The Involvement of Anti-Oxidative Response and Mitochondrial Dynamics in the Pathogenesis of Friedreich’s Ataxia: Relevance to the Development of Future Therapeutics

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I am extremely grateful for the unending love and support and encouragement from my family.
I love you all very much.

This thesis is dedicated to my family.
STATEMENT OF ORIGINALITY

This dissertation is written and submitted in accordance with the regulations for the degree of Doctor of Philosophy at the University of Sydney. I certify, to the best of my knowledge, that previously published work and materials has been appropriately acknowledged in the text. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Shannon Chiang
AUTHORSHIP ATTRIBUTION STATEMENT

This thesis contains materials published in:


Published materials in these articles are presented in *Chapter 1, Chapter 2, and Chapter 3*. Where there is joint first-authorship, I was a principal contributor, and was involved in designing and performing experiments, statistical analysis of data for the studies (Western blotting, co-immunoprecipitation, histology, PCR, transmission electron microscopy, animal treatments, *in vivo* work), and I also contributed in figure preparations and writing of manuscripts. As an equal
first-author, Dr. Anzovino was involved in the intellectual design and planning of studies, performing particular experiments (Fig. 3.2; Fig. 3.3A, C; Fig. 3.5; and Fig. 3.7), statistical analysis, and writing of the manuscript that partially contributes to the thesis in Chapter 3. Dr. Huang was also involved in designing and performing certain experiments in Chapter 3 and Chapter 4, and in writing parts of the manuscripts. Permission to include these works was granted by both Dr. Anzovino and Dr. Huang.

In addition to the above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding authors.

Shannon Chiang, 21st May 2019

As supervisors for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Des Richardson, 21st May 2019

Michael L. Huang, 21st May 2019
ABSTRACT

Friedreich’s ataxia (FA) is the most common autosomal recessive ataxia, and patients of the disease are severely afflicted with progressive neuro- and cardio-degeneration. The main cause of FA is due to the deficient expression of the mitochondrial protein, frataxin, and its deficiency has been well reported to be associated with oxidative stress and losses in energy metabolism.

The major aim of this thesis was to elucidate the molecular mechanisms involved in the dysregulated anti-oxidative response in frataxin deficiency, which is responsible for the exacerbation of oxidative stress in FA. In light of the disease-associated deficits in mitochondrial bioenergetics and stress, this thesis then sought to explore the involvement of mitochondrial dynamics in the pathogenesis of FA. Considering these two pathological aspects of the disease, the thesis further assessed the efficacy of two different treatments aimed at restoring antioxidant defence and energy metabolism in vivo in frataxin deficiency. Results from these investigations are significant due to their potential applications and relevance to the development of future therapeutics for FA patients.

This dissertation is comprised of 6 chapters: a comprehensive literature review (Chapter 1 Introduction); a materials and methods chapter (Chapter 2 Materials and Methods); 3 results chapters (Chapter 3 – 5); and a general discussion of principle findings and future directions chapter (Chapter 6 Discussion and Future Directions).
Chapter 3: Various studies in models of FA have previously reported a decrease in the expression of the master regulator of antioxidant response, nuclear factor-erythroid 2-related factor-2 (Nrf2), due to unknown mechanisms. This chapter herein, examined the Nrf2-Keap1 signalling pathway using a mouse conditional frataxin knockout (KO) model of FA, by comparing the heart and skeletal muscle in these mice. The frataxin KO hearts exhibited fatal cardiomyopathy, while the skeletal muscle was asymptomatic. These two tissue-types demonstrated contrasting molecular alterations. In the KO heart, protein oxidation and decreased GSH:GSSG ratio were observed, as well as decreased total and nuclear Nrf2 expression and increased Keap1 levels. However, the skeletal muscle did not demonstrate these alterations.

Notably, for the first time, a mechanism involving Gsk3β-signalling in the activation of nuclear Nrf2 export and/or degradation machinery was demonstrated in the KO heart. This process involved the increased activation of Gsk3β, increased Fyn phosphorylation, and the nuclear accumulation of β-TrCP to facilitate Nrf2 nuclear export. Furthermore, Nrf2-DNA-binding activity and the mRNA expression of Nrf2-targets were decreased in the frataxin KO mice. However, certain Nrf2 antioxidant targets, namely, NADPH quinone oxidoreductase-1 (Nqo1), glutathione-S-transferase-Mu1 (Gstm1), and thioredoxin reductase 1 (TxnRD1), demonstrated increased protein levels in the KO heart. In general, two potential mechanisms could be responsible for the reduced Nrf2 levels in the frataxin-deficient hearts: (1) increased cytosolic Keap1 levels, and (2) the activation of Gsk3β-signalling or the Gsk3β-Fyn axis that decreases nuclear Nrf2 levels. On the other hand, the frataxin-deficient skeletal muscle had no decrease in Nrf2 levels and had contrasting results to the heart. Collectively, these findings have revealed tissue-specific
alterations in frataxin deficiency, but more importantly, the data have uncovered potential mechanisms that could significantly dysregulate the anti-oxidative response in FA.

**Chapter 4:** The mitochondrion is an essential organelle that maintains cellular function and health through its role in energy production. The mitochondrion protects the cell from oxidative stress by maintaining its homeostasis with critically dynamic processes of mitochondrial biogenesis, fusion/fission, and mitophagy. An imbalance between oxidative stress formation and endogenous antioxidant processes can induce mitochondrial protein defects that can severely disrupt mitochondrial homeostasis. This can lead to mitochondrial dysfunction, which is accompanied by mitochondrial protein oxidation and mitochondrial DNA (mtDNA) damage, culminating in the depletion of ATP and NAD\(^+\), apoptosis and organ failure. The heart and the nervous systems, which have an abundance of mitochondria, are most vulnerable to mitochondrial protein dysfunction, as evident in a number of belligerent human disease states. FA is also regarded as a mitochondrial disease, due to the role of frataxin in mitochondrial functions. Frataxin deficiency leads to a defect in mitochondrial iron metabolism and oxidative stress that potentiates the pathology of the disease. However, alterations to mitochondrial homeostasis have not been fully elucidated in the pathogenesis of FA.

Using the aforementioned frataxin KO mice model of FA that develops dilated cardiomyopathy, a number of key observations were found in the KO hearts relative to their wild-type littermates: (1) irregular mitochondrial morphologies and abnormal structure of cristae; (2) increased Parp activation, decreased NAD\(^+\):NADH ratio and reduced Sirt1 activity, (3) increased protein markers of mitochondrial biogenesis and dynamics (both fusion and fission), and (4) increased autophagic
flux at 10-weeks of age. These novel findings demonstrate significant changes to mitochondrial homeostasis in the condition of frataxin deficiency. Not only does this illustrate the importance of maintaining mitochondrial homeostasis in cardio-degenerative diseases, but it offers the potential for the development of new treatments that target mitochondrial function.

**Chapter 5:** Results from *Chapter 3* have found multiple molecular mechanisms involved in the dysregulation of the Nrf2 signalling pathway that negatively affects the anti-oxidative response in frataxin-deficient condition. Data from *Chapter 4* have demonstrated significant alterations to mitochondrial morphologies, dynamics, and function in the frataxin KO mice with age. Taken together, these results indicate the critical involvement of oxidative stress, mitochondrial dysfunction and decreased bioenergetics in the pathogenesis of FA. Since there are currently limited treatments available for FA patients, there is an urgent need to develop new therapies that focuses on ameliorating these pathological deficits of the disease. This chapter herein examined the potential therapeutic effects of two agents, namely, N-acetylcysteine (NAC) in the supplementation of the antioxidant glutathione, and the novel compound, 6-methoxy-2-salicylaldehyde nicotinoyl hydrazine (SNH6), developed in our laboratory that has a dual-mechanism of action mediated by NAD⁺ supplementation and iron chelation.

Using the previously described MCK mouse model of FA, these animals were treated from the asymptomatic age of 4-weeks-old, up until 9-weeks of age, where the animal displays an overt dilated cardiomyopathy. In general, iron deposits, interstitial fibrosis, and enlargement of cardiac muscle fibre size were observed in histological examinations of the KO hearts treated with either NAC or SNH6. Hence, the treatments did not attenuate disease progression or prevent the
development of cardiac hypertrophy. Despite these observations, the treatment with SNH6 did significantly increase NAD$^+$ levels, and as a result, there was increased sirtuin 1 and Parp activities in the SNH6-treated KO hearts. Hence, SNH6-supplementation of NAD$^+$ potentially restored, in part, mitochondrial function and dynamics, despite that it did not increase the NAD$^+$:NADH ratio and ATP levels. Collectively, increasing endogenous antioxidant levels and NAD$^+$ supplementation are two different, but important therapeutic strategies that deserves further investigation. Particularly, the therapeutic use of the novel agent, SNH6, and the supplementation of NAD$^+$, holds promise for the development of novel therapeutic strategies for FA patients.

In conclusion, this dissertation has elucidated the molecular mechanisms involved in the dysregulation of anti-oxidative response and mitochondrial dynamics in the condition of frataxin deficiency of FA. The research in this thesis has enhanced our understanding of the pathophysiology of the disease and its associated cardiomyopathy, which offers new insights into the development of potential therapeutics. Thus, the significance of this dissertation is in its relevance to the advancement of future therapies for effective FA treatment.
ABBREVIATIONS

AIF        apoptosis-inducing factor
ALAS       aminolevulinate synthase
AMBRA1     autophagy/beclin-1 regulator-1
Ampk       AMP-activated protein kinase
Apaf1      apoptotic protease factor 1
APS        ammonium persulphate
ARE        antioxidant response element
B2m        β2-microglobulin
Bach1      BTB and CNC homolog 1
BAF        Bafilomycin A1
BCA        bicinchominic acid
BSA        bovine serum albumin
Ca$^{2+}$  cellular calcium
CAD        caspase-activated deoxyribonuclease
CIA        cytosolic iron-sulfur cluster assembly
DCYTB      duodenal cytochrome-b
DEPC       diethylpyrocarbonate
DMSO       dimethylsulphoxide
DMT1       divalent metal transporter-1
dNTPs      deoxynucleotide triphosphates
DRG        dorsal root ganglia
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Drp1</td>
<td>dynamin-related protein 1</td>
</tr>
<tr>
<td>EDS</td>
<td>electron dispersive X-ray spectroscopy</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Eif2α</td>
<td>eukaryotic translation initiation factor 2 alpha</td>
</tr>
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<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FA</td>
<td>Friedreich's ataxia</td>
</tr>
<tr>
<td>FDA</td>
<td>Federal Drug Administration</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>ferrous iron</td>
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<tr>
<td>Fe(III)</td>
<td>ferric iron</td>
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<tr>
<td>FECH</td>
<td>ferrochelatase</td>
</tr>
<tr>
<td>Fis1</td>
<td>fission protein 1</td>
</tr>
<tr>
<td>FPN1</td>
<td>ferroportin 1</td>
</tr>
<tr>
<td>FTH1</td>
<td>ferritin heavy chain subunit 1</td>
</tr>
<tr>
<td>FTL</td>
<td>ferritin light chain</td>
</tr>
<tr>
<td>FTMT</td>
<td>mitochondrial ferritin</td>
</tr>
<tr>
<td>Fundc1</td>
<td>FUN14 domain containing 1</td>
</tr>
<tr>
<td>FXN</td>
<td>frataxin gene</td>
</tr>
<tr>
<td>Gapdh</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>Gsk3β</td>
<td>glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidised glutathione</td>
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</tbody>
</table>
Gstm1  glutathione-S-transferase Mu1
H&E  hematoxylin and eosin
HCl  hydrochloric acid
Hdac1  histone deacetylase-1
HO-1  haem oxygenase-1
HPLC  high-performance liquid chromatography
i.p.  intraperitoneal injection
IRE  iron responsive element
IRP  iron regulatory protein
ISC  iron-sulfur cluster
ISCU  iron-sulfur-cluster scaffold protein
ISD11  iron-sulfur-cluster biogenesis desulfurase-interacting protein of 11kDa
Keap1  Kelch-like ECH-associated protein 1
KO  knockout
Lc3  microtubule-associated protein 1A/1B-light chain 3
LIP  labile iron pool
LIR  Lc3-interacting regions
mAconitase  mitochondrial aconitase
Mapk  microtubule associated protein kinase
MARE  Maf recognition element
MCK  muscle creatine kinase
Mff  mitochondrial fission factor
Mfn  mitofusin
MFRN  mitoferrin
MFRN1/2  mitoferrin 1 or 2
MIT  mitochondrial
MPTP  mitochondrial permeability transition pore
$M_r$  molecular weight
MRCKα  myotonic dystrophy kinase related Cdc42-binding kinase alpha
mtDNA  mitochondrial DNA
NA  nicotinic acid
NAC  $N$-acetylcysteine
NaCl  sodium chloride
NaF  sodium fluoride
NCOA4  nuclear receptor coactivator 4
ND1  NADH-ubiquinone oxidoreductase chain 1
NFS1  cysteine desulfurase
NMN  nicotinic mononucleotide
NNT  nicotinamide nucleotide transhydrogenase
NQO1  NADPH quinone oxidoreductase-1
NR  nicotinamide riboside
Nrf1  nuclear respiratory factor 1
Nrf2  nuclear factor erythroid 2-related factor 2
Nrk2  nitotinamide riboside kinase 2
o-Tyr  ortho-tyrosine
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
</tr>
<tr>
<td>Par</td>
<td>poly(ADP-ribose)</td>
</tr>
<tr>
<td>Parp</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCBP</td>
<td>poly r(C)-binding protein</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>PPAR γ-co-activator 1 alpha</td>
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<tr>
<td>PIH</td>
<td>pyridoxal isonicotinoyl hydrazone</td>
</tr>
<tr>
<td>Pink1</td>
<td>PTEN-induced putative kinase 1</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferation activator receptor</td>
</tr>
<tr>
<td>PPIX</td>
<td>protoporphyrin IX</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue deleted on chromosome 10</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 up-regulated modulator of apoptosis</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinyl difluoride</td>
</tr>
<tr>
<td>redox</td>
<td>oxidation-reduction</td>
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<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>Sirt1</td>
<td>sirtuin 1</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Sod2</td>
<td>superoxide dismutase 2</td>
</tr>
<tr>
<td>Spec. Comp.</td>
<td>specific competitor</td>
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<tr>
<td>TEMED</td>
<td>N, N,N',N'- tetramethylethylenediamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Explanation</td>
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<tr>
<td>Tf</td>
<td>transferrin</td>
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<tr>
<td>Tfam</td>
<td>mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TfR1</td>
<td>transferrin receptor 1</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TxnRD1</td>
<td>thioredoxin reductase 1</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated regions</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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LIST OF PUBLICATIONS

Publications and work in support of this thesis


LIST OF COMPETITIVE AWARDS

1. **Australian Postgraduate Award (APA)** – 2 years ($28,000 per annum) 2016-2018
   
   Competitive scholarship awarded to support PhD candidature.

2. **Postgraduate Research Support Scheme** ($1,500) 2016
   
   Competitive funding awarded to attend a domestic conference (SFRR, Queensland, Australia).

3. **Runner-up Prize for Poster Presentation** (2016) ($450)
   
   Competitive award at the Aussie-Mit Conference in Sydney.

4. **Travel Award for the 8th Joint Meeting of Society for Free Radical Research Australasia and Japan Conference** ($7000) 2017
   
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5. **Postgraduate Research Support Scheme** ($1,500) 2017
   
   Competitive funding awarded to attend an international conference (SFRR+J, Japan).

6. **Postgraduate Research Support Scheme** ($1,500) 2018
   
   Competitive funding awarded to attend an international conference (19th SFRR Biennial Meeting 2018, Lisbon, Portugal).

7. **Third-Prize for Poster Presentation** 2018
   
   Bosch Annual Scientific Symposium 2018 at the University of Sydney, Australia.
INVITED ORAL PRESENTATIONS

1. Oral Presentation at SFRRA+J 2017 (Hachioji, Tokyo, and Lake Kawaguchi, Yamanashi, Japan)

   Shannon Chiang, Michael L.-H. Huang,* and Des R. Richardson*

   Titled: “Frataxin deficiency and alterations in microRNA expression in a mouse model of Friedreich’s ataxia”

2. Oral Presentation at the 19th SFRRI Biennial Meeting 2018 (Lisbon, Portugal)

   Amy Anzovino, Shannon Chiang, Bronwyn E. Brown, Clare L. Hawkins, Des R. Richardson,* and Michael L.-H. Huang*

   Titled: “The Dys-regulation of Anti-oxidant Defense via an Impairment of Nrf2 Response in the Pathology of Friedreich’s Ataxia”

3. Oral Presentation at Bosch Young Investigators Symposium 2017 (University of Sydney, Sydney)

   Amy Anzovino, Shannon Chiang, Bronwyn E. Brown, Clare L. Hawkins, Des R. Richardson,* and Michael L.-H. Huang*

   Titled: “Molecular Alterations in a Mouse Cardiac Model of Friedreich’s Ataxia: An Impaired Nrf2 Response Mediated via Up-Regulation of Keap1 and Activation of the Gsk3β Axis”
INVITED POSTER PRESENTATIONS

1. Poster Presentation at SFRRA 2016 (Griffith University, Gold Coast, Australia)
   Amy Anzovino, Shannon Chiang, Bronwyn E. Brown, Clare L. Hawkins, Des R. Richardson,* and Michael L.-H. Huang*
   Titled: “The Effect of N-acetyl Cysteine and Sulforaphane in Modulating the Anti-oxidative Nrf2-ARE Signalling Pathway as Potential Treatments for Friedreich’s Ataxia”

2. Poster Presentation at AussieMit 2016 (Kolling Institute of Medical Research Sydney, Australia)
   Shannon Chiang, Michael L.-H. Huang,* and Des R. Richardson*
   Titled: “Frataxin Deficiency and Alterations in microRNA Expression Using a Mouse Model of Friedreich’s Ataxia”

3. The 18th Bosch Young Investigators Annual Symposium 2018 (University of Sydney, Sydney)
   Amy Anzovino, Shannon Chiang, Bronwyn E. Brown, Clare L. Hawkins, Des R. Richardson,* and Michael L.-H. Huang*
   Titled: “Molecular Alterations in a Mouse Cardiac Model of Friedreich’s Ataxia: An Impaired Nrf2 Response Mediated via Up-Regulation of Keap1 and Activation of the Gsk3β Axis”
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CHAPTER ONE

Introduction

This Chapter is adapted and modified from the following publications and work, two of which I am the first author:


1.1 Friedreich’s Ataxia

Friedreich’s Ataxia (FA) is the most common hereditary autosomal recessive ataxia characterised by progressive cardio- and neuro-degeneration due to deficient expression of the mitochondrial (MIT) protein, frataxin (Campuzano et al. 1996, Abrahao et al. 2015). The disease affects both the central and peripheral nervous systems with neurological phenotypes that result from lesions in the dorsal root ganglia (DRG), corticospinal tracts, sensory peripheral nerves, and dentate nucleus in the cerebellum (Koeppen 2011). Major neurological symptoms include gait and limb ataxia, tendon areflexia, sensory loss, dysarthria, dysphagia, and pyramidal signs (Gomes and Santos 2013, Parkinson et al. 2013a). The heart, skeleton, and the endocrine pancreas are also affected in FA and this leads to the common non-neurological symptoms, namely: cardiomyopathy, diabetes mellitus and skeletal abnormalities (Koeppen 2011, Parkinson et al. 2013a, Abrahao et al. 2015).

The estimated frequency of asymptomatic carriers is from 1:50 to 1:100 in people of European, Middle Eastern, Indian, and North African descent, with approximately one individual in 50,000 being affected by the disease (Campuzano et al. 1996, Labuda et al. 2000, Koeppen 2011, Abrahao et al. 2015). However, FA is significantly less prevalent among individuals of Asian, Saharan African, and American Indian decent (Labuda et al. 2000, Santos et al. 2010). Onset of the disease is typically around puberty with gradual progression of symptoms and shortened life-span, mostly due to cardiac complications rather than neurological causes (Parkinson et al. 2013a). Cardiomyopathy often develops after the onset of neurological features with a significant proportion of patients exhibiting cardiac hypertrophy leading to premature death (Pandolfo 2006, Parkinson et al. 2013a).
In the majority of cases, the pathogenic mutation in FA consists of a homozygous GAA trinucleotide repeat hyper-expansion in the first intron of the frataxin gene (FXN) located on chromosome 9q13 (Bidichandani et al. 1998, Koeppen 2011, Abrahao et al. 2015). Approximately 4% of cases are due to a heterozygous point mutation in the other allele (Koeppen 2011). The number of GAA repeats in healthy individuals ranges from 6-36, whereas FA patients have a range of 70-1700, most commonly between 600-900 repeats (Pastore and Puccio 2013). This pathogenic mutation causes an abnormal conformation of DNA, resulting in the transcriptional silencing of FXN (Santos et al. 2010). This leads to a reduction in its mRNA expression and its encoded protein, frataxin (Santos et al. 2010). The repression of FXN expression is due to the arrested RNA polymerase II progression and the formation of heterochromatin-like structures proximal to the region of the expansion (Kim et al. 2011, Gomes and Santos 2013). This prevents the efficient transition from initiation to elongation during FXN transcription (Kim et al. 2011, Gomes and Santos 2013). Furthermore, the expansion causes a decrease in splicing efficiency of the first intron, whereby GAA repeats bind to splicing factors that affect the turnover of intronic RNA in pre-mRNA processing, decreasing the generation of mature mRNA (Baralle et al. 2008). The overall consequence is a decrease in the expression of frataxin, which is a MIT protein known to play a significant role in MIT iron metabolism particularly, iron-sulfur cluster (ISC) biogenesis and haem synthesis (Pastore and Puccio 2013, Vaubel and Isaya 2013, Anzovino et al. 2014). In FA, frataxin deficiency plays a critical role in the pathology of the disease in which redox-active iron accumulates in the mitochondria, disrupting iron metabolism and causing oxidative stress (Gomes and Santos 2013, Abrahao et al. 2015).
This chapter will herein first discuss the molecular mechanisms of MIT iron-loading involved in the pathogenesis of FA. Since frataxin plays a major role in regulating iron metabolism and its deficiency is a primary pathological feature of the disease, it is necessary to first review the processes of cellular and MIT iron metabolism in order to understand the deleterious consequences of frataxin deficiency.

1.2 General Iron Metabolism and Homeostasis

Iron plays an essential role in many important cellular processes responsible for cell growth, proliferation and death (Silva and Faustino 2015). Such cellular processes include energy production, cellular respiration, electron and oxygen transport, MIT energy metabolism, and DNA synthesis (Richardson and Ponka 1997, Huang et al. 2011, Silva and Faustino 2015). Consequently, depletion of iron can lead to cellular death (Richardson et al. 1995, Merlot et al. 2013). However, excessive cellular iron can also cause cytotoxicity as a result of the iron-mediated generation of reactive oxygen species (ROS) (Lane et al. 2015b, Yun and Vincelette 2015). In the presence of oxygen, ferrous iron (Fe(II)) catalyses the production of cytotoxic hydroxyl radicals via the Fenton and Haber-Weiss-like reactions (Hentze and Kuhn 1996, Lawen and Lane 2013). Therefore, iron homeostasis is tightly controlled to ensure cell survival (Hadzhieva et al. 2013). Considering that many diseases, including FA, attribute their pathogenesis to the dysregulation of iron homeostasis, it is imperative to first review iron metabolism under physiological conditions in order to understand its defective alterations.
1.3 Iron Uptake

1.3.1 Dietary and Cellular Iron Uptake

In mammals, dietary non-haem iron is reduced from the ferric form, Fe(III), to the ferrous form, Fe(II), before being exported to the circulation through the enterocytes of the duodenal and upper jejuna epithelia (Dunn et al. 2007). This reduction of iron occurs at the apical surface of these enterocytes, which appears to be mediated by enterocytic ferrireductases (Dunn et al. 2007, Lawen and Lane 2013) and secreted ascorbate (Lane and Richardson 2014). The cytochrome $b_{561}$ protein, duodenal cytochrome-$b$ 4(DCYTB/CBRD1/CYB561A2), is an ascorbate-dependent oxidoreductase that is thought to be the most likely ferrireductase to be involved in the reduction of dietary iron at the brush-border of the duodenal enterocyte (McKie et al. 2001, Lawen and Lane 2013, Lane et al. 2015a). However, the importance of DCYTB in duodenal iron absorption is unclear, as it has been found to be non-essential for dietary iron uptake (Gunshin et al. 2005, Lawen and Lane 2013), suggesting that other enzymatic or non-enzymatic reductants (e.g., secreted ascorbate) may also be involved (Gunshin et al. 2005). Interestingly, studies have demonstrated that non-enzymatic ferrireduction can be achieved by ascorbate that is actively secreted from cells, which can contribute up to 50% of the reduction of low-M$_r$ iron e.g., non-haem iron (Atanassova and Tzatchev 2008, Lane and Lawen 2008, Lane et al. 2010).

Once iron is reduced to Fe(II), it is transported into the enterocyte via the divalent metal transporter-1 (DMT1) (Sassa 1976), which is expressed at the apical surface for intestinal uptake (Dunn et al. 2007). Subsequently, Fe(II) is often thought to enter the “labile iron pool” (LIP), which is a methodologically-defined chelatable-iron compartment in the cytosol that remains
poorly characterised (Dunn et al. 2007). The Fe(II) is then exported to the plasma through the basolateral membrane of the enterocyte through the iron exporter ferroportin 1 (FPN1) (Donovan et al. 2005, Lawen and Lane 2013). During its efflux, Fe(II) is subsequently oxidised to Fe(III) by the transmembrane, copper-dependent ferroxidase, hephaestin, which suggests that iron export and re-oxidation are coupled (Chen et al. 2004, Lawen and Lane 2013). The soluble plasma proteins, ceruloplasmin and transferrin (Tf), are also thought to play a role in oxidising plasma Fe(II) to Fe(III) to enhance iron efflux from cells (Richardson 1999a). The released Fe(III) in the plasma then binds to apo-Tf to form holo-Tf, which is the major plasma iron transport protein that contains two high affinity Fe(III) sites (for reviews see: (Morgan 1981, Richardson and Ponka 1997, Huang et al. 2011, Lawen and Lane 2013)). Transferrin functions to maintain the redox-inert state of iron in the circulation and facilitates its systemic distribution to tissues and cells (Ponka et al. 1998, Biasiotto et al. 2015). The cellular uptake of iron involves the binding of mono- and di-ferric Tf to transferrin receptor 1 (TfR1) on the plasma membrane, with this complex subsequently being internalized via receptor-mediated endocytosis (Fig. 1.1) (Anzovino et al. 2014).
**Figure 1.1: Simplified model of cellular iron uptake and trafficking.** Ferric iron Fe(III) binds to transferrin (Tf), which forms a complex with transferrin receptor 1 (TfR1) and enters the cytosol to form an endosome via receptor-mediated endocytosis. Fe(III) is released from the complex due to the acidic environment of the endosome, which is subsequently reduced into ferrous iron Fe(II) by the enzyme STEAP3 and is transported to the cytosol via DMT1. Fe(II) transiently resides in the labile iron pool (LIP) before being either stored by ferritin, exported by ferroportin 1 (FPN1) or taken up into the mitochondria by the mitochondrial transporter, MFRN, for iron utilisation. In addition, Sec15l1 has a role in exocytosis of the endosome in regulating Tf-mediated iron uptake.
A vacuolar-type proton-pumping ATPase instigates the acidic environment of the endosome, resulting in the reduction of the affinity of Tf for Fe(III) (Dunn et al. 2007, Anzovino et al. 2014). The NAD(P)H-dependent ferrireductase, STEAP3, is thought to play an important role in the reduction of this intra-endosomal iron from Fe(III) into Fe(II) prior to its export into the cytosol by the Fe(II)-selective proton-dependent DMT1 at the endosomal membrane (Fleming et al. 1997, Ohgami et al. 2005, Lawen and Lane 2013). This nascent cellular iron can then be utilised for cellular and MIT iron metabolism. The endosomes containing iron-free Tf (apo-Tf) and TfR1 are then recycled to the cell surface whereby apo-Tf dissociates from TfR1 at pH values close to neutrality and returns to the circulation, resuming iron transport and uptake (Fig. 1.1) (Morgan 1981, Andrews 2005, Anzovino et al. 2014).

1.4 Cellular Iron Metabolism

There are a number of proposed destinations for iron upon its exportation from the endosome to the cytosol (Fig 1.1). In both erythroid and non-erythroid cells, cytosolic iron enters a low-\( M_r \) LIP within the cytosol before entering downstream metabolic processes (Jacobs 1977, Sheftel et al. 2007). However, the nature of the LIP remains unclear with minimal low-\( M_r \) iron being found in actively metabolising reticulocytes (Richardson et al. 1996). It has been proposed that the LIP is composed of redox-active and chelatable Fe(II) that may be exchanged between metal-binding ligands for cellular processes (Kakhlon and Cabantchik 2002, Shvartsman and Ioav Cabantchik 2012).
Importantly, ROS generation in cells is dependent on the level and form of iron, which is mediated by changes in iron uptake and export, as well as iron chelation and storage in the cytosol (Kakhlon and Cabantchik 2002). Cytosolic iron can be stored within nano-cages created by heteropolymers of ferritin heavy chain (FTH1) and ferritin light chain (FTL) (Theil 2003, Arosio et al. 2009). Recently, the iron chaperones, poly r(C)-binding protein (PCBP) 1 and 2, have been shown to mediate the uptake of iron by ferritin (Leidgens et al. 2013). Moreover, PCBP2 has been demonstrated to bind to the cytoplasmic domain of endosomal DMT1, suggesting its role in the direct protein-to-protein transport of iron from the endosome to ferritin for storage (Yanatori et al. 2014). The labile iron-bound to ferritin is catalytically mineralised to become the iron core of the ferritin protein (Arosio et al. 2009). FTH1, but not FTL, has intrinsic ferroxidase activity, and the storage of iron within a redox-inert form within ferritin prevents the iron from undergoing Fenton reactions that would otherwise produce toxic free radicals (Arosio et al. 2009). Importantly, stored iron can be released from ferritin through lysosomal proteolysis (i.e., ferritinophagy), involving nuclear receptor coactivator 4 (NCOA4) and ATG8 proteins that recruit cargo-receptor complexes into autophagosomes (Mancias et al. 2014). NCOA4 is down-regulated by high cellular iron levels in a HERC2 ubiquitin ligase and proteasome-dependent manner, providing a partial explanation for how NCOA4 regulates ferritinophagy (Mancias et al. 2015).

Furthermore, cytosolic iron can also be exported from the cell via the iron exporter FPN1, which also plays a crucial role in maintaining systemic iron homeostasis (Fleming et al. 1997, Donovan et al. 2000, Donovan et al. 2005) (Fig. 1.1). Due to the necessity of iron in so many metabolic processes and the double-edged nature of iron where a deficiency or excess can be toxic, its utilisation is closely monitored through a number of regulatory systems that are discussed below.
1.5 Regulation of Iron Metabolism

The regulation of iron metabolism prevents the toxicity associated with iron deficiency, but also prevents oxidative damage by directly regulating the availability of ROS-generating redox-active iron (Huang et al. 2011). To this end, cellular iron levels are monitored and regulated by cytosolic iron regulatory proteins (IRPs) 1 and 2 that affect the expression of proteins involved in iron uptake, transport, and storage in the cytosol (Kakhlon and Cabantchik 2002). The IRPs are RNA-binding proteins that bind to iron responsive elements (IREs), which are specific sequences in the 5'- and 3'-untranslated regions (UTRs) of mRNAs that encode iron homeostatic proteins. The binding of IRPs to IREs directly control the stability or translation of their targeted mRNA, thereby regulating control over the synthesis of iron metabolism proteins (Richardson and Ponka 1997, Kakhlon and Cabantchik 2002) (Fig. 1.2).

The binding of IRPs to mRNAs containing 5'-IREs leads to translational repression, which rapidly reduces the translation of proteins responsible for iron storage (FTH1 and FTL), cellular respiration (mitochondrial aconitase (mAconitase)), haem synthesis (erythroid 5-aminolevulinate synthase), and iron export (FPN1) (Hentze et al. 1987, Hentze and Kuhn 1996, McKie et al. 2000). On the other hand, the binding of IRPs to mRNAs containing a 3'-IRE increases their stability, leading to increased translation of encoded proteins, such as TfR1 (cellular iron uptake), DMT1 (endosomal transporter of iron), and myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCKα; cytoskeletal re-organisation) (Cmejla et al. 2006).
Figure 1.2: Regulation of cellular iron metabolism by iron regulatory proteins (IRPs). In iron deplete conditions, IRPs bind to the iron response elements (IREs) at either the 5'- or 3'-UTR of the targeted mRNA. Binding of IRPs to 3'-IREs stabilises mRNA for translation while binding to 5'-IREs inhibits translation. When iron is replete, IRP1 undergoes cytosolic aconitase activity through its assembly of an iron-sulfur cluster (ISC) and is thus unable to bind IREs, whereas IRP2 undergoes proteasomal degradation. 5' IRE-containing mRNAs include ferritin heavy chain (FTH) and light chain (FTL), ferroportin 1 (FPN1), erythroid-specific δ-aminolevulinate synthase (ALAS2), and mitochondrial aconitase (mAconitase), while 3' IRE-containing mRNAs include transferrin receptor 1 (TfR1), divalent metal transporter 1 (DMT1), and myotonic dystrophy kinase-related Cdc42-binding kinase alpha (MRCKα) mRNA. The translational activity of mRNAs is dependent on the IRE binding activity of IRP1 and IRP2, which is dependent on cellular iron levels.
Cellular iron levels regulate the IRE-binding activity of IRPs, which in turn regulates the translational activity of targeted mRNAs (Hentze and Kuhn 1996). Under conditions of low cellular iron, IRP1- and IRP2-binding to IREs is increased (Hentze and Kuhn 1996) (Fig. 1.2). This promotes stability and translation of mRNAs containing 3'-IREs, which up-regulates their encoded proteins, whereas mRNAs containing 5'-IREs are translationally suppressed, leading to down-regulation of their encoded proteins (Hentze and Kuhn 1996, Chen et al. 1997). As a result, iron homeostasis is restored with reduced iron loss, enhanced iron uptake and increased iron availability for cellular metabolism (Muckenthaler et al. 2008).

The IRP-dependent IRE-binding activity of IRPs is significantly decreased under conditions of high cellular iron levels (Hentze and Kuhn 1996). This occurs by two different mechanisms, depending of the IRP involved. The IRE-binding activity of IRP1 is inactivated due to acquisition of an ISC, which converts the protein into a cytosolic aconitase (Hentze and Kuhn 1996). In the case of IRP2, IRE-binding capacity is lost due to its degradation by iron-dependent ubiquitination (Rouault 2006, Salahudeen et al. 2009, Vashisht et al. 2009) (Fig. 1.2). Hence, in iron-replete cells, the translation of the mRNA encoding for TfR1 decrease, whereas mRNAs encoding ferritin subunits and FPN1 are more rapidly translated. This response in the iron replete state maintains iron homeostasis and prevents cytotoxicity by decreasing iron uptake, and increasing iron storage and export (for reviews see: (Hentze and Kuhn 1996, Richardson and Ponka 1997).

Iron homeostasis involves the key role of the iron exporter, FPN1, which is a major target of a systemic iron regulatory system, in addition to the post-transcriptional IRP-IRE regulatory system
Systemic iron levels (e.g., plasma Tf saturation and hepatic iron levels) are predominantly “sensed” by hepatocytes of the liver, which promotes the transcription and secretion of the first described “hormone of iron metabolism”, hepcidin (Nemeth et al. 2004). Notably, hepcidin is a peptide that plays a major role in regulating the post-translational expression of FPN1 (Nemeth et al. 2004). This is thought to occur by the binding of hepcidin to FPN1, causing its cytoplasmic phosphorylation by JAK2 kinase followed by its internalization by endocytosis and subsequent degradation in the lysosome, which reduces cellular iron export (Nemeth et al. 2004, Donovan et al. 2005, Ganz 2007, De Domenico et al. 2009). This occurs in cells such as enterocytes, hepatocytes, and macrophages and effectively limits serum iron availability (Nemeth et al. 2004, Knutson et al. 2005).

In mammals, iron overload induces the secretion of hepcidin from the liver, which binds to FPN1 and down-regulates its expression on the plasma membrane, thereby reducing iron export from enterocytes or iron-storage cells (Nemeth et al. 2004, Mena et al. 2008) (Fig. 1.3). It is also known that hepcidin negatively regulates iron transport through the inhibition of apical iron uptake via the induction of DMT1 degradation (Dunn et al. 2007, Mena et al. 2008, Brasse-Lagnel et al. 2011) (Fig. 1.3). Therefore, hepcidin functions in response to changes in systemic iron levels to regulate iron uptake and maintain iron homeostasis (Ganz 2003). Conversely, during iron deficiency, hepcidin synthesis is repressed, and DMT1 expression is induced at the transcriptional level to increase systemic iron uptake (Brasse-Lagnel et al. 2011). Thus, overall, during systemic iron deficiency, iron homeostasis is maintained with enhanced iron absorption and iron accumulation, and decreased cellular iron efflux.
Figure 1.3: The role of hepcidin on cellular iron uptake in response to increased systemic iron levels. Tissue-iron overload causes the liver to secrete hepcidin into the circulation, which binds to ferroportin 1 (FPN1) and causes its internalisation for degradation in enterocytes, including macrophages. This prevents iron export, which reduces serum iron availability for cellular uptake. Hepcidin also negatively regulates the iron transporter DMT1 and the ferrireductase DCYTB via unknown mechanisms, leading to reduced dietary iron uptake.
1.6 Mitochondrial Iron Uptake and Metabolism

1.6.1 Iron Uptake by the Mitochondrion

The mitochondrion is a critical organelle for intracellular iron processing (Zhang et al. 2005, Richardson et al. 2010b). There are a number of hypotheses that explain the intracellular mechanism of iron trafficking and uptake by the mitochondrion (Anzovino et al. 2014).

Studies in erythroid cells identified the presence of low molecular weight ($M_r$) iron that was transported from one protein to another in their hydrophobic environments (Richardson et al. 1996). This protected the iron from reacting with oxygen and generate noxious ROS (Richardson et al. 1996). Such transport proteins could include chaperone molecules such as the previously described members of the PCBP class (Leidgens et al. 2013).

Later studies suggested a “kiss-and-run” hypothesis which describes the direct transport of iron from the endosome to the mitochondria in erythroid cells (Sheftel et al. 2007). With this model, the endosome transiently docks at the mitochondrion to deliver iron, avoiding the cytosolic LIP (Sheftel et al. 2007, Anzovino et al. 2014). However, the molecular mechanisms that are involved regarding this potential process remain unclear. It has also been suggested that cytosolic iron may be taken up directly by the mitochondria as “free” Fe(II), which is dependent on the MIT membrane potential (Lange et al. 1999). This would involve the passage of iron across the outer and inner MIT membranes. The MIT-specific iron transport protein, mitoferrin (MFRN), facilitates the transport of iron across the inner MIT membrane (Shaw et al. 2006) (Fig. 1.1). Interestingly, MFRN exists as two homologues, MFRN1 (SLC25A37) and MFRN2 (SLC25A28).
that are vital for MIT iron uptake in erythroid and non-erythroid cells, respectively (Shaw et al. 2006, Paradkar et al. 2009). MFRN1 interacts with the inner MIT membrane ATP-binding cassette transporter, ABCB10, to enhance its stability and efficiency for erythroid MIT iron import (Chen et al. 2009, Lawen and Lane 2013).

1.7 Mitochondrial Iron Metabolism

1.7.1 ISC biogenesis

The mitochondrion is also a major site of cellular iron utilisation. Elemental iron is utilised by the mitochondrion for three major metabolic pathways: (i) MIT ISC biogenesis; (ii) haem biosynthesis; and (iii) MIT iron storage (Fig. 1.4) (Richardson et al. 2010a, Huang et al. 2011, Lawen and Lane 2013). These metabolic processes are crucial for the vitality of the cell (Richardson et al. 2010a, Huang et al. 2011, Lawen and Lane 2013). ISCs are vital and evolutionarily conserved protein-bound cofactors, also known as prosthetic groups, that are found in a wide range of proteins found in the mitochondria, cytosol and nucleus. Many of these proteins play major roles in metabolism, such as metabolic catalysis, electron-transfer in redox reactions and the regulation of gene expression (Gerber and Lill 2002, Tong et al. 2003). Although ISCs come in different configurations, the most common in mammals are the [2Fe-2S] and [4Fe-4S] clusters in which iron ions are coordinated with cysteine residues (Gerber and Lill 2002, Lill 2009).
Figure 1.4: Mitochondrial iron uptake, utilisation, and metabolism. Cytosolic iron Fe(II) crosses the outer mitochondrial membrane via uncharacterised mechanisms. In vertebrates, Fe(II) is then transported across the inner mitochondrial membrane via the mitoferrin proteins, MFRN1 and MFRN2, found in erythroid and non-erythroid cells, respectively. In the mitochondrion, iron can be processed by three main pathways: (1) iron-sulfur cluster (ISC) biogenesis; (2) mitochondrial iron storage via sequestration of Fe(II) by mitochondrial ferritin (FTMT); and (3) haem biosynthesis. The ISC assembly machinery is composed of: cysteine desulfurase (NFS1), which retrieves sulfur (R-SH) from cysteine residues; small accessory proteins (ISD11); and the scaffolding protein ISC assembly enzyme, ISCU. An unknown ISC intermediate (?) is produced and is exported from the mitochondrion via the inner mitochondrial membrane exporter, ABCB7. The transported ISCs are subsequently utilised by the cytosolic ISC assembly system for maturation and formation of cytosolic and nuclear ISC proteins. The final enzymatic step of the haem biosynthesis pathway involves the insertion of Fe(II) into protoporphyrin IX (PPIX) by ferrochelatase (FECH) to form haem. In addition, FECH is an ISC-containing enzyme, which suggests a relationship between ISC biogenesis and haem biosynthesis.
Importantly, mammalian ISC biogenesis occurs in two different, but functionally connected compartments: the “ISC” assembly apparatus in mitochondria and the more recently-characterised cytosolic ISC assembly (CIA) system (overviewed below). The biogenesis of ISCs, in general, can occur in two phases: (1) the transient synthesis of the ISC on a scaffold assembly protein; and (2) the transfer of these ISCs to target apo-proteins (Rawat and Stemmler 2011, Lane et al. 2015b).

In the MIT ISC system, the ISC core is assembled with the cysteine desulfurase (NFS1), which extracts sulfur from cysteine residues with the aid of the cofactor, pyridoxal phosphate (Ye and Rouault 2010, Rouault 2012). In eukaryotes, the small accessory protein, ISD11, and two monomers of the dedicated ISC scaffold protein (ISCU), bind NFS1 (Raulfs et al. 2008, Rouault 2012). It is known that ISD11 functions to stabilise the cysteine desulfurase, whereas ISCU provides the backbone structure for the formation of new clusters containing covalently bound iron and inorganic sulfur (Wiedemann et al. 2006, Raulfs et al. 2008, Rouault 2012). The binding of Fe(II) to the assembly contributes to the formation of ISCU, which allows for the synthesis of ISCs (Tong and Rouault 2000, Frazzon and Dean 2003, Anzovino et al. 2014).

Of note, ISCU is found in both the mitochondria and the cytosol of mammalian cells, suggesting that this scaffolding protein may contribute to both MIT and CIA-dependent ISC biogenesis (Shi et al. 2009, Lane et al. 2015b). Furthermore, the assembly of nascent clusters relies on a source of electrons to achieve the appropriate electron configurations (Rouault 2012). In mammals, ferredoxins 1/2 and ferredoxin reductase are responsible for this activity (Sheftel et al. 2010, Shi et al. 2012). Newly synthesised ISCs are transferred from the ISCU to recipient proteins in which
the ISC functions as a cofactor for other cellular metabolic processes, such as the respiratory chain and the citric acid cycle (Huang et al. 2011, Rouault 2012, Lane et al. 2015b).

The complex logistics of this directed transfer of nascent ISCs to specific apo-proteins occurs by the concerted action of cluster-transfer proteins (Lill 2009). These proteins dissociate the nascent ISC from ISCU and ensure accurate and specific transfer to the correct apo-proteins, as well as assisting in the correct assembly and integration of the ISC at the acceptor site (Lill 2009, Rawat and Stemmler 2011, Rouault 2012). As a detailed discussion on this aspect of MIT ISC biogenesis is beyond the scope of this thesis, we refer readers to ISC-focussed reviews (Lill 2009, Rawat and Stemmler 2011, Rouault 2012).

The biogenesis of extra-MIT ISCs occurs in the CIA pathway. The CIA pathway involves distinct molecular machineries and, like MIT ISC biogenesis, occurs in two major steps that are highly conserved throughout the eukaryotes involving nine known proteins (Balk et al. 2004, Balk et al. 2005, Hausmann et al. 2005, Netz et al. 2014). Importantly, the CIA pathway has a dependence on MIT sulfur metabolism (Lill et al. 2012). Indeed, in the first step of the mammalian CIA pathway, a [4Fe-4S] cluster is assembled on a scaffolding hetero-tetrameric complex composed of the P-loop NTPases, NUBP1 and NUBP2 (Roy et al. 2003, Hausmann et al. 2005, Netz et al. 2007). This process requires a currently unknown source of sulfur (designated “X-S”), which appears to be exported from the mitochondrion by ABCB7 (Pondarre et al. 2006). Additionally, an intermembrane space sulfhydryl oxidase, GFER, may play a role in disulfide formation and the facilitation of export of this unknown intermediate to the cytosol (Mesecke et al. 2005, Qi et al.
2014). As a detailed discussion of the CIA pathway is outside the scope of this thesis, we refer readers to the following excellent reviews (Sharma et al. 2010, Netz et al. 2014).

