Development of an *in vitro* Model to Explore the Impact of mtDNA Mutations on the Metabolism and Epigenome of Myogenic Cells

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Thesis submitted for the degree of Doctor of Philosophy
Declaration

I, Benjamin John O’Callaghan confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature:

Date: 23rd of July 2020
Abstract

The work presented in this thesis describes the successful establishment of an in vitro model of mitochondrial disease using hiPSC technology and targeted differentiation towards disease-relevant skeletal muscle cell types, which was then utilised to explore the impact of mtDNA disease pathogenic variants on histone acetylation modifications.

hiPSC lines harbouring a number of different disease-associated mitochondrial DNA (mtDNA) pathogenic variants were first established, through reprogramming of mitochondrial disease patient fibroblasts. By taking advantage of the random segregation of mutant mtDNA in patient fibroblast populations, hiPSC lines with high heteroplasmy for disease modelling were successfully established, alongside isogenic control lines with low/undetectable levels. Whilst the m.8344A>G pathogenic variant is detrimental to cell reprogramming and pluripotency maintenance, the m.3243A>G does not overtly impact the reprogramming process.

A targeted differentiation protocol was then optimised in order to differentiate selected mtDNA disease hiPSC lines into disease-relevant myogenic cell types including terminally differentiated myotubes which show characteristic deficits in mitochondrial function. Whilst mid-range m.8344A>G mutation loads do not overtly impact the myogenic differentiation process, impairments in both myogenic differentiation and subsequent maturation of myotubes are observed in lines harbouring mid-range and higher m.3243A>G mutation loads. Assessments of metabolically sensitive histone acetylation modifications at early and later stages of the myogenic differentiation process revealed cell lines harbouring the m.3243A>G pathogenic variant display widespread deficits in a histone acetylation deposition and/or maintenance.

Together, the data presented in this thesis show that hiPSC-derived myogenic cell types represent a useful in vitro model for exploring early pathomechanisms contributing to myopathic phenotypes in mitochondrial disease states. These data show for the first time that mitochondrial deficits caused by mtDNA pathogenic variants can negatively impact metabolic fluxes contributing to histone acetylation deposition and associated physiologically relevant myogenic cell-identity transitions. Modulators of histone acetylation modifications might therefore represent a novel target for therapeutic intervention in human mitochondrial diseases.
Impact Statement

Mitochondrial disease patients present clinical symptoms associated with multiple organ systems, particularly tissues that have high oxidative cellular energy requirements (e.g. muscle and nervous system). At present no treatment has been shown to effectively alter the clinical course of these patients. Whilst there have been great advancements in our understanding of the biochemical deficits associated with disease-causing mtDNA pathogenic variants, downstream pathomechanisms contributing to cellular and tissue dysfunction underlying the mitochondrial disease state remain more poorly understood.

Primary patient fibroblasts and immortalised cybrid lines are the main human cell models currently used to investigate mitochondrial dysfunction caused by mtDNA pathogenic variants. Unlike the cell types affected in patients however, these cells are proliferative and rely on glycolytic metabolism for energy production. The work presented in this thesis describes the successful establishment of a more disease-relevant in vitro cell model through the use of hiPSC technology and targeted differentiation into skeletal muscle cell types often affected in mitochondrial disease patients. This work builds on knowledge spanning multiple scientific fields including hiPSC, skeletal muscle and mitochondrial biology, and the methodology implemented here serves as an excellent basis for future researchers to explore further, cellular and molecular mechanisms underscoring the myopathic phenotypes of the mitochondrial disease state.

There has been accumulating evidence that cellular metabolism and cell-identity changes are intrinsically linked, at least partly through metabolically sensitive enzymatic modifications of chromatin. The impact of disease associated impairments in mitochondrial metabolism, on such epigenetic modifications, and the cell-identity transitions driven by them, has until now not been explored in any great detail. The work described in this thesis shows that disease causing mtDNA pathogenic variants negatively impact myogenic cell-identity transitions which are not only important for developmental myogenesis, but which also underlie muscle repair and growth in adulthood. Impairments in muscle repair might therefore represent an important contributing factor to the myopathic phenotype of mitochondrial disease patients which up until now, has been a factor largely overlooked by the field. This work also shows for the first time that histone acetylation modifications which govern such myogenic cell identity transitions are impaired by disease causing mtDNA pathogenic variants, and potentially represent the
underlying pathomechanism which links impairments in mitochondrial metabolism and cell identity changes involved in muscle repair. These data open novel avenues and targets for therapeutic intervention of mitochondrial diseases, for which treatment at present remains largely symptomatic.
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Abbreviations

3PG: 3-phosphoglyceric acid
acetyl-CoA: acetyl-coenzyme A
ACLY: ATP-citrate lyase
ACS: acetyl-CoA synthetase
ACSS2: Acyl-coenzyme A synthetase short-chain family member-2
ADP: adenine nucleotide diphosphate
α-KG: α-ketoglutarate
ALK: activin receptor-like kinase
ANOVA: analysis of variance
ANT: adenine nucleotide transporter
ATF2: activating transcription factor 2
ATG8: autophagy-related protein 8
ATP: adenine nucleotide triphosphate
bHLH: basic helix-loop-helix
BMP4: Bone morphogenic protein 4
BNIP3: BCL2 interacting protein 3
bp: base pairs
BRD: bromodomain
BRD4: bromodomain-containing protein 4
CaMKIV: Ca2+/calmodulin-dependent protein kinase IV
CaN: calcineurin protein phosphatase
CCCP: carbonyl cyanide m-chlorophenylhydrazone
cDNA: complementary DNA
ChIP: chromatin immunoprecipitation
CI: OXPHOS Complex I
CIC: tricarboxylate transporter protein / citrate carrier
CII: OXPHOS Complex II
CIII: OXPHOS Complex III
CIV: OXPHOS Complex IV
CMT: Charcot Marie Tooth
coA: coenzyme A
CoQ10: coenzyme Q10
COX: cytochrome C oxidase
CRE: cAMP response element
CREB: CRE binding protein
Ct: cycle threshold
CV: OXPHOS Complex V
CXCR4: C-X-C chemokine receptor 4
CytC: Cytochrome C
DAPI: 4′,6-diamidino-2-phenylindole
DHAP: dihydroxyacetone phosphate
D-loop: displacement loop
DMEM: Dulbecco’s Modified Eagle’s Medium
DNMT: DNA methyltransferase
DRP1: dynamin-related protein 1
DSB: double-stranded breaks
dsDNA: double stranded DNA
EB: embryoid body
E-box: enhancer box
ECAR: extracellular acidification rate
EDTA: Ethylenediaminetetraacetic acid
EM: electron microscopy
ER: Endoplasmic Reticulum
ERR: estrogen-related receptor
ESC: embryonic stem cell
EtBr: ethidium bromide
ETC: electron transport chain
F6P: fructose 6-phosphate
FAD: oxidised flavin adenine nucleotide
FADH2: reduced flavin adenine nucleotide
FBS: foetal bovine serum
FCCP: Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FGF: fibroblast growth factor
FMN: Flavin mononucleotide
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GPI: Glucose-6-phosphate isomerase
gRNA: guide RNA
HAT: histone acetyltransferase
HDAC: histone deacetylase
hESC: human ESC
HIF1α: hypoxia inducible factor 1 α
hiPSC: human induced pluripotent stem cell

HMT: histone methyltransferase

hPSC: human PSC

HSP1: heavy strand promoter 1

HSP2: heavy strand promoter 2

ICM: inner cell mass

IDO1: indoleamine 2,3-dioxygenase 1

IGF1: insulin growth factor 1

IMM: inner mitochondrial membrane

IMS: intermembrane space

IST: insulin selenium transferrin

IVF: in vitro fertilisation

JHDM: jumonji C domain-containing histone demethylase

KAT: lysine acetyltransferase

KDM: lysine demethylase

KEAP1: Kelch-like ECH-associated protein 1

KLF4: Kruppel-like factor 4

KSR: knockout serum replacement

LC-MS: liquid chromatography mass spectrometry

LDH: lactate dehydrogenase

L-OPA1: long OPA1

LSP: light strand promoter

MEF: mouse embryonic fibroblasts

MEF2: myocyte enhancer factor 2
MELAS: mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes

MERRF: myoclonic epilepsy with ragged red fibres

mESC: murine ESC

MFF: mitochondrial fission protein

MFN: mitofusin

MiD: mitochondrial dynamics protein

ψm: mitochondrial membrane potential

mitoTALEN: mitochondrial targeted TALEN

MPP: mitochondrial processing protease

mPTP: mitochondrial permeability transition pore

MRF: myogenic regulatory factor

mRNA: messenger RNA

mtDNA: mitochondrial DNA

mt-EFG1: mitochondrial elongation factor G1

mt-Eftu: mitochondrial elongation factor tu

mt-IF2: mitochondrial translational initiation factor 2

mt-IF3: mitochondrial translational initiation factor 3

mt-LSU: 39s large mitochondria ribosomal subunit

mt-RF1a: mitochondrial release factor 1a

mt-rRNA: mitochondrial ribosomal RNA

mtSSBs: mitochondrial single stranded binding proteins

mt-SSU: 28S small mitochondria ribosomal subunit

mt-tRNA: mitochondrial transfer RNA

Myf5: myogenic factor 5
MyHC: myosin heavy chain
MyoD: myogenesis determination protein 1
N.D.: not described
NAD⁺: oxidised nicotinamide adenine dinucleotide
NADH: reduced nicotinamide adenine dinucleotide
NCAM: neural cell adhesion molecule
NCC: neural crest cell
NCR: non-coding region
nDNA: nuclear DNA
NFAT: nuclear factor of activated T-cells
NFE2L2: Nuclear factor erythroid-derived 2-like 2
NGS: next generation sequencing
NMP: neuromesodermal progenitor
NNMT: nicotinamide N-methyltransferase
NRF: nuclear respiratory factor
NSC: neural stem cell
OCR: oxygen consumption rate
OCT4: octamer-binding transcription factor-4
O₁H: origin for heavy-strand replication
O₁L: origin for light-strand replication
OMM: outer mitochondrial membrane
OXPHOS: oxidative phosphorylation
p38-MAPK: p38 mitogen-activated protein kinase
PARL: presenilin-associated rhomboid-like protein
PAX7: paired box protein 7
PB1: Protein polybromo-1
PBGD: porphobilinogen deaminase
PCR: polymerase chain reaction
PDGFR: platelet derived growth factor receptor
PDK: pyruvate dehydrogenase kinase
PEG: polyethylene glycol
PEP: phosphoenolpyruvate
PGC: primordial germ cell
PGC1: peroxisome proliferator-activated receptor gamma coactivator 1
Pi: inorganic phosphate
PINK1: phosphatase and tensin homologue-induced putative kinase 1
PKB: protein kinase B
POLRMT: mitochondrial RNA polymerase
POLγ: DNA polymerase gamma
PPAR: peroxisome proliferator-activated receptor
PPP: pentose-phosphate pathway
ΔP: proton motive force
PSC: pluripotent stem cell
rCRS: revised Cambridge Reference Sequence
ROS: reactive oxygen species
RFLP: Restriction fragment length polymorphism
RRF: ragged red fibre
RT-qPCR: real-time quantitative PCR
SAM: S-Adenosyl methionine
SDH: succinate dehydrogenase
SeV: sendai virus
Shh: sonic hedgehog
SIRT1: sirtuin-1
SMARCD3: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3
SOX2: sex determining region Y-box 2
TAF1: Transcription initiation factor TFIID subunit 1
TALEN: transcription activator-like effector nuclease
TCA: tricarbocyclic acid
TEM: transmission EM
TET: ten-eleven translocation methylcytosine dioxygenase
TFAM: mitochondrial transcription factor A
TFB2M: Mitochondrial Transcription Factor B2
TGFβ: transforming growth factor β
TIM: translocases of the IMM
TMRM: tetramethylrhodamine methyl ester
TOM: translocases of the OMM
TSS: transcription start site
VDAC: voltage dependant anion channels
VEGFR: vascular endothelial growth factor receptor
WES: whole exome sequencing
WT: wild-type
ZFN: zinc-finger nuclease
Chapter 1  Introduction

Whilst currently used in vitro models have provided much insight into the fundamental molecular and bioenergetic consequences of various mitochondrial diseases, downstream pathomechanisms which might be more readily targeted for therapeutic intervention are relatively poorly described. A useful model better recapitulating the in vivo setting is therefore desired for exploring disease pathomechanisms and future therapeutic interventions. It is becoming ever clear that mitochondrial metabolism can strongly influence a cell’s transcriptional profile through metabolically sensitive enzymatic epigenetic modifications, underscoring an important mechanism contributing to maintenance of cell identity and the driving of differentiation during development, tissue repair and immunity. Alterations to the epigenome could underlie a mechanistically unexplored pathomechanism contributing to the tissue-specific dysfunction of mitochondrial disease patients that warrants further investigation.

1.1 Mitochondrial Biology

Mitochondria are cytosolic organelles present in all nucleated cells of the body that are involved in a number of important cellular functions including: calcium homeostasis, reactive oxygen species (ROS) generation for cell signalling, apoptosis and many vital metabolic processes; most notably the synthesis of adenine nucleotide triphosphate (ATP) through oxidative phosphorylation (OXPHOS) (Duchen, 2004).

Whilst every one of the specialised organelles within our cells may be considered unique, the mitochondrion has a number of characteristic features that clearly distinguishes it from all others. Much of these differences can be attributed to the independent evolutionary origin of mitochondria as an aerobic α-proteobacterium that was engulfed by a primitive eukaryotic cell host (Roger, Muñoz-Gómez & Kamikawa, 2017).

Mitochondria are contained within a bacterial-like double membrane consisting of an outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) separated by an intermembrane space (IMS).
1.1.1 Outer Mitochondrial Membrane

The OMM is a simply structured and highly permeable phospholipid bilayer that defines the interface between mitochondria and the rest of the cell. Voltage dependant anion channels (VDACs), also referred to as mitochondrial porins, are the most abundant protein in the OMM and the major determinant of its permeability (Noskov et al., 2016). VDACs form large transmembrane pores that allow passive diffusion between the cytosol and IMS of: charged ions and a number of water soluble molecules including; adenosine diphosphate (ADP), ATP, inorganic phosphate (Pi), pyruvate and other metabolic substrates/products (Colombini, 2012). For this reason, the cytosol and IMS are considered electrochemically equivalent.

1.1.2 Inner Mitochondrial Membrane

The IMM is largely impermeant (i.e. expresses selective channels and carriers) by comparison, compartmentalising the mitochondrial matrix from the IMS and cytosol. The ionic impermeability of the membrane permits the generation of electrochemical gradients that give rise to a mitochondrial membrane potential ($\psi_m$). $\psi_m$ is largely set by a proton gradient established from the extrusion of H$^+$ from the mitochondrial matrix to the IMS through activity of the electron transport chain (ETC). Unlike the relatively smooth OMM, the IMM is highly convoluted, forming numerous tubular invaginations into the mitochondrial matrix known as cristae. OXPHOS complexes are highly concentrated on the cristae membrane (Cogliati, Enriquez & Scorrano, 2016), and the IMS within the cristae lumen is partially compartmentalised due to the narrow tubular junctions which join them (Santo-Domingo & Demaurex, 2012). Cristae therefore form semi-independent sub-domains which enhance ETC efficiency and OXPHOS coupling.

1.1.3 Electron Transport Chain and OXPHOS

The ETC is embedded in the IMM and consists of four protein complexes (Complexes I – IV), and two mobile electron carriers: coenzyme Q10 (CoQ$_{10}$) and cytochrome C (CytC) (Figure 1-1). Complexes I and II oxidise reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine nucleotide (FADH$_2$) coenzymes respectively. The released electrons are then shuttled to Complex III via CoQ$_{10}$ and subsequently to Complex IV via Cyt C. Complex IV catalyses the terminal redox transfer of electrons from CytC to O$_2$ forming water, and underlies the aerobic nature of the ETC. Free energy released during these sequential exergonic redox reactions is used by Complexes I, III and IV to pump H$^+$ from the mitochondrial matrix to the IMS. Under normal physiological
conditions the $\psi_m$ is therefore negative (i.e. mitochondrial matrix more negatively charged than IMS) and the chemical proton gradient inward to the mitochondrial matrix ([H$^+$]$_{\text{matrix}}$ $<$ [H$^+$]$_{\text{IMS}}$).

![Diagram of OXPHOS Complexes I-V](image)

<table>
<thead>
<tr>
<th></th>
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<td>10</td>
<td>10</td>
<td>~14</td>
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<td>subunits</td>
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**Figure 1-1 OXPHOS Complexes I-V together with the mobile electron carriers coenzyme Q10 (CoQ) and cytochrome C (Cyt C) are necessary for the synthesis of ATP via oxidative phosphorylation (see text for details). Oxidative phosphorylation is under the unique control of both nuclear DNA (nDNA) and mitochondrial (mtDNA). IMS: Intermembrane Space, IMM: Inner Mitochondrial Membrane.**

### 1.1.3.1 OXPHOS CI (NADH:Ubiquinone Reductase)

Human mitochondrial OXPHOS CI represents the largest OXPHOS complex, consisting of at least 46 subunits encoded within both the mitochondrial and nuclear genomes. Prokaryotic CI is much simpler enzyme by comparison, consisting of only 14 ‘core’ subunits which share high degree of homology to subunits of mitochondrial OXPHOS CI (Efremov, Baradaran & Sazanov, 2010; Letts & Sazanov, 2015). Subunits of both prokaryotic and eukaryotic OXPHOS CI assemble into a large L-shaped structure with a hydrophobic arm imbedded in the IMM (or bacterial membrane) and a hydrophilic arm protruding into the mitochondrial matrix (or bacterial cytoplasm) (Efremov, Baradaran & Sazanov, 2010; Letts & Sazanov, 2015). The ‘extra’ subunits present in human mitochondrial and other higher species OXPHOS CI are often termed supernumerary,
which surround the core membrane domain, and are involved in complex assembly and/or stability (Stroud et al., 2016), although the precise functional roles of many of these subunits remains to be determined (Zhu, Vinothkumar & Hirst, 2016).

Based on evolutionary origins, OXPHOS CI subunits can be subdivided into three categories that form distinct functional N-, Q- and P-modules (Hunte, Zickermann & Brandt, 2010). The N-module is positioned at the distal end of the hydrophilic arm that protrudes into the mitochondrial matrix and is the site at which NADH is bound and oxidised by a non-covalently bound flavin mononucleotide (FMN) (Hirst, 2013). The reduced FMNH$_2$ then rapidly undergoes oxidation releasing two electrons which are redox transferred through a chain of 8 iron-sulphur (FeS) clusters towards the proximal end of the hydrophilic arm. The Q-module is positioned at the proximal end of the hydrophilic arm and transfers the electrons received from NADH oxidation in the N-module to a mobile CoQ$_{10}$ (oxidised isoform ubiquinone) molecule forming the reduced isoform ubiquinol in the IMM for subsequent electron transfer to OXPHOS CIII (Alcázar-Fabra, Navas & Brea-Calvo, 2016). Alongside a number of nuclear encoded subunits, all seven mitochondrial encoded OXPHOS CI subunits contribute to the structure and function of the P-module. The P-module constitutes the hydrophobic arm imbedded in the IMM and represents the site at which the energy associated with the exergonic redox transfer of electrons from NADH to CoQ$_{10}$ is coupled with the active extrusion of 4 H$^+$ from the mitochondrial matrix to the IMS (Efremov & Sazanov, 2011; Sharma et al., 2015; Di Luca et al., 2018).

Whilst there have been great advancements in understanding the process by which OXPHOS CI assembles, including the presence of various subassembly intermediates, more recent liquid chromatography mass spectrometry (LC-MS) proteome analysis following blue-native gel extraction of stable OXPHOS CI assembly intermediates (complexome) has greatly advanced our understanding of this process (Guerrero-Castillo et al., 2017). From this data it has been shown that OXPHOS CI subunits first form major subassemblies which are then incorporated together forming the complete OXPHOS CI. One of these stable intermediates consists of subunits which form the central-proximal part of the hydrophobic P-module including 4/7 mtDNA encoded subunits (MT-ND2, MT-ND3, MT-ND4L, MT-ND6), which has been termed proximal P-module b ($P_P$-b). A second stable intermediate consisting of distal components of the P-module which sit adjacent to this $P_P$-b including MT-ND4 has been termed distal P-module a ($P_D$-a). A
third stable intermediate consisting of the most distal components of the P-module and includes MT-ND5 has been termed P<sub>D-b</sub>, which also additionally includes the NDUFB8 subunit which has been targeted as a representative measure of steady-state OXPHOS CI levels in this thesis. The hydrophilic Q-module arm also forms a stable intermediate which subsequently associates with the most proximal subunits of the P-module (P<sub>P-a</sub>) which includes MT-ND1, forming a stable Q/P<sub>P-a</sub> intermediate assembly. The P<sub>D-a</sub> and Q/P<sub>P-a</sub> intermediate assemblies can then form a larger molecular weight assembly through associations with P<sub>P-b</sub>, and subsequent associations with P<sub>D-b</sub>. The subunits which form the N-module positioned at the distal end of the hydrophilic arm also form a stable intermediate, with the association of this N-module to the large molecular weight assembly outlined above representing the final step of OXPHOS CI assembly.

1.1.3.2 OXPHOS CII (Succinate Dehydrogenase)

OXPHOS CII is the smallest of the human mitochondrial OXPHOS complexes, consisting of four protein subunits (SDHA, SDHB, SDHC and SDHD) which are all encoded by the nuclear genome. The SHDA and SDHB subunits together form the hydrophilic catalytic domain which is exposed to the mitochondrial matrix and responsible for catalysing succinate oxidation to fumarate in the TCA cycle. The SDHA is a flavoprotein with a covalently bound FAD molecule that accepts the electrons released from succinate oxidation, and SDHB contains three FeS clusters which transfer the electrons released from the subsequent oxidation of FADH<sub>2</sub> to CoQ<sub>10</sub> (ubiquinone to ubiquinol redox reaction). The SDHC and SDHD subunits together form the hydrophobic domain which anchors OXPHOS CII onto the IMM, and together with SDHA and SDHB forms the binding site for CoQ<sub>10</sub> (Sun et al., 2005). SDHB represents the subunit of the catalytic domain that interacts with the SDHC/SDHD domain (Sun et al., 2005) and is the specific subunit which has been targeted in this thesis as a representative measure of steady-state OXPHOS CII levels.

OXPHOS CII assembly occurs in a stepwise manner dependant on the initial addition of functional units to each of the SDH subunits. SDHA and SDHB heterodimerization first requires the incorporation of FAD and three FeS clusters respectively (Kim et al., 2012; Saxena et al., 2016). Whilst the exact mechanism by which SDHC and SDHD heterodimerize to form the IMM associated hydrophobic domain remain to be fully elucidated, incorporation of a heme-b molecule at the interface between SDHC and SDHD, and the subsequent association of the hydrophilic domain dimer, facilitates this
process and the subsequent stability of the assembled complex (Lemarie & Grimm, 2009; Kim et al., 2012).

1.1.3.3 OXPHOS CIII (CoQ\textsubscript{10}-Cyt C reductase)

Human OXPHOS CIII consist of 11 subunits, one of which (Cyt B) is encoded within the mitochondrial genome (\textit{MT-CYB}). Similar to the structure of OXPHOS CIII isoforms solved for other mammals and eukaryotic organisms, human OXPHOS CIII forms a functional transmembrane homodimer. Of the 11 subunits which form each half of the OXPHOS CIII dimer, only 3 of these constitute redox catalytic activity: the mitochondrial encoded Cyt B which constitutes two CoQ\textsubscript{10} binding domains and two heme-b molecules, cytochrome C1 (Cyt C1: encoded by \textit{CYC1}) which contains a heme-c (Cyt C) molecule, and the Rieske FeS protein ubiquinol-cytochrome c reductase (UQCRFS1: encoded by \textit{UQCRFS1}) which as the name suggests constitutes a Rieske 2Fe-2S iron-sulphur cluster (Fernandez-Vizarra & Zeviani, 2018; Zhao et al., 2019).

OXPHOS CIII represents a converging site at which electrons carried by the reduced CoQ\textsubscript{10} (ubiquinol) catalysed from electron entry at OXPHOS CI and CII is further channelled through the ETC and transferred to the mobile electron carrier Cyt C. This redox transfer of electrons from OXPHOS CI and CII derived ubiquinol to Cyt C is coupled with the translocation of H\textsuperscript{+} from the mitochondrial matrix to the IMS in a mechanism known as the Q cycle (Cooley, Lee & Daldal, 2009). The OXPHOS CIII catalytic Q cycle begins with the binding and oxidation of a ubiquinol at the CoQ\textsubscript{10} binding domain of Cyt B proximal to the IMS, with resultant release of 2 H\textsuperscript{+} into the IMS. The two electrons released as part of this oxidative reaction are then passed via different routes. One of the electrons is first transferred to the Rieske 2Fe-2S cluster of UQCRFS-1, before being transferred to heme-c (Cyt C) by the Cyt C1 subunit. The other electron is first transferred to the high potential heme-b (heme-b\textsubscript{H}) and then low potential heme-b (heme-b\textsubscript{L}) within Cyt B, which then catalyses the transfer of this electron to a ubiquinone bound at the second of the Cyt B CoQ\textsubscript{10} binding domains proximal to the mitochondrial matrix, forming ubisemiquinone. A second ubiquinol molecule is then bound and oxidised at Cyt B, with one electron being transferred to Cyt C as before. The second electron is passed along the heme-b molecules of Cyt B before being transferred to the ubisemiquinone still bound at the CoQ\textsubscript{10} binding domain, resulting in the addition of 2 H\textsuperscript{+}
from the mitochondrial matrix and reductive formation of a ubiquinol molecule. Thus, from each complete Q cycle, two ubiquinol molecules are oxidised, one ubiquionone molecule is reduced (net oxidation of 1 ubiquinol), 2 Cyt C molecules are reduced, 4 H⁺ are released into the IMS and 2 H⁺ are taken from the mitochondrial matrix.

In addition to the three catalytic subunits of OXPHOS CIII involved in the Q cycle as outlined above, the other 8 supernumerary nuclear encoded OXPHOS CIII subunits are essential for the functional assembly and stability of the OXPHOS CIII homodimer. Cyt B synthesised in the mitochondria is first inserted into the IMM and its two heme-b molecules incorporated. Two low molecular weight subunits UQCR7 and UQCR8 are then incorporated forming a early subcomplex which subsequently incorporates the majority of remaining OXPHOS CIII subunits including Cyt C1, and two large UQCRC1 and UQCRC2 subunits which form the hydrophobic core, the second of which was used as a representative measure of steady state levels of OXPHOS CIII in this thesis (Smith, Fox & Winge, 2012). OXPHOS CIII assemblies consisting of all subunits except UQCRFS1 and UQCR11 are sufficient for CIII homodimerization into a catalytically inactive pre-CIII₂ assembly (Fernández-Vizarra & Zeviani, 2015; Conte et al., 2015). Following addition of the 2Fe-2S iron sulphur cluster to UQCRSF1, it is subsequently incorporated into the pre-CIII₂ complex (Wagener et al., 2011), and finally UQCR11 is added (Zara, Conte & Trumpower, 2009) forming a functional and mature OXPHOS CIII₂ assembly (Fernandez-Vizarra & Zeviani, 2018).

1.1.3.4 OXPHOS CIV (Cyt C Oxidase)

Human OXPHOS CIV consists of 13 subunits, 3 core subunits (MT-CO1, MT-CO2 and MT-CO3) which are encoded by the mitochondrial genome (MT-CO1, MT-CO2 and MT-CO3 respectively), and 10 supernumerary subunits encoded within the nuclear genome. MT-CO1 is a transmembrane subunit which includes three hydrophilic pores, two of which (K and D channels) are thought to permit H⁺ entry for oxygen reduction and H⁺ transfer across the IMM (Wikström, Krab & Sharma, 2018), with the third H-channel also potentially implicated in mammalian mitochondrial H⁺ transfer (Tsukihara et al., 2003; Shimada et al., 2017), although there remains controversy surrounding the functional properties of this H channel (Maréchal et al., 2020). MT-CO1 contains a redox active heme-a (Cyt A) molecule, in addition to a binuclear centre consisting of a heme-a₃ (Cyt A³) and copper centre (Cu第八). MT-CO2 is also a IMM spanning subunit which associates closely with MT-CO1 and contains a second redox active copper centre (Cu₈). MT-CO3
is also a IMM spanning subunit, but unlike MT-CO1 and MT-CO2 does not contain any catalytically active redox centres and does not appear to be involved in H⁺ translocation. MT-CO3 is however closely associated with MT-CO1 and these interactions appear crucial for maintaining stability of the redox centres and facilitating O₂ channelling to the Cyt A3-Cu₉ catalytic site (Sharma et al., 2015).

OXPHOS CIV represents the terminal site of respiratory chain electron transfer to O₂, defining the aerobic nature of OXPHOS. Reduced Cyt C from OXPHOS CIII first associates with the IMS facing side of MT-CO2 where it is oxidised and an electron transferred to its Cu₉ redox active copper centre (Ludwig et al., 2001). The electron is then redox transferred to MT-CO1 Cyt A and then its Cyt A3-Cu₉ redox centre. A second reduced Cyt C molecule is then oxidised at MT-CO2 as before, and transferred to Cyt A, at which point the Cyt A3-Cu₉ redox centre becomes permissible to O₂ binding. Upon O₂ binding, the electron from Cyt A is transferred to oxygen resulting in the breaking of the dioxygen bond, with one oxygen atom stabilised by two electrons from the Fe²⁺ of Cyt A3, and the other by a nearby tyrosine molecule (Faxén et al., 2005). Two additional Cyt C molecules are rapidly oxidised and transferred to the activated oxygen atoms, alongside 4 H⁺ from the mitochondrial matrix through the K and D channels of MT-CO1 (Wikström, Krab & Sharma, 2018). In addition to the reduction of oxygen to water, the energy associated with the exergonic redox transfer of 4 electrons from Cyt C is also coupled with the pumping of 4 H⁺ from the mitochondrial matrix to the IMM space, through the MT-CO1 D-channel (or potentially H-channel) (Wikström, Krab & Sharma, 2018).

Similar to the assembly of other OXPHOS complexes, formation of the full 13-subunit OXPHOS CIV constitutes the sequential assembly of modules into stable subassemblies termed S1-S4, with S4 representing the fully formed complex (Nijtmans et al., 1998). MT-CO1 is first inserted into the IMM representing the stable S1 subassembly and the seed upon which all other subunits assemble, and was the specific subunit used as a measure of steady-state OXPHOS CIV levels in this thesis. Additional assembly with the nuclear encoded COX4 and COX5A subunits gives rise to the stable S2 subassembly (Nijtmans et al., 1998), and this appears to necessitate prior association of MT-CO1 Cyt A, as cells lacking the enzymes responsible for Cyt A biosynthesis show accumulation of S1 subassembly (Williams et al., 2004). The mitochondrial encoded MT-CO2 subunit then associates with the S2 subassembly, and is rapidly followed by subsequent
association of mitochondrial encoded MT-CO3 and then most of the remaining nuclear encoded OXPHOS CIV subunits (COX5B, COX6C, COX7A/B, COX7C and COX8) forming the stable S3 subassembly (Stiburek et al., 2005; Fontanesi et al., 2006). MT-CO2 CuA formation appears necessary for MT-CO2 association in the S3 subassembly, as mutations in the genes responsible for its synthesis result in reductions in S3 subassembly, and accumulation of the S2 subassembly (Stiburek et al., 2005; Williams et al., 2004). Final association of the remaining nuclear encoded OXPHOS complexes (COX6A, COX6B, COX7A/B) results in the formation of a functional S4 assembly. Although originally designated as a subunit of OXPHOS CI, NDUFA4 is in fact an additional subunit of OXPHOS CIV (Balsa et al., 2012; Pitceathly et al., 2013), which recent structural data suggesting facilitates maintenance of the active CIV monomer over its less active dimer assembly (Shinzawa-Itoh et al., 2019).

1.1.3.5 **OXPHOS CV (F\textsubscript{1}F\textsubscript{0}-ATPase)**

OXPHOS CV represents the site at which the energy stored in the H\textsuperscript{+} electrochemical gradient across the IMM generated through action of the ETC (OXPHOS Complexes I-IV: outlines above) is utilised to catalyse the synthesis of cellular energy resource in the form of ATP from ADP and Pi. OXPHOS CV consist of two main domains distinguishable not only through their differential functional properties but also hydrophilic vs lipophilic nature: a hydrophilic F\textsubscript{1} domain and hydrophobic F\textsubscript{0} domain (Collinson et al., 1994; Kagawa & Racker, 1966). OXPHOS CV consists of at least 16 subunits, 14 encoded by the nuclear genome, and 2 encoded by the mitochondrial genome, both of which contribute to the F\textsubscript{0} domain.

