# Disruption of mitochondrial redox homeostasis as a cellular signal 

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This dissertation is submitted for the degree of Doctor of Philosophy


Sidney Sussex College

## Thesis declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. It does not exceed the prescribed word limit for the relevant Degree Committee.

## Summary

## Filip Cvetko

## Disruption of mitochondrial redox homeostasis as a cellular signal

Mitochondria are crucial components of eukaryotic cells and exchange signalling molecules, metabolites, proteins and lipids with the rest of the cell. The organelle is key for energy metabolism as they provide most of the cellular ATP through oxidative phosphorylation and regulate intermediate metabolism. Mitochondria are also a major source of reactive oxygen species (ROS), which are by-products of aerobic respiration and recently recognised as important signalling molecules that control various cellular functions. To avoid the potential damaging effects of ROS, mitochondria contain protein antioxidant systems to help maintain thiol homeostasis. Mitochondria are emerging as an important redox signalling node and are involved in a myriad of signalling pathways, which have a redox component, either through a response to a particular ROS or the shift of the redox state of a responsive group. It is not surprising that mitochondria are therefore heavily regulated by retrograde signalling of the master regulator of cellular antioxidant defence, nuclear factor erythroid-derived 2-related factor 2 (Nrf2). Until now it has not been possible to disentangle the overlapping effects of mitochondrial ROS signalling compared to a redox signal stemming from disruption of mitochondrial thiol homeostasis. Furthermore, it is important to distinguish between disturbing the cytosolic and mitochondrial protein antioxidant systems. I characterised the effects of mitochondrial thiol homeostasis disruption on mitochondrial physiology with MitoCDNB, showing mitochondrial fission. I found that selective disruption of the mitochondrial glutathione pool and inhibition of its thioredoxin system led to Nrf2 activation, while using MitoPQ to enhance production of mitochondrial superoxide and hydrogen peroxide alone did not. To
further our understanding of how mitochondrial redox homeostasis is sensed in the cytoplasm and signalled to the nucleus I used an RNAseq approach to investigate the intricacies of early mitochondrial retrograde signalling.

This thesis is dedicated to my Mum and Dad

Ever Tried.
Ever Failed.
No Matter.
Try Again.
Fail Again.
Fail Better.

Samuel Beckett

## Abstract

Mitochondria are crucial components of eukaryotic cells and exchange signalling molecules, metabolites, proteins and lipids with the rest of the cell. The organelle is key for energy metabolism as they provide most of the cellular ATP through oxidative phosphorylation and regulate intermediate metabolism. Mitochondria are also a major source of reactive oxygen species (ROS), which are by-products of aerobic respiration and recently recognised as important signalling molecules that control various cellular functions. To avoid the potential damaging effects of ROS, mitochondria contain protein antioxidant systems to help maintain thiol homeostasis. Mitochondria are emerging as an important redox signalling node and are involved in a myriad of signalling pathways, which have a redox component, either through a response to a particular ROS or the shift of the redox state of a responsive group. It is not surprising that mitochondria are therefore heavily regulated by retrograde signalling of the master regulator of cellular antioxidant defence, nuclear factor erythroid-derived 2-related factor 2 (Nrf2). Until now it has not been possible to disentangle the overlapping effects of mitochondrial ROS signalling compared to a redox signal stemming from disruption of mitochondrial thiol homeostasis. Furthermore, it is important to distinguish between disturbing the cytosolic and mitochondrial protein antioxidant systems. I characterised the effects of mitochondrial thiol homeostasis disruption on mitochondrial physiology with MitoCDNB, showing mitochondrial fission. I found that selective disruption of the mitochondrial glutathione pool and inhibition of its thioredoxin system led to Nrf2 activation, while using MitoPQ to enhance production of mitochondrial superoxide and hydrogen peroxide alone did not. To further our understanding of how mitochondrial redox homeostasis is sensed in the cytoplasm and signalled to the nucleus I used an RNAseq approach to investigate the intricacies of early mitochondrial retrograde signalling.

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## Abbreviations

All abbreviations, unless listed, are as described in the "Instructions to Authors" of the Biochemical Journal (http://www.biochemj.org).

| $\Delta \Psi$ | membrane potential |
| :---: | :---: |
| $\Delta \mathrm{p}$ | proton motive force |
| $\Delta \mathrm{pH}$ | pH gradient |
| AAC | ADP/ATP carrier |
| ABC | ATP binding cassette |
| ACC | acetyl-coA carboxylase |
| ADP | adenosine-5'diphoshpate |
| AMP | adenosine-5'monophosphate |
| AMPK | AMP-activated protein kinase |
| Apaf1 | apoptotic protease activating factor 1 |
| ARE | antioxidant response element |
| ASK1 | apoptosis signal-regulating kinase 1 |
| ATF4 | activating transcription factor 4 |
| ATP | adenosine-5'triphosphate |
| BAX | Bcl-2-associated X protein |
| BCA | bicinchoninic acid |
| BCL2 | B-cell lymphoma 2 |
| BSA | bovine serum albumin |
| bZIP | basic leucine zipper domain |
| CDNB | 1-chloro-(2,4-dinitro)-benzene |
| CL | cardiolipin |


| CoA | coenzyme A |
| :---: | :---: |
| complex I | NADH:ubiquinone oxidoreductase |
| complex II | succinate dehydrogenase |
| complex III | ubiquinone:cytochrome c oxidoreductase |
| complex IV | cytochrome c oxidase |
| CREB | cAMP response element binding protein |
| Cyt c | cytochrome c |
| DAO | D-amino acid oxidase |
| DELE1 | DAP3 binding cell death enhancer 1 |
| DME | drug metabolising enzyme |
| DMEM | Dulbecco's modified eagle medium |
| DRP1 | dynamin related protein 1 |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethyleneglycoltetraacetic acid |
| EMT | apithelial mesenchymal transition |
| ER | endoplasmic reticulum |
| ETC | electron transport chain |
| FAD | flavin adenine dinucleotide (oxidised) |
| $\mathrm{FADH}_{2}$ | flavin adenine dinucleotide (reduced) |
| FBS | foetal bovine serum |
| FCCP | carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone |
| FeS | iron sulphur centre |
| FMN | flavin mononucleotide |
| FOXO | forkhead box 0 |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GCL | $\gamma$-glutamyl cysteine ligase |
| GCN2 | general control nonderepressible 2 |
| GGT | $\gamma$-glutamyl trasnpeptidase |
| GPx | glutathione peroxiredoxin |
| GR | glutathione reductase |
| Grx | glutaredoxin |

glutathione synthetase
GSH glutathione (reduced)
GSSG glutathione (oxidised)
GST glutathione S-transferase
$\mathrm{H}_{2} \mathrm{O}_{2} \quad$ hydrogen peroxide
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIF-1 $\alpha \quad$ hypoxia inducible factor $1 \alpha$
HNE 4-hydroxynonenal
HRI haem-regulated inhibitor
HRP horseradish peroxidase
IMM inter mitochondrial membrane
IMS intermembrane space
IR ischaemia-reperfusion
IRF interferon regulatory factor
ISR integrated stress response
JNK c-Jun N-terminal kinases
KEAP1 kelch-like ECH-associated protein 1
LC3 microtubule associated protein 1 light chain 3 alpha
LPS lipopolysaccharide
MAF musculoaponeurotic fibrosarcoma
MAPK Mitogen activated protein kinase
MEF mouse embryonic fibroblasts
MFN mitofusin
MnSOD manganese superoxide dismutase
MOTS-c mitochondrial ORF of the 12 S rRNA type-c
MPP mitochondria penetrating peptides
MPTP mitochondrial permeability transition pore
mTORC mammalian target of rapamycin complex
mtDNA mitochondrial DNA
NADH nicotinamide adenine dinucleotide
NADPH nicotinamide adenine dinucleotide phosphate

| NEM | N -ethylmaleimide |
| :---: | :---: |
| NFкB | nuclear factor kappa-light -chain-enhancer of activated B cells |
| NQO1 | NAD(P)H quinone dehydrogenase 1 |
| NRF1 | nuclear respiratory factor 1 |
| Nrf2 | nuclear factor erythroid 2-related factor 2 |
| Nrx | nucleoredoxin |
| $\mathrm{O}_{2}{ }^{\text {- }}$ | superoxide radical |
| $\mathrm{OH}^{\bullet}$ | hydroxyl radical |
| OMM | outer mitochondrial membrane |
| OPA1 | optic atrophy gene 1 |
| OXPHOS | oxidative phosphorylation |
| PBS | phosphate buffered saline |
| PGAM5 | Phosphoglycerate mutase 5 |
| PGC1a | peroxisome proliferator-activated receptor gamma coactivator 1-a |
| PHD | prolyl hydroxylases |
| Pi | phosphate |
| PLA2y | phospholipase A2-gamma |
| Prx | peroxiredoxin |
| PTEN | phosphatase and tensin homolog deleted on chromosome ten |
| PTM | post-translational modification |
| PTP | protein tyrosine phosphatase |
| pVHL | Von Hippel-Lindau tumour suppressor protein |
| Q | ubiquinone |
| Q $\mathrm{H}_{2}$ | ubiquinol |
| RET | reverse electron transport |
| RHM | rat heart mitochondria |
| RLM | rat liver mitochondria |
| ROS | reactive oxygen species |
| RP-HPLC | reverse phase high performance liquid chromatography |
| SDH | succinate dehydrogenase |
| SOD | superoxide dismutase |


| TBS | tris buffered saline |
| :--- | :--- |
| TGF- $\beta$ | transforming growth factor beta |
| TIM | translocase of the inner mitochondrial membrane |
| TMRM | tetramethylrhodamine |
| TNF- $\alpha$ | tumor necrosis factor-alpha |
| TOM | translocase of the outer mitochondrial membrane |
| TPB | triphenyl borate |
| TPMP | methyltriphenylphosphonium |
| TPP | triphenyl phosphonium |
| Trx | thioredoxin |
| TrxR | thioredoxin reductase |
| UPR | unfolded protein response |
| VDAC | voltage dependent anion channel |
| WB | Born energy |

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## Chapter 1. Introduction

### 1.1. General Introduction

Mitochondria played a crucial role in the development of eukaryotic life. The origins of mitochondria come from the incorporation of an $\alpha$-proteobacterium ancestor into a host cell, but the mechanism of this incorporation is under debate, with two main schools of thought regarding the endosymbiotic event: archeozoan or symbiogenesis scenarios (Gray, 2012; Lane \& Martin, 2010; Roger et al., 2017). Archeozoan scenarios involve a process where an amitochondriate eukaryotic host cell engulfs an $\alpha$-proteobacteria, whereas symbiogenesis describes a prokaryotic host cell, Archea, with subsequent compartmentalisation and development of the nucleus (Gray, 2012; Lane \& Martin, 2010). Since the incorporation of the proto-mitochondrion, genes have transferred to the host cell genome, subsequently increasing nuclear complexity and abolishing organelle autonomy (Gray, 2012; Lane \& Martin, 2010; Roger et al., 2017). An advantage of eukaryotes possessing mitochondria is the ability of sustained protein translation without bioenergetic penalty. It is thought that this led to the divergence in complexity between prokaryotes and eukaryotes, with the energy per gene expressed for eukaryotes allowing a more diverse genome (Lane \& Martin, 2010). A further benefit of the endosymbiotic relationship is the existence of the mitochondrial genome, as it provides the ability of changing gene expression, in response to the metabolic demand (Lane \& Martin, 2010; Picard et al., 2016). Consequently, many eukaryotes rely on functional mitochondria to provide an efficient means of energy production for ever changing metabolic needs (Gray, 2012; Lane \& Martin, 2010; Roger et al., 2017). Introduction of mitochondria to a cell remains a crucial event as they are vital for complex eukaryotic life, and therefore a deep understanding of mitochondrial biology is crucial.

Mitochondria are essential components of eukaryotic cells, but they do not act as solely independent isolated organelles, as they exchange molecules, from ions, small metabolites to proteins and lipids with the rest of the cell (Mottis et al., 2019). The organelle is the key for energy metabolism within the cell as they produce most of the cellular ATP through
oxidative phosphorylation (OXPHOS) and are essential for regulating intermediate metabolism. Both as a consequence of normal electron transport and during mitochondrial dysfunction, electrons can escape from the electron transport chain (ETC) to induce formation of superoxide anions by one-electron reduction of oxygen. Mitochondria are believed to be the major source of intracellular reactive oxygen species (ROS) generation.

To avoid the potential damaging effects of ROS, mitochondria contain a number of protein antioxidant systems, such as the glutathione, thioredoxin and peroxiredoxin systems, which help maintain homeostasis in the organelle through the regeneration of oxidized biomolecules and direct scavenging of the oxidizing radical (Ruszkiewicz \& Albrecht, 2015). Knowledge of the mitochondrial antioxidant systems is critical to understanding the importance of the cellular defence mechanisms (Murphy, 2012). Furthermore, the defence systems are also linked to a number of pathophysiologies, including cancer, liver disease and neurological disorders (Chang et al., 2004; Colell et al., 1998; Garcia-Ruiz \& FernandezCheca, 2006; Holzerova et al., 2016; Huang et al., 2019; Marí et al., 2013; Ruszkiewicz \& Albrecht, 2015; Zhao et al., 2002).

All of the above, illustrates how mitochondria are an important redox signalling node, as they are central to metabolism, have key roles in apoptosis, calcium homeostasis and oxygen sensing, in addition to the flux of ROS generated by the respiratory chain and other core metabolic machineries within mitochondria (Collins et al., 2012; Finkel, 2011; Murphy, 2009). Redox signalling occurs when a biological system alters in response to a change in the level of a particular reactive oxygen species (ROS) or the shift in the redox state of a responsive group such as a dithiol-disulphide couple (Collins et al., 2012). Although ROS are best known as damaging agents in pathology or the scientific "antihero", a more nuanced view has evolved.

As the mitochondrion is an active site of cellular redox homeostasis and a major source of ROS, it is not surprising that the homeostasis of the organelle can be regulated by a variety of retrograde signalling pathways such as c-Jun N-terminal kinase (JNK), nuclear factor kappa B (NF-кB) and the master regulator of cellular antioxidant defence, nuclear factor erythroid-derived 2-related factor 2, Nrf2. Nrf2 is a well-known transcription factor and a
master regulator of a variety of antioxidant and detoxifying enzymes through binding to the antioxidant response element (ARE) (Itoh et al., 1997; Ma, 2013; Tebay et al., 2015). Nrf2 regulation is mediated through irreversible alkylation of thiols by electrophiles on Kelch-like ECH-associated protein 1 (KEAP1), which subsequently releases Nrf2 from its ubiquitination pathway (Kobayashi et al., 2004).

Nrf2 has a multifaceted role in mitochondrial function (Dinkova-Kostova \& Abramov, 2015; Holmström et al., 2016). Under homeostatic conditions, Nrf2 affects the availability of substrates for respiration and ATP synthesis, mitochondrial membrane potential and fatty acid oxidation (Holmstrom et al., 2013). On the contrary, in stress, activation of Nrf2 counteracts the increased reactive oxygen species production in mitochondria, stimulates mitochondria antioxidant defences (Kasai et al., 2020; Ryoo \& Kwak, 2018) and influences mitochondrial biogenesis (Zhang et al., 2013). Nrf2 deficiency leads to impaired mitochondrial fatty acid oxidation, respiration and ATP production, and its function is suppressed in mitochondria-related disorders (Dinkova-Kostova \& Abramov, 2015). Furthermore, Nrf2 activators support mitochondrial integrity by promoting mitophagy (East et al., 2014) and inhibiting oxidative stress-mediated permeability transition pore (Greco et al., 2011).

In the next sections I outline the general principles of mitochondrial energy metabolism, mitochondrial ROS production and redox signalling, before focusing on Nrf2 - a vital player in cellular redox homeostasis and its relationship with mitochondria.

### 1.2. Mitochondrial Structure and function

Historically, the discovery of the existence of mitochondria dates back to the late 1800s, with Richard Altmann correctly distinguishing them as separate subcellular organelles (Altmann, 1890). By the early 1900s, work by Otto Warburg (Warburg, 1913) and others was crucial to determine the link between the particulate nature of the mitochondria and cellular respiration (Ernster \& Schatz, 1981). The knowledge of mitochondrial function exponentially increased, with structure-linked biochemical characterisation, establishment
of mitochondrial isolation procedures and developments in the field of mitochondrial bioenergetics; these include work on mitochondrial enzyme redox states by Chance and Williams (Chance \& Williams, 1955), as well as the well-known hypothesis from Peter Mitchell on chemiosmotic coupling (Mitchell, 1961). Over the following forty years or so, research has led to a modern understanding of the mitochondrion as an essential organelle in metabolism and a dramatic increase in the knowledge of mitochondrial biology (Ernster \& Schatz, 1981; Pagliarini \& Rutter, 2013). In addition to their fundamental functions, many roles of the 'powerhouse of the cell', particularly as a key player in a multitude of pathologies have been underappreciated until recently (Pagliarini \& Rutter, 2013).

### 1.2.1. Mitochondrial morphology and dynamics

Mitochondria are highly dynamic organelles, present in a range of morphologies and number within cells, dependent on the cellular environment. As they are vital to the homeostasis of cellular metabolism, they need to be highly responsive and adaptable. The structure of mitochondria stems from their prokaryotic origins; they are composed of two distinct phospholipid bilayer membranes, the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM), separated by the intermembrane space (IMS), with the IMM encompassing the central mitochondrial matrix (Figure 1.1) (Frey \& Mannella, 2000). The lipid composition of the IMM and OMM differs, with the IMM, thought to be a relic of their prokaryotic past, resembling prokaryotic membranes with high cardiolipin composition; whereas the OMM mirrors the composition of the endoplasmic reticulum (ER) membranes (Gray, 2012; Paradies et al., 2014).

The OMM is approximately 7 nm thick, has an equal protein:phospholipid ratio and separates the mitochondrion from the cellular cytosol (Comte et al., 1976; Frey \& Mannella, 2000; Perkins et al., 1997). The OMM is porous and freely traversed by ions and small molecules due to pore-forming membrane proteins (porins), such as the voltagedependent anion channel (VDAC), which allows access of molecules less than 6.8 kDa into the IMS (Shoshan-Barmatz et al., 2010). Due to its porosity, the IMS ion and sugar concentrations resemble the cytoplasm and there is no established membrane potential
across the outer membrane. Larger proteins must be imported by import machinery such as translocase of the outer mitochondrial membrane complex (TOM) (Wiedemann et al., 2004). Furthermore, OMM plays a key role in mitochondrial fusion and fission, cellular death, organelle biogenesis and mitochondrial quality control by mitophagy, among others (Shoshan-Barmatz et al., 2010; Walther \& Rapaport, 2009).

The IMM has a different composition from the OMM, with a much higher protein:phospholipid ratio as well as containing cardiolipin in place of cholesterol (Barbot \& Meinecke, 2016; Gray, 2012). By contrast, the inner membrane is a tight diffusion barrier to all ions and molecules and hence transport across the IMM is much more tightly regulated than the OMM (Buzhynsky et al., 2007). As a result of its ion selectivity, an electrochemical membrane potential of $\sim-180 \mathrm{mV}$ builds up across the inner mitochondrial membrane (Kühlbrandt, 2015). The IMM is also the location of the enzymes responsible for the main mitochondrial function of energy production, OXPHOS and ATP production (respiratory complexes I, II, III, IV and the $\mathrm{F}_{1} \mathrm{Fo}_{0}-A T P$ synthase) and acts as a pool of the membrane soluble electron carrier, ubiquinone (Q) (Barbot \& Meinecke, 2016; Buzhynsky et al., 2007; Kühlbrandt, 2015). Other important components in the IMM are the protein import machinery (TIM complex), as well as other ion and substrate carriers essential for the organelle's function (Kunji, 2004; Kunji et al., 2020; A. Robinson et al., 2008; Wiedemann \& Pfanner, 2017). Unlike the OMM, the IMM forms invaginations, called cristae, increasing its surface area and are predominantly the location of fully assembled complexes of the transport chain and the ATP synthase (Frey \& Mannella, 2000; Kühlbrandt, 2015). Dimers of $\mathrm{F}_{1} \mathrm{~F}_{\mathrm{o}}$-ATP synthase self-associate into rows and induce the formation of curved cristae structures (Kühlbrandt, 2015). The latter are also maintained by an array of proteins, organised into the mitochondrial intermembrane space bridging (MIB) complex, creating a scaffold between the inner and outer mitochondrial membranes (Kühlbrandt, 2015). Both the increased surface area and the proximity of electron transport chain (ETC) enzymes to ATP synthase create a suitable environment for ATP production. The intermembrane space (IMS), an approximately 20 nm gap between OMM and IMM, is the equivalent of the periplasm in the bacterial ancestors of mitochondria (Kühlbrandt, 2015). The IMS has multiple roles, including regulation of mitochondrial phospholipid metabolism (Osman et al., 2009), transport of proteins and metal ions (Terziyska et al.,
2005). In addition, a number of apoptotic factors reside in the IMS, such as cytochrome c (Cyt c), a component of the ETC, involved in shuttling electrons to complex IV (Wiedemann \& Pfanner, 2017). Release of Cyt c from the IMS leads to the activation of the intrinsic apoptotic pathway (Goldstein et al., 2000). Moreover, the IMS is also involved in the detoxification of mitochondrial ROS, through the action of cytosolic thioredoxin 1 (Trx1) (Dickson-Murray et al., 2021), glutathione peroxidase 3 (Gpx3) (Kritsiligkou et al., 2017), Glutaredoxin 1 (Grx1) (Pai et al., 2007) and $\mathrm{Cu} / \mathrm{Zn}$ Superoxide dismutase (SOD1). The latter dismutates superoxide to hydrogen peroxide and molecular oxygen, providing a line of defence against oxidative damage (Kawamata \& Manfredi, 2010).

The innermost compartment, surrounded by the IMM, is the mitochondrial matrix. It is protein dense (approximately $500 \mathrm{mg} / \mathrm{ml}$ ) and contains numerous proteins involved in various anabolic and catabolic processes, including the TCA cycle, lipid and amino acid metabolism (Frey \& Mannella, 2000; Kühlbrandt, 2015). It is topologically equivalent of the bacterial cytoplasm and distinguishes from the eukaryotic cytoplasm by its higher matrix pH (7.8-8) (Llopis et al., 1998). The latter helps creates the trans-membrane electrochemical gradient that drives ATP synthesis. The mitochondrial matrix also houses hundreds of copies of mitochondrial DNA (mtDNA), which is a circular, 16.6 kb doublestranded DNA molecule, compacted by the mitochondrial transcription factor TFAM (Kukat et al., 2015). MtDNA encodes 2 ribosomal RNAs, 22 transfer RNAs and 13 polypeptides ( 7 complex I subunits, 1 Complex III subunit, 3 complex IV subunits and $2 \mathrm{~F}_{1} \mathrm{~F}_{0}$-ATP synthase subunits) (Gustafsson et al., 2016). Complex II is the only respiratory complex that is completely nuclear encoded (Bezawork-Geleta et al., 2017). As there are considered to be 1,200 different protein types in mitochondria, it shows the importance of crosstalk and orchestration needed between the nuclear and mitochondrial genomes in order to create complete and fully functioning respiratory complexes and mitochondria (Stefely et al., 2016).

Typically, mitochondria exist within the cell as a dynamic network, constantly undergoing remodelling during fission and fusion processes to respond to cellular needs (Braschi \& McBride, 2010; Hoitzing et al., 2015). Although they are traditionally characterised as capsule-shaped from electron microscopy images, mitochondria exist as a threadlike
network throughout the cell (Friedman \& Nunnari, 2014). Fusion events are coordinated by optic atrophy gene 1 (OPA1) and Mitofusin 1 and 2 (MFN1 and MFN2) in a GTP hydrolysis-dependent manner (Franco et al., 2016; Wai \& Langer, 2016). OMM fusion is orchestrated by oligomeric complexes of MFN1 and MFN2, whereas IMM fusion is catalysed by OPA1 (Wai \& Langer, 2016). Mitochondrial fission is thought to occur as a mechanism of quality control. Outer membrane fission is performed by dynamin-related protein 1 (DRP1), which translocates from the cytosol to the OMM, where it oligomerizes into ring-like structures (Wai \& Langer, 2016). Many pro-fission proteins serve as recruitment factors for DRP1, which provides the platform for membrane constriction and scission (Mears et al., 2011). The resulting fragmented mitochondria have different properties and can proceed to fuse with other mitochondria or continue down the degradation pathway (Figure 1.2).


Figure 1.1. Structure of the mitochondria. A) Cartoon representation of mitochondria with labelled features. Image adapted from Lodish et al., 2000. B) A section through the 3D tomogram of the mitochondrion, outlining the sub-organelle structures. Image from Frey \& Mannella, 2000. C) Segmented 3D volumes of mitochondria with closely stacked, roughly parallel cristae (blue). The outer membrane is yellowish grey. Image adapted from Brandt et al., 2017.

## Spectrum of mitochondrial morphologies



Figure 1.2. Mitochondrial morphology. The morphology of mitochondria can vary significantly over a wide range spanning from hyperfused (green) or fragmented (red) mitochondria. Figure adapted from (Wai \& Langer, 2016).

### 1.2.2. Oxidative phosphorylation

Aerobic metabolism consists of many regulated pathways feeding in at various levels building a highly complex metabolic system. All of this enables the efficient production of the cellular energy currency, ATP, suitable for flexible demand (Sazanov, 2015). Oxidative phosphorylation of carbohydrates is usually preceded by glycolysis, a series of oxygenindependent enzymatic reactions, converting glucose to pyruvate and producing a net amount of 2 ATP and 2 NADH molecules. Glycolysis provides a mechanism of ATP production by substrate-level phosphorylation, without the need of oxygen availability (Stettner \& Segrè, 2013). Pyruvate is transported into the mitochondria and provides a source of reduced substrate for the TCA cycle (Nagampalli et al., 2018). Pyruvate dehydrogenase decarboxylates pyruvate and couples it to Coenzyme $A(C o A)$, producing acetyl-CoA, NADH and $\mathrm{CO}_{2}$ (Figure 1.3).

Mitochondrial acetyl-CoA is also formed from $\beta$-oxidation of fatty acids and the breakdown of amino acids, creating a pool of acetyl-CoA (Houten \& Wanders, 2010). Although betaoxidation occurs in the mitochondrial matrix, free fatty acids from the cytosol cannot
traverse the IMM and are therefore activated to fatty acyl-CoA before being transported into the mitochondria with carnitine palmitoyl transferase I and II, as well as translocase enzymes. Beta oxidation consists of four steps, where mitochondrial acyl-CoA is dehydrated by Acyl-CoA dehydrogenase, to trans- $\Delta 2$-enoyl-CoA, producing FADH ${ }_{2}$ in the process. Next, trans- $\Delta 2$-enoyl-CoA undergoes hydration and oxidation, producing 3-ketoacyl-CoA and NADH. Finally, beta-ketoacyl-CoA thiolase inserts another CoA molecule and thiolysis occurs producing a molecule of acetyl-CoA and a fatty acyl-CoA shortened by two carbons (Figure 1.3) (Röhrig \& Schulze, 2016).

The mitochondrial pool of acetyl-CoA is important to sustain the TCA cycle in the matrix (Houten \& Wanders, 2010). Acetyl-CoA enters the TCA cycle through citrate synthase catalysing the condensation of acetyl-CoA with oxaloacetate to form citrate, which is subsequently isomerised to D-isocitrate by aconitase (Figure 1.3). D-isocitrate is oxidized by isocitrate dehydrogenase to 2 -oxoglutarate ( $\alpha$-ketoglutarate), which is coupled to the production of NADH and $\mathrm{CO}_{2}$. 2-oxoglutarate is further oxidatively decarboxylated by 2oxoglutarate dehydrogenase to produce succinyl-CoA, NADH and $\mathrm{CO}_{2}$. Succinyl-CoA is hydrolysed by succinyl-CoA synthetase to succinate, coupled to GTP production. Succinate is subsequently oxidised by succinate dehydrogenase (SDH, Complex II in ETC), producing fumarate and FADH2. Fumarate is hydrated to malate by fumarate hydratase and malate is further oxidised by malate dehydrogenase to recover oxaloacetate and produce one molecule of NADH. One turn of the TCA cycle leads to the production of three NADH, one GTP, one $\mathrm{FADH}_{2}$ and two $\mathrm{CO}_{2}$ molecules. The generation of reduced equivalents underlies the higher efficiency of ATP production in aerobic conditions compared to glycolysis alone and it can be stipulated how the energetic advantage may have led to the eukaryotic development (Lane \& Martin, 2010).

A


Figure 1.3. Sourcing electrons for the electron transport chain. A) Schematic of the TCA cycle. The TCA cycle takes acetyl-CoA from sources such as glycolysis and beta- oxidation, through a series of redox reactions with electrons transferred to cofactors NAD+ and FAD. Substrate level phosphorylation also occurs, with the formation of GTP. B) Beta-oxidation of fatty acids. Fatty acids are coupled to COA and transported into mitochondria. They are subsequently oxidised, with the release of acetyl-CoA and an acyl-CoA, 2 carbons shorter in length.

In catabolic processes, carbohydrates, lipids and amino acids act as sources of energy and are broken down, with their electrons passed to the corresponding electron carriers in order to reduce either $\mathrm{NAD}^{+}$or FAD. These electron carriers are subsequently oxidised by complexes of the ETC, with increasing reduction potential of the downstream complexes leading to electrons being passed along the ETC (Sazanov, 2015). The free energy released as a result of electrons flowing down the ETC is converted by complexes I, III and IV to proton translocation across the $I M M$, into the $I M S$, generating a $\Delta \mathrm{p}$. In addition to the respiratory complexes, electron carriers, such as Q and Cyt c , are crucial in facilitating the electron movement to its terminal acceptor, oxygen, resulting in the production of water. Oxygen is indeed important in aerobic respiration as its loss disrupts electron flow and impairs ETC activity. The $\Delta \mathrm{p}$ generated through the ETC enables protons to flow back into
the matrix through the $\mathrm{F}_{1} \mathrm{~F}_{0}$-ATP synthase, which transduces the energy into ADP phosphorylation into ATP (Sazanov, 2015).

The IMM is critical for mitochondrial function as it contains the four complexes of ETC and the ATP synthase (Figure 1.4), in addition to membrane associated ETC proteins. Together, these protein complexes are responsible for ATP production by oxidative phosphorylation; coupling the oxidation of reduced substrates obtained from ingestion of food, to the production of chemical energy, ATP (Sazanov, 2015; Watt et al., 2010). The driving force for ATP synthesis, proton motive force $(\Delta p)$, is generated by the proton pumping across IMM by the respiratory chain complexes. The chemiosmotic theory, development of a $\Delta \mathrm{p}$ across a semi-permeable membrane and coupling it to produce ATP, was first proposed by Peter Mitchell (Mitchell, 1961). The $\Delta \mathrm{p}$ is composed of the membrane potential $(\Delta \psi)$, the electrical potential difference across the membrane due to the movement of positive charges and the pH gradient $(\Delta \mathrm{pH})$ from the loss of protons from the matrix (Mitchell \& Moyle, 1969). Equation 1.1 describes $\Delta p$ (in $V$ ) across the $I M M ; R=$ universal gas constant, $\mathrm{T}=$ absolute temperature and $\mathrm{F}=$ Faraday's constant.

$$
\Delta p=\Delta \psi-(2.3 R T / F) \Delta p H
$$



Figure 1.4. Mitochondrial respiratory chain. Electrons are fed into the respiratory chain by NADH onto complex I or via the FADH2 on SDH to $Q$. The electrons are transferred to oxygen via complex III, Cyt c and complex IV. Electron transfer enables proton pumping by complexes I, III and IV, generating $\Delta p$ across the $I M M$. The $\Delta p$ is utilised by the $F_{1} F_{o}$ ATP synthase to synthesise ATP from ADP and Pi. Figure adapted from (Chouchani et al., 2016).

NADH and $\mathrm{FADH}_{2}$ are the precursors for electron transport chain function and ATP production through oxidative phosphorylation. During glycolysis, cytosolic NADH is produced, but it has to be shuttled to the mitochondria in order for electrons to participate in the ETC (Sazanov, 2015). The IMM is impermeable to NADH, hence the electrons are carried via the malate-aspartate shuttle, with NADH reducing the cytosolic oxaloacetate to malate, which in turn can traverse the IMM (M. Lu et al., 2008). The NAD+/NADH couple has a standard midpoint reduction potential of -320 mV , indicating a reducing species, which favours electron donation. NADH is oxidised by complex I (NADH:ubiquinone oxidoreductase) of the ETC (Agip et al., 2018; Baradaran et al., 2013; Vinothkumar et al., 2014; J. Zhu et al., 2016). Mammalian complex I contains 45 subunits, 14 core catalytic subunits and 31 supernumerary subunits, required for a number of roles, including stabilising the complex (Agip et al., 2018; J. Zhu et al., 2016). Complex I oxidises NADH
through a flavin mononucleotide (FMN), located in the hydrophilic arm of the complex, with the electrons subsequently flowing down a series of seven iron-sulphur (FeS) clusters within the hydrophilic domain (Figure 1.5). The terminal FeS cluster, N2, has the least negative reduction potential, leading to a favourable electron transfer and reoxidation of FMN. The electrons from $N 2$ are passed onto $Q$, reducing it to ubiquinol $\left(\mathrm{QH}_{2}\right)$, together with matrix- derived protons. The free energy released from NADH oxidation to reduction of $Q$ is coupled to the pumping of 4 protons across the IMM, by complex $I$, with the exact mechanism of translocation remaining elusive (Galkin et al., 2006).
$\mathrm{FADH}_{2}$ contributes 'less energetic' electrons ( $\mathrm{E}=0 \mathrm{mV}$ ) to the ETC through succinate oxidation by SDH, electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO; beta oxidation) and glycerol-3-phosphate dehydrogenase (Sazanov, 2015). SDH provides a direct link between the TCA cycle and ETC, as electrons from succinate reduce FAD to FADH 2 , with subsequent reduction of $Q$ after electron movement through the FeS clusters of SDH (F. Sun et al., 2005). Other electrons from $\mathrm{FADH}_{2}$ sources reduce $Q$ directly. The similar reduction potentials of $Q$ and $\mathrm{FADH}_{2}$ couples correspond to no proton translocation across the IMM (Sazanov, 2015).

Electrons from $\mathrm{QH}_{2}$ are next transferred to Cyt $c$, through complex III (Cytochrome bc1 complex), via a Q-cycle mechanism (Mitchell, 1975; Sazanov, 2015). One molecule QH 2 binds to the $\mathrm{Q}_{0}$ site in Complex III, with one electron passed on to the FeS cluster of the Rieske protein and subsequent movement to cytochrome $c_{1}$ and then $C_{t} c$ in the IMS. The second electron reduces $c y t b_{L}$ haem, then cyt $b_{H}$ haem and re-reduce a $Q$ molecule, in the $\mathrm{Q}_{i}$ site, into a semiquinone radical (Figure 1.5). This process is repeated with another $\mathrm{QH}_{2}$ molecule transferring one electron to Cyt $c$ and the second electron through $c y t b_{L}$ and $b_{H}$ onto the ubisemiquinone radical. The latter together with two protons from the matrix rereduce Q to $\mathrm{QH}_{2}$, which re-enters the Q pool. During the two cycles of $\mathrm{QH}_{2}$ oxidation, the protons are transferred to the IMS, contributing to the $\Delta \mathrm{p}$ in a directly coupled manner, unlike in complex I (Moser et al., 2006).

The final step of the ETC couples the transfer of electrons from Cyt c to oxygen, producing $\mathrm{H}_{2} \mathrm{O}$, via complex IV (Cytochrome c oxidase) (Braymer \& Lill, 2017; Sazanov, 2015).

Electrons flow, one at a time, from Cyt c to CuA in Complex IV and subsequently onto haem $a_{3}-C u B$, via haem a. Oxygen, bound to haem $a^{3}$, is reduced and together with matrix protons generates $\mathrm{H}_{2} \mathrm{O}$. During this process, 2 protons are pumped across the IMM per 2 electrons in a directly coupled manner, again adding to the generation of $\Delta \mathrm{p}$.


Figure 1.5. Mechanisms of electron transfer through ETC enzyme complexes. A) Structure of murine complex I, with modelling of the 7 FeS clusters, together with the FMN and $Q$-sites. B) Schematic of the $Q$ cycle mechanism in complex III and electron transfer to oxygen by complex IV. Figure adapted from (Agip et al., 2018; Sazanov, 2015).

The generated $\Delta \mathrm{p}$ is converted to ATP production by $\mathrm{F}_{1} \mathrm{~F}_{\mathrm{o}}-$ ATP synthase (Watt et al., 2010). The latter binds ADP and inorganic phosphate $\left(\mathrm{P}_{\mathrm{i}}\right)$ in the $\beta$-subunit of the catalytic domain, which is situated in the mitochondrial matrix. The $\beta$-subunits have several conformational states, which promote either ADP and $P_{i}$ binding, ATP synthesis or ATP release. As the central stalk rotates for a full 360 -degrees, each of the three $\beta$-subunits move through all the conformational changes and 3 ATP molecules are released. As mentioned before, the driving force originates from the generated $\Delta \mathrm{p}$, more specifically from the movement of protons from the IMS to the mitochondrial matrix. It is thought that the IMS protons protonate a glutamate residue on the c ring subunits of the ATP synthase, which subsequently increases hydrophobicity of said residue and Brownian motion pushes the
residue to a more hydrophobic environment. The consequence of this movement is another c subunit glutamate free for protonation. This process continues, movement from the c ring is translated into the central stalk and pushes for conformational changes in the $\beta$-subunits and leads to ATP formation and release. At the same time the protonated c subunits move into an environment where protons are released into the mitochondrial matrix. In mammals, the c ring consists of 8 c subunits, meaning that 8 protons are needed for a 360-degree rotation and the synthesis of 3 ATP molecules, or in other words 2.7 protons are required per ATP molecule synthesised by the $\mathrm{F}_{1} \mathrm{~F}_{\mathrm{o}}$-ATP synthase (Watt et al., 2010). The synthesised ATP is transported out of mitochondria by the ADP/ATP carrier (AAC), which works by an exchange mechanism with ADP (Kunji et al., 2016). Mitochondrial phosphate carrier is responsible for the electroneutral, co-transport of Pi and a proton into the mitochondria, meaning that the synthesis of 3 ATP molecules actually require 11 protons (Kunji et al., 2016; Watt et al., 2010).


Figure 1.6. ATP synthase mechanism. A) Schematic view of ATP synthase. The solvent exposed $F_{1}$ unit consists of subunits $\alpha_{3} \beta_{3} \gamma \sigma \varepsilon$; the membrane $F_{0}$ unit consists of subunits $a b_{2} c_{8}$. B) During ATP synthesis, rotation of the $\gamma$ subunit causes sequential changes in the $\beta$ subunits. A rotation of $120^{\circ}$ changes the $\beta$ subunit that binds ADP and $P_{i}$ to form with tightly bound ATP. The subunit with tightly bound ATP then changes to a form that releases ATP, and the third subunit prepares to bind another ADP and $P_{i}$. Figure adapted from (Boyer, 1999).

### 1.2.3. Additional functions of mitochondria

Mitochondria are central to many other cellular processes not involving oxidative metabolism and ATP synthesis (Duchen \& Szabadkai, 2010; Smith et al., 2012b) (Figure 1.7).

Mitochondria are pivotal in their role of taking up excess cytosolic calcium, its storage and signalling (Paupe \& Prudent, 2018). Optimal cytosolic calcium levels (100 nM) are maintained by uptake into stores within cellular organelles, particularly the ER. ER calcium release raises the cytosolic calcium, but also leads to calcium entry into the mitochondria, as the two organelles are in close proximity and have many contact sites and tethers (Prudent \& McBride, 2017). One mechanism suggests calcium enters the mitochondrial matrix via the mitochondria calcium uniporter (MCU), where it modulates mitochondrial ATP production, enhances TCA cycle dehydrogenases and is involved in cell death (Murgia \& Rizzuto, 2015).

FeS clusters are inorganic cofactors, essential for proper functioning of virtually all biological life (Braymer \& Lill, 2017). The chemical versatility of these clusters is utilized in processes such as metabolic conversions, DNA maintenance, protein translation and electron transfers in redox reactions (Lill et al., 2012). Mitochondria or the evolutionary derived mitosomes appear to be essential for biogenesis of all cellular FeS proteins (Stehling et al., 2014). All FeS protein biogenesis is initiated by the mitochondrial iron-sulfur cluster (ISC) assembly machinery (Paul \& Lill, 2015). Haem groups, found in cytochromes, haemoglobin and myoglobin, are also synthesised and exported from the mitochondria (Ye \& Rouault, 2010).

Mitochondria exert control over cell death and survival (Wang \& Youle, 2009). They play a vital role in apoptosis, with the localisation of the apoptotic machinery such as Bax, Bak, Bcl 2 proteins to the OMM as well as triggered release of Cyt $c$ from mitochondria to the cytosol, where it forms the apoptosome with Apaf1 and pro-caspase 9, leading to caspase activation and finally cell death (Nagata, 2018).

As well as programmed cell death, the central role of mitochondria in ATP production means that damage to the organelle, such as loss of $\Delta \mathrm{p}$, will lead to necrotic cell death, due to lack of ATP and inability to sustain cell ion gradients. During necrosis, following acute damage or trauma, the loss of cellular homeostasis leads to disruptions in ion handling, affecting mitochondrial calcium load. The latter leads to opening of the mitochondrial permeability transition pore (MPTP), a large, non-specific channel within the IMM, which dissipates the proton motive force, ruptures the organelle and leads to cell death being imminent (Kwong \& Molkentin, 2015).


Figure 1.7. Auxiliary roles of mitochondria. As well as their role in OXPHOS, mitochondria have numerous other roles. Mitochondria are an important regulator of cytosolic calcium levels by taking up calcium via the MCU (A). Mitochondria are key in FeS cluster synthesis and iron homeostasis by taking up iron and incorporating it into FeS clusters (B). FeS clusters are incorporated into FeS protein within mitochondrial and extramitochondrial proteins. Mitochondria also play a role in cell death in a regulated manner by apoptosis (C) or by necrosis after acute trauma (D).

### 1.3. Mitochondrial ROS production

Our knowledge of how mitochondria can impact the rest of the cell has increased dramatically over the past few years. In addition to their well-known metabolic roles, mitochondria are now emerging as hubs for a range of signalling processes. One way of how this can occur is through the generation of ROS that radiates from the organelle as a redox signal. Mitochondria are a major source of ROS within the cell (Murphy, 2009). These ROS come primarily from the respiratory chain in the form of superoxide that then goes on to form hydrogen peroxide $\left(\mathrm{H}_{2} \mathrm{O}_{2}\right)$ (Murphy, 2009). The production of mitochondrial ROS and their roles in redox signalling and tissue damage are of interest, because of their relevance to a range of pathologies (Aldosari et al., 2018; Di Meo et al., 2016). The latter is due to the ROS overwhelming the multitude of antioxidant defences within the mitochondrial matrix. While ROS have traditionally been met with a negative outlook and observed as an unwanted consequence of mitochondrial dysfunction, their role in redox signalling in normal mitochondrial physiology are becoming apparent (Holmström \& Finkel, 2014). Additionally, as ROS can infer damage, the mitochondrial and cellular defences towards ROS are crucial.

### 1.3.1. Reactive Oxygen Species

It is important to introduce the notion of ROS, particularly why oxygen can result in the production of said species, as well as the terminology that addresses this field. Firstly, it needs to be explained that oxygen is not the only element that can generate potentially damaging molecules, as reactive chlorine, bromine, sulphur and nitrogen species exist too (Giles \& Jacob, 2002; Halliwell \& Gutteridge, 2015). Each of these groups have their own chemical and biological pathways that can interact (Murphy, 2012). The field of ROS has been the most prominently studied, but the other reactive species have been shown to be important players in cell viability and signalling (Giles \& Jacob, 2002; Halliwell \& Gutteridge, 2015; Martínez \& Andriantsitohaina, 2009; Patel et al., 1999). The term ROS also encompasses some species from the other reactive species group, namely $\mathrm{ONOO}^{-}, \mathrm{HOCl}^{\circ}$ HOBr (Halliwell \& Gutteridge, 2015). Furthermore, all reactive species are also not free
radicals and the term ROS does not simply refer to reactive free radicals but covers both radical and non-radical derivatives of oxygen. In addition, the reactivity of ROS varies considerably between species, for example whilst $\mathrm{O}_{2}{ }^{\circ}$ and hydrogen peroxide $\left(\mathrm{H}_{2} \mathrm{O}_{2}\right)$ are selective in their reactions, a species like the hydroxyl radical ( $(\circ \mathrm{OH})$ will react with nearly all chemical species (Halliwell \& Gutteridge, 2015).

### 1.3.2. Sources of mitochondrial ROS production

The single electron reduction of molecular oxygen leads to the formation of the proximal ROS, superoxide $\left(\mathrm{O}_{2}{ }^{\bullet}\right)$, within mitochondria (Murphy, 2009). This occurs at multiples sites, with some uncertainties remaining as to the main source. The current consensus implicates the FMN site of complex I is the main source of superoxide production within mitochondria (Chouchani et al., 2016; Pryde \& Hirst, 2011), with some limited production from complex III, though the physiological relevance of this is still debated (Bleier \& Dröse, 2013; Murphy, 2009). The production of superoxide by complex III occurs in response to inhibition at the internal Q-binding site by inhibitors such as antimycin A, which is unlikely to be physiological (Murphy, 2009). Whereas superoxide from complex III is released on either side of the IMM, superoxide from complex I is exclusively produced within the matrix. In addition, to the respiratory complexes, superoxide is produced due to high NADH/NAD ${ }^{+}$ ratio from the FAD containing dihydrolipoamide dehydrogenase within the pyruvate and 2-OG dehydrogenase complexes (Bunik \& Sievers, 2002; Starkov et al., 2004). Other enzymes can also interact with the Q pool and lead to superoxide production by complex I, e.g. electron transfer flavoprotein (ETF), which is reduced during fatty acid oxidation and transfers electrons to the Q pool (Murphy, 2009). The IMS can also produce superoxide, where $\alpha$-glycerophosphate dehydrogenase at the outer surface of the IMM resides., but redox signals from the matrix may enable the most direct feedback from the organelle about its metabolic status (Chouchani et al., 2016; Murphy, 2009). Furthermore, superoxide can be produced independently of the NADH or CoQ pools, by relying on enzymes using the NADPH pool. Superoxide can be formed from electrons from the NADPH pool feeding the adrenodoxin reductase/adrenodoxin/cytochrome $\mathrm{P}_{450}$ systems involved in vitamin D and bile acid synthesis (Bansal et al., 2014; Hanukoglu, 2006). However, in
many situations ROS production from these complexes is in response to damage leading to a backup of electrons that then spill out as superoxide (Murphy, 2009). When it comes to ROS as a potential redox signal, it is likely that the ROS generation would be more regulated (Holmström \& Finkel, 2014) and the superoxide generation at complex I as such can be regulated and is probably the best understood potential source of redox signalling from mitochondria (Chouchani et al., 2016; Murphy, 2009).

### 1.3.3. Superoxide production by Complex I

Complex I is the major site of superoxide production within mitochondria, with this occurring when complex I is functioning in the forward or reverse electron transport (RET) (Figure 1.8) (Murphy, 2009; Pryde \& Hirst, 2011). Superoxide production from complex I in the forward direction occurs via electron transfer from FMN to molecular oxygen, when FMN is fully reduced. The extent of superoxide production is governed by the matrix NADH/NAD+ ratio, which sets the proportion of complex I with a fully reduced FMN. Conditions in which the NADH/NAD ${ }^{+}$ratio is elevated occur when there is low ATP demand due to respiratory chain damage (loss of Cyt c, ischaemia, mutations in respiratory complexes), which reduces NADH consumption and increases the proportion of reduced FMN at complex I. Inhibition of complex I with rotenone, complex I Q-site inhibitor, increases superoxide production by this mechanism. Rotenone backs up electrons onto the FMN site, maintaining it in a reduced state. In normal physiology, the extent of superoxide production by this forward direction mechanism is likely to be low, due to a fully functioning respiratory chain and $\mathrm{F}_{1} \mathrm{~F}_{0}-$ ATP synthase, which subsequently leads to the NADH pool being oxidized.
A

| phosphate | adenine |
| :---: | :---: |
| carrier | nucelotide |
| translocase |  |


B


Figure 1.8. Forward and reverse electron transport by complex I. A) In forward electron transfer. NADH donates two electrons to complex I, which transfer along a series of Fe-S clusters to reduce $Q$ to $Q H_{2}$. When the NADH $/ N A D^{+}$ratio is high, or electron transfer to the rest of the ETC is inhibited, electrons can back up and hyper-reduce the FMN, leading to superoxide generation by reduction of $\mathrm{O}_{2}$. The redox energy difference from the transfer of electrons between $N A D H / N A D^{+}$and $Q / Q H_{2}\left(\Delta E_{h}\right)$ drives the pumping of four protons across the IMM to maintain the protonmotive force ( $\Delta p$ ) that drives ATP synthesis. For forward electron transfer, the $\Delta E_{h}$ needs to be greater than the energy required to pump the protons against the $\Delta p: 2 \Delta E_{h}>4 \Delta p$. B) The direction of electron transport can be reversed if the $\Delta p$ is high and/or the $Q$ pool is very reduced so that $4 \Delta p>2 \Delta E_{h}$. Then, electrons can flow backward through complex I and onto the FMN from where they can reduce NAD to NADH or $\mathrm{O}_{2}$ to superoxide. The red arrow in complex I indicates the direction of electron transfer. Adapted from (Chouchani et al., 2016).

As mentioned above, superoxide can also be generated from complex I by reverse electron transfer (RET) (Chouchani et al., 2016; Murphy, 2009; Robb et al., 2018). RET requires a highly reduced $Q$ pool and a high $\Delta p$ across the $I M M$, which forces electrons back from the Q pool onto complex I, reducing FMN. The reduced FMN can then either donate its
electrons to NAD ${ }^{+}$to form NADH or to $\mathrm{O}_{2}$ to form superoxide. The $Q$ pool can be reduced by electron donation by succinate, $\alpha$-glycerophosphate and fatty acid oxidation. A high $\Delta p$ is maintained by proton pumping through complex III and IV, and is enhanced by low rates of ATP synthesis. Superoxide production by RET is inhibited by rotenone, indicating that electrons enter into complex I through the Q-binding site (Chouchani et al., 2016; Murphy, 2009).