1.7.2 Haem synthesis

The mitochondrion can also utilise iron for the synthesis of haem (Fig. 1.4), which is an essential cofactor of important proteins such as hemoglobin and myoglobin (Ponka 1997, Anzovino et al. 2014). There are eight enzymes in the haem biosynthetic pathway that involves all compartments of the mitochondrion, as well as four intermediate steps in the cytosol (Ponka 1997). The first and rate-limiting enzyme of the pathway, aminolevulinate synthase (ALAS), has two variants, namely ALAS1 that is expressed ubiquitously, and ALAS2 expressed in erythroid cells (Huang et al. 2011). Notably, ALAS2 can be regulated by the IRP system due to its possession of an IRE in its 5' UTR (Wilkinson and Pantopoulos 2014). This indicates that the rate of haem synthesis is regulated by the levels of cytosolic iron in erythroid cells (Ponka 1997). The final enzyme for haem synthesis is ferrochelatase (FECH) that functions to insert iron into protoporphyrin IX (PPIX), a haem precursor, to generate haem (Fig. 1.4) (Ponka 1997, Zhang et al. 2005). For a detailed review of haem metabolism, we refer readers to the following reviews (Ponka 1997, Chiabrando et al. 2014).

1.7.3 Mitochondrial iron storage

Iron can be stored in the mitochondrion via its sequestration in MIT ferritin (FTMT) (Levi et al. 2001, Arosio et al. 2009) (Fig. 1.4). The storage of iron in FTMT prevents redox reactions that generate ROS which induce MIT oxidative damage (Arosio et al. 2009). FTMT has ferroxidase
activity that inhibits the production of free radicals, thus preventing iron toxicity (Arosio et al. 2009). FTMT is expressed in cells with high-energy requirements and consumption, such as the brain, heart, thymus, kidney, smooth muscle, and testis (Levi et al. 2001, Campanella et al. 2004). Consequently, FTMT is not expressed in tissues with iron storage functions, such as the liver and spleen (Santambrogio et al. 2007). This suggests that the level of FTMT expression is associated with oxidative metabolic activity (Santambrogio et al. 2007). Furthermore, FTMT may allow the mobility and trafficking of iron within the mitochondrion so as to prevent the formation of ROS (Arosio et al. 2009).

The 3 major pathways involved in MIT iron metabolism described above namely, haem synthesis, ISC synthesis and MIT iron storage, are affected by the deletion of the MIT protein, frataxin (Huang et al. 2009, Whitnall et al. 2012). Deficient expression of frataxin results in the devastating neuro-degenerative and cardio-degenerative disease, FA (Richardson et al. 2010a). Hence, the role of this molecule in MIT iron metabolism is critical to understand and is described in detail below.

1.8 Frataxin and its Metabolic Role

Frataxin is an evolutionarily-conserved MIT protein made of 210 amino acids and it is highly expressed in tissues that are rich in mitochondria such as the heart, skeletal muscle, liver and neurons (Campuzano et al. 1996, Richardson et al. 2010b, Anzovino et al. 2014). Frataxin is encoded by the nuclear gene FXN and is synthesised as a precursor before being transported into the mitochondria, where it is processed into its mature form (Cavadini et al. 2000, Pandolfo and Pastore 2009).
The precise role of frataxin is unclear, but many studies have indicated an important role in iron metabolism (Huang et al. 2011, Pastore and Puccio 2013, Anzovino et al. 2014). There are indications for its involvement in regulating iron utilisation by the mitochondria for ISC and haem biogenesis, and iron storage (Richardson et al. 2010b). Frataxin was initially found to be associated with the inner MIT membrane and crests, which led some investigators to suggest that frataxin could potentially regulate iron transport (Campuzano et al. 1997). However, frataxin has no apparent structural features that would enable its anchorage at the MIT membrane. Thus, it is possible that frataxin associates with inner MIT membrane-bound MIT proteins, such as FECH, to form complexes that regulate iron metabolism (Yoon and Cowan 2004, Richardson et al. 2010b). Many studies have suggested other possible roles for frataxin, including its function as an iron chaperone, a regulator of iron metabolism, or as an iron storage protein, as discussed further below (Pastore and Puccio 2013).

1.8.1 Frataxin and ISC Biogenesis

As discussed above, ISCs are prosthetic groups that are ligated to cysteine residue of proteins that are involved in a number of essential cellular processes (Rouault 2012). They are composed of non-haem iron and inorganic sulfide and are predominately found in the cubane form of four iron and four sulfur atoms, i.e., [4Fe-4S] (Rouault 2012, Lane et al. 2015b). In addition to the functions discussed above, ISCs generated in the CIA pathway, such as those found in mammalian IRP1, enable this protein to function as a metabolic sensor that helps control cellular iron homeostasis (Muckenthaler et al. 2008, Rouault 2012, Lane et al. 2015b) (also see above).
Frataxin has been suggested to act as a chaperone protein due in part to its ability to bind multiple Fe(II) ions on exposed acidic patches (Foury et al. 2007, Richardson et al. 2010b, Lane et al. 2015b). Previous studies using knockout (KO) mice (Puccio et al. 2001, Huang et al. 2009) and yeast (Foury 1999, Wang and Craig 2008) models have shown that frataxin deficiency is associated with ISC deficiency, suggesting a possible role for frataxin in ISC biosynthesis (Li et al. 1999, Muhlenhoff et al. 2002, Cook et al. 2010). Furthermore, multiple lines of evidence in yeast and human cells have shown that frataxin interacts with the core ISC assembly proteins, NFS1, ISD11 and ISCU (Gerber et al. 2003, Shan et al. 2007, Wang and Craig 2008, Tsai and Barondeau 2010, Schmucker et al. 2011). Moreover, the biophysical interaction between purified human frataxin and ISCU1 appears to be iron-dependent (Yoon and Cowan 2003, Kondapalli et al. 2008). However, whether frataxin plays a role in supplying iron for ISC biogenesis in the CIA pathway remains to be determined.

1.8.2 Frataxin and Haem Biosynthesis

The mitochondrion is the site for haem biosynthesis and this occurs in all cells, particularly hepatocytes and erythroid cells (Ponka 1997). The final step of the biosynthesis pathway involves the ISC-containing enzyme, FECH, which is a ISC-containing enzyme in human (Dailey et al. 1994), that catalyses the insertion of Fe(II) into PPIX to generate haem (Napier et al. 2005, Huang et al. 2009). Studies have suggested that frataxin plays a role in haem biosynthesis, as it has a high binding-affinity for FECH, and thus, may be capable of mediating the final step of this pathway (Yoon and Cowan 2004, Napier et al. 2005). This was demonstrated in a study examining the yeast frataxin homologue, Yfh1, where endogenous yeast FECH was down-regulated when frataxin was
depleted (Lesuisse et al. 2003). This was further supported by the observation of decreased haem levels in the frataxin-deficient yeast and mouse model (Lesuisse et al. 2003, Huang et al. 2009, Hadzhieva et al. 2013). It could be argued that frataxin deficiency indirectly caused this effect due to the requirement of FECH for the ISC moiety (Huang et al. 2011). However, as yeast FECH is not an ISC-containing protein, the down-regulation of FECH upon deletion of frataxin homologue in yeast (Lesuisse et al. 2003) supports the direct involvement of frataxin in haem biosynthesis (Huang et al. 2011).

1.8.3 Frataxin as an Iron Chaperone

It was suggested that frataxin functions as an iron chaperone because it facilitates the formation of ISCs (Foury et al. 2007). The synthesis of ISC requires the assembly of the scaffold protein ISCU that acquires sulfur and iron, via the activity of NFS1 and an unidentified donor, respectively (Anzovino et al. 2014). Therefore, frataxin has been proposed as the iron donor that presents iron to the complex (Fig. 1.5A). This was validated by structural studies on the protein, suggesting its role as an iron donor (Hradilek and Neuwirt 1987, Ponka 1999, Yoon and Cowan 2003, Gomes and Santos 2013). Comparisons between bacterial frataxin, CyaY, and known chaperone proteins, such as hscA and hscB, that are involved in ISC synthesis, show significant similarities (Vickery and Cupp-Vickery 2007, Adinolfi et al. 2009), which further supports the hypothesis that frataxin could be an iron chaperone. This role could also be relevant to haem synthesis, where frataxin has been suggested to bind to FECH to present its iron for haem synthesis (Yoon and Cowan 2004).
**Figure 1.5:** Proposed roles of frataxin. (A) an iron chaperone that facilitates the biosynthesis of iron sulfur cluster (ISC), and haem via presenting iron to ferrochelatase (FECH); (B) as a protein for iron storage.

### 1.8.4 The Role of Frataxin in Iron Storage

The hypothesis that frataxin functions as an iron storage protein was proposed due to the oligomerisation of frataxin in yeast, forming frataxin oligomers that have ferroxidase activity ([Fig. 1.5B](#)) (Park *et al.* 2003, Huang *et al.* 2011). The iron-binding capacity of yeast frataxin is high in aggregates, which are able to maintain iron in a redox inactive state, while still retaining the ability to release iron for biosynthetic pathways (Park *et al.* 2003). However, human frataxin has a lower iron-binding capacity than yeast frataxin, even under conditions of iron accumulation in the mitochondria (Cavadini *et al.* 2002). This discrepancy between the different organisms may be due to the fact that yeast, unlike higher organisms, do not express ferritin (Arosio *et al.* 2009). The role of frataxin for iron storage in the mitochondria became redundant and improbable when FTMT was discovered in human tissue (Drysdale *et al.* 2002, Huang *et al.* 2011). Furthermore,
intracellular iron levels do not influence frataxin expression in erythroid cells, which again contradicts the hypothesis that the activity of an iron storage protein should be regulated by the availability of iron in the cell (Becker et al. 2002).

1.8.5 Frataxin as an Iron Metabolic Switch

Other than its proposed roles as an iron chaperone and storage protein, frataxin has also been suggested to regulate iron metabolism by functioning as a metabolic switch (Fig. 1.6A). A previous study has demonstrated this role of frataxin in differentiating erythroid cells, in which frataxin was able to distribute iron between major MIT metabolic pathways (Becker et al. 2002). Considering the increase in MIT iron uptake or decrease iron export as a result of frataxin deficiency, it was hypothesised that decreased frataxin expression could potentially trigger a higher rate of haem synthesis in the expense of ISC synthesis (Becker et al. 2002). Therefore, frataxin could act as a metabolic switch that allocates and divides the utilisation of iron between metabolic pathways in the mitochondria (Becker et al. 2002).
Figure 1.6: Proposed roles of frataxin. (A) a metabolic switch that allocates iron utilisation between mitochondrial metabolic processes; and (B) an iron sensor that regulates ISC synthesis depending on the availability of apo-ISC protein and free iron.
This hypothesis is supported by the observation that an increased level of the haem intermediate, PPIX, leads to the down-regulation of frataxin expression and potentially a diversion of iron from metabolic pathways, such as ISC synthesis, towards haem synthesis (Becker et al. 2002). In addition, the molar ratio between frataxin and FECH affects the rate of haem synthesis (Yoon and Cowan 2004). A decrease in FECH activity is observed when frataxin levels are higher than a ratio of 1 frataxin to 1 FECH molecule, which leads to a decrease in the rate of haem synthesis (Yoon and Cowan 2004). Overall, these findings suggested that frataxin may function as a metabolic switch in regulating MIT iron metabolism, which is dependent on the expression levels of its binding ligand, such as PPIX and FECH. Further studies are clearly required to delineate this.

1.8.6 Frataxin as an Iron Sensor

It has also been hypothesised that frataxin may act as an iron sensor, particular in regulating and fine-tuning ISC synthesis (Fig. 1.6B) (Adinolfi et al. 2009). Studies examining the bacterial ortholog of frataxin, CyaY, have shown that it negatively regulates ISC synthesis when there is an excess of iron relative to the amount of available ISC apo-acceptor protein (Adinolfi et al. 2009). Considering this, without sufficient acceptor proteins, ISCs degrade rapidly, and hence, there is a need to regulate the balance between ISC synthesis and available apo-proteins (Fig. 1.6B). In the absence of a negative regulator such as CyaY, a high supply of iron with low ISC apo-acceptor availability will lead to excessive generation of unbound ISCs (Adinolfi et al. 2009). As a result, the degradation of these ISCs will generate free iron that is capable of producing toxic ROS and can lead to oxidative damage (Adinolfi et al. 2009). Based on these findings, the presence of a negative regulator is essential for monitoring ISC synthesis and balancing it with apo-protein availability. A surplus of iron would cause the binding of CyaY to the bacterial homolog of NFS1,
IscS, and lead to the suppression of ISC assembly (Adinolfi et al. 2009). In addition, the same study also proposed that the formation of iron aggregates under frataxin deficiency in FA is caused by an excess of ISCs relative to its apo-protein due to the dysregulation of ISC synthesis (Adinolfi et al. 2009). However, this observation in bacteria, contradicts the finding of low ISC protein levels in FA patients and animal models (Rotig et al. 1997, Puccio et al. 2001, Whitnall et al. 2008, Huang et al. 2009), thus arguing against this role of frataxin as a negative regulator of ISC synthesis in higher eukaryotes. Despite the uncertainty regarding the exact function of frataxin, it is evident that it plays a significant role in MIT iron metabolism.

1.9 Effect of Frataxin Deficiency

1.9.1 Dysregulated Iron Metabolism

The pathogenesis of FA is caused by frataxin deficiency that leads to severe alterations in iron metabolism (Pandolfo 2012). Frataxin deficiency leads to increase cellular and MIT iron uptake, as a consequence of suppressed utilisation of MIT iron in metabolic processes such as ISC and haem biosynthesis (Whitnall et al. 2008, Huang et al. 2009).

Notably, the muscle creatine kinase (MCK) frataxin KO mice model of FA exhibits targeted frataxin-deletion in the heart and skeletal muscle, such that it closely recapitulates the primary pathology of left ventricular cardiac hypertrophy and fatal heart failure found in FA patients (Puccio et al. 2001). Considering the significant relevance of this animal model to the study of FA pathophysiology, previous studies have shown in the MCK frataxin-deficient mice that TfR1 expression is up-regulated, while FPN1 and ferritin expression is down-regulated due to the up-
regulation of IRP2 (Fig. 1.7) (Whitnall et al. 2008, Huang et al. 2009). As a result, there is a marked influx of iron into the cardiomyocyte and a decrease in iron release and iron storage (Whitnall et al. 2008, Huang et al. 2009). Notably, the expression of the exocyst complex protein, Sec1511, is up-regulated (Lim et al. 2005, Huang et al. 2009), which could potentially aid cellular iron uptake through the intracellular cycling of Tf-containing endosomes (Lim et al. 2005, Huang et al. 2009) (Fig. 1.7).

This influx of iron into the cell leads to an increase in iron uptake by the mitochondria via an increase in the MIT transporter, MFRN2, in the MCK frataxin KO mouse (Shaw et al. 2006, Huang et al. 2009) (Fig. 1.7). Furthermore, frataxin deficiency in this latter model leads to the down-regulation of all three major iron-dependent metabolic pathways of the mitochondrion, namely: ISC synthesis, haem synthesis, and MIT iron storage (Whitnall et al. 2008, Huang et al. 2009) (Fig. 1.7). For ISC synthesis, both the mRNA and protein expression of NFS1 and ISCU1/2 were found to be down-regulated in the hearts of the MCK frataxin KO model (Huang et al. 2009) (Fig. 1.7). This is consistent with depressed expression of ISC-dependent proteins, i.e., Sdha and FECH (Sutak et al. 2008, Huang et al. 2009), and supports the hypothesis of the requirement of frataxin in ISC synthesis (Li et al. 1999, Muhlenhoff et al. 2002, Cook et al. 2010). Additionally, down-regulation of a number of key haem synthesis enzymes as well as decreased intra-cardiac haem levels were observed in the MCK KO (Schoenfeld et al. 2005, Huang et al. 2009) (Fig. 1.7). Frataxin deficiency also resulted in the down-regulation of FTMT, thereby limiting MIT iron storage (Fig. 1.7) (Huang et al. 2009).
Figure 1.7: Schematics of Friedreich’s ataxia (FA) and the effects of frataxin deficiency. In FA, there is a decrease in frataxin expression in the mitochondria that leads to severe alterations in cellular and mitochondrial (MIT) iron metabolism. Transferrin receptor 1 (TfR1) is up-regulated while ferritin and ferroportin 1 (FPN1) are down-regulated through the increased RNA-binding activity of the iron regulatory protein IRP2. These changes occur in conjunction with the up-regulation of the exocyst protein, Sec15l1, that potentially aids cellular iron uptake. There is also MIT-targeted iron trafficking evident by an increase in the MIT transporter MFRN2. However, major iron utilisation processes in the mitochondria are down-regulated, namely ISC and haem biosynthesis, and MIT iron storage via the MIT ferritin FTMT. Therefore, the suppression of these metabolic pathways, combined with the increase in iron uptake and targeting to the mitochondria, ultimately leads to MIT iron-loading observed in the FA pathogenesis.
Of interest, decreased frataxin expression in FA patients does not result in marked defects in haem synthesis in erythroid cells (Morgan et al. 1979). The reason for this interesting finding could be that it has been demonstrated that erythroid differentiation leads to a reduction in frataxin expression (Becker et al. 2002). Since a decrease in frataxin expression occurs concurrently with an increase in iron uptake, haem synthesis, and TfR1 and β-globin expression (Friend et al. 1971, Ross et al. 1972, Sassa 1976, Glass et al. 1978, Hradilek and Neuwirt 1987, Ponka 1997, Ponka 1999), this decrease in frataxin levels may facilitate these latter processes. Indeed, in erythroid cells the limiting factor for haem synthesis is iron assimilation (Ponka 1997, Ponka 1999). Thus, in erythroid cells, the reduction in frataxin expression appears coupled to the marked increase in haem synthesis that is then exported from the mitochondrion preventing toxic iron-loading (Becker et al. 2002). In contrast, in non-erythroid cells, the decreased frataxin levels lead to MIT iron-loading. However, since non-erythroid cells have minimal haem production rates, the increased iron uptake is not matched by a similar rise in haem synthesis that is critical for iron uptake into PPIX and its transport out of the mitochondrion as haem (Becker et al. 2002). Under these conditions in non-erythroid cells, iron accumulates within the mitochondrion rather than being effectively assimilated by rapid haem synthesis.

In summary, frataxin deficiency results in defective MIT-targeted iron trafficking and inhibition of MIT iron utilisation (Huang et al. 2009). In the absence of known MIT iron exporting molecules, these alterations in metabolism culminate in the accumulation of excess free iron, which cannot be exported out of the mitochondria in the form of iron metabolites, namely ISCs and haem (Fig. 1.7) (Huang et al. 2009). This leads to MIT iron-loading and the formation of inorganic iron crystallite
aggregates that contributes to the pathogenesis of FA (Fig. 1.7) (Huang et al. 2009, Whitnall et al. 2012).

1.9.2 Cellular Antioxidant Defence and FA

In addition to the deleterious effects on iron metabolism caused by frataxin deficiency, the ensuing oxidative stress is a feature of FA (Wong et al. 1999, Bradley et al. 2000, Schulz et al. 2000). This is evident by the leakage of electrons due to the respiratory chain impairment and subsequent increased formation of ROS (Armstrong et al. 2010). MIT iron accumulation in the form of an inorganic iron crystallite without a protective protein shell could be responsible, at least in part, for the oxidative stress in mitochondria of FA patients (Santos et al. 2010, Koeppen 2011, Whitnall et al. 2012). In fact, studies have demonstrated this outcome in both yeast and animal models of FA, which further illustrates the significance of frataxin in maintaining iron homeostasis for cellular function and vitality (Babcock et al. 1997, Whitnall et al. 2012). Frataxin deficiency in FA leads to the down-regulation of ISC expression that subsequently causes a dysfunctional respiratory chain (Yoon and Cowan 2003, Tsai and Barondeau 2010). This dysregulation, together with the defective utilisation of MIT iron, and presence of the redox active iron aggregates in the mitochondria, could increase the generation of ROS (Huang et al. 2009, Armstrong et al. 2010, Whitnall et al. 2012). This is supported by studies examining tissues from FA patients, as well as from animal models of FA, in which these alterations could potentiate the oxidative stress in FA (Bradley et al. 2000, Puccio et al. 2001, Simon et al. 2004, Martelli et al. 2007, Pandolfo 2012).
It is of interest to note that Myers and colleagues have indicated that the levels of the urinary oxidative biomarker, F\textsubscript{2}-isoprostanes, in FA patients were not different from controls and were not significantly associated with age, GAA repeat length, disability levels or antioxidant (Myers et al. 2008). However, these authors indicate that the lack of significant alteration in F\textsubscript{2}-isoprostanes could be due to the fact that ROS production in FA does not target membrane lipids. Furthermore, it is known that increased F\textsubscript{2}-isoprostane levels are also not observed in other neuro-degenerative conditions such as Parkinson’s disease where there is documented redox stress (Myers et al. 2008). In contrast, previous studies that examined the DNA oxidation marker, 8-hydroxy-2’-deoxyguanosine, have observed a significant increase in FA patients relative to controls (Schulz et al. 2000). Moreover, treatment with the antioxidant idebenone was able to significantly reduce 8-hydroxy-2’-deoxyguanosine levels in FA patients (Schulz et al. 2000). This finding was contrary to a later investigation that demonstrated no significant difference in this oxidative metabolite between idebenone-treated and -untreated FA patients (Di Prospero et al. 2007). In all these later studies, measurements of oxidative markers correlated poorly with disease duration or the number of GAA repeats. Therefore, while most studies identified a role for oxidative stress in models of FA and FA patients, there is still a need for identifying an appropriate biomarker for oxidative stress and understanding the mechanism of its generation.

Frataxin deficiency has also been associated with a decrease in the antioxidant response upon exposure to oxidative stress stimuli (Paupe et al. 2009). In frataxin-depleted cells of FA patients and in a yeast model of FA, the antioxidant, glutathione (GSH), was markedly decreased (Jauslin et al. 2002, Auchere et al. 2008). Notably, frataxin deficiency has also been linked to the impaired activity of the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), which is
crucial for the regulation of genes that are involved in antioxidant defence (Santos et al. 2010, D’Oria et al. 2013). This has been demonstrated in DRG of mouse models of FA, as well as in numerous other cell-types with frataxin depletion (Shan et al. 2013). However, the specific molecular mechanism of how frataxin regulates the Nrf2 anti-oxidative signalling pathway is elusive and remains to be investigated.

In general, frataxin deficiency leads to dysregulated cellular and MIT iron metabolism (Huang et al. 2009). The marked accumulation of toxic iron in the mitochondria along with a defective antioxidant response exacerbates oxidative damage that could play a role in the pathogenesis of FA (Shan et al. 2013, Anzovino et al. 2014).

1.9.3 Iron Loading and Cell Death in FA

As discussed above, the marked alterations in cellular iron metabolism in the heart leads to MIT iron accumulation in FA patients (Puccio et al. 2001, Gonzalez-Cabo and Palau 2013) and also FA models, particularly those of the heart (Huang et al. 2013), but also in neuronal tissues (Anjomani Virmouni et al. 2015, Chutake et al. 2015, Molla et al. 2016). This accumulation of iron, at least in a representative MCK mouse model of the cardiomyopathy in FA, is an inorganic iron aggregate that does not appear to be encapsulated within a protein shell and has the potential of being redox active (Whitnall et al. 2012). While the mechanism of cell death in FA remains unclear, previous studies have indicated the existence of increased levels of markers of autophagy (i.e., Lc3-II and p62) and mitophagy (i.e., FUN14 domain containing 1 (Fundc1)) as a function of age in the heart of the MCK mouse model (Huang et al. 2013). In fact, using this model, the ratio between the anti-
apoptotic protein, B-cell lymphoma 2 (BCL2), and the pro-apoptotic protein, Bax, was found to be progressively increased with age (Huang et al. 2013). Other studies using various neuronal models of FA, namely human astrocytes and human SH-SY5Y neuroblastoma cells, have also shown the association of frataxin deficiency with the up-regulation of markers of cell cycle arrest (i.e., p21) and apoptosis (i.e., Caspase-3, p53, p53 up-regulated modulator of apoptosis (PUMA), Bax) (Palomo et al. 2011, Loria and Díaz-Nido 2015).

More recently, a novel form of regulated cell death known as ferroptosis has been identified (Dixon et al. 2012, Xie et al. 2016, Latunde-Dada 2017). This form of apoptosis is morphologically and biochemically distinct from apoptosis and other processes of regulated cell death and is dependent on cellular iron levels and ROS (Dixon et al. 2012, Xie et al. 2016, Latunde-Dada 2017). Morphologically, ferroptotic cells exhibit smaller than normal mitochondria with reduction in MIT cristae and increased MIT membrane density (Xie et al. 2016). Biochemically, ferroptosis is characterised by lipid peroxidation and the accumulation of ROS from dysregulated iron metabolism (Xie et al. 2016, Latunde-Dada 2017). A decrease in cysteine uptake or the inactivation of GSH peroxidase leads to the accumulation of ROS in the form of lipid hydroperoxides, which induces ferroptosis (Xie et al. 2016, Latunde-Dada 2017). Using a conditional neuronal KO of GSH peroxidase in mouse models, studies have identified an association between ferroptosis and various neuro-degenerative diseases (Seiler et al. 2008, Wirth et al. 2010, Chen et al. 2015). Currently, the involvement of ferroptosis in the pathophysiology of the death of cells in FA is unknown and is an important aspect of this disease that needs further investigation.
1.9.4 Mitochondrial Energy Metabolism and Oxidative Stress

The mitochondrion plays a significant role within cells and is involved in many essential processes, such as energy transduction, the biosynthesis of ISC and haem, and apoptosis signalling (Huang et al. 2011). More importantly, the primary function of mitochondria is the generation of energy, which is essential for cell growth and vitality, and in maintaining cellular metabolic processes (Gonzalez-Cabo and Palau 2013). MIT respiration involves the electron transport chain located on the inner MIT membrane, which generates a gradient of protons that enables the production of energy in the form of ATP via oxidative phosphorylation (OXPHOS) (Wu et al. 2016, Serasinghe and Chipuk 2017). However, when the respiratory chain and the activity of enzymes involved in OXPHOS are disrupted, it affects the MIT membrane potential and decreases ATP production (Gonzalez-Cabo and Palau 2013).

Studies have demonstrated that over-expressing frataxin in mammalian cells can elevate cellular ATP levels, suggesting that frataxin can potentially activate OXPHOS, and its deficiency may lead to a decrease in ATP production in FA patients (Ristow et al. 2000). In a different study, frataxin silencing in human SH-SY5Y neuroblastoma cells resulted in reduced ATP production and defective energy metabolism (Bolinches-Amoros et al. 2014). Collectively, these studies demonstrate that frataxin deficiency can result in the dysfunction of mitochondria in energy metabolism, which could represent a possible pathogenic mechanism in the progression of FA.

Notably, organs and tissues, such as neurons of the central nervous system, with high-energy demands and consumption have a greater need for MIT respiratory function that involves aerobic
OXPHOS (Ikeda et al. 2014, Islam 2017, Serasinghe and Chipuk 2017) As such, the utilisation of oxygen in mitochondria for energy production is one of the main causes of oxidative stress (Gonzalez-Cabo and Palau 2013, Islam 2017). In particular, OXPHOS within the mitochondrion is the major source of endogenous ROS (Gonzalez-Cabo and Palau 2013). During energy transduction, where electrons are transferred to molecular oxygen in the MIT respiratory chain, a small proportion (1 to 5%) of electrons “leak”, resulting in the generation of superoxide anions (Valko et al. 2004, Islam 2017). Thus, decreased efficiency of the electron transport chain can lead to increased superoxide generation, causing an imbalance in cellular redox status that is evident in many neuro-degenerative diseases (Gonzalez-Cabo and Palau 2013, Islam 2017).

Considering that frataxin is associated with the inner MIT membrane, this may suggest a potential interaction of frataxin with the MIT respiratory chain (Campuzano et al. 1997, Huang et al. 2011). How frataxin affects MIT energy metabolism is unclear and remains to be elucidated. However, several studies in cellular and animal models of frataxin deficiency have shown an increase in ROS and, in certain cases, it is associated with redox-active iron deposits within the mitochondria (Puccio et al. 2001, Whitnall et al. 2012, Gonzalez-Cabo and Palau 2013). In yeast models of FA, frataxin deficiency is linked to MIT iron overload, which is responsible for the generation of toxic ROS (Babcock et al. 1997, Foury 1999). Additionally, in mouse models of FA, frataxin deficiency results in a deficit in ISC enzymes and defective haem biosynthesis (Puccio et al. 2001, Huang et al. 2009). These deficiencies are associated with MIT iron deposits and decreased activity of multiple respiratory chain complexes (Puccio et al. 2001, Simon et al. 2004, Huang et al. 2009, Huang et al. 2011). Furthermore, in depth investigations in yeast and human cells have shown that frataxin interacts with the complex II subunit of the MIT respiratory chain (Gonzalez-Cabo et al. 2014).
2009, Gonzalez-Cabo and Palau 2013). These studies suggest a possible role of frataxin in MIT respiration. Hence, disruption of the electron transport chain by frataxin deficiency may directly or indirectly affect MIT energy production and contribute to FA pathogenesis (Fig. 1.8).

**Figure 1.8: Schematic model of frataxin deficiency on mitochondrial (MIT) dysfunction in FA pathology.** Frataxin deficiency leads to a number of major MIT dysfunction that could potentially contribute to the pathogenesis of FA. These include the well-established dysregulation of MIT iron utilisation for iron-sulfur cluster (ISC) biogenesis and haem biosynthesis. These defects in MIT iron metabolism may result in a compensatory increase in cellular iron uptake and the MIT-targeting of iron to compensate for the deficiencies in ISC and haem synthesis. The marked increase in iron uptake and targeting to the mitochondria leads to iron loading of this organelle. Other studies have shown that frataxin deficiency leads to defective energy production and calcium metabolism in which the former could potentially induce oxidative stress due to the generation of superoxide, while the latter can contribute to apoptotic signalling. Moreover, the dysregulation of MIT homeostasis, notably involving the fusion/fission process, mitophagy, and MIT biogenesis, have been linked to other neuro-degenerative diseases, which suggest their possible roles in FA pathology.
Furthermore, proteomic analysis examining the hearts from MCK frataxin KO mice that develop a severe cardiomyopathy, demonstrated that components of the iron-dependent complex-I and -II of the electron transport chain and enzymes involved in ATP metabolism (creatine kinase, adenylate kinase) displayed decreased expression (Sutak et al. 2008). The frataxin KO hearts exhibited increased levels of enzymes participating in the Krebs cycle, catabolism of branched-chain amino acids, ketone body utilisation and pyruvate decarboxylation (Sutak et al. 2008). These alterations indicate metabolic compensation due to the decreased expression of MIT electron transport proteins. There was also marked up-regulation of proteins involved in stress protection, such as multiple chaperones, and proteins involved in cell structure, motility and general metabolism. All of these alterations at the protein level could be involved in the pathogenesis of the cardiomyopathy observed in the frataxin KO mouse (Sutak et al. 2008).

The association between gene expression and the progressive histopathological and functional changes in the MCK conditional frataxin KO mouse that develop a severe cardiac phenotype have been extensively examined (Huang et al. 2013). These studies examined KO mice from 3 weeks of age, when they are asymptomatic, to 10-weeks of age, when they die of the disease. Positive iron staining was identified in KO mice from 5 weeks of age, with markedly reduced cardiac function from 6 weeks (Huang et al. 2013). These studies identified in frataxin KO mice at 3 weeks of age: (1) an early up-regulation of a gene cohort responsible for stress-induced amino acid biosynthesis; and (2) increased phosphorylation of eukaryotic translation initiation factor 2α (p-Eif2α), which is an activator of the integrated stress response (Huang et al. 2013). Importantly, the Eif2α-mediated integrated stress response has been implicated in heart failure via downstream processes such as autophagy and apoptosis (Huang et al. 2013). Indeed, expression of a set of
autophagy and apoptosis markers was increased in the KO mice. Thus, the pathogenesis of cardiomyopathy in FA correlates with early and persistent Eif2α phosphorylation, which precedes activation of autophagy and apoptosis.

1.9.5 Mitochondrial Calcium Metabolism

In addition to energy production and normal MIT membrane potentials, cellular calcium (Ca$^{2+}$) homeostasis is also a vital physiological process essential for neuronal survival (Bolinches-Amoros et al. 2014). In fact, Ca$^{2+}$ levels are tightly regulated by the interactions between mitochondria and the cytosol. The MIT regulation of Ca$^{2+}$ includes Ca$^{2+}$ buffering and Ca$^{2+}$ signalling through the control of its release and uptake from cellular Ca$^{2+}$ stores (Walsh et al. 2009). MIT buffering facilitates proper Ca$^{2+}$ influx via Ca$^{2+}$ channels (Cali et al. 2012, Bolinches-Amoros et al. 2014). This is partly achieved by the communication between mitochondria and the endoplasmic reticulum network, which is the main structure for Ca$^{2+}$ storage that allows for Ca$^{2+}$ uptake by the mitochondria (de Brito and Scorrano 2008, Walsh et al. 2009, Cali et al. 2012, Serasinghe and Chipuk 2017).

Furthermore, mitochondria can influence the cytosolic Ca$^{2+}$ concentration through its uptake and release, whereby MIT ATP, NADH, ROS, and pyruvate regulate the Ca$^{2+}$ signalling machinery (Walsh et al. 2009). In addition, Ca$^{2+}$ is attracted to the negatively charged electron gradient across the MIT membrane, and subsequently, Ca$^{2+}$ is taken into the matrix through the low-affinity Ca$^{2+}$ uniport (Jeanneteau and Arango-Lievano 2016). Since the mitochondrion is the main site for ROS
generation in the cell, disruption of mitochondria and an increase in ROS can affect cellular and cytosolic Ca\(^{2+}\) concentrations.

In the nervous system, mitochondria play a role in other significant physiological functions, such as dendritic and axonal transport, maintenance of membrane potential, support of synaptic assembly, generation of the action potential, and the reutilisation and release of synaptic neurotransmitters (Bolinches-Amoros et al. 2014, Islam 2017). The MIT regulation of cellular Ca\(^{2+}\) homeostasis is essential for proper functioning of these biological processes. MIT buffering of Ca\(^{2+}\) in the synaptic cleft helps to maintain and regulate neurotransmission (Islam 2017). The process of MIT Ca\(^{2+}\) storage, uptake and release is reversible in neurons of the brain in order to maintain cellular Ca\(^{2+}\) homeostasis, which is dependent on cytosolic Ca\(^{2+}\) levels (Jeanneteau and Arango-Lievano 2016). Moreover, low Ca\(^{2+}\) levels in the MIT matrix can in turn affect the activity of the OXPHOS pathway, leading to a change in the rate of ATP production (McCormack et al. 1992, Jeanneteau and Arango-Lievano 2016). Thus, in addition to its other roles, maintaining Ca\(^{2+}\) homeostasis is a major physiological function of the mitochondria. Thus, MIT dysfunction and the resultant perturbations in Ca\(^{2+}\) homeostasis would severely affect the physiology of the nervous system.

Many studies on other neuro-degenerative diseases, such as Parkinson’s, Alzheimer’s, and Huntington’s disease, suggest that alterations in Ca\(^{2+}\) homeostasis is a hallmark of their pathologies (Cali et al. 2012). Notably, a study using cellular models of FA demonstrated that frataxin deficiency results in alterations in MIT physiology and induces endoplasmic reticulum stress, as
well as alterations in Ca\textsuperscript{2+} metabolism (Bolinches-Amoros et al. 2014). In particular, frataxin deficiency leads to a decrease in MIT Ca\textsuperscript{2+} uptake and an accumulation of Ca\textsuperscript{2+} in the cytosol (Bolinches-Amoros et al. 2014). This reduction in Ca\textsuperscript{2+} uptake capacity is also associated with inducing cellular stress responses, which could trigger autophagic signalling and cause neuronal degeneration (Bolinches-Amoros et al. 2014). This suggests that decreased frataxin levels can affect MIT Ca\textsuperscript{2+} uptake and potentially affect neural function and survival in FA (Fig. 1.8).

1.10 Cellular Antioxidant Defence

Oxidative stress is known to play a major role in the development of neurodegeneration, and previous studies in cells of FA patients have shown increased sensitivity to oxidative stress and reduced cellular antioxidant expressions (Wong et al. 1999, Schulz et al. 2000, Chantrel-Groussard et al. 2001, Shan et al.). This could be due to the dysregulation of enzymatic systems that would otherwise respond to oxidative stress and provide antioxidant protection in normal physiological conditions (Auchere et al. 2008, Shan et al. 2013).

1.10.1 Nrf2 Regulation of Antioxidant Defence System

The antioxidant transcription factor, Nrf2, regulates the expression of detoxification and cytoprotective genes that are important for cellular antioxidant defence (Calkins et al. 2009). In particular, certain Nrf2-target genes include thiol antioxidant genes such as Txnip, TxnRD, and Glrx1, and genes that contribute to the endogenous synthesis of the antioxidant GSH (Calkins et al. 2009, Shan et al. 2013). Importantly, studies in FA animal models have shown that Nrf2-mediated transcription regulates antioxidant defence against oxidative stress and neurodegeneration (Calkins et al. 2009).
**1.10.1.1 Nrf2 Anti-oxidative Signalling Pathway**

Nrf2 is part of a critical anti-oxidative signalling pathway that activates in response to oxidative stress (Kobayashi *et al.* 2004). In normal physiological conditions, Nrf2 is sequestered by Kelch-like ECH-associated protein 1 (Keap1) in the cytosol by binding to its Neh2 domain that controls the ubiquitin-dependent degradation of Nrf2 (Eggler *et al.* 2005, Calkins *et al.* 2009). The interaction between Keap1 and Nrf2 could be disrupted by endogenous and exogenous electrophilic molecules, thus enabling the nuclear translocation of Nrf2 to elicit its transcriptional activity (Baird and Dinkova-Kostova 2011). In addition, these electrophilic agents can react with the cysteine residues of Keap1, hence stabilises Nrf2 for nuclear translocation (Baird and Dinkova-Kostova 2011).

Once Nrf2 enters the nucleus, it undergoes dimerisation with small Maf proteins, which enables Nrf2 to bind to the antioxidant response element (ARE) for transcriptional activation (*Fig. 1.9*) (Itoh *et al.* 1997, Magesh *et al.* 2012). The ARE is a *cis*-acting enhancer located at the upstream regulatory regions of cyto-protective genes (Baird and Dinkova-Kostova 2011). Subsequently, the binding of the Nrf2-Maf heterodimer to the ARE would activate the transcription of Nrf2 downstream-target genes (Eggler *et al.* 2005, Baird and Dinkova-Kostova 2011). Hence, this composes the Keap1-Nrf2-ARE signalling pathway, which regulates the transcriptional expression of cyto-protective proteins and antioxidants. This signalling pathway is essential for cellular defence against oxidative stress and it is critical for the maintenance of redox homeostasis and metabolism (Baird and Dinkova-Kostova 2011, Magesh *et al.* 2012).
Figure 1.9: Keap1-Nrf2-ARE pathway and Haem-Bach1 pathway in regulating transcriptional activity of antioxidant and iron metabolism genes. The dissociation of Nrf2 from Keap1 is induced by conditions of high cytosolic haem levels, oxidative stress, and mitochondrial dysfunction. Upon translocation to the nucleus, Nrf2 binds to Maf protein and subsequently binds to ARE to promote the transcription of target genes involved in iron metabolism and antioxidant defence pathway. Bach1 competitively antagonises the association between Nrf2 and ARE. In the presence of haem, Bach1 binds to haem and is removed from ARE thereby relieving its suppressive effect on ARE and allowing Nrf2 transcriptional activation.
1.10.1.2 Haem and Bach1 in Nrf2 Signalling Pathway

In addition to Keap1, the transcription regulator protein, BTB and CNC homology 1 (Bach1), functions as a transcriptional repressor for the Nrf2 signalling pathway (Marro et al. 2010, Liu et al. 2013). Similarly to Nrf2, Bach1 forms a heterodimer with small Maf protein in the nucleus, but this antagonises the activity of Maf to bind to Maf recognition elements (MAREs), hence this interaction suppresses the transcription of ARE-dependent genes such as haem oxygenase-1 (HO-1), β-globin, and NADPH quinone oxidoreductase-1 (NQO1) (Marro et al. 2010, Magesh et al. 2012, Raval et al. 2012). However, Bach1 can be bound by haem, which removes Bach1-suppression of its target gene enhancer (Igarashi and Sun 2006). As a consequence, Bach1 disassociates from small Maf proteins thereby allowing Nrf2-ARE-mediated transcription to proceed (Igarashi and Sun 2006). Thus, Bach1 is a sensor of cellular haem levels in which haem is able to negatively regulate Bach1, which indirectly regulates Nrf2-mediated transcriptional activation (Fig. 1.9) (Igarashi and Sun 2006). Furthermore, the combined effects of this relationship between haem and Bach1 with Nrf2-mediated transcription functions to regulate cellular antioxidant defence, as well as the recycling of iron for haemoglobin and iron homeostasis (Marro et al. 2010). The inhibition of Bach1 activity in the presence of haem results in increased transcription of Fpn1 for iron export (Marro et al. 2010). Hence, the haem-Bach1 pathway regulates the availability of iron for haem synthesis, and this helps maintain iron homeostasis and the Nrf2 signalling pathway (Marro et al. 2010).

1.10.2 The Effects of Frataxin Deficiency on Nrf2

Previous studies have shown that frataxin-deficient cells from fibroblasts of FA patients exhibited heightened sensitivity to oxidative stress, which suggests a correlation between frataxin deficiency
and the regulation of antioxidant defence (Paupe et al. 2009). This is reinforced by findings that showed a defective Nrf2 signalling pathway in frataxin-deficient cells (Shan et al. 2013). It is evident that Nrf2-mediated transcription is decreased due to the significant reduction in the expression of Nrf2-target genes such as Gstm1 and Glrx for GSH synthesis, and antioxidant enzymes such as catalase and superoxide dismutase (SOD) (Shan et al. 2013). Moreover, the expression of Nrf2 mRNA was reportedly decreased in frataxin-deficient neurons (D'Oria et al. 2013).

In general, many FA studies examining FA models have demonstrated a dysregulation of the Nrf2 signalling pathway, which could result in an impaired anti-oxidative response. Hence, frataxin deficiency may have a pathological role in this defect that can lead to increased cellular susceptibility to oxidative stress and damage (D'Oria et al. 2013).

1.11 Mitochondrial Homeostasis and Dynamics

The maintenance of MIT homeostasis is critical for proper functioning of the cell. Hence, mitochondria have a network of dynamic processes that tightly regulate its homeostasis and lifecycle, namely MIT fusion and fission, mitophagy, and MIT biogenesis (Frank 2006, Chen et al. 2011, Ikeda et al. 2014). MIT fusion and fission mediates MIT quality control through regulation of its turnover via MIT biogenesis and elimination (Youle and van der Bliek 2012, Ikeda et al. 2014). Due to the exquisite dependence of high energy demanding cells, such as neurons and cardiomyocytes, on mitochondria for energy production, suggests that MIT dysfunction can lead to their demise. Thus, it is important to explore the dynamic processes and functions of the
mitochondrion in order to elucidate the impact of its dysregulation on disease-pathology such as frataxin deficiency in FA.

1.11.1 Mitochondrial DNA

The mtDNA encodes 22 transfer RNAs, two ribosomal RNAs, and 13 essential proteins of oxidative phosphorylation, the quintessential machinery responsible for ATP production (Taanman 1999). Due to limited mtDNA repair enzymes, absence of protective histone molecules and the susceptibility of mtDNA to oxidative damage, mtDNA is prone to mutations, which drives further MIT dysfunction and potentiates a vicious cycle of mtDNA damage (Trifunovic 2006, Chen and Zweier 2014, Cha et al. 2015). Mutations in mtDNA also accumulate with ageing (Corral-Debrinski et al. 1992), or are inherited in a number of human MIT diseases (Leonard and Schapira 2000). The importance of maintaining mtDNA integrity in age-related diseases is demonstrated by mice that carry a mutation in the mtDNA polymerase-γ (Polg), which disables the mtDNA proof reading activity of the enzyme (Trifunovic 2006). As a result, Polg mutant mice accumulate mtDNA mutations during mtDNA replication (Trifunovic 2006) and carry an average of 9 point mutations per 10 kb in cytochrome b, versus 1 mutation per 10 kb in control mice (Trifunovic 2006). The mutant mice develop pathologies associated with aging, including weight loss, osteoporosis, kyphosis, alopecia, cardiomyopathy, anemia and sarcopenia (Trifunovic 2006).

1.11.2 Mitochondrial Dynamics

Mitochondria are dynamic organelles that maintain their homeostasis through processes of MIT fusion and fission, MIT biogenesis, and autophagy to ensure optimal cellular and MIT function
MIT fusion is a process intended to alleviate MIT stress by diluting contents, including senescent enzymes within the partially damaged mitochondria via complementation (Fig. 1.10) (Youle and van der Bliek 2012). On the other hand, MIT fission restores normal MIT morphology and function through the removal of damaged mitochondria components via compartmentalising the damaged components into a daughter organelle for elimination during stress (Fig. 1.10) (Youle and van der Bliek 2012). In addition, MIT fission assists in the formation of new mitochondria and their distribution to daughter cells during cell division, particularly to high energy-demanding regions of cells, including neuronal axons (Youle and van der Bliek 2012, Serasinghe and Chipuk 2017).

