechanisms. One of such mechanism could be the uncoupling of oxidative phosphorylation, thus, state 3 respiration-induced TCA cycle activation (Satapati et al., 2012), or peradventure, the downregulation of ETC components due to metabolic reprogramming.
CypD deficiency positively modulates TCA cycle in the forward flux. OGDH was higher in the KO than WT. In addition, IDH3 which catalyses the irreversible conversion of isocitrate to 2-oxoglutarate was higher in both KD and KO than WT while IDH2 which catalyses the reversible reaction was not detected in HAP1 cells while observed to be lower in HeLa KD cells. Cytosolic IDH1, which catalyses the reversible conversion of isocitrate to 2-oxoglutarate was observed to be lower in KO cells while being higher in HeLa KD cells. Blue and Green arrows correspond to HAP1 KO and HeLa KD cells respectively.

Mitochondrial state 3 respiration is usually characterised by reduced $\Delta \Psi_m$ because of the flow of proton down its electrochemical gradient that occur during ADP phosphorylation at the ATP synthase. It could also be triggered by the presence of uncouplers such as FCCP. Mild inner membrane uncoupling is beneficial because it limits the production of superoxide in the mitochondria, as it reduces reverse electron transfer (RET), whereas elevated $\Delta \Psi_m$ due to increased electrochemical proton gradient, may be suggestive of reduced dissipation rate for either endogenous ATP synthesis or various PMF dependent mitochondrial activities. Reduced dissipation of the inner membrane PMF ironically is an enabling factor for ROS production either via RET-induced ROS production (Baffy, 2017) or elevated CoQH$_2$-induced one electron oxygen reduction at complex III (Murphy, 2009). Indeed, lower level of superoxide was observed in the KO mitochondria (Figures 4.9; 6.1). Notably elevated ATP level in WT when compared with KO suggest that the PMF...
dissipation in CypD deficient cells may be attributed to other endogenous inner membrane proteins that are separate from the ATP synthase (Figure 4.10).

After 24hr ATP synthase inhibition with oligomycin resulted in more death of the KO cells when compared to the WT cells (Figure 5.2). This suggests the importance of the mitochondrial TCA and ATP generation in the maintenance of survival in KO cells. This is because lower level of ATP in the KO cells might have already subjected them to more cellular stress than their WT counterpart; therefore, the total blockade of the ATP synthase with oligomycin resulted in extensive cell death in these KO cells. Moreover, lower ATP keeps the TCA cycle going in the forward flux, which corroborates the observation in the KO cells. The corollary is that ATP accumulation would halt the forward flow of the TCA by, for example, the inhibition of PDH and OGDH (Quinlan et al., 2014); this is not the case here as ATP level is low, and the TCA is in the forward flux, as also shown by reduced level of P-PDH (Figure 5.1C).

5.3.2 Linking CypD to the MPTP through its role in coupling TCA with oxidative phosphorylation

Do the results provide plausible explanations for the relationship of CypD with the MPTP? As described above, the dissipation of basal $\Delta\Psi_m$, and low superoxide and ATP levels in the CypD deficient cells suggest that CypD deficiency potentially leads to the uncoupling of oxidative phosphorylation from the TCA metabolism. Mild uncoupling of the inner mitochondrial membrane may prevent ROS-mediated MPTP formation by preventing the reverse electron transfer in the respiratory chain and oxidative burst in the mitochondria. More evidences to support this may be achieved by measuring the mitochondrial oxygen consumption, and superoxide production at complex III at CypD deficient conditions.

Interestingly, most experimental measurement of the MPTP activation are carried out under state 2 respiration, i.e. the supply of ETC substrate without the concomitant generation of ATP. This results in ROS production and
consequently, MPTP opening immediately after Ca\(^{2+}\) exposure (Briston et al., 2017). Notably, cancer cells positively correlate with CypD accumulation and the consequent ROS production is described in the previous chapters (Figures 3.8; 4.9). Interestingly, these cells also accumulate the mitochondrial chaperone HSP60 (Figure 3.8), which perhaps prevents ROS-mediated MPTP in cancer cells (Ghosh et al., 2010). Moreover, they may also have downregulated the structural components of the MPTP, as these still remain elusive.

### 5.3.3 CypD Modulates Cellular ROS in HAP1 Cells

Following the effect of CypD on mitochondrial ROS, the overall level of cellular ROS was examined in HAP1 cells. Interestingly, it was observed that CypD KD cells were characterised with significantly high level of ROS than the WT cells while KO cells had significantly low level of cellular ROS (Figure 5.4). The ROS level in the KO was consistent with the mitochondrial ROS that was observed (Figure 4.9), suggesting that this changes in ROS observed in the CypD deficient cells originates from the mitochondria.

The significantly reduced cellular ROS in KO cells may be attributed to reprogramming that results from the chronic depletion of CypD in HAP1 cells. It is, however, important to note that transient changes in ROS, i.e. from KD to KO may be cell specific and may involve the direct production of ROS from NAD(P)H oxidase, as the accumulation of NAD(P)H has been widely reported to be associated with CypD deficiency. The absence of this enzyme in other cell lines will result in the accumulation of NADPH, which is a reducing equivalent that counteract the effects of oxidative stress and its resulting cellular damage. The presence of NADPH oxidase in the mitochondria is widely debated (Zorov et al., 2014) and therefore, conclusions based on the enzyme being the source of mitochondrial ROS in CypD KD cells cannot be reached. The presence and the role of NADPH oxidase in the mitochondria of HAP1 cells would be further investigated.
5.3.4 AMPK is Activated in CypD Knock Downs

To verify that the level of cellular ATP in the cell was depleted even in the presence of enhanced TCA cycle, the level of phosphorylated AMPK (pAMPK) was probed in both HAP1 and HeLa cells. AMPK is a central player in metabolic reprogramming and undergoes a phosphorylation-dephosphorylation cycle governed by the AMP/ATP ratio. Cellular elevation of AMP consequent to ATP depletion results in the binding of AMP to the γ regulatory subunit of AMPK, thereby providing it with a conformation that is resistant to dephosphorylation and vice-versa (Mihaylova and Shaw, 2011; Oakhill et al., 2011; Xia et al., 2011). Generally, there is an inverse correlation between p-AMPK and ATP levels; high p-AMPK level implies low ATP. Indeed, in the HAP1 and HeLa KD cells, elevated levels of AMPK phosphorylated at Thr172 were observed (Figure 5.3), together with a high AMP/ATP ratio (data obtained from metabolomics studies by another member of the group). This shows that the CypD KD cells have lower energy levels.