RET has been previously thought to be an artefact of in vitro experiments, with little in vivo relevance; but it has been recently identified as a physiological process in mammals (Chouchani et al., 2014; Murphy, 2009; Scialò et al., 2017), with its role implicated in numerous pathologies (Chouchani et al., 2014, 2016), which indicates the potential for therapeutics targeting either $Q$ pool reduction, or $\Delta p$ generation, as strategies to decrease superoxide production.

For example, macrophages alter the way ROS are produced in response to activation by lipopolysaccharide (LPS) (Mills et al., 2016) by interrupting ATP production via oxidative phosphorylation (switching to glycolysis), boosting oxidation of succinate by Complex II and increasing mitochondrial membrane potential, which subsequently triggers the production of RET-ROS (Scialò et al., 2017). These results suggest that RET-ROS signalling is instrumental for metabolic and immune reprogramming of macrophages and thus initiation of an inflammatory response.

Another important physiological phenomenon that involves RET-ROS signalling is the sensing of oxygen levels by the carotid body (CB) (Fernández-Agüera et al., 2015). The chemoreceptor cells of CB are responsible for inducing a response to hypoxia and under normoxia, the CB cells use NADH and Complex I to produce low levels of forward ROS. Under hypoxia, cells undergo a metabolic shift characterized by increased use of succinate, which leads to reverse ROS production from Complex I (Fernández-Agüera et al., 2015). In this model both ROS and an increase in NADH levels are needed to trigger the hypoxia response (Scialò et al., 2017).

The dysregulation of RET-ROS signalling has pathological consequences as oxidative damage and associated cell death occurring during reperfusion, after an ischemic episode in heart of brain is caused by excess of ROS produced via RET (Chouchani et al., 2014). In the course of ischemia, succinate accumulates in tissues and during reperfusion it is rapidly oxidized by Complex II producing RET-ROS, which causes oxidative damage leading to cell death (Chouchani et al., 2014). Inhibition of Complex II with dimethyl malonate or Cl with rotenone protects the heart during ischemia-reperfusion (Chouchani et al., 2014; Prag et al., 2020b). Furthermore, antioxidants that specifically neutralize ROS produced at the lo site within Complex I also alleviate the effects of ischemia-reperfusion in the heart (Brand et al., 2016), suggesting that specific protection against RET-ROS is a viable therapeutic approach in heart attack or ischemic stroke.

### 1.3.4. Mitochondrial superoxide and hydrogen peroxide metabolism

Superoxide produced in the matrix cannot cross the IMM due to the limited membrane permeability of the anion, pKa of approximately 4.8 (Sheng et al., 2014). Nitric oxide (NO) can diffuse into mitochondria and interact with superoxide to generate the highly damaging peroxynitrite species ( $\mathrm{ONOO}^{-}$) (Murphy, 2009). Superoxide can also itself directly inactivate some FeS clusters in proteins, such as aconitase (Gardner, 2002). Generated superoxide is normally short lived, with rapid dismutation to form hydrogen peroxide $\left(\mathrm{H}_{2} \mathrm{O}_{2}\right)$, either spontaneously or enzymatically by the high concentration of matrix manganese superoxide dismutase (MnSOD) (Murphy, 2009) (Figure 1.9). Hydrogen peroxide is freely permeable to the IMM and can therefore translocate from the mitochondrial matrix to other cellular compartments, making it a viable redox signalling molecule. MnSOD is therefore not only a protector against the build-up of superoxide and consequent damage to FeS clusters, but also a key mediator in the release of redox signals (Fukai \& Ushio-Fukai, 2011). $\mathrm{H}_{2} \mathrm{O}_{2}$ has selective reactivity towards cysteine residues, to form sulfenic acid, providing a pathway for direct signal transduction (Reczek \& Chandel, 2015). $\mathrm{H}_{2} \mathrm{O}_{2}$ can also be converted via the Fenton reaction, in the presence of cuprous or ferrous ions, producing the highly reactive hydroxyl radical (`OH) (Murphy, 2012). The latter and peroxynitrite can cause oxidative damage to mitochondrial proteins, DNA and lipids,
thus disrupting normal mitochondrial function (Murphy, 2009). Unsaturated fatty acids in the IMM are susceptible to lipid peroxidation, making the IMM more permeable to protons, uncoupling OXPHOS in the process, as well as disrupting activity of other membrane proteins. Lipid peroxidation can also generate reactive species, such as 4hydroxynonenal, which has been shown to cause damage in both mitochondrial proteins and DNA (Hruszkewycz, 1988). To manage the ROS levels within cells, prevent oxidative damage to DNA, lipids and proteins, a number of matrix non-protein and protein thiols with specialised functions, including peroxiredoxin/thioredoxin and mitochondrial glutathione systems, exist to provide mechanisms of ROS defence, which are explained in greater detail below.


Figure 1.9. Mitochondrial superoxide and hydrogen peroxide metabolism. ROS is produced from a number of sources in mitochondria, including IMM, OMM, and matrix proteins. The proximal ROS, superoxide is rapidly dismutated to $\mathrm{H}_{2} \mathrm{O}_{2}$ by SOD or reacts with other radicals, forming damaging species, such as peroxynitrite (ONOO-). $\mathrm{H}_{2} \mathrm{O}_{2}$ is also able to exit mitochondria and modify thiols on cellular proteins, acting as a redox signa.
$\mathrm{H}_{2} \mathrm{O}_{2}$ can also directly damage mitochondria and cells, hence defence systems including GSH, GPx, Prx and Trx exist, to maintain redox homeostasis. TH = transhydrogenase, ICDH = isocitrate dehydrogenase. Adapted from (Murphy, 2009).

### 1.4. Mitochondrial Thiol Defences

To ensure that the level of ROS is balanced between beneficial signalling and detrimental damage, a multitude of defence mechanisms have evolved to combat the effects of ROS (Halliwell, 2009). Molecules that act against ROS are known as antioxidants. Typically, antioxidants are used by biological systems to combat the levels of oxidants and are found to be more abundant than ROS (Halliwell \& Gutteridge, 2015). The word "antioxidant" is normally used for small molecule antioxidants, such as vitamins C and E , and although they are important in the protection against ROS, arguably even more important is the area of thiol biology (Murphy, 2012). Thiols, which are present in proteins through the cysteine residue, are redox sensitive and act as part of the antioxidant pools and enzymatic reactions to ensure the balance is kept not just within the cell but also within the mitochondria (Murphy, 2012). $\mathrm{H}_{2} \mathrm{O}_{2}$ can oxidise the thiolate anion of reactive protein thiols (pKa is lower than typical thiols) creating sulfenic acids (SOH), causing a conformational change and altering activity of certain proteins (Murphy, 2009; Poole, 2015). When there is abundant $\mathrm{H}_{2} \mathrm{O}_{2}$ present, as in mitochondrial dysfunction, sulfenic acids can be hyperoxidised to sulfinic acids $\left(\mathrm{SO}_{2} \mathrm{H}\right)$ or irreversibly to sulfonic acids $\left(\mathrm{SO}_{3} \mathrm{H}\right)$. There are two mitochondrial specific systems, thioredoxin and glutathione, which represent the forefront of thiol defence mechanisms and are explained in detail below.

### 1.4.1. The thioredoxin system

The mitochondrial thioredoxin ( $\operatorname{Trx}$ ) system mainly revolves around one 12 kDa protein, which possesses a dithiol motif within the active site. The main role of this thioredoxin, Trx2, is the reduction of protein thiols in the mitochondrial matrix (Arnér \& Holmgren, 2000; Murphy, 2012). As discussed above protein thiols are the first line of defence against oxidants and require constant recycling in order to avoid irreversible modifications, such
as oxidation to sulfonic acids. In mitochondria, the reduction of protein thiols occurs by Trx2 acting as a nucleophile to attack the disulphide bond on the protein. This leads to an intermediate disulphide between one thiol of Trx2 and one of the protein thiols, which can then be further reduced by the resolving thiol of Trx2, ending in a disulphide bond on Trx2 and regenerated cysteine residues on the protein (Arnér \& Holmgren, 2000; Murphy, 2012). The now formed disulphide dithiol on Trx2 is reduced by thioredoxin reductase (TxrR2 in mitochondria), which utilises NADPH as a reducing equivalent (Arnér \& Holmgren, 2000) (Figure 1.10). In addition to the Trx2 activity in the mitochondrial matrix, there is also evidence of the cytosolic thioredoxin $\operatorname{Trx} 1$ involved in thiol reduction in the IMS (DicksonMurray et al., 2021).

Additionally, the mitochondrial $\operatorname{Trx}$ system plays a crucial role in the activity of mitochondrial peroxiredoxin (Prx) system. Peroxiredoxins are a family of thiol peroxidases that help to maintain the redox balance of the cell by reducing $\mathrm{H}_{2} \mathrm{O}_{2}$ to $\mathrm{H}_{2} \mathrm{O}$ and are recharged by thioredoxin (Chae et al., 1994; Cox et al., 2009; Murphy, 2012). Different Prx isomers are located in different subcellular compartments, with $\operatorname{Prx} 3$ and 5 are in the mitochondrial matrix. Active Prx monomers form a reversible inter-protein disulphide bridge when oxidised, which results in homodimerization and temporary inactivity. Typically, two cysteine Prxs contain an active thiol that reacts rapidly with $\mathrm{H}_{2} \mathrm{O}_{2}$ to generate sulfenic acid (-SOH), which in turn reacts with an adjacent thiol on another Prx to form a dimer. Trx2 is responsible for the reduction of this disulphide (Murphy, 2012). To highlight the importance of the Trx2 enzyme, approximately $90 \%$ of the $\mathrm{H}_{2} \mathrm{O}_{2}$ clearance within mitochondria is mediated by the $\operatorname{Prx3}$ enzyme, which is required to be reduced by the mitochondrial Trx2 system (Cox et al., 2010; Murphy, 2012). Furthermore, Trx2 is also essential for mediating Prx5 reduction, which is shown to be reactive with ONOO- (Cox et al., 2010; Hanschmann et al., 2010). Trx2 within mitochondria has many functions and acts to protect mitochondria from oxidative stress, as well as regulating the $\mathrm{H}_{2} \mathrm{O}_{2}$ output as a signalling molecule. Mouse compound knockouts of these Trx2 and TrxR2 are embryonically lethal, whereas heterozygous knockouts have no phenotypic changes, suggesting compensatory mechanisms through alternative thiol defence pathways (Conrad, 2009; Murphy, 2012; Tanaka et al., 2002). Silencing of Prx3 makes mice slightly more susceptible to oxidative stress, whereas overexpression leads to positive outcomes
in cardiac pathophysiology (Chen et al., 2008; De Simoni et al., 2008; Li et al., 2007; Matsushima et al., 2006).


Figure 1.10. Thioredoxin and peroxiredoxin system. Peroxiredoxins (Prxs) reduce $\mathrm{H}_{2} \mathrm{O}_{2}$ to $\mathrm{H}_{2} \mathrm{O}$, forming sulfenic acids (-SOH), which lead to the formation of inter-molecular homodimers linked by disulfide bridges. Hyperoxidation can lead to sulfinic acid ( $-\mathrm{SO}_{2} \mathrm{H}$ ) or sulfonic acid ( $-\mathrm{SO}_{3} \mathrm{H}$ ) modifications, which are slowly reversible (by sulfiredoxin (SRX)), or irreversible, respectively. Hyperoxidised Prxs are inactive and indicative of oxidative damage. Prx dimers are recycled by thioredoxins (Trx), which in turn are regulated by thioredoxin reductase (TR) and NADPH.

### 1.4.2. The glutathione system

Glutathione (GSH) is the most abundant low molecular weight thiol within the cell and the mitochondria (Forman et al., 2009; Murphy, 2012). The role of GSH as an antioxidant was uncovered by the Nobel Laurate, Frederick Gowland Hopkins, in 1921 (Hopkins, 1921) and he was the first to show that GSH is a tripeptide, consisting of glutamate, cysteine and glycine (Lillig \& Berndt, 2013). GSH is now understood to be a multifunctional component of the cell and an essential compound for homeostasis, particularly in redox balance and detoxification (Flohé, 2013). Current interest is now focused on GSH's role in (patho)physiology (Ballatori et al., 2009).

Glutathione exists as either a reduced (GSH) or an oxidised disulphide form (GSSG) (Figure 1.11), with the GSH being the predominant form, with a ratio of 95-99 \% GSH to $1-5 \%$ GSSG (Ballatori et al., 2009; Forman et al., 2009; Lu, 2013). The structure of GSH is interesting, because of the unusual bond between the glutamate and cysteine residues. Peptide bonds usually involve $\alpha$-carboxyl groups, but in GSH the bond is through the gamma-carboxyl group of glutamate. Consequently, it can escape degradation by omni-present proteases. Therefore, the only enzyme that degrades glutathione is gamma-glutamyltranspeptidase (GGT) that is found on the external face of most cell types (Meister \& Anderson, 1983).

Glutathione is synthesised solely in the cytoplasm in two ATP-dependent steps (Lu, 2013; Murphy, 2012). The first step of glutathione synthesis is catalysed by gamma-glutamyl cysteine ligase (GCL), which reacts glutamate and cysteine together in the rate limiting step of glutathione synthesis (Lu, 2013). The second step of the glutathione synthesis pathway is glutathione synthetase (GS) (Lu, 2013). In contrast to glutamate being easily available and having many entry routes into the cell (Newsholme et al., 2003), the availability of cysteine is another rate limiting factor in the production of glutathione. Importantly, as discussed above, glutathione synthesis is entirely cytoplasmic. All other subcellular pools, and plasma glutathione thus come from the cytoplasm of cells. Mitochondrial glutathione is transported from the cytosol with the transport mechanism being unclear (Booty et al., 2015) until recently when it has been proposed to involve SLC25A39 (Y. Wang et al., 2021).


Figure 1.11. Overview of the mitochondrial glutathione system. A) Glutathione peroxidase (Gpx) metabolises $\mathrm{H}_{2} \mathrm{O}_{2}$ to $\mathrm{H}_{2} \mathrm{O}$ using reduced glutathione (GSH). Oxidised glutathione (GSSG) is recycled back to GSH by glutathione reductase (GR). B) GSH exchanges with protein (Pr) thiols, catalysed by glutaredoxin (Grx). C) GSH reacts with electrophiles (RX), catalysed by glutathione-S-transferase (GST).

The glutathione peroxidase family is comprised of four main members, GPx1-4, that catalyse the reduction of $\mathrm{H}_{2} \mathrm{O}_{2}$ or organic hydroperoxides, to water or alcohols using GSH as the reductant (Figure 1.11) (Brigelius-Flohé \& Maiorino, 2013). GPx1-3 are active in solution, whereas GPx4 exists on the membranes to protect lipid environments from peroxidation (Brigelius-Flohé \& Maiorino, 2013). GPx members also vary in their reducing factor, such as GPx1 uses only GSH, whereas GPx3 and GPx4 use GSH, but also interact with mercaptoethanol, cysteine, DTT and protein thiols, as well as Grx and Trx (Brigelius-Flohé \& Maiorino, 2013). The interaction with $\mathrm{H}_{2} \mathrm{O}_{2}$ means that GPx family members play a critical role in the defence from toxic species, as well as redox signalling. GPx1 is found in all cells and subcellular compartments such as mitochondria. As mentioned, GSH is central to the
catalytic mechanism of GPx1. A selenium moiety on GPx reacts with a peroxide to form water and a selenium acid moiety in the active site of GPx. Two GSH molecules are then required to reduce the selenic acid, forming a glutathionylated selenium intermediate and finally a GSSG molecule, restoring the GPx active site back to normal (Figure 1.11) (BrigeliusFlohé \& Maiorino, 2013). As discussed above, mitochondrial Prx3 contributes to approximately $90 \%$ of the $\mathrm{H}_{2} \mathrm{O}_{2}$ degradation capacity within the mitochondrial matrix (Cox et al., 2010). Shut down of peroxiredoxins can result in a shift of balance towards oxidative stress, as in pathophysiology of the heart during ischaemia-reperfusion (Cox et al., 2009; Kumar et al., 2009). GPx1 and GPx4 are responsible for mitochondrial degradation of $\mathrm{H}_{2} \mathrm{O}_{2}$ and lipid peroxides, respectively, with GPx1 accounting for $9 \%$ of $\mathrm{H}_{2} \mathrm{O}_{2}$ degradation within mitochondrial matrix (Cox et al., 2010; Murphy, 2012). Knockout studies have been used to reveal the relative importance of these systems. While GPx1 knockout mice are only slightly more susceptible to damage caused by ROS-producing agents (similar to Prx3 knockout mice), GPx4 knockout mice are embryonically lethal, highlighting the importance of mitochondrial defences against ROS and specifically the vulnerability of mitochondrial membranes to oxidative stress (De Haan et al., 1998; Ho et al., 1997; Yant et al., 2003). In addition to the mitochondrial localised GPx1 (matrix) and GPx4 (mitochondrial membrane), GPx3 has been suggested to play an important role in IMS redox regulation (Kritsiligkou et al., 2017).

Glutathione can itself directly interact with oxidants, as well as the mitochondrial thiol pool. The mitochondrial matrix's increased pH is the reason for a higher likelihood of direct interactions between thiols and reactive species. Although, the direct interaction between thiols and superoxide or $\mathrm{H}_{2} \mathrm{O}_{2}$ is unlikely, due to rapid reactions with MnSOD or Prx3/GPx1, respectively, there is a huge abundance of protein thiols (Cox et al., 2010; Murphy, 2009, 2012). More relevant for direct thiol protection against reactive species are the reactions with $\mathrm{OH}, \mathrm{CO}_{3}$ and $\mathrm{NO}_{2}$ radicals (Bonini \& Augusto, 2001; Ford et al., 2002; Kissner et al., 1997). Thiols that do interact with reactive species will typically be converted to thiyl or sulfenic acids as a result, subsequently leading to the formation of irreversible modifications in oxidative environments. The latter can render thiols ineffective and result in the protein being degraded, which has been implicated in certain diseases, such as Parkinson's (Aiken et al., 2011; Davies, 2001). In order to prevent protein damage, the
intermediate stages are recycled quickly back to thiols by interacting with GSH to form a disulphide, which can then be resolved by Trx2 or glutaredoxins (Grx) (Jung \& Thomas, 1996). However, it is unclear if these mechanisms take place in mitochondria in vivo (Murphy, 2012).

As mentioned, crucial to the recycling of protein thiols is the glutaredoxin (Grx) family of enzymes (Figure 1.11), which catalyse the deglutathionylation of protein thiols that have formed disulphides with GSH, recycling the protein thiols in the process (Jung \& Thomas, 1996; Murphy, 2012). The active site has a dithiol motif whereby one cysteine deglutathionylates protein thiols due to its proximity to the GSH binding site and the other GSH molecule can then react with the glutathionylated cysteine and form GSSG, recycling the enzyme. The GSSG molecule is then recycled back to GSH by glutathione reductase using NADPH as a reducing agent (Murphy, 2012). The mitochondrial Grx, Grx2, lacks a cysteine that is normally oxidised making it less susceptible to oxidising agents within the mitochondrial matrix (Murphy, 2012). It may be responsible for buffering the GSH pool during periods of oxidative stress, as it can interact with protein-disulphides as well as with GSSG (Beer et al., 2004; Schafer \& Buettner, 2001). As well as Grx2, Grx5 is also localised to the mitochondria, but is largely involved in FeS cluster synthesis, where GSH is at the centre of the process (Ye \& Rouault, 2010). Mitochondrial glutaredoxins are clearly important in antioxidant defence and redox signalling, through interactions with the GSH pool and protein thiols.

Another protective role for glutathione is the detoxification of electrophiles and xenobiotic compounds, where glutathione is conjugated to the compound by an enzymatic process, catalysed by glutathione S-transferases (GST) (Figure 1.11) (Board \& Menon, 2013). GST’s can be found throughout the cell, in the cytoplasm and mitochondria. They are well characterized enzymes, which consist of a catalytically independent active site that binds GSH and a site that binds the hydrophobic electrophilic compounds (Laborde, 2010). The GSH acts as a nucleophile in the environment and effectively detoxifies the compounds by creating a bulky polar compound (Hayes et al., 2005). As GSTs allow the cell to nonspecifically detoxify a range of compounds, they have been aptly named "cell housekeepers" (Laborde, 2010). Furthermore, GSTs are also involved in biosynthetic processes of prostaglandins, steroids and leukotrienes, as well as modulation of the cell
during apoptosis, proliferation and glutathionylation of cysteine residues (Adler et al., 1999; Anuradha et al., 2000; Beuckmann et al., 2000; Cho et al., 2001; Johansson \& Mannervik, 2001; Romero et al., 2006; Ruscoe et al., 2001; Ryoo et al., 2004).

The ability of GSTs to conjugate electrophiles with GSH is interesting particularly in the development of cellular probes. Probes that are targeted to the cell and either modify or output information on cellular health are becoming increasingly important, especially in mitochondrial biology (Booty et al., 2019; Cairns et al., 2015; Cochemé et al., 2011, 2012; Hu et al., 2014; Pun et al., 2014). Furthermore, the development of triphenylphosphonium (TPP) as a mitochondrial targeting moiety allows compounds to be used in a wide range of systems including cell culture and animal studies. A good example is MitoCDNB, a mitochondria-targeted CDNB (1-chloro-2,4-dinitrobenzene) moiety, which is a known GST substrate. This compound accumulates rapidly in mitochondria and leads to inhibition of TrxR2 and mitochondrial GSH depletion, while keeping the cytosolic GSH pool intact (Booty et al., 2019).
(1)

(2)




Figure 1.12. GST-mediated catalysis of CDNB. GSTs can coordinate GSH using a G-site and electrophiles using an H -site. The orientation and vicinity of the two compounds then result in a nucleophilic attack of GSH on the chlorine of the CDNB, which is essentially detoxifies the compound, rendering it for further metabolism and clearance. Adapted from (Hayes et al., 2005).

Mitochondria have evolved to feature distinct thiol defence mechanisms from the rest of the cell due to the organelle being the main source of ROS in the cell and herein lies the importance of being able to study these mechanisms specifically to understand their significance in cellular thiol homeostasis in physiology and pathology.

### 1.5. Redox signalling

Redox signalling occurs when a biological system alters in response to a change in the level of a particular reactive oxygen species (ROS) or the shift in redox state of a responsive group such as a dithiol-disulphide couple (Collins et al., 2012; Finkel, 2011). ROS are usually portrayed as damaging agents in pathology, but a more sophisticated view has been since proposed (Collins et al., 2012). Some ROS, such as hydrogen peroxide, can act as signalling messengers both in the extracellular environment and within cells (Collins et al., 2012; D'Autréaux \& Toledano, 2007). Mitochondria seem to be at the centre of redox signalling within cells, partly because of the flux of the ROS superoxide generated by the respiratory chain and other core metabolic machineries within mitochondria (Collins et al., 2012). Consequently, mitochondria are at the core of multiple biological processes, and redox signals to and from this organelle are vital for integrating mitochondrial function within the cell and organism (Collins et al., 2012).

### 1.5.1. General mechanisms of redox signalling

For many years, the study of oxidants has centred around the back-and-forth battle between the production of ROS and the equally potent cellular antioxidant defence mechanisms. This view has undergone a change as a result of the growing appreciation that ROS reversibly modulate several important intercellular pathways (Holmström \& Finkel, 2014) (Figure 1.13). One of the earliest observations in support of a role for ROS in signal transduction was the observed increase in Tyr phosphorylation, occurring as a result of growth factor stimulation (PDGF or EGF), is preceded by a burst of ROS production (Bae et al., 1997; Sundaresan et al., 1995). Rather than being harmful, this rise in intracellular ROS
level was shown to be required for downstream signalling, as inhibiting the rise of ROS was shown to block the normal tyrosine kinase signalling induced by growth factor addition. The burst of Tyr phosphorylation after stimulation has been shown to involve the redoxdependent inactivation of protein Tyr phosphatases (PTPs) (Meng et al., 2002). The basis for this regulation of phosphatase activity centres on the unique chemistry of certain reactive cysteine residues found in the active sites (Finkel, 2011). Oxidation of these cysteine residues, with pKas as low as 4 due to the microenvironment, leads to inactivation of the enzyme (Denu \& Tanner, 1998). This PTP inactivation tilts the balance towards unopposed Tyr kinase activity, leading to an increase in ligand-stimulated Tyr phosphorylation. ROS can also directly affect kinase signalling, for example, the redox dependent modification of a reactive Cys residue in the cytoplasmic domain of the EGF receptor, which leads to receptor activation (Paulsen et al., 2012). In addition to $\mathrm{H}_{2} \mathrm{O}_{2}$, several other oxidant species, including GSH, hydrogen sulphide and lipid peroxides, can transiently inactivate PTPs (Frijhoff et al., 2014). These observations can be summarised in a few important points. First, by modulating reactive Cys residues ROS can both activate or inactivate target proteins. Second, there seems to be an increasing number of intracellular targets for redox-dependent signalling. For example, the role of oxidants regulating phosphatase activity extends to dual specificity phosphatases such as PTEN (Kwon et al., 2004; Leslie et al., 2003). Third, for this signalling system to function in a reversible manner, oxidized targets need to be reduced. This reversibility has been mainly studied in terms of peroxiredoxin enzymes (Holmström \& Finkel, 2014). Reversing the oxidized reactive Cys residue occurs through thioredoxin, GSH or sulphiredoxin pathways (Jeong et al., 2012). Reversible, higher-order oxidation of Prx enzymes seem to be important for peroxide mediated signalling through a phenomenon called the "floodgate model" (Holmström \& Finkel, 2014). The reversible oxidation and reduction of Prxs have also been linked to circadian rhythms (Edgar et al., 2012). Finally, as mentioned before, some degree of specificity can also be achieved by localising the production of ROS, or by confining the spread of it to intracellular regions that are enriched in redox targets (K. Chen et al., 2008; Woo et al., 2010).

Another important and emerging theme is that antioxidant proteins are not merely passive disposers of intracellular oxidants but rather active participants in redox signalling (Finkel,
2011). One of the first descriptions of this trend came from the interaction between thioredoxin (Trx) and the apoptosis signal-regulating kinase (ASK1) (Finkel, 2011; Saitoh et al., 1998). It has been previously shown that TNF, which activates ASK1 also stimulates ROS production. It was also known that ASK1 regulates the induction of downstream effectors such as the c-Jun N-terminal kinase (JNK) and the p38 MAPK pathway required for cell death. The link between ROS production and subsequent activation of ASK-1 dependent signalling appears to involve a redox-dependent interaction between ASK1 and thioredoxin. Oxidative stress leads to direct oxidation of Trx cysteines and subsequent dissociation from ASK1 (Finkel, 2011). ASK1 now free from its interaction with Trx, can now activate its downstream target. In this scenario Trx does not act as the classical antioxidant but more as a sensor of intracellular oxidants and a regulator of redox signalling (Finkel, 2011).

ROS also modulate WNT and NOTCH signalling through various mechanisms. WNT ligands stimulate NOX1-generated ROS, which in turn can oxidize the thioredoxin family member nucleoredoxin ( Nrx ) (Holmström \& Finkel, 2014). As above, the reduced form of Nrx can interact with the Wnt signalling intermediate Dishevelled (Dvl) (Funato et al., 2006). This interaction is abolished with increasing oxidant levels and is completely absent from a mutant of Nrx which lacks two reactive Cys residues (Funato et al., 2006). When Dvl detaches from Nrx it increases stabilization of beta-catenin, which can in turn interact with the members of T Cell factor (TCF) or FOXO family of transcription factors (De Keizer et al., 2011).

A general mechanism of inducing a coordinated transcriptional response to a rise in ROS levels is an intramolecular disulphide bond, which leads to locking the transcription factor in its active configuration (Holmström \& Finkel, 2014). For example, members of the forkhead box protein O (FOXO) family of transcription factors, which can regulate antioxidant defences in mammals, also seem to be direct targets of Cys oxidation (Putker et al., 2013). Cys oxidation of FOXO proteins promoted the formation of new proteinprotein interactions that occur through disulphide bridges. With increased ROS levels, FOXO4 interacts with nuclear import receptor transportin 1 in a redox dependent manner,
which subsequently leads to it localising in the nucleus and activation of the transcription factor (Holmström \& Finkel, 2014).

The intestinal microflora can modulate the activity of both nuclear factor- $\kappa B$ ( $\mathrm{NF}-\mathrm{\kappa B}$ ) and beta catenin within the epithelium in a cys oxidation-dependent process (Neish et al., 2000). The ubiquitin mediated degradation of both inhibitor of $\mathrm{kB}-\alpha(\mathrm{IkB} \alpha)$ and betacatenin is regulated, in part, by the ubiquitin -like protein NEDD8. ROS production oxidizes a crucial Cys residue in UBC12, a NEDD8-conjugating enzyme, consequently modulating the protein stability and altering both NfkB and Wnt signalling (Kumar et al., 2007).

Another example of transcriptional regulation that uses Cys oxidation in order to sense and respond to an increase in ROS levels is the mammalian Keap1-Nrf2 pathway. In this case, the target of oxidants is Keap1, a partner protein and not the Nrf2 transcription factor. Under basal conditions, Keap1 binds to Nrf2 and leads to its degradation by facilitating its constitutive ubiquitination through binding of an E3 ubiquitin ligase cullin 3(Cul3) (Holmström \& Finkel, 2014). Due to the importance of Nrf2 in oxidative stress signalling and its crosstalk with mitochondria, it will be discussed in detail later on in the introduction.


Figure 1.13. The multiple ways in which oxidant signalling can affect cellular function. Oxidation of a reactive Cys residue (SH (thiol) to SOH (sulphenic acid)) leads to an alteration in the reduction-oxidation (redox)-sensitive target that can then have myriad effects on protein stability activity, localisation or proteinOprotein interaction. DVL, Dishevelled; EGFR, epidermal growth factor receptor; NRF2, nuclear factor erythroid 2-related factor 2; NRX, nucleoredoxin; PTP, protein Tyrphosphatase; ROS, reactive oxygen species. Adapted from (Holmström \& Finkel, 2014).

As mitochondria represent the main site of ROS production in the cell, it is not surprising that their involvement is intertwined throughout cellular redox signalling pathways and I will explain their importance in the following section.

### 1.6. Mitochondrial redox signalling

To be able to discuss mitochondrial redox signalling and their involvement in cellular redox pathways it needs clarification of how mitochondrial ROS can become signals and a distinction being drawn between the mitochondrial ROS themselves diffusing out of mitochondria or inducing an intramitochondrial signalling in a way that the organelle then subsequently releases a secondary signal, which can affect the cytosolic pathways.

### 1.6.1. Mitochondrial redox signals and their regulation

Mitochondrial redox signalling is an essential cog of cellular redox signalling. In order for mitochondrial $\mathrm{H}_{2} \mathrm{O}_{2}$ to be considered a redox signal it is important to be responsive to mitochondrial status (Holmström \& Finkel, 2014). One way in which this can occur is through the alteration of the generation of superoxide at complex I as a response to changes in $\Delta \mathrm{p}$ and the redox state of the Q pool (Chouchani et al., 2016; Murphy, 2009). RET is sensitive to these factors, both of which represent central components of mitochondrial function and therefore vary in response to mitochondrial activity. For example, ROS production by RET can be enhanced when mitochondria are inactive and over-supplied with electrons and minimised when mitochondria are active (Chouchani et al., 2016; Murphy, 2009). RET could also possibly be affected by other changes to complex I, such as the proportion of complex I in deactive vs active state (Dröse et al., 2016; Gorenkova et al., 2013). Although it is currently unknown if these transitions are used to regulate ROS. Finally, complex I can exist as either an isolated complex, or as a supercomplex with other respiratory chain complexes (Blaza et al., 2014). It has been shown that changes in the extent of complex I incorporation into supercomplexes
correlates with changes in mitochondrial ROS production (Lopez-Fabuel et al., 2016; Moreno-Loshuertos \& Enríquez, 2016).

The proof of RET has been found to be biologically relevant in several scenarios. Pathologically, after blood flow is restored to ischaemic tissue superoxide production by RET has been found to contribute to tissue damage known as ischaemia reperfusion injury. The rapid oxidation of the succinate that accumulates during ischaemia favours reduction of the Q pool. The reduced Q pool favours proton pumping by complexes III and IV, helping maintain a large $\Delta \mathrm{p}$ upon reperfusion and the degradation of adenine nucleotides during ischaemia limits ADP availability upon reperfusion that would otherwise diminish $\Delta \mathrm{p}$ by stimulating mitochondrial ATP synthesis (Chouchani et al., 2016). The final result is a burst of superoxide by RET through complex I when oxygenated blood flow is restored. In addition, RET has also been shown to be involved in metabolic adaptation of immune cells during inflammation (Mills et al., 2016), the immune response to viral infection (Buskiewicz et al., 2016) and life span extension in fruit flies (Scialò et al., 2016).

Superoxide produced in the matrix is dismutated to membrane permeable $\mathrm{H}_{2} \mathrm{O}_{2}$ by the high concentration of MnSOD (Murphy, 2009). Once formed within the mitochondrial matrix, the $\mathrm{H}_{2} \mathrm{O}_{2}$ can then be regulated by its degradation or rate of release. The effects of MnSOD level of expression on cell fate is variable and poorly understood (Hart et al., 2015), suggesting that the balance between superoxide and $\mathrm{H}_{2} \mathrm{O}_{2}$ could also modulate certain signalling pathways. Matrix $\mathrm{H}_{2} \mathrm{O}_{2}$ concentration is regulated by degradation through Prx3, Prx5 and Gpx1, with Prx3 being the most significant because of its relative abundance and reactivity (Cox et al., 2010). Thus, regulating the activity of Prx3 may be a key way of modulating $\mathrm{H}_{2} \mathrm{O}_{2}$ levels within mitochondria. The activity of Prx is modulated by the ratio of its active to reversibly inactive form, due to disulphide formation (Cox et al., 2009). Extended oxidation can lead to less readily reversible thiol modifications and therefore alter the flux of $\mathrm{H}_{2} \mathrm{O}_{2}$ from the mitochondria. The activity of $\mathrm{Prx3}$ might also be affected by post-translational modification or by the extent of its oligomerisation (Collins et al., 2012). Finally, the extent of activity of all peroxidases in mitochondria is determined by the redox state of the mitochondrial NADPH pool. The mitochondrial NADPH pool itself can respond to a number of factors: the activity of transhydrogenase, isocitrate dehydrogenase and
malic enzyme, which maintain NADPH levels, the redox state of GSH and Trx systems, which are reduced by NADPH (Murphy, 2012). As we can observe there are multiple factors that can potentially regulate the steady state level of $\mathrm{H}_{2} \mathrm{O}_{2}$ in mitochondria.

### 1.6.2. Intramitochondrial redox signalling

When we are focusing on mitochondrial ROS sources initiating redox signalling, one may distinguish between intramitochondrial redox signalling, where the targets are located within the structure of the mitochondrial network and redox signalling from the mitochondria to targets in the cytosol, nucleus (retrograde redox signalling) or even targets within the extracellular matrix. For example, as superoxide is a negatively charged molecule and will not readily diffuse through membranes, it is possible that some superoxide escapes MnSOD and also act independently as a signalling molecule within the matrix, by acting on the Fe-S centre in aconitase or succinate dehydrogenase (Brand et al., 2016; Gardner, 2002; Hurd et al., 2012). Furthermore, the protonated form of superoxide, perhydroxyl radical, is uncharged and lipid-soluble, meaning it can react with polyunsaturated fatty acyl sidechains of mitochondrial phospholipids, particularly cardiolipin, to initiate cascades of lipid peroxidation formation that alter the functions of the mitochondrial inner membrane, but also interferes with mtDNA and membrane proteins (Brand, 2020). Matrix superoxide has also been suggested to activate mitochondrial uncoupling proteins through lipid peroxidation reactions (Echtay, Murphy, et al., 2002; Echtay, Roussel, et al., 2002). In addition, hydrogen peroxide can rapidly oxidize specific reactive protein thiolates, one of its targets being cysteine residues on pyruvate dehydrogenase kinase 2 , in turn inhibiting it and activating the pyruvate dehydrogenase complex and increased oxidation of pyruvate (Hurd et al., 2012). It is also important to note that reactions with superoxide and hydrogen peroxide generate secondary species, such as release of $\mathrm{Fe}^{2+}$ through oxidation of $\mathrm{Fe}-\mathrm{S}$ centres, which in turn leads to OH radicals. The latter have weak specificity and react at diffusion-limited rates with proteins, mtDNA bases to cause mutation and mitochondrial phospholipids to lead to lipid peroxidation.

### 1.6.3. Exodus of mitochondrial redox signals

The concept of mitochondrial redox signalling communicating to other cell compartments is based on the assumption that mitochondrial ROS can be relayed to the cytosol. In the cytosol, $\mathrm{H}_{2} \mathrm{O}_{2}$ could alter enzyme activity either directly through reversible oxidation of cysteine/methionine thiols, or indirectly by facilitating redox-relay interactions with Prxs and Trxs (Herrmann \& Riemer, 2012; Sobotta et al., 2015). In order for $\mathrm{H}_{2} \mathrm{O}_{2}$ itself to act as a redox signal it has to leave the mitochondria and enter the cytosol. Although it is known that isolated mitochondria can produce a flux of $\mathrm{H}_{2} \mathrm{O}_{2}$ from both the IMS and the matrix (Murphy, 2009), this has not been clearly shown in whole cells yet. The passage of $\mathrm{H}_{2} \mathrm{O}_{2}$ through plasma membranes, as a result of generation by NADPH oxidases, is mediated by aquaporins (Bienert et al., 2007; Miller et al., 2010). There have been reports of aquaporin 8 in the IMM (Calamita et al., 2005; Chauvigné et al., 2015; Marchissio et al., 2012), but these have been disputed (Yang et al., 2006). The large surface area of the IMM to the matrix volume would enable the rapid diffusion of $\mathrm{H}_{2} \mathrm{O}_{2}$ through the IMM phospholipid bilayer even in the absence of aquaporins (Yang et al., 2006). Once in the IMS $\mathrm{H}_{2} \mathrm{O}_{2}$ should be able to diffuse out of the compartment through the porins found in the OMM. Another barrier for $\mathrm{H}_{2} \mathrm{O}_{2}$ diffusion is the multitude of antioxidant defence systems in the matrix, that were explained in the previous chapters, as the levels of mitochondrial ROS required to overwhelm these systems may be high. The possibility also exists that $\mathrm{H}_{2} \mathrm{O}_{2}$ alters mitochondrial metabolism in the matrix, by altering the activity of metal-centre proteins (Winterbourn, 2013), which could lead to a generation of another stable signal that could be passed to the rest of the cell, for example an electrophile.

A series of experiments using the HyPer probes, which contain circularly permuted YFP/GFP integrated into the regulatory domain of $\mathrm{H}_{2} \mathrm{O}_{2}$-sensing protein OxyR and a chemogenetic substrate-controlled $\mathrm{H}_{2} \mathrm{O}_{2}$ generator, a yeast D -amino acid oxidase (DAO) targeted to various cell compartments (Bogdanova et al., 2017) were done recently to shed light on the transport of $\mathrm{H}_{2} \mathrm{O}_{2}$ across the mitochondrial membrane (Pak et al., 2020). $\mathrm{H}_{2} \mathrm{O}_{2}$ produced within the matrix did not exit into the cytosol, as shown with the DAO placed in the matrix and co-expressed with HyPer probes in various compartments (Pak et al., 2020). The HyPer
probes in the mitochondrial matrix were able to reflect matrix $\mathrm{H}_{2} \mathrm{O}_{2}$ production after introduction of D-Ala. In contrast to unrestricted $\mathrm{H}_{2} \mathrm{O}_{2}$ diffusion from the cytosol into the matrix, there was no observed oxidation of Hyper probes in the cytosol, indicating a restriction of diffusion of matrix produced $\mathrm{H}_{2} \mathrm{O}_{2}$ to the cytosol (Pak et al., 2020). Furthermore, inhibiting the thioredoxin systems with auranofin led to the release of mitochondrial $\mathrm{H}_{2} \mathrm{O}_{2}$ upon addition of D-Ala, which indicates that the thioredoxin system is responsible for $\mathrm{H}_{2} \mathrm{O}_{2}$ restriction within mitochondria (Pak et al., 2020). A follow up with specific inhibition of mitochondrial thioredoxin systems would elucidate the process further, as auranofin does have off-target effects, such as inhibiting proteasome activity at the level of deubiquitinases (X. Zhang et al., 2019).

The mitochondrial membranes have a unique composition, with almost $20 \%$ of the IMM comprised of cardiolipin, which can function as a mitochondrial scavenger of ROS in order to protect cells from oxidative stress via cardiolipin oxidation and degradation (Tan \& Finkel, 2020). Oxidised cardiolipin is toxic (Paradies et al., 2001, 2002) and needs to be quickly degraded by enzymes, such as phospholipase A2-gamma (PLA2y) (Liu et al., 2017) 17-beta-hydroxysteroid dehydrogenase 10 (HSD10) (Boynton \& Shimkets, 2015). From a signalling view point, the metabolism of oxidized cardiolipin generates a large group of second messengers, including oxidized fatty acid lipids and diacylglycerol (Boynton \& Shimkets, 2015; Liu et al., 2017; Tan \& Finkel, 2020), which may be involved in mediating cellular response to mitochondrial stress, especially as cardiolipin can be externalised to OMM through lipid reorganisation events and therefore presented on the cytosolic side (Tan \& Finkel, 2020).

### 1.6.4. Redox signalling from mitochondria

### 1.6.4.1. Lipid redox signalling from mitochondria

Starting with mitochondrial lipid signalling, the signalling roles of cardiolipin and its lipid metabolites have been increasingly recognized in multiple biological processes including apoptosis, autophagy and gene expression regulation (Maguire et al., 2017). All these
processes appear to be closely associated with CL oxidation and generation of bioactive lipid mediators, such as 4 -HNE, which is an important signalling molecule (M. Xiao et al., 2017). Externalized, oxidised cardiolipin at the OMM can establish a signalling platform to orchestrate cellular response to mitochondrial damage that can include apoptosis or mitophagy (Tan \& Finkel, 2020). In mitochondrial-dependent apoptosis, the downstream events such as the recruitment and activation of apoptotic initiator protease caspase-8, its substrate BH3-interacting domain death agonist (BID), and the downstream effector BCL-2-associated X (BAX) are all dependent on the presence of cardiolipin in the OMM (Gonzalvez et al., 2008; Kuwana et al., 2002; Lutter et al., 2000). The interactions between cardiolipin, BID and BAX drive formations of complexes containing BAX oligomers on the OMM surface that mediate membrane permeabilization and cytochrome c release (Kuwana et al., 2002; Lai et al., 2019). The oxidation of cardiolipin also reduces its interaction with cytochrome c, further accelerating cytochrome c release to initiate apoptosis. Furthermore, in mitophagy-promoting conditions, increased cardiolipin at the OMM functions as a signal, recognized by the autophagy market microtubule-associated-protein-1 light chain 3 (LC3). Allowing for the autophagic capture of the damaged mitochondria (Chu et al., 2013). In addition to the regulation of apoptosis by these lipid mediators, covalent modification of mitochondrial apoptosis inducing factor (AIFm2) by HNE represents an excellent example of retrograde signalling induced by mitochondrial lipid oxidation (Miriyala et al., 2016), with the HNE adduction inactivating the NADH oxidoreductase activity of AIFm2 and facilitates its translocation from the mitochondria to the nucleus. Mitochondrial lipid oxidation signalling has significant implications in common human diseases, including cancer, cardiovascular diseases and other metabolic diseases (M. Xiao et al., 2017) (Figure 1.14).


Figure 1.14. Lipid signalling in mitochondria. Mitochondrial cardiolipin and PE orchestrate multiple aspects of mitochondrial signalling and bioenergetics. These are summarized here diagrammatically. (1) Cardiolipin is synthesised from phosphatidylglycerol (PG) and remodelled at the IMM. (2) Cardiolipin acts as a ROS scavenger via oxidation and redox-mediated degradation. (3) Moderate levels of mitochondrial stress stimulate cardiolipin externalisation. (4) Exposed cardiolipin can be recognised by LC3, stimulating mitophagic clearance. (5) Severe stress triggers cardiolipin-mediated cytochrome c release and apoptosis. Adapted from (Tan \& Finkel, 2020).

### 1.6.4.2. Mitochondrial redox signalling and hypoxia

Among the most intriguing biological areas of mitochondrial redox signalling is the role of mitochondrial ROS in $\mathrm{O}_{2}$ sensing, especially during hypoxia (Brunelle et al., 2005; Collins et al., 2012; Guzy et al., 2008; Guzy \& Schumacker, 2006; Patten et al., 2010). In particular, studies are suggesting that under hypoxic conditions, mitochondria increase their superoxide production by the respiratory chain (Chandel et al., 2000; Guzy et al., 2005). The site of ROS production seems to be respiratory Complex III, as ablating components of the complex prevents the stabilisation of Hypoxia inducible factor 1 alpha (HIF-1 $\alpha$ ) (Ježek et al., 2020). HIF-1 $\alpha$ consists of a labile $\alpha$ subunit and a constitutively expressed betasubunit. Under normoxic conditions, HIF-1 $\alpha$ is constantly degraded through ubiquitination
by ubiquitin ligase Von Hippel-Lindau tumour suppressor protein ( pVHL ). A prerequisite for ubiquitination is proline hydroxylation of HIF-1 $\alpha$ by 2OG-dependent prolyl hydroxylases (PHDs) (Ivan et al., 2001; Ježek et al., 2020). Upon inactivation of PHDs, HIF1- $\alpha$ is stabilized and can lead to hypoxic induction of over 400 genes (Collins et al., 2012; Sanjuán-Pla et al., 2005). The elevated mitochondrial superoxide is converted to $\mathrm{H}_{2} \mathrm{O}_{2}$ in the mitochondrial matrix, followed by diffusion into the cytosol where it leads to stabilisation of HIF-1 $\alpha$ through ROS inactivation of PHD (Ježek et al., 2020). It has been suggested that ROS acts in numerous ways such as oxidizing the PHD's cofactor iron as well as leading to inactive PHD homodimers due to redox-induced formation of disulphide bridges (Ježek et al., 2020; Lee et al., 2016) (Figure 1.15).


Figure 1.15. ROS regulation of HIF system. The major mechanisms of initiation of HIF-mediated transcriptome reprogramming-At normoxia isoforms of hypoxia-inducible factor $\alpha$ (HIF- $\alpha$ ), such as HIF-1 $\alpha$ are constantly degraded which is ensured by ubiquitin ligase Von Hippel-Lindau tumor suppressor protein (pVHL) and the components of the proteasome complex e.g., elongin $B / C(E l o B / C)$, cullin 2 (Cul2), ring-H2 finger protein (Rbx1) or ubiquitin ligase E2. During hypoxic adaptation the lack of oxygen and mitochondrial redox signalling (or in some situations also elevation of cytosolic ROS) lead to stabilization of HIF- $\alpha$ and its binding to HIF-6 through their interaction with prolyl hydroxylases (PHDs). As a result, stabilized HIF with the help of transcription factor p300/CBP causes transcriptome reprogramming (up to 400 genes are affected) and a typical metabolic switch to Warburg phenotype, when OXPHOS is suppressed while glycolysis and lactate production is upregulated. Adapted from (Fuhrmann \& Brüne, 2017).

### 1.6.4.3. Mitochondrial redox signalling and stress pathways

An increasing number of proteins encoded by the nuclear genome have been shown to reside in, or on, mitochondria under basal conditions, whereas redox stress leads to their translocation into the nucleus to activate transcriptional stress response (Tan \& Finkel, 2020). The well-conserved oxidative stress response factor $\mathrm{Nrf2}$ is sequestered by interacting with Keap1 and the mitochondrial serine/threonine protein phosphatase PGAM5 on the surface of the OMM (Lo \& Hannink, 2008), and will be explained in detail in the next chapter. In addition, there exists the mammalian mitochondrial-encoded peptide MOTS-c, a 16-amino acid peptide, that can translocate to the nucleus upon metabolic stresses accompanied by ROS production (Kim et al., 2018). Activation of the 5'-AMPactivated protein kinase (AMPK) downstream of ROS is required for MOTS-c nuclear translocation (Kim et al., 2018). In the nucleus, MOTS-c in turn initiates the transcription of stress response genes that protect against conditions such as metabolic dysfunction (Lee et al., 2015).

Increased levels of mitochondrial ROS were also reported to activate NF-кB, thereby promoting cellular proliferation and survival in cancer cells (Formentini et al., 2012; Quirós et al., 2016). The mechanism involves ROS leading to inhibiting the phosphorylation of $1 \kappa \mathrm{~B}$ $\alpha$, which leads to its ubiquitination and degradation, subsequently allowing NF-кB to translocate to the nucleus and activate transcription of its target genes (Almeida et al., 2010; Basak \& Hoffmann, 2008; Devin et al., 2000; Zhang et al., 2016). In addition, IKK, kinase that phosphorylates lкB- $\alpha$, can also be influenced by ROS through S glutathionylation of its cysteines and subsequent inhibition (Reynaert et al., 2006).

An increase in ROS also activates the c-Jun N-terminal kinase (JNK) pathway (Quirós et al., 2016). JNK pathway involves a kinase cascade with MAP kinase family, subsequently phosphorylating JNK on critical threonine and tyrosine residues resulting in its activation, and translocation to the nucleus, where it regulates the activity of multiple transcription factors (Zhang et al., 2016). Redox regulation of JNK involves Trx1, Grx as well as its upstream kinases such as the MAP kinase family (Han et al., 2009). As mentioned in the
chapter of redox signalling, oxidation of Trx1 by ROS leads to it dissociating from ASK-1, a kinase that phosphorylates and activates JNK through MAPK kinase. ASK-1 is kept in its inactive form by association with $\mathrm{Trx1}$, which is in turn disrupted by $\mathrm{H}_{2} \mathrm{O}_{2}$ oxidising the critical thiols on Trx1 and ASK-1 self-activating. Furthermore, JNK is also inhibited through association with redox-regulated proteins such as Grx and GST (Zhang et al., 2016), which contain critical cysteines that can become oxidised and liberate JNK in the process (Han et al., 2009) (Figure 1.16).


