

An Exploration of Mitochondrially Targeted Gene Therapies for Ocular Disorders



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Declaration

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Summary

The mitochondria play a vital role in numerous fundamental processes of the cell such as ATP synthesis, cellular signalling, reactive oxygen species production, calcium regulation and apoptosis to name but a few. Given their central role in cellular homeostasis, mitochondria are increasingly being implicated in a wide array of diseases due to a broad range of primary mitochondrial mutations. Furthermore, there is increasing evidence of mitochondrial dysfunction in disorders not directly due to primary heritable mitochondrial mutations but rather as a result of dysregulation of the myriad of functions that mitochondria undertake; these disorders include Alzheimer's disease, Parkinson's disease and Multiple Sclerosis, among others.

The eye, and in particular the retina, is a highly energetic tissue requiring significant levels of ATP production to stay active and as such is exquisitely sensitive to disturbances in mitochondrial function. This can be seen in the primary heritable mitochondrial disorders that affect the eye, such as Leber Hereditary Optic Neuropathy and Dominant Optic Atrophy, however the mitochondrion's role in more complex ocular diseases such as Glaucoma and Age-related Macular degeneration has recently come to the fore.

The primary aim of the studies described in this PhD thesis is to investigate novel gene therapy approaches for modulating mitochondrial function, in particular for ocular disorders. As there are currently over 270 genes associated with inherited retinal degenerations, potential therapies that can target common aspects between these disorders will be advantageous.

In the first results chapter of this thesis, the alternative NADH dehydrogenase, *Ndi1* is investigated as a potential therapeutic for complex I deficiencies of the mitochondria. Previous work in the Farrar lab has produced an optimised version of the *Ndi1* gene which is characterised here in terms of activity levels and its ability to restore functionality in chemically induced models of complex I deficiencies. An investigation of *Ndi1* as a potential therapeutic strategy for

complex ocular disorders not primarily associated with mitochondrial dysfunction is also undertaken.

The second results chapter in the thesis focuses on the *OPA1* gene, mutations in which are classically associated with the progressive retinal degeneration Dominant Optic Atrophy. Here, the expression profile of *OPA1* isoforms in retinal tissue is examined, with two of the different isoforms of *OPA1* examined for their ability to restore functionality to *OPA1* knockout cells in a number of bioenergetic assays. These data highlight the potentially narrow therapeutic index of *Opa1*-based therapies.

In the third results chapter of the thesis, the alternative oxidase family of proteins are investigated. These proteins offer a potentially novel therapeutic approach to bypass complex III and IV deficiency in ocular disorders. First sequence data is analysed for novel putative alternative oxidase sequences. A novel alternative oxidase gene from *B. glabrata* is then examined for its ability to modulate cellular bioenergetic *in vitro*.

The data presented in this PhD thesis represent significant steps forward in the investigation of mitochondrially targeted gene therapies by providing detailed analyses of six potential novel gene therapy approaches that can modulate different aspects of cellular bioenergetics and may be of benefit in diseases with aspects of mitochondrial dysfunction.

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Abbreviations

AA	Antimycin A		HEK293	Human Embryonic Kidney cell	
AAV	Adeno-Associated Virus		HIS-tag	6x	Histidine Tag
AD	Alzheimer's Disease		IMM	Inner Mitochondrial Membrane	
AIF	Apoptosis Inducing Factor		INL	Inner	Nuclear Layer
AMD	Age-related Macular Degeneration		KO	Knockout	
AOX	Alternative Oxidase		LHON	Leber Hereditary Optic Neuropathy	
bp	base pair		MEF	Mouse Embryonic Fibroblast	
CBA	Chicken β -actin		MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes	
cds	Coding Sequence		MERRF	Myoclonic epilepsy with ragged red fibres	
CI	Complex I	NADH:Ubiquinone Oxidoreductase	Mfn	Mitofusin	
CII	Complex II	Succinate Dehydrogenase	MOI	Multiplicity Of Infection	
CIII	Complex III	Cytochrome C Reductase	mtDNA	Mitogenome	
CIV	Complex IV	Cytochrome C Oxidase	MTS	Mitochondrial Targeting Sequence	
cKO	Conditional Knockout		NARP	Neuropathy,	Ataxia and Retinitis Pigmentosa
CMV	Cytomegalovirus promoter		nde	External NDH	
CS	Citrate Synthase		NDH	NADH Dehydrogenase	
CV	Complex V	ATP synthase	ndi	Internal NDH	
Dnm1l	Dynamin 1 Like (Drp1)		OCR	Oxygen Consumption Rate	
DOA	Dominant Optic Atrophy		OMM	Outer Mitochondrial Membrane	
EAE	Experimental encephalomyelitis	autoimmune	ONL	Outer	Nuclear Layer
ECAR	Extracellular Acidification Rate		OPA1	Optic Atrophy	1
EMT	epithelial-to-mesenchymal transition		OS	Outer	Segments
ERG	Electroretinogram		OXPHOS	Oxidative Phosphorylation	
ETC	Electron Transport Chain		pAAV-MCS	mammalian expression plasmid	
FAD	Flavin Adenine Dinucleotide		PD	Parkinson's Disease	
FMN	Flavin Mononucleotide		PG	Propyl Gallate	
FPKM	Fragments per Kilobase Million				

POAG	Primary Open Angle Glaucoma	SRC	Spare Respiratory Capacity
RET	Reverse Electron Transfer	TCA cycle	Tricarboxylic Acid Cycle (Citric Acid cycle)
RGC	Retinal Ganglion Cell	TSD	Tukey's Honestly Significant Difference
ROS	Reactive Oxygen Species	UQ	Ubiquinone
ROT	Rotenone	UQ[•]	Ubisemiquinone
RPE	Retinal Pigment Epithelium	UQH₂	Ubiquinol
SD	Standard Deviation	vg	Vector Genomes
SEM	Standard Error	wt	wild-type
SNP	Single Nucleotide Polymorphism	$\Delta\psi_m$	mitochondrial membrane potential

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1. General Introduction

1.1. The Eye

“Since the earth formed more than 5 billion years ago, sunlight has been the most potent selective force to control the evolution of living organisms. Consequences of this solar selection are most evident in eyes, the premier sensory outposts of the brain.” – (Land & Fernald, 1992)

The eye is the primary light sensing organ of the animal kingdom. The importance of the eye is highlighted by its presence in around 96% of living animal species (Land & Fernald, 1992). Of course, this 96% depends on your definition of what an eye is. Here it is restricted to eyes that are capable of pattern recognition, allowing the user to, for instance, detect predators or prey and allow some manner of visual locomotion. The over-abundance of extant animals that possess eyes demonstrates their evolutionary advantage, although not all of them rely on them as heavily as we do. It follows that there are very few truly blind animals, not even the bat (Simões et al., 2019), apart from some that have lost the use of their eyes such as Mexican blind cave fish, but even these fish retain some form of photosensitivity as they can alter behaviour when in an environment with a light-dark cycle (Carlson & Gross, 2018).

The first light sensing organisms are believed to be cyanobacteria, which first harnessed the sun’s light for the process of photosynthesis some 2.4 billion years ago (Shih, 2015), and would eventually be endosymbiosed to become the chloroplast of plants at least 1 billion years ago (McFadden, 2001).

The evolution of opsins, the light sensitive molecules present in our eyes also came from a similar energy harnessing approach. Early opsins were related to G-protein coupled receptors (GPCR) which bound a light responsive molecule, such as retinal. When a photon interacts with retinal it alters its conformation. This alteration activates the bound G protein causing an

electrical signal to propagate, typically in the form of proton pumping (Grote, Engelhard, & Hegemann, 2014). This proto-opsin is believed to have been passed on through vertical evolution to the metazoa and thus is the evolutionary starting point for our eyes (Shichida & Matsuyama, 2009). These bacteriorhodopsins would initially have been arranged all over the surface of the cell, but eventually these would have coalesced to a single area termed an eye spot (Schwab, 2018). The estimate for the time it would take for this eye spot to become the “camera-eye” of similar morphology to ours, is actually comparatively short at around 364,000 years (Nilsson & Pelger, 1994), an evolutionary “blink of the eye”. The authors argue that the reason not all animals have flawless visual perception is not due to the difficulty of developing an eye, but due to the difficulty of developing a brain capable of interpreting the information provided.

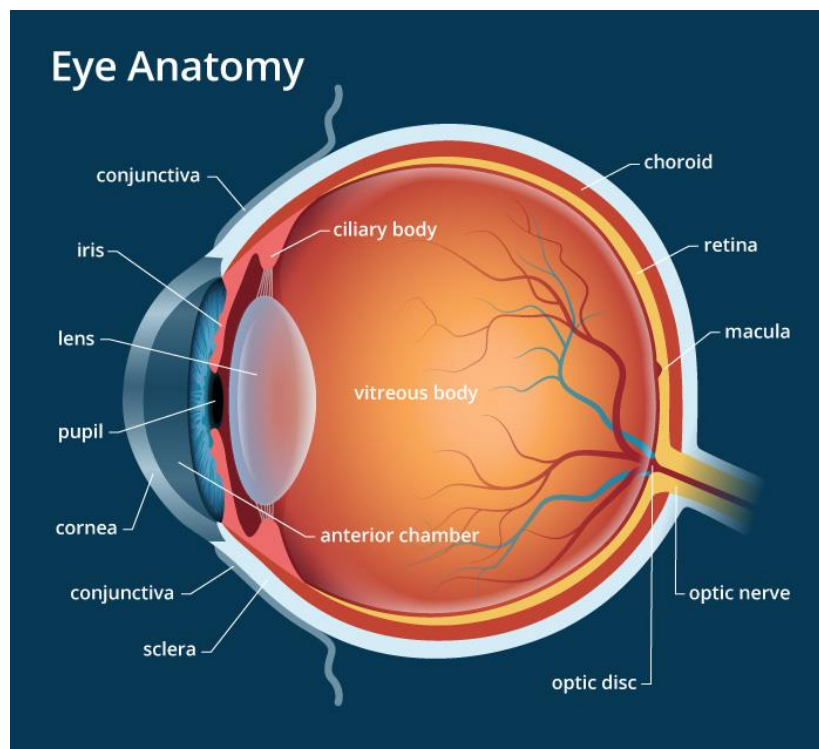


Figure 1-1: Diagram showing the structure of the human eye. Light passes through the pupil and is focused by the lens. The light sensing cells in the retina then detect this signal and convert it to electrical potential that is propagated by a series of nerve cells to the optic nerve. The optic nerve is connected directly to the brain where image processing takes place.

The eye that we possess is an immensely sophisticated organ, but the principle of a collection of light sensitive cells to allow electrical signal transduction remains the same. Figure 1-1 shows a schematic representation of the eye. Light passes from left to right through the pupil and is then focused by the lens to stimulate the light sensitive cells in the retina at the back of the eye.

The retina has evolved as a highly sophisticated tissue, with numerous cell types and layers with specialised functionalities and roles. The retina consists of over 100 distinct neuronal cell types enacting specific roles in signal transduction (Demb & Singer, 2015), but these can broadly be separated into 5 major classes; photoreceptors, horizontal cells, bipolar cells, amacrine cells and retinal ganglion cells (Hoon, Okawa, Della Santina, & Wong, 2014)(see Figure 1-2A).

The photoreceptors are the descendants of the primitive bacterial opsins mentioned earlier and are broadly divided into two categories, rods and cones, named after their physical appearance. Rod photoreceptors are highly sensitive to light which allows them sense a single photon, an ability that has been experimentally validated in human vision (Pugh, 2018). This sensitivity results in rods being predominantly the source of visual information in low light conditions. In the retina rods outnumber cones by at least 20:1 (Mustafi, Engel, & Palczewski, 2009). Cone photoreceptors require a much higher threshold of light to be activated but can produce a much faster response and thus allow much higher spatial resolution of the image. Cone photoreceptors show particular absorption spectra, and as such, can be further divided into different categories depending on the wavelengths of light they can absorb. Humans possess three types of cone receptors L, M and S cones, which correspond to long, medium and short wavelength light stimuli and thus also correspond to the colour range they can detect, red, green and blue respectively. The total number of opsin genes varies widely between species in the *Vertebrata* lineage, and primates are an interesting case. Ancestrally there were 4 different opsin genes (tetrachromacy) for cones but at the start of the mammalian lineage only two cone types remained, indicative of the nocturnal lifestyle of early mammals (Musser, 2017).

Mammals such as mice still only have 2 cone opsin genes (dichromacy) and heavily rely on rods for vision, whereas humans now possess three (trichromacy) thanks to a gene duplication event in the Old World primate lineage, allowing re-adaption for our exceptional day time vision (Dulai, von Dornum, Mollon, & Hunt, 1999). However, our colour perception pales in comparison to the mantis shrimp, which has 16 physiologically distinct photoreceptor classes allowing unparalleled levels of colour distinction (Donohue, Cohen, & Cronin, 2018).

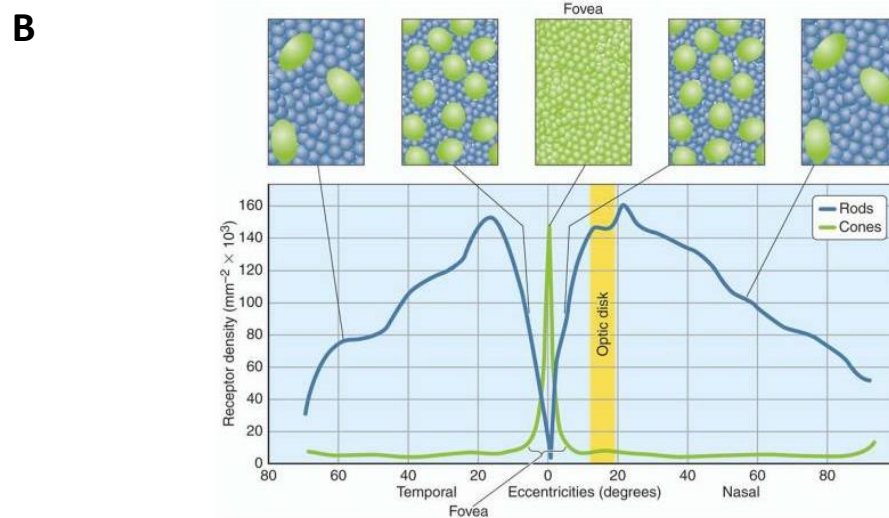
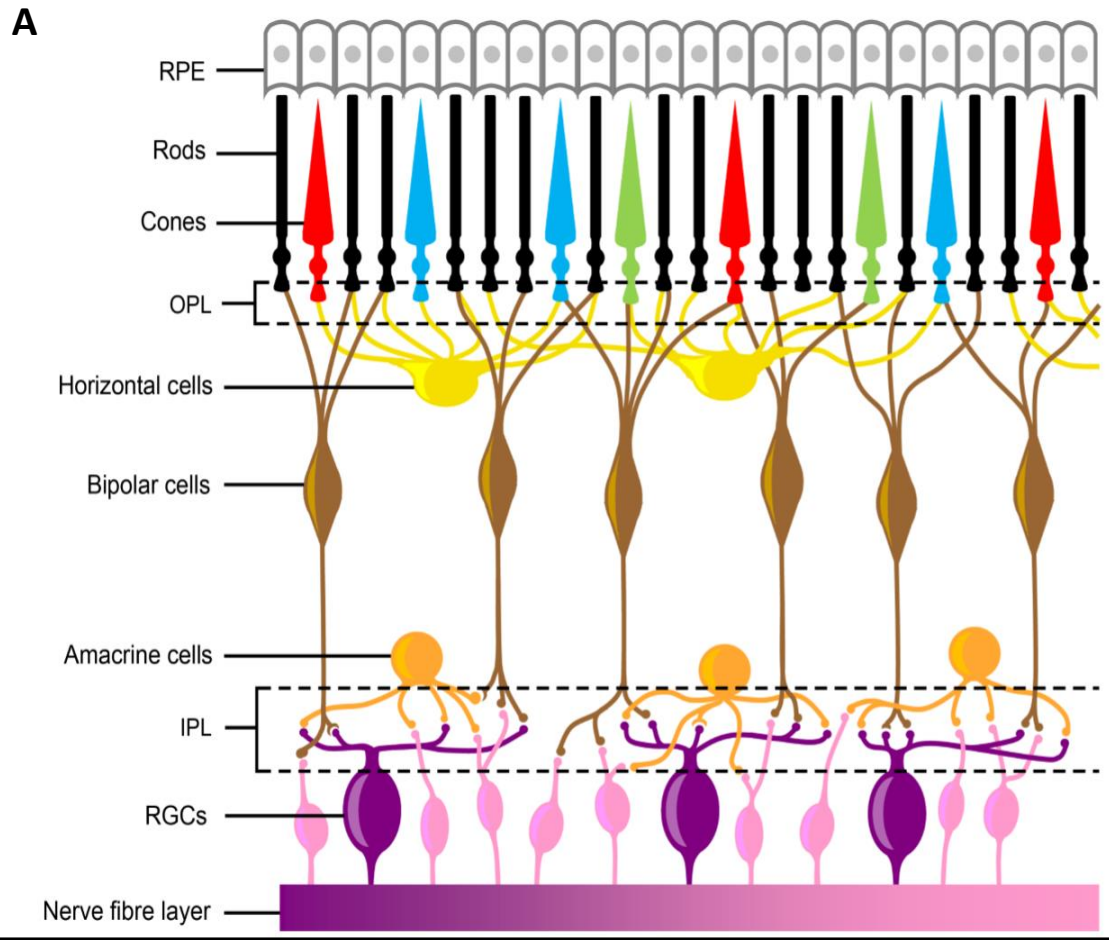


Figure 1-2: A) a diagrammatic representation of the human retina. Light transduction occurs from bottom to top as the human retina is inverse, travelling through the various neural cell layers before reaching the photoreceptors. Once a signal is detected by the photoreceptors, an electrical signal is propagated through the other neuronal cell types. Bipolar cells contact the photoreceptors directly, where horizontal cells also interact to modulate the signal transduction from the photoreceptors. This region of contact is termed the outer plexiform layer (OPL). Bipolar cells then contact retinal ganglion cells (RGCs). These RGCs propagate the signal out of the retina along the optic nerve to the brain. RGCs in turn are modulated by amacrine cells which can amplify or inhibit the signal. The region of these synaptic contacts is termed the inner plexiform layer (IPL). RPE = Retinal Pigment Epithelium. B) a schematic of the photoreceptor density across the retina. Central vision is mediated by the fovea, located in the macula, which is a very cone dense region. As we move towards the periphery of the retina rod photoreceptors start to dominate, with a much sparser distribution of cones. The panels above show a front-on view of photoreceptor distribution at specific points of the distribution. Reproduced from (Mustafi et al., 2009).

Photoreceptors are not uniformly distributed around the retina but show specific distributions (Figure 1-2B). Due to the spherical nature of the eye and the light focusing properties of the lens, light does not uniformly hit the retina. Around 75% of light focused on to retina hits the fovea, a very cone dense region located in the macula which is responsible for our sharp acute colour vision (Figure 1-1), with comparatively few cones elsewhere in the retina (Mustafi et al., 2009). As we travel in any direction away from this focal point to the peripheral retina the density of cones compared to rods diminishes as can be seen in Figure 1-2. This is effectively why you cannot distinguish things clearly in your peripheral vision but can catch motion out of the corner of your eye; this vision is rod dominated, allowing quick response with minimal input, but at the expense of acuity. Conversely it highlights the importance of the macular for allowing clear central vision, and why diseases that effect this region, such as age-related macular degeneration (AMD), are so devastating.

Once the photoreceptors have been triggered, an electrical signal propagates to the bipolar cells. Bipolar cells are broadly categorised into rod and cone bipolar cells, although 12 types have been identified currently (Demb & Singer, 2015). Each bipolar cell connects to multiple photoreceptors of the corresponding type, but there are also known to be bipolar cells that receive input from both rods and cones simultaneously (Masland, 2012). Bipolar cells are differentiated into ON and OFF types, with inhibitory or excitatory properties on the subsequent neural connections. The connections of the rod bipolar cells, which only come in the ON variety, to multiple rods amplifies the incredible sensitivity of rods by amplifying the signal for further transduction (Martemyanov & Sampath, 2017). Cones can be contacted by either ON or OFF bipolar cells, and some cones are contacted by multiple bipolar cells of both types, creating a highly dynamic network of synaptic connections (Martemyanov & Sampath, 2017).

To add to this, horizontal cells also form synaptic connections here, at the outer plexiform layer (OPL) adding a further level of excitatory regulation (Figure 1-2A). These cells connect to

multiple photoreceptors and bipolar cells in triad synaptic junctions where they modulate signal transduction by inhibiting surrounding photoreceptor signals, which is believed to help enhance spatial definition of light sources (Thoreson & Mangel, 2012).

This light induced signal continues down to the retinal ganglion cells (RGCs) which propagate the visual signal along to the optic nerve, where the signal is sent to the optical lobes of the brain for processing. Cone bipolar cells connect directly to ganglion cells, whereas rod bipolar cells connect to specialised amacrine cells that pass the signal on to retinal ganglion cells (Demb & Singer, 2015). RGCs represent the coalescing of the vast array of visual stimuli received by the photoreceptor; for the roughly 120 million photoreceptors humans possess, there are estimated to be roughly 1 million RGCs (You, Gupta, Li, Klistorner, & Graham, 2013). This is part of the reason that pathologies that effect RGCs tend to be quite rapid and catastrophic; loss of a comparatively small number of cells can render a large portion of the retinal circuitry unusable. There is an array of RGC types in the retina, 20 to 30, which have different responses to various stimuli, both electrical from the retina, and physiological in the form of increased resistance to damage (Mead & Tomarev, 2016). Retinal ganglion cells are somewhat unusual as neurons since a significant proportion of their axon is unmyelinated. Myelin is a lipid rich tissue that surrounds nerve axons, effectively insulating them and allowing electrical signals to propagate more easily along the axon. Being a fatty tissue however, myelin is opaque and as such would be detrimental to have covering the retina; there are human disorders where myelination of these RGCs causes visual impairment (X. Yang et al., 2013). To overcome this, RGCs contain vast amounts of mitochondria along the length of the axon in order to propagate a signal down the axon. This coupled with the fact that RGCs can have comparatively long axons, travelling from the periphery of the retina to the optic nerve head, means that RGCs are exquisitely sensitive to metabolic deficiency, which makes them a prime target in mitochondrial disorders (Valerio Carelli et al., 2009). Where other neuronal cells could potentially cope with a marginal decrease in mitochondrial functionality, the high energetic burden of RGCs means they cannot cope as

well, evidenced by their selective loss in a number of mitochondrial disorders such as Leber Hereditary Optic Neuropathy (LHON) and Dominant Optic Atrophy (DOA), leading to a scenario where the photosensitive retina is still functional, but the transduction of this information to the brain is prevented. These diseases will both be discussed in detail in this thesis with regards to potential gene therapy approaches for mitochondrial dysfunction.

A very important non-neuronal section of the eye is the retinal pigment epithelium (RPE), located at the back of the eye adjacent to the photoreceptor cell layer. RPE cells act as metabolic support cells for the photoreceptors. They form a tightly packed monolayer of hexagonal cells, forming a barrier between the photoreceptors and the blood vessels of the eye, enacting the blood-retinal barrier (Rizzolo, 2007). This effectively means that the RPE regulates what can reach the retina from the blood stream. RPE cells are polarised, reflecting the different functions they play on each side. On their apical side, facing the photoreceptors, the cells have microvilli which surround the outer segments of the photoreceptors, the region where the light sensitive opsins are housed. These microvilli are used to continually phagocytose the photoreceptor outer segments at a constant rate; it has been estimated that a rod completely renews its outer segments every 7-12 days (Young & Droz, 1968). This is believed to happen to prevent the build-up of dysfunction in photoreceptors, allowing them to recycle old structures and replace them with new discs and preventing the accumulation of reactive oxygen species and other harmful substances (Sparrow, Hicks, & Hamel, 2010). Under normal physiological conditions the RPE can efficiently recycle these outer segments but as age and dysfunction accumulates deposits can form at the basal side of the RPE, adjacent to the Bruch's membrane. These deposits can build to form drusen a hallmark of age-related macular degeneration (AMD), the most prevalent form of irreversible blindness in adults over 50 years old (Pennington & DeAngelis, 2016). The RPE is also an integral part of the metabolic landscape of the eye, as it is the point of contact for the photoreceptors with the circulatory system.

Recent work has demonstrated that the RPE works to shuttle glucose from the bloodstream to the photoreceptors where it is used to fuel aerobic glycolysis (non-mitochondrial ATP production) (Hurley et al. 2015). Photoreceptors in turn secrete lactate which the RPE can convert into pyruvate and use to fuel oxidative phosphorylation (OXPHOS), the ATP producing process carried out by the mitochondria. This creates an interesting metabolic balance in the retina (Kanow et al. 2017). A shift away from OXPHOS towards utilising this glucose in glycolysis in RPE cells can cause starvation of the photoreceptor layer because these RPE cells start to utilise the glucose that would otherwise have been transported towards the photoreceptor layer. The photoreceptors now produce less lactate as a result forcing the RPE to rely more heavily on glycolysis for energy (Kanow et al., 2017).

This brief introduction to the eye demonstrates the complex metabolic interplay present between the different cells of the retina. The cells here have developed specific metabolic adaptations in order to cope with the energetically demanding process of visual transduction, which leaves the eye uniquely vulnerable to mitochondrial dysfunction.

1.1.1. Inherited Retinal Degenerations and Gene Independent Therapeutics

The range of Inherited Retinal Degenerations (IRDs) is both wide and varied but they all essentially lead to the same destination, visual dysfunction. IRDs are the most common form of visual dysfunction in those of working age, with an estimated 1-3,000 to 1-5,000 individuals affected (Farrar et al., 2017). They typically result in gradual photoreceptor loss and compromised vision, although with the IRDs that will be focused on in this thesis, that is not strictly the course of disease pathology. Since the identification of the first gene and subsequently mutation to cause an IRD some 30 years ago (Bhattacharya et al., 1984; Farrar et al., 1991), there has been a high rate of discovery, with the advent of next-generation sequencing propelling the rate of IRD associated gene identification to new heights. As of March

15, 2019, 271 genes have been implicated in IRDs, according to the online database RetNet¹, demonstrating the significant genetic heterogeneity in IRDs. To further compound this, mutations in a particular gene can give rise to different IRD pathologies depending on the severity of the mutation (Carrigan et al., 2016; Kaway, Adams, Jenkins, & Layton, 2017). There is still significant progress though, with an estimated 66% of patients with an IRD having the underlying genetic cause identified (Duncan et al., 2018).

Of the genes identified to cause IRDs around 20 of them are directly involved in mitochondrial processes (RetNet). IRDs that involve mitochondrial dysfunction typically manifest as optic neuropathies such as Leber Hereditary Optic Neuropathy (LHON) and Dominant Optic Atrophy (DOA). This disease will be discussed further when we discuss mitochondrial disorders.

The wide range of potential genes associated with IRDs poses a challenge for the development of therapeutic modalities. It would be prohibitive to try and develop therapeutics for each of these genes individually which is why gene independent therapeutic approaches are an attractive option. For instance, of the genes identified as causative of IRDs, 70 of them are associated with Retinitis Pigmentosa (RP), a progressive blinding disorder that results from the slow degeneration of rod photoreceptor cells (Farrar et al., 2017). A more expedient approach to therapeutic design would be to attempt to modulate commonalities in the disease pathways, rather than each of the 70 genes individually. This has led to some novel approaches such as using CRISPR-Cas9 mediated editing to modulate rod photoreceptors to express cone photoreceptor neuroprotective factors, which has shown protection in a number of RP mouse models (Zhu et al., 2017), among other approaches.

Focus recently has been moving towards the use of therapeutic strategies that provide mitochondrial benefit as a means of protection against degeneration. It has been shown that

¹ <https://sph.uth.edu/retnet/>

modification of the NAD^+/NADH ratio may be of benefit to retinal cells. The NAD^+/NADH ratio is an indicator of the cell's redox status, and is controlled by a cell's production of NAD^+ versus its use of NADH in anabolic processes (Bilan et al. 2014). Enzymes such as *Nmnat1*, involved in NAD^+ biosynthesis, have been implicated in IRDs such as Leber Congenital Amaurosis and Cone-Rod Dystrophy (Koenekoop et al. 2012; Nash et al. 2018). Delivery of AAV2-*Nmnat1* has shown protection in the DBA/2J mouse model, which shows retinal ganglion cell death due to a glaucoma phenotype.

There has also been interest recently in trans-kingdom gene independent therapies, particularly using channelrhodopsin (ChR). This family of light -sensitive ion channels were originally identified in *Chlamydomonas reinhardtii*, a genus of photosynthetic microalgae (Hamblin & Avci, 2015). When they are ectopically expressed in ON-bipolar cells of rd1 blind mice after AAV2 delivery, they restore some level of functional vision, in the form of light perception (Macé et al., 2015). This approach has shown great promise, and is currently in phase I/II clinical trials for safety and efficacy in late-stage RP patients (www.clinicaltrials.gov, NCT02556736) (Simunovic et al., 2019).

This is just a small selection of current gene independent and trans-kingdom approaches to gene therapy for IRDs. In this thesis we will discuss three potential gene therapies that could be applicable to gene independent therapeutic approaches. These recent efforts highlight the potential utility of this type of approach.

1.2. The Mitochondria

The mitochondria are effectively the batteries of the cell, providing the bulk of the typical cell's ATP via the process of oxidative phosphorylation (OXPHOS). The mitochondrion is a subcellular organelle uniquely possessed by eukaryotes. They house the necessary enzymes involved in energy production such as those of the tricarboxylic acid cycle (TCA cycle) and OXPHOS, but are also involved in a variety of important biochemical processes such as calcium homeostasis, reactive oxygen species generation, apoptosis and endoplasmic reticulum stress responses to name but a few (Alexander M van der Bliek, Sedensky, & Morgan, 2017). The mitochondria's intractable link to an array of fundamental cell processes has led to its implication in an array of common neurological diseases such as Parkinson's disease, multiple sclerosis and Alzheimer's disease (Arun, Liu, & Donmez, 2016), as well as a slew of disorders directly attributed to mitochondrial mutations such as LHON and DOA (Finsterer, Zarrouk-Mahjoub, & Daruich, 2016). Here I aim to give a broad overview of the mitochondria and its role in the processes directly affected in the ocular disorders we are investigating, namely energy metabolism, oxidative stress and mitochondrial remodelling, and how mitochondrial haplogroups affect ocular disease pathology. In addition to this, I will outline the shared evolutionary origins of mitochondria, which I feel are important to highlight with regard to the use of trans-kingdom and trans-species gene therapy as a treatment approach.

The etymology of mitochondria comes from *mitos* (thread) and *chondrios* (granular) showing a logical but ultimately misinterpreted view of the data from the time. Carl Benda, a German microbiologist, gave mitochondria their name in 1898 where he described them as chains of 'particular bodies' most likely with a role in cellular structure (Benda, 1898). It was not until the advent of electron microscopy in the 1950's that the currently recognised structure of the mitochondrion emerged. Palade, 1953 showed that mitochondria have a highly complex internal structure comprising of an outer membrane, and an inner membrane organised into

cristae mitochondrialis. Showing remarkable prescience, Palade proposed that “cristae represent the most probable location for the postulated enzyme chains” responsible for the mitochondria’s energy output and that the enzymes involved would be “disposed in the proper succession in enzyme chains”. We now know the mitochondria to be double membraned, with a highly invaginated inner membrane creating structures known as cristae. The space inside of the inner membrane is known as the matrix.

1.2.1. The Electron Transport Chain

The mitochondrial electron transport chain (ETC) carries out the process of oxidative phosphorylation (OXPHOS) in order to produce ATP. Broadly speaking, OXPHOS is the process of transferring electrons from an electron donor through a series of large protein complexes (known as complex I, II, III and IV) located on the inner mitochondrial membrane (IMM) to O_2 to form H_2O (the oxidation part of OXPHOS). This process is exergonic, so during the transfer of these electrons the energy released is used to pump protons across the inner mitochondrial membrane to the intermembrane space. This builds up a proton gradient between the matrix of the mitochondria and the intermembrane space, effectively creating an electrochemical gradient. This build-up of protons wants to normalise across the membrane, and it is allowed to do so by flowing back through a fifth protein complex, ATP synthase or complex V. As these protons flow through complex V they are used to produce mechanical energy which converts a molecule of ADP to ATP (the phosphorylation part of OXPHOS). This general scheme holds true for mitochondria across the eukaryotes, all that changes are the specific enzymes that fit into this framework (Allison E. McDonald & Gospodaryov, 2018).

In humans (and indeed all vertebrate mitochondria) we have two entry points to the electron transport chain, through complex I (CI) or complex II (CII). CI is a huge multimeric protein complex, with 44 subunits, 7 of which are contained on the mitochondrial genome, and weighs just over a megadalton in total (Vinothkumar, Zhu, & Hirst, 2014).

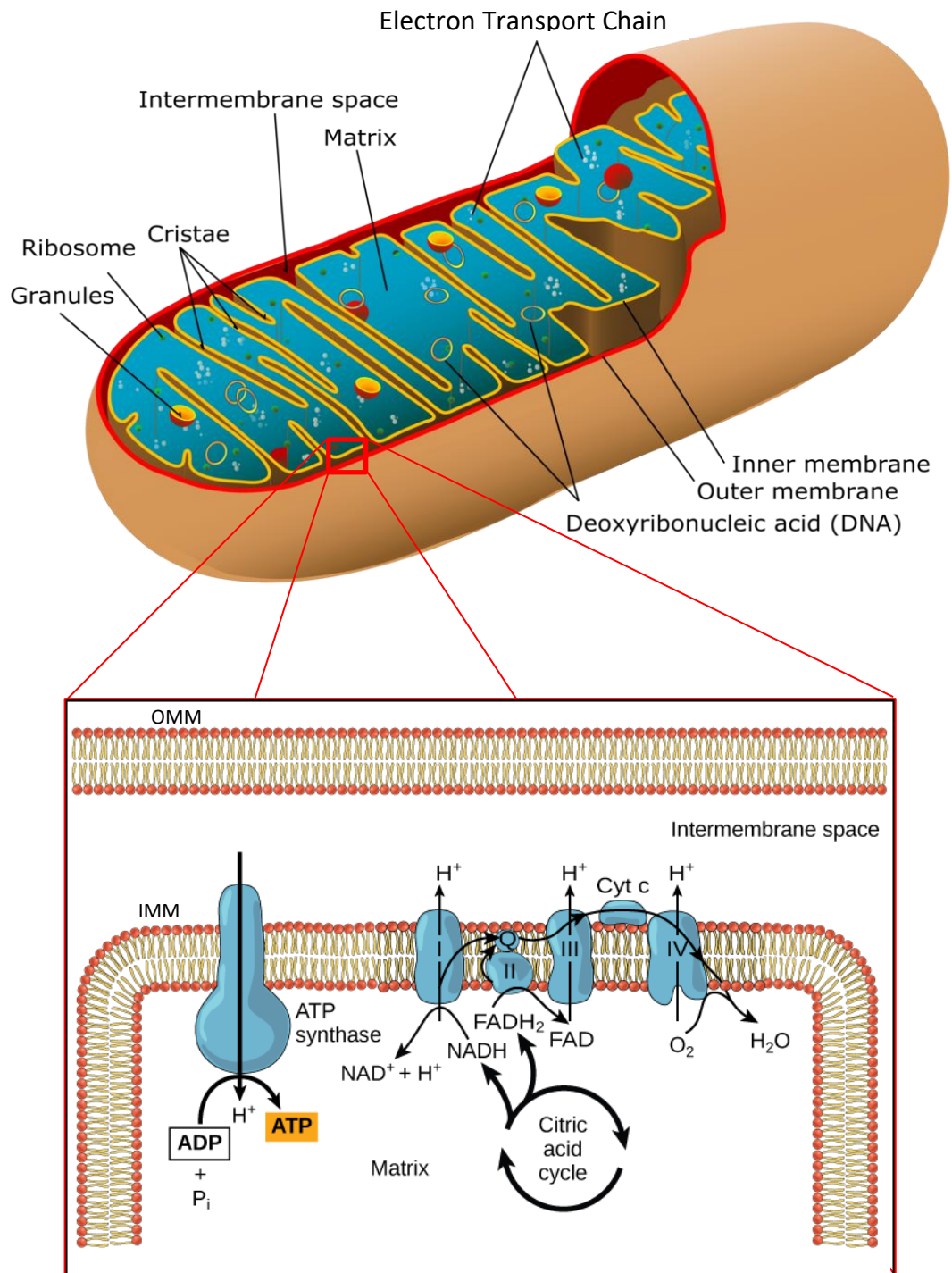


Figure 1-3: A representation of a mitochondrion, with a diagrammatic representation of the ETC. A) shows a cross section of a mitochondrion with various component parts highlighted. B) shows the area in the red box up close, highlighting the double membrane structure of the mitochondria. The ETC forms part of the inner membrane, where complexes I-IV can be seen, along with ATP synthase, in blue. The black arrows on CI-IV highlight the path that electrons take after oxidation of the electron donor, either NADH or FADH. IMM = inner mitochondrial membrane, OMM = outer mitochondrial membrane. Adapted from publicly available diagrams on Wikimedia Commons.

CI is an NADH:ubiquinone oxidoreductase, which means that it takes 2 electrons from the electron donor NADH, and reduces ubiquinone, an electron carrier, via a series of iron-sulphur clusters. It concomitantly transfers 4 protons to the intermembrane space. Alternatively, CII can act as an entry point to the ETC. CII contains just 4 subunits so is the smallest of the members of the ETC and is the only member to be entirely encoded by the nuclear genome. CII acts as part of the citric acid cycle where it acts as a succinate dehydrogenase, transferring 2 electrons from succinate through an FAD cofactor to reduce ubiquinone. CII is non-proton motive. Ubiquinone then diffuses to CIII, also known as cytochrome C reductase, where it undergoes oxidation and subsequent transferral of these electrons to cytochrome C, a second electron carrier. CIII contains 11 subunits, 10 of which are encoded by the nuclear genome. CIII contains 3 iron centres which it passes the electrons through to reduce cytochrome C. CIII pumps 2 protons. Finally, the electrons are transferred from cytochrome C to CIV, cytochrome C oxidase. CIV contains 34 subunits, with 3 of them encoded by the mitochondrial genome and pumps 4 protons with each reaction. CIV contains 2 heme groups and 3 copper ions, which it uses to bind oxygen. The electrons from cytochrome C are used to reduce molecular oxygen to form H_2O . This process of reducing O_2 also uses protons from the matrix side of the mitochondria which contributes to the proton gradient independently to the proton pumping ability of CIV. This reaction completes the electron transport chain from the electron donor NADH or succinate to oxygen.

The proton gradient that has been built up by the ETC is then used by CV, also known as ATP synthases, to produce ATP. Although CV is not technically part of the ETC, it is often considered as such due to the intrinsic link between the ETC and ATP production. The protons built up at the inter membrane space cannot cross the mitochondrial membrane without the help of an ion channel. CV can act as this ion channel. Like CI, III and IV, ATP synthase spans the inner mitochondrial membrane to create channels for the movement of protons. CV is divided into two regions, the F_1 and F_0 regions, with the F_0 region spanning the membrane and the F_1 region

being attached to this on the matrix side (Figure 1-3B). As protons pass through the F_0 channel from the intermembrane space to the matrix, the electrochemical potential is turned into mechanical energy which rotates the F_1 region. This F_1 region has three catalytic sites that bind ADP, and as they rotate they go through a series of conformational changes that result in the production of an ATP molecule (Stryer, 2010).

This electrochemical proton gradient forms part of the mitochondrial membrane potential ($\Delta\Psi_m$). In general the $\Delta\Psi_m$ is kept relatively constant, and significant deviations can be a sign of ETC dysfunction and indicative of disease (Zorova et al., 2018). As the energy required to pump protons across the inner membrane is dependent of the concentration of protons on the side being pumped to, as the protons build up in the intermembrane space it becomes harder for the ETC to pump further protons out of the matrix. This pressure is predominantly released by the action of ATP synthase, which diminishes the $\Delta\Psi_m$ by returning protons to the matrix. At equilibrium for a given amount of NADH or FADH we can predict how much ATP will be produced (2-3 molecules of ATP per NADH and 1-2 ATP molecules per FADH (Rich, 2003))

In this sense the activity level of the ETC is “coupled” to the activity of ATP synthase. Throughout this thesis “uncouplers” will be referred to, these are essentially either chemical inhibitors, mutations and adaptations that effect ETC activity or even entire proteins that can “uncouple” the relationship between ETC activity and ATP production, modifying its efficiency.

Finally, it is also worth noting that the electron transport “chain” is a bit of a misnomer. It is believed that the ETC protein complexes coalesce into super-complexes, aggregates of multiple complexes that covalently bind together. These complexes typically form between CI/III_2 , $CIII_2/CIV$ and $CI/III_2/IV$ (Dudkina, Kouřil, Peters, Braun, & Boekema, 2010; Schägger & Pfeiffer, 2000). The electron carriers are also present in these super-complexes making the $CI/III_2/IV$ a complete pathway from NADH to O_2 . The precise reason for the assembly of the super-complexes remains debated, but are posited to be increasing the stability of the individual

complexes, reducing reactive oxygen species (ROS) and potentially increasing the efficiency of electron transport (Letts & Sazanov, 2017). Super-complex formation and its role in disease is also unclear. There is a decline in super-complex formation in aging but it is unclear if this in and of itself has pathological consequences or if it is a symptom of dysfunction at large (Milenkovic, Blaza, Larsson, & Hirst, 2017).

1.2.2. Metabolic Choices – OXPHOS vs Glycolysis

ATP is the energy currency of the cell and is of paramount importance to the cell's survival. It takes an astonishing amount of ATP to fuel an entire person; a sedentary male of 70kg requires 83kg of ATP per day to provide their requisite energy! (Stryer, 2010). Broadly speaking cells have two semi-exclusive processes to choose from when it comes to ATP production, that of glycolysis or of oxidative phosphorylation outlined above. Glycolysis is the process of metabolising a glucose molecule to form two molecules of pyruvate as well as two molecules of ATP. In eukaryotic cells in the presence of oxygen pyruvate would be shuttled to the mitochondria where it is metabolised in the TCA and then used to fuel OXPHOS, which can produce a maximum of 36 ATP per glucose molecule, providing a far more efficient use (Zheng, 2012). Due to the perceived inefficiency it was believed that in humans a cellular shift to glycolysis only happened in anaerobic conditions where OXPHOS is impossible (such as during intense exercise periods), in the case of mitochondrial dysfunction where the electron transport chain is perturbed, or also in cancer, where the phenomenon is known as the Warburg effect (Zheng, 2012). Increasingly though it appears that aerobic glycolysis is the bioenergetic pathway of choice for certain cells, in particular neuronal cell types. For instance, it was mentioned earlier that photoreceptors appear to favour aerobic glycolysis (Kanow et al., 2017). There is also growing evidence that brain neurons can also switch to aerobic glycolysis (Yellen, 2018). This is believed to be due glycolysis being more readily scalable, that it can be upregulated quickly to respond to immediate metabolic demands such as transmitting signals. It takes longer for the cell to increase OXPHOS to produce more ATP than it does glycolysis. Exciting work recently

proposed that there is a physical limit to the amount of Gibb's free energy a cell can produce before it is detrimental to the cell (Niebel, Leupold, & Heinemann, 2019). The authors propose that glycolysis offers a method for cells to maximise ATP production whilst remaining under this limit when there are no constraints on metabolites. This glycolytic shift has a bearing on the metabolism of cells in culture, which will be outlined in chapter 5 in particular.

1.2.3. Mitochondrial Origins

Perhaps the single most important evolutionary event was the recruitment of the mitochondria for the host cell's bioenergetic needs, what would become one of the defining criteria for a eukaryotic cell. With the mitochondria allowing the host to utilise oxidative phosphorylation, which responsible for up to 95% of a eukaryotic cell's energy production, it is easy to see why this advance could be responsible for the explosion of diversity that followed (da Fonseca, Johnson, O'Brien, Ramos, & Antunes, 2008). There are no examples of truly complex multicellular organisms outside of the eukaryotic lineage (maybe apart from some cyanobacteria that grow in multicellular filaments containing two types of interdependent cells (Flores & Herrero, 2010)) suggesting this new energy production method allowed the development of far more complicated systems than were previously possible. This notion that mitochondria allowed complex life formation has been investigated as an energy-per-gene burden that organisms have to carry. The increased supply of energy that OXPHOS provides is estimated to permit an astonishing 200,000-fold increase of the theoretical limit of genes expressed opening the door to the incredible diversity we see in gene systems across eukaryotes (Lane & Martin, 2010). Superfluous DNA is a luxury that bacteria cannot afford, as there is an energetic cost to replicate each base, hence the typically gene dense bacterial genomes. The glut of ATP produced by mitochondria relaxes this pressure enabling genomes to grow and expand, allowing room for diversity to flourish. It has been a long held tenant of evolutionary biology that gene duplication events give freedom for rapid neofunctionalization of genes to occur (although it might not actually be this straightforward (O'Toole, Hurst, & McLysaght,

2018)). In bacteria the added energetic burden of large-scale duplication of genes would most likely prohibit this, severing an evolutionary avenue.

The widely accepted origin of mitochondria is an endosymbiotic event which engulfed an ancestral α -proteobacterium into a host cell, most likely part of the archaea kingdom (Roger, Muñoz-Gómez, & Kamikawa, 2017). This endosymbiotic theory, along with the idea that chloroplasts arose in the same manner, was first published in 1967, where it met considerable push-back for a number of years (Sagan, 1967). The α -proteobacteria family is highly diverse, including an array of endosymbionts and intracellular pathogens. Currently the closest extant α -proteobacterial ancestor of the mitochondria is believed to be *Rickettsia prowazekii*, the causative agent of a louse-borne typhus (Gray, Burger, & Lang, 1999). The *R. prowazekii* genome is roughly 1.1Mbp in size, some 70 times larger than that of our mitogenome (see the next section for an in depth analysis of mitogenome content) and encodes for 834 proteins (Andersson et al., 1998). What is particularly interesting about the *R. prowazekii* genome is that a number of genes involved in the biosynthesis of amino acids along with other vital processes found in free-living bacteria are already missing, hinting at the start of the march towards obligate endosymbiosis.

Only recently has a candidate for the ancestral archaeon been put forward. The ancestral host cell is believed to be related to Lokiarchaeota, a member of the Asgard superphylum² due to their enrichment for proteins previously considered to be eukaryote specific (Zaremba-Niedzwiedzka et al., 2017).

² Can you guess the connection between the names of the members and the phylum name? Hint: the phylum also contains Thorarchaeota, Odinararchaeota and Heimdallarchaeota. Scientists love a laugh.

1.2.4. The Mitogenome

In animals the mtDNA is arranged in a circular genome of varying sizes depending on how many of the mitochondrial genes have been transferred to the nucleus. In humans the reference mtDNA molecule is 16,569 base pairs in length (current revised Cambridge Reference Sequence, accession: AC_000021.2) and was the first mitogenome to be sequenced (Anderson et al., 1981). The human mitochondrial genome sequence contains 37 genes, 13 of which are protein coding, 22 are for tRNAs and 2 encode the mitochondrial rRNAs. The 13 proteins are all subunits of the large multimeric protein complexes that constitute the ETC the enzymatic pathway responsible for OXPHOS (Table 1-1). Mitochondria use a slightly modified version of the “universal” genetic code which makes encoding their own tRNAs necessary, an important consideration when ectopically expressing genes typically found on the mitochondrial genome. These changes in human mitochondria are detailed in Table 1-2, but they can vary between animal species’ mitochondria. The addition of AUA encoding for Methionine means that mitochondrial genes have either ATG or AUA as their start codon. The addition of the two rRNA genes means that human mitochondria remain self-sufficient for the expression of the proteins encoded by the mtDNA genome, but of course heavily reliant on the nuclear genome for the remainder of the proteins necessary to produce a mitochondrion, or even replicate the mtDNA.

Table 1-1: The 13 protein coding genes of the human mtDNA genome, as well as the electron transport chain complex to which they belong.

ETC Complex	Gene Name
NADH:Ubiquinone Oxidoreductase (complex I)	ND1, ND2, ND3, ND4, ND4L, ND5, ND6
Ubiquinol:Cytochrome C Oxidoreductase (complex III)	CytB
Cytochrome C Oxidase (complex IV)	COX1, COX2, COX3
ATP synthase (complex V)³	ATP6, ATP8

Table 1-2: Changes to the “universal” genetic code found in vertebrate mitochondria.

Triplet	Nuclear tRNA	Mitochondrial tRNA
AGA & AGG	Arginine	Stop
UGA	Stop	Tryptophan
AUA	Isoleucine	Methionine

There is a valid question as to why mitochondria (and indeed chloroplasts) still have a genome at all, why haven't all the genes been transferred to the nuclear genome? There are believed to be between 1,000 – 1,500 proteins that comprise the mitochondrial proteome, the vast majority of which are now encoded for by the nuclear genome (Calvo & Mootha, 2010). It has been shown that an evolutionarily neutral scheme, where genes would have been lost at random from the mitochondrial genome and these 13 just happen to be the ones that remain does not hold true (Johnston & Williams, 2016). The reasons that genes have been lost in the first place appear to be avoidance of Muller's ratchet, as the nuclear genome is much more carefully maintained than the mitochondrial genome, as well as enhanced likelihood of the fixation of beneficial mutations (Allen, 2015). The question remains as to why some genes have remained

³ Technically ATP synthase is not part of the ETC as it does not accept any electrons, but it is often considered as such because of its integral role in OXPHOS. It uses the H⁺ gradient generated by the other complexes to produce ATP.

at all, and there are two non-mutually exclusive prevailing theories for this. One is the CoRR hypothesis (“colocation (of gene and gene product) for redox regulation of gene expression”) which posits that the expression of the genes that remain need to be under the mitochondrion’s direct control and thus moving them to the nucleus does not offer a selective advantage (Allen, 2003). The other theory suggests that the remaining proteins could not be expressed by the nuclear genome as they are unable to avoid being incorrectly targeted to the endoplasmic reticulum (ER) due to the highly hydrophobic nature of the remaining proteins (Björkholm, Harish, Hagström, Ernst, & Andersson, 2015). To demonstrate this, the authors of this study ectopically expressed Cox1, ATP6 and CytB in the cytosol of cells and demonstrated that they would not localise to the mitochondria and instead localised to the ER. This is an important consideration when designing potential therapeutics based on existing genes in the mitochondria, as expressing

them directly in the cytoplasm of the cell may not work. To avoid this, gene therapies such as GS010 by GenSight (more on this later) added a mitochondrial targeting signal to the ND4 subunit they express in the cytoplasm.

1.3. Haplogroups

Due to the predominantly maternal inheritance of mitochondria, it is possible to trace one’s mitochondrial lineage back to a matrilineal most recent common ancestor (Cann, Stoneking, & Wilson, 1987). This “mitochondrial Eve”, a title first coined by R Lewin, 1987 lived some 150,000 years ago in central Africa according to modern estimates (Fu et al., 2013; Poznik et al., 2013). It is interesting to note that mitochondrial Eve is not fixed temporally and will continue to get ‘younger’ as currently existing mitochondrial lineages die out, making our matrilineal most recent common ancestor more recent. Since mitochondrial Eve there has been a significant increase in the diversity of mitochondrial sequences extant on Earth, owing to a number of

reasons such as the mitochondrion's comparatively high mutation rate (W. M. Brown, George, & Wilson, 1979) and the migration of people across the globe causing geographic isolation. This has led to a number of mitochondrial lineages branching from mitochondrial Eve. These different lineages are termed haplogroups (see Figure 1-4).

As mentioned previously, different haplogroups occupy different geographical regions around the globe and broadly correspond to the order in which the regions were colonised by early *Homo sapiens*. For example, the L clade, the oldest branches, are predominantly located in Sub-Saharan Africa whereas the more recently diverged haplogroups are located in areas believed to be more recently colonised such as the Americas (Figure 1-5). This has been used as further evidence for the 'out of Africa' hypothesis (Stewart & Chinnery, 2015).

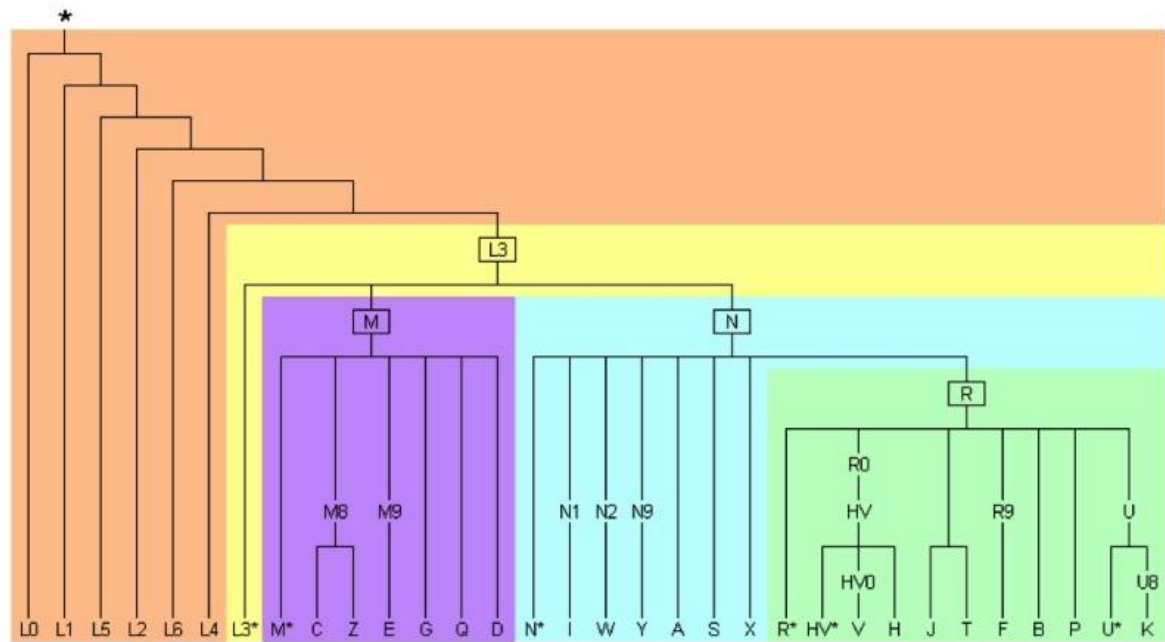


Figure 1-4: A simplified mtDNA phylogeny showing the emergence of the different haplogroups, represented here by letters at the branch ends. The * represents mitochondrial Eve. Haplogroups are named A-Z but in the order they were discovered rather than in the order of divergence. The macro-haplogroups are colour coded. Figure reproduced from (van Oven & Kayser, 2009).

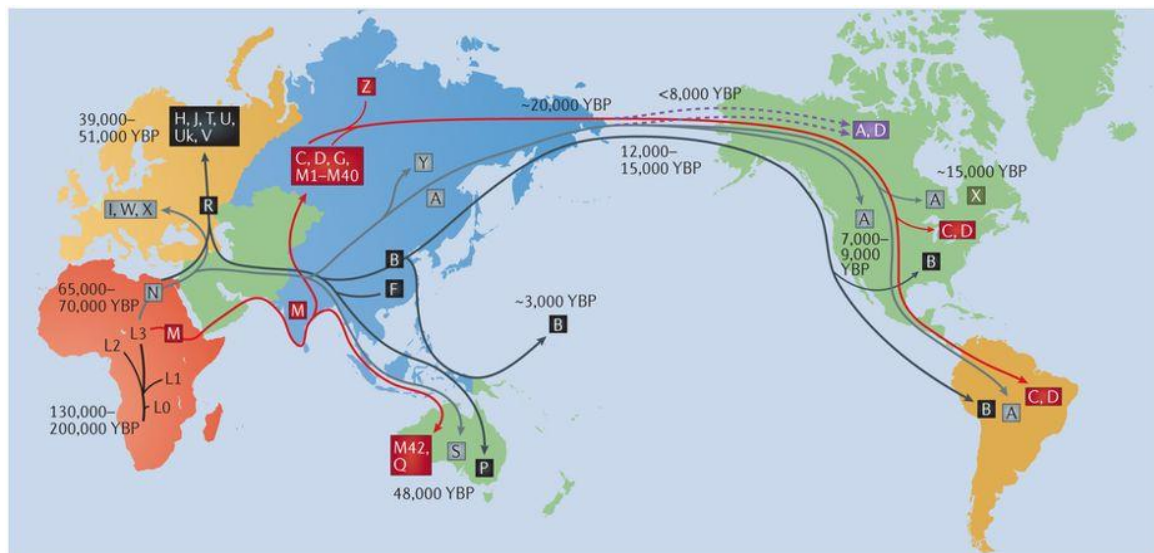


Figure 1-5: A map of predominant macro-haplogroups across the globe. The divergence of macro-haplogroups broadly correlates with the colonisation of each land mass by Homo Sapiens. YBP: Years Before Present. Map reproduced from (Stewart & Chinnery, 2015).

Haplogroups are differentiated from each other by single nucleotide polymorphisms (SNPs) located around the mitochondrial genome. Each haplogroup has 'characteristic' SNPs which define the macro-haplogroup and further SNPs which assign the mtDNA to haplogroups and even sub-haplogroups. As a single SNP can be the difference between two haplogroups and the range of potential SNPs an mtDNA genome can have is very large, it is somewhat arbitrary when one haplogroup is considered distinct from another, with potential for each individual to have their own unique haplogroup. The current standard for haplogroup and sub-haplogroup differentiation is the presence of a "relatively stable (set of) mutation(s) that are shared by at least three complete sequences" (van Oven & Kayser, 2009). Each mitochondrial haplogroup has hundreds of sub-haplogroups that have been defined (see <http://www.phylotree.org/> for an up to date list; it currently stands at approximately 5,400 haplogroups and sub-haplogroups!). It is more a case of stopping when sub-dividing haplogroups is no longer usefully reductive. As such most research investigating differences between haplogroups will stop at the level of Figure 1-4.

The question of whether there is a biochemical difference between the haplogroups has long been debated, with some rather interesting findings coming to light. Take for example the N mtDNA macrohaplogroup. This haplogroup spread northwards from Saharan Africa towards the much colder climes of northern Europe (Figure 1-5) and is the root of the common European haplogroups (see Figure 1-4 and Figure 1-5). The N macrohaplogroup is associated with two SNPs that are posited to reduce the coupling efficiency of mitochondrial OXPHOS. This requires more calories to be burned to produce an equivalent amount of ATP, which in turn causes excess heat to be produced and presumably provides a selective advantage in colder regions (Douglas C Wallace & Chalkia, 2013). Indeed it is argued that climate adaptation is majoritively through mitochondrial mutations, with little correlation between nDNA variation and climatic differences (Balloux, Handley, Jombart, Liu, & Manica, 2009).

Does your mtDNA haplogroup have consequences for disease? As newer and cheaper sequencing technology allows the haplogroup of patients to be quickly and cheaply determined, the answer to this question is being sought. Which haplogroup a person's haplotype belongs to can have consequences for disease progression (Chinnery & Gomez-Duran, 2018).

Around 60,000 years before present (YBP) the N lineage diverged from the R macro-haplogroup, which gave rise to the modern common "European" haplogroups, U, J, T, H and V (Achilli et al., 2004), with 90% of Europeans being part of either the H, V, U, J or T lineages. The remaining 10% are I, W, X and other variations thereof (Figure 1-5). More specifically up to 45% of Europeans possess the H haplogroup (Roostalu et al., 2007) roughly 9% are of the J haplogroup (Valerio Carelli et al., 2006) and 20% belong to the U haplogroup branch (Montiel-Sosa et al., 2006). As mitochondria are the main energy producers of the cell it is no surprise that SNPs in the mtDNA can lead to variations in energy production, which can have serious adverse effects on cells with high energy demands such as neurons and muscle tissue. It would be unlikely from an evolutionary stand point that significantly deleterious mutations to mtDNA would become fixed in a population and hence become a viable haplogroup, but it could be possible that a haplogroup contains "sensitising" mutations or risk factors that can subtly alter a person's cellular bioenergetics and leave them more vulnerable to deleterious mutations. For example, men with U lineage haplogroups have lower sperm motility than average, another cell type with high energy demand (Montiel-Sosa et al., 2006). Of more relevance to the subject of this PhD study, different haplotypes have been linked with the progression of Leber Hereditary Optic Neuropathy (LHON) (V. Carelli et al., 1997; Johns & Berman, 1991). LHON will be discussed in more detail later but briefly it is a disease caused predominantly by mutations in subunits of complex I of the mitochondrial electron transport chain (ETC) that leads to the death of retinal ganglion cells eventually causing blindness. There are 3 classical mutations that are associated with LHON which account for ~90% of cases in the Caucasian population; m.11778G > A:MT-ND4 (60%), m.3460G > A:MT-ND1 (15%) and m.14484T > C:MT-ND6 (15%) (Theodorou-Kanakari

et al., 2018). Intriguingly, these mutations are over-represented in patient cohorts with certain haplogroups. The m. 11778G > A mutation (known as the Wallace mutation) is associated with the J haplogroup, whereas carriers of the m.3460G > A mutation and the Uk haplogroup were at increased risk of developing the disease. Furthermore, haplogroup H is under-represented in patients and as such is believed to be protective against LHON (Hudson, Carelli, Spruijt, et al., 2007). This hints at a potential mechanism for the incomplete penetrance frequently observed with LHON. It could be that the haplogroup is a risk factor and the primary mutation acts as the final insult that causes dysfunction.

The H haplogroup has been found to be protective in a number of disorders such as LHON and AMD (Riazi-Esfahani, Kuppermann, & Kenney, 2017). Cybrids with H haplogroup mtDNA have higher levels of mtDNA, mtDNA encoded mRNAs, grow faster and have a higher oxygen consumption rate than other haplogroups (Gómez-Durán et al., 2010). This increased mitochondrial activity could act as a “buffer” whereby the increase in intrinsic activity allows a higher level of dysfunction to be tolerated before detrimental effects are experienced by the cell. Counter-intuitively to its protective role, but perhaps to be expected from its increased metabolic rate, H haplogroup cybrids are more susceptible to ROS induced cell death than J haplogroup cybrids are (Mueller et al., 2012), which manifests itself in H haplogroup people having an increased risk of ischemic cardiomyopathy, where increased ROS is a predictive marker (Fernández-Caggiano et al., 2012).

It also appears that certain SNPs can be beneficial and detrimental depending on the mtDNA haplogroup they arise on. The 3394T > C polymorphism in *ND1* causes a significant decrease in complex I activity of up to 30% in common Asian haplogroups B4c and F1, and has been associated with LHON in these populations (M. Liang et al., 2009). However, this polymorphism is highly enriched in high altitude Tibetan and Indian populations, occurring most frequently on the M9 and C4a4 haplogroups respectively. In these populations, the 3394T > C polymorphism

is correlated with higher complex I activity than the 3394T “wild-type” *ND1* in the B4c and F1 populations (F. Ji et al., 2012). Consequently, it is clear that the context of a polymorphism on the mitochondria, not just the polymorphism itself, is of vital importance to the correct interpretation of disease-causing variants. It is possible that highly targeted sequencing of a specific mtDNA region and identification of putative causative variants is not a reasonable approach without first establishing the mtDNA context of the patient.

The complexity of the relationship between haplogroups and disease alleles underscores the fact that it is rare for a mutation to cause a disorder in isolation, it is part of a much more complex network of interactions that are suitably altered by the myriad of seemingly “non-pathogenic” variants we also inherit. It seems increasingly possible that for these LHON cases mentioned, along with other complex disorders, a “multiple hit” scenario is the cause, with an accumulation of dysfunction that once past a certain threshold causes the disease phenotype to manifest. Although a mutation for a mitochondrial disorder can show high penetrance, such as the 8993T > G transversion that causes neuropathy, ataxia and retinitis pigmentosa (NARP) (Thorburn, Rahman, & Rahman, 1993), its manifestation is still predicated on the mtDNA background of the affected person (D’Aurelio, Vives-Bauza, Davidson, & Manfredi, 2010). The fact that both J and H haplogroups are protective and risk factors for different conditions highlights that mitochondria exist at a delicate equilibrium, and that going too far in any dimension can increase the likelihood of disease.

1.4. Heteroplasmy

The haplogroup of an individual is only one significant player in the manifestation of mitochondrial maladies; the heteroplasmy levels of a variant are also of importance. As previously mentioned, the mitochondrial genome is maintained at 100s to 10,000s of copies per cell (Stefano, Bjenning, Wang, Wang, & Kream, 2017). It is possible for subpopulations of mtDNA

genomes with different sequences to be present in the same cell, a state which is referred to as heteroplasmic. The differences between these mtDNA genomes can range in size from large scale deletions to much more common single nucleotide polymorphisms (Rocha et al., 2018).

Whilst heteroplasmy can arise during a person's lifetime due to mutations in the mtDNA content of an individual, it is far rarer for these mutations to reach significant levels required for detrimental effects to be actualised. It is thought that the main mechanisms for extreme alterations in heteroplasmy to occur happen at early prenatal and possibly even prefertilisation stages of development. It was observed that a *de novo* mtDNA SNP could become fixed, or homoplasmic, within 2 to 3 generations in Holstein Cattle (Olivo, Van de Walle, Laipis, & Hauswirth, 1983).

As mitochondria are primarily passed down through the matrilineal line, oocyte formation is a key stage in the development of an individual's heteroplasmy levels (Stewart & Chinnery, 2015). In a study of 84 oocytes from women harbouring a heteroplasmic 3243A > G mutation, it appeared that the levels of heteroplasmy in the woman's oocytes were not under any selection at gametogenesis as the resulting levels of heteroplasmy correlated to those expected from genetic drift (D. T. Brown, Samuels, Michael, Turnbull, & Chinnery, 2001). A genetic bottleneck occurs at this point in oocyte formation, whereby only a subset of the mother's mitochondria is passed on to the oocyte. There are three proposed mechanisms for why a certain subsection of mitochondria would be passed on to the oocyte, but these all revolve around the selection of only a subsection of available mtDNA molecules, which are then clonally expanded in the resulting oocytes (Stewart & Chinnery, 2015). A simplified example of this would be if the mother had a mutation in a 50% heteroplasmic state and the oocyte receives a random 25% of the mtDNA molecules, the heteroplasmic fraction in the oocyte could theoretically range from 0-100%. There are other factors potentially at play too, especially since the mechanism underlying the selection of mitochondria for an oocyte remains unclear. Another intriguing

scenario in the case of mtDNA deletions could be that they have an advantage when it comes to replication; with a section of mtDNA missing it presumably is quicker to replicate a mtDNA molecule with a deletion than is to replicate a wildtype molecule, providing the damaged mtDNA molecule with a selective advantage. This would be irrelevant for nuclear DNA deletions as their replication is coupled with mitosis and occurs only infrequently, but mtDNA replication occurs independently from each other molecule in the cell allowing for clonal expansion of a particular mtDNA sequence.

What about selection on a molecular level to correct for detrimental mtDNA heteroplasmies? Muller's Ratchet states that without recombination afforded through sexual reproduction, genomes of organisms that reproduce only asexually are doomed to reach "mutational meltdown"(Muller, 1932). As mitochondria do not recombine and are uniparentally inherited, it seems we would also be fated to go extinct through mtDNA mutational load. That, however, does not appear to be the case.

There is some evidence that molecular selection against severe mtDNA mutations occurs, but the extent to which it occurs remains unclear. It has been suggested that "oocyte attrition" could be a form of mtDNA selection. This is the natural ongoing process whereby a female loses oocytes from their original pool at a far higher rate than just ovulating, until the eventual onset of the menopause (Freyer et al., 2012). It has also been postulated that the high rate of early pregnancy miscarriage in humans could involve a high mtDNA heteroplasmic mutation load (Kaare et al., 2009; Seyedhassani, Houshmand, Kalantar, Modabber, & Aflatoonian, 2010). Furthermore, a recent study of primordial germ cells (PGCs; gamete precursor cells) from human female embryos showed a vast decrease in mtDNA diversity, validating the genetic bottleneck theory and also suggesting its use in molecular selection (Floros et al., 2018). Whilst sequencing these PGCs they found that the mtDNA population was reduced to a mean of ~1500 copies, roughly 1000-fold less than mature oocytes. This in turn means that the per mitochondrion level

of mtDNA was ~5 copies. Concomitantly with this, the PGCs switch from being primarily glycolytic to utilising oxidative phosphorylation. This metabolic shift would highlight any severely detrimental mitochondrial mutations and allow the cell to activate the necessary mitophagy pathways to destroy any highly dysfunctional mitochondria, and at its extreme to destroy the PGC itself (Floros et al., 2018). I feel this is akin to revving the engine a few times when you are buying a car to make sure it runs okay! It is also interesting that using a similar method examining embryos from mice, a similar but “wider” bottleneck was observed (Cree et al., 2008). In other words, the reduction of mitochondria in the PGCs was to a lesser degree in mice than humans. This, in theory, would mean that in humans there is more sensitivity in detecting mtDNA mutations, as any mutations would be present in a higher heteroplasmic fraction in the resulting oocytes, due to the narrower bottleneck.

However, it is apparent that this system, bottleneck then metabolic challenge, is imperfect due to the prevalence of mitochondrial disease. It can prevent a PGC from developing with an extreme, catastrophic level of a deleterious heteroplasmy, but it is easy to imagine a scenario with a mutation hovering around the clinical threshold not being weeded out by this process. The bottleneck and metabolic challenge hypothesis possibly explains how mitochondria escape Muller’s Ratchet. It is also useful to consider that perhaps not all mitochondrial disorders are debilitatingly detrimental to the individual cell, but that the effects of some mitochondrial disorders only manifest themselves when the entire tissue is affected. This would allow these mutations to evade the purifying selection on the single PGC level.

It is worth taking a moment to discuss when these mitochondrial mutations may occur. If a shift in heteroplasmy is disease causing and has arisen due to the genetic bottleneck, then this has happened at oogenesis, meaning that the shift has potentially occurred while the affected person’s mother is *in utero*, a foetus in her own mother. Some effort has been made to identify the rate of *de novo* mutations in mitochondrial disease, which puts it at around 25% for (likely)

pathogenic mutations in a cohort of 105 patients (Sallevelt et al., 2017). In order for these mutations to reach a clinically relevant level of heteroplasmy, it is most likely that this mutation happened near the narrowest part of the bottleneck, where the PGC is expanding its mitochondrial population from some 1500 mtDNA copies to the 1×10^6 copies found in a mature oocyte (Floros et al., 2018), and thus would reach a high heteroplasmic fraction and be most likely to cause disease. It is also possible that the mutations occur post fertilisation at an early cell division, which would allow it to spread to a significant proportion of the body, leading to mtDNA heteroplasmic mosaicism. It would be possible at this early stage for a mitochondrial mutation to reach significant levels of heteroplasmy if during cell division, there is an unequal partition of mutated and wild type mitochondria, leading to a daughter cell receiving a higher proportion of diseased mitochondria. This could be tested for in patients by analysing a number of different tissues, preferably from different early cell lineages, for the heteroplasmic mutation levels. The rate of recurrence of *de novo* mitochondrial mutations in the Sallevelt et al., 2017 study was very low, with only one case documented in a literature survey of 137 *de novo* mitochondrial mutations, this would most likely be due to germline mosaicism. Humans are estimated to have between 30-50 PGCs, that all subsequent germline cells derive from (Samuels & Friedman, 2015). It is therefore possible that if the mutation happened during the formation of the PGCs themselves then the daughter PGCs and their resulting oocytes will contain the mutation.

1.5. Mitochondrial Mutations and Reactive Oxygen Species (ROS)

It is possible that the close proximity of mtDNA to the electron transport chain and the free radicals (in particular reactive oxygen species (ROS)) that it produces leads to sporadic mtDNA mutations, a notion that has long been a pillar of the free radical theory of aging (Pinto & Moraes, 2015). It is worth noting that the original paper, "Aging: A Theory Based on Free Radical

and Radiation Chemistry” published some 60 years ago, does not mention mitochondria and at this point there was no evidence to suggest free radicals even interacted with DNA and RNA. It was purely based on chemical evidence that cells produce free radicals and that chemically speaking, free radicals are highly reactive, catalysing a large array of oxidation reactions. It

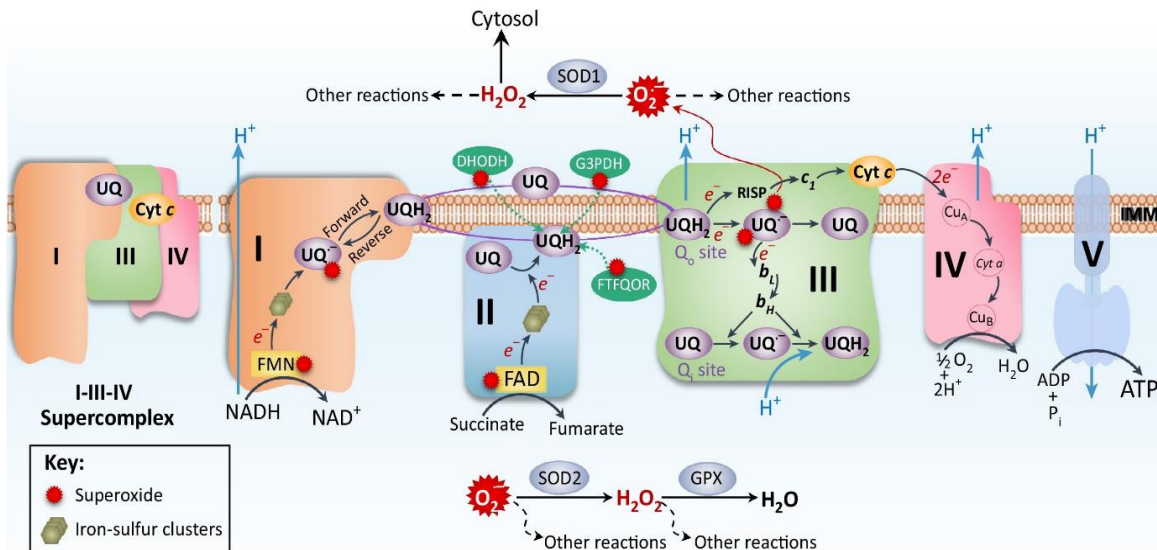


Figure 1-6: A schematic of the Electron Transport Chain (ETC) showing the flow of electrons down the chain towards H_2O via the intermediary electron carriers. Of particular note is ubiquinone in its oxidised, semi-reduced and reduced states (UQ , $UQ^{\bullet -}$ and UQH_2 respectively). It is through these carriers that the majority of ROS is produced. Reproduced from (Wang & Hekimi, 2016).

shows an impressive level of insight given a starting point obfuscated by a lack of knowledge of fundamental processes in the cell.

As previously mentioned, mitochondria are the primary source of ROS, which is produced during OXPHOS, in particular at complex I and III (Murphy, 2009). This is broadly due to either a build-up of NADH (the CI substrate) or reduced ubiquinone (the product of CI activity and the CIII substrate) which then in turn reduce O_2 to form $O_2^{\bullet -}$ (also known as superoxide) which in turn causes oxidative stress (See Figure 1-6). In brief, CI oxidises NADH to NAD^+ by passing its electrons on to flavin mononucleotide (FMN) which then passes one electron at a time up to oxidised ubiquinone (UQ) to initially form ubisemiquinone ($UQ^{\bullet -}$) then fully reduced ubiquinone (UQH_2). The fully reduced ubiquinone is then transferred to CIII which oxidises UQH_2 via $UQ^{\bullet -}$ to UQ and transfers these electrons to Cytochrome C and onwards to CIV.

CI is believed to induce ROS production in two distinct ways. Firstly $UQ^{\cdot-}$ is an unstable state and as such acts as an oxidiser which can produce superoxide molecules (Wang & Hekimi, 2016). Secondly the FMN is believed to be the main site of superoxide production by CI (Pryde & Hirst, 2011). Initially when electrons are transferred to FMN from NADH, they can be erroneously transferred to O_2 , rather than to the iron sulphur clusters, forming a superoxide anion. There is also a phenomenon known as reverse electron transfer (RET), where UQH_2 molecules are in fact oxidised by CI and their electrons are passed “uphill” to the FMN complex where they can reduce NAD^+ to NADH, but where they can also produce superoxide (Kudin, Bimpong-Buta, Vielhaber, Elger, & Kunz, 2004). It appears that RET is the most prolific producer of ROS in the cell (Brand, 2010). This has been demonstrated by the selective use of inhibitors that block the UQ-reduction site on CI, leading to a greatly diminished rate of superoxide production (Wang & Hekimi, 2016). As RET uses UQH_2 as its substrate, it is acutely sensitive to fluctuations in the redox state of the UQ pool. This can be caused by a number of metabolic pathways but is intractably linked to complex II of the ETC. In an interesting study it was hypothesised that there is build-up of succinate during ischemia due to the reversal of enzymatic processes involved in purine nucleotide breakdown. This succinate is then rapidly oxidised by CII once normoxia is returned which causes a dramatic reduction of the UQ pool, more than can be dissipated by CIII alone. The increase in UQH_2 then leads to a significant burst of ROS production due to RET (Chouchani et al., 2014).

The ROS produced by CIII is believed to be mainly due to its production of $UQ^{\cdot-}$ during its oxidation of UQH_2 . The electron transferral takes place one electron at a time between UQH_2 and CytC. CIII has two enzymatically active sites called Q_0 and Q_i (see Figure 1-6). It has been well established that Q_0 is the site of superoxide production, mainly through the use of inhibitors that selectively inhibit one of the CIII active sites (Turrens, Alexandre, & Lehninger, 1985). For instance, Antimycin A blocks the Q_i site which leads to an increase the levels of $UQ^{\cdot-}$ which in turn leads to a significant increase in superoxide levels (Cape, Bowman, & Kramer, 2007). There

is not an equivalent increase in ROS when an inhibitor of the Q_0 site is used as there is no ubiquinone produced since electron transfer to CIII is completely blocked (Cape et al., 2007). In addition to this, as one would expect, the addition of specific inhibitors of Q_0 such as myxothiazol lead to an over-reduction of the UQ pool and as such cause superoxide production from CI mediated RET (Brand, 2010).