The situation in the KO cells is more complicated. The chronic loss of CypD maintained lower ATP levels in both cellular and mitochondrial compartments than the WT (Figure 4.10). However, the level of phosphorylated AMPK was also lower in the KO than WT which contradicts the measured lower ATP level. Hence, there is a discrepancy between the ATP level and the phosphorylated AMPK level in the CypD KO cells. Recently, a novel regulation of AMPK which is independent of the cellular ATP status was reported. The glycolytic metabolite, fructose-1,6-bis-phosphate desensitises AMPK phosphorylation following binding to aldolase on the lysosomal membrane (Herzig and Shaw, 2018; Figure 5.14). Indeed, metabolomics data in our group observed elevation in glycolytic markers such as glucose-6-phosphate in the HAP1 KO cells.

Moreover, mitochondrial Ca^{2+} sequestration following CypD deficiency may also desensitise AMPK to activation by CAMKK2 whose AMPK mediated phosphorylation is dependent on the availability of cytosolic Ca^{2+}. Moreover, elevation of cellular ROS has been reported to activate AMPK which is
consistent with the level of ROS observed in both KD and KO cells. ROS can mediate the activation of pyruvate dehydrogenase kinases which prevents the metabolism of pyruvate, thus lowering the rate of oxidative phosphorylation (Hitosugi et al., 2011). In addition, ROS can also activate AMPK via ATM/LKB1 axis (Alexander et al., 2010). It is thus hypothesized that cells initially respond to the drop in ATP mediated by CypD KD by enhancing the phosphorylation of AMPK and subsequent physiological changes associated with CypD deficiency may modulate the AMPK status as CypD deficiency persists in the cell (as observed in the HAP1 KO), and these changes may vary between different cell types.

At this stage, therefore, it is safe to conclude that AMPK phosphorylation is modulated differently in the KD and KO cells. Whilst CypD deficiency in KD cells activated the AMPK following depletion of mitochondrial ATP, in the CypD KO cells, AMPK phosphorylation is complicated by other glycolytic events resulting from the adaptation to complete removal of CypD. For example, AMPK could enhance glycolysis via the upregulation of glucose transporter, Glut 4 (Habegger et al., 2012), which afterwards downregulates AMPK phosphorylation through the glycolytic intermediate, fructose 1, 6 bisphosphates. However, it is pertinent to confirm the elevation of both HDAC5 and Glut4 in both CypD KD and KO cells.

The observed lower level of ATP and elevated cell death following oligomycin treatment in CypD deficient cells when compared to WT cells does not suggest the inhibition of the ATP synthase, as it would result in a block in the respiratory chain due to proton accumulation in the intermembrane space and subsequent elevation of the $\Delta \Psi_m$ and inhibition of the TCA cycle (Zorova et al., 2018), neither of which is observed in the CypD deficient cells. Instead, this observation may depict either enhanced activities of the mitochondrial respiratory chain which is underlined by enhanced proton leak down its electrochemical gradient, mediated by unknown means, or downregulation of other ETC components that have not been investigated during this research,
hence, suppressing ATP synthesis. However, the mechanism of potential proton leakage needs to be further investigated.

5.3.5 CypD Deficiency Suppresses HIF-1α Activity in HAP1 and HeLa Cells

The activity and stabilisation of HIF-1α is negatively regulated by mitochondrial respiration, as the elevation of the TCA cycle intermediate, 2-oxoglutarate, Fe^{2+} and molecular oxygen rapidly subverts its activity when hydroxylated at Asn803 by factor inhibiting HIFs (FIH) and also its degradation via the ubiquitin proteasome system when hydroxylated at Pro402 or Pro564 by prolyl hydroxylases (PHDs). Moreover, mitochondrial ROS and elevated ΔΨ_m / proton motive force has been associated with the normoxic stabilisation of HIF-1α (Chandell et al., 2000; Martinez-Reyes et al., 2016; Iommarini et al., 2017).

Following the inhibition of proteasomal degradation by 4hrs treatment with MG132, a potent proteasome inhibitor, of both WT and CypD deficient (KD and KO) cells, the level of HIF-1α was upregulated in both KD and KO HeLa and HAP1 cells (Figure 5.5), suggesting elevated degradation through the proteasome during CypD deficiency when the inhibitor is absent. Notably, HAP1 cells that were not treated with MG132 were characterised with enhanced degradation of HIF-1α in KO cells while the KD possessed elevated HIF-1α when compared with WT (Figures 5.5). Indeed, elevated ROS has been reported to stabilise normoxic HIF-1α (Metzen et al., 2003; Jung et al., 2008) and this is consistent with the significantly higher and lower cellular ROS in the HAP1 KD and KO cells respectively when compared with the WT (Figure 5.4).

The Luciferase reporter assay was used to further assess the difference in activity of HIF-1α between WT and KD HeLa cells. In here, cells carrying construct containing HIF response element (HRE) upstream luciferase gene were cultured under normoxic and hypoxic conditions. Interestingly, luciferase activity was significantly higher during hypoxia for both WT and KD cells than
the normoxic group (Figure 5.6). However, during normoxia, luciferase activity was lower in KD cells than WT suggesting reduced HIF-1α activity in KD cells. Since it has been shown that proteasomal inhibition accumulates HIF-1α in CypD deficient cells, therefore, it is plausible that the abundance of Prolyl hydroxylases co-substrates in CypD deficient cells may contribute to HIF-1α degradation. Moreover, CypD may play some yet unidentified role in alternative pathways independent of the proteasome dependent degradation pathway of HIF-1α.

Indeed, the contributions by excessive mitochondrial 2-oxoglutarate (depicted by our metabolomics data and also mirrored by elevated OGDH) coupled with lower level of ROS may have contributed to elevated HIF-1α degradation in the CypD deficient cells.

Furthermore, the level of HIF-1α target PHD2, was observed to be higher and lower in CypD KD and KO HAP1 cells respectively (Figure 5.5A). Notably, hypoxic CA9 was significantly lower in CypD KD HeLa cells (Figure 5.6C). Indeed, the level of cellular protein is tightly regulated by the balance between protein synthesis and degradation, therefore the significant effect on the HIF targets observed in the KO are most likely associated with continuous inactivation and degradation of HIF-1α during mitochondrial metabolism.

In addition, gene expression using QPCR revealed that HIF-1α target genes such as PHD2 and PDHK1 were lower in KO than WT (Figure 5.5C). Decreased expression of these HIF-1α target genes further indicates the reduced activity of HIF-1 in KO cells which is largely attributed to the reductive environment and elevated TCA cycle intermediate (2-oxoglutarate) that is quite abundant in the KO cells. Overall, the stabilisation and transcriptional activity of HIF-1α has been shown in this study to be modulated by mitochondrial CypD and its detailed mechanisms should be further elucidated.
Phosphorylation of AMPK at Thr172 within the activation loop of its alpha subunit is catalysed either liver kinase B1 (LKB1) or calcium/calmodulin dependent protein kinase kinase 2 (CAMKK2) via modulation by AMP or Ca$^{2+}$ respectively. Reduced glycolysis results in low availability of the glycolytic intermediate, fructose 1, 6 bisphosphate (FBP) and this facilitates the interaction between the glycolytic enzyme aldolase, vacuolar ATPase (V-ATPase) and axin, thereby leading to the activation of LKB1 on the surface of the lysosome and activating lysosome bound AMPK.