The processes of fusion and fission regulate the balance between MIT biogenesis and elimination, which provides MIT quality control (Youle and van der Bliek 2012, Ikeda et al. 2014). Many studies have suggested the involvement of impaired MIT dynamics in the development of pathologies in neuro-degenerative diseases (Youle and van der Bliek 2012, Ikeda et al. 2014, Ishihara et al. 2015, Mena et al. 2015). Therefore, it is important to understand how disruptions in these processes can affect normal MIT physiology, which can contribute to the pathogenesis of diseases, particularly FA.
Figure 1.10: Mitochondrial homeostasis is dynamically maintained by the processes of mitochondrial biogenesis, mitochondrial fusion/fission, mitophagy and apoptosis. The up-regulation of Pgc1α, Nrf1, and Tfam promotes mitochondrial biogenesis. In mammals, mitochondrial fusion is facilitated by mitofusin (Mfn) 1 and 2, and Opa1 for the fusion of the outer and inner mitochondrial membranes, respectively. Mitochondrial fission involves dynamin-related protein 1 (Drp1) that interacts with fission protein 1 (Fis1), which compartmentalizes damaged mitochondrial components into daughter mitochondria for elimination via mitophagy. Decreased ATP levels and membrane potential ($\Delta\psi$), and increased ROS generation are features of damaged mitochondria. These dysfunctional mitochondria are detected by Pink1 and recruits Parkin, which initiates mitophagy and the subsequent formation of the autophagosome to degrade target mitochondria. Damaged mitochondria can also induce apoptosis through the permeabilisation of the mitochondrial membranes, release of cytochrome c that can activate caspase-mediated apoptosis, as well as the release of pro-apoptotic proteins such as apoptosis-inducing factor (AIF).
1.11.2.1 Mitochondrial Fusion

MIT fusion is a dynamic process in which two or more mitochondria fuse together in an attempt to reduce MIT stress that could be induced by senescent or damaged proteins and ROS (Fig. 1.10) (Meeusen and Nunnari 2005, Youle and van der Bliek 2012). This process enables damaged mitochondria to repair their function and prevent the accumulation of mtDNA mutations (Youle and van der Bliek 2012). MIT fusion requires a spatially coordinated fusion of the outer and inner MIT membranes that are different in electrophysiological properties, structure, and composition (Meeusen and Nunnari 2005). Notably in mammals, fusion of the outer and inner MIT membranes are facilitated by members of the membrane-anchored dynamin family, mitofusin (Mfn) 1 and 2, and the single dynamin family member, Opa1, respectively (Cipolat et al. 2004, Frank 2006, de Brito and Scorrano 2008).

1.11.2.2 Mitochondrial Fission

When MIT fusion is unable to restore MIT homeostasis in disease conditions, the dynamic nature of the MIT network shifts towards MIT fission that leads to the removal of damaged mitochondria (Fig. 1.10) (Youle and van der Bliek 2012). MIT fission compartmentalises damaged MIT components into daughter organelles that are to be removed and targeted for elimination (Youle and van der Bliek 2012). In mammals, MIT fission involves the cytoplasmic protein, dynamin-related protein 1 (Drp1), which forms a ring structure to encircle and constrict at a site on the outer MIT membrane upon its interaction with fission protein 1 (Fis1) (Smirnova et al. 2001, Youle and van der Bliek 2012). As a result, MIT fission generates smaller and spherical mitochondria, as opposed to the tubular morphologies observed from MIT fusion (Youle and van der Bliek 2012).
1.11.2.3 Mitophagy

In response to MIT stress, MIT fusion and fission also play an important role in the elimination of irreversibly damaged mitochondria through an autophagic process known as mitophagy (Lemasters 2005, Youle and van der Bliek 2012). The mechanism of mitophagy has been attributed to a number of key molecules, particularly phosphatase and tensin homologue deleted on chromosome 10 (PTEN)-induced putative kinase 1 (Pink1) and Parkin that were identified in models of Parkinson’s disease (Valente et al. 2004, Narendra et al. 2008, Scarffe et al. 2014). Pink1 is a serine/threonine kinase that specifically targets mitochondria while Parkin is an E3 ubiquitin ligase, with mutations in either genes resulting in the early-onset autosomal recessive form of Parkinson’s disease (Narendra et al. 2008, Tanaka et al. 2010). The initiation of mitophagy involves the targeting of damaged mitochondria by Pink1 that recruits and activates Parkin via its phosphorylation at Ser65 on the N-terminal ubiquitin-like domain (Figure 1.1) (Kondapalli et al. 2012, Lazarou et al. 2015).

Pink1 also phosphorylates ubiquitin at Ser65 leading to structurally distinctive properties, which allows for interactions with ubiquitin-binding proteins specific for mitophagy (Kondapalli et al. 2012, Lazarou et al. 2015, Wauer et al. 2015). The phosphorylation of Parkin and ubiquitin by Pink1 leads to the recruitment and subsequent formation of ubiquitin chains on outer MIT membrane proteins, such as Mfn1 and/or Mfn2 (Figure 1.11) (Lazarou et al. 2015). The ubiquitination of Mfn results in the inhibition of MIT fusion and the recruitment of autophagy receptors to promote mitophagy (Figure 1.11) (Ziviani et al. 2010, Scarffe et al. 2014, Lazarou et al. 2015). Therefore, the interaction between Pink1 and Parkin are critical for the initiation and regulation of mitophagy.
Figure 1.11: Pink1-Parkin mediated initiation of mitophagy and inhibition of mitochondrial fusion. Pink1 recognises damaged mitochondria that exhibit mitochondrial dysfunction often characterised by decreased membrane potential ($\Delta \psi$), increased ROS levels, and decreased ATP. As a result, Pink1 accumulates on the outer mitochondrial membrane, which can recruit and activate Parkin via its phosphorylation at Ser65 on the N-terminal ubiquitin-like domain, as well as phosphorylate ubiquitin. Phosphorylated Parkin then recruits and form ubiquitin chains on mitofusin (Mfn) located on the outer mitochondrial membrane, leading to its subsequent proteasomal degradation and inhibition of mitochondrial fusion. As such, the ubiquitination of Mfn promotes mitophagy through the recruitment of autophagy substrates and receptors such as p62, Lc3, and Fundc1 that facilitates the elimination of the targeted mitochondria.

However, Pink1-independent mechanisms may exists, as demonstrated by a recent study where Pink1-deficiency does not inhibit basal mitophagy in multiple high energy demanding tissues, including neural tissue and the heart (McWilliams et al. 2018). Over the past decade, a number of MIT-localised mitophagic markers that interact with the autophagosomal protein, microtubule-associated protein 1A/1B-light chain 3 (Lc3), have also been identified (Novak et al. 2010, Liu et al. 2012, Lazarou 2015). These include Fundc1, BNIP3, NIX, optineurin, and NDP52, that also potentiate mitophagy through their Lc3-interacting regions (LIR) in both a Pink1-dependent and
independent manner (Novak et al. 2010, Liu et al. 2012, Lazarou 2015). A recent addition to this
list of mitophagic markers is autophagy/beclin-1 regulator-1 (AMBRA1) (Di Rita et al. 2018).
AMBRA1 mediates the MIT localisation of the ubiquitin ligase HUWE1 and potentiates Mfn2
ubiquitination and degradation, but also the recruitment of autophagosome via the AMBRA1 LIR
motif (Di Rita et al. 2018).

1.11.2.4 Mitochondrial Biogenesis

In addition to the removal and processing of MIT stress, there is a need for the restoration of MIT
deficits by producing new mitochondria through MIT biogenesis. This results in the replication of
mtDNA and the synthesis and assembly of MIT components. The transcription co-activator,
peroxisome proliferation activator receptor (PPAR) γ-co-activator 1 alpha (Pgc1α), regulates MIT
biogenesis by activating a group of transcription factors, such as the nuclear respiratory factor 1
(Nrf1), and the MIT transcription factor A (Tfam) (Fig. 1.10) (Lin et al. 2005, Manoli et al. 2007,
Coppola et al. 2009, Garcia-Gimenez et al. 2011). These two transcription factors mediate the
transcription of nuclear DNA and mtDNA, respectively (Manoli et al. 2007).

A previous study has shown that Pgc1α was down-regulated in the fibroblasts and lymphoblasts
of FA patients and in neural cells and skeletal muscle of frataxin-deficient mice (Coppola et al.
2009). Interestingly, Pgc1α silencing in fibroblasts from healthy individuals and FA patients
mediated a decrease in frataxin levels (Coppola et al. 2009). However, another study found
contradictory results, where Pgc1α was significantly up-regulated in FA patient fibroblasts and
was associated with increased expression of the downstream MIT transcription factor, Tfam, and
the upstream Pgc1α activators, p38, microtubule associated protein kinase (Mapk) and AMP-activated protein kinase (Ampk) (Garcia-Gimenez et al. 2011). In addition, this latter study also found an associated increase in superoxide anion levels with increased Pgc1α expression in the fibroblasts of two FA patients, which suggests a relationship between oxidative stress and MIT biogenesis in FA (Garcia-Gimenez et al. 2011, Gonzalez-Cabo and Palau 2013). Considering that MIT biogenesis and mitophagy play significant roles in maintaining MIT homeostasis, their dysfunction and alterations in the fusion/fission process could potentially contribute to the pathophysiology of FA (Fig. 1.8).

1.11.3 Mitochondria and Apoptosis
Apoptosis is an active mechanism of programmed cell death in response to stress-inducing or regulatory signals. This process is tightly regulated to facilitate the growth, development, and replication or replacement of cells to maintain normal cellular life cycle. Impairment of MIT function and structure destabilises the cell and initiates a signalling cascade for apoptosis (Qadri et al. 2018). There are a number of mechanisms by which mitochondria induce and mediate the process of programmed cell death in mammals. This often involves the permeabilisation of the MIT membrane with the release of cytochrome c and pro-apoptotic proteins that causes a cascade of apoptotic signalling to execute apoptosis. MIT mechanisms for apoptosis can be caspase-dependent or -independent (for more detail, see (Borutaite 2010, Ulukaya et al. 2011, Shakeri et al. 2017, Kalpage et al. 2018)).
Cytochrome c is an essential component of the respiratory chain that facilitates the transfer of electrons from complex III to complex IV (Kalpage et al. 2018). MIT dysfunction, MIT membrane permeabilisation, and oxidative stress can disrupt the electron transport chain and affect cytochrome c function (Shakeri et al. 2017). In response, mitochondria release cytochrome c to the cytosol to trigger downstream activation of caspases and the formation of a caspase-activated complex, the apoptosome, which leads to apoptosis with the degradation of cellular components (Figure 1.12) (Borutaite 2010). The release of cytochrome c is mediated by protein members of the BCL2 family, such as Bak and Bax, the MIT permeability transition pore (MPTP), and MIT lipids to execute apoptosis (Figure 1.12) (Shakeri et al. 2017). Additionally, cytochrome c activates caspase-3 and -9 in the cytosol via forming the apoptosome complex by binding to and activating the apoptotic protease factor 1 (Apaf1) (Yuan et al. 2013). It is well established that the activation of caspase-3, in turn, liberates the caspase-activated deoxyribonuclease (CAD) from its inhibitor, ICAD, which results in apoptotic features of DNA fragmentation and chromatin condensation (Figure 1.12) (Li et al. 1997, Enari et al. 1998, Shakeri et al. 2017).
Figure 1.12: Mitochondrial caspase-dependent and caspase-independent mechanisms of apoptosis. Mitochondrial dysfunction leads to the permeabilisation of its membranes, which is the first step towards apoptosis. Membrane permeabilisation of the outer mitochondrial membrane is driven by mitochondrial permeability transition pore (MPTP), members of the BCL2 protein family (i.e. Bak/Bax), and mitochondrial lipid such as cardiolipin. More specifically, cardiolipin is associated with the recruitment of Bax to the outer mitochondrial membrane to trigger membrane permeabilisation. For the caspase-dependent mechanism of apoptosis, cytochrome c is released by the mitochondria to trigger the formation of the apoptosome complex by binding to and activating the apoptotic protease activating factor 1 (Apaf1). This in turn activates caspase-9 and -3, which leads to the release of CAD from its inhibitor, ICAD. This causes apoptosis resulting from DNA fragmentation and chromatin condensation. The caspase-independent mechanism involves the mitochondrial release of pro-apoptotic proteins such as apoptosis-inducing factor (AIF) into the cytosol whereby it can either directly interact with DNA or potentiate oxidative stress in the mitochondria through its release to induce apoptosis.
Oxidised lipids also play an important role in the induction of apoptosis (Gonzalvez and Gottlieb 2007, Fruhwirth and Hermetter 2008). Cardiolipin is the mitochondria-specific lipid whose oxidation results in MIT membrane permeability and the recruitment of the pro-apoptotic protein, Bax (Figure 1.12) (Gonzalvez and Gottlieb 2007, Lucken-Ardjomande et al. 2008, Dingeldein et al. 2018). Cytochrome c is normally associated with cardiolipin in the inner MIT membrane (Ott et al. 2002, Santambrogio et al. 2007). The oxidation of cardiolipin results in both MIT membrane permeabilisation and cytochrome c dissociation and release (Petrosillo et al. 2001, Ott et al. 2002, Santambrogio et al. 2007).

Alternatively, following MIT dysfunction, oxidative stress, or a decrease in ATP levels, a caspase-independent mechanism of MIT-associated apoptosis may also be induced (Borutaite 2010). This involves permeabilisation of the outer and inner MIT membranes, whereby the mitochondria releases pro-apoptotic proteins, such as apoptosis-inducing factor (AIF), into the cytosol to regulate apoptosis (Figure 1.12) (Borutaite 2010). The translocation of AIF from the mitochondria to the cytosol occurs in a BCL2-controlled manner in which cytosolic AIF can travel further into the nucleus where it causes DNA fragmentation and chromatin condensation (Figure 1.12) (Ye et al. 2002, Lorenzo and Susin 2004). Furthermore, the MIT release of AIF can also increase oxidative stress due its potential role in maintaining ROS levels generated by the respiratory chain (Figure 1.12) (Lorenzo and Susin 2004).
Previous studies on diabetic neuronal injury have also shown a MIT profile of decreased MIT membrane potential and BCL2 expression, accompanied by ROS generation and increased expression of pro-apoptotic proteins (Yang et al. 2018). Similar MIT alterations that mediate apoptosis are found in cardiac ageing and pulmonary hypertension (Marshall et al. 2018, Martin-Fernandez and Gredilla 2018). In many of these diseases, MIT oxidative stress appears to be a key feature of MIT dysfunction that drives apoptosis in disease progression.

1.12 Potential Treatment of FA

1.12.1 Antioxidants, Nrf2 and GSH Inducers

It has been shown in vivo that GSH levels can be supplemented by N-acetylcysteine (NAC), which acts on Nrf2 and elevates the biosynthesis of GSH, thus increasing antioxidant protection (Ji et al. 2010). Notably, NAC has shown potential therapeutic effects with an increase in GSH reductase activity in mouse models of Alzheimer’s disease (Huang et al. 2010). These studies have led to clinical trials investigating the effects of NAC as a GSH precursor to induce GSH synthesis in patients with Alzheimer’s disease (Johnson et al. 2012).

Nrf2 is a transcription factor responsible for the expression of genes important for antioxidant defence and maintaining homeostasis of GSH and ROS (Harvey et al. 2009, Zhuang et al. 2014). The overall effect of Nrf2 expression is an increase in reducing potential, making cells more resistant to oxidative insult (Calkins et al. 2009). Therefore, agents that promote cellular Nrf2 expression may provide a potential therapeutic avenue for the prevention of neurodegeneration in FA and other diseases, such as Parkinson’s disease and Alzheimer’s disease (Calkins et al. 2009).
There are a variety of antioxidants designed for the treatment of FA that are under development (Strawser et al. 2014). For instance, the ubiquinone analogue, idebenone, functions to scavenge free radicals in order to potentially restore MIT ATP production (Rustin et al. 1999). Studies have found that idebenone can improve cardiac and neurological functions of FA patients (Rustin et al. 1999), although clinical trials have received contradictory results (Artuch et al. 2002, Lagedrost et al. 2011). Another antioxidant, Coenzyme Q10, is a lipophilic molecule structurally similar to idebenone that functions to facilitate MIT electron transport (Parkinson et al. 2013b). In addition, α-tocopheryl quinone is a potent antioxidant that was found to cause a dose-dependent improvement in neurological functions among adult FA patients (Lynch et al. 2012). The use of antioxidants for the treatment of FA has been ongoing and further assessments are required to verify the effectiveness of these agents.

1.12.2 Treatment with Antioxidants and Iron Chelators

The potential treatment of FA with iron chelators has been assessed in previous studies (Rustin et al. 1999, Richardson 2003, Boddaert et al. 2007, Goncalves et al. 2008, Kakhlon et al. 2008). Since iron accumulates in the mitochondria of FA patients (Delatycki et al. 1999, Puccio and Koenig 2000), it has been suggested that the chelation of iron would lower its toxicity (Becker and Richardson 2001, Richardson 2003). Moreover, it has been shown that chelating and removing the MIT iron-load reduced cardiac hypertrophy in vivo (Whitnall et al. 2008). Nevertheless, chelation did not entirely reverse the phenotype, as it did not rescue the function of frataxin in MIT iron metabolism (Whitnall et al. 2008). Similarly, treatment strategies utilising antioxidants alone, including idebenone and CoQ10/vitamin E have demonstrated mixed results (Schulz et al. 2000,
Di Prospero et al. 2007, Cooper et al. 2008). Subsequent studies in FA patients demonstrated that therapies which combine the iron chelator, deferiprone, and the antioxidant, idebenone, significantly improved the heart hypertrophy and reduced the iron deposits in the dentate nucleus (Velasco-Sanchez et al. 2010). In addition, this combination treatment induced a stabilising effect in the neurologic dysfunction, as shown by improved kinetic functions in the patients (Rustin et al. 1999, Velasco-Sanchez et al. 2010). In the absence of an appropriate therapy to replace frataxin, these studies demonstrate the potential of the combination of using both an iron chelator and antioxidant for the treatment of FA. However, it is essential to further assess the effectiveness of this combined therapy in comprehensive animal and clinical studies, as it could result in new therapeutic avenues for FA patients.
1.13 Aims and Significance

1.13.1 Aims of the Thesis

The Introduction above underscored the significant involvement of oxidative stress and MIT dysfunction in the aetiology of FA. However, there are major gaps in our understanding of how these pathological features drive disease progression, especially the cardiomyopathy of FA. Specifically, the dysregulation of cellular anti-oxidative response via the Nrf2 signalling pathway is yet to be fully elucidated. The molecular mechanisms involved in MIT homeostasis are also poorly understood in the condition of frataxin deficiency.

This thesis sought to unravel these voids in our knowledge of FA pathology. Furthermore, the lack of effective treatments available for FA patients warrant for the development of novel therapeutics. Hence, by considering the relevance of the aforementioned pathological features of FA, this thesis also aimed to assess the potential application of two therapeutic strategies to treat the cardiomyopathy of this disease.

The aims of this thesis were as follow:

1. To elucidate in vivo the molecular mechanisms involved in the dysregulation of the Nrf2 signalling pathway for anti-oxidative response against oxidative stress, specifically the cause for the down-regulation of Nrf2 expression, in the frataxin-deficient heart.
2. To explore *in vivo* the dynamic MIT processes, namely, MIT fusion and fission, mitophagy, and MIT biogenesis, in the maintenance of MIT homeostasis and function in the heart under the condition of frataxin deficiency, which is associated with oxidative stress.

3. To assess the therapeutic efficacy of two agents independently, namely, NAC in the supplementation of GSH, and the novel compound SNH6 in the supplementation of NAD\(^+\) to restore energy metabolism, in an attempt to rescue FA cardiomyopathy *in vivo* over time.

**1.13.2 Significance of the Thesis**

The studies in this dissertation highlighted the importance of antioxidant defence and MIT homeostasis in maintaining cell vitality, and that the disruption of these crucial cellular and MIT functions greatly contributes to the pathogenesis of FA. Importantly, this thesis encourages the development of novel therapeutics that could target these deficits associated with frataxin deficiency to restore cardiac function. Thus, the research of this thesis not only furthered our understanding of FA pathology, but it also holds the potential in the future to translate the development of innovative therapeutics for other cardio- and neuro-degenerative diseases.
CHAPTER TWO

Materials and Methods

This Chapter is adapted and modified from the publication and work below where I am the first or equal first author:


2.1 Materials

2.1.1 Chemicals and General Reagents

Chemicals and reagents commonly used in experimental procedures of this thesis are listed below in Table 2.1.

Table 2.1: Chemicals and General Reagents.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Agarose</td>
<td>Amresco</td>
</tr>
<tr>
<td>Ammonium persulphate (APS)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Bicinchominic acid (BCA) protein assay</td>
<td>Thermo Fisher Scientific</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Deoxynucleotide triphosphates (dNTPs)</td>
<td>Promega</td>
</tr>
<tr>
<td>Diethylpyrocarbonate (DEPC)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Dimethylsulphoxide (DMSO)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>ECL chemiluminescence reagent</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Ethanol (Spectrasol grade)</td>
<td>Labtech</td>
</tr>
<tr>
<td>Ethidium bromide (EtBr)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Chemical</td>
<td>Supplier</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Formalin</td>
<td>Fornine</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Isofluorane</td>
<td>Abbott Australasia</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Methanol</td>
<td>LabServ</td>
</tr>
<tr>
<td>Non-fat milk powder</td>
<td>Diploma</td>
</tr>
<tr>
<td>Phosphatase inhibitor</td>
<td>Roche</td>
</tr>
<tr>
<td>Phosphate-buffered saline (PBS) powder</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Polyvinyl difluoride (PVDF) membrane</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>Roche</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>Amresco</td>
</tr>
<tr>
<td>Sodium fluoride (NaF)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TEMED (N, N,N',N'- tetramethylethlenediamine)</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Tris(hydroxymethyl)aminomethane (Tris)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TRizol® Reagent</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Trypan blue</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
2.1.2 Buffers and Solutions

Commonly used buffers and solutions in experimental procedures of this thesis are listed below in Table 2.2. All buffers were prepared with de-ionised water (dH₂O) with the exception for buffers and reagents used for RNA analysis that were prepared with DEPC-treated water instead. DEPC-treated water (0.1%) was left overnight before autoclaved for three times to ensure complete inactivation of DEPC.

Table 2.2: Buffers and Solutions.

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysis buffer</td>
<td>150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1.5% Triton X-100, 0.5% SDS, 1 mM EDTA, 1 mM EGTA, 0.04 mM NaF</td>
</tr>
<tr>
<td>SDS-PAGE running buffer (10x)</td>
<td>247.65 mM Tris, 1.92 M Glycine, 34.68 mM SDS, pH 8.3</td>
</tr>
<tr>
<td>Separating gel buffer</td>
<td>749.55 mM Tris, 6.94 mM SDS, pH 8.8</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>250.12 mM Tris, 6.94 mM SDS, pH 6.8</td>
</tr>
<tr>
<td>Transfer buffer (10x)</td>
<td>247.65 mM Tris, 1.92 M Glycine</td>
</tr>
<tr>
<td>Tris-buffered saline (TBS; 20x)</td>
<td>25mM Tris, 2.75 mM NaCl, pH 7.6</td>
</tr>
<tr>
<td>TBST (1x)</td>
<td>1x TBS, 0.1% (v/v) Tween-20</td>
</tr>
<tr>
<td>Tris-acetate EDTA (TAE) buffer (20x)</td>
<td>800 mM Tris, 2.2% glacial acetic acid, 100 mM EDTA</td>
</tr>
</tbody>
</table>
2.2 Animals

2.2.1 Animal Model

All experiments of this thesis were performed in the muscle creatine kinase (MCK) conditional \textit{frataxin} KO mice cardiac model of FA. These animals are transgenic C57B1/6 mice that harbour the MCK promoter-driven Cre recombinase expression such that they are homozygous for the deletion of \textit{frataxin} exon4 for the KO mice (Puccio \textit{et al.} 2001).

2.2.2 Animal Monitoring and Ethics

All animal work with experimental mice followed strict ethic guidelines approved by the University of Sydney. The animals were housed under a 12 h light-dark cycle, and routinely fed with basal rodent chow (0.02\% iron/kg) and provided water \textit{ad libitum}. All mice were monitored daily for general health, behaviour, and housing conditions, and their body weight were recorded twice weekly.

2.2.3 Necroscopy

Mice were weighed and anaesthetised with isofluorane before sacrificed by cervical dislocation at the end of experiments. Organs were removed, weighed, and processed for further analysis such as RNA and or protein isolations, histological examination (organ preserved in 10\% neutral buffered formalin), glutathione assay \textit{etc}. 

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2.2.4 Breeding and Genotyping

The MCK \textit{frataxin} KO mice model was developed and bred by Drs. H. Puccio and M. Koenig (Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM, Universite Louis Pasteur, Strasbourg, France) \cite{Puccio2001}. The mice were bred and handled according to the protocol approved by the University of Sydney’s Animal Ethics Committee, and genotyping was performed using tail DNA using standard techniques \cite{Puccio2001}.

2.3 Protein Processing and Analysis

2.3.1 Protein Extraction from Animal Tissues

Heart and skeletal muscle tissue (left and right quadriceps) from euthanised mice were harvested for protein extraction. The heart was perfused and rinsed with PBS (pH 7.4) to remove excess blood before snap frozen in liquid nitrogen and stored at -80 °C. When required for protein extraction, tissue samples were thawed on ice, then appropriate volume of ice-cold lysis buffer (\textit{Section 2.1.2}) and 4\% (v/v) protease inhibitor cocktail was added. A 10\% (v/v) solution of phosSTOP phosphatase inhibitor was added with the lysis buffer when the tissue sample was used for the detection of phosphorylated proteins. Tissue samples were homogenised on ice using a Dounce glass homogeniser in lysis solution. The lysates were then sonicated on ice for three times of ten successive 2 s short bursts for a total of thirty times, followed by centrifugation at 13,200 \textit{x g} for 40 min at 4 °C. The supernatant contains cellular proteins that were collected, and the protein concentration was determined using BCA Protein Assay (ThermoFisher Scientific; Waltham, MA, USA) \textit{via} measuring the absorbance at 562 nm using UV spectrophotometry (UV spectrophotometer UV-1800; Shimadzu; Kyoto, Japan).
2.3.2 Western Blot Analysis

Protein lysates at 50 µg protein concentration were first heat denatured at 95 ºC for 2 min in the presence of β-mercaptoethanol. Protein samples were then loaded per lane alongside protein molecular weight marker (Bio-Rad, Sydney, Australia) onto SDS-PAGE gel (8, 10, or 12%) and underwent electrophoresis at 80 V for the first 15 min and then 120 V for 60 min in 1x SDS-PAGE running buffer. Proteins were then transferred onto PVDF membrane (0.45 μm, Merck Millipore, USA) overnight at 30 V in 4 ºC. Membranes were then blocked for 1 h at room temperature in either 5% (w/v) non-fat skim milk or 5% (w/v) BSA prepared in 1x TBST on a platform rocker. Both primary and secondary antibodies were diluted in either 5% milk/TBST or 5% BSA/TBST. Membranes were incubated overnight at 4 ºC on a rocker with primary antibody, followed by three 10 min washes with 1x TBST on a rocker. The membranes were then incubated for 1 h on a rocker with secondary antibody and then washed four times in 1x TBST 5 min each. The antigen-antibody complex on the membranes were detected using electrochemiluminescence (ECL) system with either Luminata Classico Western HRP substrate, Luminata Crescendo Western HRP substrate, or Luminata Forte Western HRP substrate. The chemiluminescence signals from the developing membrane were captured using the ChemiDoc MP imaging system (Bio-rad; CA, USA).
2.3.3 Antibodies

Commonly used antibodies for experimental procedures of this thesis are listed below in Table 2.3

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog Number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Loading Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-mouse Gapdh</td>
<td>2118S</td>
<td>Cell Signaling Technology</td>
</tr>
<tr>
<td><strong>Secondary antibody conjugated with horseradish peroxidase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-mouse</td>
<td>A9917</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Goat anti-rabbit</td>
<td>A0545</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

2.4 RNA Processing and Analysis

2.4.1 RNA Isolation from Animal Tissues

Heart tissue from the MCK mice were harvested and homogenised on ice using a motorised homogeniser. The probes used for homogenisation were pre-treated with DEPC H₂O overnight and autoclaved. Total RNA was isolated from animal tissues using 1 mL volume of TRIzol® Reagent per tissue following manufacturer protocol (Invitrogen, Carlsbad, CA). Briefly, homogenised tissue samples were incubated at room temperature for 5 min, followed by 0.2 mL of chloroform added to the samples and shaken vigorously for 15 s. Samples were incubated at room temperature for another 3 min, then centrifuged at 12,000 x g for 15 min at 4 °C. The aqueous phase of the supernatant was then transferred and mixed with 0.5 mL volume of isopropanol. The samples were incubated for 10 min at room temperature followed by centrifugation where the supernatant was removed, and the RNA pellet washed in 75% sterile ethanol. The RNA pellets
were resuspended in DEPC-treated H₂O and the RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and determined using the UV-1800 spectrophotometer (Shimadzu; Kyoto, Japan).

2.4.2 Semi-Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

Isolated total RNA from Section 2.4.1 was used to perform reverse transcriptase-polymerase chain reaction (RT-PCR) using standard procedures (Anzovino et al. 2017). The RT-PCR reaction mixture of a total volume of 12.5 µL contains 0.5 µg of RNA, 2x Reaction Mix (1.6 mM MgSO₄ and 200 µM dNTP), 0.2 µM of each sense and anti-sense gene specific primers, and 1 µL of Superscript™ III RT/Platinum® Taq Mix (Invitrogen). Briefly, the reverse transcription reaction mixture was performed according to manufacturer’s protocol using a Bio-Rad PCR Thermocycler. Samples were denatured at 94 °C for 2 min, followed by amplification for 25 – 40 PCR cycles, which included a 94 °C denaturation step for 15 s, a 56-60 °C annealing step for 30 s, a 68 °C extension step for 60 s, and a final extension time at 68 °C for 5 min. The house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (Gapdh), was used as an internal control and was amplified from the same samples. Every RT-PCR performed included a negative control reaction that contains no primers. The products from RT-PCR were then mixed with 6x loading dye, and then separated via electrophoresis on a 1.5% agarose gel in 1x TAE buffer (Section 2.1.2) with 1 µg/mL concentration of EtBr. Electrophoresis was performed at 85 V for 45 min. Visualisation was conducted using the Bio-Rad ChemiDoc XRS+ System (Bio-rad) where the gels were transilluminated with UV light.
2.5 Data Analysis

2.5.1 Densitometry

Densitometric analysis was performed to quantify the intensities of data obtained from Western blot analysis, and RT-PCR. Quantification of band intensities was achieved using Quantity One software (Bio-Rad). Band intensities of target proteins were normalised relative to the internal loading control, Gapdh.

2.5.2 Statistical Analysis

Experimental data were compared using Student’s t-test. Results are expressed as mean ± standard error of the mean (SEM). Data were considered statistically significant when $p<0.05$. In figures, (* and #) refers to $p<0.05$, (** and ##) refers to $p<0.01$, and (***) and ###) refers to $p<0.001$. 
CHAPTER THREE

Molecular Alterations in a Mouse Cardiac Model of Friedreich’s Ataxia:
An Impaired Nrf2 Response Mediated via Up-Regulation of Keap1 and Activation of the Gsk3β Axis.

This Chapter is adapted and modified from the publication below where I am an equal first author:

3.1 Introduction

Friedreich’s ataxia (FA) is a cardio-degenerative and neuro-degenerative disorder caused by reduced expression of the mitochondrial (MIT) protein, frataxin (Campuzano et al. 1996, Campuzano et al. 1997). In the heart, the loss of frataxin results in a fatal cardiomyopathy leading to premature patient death (Koeppen 2011). Surprisingly, little is known about the molecular dysfunction in FA, particularly in the heart, although it is usually ascribed to MIT dysfunction (Payne et al. 2011). Electron micrographs of cardiac tissue from FA patients and from frataxin knockout (KO) mice demonstrate MIT proliferation, loss of contractile sarcomeres and distinct iron deposits within the mitochondria (Michael et al. 2006, Whitnall et al. 2012, Huang et al. 2013). It is hypothesised that the iron deposits within the highly oxidation-reduction (redox)-active environment of the mitochondrion can generate hydroxyl radicals through the Fenton reaction and cause oxidative stress (Dröge 2002). In fact, these MIT iron accumulations are not sequestered within MIT ferritin and appear as inorganic crystallites that have the potential to participate in reactive oxygen species (ROS) generation (Whitnall et al. 2012).

Oxidative stress is well described in FA (Schulz et al. 2000, Sparaco et al. 2009), with evidence of hydroxyl radical formation, oxidative damage to DNA and lipid peroxidation markers being identified in patient blood and urine samples (Emond et al. 2000, Schulz et al. 2000). Furthermore, histology of FA patient spinal cord samples demonstrates increased glutathionylation, a protein modification caused by oxidative insult (Sparaco et al. 2009). In cell culture, FA patient fibroblasts exhibit increased oxidative modifications on treatment with ferrous salts, causing impaired cytoskeletal protein function and increased sensitivity to oxidative stress (Pastore et al. 2003). When challenged with low doses of H$_2$O$_2$ or oligomycin, FA patient fibroblasts were unable to
induce superoxide dismutase (SOD) activity, resulting in higher cell lethality compared to healthy controls (Chantrel-Groussard et al. 2001). However, in these latter studies, the reason for the failed induction of SOD was not addressed. Oxidant-inactivating enzymes such as SOD, and intracellular antioxidants, including glutathione (GSH) and thioredoxin, are the primary defence mechanisms of eukaryotic cells against ROS (Kensler et al. 2007). However, perturbed antioxidant defence in the myocardium has not been investigated in the development of the fatal cardiomyopathy in FA.

The primary form of regulation of antioxidant defence occurs via de novo gene transcription (Kensler et al. 2007). Regulation of a broad range of antioxidant genes is mediated by a consensus sequence located in the promoter region known as the antioxidant response element (ARE) (Nioi et al. 2003, Nguyen et al. 2009). The AREs have been identified in hundreds of genes responsible for the detoxification of cellular ROS, collectively referred to as Phase II (detoxifying and antioxidant proteins) enzymes. The induction of the antioxidant response is controlled by the cap’n’collar bzip transcription factor, nuclear factor-erythroid 2-related factor-2 (Nrf2) (Kensler et al. 2007, Niture et al. 2014). Two post-translational mechanisms exist to regulate Nrf2 activity in both the cytosolic and nuclear compartments. These mechanisms are as follows: Kelch-like ECH-associated protein1 (Keap1)-mediated sequestration of Nrf2 in the cytosol, which targets Nrf2 for proteasomal degradation; and glycogen synthase kinase-3β (Gsk3β)-dependent regulation of nuclear Nrf2, that leads to the phosphorylation, exportation and degradation of nuclear Nrf2 (Bryan et al. 2013).
Another level of antioxidant regulation comes from the transcriptional repressor, BTB domain and CNC homolog 1 (Bach1), which competes with Nrf2 for binding to AREs (Dhakshinamoorthy et al. 2005, Kaspar and Jaiswal 2010). In unstressed conditions, Bach1 is bound as a heterodimer with small Maf proteins to AREs, preventing the transcription of Phase II genes (Dhakshinamoorthy et al. 2005, Kaspar and Jaiswal 2010). Interestingly, the activity and nuclear localisation of Bach1 is haem-regulated (Sun et al. 2004, Suzuki et al. 2004). When haem is bound to Bach1, its DNA-binding activity is decreased, which induces nuclear export of Bach1, reducing its repressive activity on the ARE (Suzuki et al. 2004). Considering that haem synthesis is markedly depressed in the heart of an FA mouse model (Huang et al. 2009), the expression and localisation of Bach1 is important to assess. Notably, a decrease in Nrf2 expression has been noted in FA models, although the mechanism involved in reducing Nrf2 levels has not been deciphered (Paupe et al. 2009, Shan et al. 2013).

To examine Nrf2 function and redox homeostasis in FA cardiomyopathy, the hearts from an established FA mouse model, the muscle creatine kinase (MCK) frataxin KO mouse, was examined in this study (Puccio et al. 2001, Huang et al. 2013). In these mice, the MCK promoter-driven CRE recombinase specifically excises frataxin in the striated muscle (i.e., both cardiomyocytes and skeletal muscle cells) (Puccio et al. 2001). By 9-weeks of age, the KO mouse progressively develops a dilated cardiomyopathy, leading to heart failure, as in the human disease (Puccio et al. 2001, Huang et al. 2013). Interestingly, and in marked contrast, despite the complete loss of frataxin, skeletal muscle pathology was not observed (Puccio et al. 2001). The MCK KO mouse mimics the altered iron metabolism observed in FA patients, including the accumulation of

In the current investigation, the MCK KO mouse was examined to assess redox stress and the antioxidant response. This study revealed significant protein and GSH oxidation associated with ROS formation in the KO relative to WT littermates in the heart, but not in the skeletal muscle. Both total and nuclear Nrf2 expression was also found to be significantly decreased in the KO relative to the WT mouse heart. On investigation of well-characterised Nrf2 regulators (Bryan et al. 2013), the current studies have demonstrated increased Keap1 expression and enhanced activation of nuclear export machinery via Gsk3β signalling, which are processes involved in decreasing cytosolic and nuclear Nrf2, respectively. These mechanisms would explain the observed decrease in nuclear Nrf2, Nrf2 ARE-binding activity and the general decrease in the mRNA levels of antioxidant genes targeted by Nrf2 in the frataxin KO heart. In contrast to the heart, there were no significant alterations to the Nrf2 pathways in the frataxin KO skeletal muscle. Paradoxically, despite the general reduction in Nrf2 downstream antioxidant response genes at the mRNA level, their protein levels were either not reduced or significantly increased. This finding indicates that other pathways could at least partially compensate for the reduced Nrf2 levels and its transcriptional activity. Nonetheless, despite this compensation, oxidative damage still occurred in the heart, indicating the potential for antioxidant therapy for the treatment of this condition.
3.2 Materials and Methods

3.2.1 Animals

Transgenic C57Bl/6 mice harbouring MCK promoter-driven Cre recombinase expression that are homozygous for deletion of Frataxin exon4 (KO) and their WT littermates were used and genotyped, as described previously (Puccio et al. 2001). All animal work was approved by the University of Sydney’s Animal Ethics Committee (Sydney, New South Wales, Australia).

3.2.2 Histology

MCK WT and KO mouse littermates were weighed and euthanised. The heart and skeletal muscle from the superficial part of the quadriceps were excised, freed from connective tissue and fat, washed in cold saline, blotted dry, weighed and fixed in 10% formalin. Muscle samples were then cut and embedded in paraffin blocks, divided into sections, and stained with Perls’ Prussian blue, Gömöri trichrome or hematoxylin and eosin (H&E). Muscle fibre size was measured by ImageJ (National Institutes of Health, Baltimore, MD) (Schneider et al. 2012). Measurements were taken from at least 3 images from different animals (5WT and 6KO) and there were 10 measurements per image.

3.2.3 Assessment of Protein Oxidation by HPLC

Measurement of tyrosine oxidation products as a marker of protein oxidation in MCK mouse heart and skeletal muscle were performed by high-performance liquid chromatography (HPLC), as performed previously (Hawkins et al. 2009). Briefly, whole heart or skeletal muscle (left and right quadriceps) from WT and KO MCK mice were perfused with PBS (pH 7.4) to thoroughly remove
blood and then immediately snap frozen in liquid nitrogen. Frozen tissue samples were then ground to a fine powder using a mortar and pestle cooled with liquid nitrogen. Samples were re-suspended in PBS before precipitation of the proteins with trichloroacetic acid (10% w/v) and acid hydrolysis of the resulting protein pellets with hydrochloric and thioglycolic acid under vacuum overnight at 110°C. Samples were then separated by HPLC, detected using a UV-Vis and fluorescence detector and analysed against analytical standards, as described (Hawkins et al. 2009).

3.2.4 Glutathione Measurement

Total, reduced, and oxidised GSH were measured using Caymans Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI), following the manufacturer’s instructions. Briefly, WT and KO whole heart or skeletal muscle (left and right quadriceps) was perfused with PBS (pH 7.4) to remove blood before extraction. Homogenisation of tissues was done on ice in 5 mL of cold MES buffer [containing 50 mM 2-(N-morpholino) ethanesulphonic acid (pH 6.0) and 1 mM EDTA] using a motorized homogeniser. Samples were then centrifuged at 10,000 x g/15 min/4 °C and the supernatant removed for de-proteination as follows. Fresh metaphosphoric acid (Sigma-Aldrich, St. Louis, MO) was prepared and added in equal volume to the sample and vortex mixed. Samples were then incubated at room temperature for 5 min and centrifuged at 1,500 x g/2 min/4 °C. The supernatant was collected and freshly prepared 4 M triethanolamine (Sigma-Aldrich) was added to the samples and mixed by vortex mixing. Samples were then diluted 1:20 with MES buffer before performing the assay. A plate reader was used to measure the absorbance, which was read at 405 nm at 5 min intervals for 30 min. GSH and oxidised GSH (GSSG) levels were expressed as a ratio.
3.2.5 Protein Extraction

Whole hearts and skeletal muscle (left and right quadriceps) from WT and KO MCK mice were perfused with PBS (pH 7.4) to remove excess blood prior to removal. Tissue was homogenised using a Dounce glass homogeniser in lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.5% (w/v) sodium dodecyl sulphate (SDS), 1 mM EDTA, 40 μM NaF, 1% (v/v) Triton X-100] containing a 1x solution of PhosSTOP (Roche Diagnostics, Risch-Rotkreuz, Switzerland) and a 1x solution of protease inhibitor cocktail (Roche Diagnostics). Lysates were then sonicated on ice and centrifuged at 13,200 x g/40 min/4 °C. The supernatant was collected, and the protein concentration was determined by the BCA Protein Assay (Pierce Biotechnology, Rockford, IL).

3.2.6 Nuclear and Cytosolic Fractions

Cytosolic and nuclear fractions were prepared from MCK whole hearts and skeletal muscle (left and right quadriceps) using NE-PER nuclear and cytosolic extraction reagents (Thermo Fisher Scientific, Waltham, MA). Fractionation was performed according to the kit instructions. Tissue samples were first homogenised in a Dounce glass homogeniser. Tissue lysates were then supplemented with a 1x solution of protease inhibitor (Roche Diagnostics) and 1x solution of PhosSTOP (Roche Diagnostics).

3.2.7 Protein Separation and Western Blot Analysis

Protein lysates were heat denatured at 95 °C/2 min in the presence of β-mercaptoethanol. Then, 50 μg of protein or molecular weight marker (Bio-Rad, Hercules, CA) were loaded onto an 8% to 12% polyacrylamide gel and separated using SDS-PAGE. Proteins were then transferred overnight
Membranes were then blocked for 1 h at room temperature in either 5% skim milk prepared in Tris-buffered saline and 0.1% Tween-20 (TBST) or 5% BSA/TBST. Primary antibodies were diluted in either 5% skim milk/TBST or 5% BSA/TBST and added to membranes and incubated overnight at 4 °C on a rocker. Antibodies used were against Nrf2 (Santa Cruz Biotechnologies, Dallas, TX; catalog number sc-722), Bach1 (Santa Cruz; catalog number sc-14700), Keap1 (ProteinTech, Rosemont, IL; catalog number 10503-2-AP), phosphorylated Gsk3β Serine 9 (Cell Signaling, Danvers, MA; catalog number 9332) and Tyrosine 216 (Abcam, Cambridge, MA; catalog number ab75745), Gsk3β (Cell Signaling; catalog number 9832), phosphorylated Fyn threonine 12 (Santa Cruz; catalog number sc-16848), Fyn (Cell Signaling; catalog number 40238), phosphorylated tyrosine (Cell Signaling; catalog number 9416), β-TrCP (Cell Signaling; catalog number 11984), Gapdh (Cell Signaling; catalog number 2118) and Hdac1 (Santa Cruz; catalog number sc-8410). Antibodies were used at 1:500-1:2000. Secondary antibodies used were goat anti-rabbit, rabbit anti-goat, goat anti-mouse (A0545, A5420, A9917, respectively; 1/10000; Sigma-Aldrich) conjugated with horseradish peroxidase.

3.2.8 Immunoprecipitation

Whole hearts were homogenised in 500 μL of ice-cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1x solution of protease and phosphatase inhibitors (Roche Diagnostics)] using a Dounce glass homogeniser. The resulting lysate was incubated for 30 min/4°C plus rotation to ensure complete lyses of cells and then centrifuged at 13,200 x g/40 min/4°C. The supernatant was collected in a new sterile Eppendorf tube and the protein
concentration was determined using the BCA protein assay (Pierce Biotechnology). Then 30 μL of Dynabeads protein G (Novex; Thermo Fisher Scientific) per sample were placed in Eppendorf tubes and washed twice with RIPA buffer. After the second wash, the beads were re-suspended in 1 mL of RIPA buffer and 4 μg of Nrf2 antibody (Cell Signaling; catalog number 12721) per tube was added. The primary antibody was conjugated to the beads after incubation for 2 h/4 °C. After incubation, the conjugated beads were collected on a magnet and the supernatant removed. The conjugated beads were washed twice with ice-cold RIPA buffer and the last wash discarded.