The hydrophillic F\textsubscript{1} domain is positioned within the mitochondrial matrix and constitutes the catalytic site at which ADP condensation with Pi to ATP takes place. Mammalian mitochondrial F\textsubscript{1} is composed of 5 different globular proteins: α (encoded by ATP5A1 and used as a representative measure of OXPHOS CV steady-state levels in this thesis), β (encoded by ATP5B), γ (encoded by ATP5C1), δ (encoded by ATP5D) and ε (encoded by ATP5E). Each F\textsubscript{1} domain consists of 3 α and 3 β subunits arranged as an alternating hexamer positioned around a central stalk consisting of an extension of the γ subunit (Abrahams et al., 1994), with the δ and ε subunits associated with the opposing end of the γ subunit (Stock, Leslie & Walker, 1999). The β subunit constitutes the catalytically active site of the F\textsubscript{1} domain and forms the majority of the ADP and Pi binding site at the interface with an adjacent α subunit (Abrahams et al., 1994).
The mitochondrial hydrophobic transmembrane Fo domain constitutes a ring of 8 c subunits (encoded by ATP5G1/2/3) associated with a single a subunit (encoded by the MT-ATP6 and MT-ATP8) which forms the H+ translocation machinery and rotor ring across the IMM (Watt et al., 2010). H+ entry through the Fo domain is coupled with rotatory movement of the central stalk through the associations of the multimeric c assembly of the Fo domain with subunits of the F1 central stalk (Stock, Leslie & Walker, 1999), which induces conformational changes in the αβ assembly of F1 that is coupled with ADP+Pi condensation to ATP (Watt et al., 2010). In order for the rotational movement of the central stalk to evoke such conformational changes within the αβ assembly, the αβ subunits must be maintained in a static state, and this is achieved through associations of some of the remaining Fo subunits which form a peripheral/stator stalk. Subunits b (encoded by ATP5F1), d (encoded by ATPH), F6 (encoded by ATP5J) and oligomycin sensitivity conferring protein (OSCP encoded by ATP5O) together form the peripheral stalk (Walker, 2013). Subunit b is the major IMM spanning component of the peripheral stalk, which associates with subunits d, F6 and OSCP through its residues which project into the mitochondrial matrix (Dickson et al., 2006). Subunit b is maintained close with the F1 through interactions with the IMM spanning subunit a of Fo (Walker & Dickson, 2006; Lee et al., 2015). Among the matrix facing components of the peripheral stalk, it is OSCP which is responsible for associating with a α subunit of F1, fixing in place the αβ assembly (He et al., 2020).

OXPHOS CV assembly begins with the separate formation of the matrix F1 αβγδε, and IMM multimeric c-ring of Fo which subsequently associate to form a IMM associated F1-c subassembly (Wittig et al., 2010). The peripheral stalk also forms an independent subassembly consisting of subunits b, d, F6 and OSCP, which subsequently associates with the supernumerary membrane associated e, f and g subunits (He et al., 2020). The peripheral stalk assembly can then associate with the F1-c subassembly (He et al., 2020). Association of the mitochondrial encoded a subunits of Fo, appears to be among the last steps of OXPHOS CV assembly as ρ0 cells lacking the mitochondrial genome show almost fully assembled complexes (Wittig et al., 2010).

1.1.3.6 OXPHOS Supercomplex (and Megacomplex)

The different components of the OXPHOS machinery are not randomly distributed throughout the IMM but instead form supramolecular complexes (Supercomplexes) with one another, in line with the intrinsic functional reliance that exists between them. Much
of the early insight surrounding OXPHOS supercomplex structures has come from blue-native gel electrophoresis with early studies revealing a number of supercomplex structures inclusive of OXPHOS CIII dimers including: OXPHOS CI with a OXPHOS CIII dimer, and OXPHOS CIV with a OXPHOS CIII dimer, in addition to a complex consisting of OXPHOS CI, CIII dimer and CIV (Schägger & Pfeiffer, 2000, 2001). This CI+CIII2+CIV complex has since been designated as the respirasome, with this supercomplex constituting all of the necessary components required for electron transfer to oxygen, coupled with H⁺ extrusion across the IMM (Javadov et al., 2021). More recently, cryo-EM has revealed the structure of a human OXPHOS megacomplex constituting two of each CI, CIII and CIV, which forms a circular structure consisting of a CIII dimer at the central core, and CI and CIV distributed nonadjacent around this (Guo et al., 2017). Using this structure, and the structure of a porcine OXPHOS CII, the authors of this study also performed molecular dynamic modelling which suggest the OXPHOS megacomplex is permissible to associations with OXPHOS CII, forming an electron transport chain supercomplex (ETCS) inclusive of all components of the ETC (Guo et al., 2017).

Whilst the exact functional importance and/or role of mitochondrial OXPHOS supercomplexes remains to be completely understood, evidence suggests that the close association of these ETC components reduced diffusion distances which facilitate more efficient electron transfer through both the Q cycle from OXPHOS CI to CIII, and cytochrome C from CIII to CIV (Sousa et al., 2016). In addition, supercomplex formation is thought to enhance overall stability of the OXPHOS complexes, with evidence from studies investigating mitochondrial disease associated pathogenic variants and knockout mice revealing dysfunction to one OXPHOS complex can result in downstream functional and stability impairments in other OXPHOS complexes. For example, loss of OXPHOS CIII also causes a concomitant reduction in OXPHOS CI (Acín-Pérez et al., 2004), and loss of OXPHOS CIV in fact appears to cause an even more substantial reduction in OXPHOS CI (Balsa et al., 2012).

1.1.3.7 Proton-Motive Force

The electrochemical proton gradient (proton-motive force; ΔP) generated by the ETC serves a number of important mitochondrial functions, including its principal role in the synthesis of ATP through OXPHOS. Under normal physiological conditions influx of protons into the mitochondrial matrix predominantly occurs through the mitochondrial
ATPase (Complex V), which utilises the energy associated with $\Delta P$ to catalyse the phosphorylation of ADP with Pi to generate ATP.

The transfer of metabolic molecules into and out of the mitochondrial matrix largely occurs on the part of the IMM parallel to the OMM (inner boundary membrane). This highly selective transport is regulated by a number of different carrier proteins including: pyruvate translocase, phosphate carrier, adenine nucleotide transporter (ANT), dicarboxylate carrier and tricarboxylate carrier. The $\psi_m$ and chemical proton gradient provide directionality to many of these transport processes, highlighting another important role of the ETC in mitochondrial function. For example, pyruvate translocase transports negatively charged pyruvate$^-$ into the mitochondrial matrix in symport with H$^+$ thus utilising the chemical aspect of $\Delta P$. Similarly, the transfer of Pi$^-$ in symport with H$^+$ via the phosphate carrier is driven solely by the chemical proton gradient. In comparison the ANT relies only on $\psi_m$ to transport ATP$^4$ out of the mitochondrial matrix in exchange for a less negatively charged ADP$^3$ molecule.

1.1.4 Mitochondrial Matrix

The mitochondrial matrix contains a large range of enzymes involved in a number of different oxidative metabolic pathways which form the OXPHOS electron donors NADH and FADH$_2$ from their oxidised NAD$^+$ and FAD isoforms respectively. These include the $\beta$-oxidation pathway of fatty acid metabolism and pyruvate dehydrogenase (PDH) oxidation of pyruvate (final product of glycolysis). Both pathways generate the intermediate metabolite acetyl coenzyme A (acetyl-CoA) which is fully oxidised in a series of enzymatic reactions known at the tricarboxylic acid (TCA) cycle to generate three NADH and one FADH$_2$ reduced coenzymes for every acetyl-CoA molecule. TCA cycle and ETC activity are therefore intrinsically linked by the FAD/FADH$_2$ and NAD$^+$/NADH redox equilibriums.

1.1.5 Mitochondrial DNA

Another unique feature of the mitochondria organelle stemming back to its bacterial origin, is the presence of a small amount of its own mitochondrial DNA (mtDNA) in the matrix, separate to that contained within the nucleus (nDNA).
1.1.5.1 Genome structure

The mitochondrial genome is a closed circle of double stranded DNA (dsDNA) consisting of 16 569 base pairs (bp) that encodes 37 genes: 13 mitochondrial OXPHOS subunits and 24 genes required for mitochondrial translation (22 transfer RNAs; mt-tRNAs, and 2 ribosomal RNAs; mt-rRNAs) (Andrews et al., 1999) (Figure 1-2). Genes are compactly encoded on both the heavier more purine rich H-strand and lighter pyrimidine rich L-strand, with no intronic regions and very few intergenic regions (Taanman, 1999). The majority of genes are encoded on the H-strand: both mt-rRNAs, 14 of the mt-tRNAs and all mitochondrial encoded OXPHOS subunits except ND6 of Complex I. mtDNA has two non-coding regions, a major non-coding region (designated NCR) and the origin for light strand replication (OL). The major NCR contains the heavy strand promoters (HSP1 and HSP2) and light strand promoter (LSP) for transcription of the respective strands. The NCR also contains the origin for heavy strand replication (OH) and a triple-stranded region of DNA known as the displacement loop (D-loop) for which the actual functional role is unknown (Nicholls & Minczuk, 2014).
Figure 1-2 A schematic map of the 16 569bp human mitochondrial genome. The outer circle represents the heavy-strand (H-strand) inner circle the light-strand (L-strand). The location of coding and non-coding regions are shown as coloured and empty boxes respectively. mt-tRNA genes (grey) are shown as the abbreviation of the amino acid for which they are primed. The direction of mtDNA replication is indicated by arrows from the origins of H-strand (O_H) and L-strand (O_L). The direction of mtDNA transcription is shown as arrows from the H-strand promoters (P_H1 and P_H2) and L-strand promoter (P_L). Figure adapted from Picard, Wallace & Burelle, 2016

1.1.5.2 Nucleoid

mtDNA is condensed into compact nucleoprotein structures known as nucleoids which represent the fundamental unit of mtDNA inheritance. High resolution microscopy has revealed that human mt-nucleoids have a relatively uniform size of ~100 nm and each contain ~1.4 mtDNA molecules on average (Kukat et al., 2011). Mitochondrial transcription factor A (TFAM) is the most abundant protein of the mt-nucleoid (Bogenhagen, 2012) and alone sufficient to induce compaction of mtDNA into nucleoid structures (Kaufman et al., 2007) through non-sequence-specific cross-strand binding and bending of the mtDNA (Kukat et al., 2015). As its name would suggest, TFAM also plays a central role in mitochondrial transcription (discussed below).
1.1.5.3 Inheritance

In humans, every mitochondrial genome is derived from those originally present in the parental oocyte but not spermatozoa, and therefore maternally inherited (Giles et al., 1980), although rare instances of paternal inheritance of pathogenic mitochondrial genomes have been described (Gustafson et al., 2002; Kraytsberg et al., 2004; Luo et al., 2018). With exception of the Chinese hamster, mammalian spermatozoa mitochondria housed in the sperm tail do enter the oocyte during fertilization (Ankel-Simons & Cummins, 1996). The mitochondrial protein prohibitin is ubiquitinated in spermatozoa and more immature spermatid cells, which is thought to target the paternal mitochondria for degradation by the oocyte upon fertilization (Thompson, Ramalho-Santos & Sutovsky, 2003). In agreement, VCP-mediated proteasomal and ubiquitin-targeted autophagy pathways are both important for selective degradation of paternal mitochondria (Song et al., 2016).

1.1.5.4 Replication

Replication of mtDNA is carried out by DNA polymerase gamma (POLγ) which is a heterotrimeric assembly consisting of a catalytic subunit (POLγA) associated with two accessory subunits (POLγB). POLγ alone is insufficient for mtDNA replication, requiring the DNA helicase TWINKLE that unwinds and separates the dsDNA, and mitochondrial single stranded binding proteins (mtSSBs) that stabilise the separated single stranded DNA (ssDNA) and protect it from degradation (Diaz & Moraes, 2008). Initiation of mtDNA replication also requires the synthesis of an RNA primer by mitochondrial RNA polymerase (POLRMT) and is therefore dependant on components of the transcription machinery including TFAM (see below). Opposing models of mtDNA replication have been proposed, but the asynchronous strand displacement model is most widely accepted. In this model, POLRMT generates an RNA primer from the LSP, initiating POLγ DNA replication of the H-strand at OH. Upon the replication machinery passing the OL, a stem loop is formed from the separated ssDNA which inhibits the binding of mtSSBs at this site. POLRMT can then bind generating an RNA primer which initiates POLγ replication of the L-strand (Gustafsson, Falkenberg & Larsson, 2016).

1.1.5.5 Transcription

In addition to its requirement for mtDNA replication, POLRMT is central to transcription of the mitochondrial genome. Two other essential components of the mitochondrial transcription machinery are TFAM and Mitochondrial Transcription Factor B2 (TFB2M)
TFAM binds upstream of the transcription start site and through direct associations and alterations to the promoters structure recruits POLRMT (Gustafsson, Falkenberg & Larsson, 2016). TFB2M binds close to the transcription start site, where it facilitates melting of the promoter region (Sologub et al., 2009). Both the H-strand and L-strand are transcribed as long polycistronic products which and then processed into smaller mono- and bi-cistronic RNA molecules (Temperley et al., 2010). This is achieved through RNase P and RNase Z mediated cleavage of mt-tRNA that concurrently liberates the flanking mt-rRNA and mt-mRNA transcripts (Ojala, Montoya & Attardi, 1981). The cleaved mitochondrial transcripts are then processed further through 3’ polyadenylation (mt-mRNA and mt-rRNA), nucleotide modifications (mt-tRNA and mt-rRNA), 3’ CCA attachment (mt-tRNA), and aminoacylation (mt-tRNA).

### 1.1.6 Mitochondrial Translation

Central to the machinery for translation of mitochondrial encoded OXPHOS subunits is the mitochondrial ribosome which consists of a 28S small subunit (mt-SSU) containing 12S mt-rRNA and a 39S large subunit (mt-LSU) containing 16S mt-rRNA. Initiation of mitochondrial protein synthesis begins with the binding of mt-SSU to the start codon and recruitment of methionine charged mt-tRNA via mitochondrial translational initiation factor 2 (mt-IF2). mt-IF2 then catalyses the release of mitochondrial translational initiation factor 3 (mt-IF3) from the mt-SSU and binding of the mt-LSU. Elongation and peptide bond formation can then proceed through recruitment of charged mt-tRNA molecules by mitochondrial elongation factor tu (mt-EFtu). Mitochondrial elongation factor G1 (mt-EFG1) catalyses the release of deacylated mt-tRNA and movement of the ribosome to the next codon. Elongation proceeds until a stop codon is reached, at which point mitochondrial release factor 1a (mt-RF1a) binds to facilitate deacylation of the terminal amino acid and release of the nascent peptide (Mai, Chrzanowska-Lightowlers & Lightowlers, 2017).

### 1.1.7 Mitochondria Dynamics and Quality Control

The mitochondrial network of a cell is highly dynamic in nature, continuously growing through mitochondrial biogenesis, degrading by mitophagy, moving along the cytoskeleton attached to motor proteins, and changing in size and complexity through opposing fission and fusion events. Mitochondrial mass and network complexity are tightly regulated in order to maintain cellular homeostasis during changes in metabolic demands and/or substrate availability. This dynamic behaviour is also crucial for
maintaining a healthy mitochondrial network, through synthesis of new mitochondrial proteins, dilution of impaired components and selective removal of damaged mitochondria as a whole.

1.1.7.1 PGC1α and Transcriptional Regulation of Mitochondrial Biogenesis

Mitochondrial biogenesis is predominantly regulated through a tightly controlled transcriptional network. Although a large number of complex transcriptional cascades are necessary, these are all chiefly governed by one family of transcription co-activators, PGC1α and PGC1β of the peroxisome proliferator-activated receptor gamma coactivator 1 family (Scarpulla, 2008), often termed the master regulators of mitochondrial biogenesis. Among some of the most important PGC1 interactors are the nuclear respiratory factors NRF1 and NRF2, which control transcription of nuclear encoded subunits contributing to all five OXPHOS complexes (e.g. NDUFB8 (CI), SDHB/C/D (CII), UQCRB (CIII), COX5B (CIV), ATP5MC1 (CV)), in addition to proteins necessary for mitochondrial protein import (TOMM20), and translation (MRPS12) (Scarpulla, 2002). The PGC1-NRF complex also initiates TFAM expression, which regulates mtDNA synthesis, transcription and stability (outlined above), therefore co-ordinating mtDNA replication and gene expression with the expression of nuclear encoded mitochondrial genes. Another important target of PGC1 coactivation is the estrogen-related receptor (ERR) family of nuclear receptors which includes ERRα, ERRβ and ERRγ. PGC1-ERR complexes contribute to activating the expression of various nuclear encoded OXPHOS complex subunits (NDUFS7, SDHA, COX5B, ATP5F1B), in addition to genes encoding enzymes of the TCA cycle (ACO2, IDH3A), β-oxidation enzymes (ACADM, CPT1B) and mitochondrial fusion proteins (MFN2) (Giguère, 2008). PGC1 proteins are named so following the initial identification that this protein interacted with the PPARγ member of the peroxisome proliferator-activated receptor (PPAR) family. Since this identification PGC1 has also been shown to be key to activation of all three PPAR family members; PPARα, PPARγ and PPARδ. The three PPARs show distinct tissue expression patterns and some different physiological functions, but all play an essential role in controlling the expression of genes associated with fatty acid uptake and β-oxidation (Wang, 2010).

1.1.7.2 GTPases Regulating Mitochondrial Fission and Fusion

The fusion of two adjacent mitochondria into a single mitochondrial compartment requires the merging of both the OMM and IMM (Figure 1-3). Fusion of the OMM is mediated by the mitofusin (MFN) transmembrane GTPase family members MFN1 and
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MFN2 embedded within the OMM. IMM fusion is instead mediated by the long transmembrane isoforms of the OPA1 GTPase (L-OPA1) situated within the IMM.

Figure 1-3 Schematic of mitochondrial fission and fusion dynamics mediated by dynamin GTPases. Mitochondrial fusion first requires the intermixing of the juxtaposing outer mitochondrial membrane (OMM) mediated by mitofusin proteins (MFN1/2) followed by inner mitochondrial membrane (IMM) fusion driven by long OPA1 isoforms (L-OPA1). Mitochondrial fission first requires the recruitment of dynamin-related protein 1 (DRP1) by mitochondrial fission protein (MFF) and mitochondrial dynamics proteins 49 and 51 (MiD49, MiD51). DRP1 oligomers constrict driving the constriction and division of the OMM and IMM. This figure was prepared using Servier Medical Art (http://smart.servier.com/).

Until recently, models of OMM fusion have been made under the assumption that MFNs are double membrane spanning proteins with both the N-terminal and C-terminal domains being cytosolic. However, recent evidence has highlighted that MFNs are instead single membrane spanning proteins with the C-terminal being localised to the IMS rather cytosol (Mattie et al., 2018). Based on this recent insight a new model of OMM fusion has been proposed. Mitochondrial fusion first necessitates tethering of adjacent mitochondria through interactions between cytosolic GTPase domains of MFNs in trans (Cao et al., 2017). GTP hydrolysis then drives MFN conformational changes that pull the juxtaposing OMMs together and facilitates fusion through lipid-destabilising interactions mediated by the cytosolic HR1 heptad repeat domain (Daste et al., 2018).
Unlike OMM fusion which requires the trans expression of MFNs on both of the juxtaposing mitochondrial membranes to be fused, IMM fusion only necessitates the presence of L-OPA1 on the IMM of one of the two fusing mitochondria (Song et al., 2009). Recent evidence shows that tethering of the IMMs between two fusing mitochondria is mediated through L-OPA1 interactions with the mitochondrial IMM specific lipid cardiolipin, rather than trans protein interactions (Ban et al., 2017). GTP hydrolysis then drives a conformational change that destabilises the lipid bilayers and facilitates mixing of the juxtaposing IMMs, although the exact mechanism remains to be determined (Tilokani et al., 2018).

The dividing of a single mitochondrial compartment into two during mitochondrial fission, also necessitates a GTPase of the dynamin family: dynamin-related protein 1 (DRP1) (Figure 1-3). Through a multi-step process reliant on DRP1 adapter proteins including mitochondrial fission protein (MFF) and mitochondrial dynamics proteins 49 and 51 (MiD49, MiD51), DRP1 is recruited to the OMM (Osellame et al., 2016). MiD49 and MiD51 inhibit the GTPase activity of DRP1 and facilitate its assembly into oligomeric ring structures, whereas MFF stimulates DRP1 GTPase activity, promoting the release of MiD49 and MiD51, and an associated conformational change that constricts the assembled DRP1 ring inducing fission (Osellame et al., 2016; Kalia et al., 2018; Fonseca et al., 2019). Prior to DRP1 recruitment and fission, mitochondria are first pre-constricted by association with the endoplasmic reticulum (ER), in order to facilitate formation of the DRP1-ring (Friedman et al., 2011; Elgass et al., 2015).

1.1.7.3 Targeted Degradation of Mitochondria Through Mitophagy

Mitophagy is the process by which entire mitochondrial organelles are targeted for autophagy-mediated degradation. Although there is an ever-increasing number of different mechanisms of mitophagy being identified, they all fundamentally rely on labelling the OMM with ligands that interact with autophagy-related protein 8 (ATG8) receptors expressed on autophagosomal membranes.

Central to the large majority of mitophagy mechanisms is the phosphatase and tensin homologue-induced putative kinase 1 (PINK1) (Eiyama & Okamoto, 2015). In functional mitochondria, PINK1 is imported into the mitochondrial matrix through the translocases of the OMM (TOM) and IMM (TIM) due to its N-terminal mitochondrial targeting sequence (MTS) and the polarised $\psi_m$. PINK1 is then cleaved by the mitochondrial
processing protease (MPP) and subsequently the IMM-associated protease presenilin-associated rhomboid-like protein (PARL) (Jin et al., 2010), releasing a PINK1 fragment into the cytosol which rapidly undergoes proteasomal degradation (Yamano & Youle, 2013) (Figure 1-4A). In dysfunctional mitochondria which generally have a depolarised $\psi_m$, PINK1 does not fully translocate across the IMM for PARL cleavage and is instead stabilised in association with the TOM (Okatsu et al., 2012, 2013) (Figure 1-4B). PINK1 then undergoes autophosphorylation and enters an activated conformation (Okatsu et al., 2012). PINK1 activation through this mechanism is therefore specific to damaged mitochondria.

Figure 1-4 A schematic of the canonical PINK1/parkin-dependant mechanism of mitophagy. (A) Functional mitochondria import phosphatase and tensin homologue-induced putative kinase 1 (PINK1) into the mitochondrial matrix where it is cleaved by mitochondrial processing protease (MPP) and presenilin-associated rhomboid-like protein (PARL). The PINK1 cleavage product is released into the cytosol and undergoes proteasomal degradation. (B) Upon mitochondrial dysfunction and $\psi_m$ depolarisation PINK1 is stabilised on the OMM and activates through autophosphorylation. PINK phosphorylates and activates parkin ubiquitin-ligase. Parkin-dependant ubiquitination and ubiquitin phosphorylation by PINK targets the mitochondria for engulfment by autophagosome membranes and subsequent degradation. Figure adapted from Eiyama & Okamoto, 2015.

The most important PINK1 target associated with canonical mitophagy is the E3-ubiquitin ligase parkin. Phosphorylation of parkin’s ubiquitin-like domain induces a conformational change which stimulates its E3 ubiquitin ligase activity and promotes its translocation to the OMM (Kondapalli et al., 2012; Okatsu et al., 2012). Activated parkin
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ubiquitinates numerous proteins present on the OMM which are then bound by ubiquitin-binding autophagy receptor proteins including p62, optineurin and NDP52. These receptors recruit autophagosome membranes to the mitochondria through interactions with autophagy associated ATG8 receptors such as LC3 and resultant envelopment of the mitochondria within an autophagosome (Pickles, Vigié & Youle, 2018). Finally, the autophagosome fuses with a lysosome resulting in degradation of the mitochondrial components.

PINK1 also phosphorylates ubiquitinated proteins on the OMM of dysfunctional mitochondria, and free moving ubiquitin within the surrounding cytosol. Inactive parkin can be activated by free moving phosphorylated ubiquitin, and parkin binds to phosphorylated polyubiquitin chains and MFN2, highlighting PINK1-dependant feed forward amplification mechanisms for parkin activation and recruitment respectively (Ordureau et al., 2014; Chen & Dorn, 2013). Phosphorylated polyubiquitin chains are also more resistant to hydrolysis by deubiquitinase enzymes, further enhancing the mitophagy stimulating signal (Palikaras, Lionaki & Tavernarakis, 2018). Mitochondria also express mitophagy receptors which directly interact with autophagosome ATG8 receptors including, BCL2 interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L), which likely facilitate autophagosome formation.

In addition to this canonical mechanism which relies on $\psi_m$ depolarisation for activation, there are a number of other signalling pathways which can promote mitophagy flux. For example, mitophagy flux increases in response to hypoxia due to the transcriptional upregulation of $BNIP3$ and $BNIP3L$ expression by hypoxia inducible factor 1 α (HIF1α) (Zhang et al., 2008; Bellot et al., 2009). A transcriptionally driven upregulation in $BNIP3L$ expression also underlies the developmentally regulated clearance of mitochondria necessary for red blood cell maturation (Schweers et al., 2007).

1.1.7.4 Regulatory Mechanisms of Mitochondrial Dynamics

Activity-Dependant Regulation of $PPARGC1A$ Expression in Muscle

As the master regulator of mitochondrial biogenesis, PGC1α expression and activity are tightly regulated to reflect the metabolic demands of a cell. PGC1α is under the control of both transcriptional and post-translational regulation, with $PPARGC1A$ gene expression and PGC1α co-activator activity, increasing in response to greater energy demands or mitochondrial dysfunction. Transcription of $PPARGC1A$ is greatly
upregulated in skeletal muscle during exercise, and the mechanisms of action governing
PPARGC1A transcriptional regulation have been most extensively studied in this setting. During exercise, the stress-induced p38 mitogen-activated protein kinase (p38-MAPK) is activated, which phosphorylates myocyte enhancer factor 2 (MEF2) and activating transcription factor 2 (ATF2), both of which increase PPARGC1A expression (Akimoto et al., 2005). PPARGC1A upregulation in muscle is also driven through Ca\(^{2+}\)-sensitive mechanisms reliant on Ca\(^{2+}\)/calmodulin-dependent protein kinase IV (CaMKIV) and calcineurin protein phosphatase (CaN). CaMKIV phosphorylates cAMP response element (CRE) binding protein (CREB) and CaN dephosphorylates MEF2, both of which then bind to upstream promoters enhancing PPARGC1A expression (Handschin et al., 2003). PGC1\(\alpha\) also transcriptionally enhances the expression of MEF2, highlighting a positive feedback loop amplifying PGC1\(\alpha\) activity (Handschin et al., 2003).

**AMPK and SIRT1 as Key Metabolic Sensors Controlling Mitochondrial Biogenesis**

AMP-activated protein kinase (AMPK) is activated in response to rises in the AMP/ATP ratio and is therefore a key indicator of a cell’s metabolic state. Upon increased cellular energy demands (e.g. during muscle contraction), or impairments in ATP production (e.g. during glucose deprivation or mitochondrial dysfunction), AMPK becomes activated to repress anabolic processes while stimulating catabolic processes (Hardie & Sakamoto, 2006). AMPK exerts both acute and chronic effects on cell metabolism by directly phosphorylating metabolic enzymes and modulating transcription factor activity respectively. AMPK exerts much of its transcriptional effects by enhancing PGC1\(\alpha\) activity (Herzig & Shaw, 2018). While this was initially thought to be through transcriptional upregulation of PPARGC1A expression (Fernandez-Marcos & Auwerx, 2011), AMPK actually phosphorylates PGC1\(\alpha\) to directly enhance its co-transcriptional activity (Figure 1-5), including positive-feedback pathways (e.g. MEF2-dependant outlined above) responsible for PPARGC1A transcription upregulation (Jäer et al., 2007). PGC1\(\alpha\) is also phosphorylated by a number of other kinases including, p38-MAPK which increases both its activity and protein stability (Puigserver et al., 2001), and protein kinase B (PKB) which decreases its coactivating activity (Li et al., 2007).
Regulation of PGC1α activity through metabolically sensitive phosphorylation and acetylation modifications. The phosphorylation of PGC1α by AMP-activated protein kinase (AMPK) enhances its co-transcriptional activity. Acetylation of PGC1α by lysine acetyltransferase 2A (KAT2A) represses PGC1α activity. Deacetylation by the sirtuin deacetylase SIRT1 derepresses PGC1α activity. KAT2A, AMPK and SIRT1 activity are sensitive to alterations in the cellular energetic state and act as sensors regulating PGC1α activity and mitochondrial biogenesis.

PGC1α is also regulated through acetylation of numerous lysine residues, which represents another metabolically-sensitive mechanism mediating its activity (Rodgers et al., 2005) (Figure 1-5). Acetylation of PGC1α by lysine acetyltransferase 2A (KAT2A), causes it to dissociate from promoter regions and accumulate in nuclear foci, therefore reducing its coactivating potential (Lerin et al., 2006). During caloric excess through high fat feeding, KAT2A expression is upregulated leading to a reduction in PGC1α activity and energy expenditure, whereas the opposite is true upon calorific restriction during fasting (Coste et al., 2008). By comparison, levels of the NAD⁺-dependent sirtuin-1 (SIRT1) deacetylase responsible for PGC1α deacetylation are increased in the fasting state, also contributing to PGC1α deacetylation and activation (Rodgers et al., 2005). KAT2A and indeed all KAT enzymes rely on acetyl-CoA as the acetylation donor. PGC1α acetylation might therefore be affected by the nuclear-cytosolic availability of acetyl-CoA and provide an additional mechanism by which PGC1α activity is regulated.
by the metabolic state of a cell. Sirtuin deacetylases rely on NAD\(^+\) cofactors for their activity and for this reason sirtuin deacetylase activity is also tightly associated with the metabolic state of a cell. In times of increased metabolic demands and/or limited metabolic substrates, ATP levels and nucleocytoplasmic acetyl-CoA availability will be reduced, resulting in enhancement of PGC1\(\alpha\) activity through AMPK phosphorylation and reduced KAT2A acetylation respectively. In addition, cellular redox status will be more oxidised resulting in an increased availability of NAD\(^+\) for SIRT1, also contributing to PGC1\(\alpha\) deacetylation. Interestingly, AMPK activation not only promotes PGC1\(\alpha\) signalling directly by phosphorylation, but also indirectly by increasing cellular NAD\(^+\) levels via for SIRT1-dependant deacetylation of PGC1\(\alpha\) (Fulco \textit{et al.}, 2008; Cantó \textit{et al.}, 2009). Together these studies highlight a number of metabolic sensors which can be rapidly activated in response to mitochondrial dysfunction and/or changes in metabolic state, synergistically promoting mitochondrial biogenesis through PGC1\(\alpha\).

\(\psi_m\) and OXPHOS Activity Regulate Fission/Fusion

Cells with high oxidative metabolism typically contain a much more fused mitochondrial network and recent evidence highlights that fusion can alone enhance mitochondrial OXPHOS and is not just correlated with oxidative activity (Yao \textit{et al.}, 2019). Although OMM fusion occurs independently of mitochondrial activity or health, IMM fusion mediated by OPA1 is sensitive to changes in both OXPHOS and \(\psi_m\). L-OPA1 isoforms can be proteolytically cleaved by two different mitochondrial matrix metalloproteases: YME1L1 and OMA1. Cleavage of OPA1 by YME1L1 is ATP-dependant and stimulates IMM fusion, therefore acting as an energy-sensing mechanism to enhance the fusion of functional mitochondria (Mishra \textit{et al.}, 2014). On the other hand, OMA1 proteolysis of OPA1 is initiated in response to oxidative stress and \(\psi_m\) depolarisation, reducing OPA1 activity and consequently attenuating mitochondrial fusion (Baker \textit{et al.}, 2014). Mitochondrial activity and cell metabolism can also influence the recruitment of DRP1 and consequently mitochondrial fission. The DRP adaptor protein MiD51 prevents DRP1 oligomerization and GTP hydrolysis, but this inhibitory function is liberated upon binding of ADP (Losón \textit{et al.}, 2014). In addition AMPK phosphorylation of MFF, promotes DRP1 recruitment (Toyama \textit{et al.}, 2016). Activation of Ca\(^{2+}\)-sensitive calcineurin and dephosphorylation of DRP1 enhances mitochondrial fission. DRP1 activity is therefore indirectly linked to \(\psi_m\) depolarisation, due to elevations in cytosolic Ca\(^{2+}\) which accompany attenuated \(\psi_m\)-dependant Ca\(^{2+}\) buffering mechanisms (Cereghetti \textit{et al.}, 2008). Together these sensors of mitochondrial function, facilitate the fusion of active
and healthy mitochondria while segregating inactive and unhealthy mitochondria for targeted degradation through mitophagy (Mishra et al., 2014).