It has been shown that the ROS production from chemically induced CI dysfunction can be modulated by tweaking the NADH/NAD⁺ ratio in cardiac mitochondria (Korge, Calmettes, & Weiss, 2016). Similarly, it has long been known in diseases associated with CI dysfunction, such as LHON and Parkinson's Disease (PD), that there is significant increase in the oxidative stress via increased levels of ROS (Zhuo, Luo, & Zhang, 2012). It has also been demonstrated that ROS accumulates as an organism ages. In rats a significant increase in ROS levels has been demonstrated by "middle age", and increases further the older the rat becomes (Luceri et al., 2018). In humans the accumulation of plasma-protein oxidation is also age dependant (Pandey, Mehdi, Maurya, & Rizvi, 2010).

The question remains though, is ROS a symptom or a cause of mitochondrial dysfunction? With regard to aging it does not appear to be clear cut. A number of factors fit this theory, such as the proximity of mtDNA to the ETC and the known mutagenic power of ROS. Superoxide molecules can interact with DNA to produce 8-oxo-2'-deoxyguanosine (8OHdG) which is used as a marker for oxidative damage and is consistently shown to be correlated with an accumulation of mtDNA mutations (Pinto & Moraes, 2015). Work in mice has also shown that a mitochondrially targeted catalase (mCAT), which can detoxify H_2O_2 , demonstrated an increase in lifespan of 18% (Schriner et al., 2005). An equivalent increase was not seen when the catalase was targeted to the nucleus or the peroxisome.

1.5.1. The Duality of ROS

ROS is increasingly being associated with having a hormetic response (Hood, Zhang, Mowry, Hyatt, & Kavazis, 2018). This is toxicologist's term meaning that something shows a biphasic dose response curve. In practicality this means that at low doses the effector, be it drug or process, has a beneficial response, but as the dose increases this turns to an inhibitory or toxic effect. This is believed to be the response curve of the majority of therapeutic drugs (Calabrese, 2008). A little goes a long way, as it were.

It has long been known that even under normal physiological conditions ROS is produced by electron escape from the ETC. Under normal conditions ROS appears to be an important signalling molecule that has been evolutionarily conserved since prokaryotes (which is particularly striking since they do not have mitochondria) where ROS has been shown to directly activate transcription factors for adaption to stress (Schieber & Chandel, 2014). This is typically mediated through the enzymatic detoxification of superoxide into H_2O_2 by Superoxide Dismutase (SOD) proteins, and these H_2O_2 molecules go on to reduce cysteine residues in a vast array of signalling proteins (Kiley & Storz, 2004). ROS signalling has been shown to induce mitochondrial biogenesis through phosphorylating NRF-1 which in turn activates TFAM transcription (TFAM being part of the mtDNA replication pathway) (Sano & Fukuda, 2008). As mentioned previously, a substantial proportion of superoxide produced by the cell comes from Reverse Electron Transfer (RET) (Brand, 2010). A recent study on *Drosophila* has demonstrated that increasing ROS production specifically via the RET pathway extends lifespan and ameliorates pathogenesis induced by severe oxidative stress (Scialò et al., 2016). To achieve higher levels of RET, allotopic expression of Ndi1 was utilised. Ndi1 is an alternative NADH dehydrogenase found in *S. cerevisiae*. It acts as an equivalent of CI in *S. cerevisiae* mitochondria. We will discuss Ndi1 and its properties in much, much more detail further on. In aged *Drosophila* the allotopic expression of Ndi1 caused an increase in the levels of ROS detected, similar to levels of ROS detected after treatment of flies with Rotenone, a potent and specific inhibitor of

CI. The increased ROS due to Ndi1, but not that of Rotenone extended the lifespan of these flies. It is also important to note that knockdown of CI subunits, whilst still expressing Ndi1, led to a reduction in the levels of ROS produced but not to an increase in lifespan (Scialò et al., 2016). Furthermore, the allotopic expression of mCAT, a mitochondrially targeted catalase, actually decreased lifespan in this study, indicating that in healthy cells, too much reduction in ROS levels is actually detrimental. These findings propose an interesting model for the disease pathology of CI disorders. Perhaps the loss of CI activity in the forward sense leading to reduced ATP production is only part of the problem, with an inability of CI to perform RET with its resultant ROS signalling preventing protective pathways to be activated.

ROS signalling is believed to help in the recruitment of Parkin/PINK1 to the mitochondria, which is a key step in the mitophagy process (Xiao, Deng, et al., 2017). Mitophagy is the process whereby dysfunctional mitochondria are degraded and potentially recycled by the cell (Ashrafi & Schwarz, 2013). Mitophagy shall be discussed later on, but in the context of ROS, it is a possible link between elevated ROS levels and a cytoprotective effect. In a second study by the same group, it was found that depolarisation of the mitochondria did not trigger Parkin/PINK1 mediated mitophagy unless there was a concomitant ROS burst (Xiao, Goh, et al., 2017).

However there is a limit to which ROS production provides benefit, as evidenced by the large body of evidence associating excess ROS and cellular toxicity, especially in neurodegenerative diseases (Manoharan et al., 2016). For instance there is a lot of data demonstrating an increase in oxidative damage mediated by ROS in Alzheimer's Disease which leads to increased neuronal damage in the brain (A. Massaad, 2011). Likewise in Parkinson's disease the mitochondrial dysfunction seen in patients leads to increased ROS levels and its subsequent damage in the brains of patients (Puspita, Chung, & Shim, 2017). Prion disease toxicity has been linked to the excessive generation of ROS as well (Sonati et al., 2013). ROS accumulation leads to DNA, protein

and lipid damage through the highly oxidising nature of ROS which in turn can seriously disrupt cellular functions and eventually lead to cellular apoptosis.

There have been links drawn between ocular disease and overaccumulation of ROS also. For instance, HIF1 α is stabilised under hypoxic conditions where it promotes angiogenesis. This has been of particular interest to researchers studying eye pathologies associated with retinal neovascularisation such as AMD and Diabetic Retinopathy where HIF1 α suppression has been posited as a therapeutic approach (Zeng et al., 2017). ROS has been shown to stabilise HIF1 α by its inhibition of prolyl hydroxylases that would normally negatively regulate HIF1 α by targeting it for degradation by ubiquitinating it (Semenza, 2012). Oxidative stress has been shown to introduce mitochondrial DNA damage in human RPE cells too, further implicating aberrant ROS signalling in the disease progression of AMD (F.-Q. Liang & Godley, 2003). Furthermore, in diseases such as LHON (LHON will be discussed in detail in the introduction to chapter 3) oxidative stress caused by exogenous factors has been linked to disease onset and severity (Patrick Yu-Wai-Man & Chinnery, 2016). The retinal ganglion cell layer has also been linked with particular susceptibility to ROS induced damage, with evidence not only from LHON patients but also from glaucoma, another disease with preferential loss of RGCs (Ung et al., 2017) where one of the earliest sign of RGC degeneration is a burst in ROS production (Liu et al., 2007; Masuda, Shimazawa, & Hara, 2017).

Reactive oxygen species production is certainly part of normal physiological cellular processes, and there is ample evidence showing that a certain level of ROS production may even be beneficial to the organism at large (e.g. (Scialò, Fernández-Ayala, & Sanz, 2017)). On the other hand, there is clear data corroborating increased ROS levels with disease pathology in an array of disorders (e.g. (Ung et al., 2017)) including ocular diseases. These data suggest that a decrease in ROS in these ocular diseases could be a protective measure.

1.6. Mitochondrial Remodelling

1.6.1. Mitochondrion or Mitochondria?

Mitochondrial fission and fusion are tightly regulated processes, that are constantly ongoing in cells. *In vitro* imaging studies have shown that fission and fusion can happen within seconds (Dalmasso, Marin Zapata, Brady, & Hamacher-Brady, 2017). This process allows mixing of mitochondrial contents and maintenance of a contiguous mitochondrial population in the cell. It has been demonstrated that mitochondria with increased interconnection of mitochondria (more networked) are more efficient at producing ATP via aerobic respiration (Mishra & Chan, 2016; Westermann, 2012). The increased network of mitochondria can more efficiently share imported metabolites as well as mtDNA gene products. Forcing cells to grow in conditions where aerobic forms of ATP production are favoured, such as growth in galactose containing media, induces an elongation of the mitochondrial network (Mishra & Chan, 2016). It also acts as a method to normalise mitochondrial dysfunction and mutation accumulation throughout life, to attempt to prevent a specific sub-population of accruing high levels of dysfunction, a process known as “functional complementation”. It has been shown that blocking mitochondrial fusion leads to mtDNA depletion (Chen et al., 2010; Meyer, Leuthner, & Luz, 2017). The process of fission also plays a role in mtDNA maintenance where highly dysfunctional portions of the mitochondrial network are selectively fissioned, allowing them to be isolated and targeted for decommissioning via mitophagy. An interesting study modulated parameters involved in fission, fusion and mitophagy pathways and monitored the fate of mtDNA mutations in these cells. It appears there is a ‘quasi-arms race’ that occurs where the mitochondria are restructured to isolate the dysfunctional mitochondria whilst minimising fusion of mutant mitochondria with non-mutated mitochondria which would lead to the propagation of the mutated mtDNA, leading to the notion of a fission/fusion trade off (Tam, Gruber, Halliwell, & Gunawan, 2015).

This is also presumably the route of clonal expansion of heteroplasmic mutations. Mitochondrial fission and fusion is also required for trafficking of mitochondria along axons away from the soma towards the synapses and vice versa (Sheng & Cai, 2012). Recent work has outlined mitochondrial fission and transport along the axon back to the soma as a signalling mechanism between the synapses and the cell body in astroglia (Göbel, Motori, & Bergami, 2018). Fission of course is also necessary for the compartmentalisation of mitochondria for cell divisions as well (Mishra & Chan, 2014).

Mitochondria also remodel in response to cellular stressors. This can actually occur in both directions, with mitochondrial stress causing either hyper-fusion of the mitochondrial network or fragmentation. For instance ultra-violet (UV) damage, certain chemical stressors and nutritional deprivation can cause mitochondrial remodelling (Meyer et al., 2017). This is presumably to allow the cell to try and maximise bioenergetic fitness by increasing OXPHOS to maximise ATP production. However there appears to be a fine line when it comes to regulating this process, as hyper-fusion in times of mitochondrial dysfunction would be detrimental as mitophagy relies on fission to be able to act (Pryde, Smith, Chau, & Schapira, 2016). As such when sufficient dysfunction arises, AMPK activation phosphorylates mitochondrial fission factor (MFF) which increases the rate of fission (Herzig & Shaw, 2018). High levels of fission can facilitate cytochrome C release and subsequently apoptosis due to the cleavage of pro-fusion proteins and subsequent loss of mitochondrial membrane potential (A. M. van der Bliek, Shen, & Kawajiri, 2013). Reactive oxygen species (ROS) can initiate mitochondrial fission, where low concentrations initiate mitochondrial fission but higher quantities are cytotoxic to myofibers (Fan, Hussien, & Brooks, 2010). It is possible that the hormetic response to low-level ROS may involve mitochondrial fission activating mitophagy pathways, thereby acting as extra quality control for the mitochondrial network at large.

An increase in network fragmentation is correlated with disease. In a study of muscle fibres from patients suffering from mitochondrial myopathies there was a 3.6-fold increase in the proportion of mitochondria considered to be “simple” in patients from 10% to 46% of total mitochondria (simple here meaning below the 10th percentile of mitochondrial morphological complexity in the control population) (Vincent *et al.*, 2019). This would indicate a more fragmented mitochondrial network in these patients, who were suffering from a range of common mitochondrial myopathies (mtDNA deletions, MERRF and MELAS). No differences were found in mitochondrial volume between patient samples and controls, ruling out a difference in mitochondrial biomass being the the reason for an increase in simplistic mitochondria. This suggests that the absolute number of mitochondria in a cell may be uninformative, and it is the network morphology that has a bigger impact on the phenotype of the cell. In Figure 1-7 we can see the mitochondrial networks of two mouse fibroblast cells stained using mitotracker orange. The cell in A shows a highly elongated tubular network, a sign of robust mitochondrial bioenergetics whereas the cell in B shows a fragmented network, a sign of dysfunction and potentially the beginning of apoptosis.

A brief overview of the key proteins involved in mitochondrial network remodelling as well as their link to ocular disorders is provided below.

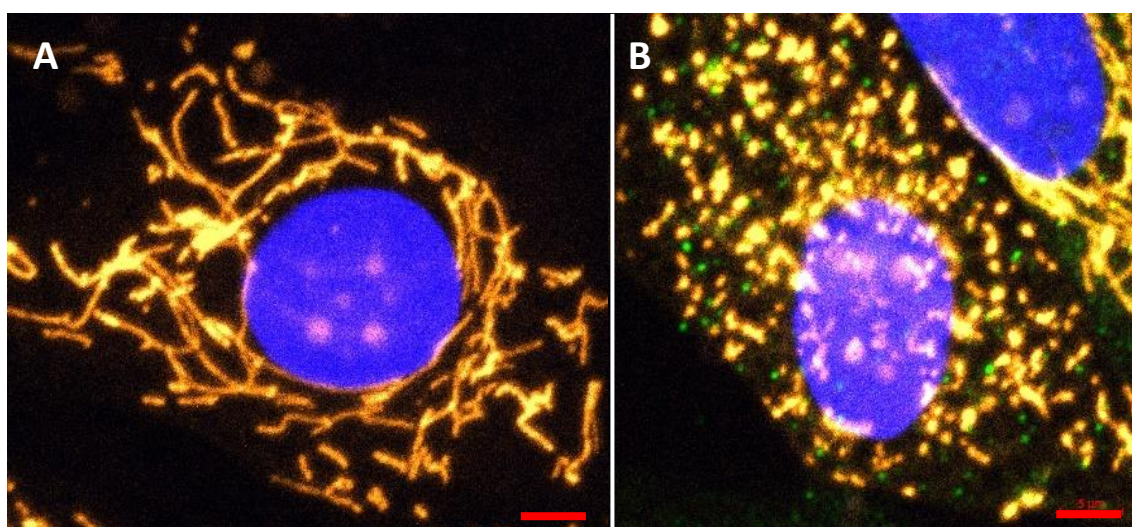


Figure 1-7: Mouse fibroblast cells with the mitochondria stained using mitotracker orange. A) the cell has a highly elongated tubular network, a sign of bioenergetic health, whereas the cell in B) has a highly fragmented network, indicative of poor mitochondrial bioenergetics and dysfunction. Scale bar in red is 5µm.

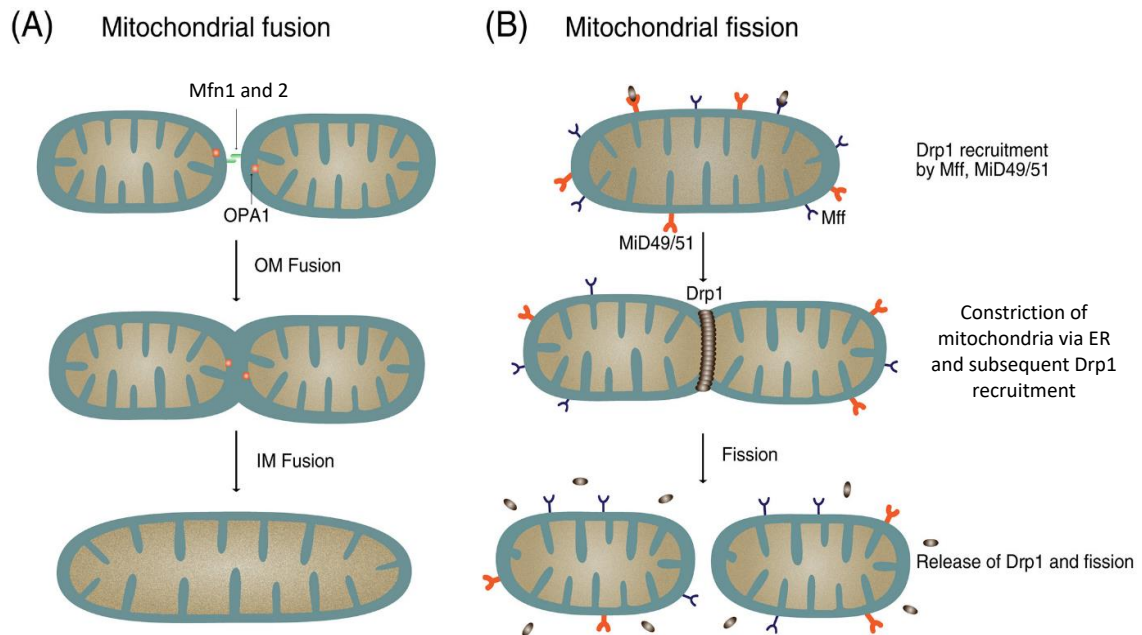


Figure 1-8: Schematic of mitochondrial fission and fusion. A) During mitochondrial fusion, the presence of Mfn1 and 2 acts to tether together two mitochondria. The formation of a “docking ring” where the two opposing mitochondrial outer membrane touch precipitates the fusion of the two outer membranes. The presence of OPA1 then allows the fusion of the inner membranes. B) Fission of the mitochondria is mediated by the recruitment of Drp1 to the outer mitochondrial membrane. When the endoplasmic reticulum constricts the mitochondrial tubule a ring of Drp1 protein can then encircle the tubule. Activation of Drp1 GTPase causes this ring to constrict and pinch apart the mitochondrial tubule, resulting in fission. Mfn – mitofusin, OM – outer membrane, IM – inner membrane, ER – endoplasmic reticulum. Adapted from (Chandhok et al., 2018).

1.6.2. Fission

Drp1, encoded by the *Dnm1l* gene, is responsible for fission of the mitochondrial network (Smirnova, Shurland, Ryazantsev, & van der Bliek, 1998). It is a cytosolic protein that localises to the outer mitochondrial membrane (OMM) to promote fission. Drp1 has multiple isoforms which lead to either short or long forms of Drp1 protein, which differ in activity levels (Macdonald et al., 2016). Drp1 recruitment to the mitochondria is mediated by a number of OMM-anchored receptor proteins such as mitochondrial fission factor (Mff) amongst others, which appear to play roles in different fission inducing responses (Gandre-Babbe & van der Bliek, 2008). Deletion of Mff causes a drastic decrease in mitochondrial fission levels and leads to a highly interconnected ‘net-like’ network in mutant cells, due to inefficient recruitment of Drp1 protein (Losón, Song, Chen, & Chan, 2013). Once recruited to the OMM Drp1 protein forms

a ring around the mitochondrial tubule to undergo fission, where subsequent GTP hydrolysis contracts Drp1 protein and tightens the ring, effectively pinching apart the mitochondrial tubule (Lackner et al., 2009). It appears that initial formation of the Drp1 ring around the mitochondria is mediated by the endoplasmic reticulum (ER) looping around the mitochondria and constricting the mitochondrial tubule to a size amenable to Drp1 collar formation (Friedman et al., 2011). It is unclear how the recruitment of these ER tendrils to the mitochondria is enacted, but it appears to be mediated by two proteins involved in actin recruitment, cofilin1 and Spire1C, with Spire1C initiating ER constriction of mitochondrial tubules and cofilin1 dispersing them (Manor et al., 2015; Rehklau et al., 2017). However, these studies focused on the structural aspects of these two proteins and did not comment on changes regarding metabolic dynamics in the cell.

Drp1 long isoforms have been shown to have reduced fission activity and are predominantly expressed in neuronal tissue (Macdonald et al., 2016; Uo et al., 2009). This suggests that fission is somewhat regulated against in neuronal tissue, favouring the more active tubular mitochondrial network morphology.

1.6.3. Fusion

Mfn1 and Mfn2 are localised to the outer mitochondrial membrane (OMM) and are part of the machinery that regulates the process of mitochondrial fusion. The Mfns can interact in either homotypic or heterotypic arrangements to promote fusion (Chen et al., 2003). However, knockouts of either Mfn1 or Mfn2 produced different phenotypes; Mfn1 knockout cells had uniformly fragmented mitochondria, whereas Mfn2 knockout cells had fragmented mitochondria of varying sizes, some of which were larger than mitochondrial tubules in wt cells (Chen et al., 2003). This highlights that despite homotypic interactions being possible, individually, mitofusin proteins are insufficient to rescue mitochondrial network morphology alone. Subsequent work has also shown that Mfns need to be present on both mitochondria for

them to fuse (Koshiba et al., 2004). If Mfn KO cells are hybridised to wt cells, then no fusion of the opposing mitochondria occurs. However, if either Mfn1 or 2 is returned to the KO cell line then they can fuse with the wt mitochondria again (Koshiba et al., 2004). Mfn1 and 2 have a similar structure, with the main action happening as a result of a 'dimeric antiparallel coiled coil', a shape that looks remarkably similar to two corkscrews fighting each other, forming between Mfn proteins on opposing mitochondrial outer membranes (Koshiba et al., 2004). Once Mfns interact *in trans* the two mitochondria are deemed to be tethered. Incredible cryo-electron microscopy images by Brandt et al., (2016) have helped elucidate the steps involved in mitochondrial outer membrane fusion. Initially two mitochondria come into close proximity with one another (Koshiba et al., 2004 estimated the length of the coiled coil in Mfn1 to be $\sim 95\text{\AA}$, Brandt et al., (2016) estimated the distance between membranes to be $\sim 60\text{\AA}$ at the time of fusion) and are tethered to each other through the action of the Mfn proteins. GTP hydrolysis causes a conformational change in the Mfn proteins, pulling the two mitochondrial outer membranes into close proximity (Lee & Yoon, 2016). The two mitochondria now have a large surface area in contact with one another and are deemed to be 'docked'. A 'docking ring' of proteins forms around this junction, thought to be a number of small GTPases. Formation of the initial fusion pore occurs near the periphery of the docking ring, where the density of the docking ring starts to diminish (Brandt et al., 2016). This then leads to full fusion of the outer membranes, as the docking ring disperses.

Over expression of Mfn2 in HeLa cells causes mitochondria to aggregate in peri-nuclear clusters (Rojo, Legros, Chateau, & Lombès, 2002). This clustering occurs when the GTPase domain is deleted. This would suggest the mitochondria are tethered together like during the preliminary stages of mitochondrial fusion, rather than forming a hyper-fused network. Analogous to mitochondria having too much 'Velcro' on their surfaces, causing the mitochondria to amass.

This of course is just outer membrane fusion, in order to fuse the inner membranes Optic Atrophy 1 (OPA1) is required. OPA1 is a dynamin-related GTPase found located to the inner mitochondrial membrane (IMM). The mature OPA1 protein has a transmembrane domain that anchors it to the IMM with the majority of the protein extending to the inter membrane space (Aurélien Olichon et al., 2002). It can then undergo post-translational cleavage to produce either long or short versions of the isoform (more detail on this will be provided in Chapter 4), both of which are required for fusion of the IMM (Lee & Yoon, 2016). OPA1 is predicted to be similar in 3D structure to Drp1 which is interesting given the opposing roles they play in fission and fusion, and as such is believed to assemble in a spiral pattern around the membrane to promote the tubular structure of the mitochondria (Ferguson & De Camilli, 2012). The precise structural interactions between OPA1 protein versions to promote IMM have not yet been fully elucidated, but it is known that only one membrane needs to have OPA1 expression in order for fusion to occur (Song, Ghochani, McCaffery, Frey, & Chan, 2009). This means if an OPA -/- cell and a wild type cell were to be hybridised they would be able to fuse inner mitochondrial networks, which is in contrast to the mitofusins, as mentioned above. This study also showed that the steps in mitochondrial fusion occur sequentially and independently, with mitofusin mediated docking occurring first with subsequent OMM fusion happening in OPA -/- cells, but without OPA mediated IMM fusion the mitochondrial network eventually fragments (Song et al., 2009).

1.6.4. Mitophagy

In the context of mitochondrial remodelling mitophagy represents another important process, and moreover, has increasingly been implicated in ocular diseases such as LHON and AMD (Hyttinen, Viiri, Kaarniranta, & Błasiak, 2018; Sharma, Tiwari, Rai, & Bai, 2018). Mitophagy is the process of “mitochondrial autophagy”, where aged and dysfunctional mitochondria are

targeted for degradation via lysosomes. This process plays an important role in maintaining cellular homeostasis (Rodger, McWilliams, & Ganley, 2018). For instance, mitochondria that have lost their membrane potential, are producing excessive ROS, have impaired mitochondrial dynamics, can be specifically targeted for mitophagy which allows their clearance and stops any potential negative effects they can cause to the cell at large (McWilliams & Muqit, 2017). As mentioned earlier with regard to the fusion fission trade-off in cells, fission allows the isolation of dysfunctional mitochondria and their subsequent mitophagy (Tam et al., 2015). This in turn can prevent the clonal expansion of mtDNA mutations, as long as these mitochondria can be targeted for degradation before fusion allows the propagation of these mutations throughout the general mitochondrial population of the cell.

The most well-known players in mitophagy are PINK1 and Parkin, which have been implicated in cases of early onset familial parkinsonism (Kitada et al., 1998; E. M. Valente et al., 2004), where mutations disrupt the ability of these cells to target mitochondria for mitophagy. Under normal physiological conditions PINK1 levels remain low as it is targeted for cleavage continuously in the mitochondria, until it is stabilised by binding to the OMM of depolarised mitochondria. Once stabilised on the OMM it recruits Parkin which acts to ubiquitinate mitochondria targeted for degradation. A phagosome then forms around these mitochondria which are then destroyed (McWilliams & Muqit, 2017; Rodger et al., 2018).

1.6.5. Mitochondrial remodelling proteins in ocular disease

As the processes of fission and fusion are opposing, dynamic processes key to the metabolic fitness of the cell, it is easy to imagine significant disruption of these processes would lead to disease. Acquired dysfunction to mitochondrial dynamics have been implicated in a wide array of diseases, or at least perturbed mitochondrial dynamics have been observed in diseases as varied as hypertension, obesity, cancers, ischemia reperfusion injury, Alzheimer's Disease and

Diabetes (Archer, 2013; Higgins & Coughlan, 2016). However, there is a smaller selection of diseases directly associated with mutations in the mitochondrial remodelling proteins mentioned above.

MFN2 is specifically associated with Charcot-Marie-Tooth disease type 2A (CMT2), which accounts for roughly 20-30% of all cases of CMT2 (Tan et al., 2016). CMT2 is relatively common, with an estimated prevalence of $\sim 1/7500$ (Meyer et al., 2017). Mutations are almost exclusively dominant, but there are rare cases of compound heterozygous and homozygous mutations following a recessive pattern of inheritance. In contrast, thus far to my knowledge MFN1 mutations have not been associated with disease. As MFN1 is expressed constitutively throughout the body, and to a higher degree than MFN2, it is conceivable that MFN1 mutations are embryonic lethal, a notion that is backed up by knock-out studies in animals (Filadi, Pendin, & Pizzo, 2018). In contrast to this, MFN2 protein expression appears to dominate in neural tissue (Kawalec, Zabłocka, Kabzińska, Neska, & Beręsewicz, 2014). Indeed, deleting MFN2 from neurons leads to erroneous trafficking of mitochondria, which correlates with the peripheral neuropathy experienced by patients with CMT2 (Chandhok, Lazarou, & Neumann, 2018). Patients with CMT2 can often show signs of optic atrophy comorbidly, but this is not always seen and has not been shown to be the only clinical manifestation of a patient with MFN2 mutations (Filadi et al., 2018), suggesting an increased severity of phenotype compared to OPA1 mutations.

Mutations in DRP1 have been described that cause disease, but these are comparatively rare, with only a handful of family specific mutations having been described that typically cause Encephalopathy due to defective Mitochondrial and Peroxisomal Fission-1 (EMPF1), a disease that causes microcephaly, abnormal brain development and lactic acidosis and typically leads to death at a young age (Sheffer et al., 2016; Vanstone et al., 2016; Yoon et al., 2016). These cases were all caused by *de novo* heterozygous mutations. Interestingly from the point of view

of the current study is the recent discovery of 3 unrelated families who had dominant optic atrophy (DOA) due to mutations in *DRP1* (Gerber et al., 2017). This is a very interesting finding as it implies that RGCs are at risk from both too much (*DRP1* mutations) and too little (*OPA1* mutations) mitochondrial fusion, highlighting the sensitive balance needed in healthy cells.

OPA1 of course is named after the condition in which it is implicated, optic atrophy, which will be the focus of chapter 4. *OPA1* mutations correspond to roughly 75% of patients with DOA, a disease which leads to the specific loss of RGCs causing blindness, with an estimated prevalence of between 1-10,000 to 1-30,000 (Lenaers et al., 2012; Patrick Yu-Wai-Man et al., 2010). Mutations in *OPA1* lead to fragmentations of the mitochondrial network due to the inability of cells to correctly fuse their IMM. A proportion of patients (~20%) harbouring *OPA1* mutations go on to develop DOA plus, which is a syndromic disorder involving multi-systemic features, peripheral neuropathy and more acute vision loss (P Yu-Wai-Man, Bailie, Atawan, Chinnery, & Griffiths, 2011). It is interesting to consider the *MFN2* OMM fusion dysfunction leading to peripheral neuropathy and sometimes an ocular atrophy phenotype with *OPA1* IMM fusion dysfunction leading to ocular atrophy and sometimes a peripheral neuropathy phenotype.

The role of mitophagy in ocular pathologies has been highlighted in recent studies of LHON (Sharma et al., 2018; J. Zhang, Ji, Lu, et al., 2018). Mitophagy is significantly compromised in cybrid cell models containing different canonical LHON mutations. When mitophagy is induced pharmacologically in these cells there is a significant decrease in mitochondrial defects associated with LHON due to selective clearance of damaged mtDNA (Sharma et al. 2018). As the cybrid lines used were homoplasmic for causative mutations, it would be interesting to see if an increase in mitophagy in heteroplasmic tissue might cause an enrichment for mitochondria containing wt mtDNA. In the AMD retina, there is an accumulation of mtDNA damage in RPE cells. However, it seems that neural retina may not suffer mtDNA damage to the same extent as RPE (Terluk et al. 2015). These observations raise two possibilities; in wt photoreceptors there

may be a higher rate of mitophagy than in RPE cells that keeps mtDNA damage in check, and that increasing mitophagy may be an avenue for AMD therapies (Hyytinen et al. 2018).

Evidence suggesting mitophagy as a key player in neural protection has been provided by Parkinson's disease, where dysregulation of PINK1/Parkin-mediated mitophagy is linked to disease aetiology (Bingol et al., 2014; Harper, Ordureau, & Heo, 2018). For example, *Drosophila* treated with paraquat (a superoxide producer) showed protection of neurons when Parkin-mediated mitophagy proceeded due to knockdown of USP30. USP30 is a mitochondrially targeted deubiquitinase that acts as a checkpoint for mitophagy, preventing mitophagy until a requisite level of dysfunction and therefore ubiquitination is reached (Ganley, 2018). Given that some of the same molecular machinery is expressed in retina, the therapeutic potential of modulating mitophagy in retinal cells is being explored (Huang et al., 2018).

Recent work has indicated that epithelial-to-mesenchymal transition (EMT) may play a role in AMD and that it is linked to mitochondrial damage and mitophagy in RPE cells (Guerra et al. 2017). EMT has been thoroughly investigated in cancer metastasis and has been associated with mitochondrial dysfunction (Guerra et al. 2017). A correlation has been shown between low mtDNA content and expression of EMT-related genes (*ESRP1*, *SNAI1* and *TGFB1*) (Weerts et al. 2016). In AMD, an association between dysfunctional lysosome clearance and EMT has been established (Ghosh et al. 2018). β A3/A1-crystallin deficiency due to *Cryba1* loss of function caused murine RPE cells to undergo type 2 EMT. β A3/A1-crystallin had been shown previously (Valapala et al. 2014) to be essential for normal lysosomal processes such as autophagy, and therefore mitophagy and hence potential links can be drawn between impaired mitophagy and EMT in AMD. The incomplete clearing of lysosomes also leads to a build-up of lipofuscin in RPE, a classic marker for AMD (Hyytinen et al. 2018). Although the linkages between the control of mitophagy and retinal disease are just emerging, it is likely to be a growing field that will reveal novel therapeutic targets in the future.

1.7. Diseases of Interest

This thesis focuses on novel therapeutic interventions for a number of ocular diseases which will be introduced here.

1.7.1. LHON: Epidemiology

Leber Hereditary Optic Neuropathy (LHON) is often considered the archetypal mitochondrial disease it is worth discussing some of its particulars and peculiarities. LHON was first identified by the German physician Leber in the late 19th century. It is a maternally inherited disease that causes progressive vision loss. LHON is considered to be one of the most prevalent mitochondrial disorders, affecting somewhere in the region of 1:30,000 to 1:50,000 people (Sadun, Morgia, & Carelli, 2011). LHON is thought to be under-diagnosed which, coupled with incomplete penetrance, suggests the proportion of mutation carriers may be closer to 1:7000 people (Douglas C. Wallace & Lott, 2017).

LHON manifests during the second or third decade of life, with a median age of onset in the early twenties, depending on the specific mutation the patient possesses. It is characterised by painless, subacute visual loss that occurs bilaterally but typically with a staggered onset of symptoms of up to six months between eyes (Patrick Yu-Wai-Man & Chinnery, 2016). This staggered onset of disease between the two eyes offers a unique opportunity for therapeutic intervention. As the occurrence of unilateral LHON is very rare (Patrick Yu-Wai-Man & Chinnery, 2016), it would be possible to prophylactically deliver a therapeutic to the contralateral eye. Visual function decreases over the course of several weeks to levels around 20/200, but complete blindness as a result of LHON is rare (McClelland, Meyerson, & Van Stavern, 2015).

LHON sufferers have a characteristic central scotoma accompanied by Retinal Nerve Fibre Layer (RNFL) cell death. Retinal Ganglion Cells (RGCs) show particular susceptibility to death in LHON

patients. As RGCs conduct the visual signals from the photoreceptors towards the optic nerve, sufficient damage causes an interruption in signal transduction towards the optic nerve resulting in blindness whilst the photoreceptors remain fully functioning. The RGC layer is believed to be most vulnerable to mitochondrial dysfunction due to its morphology. Myelin is a lipid rich tissue that surrounds nerve axons, effectively insulating them and allowing electrical signals to propagate more easily along the axon. Being a fatty tissue however, myelin is opaque and as such it would be detrimental to have it covering the retina; there are human disorders where myelination of these RGCs causes visual impairment (X. Yang et al., 2013). This unmyelinated portion of the RGC axon requires significant amounts of energy to propagate an action potential and as such has a high energy demand, a fact borne out by the higher distribution of mitochondria in the unmyelinated portion of the RGCs than in the myelinated portion within the optic nerve (Fraser, Biousse, & Newman, 2010). Some mammals, such as the rabbit, have RGC myelination in the retina. It would be interesting to see how susceptible their RGCs are to complex I insults.

1.7.2. LHON: Genetics

Initially, LHON was erroneously thought to be X-linked due to its inheritance pattern. It was not until 1988 that a genetic basis for LHON was identified. The 11778G > A mutation causing LHON was the first disease causing mitochondrial mutation to be identified (D C Wallace et al., 1988), and is now colloquially referred to as the “Wallace mutation”. In the European population, 3 mutations account for between 90-95% of all LHON cases; 3460G > A, 11778G > A and 14484T > C (Theodorou-Kanakari et al., 2018). 11778G > A on its own accounts for roughly 70% of cases. It is interesting to note that the mutational breakdown is different in different populations. For instance, around 90% of LHON patients in Asian countries such as Japan, China and Thailand possess the 11778G > A mutation with the other two common European mutations being considerably rarer (Manickam, Michael, & Ramasamy, 2017). This is quite likely due to the different mtDNA haplogroups that are most common in each of the populations in question for

two reasons. First, at least one of the ten most common pathogenic mtDNA mutations (all three of the main LHON mutations are on the list) is believed to occur *de novo* in around 1:200 live births (Elliott, Samuels, Eden, Relton, & Chinnery, 2008) so it is unlikely to be a founder effect causing the shift in frequency between the populations. Second, we know that the different LHON mutations show different penetrance depending on the haplogroup they are found on, for instance 3460G > A is associated with the Uk haplogroup in Europe. This haplogroup is very rare in Asian populations, so the frequency of the mutation could still be the same in both populations, but less likely to manifest due to the difference in haplogroups (see the section on haplogroups for more information).

In total there are now at least 18 mtDNA mutations associated with LHON, which have been found in all of the 7 complex I ND subunits encoded for by the mitochondrial genome (Douglas C. Wallace & Lott, 2017). It is interesting that no mutations causing LHON have been discovered in the numerous CI subunits encoded for by the nuclear genome thus far. As mentioned previously CI is a huge multimeric protein complex with around 44 subunits (Balsa et al., 2012). Of these subunits there are 14 core subunits, that play key roles in the enzymatic activity of CI, and 30 supernumerary subunits that help with assembly, stabilization and location of the mature complex I (Vinothkumar et al., 2014). A search through the Online Mendelian Inheritance in Man (OMIM; <https://www.omim.org/>) database shows no correlation with any of the non-mitochondrial CI subunits and LHON. It is fun to speculate as to why this is the case. Mutations in nDNA CI subunits are often associated with catastrophic disorders such as Leigh syndrome and other Complex I Deficiency Disorders, which usually have a very poor prognosis for the affected, with 75% of the affected dying before the age of 10 (Rodenburg, 2016). By comparison, LHON causing mutations have a milder phenotype, apart from rare cases where LHON associated mtDNA mutations such as the 11778G > A present at homoplasmic levels causing Leigh syndrome, but these appear to happen only when a sufficiently sensitised genetic background is present (Fruhman et al., 2011; Paquay et al., 2014), the prognosis is substantially

better for patients with LHON. The nDNA CI disease causing mutations are autosomal recessive, but have been identified as compound heterozygotes (Rodenburg, 2016), indicating a loss of function. So, with this in mind, it appears that the reason nDNA CI mutations do not cause LHON may be due to the severity of the mutations identified in these nuclear genes. Disease manifestation for nDNA mutations only occur when homozygous, where there is a major change in function. On the other hand, mtDNA mutations causing LHON can have a broad range of mutational load due to heteroplasmy, allowing for more subtle phenotypes to be seen. Also, any nDNA mutations will be present to the same frequency throughout the individual, barring any mosaicism, whereas mtDNA heteroplasmy levels are different depending on the tissue further adding to the modulation of the phenotype. Of course, there is no reason why nDNA CI subunit polymorphisms cannot modulate the overall sensitivity of CI to typically less damaging mtDNA mutations, and perhaps this is part of the reason behind the incomplete penetrance of LHON. Useful insight could be gained by sequencing the nDNA encoded CI subunits of LHON families with widely variable phenotypic expression. It is also worth bearing in mind that any truly catastrophic mutations of complex I would be embryonic lethal due to the integral role it plays in energy production, so all of these disease-causing mutations must have more subtle effects on the overall efficiency of complex I.

One of the most enduring questions about LHON is that of its variable penetrance and sexual dimorphism. Each of the primary mutations has a unique male:female ratio (see Table 1.3). Most remarkably these all show a skew towards male penetrance (Manickam et al., 2017). Even when the mutations are homoplasmic throughout a family only some 50% of males and 10% of females go on to develop LHON (Caporali et al., 2017).

Table 1-3: The most 3 most common LHON mutations as well as some selected biochemical and penetrance information for each. OCR: Oxygen Consumption Rate Data from (Abu-Amero, 2011; Douglas C. Wallace & Lott, 2017; Patrick Yu-Wai-Man & Chinnery, 2016)

Mutation	Gene	CI activity (% of Control activity)	OCR (% of Control Activity)	Male:Female Ratio	Penetrance in males (homoplasmic)
3460G > A	ND1	20-40%	65-70%	3:1	40-80%
11778G > A	ND4	50-100%	50-70%	6:1	82%
14484T > C	ND6	35-100%	80-90%	8:1	68%

1.7.3. LHON: The Male-Female Divide

It is unclear as to what causes this sexual dimorphism in patients. There is evidence that maternal relatives of LHON patients have some level of visual impairment such as alterations in electroretinogram (ERG) responses (Salomão et al., 2004) but there is no progression to the LHON disease phenotype. The first place to look when sexual dimorphism occurs is of course the sex chromosomes. Some linkage studies have implicated regions on the X chromosomes (Yanli Ji et al., 2010; Shankar et al., 2008). There are two supernumerary CI subunits located on the X chromosome which one would imagine would be prime targets for nuclear modifier genes, but a large study of Italian LHON sufferers found no evidence for a link between NDUFB11 expression and LHON penetrance (Petruzzella et al., 2007). The logic behind there being a recessive modifier on the X chromosome causing the gender skew would dictate that females would only be susceptible if the modifier were homozygous or if there were skewed X inactivation in the affected. The possibility of skewed X-inactivation has been investigated with little evidence so far to support it, even in post-mortem retinal and optic nerve tissue (Hudson, Carelli, Horvath, et al., 2007; Pegoraro et al., 2003). In contrast to this, a number of autosomal genes have been linked to LHON progression, which will be discussed further on. Currently X-linked modifiers seem unlikely to play a big role in the variability between sexes.

Intriguing links have been drawn between LHON penetrance and female hormones. Oestrogen is believed to be protective via two mechanism; it causes the upregulation of superoxide

dismutase (SOD) which is a potent ROS scavenger (Unfer et al., 2015), and it is also believed to directly scavenge ROS to some extent (Fidarov et al., 2015). Oestrogen is known to bind to mitochondria where it increases MnSOD expression which is a potent anti-apoptotic factor. Subsequent to this, studies of cybrid cells harbouring 11778G > A showed that treatment with a number of phytoestrogens (plant-based Oestrogen analogues⁴) rescued OCR to normal levels and reduced ROS levels with a concomitant up-regulation of a number of protective mitochondrial genes (Pisano et al., 2015). In addition to this, 17 β -oestradiol treatment of LHON cybrids led to an increase in mitochondrial biogenesis, demonstrating another putative protective factor (discussed further below)(C. Giordano et al., 2011). A caveat to this is that if oestrogen is preventing LHON in female mutation carriers, one might expect there to be a significant increase in LHON cases in post-menopausal women whose oestrogen levels naturally drop. A study of late-onset LHON cases found no difference in the sex bias compared to patients with a more typical age of onset (Dimitriadis et al., 2014).

1.7.4. LHON: The Variable Penetrance Conundrum

The link between LHON and certain environmental risk factors may also be influential in determining penetrance. Multiple studies have found that amongst carriers of the three common LHON mutations, increased alcohol and tobacco consumption can double the likelihood of disease (Kirkman et al., 2009; Sadun et al., 2003). It is not just tobacco smoke, but also smoke from fires in general, that can increase the chance of LHON development (Sanchez, Smith, Carelli, Sadun, & Keltner, 2006).

⁴ Due to the similarities of phytoestrogens to oestrogen, and their high prevalence in soy-based foods, a staple of the modern American diet, there is a wonderful conspiracy theory that supposes that consumption of these foods is leading to the mass feminisation of the male population, aka soy-boys. Unsurprisingly, this has recently been debunked (Segovia-Siapco, Pribis, Oda, & Sabaté, 2018).

Drugs that have mitochondria-related side-effects that alter ETC bioenergetics, such as chloramphenicol, have also been associated with influencing disease penetrance (Fraser et al., 2010). These data suggest that mitochondrial health exists in a fine balance, with insults that are normally well tolerated by healthy individuals potentially causing significant dysfunction in LHON carriers.

Another potential factor in the incomplete penetrance of LHON is the mitochondrial content of the cells themselves. We know that not all people who have an LHON mutation, even at homoplasmic levels, experience loss of vision (Caporali et al., 2017). Studies of mtDNA copy number (used as a surrogate for mitochondrial biomass) between affected, carriers and non-carriers show marked differences. A large study of mtDNA copy number of white blood cells in LHON families showed that non-carriers had the lowest mtDNA copy number, next were the affected, with carriers having the highest mtDNA copy number; roughly double the amount of affected and nearly three times that of non-carriers (C. Giordano et al., 2014). This finding suggests there is a compensatory expansion of the mitochondrial biomass in order to counter the dysfunction caused by the primary LHON mutation. This scenario holds true for the three most common LHON mutations (Bianco et al., 2018; C. Giordano et al., 2014). Recently it has been found that carriers with heteroplasmic mutations also have increased mtDNA copy number compared to heteroplasmic affected people (Bianco et al., 2017). There is no clearly identified mechanism behind this compensatory increase in mtDNA copy number. There is compelling evidence linking defects in mitochondrial biogenesis with diseases, particularly in neurodegenerative diseases (Golpich et al., 2017). However there has been no robust genetic evidence linking any SNPs in some of the genes most commonly associated with mitochondrial biogenesis such as TFAM and PGC-1 α with LHON so far (Caporali et al., 2017; C. Giordano et al., 2014). Data from Giordano *et al.*, 2014 showed that increase in mtDNA copy number came concomitant with an increase in expression of a number of mitochondrial proteins. We can contrast this with a number of recent studies that have implicated two separate mitochondrial

tRNA mutations with LHON (Yanchun Ji et al., 2017; J. Zhang, Ji, Liu, et al., 2018). These studies both showed a number of unrelated families that have LHON whilst lacking common primary LHON mutations. Cybrids generated with the 15927G > A mutation showed decreases in mtDNA encoded proteins with ND1 showing the largest decrease of those tested (J. Zhang, Ji, Liu, et al., 2018).

Genetic studies have also sought to establish a basis for the incomplete penetrance of LHON. As discussed previously, mitochondrial haplogroups can influence the penetrance of LHON (see Haplogroups section). Indeed LHON was the first disease in which the link between haplogroups and disease progression was established (M. D. Brown, Sun, & Wallace, 1997). There is also evidence for non-haplogroup associated mtDNA variations in CI subunits being associated with LHON (Jiang et al., 2016) and even other ETC complexes such as CytB of CIII (K. Ji et al., 2014). Beside the above, genetic modifiers of LHON remain elusive. There is conflicting evidence associating SNPs in PARL, an inner mitochondrial membrane protein involved with the PINK1 mitochondrial quality control pathway, with LHON penetrance. Two studies have shown PARL SNPs to be enriched in patients from an Italian-Brazilian family and Thai patients in general (C. Giordano et al., 2014; Istikharah et al., 2013), whereas there is no association with Chinese patients (A.-M. Zhang, Jia, Zhang, & Yao, 2010). Interestingly these were separate groups of SNPs, with the Thai family's being intronic and the Italian family's being in the promoter region. A severe loss of function mutation in PARL has previously been associated with PD, suggesting an interesting link between the two disorders, with minor dysfunction possibly sensitizing for bioenergetic defects and major mutations causing catastrophic neuronal damage (Shi et al., 2011).

Despite the aforementioned mtDNA mutations that exacerbate LHON dysfunction, the question of whether primary LHON mutations work in a synergistic matter is also up for debate. Two studies offer interesting examples. Firstly, one study found an LHON patient that harbours three

individually pathogenic mutations, 11778G > A, 11253T > C and 14484T > C, on the one mtDNA molecule, a particularly rare occurrence. Cybrid cell studies were utilised, which involve depleting a chosen cell line of its endogenous mitochondria and then repopulating them with mtDNA from a patient in order to isolate the impact of mtDNA without the variation caused by differing nDNA backgrounds. Over a range of biochemical tests, the triple mtDNA mutant cybrids showed no increase in dysfunction when compared to cybrids containing one of the three mutations (Cruz-Bermúdez et al., 2016). A study of a family harbouring both the 11778G > A and 3460G > A mutations also saw no signs of increased disease severity or biochemical defect (Tonska et al., 2008). In contrast to the Cruz-Bermúdez *et al.*, 2016 study, it has been noted that combinations of individually non-pathogenic mutations can cause LHON (Caporali et al., 2018). It is important to note though that the three mutations identified to work synergistically to cause LHON are all on the same subunit of LHON, ND6, whereas 11778G > A and 14484T > C are of course on different subunits.

Amazingly though, in families with both the 11778G > A and 14484T > C mutations at near homoplasmic levels there is a reversal of the sexual dimorphism with 5:1 females to males displaying LHON phenotype (Catarino et al., 2017). This could be a quirk of statistics, as there are only seven families characterised with both primary mutations present at similar levels, but it would be fascinating to tease out the mechanism behind this if it holds true. What is of note however, is that fibroblasts derived from these double hit patients did show significant CI dependent OCR decreases compared to patient fibroblasts with a single homoplasmic mutation (Catarino et al., 2017). This would suggest that these mutations can work synergistically *in vivo* but not in certain cybrid models, underscoring the need for the use of multiple experimental models to obtain a more complete picture.

1.7.5. AMD and mitochondria

In chapter 3 of this thesis the use of the yeast gene Ndi1 as a potential therapeutic intervention for complex visual disorders will be investigated. In particular the potential use of Ndi1 will be investigated in models of Age-related macular degeneration (AMD). AMD is a progressive blinding disease that primarily effects central vision. It is one of the leading causes of blindness in the developed world, affecting 10% of people over the age of 65 and 25% of over 75's (Al-Zamil & Yassin, 2017). There is no clear-cut disease aetiology for AMD. In general the cause of AMD is believed to be mounting dysfunction in the retinal pigment epithelium (RPE) cell layer, associated with photoreceptor degeneration, thickening of the Bruch's membrane with drusen, and damage to the choroidal layer of the eye (Gheorghe, Mahdi, & Musat, 2015). AMD is also broadly categorised as either dry AMD with extensive geographic atrophy or wet AMD displaying neovascularisation of retinal blood vessels, leading to severe disruption of vision (Toomey, Johnson, & Bowes Rickman, 2018). Early AMD shows signs of rod photoreceptor dysfunction, with late-stage more commonly characterised by central scotoma and the loss of central vision (Owsley, Huisinigh, Clark, Jackson, & McGwin, 2016) .

More than 50 genetic loci have so far been associated as risk factors for the development of AMD pathology (García-Layana, Cabrera-López, García-Arumí, Arias-Barquet, & Ruiz-Moreno, 2017), one of the most strongly associated being complement factor H (CFH; this will be discussed in more detail further on). These genes are all risk-factors rather than directly causative mutations.

The exact disease pathogenesis is still debated. One of the earliest clinical signs of AMD is the appearance of drusen between the Bruch's membrane and the RPE cell layer, but increasing evidence suggests that drusen may to some extent be part of normal aging (Khan et al., 2016). Drusen deposit size, along with the loss of pigmentation of the RPE is monitored through fundus imaging and used to characterise the presence and severity of disease progression (Ferris et al.,

2013). The over-arching theory is that RPE dysfunction prevents the correct phagocytosing of photoreceptor outer-segments leading to photo receptor damage and eventual vision loss.

OXPHOS defects have been identified in RPE cells from patients with AMD (Ferrington et al. 2017). A manifestation of this is a significant decrease of the maximal OCR in cells (Ferrington et al. 2017). The use of the Seahorse Extracellular Flux Analyzer allows the examination of a number of mitochondrial related biomarkers. Amongst these is the 'spare respiratory capacity' which is defined as the difference between basal OCR and maximal OCR after mitochondrial uncoupling. Ferrington et al. (2017) showed that despite clear respiratory defects in AMD retinal pigment epithelium (RPE) versus healthy donor RPE, there is still a large spare respiratory capacity in AMD RPE cells. This observation may suggest that these cells in principle should be able to increase their OCR to cope with metabolic stresses. This is in contrast to Kooragayala et al. (2015) who have shown very limited spare respiratory capacity in retinal punches from mice and have hypothesised that the retina's susceptibility to mitochondrial dysfunction is due to an inability to modulate OXPHOS to meet metabolic demands. Given the findings from such studies, there may be therapeutic benefit from investigating how to drive AMD RPE cells towards the utilisation of this spare respiratory capacity.

As mentioned in the introduction, under normal physiological conditions in the mammalian retina, the RPE shuttles glucose from periphery blood vessels to photoreceptors where it undergoes aerobic glycolysis (Hurley et al. 2015). Photoreceptors in turn secrete lactate which the RPE can convert into pyruvate and use to fuel OXPHOS. This creates a metabolic balance in the retina (Kanow et al. 2017). A shift away from OXPHOS towards glycolysis in RPE cells can cause starvation of the photoreceptor layer because these RPE cells start to utilise the glucose that would otherwise have been transported towards the photoreceptor layer. The photoreceptors now produce less lactate as a result, forcing the RPE to rely more heavily on glycolysis for energy (Kanow et al. 2017). In addition to this, Kanow et al (2017) show that

human foetal RPE cells can use lactate as a fuel source and incorporate it into the TCA cycle much quicker than glucose. In light of this, it would be interesting if a study was undertaken to measure the difference in OCR in patient derived RPE cells when they are forced to use lactate as their primary fuel source.

In addition, it has been established that mitochondrial haplogroups can also play a role in AMD penetrance, with haplogroup J, U and T being associated with an increased likelihood of developing the disease (Riazi-Esfahani et al., 2017; Udar et al., 2009). This indicates a direct link between mitochondrial physiology and AMD progression, or at least protection from progression.

So with this in mind, there may be potential for Ndi1-based therapeutics to be of benefit in AMD, not necessarily from a complex I deficiency point of view per se, but via helping maintain levels of OXPHOS in cells which might otherwise start shifting towards glycolysis. If metabolic dysfunction is a key component of AMD, then providing Ndi1 protein to promote OXPHOS could potentially be of benefit to these cells. In order to investigate Ndi1 as a potential therapeutic modality in AMD we decided to investigate its use in the CFH $-/-$ mouse model of AMD. While there are no murine models that completely recapitulate AMD, the rationale underlying the use of this model in this study is detailed below.

1.7.6. AMD: Complement Factor H and the CFH $-/-$ mouse model

There are currently around 50 genetic loci associated with AMD, which are estimated to explain approximately 27% of disease variability and at least 50% of the heritability of AMD (Mitchell, Liew, Gopinath, & Wong, 2018). Amongst these a number of loci involved in the complement pathway have been identified. The complement system is part of the innate immune system and acts to 'complement' the adaptive immune system (Janeway, Travers, Walport, &

Shlomchik, 2001). The complement pathway has three branches, the classical, alternative and lectin pathways and genes in the alternative pathway in particular have been implicated in AMD, namely CFH, C3 and C2 (García-Layana et al., 2017). In particular the complement factor H (CFH) polymorphism Y402H has been implicated in almost 60% of AMD cases at the population level (Thakkinstian et al., 2006). At the risk of over simplifying a highly complicated immune pathway, CFH acts as a negative regulator of the alternative complement cascade. The alternative pathway is driven by the hydrolysis of C3, which either occurs due to the binding of cell surface markers on a pathogen, or spontaneously at a low rate. C3, through a pathway of proteolytic cleavage events, can increase expression of itself in an “amplification loop” (Toomey et al., 2018). Unmitigated C3 activation leads to inflammation due to activation of an immune response, which if left uncontrolled, can lead to inflammatory damage to tissues, which is seen in patients with AMD (Kauppinen, Paterno, Blasiak, Salminen, & Kaarniranta, 2016). The role of CFH is to stop excessive amplification of C3 and the subsequent immune activation by binding to and inhibiting C3. It was originally thought that the Y402H polymorphism causes reduced affinity of CFH for C3, thus improperly regulating the inflammatory cascade, but studies have shown that Y402H CFH regulates C3 just as well as wild-type CFH (Kelly et al., 2010). However it does appear that the Y402H variant is implicated with poorer binding abilities to oxidative stress markers such as malondialdehyde, a marker of lipid peroxidation (Weismann et al., 2011). Further work suggests that this inability to bind oxidative stress markers correctly in turn leads to aberrant activation of microglia resulting in increased oxidative stress in the retina of mice (Aredo et al., 2015). With oxidative stress being linked to disease pathology, we sought to investigate if protecting mitochondrial function via Ndi1 could help alleviate potential oxidative damage in a mouse model of CFH deficiency.

A *Cfh* ^{-/-} mouse model has been generated, on the C57 background, that shows reduced scotopic electroretinograms as well as severe disorganisation of photoreceptor outer segments in aged mice (Coffey et al., 2007), consistent with the idea that CFH plays a role in the retinal

degeneration seen in AMD patients with CFH polymorphisms. Unexpectedly however, these mice showed a thinning of the Bruch's membrane, which is inconsistent with what is seen in patients with AMD. This inconsistency has been challenged in recent years, with other groups demonstrating thickened Bruch's membrane in mice (Toomey, Kelly, Saban, & Bowes Rickman, 2015). Intriguingly it seems that exogenous factors can mediate the variable phenotype seen in the Bruch's membrane and subsequent sub-laminar deposits. When bred in a pathogen free environment this thinning of the Bruch's membrane phenotype is seen, whereas if they are allowed to develop in an open environment thickening of the membrane is observed (Hoh Kam, Morgan, & Jeffery, 2016).

The ERG data for this mouse model in previous studies has been lacking statistical power to potentially elucidate more subtle differences in phenotype due to small numbers of mice tested. Here we sought to investigate the levels of dysfunction at earlier time points than the two years used in the original study (Coffey et al., 2007), to try to better model the progression of dysfunction over time in these animals.

The *Cfh* ^{-/-} mouse model has been linked with mitochondrial dysfunction previously, through decreased ATP levels in aged *Cfh* ^{-/-} mice compared to aged matched C57 mice (Calaza, Kam, Hogg, & Jeffery, 2015), however as visual acuity was not simultaneously tested this could be due to cell death resulting in decreased ATP levels across the retina. The levels of reactive oxygen species had not been assessed in these mice previously, and hence in the current study we sought to investigate this in *Cfh* ^{-/-} mouse retinæ. The treatment of cells with exogenous CFH causes an increase in mitochondrial SOD2 expression, which is a powerful antioxidant (Olivar et al., 2016), so perturbed CFH signalling could lead to some dysregulation of the antioxidant defence system.

Very recently, and as such after the start of this study, clear mitochondrial decline has been noted early on in the retina of *Cfh* ^{-/-} mice (Sivapathasuntharam et al., 2019). When comparing

mtDNA copy number levels at one month there was an average decline, but this did not reach statistical significance ($p = .055$, $n = 4$). The mitochondria in the photoreceptors and RPE of three-week *Cfh* $-/-$ mice displayed an abnormal morphology compared to C57, with mitochondria appearing swollen, commonly associated with senescence. Analysis of retinal layers at birth showed that *Cfh* $-/-$ mice have significantly thinner retinae than C57 mice (Sivapathasuntharam et al., 2019). This is intriguing data as it suggests that CFH expression may play a role in retinal development, outside of any negative effects it may have over the long-term course of life and may predispose those with CFH dysfunction to AMD.

The link between mitochondria and CFH is not clear currently, but there has been data associating mitochondria with the complement pathway, and innate immunity at large (West, Shadel, & Ghosh, 2011). ROS signalling is key to this defence pathway, where increased ROS causes signal cascades activating pro-inflammatory cytokines via NF- κ B signalling (Morgan & Liu, 2011). One hypothesis may be that unabridged immune responses due to the lack of the inhibitory effect of CFH on the complement pathway leads to uncontrolled ROS signalling mediated by the mitochondria, causing a cascade of damage. It is an interesting thought that the mitochondria, which itself must have one point been a foreign entity in the host cell, is now intrinsically linked to the host's defence from further invaders.

Armed with the increasing weight of data showing RPE and retinal metabolism playing a role in AMD dysfunction, along with the identification of a potential causal role of oxidative stress and non-immunological process in CFH that mediate AMD pathology, we wanted to investigate if mitochondrial protection mediated by Ndi1 protein expression could be of benefit in this model.

As detailed in this chapter of the thesis, the functionality of candidate Ndi1 and optNdi1 therapeutics were explored in a wide range of assays to assess function. In addition, robust measures to evaluate *in vitro* the potencies of virally delivered (AAV) ndi1 and optNdi1 gene therapies were devised and validated. Finally, AAV-mediated optNdi1 gene therapies were

evaluated *in vivo* in the *Cfh* $-/-$ murine model which presents with an ocular phenotype simulating aspects of AMD.

1.7.7. Dominant Optic Atrophy (DOA)

Dominant Optic Atrophy (DOA), also known as Kjer's optic atrophy after a Danish ophthalmologist (Kjer, 1959), is an inherited blinding disease that primarily targets the retinal ganglion cells (RGC). DOA has an estimated prevalence of between 1-10,000 to 1-30,000, making it one of the most common optic neuropathies (Lenaers et al., 2012; Patrick Yu-Wai-Man et al., 2010). The higher end of the frequency estimation is from Denmark, where a founder effect is believed to be responsible for the high prevalence (Lenaers et al., 2012). DOA, being an optic neuropathy has a number of similarities to LHON; it typically manifests in the first or second decade of life, with progressive bilateral visual loss with central scotomas, decreased thickness of the retinal nerve fibre layer (RNFL) and optic nerve damage (Chun & Rizzo, 2017). Like LHON, DOA also displays incomplete penetrance with variable phenotypic expression between families, with visual dysfunction of patients ranging from light perception only to 20/20 central vision (Amati-Bonneau et al., 2009). Perhaps somewhat in line with severe complex I disorders resulting in Leigh syndrome (Paquay et al., 2014), around 20% of patients show a multi-system disorder characterised by hearing loss, ataxia, myopathy and peripheral neuropathy called DOA plus (Skidd, Lessell, & Cestari, 2013). Of course, this phenotypic heterogeneity cannot be due to heteroplasmy, where a large proportion of the variability seen in LHON arises from, as DOA causing mutations arise in nuclear encoded genes, suggesting that other genetic variance play a role in determining penetrance. Estimates of penetrance of familial DOA mutations demonstrated that the specific mutation accounts for a large proportion of the variability, with some mutations showing 100% penetrance, compared to just 66% for others, with an average penetrance of ~90% (Cohn et al., 2007). It also appears currently to be impossible to fully predict

if a given mutation will result in DOA plus instead of just DOA, suggesting that disease severity is multi-factorial.

Currently there are 7 genes and a further 4 genetic loci associated with optic atrophy, with three of those forms of optic atrophy following an autosomal recessive inheritance pattern (see table 1-4). Mutations in the OPA1 gene accounts for the vast majority of cases, estimated to be between 65-90% of DOA (Chun & Rizzo, 2017) and correspondingly is also the most well understood of the disease causing genes. Perhaps what is most interesting is that these mutations in multiple genes all cause the same disease phenotypically, optic atrophy, but do not all cause the disease through the same apparent mechanism. A stark example of this is that OPA1 and OPA5 cases effectively have opposite effects on mitochondrial morphology, where OPA1 mutations cause mitochondrial fragmentation and OPA5 – Dnm1L mutations cause excessive mitochondrial elongation (Gerber et al., 2017). The authors of the study did not perform any metabolic analysis on cells derived from these patients, so it remains unclear as to what effect(s) the mutation has on OXPHOS. This is however quite an insightful finding and highlights the acute sensitivity of RGCs to mitochondrial network morphology.

Table 1-4: Genes and genetic loci currently associated with optic atrophy (OPA).

	Gene	Inheritance	Function	Frequency	Reference
OPA1	OPA1	Dominant	Mitochondrial fusion	65-90%	(Delettre et al., 2000b)
OPA2	unknown	X-linked	Not mapped	3 families	(Katz et al., 2006)
OPA3	OPA3	Dominant Recessive	IMM protein; function unknown	1-2%	(Anikster, Kleta, Shaag, Gahl, & Elpeleg, 2001)
OPA4	unknown	Dominant	Not mapped; ~18q12	1 family	(Kerrison et al., 1999)
OPA5	DNM1I	Dominant	Mitochondrial fission	3 families	(Gerber et al., 2017)
OPA6	unknown	Recessive	Not mapped; ~8q21-q22	1 family	(Barbet et al., 2003)
OPA7	TMEM126A	Recessive	IMM protein; function unknown, similar structure to ETC assembly factors;	10 families	(Hanein et al., 2009)
OPA8	unknown	Dominant	Not mapped; ~16q21-q22; no ETC dysfunction, elevated levels of fusion	1 family	(Valerio Carelli et al., 2011)
OPA9	ACO2	Recessive	TCA cycle enzyme;	1 family	(Metodiev et al., 2014)
OPA10	RTN4IP1	Recessive	Mitochondrial localisation; Involved in UV light responses and RGC development;	4 families	(Angebault et al., 2015)
OPA11	YME1L1	Recessive	OPA1 proteolytic processing	1 family	(Hartmann et al., 2016)

Around 370 variants have been identified in the OPA1 gene, with ~65% confirmed pathogenic (Del Dotto, Fogazza, Musiani, et al., 2018), displaying significant mutational heterogeneity. This can be further categorised as follows: 44% of mutations in the coding regions, 27% are splice variants and the remainder (29%) are small exonic duplications and deletions (Ferré et al., 2015).

The main pathological mechanism appears to be haploinsufficiency of OPA1 protein, with ~50% of pathogenic mutations introducing a premature stop codon (Del Dotto, Fogazza, Lenaers, et al., 2018). Pathogenic mutations are present on almost all exons. For up to date OPA1 mutation statistics visit MITOdyn at http://mitodyn.org/home.php?select_db=OPA1.

OPA1 has already been mentioned with regard to its role in inner mitochondrial membrane (IMM) fusion (see section 1.4.3.), but in chapter 4 the role of OPA1 in DOA will be investigated much further using a number of DOA cell models, as well as the potential use of OPA1 as a therapeutic treatment using AAV-mediated gene delivery.

1.8. Therapeutic Approaches to Mitochondrial Disorders

A number of avenues have been investigated for mitochondrial therapeutics, but currently the only ones to make it to market are drug treatments. Although of course we are not investigating drug-based therapeutics, it is informative to see what has shown efficacy and what pathways these drugs have targeted in order to elucidate potential targets for gene therapy intervention. Table 1-5 is an inexhaustive list of current non-gene-therapy based treatments for mitochondrial dysfunction. These therapeutic agents tend to target common aspects of mitochondrial physiology; ETC function, antioxidants and mitochondrial biogenesis are predominant targets (El-Hattab, Zarante, Almannai, & Scaglia, 2017).

Ubiquinone (UQ; CoQ₁₀), the electron shuttle between complex I & II and complex III, has been a primary focus of potential therapeutics since the 1980s, when clinical trials were conducted on patients with cardiomyopathy after it was discovered that patients with more severe cases had reduced ubiquinone levels in heart muscle (Folkers, Vadhanavikit, & Mortensen, 1985). Unfortunately, to date, ubiquinone supplementation has only shown disease amelioration in ubiquinone deficiency (Glover et al., 2010). It is worth, however, talking about a successor to ubiquinone therapy, idebenone, which is a synthetic analogue of ubiquinone with a smaller alkyl tail. Despite being an electron carrier like ubiquinone, there is some discussion in the field as to how idebenone mediates its therapeutic benefit. For instance, it appears that idebenone partially inhibits complex I activity, but is very effective at transferring electrons from complex II, giving it potent antioxidant activities (Jaber & Polster, 2015). Idebenone is also readily reduced by certain cytosolic proteins called NADH:quinone dehydrogenases⁵ and then shuttled to the mitochondria where it can transfer electrons directly to CIII (Haefeli et al., 2011). Despite initial positive indications for idebenone in a number of clinical trials for diseases such as

⁵ It is important to highlight the difference between the NADH:quinone dehydrogenases and NADH:ubiquinone dehydrogenases mentioned below. The former accepts smaller quinones such as CoQ₁ (MW: 250.29g/mol) or idebenone (MW:338.44g/mol) but the not larger quinone CoQ₁₀ (ubiquinone; MW: 863.49g/mol) which is only reduced by the latter.

Alzheimer's disease and Friedrich's ataxia, this has been marred by a number of ensuing drug recalls and withdrawals due to inconsistent results (Kearney, Orrell, Fahey, Brassington, & Pandolfo, 2016; Thal et al., 2003). Of interest to us, idebenone has been approved for LHON treatment under the brand name Raxone, but there remains a good deal of uncertainty as to how much benefit can be achieved with it (Patrick Yu-Wai-Man, Soiferman, Moore, Burté, & Saada, 2017). A caveat to the mixed results idebenone has shown is the fact that it has very poor bioavailability; after oral dosing idebenone undergoes significant first pass metabolism in the liver with only 1% reaching systemic circulation (Becker, Bray-French, & Drewe, 2010). Current work is being undertaken to increase the bioavailability of idebenone through various delivery mechanisms, such as the use of liposomes, and chemical modifications of its side chain (Montenegro, Turnaturi, Parenti, & Pasquinucci, 2018). Despite the above, idebenone highlights that mitochondrial therapeutics modifying ETC dynamics may have great potential to provide benefit, providing they can meet the bioenergetic demands of the cell. If we acknowledge that Idebenone's success is likely due to its increase of electron flow down the ETC and antioxidant properties, and that its failure has been inconsistent delivery, then it highlights the opportunity for mitochondrial gene therapies that modulate the ETC such as *Ndi1* and AOX explored in Chapter 3 and 5 of this thesis.

It is promising that some of the drugs listed in Table 1-5 target genes that have been linked to potential gene therapies, or at least modulation through gene therapy. RTA 408 targets mitochondrial dynamics by upregulating Nrf2 expression leading to an increase in mitochondrial biogenesis. AAV2 delivery of Nrf2 has also shown benefit in the mouse EAE model against optic neuritis, where it protected RGCs from degeneration (McDougald, Dine, Zezulín, Bennett, & Shindler, 2018). This shows a convergence of chemical and genetic intervention and posits that dual interventions could be beneficial or even provide greater benefit together. For example, *Ndi1* coupled with idebenone could counter the over-reduction of the ubiquinone pool that can accompany *Ndi1* overexpression by providing an exogenous source of electron carriers. It could

be hugely beneficial to examine potential combinatorial therapies, of gene therapy and drug, in disease model systems.

Table 1-5: A list of some of the non-gene therapy related treatment modalities under investigation for mitochondrial disorders. Adapted and updated from (El-Hattab et al., 2017).

	Effect on mitochondrial function	Diseases for which agents are used
Agents increasing electron transfer chain function		
CoQ10	Improving the efficacy of electron transfer through ETC	Primary CoQ10 deficiency
Idebenone	A CoQ10 analog with higher efficacy	LHON
Riboflavin	A flavoprotein precursor that is a key building block in complexes I and II	Acyl-CoA dehydrogenase-9 deficiency and multiple acyl-CoA dehydrogenase deficiency
Dichloroacetate	Increasing pyruvate dehydrogenase activity, thereby increasing the catabolism of pyruvate to acetyl-CoA	Congenital lactic acidosis
Nicotinamide riboside	An intermediate in the NAD ⁺ salvage pathway, modulating NAD ⁺ /NADH ratio can be protective	Li-Fraumeni syndrome, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT03789175)
Thiamine	Enhancing pyruvate dehydrogenase activity, thereby increasing the catabolism of pyruvate to acetyl-CoA	Leigh disease and thiamine transporter deficiency
Energy Buffer		
Creatine monohydrate	Acting as an intracellular buffer for ATP and an energy shuttle for high energy phosphates movement from mitochondrial to cytoplasm	Mitochondrial myopathies
Antioxidants		
Lipoic acid	Providing antioxidant action and being an essential factor for pyruvate and ketoglutarate dehydrogenases	MELAS and other mitochondrial diseases
RP103	Increasing intracellular glutathione levels by increasing cysteine availability	Leigh and other mitochondrial diseases, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT02023866)

EPI-743	Protecting against excessive ROS and restoring reduced intracellular glutathione	Leigh disease, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT02352896) Mitochondrial diseases, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT01642056)
Agents enhancing mitochondrial biogenesis		
Bezafibrate	Activating PPAR which activates PCG-1 α pathway and induces mitochondrial biogenesis	Mitochondrial myopathy, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT02398201)
Epicatechin	Enhancing mitochondrial biogenesis	Friedreich ataxia, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT02660112)
RTA 408	Activating Nrf 2 which stimulates mitochondrial biogenesis	Mitochondrial myopathy, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT02255422)
Physical Intervention		
Autologous transplantation	Mitochondria derived from patients own tissue (skeletal muscle typically) are transplanted into tissue experiencing ischemia.	Myocardial Ischemia, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT02851758)
MNV-BLD (CD34+ cells enriched with blood derived mitochondria)	Enrichment for autologous haematopoietic stem cells with normal mitochondria to re-seed patient	Pearson Syndrome, ongoing clinical study (https://clinicaltrials.gov/ct2/show/NCT03384420)

1.8.1. Gene Therapy Targeted to the Mitochondria

The era of retinal gene therapy in human patients reached a key milestone in 2008 with the publication of the safety and efficacy data of the first ocular gene therapy trials for a condition termed Leber Congenital Amaurosis (Bainbridge et al., 2008; Maguire et al., 2008). The outcomes from these studies were two-fold, that gene therapy was both potentially safe and a viable means of treating ocular disease.

Now, just over a decade later, there are 156 gene therapy trials either ongoing or recruiting according to www.clinicaltrials.gov and www.clinicaltrialsregister.eu, with 42 of these tackling ocular disorders. Furthermore, it is an exciting time in the field of mitochondrial therapeutics. There are currently 54 clinical trials listed as active and about 200 more currently recruiting for participants investigating potential mitochondrial therapies. In the Venn diagram intersection of ocular mitochondrial gene therapies there are only three gene therapy trials for mitochondrial disorders, which are in fact the only mitochondrially targeted active gene therapy trials. These clinical trials all use AAV vector delivery of ND4 for the treatment of LHON (see Table 1-6). Despite the limitations of using a gene specific approach to treatment, it is logical that ND4 was the first gene to target for LHON as the majority (~60%) of LHON cases are caused by the 11778G > A mutation in ND4, also known as the Wallace mutation (D C Wallace et al., 1988). Furthermore, at only around 1.4kb in size, the ND4 gene is a very suitable size for incorporation into an AAV vector. All three of these studies have recoded ND4 for translation in the cytoplasm using the “universal” genetic code rather than the mitochondrial genetic code and attached a mitochondrial localisation signal (MLS) These MLSs are from different endogenously expressed mitochondrially targeted proteins.

All three studies have reported outcomes. Firstly the group behind NCT03153293 have shown no serious adverse effects in nine participants over a three year observational period (S. Yang et al., 2016).

Trial Identifier	Treatment	Dose	Route	Phase
NCT03153293	rAAV2-ND4	1e10vg (50µl)	Intravitreal	II & III
NCT03293524*	GS010 (rAAV2-ND4)	9e10vg (90µl)	Intravitreal	III
NCT02161380	scAAV2-P1ND4v2	5e9vg (200µl) 2.46e10vg (200µl) 1e11vg (200µl)	Intravitreal	I

*Table 1-6: A list of the currently active mitochondrially targeted gene therapies, all of which are for the ND4 subunit of complex I according to www.clinicaltrials.gov. * Please note that the GS010 study has multiple study arms and as such multiple Trial Identifiers, all of which are discussed in the main text. Only one was included here for brevity.*

Five of the study patients, who had unilateral injections of rAAV-ND4, experienced an increase in visual acuity and only one patient showed a decrease. This patient was the only one to receive a bilateral injection of the therapeutic. The NCT03293524 group, a biopharma company called GenSight biologics, have also had success with their ND4 treatment. The initial phase I of LHON patients in France showed no serious adverse effects. They are now currently running two trials called Rescue (NCT02652767) and Reverse (NCT02652780). Patients received a unilateral injection of GS010 either within 6 months of disease onset (Rescue) or 7 months to 1 year of onset (Reverse). 72-week data from the Reverse study has been published on the company's website (www.gensight-biologics.com) and showed an average change from baseline of +15 letters across the 37 patients enrolled in the study. The 48-week data for the Rescue study is due in 2019. GenSight are currently recruiting for a trial of bilateral GS010 injections of patients up to 1 year from disease onset called Reflect. The final study group, NCT02161380, have also posted positive preliminary results for their scAAV2-P1ND4v2 study. Of the five patients, there were no serious adverse events observed and there was no vision loss after 180 days. Two of the patients experienced an increase of visual acuity over the time frame of the study (Feuer et al., 2016).

A very interesting phenomenon was observed during the three studies where the uninjected eye also showed an improvement in visual acuity over the time course of the trial. Spontaneous recovery of visual acuity in LHON has been previously described, with 4-33% of patients carrying the 11778G > A mutation showing recovery (Lam et al., 2014). A follow up study to Yang *et al.*

(2016) showed that there was no significant difference in the visual field between the treated and untreated eyes after intervention, but there were significant differences between the eyes pre and post-intervention (Yuan et al., 2018). Spontaneous recovery was ruled out as the progenitor of the benefits seen in their trial as the time frame for the viral mediated visual recovery was at a consistent time post intervention, whereas the timing of spontaneous recovery is inherently random. The level of recovery of the contralateral eye is quite striking in the GenSight study, with patients demonstrating an improvement of +12 letters in the sham injected eye, compared to +15 letters in the treated eye. GenSight are currently running a natural history study of LHON progression in 50 patients (called Reality) and it will be interesting to see what spontaneous rates of recovery they find.

Some effort has gone into trying to elucidate the mechanism of visual acuity recovery in the untreated eye. It appears that the untreated eye in patients shows a characteristic pattern of visual field recovery, starting at the nasal side and progressing towards the temporal side (Yuan et al., 2018). Intriguingly this is the opposite direction in which visual field defects occur during LHON progression. It has been suggested that some manner of signalling can occur between our two eyes and that this is behind the recovery of the untreated eye. In rats treated unilaterally with a variety of AAV vectors, it was shown that proteins do not transfer from one retina to the other, but a nerve tracer dye showed that some neuronal projections crossed the optic chiasma from one eye to the other, suggesting there could be direct neuronal signalling between eyes (S. Yang et al., 2015). It is possible that the injection into one eye creates some manner of signalling response that can enact change in the second eye. It is possible that the restoration of mitochondrial competency of the optic nerve elicits a benefit in the visual transduction of the contralateral eye. It will also be very interesting to see if other disorders involving RGCs and the optic nerve, have a similar recovery in the untreated eye after gene therapy intervention, or if this is a phenomenon unique to LHON.