Figure 1.16. The retrograde response. Defects in oxidative phosphorylation (OXPHOS) and mitochondrial DNA (mtDNA), caused by damage in the electron transport chain (ETC) and by mutations, respectively, activate a retrograde response to the nucleus that can be triggered by a decrease in the level of ATP, increased signalling by reactive oxygen species (ROS) or the release of Ca2+ from mitochondria. Low ATP levels activate AMPactivated protein kinase (AMPK), which stimulates mitochondrial biogenesis and quality control. An increase in ROS also activates anterograde regulation through AMPK or the c-Jun N-terminal kinase (JNK) pathway, by activating PPARy co-activator 1 1 (PGC1 $\alpha$ ). Increased levels of ROS inhibit the kelch-like ECH-associated protein 1 (KEAP1)-mediated proteasomal degradation of nuclear factor erythroid 2-related factor 2 (NFE2L2) and facilitates the translocation of NFE2L2 to the nucleus and the subsequent activation of an antioxidant response. Loss of the mitochondrial membrane potential $(\Delta \psi m)$ results in the release of $\mathrm{Ca}^{2+}$ from mitochondria, inducing the expression of genes for calcium metabolism and glycolysis through two mechanisms. First, calcineurin translocates to the nucleus with nuclear factor-кB (NF-кB), which can also be activated by ROS, and with nuclear factor of activated $T$ cells (NFATC). Alternatively, $\mathrm{Ca}^{2+}$ can activate several
kinases, such as protein kinase C (PKC), JNK-p38 and $\mathrm{Ca}^{2+} /$ calmodulin-dependent protein kinase type IV (CAMKIV), which, in turn, activate different transcription factors such as early growth response protein 1 (EGR1), ATF2, cAMP response element-binding protein (CREB), CCAAT/enhancer-binding protein- $\delta$ (CEBP $\delta$ ) and CEBP homologous protein (CHOP). The release of $\mathrm{Ca}^{2+}$ can also activate anterograde regulation through $\mathrm{Ca}^{2+} /$ calmodulin-dependent protein kinase kinase-8 (CAMKKB). Adapted from (Quirós et al., 2016).

In mammalian cells, several mitochondrial insults, including proteotoxic stress and ROS, signal through the integrated stress response (ISR), which is a general cellular response that modulates global protein synthesis (D'Amico et al., 2017; Mottis et al., 2019; PakosZebrucka et al., 2016; Quirós et al., 2016). The key component of this stress response is the $\alpha$-subunit of eukaryotic translation initiation factor 2 (eIF2- $\alpha$ ) (Quirós et al., 2016). Phosphorylation of eIF2- $\alpha$ occurs by several kinases, with mitochondrial ROS suggested to act through the general control non-derepressible 2 (GCN2) kinase (Quirós et al., 2016). A further mechanism involving mitochondrial stress (induced by oligomycin) stimulating OMA-1-dependent cleavage (mitochondrial-stress-activated protease) of DELE1 (protein in the $I M M$ ) and leading to accumulation of DELE1 in the cytosol, where it interact with HRI and activates elF2- $\alpha$ through its kinase activity (Guo et al., 2020). Phosphorylation of elF2$\alpha$ globally inhibits protein synthesis but concurrently facilitates the specific expression of stress response genes such as ATF4 (Donnelly et al., 2013; Palam et al., 2011). ATF4 then induces expression of stress proteins, such as CHOP, FGADD34, ATF3 among many to restore proper cellular function (Quirós et al., 2016) (Figure 1.17).


Figure 1.17. The integrated stress response. The central regulation node of the integrated stress response (ISR) is the eukaryotic translation initiation factor eIF2 $\alpha$, which, when phosphorylated, inhibits cytosolic translation. Four different kinases are known to phosphorylate eIF2 $\alpha$ in response to various stresses: general control non-derepressible 2 (GCN2) is activated by amino acid starvation; PKR-like ER-kinase (PERK) is activated following endoplasmic reticulum (ER) stress; protein kinase double stranded RNA-dependent (PKR) is activated by double-stranded RNA (dsRNA) following viral infection; and haem-regulated inhibitor (HRI) is activated by heavy metals and haem deficiency. Defects in the electron transport chain (ETC), reactive oxygen species (ROS) and mitochondrial proteotoxic stresses can activate GCN2, PERK or HRI, depending on the context. Phosphorylation of eIF2 $\alpha$ promotes the selective translation of the transcription factor ATF4, which, in turn, promotes the expression of CEBP homologous protein (CHOP), growth arrest and DNA damageinducible protein 34 (GADD34), ATF3, tribbles homologue 3 (TRIB3) and immunoglobulin heavy chain-binding protein (BIP), as well as other transcription factors, to restore cellular homeostasis. However, apoptosis can also ensue in the case of irreversible cellular damage. In yeast, under conditions of mitochondrial stress, when protein import to mitochondria is slowed, newly translated mitochondrial polypeptides accumulate in the cytosol, inducing mitochondrial precursor over-accumulation stress (mPOS). This accumulation blocks translation and triggers an unfolded protein response activated by mis-targeting of proteins (UPRam), which activates the proteasomal degradation pathway and induces the expression of gene sets that are involved in restoring cellular homeostasis. MTS, mitochondrial targeting sequence. Adapted from (Quirós et al., 2016).

### 1.6.4.4. Mitochondrial redox signalling and epigenetic changes

Mitochondria can also transmit stress signals that result in epigenetic modifications, such as histone acetylation and DNA methylation. A mild increase in mitochondrial ROS, especially at the early stages of life, has been shown to be beneficial to multiple organisms from yeast to humans. Although it remains unclear in many cases as to how the mitochondrial stress is transmitted to the epigenome, some downstream mitochondrial ROS effects have been identified in yeasts and worms, with conserved homologs in mammals (Tan \& Finkel, 2020). For instance, in murine cells, the heterogeneous ribonucleoprotein A2 (hnRNPA2) acetylates lysine 8 of histone G4 at mitochondrial stressresponsive promoters in an acetyl-coenzyme A-dependent manner to activate gene transcription (Guha et al., 2016). Another example are the yeast homologues of mammalian ataxia telangiectasia mutated (ATM) and Chk2, serine/threonine protein kinases Tel1p and Rad35p (Schroeder et al., 2013), which promote yeast chronological life span in response to mitochondrial ROS by inactivating histone H3K36 demethylase Rph1p, leading to methylation and suppression of subtelomeric regions (Schroeder et al., 2013).

### 1.7. Nrf2

As mentioned before, Nrf2 is situated at the centre of cellular oxidative stress signalling and the antioxidant response, while also involved in an active crosstalk with mitochondria and therefore warrants a deep dive into its mechanism of action.

Nuclear factor-erythroid 2 p45-related factor 2 (NFE2L2 or Nrf2) is a master regulator of cellular redox homeostasis (Hayes \& Dinkova-Kostova, 2014). Nrf2 is a member of the cap'n'collar (CNC) subfamily of basic region leucine zipper (bZIP) transcription factors (Ma, 2013). There are 3 other CNC-bZIP transcription actors in mammals, Nrf1, Nrf3 and NF-E2 p45 (Sykiotis and Bohmann, 2010; Chevillard and Blank, 2011). It was first described in the laboratory of Yuet Wai Kan as a cDNA clone that encoded a protein that could bind a tandem repeat of the consensus site for the transcription factors activating protein (AP-1) and nuclear factor-erythroid 2 (NF-E2) (Moi et al., 1994 from, Hayes and Dinkova-Kostova,
2014). Nrf2 has been found to mediate induction of a set of drug-metabolizing enzymes (DMEs), such as GST and NADPH:quinone oxidoreductase 1 (NQO1), by antioxidants and electrophiles in addition to numerous enzymes in control of oxidant homeostasis (Ma, 2013). The induction requires a common DNA sequence called antioxidant response element (ARE) that resembles the NFE2-binding motif (Nguyen et al., 2003).

Transfection experiments identified ARE, as a 41-base-pair enhancer sequence upstream of various genes involved in oxidative stress with a core 16-base pair consensus sequence 5'-TMAnnRTGAYnnnGCR-3' (M = A or C, R = A, $\mathrm{Y}=\mathrm{C}$ or T, W = A or T) (NIOI et al., 2003; Wasserman \& Fahl, 1997). ARE is similar to the NFE2-binding motif 5'-TGXTGAGTCAC-3' and in part to the Maf (musculoaponeurotic fibrosarcoma) protein recognition element (MARE),
$5^{\prime}$-TGCTGA ${ }^{G} / c$ (or $\left.{ }^{G C} / c G\right)$ TCAAGCA-3', recognized by Maf heterodimers (Ma, 2013). As described before Nrf2 has a characteristic CNC bZIP domain in the C-terminal region, with the leucine zipper domain contributes to obligatory heterodimerization with small Maf proteins and the basic region to DNA binding (Ma, 2013). Nrf2 is widely expressed in human and mouse tissue and works as a transcription factor in heterodimers with the small musculoaponeurotic fibrosarcoma (Maf) proteins, MafF, MafG, MafK (Motohashi et al., 2004). Mafs contain a bZIP domain for DNA binding and dimerization but lack transcription activation domains themselves and therefore act as transcription repressors upon selfdimerization (Ma, 2013). bZIP proteins including AP-1, NFE2, Nrf1, Nrf2, Nrf3, Bach1 and 2, small Mafs and CREB/ATF exhibit overlapping binding activities toward DNA elements. Cross-interaction among the bZIP proteins is widespread and occurs through overlapping DNA binding and heterodimerization domains, which leads to expansion of the repertoire of target genes of individual bZIP proteins.

Nrf2 is a modular protein and each of its seven domains are called the Nrf2-ECH homology (Neh) domains and fulfil a distinct function (Baird \& Dinkova-Kostova, 2011; Hayes et al., 2010; Wang et al., 2013). The Neh1 domain comprises of the previously described CNCbZIP region that dimerizes with Maf proteins and binds DNA (Hirotsu et al., 2012). The Neh2 domain negatively controls Nrf2, because it recruits Keap1, a dimeric redox sensitive substrate adaptor for the Cullin (Cul)3-RING (really interesting new gene)-box protein
(Rbx)1 ubiquitin ligase complex (CRL ${ }^{\text {Keap1 }}$ ) (Ogura et al., 2010), through its DLG and ETGE motifs (Fukutomi et al., 2014; McMahon et al., 2006; Tong et al., 2006) (Figure 1.18). Neh3 domain is found in the C-terminal region and is a transactivation domain recruiting chromoATPase/helicase DNA-binding protein (CHD)6 (Hayes \& McMahon, 2009). Neh4 and Neh5 represent further transactivation regions capable of recruiting the cAMP response element-binding protein (CREB)-binding protein (CBPP) and receptor-associated coactivator (RAC) 3 (Kim et al., 2013). The Neh6 domain negatively control Nrf2 through recruitment of dimeric beta-transducin repeat-containing protein (beta-TrCP) (Chowdhry et al., 2013; Rada et al., 2011, 2012), which acts as a substrate adaptor for the S-phase kinase-associated protein 1 (Skp1)-Cul1-Rbx1-core E3 complex (SCF ${ }^{\text {beta-TrCP }}$ ) (Suzuki et al., 2000; Wu et al., 2003). Lastly, Neh7 domain mediates the repression of Nrf2 through a physical association with retinoid $X$ receptor (RXR)- $\alpha$ (Wang et al., 2013).


Figure 1.18. Keap1 and Nrf2 structure. A) Domain structures of the Keap1, Nrf2, and Nrf2 Neh2 proteins. B) Structure of the Keap1-Nrf2 complex. Adapted from (Lee \& Hu, 2020).

### 1.7.1. Nrf2 and Keap1

Nrf2 mRNA is expressed broadly and independently of inducers, suggesting a posttranscriptional mechanism for Nrf2 activation. Mechanistically, activation of Nrf2 for induction is twofold: suppression under basal condition and activation by inducers. Under normal homeostatic conditions, Nrf2 is maintained at a low level because it is targeted constitutively for proteasomal degradation by ubiquitination through its association with

Keap1 (Kelch-like erythroid cell-derived protein with CNC homology-associated protein 1). Keap1 was identified as a novel Nrf2-binding protein from yeast two-hybrid screening using the inhibitory Neh2 domain as a bait (Itoh et al., 1999). It contains two known proteininteracting domains: the BTB (bric-a-brac, tramtrack, broad-complex) domain in the N terminal region and the Kelch repeats in the C terminal region (DGR domain) (Ma, 2013). The BTB domain mediates homodimerization and binding of Keap1 to Cullin (Cul) 3, a scaffold protein of Nrf2 ubiquitin ligase (E3), while DGR mediates binding of Keap1 with Nrf2 Neh2 (X. Li et al., 2004; Ogura et al., 2010; Padmanabhan et al., 2006). Between the two domains, there is a cysteine rich intervening (IVR) or linker region (LR). Similar to Nrf2, Keap1 is expressed broadly in tissues and resides in the cytoplasm (Figure 1.19).

The view that protein turnover is a major mechanism of Nrf2 regulation is supported by the observation that cycloheximide inhibition of protein synthesis totally blocked the basal and induced expression of ARE-controlled DMEs (Ma, 2013). Nrf2 is found at a low protein level in many cell types, with a half-life of $\sim 20$ min due to rapid degradation by proteasomes (He et al., 2006; Kobayashi et al., 2004; Zhang et al., 2004). Its degradation is triggered by polyubiquitination through Keap1/Cul3 ubiquitin ligase, with Keap1 acting as a substrate adaptor bringing Nrf2 to the E3 complex, through binding to Nrf2 with its DGR domain and to Cul3 with its BTB domain. RING box protein 1 recruits the catalytic function of ubiquitinconjugating enzyme (e2) by binding to Cul4 C-terminal region. E2 then catalyses polyubiquitination of Nrf2 on the lysine residues found in Neh2 domain. Disruption of Keap1 in mice or knockdown of Keap1 in human cells are both sufficient to increase the abundance and activity of the transcription factor (Devling et al., 2005; Wakabayashi et al., 2003). Furthermore, somatic mutations in Keap1 result in upregulation of Nrf2 in tumours (Hayes \& McMahon, 2009), as does hypermethylation of the Keap1 promoter in patients with lung, breast and colon cancer (Barbano et al., 2013; Hanada et al., 2012; Muscarella et al., 2011).


Figure 1.19. Nrf2-Keap1 system. The Keap1-Nrf2-ARE signalling in basal and oxidative stress conditions. There are three proposed mechanism models for dysregulation of the KEap1-Nrf2 complex by cysteine modification in the KEap1 protein: the "Keap1-Cul3 dissociation model," the "hinge and latch model" and the "conformation cycling model". Adapted from (Lee \& Hu, 2020).

The mechanism of interaction between Keap1 and Nrf2 was revealed from structural studies of the two proteins. The Neh2 region of Nrf2 has been shown to be intrinsically disordered with some secondary structure with nuclear magnetic resonance spectroscopy (Tong et al., 2006). Two conserved sequences, ETGE and DLG motif, were discovered on either side of a conserved $\alpha$ helix, which is found in the centre of the rod-shaped Neh2. The overall structure of mouse Keap1 resembles a cherry-bob, where two Keap1 molecules form a dimer with two large spheres attached by short linker arms to the sides of a small forked-stem structure (Ogura et al., 2010). Neh2 interacts with Keap1 DGR domains in a ratio 1:2 through its ETGE and DLG motifs (Lo et al., 2006; Padmanabhan et al., 2006; Tong et al., 2006). In this two-site binding, or hinge and latch model (Ma, 2013), Keap1 recruits newly synthesised Nrf2 by binding to Neh2. The $\alpha$-helix between DLG and ETGE is
presented to E2 with six lysine residues facing the enzyme for polyubiquitination (Ma, 2013).

### 1.7.2. Nrf2 regulation

### 1.7.2.1. Nrf2 regulation via Keap1

Modification of Keap1 by electrophiles does not result in release of Nrf2, but rather, inducing agents cause conformational changes in Keap1 leading to the inability of regenerating free Keap1, which could bind newly synthesised Nrf2. The latter is then permitted to translocate directly to the nucleus (Hayes \& Dinkova-Kostova, 2014). A variety of chemicals, including phytochemicals and derivatives (CDDO, sulforaphane (SFN)), therapeutics (oltipraz, auranofin), environmental agents (paraquat, arsenic), and endogenous chemicals (NO, 15d-PGJ2, nitro-fatty acids, and 4-hydroxynonenal (4-HNE)), induce ARE genes through Nrf2 (Dinkova-Kostova et al., 2004; Ma \& He, 2012). Although they are structurally diverse and have very few common properties, they are able to modify -SH groups at rates closely correlating with their potency of NQO1 induction (DinkovaKostova et al., 2001). Binding studies of inducers to Keap1 cysteine thiols were provided using labelled inducers, ultraviolet spectroscopy, mass spectrometry and mutational studies (Dinkova-Kostova et al., 2001; Eggler et al., 2005; He \& Ma, 2010; Hong et al., 2005). As discussed before, the IVR region on Keap1 protein contains multiple cysteines that are labelled by inducers, such as C273, C288 and C297. Mutational studies have confirmed that C151, C273 and C288 are critical for Nrf2 regulation. C272 and C288 are required for suppression in the basal condition, while C151 is needed for activation by electrophiles (Yamamoto et al., 2008). C288 has been shown to be a sensor for 4-HNE, C151 similarly for NO and SFN, whereas the couple C226 and C613 a $\mathrm{Zn}^{2+}$ and $\mathrm{H}_{2} \mathrm{O}_{2}$ sensor (McMahon et al., 2010; Suzuki et al., 2019). These have been organised into classes: Class I (C151), Class II (C288), Class III (C151,273,288), Class IV independently from these, C226,613,622,624 $\mathrm{H}_{2} \mathrm{O}_{2}$ through disulphide bridges, Class V (Keap1-Nrf2 binding site) (Suzuki et al., 2019) (Figure 1.20). While Keap1 contains at least 25 cysteine residues, Nrf2 has only 6 (human)
or 7 (mouse and rat) cysteine residues. These are highly conserved and can also lead to Nrf2 activation through arsenic as an example (He \& Ma, 2009).


Figure 1.20. Model of the multiple stress sensing mechanism acting through Keap1. There are five classes of chemical inducers of Nrf2: Class I, Cys-151-preferring; Class II, Cys-288-preferring; Class III, Cys-151/Cys-273/Cys-288-selective; Class IV, Cys226/613/622/624-selective; Class V, non-electrophilic. Chemical representatives of each class are shown. Abbreviations used are as follows: DMF, dimethyl fumarate; SFN, sulforaphane; NO, nitric oxide; 4-HNE, 4-hydroxynonenal; 15d-PGJ2, 15-deoxy-412,14prostaglandin J2; H2O2, hydrogen peroxide. Adapted from (Suzuki et al., 2019).

Inducers that bind to Keap1 IVR cysteines that are spatially close to Keap1 DGR, disrupt DGR-DLG binding, but not the DGR-ETGE binding, leads to prevention of Nrf2 ubiquitination and "dead" Keap1 complex. Other inducers such as toxic metals (As, Cd, Cr) dissociate Nrf2 from Keap1 to stabilize Nrf2 (He et al., 2006, 2007, 2008). Modification of C151, which is close to the BTB region, disrupts Keap1-Cul3 binding, again inhibiting Nrf2 ubiquitination.

Mst1 and Mst2, mammalian Hippo kinase homologs, known for inhibiting cell proliferation and promoting apoptosis during tissue development and regeneration (Galan \& Avruch, 2016; Moya \& Halder, 2016; Pan, 2010), have been suggested to cause phosphorylation of Keap1 within or next to the BTB domain (Wang et al., 2019). This subsequently leads to the
inability of Keap1 to form dimers or polymers and to target Nrf2 for degradation (Wang et al., 2019).

There have been several other mechanisms of Nrf2 regulation described in addition to inducer-cysteine thiol interaction. Moreover, some proteins, such as p53 regulated p21, Bc-Xl-interacting protein PGAM5 and autophagy substrate p62 can bind KEAP1 DGR in a similar manner to Nrf2, subsequently leading to disruption of Nrf2-Keap1 binding and its persistent activation (Chen et al., 2008; Komatsu et al., 2010; Lo \& Hannink, 2008). The binding of some of these competitor proteins to Keap1 is regulated, which allows Nrf2 activity to be increased through crosstalk with other pathways. The competitor protein PGAM5 has the ability to inhibit Keap1 and also might be influenced by mitochondrial activity as it associates with the organelle (Lo \& Hannink, 2008). PGAM5 exhibits phosphatase activity and is associated with tumor necrosis factor (TNF)- $\alpha$-stimulated necrosis, suggesting that TNF- $\alpha$ regulates Nrf2 activity via PGAM5 (Wang et al., 2012). The autophagy receptor p62 contains an STGE motif for instance, but it becomes a potent inhibitor of Keap1 following phosphorylation of Ser-351 by mammalian target of rapamycin complex (mTORC)1 (Copple et al., 2010; Ichimura et al., 2013; Jain et al., 2010; Komatsu et al., 2010; Lau et al., 2010). Furthermore, this is augmented through Sestrin-1 and Sestrin-2 binding Keap1 and leading to its degradation under fasting and re-feeding conditions (Bae et al., 2013).

Park7, multifunctional protein associated with Parkinson's disease (PD) and cancers, interferes with Nrf2-Keap1 binding, hence stabilizes Nrf2 to protect against oxidative stress in dopaminergic neurons and cancer (Clements et al., 2006). Furthermore, tumour cells cause the Nrf2 pathway to be persistently activated through mutations and epigenetic mechanisms (Taguchi et al., 2011).

### 1.7.2.2. Nrf2 regulation via post translational modifications of Nrf2

Covalent modification of Nrf2 by phosphorylation/dephosphorylation and acetylation/deacetylation affects the nuclear translocation/export, transcription activation and degradation of Nrf2 in response to inducing signals (Apopa et al., 2008; Cullinan \& Diehl, 2004; Huang et al., 2002; Kawai et al., 2011; Salazar et al., 2006).

There are also several serine residues located in Neh2 that can be phosphorylated by GSK3 , creating a recognition motif for $\beta-\operatorname{TrCP}$. The latter is an adaptor protein for the Cul1dependent SCF E3 and hence leads to the ubiquitination and proteasomal degradation of Nrf2 independently of Keap1 (Rada et al., 2011). This mode of regulation is often overlooked, because GSK-3 is inhibited under conventional cell culture conditions (medium containing $10 \%$ fetal bovine serum) by growth factor signalling through the phosphatidylinositide 3-kinase (PI3K)-protein kinase B (PKB/Akt) pathway (Hayes \& Dinkova-Kostova, 2014). Therefore, the constitutive activity of GSK-3 is inhibited by PKB/Akt phosphorylation, and stimulation of PI3K prevents antagonism of Nrf2 by GSK-3.

There have also been studies showing the possibility of Nrf2 being targeted for degradation inside the nucleus. Nrf2 has been reported to be polysumoylated by small ubiquitin-like modifier (SUMO)-1 and SUMO-2, which target the transcription factor to promyelocytic leukemia nuclear bodies (Malloy et al., 2013). Within the nucleus the polysumoylated Nrf2 can be further ubiquitinated by the SUMO-specific RING finger protein 4 (RNF4).

In addition to polyubiquitination and phosphorylation of Nrf2, multiple Lys residues in the Neh1 domain are acetylated by p300/CBP following the treatment with arsenite, and this increases the specificity of Nrf2-Maf binding to ARE sequences and induction of target genes (Sun et al., 2009).

### 1.7.2.3. Nrf2 regulation via Nrf2 expression


#### Abstract

Although Nrf2 is a ubiquitously expressed transcription factor, its expression can decrease substantially due to CpG sequences in its promoter that have been shown to be hypermethylated in prostate tumorigenesis (Yu et al., 2010). Nrf-2 can be transcriptionally activated by nuclear factor (NF)-кB (Rushworth et al., 2012) and PPAR- $\alpha$ (Kulkarni et al., 2014; Zhang et al., 2013). Furthermore, its transcriptional start site (TSS) contains Jun and Myc binding sites (DeNicola et al., 2011).


### 1.7.3. Nrf2 target genes

Besides regulating the basal and inducible expression of drug-metabolizing enzymes, Nrf2 also controls the key components in endogenous antioxidant systems. Genome-wide searches for Nrf2 targets have led to the identification of an array of ARE genes, providing an explanation for the multiple roles Nrf2 plays (Hayes et al., 2010; Kensler et al., 2007; Ma \& $\mathrm{He}, 2012$ ). A central theme emerging from the identification of these genes is their ability to provide resistance to oxidants and electrophiles and they can be placed in 3 major groups: genes that regulate drug metabolism and disposition, antioxidant defence and oxidant signalling (Ma, 2013) (Figure 1.21). Furthermore, Nrf2 also regulates proteosomal protein degradation (Kwak et al., 2003), cell proliferation (Malhotra et al., 2010) and metabolic programming (Mitsuishi et al., 2012).

It has been suggested that induction of drug metabolism and transport through ARE could have originated as a strategy of detoxification of endogenous oxidants and electrophiles, but evolved as an adaption to environmental toxicants (Ma, 2013). Nrf2 can regulate the metabolic fate of numerous pro-oxidants and electrophiles in the body, by controlling the basal and induced expression of DMEs and transporters (Ma, 2013). ARE-regulated DMEs can catalyse a collection of heterogeneous reactions, including oxidation by CYP2A5, reduction by NQO1, conjugation by UGTs and nucleophilic trapping reactions by GSTs. Furthermore, drug transporters such a MRP2 and MRP3, transport drugs and metabolites out of cells (Ma, 2013). Nrf2 directly affects the homeostasis of ROS and RNS by regulating
the antioxidant defence systems through several mechanisms. Among the proteins that contribute to intracellular glutathione homeostasis, Nrf2 control the expression of the xCT subunit of system xc-, which imports cystine/glutamate into cells (Lewerenz et al., 2013), along with the glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits that catalyse the rate-limiting step in glutathione synthesis (Higgins et al., 2009). Furthermore, it controls the expression of glutathione peroxidase (GPx), which during the reduction of peroxides produces GSSG and glutathione reductase (GSR), which reduces GSSH, thereby maintaining intracellular levels of GSH. Besides the GSH-based antioxidant system, Nrf2 also controls the expression of thioredoxin, thioredoxin reductase, sulfiredoxin, peroxiredoxins, all of which reduce oxidised protein thiols (Abbas et al., 2011; Agyeman et al., 2012; Chorley et al., 2012; Hawkes et al., 2014; Jeong et al., 2012; Lu \& Holmgren, 2014; MacLeod et al., 2009; Malhotra et al., 2010). As many drug metabolizing enzymes need NADPH as a cofactor, it is notable that the NADPH-generating enzymes glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (PGD), isocitrate dehydrogenase (IDH) 1 and malic enzyme (ME)1 are all regulated by Nrf2 (Lee et al., 2003; Mitsuishi et al., 2012; Singh et al., 2013; Wu et al., 2011). It also induces metalchelation through MT1, MT2 and ferritin as well as regulation of stress response proteins (HO-1) (Ma, 2013). Many of the antioxidant enzymes/proteins regulated by Nrf2 localize in specific compartments within the cell to regulate the redox signalling in their local environment.


Figure 1.21. Nrf2 is the master regulator of cellular redox, energy metabolism and iron homeostasis. The main energy source of the cell, glucose enters catabolic processes in glycolysis followed by TCA or the anabolic chain of PPP. Nrf2 regulates rate-limiting enzymes to control the flow of substrates to these pathways. Oxidative phosphorylation is the main contributor to ROS. Cellular redox status is managed by enzymes such as SOD1 and SOD2 that converts superoxide to peroxides which eventually neutralized by catalases and peroxidases. Nrf2 also regulates the transcription of enzymes involved in glutathione synthesis, the main cellular reducing currency. Iron is another source of cellular ROS, managed by Nrf2 transcribed transporters. Enzymes whose transcription is directly regulated by Nrf2 is highlighted in yellow. 1, 2, 3, 4 and 5: mitochondrial complexes 1, 2, 3, 4 and 5; Cat: catalase; C: cytochrome C; FeS: iron-sulfur clusters (ISCs); vGCL:vglutamate-cysteine ligase;GS; GSR: glutathione reductase; GPX: glutathione peroxidase; HMOX1: heme oxygenase 1; Me1: malic enzyme 1; PDH: pyruvate dehydrogenase; PDK1: pyruvate dehydrogenase kinase 1; PPP: pentose phosphate pathway; PK: pyruvate kinase; PRX: peroxidase; SLC25: SLC25A37 and SLC25A28: mitoferrin; SOD1/2: superoxide dismutase-1 or 2; TCA: tricarboxylic cycle; U: ubiquinone; XCT: SLC7A11. Adapted from (Ammal Kaidery et al., 2019).

### 1.7.4. Nrf2 role in mitochondrial function

The function of Nrf2 as a master regulator of cellular redox homeostasis is widely recognized. Nrf2 has an important role in GSH biosynthesis, with regulating the gene expression of both catalytic and regulatory subunits of GCLC (Wild et al., 1999) as well as the xCT subunit of xc- system, which imports cystine into the cells (Sasaki et al., 2002). In the cell, cystine undergoes the conversion to cysteine, a precursor for GSH biosynthesis. As mentioned before, glutathione is synthesised exclusively in the cytosol and then transported into mitochondria, which means that Nrf2 is inherently regulating the mitochondrial GSH pool as well. Furthermore, Nrf2 provides the means for maintenance of glutathione in the reduced state, by coordinated transcriptional regulation of glutathione reductase (Harvey et al., 2009; MacLeod et al., 2009), which reduces GSSG to GSH using NADPH as a reducing equivalent. NADPH is produced by four principal NADPHgenerating enzymes, malic enzyme 1 (ME1) isocitrate dehydrogenase 1 (IDH1), glucose6phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (PGD), all of which are regulated by Nrf2 (Dinkova-Kostova \& Abramov, 2015; Lee et al., 2003; Mitsuishi et al., 2012; Wu et al., 2011). In systems where Nrf2 has been disrupted, the levels of GSH and NADPH are lower, whereas Nrf2 activation leads to GSH and NADPH upregulation (Benedict et al., 2012; Higgins \& Hayes, 2011; Wakabayashi et al., 2004).

Nrf2 is also critical for mitochondrial specific redox homeostasis, by regulating the expression of mitochondrial proteins thioredoxin reductase-2, peroxiredoxin 3 and 5 , and SOD2 (Kasai et al., 2020; Ryoo \& Kwak, 2018). Nrf2-KO cells had higher rates of mitochondrial ROS production and decreased total mitochondrial NADH pool (Holmstrom et al., 2013). Studies have shown the absence of Nrf2 cause impaired activity of complex I through substrate limitation and consequently increased mitochondrial ROS production, possibly through reverse electron flow from complex II (Dinkova-Kostova \& Abramov, 2015).

Under conditions of oxidative stress, the increased ROS production is counteracted by the Nrf2-dependent transcriptional upregulation of uncoupling protein 3 (UCP3), which
subsequently increases proton conductance of the mitochondrial inner membrane, decreasing the production of superoxide (Anedda et al., 2013). Recently, it has been shown that the lipid peroxidation product, 4-hydroxy-2-nonenal mediates the Nrf2-dependent upregulation of UCP3 in cardiomyocytes (López-Bernardo et al., 2015).

Nrf2 deficiency results in a decreased efficiency of oxidative phosphorylation, whereas Nrf2 activation has the opposite effect (Holmstrom et al., 2013). ATP is produced primarily in glycolysis, under conditions of Nrf2 deficiency, and used in part by $\mathrm{F}_{1} \mathrm{~F}_{0}$-ATPase to maintain the mitochondrial membrane potential (Dinkova-Kostova \& Abramov, 2015).

The effect on Nrf2 deficiency on the mitochondrial membrane potential is more pronounced in cells incubated in a medium lacking glucose (Holmstrom et al., 2013), suggesting that Nrf2 affects fatty acid oxidation. Nrf2 is known to downregulate transcription of acetyl-CoA carboxylase (ACC) (Yates et al., 2009), leading to less acetyl-CoA funnelled to malonyl-CoA, which in turn inhibits carnitine palmitoyltransferase (CPT), decreasing transport of fatty acids into the mitochondria (Dinkova-Kostova \& Abramov, 2015). Furthermore, Nrf2 positively regulates CD36 (Maruyama et al., 2008), a fatty acid translocase on the plasma and mitochondrial membranes. Nrf2 is therefore seen as an enhancer of mitochondrial fatty acid oxidation (Dinkova-Kostova \& Abramov, 2015).

It has been reported that livers of Nrf2-KO mice have a lower mitochondrial content (Zhang et al., 2013). Although the underlying mechanisms are complex and need further elucidation, studies indicate that Nrf2 affects mitochondrial biogenesis by influencing the expression levels of critical transcription factors (such as nuclear respiratory factor 1 and PGC1- $\alpha$ ) and coactivators, as well as by enhancing nucleotide biosynthesis through the pentose phosphate pathway (Dinkova-Kostova \& Abramov, 2015) (Figure 1.22).


Figure 1.22. Nrf2 and mitochondrion function. Upon Nrf2 activation and subsequent translocation to the nucleus it activates transcription of genes that influence a variety of mitochondrial functions, such as mitochondrial ROS defence, mitochondrial biogenesis, quality control as well as upregulation of mitochondrial metabolism through oxidative phosphorylation and fatty acid oxidation.

### 1.7.5. Crosstalk between mitochondria and Nrf2

As explained in the previous section, Nrf2 is instrumental in a variety of mitochondrial functions and defences. Hence, it is not surprising that there is much crosstalk between mitochondria and the Nrf2 pathway, some of which is explained below.

Nrf2-Keap1 forms a ternary complex with PGAM5, which has been shown to be localized at the mitochondrial outer membrane through the PGAM5 N -terminal mitochondriallocalization sequence, which may allow Nrf2 to directly sense and respond to mitochondrial signals such as mitochondrial ROS (Lo \& Hannink, 2008). PGAM5 is also known as the convergent point for multiple necrosis pathways, with PGAM5 knockdown leading to attenuated necrosis induced by TNF- $\alpha$ as well as ROS and calcium ionophore (Z. Wang et al., 2012). Upon necrosis induction, PGAM5 also recruits the mitochondrial fission factor Drp1 and activates its GTPase activity by dephosphorylating the serine 637 site of Drp1 (Z. Wang et al., 2012). This subsequently leads to mitochondrial fragmentation, an early and obligatory step for necrosis execution (Z. Wang et al., 2012).

Nrf2 inhibits the expression of TXNIP, which subsequently reduces the concentration of TXNIP in the cytosol and mitochondria, leading to free thioredoxin. Reduced thioredoxin can bind to ASK1 and inhibit ASK-1 dependent mitochondrial apoptosis (Ma, 2013).

Upon oxidative stress, DJ-1 (Parkin 7) translocates into the mitochondria in order to protect the organelle (Junn et al., 2009). Loss of Parkin 7 has been correlated with increased ROS production, mitochondrial damage, autophagy, and mitophagy, as well as accompanied with downregulation of Nrf2 and ARE genes (Clements et al., 2006; McCoy \& Cookson, 2011). Parkin7 has been shown to activate Nrf2 by interfering with Nrf2-Keap1 binding, while Nrf2 may upregulate Parkin7 expression through an ARE like enhancer sequence of Parkin7 (Clements et al., 2006).

Recently an unknown aspect of antioxidant signalling involved in host defence was uncovered through the role of Mst1/2, hippo like kinases, in sensing ROS and enhancing the stability and activity of Nrf2 (Wang et al., 2019). Mitochondrial ROS led to recruitment of Mst1/2 to the mitochondrial membrane and subsequent activation, which in turn regulated Nrf2 protein stability (Wang et al., 2019). Activated Mst1/2 phosphorylated Keap1 and prevented its polymerisation and association with Nrf2 (Wang et al., 2019). Furthermore, the antioxidant N -acetyl-cysteine (NAC) disrupted the association of Mst1/2 with mitochondria (Wang et al., 2019).

Various mitochondrial abnormalities can be signalled to the cytosol by non-ROS-related mechanisms as seen in mitochondrial retrograde signalling, such as in the Caenorhabditis elegans ATFS-1 system, where a drop in membrane potential inhibits peptide transport activity and proteolysis, subsequently resulting in nuclear translocation of ATFS-1 (Jovaisaite et al., 2014). It has also been reported that alterations in complex I activity determine Nrf2 gene expression via ERK5-myocyte enhancer factor 2 (MEF2) pathway in a ROS-independent manner (Khan et al., 2018; Tsushima et al., 2020). In contrast to exogenous electrophiles and ROS, the mechanism of Nrf2 activation by mitochondrial ROS production is more complicated considering that superoxide and $\mathrm{H}_{2} \mathrm{O}_{2}$ produced in the mitochondrial matrix do not easily diffuse into the cytoplasm (Hinchy et al., 2018). It is known that mitochondrial ROS activate Nrf2 (Kasai et al., 2020). Low extracellular
cysteine/cystine ratio increased both cellular and mitochondrial ROS levels and lead to activation of Nrf2 in NIH 3 T3 MEFs (Imhoff \& Hansen, 2009). Mitochondria-specific thioredoxin-2 transgenic MEFs have elevated cellular ROS but suppressed Nrf2 activation, further pointing to importance of mitochondrial ROS (Imhoff \& Hansen, 2009). The expression of transgenic catalase fused to a mitochondrial targeting signal (mtCAT) in Down syndrome cells reduced both cytoplasmic and mitochondrial ROS and restored mitochondrial dysfunction as well as Nrf2 function (Zamponi et al., 2018).

Physical exercise activates similar cellular responses as mitohormesis. Mitohormesis caused by mild metabolic stress elicits an antioxidant defence response through Sirt3, PGC1- $\alpha$; whereas the molecular mechanisms by which Nrf2 activation is achieved and maintained for a prolonged period remain to be clarified (Kasai et al., 2020). There have been many studies that show Nrf2 being activated in response to exercise in both cardiac and skeletal muscle (Done \& Traustadóttir, 2016). Acute exercise activates Nrf2 via NOX4 expression in the heart, via mitochondrial localisation and stimulation of mitochondrial ROS (Kasai et al., 2020). Furthermore, mitochondrial defects in the hearts of Nrf2-KO mice were rescued by the mitochondria-specific antioxidant mitoquinone (MitoQ) (Kasai et al., 2020). In oxidative muscles, it has been shown that p62 regulates Nrf2 activation in response to acute exercise stress (Yamada et al., 2019).

In certain pathological conditions, mitochondrial ROS can also inactivate Nrf2 (Kasai et al., 2020), with accumulating evidence to date that Nrf2 is repressed in various tissues in diabetic patients (Cheng et al., 2013; Rabbani et al., 2019; Smith \& Murphy, 2010; Zuo et al., 2019). Although, the precise mechanisms are still not currently clear. In diabetic kidney disease, PINK1/Parkin-mediated mitophagy and Nrf2 down regulation can be restored by MitoQ (L. Xiao et al., 2017).

As described before, in terms of mitochondrial homeostasis regulation, ATF4 is activated downstream of various mitochondrial perturbations (Kasai et al., 2019). ATF4 activation by stress signals is mediated by a conserved signalling pathway designated as the integrated stress response (ISR), which is triggered by Ser51 phosphorylation of eukaryotic initiation factor (eIF) 2- $\alpha$ by various kinases, leading to global translation inhibition and selective
translation of ATF4 (Pakos-Zebrucka et al., 2016). There have been many reports describing the Nrf2 and ATF4 crosstalk in mitochondrial retrograde signalling (Kasai et al., 2020), as well as ATF4 acting as a downstream effector of Nrf2 in cancer cells. It has been shown that Nrf2 physically binds ATF4 and that ATF4-Nrf2 heterodimers can bind to AREs (He et al., 2001). Nrf2 and ATF4 cooperatively work in the redox homeostasis regulation of cells (Kasai et al., 2020). The cooperative regulation is extensive for genes involved in GSH and NADPH synthesis specifically, such as GCLC, GLCM, GSS and xCT (Kasai et al., 2020).

The crosstalk between mitochondria and the Nrf2 signalling pathway involves a plethora of different mitochondrial signals and is interconnected with a variety of cellular outcomes such as mitohormesis, apoptosis, necrosis and inflammation, with an abundance of future research opportunities to elucidate this connection further.

### 1.8. Targeted mitochondrial delivery of compounds

Mitochondria targeting of small molecule probes provides an elegant solution of studying mitochondrial physiology and pathophysiology in cells and in vivo. The difficulty resides in ensuring compounds are directly targeted to the mitochondria, limiting their entry into other organelles. One of the key issues surrounding such a delivery is a compound's biological membrane permeability (Rautio et al., 2008). Both the cellular membrane and the IMM are critical for entry of molecules into the mitochondria (Smith et al., 2012b) and therefore, to be a successful mitochondria-targeting compound, it must be able to cross not only the plasma membrane but also the IMM (Jean et al., 2016; Ross et al., 2006, 2008; Yamada \& Harashima, 2008).

### 1.8.1. Mitochondria-targeting techniques

One approach to delivery of compounds to mitochondria is by the use of mitochondriapenetrating peptides (MPPs), which consist of cationic and hydrophobic amino acids that can target and penetrate the mitochondrial membrane (Horton et al., 2008; Jean et al.,
2016). The sequences and the amino acids within the MPPs can be altered, therefore generating tuned compounds and offsetting the properties of the bioactive compounds to be delivered (Jean et al., 2016). Although the exact mechanism of how MPPs are taken up by the mitochondria is unknown, they have been shown to localise within the mitochondrial matrix in response to the $\Delta \psi_{\mathrm{m}}$ (Marbella et al., 2013).

Another mechanism of targeting compounds to mitochondria is via the use of mitochondria targeting peptides. The first of these synthesised, named Szeto Schiller (SS) peptides, are made up of small aromatic and cationic amino acids (4-20 amino acids in length, which are able to permeate biological membranes (Dai et al., 2014; Szeto \& Schiller, 2011). These SSpeptides have been found to preferentially localise to mitochondria, despite their relatively polar, water soluble characteristics. Although SS-peptides have a net positive charge of +3 at physiological pH , their uptake is independent of the $\Delta \psi_{\mathrm{m}}$, meaning that they are not localised within the mitochondrial matrix. Subsequently, $85 \%$ of the SS-peptide, SS-02, was shown to be localised to the IMM (Dai et al., 2014; Ross \& Murphy, 2004). As they are localising to the IMM and not the mitochondrial matrix, this places a constraint on their use (Dai et al., 2014).

A different approach is the use of segments of gramicidin $S$ (hemigramicidin) as the mitochondria-targeting moiety (Fink et al., 2007). Gramicidin is a pore-forming antibiotic, and its bacterial-targeting properties were utilised to access the IMM. Similarly, to the SSpeptides its uptake is independent of the $\Delta \psi_{\mathrm{m}}$ and does not deliver compounds to the mitochondrial matrix. Rather, the payload is targeted to the IMM (Fink et al., 2007; Jiang et al., 2008; Smith et al., 2012b).

Although, these approaches have shown promise, with both MPPs and SS-peptides successfully delivered compounds in vivo, the unknown uptake mechanism requires further work to understand the nature of the interactions with the IMM (Jean et al., 2016; Szeto \& Schiller, 2011).

### 1.8.2. TPP platform for mitochondria-targeting

The bio-orthogonal approaches make use of the large mitochondrial membrane potential, approximately -180 mV , spanning the IMM (Ross et al., 2006, 2008; Smith et al., 2012b). The work on liphophilic cations as an approach of targeting compounds using the $\Delta \psi_{\mathrm{m}}$ began in the lab of Skulachev, with the use of the lipophilic dibenzylammonium cation, which was used to prove the existence of the mitochondrial membrane potential (Liberman et al., 1969). The use of lipophilic cations in mitochondrial biology is currently commonplace, with the use of fluorescent derivatives in measuring membrane potential (MitoTracker and tetramethylrhodamine (TMRM). The lipophilic cations of choice for targeting mitochondria are the triphenylphosphonium (TPP) cations. Murphy championed the use of TPP as a targeting strategy and enabled conjugation to numerous bioactive small molecules for delivery into the mitochondrial matrix (Chouchani et al., 2013; Cochemé et al., 2011; Robb et al., 2015; Smith et al., 2012b). Many of the mitochondria targeted molecules featured in this thesis utilise the TPP-targeting moiety, therefore I will explain the basis by which TPP molecules are effective at mitochondria-targeting (Figure 1.23).


Figure 1.23. Uptake of triphenylphosphonium cations by mitochondria within cells. This schematic shows the uptake of a triphenylphosphonium moiety attached to a moiety $(X)$ into the cytoplasm from the extracellular environment driven by the plasma membrane potential. From the cytoplasm the compound is further accumulated into mitochondria, driven by the mitochondrial membrane potential. The mitochondrial
and plasma membrane potentials $(\Delta \psi)$ are indicated. The moiety, $X$, could be an antioxidant or a probe of mitochondrial function. Adapted from (Murphy, 2008).

As described previously, the proton translocation across the IMM creates the $\Delta \mathrm{p}$, consisting of the pH gradient and membrane potential (Mitchell, 1961). The difference in charge across the membrane, generates an electrical driving force and the ion concentration gradient creating a chemical driving force, therefore the electrochemical driving force by which TPP cations are accumulated (Ross et al., 2005).

The movement on an ion across a membrane involves diffusion down a concentration gradient, as well as the movement of charge. The build-up of electrical force counterbalances the diffusion of the ion and when they are in equilibrium, net ion diffusion comes to a halt. This equilibrium of two forces is dependent on the concentration of the ion on either side of the membrane and governed by the Nernst equation:

$$
\Delta \psi=(2.3 R T / n F) \log _{10}\left([\text { ion }]_{\text {in }} /[\text { ion }]_{o u t}\right)
$$

The Nernst equation describes the $\Delta \psi$-dependent distribution of ions across a membrane, with $\mathrm{R}=$ universal gas constant, $\mathrm{T}=$ absolute temperature, $\mathrm{n}=$ number of charges and $\mathrm{F}=$ Faraday's constant. The previously described proton translocation across the IMM creates a negatively charged matrix, hence a negative equilibrium potential. Lipophilic cations will equilibrate with the membrane potential, with the extent given by the Nernst equation. For a single-charged species, the $2.3 \mathrm{RT} / \mathrm{nF}$ terms give $59.5-61.5 \mathrm{mV}$ at physiological temperature. Subsequently, there will be approximately a 10 -fold accumulation of the lipophilic cation into the mitochondrial matrix per 60 mV of membrane potential. With the mitochondrial membrane potentials being in the region of 140 to 180 mV , this implies an accumulation potential of several hundred-fold within the matrix (Ross et al., 2005). Hence, the TPP cation utilises the electrochemical driving force to accumulate molecules directly into the matrix, in a membrane potential manner. The plasma membrane potential ranges depending on the cell type from 5 to 90 mV (Yang \& Brackenbury, 2013), meaning that the accumulation of positively charged TPP moiety will also occur from the extracellular space to the cytoplasm (Ross et al., 2005).

While TPP lipophilic cations uptake readily across the IMM despite their inherent positive charge, this greatly differs to hydrophilic cations, which require carriers for uptake. This is due to large activation energies required to move from the aqueous medium to the hydrophobic phospholipid membrane. The electrostatic energy component of the activation energy is the Born energy ( Wb ), which is the energy required to remove the aqueous solvation shell for movement of the ion into the hydrophobic core (Ross et al., 2005). Wb is inversely proportional to the ionic radius and the large distribution of the positive charge across TPP considerably lowers Wb , hence reducing the repulsive forces compared to a hydrophilic cation. There is a further component of the activation energy that arises from non-charge related interactions of the molecule, namely the hydrophobic interactions between molecule and membrane. This hydrophobic interaction is an attractive force due to increase in entropy from the molecule losing its solvation shell and as the TPP has a large hydrophobic surface area it leads to a greater hydrophobic interaction and further reduction of the activation energy (Ross et al., 2005). The mechanism of TPP transport across the IMM follows the energy diagram, adsorption to the membrane favours hydrophobic interactions and creates a potential energy well, where free energy required for the movement is at a minimum, as the attractant forces are dominating. As TPP moves through the membrane the work required increases due to the increased electrostatic repulsive forces, but it still a rapid movement. At the opposite side of the membrane it encounters another potential energy well, desorbs and enters the mitochondrial matrix (James et al., 2007; Ross et al., 2005) (Figure 1.24).


B


Figure 1.24. Crossing the IMM by TPP. A) Schematic of TPP crossing the IMM, with the potential energy required to cross described B). Figure adapted from (Ross et al., 2005).

The three characteristics that make TPP a robust mitochondria-targeting moiety are the positive charge is spread across the molecule by inductive effects, rather than being concentrated on one atom (phosphorus), the lipophilicity of three phenyl groups provides a large hydrophobic surface area, aiding membrane transport and lastly the phenyl groups also provide a steric hindrance to water, shielding the phosphorus atom from solvation. Subsequently, the free energy to remove solvating water molecules for movement into the lipophilic membrane is greatly reduced, compared to other charged molecules (Robb et al., 2015; Ross et al., 2005; Smith et al., 2012). Recent reports have shown that modifying the TPP group by replacing hydrogens in the phenyl groups with methyl groups improves the efficiency of cation uptake into mitochondria. Furthermore, increase in linker length has the potential for increasing the solvent-accessible surface area and volume per charge of the cation improve accumulation into mitochondria (Prag et al., 2020a). All in all, the TPPconjugated bioactive compounds are readily and rapidly taken up into mitochondria in vitro, as well as in vivo (Porteous et al., 2010; Smith et al., 2003).