1.2 Mitochondrial Disease

Mitochondrial diseases are a heterogeneous group of disorders caused by primary genetic dysfunction of mitochondrial OXPHOS. Mitochondrial disease can present at any age, with a multitude of different symptoms and show almost any inheritance pattern. This heterogeneity makes both diagnosis and treatment/management difficult, often necessitating collaborative approaches between specialists across medical fields. Whilst there has been great improvement in mitochondrial disease diagnosis as a result of better clinical phenotypic descriptions and advancements in genetic screening, no therapeutic intervention has been shown to effectively alter the clinical course and instead treatment remains largely symptomatic (Poole, Hanna & Pitceathly, 2015).

1.2.1 Clinical Phenotype and Diagnosis

Patients present with a wide variety of symptoms that can be associated with any organ system (Figure 1-6), highlighting the ubiquitous importance of mitochondria in all nucleated cells of the body. Tissues with high oxidative metabolic requirements such as skeletal muscle and nervous system are particularly vulnerable (Mckenzie, Liolitsa & Hanna, 2004) and for this reason mitochondrial diseases are often termed encephalomyopathies (DiMauro et al., 2013). Initial diagnosis can prove difficult due to variability in clinical presentations, but a strong myopathic and neuropathic phenotype with concurrent involvement of multiple organs is highly suggestive of mitochondrial disease. Furthermore, several distinct but overlapping mitochondrial syndromes with a particular set of primary features have been described which can assist in clinical diagnosis, e.g. mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS), and myoclonic epilepsy with ragged red fibres (MERRF). Canonical features of MELAS include seizures, stroke-like episodes, encephalopathy, cortical vision loss, hearing impairment and myopathy (with lactic acidosis and ragged-red fibres; RRFs). Myopathy with RRFs is also a defining feature of MERRF syndrome alongside myoclonus, generalized epilepsy and ataxia.
Mitochondrial diseases can manifest a large variety of symptoms often associated with multiple organ systems simultaneously. Tissues that contain cells with high energy requirements utilising oxidative metabolic pathways are particularly vulnerable. Diagram obtained from (Gorman et al., 2016).
1.2.1.1 Muscle Histopathology Remains Key Hallmark for Mitochondrial Disease Diagnosis

As one of the primary tissues affected in mitochondrial diseases, muscle histopathology remains one of the gold standard approaches taken towards suspected mitochondrial disease diagnosis (DiMauro & Paradas, 2014). Ragged red-fibres relate to a histochemical observation in mitochondrial disease muscle biopsies stained with modified Gomori trichrome which reveal accumulation of subsarcolemma mitochondria, presumably as an attempted compensatory response to mitochondrial dysfunction (Figure 1-7). Other histological muscle biopsy observations suggestive of mitochondrial disease include strong succinate dehydrogenase (SDH; Complex II) stained fibres also indicative of mitochondrial proliferation, and fibres negative for cytochrome C oxidase (COX; Complex IV) staining indicative of impaired mitochondrial protein synthesis (McFarland, Taylor & Turnbull, 2010). A strong SHD fibre that is concomitantly negative for COX staining is a characteristic histopathological feature of mitochondrial disease, particularly those of mtDNA origin (Alston et al., 2017). Biochemical assays of OXPHOS complex activities can also be a useful tool for diagnosis and provide better insight into the specific OXPHOS complexes affected. More recently, a multiplex immunofluorescence assay that permits the simultaneous assessment of assembled OXPHOS complexes I and IV in patient muscle biopsies has been described (Rocha et al., 2015). In addition to providing better reliability and higher throughput, this histological technique permits better identification of isolated CI defects (Ahmed et al., 2017). Similar assays which utilise antibodies targeting other OXPHOS complexes might also prove useful for histopathological diagnoses of mitochondrial diseases predominantly affecting other OXPHOS complexes.
Figure 1-7 Example images showing typical histopathological staining patterns of muscle fibres from a healthy control (A and B) and mitochondrial disease patient (C-F; m.8344A>G MERRF patient) muscle biopsy. All healthy control muscle fibres stain positive for succinate dehydrogenase (SDH) (A) and cytochrome c oxidase (COX) (B). Staining of mitochondrial disease patient muscle fibres with the modified Gomori trichrome stain (C) reveals the presence of subsarcolemma mitochondria in ragged red fibres (RRFs) (arrows). These RRFs (arrows) also show strong SDH staining (D) suggestive of an increase in mitochondrial content but are negative for COX staining (E). COX staining also reveals the mosaic pattern of COX negative muscle fibres (indicated by *) (E) which generally show increased SDH staining and appear blue following combined SDH/COX staining (F). Figure adapted from DiMauro & Paradas, 2014.
1.2.1.2 Genetics

The mitochondrial genome encodes subunits of all OXPHOS complexes except Complex II (Signes & Fernandez-Vizarra, 2018), in addition to the RNA machinery required for their translation (Figure 1-1). The numerous other (~1500) mitochondrial proteins are encoded by the nuclear genome including: the majority of OXPHOS subunits, proteins involved in the trafficking and assembly of OXPHOS subunits and proteins involved in mtDNA maintenance and/or expression (Calvo & Mootha, 2010).

Nuclear Causes of Mitochondrial Disease

Pathogenic genetic variants in over 250 nDNA encoded mitochondrial proteins have been associated with mitochondrial disease (Mayr et al., 2015). In these cases, disease shows a classical recessive Mendelian inheritance that presents as a severe and rapidly progressing phenotype in early childhood (<16 years) (Lightowlers, Taylor & Turnbull, 2015). Minimum symptomatic prevalence of childhood-onset mitochondrial disease is approximately 5.0 per 100,000, with patients possessing pathogenic variants in the nuclear genome showing earlier onset than those with pathogenic variants of the mitochondrial genome (Skladal, Halliday & Thorburn, 2003). As with other inherited disorders, prevalence of mitochondrial diseases is greater within populations of high consanguinity such as the Australian-Lebanese population (58.6:100,000) (Skladal, Halliday & Thorburn, 2003).

MtDNA Disease

By comparison, mitochondrial disease caused by mtDNA pathogenic variants (mtDNA disease) show maternal inheritance and generally present later in childhood or adulthood. Minimum symptomatic prevalence of adult-onset mitochondrial disease is approximately 12.5 per 100,000, with the large majority of these cases been caused by pathogenic variants in the mitochondrial genome (9.6:100,000) rather than nuclear genome (2.9:100,000) (Gorman et al., 2015). Maternal inheritance alongside a myopathic associated phenotype is a highly specific suggestion of mtDNA disease, but mtDNA heteroplasmy and germline bottlenecks (see below) can often make this maternal inheritance pattern less evident.

mtDNA pathogenic variants can be broadly classified into three categories: 1) large-scale deletions, 2) variants which affect protein translation (mt-rRNA and mt-tRNA) and 3) variants which affect OXPHOS subunit structure (mt-mRNA) (Gorman et al., 2016).
Pathogenic variants in protein coding genes typically result in an isolated dysfunction to the OXPHOS complex it constitutes. Pathogenic variants within mt-rRNA and mt-tRNA have the potential to effect translation of all mitochondrial encoded subunits and consequently cause more universal OXPHOS dysfunction. Interestingly a disproportional amount of pathogenic variants are in mt-tRNA genes (McFarland, Taylor & Turnbull, 2010) which includes the m.3243A>G MT-TL1 pathogenic variant affecting mt-tRNALeu(UUR) and m.8344A>G MT-TK pathogenic variant affecting mt-tRNA^Lys.

The m.3243A>G pathogenic variant was initially identified in cohorts of MELAS patients (Goto, Nonaka & Horai, 1990; Kobayashi et al., 1990) and remains the most common cause of MELAS, accounting for approximately 80% of cases (El-Hattab et al., 2015). However, only ~18% of mtDNA disease patients harbouring the m.3243A>G pathogenic variant present as MELAS, with maternally inherited deafness and diabetes (MIDD) instead being the most common clinical syndrome recognised (~41%) (Nesbitt et al., 2013). m.3243A>G is by far the most common pathogenic variant identified, accounting for ~64% of mtDNA disease, and ~28% of all mitochondrial disease diagnoses (Gorman et al., 2015).

The m.8344A>G pathogenic variant was first identified in a cohort of MERRF patients (Shoffner et al., 1990) and remains the most common cause of MERRF syndrome to date (~80 %) (DiMauro & Hirano, 2015), but only accounts for <2% of total mitochondrial disease diagnoses (Gorman et al., 2015). The large majority of mtDNA patients harbouring m.8344A>G present with a phenotype that meets criteria for MERRF diagnosis (~60-70%), but rarely display all canonical features (Mancuso et al., 2013; Altmann et al., 2016). Whilst the MERRF acronym highlights epilepsy among the most common features of this syndrome, patients more commonly display an ataxic rather than generalised epileptic phenotype (Mancuso et al., 2013). Similar to m.3243A>G, m.8344A>G can also be associated with a wider phenotypic range including isolated myopathy with lipomatosis (Gámez et al., 1998; Muñoz-Málagà et al., 2000) and Leigh syndrome (Han et al., 2014; Shen et al., 2018).

**Genetic Diagnosis**

The m.3243A>G and m.8344A>G pathogenic variants, and MELAS and MERRF syndromes provide excellent examples of the complications facing mitochondrial disease diagnosis and genotype-phenotype correlations. One genotype can present multiple
different clinical phenotypes, and one clinical phenotype can be associated with several underlying genetic causes. Nevertheless, an accurate clinical profile together with specific histological and biochemical observations can guide targeted genetic analysis. In suspected cases of mtDNA disease (e.g. strong maternal inheritance and/or typical syndrome phenotype), common mtDNA pathogenic variants such as m.3243A>G and m.8344A>G are prioritised, followed by sequencing of the entire mitochondrial genome. For situations in which no pathogenic mtDNA variant is identified and/or Mendelian inheritance is evident, targeted sequencing of nuclear encoded mitochondrial genes individually or as part of a next generation sequencing (NGS) panel is performed. Histological and/or biochemical assessments of OXPHOS complexes can be informative for targeting genes associated with the function or assembly of the specific complexes impaired. Whole exome sequencing (WES) may then be considered, with published data highlighting a surprisingly high diagnostic rate of up to 60% in cohorts which have already undergone considerable genetic evaluation including exclusion of a mtDNA basis to the disease (Taylor et al., 2014).

1.2.2 MtDNA Heteroplasmy

Mitochondria have multiple copies of the mitochondrial genome, and each cell has numerous mitochondria. For this reason cells from mitochondrial disease patients can harbour both wild-type (WT) and mutant mtDNA (i.e. heteroplasmy) or entirely mutant mtDNA (homoplasmy), with the proportion of mitochondrial genomes containing a specific mtDNA pathogenic variant known as the mutation load (Stewart & Chinnery, 2015).

1.2.2.1 Heteroplasmic Shifts Caused by Mitochondrial Segregation and Clonal Expansion

Mutation load can drift in individual tissues over time through vegetative segregation in mitotic cells and relaxed replication in post-mitotic cells (Stewart & Chinnery, 2015). Vegetative segregation occurs due to the random, unequal partitioning of mutant and WT mtDNA during cell division such that two daughter cells can receive different proportions of mutant vs WT mitochondria. If the mutation load of the daughter cells reaches a threshold sufficient to exert a positive or negative effect on cell proliferation, heteroplasmy in the cell population will gradually increase or decrease respectively. The m.3243A>G but not m.8344A>G mutation load decreases in patient blood over time and it is thought that this occurs due to vegetative segregation and negative selective pressure.
in proliferating blood stem cells (Rajasimha, Chinnery & Samuels, 2008). This also highlights the importance of conducting sequential genetic testing for mtDNA disease in post-mitotic tissues such as muscle following a negative blood result in highly suspected mtDNA disease cases. Mitochondria are continuously proliferating and dividing even in post-mitotic tissues. It is possible that by random chance, mtDNA with/without specific pathogenic variants can be replicated more often and thus lead to changes in mutation load within an individual cell. This relaxed replication can lead to clonal expansion of mtDNA pathogenic variants in individuals throughout life and cause OXPHOS dysfunction in previously unaffected tissues (Elson et al., 2001). Relaxed replication also likely underlies the clonal expansion of mitochondrial genomes possessing deletions at specific foci within individual muscle fibres (Vincent et al., 2018).

### 1.2.2.2 Mitochondrial Bottleneck During Germline Transmission

In addition to gradual drifts of mtDNA mutation load in individuals over time, a “bottleneck” causes marked changes in heteroplasmy during germline transmission (Wallace & Chalkia, 2013). Initial studies with mice had shown that a reduction in mtDNA copy number in primordial germ cells (PGCs) (Cree et al., 2008) and/or selective replication of a small fraction of mtDNA in primordial follicle cells (Wai, Teoli & Shoubridge, 2008) may be responsible for this bottleneck. More recently this reduction in mtDNA copy number has also been shown in human PGCs (Floros et al., 2018). Although this bottleneck can give rise to oocytes with above threshold mutation loads, non-synonymous pathogenic variants are in fact negatively selected against during PGC development, highlighting an evolutionary mechanism to prevent accumulation of pathogenic mtDNA variants within a population.

### 1.2.2.3 Mutation load Threshold Effect

Mutation load sometimes correlates with disease severity, however a very high heteroplasmy level (>90%) is generally required for mitochondrial function to be sufficiently impaired for disease presentation (Rossignol et al., 2003; Stewart & Chinnery, 2015). While heteroplasmy levels measured from bulk tissues of affected patients are often less than 90%, this is likely due to the heterogenous nature of mutation loads between individual cells, with proportions of cells been both above and below the detrimental threshold level. Indeed, individual muscle fibres from mtDNA disease patients harbouring m.3243A>G or m.8344A>G show a large range of different mutation loads, with higher mutation loads (>90%) being evident in muscle fibres that show
histological abnormalities (e.g. RRFs or negative COX staining), and much lower loads being evident in apparently normal muscle fibres (Petruzzella et al., 1994; Moslemi et al., 1998; Koga et al., 2000).

The severity, progression and onset of mtDNA disease caused by m.3243A>G or m.8344A>G are at least partially correlated with mutation load (Mancuso et al., 2014; Pickett et al., 2018; Grady et al., 2018; Hammans et al., 1993). Patients with identical heteroplasmy levels can express very different phenotypes, and some individuals with high load are relatively asymptomatic (Boggan et al., 2019; Altmann et al., 2016), suggesting additional contribution from environmental or genetic modifiers.

**Figure 1-8** The threshold effect of heteroplasmic mtDNA pathogenic variants. The proportion of mutant mitochondrial genomes within an individual cell of a heteroplasmic mtDNA disease patient can vary from entirely healthy (0%) to homoplasmic (100%) levels. At heteroplasmy levels <90%, mitochondrial function is maintained at sufficient levels for apparently normal cellular function. However, at heteroplasmy levels >90% compensatory mechanisms are insufficient and phenotypic abnormalities underlying the mitochondrial disease state occur.

### 1.3 In Vitro Models of mtDNA Disease

Establishing model systems that appropriately recapitulate human diseases has always proven difficult and this is especially true for mitochondrial diseases caused by mtDNA pathogenic variants. Advancements in gene-editing technologies has permitted the manipulation of many nuclear encoded genes for developing both in vivo animal models and in vitro cell models of various human diseases, including those caused by pathogenic variants in nuclear encoded mitochondrial genes (Thompson et al., 2020). Manipulating the mitochondrial genome has proven to be much more difficult in comparison (Gammage, Moraes & Minczuk, 2018), and for this reason researchers have instead
selected for cells or organisms with native mtDNA pathogenic variants, and/or transferred mutant mtDNA of interest through whole mitochondrial transfer (Wilkins, Carl & Swerdlow, 2014). With the advent of human induced pluripotent stem cell (hiPSC) technology it has become possible to directly reprogram primary human cells harbouring disease causing mtDNA pathogenic variants back into a pluripotent state, providing an inexhaustible supply of patient specific cell progeny for differentiation into disease relevant cell types. Hence, hiPSC modelling not only circumvents the problems associated with mtDNA manipulation but also provides a more abundant source of otherwise inaccessible human cell types for mitochondrial disease research purposes.

1.3.1 Road-Block Preventing Editing of mtDNA for Heteroplasmic Disease Modelling

There has been great progress in targeting nucleotide sequence specific enzymes including: restriction endonucleases (Srivastava & Moraes, 2001), zinc-finger nucleases (ZFNs) (Gammage et al., 2014) and transcription activator-like effector nucleases (TALENs) (Bacman et al., 2013), to the mitochondria in order to initiate double-stranded breaks (DSBs) at specific mtDNA haplotypes/pathogenic variants. Unlike the nucleus, mitochondria lack efficient DSB repair mechanisms and for this reason mitochondrial genomes cut by such constructs rapidly undergo degradation. Whilst these tools offer great promise as therapeutic interventions to shift mutant heteroplasmy towards WT homoplasmy, they alone are unable to introduce or correct single-nucleotide changes in mtDNA encoded genes for disease modelling purposes. For this same reason, CRISPR-associated protein 9 (Cas9) technology remains insufficient for mtDNA editing purposes and is additionally faced with challenges in introducing the necessary guide RNA (gRNA) components into the mitochondrial matrix (Gammage, Moraes & Minczuk, 2018). Concurrent targeting of DSB repair machinery to the mitochondria might represent a strategy that permits single-nucleotide editing of mtDNA in the future.

1.3.2 Taking Advantage of Primary Cells from mtDNA Disease Patients

Due to the challenges of introducing single nucleotide changes into the mitochondrial genome at present, currently the most efficient way to establish mtDNA disease models is by selection of mitochondria with pathogenic variants in the mitochondrial genome. Cells cultured from patients with mtDNA disease are the primary source of mutant mitochondria and include: dermal fibroblasts, lymphoblasts and myoblasts. In addition to the use of patients’ cells directly as an in vitro model of mitochondrial disease, the
mitochondria contained within them have also been transferred to cell types of interest to create cytoplasmic hybrid cells.

### 1.3.2.1 Primary Fibroblasts, Lymphoblasts and Myoblasts

Fibroblasts and lymphoblasts are among the most readily available proliferative cell types for diagnostic and research purposes, obtained with relative ease through culturing outgrowth from a minimally invasive punch skin biopsy explant and centrifugation purification from whole blood respectively. Establishing a myoblast culture is more difficult by comparison, requiring a more invasive muscle biopsy and slower proliferating explant outgrowth that typically necessitates purification to exclude fibroblasts and other contaminating cell types (Spinazzola & Gussoni, 2017). Although the use of these primary cell types has several limitations that will be discussed below, they have given much insight into the biochemical and cellular pathomechanisms of mtDNA disease and served as a useful platform for the discovery/testing of compounds and intervention strategies with potential therapeutic effect. Due to the great abundance of literature describing the use of these cell types for mitochondrial disease research, this section will focus specifically on cells possessing the m.3243A>G pathogenic variant in mt-tRNA\(_{\text{Leu(UUR)}}\) or m.8344A>G pathogenic variant in mt-tRNA\(_{\text{Lys}}\) most relevant to the work presented in this thesis.

**Use of Primary Models to Explore mtDNA Disease Pathomechanisms**

In one of the first studies to utilise mtDNA disease patient fibroblasts Moudy et al. showed cells from 5 different MELAS patients with 3 different mt-tRNA pathogenic variants (3 m.3243A>G, 1 m.3271T>C and 1 m.8344A>G) have a more depolarised \(\psi_m\) and accompanying impairments in intracellular Ca\(^{2+}\) buffering (Moudy et al., 1995). mtDNA disease fibroblasts with heteroplasmic m.3243A>G or m.8344A>G also display reduced OXPHOS complex activities, particularly CI, and a reduction in cellular oxygen consumption rate (OCR) (James et al., 1996) confirming ETC dysfunction as the root mechanism underlying disease. In cultured myoblasts from a MERRF patient which harbour m.8344A>G, synthesis of mtDNA encoded subunits of OXPHOS CI and CIV was shown to be impaired (Hanna et al., 1995). By taking advantage of the natural heterogeneity of m.8344A>G mutation load in individual myoblasts, the researchers established clonal myoblast lines with differing mutation loads and additionally showed the severity of this translation impairment was correlated with mutation load and detectable at loads as low as 35 %.
In a recent publication, precise live cell fluorescent imaging techniques were used to probe more deeply the bioenergetics consequences of m.3243A>G and m.8344A>G in mtDNA disease fibroblasts (Kovac et al., 2019). Unlike previous studies, $\psi_m$ was only slightly more depolarised in m.3243A>G and m.8344A>G fibroblasts. However, assessing changes in $\psi_m$ following the sequential addition of mitochondrial toxins: oligomycin A (CV inhibitor) and rotenone (CI inhibitor), revealed a reduction in the contribution of CI and compensatory contribution of CV working in reverse to $\psi_m$ polarisation. The reductions in CI activity were supported by reduced NADH redox indices (basal level of NAD+/NADH pool in NAD+ state). Impairments in mitochondrial Ca$^{2+}$ handling were also confirmed, in addition to an increased propensity for mitochondrial permeability transition pore (mPTP) opening in response to repetitive Ca$^{2+}$ transients, imitating those observed in active neurons and myocytes.

Intracellular ATP concentration and intracellular ATP/ADP ratio are surprisingly unaffected in fibroblasts with the m.3243A>G or m.8344A>G pathogenic variants, but this is due to the relatively low energy requirements of fibroblasts under basal conditions (James et al., 1999). Disruption of monovalent cation gradients using 10 ng/ml gramicidin, increases the demand for the plasma membrane Na/K$^+$ ATPase transporter and results in a lower ATP/ADP ratio in mtDNA disease fibroblasts, yet at the same gramicidin concentration the ATP/ADP ratio of control fibroblasts is unaffected (James et al., 1999). In accordance with this, lymphoblasts harbouring m.3243A>G have reduced ATP synthesis rates (Marriage et al., 2003) and cellular ATP levels (Kariya et al., 2005). Interestingly, the ATP/ADP ratio of myoblasts possessing m.3243A>G is largely unaffected, but significant reductions in cellular ATP levels and total ATP+ADP pool are observed (Rusanen, Majamaa & Hassinen, 2000).

Fibroblasts fostering a number of different mtDNA pathogenic variants including m.3243A>G and m.8344A>G display elevated levels of ROS and oxidative protein damage (Wu et al., 2010; Voets et al., 2012), confirming an additional cellular pathomechanism contributing to disease. In line with this, patient myoblasts harbouring m.3243A>G have increased superoxide dismutase and catalase enzyme activities (Rusanen, Majamaa & Hassinen, 2000) presumably as a compensatory cellular response to protect from elevated ROS levels.

Mitochondrial metabolism and health are closely linked to the dynamic structure of the mitochondrial network through control of mitochondrial fission and fusion.
Pharmacological dissipation of $\psi_m$ and/or increases in ROS levels both impair mitochondrial fusion events, and promote a more punctate rather than tubular mitochondrial network (Legros et al., 2002; Jendrach et al., 2008). In line with this, fibroblasts harbouring m.3243A>G or m.8344A>G have a punctate network consisting of more swollen and globular mitochondria (De la Mata et al., 2012; Lin et al., 2019b). Similar alterations in the structure of the mitochondrial network are also observed in cultured myoblasts possessing m.3243A>G (Sládková et al., 2015). In relation to alterations in the structure of the mitochondrial network, patient fibroblasts harbouring m.3243A>G show greater autophagic activity and colocalisation of small globular mitochondria with autophagy markers, indicative of an increase in mitophagic flux (Cotán et al., 2011). Inhibition of autophagy specifically reduces the viability of patient fibroblasts harbouring m.3243A>G but not control fibroblasts, revealing increased mitophagic flux is serving a protective function in these cells. Culturing heteroplasmic patient fibroblasts possessing m.3243A>G under energetically stressing conditions reduces m.3243A>G load in a mitophagy dependant manner (Diot et al., 2015), suggesting this raised mitophagic flux is specifically targeted against mitochondria harbouring m.3243A>G. A similar increase in mitophagic flux is also observed in patient fibroblasts harbouring m.8344A>G (De la Mata et al., 2012).

**Testing and Validation of Therapeutic Strategies in Patient Cells**

In addition to improving our understanding of the cellular consequences caused by mtDNA pathogenic variants, primary cells from mtDNA disease patients have also served as useful models for testing and validating potential therapeutic strategies.

Due to its important role in mitochondrial ETC function and ability to scavenge ROS, CoQ$_{10}$ has been recommended as a supplement to mitochondrial disease patients for a number of years, although there still remains limited evidence of clinical efficacy (Pfeffer et al., 2012). Patient fibroblasts harbouring m.3243A>G or m.8344A>G show evidence of CoQ$_{10}$ deficiency and supplementation of culture media with exogenous CoQ$_{10}$ was shown to fully or partially restore numerous measures of mitochondrial dysfunction (Cotán et al., 2011; De la Mata et al., 2012), suggesting that at least in these patients specifically, CoQ$_{10}$ supplementation might be of some therapeutic benefit. Besides replicating the beneficial effect of CoQ$_{10}$ supplementation, a subsequent study showed beneficial effects of riboflavin (vitamin B2) on fibroblasts harbouring m.3243A>G (Garrido-Maraver et al., 2012). As the sole precursor for flavin cofactors that are
indispensable for the function of numerous mitochondrial enzymes including subunits of the ETC and antioxidant enzymes (O’Callaghan, Bosch & Houlden, 2019), riboflavin is probably exerting its beneficial effect in similar ways to that of CoQ_{10} described above. Resveratrol is a polyphenol that increases PGC1α signalling and mitochondrial biogenesis (amongst other mechanisms), which improves the respiration of patient fibroblasts fostering m.3243A>G or m.8344A>G (Mizuguchi et al., 2017), highlighting enhancement of mitochondrial biogenesis as a potential therapeutic intervention. Using a more novel strategy, WT donor mitochondria conjugated to a cell-penetrating peptide have been shown to be successfully incorporated in fibroblasts harbouring m.8344A>G and at least partially rescue various measures of mitochondrial dysfunction and cell viability (Chang et al., 2013a, 2013b).

1.3.2.2 Cytoplasmic Hybrids (Cybrids)

Primary cells taken from patients have provided great insight into underlying pathomechanisms of various human diseases, but the relatively slow proliferation rates, low protein contents and limited number of passages they can be expanded before entering senescence limits the material available for research purposes. Immortalisation by transformation with pro-oncogenic viruses has been one strategy successfully utilised by researchers to overcome some of these problems (Reddy et al., 2015; Chin et al., 2018). In relation to mtDNA disease however, the most widely used method for establishing a proliferative cell line harbouring mutant mtDNA of interest has been through creation of cytoplasmic hybrid (cybrid) cells. King and Attardi’s landmark paper describing the establishment of human cybrid cells (King & Attardi, 1989) was published only one year after the first descriptions of disease causing pathogenic variants in the mitochondrial genome (Holt, Harding & Morgan-Hughes, 1988; Wallace et al., 1988), and have remained one of the go to cell models of mtDNA disease ever since.

Cybrid Cell Generation

Cybrid cells are created by fusing cytoplasts from mtDNA disease patient cells with a proliferative cell line (typically of cancerous origin) that has been depleted of mtDNA (termed ρ0 cells) (Figure 1-9). To generate human ρ0 cells, a cell line is cultured in the presence of a low concentration of ethidium bromide (EtBr) DNA intercalating dye or other mtDNA replication inhibitor, sufficient to completely inhibit mtDNA replication but which does not impact nDNA replication (King & Attardi, 1996). ρ0 cells are auxotrophic for pyrimidines as a consequence of inefficient ETC coupling with important
pyrimidine biosynthetic steps, and pyruvate due to deficient maintenance of cellular redox status for non-oxidative metabolism (King & Attardi, 1989). For these reasons ρ0 cell generation and culture requires media to be additionally supplemented with uridine (50 μg/ml) and pyruvate (0.5 mM) respectively. Cells obtained from mtDNA patients are enucleated by density centrifugation in the presence of cytochalasin (actin filament destabiliser), giving rise to patient-derived cytoplasts containing the mutant mitochondria of interest. Platelets are now commonly used as the donor cytoplasm as they naturally lack a nucleus and can be isolated from whole blood samples with relative ease (Chomyn et al., 1994). The cytoplasts and ρ0 cells are then fused most commonly using polyethylene glycol (PEG), and individual clones expanded in order to generate stable cybrid lines containing the mutant mtDNA of interest.

![Diagram](image)

**Figure 1-9 Schematic overview of cybrid cell generation.** Enucleated cytoplasts from a mtDNA disease patient are fused with immortalised cells lacking mtDNA (ρ0). Fused cells are then clonally expanded to obtain isogenic lines with various heteroplasmy levels.

**Use of Cybrids for mtDNA Disease modelling**

Besides their greater proliferative potential, cybrid cell lines offer several other benefits over primary patient cells for studying mtDNA diseases. This includes the ability to establish lines with various levels of mtDNA mutation load for probing the threshold effect of a specific mtDNA pathogenic variant. In addition, cell lines with higher levels of mtDNA mutation load including homoplasmy can be obtained which might otherwise not be present in a primary cell culture. Furthermore, cybrid cell lines remove any confounding factors associated with nuclear gene variations and thus allow for better controlled studies focused on the specific mtDNA pathogenic variant of interest. This can
also be considered a limitation to the use of cybrid cell models however, as nuclear variation is likely an important factor contributing/modifying patient phenotype (Chen, Chen & Guan, 2015). In line with this, cybrid cells represent an excellent tool for investigating nuclear-mitochondrial compatibility which has become particularly important to understand given recent advancements in mitochondrial replacement strategies being offered to mtDNA disease females as part of \textit{in vitro} fertilisation (IVF) procedures (Herbert & Turnbull, 2018). Studies in inbred mouse strains show nuclear-mitochondrial compatibility to be crucial for fertilised egg viability (Ma \textit{et al.}, 2016), in more diverse outbred species (including humans) such incompatibility is much more unlikely (Eyre-Walker, 2017), but might still warrant further investigations for improving IVF efficiency.

**Novel Observations From mtDNA Disease Cybrids**

Alongside the use of primary patient cells, cybrids have been instrumental in delineating the bioenergetic and cellular consequences of various mtDNA pathogenic variants and testing of potential therapeutic strategies. For brevity, only studies which describe novel observations utilising cybrid cells harbouring the m.3243A>G or m.8344A>G pathogenic variants will be discussed.

Using cybrid cells, direct consequences of the m.3243A>G and m.8344A>G pathogenic variants on mt-tRNA stability and function have been revealed. Consistent between publications, cybrid clones possessing high m.3243A>G heteroplasmy have reduced levels of leucine loaded mt-tRNA$_{Leu(UU\text{R})}$ due to a reduction in the total amount of mt-tRNA$_{Leu(UU\text{R})}$ and additionally the proportion aminoacylated (Janssen, Maassen & Van Den Ouweland, 1999; Chomyn \textit{et al.}, 2000; Park, Davidson & King, 2003). The m.3243A>G pathogenic variant also interferes with the post-transcriptional addition of taurine to the mt-tRNA$_{Leu(UU\text{R})}$ wobble anticodon uridine (Figure 1-10; $\tau_m^5\text{U}$) (Yasukawa \textit{et al.}, 2000, 2005), which is a modification necessary for efficient decoding of UUG leucine codons (Kirino \textit{et al.}, 2004). An initial publication suggested m.8344A>G also reduces mt-tRNA$_{Lys}$ stability and its aminoacylation with lysine (Antonio Enriquez \textit{et al.}, 1995), but such impairments were not always observed in subsequent studies (Borner, 2000; Yasukawa \textit{et al.}, 2001). m.8344A>G does however negatively impact mt-tRNA$_{Lys}$ recognition of lysine codons (Yasukawa \textit{et al.}, 2001) due to impaired post-transcriptional wobble modification of the third anticodon uridine of mt-tRNA$_{Lys}$ (Fig. x; $\tau_m^5\text{s}^2\text{U}$) (Yasukawa \textit{et al.}, 2005).
Figure 1-10 Cloverleaf secondary structures of mt-\text{tRNA}^{Leu(UUR)} and mt-\text{tRNA}^{Lys} showing the positions of the m.3243A>G and m.8344A>G pathogenic variants respectively. Post-transcriptionally modified nucleotides are indicated by: \textit{m}^{1}A (1-methyladenosine), \textit{t}^{6}A (N6-threoninocarbonyladenosine), \textit{m}^{1}G (1-methylguanosine), \textit{m}^{2}G (2-methylguanosine), Ψ (pseudouridine), \textit{T} (ribothymidine), \textit{U} (dihydrouridine), \textit{m}^{2}C (5-methylcytidine), \textit{τm}^{5}U (5-taurinomethyluridine) and \textit{τm}^{5}s^{2}U (5-taurinomethyl-2-thiouridine). Figure adapted from Yaskuawa et al., 2005

Taking advantage of the ability to establish cybrid cell lines with differing mutation loads, researchers have explored the threshold effect revealing that a surprisingly high proportion (>90%) of m.3243A>G (Chomyn et al., 1992) or m.8344A>G (Chomyn et al., 1994) is required to sufficiently impair mitochondrial translation and negatively impact cellular respiration.