Then, 500 μg of protein lysate was added per tube of conjugated beads and the volume was increased to 1 mL. Lysates and antibody-conjugated beads were incubated at 4 °C plus rotation overnight. The next day, the beads were collected on a magnet and the supernatant was discarded. The beads were washed 3 times with ice-cold RIPA buffer and then re-suspended in 35 μL of 1x loading dye plus β-mercaptoethanol. The samples were incubated at 5 min/95 °C to disassociate the complexes from the beads. The beads were then collected on the magnet, the supernatant was separated on a 10% polyacrylamide gel and Western blot was performed, as described above.

3.2.9 Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) reactions were conducted in 20 mM HEPES (pH 7.9) containing 1 mM EDTA, 50 mM KCl, 5 mM MgCl2, 4% (v/v) glycerol, 1 mM dithiothreitol, 3 μg/mL poly(dI-dC), 36-bp end-labelled quinone 1 (Nqo1) ARE sequence (5’-AGTCTAGAGTCACAGTGAGTGCCAAAATTTGAGCC-3’, corresponding to nt-451 to -416) and 20 μg of nuclear protein. In competition experiments, a 200-fold molar excess of unlabelled
36-bp Nqo1 ARE sequence was also included in the reaction. Reactions were incubated for 30 min/20 °C before being subjected to electrophoresis under native conditions using a 6% (w/v) poly-acrylamide (1:75 bis-acrylamide:acrylamide) gel in 0.5x TBE buffer at 4 °C. Gels were then transferred onto Immobilon-Ny+ membrane and detected by Chemiluminescent Nucleic Acid Detection Module Kit (Thermofisher Scientific, catalog number 89880).

3.2.10 RNA Isolation from Tissue

Whole hearts from WT and KO MCK mice were homogenised using a motorized homogeniser. Homogeniser probes had been treated in DEPC water overnight and sterilised before use. RNA extraction was done using TRIzol® Reagent, following manufacturer’s protocol (Invitrogen, Carlsbad, CA) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

3.2.11 Quantitative Real-Time RT-qPCR

Total mRNA was obtained from whole hearts and used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was performed using the Roche Lightcycler 480 (Roche Diagnostics). Taqman probes (Thermo Fisher Scientific) targeting mouse Nrf2 (Mm00477784_m1), Nqo1 (Mm01253561_m1), TxnRD1 (Mm00443675_m1), Gstm1 (Mm00833915_g1), Sod2 (Mm01313000_m1), catalase (Mm00437992_m1) and Gapdh (Mm99999915_g1) were used. Standard curves were generated for each probe and samples were fitted to the linear portion of the curve. Data was analysed using Genex Software (MultiD Analyses, Göteborg Sweden).
3.2.12 Statistical Analysis

Data were compared using Student’s $t$-test. Data were considered statistically significant when $p<0.05$. Results are expressed as mean ± SEM.
3.3 Results

3.3.1 Identification of Oxidative Stress in the Frataxin-Deficient Heart, but not the Skeletal Muscle

Oxidative stress is hypothesised to play a role in the pathogenesis of FA (Schulz et al. 2000, Chantrel-Groussard et al. 2001, Shan et al. 2013). Previous examination of the iron deposits in the cardiac mitochondria of 9-week-old MCK KO mice revealed that they were distinct from the iron bound by the iron storage protein ferritin (Whitnall et al. 2012). In fact, this MIT iron was demonstrated to be in the form of an inorganic iron crystallite that could potentially be redox-active (Whitnall et al. 2012). This is particularly significant, as non-ferritin-bound iron can result in cytotoxic ROS generation (Dröge 2002).

The MCK KO mouse, which specifically exhibits deletion of frataxin within striated muscle, namely, the heart and skeletal muscle, was utilised for this current investigation (Puccio et al. 2001, Huang et al. 2013). Despite frataxin deletion in both tissues, it has been reported that there is a remarkable difference in terms of the pathology observed (Puccio et al. 2001), but also the expression of proteins involved in cellular iron metabolism (Whitnall et al. 2012). However, there has been no direct comparison of the histology between the heart and skeletal muscle, their relative redox stress status, or related molecular alterations. Hence, these aspects were examined herein.

Initial studies assessed the gross histological alterations in the heart and skeletal muscle of the MCK WT and KO mice at 9-weeks of age, when there is a pronounced phenotype of cardiac hypertrophy (Puccio et al. 2001, Huang et al. 2013). Using Perls’ Prussian blue, Gömöri trichrome
and H&E staining, the 9-week-old MCK KO heart demonstrated: (1) myofibre hypertrophy with iron accumulation (see arrows; Fig. 3.1A, D); (2) interstitial fibrosis (see arrows; Fig. 3.1B, E); and (3) myofibrillar disarray (Fig. 3.1C, F) relative to the WT littermates, as reported previously (Puccio et al. 2001, Huang et al. 2013). In contrast, despite complete frataxin loss (Puccio et al. 2001, Huang et al. 2013), the quadriceps skeletal muscle in the 9-week-old MCK KO mouse showed no histopathological alterations relative to the WT mice (Fig. 3.1G-L) (Puccio et al. 2001), as found for FA patients (Harding 1981, Rotig et al. 1997). Notably, while the cross-sectional area of the muscle fibres was found to be significantly (p<0.001) greater in the heart muscle of the KO mice (562 ± 42 µm²) relative to WT mice (310 ± 30 µm²), the skeletal muscle of KO mice was significantly (p<0.001) smaller (880 ± 98 µm²) compared to their WT littermates (2057±190 µm²; Fig. 3.1M). However, as demonstrated previously, the MCK KO mouse markedly loses body weight relative to the WT control (Puccio et al. 2001, Whitnall et al. 2008) As a consequence, the heart to body weight ratio was significantly (p<0.001) higher in the KO (1.27 ± 0.08%; n = 6) relative to the WT littermates (0.54 ± 0.01%; n = 4; Fig. 3.1N). In contrast to the heart, the skeletal muscle (quadriceps) to body weight ratio was not significantly (p>0.05) altered between the KO and WT mice (Fig. 3.1N). This observation demonstrates that, although there was a marked reduction in skeletal muscle fibre area in KO relative to WT mice (Fig. 3.1M), this decrease was proportional to the loss of body weight in KO littermates (Fig. 3.1N).
Figure 3.1: Pronounced histopathology of the heart, but not the skeletal muscle of MCK KO mice. Histological staining of: (A-F) heart and (G-L) skeletal muscle from 9-week-old WT (A-C; G-I) and KO (D-F; J-L) littermates. The onset of FA cardiac histopathological features can be observed at 9-weeks of age, when the KO mice succumb to cardiomyopathy. Hearts and skeletal muscle were stained with Perls’ Prussian blue (left images: A, D, G, J), Gömöri trichrome (middle images: B, E, H, K) and H&E staining (right images: C, F, I, L). Arrows in: (D) denote iron-positive cardiomyocytes; (E) indicates myocardial fibrosis, as depicted by the blue/gray staining. In the heart, (A) and (D) are transverse sections (scale bar: 40 µm), while (B) and (C) (scale bar: 100 µm), and (E) and (F) (scale bar: 40 µm) are longitudinal sections. In the skeletal muscle, all sections (G-L) (scale bar: 100 µm) are transverse sections. Representative histological staining are shown (n = 5-6 in each group). Analysis of (M) muscle fibre size and (N) muscle:body weight (%) between the heart and skeletal muscle of 9-week-old WT and KO littermates. Figures are mean ± SEM (n = 5-6 in each group). ***, p<0.001 denotes significance compared to the WT sample.
Cardiac histopathology at end-stage (9 weeks)

Perls’

Gömöri Trichrome

H&E

A. WT

B. KO

D. WT

E. KO

F. WT

G. KO

H. WT

I. KO

J. WT

K. KO

L. WT

M. KO

N.

Muscle:Body Weight (%)

WT KO WT KO

Muscle Fibre Size (µm²)

WT KO WT KO

Heart

Skeletal Muscle

Muscle Body Weight (%)
Considering these observations, we then assessed redox stress in the whole heart compared to quadriceps skeletal muscle from WT and KO littermates at 9-weeks of age (Fig. 3.2). This was achieved by utilising HPLC, which is a sensitive method for the detection and quantification of protein oxidation products (Hawkins et al. 2009). In addition, our studies also examined the major intracellular antioxidant, GSH, and its oxidised counterpart, GSSG, via an established method (Owen and Butterfield 2010).

Whole hearts from 9-week-old MCK WT and KO mice were assayed by HPLC and a significant ($p<0.01; n = 12$) increase in ortho-tyrosine (o-Tyr) relative to Tyr was identified in the hearts of KO mice compared to WT mice (Fig. 3.2A). This observation indicated increased protein oxidation that has been reported in other disease states and which could occur through generation of highly reactive hydroxyl radicals (Davies et al. 1999, Huang et al. 2010).

We then examined the levels of the antioxidant, GSH (Anderson 1998), and demonstrated that in the heart of 9-week-old KO mice, relative to WT littermates, there was: (1) a significant ($p<0.01$) increase in total glutathione (i.e., GSH + GSSG; Fig. 3.2C); (2) a significant ($p<0.01$) increase in GSSG (Fig. 3.2E); and (3) no significant change in GSH levels in the heart of KO mice (Fig. 3.2G). These alterations in GSH resulted in a significant ($p<0.01$) decrease in the ratio of GSH:GSSG in the heart of the MCK KO mice relative to their WT littermates, indicating increased GSH oxidation (Fig. 3.2I).
Figure 3.2: Increased levels of markers of oxidative stress in the heart, but not the skeletal muscle of MCK frataxin WT and KO mice. (A, C, E, G, I) Heart and (B, D, F, H, J) skeletal muscle of the MCK *frataxin* KO mice relative to WT littermates were examined at 9-weeks of age for: (A, B) o-Tyr/Tyr ratio that indicates protein oxidation using HPLC; and (C-J) glutathione (GSH) oxidation by examining: (C, D) total GSH, (E, F) oxidised GSH (GSSG), (G, H) GSH, and (I, J) GSH/GSSG ratio. Values for total GSH, GSSG and GSH were expressed as μmol per gram of tissue. Figures are mean ± SEM (3 experiments). *, p<0.05; **, p<0.01; ***, p<0.001 denotes significance compared to the WT sample.
In contrast to the oxidative stress in the heart, examination of skeletal muscle demonstrated a slight, non-significant \((p>0.05)\) decrease in \(\sigma\)-Tyr relative to Tyr in the KO relative to WT mice (Fig. 3.2B). In terms of GSH status, in the skeletal muscle of KO mice relative to WT mice, we observed the following: (1) a slight, but significant \((p<0.01)\) decrease in total glutathione \((i.e., \text{GSH} + \text{GSSG}; \text{Fig. 3.2D})\); (2) a significant \((p<0.001)\) decrease in GSSG levels (Fig. 3.2F); and (3) no significant \((p>0.05)\) change in GSH levels (Fig. 3.2H). These changes in GSH and GSSG levels resulted in a significant \((p<0.05)\) increase in the GSH:GSSG ratio (Fig. 3.2J) in the skeletal muscle of KO compared to WT mice. Collectively, these data in Fig. 3.2 demonstrate that despite frataxin deficiency in both the heart and skeletal muscle of MCK KO mice (Puccio et al. 2001, Whitnall et al. 2012), oxidative stress is evident in the heart, but not the skeletal muscle.

### 3.3.2 Decreased Total Nrf2 Expression is Observed in the KO Heart and Corresponds with Increased Keap1 Expression

The so-called “master regulator” of antioxidant gene transcription, Nrf2, has become a focus in terms of understanding the cellular response to oxidative stress, with some recent reports of defective Nrf2 responses in multiple FA models (Paupe et al. 2009, D'Oria et al. 2013, Shan et al. 2013), but not the heart or skeletal muscle. Indeed, previous studies in cellular models of FA have reported that a decrease in Nrf2 nuclear translocation and nuclear levels of this protein could be responsible for the blunted antioxidant response to frataxin deficiency (Paupe et al. 2009, D'Oria et al. 2013). Considering these investigations and the results in Fig. 3.2, our studies then examined if Nrf2 protein expression was affected in the heart relative to the skeletal muscle in MCK KO and WT mice (Fig. 3.3).
Figure 3.3: Western blot analysis showing alterations in expression of Nrf2, Bach1 and Keap1 in the heart, but not the skeletal muscle of MCK frataxin KO mice. (A, B) Total protein and (C, D) cytosolic and nuclear lysates from: heart and skeletal muscle of the MCK frataxin WT and KO mice. The MCK frataxin KO mice relative to WT littermates were examined at 9-weeks of age. Western blot analysis of frataxin, Nrf2, Bach1 and Keap1 expression in the heart and skeletal muscle. In (A, B), Gapdh was used as a protein-loading control and implemented for normalisation of protein expression. In (C, D), Gapdh and Hdac1 were used to assess the cytosolic and nuclear fractions, respectively, and implemented for normalisation of protein loading. Western blot analysis shown are typical of 3-4 experiments and the densitometry is mean ± SEM (3-4 experiments). **, *p*<0.01; ###, *p*<0.001 denotes significance compared to the WT or WT cytosolic sample. #, *p*<0.05; ##, *p*<0.01; ###, *p*<0.001 denotes significance compared to the WT nuclear sample.
In the heart, Western blot analysis using total protein lysates from 9-week-old MCK mice confirmed that frataxin expression was almost ablated in the KO compared to WT littermates (Fig. 3.3A). Total ablation of cardiac frataxin was not observed, as the heart tissue sample is composed of a small proportion of fibroblasts, nerves, endothelial cells, where the frataxin gene remains intact. Our studies showed, using total protein lysates, that Nrf2 expression in the KO heart was significantly ($p<0.001$) decreased relative to the WT heart (Fig. 3.3A). Similarly, the ARE transcriptional repressor, Bach1, which competes for ARE-binding with Nrf2 (Dhakshinamoorthy et al. 2005, Kaspar and Jaiswal 2010), was also significantly ($p<0.01$) decreased in the KO mice compared to WT littermates (Fig. 3.3A). These data suggest the Bach1-mediated transcriptional repression of ARE-containing genes (Dhakshinamoorthy et al. 2005) may not be as marked in the KO relative to the WT heart. Moreover, reduced Bach1 in the KO may be attributable to decreased Nrf2 levels, because Bach1 is positively regulated by Nrf2 as part of a feedback-inhibitory mechanism (Jyrkkänen et al. 2011). In fact, Bach1 has been reported to contain an ARE in its promoter region and is a transcriptional target of Nrf2 (Jyrkkänen et al. 2011).

Since Keap1 regulates Nrf2 by binding to its C-terminal and sequestering it within the cytosol under physiological conditions (Lee et al. 2007), we examined Keap1 levels to determine if it could be a mechanism responsible for the observed decrease in total Nrf2 levels. Interestingly, Keap1 was markedly and significantly ($p<0.001$) increased in the KO relative to the WT in these total heart lysates from 9-week-old mice (Fig. 3.3A), suggesting Keap1-mediated degradation of Nrf2 (Bryan et al. 2013). As cardiomyopathy and functional deficits are only apparent in the MCK KO mice from 7 weeks of age (Puccio et al. 2001, Huang et al. 2013), studies also assessed alterations in Nrf2, Bach and Keap1 expression in these mice at 4-weeks of age where there is no
gross phenotype (Puccio et al. 2001, Huang et al. 2013). These studies demonstrated a slight, but not significant ($p>0.05$), decrease in Nrf2 expression between 4-week-old KO and WT hearts (Fig. 3.4). In contrast, a significant ($p<0.001$) decrease in Bach1 and a significant ($p<0.01$) increase in Keap1 were observed in the KO relative to the WT hearts (Fig. 3.4). This increase in Keap1 may be responsible for the slight decrease in Nrf2 (Lee et al. 2007). Hence, Keap1 appeared to be an early molecular marker of the dysfunctional Nrf2 pathway in KO mice.

Figure 3.4: Western blot analysis showing alterations in expression of Nrf2, Bach1 and Keap1 in the heart, but not the skeletal muscle of MCK frataxin KO mice at 4-weeks of age. Western blot analysis (total protein) demonstrating the expression of Nrf2, Bach1, and Keap1 in 4-week-old heart (left panel) and skeletal muscle (right panel) of MCK frataxin knockout (KO) mice. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a protein-loading control and implemented for normalisation of protein expression. Western blot analysis shown is typical of three to four experiments. Data are expressed as means ± SEM. n = 3 to 4 experiments (left and right panels). **$p<0.01$, ***$p<0.001$ versus wild-type (WT).
As a relevant comparison to the heart, we also examined Nrf2, Bach1 and Keap1 expression in skeletal muscle from 9-week-old mice, as frataxin is also deleted in this tissue (Fig. 3.3B) (Puccio et al. 2001). When examining skeletal muscle, frataxin expression was confirmed to be markedly ablated in the KO relative to WT littermates (Fig. 3.3B), as shown previously (Puccio et al. 2001, Whitnall et al. 2012). Again, as discussed above for the heart, total ablation of frataxin could not be expected, as the total skeletal muscle lysate contains a small proportion of other cell-types (e.g., fibroblasts and endothelial cells), which do not harbor the frataxin deletion (Puccio et al. 2001, Huang et al. 2013). In clear contrast to the heart of the MCK KO mouse (Fig. 3.3A), there was no significant ($p>0.05$) alteration of Nrf2, Bach1, or Keap1 expression between the MCK KO and WT littermates, despite the deficiency of frataxin expression (Fig. 3.3B). No significant alteration in Nrf2 or Bach1 expression was observed in the skeletal muscle at 4-weeks of age (Fig. 3.4). On the other hand, a significant ($p<0.01$) decrease in Keap1 was observed (Fig. 3.4). In summary, the alteration in Nrf2 expression appears to be tissue-specific and was only observed in the 9-week-old mouse heart (Fig. 3.3A), where a pronounced phenotype is apparent (Puccio et al. 2001, Whitnall et al. 2012).

3.3.3 Nuclear Nrf2 Expression is Decreased in the 9-Week-Old KO Heart

Considering the following: (1) pro-oxidant environment in the KO heart (Fig. 3.2A, C, E, G, I); (2) the reduced total Nrf2 in the KO heart (Fig. 3.3A); and (3) the reported impaired Nrf2 nuclear translocation in cellular FA models (Paupe et al. 2009, D'Oria et al. 2013), we next examined the nuclear levels of Nrf2 in the KO versus WT heart (Fig. 3.3C).
Nuclear and cytosolic fractions were prepared from MCK mouse hearts and examined by Western blot analysis for the subcellular distribution of Nrf2 (Fig. 3.3C). In these studies, Gapdh (a cytosolic marker) and histone deacetylase-1 (Hdac1, a nuclear marker) were examined to ensure fraction identity and to assess the possibility of fraction cross-contamination (Fig. 3.3C). The expression of Nrf2 was predominantly nuclear in the heart of both WT and KO mice, with Nrf2 nuclear expression being significantly ($p<0.01$) reduced in the KO relative to the WT littermates (Fig. 3.3C). Similarly, Bach1 was also significantly ($p<0.001$) reduced in both the cytosolic and nuclear fractions of the KO relative to WT mice (Fig. 3.3C). Conversely, Keap1 was predominantly cytosolic as expected, (Itoh et al. 1999) with a significant ($p<0.01$-0.05) increase in its expression in both cytosolic and nuclear fractions being evident in the KO heart, relative to their WT counterparts (Fig. 3C). Considering the presence of nuclear Keap1, this observation can be explained by the following: (1) the translocation of Keap1 into the nucleus where it can complex with Nrf2 to facilitate Keap1-Nrf2 nuclear export and subsequent degradation (Velichkova and Hasson 2005, Roy Chowdhury et al. 2014); and/or (2) the slight crossover of the nuclear with the cytosolic fraction, as shown by marker analysis (Fig. 3.3C).

Interestingly, cellular fractionation of skeletal muscle at 9-weeks of age demonstrated no significant ($p>0.05$) alteration in the cytosolic or nuclear expression of Nrf2, Bach1 or Keap1 between WT and KO mice (Fig. 3.3D). Despite harbouring the same frataxin deficiency (Puccio et al. 2001, Huang et al. 2013), this marked difference in Nrf2 pathway regulation between the heart and skeletal muscle, demonstrates the clear tissue-specific effects of frataxin.
In summary, in the heart of MCK KO mice, there is a decrease in total Nrf2 expression that could be mediated by the increase in cytosolic Keap1. In contrast, despite the knockout of frataxin in the skeletal muscle, there was no significant \( p>0.05 \) alteration in the expression of Nrf2, Bach1, or Keap1. This tissue specificity is of considerable interest, because systemic frataxin deficiency in FA patients only causes a pathologic phenotype in certain tissues, with the heart being markedly affected (Coppola \textit{et al}. 2009, Whitnall \textit{et al}. 2012).

3.3.4 The Gsk3β-Mediated Pathway that Decreases Nuclear Nrf2 is Activated in the Heart of Frataxin KO Mice

In addition to the potential role of Keap1 in decreasing Nrf2 in the heart of MCK KO mice (Fig. 3.3A, C), Keap1-independent mechanisms of Nrf2 down-regulation involve Nrf2 phosphorylation by Gsk3β-dependent pathways that regulate Nrf2 nuclear localisation (Jain and Jaiswal 2007, Rada \textit{et al}. 2011, Rada \textit{et al}. 2012, Chowdhry \textit{et al}. 2013). These mechanisms are examined in Fig. 3.5, as they could also explain the decreased nuclear and total Nrf2 levels observed in the MCK KO heart.
Figure 3.5: Western blot analysis demonstrating that the Gsk3β-mediated nuclear Nrf2 export/degradation machinery is activated in the heart of MCK KO relative to the WT mice. MCK frataxin KO mice relative to WT mice were examined at 9-weeks of age. (A) Western blot and densitometric analysis of phosphorylated (Ser9 and Tyr216) relative to total Gsk3β expression in total heart lysate. Gapdh was used as a loading control and implemented for normalisation of protein-loading. (B) Western blot and densitometric analysis of the expression and subcellular localisation of phosphorylated Gsk3β (Ser9 and Tyr216) relative to total Gsk3β expression, β-TrCP expression, and phosphorylated (pThr12) and total Fyn expression in the cytosolic and nuclear heart fractions. Gapdh and Hdac1 were used as fractionation controls for cytosolic and nuclear fractions, respectively, and implemented for normalisation of protein expression. (C) Immunoprecipitation of Nrf2 followed by western blot and densitometric analysis of phosphorylated Tyr (pTyr) levels of Nrf2 using an anti-phospho-Tyr antibody. Western blot analyses shown are typical of 3-4 experiments and densitometry is mean ± SEM (3-4 experiments). *, p<0.05; **, p<0.01; ***, p<0.001 denotes significance compared to the WT or WT cytosolic sample. #, p<0.05; ##, p<0.01; ###, p<0.001 denotes significance compared to the WT nuclear sample.
A. Heart

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B. Heart

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C. Heart

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First, our studies examined the phosphorylation status of Gsk3β, which governs its kinase activity and ability to effect downstream targets such as Nrf2 (Rada et al. 2011, Rada et al. 2012, Chowdhry et al. 2013). Both the inactivating (Ser9) and activating (Tyr216) phosphorylation sites of Gsk3β (Grimes and Jope 2001) were examined by Western blot analysis in total cell lysates from MCK WT and KO hearts (Fig. 3.5A). The pSer9/Gsk3β ratio was significantly \(p<0.05\) decreased in the KO relative to WT hearts, whereas the pTyr216/Gsk3β ratio was significantly \(p<0.01\) increased (Fig. 3.5A). In contrast, cellular expression of total Gsk3β was not significantly \(p>0.05\) altered in the KO versus the WT littermates (Fig. 3.5A). Collectively, these observations indicate that Gsk3β is activated \(i.e.,\) phosphorylated at Tyr216 in the heart of KO mice relative to their WT littermates.

More important, as Gsk3β-dependent mechanisms regulate Nrf2 expression in the nucleus (Jain and Jaiswal 2007, Rada et al. 2011, Rada et al. 2012, Chowdhry et al. 2013), we then performed cellular fractionation studies of WT and KO heart lysates to examine the phosphorylation status and subcellular localisation of Gsk3β (Fig. 3.5B). Similar to Fig. 3.3C and D, the expression of Gapdh and Hdac1 were examined as cytosolic and nuclear fraction markers, respectively. The pSer9/Gsk3β ratio was significantly \(p<0.05\) decreased in both the cytosolic and nuclear fractions (Fig. 3.5B). On the other hand, the pTyr216/Gsk3β ratio was slightly \(p>0.05\) increased in the cytosolic fraction in the KO versus WT, but significantly \(p<0.01\) increased in the nuclear fraction in the KO mice relative to the WT (Fig. 3.5B). In accordance with the results from the whole heart lysates (Fig. 3.5A), total Gsk3β expression was not significantly \(p>0.05\) altered in both the cytosolic and nuclear fractions (Fig. 3.5B).
Activation of Gsk3β has been reported to directly phosphorylate Nrf2 at Ser338 (Chowdhry et al. 2013), which leads to recruitment of the β-transducin repeat containing E3 ubiquitin protein ligase (β-TrCP) (Rada et al. 2011, Rada et al. 2012). This protein is a substrate recognition subunit of the Skp1-Cul1-Rbx/Roc1 E3 ubiquitin ligase complex that targets Nrf2 phosphorylated at Ser338 for ubiquitination and subsequent nuclear export and/or degradation (Rada et al. 2011, Rada et al. 2012, Chowdhry et al. 2013). In the current studies, β-TrCP was observed to be predominantly localized in the nucleus of the KO heart, with a pronounced and significant ($p<0.01$) increase in nuclear β-TrCP expression in the KO mice compared to their WT littermates (Fig. 3.5B). Combined with the observed increased activation of Gsk3β in the KO mouse heart (Fig. 3.5A, B), these studies suggest increased Gsk3β-mediated Nrf2 phosphorylation leading to β-TrCP nuclear recruitment and subsequent Nrf2 nuclear export/degradation (Rada et al. 2011, Rada et al. 2012, Chowdhry et al. 2013).

Another Gsk3β-dependent mechanism of Nrf2 down-regulation involves the controversial finding that phosphorylation of Nrf2 at Tyr568 leads to its nuclear efflux and degradation (Jain and Jaiswal 2006, Jain and Jaiswal 2007). This mechanism involves Gsk3β (Tyr216 phosphorylated form) activating the Src tyrosine kinase, Fyn, via threonine phosphorylation (Jain and Jaiswal 2007, Shang et al. 2015). This modification then results in the translocation of activated Fyn into the nucleus (Jain and Jaiswal 2007, Mobasher et al. 2013, Shang et al. 2015), where it has been suggested to phosphorylate the Nrf2 Tyr568 residue (Jain and Jaiswal 2006, Jain and Jaiswal 2007). Although the phosphorylation of Tyr568 is disputed (Jain and Jaiswal 2006), the increased phosphorylation of Nrf2 tyrosine residues and Gsk3β-Fyn activation have been linked to enhanced nuclear export by other investigators (Jain and Jaiswal 2007, Mobasher et al. 2013, Shang et al.
To further examine and dissect the mechanism of the decreased nuclear Nrf2 levels in frataxin KO mice, the subcellular localisation and phosphorylation of Fyn were assessed.

Importantly, phosphorylation of Fyn at Thr12 has been demonstrated to activate both its tyrosine kinase activity and translocation into the nucleus (He et al. 2005, He et al. 2008), which may then lead to the phosphorylation Nrf2 (Jain and Jaiswal 2007). Considering this, Fyn phosphorylation at Thr12 was examined (Fig. 3.5B). As expected from the activation of Gsk3β, the cytosolic and particularly the nuclear levels of the phosphorylated Fyn (Thr12)/Fyn ratio were significantly ($p<0.001$-$0.01$) elevated in the KO relative to WT littermate hearts (Fig. 3.5B). Consistent with the nuclear accumulation of phosphorylated Fyn (Jain and Jaiswal 2007), nuclear expression of total Fyn was significantly ($p<0.01$) greater in the KO mice relative to WT littermates (Fig. 3.5B).

Considering the elevated Fyn phosphorylation in the KO heart, studies then examined the phosphorylation of Nrf2 tyrosine levels by probing immunoprecipitated Nrf2 using an anti-pTyr antibody (Fig. 3.5C). This assessment of general tyrosine phosphorylation has been previously implemented by others to gauge Fyn kinase activity in this context (Jain and Jaiswal 2007, Mobasher et al. 2013). In addition, examination of general Tyr phosphorylation was also deemed appropriate, considering: (1) that there is no commercially available antibody against phosphorylated Tyr568; and (2) the role of Tyr568 phosphorylation in Nrf2 regulation remains unclear (Jain and Jaiswal 2006). Our studies revealed a pronounced and significant ($p<0.001$) increase of Nrf2 Tyr phosphorylation in the immunoprecipitate from KO hearts relative to the
hearts from their WT littermates (Fig. 3.5C). These data are consistent with the phosphorylation of Nrf2 Tyr residues by the Gsk3β-Fyn axis in the KO mice, which could then be targeted for nuclear export (Jain and Jaiswal 2007).

Collectively, these data in Fig. 3.5 demonstrate activation of the Gsk3β-mediated nuclear export/degradation machinery of Nrf2 via the β-TrCP and/or Fyn mechanisms in the heart of KO mice relative to their WT littermates.

### 3.3.5 The Gsk3β-Mediated Mechanism for Decreasing Nuclear Nrf2 is Inactivated in the Skeletal Muscle of the KO Mice

As a relevant comparison to the heart (Fig. 3.5), the levels of phosphorylated Gsk3β (inactivating pSer9 and activating pTyr216) were also examined in the skeletal muscle of MCK mice by Western blot analysis (Fig. 3.6). In contrast to the total heart lysate (Fig. 3.5A), the pSer9/Gsk3β ratio in the total skeletal muscle lysate was slightly, but significantly ($p < 0.05$), increased in the KO relative to WT mouse, whereas the pTyr216/Gsk3β ratio was not significantly ($p > 0.05$) altered (Fig. 3.6A). Similar to the heart, total cellular Gsk3β expression in skeletal muscle was not significantly ($p > 0.05$) altered in KO mice relative to their WT littermates (Fig. 3.6A). These observations suggest a tissue-specific alteration between the heart and skeletal muscle of KO MCK mice, namely a decrease of the inactivating Ser9 phosphorylation of Gsk3β in the heart, whereas there was increased Ser9 phosphorylation in the skeletal muscle indicating Gsk3β inactivation.
Figure 3.6: Western blot analysis demonstrating that the Gsk3β-mediated nuclear Nrf2 export/degradation machinery is inactivated in the skeletal muscle of MCK KO relative to the WT mice. MCK \textit{frataxin} KO mice relative to WT mice were examined at 9-weeks of age. (A) Western blot and densitometric analysis of phosphorylated (Ser9 and Tyr216) relative to total Gsk3β expression in total skeletal muscle lysate. Gapdh was used as a loading control and implemented for normalisation of protein-loading. (B) Western blot and densitometric analysis of phosphorylated Gsk3β (Ser9 and Tyr216) relative to total Gsk3β expression, β-TrCP expression and phosphorylated (pThr12) and total Fyn expression in the cytosolic and nuclear skeletal muscle fractions. Gapdh and Hdac1 were used as fractionation controls for cytosolic and nuclear fractions, respectively, and implemented for normalisation of protein expression. (C) Immunoprecipitation of Nrf2 followed by western blot and densitometric analysis of phosphorylated Tyr (pTyr) levels of Nrf2 using an anti-phospho-Tyr antibody. Western blot analyses shown are typical of 3-4 experiments and densitometry is mean ± SEM (3-4 experiments). * $p<0.05$; **, $p<0.01$ denotes significance compared to the WT or WT cytosolic sample. ##, $p<0.01$ denotes significance compared to the WT nuclear sample.
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C. Skeletal Muscle

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To enable a comparison with the heart (Fig. 3.5B), the subcellular localisation and/or phosphorylation of Gsk3β, β-TrCP and Fyn was examined by performing cellular fractionation studies in WT and KO skeletal muscle lysates (Fig. 3.6B). As in the studies above examining the heart, the cytosolic and nuclear fraction markers, Gapdh and Hdac1, respectively, were assessed as relative fraction controls. In the skeletal muscle, the inactivating pSer9/Gsk3β ratio was significantly ($p<0.01$) increased in the nuclear fraction of the KO mouse relative to the WT (Fig. 3.6B). In contrast, the activating pTyr216/Gsk3β ratio demonstrated no significant ($p>0.05$) change in both the nuclear and cytosolic fractions in the KO mice relative to their WT littermates (Fig. 3.6B). The total Gsk3β expression was not significantly ($p>0.05$) altered in both the cytosolic and nuclear fractions in the skeletal muscle (Fig. 3.6B).

Examination of β-TrCP levels in the skeletal muscle demonstrated that in KO mice, there was a significant ($p<0.01$) decrease in the expression of both the cytosolic and nuclear β-TrCP relative to their respective levels in WT littermates (Fig. 3.6B). This finding was in marked contrast to the increased β-TrCP levels in the heart nuclear fraction of the KO mice relative to the WT (Fig. 3.5B) and may be caused by the increased inhibition of Gsk3β in the KO skeletal muscle (Fig. 3.6A, B).

Furthermore, in terms of Fyn phosphorylation status at Thr12, in contrast to the heart (Fig. 3.5B), there was no significant ($p>0.05$) alteration in the nuclear and cytosolic fractions of KO skeletal muscle relative to the WT (Fig. 3.6B). Studies then examined the phosphorylation of Nrf2 by immunoprecipitation (as in Fig. 3.5C) and this demonstrated no significant ($p>0.05$) change of Nrf2 Tyr phosphorylation from KO relative to WT skeletal muscle (Fig. 3.6C). These data are in
contrast to the results assessing the heart in Fig. 3.5C, indicating that the Gsk3β-Fyn axis has not been activated in the skeletal muscle of the KO relative to the WT. Collectively, these results again demonstrate an opposite and tissue-specific effect of frataxin deletion in the skeletal muscle compared to the heart of KO mice.

3.3.6 Decreased Nrf2 Binding to the ARE Attenuates the Antioxidant Response in KO Hearts

Considering the marked histopathology in the heart (Fig. 3.1A-F), the observed alterations in oxidative markers (Fig. 3.2A, C, E, G, I), and the marked reduction in Nrf2 expression in the total (Fig. 3.3A) and nuclear fractions (Fig. 3C) of the KO mouse heart, we then examined the ARE-binding activity in heart nuclear lysates using an electrophoretic mobility shift assay (EMSA; Fig. 3.7A). As demonstrated in Fig. 3.7A, although there was a specific band representing protein-binding to the ARE probe sequence in the WT nuclear sample, the binding was significantly ($p<0.01$) reduced in the KO mice (Fig. 3.7A). A 200-fold excess of unlabelled ARE nucleotide sequence acting as a specific competitor (Spec. Comp.) was able to significantly ($p<0.001-0.01$) reduce the detected protein-bound ARE probe in both WT and KO samples, demonstrating the specificity of the protein DNA-binding activity (Fig. 3.7A). Hence, in agreement with the Western blot results indicating decreased Nrf2 in the nucleus of the KO heart (Fig. 3.3C), these studies demonstrated that Nrf2 DNA-binding activity was markedly reduced in the MCK KO relative to WT littermates. This observation suggested a potential reduction in Nrf2 antioxidant target gene mRNA levels in the KO heart.
Figure 3.7: EMSA analysis demonstrates a decrease in ARE-binding and also a decrease in mRNA expression of Nrf2 and four out of five target genes in the MCK KO heart relative to the WT. Heart tissue from MCK frataxin KO mice relative to WT mice were examined at 9-weeks of age. (A) Nuclear protein lysates from heart tissues of MCK WT and KO mice were prepared by cellular fractionation. The nuclear lysates (20 μg) were then incubated with a 36-bp biotin-labelled probe containing the mouse Nqo1 ARE sequence (5’-AGTCTAGAGTCACAGTGAGTGCCAAAATTTGAGCC-3’, corresponding to nt-451 to -416) in the presence or absence of the non-labelled Nqo1 ARE sequence (i.e., Specific Competitor probe; Spec. Comp.). EMSA analysis shown are typical of 3 experiments and densitometry is mean ± SEM (3 experiments). **, *p*<0.01; ***, *p*<0.001 relative to the WT; ††, *p*<0.01 relative to the KO. Red and black arrows pointing to the gel indicate protein-bound ARE probe and free probe, respectively. Dotted line on densitometric analysis indicates 100%. (B) Quantification of the mRNA levels of ARE-containing genes: Nrf2, Nqo1, TxnRD1, Gstm1, Sod2 and catalase, were examined by RT-qPCR and normalised to Gapdh mRNA expression. Data are expressed as log2 ± SEM (*n* = 5-6 mice/genotype, 3 experiments). *, *p*<0.05 denotes significance compared to the WT sample.
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B. Heart

Gene/Gapdh relative to WT (log2)

- Nrf2
- Nqo1
- TxnRD1
- Gstm1
- Sod2
- Catalase

- *: p < 0.05
- **: p < 0.005
- ***: p < 0.001
- ##: p < 0.01
To examine this latter possibility, we then assessed by real-time quantitative RT-qPCR the expression of important cellular antioxidant gene targets of Nrf2 that contain an ARE within their promoter region (Fig. 3.7B). These target genes included the following: Nrf2, \( NAD(P)H \) dehydrogenase, quinone1 (Nqo1), thioredoxin reductase1 (TxnRD1), glutatione-S-transferase Mu1 (Gstm1), superoxide dismutase2 (Sod2) and catalase (Chanas et al. 2002, Hintze et al. 2003, Nioi et al. 2003, Reisman et al. 2009). Despite the oxidative stress in the heart (Fig. 3.2A, I), and in agreement with the reduced total and nuclear Nrf2 protein levels (Fig. 3.3A, C) and Nrf2-ARE-binding activity (Fig. 3.7A), we observed a significant \((p<0.05)\) decrease in Sod2 mRNA expression, together with a non-significant \((p>0.05)\) decrease in the levels of Nrf2, TxnRD1, Gstm1 and catalase mRNA in the KO mice compared to their WT littermates (Fig. 3.7B). Only Nqo1 mRNA levels were slightly, but significantly \((p<0.05)\), increased in the KO mice relative to WT littermates (Fig. 3.7B). Notably, our previous microarray analysis examining MCK KO and WT mice (Gene Expression Omnibus data set GSE31208; http://www.ncbi.nlm.nih.gov/geo/) (Huang et al. 2009) also identified a significant \((p<0.01)\) decrease in Sod2 mRNA expression in the KO, whereas no other antioxidant genes were significantly altered. The decrease in Sod2 mRNA levels is in agreement with previous studies in mouse models of FA (Chantrel-Groussard et al. 2001, Seznec et al. 2005, Sandi et al. 2014), and consistent with finding that oxidative challenge is unable to induce Sod2 in cells from FA patients (Chantrel-Groussard et al. 2001, Jiralerspong et al. 2001). Taken together, despite the marked pathology and increased oxidative products in the KO heart (Fig. 3.1A-F, Fig. 3.2A, C, E, G, I), the mRNA expression of Nrf2 and four out of its five target genes were not up-regulated in response to oxidative stress.
Intriguingly, despite the general decrease in mRNA levels, further examination of these downstream Nrf2 antioxidant targets at the protein level demonstrated contrasting results (Fig. 3.8). In the heart, there was a significant \( p<0.001 \) increase in the expression of Nqo1, Gstm1 and TxnRD1 in the KO mice compared to their WT littermates, while the expression of Sod2 and catalase were unchanged (Fig. 3.8A, B). Notably, in yeast models of frataxin deficiency, Sod2 protein levels were also unchanged despite high MIT iron (Yang et al. 2006). In the skeletal muscle of KO mice, where no significant oxidative stress (Fig. 3.2B, J) or alteration in Nrf2 levels (Fig. 3.3B, D) was observed, there was a significant \( p<0.001-0.01 \) increase in the protein levels of Nqo1, Gstm1 and catalase relative to WT littermates (Fig. 3.8A, C). In contrast, Sod2 and TxnRD1 protein expression remained unchanged (Fig. 3.8A, C). The reason for the increased expression of Nqo1, Gstm1 and catalase in the KO skeletal muscle was unclear.
Figure 3.8: Western blot analysis demonstrates differential expression profile of antioxidant proteins in the heart and skeletal muscle of MCK WT and KO mice. MCK frataxin KO mice relative to WT mice were examined at 9-weeks of age. Western blot (A) and densitometric analysis (B, C) of Nqo1, Gstm1, Sod2, TxnRD1 and catalase expression in total heart and total skeletal muscle lysate. Gapdh was used as a loading control and implemented for normalisation of protein-loading. Western blot analysis shown are typical of 3-4 experiments and densitometry is mean ± SEM (3-4 experiments). **, p<0.01; ***, p<0.001 denotes significance compared to the WT sample.
A. Heart and Skeletal Muscle

- Nqo1
- Gstm1
- Sod2
- TxnRD1
- Catalase
- Gapdh

ko relative to WT (a.u.)

B. Heart

Protein/Gapdh

ko relative to WT (a.u.)

***

Nqo1 Gstm1 Sod2 TxnRD1 Catalase

C. Skeletal Muscle

Protein/Gapdh

ko relative to WT (a.u.)

***

Nqo1 Gstm1 Sod2 TxnRD1 Catalase

Nrf2-ARE mediated antioxidant proteins
3.4 Discussion

Our previous study demonstrated the marked functional and molecular alterations in the MCK KO heart relative to the WT heart, with the pathogenesis of the cardiomyopathy correlating with early and persistent eIF2α phosphorylation, which precedes activation of autophagy and apoptosis (Huang et al. 2013). From our current investigation of Nrf2 signalling in the frataxin-deficient heart, we have demonstrated for the first time the mechanism responsible for the decrease of Nrf2 after loss of frataxin in the heart (Fig. 3.9). This response in the heart appears paradoxical in the face of the observed oxidative stress that is evident from protein oxidation and GSH measurements (Fig. 3.2A, C, E, G, I) and previous studies demonstrating the importance of Nrf2 in cardio-protection from oxidative damage in vivo (Li et al. 2009, Wang et al. 2014). Moreover, a tissue-specific effect was observed, although frataxin deletion also occurred in the MCK skeletal muscle, no oxidative stress, histological abnormalities, or Nrf2 dysfunction was observed. These observations are in good agreement with the tissue-specific characteristics of FA as reported by others in patients (Harding 1981, Rotig et al. 1997) and in the MCK mouse model (Puccio et al. 2001). In fact, previous studies have demonstrated that, in striking contrast to the heart, the skeletal muscle of the MCK model does not show any histological, ultrastructural, or biochemical defect, despite extensive Cre recombination (Puccio et al. 2001). The differential between the heart and skeletal muscle may be attributable to the generally known fact that the heart relies on OXPHOS via the mitochondrion, with an almost exclusive dependence on aerobic metabolism (Berg J.M. 2002). In contrast, the skeletal muscle is more dependent on cytosolic anaerobic glycolysis for its energy requirements (Berg J.M. 2002). Thus, it can be suggested the MIT dysfunction caused by frataxin deficiency has a greater impact on the metabolism of the heart relative to the skeletal muscle (Richardson et al. 2010b, Huang et al. 2011, Vaubel and Isaya 2013).
Figure 3.9: Schematic illustrating the mechanisms mediating the impaired Nrf2 response in the heart of MCK frataxin KO mice. (1) Frataxin deficiency results in mitochondrial iron accumulation (Whitnall et al. 2008, Huang et al. 2009, Whitnall et al. 2012) that could increase ROS generation and the production of oxidative products (Fig. 3.2A, C, E, G, I). (2) Despite the presence of oxidative stress in the MCK KO heart, increased cytosolic Keap1 expression (Fig. 3.3A, C) could result in Keap1-mediated proteasomal degradation of Nrf2 (Bryan et al. 2013), decreasing its levels (Figure 3.3A, C). (3) Reduced Nrf2 expression leads to decreased Nrf2 nuclear levels (Fig. 3.3C). (4) Within the nucleus, increased Gsk3β activation (Tyr216 phosphorylation; Fig. 3.5B) may result in (5) direct Gsk3β-mediated Nrf2 phosphorylation (Ser338) (Chowdhry et al. 2013) and (6) subsequent nuclear accumulation of β-TrCP (Figure 3.5B) that facilitates the decrease in nuclear Nrf2 (Figure 3.3C) via the known processes of nuclear export and/or degradation (Bryan et al. 2013). (7) Alternatively, activated Gsk3β could increase phosphorylation of Fyn (Thr12; Figure 3.5B) to enhance Fyn tyrosine kinase activity (He et al. 2005, He et al. 2008). (8) This in turn, mediates Tyr phosphorylation of Nrf2 (Figure 3.5C), leading to a decrease in nuclear Nrf2 (Figure 3.3C), via its export (Jain and Jaiswal 2007). (9) These mechanisms culminate in decreased ARE-binding activity (Figure 3.7A) and a slight, but general, decrease in the expression of ARE-containing genes (Figure 3.7B) observed in the MCK frataxin KO heart.
Dysfunctional Nrf2 Signalling in the Frataxin-Deficient Cardiomyocyte

1. o-Tyr
2. GSH/GSSG
3. Keap1
4. Nrf2 degradation
5. Nrf2
6. Mitochondrial Iron Accumulation

Antioxidant Response Element (ARE)
Despite oxidative stress in the MCK KO heart, our data demonstrate decreased total cellular and nuclear Nrf2 levels that correspond with increased Keap1 at 9-weeks of age (Fig. 3.3A, C). This observation suggests classical Keap1-mediated degradation of cytosolic Nrf2 (Bryan et al. 2013), which has not been previously characterised after frataxin deletion. Our studies, for the first time, demonstrate a significant increase in Keap1 in the frataxin-deficient heart that is well known to result in decreased cytosolic Nrf2 levels (Bryan et al. 2013). Previous investigations using various models of frataxin deficiency have either: (1) not assessed Keap1 expression (D’Oria et al. 2013); (2) indicated no alteration in Keap1 (Shan et al. 2013); or (3) reported an alteration in cellular distribution of Keap1, but without increased expression (Paupe et al. 2009). Moreover, the observed increase of Keap1 in 4-week-old KO heart, where there is no morphological or functional cardiac pathology (Huang et al. 2013), suggests the dysregulation of the Nrf2 pathway occurs relatively early and is not a consequence of the marked cardiomyopathy at 9-weeks of age.