1.8.2. Viral Vectors

Since the birth of the gene therapy field the question has been how to efficiently and safely deliver this exogenous genetic material. The idea of gene therapy was first posited in the 1960's after pioneering work involving the delivery of exogenously supplied DNA to cure an auxotrophic mutation found in Lesch-Nyhan patients in a mammalian cell line (Szybalska & Szybalski, 1962). The success of this experiment demonstrated the power and potential for this method to cure any disease that the gene could be identified for, or at least that was the sentiment at the time. Work was undertaken to improve the safety and efficacy vectors used to transfer genes to patients, especially after a series of tragic events related to the viral vector chosen for patient intervention (Thomas, Ehrhardt, & Kay, 2003). There are a wide variety of viral vectors currently under investigation, all with their respective strengths and weaknesses depending on the target tissue and the gene cassette to be delivered. Broadly speaking, there are two vector families that have been the focus of the most research, adenoviruses and retroviruses.

Retroviruses, and in particular lentivirus, have been used in a number of studies. Retroviruses contain a ssRNA genome and have a large packaging capacity for foreign DNA of up to 8kb. These viruses are integrative, which means that the foreign DNA is transcribed into the nuclear DNA of any cells that are infected. If this happens at random, as it did in early retroviral vectors, it is easy to visualise the detrimental effects this could have on a cell through endogenous gene disruption, and indeed this has resulted in serious adverse effects in patients receiving lentiviral therapies (Hacein-Bey-Abina et al., 2008). Work has subsequently been carried out to reduce these off-target effects by designing viruses that preferentially integrate at certain genomic loci. This has decreased the risk of unpredictable genomic integration, but not entirely eliminated it (Vranckx, Demeulemeester, Debyser, & Gijssbers, 2016). However, this integration is necessary for the long-term expression of the transgene in actively dividing cells, as all daughter cells will contain a copy of the transgene which is hugely advantageous when targeting fast growing cell

populations. Lentivirus cellular tropism is typically quite promiscuous, with expression to specific cell types being achieved by the selection of cell type specific promoters, although work has been carried out to increase cellular specificity by pseudo-typing the lentiviral vector (Cronin, Zhang, & Reiser, 2005).

Adenoviruses are non-enveloped dsDNA viruses that have an icosahedral capsid shape and so-called due to their original isolation from human adenoids in the 1950's. They have a packaging capacity of around 7.5kb of foreign DNA, which after cellular infection exists episomally, removing the dangers inherent with genomic integration, but precluding long term expression in replicative cell populations (Lundstrom, 2018). Perhaps the most notorious serious adverse event of all virally mediated gene therapy interventions happened in a study using adenovirus to treat ornithine transcarbamylase deficiency. Only 4 hours after adenovirus treatment, Jesse Gelsinger developed a severe immune reaction to the virus, and died of multiorgan failure within four days. The cause of death is believed to be a severe immune response to adenovirus (NIH, 2002). This was believed to be elicited by previous exposure to adenovirus directly, rather than its genetic cargo (Thomas et al., 2003). This led to the development of non-immunogenic adenoviruses, a number of which are in use in clinical trials currently.

The Adeno-Associated Viruses (AAVs) are a family of ssDNA viruses with an icosahedral capsid that are found naturally in a significant proportion of the population (Lai, Lai, & Rakoczy, 2002). Their presence is believed to be benign, with no disease pathologies associated with naturally occurring AAV infections (Mingozzi & Büning, 2015). AAV vectors have a reduced capacity of around 4.7kb compared to the prior two categories of vector described (Wu, Yang, & Colosi, 2010). Although this is a reduction in the genetic payload of the vector, it also correlates with a decrease in the size of the virus itself, with the virus being ~25nm in size versus lentivirus which is ~80nm in size, which allows more concentrated solutions of AAV particles. However, AAV vectors deliver their exogenous DNA as episomal circular DNA molecules without genomic

integration. The circular nature of the DNA molecule is thanks to two inverted tandem repeats (ITRs) that recombine after cellular infection to produce a single circular molecule. The non-integrative nature of AAV limits their use to less proliferic cell types, which is why the post-mitotic cells of the eye are a very suitable target. AAVs have been identified to have at least 12 naturally occurring serotypes, with AAV2 being the first to be isolated and used for gene therapy (Colella, Ronzitti, & Mingozzi, 2018). The naming convention for AAV vectors is to give the serotype number from which the vector genome is derived and then the number from which the capsid is derived, so AAV2/8 has the genomic structure (ITRs) of AAV2 but the capsid of AAV8.

These serotypes are an important aspect of AAV gene therapy design as by employing specific serotypes, specific cell populations can be targeted. Different serotypes have specific amino acid substitutions on the capsid protein that allows the recognition and binding of the AAV virus to cell surface receptors specific to certain cell populations. There is significant variation in the sequences of these serotypes, some natural and some engineered, that means there are over 100 different serotypes of AAV to choose from (Colella et al., 2018). This diversity has allowed unprecedented cell type targeting, where for instance specific cell sub-populations can be focused on in the eye (Surace & Auricchio, 2008). There have been a number of studies undertaken to identify which serotype shows the highest affinity for various ocular cell types.

The AAV2/2 serotype has been widely used for therapeutic interventions and is the serotype of all three of the ocular mitochondrial gene therapies mentioned above. This is because when AAV2/2 is injected intravitreally, robust transduction of the inner nuclear layer and in particular the ganglion cell layer is seen (R. R. Ali et al., 1998; Chadderton et al., 2013). As the RGCs are acutely vulnerable to LHON related dysfunction, it is clear why AAV2/2 has been the serotype of choice for these studies. Injection of AAV2/2 subretinally allows robust transduction of RPE and photoreceptors (R. Ali et al., 1996). Work has been undertaken to modify the AAV2 capsid proteins by the introduction of a number of tyrosine residues. In a “triple-tyrosine”, AAV2/2-

3tyr vector, tropism for the photoreceptor cell layer has been increased, with somewhat reduced RPE transduction compared to wild type AAV2/2 (Petr-Silva et al., 2011). The AAV2/8 serotype has also shown promising results in studies of cellular tropism, demonstrating high levels of transduction of RPE cells and photoreceptors after subretinal injection (Allocca et al., 2007; Palfi et al., 2015). It has been shown that for similar doses, AAV2/8 provides a higher rate of transduction of these cell types than AAV2/2 (Natkunarahaj et al., 2008).

Specifically in relation to mitochondrially targeted gene therapies delivered with AAV vectors, recent work has been undertaken to design an AAV serotype that once endocytosed by the cell will be targeted to the mitochondria by the addition of an MTS to the capsid. This would allow the delivery of the genetic payload directly to the mitochondria. This approach has been used to treat an ND4 deficient mouse model (Yu et al., 2012) and represents an interesting approach to mitochondrially targeted therapeutics. If expanded upon, this could help circumvent some of the issues with delivering the mitochondrially encoded proteins ectopically due to their propensity for being mistargeted to the ER (Björkholm et al., 2015), as mentioned earlier.

1.8.3. Mitochondrial Replacement Therapy

Perhaps one of the most promising therapeutic modalities for mitochondrial disorders currently is that of mitochondrial replacement therapy (MRT). MRT is essentially a form of *in vitro* fertilisation whereby a mother who is at risk of passing on a mitochondrially encoded pathology can safely receive a fertilised egg that contains her own nuclear DNA whilst containing the mitochondrial DNA from a third-party donor, hence the unflattering but not inaccurate term of “three parent babies”. This is achieved by enucleating the affected mother’s egg and transferring the nucleus to a donor egg containing healthy mitochondria that has been enucleated. This hybrid egg is then fertilised *in vitro* and implanted as would normally happen with IVF (Amato, Tachibana, Sparman, & Mitalipov, 2014). The first baby born via this technique

was successfully delivered in 2016, to a mother in the USA who carried a Leigh syndrome mutation⁶. There was some consternation at the time because the medical team who carried out the procedure travelled to Mexico to actually perform the procedure. Foreshadowing perhaps, of the furore around the Crispr procedure carried out on twins born in China late last year.

The MRT procedure has now been legalised in a handful of countries including the UK (where the first women to be given the treatment both have MERRF) but currently remains unapproved in the USA. This is an incredibly powerful tool in the fight against mitochondrial disorders and one that should have a positive effect on relieving mitochondrial disease burden eventually. In an ideal world where everyone knows their genetic make-up, it would all but eradicate mitochondrial disorders, or at least prevent their continuation from one generation to the next. There are caveats, of course. The *de novo* mutation rate of mitochondria has been demonstrated to be high and MRT would do nothing to change disease resulting from such mutations (Elliott et al., 2008). In a similar vein mothers are very frequently undiagnosed carriers, especially with LHON for instance, so it may be that MRT is offered on the diagnosis of another family member. Mitochondrial retrograde signalling, (from the mitochondria to the nucleus) is currently poorly understood, meaning that there could be unforeseen consequences of providing exogenous mitochondria (Cardamone et al., 2018). There is also a possible ethical quandary regarding the mitochondrial haplogroup of the donor mitochondria. Should it be matched to the parent? However, what if for example, this haplotype is a known risk haplogroup for AMD? There will likely be associated ethical considerations with MRT.

There has been some data suggesting that mitochondrial control region, the D-loop, polymorphisms affect the outcome of MRT in embryonic stem cells derived from MRT oocytes, where over subsequent cell divisions a reversion to the mother's mtDNA molecule was noted,

⁶ <https://www.bbc.com/news/health-37485263>

despite estimating only a 1% carry over of mitochondria from the original donor egg (Tachibana, Kuno, & Yaegashi, 2018). Further investigation of control loop - nuclear interactive regulation of mitochondrial replication is needed.

2. Methods

2.1. Cloning methods

Throughout this thesis around 20 different expression plasmids were created to allow a detailed analysis of effects of ectopically expressing the genes in question. This section will detail the general scheme of cloning for the plasmids created, with a full listed of created plasmids in Table 2-2. Sequences for the transgenes are in the Appendix (section 7.1).

2.1.1. Restriction Digest

DNA to be digested was cut using restriction endonucleases provided by NEB. For cloning typically 10µg of DNA was used, to ensure a sufficient amount is retained throughout the cloning steps. A 50µl digest would be set up as per the scheme in (Table 2-1). All digests were at 37°C for 3 hours. If multiple enzymes were to be used for a double digest the total volume of enzyme was not allowed to exceed 10% of total volume as inhibition by the storage glycerol can occur.

Table 2-1: General template for enzymatic digestion.

	Volume
DNA (10µg)	X
Enzyme	2µl
Buffer	5µl
H₂O	Up to 50µl

In order to remove any buffer and enzyme from any reactions, both the backbone and the insert were then either cleaned using the PCR purification kit (Thermo Fischer; K0702) as per the manufacturers protocol, or were gel purified. For gel purification, the DNA to be isolated was run on a 1.5% agarose gel. The band of the desired DNA product was then excised from the gel and the DNA was extracted using a gel extraction kit (Thermo Fischer; K0691) as per the manufacturers protocol.

All of the transgenes that were ordered for synthesis were designed with flexibility in mind, with multiple restriction sites added before the start codon and after the stop codon to allow for cloning to and from different vectors. All transgenes used the minimal Poly A sequence

2.1.2. DNA ligation

Once the insert and backbone had been purified the next step was to ligate them together. Depending on the nature of the vector backbone and the insert a number of steps could be taken to ensure the success of the ligation process. If the backbone was likely to self-ligate, it could be dephosphorylated using Calf-Intestinal Phosphatase (NEB; M0290S; manufacturers protocol) to prevent this. If either the insert or the backbone had incompatible DNA overhangs these could be enzymatically removed using Klenow (NEB; M0210S; manufacturers protocol).

Special consideration was taken when using PCR cloned fragments for ligations, such as the removal of the HIS-tags in the OPAiso1, OPAiso7, BgAOX and CiAOX constructs. Standard PCR primers are not phosphorylated, so a PCR product cannot be directly ligated into a backbone unless there is a phosphate group present on the backbone. However, if the backbone is blunt cut to accept the PCR insert, there will be a high degree of false positives due to self-complementarity of the backbone. To avoid this, two approaches were taken. Either the backbone was dephosphorylated and the PCR insert was phosphorylated using a T4 kinase (NEB; M0201S), or restriction sites were incorporated into the PCR primer design to allow digestion of the PCR product. Enzymatic digestion leaves phosphate groups and allows subsequent ligation.

Ligation of the insert to the vector backbone was carried out using T4 DNA ligase (Roche; 10481220001) as per the manufacturers protocol. Vector and insert concentrations were first estimated by agarose gel and used to estimate ratios of insert:vector, typically in the region of 10:1, but was modified if unsuccessful at first. Vector only and no ligase controls were also set up. Ligations were performed at 16°C overnight.

2.1.3. Plasmid transformations

The (hopefully) ligated plasmid was then used for transformation of chemically competent *E. coli* XL1-Blu bacteria. Briefly, half the volume of ligated product was added 200µl of competent cells and incubated on ice for 20 minutes. Cells were then heat shocked at 42°C for 2 minutes, before 1ml of LB media was added at the tubes were placed in an orbital shaker for 1 hour. After this 200µl of were plated on agar plates with appropriate antibiotics and placed at 37°C overnight.

Plates were examined for colonies with successful candidates used to inoculate 5ml of LB and were incubated at 37°C overnight with appropriate antibiotics. This was used as culture for a mini-prep plasmid DNA extraction. Up to 24 mini-preps were run simultaneously, depending on the number of putative colonies identified using the manufacturers protocol (Thermo Fischer; K0503). Extracted plasmid was then subjected to a test digest to identify the presence or absence of the insert. Any candidate insert-containing plasmids were then sent for initial Sanger sequencing to confirm the presence of absence of the insert. If positively identified as containing the insert the resulting plasmid was then grown in either a maxi-prep (Qiagen; 12165), for cell-based plasmid studies, or using an endotoxin free mega-prep (Qiagen; 12181) for viral production. After isolation of the plasmid Sanger sequencing was used to confirm the integrity of the transgene sequence.

Table 2-2: Plasmid constructs created for this thesis

Name	Insert	Backbone	Uses
OPAiso1	CMV-OPAiso1	pAAV-MCS	OPAiso1 expression plasmid.
OPAiso7	CMV-OPAiso7	pAAV-MCS	OPAiso7 expression plasmid. Insertion of exon 5b.
pcOPAiso1	CMV-OPAiso1	pcDNA3.1(+)	Stable cell line generation.
pcOPAiso7	CMV-OPAiso7	pcDNA3.1(+)	Stable cell line generation.
OPAiso1-dsRed	CMV-OPAiso1 CBA-Mito-dsRed	pAAV-MCS	Localisation studies and early fission and fusion.
OPAiso7-dsRed	CMV-OPAiso1 CBA-Mito-dsRed	pAAV-MCS	Localisation studies and early fission and fusion.
OPAiso1-noHIS	CMV-OPAiso1	pAAV-MCS	PCR cloned to remove the HIS tag for functional assays.
OPAiso7-noHIS	CMV-OPAiso1	pAAV-MCS	PCR cloned to remove the HIS tag for functional assays.
CiAOX	CMV-CiAOX	pAAV-MCS	CiAOX expression plasmid.
BgAOX	CMV-BgAOX	pAAV-MCS	BgAOX expression plasmid.
optCiAOX	CMV-optCiAOX	pAAV-MCS	Codon optimised version of CiAOX for expression analysis.
optBgAOX	CMV-optBgAOX	pAAV-MCS	Codon optimised version of BgAOX for expression analysis.
pcCiAOX	CMV-CiAOX	pcDNA3.1(+)	Stable cell line generation.
pcBgAOX	CMV-BgAOX	pcDNA3.1(+)	Stable cell line generation.
CiAOX-noHIS	CMV-CiAOX	pAAV-MCS	PCR cloned to remove the HIS tag for functional assays.
BgAOX-noHIS	CMV-BgAOX	pAAV-MCS	PCR cloned to remove the HIS tag for functional assays.
Ndi1	CBA-Ndi1	pAAV-MCS	Ndi1 expression plasmid.
optNdi1	CBA-optNdi1	CHOP	optNdi1 expression plasmid.
optNdi1-HA	CBA-optNdi1	pcDNA3.1(-)	HA-tag expression plasmid.

2.2. Spectrophotometric Assays of Mitochondrial Function

2.2.1. NADH oxidation assay – reagent preparation

Potassium phosphate buffer (0.5 M, pH 7.5)

Titrate 0.5 M potassium phosphate dibasic (Merck; P2222) with 0.5 M potassium phosphate monobasic (Merck; P5655) up to a pH of 7.5. Store at 4 °C for up to 2 months.

Ubiquinone₁ solution (10 mM)

Dissolve 2mg of ubiquinone₁ (Merck; C7956) in 0.8ml of absolute ethanol and store it in 100μl aliquots at –20°C for several months.

Bovine Serum Albumin (50 mg ml⁻¹)

Dissolve 250mg of defatted BSA (Merck; A7030) in 5ml of distilled water and store it in 1ml aliquots at 4°C for up to 1 month.

Potassium Cyanide (KCN) solution (10 mM)

Dissolve 6.5mg of KCN (Merck; 60178) in 10ml of distilled water under a fume hood. Must be prepared fresh.

NADH solution (10 mM)

Dissolve 7.5mg of NADH (Merck; N8129) in 1ml of distilled water, must be prepared fresh.

Rotenone solution (1 mM)

Dissolve 3.94mg of rotenone (Merck; R8875) in 10ml of absolute ethanol and store it in 1ml aliquots protected from light at –20°C for several months.

Flavone (5 mM)

Dissolve 11.1mg of flavone (Merck; F2003) in 10ml of absolute ethanol and store in 1ml aliquots at –20°C for several months.

2.2.2. NADH oxidation assay

This procedure is an expanded version of the one published in (Spinazzi, Casarin, Pertegato, Salviati, & Angelini, 2012). First mitochondria were extracted as per the protocol in Mechanical Extraction of Whole Mitochondria from Cultured Cells (see 2.8.4.). Mitochondrial samples were subjected to 3 liquid nitrogen flash-freeze thaw cycles prior to use.

The spectrophotometer (Shimadzu UV-mini 1280) was set to time course mode, with the wavelength at 340nm and was blanked using distilled water. Mitochondrial sample was added to a 1ml cuvette, and the total volume was brought to 774 μ l with distilled water. The volume of sample depended on the concentration of mitochondria in the extracted sample. If a single confluent HEK293 containing well from a 6 well plate was used, such as during the MOI dosage test, 100-200 μ l of sample was routinely used. From larger plates as little as 12.5 μ l was used successfully. The goal was to have enough sample to allow a robust linear trace to be observed. 100 μ l of potassium phosphate buffer, 60 μ l of fatty acid-free BSA, 30 μ l of KCN and 10 μ l of NADH were then added to the cuvette. The cuvette was then parafilmed and inverted to mix contents. This cuvette was then read for ~1min or until the trace was steady. To start the reaction 6 μ l of ubiquinone was added to the cuvette and mixed by inversion. The absorbance was then measured for 90 seconds. The absorbance should decrease over time due to NADH oxidation activity in the sample. If the trace is not linear at this point the reaction needs to be repeated using a lower volume of mitochondrial sample. This rate represents complex I and Ndi1 activity (if present). Next 10 μ l of rotenone was added and mixed by inversion, and the absorbance was measured for a further 90 seconds. The trace now represents Ndi1 activity levels, or background levels if Ndi1 is not present. 10 μ l of flavone is then added and mixed by inversion, with the absorbance being measured for a further 60 seconds. Flavone inhibits Ndi1 and will be a measure of background activity. The addition order of rotenone or flavone can be reversed without affecting the rates obtained by the assay. To analyse the traces the slopes of each section of the trace were calculated, using the UVprobe software supplied with the Shimadzu UV-mini 1280. The rate of background NADH oxidation (all inhibitors present) was subtracted from the rate after ubiquinone and both inhibitors. The subtraction of activity after rotenone addition from activity after ubiquinone addition can be used to calculate complex I activity, or Ndi1 activity if the rate after flavone addition is used.

2.2.3. Citrate Synthase assay – reagent preparation

Tris (200 mM, pH 8.0) with Triton X-100 (0.2% (vol/vol))

Dissolve 1.21g of Tris in 40ml of distilled water, adjust the pH to 8.0 with HCl, add 0.1ml of Triton X-100 and adjust the volume to 50ml. Store at 4 °C up to 2 months.

DTNB solution (1 mM)

Dissolve 7.9mg of DTNB (Merck; D8130) in 20 ml of 100mM Tris (pH 8.0).

Acetyl CoA solution (10 mM)

Dissolve 10 mg Acetyl CoA (Merck; A2056) in 1,235µl of distilled water. Store at –80°C in 200µl aliquots for several months.

Oxaloacetic acid solution (10 mM)

Dissolve 6.6mg of oxalacetic acid (Merck; O4126) in 5ml of distilled water.

2.2.4. Citrate Synthase assay

The spectrophotometer (Shimadzu UV-mini 1280) was set to time course mode, with the wavelength at 412nm and was blanked using distilled water. Mitochondrial sample was added to a 1ml cuvette, and the total volume was brought to 320µl with distilled water. Typically, 50µl of sample provided a strong linear trace. 500µl of Tris-Triton, 100µl of DTNB and 30µl of Acetyl CoA were added to the cuvette and mixed by inversion. Baseline activity was then monitored at 412nm for 90 seconds. The reaction was started by the addition of 50µl of the oxaloacetic acid solution and was monitored for 2 minutes. The rate of increase in absorbance corresponds to the level of citrate synthase activity.

2.3. Tissue Culture

2.3.1. Thawing Cells

The appropriate media was prewarmed in the 37°C water bath. An appropriate sized flask was selected depending on the cell number and type frozen e.g. 2×10^6 HEK 293 cells could be plated directly into a T75. 15 minutes prior to thawing 10ml of complete media was added to the flask and was placed in the 37°C CO₂ incubator to allow the pH to normalise in the media.

The vial of cells was placed in the 37°C water bath and allowed to partially thaw, until the contents can be moved in the vial. In the laminar hood the contents of the vial are decanted into a 15ml tube. To this 9ml of prewarmed media was added, dropwise at the start to avoid osmotic shock. The 15ml tube was then spun down at 300 x g for 5 minutes. The supernatant was decanted and the cell pellet was resuspended using the prewarmed media from the flask. Cells were placed in the 37°C CO₂ incubator and monitored daily.

2.3.2. Passaging Cells

Cells were typically split when they reached 70-90% confluency, depending on the cell line being used. Briefly, for a 15cm plate, the media was removed from the cell plates and were washed once with 6ml PBS. 2ml of prewarmed Trypsin was added to the plate and returned to the 37°C CO₂ incubator. The plate was inspected after 3 minutes. Once cells were fully detached 8ml of prewarmed media was added to the plate and the cells were resuspended. For routine passaging cells were split 1:20 to 1:3 to new 15cm plates, with the media volume increased to 20ml with fresh prewarmed media.

2.3.3. Cell Counting

Cells were treated as per passaging cells, but after trypsinisation cells were transferred to a 50ml tube and gently but thoroughly resuspended. Three 7µl aliquots of cell were removed for analysis. An equal volume of Trypan-Blue was added to each then 10.5 µl was loaded onto a Luna Cell Counter slide. The live cell count was recorded and used for seeding calculations.

Alternatively, a haemocytometer can be used for cell counting. After the addition of Trypan-Blue to the aliquots of cells, 14µl was loaded onto the edge of the coverslip on a haemocytometer. Cells were observed on an inverted microscope and were counted, including cells on the top and left edges. The count was multiplied by 2×10^4 to obtain the total count per ml.

Cell counting was done prior to seeding cells into tissue culture dishes of various sizes. Table 2-3 details a number of cell lines and the typical number of cells seeded for a given dish size. These numbers were used as a starting point and could be modified depending on the downstream assays the cells would be subjected to.

	96 well	Seahorse XF96 (80µl)	Chamber slide	24 well	6 well	T25	T75	15cm Dish
HEK 293	2×10^4	2.5×10^4	2.5×10^4	1×10^5	5×10^5	5×10^5	1×10^6	2×10^6
Hela	2×10^4	2.5×10^4	2.5×10^4	1×10^5	5×10^5	5×10^5	1×10^6	2×10^6
MEF	1.5×10^4	2×10^4	2×10^4	5×10^4	$1-5 \times 10^5$	$1-5 \times 10^5$	1×10^6	1×10^6
OPA -/-	2×10^4	2.5×10^4	2.5×10^4	1×10^5	5×10^5	5×10^5	1×10^6	2×10^6
1^{ry} Fibro	1×10^4	n/a	1.5×10^4	n/a	n/a	1×10^5	1×10^6	n/a

Table 2-3: Cell seeding numbers for commonly used plates. Cells from a similar background to these cells can use these numbers also e.g. MEF, NIH3T3 and OPA^{-/-} can be seeded at the same densities.

2.3.4. Cell Stock Freezing

Cells were treated the same as passaging but after trypsinisation cells were transferred into a 50ml tube and counted. The tube was spun down at 300 x g for 5 minutes. The pellet was resuspended in 9:1 FBS:DMSO to give $1-2 \times 10^6$ cells per ml. 1ml of cells was added per cryo-vial and transferred to the -80°C freezer in a Mr. Frosty.

2.4. Cell Transfections

2.4.1. Lipofectamine 2000

Standard DNA plasmid transfections were carried out using Thermo Fisher Lipofectamine 2000 (product no. 11668019) as per the manufacturers protocol with slight refinements depending on the cell type being transfected. Table 2-4 shows required reagent volumes for HEK 293 cells in various plate sizes. These values were used as a starting point for cellular analysis, specific values need to be empirically tested for each cell type.

	96 well	24 well	6 well
DNA per well...	100 ng	500 ng	2500 ng
...diluted in x μ l Optimem	5 μ l	25 μ l	125 μ l
Lipofectamine 2000 per well...	.25 μ l	1 μ l	5 μ l
...diluted in x μ l Optimem	5 μ l	25 μ l	125 μ l
Volume of DNA-lipid complex added	10 μ l	50 μ l	250 μ l

Table 2-4: Typical volumes of reagents used for DNA plasmid transfection of HEK 293 cells.

Two tubes are prepared per DNA plasmid treatment, one containing Optimem and DNA, the other Optimem and Lipofectamine 2000 and are incubated at room temperature for 5 minutes. The contents of each tube pair are mixed together and incubated for a further 20 minutes at room temperature. The DNA-lipid complex is then added to the cell wells and returned to the 37°C CO₂ incubator. Cells were typically analysed 24 hours post for RNA or 48 hours post for protein.

It is important to have a positive transfection control to validate the success of the transfection, and to treat control cells with either an unrelated plasmid or lipofectamine alone to simulate the stress of plasmid transfection.

2.4.2. PEI Transformation

For transfection of larger plate sizes PEI based transfection can be used. PEI (MW:25,000; Polysciences; 23966-1) is dissolved at 1g per 1L of deionised water, pH7 (Reed, Staley, Mayginnes, Pintel, & Tullis, 2006). Per 15 cm dish, 12µg of DNA plasmid is diluted in 1ml of buffer. 1ml of PEG reagent is added to this and is incubated at room temperature for 8 minutes. 2ml of this is plated. The media is replaced after 6 hours to avoid PEI induced toxicity.

2.5. Cell Preparations for Immunohistochemistry

2.5.1. Paraformaldehyde Fixation and Immunohistochemical Staining of Cells

Cells were seeded on coverslips in 24 well plates, with appropriate transfection carried out 24/48hrs prior to fixation. Growth media was removed from cells and 250ul of 3.7% paraformaldehyde (pfa) solution was added. The plate was incubated for 20 minutes at room temperature. The pfa solution was removed then washed 3 times with 250ul PBS. Plates can be parafilmed and stored at 4C. Cells were then blocked with 5% Donkey Serum and .3% triton in PBS at room temperature for 2 hours. Blocking solution was then removed and an appropriate primary antibody was diluted in 250ul of blocking solution and was added to cells at 4C overnight or room temperature for 2 hours. Cells were then washed 3 times with PBS and an appropriate secondary antibody was diluted in blocking solution and incubated at room temperature for 2 hours. Cells were then washed three times with PBS (if signs of cell monolayer detaching washes can be reduced). OPTIONAL: Cells can be stained with DAPI nuclear stain, diluted 1:10,000 in PBS, and incubated at room temperature for no longer than 15 minutes, and washed in PBS 3 times. Coverslips are then removed from the plate wells using tweezers and dab dried on the non-cell side to remove any remaining PBS. A drop of mounting media is placed on a microscope slide and the coverslip is lowered at an angle, cell side down, on to the slide. Slides are then allowed to dry before imaging.

2.5.2. Mitotracker staining of cells

Cells were seeded on coverslips in a 24 well plate, with appropriate transfection carried out 24/48hrs prior to staining with Mitotracker CMTM Orange (M7510, Thermo

Fischer). A stock solution of Mitotracker was reconstituted to 1mM with DMSO. The stock solution was then diluted to a working concentration of 100nM – 500nM in pre-warmed complete media. Growth Media was removed from cells and 1ml of Mitotraker staining solution was added. Cells were incubated at 37C for 30 minutes. Cells were then washed once in complete media or were fixed and stained as previously described.

2.6. Stable Cell Line Generation

2.6.1. HEK 293 G418 Kill Curve

In order to effectively select for cells with G418 it is important to establish what the effective dose is. Appropriate cells were plated into a 24 well plate 24 hours prior to the assay (see Table 2-3). The media on the cells was replaced with media containing G418 at a range of concentrations from 0 – 1000µg/ml, with each concentration in duplicate wells. Cells are monitored over the next 7 days to establish the low, optimal and high doses. The low dose is the highest antibiotic concentration where there is no visual toxicity. The optimal dose is the lowest dose where all cells are dead after 7 days. The high dose is the dose where visual toxicity is apparent after 2-3 days. Care must be taken that, at higher doses of G418, toxicity due to excessive pH changes is not mistaken for the action of the antibiotic.

2.6.2. pcDNA 3.1 Stable Cell Line Generation

HEK 293 cells were plated in 6 well plates and transfected as per the lipofectamine 2000 protocol with a pcDNA 3.1 plasmid containing the gene of interest. This plasmid contains the Neomycin resistance gene under the control of a mammalian specific promoter allowing the selection of transfected cells, as well as an SV40 origin which allows the plasmid to be replicated in mammalian cells. 48hrs after the initial selection the cell media was replaced with media containing 400µg/ml of G418 (optimal dose for HEK293 cells identified from the kill curve). Care was taken to not allow cells to reach confluency as the antibiotic has most effect on actively dividing cells and media was changed to fresh G418 media every 3 – 4 days. Cells were maintained with G418 in the media until no more cell death appears to be attributable to the G418. At this point cells can be passaged into media containing the high dose of G418 to test cell survival. If minimal cell death is observed a stable cell population has been produced. Carry

out protein and RNA analysis to confirm expression of the gene of interest. The stable cell line can be passaged routinely in the presence of G418.

2.6.3. HEK 293 p0 Stable Cell Line Generation

Cells were treated as outlined in Hashiguchi and Zhang-Akiyama (2009) with some slight modifications. Briefly, HEK 293 cells were plated into 15cm dishes and 24hrs later the cell media was replaced with media containing 200ng/ml of ethidium bromide (EtBr) and 50µg/ml of uridine. During the process cell growth rate decreases due to an increased reliance of the cells on glycolysis, which in turn causes a higher rate of media acidification. Cells were monitored closely, and their media was replaced every 2-3 days with fresh media containing 200ng/ml EtBr and 50µg/ml uridine. Major cell death starts after 5-7 days, and cells were routinely passaged when 80-90% confluency was observed. It can take up to a month after initial treatment for cells to reach confluency. The EtBr and uridine are continually supplemented to the media to maintain the selective pressure, but EtBr was removed prior to any DNA plasmid transfections.

To validate the p0 status of the HEK 293 cells qPCR analysis was used. Table 2-5 shows two sets of primers, one targeted to the nuclear genome and the other to the mitochondrial genome. DNA was extracted from cells using the Thermo Scientific GeneJET Genomic DNA Purification Kit (K0721) as per the manufacturers protocol.

Nuclear Genome	Mitochondrial Genome
B2 microglobulin F	tRNA Leu F
5' - TGCTGTCTCCATGTTTGATGTATCT	5' - CACCCAAGAACAGGGTTTGT
B2 microglobulin R	tRNA Leu R
5' - TCTCTGCTCCCCACCTCTAAGT	5' - TGGCCATGGGTATGTTGTTA

Table 2-5: Primers for qPCR analysis of p0 mitochondrial content.

2.7. Primary Fibroblast Cell Culture from Skin Biopsy

2.7.1. Preparation of Geltrex Plates

Geltrex base layer was added to 4 wells per punch to help the skin biopsies adhere to the culture plates. Aliquots of Geltrex were thawed overnight at 4°C before being diluted 1:100 with base DMEM. 350µl of 1x Geltrex was added per well (4 well plate; 1.5ml per well of a 6 well plate). Plates can be paraflimed and stored at 4°C for a week or placed in the 37°C CO₂ incubator for at least 1 hour to allow gelling. Plates were then kept at room temperature for an hour before use.

2.7.2. Skin Biopsy handling and Plating

Patient biopsies were taken from the upper forearm using a 3mm single use sterile biopsy punch (Integra Miltex; 12-460-406). Biopsy pieces were then placed in transportation media (DMEM, 1% pen/strep, Gentamycin 50 µg/ml) and kept on ice until they arrived at the lab.

Biopsy pieces were transferred to the laminar flow hood then washed in PBS – 1% pen/strep. Biopsies were dissected into ~4-6 pieces using a sterile scalpel blade in prewarmed complete media DMEM + Glutamax, 20% FBS, 1%, sodium pyruvate 1% non-essential amino acids, 1% pen/strep, 1% Fungizone). The Geltrex media was aspirated from prepared plates and replaced with 300µl of complete media. A single biopsy piece was added per well. The media level in the well was reduced until the top of the biopsy piece is exposed. Any remaining wells of plate cavity were filled with sterile PBS. The plate was placed in to the 37°C CO₂ incubator. Plates were monitored daily, with a half media change every 2-3 days. Once cell outgrowth is observed the media volume can be increased to completely cover the biopsy piece.

Biopsy attachment was typically observed after 1-2 days. Cell outgrowth starts ~5-7 days later, with a confluent monolayer achieved between 20-30 days after plating, depending on initial plate size.

2.7.3. Primary Fibroblast Passaging

Once primary fibroblast cultures reached the outer edges of the initial culture dish cells were split. Briefly, cells were washed once with PBS. 500µl of pre-warmed TrypLE Express was added and cells were returned to the 37°C CO₂ incubator and monitored every 5 minutes for cell detachment. 2.5ml of pre-warmed fibroblast media was added to the plate and cells were gently pipetted up and down to before being transferred to a 15ml red cap tube and spun down at 100 x g for 5 minutes. Cells were resuspended with media and transferred to a T25 flask. The total media volume was increased to 5ml.

Once cells reach 80-90% confluency they can be passaged again. Typically, this was to 3x T25 flasks. When cells reach confluency again two flasks were frozen down for stocks. Cells were routinely split at 1:3

2.7.4. Primary Fibroblast Freezing

Cells were treated the same as passaging but at the pellet resuspension step 1ml of warmed 9:1 FBS:DMSO was added (larger volume for T75 freezing). 1ml of cells was added per cryo-vial and transferred to the -80°C freezer in a Mr. Frosty.

2.7.5. Mycoplasma Testing

Mycoplasma are a genus of bacteria lacking a cell wall, so difficult to target using conventional tissue culture antibiotics such as penicillin. Serial mycoplasma infections have been known to retard growth and alter the physiology and metabolism of the cell line being studied (Nikfarjam & Farzaneh, 2012). As such it is important to monitor cell cultures for mycoplasma infection.

Visual inspection can be used as a preliminary diagnosis. After fixing and staining cells with DAPI as per the protocol above, small extra cellular specs of DAPI fluorescence can be seen if a significant mycoplasma infection is present. The only definitive test however is attempted PCR based amplification of mycoplasma DNA from a sample of the media the cells are growing in. For this we used “LookOut Mycoplasma PCR Detection Kit” (MP0035) from Merck. Briefly, the kit contains Rehydration Buffer, which contains primers to amplify mycoplasma DNA. To this 2 μ l of media from the cell line to be tested was added. This media was aspirated just prior to splitting the cell line in question, so there was maximal growth time for the mycoplasma. Finally, 1 unit of HotFire DNA polymerase was added and the PCR was run using the manufacturers specifications. The resulting gel was run on a 1.2% agarose gel. A band at ~480bp indicates a successful PCR reaction, with a band at ~250bp indicating contamination with mycoplasma.

2.8. Cellular extractions

2.8.1. Protein Extraction from Cultured Cells

Cells were grown on plates and treated as desired prior to harvesting. Typically, cells would be transfected 48 hours prior to harvesting, and a minimum of 4 wells of a 24 well plate would be harvested.

The lysis buffer used for extraction varies depending on the subcellular localisation of the protein as well as the down-stream applications the proteins will be used for. RIPA buffer is widely used for extraction of cytosolic proteins as well mitochondrially localised proteins so is predominately used here.

For every 10ml of RIPA extraction buffer (150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0) a Complete Mini protease inhibitor cocktail tablet (Roche, 11836153001) is added. The RIPA + protease inhibitor is kept on ice throughout and can be stored at -20°C. The media is removed from the cell plates and washed twice with ice-cold PBS. RIPA +protease inhibitor is added to the plate (1ml for a well of a 6 well plate) and cells are detached using a cell scraper. The cell lysate is aspirated and transferred to a 1.5ml Eppendorf tube and pipetted up and down repeatedly to help disrupt cells. The tubes were incubated on ice for 30 minutes and vortexed every 10 minutes. If a large number of cells are being disrupted in a small volume cells can be passed through a 21-gauge needle repeatedly. Cells were then centrifuged at 10,000 x g for 15 minutes at 4°C to pellet cell debris. The supernatant was aliquoted and stored at -80°C.

Protein quantification was carried out on the Qubit Fluorometer using the Qubit protein assay kit (Q33211)

2.8.2. RNA Extraction from Cultured Cells

RNA extraction from cells was carried out using Qiagen's RNeasy mini kit (74104). 4 wells of a 24 well plate were normally used. Cells were treated as per the manufacturers protocol with slight modification at the DNase step, where cells were incubated at room temperature for 3 hours with DNase on the column. RNA was eluted in nuclease free water and stored at -20°C.

RNA was quantified on the NanoDrop ND-1000 Spectrophotometer.

2.8.3. DNA Extraction from Cultured Cells

Nuclear and mitochondrial DNA was extracted using Thermo Scientific GeneJET Genomic DNA Purification Kit (K0721) as per the manufacturers protocol. At least 1×10^6 cells were used for the extraction.

DNA was quantified on the NanoDrop ND-1000 Spectrophotometer.

2.8.4. Mechanical Extraction of Whole Mitochondria from Cultured Cells

Mitochondrial extraction from tissue culture cells is based on Spinazzi *et al.*, 2012 with modifications and is detailed in SOP F-30-040-00.

Cells are grown to near confluency in culture. Plate sizes from a well of a 6 well to a 15cm dish have been used. The media was removed from each plate and retained in separate 15ml tubes. Cells are trypsinised and the removed media is used to inactivate the trypsin. Detached cells were spun down at 1000 x g for 5 minutes. The media was then removed and the cell pellets were resuspended in ice-cold PBS and spun down at 1000 x g for 5 minutes, twice. After the final wash, the PBS was completely removed and the cell pellets were flash frozen in liquid nitrogen and kept on ice. Pellets were resuspended one by one in ice-cold 10mM Tris-HCL isotonic buffer pH 7.6 and transferred to a pre-cooled 2ml Glass-Teflon Dounce Homogeniser. Cells were

homogenised by up-down strokes of the pestle, the number of which depended on the cell type being used. Table 2-6 details the number of strokes for some commonly used cells types.

Cell Type	Number of Strokes
HEK293	30
HeLa	60
NIH 3T3	80

Table 2-6: Stroke number for Dounce Homogenisation of different cell types.

The cell homogenate was transferred to a 1.5ml Eppendorf and 200µl of 1.5M sucrose was added to minimise osmotic shock of the mitochondria. Clean the homogeniser thoroughly between each sample using detergent and ice-cold deionised water. Once all samples have been homogenised they were centrifuged at 600 x g at 2°C for 10 minutes to pellet larger cell debris. The supernatant can be kept at this point as a crude mitochondrial fraction, or a purer fraction can be obtained by centrifuging the supernatant at 14,000 x g at 2°C for 10 minutes. The supernatant is then discarded, and the remaining pellet is resuspended in 500µl of ice-cold 10mM Tris-HCL. Extracted Mitochondria can be flash frozen in liquid nitrogen and stored at -80°C for several months.

2.9. Live Cell Assays

2.9.1. Reactive Oxygen Species Cell Culture Assay

Cells are plated in a clear bottomed black walled polystyrene sterile 96 well plate (Falcon, 353219). Cell number at seeding depends on treatment of cells prior to running the assay. For HEK 293 cells, 2.5×10^4 if assay is to be performed 24 hours later, 1×10^4 if cells are to be transfected first. On the day of the assay cells were inspected for a confluent monolayer without any significant gaps. Cells are then treated with any compounds for the desired period of time by adding directly to the tissue culture medium and returned to the 37°C CO₂ incubator. After incubation DCFDA (Thermo, C10444; Abcam ab113851) was added to the cells to a final concentration of 5µM and returned to the 37°C CO₂ incubator for 30 minutes, taking care not to stain wells for background fluorescence quantification. Cells were then washed 3 times with PBS, with extreme care taken to not disrupt the cell monolayer. Cells were then imaged on the FLUOstar OPTIMA Microplate Reader using the bottom optic. Unstained wells were set as 5% of gain for the analysis. Cells can also be fixed in these wells after analysis for visual inspection of the fluorescence.

2.9.2. ROS assay for *ex vivo* retinal tissue

Mice were treated and aged for the appropriate times for the study in question. Aged mice were then enucleated and the retinae were dissected out. The retinae were then dissociated by incubation with 1ml trypsin (1mg/ml in HBSS) for 20 minutes at 37°C with the addition of 10µl DNase I (10mg/ml stock) after 5 minutes of incubation. Dissociated retinae were then incubated with 20µM DCFDA for 2 hours at 37°C with frequent agitation. Immediately before flow cytometry analysis DRAQ5 was added to stain live cells. Cells were gated for high DRAQ5 fluorescence, with 10,000 events being counted in this gate.

2.9.3. ATP Cell Culture Assay

Cells are plated in a white walled polystyrene sterile 96 well plate (Falcon, 353296). Cell number at seeding depends on treatment of cells prior to running the assay. For HEK 293 cells, 2.5×10^4 if assay is to be performed 24 hours later, 1×10^4 if cells are to be transfected first. Care must be taken to include wells without cells for blanks and standards. Cells are then treated with any compounds for the desired period of time by adding directly to the tissue culture medium and returned to the 37°C CO₂ incubator. The assay is carried out as per Abcam Luminescent ATP Detection Assay Kit (ab113849) with minor modifications. Briefly, the cell media is removed and 100µl of fresh pre-warmed media is added to each well. 50µl of detergent is added to each well and vortexed at 700 rpm for 5 minutes. A clear plastic adhesive lid is placed on the plate to prevent well crossover. 50µl of substrate solution is added to each well and the plate is vortexed for a further 5 minutes with an adhesive lid. The plate is then covered in tin foil and allowed to dark adapt for 10 minutes before the luminescence is measured on the FLUOstar OPTIMA Microplate Reader. A blank well is set as 5% of gain, or alternatively if a standard curve is used, the top point can be set to 95% gain.

2.9.4. Mitochondrial Fission and Fusion Assay

Cells were seeded into a 35mm µ-Dish (iBidi, 81156) 72 hours prior to the assay. 24 hours later cells were transfected with PA-GFP and a plasmid containing mito-dsRED plus the gene of interest. The presence of mito-dsRED allows successfully transfected cells to be identified. PA-GFP encodes for a mitochondrially targeted photoactivatable GFP which has minimal fluorescence until it is activated with UV light. This causes a conformational change in the PA-GFP and causes the protein to fluoresce brightly. The analysis is performed on a Leica SP8 gated STED confocal microscope which has an incubator on the microscope stage to allow the cells to

be maintained at 37°C and 5% CO₂. Cell dishes were placed in the incubated stage and given 10 minutes to equilibrate to temperature in order to minimise focal drift. Five mito-dsRED positive fields of view (FOV) are then identified, and their stage co-ordinates marked. Z-stack limits are then set for each of the target cells. The five FOV are imaged as T-1. A 2.5µm x 2.5µm region of interest (ROI) is then marked on an area rich in mitochondria in each cell. The microscope is then set to illuminate only the ROI (background set to 0%) with the UV lamp, with lamp power set to 40%. Sequentially, all five cells are then illuminated with the UV lamp in the predefined ROI for 5-10 seconds depending on the level of protein and therefore fluorescence seen. Cells are imaged immediately as T0. Cells are imaged further every 15 minutes for a total of 45 minutes. Care must be taken to ensure the z-stack focus does not shift considerably between timepoints.

Analysis of the images is completed using Leica Application Suite X. Two 2.5µm x 2.5µm ROIs are taken for each cell at each timepoint, one measuring the fluorescent intensity of the area that received UV illumination, and a second ROI elsewhere in the cell that did not receive any illumination. The intensity levels for the green channel (PA-GFP) are recorded. The green value in the unilluminated ROI for a given cell can be used to normalise the intensity levels of the cell to other cells, accounting for fluctuations in protein expression between cells. The overall level of fission and fusion is taken as the percentage of green signal that remains in the UV illuminated ROI after 45 minutes, when compared to the cell just after the ROI is illuminated.

2.9.5. Peredox Assay

Peredox is a fluorescent protein that can be used to assay the cytosolic NADH/NAD⁺ ratio in live cells (Hung, Albeck, Tantama, & Yellen, 2011). Briefly, Peredox is a circularly permuted GFP with a bacterial NADH-binding protein attached. Once NADH binds to Peredox, a conformational change takes place and the two halves of the circularly permuted GFP come into proximity with

each other allowing fluorescence to be emitted. It is also possible for NAD⁺ to compete with NADH to bind Peredox, but no conformational change takes place and thus no fluorescence is emitted. This means that as the NADH/NAD⁺ ratio changes in a cell, the relative levels of fluorescence will change too. Peredox is also fused to mCherry which allows normalisation of the green signal to total Peredox protein levels.

For this work, cells grown in media without phenol red were transfected with the pcDNA3.1-Peredox-mCherry plasmid (a gift from Gary Yellen (Addgene plasmid # 32383; <http://n2t.net/addgene:32383>; RRID: Addgene_32383)) using the standard Lipofectamine 2000 protocol, outlined previously. The cells were left for 48hrs. Cells were imaged on the confocal microscope using the heated stage with CO₂ at 5%, to maintain physiological conditions throughout the assay. As peredox is a highly dynamic sensor of the NADH/NAD⁺ ratio, the media cannot be changed prior to imaging if continuing ratios of NADH to NAD⁺ are to be investigated.

Cells were imaged using the appropriate green (ex:400nm em:510nm) and red (ex:575nm em:610nm) lasers and filters. Random fields were selected by visualisation in the red channel only. Image analysis was carried out on ZEN blue edition software by Carl Zeiss AG. An ROI was drawn in each cell and the intensity of the green and red channels was recorded. Care was taken not to record any overexposed cells. The green levels (NADH content) were then normalised to the red levels (Peredox protein content) in each cell.

2.9.6. TMRE Staining for Mitochondrial Content

Tetramethylrhodamine, ethyl ester (TMRE) is a fluorescent dye that is sequestered into the mitochondria of cells. Retention of TMRE is dependent on mitochondrial membrane potential. This means that only active mitochondria with robust membrane potential are stained by TMRE

and as such it can be used as a marker of cell mitochondrial health. It is not well retained upon fixation of stained cells, so its use is limited to live cell assays.

TMRE live Imaging

Cells were seeded into an appropriate dish for live imaging, typically μ -Slide 8 well chamber slides (ibidi; 80826). On the day of the assay the culture media was aspirated from the cells and media containing 200nM TMRE was added. The plate was returned to the tissue culture incubator for 20 minutes. Afterwards cells were washed once with PBS and warm complete media without phenol red was added to the plate. Cells were then imaged using confocal microscopy (ex:549 em:575) to examine mitochondrial health.

TMRE Flow Cytometry

Cells were typically grown and treated in 6 well plates. Cells were trypsinised as per the cell passaging protocol and resuspended to achieve $\sim 10^6$ cells per ml. Stock TMRE was added to the tubes containing suspended cells to a final concentration of 50nM. These tubes were incubated in a 37°C water bath for 30 minutes, with occasional agitation. DRAQ5 was added just prior to analysis as a live/dead stain. Samples were then analysed on the BD Accuri C6 flow cytometer using FL2 for TMRE and FL4 for DRAQ5. For each sample 10,000 events in a highly DRAQ5 positive gate were recorded.

2.9.7. MTS assay

The MTS assays were carried out as per the manufacturer's specifications (Promega; G3580). Briefly, cells were grown in a 96 well plate and treated with the relevant experimental parameters. MTS reagent was added to complete cell culture media at a concentration of 20 μ l per 100 μ l. The growth media in the 96 well plate was aspirated then the MTS media was added. The plate was then returned to the 37°C cell culture incubator for 1 hour before being measured via plate reader at 490nm.

2.10. Seahorse XFe Analyzer Assays

2.10.1. Overview

The Seahorse XF Analyzer is a powerful tool that can be used to measure the Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) of tissue culture samples and, potentially, *ex vivo* tissue for experimental animals. During the course of my Ph.D. I used two formats of the machine, the XFe24 and the XFe96, which as the name suggests use either a 24 well plate or 96 well plate format. As the only significant differences between the protocols for using the two machines are the volumes of media and reagents used, for expedience's sake I will just outline the protocols for the XFe96.

The Seahorse XFe96 is a temperature-controlled analyser that uses a sensor cartridge to measure and record the levels of oxygen and H^+ in the media of a cell sample. The running procedure of the Seahorse is typically broken into cycles with two sections, mix and measure, each section lasting 3 minutes. This cycle is then repeated until the end of the run. During the mix cycle the sensor cartridge is repeatedly gently raised and lowered in the cell media to mix its contents, and to reoxygenate the media. During the measurement stage the sensor cartridge is lowered to just 200 microns above the cell layer, creating a transient "micro chamber". It is at this point that the fluorescent probes interact with O_2 and H^+ in the media, giving a reading that is remeasured repeatedly over the 3 minute measure step in order to produce a rate. Because of the creation of this micro chamber there is only a finite amount of O_2 in the media and so it is important that a suitable number of cells are seeded; too many and all the oxygen in the chamber is no longer in excess, leading to artificially deflated OCR measurements.

The exciting part happens when you utilise the injection ports, of which there are 4 (A, B, C and D), to provide reagents that can alter and augment the OCR and ECAR of the sample. Throughout the course of this work I used a number of different reagents and injection schemes. Here I will

outline them, what reagents are used and the rationale behind what information these assays elucidate about cellular metabolism. All of the reagent concentrations need to be titrated to give the optimal response depending on the cell type and seeding density and as such I will only detail the concentrations I most commonly used.

2.10.2. Assay Preparation: 24hrs+ Beforehand

Cells are seeded in a Seahorse cell culture plate a suitable amount of time before the assay, depending on whether or not cells need to be pre-treated, such a plasmid transformation. For the XFe96, seeding densities used were the same as a standard 96 well tissue culture plate (see above) except that the cells were initially plated in 80µl of media to allow optimal seeding of the cells in the plate. The four corner wells are left unseeded but have 80ul of the media used to seed added. These wells will act as blanks. Any unused wells can also act as blanks, provided media is added at the time of seeding.

A sensor cartridge was hydrated by adding ~200µl of XF Calibrant Buffer and was placed into a 37°C non-CO₂ incubator overnight (min 8hrs, max 3 days (Seahorse Representative, personal correspondence). Care needs to be taken to ensure there are no bubbles in the wells that may interact with the probes.

2.10.3. Assay Preparation: Day of Assay

The assay needs to be run in a buffer that does not contain any strong buffering chemicals typically found in tissue culture media such as sodium bicarbonate. It is also preferable to use media that does not contain phenol red as it interacts with the pH probe giving marginally altered readings (this is only really a concern when running an assay that quantifies ECAR, rather than just providing the rate, such as the ATP rate assay). The pH of the media also needs to be

carefully determined, with pH 7.4 being optimal. Thankfully Seahorse provide a buffer-less, phenol red-less base media at pH 7.4 (103575-100; Agilent). All that needs to be added is any necessary energy sources (Glucose, Galactose and Sodium Pyruvate typically) and L-Glutamine.

Any chemicals to be injected are also prepared using the Seahorse assay media, care needs to be taken to use the appropriate media for the test condition of the well if multiple medias are being prepared (ie don't use glucose containing media to inject into a galactose containing well).

Prior to the assay, the media the cells were plated in is replaced with 37°C assay media. The cells are washed with the assay media twice before the total volume of media is increased to 180µl. Typically during the wash process I would not completely remove the media, leaving ~50µl each time to avoid damaging the cells with the pipette tips. This cell plate is then placed in a 37°C non-CO₂ incubator for 1 hour. Once the injection ports of the sensor cartridge have been filled, the plate can be loaded into the machine. The injections are 10x so the volume increases with each injection: 20µl, 22µl, 25µl and 28µl.

2.10.4. Mito Stress Test

Injection Port	Reagent	Concentration (in port;10x final)
A	Oligomycin	10 μ M
B	FCCP	5 μ M
C	Rotenone/Antimycin A	5 μ M

Seahorse base media supplemented with 100mM Pyruvate, 200mM L-Glutamine and either 2.5M Glucose or 2.5M Galactose, depending on what is being investigated. Figure shows an idealised OCR trace when using the Mito Stress test kit. The initial measurements show the basal OCR, indicative of resting state respiration. Oligomycin is an inhibitor of ATP synthase (Complex V; CV). It effectively causes a back-log of H⁺ ions that have been pumped out of the mitochondria due the activity of the ETC, but which cannot return through the canonical pathway via CV. Thus, it becomes more difficult for protons to be pumped out to the intermembrane space against the H⁺ gradient and as a result the OCR drops. The addition of FCCP acts as an “uncoupler”, which uncouples proton pumping from passing electrons down the ETC. FCCP achieves this by transporting H⁺ ions through the mitochondrial membrane, essentially bypassing the inhibited CV and relieving the H⁺ build up. This allows the ETC to operate at maximal capacity, giving the highest OCR. After this the Rotenone/Antimycin A mixture is injected which inhibits both CI and CIII, respectively. This disables electron flow down the ETC and so the OCR is at its lowest rate, showing only non-ETC related oxygen consumption.

The Mito Stress test is a very useful starting point when investigating mitochondrial deficiencies. It gives an overview of the energy profile of a cell (or tissue) and can highlight any potential areas where dysfunction may be arising or affecting. For example, a decreased spare respiratory

capacity can indicate a cell under stress and an increase in ECAR can indicate a shift to aerobic glycolysis to compensate.

2.10.5. Glycolysis Stress Test

Injection Port	Reagent	Concentration (in port; 10x final)
A	Glucose	100mM
B	Oligomycin	10 μ M
C	2-DG	500mM

The Glycolysis Stress test uses Seahorse base media supplemented with just 200mM L-Glutamine. Figure 2- shows the idealised ECAR trace for the glycolysis stress test. Due to the absence of any glucose at the start of the assay, there is a background rate of non-glycolytic Acidification. The addition of glucose allows glycolysis to take place, with its concomitant increase pH, so the ECAR increases. The addition of oligomycin inhibits CV and thus forces the cell to shift its reliance away from oxphos to aerobic glycolysis to produce the ATP required for the cell. This results in an increase in the ECAR to maximal levels. Finally, the addition of 2-Deoxy-Glucose (2-DG) inhibits glycolysis resulting in a return to the background rates of glycolysis. 2-DG has a -H group instead of the -OH group found in glucose. This means it cannot undergo isomerization by Glucose-6-phosphate isomerase, the second step in glycolysis and so acts to competitively inhibit glycolysis.

The glycolytic stress test can be informative when attempting to rescue cells that have oxphos dysfunction. There is often a compensatory increase in the rate of aerobic glycolysis when a cell is not producing a sufficient amount of ATP from oxphos alone. As such, monitoring the glycolytic rate can inform whether a cell has indeed modulated its metabolism to assuage detrimental oxphos mutations and inhibition.

Seahorse XF Cell Mito Stress Test Profile

Mitochondrial Respiration

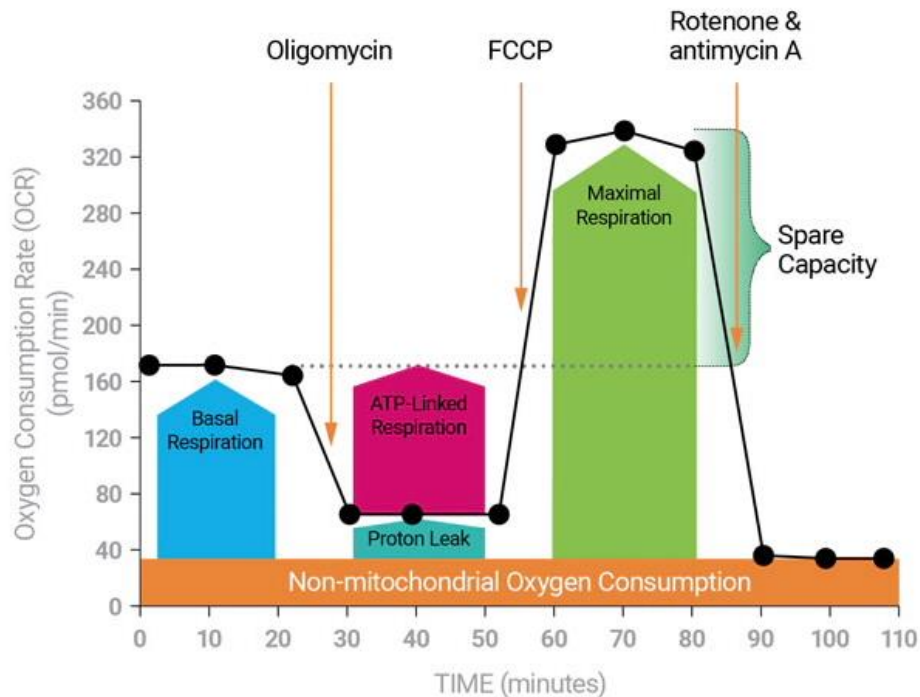


Figure 2-1: An idealised OCR trace of response to using the mitochondrial stress test. A number of calculations can be run on this, including identifying spare respiratory capacity. Reproduced from the Agilent website.

Seahorse XF Glycolysis Stress Test Profile

Glycolytic Function

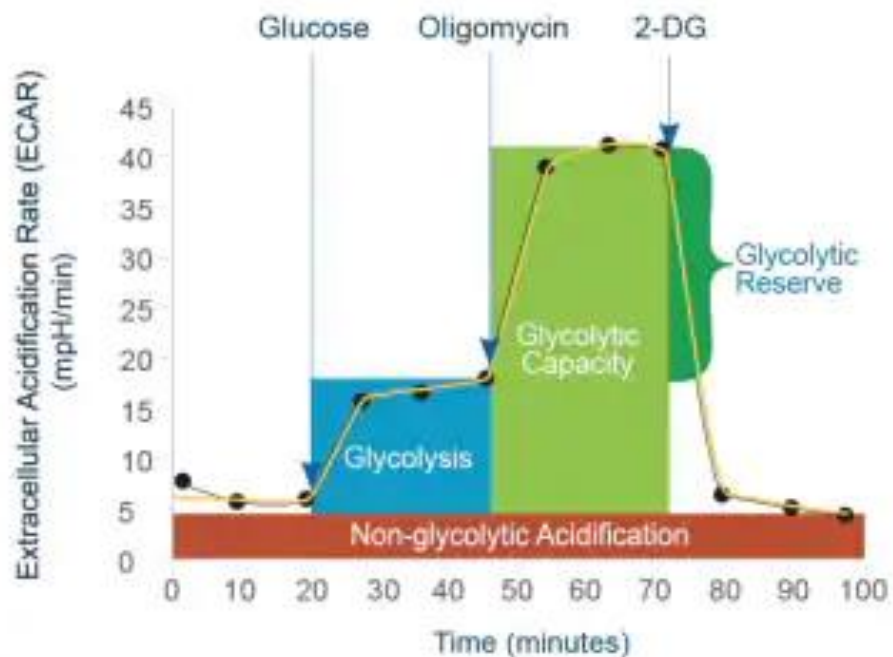


Figure 2-2: An Idealised ECAR trace of response to using the glycolytic stress test. Measuring the ECAR in cells gives an estimate of the rate of aerobic glycolysis. Reproduced from the Agilent website.

2.10.6. ATP Rate Assay

Injection Port	Reagent	Concentration
A	Oligomycin	15 μ M
B	Rotenone/Antimycin A	5 μ M

For this assay base media with 10mM glucose, 1mM pyruvate and 2mM glutamine is used. As this assay attempts to give quantifiable results it is also important to ensure the media is pH 7.4, does not have phenol red, and that the buffering factor is known (see below for buffering factor calculations). The first injection of Oligomycin decreases OCR but ECAR increases to compensate for the loss of mitochondrial ATP production. Next the addition of Rotenone completely inhibits mitochondrial respiration, leaving the cells completely dependent on aerobic glycolysis for ATP production (see Figure 2-3).

By comparing the OCR and the ECAR it is then possible to calculate the amount of ATP being generated by each pathway, oxphos and glycolysis. This provides a more in depth analysis of the cells reliance on a certain pathway that either the Mito Stress test or the Glycolysis Stress test. Furthermore, the accurate quantification of ATP production in pmol/min allows direct comparison of different test runs.

2.10.7. Glycolytic Rate Assay

Injection Port	Reagent	Concentration
A	Rotenone/Antimycin A	5 μ M
B	2-Deoxy-Glucose	500mM

For this assay base media with 10mM glucose, 1mM pyruvate and 2mM glutamine is used. As this assay attempts to give quantifiable results it is also important to ensure the media is pH 7.4, does not have phenol red, and that the buffering factor is known. The initial Rot/AA injection forces cells to utilise glycolysis for ATP production. The 2-DG injection then inhibits glycolysis allowing for quantification of the background ECAR (See Figure 2-4).

This assay allows the rate of glycolysis to be accurately quantified in the form of a Proton Efflux Rate in pmol/min. Furthermore, it allows the dissection out of some potentially confounding results from the glycolysis data. Under normal conditions there are two main contributors to the ECAR in a cell sample. The first, of course, is glycolysis. The second is mitochondrial-associated acidification. This occurs when the pyruvate produced by glycolysis is not converted to lactate by lactate dehydrogenase (LDH) but is instead used to fuel the TCA cycle. This use of pyruvate in turn produces CO₂ and water. It is then possible for the CO₂ to acidify the extracellular media via carbonic acid. This means that measuring ECAR alone without correcting for mitochondrial-associated acidification leads to an over estimation of the true glycolytic rate of the cells.

2.10.8. Calculation of Media Buffer Factor

The ECAR measurement is in terms of milli-pH per minute (mpH/min), which is a qualitative measure. In order to convert this into the quantitative Proton Efflux Rate (PER), we need to

calculate the buffering factor of the media the assay is carried out in. Calculation of the PER is essential for the ATP Rate assay and the Glycolytic Rate assay.

The buffering factor is expressed in terms of H^+ mM/pH, or the amount of H^+ ions needed to change the pH by 1 unit. In order to calculate this a number of wells are set aside during a seahorse run, typically 4-8 wells. 175 μ l of assay media was added to these wells and 25 μ l of 5mM HCL was added to injection ports A, B and C. The assay is then run as normal for the rest of the plate (if an assay using only 2 injections is being used, such as the glycolytic rate assay, it is important to add a third injection step to the protocol). The resulting data can then be inputted to the Seahorse XF Buffer Factor Calculator which outputs the buffer factor for the assayed media.

2.10.9. Normalisation

Runs were normalised to protein content unless otherwise stated. After the seahorse run has completed, the media is carefully aspirated from the plate, taking care not to disturb the cell monolayer which can be fragile due to the addition of numerous mitochondrial inhibitors during the assay. At this point the plate can be stored at -20°C for future quantification.

When ready for quantification, 100 μ l of RIPA protein lysis buffer is added to each well and is pipetted up and down repeatedly to lyse cells. 10 μ l from each well is then transferred to a new plate containing 300 μ l Bradford reagent per well. The assay is then carried out as per the manufacturers protocol (Thermo Fisher – Cat. No. 23246).

Protein concentration values can then be inputted via the Wave software used for Seahorse data analysis in order to normalise the seahorse runs to protein content.

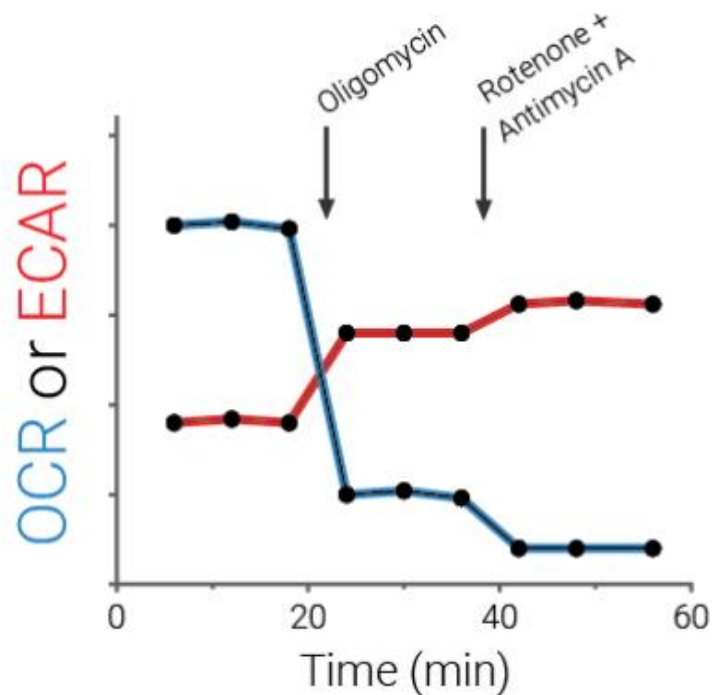


Figure 2-3: Representative trace from an ATP Rate assay. The blue line represents OCR and the red line ECAR. As the OCR decreases due to the addition of various inhibitors, the ECAR increases due to increased glycolysis which maintains ATP production rates. Reproduced from the Agilent website.

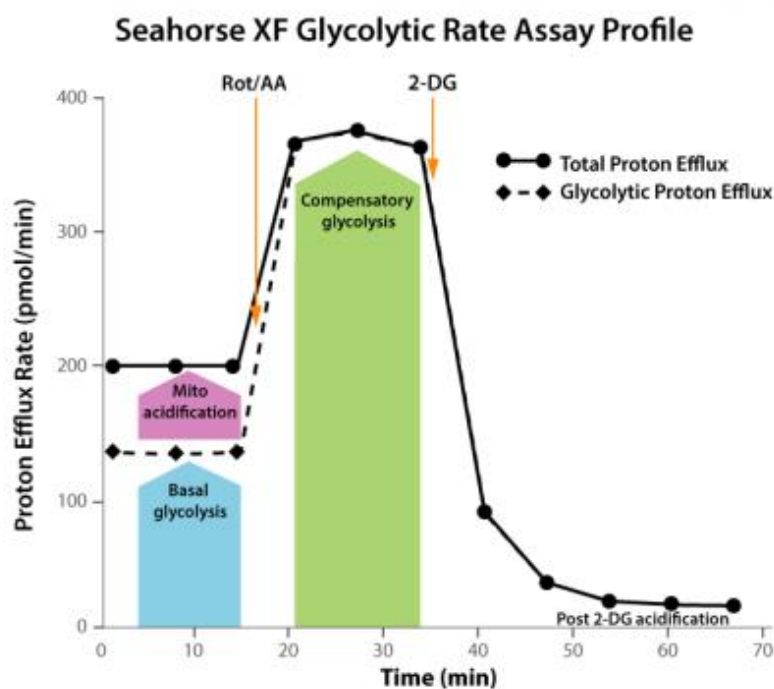


Figure 2-4: Representative ECAR trace from a Glycolytic Rate Assay. Here the solid line shows the total ECAR, whereas the dashed line shows the true glycolytic rate after correcting for mitochondrial-associated acidification. Note that the two traces overlap after the addition of the oxphos inhibitors Rot/AA. Reproduced from the Agilent website.

2.11. Electroretinogram (ERG)

ERG analysis allows us to interrogate the electrophysiology of the eye in question, giving us a detailed read out of the electrical activity of the eye. This is achieved by providing controlled pulses of light to the eye of a mouse to stimulate the light sensitive cells in the retina, thus causing them to emit an electrical signal. This electrical signal can then be measured via electrodes which can allow the detailed interrogation of the functionality of specific cell populations in the retina, and also how well the circuitry of the neural retina is functioning as a complete unit. ERGs are a particularly sensitive methodology for measuring visual function as they can identify and quantify subtle differences in the functionality of the cells of the retina which might not be apparent by other means such as examining the gross morphology of the retina through histology. Using this technique, it is possible to measure the functional consequences of any potential therapeutics *in vivo* by, for instance, treating one eye with AAV and comparing it to the untreated eye.

A sample ERG trace can be seen in figure 2-5, identifying the main components of the ERG trace, the a-wave and the b-wave. At N the light stimulus is administered to the animal. Initially the hyperpolarization of cells causes a dip in the amplitude of the trace, which corresponds to the hyperpolarisation of the cells being interrogated. The difference between the trace at T=0 and the bottom of this dip is the a-wave. The a-wave is followed by the depolarisation of cells in the retina which causes a jump in the amplitude of the trace. The amplitude between the bottom of the a-wave to the peak of this trace is known as the b-wave. The trace then proceeds to return to baseline over time. Of particular interest to the work in this thesis, the amplitude of the b-wave can be used as a proxy for the functionality of the retina, with larger b-waves suggesting better visual function.

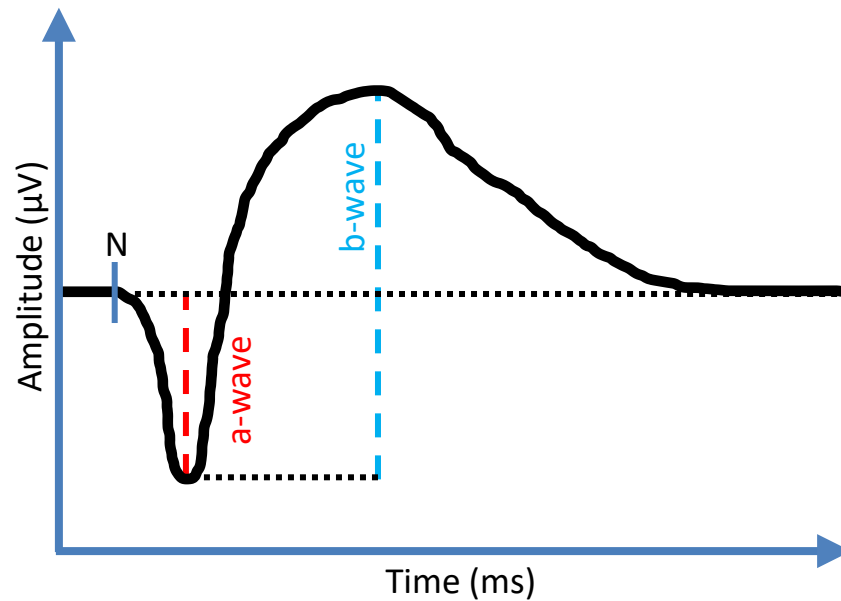


Figure 2-5: A sample ERG trace showing an idealised response to a light stimulus delivered at N. This light stimulus causes an initial hyperpolarisation of retinal cells causing a decrease in amplitude. The difference between the amplitude at T=0 and the lowest point of the trace is the a-wave. The amplitude of the trace then increases with the depolarisation of retinal cells. The difference between the lowest point of the trace and the peak of the trace is the b-wave.

The specific cells activated in the retina depend on the light stimulus delivered to the eye and whether the mouse was dark adapted or not prior to the ERG. This in turn alters the shape of the trace.

To carry out ERG analysis, mice are dark adapted overnight. The following day the mice are sedated with an intraperitoneal injection of ketamine and xylazine. Pupils were dilated via administration of 1% cyclopentolate and 2.5% phenylephrine drops. Reference and ground electrodes were placed sub-dermally ~1mm from the corner of the eye and at the base of the tail. ERG responses are recorded simultaneously from both eyes using gold wire electrodes positioned to touch the cornea. Visidic drops are administered to the eyes to maintain hydration and electrical contact. Mice are placed on a heating pad to insure they do not drop below 37°C whilst sedated. The mouse is then placed inside the Ganzfeld dome of the ERG machine, where

pulses of light are flashed, and the electrical response of the retina is measured and analysed using the RetiScan RetiPort electrophysiology unit (Roland, Brandenburg, Germany).

Figure 2-6 shows sample traces from a 1-month old C57 mouse. The dark-adapted ERG is termed the scotopic ERG, and is rod dominated. This is measured using a dim white light flash of -25dB from maximal 3 candelas/m²/s (Figure 2-6 A). Maximum responses are measured with a bright flash (full intensity 3 candelas/m²/s) causing both cones and rods to signal (Figure 2-6 B).

Mice were then light adapted for 10 minutes in light of 30 candelas/m². The single flash cone (SFC) provides one quick pulse of light (3 candelas/m²/s) that specifically interrogates the cone cell response as they are more sensitive to quick pulses of light (Figure 2-5 C). Finally, a flickering light source (10Hz, 3 candelas/m²/s) allows the interrogation of repeat stimulus to the retina and is cone dominated as rods cannot recover quick enough to be stimulated again (Figure 2-5 D).

For the analysis of Ndi1 as a potential therapeutic intervention for complex disorders (Section 3.2.6), the rod b-wave, max a-wave, max b-wave, single flash cone b-wave and 10Hz flicker b-wave were used as these measures give an overview of photoreceptor functionality. Each eye was measured as a separate unit. When mice were treated in one eye and not the other the data was treated as paired for statistical analysis purposes.

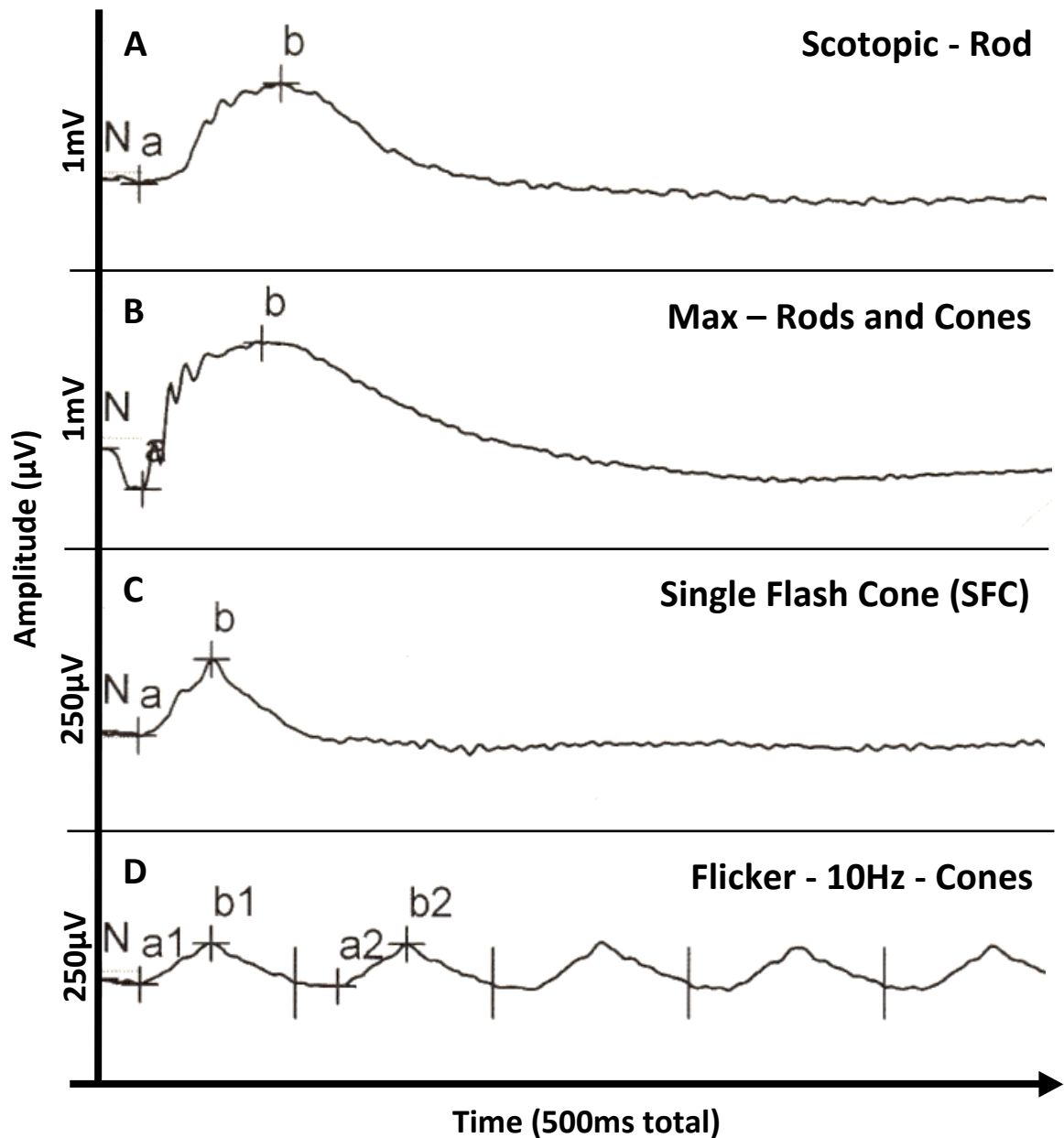


Figure 2-6: Four traces demonstrating the different ERG methods used to interrogate retinal function in this thesis. The ERG traces here were carried out on a 1 month old C57 mouse. A) The dark adapted, scotopic, trace which is produced by dim flashes of light that specifically excite rod photoreceptors. B) A much brighter flash is used for the Max trace which shows increased a and b-waves compared to A), as both rods and cones are stimulated. C) The photopic single flash cone (SFC) response. This is a quick flash of light that only cones respond to. D) 10Hz Flicker trace N corresponds to the light stimulus. Lower case a and b indicate the computationally determined lowest and highest point of each trace, used for calculating the a and b-wave responses. Note A) and B) are on the same scale of 1mV with C) and D) on the same scale of 250μV.