Cybrids harbouring m.3243A>G have also been established from cancerous/immortalised cell lines with identities resembling tissues typically affected in mitochondrial disease. Muscle-like rhabdomyosarcoma (Vergani et al., 2007) and neuronal-like neuroblastoma (Desquiret-Dumas et al., 2012) cybrids with near homoplasmic levels of m.3243A>G show evidence of oxidative stress and, abnormalities in mitochondrial distribution, morphology and ultrastructure. Interestingly, these abnormalities were only observed in fused myotube-like rhabdomyosarcoma cybrids and not the proliferative myoblast-like progeny, highlighting the importance of performing phenotypic assays on disease appropriate cell-types. Using immortalised brain capillary endothelial cybrids,
mitochondrial dysfunction caused by m.3243A>G has also been shown to diminish the integrity of the blood brain barrier (Davidson, Walker & Hernandez-Rosa, 2009).

**Testing and Optimisation of Potential Therapeutic Strategies in mtDNA Cybrids**

Using cybrids harbouring m.8344A>G (Kolesnikova et al., 2004), researchers showed that modified yeast mt-tRNA^{Lys} derivatives expressed in the nucleus are successfully imported into the mitochondria, aminoacylated, and improve mitochondrial translation. This strategy was subsequently used by the same group to design a yeast mt-tRNA derivative recapitulating mt-tRNA^{Leu} function in order to improve mitochondrial translation in cybrids harbouring m.3243A>G (Karicheva et al., 2011). In both instances, a beneficial impact on various measures of mitochondrial function including ψ_m and ETC enzyme activities accompanied these improvements in mitochondrial translation.

Mitochondrial targeted TALENs (mitoTALENs) that specifically cleave mutant mitochondrial genomes represents a promising therapeutic strategy for reducing mutation load in patients with heteroplasmic mtDNA diseases. Using cybrids cells harbouring heteroplasmic levels of m.8344A>G, researchers showed successful targeting of mitoTALENs specific for m.8344A>G to the mitochondria and accompanying decrease in mutation load (Hashimoto et al., 2015; Pereira et al., 2018). The reductions in m.8344A>G mutation load resulted in improvements in activity of OXPHOS CIV and overall cellular respiration.

**Recent Evidence Showing Epigenetic Aberrations in mtDNA Disease Cybrids**

Recently, publications from the Wallace laboratory have utilised cybrid cells with a range of different m.3243A>G heteroplasmy levels from 0 % to 100 %, to explore mutation load dependant changes on the cellular transcriptome and epigenome.

Whilst increasing m.3243A>G heteroplasmy causes a staggered decrease in levels of mtDNA encoded OXPHOS complex subunits, sharp and distinct changes in transcriptional profiles occur (Picard et al., 2014). Principal component analysis revealed four distinct gene expression profiles which could be separated into groups based on cybrid heteroplasmy level: 0%, 20-30%, 50-90% and 100%. Among the defining differences, 20-30% cybrids showed downregulation of mTOR and other growth signalling pathways and 50-90% cybrids showed an increase in expression of glycolysis and mitochondrial genes (both mtDNA and nDNA encoded). 100% cybrids were
comparable to 50-90% in many ways, including increased expression of mitochondrial genes, but there were notable differences which included limited induction of glycolytic gene expression. The authors also noted changes in the expression and activity of numerous epigenetic modifying enzymes, with differences in histone acetyltransferase (HAT) and Zn\(^{2+}\)-dependant (class I/II) histone deacetylase HDAC (HDAC) activity being most pronounced.

Using LC-MS the cellular levels of various intermediate metabolites and epigenetic histone modifications have been simultaneously measured in cybrids with different m.3243A>G heteroplasmy (Kopinski et al., 2019). Using this technique, correlations were made between different TCA cycle intermediates and specific histone modifications. Among the most interesting observations, levels of: H3K9ac, H3K14ac, H3K18ac, H3K27ac, H4K8ac and H4K16ac were all positively correlated with citrate and acetyl-CoA levels. With increasing m.3243A>G heteroplasmy level there was a decline in cellular citrate and acetyl-CoA levels, presumably because of impaired oxidative metabolism. This was accompanied by reduced levels of the various histone acetylation marks outlined above, with levels of H4 acetylation modifications in particular showing a negative association with increasing m.3243A>G heteroplasmy. Using heavy isotope glucose labelling and flux analysis, mitochondrial derived acetyl-CoA was shown to serve as a substrate for H4 acetylation, and this contribution was reduced in cybrids with \( \geq 90\% \) levels of m.3243A>G. The NADH autofluorescence lifetime is an indirect measure of the NAD\(^{+}\)/NADH redox index when it is assumed that the total NAD(H) pool remains stable. Using this technique, it was shown that the NADH lifetime declined with increasing m.3243A>G mutation load, but at heteroplasmy levels \( \geq 70\% \) a compensatory increase in the expression of NAD de novo synthesis genes was observed, resulting in an apparent increase in NADH lifetime at these higher heteroplasmy levels. Even though no certainty in the absolute availability of NAD\(^{+}\) for nuclear class III sirtuin HDACs can be made, these data suggests NAD\(^{+}\) levels might be largely maintained or in fact greater in cells with high m.3243A>G burden. Relevant to the work in this thesis, these publications highlight histone acetylation as an epigenetic modification sensitive to mitochondrial dysfunction, at least partly through regulation of nuclear acetyl-CoA availability for histone acetyltransferase enzymes, with alterations in nuclear NAD\(^{+}\) availability for sirtuin enzymes possibly contributing in concert.
1.3.3 Limitations of Commonly Used In Vitro Cell Models of mtDNA Disease

While primary human cells and cancerous cybrid cell lines have given much insight into underlying mitochondrial, bioenergetic and in some instances novel downstream pathomechanisms of mtDNA disease, there are several important limitations associated with their use which must be considered. Some of these limitations are technical and not specific for mtDNA disease including the slow proliferation rate of primary cells, cytogenetic abnormalities associated with cancerous cell lines and lack of disease-relevance.

In relation to mtDNA disease there are additional factors that must be considered. Unlike the tissues and cell types typically affected in mtDNA disease patients, proliferative primary cells and cancerous cell lines rely predominantly on glycolysis to meet their energetic and biosynthetic demands (Ghesquière et al., 2014). The downstream phenotypes observed in these cell types might be of little relevance to the in vivo patient setting and present false negatives due to minimal reliance on oxidative mitochondrial metabolism. This is particularly well illustrated by a publication described in 1.3.2.2, which showed phenotypic impairments in more terminally differentiated myotube-like cells that were not also observed in the proliferative myoblast-like progeny (Vergani et al., 2007). Indeed, it is often necessary to place glycolytic favouring cell types under some form of stress that promotes the use of oxidative metabolism in order to reveal a cellular phenotype, such as culturing in galactose rather than glucose containing media (Diot et al., 2015), or disrupting electrochemical gradients across the plasma membrane (James et al., 1999). Clonally established cell lines from heteroplasmic mtDNA disease patient biopsies do permit investigations into the impact of different mtDNA mutation loads whilst maintaining a patient’s nuclear background. However, the limited proliferative potential of primary cells, particularly clonally expanded cells, restricts the number and range of experimental assays that can be performed.

1.4 Patient Derived Human Induced Pluripotent Stem Cells as a Better Model for mtDNA Disease

Human induced pluripotent stem cells (hiPSCs) are an artificial stem cell population generated by a process known as cell reprogramming. Through the forced expression of four transcription factors (sex determining region Y-box 2, SOX2; octamer-binding transcription factor-4, OCT4; Kruppel-like factor 4, KLF4 and c-Myc), terminally
differentiated somatic cells (e.g. dermal fibroblasts from skin biopsies) can be pushed backwards in development to a pluripotent state resembling that of human embryonic stem cells (hESCs) (Takahashi et al., 2007). hiPSCs can proliferate indefinitely and by definition differentiate into cell-types from of all three germ layers (mesoderm, ectoderm and endoderm).

Establishment of hiPSCs from mtDNA disease patients offers numerous benefits over other in vitro cell modelling systems including: potential to culture an almost unlimited amount of cell material for research assays, ability to obtain typically affected disease relevant human cell-types, maintenance of a patient’s specific nuclear background and, better recapitulation of the mitochondrial and cellular impairments observed in vivo.

1.4.1 Pluripotent Stem Cell Metabolism

Although OXPHOS is important for the survival and function of the large majority of somatic cells, hiPSCs and hESCs (collectively human pluripotent stem cells; hPSCs) principally rely on glycolysis to support the energy demands and biosynthetic pathways necessary for growth and proliferation¹. Even under culture conditions at atmospheric oxygen concentrations (21% O₂), glycolytic flux remains high (Kondoh et al., 2006; Folmes et al., 2011), resembling the aerobic glycolytic metabolism (Warburg effect) of the blastocyst’s inner cell mass (ICM) (Gardner & Harvey, 2015). In line with this, hPSCs contain a relatively fragmented mitochondrial network localised to the perinuclear region of the cell, with electron microscopy (EM) imaging revealing mitochondria that have a globular structure with poorly developed cristae (Prigione et al., 2010; Folmes et al., 2011; Varum et al., 2011), similar to what is observed in the ICM of the early blastocyst (Sathananthan & Trounson, 2000).

1.4.2 Aerobic Glycolysis Maintains Pluripotency

Inhibiting OXPHOS by culturing cells under more restricted physiological oxygen conditions (5% O₂) (Ezashi, Das & Roberts, 2005), through pharmacological inhibition of OXPHOS CIII (Varum et al., 2009) or by decoupling ψₘ with high concentrations of carbonyl cyanide m-chlorophenylhydrazone (CCCP) protonophore (Mandal et al., 2011) hPSCs cultured in vitro retain a more primed identity reminiscent of post-implantation epiblast stem cells. There have been exciting advancements in pushing hPSCs further backwards in development towards a more naive, preimplantation ground state comparable to mouse embryonic stem cells. However, this section will only focus on primed hPSCs relevant to the work in this thesis and cautiously avoid observations made in naïve mouse and human PSCs.
promotes hPSC pluripotency. By comparison, enhancing mitochondrial biogenesis and presumably OXPHOS activity induces hPSC differentiation (Prowse et al., 2012). Conversely, inhibiting glycolysis with pharmacological inhibitors of Glucose-6-phosphate isomerase (GPI) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) glycolytic enzymes impairs pluripotency and induces differentiation (Moussaieff et al., 2015; Cliff et al., 2017). Indoleamine 2,3-dioxygenase 1 (IDO1) is the rate-limiting enzyme of the kynurenine pathway which breaks down tryptophan to generate cytosolic NAD\(^+\) required for maintaining glycolytic flux. In order to maintain a high glycolytic flux hPSCs have upregulated expression levels of IDO1 (Liu et al., 2019). Together these studies indicate that the high rates of aerobic glycolysis and minimal OXPHOS activity are not just a consequent feature of hPSCs, but in fact causative of the pluripotent state.

### 1.4.3 The Hidden Activity of Mitochondrial ETC in hPSCs

Given the relatively low respiratory activity of hPSCs and high glycolytic fluxes, the importance of mitochondrial metabolism in hPSCs has previously been overlooked. Interestingly, the basal activity of OXPHOS complexes I-IV of hPSCs is comparable to that of fibroblasts when normalised to mitochondrial mass, but unlike fibroblasts, hPSCs have limited reserve respiratory capacity (i.e. are operating at maximal respiratory rate under basal conditions) (Zhang et al., 2011). Chronic exposure to CCCP greatly attenuates hPSC proliferation (Mandal et al., 2011), indicating ETC activity and mitochondrial function are in fact necessary for hPSC biosynthesis, growth and/or division.

Although mitochondrial ETC activity of hPSCs is high, it is largely uncoupled from glucose oxidation pathways due to low expression levels of early TCA cycle enzymes responsible for the sequential conversion of mitochondrial acetyl-CoA (entry point of glycolysis derived pyruvate) to \(\alpha\)-ketoglutarate (\(\alpha\)-KG) (Tohyama et al., 2016). hPSCs do show active oxidative glutaminolysis pathways however, and withdrawal of glutamine from culture media severely impacts hPSC survival and proliferation (Tohyama et al., 2016; Zhang et al., 2016). Glutamine is first metabolised into glutamate by cytosolic glutaminase, before entering the mitochondria where it undergoes oxidative deamination to \(\alpha\)-KG. \(\alpha\)-KG can then be oxidised further in the latter part of the TCA cycle, bypassing the requirement of early TCA cycle enzymes which hPSCs do not express at considerable levels. In line with this, withdrawal of glutamine but not glucose from culture media reduces levels of late stage TCA cycle intermediates including succinate, fumarate and
malate, and heavy isotope labelling experiments show predominant contribution of glutamine but not glucose derived carbons to these intermediates (Tohyama et al., 2016). The majority of citrate in hPSCs is derived from glucose, as indicated through heavy isotope labelled glucose carbon incorporation into malonyl-CoA during lipid anabolism (Tohyama et al., 2016). The limited contribution of glutamine derived carbons to cytosolic citrate is probably due to increased export of malate and oxaloacetate intermediates from the mitochondria by the mitochondrial uncoupling protein UCP2 (Vozza et al., 2014), which is expressed at high levels in hPSCs (Zhang et al., 2011). Interestingly, this oxidative glutaminolysis activity of hPSCs appears to be strongly influenced by culture conditions. In the presence of exogenous lipids, glutamine oxidation is attenuated (Zhang et al., 2016). Together these studies show that mitochondria within hPSCs are active and necessary but optimised to differentially metabolise glucose and glutamine. Oxidative glutaminolysis pathways generate most TCA cycle intermediates and maintain $\psi_m$ polarisation, whereas glucose derived pyruvate is partially oxidised and only contributes to generation of citrate.

### 1.4.4 Metabolic Switch During Reprogramming to Pluripotency

In addition to the importance of hPSC metabolism for maintenance of the pluripotent state, a defining shift in cellular metabolic fluxes occurs during the reprogramming of somatic cells to iPSCs. The expression of glycolytic genes and glycolytic flux activity are upregulated shortly after reprogramming induction (Prigione et al., 2014) and this precedes expression of pluripotency markers (Folmes et al., 2011). Attenuating glycolytic flux with inhibitors of GPI or GAPDH greatly impairs reprogramming efficiency (Folmes et al., 2011; Panopoulos et al., 2012), indicating this increase in glycolytic flux is necessary for pluripotency acquisition. Conversely, addition of the glycolysis intermediate fructose 6-phosphate (F6P), increases glycolytic fluxes and improves reprogramming efficiency (Panopoulos et al., 2012). In relation to this, somatic cells which favour glycolytic metabolism show a better reprogramming efficiency than more oxidative cell types (Panopoulos et al., 2012). Beside an upregulation in glycolytic flux there is an accompanying decline in basal and maximal cellular respiration detectable 8 days after reprogramming induction (Mathieu et al., 2014). In line with this, a transient upregulation of autophagy occurs after reprogramming induction which is at least partly driven by the reprogramming factor SOX2 (Wang et al., 2013). Inducing mitophagy flux through pharmacological AMPK activation enhances reprogramming efficiency, conversely pharmacological inhibition of autophagy greatly attenuates reprogramming
O’Callaghan, B.J. (Ma et al., 2015b). This mitophagy induction is not reliant on $\psi_m$ depolarisation, and instead mediated through non-canonical $\psi_m$-independent but BNIP3L and PINK1 dependant mechanisms (Xiang et al., 2017; Vazquez-Martin et al., 2016). In line with this important change in mitochondrial dynamics during reprogramming, inhibition of mitochondrial fusion through MFN knockdown enhances reprogramming efficiency (Son et al., 2015), while pharmacologically inhibiting DRP1 and fission impairs reprogramming efficiency (Vazquez-Martin et al., 2012).

### 1.4.4.1 Importance of Transient Oxidative Stress and Hypoxic Signalling Pathways Early in Reprogramming

Hypoxia inducible factors (HIFs) are a family of transcription factors stabilised and activated in response to low oxygen availability (hypoxia). Under conditions of available oxygen (normoxia), HIFα subunits are hydroxylated by oxygen-dependant prolyl hydroxylases (PHDs), targeting them for ubiquitin-mediated degradation. Under conditions of restricted oxygen availability (hypoxia) this hydroxylation reaction is attenuated, permitting HIFα escape from proteasomal degradation, heterodimerization with HIF1β and translocation to the nucleus for binding to target gene hypoxic responsive elements (HREs) (Semenza, 2012). HIFs coordinate the activated expression of numerous genes associated with an adaptive response to limited oxygen availability, including most glycolytic enzymes (Dengler, Galbraith & Espinosa, 2014).

Culturing cells under lower, more physiological O$_2$ concentrations (5%) (Yoshida et al., 2009; Mathieu et al., 2014) or through pharmacological stabilisation and activation of HIFs (Prigione et al., 2014) increases glycolytic metabolism, decreases oxidative metabolism and improves reprogramming efficiency. Remarkably, genetic knockdown of HIF1α or HIF2α not only impairs reprogramming, but in fact prevents pluripotency acquisition entirely, even under supraphysiological oxygen concentrations (Prigione et al., 2014; Mathieu et al., 2014). HIFs are therefore a key underlying component of the signalling pathways underlying the shift from oxidative to glycolytic metabolic states, even under high oxygen conditions.

Unexpectedly, very high oxidative metabolic fluxes play a transient but essential role early during reprogramming, that is closely linked with activation of HIF signalling and the consequential shift towards glycolytic metabolism. Shortly after reprogramming induction, a transient increase in oxidative mitochondrial activity occurs (Prigione et al.,
driven by estrogen-related receptor α (ERRα) working in concert with the master regulator of mitochondrial biogenesis PGC1α (Kida et al., 2015). Inhibition of mitochondrial ETC function using the Complex IV inhibitor rotenone during the critical period in which this oxidative burst occurs but not after, significantly reduces reprogramming efficiency (Kida et al., 2015).

Nuclear factor erythroid-derived 2-like 2 (NFE2L2) is a transcription factor which acts as the master regulator of a cellular antioxidant response. Under homeostatic conditions of low cellular ROS levels, NFE2L2 associates with and is ubiquitinated by Kelch-like ECH-associated protein 1 (KEAP1), targeting it for degradation. Upon exposure to higher cellular levels of ROS, KEAP1 cysteine residues are modified resulting in dissociation of NFE2L2, its escape from ubiquitin targeted degradation, and translocation to the nucleus (Deshmukh et al., 2017). NFE2L2 binds to antioxidant responsive elements (AREs) at a number of gene targets associated with protective responses to oxidative stress, among which is HIF1α (Lacher et al., 2018). Cellular ROS additionally inhibits PHD hydroxylation of HIFα subunits and therefore stabilises the HIFα protein post-translationally (Guzy et al., 2005). Interestingly, recent evidence suggests elevated mitochondrial ROS production is in fact the underlying mechanism by which HIF signalling is activated in response to hypoxia (Martínez-Reyes et al., 2016). Upon reprogramming induction a transient increase in NFE2L2 activity is observed which coincides with elevated cellular ROS caused by the burst in oxidative activity (Hawkins et al., 2016). This increase in NFE2L2 activity precedes activation of HIF1α signalling suggesting post-transcriptional rather than post-translational regulation underlies the HIF1α signalling activation. Inhibiting NFE2L2 stabilisation through KEAP1 overexpression, prevents the elevation of HIF1α signalling and consequently impairs reprogramming efficiency (Hawkins et al., 2016). The oxidative burst therefore appears necessary for ROS-dependant NFE2L2 activation and consequential HIF1α stabilisation underlying the metabolic shift during reprogramming.

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2 Nuclear factor erythroid-derived 2-like 2 is often referred to as NRF2, including within the literature cited here. It is instead abbreviated to NFE2L2 in this thesis, in order to distinguish it from nuclear respiratory factor-2.
1.4.5 Metabolic Requirements for hPSC Proliferation and Pluripotency

1.4.5.1 Metabolic Fluxes Maintaining Biosynthetic Pathways

As a rapidly proliferating cell type, hPSCs require an abundant and continuously replenishing source of metabolic intermediates for the biosynthesis of lipid membranes, proteins and nucleotides. The pentose-phosphate pathway (PPP) is crucial for de novo lipid and nucleotide biosynthesis and hPSCs express PPP-related genes at high levels (Varum et al., 2011). In line with this, heavy isotope-labelled glucose tracing reveals that hPSCs have a highly active PPP (Hawkins et al., 2016), with the large majority of ribose (Zhang et al., 2011), ribonucleotides (Gu et al., 2016) and NADPH (required for nucleotide and fatty acid synthesis) (Zhang et al., 2016) being derived from glucose flux through the PPP. hPSCs also synthesise serine, glycine and other non-essential amino acids through glucose derived intermediates of the PPP (Gu et al., 2016). Heavy-isotope glucose labelling has also shown hPSCs have highly active de novo lipid synthesis pathways reliant on mitochondrial derived citrate (Tohyama et al., 2016), particularly when cultured in media with limited exogenous lipids (Zhang et al., 2016). Proliferative cell types require the de novo synthesis of aspartate from TCA cycle derived oxaloacetate in order to support protein and nucleotide biosynthesis (Sullivan et al., 2015; Birsoy et al., 2015). Heavy-isotope glutamine labelling shows high contribution of glutamine carbons to hPSC aspartate pools (Zhang et al., 2016) and is likely part of the necessary requirement of oxidative glutaminolysis for hPSC survival and proliferation.

1.4.5.2 Cell Metabolites Modulate the Epigenetic Landscape Underlying Pluripotency

All cells of the body contain the entire genome, yet the alternate functions of pluripotent stem cells, adult stem cells and terminally differentiated cells require the expression of only a select number of these genes. An important layer of this transcriptional regulation is through controlled epigenetic changes which include enzymatic chromatin modifications (Li, Liu & Belmonte, 2012). A number of chromatin modifying enzymes are reliant on intermediate products of metabolism (Gut & Verdin, 2013) and represent a functional link between a cell’s metabolic state and transcription profile.

There is accumulating evidence that cell metabolism influences enzymatic chromatin modifications in hPSCs and consequently differentiation or maintenance of pluripotency. In general, hPSC chromatin is in a much more open and transcriptionally permissive structure (euchromatin) compared to that of somatic cells which is predominantly compacted (heterochromatin). This open structure is associated with increased levels of
transcriptionally active associated epigenetic modifications including histone acetylation and H3K4me3, and smaller regions of inactive H3K9me3 and H3K27me3 marks. Beyond the requirements for ATP production and biosynthesis pathways, hPSC metabolism likely plays a crucial role in maintaining levels of intermediate metabolites which serve as substrates for epigenetic modifying enzymes underlying the acquisition and/or maintenance of pluripotency gene expression.

**Pluripotency Requires Metabolically Sensitive Chromatin Methylation Modifications**

Methionine is an essential amino acid which is metabolised into S-Adenosyl methionine (SAM), an important methyl-donor for post-translational modification of numerous proteins including histone methylation by histone methyltransferases (HMTs) and DNA methylation by DNA methyltransferases (DNMTs). hPSCs show particularly high rates of methionine metabolism, and SAM is crucial for hPSC self-renewal and survival (Shiraki et al., 2014). Withdrawal of methionine from hPSC culture media results in a reduction in intracellular SAM and accompanying reduction in global DNA methylation and histone H3K4me3 but not H3K9me3, H3K27me3 or H3K36me3 (Shiraki et al., 2014). Short-term removal of methionine enhances subsequent differentiation induction (Shiraki et al., 2014), indicating SAM-dependant epigenetic modifications are important for maintenance of pluripotency but not early lineage-commitment. In line with this, methionine uptake and metabolism are substantially reduced following differentiation into definitive mesoderm. H3K4me3 is an epigenetic mark that is typically associated with promoters and enriched at the transcription start site (TSS) of actively transcribing genes (Guenther et al., 2007). In hPSCs H3K4me3 levels are enriched at the promoters of key pluripotency markers, with the *NANOG* promoter showing exceptionally high levels (Pan et al., 2007). In line with this, *NANOG* expression is particularly sensitive to methionine depletion, whereas *POU5F1* (encoding OCT4) is mostly resistant in comparison (Shiraki et al., 2014). DNA methylation is largely considered to be a repressive modification at promoter regions, but can also serve to enhance gene expression when present within gene bodies (Jones, 2012). hPSCs show a relatively high abundance of gene body methylation that is positively correlated with gene expression (Lister et al., 2009). Modulation of SAM levels in hPSCs might therefore be important for maintaining promoter H3K4me3 activation of pluripotency gene expression, gene body DNA methylation for enhancement of pluripotency associated gene expression and promoter DNA methylation for repression of lineage-committed gene expression. Overexpression of nicotinamide N-methyltransferase (NNMT) which uses SAM as a
methyl donor results in a reduction in H3K27me3 levels (see supplementary figure from Sperber et al., 2015) which contradicts observations made in the study outlined above in which only H3K4me3 appeared sensitive to SAM perturbations. The reason behind this is difficult to determine but likely due to the differences in strategies utilised for altering intracellular SAM availability.

The converse active demethylation of DNA and histone lysine residues is mediated by the ten-eleven translocation methylcytosine dioxygenase (TET) and jumonji C domain-containing histone demethylase (JHDM) hydroxylase families respectively. Both TET and JHDM are reliant on α-KG for their demethylase activity and therefore provide an additional link between epigenetic methylation modifications and cell metabolism. As described in 1.4.3, hPSCs have active oxidative glutaminolysis pathways which are important for maintaining cellular levels of late stage TCA cycle intermediates including α-KG. Early during differentiation induction, cells increase glutamate uptake and utilisation which results in an increase in cellular α-KG levels (TeSlaa et al., 2016). In line with the importance of histone and DNA methylation for pluripotency, supplementation of hPSC culture media with a cell-permeable precursor of α-KG enhances hPSC differentiation to all three germ layers and is accompanied by decreases in all histone trimethylation modifications assessed (H3K4me3, H3K9me3, H3K27me3, H3K36me3), and decreased DNA methylation levels (TeSlaa et al., 2016). Conversely, inhibiting α-KG dependant TET and JHDM enzymes by increasing cellular succinate levels, attenuates hPSC differentiation (TeSlaa et al., 2016). H3K36me3 is found primarily within gene bodies of actively transcribed genes and is associated with transcription elongation (Guenther et al., 2007). In hPSCs, H3K36me3 is positively correlated with gene expression and likely important for activating pluripotency gene expression cooperatively with H3K4me3 (Hawkins et al., 2010). H3K9me3 and H3K27me3 are found primarily within promoter regions, but unlike H3K4me3 are typically associated with transcription repression and heterochromatin. Although hPSCs contain smaller regions of H3K9me3 and H3K27me3 relative to somatic cell counterparts (Hawkins et al., 2010), these repressive marks, along with promoter DNA methylation, are probably important for suppressing the expression of differentiation and lineage-commitment genes. Interestingly, in hPSCs the large majority of promoters associated

Note, the KDM1 lysine-demethylase family which includes KDM1A and KDM1B demethylate mono- and di- methylated H3K4 and H3K9 histone residues in a FAD-dependant manner not reliant on α-KG have not been described here.
with repressive H3K27me3 mark also contain the transcription activating H3K4me3 modification, yet expression of the associated genes is relatively low (Pan et al., 2007; Hawkins et al., 2010). This “bivalent” mark is associated with genes that are involved in development and early-lineage commitment and thought to poise them for activation through H3K27me3 demethylation or for stable repression through H3K4me3 demethylation, following differentiation cues (Pan et al., 2007; Hawkins et al., 2010). Another important target of H3K27me3 mediated suppression is EGLN1 which encodes the PHD2 enzyme responsible for hydroxylating HIF1α to target it for degradation (Sperber et al., 2015).

Glucose-Derived Citrate is Crucial for Histone Acetylation

As recounted above, hPSCs partially oxidise glucose to maintain cellular citrate levels for de novo lipid synthesis pathways. Citrate is first transported from the mitochondria via the tricarboxylate carrier expressed on the IMM before being converted into acetyl-CoA via ATP-citrate lyase (ACLY). In a landmark paper, Wellen et al. showed that ACLY derived acetyl-CoA is also required for histone acetylation modifications mediated by HATs. ACLY is localised to both cytoplasmic and nuclear compartments, and siRNA knockdown of ACLY reduces global histone acetylation modifications (Wellen et al., 2009). Furthermore, withdrawal of glucose from culture media reduces the acetylation of histone H3, providing one of the first pieces of evidence that epigenetic modifications can be affected by changes in cellular metabolic fluxes. More recently, it has been shown that besides lipid biosynthesis, the active production of citrate from glucose is also necessary for maintaining histone acetylation modifications that underlie the pluripotent state. Pharmacological inhibition of glycolytic enzymes or ACLY decreases pluripotency, whereas increasing pyruvate flux into the mitochondria for conversion to citrate through pyruvate dehydrogenase kinase (PDK) inhibition enhances pluripotency (Moussaieff et al., 2015). Supplementation of culture media with acetate which is converted into acetyl-CoA by acetyl-CoA synthetase (ACS) enhances pluripotency marker expression and attenuates spontaneous differentiation. Maintenance of nucleocytoplasmic acetyl-CoA for histone acetylation is therefore one of the crucial requirements of glucose-citrate flux in hPSCs. In line with the importance of histone acetylation for pluripotency, inhibition of Zn²⁺-dependent (Class I, II and IV) HDACs enhances the reprogramming of somatic cells, whereas inhibition of KAT3A and KAT3B HATs prevents somatic cell reprogramming (Mali et al., 2010). Furthermore, overexpression of the KAT8 HAT
improves reprogramming efficiency, whereas knockdown of KAT8 impairs somatic cell reprogramming (Mu et al., 2015).

In addition to Zn²⁺-dependant HDACs, histones are also deacetylated by NAD⁺-dependant sirtuin (Class III) HDACs. NAD⁺ availability is dependent on the redox status of the cell and might therefore be an additional mechanism linking cellular metabolism with epigenetic acetylation modifications. hPSCs have very active NAD⁺ synthesis pathways and maintain high NAD⁺ levels for glycolytic flux (Liu et al., 2019), but the importance of sirtuin deacetylation of histone residues in pluripotency remains poorly described. As hPSCs are enriched for highly acetylated euchromatin, it seems unlikely histone deacetylation by sirtuins is playing any considerable role in maintenance of the pluripotent state, and in fact be considered to negatively impact pluripotency. SIRT1 is expressed at high levels in hPSCs however and is under the direct transcriptional control of the pluripotency factor OCT4 (Zhang et al., 2014; Jang et al., 2017) suggesting it might have an important role in the pluripotent state. In addition to histone acetylation targets, SIRT1 also deacetylates lysine residues on the p53 tumour suppressor which serve to reduce its activity. In hPSCs p53 activates expression of lineage-commitment genes and pro-apoptotic genes, and SIRT1 is necessary to prevent p53 activity from exerting these effects (Zhang et al., 2014; Jang et al., 2017).

1.4.6 hiPSCs Harbouring mtDNA Mutations

1.4.6.1 mtDNA Genotype as Important Contributor of Healthy hiPSCs Pluripotency

Interestingly, the first description of hiPSCs possessing mtDNA mutations came from a 2011 study which sequenced the mitochondrial genomes of hiPSC lines reprogrammed from fibroblasts of young and apparently healthy individuals (Prigione et al., 2011). The large majority of homoplasmic and heteroplasmic mutations (differing from the revised Cambridge Reference Sequence (rCRS): GenBank NC_012920) identified in hiPSC lines are also present in the bulk fibroblast population from which they are reprogrammed and are generally common variants not previously associated with disease. Several variants present at heteroplasmic levels in the bulk fibroblast populations were at higher or lower levels in hiPSC lines derived, in some instances up to homoplasm or complete absence. Moreover, homoplasmic and heteroplasmic mutations were identified in hiPSC lines which were undetectable in the bulk fibroblast population, suggesting that they may have arisen de novo during the reprogramming process.
However more recent evidence suggests that the vast majority of mtDNA mutations present in hiPSC lines from healthy donors are in fact present in the bulk fibroblast population but can be at low levels which make detection technically difficult. Sequencing of clonally expanded fibroblasts from the bulk population reveals a greater number of heteroplasmic mutations, including mutations present in hiPSC lines which were otherwise not detected from sequencing of the bulk population (Kang et al., 2016). More sensitive NGS technologies have shown all mtDNA mutations present in established hiPSC lines are also present within the bulk fibroblast population (Perales-Clemente et al., 2016). This evidence points towards the mitochondrial genomic background of a hiPSC line stemming from that of the individual fibroblast within the heterogenous bulk population from which it arises.