### 1.8.3. TPP linked probes

TPP has been conjugated to a number of molecules and is implicated in a range of uses. TPP-conjugates include low molecular weight antioxidants, probes and a number of other bioactive compounds. The development of TPP-conjugated quinone (MitoQ), a mitochondria-targeted antioxidant, followed the ubiquitous presence of oxidative stress in numerous pathologies and the ineffectiveness of non-targeted antioxidants (Kelso et al., 2001). MitoQ prevents against lipid peroxidation and has been shown to be effective both in vitro and in vivo (Kelso et al., 2001; Rossman et al., 2018; Smith \& Murphy, 2010). Furthermore, it has been translated to use in humans, where it was found to reduce liver damage in chronic hepatitis C infected patients (Gane et al., 2010). Although MitoQ did not indicate any effect on the progression of Parkinson's disease in patients, it was well tolerated over the 12 months period and hence indicates a good safety profile for TPPconjugated compounds in humans (Snow et al., 2010). These results warrant future exploration into the use of mitochondria-targeted antioxidants in the treatment of human pathologies with several clinical trials currently underway. Recently, it has been shown that chronic supplementation with MitoQ improves vascular function in healthy older adults (Rossman et al., 2018).

Mitochondria-targeted probes are another use of the TPP targeting platform, particularly in the field of mitochondrial ROS measurements, such as MitoSOX, MitoNeoD and MitoB (Cochemé et al., 2011; Shchepinova et al., 2017). MitoB is a mitochondria-targeted arylboronic acid which can react with hydrogen peroxide, producing a stable phenol product (MitoP), with the ratio between MitoB:MitoP indicative of the oxidative state of the cell (Cochemé et al., 2011).

Mitochondria-targeted compounds also offer a chemical biology approach to targeting and stimulating mitochondrial dysfunction in order to aid investigation of mitochondrial biology. A mitochondria-targeted redox cycler, MitoParaquat (MitoPQ), which comprises of a TPP conjugated to a paraquat moiety, has been characterised in order to accurately assess the role of mitochondrial superoxide in cells and in vivo (Robb et al., 2015). MitoPQ
accumulates selectively in the mitochondrial matrix and produces superoxide by redox cycling at the flavin site of complex I, selectively increasing superoxide production within mitochondria. Therefore, it provides a useful tool to investigate the many roles of mitochondrial superoxide in pathology and redox signalling in cells and in vivo, which will also be further explored in this thesis.

To investigate mitochondrial thiol redox state irrespective of the cytosolic thiol redox state, 1-chloro-2,4-dinitrobenzene (CDNB) moiety, a glutathione S-transferase substrate and TrxR inhibitor was conjugated to TPP, resulting in MitoCDNB. The latter is taken up by mitochondria where it selectively depletes the mitochondrial GSH pool, catalysed by glutathione S-transferases and directly inhibits mitochondrial TrxR2 and peroxiredoxin 3 (Booty et al., 2019). MitoCDNB inactivates mitochondrial thiol redox homeostasis both in isolated cells and in vivo, without affecting that of the cytosol. Consequently, MitoCDNB enables assessment of the biological importance of mitochondrial thiol homeostasis in reactive oxygen species production, organelle, dynamics, redox signalling in cells and in vivo, much of which was investigated as part of this thesis.

Mitochondria-targeting platforms provide a valuable method to deliver a multitude of bioactive compounds, with minimal interference from the TPP targeting moiety attached. Hence, this opens door for the use of mitochondria-targeted strategies to deliver molecules in numerous pathologies, stemming from mitochondrial dysfunction, as well as aiding in the investigation of mitochondrial dysfunction to a greater depth.

### 1.9. Summary

Above, I have introduced the multitude of vital roles mitochondria play within the cell and highlighted the importance of mitochondria to complex life as we know it. As a by-product of energy metabolism, mitochondria can also produce ROS from several sources within mitochondria, including from the respiratory complexes of the electron transport chain (Murphy, 2009). In order to deal with ROS, there are diverse antioxidant defence systems within the mitochondrial matrix consisting of the glutathione, thioredoxin and
peroxiredoxin systems (Ruszkiewicz \& Albrecht, 2015). Disruption of these systems has been implicated in numerous pathologies, and therefore requires greater understanding.

If ROS overwhelm the multitude of mitochondrial antioxidant systems, it leads to oxidative damage which can contribute to cell death (Smith et al., 2012). ROS may also behave as signalling molecules, by reversibly oxidising proteins at their cysteine residues that have an influence on their function (Holmström \& Finkel, 2014). Historically, mitochondrial ROS production has been regarded as an unregulated process, exclusive to dysfunctional mitochondria, but in recent years that view has been changing, and mitochondrial ROS have been proposed to behave as a biologically relevant signal. Mitochondrial ROS produced by RET is dependent on a highly reduced mitochondrial Q pool and high mitochondrial $\Delta p$ (Chouchani et al., 2016), and reported to play a role in diverse situations, such as metabolic adaptation of immune cells during inflammation (Mills et al., 2016), lifespan extension (Scialò et al., 2016) as well as being a key source of ROS during ischaemia-reperfusion (IR) injury (Chouchani et al., 2014).

Mitochondria actively influence other cellular components and cytosolic pathways, creating a mito-cellular communication network based on a variety of signals (Mottis et al., 2019). This network allows them to stay in tune with cellular needs, nuclear transcriptional programs and adapt to various stresses, hence alleviate mitochondrial dysfunction. In mammals, increases in ROS levels induce a retrograde signal to activate detoxification enzymes and antioxidant proteins in mitochondria and the cytosol (Chen \& Kunsch, 2004; Kops et al., 2002; Lu et al., 2012; Tan et al., 2008) through the nuclear factor erythroid 2related factor 2 (Nrf2) binding to antioxidant response elements (Nguyen et al., 2009). Increased levels of mitochondrial ROS have also been shown to activate NF-кB, subsequently promoting cellular proliferation and survival in cancer cells (Formentini et al., 2012). OXPHOS genes and mitochondrial biogenesis is induced by promoting JNK-PGC1 $\alpha$ signalling (Chae et al., 2013). Mitochondrial ROS can also influence metabolic reprogramming through mitochondrial uncoupling and AMPK activation (Shi et al., 2015).

One way to explore how mitochondrial ROS signalling is affecting the cell is to explore mitochondrial dysfunction stemming from disrupting mitochondrial antioxidant systems,
which usually behave as guardians of ROS homeostasis. After consideration of the current literature knowledge, it is clear that the area needs further work, as it has been difficult to selectively target mitochondrial thiol homeostasis without disrupting the cytosolic counterparts. Therefore, the main focus of this thesis is to build upon previous work in the field and work towards characterising the effects of mitochondrial thiol homeostasis on retrograde signalling. This goal was divided into three individual but interrelated sub projects, which I discuss in detail in the following chapters, and introduce briefly below.

### 1.10. Aims of the thesis

The overall aim of this thesis is to develop a deeper understanding of mitochondrial thiol redox homeostasis and how it is signalled from the mitochondria to other parts of the cell. This has the potential to impact greatly the field of mitochondrial signalling upon dysfunction and how it can contribute to various pathologies. The main point of focus throughout this thesis are as follows:

1. To understand the role of mitochondrial thiol homeostasis and the consequences of its disruption to mitochondrial physiology.
2. To understand the role of mitochondrial thiol homeostasis and mitochondrial superoxide production in the activation of Nrf2 signalling.
3. To elucidate the mechanism of early mitochondrial retrograde signalling following mitochondrial dysfunction caused by mitochondrial targeted molecules.

The above aims, which encompassed most of the thesis, utilised mitochondria-targeted TPP-compounds in order to target the mitochondria specifically. In addition, a further minor aim was:
4. to characterise the use of tetraphenyl boronate (TPB) as a non-mitochondriatargeting moiety for a new class of cellular probes.

In the following chapters, the initial work will focus on the impact of disruption to thiol metabolism on mitochondrial physiology. In particular, I will first investigate ROS production within mitochondria and its impact on mitochondrial morphology and dynamics (Chapter 3). Next, the MitoCDNB control compound will be introduced and characterised to provide more robust comparison for future experiments. Then focus is shifted to study mitochondrial thiol dysfunction and enhanced superoxide production and their effect on the Nrf2 signalling pathway in vitro. Subsequently Nrf2 protein levels, subcellular localisation as well as downstream targets will be assessed to understand the effects of MitoCDNB and MitoPQ. After this, possible mitochondrial signals that activate the Nrf2 pathway will be investigated (Chapter 4). Then, mitochondrial redox homeostasis in cross talk signalling to the nucleus to regulate transcription, was investigated by using a full transcriptome approach to probe the effects of mitochondrial thiol redox homeostasis on the expression of various clusters of genes (Chapter 5). Finally, I will also describe preliminary work on fluorescently labelled small molecules designed to be excluded from the mitochondria in order to aid our investigation of the intricacies of sub-cellular physiology. Here the focus is to explore the cell uptake and sub-cellular distribution of fluorescent TPB probes, TPBCoumarin and TPBBODIPY (Chapter 6).

## Chapter 2. Materials and methods

### 2.1. Chemicals and consumables

All reagents were purchased from Merck (formerly Sigma Aldrich), UK unless otherwise specified.

Stock solutions were stored at $-20^{\circ} \mathrm{C}$, unless otherwise stated.

The pH of solutions was measured and corrected at the temperature of use (as indicated), using a calibrated pH meter (3310; Jenway, UK).

Milli-Q filtered water (resistivity of $18.2 \mathrm{M} \Omega . \mathrm{cm}$ at room temperature (RT)) (Merck, UK) was used in all experiments unless specified.

All compounds specifically synthesised for use in this thesis were synthesised by Dr Stuart T. Caldwell, in the lab of Professor Richard C. Hartley (WestCHEM School of Chemistry, University of Glasgow, UK).

### 2.1.1. Antibodies

All antibodies were polyclonal antibodies unless otherwise stated as being monoclonal.

### 2.1.1.1. Primary antibodies and Western blot working dilutions

rabbit anti-Tom20: Santa Cruz Biotechnology (sc-11415), 1:1,000
rabbit anti-GAPDH: Sigma Aldrich (Merck) (G9545), 1:5,000
rabbit anti-Nrf2: Cell Signaling Technologies (12721), 1:1,000
mouse anti-Drp1: BD Biosciences (611113), 1:1,000
rabbit anti-GSS: Abcam (ab133592), 1:1,000
rabbit anti-GCLC: Abcam (ab190685), 1:1,000
mouse anti-HO-1: Abcam (ab13248), 1:1,000
mouse anti-Histone 4: Cell Signaling Technology (2935), 1:1,000
mouse anti-Tubulin: Abcam (ab56676), 1:1,000
mouse anti-Vinculin: Abcam (ab130007), 1:1,000
rabbit anti-Drp1 phospho Ser-616: Cell Signaling Technologies (3455S), 1:1000
rabbit anti-Drp1 phospho Ser-637: Cell Signaling Technologies (4867S), 1:1000

### 2.1.1.2. Secondary antibodies and Western blot working dilution

IRDye ${ }^{\circledR} 800$ goat-anti-rabbit lgG (H\&L): Rockland Antibodies and Assays (611-132-003), 1:20,000

IRDye ${ }^{\circledR}$ 680RD goat-anti-mouse IgG (H\&L): LI-COR Biosciences (826-68070), 1:20,000

Horseradish peroxidase-conjugated anti-rabbit lgG (H+L): Promega (W4011), 1:3,000

Horseradish peroxidase-conjugated anti-mouse IgG (H+L): Promega (W4021), 1:3,000

### 2.2. Commonly used solvents and buffers

### 2.2.1. Mitochondrial isolation buffers

STE buffer - 220 mM sucrose, 5 mM Tris- HCl pH 7.4 at $4^{\circ} \mathrm{C} 1 \mathrm{mM}$ ethylene glycol-bis( $\beta$ aminoethyl ether)- $\mathrm{N}, \mathrm{N}, \mathrm{N}^{\prime}, \mathrm{N}^{\prime}$-tetraacetic acid (EGTA).

STEB buffer - STE buffer supplemented with $0.1 \%(w / v)$ fatty-acid free bovine serum albumin (BSA).

### 2.2.2. Mitochondrial incubation buffers

Potassium Chloride ( KCl ) buffer - 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) ( pH 7.2 at $37^{\circ} \mathrm{C}, 120 \mathrm{mM} \mathrm{KCl}, 5 \mathrm{mM}$ ethylenediaminetetraacetic acid (EDTA).

50 mM potassium phosphate buffer - $50 \mathrm{mM} \mathrm{KH} \mathrm{KO}_{4} \mathrm{pH} 7.4$ at $37^{\circ} \mathrm{C}, 1 \mathrm{mM}$ EGTA.

### 2.2.3. Reversed phase-high performance liquid chromatography (RPHPLC) solutions

HPLC buffer A - 99.9 \% (v/v) Milli-Q water $+0.1 \%(v / v)$ trifluoroacetic acid (TFA).

HPLC buffer B - 99.9 (v/v) HPLC grade acetonitrile (Fisher Scientific, UK) + $0.1 \%(v / v)$ TFA.

### 2.2.4. Western blotting solutions

Phosphate buffered saline (PBS) - $1.8 \mathrm{mM} \mathrm{KH}_{2} \mathrm{PO}_{4} \mathrm{pH} 7.4$ at $\mathrm{RT}, 137 \mathrm{mM} \mathrm{NaCl}, 2.7 \mathrm{mM} \mathrm{KCl}$, $10 \mathrm{mM} \mathrm{Na} 2 \mathrm{HPO}_{4}$.

PBST - PBS supplemented with 0.05 \% (v/v) Tween-20.

Tris-buffered saline (TBS) - 50 mM Tris- HC pH 7.4 at RT and 150 mM NaCl .

TBST - TBS supplemented with $0.1 \%(v / v)$ Tween-20.

4x loading buffer - 200 mM Tris pH 6.8 at RT, $8 \%(w / v)$ SDS, $0.4 \%(w / v)$ bromophenol blue, 40 \% (v/v) glycerol, 400 mM DTT.

SDS-PAGE running buffer - 192 mM glycine, 25 mM Tris, 0.1 \% (w/v) SDS (pH unadjusted).

Transfer buffer - 192 mM glycine, 25 mM Tris base, $20 \%(\mathrm{v} / \mathrm{v})$ methanol ( pH unadjusted).

### 2.2.5. Live-cell microscopy solutions

KREBS buffer - $126 \mathrm{mM} \mathrm{NaCl}, 2.5 \mathrm{mM} \mathrm{KCl}, 1.2 \mathrm{mM} \mathrm{NaH}{ }_{2} \mathrm{PO}_{4}, 25 \mathrm{mM} \mathrm{NaHCO} 3,1.2 \mathrm{mM}$ $\mathrm{MgCl}_{2}, 2.5 \mathrm{mM} \mathrm{CaCl} 2 \mathrm{pH}$ adjusted to 7.2 at RT.

### 2.3. Protein Concentration

### 2.3.1. Bicinchoninic acid (BCA) assay

The protein concentration of samples was determined using BCA assay by comparison to bovine serum albumin standards (BSA) (ranging from 0 to $2 \mathrm{mg} / \mathrm{ml}$ of protein). In brief, samples and BSA standards were plated in triplicate $10 \mu$ l aliquots on a 96 -well plate. Then, 50 parts reagent $\mathrm{A}(1 \% \mathrm{w} / \mathrm{v}) \mathrm{BCA}, 2 \%(\mathrm{w} / \mathrm{v}) \mathrm{Na} 2 \mathrm{CO} 3, \mathrm{pH} 11.25)$ were mixed with 1 part reagent $\mathrm{B}(4 \%(\mathrm{w} / \mathrm{v}) \mathrm{CuSO} 4.5 \mathrm{H} 20)$ and $190 \mu \mathrm{l}$ added to each well. The plate was then incubated at $37^{\circ} \mathrm{C}$ for 30 min , and absorbance was measured at 562 nm on a Spectramax Plus 382, Molecular Devices, UK.

### 2.3.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Cell lysates (mass of protein as indicated in the individual experiment) were heated in an appropriate volume of 4 X Laemmli sample buffer ( 125 mM Tris- HCl pH 6.8 , 4 \% (w/v) sodium dodecyl sulphate (SDS), $40 \%(\mathrm{v} / \mathrm{v})$ glycerol, 25 mg bromophenol blue and freshly added reducing agent ( 50 mM DTT or 10 mM TCEP)) for 5 min at $95^{\circ} \mathrm{C}$. Then, samples were loaded on a Mini-PROTEAN ${ }^{\circledR}$ TGX ${ }^{\text {TM }}$ Precast $7.5 \%, 10 \%, 12 \%$ or 4-20 \% gradient gels (BIORAD), depending on the molecular weight of the target protein(s). Prepared samples (10$50 \mu \mathrm{~g}$ ) were subjected to electrophoresis at 100 V using a running buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1 \% (w/v) SDS. To estimate proteins molecular weight, $6 \mu$ l of known molecular size markers (Precision Plus Protein Dual Color standards from BioRAD) were loaded and electrophoresed alongside the samples.

### 2.3.3. Western Blotting

SDS-PAGE gels were transferred to pre-activated (placed in methanol for 15 s) Immobilon ${ }^{®_{-}}$ FL Polyvinylidene difluoride (PVDF) membranes (Merck Millipore: IPFL00010) by wet transfer method in 25 mM Tris, 192 mM glycine, 20 (v/v) methanol, pH 8.4 (unadjusted) at 100 V for 1 h at $4^{\circ} \mathrm{C}$. Post-transfer, the membranes were incubated in Odyssey ${ }^{\circledR}$ Blocking Buffer (PBS) (LI-COR Biosciences: 927-40003) for 1 h at RT with gentle shaking (Bibby Stuart Platform Rocker STR6 at 30 revolutions/min (rpm)). Then, membranes were incubated overnight at $4^{\circ} \mathrm{C}$ in primary antibodies in $4 \%(v / v)$ blocking buffer in PBST $(137 \mathrm{mM} \mathrm{NaCl}$, $2.7 \mathrm{mM} \mathrm{KCl}, 10 \mathrm{mM} \mathrm{Na} 2 \mathrm{HPO}_{4}, 1.8 \mathrm{mM} \mathrm{KH}_{2} \mathrm{PO}_{4}, 0.05 \%(\mathrm{v} / \mathrm{v})$ Tween-20) or TBST (20 mM Tris, $150 \mathrm{mM} \mathrm{NaCl}, 0.1$ \% (v/v) Tween-20) (both pH 7.4 at RT. After several washing steps using PBST or TBST, membranes were incubated in secondary antibodies in $4 \%(v / v)$ blocking buffer/PBST or TBST for 1 h at RT, in the dark. Both the primary and secondary antibody dilutions used were specific for the antibody, see 2.1.1. Antibodies. All primary antibodies were raised in rabbit or mouse. All secondary antibodies were raised in goat and were specific for either rabbit or mouse IgG. Membranes were washed before and after secondary antibody incubations in PBST or TBST at RT (4-5 buffer changes in 1 h). Incubations and washings were performed with gentle shaking ( 30 rpm ). Final washings were performed in PBS or TBS (without Tween-20). The signal intensities of target bands were measured as fluorescence emission at 680 nm or 800 nm (depending on the secondary antibody used) with the Odyssey ${ }^{\circledR}$ CLx Infrared Imaging System or developed using enhanced chemiluminescence (Amersham ECL Prime; GE Healthcare, UK). The signal intensities of target bands were quantified with LI-COR Biosciences Image StudioTM Lite software and were normalised against those of a suitable protein loading control, such as a protein located in the same cell sub-compartment as the target protein, whose levels in cells tend to remain stable and for which there is a sensitive and specific primary antibody. For normalisation, the signal intensity of the target band was divided by the signal intensity of the control band of the same lane so that small differences in total protein loaded between samples did not affect the quantification of the target band. Transfer buffer was re- used up to five times and stored at $4^{\circ} \mathrm{C}$. Blocking buffers and antibody buffers were reused up to five times and up to three times, respectively, and stored at $-20^{\circ} \mathrm{C}$.

### 2.4. Mammalian Cell culture

### 2.4.1. Cell culture media, buffers, reagents and cell lines

Standard medium: Dulbecco's Modified Eagle's Medium (DMEM) (GibcoTM): high glucose $(4.5 \mathrm{~g} / \mathrm{I})$ or low glucose ( $1.0 \mathrm{~g} / \mathrm{I}$ ), sodium pyruvate ( $110 \mathrm{mg} / \mathrm{ml}$ ); supplemented with $10 \%$ (v/v) fetal bovine serum (FBS) (E.U.-approved, South American origin, GibcoTM), $100 \mathrm{U} / \mathrm{ml}$ penicillin and $100 \mu \mathrm{~g} / \mathrm{ml}$ streptomycin (GibcoTM);

Cell freezing medium: 90 \% (v/v) FBS, 10 \% (v/v) dimethyl sulfoxide (DMSO) (Fisher BioReagents);

Phosphate-buffered saline (PBS): Dulbecco's phosphate-buffered saline (GibcoTM) without calcium, magnesium or phenol red;

Trypsin: Trypsin-EDTA (0.25 \%), phenol red (GibcoTM);

Cell lines: C2C12 - mouse myoblast cell line (ECACC: 91031101); HeLa - human cervix epitheloid carcinoma (ECACC: 93021013); Cos7 - simian kidney SV40 transformed cells (ECACC: 87021302), Hepa1c1c7 - mouse liver hepatoma cell line (ECACC: 95090613). MEF WT, Keap1 ${ }^{\text {C1515 }}$ and Keap1C ${ }^{2265 / C 6135}$ MEF cells were kindly provided by our collaborator, Albena T. Dinkova-Kostova.

### 2.4.2. Growth and maintenance of cell lines

Cell lines were typically cultured in T75 or T125 (Thermo Fisher Scientific, Nunclon ${ }^{\text {TM }}$ Delta treated, vented) flasks in standard DMEM medium (high glucose), unless otherwise stated. Cells were incubated in a controlled atmospheric environment ( $5 \% \mathrm{CO}_{2} / 95 \%$ air humidity) at $37{ }^{\circ} \mathrm{C}$ and routinely subcultured (at $\sim 70 \%$ confluency). For subculturing, standard medium and PBS were pre-warmed from $4^{\circ} \mathrm{C}$ to $37^{\circ} \mathrm{C}$ in a water bath and trypsin was prewarmed from $4^{\circ} \mathrm{C}$ to RT . In the cell culture hood, the medium was aspirated, and cells were washed in 5 ml PBS. PBS was aspirated, then 5 ml of trypsin was added to cells. For adherent cells (e.g. C2C12 cells), excess trypsin was aspirated, leaving ~ 1 ml trypsin and cells were
incubated at $37^{\circ} \mathrm{C}$ for $3-5 \mathrm{~min}$. Cells were then resuspended by gentle pipetting up and down after the addition of 9 ml of standard medium. Fresh T75 or T125 flasks (containing 10 ml or 25 ml standard medium, respectively) were seeded with a $1 / 10-1 / 50$ dilution of cell suspension. Media in sub-confluent flasks was replaced every 2-3 days.

### 2.4.3. Cellular Fractionation

C2C12 cells were grown up to $20 \times 10^{6}$ in 150 mm dishes (Thermo Fisher Scientific). After appropriate incubations, cells were detached using trypsin, pelleted ( $150 \times \mathrm{g}, 3 \mathrm{~min}, \mathrm{RT}$ ) and resuspended in 1 mL Fractionation buffer (FB) ( 220 mM D-Mannitol, 70 mM Sucrose, 1 mM EDTA, 10 mM Hepes). Cells were homogenised either by using a tight-fitting Dounce homogeniser (Wheaton, USA) or being passed through a 27-gauge needle 10 times, and a sample was taken as a whole-cell fraction. The homogenate was centrifuged ( $720 \times \mathrm{g}, 5$ $\min , 4^{\circ} \mathrm{C}$ ), with the supernatant $(\mathrm{S} 1)$ stored and the nuclear pellet resuspended and passed through a 25 -gauge needle 10 times. The subsequent homogenate was centrifuged again ( $720 \times \mathrm{g}, 5 \mathrm{~min}, 4^{\circ} \mathrm{C}$ ), with the supernatant discarded and the pellet containing nuclei was kept as the nuclear fraction. The supernatant (S1) containing mitochondria, membranes and cytoplasm was recentrifuged ( $800 \times \mathrm{g}, 10 \mathrm{~min}, 4^{\circ} \mathrm{C}$ ) and the pellet discarded, with this step repeated x 3 or until no pellet was observed. The resulting supernatant was subsequently centrifuged ( $9000 \times \mathrm{g}, 10 \mathrm{~min}, 4^{\circ} \mathrm{C}$ ), thereafter collecting the supernatant $(S 2)$ and resuspending the pellet ( P ) in 1 mL of FB . The centrifugation step was repeated on the supernatant. The resuspended pellet (P) was centrifuged ( $9000 \times \mathrm{g}, 15 \mathrm{~min}, 4^{\circ} \mathrm{C}$ ), the supernatant was discarded, and the pellet was resuspended in $50 \mu \mathrm{FB}$ (crude mitochondrial fraction). The supernatant (S2) was subjected to an ultracentrifugation step ( $109,000 \times \mathrm{g}, 1 \mathrm{~h}, 4^{\circ} \mathrm{C}$ ) and the resulting supernatant was collected as the cytosolic fraction. The protein concentration of each fraction was measured with a BCA assay (Thermo Fisher Scientific).

### 2.5. Flow cytometry

### 2.5.1. Mitochondrial ROS measurements with Flow cytometry analysis

C2C12 and HeLa cells were cultured in full growth medium (DMEM). For flow cytometric measurements, the cells were harvested from the cultures and suspended at $0.5 \times 10^{\circ}$ cells $/ \mathrm{ml}$ of DMEM in an Eppendorf tube supplemented with MitoSOX ( $5 \mu \mathrm{M}$; Thermo Fisher Scientific) and the tubes were shaken at $37^{\circ} \mathrm{C}$ water in a bath ( 20 min , in the dark). The cells were then pelleted ( $150 \times \mathrm{g}, 3 \mathrm{~min}, \mathrm{RT}$ ) and resuspended in pre-warmed DMEM. The cells, at least 10,000 in total, were analysed using a BD LSRFortessa cell analyser (BD Biosciences). MitoSOX Red was excited by a laser at 532 nm and data was collected at forward scatter (FSC), side scatter (SSC), 586/15 (MitoSOX) and 510/20 detector. The data were analysed using FlowJo software (version 10.4.2). Median fluorescence intensity (MFI) was quantified as a measure of mitochondrial reactive oxygen species production.

### 2.5.2. Cellular ROS measurements with Flow cytometry analysis

C2C12s were seeded at $0.5 \times 10^{6}$ cells per ml. Thirty minutes before the end of the stimulation, CellROX ( $1 \mu \mathrm{M}$ ) was added directly into the cell culture medium. Cells were incubated in the dark at $37^{\circ} \mathrm{C}$ for 30 min . Fifteen minutes into the incubation with CelliROX, SYTOX Red ( $1 \mu \mathrm{M}$ ) was added into the cell culture medium. Cells were washed with PBS, scraped in PBS ( 0.5 ml ), and transferred to polypropylene FACS tubes. At least 10,000 total events were acquired using a BD LSRFortessa cell analyser (BD Biosciences). CellROX Green and SYTOX Red were excited by a laser at 488 nm and 640 nm , respectively, and the data were collected at forward scatter (FSC), side scatter (SSC), 530/30 nm (CellROX) and 660/20 (SYTOX) detector. The data were analysed using FlowJo software (version 10.4.2), with the cell debris, represented by forward and side scatter, gated out for analysis. Median fluorescence intensity (MFI) of the 530/30 nm channel was measured as a measure of cellular oxidative stress.

### 2.6. Measurement of Glutathione

### 2.6.1. GSH recycling assay

Measurements of total glutathione were conducted using an established glutathione recycling assay as depicted in Figure 2.1 (Griffith, 1980; Tietze, 1969). Whole-cell or mitochondrial fractions pellets were resuspended in $100 \mu \mathrm{l}$ of $5 \%(\mathrm{w} / \mathrm{v})$ sulfosalicylic acid and centrifuged at 16000 xg for 10 min at $4^{\circ} \mathrm{C}$. Subsequently, $10 \mu \mathrm{l}$ of supernatant was analysed for total glutathione on a 96-well plate by comparing against GSH standards ( 0 , $0.1,0.2,0.3,0.4,0.5,0.6$ and 0.7 nmoles) in $5 \%(w / v)$ sulfosalicylic acid. Samples and standards were incubated with NAD(P)H ( 0.5 mM ), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB; 0.5 mM ) and glutathione reductase from baker's yeast ( $4 \mathrm{U} / \mathrm{ml}$ ). Reduction of DTNB to 2-nitro-5-thiobenzoate (TNB) was monitored at 412 nm over 20 min using a 96 -well plate spectrophotometer (SpectraMax Plus 384; Molecular Devices, UK). The sample kinetic rates were compared to GSH standards.


Figure 2.1. Glutathione recycling assay.
Total glutathione is assessed by measurement of TNB production and subsequent absorption at 412 nm . Glutathione reacts with DTNB to form TNB and GS-TNB, which can be further reduced by an additional glutathione molecule to form GSSG and TNB. The GSSG can then be recycled back to 2GSH by glutathione reductase, using NADPH as an electron donor.

### 2.7. Microscopy

### 2.7.1. Mitochondrial ROS measurements with live-cell microscopy

C2C12 cells were plated at 75,000 cells in 27 mm Nunc Glass Bottom Dishes (Thermo Fisher Scientific) 24 h before live-cell microscopy. Cells were washed x 2 with pre-warmed PBS and incubated with MitoSox ( $5 \mu \mathrm{M}$; Thermo Fisher Scientific) containing full growth medium (DMEM with high glucose ( $4.5 \mathrm{~g} / \mathrm{L}$ D-glucose), $10 \%$ (v/v) fetal bovine serum (FBS), $100 \mathrm{U} / \mathrm{mL}$ penicillin and $100 \mu \mathrm{~g} / \mathrm{mL}$ streptomycin) for 10 min at $37^{\circ} \mathrm{C}$ in $95 \%$ humidity and 5 \% CO2. Cells were washed $x 3$ with Krebs buffer ( $118 \mathrm{mM} \mathrm{NaCl}, 4.7 \mathrm{mM} \mathrm{KCl}, 1.2 \mathrm{mM}$ $\mathrm{MgSO}_{4} 1.25 \mathrm{mM} \mathrm{CaCl} 2,1.2 \mathrm{mM} \mathrm{KH}{ }_{2} \mathrm{PO}_{4}, 25 \mathrm{mM} \mathrm{NaHCO}$ live-cell imaging platform ( $37{ }^{\circ} \mathrm{C}$ at $95 \%$ humidity and $5 \% \mathrm{CO}_{2}$, OKO lab) on a Nikon $\mathrm{N}-\mathrm{SIM}$
microscope. Images were acquired using a 355 nm (DAPI) and 568 nm (MitoSOX) laser and corresponding filters.

### 2.7.2. Immunocytochemistry

C2C12 cells were plated at 15,000 cells/well in 24 -well plates containing glass coverslips. After 16 hours, the compounds of interest were added. At the end of the incubation, the media was removed, cells were washed twice in PBS (Gibco), treated with 4 \% (w/v) paraformaldehyde for 15 mins at $37^{\circ} \mathrm{C}$, followed by three 5 min washes with PBS. Cells were then permeabilised with $0.1 \%(v / v)$ Tritonx100 in PBS for 10 min at RT, washed with PBS ( $3 \times 10 \mathrm{~min}$ ), blocked with $10 \%(\mathrm{w} / \mathrm{v})$ BSA in PBS for 20 min at RT, and then incubated with a primary antibody in $5 \%(\mathrm{w} / \mathrm{v})$ BSA in PBS for 2 h at RT. The primary antibody solution was removed, cells washed with PBS ( $3 \times 10 \mathrm{~min}$ ) and incubated with secondary antibodies in $5 \%(\mathrm{w} / \mathrm{v})$ BSA in PBS for 1 h at RT. This solution was then removed, and cells were washed with PBS ( $3 \times 10 \mathrm{~min}$ ). Coverslips were then mounted onto slides using Dako Mounting Medium ( $5 \mu \mathrm{~L} /$ coverslip; Agilent) and analysed using Andor Dragonfly spinning disk confocal microscope equipped with a Nikon Plan-Apochromat 100x/1.45 NA oil immersion objective. Z-stacks were acquired at $0.2-\mu \mathrm{m}$ steps.

For the mitochondrial morphology experiments the primary antibody used was rabbit antiTOMM20 (1:1000), and the secondary antibody was AlexaFluor594 or AlexaFluor488, both goat anti-rabbit (1:1000).

For the Nrf2 localisation experiments, the primary antibody used was rabbit anti-Nrf2 (1:1000) and the secondary antibody was AlexaFluor488 goat anti-rabbit (1:1000). Nuclei were stained with DAPI (Merck).

For lipid peroxidation experiments, mouse anti-HNE (1:1000) and the secondary antibody was AlexaFluor594 goat anti-mouse was used.

Imaging was done using 355 (DAPI), 488 (Nrf2, Tomm20) and 568 (Tomm20, HNE) nm lasers and corresponding filters.

### 2.7.3. Live cell imaging with TPB probes

C2C12, HeLa and Cos7 cells were seeded at 50,000 cells per 27 mm glass-bottomed dishes (ThermoFisher Scientific \#150682) in complete DMEM (high glucose + 10 \% dFBS + 1 \% PenStrep) and grown overnight. The following day, cells were washed twice with 1 ml complete FluoroBrite ${ }^{\mathrm{TM}}$ DMEM (ThermoFisher Scientific A1896701) and in some cases incubated with $20 \mu \mathrm{M}$ PitStop2 (Abcam) for 30 min . After this incubation, cells were placed in a Zeiss LSM880 confocal system equipped with a Zeiss Plan-Achromat 63x/1.4NA oil immersion objective. Cells were incubated with TPBCoumarin or TPBBODIPY (both 100 nM ) as well as MitoTracker Deep Red and LysoTracker DN99 (both 500 nM ).

3D images were acquired every 5 sec for 10 min using a 405 nm (TPBCoumarin), 488 nm (TPBBodipy), 568 nm (Lysotracker) or 633 nm (MitoTracker) laser and corresponding filters.

### 2.7.4. Image analysis

For measurement of mitochondrial ROS with MitoSOX cells were imaged at indicated time points before and after the addition of MitoParaquat and the fluorescence intensity was quantified in at least 30 cells per condition, with the experiment replicated at least three times.

For the mitochondrial morphology experiments, representative images for each condition were taken from > 30 cells and sorted through a blinded process into tubular, intermediate and fragmented mitochondrial categories. This process was repeated on three separate cell passages to ensure biological replication.

Nrf2 localisation was determined by counting the number of cells that exhibited cytosolic or nuclear distribution of Nrf2, >30 cells were counted for each condition. This process was repeated for three biological replicates.

For lipid peroxidation, anti-HNE staining was measured by quantifying the fluorescent intensity of at least 30 cells per condition.

When visualising TPB compounds the fluorescence intensity of cells ( $>30$ ) was measured for different conditions and repeated three times with biological replicates. The colocalisation studies involving Lysotracker and Mitotracker with corresponding TPB compounds were done using merged images.

Image analysis was done using FIJI (ImageJ) and Imaris v.9.1.2 software (Bitplane AG, Zurich, Switzerland).

### 2.8. Animals

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and the University of Cambridge Animal Welfare Policy. Female Wistar rats and male or female C57BL/6 mice (both Charles River Laboratories, UK) were maintained in pathogen-free facilities with ad libitum chow and water until 8-12 weeks old for experimental use.

### 2.8.1. Mitochondrial isolations

The basis of our preparation of isolated mitochondria comes from a differential centrifugation method by Chappell and Hansford (1972). Rats were culled by stunning and cervical dislocation, in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. Tissues were immediately excised and kept in cold STE or STEB buffer on ice, ready for mitochondrial isolation. All steps were carried out at $4^{\circ} \mathrm{C}$ and the equipment used during the isolation were detergent-free and rinsed with ice-cold STE buffer. Mitochondrial protein concentrations were determined using the BCA assay kit (Thermo Fisher Scientific, UK).

### 2.8.2. Isolation of rat liver mitochondria

The liver was rinsed thoroughly with ice-cold STE before connective tissue and fat were removed and the liver minced finely using a razor blade. The minced tissue was washed multiple times in STE buffer, before transferring to a 55 ml Potter-Elvehjem tissue grinder (Wheaton, USA). The tissue was homogenised in 40 ml STE buffer with 3 strokes of a loosefitting PTFE pestle (Wheaton, USA) before homogenising with 8 strokes of a tight-fitting PTFE pestle (Wheaton, USA). The resulting homogenate was split between two 50 ml centrifuge tubes, 20 ml of STE added to each, balanced and centrifuged ( $1,000 \mathrm{xg}, 3 \mathrm{~min}$, $4^{\circ} \mathrm{C}$ ) to pellet blood and cell debris. The supernatants from both tubes were transferred to a fresh 50 ml centrifuge tubes and re-centrifuged ( $10,000 \times \mathrm{g}, 10 \mathrm{~min}, 4^{\circ} \mathrm{C}$ ) to pellet mitochondria. The pellet was once again resuspended in fresh STE buffer and recentrifuged. The final mitochondrial pellet was resuspended in 7 ml of STE buffer and kept on ice. Concentrations for rat liver mitochondrial isolations were roughly $30 \mathrm{mg} / \mathrm{ml}$.

### 2.8.3. Mitochondrial uptake of mitochondria-targeted compounds

Rat liver mitochondria ( 0.5 mg protein $/ \mathrm{ml}$ ) were incubated in 2 ml of KCl buffer ( pH 7.2 unless otherwise stated, $37^{\circ} \mathrm{C}$ ) supplemented with compound and internal standard ( $5 \mu \mathrm{M}$ each, unless otherwise indicated) in a shaking heat block (1,000 rpm; Eppendorf, UK). Mitochondria were energised with either: succinate ( 10 mM ) and rotenone ( $4 \mu \mathrm{~g} / \mathrm{ml}$ ), glutamate and malate ( 10 mM each). Uptake of compounds was assessed after 5 min of incubation. FCCP $(1 \mu \mathrm{M})$ or nigericin ( 100 nM ) were added at the beginning of incubations to assess the uptake of compounds with a depleted or maximal mitochondrial membrane potential, respectively, both with 5 min incubations. After appropriate incubation time, samples were rapidly cooled on ice before pelleting mitochondria by centrifuging (10,000 $\mathrm{xg}, 5 \mathrm{~min}, 4 \mathrm{C}$ ). $750 \mu \mathrm{l}$ supernatant was removed and snap-frozen in liquid nitrogen before discarding the remaining supernatant and drying the mitochondrial pellet. Mitochondria were extracted with the dropwise addition of $250 \mu$ HPLC buffer B while vortexing. Extracted mitochondria were centrifuged ( $17,000 \times \mathrm{g}, 10 \mathrm{~min}, 4 \mathrm{C}$ ) with the subsequent supernatant collected and snap-frozen in liquid nitrogen. Samples were stored at -80C until
further analysis. Before analysis, samples were thawed and diluted to a final concentration of $25 \%$ HPLC buffer B ( $250 \mu$ I HPLC buffer B added to supernatants and $750 \mu \mathrm{l}$ HPLC buffer A added to mitochondrial matrix extracts) before filtering and analysing it by RP-HPLC.

### 2.9. Reversed phase-high performance liquid chromatography (RP-HPLC)

RP-HPLC was used to characterise the TPP-linked compounds, similar to the protocol used by Robb et al. (2015). The standard procedure for separating compounds by RP-HPLC was used in all cases and is outlined below. Prior to running samples, HPLC buffers were sonicated and lines primed (flow rate at $10.0 \mathrm{ml} / \mathrm{min}$ ) to remove air bubbles. The HPLC column was washed and equilibrated by running the following gradient twice, at a flow rate of $1.0 \mathrm{ml} / \mathrm{min}$ : 0-15 min: $5 \%$ HPLC buffer B, $15-30 \mathrm{~min}$ : $100 \%$ HPLC buffer B, $30-45 \mathrm{~min}: 5 \%$ HPLC buffer B. Samples were prepared in 1 ml volume, with $25 \%$ acetonitrile $+0.1 \%$ TFA and stored at - $80^{\circ} \mathrm{C}$ until analysis. Before separating by RP-HPLC, samples were thawed and filtered using either a $0.22 \mu \mathrm{~m}$ syringe-driven filter unit (Merck Millipore, UK) or a vacuum-driven, 1 ml volume 96 -well high-density polyethylene filter unit ( $0.25 \mu \mathrm{~m}$ pore size; Porvair Sciences, UK). A total of $800 \mu \mathrm{l}$ filtrate was loaded into a 2 ml sample loop and compounds were separated using a Jupiter 300 Å C18 RP-HPLC column (Phenomenex, UK) attached to a Widepore C18 guard column (Phenomenex, UK) using a Gilson 321 pump (Gilson, UK). Compounds were eluted using gradient elution with mobile phase composition consisting of HPLC buffer A and HPLC buffer B. Compounds were eluted at a flow rate of $1.0 \mathrm{ml} / \mathrm{min}$ with a gradient of 0-2 min: $5 \%$ HPLC buffer B, 2-17 min: 5-100\% HPLC buffer B, 17-19 min: 100\% HPLC buffer B, 19-22 min: 100-5\% HPLC buffer B and UV absorbance monitored at 220 nm with a Gilson 151 UV/Vis spectrophotometer (Gilson, UK).

### 2.10. Transcription analysis

### 2.10.1. RNA extraction

For RNA extraction from tissues, the mouse liver's medial lobe and the mouse heart were collected into RNAlater (Invitrogen). 30 mg of tissue was retrieved from the storage solution, placed in PureLink RNA Mini Kit lysis buffer (Invitrogen) in a Precellys tube (Soft tissue homogenising CK14-2ml; Bertin Instruments) and homogenised twice using a Precellys 24 tissue homogeniser (6,500 rpm, 15 seconds: Bertin Instruments). RNA was extracted using a PureLink RNA Mini Kit (Invitrogen) with the RNA eluted into $80 \mu$ of RNAfree water and immediately placed on ice. The 260:280 and 260:230 ratios were assessed using a NanoDrop spectrophotometer. The RNA concentration and RNA integrity number (RIN) were determined using the Agilent 4200 TapeStation System and RNA ScreenTape (Agilent Technologies), and samples with a RIN > 8 were used for downstream analysis.

### 2.10.2. RNA sequencing

mRNA was purified from total RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). First- and second-strand cDNA was synthesised using NEBNext synthesis modules (New England Biolabs) and purified using Agencourt AMPure XP beads (Beckman Coulter). RNA library preparation was done using NEBNext Ultra RNA Library Prep kit for Illumina (New England Biolabs). Libraries were pooled and sequenced on a HiSeq X Ten platform (Illumina) with a paired-end 150 bp (PE 150) sequencing strategy by Novogene.

### 2.10.3. RNASeq analysis

Following sequencing BCL files were demultiplexed with CASAVA (Illumina) to FASTQ files using the NIAMS cluster. These were processed to remove low-quality reads and adapter sequences using TrimGalore!, and the quality of the sequencing data was confirmed using

FASTQC. Reads were aligned to the mm10 genome using hisat 2 and a count table produced using Feature counts from RSubread. Normalisation and differential expression analysis was carried out using DESeq2 within the R statistical environment. For comparison of all the samples the differential expression between various conditions and control (sham injection) was calculated using a negative binomial distribution model, from which the package DESeq2 estimates dispersion and logarithmic fold changes. In the comparisons with two different tissues (heart and liver) the design formula used for modeling took into consideration of the effects from just the conditions irrespective of the tissue. For plotting of the QC graphs and data plots the following packages within R were used: DESeq2, ggplots, fgsea and pheatmap. Gene ontology and upstream transcription factor analysis was carried out using the g:Profiler (Raudvere et al., 2019), a web server for functional enrichment analysis and conversions of gene lists. All packages are publicly available for download from the CRAN and Bioconductor servers.

The resulting data has been uploaded to PubMED GEO under GSE117425.

### 2.11. NQO1 Assay

To measure NQO1 activity, inducer potency was quantified by use of the NQO1 bioassay in Hepa1c1c7 mouse hepatoma and MEF cells (Fahey et al., 2004; Prochaska \& Santamaria, 1988). Cells ( $1 \times 10^{4}$ per well of a 96 -well plate) were grown for 24 h and exposed ( $\mathrm{n}=8$ ) to serial dilutions of compounds for the time periods indicated in the figure legends. NQO1 enzyme activity was quantified in cell lysates using menadione as a substrate. Protein concentrations were determined in aliquots from the same cell lysates by the bicinchoninic acid (BCA) assay (Thermo Scientific). The CD value was used as a measure of inducer potency.

### 2.12. Synthesis of MitoCDNB Control



Diisopropylamine ( $598 \mu \mathrm{~L}, 3.34 \mathrm{mmol}, 2.0 \mathrm{eq}$ ) was added to a stirred solution of 5 -chloro-2,4-dinitrotoluene ( $397 \mathrm{mg}, 1.83 \mathrm{mmol}, 1.1 \mathrm{eq}$ ) and (4-aminobutyl)triphenylphosphonium bromide ( $692 \mathrm{mg}, 1.67 \mathrm{mmol}, 1.0 \mathrm{eq}$ ) in dry acetonitrile ( 10 mL ) at RT under argon. The solution was stirred overnight then extracted into $\mathrm{CH}_{2} \mathrm{Cl}_{2}(30 \mathrm{~mL})$. The organic layer was washed with 1 M hydrochloric acid $(2 \times 100 \mathrm{~mL})$, dried over sodium sulfate and concentrated under vacuum. The residue was purified by column chromatography using a 12 g Agela cartridge eluting with $\mathrm{CH}_{2} \mathrm{Cl}_{2}: \mathrm{MeOH}$ (100:0 increasing to 90:10 over 10 column volumes) to give the phosphonium salt as a yellow solid foam ( $638 \mathrm{mg}, 69 \%$ ). This was done by Dr Stuart Caldwell in Dr Richard Hartley's lab (School of Chemistry, University of Glasgow, UK).
$\delta_{\mathrm{H}}\left(400 \mathrm{MHz}: \mathrm{CDCl}_{3}\right): 1.74-1.84\left(2 \mathrm{H}, \mathrm{m}, \mathrm{PCH}_{2} \mathrm{CH}_{2}\right), 2.08-2.15\left(2 \mathrm{H}, \mathrm{m}, \mathrm{NCH}_{2} \mathrm{CH}_{2}\right), 2.71(3 \mathrm{H}, \mathrm{s}$, $\mathrm{Me}), 3.80\left(2 \mathrm{H}, \mathrm{m}, \mathrm{NHCH}_{2}\right), 4.05-4.12\left(2 \mathrm{H}, \mathrm{m}, \mathrm{PCH}_{2}\right), 7.25(1 \mathrm{H}, \mathrm{s}, \mathrm{H}-6), 7.64-7.69(6 \mathrm{H}, \mathrm{m}, \mathrm{ArH})$, 7.74-7.79 (3H, m, ArH), 7.86-7.91 (6H, m, ArH), $8.29(1 \mathrm{H}, \mathrm{t}, \mathrm{J}=5.1 \mathrm{~Hz}, \mathrm{NH}), 8.97(1 \mathrm{H}, \mathrm{s}, \mathrm{H}-$ $3)$.
$\delta_{\mathrm{c}}\left(101 \mathrm{MHz}: \mathrm{CDCl}_{3}\right): 19.83\left(\mathrm{~d}, \mathrm{~J}=3.9 \mathrm{~Hz}, \mathrm{CH}_{2}\right), 22.27\left(\mathrm{~d}, \mathrm{~J}=50.6 \mathrm{~Hz}, \mathrm{CH}_{2}\right), 22.27\left(\mathrm{CH}_{3}\right), 29.07$ (d, J=17.1 Hz, CH2 $), 42.44\left(\mathrm{CH}_{2}\right), 117.83(\mathrm{CH}), 118.34(\mathrm{~d}, \mathrm{~J}=85.9 \mathrm{~Hz}, \mathrm{C}), 125.79(\mathrm{CH}), 128.59$ (C), $130.60(d, J=12.6 \mathrm{~Hz}, \mathrm{CH}), 133.84(\mathrm{~d}, J=10.0 \mathrm{~Hz}, \mathrm{CH}), 135.15(\mathrm{~d}, J=3.0 \mathrm{~Hz}, \mathrm{CH}), 137.06$ (C), 143.81 (C), 147.25 (C).
$\delta_{p}\left(162 \mathrm{MHz}: \mathrm{CDCl}_{3}\right): 24.66(\mathrm{~s})$.
$\mathrm{m} / \mathrm{z}(E S I)$ : Found: 514.1881. $\mathrm{C}_{29} \mathrm{H}_{29} \mathrm{O}_{4} \mathrm{~N}_{3} \mathrm{P}$ requires $\mathrm{M}^{+}, 514.1890$.

### 2.13. Statistical analysis

Data presented in this thesis are represented as mean $\pm$ S.E.M. (or S.D. when indicated), with ' $n$ ' values representing either the number of independent experiments or biological replicates as indicated in figure legends. Statistical analysis was assessed using Prism 7.0 (Graphpad, USA). Comparisons between two datasets were assessed via two-tailed unpaired Student's t-tests, assuming equal variance. For comparison of multiple datasets, one or two-way ANOVA was used, with the appropriate correction for multiple comparisons. The p-value, or associated probability, was considered significant if $* \mathrm{p}<0.05$; ${ }^{* *} \mathrm{p}<0.01 ;{ }^{* * *} \mathrm{p}<0.001,{ }^{* * * *} \mathrm{p}<0.0001$.

### 2.14. Collaborative experiments

Where experiments have been conducted by or with collaborators, these are explicitly stated in the figure legends IV injections were performed by Ms Olga Sauchanka and Dr John Mulvey in Dr Thomas Krieg's lab (Department of Medicine, University of Cambridge, UK). Maureen Higgins performed NQO1 assays in the lab of Dr Albena Dinkova Kostova (Jacqui Wood Cancer Centre, University of Dundee, UK). John Ferdinand performed processing of the RNA sequencing pre-analysis in the lab of Dr Menna Clathworthy (Laboratory of Molecular Biology, University of Cambridge, UK).