2.12. Data Handling and Statistical Analysis

Individual experimental units (n) are defined throughout the results section of this thesis but in general are separate units which have been individual subjected the experimental parameters in question. For instance, a well treated with a plasmid transfection for a Seahorse experiment or an eye treated with virus would be an individual n.

All data was collated, and raw numbers were inputted into either Microsoft Excel or R Studio for data analysis. Data was checked for normality by plotting the overall distribution and comparing mean and median statistics. If normality was assumed, then a Student's t-test was carried out when comparing two groups. If more than two groups were being analysed the ANOVA was used, with Tukey's Honestly Significant Difference (HSD) post-hoc analysis, $\alpha = .05$, used to identify pairings with significantly different means.

If the data could not be confirmed as being normal, then non-parametric statistical methods were employed. This was either the Krushkal-Wallis test for testing the relationships between more than two non-parametric groups, or the paired Wilcoxon test for analysis of paired non-parametric data.

All graphs in this thesis were produced using either Microsoft Excel or ggplot2 in R Studio.

3. Investigating Ndi and optNdi1 gene therapies for the treatment of LHON and complex ocular disease.

3.1. Introduction

3.1.1. Alternative NADH:ubiquinone Oxidoreductases

In mitochondria, NADH generated by the cell (typically through the citric acid cycle) is oxidised by an NADH:ubiquinone oxidoreductase (NADH dehydrogenase; NDH) and its electrons are transferred to an electron carrier, ubiquinone. In human mitochondria, and indeed all mammalian mitochondria investigated so far, this is carried out via complex I. In plants, yeast and bacteria however, there exists a class of alternative NDH which are also capable of fulfilling this role (Stefan J. Kerscher, 2000). These alternative NDH take two forms, internal and external. The internal versions (ndi), such as *Ndi1*, are located on the inner mitochondrial membrane facing in towards the mitochondrial matrix and metabolise NADH produced by the Citric Acid cycle (TCA cycle). The external versions (nde) are located on the outer mitochondrial membrane and can oxidise NADH from cytoplasmic sources, such as glycolysis. The localisation appears to be the biggest difference between ndi and nde, with work showing that retargeting the external *NDH2* to the internal mitochondrial membrane of *Y. lipolytica* can compensate for rotenone induced complex I dysfunction (S J Kerscher, Eschemann, Okun, & Brandt, 2001). The same study also showed no observable negative effects to growth or lifespan when the *NDH2* gene was deleted entirely.

Studies have shown that alternative NADH:ubiquinone oxidoreductases are present in members of all the phylogenetic kingdoms, including animalia (Matus-Ortega et al., 2011). The animals that contain NDH enzymes according to this study are *T. adhaerens*, *N. vectensis*, *H. magnipapillata*, *C. intestinalis*, and *S. purpuratus*. *T. adhaerens* is the last extant member of the Placozoa, placed near the root of the metazoan lineage. The next two are sea anemones, followed by a sea urchin and finally the sea squirt *C. intestinalis*, an animal we will hear a lot more about in chapter 5 of this PhD thesis regarding alternative oxidases. It is likely that other examples of NDH enzymes will be found in the animal kingdom with an increase in the number

of whole genome sequences available from a more diverse cohort of species. It does seem unlikely though that any “higher” animals have NDH enzymes. A hypothesis for the loss of NDH enzymes in animals is that these enzymes are non-proton motive (Matus-Ortega et al., 2011). Unlike endogenous complex I, they do not shuttle enzymes across the mitochondrial inner membrane as they oxidise NADH, and thus do not directly contribute to ATP synthesis. They are in fact uncouplers of mitochondrial respiration. In more energy demanding organisms this metabolic uncoupling could act as a disadvantage, whereas in sedentary filter feeders such as the aforementioned animals, a certain degree of inefficient metabolism can be tolerated when weighed against the benefits of having an NDH enzyme.

However, the picture is not quite as clear cut as stated above. More recent work has identified two proteins, the rather unfortunately named Apoptosis-inducing factor (AIF) and AIF-homologous mitochondrion-associated inducer of death (AMID), that could function as mammalian NDH enzymes. Research has shown that they can have NDH activity when expressed in *E. coli* and using *in vitro* reconstituted mitochondrial membranes (Elguindy & Nakamaru-Ogiso, 2015). Under normal physiological conditions AIF is located in the intermembrane space or loosely associated to the outer membrane of the mitochondria, and got its name from the caspase dependant translocation of AIF to the nucleus upon the initiation of apoptosis (Susin et al., 1999). AIF also appears to play a role in stabilising the assembly of the ETC complexes. A mouse model of AIF deficiency causes downregulation of ETC complexes, in particular CI, which leads to photoreceptor and retinal ganglion cell death. The retinal phenotype can be rescued by administration of methylene blue (MB), which is a surrogate electron carrier (Mekala et al. 2019). It appears that AIF now functions in OXPHOS via the assembly and stabilization of the ETC components and modulation of the structure of mitochondria rather than carrying out an NDH activity specifically, and that its apoptotic role is secondary to its primary role in mitochondrial bioenergetics (Bano & Prehn, 2018). The decrease in CI activity in AIF deficient mice is believed to be due to either decreased biogenesis or

stabilisation of CI subunits (Vahsen et al., 2004). This raises an interesting possibility that the NADH oxidation activity of AIF has evolved from having a direct metabolic role like ancestral NDH proteins, oxidising NADH to reduce ubiquinone, to an indirect role of upregulating or stabilising CI production in mammalian mitochondria. Further weight is given to the importance of the NADH binding domain of AIF when you consider its disease-causing mutations. It appears that only drastic decreases in expression levels or elimination of the NADH redox activity of AIF causes severe neurodegeneration (Sevrioukova, 2016). For instance the G308E mutation causes almost complete ablation of the NADH oxidation capabilities of AIF and is prenatally fatal as a result (Berger et al., 2011). Furthermore the harlequin mouse (so called due to its pattern of hair loss) shows a ~80% reduction in AIF expression and as a result shows significant increases in oxidative stress and neuronal damage in the brain and the retina (Klein et al., 2002). Intriguingly recent work has shown that there is a brain specific isoform of AIF called AIF2. KO of this isoform was well tolerated by mice under normal conditions but led to an increase in brain damage through ischemia reperfusion injury (Rodriguez et al., 2019). However, there was no increase in apoptotic pathways or decrease in ETC subunits but there was a significant increase in oxidative stress, indicating that AIF2 either can act as a free radical scavenger or it plays a hereto unidentified role in mitochondrial homeostasis. The separation of the redox and apoptotic activities of AIF has been achieved by altering a cleavage site and effectively anchoring AIF to the mitochondrial inner membrane, which corrected mitochondrial defects in neurons of an AIF-null mouse (Cheung et al., 2006). This raises an interesting possibility that one could disable or at least abrogate the apoptotic role of AIF and maintain the redox activity that appears to be beneficial for the mitochondria. It remains to be seen if AIF (and its far less studied family member AMID) could be used as an NDH in mammalian mitochondria, perhaps by retargeting away from its current location in the intermembrane space (Susin et al., 1999) to the inner surface of the mitochondrial inner membrane to keep it in close proximity with the NADH pool of the mitochondrial matrix and the other members of the ETC.

Why not chose a proton pumping NADH:ubiquinone oxidoreductase for the gene therapy? Due to the complexity of the chemistry involved in pumping protons across the inner mitochondrial membrane concomitantly with NADH oxidation, it appears there is a minimal limit to the size the protein or protein complex can be. The *E. coli* complex I, encoded by the *nuo* operon is considered to be “a structural minimal form of the proton-translocating NADH:ubiquinone oxidoreductase” (Schneider et al., 2008). This *nuo* operon still contains 14 subunits and the coding sequence spans some 15kb (Weidner et al., 1993). This is of course far too large for AAV vector delivery methods whose current limit stands at around 4.8kb (Wu et al., 2010).

3.1.2. Ndi1

It was more than fifty years ago that the existence of Ndi1 was first suggested, due to the notable absence of a rotenone sensitive NADH oxidoreductase, like complex I, in the mitochondria of the yeast, *S. carlsbergensis* (the yeast responsible for Carlsberg lager, for better or for worse) (Ohnishi, Kawaguchi, & Hagihara, 1966). These *S. carlsbergensis* mitochondrial extracts were prepared by using “snail gut juice” and make me glad I conduct my research in this day and age. Further work in the closely related yeast *S. cerevisiae* elucidated that there were at least two proteins that carried out this NADH oxidation role, one located in between the inner and outer mitochondrial membranes, Nde1, and one located in the inner membrane, Ndi1 (de Vries & Grivell, 1988). The Ndi1 gene was first identified in the early 90s (Marres, de Vries, & Grivell, 1991). Ndi1 is a nuclear gene and as such has a mitochondrial localisation signal to aid import into the mitochondria. A few years later it was noted that allotopic expression of Ndi1 in *E. coli* conferred rotenone resistance to the bacteria, showing that Ndi1 can be incorporated in the *E. coli* electron transport chain (Kitajima-Ihara & Yagi, 1998). A few months after that, the same group successfully demonstrated that Ndi1 can localise to the mitochondria and incorporate in the electron transport chain of mammalian cells (B B Seo et al., 1998), thus initiating interest in Ndi1 as a potential therapeutic for complex I disorders. Further work showed that Ndi1 could be successfully delivered via AAV to HEK293 cells where its expression

protected from rotenone induced complex I dysfunction (Byoung Boo Seo, Wang, Flotte, Yagi, & Matsuno-Yagi, 2000). Subsequent to this, complex I dysfunction mediated by deletion of the ND4 gene in mitochondria was compensated for by Ndi1 expression (Bai et al., 2001). All these data pointed towards the utility of Ndi1 in complex I disorders.

For the next few years, the focus of Ndi1 research shifted towards its potential utility in Parkinson's Disease (PD). At this point the aetiology of PD was starting to be investigated, and the evidence seemed to point towards mitochondrial dysfunction (Parker, Boyson, & Parks, 1989), in particular Complex I dysfunction (Mizuno et al., 1989; Parker et al., 1989). Chemically induced PD was initially noted during studies of a synthetic opioid drug MPPP. Unbeknownst to chemists at the time, an impurity in the form of MPTP can also be produced which on its own is not toxic, but can be transported across the blood-brain barrier and taken up by neurons where the neural specific MOA-B enzyme converts it to MPP⁺ which is highly toxic to cells due to inhibition of CI (Nicklas, Vyas, & Heikkila, 1985). This links specific mitochondrial inhibition of CI in neurons with PD. Exposure to MPTP in humans (mainly through illicit use of MPPP) causes an extremely rapid onset PD (Davis et al., 1979). The establishment of a link between pesticide exposure, commonly experienced by farmers, and PD suggested a link to mitochondrial dysfunction as a majority of pesticides target respiration or cause extreme oxidative stress (Gorell et al., 1998). Research into chemically induced animal models of PD led to rotenone induced PD. Rats were continuously perfused with 2-3mg/kg of rotenone per day, which caused damage without being lethal, at least over the 5-week study period. These rats showed systemic CI dysfunction, highly selective nigrostriatal dopaminergic degeneration and the accompanying motor function disruption considered hallmarks of PD (Betarbet et al., 2000). As Ndi1 is a rotenone insensitive CI substitute it seemed logical to investigate its therapeutic potential in PD.

The transient expression of Ndi1 in human neuroblastoma cells via plasmid transfection countered the damage caused by rotenone, protecting cells from death (Sherer et al., 2003).

Subsequent work packaged *Ndi1* into an AAV vector and demonstrated it could be delivered to mouse muscle and neuronal tissue where it can safely and sustainably express (Boo Seo et al., 2004). A follow-up study by the same group demonstrated that AAV-Ndi1 could protect mice in an acute MPTP induced model of PD (Byoung Boo Seo, Marella, Yagi, & Matsuno-Yagi, 2006). This was the first study to demonstrate that Ndi1 could be used to counteract CI dysfunction *in vivo*. Treated rodents received unilateral injections of rAAV-Ndi1 into the substantia nigra area of the brain followed by intraperitoneal injection of MPTP. In the treated side there was a significant increase in NADH oxidation activity as well as protection of tyrosine hydroxylase positive cells, a key enzyme in the dopamine production pathway and marker for neuronal tissue. This in turn led to maintenance of roughly 50% of the wild type levels of dopamine in the treated hemisphere of the substantia nigra (Byoung Boo Seo, Nakamaru-Ogiso, Flotte, Matsuno-Yagi, & Yagi, 2006). Ndi1 went on to show promising results in the chronic rotenone and MPTP models of PD as well, where brain function and motor function showed some preservation (Barber-Singh et al., 2009; Marella et al., 2008).

3.1.3. Ndi1 Therapy for Complex I Disorders

Fulfilling the role of NADH:Ubiquinone oxidoreductase, Ndi1 is a clear candidate therapeutic for complex I disorders. With this in mind, focus shifted to the use of Ndi1 in Leber Hereditary Optic Neuropathy (LHON).

It is clear that while there is significant genetic heterogeneity with LHON, complex I dysfunction is the common factor. Development of a therapeutic for each of these genetic insults would be extremely time-consuming, especially when considering that some mutations appear to be geographically specific or even family specific. With this in mind, a mutation independent therapeutic, such as Ndi1 in principle could have great potential. Initial work investigating this was carried out on a 134B cybrid cell line with 11778G > A patient mitochondria (Park, Li, & Bai,

2007). The Ndi1 plasmid transformed cells showed correction for the LHON associated drop in OCR, as well as decreases in ROS and apoptosis induced by known mitochondrial stressors. A rather interesting approach to modelling LHON was utilised involving rotenone releasing microspheres injected into the superior colliculus, the region of the brain that receives impulses from the optic nerve, which leads to demyelination of the optic nerve, significant decreases in retinal thickness and a complete loss of optokinetic response (OKR) in these animals by four weeks. The injection of rAAV5-Ndi1 concomitantly, as well as two weeks post rotenone injection to the super colliculus showed significant protection of the OKR in these animals, as well as increased RGC counts compared to rotenone treated animals (Marella, Seo, Thomas, Matsuno-Yagi, & Yagi, 2010). This study provided evidence of the efficacy of Ndi1 as a potential LHON therapeutic, however the delivery method, direct injection into the superior colliculus, is not a viable routine procedure for patients. In contrast, our group investigated ocular delivery of Ndi1 for rotenone induced LHON (Chadderton et al., 2013). Mice were injected intravitreally (IV) with AAV2/2-Ndi1 and showed robust transduction of RGCs, the cell type most vulnerable in LHON. Three weeks post AAV2/2-Ndi1 or AAV2/2-eGFP transduction, rotenone was injected intravitreally. It was found that Ndi1 expressing animals maintained significant levels of RGCs compared to eGFP treated animals. The correlated with a functional improvement of OKR in rotenone + Ndi1 treated animals versus rotenone + eGFP animals and additionally improvements as evaluated by MRI (Chadderton et al., 2013).

Subsequent research in our lab has led to the development of an optimised version of Ndi1, entitled optNdi1. This optimisation process was started by a previous Ph.D student, Dr. Mathew Carrigan. For the creation of optNdi1, the Ndi1 sequence was optimised for expression in human systems. This involved modifying the codon sequence to better reflect human codon biases instead of *S. cerevisiae*, as well as the addition of a Kozak sequence. The sequence was also analysed for any regions of predicted immunogenicity, with a number of sequences with conservative amino acid changes to minimise such potential immunogenicity, evaluated for

preserved functionality *in vitro* (unpublished data, see Dr. Carrigan's thesis; manuscript in preparation). Additionally, work on the OCR compared to wt Ndi1 was carried out by our lab. A large focus of the first results chapter of this PhD thesis is on the biochemical effects of optimising Ndi1, evaluating optNdi1 in a number of cell-based assays to assess its functionality compared to the wild-type Ndi1. Furthermore, the function and efficacy of Ndi1 and optNdi1 constructs engineered into AAV vectors were also evaluated. In order to help examine the relative potency of these potential gene-based therapeutics, a novel activity-based dose curve assay was developed to investigate the minimal and maximal ranges of activity of optimised Ndi1 *in vitro*.

3.1.4. Ndi1 Therapy for Complex Disease

Increasingly mitochondrial dysfunction is being implicated in a range of complex disorders, not classically associated with mitochondrial mutations. Mitochondria have long been associated with systemic highly debilitating diseases such as MELAS, MERRF and NARP (Molnar, 2018). However, focus has recently turned to diseases with more idiopathic origins, where mitochondrial dysfunction may be secondary to the primary insult but leads to exacerbation of the disease phenotype. For example, mitochondrial dysfunction is increasingly being linked to neurological disorders such as schizophrenia and bipolar disorder. For bipolar disorder, evidence suggests that mice who accumulate mtDNA deletions show spontaneous depressive episodes, and an accumulation of mtDNA mutations has been identified in some patients with the disease (Kato, 2017). More specifically, complex I deficiency has been argued to be a key player in disease aetiology in schizophrenia (Ben-Shachar, 2017). Intriguingly this group argues that dopamine actually directly inhibits CI activity, acting to self-limit dopaminergic neuronal activity (Brenner-Lavie, Klein, & Ben-Shachar, 2009). This is an interesting potential link as to possibly why in Parkinson's disease it is dopaminergic neurons that die first due to CI insult, if

they already have reduced levels of CI activity due to dopamine inhibition then further insult would be less well tolerated. However, it must be noted that the neuronal loss seen in Parkinson's disease is not limited to dopaminergic neurons (Sulzer & Surmeier, 2013).

Mitochondrial dysfunction has been linked to diseases not classically considered to be mitochondrial disorders, and ocular diseases are no exception. For instance, studies have recently shown that modification of the NAD⁺/NADH ratio may be of benefit to protecting retinal cells. The ratio of NAD⁺/NADH ratio is an indicator of the cells redox status and is controlled by a cells production of NAD⁺ versus its use of NADH in anabolic processes such as ATP generation (Bilan et al. 2014). Enzymes such as Nmnat1, an enzyme involved in NAD⁺ biosynthesis, have been implicated in inherited retinal degenerations such as Leber Congenital Amaurosis and Cone-Rod Dystrophy (Koenekoop et al. 2012; Nash et al. 2018). In addition, Nmnat1 conditional KO mice show severe early onset loss of photoreceptors (Eblimit et al. 2018) and Williams et al. (2017) have reported that AAV2-Nmnat1 can protect retinal ganglion cells in the DBA/2J mouse model of glaucoma. Given such observations, it would be informative to test OXPHOS activity and ATP levels in Nmnat1 deficient cells, especially in light of the fact that patients with clinically diagnosed Nmnat1 mutations only show pathological effects in photoreceptor cells (Eblimit et al. 2018).

3.2. Results

3.2.1. Ndi1 optimisation process

Previously published work in our lab has shown Ndi1 can be used as a therapeutic approach for a rotenone-induced mouse model of LHON (Chadderton et al., 2013). Intravitreal injection of AAV2-Ndi1 significantly reduced retinal ganglion cell (RGC) death and showed significant preservation of retinal function. Subsequent work in our lab by Dr. Matthew Carrigan led to the creation of an optimised version of the Ndi1 gene, entitled optNdi1. The optimisation process focused on changing the codon usage of the gene to more accurately represent the human codon bias. A Kozak sequence was also added to improve translational activity of the mRNA transcript. This process led to a significant increase in the RNA expression levels, which also led to an increase in protein expression levels *in vitro* (Figure 3-1).

It was important to investigate how much this optimisation process changes the efficacy of Ndi1 as a potential therapeutic *in vitro*, to develop a potency assay for its activity and to evaluate its expression *in vivo* and its potential use as a therapeutic in a mouse model of complex ocular disease.

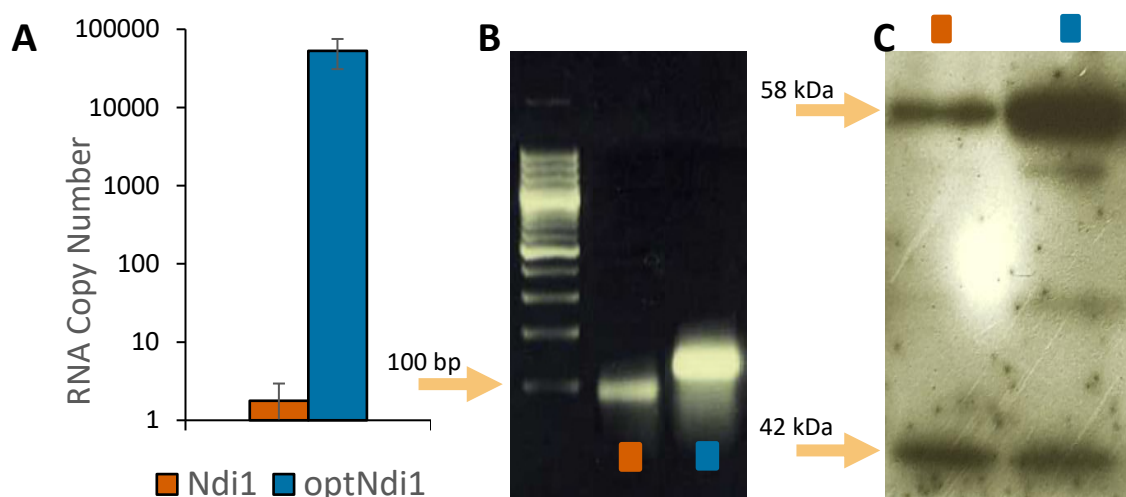


Figure 3-1: Previous analysis on plasmid transfected HEK293 cells comparing Ndi1 with optNdi1. A) RT-PCR analysis of RNA content between Ndi1 and optNdi1 transfected cells. There is a massive increase in RNA transcript levels between the two constructs. B) An agarose gel of the RNA from A) showing the abundance of optNdi1 transcript compared to Ndi1. C) Western blot analysis of Ndi1 and optNdi1. The increase in RNA levels leads to a substantial increase in protein levels, as seen in the upper bands. The two lower bands correspond to β -actin levels.

3.2.2. NADH oxidation activity

The primary role of Ndi1 *in vivo* is to catalyse the oxidation of NADH, and to transfer these electrons to reduce ubiquinone. This, of course, is the same physiological role that Complex I carries out in the mitochondria. As such, it was reasoned that we could modify existing biochemical assays to measure the activity levels of Ndi1 and optNdi1. The assay I developed is based on the spectrophotometric assay of CI activity (Spinazzi et al., 2012). Briefly, this process involves first isolating mitochondria from the sample to be tested. These mitochondria are then subjected to multiple freeze-thaw cycles to disrupt the mitochondrial membranes. This serves two purposes, it allows direct access of substrates and inhibitors to the electron transport chain (ETC) and also uncouples the enzymatic activity of the canonical members of the ETC from proton pumping. This allows specific interrogation of the NADH oxidation activity of CI, which is achieved by measuring the decrease in absorbance at 340nm, as a shift in the absorbance spectrum peaks of the molecule are seen when NADH is oxidised to NAD⁺. To measure CI activity accurately, the rate of decrease in the absorbance at 340nm is measured, then rotenone is added to inhibit CI. The rate of rotenone insensitive decrease in absorbance is subtracted from the first rate to give the CI activity rate. For this assay to be useable for investigating Ndi1 activity required the addition of a specific inhibitor of Ndi1. Flavone was chosen to fulfil this role, as it is a specific inhibitor of Ndi1 but not of CI. By adding flavone either before or after rotenone it is possible to interrogate how much of the enzymatic rate was due to either CI, Ndi1 or background. It is also important to note that the rate, the change in absorbance over time, is the important metric, not the absolute value of the absorbance.

Figure 3-2A shows three representative traces from the NADH oxidation assay. When running the assay, ubiquinone is added in as the electron acceptor which starts the reaction. The next addition is of rotenone, this inhibits CI activity. Finally, flavone is added which inhibits Ndi1 activity. The order of the inhibitors can be reversed to inhibit Ndi1 activity first without affecting overall results.

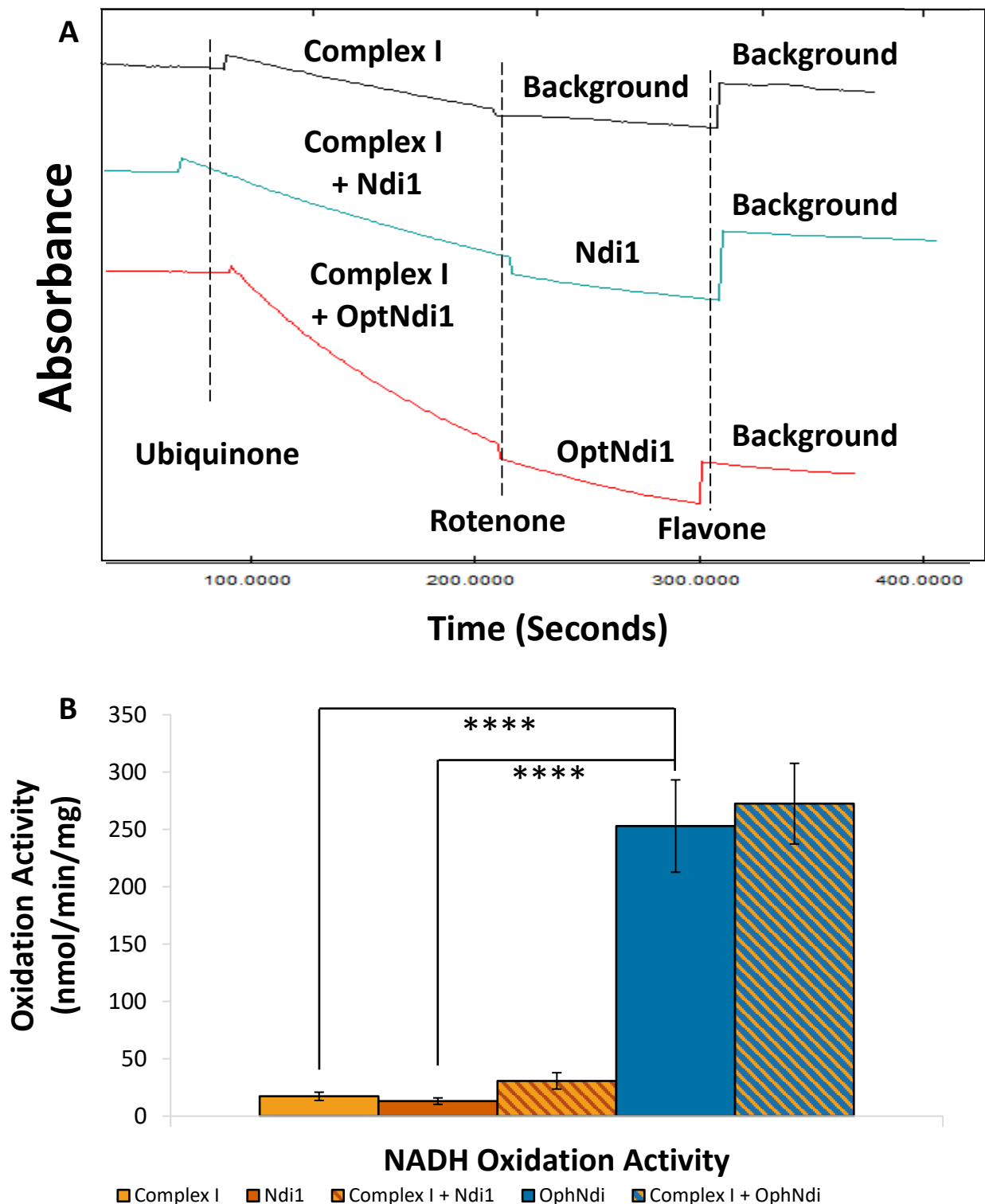


Figure 3-2: Development of an NADH oxidation assay to measure Ndi1 functionality. A) three representative traces produced by the NADH oxidation assay. Baseline activity is monitored until the trace appears stable, the ubiquinone is added to start the reaction. The absorbance then starts to decrease, proportionally to the amount of NADH oxidation. The addition of rotenone then inhibits complex I, allowing us to measure only Ndi1 activity. The addition of flavone inhibits Ndi1 and the rate of background NADH activity is then measured. The addition of the inhibitors can be reversed. B) A bar graph representing NADH oxidation activity of plasmid transfected HEK293 cells expressing either Ndi1, optNdi1 or eGFP as control. Cells expressing optNdi1 exhibit significantly higher levels of NADH oxidation activity when compared to either complex I or Ndi1 expressing cells alone (Student's t-test, $p < .0001$, $n = 5$ separate transfections). To demonstrate the robustness of the assay, complex I plus either Ndi1 or optNdi1 rates of activity were measured (hatched bars), and these were found to be very similar to the cumulative value of complex I and Ndi1 or optNdi1 when measured individually.

Initial analysis was carried out on HEK293 cells transiently transfected using plasmids containing either Ndi1, optNdi1 or eGFP (see Methods section 2.1 for cloning and Appendix 7.1 for sequences). Plasmid quantities were normalised for copy number, as the backbone of the optNdi1 plasmid was considerably larger than that of the other two. All genes were driven by the CMV constitutive promoter. Transfections were carried out 48 hours prior to extraction of the mitochondria. In Figure 3-2A the black trace corresponds to eGFP transfected cells, the blue trace to Ndi1 transfected cells and the red trace to optNdi1 transfected cells. It is important to note that optNdi1 transfected samples showed such high levels of NADH oxidation activity, this red trace is using 10x less sample than the other two traces. The amount added was diluted in order to obtain a linear rate of activity for subsequent measurement. Activity rates are then calculated and normalised to protein content.

Figure 3-2B shows these NADH oxidation rates in nmol/min/mg. We can see from this that optNdi1 transfected cells have a vastly increased ability to oxidise NADH compared to eGFP and Ndi1 transfected cells. optNdi1 activity averaged at 253.9 nmol/min/mg (n=5 separate plasmid transfections) whereas control cells had an activity rate of 17.2 nmol/min/mg (n=5 separate plasmid transfections). This equates to an almost 15-fold increase in NADH oxidation activity in optNdi1 transfected cells compared to CI levels alone. Ndi1, in contrast, showed slightly lower average activity levels compared to CI, of 13 nmol/min/mg (n=5 separate plasmid transfections), however this was not significantly different to CI activity levels (Student's t.test, $p > .05$). The striped bars in Figure 3-2B represent activity rates of either Ndi1 or optNdi1 plus CI, showing that they can work additively to increase the overall NADH oxidation activity of the cell. The Ndi1 optimisation process has clearly enabled previously unachievable levels of activity in mammalian cells.

3.2.3. optNdi1 and Ndi1 show benefit in cell models of complex I disorders

To investigate whether the increased activity levels in cells translated to an increased functional benefit we employed a number of biochemical assays to examine mitochondrial health.

In order to ensure that the optimisation process had not perturbed the mitochondrial localisation of the protein, or indeed that the higher levels of protein did not have observable negative effects on mitochondrial morphology, immunocytochemistry analysis of HEK293 cells was carried out.

HEK293 cells were seeded onto coverslips then transfected with a HA-tagged version of optNdi1 (see Methods 2.1 for cloning and plasmid uses, 2.4.1 for transfections) In order to confirm mitochondrial localisation cells were co-transfected with mito-dsRed (Addgene). This is a red fluorescent protein with a mitochondrial targeting sequence (MTS) attached that shows strong sub-cellular localisation to the mitochondria. Cells were fixed (Methods 2.5.1) then probed with an anti-HA tag antibody.

Figure 3-3A to D shows representative images of these optNdi1 cells. In Figure 3-3A we can see the pattern of expression of the mitochondrially targeted mito-dsRed. Panel B shows that the HA antibody, here staining optNdi1, also shows a distinct expression pattern within the transfected cells. When these two channels are merged, in panel C, we can see clear mixing of the red and green signals, suggesting colocalization of the two proteins, and therefore continued localisation of optNdi1 to the mitochondria. The close up of the cell from the orange box in C allows us to more clearly see the mitochondrial morphology, which continues to show tubular morphology. This holds true for the vast majority of cells positive for optNdi1 suggesting that the increased levels of Ndi1 protein expression associated with the optimisation process have not perturbed normal mitochondrial morphology. To ensure that the mitochondrial localisation signal on mito-dsRed is not somehow affecting mitochondrial localisation of optNdi1, we also co-transfected some cells with optNdi1 and eGFP (Figure 3-3E to F). When

ophNdi1 is now labelled with cy3 (red fluorescence), a clear subcellular compartmentalisation of optNdi1 signal is seen (Figure 3-3F)

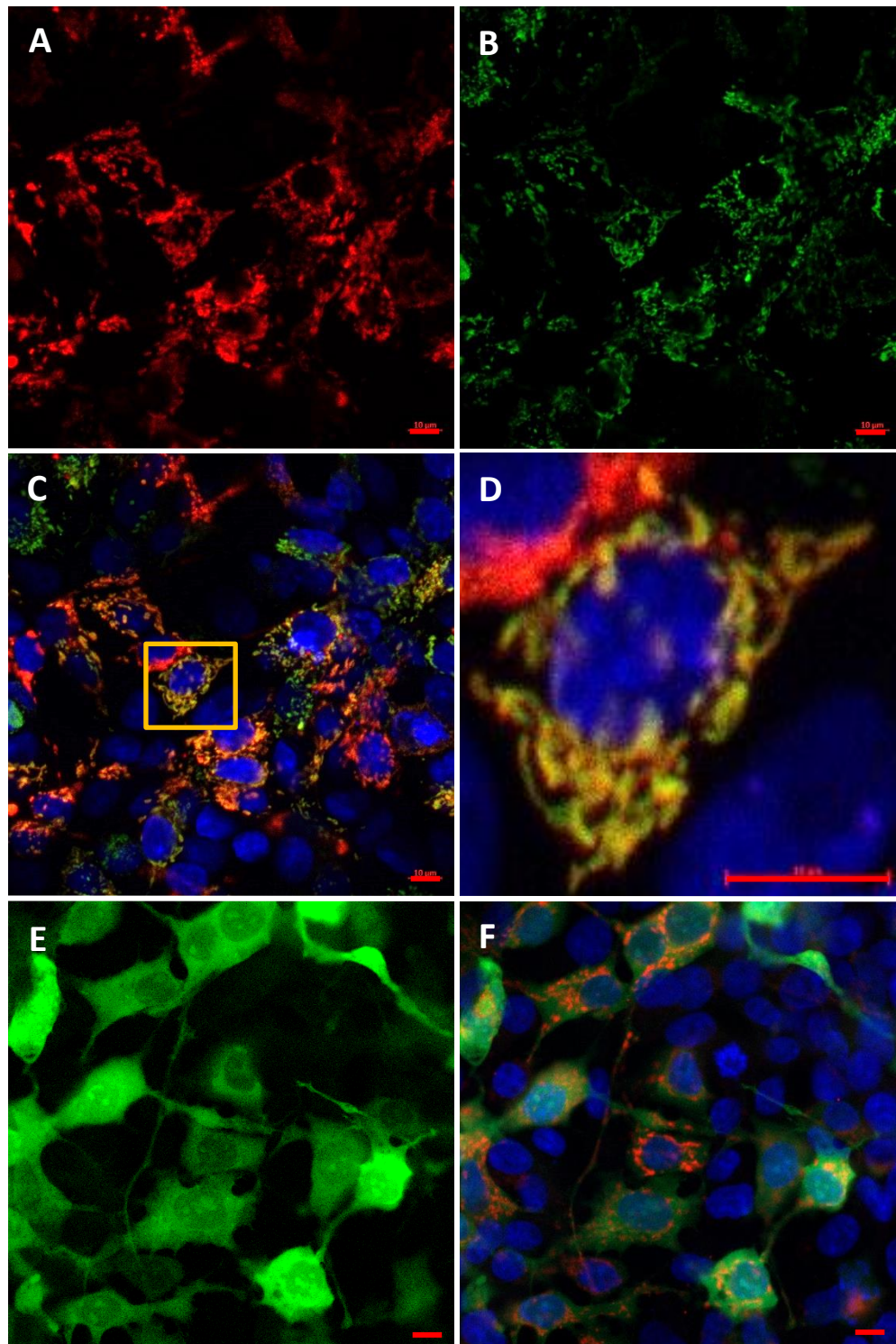


Figure 3-3: Representative images of the mitochondrial localisation of optNdi1 in transiently transfected HEK293 cells. A) mito-dsRed signal shown in red shows a distinct pattern of expression in cells, corresponding to the mitochondria. B) Ndi1 antibody staining optNdi1, also shows compartmentalisation of signal. C) A merge of panels A) and B) which shows colocalization of the two signals as denoted by the orange colour. Nuclei are stained with DAPI. D) is a higher magnification image of the orange box from panel C) showing retention of the tubular mitochondrial network. E) HEK293 cells transfected with both optNdi1 and eGFP, here showing just eGFP signal which presents as a diffuse pattern throughout the cytosol. F) is a merge of E) as well as ophNdi1 signal in red. ophNdi1 clearly has a distinct subcellular pattern to its expression. DAPI staining nuclei are in blue. Scales bars in red represent 10 μ m.

Next, Ndi 1 and optNdi1 were tested in a number of biochemical assays to test their effectiveness at coping with mitochondrial insults, in particular their ability to protect the cell in cases of complex I dysfunction. For ATP quantification (Methods 2.9.2.), HEK293 cells were seeded in appropriate white-walled 96 well plates and grown in glucose containing media. They were transfected with either Ndi1, optNdi1 or eGFP as a control 24 hours later. 24 hours after transfection they were treated with 5nM rotenone either 24 or 2 hours before the assay was performed, with a group of cells not receiving rotenone for each transfection group. When compared to non-rotenone treated cells there was very little decrease in the ATP levels in the three treatments at either time point (75-90% of the non-rotenone treated cells, Figure 3-4A). There was however a modest significant increase in protection in the cells transfected with either Ndi1 or optNdi1 when compared to eGFP transfected cells (ANOVA, Tukey HSD, $\alpha = .05$, $n = 6$ separate transfections, Figure 3-4A). There was no difference between Ndi1 and optNdi1. The minor decrease in ATP levels after rotenone in glucose is presumably due to HEK293 cells relying predominantly on glycolysis for energy production, so despite losing OXPHOS capacity, there is little detriment to the cell (see the AOX chapter for more on this phenomenon).

To interrogate this further, cells were grown in galactose media instead of glucose, which forces the cells to be dependent on exogenously supplied pyruvate for ATP production, causing them to rely on mitochondrial OXPHOS. Cells were set up in the same manner, but the rotenone timepoints were increased to include 24, 4, 2 and 1 hours. In this experiment a substantial reduction in ATP levels compared to untreated cells was observed (Figure 3-4B). 1-hour post rotenone treatment eGFP cells contained only 5% of the level of ATP of non-rotenone treated cells. Both Ndi1 and optNdi1 showed significant levels of protection of ATP levels compared to eGFP treated cells at all time points (ANOVA, Tukey HSD, $\alpha = .05$, $n = 6$ separate transfections). However, there is still a sizeable reduction in ATP levels in Ndi1 and optNdi1 expressing cells compared to non-rotenone treated cells. Complete inhibition of complex I is an extreme scenario which would not be found in actual disease situations.

I also investigated Ndi1 and optNdi1's ability to protect cells from ROS generation due to complex I inhibition Figure 3-4C and D (see Methods 2.9.1.). First cells were grown in glucose media and transfected with either Ndi1, optNdi1 or mock-transfected (using lipofectamine but no plasmid DNA). The control plasmid for the ATP assay, eGFP, could not be used as DCFDA also fluoresces in the green channel. Cells were treated with 5nM rotenone for 24 hours prior to analysis. Cells were then stained using DCFDA (see Methods 2.9.1), which fluoresces upon activation by ROS. Ndi1 and optNdi1 cells actually show significantly increased levels of ROS compared to the mock-transfected cells, either with 24-hour rotenone treatment or untreated (ANOVA, Tukey HSD, $\alpha = .05$, $n = 6$ separate transfections). This was somewhat unexpected but can potentially be explained via two mechanisms, either reverse electron transfer (RET) ROS production facilitated by the presence of Ndi1 or, more straight-forwardly, increased use of the ETC and thus more ROS resulting through its continued use.

When grown in galactose, forcing the utilisation of the ETC, the situation changes (Figure 3-4D). The non-rotenone treated cells expressing either Ndi1 or optNdi1 now show a significant reduction in ROS compared to mock transfected cells. This is potentially due to the presence of Ndi1 helping the ETC to work more efficiently when its use is required due to the provision of galactose media. After poisoning with rotenone, optNdi1 transfected cells show the most protection from increased ROS levels. Ndi1 only shows a significant decrease compared to the control when untreated although was approaching significance at 4 hours ($p = .06$), whereas optNdi1 treated cells show significantly decreased ROS levels compared to mock-transfected cells at all timepoints, and at all poisoned time-points when compared to Ndi1 (ANOVA, Tukey HSD, $\alpha = .05$, $n = 6$ separate transfections). These data demonstrate the ability of Ndi1 and in particular optNdi1 to protect from ROS production due to electron transport chain dysfunction. Mock-transfected cells were too compromised to achieve reliable results at a 24-hour time point of rotenone treatment as the mock-transfected cells were no longer adherent due to excessive cell death.

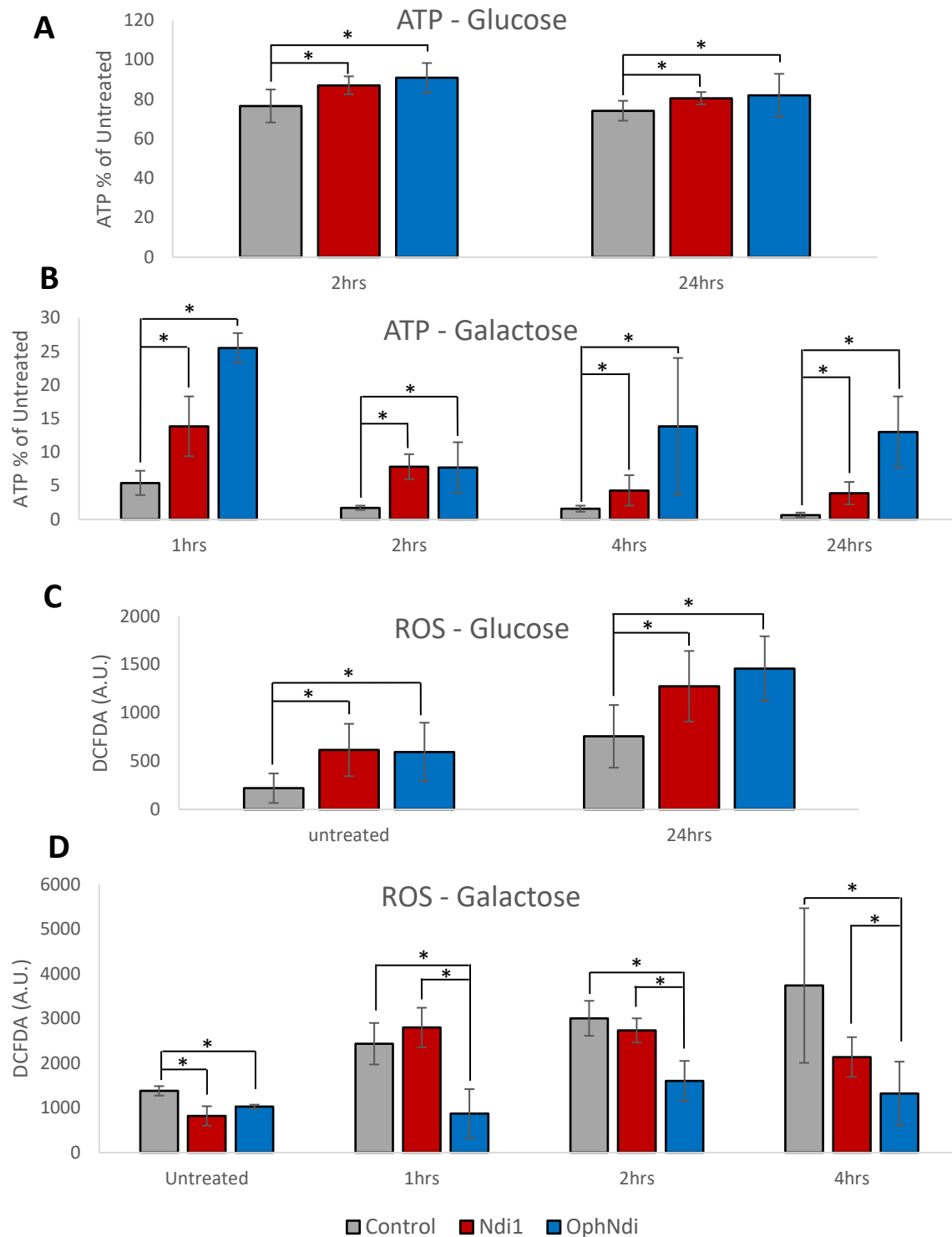


Figure 3-4: HEK293 cells transfected with either Ndi1 or optNdi1 or mock-transfected, grown in either glucose or galactose media. A) Under normal culture conditions, with an excess of glucose, rotenone inhibition of CI for 2 or 24 hours has little effect on cellular ATP levels, however Ndi1 or optNdi1 transfected cells showed a modest significant increase in ATP levels. B) When forced to use galactose both Ndi1 and optNdi1 offer significant protection of ATP levels compared to control cells, with optNdi1 providing significant benefit over Ndi1 at 24 hours. C) ROS analysis using DCFDA shows elevated levels in treated cells compared to mock-transfected cells when grown in glucose media. As the ETC is the primary source of ROS this could be due to transfected cells using OXPHOS for ATP production. D) When forced to use galactose optNdi1 shows significant protection against ROS production after rotenone inhibition (ANOVA, Tukey HSD, $\alpha = .05$).

To further investigate the source of ATP production after rotenone poisoning in glucose media, HEK293 cells transfected with either optNdi1, Ndi1 or eGFP. The lower the pH of the cell media, the more reliance on glycolysis the cells in question have, as lactic acid is a by-product of glycolysis. OptNdi1 maintained a higher media pH (pH6.82) compared to Ndi1 (pH6.61) and eGFP (pH6.64), providing evidence that optNdi1 cells can utilise non-glycolytic sources of energy production to maintain cellular ATP levels (ANOVA, Tukey HSD, $\alpha = .05$, $n = 4$ separate transfections, Figure 3-5A).

HEK293 transfected with optNdi1, Ndi1 or eGFP show different growth rates when forced to use galactose as their primary energy source. Cells were seeded at 1×10^5 in galactose containing media, then growth rate was monitored for 96 hours, with cells being counted every 24 hours. OptNdi1 and Ndi1 transfected cells showed a significantly increased cell number after 96 hours of growth in the reduced media, suggesting these cells can better utilise the OXPHOS pathway when forced to do so (ANOVA, $p < .05$, $n = 3$ separate transfections, Figure 3-5B).

To test whether the bioenergetic benefits observed also translated to the oxygen consumption rate of cells transfected with Ndi1, optNdi1 or eGFP, the Seahorse XFe24 was utilised to measure oxygen consumption rate, normalised to cell number (Figure 3-5C). This was a non-standard assay, using 5nM well concentration of rotenone as the first injection to inhibit complex I and 5nM of Flavone dissolved in DMSO as the second injection to inhibit Ndi1. Average baseline levels of oxygen consumption were increased in the optNdi1 and Ndi1 cells compared to eGFP treated cells suggesting increased OCR under normal physiological conditions. After the addition of rotenone, eGFP cells had a significant decrease in OCR compared to the other two transfected cell populations (to 9% of baseline), with optNdi1 maintaining the highest levels of OCR (58% of baseline) and with Ndi1 treated cells retaining 30% of baseline activity. The addition of flavone to inhibit Ndi1 enzymatic activity brought all three traces to a consistent background level of OCR. The bar chart shows the OCR of the three treatments at measurement 8. Ndi1 showed a significant protection of OCR above eGFP transfected cells, and optNdi1 showed a significant increase in this protection level compared to the other two treatments (Student's t-test, $n = 5$ wells, $p < .001$, Figure 3-5D).

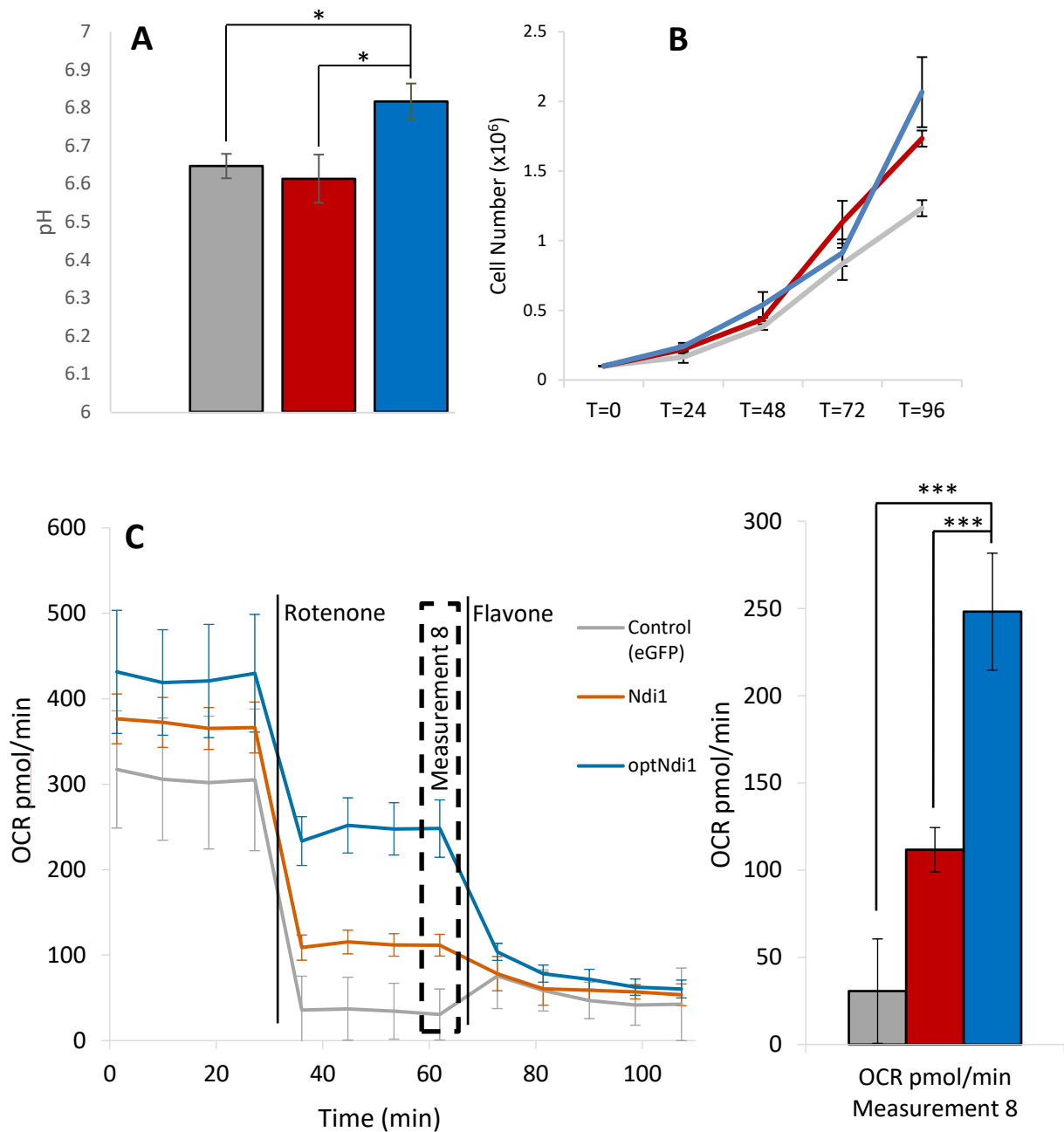


Figure 3-5: Bioenergetic analysis of HEK293 cells transfected with either Ndi1, optNdi1 or eGFP. A) pH changes after rotenone poisoning of transfected cells. optNdi1 transfected cells maintain a significantly higher pH after poisoning, suggesting less reliance on glycolysis in these cells. B) When given galactose containing media cells transfected with either Ndi1 or optNdi1 show a significant increase in cell number after 96 hours of growth (ANOVA, $p < .05$, $n = 4$ wells). No other time points were significantly different. C) Seahorse XFe24 analysis of OCR of transfected HEK293 cells. Ndi1 or optNdi1 cells showed an average increase in OCR before rotenone treatment, but optNdi1 demonstrated a significant level of protection of OCR after treatment with rotenone compared to either eGFP control cells or Ndi1 transfected cells (Student's t test, $n = 5$ wells, $p < .001$).

3.2.4. Development of an *in vitro* potency assay for optNdi1 viral gene therapy

While transfection of cells with plasmids provides a means to undertake an array of assays to investigate many features of Ndi1 and optNdi1-based therapeutic approaches, ultimately it is proposed to provide long term expression of the transgene using adeno associated virus (AAV) for gene delivery. However, in order for a virally delivered gene therapy to be of value it is vital for there to be reproducibility of the levels of expression and activity when used. Therefore, generating a robust potency assay would allow viral doses and potential for therapeutic efficacy to be better matched between AAV vector batches and also give a better understanding of the range of efficacy within cells. A potency assay would also provide us with the ability to plot a dose response curve for this novel candidate therapy which would be beneficial for identifying what kind of relationship there is between dose and therapeutic activity.

To undertake these investigations optNdi1 was engineered into an AAV serotype virus. The AAV2 serotype was chosen for this study due to its known ability to transfect retinal cells *in vivo*, but also because of its efficient transduction of HEK293 cells *in vitro*. In order to model this, we utilised the NADH oxidation assay detailed previously (Methods section 2.2). HEK293 cells were seeded in 6 well plates at a density of 5×10^5 cells per well. 24 hours later 2 wells were trypsinised and used for counting to estimate the number of cells in the remaining wells. At this point AAV2-optNdi1 was added to the wells at 6 different multiplicities of infection (MOI). The MOI is a measure of how many viral particles are added per cell. We started by using 5 different MOIs in 5x increments from 1.6×10^2 viral genomes (vg) to 1×10^5 vg per well. After initial tests were undertaken, we established that the 1×10^5 vg dose was not saturating transgene activity levels (as evaluated by NADH oxidation assay), so we increased the dose by 5x again to 5×10^5 vg. Additionally, one well was lipofectamine transfected with optNdi1 plasmid to act as positive control, and another well was left untreated to act as a negative control. After 48 hours whole mitochondria were extracted from the cells in each well via mechanical methods using a Dounce

homogeniser (see methods, section 2.8.4 for further details). These mitochondrial samples were then run through the NADH oxidation assay as previously described. Figure 3-6A shows the NADH oxidation traces for a set of the 6 MOIs. The traces decrease in MOI from top to bottom, with the top two MOIs 5×10^5 vg and 1×10^5 vg using half the sample quantity as the lower four traces, as the larger volume of sample was too active and resulted in a non-linear trace. If each of the samples are compared, a difference in the slope of the traces after rotenone addition can be seen. To explore the robust nature of the assay, each sample of extracted mitochondria was run three times in the NADH oxidation assay, and each MOI was repeated six times.

Two methods of normalisation were tested to see what effect they had on the data; either protein concentration of the extracted mitochondrial sample (Qubit assay) or Citrate Synthase activity levels (CS) were used. CS is an enzyme that acts as the gateway to the citric acid cycle, and has previously been shown to be a good biomarker for mitochondrial content (Spinazzi et al., 2012; Vigelsø, Andersen, & Dela, 2014). Figure 3-6B shows a comparison of the average activity levels of the same samples when normalised to each of these markers (n=6 separate transfections).

In Figure 3-6C we can see the curves plotted by these data normalised to either protein content or CS activity. Both show a tendency to a sigmoidal dose curve, suggesting that higher levels of virus are leading to a saturation of NADH oxidation activity. The two normalisation methods show very similar relative activity levels at lower MOIs but as this increases CS normalisations starts to predict a saturation of NADH activity, with 1×10^5 vg and 5×10^5 vg showing almost no difference in activity levels. In comparison, when protein levels are used there is 33% increase in activity between the two MOIs.

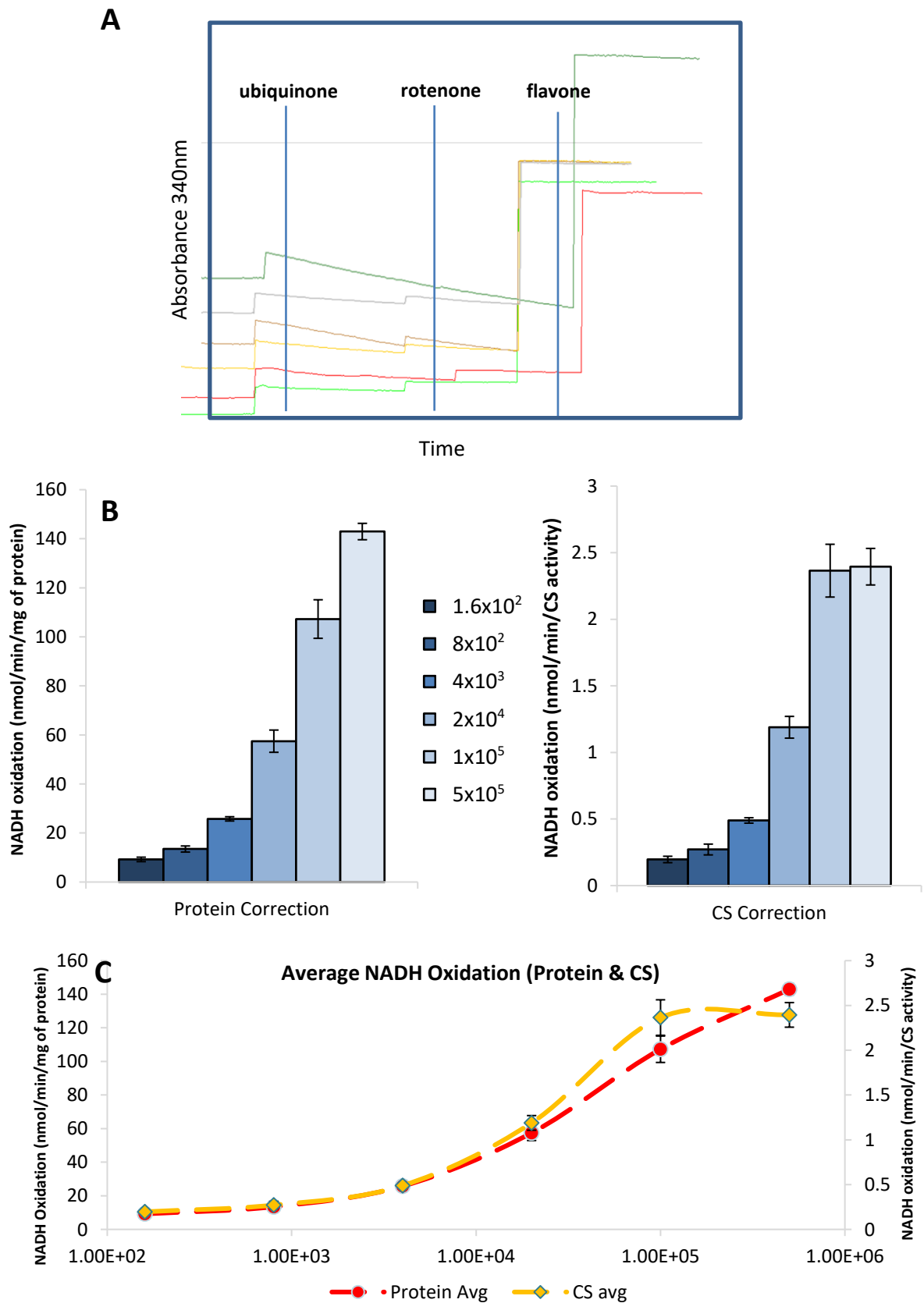
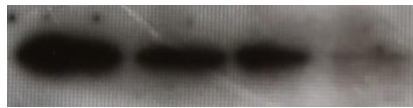


Figure 3-6: Development of an *in vitro* potency assay for optNdi1 activity levels using virally transduced HEK293 cells. A) The NADH oxidation activity traces from one full set of MOIs, starting with 5x10⁵ at the top in dark green descending to 1.6x10² at the bottom in light green. The top two traces use half the volume of samples as the lower four. It is the rate of change after the additions we are interested in, not the absolute value of the absorbance. B) The average activity levels of each MOI (n=6 separate transfections) normalised to either protein content or citrate synthase activity. C) The averages plotted on a scatter graph suggest a sigmoidal dose curve and demonstrate a similar increase in activity level at the lower MOIs but diverge somewhat at the higher levels when data is normalised using protein concentration or CS (error bars $\pm$ SEM).

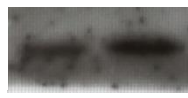
optNdi1



5×10^5



1.6×10^2



β -Actin

Figure 3-7: Western blot analysis of the 6 optNdi1 MOIs using anti-Ndi antibody on the top row, with 5×10^5 in the first lane and 1.6×10^2 in the last lane and anti- β -actin on the bottom row. A clear stepwise decrease in protein expression is seen depending on the MOI used. No detectable protein is seen in the last lane, but its activity can be measured using the NADH oxidation assay, highlighting its sensitivity.

To correlate these MOI dependent changes in activity levels Western Blot analysis was carried out on cells transfected with each of the 6 MOIs (4-12% denaturing gel; Figure 3-7). There is a clear stepwise decrease in protein levels as the MOI is decreased, with the bottom two MOIs no longer providing levels of protein expression detectable by western blot ($n = 3$ separate blots).

To investigate whether these data do actually fit a logistic (sigmoidal) distribution, a least-squares regression was performed on the data, then the resulting line was plotted against the average values for each MOI (Figure 3-8A and B). The dashed line shows the predicted extension of curve as far as 4×10^7 vg MOI using the equation for the best fit line. The lines both suggest that the NADH oxidation levels are nearing saturation, that an increase in MOI will not yield much in terms of increased activity. Pearson's correlation coefficient between the observed activity levels and those predicted by the sigmoidal functions was very high ($r^2 > .99$ for both) suggesting these curves match observed data well.

However, when subsequently testing the sigmoidal models with activity levels from known viral MOIs, both showed inconsistencies. In Figure 3-8 A and B the yellow triangles represent the predicted MOIs of 2×10^4 vg and 1×10^5 vg when using only the equation given by the sigmoidal curve. The protein estimation curve estimated 1.12×10^4 vg and 6.4×10^4 vg, whereas citrate synthase estimated 2.18×10^4 vg and 4×10^5 vg ($n=3$ separate transfections). Although an underestimate, protein normalisation gives much better preservation of the 5-fold increase in MOI between the two doses. It seems that normalising data with citrate synthase values may result in vastly overestimating the MOI in our model, most likely due to the steep change in the

curve between the 1×10^5 vg and 5×10^5 vg values, as can be seen in Figure 3-8B. This leads to a predicted 18.5-fold increase in MOI between the two. It would be interesting to repeat this with both higher and lower MOIs; the lower MOIs to estimate if the lower parts of the curve may be more accurate in its estimations, where the two methods of normalisations predict more similar NADH oxidation activity levels. The addition of higher MOIs would allow extension of the sigmoidal curve, to see if we truly have reached saturation. This experiment would be unlikely to be carried out in practice in an academic laboratory such as ours, as an MOI that high would unfortunately require excessive amounts of AAV vector, to the point where at the concentrations we have, the cell media would effectively be replaced with virus solution.

Of course, the NADH oxidation assay does not measure activity as part of the ETC. To investigate whether a similar dose response is seen in other mitochondrial biomarkers the Seahorse XFe24 assay was used to measure OCR after viral transduction in HEK293 cells (Figure 3-8C). Four MOIs were used, chosen to mirror the values in the top half of the sigmoidal dose curve: 2.5×10^4 vg, 7.5×10^4 vg, 1.5×10^5 vg, and 2.25×10^5 vg. Cells were seeded at 2×10^4 cells per well then transduced 24 hours later, allowing a further 48 hours before the assay was undertaken. Results were normalised to cell number. After four baseline measurements, rotenone was injected, inhibiting endogenous complex I activity, which can be seen in the steep decline in OCR of the control cells. After four more measurements flavone was added which inhibits optNdi1 activity, with all traces now dropping to a background level.

There is a clear increase in OCR as the MOI increases, but this reaches saturation at the higher two MOIs, which are not statistically different from each other (Student's t-test, $p > .05$). This mirrors the upper portion of the sigmoidal dose curve obtained from the NADH oxidation assay.

Demonstrating an effective dose range for this AAV-optNdi1 therapeutic (and likewise for any candidate therapeutic) is important for the design of future studies, both *in vitro* and *in vivo*, and moreover, to ensure reliability of data between different batches of AAV-optNdi1 vectors.

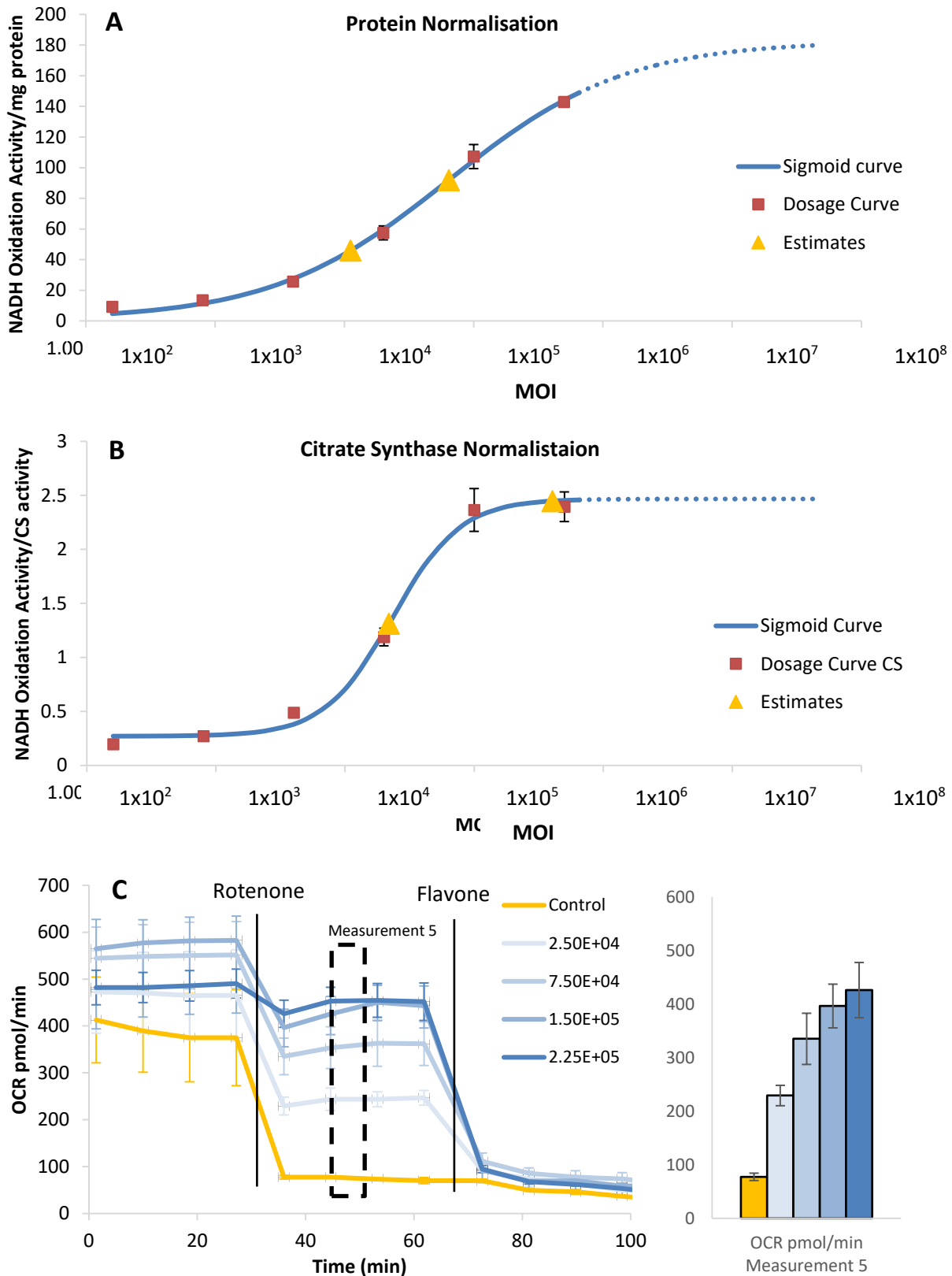


Figure 3-8: Analysis of the *optNdi1* NADH activity dose curves. A) and B) show the best fit sigmoidal curve through the data for the average NADH oxidation activity normalised to either A) protein content or B) citrate synthase activity. The curve was drawn using the least squares regression method. Both curves have an $r^2 > .99$. The yellow triangles represent NADH oxidation activity levels for three independently tested replicates of 1×10^5 vg and 2×10^4 vg MOI matched to the curve using the function of the sigmoidal curve. Protein gives a modest underestimation whereas citrate synthase gives correct results for 2×10^4 vg but vastly overestimates the 1×10^5 vg MOI. C) Seahorse XFe24 data of an AAV2-*optNdi1* dose curve. This also shows saturation of OCR towards the higher MOIs. The bar chart shows the MOIs at measurement 5. (all error bars $\pm$ SEM)

3.2.5. optNdi1 increases protein expression *in vivo*

To investigate the relative expression of Ndi1 and optNdi1 *in vivo*, C57 mice were subretinally injected with 1×10^9 vg of AAV2/2-Ndi1 or AAV2/2-optNdi1. One-month post injection the mice were sacrificed and enucleated. The eyes were dissected and retinal tissue was extracted. Preliminary Western Blots were carried out on single whole retinas, but this did not give clear levels of expression for each construct with the anti-Ndi1 antibody. To overcome this, 3 retinae were pooled, and mitochondria were extracted as per section 2.8.4. but were resuspended in 25 μ l total. The protein concentration was measured by Qubit Fluorometer protein quantification assay (Thermo; Q33211) and equal amounts were loaded for Western Blot analysis (4-12% denaturing gel) with HEK293 cells transfected with 2×10^4 AAV-optNdi1 as control (see Figure 3-9). For an equal amount of retinal mitochondrial protein there is a large increase in optNdi1 (lane 4) protein expression compared Ndi1 (lane 3). The identity of the lower band in the two retinal samples is unclear but appears to be retinal specific as it is not present in the HEK293 2×10^4 transduced lane (5).

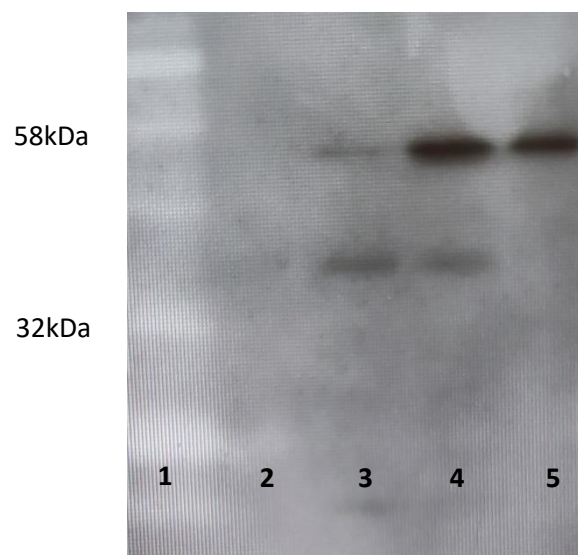


Figure 3-9: Western Blot analysis of retinae subretinally injected with either AAV2/2-Ndi1 (lane 3) or AAV2/2-optNdi1 (lane 4). Lane 1 shows the protein ladder, lane 2 is HEK293 cells without Ndi1 transduction and lane 4 is HEK293 cells with 2×10^4 MOI AAV2/2-optNdi1. The upper bands correlate to Ndi1 protein, with higher levels expressed in AAV2/2-optNdi1 transduced retinae compared to AAV2/2-Ndi1 retinae, for an equal amount of mitochondrial protein.

3.2.6. Ndi1 and optNdi1 as a potential therapeutic in the *Cfh* ^{-/-} mouse model of Age-Related Macular Degeneration

In order to investigate if the utility of Ndi1 and optNdi1 expands outside what are thought of as classical complex I disorders such as LHON (Chun & Rizzo, 2017), we decided to investigate its use in the *Cfh* ^{-/-} mouse model of age-related macular (AMD). This model shows a progressive visual loss over the course of 2 years with a reduction in electroretinography (ERG) amplitudes, disorganisation of photoreceptor outer segments as well as an increase in auto-fluorescent subretinal deposits (Coffey et al., 2007). As mitochondrial decline has been implicated in the disease aetiology of *Cfh* ^{-/-} mice (Calaza et al., 2015), we sought to investigate if Ndi1 and optNdi1 could provide protection in the *Cfh* ^{-/-} mouse model of AMD.

Initially we sought to establish mitochondrial biomarkers for investigation in this murine model. There is a link emerging between reactive oxygen species (ROS) and the dysregulation of CFH, and subsequently the disease pathology of AMD (Aldosari, Awad, Harrington, Sellke, & Abid, 2018; Marazita, Dugour, Marquioni-Ramella, Figueroa, & Suburo, 2016). As such, we sought to investigate oxidative stress in the eyes of both young and old *Cfh* ^{-/-} mice compared to age matched C57 mice, as the *Cfh* ^{-/-} is on a C57 background.

To estimate ROS levels in the retina of *Cfh* ^{-/-} mice we used DCFDA staining, a molecule that only exhibits fluorescence when it comes into contact with ROS. Eyes were enucleated from 2 month old *Cfh* ^{-/-} and C57 mice as well as 2 year old *Cfh* ^{-/-} and C57 mice.

Surprisingly, it was young *Cfh* ^{-/-} mice that had the highest mean DCFDA fluorescence level, indicating they have the highest levels of ROS, when compared to either aged *Cfh* ^{-/-} mice or young and aged C57 mice (n=4 eyes, ANOVA, p < .05). Post-hoc analysis shows that young CFH have a significantly higher mean than old CFH (p < .05).

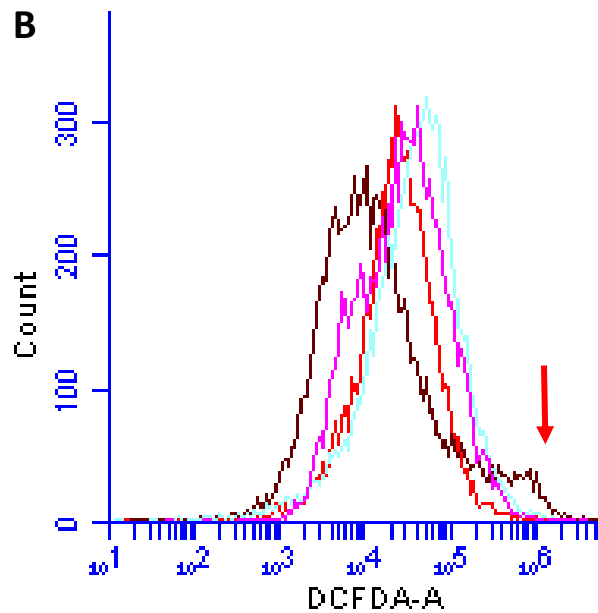
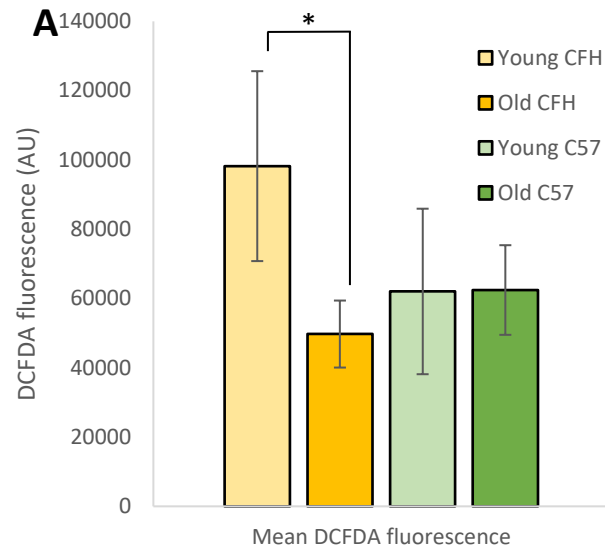


Figure 3-10: Reactive Oxygen Species levels in retinae show differences between old and young *Cfh* ^{-/-} or C57 mice. A) Mean DCFDA fluorescence show that young (2 month) *Cfh* ^{-/-} mice have significantly higher ROS levels than old (24months) *Cfh* ^{-/-} mice (ANOVA, Tukey HSD post-hoc, $\alpha = .05$, $n = 4$ eyes). B) Representative DCFDA fluorescence plots demonstrates the log-normal distribution. The dark trace represents young *Cfh* ^{-/-} which show a peak shifted to the left. However, there is a sub-population of cells with very high ROS levels not seen in the other traces, indicated by the red arrow. These cells are what increases the mean DCFDA fluorescence of young *Cfh* ^{-/-} cells above the means of the other mice.