ROS increase can also mediate the phosphorylation and activation of AMPK via ATM/LKB1 axis. LKB1 is phosphorylated by ATM at Thr366.

Binding of AMP to the γ subunit of AMPK changes its conformation, thus, enabling phosphorylation by LKB1. Decrease in intracellular ATP and increase in both ADP and AMP prevents the dephosphorylation of AMPK at Thr172. Broken arrows represent unknown mechanisms. Image was adapted from Steinberg and Carling, 2019.

**Figure 5.14: Regulation of AMPK by ATP and Glycolysis.** Phosphorylation of AMPK at Thr172 within the activation loop of its alpha subunit is catalysed either liver kinase B1 (LKB1) or calcium/calmodulin dependent protein kinase kinase 2 (CAMKK2) via modulation by AMP or Ca$^{2+}$ respectively. Reduced glycolysis results in low availability of the glycolytic intermediate, fructose 1, 6 bisphosphate (FBP) and this facilitates the interaction between the glycolytic enzyme aldolase, vacuolar ATPase (V-ATPase) and axin, thereby leading to the activation of LKB1 on the surface of the lysosome and activating lysosome bound AMPK. ROS increase can also mediate the phosphorylation and activation of AMPK via ATM/LKB1 axis. LKB1 is phosphorylated by ATM at Thr366. Binding of AMP to the γ subunit of AMPK changes its conformation, thus, enabling phosphorylation by LKB1. Decrease in intracellular ATP and increase in both ADP and AMP prevents the dephosphorylation of AMPK at Thr172. Broken arrows represent unknown mechanisms. Image was adapted from Steinberg and Carling, 2019.

### 5.3.6 CypD is Required for Lipid Droplet Formation in HAP1 and HeLa Cells

Glutaminolysis is the hallmark of rapidly proliferating cells which is often characterised with reductive carboxylation, hence lipid biosynthesis (Yang et al., 2017). Keeping in mind that both HAP1 and HeLa cells are rapidly proliferating cells, therefore the size of cellular lipid droplets in these cell lines was used to distinguish the rate of reductive carboxylation between CypD sufficient and deficient cells. Indeed, deficient HAP and HeLa cells showed significantly lower level of lipids and droplet sizes when compared with their...
WT counterpart (Figure 5.7). This result may suggest that CypD deficient HAP1 and HeLa cells prioritised lipid metabolism, hence, mitochondrial respiration over lipid biosynthesis. Interestingly, HAP1 cells showed undetectable NADP\(^+\) dependent IDH2 by western blot. Moreover, HAP1 KO cells were also characterised with reduced level of cytosolic NADP\(^+\) dependent IDH1 (Figure 5.1). These enzymes are responsible for both mitochondrial and cytosolic reductive carboxylation respectively (Du et al., 2016). Therefore, WT cells, due to reduced respiratory demand exemplified by elevated mitochondrial O\(_2\)\(^•−\) and ΔΨ\(_m\) / proton motive force, may export 2-oxoglutarate derived from mitochondrial glutaminolysis from the mitochondria via the 2-oxoglutarate carrier for cytosolic reductive carboxylation to citrate by IDH1, hence, lipid synthesis and elevation of lipid droplets. Indeed, the level of IDH1 was significantly higher in the WT HAP1 cells (Figure 5.1) suggesting the possible need for reductive carboxylation.

Another plausibility for lipid droplets formation is via the HIF-1α axis. HIF-1α has been reported to contribute to the formation of lipid droplets by repressing the expression of mitochondrial carnitine palmitoyl transferase 1A (CPT1A), which is a protein that facilitates the transport of fatty acid into the mitochondria prior to β oxidation (Du et al., 2017; Mylonis et al., 2019). Therefore, lower level of lipid droplets observed in KD Hela and KO HAP1 cells also suggested reduced activity of HIF-1α, as confirmed by reduced normoxic protein level of HIF-1α in HAP1 KD and KO cells (Figure 5.5). HIF-1α represses mitochondrial pyruvate and fatty acid metabolism, therefore, HAP KO and HeLa KD cells are characterised with elevated level of mitochondrial metabolism (Figure 5.1). This suggests that the increased HIF-1α activity in the WT resulted in reduced mitochondrial metabolism, hence the accumulation of neutral lipids for droplet formation. Moreover, the elevated level of ATP in the WT, as discussed in chapter 4 (Figure 4.10, Table 4.1) will assist the carboxylation of acetyl-CoA to malonyl-CoA. More importantly, the protein level of CPT1A should be investigated to verify the level of mitochondrial fatty acid importation between WT and deficient cells.
It is also important to note that lipid droplets are regulated by the ROS/AMPK/mTORC1 axis, as elevated activities of mTORC1 results in the inhibition of autophagy, thereby preventing the turnover of lipids for droplets formation (Madeira et al., 2015; Yang et al., 2020). Data obtained in this project revealed the elevation of mTORC1 activities in HAP1 KO cells with corresponding stabilisation of the autophagic LC3b-I protein (Figure 5.9; 5.12), suggesting that the inhibition of autophagy in this cell are mTORC1 dependent. Interestingly, KO cells were also characterised with significantly reduced level of cellular ROS and p-AMPK. Contrarywise, KD cells were characterised with elevated level of ROS, p-AMPK and the subsequent activation of autophagy, marked by reduced LC3b-I. Hence, droplets sizes were higher (although not significant) than their WT counterpart. these further validates the inhibition of autophagy via the ROS/AMPK/mTORC1 axis in KO cells, hence, reduced formation of lipid droplets. Likewise, the mTORC1 pathway in HeLa KD cells resulted in their reduced droplet sizes when compared to their WT.

5.3.7 CypD Deficiency Differentially Regulates PI3K/AKT and MEK/ERK Pathways in Different Cell Lines

Phosphorylation of AKT is tightly regulated by the activities of the intracellular kinases and phosphatases. Upstream activating kinases are PIP3-dependent kinase (PDK1) and the mTORC2. Indeed, these are further regulated upstream by receptor tyrosine kinases and G protein coupled receptor associated kinases. Regulating phosphatases include tyrosine phosphatase such as phosphatase and tensin homolog deleted on chromosome ten (PTEN) and protein phosphatase 2 (PP2A) to mention a few (Carracedo and Pandolfi, 2008).