The expression of Bach1, which is another key regulator of Nrf2 activity, was decreased in the frataxin-deficient heart. In the absence of haem, Bach1 acts as a repressor of Nrf2-DNA binding activity (Suzuki et al. 2004), and as such, it is likely the decrease in nuclear Bach1 in the KO heart (Fig. 3.3C), should facilitate access of Nrf2 to AREs. Nonetheless, the decreased Bach1 expression observed in the heart was surprising, as haem synthesis and haem levels are depressed in the heart of MCK KO mice (Huang et al. 2009), which should have led to increased nuclear Bach1 levels (Suzuki et al. 2004). Indeed, it is well known that upon haem-binding to Bach1, its DNA-binding activity is reduced, and nuclear export increased (Suzuki et al. 2004).
Apart from the dysfunction in the classical Keap1/Bach1 system for controlling Nrf2 activity in the KO heart, we demonstrate for the first time, activation of Nrf2 nuclear export/degradation machinery via Gsk3β-mediated Nrf2 phosphorylation either directly, or through Fyn kinase (Jain and Jaiswal 2007, Rada et al. 2011, Rada et al. 2012, Chowdhry et al. 2013). In fact, in the KO heart, there was increased levels of the activating phosphorylation of Gsk3β (Tyr216; Figs. 3.5A, B) and Fyn kinase (Thr12; Fig. 3.5B), as well as increased Tyr phosphorylation of Nrf2 (Fig. 3.5C), that is known to result in Nrf2 nuclear export (Jain and Jaiswal 2007, Mobasher et al. 2013). This effect, in conjunction with the enhanced β-TrCP expression (Fig. 3.5B), could facilitate the degradation of nuclear Nrf2 by acting as a substrate for the E3 proteasome complex of Cullin-1 and Rbx1 (Rada et al. 2011). Collectively, the alterations in the Nrf2 pathway by these mechanisms could be responsible for the decreased Nrf2 protein levels, reduced ARE-binding and the generally depressed expression of ARE-containing antioxidant defence genes in the frataxin-deficient heart.

Despite Nrf2 down-regulation in the heart (Fig. 3.3A, C) and a general decrease in the mRNA levels of its downstream antioxidant targets (Fig. 3.7B), the protein levels of some of these antioxidant targets were increased in the KO mice (Fig. 3.8A-C). A lack of correlation between mRNA and protein expression is well known in the literature and is attributable to the existence of post-transcriptional mechanisms. These can include: (1) RNA-binding proteins, such as iron regulatory protein-1(Hentze and Kuhn 1996, Richardson and Ponka 1997) and others (Aguilera et al. 2003), that can modulate mRNA stability and translation; and (2) microRNAs (Tabernero et al. 2016), which silence mRNA translation and can lead to little correlation between mRNA levels and protein expression. In addition, there are also post-translational mechanisms that can rapidly degrade proteins (e.g., proteasome and also the lysosome via autophagy (Sandri 2013)), which can
also lead to a lack of correlation between mRNA and protein levels. Furthermore, these processes can be dysregulated during disease (Rubinsztein 2006, Sandri 2013), leading to additional complexity in terms of understanding the balance between mRNA and protein expression.

Even with the marked down-regulation of Nrf2 in the heart, there was still appreciable mRNA levels of its target effector genes with some of these being up-regulated (i.e., Nqo1). This interesting finding may be attributable to the activity of other transcription factors (e.g., peroxisome proliferator-activated receptor γ and FOXO) that actively target these critical Nrf2 downstream effectors (Greer and Brunet 2005, Ding et al. 2007, Okuno et al. 2010) and could potentially compensate for Nrf2 dysfunction. In fact, our previous studies examining the MCK heart demonstrated that the mRNA and protein expression of HO-1, which is also an ARE-containing gene and Nrf2 target (Rada et al. 2012), was markedly and significantly increased (Huang et al. 2009) despite the marked depression of Nrf2 activity demonstrated herein. Together, these observations indicate that other mechanisms can at least partially compensate for the depression in Nrf2 levels. Nonetheless, despite the compensation observed in terms of antioxidant response, an overall state of oxidative stress was evident in the KO heart, as demonstrated by increased levels of the phenylalanine oxidation product, o-Tyr, and the depressed GSH/GSSG ratio (Fig. 3.2).

Collectively, the current study provides a rationale for antioxidant supplementation to enhance cardiac GSH (thereby increasing the GSH/GSSG ratio) through administration of antioxidants, such as N-acetylcysteine (NAC) (Prescott et al. 1977, Santos et al. 2001, Ji et al. 2010). Notably, NAC has been demonstrated to increase Nrf2 expression in vivo (Ji et al. 2010, Zhang et al. 2014),
which should bolster antioxidant defence. This is important as the Nrf2 response is decreased in the heart, which leads to oxidative stress (Fig. 3.2), probably because of a less than adequate response of the battery of antioxidant response proteins downstream of Nrf2. Enhancing the up-regulation of these proteins (e.g., catalase and SOD, which did not display any significant increase in levels) (Fig. 3.8, A-C) could be important to target. In addition, NAC is a Federal Drug Administration (FDA)-approved drug (Prescott et al. 1977) and has been shown to have protective effects against cytotoxicity in non-differentiated frataxin-deficient cell-types, due to its ability to prevent ROS-induced cytotoxicity (Santos et al. 2001). This is significant, as previous studies in yeast mutants lacking frataxin have also shown that NAC supplements cellular GSH and prevents iron-induced toxicity, with cell survival increasing by 2–4 orders of magnitude (Karthikeyan et al. 2002).

Considering the potential role of iron in the oxidative stress and pathogenesis of FA, and the beneficial effects of NAC in cellular systems of this disease, a potential therapeutic modality could include the combination of NAC with chelators (e.g., pyridoxal isonicotinoyl hydrazone (PIH)) that have been demonstrated to mobilise MIT iron accumulation and inhibit oxidative stress (Richardson et al. 2001, Lim et al. 2008, Whitnall et al. 2008). This could lead to a rationalised treatment for FA in the absence of a therapy to replace frataxin function. This is particularly relevant, because of the following: (1) NAC can rescue low GSH levels (Johnson et al. 2012), which were demonstrated to be low in the heart of the MCK mouse (Figure 3.2I) and prevent deleterious ROS-induced tissue damage; (2) in addition to its ability to directly react with ROS to prevent their deleterious activity, NAC also restitutes GSH (Elbini Dhouib et al. 2016), which could be crucial because GSH plays a role in ISC assembly (Kumar et al. 2011, Wang et al. 2012),
a key defect in FA (Vaubel and Isaya 2013); and (3) NAC has been shown to increase Nrf2 expression (Ji et al. 2010, Zhang et al. 2014). Together, by removing an important oxidative insult and supplementing GSH, NAC could ameliorate ROS generation, bolster antioxidant defence by increasing Nrf2 expression, and aid the synthesis of critical ISC.

Although there is clear evidence demonstrating that frataxin deficiency results in increased ROS generation (Schulz et al. 2000, Sparaco et al. 2009), the reason why a loss of frataxin leads to a decrease in Nrf2 expression remains uncertain. One possible mechanism could be related to the recent finding that intracellular iron levels can be increased through Nrf2 degradation and decreased Nrf2-induced expression of the iron export protein, FPN1 (Yang et al. 2017). This is relevant, because FPN1 is involved in iron release from cells, and we previously demonstrated the frataxin KO heart is in a cytosolic iron-deficient state, where FPN1 expression is decreased relative to WT mice (Huang et al. 2009). Such a decrease in FPN1 expression could reduce iron export, and thus, aid in preventing the iron-deficit (Huang et al. 2009). Hence, the inhibition of Nrf2 expression observed here may be part of an attempt by cellular regulatory mechanisms to decrease FPN1 expression to reduce cellular iron efflux, and thus, restore intracellular iron levels in the absence of frataxin.

In summary, our study have demonstrated for the first time, that frataxin deficiency in the heart results in significant alterations in the cellular redox-homeostasis mediated by Nrf2. Furthermore, this is the first investigation to dissect the mechanism of how loss of frataxin in the heart results in Nrf2-deficiency, namely through: (1) increased cytosolic Keap1 levels and (2) activation of nuclear Nrf2 export/degradation machinery via Gsk3β signalling. These effects lead to a general
decrease in Nrf2-binding to the ARE of target genes involved in antioxidant defence. Hence, despite evidence of marked redox stress in the frataxin-deficient heart, the major antioxidant defence mechanism mediated via Nrf2 is dysfunctional and this could play a role in the cardiac pathology observed in FA.
CHAPTER FOUR

Characterisation of Mitochondrial Dysfunction in a Mouse Model of Dilated Cardiomyopathy:
Impaired NAD\(^+\) Metabolism and Increased Mitochondrial Fission, Fusion, Biogenesis and Autophagic Flux.

This Chapter is adapted from the manuscript below that is currently in preparation, where I am the first author:

**Chiang, S.***, Braidy, N., Richardson, D.R., Huang, M.L. Characterisation of Mitochondrial Dysfunction in a Mouse Model of Dilated Cardiomyopathy: Impaired NAD\(^+\) Metabolism and Increased Mitochondrial Fission, Fusion, Biogenesis and Autophagic Flux. American Journal of Pathology, 2019. [Manuscript in Preparation, 2019]
4.1 Introduction

Frataxin is a nuclear-encoded, mitochondrial (MIT) protein. In humans, its deficiency causes the human neuro- and cardio-degenerative condition, Friedreich’s ataxia (FA), which is characterised by progressive ataxia and fatal cardiomyopathy (Huang et al. 2011, Huang et al. 2013). Frataxin plays a pivotal role in MIT iron metabolism, especially iron-sulfur cluster (ISC) and haem synthesis (Huang et al. 2009, Richardson et al. 2010a, Huang et al. 2011). This is of particular importance, since many components within the mitochondrial respiratory complexes require ISC proteins or haem prosthetic groups for their activity, in particular, Complex I-III (Kim et al. 2012).

Accordingly, frataxin deficiency results in significant inhibition of Complex I-III (Rotig et al. 1997). Yet, despite reduced MIT iron utilisation for ISC and haem synthesis, MIT iron influx continues (Huang et al. 2009), causing the excessive MIT iron-loading in this disease. Moreover, a recent study from our laboratory (Whitnall et al. 2012) demonstrated that the frataxin-deficient mitochondria contained non-ferritin-bound iron aggregates, which, without the protective ferritin shell, may potentiate oxidative damage to MIT proteins and MIT DNA (mtDNA), leading to MIT dysfunction and mtDNA loss (Karthikeyan et al. 2002).

Damage to mtDNA severely affects MIT function due to the 13 mtDNA-encoded proteins vital for the electron transport chain (Schon et al. 2012). In FA patients, mtDNA was found to be decreased in the heart and cerebellum (Bradley et al. 2000). In yeast models of FA, increased mtDNA lesions and mtDNA loss was observed subsequent to iron-loading (Wilson and Roof 1997, Karthikeyan et al. 2002). Therefore, the MIT redox-active iron likely contributes to mtDNA degradation via
reactive oxygen species (ROS) generation. Indeed, as evidence of increased oxidative stress in frataxin deficiency, our published studies demonstrated a significant increase in protein oxidation and a decrease in reduced glutathione (GSH) to oxidised GSH (GSSG) ratio in the heart of muscle creatine kinase (MCK) frataxin KO mice (Anzovino et al. 2017).

Generally, increased ROS production and oxidative DNA damage lead to hyperactivation of the NAD$^+$-dependent ‘nick sensor’ poly(ADP-ribose) polymerase (Parp), MIT dysfunction, depletion of cellular ATP and NAD$^+$ levels, and the release of pro-apoptotic proteins culminating in cell death via energy restriction (Kim et al. 2007b, Nunnari and Suomalainen 2012, Bai et al. 2015). Thus, the selective recycling of defective mitochondria by MIT-specific autophagy (i.e., mitophagy) is essential to prevent these detrimental effects (Kim et al. 2007b). Mitophagy is mediated by the binding of the auto-phagosomal protein, Lc3, to outer MIT membrane adapters, such as Fundc1 (Lazarou 2015).

Our previous studies demonstrated a progressive increase in Fundc1 expression in the MCK frataxin KO mouse model (Huang et al. 2013). However, a progressive accumulation of the autophagic substrates, Lc3-II and p62, was also observed in the KO mice compared to WT littermates with increasing age (Huang et al. 2013). This latter finding is interesting, because under normal physiological conditions, these proteins are efficiently degraded by autophagy (Mizushima et al. 2010, Klionsky et al. 2016). This accumulation of autophagic substrates could suggest decreased autophagic flux in the heart of KO mice, given the activated autophagic initiation machinery (i.e., increased Atg3 and decreased Lc3-I), but an inhibition of lysosomal-mediated
autophagic degradation (*i.e.*, accumulation of Lc3-II and p62, which are efficiently degraded by the lysosome (Huang *et al.* 2013)).

Mitochondria are dynamic organelles that constantly undergo fission or fusion, which is collectively known as MIT network dynamics (Chen and Chan 2006, Frank 2006). In tissues with a high energy demand, such as the heart and neurons, MIT dynamics plays major roles in governing their viability (Kim *et al.* 2007b). As both neural and cardio-degeneration are major pathologies of FA, the alterations in MIT dynamics are key factors that require intense investigation. Recent studies in Parkinson’s disease have identified two major regulators of MIT dynamics and mitophagy, namely: Parkin, an E3 ubiquitin ligase whose mutation causes the most common autosomal recessive Parkinson’s disease; and Pink1, a Ser/Thr kinase that associates with damaged mitochondria to recruit and activate Parkin (Lazarou *et al.* 2015). Activated Parkin poly-ubiquitinites outer MIT membrane proteins, such as mitofusin (Mfn). This leads to Mfn degradation, inhibition of fusion with healthy mitochondria, and ensuing mitophagic degradation (*via* autophagic adapters and Lc3) (Scarffe *et al.* 2014, Lazarou 2015). Conversely, MIT fission is mainly facilitated by the adapter-mediated targeting of dynamin-related protein 1 (Drp1) to MIT constriction sites, where it constricts the outer MIT membrane and causes MIT fission (Palmer *et al.* 2013).

In terms of regenerating new mitochondria to restore MIT deficit, the peroxisome proliferation activator receptor (PPAR) γ-coactivator 1α (Pgc1α) is the major transcriptional regulator of MIT biogenesis (Finck and Kelly 2006). Pgc1α activates a group of transcription factors, including
nuclear respiratory factors 1 and 2 (Nrf1/2) and the MIT transcription factor A (Tfam), which coordinates the transcription of nuclear DNA and mtDNA encoded MIT proteins, respectively (Finck and Kelly 2006, Manoli et al. 2007). The activity of Pgc1α is predominantly regulated post-translationally by two important processes: (1) the phosphorylation of Pgc1α by AMPK and (2) the deacetylation of Pgc1α by the nuclear NAD⁺-dependent histone deacetylase, Sirt1 (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011). The activation of Sirt1 is driven by an overall increase in NAD⁺:NADH ratio, which results in Sirt1-mediated deacetylation of Pgc1α, thereby activating Pgc1α transcriptional activity (Fernandez-Marcos and Auwerx 2011). As such, Sirt1 is highly dependent on NAD⁺ levels to regulate MIT metabolism for energy production (Fernandez-Marcos and Auwerx 2011). Furthermore, Ampk can indirectly regulate Pgc1α through Sirt1 by increasing NAD⁺ levels (Canto et al. 2009), although the mechanism is unclear. These NAD+-dependent post-translational modifications of Pgc1α mediated by Ampk and Sirt1 activities demonstrate their importance in stress response and energy sensing by the cell, and hence, their roles in MIT biogenesis and function.

The disruption of the MIT respiratory chain is a well-studied cellular pathology in FA that inhibits cellular energy production (Huang et al. 2009, Richardson et al. 2010a, Huang et al. 2011). However, the mitochondria is a highly dynamic organelle that are well-known for its ability to remediate MIT dysfunction through the processes of MIT biogenesis, dynamics, and mitophagy, collectively referred to as the MIT homeostasis (Meeusen and Nunnari 2005, Youle and van der Bliek 2012). Importantly, perturbations in these processes have not been fully elucidated in FA, but are known to be involved in other cardiac and neural diseases (Johri and Beal 2012, Ikeda et al. 2014, Bhat et al. 2015). Therefore, this chapter aims to elucidate the role that MIT homeostasis
plays in the pathogenesis of FA. Herein, using the MCK conditional frataxin KO mouse that exhibits a fatal dilated cardiomyopathy (Puccio et al. 2001), my studies have identified a marked proliferation of mitochondria relative to the WT controls. These mitochondria in the KO demonstrated abnormal, ill-defined, cristae that were markedly condensed and were associated with markers of MIT dysfunction, such as increased Pink1 and Parkin. The MIT proliferation was associated with increased MIT biogenesis, increased fission, increased mitophagic flux, increased Parp activity and decreased NAD⁺:NADH ratio. Therefore, maintenance of intracellular NAD⁺ levels and/or inhibition of Parp-mediated NAD⁺ depletion may represent an important therapeutic strategy for the treatment of MIT myopathy and impaired cardiac function in FA, by improving MIT function.
4.2 Materials and Methods

4.2.1 Animals

For all of the following studies, the MCK conditional frataxin KO mice model was used and genotyped, as described previously in Section 2.2.1 (Puccio et al. 2001). All animal work was approved by the University of Sydney’s Animal Ethics Committee (Sydney, New South Wales, Australia).

4.2.2 Transmission Electron Microscopy (TEM)

MCK WT and KO mouse littermates were anesthetized by isoflurane and trans-cardiac perfusion was performed with K buffer (2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4) to fix the heart and remove excess blood (Shami et al. 2014). Heart was then excised and fixed whole overnight in K buffer before careful dissection into 1 mm³ pieces and further fixed 1 h/room temperature. Samples will then be washed three times with 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide for 1 h/room temperature.

Subsequently, samples were then dehydrated gradually in 30%, 50%, 70%, 95% and 100% absolute ethanol, followed by gradual resin infiltration in 25%, 50%, 75% and 100% medium density Epon resin (in 100% absolute ethanol) for 3 h, 12 h, 4 h and 12 h, respectively, at room temperature. Samples were then embedded in medium density Epon resin and polymerised at 60 °C/12 h following standard protocols for TEM sample preparation (Shami et al. 2014). Ultra-thin sections (70 nm) were collected on 200 nm copper mesh. Sample sections were post-stained with
2% uranyl acetate and 2% lead citrate for 10 min/room temperature each. The TEM micrographs were captured using a JEOL JEM-1400 transmission electron microscope (JEOL) operated at 120 kV. TEM Image analyses were examined using ImageJ (National Institutes of Health, Baltimore, MD) (Schneider et al. 2012). Measurements were taken from at least 3 images per mice (6 WT and 6 KO) and more than 100 mitochondria per image. Average number of mitochondria was measured by quantifying the number of mitochondria per image (Koopman et al. 2006, Picard et al. 2013). The mitochondria:myocyte area ratio was calculated by normalising the total area occupied by the mitochondria per cardiomyocyte by the total area of each cardiomyocyte. The mitochondrion cross-sectional area was quantified from the cross-sectional area of mitochondria per image. MIT aspect ratio was calculated by measuring the longest axis divided by the shortest axis per mitochondrion, as performed previously (Koopman et al. 2006, Picard et al. 2013).

4.2.3 Protein Isolation and Western Blot Analysis

Whole hearts from WT and KO MCK mice were homogenised for protein extraction, and Western blot analysis was performed with tissue lysates according to previously described procedures (Anzovino et al. 2017). Antibodies used were against Pgc1α (Abcam, Cambridge, MA; catalog number ab54481), Nrf1 (Abcam; catalog number ab34682), Tfam (Abcam; catalog number ab131607), Ampk (Abcam; catalog number ab32047), pAmpk (Cell Signaling, Danvers, MA; catalog number 2535S), Sirt1 (Abcam; catalog number ab7343), Paris (Abcam; catalog number ab130867), Anti-acetyl lysine (Abcam; catalog number ab80178), Mfn1 (ProteinTech, Rosemont, IL; catalog number 13798-1-AP), Opa1 (Abcam; catalog number ab42364), Drp1 (Abcam; catalog number ab56788), Fis1 (ProteinTech; catalog number 10956-1-AP), Mff (Abcam; catalog number ab81127), Pink1 (Novus Biology, Centennial, CO; catalog number NB100-493), Parkin (Abcam;
catalog number ab77924), Lc3 (MBL, Woburn, MA; catalog number PD014), Fundc1 (Aviva Systems Biology, San Diego, CA; catalog number ARP53280_P050), Sqstm1/p62 (Abcam; ab56416), Gapdh (Cell Signaling; catalog number 2118S), Tomm20 (Abnova, Walnut, CA; catalog number H00009804-M01), Hsp90 (Abcam; catalog number ab13495). Antibodies were used at 1:500 – 1:1000. Secondary antibodies used were goat anti-rabbit, goat anti-mouse (catalog number A0545 and A9917, respectively; 1/10,000; Sigma-Aldrich) conjugated with horseradish peroxidase.

4.2.4 Genomic DNA Isolation

For whole genome DNA extraction, approximately 20 mg of heart tissue from WT and KO MCK mice were cut into small pieces for isolation using the Wizard® SV Genomic DNA Purification System as per the manufacturer’s instructions (Promega, Madison, WI). Genomic DNA concentration was measured on a Nanodrop 1000 spectrophotometer.

4.2.5 Digital Droplet PCR (ddPCR)

The ddPCR studies were conducted following previous protocol with modifications (Li et al. 2018). Briefly, 100 pg of genomic DNA was used in a 20 μL duplex reaction containing ddPCR Supermix (BioRad), 5 U HaeIII restriction enzyme (NEB, Ipswich, MA) and 1 μL of the nuclear and mtDNA probes. For the nuclear genome probe, β2-microglobulin (B2m) probe (HEX; Assay ID: dMmuCNS576316288, Bio-Rad) was used. For mtDNA probes, NADH-ubiquinone oxidoreductase chain 1 (ND1; FAM; Assay ID: dMmuCNS343824284), was used (Bio-Rad). Standard ddPCR droplet generation procedure was conducted following the manufacturer’s instructions (Bio-Rad). Following droplet generation, 40 μL of dropletised reaction was transferred
to a 96-well PCR plate, heat sealed, and cycled with the following programs: 95 °C/10 min, 40 cycles of 94 °C/30 s and 60 °C/60 s, followed by 98 °C/10 min and hold at 4 °C. A ramp rate of 2 °C/s was used. Dropletised PCR products were analyzed on the QX100 Droplet Reader (Bio-Rad).

4.2.6 RNA Isolation from Tissue

Whole hearts from WT and KO MCK mice were homogenised on ice using a motorised homogeniser. Homogeniser probes had been treated in DEPC water overnight and sterilised prior to use. The RNA extraction was done using TRI® Reagent following the manufacturer’s protocol (Invitrogen, Carlsbad, CA) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

4.2.7 RT-qPCR

Total mRNA was obtained from whole hearts and used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was performed using the Roche Lightcycler 480 PCR Machine (Roche Diagnostics). Taqman probes (Thermo Fisher Scientific) targeting mouse Nrf1 (Mm00447996_m1), Tfam (Mm00447485_m1) and Gapdh (Mm99999915_g1) were used. Briefly, following the manufacturer’s protocol, the reverse transcription reaction mixture was performed by the following conditions in a Bio-Rad PCR Thermocycler: 25 °C/10 min, 37 °C/120 min, 85 °C/5 min and hold at 4 °C. For qPCR reaction, 100 ng of cDNA were then cycled with the following programs: 50 °C/2 min, 95 °C/10 min and 40 cycles of 95 °C/15 s, 60 °C/1 min and hold at 4 °C, with a ramp rate of 1.6 °C/s. Standard curves were generated for each probe and samples were fitted to the linear portion of the
curve. Data was analysed by the \( \Delta \Delta CT \) method using Genex Software (MultiD Analyses, Göteborg Sweden).

### 4.2.8 Mitochondrial Isolation

Whole hearts from WT and KO MCK mice were extensively perfused \textit{in vivo} with ice-cold PBS (pH 7.4) to remove excess blood prior to removal. Tissue was weighed then mitochondria isolated using the MIT isolation kit for tissue following manufacturer’s instructions (Thermo Scientific, catalog number 89801).

### 4.2.9 Nuclear and Cytosolic Fractions

Cytosolic and nuclear fractions were prepared from MCK whole hearts and skeletal muscle (left and right quadriceps) using NE-PER nuclear and cytosolic extraction reagents (Thermo Fisher Scientific, Waltham, MA). Fractionation was performed according to the kit instructions. Tissue samples were first homogenised in a Dounce glass homogeniser. Tissue lysates were then supplemented with a 1x solution of protease inhibitor (Roche Diagnostics) and 1x solution of PhosSTOP (Roche Diagnostics).

### 4.2.10 Immunoprecipitation

Whole hearts were homogenised in 500 μL of ice-cold RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 1x solution of protease and phosphatase inhibitors (Roche Diagnostics)] using a Dounce glass homogeniser. Immunoprecipitation was performed as described previously (Anzovino \textit{et al.} 2017),
but using a Pgc1α antibody. Briefly, the tissue homogenate was centrifuged at 13,200 \times g/4 ^\circ \text{C}/40 \text{ min. The supernatant was collected in a new sterile Eppendorf tube and the protein concentration was determined using the BCA protein assay (Pierce Biotechnology). Then 50 \mu L of Dynabeads protein G (Novex; Thermo Fisher Scientific) per sample were placed in Eppendorf tubes and washed twice with RIPA buffer. After the washes, the beads were re-suspended in 500 \mu L of RIPA buffer and 5 \mu g of Pgc1α antibody (Abcam, catalog number ab54481) per tube was added. The primary antibody was conjugated to the beads after incubation overnight at 4 ^\circ \text{C}. Next day, the conjugated beads were collected on a magnet and the supernatant removed. The conjugated beads were washed twice with ice-cold RIPA buffer and the last wash discarded. Then, 500 \mu g of protein lysate was added per tube of conjugated beads and the volume was made to 100 \mu L with RIPA buffer. Lysates and antibody-conjugated beads were incubated at 4 ^\circ \text{C}/1 \text{ h plus rotation. The beads were then collected on a magnet and the supernatant was discarded. The beads were washed 3 times with ice-cold RIPA buffer and then re-suspended in 35 \mu L of 1x loading dye plus \beta-mercaptoethanol. The samples were incubated at 95 ^\circ \text{C}/5 \text{ min to disassociate the complexes from the beads. The beads were then collected on the magnet and Western blot analysis was performed, as was previously described (Anzovino et al. 2017).}

4.2.11 NAD^+, NADH and NAD^+:NADH Ratio Assessment

Whole hearts from WT and KO MCK mice were perfused with PBS (pH 7.4) to remove excess blood prior to removal and NAD^+ and NADH levels were extracted from heart homogenates, as previously described (Herrera-Marschitz et al. 2018). Briefly, 100 \mu L of homogenate was added to 20 \mu L of \([\text{D}_4]\)-NAM in 5% formic acid (v/v). Samples were then mixed with 300 \mu L of acetonitrile, vortexed for 15 s, and then centrifuged, and dried via speed vacuum overnight at
37 °C. Samples were then reconstituted in 100 μL of 10 mM ammonium acetate with 0.1% formic acid. Separation and quantitation of NAD\(^+\) and NADH were performed with a QTRAP 5500 mass spectrometer (Sciex, Victoria, Australia) operated in positive ion multiple reaction monitoring mode, as described previously (Herrera-Marschitz et al. 2018). NAD\(^+\) and NADH were separated on a Phenomenex NH\(_2\) column (150 mm x 2 mm x 3 μm; Phenomenex, Torrance, CA) using the same gradient and mobile phase as described for the acid separation (Herrera-Marschitz et al. 2018). The analytes were separated using a binary solvent gradient consisting of 5 mM NH\(_4\)OAc (pH 9.5) adjusted with ammonia (mobile phase A) and acetonitrile (mobile phase B) with a flow rate of 250 μL/min. The initial solvent composition at injection was 25% A, followed by a 2 min gradient to 45% A and a fast gradient ramp to 80% A (0.1 min), which was maintained for 5.9 min. Then, A was increased again to 95% (2 min), held for 13 min and then reverted to initial conditions (0.1 min) for equilibration, with a total run time of 30 min. The % coefficient of variation ranges from 2.8-15.8%, with the limit of quantification being 0.1-0.2 pmol for NAD\(^+\) and NADH, respectively (Bustamante et al. 2017).

4.2.12 ATP Assessment

The levels of intracellular ATP were measured from harvested heart tissues from WT and KO MCK mice, and the assessment was performed using the luciferin-luciferase assay system (Promega, catalog number P1041 and E1500), as previously described (Harper et al. 1998, Braidy et al. 2014). In brief, tissue was homogenised in 250 μL of extraction solution (10 mM KH\(_2\)PO\(_4\), 4 mM MgSO\(_4\), pH 7.4), heated for 4 min at 98 °C, and then placed on ice. 50 μL of sample was then added to a 100 μL reaction solution (50 mM NaAsO\(_2\), 20 mM MgSO\(_4\), pH 7.4, and 800 μg of luciferin/luciferase enzyme). Luminosity at 259 nm was measured with an extinction coefficient
of 15,400 M⁻¹.cm⁻¹ using a Turner Designs TD 20/20 luminometer (Stratec Biomedical Systems, Germany) (Braidy et al. 2014). ATP levels were normalised to protein concentration using the Bradford protein assay (Bradford 1976).

### 4.2.13 Sirt1 Deacetylase Activity

Sirt1 deacetylase activity was evaluated in nuclear extracts from the heart using the Cyclex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CycLex, Nagano, Japan). The final reaction mixture (100 µL) contained 50 mM Tris-HCl (pH 8.8), 4 mM MgCl₂, 0.5 mM DTT, 0.25 mA/ml Lysyl endopeptidase, 1 µM Trichostatin A, 200 µM NAD⁺, and 5 µL of nuclear sample. After mixing thoroughly, samples were incubated for 10 min at room temperature and the fluorescence intensity (ex. 340 nm, em. 460 nm) was measured every 30 s for a total of 60 min immediately after the addition of fluorosubstrate peptide (20 µM final concentration) using a Fluostar Optima Fluorometer (NY, USA) and normalised by the protein content. The results are reported as relative fluorescence (FU)/mg of protein.

### 4.2.14 Parp Activity Assay

Parp is the major consumer of NAD⁺ in the nucleus (Durkacz et al. 1980, Soane et al. 2007, Cerutti et al. 2014). Previous studies have utilised nuclear NAD⁺ consumption as an accurate indicator of Parp activity (Putt and Hergenrother 2004, Putt et al. 2005, Braidy et al. 2014, Herrera-Marschitz et al. 2018). Hence, NAD⁺ consumption activity was measured in nuclear extracts from the heart, as previously described (Putt et al. 2005). The final reaction mixture contained 10 mM MgCl₂, Triton X-100 (1%), and 20 µM NAD⁺ in 50 mM Tris buffer, pH 8.1. The plate was then incubated
for 1 h/37 °C and the amount of NAD⁺ consumed was measured as described above. NAD⁺
consumption was normalised to protein concentration using the Bradford protein assay (Bradford
1976). The results were expressed as µg NAD⁺ consumed/h/mg nuclear protein.

4.2.15 Statistical Analysis
Data were compared using Student’s t-test, or one-way analysis of variance (ANOVA). Data were
considered statistically significant when p<0.05. Results are expressed as mean ± SEM (3-6
experiments).
4.3 Results

4.3.1 Morphology of Mitochondria in 4-Week and 10-Week-Old Frataxin-Deficient Heart

Deficiency of the MIT protein, frataxin, causes severe cardiomyopathy in FA (Puccio et al. 2001, Richardson et al. 2010a). In fact, cardiomyocytes have significantly greater number of mitochondria than other tissue-types and are exquisitely dependent on mitochondria for ATP production (Chen and Zweier 2014). Consequently, cardiomyocytes are particularly susceptible to the generation of mitochondrial ROS (Chen and Zweier 2014). Our previous studies identified marked MIT defects in the regulation of highly redox active iron in the heart from MCK KO cardiac mouse model (Huang et al. 2009, Huang et al. 2011, Whitnall et al. 2012, Huang et al. 2013, Anzovino et al. 2017), which can result in a vicious cascade of oxidative damage hindering MIT function (Bradley et al. 2000, Karthikeyan et al. 2002). While MIT homeostasis is essential in preventing these detrimental effects (Kim et al. 2007b), the alterations in MIT homeostasis remains largely uncharacterised in the cardiomyopathy of FA. To investigate the effects of cardiac frataxin deficiency on MIT homeostasis, we first examined MIT morphology by TEM using the heart of the well-established MCK KO mouse versus their WT littermates.

The KO mice at 4-weeks of age was previously shown to exhibit no gross, histological, or functional cardiac defects relative to their WT counterparts (Anzovino et al. 2017), while marked pathologies in these aspects had developed by 10-weeks of age (Puccio et al. 2001, Huang et al. 2013). As such, 4- and 10-week-old MCK mice were examined in this study to show disease progression in the heart at the ultrastructure level by comparing the KO with their respective WT littermates. This complements our previous studies that also utilise these two ages to characterise the pathological progression at the molecular, functional, and gross histological levels (Huang et
al. 2013). Both transverse (Fig. 4.1) and longitudinal (Fig. 4.3) sections of MCK mouse hearts (WT and KO) were examined in both age groups to provide a perspective of the horizontal and vertical planes of the myocyte and its mitochondria. In these studies, sections of the left ventricle were examined as the heart in the MCK *frataxin* KO demonstrates defective systolic function, as characterised by reduced fractional shortening, enlargement of left ventricular end-diastolic and end-systolic dimensions, and fibrosis and iron deposits within this region (Huang *et al.* 2013).

### 4.3.2 Transverse TEM Sections of Cardiomyocytes

Examining transverse sections of the left ventricular cardiomyocytes, the 4-week-old KO mice showed some observable difference in MIT morphology relative to their WT littermates (Fig. 4.1A). An interesting feature of the mitochondria from the KO mice at this age was the presence of condensed MIT cristae that was not observed in the WT (see white arrows; Fig. 4.1A), suggesting an alteration in MIT structure and function. Similarly, transverse sections of 10-week-old KO heart demonstrated an increased abundance of mitochondria that was not observed in the 4-week KO, with the loss of clear cristae structure and increased condensation of MIT cristae (see white arrows; Fig. 4.1B), relative to WT mice where defined cristae are observed.

Furthermore, examining the spatial distribution of the mitochondria within cardiomyocyte from WT and KO mice at 4-weeks of age, the mitochondria appear as predominantly isolated organelles distributed amongst the myofibres (Fig. 4.1A). In clear contrast, the 10-week-old KO mice exhibit marked MIT proliferation where these organelles appear together as disorganised masses. This morphology contrasts to the 10-week-old WT hearts that show similar morphology to the 4-week-old WT hearts. Notably, in contrast to the 4-week-old WT and KO mitochondria and 10-week-old
WT mice, in the 10-week-old KO mice, electron-dense accumulations consistent with iron deposits can be observed (black arrows; **Fig. 4.1B**), as previously observed in this model (Puccio et al. 2001, Whitnall et al. 2012, Anzovino et al. 2017). In fact, analysis of the MIT electron-dense accumulations in the 10-week-old KO (white arrow, **Fig. 4.2A**) using energy dispersive X-ray spectroscopy (EDS) confirms X-ray energy emission specific for elemental iron, with Kα of 6.4 keV (**Fig. 4.2B**). Analysis of electron scatter of the iron aggregate did not observe any identifiable diffraction patterns, suggesting that the iron accumulation was amorphous in composition.

Quantitative assessments were performed to delineate the morphological differences in mitochondria described above in the transverse heart sections (**Fig. 4.1C-F**). For these assessments, approximately 250 mitochondria across 3- to 5-different TEM images of each specimen of WT and KO mouse in each age group were used. In agreement with the observed MIT proliferation in the KO at 10-weeks (**Fig. 4.1B**), the average number of mitochondria per field of view was significantly ($p<0.001$) increased in the 10-week-old KO relative to the WT, while there was no significant change between the KO and WT mice at 4-weeks of age (**Fig. 4.1C**). When comparing between age groups, there was no significant difference between the WT s, whereas average MIT number per field of view in the 10-week-old KO was markedly ($p<0.001$) increased relative to the 4-week-old KO heart (**Fig. 4.1C**).
Figure 4.1: Qualitative and quantitative assessment of MIT morphologies in transverse TEM sections of the heart of MCK wild-type (WT) and knockout (KO) littermates. Examining TEM, transverse-sections of the heart from WT and KO MCK littermates at: (A) 4-weeks and (B) 10-weeks of age were assessed. Mitochondria (m) were found within the myocyte with condensed MIT cristae (white arrows) at both 4- and 10-weeks of age in the KO being identified, while iron aggregates were found in KO mitochondria only at 10-weeks of age (black arrows). Quantitatively, the average number of mitochondria (C), mitochondria:myocyte area ratio (%; D), mitochondrial cross-sectional area (µm²; E), and MIT aspect ratio (F) were assessed between the KO and WT at 4- and 10-weeks of age. Data are mean ± SEM, (n = 6) in each age group. *p<0.05, **p<0.01, ***p<0.001 between 4- and 10-week-old WTs and KOs. #p<0.05, ##p<0.01, ###p<0.001 versus WT. a.u., arbitrary unit. n.s., not significant. I acknowledge the facilities, and the scientific and technical assistance, of the Australian Microscopy & Microanalysis Research Facility at the University of Sydney.
A 4 weeks WT 4 weeks KO

Zoom

500 nm
Zoom

2 μm 2 μm

500 nm
m m m

10 weeks WT 10 weeks KO

B 10 weeks WT 10 weeks KO

500 nm
Zoom

500 nm
Zoom

m m m

C

Average number of mitochondria

Mitochondria/myocyte area ratio

Mitochondrion cross-sectional area (μm²)

Mitochondrial aspect ratio (a.u.)

D

E

F

0 100 200 300 400 500 600

n.s. ***

WT KO

WT KO

WT KO

WT KO
Figure 4.2: Energy dispersive X-ray spectroscopy (EDS) analysis of iron accumulations within the mitochondria of 10-week-old hearts from MCK KO mice. (A) TEM micrograph of the region subjected to EDS analysis. (B) Corresponding X-ray distribution map for iron Kα (6.4 keV) over identical field as shown in (A). The white arrows in (A) and (B) indicate the electron-dense iron accumulations. Scale bar is 1 μm as indicated in (A) and (B). I acknowledge the facilities, and the scientific and technical assistance, of the Australian Microscopy & Microanalysis Research Facility at the University of Sydney.
The total mitochondria area to myocyte area ratio of 4- and 10-week-old were significantly ($p<0.01$-$p<0.05$) increased in the KO mice relative to the WT (Fig. 4.1D). However, there was no significant change to this ratio between the 4- and 10-week WT mice, while there was a significant ($p<0.01$) increase in the mitochondria area to myocyte area ratio in the 10-week-old KO relative to the 4-week-old KO (Fig. 4.1D). Notably, the average area cross-sectional area of individual mitochondrion (MIT area; $\mu m^2$) was significantly ($p<0.05$) increased in the 4-week-old KO relative to the 4-week-old WT, while a marked ($p<0.05$) decrease in MIT area occurred comparing the 10-week-old KO relative to its 10-week-old WT littermates (Fig. 4.1E). Interestingly, the average MIT area was significantly ($p<0.05$) increased for the WTs as a function of age, while it was significantly ($p<0.05$) decreased for the KOs with advancing age (Fig. 4.1E).

The fourth quantitative assessment is the MIT aspect ratio, which is a measure of MIT shape by assessing the ratio between the length of the major axis relative to the length of the minor axis of an individual mitochondrion (Picard et al. 2013). It was found in the transverse sections of the heart that the average MIT aspect ratio was not significantly different between the 4-week-old KO relative to their WT, while there was a significant ($p<0.01$) increase in the 10-week-old KO mice relative to the WT (Fig. 4.1F). This finding indicates that the mitochondria in 10-week-old KO mice are becoming more elongated in the transverse plane. Furthermore, the average MIT aspect ratio of the 10-week-old WT mice was significantly ($p<0.001$) decreased when compared to the 4-week-old WT, while an opposite effect was observed in the KO mice, with a significant ($p<0.01$) increase in the average MIT aspect ratio with age (Fig. 4.1F).
The analysis of the transverse sections of the MCK KO and WT cardiomyocytes indicated that there were: (1) proliferation of mitochondria, as indicated by an increased number of mitochondria (Fig. 4.1C); (2) an increased mitochondria area to cardiomyocyte area ratio (Fig. 4.1D); (3) decreased mitochondrion cross-sectional area (Fig. 4.1E); and (4) an elongation of mitochondria (Fig. 4.1F).

4.3.3 Longitudinal TEM Sections of Cardiomyocytes

For longitudinal sections of the left ventricular cardiomyocytes (Fig. 4.3), there were similar morphological changes in KO relative to their WT littermates in both age groups, identified in the previously described transverse sections (Fig. 4.1). Similarly, condensed MIT cristae can be observed in the 4-week-old KO compared to their WT littermates (Fig. 4.3A), and by 10-weeks of age, the amount of mitochondria with condensed cristae was increased and a greater loss of cristae structure was observed in the KO relative to the WT (Fig. 4.3B). Moreover, electron-dense accumulation consistent with iron deposits were also present in the 10-week-old KO mice (black arrows; Fig. 4.3B). The mitochondria of 4-week-old WT and KO hearts run distinctively parallel to the myofibrils (Fig. 4.3A). In contrast, mitochondria of 10-week-old KO mice are far less organised, and appear to collect in large clusters compared to the 10-week-old WT (Fig. 4.3B). Furthermore, there appears to be an increase in MIT number in the KOs with age (Fig. 4.3A, B).
Figure 4.3: Qualitative and quantitative assessment of MIT morphologies in longitudinal TEM sections of the heart of MCK WT and KO littermates. Using TEM, longitudinal sections of the heart from MCK WT and KO littermates at: (A) 4-weeks and (B) 10-weeks of age were assessed. Mitochondria (m) were observed in the myocytes where condensation of MIT cristae (white arrows) were identified at 4- and 10-weeks of age, while MIT iron deposits (black arrows) were present at 10-weeks of age only. In addition, the nucleus is marked by N. Quantitatively, the average number of mitochondria (C), mitochondria:myocyte area ratio (%; D), mitochondrion cross-sectional area (µm²; E), and MIT aspect ratio (F) were assessed between the hearts of KO and WT littermates at 4- and 10-weeks of age. Results are as mean ± SEM, n = 6 in each age group. *p<0.05, **p<0.01, ***p<0.001 between 4- and 10-week-old WTs and KOs. #p<0.05, ##p<0.01, ###p<0.001 versus WT. a.u., arbitrary unit. n.s., not significant. I acknowledge the facilities, and the scientific and technical assistance, of the Australian Microscopy & Microanalysis Research Facility at the University of Sydney.
Average number of mitochondria
Mitochondria:myocyte area ratio
Mitochondrion cross-sectional area (μm²)
Mitochondrial aspect ratio (a.u.)

4 weeks WT
4 weeks KO

10 weeks WT
10 weeks KO

A
B
C
D
E
F

n.s.
*
**
###
##
***
**

4 weeks
10 weeks

WT
KO

0
50
100
150
200
250
300

0
20
40
60
80
100

0
0.4
0.8
1.2
1.6

0
2
4
6

4 weeks
10 weeks

WT
KO

n.s.
*
**
###
##
***
**
Quantitatively, in longitudinal heart sections, there was a significant ($p<0.001$) increase in the number of mitochondria in the 10-week-old KO relative to the WT, while there was no significant change at 4-weeks of age between the WT and KO mice (Fig. 4.3C). Interestingly, the MIT number was significantly ($p<0.01$-$p<0.05$) increased for the WTs and KOs with age (Fig. 4.3C). In accordance with the observation of MIT proliferation in the longitudinal sections, the mitochondria area to myocyte area ratio was significantly ($p<0.01$-$p<0.05$) increased in both 4- and 10-week-old KO relative to their WT littermates (Fig. 4.3D). However, this ratio was unchanged in the WTs between both age groups, whereas there was a marked ($p<0.01$) increase between 4- and 10-week-old KO mice (Fig. 4.3D).

Interestingly, the mitochondrion cross-sectional area in cardiomyocytes was significantly ($p<0.05$) increased in the 4-week-old KO mice relative to the WT, while there was a marked and significant ($p<0.001$) decrease for the KO mice at 10-weeks of age compared to the WT (Fig. 4.3E). Notably, the MIT area was significantly ($p<0.001$) increased in the WTs with age, while it was significantly ($p<0.05$) decreased for the KOs (Fig. 4.3E). Finally, the MIT aspect ratio was significantly ($p<0.001$) decreased in the 4-week-old KO cardiomyocytes relative to the WT, whereas it was significantly ($p<0.01$) increased at 10-weeks of age when compared to their 10-week-old WT littermates (Fig. 4.3F). The MIT aspect ratio was also significantly ($p<0.001$) decreased between the WTs with increasing age, and significantly ($p<0.01$) increased between the 4- and 10-week-old KOs (Fig. 4.3F).