# Chapter 3. Characterisation of the effect of mitochondrial thiol disruption by MitoCDNB 

### 3.1. Introduction

There are many mitochondrial thiol redox systems that are independent from those in the cytosol (Booty et al., 2015; Griffith \& Meister, 1985; Mårtensson et al., 1990) and include the organelle's GSH and Trx systems, both of which are critical for cell viability and function (Fernández-Checa et al., 1998; Go \& Jones, 2013; Murphy, 2012). Furthermore, disruption of these systems is frequently associated with a range of pathologies (Go et al., 2013; Hayes et al., 2005). Mitochondrial GSH is synthesised in the cytosol and then transported from the cytosol into the mitochondrial matrix where it is maintained in a reduced state by glutathione reductase (Lu \& Holmgren, 2014; Murphy, 2012). GSH is used by glutathione peroxidases 1 and 4, glutathione-S-transferases and glutaredoxin-2 to protect against ROS, electrophiles, xenobiotics and protein thiol oxidation (Brigelius-Flohé \& Maiorino, 2013; Lu \& Holmgren, 2014; Murphy, 2012). The mitochondrial Trx system consists of Trx2 and thioredoxin reductase 2, which maintains Trx2 in a reduced state by using mitochondrial NADPH as a substrate (Arnér \& Holmgren, 2000; Lillig \& Holmgren, 2007). Trx2 maintains the activities of the peroxidase PRX3 and of methionine sulfoxide reductases, while also directly reducing protein disulfides (Cox et al., 2010; Lillig \& Holmgren, 2007; Lu \& Holmgren, 2014).

In order to explore the significance of mitochondrial thiol redox homeostasis in health and different pathologies, it is important to be able to manipulate it acutely in cells and in vivo independently of the cytosolic and other thiol redox states. A genetic approach to achieve this goal has limitations, as the deletion of some genes encoding for mitochondrial proteins
such as Trx2 (Nonn et al., 2003), GPx4 and TrxR2 (Conrad, 2009) implicated in thiol redox homeostasis are embryonically lethal, while other GPXs are known to be localised in both the mitochondria and the cytosol (Kelner \& Montoya, 2000). Consequently, a chemical biology approach to independently target the mitochondrial GSH and Trx system has been developed in the form of MitoCDNB (Booty et al., 2019).

1-chloro-2,4-dinitrobenzene (CDNB) is commonly used to deplete GSH, through its actions as a glutathione S-transferase substrate that catalyses the conjugation of GSH to CDNB to form glutathione dinitrobenzoate (GS-DNB) (Han et al., 2003; Nkabyo et al., 2002). In addition, CDNB inhibits the mammalian TrxR by irreversibly conjugating the active site selenol in TrxR (Urig et al., 2006). There have been many reports of CDNB being used to deplete GSH and inactivating TrxR, but these actions occur throughout the whole cell and are not organelle specific. Therefore, CDNB was modified in order to selectively target the mitochondria by conjugating it to the triphenylphosphonium (TPP) cation to make MitoCDNB (Booty et al., 2019) (Figure 3.1).

The TPP-conjugation concept is well-established in targeting various molecules to mitochondria in cells and in vivo (Murphy \& Smith, 2007; Smith et al., 2011, 2012b; Smith et al., 2003). Selective mitochondrial accumulation is achieved by utilizing the mitochondrial membrane potential, 140-160 mV in vivo (Smith et al., 2011), resulting in accumulation of TPP cations of several hundred-fold within mitochondria. Hence, TPP compounds can selectively affect mitochondria processes, while the rest of the cell is unaffected.


Figure 3.1. Molecular structure of MitoCDNB.

In earlier work (Booty et al., 2019) have shown that MitoCDNB accumulates up to 1000fold in isolated mitochondria, consistent with the magnitude of the mitochondrial membrane potential ( $\sim 180 \mathrm{mV}$ ), and once within mitochondria it reacts with GSH to form the membrane-impermeant MitoGSDNB (Booty et al., 2019). A rapid reaction of the two was initiated in the presence of recombinant GST, or by an isolated mitochondrial matrix extract due to the presence of endogenous GSTs. MitoCDNB also inhibited recombinant mammalian cytosolic TrxR1 and mitochondrial TrxR2 through alkylation of the active site selenol. Furthermore, it also alkylated active thiols on Prx3 leading to inhibition of these proteins. Therefore, MitoCDNB can both deplete GSH and inhibit the mitochondrial TrxR2, selectively depleting mitochondrial thiol defense mechanisms. It exhibited minimal impact on either mitochondrial respiration or mitochondrial membrane potential, keeping its effects on mitochondrial function secondary to disruption of GSH and Trx systems at working concentrations (Booty et al., 2019). MitoCDNB had minimal effects on cell growth and was stable in cell media. Cells incubated with MitoCDNB showed substantial decrease in the amount of MitoCDNB, whilst MitoGDSNB accumulated in the medium after incubation. Therefore, MitoCDNB is taken up by cells and rapidly reacts with GSH to form MitoGSDNB before excretion (Figure 3.2). In cells, MitoCDNB diminished mitochondrial GSH content while leaving the cytosolic levels of GSH intact, which is the opposite of CDNB's effect. Furthermore, within cells it led to inhibition of mitochondrial TrxR2 and alkylation of active cysteines on mitochondrial Prx3. After this, the study successfully showed that the same pattern of selective reactivity with mitochondrial thiol redox systems in vivo. To show this, MitoCDNB was injected IV and it was shown that MitoCDNB was readily taken up by mitochondria in tissues within mice, there it formed MitoGSDNB which was then rapidly excreted from the cells and the body through the urine. MitoCDNB injections specifically depleted the mitochondrial GSH pool in the liver, which returned to control levels 7 days post injection. In contrast, the total GSH levels in the heart tissue were not depleted, rather increased 24 h after MitoCDNB injection. With regards to TrxR2 activity, MitoCDNB injection led to inhibition of it in mitochondria compared to the cytosol, with 7 days needed for about 70\% recovery. Furthermore, there was a marked increase in expression of GCL, the rate limiting enzyme for GSH synthesis, as well as GSS (Figure 3.2).


Figure 3.2. Mode of MitoCDNB action. The mitochondria-targeting TPP of MitoCDNB leads to its selective accumulation within the mitochondrial matrix in vitro and in vivo, driven by the plasma and mitochondrial membrane potentials. Within mitochondria the CDNB moiety acts as a GST substrate to deplete GSH, and also a TrxR2 inhibitor. Thus, MitoCDNB selectively disrupts mitochondrial thiol homeostasis through its effects on the GSH and Trx systems. Taken from (Booty et al., 2019).

MitoCDNB selectively disrupts the mitochondrial GSH and Trx systems, enabling us to explore the many roles of mitochondrial thiol homeostasis in health and disease. While the mitochondrial GSH and Trx systems are thought to play a key role in preventing pathologies, such as mitochondrial dysfunction, neurological disorders, ischaemia/reperfusion injury, alcoholic liver disease, diabetes and thyroid diseases (Booty et al., 2015; Fernández \& Videla, 1996; Hayes et al., 2005; Jung \& Thomas, 1996; Sies et al., 2017), the independent role of the mitochondrial pool has not been investigated. With the development of MitoCDNB it is now possible to assess the role of mitochondrial thiol redox homeostasis independently of that in the cytosol.

Much of the work of this chapter has been published in Cell Chemical Biology (Booty et al., 2019).

### 3.2. Chapter hypothesis

The hypothesis to be tested in this chapter is that the selective disruption of mitochondrial thiol homeostasis, through mitochondrial glutathione depletion and inhibition of mitochondrial TrxR2, leads to adverse effects on mitochondrial physiology and also alters mitochondrial redox signalling pathways.

### 3.3. Aims and rationale

In this chapter, I aim to understand the role of mitochondrial thiol homeostasis and the consequences of its disruption towards mitochondrial physiology. The initial work will focus on ROS production within mitochondria through microscopy and flow cytometry. Subsequently mitochondrial morphology and dynamics will be assessed in order to establish the impact of disruption to thiol metabolism on mitochondrial physiology. After this, as mitochondrial redox homeostasis has been implicated in cross talk signalling to the nucleus to regulate gene expression at the level of transcription, a full transcriptome approach will be taken in order to probe the effects of mitochondrial thiol redox homeostasis on the expression of various clusters of genes.

### 3.4. Results

### 3.4.1. Interplay between mitochondrial thiol homeostasis and mitochondrial ROS production

Mitochondrial thiol defence mechanisms, such as mitochondrial GSH and TrxR2 are important in degrading $\mathrm{H}_{2} \mathrm{O}_{2}$ and its subsequent products in the mitochondrial matrix through glutathione peroxidases and peroxiredoxins. MitoCDNB has been previously shown to deplete mitochondrial glutathione content and to inhibit TrxR2 activity selectively within cells and in vivo without altering the cytosolic thiol redox homeostasis (Booty et al.,
2019). Therefore, it is important to investigate the interplay between disruption of mitochondrial thiol defence mechanisms and mitochondrial ROS production and degradation.

ROS production was assessed by measurement of fluorescence intensity of the mitochondrial indicator MitoSOX Red with both live-cell imaging and flow cytometry.


MitoSoxRed, $\mathrm{R}=-\left(\mathrm{CH}_{2}\right)_{4}{ }^{+} \mathrm{P}(\mathrm{Ph})_{3}$





Figure 3.3. A schematic illustration of the oxidation of MitoSOX probe. The positive charge of the TPP moiety of MitoSOX is responsible for its intra mitochondrial accumulation. MitoSOX reacts with superoxide to form a radical cation that then reacts with superoxide to form a hydroxyperoxide adduct that rearranges to a quinone imine form and then to a 2-hydroxy derivative (MitoSOX-OH). However, other one-electron oxidants can also generate the radical cation that can be further oxidized, or disproportionate to MitoSOX. Taken from (Shchepinova et al., 2017).

MitoSOX Red (Figure 3.3) is a mitochondrial ROS indicator, a fluorogenic dye for highly selective detection of ROS in the mitochondria of live cells (Zielonka \& Kalyanaraman, 2010). MitoSOX Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria due to its TPP as described before (Kauffman et al., 2016; K. Robinson et al., 2008). Once in the mitochondria, MitoSOX Red reagent is oxidized by superoxide as well as other oxidants (Figure 3.3) and exhibits red fluorescence (Zielonka \& Kalyanaraman, 2010). Fluorescence imaging of MitoSOX-stained cells has been claimed as a selective assay
for intracellular and intra-mitochondrial ROS production (Kauffman et al., 2016; K. Robinson et al., 2008). Therefore, the effect of MitoCDNB on the oxidation of MitoSOX is a reasonable readout for the effect of MitoCDNB on mitochondrial $\mathrm{H}_{2} \mathrm{O}_{2}$ production or degradation, as this would be observed as an increase or decrease of oxidants.

ROS production in mitochondria in live cells was assessed by measurement of relative fluorescence intensity using confocal microscopy images (Figure 3.4 A ). C2C12 cells, cultured at sub-confluency levels, were incubated with $5 \mu \mathrm{M}$ MitoSOX and either vehicle ( $0.1 \%$ ethanol) or $10 \mu \mathrm{M}$ MitoCDNB prior to treatment with $5 \mu \mathrm{M}$ MitoPQ for 30 min . MitoPQ is a mitochondrial targeted redox cycler, which leads to elevated levels of superoxide production at Complex I (Robb et al., 2015).

The concentration of MitoCDNB and MitoPQ were chosen due to previous publications and experiments done (Booty et al., 2019; Robb et al., 2015). These experiments with MitoCDNB showed that these concentrations led to the effective disruption of the mitochondrial thiols systems, without killing the cells, while these levels of MitoPQ generated large amounts of superoxide within the mitochondrial matrix that was rapidly converted to $\mathrm{H}_{2} \mathrm{O}_{2}$, thus enabling the ability of the mitochondrial thiols systems to degrade $\mathrm{H}_{2} \mathrm{O}_{2}$.

Relative fluorescent intensity of MitoSOX was measured before and after 30 min of addition of MitoPQ and again after 30 min of incubation with MitoPQ. Cells incubated with MitoCDNB exhibited a 70\% increase in MitoSOX fluorescence intensity compared to the vehicle with just MitoSOX (Figure 3.4 B). Elevation of superoxide, and thus hydrogen peroxide levels by the addition of MitoPQ led to a further increase in MitoSOX fluorescence.


Figure 3.4. MitoCDNB leads to elevated MitoSOX fluorescence. (A) Representative maximum projections of superoxide production measured by MitoSOX fluorescence in C2C12 myoblasts at 0 min or 30 min after addition of $10 \mu \mathrm{M}$ MitoPQ. Myoblasts were incubated with MitoSOX ( $5 \mu \mathrm{M}$ ) and either $0.1 \%$ ethanol (control) or MitoCDNB $(10 \mu M)$ prior to MitoPQ addition. Red is oxidized MitoSOX and blue is DAPI nuclear staining. Scale bar $=10 \mu \mathrm{~m}$. (B) Change in MitoSOX fluorescence over time in C2C12 cells treated with $0.1 \%$ ethanol
(control) or MitoCDNB $(10 \mu M)$ before addition of MitoPQ (10 $\mu M$ ). Data are means $+/-$ SEM, $n=4$. Student $t$ test: ${ }^{*} p<0.05,{ }^{* *} p<0.01,{ }^{* * *} p<0.001,{ }^{* * * *} p<0.0001$.

To complement the live cell imaging results, a MitoSOX-based flow cytometry protocol for detecting mitochondrial ROS formation was adapted from Kauffman et al., 2016. After optimisation of the procedure, it was used as an additional measure of mitochondrial ROS levels in cells. The flow cytometer enables real-time measurement of the fluorescence emission from a sample of many cells, which is reported as dot plots as shown in Figure 3.5. The gating strategy (Figure 3.5) illustrates how live, single and fluorescence-positive cells were distinguished from dead debris, doublet cells as well as from non-fluorescent cells. The histogram (Figure 3.5) shows the counts of fluorescence emission and the fluorescence intensity at 582 nm .


Figure 3.5. Representative gating strategy for FACS analysis of mitochondria ROS production in C2C12 cells (image representative of 5 independent experiments), which were stained with MitoSox Red ( $5 \mu \mathrm{M}$ ) and treated with $0.1 \%$ ethanol (control), MitoCDNB $(10 \mu M)$, TPMP (10 $\mu \mathrm{M}$ ) and CDNB ( $10 \mu \mathrm{M}$ ). The latter two serve as important controls for the TPP and CDNB moiety of MitoCDNB, respectively.

C2C12 and HeLa cells were scraped and resuspended in $1 \times 10^{6}$ cells per mL in DMEM and incubated with Vehicle ( $0.1 \%$ Ethanol), $10 \mu \mathrm{M}$ MitoCDNB, $10 \mu \mathrm{M}$ TPMP or $10 \mu \mathrm{M}$ CDNB for 30 min before MitoSOX was added at the concentration of $5 \mu \mathrm{M}$ for an additional 30 min . Cells were pelleted and resuspended in PBS into FC tubes for Flow cytometry. The median fluorescence intensity detected by the MitoSOX-based flow cytometry in C2C12 and HeLa cells are represented as bar graphs in Figure 3.6 and 3.7, respectively. No differences in fluorescence intensity are observed on addition of TPMP and CDNB to the cells while incubating with MitoSOX Red. However, there are large differences in fluorescence with MitoCDNB addition, which was reproducible in both cell lines.


Figure 3.6. MitoSOX fluorescence in C2C12 cells using FACS. Change in superoxide production quantified using median fluorescence intensity from FACS measurements of C2C12 cells stained with MitoSOX Red and treated with $0.1 \%$ ethanol (control), MitoCDNB (10 $\mu \mathrm{M}$ ), TPMP (10 $\mu \mathrm{M}$ ) and CDNB (10 $\mu \mathrm{M}$ ). Data are mean $+/-$ SEM, $n=6$. One-way ANOVA (Tukey's post hoc correction for multiple comparisons): * $p<0.05,{ }^{* *} p<0.01$, ${ }^{* * *} p<0.001,{ }^{* * * *} p<0.0001$.


Figure 3.7. MitoSOX fluorescence in HeLa cells using FACS. Change in superoxide production quantified using median fluorescence intensity from FACS measurements of HeLa cells stained with MitoSOX Red and treated with $0.1 \%$ ethanol (control), MitoCDNB (10 $\mu \mathrm{M}$ ), TPMP ( $10 \mu \mathrm{M}$ ) and CDNB $(10 \mu \mathrm{M})$. Data are mean $+/-$ SEM, $n=3$. One-way ANOVA (Tukey's post hoc correction for multiple comparisons): ${ }^{*} p<0.05,{ }^{* *} p<0.01,{ }^{* * *} p<$ 0.001 , $^{* * * *} p<0.0001$.

Our investigations of ROS production within mitochondria using the MitoSOX Red reagent with live cell microscopy and flow cytometry clearly suggested that MitoCDNB increases ROS levels in cells, even without additional stressors such as MitoPQ, which has been shown previously (Booty et al., 2019). TPMP and CDNB on the other hand show no effect on mitochondrial ROS production under the same conditions, confirming that the differences were not due to an effect from the TPP moiety of MitoCDNB alone (Reily et al., 2013), or cytosolic glutathione depletion (due to CDNB) and therefore the effect seen is in fact specific to disruption of mitochondrial thiol defenses.

Experiments with isolated heart mitochondria (Booty et al., 2019) have shown that there is no increase in $\mathrm{H}_{2} \mathrm{O}_{2}$ release from mitochondria after incubation with MitoCDNB. However, when $\mathrm{H}_{2} \mathrm{O}_{2}$ efflux was amplified using MitoPQ, which generates superoxide
within mitochondria and from this $\mathrm{H}_{2} \mathrm{O}_{2}$ (Robb et al., 2015), $\mathrm{H}_{2} \mathrm{O}_{2}$ efflux was further enhanced by MitoCDNB (Booty et al, 2019). In cells, experiments using MitoSOX did show that MitoCDNB leads to an increased level of mitochondrial ROS, but it is unsure if this also leads to a cytosolic increase in ROS as $\mathrm{H}_{2} \mathrm{O}_{2}$. Furthermore, previous cell experiments have shown that MitoPQ also does not lead to a release of $\mathrm{H}_{2} \mathrm{O}_{2}$ in the cytosol but does increase mitochondrial ROS (Hinchy et al., 2018). Similarly, experiments involving HyPer probes and mitochondrial D -amino acid oxidases have shown that hydrogen peroxide is contained in the mitochondrial matrix (Pak et al., 2020).

Thus, selectively depleting the mitochondrial thiol system with MitoCDNB does impact the levels of mitochondrial ROS, but it is yet unclear if it affects $\mathrm{H}_{2} \mathrm{O}_{2}$ release to the cytosol. It is important to note that even if the release is minimal hydrogen peroxide can elicit local effects.

### 3.4.2. MitoCDNB acutely alters mitochondrial morphology

As mentioned before in the introduction, mitochondria are highly dynamic organelles, constantly moving, fusing and dividing in response to specific cellular needs (Pernas \& Scorrano, 2016). Cellular redox homeostasis has been recently linked with mitochondrial dynamics (Willems et al., 2015), where for example cytosolic accumulation of GSSG has led to mitochondrial hyperfusion (Shutt et al., 2012). However, the specific contribution of mitochondrial matrix thiol redox state to mitochondrial dynamics has been difficult to assess as existing methods that alter mitochondrial thiol redox state also affect the cytosolic redox state (i.e. CDNB). Therefore, MitoCDNB gives us the opportunity to specifically assess if mitochondrial thiol redox alterations alone can affect mitochondrial plasticity and morphology.

Live cell imaging of C2C12 cells transiently expressing the mitochondrial marker, MitoGFP, showed no mitochondrial fragmentation in cells with vehicle ( $0.1 \%$ ethanol), or with cells incubated with $10 \mu \mathrm{M}$ TPMP, which served as a control of the TPP moiety of MitoCDNB and
its effect on mitochondrial membrane potential. In contrast, addition of MitoCDNB for 30 min led to a rapid fragmentation of the mitochondrial network (Booty et al., 2019).

The preliminary experiments involving immunocytochemistry had been done with C2C12 myoblasts, where they were exposed to either vehicle ( $0.1 \%$ ethanol) or $10 \mu \mathrm{M}$ MitoCDNB for 1, 2 or 4 h . This was done in order to establish a time frame for future more in-depth experiments. Figure 3.8 shows that the highest level of mitochondrial fragmentation can be observed after a 4 h period and thus I focused on this time point for subsequent experiments.


Figure 3.8. Representative maximum projection images of C2C12 mouse myoblasts immunostained for Tom20 and analysed by confocal microscopy to visualize mitochondria. The top row represents the Vehicle ( $0.1 \%$ Ethanol) condition and the bottom row MitoCDNB ( $10 \mu \mathrm{M}$ ) at 1,2 and 4 hours of incubation. A magnified image is shown for the 4 h time point to show morphology. Scale bar represents $20 \mu \mathrm{~m}$.

To assess mitochondria dynamics, immunocytochemistry was again employed to observe mitochondrial morphology. C2C12 myoblast cells were exposed to increasing concentrations of MitoCDNB, TPMP, CDNB and FCCP ( 1,5 and $10 \mu \mathrm{M}$ ) for 4 h before fixation on coverslips and immunofluorescently stained for the mitochondrial outer membrane marker TOMM20. Representative images are shown in Figure 3.9. Cells were examined and the mitochondria grouped into three groups: fragmented, intermediate and tubular as
seen in Morita et al., 2017 to qualitatively assess their morphology. It was expected that most mitochondria would present the intermediate and tubular morphology, while a low amount of cells would have a fragmented morphology.


Figure 3.9. Investigation of mitochondrial morphology in C2C12 cells. (Top) Representative maximum projection images of C2C12 myoblasts immunostained for Tom20 and analysed by confocal microscopy to
visualise mitochondria. Mitochondrial morphology was graded as tubular, intermediate or fragmented as representative images show. Immunofluorescence imaging of C2C12 myoblasts incubated with either 0.1\% ethanol (control) or various concentrations (1,5 and $10 \mu M$ ) of MitoCDNB, TPMP, CDNB and FCCP for 4 hours. Images are representative of three independent experiments. Scale bar $=10 \mu \mathrm{~m}$.

In the vehicle condition ( $0.1 \%$ ethanol) most of the cells exhibited mitochondria with an intermediate morphology, with a small percentage of them having either tubular or fragmented mitochondria. FCCP, a known uncoupler, served as the positive control with all of the cells showing complete fragmentation of mitochondria at the highest concentration, $10 \mu \mathrm{M}$. This is due to the fact that FCCP leads to depolarisation of the mitochondrial membrane potential, which is a known signal for fragmentation (Miyazono et al., 2018). Increasing concentration of MitoCDNB (Figure 3.10) was followed by a proportional increase in cells containing fragmented mitochondria, whereas TPMP did not have a similar correlation at the tested concentration. The CDNB condition, which leads to nonspecific lowering of whole cell GSH levels, also resulted in a fragmented mitochondrial morphology.


Figure 3.10. Quantification of mitochondrial morphology in C2C12 myoblasts. Cells were treated with $0.1 \%$ ethanol (control) and increasing concentrations (1,5 and $10 \mu \mathrm{M}$ ) of MitoCDNB, TPMP, CDNB and FCCP. Data shown as mean +/- SEM from 3 independent experiments, 100 cells were counted for each condition. Twoway ANOVA (Tukey's post hoc correction for multiple comparisons): * $p<0.05,{ }^{* *} p<0.01,{ }^{* * *} p<0.001$, **** $p<0.0001$.

These experiments have showed that mitochondrial morphology becomes fragmented in C2C12 myoblast cells when the cells are incubated with MitoCDNB for a certain period, 4 h and suggests that disrupting mitochondrial thiol homeostasis affects the dynamics of mitochondrial morphology. In order to investigate the mechanism of these alterations, the involvement of Drp1, a known fission protein factor, had to be explored.

### 3.4.3. Involvement of Drp1 in the mitochondrial fragmentation upon MitoCDNB exposure

Mitochondria exist in a dynamic network and their fusion and fission are highly regulated processes (Scott \& Youle, 2010). Fusion events of OMM are coordinated by optic atrophy gene 1 (OPA1) and IMM by Mitofusin 1 and 2 (MFN1 and MFN2) in a GTP hydrolysis dependent manner (Franco et al., 2016; Wai \& Langer, 2016). Outer membrane fission is performed by dynamin-related protein (DRP1), which translocates from the cytosol to the OMM, where it oligomerizes into ring-like structures (Wai \& Langer, 2016). One of the reasons that mitochondrial fission is thought to occur is as a mechanism of quality control to repair damaged components of the mitochondria, which allows for segregation of damaged mitochondria via the fission process, while the fission process allows for exchange of material between healthy mitochondria (Twig et al., 2008; van der Bliek et al., 2013). Many pro-fission proteins serve as recruitment factors for Drp1, which provides a platform for membrane constriction and scission (Mears et al., 2011). The resulting fragmented mitochondria have different properties and can either proceed to fuse with other mitochondria or continue down the degradation pathway.

Parallel changes in ROS levels and mitochondrial morphology have been reported in many experimental studies and led to a hypothesis that ROS is involved in short-term regulation of mitochondrial morphology and function via non-transcriptional pathways, but through local regulation of mitochondrial fusion and fission proteins (Willems et al., 2015).

In order to investigate the involvement of Drp1 in the mitochondrial fragmentation observed during disruption of mitochondrial thiol homeostasis by MitoCDNB, a Drp1
knockout HeLa cell line was produced using RNAi by our collaborators (Nagashima et al., 2020). As before, HeLa cells lacking Drp1 as well as WT HeLa cells were subjected to MitoCDNB $(10 \mu \mathrm{M})$ and vehicle ( $0.1 \%$ ethanol) for 4 h , to ease comparison with the timeline with C2C12 cells, and stained for the mitochondrial membrane marker TOMM2O. Representative images are shown in Figure 3.11.


Figure 3.11. Mitochondrial morphology of Drp1 KO cells. Representative maximum projection images of WT and Drp1 knockout HeLa cells immunostained for Tom20 and analysed by confocal microscopy to visualise mitochondrial morphology. Immunofluorescence imaging of cells was done after vehicle (0.1\% Ethanol) or 10 $\mu M$ MitoCDNB for 4 h. Images are representative of three biological replicates. Scale bar represents $20 \mu \mathrm{~m}$.

Wild type HeLa cells exhibited similar fragmented mitochondrial morphology upon MitoCDNB exposure as C2C12 myoblasts above when compared to the cells treated with vehicle. In the Drp1 KO HeLa cells treated with vehicle, mitochondria appear in a hyperfused (elongated) morphology, as described before (Tilokani et al., 2018). The described morphology does not change when the cells are incubated with MitoCDNB, suggesting a possible role of Drp1 in mitochondrial fragmentation upon disruption of mitochondrial thiol homeostasis.

In order to further elucidate the mechanism of the involvement of Drp1 in the observed mitochondrial fragmentation, cellular localisation of Drp1 was assessed to determine if

Drp1 transitions between the cytosol and mitochondria (Wai \& Langer, 2016) upon incubation with MitoCDNB. C2C12 myoblasts were treated with vehicle ( $0.1 \%$ Ethanol), TPMP (10 $\mu \mathrm{M}$ ), MitoCDNB $(10 \mu \mathrm{M})$ for 4 h and subsequently lysed and fractioned into cytosolic and mitochondria fractions using a differential centrifugation method. Drp1 subcellular localisation was then assessed by western blotting for Drp1 in the specific cellular fractions (Figure 3.12).


Figure 3.12. Drp1- localisation western blotting. Representative western blots of Drp1 protein levels in different cellular fractions of C2C12 myoblasts, (W whole cell extract; C cytosolic extract; $M$ mitochondrial extract), which were incubated for 4 hours with $0.1 \%$ ethanol (control), $10 \mu M$ TPMP, $10 \mu M$ MitoCDNB. GAPDH and TOM20 represent cytosolic and mitochondrial loading controls, respectively. The western blots are representative of three independent experiments.

The experiment (Figure 3.12) did not suggest any Drp1 transition between the cytosol to the mitochondria, which has been robustly shown to be one of the first steps in the Drp1 fragmentation pathway (Estaquier \& Arnoult, 2007; Wai \& Langer, 2016; Willems et al., 2015). In C2C12 myoblasts Drp1 appears to be already present in the mitochondrial fraction in the vehicle treatment and could therefore suggest that Drp1 is already in a primed position but that further signals are necessary to alter mitochondrial morphology.

Post-translational modification of the core protein machinery involved in fission have been extensively studied over the last 10 years (Chang \& Blackstone, 2010; Santel \& Frank, 2008;

Tilokani et al., 2018). Drp1 phosphorylation has been the most studied and phosphorylation at serine 616 and serine 637 are considered as pro-fission and pro-fusion forms, respectively. Drp1 is phosphorylated at serine 616 by cdk1/cyclin B kinase (Taguchi et al., 2007) or during cell death by protein kinase C (Qi et al., 2011), Ca ${ }^{2+}$-/calmodulin -dependent kinase II CaMKII (Kim et al., 2016; S. Xu et al., 2016) and by ERK-1/2 during cancer cell invasion (Kashatus et al., 2015; Serasinghe et al., 2015). On the other hand, protein kinase A, recruited to mitochondria through A kinase-anchoring protein (AKAP1), phosphorylates Drp1 on residue 637 inhibiting fission and protecting from autophagosomal degradation during nutrient deprivation (Gomes et al., 2011) and cell death, meaning that phosphorylation desensitizes the cells to apoptotic insults (Chang \& Blackstone, 2007; Cribbs \& Strack, 2007). Dephosphorylation of this residue is carried out by PGAM5 during necrosis (Z. Wang et al., 2012) or calcium-dependent phosphatase calcineurin during cell death (Cereghetti et al., 2008; Cribbs \& Strack, 2007; Slupe et al., 2013).

In order to investigate more about the mechanism of Drp1 activated fragmentation by MitoCDNB, the phosphorylation status of Drp1 was checked. C2C12 cells were treated with vehicle ( $0.1 \%$ ethanol), $10 \mu \mathrm{M}$ TPMP or $10 \mu \mathrm{M}$ MitoCDNB for 4 h and lysed with a RIPA buffer and phosphatase inhibitors in order to preserve the phosphorylation status of proteins. Western blotting experiments were done with anti-phospho Ser 616 and antiphospho Ser 637 Drp1 antibodies. Drp1 phosphorylation level at serine 616 remains unchanged when compared to vehicle (Figure 3.13), whereas both TPMP and MitoCDNB lead to a decrease of serine 637 phosphorylation of Drp1 (Figure 3.14). Further experiments with positive controls of the post-translational modifications are necessary.


Figure 3.13. Drp1 phosphorylation at serine 616. (Left) Representative western blots of phosphorylated Drp1 (serine 616) and Drp1 protein levels in C2C12 myoblasts, which were incubated for 4 hours with $0.1 \%$ ethanol (vehicle), $10 \mu M$ TPMP and $10 \mu M$ MitoCDNB. GAPDH represents the loading control. (Right) Quantification of Drp1 Ser616 phosphorylation level was measured relative to the Drp1 and GAPDH protein level and then compared to the vehicle condition. Data are mean $+/-$ SEM, $n=3$.



Figure 3.14. Drp1 phosphorylation at serine 637. (Left) Representative western blots of phosphorylated Drp1 (serine 637) and Drp1 protein levels in C2C12 myoblasts, which were incubated for 4 hours with $0.1 \%$ ethanol (vehicle), $10 \mu \mathrm{M}$ TPMP and $10 \mu \mathrm{M}$ MitoCDNB. GAPDH represents the loading control. (Right) Quantification of Drp1 Ser637 phosphorylation level was measured relative to the Drp1 and GAPDH protein level and then compared to the vehicle condition. Data are mean $+/-$ SEM, $n=3$.

The experiments above have shown that the observed mitochondrial fragmentation after disruption of mitochondrial thiol homeostasis with MitoCDNB involves Drp1, which is a well characterised fission protein. In C2C12 cells Drp1 was found to be already localised at the mitochondrial membrane. The investigation into post translational modifications on Drp1, more specifically the phosphorylation of serine 616 and 637 did not lead to conclusive results and suggested that other modifications, such as SUMOylation might be involved.

### 3.4.4. Effect of MitoCDNB on the mouse transcriptome

MitoCDNB injection into a mouse led to an increase in the expression of GCLC in the liver at 24 h post injection (Booty et al., 2019). As MitoCDNB has shown to only affect the thiol redox status of the mitochondrial matrix, this finding suggested that alterations to mitochondrial thiol redox state are communicated to the nucleus and subsequently affect transcription. In order to assess this possibility in more detail we assessed the effect of MitoCDNB on transcription within mice in vivo. Mouse livers ( $\mathrm{n}=6$ per condition) were isolated 1 and 4 hr after MitoCDNB or vehicle injections, homogenized and followed by RNA extraction. The transcriptomes were analysed using RNA sequencing.

Following sequencing, the resulting FASTQ files were processed to remove low quality reads and adapter sequences using TrimGalore! and quality of the sequencing data was confirmed using FASTQC. Reads were aligned to the mm10 genome using hisat2 and a count table produced using Feature counts from RSubread. Normalisation and differential expression analyses were carried out using DESEQ2 within the R statistical environment. Principal component analysis (PCA) was used as an unbiased approach to dimensionality reduction in order to observe if there is any grouping in our experiment (Figure 3.16). The PCA analysis was done using the top 500 most variably expressed genes and plotted in Figure 3.16 as Principal component 2 (PC2) vs Principal component 1 (PC1). The observed groupings by condition are reasonable, with the highest difference, as well as the best grouping, coming from the condition of 1 h after MitoCDNB injection.


Figure 3.16. Principal component analysis of effect of MitoCDNB on RNA expression levels. Mice were administered either MitoCDNB ( $5 \mathrm{mg} / \mathrm{kg}$ ) or vehicle by tail vein injection and then liver was isolated 1 and 4 h after injection and underwent transcriptome analysis with RNA seq. The transcriptomes were then subjected to principal component analysis using the top 500 most variably expressed genes. PC1 and PC2 are shown here. The samples are coloured by their condition. $N=6$.

In order to observe which genes were responsible for the observed $34.94 \%$ variance in PC1, a heatmap of the top 25 positive (Figure 3.17) and 25 negative (Figure 3.18) correlated genes was generated in the $R$ environment. Both of these heatmaps suggest that there are no anomalies within the genes that are causing the variance in PC1. For example, if the
responsible genes would be highly tissue-specific to a non-liver tissue that would possibly suggest a contamination in our extraction methods.


Figure 3.17. Heatmap of the 25 positively correlated genes, which are causing the $\mathbf{3 4 . 8 4 \%}$ variance in PC1. The legend on the right corresponds to the compound injected into the mice and the length of the incubation. The $z$-score ranges from 2 to -2 and corresponds to the log 2 fold change of the specific genes.


Figure 3.18. Heatmap of the 25 negatively correlated genes, which are causing the $\mathbf{3 4 . 8 4 \%}$ variance in PC1. The legend on the right corresponds to the compound injected into the mice and the length of the incubation. The $z$-score ranges from 2 to -2 and corresponds to the log2 fold change of the specific genes.

The differential expression analysis was performed using the R package DESEQ2 with the MitoCDNB condition compared to the vehicle condition at either 1 or 4 h . The differential transcriptome analysis showed a dramatic change in transcription in the liver 1 hr after MitoCDNB injection compared to the sham vehicle injection (Figure 3.19). The results were surprising as over 592 genes were shown to be significantly downregulated and 474 genes were significantly upregulated (Figure 3.19). Interestingly, 4 hr after the injection
the transcriptome changes were far less than at 1 hr , as there was only a total of 351 genes significantly differentially expressed at $4 \mathrm{hr}-149$ genes were downregulated, and 202 genes were upregulated (Figure 3.20). This in turn suggested that the MitoCDNB effect on the mouse transcriptome in the liver is more profound in the early stages after administration.


Figure 3.19. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB 1 hr after injection compared with vehicle - using differential expression analysis with DESEQ2 in the R environment. The negative $\log 10$ of $p$ value is portrayed on the $y$ axis and the log 2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$.


Figure 3.20. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB 4 hr after injection compared with vehicle - using differential expression analysis with DESEQ2 in the $R$ environment. The negative log10 of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$.

In order to further explore the differential expression transcriptome, a heatmap of top 20 genes that were upregulated and downregulated in their expression at 1 hr (Figure 3.21) and 4 hr (Figure 3.22) of MitoCDNB injection compared with vehicle injection was generated.


Figure 3.21. Heatmap of changes in expression for the $\mathbf{2 0}$ most upregulated and downregulated genes $\mathbf{1}$ hr after MitoCDNB injection compared to vehicle injection - using differential expression analysis with DESEQ2 in the $R$ environment. The legend on the right corresponds to the compound injected into the mice and the length of the incubation. The $z$-score ranges from 3 to -3 and corresponds to the log2 fold change of the specific genes.


Figure 3.22. Heatmap of changes in expression for the $\mathbf{2 0}$ most upregulated and downregulated genes $\mathbf{4 h r}$ after MitoCDNB injection compared to the vehicle injection - using differential expression analysis with DESEQ2 in the $R$ environment. The legend on the right corresponds to the compound injected into the mice and the length of the incubation. The $z$-score ranges from 4 to - 4 and corresponds to the log2 fold change of the specific genes.

To better our understanding of the differentially expressed transcriptome, a pathway analysis was performed using Gene Set Enrichment Analysis (GSEA), which evaluates microarray data at the level of gene sets (Subramanian et al., 2005). The gene sets are defined based on prior biological knowledge. The goal of GSEA is to determine whether the
members of a particular gene set occur at the top or bottom of the ranked members list of your transcriptome gene set.

The genes were ranked by the inverse of their $p$ value multiplied by the sign of the log fold change (1/pvalue*(abs(log2Foldchange))/log2Foldchange); large positive numbers meant significantly up regulated genes; large negative numbers are significantly downregulated genes.

After the genes were ranked, they were run against the hallmarks pathway dataset using the GSEA java applet. The pathway analysis of the 1 h and 4 h post MitoCDNB injection compared to vehicle injections are represented in Figure 3.23 and 3.24, respectively. It is clear that there is a significant difference between the MitoCDNB effect at 1 hr compared to the 4 hr post injection. The first observation is that while the pathways are mainly upregulated at 1 hr post injection, they are essentially all downregulated at 4 hr , therefore exhibiting an almost complete reverse outcome. The upregulated pathways at 1 hr post injection are involved in various signalling pathways: TNF- $\alpha$ signalling via NFкB, Hypoxia, IL2 STAT5 signalling, p53 pathway, KRAS signalling, inflammatory response and TGF- $\beta$ signalling; as well as metabolism: cholesterol homeostasis, oxidative phosphorylation and xenobiotic metabolism (Figure 3.22).


Figure 3.23. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between 1 hr post MitoCDNB injection and vehicle. The blue and red dots correspond to either downregulated or upregulated pathways, respectively. The size of the dots corresponds to the FDR-adjusted $p$ value, hence its statistical significance.

MitoCDNB at 4 hr post injection leads to reversal and therefore downregulation of signalling pathways: IFN- $\gamma$ response, JAK STAT3 signalling, TNF- $\alpha$ signalling via NF-кB and inflammatory response. Surprisingly, the only pathway that stays upregulated is the one corresponding to oxidative phosphorylation.


Figure 3.24. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between 4 hr post MitoCDNB injection and vehicle. The blue and red dots correspond to either downregulated or upregulated pathways, respectively. The size of the dots corresponds to the FDR-adjusted $p$ value, hence its statistical significance.

This analysis further indicates that the changes in transcription induced by MitoCDNB injection were far greater at 1 hr after injection compared with 4 h . Together these findings show that there is a rapid transcriptional response to changes in mitochondrial thiol redox state and therefore MitoCDNB is an important tool in investigating the retrograde signalling pathways that link mitochondria to the nucleus.

### 3.5. Discussion

In this chapter I have characterised MitoCDNB, a CDNB moiety bound to a TPP cation, which targets the mitochondrial GSH and Trx systems irrespective of its cytosolic counterparts. MitoCDNB is a novel chemical biology tool to aid in the investigation of the mitochondrial thiol systems, as they are thought to be a key determinant of cell function and its stress response. Furthermore, the introduction of MitoCDNB enabled the selective alteration of mitochondrial thiol redox state and facilitated understanding its role in many areas, including mitochondrial ROS production, redox signalling, and dynamics.

Mitochondrial thiol defence mechanisms are important in degrading $\mathrm{H}_{2} \mathrm{O}_{2}$ and its subsequent products in the mitochondrial matrix through SOD2 and Prxs. To investigate the interplay between disruption of mitochondrial thiol defence mechanisms and mitochondrial ROS production, the latter needed to be sufficiently measured. MitoSOXbased assays in mouse myoblasts suggested that MitoCDNB leads to an increase in ROS levels in mitochondria. A result that is exacerbated by the addition of MitoPQ, a mitochondrial redox cycler. $\mathrm{H}_{2} \mathrm{O}_{2}$ efflux measurements in isolated heart mitochondria led to the conclusion that MitoCDNB alone is not sufficient to be translated to a burst of cellular $\mathrm{H}_{2} \mathrm{O}_{2}$, but it does occur when further enhanced with MitoPQ (Booty et al., 2019). How mitochondria produce flux of hydrogen peroxide in whole cells has not been clearly shown yet. Experiments with D-amino acid oxidases and HyPer probes showed that hydrogen peroxide is mostly confined to the mitochondrial matrix with little flux to the cytosol (Pak et al., 2020). It is also important to note that the multitude of antioxidant defence systems act as sinks and therefore limit diffusion and possibly detection of hydrogen peroxide.

Mitochondria are constantly fusing and dividing in response to specific cellular needs (Pernas \& Scorrano, 2016). It has been proposed that cellular redox homeostasis is linked with mitochondrial dynamics (Shutt et al., 2012), due to the observation that cytosolic accumulation of oxidised GSH led to mitochondrial hyperfusion. MitoCDNB gave us the opportunity to specifically assess if alterations in mitochondrial thiol redox homeostasis alone can affect mitochondrial morphology. Mouse myoblast mitochondria did undergo a
morphological change as they exhibited a fragmented, circular shape after MitoCDNB incubations. The observed fragmentation increased in a dose dependent manner. The possible explanation for MitoCDNB induced mitochondrial fission is quality control, as fission is known to be an important part of the process (Ni et al., 2015).

Mitochondrial fusion and fission are highly regulated processes, each involving its own distinct protein machinery and regulation (Pernas \& Scorrano, 2016). DRP1 is a protein involved in outer membrane fission, translocating from the cytosol to the mitochondrial membrane, where it oligomerizes into ring-like structures (Wai \& Langer, 2016). MitoCDNB did not have an effect on mitochondrial morphology in the cells lacking DRP1, suggesting the latter's importance in the observed fragmentation of mitochondria upon disruption of mitochondrial thiol homeostasis. In addition, MitoCDNB did not influence the subcellular localisation of DRP1, as it is already present in the mitochondrial fraction in mouse myoblasts. Post-translational modifications (PTM) are an integral regulatory mechanism of DRP1 mediated mitochondrial fission (Tilokani et al., 2018). Although MitoCDNB incubations led to DRP1 being less phosphorylated at serine 637, it was not a MitoCDNB specific effect and could be due to mitochondrial membrane potential effects. There are further regulatory PTMs of DRP1 such as GlcNAcylation, S-nitrosylation as well as SUMOylation (Tilokani et al., 2018), which would be interesting to investigate in the future to elucidate the final mechanism of MitoCDNB stimulated mitochondrial fragmentation.

Mouse injections of MitoCDNB led to an increase in expression of GCLC, a rate-limiting enzyme in GSH synthesis, at 24 hours post injection (Booty et al., 2019). To fuel the curiosity of how an effect on thiol redox status of the mitochondrial matrix can be communicated to the nucleus and affect transcription, the mouse liver transcriptome was assessed using RNA sequencing. The differential transcriptome analysis showed dramatic change in transcription in the liver 1 hr after MitoCDNB injection with a lower effect observed after 4 hours. Furthermore, GSEA pathway analysis suggested that MitoCDNB mainly leads to upregulation of various pathways in the early stages, but the reversal holds true for the later time point, with most of the pathways being downregulated. This is indicative of a stress-like response to the compound. The only outlier pathway that stays upregulated in both conditions is the oxidative phosphorylation pathway (hallmarks), which features
genes involved in the mitochondrial ETC, as well as some mitochondrial genes responsible for redox homeostasis, such as peroxiredoxin 3 and glutathione peroxidase 4.

In the early stress-like response after MitoCDNB injection, the TNF- $\alpha$ induced NF-кB signalling pathway had been singled out with the GSEA as the most apparently upregulated pathway. TNF family of cytokines induce rapid transcription of genes regulating inflammation, cell survival, proliferation and differentiation, mainly through the activation of the NF-кB pathway (Hayden \& Ghosh, 2014). The NF-кB family consists of 5 related proteins, p50, p52, p65, RelB and c-Rel, that share a Rel homology domain (RHD) and bind the DNA as homo or heterodimers (Hayden \& Ghosh, 2014). In the "resting" state cytosolic NF-кB dimers are prevented from binding target sites through associates with the inhibitor of $\kappa B$ ( $1 \kappa B$ ) proteins (Hayden \& Ghosh, 2012). While there are many post-translational modifications involved in regulation of IкB proteins, other important modifications involve the NF-KB subunits and have a profound effect on its function (Christian et al., 2016). Further experiments are needed to investigate the possible link between the disruption of mitochondrial thiol redox homeostasis and this inflammatory pathway.

Overall, the novel chemical biology approach to studying mitochondrial thiol homeostasis will provide insights and enhance our ability to address many critical biochemical questions, including ischaemia-reperfusion injury, xenobiotic detoxification and neurodegenerative diseases. Furthermore, it indicates how chemical biology approaches coupled with intracellular targeting can be used to gain insight into organelle function and signalling in cells and in vivo. The next step is to synthesise an inactive control compound, which would mimic MitoCDNB's mitochondrial accumulation but not disrupt the mitochondrial thiol homeostasis.

# Chapter 4. Interplay between Nrf2 activation and mitochondrial dysfunction 

### 4.1. Introduction

Cells are equipped with elaborate defence systems that allow them to maintain homeostasis in the face of physiological stress, such as oxidative stress and damage, which are involved in the development and progression of many diseases (Finkel \& Holbrook, 2000; Sies et al., 2017). In the previous chapter, I have mentioned that after treatment with MitoCDNB transcriptional changes of hepatic GCLC, a bottleneck enzyme for GSH synthesis, were observed (Booty et al., 2019). Expression of GCLC is under the control of the transcription factor Nrf2, which plays a central role in the cytoprotective response to oxidative stress and damage (Itoh et al., 1997; Yamamoto et al., 2018). In addition to its role in overall cellular redox homeostasis, Nrf2 is also critical for the maintenance of mitochondrial antioxidant defences and organelle redox homeostasis (Dinkova-Kostova \& Abramov, 2015). This is of particular importance because mitochondria are a major source of hydrogen peroxide due to superoxide production from respiratory complexes, especially complex I (Chance et al., 1979; Murphy, 2009). Within the mitochondrial matrix, this superoxide is rapidly converted by MnSOD to hydrogen peroxide, which can both contribute to oxidative damage in a range of pathologies, but also acts as a signalling molecule that transduces redox signals through modifying the activity of redox-sensitive proteins (Collins et al., 2012; Finkel, 2011; Janssen-Heininger et al., 2008).

During unstressed conditions, Nrf2 protein levels are maintained relatively low, which is due to its constitutive ubiquitination mediated by Keap1 (Kelch-like ECH-associated protein 1), an adaptor component of a Cul3 (Cullin 3)-based ubiquitin E3 ligase complex, which targets Nrf2 for proteasomal degradation (Itoh et al., 1999; Kobayashi et al., 2004). Upon exposure to electrophiles such as sulforaphane (SFN) or oxidants such as hydrogen peroxide, specific cysteine sensors in Keap1 are modified (Dinkova-Kostova et al., 2002; McMahon et al., 2010; Saito et al., 2015). This inhibits the ubiquitination of Nrf2, which in
turn leads to its stabilization and its accumulation (Taguchi et al., 2011). Subsequently, Nrf2 translocates to the nucleus where it acts as a transcription factor, binding to antioxidant response elements (ARE) in the promotor regions of Nrf2-target genes, upregulating the expression of a series of antioxidant genes (Ishii et al., 2000; Suzuki et al., 2013).

Nrf2 activity enhances the expression of antioxidant systems (Hayes \& Dinkova-Kostova, 2014; Kasai et al., 2020), which include glutathione synthesis (MacLeod et al., 2009), GR (Agyeman et al., 2012), GPXs (Hirotsu et al., 2012), PRX3 (Malhotra et al., 2010; Miyamoto et al., 2011; I. Ryoo \& Kwak, 2018) and TRXR2 (Malhotra et al., 2010; Ryoo \& Kwak, 2018). Nrf2 activity also affects mitochondrial biogenesis by influencing expression of critical transcription factors, such as Peroxisome Proliferator Activated Receptor Gamma (PPARG) (Chorley et al., 2012). Nrf2 deficiency leads to mitochondrial damage (Holmstrom et al., 2013), as the above-mentioned responses enable mitochondria to adapt to elevated oxidative stress and damage. Hence, it is widely assumed that $\mathrm{Nrf2}$ is upregulated in response to mitochondrial oxidative stress and damage (Kasai et al., 2020). However, the specific mechanistic details by which mitochondrial oxidative stress and damage activate Nrf2 are still unclear. Possibilities include that elevated mitochondrial superoxide production generates hydrogen peroxide that goes from the mitochondria to the cytosol to activate Nrf2 directly or indirectly. Alternatively, the redox changes within mitochondria may lead to secondary signals to the cytosol that then activate Nrf2. There are also suggestions that mitochondrial dysfunction may activate Nrf2 through formation of a complex with Keap1 and the mitochondrial outer membrane serine/threonine protein phosphatase, PGAM5 (Kasai et al., 2020; Lo \& Hannink, 2008; O’Mealey et al., 2017). This Nrf2-Keap1-PGAM5 complex has been proposed to act as a hub for ROS-induced necrosis and as an activator of mitochondrial fragmentation, mediated through dephosphorylation of DRP1 (W. Wang et al., 2012). However, whether there are distinctive, mitochondrial and cytosolic pools of Nrf 2 with specified roles is unclear.