1.4.6.2 MtDNA Disease hiPSCs

Over the past 17 years there has been an increasing number of publications reporting on the derivation of hiPSCs with disease causing mutations in the mitochondrial genome (Summarised in Table 1-1).
Table 1-1 Heteroplasmy levels of all hiPSC lines harbouring disease causing mtDNA mutations described to date

<table>
<thead>
<tr>
<th>mtDNA Mutation</th>
<th>Patient Phenotype</th>
<th>Reprogramming Conditions</th>
<th>Media Suppl.</th>
<th>Reprogramming Efficiency</th>
<th>Somatic Cell Heteroplasmy (Bulk)</th>
<th>hiPSC Clone Heteroplasmy</th>
<th>hiPSC Mutation load Stability</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MT-RNR2</strong> m.2336T&gt;C (16s mt-rRNA)</td>
<td></td>
<td>Feeder, pMXs retroviral delivery (OSKM), urine epithelial cells</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Homoplasmic</td>
<td>Homoplasmic</td>
<td>Stable</td>
<td>(Li et al., 2018)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>Patient 1: DM Patient 2: DM, SNHL, CM</td>
<td>Feeder, pMXs retroviral delivery (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Patient 1: 18% Patient 2: 24%</td>
<td>Patient 1: 0-87% Patient 2: 0-83%</td>
<td>Stable</td>
<td>(Matsubara et al., 2018; Fujikura et al., 2012)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>Patient 1: MIDD Patient 2: MIDD + ataxia Patient 3: CM</td>
<td>Feeder, pMSCV retroviral delivery (OSKM), fibroblasts</td>
<td>50µg/ml uridine</td>
<td>Normal</td>
<td>Patient1: 22% Patient 2: 21% Patient 3: 35%</td>
<td>0-90% (specific patient origin N.D.)</td>
<td>Stable</td>
<td>(Hämäläinen et al., 2013)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>Feeder, Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>50µM uridine</td>
<td>N.D.</td>
<td>29%</td>
<td>0-100%</td>
<td>N.D.</td>
<td>(Ma et al., 2015a)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>N.D.</td>
<td>Feeder, episomal plasmid transfection (OSKMN), fibroblasts</td>
<td>N.D.</td>
<td></td>
<td>Patient1: 99% Patient 2: 69% Patient 3: 55%</td>
<td>Patient1: 45-100% Patient 2: 5-100% Patient 3: 0-100%</td>
<td>Stable but increases from 70% to 90% also observed.</td>
<td>(Yokota et al., 2015)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>Feeder, pMSCV retroviral delivery (OSKM), fibroblasts</td>
<td>N.D.</td>
<td></td>
<td>78%</td>
<td>4-99%</td>
<td>Stable</td>
<td>(Kodaira et al., 2015)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>Patient 1: MELAS Patient 2: MELAS Patient 3: MELAS</td>
<td>Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>50µg/ml uridine</td>
<td>Normal</td>
<td>Patient 1: 20% Patient 2: 45% Patient 3: 45%</td>
<td>Patient 1: 0-100% Patient 2: 0-85% Patient 3: 0-90%</td>
<td>General loss of ~12% over 6 passages when heteroplasmy is &gt;80%.</td>
<td>(Perales-Clemente et al., 2016)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>Patient 1: R, DM, SNHL</td>
<td>Feeder, Cytotune-IPS Sendai virus (OSKM), fibroblasts</td>
<td>50µg/ml uridine</td>
<td>Reprogramming clonal fibroblasts from Patient 2 and Patient 1: 70% Patient 2: 66%</td>
<td>Patient 1: 1-85% Patient2: 0-2%</td>
<td>Stable</td>
<td>(Chichagova et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>Mutation</td>
<td>Patient</td>
<td>Transfection Method</td>
<td>Treatment Details</td>
<td>Result 1</td>
<td>Result 2</td>
<td>Result 3</td>
<td>Notes</td>
<td></td>
</tr>
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<td>-------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA^Leu(URR))</td>
<td>Patient 1: N.D. Patient 2: N.D.</td>
<td>Feeder, episomal plasmid transfection (OSKMN), myoblasts</td>
<td>50µg/ml uridine</td>
<td>N.D.</td>
<td>Patient 1: N.D. Patient 2: N.D.</td>
<td>Patient 1: 40% and 90% Patient 2: &gt;90% Stable</td>
<td>(Yokota et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA^Leu(URR))</td>
<td>N.D.</td>
<td>Feeder-free, episomal plasmid transfection (OSKMN), fibroblasts</td>
<td>N.D.</td>
<td>Normal</td>
<td>Homoplasmic</td>
<td>Homoplasmic Stable</td>
<td>Most lines stable but some lines showed drops in heteroplasmy of 20-40% over 30 passages. (Mizuguchi et al., 2017)</td>
<td></td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA^Leu(URR))</td>
<td>MELAS</td>
<td>Feeder-free, Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>N.D.</td>
<td>90%</td>
<td>83-95% Stable</td>
<td>(Yang et al., 2018)</td>
<td></td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA^Leu(URR))</td>
<td>MELAS</td>
<td>retroviral delivery (OSKM), fibroblasts</td>
<td>50µg/ml uridine</td>
<td>N.D.</td>
<td>Patient 1: 89% Patient 2: N.D.</td>
<td>Patient 1: 0%, 71%, 83% Patient 2: 0% and 84% Stable</td>
<td>(Klein Gunnewiek et al., 2019)</td>
<td></td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA^Leu(URR))</td>
<td>MELAS</td>
<td>Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>N.D.</td>
<td>95%</td>
<td>0% and 85% N.D.</td>
<td>(Lin et al., 2019a)</td>
<td></td>
</tr>
<tr>
<td>MT-TW m.5541C&gt;T (mt-tRNA^Trp)</td>
<td>MELAS</td>
<td>Feeder, episomal plasmid transfection (OSKMN), myoblasts</td>
<td>N.D.</td>
<td>Normal</td>
<td>Homoplasmic</td>
<td>Homoplasmic Stable</td>
<td>(Hatakeyama et al., 2015)</td>
<td></td>
</tr>
<tr>
<td>6.0kb single large-scale mtDNA deletion (m.Δ7777:13794)</td>
<td>PS</td>
<td>Feeder, Cytotune-IPS Sendai virus (OSKM), fibroblasts</td>
<td>50µg/ml uridine</td>
<td>N.D.</td>
<td>17%</td>
<td>&lt;10%, 30%, 65% Rapid increase from &lt;10% and 30% towards 65% during culture. 65% maintained.</td>
<td>(Russell et al., 2018)</td>
<td></td>
</tr>
<tr>
<td>MT-TK m.8344A&gt;G (mt-tRNA^Lys)</td>
<td>MERRF</td>
<td>Feeder, pMXs retroviral delivery (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>Impaired (Patient 1 and 2)</td>
<td>Patient 1: 90% Patient 2: 60%</td>
<td>Patient 1: 0% and 70% Patient 2: 60% Patient 1: 70% heteroplasmy levels declined to 50% after 20 passages Patient 2: Stable</td>
<td>(Chou et al., 2018; Chen et al., 2018b; Chou et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>MT-TK m.8344A&gt;G (mt-tRNA&lt;sup&gt;53&lt;/sup&gt;)</td>
<td>N.D.</td>
<td>Feeder-free, episomal plasmid transfection (OSKMN), fibroblasts</td>
<td>N.D.</td>
<td>Impaired</td>
<td>Homoplasmic</td>
<td>Homoplasmic</td>
<td>Stable</td>
<td>(Mizuguchi et al., 2017)</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>LS</td>
<td>Feeder free, mRNA transfection (OSKM) + microRNA, fibroblasts</td>
<td>N.D.</td>
<td>Impaired</td>
<td>85%</td>
<td>Single clone: 85%</td>
<td>Stable</td>
<td>(Grace et al., 2019)</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>LS</td>
<td>Feeder, Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>50µM uridine</td>
<td>N.D.</td>
<td>Patient 1: 52% Homoplasmic</td>
<td>Patient 1: 0-87% Homoplasmic</td>
<td>N.D.</td>
<td>(Ma et al., 2015a)</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>LS</td>
<td>Feeder, pMSCV retroviral delivery (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>N.D.</td>
<td>&gt;90%</td>
<td>&gt;90%</td>
<td>N.D.</td>
<td>(Galera-Monge et al., 2019; Galera-Monge et al., 2016b)</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>N.D.</td>
<td>Feeder-free, episomal plasmid transfection (OSKMN), fibroblasts</td>
<td>N.D.</td>
<td>Normal</td>
<td>Homoplasmic</td>
<td>Homoplasmic</td>
<td>Stable</td>
<td>(Mizuguchi et al., 2017)</td>
</tr>
<tr>
<td>MT-ATP6 m.9185T&gt;C (mt-ATP6 p.Leu220Pro)</td>
<td>Patients 1-3: PP</td>
<td>Feeder, episomal plasmid transfection (OSKMNLS) or pMXs retroviral delivery (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Patients1-3: Homoplasmic</td>
<td>Patients1-3: Homoplasmic</td>
<td>Stable</td>
<td>(Lorenz et al., 2017)</td>
</tr>
<tr>
<td>2.5kb single large-scale mtDNA deletion (m.Δ10949–13449)</td>
<td>PS</td>
<td>Feeder, retroviral delivery (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>Impaired with fewer colonies observed and with delayed emergence.</td>
<td>60-80%</td>
<td>55-70%</td>
<td>Maintained in one line but lost in two lines.</td>
<td>(Cherry et al., 2013)</td>
</tr>
<tr>
<td>MT-ND4 m.11778G&gt;A (mt.-ND4 p.Arg340His)</td>
<td>LHON</td>
<td>Feeder, Cytotune-IPS 2.0 Sendai virus (OSKM), Peripheral blood mononuclear cells</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Homoplasmic</td>
<td>Homoplasmic</td>
<td>Stable</td>
<td>(Lu et al., 2018; Yang et al., 2019; Wu et al., 2018)</td>
</tr>
<tr>
<td>MT-ND5 m.13513G&gt;A (mt.-ND5 p.Asp393Asn)</td>
<td>MELAS</td>
<td>Feeder, lentiviral delivery (OSKM)</td>
<td>200µM uridine</td>
<td>N.D.</td>
<td>47%</td>
<td>0-46%</td>
<td>Slow decline from 50% to undetectable levels over a year in culture.</td>
<td>(Folmes et al., 2013)</td>
</tr>
<tr>
<td>MT-ND5 m.13513G&gt;A (mt-ND5 p.Asp393Asn)</td>
<td>LS</td>
<td>Feeder, Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>50μM uridine</td>
<td>N.D.</td>
<td>84%</td>
<td>0-100%</td>
<td>N.D.</td>
<td>(Ma et al., 2015a)</td>
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</tr>
<tr>
<td>MT-ND5 m.13513G&gt;A (mt-ND5 p.Asp393Asn)</td>
<td>LS</td>
<td>Feeder, Cytotune-IPS 2.0 Sendai virus (OSKM), fibroblasts</td>
<td>N.D.</td>
<td>N.D.</td>
<td>56%</td>
<td>16-45%</td>
<td>Modest fall of ~10% over 30 passages</td>
<td>(Galera-Monge et al., 2016a; Galera-Monge et al., 2019)</td>
</tr>
<tr>
<td>MT-ND5 m.13514A&gt;G (mt-ND5 p.Asp393Gly)</td>
<td>LS</td>
<td>Feeder, Cytotune-IPS Sendai virus (OSKM), fibroblasts</td>
<td>50μg/ml uridine</td>
<td>N.D.</td>
<td>55%</td>
<td>37% and 58%</td>
<td>Stable</td>
<td>(Russell et al., 2018)</td>
</tr>
</tbody>
</table>

Abbreviations: Not described, N.D.; Cardiomyopathy, CM; diabetes mellitus, DM; sensorineural hearing loss, SNHL; maternally inherited deafness and diabetes, MIDD; mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes, MELAS; myoclonic epilepsy with ragged red fibres, MERRF; Leigh’s syndrome, LS; periodic paralysis, PP; Pearson’s Syndrome, PS; Leber's hereditary optic neuropathy, LHON; OCT4 SOX2 KLF4 c-MYC, OSKM; OCT4 SOX2 KLF4 l-MYC NANOG, OSKMN; OSKM + LIN28A, OSKML; OSKML + SVLT, OSKMLS;
1.4.6.3 Heteroplasmy Segregation During Clonal hiPSC Derivation

Consistent between studies with different mutations, cell-types and reprogramming methods, hiPSC clones obtained from reprogramming of bulk patient cell populations with heteroplasmic levels of mtDNA pathogenic variant show a range of different mutation loads. Heteroplasmy often ranges from undetectable levels to near homoplasmic thus permitting hiPSC lines with and without pathogenic variant to be obtained, bypassing the requirement for gene-editing to obtain isogenic controls. Initial studies showed bimodal segregation of m.3243A>G during reprogramming from a bulk fibroblast population containing a range of intermediate mutation loads (determined through clonal expansion of fibroblasts) (Hämäläinen et al., 2013). mtDNA content is much lower in hiPSCs compared to fibroblasts and for this reason the authors suggested a genetic bottleneck not dissimilar to that observed during germline transmission might be occurring during reprogramming. Subsequent studies of the m.3243A>G and m.13513G>A pathogenic variant affecting mt-ND5 did not replicate this finding however and instead suggest the mutation load of hiPSC clones obtained is representative of that in individual fibroblasts of the bulk population (Yokota et al., 2015; Ma et al., 2015a). This is further supported by more recent studies highlighting the heterogenous population of mtDNA heteroplasmy levels in individual fibroblast clones from healthy patients which underlies the mtDNA variations in hiPSCs derived from them (Kang et al., 2016). In comparison, all hiPSC lines derived from patient cells with homoplasmic or very high levels of mtDNA pathogenic variant show similarly high/homoplasmic levels.

1.4.6.4 Negative Selection Pressures During Reprogramming

Whilst it is possible to retain mtDNA mutations at high heteroplasmy during reprogramming, it has been reported that bulk fibroblasts with heteroplasmic or homoplasmic levels of m.8344A>G (Chou et al., 2016; Mizuguchi et al., 2017), heteroplasmic m.10949–13449del (Cherry et al., 2013), or heteroplasmic m.3243A>G (Chichagova et al., 2017) show reduced reprogramming efficiencies. In an excellent study which took advantage of the heterogenous levels of heteroplasmy in individual fibroblasts of a bulk population, clonally expanded fibroblasts with >90% m.3243A>G were shown to be poorly reprogrammed whilst isogenic clonal lines <90% m.3243A>G were reprogrammed relatively efficiently (Yokota et al., 2015). It is likely that the poor reprogramming efficiency of individual cells with high heteroplasmy levels underlies a
negative selection pressure which accounts for the generally lower mutation loads of hiPSC clones derived from bulk fibroblasts than would be expected by chance.

1.4.6.5 Shifts in Heteroplasmy during hiPSC Culture

Another important property to consider in relation to mtDNA mutations is the potential for drifts in heteroplasmy level to occur through vegetative segregation or clonal expansion. Heteroplasmy of m.3243A>G during hiPSC culture have been the most reported and generally show no substantial changes across passages, although both increases and decreases have been observed (See Table 1-1). Decreases in the heteroplasmy levels of m.13513G>A, m.10949–13449del and m.8344A>G have also been described during hiPSC culture. These declines in heteroplasmy level might be caused by negative selection at the individual mitochondrial level (e.g. by preferential mitophagy against mutant mitochondria) or through selective pressures at the cellular level following random segregation of mutant load above a critical threshold to impair hiPSC proliferation or survival. More recently, the mutation load of a 6.0kb single large-scale mtDNA deletion (m.Δ7777:13794) has been shown to rapidly increase during hiPSC culture, being limited only by reaching a sufficiently detrimental level to impair cell viability (Russell et al., 2018). This clonal expansion is likely due to a greater replication rate of the substantially smaller mutant mitochondrial genome.

1.4.6.6 Heteroplasmy Changes During Differentiation

In general, no substantial alterations in heteroplasmy level have been noted upon cell differentiation, with successfully differentiated cell types showing comparable mutation loads to that of the hiPSCs they were derived from (Table 1-2). There has been one report of m.3243A>G load decreasing from 90% to 60% in 1/3 cardiomyocyte differentiation inductions however (Yokota et al., 2017). There have been a number of reports of specific lines poorly differentiating or failing to differentiate entirely however, and this has generally been correlated with high mutation loads. Although hiPSCs harbouring homoplasmic levels of m.3243A>G could be efficiently differentiated into neural stem cells (NSCs), the same lines failed to differentiate into cardiomyocytes due to reduced cell viability (Ma et al., 2015a). hiPSCs possessing >90% m.3243A>G heteroplasmy (Yokota et al., 2017), homoplasmic m.8993T>G and 35% m.13513G>A heteroplasmy (Galera-Monge et al., 2019) also show impaired cardiomyocyte differentiation efficiencies. Although differentiation into NSCs and neural crest cells (NCCs) is largely unaffected by >90% m.3243A>G or homoplasmic m.5541C>T, subsequent terminal
differentiation into mature neurons is substantially impaired due to reduced cell viabilities (Yokota et al., 2017; Hatakeyama et al., 2015). Lower differentiation efficiencies have also been described for homoplasmic m.11778G>A hiPSCs into retinal ganglion cells (Yang et al., 2019) and 83% m.3243A>G hiPSCs into excitatory cortical layer 2/3 neurons (Klein Gunnewiek et al., 2019).

1.4.6.7 in vitro Phenotypes

Although hiPSCs are less reliant on OXPHOS and considered to have more immature mitochondria, impairments in mitochondrial function have been described in hiPSCs harbouring several different mtDNA mutations. Differentiation towards disease-relevant and more oxidative demanding cell-types has in some instances revealed otherwise hidden mitochondrial and/or cellular impairments (summarized in Table 1-2).

Mitochondrial Dysfunction in m.3243A>G hiPSCs

As the most prevalent disease causing mtDNA mutation, m.3243A>G has been the most studied mtDNA pathogenic variant in hiPSCs. Typical for m.3243A>G in other cell types, reductions in protein levels of assembled OXPHOS CI and CIV are seen in hiPSCs with 85% heteroplasmy (Lin et al., 2019a) but normal expression levels have been observed in hiPSC lines with similar or lower mutation loads (Hämäläinen et al., 2013). More consistently, at >70% m.3243A>G mutation loads impairments in OXPHOS CI activity are described (Matsubara et al., 2018; Yokota et al., 2015). These impairments are often associated with reductions in basal and maximal OCR measurements (Ma et al., 2015a; Matsubara et al., 2018; Yang et al., 2018) but normal OCR measurements at >90% heteroplasmy have also been described (Yokota et al., 2017). In line with these signs of mitochondrial dysfunction, hiPSCs with 85% m.3243A>G mutation load have a more depolarised $\psi_m$ and elevated ROS levels which is associated with increased mitophagic flux (Lin et al., 2019a).
Table 1-2 In vitro phenotypes of hiPSCs and differentiated progeny harbouring mtDNA disease mutations published to date.

<table>
<thead>
<tr>
<th>mtDNA Mutation</th>
<th>Patient Phenotype</th>
<th>hiPSC Phenotype</th>
<th>Differentiation Efficiency</th>
<th>Mutation load after Differentiation</th>
<th>Differentiated Cell Phenotypes</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MT-RNR2</strong> m.2336T&gt;C (16s mt-rRNA)</td>
<td>CM</td>
<td>Normal pluripotency and mitochondrial morphology.</td>
<td>Normal EB formation. Efficient differentiation into cardiomyocytes.</td>
<td>Homoplasmic</td>
<td>Cardiomyocytes: Increased cell size and expression of hypertrophic cardiomyocyte associated proteins. Increased mtDNA copy number. Fragmented, round mitochondrial structure with poorly developed cristae. Reduction in 16s mt-rRNA expression and mt-rRNA binding proteins. Reductions in mitochondrial encoded OXPHOS complex translates. Reduction in $\psi_m$ and cellular ATP:ADP ratio. Increase in $[\text{Ca}^{2+}]$ and associated decrease in L-type $\text{Ca}^{2+}$ current. Prolonged AP durations and increase in delayed after depolarisations failing to trigger AP firing.</td>
<td>(Li et al., 2018)</td>
</tr>
<tr>
<td><strong>MT-TL1</strong> m.3243A&gt;G (mt-tRNALeu(URR))</td>
<td>Patient 1: DM</td>
<td>Normal Pluripotency. No differences in mtDNA content. Reductions in OXPHOS CI activity at high heteroplasm (Patient 1 77% and 91%, Patient 2 85%) but not at midrange heteroplasm (Patient 2 70%). Reductions in basal OCR and OXPHOS-dependent ATP production (Patient 1 77% and 91%) and maximal OCR (Patient 1 91%).</td>
<td>Normal EB formation.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>(Matsubara et al., 2018; Fujikura et al., 2012)</td>
</tr>
<tr>
<td>Mutation</td>
<td>Patient 1</td>
<td>Patient 2</td>
<td>Patient 3</td>
<td>OXPHOS Activity</td>
<td>Differentiation</td>
<td>Heteroplasmy</td>
</tr>
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<td>-------------</td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA&lt;sub&gt;Leu&lt;/sub&gt;&lt;sup&gt;(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>MELAS</td>
<td>MELAS</td>
<td>Normal levels of assembled OXPHOS CI, CIII, CIV and CV but increased CII in cells with &gt;80% heteroplasmy.</td>
<td>Efficient differentiation into neurons and astrocytes.</td>
<td>Heteroplasmy maintained</td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA&lt;sub&gt;Leu&lt;/sub&gt;&lt;sup&gt;(URR)&lt;/sup&gt;)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Normal pluripotency. Trend towards lower OXPHOS CI and CIV activities in homoplasmic lines (Patients 1, 2, 3).</td>
<td>Normal EB formation.</td>
<td>N.D.</td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA&lt;sub&gt;Leu&lt;/sub&gt;&lt;sup&gt;(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>MELAS</td>
<td>MELAS</td>
<td>Normal pluripotency. Normal basal and maximal OCR (Patient 1 80%).</td>
<td>Efficient NPC and fibroblast differentiation of homoplasmic cells but impaired cardiomyocyte differentiation due to low cell viability.</td>
<td>Homoplasmic</td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA&lt;sub&gt;Leu&lt;/sub&gt;&lt;sup&gt;(URR)&lt;/sup&gt;)</td>
<td>Patient 1: MELAS, Patient 2: MELAS, Patient 3: MELAS</td>
<td>MELAS</td>
<td>MELAS</td>
<td>Normal pluripotency. Normal basal and maximal OCR (Patient 1 80%).</td>
<td>Efficient cardiomycocyte differentiation efficiency (Patient 1 80%).</td>
<td>Heteroplasmy maintained</td>
</tr>
<tr>
<td>MT-TL1 m.3243A&gt;G (mt-tRNA&lt;sub&gt;Leu&lt;/sub&gt;&lt;sup&gt;(URR)&lt;/sup&gt;)</td>
<td>Patient 1: N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Normal pluripotency. Reduction in OXPHOS CI activity but no change in OCR.</td>
<td>Normal EB formation. Impaired cardiomycocyte differentiation at very high heteroplasmy levels (Patient 2 &gt;90%) but normal differentiation efficiency at lower levels (Patient 1 40% and 90%). Impaired neuronal differentiation efficiency at &gt;90% heteroplasmy.</td>
<td>Maintained but selection against &gt;90% heteroplasmy.</td>
</tr>
<tr>
<td>MT-TLI m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>N.D.</td>
<td>Normal pluripotency</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>(Mizuguchi et al., 2017)</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
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</tr>
<tr>
<td>MT-TLI m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>Normal Pluripotency. Reduction in basal and maximal OCR at 90% heteroplasmy</td>
<td>Normal EB formation. Efficient differentiation into NPC.</td>
<td>Heteroplasmy maintained</td>
<td>NPCs: Reduction in basal and maximal OCR at 90% heteroplasmy</td>
<td>(Yang et al., 2018)</td>
</tr>
<tr>
<td>MT-TLI m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>Normal pluripotency</td>
<td>Reduced excitatory cortical Layer 2/3 neuron differentiation efficiency.</td>
<td>Slight increase from 71 to 85% heteroplasmy</td>
<td>Excitatory cortical neurons (Patient 1 71%): Reduced basal and maximal OCR. Reduced mitochondrial content in proximal and distal neurites. Reduced dendritic length, structure complexity and synaptic density. Reduction in spontaneous excitatory post synaptic current frequency but not amplitudes.</td>
<td>(Klein Gunnewiek et al., 2019)</td>
</tr>
<tr>
<td>MT-TLI m.3243A&gt;G (mt-tRNA&lt;sup&gt;Leu(URR)&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>Normal pluripotency. Reductions in OXPHOS CI and CIV levels. Reduction in ψm and increase in mitochondrial ROS production. Increased mitophagy flux under basal and stressed conditions. Reduced cellular ATP levels and viability.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>(Lin et al., 2019a)</td>
</tr>
<tr>
<td>MT-TW m.5541C&gt;T (mt-tRNA&lt;sup&gt;Trp&lt;/sup&gt;)</td>
<td>MELAS</td>
<td>Normal pluripotency</td>
<td>Normal EB formation. Efficient differentiation into neural stem cells and neural crest cells but terminal differentiation into mature central and peripheral neurons respectively was substantially impaired.</td>
<td>Homoplasmic</td>
<td>Viability of neuronal inductions impaired during long term culture.</td>
<td>(Hatakeyama et al., 2015)</td>
</tr>
</tbody>
</table>
| Mutation                        | Condition | Effect on 
<table>
<thead>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-TK m.8344A&gt;G (mt-tRNA&lt;sup&gt;39&lt;/sup&gt;)</td>
<td>PS</td>
<td>Normal pluripotency. Reduced basal OCR (Patient 1 70%, Patient 2 60%), reduced maximal OCR (Patient 1 60%), and increased ROS production with associated increase in catalase antioxidant expression (Patient 1 70%, Patient 2 60%). Elevated expression of mitochondrial fission proteins and more fragmented mitochondrial network (Patient 1 70%, Patient 2 60%).</td>
</tr>
<tr>
<td>MT-TK m.8344A&gt;G (mt-tRNA&lt;sup&gt;39&lt;/sup&gt;)</td>
<td>N.D.</td>
<td>Normal pluripotency</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>LS</td>
<td>Normal pluripotency</td>
</tr>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>LS</td>
<td>Normal pluripotency</td>
</tr>
</tbody>
</table>
| MT-ATP6 m.8993T>G (mt-ATP6 p.Leu156Arg) | LS        | Normal pluripotency | Normal EB formation. Failure to differentiate into cardiomyocytes. | Maintained | N.D. | (Galera-Monge et al., 2019; Galera-
<table>
<thead>
<tr>
<th>Mitochondrial mutation</th>
<th>Patients</th>
<th>Pluripotency</th>
<th>EB formation</th>
<th>Differentiation</th>
<th>OCR/ATP levels</th>
<th>Mitochondrial morphology/Other findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-ATP6 m.8993T&gt;G (mt-ATP6 p.Leu156Arg)</td>
<td>N.D.</td>
<td>Normal pluripotency</td>
<td>N.D.</td>
<td>Maintained</td>
<td>N.D.</td>
<td>N.D. (Mizuguchi et al., 2017) NPCs: Normal mtDNA content and mitochondrial morphology. No change in OCR or cellular ATP levels. Reduction in ATP synthesis rate. Partial membrane depolarisation. $\psi_m$ hyperpolarisation, reduced glutamate and Ca$^{2+}$ evoked calcium release from intracellular stores, and reduced release of Ca$^{2+}$ following $\psi_m$ uncoupling.</td>
</tr>
<tr>
<td>MT-ATP6 m.9185T&gt;C (mt-ATP6 p.Leu220Pro)</td>
<td>Patients 1-3: PP</td>
<td>Normal pluripotency.</td>
<td>Normal EB formation. Efficient differentiation into NPCs.</td>
<td>Maintained</td>
<td>N.D.</td>
<td>N.D. (Mizuguchi et al., 2017) NPCs: Normal mtDNA content and mitochondrial morphology. No change in OCR or cellular ATP levels. Reduction in ATP synthesis rate. Partial membrane depolarisation. $\psi_m$ hyperpolarisation, reduced glutamate and Ca$^{2+}$ evoked calcium release from intracellular stores, and reduced release of Ca$^{2+}$ following $\psi_m$ uncoupling.</td>
</tr>
<tr>
<td>2.5kb single large-scale mtDNA deletion (m.Δ10949–13449)</td>
<td>PS</td>
<td>Slower growth rate and more depolarised $\psi_m$ at 30% heteroplasmy. Reduced basal and maximal OCR at 64% heteroplasmy but normal at 14% heteroplasmy.</td>
<td>Efficient erythroid precursor differentiation but trend towards reduced efficiency at &gt;10% heteroplasmy.</td>
<td>Maintained</td>
<td>Erythroid precursors showed increase pathological iron depositions at 10% and particularly 50% heteroplasmy.</td>
<td>(Lorenz et al., 2017)</td>
</tr>
<tr>
<td>MT-ND4 m.11778G&gt;A (mt.-ND4 p.Arg340His)</td>
<td>LHON</td>
<td>Normal pluripotency</td>
<td>Normal EB formation. Reduced efficiency of differentiation into RGCs.</td>
<td>Maintained</td>
<td>RGCs: Increased mitochondrial content and PPARG1A expression but reduced basal and maximal OCR. Impaired neurite outgrowth and reduced electrophysiological activities (no AP firing during sustained current injection). Glutamatergic AMPAR-dependent signalling impaired with reduced GluR1 and GluR2 expression, and impaired GluR2 cell-surface trafficking.</td>
<td>(Lu et al., 2018; Yang et al., 2019; Wu et al., 2018)</td>
</tr>
<tr>
<td>MT-ND5 m.13513G&gt;A (mt.-ND5 p.Asp393Asn)</td>
<td>MELAS</td>
<td>Normal pluripotency.</td>
<td>Normal basal and maximal OCR (46% vs 37% heteroplasmy)</td>
<td>More efficient EB formation at 7% vs 37% heteroplasmy.</td>
<td>N.D.</td>
<td>N.D. (Folmes et al., 2013)</td>
</tr>
<tr>
<td>MT-ND5 m.13513G&gt;A (mt.-ND5 p.Asp393Asn)</td>
<td>LS</td>
<td>Normal pluripotency</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D. (Ma et al., 2015a)</td>
<td></td>
</tr>
<tr>
<td>Abbreviations: Not described, N.D.; Cardiomyopathy, CM; diabetes mellitus, DM; sensorineural hearing loss, SNHL; maternally inherited deafness and diabetes, MIDD; mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes, MELAS; myoclonic epilepsy with ragged red fibres, MERRF; Leigh’s syndrome, LS; periodic paralysis, PP; Pearson’s Syndrome, PS; Leber’s hereditary optic neuropathy, LHON; neural progenitor cells, NPCs; inner-hair cells, IHCs; embryoid body, EB; action potential, AP;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| MT-ND5 m.13513G>A (mt-ND5 p.Asp393Asn) | LS | Normal pluripotency | Normal EB formation. Successful cardiomyocyte differentiation at 12% but not 35% heteroplasmy. Due to impaired epithelial-mesenchymal transition (assessed by Brachyury induction after 24hr CHIR99021 stimulation) and increased apoptosis. Reduction of 35% heteroplasmy to 31% heteroplasmy by extended culture permitted cardiomyocyte differentiation. | Enriched cardiomyocytes successfully differentiated from 12% heteroplasmy maintain heteroplasmy level. | Mutation load dependent decrease in spontaneous cardiomyocyte beating frequency and beating strength. High heteroplasmy favoured spontaneous differentiation into neuroectoderm lineage. | (Galera-Monge et al., 2016a; Galera-Monge et al., 2019) |

| MT-ND5 m.13514A>G (mt-ND5 p.Asp393Gly) | LS | Normal EB formation. | N.D. | N.D. | Normal EB formation. | (Russell et al., 2018) |
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Phenotype of Disease-Relevant Differentiated Cell Progeny with m.3243A>G

Neuropathy is a relatively common phenotype associated with m.3243A>G with infarct areas in cortical and subcortical white matter regions also observed in more severe MELAS cases. Differentiation of hiPSCs harbouring m.3243A>G has revealed several mitochondrial and cellular impairments of relevance to the in vivo setting. Following neuronal differentiation a substantial and isolated reduction in OXPHOS CI protein is observed in neurons with >80% m.3243A>G heteroplasmacy (Hämäläinen et al., 2013). Whilst OXPHOS CII and CIV are distributed evenly throughout the mitochondrial network of these neurons, OXPHOS CI is instead present in perinuclear mitochondrial foci that stain positive for mitophagy markers. Neural progenitor cells with >90% heteroplasmacy also show reductions in basal and maximal OCR (Ma et al., 2015a; Yang et al., 2018). As already described, NSCs can be efficiently generated from hiPSCs with >90% m.3243A>G, but only cell lines with <90% heteroplasmacy can then be successfully terminally differentiated into post-mitotic neurons (Yokota et al., 2017). Terminally differentiated neurons with 90% m.3243A>G mutation load do not show any impairments in OCR, suggesting mutation loads detrimental to cellular respiration are selected against during terminal differentiation from glycolytic favouring NPCs to more oxidative metabolising post-mitotic neurons. In line with this, heteroplasmacy levels of neurospheres differentiated from 90% m.3243A>G hiPSCs which died upon terminal neuron differentiation were generally higher than the remaining surviving neurospheres (Yokota et al., 2017). In a more recent study, differentiated excitatory layer 2/3 cortical neurons with 85% m.3243A>G heteroplasmacy do show reduced basal and maximal OCR however, and this was accompanied by reductions in mitochondrial content at both proximal and distal neurite processes (Klein Gunnewiek et al., 2019). This study also showed morphological aberrations in neuronal structure, with neurons harbouring m.3243A>G having reduced dendritic length, structure, complexity and synaptic density. In line with reduced synaptic densities, neurons with 85% m.3243A>G heteroplasmacy show reduced spontaneous excitatory postsynaptic current (sEPSC) firing frequencies and accompanying reduction in network synchronicity and overall activity.