Phosphorylated AKT at Thr308 was observed to increase with decreasing level of CypD in HAP1 cells when assessed in WT, KD and KO indicating direct effect of CypD at this phosphorylation site. However, phosphorylation on Ser473 was slightly lower in both KD and KO than the WT HAP1 cells (Figure 5.8A). PDK1 is responsible for Thr308 phosphorylation while the mTORC2 is
responsible for phosphorylation at Ser473. More importantly, phosphorylation of both sites on AKT results in the fully active protein, although Ser473 phosphorylation is dispensable for mTORC1 regulation (Vadlakonda et al., 2013). Activated AKT regulates a plethora of downstream pathways such as enhancing Glut4 translocation to the plasma membrane (Sano et al., 2003), inhibition of phosphoenolpyruvate carboxykinase (Burgering, 2008); these enhances glucose metabolism in the forward direction. Interestingly, in our metabolomics data, we also observed enhanced intracellular glucose in CypD KO HAP1 cells.

Although, phosphorylated AKT at Thr308 was not detected in both WT and KD HeLa cells, p-GSK 3β at Ser9 which is downstream the PI3K/AKT pathway, was detected to be upregulated in CypD KD cells (Figure 5.8D). It is unclear why phosphorylation at Thr308 was not detected in the cell line, perhaps longer exposure time during western blot image acquisition may help solve the problem in future experiments. Notably, p-GSK 3β was downregulated in HT1080 cells (Figure 5.8E). These results show that the modulation of mitochondrial CypD differentially regulates different cells depending on the metabolic background of the cell. However, it is suspected that the differential regulation of these pathways by mitochondrial CypD is dependent on either the deregulation of their receptor protein or the second messenger associated with these receptors.

The TCA cycle metabolite, succinate, can regulate G-protein coupled receptors / AKT phosphorylation at Thr308 via the succinate receptor, GPR91 (He et al., 2004; Robben et al., 2009; Vargas et al., 2009; Aguiar et al., 2010; Aguiar et al., 2014). GPCR can activate ras protein which in turn activates PI3K, converting Phosphoinositide 4, 5 bisphosphates to phosphoinositide 3,4,5 trisphosphate, thereby activating PDK/AKT. Also, GPCR paired with Gαs as their intracellular second messenger have been reported to inhibit the AKT via PKA activation (Lou, et al., 2002; Jewell et al., 2019). This type of GPCR/Gαs pairing have also been reported to be abundant in U2OS cells (Jewell et al., 2019); interestingly, inactivation of mTORC1 in CypD deficient
U2OS cells has been observed in our lab (unpublished data). It is plausible that this regulatory pattern may also exist in the HT1080 cells, but it requires further validation.

CypD KO was also observed to modulate the level of ERK (Figure 5.8B). Indeed, phosphorylated ERK1/2 at both Thr202/Tyr204 are important activators of the mTORC1 which has been reported in here to characterise the KO cells. Although the level of this protein was not validated in HeLa cells and CypD KD cells, however, the ERK pathway plays significant role in other downstream signalling pathways that may have redefined the metabolic profile of the HAP KO cells. These evidences reiterate the role of CypD deficiency in the regulation of the PI3K/AKT pathway and modulation of these pathways by the mitochondria is subject to the physiological background of the cell.

### 5.3.8 CypD Deficiency Activates mTORC1 Pathway in HAP1 and HeLa Cells

Following the activation of AKT and ERK pathway in chronic CypD deficient HAP1 cells (i.e. in KO cells), it was important to see if these would translate into changes in the mTORC1 pathway, as it is the central hub of cellular metabolism, where many pathways converge and can therefore provide great insight into the metabolic state of the cell (Valvezan and Manning, 2019). Interestingly, CypD deficiency in HAP1 KO revealed increase in the mTORC1 pathway. This was evidenced by the elevation of phosphorylated Thr389 of p70S6K and Ser235/236 of ribosomal S6 protein (Figure 5.9A) which are established downstream effectors of the mTORC1 pathway (Berven and Crouch, 2000; Iijima et al., 2002; Iwenofu et al., 2008; Sardiña et al., 2019). Indeed, this is also consistent with downregulated AMPK which is an established mTORC1 antagonist (Laplante and Sabatini, 2013). Since mTORC1 activity promotes cell growth, its upregulation in KO cells was also visible, as KO cells formed larger cell colonies in clonogenic assays when compared to their WT counterpart (Figure 5.2).
Furthermore, to confirm the effect of CypD KD on the mTORC1, two cell types were examined; HeLa and HT1080 cells. Surprisingly, two different effects were observed in the two cell types. CypD KD in HeLa cells resulted in the upregulation of the mTOR pathway (Figure 5.9B), whereas in HT1080 cells, mTORC1 activity was downregulated (Figure 5.9C). Since the mTORC1 is usually downstream AKT signalling, different effects on the AKT may arise from the KD of CypD in different cell lines. Indeed, the upregulation of mTORC1 has previously been reported in CypD deficient cells (Klawitter et al., 2017b), however, the inhibition of mTORC1 in HT1080 cells observed in this result is quite novel. Besides, it has been previously reported that multiple pathways such as AKT, ERK and other still elusive mechanisms converge at mTORC1 (Asati et al., 2016).

Moreover, mTORC1 inhibition in HT1080 cells may also be mediated via 2-hydroxyglutarate, as this cell possessed mutations in IDH1 (R132H) which confers the enzymes with neomorphic function of converting 2-oxoglutarate to the oncometabolite, 2-hydroxyglutarate. This oncometabolite has been recently implicated in the inhibition of the mTORC1 either directly or indirectly (Fu et al., 2015; Han et al., 2019). Elevated glutaminolysis accompanied by CypD KD is hypothesized in this study, and this can favour the elevation of 2-hydroxyglutarate, HIF-1α stabilisation and dephosphorylation of AMPK via fructose 1,6 bisphosphate (Zhang et al., 2017) in HT1080 cells. Indeed, dephosphorylation of AMPK was observed in the KD (data not shown), which ideally should favour mTORC1 activation, however, mTORC1 activity was repressed in the CypD KD HT1080 cells (Figure 5.9C). mTORC1 repression in the KD of HT1080 cells correlates with the reduced activation of the AKT pathway in this CypD phenotype (Figure 5.9D).

Of note, HeLa KD cells were also characterised with elevated p-AMPK than their WT, and this is inconsistent with the negative regulation of mTORC1 by AMPK (Hindupur et al., 2015). This indicates that the mTORC1 inhibition observed in HT1080 cells and activation observed in both HAP1 and HeLa
cells are regulated via different mechanisms. It is therefore important to verify which pathway exactly controls the regulation of mTORC1 in these cell lines.

Previous reports have shown that the perturbation of mitochondrial $\Delta \Psi_m$ can affect the mTORC1 pathway (Schieke et al., 2006). This phenomenon is hypothesized to likely occur via the alteration and re-wiring of metabolism that results to increased demand for energy production which leads to enhanced amino acid metabolism. As it is known, essential and some non-essential amino acids drive mTORC1-lysosomal colocalization (Dalle Pezze et al., 2016).