In exploring how mitochondrial oxidative stress and damage activate Nrf2 (Kasai et al., 2020) it has not been possible to distinguish between the effects of superoxide and hydrogen peroxide generation and redox changes in mitochondria independently from those in the rest of the cell. Furthermore, many Nrf2 activators cause changes in both
superoxide and hydrogen peroxide levels and in thiol homeostasis. However, these pathways interact closely and changes in thiol homeostasis can affect hydrogen peroxide levels, while conversely, increased levels of hydrogen peroxide can alter the thiol redox state via peroxidases (Dinkova-Kostova \& Abramov, 2015; Suzuki et al., 2013; Yamamoto et al., 2018). However, Nrf2 is regulated in different ways by these effectors, in part through the differential reactivity of particular thiols on Keap1 (Suzuki et al., 2019), suggesting there is a different effect of ROS such as hydrogen peroxide and thiol redox alterations on Nrf2. Therefore, I aim to address the role in Nrf2 activation of mitochondrial redox changes independently of those from the cytosol, while also distinguishing between the impact of mitochondrial superoxide and hydrogen peroxide production and that of thiol redox changes. To do this, I used two mitochondria-targeted redox active agents. To investigate thiol redox state, the previously characterised mitochondria-targeted disrupter of thiol redox homeostasis MitoCDNB, a 1-chloro-2,4-dinitrobenzene (CDNB) derivative was used. MitoCDNB is selectively taken up by mitochondria within cells where it selectively depletes mitochondrial GSH largely, by acting as a substrate for mitochondrial GSTs while also inhibiting the mitochondrial Trx system by inhibiting thioredoxin reductases was used (Figure 4.1) (Booty et al., 2019). In addition, MitoPQ, a mitochondria-targeted redox cycler was used. MPQ is selectively taken up by mitochondria where the Paraquat moiety reacts with the complex I flavin to selectively increase superoxide production by redox cycling, and thus increase hydrogen peroxide within the mitochondrial matrix (Figure 4.1) (Antonucci et al., 2019; Hinchy et al., 2018; Robb et al., 2015).

Much of the work from this chapter has been published in the Journal of Biological Chemistry (Cvetko et al., 2020).

A


B


Figure 4.1. Schematic of activity of MitoCDNB and MitoPQ and their controls. A, TPP moiety in MitoCDNB leads to its selective accumulation within the mitochondrial matrix, driven by the plasma and mitochondrial membrane potentials. Within the mitochondria the CDNB moiety acts as a GST substrate to deplete GSH, and it is also a TrxR2 inhibitor, leading to the disruption of mitochondrial thiol redox defence homeostasis. We hypothesize this could lead to Nrf2 activation and its nuclear localization. Its inactive control compound, MitoCDNB Ctrl accumulates in the mitochondrial matrix but does not lead to GSH depletion or TrxR2 inhibition. B, MitoPQ is composed of a redox cycling paraquat moiety and a hydrophobic carbon chain linking it to a mitochondria-targeting triphenylphosphonium cation. MitoPQ is accumulated by mitochondria driven by the plasma and mitochondrial membrane potentials. Within the matrix, MitoPQ is reduced to a radical
monocation by one-electron reduction at the flavin site of complex I, which subsequently interacts rapidly with $\mathrm{O}_{2}$ to generate superoxide $\left(\mathrm{O}_{2}^{*}\right)$. We hypothesize the ability of mitochondrial specific $\mathrm{O}_{2}{ }^{*-}$ production to activate the Keap1/Nrf2 pathway and lead to Nrf2 nuclear localization. The inactive control compound, MitoPQ Ctrl selectively accumulates in the matrix, but does not act as a redox cycler, and therefore is unable to produce $\mathrm{O}_{2}{ }^{\circ}$. Taken from (Cvetko et al., 2020).

### 4.2. Chapter hypothesis

My hypothesis for this chapter is that inducing mitochondrial dysfunction through two independent chemical biology approaches, would provide insights into the redox signaling mechanisms that underlie activation of Nrf2 by mitochondrial oxidative stress and damage.

### 4.3. Aims and rationale

In this chapter, I aim to understand the role of mitochondrial thiol homeostasis and mitochondrial superoxide production in activation of Nrf2 signalling. Initially, the MitoCDNB Control compound will be introduced and characterised to provide more robust experiments. Subsequently Nrf2 protein levels, subcellular localisation as well as downstream targets will be assessed to understand the effects of MitoCDNB and MitoPQ incubations. After this, possible mitochondrial signals that instigate the pathway will be investigated.

### 4.4. Results

### 4.4.1. MitoCDNB Control characterisation

As mentioned in the previous chapter's discussion, a better control compound than TPMP is necessary to assess MitoCDNB and its effect moving forward. TPMP does match MitoCDNB's charge but not its hydrophobicity, which could have a specific effect on its own and also enhance the uptake of the molecule by mitochondria, leading to effects that are
independent of thiol modification. The solution for this would be a chemically similar molecule but in its inactive form (Figure 4.2). MitoCDNB Ctrl compound was synthesised by our collaborators at the University of Glasgow and consists of the triphenylphosphonium cation, which leads to the mitochondrial accumulation due to its charge and the mitochondrial membrane potential, a linker region and a 2,4-dinitrobenzene moiety, which is the CDNB moiety of MitoCDNB but contains a methyl group instead of the $\mathrm{Cl}^{-}$leaving group, thus making it inactive in reacting with thiols or selenols (Cvetko et al., 2020) (Figure 4.2).

MitoCDNB Control



## MitoCDNB



TrxR2 $\boldsymbol{T}_{\text {Inhibition }}^{\text {of } T_{\text {IrxR2 }}}$ GST, GSH

Figure 4.2. Mechanism of MitoCDNB Ctrl compared to MitoCDNB. MitoCDNB Ctrl is missing the Cl-leaving group on the CDNB's moiety of MitoCDNB (blue bracket), which is replaced with an inert methyl group that is non-reactive. This leads to no reaction with GST, GSH or TrxR2 and no subsequent lowering of mitochondrial GSH or inhibition of TrxR2, which is shown with MitoCDNB.

First, the mitochondrial uptake of the Ctrl compound had to be established, which was predicted from the positive charge of TPP moiety and the negative mitochondrial membrane potential across the inner mitochondrial membrane. To do this, isolated rat liver mitochondria were incubated in KCl buffer with succinate ( 10 mM ) and rotenone (4 $\mu \mathrm{g} / \mathrm{ml}$ ) added to drive respiration through complex II and inhibit complex I, respectively. MitoCDNB Ctrl and MitoCDNB were added at $5 \mu \mathrm{M}$ with or without FCCP ( $0.5 \mu \mathrm{M}$ ) and
incubated for 5 min at $37{ }^{\circ} \mathrm{C}$. As a known uncoupler, FCCP diminishes the mitochondrial membrane potential and should therefore impede the uptake of TPP compounds. Mitochondria were pelleted and analysed for TPP-containing compounds by RP-HPLC. TPMP ( $5 \mu \mathrm{M}$ ) was used as an internal standard in order to help with the quantification of peaks by RP-HPLC (Figure 4.3). MitoCDNB Ctrl compound was observed in the mitochondrial pellet similarly to MitoCDNB and the uptake was diminished when FCCP was present, which confirms the uptake of these TPP compounds is dependent on mitochondrial membrane potential (Figure 4.3).


Figure 4.3. Uptake of MitoCDNB Ctrl. MitoCDNB Ctrl uptake by isolated mitochondria. Rat liver mitochondria (1 mg/ml) were incubated in KCl buffer with succinate (10 mM), rotenone ( $4 \mu \mathrm{~g} / \mathrm{ml}$ ), TPMP (5 $\mu \mathrm{M}$ ) and MitoCDNB or MitoCDNB Ctrl (both at $5 \mu M$ ) $+/-\operatorname{FCCP}(0.5 \mu M)$ at $37^{\circ} \mathrm{C}$ for 5 min and the mitochondrial pellet analyzed for TPP-containing compounds by RP-HPLC with peak area recorded. All data are mean +/- SD of three independent experiments. P Values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p$ < 0.05).

After the uptake of the MitoCDNB Ctrl was shown confirming the inactivity of its CDNB moiety is essential for it to be a suitable Control compound. Isolated rat liver mitochondria
in a KCl buffer with succinate ( 10 mM ) and rotenone ( $4 \mu \mathrm{~g} / \mathrm{ml}$ ), were incubated with MitoCDNB Ctrl and MitoCDNB (both $5 \mu \mathrm{M}$ ) for 5 min at $37^{\circ} \mathrm{C}$. Both the mitochondrial pellet and supernatant were analyzed for GSH adducts at 340 nm by RP-HPLC. As before, TPMP $(5 \mu \mathrm{M})$ was used as the internal standard for peak quantification. MitoCDNB exhibited a high GSH-adduct peak, which was present in the mitochondrial pellet and supernatant, as shown before (Booty et al., 2019), whereas there were no GSH adducts observed with MitoCDNB Ctrl (Figure 4.4). This experiment demonstrates that the CDNB moiety of MitoCDNB Ctrl is inactive and is therefore not expected to lead to any mitochondrial thiol disruptions as observed with MitoCDNB.


Figure 4.4. MitoCDNB and MitoCDNB Ctrl - GSH adduct. Rat liver mitochondria ( $1 \mathrm{mg} / \mathrm{ml}$ ) were incubated in $K C l$ buffer with succinate ( 10 mM ), rotenone ( $4 \mu \mathrm{~g} / \mathrm{ml}$ ), TPMP ( $5 \mu \mathrm{M}$ ) and MitoCDNB or MitoCDNB Ctrl (both at $5 \mu \mathrm{M}$ ) at $37^{\circ} \mathrm{C}$ for 5 min . The mitochondrial pellet and supernatant were analyzed for GSH adducts by RPHPLC with peak area quantified. All data are mean +/-SD of three independent experiments. P Values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant $p$ values are shown (statistical significance corresponds to $p<0.05$ ).

Before MitoCDNB Ctrl was used in cell experiments, a working concentration had to be determined to be comparable to MitoCDNB. C2C12 mouse myoblasts were incubated with
increasing concentrations of MitoCDNB Ctrl (up to $100 \mu \mathrm{M}$ ) and their cell growth observed with an Incucyte Zoom imager (Figure 4.5). C2C12 cells grew normally up to $20 \mu \mathrm{M}$ MitoCDNB Ctrl (Figure 4.5) and therefore confirmed that the compound can be used at 10 $\mu \mathrm{M}$ concentrations for the cell experiment similar to MitoCDNB. The above-mentioned experiments detailing the characterisation of the MitoCDNB Ctrl compound confirmed that it could be used as a control to MitoCDNB in further experiments.


Figure 4.5. Cell growth in the presence of MitoCDNB Ctrl. C2C12 cells were grown in DMEM $+10 \%$ FBS for 30 hours in an Incucyte Zoom imager. MitoCDNB Ctrl was added at concentrations of $0.5 \mu M, 1 \mu M, 5 \mu M, 10$ $\mu \mathrm{M}, 20 \mu \mathrm{M}, 50 \mu \mathrm{M}$ and $100 \mu \mathrm{M}$. Control cells had an addition of equivalent volume of ethanol (control). Cell growth was monitored by an Incucyte Zoom imager. Data are means +/- SD of 3 biological replicates.

### 4.4.2. Selective disruption of mitochondrial thiol homeostasis stabilises Nrf2 protein and leads to its nuclear translocation

The transcription factor Nrf2 plays a central role in the cytoprotective response to oxidative stress and damage (Itoh et al., 1997; Yamamoto et al., 2018). In unstressed conditions, Nrf2 protein levels are maintained relatively low due to constant ubiquitination mediated by Keap1 (Itoh et al., 1999; Kobayashi et al., 2004). In addition to its role in overall cellular redox homeostasis, Nrf2 is also critical for the maintenance of mitochondrial antioxidant
defenses and redox homeostasis (Dinkova-Kostova \& Abramov, 2015). It is widely assumed that Nrf2 is upregulated in response to mitochondrial oxidative stress and damage (Kasai et al., 2020). However, the mechanistic details of this are still unclear. Possibilities include that elevated mitochondrial superoxide production leads to hydrogen peroxide going from mitochondria to the cytosol and activating Nrf2 or that redox changes within mitochondria lead to secondary signals to the cytosol that then activate Nrf2.

The first question was if selective disruption of mitochondrial thiol redox homeostasis activated Nrf2. To do this we used the mitochondria-targeted thiol reagent MitoCDNB, its control compound MitoCDNB Ctrl and a known Nrf2 activator, $\mathrm{H}_{2} \mathrm{O}_{2}$. C 2 C 12 mouse myoblasts cells were incubated with either Vehicle ( $0.1 \%$ ethanol), $\mathrm{H}_{2} \mathrm{O}_{2}(100 \mu \mathrm{M}, 30 \mathrm{~min}$ ), MitoCDNB ( $10 \mu \mathrm{M}, 1$ and 4 h ) and MitoCDNB Ctrl ( $10 \mu \mathrm{M}, 1$ and 4 h ) in order to determine if MitoCDNB does lead to Nrf2 stabilisation, which is a key step towards its activation (Figure 4.6). Under control conditions, the Nrf2 protein is present at low levels and is only detected in the cytosol of C 2 C 12 mouse myoblasts. Treatment with $\mathrm{H}_{2} \mathrm{O}_{2}$ as a positive control increased the protein levels of Nrf2 within the cell around 2-fold compared to the vehicle condition (Figure 4.6). Similarly, MitoCDNB led to the stabilisation of the Nrf2 protein in the cytosol at 4 h of incubation, but not at the earlier 1 h time point. The lag in the response may be explained as being due to the time taken for MitoCDNB to deplete mitochondrial GSH levels and to disrupt the mitochondrial thioredoxin system (Booty et al., 2019). MitoCDNB Ctrl compound had no effect on Nrf2 protein levels (Figure 4.6), helping us determine whether the effect of MitoCDNB was due to its reaction with mitochondrial thiols, or by a non-specific disruption of mitochondria by accumulation of the TPP targeting group.



Figure 4.6. MitoCDNB stabilizes Nrf2 protein levels. (Top) C2C12 cells were incubated with Vehicle (0.1 \% ethanol; Veh), $\mathrm{H}_{2} \mathrm{O}_{2}(100 \mu \mathrm{M}$ for 30 min$)$, MitoCDNB ( $10 \mu \mathrm{M}$ for 1 and 4 h ) or MitoCDNB Ctrl ( $10 \mu \mathrm{M}$ for 1 and 4 h). Protein levels were then assessed by western blotting for Nrf2 (top) and GAPDH (Bottom) in whole cell lysates. Western blot band intensities were quantified (below) and normalised to GAPDH and subsequently to the vehicle condition. All data are mean +/- SD. Blots are representative of three independent experiments. $P$ values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant $p$ values are shown (statistical significance corresponds to $p<0.05$ ).

The next step was to determine the subcellular localisation of the Nrf2 protein. For this, 2 different experimental methods were employed, an immunocytochemistry approach as well as subcellular fractionation. In the immunocytochemistry experiment, C2C12 cells were fixed after incubation with conditions as in Figure 4.6, and subsequently Nrf2 protein was labelled with fluorescent antibodies, while DAPI was used for nucleus labelling. For a complementary method, C2C12 cells were fractioned into nuclear and cytosolic fractions after incubation with MitoCDNB and MitoCDNB Ctrl (both $10 \mu \mathrm{M}$ ) for 4 h . The Nrf2 protein
was then assessed by western blotting. Treatment with $\mathrm{H}_{2} \mathrm{O}_{2}(100 \mu \mathrm{M}, 30 \mathrm{~min})$ led to redistribution of the stabilised Nrf2 protein compared to the vehicle condition, as assessed by immunocytochemistry (Figure 4.7). MitoCDNB, but not its control compound, similarly induced the nuclear localisation of Nrf2, as shown with both immunocytochemistry (Figure 4.7) and by sub-cellular fractionation (Figure 4.8), with nearly $90 \%$ of cells having a clear nuclear distribution of Nrf2 upon MitoCDNB treatment.



Figure 4.7. MitoCDNB leads to nuclear translocation of Nrf2. 3D maximum projection images (top) showing fluorescence obtained with C2C12 cells with DAPI nuclear staining (first row), immunocytochemistry for Nrf2 (second row) and composite merge of the two fluorescent channels (third row) after treatment of $0.1 \%$ ethanol (vehicle), $100 \mu \mathrm{M} \mathrm{H}_{2} \mathrm{O}_{2}$ for $30 \mathrm{~min}, 10 \mu \mathrm{M}$ MitoCDNB or $10 \mu \mathrm{M}$ MitoCDNB Ctrl (both 4 h ). Scale bars = $20 \mu \mathrm{~m}$. Nuclear distribution (below) is presented as mean \% of all cells +/-SD. Data are from 3 independent experiments; 30 cells were counted for each condition. P values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).



Figure 4.8. Nrf2 subcellular localisation. C2C12 cells were incubated for $4 h$ with $10 \mu \mathrm{M}$ of either MitoCDNB Ctrl or MitoCDNB and fractioned into the cytosolic and nuclear fractions. Protein was then assessed by western blotting for Nrf2 (top), alpha-Tubulin (middle) and Histone-4 (bottom). All data are mean $+/-$ SD. Blots are representative of three independent experiments. P values were calculated using a two-tailed, unpaired Student's T test. Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

### 4.4.3. Selective disruption of mitochondrial thiol homeostasis activates Nrf2 downstream signalling

As shown above, MitoCDNB led to stabilisation of Nrf2 and its nuclear translocation, and this was due to its thiol reactivity and not to a non-specific interaction with mitochondria due to the TPP moiety, as no effects were seen with MitoCDNB Ctrl. The next step was to
explore if the nuclear accumulation of the Nrf2 protein by MitoCDNB activates transcription of Nrf2-dependent genes and hence its downstream signalling following binding to the ARE. Therefore, the level of certain proteins known to be under the Nrf2 control via the ARE, were assessed.

C2C12 mouse myoblast cells were incubated with MitoCDNB and its control compound, MitoCDNB Ctrl (both $10 \mu \mathrm{M}$ ) and lysed after 8, 12 or 24 h of incubation. The protein was extracted and assessed using western blotting for GCLC, GSS and HO-1, representing the downstream targets of Nrf2, and GAPDH, which is used as a loading control (Figure 4.9). Immunoblotting showed that MitoCDNB, but not its corresponding control compound, led to a time-dependent increase in the levels of the Nrf2 downstream targets, GCLC, GSS and HO-1. The latter was increased at 8 - and 12 h post-exposure, while GCLC and GSS levels increase later, at 12 h point (Figure 4.9).


Figure 4.9. MitoCDNB induces protein transcription of Nrf2 downstream targets in C2C12 cells. (Top) Induction of Nrf2 downstream targets (GCLC, GSS, HO-1) after treatment with $10 \mu \mathrm{M}$ MitoCDNB and

MitoCDNB Ctrl for 8, 12 or 24 h. Protein levels were assessed by western blotting for GCLC, GSS, HO-1 and GAPDH. (Bottom) Relative fold induction was obtained as compared to the untreated/MitoCDNB Ctrl and GAPDH western blots. All data are mean +/- SD. Blots are representative of three independent experiments. $P$ values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

To further confirm Nrf2 activation of transcription, the activity of NQO1, another downstream target of Nrf2 was assessed using a quantitative NQO1 inducer assay (Fahey et al., 2004; Prochaska \& Santamaria, 1988) by our collaborators in Dundee. The activity of this $N A D(P) H: q u i n o n e ~ o x i d o r e d u c t a s e ~ e n z y m e, ~ w h i c h ~ i s ~ i n v o l v e d ~ i n ~ d e t o x i f i c a t i o n ~$ pathways, is a particularly sensitive indication of Nrf2 activation, as transcription of its gene is primarily regulated by Nrf2 and widely recognized as one of its classical targets (DinkovaKostova \& Talalay, 2010). The potency of MitoCDNB was defined as the Concentration required to Double (CD) the NQO1 Enzyme specific activity. Hepa1c1c7 were incubated with MitoCDNB, or MitoCDNB Ctrl, for 24 h , and the NQO1 activity was assessed. MitoCDNB elicited a pronounced concentration dependent NQO1 induction with a CD value of 12.5 $\mu \mathrm{M}$, which facilitates the comparison of its potency with other inducers (Figure 4.10), whereas MitoCDNB Ctrl had no effect.


Figure 4.10. MitoCDNB leads to an increase in NQO1 activity. NQO1 activity in mouse Hepa1c1c7 cells treated with increased concentrations of MitoCDNB or MitoCDNB Ctrl for 24 h. Mean values of 8 independent
experiments are shown. This work has been done by Maureen Higgins in the lab of Albena T. Dinkova-Kostova at the University of Dundee.

The above experiments led to the conclusion that the selective disruption of mitochondrial thiol homeostasis by MitoCDNB leads to Nrf2 stabilisation, its translocation to the nucleus, where it activates the expression of its target genes, such as GCLC, GSS and HO-1. In addition, MitoCDNB also increases the activity of NQO1, further confirming the activation of Nrf2 signalling axis. Next, I will look into Nrf2 activation upon elevated mitochondrial superoxide production with MitoPQ, a mitochondrial targeted redox cycler (Robb et al., 2015).

### 4.4.4. Selective generation of superoxide within mitochondria does not activate Nrf2

It has been previously shown that increasing mitochondrial oxidative damage activates the Nrf2 pathway (Kasai et al., 2020). One of the proposed mechanisms suggested that oxidative damage and stress activated Nrf2 through kinase dependent mechanisms such as the macrophage stimulating (Mst)1 and Mst2 systems (Wang et al., 2019). However, it was still unclear whether mitochondrial superoxide production alone could activate Nrf2 by generating enough hydrogen peroxide, which would be released as a redox signal from the organelle to the cytosol. Or whether the redox signal was secondary to intramitochondrial alterations. Therefore, the targeted redox cycler MitoPQ was used to generate superoxide in a selective fashion inside mitochondria (Robb et al., 2015), without any further effects on other mitochondrial processes, or the cytosolic redox environment of C2C12 cells (Hinchy et al., 2018). The effects of MitoPQ were compared with its inactive control compound, which is taken up by mitochondria within cells but does not generate superoxide (Antonucci et al., 2019).

C2C12 mouse myoblast cells were normally treated with $5 \mu \mathrm{M}$ MitoPQ and its control compound, as this concentration of MitoPQ has been shown to robustly increase superoxide production within mitochondria, but not in the cytosol, in C2C12 cells (Robb et al., 2015). The latter was examined again with a flow cytometry-based measurement of
cytosolic oxidative stress with the CellROX Green dye (ThermoFisher Scientific). C2C12 cells were treated with vehicle ( 0.1 \% ethanol), MitoPQ or MitoPQ Ctrl (both $5 \mu \mathrm{M}$ ) for 4 h , with the CellROX dye added in the last 30 minutes of incubation. Tert-Butyl hydroperoxide (TBHP) was used as a positive control at $250 \mu \mathrm{M}$. The experiment showed that neither MitoPQ nor its control compound, MitoPQ Ctrl, induced oxidative stress within the cytosol (Figure 4.11).


Figure 4.11. MitoPQ does not induce cytosolic oxidative stress. Cellular $\mathrm{H}_{2} \mathrm{O}_{2}$ levels were assessed with CellROX Green using flow cytometry in C2C12 cells incubated with vehicle ( $0.1 \%$ ethanol), TBHP (250 $\mu \mathrm{M}-$ positive control), MitoPQ ( $5 \mu \mathrm{M}$ ) or MitoPQ ctrl $(5 \mu \mathrm{M})$ for 4 h . All data are mean +/- SD. P values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant $p$ values are shown (statistical significance corresponds to $p<0.05$ ).

To explore, if enhanced mitochondrial superoxide levels lead to activation of the Nrf2 signalling pathway, the stabilisation of Nrf2 protein levels needed to be seen in the cells upon MitoPQ incubation. With that in mind, C2C12 mouse myoblast cells were incubated with vehicle ( $0.1 \%$ ethanol), $\mathrm{H}_{2} \mathrm{O}_{2}$ ( $100 \mu \mathrm{M}$ for 30 min ), MitoPQ or MitoPQ Ctrl (both $5 \mu \mathrm{M}$ for 1 and 4 h). Protein levels were assessed by western blotting for Nrf2 and GAPDH in whole cell lysates (Figure 4.12). After quantification and normalisation of western band
intensities it became apparent that MitoPQ did not cause an increase in Nrf2 protein levels (Figure 4.12), hence it did not stabilise Nrf2.


Figure 4.12. MitoPQ does not stabilize Nrf2 protein levels. (Left) C2C12 cells were incubated with Vehicle (0.1 \% ethanol; Veh), $\mathrm{H}_{2} \mathrm{O}_{2}(100 \mu \mathrm{M}$ for 30 min ), MitoPQ ( $5 \mu \mathrm{M}$ for 1 and 4 h ) or MitoPQ Ctrl ( $5 \mu \mathrm{M}$ for 1 and 4 h ). Protein levels were then assessed by western blotting for Nrf2 (top) and GAPDH (Bottom) in whole cell lysates. Western blot band intensities were quantified (right) and normalised to GAPDH and subsequently to the vehicle condition. All data are mean $+/-S D$. Blots are representative of three independent experiments. $P$ values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant $p$ values are shown (statistical significance corresponds to $p<0.05$ ).

The next step was to determine if the subcellular localisation of the Nrf2 protein had been altered. As before for MitoCDNB, 2 different experimental methods were employed, an immunocytochemistry approach as well as subcellular fractionation. In the immunocytochemistry experiment, C2C12 mouse myoblast cells were fixed after incubation with conditions similar to those in Figure 4.12 (without the 1 hr condition for MitoPQ and MitoPQ Ctrl). The Nrf2 protein was subsequently labelled with fluorescent antibodies, with the DAPI dye used for nucleus labelling (Figure 4.13). Treatment with $\mathrm{H}_{2} \mathrm{O}_{2}$ ( $100 \mu \mathrm{M}, 30 \mathrm{~min}$ ) led to redistribution of the stabilised $\mathrm{Nrf2}$ protein compared to the vehicle condition (Figure 4.13). Incubation with MitoPQ did not lead to the same redistribution (Figure 4.13) and the Nrf 2 levels were considerably lower than with $\mathrm{H}_{2} \mathrm{O}_{2}$, confirming the conclusion from the previous experiment about the lack of an effect of MitoPQ on Nrf2 stability. For complementary method, C2C12 cells were fractioned into nuclear and cytosolic fractions after incubation with MitoPQ and MitoPQ Ctrl (both $5 \mu \mathrm{M}$ ) for 4 h . The Nrf2 protein was then assessed by western blotting. MitoPQ did not lead to more nuclear localisation of Nrf2 compared to MitoPQ Ctrl, as assessed by cell sub fractionation followed by immunoblotting (Figure 4.14 A ). As a positive control the experiment was also repeated with a known Nrf2 activator, SFN (Figure 4.14 B ), which did show a large nuclear localisation of the Nrf2 protein. Therefore, MitoPQ did not elicit either Nrf2 stabilisation or its nuclear localisation, which is necessary for Nrf2 signalling to occur.


Figure 4.13. MitoPQ does not lead to nuclear translocation of Nrf2. 3D maximum projection images (left) showing fluorescence obtained with C2C12 cells with DAPI nuclear staining (first row), immunocytochemistry for Nrf2 (second row) and composite merge of the two fluorescent channels (third row) after treatment of 0.1 \% ethanol (vehicle), $100 \mu \mathrm{M} \mathrm{H} \mathrm{H}_{2} \mathrm{O}_{2}$ for $30 \mathrm{~min}, 5 \mu \mathrm{M}$ MitoPQ or $5 \mu \mathrm{M}$ MitoPQ Ctrl (both 4 h ). Scale bars $=20$ $\mu m$. Nuclear distribution (right) is presented as mean \% of all cells +/- SD. Data are from 3 independent experiments; 30 cells were counted for each condition. $P$ values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

A



B


Figure 4.14. Nrf2 subcellular localisation. (A) C2C12 cells were incubated for $4 h$ with $5 \mu \mathrm{M}$ of either MitoPQ Ctrl or MitoPQ and with vehicle ( 0.1 \% ethanol) or SFN ( $5 \mu \mathrm{M}$ ) (B) The cells were fractioned into the cytosolic and nuclear fractions. Protein was then assessed by western blotting for Nrf2 (top), alpha-Tubulin (middle) and Histone-4 (bottom). All data are mean +/-SD. Blots are representative of three independent experiments. $P$ values were calculated using a two-tailed, unpaired Student's $T$ test. Individual significant $p$ values are shown (statistical significance corresponds to $p<0.05$ ).

As before with MitoCDNB, it was important to confirm that MitoPQ does not induce the transcription of Nrf2-dependent genes via the ARE, which would be expected as MitoPQ did not lead to either Nrf2 stabilisation or its translocation to the nucleus. As before, the target genes that were investigated were the protein levels of GCLC, GSS and HO-1, as well as the activity of the NQO1 enzyme. C2C12 mouse myoblast cells were incubated with MitoPQ and MitoPQ Ctrl (both $5 \mu \mathrm{M}$ ) for 8,12 and 24 h (Figure 4.15). Relative fold induction of western blot bands showed that MitoPQ did not lead to a different expression profile of GCLC, GSS or HO-1 compared to its control, MitoPQ Ctrl (Figure 4.15). Furthermore, our collaborators in Dundee used the quantitative NQO1 inducer assay (Fahey et al., 2004; Prochaska \& Santamaria, 1988) to explore any changes in specific activity of NQO1.

Hepa1c1c7 cells were exposed to a range of concentrations of MitoPQ and MitoPQ Ctrl for 24 h , before measuring the NQO1 activity in the cells (Figure 4.16). Similarly, MitoPQ and MitoPQ Ctrl did not lead to a change in NQO1 activity, which is corroborated by the substantial response to the positive control SFN (Figure 4.16). As expected, because MitoPQ failed to stabilise and lead to nuclear translocation of the Nrf2 protein, it further failed to induce the protein expression and activity of its downstream targets.


Figure 4.15. MitoPQ does not induce protein transcription of Nrf2 downstream targets in C2C12 cells. (Top) Induction of Nrf2 downstream targets (GCLC, GSS, HO-1) after treatment with $5 \mu \mathrm{M}$ MitoPQ and MitoPQ Ctrl for 8, 12 or 24 h . Protein levels were assessed by western blotting for GCLC, GSS, HO-1 and GAPDH. (Bottom) Relative fold induction was obtained as compared to the untreated/MitoPQ Ctrl and GAPDH western blots. All data are mean +/- SD. Blots are representative of three independent experiments. P values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).


Figure 4.16. MitoPQ does not lead to a change in NQO1 activity. NQO1 activity in mouse Hepa1c1c7 cells treated with a range of concentrations of SFN, MitoPQ, MitoPQ Ctrl for 24 h. Mean values of 8 independent experiments are shown. This work has been done by Maureen Higgins in the lab of Albena T. Dinkova-Kostova at the University of Dundee.

It could be speculated that the lack of effect of MitoPQ on Nrf2 signalling was due to a low and insufficient levels of MitoPQ used in the cell experiments. Therefore, C2C12 cells were incubated with a 5- to 10-fold increasing concentrations of MitoPQ, as well as with vehicle ( 0.1 \% ethanol) and SFN ( $5 \mu \mathrm{M}$ ) for 4 h (Figure 4.17). Even the highest concentration of MitoPQ $(50 \mu \mathrm{M})$, did not lead to enhancement of NRf2 levels, indicating that the lack of an effect on Nrf2 is not due to insufficient MitoPQ.


Figure 4.17. High dose of MitoPQ does not induce Nrf2 signalling. C2C12 cells were incubated with Vehicle ( 0.1 \% ethanol), SFN ( $5 \mu \mathrm{M}$ ) and MitoPQ (5, 25, $50 \mu \mathrm{M}$ for 4 h) and MitoPQ Ctrl (5, 25, $50 \mu \mathrm{M}$ for 4 h). Protein levels were then assessed by western blotting for Nrf2 (top) and GAPDH (bottom). Blots are representative of three independent experiments.

All in all, from these experiments it can be concluded that in these conditions the selective production of superoxide and hydrogen peroxide within the mitochondrial matrix by MitoPQ does not lead to activation of Nrf2.

### 4.4.5. Both MitoCDNB and CDNB lead to Nrf2 activation

From the previous experiments it was concluded that MitoCDNB, but not MitoPQ, led to Nrf2 stabilisation, nuclear localisation and enhanced expression of its downstream targets Previously (Booty et al., 2019) it has been shown that MitoCDNB selectively disrupts mitochondrial thiol antioxidant defenses as well as depleting mitochondrial GSH, which can disrupt mitochondrial thiol redox homeostasis, while the cytosolic GSH levels remained unchanged. In contrast, CDNB is known to primarily disrupt cytosolic thiol antioxidant defenses (Booty et al., 2019).

In order to determine, if the Nrf2 signalling activation is only due to the selective mitochondrial effect of MitoCDNB, or if CDNB has a similar effect, experiments on Nrf2 stabilisation, nuclear localisation and expression of downstream targets had to be carried out. C2C12 mouse myoblast cells were incubated with vehicle ( 0.1 \% Ethanol), MitoCDNB (10, 5 and $1 \mu \mathrm{M}$ ) and CDNB ( $5 \mu \mathrm{M}$ ) for 4 h . Protein levels of Nrf2 were assessed by western blotting and the bands (Vehicle, MitoCDNB $10 \mu \mathrm{M}, \mathrm{CDNB}, 5 \mu \mathrm{M}$ ) were quantified using

GAPDH as a loading control (Figure 4.18). The experiment showed that CDNB does lead to Nrf2 protein stabilisation as observed with MitoCDNB before.


Figure 4.18. CDNB leads to Nrf2 stabilisation. (Left) C2C12 cells were incubated with vehicle ( $0.1 \%$ ethanol), MitoCDNB (10, 5 and $1 \mu \mathrm{M}$ for 4 h ) and CDNB ( $5 \mu \mathrm{M}$ for 4 h ). Protein levels were then assessed by western blotting for Nrf2 (top) and GAPDH (bottom) in whole cell lysates. Western blot band intensities were quantified for vehicle, MitoCDNB $(10 \mu M)$ and CDNB $(5 \mu M)$ (right) and normalised to GAPDH and subsequently to the vehicle condition. All data are mean +/- SD. Blots are representative of three independent experiments. $P$ values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

Next, Nrf2 nuclear translocation was examined with an immunocytochemical approach, where C2C12 cells were incubated with Vehicle ( $0.1 \%$ Ethanol), MitoCDNB ( $10 \mu \mathrm{M}$ ) and CDNB $(5 \mu \mathrm{M})$ for 4 h , fixed and stained for Nrf2 and DAPI (Figure 4.19). Again, CDNB led to a similar protein localisation as MitoCDNB, where most of the Nrf2 protein is observed in the nucleus, where Nrf2 acts as a transcription factor.


Figure 4.19. CDNB causes Nrf2 to translocate to the nucleus. 3D maximum projection images showing fluorescence obtained with C2C12 cells with DAPI nuclear staining (first row), immunocytochemistry for Nrf2 (second row) after treatment of 0.1 \% ethanol (vehicle), $10 \mu \mathrm{M}$ MitoCDNB or $5 \mu \mathrm{M}$ CDNB (both 4 h). Scale bars $=20 \mu \mathrm{~m}$. The images are representative images from 3 independent experiments.

As before, the previous experiments suggested that if Nrf2 protein was stabilised and translocated to the nucleus, it should be able to activate its downstream targets via ARE. Therefore, NQO1 activity was examined with the use of the quantitative NQO1 inducer assay
(Fahey et al., 2004; Prochaska \& Santamaria, 1988) by our collaborators in Dundee. For this experiment, Hepa1c1c7 were exposed to increasing concentrations of CDNB and MitoCDNB and showed that both molecules induce NQO1 activity (Figure 4.20).


Figure 4.20. CDNB increases NQO1 activity. NQO1 activity in mouse Hepa1c1c7 cells treated with a range of concentrations of MitoCDNB and CDNB for 24 h. Mean values of 8 independent experiments are shown. This work has been done by Maureen Higgins in the lab of Albena T. Dinkova-Kostova at the University of Dundee.

Here, it was shown that CDNB follows a similar path to MitoCDNB and leads to Nrf2 signalling activation, even though the different molecules deplete GSH and disrupt thiol redox homeostasis in particular cell parts, CDNB affects the whole cell, while MitoCDNB exerts its activity mainly in the mitochondria (Booty et al., 2019). As both molecules act as GST substrates and deplete GSH (Booty et al., 2019), it is important to assess if the GSH levels are in any way involved in activating Nrf2.

### 4.4.6. NAC prevents MitoCDNB-mediated Nrf2 activation

As MitoCDNB acts in part by depleting mitochondrial GSH levels, the next step was to examine, if that is involved in activating the Nrf2 signalling pathway. In order to explore this part of the story, the effect of N -acetyl -L-cysteine (NAC) on Nrf2 activation by MitoCDNB was assessed. NAC has been characterised as a GSH precursor that also increases cell thiol levels and can thereby directly ameliorate the cellular oxidative stress that impacts thiols (Mukherjee et al., 2007).

First, it was explored if NAC has an effect on the nuclear localisation of Nrf2, caused by MitoCDNB. The C2C12 mouse myoblast cells were incubated with NAC ( 1 mM ), MitoCDNB Ctrl ( $10 \mu \mathrm{M}$ ), MitoCDNB ( $10 \mu \mathrm{M}$ ), and with NAC ( 1 mM ) for 1 h before, or simultaneously with MitoCDNB ( $10 \mu \mathrm{M}$ ) (Figure 4.21). After incubation for 4 h , cells were fixed and stained with fluorescent antibodies against Nrf2 and with DAPI as the nuclear dye. As expected, cells incubated with NAC and MitoCDNB Ctrl did not exhibit any nuclear localisation of Nrf2 (Figure 4.21). Using this protocol, both pre- and co-treatment of the cells with NAC and MitoCDNB prevented the induction of Nrf2 localisation by MitoCDNB, which was indicated by low amounts of cells showing a clear nuclear localisation compared to MitoCDNB incubation alone (Figure 4.21).


Figure 4.21. NAC prevents Nrf2 nuclear localisation caused by MitoCDNB. 3D maximum projection images (left) showing fluorescence obtained with C2C12 cells with DAPI nuclear staining (first row), immunocytochemistry for Nrf2 (second row) after treatment with 1 mM NAC (4h), $10 \mu M$ MitoCDNB Ctrl (4 h), $10 \mu \mathrm{M}$ MitoCDNB ( 4 h ), 1 mM NAC $+10 \mu \mathrm{M}$ MitoCDNB (pre-treatment for 1 h with NAC) and 1 mM NAC + $10 \mu \mathrm{M}$ MitoCDNB (co-treatment). Scale bars $=20 \mu \mathrm{~m}$. Nuclear distribution (right) is presented as mean \% of all cells +/-SD. Data are from 3 independent experiments; 30 cells were counted for each condition. $P$ values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

As NAC is a known antioxidant, the "rescue" observed in the above-mentioned experiment could be due to its overall cellular antioxidant effect or a direct action on the Keap1-Nrf2 system. Therefore, a similar experiment setup was employed using SFN as the Nrf2
activator, which is known to directly affect the Keap1-Nrf2 association by interacting with thiols on the surface of Keap1. C2C12 cells were incubated with NAC ( 1 mM ) and SFN (5 $\mu \mathrm{M}$ ) for 4 h , as well as NAC addition to the SFN condition either as a pre- or co-treatment (Figure 4.22). The immunocytochemistry approach showed that the nuclear localisation of Nrf2 caused by SFN, persisted even after the addition of NAC during the incubation (Figure 4.22). Therefore, NAC did not have an influence on SFN-induced Nrf2 nuclear localisation, suggesting that its overall cellular antioxidant effect is not enough to reverse Nrf2 activation by this activator.


Figure 4.22. NAC does not reverse SFN induced Nrf2 nuclear localisation. 3D maximum projection images showing fluorescence obtained with C2C12 cells with DAPI nuclear staining (first row), immunocytochemistry for Nrf2 (second row). C2C12 cells are incubated with NAC (1 mM) and SFN (5 $\mu \mathrm{M}$ ) for 4 h . A nuclear distribution is seen with SFN ( $5 \mu \mathrm{M}$ ). After pre-treatment or co-treatment with NAC (1mM) the nuclear distribution stays the same. Scale bars $=20 \mu \mathrm{~m}$. The images are representative images from 3 independent experiments.

As NAC prevented the induction of nuclear localisation of Nrf2 by MitoCDNB, the next step was to explore if it has an effect on the MitoCDNB- mediated induction of Nrf2 downstream targets, such as NQO1. The latter's activity was examined with the use of the quantitative NQO1 inducer assay (Fahey et al., 2004; Prochaska \& Santamaria, 1988) by our collaborators in Dundee. For this experiment, Hepa1c1c7 were exposed to increasing
concentrations of MitoCDNB alone, or with 1 mM NAC introduced at 1 h before or at the same time as MitoCDNB (Figure 4.23) and incubated for 24 h . The MitoCDNB-mediated induction of NQO1 was diminished (Figure 4.23) in both pre- and co-treated cells with NAC. Furthermore, in order to explore if NAC does not have an effect on the NQO1 inducer assay, Hepa1c1c7 cells were also treated with a range of concentrations of NAC for 24 h and NQO1 activity measured (Figure 4.24). Treatment with NAC at concentrations up to 10 mM had no effect on the activity of NQO1 (Figure 4.24). These experiments showed that NAC does prevent MitoCDNB-mediated Nrf2 nuclear localisation as well as its downstream transcriptional activation.


Figure 4.23. NAC diminishes MitoCDNB-mediated induction of NQO1. NQO1 activity in mouse Hepa1c1c7 cells treated with MitoCDNB (10 $\mu \mathrm{M}$ ), NAC (1 mM pre-treatment) + MitoCDNB (10 $\mu \mathrm{M}$ ) or NAC (1mM) + MitoCDNB (10 $\mu \mathrm{M}$, co-treatment) for $24 h(n=8)$. Mean values are shown. This work has been done by Maureen Higgins in the lab of Albena T. Dinkova-Kostova at the University of Dundee.


Figure 4.24. NAC has no effect on NQO1 activity. NQO1 activity in mouse Hepa1c1c7 cells treated with NAC for $24 h(n=8)$. Mean values are shown. This work has been done by Maureen Higgins in the lab of Albena $T$. Dinkova-Kostova at the University of Dundee.

Next, it needed to be examined whether the NAC-mediated prevention of Nrf2 activation by MitoCDNB, could be due to a direct interaction between NAC and MitoCDNB. To explore this, MitoCDNB $(10 \mu \mathrm{M})$ and NAC ( 10 mM ) were incubated in KCl buffer in the presence or absence of GST ( $10 \mu \mathrm{~g}$ ) for 1 and 24 h before being analyzed by RP-HPLC with MitoCDNB peak area recorded (Figure 4.25). TPMP (10 $\mu \mathrm{M}$ ) was used as an internal standard to normalize the peak areas. Figure 4.25 showed that NAC and MitoCDNB do not interact directly and suggests that NAC prevents the MitoCDNB-mediated Nrf2 activation by boosting thiol defences within the cell.


Figure 4.25. NAC does not react with MitoCDNB. MitoCDNB $(10 \mu \mathrm{M})$ and NAC (10 mM) were incubated in KCl buffer with and without GST $(10 \mu \mathrm{~g})$ for 1 and 24 h before being analysed by RP-HPLC with MitoCDNB peak area recorded. The peak areas were normalized to the peak area of the internal standard TPMP (10 $\mu$ M). All data are mean $\pm$ SD. P values were calculated using a two-tailed, unpaired Student's t-test student $T$-test. Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

In order to investigate whether NAC does prevent MitoCDNB-mediated Nrf2 activation by boosting thiol defences within the cell, the effect of NAC on cellular GSH levels was explored. Whole cell total GSH levels were examined using the GSH recycling assay as described before (Akerboom \& Sies, 1981; Scarlett et al., 1996). C2C12 mouse myoblast cells were incubated for 1 h with Vehicle ( $0.1 \%$ ethanol), CDNB ( $10 \mu \mathrm{M}$ ) and MitoCDNB (10 $\mu \mathrm{M}$ ) on their own or with NAC ( 1 mM ) added. Whole cell extracts were treated with sulfosalicylic acid to precipitate the protein and stabilise GSH. Next, the resulting supernatant was analysed by the GSH recycling assay and data shown as glutathione equivalents (GSH $+2 \times$ GSSG) normalized to protein content (Figure 4.26). It was observed that NAC prevents depletion of whole cell GSH by CDNB and MitoCDNB (Figure 4.26). Next, the mitochondrial GSH levels were measured with the same GSH recycling assay, but with isolated mitochondria from C2C12 cells as the starting material. Mouse C2C12 cells were incubated with MitoCDNB ( $10 \mu \mathrm{M}$ ) with or without NAC ( 1 mM ) being present for 6 h
(Figure 4.27) and mitochondria isolated using the hypotonic method described in (Booty et al., 2019). This experiment showed that NAC increased mitochondrial GSH even in the presence of MitoCDNB (Figure 4.27).


Figure 4.26. NAC prevents cellular GSH depletion by CDNB and MitoCDNB. C2C12 cells were incubated for 1 $h$ with Vehicle ( $0.1 \%$ ethanol), CDNB $(10 \mu M)$ and MitoCDNB $(10 \mu M)+/-N A C(1 m M)$. All data are mean $\pm S D$. P values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).


Figure 4.27. Rescue of mitochondrial GSH with NAC. C2C12 cells were incubated with MitoCDNB ( $10 \mu \mathrm{M}$ ) +/NAC ( 1 mM ) for 6 h , mitochondria were then isolated and GSH measured. All data are mean $\pm$ SD. P values were calculated using a two-tailed, unpaired Student's t-test student $T$-test. Individual significant $p$ values are shown (statistical significance corresponds to $p<0.05$ ).

With these experiments it is evident that NAC prevents MitoCDNB-mediated Nrf2 nuclear localisation (Figure 4.21) and activation of its downstream targets (Figure 4.23), without affecting SFN-induced Nrf2 activation (Figure 4.22). This is not due to a direct reaction between MitoCDNB and NAC (Figure 4.25), as was shown by RP-HPLC. In addition, NAC seems to prevent both cellular GSH depletion by CDNB (Figure 4.26) and mitochondrial GSH depletion caused by MitoCDNB (Figure 4.27). All in all, this suggests that NAC prevention of Nrf2 activation caused by MitoCDNB, is due in part to the replenishment of mitochondrial GSH levels by NAC and not just its overall cellular antioxidant effect as it does not have an effect on SFN-induced Nrf2 activation. This is not surprising as NAC has been characterised as a GSH precursor (Mukherjee et al., 2007).

### 4.4.7. Possible mitochondrial signals eliciting Nrf2 activation

The above-mentioned experiments have shown that MitoCDNB, but not MitoPQ leads to activation of the Nrf2 signalling pathway in the cell, and that mitochondrial GSH depletion is one of the key steps in this mechanism. It is however necessary to explore the notion of a potential mitochondrial signal further. To investigate further how MitoCDNB would be able to affect the cytosol, CellROX was used, a fluorescent probe that responds to a wide range of oxidative processes, enabling us to measure changes in whole cell oxidative stress. C2C12 cells were incubated with vehicle ( $0.1 \%$ ethanol), MitoCDNB ( $10 \mu \mathrm{M}$ ) or MitoCDNB Ctrl ( $10 \mu \mathrm{M}$ ) for 4 h , before CellROX was added for 30 min (Figure 4.28). TBHP ( $250 \mu \mathrm{M}$ ) was used as a positive control. Flow cytometry was used to assess the amount of highly fluorescent cells, a readout of whole cell oxidative stress (Figure 4.28). MitoCDNB did not elevate CellROX fluorescence, suggesting that it does not lead to whole cell oxidative stress (Figure 4.28). In the previous chapter it was shown that MitoCDNB does lead to elevated mitochondrial ROS, measured with MitoSOX fluorescence, but this could be a localised signal that does not diffuse throughout the cell.


Figure 4.28. MitoCDNB does not induce cytosolic oxidative stress. Cellular $\mathrm{H}_{2} \mathrm{O}_{2}$ levels were assessed with CellROX Green using flow cytometry in C2C12 cells incubated with vehicle ( $0.1 \%$ ethanol), TBHP (250 $\mu \mathrm{M}-$ positive control), MitoCDNB (10 $\mu \mathrm{M}$ ) or MitoCDNB Ctrl $(10 \mu \mathrm{M})$ for 4 h. All data are mean +/-SD. P values were calculated using one-way ANOVA (Tukey's post hoc correction for multiple comparisons). Individual significant p values are shown (statistical significance corresponds to $p<0.05$ ).

One of the mitochondrial signals that could be a potential signal to activate the Keap1-Nrf2 pathway is lipid peroxidation (López-Bernardo et al., 2015), which can occur as a byproduct of disrupted mitochondrial thiol homeostasis and elevated mitochondrial ROS. Measuring 4-hydroxy-nonenal (HNE), a lipid peroxidation product), is one way of determining if cells have undergone lipid peroxidation. Here, HNE was measured with immunoblotting with anti-HNE antibodies for whole cell extracts, as well as immunocytochemistry and measuring fluorescence intensity of cell images taken with a confocal microscope. (Figure 4.29). MitoCDNB did seem to elicit a change in the levels of HNE amount present in the cells (Figure 4.29 A). As there is no good positive control in the experiment, it is currently unclear if the observed increase is robust. Furthermore, when looking at whole cell extracts with immunoblotting, only the positive control (addition of $10 \mu \mathrm{M}$ HNE to the cells for 4 h ) showed a small increase in proteins with an HNE adduct (Figure 4.29 B). Thus, these results are not definitive, and more work with alternative and more sensitive methods to assess lipid peroxidation is required.