Interestingly there appears to be a population of highly ROS positive cells in the young *Cfh*^{-/-} retinæ (Figure 3-10B). This highly ROS positive sub population was seen in all 2 month *Cfh*^{-/-} retinæ tested (n = 4 eyes) but not in the other mice tested (n = 12 eyes) One hypothesis is that if these cells were more prone to death it could be that this population is absent in the aged retina and thus leads to the decrease in the mean ROS seen in the 2 year old mice. Although the medians are not significantly different from each other (Kruskal-Wallis, p = .054), there is a trend towards older eyes having higher median ROS levels than the young eyes and old *Cfh*^{-/-} mice having higher median ROS levels than old wild type C57 mice (data not shown). An increase in the numbers of animals evaluated should allow more definitive conclusions to be drawn. However, from these analyses, ROS levels do seem to change over time as these mice age and as such we decided that ROS levels would be worth further investigation with regards to disease progression in the *Cfh*^{-/-} mouse model.

To investigate Ndi1 and optNdi1 as potential therapeutics in the *Cfh*^{-/-} mouse model, we generated a number of AAV vectors with different serotypes in order to increase the flexibility of target cell type that could be transduced by virus. This was primarily because it is as yet unclear where Ndi1 expression would provide the most benefit in the *Cfh*^{-/-} model. Initially two AAV vectors were focused on AAV2/8 – Ndi1 and AAV2/2-3tyr-optNdi1. The choice of these AAV serotypes was based on prior studies in murine retina (Palfi et al., 2015). When injected subretinally, AAV2/8 has been shown to efficiently transduce the RPE cell layer as well as photoreceptor cells in mice (Allocca et al., 2007), and is believed to do so to a higher efficiency than AAV2/2 at the same dose (Natkunarahaj et al., 2008). Subretinal injection of AAV2/2-3tyr shows very strong transduction for photoreceptors and also retinal ganglion cells, but comparatively little tropism for RPE cells (Petr-Silva et al., 2011).

To investigate the cellular tropisms of these AAV vectors in our hands AAV2/8-eGFP and AAV2/2-3tyr-eGFP vectors were produced. AAV2/8-eGFP was subretinally injected into C57

mice. Eyes were enucleated one-month post injection followed by paraformaldehyde fixation and cryosectioning. Sections were stained using DAPI to highlight nuclei, and then imaged to examine eGFP native fluorescence. With AAV2/8-eGFP (Figure 3-11), there is strong eGFP signal in the photoreceptor layer with transduction seen in the outer segments as well as the outer nuclear layer. There is also transduction of the RPE cell layer seen which corroborates well with previous studies (Allocca et al., 2007). Unfortunately, the AAV2/2-3tyr-eGFP viral preparation did not yield a high enough titre for strong transduction of the retina. The viral preparation has been repeated but animals were not ready for examination at the time of writing.

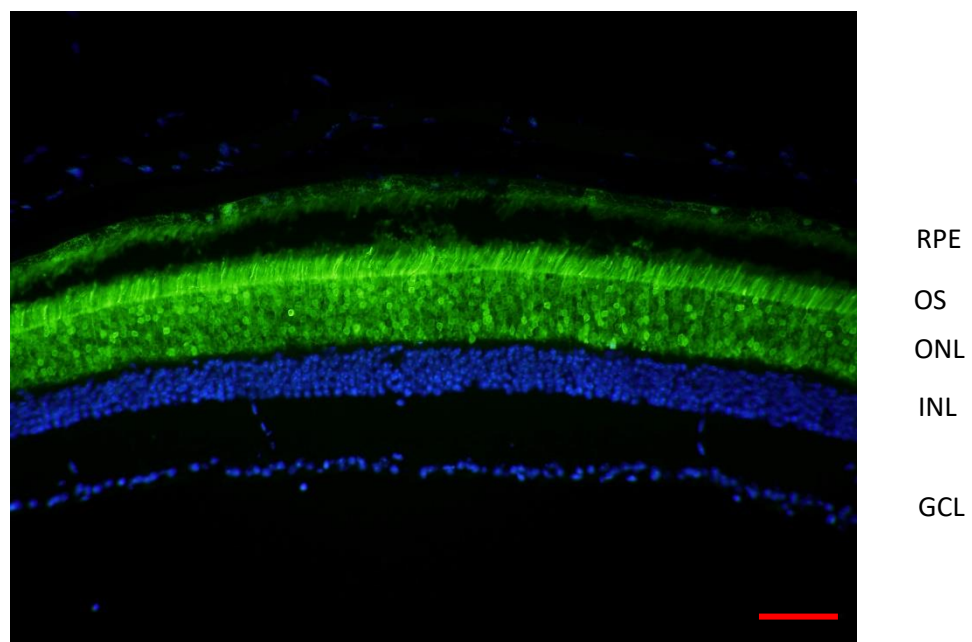


Figure 3-11: Subretinal injection of AAV2/8-eGFP to monitor cellular tropism. Strong eGFP fluorescence is seen in the photoreceptor outer-segments (OS) as well as the outer nuclear layer (ONL). The RPE also shows good eGFP expression levels suggesting that AAV2/8 transduces these cell types efficiently. INL = inner nuclear layer, GCL = ganglion cell layer. Scale bar = 50µm

To investigate the effects of Ndi1 and optNdi1 on ROS *in vivo* in the *Cfh*^{-/-} mouse model, we unilaterally sub-retinally injected 2.4×10^9 vector genomes (vg) of either AAV2/8-Ndi1 or AAV2/2-3tyr-optNdi1. At 5 months we enucleated the eyes and carried out the DCFDA ROS assay as described previously, comparing the treated and untreated eyes with each other. The treated *Cfh*^{-/-} eyes showed a decrease in mean DCFDA levels when compared to uninjected *Cfh*^{-/-} eyes. AAV2/8-Ndi1 showed a 51% decrease in ROS levels, with AAV2/2-3tyr-optNdi1 showing a 64% decrease when compared with the uninjected eyes (Figure 3-12). Normality was assumed for all distributions as the mean and median for the traces were similar, indicating a lack of skew to the data. For statistical analysis the data from all uninjected eyes (i.e. from both treatment groups) were pooled. Both treatments showed a significant decrease in mean and median ROS levels after ANOVA post-hoc analysis (Tukey HSD, $\alpha = .05$) when compared to the uninjected eyes. However, this is one indicator of a possible effect of delivery of AAV-optNdi1 and Ndi1 vectors. A reduction in ROS does not necessarily equate to a functional benefit, hence to begin to assess the possible functional benefit of this, we carried out electroretinography (ERG) analysis on another cohort of AAV treated *Cfh*^{-/-} mice.

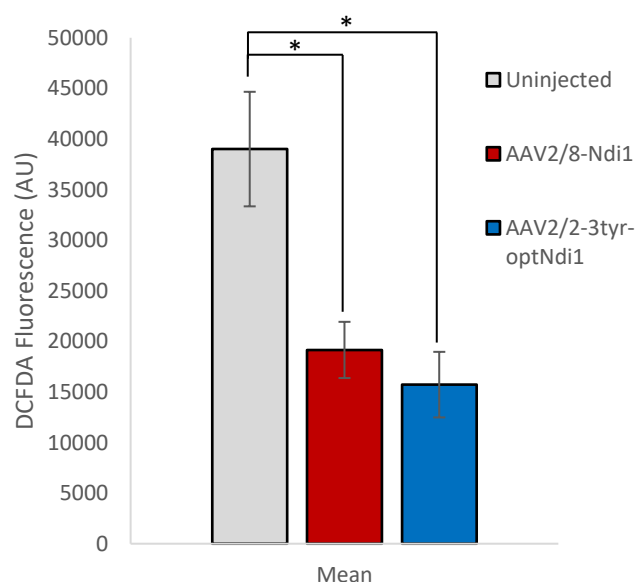


Figure 3-12: Mean DCFDA fluorescence in 5-month old *Cfh*^{-/-} mice, either treated with AAV2/8-Ndi1, AAV2/2-3tyr-optNdi1 or untreated. ANOVA showed a significant decrease in DCFDA fluorescence when eyes were treated with either of the AAV constructs. Tukey HSD post-hoc analysis indicated AAV2/8-Ndi1 ($n=10$ eyes) and AAV2/2-3tyr-optNdi1 ($n=8$ eyes) to be significantly different to untreated eyes ($n=18$). ($\alpha = .05$, error bars $\pm$ SEM).

We first sought to characterise the ERG deficit in aged *Cfh*^{-/-} mice compared to C57 wild type mice, at 9 months and 12 months (Figure 3-13 A and B, as detailed in Methods section 2-11). At 9 months there is a trend towards decreased function in the ERG parameters measured, but the only reaches significance when comparing the max A-wave response (*Cfh*^{-/-} n = 6 eyes, C57 n = 18 eyes, Wilcoxon, p < .05). The max A-wave is a measure of rod and cone function, but under dark-adapted conditions rods predominate. The non-significance of the rod response suggests that the decrease in the A-wave could be due to cone photoreceptor dysfunction. At the 12-month timepoint there is a significant difference between the C57 and *Cfh*^{-/-} genotypes in three of the metrics measured, suggesting cumulative damage that effects visual function over time in the *Cfh*^{-/-} mice.

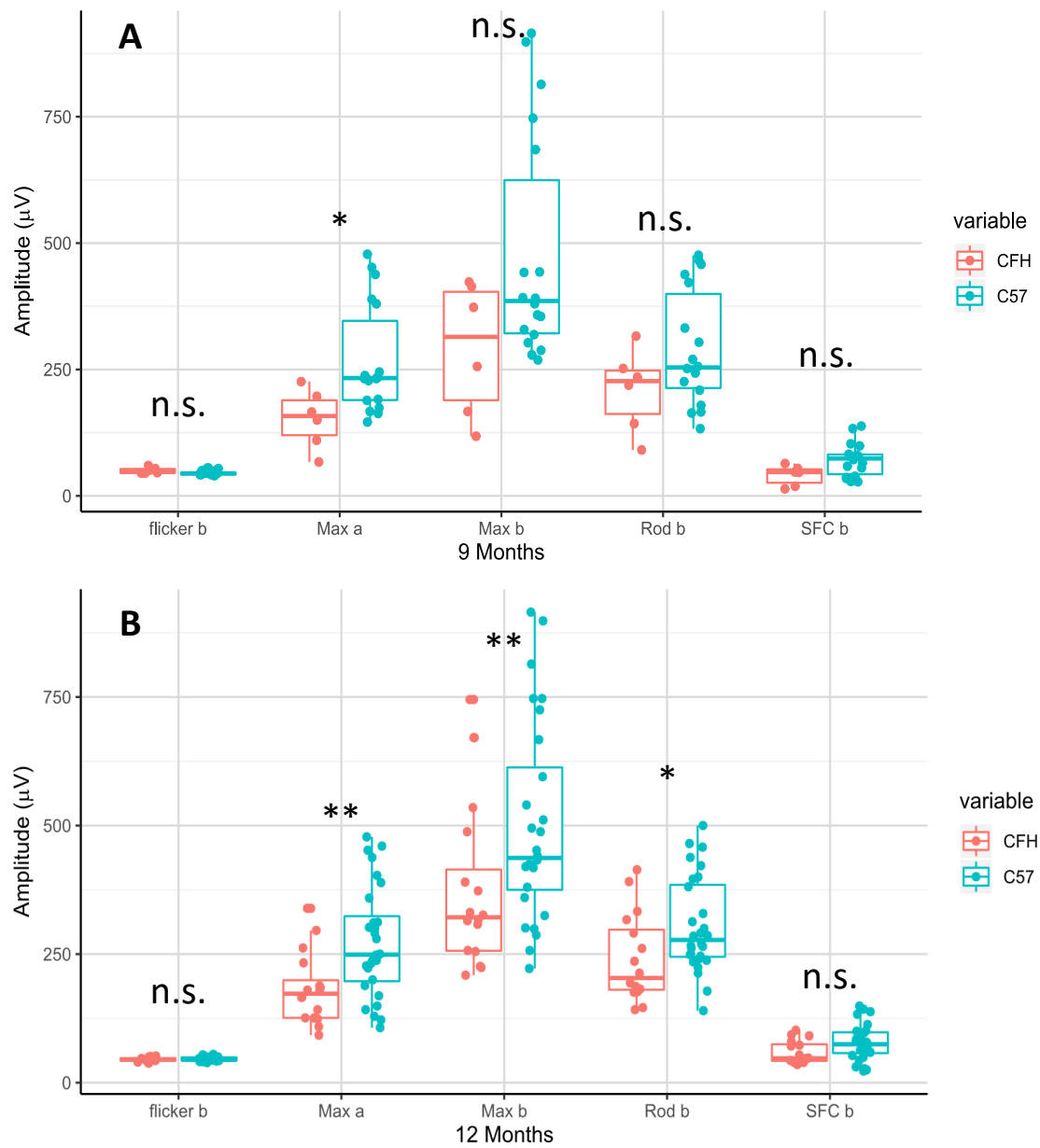


Figure 3-13: ERG analysis of C57 and Cfh $^{-/-}$ mice. A) 9 month and B) 12-month ERG analysis of C57 and CFH $^{-/-}$ mice. We see a decrease in average amplitude (μV) response across all time points, but this only reaches significance in the 9-month animals in the Max A response (Wilcoxon, CFH $n = 6$ eyes, C57 $n = 18$). As this measures rods and cones, but the rod response is not significantly decreased, there could be a decrease in cone function in these animals. At 12 months there is a significant decrease in max a, max b and rod b responses (Wilcoxon, CFH $n = 16$ eyes, C57 $n = 28$ eyes).

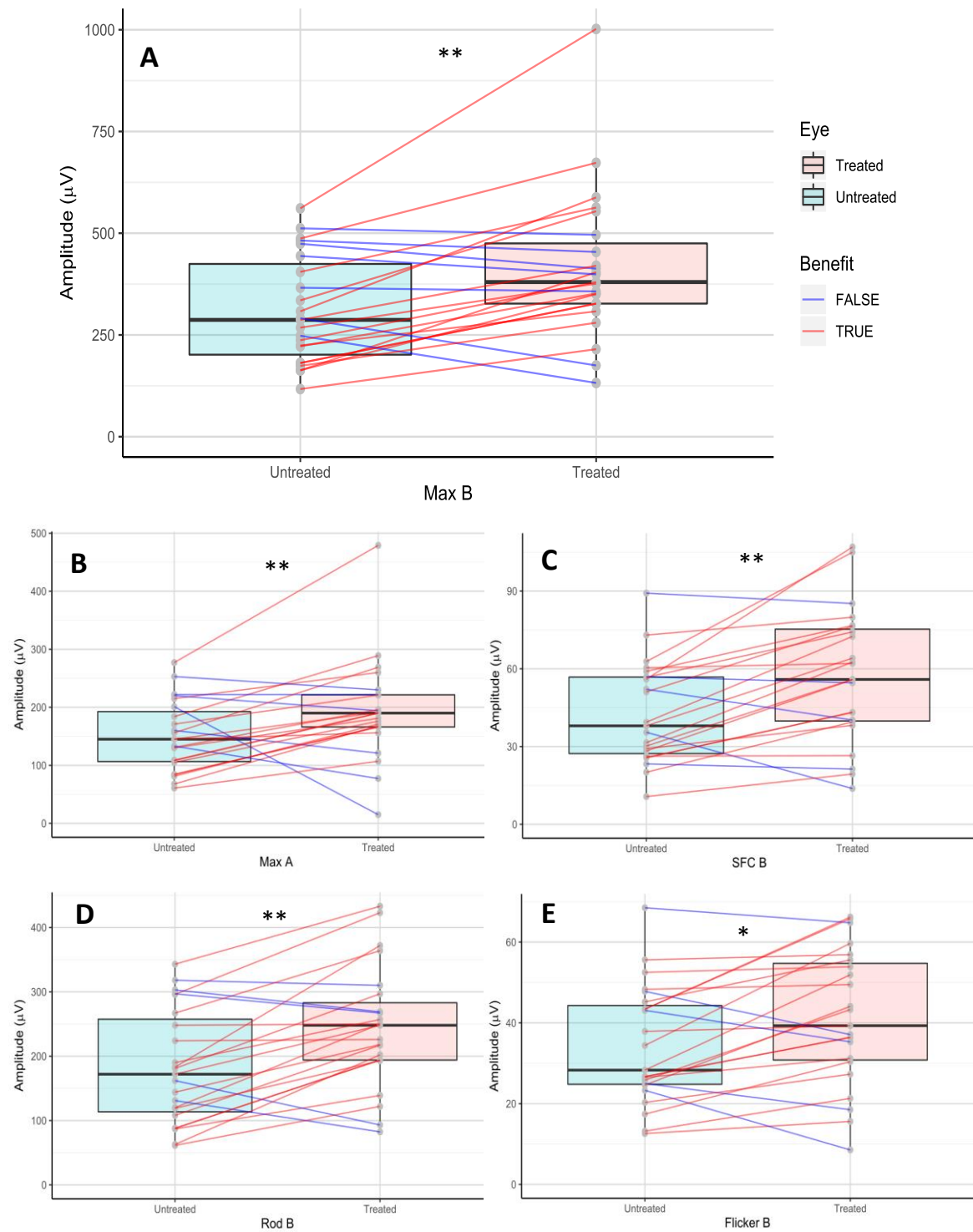


Figure 3-14: ERG analysis of treated and untreated eyes from *Cfh*^{-/-} mice aged to 9 months. Mice were injected with AAV2/8-Ndi1 (7.5×10^8 vector genomes, red box-plot) into one eye at 2 months, then aged to 9 months before ERG analysis. The fellow eye was left untreated (blue box-plot). The boxplots here show paired data, with each treated and untreated eye of an individual mouse linked by a red or blue line. If the treated eye showed an increase in ERG function over the untreated eye then the line is red. If there is a decrease in ERG function between the treated and the untreated eye, then the line is blue. All ERG functions measured showed a significant increase in function after treatment when compared to the untreated partner eye (Wilcoxon paired test, $n = 19$ pairs of eyes). This suggests an increase of visual function in these eyes when treated with this vector combination. SFC = Single Flash Cone. Flicker was 10Hz.

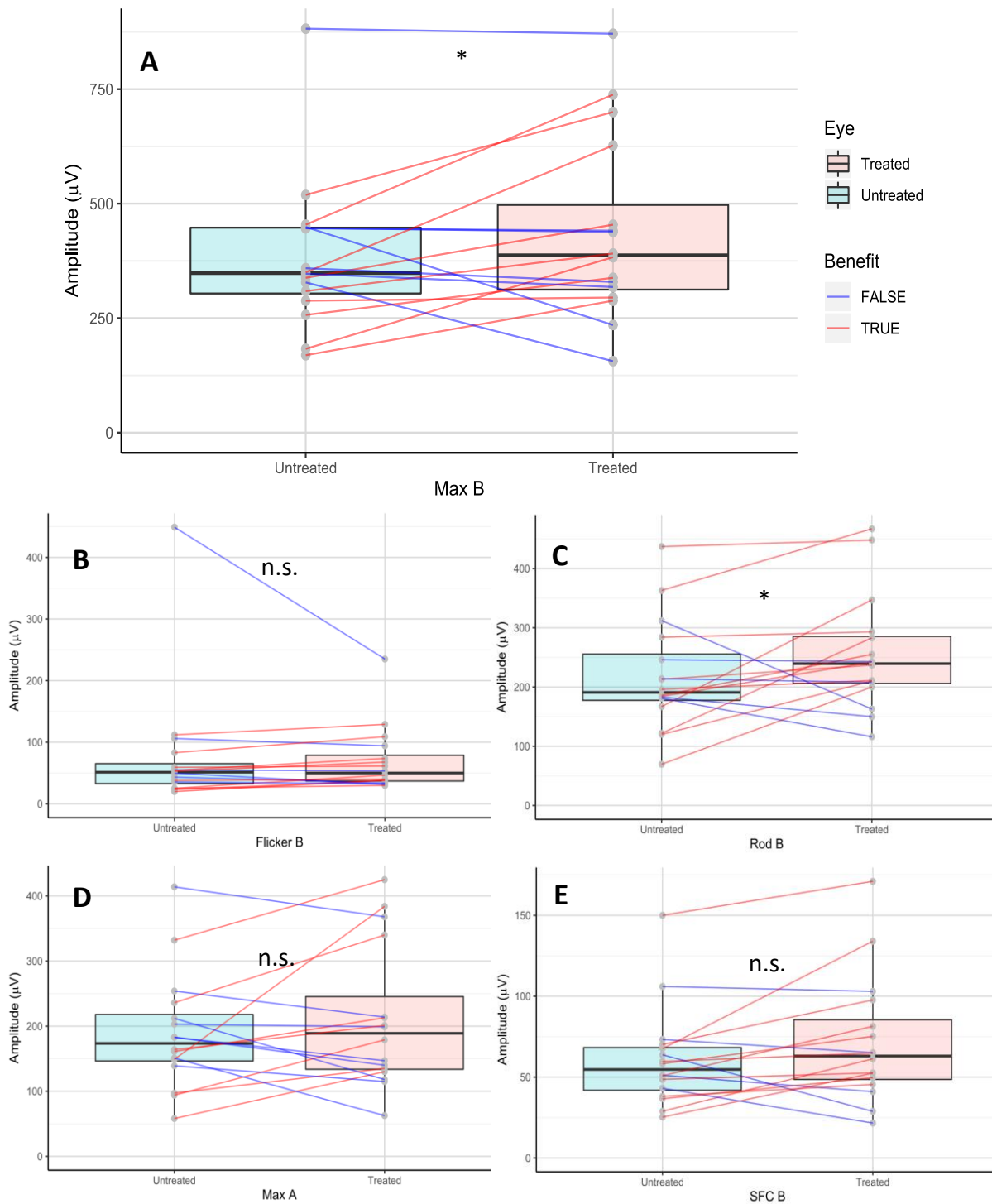


Figure 3-15: ERG analysis of treated and untreated eyes from *Cfh*^{-/-} mice aged to 9 months. Mice were injected with AAV2/2-3tyr-optNdi1 (4.7×10^8 vector genomes, red box-plot) into one eye at 2 months, then aged to 9 months before ERG analysis. The fellow eye was left untreated (blue box-plot). The boxplots here show paired data, with each treated and untreated eye of an individual mouse linked by a red or blue line. If the treated eye showed an increase in ERG function over the untreated eye then the line is red. If there is a decrease in ERG function between the treated and the untreated eye, then the line is blue. Max B and Rod B ERG traces showed a significant increase in function after treatment when compared to the untreated partner eye (Wilcoxon paired test, $n = 14$ pairs of eyes). This suggests a specific benefit to rod cells using this vector combination. SFC = Single Flash Cone. Flicker was 10Hz.

In order to examine the potential therapeutic power of Ndi1 and optNdi1 in the *Cfh*^{-/-} model a range of experimental cohorts were set up. These mice were treated in one eye with the vector to be examined with the other eye left untreated at the age of 2 months, prior to the onset of significant visual degeneration. These animals were then aged until 9 months where they were subjected to ERG analysis. For analysis purposes, the treated and untreated eyes of an individual mouse were treated as paired data with the goal of comparing the mean ERG responses between treated and untreated eyes.

When initially carrying out the ERGs of mice injected with the 2.4×10^9 vg of AAV2/2-3tyr-optNdi1 used for the DCFDA study, we found there was a significantly reduced ERG response when compared with the untreated eyes at 9 months, which was 7 months after injection. It would seem that despite success with the reduction of ROS seen at the 5-month time-point (using a different cohort of *Cfh*^{-/-} mice as for DCFDA assay the mouse must be sacrificed) there was a deleterious effect on retinal health in these animals (data not shown). This is believed to be due to over dosing and highlights the importance of dosage studies and potency assays which provide information of levels of expression between AAV batches and viral serotype. The hypothesis is that this mouse cohort effectively received too high a dose of optNdi1 protein causing dysfunction rather than the desired protection.

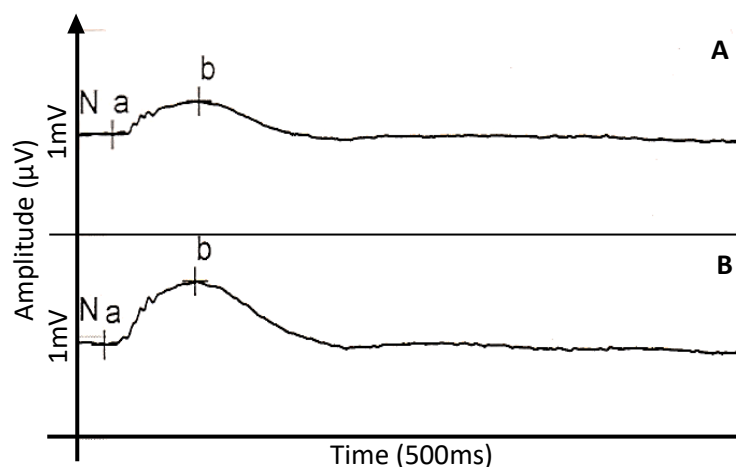


Figure 3-16: Two sample Rod ERG traces of a pair of eyes from a *Cfh*^{-/-} mouse treated with 7.5×10^8 vg of AAV2/8-Ndi1 in one eye. A) shows the untreated eye with a b-wave of 163µV. B) shows the treated eye with a b-wave of 312µV. This suggests that the Ndi1 treatment showed protective benefit in this individual.

The initial study with 2.4×10^9 vg of the AAV2/8-Ndi1 vector resulted in mice with measurable ERGs (again evaluated 7 months post injection of virus), however this mouse cohort did not show a significant benefit in treated eyes after ERG compared to the untreated eye (Wilcoxon paired test, $n = 12$ pairs of eyes, $p > .05$). Given these findings, the decision was made to lower the doses in order to find the appropriate dose range. New doses were set at 7.5×10^8 vg for AAV2/8-Ndi1 and 4.7×10^8 vg for AAV2/2-3tyr-optNdi1.

At 9 months of age (7 months post injection of vector), ERG analysis was performed on both sets of mice, both of which showed much more promising responses than at the higher doses (Figure 3-14, Figure 3-15 and Figure 3-16). Treated right eyes showed a trend towards increased amplitude across all ERG parameters measured, when compared to the untreated left eye. At the lower dose, AAV-2/8-Ndi1 showed a significant improvement in the treated eye versus the untreated eye (Wilcoxon paired test, $n = 19$ pairs of eyes, * $p < .05$, ** $p < .01$). It appears, as evaluated by ERG, that in these mice the health of the retina has improved after treatment. In these treated *Cfh* $-/-$ mice there is a significant increase in the amplitude of response from both rods (measured by Rod B, Max A and Max B) and cones (measured by single flash cone (SFC), Flicker and Max B).

In addition, AAV-2/2-3tyr-optNdi1 at the lower dose also shows some ERG improvement. The rod response is significantly improved, as is the max b wave (Wilcoxon paired test, $n = 17$ pairs of eyes, * $p < .05$). The increase in rod response but not in max A suggests there may still be cone dysfunction in these animals that the therapy has not corrected; further optimisation of vector dose will be required. The increase in max b wave suggests better connectivity of the retina is maintained, as it is inner retina cells such as bipolar cells that predominate. The data thus far while encouraging represents a preliminary evaluation, we hope to expand these data with further time points and histological analysis. Future studies are discussed below.

3.2.7. optNdi1 can increase Oxygen Consumption Rates in Patient Derived Primary Dermal Fibroblasts

Rescuing function in animal models is only part of the story, for a therapeutic to be viable it needs to demonstrate efficacy in models recapitulating the phenotype of patients affected with the disease in question. To this end we sought to establish a cell bank of primary dermal fibroblasts derived from patients as well as sex and age matched controls with which to compare mitochondrial functionality and test potential therapeutic interventions. All participants had previously been screened for the presence or absence of LHON causing mutations through the Target 5000 program in the Farrar lab.

Initially patient and age matched control fibroblasts were analysed to see if a functional deficit in OCR could be identified. Two affected patients (11778 G>A, aged 36 and 40), one carrier (no visual acuity loss at time of analysis, 11778 G>A, aged 33) and two control lines (aged 32 and 35) were used for a Seahorse XFe96 mitochondrial stress test (See Figure 3-16A, methods section 2.10).

After normalisation for cell number there is a clear separation of the OCR of the affected LHON fibroblast samples when compared to the carrier and the unaffected controls, which show between 2-3 fold higher OCR (Figure 3-16A, n = 8 wells). These data demonstrate that there is a measurable deficit in OCR of patient derived fibroblast, and therefore a potential parameter than could be modulated be a therapeutic entity, such as optNdi1.

To test this, these patient and control derived fibroblasts were examined with a Seahorse XFe96 mitochondrial stress test but were transduced with AAV2/2 optNdi1 at a range of MOIs (1e4, 5e4, 1e5 and 5e5) 48 hours prior to the assay as a dose finding study. Both patient and control cells treated with optNdi1 showed a significant increase in OCR post-rotenone compared to untreated cells, but only at the 1e5 MOI (ANOVA Tukey HSD, $\alpha = .05$, n = 4 wells, see Figure 3-16B). At the 1e5 MOI patient cells also showed a significant increase in basal OCR compared to

untreated cells, which in turn was no longer significantly reduced compared to the basal OCR of control cells (ANOVA Tukey HSD, $\alpha = .05$, $n = 4$ wells). There was no significant difference in the maximal OCR of treated vs untreated patient cells, however there was no longer any significant difference in the maximal OCR of treated patient cells compared to untreated control cells (ANOVA Tukey HSD, $\alpha = .05$, $n = 4$ wells).

These data suggest there is potential for therapeutic benefit to the OCR of patient cells treated with optNdi1, however this is only a pilot study and will need to be broadened to include more patients (especially those with LHON mutations other than 11778G>A) and controls before any definitive conclusions can be drawn.

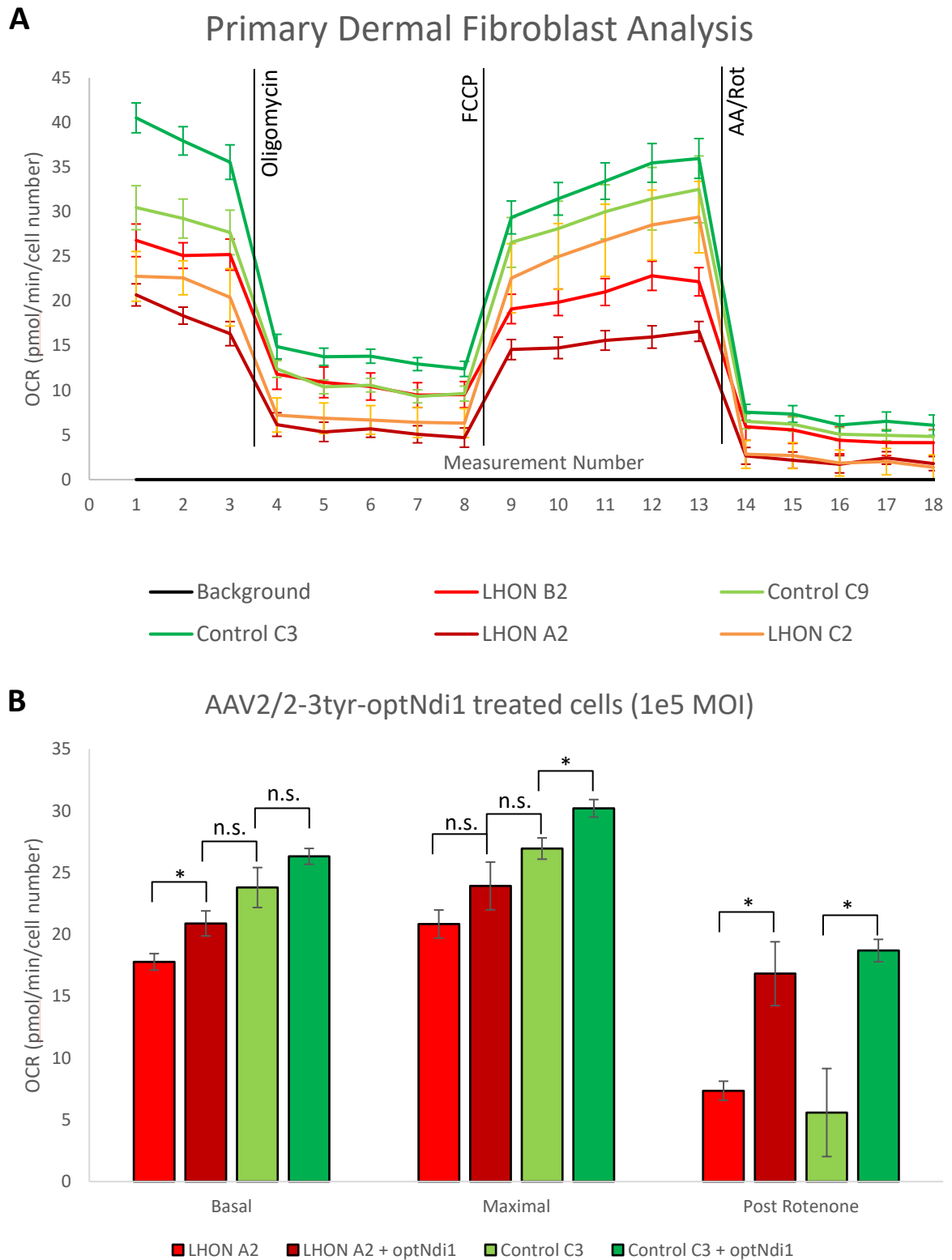


Figure 3-17: Seahorse XFe96 analysis of LHON patient and control derived fibroblasts. A) A Seahorse mitochondrial stress test of two affected patients (A2 and B2), one unaffected carrier (C2) and two age matched controls (C3 and C9). After normalisation to cell number there is a clear separation in the OCR of patient and control cells, with the carrier showing an intermediate OCR. This is indicative of mitochondrial dysfunction in these cells. B) A Seahorse mitochondrial stress test of LHON A2 cells and control C3 cells treated with 1e5 MOI of AAV2/2-3tyr-optNdi1 48 hours prior to the assay. Both cell types treated with AAV demonstrated significant oxygen consumption post rotenone compared to untreated cells indicating optNdi1 activity in these cells. Treated patient cells also showed recovery of oxygen consumption to untreated control levels. (ANOVA Tukey HSD, $\alpha = .05$, $n = 4$ wells).

3.3. Discussion and future studies

The alternative NADH:ubiquinone oxidoreductase Ndi1 has shown promise in a number of models of complex I disorders to date (Chadderton et al., 2013; Byoung Boo Seo, Marella, et al., 2006). To optimise this candidate trans kingdom gene therapy, the yeast Ndi1 gene has been modified using human codons and moreover an amino acid alteration introduced to reduce potential immunogenicity. Subsequent to the implementation of these optimisations to Ndi1 constructs, it was important to compare the characteristics of the original Ndi1 construct to the optimised version. Of note, it has been shown in this study that the optimisation process has enabled increased expression of the transgene, and that this translates to better utility in a number of biochemical assays *in vitro*. In addition, we have also demonstrated that there is potential for Ndi1 protein to have beneficial effect in a mouse model of AMD, underscoring the utility of mitochondrial protection in diseases not classically thought to be mitochondrial in origin.

3.3.1. Optimisation of Ndi1 has significant benefits

The Ndi1 construct optimisation process had a significant effect on expression levels at the RNA level in transfected HEK293 cells (Figure 3-1A). It seems likely that this increase is not solely due to the codon optimisation process per se, or the addition of a Kozak sequence, but hypothetically could be due to the elimination of an endogenous RNAi regulation site. This seems plausible as similar attempts at optimising other trans kingdom gene therapies have not yielded the same extraordinary expression level increases. For instance, in the alternative oxidase (AOX) study in chapter 6 of this thesis, optimisation of either the *C. intestinalis* or *B. glabrata* AOX genes did not lead to the same comparative increases in expression. A caveat to this is that the codon usage bias may be more significantly altered between the yeast *S. cerevisiae* and the animal lineage *C. intestinalis* and *B. glabrata*, so more benefit could be obtained purely from the optimisation process. However, this hypothesis could not be explored

as codon bias data are not available for *C. intestinalis* and *B. glabrata*. Either way, the subsequent increase in expression provides significantly more flexibility when it comes to dosing Ndi1 protein for its use as a therapeutic. For example, for a given amount vector, optNdi1 provides more protein, allowing a lower dose to be administered to achieve the same levels of expression and efficacy. This is an important consideration for potential gene therapies, as the least amount of disruption possible to the target tissue is desirable, and the ability to deliver equivalent effective doses in a lower volume would be beneficial to this. Furthermore, where viral vectors are used for gene delivery, using lower doses of virus to achieve the same level of transgene expression would aid in minimising potential immunological responses to the virus (Bessis, GarciaCozar, & Boissier, 2004).

The generation and development of the NADH oxidation assay to allow the measurement of the specific activity levels of Ndi1 will be valuable for interrogating treatment efficacy in cells. This assay enables an estimation of the maximum NADH oxidative capacity that these cells can obtain, free from any rate limiting effects caused by mitochondrial physiology. Of note there is a large increase in the enzymatic activity in HEK293 cells transfected with the optNdi1 construct compared to plasmid copy number matched Ndi1 (Figure 3-2). This is presumably due to the increased protein expression mediated by the optimisation process, which has provided a ~20-fold increase in activity compared to the original Ndi1 construct. Again, inclusion of this optimisation gives access to a much wider potential therapeutic range. The non-optimised Ndi1 only shows activity levels comparable to endogenous complex I NADH oxidation activity. Whilst a 20-fold higher NADH oxidation activity in a cell may be too much (and perhaps the mechanism of toxicity in the over-dosed AAV2/2-3tyr-optNdi1 *Cfh* ^{-/-} mice), there is scope for levels of activity higher than endogenous complex I levels to be beneficial, especially when we consider that Ndi1 protein is non-proton pumping. If focus on neurons as an example, it appears that complex I is the rate limiting step in OXPHOS (Telford, Kilbride, & Davey, 2009). As such, increasing NADH oxidation and subsequent transfer of electrons further down the ETC should

increase the ability of cells to pump protons from complexes III and IV, in turn generating more ATP, thus potentially counteracting the by-passing of CI proton pumping. One could hypothesise that optNdi1 could out-compete complex I for NADH and thus reduce the proton pumping of the ETC by 4 out of 10 protons per molecule of NADH, however the impact of this could be mitigated as long as NADH is in excess and CIII and CIV do not get overwhelmed. The ability of optNdi1 expressing cells to grow better in galactose containing media suggests that Ndi1 protein can work synergistically rather than antagonistically to the canonical ETC (Figure 3-5B).

Preliminary work has been carried out on using this assay to study Ndi1 and optNdi1 treated retinæ *ex vivo*, however thus far we have had issues measuring the endogenous complex I levels above that of background. This would not allow us to obtain an accurate portrayal of the therapeutic benefit, or indeed the potential deficiency in the first place. Alternative spectrophotometric assays are being investigated.

The enhanced protein expression driven by the optimisation process seems to have a noticeable effect on the ability of Ndi1 to compensate for complex I dysfunction, as seen in the bioenergetics assays (Figure 3-4 and 3-5). These assays also demonstrate that optNdi1 and Ndi1 do not negatively perturb the cell's normal bioenergetics, perhaps apart from the increase in ROS seen in untreated and rotenone treated HEK293 cells grown in glucose media. An increase of ROS in cells, and *Drosophila*, ectopically expressing Ndi1 has been shown previously (Alberto Sanz, 2016; Scialò et al., 2017). This has been linked to the phenomenon of reverse electron transport (RET), whereby a highly reduced quinone pool, induced by the presence of Ndi1, transfers electrons back through CI forming ROS. This in turn is believed to have a protective role *in vivo*, as increased RET has been associated with an increase in lifespan in *Drosophila*, which is ablated if a mitochondrially targeted catalase is co-expressed (Scialò et al., 2016). However, if RET is solely responsible for the increase in ROS we see in the glucose ROS HEK293 cells, we would expect the ROS increase to be proportional to the amount of Ndi1 protein

present, i.e., optNdi1 cells should exhibit more RET than Ndi1 expressing cells. In this ROS assay we do not see a significant increase between the two Ndi1 constructs (Figure 3-4C). Of course, there could be other rate limiting factors preventing increased Ndi1 protein leading to RET, but I think it is also plausible that generally increased mitochondrial activity in the HEK293 cells due to the presence of Ndi1 could lead to more ROS production, as the mitochondria is the primary source of ROS in the cell (Murphy, 2009). As the mock-transfected cells would primarily just use glycolysis to produce ATP, the addition the Ndi1 protein could shift their metabolism partially back towards OXPHOS even in excess glucose. Further weight is given to this theory by the increase in average basal OCR of the cells when examined in the Seahorse XFe24 (Figure 3-5C). These cells were grown in glucose media prior to the assay, and glucose was provided during the assay itself.

The marginal, yet significant, increase in ATP levels in glucose grown cells expressing Ndi1 and optNdi1 when poisoned with rotenone also suggests the continued use of OXPHOS could be providing the extra ROS seen in the glucose grown cells. It also underlines how little HEK293 cells utilise mitochondrial OXPHOS for ATP production when grown in an excess of glucose, as rotenone treated eGFP expressing cells retained an average of 75% the levels of ATP seen in untreated cells.

When HEK293 cells were forced to use their mitochondria for ATP production via galactose media, Ndi1 and optNdi1 plasmids offered significant protection against CI inhibition, allowing maintenance of cellular ATP levels significantly higher than those of eGFP transfected cells. After 24 hours optNdi1 showed significantly higher levels of ATP than did Ndi1 transfected cells, suggesting a dose response in these cells. When compared to wild-type levels, the rescue was marginal however, but this may at least in part be explained by transfection efficiency. HEK293 cells were routinely transfected with eGFP to monitor transfection efficiencies which were typically in the region of 50-60%. Plasmid transfection only ever transfects a proportion of cells

and as such only this subpopulation would be protected in this assay. The generation of a stable cell line would be useful in more precisely quantifying the levels of ATP protection achievable when using Ndi1 as the sole NADH:ubiquinone oxidoreductase.

It is interesting that optNdi1 is significantly better at protecting from ROS than Ndi1 appears to be, when cells are forced to use galactose. However, during the time course of the experiment, Ndi1 becomes significantly better than mock-transfected cells (Figure 3-5D). It could be that the initial insult is better dealt with by optNdi1 due to its increased expression levels, but that Ndi1 still allows cells to live sufficiently long to cope with the elevated ROS production.

The protection of OCR after rotenone treatment of HEK293 Ndi1 and optNdi1 cells is substantial (Figure 3-5C) and hints at a potentially wide window of CI dysfunction that Ndi1 protein could potentially mitigate. It would be interesting to see if this increase in OCR can be sustained by cells for prolonged periods of time, as the Seahorse only measures OCR over a relatively brief period of time. Again, a stable cell line would help us to investigate this further, for instance poisoning a cell population with Ndi1 and optNdi1 stably expressed and then subsequently measuring the OCR the following day. However, it is important to remember that in actual disease aetiology, there will never be complete loss of function of CI activity as is seen with rotenone poisoning, so from an academic point of view the proposed experiments would help us better understand how Ndi1 fits into the canonical ETC, but perhaps would not be as directly relevant to patients.

What would be informative to look at is the P/O ratio, or respiratory control ratio (RCR) in cells expressing Ndi1 protein. This is essentially a measure of the efficiency of the ETC, how much oxygen is consumed to phosphorylate a known amount of ADP to ATP. As the non-proton motive Ndi1 protein can potentially act as an uncoupler, it would be informative to investigate the RCR in cells expressing the two Ndi1 constructs. As the ETC becomes increasingly uncoupled, the amount of oxygen consumed to reduce this known quantity of ADP would increase. As Ndi1

protein levels increase there is increased potential to uncouple the ETC, so this would be an important metric to quantify. For instance, as we increase viral MOI, as in the NADH dosage curve, would we significantly alter the P/O ratio in these cells or can the potential for increased CIII and CIV activity counteract the bypassing of CI. Unfortunately, we do not currently have the set up required in our lab to test this, namely a Clark-type electrode oxygraph, however it is something to investigate going forward.

3.3.2. Potency assays are informative for the design of therapeutic interventions

The dosage curve will prove useful for testing efficacy and potency matching future viral preparations. Currently we titre match viral preparation via real-time PCR analysis of each viral fraction to provide a viral genome quantity. The assays generated and developed in this study with associated dosage curves will allow a further level of normalisation between AAV preparations in terms of the efficacy levels provided by the vector. This is essential if one is to robustly compare data sets between experiments whether from in vitro or in vivo experiments or preclinical or clinical studies. The sigmoidal dose curve that the increasing viral MOIs produces is also informative. It demonstrates that there is a range where comparatively minor changes in dose can lead to much more substantial levels of activity. Both curves have mid points around the 2×10^4 MOI. At this point, a ± 2 x change in the quantity of virus added at this point would lead to roughly a $\pm 30\%$ change in activity levels for protein normalisation or a $\pm 40\%$ change for citrate synthase (CS) normalisation. In comparison, a 5x increase in MOI at the low end 1.6×10^2 to 8×10^2 achieves similar increase in activity levels.

The disparity between the activity levels normalised to CS activity and protein content is interesting especially as they follow very similar trajectories at the lower end of the dose curve. Citrate synthase is the first step in the citric acid cycle, where the cell utilises glycolytic pyruvate

to produce NADH for utilisation in OXPHOS. High levels of NADH negatively regulate CS activity. It is possible that the presence of Ndi1 at the higher doses is sufficiently altering the NADH levels in the mitochondria, via NADH oxidation, which in turn increases the activity of CS in these mitochondria. As CS activity level was being used to normalise, an increase in CS activity due to lessened inhibition would result in an apparent decrease in the corresponding NADH oxidation activity. CS activity levels are considered to be a good biomarker for mitochondrial content (Larsen et al., 2012), so alternatively the inconsistencies observed in this study could be due to experimental error, increasing the replicates further may help to identify the true population mean more accurately.

It could also be possible that there is increased mitochondrial biogenesis in cells with more robust OXPHOS due to the presence of high levels of Ndi1 protein, which again would also increase CS activity in proportion to this. This could be investigated by measuring mtDNA content in these cells to evaluate if increased Ndi1 protein leads to an increase in mitochondrial mass and indeed it is our intention to undertake this assay in the future. Currently it seems that protein normalisation gives the better fitting curve and that for a given amount of extracted mitochondrial protein NADH oxidation activity in these transduced cells remains largely consistent.

3.3.3. Ndi1 and optNdi1 show promise for complex disorders

The research that has been undertaken in the *Cfh* ^{-/-} mouse model of AMD is demonstrating some exciting preliminary results. The protection of visual function in these mice, as evaluated by ERG benefit and the decrease in cellular ROS levels are both positive outcomes for the use of Ndi1 as a potential therapeutic in this model, which demonstrates the first example of Ndi1-mediated benefit outside a primary mitochondrial disorder due to complex I deficiency such as LHON, with the exception of one study demonstrating benefit related to delivery of Ndi1 for an ocular phenotype in a murine MS model (Talla et al., 2013). Furthermore, this study

demonstrates the potential benefit of modulating mitochondrial function for disorders such as AMD, whether using Ndi1 or other candidate therapies. I believe the therapeutic benefit observed in this study could be from two potential mechanisms, or possibly a combination of both; either metabolically, through decreasing ROS and increasing ATP production ability, or through prophylactic hormesis via reverse electron transfer (RET). Mitochondrial dysfunction has been linked to the visual loss in *Cfh*^{-/-} mice, where ATP levels in *Cfh*^{-/-} retinæ were significantly lower than those of C57 mice at 8 months (Calaza et al., 2015). It is conceivable that, as we saw in HEK293 cells, that the expression of Ndi1 protein results in the increased generation of ATP allowing prolonged survival of retinal cells. In this regard it is interesting to note that in recent studies it has been found that mitochondrial haplogroup J carriers, who are at higher risk of developing AMD, show decreased levels of ATP and even decreased CFH levels (Riazi-Esfahani et al., 2017). These observations support the view that there is a link between CFH and mitochondrial ETC dysfunction.

It is also possible that the presence of Ndi1 is triggering RET in the RPE and photoreceptors transduced by our viruses, as the presence of ectopic Ndi1 has been shown to induce RET, which in turn has a protective effect on longevity in *Drosophila* (Scialò et al., 2016). RET is believed to trigger protective mechanisms in cells, by locally increasing ROS produced through CI, although the presence and relevance of this phenomenon is still debated (Scialò et al., 2017). To dissect this further in our model, a potential method could be to overexpress other ubiquinone reducing enzymes, such as CII maybe, to see if Ndi1 is beneficial because of its ability to bypass CI, or if it is due to over-reduction of the ubiquinone pool and the subsequent initiation of RET and its protective properties. The potential of Ndi1 to provide benefit in disorders not directly linked to CI dysfunction is an intriguing possibility and opens the door to an array of other candidate mitochondrial disorders where the protective benefits of Ndi1 could be useful to employ.

The recent demonstration of mitochondrial dysfunction early on in *Cfh*^{-/-} (Sivapathasuntharam et al., 2019) correlates well with our finding that mitochondrial protection mediated by Ndi1 and optNdi1 can provide functional benefit in the *Cfh*^{-/-} model. We also hope to perform electron microscopy on some of the aged, treated and untreated *Cfh*^{-/-} mice to see if there is any reversion of the changes to mitochondrial morphology seen. Significant structural aberration of the retina was also seen in the original paper describing the *Cfh*^{-/-} mouse model, with disorganisation of the photoreceptor outer segments (Coffey et al., 2007). In the original paper, significant ERG differences were only seen at around two years of age, whereas we have shown statistical differences from as early as 9 months (Figure 3-13). We believe this is due to our elevated numbers compared to the original study; n = 4 (Coffey et al., 2007) vs n ≥ 14 in our study.

There has been suggestion that the growth environment of the *Cfh*^{-/-} mice affects the severity of the phenotype of the mice, where mice kept in an open environment develop a more severe phenotype than those in specific-pathogen free (SPF) facilities (Hoh Kam et al., 2016). As our mice and those in the original study were housed in an SPF environment, we believe them to be comparable. Given the recent developments with regards a potential bacterial involvement in the development of Alzheimer's disease and the role of *P. gingivalis* (Dominy et al., 2019) it would be interesting to parse if the worsening of the *Cfh*^{-/-} phenotype is triggered by a particular bacterium in these open environment mice, or if the *Cfh*^{-/-} mice just need an immunogenic trigger to initiate this increase in the detrimental phenotype.

3.3.4. Future translatability of Ndi1 as a gene therapy intervention

Although Ndi1 expression shows benefit in the *Cfh*^{-/-} model of AMD, further examination will need to be done in order to see if this is translational to other models of AMD and further down the line hopefully translatable to patients. For future studies with our *Cfh*^{-/-} mouse model it would be beneficial to run blinded studies, where the identity of the treated mice remains

unknown to the researcher examining the experimental cohort. This could be of particular value if mice were then used for animal experiments involving participation from the animal such as measurement of the optokinetic response. This would allow the removal of potential biases from knowing which animals have been treated before the running of the experiments themselves and will be implemented where possible going forward.

In future we also hope to be able to interrogate RPE cells directly in tissue culture. With the amazing detailing of the metabolic processes in the eye having demonstrated the reliance of RPE on OXPHOS and retina on aerobic glycolysis (Hurley, Lindsay, & Du, 2015; Kanow et al., 2017), it would be very interesting to see how Ndi1 fits into this framework. If the decline in RPE OXPHOS dependency does lead to starvation of the photoreceptors, it is conceivable that the presence of Ndi1 could promote the continued use of the mitochondria for ATP production in RPE cells, and potentially protect from this metabolic shift induced starvation. RPE tissue culture would help focus in on the role of Ndi1 in these cells.

The work using patient and control derived fibroblasts provides an insight into the translatability of Ndi1 based therapeutic interventions (Figure 3.16A and B). The work here demonstrates that there is a deficit in oxygen consumption in cells with the LHON associated 11778G>A mutation, with both patient lines examined, and a carrier line, showing a lower OCR compared to age matched controls. Treatment with optNdi1 can rescue this deficit in oxygen consumption (Figure 3.16B). This is a promising finding that suggests that optNdi1 could be beneficial in LHON. However, this is only a preliminary study and would need to be expanded before any definitive conclusions can be drawn. An increase of patient samples would be necessary, as well as samples from patients with mutations other than the Wallace 11778G>A mutation. But as this mutation accounts for a significant proportion of LHON patients it is a good place to start. Further work also needs to be carried out to establish if this increase in OCR in patient line also

translates to benefit in other mitochondrial biomarkers such as increased ATP levels and if this translates to increased survival of cells.

4. Investigating OPA1 Gene Therapy for Dominant Optic Atrophy

4.1. Introduction

4.1.1. OPA1 structure and function

OPA1 is a mitochondrially targeted GTPase that plays a pivotal role in IMM fusion with mutations causing severe mitochondrial dysfunctionality. Apart from mediating fusion in mitochondria, OPA1 also plays a role in maintenance of mtDNA levels, as patients harbouring OPA1 mutations show lower mtDNA levels (Vidoni, Zanna, Rugolo, Sarzi, & Lenaers, 2013). It is up for debate however whether this is a direct result of OPA1 mutation or due to a lack of mitochondrial fusion. It appears that a lack of OPA1 does disrupt the distribution of mtDNA, leading to aggregation of mtDNA nucleosomes (Elachouri et al., 2011). Since similar accumulation of mtDNA mutations occur in MFN2 patients, it seems likely that the process of fusion itself is needed for mitochondrial mtDNA maintenance (Rouzier et al., 2012). OPA1 mutations have also been shown to cause serious disorganisation of the mitochondrial ultrastructure, with cristae structure being compromised, a process which is believed to be independent of its role in IMM fusion (Frezza et al., 2006). OXPHOS in OPA1 deficient cells is decreased, not just because of the lack of fusion, but there is also evidence that OPA1 interacts directly with members of the ETC to stabilise them, with OPA1 depletion leading to a decrease in CIV activity specifically (Agier et al., 2012; Takahashi, Ohsawa, Shirasawa, & Takahashi, 2017).

OPA1 possess an MTS and localises to the IMM, with the majority of the protein facing into the inter-membrane space (Aurélien Olichon et al., 2002). This is presumably to allow it to come into contact with other mitochondrial IMMs to mediate the process of membrane fusion. OPA1 is a dynamin-related protein, so is similar in structure to Drp1, the mediator of mitochondrial fission (Ferguson & De Camilli, 2012). OPA1 is thought to be the human orthologue of the yeast MGM1 gene, and likewise has a transmembrane domain, which anchors it to the IMM, a coiled-coil domain, then A GTPase domain, a middle domain and a coiled-coil GTPase effector domain

(GED) (Delettre et al., 2000a). The OPA1 gene consists of 31 exons, which produce 8 mRNA isoforms, which are produced by the differential splicing of exons 4, 4b and 5b (A Olichon et al., 2007) (see Figure 4-1).

These eight isoforms are all transported to the mitochondria, where the MTS is cleaved and the transmembrane domain attaches the protein to the IMM (Aurélien Olichon et al., 2002). This is termed the long isoform or l-form of OPA1. These l-forms can then be cleaved at either S1 or S2, present on exons 5 or 5b respectively, by either OMA1 or YME1L, respectively. This releases a short isoform, or s-form, of the protein into the intermembrane space (A Olichon et al., 2007). Any isoform that contains the 4b exon is completely processed into s-form (Song, Chen, Fiket, Alexander, & Chan, 2007). This means that exon 4b containing isoforms produce only s-forms of OPA1 protein, whereas the others produce both l and s-forms. These l and s-forms are believed to produce distinct physiological functions and a lot of work has gone into deciphering the different roles of each of the isoforms (Del Dotto et al., 2017).

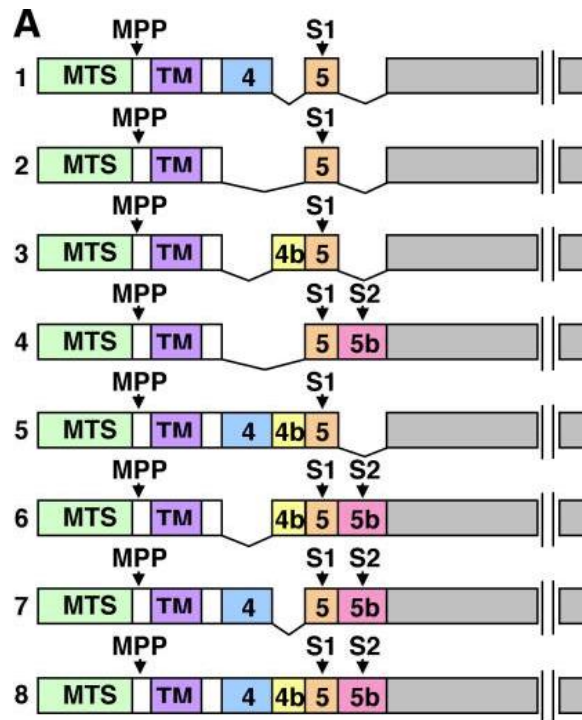


Figure 4-1: The eight different OPA1 isoforms, produced by differential splicing of exons 4, 4b and 5b, shown in blue, yellow and pink respectively. The mitochondrial targeting sequence (MTS) is shown in green and is cleaved by MPP. The purple box is the transmembrane domain (TM), which allows the protein to be anchored to the mitochondrial IMM. S1 and S2 correspond to proteolytic cleavage sites, with site S1 being cleaved by OMA1 and site S2 being cleaved by YME1L. Figure taken from (Z. Zhang et al., 2011).

Expression of any of the eight isoforms was sufficient to restore mtDNA levels, reorganise mitochondrial cristae, and boost the function of the ETC. However, the presence of both I and s-forms of OPA1 are needed to restore fusion and thus correct network morphology to the mitochondria (Del Dotto et al., 2017). This would suggest that the most beneficial isoform(s) to take forward for potential therapeutic intervention would be one that produces both I and s-forms of OPA1 protein. Intriguingly the same study also found that a mutated OPA1 isoform that could not be proteolytically processed and thus only produced I-form protein, could restore fusion to the mitochondrial network, but not restoration of the tubular morphology, suggesting network fusion and maintenance are somewhat distinct processes (Del Dotto et al., 2017).

The finding that the different isoforms provide overlapping but distinct functions in the mitochondria highlights the need to better understand the isoform expression in the target tissue, in this case the retina. Different tissues have been shown to have different patterns of

expression with isoforms 1 and 7 being predominant throughout the body, but isoform 5 being the most highly expressed in brain tissue (A Olichon et al., 2007). However, to our knowledge no direct analysis has been made of retinal tissue to identify the OPA1 isoform expression pattern. In the current study we aimed to resolve this by interrogating publicly available RNAseq data to analyse OPA1 isoform expression.

4.1.2. DOA therapeutic approaches

Currently there is no effective treatment for DOA, meaning there is a substantial clinical need being unmet. Recently though, there have been some promising results providing hope for the development of DOA therapeutics.

The fact that most mutations in the OPA1 gene are point mutations would suggest that it could potentially lend itself to therapeutic intervention with a genome editing strategy using, for example, CRISPR/Cas editing or other strategies to suppress expression of the target mutant allele in a mutation-specific manner such as RNA interference (RNAi). These approaches have been used with some success for a number of inherited ocular conditions. By way of example, a missense mutation in retinitis pigmentosa has been corrected in a mouse model after sub-retinal injection of mutation specific CRISPR/Cas therapy (Bakondi et al., 2016), representing one of a number of recent studies. However, given the wide array of mutations in DOA, with many of them being family specific, it is unclear if it will be feasible to design CRISPR/Cas or RNAi strategies for all of them. If clinical trials need to be carried out for each mutation it would surely be prohibitive in terms of time and cost to use a mutation-specific therapeutic strategy for the OPA1 gene, given that there is such mutational heterogeneity in DOA. However, CRISPR/Cas editing may remain a potentially effective therapeutic approach for dominant negative mutations in OPA1, as likewise RNAi-mediated OPA1 suppression. Potentially an edit and replace, or RNAi suppression and replace approach, could be adopted for OPA1-linked DOA thereby overcoming mutational heterogeneity much like that utilised previously for dominant

forms of retinitis pigmentosa (Millington-Ward et al., 2011; O'Reilly et al., 2007). This strategy utilises suppression/editing of the target gene, whilst also involving delivery of a codon-modified copy of the gene encoding wild type protein but engineered to be resistant to suppression/editing using the degeneracy of the genetic code (Millington-Ward et al., 2011).

Whilst such a novel strategy circumvents the mutational heterogeneity in some dominantly inherited diseases, it is notable that for dominant OPA1-linked DOA a simple gene replacement approach may be possible. As the majority of mutations are believed to cause haploinsufficiency (Del Dotto, Fogazza, Lenaers, et al., 2018), OPA1-linked DOA is a prime candidate for virally-mediated gene replacement therapy. The full length OPA1 gene would be far too large to deliver via viral interventions, in particular using AAV, but the cds is around 3kb for isoform 1. The cds length of course changes depending on the specific isoform chosen. Given size restrictions for AAV-delivered transgenes the choice of OPA1 isoform becomes more important, as we can currently only package a single isoform for therapeutic intervention using a single AAV.

At the outset of this project, OPA1 mediated interventions had predominantly been used for disorders other than DOA. Although this seems a little counterintuitive, it has led to some interesting findings. A mouse line was generated where the OPA1 isoform 1 was under the control of the β -actin promoter and was targeted to a permissible site on the X-chromosome, leading to mild OPA1 over-expression (Cogliati et al., 2013). These mice were viable and fertile, showing in principle that a degree of OPA1 over-expression can be tolerated organism wide. This led to a significant increase in super-complex formation and tightness of cristae junctions. These increases in super-complex formation were not a result of increased mtDNA and subsequent mitochondrial protein increase, as levels of both stayed the same when compared to wt mice (Cogliati et al., 2013). Further work using this OPA1 mouse model demonstrated that the mouse was also protected from reperfusion ischemia damage, liver cellular apoptosis due

to Fas-activation as well as being able to ameliorate deterioration in the *Ndufs4*^{-/-} and *Cox15*⁷ mouse models (Civiletto et al., 2015a; Varanita et al., 2015). The mechanism of benefit derived from OPA1 overexpression is likely two-fold. First, as these mouse models involve assembly factors of CI and CIV respectively, it appears that OPA1 can help with the stabilisation of these complexes *in vivo* and their subsequent formation into respiratory super-complexes, allowing continued respiration in these compromised cells. Secondly, OPA1 overexpression is anti-apoptotic due to its cristae tightening capabilities thereby preventing the release of pro-apoptotic markers such as cytochrome-c. This effectively raises the threshold of dysfunction required prior to apoptosis being initiated. This aspect of OPA1 over-expression is desirable when targeting a sub-population of cells prone to apoptosis due to dysfunction, but such organism wide anti-apoptotic stimuli can have detrimental effects, namely prevention of apoptosis in cells that need to be removed. This indeed is borne out by the observation of an increase in sporadic cancer rates in OPA1 overexpression mice bred onto one of the two genetic backgrounds investigated (Varanita et al., 2015). However, it is possible that this anti-apoptotic effect might be a boon to a post-mitotic tissue like the retina allowing protection of severely compromised cells that cannot be replaced by cell division. The role of OPA1 in tumour-genesis has been highlighted recently in a study of tumour suppressors. KIF1B β is a tumour suppressor that activates YME1L, the protease that cleaves at S2, and is frequently dysfunctional in neuroblastomas. This activation of YME1L leads to cleavage of OPA1 I-forms causing fragmentation of the mitochondrial network which leads to cell death (Ando et al., 2019). As the S2 site is only on exon 5b this again demonstrates the need for careful selection of OPA1 isoform. Furthermore, it has been proposed that OMA1, the other OPA1 protease that targets site S1 is a target of P53 activation, again leading to OPA1 I-form cleavage and then apoptosis (Alavi, 2019).

⁷ This *Cox15* mouse model is the same as the one mentioned in the AOX chapter with regards to systemic CiAOX expression.

Subsequent to the completion of the work detailed in this chapter of the thesis, recent progress has been made in AAV-OPA1 delivery for treatment of DOA. Using a mouse model containing a known human pathological mutation in exon 27 of the OPA1 gene, AAV2/2-OPA1 isoform 1 driven by the CMV promoter showed significant protection of RGCs (Sarzi et al., 2018). This RGC protection did not fully translate to increased visual acuity however, with only a small benefit recorded. The authors believe this could be rectified with increased viral dose potentially reaching more RGCs. In addition, it is notable that AAV2/2-OPA1 has also shown to be beneficial in a laser induced model of glaucoma in rats, where it also provided protection of RGCs (Hu, Dai, Zhang, Shang, & Sun, 2018). OPA1 overexpression attenuated Bax expression in RGCs preventing the initiation of apoptosis in these cells. There was an OPA1 mediated increase in the levels of Parkin in these rat RGCs, which led to an increase in mitophagy. Visual function was not probed in these rats, however, the ability to protect RGCs from death represents a promising start and highlights the potential value of OPA1-based gene therapies.

Recent work has implicated OPA1 in other ocular diseases. It appears that OPA1 is upregulated in RPE cells of a zebrafish model of retinal degeneration, which is believed to be as a protective mechanism (Toms et al., 2019). There is also a dramatic decrease in OPA1 protein expression, and subsequent increase in Drp1 protein expression after exposure of ARPE-19 cells after treatment of blue light, with blue light being a known exacerbator of AMD (Alaimo et al., 2019). This led to marked fragmentation of the mitochondrial network and subsequent cell death, due to the activation of OMA1, the protease that cleaves OPA1 I-forms at the S1 cleavage site.

These data suggest the potential utility of virally delivered OPA1 as a potential method of treatment for optic atrophy, as well in other ocular diseases where mitochondrial dynamics are perturbed. Although progress has been made recently in OPA1 related therapeutics, there is still scope for further investigations of OPA1 isoforms as therapeutic strategies to combat aberrant mitochondrial dynamics. As there has not been any definitive elucidation of the OPA1 isoform

expression pattern in human retinal tissue, we first set out to answer this question using publicly available RNAseq data. Using this information, we then wanted to design potential OPA1 isoform specific constructs to test in an array of *in vitro* based functional assays to see if any difference in therapeutic potential of these OPA1 isoforms could be identified.

4.2. Results

4.2.1. RNAseq Analysis of Retinal Tissue

The OPA1 gene has been identified to have a number of isoforms which have been shown to be expressed to different extents across tissue types. Initial proteomic work has identified OPA isoform 1 as the primary isoform present in mouse tissue (Akepati et al., 2008). This was done through Western Blot analysis. To the best of our knowledge the question of which isoform is primarily expressed in the healthy human retina has not been investigated. In order to address this, we analysed some publicly available RNA-seq data from retinal tissue. The original RNA-seq was carried out on 8 healthy post-mortem retinæ (Li et al., 2014) but the data is available on the NCBI Gene Expression Omnibus (GEO, accessed 14/07/2017; Accession: GSE94437). Samples used were from punches of the macular retina. Briefly the raw sequence data provided was converted to fastq format and aligned to the human genome (hg38) using STAR (Dobin et al., 2013). These aligned reads were then converted to Fragments Per Kilobase of transcript per Million mapped reads (FPKM) which gives a normalised count of reads for each gene, taking into account the length of the gene and the depth of sequencing.

The output from the 8 samples was initially probed for markers for specific cell lineages to ensure the data came from a retinal tissue sample. Myoglobin, haemoglobin, and T-cell receptor proteins all returned less than one FPKM, whereas the cone opsins, and in particular rhodopsin showed highly levels of expression, with rhodopsin FPKM values of between 2,000 to 4,000. This means at the high end 1 out of every 250 reads is for rhodopsin in these samples. Next, the expression of the different OPA1 isoforms in these samples was compared, using the curated list of OPA1 transcripts from the Ensembl database⁸ (see Figure 4-2A) Isoform 1

⁸

https://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000198836;r=3:193648766-193659514;t=ENST00000482865 – accessed 14/07/2017

(ENST00000392438.7) showed by far the highest FPKM, with an average of 12.4 FPKM, followed by isoform 7 (ENST00000361908.7), with an average of 3.4 FPKM. With an average total of 24.4 FPKM attributed to the OPA1 gene, meaning in this analysis 50.7% of OPA1 gene transcripts are isoform 1 and 13.9% from isoform 7. This is in agreement with previous data suggesting isoform 1 and 7 are the predominant isoforms in other tissues. The “no protein 2” (ENST00000482865.1) isoform is intriguing as it shows high levels of expression with a high degree of variation between samples. This transcript spans exons 18-23 of OPA1 gene with some retained intronic sequence and was computationally annotated by the HAVANA project. No data on function exists so it is unclear if it is of biological relevance, but it could possibly be a non-coding RNA.

Overall OPA1 gene expression when compared to other proteins associated with mitochondrial remodelling was also investigated (see Figure 4-2B). At the RNA transcript level at least, these proteins are expressed at broadly similar levels to each other. The consensus in the field is that Dnm1l works antagonistically to promote fission in mitochondria while the mitofusins and OPA work to fuse mitochondrial networks. It is conceivable that relative levels of these proteins are important to maintain the correct mitochondrial morphology. Analysis of the correlation between the levels of each of these proteins did not reach significance with the 8 macular retina samples. However, increasing the size of the dataset (n=16 samples) by inclusion of peripheral retinal samples sequenced by the same group (Li et al., 2014) showed that there is a significant positive correlation between the mitochondrial remodelling proteins (apart from Mfn1) after correction for multiple testing (Bonferroni method), but not when compared to the β -actin house-keeping gene in these samples (see Table 4-1 and Figure 4-2C & D).

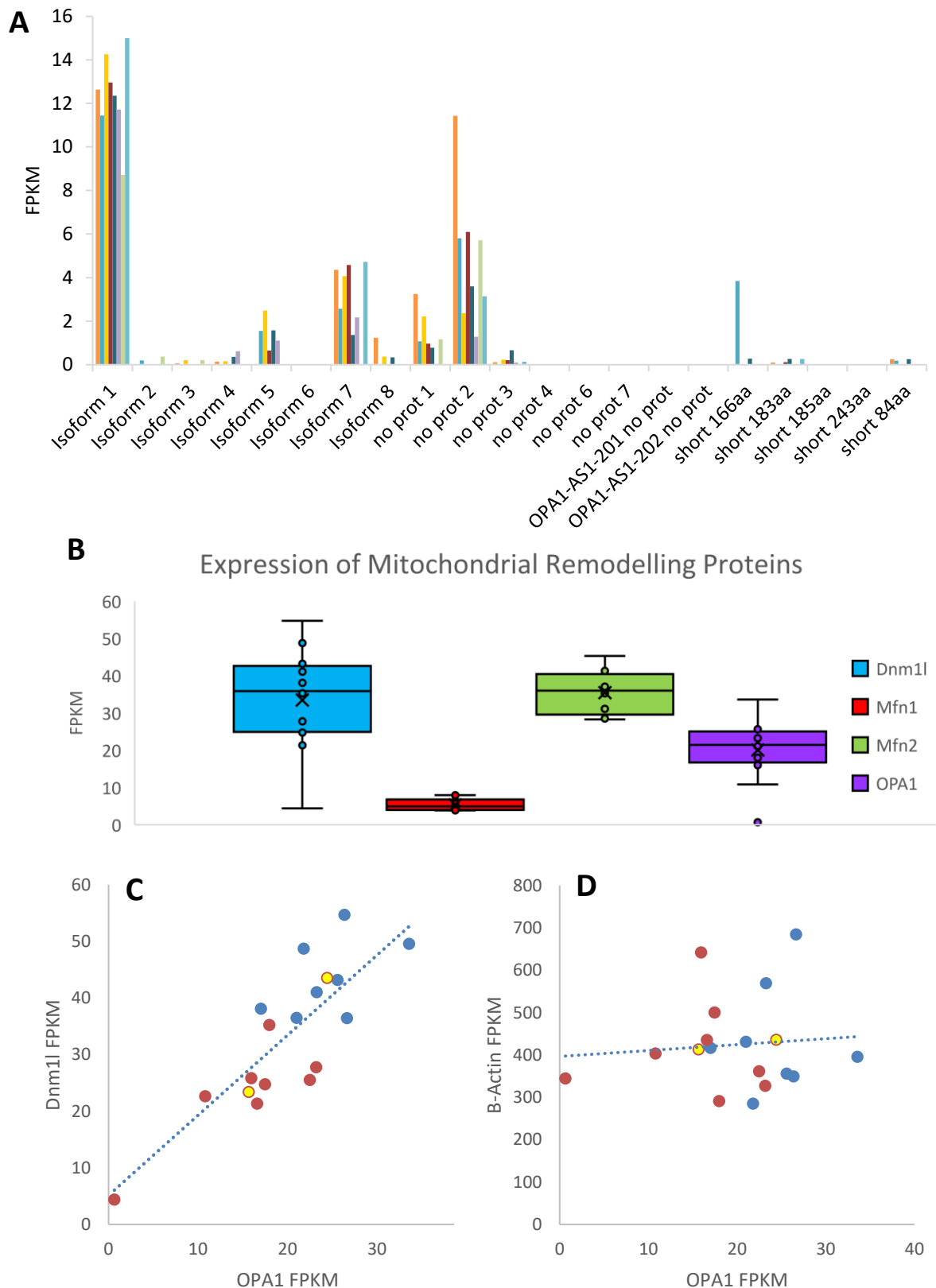


Figure 4-2: A) Shows the levels of the different OPA1 isoforms in FPKM across 8 macular retina RNA-seq samples. Of the classic protein producing isoforms 1 and 7 show the highest average expression with 12.4 and 3.4 FPKM respectively. B) Shows the FPKM values for 4 of the primary mitochondrial remodelling proteins in 16 retinal tissue samples. They demonstrate broadly similar expression levels for RNA. Two representative correlation plots are seen in C), a plot of OPA1 vs Dnm1l levels and D) OPA1 vs B-Actin. A significant positive correlation can be seen in C) but there is no correlation seen in D). However there appears to be broadly different levels of these proteins when grouped by peripheral retina (red dots) or macular retina (blue dots). The two yellow dots represent the means of each group.

Table 4-1: A table of Pearson's correlation coefficient (*r*, below the blue boxes) and *p*-value (above the blue boxes) between mitochondrial remodelling proteins with β -actin as reference. Orange shaded signifies significance. $\alpha = .0166$.

	<i>Dnm1l</i>	<i>Mfn1</i>	<i>Mfn2</i>	<i>OPA1</i>	<i>B-Actin</i>
Dnm1l		0.1177	0.0144	9.45E-05	0.7408
Mfn1	0.40701843		0.5505	0.3286	0.0825
Mfn2	0.597740943	-0.16133184		0.0022	0.5268
OPA1	0.821703179	0.261119352	0.705925772		0.7456
B-Actin	-0.089796863	-0.44709986	0.170883392	0.088087038	

Interestingly, if we stratify the RNAseq data by the area the sample was taken from we can see clustering of two distinct groups. In Figure 4-2C and D samples from peripheral retina are coloured red, with macular retina samples coloured blue. There is clear clustering of the data with peripheral retina having in general lower levels of expression of the mitochondrial markers than macular retina. However, this is also true of β -actin expression so it can't be ruled out that there is just lower global gene expression in the peripheral retina samples.

4.2.2. Cloning (woes) of OPA isoform 1 & 7

As the RNA-seq analysis had shown that isoforms 1 and 7 were the most highly expressed of the OPA1 gene, it was decided that these would be taken forward for further study. The decision was made to codon optimise the sequence (using the GeneArt optimisation algorithm) in order to achieve higher levels of expression, such as was the case with Ndi1 and optNdi1, as detailed in Chapter 3, as well as the addition of a HIS tag at the 3' end of the sequence, just prior to the stop codon. As previously discussed, the difference between OPA1 isoform 1 and isoform 7 is the addition of the exon 5b (see Figure 4-1). As such, on a cost-per-base basis it was reasoned the most astute way forward was to get OPA isoform 1 synthesized and then clone in the extra exon. The sequence was sent to IDT for synthesis but unfortunately returned with the last 200bp missing. This could possibly be due to instability near the 3' end caused by a dip in the GC content of the sequence (data not shown). IDT would not attempt to synthesis the sequence again without changing the sequence, so it was decided to ask them to synthesize a smaller 300bp fragment that would complete the optimised OPAiso1 sequence. This fragment was designed to be compatible with an endogenous restriction site present near to the break point of the OPAiso1 sequence. Due to there only being one restriction site it would be possible for the end fragment to be inserted in the correct orientation or "upside-down".