Cardiomyopathy is also a common feature of patients harbouring high m.3243A>G mutation loads and for this reason differentiation into cardiomyocytes has also received attention. As described above, hiPSCs with >90% m.3243A>G mutation load show poor viability during differentiation into cardiomyocytes. Successfully differentiated myocytes with 90% m.3243A>G mutation loads show normal basal and maximal OCRs, and
unaffected spontaneous beating function (Yokota et al., 2017), suggesting m.3243A>G loads detrimental to cellular respiration and cardiomyocyte function are selected against during differentiation into these more oxidative demanding cells. Reductions in basal and maximal OCR have been observed in cardiomyocytes with 80% m.3243A>G heteroplasmym however (Perales-Clemente et al., 2016). Interestingly these respiratory impairments were not also observed in the parental hiPSCs, highlighting disease relevant cell-type specific dysfunction.

Increased ROS and Respiration Impairments in Disease-Relevant hiPSC-Derived Cells with m.8344A>G

Cardiomyopathy is also a common feature of mtDNA disease patients harbouring high levels of m.8344A>G. While OCR impairments are sometimes observed in cardiomyocytes with >80% m.3243A>G mutation load, reductions in basal and maximal OCR are seen in differentiated cardiomyocytes with 40% m.8344A>G heteroplasmym level (Chou et al., 2016). This suggests m.8344A>G is more potent than m.3243A>G, i.e. the mutation load threshold for m.8344A>G is lower than m.3243A>G. Similar reductions in OCR measurements are also observed in NPCs differentiated from the same hiPSCs with 40% m.8344A>G heteroplasmym (Chou et al., 2016). Cardiomyocytes, NPCs and inner-hair cells with 40% m.8344A>G mutation load also show increased levels of ROS and compensatory elevation in catalase antioxidant expression (Chou et al., 2016; Chen et al., 2018b). The mitochondrial dysfunction observed in cardiomyocytes and NPCs with 40% m.8344A>G heteroplasmym causes the mitochondrial network to become much more fragmented and globular, and is associated with an increased expression of mitochondrial fission proteins. A reduction in cilium length and bundling is also observed in inner-hair cells with 40% heteroplasmym, indicating mitochondrial dysfunction is likely having a negative impact on cellular functionality.

Taken together these studies suggest that functional impairments in mtDNA disease hiPSCs are insufficient to entirely prevent their derivation, culture, and differentiation. Upon differentiation to disease relevant cells which favour oxidative metabolism and are more reliant on mitochondrial function, previously hidden cellular dysfunction can be revealed due to a lower mtDNA mutation load threshold.
1.5 Metabolic Flux Changes Drive Cell-Fate Decisions Through Metabolically Sensitive Epigenetic Modifications

As described in 1.4.5.2, the metabolic profile of hPSCs is crucial for maintenance of the pluripotency state, at least partly through regulation of metabolically sensitive enzymatic chromatin modifications. In addition to maintenance of pluripotency, there is accumulating evidence that switches in cell metabolism are responsible for driving epigenetic modifications which underly changes in cell identity during development, tissue repair, immune system activation and differentiation of hPSCs in vitro.

1.5.1 Metabolic Flux Changes Necessary for Lineage-Specific hPSC Differentiation

Until recently, consensus in the field was that a switch from aerobic glycolysis to oxidative favouring metabolism is necessary for driving hPSC differentiation. This stems from early observations outlined in 1.4.2 which showed spontaneous differentiation is accompanied by an increase in mitochondrial OXPHOS and general decrease in glycolytic flux. Furthermore, enhancing mitochondrial OXPHOS or inhibiting glycolysis pushes hPSCs towards spontaneous differentiation. While it is clear aerobic glycolysis and reduced oxidative activity is indispensable for maintenance of pluripotency, loss of pluripotency does not alone define cell differentiation.

Recently, metabolic flux changes which occur early during the lineage specific differentiation of hPSCs to the three different germ layers have been assessed (Cliff et al., 2017). In line with general consensus, glycolytic flux decreases and oxidative activity increases during targeted differentiation towards mesoderm and definitive endoderm lineages. Glycolytic flux and oxidative activity of cells targeted towards early neuroectoderm (NPCs and NCCs) however, remains at similar levels to hPSCs. In line with this, mesoderm and definitive endoderm differentiation is unaffected by glycolysis inhibitors whereas NPC and NCC differentiation efficiency is substantially impaired. Furthermore, promoting glucose oxidation through PDK inhibition enhances early mesoderm and endoderm but not early ectoderm differentiation. This maintenance of a hiPSC like metabolic state in early neuroectoderm is largely driven by continued N-Myc transcription activating activity, whereas N-Myc is strongly downregulated during early mesodermal and early endodermal transitions. The subsequent developmental transition into floor plate cells is however accompanied by a reduction in N-Myc activity and switch
from glycolytic to more oxidative favouring metabolism (Zheng et al., 2016; Cliff et al., 2017).

In a more recent study, oxidative fluxes were shown to be specifically elevated following differentiation to early mesoderm but not early endoderm and ectoderm lineages (Lu et al., 2019). While glucose consumption remained high in early ectoderm, glucose utilisation was much lower in both early endoderm and mesoderm. Instead an increased glutamine flux through oxidative glutaminolysis was observed in early mesoderm and endoderm (less so) compared to early ectoderm. In line with this metabolic flux change being an important contributor to lineage-commitment, withdrawal of glutamine from media during embryoid body formation resulted in a reduction in the expression of early mesoderm but not ectoderm markers, and specifically reduced cell viability of mesoderm-specific but not ectoderm-specific differentiation.

Together these studies highlight that while a switch from glycolytic to oxidative metabolism is important for the early specification of endoderm and mesoderm lineages, early ectoderm differentiation requires maintenance of high glycolytic fluxes, with a subsequent oxidative switch being important during transition to neural plate cells. It will be interesting to see whether this differential metabolism of early ectoderm compared to early mesoderm and endoderm lineages is necessary for alternative regulation of epigenetic marks defining this state.

1.5.2 Metabolically-Sensitive Epigenetic Modifications During Cell Identity Changes

In addition to the contribution of metabolic flux changes driving the loss of metabolically-sensitive epigenetic modifications underlying pluripotency described in 1.4.5.2, there is accumulating evidence that metabolic flux changes are also important for driving changes in metabolically-sensitive modifications during cell-fate transitions of adult stem cells.

1.5.2.1 Metabolic Control of Immunity Through Epigenetic Mechanisms

Cells of the haematopoietic-lineage are currently the most well studied in relation to metabolic control of cell-fate decisions. Naïve T-cells show minimal glycolytic activity and instead utilise fatty acids through mitochondrial oxidation to support their energy demands (Jung, Zeng & Horng, 2019). Following activation, glucose uptake and utilisation increases, which is at least partly induced through upregulation of the GLUT1 glucose transporter (Jacobs et al., 2008). This is accompanied by increases in oxidative
and glycolytic fluxes, but overall decline in the OCR:ECAR ratio indicating a switch towards predominantly aerobic glycolysis favouring metabolism (Macintyre et al., 2014), reminiscent of that observed in human ESCs. This metabolic switch causes an elevation in cellular acetyl-CoA levels and resultant increase in H3K9ac deposition at enhancer and promoter regions upstream of the *Ifng* locus which are crucial for initiating its transcription during CD4⁺ T-cell activation (Peng et al., 2016).

Metabolically driven alterations in histone methylation modifications are also important for T-cell fate transitions. Activated CD8⁺ T-cells show active glutaminolysis pathways which give rise to high cellular levels of 2-hydroxyglutarate (generated from glutamine-derived α-KG by LDHA) (Tyrakis et al., 2016). 2-hydroxyglutarate is a natural inhibitor of αKG-dependent enzymes including the JHDM enzymes responsible for histone demethylation. Elevations of 2-hydroxyglutarate during CD8⁺ T-cell activation inhibit JHDM demethylases resulting in global elevations of the transcriptionally inactive H3K27me3 mark necessary for maintaining CD8⁺ memory T-cell identity. In addition, CD62L is an important cell surface molecule of CD8⁺ memory T-cells, and 2-hydroxyglutarate increases levels of the transcriptionally active H3K4me3 at the promoter of *CD62L*. Whereas DNA methylation and H3K27me3 appear to be associated with memory T-cell fate, an increase in α-KG-dependant demethylation of DNA and H3K27me3 is instead associated with differentiation of CD4⁺ and CD8⁺ T-cells towards an effector identity. Cellular levels of α-KG are much higher in T-cells differentiated towards an effector cell identity compared to those differentiated towards a memory cell fate (Chisolm et al., 2017). This elevation in glutaminolysis derived α-KG is important for JHDM and TET dependant removal of the transcriptionally repressive H3K27me3 and DNA methylation marks respectively at promoter regions of genes associated with T effector cell function. 2-hydroxyglutarate is also a natural inhibitor of the α-KG dependant TET enzymes responsible for DNA demethylation. Elevations in glutamine-derived 2-hydroxyglutarate occur during the differentiation of CD4⁺ cells towards a helper Th17 cell fate and are necessary for inhibiting TET-dependant demethylation at the *Foxp3* promoter which would otherwise drive a regulatory Treg cell identity (Xu et al., 2017).
1.5.2.2 Metabolic Regulation of Histone Acetylation and Deacetylation Underlies Cell Identity Transitions During Muscle Repair and Differentiation

Of great relevance to the work presented in this thesis, several recent studies have indicated changes in cell metabolism are particularly important for driving epigenetic changes underlying myogenic differentiation and the activation of satellite cells, the adult stem cell niche of skeletal muscle.

In a landmark paper Ryall et al., showed for the first time that changes in cell metabolism could alter histone acetylation changes through modulation of NAD$^+$-dependant sirtuin HDACs (Ryall et al., 2015). Reminiscent of activated T-cells and hPSCs, the activation of murine paired box protein 7 positive (PAX7$^+$) satellite cells from a quiescent to highly proliferative state is accompanied by a switch from oxidative metabolism to elevated glycolytic flux. This results in a substantial decrease in NAD$^+$ availability due to limited mitochondrial ETC oxidation of NADH for NAD$^+$ regeneration. Among its many targets, SIRT1 is responsible for the active deacetylation of H4K16ac. H4K16ac is a mark that is associated with active enhancers and promoters, and is enriched at the TSS of actively transcribed genes (Taylor et al., 2013). Ryall et al. showed that during satellite cell activation there is a global increase in H4K16ac but with particular enrichment at genes highly expressed in activated satellite cells, including the master regulator of myogenesis, myogenesis determination protein 1 (MyoD). $SIRT1^{KO}$ promotes satellite cell activation and MyoD expression and facilitates H4K16ac increases at many of the genes associated with activated satellite cells, highlighting this regulation is SIRT1 dependant. Pushing satellite cells towards a more oxidative state by culturing in galactose rather than glucose, increases the NAD$^+$:NADH ratio, reduces H4K16ac levels and attenuates satellite cell activation and MyoD expression, revealing the metabolic regulation of this process.

Elevations in global H3K9/14ac have also been shown to occur during murine satellite cell activation (Das et al., 2017). In addition, H3K9/14ac and H3K27ac increase at the promoters of $MYOD1$/MyoD during the early stages of human myoblast and C2C12 myoblast differentiation towards myotubes. These increases in histone acetylation modifications, expression of various myogenic differentiation markers including $MYOD1$/MyoD, and overall myogenic potential can be attenuated by $ACLY$ knockdown highlighting the regulatory effect of nucleocytoplasmic acetyl-CoA availability during satellite cell activation and muscle differentiation. Interestingly, $ACLY$ knockdown in human myoblasts attenuates the expression of proteins associated with fast skeletal
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muscle fibre types but increases slow muscle fibre type proteins (Das et al., 2017), highlighting an ACLY dependant regulation of later stages of myogenic differentiation relating to fibre-type identity. Elevations in nucleocytoplasmic acetyl-CoA during myocyte terminal differentiation therefore have the potential to favour differentiation into glycolytic fast fibre-types over oxidative slow fibre types.

Elevations in global H3K9ac and H4K16ac following satellite cell activation have been confirmed in a more recent study which also showed levels of H3K18ac remain relatively unchanged (Yucel et al., 2019). However, following differentiation into myogenin positive (MyoG+) myocytes, there is a substantial reduction in global levels of all histone acetylation marks assessed, including H3K18ac, H3K9ac, H3K18ac, H4K16ac, and H2BK5ac. Assessment of histone acetylation status in cultured primary myoblasts comparing self-renewing MyoG- with differentiating MyoG+ myoblasts confirmed these reductions in H3K9/14ac, H3K18ac, H4K16ac, and H2BK5ac, and showed similar reductions in H3K27ac and H3K56ac levels also occur. Both oxidative and glycolytic fluxes decrease shortly after myoblast differentiation induction and this leads to a reduction in glucose flux contributing to cellular citrate levels available for HATs. This decline in mitochondrial activity is regulated by enhanced mitochondria fission, with DRP1 inhibition resulting in increased histone acetylation levels and attenuated myoblast differentiation. In line with this, enhancing glucose-derived pyruvate entry into the TCA cycle by inhibiting PDK, increases histone acetylation levels and attenuates myoblast differentiation. Assessment of locus specific chromatin immunoprecipitation sequencing (ChIP-Seq) data comparing C2C12 myoblasts and differentiating myotubes revealed that while the global levels of various histone acetylation marks do indeed decrease, deposition at promoter regions of myogenic differentiation related genes increases, in accordance with histone acetylation marks being associated with active gene transcription.

Together these studies show that histone acetylation modifications transiently change during the activation and subsequent differentiation of satellite cells, and that metabolism is an important factor driving these histone acetylation modifications during myogenic cell-identity transitions. Satellite cell activation is accompanied by a switch towards aerobic glycolysis and decline in mitochondrial activity which drives the conversion of glucose towards citrate and nucleocytoplasmic acetyl-CoA available for HAT-dependant histone acetylation modifications, while simultaneously limiting NAD+ availability for
SIRT1-dependant deacetylation. By comparison, the differentiation of myoblasts towards myotubes is accompanied by decreases in histone acetylation levels which are driven by an overall reduction in glucose utilisation and mitochondrial metabolism generating citrate, limiting the availability of nucleocytoplasmic acetyl-CoA for HATs.

1.6 Myogenesis and In Vitro Myogenic Differentiation

In 1987 Lassar et al. showed that transfection of mouse embryonic fibroblasts (MEFs) with cDNA encoding MyoD was sufficient to transdifferentiate somatic cells directly into myoblasts (Lassar, Paterson & Weintraub, 1986; Davis, Weintraub & Lassar, 1987), representing the first description of lineage reprogramming in which one cell could be pushed into an alternate cell-identity. While this pioneering work opened a whole new field of research into forced cell-identity changes, the successful differentiation of hPSCs into a myogenic lineage has proven much more difficult to establish when compared with other lineages. Recent work has taken advantage of the knowledge basis surrounding myogenesis during development in order to overexpress key myogenic associated transcription factors and/or recapitulate developmental signalling gradients for successful differentiation of myogenic cell types from hPSCs in vitro.

1.6.1 Myogenic Regulatory Factors

Myogenic regulatory factors (MRFs) are a family of four transcription factors central to the process of myogenesis: MyoD, MyoG, myogenic factor 5 (Myf5) and myogenic factor 6 (Myf6; originally designated myogenic regulatory factor 4, MRF4). Like MyoD, overexpression of myogenin (Wright, Sassoon & Lin, 1989), Myf5 (Braun et al., 1989) or Myf6 (Rhodes & Konieczny, 1989) is sufficient for transdifferentiation of somatic cells into myoblasts, and represents the most defining feature of the MRF family. MRFs originate from duplication of a single ancestral gene and remain structurally homologous with all four containing highly conserved basic helix-loop-helix (bHLH) domains indispensable for their promyogenic activity (Asfour, Allouh & Said, 2018). MRFs can homodimerize but preferentially heterodimerize with E2A proteins belonging to the ubiquitously expressed class I bHLH family through interactions between helix-loop-helix regions (Berkes & Tapscott, 2005). Active MRF/E2A heterodimers bind to promoter and enhancer regions of muscle-specific target genes through recognition of CANNTG sequence motifs known as enhancer boxes (E-boxes) (Davis et al., 1990; Lassar et al., 1991). MRFs control the expression of distinct but overlapping sets of gene targets, and additionally show autoregulatory and cross-regulatory mechanisms (Asfour,
Although MyoD\textsuperscript{−/−} or Myf5\textsuperscript{−/−} knockout mice do not show any obvious impairments in developmental myogenesis, simultaneous knockout of MyoD and Myf5 prevents the generation of cells from the myogenic lineage entirely (Rudnicki et al., 1993). For technical reasons the MyoD\textsuperscript{−/−}/Myf5\textsuperscript{−/−} double knockout mice also fail to express MRF4 during development, but in a follow-on study which maintained MRF4 expression (Kassar-Duchossoy et al., 2004), myoblasts did develop suggesting MRF4 is also important for determining the myogenic lineage. These studies also highlight the redundant roles of MyoD, Myf5 and MRF4 during myogenic specification. By comparison, MyoG\textsuperscript{−/−} single knockout mice maintain development of myoblasts but show severe impairments in downstream skeletal muscle formation, which suggests myogenin is more important for driving terminal myogenic differentiation rather than early determination of the myogenic lineage (Hasty et al., 1993; Nabeshima et al., 1993). MyoD, Myf5 and MRF4 are therefore considered to be serving as myogenic determination factors whereas myogenin functions as a myogenic differentiation factor.

### 1.6.2 Myogenesis During Embryonic Development

Skeletal muscle cells originate from the paraxial mesoderm which forms from the primary mesenchyme as two bilateral strips at either side of the neural tube (Chal & Pourquié, 2017) (Figure 1-11). Formation of the paraxial mesoderm is largely driven by canonical Wingless-related integration site (Wnt) signalling and fibroblast growth factor 2 (FGF2; basic FGF) signalling which patterns the specification of Brachyury\textsuperscript{+}/SOX2\textsuperscript{+} neuromesodermal progenitors originating from the anterior primitive streak towards a mesodermal rather than neuronal fate (Garriock et al., 2015). Bone morphogenic protein 4 (BMP4) released by the posterior primitive streak (and lateral mesoderm itself) promotes lateral mesoderm patterning. Noggin released from the notochord binds to and inhibits BMP4 and thus prevents this mediolateral transition, maintaining the paraxial mesoderm identity proximal to the neural tube (Reshef, Maroto & Lassar, 1998). The paraxial mesoderm is segmented into repetitive somite structures through the periodic release of Notch, Wnt and FGF2 from proximal cells of the paraxial mesoderm (Hubaud & Pourquié, 2014). Somites are patterned into distinct sclerotome and dermomyotome compartments by Wnt and sonic hedgehog (Shh) signalling gradients. Sclerotome differentiation is driven by Shh released from the notochord whereas Wnt released by the neural tube and ectoderm drives dermomyotome patterning (Yusuf & Brand-Saberi, 2006). Cells of the dermomyotome express the PAX3 paired box transcription factor which although not specific for the myogenic lineage, is expressed in all myogenic
precursors (Buckingham & Rigby, 2014). PAX3+ cells move from the dermomyotome and rapidly downregulate PAX3 differentiating into Myf5+ and MyoD+ myoblasts which form a structure known as the myotome which represents the first embryonic skeletal muscles. PAX3+ cells continue to enter the myotome, differentiate into Myf5+/MyoD+ myoblasts and subsequently upregulate myogenin which drives differentiation into myocytes (Chal & Pourquié, 2017). Knockout mice studies suggest hepatocyte growth factor (HGF) released by mesodermal cells in developing limb buds are crucial for the delamination of PAX3+ cells from the dermomyotome and migration into the myotome and developing muscles through activation of tyrosine-protein kinase Met (c-Met; HGF receptor) (Dietrich et al., 1999). Cells within the central part of the dermomyotome coexpressing PAX3 with PAX7, migrate into the developing muscle and fuse with other myocytes contributing to a second wave of myogenesis that gives rise to multinucleated myofibres (Relaix et al., 2005; Hutcheson et al., 2009; Pourquié, Al Tanoury & Chal, 2018). A subset of these PAX7+ cells also enter a quiescent state and form the adult stem cell pool of skeletal muscle (satellite cells) important for postnatal muscle growth, repair and regeneration (Relaix et al., 2005; Pourquié, Al Tanoury & Chal, 2018). In addition to its role in dermomyotome delamination, HGF signalling has also been shown to promote satellite cell activation and proliferation of myoblast progeny while simultaneously attenuating myoblast differentiation into myocytes (Tatsumi et al., 1998; Miller et al., 2000). It is likely HGF is serving a similar role promoting myoblast proliferation in developing muscles. Insulin growth factor 1 (IGF1) and FGF2 have also been shown to enhance the proliferation of satellite cells and myoblast progeny (Allen & Boxhorn, 1989; Doumit, Cook & Merkel, 1993) and might play similar roles during muscle development. While FGF2 and HGF negatively regulate myoblast differentiation and therefore specifically enhance satellite cell activation and myoblast proliferation, IGF1 also stimulates the differentiation of myoblasts into myocytes (Allen & Boxhorn, 1989) through upregulation of myogenin (Florini, Ewton & Roof, 1991).
Figure 1-11 Schematic of skeletal myogenesis during embryonic development showing one of the two bilateral primary mesenchyme strips. SOX2+/Brachyury+ neuromesodermal progenitors (NMPs) originating from the primitive streak are patterned into mesodermal progenitor cells (MPCs) through WNT and FGF-2 signalling. Noggin released by the notochord inhibits BMP4 signalling which would otherwise promote subsequent patterning towards a more lateral rather than paraxial mesoderm (a.k.a. presomitic mesoderm; PSM) fate. PAX3+ and PAX7+ skeletal muscle precursors originate from the dermomyotome compartment of the somite and migrate as myoblasts into the myotome, differentiating into myocytes and myotubes which form the first skeletal muscles of the developing embryo. Figure adapted from Pourquié, Al Tanoury & Chal, 2018.

1.6.3 Differentiation of Skeletal Muscle Cell Types from hPSCs

There are currently two strategies that have been utilised independently and jointly to direct hPSC differentiation towards skeletal muscle cell types: 1.) direct reprogramming through overexpression of transcription factors associated with the myogenic lineage, 2.) directed differentiation by sequentially recapitulating developmental signalling gradients and pathways active during embryonic myogenesis.

1.6.3.1 Direct Reprogramming of hPSCs into Myogenic Cells

As one of the key transcription factors controlling myogenesis, and the early descriptions of direct reprogramming of somatic cells to myoblasts, overexpression of MyoD has being one of the most commonly utilised strategies for differentiation of hPSCs into myogenic cell types. Using hPSCs stably transduced/transfected with doxycycline-inducible MyoD-overexpression vectors it has been shown that MyoD overexpression for >5 days is alone sufficient for differentiation of hPSCs into myoblast-like cells which express endogenous MyoD and myogenin and have the potential to form myosin heavy chain positive (MyHC+) myocytes and multinucleated myotubes (Rao et al., 2012; Tanaka et al., 2013). Due to poor transduction efficiency of hPSCs grown as compact colonies and poor survival of hPSCs in dissociated cell culture, priming differentiation towards mesenchymal cells before transduction with an adenovirus expressing MyoD has
also been successful for inducing hPSC myogenic differentiation (Goudenege et al., 2012). Simultaneous treatment with retinoic acid during induction of MyoD overexpression, which presumably facilitates mesodermal fate transition, also improves cell survival during early differentiation induction (Pawlowski et al., 2017). While MyoD can directly force undifferentiated hPSCs towards a myogenic cell fate, the efficiency of this directed differentiation is much lower for hPSCs compared to fibroblasts, which is at least partly due to low expression of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3 (SMARCD3) (Albini et al., 2013). SMARCD3 is a component of the SWI/SNF chromatin remodelling complex important for the ATP-dependant destabilisation of histone-DNA interactions, which among its many roles is necessary for remodelling chromatin to a more open conformation at MyoD target sites (Albini et al., 2013). Chromatin remodelling must therefore occur before MyoD can exert its pro-myogenic transcriptional activity and likely explains why additional priming factors facilitate MyoD-induced differentiation of hPSCs. In relation to the importance of chromatin remodelling during myogenic differentiation, overexpression of the JHDM Lysine demethylase 6B (KDM6B), which demethylates the repressive H3K27me3 bivalent mark, also enhances MyoD driven myogenic differentiation of hPSCs (Akiyama et al., 2016).

In addition to MyoD, the overexpression of Myf5 in hPSCs primed towards differentiation through embryoid body formation can also successfully drive hPSC myogenic differentiation, in line with its MRF function (Iacovino et al., 2011). In addition to MRFs, overexpression of the myogenic precursor associated PAX7 transcription factor in hPSC derived embryoid bodies has also been a successful strategy for driving myogenic differentiation (Darabi et al., 2012; Skoglund et al., 2014).

1.6.3.2 Recapitulating Developmental Myogenesis In Vitro

Initial myogenic differentiation strategies took advantage of the relatively untargeted spontaneous differentiation of hPSCs towards the mesenchymal lineage and subsequent isolation of cells directing towards a myogenic identity. Co-culturing hPSCs with the OP9 immortalised mesenchyme cell line or inducing spontaneous differentiation in serum containing media when seeded at low density promotes mesenchyme identity acquisition of hPSCs (Barberi et al., 2005, 2007). In addition, inhibition of transforming growth factor β (TGFβ) receptor signalling during embryoid body formation and outgrowth also facilitates mesenchymal identity acquisition, likely by inhibiting the spontaneous
transition towards endoderm identities (Mahmood et al., 2010). Retinoic acid signalling also enhances spontaneous embryoid body differentiation toward PAX3+ myogenic cell precursors (Ryan et al., 2012). Selecting cells expressing the CD73 cell surface marker through FACS permits isolation of a relatively pure population of mesenchymal precursor cells (Barberi et al., 2005, 2007). Continued culture of these CD73+ mesenchymal precursors in serum containing media promotes spontaneous differentiation into endodermal and mesodermal cell types including myogenic precursors which can then be purified through FACS for the embryonic skeletal muscle marker neural cell adhesion molecule (NCAM) (Barberi et al., 2007). FACS for platelet derived growth factor receptor alpha (PDGFRα) positive and vascular endothelial growth factor receptor 2 (VEGFR2) negative paraxial mesoderm cells from spontaneously differentiating hPSCs has also been utilised to select for cells with promyogenic identity (Sakurai et al., 2012).

Following on from this work, researchers began to sequentially apply activators and inhibitors of signalling pathways to adherent hPSC cultures in order to promote cell identity transitions similar to those occurring in vivo during myogenesis (Figure 1-12), significantly improving myogenic differentiation efficiency.

**Figure 1-12** Schematic showing the signalling pathways and cell-identity transitions which have been sequentially recapitulated for in vitro myogenic differentiation of hPSCs. Exogenous activation of WNT and FGF2 signalling pathways drives the early differentiation of hPSCs into neuromesodermal precursors (NMPs) and subsequently paraxial mesoderm (a.k.a. presomitic mesoderm; PSM). Coapplication of BMP4 receptor antagonists has been used to prevent mediolateral transition away from the paraxial mesoderm and enhance myogenic identity acquisition. Promyogenic growth factors (e.g. HGF, IGF1, FGF2) have then been added in order to facilitate the proliferation of myoblast progeny. Antagonists of TGFβ receptors have also been applied at later stages in order to facilitate somitic commitment of paraxial mesoderm cells. Figure adapted from Pourquié, Al Tanoury & Chal, 2018.

Due to the crucial role of canonical Wnt signalling for patterning cells into neuromesoderm and subsequently paraxial mesoderm in conjunction with FGF2, Wnt
signalling activation through inhibition of glycogen synthase kinase 3 beta (GSK3β) and successive application of FGF2 has been particularly advantageous in improving myogenic differentiation efficiency. In one of the first studies to utilise this strategy, treatment of undifferentiated hPSCs with the GSK3β inhibitor CHIR99021 (3µM) for 4 days (d0-4) followed by FGF2 (20 ng/ml) for 2 weeks (d4-d18) was shown to give rise to a much higher proportion of PAX3+/PAX7+ myogenic precursors (>18% at d35) and MyHC+ myocytes (>8% at d35) than had been previously achieved (Borchin, Chen & Barberi, 2013). FACS purification of cells positive for C-X-C chemokine receptor 4 (CXCR4) and/or c-MET (HGF-R) was still required however, in order to isolate a pure myogenic cell population. Treatment of more sparsely seeded hPSCs with higher 10µM CHIR99021 for 2 days (d0-2) followed by FGF2 application for 8 days (d12-20) considerably increased the efficiency of this myogenic differentiation strategy with >90% of cells showing myogenic identity after prolonged culture (43% PAX7+ and 47% MyHC+ at d50), removing the requirement for FACS purification (Shelton et al., 2014, 2015).

In order to accelerate myogenic transitions and improve the purity of differentiated myogenic cultures, additional growth factors and signalling pathways have been modulated in more recent myogenic differentiation strategies in order to more fully recapitulate in vivo myogenesis. During initial Wnt signalling activation through application of CHIR99021, the BMP4 receptor antagonist LDN193189 (inhibitor of ALK2, ALK3 and ALK6 members of the activin receptor-like kinase family) has been applied in order to inhibit BMP4 signalling which would otherwise drive the differentiation of hPSC-derived mesoderm precursors towards a lateral rather than paraxial mesoderm fate (Chal et al., 2015, 2016). While this differentiation protocol by Chal et al. used lower CHIR99021 concentrations (3µM) than that of Shelton et al. outlined above, the duration of this CHIR99021 stimulation of Wnt signalling was extended to 6 days (d0-6). Following on from this initial patterning towards paraxial mesoderm and myogenic precursor cells, application of FGF2, HGF and IGF1 promyogenic growth factors in a serum containing medium can promote the expansion of myogenic precursor cells (Chal et al., 2015, 2016). Interestingly, a population of MyoD+ (Shelton et al., 2019) and non-proliferative (Ki67-) (Chal et al., 2015, 2016; Shelton et al., 2019) PAX7+ cells is maintained associated with terminally differentiated myotubes in these cultures, not dissimilar to the resident population of PAX7+ satellite cells of skeletal muscle in vivo. In line with this, gene expression profiling suggests hPSC
derived myogenic cultures with high proportions of PAX7+ cells are more similar to quiescent or early activated mouse satellite cells rather than late activated satellite cells (Shelton et al., 2019). More recently, inhibiting TGFβ signalling using antagonists of TGFβ receptors (ALK4, ALK5 and ALK7) has also been shown to improve myogenic differentiation efficiency by enhancing somatic commitment of hPSC derived paraxial mesoderm precursors (Xi et al., 2017; Sakai-Takemura et al., 2018; Hicks et al., 2018).

1.7 Potential Impact of Disease Causing MtDNA Mutations to the Myogenic Differentiation of hPSCs Due to Epigenetic Aberrations

hiPSCs harbouring high mtDNA mutation loads sometimes show impaired differentiation efficiencies associated with reduced cell viabilities (see 1.4.6.7). This might be solely due to the inability of mutant mitochondria to meet the more oxidative energy demands of differentiated cell progeny. The potential impact of mitochondrial dysfunction to metabolically sensitive epigenetic modifications underlying cell identity changes during differentiation of mtDNA disease hPSCs have, however, been relatively overlooked.

Cellular metabolic fluxes are important for determining germ layer specification during hPSC differentiation, with oxidative mitochondrial function being particularly important for mesodermal but not neuroectodermal specification (see 1.5.1). In line with this, hPSCs with high levels of m.13513G>A show impairments in epithelial to mesenchymal transition, instead favouring a neuroectodermal lineage commitment (Galera-Monge et al., 2019). Mitochondrial activity and associated TCA fluxes are crucial for modulating histone acetylation levels (Martínez-Reyes et al., 2016), with mtDNA disease cybrids showing alterations to various histone acetylation modifications (see 1.3.2.2). Furthermore, remodelling of mitochondrial metabolic activity is central to histone acetylation changes occurring during various stages of the myogenic programme (see 1.5.2.2). Disease causing mtDNA mutations therefore have the potential to impact chromatin modifications underlying mesodermal and myogenic cell identity acquisition/transitions and could represent an important pathomechanism contributing to tissue-specific dysfunction in mitochondrial disease patients that warrants further investigation.
1.8 Aims and Hypothesis

The overall aims of this research project are: 1) To develop an *in vitro* model of mitochondrial disease using hiPSC-derived myogenic cells harbouring the m.3243A>G and m.8344A>G mt-tRNA mutations associated with mitochondrial disease, 2) To investigate the functional consequences of these mutations in the developed cell model with a particular focus on how they might impact the metabolic and epigenetic profile.