Figure 4.29. Lipid peroxidation levels after MitoCDNB incubation. (A) 3D maximum projection images showing fluorescence obtained with C2C12 cells with immunocytochemistry for HNE. C2C12 cells were incubated with vehicle ( 0.1 \% ethanol), or either $10 \mu \mathrm{M}$ MitoCDNB or MitoCDNB Ctrl for 4 h . Scale bars $=20$
$\mu m$. The images are representative images form 3 independent experiments. All data are mean $+/-$ SD. (B) Representative western blot image of immunoblotting against HNE in whole cell extracts. C2C12 cells were incubated with Vehicle ( 0.1 \% ethanol), MitoCDNB Ctrl (10 $\mu \mathrm{M}$ ), MitoCDNB ( $10 \mu \mathrm{M}$ ) or HNE (10 $\mu \mathrm{M}$ ) for 4 h.

### 4.4.8. MitoCDNB activates Nrf2 via altering sensor thiols on Keap1

The above analysis indicates that MitoCDNB, but not MitoPQ, generates signals that activate Nrf2. Under homeostatic conditions, Nrf2 is found bound to Keap1 and targeted for ubiquitination and proteolysis by the proteasome. The canonical pathway for the activation of Nrf2 by oxidants or electrophiles is via the reaction with thiols on Keap1, with specific thiols having particular reactivity with different species. This reactivity disrupts the substrate adaptor function of Keap1 and enable Nrf2 to escape the cycle of ubiquitination, migrate to the nucleus and activate gene expression. To investigate the involvement of Keap1 in the mechanisms of Nrf2 activation by MitoCDNB, we used mouse embryonic fibroblast (MEF) cells expressing two different Keap1 cysteine mutants or their wild-type counterparts (Suzuki et al., 2019). Specifically, these mutants were Keap1 ${ }^{\text {C151S }}$ and Keap1 ${ }^{\text {C2265/C613S }}$ (Figure 4.30A). Cys151 on Keap1 is the main sensor for SFN, HNE and NO, while Cys 226 and Cys 613 respond to $\mathrm{H}_{2} \mathrm{O}_{2}$ (Figure 4.30A) (Dayalan Naidu \& DinkovaKostova, 2020). In order to explore mechanisms of Nrf2 activation by MitoCDNB via Keap1, the NQO1 inducer assay was used (Fahey et al., 2004; Prochaska \& Santamaria, 1988) by our collaborators in Dundee. The MEF cells were incubated with a range of MitoCDNB concentrations and NQO1 activity was measured 24 h later (Figure 4.30B). In wild-type cells, MitoCDNB induced NQO1 in a concentration-dependent manner, but this induction was diminished in MEF cells expressing either of the Keap1 mutants (Figure 4.30B). In Figure 4.30C, the similar experiment was done using the classical Nrf2 activator SFN and NQO1 activation was measured. The Keap1 ${ }^{\mathrm{C1515}}$ mutant cells had greatly reduced the ability of $\mathrm{Nrf2}$ activation by SFN in comparison with their wild-type or Keap1C2265/C613S counterparts, in agreement with previous reports that Cys 151 is the main sensor for SFN (Figure 4.30C) (Saito et al., 2015; Zhang \& Hannink, 2003). Interestingly, CDNB did not lead to any induction of NQO1 in MEF cells expressing either WT or Keap 1 cysteine mutants. (Figure 4.30D). The use of Keap1 mutants confirms that MitoCDNB activates Nrf2 through
multiple cysteine sensors in Keap1, and further suggests the involvement of both electrophiles and oxidants as potential mediators.

A


B

C

D


Figure 4.30. MitoCDNB induction of Nrf2 is diminished in MEF cells expressing Keap1 cysteine mutants. (A) Schematic representation of different classes of Nrf2 inducers acting on specific cysteines on Keap 1, adapted from (Suzuki et al., 2019). (B) NQO1 activity in Keap1 ${ }^{\text {WT }}$, Keap1 $1^{\text {C151S }}$ and Keap1 ${ }^{\text {C2265/C613S }}$ MEF cells treated with MitoCDNB for $24 h(n=8)$. (C) NQO1 activity in Keap1 ${ }^{\text {WT }}$, Keap1 $1^{\text {C151S }}$ and Keap1 ${ }^{\text {C2265/C613S }}$ MEF cells treated with

SFN for $24 h(n=8)$. (D) NQO1 activity in Keap1 ${ }^{\text {WT }}$, Keap1 $1^{\text {C1515 }}$ and Keap1 ${ }^{\text {C2265/C613S MEF cells treated with CDNB }}$ for 24 ( $n=8$ ). Mean values are shown. This work has been done by Maureen Higgins in the lab of Albena $T$. Dinkova-Kostova at the University of Dundee.

### 4.5. Discussion

Nrf2 is known for its central role in the cytoprotective response to oxidative stress and is critical for the maintenance of mitochondrial redox homeostasis (Dinkova-Kostova \& Abramov, 2015; Kasai et al., 2020). However, how mitochondrial oxidative stress and damage generate the signals that lead to Nrf2 activation in the cytosol is not yet completely understood. To address this, selective chemical biology approaches were used to investigate separately the effects of mitochondrial superoxide and hydrogen peroxide production and disruption of thiol redox homeostasis. It was found that disruption of the mitochondrial thiol homeostasis with MitoCDNB activated Nrf2, while enhancing mitochondria superoxide production with MitoPQ did not (Cvetko et al., 2020).

MitoCDNB has been previously shown to selectively deplete the mitochondrial glutathione pool and inhibit TrxR2 in cells and in vivo (Booty et al., 2019). This chapter showed that in C2C12 mouse myoblast cells MitoCDNB increases the levels of Nrf2, which then translocated to the nucleus where it induced the expression of Nrf2 downstream targets such as HO-1, GCLC, GSS and NQO1. By contrast, MitoPQ, a mitochondria-targeted redox cycler does not stabilize Nrf2, or lead to its translocation into the nucleus, where it could act as a transcription factor. MitoPQ is a mitochondria-targeted redox cycler and generates superoxide selectively within the mitochondria, which is in turn rapidly dismutated to $\mathrm{H}_{2} \mathrm{O}_{2}$ (Robb et al., 2015). The latter is formed locally and does not diffuse out of mitochondria within the cells, which provides a possible explanation for why we do not observe Nrf2 activation. In addition, although there have been studies linking mitochondrial hydrogen peroxide with Nrf2 activation (Kasai et al., 2020), such a link has not been shown directly. The experiments with MitoPQ indicate that elevating superoxide and hydrogen peroxide within mitochondria, in the absence of other changes, is not capable of generating a hydrogen peroxide signal from the mitochondria to the cytosol in order to activate Nrf2. It is important to note that in disrupting the thiol-based antioxidant defences, and
peroxidases within mitochondria that MitoCDNB may also lead indirectly to an elevation of mitochondrial hydrogen peroxide levels in the presence of other sources of superoxide (Booty et al., 2019). Therefore, it is possible that elevated mitochondrial hydrogen peroxide production in conjunction with the disruption of matrix thiol redox homeostasis, could lead to a mitochondria-generated hydrogen peroxide signal reaching the cytosol and activating Nrf2.

In this chapter, it has also been shown that CDNB is an Nrf2 activator, as it increases the protein levels of Nrf 2 and leads to its translocation to the nucleus, but it is much more toxic than its mitochondria-targeted counterpart MitoCDNB. The difference is mainly due to the fact that CDNB affects global cellular GSH levels, those within the cytosol and mitochondria, due to the import of GSH from the cytosol to the mitochondria. There have been studies showing that global GSH depletion leads to activation of Nrf2 (Lee et al., 2008). It is also possible that CDNB may directly react with cysteines in Keap1, as CDNB is an electrophile (Spencer et al., 1991).

These experiments have also shown that pre- or co-treatment with NAC blocks the MitoCDNB-mediated stabilization and nuclear translocation of Nrf2, and transcription of its downstream targets. NAC is generally regarded as a GSH precursor and therefore leads to an increase in GSH levels (Mukherjee et al., 2007), but also increases levels of $\mathrm{H}_{2} \mathrm{~S}$ (Ezeriṇa et al., 2018), which could contribute to its effects on MitoCDNB interactions with Nrf2 (Hourihan et al., 2013; G. Yang et al., 2013). Furthermore, Nrf2 activation through MitoCDNB leads to an increase in transcription of enzymes involved in glutathione metabolism, GCLC and GSS. Therefore, the experiments suggest that the mitochondrial glutathione depletion is a key signal for Nrf2 activation. This conclusion is also strengthened by the fact that MitoCDNB leads to Nrf2 activation after 4 h , but not 1 h , which means that mitochondrial GSH depletion has to be underway (Booty et al., 2019)

MitoCDNB appears to activate Nrf2 through a Keap1-dependent mechanism that involves both Cys151 as well as the Cys226/Cys613 sensors. These residues have been involved in mediating Nrf2 activation through Keap1 by inducers such as SFN, HNE and $\mathrm{H}_{2} \mathrm{O}_{2}$ (Suzuki et al., 2019). Interestingly, at non-toxic concentrations, CDNB does not lead to any NQO1
induction in MEF cells expressing either WT or Keap1 cysteine mutants, which suggests the effect of MitoCDNB is unrelated to any of its (minimal) direct cytosolic activity. Furthermore, as MitoCDNB seems to activate Nrf2 through more than one sensor, it might be that the emitted mitochondrial signal is a combination of different factors. Although MitoCDNB did lead to an increase in mitochondrial ROS (Booty et al., 2019), there was no increase on whole cellular oxidative stress as measured by CellROX or leakage of hydrogen peroxide from isolated mitochondria (Booty et al., 2019). Furthermore, lipid peroxidation and its product 4-HNE would be another possible mitochondrial signal, but the experiments shown in this chapter have not shown a marked increase in HNE levels following MitoCDNB incubations. However, these findings do not preclude the possibility of mitochondrial hydrogen peroxide or mitochondrial lipid peroxidation acting on Keap1 in the close vicinity to the mitochondria.

In the previous chapter it was shown that MitoCDNB leads to mitochondrial fragmentation (Booty et al., 2019) that involves Drp1. Interestingly, it has been previously shown that Nrf2 activation facilitates the proteasomal degradation of Drp1 (Sabouny et al., 2017). Additionally, PGAM5 has been shown to act as a signalling hub, which brings the Keap1Nrf2 complex to the mitochondria by forming a tripartite Keap1-PGAM5-Nrf2 complex (Lo \& Hannink, 2008; O’Mealey et al., 2017).

To summarise this chapter, the selective disruption of mitochondrial thiol metabolism generates signals that then move from the mitochondria to the cytosol to interact with Keap1 cysteines to activate the Nrf2 signaling pathway. The nature of these signals and how they can exit mitochondria to interact with specific thiols on Keap1 should be explored in future studies.

# Chapter 5. A bioinformatic analysis of early mitochondrial retrograde signalling 

### 5.1. Introduction

In the previous chapters, I have explored mitochondrial dysfunction, specifically disruption of thiol redox homeostasis and enhanced mitochondrial superoxide production, and their effects on mitochondrial morphology, dynamics as well as the interplay with a main antioxidant transcription control pathway, the Nrf2 pathway. In this chapter I take an in vivo systemic approach using transcriptional analysis to investigate the plethora of potential retrograde signalling pathways that may accompany the above-mentioned mitochondrial dysfunction.

Mitochondria are the heart of metabolism, as they produce most of the cellular ATP through oxidative phosphorylation and are essential for a myriad of other metabolic networks, as well as contributing to both apoptotic and necrotic cell death (Murphy, 2016; Murphy \& Hartley, 2018). Mitochondria are also signalling organelles that continuously communicate with the nucleus in order to achieve coordination of metabolic processes (Chandel, 2015; Quirós et al., 2016). Further, mitochondria actively influence other cellular components and cytosolic pathways, creating a mito-cellular communication network based on a variety of signals (Mottis et al., 2019). This network allows mitochondria to adapt and respond to cellular needs and adapt to various stresses, both in the short- and long-term, often through alteration to nuclear transcriptional programs. These outcomes can evolve in order to avoid or alleviate mitochondrial dysfunction as well as to optimise the integration of mitochondrial function into that of the rest of the cell. Therefore, mitochondria generate a wide range of retrograde signals, through which they modulate cellular and organismal activities and protect against mitochondrial dysfunction by activating expression of nuclear genes involved in metabolic reprogramming or stress defence (Jazwinski, 2013; Quirós et al., 2016). The production of mitochondrial ROS occurs as a consequence of aerobic metabolism, but they are increasingly viewed less as just by-
products of metabolism and more as important signalling molecules (Schieber \& Chandel, 2014). Mitochondrial ROS function directly by regulating redox biology and as signalling molecules in numerous cellular processes both under physiological and stress conditions (Schieber \& Chandel, 2014; Shadel \& Horvath, 2015).

As described in previous chapters, our lab has developed chemical biology approaches in order to be able to selectively study certain mitochondrial perturbations. To investigate how mitochondrial thiol redox state influences retrograde signalling, I used the mitochondria-targeted disruptor of thiol redox homeostasis, MitoCDNB, a 1-chloro-2,4dinitrobenzene (CDNB) derivative that is selectively taken up by mitochondria within cells, where it depletes mitochondrial GSH largely, but not solely, by acting as a substrate for mitochondrial GSTs while also inhibiting the mitochondrial Trx system by inhibiting thioredoxin reductase (Figure 5.1 A) (Booty et al., 2019; Cvetko et al., 2020). In addition, I used MitoPQ, a mitochondria-targeted redox cycler, which is selectively taken up by mitochondria where the Paraquat moiety reacts with complex I flavin to selectively increase superoxide production by redox cycling, and thus increase hydrogen peroxide levels within the mitochondrial matrix (Figure 5.1 B) (Antonucci et al., 2019; Hinchy et al., 2018; Robb et al., 2015). Similar to chapter 4, the control compounds, MitoCDNB and MitoPQ Control, were used as inactive versions of the compounds in question.

In mammals increases in ROS levels that cause damage induce a retrograde signal to activate the expression of detoxification enzymes and antioxidant proteins in mitochondria and the cytosol (Chen \& Kunsch, 2004; Kops et al., 2002; Lu et al., 2012; Tan et al., 2008). This activation is mediated by a number of transcription factors. One important example is the nuclear factor erythroid 2-related factor 2 (Nrf2) (Nguyen et al., 2009), which was investigated in chapter 4. But there are many other pathways that have been identified to play a part in retrograde signalling. Increased levels of mitochondria ROS have been reported to activate $N F-\kappa B$, thereby promoting cellular proliferation and survival in cancer cells (Formentini et al., 2012). OXPHOS genes and mitochondrial biogenesis are induced by promoting JNK-PGC1 $\alpha$ signalling (Chae et al., 2013). Mitochondrial ROS can also influence metabolic reprogramming through mitochondrial uncoupling and AMPK activation (Shi et al., 2015), as well as mitochondria fuel switching by mediating complex II phosphorylation
(Acín-Pérez et al., 2014). Lastly, moderate levels of ROS are also crucial transducers of mitochondrial stress that induce longevity in a range of organisms (Ristow, 2014).

Although retrograde signalling has been studied to a certain extent involving mitochondrial ROS, it has not been possible to do so regarding disruption of mitochondrial thiol redox homeostasis. Therefore, to expand this body of work I moved into in vivo experiments, as my previous chapters involved mostly in vitro experiments. In chapter 3, I have touched upon the transcriptomic analysis, which was important to highlight the enormity of changes one can expect and the possible timeline of experiments to explore, but it lacked a control compound and therefore a certain robustness. RNAseq is a great tool for the transcriptomic analysis in tissues as it is considered unbiased, has a high sensitivity for genes expressed in either low or high level, as well as a high dynamic range of expression levels, coupled with low technical variation and high levels of reproducibility.

To shed light on these retrograde mitochondrial dysfunction pathways, mice were challenged with the mitochondria-targeted compounds, MitoCDNB and MitoPQ, as well as their inactive control compounds, MitoCDNB Ctrl and MitoPQ Ctrl, respectively (Figure 5 C). 1 h after injection, mouse liver and heart tissues were excised and preserved in RNAlater to prepare for future RNA extraction and sequencing (Figure 5 C ). Sequencing files were processed with TrimGalore! to remove low-quality reads and adapter sequences, aligned to the mm10 genome using hisat2 and a count table was produced using RSubread. The normalisation and differential expression analysis were subsequently carried out using DESeq2 within the R statistical environment (Figure 5 D). With the differential expression analysis, I set out to explore the main differences of MitoCDNB and MitoPQ compared to their respective controls in different tissues, as well as the common differences arising in both tissues. In addition, I investigated the contrast between disruption of mitochondrial thiol redox homeostasis and enhanced mitochondrial superoxide production, which already started in chapter 4. Lastly, the experimental setup was an opportunity to study the TPP effect on retrograde signalling, which is a central component of our chemical biology approach.

A



B



D

## Sequence reads



Figure 5.1. Schematic representation of our compounds, their effect and our experimental design. $A$, MitoCDNB and MitoCDNB Ctrl compounds accumulate in the mitochondria due to their TPP moiety. There only the active compound, MitoCDNB, leads to GSH depletion and inhibition of thioredoxin and peroxiredoxin
systems. B, MitoPQ and MitoPQ Ctrl compounds accumulate in the mitochondria due to the mitochondria targeting TPP moiety. Once in the mitochondria, MitoPQ but not its inactive control compound, leads to enhanced production of superoxide at the flavin site of Complex I. C, The experimental workflow consists of mice injected with vehicle ( $0.1 \%$ ethanol), MitoCDNB or MitoCDNB Ctrl (both $5 \mathrm{mg} / \mathrm{kg}$ ), MitoPQ or MitoPQ Ctrl (both 2.5 nmol ). After 1 h mice were culled with the liver and heart excised and stored in RNAlater to preserve RNA. Subsequently, RNA isolation and sequencing was done. D, Following on from the sequencing, the reads had to be trimmed of adaptor sequences by TrimGalore!, aligned to the up-to-date mouse genome mm10 via hisat2, from which the count table was produced by RSubread and finally the normalisation and differential expression analysis was carried out by DESeq2 in the $R$ environment.

### 5.2. Chapter hypothesis

The hypothesis to be tested in this chapter is that the enhanced mitochondrial superoxide production and/or disruption of mitochondrial redox homeostasis led to retrograde signalling to the nucleus.

### 5.3. Aims and rationale

In this chapter, I aim to identify the variety of early mitochondrial retrograde signalling pathways following mitochondrial dysfunction caused by mitochondria-targeted molecules using RNAseq. The initial work will focus on the effect of either MitoCDNB or MitoPQ compared to their respective controls in mouse heart and liver, as well as exploring tissue similarities and differences. Subsequently the transcriptional changes of MitoCDNB and MitoPQ will be explored. After this, the transcriptome approach will be used to investigate the underlying effect of TPP-compounds in these experiments.

### 5.4. Results

### 5.4.1. MitoCDNB specific effect

At the end of chapter 3, I have discussed a preliminary in vivo transcriptomic analysis accompanying the disruption of mitochondrial thiol redox homeostasis, from which I observed a markedly larger effect after 1 h than after 4 h . Hence, here I have focussed on the 1 h time point moving forward with this larger analysis. Furthermore, as I have mentioned there was no inactive control compound in that experiment, which limited the interpretation of the results.

To investigate the early retrograde signalling that occurs after dysfunction of mitochondrial thiol defences, I have employed a differential expression analysis using the commonly used DEseq2 package in R. Firstly, I checked the genes that were differentially expressed when comparing the conditions MitoCDNB and vehicle, or MitoCDNB Ctrl and vehicle. Subsequently, the conditions MitoCDNB and MitoCDNB Ctrl were compared. This type of analysis was done on sequencing data from liver and heart, before another analysis which looked at the same differential expression comparisons but irrespectively of tissues in order to investigate the common changes.

### 5.4.1.1. MitoCDNB-specific effect in liver

I first explored the transcriptomic changes occurring under specific mitochondrial disruption of thiol homeostasis, caused by MitoCDNB in the liver. Compared to the vehicle ( 0.1 \% ethanol) injection both MitoCDNB and MitoCDNB Ctrl elicited large transcriptome changes in the liver (Figure 5.2 and 5.3) as shown with volcano plots. In the case of MitoCDNB vs Vehicle there were 267 genes upregulated and 168 genes downregulated, whereas when MitoCDNB Ctrl vs Vehicle were compared there were 320 genes upregulated and 223 genes downregulated. However, the differential expression analysis using DESeq2 of MitoCDNB compared to MitoCDNB Ctrl did not lead to many significant
(FDR < 0.05 ) hits in the liver (Figure 5.4). With just 1 gene upregulated (Ddias) and downregulated (Jun). One reason for this could be the short timeline of 1 h , meaning that MitoCDNB did not have enough time to show its effect, and therefore the result is that both compounds have a very similar outcome, may be due in large part to their mitochondria targeting alone, separate from the effect of MitoCDNB on mitochondrial thiol homeostasis.


Figure 5.2. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB compared with vehicle in the liver - using differential expression analysis with DESeq2 in the R environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly
expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted p value <0.05. $N=6$ per condition.


Figure 5.3. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB Ctrl compared with vehicle in the liver - using differential expression analysis with DESeq 2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the $\log 2$ fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$. $N=6$ per condition.


Figure 5.4. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB compared with MitoCDNB Ctrl in the liver - using differential expression analysis with DESeq2 in the R environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log 2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value <0.05. $N=6$ per condition.

Another option to investigate this dataset is pathway enrichment analysis, which helps gain mechanistic insight into gene lists that are generated from differential expression analysis. I opted for Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005), which is a computational method that determines if biological pathways are enriched in a gene list
more than would be expected by chance. In order to do this, we rank the genes by the inverse of their $p$ value multiplied by the sign of the log fold change
 significantly up regulated genes; large negative numbers are significantly downregulated genes - as shown in chapter 3. Utilising the GSEA Hallmark pathway analysis on the differentially expressed analysis of MitoCDNB and MitoCDNB Ctrl showed that in the liver, MitoCDNB specifically led to upregulation of the Epithelial Mesenchymal Transition (EMT) pathway and downregulation of TNF- $\alpha$ signalling via NfкB, TGF $\beta$ and p53 pathways (Figure 5.5) over its control compound. This suggests that the MitoCDNB Ctrl compound has a more inflammatory/stress effect compared to MitoCDNB. Whereas disruption of mitochondrial thiol redox homeostasis in liver leads to upregulation of EMT, which is a well-established tumorigenesis pathway as it leads to metastasis and invasion of tissues.

Hallmark pathways NES from GSEA


Figure 5.5. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between MitoCDNB and MitoCDNB Ctrl in the liver. The green and red bars correspond to either statistical significance or non-significance (due to FRD-adjusted p value), respectively. The bars at the top represent upregulation (positive normalized enrichment scores, NES) and the bars at the bottom represent downregulation (negative normalized enrichment scores, NES).

### 5.4.1.2. MitoCDNB-specific effect in heart

Next, I moved to the analysis of the heart tissue with regards to the specific transcriptomic changes that accompany the effects of MitoCDNB-induced mitochondrial dysfunction. This analysis showed a difference from the observed effect in the liver, with smaller transcriptomic changes when MitoCDNB or MitoCDNB Ctrl conditions were compared to vehicle ( 0.1 \% ethanol), which can be seen from the volcano plots (Figure 5.6 and 5.7 ) and the number of differentially expressed genes, with 67 downregulated, and 73 upregulated in MitoCDNB compared to vehicle; while there were 68 downregulated and 60 upregulated when MitoCDNB Ctrl was compared with vehicle. Similar, to the liver, the differential expression analysis using DESeq2 of MitoCDNB compared to MitoCDNB Ctrl did not lead to any significantly differentially expressed genes (Figure 5.8).


Figure 5.6. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB compared with vehicle in the heart - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted p value <0.05. $N=6$ per condition.


Figure 5.7. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB Ctrl compared with vehicle in the heart - using differential expression analysis with DESeq2 in the $R$ environment. The negative log10 of $p$ value is portrayed on the $y$ axis and the log 2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$. $N=6$ per condition.


Figure 5.8. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB compared with MitoCDNB Ctrl in the heart - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$. $N=6$ per condition.

The GSEA analysis of MitoCDNB compared to its inactive version in heart (Figure 5.9) led to a reversed result, this time TNF- $\alpha$ signalling via NfкB, TGF $\beta$ and p 53 pathways were upregulated in conjunction with an inflammatory response, interferon gamma response, angiogenesis and the EMT pathways. In addition, in the heart MitoCDNB seemed to downregulate mostly metabolic pathways such as Oxidative phosphorylation, Fatty acid
metabolism, adipogenesis and bile acid metabolism. Therefore, the results suggest that disruption of mitochondrial thiol redox homeostasis in the heart leads to a more inflammatory and stress-related signalling, the opposite of what was observed in the liver. Furthermore, the metabolic pathways that are downregulated, could in turn suggest less mitochondrial activity.


Figure 5.9. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between MitoCDNB and MitoCDNB Ctrl in the heart. The green and red bars correspond to either statistical significance or non-significance (due to FRD-adjusted $p$ value), respectively. The bars at the top represent upregulation (positive normalized enrichment scores, NES) and the bars at the bottom represent downregulation (negative normalized enrichment scores, NES).

### 5.4.1.3. Common MitoCDNB effects in both tissues

After I explored the effects of MitoCDNB relative to its control in both liver and heart, the focus moved to identifying the common effects of MitoCDNB in both tissues. This was done using a different study design ( $\sim$ Tissue + Condition) in the DESeq2 package, which tests the effect of conditions, controlling for the effect of the tissue, therefore the algorithm returns the fold-change result only from the effect of conditions. Next, the differential expression of MitoCDNB compared to vehicle, as well as its control compound, was carried out and a similar picture emerged with MitoCDNB (upregulated: 238; downregulated: 195) and MitoCDNB Ctrl (upregulated: 211; downregulated: 218) eliciting large transcriptome effects when compared to vehicle (Figure 5.10 and 5.11 ), but not one significantly differentially expressed gene when compared with each other (Figure 5.12).


Figure 5.10. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB compared with vehicle irrespective of tissue - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$. $N=12$ per condition.


Figure 5.11. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB Ctrl compared with vehicle irrespective of tissue - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the $\log 2$ fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value < 0.05. $N=12$ per condition.


Figure 5.12. Volcano plot of the significance of the transcriptional changes caused by MitoCDNB compared with MitoCDNB Ctrl irrespective of tissue - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the $\log 2$ fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value $<0.05$. $N=12$ per condition.

The GSEA hallmark pathway analysis (Figure 5.13) suggested that the MitoCDNB effect, compared to that of MitoCDNB Ctrl, was conserved across tissues and led to upregulation of EMT, angiogenesis, cholesterol, homeostasis, apical junction, estrogen response, coagulation and KRAS signalling upregulation pathways, but that no pathways were significantly downregulated.

Hallmark pathways NES from GSEA


Figure 5.13. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between MitoCDNB and MitoCDNB Ctrl irrespective of tissue. The green and red bars correspond to either statistical significance or non-significance (due to FRD-adjusted p value), respectively. The bars at the top represent upregulation (positive normalized enrichment scores, NES) and the bars at the bottom represent downregulation (negative normalized enrichment scores, NES).

To summarise, the acute disruption of mitochondrial thiol defences by MitoCDNB leads to regulation of a variety of pathways. Interestingly, in liver the inflammatory and stressrelated response pathways are downregulated, whereas the reverse is observed in the heart, where these pathways are upregulated, along with others. In the heart, an effect is also observed on the downregulation of metabolism, which could be a response to the
activity and/or health of mitochondria. In both tissues, the EMT pathway, a known pathway mainly involved in tumorigenesis and metastasis is upregulated, this was also shown when common effects of both compounds on this pathway were assessed.

### 5.4.2. MitoPQ-specific effect

Next, I wanted to investigate the early retrograde signalling that occurs upon enhanced production of mitochondrial superoxide. In chapter 4, with regards to Nrf2 signalling pathway, I observed a marked difference between disrupting mitochondrial thiol defences and enhancing mitochondrial superoxide production. To that end, I have employed a transcriptomic analysis using RNAseq and differential expression analysis using the DESeq2 package in R. Initially, I investigated the genes that were differentially expressed when comparing the conditions using MitoPQ, a known mitochondria selective redox cycler (Robb et al., 2015), and vehicle, or MitoPQ Ctrl and vehicle. Subsequently, the conditions utilising MitoPQ and its inactive version, MitoPQ Ctrl were compared. This type of analysis was done in both liver and heart, before another analysis design was used that looked for the common changes in both tissues in response to these interventions.

### 5.4.2.1. MitoPQ-specific effect in liver

I first explored the transcriptomic changes following enhanced mitochondrial superoxide production, due to the injection of MitoPQ, in liver. Volcano plots of our differential expression analysis showed that MitoPQ and MitoPQ Ctrl elicited a few transcriptome changes when compared to the vehicle ( $0.1 \%$ ethanol) in liver (Figure 5.14 and 5.15). MitoPQ led to 21 upregulated and 42 downregulated genes, whereas MitoPQ Ctrl resulted in 24 upregulated and 18 downregulated genes. Comparison of MitoPQ with its inactive control compound, MitoPQ Ctrl, led to some transcriptionally significant genes in liver (Figure 5.16) - 12 upregulated and 55 downregulated genes.


Figure 5.14. Volcano plot of the significance of the transcriptional changes caused by MitoPQ compared with vehicle in the liver - using differential expression analysis with DESeq2 in the R environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted p value <0.05. $N=6$ per condition.


Figure 5.15. Volcano plot of the significance of the transcriptional changes caused by MitoPQ Ctrl compared with vehicle in the liver - using differential expression analysis with DESeq2 in the R environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the $\log 2$ fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value < 0.05. $N=6$ per condition.


Figure 5.16. Volcano plot of the significance of the transcriptional changes caused by MitoPQ compared with MitoPQ Ctrl in the liver - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log 2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted p value <0.05. $N=6$ per condition.

A similar GSEA hallmark pathway analysis as done above for MitoCDNB, showed that enhanced mitochondrial superoxide production affects upregulation of EMT, peroxisome, fatty acid metabolism, myogenesis, adipogenesis and IL-6-JAK-STAT3 signalling pathways, whereas no downregulation of any pathway was observed (Figure 5.17). This suggests that although the inactive MitoPQ Ctrl does have somewhat of an effect on the transcriptomic
profile in the liver, MitoPQ leads to upregulation of cancer specific pathways, such as EMT and II6-JAK-STAT3. The latter is a principal signalling pathway and has a key role in growth and development of many human cancers, when STAT translocates to the nucleus it induces the expression of pro-survival and proinflammatory genes (Johnson et al., 2018).


Figure 5.17. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between MitoPQ and MitoPQ Ctrl in the liver. The green and red bars correspond to either statistical significance or non-significance (due to FRD-adjusted $p$ value), respectively. The bars at the top represent upregulation (positive normalized enrichment scores, NES) and the bars at the bottom represent downregulation (negative normalized enrichment scores, NES). $N=6$ per condition.

### 5.4.2.2. MitoPQ-specific effect in heart

Next, I moved to the analysis of the heart tissue with regards to the specific transcriptomic changes that accompany the effects of MitoPQ, due to its enhanced mitochondrial superoxide production. This analysis showed a difference from the observed effect in the liver, with smaller transcriptomic changes when MitoPQ or MitoPQ Ctrl were compared to vehicle ( 0.1 \% ethanol), which can be seen from the volcano plots (Figure 5.18 and 5.19). There were also differences in the number of differentially expressed genes, with 20 downregulated and 10 upregulated in MitoPQ compared to vehicle; while there were 6 downregulated and 15 upregulated when MitoPQ Ctrl was compared with vehicle. As in the liver, the differential expression analysis using DESeq2 of MitoPQ compared to MitoPQ Ctrl led to a few differentially expressed genes - 21 upregulated and 18 downregulated genes (Figure 5.8).


Figure 5.18. Volcano plot of the significance of the transcriptional changes caused by MitoPQ compared with vehicle in the heart - using differential expression analysis with DESeq 2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value <0.05. $N=6$ per condition.


Figure 5.19. Volcano plot of the significance of the transcriptional changes caused by MitoPQ Ctrl compared with vehicle in the heart - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the $\log 2$ fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted p value <0.05. $N=6$ per condition.


Figure 5.20. Volcano plot of the significance of the transcriptional changes caused by MitoPQ compared with MitoPQ Ctrl in the heart - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log 2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value <0.05. $N=6$ per condition.

The GSEA analysis of enhanced mitochondrial superoxide production, comparing MitoPQ to its inactive version, in heart (Figure 5.21) led to downregulation of adipogenesis, cholesterol homeostasis and MTORC1 signalling pathways; whereas upregulation of TGF $\beta$ signalling, mitotic spindle, TNF $\alpha$ signalling via NFкB, myogenesis and EMT pathways was
observed (Figure 5.21). This suggests that enhanced mitochondrial superoxide production results in downregulation of metabolism associated pathways in the heart, which could be explained by perturbations of mitochondrial activity. The upregulated pathways can be observed as a sign of cell stress, as many of them are involved in inflammatory, stress and cancer responses.


Figure 5.21. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between MitoPQ and MitoPQ Ctrl in the heart. The green and red bars correspond to either statistical significance or non-significance (due to FRD-adjusted $p$ value), respectively. The bars at the top represent upregulation (positive normalized enrichment scores, NES) and the bars at the bottom represent downregulation (negative normalized enrichment scores, NES).

### 5.4.2.3. Common MitoPQ effects in both tissues

As a next step, my focus shifted to exploring the common effects of MitoPQ across both liver and heart tissues. As described above, this was done using a different study design ( $\sim$ Tissue + Condition) in the DESeq2 package, which tests the effect of conditions, controlling for the effect of the tissue. Therefore, the algorithm returns the fold change result only due to the effect of conditions, MitoPQ, MitoPQ Ctrl and vehicle in this instance. Next, the differential expression of MitoPQ compared to vehicle as well as its control compound was carried out and resulted in MitoPQ (upregulated: 30; downregulated: 67) and MitoPQ Ctrl (upregulated: 9; downregulated: 19) eliciting some transcriptome effects when compared to vehicle (Figure 5.22 and 5.23 ). There were a number of differentially expressed genes when MitoPQ and MitoPQ Ctrl were compared with each other - 40 upregulated and 86 downregulated genes (Figure 5.24).


Figure 5.22. Volcano plot of the significance of the transcriptional changes caused by MitoPQ compared with vehicle irrespective of tissue - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$. $N=12$ per condition.


Figure 5.23. Volcano plot of the significance of the transcriptional changes caused by MitoPQ Ctrl compared with vehicle irrespective of tissue - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the log2 fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDRadjusted $p$ value $<0.05$. $N=12$ per condition.


Figure 5.24. Volcano plot of the significance of the transcriptional changes caused by MitoPQ compared with MitoPQ Ctrl irrespective of tissue - using differential expression analysis with DESeq2 in the $R$ environment. The negative $\log _{10}$ of $p$ value is portrayed on the $y$ axis and the $\log 2$ fold change is shown on the $x$ axis. Significantly expressed genes are represented with the red dots. Statistical significance is set as the FDR-adjusted $p$ value $<0.05$. $N=12$ per condition.

The GSEA pathway analysis (Figure 5.25) showed that no pathways were significantly changed and conserved throughout the tissues, suggesting that the tissue-specific effects are much larger. Furthermore, it also suggests that the many significantly differentially expressed genes shown above are found in different pathways and only incur small fold changes.

Hallmark pathways NES from GSEA


Figure 5.25. GSEA pathway analysis of hallmarks pathways for the differentially expressed genes between
MitoPQ and MitoPQ Ctrl irrespective of tissue. The green and red bars correspond to either statistical significance or non-significance (due to FRD-adjusted p value), respectively. The bars at the top represent upregulation (positive normalized enrichment scores, NES) and the bars at the bottom represent downregulation (negative normalized enrichment scores, NES).

To summarise, the enhanced production of mitochondrial superoxide leads to the regulation of a variety of pathways. Interestingly, in both tissues it leads to upregulation of the inflammatory and stress-related response pathways. In the heart, the effect is also observed on the downregulation of metabolism, which could be due to the activity and health of mitochondria. As with disrupting mitochondrial thiol redox homeostasis, in both
tissues, the EMT pathway, a known pathway mainly involved in tumourigenesis and metastasis is upregulated, further corroborating the important role of mitochondrial dysfunction in cancer.

### 5.4.3. MitoCDNB vs MitoPQ

In the previous sections I looked at the specific effects on retrograde signalling resulting from the disruption of mitochondrial thiol redox homeostasis or by elevated mitochondrial superoxide production in liver and heart. In this section, I will investigate the similarities and differences between these two modes of mitochondrial disruption on the retrograde signalling pathways.

In order to compare the different expression changes, I first had to establish the specific genes that are transcribed differentially, due to the active compounds (MitoCDNB, MitoPQ) versus the controls (MitoCDNB Ctrl and MitoPQ Ctrl). To do so, I first took all of the significant gene-expression changes by compounds (active and inactive) when compared to the vehicle condition in each tissue. Next, just genes that altered their expression significantly due to the active compounds vs the control compounds, MitoCDNB and MitoPQ were then compared to each other using Venn diagrams, and the specific genes analysed by g:Profiler (Figure 5.26). The latter is a web server for functional enrichment analysis and conversions of gene lists, which I used mainly for gene ontology and upstream transcription factor analysis (Raudvere et al., 2019). This type of analysis was done for liver, heart and also for common features across both tissues.


Figure 5.26. A schematic representation of steps taken to specifically compare the effects of MitoCDNB and MitoPQ. I started with the differentially expressed genes from DESeq2 between conditions (MitoCDNB, MitoCDNB Ctrl, MitoPQ, MitoPQ Ctrl) and vehicle. Next, the genes were selected that are only differentially
expressed in the active compound (MitoCDNB, MitoPQ), by using a Venn diagram to compare the active and inactive compounds. Subsequently, the differentially expressed genes from just the active compounds were compared together using a Venn diagram in each tissue. Finally, gene ontology and upstream transcription factor analysis was done using g:Profiler.

### 5.4.3.1. MitoCDNB vs MitoPQ in liver

Firstly, the effect of MitoCDNB and MitoPQ was checked in the liver. As the Venn diagram shows (Figure 5.27 A ), there are 127 genes that are specific for MitoCDNB and 58 genes that are specific for MitoPQ effects in liver. Interestingly there is only one gene in the intersection between both conditions.

Next, the MitoCDNB-specific genes for the liver were analysed with g:Profiler (Figure 5.27 B). Gene ontology analysis of biological processes of suggested that the genes are involved in signal transduction in response to DNA damage, cellular macromolecule metabolic processes and regulation of protein neddylation. The TRANSFAC analysis of upstream transcription factors suggested the involvement of ZF5, E2F-1, IRF4, BCL6B, BEN, CREB, SP1 among others.

Similarly, MitoPQ specific genes for the liver were analysed with g:Profiler (Figure 5.27 C). The main biological processes involved with the MitoPQ effect in liver are regulation of nitrogen compound metabolic processes, primary metabolic processes, macromolecule metabolic processes, cellular metabolic processes. The KEGG pathway suggested the involvement of FoxO signalling pathway and pathways involving infection/inflammation. The involved upstream transcription factors are Osx, ZBP89, BCL6B, BEN, ZF5 among others.


Figure 5.27. MitoCDNB and MitoPQ effect in liver tissue. A) Venn diagram of specific differentially expressed genes of MitoCDNB and MitoPQ in liver. MitoCDNB (B) and MitoPQ (C) specific genes underwent g:Profiler analysis, which is shown using plots, where the $x$-axis contains gene ontology (molecular function - red, biological processes - yellow), KEGG pathways-purple and transcription factor-blue analysis, and the $y$-axis represents -log(FDR).

In the liver, the specific effects of MitoCDNB and MitoPQ are very distinct as the differentially expressed genes show almost no overlap. Furthermore, the gene ontology analysis suggested that disruption of mitochondrial thiol redox homeostasis by MitoCDNB leads to biological processes such as signal transduction after DNA damage, whereas mostly metabolic processes are altered by MitoPQ-induced enhancement of mitochondrial superoxide levels. With regards to the upstream transcription factors, there is some overlap between MitoPQ and MitoCDNB, with the involvement of factors ZF5, BCL6B and BEN in both even though the differentially expressed genes differ between the conditions. ZF5 and BCL6B are known to be involved in cell cycle regulation during development (Chevrier \& Corcoran, 2014). The MitoCDNB condition also revealed involvement of upstream transcription factors such as IRF4, CREB and E2F. IRF4, interferon regulatory factor 4, is in a family of transcriptional factors that play pivotal roles in many aspects of the immune response, development and differentiation. It has recently been implicated in regulation of mitochondrial homeostasis (Low et al., 2019). CREB, cAMP-responsive element-binding protein, activation has been shown to be induced by mitochondrial dysfunction, using oligomycin (Arnould et al., 2002). Furthermore, CREB is one of the
transcription factors that synergistically associates with Nrf2 (Katoh et al., 2001), which I have shown in chapter 4 is activated by MitoCDNB-dependent mitochondrial dysfunction.

### 5.4.3.2. MitoCDNB vs MitoPQ in heart

Next, the effects of MitoCDNB and MitoPQ were compared in the heart. As the Venn diagram shows (Figure 5.28 A ), there are 59 genes that are specifically affected by MitoCDNB and 26 genes that are specifically affected by MitoPQ in the heart. Interestingly there are only 2 genes in the intersection between both conditions.

When the MitoCDNB-specifically altered genes for the heart were analysed with g :Profiler, there was no significant output in any of the aforementioned categories. MitoPQ-specific genes for heart tissue also did not give more insight beyond an involvement of biological processes such as regulation of nucleobase-containing compound metabolic process, response to glucocorticoid, corticosteroids, steroid hormones, lipids and regulation of hematopoiesis (Figure 5.28 B).


B


Figure 5.28. MitoCDNB and MitoPQ effect in heart tissue. A) Venn diagram of specific differentially expressed genes of MitoCDNB and MitoPQ in heart. MitoPQ (B) specific genes underwent g:Profiler analysis, which is shown using a plot, where the $x$-axis contains gene ontology (molecular function - red, biological processes yellow), KEGG pathways-purple and transcription factor-blue analysis, and the $y$-axis represents -log(FDR).

Overall, the effect of both MitoPQ and MitoCDNB in the heart is a lot lower compared to in the liver, as shown in the previous section. Nonetheless, there is still only minimal overlap between the differentially expressed genes for MitoCDNB and MitoPQ conditions in both tissues. The fact that g :Profiler analysis does not show many results could be due to the low number of genes affected in each of the conditions, as well as their relative variety.

### 5.4.3.3. Common MitoCDNB vs MitoPQ effect in both tissues

Finally, the effects of MitoCDNB and MitoPQ on gene expression, irrespective of the tissues, was checked. For this, the resulting genes were taken from the differential expression analysis, where the effect of the tissue was controlled for, and therefore the fold change results only from the effect of the conditions. After this, the analysis was the same as described in Figure 5.26.

As the Venn diagram shows (Figure 5.29 A), there are 180 genes that are specifically affected by MitoCDNB and 89 genes that are specifically affected by MitoPQ. Interestingly, as observed above there are only 2 genes in the intersection of the diagram that are affected by both interventions.

Biological processes, identified through gene ontology, specific for MitoCDNB (Figure 5.29 B) are nitrogen compound metabolic process, response to peptide, cellular macromolecule metabolic process among others. Gene ontology was also used to explore the molecular function and the results are glucocorticoid receptor binding, ligand-activated transcription factor activity and nuclear receptor activity. MitoCDNB led to changes in the expression of genes regulated by many transcription factors, some of which are E2F, E2F-1, E2F-2, SREBP, GKLF and ZF5.

MitoPQ specific biological processes consist of a regulation of a variety of metabolic processes (nitrogen compound metabolic process, nucleic acid metabolic process, RNA metabolic process, cellular metabolic process), cell growth and RNA biosynthetic process to name a few. The analysis of KEGG pathways with g :Profiler has also recognised the involvement of NFאB, NOD-like receptor and TNF signalling pathways as well as infection
pathways. The upstream transcription factors that could potentially explain the specific genes observed with the condition of enhanced mitochondrial superoxide levels are E2F, ZF5, SP100, IRF4, AP-2, GKLF, Kaiso among others (Figure 5.29 C).


Figure 5.29. MitoCDNB and MitoPQ effects irrespective of tissue. A) Venn diagram of specific differentially expressed genes of MitoCDNB and MitoPQ irrespective of tissue. MitoCDNB (B) and MitoPQ (C) specific genes underwent g:Profiler analysis, which is shown using plots, where the $x$-axis contains gene ontology (molecular function - red, biological processes - yellow), KEGG pathways-purple and transcription factor-blue analysis, and the $y$-axis represents -log(FDR).

To summarise the comparison of disruption of mitochondrial thiol redox homeostasis and enhanced mitochondrial superoxide production, showed that the most striking feature is that there are almost no overlapping genes regulated in similar ways in either of the tissues, which means that the effects from the two interventions are distinct, at least at the early time points.

Both conditions have shown effects on metabolic processes, with MitoPQ interestingly, mainly affecting the RNA metabolic processes. Furthermore, the MitoPQ condition has shown involvement of signalling pathways such as NFKB and TNF, which was shown by the GSEA analysis as well. There are some upstream transcription factors that have been discussed before in previous sections, hence I will explore the connection with GKLF and E2F family of transcription factors, which are implicated in both conditions. GKLF, gut
enriched Kruppel-like factor, is involved in redox-sensitive growth arrest of cells through its activation of the p53 pathway (Wassmann et al., 2007) as well as a nodal transcriptional regulator of mitochondrial homeostasis (Liao et al., 2015). Lastly, the E2F family of transcription factors are mostly known as the key downstream target of Retinoblastoma tumour suppressor protein (pRB), regulating cell cycle progression and apoptosis, subsequently they are inactivated in various human cancers (Benevolenskaya \& Frolov, 2015). The E2F transcription factors have been proposed to have an emerging role in regulation of mitochondrial functions through direct binding to the promoter regions and through interactions with Nrf1, Nrf2, PPAR, which are all key regulatory factors of mitochondrial biogenesis (Benevolenskaya \& Frolov, 2015).

### 5.4.4. Effects of TPP on transcription

Lastly, I was interested in the "TPP" effect of our compounds, as all of them have a mitochondria-targeting TPP-moiety, which due to its accumulation may result in some of the mitochondrial specific effects that are observed. In order to investigate this effect, I first had to establish the specific genes that are transcribed differentially due to both active (MitoCDNB, MitoPQ) and inactive (MitoCDNB Ctrl, MitoPQ Ctrl) versions of the compounds, and then subsequently investigate the significantly expressed genes in the intersection of these compounds further via $\mathrm{g}:$ Profiler (Figure 5.30). This is because the genes at the intersection are the ones that are affected by both compounds, both active and inactive, and from that perspective should to some extent display the effect on gene-expression of the TPP-moiety. This effect was explored for both pairs of molecules (MitoCDNB and MitoPQ), as the molecules themselves have very different chemical properties, which can influence the outcome.

Compare the
differentially
expressed genes
for active and
inactive
compounds

Select the differentially expressed genes in the cross section

Analysis of the genes with G:profiler

Figure 5.30. A schematic representation of steps taken to investigate the "TPP" effects of mitochondria targeted compounds. I started with the differentially expressed genes from DESeq2 between conditions (MitoCDNB, MitoCDNB Ctrl, MitoPQ, MitoPQ Ctrl) and vehicle. Next, the differentially expressed genes for active and inactive compounds were compared using a Venn diagram (MitoCDNB vs MitoCDNB Ctrl; MitoPQ vs MitoPQ Ctrl). Subsequently, the differentially expressed genes in the intersection of the Venn diagram in each tissue were selected. Finally, gene ontology and upstream transcription factor analysis was done using g:Profiler.

### 5.4.4.1. TPP effect of the MitoCDNB molecules

Firstly, the TPP effect from the MitoCDNB pair of molecules was investigated. This was done for the upregulated genes from the comparisons in liver and heart, as well as irrespective of tissue.

As described above, the differentially expressed genes from MitoCDNB and MitoCDNB Ctrl in liver were compared in a Venn diagram (Figure 5.31 A ), with 307 genes falling into the intersection, which are the interesting ones for the analysis with g :Profiler. As observed in Figure 5.31 B there are numerous results, and I will only discuss a select few here. The gene ontology-based analysis for molecular function in liver led to mainly terms involving DNA binding, transcription activity and MAP kinase phosphatase activity. The "TPP"-effect mainly involved biological processes such as metabolism, gene expression and RNA biosynthetic processes; and led to changes in KEGG pathways such as MAPK, FoxO and TNF signalling pathways. The upstream transcription factors found to be responsible for the differentially expressed genes were E2F-1/3/4, CREB, ATF-1/2, ATF4, Kaiso, CREB, E2F and ZF5 among others.


Figure 5.31. TPP effect in liver tissue. A) Venn diagram of specific differentially expressed genes of MitoCDNB and MitoCDNB Ctrl in liver. B, The common specific genes underwent g:Profiler analysis, which is shown using a plot, where the $x$-axis contains gene ontology (molecular function - red, biological processes - yellow), KEGG pathways-purple and transcription factor-blue analysis, and the $y$-axis represents - $\log (F D R)$.

Next, the same approach was taken for the differentially expressed genes that are found in the intersection of MitoCDNB and MitoCDNB Ctrl from the heart tissue, which comprises a total of 79 genes (Figure 5.32 A ). Interestingly, the number of genes of interest here is almost four times lower than found in the liver shown above. This is also due to the fact that there were a lot less differentially expressed genes in the heart tissue from these conditions to begin with. In the heart, the "TPP"-effect was correlated with the molecular function of transcription regulator inhibitor activity, biological processes involved in negative regulation of biosynthetic processes, cell differentiation, transcription and development (Figure 5.32 B). Furthermore, the KEGG pathway that stood out was the Notch signalling pathway. The upstream transcription factors that were identified from the TRANSFAC database were E2F, ZF5, E2F-1, LKLF, and CPBP among others.