Collectively, our TEM assessment of transverse and longitudinal sections of left ventricular cardiomyocytes demonstrates marked alterations in MIT morphology as indicated by: (1) an
abnormal proliferation of mitochondria in the KOs relative to the WTs that progressively increased the mitochondria:myocyte ratio; and (2) mitochondria with reduced cross-sectional area that are markedly elongated in both anatomical planes in the KOs compared to the WT littermates (Fig. 4.1, 4.3). These pronounced alterations in MIT morphologies in cardiomyocytes of the diseased heart would likely be driven by marked molecular alterations in MIT homeostasis in the KOs and cause significant alterations to MIT function that may contribute to the pathogenesis of the disease (Chen and Chan 2006, Finck and Kelly 2006, Frank 2006, Kim et al. 2007b, Scarffe et al. 2014, Lazarou et al. 2015).

4.3.4 Dysfunctional Alterations in Mitochondrial Biogenesis in the Frataxin KO Heart

The observed increase of proliferation of mitochondria in the MCK heart could be due to enhanced MIT biogenesis, in an attempt to increase MIT mass to attenuate the MIT deficits in disease states (Finck and Kelly 2006). Accordingly, the markers of MIT biogenesis were examined, including the primary transcriptional regulator of MIT biogenesis, Pgc1α, which plays a role in the transcriptional activation of Nrf1 and Tfam (Finck and Kelly 2006, Manoli et al. 2007). Both of the latter are transcription factors essential for nuclear DNA and mtDNA transcription of MIT proteins, respectively (Finck and Kelly 2006, Manoli et al. 2007).

Using western blot analysis, the total protein expression of Pgc1α, Nrf1, and Tfam were assessed in the hearts of 4- and 10-week-old MCK mice (Fig. 4.4A). In both age groups, Pgc1α levels were significantly ($p<0.001-0.05$) increased in the KO versus WT mice (Fig. 4.4A). Consistent with this observation, there were also significant ($p<0.01-0.05$) increases in Nrf1 and Tfam protein levels in the KO mice of both age groups, relative to the respective WTs (Fig. 4.4A). These results
suggest that MIT biogenesis is up-regulated in the frataxin KO heart, which could explain the observed increase in MIT number (Fig. 4.1, 4.3).
Figure 4.4: Examination of (A) mitochondrial biogenesis, (B) mtDNA copy numbers, (C) gene expressions, (D) regulation of Pgc1α, (E) mitochondrial bioenergetics, (F) ATP levels, (G) Parp activity, (H) Sirt1 activity, and (I) Pgc1α acetylation in the heart of MCK WT and KO littermates. (A) Total protein expression of MIT biogenesis markers (Pgc1α, Nrf1, Tfam), (B) quantitation of MIT DNA (mtDNA) copies of ND1 relative to the single copy nuclear DNA B2m gene, as performed by digital droplet PCR, (C) Nrf1 and Tfam gene expressions, and (D) Pgc1α regulatory markers (Ampk, Paris, and Sirt1), were assessed in 4- and 10-week-old MCK mice. (E) Hearts from MCK WT and KO littermates at 10-weeks of age were assessed for NAD\(^+\) and NADH levels, and the NAD\(^+\):NADH ratio, as well as (F) ATP levels, (G) nuclear NAD\(^+\) consumption as a measure of Parp activity, and (H) Sirt1 activity by standard methods (see Materials and Methods). (I) Immunoprecipitation of Pgc1α with detection of acetyl-lysine in the immunoprecipitated using lysates from MCK WT and KO littermates at 4- and 10-weeks of age. The densitometry data are mean ± SEM (\(n = 4\)-6 experiments). *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) versus WT. a.u., arbitrary unit. n.s., not significant.
4.3.5 Assessment of Mitochondrial DNA Copy Numbers in the \textit{Frataxin} KO Heart

Increased MIT number (Fig. 4.1, 4.3) and enhanced MIT biogenesis signalling (Fig. 4.4) could potentially lead to an increase in mtDNA copy number per cardiomyocyte in the KO relative to the WT littermates, respectively. To directly examine mtDNA copy number in these animals with frataxin deficiency, the MIT encoded genes, \textit{NADH-ubiquinone oxidoreductase chain 1} (\textit{ND1}), was assessed by the digital droplet PCR method at 4- and 10-weeks of age (Fig. 4.4B). The copy numbers of the mtDNA \textit{ND1} gene was normalised to the single copy nuclear gene, \textit{B2m}, to deduce mtDNA copies per copy of nuclear DNA (Phillips \textit{et al.} 2014, Li \textit{et al.} 2018). At 4-weeks of age, normalised \textit{ND1:B2m} copy numbers was not significantly ($p>0.05$) altered in the KO relative to the WT (Fig. 4.4B). Interesting, by 10-weeks of age, the mtDNA copy number of \textit{ND1} was significantly ($p<0.05$) increased in the KO relative to the WT (Fig. 4.4B). This is a significant observation because the mtDNA \textit{ND1} gene is situated in a region of the mtDNA sequence that is less susceptible to deletion (Li \textit{et al.} 2018), and therefore its increase in mtDNA copy number correlates with the increase in MIT number in the KO hearts.

4.3.6 Alterations in \textit{Nrf1} and \textit{Tfam} gene expression in the \textit{Frataxin} KO Heart

MIT biogenesis is potentially up-regulated due to the observed increase in Pgc1\(\alpha\) protein levels in the KO mice (Fig. 4.4A), which could lead to an increase in the transcription of its downstream target genes, namely, \textit{Nrf1} and \textit{Tfam}. As such, the transcription levels of \textit{Nrf1} and \textit{Tfam} were assessed in the MCK mice using RT-qPCR. At 4-weeks of age, \textit{Nrf1} mRNA levels were significantly ($p<0.05$) decreased, while \textit{Tfam} was unchanged ($p>0.05$) in the KO relative to the WT (Fig. 4.4C). By 10-weeks of age, both \textit{Nrf1} and \textit{Tfam} mRNA levels were significantly ($p<0.001$) decreased in the KO (Fig. 4.4C). This finding does not correspond with the observed
increase in Nrf1 and Tfam total protein levels in the KO heart (Fig. 4.4A). However, their decreased mRNA levels suggest that Pgc1α transcriptional activity could be reduced despite an increase in Pgc1α total protein levels in the KO (Fig. 4.4A). In order to assess the activity of Pgc1α, it is necessary to investigate the regulators of Pgc1α and MIT biogenesis, which are involved in the sensing of cellular energy levels, and hence, MIT function.

4.3.7 The Regulation of Pgc1α in the Frataxin KO Heart at Both 4-Weeks and 10-Weeks of Age

Pgc1α can be transcriptionally up-regulated by Ampk and repressed by Paris, while Pgc1α activity can be further post-translationally regulated by Ampk-mediated phosphorylation and deacetylation by Sirt1 (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011, Mihaylova and Shaw 2011, Shin et al. 2011). Both Ampk and Sirt1 also play a role in modulating NAD+ levels in response to changing cellular energy status to regulate Pgc1α activity (Zong et al. 2002, Canto et al. 2009). Ampk can increase NAD+ levels in response to low energy levels (i.e., high AMP:ATP ratio; (Canto et al. 2009), while increased NAD+ levels activate Sirt1, which deacetylates, and thus, activates Pgc1α transcriptional activity (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011). As such, phosphorylated Ampk (pAmpk) at Thr172, total Ampk, Paris, and Sirt1 expression were examined in these animals to assess the regulation of Pgc1α. In the 4-week-old mice, the expression of pAmpk and total Ampk were significantly (p<0.001-0.05) increased in the KO relative to the WT (Fig. 4.4D). However, the pAmpk to total Ampk ratio was not significantly (p>0.05) altered, which suggests that Ampk activity was unchanged in the 4-week-old KO mice (Fig. 4.4D).
The levels of Paris were not significantly (p>0.05) changed, while Sirt1 was significantly (p<0.05) decreased in the KO hearts at 4-weeks of age (Fig. 4.4D). By 10-weeks of age, while pAmpk and total Ampk levels remained significantly (p<0.01) increased, the pAmpk to Ampk ratio was also significantly (p<0.05) increased in the KO relative to the WTs (Fig. 4.4D). This Ampk activation in the KO, together with the significant (p<0.01) down-regulation of the Pgc1α transcriptional repressor, Paris (Fig. 4.4D), could explain the observed up-regulation of Pgc1α protein (Fig. 4.4A). However, Sirt1 expression was significantly (p<0.01) decreased in the 10-week-old KO (Fig. 4.4D), which could result in reduced Pgc1α deacetylation. Since Sirt1 activity is NAD⁺-dependent (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011), it is necessary to assess NAD⁺ levels to examine the Sirt1 activity.

4.3.8 Decreased NAD⁺:NADH Ratio, ATP levels, and Sirt1 Activity in the 10-Week Old Frataxin KO Heart

Previous studies have shown that an imbalance of NAD⁺ levels can alter MIT biogenesis in order to restore MIT function and homeostasis (Canto et al. 2009, Long et al. 2015). In fact, the NAD⁺-dependent deacetylation of Pgc1α by Sirt1 is a major regulator of MIT biogenesis (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011). Interestingly, NAD⁺ levels were significantly (1.11 ± 0.08 vs. 2.20 ± 0.10 μg/mg protein; p<0.001) decreased, while NADH level was significantly (3.16 ± 0.08 vs. 1.42 ± 0.14 μg/mg protein; p<0.001) increased in the 10-week-old KO mice relative to the WT (Fig. 4.4E). As a result, the NAD⁺:NADH ratio was significantly (0.35 ± 0.02 vs. 1.57 ± 0.11; p<0.001) decreased in the KO mice versus the WT (Fig. 4.4E). These results suggest that upon frataxin deficiency, there was decreased NAD⁺ generation and/or increased consumption of NAD⁺, or impaired oxidation of NADH to NAD⁺. This observation can be due to
the presence of a greater number of defective mitochondria that are unable to produce, utilise, and/or recycle NAD\(^+\) effectively. This imbalance of NAD\(^+\) levels is also an indication of MIT dysfunction, where the vital metabolic processes for ATP production and energy metabolism could be dysfunctional in the KO mice. Indeed, the level of ATP was markedly \(p<0.001\) decreased in KO mice \textit{versus} the WT\(s\) (Fig. 4.4F). A reduced ATP level would also support the observed increase in Ampk phosphorylation in the KO (Fig. 4.4D).

The availability of NAD\(^+\) within subcellular pools is greatly influenced by the activity of several NAD\(^+\)-dependent enzymes, such as the poly(ADP)-ribose polymerase (Parp) (Canto and Auwerx 2011b, Chiarugi \textit{et al.} 2012, Houtkooper and Auwerx 2012). Activation of Parp in response to oxidative DNA damage (Oei and Shi 2001, Chiarugi and Moskowitz 2003, Wang \textit{et al.} 2009), could potentially contribute to the depletion of NAD\(^+\) in the KO mice. The assay used in this study for nuclear NAD\(^+\) consumption strongly reflects the activity of Parp, as it is the highest consumer of NAD\(^+\) in mammalian tissue (Durkacz \textit{et al.} 1980, Soane \textit{et al.} 2007, Cerutti \textit{et al.} 2014). Interestingly, Parp activity was found to be markedly \(p<0.001\) increased in the 10-week-old KO relative to the WT (Fig. 4.4G), which suggests increased NAD\(^+\) consumption due to increased oxidative DNA damage. Thus, this may lead to the decreased Sirt1 activity and the subsequent activation of Pgc1\(\alpha\) (Bai \textit{et al.} 2011, Fang \textit{et al.} 2014). Furthermore, increased poly(ADP-ribose) (Par) formation plays a role in energy derangement upon genotoxic stress and increased Par synthesis can affect epigenetic regulation of gene expression (Kraus and Lis 2003, Kraus 2008).

Sirt1 is another important NAD\(^+\)-dependent enzyme that is able to deacetylate and thereby modulate the activity of a wide variety of proteins involved in MIT oxidative metabolism and cell
fate decision, including Pgc1α (Imai and Guarente 2010). Therefore, the observed decrease in NAD$^+$ levels (Fig. 4.4E) could lead to a decrease in Sirt1 activity (Imai and Guarente 2010). Using fluorometry, Sirt1 activity was found to be significantly ($p<0.01$) decreased in the KO hearts relative to the WTs (Fig. 4.4H), which could affect the Sirt1-catalysed deacetylation of Pgc1α, and thereby reduce Pgc1α transcriptional activity (Fig. 4.4C) despite the markedly increased Pgc1α protein expression (Fig. 4.4A) (Canto and Auwerx 2009).

### 4.3.9 Increased Pgc1α Acetylation in the Frataxin KO Heart

Considering that Pgc1α can be activated post-translationally through its deacetylation by Sirt1, and that Sirt1 levels and activity were significantly decreased in the KO (Fig. 4.4D, H), immunoprecipitation of Pgc1α followed by western blot analysis for acetyl-lysine levels were conducted in the MCK WT and KO heart lysates to assess Pgc1α activation. At 4-weeks of age, the levels of Pgc1α acetyl-lysine was significantly ($p<0.01$) increased in the KO relative to the WT when normalised to total Pgc1α (Fig. 4.4I). The levels of Pgc1α acetyl-lysine was also significantly ($p<0.001$) increased in the 10-week-old KO (Fig. 4.4I). These findings suggest increased inactivation of Pgc1α in the KO with age despite an increase in Pgc1α total protein levels at both age groups (Fig. 4.4A). This in turn, could potentially explain the decrease in Nrf1 and Tfam gene expression in the 10-week-old KO (Fig. 4.4C). Previous studies have reported that increased MIT protein acetylation is associated with a decrease in cardiac function in models of FA (Wagner et al. 2012, Stram et al. 2017).
4.3.10 Increased Protein Expression of Mitochondrial Fusion and Fission Proteins in the Frataxin KO Heart at Both 4-Weeks and 10-Weeks of Age

The observed MIT elongation in the KO mice (Fig. 4.1, 4.3), could be explained by changes in the regulation of MIT dynamics, namely, MIT fusion and MIT fission. Under MIT stress, MIT fusion is a dynamic process that could alleviate the accumulated MIT ROS and mtDNA damage via complementation to restore adequate MIT function, such as energy production (Youle and van der Bliek 2012). In mammals, fusion of the outer and inner MIT membranes is facilitated by Mfn1/2, and the dynamin family member, Opa1, respectively (Cipolat et al. 2004, Frank 2006, de Brito and Scorrano 2008). Alternatively, MIT fission compartmentalises damaged MIT components into daughter organelles that could be removed by selective mitophagy (Youle and van der Bliek 2012). In mammals, MIT fission involves the cytoplasmic protein, dynamin-related protein 1 (Drp1), which forms a ring structure to encircle the constriction site on the outer MIT membrane upon its interaction with fission protein 1 (Fis1) and MIT fission factor, Mff (Smirnova et al. 2001, Youle and van der Bliek 2012).

In the 4-week-old mice, the total cellular expression of Mfn1 and Opa1 were significantly (p<0.001-0.05) increased in the KO heart relative to the WT (Fig. 4.5A). The protein markers for MIT fission, Drp1 and Fis1, were significantly (p<0.01) increased, whereas Mff was significantly (p<0.01) decreased in the KO versus the WT (Fig. 4.5A). Interestingly, the total protein expression of the outer MIT membrane marker, Tomm20, was significantly (p<0.01) increased in the KO relative to the WT (Fig. 4.5A), which corresponds to the increased MIT area in the frataxin-deficient heart at 4-weeks (Fig. 4.1E, 4.3E).
Figure 4.5: Western blot analysis demonstrating alterations in the expression of MIT fusion and MIT fission markers in the heart of MCK WT and KO littermates. (A) Total protein expression of MIT fusion markers (Mfn and Opal) and MIT fission markers (Drp1, Fis1, and Mff) were assessed in 4- and 10-week-old mice. The levels of Tomm20 and Gapdh were assessed as a MIT marker and protein-loading control, respectively. (B) The MIT fraction of these hearts was also assessed for these markers of MIT dynamics in littermates from both age groups. Hsp90 and Tomm20 were used as protein-loading controls for cytoplasmic and MIT fractions, respectively. The densitometry data are mean ± SEM (n = 4 to 6 experiments). *p<0.05, **p<0.01, ***p<0.001 versus WT or WT cytoplasmic. a.u., arbitrary unit. n.s., not significant.
In general, these alterations in the protein levels of these markers of MIT dynamics suggest disturbances to MIT dynamics and MIT homeostasis as early as 4-weeks of age in the MCK KO mice. When compared to the 10-week-old heart, total protein expression of protein markers for MIT fusion (Mfn1 and Opa1) and MIT fission (Drp1, Fis1, and Mff) were significantly \((p<0.001-0.05)\) increased in the KO relative to their WT littermates (Fig. 4.5A). In particular, the increase in Opa1 levels were substantially greater in the 10-week-old KO mice compared to 4-weeks, and Mff was increased at 10-weeks in contrast to the decrease at 4-weeks of age (Fig. 4.5A). A comparable significant \((p<0.01)\) increase in Tomm20 was also observed in the 10-week-old KO relative to WT littermates (Fig. 4.5A), which could be due to the increases in the number of mitochondria in the 10-week-old heart (Fig. 4.1C, 4.3C).

Considering the results above examining total cellular protein lysates, a MIT fraction was prepared to assess the markers of fusion and fission in the hearts of 4- and 10-week-old KO and WT mice (Fig. 4.5B). For MIT fractionation, it is important to normalise protein expression to Tomm20 as the MIT marker, to reflect the relative expression of these proteins in the mitochondria. Of note, the Tomm20 expression in the MIT fraction was also significantly \((p<0.01-0.05)\) increased in the KO relative to the WT at 4- and 10-weeks of age (Fig. 4.5B). In contrast, levels of the cytoplasmic marker, Hsp90, were very low, which indicated minimal contamination of the MIT fraction in both age groups (Fig. 4.5B). Furthermore, Hsp90 levels did not significantly \((p>0.05)\) alter between the KO and WT mice at 4- and 10-weeks of age.

In 4-week-old mice, the MIT fraction of Mfn1 was significantly \((p<0.05)\) increased, while Opa1 was unchanged in the KO relative to the WT (Fig. 4.5B). Similar to their total cellular expression
at 4-weeks of age, the MIT fraction of the fission markers, Drp1 and Fis1, were significantly 
($p<$0.01-0.05) increased, whereas Mff was markedly ($p<$0.001) decreased in the KO (Fig. 4.5B).
By 10-weeks of age, the MIT fraction of the MIT fusion proteins (Mfn1 and Opa1) and MIT fission
proteins (Drp1, Fis1, and Mff) were significantly ($p<$0.001-0.05) increased in the KO hearts (Fig.
4.5B). Notably, the detection of the cytosolic Drp1 protein and its increased expression in the MIT
fraction of the KO mice correspond to the recruitment of the MIT fission machinery, such as Fis1
(Smirnova et al. 2001, Youle and van der Bliek 2012).

These findings collectively demonstrate that there are marked alterations in the expression of
proteins regulating MIT dynamics. Specifically, there is an overall up-regulation for both MIT
fusion and fission even as early as 4-weeks of age in the MCK mice. These molecular alterations
in MIT dynamics could contribute to the observed MIT elongation (Fig. 4.1F, 4.3F) as a result of
MIT fusion, as well as MIT proliferation (Fig. 4.1C, 4.3C) due to increased MIT fission.
4.3.11 Increased Pink1 and Parkin Protein Expression in the Frataxin KO Heart Indicate MIT Dysfunction

As depicted in Figure 4.6A, Pink1 and Parkin are critical sensor of MIT dysfunction and regulators of MIT dynamics, which makes them important markers of MIT health, function, and quality control (Scarffe et al. 2014). In addition to MIT dynamics, Pink1 and Parkin also regulate the MIT-specific autophagic process known as mitophagy (Scarffe et al. 2014). In response to decreased MIT membrane potential and MIT damage, Pink1 translocates to the mitochondria and recruits and activates Parkin through its phosphorylation (Scarffe et al. 2014, Lazarou et al. 2015). Phosphorylated Parkin in turn targets Mfn for poly-ubiquitination and subsequent degradation to inhibit MIT fusion (Scarfe et al. 2014, Lazarou et al. 2015) (Fig. 4.6A). Hence, the expression and localisation of Pink1 and Parkin regulates MIT dynamics and facilitate mitophagic processing of damaged mitochondria (Fig. 4.6B). Therefore, these two proteins were examined.

Both Pink1 and Parkin total protein expression at 4-weeks and 10-weeks of age were significantly (p<0.001-0.05) increased in the KO hearts relative to the WT (Fig. 4.6B). Moreover, utilising the same MIT fractionation method as in Fig. 4.5B, strong levels of Pink1 and Parkin were detected in the MIT fractions, which were significantly (p<0.01-0.05) increased in the KO for both age groups (Fig. 4.6C). These results indicate that Pink1 and Parkin were localised to the mitochondria, which suggests MIT dysfunction in the frataxin KO heart.
Figure 4.6: Increased levels of markers of MIT dysfunction in the heart of MCK KO mice relative to their WT littermates. (A) Pink1 and Parkin are involved in recognising and targeting dysfunctional mitochondria through their interaction with Mfn, leading to its ubiquitination and subsequent proteasomal degradation. Thus, these later processes inhibit MIT fusion and promote mitophagy. (B, C) Western blot analysis demonstrates increased total cell Pink1 and Parkin expression (B), as well as their MIT fractions (C), in the 4- and 10-week-old KO relative to their WT littermates. Gapdh was used as a loading control for total protein-loading in (B), while Hsp90 and Tomm20 were used as protein-loading controls for cytoplasmic and MIT fractions, respectively, in (C). The densitometry data are mean ± SEM (n = 3-6) experiments. *p<0.05, **p<0.01, ***p<0.001 versus WT or WT cytoplasmic. a.u., arbitrary unit. n.s., not significant.
4.3.12 Inhibition of Lysosomal Degradation by Bafilomycin A1 Demonstrated Defective Autophagic Flux in the Frataxin KO Heart

Our previous studies indicated an increase in autophagy and mitophagy in MCK mice after demonstrating the up-regulation of the autophagic markers, Lc3 and Atg3, and the mitophagy marker, Fundc1 (Huang et al. 2013). As previously demonstrated (Huang et al. 2013), while there was no significant ($p>0.05$) alteration in total Fundc1 protein expression in the KO mice at 4-weeks of age, there was a marked ($p<0.001$) increase by 10-weeks of age relative to their WT littermates (Fig. 4.7A). Importantly, MIT fractionation experiments further confirmed that Fundc1 expression in the MIT fraction to be significantly ($p<0.05$) increased in both 4-week and 10-week-old KO hearts (Fig. 4.7B).

Considering the marked alterations in key proteins involved in MIT dynamics and mitophagy, it was important to investigate the rate of MIT elimination by autophagy in these animals. Specifically, the autophagic flux between WT and KO mice was assessed, as an indication of the capacity of the heart to undergo autophagic degradation (Mauvezin and Neufeld 2015). To assess this in vivo, 10-week-old MCK mice were administered Bafilomycin A1 (BAF; 6 mg/kg, i.p.), that inhibits autophagosomal-lysosomal fusion and thereby inhibits the degradation of the autophagic cargo (Mauvezin and Neufeld 2015). The classical autophagic markers, Lc3-II and p62, as well as the MIT protein, Mfn1, were then assessed in the BAF-treated mice versus those treated with the vehicle alone (saline; Fig. 4.7C).
Figure 4.7: Increased levels of the mitophagic marker, Fundc1, in the heart of 10-week-old MCK KO mice relative to WT littermates and increased autophagic flux upon Bafilomycin A1 (BAF) treatment in the heart of 10-week-old MCK KO mice in vivo. (A, B) Western blot analysis demonstrates increased total cell Fundc1 expression in the KO at 10-weeks of age (A), as well as its increased levels in a MIT fraction (B) of the KO at 4- and 10-weeks of age, relative to their WT littermates. Gapdh was used as a loading control for total protein-loading in (A), while Hsp90 and Tomm20 were used as protein-loading controls for cytoplasmic and MIT fractions, respectively, in (B). (C) BAF inhibits autolysosomal degradation of autophagic components i.e., Lc3-II and p62. Western blot analysis of Lc3-I, Lc3-II, and p62 in the heart from MCK WT and KO littermates at 10-weeks of age were assessed after treatment for 30 min with or without BAF (6 mg/kg; i.p.). The Lc3-II:Lc3-I ratio demonstrates increased accumulation of the autophagic substrates, Lc3-II and p62, in both WT and KO mice relative to the WT vehicle control. Densitometry data and the Lc3-II:Lc3-I ratio are as mean ± SEM (n = 6 mice/group). *p<0.05, **p<0.01, ***p<0.001 relative to the vehicle control WT. #p<0.05, ##p<0.01, between WT and KO groups. a.u., arbitrary unit. n.s., not significant.
Previous work from our laboratory have demonstrated in naïve MCK mice that while the WT mice express both Lc3-I (18 kDa) and Lc3-II (16 kDa), in the KO mice Lc3-II is the major form observed (Fig. 4.7Ci-iii) (Huang et al. 2013). This finding in the KO mice may be related to the increased conversion of Lc3-I to -II, which indicates increased autophagosome levels (Mizushima et al. 2010). This alteration results in a significantly ($p<0.01$) increased Lc3-II:Lc3-I ratio (Fig. 4.7Civ) in the KO mice relative to WT littermates (Huang et al. 2013). Inhibition of autophagosomal degradation by BAF led to further significant ($p<0.01$) increase in Lc3-II levels without affecting Lc3-I expression in both the WT and KO mice relative to the vehicle control (Fig. 4.7Ci-iii). Taken together, the Lc3-II:Lc3-I ratio in the BAF-treated animals demonstrated a small, but significant ($p<0.01$) increase in the WT mice, while there was a surprisingly marked and significant ($p<0.05$) increase in the KO mice, relative to their vehicle-treated counterparts (Fig. 4.7Civ).

As demonstrated previously in naïve MCK WT and KO mice (Huang et al. 2013), the expression of p62 is significantly ($p<0.001$) elevated in vehicle control KO mice relative to WT littermates (Fig. 4.7Ci,v). Similar to Lc3-II:Lc3-I ratio, BAF treatment also led to a significant ($p<0.05$) increase in p62 expression in the WT and KO mice relative to the vehicle-treated counterparts (Fig. 4.7Ci,v). To confirm that mitophagic degradation also occurs via autophagic processing, Mfn1 expression in BAF-treated MCK mice was then assessed (Fig. 4.7Ci,vi). The expression profile of Mfn1 was found to be similar to both the Lc3-II:Lc3-I ratio and p62 expression, where BAF-treatment resulted in a further significant ($p<0.05$) enhancement of Mfn1 level in the KO mice relative to the vehicle treatment (Fig. 4.7Ci,vi).
Collectively, these observations demonstrate that: (1) Mfn1 is a substrate of autophagic degradation in the KO mice and indicates the involvement of mitophagy; (2) the observed increased accumulation of Lc3-II, p62 and Mfn1 in the KO mice following BAF-treatment indicates that the capacity to degrade these autophagic substrates was maintained in these animals; and (3) the heightened accumulation of these proteins in the BAF-treated KO mice relative to their WT littermates suggests enhanced autophagic activity, i.e., autophagic flux, in the KO relative to the WT.
4.4 Discussion

In patients with FA, cardiomyopathy is the major cause of death and MIT dysfunction is probably the main contributing factors (Lagedrost et al. 2011, Lane et al. 2013, Ting et al. 2014). Indeed, MIT dysfunction and disruption in MIT dynamics and turnover has been shown to play key roles in the pathogenesis of cardiomyopathy in other disease states (Karthikeyan et al. 2002, Huang et al. 2009, Whitnall et al. 2012). In the current study, the well-characterised MCK conditional frataxin KO mouse model (Puccio et al. 2001, Whitnall et al. 2008, Huang et al. 2009, Whitnall et al. 2012, Huang et al. 2013, Anzovino et al. 2017) was utilised as a model of the fatal cardiomyopathy in FA to examine alterations in MIT morphology, biogenesis, dynamics and mitophagy to further understand and dissect the disease process. These aspects have not been assessed previously in the MCK KO and were crucial in order to understand the pathophysiology of this condition.

In the current study, TEM ultrastructural assessment of the left ventricular cardiomyocytes from KO mice demonstrates extensive proliferation of structurally abnormal mitochondria, with iron deposits being a distinct feature in the 10-week-old KO mice (but not in the 4-week-old KO) relative to their WT littermates (Fig. 4.1, 4.3). Within the MIT matrix, condensations of MIT cristae were also observed in the KO at both 4- and 10-weeks of age. In both the transverse and longitudinal planes of the KO mice, thin, elongated and entangled mass of mitochondria can be seen that markedly displaced the cardiac myofibres (Fig. 4.1, 4.3). This ultrastructural feature was in stark contrast to the distinctly well-organised mitochondria arranged parallel to the length of the myofibre observed in the WT. This morphological characteristic in the KO resembles neonatal mitochondria or mitochondria with disrupted Parkin-mediated mitophagy (Gong et al. 2015).
Indeed, the present studies and others have previously demonstrated the activation of fetal gene program in the heart of the MCK KO mice (Huang et al. 2013). These morphological and molecular alterations may underline the critical bioenergetics deficits observed in this model.

Increased MIT number in the KO suggests increased MIT biogenesis, which was confirmed by the up-regulation of Pgc1α, Nrf1, and Tfam (Fig. 4.4A). This was further supplemented by the examination of the mtDNA ND1 gene copy numbers, which demonstrated a significant increase in the KO at 10-weeks of age relative to the WT (Fig. 4.4B). Therefore, this increase in mtDNA copy number correlates with the observed increase in MIT number in the KO mice. This elevation of MIT number could be an indication of increasing MIT and cellular energy demands as a result of MIT dysfunction in frataxin deficiency.

Indeed, our studies demonstrated a bioenergetic deficit, as shown by markedly decreased NAD⁺ level and increased NADH levels that resulted in a decrease in the NAD⁺:NADH ratio, as well as a prominent reduction in ATP levels in the 10-week-old MCK KO mice (Fig. 4.4E, F). Under physiological conditions, the NAD⁺:NADH ratio is stabilised by the concerted effort of MIT Complex I, which initiates the electron transport chain with the oxidation of NADH to NAD⁺, and the MIT enzyme nicotinamide nucleotide transhydrogenase (NNT), which catalyses the following reaction: NADH + NADP⁺ ⇌ NAD⁺ + NADPH (Olgun 2009). The activity of NNT is dependent on an intact respiratory chain, which provides an efficient electrochemical proton gradient necessary for optimal enzyme activity (Rydstrom 2006). In fact, diminished MIT Complex I activity is a prominent cellular pathology in FA (Rotig et al. 1997, Sutak et al. 2008). Therefore,
insufficient NADH oxidation by both MIT Complex I and NNT, together with the observed
increase in NAD\(^+\) consumption by NAD\(^+\)-dependent enzymes such as Parp and Sirt1 (Fig. 4.4G, H), could result in the observed NAD\(^+\) imbalance and potentiate the decrease in ATP production from MIT OXPHOS.

Moreover, the activation of Ampk observed in the KO mice could positively regulate the NAD\(^+\) salvage pathway to replenish intracellular NAD\(^+\) pool (Fulco et al. 2008, Canto et al. 2009). However, previous study utilising the MCK frataxin KO mice has identified a widespread up-regulation of NAD\(^+\)-reducing enzymes involved in the citric acid cycle, the catabolism of branched-chain amino acids and ketone body, and pyruvate decarboxylation (Sutak et al. 2008). The cumulative activity of the above processes would result in the overt depletion of NAD\(^+\), and an accumulation of NADH levels observed in the KO mice.

Pgc1\(\alpha\) is part of a major pathway that have significant relevance to the protection of cardiomyocytes (Lehman et al. 2000). Previous studies have demonstrated that genetic suppression or pharmacological inhibition of Parp enhances Sirt1-dependent Pgc1\(\alpha\) activation, which subsequently increases MIT content and ATP production (Bai et al. 2011). Since Sirt1 requires NAD\(^+\) for its activation and subsequent deacetylation of Pgc1\(\alpha\) (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011), the observed decrease in NAD\(^+\) levels (Fig. 4.4E) corresponds with the decreased Sirt1 expression (Fig. 4.4D) and activity (Fig. 4.4H) in the KO mice, and correlates with the increased Pgc1\(\alpha\) acetylation at 10-weeks of age (Fig. 4.4I). A pathological association has been observed between increased MIT protein acetylation with the development of
Given the importance of NAD$^+$ metabolism in cardiac function, NAD$^+$ supplementation is likely to be beneficial in the treatment of FA. Supplementation with NAD$^+$ precursors have been reported to increase the activity of several sirtuins, including Sirt1, and the MIT sirtuins – Sirt3, Sirt4 and Sirt5 (Kane and Sinclair 2018). Over-expression of the latter sirtuins has been shown to ameliorate cardiac stress (de Moura et al. 2014). Cardiac stress may be associated with increased acetylation status of several MIT protein which may be indicative of depletion of intracellular NAD$^+$ levels and impaired sirtuin activity (Hershberger et al. 2017). Increased protein acetylation has also been previously reported in the failing human heart (Horton et al. 2016, Lee et al. 2016), and our current findings have significant clinical relevance to improving MIT abnormalities in the heart under pathological conditions.

Previous findings have reported increased mRNA abundance of both nuclear- and MIT-encoded respiratory complex subunits in mice receiving a Parp inhibitor (Felici et al. 2014). This is likely to be due not only to the activation of the Pgc1α-dependent transcriptional cascade, but also due to the direct inhibition of Parp on nuclear transcription. It is well established that poly(ADP-ribose) polymers can epigenetically regulate the transcription of several genes by directly interacting with gene promoters and basal transcriptional machinery (Kraus 2008). Furthermore, the activity of several transcription factors that are important for optimal MIT OXPHOS and mtDNA replication, including Nrf1, are negatively regulated by Parp activity (Hossain et al. 2009). Therefore, inhibition of Parp and/or supplementation with NAD$^+$ precursors, may promote Pgc1α-dependent
MIT biogenesis.

The presence of MIT dysfunction in the MCK KO mice could result from alterations in MIT dynamics, namely, MIT fusion and fission. Considering in the 4-week-old KO mice that: (1) while the average number of mitochondria were unchanged (Fig. 4.1C, 4.3C); (2) the MIT aspect ratio was unchanged transversely (Fig. 4.1F), but was markedly decreased longitudinally (Fig. 4.3F); (3) the average MIT area increased; and also (3) the mitochondrion cross-sectional area was increased both transversely and longitudinally (Fig. 4.1E, 4.3E); and (4) the mitochondria area to myocyte area ratio was increased both transversely and longitudinally (Fig. 4.1D, 4.3D), together suggests that the mitochondria are elongated and volumetrically larger in the KO than WT at 4-weeks of age. This increase in size is potentially due to increased MIT mass mediated through MIT biogenesis (Westermann 2010) (Fig. 4.4) and/or increased MIT fusion (Fig. 4.5), which resulted in no significant change to the average MIT number at this age. This unchanged level of MIT number also suggests that MIT fission was less pronounced in the KO at 4-weeks of age, despite the up-regulation of Drp1 and Fis1 relative to the WT.

There was also a distinctive decrease in Mff expression in the 4-week-old KO compared to its increase in the KO at 10-weeks of age relative to their age-matched WT littermates. The function of Mff has been attributed to a similar role to Fis1 in the recruitment of Drp1 to the outer MIT membrane in order to induce MIT fission (Zhang et al. 2016). Previous in vivo studies have examined the effects of inhibiting the interaction between Mff and Drp1, and found impaired MIT fission and function, resulting in elongated mitochondria (Kornfeld et al. 2018). Furthermore,
knockdown of Mff \textit{in vitro} is associated with an elongated MIT morphology, while over-expression led to MIT fragmentation characteristic of MIT fission (Zhang \textit{et al.} 2016). The quantitative assessment of mitochondria in the 4-week-old MCK KO indicated that the decrease in Mff led to less MIT fission as expected, but elongation was not observed, potentially due to the concurrent increase in biogenesis and fusion. Overall, at 4-weeks of age, our molecular studies demonstrated up-regulation of only a subset of markers of MIT fusion and fission in the MIT fraction, while all are induced in the KO by 10-weeks of age. In combination with the quantitative assessment of MIT morphology, this indicated less robust alterations in MIT dynamics in this younger age group.

Relative to the 4-week-old KO, quantification of MIT ultrastructure in the 10-week-old KOs shown in Figure 4.1 and 4.3 demonstrate an increased MIT aspect ratio that suggests elongation of the organelles, and reduced mitochondrion cross-sectional area in both the transverse and longitudinal planes \textit{versus} the WT. The observed MIT elongation could be explained by an up-regulation of MIT fusion machinery, while the reduction in MIT area could be due to the observed elevation of MIT fission machinery. In fact, both MIT fusion and fission are enhanced, with all MIT dynamic markers significantly up-regulated in the 10-week-old KO (Fig. 4.5). The increased MIT fission and biogenesis contributes to the observed increase in MIT number at this age. Thus, MIT fusion and fission occur concurrently by 10-weeks of age, altering MIT morphology, function, and number, and could be integral to the pathogenesis of the cardiomyopathy due to the loss of frataxin.
It is well known that MIT fusion and fission are critically involved in the regulation of MIT morphology and function (Westermann 2010, Youle and van der Bliek 2012). The fusion of mitochondria is presumably an attempt to rescue MIT component and mtDNA damage, and increase MIT oxidative capacity, in this case, to restore cardiac energetics (Youle and van der Bliek 2012). However, an over-expression or presence of MIT fusion machinery has been reported to generate an over-abundance of defective mitochondria, which may promote cardiomyopathy (Bhandari et al. 2014, Tahrir et al. 2018). Alternatively, the deletion or silencing of Mfn1/2 results in MIT fragmentation, and dilated cardiomyopathy with hypertrophy in other disease models (Dorn et al. 2011). This suggests an interplay between MIT fusion and fission in the regulation of MIT morphology. The mitochondria in frataxin-deficient yeasts also demonstrated a MIT fission morphology that is associated with oxidative stress (Lefevre et al. 2012). Furthermore, in models of ischemia and reperfusion injury, the MIT fission machinery is up-regulated and leads to fragmentation of dysfunctional mitochondria (Samangouei et al. 2018). From this preceding analysis, it is evident that the dysregulation of either MIT fusion and/or fission affects MIT morphology, and thus, a balance between these two dynamic processes are essential for proper mitochondria and cellular health, as well as cardiac function. Hence, the alterations in MIT dynamics in our frataxin-deficient MCK mice, which corresponds with the observed changes in MIT morphology, demonstrated a severe dysregulation in the maintenance of MIT homeostasis that contributes to the cardiac pathology of FA.

Additionally, markers of MIT dysfunction that are involved in MIT quality control, namely Pink1 and Parkin, were found to be up-regulated in the KO mice at both 4- and 10-weeks of age (Fig. 4.6), which further indicates an increase in the number of dysfunctional mitochondria. In
particular, the increased localization of these proteins to the mitochondria indicates the presence of MIT damage and also the induction of mitophagy. The latter is consistent with our published data showing an increase in the mitophagy biomarker, Fundc1, in MCK KO mice (Huang et al. 2013). Of interest, despite the up-regulation of Pink1 and Parkin, which are involved in the Mfn1 degradation that plays a key role in inducing MIT fusion, the expression of Mfn1 was up-regulated in the KO mice at both age groups (Fig. 4.5). As such, MIT degradation regulated by Pink1 and Parkin occurs concurrently with the up-regulation of MIT fusion in an attempt to increase MIT capacity. This response is probably an attempt to increase cardiac energetics through the enhancement of MIT capacity due to the frataxin-induced loss of ISC synthesis (Rouault 2012, Abeti et al. 2016) that is required for electron transport and cellular respiration.

The elimination of defective mitochondria is critical for restoring MIT homeostasis, and given the observed up-regulation of MIT fission and quality control markers in the 10-week-old KO mice, these suggest that damaged mitochondria are being targeted for degradation. Our previous studies identified a pronounced increase in the autophagic substrates, Lc3-II and p62, in the KO relative to the WT and suggested two possibilities, namely: (1) an increased requirement for autophagic degradation; and/or (2) an inhibition of autophagic degradation resulting in their accumulation (Huang et al. 2013).

The current investigation, using the lysosomotropic agent BAF in vivo delineate these two outcomes and demonstrated an increased autophagic flux in the KO at 10-weeks of age relative to the vehicle at this age, rather than an inhibition of autophagosomesal degradation. In fact, the
heightened levels of autophagic substrates, namely Lc3-II and p62, concord with the former possibility that there is an increased autophagic demand in the KO relative to the WT. For the first time, our study demonstrates that mitophagy and the degradation of MIT substrates are heightened in the KO mice. This is supported by the observed increase in the level of the mitophagic marker, Fundc1, and the accumulation of Mfn1 in the BAF-treated KO mice. Therefore, these findings indicate increased MIT turnover, potentially as an attempt to reconstitute MIT energetics in the KO mice. Despite this, MIT morphology and function were not rescued, demonstrating that the metabolic defect due to frataxin deficiency could not be overcome.

In summary, the alterations in MIT morphology, biogenesis, dynamics and quality control are markedly altered in response to the loss of frataxin in the MCK KO mouse (Fig. 4.8). At 4-weeks of age, while the deletion of frataxin leads to no effect on cardiac function (Puccio et al. 2001, Huang et al. 2013), increased biogenesis and/or fusion results in larger mitochondria without any alteration in number versus WT littermates. This probably compensates for the decreased levels of ISC-containing proteins critical for bioenergetics and respiration. In contrast, at 10-weeks of age, when the dilated cardiomyopathy is obvious and results in marked alterations in cardiac function (Puccio et al. 2001, Huang et al. 2013), there is pronounced fusion and fission leading to greater numbers of smaller yet elongated mitochondria relative to the WT (Fig. 4.8).
Figure 4.8: Summary schematic illustrating the effects of frataxin deficiency in the heart on the development of mitochondrial (MIT) homeostatic dysfunction with increasing age. In FA, frataxin deficiency in the heart leads to MIT iron (Fe) accumulation that causes MIT stress, which impairs MIT homeostasis and function. Phenotypic and molecular changes to MIT dynamics were observed in the frataxin-deficient hearts of mice, which demonstrated a disease progression of MIT dysfunction from 4-weeks of age that exhibits an asymptomatic phenotype to 10-weeks of age with pronounced dilated cardiomyopathy. MIT dysfunction is evident by the significant decrease to the NAD⁺:NADH ratio in the 10-week-old KO mice relative to the WT. Markers of MIT biogenesis and its regulation (Pgc1α, Nrf1, Tfam, Aampk, Paris, and Sirt1), MIT fusion (Mfn1 and Opa1), and MIT dysfunction (Pink1 and Parkin) were up-regulated while markers of MIT fission (Drp1, Fis1, and Mff) and mitophagy (Fundcl and Lc3-II) were altered at 4-weeks of age. However, all of these markers of MIT dynamics were significantly up-regulated by 10-weeks of age. More importantly, the molecular changes to MIT dynamics coincide with the observed abnormalities in MIT morphology as early as 4-weeks of age, which was substantially different and evidently more defective by 10-weeks. Thus, frataxin deficiency leads to a progressive dysfunction of MIT dynamics, which plays a role in the development of FA cardiac pathology.
Progressive MIT Homeostatic Dysfunction in the Frataxin-Deficient Heart with Increasing Age

Frataxin

Fe
Fe
Fe

MIT Iron Accumulation

MIT Stress

4 Weeks of Age
Asymptomatic Phenotype

Progressive MIT Dysfunction

10 Weeks of Age
Dilated Cardiomyopathy

NAD⁺:NADH

Abnormal MIT morphology as early as 4 weeks of age

Significant alterations in MIT Morphology by 10 weeks of age

↑Pgc1α  ↑Ampk  ↑Nrf1  ↓Sirt1  ↑Tfam  ↑Pgc1α  ↑Ampk  ↑Nrf1  ↓Sirt1

↑Pink1  ↑Parkin  ↑Pink1  ↑Parkin

↑Autophagic Flux

↑Mitophagy

↑Fundc1 ⇔Lc3-II  ↑Fundc1  ↑Lc3-II  ↑Fundc1  ↑Lc3-II

↑Mfn1  ↑Opa1  ↑Mfn1  ↑Opa1

MIT Biogenesis

↑Drp1  ↑Fis1  ↓Mff  ↑Drp1  ↑Fis1  ↓Mff

MIT Fission

MIT Fusion

MIT Biogenesis

MIT Fission

MIT Fusion

Abnormal MIT morphology as early as 4 weeks of age

Significant alterations in MIT Morphology by 10 weeks of age
At both ages, evidence of dysfunctional mitochondria was apparent due to increased Pink1 and Parkin levels, and the removal of these defective mitochondria is enhanced given the observed increase in mitophagic marker, and increased autophagic flux at 10-weeks of age (Fig. 4.8). As a result, MIT homeostasis is impaired in these frataxin-deficient animals, hence the accumulative effect of MIT dysfunction could be responsible for the progressive loss of cardiac function that contributes to the pathophysiology of FA (Fig. 4.8).