To further confirm the role of mitochondrial metabolism in the activation of mTORC1 in HeLa cells, immunofluorescence was used to check for lysosomal-mTOR co-localisation, as mTORC1 must be localised on the lysosome for activation by RHEB (Ras homologue enriched in brain; Jewell et al., 2013). CypD KD cells were characterised with the elevation of mTOR aggregates which were colocalised with the lysosome (Figure 5.10). Of note, amino acids such as glutamine, alanine and leucine among a few are crucial for mTORC1-lysosomal conjugation by inhibiting sestrin2 (Wolfson and Sabatini, 2017). This suggests the importance of amino acid influx, which may have resulted from the need for elevated TCA cycle in CypD deficient HeLa cells. Unfortunately, attempt to check for LAMP2-mTOR colocalization in HAP1 and HT1080 cells were unsuccessful. However, they remain top priority in future experiments.

5.3.9 CypD Maintains Survival via Enhanced Autophagy and mTORC2 Stabilisation in HAP1 Cells

Autophagy is a cell rescue mechanism whereby damaged / compromised organelles and macromolecules are quickly degraded and recycled via the autophagosome. However, it must be controlled, as deficient or excessive autophagic process may pose debilitating effect on the wellbeing of the cell (Madeira et al., 2015).
It was observed that different forms of death stimuli such as treatment with H$_2$O$_2$ increased cell death significantly in these cell types (Figure 4.13). This is because of the low level of autophagic process in the KO, which directly stems from their increased mTORC1 activation (Figure 5.9; 5.10). Indeed, mTORC1 prevents the activation of autophagy which is required for survival when cells are subjected to environmental stressors that compromises their macromolecular or organellar content. Indeed, CypD KO HAP1 cells were observed to be frail when compared with their WT counterpart. In this light, it is hypothesized that HAP KD cells would respond better to stress stimuli like H$_2$O$_2$ than WT because of their significantly increased autophagic activities (Figure 5.12).

mTORC2 is reported to regulate cell survival and proliferation through the phosphorylation of AKT, SGK1, PKC and FOXO (Tato et al., 2011). Phosphorylation of AKT at Ser473 by mTORC2, otherwise known as PDK2 was not different between WT and CypD deficient cells of HAP1 cells when assessed. However, an unexpected downregulation in CypD deficient HAP1 was observed when cells were subjected to hypoxia (1% O$_2$) for 24 hrs (Figure 5.8A).

Little is known about the regulation of mTORC2 as opposed to the much larger pile of information surrounding mTORC1. Perhaps, the only known regulation of mTORC2 is tightly associated with PI3K. Notably, mSIN1, one of the protein members of mTORC2 possesses a PH domain that binds plasma membrane immediately after the formation of PIP3 and positions it for AKT phosphorylation (Gan et al., 2011). To verify that the reduction of mTORC2 activity in CypD deficient cells was in relation to the plasma membrane PIP3 status, another pathway associated with PIP3, phosphorylated AKT at Thr308, was determined during hypoxia in both HAP and HeLa cells. As reported earlier (Figure 5.8A, D), CypD deficiency, resulted in drastic reduction of p-AKT during hypoxia in both HAP1 and HeLa cells, suggesting that cellular stress may have negative effect on PIP3 in CypD deficient cells. Indeed, stress induced ROS production has been shown to stabilise the AKT pathway via
downregulation of PTEN (Koundouros and Poulakiannis, 2018). Therefore, the reductive environment generated during CypD deficiency may destabilise the AKT pathway during cellular stress. However, further work is required on the effect of CypD on mTORC2 stabilisation.

5.3.10 CypD Deficiency Differentially Regulates NF-κB in HAP1 and HeLa Cells

NF-κB is a stress activated pathway that regulates different pathways involved in adaptation to survival especially in cancer cells. Hap1 KO cells were characterised with the activation of the NF-κB pathway, as revealed by higher level of P-IκB-α at Ser32/36 and reduced level of the unphosphorylated / total form of the protein (Figure 5.1). Phosphorylation of IκB-α has been reported to precede its ubiquitination and proteasomal degradation (Peng et al., 2010; Yao et al., 2018). Hence, the dimerization (i.e. p65/p50) and nuclear translocation of the NF-κB pathway.

Apart from the known activating ligands that regulates NF-κB (TNF-α, lipopolysaccharide, or IL-1 mediated pathway), phosphorylation and activation of the NF-κB pathway can also be mediated MEK/ERK pathway (Peng et al., 2010). Indeed, elevation of the ERK pathway observed in the KO cells is sufficient for NF-κB activation (Figure 5.8C). It is quite important to also verify other players involved in NF-κB activation the KO cells.

More importantly, NF-κB activation should also be verified in the HAP1 KD cells, as it was difficult carrying out an siRNA KD on the HAP1 cells at the time of the experiment. Studies in HAP1 KD will fully delineate the events that precede the overall metabolic reprogramming observed in the HAP1 KO cells. However, it is plausible to assume that the NF-κB pathway was elevated in the KD because of the high ROS that was observed in KD cells (Figure 5.4). This is because ROS is an important activator of the NF-κB pathway (Siomek, 2012).
CypD KD in HeLa cells was unsurprisingly characterised with reduced NF-κB activation, as depicted with elevation in cellular IκB-α (Figure 5.2). Notably, there was no significant difference in the level of phosphorylated IκB-α. Although cellular ROS was not measured in HeLa cells, however, mitochondrial ROS can be used as a readout for the ROS in the cell, since the mitochondria is a major supplier of cellular ROS (Murphy, 2009). Notably, mitochondria ROS in KD HeLa cells were significantly lower than the WT, and this result corresponds to the stabilisation of IκB-α observed in the KD cells.

5.4 Conclusion
The metabolic reprogramming associated with CypD deficiency highlighted the role of CypD not only in mitochondrial health but also in the general cellular wellbeing. This chapter emphasized the effects of CypD on the mitochondrial TCA cycle and monitored the accompanying cellular effects which reflect on the state of the TCA. In the CypD-deficient HAP1 and Hela cells, elevated level of TCA cycle key enzymes such as OGDH and IDH3 were respectively observed, while also considering the phosphorylation status of PDHE1α in HAP1 cells. Also, normoxic HIF-1α activity, known for inhibiting the flux through the TCA was downregulated in CypD deficient cells due to decreased ROS and increased 2-oxoglutarate, except in the HAP KD which was characterised with elevated ROS. Physiological evidence of this was observed by the lower level of lipid droplets in CypD KO and KD HAP1 and HeLa cells respectively. These data together with undiscussed data obtained from our lab suggest that when CypD is present, it acts by reducing the forward flux of the TCA, especially when the carbon is sourced from glucose metabolism.

Interestingly, decreased mitochondrial ATP coupled with differential level of mitochondrial and cellular ROS resulted in the differential expression of p-AMPK in both KD and KO cells suggesting that metabolic reprogramming might have occurred via ROS/AMPK signalling, and that elevation of these signalling molecules in the HAP KD might have initiated the changes in overall metabolism and signalling. Because these signals are transient, response by
elevated glucose metabolism immediately suppresses both AMPK and ROS via the glycolytic intermediate. However, effects from upregulated glycolysis, coupled with reduced mitochondrial ATP as observed in the previous chapter, enhances the mitochondrial TCA cycle anaplerotic supply. This results in the further activation of other pathways such as NF-κB and mTORC1, thereby overwhelming the cell and inducing possibly stress responses, hence, making the cells susceptible to stress induced death. Enhanced TCA cycle requires further verification which can be carried out using $^{13}$C isotopic labelling.