However, when it came to cloning this new end fragment into the incomplete OPAiso1 plasmid, significant problems were encountered (see section 2.1 of Methods for cloning). After transformation of the plasmid, any viable colonies were mini-prepped to isolate the plasmid and digested for the presence of a new restriction site present in the inserted fragment. Of the 150 plus colonies mini-prepped and digested from numerous ligation attempts, 40 were identified to contain the fragment and were sent for sequencing. Of this only 2 had the fragment in the correct orientation with no changes to the coding sequence but both had the same deletion of three bases when compared to the original sequence. As these were immediately after the stop

codon, in a restriction site added to aid cloning before the poly A sequence, it was considered to be tolerable (see Appendix 7.1 to see all sequences and for some plasmid maps). It appears there may be a genomic instability near the 3' end which makes the cloning more prone to errors than typically experienced as witnessed by the number of small deletions, typically located at the same sites throughout the cds, seen in the samples sent for sequencing. Cloning was continued and the 5b exon was added to this vector to create OPAiso7; this step was achieved without any difficulties, it took 1 week for this cloning step versus 4 months for the previous one, further suggesting an instability at the 3' end of the cds. Finally, the two completed Opa1 isoforms were transferred to the pAAV-MCS expression vector where they are driven by the CMV constitutive promoter. These two plasmids will from here-on be referred to as OPAiso1 and OPAiso7. Sequences of these can be found in the appendix, with a full list of constructed plasmids in the materials and methods section.

4.2.3. Optimisation does not perturb mitochondrial localisation

As a first step towards investigating functionality we wanted to confirm the continued subcellular localisation of OPAiso1 and 7 to the mitochondria. Initially OPAiso1 and OPAiso7 were transiently transfected into HEK293 cells, along with mito-dsRed. This plasmid encodes for the dsRed fluorescent protein which has a mitochondrial targeting sequence (MTS) appended to it. Co-transfection of the two plasmids showed limited expression of mito-dsRed, despite this approach having previously been successful for the optNdi1-HA co-localisation study. This was repeated multiple times, but double transfection never led to useable levels of mito-dsRed expression. Initially to circumvent this a dual expression plasmid was created, with each OPA isoform cloned on to the same plasmid as mito-dsRed to ensure cells positive for mito-dsRed would also express an OPA isoform (see methods 2.1 for cloning and table of constructs).

However, mito-dsRed levels were still very weak after multiple transfection attempts and thus uninformative for localisation studies.

As an alternative, HEK293 cells were transfected with one of the OPA plasmids, then 48 hours later were stained using mitotracker orange (ThermoFisher Scientific; M7510 using the method detailed in section 2.5.2). Cells were subsequently aldehyde fixed and were probed with a HIS-tag antibody (abcam; ab9108) as well as DAPI stained for nuclei (see Figure 4-3, details on fixation and staining in section 2.5). In Figure 4-3A a distinct tubular network of mitochondria can be seen, indicating healthy network morphology as well as mitochondrial membrane potential, as mitotracker orange is only sequestered while there is an active mitochondrial membrane potential. Figure 4-3B shows the HIS-tag antibody, here visualised with an alexa-488 secondary antibody, which shows a similar pattern of expression to mitotracker orange. This is more clearly seen in the Figure 4-3C row where a merge of the mitotracker orange and HIS-tag signals are shown. Finally, Figure 4-3D shows an intensity plot that follows the red arrows in Figure 4-3C from left to right. Each colour trace corresponds to the intensity of that signal along the line, with red for mitotracker, green for OPAiso 1 & 7 and blue for DAPI. There is clear overlap of the mitotracker and OPAiso peaks along the line, in contrast to the spike in blue signal when the line crosses the nucleus. This indicates that there is strong co-localisation of optimised OPAiso1 and OPAiso7 to the mitochondria of cells.

OPAiso1

OPAiso7

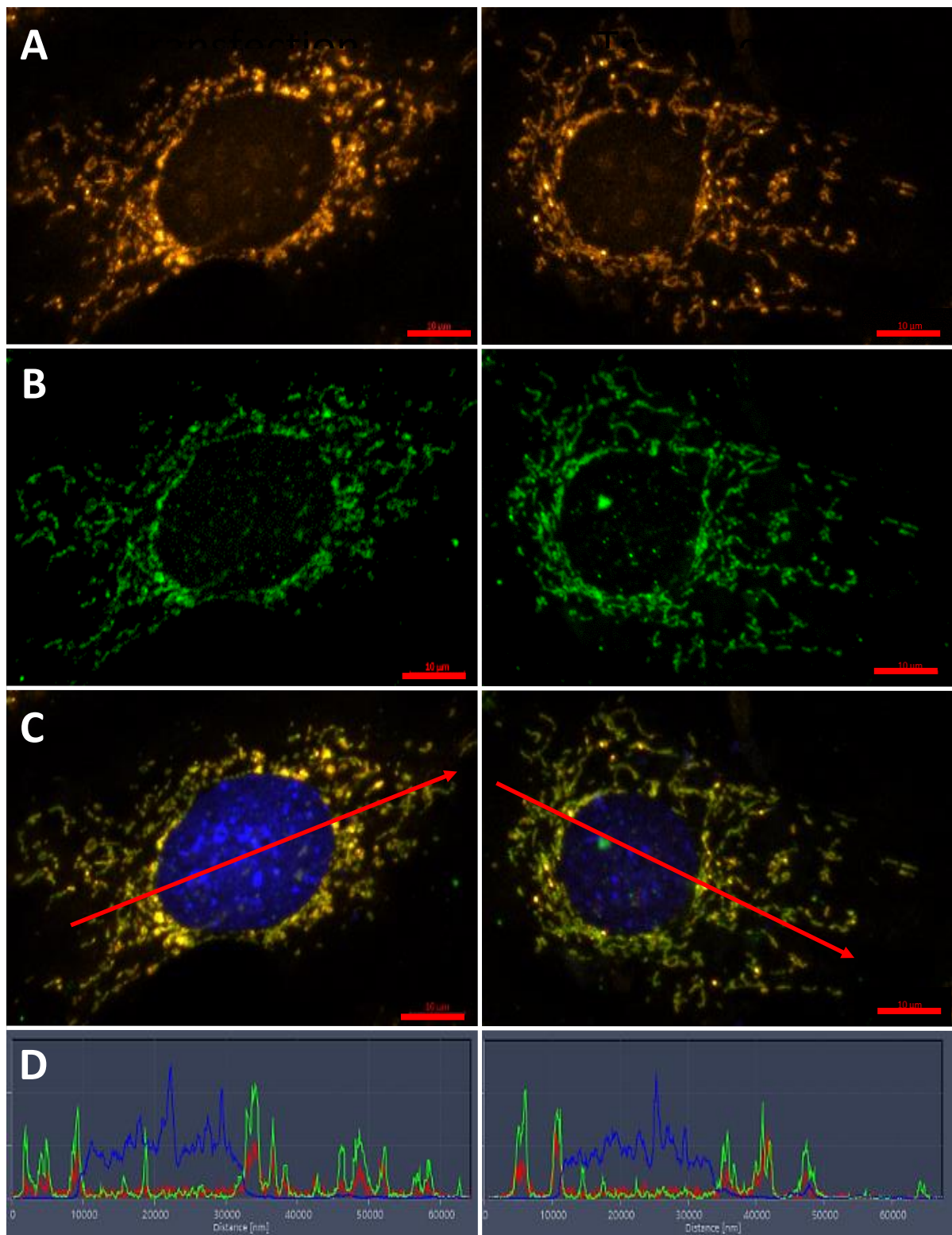


Figure 4-3: Colocalisation study of HEK293 cells transfected with either OPAiso1 or OPAiso7. Row A shows cells stained with mitotracker. Row B shows HIS-tagged OPA iso 1 or 7 in green. Row C shows a merge of rows A and B with DAPI in blue. The red arrow corresponds to the intensity plot shown in D. Clear colocalization of the peaks of red and green fluorescence can be seen. Images obtained via confocal microscopy. All scale bars are 10 μm.

4.2.4. OPAiso1 and OPAiso7 can restore mitochondrial morphology

Mitochondrial localisation does not indicate function, so work was now undertaken to establish if these optimised OPA isoforms could restore mitochondrial network morphology. Initial experiments were carried out using OPA-null Mouse Embryonic Fibroblast (MEF) cells (ATCC CRL-2995). These cells display a highly characteristic mitochondrial morphology with extreme fragmentation of the mitochondrial network (Figure 4-4D). Visually OPA ^{-/-} cells look distinct from MEF cells, they are much smaller, not showing any of the characteristic elongation typically seen by fibroblast cell lines. To this end seeding for confluency requires more cells than would typically be seeded for MEF cells (see methods, 2.3.3). As such these OPA ^{-/-} cells were largely treated as being distinct to commonly used MEF cell lines such as NIH-3t3 cells.

OPA ^{-/-} cells were transiently transfected with plasmids containing either OPAiso1 or OPAiso7 and stained with mitotracker orange before fixation to reveal mitochondrial morphology (methods section 2.5). Figure 4-4 shows representative confocal images of these cells. Clear rescue of the tubular mitochondrial network morphology was seen in cells expressing OPAiso1 or OPAiso7 when compared to OPA ^{-/-} cells (see Figure 4-4) across multiple repeated transfections. Mock transfected OPA ^{-/-} cells (lipofectamine treated but no plasmid supplied) show no alteration of the punctuate morphology (Figure 4-4A & D). Rescued cells however, demonstrated a clear ability to remodel mitochondria by fusion, allowing cells that otherwise have punctuate diffuse mitochondria to regain a network of mitochondria. (Figure 4-6 and also Figure 1-7 shows wt MEF mitochondrial morphology).

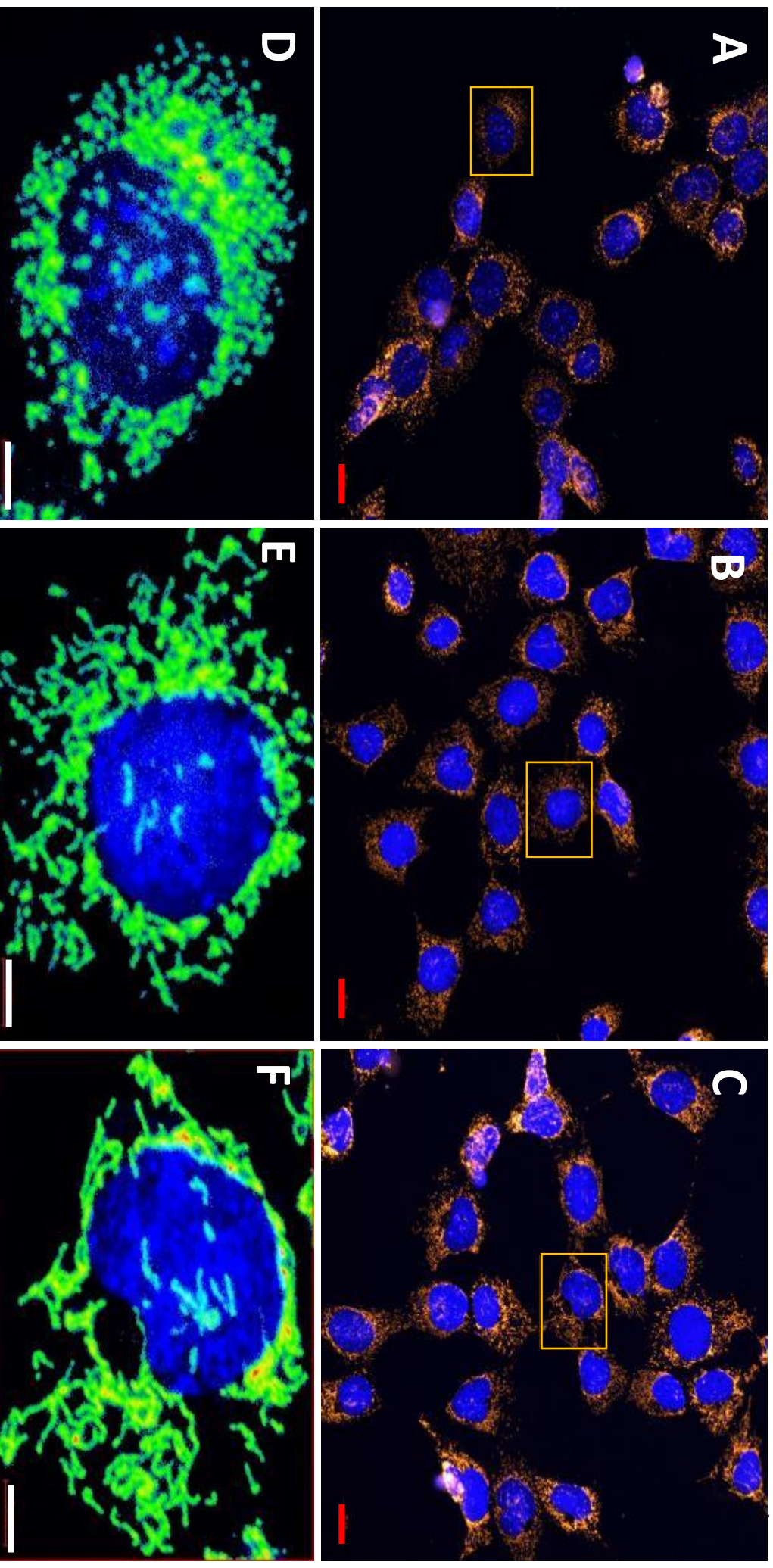


Figure 4-4: Representative images of *OPA*^{-/-} cells transiently transfected with *OPAiso1* or *OPAiso7* showing rescue of mitochondrial network morphology. A) Shows *OPA*^{-/-} cells mock transfected. There is no rescue of mitochondrial morphology and as such the mitochondria demonstrate a characteristic punctate morphology as seen in D), the area in the yellow box enlarged from A). The expression of either *OPAiso1* B) and E), or *OPAiso7* C) and F) is sufficient to rescue the tubular mitochondrial morphology seen in wild type cells. Images obtained via confocal microscopy, bottom row images have been false coloured to highlight the mitochondrial morphologies. Red scale bar = 25 μm, white scale bar = 5 μm.

Efforts were made to quantitatively analyse the observed changes to mitochondrial the mitochondrial network observed in these cells but at the time of writing these were not successful to a useable degree. The software approaches used could not differentiate the highly fragmented mitochondria characteristic of OPA $-/-$ cells. This is presumably as a consequence of mitochondria used to develop various quantification software typically having a network of elongated tubes, opposed to the punctuate mitochondria observed in OPA $-/-$ cells. This is further compounded by the aggregation of mitochondria seen in OPA $-/-$ cells, such as in Figure 4-4D being recognised consistently as one giant mitochondria, which in turn gives a completely inaccurate and inconsistent evaluation of the mitochondrial network in these cells. This analysis was attempted with two ImageJ plugins (MiNA (A. J. Valente, Maddalena, Robb, Moradi, & Stuart, 2017) and MitoLoc (Vowinckel, Hartl, Butler, & Ralser, 2015)) but neither gave a satisfactory predicted networks for OPA $-/-$ cells in our hands.

It is possible that higher resolution confocal images at a higher magnification would allow easier distinction of individual mitochondria, as well as using automated z-stacks to provide a fuller picture of the total mitochondrial network in these cells. Also sophisticated deconvolution software or more powerful imaging software such as Imaris may be able to successfully identify individual mitochondria, but currently we do not have access to such tools. As such the classification of mitochondria in this thesis is qualitative, with only mitochondria with evidence of branching mitochondrial tubules being considered rescued, such as those in Figure 4-4E and F. Future work will investigate more robust methods of mitochondrial network quantification.

4.2.5. OPA isoform expression levels are important for mitochondrial morphology

While clear evidence of rescue using OPAiso1 or OPAiso7 was obtained, what was disappointing was the quantity of rescued cells after OPAiso1 or OPAiso7 transfection. Cells showing rescued morphology were the exception rather than the rule, with an estimate of only ~2% of cells showing the rescued phenotype across five separate transfections. The lack of phenotypic correction was also seen in early Seahorse runs monitoring oxygen consumption rate (OCR) in transfected vs untransfected OPA^{-/-} cells, with no change seen between OPA^{-/-} cells and either of the two OPA isoforms (see Appendix chapter 7). This was initially thought to be due to low transfection levels because of the lack of mito-dsRed signal acquired as previously mentioned. To investigate this a number of approaches were utilised. OPA^{-/-} cells were transfected with either of the two OPA plasmids, stained with mitotracker orange and subsequently probed with anti-HIS tag antibody to identify OPA isoform expressing cells. The percentage of HIS positive cells was far higher than the number of cells that showed mitochondrial network rescue, with ~2% rescued versus an average of 50% HIS positive cells across three separate transfections. It appeared that cells that were highly HIS positive as evaluated semi-quantitatively by immunocytochemistry, and therefore likely expressing OPAiso1 or 7 to a high degree, displayed a unique mitochondrial phenotype, with mitochondrial rounding and aggregation of the mitochondria around the nucleus (see Figure 4-5). Figure 4-5A shows three cells transiently transfected with OPAiso7 with differing mitochondrial network morphologies; the cell to the west shows no rescue of the network, the cell to the south shows a good tubular morphology and the northern cell shows highly rounded, aggregated mitochondria. This phenotype was the most common mitochondrial phenotype seen in HIS positive transiently transfected cells. This was repeated using multiple lipofectamine and DNA concentrations in an attempt to maximise transfection rates. When we inspect the intensities of the anti-HIS-OPA green signal we can see that the northern cell is highly OPAiso7 positive, whereas the southern cell with rescued

morphology shows far lower levels of signal which are higher than the unrescued signal. It does not appear that this signal is increasing due to the aggregation of the mitochondria as the mitotracker orange signal intensity remains comparatively constant across the three cells (~55 arbitrary units; see Figure 4-5D). A similar relationship between expression levels and OPAiso1 is also seen with overexpression leading to this phenotype. These data suggest the existence of a 'sweet spot' for OPA expression, where too little has no effect on network morphology but too much causes extreme aggregation of mitochondria.

The possibility that the HIS tag was interfering with the OPA isoforms' function was investigated. Protein conformation with and without the HIS tags was computationally modelled using the I-TASSER suite of protein analysis software (J. Yang et al., 2015). It was predicted that the addition of the HIS-tag (6x Histidine residues) could cause a structural change in the predicted protein structure when compared to sequences without the HIS tag which in turn could affect function (see Appendix chapter 7). PCR cloning was used to remove the HIS tags from both isoforms as per the cloning methods outlined in 2.1. No difference to the extent of the of mitochondrial network morphology was observed after transient transfection, as monitored via mitotracker staining, with an estimate of around 2% of cells showing rescued mitochondrial network morphology and around 50% showing the overexpression phenotype. Given the apparent equivalence of both sets of constructs the HIS tagged versions were continued with as it allowed clearer identification of OPA1 expressing cells.

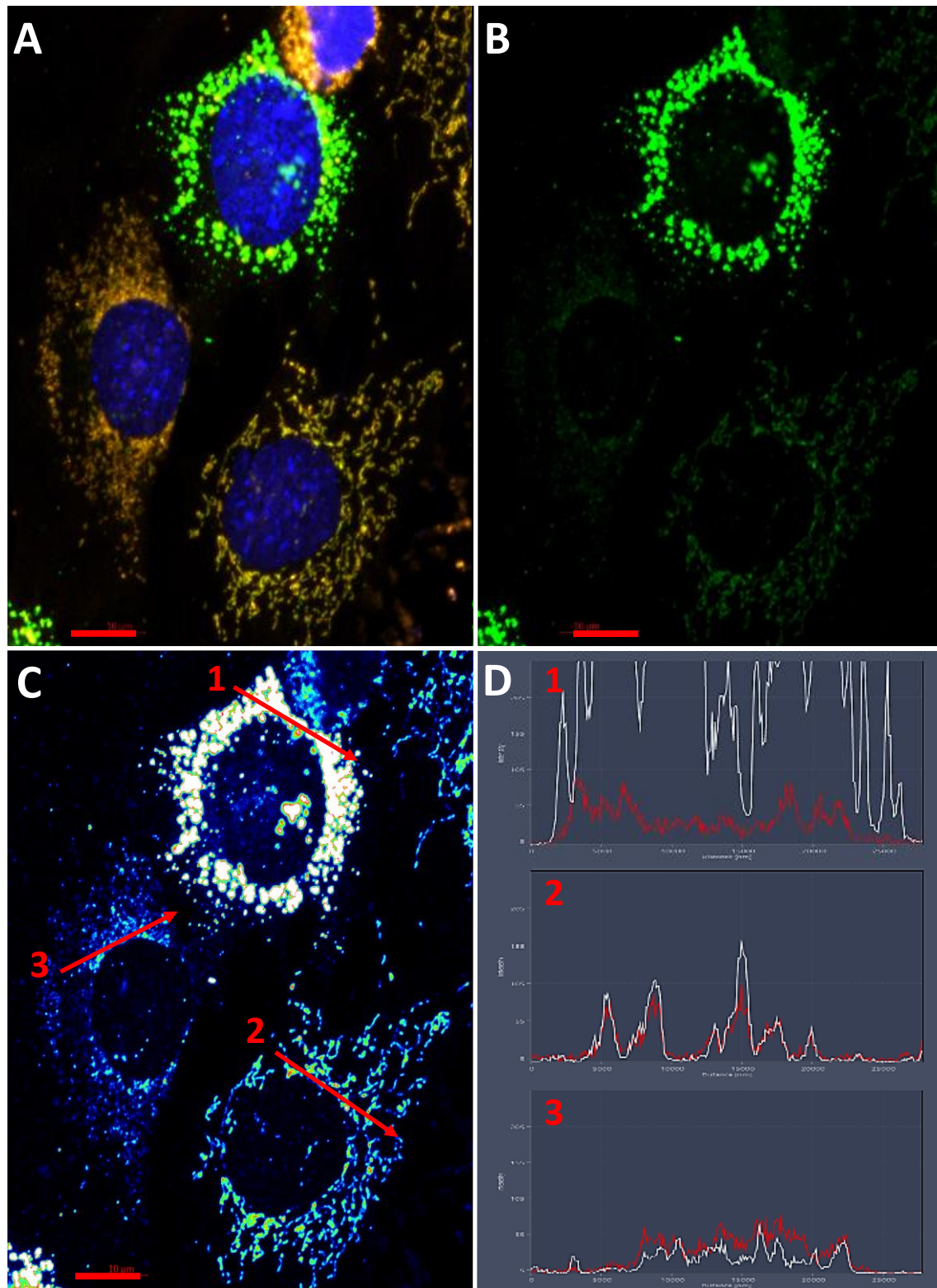


Figure 4-5: Representative images of OPA^{-/-} cells transfected with OPAiso7 showing different mitochondrial morphology depending on the level of expression. A) Shows a merge of mitotracker orange and anti-HIS-OPAiso7 (green). The different degrees of colour mixing suggest changes in expression level of OPAiso7. B) Shows only the green channel. The northern cell clearly shows extremely high levels of OPA expression, the southern cell shows an intermediate amount, and the western cell shows almost no green signal. C) The green signal has been converted to a heat map of intensity going from low (blue) through the spectrum to high (white). D) Shows the intensity traces along the red arrows from C). The white trace corresponds to OPA signal, the red trace for mitotracker orange. The mitotracker signal remains at a constant level where as OPA signal intensity varies significantly. It appears that there may be a 'sweet spot' for OPA expression to rescue mitochondrial network morphology. Scale bar = 10 μm.

To investigate if the overexpression phenotype of the OPA $-/-$ cells is not unique to them due to the OPA knockout compromising the cells in a here-to-unforeseen manner, wild type MEF cells were transiently transfected with both of the OPA isoforms. MEF cells that were highly positive for HIS-OPA signal also showed this aggregation of the mitochondrial network (see Figure 4-6). As in Figure 4-5, cells demonstrated a range of phenotypes dependant on the levels of HIS-OPA signal observed, with cells possessing moderate levels of signal showing a ‘hyper-fused’ mitochondrial network. As before, this was the case when either OPA isoform was expressed.

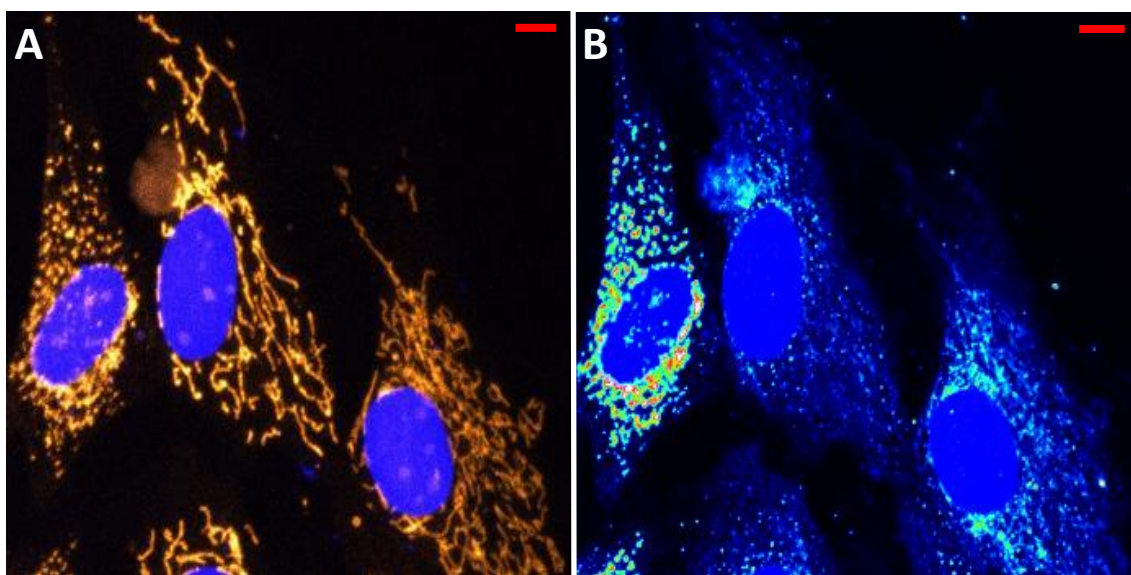


Figure 4-6: Transient transfection of MEF cells with OPAiso1. A) Mitotracker orange signal with DAPI stained nuclei shows clear differences in mitochondrial network morphology. B) OPAiso1 signal visualised as a heat map. The centre cell is representative of normal MEF mitochondrial morphology. Varying levels of OPAiso1 cause different phenotypes, with overexpression in the left-hand cell leading to punctuate mitochondria and with a moderate increase in the right most cell leading to a hyperfused network. Scale bar = 10 μ m.

It appears that reasonably high levels of transduction and expression in OPA $-/-$ cells is achieved, but the majority of these cells were displaying the overexpression phenotype. This mean that looking at the mitochondrial morphology alone of the transiently transfected OPA $-/-$ cells was not indicative of expression levels. In an attempt to modulate the expression levels of the OPA isoforms in OPA $-/-$ cells, cell lines that would stably express the OPA isoforms were generated. This was achieved by cloning OPAiso1 and OPAiso7 into the pcDNA 3.1 vector

(Thermo Fisher Scientific; V79020, cloning outlined in 2.1, plasmid map in Appendix). This vector contains both a SV40 origin, to allow replication in mammalian cells, as well as a Neomycin selectable marker. Addition of 500µg/ml G418 (geneticin) to the culture media selects for cells that contain the plasmid (see Methods 2.6). The gene of interest is not integrated into the genome of the host cell but is maintained as a plasmid in the cytosol of the cell. Unlike transient transfection, which lose copy numbers due to dilution of the non-replicating plasmid, the pcDNA plasmid can be replicated and thus passed on to daughter cells continually. For these experiments OPA ^{-/-} cells transformed with empty pcDNA 3.1 vector were placed under selection to cause retention of the plasmid and were subsequently used as controls (pcOPA ^{-/-}).

The mitochondria seen in the overexpression phenotype bear a striking similarity to that seen when a cell is undergoing apoptosis (see Appendix 7.4) where the mitochondria also aggregate and show a distinct rounding prior to depolarisation of the mitochondria which releases a number of proteins such as Smac/DIABLO and Omi/HtrA2, which are normally associated with the mitochondrial inter membrane space. These in turn activate caspases, or inhibit inhibitors of caspases, which cause the cell to initiate apoptosis (Elmore, 2007). When we fix cells we only receive a snapshot in time and as such it is possible that all of the cells we have imaged so far have been on the cusp of apoptosis. Furthermore, if a narrow optimal zone of expression exists, a 'goldilocks zone', it should be possible to identify cells that go from a fragmented to a tubular network and from a tubular network to the aggregated network. To examine this, an imaging time-course was set up. OPA cells stably expressing either OPAiso7 (pcOPAiso7) were co-transfected with mito-dsRed. After 24 hours to allow dsRed expression, cells were imaged every 15 minutes over a thirteen-hour period giving 52 images. The microscope stage was maintained at 37°C with 5% CO₂ to mirror typical culture conditions. In Figure 4-7 you can see three cells I have chosen to focus on, all from the pcOPAiso7 time course.

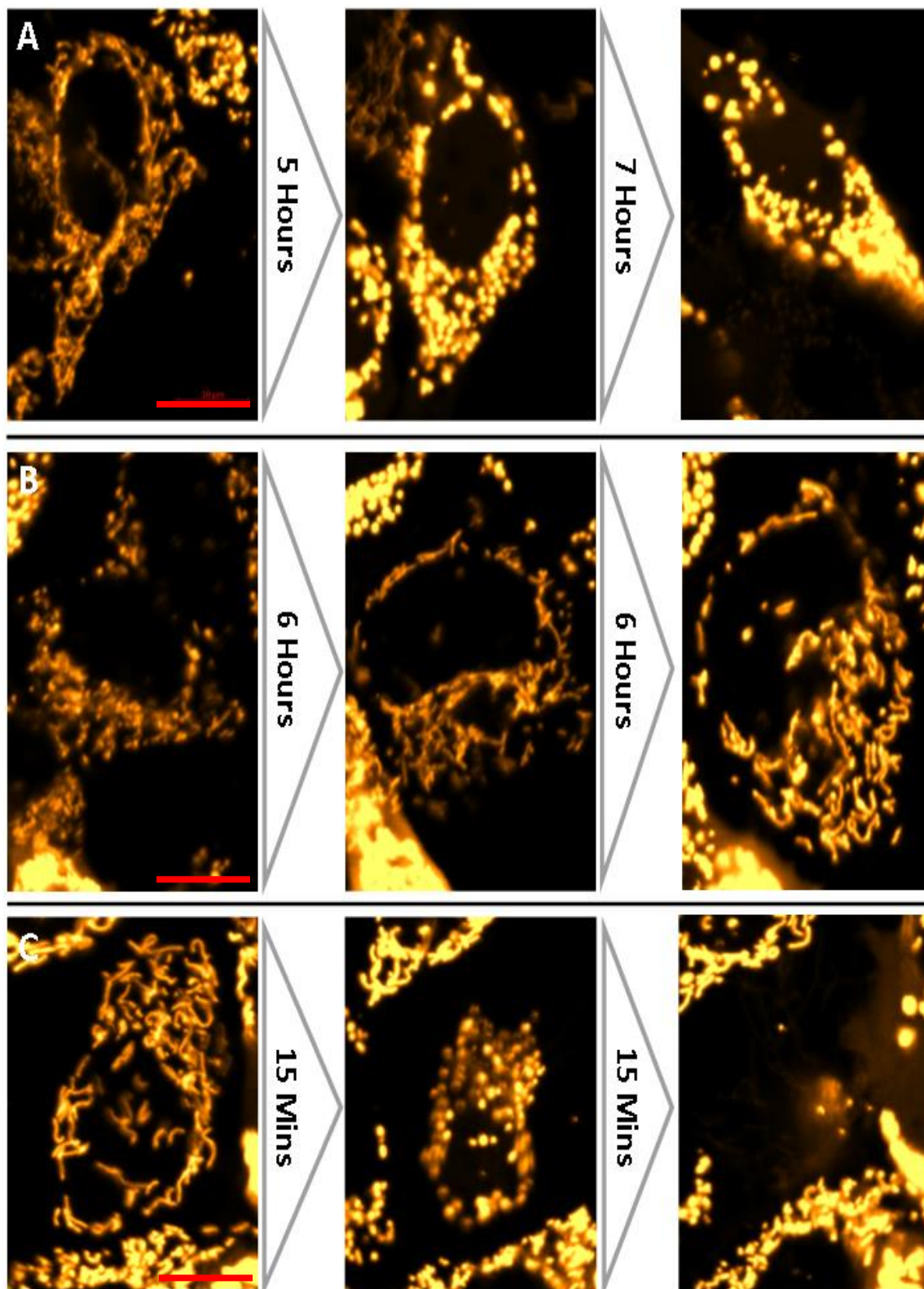


Figure 4-7: Snapshots of specific cells during the 13-hour live cell pcOPAiso7 experiment, grown in galactose. Cells were OPA $-/-$ cells stably expressing OPAiso7, transfected with mito-dsRed 24 hours previously. Row A) shows a cell with a tubular mitochondrial network transition to the overexpression phenotype. The over expression phenotype appears to not be detrimental to the cell as it was still alive for at least 7 hours after the transition. Row B) shows a cell transition from a punctuate mitochondrial network, typical of unrescued OPA $-/-$ cells, to an extensive tubular network similar to wt MEF cells. Row C) shows a cell undergoing the initial stages of apoptosis. Initially the mitochondrial network is tubular indicating OPAiso7 rescue. It then resembles that of the overexpression phenotype in row A) but the transition happens much more rapidly and subsequently leads to the depolarisation of the mitochondrial network, presumably due to MOMP (indicated by loss of mitotracker signal).

In row A of Figure 4-7 you can see a cell transition from a rescued, tubular network to the aggregated overexpression phenotype. Conformation of the presence of OPA is not possible in this assay since it is in living cells, but thus far no substantial rescue of mitochondrial morphology has been seen without the presence of either of the two OPA isoforms. The conformational change takes time to be established, with 5 hours elapsing between the tubular network and the full aggregation seen in the centre panel of row A. Furthermore, the cell remains alive and maintains its mitochondrial membrane potential for at least 7 more hours. This is in direct contrast to row C. Here the cell shows an extensive tubular network but just 15 minutes later (1 image cycle) there is condensation of the mitochondrial network followed 15 minutes later by dissipation of the dsRed signal, presumably due to mitochondrial outer membrane permeabilization (MOMP). The rapid events suggest that the cell is undergoing apoptosis.

The cell in row B demonstrates a slow reorganisation of the mitochondrial network. At the outset the cell has a punctuate network typical of unrescued cells. 6 hours later, the network is showing signs of rearrangement, until a further 6 hours later we see clear tubular mitochondria. This suggests that OPA accumulation may be directly responsible for the remodelling of the mitochondrial network in a dosage sensitive manner. Barring any compensatory increase in mitofusin content, the other proteins responsible for mitochondrial fusion, it appears that as OPA accumulates it increases the rate of fusion of the mitochondria and thus the complexity of the network, until eventually the state of overexpression is reached. This could explain why only a proportion of cells imaged have the correct mitochondrial morphology, as it would seem that may be only a window in time where there are the correct levels of OPA1 protein to counteract the fission enacted by Drp1.

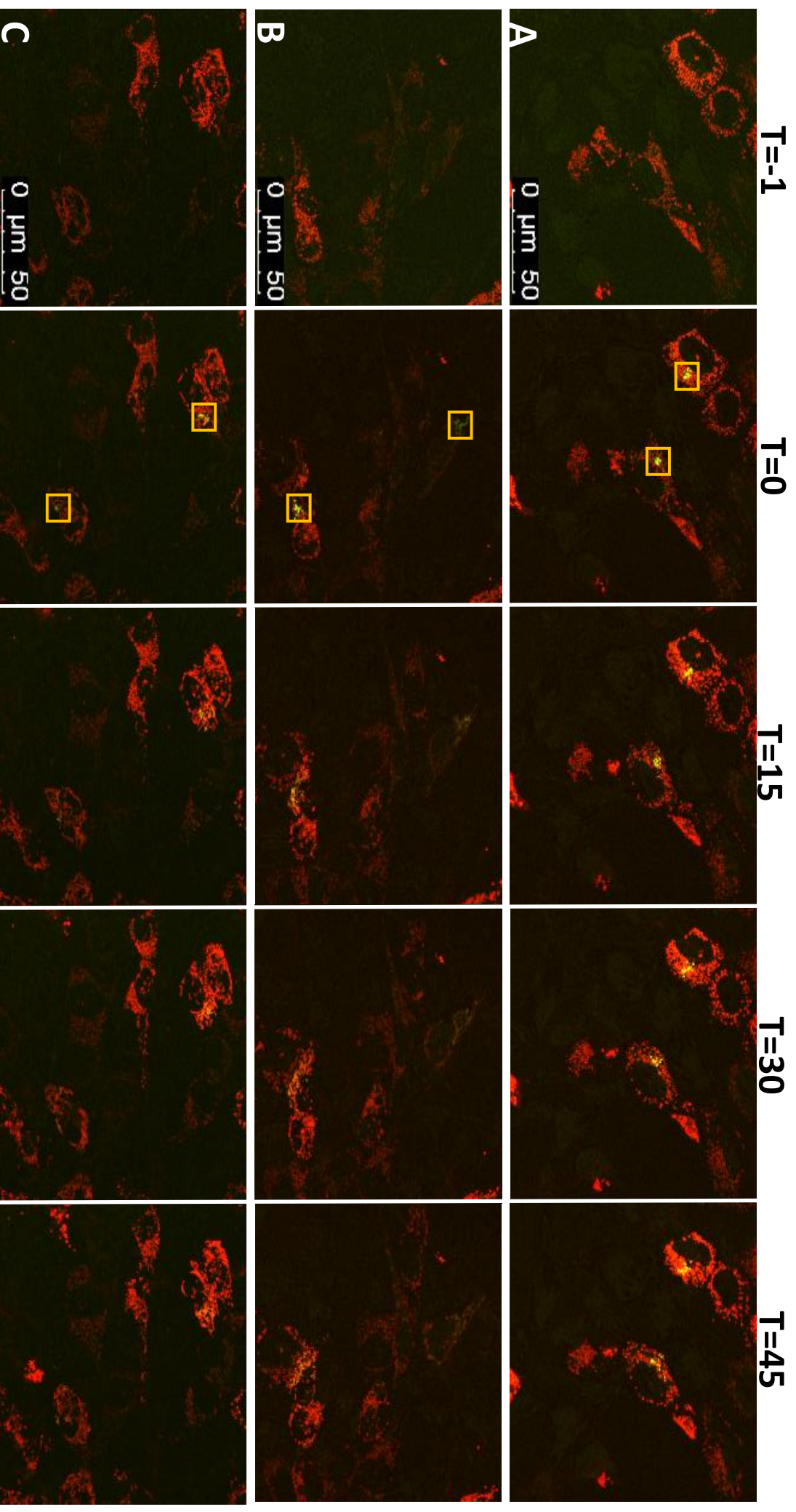
Unfortunately, due to technical limitations this experiment was only carried out once and at high magnification. This prevents broad generalisations from being made, but if we consider the 40 or so cells captured in this time course there was a range of mitochondrial stages seen, but

around half of the cells showed punctuate mitochondria and did not transition from one mitochondrial phenotype to another.

4.2.6. OPAiso1 & 7 allow dynamic fission and fusion

The restoration of the mitochondrial network morphology is important but without the correction of the patients' biochemical defects it would likely not provide much benefit. To this end, some biochemical markers were investigated. First the ability of OPAiso 1 and 7 to restore dynamic modulation of the mitochondrial network was examined. Mitochondria are remodelled at high speed as metabolic demands shift within the cell, this means that mitochondria constantly fission and fuse with one another. The rate of this dynamic remodelling can be investigated by the use of a photo-activatable green fluorescent protein (pa-GFP) targeted to the mitochondria, which does not fluoresce until it is activated via an ultra-violet light source. See the methods section 2.9.3 for a full breakdown of how the assay is run and quantified but in brief, cells stably expressing either OPAiso1 or 7 were co-transfected with mito-dsRed (to allow identification of transfected cells) and pa-GFP. One identified, a 2.5µm region of interest (ROI) was illuminated using the 408nm laser, causing the pa-GFP signal localised to the mitochondria in that specific ROI to become visible. It is then possible to monitor the rate of dissipation of the pa-GFP signal, which will dissociate quickly with more fission and fusion activity (see Figure 4-8).

This experiment is run over the course of 45 minutes, with a z-stack through the cells taken at T=-1, just after activation of paGFP at T=0, and then every 15 minutes subsequently. It is possible to image 5 fields of view (FOV) in under a minute, and each FOV can contain multiple ROIs and therefore multiple cells. The paGFP signal intensity is then monitored and given as a percentage of signal reduction compared to T=0. See methods for more details.



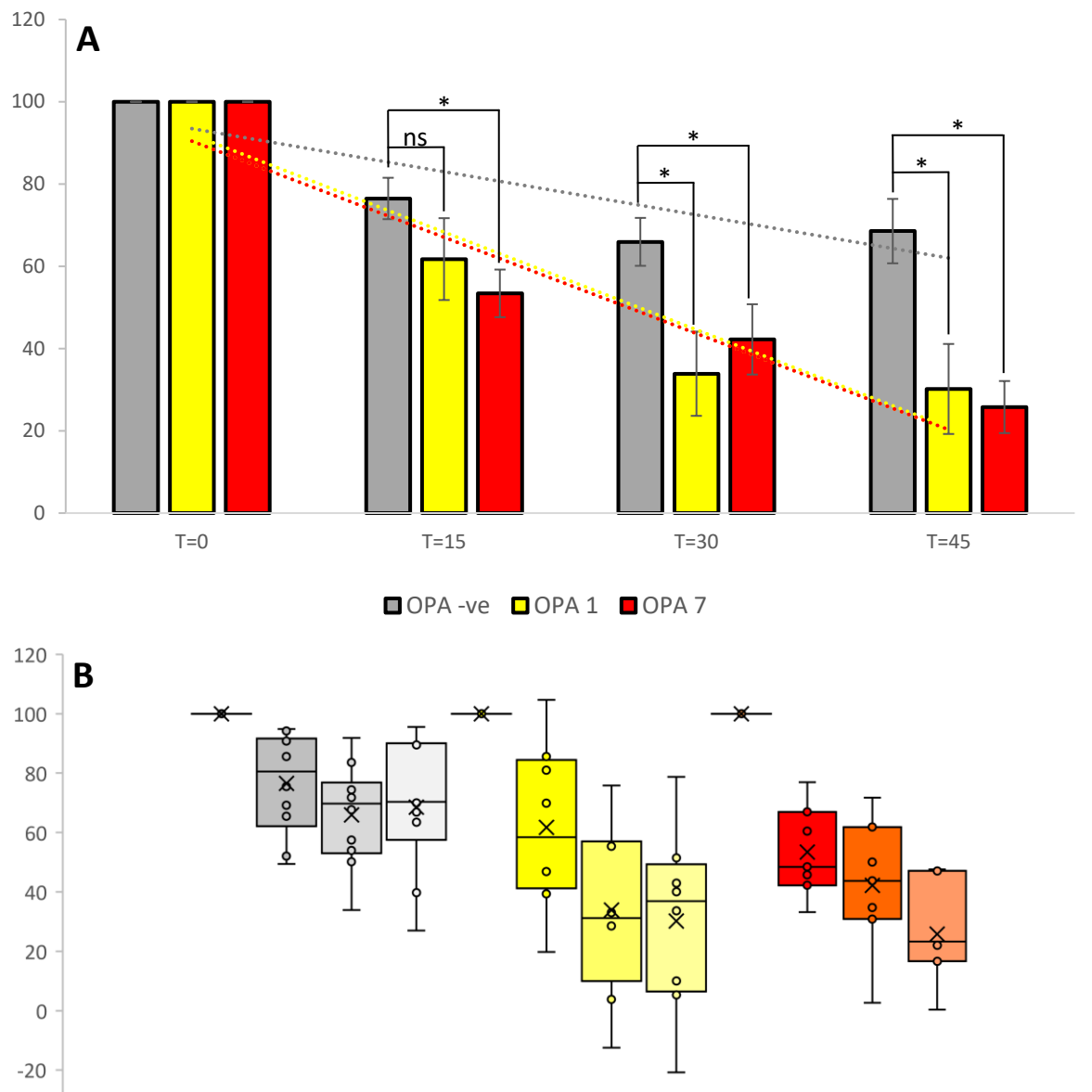


Figure 4-9: Graphs representing the percentage of paGFP signal remaining at each timepoint compared to T=0. A) shows the three cell types grouped by timepoint. Cells stably expressing pcOPAiso1 (yellow; n=8 cells) and pcOPAiso7 (red; n=7 cells) show a significant decrease in paGFP fluorescence at all time points when compared to pcOPA -/- (grey; n=10 cells) apart from T=15 pcOPAiso1. The trendlines suggest a trend for quicker decrease in rescued cells versus pcOPA -/- cells. B) The box plots show the distribution of results across the 4 timepoints, T=0,15,30,45, grouped by OPAiso expression. Across the cells tested OPAiso1 rescued cells showed the highest variance in results. There is no significance between pcOPAiso1 and 7 at any timepoints. (ANOVA Tukey HSD; $\alpha = .05$; error bars $\pm$ SEM).

In Figure 4-8A we have a time course for two pcOPA $-/-$ cells. After initiation of the pa-GFP signal at T=0 (highlighted by the yellow box) you can see there is little diminishing of intensity over the following time points, especially when compared with rows B and C. This would be indicative of diminished levels of fusion not allowing the dispersal of the paGFP signal.

Figure 4-8B and C show pcOPAiso 1 & 7 respectively. In both of these the paGFP signal is strong at T=0 but there is a marked decrease in signal intensity over time. This can be seen more clearly in Figure 4-9A and B which detail the quantification of the paGFP signal. There is a significantly larger decrease in the levels of paGFP in the rescued cells compared to pcOPA $-/-$ at almost all time points. The rate of the dispersal of paGFP signal appears consistent with pcOPAiso 1 and 7 having almost identical slopes for their trendlines, with -23.732 and -23.382 respectively, compared to -10.487 for pcOPA $-/-$. In other words the two rescued cell lines show a decrease of $\sim 24\%$ of the original every 15 minutes, compared to only $\sim 11\%$ for pcOPA $-/-$. Of course this is just a linear trendline giving the average change, if we look at Figure 4-9A we see that the greatest change in intensity occurs in the 15 minutes after the initial paGFP activation.

These data demonstrate the capacity of OPAiso1 and 7 to restore a degree of dynamic mitochondrial modulation to OPA $-/-$ cells. It would have been favourable to have wild type MEF cells as a positive control to understand the rate of fission and fusion when mitochondria are fully functional. This assay will be expanded to include wt MEFs in the future, especially when expression levels of the OPAiso1 and 7 cells have been optimised.

4.2.7. OPAiso1 & 7 increase the metabolic fitness of OPA -/- cells

OPA -/- cell show severe deficiencies in mitochondrial dependant metabolic processes. In order to cope with a changing environment and different energy sources, cells can restructure their mitochondrial network (Gottlieb & Bernstein, 2016). In order to challenge these cells metabolically the glucose in the cell's media was replaced with galactose. Galactose cannot be used as an input for glycolysis, which is often the preferred method of ATP production in immortalised cells in culture. This in turn means that the cells must rely on OXPHOS as the primary producer of cellular ATP, mainly through metabolising sodium pyruvate provided exogenously to the cells. pcOPA -/-, pcOPAiso1 and pcOPAiso7 cells were grown in either glucose or galactose media for a number of passages, being counted and reseeded at 1e6 each split. No significant difference was seen in the growth rates of the three cell lines in glucose media when compared to each other, but in galactose media there was a significant decline in the proliferative ability of the pcOPA -/- cells compared to the cell lines expressing each of the OPA1 isoforms, with a significant decrease in cell number by the second split, 96 hours in culture ($p < .01$; $n=3$ separate seedings for each cell line) (see Figure 4-10A and B). The rescued cells showed no signs that growth in galactose was detrimental to their proliferation rates, showing similar cell numbers after 48 hours in culture with either glucose or galactose. pcOPA -/- cells however showed arrest of cell proliferation in galactose media with numbers actively declining after 192 hours in culture.

Cells were also examined for TMRE staining intensity as a marker for mitochondrial potential. TMRE is a live cell stain for mitochondria that is retained while there is active mitochondrial potential in a potential dependant manner, the higher the signal, the higher the mitochondrial potential. Cells were stained with TMRE and DRAQ5 (a marker for live cells) prior to analysis by flow cytometry, with 20,000 events in a highly DRAQ5 positive gate being analysed for TMRE levels in each cell population. See section 2.9.5 for detailed methodology.

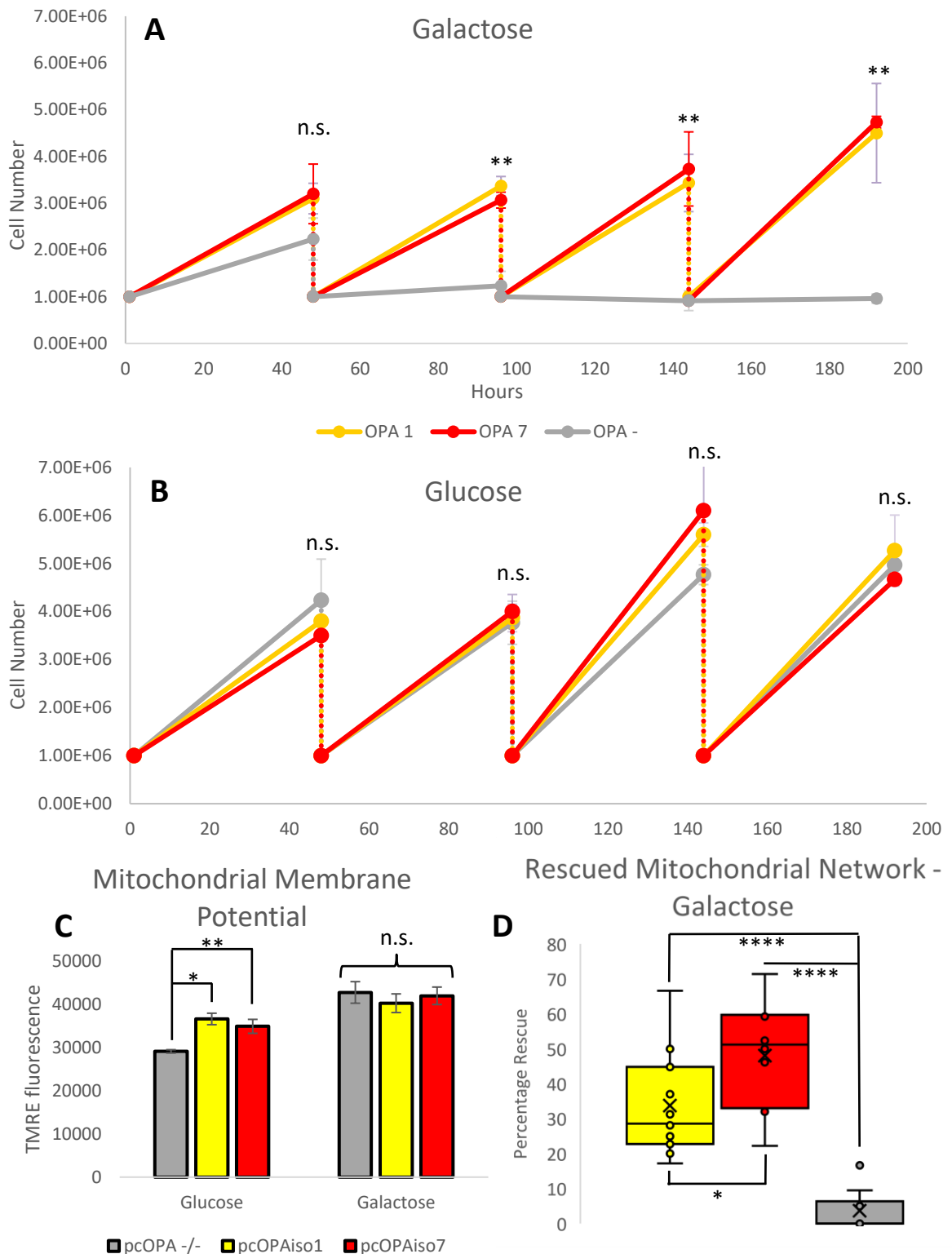


Figure 4-10: Bioenergetic responses to changing metabolic demands. The growth rate for pcOPA ^{-/-}, pcOPAiso1 and 7 in media containing either galactose A) or glucose B). Rescued cells show significantly better survival after 96 hours in galactose media, but no change in proliferation rate in glucose containing media. This indicates an ability for the rescued cells to modulate their metabolism in response to different energy sources. C) TMRE fluorescence intensity significantly increases in pcOPAiso1 and 7 cells compared to pcOPA ^{-/-} when grown in glucose but not when grown in galactose for 48 hours. This signifies higher levels of mitochondrial activity in glucose grown rescued cells, but that all cells increase membrane potential in galactose. D) pcOPAiso1 and 7 show a far higher proportion of cells with a tubular mitochondrial morphology than unrescued cells. Interestingly pcOPAiso7 appears to have significantly more mitochondrial network rescue than pcOPAiso1, the first assay to show a statistical difference between the two isoforms. (* = p < 0.05, ** = p < 0.01, **** = p < 0.0001)

When cells were grown in glucose for 48 hours, there was a significant increase in mitochondrial potential in the pcOPAiso1 and 7 cells compared to pcOPA^{-/-}. The pcOPAiso1 cells showed a 25% increase in TMRE staining (n=3 separate plates, p<.01) and pcOPAiso7 showed a 20% increase (n=3 separate plates, p<.05) when compared to pcOPA^{-/-} cells. Interestingly, there was no significant increase in mitochondrial potential when the cells were grown in galactose prior to analysis (n=3 separate plates of each genotype, p=.43 one-way ANOVA) (see Figure 4-10). This could be due to the fact that up to 48 hours growth in galactose, cell numbers are not significantly different from each other (see Figure 4-10A) and subsequent to 48 hours it could be that pcOPA^{-/-} cells with the highest mitochondrial potential would be the cells that survive the longest masking potential differences in TMRE in the whole cell population. As galactose puts pressure on pcOPA^{-/-} cells, would we could be artificially selecting for those unrescued cells with a better capacity to withstand the change in metabolic demands caused by galactose. This potentially explains the jump in TMRE staining levels between glucose and galactose grown OPA^{-/-} cells also.

When we examine the mitochondrial network morphology however after 48 hours of growth in galactose the pcOPAiso1 and 7 cells show significant levels of rescued network morphology as observed by mitotracker orange staining. 10 FOV were chosen blind from 3 separate transfections using DAPI staining as a guide, then network morphology was examined in these cells. If a cell showed restoration of a tubular network by the presence of mitochondrial tubules showing evidence of networking via visual inspection, it was counted as rescued. In total 33.8% of pcOPAiso1 cells (n=274 cells), 48.1% of pcOPAiso7 (n=241 cells) and 3.6% of pcOPA^{-/-} (n=163 cells). 1 vs ^{-/-} (p<.0001) and 7 vs ^{-/-} (p<.0001) were highly significant but interestingly 1 vs 7 (p<.05) was also significant (All comparisons via ANOVA, Tukey HSD post-hoc). This is the first significant difference noted between the two isoforms. Stably transformed OPA cells show a far higher percentage of rescued cells than transient transfection did, this may be due to the lower levels of protein expression per cell achieved when cells are stably transformed.

It is also worth noting the signs of network remodelling in the pcOPA $-/-$ cells too. Although very infrequent, around 3% of cells showed a modest degree of network remodelling, which was never seen in OPA $-/-$ cells grown in glucose. This is most likely due to the pcOPA $-/-$ cells attempting to cope with the metabolic constraint of galactose. The presence of the two mitofusin proteins would allow fusion of mitochondrial outer membranes but the inability of cells to fuse the inner membrane due to lack of OPA1 would most likely destabilise these structures and cause re-fragmentation of the network.

4.2.8. Cells expressing OPAiso1 or 7 move away from aerobic glycolysis as their primary energy source

As there was a change in mitochondrial membrane potential between rescued and unrescued cells when grown in glucose this suggested more activity of the electron transport chain. To examine this, we examined the reliance of these cells on aerobic glycolysis for ATP production.

Glycolysis involves the catabolism of glucose molecules to form two pyruvate molecules, in the process converting the released free energy into 2 ATP molecules and 2 NADH molecules. In actively respiring cells these pyruvate molecules would be converted to acetyl CoA and then fed into the Krebs cycle to generate more NADH and ATP. In anaerobic conditions, or when mitochondrial dysfunction prevents efficient aerobic glycolysis, there is a build-up of pyruvate. This pyruvate is in turn fermented to lactic acid, this leads to extracellular acidification which can be measured as a change in pH of the culture medium.

As such cells expressing either pcOPA $-/-$, iso1 or iso7 were uniformly seeded at 1×10^6 cells per plate and grown in glucose rich culture medium. The pH of the media was measured after 48 hours, when cells neared confluency. The culture media was significantly more acidic in pcOPA $-/-$ cells compared to pcOPAiso 1 and 7, with a shift from pH7.77 to pH7.51 (n=6 separate plates,

$p < .0001$ for both, see Figure 4-11A). No shift in pH is seen when cells are grown in galactose as it is refractory to glycolysis.

When pyruvate builds up it is converted to lactate by the lactate dehydrogenase enzyme in a reaction that also converts NADH to NAD⁺. This reaction is in equilibrium so changes in either the lactate:pyruvate or NADH:NAD⁺ ratios changes the other. When cells are highly glycolytic an increase of the NADH content in the cytosol of the cell is seen, thus altering the NADH:NAD⁺ ratio. This can be measured by using a fluorescent protein peredox (Hung et al., 2011). Briefly, this protein can be bound by either NADH or NAD⁺ with equal proclivity. Only when NADH binds does the protein undergo a conformational change causing green fluorescence. This peredox protein is also linked to an mCherry fluorescent protein allowing measurement of peredox protein expression levels in the cell. Therefore, the amount of green signal corresponds to the amount of NADH which is then normalised to the red signal as a measure of total fluorescent protein, allowing an estimation of the NADH/NAD⁺ ratio in the cell. See methods 2.9.5. for more detail on the experimental procedure, and the appendix 7.5 for an image of cells expressing peredox.

When grown in glucose media for 48 hours, pcOPAiso1 and 7 showed a highly significant decrease in intracellular NADH/NAD⁺ ratio compared to pcOpa^{-/-} cells, indicating a decrease in reliance on glycolysis ($n=55$ cells across 3 transfections, $p < .0001$ for both) (see Figure 4-11B).

Using the Seahorse XFe96 we can dissect the reliance that cells place on either the mitochondria or glycolysis for their ATP production via the “ATP rate assay” (see Methods 2.10.6 for set up). Briefly, the ATP rate assay estimates mitochondrial oxygen consumption rates (OCR) and glycolytic extra-cellular acidification rates (ECAR) as a means of estimating ATP production via mitochondria or glycolysis. Using this recently developed assay from Agilent, we can see that pcOPAiso1 and 7 cells shift their reliance away from glycolysis for the majority of their ATP demands, even in the presence of glucose as the primary energy source (Figure 4-11C). pcOPA

-/- have an estimated ratio of 0.69 for mitochondrial vs glycolytic ATP production, whereas for pcOPAiso 1 and 7 it is 1.5 and 1.3 respectively. There is also an expansion in the proportion of ATP generated from the mitochondria when cells are grown in galactose exclusively as would have been anticipated.

The energetic map in Figure 4-11D shows the shift in metabolic reliance of these cells. Rescued OPA -/- cells in galactose have the capacity to increase mitochondrial respiration by ~75% to meet metabolic demands.

Figure 4-11E is a measure of the ATP content of cells grown in glucose or galactose for 72 hours prior to testing. ATP was quantified using a luminescence-based assay and normalised to protein content. When galactose is the primary energy source, there is unsurprisingly a significant increase in the ATP levels in rescued cells compared to pcOPA -/- (n=10 wells, $p < .0001$ for both). What is interesting though is the reduction in ATP levels of the rescued cells when compared to pcOPA -/- in glucose containing media. This is also significant (n=10 wells, $p < .0001$ for both) and suggests there could be a slight burden to maintaining mitochondria in terms of ATP, but this would need to be repeated to confirm this. In other words, if glucose is in excess, being solely glycolytic could be beneficial to the individual cell as it can produce ATP at a faster rate but less efficiently via glycolysis than it can through OXPHOS. It would be interesting to restrict glucose levels to a minimum, whereby we might see the extra effort mitochondrial OXPHOS requires being bioenergetically favourable.

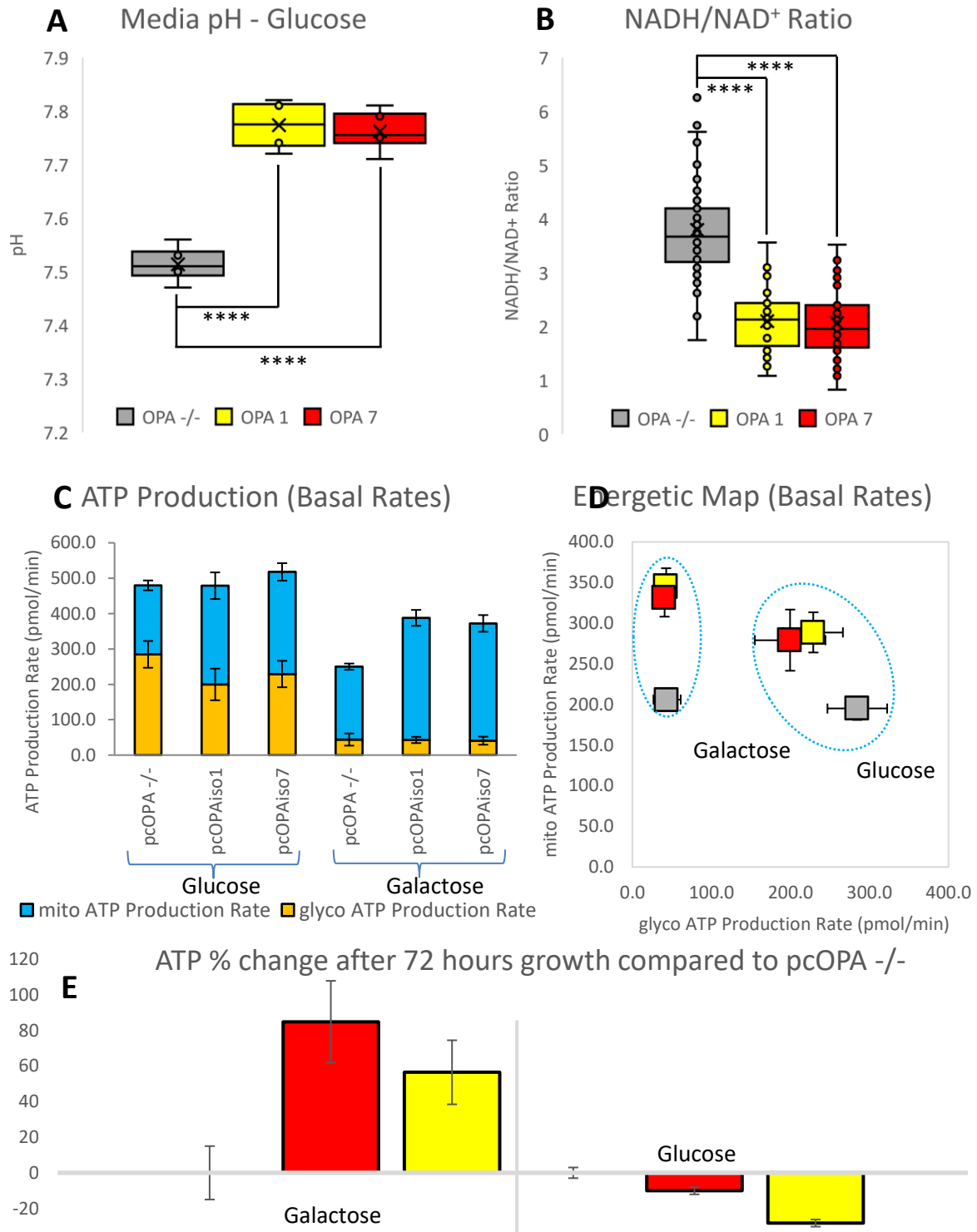


Figure 4-11: pcOPAiso 1 and 7 cells shift away from glycolysis towards mitochondrial OXPHOS. A) details the change in pH over 48 hours of culture in the presence of glucose. pcOPA -/- cells rely heavily on glycolysis for ATP generation and thus shift the pH of the media to be more acidic. This can also be seen in B), which is a measure of the cytosolic NADH/NAD⁺ ratio. There is a significantly higher NADH/NAD⁺ ratio in pcOPA -/- cells, indicative of increased glycolysis. C) and D) detail ATP rate assay information from the seahorse XFe96. Cells were seeded 12 hours before the assay having been grown in either glucose or galactose for 48 hours previously. C) shows the ratio of mitochondrial vs. glycolytic ATP production which is .69 for pcOPA -/-, 1.5 for pcOPAiso1 and 1.3 for pcOPAiso7 in the presence of glucose. When galactose is present rescued cells show ability to expand their use of mitochondria for ATP production. D) provides an energetic map of mitochondrial vs glycolytic ATP production, grouped by growth medium. E) Cells grown in galactose or glucose media were assayed for ATP content, expressed as a percentage of pcOPA -/- ATP content, normalised to protein content. The error bars on the grey line represent pcOPA -/- variation for each media. All comparisons in E) are highly significant (n=10 wells, p<.0001). Error bars ±SD.

After repeated passaging in either glucose or galactose media we can examine more long-term shifts (over 1 week) in cellular bioenergetics by utilising assays such as the Mitochondrial Stress Test on the Seahorse XFe96 (see section 2.10.4 for assay details). Using this assay allows us to investigate the oxygen consumption rate (OCR) as well as getting a sense for mitochondrial health in general. Briefly Sequential addition of various compounds targeting different parts of the ETC enables mitochondrial respiration to be elucidated. Oligomycin is used to inhibit ATP synthase; decrease in OCR following oligomycin addition provides a quantification of mitochondrial respiration due to cellular ATP production. FCCP is an uncoupling agent that dissipates the proton gradient and disrupts the mitochondrial membrane potential, resulting in an uninhibited electron flow through the ETC and providing an estimate of the maximal oxygen consumption possible. Finally, a mix of rotenone (complex I inhibitor) and antimycin A (complex III inhibitor) inhibits mitochondrial OXPHOS and enables an estimate of non-mitochondrial background respiration in cells.

In Figure 4-12 we can see a selection of these metrics. The OCR of rescued cells is significantly higher than that of unrescued pcOPA $-/-$ cells, normalised to cell number. These cells had been grown in galactose or glucose for a number of passages beforehand (at least 192 hours in culture). In Figure 4-12A we can see the changes in OCR across the course of the Mito Stress Test. The traces in grey and blue are the pcOPA $-/-$ cells. The light grey trace was given galactose media only at the start of the assay. In comparison the cells that had been routinely cultured in galactose are represented by the blue trace, which is only just above baseline. These cells were clearly severely compromised by prolonged growth in galactose.

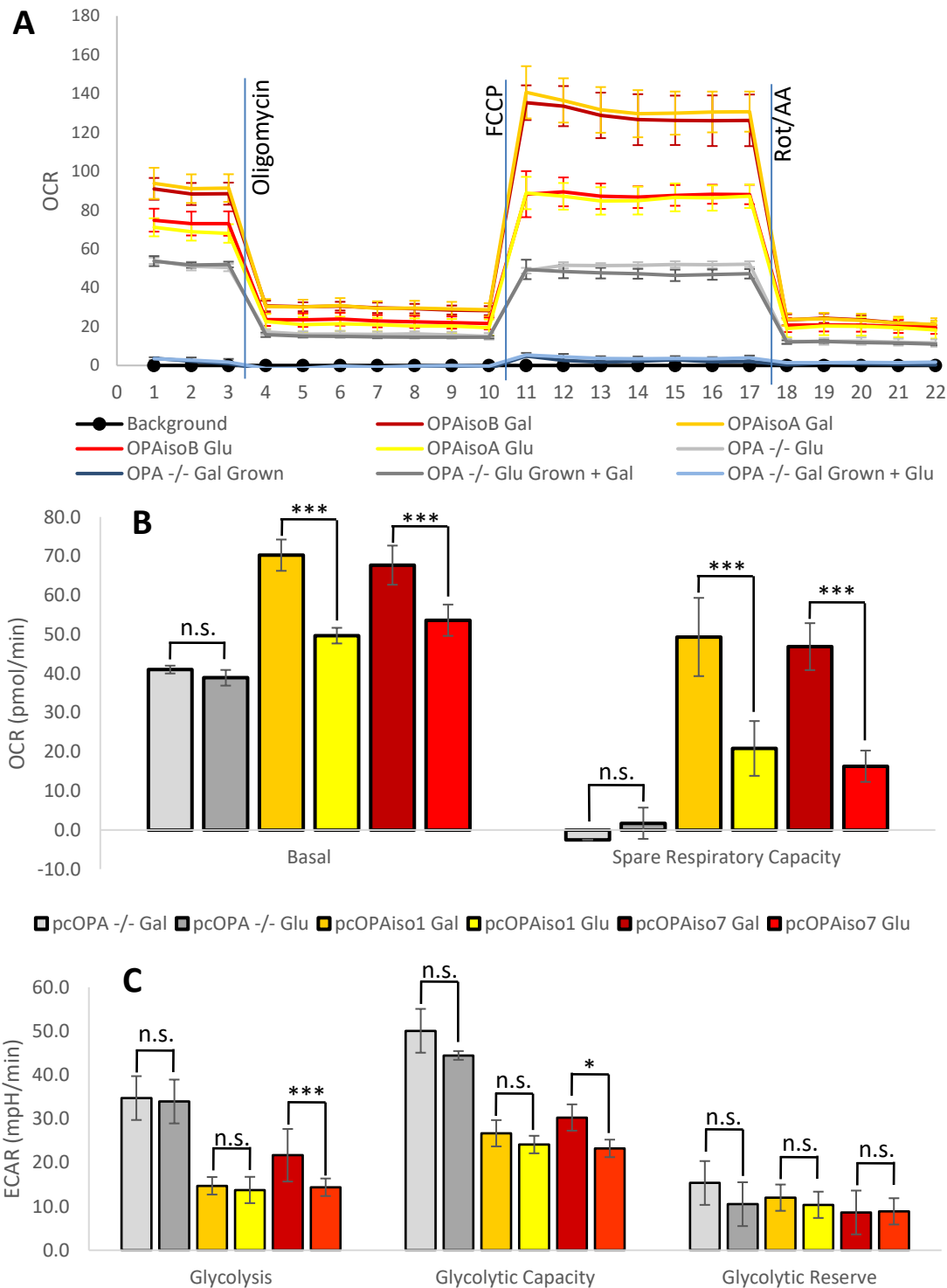


Figure 4-12: Analysis of long term effects of culture with glucose or galactose on pcOPAiso1, 7 and -/- cells. A) Shows an OCR trace from a Seahorse XFe96 mito stress test kit. Yellow traces correspond to pcOPAiso1, red traces to iso7 and grey traces to -/-. The blue trace near the baseline reading is that of pcOPA -/- cells cultured in galactose only for 192 hours. The remaining cells are effectively quiescent. These means that the pcOPA -/- results used are from the immediate switch galactose prior to the assay being run. pcOPAiso1 and 7 are from 192 hours culture in the two medias. As such only comparisons within groups are made. B) The basal OCR and spare respiratory capacity (SRC) of rescued cells. Prolonged culture in galactose media leads to a significant increase in both basal OCR and SRC, indicating strong remodelling of the mitochondria to favour OXPHOS in galactose grown rescued cells. C) pcOPAiso7 cells have significantly increased levels of basal glycolysis and glycolytic capacity after growth in galactose, but not in glycolytic reserve. (n=8 wells for all comparisons, error bars are $\pm$ SD)

The bright red and yellow traces, in the middle for OCR, are pcOPAiso1 and 7 grown in glucose. The darker coloured red and yellow traces with maximal OCR are pcOPAiso1 and 7 grown in galactose. Prolonged culture in galactose significantly increases both the basal and maximal oxygen consumption rates of pcOPAiso1 and 7 compared to glucose only grown cells expressing the same OPA1 isoforms. This increase is absent in pcOPA^{-/-} cells. There is clear metabolic plasticity seen in rescued cells.

In Figure 4-12B we can see that cells have a large increase in their spare respiratory capacity (SRC). This is calculated as the difference between basal cell respiration and the maximal rate after FCCP mitochondrial uncoupling. The OPA^{-/-} cells have little to no SRC, indicating cells are running at the maximum capacity, indicating a respiratory restraint on them. In comparison the pcOPAiso1 and 7 cells both show ~70% extra SRC when grown in galactose and ~35% extra SRC when grown in glucose. As these cells were continually cultured in the two medias beforehand, this demonstrates longer term restructuring of the mitochondria between glucose and galactose grown cells, giving the galactose grown cells a significant boost in spare respiratory capacity. From a functional point of view, larger SRC allows a cell to cope better with mitochondrial insults, giving it a more effective buffer in its energy production. Of course, as pcOPA^{-/-} cells grown in galactose long term showed almost no OCR the comparison in Figure 4-12B of pcOPA^{-/-} in galactose and glucose (grey traces) only serves to show no changes are evident immediately upon the switch in metabolic demands.

Utilising the Glycolytic Stress Test on cells in long term glucose or galactose media, we can see if the glycolytic capacity of these cells is altered by prolonged exposure to different fuel sources. For full details on this assay see the methods section 2.10.5, but briefly, the Extra Cellular Acidification Rate (ECAR) of cells is first monitored without a fuel source, then glucose is added to measure the cell's basal glycolytic rate (as evaluated by acidification of the medium). Next oligomycin is added which inhibits ATP synthase, blocking the ETC by preventing proton

pumping thus increasing glycolysis to its maximum capacity. Glycolysis is then competitively inhibited by addition of 2-DG.

We can see in Figure 4-12C the rate of glycolysis only increases significantly in pcOPAiso7 after long term galactose culturing. Likewise, only pcOPAiso7 showed a significant difference in glycolytic capacity, which is the total ECAR after addition of oligomycin. What did not change however is the glycolytic reserve, which is the difference between the basal rate of glycolysis and the maximal glycolytic capacity. As these cells are normalised to cell number seeded it is possible that an error occurred then, but one would expect all metrics to increase proportionally if so.

Overall, Figure 4-12 shows us that prolonged culture of the pcOPAiso 1 and 7 cells in media enforcing different metabolic constraints can significantly increase the OXPHOS spare respiratory capacity, showing that rescued cells have the power to modulate mitochondria beyond what is needed purely to survive in the restrictive media. We also see that this does not translate to extra spare glycolytic capacity but does demonstrate that rescued cells still have the same ability to utilise glycolysis as unrescued cells.

To examine the degree of rescue achieved with pcOPAiso1 and pcOPAiso7 compared to wild type MEF cells, a Seahorse XFe96 mitochondrial stress was performed (Methods 2.10.4). All cells were grown in galactose media 48 hours before the assay. After normalisation to cell number, there was no significant difference in basal OCR when comparing pcOPAiso1, pcOPAiso7 and wild type MEF cells (ANOVA, $n = 8$ wells, $p > .05$). In comparison OPA $-/-$ cells showed significantly reduced basal OCR compared to the other groups (TSD, $n = 8$ wells, $\alpha = .05$). However, when we compare the spare respiratory capacity percentage (the percentage increase between basal and maximal respiration) MEF cells perform significantly better than either OPAiso1 and OPAiso7 (TSD, $n = 8$ wells, $\alpha = .05$). MEF cells have an average 116% spare capacity, indicating they can more than double their respiratory activity if needed. In comparison pcOPAiso1 and pcOPAiso7

demonstrate a 68% and 54% increase in OCR, roughly half that of MEF cells. This however is a large increase over OPA $-/-$ cells which show almost no capacity to increase OCR levels, showing just an average 1% increase over basal levels. These results demonstrate that pcOPAiso1 and pcOPAiso7 haven't achieved wild type MEF levels of metabolic plasticity but can maintain basal levels equivalent to them. This represents a positive step towards a therapeutic strategy for OPA1 deficient cells.

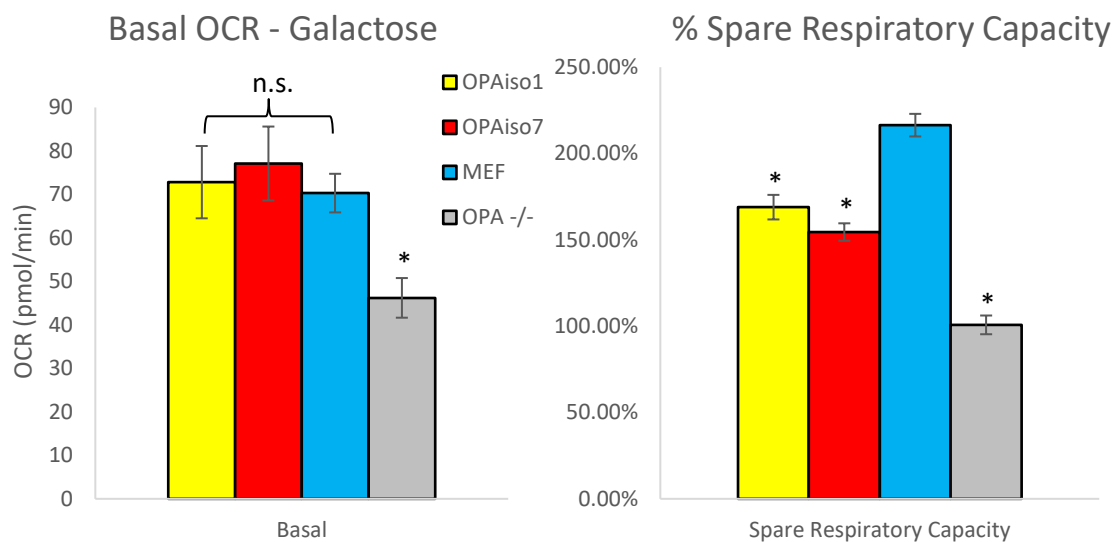


Figure 4-13: Seahorse XFe96 mitochondrial stress test comparing galactose grown pcOPA $-/-$, iso1, iso7 and MEF cells after normalisation for cell number. The basal OCR of rescued OPA cells is not significantly different from that of MEF cells grown in galactose media (ANOVA, $n = 8$ wells, $p > .05$). In comparison OPA $-/-$ cells showed a significantly decreased OCR (TSD, $n = 8$ wells, $\alpha = .05$). Wild-type MEFs demonstrate a significantly higher spare respiratory capacity (SRC) when compared to rescued or unrescued OPA cells (TSD, $n = 8$, $\alpha = .05$) but rescued pcOPAiso 1 and 7 cells show significant rescue of the SRC when compared to OPA $-/-$ cells.