Figure 5.32. TPP effect in heart tissue. A) Venn diagram of specific differentially expressed genes of MitoCDNB and MitoCDNB Ctrl in heart. B) The common specific genes underwent g:Profiler analysis, which is shown using a plot, where the $x$-axis contains gene ontology (molecular function - red, biological processes - yellow), KEGG pathways-purple and transcription factor-blue analysis, and the $y$-axis represents -log(FDR).

As before, it is also necessary to look at the "TPP" effect to see if the differentially expressed genes from MitoCDNB and MitoCDNB Ctrl are not affected by the tissue. In this situation, there are 262 genes found in the intersection of the conditions (Figure 5.33 A). The gene ontology analysis for molecular functions showed that the main terms correlating with our genes of interest are DNA binding, transcription factor activity, MAP kinase phosphatase activity, kinase and protein binding (Figure 5.33 B). The resulting biological processes terms show a similar picture as before with many of them corresponding to metabolic processes or regulation of transcription processes. The "TPP" effect of our MitoCDNB compounds, irrespective of tissue influence, suggest that MAPK, TNF, FoxO and NFкB signalling pathways are involved. Finally, there are many identified upstream transcription factors that could explain the expression of our genes of interest such as E2F, ZF5, E2F-1/3/4, BEN, IRF4, Kaiso, BCL6B, CREB, ATF-1/2/4 and ATF4 among others (Figure 5.33 B).


Figure 5.33. TPP effect irrespective of tissue. A) Venn diagram of specific differentially expressed genes of MitoCDNB and MitoCDNB Ctrl irrespective of tissue. B) The common specific genes underwent g:Profiler analysis, which is shown using a plot, where the x-axis contains gene ontology (molecular function - red, biological processes - yellow), KEGG pathways-purple and transcription factor-blue analysis, and the y-axis represents - log(FDR).

To summarise, the effect of the TPP-based compounds on signalling affects the MAPK, FoxO and TNF pathways, especially in the liver, but they are also observed when common effects irrespective of tissue are investigated. All these pathways are well characterised and involved in numerous cellular processes. FoxO signalling is known to protect mitochondria by activating mitochondrial antioxidant enzymes (Kim \& Koh, 2017). The upstream transcription factors suggest similar ones that we have described above such as E2F family, CREB, ZF5, IRF4, with a lot more potential hits coming from the liver tissue, but again that is possibly due to the lower amount of differentially expressed genes in the heart to begin with. An interesting one is ATF4, activating transcription factor 4, which has been suggested as the main regulator of the mitochondrial stress response, and also related to the integrated stress response (Quirós et al., 2017).

### 5.4.4.2. TPP effect from the MitoPQ molecules

As a next step, a similar approach to investigating the "TPP"-effect was undertaken for the compounds that elicit enhanced mitochondrial superoxide production. MitoPQ and MitoPQ Ctrl it led to a very small number of genes at the intersection (Figure 5.34), which might suggest that the effect of the active and inactive compounds, MitoPQ and MitoPQ Ctrl, is very different.
A

B

C


Figure 5.34. Venn diagrams of specific differentially expressed genes of MitoPQ and MitoPQ Ctrl in liver (A), heart (B) and irrespective of tissue (C).

### 5.5. Discussion

Mitochondria are known to have a central function in cellular communication and signalling. Moreover, recently it has become clear that some of these signals are redoxrelated, and that ROS signals are no longer viewed as just the harmful molecules that cause dysfunction. Up until now, it has not been able to study retrograde redox signalling, after targeted mitochondrial disruptors used in vivo. Here I used a chemical biology approach with MitoCDNB, a known disruptor of mitochondrial thiol redox homeostasis and MitoPQ, a mitochondria-targeted redox cycler that enhances mitochondrial superoxide production. Mice were injected with both active and inactive compounds, as well as a vehicle control, RNA isolated from liver and tissue and a transcriptomic analysis using RNAseq.

This chapter showed that disruption of mitochondrial thiol defences resulted in the differential expression of many genes when compared to vehicle, but the difference was small compared with its control compound in both liver and heart. Nonetheless, it provides an important insight into signalling pathways that are involved in this type of mitochondrial dysfunction. Interestingly, the GSEA pathway analysis showed that in the liver the inflammatory and stress-related pathways, such as TNF- $\alpha$ signalling via NFкB, TGF $\beta$ and p53 pathways were downregulated, whereas in the heart the opposite, upregulation was observed. This strikingly different result can be explained by the different roles the tissues play, with tissue specificity of mitochondrial biology and antioxidant defences. In the heart tissue, it was observed that metabolic pathways, such as OXPHOS, fatty acid metabolism and adipogenesis, were mostly downregulated, which could be due to a disruption of mitochondrial function. The main similar effect in both tissues was the upregulation of EMT, the epithelial mesenchymal transition, which normally occurs as part of tumourigenesis and renders cells resistant to therapies targeted to primary cancers. The mechanistic link between metastasis and mitochondrial dysfunction is gradually emerging (Guerra et al., 2017). It has been previously discovered that mitochondrial dysfunction due to deregulated mitophagy, depletion of the mitochondrial DNA, or defects in Krebs cycle enzymes activate the EMT pathway (Guerra et al., 2017). A tentative conclusion from the experiments shown in this chapter is that mitochondrial dysfunction due to disrupting mitochondrial antioxidant defences could be a new mechanism that leads to EMT and this idea should be investigated further.

A similar approach was taken in investigating enhanced mitochondrial superoxide production via a mitochondrial-targeted redox cycler, MitoPQ (Robb et al., 2015). There were a lot of differentially expressed genes when comparing MitoPQ/MitoPQ Ctrl and vehicle, and this was translated to the active vs inactive compound comparison. The GSEA pathway analysis showed that enhanced mitochondrial superoxide production led to upregulation of EMT, similarly, to disrupting mitochondrial thiol defences in both liver and heart tissue. This further adds to the variety of mitochondrial dysfunctions that are potentially involved in metastasis and tumorigenesis (Guerra et al., 2017). Apart from the EMT, there are some tissue specific pathways that are involved with the MitoPQ condition. In the liver, there is no significant downregulation of gene expression, but upregulation of
gene expression occurs for both metabolic pathways (adipogenesis, fatty acid metabolism) and another cancer associated pathway, the IL-6-JAK-STAT3 signalling pathway. The latter has been previously implicated to respond to mitochondrial ROS signalling (Abid et al., 2020). In the heart, distinct from the liver there was slight downregulation of metabolic pathways (adipogenesis) and MTORC1 signalling. There was also an upregulation of the inflammatory and stress-related pathways, such as TNF- $\alpha$ signalling via NFкB and TGF $\beta$.

Another approach was to look for specific effects of enhanced mitochondrial superoxide production and disruption of mitochondrial antioxidant defences, where only differentially expressed genes for the active compounds (MitoPQ and MitoCDNB) were compared using a Venn diagram and analysed with g:Profiler. One of the most striking features is that there are almost no overlapping differentially expressed genes in either of the tissues, which suggests that the effects of the two interventions tested are largely separate at least at the early time point investigated here. In the heart specifically, there were not many results for either effect due to the low amount of differentially expressed genes to start with. The gene ontology analysis showed many molecular functions and biological processes involved with the conditions that have been described in the previous section. Interestingly, the differences in activation of upstream transcription factors were not as large as would be expected from the almost zero overlap between the genes showed with the Venn diagrams, which is more indicative of the promiscuous nature of these transcription factors. In the liver, both MitoCDNB and MitoPQ conditions involved transcription factors ZF5 and BCL6B, known to be involved in cell cycle regulation during development (Chevrier \& Corcoran, 2014). In addition to those, there were some MitoCDNB specific transcription factors in the liver such as E2F, IRF4 and CREB. The latter two are known to play pivotal roles in mitochondrial homeostasis (Arnould et al., 2002; Low et al., 2019). Furthermore, CREB is known to act synergistically with Nrf2 (Katoh et al., 2001), which has already been shown to be activated by disrupting mitochondrial antioxidant defences. When I investigated the specific effects of MitoCDNB and MitoPQ irrespective of the tissue, both conditions had gene ontology results regarding metabolic processes, with MitoPQ mainly affecting RNA metabolic processes. Apart from the transcription factors already stated, there was involvement of GKLF, which is a regulator of mitochondrial homeostasis (Liao et al., 2015) and which leads to redox-sensitive growth arrest of cells by activating the p53
pathway (Wassmann et al., 2007). A common factor in these experiments has been cell cycle/growth regulation, and therefore it is not surprising that E2F family of transcription factors are involved in both conditions. Recently their emerging role has been proposed for regulation of a variety of mitochondrial functions, through Nrf2, Nrf1 and PPAR (Benevolenskaya \& Frolov, 2015).

Lastly, I was interested in investigating the effect that the mitochondria targeting moiety of these TPP-based compounds had on cell signalling. This was done by exploring the differentially expressed genes in common between the inactive and active version of our compounds, MitoCDNB and MitoPQ. The latter comparison did not lead to any results with the $\mathrm{g}:$ Profiler analysis as the intersection gene number was small. When comparing MitoCDNB and MitoCDNB Ctrl the common genes were enough to provide some insights into the proposed "TPP" effect. In the liver and common across both tissues MAPK, TNF and FoxO signalling pathways stood out, with the latter being known to protect mitochondria through activating mitochondrial antioxidant enzymes (Kim \& Koh, 2017). With regards to the upstream transcription factors, most of them I discussed above in the previous paragraphs such as E2F family, CREB, ZF5, IRF4 among many. Multiomic approaches to mitochondrial dysfunction in cells have identified ATF4, as one of the main regulators of the mitochondrial stress response (Quirós et al., 2017) and it was interesting to see the "TPP" effect also involving this transcription factor.

In summary, this study investigated early redox signalling due to mitochondrial dysfunctions, such as enhanced mitochondrial superoxide production and disruption of mitochondrial thiol redox homeostasis. Normally, with the bioinformatic approaches there are a lot of results, but it is important to be aware of the limitations of the study in order for the conclusions to be assessed. Due to the RNAseq results observed in Chapter 3 the early time point of 1 hour was chosen here and moving forward it would be interesting to explore other later time points, especially with MitoCDNB/MitoCDNB Ctrl, as GSH depletion has been shown to increase. Furthermore, to enhance our understanding of mitochondrial redox dysfunction the additive effects of both compounds needs to be assessed in the future to observe any possible synergism.

# Chapter 6. Developing non-mitochondrial sub-cellular targeting of small molecules 

### 6.1. Introduction

Throughout this thesis, a mitochondria-targeted chemical biology approach was used to assess the disruption of mitochondrial thiol redox homeostasis and hydrogen peroxide production and their effects on cellular redox signalling. The ability to direct molecules to a specific location within the cell, or even to exclude them from an undesirable location, facilitates the development of bioactive and probe compounds, such as fluorescent markers. The molecules that are targeted to the mitochondrial matrix by conjugation to the lipophilic triphenylphosphonium cation such as, MitoCDNB and MitoPQ, are examples (Booty et al., 2019; Murphy \& Hartley, 2018; Robb et al., 2015; Smith et al., 2012a; Yousif et al., 2009). The triphenylphosphonium group enables rapid crossing of biological membranes by lowering the activation energy for movement through the membrane core (Ross et al., 2005; Zielonka et al., 2017) due to its large hydrophobic surface area. In addition, the extensive accumulation within the mitochondrial matrix is due to the positive charge of the cation and the large negative membrane potential across the mitochondrial membrane (Murphy \& Hartley, 2018; Ross et al., 2005; Zielonka et al., 2017).

It would also be useful to deliver molecules to other cellular compartments in a similar way, hence the conjugation to lipophilic anions was explored. With the archetypal lipophilic cation being tetraphenylphosphonium (TPP), the corresponding lipophilic anion is tetraphenylborate (TPB). Apart from the charge, these molecules share similarities and have identical radii (4.2 Å) (Flewelling \& Hubbell, 1986a, 1986b). In order to facilitate biological uses, both are water soluble, although TPB has a more negative free energy of hydration, meaning it is hydrated more (Scheu et al., 2014).

When permeating membranes, lipophilic ions normally bind to a potential energy well (created by favouring interactions) on the membrane surface, with the repulsive forces increasing as the ions move through the membrane, before flipping to the corresponding potential energy well on the other side of the membrane. With this they traverse the activation energy barrier of the membrane core (Figure 6.1). Therefore, TPB rapidly permeates phospholipid bilayers (Benz, 1988; Flewelling \& Hubbell, 1986a, 1986b; Rokitskaya et al., 2019; L. Wang, 2012) and its distribution is determined by the membrane potential (Grinius et al., 1970). One would expect that this would lead to its exclusion from the cell and the mitochondrial matrix, due to TPB being an anion and the negative inside plasma membrane potential.

Flip Across


Figure 6.1. Membrane permeation by a TPB lipophilic anions. Taken from (Gaddale Devanna et al., 2021).

Lipophilic tetraarylborate (TPB) anions have been widely used in analytical chemistry (Flaschka \& Barnard Jr, 1960), catalysis (Riddlestone et al., 2018) and as substrates for oxidative and Suzuki-type cross-coupling (Hussain et al., 2016; Music et al., 2020). Despite the above-mentioned similar structure to TPP, and their complementary response to $\Delta \psi$, the location of TPB-conjugated compounds has not been explored. As the negative $\Delta \psi$ across the plasma membrane is unfavourable for intracellular delivery, one prediction is that lipophilic anions, such as TPB, should be excluded from cells, making these potential probes for the extracellular environment. Only one exploration of lipophilic anion distribution within cells has been done to date by conjugation of the monocarborane (1-
carba-closo-dodecaborate) lipophilic anion to a porphyrin (Moisenovich et al., 2010; Rokitskaya et al., 2012). Surprisingly, the monocarborane conjugate was not excluded rather it was taken up by cells (Moisenovich et al., 2010), although the mechanism of uptake was not investigated. Hence, there was an open question of whether the TPB anions would actually be able to exclude probes from the cell, or alternatively they could potentially be used to deliver compounds selectively to specified compartments, despite their negative charge, within the cell.

To assess the functional properties and intracellular distribution of TPB compounds our collaborators in Glasgow synthesized a series of TPB derivatives (Gaddale Devanna et al., 2021), such as TPBM, where a sulfonamide and morpholine groups were added; TPBE where TPB was conjugated to the chromanol group of $\alpha$-tocopherol through sulfonamide and two fluorescent probes TPBCoumarin and TPBBODIPY, to allow for imaging and hence investigation into subcellular localisation (Figure 6.2).

## TPBCoumarin



TPBBODIPY


Figure 6.2. Chemical structures of TPBCoumarin and TPBBODIPY. Taken from (Gaddale Devanna et al., 2021).

The investigation of TPB-conjugates distributing across membranes in response to voltage was done by a collaborator using a phospholipid black lipid membrane (BLM) system. TPBM led to greater membrane disruption compared to TPB, which was caused by its enhanced lipophilicity. Therefore to estimate the amount of compound crossing the bilayer in response to voltage (Gaddale Devanna et al., 2021) measured transient ionic currents induced before/after addition of the compounds. In the absence of compounds, the transient currents are determined by the membrane's inherent dielectric properties. When the TPB-conjugates are present the transient currents are proportional to the compound concentration, due to the charge transfer from the lipophilic anions crossing the bilayer. From this, TPBM was shown to induce the largest charge flux at a concentration of 100 nM , whereas higher concentrations caused non-specific conductance. These results indicated that TPB-conjugates do cross a BLM in a voltage-dependent manner.

Next, it was assessed whether TPB-conjugates cross biological membranes in response to a $\Delta \psi$. This was done by a collaborator using sub-mitochondrial particles (SMPs), which are inverted mitochondrial inner membrane vesicles. NADH driven proton pumping by the respiratory chain leads to a positive-inside $\Delta \psi$, which should drive uptake of lipophilic anions (Grinius et al., 1970). An ion-selective electrode (ISE) was used to measure the TPB concentration (Shoukry et al., 1987) in the vesicle. The rapid uptake of TPB was seen upon induction of $\Delta \psi$ by NADH. This was reversed by dissipating the $\Delta \psi$ with FCCP. Furthermore, TPBM and TPBE also showed uptake into SMPs in response to $\Delta \psi$. The greater hydrophobicity of TPBM and TPBE enhanced their adsorption to the membranes.

TPB conjugates have a higher membrane conductivity compared to TPP and a greater binding constant for the membrane surface due to their interaction with the dipoles inherent in the membranes (Flewelling \& Hubbell, 1986a, 1986b; Honig et al., 1986; L. Wang, 2012). The addition of a hydrophobic linker increases this membrane adsorption further and needs to be accounted for when making probes with TPB. The next experimental steps would be to investigate the uptake and distribution of TPB-conjugates within cells.

Work from this chapter has been published in (Gaddale Devanna et al., 2021).

### 6.2. Chapter hypothesis

My hypothesis for this chapter is that TPB-conjugates can be used as probes with a unique distribution.

### 6.3. Aims and rationale

In this chapter, I aim to characterise the use of TPB as a target moiety for probes. Initially, the cell uptake and sub-cellular distribution of fluorescent TPB probes, TPBCoumarin and TPBBODIPY will be explored using live cell microscopy. Subsequently, the mechanisms of uptake will be assessed using various cell biology approaches.

### 6.4. Results

### 6.4.1. TPBCoumarin uptake and cell distribution

In order to investigate the behaviour of TPB-conjugates in living cells, the fluorescent compounds TPBCoumarin and TPBBODIPY were synthesised. This meant live-cell fluorescent microscopy methods could be used to study the uptake and distribution of the compounds in question.

C2C12 mouse myoblast cells were plated in glass Nunc dishes (ThermoFisher Scientific) the day before the experiments. As live cells were visualised, the dishes were placed on temperature and $\mathrm{CO}_{2}$ controlled platforms prior to imaging. Cells were then incubated with 100 nM TPBCoumarin and visualised with Coumarin specific settings ( 343 nm laser) after 10 min (Figure 6.3 A). Despite its negative charge, TPBCoumarin (green) was shown to be rapidly taken up by cells (Figure 6.3 A ) and distributed throughout the cytosol with the cells
showing punctate staining. To explore the distribution further, cells were stained with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta) for 5 min after the initial 10 min incubation with TPBCoumarin (Figure 6.3 A and B). The results showed TPBCoumarin mainly colocalising with lysosomes and not with mitochondria (Figure 6.3 B and C ). The lysosome colocalization explained the observed punctate staining.


Figure 6.3. Cell distribution of TPBCoumarin in mouse myoblasts. (A) C2C12 cells were incubated with 100 nM TPBCoumarin (green). Distribution was imaged 10 min after addition. Cells were further incubated with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta). Colocalisation of MitoTracker with TPBCoumarin (B) is shown in orange and colocalisation of LysoTracker with TPBCoumarin (C) is shown in white. Representative images are from 3 different biological experiments. Scale bar $=20 \mu \mathrm{~m}$.

Next, the uptake and distribution of TPBCoumarin was investigated in HeLa cells. As mentioned above, to prepare for live cell imaging, cells were seeded onto glass dishes and kept in a temperature and $\mathrm{CO}_{2}$ regulated platform for the duration of the imaging. HeLa cells were incubated with 100 nM TPBCoumarin and imaged after 10 min . Similar to C2C12 cells TPBCoumarin was rapidly taken up by cells and distributed in a particular punctate pattern (Figure 6.4 A). Parallel staining with MitoTracker and LysoTracker further suggested
colocalization of TPBCoumarin and Lysotracker and therefore a lysosomal distribution (Figure 6.4 B and C ).


Figure 6.4. Cell distribution of TPBCoumarin in HeLa cells. (A) HeLa cells were incubated with 100 nM TPBCoumarin (green). Distribution was imaged 10 min after addition. Cells were further incubated with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta). Colocalisation of MitoTracker with TPBCoumarin $(B)$ is shown in orange and colocalisation of LysoTracker with TPBCoumarin $(C)$ is shown in white. Representative images are from 3 different biological experiments. Scale bar $=20 \mu \mathrm{~m}$.

Finally, to confirm the previously observed uptake and distribution of TPBCoumarin in C2C12 and HeLa cells, Cos7 cells were tested next. As discussed before, Cos7 cells were imaged on a temperature and $\mathrm{CO}_{2}$ regulated platform in order to maintain favourable conditions for the live cells. After 10 min of TPBCoumarin ( 100 nM ) incubation, Cos7 cells exhibited cellular punctate staining (Figure 6.5 A ) as observed before with C2C12 and HeLa cells (Figure 6.3 and 6.4). Staining with both MitoTracker and Lysotracker confirmed the lysosomal colocalization of TPBCoumarin.


Figure 6.5. Cell distribution of TPBCoumarin in Cos7 cells. (A) Cos7 cells were incubated with 100 nM TPBCoumarin (green). Distribution was imaged 10 min after addition. Cells were further incubated with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta). Colocalisation of MitoTracker with TPBCoumarin (B) is shown in orange and colocalisation of LysoTracker with TPBCoumarin (C) is shown in white. Representative images are from 3 different biological experiments. Scale bar $=20 \mu \mathrm{~m}$.

These experiments with TPBCoumarin convincingly showed that the negative charge of the TPB moiety does not impair its uptake into the cells. Furthermore, the observed distribution of TPBCoumarin is not random, but rather exhibits a punctate staining throughout the cell. The latter corresponds to lysosomal staining as shown with colocalization studies with the Lysotracker probe. The uptake and distribution of TPBCoumarin were not unique to a cell type, as the same results were observed in C2C12, HeLa and $\operatorname{Cos} 7$ cells.

### 6.4.2. TPBBODIPY uptake and cell distribution

To test the uptake and cell distribution of TPB-conjugates further, another fluorescent probe was used, TPBBODIPY. C2C12 mouse myoblast cells were incubated with 100 nM TPBBODIPY in glass dishes on a temperature and $\mathrm{CO}_{2}$ regulated platform and imaged after

10 min with the corresponding BODIPY setting ( 488 nm laser) (Figure 6.6 A). TPBBODIPY was rapidly taken up by the cells, despite its negative charge and similarly to TPBCoumarin exhibited a punctate staining (Figure 6.6 A). Parallel staining with MitoTracker and LysoTracker (both 500 nM ) for 5 min after TPBBODIPY incubation suggested a similar lysosomal colocalisation of TPBBODIPY in C2C12 mouse myoblasts (Figure 6.6 B and C).


Figure 6.6. Cell distribution of TPBBODIPY in mouse myoblasts. (A) C2C12 cells were incubated with 100 nM TPBBODIPY (green). Distribution was imaged 10 min after addition. Cells were further incubated with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta). Colocalisation of MitoTracker with TPBBODIPY (B) is shown in orange and colocalisation of LysoTracker with TPBBODIPY (C) is shown in white. Representative images are from 3 different biological experiments. Scale bar $=20 \mu \mathrm{~m}$.

Next, the uptake and cell distribution of TPBBODIPY was investigated in HeLa cells, to explore if the observed distribution was unique to C2C12 cells. As before HeLa cells were incubated with 100 nM TPBBODIPY on a temperature and $\mathrm{CO}_{2}$ regulated platform and imaged after 10 min of incubation. HeLa cells showed a similar rapid uptake and punctate staining distribution. across the cytosol (Figure 6.7 A). Colocalisation studies with MitoTracker (Figure 6.7 B) and LysoTracker (Figure 6.7 C) suggested a lysosomal distribution of TPBBODIPY in HeLa cells.


Figure 6.7. Cell distribution of TPBBODIPY in HeLa cells. (A) HeLa cells were incubated with 100 nM TPBBODIPY (green). Distribution was imaged 10 min after addition. Cells were further incubated with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta). Colocalisation of MitoTracker with TPBBODIPY (B) is shown in orange and colocalization of LysoTracker with TPBBODIPY (C) is shown in white. Representative images are from 3 different biological experiments. Scale bar $=20 \mu \mathrm{~m}$.

Finally, to confirm the TPBBODIPY behaviour when exposed to a live cell environment, Cos7 cells were used to investigate the uptake and distribution of TPBBODIPY. As mentioned above, the experimental setup consisted of live Cos7 cells in glass dishes on temperature and $\mathrm{CO}_{2}$ regulated platforms for optimal live cell imaging. TPBBODIPY was added at 100 nM and the cells were imaged after 10 min (Figure 6.8 A ), which led to a rapid uptake and punctate staining of TPBBODIPY. When compared to MitoTracker staining of Cos7 cells there was no mitochondrial colocalisation observed (Figure 6.8 B), while LysoTracker staining showed mainly lysosomal colocalisation of TPBBODIPY (Figure 6.8C).


Figure 6.8. Cell distribution of TPBBODIPY in Cos7 cells. (A) Cos7 cells were incubated with 100 nM TPBBODIPY (green). Distribution was imaged 10 min after addition. Cells were further incubated with 500 nM MitoTracker (red) and 500 nM LysoTracker (magenta). Colocalisation of MitoTracker with TPBBODIPY (B) is shown in orange and colocalisation of LysoTracker with TPBBODIPY (C) is shown in white. Representative images are from 3 different biological experiments. Scale bar $=20 \mu \mathrm{~m}$.

These experiments further confirmed that TPB-conjugates are readily and rapidly taken up into cells and mainly exhibit a lysosomal distribution. This has been shown for TPBCoumarin as well as for TPBBODIPY and this uptake is not unique to any cell type, as the experiments have been replicated in C2C12, HeLa and Cos7 cells. The punctate staining of TPBconjugates after cell uptake could suggest an uptake by endocytosis with the initial localisation within endosomes which then fuse with lysosomes (Doherty \& McMahon, 2009).

### 6.4.3. Mechanism of cellular uptake of TPB-conjugates

In the beginning of this chapter, TPB-conjugates were discussed as a different approach to controlling the location of compounds. TPBCoumarin and TPBBODIPY were rapidly taken up by cells, despite their negatively charged TPB moiety, and exhibited a lysosomal
distribution within the cell. The lysosomal localisation of the compounds sparked our interest into the possible mechanism of cellular uptake. From the literature uptake by endocytosis seemed most likely. In order to investigate this potential involvement of endocytosis in the uptake of TPB-conjugates, an inhibitor of endocytosis, Pitstop 2, was used.

Pitstop 2 acts via blocking ligand access to the clathrin terminal domain and stalls clathrincoated pit (CCP) dynamics. It is a selective inhibitor of both clathrin-dependent and independent endocytosis (Von Kleist et al., 2011). C2C12 mouse myoblast cells were incubated with $20 \mu \mathrm{M}$ of either Pitstop2 or vehicle ( $0.1 \%$ ethanol) for 30 min before the addition of 100 nM of TPBCoumarin (Figure 6.9) or TPBBODIPY (Figure 6.10). In both cases, there was an observed substantial decrease of fluorescent intensity of both TPBCoumarin (Figure 6.9) and TPBBODIPY (Figure 6.10) in the cell cytosol. The quantification of fluorescent intensity was done by measuring the intensity of 30 cells per experiment and the results of 3 independent experiments are shown in Figure 6.9 B for TPBCoumarin and Figure 6.10 B for TPBBODIPY. These results further suggest that the rapid uptake of the TPB-conjugates into the cell is due to endocytosis followed by redistribution to the lysosomes. In addition, Dynasore, a noncompetitive inhibitor of dynamin-dependent endocytosis (Kirchhausen et al., 2008) was used with a less prominent but consistent result (data not shown).


Figure 6.9. Pitstop2 diminishes TPBCoumarin uptake into mouse myoblasts. (A) Representative images of C2C12 cells that were incubated with $20 \mu \mathrm{M}$ Pitstop2 or vehicle ( $0.1 \%$ ethanol) for 30 min before addition of 100 nM TPBCoumarin. (B) Bar chart showing the fluorescent intensity of the cells incubated with either vehicle or Pitstop2. Scale bar $20 \mu \mathrm{~m}$. 30 cells were counted per experiment and the bar chart shows mean $+/-$ SEM of 3 independent experiments. ${ }^{* * * * p}<0.0001$ by Student's $t$ test.


Figure 6.10. Pitstop2 diminishes TPBBODIPY uptake into mouse myoblasts. (A) Representative images of C2C12 cells that were incubated with $20 \mu \mathrm{M}$ Pitstop2 or vehicle ( $0.1 \%$ ethanol) for 30 min before addition of 100 nM TPBBODIPY. (B) Bar chart showing the fluorescent intensity of the cells incubated with either vehicle or Pitstop2. Scale bar $=20 \mu \mathrm{~m} .30$ cells were counted per experiment and the bar chart shows mean $+/-$ SEM


TPB-conjugates were shown to enter the cells via endocytosis and are then preferentially accumulated in the lysosomes after 10 min of incubation. The next step was to investigate if the TPB compounds then stay in the lysosomes or if they diffuse to the cytosol later on. In order to explore this, cells were visualised 1 hour (Figure 6.11) after incubation of 100 nM TPBCoumarin and washing steps after 10 min . The results clearly show that TPBCoumarin is still present in punctate staining in the cytosol and therefore diffusion from the lysosomes has not occurred to any distinguishable degree.


Figure 6.11. TPBCoumarin distribution after washing and longer exposure. 3D maximum projection images of C2C12 cells that were incubated with 100 nM TPBCoumarin, washed after 10 min and imaged after 1 h . The images are representative of 3 independent experiments. Scale bar $=20 \mu \mathrm{~m}$.

From the previous experiment it was clear that the TPBCoumarin probe did not diffuse out of lysosomes to any measurable extent. This could be a consequence of the low lysosomal pH or TPBCoumarin sticking to lysosomal membranes. For this purpose, Chloroquine was used as it is a weak base and passively diffuses into the acidic compartments of the cells and gets trapped there (Xue et al., 2014). This accumulation leads to an increase in lysosomal pH . If the accumulation of TPBCoumarin is dependent on the low pH of lysosomes, the addition of chloroquine should be able to decrease it. In line with this thinking C2C12 mouse myoblasts were treated with $50 \mu \mathrm{M}$ chloroquine for 4 hours before incubation with TPBCoumarin and visualisation after 10 min (Figure 6.12). The results did suggest a lowering of fluorescent intensity due to chloroquine treatment, but the punctate staining was nonetheless still observed (Figure 6.12). One of the explanations is that the TPBCoumarin lysosomal accumulation is explained due to the low pH of lysosomes to some extent.

## Vehicle



## Chloroquine



Figure 6.12. pH dependence of lysosomal TPBCoumarin accumulation. 3D maximum projection images of C2C12 cells that were incubated with either $50 \mu \mathrm{M}$ chloroquine or vehicle ( $0.1 \%$ ethanol) for 4 h prior to incubation with 100 nM TPBCoumarin for 10 min and then imaged. The images are representative of 3 independent experiments. Scale bar $=20 \mu \mathrm{~m}$.

### 6.5. Discussion

Lipophilic cations, such as TPP, have been used extensively to explore delivery of molecules to cell compartments. Most notably the molecules targeted to the mitochondrial matrix are often conjugated to TPP moieties (Murphy \& Hartley, 2018; Smith et al., 2012a; Yousif et al., 2009). The TPP group enables rapid crossing of biological membranes by lowering the activation energy of movement through the membrane core in addition to the positive charge of the cation which aids the extensive accumulation both across the cell membrane and the mitochondrial membrane (Ross et al., 2005; Smith et al., 2012a; Zielonka et al., 2017). With TPP being the archetypal lipophilic cation, TPB is the corresponding lipophilic anion. These molecules have similar radii and lipophilicity but have opposite charges (Flewelling \& Hubbell, 1986a, 1986b). In this chapter it is shown that despite the negative charge, as well as the negative-inside $\Delta \psi$ across the plasma membrane, TPB probes are taken up by cells and display a novel intracellular distribution.

In addition to the TPB probes' rapid uptake into the cells, the intracellular distribution showed that they accumulate preferentially in the lysosomes. Normally, most lysosome targeting is achieved through ion-trapping by protonation of a weak base in the acidic lysosomes (W. Xu et al., 2016; H. Zhu et al., 2016). As TPBBODIPY and TPBCoumarin accumulate in the same way it suggests that TPB-conjugates do not require such a protonation site, because TPBCoumarin does have an amine group, which could be potentially protonated but TPBBODIPY does not. Furthermore, TPBCoumarin and TPBBODIPY showed a similar distribution in both HeLa and Cos7 cells meaning that the unique distribution is not dependent on the cell type.

The rapid endocytotic uptake is likely to be due to the strong binding of the TPB moiety to the potential energy well on the membrane surface. Since TPB-conjugates rapidly permeate the phospholipid bilayers of SMPs, there is also likely to be some uptake into cells directly through the plasma membrane. The fact that TPB probes accumulated in cells rapidly in a short amount of time ( 10 min ), which is similar to MitoTracker and LysoTracker probes, supports the previous conclusion. Despite this, the plasma membrane potential ( $30-60 \mathrm{mV}$, negative inside) should disfavour such transfer.

The TPB-conjugates that do enter the cells are directed to the lysosomes, which could be a response to the proposed positive-inside potential across the lysosomal membrane ( $\mathrm{H} . \mathrm{Xu}$ \& Ren, 2015). This is also in agreement with chloroquine leading to a decrease in overall intensity of TPB conjugates in lysosomal compartments. The lack of observed colocalisation with mitochondria is consistent with the expected thousand-fold exclusion due to the large $\Delta \psi$ (150-180 mV, negative inside).

Overall, this chapter has shown that a new targeting group that directs small molecules to the endosomal and lysosomal compartments within the cell has emerged. This method complements the current methods that employ ion-trapping of weak bases. In the future, by manipulating hydrophobicity, incorporating cleavable linkers and membrane impermeant moieties it will be possible to fine tune the location and kinetics of the cell distribution of bioactive molecules. Such an approach provides new opportunities to
selectively manipulate and report on cell processes to give a better understanding of the role of lysosomes in autophagy for example (Wong et al., 2017; Yim \& Mizushima, 2020).

## Chapter 7. General Discussion and Future

## Work

The work in this thesis has focused on mitochondrial redox homeostasis and its implications for cellular redox signalling. Mitochondria are a part of the mitocellular communication network based on a variety of signals they release and react to (Mottis et al., 2019). This network allows them to respond to cellular needs, nuclear transcriptional programmes and adapt to various stresses, and hence alleviate mitochondrial dysfunction. The latter has been an important area of study as mitochondrial function is a prominent feature in a variety of pathologies (Finkel \& Holbrook, 2000; Sies et al., 2017).

In order to specifically study mitochondrial redox homeostasis independently of that of the whole cell, it is necessary to be able to selectively alter mitochondrial systems. That was the reasoning behind the development of MitoCDNB, a selective mitochondrial targeted CDNB (Booty et al., 2019). The TPP-based moiety preferentially targets mitochondria due to the organelle's negative membrane potential, where MitoCDNB leads to GSH depletion and inhibition of the thioredoxin and peroxiredoxin systems, effectively disrupting the thiol dependent mitochondrial antioxidant defences. Mitochondria are a major site of ROS production in the cell (Murphy, 2009), and their thiol-dependent defence mechanisms are important to contain and reverse the effect of the redox changes in the organelle. MitoCDNB treated mouse myoblasts showed an increase in ROS levels in mitochondria, which was exacerbated by the addition of mitochondria-targeted redox cycler, MitoPQ (Robb et al., 2015). In isolated heart mitochondria, there was no observed burst of hydrogen peroxide with MitoCDNB, but it did occur when mitochondrial superoxide production was enhanced with MitoPQ. Interestingly, neither of the TPP-compounds led to an increase in cellular ROS alone, when measured in cells with CellROX, however further experiments will be necessary with both reagents combined to expand on these previous observations. How, or indeed whether, mitochondria produce a flux of hydrogen peroxide in whole cells has not been clearly shown. It is believed that the passage of hydrogen
peroxide through the plasma membrane occurs via aquaporins, but a similar mechanism proposed for hydrogen peroxide to transverse the IMM has been disputed (Yang et al., 2006), and seems unlikely. Furthermore, the multitude of antioxidant defence systems in the matrix are another important sink for hydrogen peroxide, which would also limit diffusion. Experiments using targeted hydrogen peroxide generation via yeast $D$-amino acid oxidase in the mitochondrial matrix, paired with the targeted sensing protein, HyPer, in both the mitochondrial matrix and cytosol indicated that hydrogen peroxide is mostly confined to the matrix (Pak et al., 2020). Interestingly, they also showed that inhibiting the whole cell thioredoxin systems by auranofin led to hydrogen peroxide release from the mitochondrial matrix, which suggests the potential regulation of hydrogen peroxide release to the rest of the cell by mitochondrial thiol defences. Further experiments are needed to understand if the effect that I have observed with MitoCDNB is primarily due to depletion of the mitochondrial glutathione pool, inhibition of the thioredoxin system (as observed above), or a combination of both. One possible route to distinguish between these possibilities could be to use a TrxR2 knockout cell model or small molecule inhibitors of thioredoxin systems (Conrad et al., 2004; Hellfritsch et al., 2015) and observe the effect of MitoCDNB, which would eliminate its effect on the thioredoxin part of the mitochondrial antioxidant defence systems. A further investigation would need to consider the limited spatial release of hydrogen peroxide from mitochondria, which might not fully diffuse through the cell but could act as a local redox signal, nonetheless. The tentative conclusions that I can draw is that disrupting mitochondrial thiol antioxidant defence systems might lead to an increase in mitochondrial ROS due to it being constantly produced at a certain level, but it is not necessary enough to lead to hydrogen peroxide diffusing into the rest of the cell until the ROS levels reach a certain level, as shown with MitoPQ.

Mitochondria are highly dynamic organelles going through division and fusion processes in response to cellular needs, or as part of mitochondrial quality control. Recently, it has been proposed that cellular redox homeostasis is linked with mitochondrial dynamics (Willems et al., 2015). Prior to the development of MitoCDNB, it had been difficult to investigate the specific contribution of the mitochondrial redox homeostasis as the existing methods had an overall effect on whole cell thiol redox homeostasis. I showed that in mouse myoblast cells exposed to MitoCDNB, mitochondria undergo fission and present a fragmented
morphology. The latter has been previously associated with nutrient excess, impaired OXPHOS, severe stress and a part of the induction of mitochondrial quality control processes (Wai \& Langer, 2016). It is expected that the loss of mitochondrial thiol defences would lead to mitochondrial stress and subsequently some type of mitochondrial quality control and fission. The fragmented mitochondria can then either fuse with their "healthy" counterparts or follow the mitophagy route. My experiments also included some preliminary investigations into the mechanism of the observed fragmentation and suggested the involvement of Drp1. This is a known master regulator of mitochondrial dynamics and is involved in membrane fission through the formation of ring-like oligomer structures that encircle the mitochondria upon activation. It appears that disrupting mitochondrial redox homeostasis does not change Drp1 sub-cellular localisation in the myoblast cells. Furthermore, the phosphorylation status of certain Drp1 serine residues (Ser637 or Ser616), both of which have previously been reported to be regulatory pathways of Drp1, has not been conclusive. Drp1 is known to be recruited to the mitochondria by PGAM5, a mitochondrial phosphatase located in the inner membrane, which translocates to the outer membrane upon dysfunction (Cheng et al., 2021; Z. Wang et al., 2012). Upon recruitment PGAM5 triggers Drp1 dephosphorylation at Ser-637 and promotes mitochondrial fission (Yu et al., 2020). To explore this mechanism further, mitochondrial morphology experiments in PGAM5 knockout cell lines need to be carried out. PGAM5 has been suggested as a signalling hub in necrosis as it interacts with many proteins regulating mitochondrial turnover, such as Pink1. There are also other post-translational modifications of Drp1, such as SUMOylation, S-nitrosation, GlcNAcylation, which would also need to be surveyed in order to explore the underlying fission pathway further.

Mouse injection with MitoCDNB led to increased expression of GCLC, a rate-limiting enzyme in GSH synthesis, which is probably due to the lowering of GSH content in the cells and tissues by MitoCDNB (Booty et al., 2019). As in the case with many other antioxidant genes, GCLC is under the transcriptional control of Nrf2, which is known for its central role in the cytoprotective response to oxidative stress. Furthermore, Nrf2 is involved in stimulating mitochondrial biogenesis, regulating mitochondrial metabolism and in the maintenance of mitochondrial redox homeostasis. Hence, it is a clear potential downstream target for regulation by mitochondrial redox dyshomeostasis or dysfunction.

To make these experiments as robust as possible an inactive MitoCDNB Ctrl compound was characterised, which localised to mitochondria but lacks the active site, so does not affect mitochondrial thiols. My results suggest that MitoCDNB does lead to Nrf2 pathway activation in cells, through stabilising Nrf2, enabling it to localise to the nucleus. Under homeostatic conditions, Nrf2 is maintained at low levels, because it is targeted constitutively for proteasomal degradation by ubiquitination through its association with Keap1. Disruption of mitochondrial redox homeostasis in cells also lead to increase expression of downstream targets of Nrf2, such as GCLC, GSS, HO-1 and NQO1, further confirming the pathways activation.

Interestingly, enhanced mitochondrial superoxide production with MitoPQ does not lead to Nrf2 activation, which provides an important new insight into how mitochondrial ROS connects to the Nrf2 pathway. Although there have been studies implying that mitochondrial hydrogen peroxide induces Nrf2 signalling (Kasai et al., 2020), such a link has not been shown directly. My experiments with MitoPQ suggest that in the absence of any other changes, the elevation of superoxide and hydrogen peroxide within mitochondria is not capable of generating a hydrogen peroxide signal from the mitochondria to the cytosol in order to activate Nrf2. As discussed above, it has been suggested that mitochondrial thiol defences such as the thioredoxin system need to be disrupted before the mitochondria are able to release much ROS (Pak et al., 2020). It would therefore be interesting in the future to try both MitoCDNB and MitoPQ in combination for their effect on the Nrf2 pathway. Furthermore, as MitoCDNB does activate the Nrf2 pathway, it would be interesting to investigate if this is a consequence of it depleting the mitochondrial glutathione pool, or due to its inhibition of the mitochondrial thioredoxin system and subsequently the peroxiredoxins. It would be interesting to see the effect of MitoCDNB on the Nrf2 pathway in cells lacking TrxR2, which would have the potential to clarify this point. As the mitochondrial thioredoxin system has been linked to retaining mitochondrial ROS in the organelle, it may be that thioredoxin system has a bigger role in the Nrf2 activation compared to the glutathione levels. However, as MitoCDNB alone does not lead to mitochondrial ROS release in our systems, this does not seem to be the case. It is also important to point out that MitoCDNB effectively activated the Nrf2 signalling pathway after 4 h but not after 1 h , which mimics the time needed to achieve a substantial GSH
depletion in these cells (Booty et al., 2019; Cvetko et al., 2020). Furthermore, I was able to block the MitoCDNB effect on Nrf2 with N -acetyl cysteine addition, a well-known cellular antioxidant as well as a GSH precursor. This, coupled with the fact that N -acetyl cysteine prevented mitochondrial GSH depletion that occurs with MitoCDNB, does suggest an important part for the glutathione system. However, N -acetyl cysteine is an overall cellular antioxidant and could possibly have an effect through stopping any Nrf2 activating signals from escaping from mitochondria.

Depletion of mitochondrial glutathione does have an important effect on the efficiency of mitochondrial glutathione peroxidases, which are instrumental in degrading lipid peroxidation products in the organelle. To explore this, I investigated 4-hydroxynonenal (HNE), which is a known lipid peroxidation product and Nrf2 activator in the cell. The results were not conclusive and further work needs to be done, such as experiments targeting HNE release from mitochondria and using mitochondrial glutathione peroxidase knockout cell lines. Although the results do not conclusively point to a particular mitochondrial signal, such as hydrogen peroxide or HNE, work with our collaborators showed that MitoCDNB activation of Nrf2 does involve the cysteine residues on Keap1. Interestingly, MitoCNB seemed to activate Nrf2 through more than one site on Keap1. In addition to binding Drp1, PGAM5 also serves as a signalling hub as it exists in a ternary complex containing Keap1 and Nrf2 at the mitochondrial outer membrane (Cheng et al., 2021). This has been suggested to provide a molecular framework for understanding mechanistically how Nrf2 is regulated in response to changes in mitochondrial function (Lo \& Hannink, 2008). Furthermore, the existence of this signalling hub may also provide a plausible connection between the changes in mitochondrial morphology and Nrf2 activation following on from the disruption of mitochondrial redox homeostasis represented here.

Mitochondria are signalling organelles that continuously communicate with the nucleus, influence other cellular components and cytosolic pathways, thereby creating a mitocellular communication network (Mottis et al., 2019; Quirós et al., 2016). To do this mitochondria must generate a wide range of retrograde signals, through which they modulate cellular and organismal activities and protect against mitochondrial dysfunction by acting upon nuclear gene expression involved in metabolic reprogramming or stress
defence (Jazwinski, 2013; Quirós et al., 2016). Retrograde signalling has been studied to a certain extent involving mitochondrial ROS, but it has not been possible to do this after disruption of mitochondrial thiol redox homeostasis. To shed light on these early retrograde mitochondrial dysfunction pathways, mice were challenged with the mitochondria-targeted compounds, MitoCDNB and MitoPQ, as well as their control compounds, MitoCDNB Ctrl and MitoPQ Ctrl, and a transcriptomic analysis carried out on liver and heart tissue.

With regards to the GSEA pathway analysis (Subramanian et al., 2005), disruption of mitochondrial thiol redox homeostasis led to downregulation of inflammatory and stressrelated pathways in the liver, but in contrast upregulation was observed in the heart. This result is most likely related to the different physiological roles these tissues play, which lead to quite different demands on their antioxidant defences and mitochondrial function. In addition, in the heart, but not in the liver, metabolic pathways were mostly downregulated, which suggests that disruption of mitochondrial antioxidant defences influenced mitochondrial metabolic activity. Enhanced mitochondrial superoxide production showed upregulation of the inflammatory and stress-related pathways and downregulation of some metabolic pathways in the heart. In the liver, upregulation in response to elevated superoxide was observed with metabolic pathways, as well as in a cancer-associated pathway, II6-JAK-STAT3, which has been recently implicated in the response to mitochondrial ROS signalling (Abid et al., 2020).

Interestingly, a common effect observed in response to both forms of mitochondrial redox disruption in the two tissues was the upregulation of the epithelial mesenchymal transition (EMT), an important step in tumourigenesis and metastasis. The link between EMT and mitochondrial dysfunction has been gradually emerging over the past few years (Guerra et al., 2017), with deregulated mitophagy, depletion of mitochondrial DNA and defects in TCA cycle all being proposed as activators of the pathway. It is possible that transient transition to a mesenchymal phenotype, confers a survival advantage under mitochondrial redox stress and hence EMT could represent a strategy that cells use to adapt better to altered microenvironments. The observation of mitochondrial dysfunction driving EMT is important as it also suggests novel therapeutic scenarios, whereby targeting effectors of
the mitochondrial retrograde signalling could potentially block EMT with the aim of limiting or preventing cancer metastasis. However, these tentative conclusions generated from this transcriptomic study need to be considerably extended by future follow up experiments in order to understand the interplay between mitochondrial thiol redox homeostasis disruption and EMT, as well as to identify the molecular determinants that link the two processes.

A striking feature of the comparison between the effect of MitoCDNB and MitoPQ was that there was little overlap in differentially expressed genes. This suggests that the effects of the two interventions tested are distinct, at least at the early time point investigated here. Despite this, upstream transcription factor analysis showed similarities in the transcription factors involved, which is indicative of their promiscuous nature. In the liver, both MitoCDNB and MitoPQ activated transcription factors involved in cell cycle regulation during development, suggesting that mitochondrial dysfunction may lead to changes in the normal cell cycle routine. MitoCDNB-specific transcription factors in the liver were IRF4 and CREB, both of which are known to play pivotal roles in mitochondrial homeostasis (Arnould et al., 2002; Low et al., 2019). Furthermore, CREB can act synergistically with Nrf2 (Katoh et al., 2001). In both conditions and across tissues, the E2F family of transcription factors were activated, consistent with mitochondrial disruption altering cell cycle regulators. Many roles have been proposed to link E2F transcription factors and mitochondrial functions (Benevolenskaya \& Frolov, 2015), which suggests that this pathway should be explored further in the future with more experiments to determine the mechanistic details connecting mitochondrial redox processes and this family of transcription factors.

Lastly, the transcriptomic analysis, also provided further insight into the acute effect of the TPP-moiety on gene expression and retrograde signalling. All the compounds investigated were targeted to mitochondria with the TPP group, which accumulates in the organelle due to the mitochondrial membrane potential. One of the ways of exploring the TPP-effect is to investigate the common differentially expressed genes between the active and inactive compounds, MitoCDNB and MitoCDNB Ctrl, respectively. Gene ontology analysis common across both tissues suggested involvement of MAPK, TNF and FoxO signalling pathways, with the latter being of particularly interest as it is known to activate expression of many mitochondrial antioxidant enzymes in order to protect mitochondria (Kim \& Koh, 2017).

The transcription factor analysis of the TPP effect across tissues generated a range of results and some of them were already observed in other conditions, but one particularly interesting result was the activation of the ATF4 signalling pathway. This pathway has recently been identified as one of the main regulators of the mitochondrial stress response (Quirós et al., 2017), which warrants further exploration of the acute effect of the TPPmoiety as a tool to explore the mitochondrial stress response.

Although some tentative conclusions have been drawn from the transcriptomic analysis, future experiments are required, in particular these should be focused on the longer timepoints after injections with MitoCDNB or MitoPQ, in addition to the further validation of the signalling pathways that are suggested to be involved.

Overall, the work outlined in this thesis contributes to the greater understanding of the role mitochondrial redox homeostasis plays in cellular redox signalling. In particular, I have shown that it is possible to study the mitochondrial glutathione and thioredoxin antioxidant systems and their dysfunction independently from their cytoplasmic counterparts. This work complements the growing knowledge base supporting the view that mitochondrial ROS are not solely a pathological signal, but also act as redox signals. Therefore, this thesis provides a platform for future studies to further expand our view of mitochondrial biology and its implications in physiology and pathology.

## Chapter 8. References

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