Novel findings in this chapter are the dissipation of lipid droplets, repression of HIF-1α activity, the varying activation of NF-κB associated with CypD deficiency in both HAP and Hela cells despite the elevation of the mTORC1.
Chapter Six

Overall Discussion and Conclusion
6 Overall Discussion and Conclusion

The strength of this thesis lies in being able to perform many of the cellular experiments using CypD KD as well as KO cells. The dynamic phosphorylation status of AMPK and the level of cellular ROS – being higher than WT in the transiently knocked down HAP1 cells, and lower in the HAP1 KO cells - provides an explanation for many of the observations made here and is summarised in Figures 6.1 and 6.2. It was hypothesized that AMPK in synergy with ROS upregulates glycolysis as well as autophagic macromolecular turnover in addition to modulating wide range of different signalling pathways. However, with persistent insult in the mitochondria due to CypD deficiency as observed in the KO, other stress activated pathways that promotes the overall cellular survival instincts were turned on.

Figure 6.1: Speculative Effect of CypD on Mitochondrial Metabolism. Based on the results, it was deduced that CypD has a repressing effect on three key mitochondrial enzymes; Nicotinamide nucleotide transhydrogenase (NNT), pyruvate dehydrogenase complex (PDH) and glutaminase which is involved in glutamine metabolism. These results in reduced pyruvate metabolism and the accumulation of lactate and lipid droplets, while subsequently reducing the level of some TCA cycle metabolites such as 2-oxoglutarate and succinate. However, upon stimulation of lipolysis, the resulting elevated membrane potential coupled with reduced NADPH results in increased ROS and ATP production in the mitochondria. Abbreviated molecules are represented in the list of abbreviations.
Cytochrome c (CypD) ablation results in the accumulation of mitochondrial NAD(P)H, and decreased ROS production, $\Delta \psi_m /$ PMF and ATP synthesis. NAD(P)H accumulation are products of enhanced mitochondrial TCA which is implied by the accumulation of OGDH (KO) and IDH3 (KO and KD). Mitochondrial NADH export results in global lysine acetylation via the inactivation of NAD$^+$ dependent deacetylases. Downregulated ROS together with elevated 2-oxoglutarate enhanced the degradation of HIF-1$\alpha$ which also results in the depression of lipid droplets. TCA anaplerotic intermediates such as glutamine and succinate results in the activation of AKT and mTORC1 pathway, however, this is thought to be cell specific, as succinate can also trigger the activation of inhibitory receptors of the AKT pathway. Furthermore, decreased ATP results in the transient activation of AMPK which is hypothesized to initiate metabolic reprogramming. Red and blue arrows represent changes in CypD KO and KD respectively.

Effects of these resulted in increased PI3K/AKT, mTORC1, NF-$\kappa$B pathways and elevated cellular NADPH levels, while inactivating and degrading HIF-1$\alpha$ as a result of reduced mitochondrial ROS and elevated glutamine derived TCA cycle 2-oxoglutarate. The drastic changes in signalling pattern observed here which are associated with chronic CypD deficiency undoubtedly confirms CypD as being at the hub of mitochondrial metabolism, controlling the activities of very important mitochondrial functions.

In addition to the adaptive OGDH upregulation and reduced HIF-1$\alpha$ activity, the dependence of HAP1 KO cells on mitochondrial metabolism was reflected by elevated TCA cycle and increased cell death following ATP synthase inhibition as revealed by clonogenic assay while the reduced level of neutral

Figure 6.2: Overall Summary of CypD Deficiency. CypD ablation results in the accumulation of mitochondrial NAD(P)H, and decreased ROS production, $\Delta \psi_m /$ PMF and ATP synthesis. NAD(P)H accumulation are products of enhanced mitochondrial TCA which is implied by the accumulation of OGDH (KO) and IDH3 (KO and KD). Mitochondrial NADH export results in global lysine acetylation via the inactivation of NAD$^+$ dependent deacetylases. Downregulated ROS together with elevated 2-oxoglutarate enhanced the degradation of HIF-1$\alpha$ which also results in the depression of lipid droplets. TCA anaplerotic intermediates such as glutamine and succinate results in the activation of AKT and mTORC1 pathway, however, this is thought to be cell specific, as succinate can also trigger the activation of inhibitory receptors of the AKT pathway. Furthermore, decreased ATP results in the transient activation of AMPK which is hypothesized to initiate metabolic reprogramming. Red and blue arrows represent changes in CypD KO and KD respectively.
lipids in droplets were consequences of elevated mTORC1, reduced HIF-1α, and the downregulation of autophagy (autophagy is inversely related to mTORC1). Indeed, parallel metabolomics data obtained from our lab throws more weight on enhanced mitochondrial metabolism in the KO.

The extramitochondrial effect of CypD has revealed that TCA cycle anaplerosis is rapidly elevated following CypD deficiency. In this light, reduced mitochondrial ATP increasingly supported the possible uncoupling of oxidative phosphorylation in the inner mitochondrial membrane, or the downregulation of yet unidentified ETC components. Paradoxically, hyperpolarisation of the inner mitochondrial membrane is one of predisposing factors to the MPTP activation. However, mild uncoupling of oxidative phosphorylation in the inner mitochondrial membrane is reported to prevent mitochondrial ROS formation via preventing both reverse electron transfer and CoQH₂ accumulation. Moreover, mild uncoupling also results in decreased matrix pH, which is a strong inhibitor of the MPTP.

Interestingly, this study found reduced level of both mitochondrial O₂⁻⁻ and PMF (ΔΨₘ) in CypD deficient HeLa and HAP1 KO experimental models. It is also not surprising that it was discovered that cancer cells accumulate more CypD than non-transformed cells, and that excessive ROS production in these cells drives and aids their neoplastic agenda. Since HIF-1α and ROS are associated with lipid droplet formation, it is indeed interesting that CypD KO HAP1 and KD HeLa cells resulted in significantly lower lipid droplets. Although the cause of elevated ROS in HAP1 KD cells is still unknown. However, this novel finding may have technically placed CypD on the target list of proteins to consider when designing anti-cancer drugs.

Of note, the activation of the PI3K/AKT and mTORC1 in HAP1 and HeLa cells during CypD deficiency may not bring pleasant news to being potentially therapeutic against cancer. However, it should also be considered that stress factors such as hypoxia rapidly downregulated the PI3K/AKT pathway. Interestingly, since the core cells of tumour tissues are hypoxic, therefore, the
repression of CypD in these cells may have a huge positive impact on drug-induced tumour resection. However, effects on multiple cancer cell lines should be tested, moreover, the effect of CypD depletion on cancer specific pathways such as NF-κB should also be tested in hypoxia.