4.3. Discussion and Future Prospects

The data presented in this chapter of the thesis represent an important step towards the development of a potential gene therapy for DOA, primarily focusing on how cells react to exogenously provided OPA1 gene isoforms and using a diverse array of assays to explore this. Furthermore, in this study careful thought has also been given to the development of the types of assays and end-points that we may wish to examine when investigating the effects of delivery of OPA1 gene therapies to patient-derived cell models in the future.

4.3.1. Endogenous expression of OPA1 is correlated with other mitochondrial remodelling proteins

The initial analysis of RNAseq data suggested that isoform 1 and 7 were the most highly expressed of the eight protein producing OPA1 isoforms in retinal tissue. Previous studies of the expression patterns of OPA1 isoforms have shown that systemically isoform 1 and 7 are the most highly expressed, however there are marked differences from organ to organ (A Olichon et al., 2007). Human ocular tissue was not examined in this previous study, but brain tissue was and surprisingly almost no signal for isoform 7 was found, instead isoform 5 was the predominant isoform, with isoform 1 coming in second. This is interesting considering the neural retina is often considered an extension of the brain (London, Benhar, & Schwartz, 2013). In contrast to brain tissue, if we look at Figure 4-2A we can see that isoform 5 is the third most highly expressed isoform in our retinal samples. The difference in composition between isoform 5 and isoforms 1 and 7 is the addition of exon 4b, which allows complete processing of isoform 5 to short protein forms. In contrast, isoforms 1 and 7 are processed into both long (l-form) and short form (s-form). Some beautiful work has previously been carried out to examine the roles of each isoform and the l and s-forms of OPA1 proteins, and it appears that isoform 5 has very limited ability to remodel the mitochondrial network (Song et al., 2007). The s-forms have been shown to be more efficient than l-forms alone in restoring energetic competence (Del Dotto et

al., 2017). It is important to remember that isoforms 1 and 7 are also cleaved into l and s-forms and so can perform both roles, it is only isoforms with exon 4b that are completely cleaved into short isoforms. Based on our RNAseq of actual retinal tissue from 16 healthy donors, we considered that the choice to proceed with isoforms 1 and 7 was the correct one, although further study into combinatorial effects of isoforms *in vivo* may yield interesting results and findings that might influence this choice. Given that as previously discussed, it is believed that OPA1 mutations tend to cause DOA through haploinsufficiency (Lenaers et al., 2012), it could be informative to examine the OPA1 isoform distribution of affected patients, and even between DOA and DOA plus sufferers. It may be that haploinsufficiency could cause a shift in the expression of OPA1 isoforms to try cope with the deficiency. Such studies have not as yet been undertaken, however, the availability of patient-derived cell and organoid models of DOA disease will aid in addressing such issues in the future.

The non-coding RNA landscape of these samples is also interesting. The high level of expression from 'no prot 2' is possibly just an artefact, stemming from mis-annotation of it as a relevant transcript by the HAVANA project. The sequence is classed by ensemble as a retaining intronic sequence and spans from exon 16 to 21. Without further investigation it is impossible to know if this is a biologically relevant transcript. OPA1-antisense1 has been previously described and attempts have been made to link variants in OPA1-AS1 to disease aetiology, although no link has been published. The fact that no reads were identified as being from OPA1-AS1 in these samples, albeit just a small number of retinal samples, casts some doubt on the biological relevance, certainly for retina, of the no prot 2 isoform.

Our RNAseq analysis also highlighted the correlation between the transcript levels of the major mitochondrial remodelling proteins. This correlation was particularly strong for OPA1 and Dmn1l transcript levels (Figure 4-2C). Of course, there are multiple layers of regulation in cells, so it is possible that post-translational modifiers nullify this correlation, but there has been

previous work showing that protein levels of these mitochondrial remodelers appear to correlate with each other (Del Dotto et al., 2017). This underscores the close relationship between fission and fusion processes in the cell and suggests that we may have observed the overexpression phenotype of mitochondrial bunching (Figure 4-5) due to unbalancing of this equilibrium.

It was unexpected that mitofusin 1 did not correlate with the other mitochondrial remodelling proteins. It plays an integral role in the fusion of the outer mitochondrial membrane and although mitofusin 1 and 2 are ~80% homologous, they are not redundant for each other during development but can both independently restore network tabulation in double (mitofusin1 and 2) knockout cells (Schrepfer & Scorrano, 2016). Mitofusin 1 deletion prevents OPA1 mediated tabulation of the mitochondrial network, but not deletion of mitofusin 2, suggesting there is direct interaction of the two proteins (Cipolat, Martins de Brito, Dal Zilio, & Scorrano, 2004), but no correlation at the mRNA expression level for the two, at least in the current analysis. There have been limited studies on mitofusin 1 overexpression in healthy cells, but it does lead to hyperfusion of the mitochondrial network and the perinuclear overexpression phenotype (Santel & Fuller, 2001), but not to bioenergetic increases (Lally et al., 2013). This suggests that mitofusin 1 levels need to be well regulated and kept consistent. It is important to note that mitofusin 1 and 2 show different tissue specificities, with mitofusin 1 being predominant in liver, pancreas and testis but mitofusin 2 being predominant in heart, skeletal muscle, brain and now, according to our analysis here at least, retina (Schrepfer & Scorrano, 2016). This difference in tissue expression could explain the lack of correlation observed with mitofusin 1, perhaps in tissues where predominant expression of mitofusin 1 is seen there would be a correlation.

4.3.2. Design considerations for a viable OPA1 based gene therapy

In the current study, the combination of both optimising the OPA1 isoforms and putting it under the control of the relatively strong ubiquitous CMV promoter would have led to close to the

maximal expression levels of OPA1 achievable via transient transfection. Furthermore, the sequence optimisation may also abrogate any post-transcriptional regulation that may occur to wild-type OPA1 transcripts.

Using assays to evaluate mitochondrial morphology, the effects of OPA1 delivery and potential overexpression have been explored in vitro. For example, it seems that the mitochondria that show the distinctive perinuclear clustering are highly positive for the HIS-tagged OPA1 constructs (Figure 4-5). Transient transfection of MEF cells with the two OPAiso constructs led to high levels of this overexpression phenotype, and consequently no sign of rescue in the Seahorse XFe96 analysis of oxygen consumption (Appendix 7.2). The use of the time-lapse study of pcOPAiso7 cells (Figure 4-8), suggests that this perinuclear mitochondrial organisation is not due to an immediate and acute toxicity of OPA1 over-expression, but only arises after sufficient levels of OPA1 protein are produced, possibly levels at which mitochondrial fission may no longer counteract the fusion of the mitochondrial network. Although this time-lapse also showed that apoptosis is distinct from this overexpression phenotype, it is not possible to rule out that OPA1 overexpression just prevents these cells from apoptosing when they receive the signal to do so, rather than just being a stable rearrangement of the mitochondria. For instance, it has been observed that OPA1 expressing cells can prevent cytochrome C release in cells that have been chemically treated, thereby preventing apoptosis from continuing on (Frezza et al., 2006). OPA1 achieves this by forming dimers across the mitochondrial cristae, the more OPA1 present, the tighter these junctions are thought to be. This anti-apoptotic activity is also independent of OPA1's fusion promoting activity, as seen by mitofusin 1 knockout cells having a higher resistance to apoptosis when overexpressing OPA1, despite possessing a completely fragmented mitochondrial network (Frezza et al., 2006). So, it could be possible that this perinuclear arrangement of mitochondria is due to cells initiating apoptosis but being unable to release cytochrome C and thus being unable to continue with cell death. Under normal physiological circumstances, proapoptotic proteins such as BAK and BAX are normally found in

the cytosol but associate with the mitochondria upon the initiation of apoptosis. Staining for these proteins should help to decipher whether the perinuclear mitochondrial arrangement is associated with prevention of apoptosis by OPA1 or purely the result of OPA1 overexpression. It also seems likely that if the mitochondrial clustering is independent of apoptosis that this phenotype is not terminal (or at least not immediately terminal) to the cell as some cells in the time-lapse study showed this phenotype for the duration of the study, close to 13 hours (Figure 4-7).

It would also be interesting to explore if this phenotype is reversible. Could activation of pro-fission proteins recover the phenotype after it occurs? Or perhaps increasing mitochondrial biogenesis leading to a larger area for the OPA1 protein to be dispersed? Controlling OPA1 expression via an inducible promoter would be informative; once the perinuclear mitochondrial phenotype is observed, using this approach it would be possible to withdraw the activator of protein expression and then establish if the phenotype would be reversed. The differentiation of the apoptotic and overexpression phenotypes could assuage some fears relating to the future use of OPA1-based gene therapies.

In order for exogenously delivered OPA1 to become a viable candidate therapeutic, the overexpression phenotype needs to be thoroughly understood. Making cells resistant to apoptosis is not necessarily a disadvantageous characteristic, especially in a post-mitotic tissue such as the retina, however if the OPA1 overexpression phenotype were to cause bioenergetic dysfunction, the potential harm may outweigh the possible benefit. As mentioned above, another issue that should be explored given the codon optimisation of the optOPA1 construct is the possibility of optOPA1 missing out on any post-transcriptional modifications and thereby such sequence changes in optOPA1 playing a significant role in OPA1 protein levels. Irrespective of this possibility which may influence levels of OPA1 protein, the most straightforward change that can be implemented to alter OPA1 protein expression would be the particular promoter

sequence employed for transgene expression. The CMV promoter is known to be a strong constitutive promoter sequence, with consistently high levels of expression in multiple tested cell types (Damdindorj et al., 2014; Qin et al., 2010). Other studies have steered clear of the CMV promoter, using comparatively weaker promoters, such as the LTR promoter (Del Dotto et al., 2017), among others. Furthermore, the cells examined in the Del Dotto study were created using retroviral vectors to produce stably expressing cell lines. This method in principle allows finer tuning of expression as the genomic region where the vector integrates also determines the extent to which the transgene is expressed. Selection and clonal expansion enable the production of a cell population with near identical expression levels. This is in contrast with the study described in this chapter, where episomal plasmid DNA in the form of the pcOPA vectors was used. These are maintained in the population via selection, but the copy number per cell can vary and this cannot be selected for via clonal expansion as daughter cells can inherit non-uniform quantities of plasmid. However, to an extent this mirrors the lentivirus vs. AAV debate; the risks of stable genome integration vs. AAV's non-replicative capacity and the situation that might occur in AAV-mediated gene therapies where tight control on levels of transgene expression at the individual cell level may be difficult to achieve as, for example, in part it will depend on the number of AAV vectors that transduce individual cells.

We must also remember that in patients we would be dealing with haploinsufficiency compared to complete knock-out seen in the OPA $-/-$ cells used in this study. This would in theory narrow the permissible expression range for exogenously delivered OPA1 protein before overexpression may be experienced. Promoter selection may potentially deal with this issue, as transgenic mice constitutively expressing OPA1 under the control of the human β -actin promoter can ameliorate the phenotype of mitochondrial disease caused by either *Ndufs4* deletion or *Cox15* muscle deletion (Civiletto et al., 2015b). These mice have constitutive OPA1 isoform 1 overexpression yet remain fertile, have a normal lifespan and are more resistant to a range of tissue insults (Varanita et al., 2015). These data demonstrate the value of OPA1-based

therapies, and moreover suggest that it is possible to achieve bioenergetically favourable and physiologically safe levels of OPA1 expression in tissues that already have wild-type levels of the protein. Of course, there is a caveat to this in terms of these murine studies. The researchers who bred this human β -actin promoter driven OPA1 mouse did so on two backgrounds, C57BL6 and Sv129. On the Sv129 line there was an increase in the rate of spontaneous cancer formation, highlighting the ever-present risk of modulating apoptotic factors.

Potentially the use of the endogenous OPA1 promoter could circumvent the need to titrate OPA1 expression levels so finely, as possibly the cell could do this regulation independently (auto regulate). There has been a rather scant characterisation of the OPA1 endogenous promoter to date, however it is believed to be around 700bp in length (Bosley et al., 2011). Therefore, this promoter in principle is small enough to fit with the OPAiso1 or 7 cds into an AAV vector, which could be a potential avenue for exploration to optimise control of expression of AAV-OPA1 delivered transgenes. However, to our knowledge nobody has tried to utilise this upstream promoter region for expression. I have analysed some publicly available ATAC-seq data, which allows regions of open chromatin to be identified, a marker of transcriptional activity, and the OPA1 promoter region may be potentially quite large with putative enhancer sequences far upstream. As enhancers can play key roles in tissue specific gene expression and thus regulation (Ko, Oh, & Yoo, 2017), it could potentially become quite complicated to piece together a usefully regulated endogenous promoter, albeit clearly of significant value.

The decrease of OPA1 expression we achieved in our study through the generation of the pcOPAiso plasmids led to the ability to examine a whole array of metabolic markers and phenotypes. However, in terms of mitochondrial network rescue, thus far wild-type levels were not achieved. Using the approach adopted, we reached a zenith of 60% of cells with at least partially rescued mitochondrial morphology by growing pcOPAiso7 cells in galactose (Figure 4-10D). While clearly this is a significant rescue, it is not near to 90%+ of cells with strongly

tubular networks seen in wild type MEFs. It may be that the reason for this is one of timing. It appears that OPA1 accumulates over time and as it does it alters mitochondrial morphology from unrescued, to rescued, to overexpression. We can see this in the time-lapse images (Figure 4-7). So it could be a case that all of the transfected cells will reach a point where the network is rescued, it just depends on when the cells are examined.

4.3.3. Metabolic benefits from OPA1 ectopic expression

When cells did show a restoration in mitochondrial network morphology, these cells showed a significant capacity to dynamically modify their mitochondrial networks, as demonstrated by the fission and fusion assay (Figure 4-9). Future work should include wild type MEFs to see if this increased fusion activity is approaching levels seen in healthy cells.

Galactose, or other metabolites that force the use of OXPHOS, prime cells to remodel their mitochondrial networks (Schrepfer & Scorrano, 2016) which typically increases the tubulation of the mitochondria in cells. This metabolic challenging of our stable pcOPAiso 1 and 7 cells gave the largest window for finding rescued cells and thus the highest number of rescued cells. It was also much more uncommon to find an overexpressing cell during these metabolic challenges.

The restoration of basal OCR to that seen in wildtype MEFs is a promising result for the development of OPA1 based therapeutics (Figure 4-13). This demonstrates that a significant proportion of the dysfunction in OPA^{-/-} cells can be ameliorated by OPAiso1 and 7 based gene therapies. pcOPAiso1 and 7 cells were not as able to modify spare respiratory capacity as were wildtype MEF cells, but the restoration of around half this capacity is still a significant improvement over OPA^{-/-} cells.

4.3.4. Where next? Ongoing and future work

The reason that the difficulties experienced with cloning near the start of this chapter of the thesis were highlighted is because of issues we have had further down the line with AAV

production. For optOPAiso1 or 7 to become a potential gene therapy for DOA or other target conditions, we need to be able to package and deliver the OPA1 gene in a suitable viral vector. Our lab specialises in AAV production and so we produced two AAV vectors, one for each OPA1 isoform, using the AAV 2/2 triple tyrosine capsid (Zhong et al., 2008). Titration of the virus gave 1×10^{11} viral genomes (vg) per ml and 1×10^{12} vg per ml for AAV2/2-3y-OPAiso1 and 7 respectively. However, to date transduction studies of these OPA1 viruses on HEK293, OPA^{-/-} MEFs and wild type MEFs yielded no detectable OPA1 protein by either immunocytochemistry or Western blot. Preliminary data using multiple sets of primers to titrate the viruses, targeting different regions of the packaged OPA1 transgene using an early (5'), middle and late (3') set of primers, showed that the late set (most 3' primer pair) consistently gave lower values for genome titres than the other primer sets. Further analysis will definitely be required and is being undertaken, however these preliminary data hint that instability at the 3' end of the sequence (which may have led to the cloning challenges detailed here), could also be potentially affecting viral packaging and resulting in partial OPA1 genes being packaged. The root cause of this is currently being investigated in order to produce viable AAV-OPA1iso constructs to be used in our future studies.

It is worth highlighting again that recent studies suggest that the future potential for OPA1 viral delivery as a therapeutic is encouraging. Thanks to the recent work in a DOA mouse model (Sarzi et al., 2018), it would seem that virally delivered OPA1 may be a viable therapeutic avenue. This mouse model harbours a 2708 > 2711 deletion in one OPA allele, which has been found in human DOA patients. Intravitreal injection of AAV2/2-OPA1 isoform 1 showed protection of retinal ganglion cells and some preservation of the optokinetic response. This OPA1 gene therapy was driven by the CMV promoter, suggesting at least that RGCs may survive better with overexpression of OPA1 than with haploinsufficiency (OPA1^{+/-}). Levels of expression were not compared to wild type mice after therapeutic intervention. Furthermore, the study suggests that having some level of OPA expression before administration of an AAV-OPA1 therapy may allow the cell to cope better with the addition of more OPA protein.

The decision as to which OPA1 isoform of the two studied here will be brought forward still needs to be investigated. It remains to be fully established whether there may be a particular benefit to using one OPA1 isoform over the other. *In vivo* there is clearly a complicated array of isoforms expressed in different tissues but here, using the two most commonly expressed OPA1 isoforms, 1 and 7, we saw little functional difference between the two, apart from in counts of rescued mitochondria and in glycolytic activity measured via the Seahorse XFe96 (Figure 4-10D and Figure 4-12C). As previously mentioned, the difference between the two isoforms is the addition of the 5b exon in isoform 7, which creates an extra cleavage site for proteolytic modification. The exon 5b is believed to play a role in the apoptotic regulation processes of the OPA1 protein (A Olichon et al., 2007), but most studies show little difference between the two isoforms in terms of mitochondrial morphology restoration and respiratory activity (Del Dotto et al., 2017; Song et al., 2007). To the best of our knowledge, glycolysis has not been probed before in cells with specific OPA1 isoforms. The increase in the glycolytic ability of pcOPAiso7 cells after prolonged growth in galactose is somewhat counter-intuitive (Figure 4-12C). Inspecting the ECAR of pcOPAiso1 and 7 from the Mito Stress test data in Figure 4-12A after Oligomycin, there is a significant difference between the two ($p < .05$), but no difference in the OCR suggesting there could be a difference in glycolytic regulation between the two isoforms. Further analysis would be required to ensure this is a robust finding, but it is possible that pcOPAiso7 cells may be bioenergetically more active in general. Glycolysis is the primary source of pyruvate that is shuttled into the Krebs cycle which in turn produces the NADH necessary for OXPHOS. It is undeniable that increased OPA1 expression when cells have metabolic freedom to choose between OXPHOS and glycolysis leads to an increase in OXPHOS, it is intriguing to speculate that OPA1 isoforms may play a role in glycolysis regulation when metabolic choices are limited.

The next step for the current OPA1 study will be test our construct in patient-derived cell models of disease. To achieve this the appropriate approvals from the clinical sites had to be obtained

which took somewhat longer than anticipated. In this regard however, we have recently developed methodologies to grow patient derived fibroblasts from skin punch biopsies. Indeed, as part of this study I have recently generated both patient-derived DOA and control cell lines. These cell lines have not as yet been used for evaluation of mitochondrial function employing the array of assays developed as part of this PhD study. Once the cell lines have been fully characterised, we plan to treat these cells with our AAV-OPA1 candidate therapeutics to attempt to ameliorate the disease phenotype.

In summary, the work described in this chapter of the PhD thesis has resulted in the establishment of a wide array of assays, which we have previously never used in our lab, that enable evaluation of mitochondrial function and dynamics. Establishment of such assays is essential prior to investigation of the potential value of OPA-based gene therapies. Furthermore, in this chapter of the thesis, a detailed analysis of mitochondrial dysfunction in OPA1 $-/-$ MEFs has been undertaken and the potential value of the delivery of different isoforms of OPA1 to correct this dysfunction has been evaluated. Of note, significant effects on mitochondrial morphology, dynamics and function were observed. The array of molecular tools developed during this study will enable the future detailed characterisation of patient-derived cell models of disease and a thorough investigation of candidate OPA1-based therapeutics in these disease models.

5. Identification and Functional Analysis of a Novel Alternative Oxidase Gene.

5.1. Introduction

The electron transport chain (ETC) no longer seems to be the most accurate description of the array of multi-subunit protein complexes that shuttle electrons from NADH or succinate down towards oxygen. It appears they are more like the electron transport web, with multiple entry points, complex I, complex II, Ndi1 etc, and also multiple exit points in the form of complex IV and the alternative oxidases (AOX). These AOX proteins act as part of the alternative electron transport chain, where they oxidise reduced ubiquinone, much like CIII, but transfer the electrons directly to oxygen for the formation of H₂O (see Figure 5-1). The positioning of AOX proteins at this point in the chain, allows them to potentially allow the bypassing of CIII and CIV dysfunction. The inhibition of CIII and CIV leads to a blockage of the ETC leading to over reduction of the ubiquinone pool, a phenomenon known to cause excessive ROS production and be highly detrimental to the cell (Dominiak, Koziel, & Jarmuszkiewicz, 2018). The drawback of AOX use however, is that like Ndi1, they are non-proton motive and as such act as uncouplers of the ETC. There is exciting potential utility for AOX proteins in mitochondrial dysfunction, and the aim of this chapter is to investigate a novel AOX protein and its benefit in cell models of mitochondrial dysfunction.

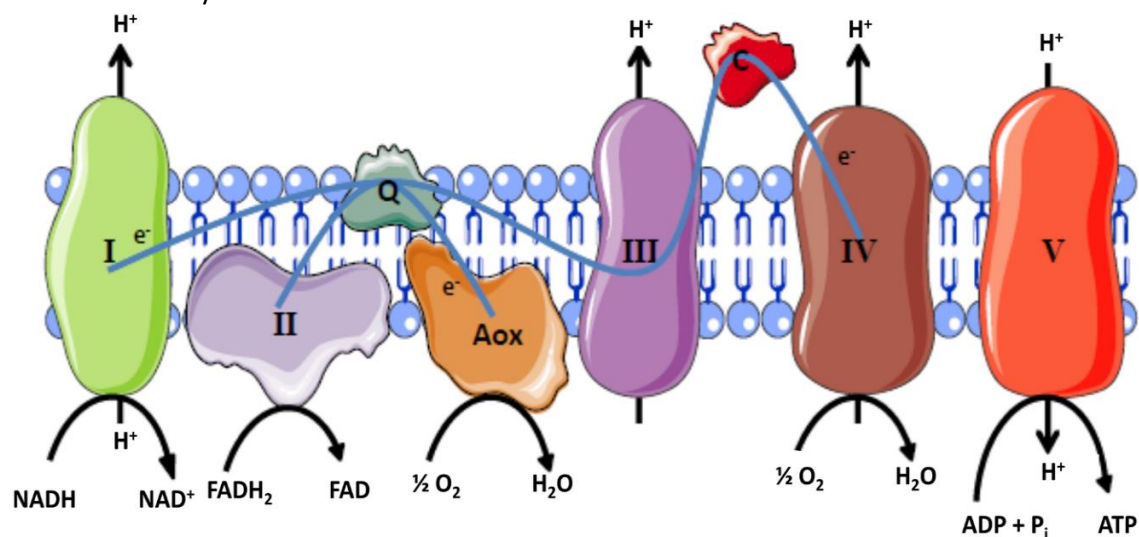


Figure 5-1: A schematic of the electron transport chain, with the canonical protein complexes as well as the alternative oxidase (AOX) protein represented in orange. With the presence of the AOX protein, electrons now have two routes they can follow, allowing the potential for AOX proteins to alleviate blockage of the ETC by CIII and CIV dysfunction. Note that the AOX gene is non-proton motive. Q = Ubiquinone, C = Cytochrome C. Repurposed from (Garcia-Neto, Cabrera-Orefice, Uribe-Carvajal, Kowaltowski, & Alberto Luévano-Martínez, 2017).

5.1.1. AOX background

It was in the 1970's when the presence of cyanide and antimycin A resistant mitochondrial respiration was first noted in the extract mitochondria of a number of plant species (Bahr & Bonner, 1973; Wilson, 1970). Cyanide and antimycin A are specific inhibitors of CIV and CIII respectively. This led to the notion of an alternative mitochondrial pathway, an alternative ETC, present in plant mitochondria (Henry & Nyns, 1975). This was first noticed in thermogenic plants (plants that have the ability to raise their temperature above that of the surrounding environment ("warm-blooded plants")) and it is the presence of the AOX protein that is in part responsible for this process. This is because the energy generated via AOX oxidation of ubiquinone is released as excess heat, instead of being used for mechanical work in the form of protein pumping (Wagner, Krab, Wagner, & Moore, 2008). The levels of heat generation are quite impressive, with some plants reaching up to 35°C above ambient levels and using equivalent amounts of oxygen as small mammals of the same weight (Knutson, 1974). There are some great thermal imaging pictures of these thermogenic plants glowing compared to the surrounding environment on the internet. This thermogenicity is believed to be used to volatise malodorous compounds to attract pollinators (Wagner et al., 2008).

It is important to remember though, that thermogenic plants are only a handful of species, whereas AOX genes have subsequently been identified in all higher plants, including *A. thaliana* (Polidoros, Mylona, & Arnholdt-Schmitt, 2009), suggesting that they play a more fundamental role in the physiology of these species, unrelated to extensive thermogenesis. In plants at least, it seems that AOX genes are differentially regulated to cope with stressors. For instance, in *A. thaliana* the expression levels of the AOX family of genes fluctuates depending on the stressors applied to the plants, whereas the components of the canonical ETC remain largely unchanged (Clifton et al., 2005). The expression of AOX genes also help protect plants during times of

metabolic crisis such as drought, where plants with AOX knockdown showed severe compromise of their respiration ability (Vanlerberghe, Martyn, & Dahal, 2016).

AOX genes have been found in fungi, protists and bacteria as well, but they are not as prevalent as in plant species (Veiga, Arrabaca, & Loureiro-dias, 2003). It is interesting to note that in almost all the yeast species that have lost complex I and thus rely on an alternative NDH gene, such as *S. cerevisiae*, there are no AOX genes (Veiga, Arrabaca, SANSONETTI, et al., 2003). This suggests that without the proton motive abilities of CI, some of the benefit of possessing an AOX is lost, perhaps due to the complete uncoupling of the ETC and ATP production if both proteins are present without CI.

An interesting quirk of evolution suggests that AOX genes in plants and yeast work to fulfil somewhat antagonistic roles. Plants induce AOX expression in response to biotic stresses, such as yeast infection of tissue, to cope with ROS production caused by pathogens and toxins (Polidoros et al., 2009). Conversely it seems that the presence of AOX genes in yeast protect the yeast from the antibiotic defences of the host plants, especially as cyanide production is a defence mechanism in a number of plant species (Rogov & Zvyagilskaya, 2015). There does seem to be an enrichment for pathogenic organisms to contain AOX genes, but again this could be due to bias in the dataset towards sequencing clinically and commercially relevant organisms (Allison E. McDonald & Vanlerberghe, 2006). Though there is mounting evidence that AOX expression can provide survival advantages in harsh environments, such as human gut colonizers that express AOX proteins to cope with the transient oxygen fluctuations, and is believed to act as protection to potential bacteria that cause IBS (Tsaousis et al., 2018). Findings like these have prompted the investigation of the AOXs as being potential antimicrobial targets as their targeted inhibition should in theory reduce collateral damage in the non-AOX expressing host whilst compromising the pathogen (Ebiloma, Balogun, Cueto-Díaz, de Koning, & Dardonville, 2019).

It was not until the 90's that there was identification of cyanide resistant respiration in the Animalia kingdom, but the confirmation of the presence of an AOX gene did not come until *in silico* analysis of sequence data in 2004, where AOX genes were identified in four species from 3 phyla (A. McDonald & Vanlerberghe, 2004). 28 animal species across 9 phyla have been identified to contain AOX genes (Allison E. McDonald, Vanlerberghe, & Staples, 2009). Interestingly, AOX containing animals tend to be members of the basal animal phyla, suggesting that the ancestral state was to possess an AOX gene, and that the gene has subsequently been lost in higher animals (Allison E. McDonald & Vanlerberghe, 2006). The vast majority of animal phyla with identified AOX genes also tend to be sessile, and located in aquatic environments, such as *C. intestinalis* as well as corals and sponges, and a number of *Mollusca* like the pacific oyster. Parallels could be drawn between the widespread presence of AOX genes in plants and those animals found to contain AOX genes, with both needing to withstand a fluctuating environment (such as oxygen concentration in aquatic environments) and increased vulnerability to pathogens due to their immobile nature. Furthermore, evolutionary analysis has suggested that the AOX gene was present in the last common ancestor of all the AOX carrying organisms elucidated so far (Pennisi et al., 2016), so conserved functionality may be anticipated to a degree. The catalytic core of the AOX genes is highly conserved across all species, but the N-terminus region is highly divergent, suggesting maintained function but potentially differential regulation of AOX genes in different species.

Unfortunately, due to the nature of the animals so far identified as carrying AOX genes, there is a paucity of direct experimental analysis of AOX expression *in vivo*. Although in the original paper identifying AOX sequences in animals, the authors went to the "local" fishmongers, bought some pacific oysters, and proceeded to analyse different tissues for AOX expression, demonstrating mRNA and protein expression of the putative AOX gene (A. McDonald & Vanlerberghe, 2004). Further than this however there is no data regarding expression profiles or conditions in these animals. It would be informative to have a better understanding of how

AOX proteins are utilised in the animal kingdom, but it seems unlikely anyone will set up a sea squirt lab any time soon.

In totality, the consensus in the field is that a major role of AOX proteins is to act as antioxidant defence (preventing ROS production from CIII and over reduction of the quinone pool), as a thermogenic protein to some degree, and as a stress induced defence mechanism against environmental stressors both biotic and abiotic (Pennisi et al., 2016; Rogov & Zvyagilskaya, 2015; Saha, Borovskii, & Panda, 2016).

5.1.2. Alternative Oxidases as a therapeutic approach

It was not long after the identification of putative animal AOX genes that one was first tested *in vitro*. Out of the four AOX genes identified at first it was the *C. intestinalis* AOX gene that was chosen, due to its taxonomic locus at the base of the chordates (Hakkaart, Dassa, Jacobs, & Rustin, 2006). By this point earlier attempts had been made to ectopically express AOX genes by this group, focusing on the *A. thaliana* AOX gene, which had led to “uncontrolled lethality” in cells (Hakkaart et al., 2006). Using HEK293 cells the *C. intestinalis* gene was placed under the control of a doxycycline inducible promoter and chromosomally integrated, to create an inducible AOX expressing cell line. Subsequent analysis showed mitochondrial localisation of the protein and demonstrated functional integration into the electron transport chain, demonstrated by cyanide resistant respiration. Further analysis demonstrated a lack of acidification of the tissue culture media after treatment with cyanide, which suggests less reliance on glycolysis for energy production after disruption of the electron transport chain (Hakkaart et al., 2006). In this study, they found no growth rate changes in cells expressing the *C. intestinalis* AOX compared to those not induced to express. It was postulated that the AOX remained inactive unless a certain level of reduction in the quinone pool was reached. This is primarily believed to be due to the AOX protein having a lower affinity for reduced ubiquinone compared to CIII (Moore et al., 2013). This means that AOX protein should not compete with

CIII under normal physiological conditions and should only act as a form a safety valve only activated by sufficient perturbation in CIII and CIV activity.

This work was built upon to demonstrate that the *C. intestinalis* AOX protein can in part compensate for CIV deficiency in cells (Dassa et al., 2009). The ectopic expression of the AOX gene demonstrated utility in both HEK293 cells with COX10 knockdown via shRNA and patient derived skin fibroblasts harbouring a COX15 pathogenic mutation, which were transduced via lentivirus to allow genomic integration.

To investigate if the expression of the *C. intestinalis* AOX gene has detrimental effects on an organismal level, *Drosophila* with an inducible AOX transgene were generated (Fernandez-Ayala et al., 2009). These flies appeared to be protected from the lethality of various CIV genetic insults as well as showing restoration of motor function in a genetic model of Parkinsonism. The growth rate of the flies appeared normal, as did their fertility. However, the benign effect on normal physiology of *C. intestinalis* has recently been called into question, with further analysis of *Drosophila* constitutively expressing the *C. intestinalis* AOX gene showing reduced fertility rates. This appears to be due to decreased production of mature sperm (Saari, Andjelković, Garcia, Jacobs, & Oliveira, 2017). It appears that in this study the presence of AOX protein disturbs mitochondrial function either through ATP synthesis dysregulation or potentially through redox or calcium signalling.

Placing the *C. intestinalis* AOX in a number of genetic models of disease has demonstrated some exciting results, but there is an inconsistency in the precise manifestations of rescue. For instance in a *Drosophila* model of neuronal mitochondrial dysfunction, where the catalytic subunit of mtDNA polymerase γ was downregulated leading to premature aging and dopaminergic neuron loss, the addition of *C. intestinalis* was found to be highly protective (Humphrey et al., 2012). The AOX ectopic expression fully recovered the ATP deficit seen in the mutant flies, as well as preventing the dopaminergic neuronal cell loss. However, when the

downregulation of the mtDNA pol γ subunit, or of Twinkle was expanded to the entire organism, AOX was unable to provide any significant rescue of the phenotype, and even appeared to increase developmental lethality in these flies (Rodrigues, Camargo, Andjelković, Jacobs, & Oliveira, 2018). This developmental lethality, or at least arrest has been seen before in *Drosophila* when constitutively expressing AOX, but this appears only to be the case in mitochondrially deficient flies, as demonstrated when using the *tko*^{25t} *Drosophila* strain, who harbour a mutation in mitochondrial ribosomal proteins (Kemppainen, Kemppainen, & Jacobs, 2014). AOX expression was well tolerated in adult flies but did nothing to relieve the mutant phenotype. It appears that AOX protein expression dysregulates mitochondrial signalling at early stages of development that prove lethal to the organism, whereas it can be well tolerated in the adult animal. This is interesting in the context of recent body of evidence placing mitochondrial homeostasis as being a big player in stem cell renewal and commitment. It is proposed that when a stem cell divides into its two daughter cells, one of them receives a different cohort of mitochondria, leading to commitment of the cell, opposed to renewal of the stem cell phenotype (H. Zhang, Menzies, & Auwerx, 2018). It appears that in human and mouse embryonic stem cells at least there is a heavy reliance on glycolysis (Takehara, Teramura, Onodera, Hamanishi, & Fukuda, 2012; Zhou et al., 2012), so it is not inconceivable that AOX expression in mitochondrially compromised *Drosophila* can lead to disruption of the normal mitochondrial signalling in stem cell division and differentiation leading to this early lethality. Indeed it has been shown that treating pluripotent stem cells with antioxidants, at doses well tolerated by other cell types, disrupts their ability to self-renew (Hämäläinen et al., 2015).

Work has proceeded with the generation of mice expressing the *C. intestinalis* AOX protein. Initially this was achieved using lentiviral transduction which led to inconsistent integration across tissues leading to some variability in the phenotypes observed (El-Khoury et al., 2013). The weight of the AOX mice was significantly reduced at 6 days of age, and interestingly the body temperature was marginally but significantly raised in the AOX expressing mice, reprising

the role of the thermogenic AOX protein. By two months however, there was no significant difference in weight. An effort was made to create a more “genetically tractable” mouse model by specifically targeting the *Rosa26* locus in mouse embryonic stem cells (ESC), and line generation after this (Szibor et al., 2017). This is worth noting as under normal mitochondrial physiology in mouse ESCs, it appears that AOX expression is well tolerated, compared to the mitochondrially challenged *Drosophila* lines mentioned earlier. Analysis of these mice showed no significant changes in an array of physiological markers such as body weight, temperature or heart performance and suggests that the AOX remains inert under standard physiological parameters (Szibor et al., 2017). This AOX expression conferred the transgenic mice very impressive resistance to cyanide after intraperitoneal injection, with 100% of animals withstanding the LD:50 quantity of cyanide.

This mouse has subsequently been crossed to a number of mouse models of mitochondrial disease showing some promising results. In a mouse model of GRACILE (Growth Retardation, Aminoaciduria, Cholestasis, Iron overload, Lactic acidosis, and Early death) syndrome, which harbours a mutation in the *BCS1L* gene (an assembly factor of CIII), there was a 3-fold extension of lifespan when these mice co-expressed the *C. intestinalis* AOX protein (Rajendran et al., 2019). Intriguingly, the AOX expressing mice did not show rescue of the growth retardation or other metabolic markers such as the glucose:lactate ratio. The protection the AOX mice exhibited appears to derive from the alleviation of the cardiac phenotype, along with protection in the kidney and liver. This appears to be due to the stabilisation of the cardiac mitochondrial ETC, allowing wild-type levels of CI and CII activity. There was a significant decrease in ROS production in heart and kidney tissue of these animals also (Rajendran et al., 2019).

However, there have been some contradictory results with the AOX mouse also. A COX15 muscle-specific knockout mouse showed increased lethality with the presence of the *C. intestinalis* AOX protein, with median survival reduced from 150 days for the KO mouse to just

60 days with the presence of the AOX (Dogan et al., 2018b). It's important to note that COX15 deficient patient cells were the first human patient cells treated with AOX, as mentioned previously, and showed positive therapeutic benefit (Dassa et al., 2009). This highlights the difference between cellular studies and whole organism therapeutic strategies. The reason for the enhanced dysfunction is believed to be due to decreased ROS production due to the AOX activity, which dysregulates the protective hormetic response to ROS mediated by the AMPK/PGC-1 α . This AMPK/PGC-1 α pathway is typically activated due to increased ROS signalling, the authors argue, and would normally lead to a compensatory increase in mitochondrial biogenesis. The expression of the AOX protein may abrogate the hormetic response to ROS in this mouse model. It is important to note that this model would not be directly be representative of COX15 mutations seen in patients, where significant phenotypic variability is seen, typically manifesting as Leigh syndrome with marked differences in CIV activity in different tissues (Bugiani, Tiranti, Farina, Uziel, & Zeviani, 2005). That is to say that this model doesn't particularly represent COX15 deficiency as a specific disease, but it does highlight that redox signalling can serve a beneficial role in disease progression and is not necessarily just a marker of damage.

The disparity between these two studies, that of GRACILE CIII disorder and COX15 CIV disorder highlights some unanswered questions about mitochondria, their role in pathogenesis and the differing ways cells compensate for dysfunction of the mitochondrial ETC. One would imagine that as AOX acts on the ETC upstream of CIII and CIV that dysfunction in either would be similarly dealt with by the presence of AOX protein. The difference here could come from the severity of the mutations these mice have, with the COX15 knockout being much more severe in terms of bioenergetic consequences.

Outside of direct mitochondrial mutations the *C. intestinalis* AOX has shown some promise. For instance in models of cigarette smoke damage, ectopically expressing AOX protein has shown

to be beneficial, increasing cell survivability, preventing apoptosis and concomitantly reducing ROS production (L. Giordano et al., 2018). When interrogating a *Drosophila* model that expresses human Amyloid- β protein, the expression of the *C. intestinalis* AOX gene was protective, showing a reduction of Amyloid- β build up and partially compensated for the decreased lifespan seen in the mutant flies (El-Khoury et al., 2016). This is believed to be due to decreasing ROS production, where ETC deficiency has previously been shown to increase Amyloid- β production in cells, and been linked to Alzheimer's disease progression (Du et al., 2010; Onyango, Dennis, & Khan, 2016).

There is now an increasing weight of data identifying the alternative oxidase as a potential therapeutic in a wide range of disease models. There is scope for the AOX protein to be used to alleviate mitochondrial dysfunction, particularly stemming from CIII and CIV, but also in disparate cases of toxicity induced by ROS. Currently all of the ectopic expression on AOX proteins has been carried out using the *C. intestinalis* version, which at the time of choosing was one of only four putative AOX genes identified. At the outset of this project, the last attempt to identify AOX genes in the *Animalia* kingdom was in 2009 (Allison E. McDonald et al., 2009) identifying 28 species in total. Given the rate at which new sequence data becomes available from a vast array of species, it was thought prescient to re-examine the distribution of AOX genes in these newly sequenced species. Furthermore, as there is currently only a limited number of AOX genes used ectopically, the possibility of identifying a novel AOX for use as a therapeutic was also investigated, with selection based on a consideration of the host species. Once identified, this novel AOX was investigated for expression and functionality *in vitro* and was tested in chemically induced models of mitochondrial deficiency

5.2. Results

5.2.1. Alternative Oxidase Sequence Analysis and Cloning

As detailed in the introduction to this chapter, the alternative oxidase of choice for the last number of years has been that of the sea squirt *Ciona intestinalis*. Interest in AOX proteins derives particularly from their candidature as a bypass of complex III and complex IV and thereby potentially acting as a novel therapeutic entity for mitochondrial deficiencies. The ectopic expression of the *C. intestinalis* AOX (referred to from here-on as CiAOX) has been used in a number of studies with varying degrees of success. It has been shown to ameliorate CIV deficiency and even lessen Amyloid-Beta accumulation (El-Khoury et al., 2016, 2014). In contrast, a mouse constitutively expressing CiAOX shows little physical difference to wild-type mice (Szibor et al., 2017) but has worsened mitochondrial complex IV deficiencies in COX15 knockout mice causing increased rates of mortality (Dogan et al., 2018b) and reduced fertility in *Drosophila* (Saari et al., 2017). With reference to the COX15 CIV deficient mouse, these results have been put down to CiAOX working too effectively at removing the blockade at CIV leading to extreme dysregulation of redox signalling in the cell. This is similar to the reason why previous attempts at using plant AOX enzymes had in human cells led to uncontrolled lethality, they may in essence be too active (Hakkaart et al., 2006). This led us to wonder if any other animal species may have an AOX gene that may have been more suited for a biology more similar to ours. Previous work has been undertaken to examine the extent of AOX genes across the animal kingdom (A. E. McDonald, Vanlerberghe, & Staples, 2009), however these data are now 10 years old. With the deluge of animal sequences now available we decided to re-examine this question, with an eye on new potential AOX's for potential use in gene therapy.

Using the Basic Local Alignment Search Tool (BLAST) we queried available sequence data with a number of established full length AOX protein sequences such as those from *C. intestinalis* and *A. thaliana*. As expected, *C. intestinalis* better predicted animal species and was used for further

analysis. Using this method, we identified 27 species (see Appendix 7.6) that have predicted AOX genes within the *Animalia* kingdom after exclusion of previously documented AOX genes. All of these sequences were predicted AOX's via NCBI's Gnomon tool. Gnomon is a eukaryotic gene prediction tool that automatically scans genome sequence data for similarities to known eukaryotic genes and tags them as potential homologs.

Of the 25 animal species identified with these predicted AOX genes, 17 were Bilateria, including 2 Chordata members, *C. intestinalis* being one. Out of all AOX containing species, this would represent the branch with the most recent Last Common Ancestor (LCA) to *H. sapiens* (see Figure 5-2A for a taxonomic tree of all species identified to contain predicted AOX sequences in this study. This is based on the NCBI taxonomic tree builder, and not on sequence data directly (see appendix section 7.6 for a table of all the species identified in this study). If, however, we examine the sequence similarities of these putative AOX genes we find a different relationship between the sequences. In Figure 5-2B we have a tree of pairwise sequence similarity comparisons. From this we can see that we lose the taxonomic distribution of the AOX genes. If we look at the other species within the Chordata, boxed in blue, we see that they are now arranged considerably more distantly from each other than when solely looking at taxonomic data. It appears that sequence conservation is not high within this taxonomic clade. If this is the case, there is not necessarily any benefit to choosing an AOX sequence from an animal with a more recent LCA. As such we investigated other methods of selecting a candidate, namely environmental selection. The species denoted with an asterisk will be mentioned in the discussion.

These species span a wide range of phyla across the Metazoa but there are some interesting commonalities amongst their habitats. All of the species identified here (and in previous studies of animal AOX genes (A. E. McDonald et al., 2009)) live in aquatic habitats and the vast majority of them live in marine environments. Animals with AOX sequences also tend to be sedentary,

like *C. intestinalis*, a sea squirt that attaches itself to driftwood and other debris found in our oceans or a number of corals such as *O. faveolata*, *A. digitifera* and *S. pistillata*.

The only two animals on the list that were not marine animals were also not sedentary, these were *P. canaliculate* and *B. glabrata*, both of which are freshwater snails, part of the Mollusca family. Although perhaps not the most well-known of snails, they have both been the subject of recent *Nature* papers (Adema et al., 2017; Q.-Q. Yang, Liu, He, & Yu, 2018). They are both air-breathing and can at least live parts of their life terrestrially. *B. glabrata* was chosen for further molecular characterisation out of the two as at the time of starting this project there was more data available about the species. The *B. glabrata* snail has been of interest to biologists for some time now as it is a host for *Schistosoma mansoni* which causes schistosomiasis in humans, the helminth disease with the highest rates of morbidity and mortality (Bridger, Brindley, & Knight, 2018), but until now there had been no study of its predicted AOX gene, referred to as BgAOX from here on.

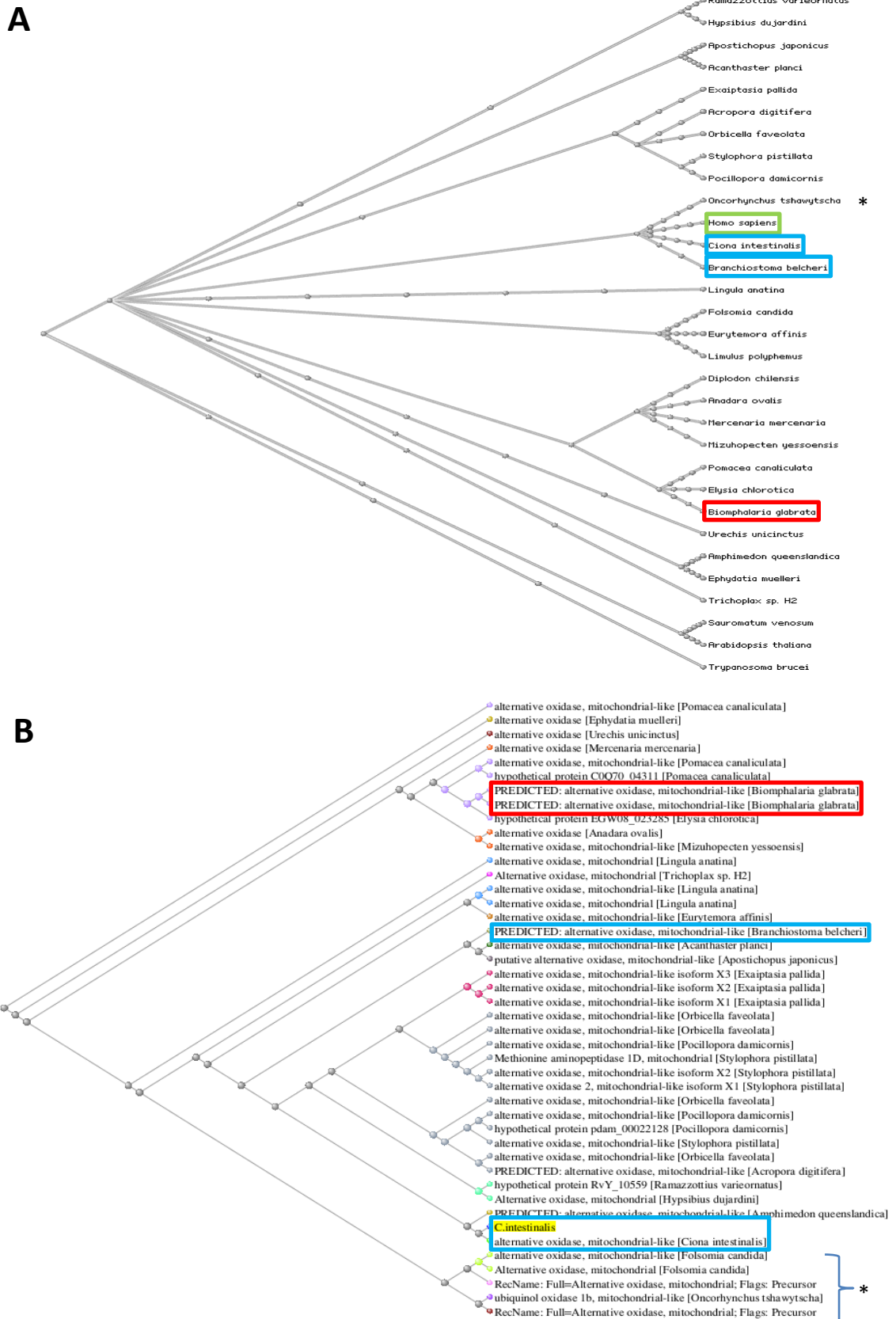


Figure 5-2: Slanted cladograms of A) species containing predicted AOX sequences and B) the sequence similarity of these predicted AOX sequences. A BLAST search of the *C. intestinalis* AOX sequence was run to identify novel predicted AOX genes. The blue box highlights *C. intestinalis* and the other species in the same taxon as it. *B. glabrata* is shown in the red box and the green box in A) shows the location of *H. sapiens*. The species denoted with an * will be dealt with in the discussion.

CiAOX is 369 amino acids long and BgAOX is 340. Using the EMBOSS Water tool hosted by EMBL, the protein sequences have only 45.3% identity and 64.8% similarity (compared to 49.4% identity and also 64.8% similarity for CiAOX and its taxonic sister, *B. belcheri*). Very intricate work has been undertaken previously detailing the highly conserved amino acid residues found in all active AOX proteins (Moore et al., 2013). Using these data, I cross-referenced the highly conserved amino acids with those in the BgAOX sequence (Figure 5-3). All of the functionally important residues are completely conserved across the known, functionally active, AOX enzymes, as well as in BgAOX. This is quite remarkable considering the high level of divergence seen in the N-terminus sequence.

In order for the putative BgAOX to be functional, the protein needs to be imported to the mitochondria. Mitochondrial Targeting Sequences (MTS) are located at the N-terminus, which is the variable region of these AOX protein sequences. We carried out an *in silico* analysis of the likelihood of mitochondrial import of BgAOX using two mitochondrial localisation predictor tools, TargetP 1.1 (Emanuelsson, Nielsen, Brunak, & von Heijne, 2000) and TPpred2 (Savojardo, Martelli, Fariselli, & Casadio, 2014). We know CiAOX is transported to the mitochondria from previous studies of the protein (Hakkaart et al., 2006), so its sequence was used as a positive control for these software. TargetP 1.1 gave CiAOX a probability of .716 of mitochondrial import, and TPpred2 predicted the MTS cleavage site to be between 21 and 57 residues into the sequence. TargetP 1.1 gave BgAOX a probability of .807 of mitochondrial import, and TPpred2 predicted the MTS cleavage site to be between the 57 and 61 residues of the protein. For comparison, TargetP 1.1 gives β -actin a probability of .089 of being mitochondrially targeted, and TPpred2 detected no MTS.

A.thaliana	-----0
S.venosum	-----MMSSRLVGTALCRQLSHVPVPQYLP25
T.brucei	-----MFRNH-----ASRITAA-----12
C.intestinalis	-----MLSTGSKTFL-----FRPFLGSCHALQSGKLPCSNLHTTPTKITVKRYLV45
U.unicinctus	MMARVTVRLLAKDSHTILSQAVRQMIPYAASHNALITSMPQ-----42
B.glabrata	-MNRVSI-----IRGFTKFG-SSNSIASLLPS-----25
A.thaliana	-----LG-2
S.venosum	ALRPTADTASSLLHGCSAAAPAQ-----RAGLWPPSWFSP60
T.brucei	AAPW-----VLRTACRQKS-----DAKTPVWGHTQLNR40
C.intestinalis	GYSWSTQPHSRLHSCQQLKIDDKNK-SEHF---KI---ETND---STDEPNIEVENFPH95
U.unicinctus	VYAYSTQTRSL-----NNKAKESVNLGP-HIQ-ENLKKFREGSHENVSPPEELQH90
B.glabrata	TFNTHKNGKLL---LCQRTGFRTSFIESYQFVVVQIRGIADDKQTETKYDKLPNPSDLAH82
:	
A.thaliana	--EKTPMKEEDANQKKTENESTGGDAAGGN--NKGDKGIASYWGVEPNKITKEDGSEWKW58
S.venosum	PRHASTLSAPAQDGGKEKAAGTAGKVPPGEDGGAKEAVVSYWAVPPSKVSKEDGSEWRW120
T.brucei	LSF-----LETVPVW---PLRVSEDESERPTWS-66
C.intestinalis	FREAKKAKET-----QKGSSLAEE---AEEHPDVEEGRAMQDGGYRLPHPIWH-139
U.unicinctus	FRKSTEEGV-----KNGGPEEEKPPMGAVALPHPIWS-122
B.glabrata	FRSSSSPPV-----KKDDKEKDNPPIGMYAMPHPPIWS-114
*	
A.thaliana	NCFRPWETYKADITIDLKHHVPTTFLDRIAYWTVKSLRWPTDLFFQRRY-----108
S.venosum	TCFRPWETYQADLSIDLHKKHVPPTILDKLALRTVKALRWPTDIFQRRY-----170
T.brucei	-----LPDIENVAITHKKPNGLVDTLAYRSVRTCRWLFDTFSLYRF---GSITES113
C.intestinalis	-----KQELESVRISHRPPVGKVDKLAYYSVQLLRTGDFVFSGYTLGTYTGRLEDEK190
U.unicinctus	-----EEELHSVHVTHRNPEGIVDKIAYMGVKFTRGCYDFVSGYSR---GRQDEK169
B.glabrata	-----EDQLHNVEITHKVPGEIIDLKAYKTQVILRSADFLLSGFKH---GETNEK161
:: *: * *: * *: * * .	
A.thaliana	--GCRAMMLETVAVPGMVGGMLLHCKSLRFEQSGGWIKALLEAEANERMHMLTMFMEVA166
S.venosum	--ACRAMMLETVAVPGMVGGVLLHLKSLRFEHSGGWIRALLEAEANERMHMLTMFMEVA228
T.brucei	KVISRCLFLETVAGVPGMVGGMLRLHSSLYMTRDKGWINTLLEAEANERMHMLTMFIELR173
C.intestinalis	QWVKRIIFLETVAGVPGMVGMVRLVSLRLKRDHGWHTLLEAEANERMHMLTMAMRIA250
U.unicinctus	MWVSRLCFLETVAGVPGMVAAMVRLTSLRKMRDHGWHTLLEAEANERMHMLMVLQKLK229
B.glabrata	KWINRICFLETVAGVPGMVAAMMRHMRSLRMQRDQGWHTLLEAEANERMHMLLTALQLK221
* :***:*.*****.: : * *** : :. ***.:** *****.: ::	
A.thaliana	KPKWYERALVITVQGVFFNAYFLGYLISPKFAHRMVGYLEEEAIHSYTEFLKELDKGNIE226
S.venosum	QPRWYERALVLAVQGVFFNAYFLGYLLSPKFAHRVVGYLEEEAIHSYTEFLKDIDSGAIQ288
T.brucei	QPLPLRVSIITQAIMYFLLLVAYVISPRFVHRFVGYLEEEAVITYTGMRAIDEGRLR233
C.intestinalis	NPGIIMRTSIVVAQGIFVSGFSLAYLISPRFCHRFVGYLEEEAVKTYTHCLEELDSGNLK310
U.unicinctus	QPSLFFRLGVMVTQGVFVSGFSVAYMLSPRLCHRFVGYLEEEAVITYTKLLKEIDSGAMQ289
B.glabrata	QPSLLFRLCVIGSQGFFVTWFGISYILSPKYCHRFVGYLEEEAVKTYTNCLKDIEQGNMK281
:* * : : *.: : .:*:***: **.******: **: .: :.* ::	
A.thaliana	---NVPAPAIIDYWRLPADATLRDVMVVRADAEAHHRDVNHFASDIHQRELKEAPAP283
S.venosum	---DCPAPAIALDYWRLPQGSTLRDVVTVVADAEAHHRDVNHFASDVHYQDLELKTTPAP345
T.brucei	PT-KNDVPEVARVYNLSKNATFRDLINVIADAEAHHRVNVHTFADMHEKRLQNSVNPV292
C.intestinalis	MWCRMKAPEIAVEYWKLPDDAMMRDVLAIADAEAHHRSVNHLGSRKPDEQ---NPYP366
U.unicinctus	HWNTLPGPDVAISYWKLRPGAAMKDVLAIADAEAHHRVNVHLSLKDIDDY----NPYK345
B.glabrata	HWKTQPAPDVAVRYWKLPQDATMKDVLAIADAEAHHRVNVHCLASLRKDDF----NPYK337
* :* **.* .: :*: :. :*****.* ** .. : . *	
A.thaliana	IGYH-----287
S.venosum	LGYH-----349
T.brucei	VLKKNPEEMYSNQPSGKTRTDFGSEGAKTASNVNKHV329
C.intestinalis	PGQ-----369
U.unicinctus	PGQ-----348
B.glabrata	PGH-----340

Figure 5-3: Clustal OMEGA alignment of known AOX sequences with that of BgAOX. Functionally important residues as per Moore et al., 2013 are coloured in blue. These residues are completely conserved across all AOXs tested, including the novel BgAOX.

5.2.2. AOX cloning and expression analysis

After the *in silico* analysis of BgAOX, there was deemed enough potential for this candidate AOX to evaluate the sequence *in vitro*. The BgAOX gene sequence was ordered from Integrated DNA Technologies (IDT), as well as the CiAOX sequence to act as positive control. As there are no commercially available antibodies for either AOX we added a HIS tag to the 3' end of each sequence, just before the stop codon. Once received, the genes were cloned into the pAAV-CMV vector (see methods section 2.1 for plasmid and cloning details, full sequences for the two genes can be found in the appendix) for expression in mammalian cells.

Upon transiently transfecting the two plasmids into HEK293 cells, RNA and protein were extracted for further analysis. Robust expression was seen at the RNA level, after being normalised to HRPT RNA expression levels (Figure 5-4A) (Student's t-test, $n = 3$, $p > .05$).

Western blot analysis of HEK293 cells transiently transfected with CiAOX and BgAOX can be seen in Figure 5-4B and C. In 3B we can see blots probed with HIS tag antibody (ab9108, abcam) to show AOX expression and β -actin antibody (A5316, Sigma-Aldrich). The predicted molecular weight of CiAOX is 42.2kDa and of BgAOX is 39.2kDa (ExPASy webtool⁹). The two protein bands that correspond to the AOX proteins are of lower molecular weight than expected compared to the protein marker (P7712, NEB), additionally β -Actin is at 42kDa as expected (see Figure 5-4B). The lower size of the AOX bands is most likely due to the cleavage of the MTS from the mature polypeptide after its import to the mitochondrion. Figure 5-4C shows a Western without β -actin staining and we can see that there is a faint band above both the CiAOX and BgAOX bands (grey arrowhead), which would correspond to non-processed AOX protein. Using the estimated values for the MTSs of the two proteins obtained from the *in silico* analysis, we estimate processed weights of ~32kDa for BgAOX and ~36kDa for CiAOX, which match with the bands

⁹ https://web.expasy.org/compute_pi/

seen in the Western analysis. Protein expression levels appear to be broadly similar between the two constructs, when compared to β -actin expression levels.

To ensure that BgAOX was in fact targeted to the mitochondria I performed a colocalization study. HEK293 cells were transfected with either of the two AOXs as well as mito-dsRed, a mitochondrially targeted red fluorescent protein. Representative images depicting mitochondrial localisation of both AOX proteins are provided in Figure 5-4. In Figure 5-4D and E, the first panel corresponds to mito-dsRed signal showing clear sub cellular compartmentalisation. The last panel shows AOX protein using the HIS tag antibody with a green secondary antibody (Alexa Fluor 488, Thermo Fisher; Z25302; section 2.5.1 of Methods). In the merge of the two images in the centre panel we can see that there is clear colocalization of the two proteins, the mito-dsRed and AOX. All cells that were positive for both mito-dsRed signal and AOX protein demonstrated colocalization, over multiple repeats. Furthermore, cells not expressing AOX showed no staining for the anti-HIS antibody. In combination these data demonstrate that BgAOX can be ectopically expressed, processed and imported into the mitochondria.

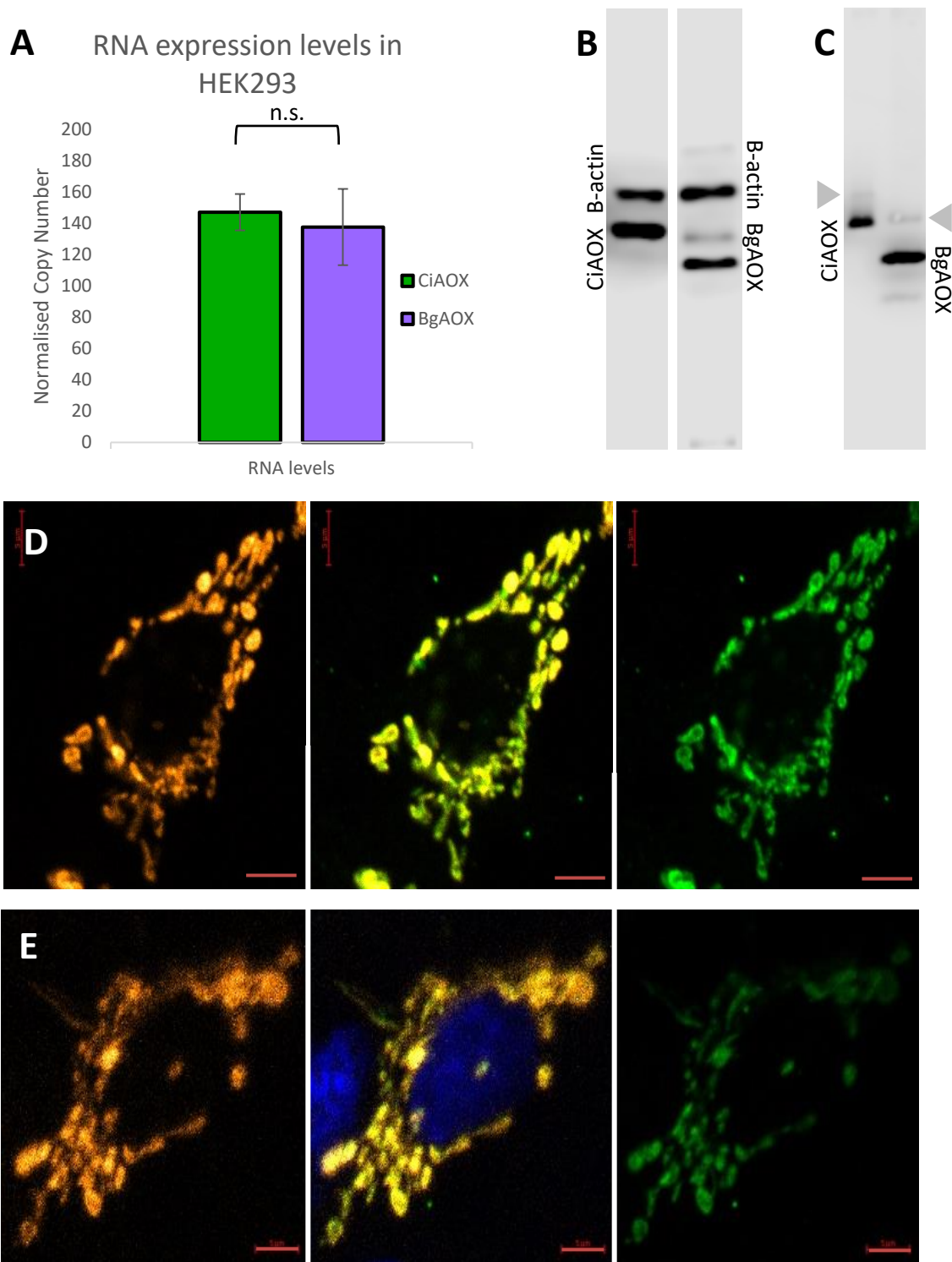


Figure 5-4: Expression analysis of CiAOX and BgAOX using transiently transfected HEK293 cells. A) RT-PCR analysis of transcript levels showing mRNA copy number normalised to HPRT mRNA levels (n.s.= no significance). B) Western blot analysis CiAOX and BgAOX, using anti HIS and anti β -actin. Protein expression levels seem broadly similar between the two plasmids. C) Western without β -actin antibody, grey arrows denote uncleaved protein before its import to the mitochondria. D) Localisation study in HEK293 cells transfected with BgAOX and mito-dsRed and E) with CiAOX and mito-dsRed. The first panel shows mito-dsRed signal, the third panel shows HIS-AOX signal. The middle image shows a merge of the two. All scale bars = 5 μ m.

5.2.3. CiAOX and BgAOX are both functionally active in mammalian cells

Being targeted to the mitochondria is one thing but demonstrating that the protein is functional is another. Exploring this aspect of BgAOX proved to be much more complicated than originally anticipated. Initially I had hoped to assay the enzymatic activity of the AOXs spectrophotometrically by developing an assay similar to that employed in the Ndi1 study in chapter 3 of this thesis, using isolated mitochondria. However, this was found to have limited success. Traditional assays for CIII activity, where an AOX protein would join the ETC, measure the change in absorbance of cytochrome c after it is reduced by CIII (eg. (Spinazzi et al., 2012)). This would not work for assaying AOX activity as they just oxidise ubiquinone and transfer these electrons to form O₂. I attempted to monitor the oxidation of ubiquinone, the electron carrier that is reduced by CI (or by Ndi1 for that matter) at 290nm, where the absorbance spectra of the reduced and oxidised forms of ubiquinone differ, using specially constructed UV cuvettes. This assay worked for CiAOX, giving a reproducible and measurable rate of activity, but the rate was lower for BgAOX even when a large quantity of sample was used (See Appendix 7.7 for method and some sample traces). As a result, this assay was deemed not to be sensitive enough to show any activity in the BgAOX samples above background.

This initial lack of observable AOX activity appears to be due to the Crabtree Effect. Normally associated with cancer where it is known as the Warburg effect, it was in fact initially discovered when, in the presence of high glucose levels, *S. cerevisiae* use less efficient glycolysis to produce ATP even when aerobic respiration would presumably be more productive. Very recently an interesting paper has sought to answer why this seemingly paradoxical shift occurs (Niebel et al., 2019). Essentially there is a limit to the amount of Gibbs free energy a cell can maintain before it is physiologically detrimental. This means that as the environment gets more energy rich, cells cannot just keep scaling-up OXPHOS to utilise all of this excess in metabolites to maximise growth rate, they would exceed this Gibbs free energy limit. Instead they modulate

their metabolism towards glycolysis to produce maximal levels of ATP whilst remaining under this Gibbs free energy limit. As mentioned, this has been noted in cancer, and it also holds to be the case for some immortalised cell lines such as the HEK293 cells being used in the current study. Essentially what this means is that cell lines such as HEK293 and HeLa do not use their mitochondria to much extent unless forced to do so, making measurements of subtle changes in mitochondrial dynamics more difficult to observe. This is in contrast to the studies in the OPA1 chapter of the thesis, where mitochondrial dysfunction caused the cell to rely upon glycolysis for energy production. HEK293 cells do not have compromised mitochondria, they just do not predominantly use them.

In order to force the transfected HEK293 cells to utilise their mitochondria for energy production, cells were grown in galactose containing media. As mentioned in the OPA1 chapter, galactose containing media (free from glucose) forces cells to metabolise exogenously supplied sodium pyruvate as the primary energy source. In Figure 5-5A we can see a modified SeahorseXFe96 mitochondrial stress test (methods 2.10) analysis of cells transfected with BgAOX, CiAOX and eGFP as a control, grown in different medias.

All of the cells have a relatively consistent basal OCR (normalised to cell number), measurements 1-3. The maximal respiration however is significantly different between the AOX transfected cells and the eGFP transfected cells when grown in galactose, perhaps suggesting AOX expression effects mitochondrial respiration detrimentally at maximal respiration rates (Figure 5-5B). When we look after injection of Antimycin A, a specific inhibitor of CIII we see that it is only the BgAOX and CiAOX transfected cells grown in galactose that maintain OCR, which maintain rates at 39% and 56% of basal OCR respectively and rates that are significantly increased to the eGFP transfected control cells (Figure 4B). In contrast, this increase in OCR in both BgAOX and CiAOX transfected cells is not seen in the glucose grown AOX transfected cells.

The data demonstrate that BgAOX is capable of functioning as an alternative oxidase in a human cell line and is, in our opinion, the first demonstration of the function of BgAOX.

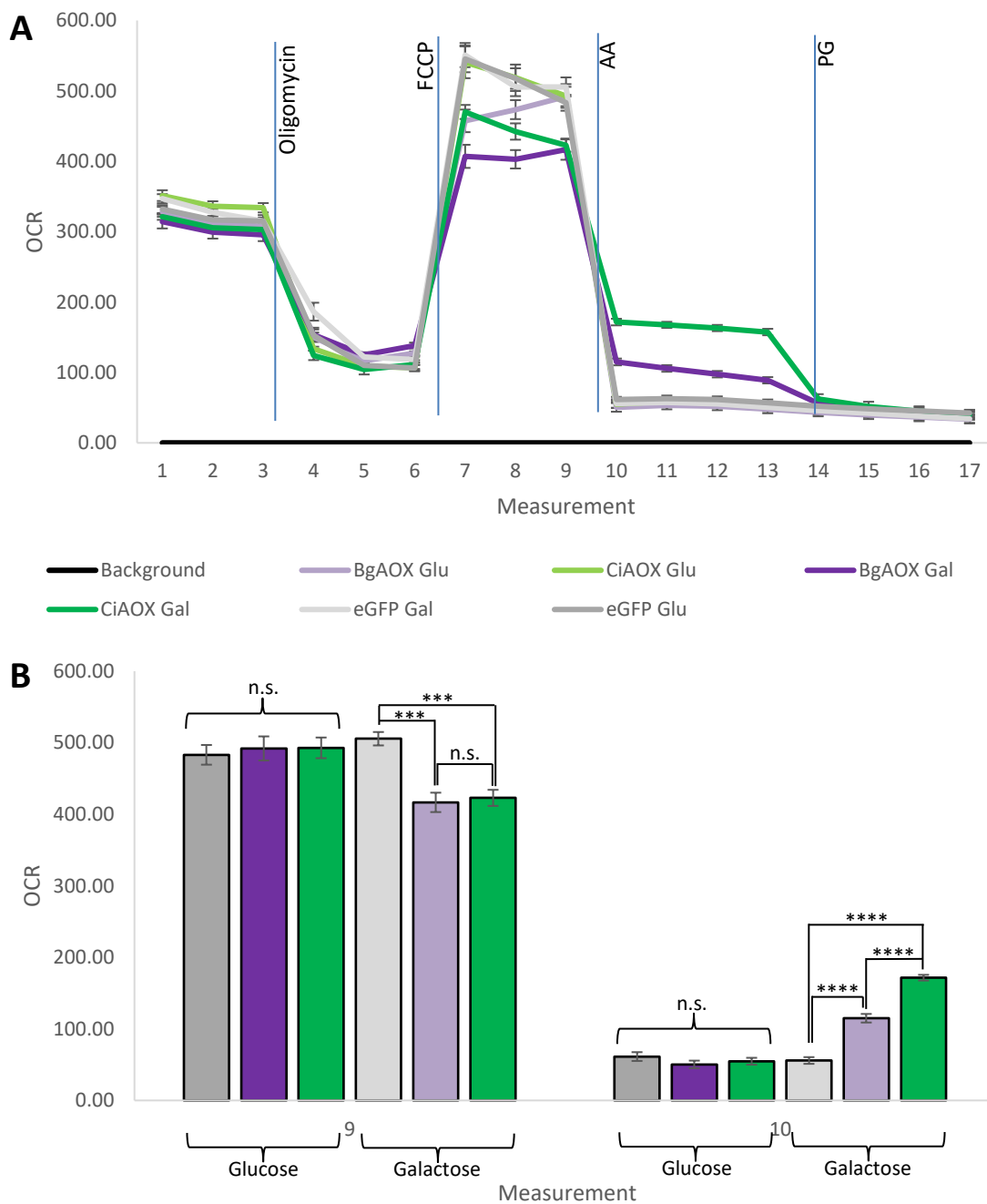


Figure 5-5: Seahorse XFe96 OCR data for HEK293 cells transfected with AOX genes and grown in galactose containing media. A) OCR of cells transfected with either eGFP, BgAOX and CiAOX then grown in either glucose or galactose media. Blue lines represent injections of different chemicals. AA = Antimycin A and PG = propyl gallate, inhibitor of AOX proteins. OCR was normalised to cell count (Error bars = $\pm$ SEM). B) Analysis of measurements 9 and 10, before and after AA inhibition of CIII. Glucose grown cells show no significant changes in OCR between treatments either before or after AA inhibition (one-way ANOVA, $p > .05$, $n=16$ wells). In comparison, growth in galactose showed a significant decrease in maximal OCR before inhibition with AA. Post AA inhibition CiAOX and BgAOX maintained significantly higher OCRs, approximately 2 and 3 fold higher than eGFP transfected cells respectively (Student's t-test, $p < .0001$, $n = 16$ wells)

5.2.4. Long term AOX expression is tolerated by cells

To investigate if expressing an alternative oxidase gene for prolonged periods of time is tolerated by HEK293 cells, we generated three cell lines stably expressing either BgAOX, CiAOX or empty pcDNA 3.1 plasmid. These cells were created by cloning the AOX genes into the pcDNA 3.1 expression plasmid under the control of the CMV promoter, similar to the process employed for the work presented in the OPA1 chapter. For details of construct generation, see section 2.1 of materials and methods. Once cloned, HEK293 cells were transfected with these AOX plasmids using the method in section 2.6 and after establishment of a kill curve for HEK293 cells, were treated with Geneticin (G418) in order to select for cells containing the plasmid.

Transformed cells were grown for multiple passages and tested positive for AOX activity using the Western blot analysis (Figure 5-6A). However, it was noticed that after multiple rounds of passaging it appeared that pcCiAOX cells were growing at a slower rate compared to pcBgAOX and pc -ve cells. This appeared to be a subtle phenotype as cells seeded the same density appeared to have the same cell number by the next split 48 hours later, but if cells were passaged multiple times using a ratio split, pcCiAOX expressing cells fell out of sync with the other two cell lines in terms of reaching confluency. This occurred regardless of media content but was most profound in galactose media. To examine this, an MTS assay was performed on cells cultured in galactose media for 96 hours, after seeding at a 1×10^4 cells per well. There was a small but significant decrease in MTS activity in pcCiAOX cells compared to pcBgAOX and pc -ve cells (Figure 5-6B). The decrease in cell growth rate in the pcCiAOX cells was deemed acceptable for continuation with other assays based on cell number as the effects were only seen after an extended period in culture.