Collectively, the present studies have elucidated the involvement of MIT dysfunction and dysregulation in the pathophysiology of the cardiomyopathy in frataxin deficiency (Fig. 4.8). More importantly, the progressive change in MIT ultrastructure and molecular markers of MIT dynamics in the well-characterised MCK frataxin KO mice model indicated significant alteration in MIT homeostasis in FA cardiac pathology. Furthermore, the studies have provided potential molecular targets for the development of future therapeutics that aims restore MIT function and the subsequent bioenergetics of the heart. Thus, this avenue of MIT-based research deserves extensive investigation to further our understanding of FA pathology and its implication for treatments.
CHAPTER FIVE

Investigating the Potential of the GSH Supplementing Agent, $N$-acetylcysteine, and the novel NAD$^+$ Supplementing Agent, SNH6, as Therapeutic Strategies in the Treatment of Friedreich’s Ataxia

This Chapter is adapted from the manuscript below that is currently in preparation for publication, where I am the first author:

5.1 Introduction

There are currently no cure or effective therapeutic strategies for the inherited and debilitating neuro- and cardio-degenerative disease, Friedreich’s ataxia (FA) (Pandolfo 2003, Richardson 2003, Strawser et al. 2014). The main cause of FA is due to the deficient expression and generation of the mitochondrial (MIT) protein, frataxin, which is known to play significant roles in the regulation of iron in the cell, as well as the biogenesis of iron-sulfur cluster (ISC) for ISC-containing enzymes (Puccio et al. 2001, Huang et al. 2009, Richardson et al. 2010a, Whitnall et al. 2012, Anzovino et al. 2014).

Many studies including those from our laboratory have established that frataxin deficiency leads to toxic MIT iron-loading that results in the production of reactive oxygen species (ROS) and causes oxidative stress (Whitnall et al. 2012, Anzovino et al. 2017). However, the well-known Nrf2-ARE signalling pathway for antioxidant defence is dysregulated when frataxin is deficiently expressed, which potentiates oxidative damage, as shown in various FA models (Paupe et al. 2009, D'Oria et al. 2013, Shan et al. 2013), including in the well-established cardiac model of FA, the MCK *frataxin* KO mouse model (*Chapter 3*) (Anzovino et al. 2017). Frataxin deficiency is also associated with MIT dysfunction, in particular, the disruption of energy transduction by the electron transport chain of the mitochondria, and impaired ATP synthesis for energy production *via* oxidative phosphorylation (OXPHOS) (Lodi et al. 1999, Heidari et al. 2009, Nunnari and Suomalainen 2012, Gonzalez-Cabo and Palau 2013).
Considering the above, the pathophysiological features of FA, namely, oxidative stress, iron accumulation, and energy metabolism, are all important to consider for the design of novel therapeutic strategies. While there is the future prospect for gene therapy as an avenue for the replacement of frataxin in humans to repair primary defects, it is currently not yet possible. Thus, there is urgent need for the development of treatments to alleviate disease progression by targeting the pathological features of FA. Importantly, all therapeutic strategies that show promise must be carefully examined and require critical scrutiny to ensure the safety and effectiveness of the treatments.

Oxidative stress plays a major role in the pathogenesis of FA, and this is well-established from previous studies including our examination using the MCK \textit{frataxin} KO mouse model of FA (Whitnall \textit{et al.} 2012, Huang \textit{et al.} 2013, Vaubel and Isaya 2013, Anzovino \textit{et al.} 2017). The dysregulation of the Nrf2 anti-oxidative signalling pathway results in a loss of the endogenous antioxidant, glutathione (GSH), which contributes to redox stress (Bulteau \textit{et al.} 2012, Shan \textit{et al.} 2013, Anzovino \textit{et al.} 2017, Abeti \textit{et al.} 2018). This is critical to consider, since decreased Nrf2 levels would result in the decrease of multiple Nrf2-target genes that plays important role in antioxidant defence and GSH metabolism (Itoh \textit{et al.} 1997, Bulteau \textit{et al.} 2012, Shan \textit{et al.} 2013).

Findings in \textit{Chapter 3} further demonstrated this dysfunction in the MCK mouse model where there was decreased GSH/GSSG ratio and increased protein oxidation when frataxin is deficient in the hearts of the MCK mice (Johnson \textit{et al.} 2012, Anzovino \textit{et al.} 2017). Moreover, GSH plays a crucial role in ISC metabolism and assembly (Kumar \textit{et al.} 2011, Wang \textit{et al.} 2012, Srinivasan \textit{et al.} 2012).
al. 2014), which is defective in FA and in our MCK KO mice (Sutak et al. 2008, Huang et al. 2009, Vaubel and Isaya 2013). Therefore, GSH-depletion potentially as a result of Nrf2 dysregulation, could be a significant factor in the pathogenesis of FA in addition to oxidative stress, where loss of GSH not only reduces antioxidant defence, but also impairs the maturation of ISC protein (Kumar et al. 2011, Srinivasan et al. 2014).

The deleterious impact of oxidative stress in FA has resulted in antioxidant supplementation becoming an important part of drug design for FA treatments. Previous studies in yeast mutants lacking frataxin showed that N-acetylcysteine (NAC) increases GSH and prevents iron-induced toxicity, with survival increasing 2–4 orders of magnitude (Karthikeyan et al. 2002). In a separate study, incubation of frataxin-deficient pluri-potent, embryonic P19 cells with NAC, resulted in cellular rescue and increased total cell numbers (Santos et al. 2001). Similarly, motor neurons in NSC34 mouse with 70% residual frataxin experienced a partial rescue in proliferation after incubation with GSH (Carletti et al. 2014). In addition, NAC could increase Nrf2 levels in vivo (Ji et al. 2010, Zhang et al. 2014), and GSH is protective against free radical cytotoxicity in frataxin-deficient cells (Santos et al. 2001). Since NAC is an FDA approved drug (Prescott et al. 1977), it has the clinical potential to be rapidly deployed for the treatment of FA if found to be effective. The antioxidant effect of NAC to increase GSH could potentially inhibit oxidative stress and protect against further damage in the frataxin-deficient MCK mice where Nrf2 levels and its downstream signalling are decreased (Paupe et al. 2009, Johnson et al. 2012, Messier et al. 2013).

Thus, we aim to assess the effectiveness of NAC as a treatment to rescue the frataxin-deficient hearts of the MCK mice by examining their organ to body weight ratios and cardiac
histopathology, which are both important indicators of disease severity by 9 to 10-weeks of age (Anzovino et al. 2017).

Previous studies have also reported that MIT dysfunction is associated with frataxin deficiency in which defects to the MIT respiratory chain is responsible for the decrease in energy production (Abeti et al. 2016, Chiang et al. 2017). Specifically, NAD$^+$ is an essential metabolic cofactor involved in energy metabolism (Srivastava 2016), and the impaired activity of the MIT respiratory Complex I can lead to an imbalance of NAD$^+$ and NADH levels, which can cause bioenergetics deficiency and oxidative stress (Heidari et al. 2009, Srivastava 2016). Indeed, previous studies in FA have shown that the loss of frataxin expression results in decreased NAD$^+$:NADH ratio, increased oxidative stress, and impaired energy production (Schulz et al. 2000, Lodi et al. 2001, Wagner et al. 2012, Stram et al. 2017). Consistent with those studies, our data in Chapter 4 also demonstrated decreased NAD$^+$ levels and NAD$^+$:NADH ratio in the MCK KO mice.

Hence, reconstituting the levels of NAD$^+$ could potentially enhance MIT function through the activation of MIT biogenesis and affecting MIT dynamics (Canto et al. 2012, Long et al. 2015). As such, the biosynthesis of NAD$^+$ is important to consider. Other than the \textit{de novo} synthesis of NAD$^+$ from L-tryptophan, the NAD$^+$ salvage pathway is the major source of its synthesis, which involves an array of enzymatic reactions with intermediate precursors, such as nicotinic acid (NA) (Srivastava 2016). For more detailed description of the NAD$^+$ salvage pathway, please see the following articles (Bogan and Brenner 2008, Houtkooper \textit{et al.} 2010, Canto \textit{et al.} 2015, Srivastava 2016).
We herein assessed the efficacy of supplementing NAD\(^+\) to alleviate the cardiac pathology in the frataxin-deficient MCK mice by using a compound we have designed and patented as a “first in class” novel therapeutic, namely, 6-methoxy-2-salicylaldehyde nicotinoyl hydrazine (SNH6) (Fig. 5.1). This compound is well-tolerated with an IC\(_{50}\) values of 18 \(\mu\)M (unpublished data), which are comparable to the clinically used chelator, DFO, and effectively chelate iron and enhance the NAD\(^+\):NADH ratio (Palanimuthu et al. 2018, Wu et al. 2019). This agent also fulfil Lipinski’s Rules for bioavailability (Lipinski 2004), and blood brain barrier permeation (Clark 1999). Another special property is that our agent acts as a lipophilic transport scaffold to enable safe and effective permeation of NAD\(^+\) (Palanimuthu et al. 2018, Wu et al. 2019), as demonstrated for other drugs (Ellis et al. 2014). As iron chelation attenuates cardiomyopathy in MCK mice (Whitnall et al. 2008), this “Dual Action” property of the compound in which it is able to remove redox active iron (Huang et al. 2009), while providing biochemical rescue through the supplementation of NAD\(^+\) are crucial to assess (Palanimuthu et al. 2018, Wu et al. 2019).
**Figure 5.1:** Chemical structure of SNH6. The novel compound, 6-methoxy-2-salicylaldehyde nicotinoyl hydrazine (SNH6), developed in our laboratory has a chemical structure that pertains two moieties designed for iron chelation, and NAD$^+$ donation. Designed and synthesised by my Auxiliary Supervisor, Dr. Danuta Kalinowski.

Considering that oxidative stress, MIT iron-loading, and defective energy production are major pathophysiological features of FA, the current study examined the therapeutic potential of tackling these pathogenic mechanisms *in vivo* using the MCK frataxin KO mouse model. These strategies include the antioxidant NAC and our specifically designed agent, SNH6, which utilises iron chelation and NAD$^+$ supplementation, in order to mitigate the progression of the cardiomyopathy in FA.
5.2 Materials and Methods

5.2.1 Animals
The MCK conditional frataxin KO mice model of FA described in Section 4.2.1 was utilised for all subsequent studies, and genotypes according to previously described procedures (Puccio et al. 2001). All animal work was approved by the University of Sydney’s Animal Ethics Committee (Sydney, New South Wales, Australia).

5.2.2 Reagents and Treatments
NAC was dissolved in vehicle (10% DMSO, 0.9% saline) at concentrations of 150 mg/kg and 1500 mg/kg. This study introduces the novel therapeutic compound, SNH6, designed and synthesised in our lab to chelate iron and increase NAD\(^+\) levels. Freshly synthesized SNH6 was dissolved in vehicle (10% DMSO, 30% cremophore EL, in 0.9% saline) at a concentration of 20 mg/kg. Treatment of NAC was performed via intraperitoneal injection (i.p.), while SNH6 was performed via oral gavage on MCK mice \((n = 3–6/WT and KO group/treatment group)\) starting at 4-weeks of age for 4 to 5 consecutive weeks, 5 times a week. The body weight of the animals was measured every second day, and daily for the last two weeks of treatment. By the end of the treatments, animals were sacrificed for their organs (heart, skeletal muscle, liver, and brain). Organ weight was measured, and histopathological assessment was performed on heart and skeletal muscle tissues.
5.2.3 Histology

The MCK WT and KO mouse littermates were weighed and euthanised after 4 to 5 weeks of treatment. For the mice treated with NAC or SNH6, the heart and skeletal muscle from the superficial part of the quadriceps were excised, freed from connective tissue and fat, washed in cold saline, blotted dry, weighed and fixed in 10% formalin. Muscle samples were then cut and embedded into paraffin blocks, divided into sections, and stained with Perls’ Prussian blue (Perls’), Gömöri trichrome, or hematoxylin and eosin (H&E). The muscle fibre size of the heart and skeletal muscle was measured by ImageJ (NIH, Baltimore, MD; http://imagej.nih.gov/ij) (Schneider et al. 2012). Measurements were taken from at least three images from WT and KO mice of each treatment group, and there was a minimum of 20 measurements per image.

5.2.4 NAD\(^+\), NADH and NAD\(^+\):NADH Ratio Assessment

Heart tissue from WT and KO MCK mice were harvested as described in Section 2.3.1. Extraction of NAD\(^+\) and NADH levels were performed from 100 μL of homogenates according to Section 4.2.11 following previously described procedures (Herrera-Marschitz et al. 2018).

5.2.5 Measurement of ATP Levels

The levels of intracellular ATP were measured from harvested heart tissues from WT and KO MCK mice, and measurements were performed according to previously described protocols in Section 4.2.12 (Braidy et al. 2014).
5.2.6 Sirt1 Deacetylase Activity

The activity of Sirt1 deacetylase was determined from heart tissue nuclear extracts using the Cyclex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit (CycLex, Nagano, Japan). The procedure was performed according to Section 4.2.13. Results were presented as relative fluorescence (FU)/mg of protein.

5.2.7 Parp Activity Assay

Considering that the NAD⁺-consuming enzyme, poly-ADP-ribose polymerase (Parp), is the major consumer of NAD⁺ in mammalian tissue (Durkacz et al. 1980, Soane et al. 2007, Cerutti et al. 2014), nuclear NAD⁺ consumption was determined to assess Parp activity using the previously described assay Section 4.2.14 (Putt et al. 2005). Previous studies have also noted this methodology to be a reliable measure of Parp activity (Putt and Hergenrother 2004, Putt et al. 2005, Braidy et al. 2014, Herrera-Marschitz et al. 2018). In brief, the final reaction mixture comprised of 10 mM MgCl₂, Triton X-100 (1%), and 20 µM of NAD⁺ in 50 mM Tris buffer (pH 8.1). The plate was then incubated for 1 h, and the amount of NAD⁺ consumed was measured as described above. Results were expressed as µg NAD⁺ consumed/h/mg nuclear protein.

5.2.8 Statistical Analysis

Data were compared using Student’s t-test, and one-way analysis of variance (ANOVA) and post hoc Tukey’s multiple comparison tests to determine statistical significance between treatment groups. Data were considered statistically significant when p<0.05. Results are expressed as mean ± SEM.
5.3 Results

5.3.1 Assessing of the Effectiveness of NAC Treatment to Attenuate FA Cardiomyopathy in the MCK Mice

Data from Chapter 3 have demonstrated the significant role of oxidative stress in the pathogenesis of FA (Anzovino et al. 2017). In particular, the dysregulation of the Nrf2-Keap1 signalling pathway could potentially exacerbate oxidative damage (Anzovino et al. 2017). As such, restoring cellular antioxidant defence to combat oxidative stress is a considerable therapeutic strategy that has been investigated in other neuro- and cardio-degenerative diseases (Bavarsad Shahripour et al. 2014, Abeti et al. 2018, Alhusaini et al. 2018, Gureev and Popov 2019). In this study, the effectiveness of the GSH-induced agent, NAC, was assessed in vivo to reduce or ameliorate the FA cardiomyopathy using the well-established MCK mouse model of FA.

5.3.1.1 Body Weight and Organ to Body Weight Ratio of NAC-Treated MCK Mice

The MCK mice at 4-weeks of age were distributed into three treatment groups: (1) vehicle control, (2) NAC at 500 mg/kg, and (3) NAC at 1500 mg/kg, such that the groups have comparable age and initial body weight. Treatments were administered via i.p. injection once per day, 5 days a week for 5 consecutive weeks, and the body weight of the animals was measured every second day and daily by the final two weeks. The body weight of these animals as a percentage across the 36-day treatment is shown in Fig. 5.2A for the vehicle control group, Fig. 5.2B for NAC treatment at 500 mg/kg, and Fig. 5.2C for the NAC-treated group at 1500 mg/kg. In combination, the average body weight of WT mice and KO mice across all groups with age is shown in Fig. 5.2D and Fig. 5.2E, respectively.
Figure 5.2: Decreased average body weight and increased heart to body weight ratio, but not the skeletal muscle in the MCK KO mice after treatment with NAC. MCK mice were treated (i.p.) with either saline, NAC (500 mg/kg), or NAC (1500 mg/kg), starting at 4-weeks of age, 5 times a week, for 5 consecutive weeks. The body weight of the animals was measured every second day, and daily for the last two weeks over the entire course of the treatments for: (A) the vehicle control group, (B) NAC (500 mg/kg) treatment group, and (C) NAC (1500 mg/kg) treatment group. The change in body weight over time of WT mice (D) and KO mice (E) of each treatment were presented together. (F) By the end of the treatments, the average body weight was examined for all animals at 9-weeks of age as a percentage. (G) Heart and (H) skeletal muscle to body weight ratio was determined for WT and KO mice from all three treatment groups. Figures are mean ± SEM (n = 3-5 per WT/KO in each group). *, p<0.05 between WT and KO mice of each group. ###, p<0.001 denotes significance versus WT mice of their respective treatment group. a.u., arbitrary unit. n.s., not significant.
WT Mice of Each Treatment

KO Mice of Each Treatment

Average Body Weight %

Heart/Body Weight Ratio (a.u.)

Skeletal Muscle/Body Weight Ratio (a.u.)
In the vehicle control group, while the WT mice showed a gradual increase in body weight with age, the KO mice had a progressive decline in body weight as early as day 8 of treatment relative to the WT (Fig. 5.2A). By the end of the treatment at day 36, the average body weight of the KO mice was significantly ($p<0.05$) decreased compared to the WT (Fig. 5.2A). Both WT and KO mice treated with NAC at 500 mg/kg demonstrated a progressive increase in their average body weight from day 1 to approximately day 20 (Fig. 5.2B). However, the average body weight of the KO mice in this treatment group began to decrease after day 20 relative to the WT, and it was significantly ($p<0.05$) decreased by day 36 (Fig. 5.2B, F). Similarly, the average body weight of the WT mice treated with NAC at 1500 mg/kg increased with age, whereas the average body weight of the KO mice progressively decreased from day 20 (Fig. 5.2C). In fact, the average body weight percentage of the KO mice in this treatment group was significantly ($p<0.05$) decreased by the end of the treatment relative to the WT (Fig. 5.2C, F).

In combination, the average body weight of the WT mice from all three treatment groups progressively increased with age where there was no significant ($p>0.05$) difference between the WT mice of both NAC treatment groups relative to the vehicle control by the end of the treatments (Fig. 5.2D, F). In contrast, the KO mice from all three treatment groups showed a progressive decline in their average body weight with age (Fig. 5.2E). Notably, the KO mice in NAC (500 mg/kg) and NAC (1500 mg/kg) treatment groups demonstrated an overall higher average body weight percentage between day 12 and 36 relative to the KO mice in the vehicle control (Fig. 5.2E). Despite this observation during this period, there were no significant ($p>0.05$) differences between the average body weights of the KO mice in both NAC treatment groups relative to the
vehicle control KO mice (Fig. 5.2E, F). This result suggests that the NAC treatments were able to temporarily prevent body weight loss in the KO mice, but it did not rescue the animals in terms of body weight by the later time points in comparison to the vehicle control.

Considering that cardiac hypertrophy is a marked phenotype of the MCK mice, as was reported in previous studies (Puccio et al. 2001, Whitnall et al. 2008, Anzovino et al. 2017), as well as in FA patients (Weidemann et al. 2012, Weidemann et al. 2015, Hanson et al. 2019), it was important to examine the effects of NAC on the pathological development of the heart. As such, the organ to body weight ratio for the heart, as an assessment of cardiac hypertrophy, and for the skeletal muscle as a negative control (Anzovino et al. 2017), was assessed in these treated animals. Since the vehicle control MCK KO mice had markedly decreased average body weight percentage by the end of the treatment (Fig. 5.2F), as a result, the heart to body weight ratio was significantly (p<0.001) higher in the KO relative to the WT in vehicle control (Fig. 5.2G).

However, the KO mice from both NAC treatment groups also demonstrated a significantly (p<0.001) higher heart to body weight ratio compared to their WT littermates (Fig. 5.2G). Moreover, this increase in heart to body weight ratio of the NAC-treated KO mice was not significantly (p>0.05) altered relative to the KO in vehicle control (Fig. 5.2G). Therefore, NAC treatments at both concentrations did not significantly improve the development of cardiac hypertrophy in the KO mice. In contrast to the heart, the skeletal muscle to body weight ratio of the KO mice from all treatment groups demonstrated no significant (p>0.05) difference when compared to their respective WT littermates (Fig. 5.2H). This finding is consistent with the
previous observation in the same mouse model of FA (Anzovino et al. 2017), but more importantly, NAC treatments did not alter the skeletal muscle to body weight ratio in these animals.

5.3.1.2 Cardiac and Skeletal Muscle Histopathology of the NAC-Treated MCK Mice by 9-weeks of Age

Previous studies have examined the gross histology of the heart and skeletal muscle of the MCK mice at 9-weeks of age and demonstrated marked cardiac hypertrophy (Puccio et al. 2001, Huang et al. 2013, Anzovino et al. 2017). Using a similar analysis, Perls’ Prussian blue, Gömöri trichrome, and H&E staining were used to provide a qualitative assessment of the cardiac histopathology of these NAC-treated mice at 9-weeks of age. Perls’ staining demonstrated iron accumulation in the cardiomyocytes of the vehicle control KO mice as indicated by arrows in Fig. 5.3D, relative to the WT control (Fig. 5.3A). However, the NAC-treated KO cardiomyocytes also have presence of iron deposits in the myocytes (Fig. 5.3J, P) when compared to their WT littermates (Fig. 5.3G, M). This observation suggests that the treatments with NAC did not ameliorate iron accumulation when frataxin is deficient. Furthermore, Gömöri trichrome staining showed consistent interstitial fibrosis in the KO of all treatment groups (arrows; Fig. 5.3E, K, Q) relative to their WT littermates (Fig. 5.3B, H, N). H&E staining also demonstrated myofibrillar disarray in the KO hearts from all treatment groups (Fig. 5.3F, L, R) compared the WTs (Fig. 5.3C, I, O). In culmination, the 5 weeks of NAC treatments did not significantly improve the cardiac histopathology of the 9-week-old KO mice.
Figure 5.3: Pronounced histopathology observed in the heart of the NAC-treated MCK KO mice by 9-weeks of age. Histological staining of the heart from 9-week-old WT and KO mice at the end of a 5-week long treatment with either: (A-F) saline as the vehicle control group, (G-L) NAC at 500 mg/kg, or (M-R) NAC at 1500 mg/kg. Transverse sections of the hearts were stained with Perls’ Prussian blue (left images: A, D, G, J, M, P; scale bar: 50 µm), Gömöri trichrome (middle images: B, E, H, K, N, Q; scale bar: 100 µm) and H&E staining (right images: C, F, I, L, O, R; scale bar: 50 µm). Arrows in: (D, J, P) denote iron deposits in cardiomyocytes, and (E, K, Q) indicates myocardial fibrosis. As such, the cardiac histopathological features of FA that are commonly observed at 9-weeks of age were also present in the hearts of the NAC-treated KO mice, which suggests that they exhibit cardiomyopathy. Images displayed are representative histological staining ($n = 3$-$5$ in each treatment group).
Cardiac histopathology at end-stage (9 weeks)

Perls’ Gömöri Trichrome H&E

A B C
D E F
G H I
J K L
M N O
P Q R

Vehicle Control

WT KO

NAC 500 mg/kg

WT KO

NAC 1500 mg/kg
Similar to a previous report from our lab as described in Chapter 3 (Anzovino et al. 2017), as well as in FA patients (Harding 1981, Rotig et al. 1997), the skeletal muscle in the 9-week-old KO mice showed no histopathological alterations relative to the WT mice (Fig. 5.4). Specifically, Perls’ Prussian blue, Gömörí trichrome, and H&E staining, did not show iron accumulation (Figure 5.4A, D, G, J, M, P), interstitial fibrosis (Fig. 5.4B, E, H, K, N, Q), nor myofibrillar disarray (Fig. 5.4C, F, I, L, O, R) in the KOs relative to the WTs at 9-weeks of age, respectively. In contrast to the heart, the skeletal muscle is unaffected despite the loss of frataxin, which is potentially due to the difference in energy metabolism between the two tissue types (Berg J.M. 2002, Hargreaves and Spriet 2017).

Specifically, the heart is known to rely heavily on aerobic metabolism and MIT OXPHOS, whereas the skeletal muscle can depend on cytosolic anaerobic glycolysis to meet energy demands (Berg J.M. 2002). The overall histopathology of the heart and skeletal muscle in the MCK mice is consistent with previous findings (Puccio et al. 2001, Huang et al. 2013, Anzovino et al. 2017). More importantly, the diseased histopathology of the KO hearts was retained despite treatment with NAC, suggesting that the enhancement of antioxidant defence by bolstering GSH levels alone to combat oxidative stress is insufficient to attenuate disease progression caused by frataxin deficiency.
Figure 5.4: No histopathological alterations of the skeletal muscle of NAC-treated MCK KO mice at 9-weeks of age. Histological staining of the skeletal muscle from 9-week-old WT and KO mice at the end of a 5-week long treatment with either: (A-F) saline as the vehicle control group, (G-L) NAC at 500 mg/kg, or (M-R) NAC at 1500 mg/kg. Transverse sections of the skeletal muscle were stained with Perls’ Prussian blue (left images: A, D, G, J, M, P; scale bar: 50 µm), Gömöri trichrome (middle images: B, E, H, K, N, Q; scale bar: 100 µm) and H&E staining (right images: C, F, I, L, O, R; scale bar: 50 µm). In contrast to the heart, the skeletal muscle of the KO mice with and without treatment with NAC has no myocardial fibrosis or depositions of iron in the myocytes. Representative histological staining images are shown (n = 3-5 in each treatment group).
Skeletal muscle histopathology at end-stage (9 weeks)

Perls' Gömöri Trichrome H&E

WT
KO

Vehicle Control

NAC 500 mg/kg

NAC 1500 mg/kg
Quantitatively, the cross-sectional area of the cardiac muscle fibres was found to be significantly ($p<0.001$) greater in the hearts of the KO mice (460–493 μm²) relative to the WT mice (311–324 μm²) for all three treatment groups (Fig. 5.5A). Notably, the cardiac muscle fibre size was not significantly ($p>0.05$) changed in the hearts of the NAC-treated KO mice relative to the vehicle control KO mice (Fig. 5.5A). This is consistent with the previously described unaltered histopathology of the NAC-treated hearts (Fig. 5.3). This observation in conjunction with the loss of body weight and increased heart to body weight ratio of the KO littermates (Fig. 5.2F, G), demonstrates the development of cardiac hypertrophy in the KO hearts despite treatment with NAC. As for the cross-sectional area of the skeletal muscle fibres, it was markedly ($p<0.001$) lower in the KO (785–814 μm²) relative to the WT mice (1877–2139 μm²) for all three treatment groups (Fig. 5.5B). This finding shows the significant reduction in skeletal muscle fibre area, which could be proportion to the loss of body weight in the KO mice (Fig. 5.2F).
Figure 5.5: Increased cardiac muscle fibre size and decreased skeletal muscle fibre size of the NAC-treated MCK KO mice at 9-weeks of age. The muscle fibre size of (A) the heart and (B) the skeletal muscle of 9-week-old WT and KO mice were measured and compared across the three treatment groups: vehicle control, NAC at 500 mg/kg, and NAC at 1500 mg/kg. Considering the significant decrease in average body weight of the NAC-treated KO mice, their increase in muscle fibre size of the heart suggests the development of cardiac hypertrophy by the end of the treatments. Figures are mean ± SEM (n = 3-5 in each treatment group). ###, p<0.001 denotes significance compared to the WT mice of their respective treatment group. n.s., not significant.
5.3.2 Assessing of the Effectiveness of SNH6 Treatment to Attenuate FA Cardiomyopathy in the MCK Mice

In FA, frataxin deficiency leads to MIT dysfunction due to defects to the electron transport chain as a result of impaired ISC biosynthesis (Puccio et al. 2001, Huang et al. 2009, Richardson et al. 2010a, Whitnall et al. 2012, Anzovino et al. 2014). Consequently, cardiac oxidative metabolism and energy production are disrupted, which manifests into the well-known cardiomyopathy of FA (Puccio et al. 2001, Heidari et al. 2009, Huang et al. 2011). Previous studies have examined the cardio-protective effects of NAD⁺ supplementation in other cardiomyopathies (Xu et al. 2015, Mericskay 2016, Berthiaume et al. 2017), in order to rescue MIT function through the reconstitution of metabolic NAD⁺ levels. Using our novel “Dual Action” therapeutic compound, SNH6, designed to supplement NAD⁺ and chelate iron, we assessed its effectiveness to ameliorate the cardiomyopathy in the MCK mice.

5.3.2.1 Body Weight and Organ to Body Weight Ratio of SNH6-Treated MCK Mice

The MCK mice at 4-weeks of age were sorted into the vehicle control and SNH6 treatment groups, with comparable group age and initial body weight. The treatments were performed via oral gavage once per day, 5 days a week for approximately 5 consecutive weeks, and their body weight was measured every second day. The average body weight of these animals as a percentage across the 29-day treatment is shown in Fig. 5.6A.
Figure 5.6: Decreased average body weight and increased heart to body weight ratio in the MCK KO mice after treatment with SNH6. MCK mice were treated via oral gavage starting at 4-weeks of age, 5 times a week, for 4 consecutive weeks. (A) Their body weights were measured every second day over the duration of the treatments for the vehicle control group, and SNH6 (20 mg/kg) treatment group. (B) By the conclusion of the treatments, the average body weight was evaluated for all animals at 9-weeks of age as a percentage. (C) Heart, (D) liver, and (E) brain to body weight ratio was determined for WT and KO mice from both treatment groups. Figures are presented with mean ± SEM (n = 5-6 per WT/KO in each group). ##, p<0.01, ###, p<0.001 denotes significance versus WT mice of their respective treatment group. a.u., arbitrary unit. n.s., not significant.
The vehicle control and SNH6-treated WT mice showed a progressive increase in their body weight with age (Fig. 5.6A). However, the body weight of the KO mice of both treatment groups peaked at day 11 and began to decrease by day 12 (Fig. 5.6A). By the end of the treatments at day 29, the body weight of the KOs has almost returned to their initial weight at day 1 (Fig. 5.6A). In fact, at 9-weeks of age, the average body weight in percentage of the KO mice was markedly (p<0.01-0.001) lower relative to the WT of their respective treatment groups (Fig. 5.6B). More importantly, the average body weight of the SNH6-treated KO was not significantly (p>0.05) altered relative to the vehicle control KO (Fig. 5.6B). This observation suggests that the treatment with SNH6 did not improve the weight of the animals with frataxin deficiency, and the associated cardiomyopathy is potentially unresolved. In fact, the heart to body weight ratio, as an indicator of cardiac hypertrophy, of the KO mice from both treatment groups were markedly increased (p<0.001) compared to their respective WT littermates (Fig. 5.6C), and this ratio was not significantly (p>0.05) altered between the SNH6-treated KO and the vehicle control KO (Fig. 5.6C). This finding further suggests that despite treatment with SNH6, the KO mice had developed end-stage cardiac hypertrophy at a similar rate to the vehicle control KO by 9-weeks of age.

As relevant physiological controls, the liver and brain tissues of these animals were harvested, and the organ weights were measured as a ratio to the average body weight. The liver to body weight ratio for vehicle control and SNH6-treated KO at 9-weeks of age were significantly (p<0.001) reduced relative to WT of their respective treatment groups (Fig. 5.6D). This reduction in liver weight could potentially be attributed to the loss of body weight in the KO mice by this later age. On the contrary, the brain to body weight ratio of the KO from both treatment groups was significantly (p<0.001) increased relative to their WT littermates (Fig. 5.6E). This latter
observation is probably due to the concurrent decrease in body weight in the KO, while the weight and size of their brain is maintained throughout disease progression.

5.3.2.2 Cardiac Histopathology of SNH6-Treated MCK Mice by 9-weeks of Age

A gross histopathological examination of the 9-week-old hearts by the end of the treatments with either saline or the NAD$^+$ supplementation compound, SNH6, was performed to provide a qualitative assessment of the cardiac pathology. Consistent with previous findings (Puccio et al. 2001, Huang et al. 2013, Anzovino et al. 2017), Perls’ staining showed iron deposits in the cardiomyocytes of the vehicle control KO mice (arrows; Fig. 5.7D), while the vehicle control WT mice have no such depositions (Fig. 5.7A). However, similar to the vehicle control KO, the SNH6-treated KO mice also have iron accumulation in the myocytes in comparison to the SNH6-treated WT that has no iron deposits (arrows; Fig. 5.7G, J). Furthermore, Gömöri trichrome staining showed presence of interstitial fibrosis in the KO of both vehicle control and SNH6 treatment groups (arrows; Fig. 5.7E, K).

These observations are indicative of cardiac dysfunction potentially due to cardiac hypertrophy, and it was not ameliorated despite treatment with SNH6. Moreover, H&E staining showed myofibrillar disarray in the KO hearts of both vehicle control and SNH6 treatment groups (Fig. 5.7F, L) when compared to their respective WT hearts (Fig. 5.7C, I), potentially due to hypertrophy of the hearts and the resultant fibrosis. In addition, the muscle fibre size of the cardiomyocytes in the vehicle control and SNH6-treated KO mice were markedly ($p<0.001$) increased relative to their respective WT littermates (Fig. 5.7M). Similar to the previous
assessments in the NAC-treated mice (Fig. 5.5A), this finding indicates that the animals developed cardiac hypertrophy despite of the treatment with SNH6. Overall, the 4 weeks of treatment with SNH6 did not significantly alter the cardiac histopathology of the KO mice in comparison to the vehicle control KOs by 9-weeks of age.
Figure 5.7: Pronounced histopathology of the heart and increased cardiomyocyte fibre size in the SNH6-treated MCK KO mice by 9-weeks of age. Histological staining of the heart from 9-week-old WT and KO mice at the end of a 4-week long treatment with either: (A-F) saline as the vehicle control group, or (G-L) SNH6 at 20 mg/kg. Transverse sections of cardiomyocytes were stained with Perls’ Prussian blue (left images: A, D, G, J; scale bar: 50 µm), Gömöri trichrome (middle images: B, E, H, K; scale bar: 100 µm) and H&E staining (right images: C, F, I, L; scale bar: 50 µm). Arrows in: (D, J) indicates deposits of iron in cardiomyocytes, and (E, K) demonstrates myocardial fibrosis. (M) The cardiac muscle fibre size of 9-week-old WT and KO mice from both treatment groups were measured and compared. It is evident that the SNH6-treated KO mice resembles the histopathological features of FA shown in the vehicle control KO mice. In addition, the KO mice treated with or without SNH6 also demonstrated cardiac hypertrophy by the end of the treatments due to this increase in cardiomyocyte fibre size. Images shown are representative histological staining (n = 5-6 in each treatment group). Figures are mean ± SEM. ###, p<0.001 indicates significance compared to the WT mice of their respective treatment group. n.s., not significant.
Cardiac histopathology at end-stage (9 weeks)

Perls’ Gömöri Trichrome H&E

A B C
D E F
G H I
J K L

Vehicle Control

WT KO

SNH6

WT KO

Cardiac Muscle Fibre Size (µm²)

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Vehicle Control

SNH6

Cardiac Muscle Fibre Size (µm²)

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5.3.2.3 Increased NAD+ Levels and Decreased NAD+:NADH Ratio in the SNH6-Treated MCK Mice by 9-weeks of Age

The SNH6 compound was designed to chelate iron, as well as to supplement NAD+ by providing the precursor, nicotinic acid (NA), that would be utilized through the NAD+ salvage pathway to synthesis NAD+ (Srivastava 2016). After orally treating the MCK mice with SNH6 for approximately 5 weeks, NAD+ levels were significantly \((p<0.05)\) increased in the SNH6-treated WT mice relative to the vehicle control WT mice, which demonstrates NAD+ induction (Fig. 5.8A). As reported previously in Chapter 4, NAD+ levels were markedly \((p<0.001)\) decreased in the vehicle control KO and SNH6-treated KO relative to the respective WT mice (Fig. 5.8A). However, NAD+ levels in the SNH6-treated KO mice were significantly increased compared to the vehicle control KO (Fig. 5.8A), and hence, this demonstrates that SNH6 treatment was able to induce NAD+ levels. As for NADH levels, it were significantly \((p<0.05)\) increased in the SNH6-treated WT mice compared to the vehicle control WT mice, while the NADH levels were unchanged \((p>0.05)\) in the SNH6-treated KO mice relative to the vehicle control KO (Fig. 5.8B).

In fact, the NADH levels were not significantly \((p>0.05)\) altered in the KO mice (Fig. 5.8B). Overall, despite NAD+ supplementation, the NAD+:NADH ratio was significantly \((p<0.01)\) decreased in the SNH6-treated KO mice relative to the SNH6-treated WT, and it was not significantly \((p>0.05)\) altered between the KOs from the vehicle control and SNH6 treatment groups (Fig. 5.8C). While SNH6 did promote NAD+ levels in the KO mice (Fig. 5.8A), the increase in NAD+ was insufficient to rescue the pathogenic decrease in NAD+:NADH ratio. This suggested that MIT and cardiac function remained defective in the frataxin-deficient KO mice after SNH6 treatment. Nonetheless, SNH6 can effectively increase NAD+ levels in MCK mice,
and hence, supplement for the NAD\(^+\) salvage pathway. This is demonstrated by data from the SNH6-treated WT mice that showed a significant \((p<0.05)\) decrease in NAD\(^+\):NADH ratio compared to the vehicle control WT (Fig. 5.8C), which is potentially due to an enhanced consumption of NAD\(^+\) in response to excessive NAD\(^+\) supplementation by SNH6.

5.3.2.4 Decreased ATP Levels, Increased Parp Activity, and Decreased Sirt1 Activity in the SNH6-Treated 9-week-old MCK Mice

Impaired MIT function, especially defects in OXPHOS, have been reported in FA patients in which the production of ATP is reduced (Heidari et al. 2009). In fact, a decrease in NAD\(^+\) levels as a result of defective OXPHOS can lead to a reduction in ATP production (Braidy et al. 2011, Zhou et al. 2015). As such, ATP levels were assessed in the MCK mice upon the 29-day treatment with either saline or our therapeutic compound, SNH6, that could increase NAD\(^+\) levels as shown in Fig. 5.8A. The levels of ATP were significantly \((p<0.05)\) increased between the vehicle control and SNH6-treated WT mice, while it was not significantly \((p>0.05)\) changed by SNH6-treatment in the KO mice (Fig. 5.8D). Furthermore, ATP levels were markedly \((p<0.001)\) decreased in the KO relative to the WT among their respective treatment groups (Fig. 5.8D). This observation demonstrates that ATP levels were reduced in the frataxin-deficient mice, and that the treatment with SNH6 did not significantly improve ATP levels.
Figure 5.8: Increased NAD\(^+\) levels and overall decreased NAD\(^+\):NADH ratio, reduced ATP levels, increased Parp activity and decreased Sirt1 activity in the SNH6-treated MCK KO mice by 9-weeks of age. After treatment with either saline or SNH6 (20 mg/kg), (A) NAD\(^+\) levels, (B) NADH levels, (C) NAD\(^+\):NADH ratio, (D) ATP levels, (E) Parp activity, and (F) Sirt1 activity were evaluated for the 9-week-old WT and KO mice of both treatment groups. Analysis of NAD\(^+\), NADH, and ATP levels were expressed as µg/mg of protein. Parp activity was presented as nuclear NAD\(^+\) consumption (µg NAD\(^+\) consumed/h/mg of protein), and results for Sirt1 activity was expressed as relative fluorescence unit (FU)/mg of protein. Figures are mean ± SEM (n = 5-6 in each treatment group). *, p<0.05, ***, p<0.001 denotes significance between WTs and KOs of the two treatment groups. ##, p<0.01, ###, p<0.001 indicates significance compared to the WT mice of their respective treatment group. n.s., not significant.
Since SNH6 was able to increase NAD$^+$ levels in the hearts of the MCK mice (Fig. 5.8A), this increased NAD$^+$ availability could potentially influence the activity of Parp (Canto and Auwerx 2011b, Chiarugi et al. 2012, Houtkooper and Auwerx 2012). Parp is a major NAD$^+$-consuming enzyme and its hyperactivation is a response to oxidative DNA damage in order to induce DNA repair processes (Oei and Shi 2001, Chiarugi and Moskowitz 2003, Wang et al. 2009). The assay used in this study measures nuclear NAD$^+$ consumption in the heart to reflect Parp activity, as Parp is known to be the highest consumer of NAD$^+$ in the nucleus (Durkacz et al. 1980, Soane et al. 2007, Cerutti et al. 2014). Indeed, Parp activity was markedly ($p<0.001$) increased in the vehicle control and SNH6-treated KO mice compared to WT mice of their respective treatment groups (Fig. 5.8E).

Increased NAD$^+$ consumption due to Parp activity may explain the significant decrease in NAD$^+$ levels in KO mice (Fig. 5.8A). More importantly, Parp activity was also markedly ($p<0.001$) increased in the SNH6-treated KO mice relative to the vehicle control KO (Fig. 5.8E). This result agrees with the increase in NAD$^+$ levels following SNH6 treatment (Fig. 5.8A), and suggests improved availability of NAD$^+$ that fuels Parp activity (Liu et al. 2009, Alano et al. 2010). Similarly, this could also be observed in the SNH6-treated WT mice, whereby Parp activity was significantly ($p<0.001$) increased compared to the vehicle control WT (Fig. 5.8E). This finding suggests increased NAD$^+$ consumption potentially due to the greater availability of NAD$^+$ supplemented by SNH6 between the WT mice (Fig. 5.8A). The treatment with SNH6 results in increased Parp activity in MCK mice, and consequently suggests an enhanced consumption of NAD$^+$ by Parp that could potentially promote further DNA repair in frataxin-deficient mice.
Members of the sirtuin family regulate vital pathways for energy sensing and energy production, including MIT respiratory complexes (Fernandez-Marcos and Auwerx 2011, Imai and Guarente 2014). The activation of Sirt1 is regulated by the availability of NAD$^+$ (Fernandez-Marcos and Auwerx 2011, Imai and Guarente 2014), and Parp can also affect Sirt1 activity and indirectly influence MIT function (Bai et al. 2011, Bai and Canto 2012). As such, the activity of Sirt1 was examined in the SNH6-treated MCK mice. Sirt1 activity was significantly ($p<0.01$) decreased in the KO relative to the WT mice in both the vehicle or SNH6 treatment groups (Fig. 5.8F). This finding corresponds to a decreased NAD$^+$:NADH ratio in the vehicle control and SNH6-treated KO mice (Fig. 5.8C).

Interestingly, Sirt1 activity was significantly ($p<0.05$) increased in SNH6-treated KO mice relative to the vehicle control KO mice (Fig. 5.8F). This observation suggests that the supplementation of NAD$^+$ bolstered Sirt1 activation and potentially improved MIT function in the KO animals. However, the SNH6-treated WT mice demonstrated a slight but significant ($p<0.05$) decrease in Sirt1 activity relative to the vehicle control WT mice (Fig. 5.8F). A possible explanation may be due to the increased Parp activity by SNH6-treatment in the WT mice (Fig. 5.8E), which could reduce the availability of NAD$^+$, and decrease the activation of Sirt1. Alternatively, a further increase in MIT biogenesis or function via Sirt1 activation may not be needed in healthy WT mice. Furthermore, Sirt1 activity in the SNH6-treated WT mice was only slightly lower than the vehicle control WT (Fig. 5.8F). Hence, SNH6 might not have significantly impacted MIT function in the WT mice. Nonetheless, Sirt1 activity was significantly increased by SNH6-treatment in the KOs, therefore, the supplementation of NAD$^+$ could potentially improve MIT biogenesis and cardiac bioenergetics.
5.4 Discussion

Treatments to alleviate the severe cardiomyopathy and cardio-degeneration of FA are limited, and previous studies have outlined various avenues of treatments to improve cardiac function for FA patients (Whitnall et al. 2008, Velasco-Sanchez et al. 2010). Considering that oxidative stress is a major contributing factor of the disease (Whitnall et al. 2012, Anzovino et al. 2017, Abeti et al. 2018), as shown in Chapter 3, antioxidant supplementation could be a potential therapeutic strategy. In particular, treatment with NAC offers the opportunity to bolster antioxidant defence against redox stress by acting as a precursor for GSH synthesis (Ji et al. 2010, Bavarsad Shahripour et al. 2014, Zhang et al. 2014). Our finding of decreased GSH level in the MCK mouse model of FA further encourages the possibility for GSH supplementation to attenuate the impaired Nrf2-ARE signalling pathway for antioxidant defence (Anzovino et al. 2017). Indeed, NAC has previously been shown to increase Nrf2 levels in disease models (Ji et al. 2010, Zhang et al. 2014). Hence, the therapeutic benefit of NAC was assessed in the MCK mice by examining the histopathology of the hearts in comparison to the non-pathological skeletal muscle.

The body weight and organ to body weight ratio for the heart are indicators of disease progression and the advancement of cardiac hypertrophy in MCK KO mice, which is a prominent feature of FA (Whitnall et al. 2008, Anzovino et al. 2017). Our studies with NAC demonstrated that despite the slight increase in the average body weight of the NAC-treated KO mice at around day-20 (Fig. 5.2E), their average body weight was not significantly different compared to the vehicle control KO mice throughout the treatment (Fig. 5.2E). Moreover, by 9-weeks of age, the average body weight was unaltered between the NAC-treated and vehicle control KO mice (Fig. 5.2F). This finding suggests that the NAC-treatment did not prevent weight loss associated with frataxin...
deficiency. In fact, the NAC-treated KO mice had similar heart to body weight ratio as the vehicle control KO mice, which was significantly greater than the WT mice (Fig. 5.2G). Hence, despite enhancing antioxidant defence with NAC, the KO mice still proceed to develop cardiac hypertrophy. This is further demonstrated by the increased cardiac muscle fibre size in the NAC-treated and vehicle control KO mice relative to their respective WTs (Fig. 5.5A).