This study has also demonstrated that CypD may contribute to mitochondrial oxidative stress by depleting mitochondrial NADPH via its ability to form stable sulfenic acid on its outer Cys203. This electrophilic specie may spontaneously form intermolecular disulphide bond with susceptible neighbouring proteins. This is a potential NADPH distractor, as the two main antioxidant systems, glutathione and thioredoxin are channelled into reducing the disulphide bond, the formation of sulfenic acid reoccurs, hence, a vicious cycle. This bivalent effect of CypD on ROS production and scavenging might have been the mechanism for ROS-induced MPTP activation. Indeed, CypD inhibition prevents ischemia reperfusion injury. It is plausible to assume that the stabilisation NADPH observed during CypD deficiency may have prevented the ROS-induced MPTP opening that occurs during ischemia-reperfusion.

Previous reports have shown that CsA, an inhibitor of CypD can independently induce ROS production in isolated mitochondria. Interestingly, it was observed in this study that CsA reduced the level of mitochondrial NAD(P)H in succinate stimulated mitochondria in HAP1 cells. Since ROS dissipates the NADPH system, therefore the changes in the WST-1 detected reductive equivalent is most likely NADPH and not NADH. In this light, the elevation of the NAD(P) reductive equivalent observed in the KO is most likely NADPH. Since the HAP1 cells are deficient in NADP-dependent IDH2 when compared with other cell counterparts such as HeLa and HT1080 cells (Table 5.1), it is safe to assume that the NADPH is mainly generated from the nicotinamide nucleotide transhydrogenase (NNT). Taken together, CypD deficiency may have enhanced the activity of NNT in the direction of NADPH production and thus, dissipate the inner mitochondrial ΔΨm, implying that CypD represses the activity of NNT; this is a hypothesis that would be worth testing in future work.
Other mechanisms of uncoupling remain elusive. However, it is possible that CypD help regulate the activities of other proteins that may be directly or indirectly involved in the inner mitochondrial uncoupling, such as ANT, UCP, NNT and others. Apart from ANT, the role of other mitochondrial uncoupler proteins on the MPTP has not been explored. Different studies have suggested a CypD-ANT interaction, albeit its role in the activation of the MPTP has been debunked because of the persistence of the MPTP in the absence of ANT. However, more explicit work is required to understand the effect of CypD on the function of the other potential uncouplers, and coupling and functioning of oxidative phosphorylation, as this may also be pertinent to understanding the role of CypD in MPTP activation.

In addition, the abundance other metabolites capable of interfering and suppressing the mitochondrial membrane potential such as intra-mitochondrial succinyl-CoA should be investigated as these are capable of preventing ROS or Ca²⁺ induced activation of the MPTP.
Chapter Seven

Future Work
7 Future work

The role of CypD in mitochondrial inner membrane uncoupling

Canonical regulation of the MPTP has been shown over the years to be mediated by ANT which incidentally accounts for over 50% percent of proton leak across the inner mitochondrial membrane. Indeed, its two inhibitors have been reported to show varying effects on the MPTP activation. Carboxyatractyloside, that locks ANT in the cytosolic form - 'c' conformation, is an activator of the MPTP while Bongkrekic acid which locks ANT to the matrix - 'm' conformation is an inhibitor of the MPTP (Bernardi, 2013). It is however, not known if these two conformational changes of the ANT affect the proton motive force of the inner mitochondrial membrane. In this experiment, we will be investigating the effect ANT inhibition alongside other known proteins that mediates mitochondrial uncoupling such as NNT and UCPs on the status of the inner mitochondrial membrane and their possible dependence on CypD.

Superoxide produced at complex 3 inhibition

Complex III is an important focal point of ROS production in the mitochondria. However, much is not known about its modulation physiologically. Often, MPTP studies in isolated mitochondria are carried out with the blockade of complex I and its associated ROS with rotenone, but ROS production at complex III is completely ignored in these experiments. It is therefore important to investigate the production of ROS at complex III during mitochondrial Ca\(^{2+}\) overload in order to delineate the exact specificity of the MPTP - Is Ca\(^{2+}\) inducing ROS production for MPTP activation? Since we already have a working model of mitochondrial Ca\(^{2+}\) sequestration in cells - thapsigargin-induced mitochondrial Ca\(^{2+}\) overload, with NADH as readout for increased Ca\(^{2+}\)-induced dehydrogenase activities in the mitochondria, then mitochondrial \(O_2^{\cdot-}\) and \(\Delta\Psi_m\) can be measured in CypD WT and KO cells grown in the presence of succinate and complex I inhibition, as this would depict ROS production mostly at complex III. Understanding the role of CypD on complex III ROS will further increase the current knowledge on the MPTP.
The role of CypD on succinate induced GPCR signalling

The role of succinate as an extracellular cytokine is being increasingly studied in different cell systems. Metabolomics studies by our group observed elevated level of succinate in CypD KO cells. It is therefore pertinent to study the role of CypD succinate signalling, as this might be another important mechanism of metabolic reprogramming observed in the KO cells. The succinate receptor GPR91 will be downregulated in both CypD WT and KO and downstream pathways such as AKT, JAK/STAT, NF-κB and ERK will be investigated.

The role of CypD in AMPK regulation

Despite the reduced level of ATP observed in both CypD KD and KO HAP and HeLa cells, only KD cells showed elevation of pAMPK while KO depicted reduced level of pAMPK. Since elevated glycolysis has been shown to dephosphorylate AMPK via fructose 1, 6 bisphosphate-aldolase interaction, independently of the ATP/AMP status, it is important to verify the dynamics of CypD regulated glycolysis by observing the level of aldolase, CAMKK2, HDAC5 de-nuclearization and Glut 4 expression in CypD WT, KD and KO cells. This would further verify the energy requirement of CypD deficient cells.

mTOR-LAMP2 co-localisation in HAP and HT1080 cells

Amino acids and upstream kinases such as AKT and ERK work synergistically to activate mTORC1. This is because, active mTORC1 only work when situated on the lysosome and amino acids activates their sequestration to the lysosome. Therefore, the absence of amino acids even when upstream activators are present will result in the repression of mTORC1. It is indeed important to fully understand the role of CypD in modulating mTORC1 co-localisation to the lysosome via either amino acid metabolism or lysosomal biogenesis. Indeed, we observed enhanced mTORC1-lysosomal colocalization in HeLa KD cells, however, this ought to be verified in other cell lines as well, especially, HAP1 and HT1080 where varying activities of mTORC1 was observed. Further questions will be to know if this effect is
associated with the status of the mitochondrial inner membrane or a generic effect associated with mitochondrial health.
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Appendices
9 Appendices

TCA Cycle metabolites Modulates Ca\(^{2+}\)- Induced Mitochondrial Swelling

This experiment was intended to understand the role of mitochondrial metabolism on Ca\(^{2+}\) overload-induced mitochondrial swelling. Different TCA cycle metabolites modulated Ca\(^{2+}\)-induced swelling in isolated rat liver mitochondria. Acetyl-CoA and Citrate conferred repression on mitochondrial swelling while 2-oxoglutarate and succinyl-CoA enhanced mitochondrial swelling (Figure 9.1).