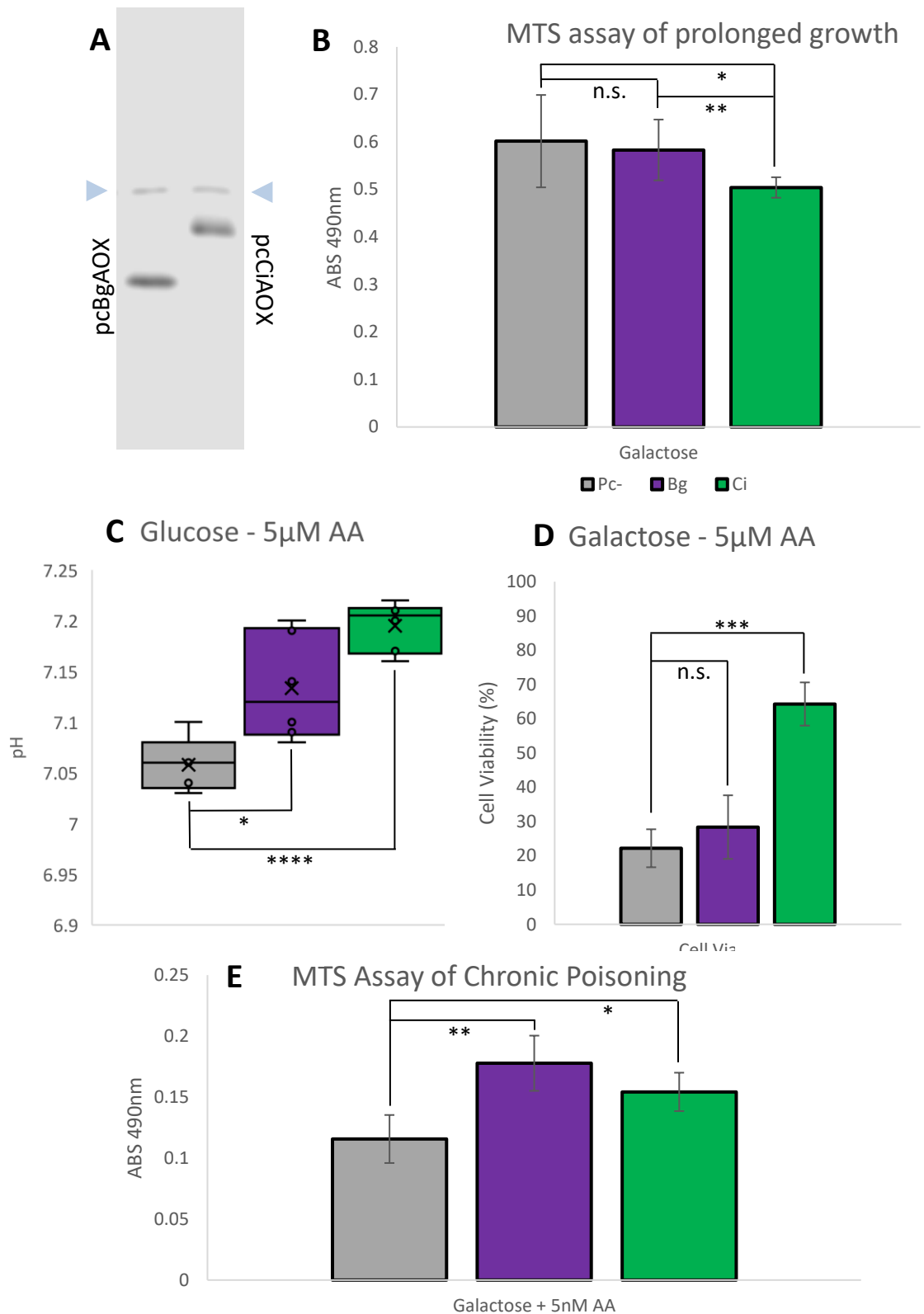


Figure 5-6: Analysis of the effect of stably expressing AOX proteins on the growth of HEK293 cells. A) Western blot of stable cell lines, blue arrow denotes β-actin. B) Prolonged growth (96 hours) in galactose shows pcCiAOX carries a small penalty on growth rate. There is a significant decrease in MTS activity levels in pcCiAOX compared to both pcBgAOX and pc -ve cells. (n = 8 wells) C) After 48 hours inhibition with 5μM AA in glucose media, cells stably expressing either pcBgAOX or pcCiAOX demonstrate higher media pH levels, indicating less reliance on glycolysis for energy production in the treated cells. (n = 6 separate treatments) D) pcCiAOX shows significantly better protection of cell viability after acute poisoning with 5μM AA in galactose media as measured by trypan-blue exclusion. (n = 4 separate treatments) E) MTS assay of chronic AA poisoning (5nM) after 48 hours of growth. Both pcBgAOX and pcCiAOX showed significant increases in MTS activity compared to pc -ve cells. The difference between pcBgAOX and pcCiAOX was approaching significance. (error bars = ±SD)

If the AOXs are active as part of the electron transport chain in these stably expressing cells, then we should be able to increase their survival after inhibiting of the ETC. Antimycin A (AA) is a specific inhibitor of CIII but does not affect the activity of AOXs, so by using this we should be able to monitor continued AOX activity.

Cells were grown in either glucose or galactose containing media then were poisoned with AA. For cells grown in Glucose there was little cell death after administration of AA regardless of how high the concentration was. For example, cells were able to tolerate 5 μ M concentration of AA without detriment. This is because, as previously mentioned, these cells can rely on glycolysis for energy metabolism. However, we can measure the change in pH as a proxy for glycolytic rate. In Figure 5-6C we can see that after 24 hours of 5 μ M AA, the media in pc -ve cells is significantly more acidic than that of either pcBgAOX or pcCiAOX, indicating a heavier reliance of these cells on glycolysis (Figure 5-6B). The presence of the AOX protein allows the other two cell lines to at least partially use the ETC for energy generation, despite the inhibition of CIII and CIV.

When cells are grown in galactose and challenged with 5 μ M there is significant cell death after 24 hours. Viability would typically be ~95% for glucose grown cells even after AA poisoning as measured by trypan-blue exclusion. Instead in galactose we had 22.2%, 28.5% and 64.3% viability for pc -ve, pcBgAOX and pcCiAOX after 24 hours with 5 μ M AA (see Figure 5-6D). No significant protection was seen when comparing pc -ve cells and pcBgAOX. The acute insult of the high AA concentration is too severe for pcBgAOX to handle, whereas pcCiAOX shows significantly better survival than either of the other two.

To model more chronic dysfunction, the AA concentration in galactose media was titrated down to the point where no significant levels of cell death were seen in pc -ve cells, but where clear growth retardation was witnessed. This was achieved with concentrations of $\leq$ 5nM AA. After 48 hours growth in galactose media with 5nM AA both pcBgAOX and pcCiAOX showed significantly

higher activity in the MTS assay ($p < .01$ and $p < .05$ respectively, $n=8$ wells), indicating higher levels of cell viability compared to pc -ve cells (Figure 5-6E). This equated to a 53.7% and 33.4% increase in cell viability for pcBgAOX and pcCiAOX respectively compared to pc -ve. The comparison between the pcBgAOX and pcCiAOX cells was approaching significance, $p = .055$. It appears that BgAOX is as capable as CiAOX at mitigating the damage done by chronic CIII inhibition, again providing evidence of BgAOX's functionality as a candidate AOX.

5.2.5. Optimisation of AOXs gives increased protein expression

As these two gene are from different species with different codon biases compared to human cells, it was investigated whether codon optimising the AOX coding sequence would yield any increase in protein expression levels. Codon optimisation of these genes was achieved using GeneArt's online optimisation tool for human cells and the optimised genes were synthesised by IDT. The optimised nucleotide sequences are both ~75% identical to the wild-type sequences. Once received, the optimised AOX genes were cloned into the pAAV-CMV expression plasmid and are referred to as optBgAOX and optCiAOX from here on. See section 2.1 for cloning methods and appendix for sequences.

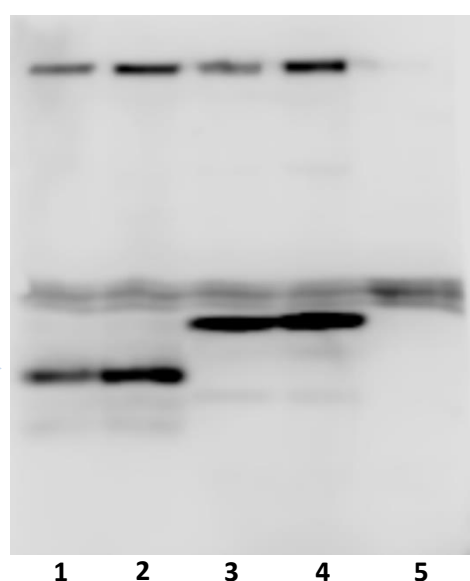


Figure 5-7: Western Blot Analysis of different AOX constructs. Lane 1 shows BgAOX, lane 2 shows optBgAOX, lane 3 shows CiAOX, lane 4 shows optCiAOX and lane 5 shows mock transfected HEK293 cells. The blue arrow indicates β -actin expression. We can see that there is a modest increase in the density of the lower, AOX bands in lanes 2 and 4 when compared to their unoptimized counterparts in lanes 1 and 3 respectively. The identity of the top most row of bands is unclear. It is quite possibly some form of AOX related product as it does not appear in the untransfected lane 5. It also appears to be denser in the two optAOX lane, 2 and 4, compared to lanes 1 and 3. It is possibly an AOX dimer.

Western Blot (4-12% gel) densitometry analysis suggested a significant increase of 1.5-fold for optBg and a 2-fold increase in protein expression when compared to the unoptimised constructs after transient transfection of plasmids in HEK293 cells, across three transfections (Figure 5-7 shows one of these, $n = 3$ separate transfections, Student's T-test $p < .05$). There was also a band of much higher molecular weight present in the transfected cells but not in untransfected HEK293 cells. The identity of the band is unclear, but as it also increased in intensity with the optimisation process it is possible it is AOX related. AOX works in dimers, it is possible that it is a dimer, however this possibility seems unlikely as the experiment was undertaken using a denaturing gel.

Despite the increase in OptAOX protein levels, there was no significant increase in the OCR of transiently transfected HEK293 cells when compared to BgAOX and CiAOX (Figure 5-8; methods 2.10), but this was in glucose grown cells. This needs to be repeated in cells grown in galactose. This suggests either the protein increase is too modest for a physiological change to be measured, or that there is a rate limiting factor other than protein levels preventing AOX activity from being increased.

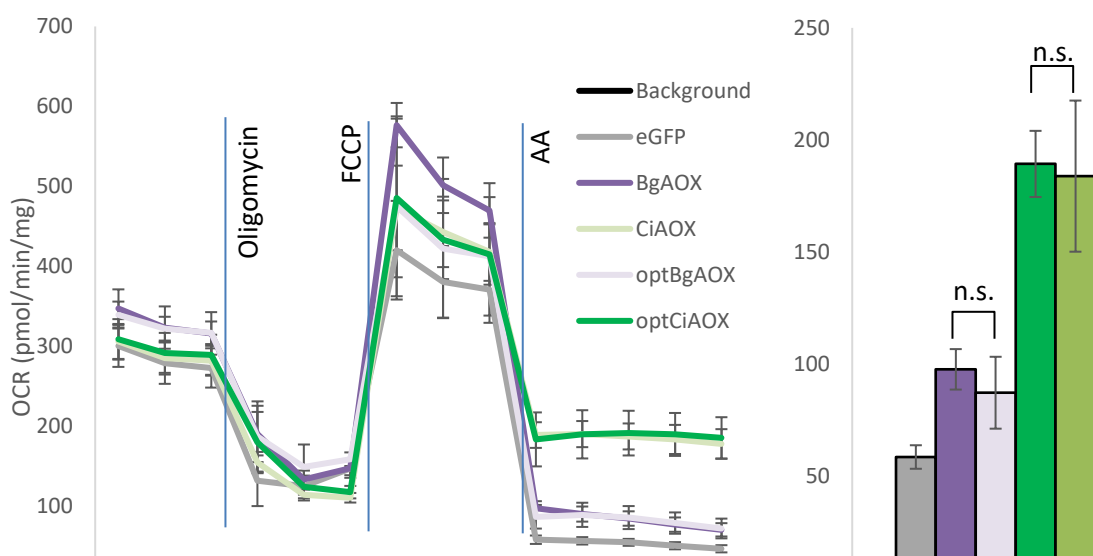


Figure 5-8: Seahorse XFe96 analysis of oxygen consumption in cells transfected with the four AOX constructs, with eGFP as control, grown in galactose. There is no significant increase in OCR between the optimised and unoptimized versions of the AOX proteins. (Student's t-test, $n=8$ wells, $p > .05$) AA = Antimycin A

5.2.6. AOX and Ndi1 expression are sufficient for oxygen consumption in $\rho 0$ cells

As alternative enzymes of the ETC, both AOXs and Ndi1 fulfil roles otherwise undertaken by CI, II, III or IV. To this end we wanted to investigate if an AOX together with our optNdi1 could potentially reconstitute an electron transport chain in cells, from the oxidation of NADH to the reduction of oxygen. In order to evaluate this, we generated a $\rho 0$ (rho zero) cell line from HEK293 cells (see section 2.6.3 of methods). $\rho 0$ cells contain no mitochondrial DNA and as a result OXPHOS in these cells is completely compromised. They do of course still contain mitochondria, as the vast majority of mitochondrial proteins are encoded for by the nuclear genome, but all of the key enzymatic proteins encoded by the mitogenome are lost and as a result they rely entirely upon glycolysis for ATP generation, with only the addition of uridine necessary to ensure their survival. This is because mitochondrial CIII activity is required for *de novo* uridine biosynthesis as the enzyme dihydroorotate dehydrogenase (DHODH) reduces ubiquinone, and if there is a blockade of ubiquinone oxidation, DHODH can no longer carry out its role in uridine biosynthesis (Löffler, Jöckel, Schuster, & Becker, 1997). This actually points at a potential utility of AOX therapy, if uridine biosynthesis is blocked by CIII dysfunction, AOX could potentially help alleviate this.

In order to produce $\rho 0$ cells, HEK293 cells were grown in the presence of ethidium bromide (EtBr) for a number of weeks (see methods 2.6.3. for further details). The EtBr preferentially intercalates with the mitogenome as it is less protected by histones and also replicated more frequently, leaving it more vulnerable to EtBr treatment. After treatment for 1 month, cells were assayed for the presence of mitochondrial DNA, proteins and activity.

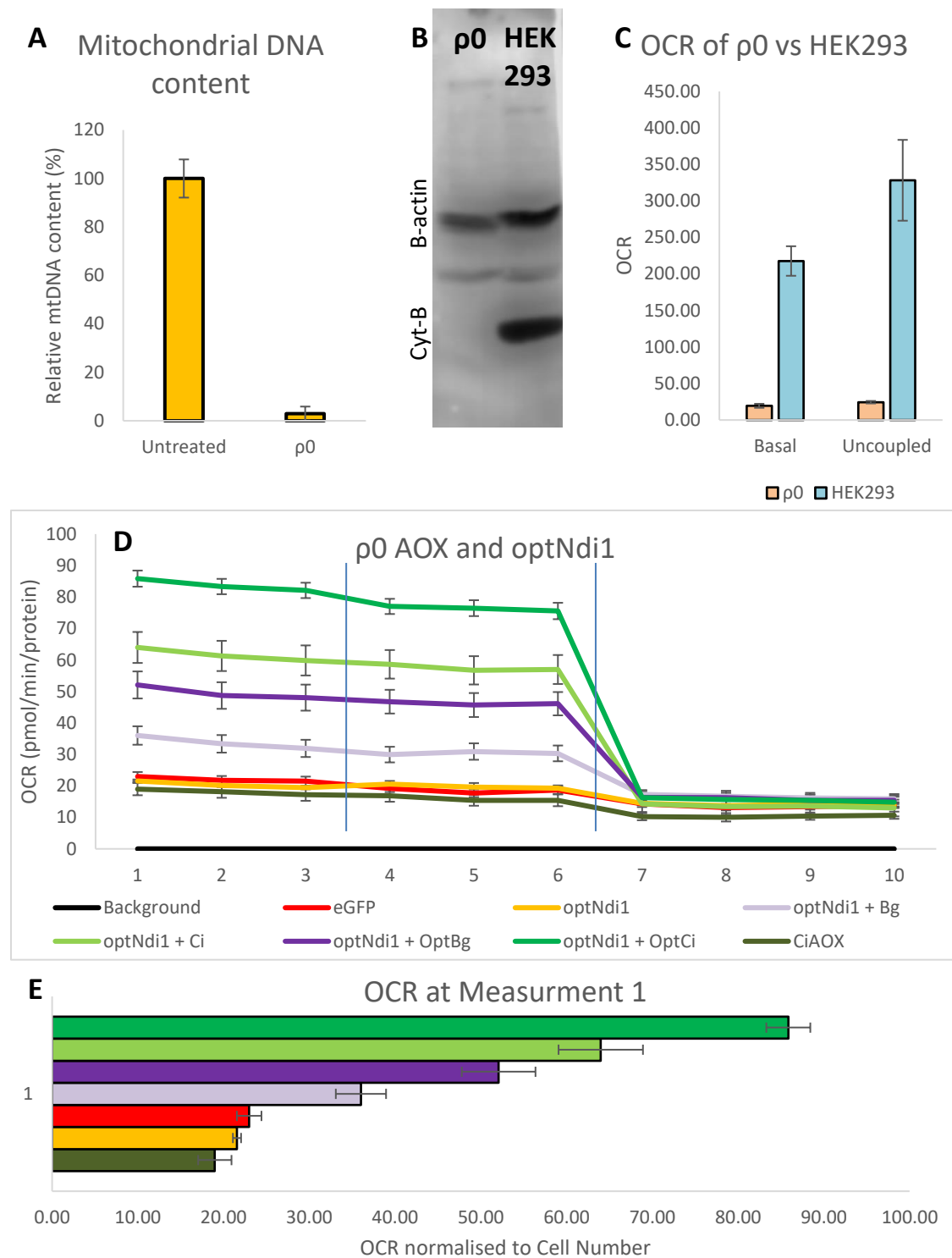


Figure 5-9: Analysis of p0 cells. A) shows real-times PCR data comparing the proportions of mtDNA using primers against tRNA-Leu in HEK293 cells to p0 cells using the B2-microglobulin gene for normalisation. There is a decrease of mtDNA in p0 cells to 3% of that of untreated cells. (n=3 repeats) B) Western blot analysis of the mitochondrially encoded Cyt-B gene comparing p0 and HEK293 cells. The upper bands are β -actin, with the lower band corresponding to Cyt-B protein. There is an absence of the Cyt-B band in p0 cells. C) Comparison of the basal and uncoupled OCR in p0 and HEK293 cells. Due to the lack of mitochondrially encoded subunits of the ETC, p0 cells show severely compromised OCR. (n=8 wells) D) Seahorse XFe96 analysis of OCR in p0 cells doubly transfected with optNdi1 and an AOX gene, with singly transfected p0 cells as controls. There was a clear rescue of OCR in doubly transfected cells. OCR was normalised to cell numbers. Blue lines correspond to injections, the first is Rot + AA and the second is Flavone + PG. (error bars = $\pm$ SEM) E) OCR measurements from time-point 1 in D). No significant increase in OCR was seen in cells singly transfected with either ciAOX, optNdi1 or eGFP (one-way ANOVA, $p > .05$) Comparisons between an AOX and its optimised version were significant (Student's t-test, $p < .01$, n=8 wells) but not between optBgAOX and CiAOX (Student's t-test, $p > .05$, n=8 wells)

Total cellular DNA was extracted from p0 cells and untreated HEK293 and subjected to real-time PCR using a set of primers for the nuclear gene B2-microglobulin and the mitochondrial tRNA gene mt-Leu. Delta-delta-ct analysis showed that there was decrease in mitochondrial DNA copy number to around 3% that of untreated HEK293 cells (Figure 5-9A), indicating a 33-fold decrease in mitogenome copy number. Western blot analysis of these cells showed an absence of Cyt-B protein expression, the mitochondrial encoded subunit of CIII (Figure 5-9B). The p0 cell line also shows severe deficiency in OCR, including basal and uncoupled OCR, when measured by seahorse XFe96 (Figure 5-9C).

In order to investigate if optNdi1 and an AOX alone can reconstitute oxygen consumption in cells, I uniformly seeded p0 cells into the assay plate, then transfected them with different combinations of optNdi1 with BgAOX, CiAOX, optBgAOX or optCiAOX plasmids and used cells singly transfected with eGFP, optNdi1 and CiAOX as controls. We can see in Figure 5-9D that each of the AOXs in combination with optNdi1 can reconstitute a quasi-ETC that can oxidise NADH and transfer those electrons to reduce oxygen. There is also clear separation in terms of efficacy of each of the different AOX constructs. The Ci AOXs are the most effective at increasing OCR, whereas the Bg AOXs are 56.3% and 60.7% as active as the equivalent unoptimized or optimised CiAOX, respectively. The blue lines in Figure 5-9D represent injection points. The first is injecting Rotenone and Antimycin A, demonstrating that the OCR is not due to residual endogenous ETC activity. This is also true for the singly transfected p0 cells. The addition of flavone and propyl-gallate, inhibitors of optNdi1 and AOXs respectively, leads to a decrease in OCR to background levels, demonstrating it is indeed the addition of these two proteins that is responsible for the increase in OCR. Comparisons between unoptimised and optimised AOXs were highly significant for both BgAOX and CiAOX (students t-test, $p < .01$, $n=8$ wells). The comparison between optBgAOX and CiAOX was not significantly different but was approaching significance (students t-test, $p < .1$, $n=8$ wells). In the singly transfected samples (eGFP, optNdi1 and CiAOX) there was no significant rescue of the OCR when comparing the three (one-way

ANOVA, $p > .05$, $n=8$ wells). It appears that the increase in protein levels seen after optimising the proteins translated to an increase in activity in p0 cells, suggesting there is another limiting factor that meant there was no observable OCR increase in HEK293 cells (contrast Figure 5-8 and Figure 5-9). It is possible that the variation seen is purely due to transfection efficiencies of these plasmids, this would need to be repeated again to ensure that this is not the case.

5.3. Discussion and Future Prospects

Here we have shown for the first time that *Biomphalaria glabrata* has a functioning alternative oxidase (AOX) gene, that this gene can function in a human cell line to mitigate CIII dysfunction and that its coding sequence can be optimised for greater expression. Furthermore, we have demonstrated that, in conjunction with optNdi1, AOX proteins can restore oxygen consumption to p0 mitochondrially deficient cells.

5.3.1. The identification of a novel animal AOX

Although initially believed to only be present in plant species and lower eukaryotes, it appears that there is quite a diversity of AOX genes in animal species too (A. E. McDonald et al., 2009; Allison E. McDonald & Gospodaryov, 2018). It is interesting to consider the presence of AOX genes as they can act as uncouplers of the ETC and ATP production. This is due to AOX proteins offering an alternative pathway for electrons to flow down the ETC; instead of ubiquinone being oxidised by CIII, with its concomitant proton pumping, AOX proteins oxidise ubiquinone and reduce oxygen directly, effectively skipping half of the ETC. This in turn prevents CIII and CIV from proton pumping, diminishing the proton gradient and decreasing the ATP production rate of the cell, a process known as mitochondrial uncoupling. Uncontrolled uncoupling would be a disadvantage and would need to be tightly regulated by a cell to avoid detrimental effects. Evidence of this can be seen in the 'uncontrolled lethality' of mammalian cells expressing plant AOX genes (Hakkaart et al., 2006).

This means in order to be a viable therapy it would be necessary that AOXs only operate under circumstances of extreme reduction of the mitochondrial ubiquinone pool, effectively only operating under circumstances of significant CIII or CIV dysfunction. Our work here increases the available roster of AOX genes from which to choose a candidate therapy from. While these initial experiments suggest that BgAOX appears to be less active than CiAOX in terms of OCR after acute Antimycin A treatment (see Figure 5-5 and Figure 5-6), however given that CiAOX

has been shown to be detrimental in certain circumstances (Dogan et al., 2018b; Saari et al., 2017), it could be that decreased, but functional, activity levels could be beneficial in CIII and CIV deficiencies. This is further reinforced by the significant levels of protection seen in pcBgAOX cells given a low, long term dose of AA Figure 5-6D, with an almost significant trend towards better protection than that provided by pcCiAOX. While many of these results are interesting, it is notable that some of the differences observed between the pcBgAOX and pcCiAOX constructs and encoded proteins may be a function of the assays employed and the quantity of transgene expression achieved and hence further experimentation with additional quantification of expression of these target proteins would be of value.

It seems as a consequence of the scientific community's efforts to sequence more and more species yields further full genome sequences, we will likely discover new animal species containing alternative oxidase proteins. It would be revealing to be able to pin point when certain phyla lost AOX genes. Currently species in the *Vertebrata* lineage, amongst others, have not been identified to contain AOX genes, suggesting an ancestral gene loss event. When I first carried out the sequence analysis of putative AOX genes, I became very excited when I saw that *Oncorhynchus tshawytscha*, the Chinook salmon, denoted with an asterisk in Figure 5-2, had been identified as containing a potential AOX gene. If it held out to be true, this would have been the first example of a member of the *Vertebrata* lineage containing an AOX gene. Further analysis revealed that the AOX sequence was in fact closely related to that of *Acidovorax*, a genus of Proteobacteria. This means that, excluding the possibility of horizontal gene transfer, the presence of this AOX sequence may in fact be due to bacterial contamination of the sample that was sequenced! Closer inspection of the tree in Figure 5-2B revealed that the outgroup highlighted by the open bracket is in fact entirely made up of bacterial contamination in the samples used for genome sequencing of these species. There is no mention of this mis-annotation of sequences in the paper discussing the *O. tshawytscha* genome (Christensen et al.,

2018). This highlights the dangers of automated computational annotation of sequence data, and sub-optimal sample sterility.

I believe that due to the lack of sequence homology between the valid *Chordata* AOX sequences, choosing an AOX gene from a more distantly related species was a valid choice. Environmental adaptation, as well as stationary or motile lifestyles would affect metabolic rates and thus lead to changes in the proteins directly involved in these processes. Without detailed knowledge of the metabolic rates of both *C. intestinalis* and *B. glabrata* it is only speculation, but it is plausible that if *B. glabrata* has higher metabolic demands it would have a less active AOX protein than that of *C. intestinalis*. There is also believed to be a role for AOX proteins in thermogenesis in plants (Moore et al., 2013). If this is also the case in animal species it is plausible that *C. intestinalis* in colder, high latitude oceans could need a more active and thus more thermogenic AOX than that of the tropically located *B. glabrata*. A number of pieces of evidence are missing which would enable confirmation of this, for example, 1) can one correlate AOX activity with the temperature of the environment the animal species lives in? And 2) are these AOX genes constitutively active or only turned on when certain triggers are met, such as excess ROS production from CIII deficiency? Further examination of the diversity of animal AOX sequences may shed some light on potential common regulatory elements. For instance there is a C-terminal amino acid sequence that is unique to animals, but its function is currently unclear (A. E. McDonald et al., 2009).

There is currently no transcriptomic data for the two species examined so the expression profile of the AOX genes is not clear. It is possible that although they are clearly functional proteins, they could be silenced by promoter mutations meaning they are not actively expressed in the organisms. Although this seems unlikely due to the evolutionary constraint on the functionally important residues of the AOX genes, seen in Figure 5-3. A further caveat to this is that we cannot definitively say that the AOX activity of these two genes is different just because of

increased CiAOX protein levels compared to BgAOX, rather than an inherent biochemical difference. The Western blot analysis of the proteins suggests there could be a slight difference in expression levels, but for the stably expressing cells protein levels seem equivalent (Figure 5-6A). The only way to truly parse this out would be to produce purified protein for the two AOXs and analyse them enzymatically.

It was also be interesting to investigate mutations of the AOX genes in these species. Is a loss-of-function of the AOX gene in these species lethal? Does it only show effects when stress is induced? There is in fact a *B. glabrata* embryonic cell line which would be a great tool for investigating the normal physiological role of the AOX proteins *in vitro* but unfortunately this line has been discontinued by ATCC (Laursen & Yoshino, 1999). In order to answer some of the more fundamental questions about AOX activity *in vivo* in these animals we currently lack the necessary tools to do so.

5.3.2. The *Biomphalaria glabrata* is functional active

In this study we have shown that in principle the putative BgAOX gene is in fact functional in mammalian cells. It is correctly localised to the mitochondria (Figure 5-4) where it is correctly processed to cleave the mitochondria targeting sequence (Figure 5-4C), in a similar fashion to the CiAOX enzyme. The BgAOX can also be functionally utilised to rescue cells in chemically induced models of CIII and CIV dysfunction (Figure 5-6). It is also possible for doubly transfected optNdi1 and an AOX protein to restore oxygen consumption to p0 cells. It is quite remarkable to think that 2 proteins can reconstitute the oxygen consumption of cells, when in wild type cells this is achieved by over 90 proteins arranged into three large protein complexes. Of course, the most significant difference between these two systems is that using the optNdi1 – AOX alternative, the ETC is non-proton motive. Although it can reduce oxygen to water like the canonical ETC, a cell cannot produce ATP aerobically using just this system. The potential utility for AOX as a candidate therapeutic comes from its ability to mitigate the damaging effects of

CIII and CIV dysfunction *in vivo*, not from its ability to replace their functionality. This makes it somewhat different to the alternative NADH:ubiquinone oxidoreductase based therapies such as Ndi1, where, assuming NADH is in excess, increased Ndi1 protein levels increase the ability of cells to shuttle electrons down the ETC, potentially increasing the rate of proton pumping from the subsequent complexes in the chain. In contrast, AOX based therapies however can only ever decrease the amount of proton pumping the remainder of the ETC can do, and as such act as uncouplers. This is why controlling AOX activity levels would be of paramount importance, and why a seemingly less active version such as BgAOX may have an advantage over the traditionally used CiAOX.

AOX proteins are believed to have a lower affinity for reduced ubiquinone than CIII does (Andjelković et al., 2016), this underpins the reason why the constitutive expression of an AOX protein is not lethal (excluding the plant AOXs with higher affinity). Again, if we assume the protein levels are not solely responsible for the differences in activity levels (Figure 5-6A), then a reduced affinity of BgAOX for reduced ubiquinone could be responsible for the change in activity levels. A lower affinity of an AOX protein for ubiquinone could be beneficial as it would effectively decrease the competition rate between the AOX and CIII, in principle enabling the uncoupling effect of AOX expression to only be seen when there are higher levels of CIII or CIV dysfunction.

Our results demonstrating a reduced growth rate of CiAOX expressing cells compared to BgAOX Figure 5-6B represent the first data to our knowledge to demonstrate a detrimental effect associated with constitutive CiAOX expression in terms of cell growth rate. The original CiAOX paper tested HEK293 cells transiently transfected with CiAOX using doxycycline inducible promoter and saw similar growth curves to wild type HEK293 cells over 192 hours (Hakkaart et al., 2006). The differentiators between that study and our study include stable expression of CiAOX and utilisation of the stronger CMV promoter but possibly most importantly the forcing

of cells to use OXPHOS via galactose media. As we also see decreased AOX activity in HEK293 cells grown in glucose Figure 5-5, it makes sense that these growth penalties are only apparent in galactose containing media. It is possible the use of HEK293 cells hides some of the metabolic consequences of AOX expression, as they have an altered metabolism compared to primary cells. Of course importantly the demonstration that CiAOX expression in a mouse model only gives “minor, biologically inconsequential effects” suggests that usable levels of CiAOX expression are achievable *in vivo* (Szibor et al., 2017).

5.3.3. The AOX protein as a therapeutic agent: looking forwards

The question remains as to what disorders AOX based therapeutics may show benefit in? If we look at the literature, previous studies have had success using AOX proteins (almost exclusively CiAOX) for mitochondrial disorders, as previously mentioned. For instance a cardiomyopathy mouse model, where an accessory subunit of CIII is mutated, mating with a CiAOX expressing mouse led to progeny with far greater survival rates than the mutated mice due to mitigation of ROS and apparent increases in CI and CII related activity (Rajendran et al., 2019). This is presumably due to the AOX dependent oxidation of the over-reduced quinone pool, allowing CI and CII to function at higher rates again. Another interesting use of AOXs shows that they can prevent lethality due to bacterial sepsis by preventing ROS production and thus preventing overzealous immune responses (Mills et al., 2016).

So far there has been no attempts made to use an AOX to investigate therapies for a primary ocular disorder or ocular phenotype associated with a syndrome. There is a scarcity of ocular specific diseases linked with deficiency in CIII or CIV. Those that are seen tend to be comparatively rare mutations in the mitochondrially encoded subunits of CIII and CIV that cause, or at least sensitise for the development of LHON (M. D. Brown et al., 1992; Kumar, Tanwar, Saxena, Sharma, & Dada, 2010; Qian, Zhou, Liang, Qu, & Guan, 2011). Rather than the localised

dysfunction seen in LHON causing Complex I disorders, Complex IV deficiency disorders tend to have multisystemic dysfunctions and are frequently associated with Leigh syndrome, which as previously mentioned is only associated with the most severe complex I mutations (DiMauro, Tanji, & Schon, 2012). Leigh syndrome does have visual dysfunction but that is only part of the multisystemic disease witnessed. It appears that CIV mutations do not have the subtlety of phenotype that allows specific organs to be affected preferentially as is seen in CI disorders. The CIII related LHON mutations could be valid targets for AOX intervention, as they are believed to be the result of CIII induced ROS production, which AOX proteins have been shown to mitigate (Bénit, Lebon, & Rustin, 2009). Likewise, the ocular disease phenotypes that are part of some multisystem disorders also represent potential therapeutic targets for AOX-based therapies. For example, a gene therapy targeted to tissue specific phenotypes associated with syndromic disease are in development for the ocular phenotype associated with MPSI (Vance et al., 2016). Likewise, Ndi1 has previously been investigated as a potential therapeutic strategy for the ocular dysfunction in MS (Talla et al., 2013).

In principle, it is possible that an AOX protein could be used in conjunction with optNdi1 as a novel therapeutic approach for mitochondrial tRNA deficiency MELAS syndrome (Mitochondrial Encephalopathy, Lactic acidosis, and Stroke-like episodes). MELAS is primarily caused by the 3243 A > G mutation (~80% of patients) which falls in the MT-TL1 gene, encoding a mitochondrial tRNA for leucine. This effectively causes a depletion of mitochondrially encoded proteins and results in multisystem failure (El-Hattab, Almannai, & Scaglia, 2018). A major clinical phenotype of MELAS is lactic acidosis, or a build-up of lactic acid in the body. This is due to the overreliance of the body on glycolysis for the production of ATP and due to the mitochondrial dysfunction underutilisation of the pyruvate that this produces, in turn leading to increased rates of lactic acid interconversion. The addition of the optNdi1 and BgAOX genes in principle should at least partially reinstate the use of the pyruvate through the reduction of NADH in the mitochondria by re-establishing an alternative electron transport chain. It is also

possible that the propping up of the ETC might allow better utilisation of the remaining mitochondrial function. We hope to test this hypothesis in patient fibroblasts in the future and are in the process of generating patient-derived cell lines for this purpose. The time limiting factor has been obtaining appropriate ethics to undertake these studies which is now in place.

I believe that the work presented in this chapter on investigating the BgAOX enzyme provides an increase to the potential of AOX-based candidate therapeutics at the disposal of researchers. If the assertion that BgAOX has different enzyme kinetics to the widely used CiAOX is further explored and validated, this in principle could enable the tailoring of these therapeutics to specific needs. Perhaps the less active BgAOX could be used successfully in milder cases of mitochondrial dysfunction. Furthermore, the AOX construct optimisation process has demonstrated further control can be exerted via the levels of protein expressed allowing variations in expression from the same expression cassette (Figure 5-9). The future investigation of different promoters, perhaps metabolic stress induced promoters and/or tissue specific promoters, could provide the specific expression of AOX proteins only when and/or where needed. Whatever the case, further research is required to fully understand how this interesting group of proteins fit into the metabolic landscape when expressed ectopically in mammalian cells.

6. General Discussion

The research presented in this PhD thesis provides significant steps forward in the search for potential mitochondrial therapeutics and should pave the way forward for their potential use in cell and or animal models of mitochondrial disorders. This work can be considered as a preliminary proof-of-concept study aimed at exploring a number of novel candidate gene therapy approaches and their effect(s) on modulating mitochondrial function and dynamics. Increasingly mitochondrial dysfunction is being implicated in an array of neurological disorders such as Alzheimer's, Parkinson's and even Huntington's disease (Arun et al., 2016), among many others. In addition to this mitochondrial dysfunction is being associated with ocular disorders outside of those with primary mitochondrial mutations (such as LHON), for instance there is a growing body of evidence associating mitochondrial dysfunction with AMD and diabetic retinopathy disease progression (Kamel, Farrell, & O'Brien, 2017). These recent findings highlight the importance of mitochondria to health in general and suggests that targeting the mitochondria in a variety of disorders could be a fruitful pursuit in terms of generating novel therapeutic approaches.

6.1.1. Ocular disorders and the mitochondria

The association of over 270 genes with inherited retinal degeneration (www.RetNet.org) demonstrates the multiple pathways that can lead to retinal dysfunction. The fact that a diverse range of 70 or so genes can all lead to Retinitis Pigmentosa for instance (Farrar et al., 2017), suggests that there are potentially some common pathways that could be targeted as a therapeutic modality.

Despite this genetic heterogeneity, there is emerging evidence of common pathways of disease in neurodegenerative disorders including IRDs despite there being different underlying genetic causes

(Luu & Palczewski, 2018; Mirzaei et al., 2017). Indeed, the immense genetic heterogeneity in Mendelian forms of ocular disease, has stimulated the ocular research community to focus on common pathways of disease between different retinal degenerations. Moreover, many of the disease mechanisms in operation in Mendelian ocular disorders are mirrored in common multifactorial conditions such as glaucoma and AMD, affecting 1-3% of people over 40 years of age and approximately 10% of people over 65 years respectively and causing a devastating and sometimes rapid vision loss. Disease mechanisms include mitochondrial dysfunction, oxidative and ER stress, the unfolded protein response (UPR), stimulation of cell death programs and inappropriate aggregation of endogenous proteins, all providing potential targets for gene-independent therapies (Athanasίου et al., 2013; Hyttinen et al., 2018; Liao et al., 2017; Puspita et al., 2017; Williams et al., 2017). Identifying the commonalities in these diseases is important to aid in the search for potential therapeutics with broad applicability. Mitochondria seem like one of the promising targets for this, considering how closely intertwined mitochondria are with an array of fundamental cell processes such as ATP production and apoptosis. The ability to modulate these processes could in principle have far reaching benefits.

However, there are currently very few therapeutic interventions open to people with either primary or secondary mitochondrial dysfunction disorders (El-Hattab et al., 2017). All mitochondrially targeted therapeutics to make it to market so far have been drug based and some of these have been dogged by inconsistent outcomes and unclear levels of efficacy. For example, idebenone, which has had its license for use revoked in Canada for Friedreich's Ataxia, a disease of the electron transport chain, due to lack of a significant benefit compared to placebo (Kearney et al., 2016). There are also concerns about the use of idebenone for treatment of LHON, with results proving to be inconsistent (Patrick Yu-Wai-Man et al., 2017). Positive work has been undertaken to try remedy the bioavailability of idebenone which could significantly improve its protective ability in patients (Montenegro et al., 2018). The number of clinical trials for drug-based interventions for mitochondrial disease is very promising (see table

1-3), especially those that act as general modifiers of mitochondrial parameters such as increasing ETC function, as there is potentially broad applicability of these therapeutics.

With respect to gene therapies targeting mitochondrial disorders, the various ND4 mitochondrial gene therapies are showing some very promising results (see table 1-4) also. The positive preliminary data for the GenSight's clinical trials with AAV-ND4 (currently in Phase III) provides a very optimistic outlook for LHON patients with ND4 mutations which given a market approval in the not too distant future, would represent another extremely positive development for the field of ocular gene therapies. Currently though this therapeutic will only be able to tackle ND4 disorders directly which, while representing a significant unmet clinical need, still leaves a lot of LHON patients to be treated. We believe that an optimal approach to surmount this is the design and implementation of gene independent therapeutic approaches.

6.1.2. A new approach: Trans-kingdom gene therapy

Trans-kingdom therapeutics are having a boon period currently with the launch of channel rhodopsin based clinical trials for late stage Retinitis Pigmentosa patients ((Macé et al., 2015) NCT02556736 and NCT03326336). These developments have opened the door for other potential trans-kingdom therapeutic approaches. The consistency of the mitochondrial genome and protein content throughout the Eukaryotes demonstrates the level of evolutionary conservation that has taken place, further highlighting the fundamental role mitochondria play in cell health. These similarities support the idea of trans-kingdom and trans-species gene therapies targeting mitochondria as a plausible approach. In this regard it is almost certain that an ancestor of humans contained both an alternative NADH:ubiquinone dehydrogenase such as Ndi1 and an alternative oxidase such as CiAOX (Allison E. McDonald & Gospodaryov, 2018) lending weight to their potential value as mitochondrial therapeutics. There is still a good deal of study required to investigate what effects the integration of alternative electron transport chain enzymes has on the myriad of processes that the mitochondria play a role in. It is notable

that ectopic expression of either Ndi1 or CiAOX proteins has been found to be well tolerated in experimental situations with the creation of constitutively expressing flies and mice living apparently normal healthy lives (A. Sanz et al., 2010; Szibor et al., 2017). However, these therapeutic strategies are also not without their potential hazards. In particular the AOX proteins will need considerable further investigation to ensure that these proteins do not cause detrimental effects to the organism, for example, in light of the contradictory results obtained in COX15 mice and cells, where cells were protected but the lethality of the mutation in mice was increased (Dassa et al., 2009; Dogan et al., 2018a). There is clearly still a lot to be understood about redox signalling and how it influences survivability of certain pathologies. It is also a stark reminder that unfortunately animal studies can be necessary to fully elucidate the impact that a potential therapeutic can have.

6.1.3. Ndi1 and optNdi1 gene therapies show promise in complex disease

The work presented in this PhD thesis provides significant progress towards better understanding some of these novel therapeutic approaches targeted to mitigate mitochondrial dysfunction. In chapter 3 of the thesis the potential of Ndi1 therapies, and its optimised version optNdi1, were investigated. There is clear utility in investigating the optimisation of trans-kingdom therapeutics for expression in other organisms, for example, employing codon optimisation for expression in mammalian cells. In this study, the significant increase in gene expression levels achieved from optNdi1 constructs compared to Ndi1 constructs both *in vitro* and *in vivo* will in principle allow the investigation of a much wider window of therapeutic dose. The increased transgene expression achieved from optNdi1 should enable lower doses of AAV to provide the same level of the Ndi1 therapeutic protein, thereby minimising potential immune responses that can be associated with AAV delivery (Reichel et al., 2017). While titre assays provide a view of the genomic titre of a batch of AAV vector, the development of the potency assay are essential to ensure that the AAV-based gene therapy is functional. In the current study a robust novel potency assay has been developed to evaluate Ndi1 function that will be of use

when comparing various modified version of the Ndi1 protein, and moreover, when comparing different AAV preparations for function/efficacy. This NADH oxidation assay has been developed using *in vitro* material from cells and works is extremely effective as a robust potency assay. We plan to try to expand this assay to also use *ex vivo* tissue to get a clearer understanding of whether the increased protein *in vivo* leads to increased activity levels associated with this novel gene therapy. With this in mind we have been testing the use of *ex vivo* tissue in the Seahorse XFe96, in particular RPE and retinal samples. Currently we have some promising data recording OCR data from RPE cells however there are still some technical limitations to overcome and hence these data were not presented in the thesis. If this can provide robust reproducible data, it will provide an unprecedented level of metabolic detail to be obtained and give insights into where Ndi1 protein is providing benefit *in vivo*.

These insights would be particularly informative for the *Cfh* ^{-/-} mouse study of AMD. The initial results summarised in this PhD thesis show promise. Given the data that is currently linking AMD to mitochondrial dysfunction (Ferrington et al., 2017; Kamel et al., 2017; Riazi-Esfahani et al., 2017), it seems plausible that mitochondrial bioenergetic modulation should be able to provide benefit in AMD. The *Cfh* ^{-/-} model is of course only one possible model of AMD disease progression, therefore further studies are of course warranted to demonstrate the broad applicability of Ndi1-based treatments in AMD. As AMD is a multifactorial disease it would be interesting to see if Ndi1 therapy could provide benefit in models of AMD that recapitulate other aspects of AMD not seen in the *Cfh* ^{-/-} model. For instance, it would be of value to use a model with choroidal neovascularisation (CNV), for example, the laser induced CNV mouse model. This is a common phenotype seen in patients and hence should be investigated to establish if Ndi1 therapies can provide beneficial effects. Additional research to establish a therapeutic window would also be required, as the optimal time for intervention could be variable between models and indeed between mouse and human. In the study presented here we are intervening before obvious defects are seen phenotypically in *Cfh* ^{-/-} mice bred in specific pathogen free facilities.

The optNdi1 therapy of course also has applications for primary complex I deficiencies. Notable examples include the primary mitochondrial disorder, LHON, for which optNdi1-based therapies are under development in the Farrar lab. The preliminary work on patient derived fibroblasts described here shows some exciting potential for AAV delivered optNdi1 to rescue oxygen consumption in cells with LHON mutations. This work needs to be expanded to include other LHON causing mutations as well as other mitochondrial biomarkers before definitive conclusions can be drawn, but acts as an exciting demonstration of the potential benefit Ndi1 based therapies can have in complex I disorders.

Of further note, the increased expression achieved with optNdi1 should allow hitherto unobtainable levels of therapeutic intervention and could warrant reinvestigation of Ndi1 as a therapeutic strategy for diseases such as Multiple Sclerosis (MS) that has been identified as having a complex I mitochondrial deficiency (Paraboschi et al., 2015), and moreover where Ndi1 (non-optimised) has previously shown some benefit in tackling the ocular phenotype in the EAE model of MS (Talla et al., 2013). It is possible the increased potency of optNdi1 could allow more widespread systemic delivery of effect therapeutic doses. It is planned in the future to explore the therapeutic potential of optNdi1 to modulate some of these complex I deficiency disorders, particularly in LHON patient derived fibroblasts.

6.1.4. OPA1 based gene therapy shows potential to rescue mitochondrial deficits but needs tight control of expression

In chapter 4 of the PhD thesis, the development of two OPA1 isoforms as potential therapeutics was investigated. This work focused on multiple aspects of mitochondrial bioenergetics using an array of different methodologies. Initially two OPA1 isoforms were codon optimised and their mitochondrial localisation confirmed post optimisation. Provision of these two OPA1 isoforms, OPAiso1 and OPAiso7 to OPA1^{-/-} MEF cells, showed significant improvement when compared to control OPA^{-/-} cells in all of the parameters investigated including rescue of mitochondrial

morphology. This also extended to restoration of basal OCR to wild-type MEF levels. There still remains a significant difference in the OPAiso1 and 7 treated OPA1-/- cell's spare respiratory capacity compared to wild type MEFs, however, this still represented a significant increase over the OPA1 -/- MEF cells. Given that OPA1 mutations are involved in 60-90% of DOA cases, these findings represent a positive step towards the development of a therapeutic strategy for DOA.

It seems likely that an important hurdle to overcome for OPA1-based therapies is that of the over expression phenotype that can be seen in cells in this study and others previously (Civiletto et al., 2015b). This overexpression did not appear toxic to cells and was possibly even protective against apoptosis in this study, however the lack of improvement in OCR of cells transiently expressing either OPA isoform (when the largest accumulation of the overexpression phenotype was observed) suggests that these cells were not metabolically active. This is a concern for the delivery of a potential therapeutic. The survivability of cells is not of benefit if they exist as somewhat of a zombie population, unable to carry out their normal physiological roles. This may possibly why there was only limited rescue of visual function in DOA mice injected with AAV2-CMV-OPA1 even though there was a statistically significant increase in RGC numbers (Sarzi et al., 2018). It may be that the therapeutic could be protecting the cells from OPA1 deficient apoptosis, but not rescuing the cellular functionality. In contrast, in the study described in Chapter 4 using OPA1 -/- MEFs a clear increase in metabolic fitness associated with delivery of both OPA1 isoforms was observed. The differences between the outcome of delivery of OPA1 therapies in the *in vitro* study in this PhD thesis and the recent *in vivo* study by Sarzi et al. 2018 warrants further investigation.

Two approaches for optimising OPA1-based therapies for *in vivo* utility could be investigated. Firstly, it might be possible to utilise the endogenous OPA1 promoter to achieve optimal regulatory control of OPA1 gene therapeutics. The OPA1 promoter in principle could be identified and the promoter sequence cloned for use in an OPA1 gene therapy vector.

Alternatively, a CRISPR-based genome editing strategy could be adopted. For example, using the recently developed Tild-CRISPR, the authors claim to be able to successfully insert up to a 6kb transgene at a location of your choosing (Yao et al., 2018). As the OPA1 iso1 and 7 coding sequence is just over 3kb, CRISPR could potentially be used to hijack the endogenous OPA1 promoter by inserting the transgene just 5' of the endogenous OPA1 transcriptional start site.

Secondly, it may be worth investigating one of the OPA1 isoforms that only produces short-forms of OPA1 protein as a therapeutic approach. Investigations of the functionality of the OPA1 isoforms suggested that whilst not able to restore mitochondrial fusion without the long OPA1 isoforms present, the short isoforms (s-form) were able to restore mtDNA levels and super-complex stabilisation (Del Dotto et al., 2017). It is possible that provision of the s-form could circumvent the need for fine tuning of the delivered dose of OPA1 protein, there is no data currently for whether an overexpression phenotype is witnessed when an s-form is overexpressed.

Although there is clear a need for OPA1 therapy directly for DOA, as OPA1 is the causative gene in 60-90% of cases, there is emerging evidence that OPA1 is cytoprotective and it too may be a novel gene independent therapeutic modality. For instance, the use of AAV2-OPA1 in a laser-induced model of glaucoma (Hu et al., 2018) to protect RGCs has been recently demonstrated. This study showed significant increases in RGC survival, however there were no data for visual function provided, so it is not possible to get a full appraisal of the potential benefit of the OPA1 therapy in this glaucoma model. Additionally, OPA1 expression has also provided significant protection in two mouse models of myopathic mutations (Civiletto et al., 2015a). In these mice dosage was maintained constant using the human β -actin promoter and mitochondrial morphology showed normal mitochondrial network organisation, demonstrating that safe and effective levels of OPA1 protein are possible to express.

These data begin to pave the way for the use of OPA1 ectopic expression as a potential therapeutic for mitochondrial dysfunction in multiple disease targets. In some regards OPA1 may be the therapeutic with the broadest applicability out of all those investigated as part of my PhD study, as in principle it has the ability to fine tune mitochondrial dynamics, and support cells that suffer from a range of disparate genetic abnormalities. A recent study indicating the dysregulation of OPA1 during blue light mediated RPE damage also supports the potential use of OPA1-based therapies in AMD (Alaimo et al., 2019). Furthermore, it is also plausible that OPA1 could work for other primary mitochondrial disorders such as LHON, where stabilisation of the ETC may be sufficient to overcome deficiency caused by complex I dysfunction. There is enough recent evidence emerging in support of OPA1 as a potential therapeutic for mitochondrial disorders that further investigation in model systems is warranted.

6.1.5. A novel Alternative Oxidase protein provides new therapeutic options

In chapter 5 of this thesis the alternative oxidase (AOX) is investigated as a potential strategy to bypass chemically induced complex III inhibition. The identification and subsequent testing of BgAOX is, to the best of our knowledge, the second animal AOX to be proven to be functional in ectopic expression studies, after CiAOX. Preliminary analysis undertaken in the current study suggests that BgAOX may be less active than its CiAOX counterpart, however, in depth biochemical analysis on isolated protein would be needed to fully examine the enzyme kinetics of each AOX protein. In principle, AOX proteins offer a promising therapeutic approach for the treatment of mitochondrial disorders, in particular those involving CIII and CIV deficiency.

The reconstitution of the electron transport chain (ETC) using the AOX genes and optNdi1 in combination was academically an interesting result; a reconstitution of the ETC achieved using just two trans kingdom genes when the same functions are encoded by over 90 mammalian genes. The clinical applicability of this approach is somewhat unclear as yet. It is certainly

possible that under normal physiological conditions that co-expression of both Ndi1 and an AOX simultaneously could prove detrimental to the organism, as has been found in a *Drosophila* study (A. Sanz et al., 2010), but to uncoupling of the ETC. However in the wild, organisms that typically express an AOX also express an alternative NADH:ubiquinone dehydrogenase, such as *C. intestinalis* (Allison E. McDonald & Gospodaryov, 2018), hence under certain circumstances it is clearly well tolerated by the organism. The question remains though as to how much AOX expression levels need to be regulated in order not to cause detrimental effects for the host. In this regard, promising results with a constitutively AOX expressing transgenic mouse shows that safe levels of AOX expression can be achieved *in vivo* in mammals (Szibor et al., 2017). In the current study an AOX gene from a second animal species has been shown to be functional *in vitro* thereby adding to the pantheon of candidate AOX therapeutics that are available for evaluation. This can potentially help with enabling the choice of the best AOX gene for the circumstances.

6.1.6. Future directions

The next steps for all the novel therapeutic strategies developed during the course of these PhD studies is their evaluation in various cell and or animal model systems. Our laboratory is currently extending its model systems with the addition of patient-derived cell models of disease, initially focusing on fibroblasts. At the time of writing we have recruited patients with mitochondrial mutations and have generated a number of patient and control cell lines. The assays developed during this PhD will be used to characterise the mitochondrial dysfunction present in these cell models. It is planned to expand our analysis of these innovative candidate therapeutic approaches to combat the mitochondrial dysfunction in these cell models of mitochondrial disease. The research described in this PhD thesis has relied heavily on cell-based assays using immortalised cell lines to simulate the dysfunction experienced by the patients, but as mentioned previously, these cells can be challenging to work with owing to their shifted metabolism. Putting patient derived cells through the assays developed and described in this

thesis will allow us to perform a detailed investigation of how cells that are affected by mitochondrial disorders will react to the putative therapeutics. This will be especially telling for the OPAiso1 and OPAiso7 therapeutics as they will be delivered to a cell that is haploinsufficient rather than a complete knock-out (as was the case with the OPA1^{-/-} MEFs). This type of study has yet to be undertaken in the literature and is certainly the next avenue of investigation for candidate DOA therapies.

The results from the study presented in this PhD thesis details the start of a hopefully fruitful search for novel treatments of mitochondrial disorders, where currently there is a distinct lack of treatment options. Our labs previous successes with the production and utilisation of AAV vectors to deliver transgenics (Chadderton et al., 2013; Millington-Ward et al., 2011) means that we are in a good position to implement and test these therapeutic strategies. The role of perturbations in mitochondrial function in an array of common pathologies is only just starting to come to the fore and with the candidate therapeutics outlined in this PhD thesis, along with additional therapeutic approaches being explored by other teams, hopefully there is a bright future ahead for mitochondrially targeted gene therapies.

7. Appendix

7.1.1. Construct and Plasmid Sequences

Chapter 3 sequences (Ndi)

>blue (optNdi1) -red (CMV) -green (PolyA)

```
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GAT

Yellow highlights start and stop codons, red indicates a cloning site, green highlights the minPolyA.

>Ndi1

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Chapter 4 sequences (OPA)

This is the sequence ordered from IDT. Yellow indicates start and stop codons, exons alternate in blue and black. Red indicates general restriction sites, green indicate sites used to clone in exon 5b and cyan indicates the site used to clone in the end piece. Magenta shows the HIS tag and dark green identifies the min-PolyA sequence. Dark blue indicates where the IDT sequence stopped.

>OPA1_Iso_1_HIS_minPolyA

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Sequence to ordered to clone in exon 5b to complete OPA1 isoform 7.

>huOPA1_Exon5b

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Sequence originally planned to be used for cloning out the HIS tag.

>huOPA1_seqend_noHIS

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The Sequence fragment ordered to patch up the incomplete OPAiso1 sequence.

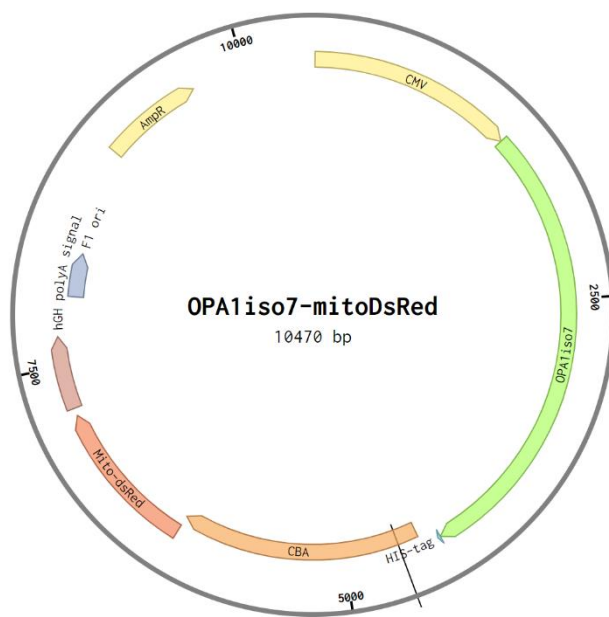
>IDT ordered fragment

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OPA1iso7 complete sequence with exon 5b in dark blue.

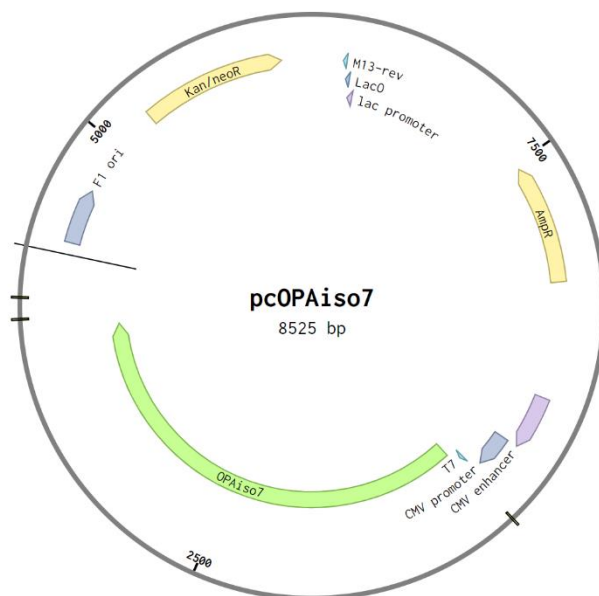
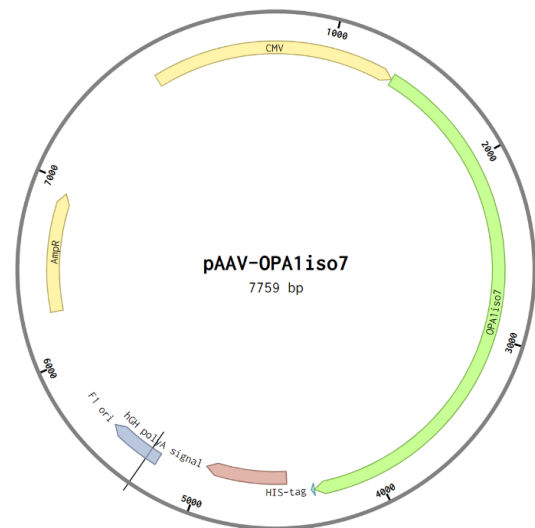
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Left shows a plasmid map for the OPAiso7-mitoDsRed dual expression plasmid. The OPAiso7 gene was cloned out to create the other dual expression plasmids.

Right shows a plasmid map for the pAAV-MCS expression plasmid. The position where OPAiso7 is situated is where the gene to be expressed was cloned in and out.



Right shows a plasmid map for the pcDNA3.1(+) expression plasmid. The position where OPAiso7 is situated is where the gene to be expressed was cloned in and out. The presence of the F1 origin and the Kan/Neo resistance gene allowed for stable expression and selection of the plasmid.

Chapter 5 sequences (AOX)

The AOX gene sequences ordered for synthesis. Red shows restriction sites, yellow shows transcription start and stop sites and green shows the minPolyA. The restriction site in blue in each sequence shows the site used during PCR cloning of the HIS tags into the sequence.

>CiMCS1

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7.1.2. OPA transient Transfection OCR

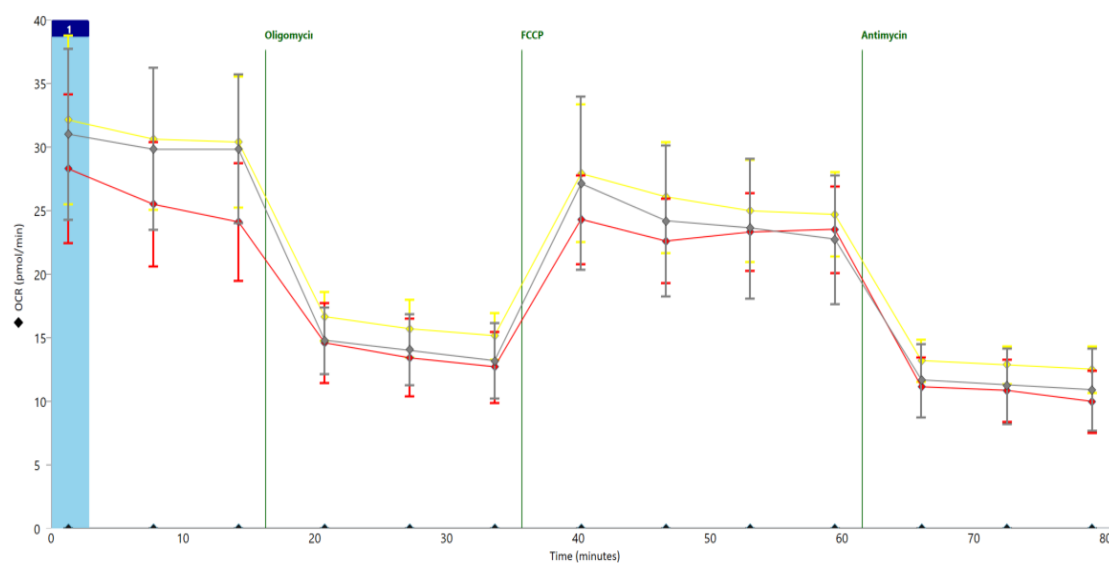


Figure 7-1: Seahorse XFe96 assay on transiently transfected OPA^{-/-} cells expressing either OPAiso1 (yellow), OPAiso7 (red) or mock transfected (grey). There was no statistically significant changes in OCR at any timepoints, $n = 16$.

7.1.3. OPAisoform protein modelling

Protein modelling of the OPA1 isoforms was achieved using the I-TASSER web-server (J. Yang et al., 2015). Figure 7.2 shows OPA isoform 1 either with (A) or without (B) the 6x HIS tag. There are clearly potential structural differences but a caveat to this is that the proteins are modelled in a free environment, not taking into account the potential for membrane binding to alter structure of indeed cleavage of the MTS. So as such this is not necessarily representative of native structure but highlights the potential perturbing effect the addition of the HIS tag could have on OPA1 protein structure. The differences did not alter with the presence of the 5b exon (data not shown).

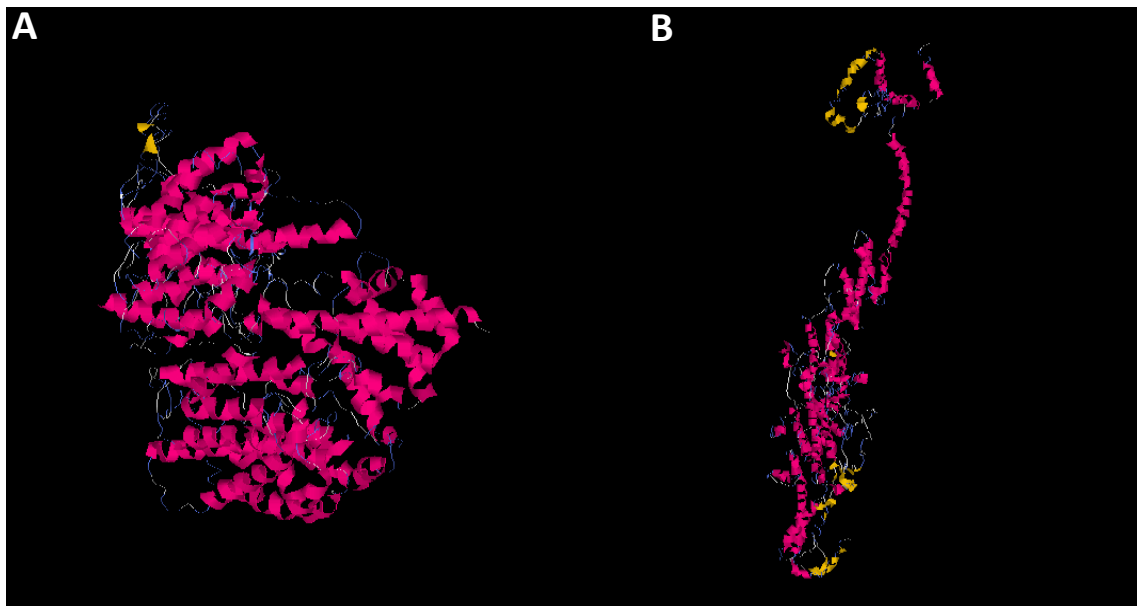


Figure 7-2: Two protein models of OPAiso1 either with (A) or without (B) the 6x HIS tag. The predicted structures show large potential differences.

7.1.4. Apoptosis leads to Mitochondrial Morphology Changes

Mitochondrial networks remodel upon the activation of apoptosis. In Figure 7.3, reproduced from (Brooks et al., 2007), overexpression of two apoptosis inducing proteins Bax and Bak led to an aggregation of the mitochondrial network, phenotypically similar to that seen when overexpressing OPA1 isoforms at high levels. The authors also saw the same mitochondrial bunching upon treatment with azide, an inducer of cell death. It would be interesting to stain OPA1 overexpressing cells for apoptosis associated proteins to further investigate this phenomenon.

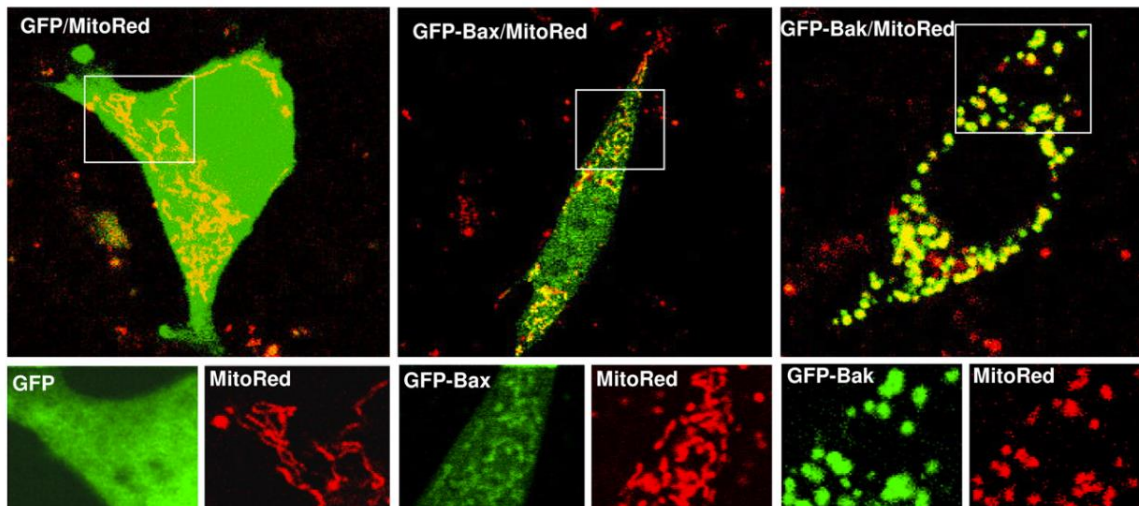


Figure 7-3: Mitochondrial remodelling due to the overexpression of apoptosis inducing proteins. There is clear aggregation of mitochondria with the expression of BAX and BAK, which looks phenotypically similar to what was seen in the OPA1 over-expressing cells in chapter 4. Reproduced from (Brooks et al., 2007).

7.1.5. OPA Peredox Figure

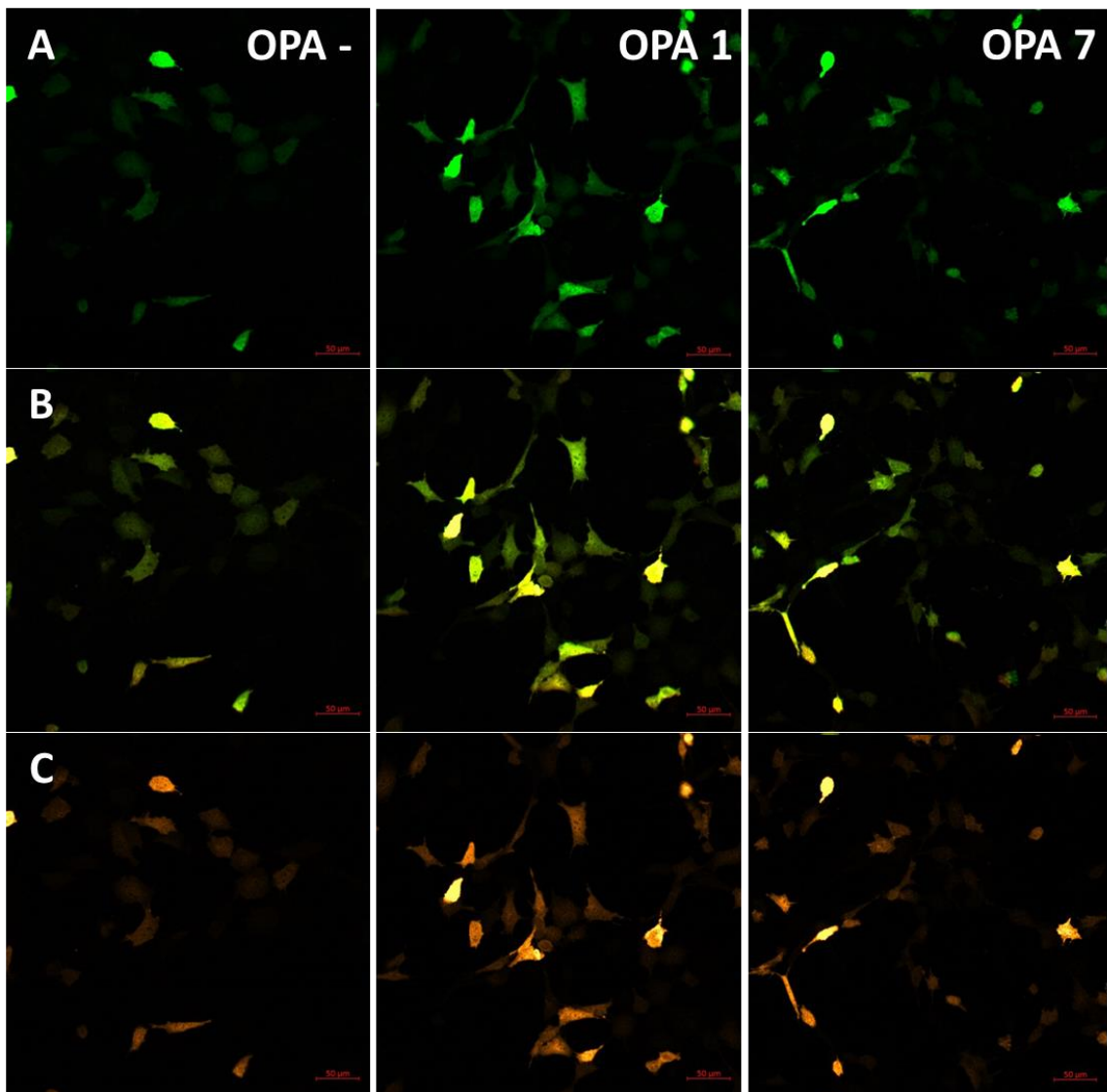


*Figure 7-4: Representative images from Peredox analysis of OPA MEF cells expressing either OPA iso 1 or 7 compared to OPA -/-. Cells used were stably expressing OPA 1, 7 or -ve and were transiently transfected with the Peredox plasmid. Row **A** shows GFP fluorescence, which is dependent on NADH levels. Row **C** shows mCherry expression for protein normalisation. Row **B** shows a merge of **A** and **C**. OPA 1 & 7 cells show much yellower cells in row **B**, indicating a lower NADH/NAD⁺ ratio. **D** shows the ratio of green to red fluorescence, indicative of the cytoplasmic NADH/NAD⁺ ratio. (n=55 treated cells).*

7.1.6. Identified AOX expressing animal Species

Species	Common Name
<i>Ciona intestinalis</i>	Sea Squirt
<i>Branchiostoma belcheri</i>	Lancelet
<i>Oncorhynchus tshawytscha</i>	Chinook Salmon
<i>Apostichopus japonicus</i>	Sea Cucumber
<i>Acanthaster planci</i>	Crown of Thorns Starfish
<i>Lingula anatina</i>	Lamp Shell
<i>Urechis unicinctus</i>	Penis Fish
<i>Elysia chlorotica</i>	Green Sea Slug
<i>Biomphalaria glabrata</i>	Ram's Horn Snail
<i>Pomacea canaliculata</i>	Channelled Applesnail
<i>Mizuhopecten yessoensis</i>	Scallop
<i>Anadara ovalis</i>	Blood Ark Clam
<i>Mercenaria mercenaria</i>	Hard Clam
<i>Diplodon chilensis</i>	Pearly Mussel
<i>Eurytemora affinis</i>	Calanoid Copepod
<i>Folsomia candida</i>	Springtail
<i>Limulus polyphemus</i>	Horseshoe Crab
<i>Ramazzottius varieornatus</i>	Tardigrade
<i>Hypsibius dujardini</i>	Tardigrade
<i>Exaiptasia pallida</i>	Anemone
<i>Pocillopora damicornis</i>	Coral
<i>Stylophora pistillata</i>	Coral
<i>Acropora digitifera</i>	Coral
<i>Orbicella faveolata</i>	Coral
<i>Amphimedon queenslandica</i>	Sponge
<i>Ephydatia muelleri</i>	Sponge
<i>Trichoplax</i> sp. H2	Placozoan

7.1.7. AOX Spectrophotometric Assay

This assay was based on the CIII assay from (Spinazzi et al., 2012) but modified to measure the oxidation of UQH to UQ. This required special UV cuvettes to monitor the change in absorbance at 290nm. Briefly the 535µl of distilled water, 50µl of potassium phosphate buffer (0.5 M, pH 7.5), 75µl of oxidized cytochrome c, 50µl of KCN (10 mM), 20µl of EDTA (5mM, pH 7.5), 10µl of Tween-20 (2.5% (vol/vol)). 250µl of sample was used for the analysis and could have been increased but as a mitochondrial extraction gives a total of 500ul of mitochondria it is felt most astute to allow extra for repetition of the assay. Baseline was monitored for 1 minute at 290nm. The addition of 10µl of 10mM decylubiquinol starts the reaction, mixed by inversion. The absorbance was then monitored for around 3 minutes. Next 10µl of 18mM Antimycin-A solution was added to stop the reaction, with absorbance monitored for a further 100 seconds. Below are two representative traces from the assay. Background levels prevented accurate measurement of BgAOX activity (n = 4). This assay wasn't tested after the discovery of the affect that galactose has on activity, so it is possible that it would be measurable after growth in galactose.

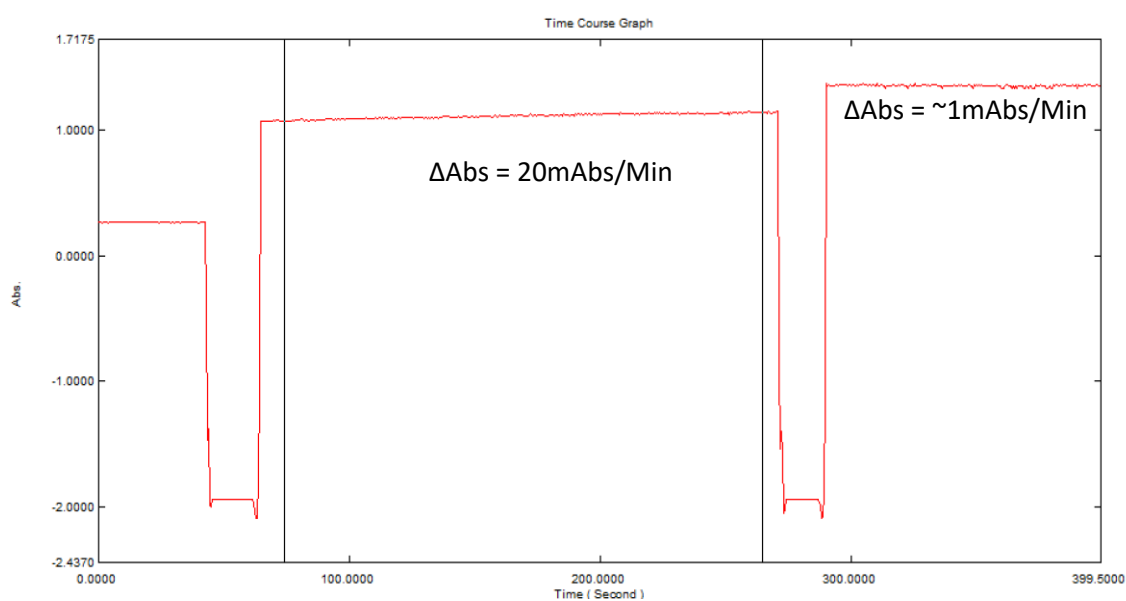


Figure 7-4: CiAOX spec assay. There was a measurable rate for CiAOX, which could be used to quantify activity. Rates shown are for the section between the black lines after decylubiquinone addition.

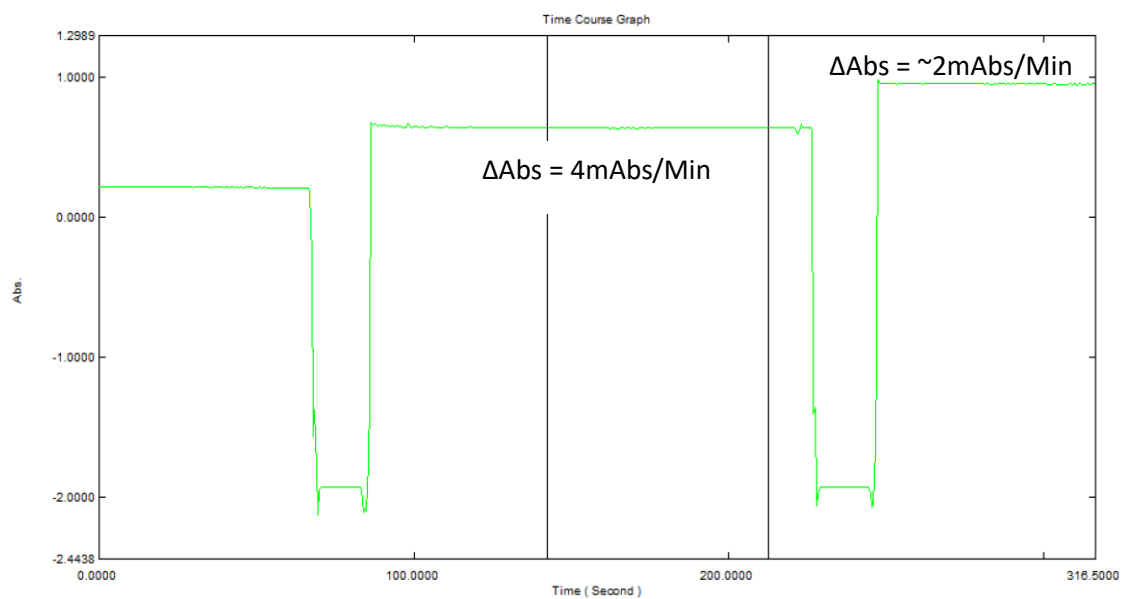


Figure 7-5: BgAOX spec assay. Background levels were very similar to the measured activity levels, making reliable measurements difficult to make.

8. References

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