The 9-week-old NAC-treated KO mice have iron deposits in their cardiomyocytes that is comparable to the vehicle control KO (Fig. 5.3). As such, treatment with NAC at concentrations of either 500 or 1500 mg/kg did not prevent the accumulation of redox active MIT iron in the myocytes, and thus suggests that oxidative stress was not attenuated. In contrast, the skeletal muscle was unaffected by the treatments with NAC, as demonstrated by the unchanged skeletal muscle to body weight ratio and histopathology of the tissue (Fig. 5.2H, Fig. 5.4). This result is consistent with previous reports (Anzovino et al. 2017), and although frataxin is deficient in the skeletal muscle similar to the heart, this discrepancy between the tissue-types could be due to their difference in energy metabolism in which the skeletal muscle may rely on anaerobic means without utilising OXPHOS (Berg J.M. 2002, Hargreaves and Spriet 2017).

The treatments with NAC did not improve the cardiac phenotype or histopathology of the MCK KO mice by 9-weeks of age. Therefore, our studies suggest that GSH supplementation alone via NAC-treatment is insufficient to elicit protective effects. Nonetheless, there are previous studies that have demonstrated the therapeutic potential of NAC in the treatment of FA (Banaclocha 2001, Johnson et al. 2012), and oxidative stress remains a deleterious component in its pathogenesis.
(Chiang et al. 2016). Thus, therapeutic strategies to improve antioxidant defence possibly through the induction of antioxidant levels or activation of the Nrf2 pathway deserves further investigation.

Besides oxidative stress, deficits in MIT bioenergetics due to MIT dysfunction is another key factor in the pathogenesis of FA (Lodi et al. 1999, Heidari et al. 2009, Nunnari and Suomalainen 2012, Gonzalez-Cabo and Palau 2013). This is evident from reports of NAD\(^+\) and ATP deficiencies in cases of FA (Heidari et al. 2009, Wagner et al. 2012). In conjunction with the findings in Chapter 4 where NAD\(^+\) levels and the NAD\(^+\):NADH ratio were decreased in the MCK KO mice, we assessed the therapeutic potential of the novel compound, SNH6, designed in our laboratory to supplement NAD\(^+\) and chelate iron. This therapeutic strategy was assessed herein to alleviate the cardiac pathology of FA.

In general, throughout the course of the treatments, the average body weight of the SNH6-treated KO mice was similar to the vehicle control KO and were significantly less than their respective WT littermates (Fig. 5.6A, B). The heart to body weight ratio and cardiac muscle fibre size were also markedly increased in the 9-week-old vehicle control and SNH6-treated KO mice (Fig. 5.6C, Fig. 5.7M), which suggests that the frataxin-deficient mice developed cardiac hypertrophy despite having treated with SNH6. Furthermore, iron deposits were present in the SNH6-treated KO mice similar to the vehicle control KO (Fig. 5.7). Thus, treatment with SNH6 did not remove sufficient amounts of toxic iron in cardiomyocytes.
Nonetheless, SNH6 was able to increase NAD$^+$ levels as shown in Fig. 5.8A, where the NAD$^+$ levels were significantly increased by SNH6-treatment in both WT and KO mice. Interestingly, NADH levels were also increased in the SNH6-treated WT mice relative to the vehicle control WT, which resulted in an overall decrease in NAD$^+$:NADH ratio between the WTs (Fig. 5.8B, C). A possible explanation for this observation could be that the increased NAD$^+$ supplementation by SNH6 fuelled the reduction of NAD$^+$ to NADH in the WT mice. Glycolytic metabolism could potentially be enhanced upon NAD$^+$ supplementation, which would increase the turnover of NAD$^+$ to NADH in the WT mice, and consequently increase ATP production (Xiao et al. 2018). Considering this, ATP levels were increased by SNH6-treatment in the WT mice (Fig. 5.8D), and hence, NAD$^+$ supplementation was able to improve energy metabolism in normal physiological conditions in these animals.

Alternatively, the decreased NAD$^+$:NADH ratio in the SNH6-treated WT mice relative to the vehicle control WT could be the result of increased NAD$^+$ consumption. This was supported by the assessment of nuclear NAD$^+$ consumption, as a measure of Parp activity, which was markedly increased by SNH6-treatment in the WT mice (Fig. 5.8E). Therefore, this finding suggests increased NAD$^+$ consumption by Parp in the SNH6-treated WT mice. This would also explain the decrease in Sirt1 activity by SNH6-treatment in the WT mice (Fig. 5.8F), as the increased consumption of NAD$^+$ would reduce the availability of NAD$^+$, thereby decreases Sirt1 activation (Heidari et al. 2009, Wang et al. 2013, Srivastava 2016).
Similarly, NAD$^+$ supplemented by SNH6 could potentially be consumed by increased Parp activity in the SNH6-treated KO mice (Fig. 5.8A, E). In fact, this increase in Parp activity is significantly greater in the SNH6-treated KO mice than in the SNH6-treated WT mice (Fig. 5.8E). This observation suggests that NAD$^+$ supplementation was able to induce Parp activity, and that the KO mice possess a greater capacity to consume NAD$^+$ by Parp than WT mice. Furthermore, this would explain the relatively minor increase in NAD$^+$ levels in the KO versus the WT mice by SNH6-treatment (Fig. 5.8A). Thus, the NAD$^+$:NADH ratio remains unchanged by SNH6-treatment in the KO mice (Fig. 5.8C). Studies in other disease models have previously shown an association between the activation of Parp1 and MIT dysfunction where Parp1 hyper-activation can lead to decreased NAD$^+$ levels and reduced Sirt1 activity accompanied by impaired oxidative metabolism (Fang et al. 2014, Bai et al. 2015, Martire et al. 2015). Specifically, Sirt1 and Parp1 has been shown to compete for the same pool of NAD$^+$ for many major metabolic processes (Canto and Auwerx 2011a, Martire et al. 2015). As such, the activation of Parp1 could out-compete Sirt1, leading to NAD$^+$ depletion, and result in the suppression of Sirt1 activity (Bai et al. 2011, Martire et al. 2015). Therefore, an increased consumption of NAD$^+$ by the enhanced activity of Parp upon treatment with SNH6 could potentially explain the observed decrease in Sirt1 activity in the hearts of the KO mice relative to the WT (Fig. 5.9).
Figure 5.9: Schematic of the effects of SNH6-supplementation of NAD\(^+\) in the heart of the MCK KO mice. (1) Frataxin deficiency is associated with decreased NAD\(^+\) levels and increased NADH levels, resulting in an overall decrease in NAD\(^+\):NADH ratio in the untreated KO hearts relative to their WT littermates. Upon treatment with SNH6, the KO hearts exhibit (2) increased NAD\(^+\) levels, (3) which induced the activity of the NAD\(^+\)-dependent enzyme, Sirt1, relative to the SNH6-treated WT mice. (4) Parp activity was also increased when the animals were treated with SNH6. (5) This increase in Parp activity increases NAD\(^+\) consumption and could deplete the NAD\(^+\) pool in the KO mice relative to the WT. As a result, (6) Parp activity potentially competed against Sirt1 for NAD\(^+\) consumption, and thereby limited the increase in Sirt1 activity by SNH6-treatment in the KO mice. Nonetheless, SNH6-supplementation of NAD\(^+\) partially increases Sirt1 activity in the KO hearts relative to the vehicle control KO. This has the potential to restore Sirt1 deacetylase activity, and thereby activate Pgc1\(\alpha\) to improve mitochondrial biogenesis and function in frataxin deficiency.
Of note, increased Parp activity in the SNH6-treated mice suggests a promotion of DNA repair (Canto and Auwerx 2011a, Canto et al. 2015). Considering that frataxin deficiency is associated with high levels of DNA damage (Haugen et al. 2010, Thierbach et al. 2010, Shen et al. 2016), a possible increase in DNA repair could be beneficial to the KO mice. However, massive Parp activation can also lead to necrotic cell death, as it significantly depletes NAD$^+$ and ATP levels (Pieper et al. 1999, Putt et al. 2005, Canto et al. 2015). Our data did not demonstrate further depletion of NAD$^+$ and ATP in the SNH6-treated mice, which suggests that the activation of Parp is unlikely to induce cell death upon treatment (Fig. 5.8A, D, F). Moreover, the histological assessments did not indicate further tissue damage in the SNH6-treated KO hearts compared to the WT. Nevertheless, the distinctive role of Parp in cell survival and cell death upon treatment with SNH6 would be important to assess in the MCK mice for future studies.

In addition, the supplementation of NAD$^+$ by SNH6 did not restore energy levels, as evident by the significant decrease in ATP levels in the SNH6-treated KO mice (Fig. 5.8D). Nonetheless, Sirt1 activity was greater in the SNH6-treated KO mice relative to the vehicle control KO (Fig. 5.8F), and while it is significantly less than the SNH6-treated WT mice, this observation suggests that SNH6 increased the availability of NAD$^+$ such that Parp did not fully out-compete Sirt1 in the KO mice, and hence allowed for Sirt1 activation (Fig. 5.9), which could potentially improve MIT function.

Moreover, the mechanism by which SNH6 increases NAD$^+$ levels involve the supplementation of the NAD$^+$ precursor, NA, hence the treatment relies on the NAD$^+$ salvage pathway to restore its
levels. However, while SNH6 did successfully supplement NAD\(^{+}\), our studies suggest that this approach to provide NA alone might not have been sufficient to restore cardiac function and protect against disease progression of the heart in the MCK KO mice. This further suggests that the NAD\(^{+}\) synthesis pathway could be dysregulated when frataxin is deficiently expressed. Thus, it is essential to further elucidate the complex involvement of NAD\(^{+}\) synthesis in the regulation of Sirt1 activity and MIT function, and hence, their roles in FA cardiomyopathy.

In summary, the current study assessed two potential therapeutic compounds, NAC and SNH6, independently to ameliorate the cardiomyopathy of FA in the MCK frataxin-deficient mice. Despite 4-5 weeks of rigorous treatments, neither the supplementation of GSH with NAC, or the supplementation of NAD\(^{+}\) with SNH6, did not sufficiently rescue either: (1) the characteristic cardiac hypertrophy, (2) the iron-loading of cardiomyocytes, or (3) restore the NAD\(^{+}\):NADH ratio in frataxin-deficient mice. These findings suggest that the independent supplementation of intrinsic antioxidant levels by NAC or the reconstitution of NAD\(^{+}\) by SNH6 did not rescue the end-stage cardiomyopathy in these animals. Nonetheless, these findings did not preclude the possibility that these compounds may have cardio-protective effects during the course of the treatments. This is evident in previous studies where the treatments with antioxidants, iron chelators, or NAD\(^{+}\) supplementing agents in the MCK mice model and FA patients demonstrated transient cardiac improvements without fully restoring cardiac function (Seznec et al. 2004, Whitnall et al. 2008, Velasco-Sanchez et al. 2010, Lagedrost et al. 2011, Elincx-Benizri et al. 2016, Stram et al. 2017). Indeed, the clinically used treatment for FA, idebenone, also only delayed the onset of left ventricular cardiomyopathy in the MCK mice without rescuing the end-stage heart failure (Seznec et al. 2004). Therefore, it is important to further investigate the complete therapeutic potential of
NAC and SNH6 in the frataxin-deficient mice by examining their cardiac function throughout the NAC/SNH6 treatment regime, in order to understand the possible cardio-protective effect of these compounds.
CHAPTER SIX

General Discussion and Future Directions
Discussion Prelude

FA is the most common autosomal recessive ataxia in the world, and at present, the majority of available treatments have limited effectiveness (Campuzano et al. 1996, Strawser et al. 2014, Abrahao et al. 2015). Therefore, it is important to understand the molecular mechanisms and the associated functional deficits that afflict this disease in order to development of effective and targeted therapies. This thesis aimed to address this issue by elucidating the intrinsic molecular mechanisms of antioxidant defence and MIT homeostasis in which their dysregulations have been attributed to the pathogenesis of the disease. The thesis then aimed to target these dysfunctions by examining the therapeutic efficacies of NAC and SNH6 to ameliorate the cardiomyopathy of FA. The well-characterised and accepted MCK conditional frataxin KO mice cardiac model of FA was used for all of the studies presented in this thesis.

Oxidative stress is a fundamental pathological feature of many neuro-degenerative and cardiac diseases including FA (Gomes and Santos 2013, Bhat et al. 2015, Martin-Fernandez and Gredilla 2018). Considering the associated down-regulation of Nrf2 with frataxin deficiency, Chapter 3 explored the molecular mechanisms responsible for the depressed expression of Nrf2 and its impact on the regulation of antioxidant defence against oxidative stress in a cardiac setting. Chapter 4 then investigated the role of MIT dysfunction in the pathogenesis of FA, as it has become an increasingly important aspect for many disease pathologies (Bhat et al. 2015, Berthiaume et al. 2017). For the first time, the ultrastructure of the frataxin-deficient cardiomyocytes was studied in an FA mice model and the study dissected the molecular mechanisms that governed MIT dynamics in frataxin deficiency. Following from the major findings of the thesis, Chapter 5 then assessed the therapeutic application of antioxidant GSH and
NAD⁺ supplementations independently to attenuate oxidative stress and to rescue the cardiac phenotype of FA.

The findings of this thesis enhanced our understanding of the various molecular faculties in the pathogenesis of FA, specifically the impaired intrinsic response of antioxidant defence, the dysregulation of MIT dynamics, and bioenergetic deficits. Hence, this work holds great significance by underlining potential avenues of approach for the development of novel FA therapies. The following encompasses a summary of the principle findings from each thesis chapter, discussions on the different avenues for future research, and a conclusion to outline the significance of this thesis.

6.1 Principle Findings

6.1.1 Summary of Principle Findings in Chapter 3

FA is well established in the literature to be caused by the deficient expression of the MIT protein, frataxin (Campuzano et al. 1996, Campuzano et al. 1997, Anzovino et al. 2014). The associated dysregulation of iron metabolism and ISC synthesis results in the harmful MIT iron accumulation that fuels the generation of ROS and causes oxidative stress in FA (Dröge 2002, Sparaco et al. 2009, Whitnall et al. 2012). A dysregulation of antioxidant defence has been implicated to the potentiation of oxidative damage, especially due to reports of decreased Nrf2 protein expression in frataxin deficiency (D’Oria et al. 2013, Shan et al. 2013, Anzovino et al. 2014). Hence, Chapter 3 aimed to dissect the different mechanisms involved in this decrease of Nrf2 and the subsequent dysregulation of the Nrf2-ARE anti-oxidative signalling pathway in FA.
The initial findings highlighted the presence of oxidative stress with increased protein oxidation and decreased GSH:GSSG ratio due to an inhibited anti-oxidative response in the hearts of the MCK frataxin-deficient mice. The assessment of the animals’ cardiac histopathology at 9-weeks of age also demonstrated the phenotypic cardiac hypertrophy of FA. Molecular studies then illustrated the dysregulation of the Nrf2 signalling pathway in which there was an inverse relationship between the protein expression of Nrf2 and Keap1 in the cytosol and nucleus. Specifically, the observed increase in Keap1 could potentially be responsible for the decrease in Nrf2 due to Keap1-mediated sequestration of Nrf2 leading to its subsequent proteasomal degradation. In addition, Bach1, which is a repressor of Nrf2-DNA binding activity (Suzuki et al. 2004), have decreased protein expression in the nucleus of frataxin KO hearts potentially to encourage access of Nrf2 to AREs to activate antioxidant defence. Despite this, there was reduced Nrf2-ARE binding, which further suggests a dysfunction in the Nrf2 pathway. However, while the mRNA levels of Nrf2 downstream antioxidant response genes were generally reduced, their protein levels were found to be not significantly altered or even exhibited an increase. This finding suggests other potential pathways could be at play to compensate the reduced activation of the Nrf2 pathway. Nevertheless, this possible compensation did not prevail and the ensuing oxidative damage results in the observed cardiac hypertrophy in the MCK mice model of FA.

Apart from the dysregulation of Nrf2 activity by Keap1, the current study demonstrated for the first time the activation of Nrf2 nuclear export/degradation machinery via the phosphorylation of Nrf2 either directly by Gsk3β or indirectly through Fyn kinase (Jain and Jaiswal 2007, Rada et al. 2011, Rada et al. 2012, Chowdhry et al. 2013). Specifically, there were increased phosphorylated
activation of Gsk3β (Tyr216) and Fyn kinase phosphorylation, along with increased Tyr phosphorylation of Nrf2 that was known to result in its nuclear export (Jain and Jaiswal 2007, Mobasher et al. 2013). Notably, there was also enhanced nuclear protein expression of β-TrCP that facilitates the nuclear exportation of Nrf2. In contrast to the heart, the skeletal muscle of the MCK mice demonstrated non-pathological molecular alterations despite that frataxin is deficiently expressed in this tissue type similar to the heart. These findings are consistent with previous studies that have also reported the skeletal muscle of the MCK mice model to have no defects in its histology, ultrastructure, or biochemical properties (Puccio et al. 2001). This tissue-specific effect could be attributable to their difference in energy metabolism in which the heart relies strongly on OXPHOS by mitochondria, while the skeletal muscle may utilise anaerobic glycolysis to satisfy energy requirements (Berg J.M. 2002). Therefore, the frataxin-deficient heart could suffer greater cardiac metabolic deficits from the associated MIT dysfunction in comparison to the skeletal muscle.

Overall, this chapter have unravelled four significant processes involved in the down-regulation of Nrf2 with its nuclear efflux and subsequent degradation, as well as its reduced induction of antioxidant defence in the frataxin-deficient heart. Namely, these processes include: (1) increased Keap1 protein expression, (2) increased Gsk3β activation, (3) phosphorylation of Fyn, and (4) the nuclear accumulation of β-TrCP. Furthermore, these studies have accentuated the significance of oxidative stress in the cardiac pathophysiology of FA, and thus, it is important to consider antioxidant supplementation as a potential therapy for this disease.
6.1.2 Summary of Principle Findings in Chapter 4

Mitochondria is a major source of oxidative stress due to the metabolic processes it utilises for energy production in which defects to the ETC can lead to the over-generation of ROS (Nunnari and Suomalainen 2012, Bhat et al. 2015). Many studies on neurodegeneration and cardiomyopathies have highlighted the significance of MIT dysfunction in the pathology of these diseases (Bhat et al. 2015, Berthiaume et al. 2017). In FA, the synthesis of ISC is impaired due to frataxin deficiency (Richardson et al. 2010b, Whitnall et al. 2012, Huang et al. 2013). As a result, Complex I activity of the MIT respiratory chain is reduced, intracellular ATP levels is decreased, and the imbalance between NAD$^+$ to NADH levels leads to excessive ROS production and causes oxidative stress (Heidari et al. 2009, Delgado-Camprubi et al. 2017). Considering the importance of MIT homeostasis in cell vitality and its relationship with oxidative stress in disease states, Chapter 4 aimed to elucidate the role of MIT dysfunction in the pathology of FA.

Using TEM, the studies in this thesis demonstrated ultrastructural alterations in the cardiomyocytes of an FA mice model with age. At 4-weeks old, the frataxin-deficient mice exhibited asymptomatic cardiac phenotype with potential increase in the size of mitochondria. However, by 10-weeks of age, the animals developed the characteristic dilated cardiomyopathy of FA with marked ultrastructural changes indicating MIT proliferation and dysfunction. In fact, distinguishable MIT iron deposits and condensation of MIT cristae were noticeable pathological features of the KO mice.
Furthermore, the MIT morphology at this later age suggests increased MIT dynamic processes, such as MIT fusion and fission that resulted in the concurrent elongation and fragmentation of the organelles. Subsequent molecular studies supported these observations in which there was an overall up-regulation of protein markers of MIT biogenesis (Pgc1α, Nrf1, and Tfam), MIT fusion (Mfn1 and Opa1), and MIT fission (Drp1, Fis1, and Mff) in the frataxin-deficient hearts. Increased biogenesis of de novo mitochondrion could potentially replace the functional deficits of disabled mitochondria (Chen and Chan 2006, Frank 2006). The fusion of mitochondria may rescue mtDNA damage and MIT oxidative capacity to restore cardiac energetics, while fission of mitochondria could lead to the compartmentalisation of defective mitochondria for removal via mitophagy (Chen and Chan 2006, Frank 2006, Youle and van der Bliek 2012). Hence, these accumulative alterations of MIT dynamic processes could be an attempt to compensate the loss of cardiac function due to the continuing oxidative insult caused by the accumulation of iron in frataxin-deficient mitochondria.

Consistent with previous studies in FA (Kim et al. 2007b, Heidari et al. 2009, Nunnari and Suomalainen 2012), the present data also showed decreased NAD⁺ levels and NAD⁺:NADH ratio, as well as reduced ATP levels in the KO hearts, thus, demonstrating MIT dysfunction and a loss in cardiac energetics. These findings aligned with the observed decrease in Sirt1 activity due to its dependence on NAD⁺ levels, which would lead to decreased deacetylation of Pgc1α and consequently reduce Pgc1α activation (Kim et al. 2007a, Canto et al. 2009). Increased Parp activity was found in the KO hearts, and this would also decrease the activity of Sirt1 and Pgc1α (Canto and Auwerx 2011a, Fang et al. 2014). Furthermore, the present immunoprecipitation studies indeed showed increased acetylation of Pgc1α, which correlates with previous reports that hyper-

Moreover, the current studies demonstrated increased Ampk activation and decreased Sirt1 and Paris protein expressions. Activated Ampk could be a response to the decrease in intracellular ATP content in the KO hearts, and the activation of Ampk is known to facilitate the phosphorylation of Pgc1α to assist its activation (Canto et al. 2009, Mihaylova and Shaw 2011). However, decreased Sirt1 levels would lead to reduced deacetylation of Pgc1α, hence suppressing its activation (Canto et al. 2009, Fernandez-Marcos and Auwerx 2011). In contrast, the decrease in Paris levels suggests reduced transcriptional suppression of Pgc1α and its downstream targets, Nrf1 and Tfam (Shin et al. 2011). Collectively, the regulation of Pgc1α and its activity is dysregulated in the MCK mice and despite the possible decrease in its activation, the frataxin-deficient cardiomyocytes continued to proliferate defective mitochondria that would further exacerbate oxidative stress and bioenergetic deficiencies.

In light of the enhanced yet dysregulated alterations in MIT dynamics, it is necessary to investigate the elimination of defective mitochondria through the MIT-targeted autophagic process known as mitophagy (Kim et al. 2007b, Lazarou 2015). The two critical regulators of MIT dynamics and mitophagy, Pink1 and Parkin (Lazarou et al. 2015), were up-regulated in the hearts of the KO mice, suggesting that the process for targeting mitochondria for degradation is functional. This is consistent with previous data from our laboratory that demonstrated increased levels of the mitophagic marker, Fundc1, and a progress accumulation of autophagic substrates, Lc3-II and p62,
in the KO mice with increasing age (Huang et al. 2013). As such, it was proposed that the autophagic machinery was active, but the lysosomal-mediated autophagic degradation may be inhibited so that these autophagic substrates would accumulate (Huang et al. 2013). The present studies solved this issue by demonstrating that there was in fact enhanced autophagic flux in the frataxin-deficient hearts. Specifically, upon lysosomal inhibition with BAF treatment of the animals, autophagic substrates, Lc3-II and p62, including Mfn1 as an indicator for the involvement of mitophagy, were all markedly accumulating in the KO compared to the WT. Thus, there was an increased capacity of autophagic and mitophagic degradation, which suggests increased removal of defective mitochondria and potentially reducing MIT stress as an attempt to restore MIT homeostasis. However, despite this enhanced response, the cell was unable to overcome the accumulative deficits presented by the frataxin-deficient mitochondria.

In general, the studies in this thesis have demonstrated for the first time, altered MIT morphologies, impaired bioenergetics, dysregulation of MIT dynamics, and enhanced mitophagy in the MCK mice model. Collectively, the dynamic network of mitochondria displayed their attempt to compensate the oxidative damage caused by frataxin deficiency. Nonetheless, MIT dysfunction have perpetuated disease progression, resulting in a loss of cardiac energetics. Hence, it is important to consider the development of therapies that aims to restore MIT function and energy metabolism in order to rescue the cardiomyopathy in FA.
6.1.3 Summary of Principle Findings in Chapter 5

At present, there are limited treatments available for FA patients, hence there is an urgent need for the development of novel therapies. Considering data in Chapter 3 and Chapter 4, oxidative stress and MIT dysfunction were shown to be major contributing factors in the pathophysiology of the disease, and therefore there is great potential for the design of therapeutic strategies aimed at attenuating these defects (Anzovino et al. 2017, Chiang et al. 2017). Our previous findings in the MCK frataxin KO mice demonstrated decreased endogenous levels of the antioxidant GSH, increased protein oxidation, and the dysregulation of the Nrf2 signalling pathway with decreased Nrf2 nuclear and cytosolic protein levels (Anzovino et al. 2017). In addition, antioxidant supplementation with the GSH precursor, NAC, has been reported to have protective effects in other disease models (Prescott et al. 1977, Santos et al. 2001, Ji et al. 2010). In fact, it has been shown in previous studies that NAC was able to improve Nrf2 levels and antioxidant defence (Santos et al. 2001, Ji et al. 2010, Zhang et al. 2014). Thus, Chapter 5 assessed the efficacy of NAC to bolster GSH levels in order to enhance antioxidant defence and rescue the cardiomyopathy in FA.

After approximately 5 weeks of oral treatment with NAC, the results demonstrated no significant alterations in terms of the cardiac histopathology of the frataxin-deficient mice. The animals retained the phenotypic iron deposits and interstitial fibrosis in the NAC-treated cardiomyocytes, and the hearts exhibited the severe end-stage cardiac hypertrophy by 9-weeks of age. While the average body weight of the frataxin-deficient mice was slightly rescued during the course of the treatment with NAC, the supplementation with GSH ultimately did not prevent the cardiomyopathy from developing. It is possible that the strategy to reconstitute endogenous GSH levels alone was insufficient to inhibit oxidative stress. Nonetheless, the dysregulated anti-
oxidative response in the KO mice would potentiate the oxidative insult to the heart, and oxidative stress would continue to be a highly destructive in frataxin deficiency (Anzovino et al. 2017). Hence, the therapeutic avenue to induce antioxidant defence or to stimulate the Nrf2 signalling pathway remains to be important strategies for the treatment of this disease.

Alternatively, in light of MIT dysfunction discussed in Chapter 4, restoring the energetic deficits in frataxin deficiency was another strategy examined in this chapter. Impaired energy metabolism can be caused by deficits to the MIT respiratory chain in which the imbalance of NAD\(^+\) and NADH levels can result in decreased energy production and enhance redox stress (Heidari et al. 2009). Findings in Chapter 4 were consistent with other studies in FA that have demonstrated the association between frataxin deficiency and a decrease in NAD\(^+\):NADH ratio, which consequently leads to oxidative stress and energy loss (Schulz et al. 2000, Lodi et al. 2001, Wagner et al. 2012).

These observations form the rationale for the current investigation examining the therapeutic efficacy of the compound, SNH6, that was designed in our laboratory to not only chelate redox-active iron, but also to increase NAD\(^+\) levels through its supplementation of the NAD\(^+\) precursor, NA. Unfortunately, the treatment studies with SNH6 demonstrated no significant improvement to the cardiomyopathy in the MCK KO mice. Their cardiac histopathology exhibited iron accumulation in the cardiomyocytes, suggesting that the chelation provided by the compound was insufficient prevent oxidative stress. The increase in cardiomyocyte fibre size and the average heart to body weight ratio also demonstrated the classic FA phenotype of cardiac hypertrophy.
Furthermore, ATP levels remain decreased after the treatment with SNH6, which suggested that it did not restore energy metabolism. Despite these observations, SNH6 was able to increase NAD$^+$ levels in the frataxin-deficient heart. Although, it was unable to increase the NAD$^+:$NADH ratio, it was evident that the increase in Parp activity could be responsible for an increased consumption of NAD$^+$ upon its supplementation by SNH6. Indeed, previous studies have shown that the hyper-activation of Parp can lead to decreased NAD$^+$ levels and diminished Sirt1 activity, hence suppressing sirtuin-mediated induction of MIT biogenesis (Bai et al. 2011, Fang et al. 2014, Bai et al. 2015, Martire et al. 2015). As such, increased Parp activity is associated with MIT dysfunction, and this has been reported in other disease models (Fang et al. 2014, Bai et al. 2015). Regardless, Sirt1 activity was increased in the frataxin-deficient hearts by treatment with SNH6, however, it did not return to nominal levels. This finding suggests that the compound had potentially induced the Sirt1-Pgc1α axis for MIT biogenesis to repair its dysfunction.

In conclusion, the assessments of NAC and SNH6 as potential therapies have demonstrated that their independent administration was insufficient to ameliorate the cardiomyopathy in FA. Yet, it remains true that oxidative stress, MIT dysfunction, and impaired energy metabolism are critical pathological factors of FA. Thus, further studies and optimisation (including their possible combination to induce synergistic effects) are required to improve these avenues of treatment that exploits these pathological defects.
6.2 Future Directions

6.2.1 The Regulation of Nrf2 and Keap1 by p62 in FA

Previous reports have found an interplay between antioxidant defence and autophagy in response to oxidative stress (Ichimura et al. 2013, Jiang et al. 2015). The interactions involve the Nrf2-Keap1 signalling pathway with the autophagic adaptor p62 in which Keap1 and p62 can regulate Nrf2 (Komatsu et al. 2010, Taguchi et al. 2012, Ichimura et al. 2013). The binding of p62 to the Nrf2-binding site of Keap1 was found to competitively inhibit the interaction between Keap1 and Nrf2 (Komatsu et al. 2010, Ichimura et al. 2013). Hence, Keap1 sequestered by p62 reduces Keap1 binding to Nrf2, thereby increases Nrf2 signalling (Jiang et al. 2015).

In fact, phosphorylation of p62 increases its affinity for Keap1, hence p62 phosphorylation can prevent Keap1-mediated Nrf2 degradation and up-regulate Nrf2 targets for antioxidant defence (Ichimura et al. 2013). This increase in Nrf2 activity can in turn induce p62, which serves as a positive feedback loop to encourage autophagy in response to oxidative stress (Jain et al. 2010). In addition, p62 was found to also regulate the turnover of Keap1 (Copple et al. 2010, Jiang et al. 2015). Hence, in the presence of oxidative stress and an increase in autophagic processes, p62 can activate Nrf2 signalling by sequestering Keap1 and controlling its availability, therefore eliciting Nrf2-mediated cyto-protection (Jiang et al. 2015).

Results in Chapter 3 demonstrated increased Keap1 and decreased Nrf2 cytosolic expression in the KO hearts. Interestingly, consistent with previous findings (Huang et al. 2013), data in Chapter 4 have also shown increased p62 expression in the hearts of the KO mice, with increased
autophagic flux that indicates enhanced autophagy. Hence, it is possible that there is a disconnect between p62 and its interaction with Keap1 in which the observed increase in autophagy in these animals did not lead to further oxidative-stress response by the Nrf2-Keap1 pathway. In other words, p62-mediated Keap1 turnover could be impaired, and as a result, allows Keap1 to target Nrf2 for degradation. However, the phosphorylation status of p62 was not examined in the animals, therefore it is important to further assess the phosphorylation of p62, and its binding affinity to Keap1. As such, a dysregulation of the p62-Keap1 axis could be another important mechanism responsible for the subdued expression of Nrf2 and its downstream targets in the MCK mice. This potential future investigation could unveil an interesting pathological defect between antioxidant defence and autophagy in frataxin deficiency that contributes to FA pathology.

6.2.2 Examination of Energy Metabolism in Cardiac and Skeletal Muscle of the MCK frataxin KO Mice

An intriguing result from Chapter 3 is the tissue-specific effects of frataxin deficiency on the heart and the skeletal muscle in the MCK mice. Our studies showed that cytosolic and nuclear expression of Nrf2 was decreased in the heart, but not in the skeletal muscle despite that frataxin was deficiently expressed in both tissue types. Importantly, the heart demonstrated a dysregulated Nrf2 signalling pathway with increased protein oxidation and reduced GSH levels, whereas the skeletal muscle had neither such dysregulation nor suffer from oxidative stress. Hence, it is proposed that this tissue-specific effects of frataxin deficiency is due to the difference in energy metabolism of the tissues whereby the cardiac muscle is highly reliant on aerobic metabolism i.e. OXPHOS for energy production, while the skeletal muscle may utilise anaerobic glycolysis (Berg J.M. 2002).
The literature have widely reported impair MIT respiration and decreased ATP generation in the condition of frataxin deficiency and in FA patients (Heidari et al. 2009, Anzovino et al. 2014, Strawser et al. 2014). The findings in Chapter 4 further reinforced these defects in the KO heart, which demonstrated decreased ATP and NAD$^+$ levels. Interestingly, previous reports have found that the skeletal muscle of FA patients have lower bioenergetics and decreased ATP levels possibly due to impaired OXPHOS (Ristow et al. 2000, Nachbauer et al. 2013). Yet, the skeletal muscle of the MCK mice did not demonstrate any defects in biochemical properties (Puccio et al. 2001) or histology, as shown in Chapter 3. In addition, the skeletal muscle in frataxin-deficient condition also revealed increased lipogenesis consistent with insulin resistance (Coppola et al. 2009). Taken together, it is possible that the cardiac and skeletal muscle of the MCK mice would display different bioenergetics profile in terms of glycolysis, lipid metabolism, and OXPHOS, which deserves further investigation.

Previous studies of different pathophysiological conditions demonstrated that severe functional deficiency of the MIT respiratory chain and decreased OXPHOS gene expression could lead to a potential shift towards anaerobic metabolism for cell survival (Levanets et al. 2011, Moran et al. 2012). Therefore, in light of MIT dysfunction and the potential decrease in OXPHOS activity in the KO hearts, it is possible that the cardiac muscle may enhance its glucose metabolic capacity to compensate the loss of energy production. In addition, previous studies have shown that the MIT capacity of skeletal muscle was reduced in FA patients, with reduced muscle endurance and a decreased rate of oxygen consumption by the tissue (Bossie et al. 2016). As such, the skeletal muscle could also suffer from MIT dysfunction, and possibly, over-exert anaerobic metabolism for energy production, leading to the loss of muscle endurance, causing muscle weakness and
fatigue. In fact, alterations in energy metabolism in the heart and skeletal muscle have not been thoroughly examined in FA, hence it would be of interest to investigate the effects of frataxin deficiency on the energy capacity of these tissue types.

Moreover, the proliferator-activated receptor gamma (PPARγ) pathway that regulates Pgc1α and MIT biogenesis has been reportedly down-regulated in animal models of FA and in FA patients (Coppola et al. 2009, Marmolino et al. 2010). In particular, the Pgc1α activity was down-regulated in the frataxin-deficient skeletal muscle, as evident by the increased expression of genes involved in lipogenesis that would have otherwise been repressed by Pgc1α (Coppola et al. 2009). In fact, Pgc1α down-regulation and the simultaneous up-regulation of the transcription factor, Srebp1, is known to occur in diabetes and insulin resistance (Mootha et al. 2003, Coppola et al. 2009), which is associated with decreased OXPHOS (Coppola et al. 2009). Hence, the dysregulation of the PPARγ- Pgc1α pathway not only increases the risk of diabetes for FA patients (Coppola et al. 2009), but that this pathway could be responsible for tissue-specific differences in energy metabolism (Lehman et al. 2000). The binding of Pgc1α to tissue-specific transcription factors could potentially regulate the activation of different metabolic processes for different tissue types (Lin et al. 2005, Finck and Kelly 2006). Thus, the regulation of energy metabolism and MIT biogenesis via the PPARγ- Pgc1α pathway is important to investigate in order to dissect the metabolic differences between the heart and the skeletal muscle in the MCK mice.
6.2.3 Potential Therapy with Iron Chelation and Combination Treatments for FA Patients

In the MCK mice cardiac model of FA, the accumulation of iron in the mitochondria is known to play a significant role in ROS generation and in potentiating oxidative stress (Alper and Narayanan 2003, Richardson 2003, Richardson et al. 2010a, Huang et al. 2013). The cardiac histopathological data shown in Chapter 3 and Chapter 5, and the EDS data in Chapter 4, further demonstrated the prevalence of MIT iron in the frataxin-deficient heart. Hence, another avenue in the development of new therapy for FA involves the targeted chelation of MIT iron. The well-characterised iron-loading in mitochondria in the hearts of the MCK mice can lead to severe defects in MIT function (Whitnall et al. 2008, Richardson et al. 2010b, Huang et al. 2011, Huang et al. 2013). Using Mössbauer spectroscopic analysis and TEM, the iron was previously found to be non-ferritin bound and is in the form of ferric iron with high spin that exists without a protein shell that prevents against ROS-mediated oxidative damage (Whitnall et al. 2012). Hence, this precipitation of iron in the mitochondria potentiates oxidative stress, and as such, this warrants for the targeted removal of redox-active MIT iron.

A specialised group of low molecular weight, lipophilic ligands of the pyridoxal isonicotinoyl hydrazone (PIH) class has been examined to target iron accumulation in the mitochondria (Richardson and Ponka 1998, Richardson et al. 2001). In terms of mechanism, PIH is able to permeate biological membranes, including the mitochondrion, and effectively chelate the iron accumulated in the reticulocytic mitochondria after inhibition of MIT haem synthesis (Ponka et al. 1982). Further studies demonstrated that PIH and several of its analogues were able to effectively remove MIT iron and inhibit oxidative stress (Becker and Richardson 1999, Richardson et al. 2001, Lim et al. 2008). In fact, these latter agents were notably superior to desferrioxamine (DFO)
(Becker and Richardson 1999), which is a clinically used iron chelator used for treating iron overload disease (Richardson 1999b).

Hence, the use of iron chelators is a potential therapeutic strategy for FA. This has been previously assessed in vivo to remove MIT iron-loading in order to reduce cardiac hypertrophy (Whitnall et al. 2008). Notably, the chelation of iron did not reverse the cardiac phenotype caused by frataxin deficiency, nor did it restore frataxin function in MIT iron metabolism (Whitnall et al. 2008). Interestingly, subsequent studies in FA patients have demonstrated that a combination therapy with iron chelator, deferriprone, and the antioxidant, idebenone, was able to improve cardiac hypertrophy while also reducing the presence of iron deposits in the dentate nucleus (Velasco-Sanchez et al. 2010). Furthermore, this combination of treatments stabilises neurological dysfunctions in patients (Rustin et al. 1999, Velasco-Sanchez et al. 2010). Therefore, given the current unavailability of frataxin-replacement therapies, these studies have demonstrated the potential of combining iron chelation therapy with antioxidant supplementation to treat FA patients. Thus, this combined therapy holds promise for future research, and extensive animal and clinical studies are required to better evaluate its effectiveness, as it could become a novel therapeutic strategy for the treatment of FA.

6.2.4 The Role of Parp in MIT Dysfunction and the Potential Application of Parp1 Inhibition in the Treatment of FA

Observations from Chapter 4 suggested that the increased activity of Parp could be responsible for depleting cardiac NAD+ levels in the KO mice potentially due to the enhanced consumption of
NAD\(^+\) mediated by Parp. Consequently, this increase in Parp activity could result in the decreased Sirt1 activity in the frataxin-deficient hearts. Present findings are consistent with other studies in which a link was drawn between Parp activation and the development of chronic diseases that exhibits MIT dysfunction, including neuro-degenerative diseases (Kauppinen and Swanson 2007, Martire et al. 2015). In particular, past studies on Alzheimer’s disease and Parkinson’s disease demonstrated that Parp1 plays a significant role in regulating cellular and MIT energetic metabolism in which Parp1 activation leads to NAD\(^+\) depletion (Soane et al. 2007, Alano et al. 2010, Martire et al. 2015). Considering that Sirt1 and Parp1 compete for the same pool of NAD\(^+\) (Canto and Auwerx 2011a, Martire et al. 2015), activation of Parp1 may out-compete Sirt1 for the consumption of NAD\(^+\) and could result in a loss of Sirt1 activity (Canto and Auwerx 2011a, Bai and Canto 2012, Martire et al. 2015). Hence, increased Parp activity affects the regulation of Sirt1 via the depletion of NAD\(^+\), thus it can potentiate MIT dysfunction and bioenergetic deficits in the frataxin-deficient heart.

Interestingly, a number of in vitro and in vivo studies have previously shown that pharmacological inhibition of Parp1 increases NAD\(^+\) content and Sirt1 activity and enhances oxidative metabolism (Bai et al. 2011, Cerutti et al. 2014, Fang et al. 2014). Therefore, Parp1 inhibition is a potential therapeutic strategy to increase NAD\(^+\) availability and activate Sirt1 to regulate MIT function and energy homeostasis (Bai et al. 2011). Another study has assessed the effectiveness of Parp1 inhibition on microglial response in an FA animal model and demonstrated that neurobehavioral impairments were attenuated upon treatment (Shen et al. 2016). This shows that Parp1 inhibitors have promising therapeutic application for FA therapy. However, this avenue of approach has not been thoroughly assessed in the context of the cardiomyopathy in FA.
Hence, it is noteworthy to assess the efficacy of Parp1 inhibitors to restore cardiac energetics and MIT function in the cardiac MCK mice model. Future studies may include in-depth examination of the effects of Parp1 inhibition on the regulation of Sirt1 and Pgc1α on MIT biogenesis. In relation to energy metabolism, ATP production, NAD\(^+\) levels, and the activity of the MIT respiratory chain should also be assessed upon inhibiting Parp1 activation. In addition, since oxidative stress to the mitochondria can trigger Parp1 metabolic activity (Soane et al. 2007), the impact of Parp1 inhibition on ROS generation, endogenous antioxidant levels, and the activation of anti-oxidative response should be considered.

### 6.2.5 Potential Therapy with NR or NMN Supplementation for FA Patients

It is evident that NAD\(^+\) plays an important role in cellular energy homeostasis and that the depletion of NAD\(^+\) would lead to a loss in MIT bioenergetics and energy production (Canto et al. 2015). Studies in *Chapter 5* attempted to restore NAD\(^+\) levels with the novel compound, SNH6, that donates the NAD\(^+\) precursor, NA. Although the treatment with SNH6 did successfully increase NAD\(^+\) levels, the supplementation of NAD\(^+\) was insufficient to restore cardiac energetics, which suggests that the NAD\(^+\) salvage pathway could be dysregulated in the frataxin-deficient heart. Therefore, it is important to consider other metabolic “players” that are involved in the synthesis of NAD\(^+\) that may also contribute to its depletion, and subsequently affect Sirt1 activity and MIT energy metabolism.
Other studies have examined the effectiveness of supplementing NAD\(^+\) intermediates, such as nicotinamide riboside (NR), to boost NAD\(^+\) levels and rescue MIT deficits in different disease models (Karamanlidis et al. 2013, Xu et al. 2015). For instance, NR has been shown to protect murine DRG neurons from axonopathy via the induction of the enzyme, nicotinamide riboside kinase 2 (Nrk2) (Bogan and Brenner 2008). Supplementation of NR also demonstrated potential restoration of cognitive function in an Alzheimer’s mouse model (Karamanlidis et al. 2013), and ameliorate autophagic defects and extended the lifespan of a cardiac mouse model of cytosolic iron-deficiency (Xu et al. 2015). A previous publication from our laboratory identified a pronounced and early induction of Nrk2 in the MCK frataxin KO mouse (Huang et al. 2013). This dramatic increase in Nrk2, in conjunction with the previously described significant deficit of NAD\(^+\) levels in the heart as shown in Chapter 4, suggests a critical need for NR (Xu et al. 2015). As such, NR supplementation could be a potential therapeutic approach in addition to NA administration to rescue MCK mice.

Similarly, studies have found therapeutic benefits with supplementing the NAD\(^+\) precursor, nicotinic mononucleotide (NMN), in which the reconstitution of NAD\(^+\) levels could enhance Sirt1 activation in various disease models (Yoshino et al. 2011, Gomes et al. 2013). In a mouse model of heart failure, NMN supplementation increased NAD\(^+\) levels and was able to normalize NAD\(^+\):NADH ratio with the potential to improve MIT function (Karamanlidis et al. 2013). In particular, another study has assessed the therapeutic efficacy of NMN supplementation on the cardiomyopathy of FA and demonstrated cardio-protection in a Sirt3 dependent-manner, which improved energy metabolism (Martin et al. 2017). Therefore, the strategy to supplement NMN to
bolster NAD$^+$ content may provide direct biochemical rescue and boost MIT dynamics to rescue MIT function and potentially alleviate the cardiac pathology in FA.
6.3 Concluding Remarks

The studies of this thesis have expanded our understanding of the molecular mechanisms involved in the dysregulation of antioxidant defence and MIT homeostasis in the pathogenesis of FA. In particular, the direct and indirect roles of Gsk3β and Fyn in the nuclear export and subsequent degradation of Nrf2 were novel findings that elucidated the pathological decrease of Nrf2 in frataxin deficiency. Morphological and molecular examinations of MIT dynamics have also highlighted, for the first time in a cardiac mice model of FA, the important role of MIT dysfunction in the cardiac pathology of this disease. These findings coupled with the present treatment studies with NAC and the novel compound, SNH6, have illuminated the significant relevance of the aforementioned therapeutic avenues for the treatment of FA cardiomyopathy. This necessary and urgent endeavour could benefit from the important findings in this thesis, and hence, encourage the design and development of innovative treatments for FA patients.
CHAPTER SEVEN

References
References


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