![Graphs showing modulation of Ca\(^{2+}\)-induced mitochondrial swelling by TCA cycle metabolites.](image)

**Figure 9.1: Modulation of Ca\(^{2+}\)-induced mitochondrial swelling by TCA cycle metabolites.**

(A) Pyruvate has no effect on Ca\(^{2+}\)-induced mitochondrial swelling. (B) Acetate and acetyl-CoA repressed Ca\(^{2+}\)-induced mitochondrial swelling. (C) Citrate repressed Ca\(^{2+}\)-induced mitochondrial swelling while CsA completely inhibited swelling. 2-oxoglutarate and succinyl-CoA enhanced swelling. (D) Acetate, acetyl-CoA and citrate inhibited swelling by 1.2-, 1.7- and 1.7-fold respectively and inhibition by acetyl-CoA and citrate were significantly (P<0.05) higher than acetate. N=3, * represents p<0.05.

Succinyl CoA Represses CypD-CsA Interaction

This experiment was intended to evaluate the effect of mitochondrial metabolism on CypD interaction in the mitochondria. CsA was used as proxy
for CypD binding partners. Differential scanning fluorimetry revealed that succinyl-CoA incubation with recombinant CypD represses the CypD-CsA binding by reducing the melting temperature by 2°C. Whereas, other mitochondrial metabolites showed no effect on CsA binding (Figure 9.2).

Figure 9.2: Succinyl-CoA represses CypD-CsA interaction. (A) Differential scanning fluorimetry reveal that CsA increases the melting temperature of CypD and 200µM of acetate, succinate, GSH and Ca²⁺ have no effect on CypD-CsA interaction. (B) Differential scanning fluorimetry reveal that succinyl-CoA represses CypD-CsA interaction while acetyl-CoA, citrate and alphaketoglutarate has no effect. (C) succinyl-CoA suppresses the melting temperature of CypD-CsA interaction significantly by 2°C. (D) Table shows the change in melting temperature of CypD resulting from CsA binding and the effect of mitochondrial metabolites. Concentration of CypD and CsA used are 5µM and 10µM respectively. N=3, * represents p<0.05.

CypD Does not Modulate PTPMT1 Activity In Vitro

This experiment was intended to understand the role of CypD in the maintenance of protein stability during oxidative stress. It was hypothesized that during oxidative stress, CypD maintains the stability of MPTP constituents during oxidative stress, hence, ensuring the formation and activation of the MPTP. PTPMT1 was used as a proxy for protein oxidative modification, as it
is redox sensitive. \( \text{H}_2\text{O}_2 \) induced the loss of activity of PTPMT1. However, CypD did not restore activity loss induced by \( \text{H}_2\text{O}_2 \) (Figure 9.3).

**Figure 9.3: PTPMT1 activity is not modulated by CypD.** (A) Absorbance trace of PNPP hydrolysis by PTPMT1 (5µM). (B) Change in absorbance at 15min. Test groups are treated with \( \text{H}_2\text{O}_2 \) (5µM) and CypD (5µM) or GSH (20µM) are added to rescue ROS-induced PTPMT1 inactivation. N=3, * represents p<0.05.

**CypD Stabilises Amyloid \( \beta \) Aggregates in HAP1 Mitochondria**

Amyloid \( \beta \) oligomers were observed to be significantly reduced in CypD KO isolated mitochondria when compared to their WT counterpart. The mechanism by which CypD stabilises the oligomeric form is unknown, and it should be verified if CypD directly or indirectly enhance this oligomeric state (Figure 9.4).

**Aconitase localises to the nucleus of both CypD WT and KO HeLa cells**

Small fraction of mitochondrial Aconitase was observed to localise to the nucleus of both WT and KO HeLa cells. However, no detectable nuclear localisation was observed for OGDH. Nuclear aconitase was not different between WT and KO HeLa cells (Figure 9.5).
Dot blot represents the amount of amyloid β fibrils and its oligomeric form in isolated CypD WT and KO mitochondria. Oligomeric amyloid β was 2-fold higher in the WT than KO mitochondria. 20µg of mitochondrial protein lysate was spotted on PVDF membrane prior to incubation in their respective primary antibody (1:1000) at 4°C overnight. N=2. * represents p<0.05.

Mitochondrial OGDH and Aconitase are more localised in the nucleus of HT1080 cells

Mitochondrial colocalization of OGDH and aconitase were found to be localised in the nuclear compartment of HT1080 cells. However, CypD KD cells were characterised with elevated nuclear colocalization of these mitochondrial enzymes (Figure 9.6).
Figure 9.5: Nuclear localisation of mitochondrial aconitase in HeLa cells.

(A) Mitochondrial OGDH was not found to be localised to the nucleus. ATP synthase b subunit was used as mitochondrial control in panel 2 and panel 3 is the merged image of both 1 and 2. (B) Mitochondrial aconitase 2 (Panel 1) was partly found in the nucleus of both WT and KD HeLa cells. HSP60 was used as mitochondrial control in panel 2 and panel 3 represents the merged images acquired. (C). There was no significant difference in the nuclear aconitase between WT and KD HeLa cells. Red and yellow arrows highlight the nucleus while green arrows were used to highlight the mitochondria. Cells were fixed in 3.7% PFA. Primary antibodies (1:200), Secondary antibodies (Alexa Fluor 555 and Alexa Fluor 488; 1:500). N=1, n > 15. Image analysis was done with Image J.
Figure 9.6: Nuclear localisation of mitochondrial OGDH and Aconitase in HT1080 cells.

(A) Mitochondrial OGDH was found to be localised to the nucleus (panel 1). ATP synthase b subunit was used as mitochondrial control in panel 2. Panel 3 and 4 represents the merged image of both 1 and 2 with and without nuclear stain respectively. (B) Mitochondrial aconitase 2 (Panel 1) was found in the nucleus of both WT and KD HeLa cells. HSP60 was used as mitochondrial control in panel 2. panel 3 and 4 represents the merged images acquired with and without nuclear stain respectively. (C) There was no significant difference in the nuclear OGDH between WT and KD HeLa cells. However, nuclear aconitase was significantly higher in the KD than WT cells. Red and yellow arrows highlight the nucleus while green arrows were used to highlight the mitochondria. Cells were fixed in 3.7% PFA. Primary antibodies (1:200), Secondary antibodies (Alexa Fluor 555 and Alexa Fluor 488; 1:500). N=1, n > 15. Image analysis was done with Image J.