In Chapter 3 the primary aim was to reprogram a number of different mtDNA disease patient fibroblast lines to hiPSCs and thoroughly characterise them. As a more glycolytic favouring cell type, suprathreshold levels of mtDNA pathogenic variants were expected in derived hiPSC lines. Based on previous reports in the literature, hiPSC lines with a range of different mtDNA mutation loads, representative of the bulk fibroblast population were expected from reprogramming of heteroplasmic patient fibroblast lines. Due to the importance of mitochondrial function during the reprogramming process and subsequent proliferation of pluripotent cells, impairments in establishment of hiPSC lines with high mtDNA mutation loads were also hypothesised for reprogramming of patient fibroblasts harbouring mtDNA pathogenic variants.

In Chapter 4 the primary aim was to investigate any mitochondrial and/or associated cellular detriments in the hiPSCs derived in Chapter 3, primarily in order to guide selection of the most appropriate hiPSC lines for downstream disease modelling purposes upon differentiation into myogenic cell types. As hiPSCs are more reliant on glycolytic function for meeting their energy requirements, minimal detriments in measure of mitochondrial biophysiology might be expected. Given detriments in mitochondrial function of cybrid cell lines harbouring the majority of mtDNA pathogenic variants investigated here have already been described in the literature, impairments in steady-state levels of OXPHOS complexes were hypothesised. Reductions in steady-state levels of OXPHOS CI and OXPHOS CV specifically were expected in MELAS2 and CMT hiPSC lines respectively, with suprathreshold levels of m.13528A>G and m.13565C>T *MT-ND5*, and m.9185T>C *MT-ATP6* pathogenic variants respectively. More global reductions in the entirely mitochondrial encoded OXPHOS complexes CI, CIII, CIV and CIV were hypothesised for MELAS1 and MERRF hiPSC lines with suprathreshold levels of m.3243A>G *MT-TL1* and m.8344A>G *MT-TK* mt-tRNA pathogenic variants respectively, with OXPHOS CI and CIV expected to be particularly vulnerable given previous reports of these particular pathogenic variants. Concordant impairments in
maintenance of the $\psi_m$ were hypothesised, and pathogenic variants causing reductions in particular OXPHOS complexes hypothesised to alter the sensitivity of $\psi_m$ maintenance to mitochondrial toxins targeting the respective OXPHOS complexes. Further downstream, alterations in levels of mitochondrial-derived metabolic intermediates and metabolic flux pathways were also hypothesised in hiPSC lines with suprathreshold levels of mtDNA pathogenic variants.

In Chapter 5 the primary aim was to successfully differentiate selected hiPSC lines with/without mtDNA pathogenic variants into disease relevant skeletal muscle cell types, and to identify any mtDNA pathogenic variant associated mitochondrial and/or downstream cellular phenotypes. It was hypothesised that mitochondrial deficits not previously observed in the glycolytic favouring hiPSC progeny and/or more severe mitochondrial deficits than the hiPSC progeny, would be observed in the more oxidatively favouring, terminally differentiated myogenic cell cultures. This included more severe reductions in steady-state levels of specific OXPHOS complexes and more severe reductions/impairments in $\psi_m$ maintenance. Given the importance of mitochondrial metabolism and associated cellular metabolic flux changes for driving mesodermal and latter myogenic cell-identity transitions, it was also hypothesised that impairments in myogenic differentiation efficiency and subsequent maturation of myogenic cell cultures would also be observed for hiPSC lines with suprathreshold levels of mtDNA pathogenic variants.

In Chapter 6 the primary aim was to determine whether mtDNA pathogenic variants and associated metabolic disturbances alter levels of metabolically sensitive histone acetylation modifications. Given the importance of mitochondrial derived co-substrates for both histone acetylation deposition (citrate/acyetyl-CoA) and removal ($\text{NAD}^+$), impairments in global levels of histone acetylation were hypothesised. It was hypothesised that histone acetylation detriments would be particularly evident during or shortly after cell-identity transitions heavily reliant on changes in the histone acetylation landscape including pluripotency to mesodermal differentiation and terminal myogenic differentiation.
Chapter 2  Methods

2.1  Cell Culture

Human cells with dysfunctional OXPHOS require uridine and pyruvate supplementation to support cell proliferation (King & Attardi, 1989). In order to reduce any negative selection pressures which might affect heteroplasmy levels, all cell culture media formulations contained sodium pyruvate (>1mM) and were additionally supplemented with 50 μg/ml uridine (Sigma).

2.1.1  Human Dermal Fibroblasts

Human dermal fibroblast lines were established from explant outgrowth of skin biopsies taken from one female control patient (WT), a male MELAS patient with \textit{MT-TL1} m.3243A>G pathogenic variant affecting \text{mt-tRNA}^{\text{Leu(UUR)}} (MELAS1), three unrelated female MERRF patients with \textit{MT-TK} m.8344A>G pathogenic variant affecting \text{mt-tRNA}^{\text{Lys}} (MERRF1-3), a MELAS patient with \textit{MT-ND5} m.13528A>G (p.T398A) and m.13565C>T (p.S410F) missense pathogenic variants affecting MT-ND5 (MELAS2), and a Charcot-Marie-Tooth type 2 patient with \textit{MT-ATP6} m.9185T>C (p.Leu220Pro) missense pathogenic variant affecting mt-ATP6 (CMT). Available clinical details of these mitochondrial disease patients are presented in Table 2-1. Human cells were obtained from the MRC Centre for Neuromuscular Diseases Biobank. Tissue sampling was approved by the NHS National Research Ethics Service, Hammersmith and Queen Charlotte’s and Chelsea Research Ethics Committee: Setting up of a Rare Diseases biological samples bank (Biobank) for research to facilitate pharmacological, gene and cell therapy trials in neuromuscular disorders (REC reference number 06/Q0406/33) and the use of cells as a model system to study pathogenesis and therapeutic strategies for Neuromuscular Disorders (REC reference 13/LO/1826), in compliance with national guidelines regarding the use of biopsy tissue for research. All patients or their legal guardians gave written informed consent.

Table 2-1 Available clinical details of the patients whose cells were used in this study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>mtDNA Mutation</th>
<th>Heteroplasmy</th>
<th>Clinical Syndrome</th>
<th>Symptoms/Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MELAS1</td>
<td>Male</td>
<td>\textit{MT-TL1} m.3243A&gt;G \text{mt-tRNA}^{\text{Leu(UUR)}}</td>
<td>17 % blood 85 % urine</td>
<td>Mixed MELAS/MERRF</td>
<td>Myoclonic epilepsy Stroke-like episodes Ataxia Left occipital lobe infarction detected by MRI</td>
</tr>
<tr>
<td>Patient</td>
<td>Sex</td>
<td>mtDNA Mutation</td>
<td>Heteroplasmy</td>
<td>Clinical Syndrome</td>
<td>Symptoms/Observations</td>
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</tr>
<tr>
<td>MERRF1</td>
<td>Female</td>
<td>MT-TK m.8344A&gt;G mt-tRNA&lt;sub&gt;Lys&lt;/sub&gt;</td>
<td>87 % blood 93 % urine 93 % muscle</td>
<td>MERRF</td>
<td>Myoclonic epilepsy  Cerebellar Ataxia  Axonal peripheral neuropathy  Hearing Impairment  RRF and motor denervation detected on muscle biopsy  Cerebellar atrophy detected on MRI</td>
</tr>
<tr>
<td>MERRF2</td>
<td>Female</td>
<td>MT-TK m.8344A&gt;G mt-tRNA&lt;sub&gt;Lys&lt;/sub&gt;</td>
<td>N/A</td>
<td>MERRF</td>
<td>N/A</td>
</tr>
<tr>
<td>MERRF3</td>
<td>Female</td>
<td>MT-TK m.8344A&gt;G mt-tRNA&lt;sub&gt;Lys&lt;/sub&gt;</td>
<td>70 % urine</td>
<td>MERRF</td>
<td>Gait and limb ataxia  Proximal weakness  Myalgia  Myoclonic epilepsy</td>
</tr>
<tr>
<td>MELAS2</td>
<td>Female</td>
<td>MT-ND5 m.13528A&gt;G (p.T398A) and m.13565C&gt;T (p.S410F) mt-ND5</td>
<td>100 % blood 100 % muscle</td>
<td>MELAS</td>
<td>Myoclonic epilepsy  Stroke-like episodes  Myopathy  Peripheral Neuropathy  Deafness  COX fibres and RRFs detected on muscle biopsy  Right occipital lobe infarction detected by MRI</td>
</tr>
<tr>
<td>CMT</td>
<td>Female</td>
<td>MT-ATP6 m.9185T&gt;C p.Leu220Pro mt-ATP6</td>
<td>100 % blood</td>
<td>CMT2</td>
<td>Peripheral Neuropathy  Migraine  Diabetes Mellitus  Motor denervation detected on muscle biopsy  Cerebellar atrophy detected on MRI</td>
</tr>
</tbody>
</table>

Fibroblasts were cultured in mouse embryonic fibroblasts (MEF) medium which consisted of Dulbecco’s Modified Eagle’s Medium: Nutrient Mixture F12 supplemented with Glutamax (DMEM/F12; Gibco) and 10 % v/v fetal bovine serum (FBS; Gibco). Cells were maintained in a humidified incubator at 37 °C, 95% air/5% CO₂ gas mixture and a full medium change was performed every 3-5 days. Cells were grown until ~80 % confluent before passaging with 0.25 % w/v trypsin-EDTA (Gibco) and reseeded at 1:2 split ratio.

2.1.2 Reprogramming of Human Dermal Fibroblasts to hiPSCs

Fibroblasts were reprogrammed into hiPSCs through non-integrative delivery of hOCT4, hSOX2, hKLF4 and hc-MYC using the CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermofisher). Reprogramming of control, MELAS1, MELAS2, MERRF1 and CMT was performed under feeder conditions. MERRF1, MERRF2 and MERRF3 fibroblasts
were subsequently reprogrammed under feeder-free conditions. Both methods are very similar and any differences will be highlighted. At all stages, cells were maintained in a humidified incubator at 37 °C, 95% air/5% CO₂ gas mixture.

2.1.2.1 Transduction with Reprogramming Factors

One day prior to reprogramming induction (d-1): 4x10⁵ fibroblasts at a passage number <10 were seeded into a single well of a 6-well plate and cultured for 24 hrs in MEF medium. The following day (d0): a transduction mixture was prepared in 2 ml of MEF medium consisting of CytoTune 2.0 Sendai vectors hKOS, hc-Myc and hKLF4 at a multiplicity of infection (MOI) ratio of 1:1:0.6. The hKOS vector encodes a polycistronic transcript consisting of hKLF4, hOCT4 and hSOX2. MOI refers to the number of cell infectious units (CIU) of virus to be added per individual fibroblast cell, therefore 4x10⁵ CIU of hKOS and hc-Myc, and 2.4x10⁵ CIU of hKLF4 were required per well of reprogramming. MEF medium was removed from the fibroblasts and replaced with the prepared transduction mixture. 24 hrs later (d1): the transduction mixture was removed and cells washed once with D-PBS before adding fresh MEF medium. Cells were then cultured for 5 days without medium change. On d6 the transduced fibroblasts were split with 0.05% w/v trypsin-EDTA (Gibco) and seeded in MEF medium onto a 10 cm dish prepared with irradiated CF1 MEFs (GlobalStem) or coated with Matrigel (Corning). Preparation of feeder dishes and Matrigel coated dishes is outlined below in 2.1.3.

2.1.2.2 Feeder-Dependant Conditions

24 hours after seeding of the transduced fibroblasts onto MEFs (d7), medium was changed to a knockout serum replacement (KSR) feeder medium consisting of DMEM/F12 supplemented with 10 % v/v KSR (Gibco), 1x Minimum Essential Medium Non-Essential Amino Acids (NEAA; Gibco), 55 mM β-mercaptoethanol (Gibco) and 4 ng/ml human fibroblast growth factor 2 (hFGF-2; R&D Systems). KSR feeder medium was changed daily until hiPSC colonies with a diameter of ~2-4 mm were visible (3-4 weeks).

2.1.2.3 Feeder-Free Conditions

24 hours after seeding of the transduced fibroblasts onto Matrigel coated dishes (d7) MEF medium was replaced with mTeSR1 medium (STEMCELL Technologies), and changed daily thereafter until hiPSC colonies with a diameter of ~2-4 mm were visible (4-6 weeks).
2.1.2.4 Isolation of Clonal hiPSC Lines

Isolated hiPSC colonies (~5 mm diameter) which presumably arise from proliferation outgrowth of a single transduced and reprogrammed fibroblast were visualised using an inverted microscope and mechanically divided into at least four equally sized clumps using a 10 μl pipette tip. The clumps were then transferred to a single well of a 24-well plate prepared with feeders or Matrigel as described in 2.1.3. KSR feeder medium or mTeSR1 was additionally supplemented with 10 μM Rho-associated, coiled-coil containing protein kinase inhibitor (ROCKi) Y-27632 (Sigma) to promote the survival of dissociated cells. Medium was replaced the following day without the addition of ROCK inhibitor and daily thereafter until ~60 % confluency. hiPSCs reprogrammed under feeder-dependant conditions were then transferred to standard feeder-free conditions (described below). hiPSC colonies were dissociated from the feeder layer by incubation with 1mg/ml collagenase IV (Gibco) and 1mg/ml dispase (Gibco) for approximately 30 min at 37 °C. hiPSC lines obtained were then cultured and expanded as described in 2.1.3.

2.1.3 hiPSC Culture

General hiPSC maintenance was performed under feeder-free conditions on Matrigel coated 6-well plates with mTeSR1 medium that was changed daily. hiPSC colonies were grown until ~60 % confluent before splitting with 0.5 mM ethylenediaminetetraacetic acid (EDTA; Gibco) and seeding ~15 hiPSC aggregates onto each well of a 6-well plate. Spontaneously differentiating regions of hiPSC cultures were highlighted using a microscope objection marker and removed by aspiration prior to passaging.

2.1.3.1 Preparation of MEF Feeder Plates

Feeder plates were prepared one day prior to splitting of cells as follows. Each well/dish was coated with 0.1 % w/v gelatin at room temperature (RT) for >3 hrs. Irradiated MEFs were then seeded in MEF medium onto each gelatin coated dish at a density of 1.3x10⁴/cm² (i.e. 1x10⁶ cells per 10 cm dish) and cultured overnight.

2.1.3.2 Preparation of Matrigel Coated Plates

Matrigel coated plates were prepared on the day of splitting as follows. Matrigel was thawed on ice overnight at 4 °C and diluted to 90 μg/ml with ice-cold DMEM/F12. 90 μg/ml Matrigel was then used to coat plates at 37 °C for >1 hr.
2.1.4 Myogenic Differentiation of hiPSCs

hiPSCs were differentiated into myogenic cells by adapting recently published trans-gene free protocols which combines defined factors to recapitulate developmental signalling gradients occurring in vivo during myogenesis (Shelton et al., 2014, 2015; Chal et al., 2015, 2016) (Figure 2-1). Cells were cultured in a humidified incubator at 37 °C, 95% air/5% CO₂ mixture throughout the differentiation process.

Figure 2-1 Schematic overview of protocol used for the myogenic differentiation of hiPSCs. hiPSCs were plated as small cell clumps in mTeSR1 media supplemented with ROCK inhibitor (ROCKi) one day prior (day -1) to differentiation induction (day 0). hiPSCs are first patterned towards a paraxial mesoderm lineage via Wnt signalling activation using the GSK3β inhibitor CHIR99021 (C) and BMP4 signalling inhibition using the BMP4R inhibitor LDN193189 (L). The proliferation of myogenic progenitor cells is then enhanced by culturing in knockout serum replacement (K) media supplemented with pro myogenic growth factors FGF-2 (F), HGF (H) and IGF1 (I). Terminal differentiation into multinucleated myotubes is achieved by culturing in an insulin-selenium-transferrin (IST) media without serum supplementation.

2.1.4.1 Seeding and Patterning Towards Myogenic Identity

hiPSCs were dissociated into small clumps of 3-6 cells by incubation with TrypLE™ Express (Thermofisher) (~3min, RT). The cell pellet was then resuspended gently in mTeSR1 supplemented with 10 μM Y-27632 ROCKi for counting. Cells were seeded onto Matrigel coated dishes in mTeSR1 with 10 μM ROCKi. A number of different seeding densities and culture dish sizes were trialled with varying success, but most consistent differentiations were achieved seeding in a 6-well format at 1x10⁴ cells/cm² (i.e. 1x10⁵ cells per well).

24 hours later (d0) medium was changed to a CL medium consisting of DMEM/F12 supplemented with Insulin-Selenium-Transferrin (IST; Gibco), 3 μM CHIR99021 (inhibitor of glycogen-synthase kinase-3, GSK-3; Tocris Bioscience) and 500 nM
LDN193189 (antagonist of BMP4Rs; Miltenyi Biotec). In cases of poor cell attachment and/or survival following seeding, fresh mTeSR1 was instead added to extend the recovery time for an additional 12-36 hrs. Alternatively, a slightly lower concentration of CHIR99021 (1.5 μM) was used for this initial 24 hr period, as it was noted that this assisted in survival of less confluent cultures. GSK-3β inhibition activates canonical Wnt signalling and together with inhibition of BMPR signalling promotes differentiation towards neumonesodermal and subsequently paraxial mesoderm identities. CL medium was changed every 24 hr for the next two days (d1 and d2). CL medium was additionally supplemented with 20 ng/ml hFGF-2 (CLF medium) for the next three days (d3-5) in order to support the growth and proliferation of paraxial mesoderm cells. The medium was then changed for the following two days (d6 and d7) to a HIFL medium consisting of DMEM-F12 supplemented with IST, 10 ng/ml human hepatocyte growth factor (hHGF; Peprotech), 2 ng/ml human insulin growth factor-1 (hIGF-1; R and D Systems), 20 ng/ml hFGF-2 and 500 nM LDN193189, facilitating patterning into cells with myogenic identity. K-I medium consisting of DMEM-F12 supplemented with 15% KSR and 2 ng/ml hIGF-1 was then added for the next four days (d8-11). Cells were then (from d12) cultured in KI medium additionally supplemented with 10 ng/ml HGF (KIH medium). The myogenic factors HGF, IGF-1 and FGF-2 promoted myogenesis and the proliferation of myogenic precursors. KIH was fully changed every 24 hrs until d14 at which point KIH was changed every 2-3 days depending on culture confluency.

2.1.4.2 Splitting and Expanding Proliferative Myogenic Cells

Differentiating myogenic cultures were dissociated by incubation with 1mg/ml collagenase and 1mg/ml dispase in DMEM/F12 for approximately 30-40 min at 37 ºC before gently triturating with a 1 ml pipette tip. Complete dissociation of the culture was rarely achieved and it was noted that large clumps resistant to collagenase/dispaase often expanded cells with a more neural like morphology. For this reason, large clumps of cells were sedimented by gravity and only cells in suspension seeded for myogenic cell expansion. Myogenic cultures were passaged for expansion between d20 and d35, most commonly on d25. Cells were seeded in DMEM/F12 supplemented with 15 % KSR onto Matrigel coated plates. Several different seeding densities were trialled for this expansion step but 1x10^5 cells/cm^2 supported cell proliferation best. The next day medium was changed to KIH and every 2-4 days thereafter until cells reached very high confluency ~7-10 days later.
2.1.4.3 Terminal Differentiation into Multinucleated Myotubes

Expanded myogenic cultures were passaged using collagenase/dispase and seeded as described above. The following day after seeding, cells were changed into fusion media consisting of DMEM/F12 supplemented with IST, or DMEM/F12 supplemented with 3% v/v KSR, which promoted cells to exit the cell-cycle and fuse into multinucleated myotubes. Fusion media was changed every 2-4 days thereafter until cells were taken for assays (7-14 days post seeding). A number of different seeding densities were trialled for this terminal differentiation step but 4x10^4 cells/cm^2 appeared to promote terminal differentiation most consistently.

2.1.5 Mycoplasma Testing

Cell lines in culture were routinely tested for mycoplasma contamination on a 1-2 month basis using the bioluminescence based MycoAlert™ Mycoplasma Detection Kit (Lonza) as per the manufacturer’s instructions. Briefly, a 500ul sample of cell culture media incubated with cells for >24 hr was taken into a sterile Eppendorf and centrifuged at 500g for 10min in order to pellet detached cells and cell debris. 50ul of this supernatant was then transferred to a white-bottom 96-well plate alongside wells containing 50ul MycoAlert™ Buffer (negative control) or 50ul of MycoAlert™ positive control. 50ul of MycoAlert™ reagent was then added to each well and a background luminescence reading performed using the FLUOstar Omega microplate reader (BMG Labtech). 50ul of MycoAlert™ substrate was then added to each well and repetitive reads of bioluminescence performed across a >1hr period. Increasing luminescence signal towards a plateau was observed in the positive control whereas no such luminescence is observed in samples without mycoplasma contamination. Particular focus was made towards regular mycoplasma testing of hiPSC lines used for downstream experiments and differentiation processes. No mycoplasma contamination was observed in any of the cells cultured throughout the duration of this research project.

2.2 Molecular Biology

2.2.1 Assessment of mtDNA Mutation load

DNA including both gDNA and mtDNA was extracted from cells using the Wizard Genomic DNA Purification Kit (Promega).
2.2.1.1 Dye-Terminator Sequencing Quantification

Fragments of mtDNA flanking the respective pathogenic variant sites were amplified in 25 µl polymerase chain reactions (PCRs) using M13 tailed primer pairs (5µM) (Table 2-2) and FastStart Taq DNA polymerase master mix (Roche).

Table 2-2 M13 Tailed mtDNA PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Product Length / bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-TL1_F</td>
<td>TGTAAAACGACGGCCAGTCTACGTGATCTGAGTTTC</td>
<td>296</td>
</tr>
<tr>
<td>MT-TL1_R</td>
<td>CAGGAAACAGCTATGACCGTAGTATGATATATAGCGCTAGA</td>
<td></td>
</tr>
<tr>
<td>MT-TK_F</td>
<td>TGTAAAACGACGGCCAGTACAGTTTCATGCCCCATCGTC</td>
<td>351</td>
</tr>
<tr>
<td>MT-TK_R</td>
<td>CAGGAAACAGCTATGACCGTAGTATGCGCTTTGGTGAGGGAG</td>
<td></td>
</tr>
<tr>
<td>MT-ND5-F</td>
<td>TGTAAAACGACGGCCAGTGGCGCTATCACCCACTC</td>
<td>487</td>
</tr>
<tr>
<td>MT-ND5-R</td>
<td>CAGGAAACAGCTATGACCCCTAGTAAGGGGTGGGGAAGCG</td>
<td></td>
</tr>
<tr>
<td>MT-ATP6_F</td>
<td>TGTAAAACGACGGCCAGTGACACCTACACCCCTATT</td>
<td>474</td>
</tr>
<tr>
<td>MT-ATP6_R</td>
<td>CAGGAAACAGCTATGACCGGTGTACATCGCGCCATCAT</td>
<td></td>
</tr>
</tbody>
</table>

A typical PCR temperature cycle (Table 2-3) was performed using a Geneamp 9700 PCR machine (Applied Biosystems).

Table 2-3 mtDNA PCR Temperature Cycle

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>15 min</td>
<td>x1</td>
</tr>
<tr>
<td>95</td>
<td>30 s</td>
<td>x30</td>
</tr>
<tr>
<td>52</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>45 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>x1</td>
</tr>
</tbody>
</table>

5 µl of the amplified mtDNA product was then cleaned enzymatically using 2 ul of Exo-Fast at 37 °C for 30 min followed by a 15 min denaturing step at 80 °C. Exo-fast contains 1 U/ul exonuclease I (Exo-I; Thermo Scientific) and 0.2 U/µl fast alkaline phosphatase (fast-AP; Thermo Scientific) to remove ssDNA including unused primers and unused deoxynucleotide triphosphates (dNTPs) respectively. 10 µl dye-terminator sequencing reactions were then performed on 1 µl of cleaned amplification product using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and M13 sequencing primers (0.32µM) (Table 2-4) in both the forward and reverse directions.

Table 2-4 M13 Sequencing Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13_F</td>
<td>TGTAAAACGACGGCCAGT</td>
</tr>
<tr>
<td>M13_R</td>
<td>CAGGAAACAGCTATGACC</td>
</tr>
</tbody>
</table>
The recommended PCR temperature cycle (Table 2-5) was performed using a Geneamp 9700 PCR machine.

*Table 2-5 Dye-Terminator Sequencing PCR Temperature Cycle*

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Time</th>
<th>Cycles</th>
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<tbody>
<tr>
<td>96</td>
<td>10 s</td>
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</tr>
<tr>
<td>50</td>
<td>5 s</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>4 min</td>
<td></td>
</tr>
</tbody>
</table>

Excess fluorescently labelled dideoxynucleotide triphosphates (ddNTPs) were removed by filtration through G50 Sephadex columns (Sigma) on 0.66 mm glass fibre FiltrEX™ plates (Corning) before sequencing analysis on a 3730xl DNA Analyser (Applied Biosystems). Mutation load was calculated by measuring the relative chromatogram peak heights for the WT and mutant nucleotides using Seq Scanner 2 software (Applied Biosystems).

2.2.1.2 PCR-RFLP Analysis of m.3243A>G

A 199bp fragment of the mitochondrial genome spanning the m.3243A>G variant site were amplified in 12.5 µl PCRs using a forward primer 5’ modified with 6-FAM fluorophore and unmodified reverse primer (each 1.2 µM) (Table 2-6) and FastStart Taq DNA polymerase master mix (Roche) (Table 2-7). 4ul of this PCR product was then restriction digested in a 20ul reaction consisting of 1U/µl HaeIII (Promega), 1x Buffer C (Promega) at 37 °C for 2hr. 1ul of the digest product was then denatured with 12ul HiDi Formamide (Thermofisher) and 0.3ul GeneScan 500 LIZ (Thermofisher) at 95 °C 3 min, followed by snap cool on ice. Denatured digest product was then subjected to fragment analysis on a 3730xl DNA Analyser. Mutation load was calculated by measuring the area under the curves of the WT 6-FAM product (162bp) and m.3243A>G variant 6-FAM product (90bp) using Peak Scanner 2 software (Applied Biosystems)

*Table 2-6 RFLP Primers Used for m.3243A>G Heteroplasmy Assessment*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Product Length / bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>3243_RFLP_F</td>
<td>[6FAM]CACAAGCGCCTCCC</td>
<td>199</td>
</tr>
<tr>
<td>3243_RFLP_R</td>
<td>GCGATTAGAATGGGTACAA</td>
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### Table 2-7 RFLP PCR Cycle

<table>
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<tr>
<th>Temperature / °C</th>
<th>Time</th>
<th>Cycles</th>
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<tbody>
<tr>
<td>95</td>
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<td>95</td>
<td>30 s</td>
<td>x23</td>
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<tr>
<td>59</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>x1</td>
</tr>
</tbody>
</table>

#### 2.2.2 Reverse Transcription

RNA was extracted and purified using the Quick-RNA Miniprep Kit as described in the manufacturers protocol, including the optional on-column DNAse treatment for removal of contaminating gDNA (Zymo Research). RNA concentration was quantified by measuring 260 nm absorbance using a nanodrop spectrophotometer. A complementary DNA (cDNA) library was immediately created from 500 ng of the RNA in a reverse transcription (RT) reaction using the SuperScript II (SSII) Reverse Transcriptase. 500 ng of RNA was first denatured in 10 µl of H₂O with 0.25 µg of random primers (Promega) at 65 °C for 5 min before snap cooling on ice. The denatured RNA and random primer mix was then made up into a 20 µl RT mix consisting of 500 ng RNA, 0.25 µg random primers, 1x first synthesis buffer, 10 mM Dithiothreitol (DTT), 0.5 mM dNTPs (C, G, A and T), 1 U/µl RNAseOUT and 1.25 U/µl SSII reverse transcriptase (all Thermo Scientific). Reverse transcription was catalysed by incubation at 42 °C for 1 hr followed by a 15 min denaturing step at 70 °C. A 20 µl RT− control reaction was also performed for each RNA sample which lacked the SSII reverse transcriptase enzyme in order to assess any remaining gDNA contamination within the sample. The cDNA was then diluted 1:30 in H₂O in order to maintain an appropriate cycle threshold (Cₜ) range.

#### 2.2.3 RT-qPCR

Real-time quantitative PCR (RT-qPCR) was performed using the QuantStudio 7 Flex Real-Time PCR System (Thermo Scientific) in 15 µl reactions with 5 µl of 1:30 diluted cDNA (as prepared in 2.2.2), gene-specific primer pairs (0.5µM) (Table 2-8) and fast SYBR Green (Thermo Scientific) as the reporter. RT-qPCR primer pairs were designed such that the annealing temperature was approximately 55 °C, giving an ~100-300bp product which spanned across two exons (primer annealing across exon-exon junction was attempted wherever possible). Newly designed primer pairs were assessed for off-target effects and/or primer interactions using the melting/dissociation curve. All cDNA
samples were run in duplicate or triplicate and quantification of expression levels was calculated using the mean Ct. The relative expression for genes of interest was calculated for each sample using the ΔCt method.

Relative expression = $2^{(CT(\text{gene of interest}) - CT(\text{housekeeping gene}))}$

Table 2-8 RT-qPCR Primer Pairs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Product Length / bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMBS_F</td>
<td>GGAGCCATGTCCTGTAACGG</td>
<td>143</td>
</tr>
<tr>
<td>HMBS_R</td>
<td>CCAACGGAATCAGCCTCTCT</td>
<td></td>
</tr>
<tr>
<td>GAPDH_F</td>
<td>TGGTGCTGAGTATGTCGAGT</td>
<td>292</td>
</tr>
<tr>
<td>GAPDH_R</td>
<td>AGTCTCTGAAGTGCGATG</td>
<td></td>
</tr>
<tr>
<td>LIN28A_F</td>
<td>GGAAGAGCATGCGAAGCG</td>
<td>91</td>
</tr>
<tr>
<td>LIN28A_R</td>
<td>GTGGCACGCTTGCATTCTTG</td>
<td></td>
</tr>
<tr>
<td>REX1_F</td>
<td>CCGAGACACGTCTGTCGG</td>
<td>125</td>
</tr>
<tr>
<td>REX1_R</td>
<td>AGGCCCTTCCGCAACCTCTA</td>
<td></td>
</tr>
<tr>
<td>DNMT3B_F</td>
<td>ATAAGTCGAGGTGCGTCGT</td>
<td>202</td>
</tr>
<tr>
<td>DNMT3B_R</td>
<td>GCACACATCTGAAGCCATT</td>
<td></td>
</tr>
<tr>
<td>SOX2_F</td>
<td>ATGTCCAAGCACTACAGAG</td>
<td>141</td>
</tr>
<tr>
<td>SOX2_R</td>
<td>GCACACCCTCCTTCTCCC</td>
<td></td>
</tr>
<tr>
<td>OCT4_F</td>
<td>CCTAATCCACTGCACTGTA</td>
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<tr>
<td>OCT4_R</td>
<td>CAGGTTCCTTCCCTCTAGCT</td>
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</tr>
<tr>
<td>NANOG_F</td>
<td>GCTTGCCCTGCTTGGAGCA</td>
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</tr>
<tr>
<td>NANOG_R</td>
<td>TTCTTGACCGAGACCTTGC</td>
<td></td>
</tr>
<tr>
<td>DES_F</td>
<td>GAGATCGCCTCAACGGTGACA</td>
<td>112</td>
</tr>
<tr>
<td>DES_R</td>
<td>GAGGCAGAGTAGGTCTGGA</td>
<td></td>
</tr>
<tr>
<td>TTN_F</td>
<td>GCCACCAATGGATCTGGACA</td>
<td>114</td>
</tr>
<tr>
<td>TTN_R</td>
<td>GCTTCCCTTGTCCAGGTCA</td>
<td></td>
</tr>
<tr>
<td>PAX7_F</td>
<td>ACCCCTGCTAACCACATC</td>
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</tr>
<tr>
<td>PAX7_R</td>
<td>GCCGCAAAAGAATCTTGGGAGAC</td>
<td></td>
</tr>
<tr>
<td>MYH1_F</td>
<td>CCCTACAAGTGTTGCGGAGT</td>
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<tr>
<td>MYH1_R</td>
<td>CTTCCCTGCACCCATCTCC</td>
<td></td>
</tr>
<tr>
<td>MYH2_F</td>
<td>AGAAACCTGCGATGACCTAGA</td>
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<tr>
<td>MYH2_R</td>
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</tr>
<tr>
<td>MYH3_F</td>
<td>TTGATGCAAGACGATCTGCT</td>
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<tr>
<td>MYH3_R</td>
<td>GGGGGTTCTCATGGCGTACAC</td>
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</tr>
<tr>
<td>MYH7_F</td>
<td>ACTGCGAGAGCGAGTATG</td>
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</tr>
<tr>
<td>MYH7_R</td>
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</tr>
<tr>
<td>MYH8_F</td>
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<tr>
<td>MYH8_R</td>
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<td></td>
</tr>
<tr>
<td>PPARGC1A_F</td>
<td>GTCAACCACCAATCTCTAT</td>
<td>131</td>
</tr>
<tr>
<td>PPARGC1A_R</td>
<td>ATCTACTGCGCTGAAGCCCT</td>
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<tr>
<td>TFAM_F</td>
<td>ATGGCGTTTCTCCGAAGCAT</td>
<td>317</td>
</tr>
<tr>
<td>TFAM_R</td>
<td>TCCGCCCTATAAGCATCTTG</td>
<td></td>
</tr>
</tbody>
</table>
2.2.4 *RT-qPCR Measurements of mtDNA Content*

DNA including both gDNA and mtDNA was extracted from cells using the Wizard Genomic DNA Purification Kit (Promega). DNA concentration was quantified by measuring 260 nm absorbance using a nanodrop spectrophotometer. A DNA dilution of 5ng/µl was then prepared. RT-qPCR was performed using the QuantStudio 7 Flex Real-Time PCR System (Thermo Scientific) in 15 µl reactions with either 1ul of 5ng/µl DNA (nDNA targets, APP and/or B2M) or 4ul of 5ng/µl DNA (mtDNA targets, MT-CYB and/or MT-ND1, MT-ND4) and nDNA/mtDNA specific primer pairs (0.5µM) (Table 2-9) and fast SYBR Green (Thermo Scientific) as the reporter. Each reaction was run in duplicate or triplicate and quantification of relative mtDNA content was calculated using the \( \Delta \Delta Ct \) method where \( \Delta Ct = Ct(nDNA) - Ct(mtDNA) \). For experiments including both APP and B2M nDNA primer pair targets, \( Ct(nDNA) \) represents the geometric mean of the \( Ct(\text{APP}) \) and \( Ct(\text{B2M}) \)

Relative mtDNA content = \( 2^{-\Delta \Delta Ct(\text{control})} \cdot \Delta Ct(\text{mutant}) \)

*Table 2-9 mtDNA Content RT-qPCR Primer Pairs*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Product Length / bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M_F</td>
<td>CACTGAAAAAGATGAGTATGCC</td>
<td>231</td>
</tr>
<tr>
<td>B2M_R</td>
<td>AACATCCCTGACAATCCC</td>
<td></td>
</tr>
<tr>
<td>APP_F</td>
<td>TGTTGTGCTCTCCCAGGTCTA</td>
<td>80</td>
</tr>
<tr>
<td>APP_R</td>
<td>CAGTTCTGAGATGTCAGTG</td>
<td></td>
</tr>
<tr>
<td>MT-CYB_F</td>
<td>GCTGCCTGATCCTCCAAAT</td>
<td>132</td>
</tr>
<tr>
<td>MT-CYB_R</td>
<td>AAGGTACGCTGATGATTAGCC</td>
<td></td>
</tr>
<tr>
<td>MT-ND1_F</td>
<td>ACGCCATAAAACTCTTCACAAAG</td>
<td>111</td>
</tr>
<tr>
<td>MT-ND1_R</td>
<td>GGGTTCATAGTAAAGAGCGATGG</td>
<td></td>
</tr>
<tr>
<td>MT-ND4_F</td>
<td>ACCGTGGCATTACTACCCCGAT</td>
<td>107</td>
</tr>
<tr>
<td>MT-ND4_R</td>
<td>AGTGCATGATAGGGGAAGG</td>
<td></td>
</tr>
</tbody>
</table>

2.2.5 *RT-PCR Assessment of SeV Transgene Clearance*

cDNA was prepared from hiPSC RNA extracts as described in 2.2.2. 25 µl RT-PCR reactions consisting of FastStart Taq DNA polymerase master mix, 5ul of hiPSC cDNA dilution and 5µM primer pairs (Table 2-10). A RT-PCR reaction with primer pairs specific for the HMBS housekeeping gene served as a positive control for the reaction.
Table 2-10 Sendai Viral Transgene Specific RT-qPCR Primer Pairs

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Product Length / bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMBS_F</td>
<td>GGAGCCATGTCTGTAACGG</td>
<td>73</td>
</tr>
<tr>
<td>HMBS_R</td>
<td>CCACGCGAATCCTCTCATCT</td>
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</tr>
<tr>
<td>Backbone_F</td>
<td>GGATCAGATGCGATGACGACG</td>
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<tr>
<td>Backbone_R</td>
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<tr>
<td>hKOS_F</td>
<td>ATGCACCGCTACGACGACGAGCGG</td>
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<td>hKOS_R</td>
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<tr>
<td>hKLF4_F</td>
<td>TTCCTGCATGCCAGGGAGGCC</td>
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<td>hKLF4_R</td>
<td>ATGTATCGAGGTGCTCAA</td>
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</tr>
<tr>
<td>hc-Myc_F</td>
<td>TAACTGACTAGCAGGCTGTCG</td>
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</tr>
<tr>
<td>hc-Myc_R</td>
<td>TCCACATACAGTCTGGATGATG</td>
<td></td>
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</tbody>
</table>

A typical PCR cycle was performed using a Geneamp 9700 PCR machine (Table 2-11)

Table 2-11 Sendai Viral RT-PCR Temperature Cycle

<table>
<thead>
<tr>
<th>Temperature / °C</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>15 min</td>
<td>x1</td>
</tr>
<tr>
<td>95</td>
<td>30 s</td>
<td>x30</td>
</tr>
<tr>
<td>55</td>
<td>30 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>45 s</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>x1</td>
</tr>
</tbody>
</table>

RT-PCR products were separated through 2% w/v agarose tris-acetate-EDTA (TAE) gel electrophoresis and visualised through UV302 transillumination using the Sybr Safe nucleic acid dye (Thermofisher).

2.2.6 Cell Fixation and Immunofluorescence

Cells were fixed for immunostaining with 4% paraformaldehyde (PFA; Sigma) in D-PBS (15 min, RT). Cells were then washed 3 times with D-PBS before incubation in a blocking and permeabilisation solution consisting of D-PBS with 0.1% w/v Triton X-100 and 10% v/v serum. The serum was obtained from the same species in which the 2°-antibodies used were raised. Cells were then incubated with 1°-antibody (in Dako antibody diluent (Agilent) (RT, 1-2hr or 4 °C, overnight) before 5 washes with PBS. Cells were then incubated with fluorophore conjugated 2°-antibody in antibody diluent (RT, 1hr) before 3 washes with PBS. Nuclei were then counter-stained with 0.1 μg/ml 4’,6-diamidino-2-
phenylindole (DAPI) in PBS (RT, 15 min), followed by 5 washes with D-PBS. Cells stained in culture dishes were imaged in D-PBS. Cells cultured on coverslips were mounted to glass slides using Dako fluorescence mounting medium (Agilent).

Images were captured using an Olympus IX71 inverted microscope with Hamamatsu Orca R2 monochrome camera, or Zeiss LSM 700 upright confocal microscope. Imaging acquisition parameters including laser intensities, pin-hole diameters, acquisition times, pixel resolution and gain settings were kept constant for each experiment. Images were processed using ImageJ FIJI software and alterations to brightness and/or contrast made for image presentation purposes kept consistent between images from the same experiment.

Table 2-12 Antibodies Used for Immunofluorescence

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Species Raised</th>
<th>Dilution</th>
<th>Fluorophore</th>
<th>Company</th>
<th>Cat. No.</th>
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</thead>
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<tr>
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<td></td>
<td>Abcam</td>
<td>ab18976</td>
</tr>
<tr>
<td>OCT4</td>
<td>Mouse monoclonal IgG2b</td>
<td>1:200</td>
<td></td>
<td>Stem Cell Technologies</td>
<td>3A2A20</td>
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<tr>
<td>NANOG</td>
<td>rabbit monoclonal</td>
<td>1:300</td>
<td></td>
<td>Abcam</td>
<td>ab109250</td>
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<tr>
<td>TRA-1-60</td>
<td>mouse monoclonal IgM</td>
<td>1:200</td>
<td></td>
<td>Santa Cruz</td>
<td>SC-21705</td>
</tr>
<tr>
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<td>mouse monoclonal IgG1</td>
<td>1:300</td>
<td></td>
<td>Dako</td>
<td>M0760</td>
</tr>
<tr>
<td>Titin</td>
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<td>1:300</td>
<td></td>
<td>DSHB</td>
<td>9 D10-c</td>
</tr>
<tr>
<td>PAX7</td>
<td>rabbit polyclonal</td>
<td>1:200</td>
<td></td>
<td>Thermofisher</td>
<td>PA1-117</td>
</tr>
<tr>
<td>Pan-MyHC</td>
<td>mouse monoclonal IgG2b</td>
<td>1:300</td>
<td></td>
<td>DSHB</td>
<td>MF 20</td>
</tr>
<tr>
<td>MyoD</td>
<td>mouse monoclonal IgG1K</td>
<td>1:200</td>
<td></td>
<td>Dako</td>
<td>5.8A</td>
</tr>
<tr>
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<td>rabbit polyclonal</td>
<td>1:200</td>
<td></td>
<td>abcam</td>
<td>ab15580</td>
</tr>
<tr>
<td>Myogenin</td>
<td>mouse monoclonal IgG1K</td>
<td>1:200</td>
<td></td>
<td>Dako</td>
<td>F5D</td>
</tr>
</tbody>
</table>
**MyHC (Embryonic)**
- Mouse monoclonal IgG1 1:50 Leica

**MyHC (Neonatal)**
- Mouse monoclonal IgG1 1:50 Leica

**MyHC (Fast)**
- Mouse monoclonal IgG1 1:100 Leica

**MyHC (Slow)**
- Mouse monoclonal IgG1 1:100 Leica

**H4K16ac**
- Rabbit polyclonal 1:1000 EpiGentek

**TOMM20**
- Rabbit polyclonal 1:1000 Abcam

**mouse IgM**
- Goat 1:1000 Alexa Fluor 488 ThermoFisher

**mouse IgM (mu chain)**
- Goat 1:1000 Alexa Fluor 568 ThermoFisher

**mouse IgG (H+L)**
- Goat 1:1000 Alexa Fluor 488 ThermoFisher

**mouse IgG1 (y1)**
- Goat 1:1000 Alexa Fluor 488 ThermoFisher

**mouse IgG2b (y2b)**
- Goat 1:1000 Alexa Fluor 594 ThermoFisher

**Rabbit IgG (H+L)**
- Donkey 1:1000 Alexa Fluor 488 ThermoFisher

**rabbit IgG (H+L)**
- Donkey 1:1000 Alexa Fluor 568 ThermoFisher

**rabbit IgG (H+L)**
- Goat 1:1000 Alexa Fluor 647 ThermoFisher

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2.2.7 **TMRM Measurements of $\psi_m$**

$\psi_m$ was assessed using live confocal imaging of the cell-permeant, fluorescent dye tetramethylrhodamine methyl ester (TMRM). TMRM is a positively charged molecule which accumulates in the negatively charged environment of the mitochondrial matrix. When used at a low concentration (25 nM) TMRM fluorescence within mitochondria is proportional to $\psi_m$, i.e. a reduction in TMRM intensity corresponds to mitochondrial depolarisation.

Cells were incubated with 25 nM TMRM (ThermoFisher) diluted in Hank's Balanced Salt Solution (HBSS; Gibco) for 40 min at RT. Fresh HBSS with TMRM was added shortly before imaging. Images were captured using a Zeiss LSM 700 upright confocal using the manufacturers predefined settings for TMRM. Laser intensity was kept to a minimum...
(<0.3%) in order to avoid photobleaching/phototoxicity. Digital gain was adjusted to increase the dynamic range of detection whilst ensuring saturation of the signal was not occurring. For measurements of basal $\psi_m$, z-stack images consisting of 2 μm slices were taken. All laser and detection settings were kept constant for each experimental imaging session. Basal $\psi_m$ quantification was made from a maximum intensity projection. Quantification was performed using ImageJ FIJI software. Mean pixel intensity was quantified with a minimum intensity threshold set such that non-mitochondrial fluorescence was excluded from the analysis. The minimum intensity threshold chosen was kept constant between images taken during the same experimental session. In order to assess the contribution of various OXPHOS complexes to $\psi_m$ maintenance, cells were incubated with 25 nM TMRM as described above and time-course imaged whilst sequentially adding mitochondrial toxins. A single 2 μm focal plane positioned to include in-focus mitochondria was imaged every 5-10 s. Baseline measurement was first established (>4 timepoints with apparently stable TMRM intensity) before addition of 10μg/ml oligomycin-A (Sigma) in order to inhibit OXPHOS CV. In healthy cells, CV is normally utilising the $\psi_m$ to synthesise ATP, thus a slight hyperpolarisation of $\psi_m$ is expected after inhibition. In diseased or stressed cellular states, CV can work in reverse (i.e. utilise ATP to pump $H^+$ from the mitochondrial matrix) and CV inhibition would instead cause $\psi_m$ depolarisation. Following stabilisation of the CV inhibited state (>4 stable timepoints), 5μM rotenone (Sigma) was added in order to inhibit CI. Following stabilisation of the CI inhibited state (>4 stable timepoints) 1μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) protonophore was then added in order to dissipate the mitochondrial $H^+$ gradient causing complete $\psi_m$ depolarisation. Quantification was performed using ImageJ FIJI software. Mean pixel intensity was quantified with a minimum intensity threshold set such that non-mitochondrial fluorescence was not included. TMRM intensity values were normalised to the basal TMRM intensity (set as 100%) and TMRM intensity following FCCP induced $\psi_m$ depolarisation (set as 0%).

### 2.2.8 Electron Microscopy

Cells were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer and 5 mM CaCl$_2$. An equal volume of fixative was first added to the cell medium for 5 min before replacing with 100 % fixative. Fixed cells were stored at 4 °C in fixative solution before further processing by Kerrie Venner from the National Hospital for Neurology and
Neurosurgery (NHNN) Neuropathology diagnostic laboratory. Images were captured on a Philips CM10 transmission electron microscope.

2.2.9 Oxygen Consumption Rate Measurements.

OCR and extracellular acidification rate (ECAR) were measured using a Seahorse Bioscience XF24 Extracellular Flux Analyser (Seahorse Bioscience). Cells were incubated in assay medium (DMEM without sodium bicarbonate supplemented with 110 μg/ml sodium pyruvate, 5.5 mM glucose (all Sigma) and 1x Glutamax (Gibco), pH = 7.4) prior to OCR measurements (37 °C, 1 hr, non-CO₂ incubator). Basal OCR and ECAR was first measured before the sequential addition of 1μM oligomycin-A, 1 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and 1 μM antimycin-A (all Sigma). OCR measurements in the presence of these toxins allows a number of respiratory chain functional parameters to be calculated including: ATP production, maximal respiration and spare respiratory capacity, which will be discussed in the respective results section. OCR and ECAR measurements were normalised to total protein content measured through Bradford Assay (BioRad).

2.2.10 Whole Cell Protein Lysates for Western Blot

Cells were first washed with ice-cold D-PBS and then immediately incubated at -80 °C for >1hr. Alternatively cells were enzymatically detached from the cell culture dish and pelleted, before washing with ice-cold D-PBS and incubating at -80 °C for >1hr. Cells on culture dishes were scraped to one side of the culture dish whilst on ice and lysed in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer consisting of 150 mM NaCl, 50 mM Tris-HCl (pH = 8), 1% w/v NP-40, 0.5% w/v sodium deoxycholate and 0.1% w/v sodium dodecylsulphate (all Sigma). Protease and phosphatase inhibitor cocktail (1x; Sigma) was freshly added to the lysis buffer before use. Lysed cells were transferred to ice-cold 1.5 ml Eppendorf tubes, and incubated on ice for 1 hr with intermittent vortexing (1 min vortex every 20 min). Cell debris was then pelleted by centrifugation (20,000g, 10 min, 4 °C) and the supernatant containing the protein lysate transferred to a clean pre-chilled Eppendorf tube. Protein concentration was quantified through a Bradford assay (BioRad) or Pierce bicinechonic acid (BCA) assay (Thermofisher) following the manufacturers protocol. Briefly, 1 μl of cell lysates were added in triplicate into individual wells of a 96-well plate. Triplicates of known bovine serum albumin (BSA; Thermofisher) concentration were also added into the 96-well plate in order to generate a standard curve. Triplicate wells containing 1 μl of lysis buffer served as blanks. 200 μl
of Bradford assay or Pierce BCA assay reagent were then added to each sample. Absorbance was measured at 590 nm (Bradford) or 562 nm (BCA) using a 96-well plate reader. Protein concentration was calculated by comparing the absorbance of the protein samples to the known BSA standard curve.

2.2.11 Histone Extraction for Western Blot

Histone extracts were prepared from cells through nuclear isolation and acid extraction using the EpiQuik Total Histone Extraction Kit as described in the manufacturers protocol. Briefly, cells were enzymatically detached from the cell culture dish, washed with ice-cold D-PBS and pelleted by centrifugation. Cells were then resuspended in ice-cold pre-lysis buffer (a cell lysis buffer maintaining nuclear integrity) at a concentration of \(5 \times 10^7\) cells / ml before incubating on ice for 10 min. Nuclei were then pelleted by centrifugation (10,000g, 1 min, 4 °C) and resuspended in lysis buffer at a concentration of \(1 \times 10^7\) cells / ml before incubating on ice for 30 min. Nuclear debris was then pelleted by centrifugation (13,500g, 5 min, 4 °C) and the supernatant containing extracted histones transferred to an Eppendorf tube containing 0.3 volumes of a balance buffer with freshly added DTT in order to neutralise the acid. Histone extractions were quantified by measuring 280 nm absorbance assuming a BSA extinction coefficient (6.67) using a nanodrop spectrophotometer.

2.2.12 Polyacrylamide Gel Electrophoresis and Western Blotting

Equal amounts of protein (20-40µg) or histones (10µg) were denatured in 1x NuPAGE LDS Sample Buffer (Thermofisher) with 10 mM DTT by heating at 95 °C for 5 min. For assessment of mitochondrial OXPHOS complexes, samples were instead heated to 37 °C in order to maintain the detection of heat-labile MT-CO1 subunit of OXPHOS CIV. Samples were then loaded into NuPAGE 4-12 % Bis-Tris polyacrylamide gels (Invitrogen) and separated by polyacrylamide gel electrophoresis (PAGE) in NuPAGE MOPS SDS Running Buffer using a Bolt mini gel tank (all Thermofisher). Polyacrylamide gels were equilibrated by running empty at 100 V for 10 min prior to sample loading. Samples were first stacked into the gel by running at 70 V for 20 min, before separating at 100 V. Separated proteins were then electrophoretically wet transferred to 0.22 µm nitrocellulose membranes (Biorad) using a genie blotter system (Idea Scientific) and transfer buffer consisting of 1x tris/glycine buffer with 20% v/v methanol (both Sigma) (18 V, 1.5 hr, 4 °C). Gels and nitrocellulose membranes were washed in transfer buffer prior to electrophoretic transfer (10 min, RT). Following
transfer, membranes were washed in tris-buffered saline (TBS; 50 mM Tris-HCl 150 mM HCl, pH = 7.5) with 0.1% w/v tween-20 (TBS-T, all Sigma) (10 min, RT). Membranes were then stained with 50% v/v Ponceau S solution (Sigma) diluted in TBS-T in order to assess transfer efficiency and guide cutting of membranes for simultaneous assessment of multiple proteins with differing molecular weights. Membranes were then washed 3 times with TBS-T (10 min, RT) before blocking in TBS-T with 5% w/v milk (1 hr, RT). Membranes were then incubated with 1° antibodies (Table 2-13 diluted in 1 % w/v milk TBS-T (4 °C, overnight). Membranes were washed 3 times with TBS-T (10 min, RT) and then incubated with horseradish peroxidase conjugated 2° antibodies (Biorad) in 1 % w/v milk TBS-T (1-2 hr, RT). western blots were developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Life Sciences) and imaged using a ChemiDoc Touch imaging system (BioRad).
Table 2-13 Primary Antibodies Used for Immunoblotting

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Species Raised</th>
<th>Dilution</th>
<th>Company</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-Actin</td>
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<td>1:5000</td>
<td>Sigma</td>
<td>A2228</td>
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<tr>
<td>TOMM20</td>
<td>rabbit</td>
<td>1:1000</td>
<td>abcam</td>
<td>ab78547</td>
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<tr>
<td>Mito OXPHOS Cl-CV</td>
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<td>AMPKα</td>
<td>rabbit</td>
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</tr>
<tr>
<td>Phospho-AMPKα (Thr172)</td>
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<td>#2535</td>
</tr>
<tr>
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<tr>
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</tr>
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<td>rabbit</td>
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<td>EpiGentek</td>
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<td>EpiGentek</td>
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</tr>
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</table>

2.2.13 Native Chromatin Immunoprecipitation and ChIP-Seq Library Preparation

A number of different conditions were tested for native chromatin immunoprecipitation (ChIP) but the following procedure gave the most optimal results.

Cells were enzymatically detached from the culture vessel and pelleted in a falcon tube (500 g, 4 min, RT). Cells were then washed twice in 5 ml of ice-cold D-PBS with
centrifugation (500 g, 4 min, 4 °C). ~1x10^7 cells were then resuspended gently in 2 ml of NBA buffer (85 mM NaCl, 5.5 % w/v sucrose, 10 mM TrisHCl (pH 7.5), 0.2 mM EDTA, 0.2 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM DTT, 1x Complete Mini EDTA-free protease inhibitors). 2 ml of NBA buffer with 0.1 % NP40 (NBB buffer) was then added before incubating on ice for 3 mins to gently lyse the cells. Nuclei were pelleted by centrifugation (1000g, 3 min, 4 °C), washed in NBR buffer (85 mM NaCl, 5.5 % w/v sucrose, 10 mM TrisHCl (pH 7.5), 3 mM MgCl2, 1.5 mM CaCl2, 0.2 mM PMSF, 1 mM DTT, 1x protease inhibitors), and centrifuged (2000g, 3 min, 4 °C). Nuclei were resuspended in NBR buffer and DNA content quantified by measuring 260nm absorbance using a nanodrop spectrophotometer. Nuclei were diluted to a DNA concentration of 0.5 µg/ml and RNA digested with 10 µg/ml RNase A (5 min, 20 °C; Sigma). Chromatin was fragmented into fragments associated with 1-3 nucleosomes by digestion with 0.1 U/µl micrococal nuclease (MNase; Sigma) for 15 mins, 20 °C. MNase digestion was stopped by addition of equal volume ice-cold STOP buffer (215 mM NaCl, 10 mM TrisHCl (pH 8), 20 mM EDTA, 5.5 % w/v sucrose, 2 % w/v Triton X-100, 0.2 mM PMSF, 1 mM DTT, 2x protease inhibitors). Nuclei were incubated on ice overnight to slowly release the fragmented chromatin. Nuclei debris was pelleted by centrifugation (20 000g, 5 min, 4 °C) and the supernatant containing cleared chromatin transferred to a new Eppendorf tube.

For each chromatin immunoprecipitation (ChIP), 20 µl of magnetic protein A Dynabead slurry (Thermofisher) were coupled with 1 µg of antibody raised against the histone modification of interest as follows. Using a magnetic stand 20 µl of bead slurry was washed twice with 500 µl of blocking solution (5mg/ml BSA, 0.1 mM PMSF). The protein A beads were then resuspended in 250 µl of blocking solution with 1 µg of antibody and rotated (>2 hrs, 4 °C). Antibody bound beads were then washed with 250 µl of blocking solution.

150 µg of released chromatin was then added to the antibody bound beads and rotated (>3 hrs, 4 °C). 15 µg of chromatin was retained as 10 % input. The chromatin bound beads were then washed 3 times with 200 µl of wash buffer (200 mM NaCl, 10 mM TrisHCl (pH 8), 2 mM EDTA, 1 % w/v Triton X-100) by rotation (10 mins, 4 °C). A final wash in TE buffer (10 mM TrisHCl (pH 8), 1 mM EDTA) was carried out at room temperature. Chromatin was released from the beads with elution buffer (0.1 M NaHCO3, 1 % SDS; 37 °C, >15 mins) and treated with 10 µg/ml proteinase K to digest the histones (55 °C,
overnight). ChIP and 10% input DNA were purified using a QIAquick PCR purification kit (Qiagen) and eluted in 20 μl of nuclease free dH2O.

ChIP and 10% input DNA were then quantified using the Qubit dsDNA high sensitivity assay kit and Qubit 2.0 fluorometer. A next generation sequencing (NGS) library was then prepared from 100 pg of ChIP and 10% input DNA samples using the Accel-NGS 2S Hyb DNA Library Kit and 2S Indexing Kit for Illumina (Swift Bio) following the manufacturers protocols. Briefly, the MNase digested dsDNA was first repaired through a 5’ dephosphorylation phosphatase reaction followed by an end-repair polymerisation reaction. The repaired dsDNA strands were then ligated with sample specific P7 index adapters for multiplexing purposes. The P7 indexed dsDNA was then ligated with independent strand specific P5 adapters (two independent P5 adapters per dsDNA molecule). A PCR reaction was then performed in order to amplify the indexed library.

2.2.14 Glucose Flux Analysis of hiPSCs

1x10^6 hiPSCs were seeded onto Matrigel coated 10cm dishes in mTeSR1 culture media (d0). 48 hours later (d2), medium was changed to basic DMEM supplemented with 5.5 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, IST, 1% w/v L-ascorbic acid 2-phosphate sesquimagnes, 25ng/ml FGF and 1.74ng/ml TGF beta. 24 hours later (d3), the media was then changed to a basic DMEM supplemented with 5.5 mM radiolabelled [U-\(^{13}\)C]-glucose. After 2 hours of radiolabel incorporation, metabolites were extracted in a cold room with ice-cold solutions as follows. Medium was removed and cells washed 3 times with D-PBS. 300 μl of double distilled H₂O (ddH₂O) containing 1 nM scyllo-inositol internal standard was then added to the centre of the dish. 300 μl of methanol was then added and cells scraped from the culture surface. The cell suspension was then transferred to an Eppendorf tube with 300 μl of chloroform. 600 μl of methanol was then added to the culture dish to wash off any remaining cells and transferred to the same Eppendorf tube before vortexing briefly. Cells were incubated for 1 hr at 4°C which included three 8 min pulses of sonication in order to lyse the cells and release metabolites. The extracted metabolites were stored at -20 °C before being further processed and analysed by collaborators at the Francis Crick Institute’s metabolomics core facility through gas chromatography-mass spectrometry.
Chapter 3  Derivation of hiPSCs with mtDNA Disease Mutations

3.1 Overview

The first step towards generation of a disease-relevant in vitro model of mitochondrial disease skeletal muscle required generation of hiPSCs harbouring mtDNA disease mutations. The reprogramming process and established hiPSCs can also serve as useful cellular models for exploring the impact of mtDNA mutations on cellular metabolism and associated metabolically sensitive epigenetic modifications.

While hiPSCs preferentially utilise glycolysis in order to meet cellular energy demands recent progress in the field highlights the crucial role of mitochondrial metabolism towards the acquirement and maintenance of the pluripotent state (see Introduction section 1.4 for in-depth overview). Derivation of hiPSCs from patients with a mitochondrial phenotype might therefore be met with additional challenges than those already faced during the relatively inefficient process of reprogramming cells from healthy control individuals and patients with non-mitochondrial related diseases. In relation to the reprogramming of primary patient cell lines harbouring heteroplasmic mtDNA disease mutations, the stochastic nature of the mitochondrial mutation load within individual cells of the bulk population means that derivation of only a few hiPSC clone(s) is unlikely to be sufficient for obtaining cell lines with mutation loads appropriate for disease modelling purposes alongside simultaneous establishment of isogenic control clones. In addition, negative selection pressures towards individual cells with high heteroplasmic or homoplasmic mutation loads might further complexify establishment of hiPSC lines with suprathreshold heteroplasmcy levels for disease modelling.

3.1.1 Reprogramming Strategy

The CytoTune-iPS 2.0 Reprogramming Kit licensed by Thermofisher utilises a non-integrating Sendai viral vector in order to introduce the four Yamanaka factors (hOCT4, hSOX2, hKLF4 and hc-MYC; collectively OSKM) most commonly used for driving somatic cell reprogramming. This Sendai virus-based kit has a number of advantages over other delivery strategies such as adenoviral vectors, retroviral vectors, episomal vectors and mRNA transfection. These include the absence of host genome integration potential, requirement of only one transduction and relatively high reprogramming efficiency.
O’Callaghan, B.J.

(commonly reported up to 0.1%) (Schlaeger et al., 2015). For these reasons the CytoTune 2.0 kit was chosen to ensure a sufficient number of hiPSC clones would arise for selection and subsequent expansion in order to facilitate the likelihood cell lines with an appropriate range of mutation loads could be selected.

3.1.2 Selection of Fully Reprogrammed Clones and Pluripotency Assessment

Although the delivery of reprogramming factors to somatic cells is probably an influential factor, the inefficiency of the reprogramming process is largely due to the unlikeliness of cells progressing through a number of essential epigenetic and transcriptional phases. Two mechanistic models seeking to explain this inefficiency of the reprogramming process were originally proposed: an elite model and stochastic model (Yamanaka, 2009).

The elite model postulates that within a given somatic cell population there exists only a few cells which are permissible to the reprogramming process. Heterogenous somatic cell populations do include cells with adult stem cell identity (Goodell, Nguyen & Shroyer, 2015) which at least in some instances appear more permissible for reprogramming to the pluripotent state (Guo et al., 2014). However, reprogramming of a wide variety of terminally differentiated somatic cells to a pluripotent state has been achieved and long-term pluripotency factor overexpression in clonal B-cells and monocytes has been shown to result in successful acquisition of pluripotency by almost every clonal cell line (Hanna et al., 2009). While a heterogenous cell population might contain individual cells with intrinsically higher susceptibility to pluripotency acquisition, this evidence suggests every cell retains a stochastic probability for reprogramming.

The stochastic model which is more widely accepted instead postulates that the low efficiency of cell reprogramming is caused by the random but rare chance numerous cellular events required for acquiring pluripotency correctly occur in parallel and/or sequentially. Among the necessary events occurring during the early phase of reprogramming is the silencing of somatic cell genes through reprogramming factor driven changes in the epigenetic landscape, both directly through binding of reprogramming factors to the enhancers/promoters of somatic cell genes, and indirectly through activation of secondary genes and redistribution of somatic gene transcription factors (Li et al., 2017; Knaupp et al., 2017; Chronis et al., 2017). Due to impermissive heterochromatin positioned at pluripotency genes, reprogramming factor binding at the promoters and subsequently enhancers of these genes necessary for inducing their
expression is a very inefficient process, and likely represents one of the major roadblocks preventing efficient somatic cell reprogramming (Knaupp et al., 2017; Soufi, Donahue & Zaret, 2012). Following on from this relatively inefficient ‘first wave’ of pluripotency gene expression, a second more deterministic induction of late pluripotency genes including endogenous SOX2 occurs (Chung et al., 2014; Polo et al., 2012).

A proportion of cells which have silenced the somatic gene expression profile and at least partially initiated the stochastic induction of early pluripotency genes subsequently fail to instigate the second phase of late pluripotency gene expression and are considered partially reprogrammed cells (Takahashi & Yamanaka, 2016; Polo et al., 2012). Following cessation of exogenous pluripotency/reprogramming factor expression, partially reprogrammed cells will often exit this transient pluripotent-like state and differentiate into cells evidently discernible from true PSCs. Partially reprogrammed cells can retain a PSC-like morphology and express a number of different pluripotency associated markers however (Chan et al., 2009; Chung et al., 2014; Buganim et al., 2012). Picking colonies with a typical PSC-like morphology is therefore a good strategy for exclusion of partially reprogrammed cells but not 100% efficient.

Even with non-integrative reprogramming strategies including Sendai virus-based delivery methods, it is important to ensure that apparently stable hiPSC lines are no longer expressing exogenous reprogramming factors which might otherwise be masking incomplete cell reprogramming. In relation to this, is it also necessary to ensure that picked colonies expresses a panel of endogenous pluripotency transcription factors and pluripotency markers, with immunofluorescence staining being particularly informative of homogenous expression across all cells within the entire population.

As a consequence of the reprogramming process, clonal expansion and/or extended culture of cells in vitro, hiPSC clones can also show genetic variations/abnormalities which might confound the phenotypic behaviour of the cells and their differentiated progeny (Yoshihara, Hayashizaki & Murakawa, 2017). For this reason, it is important that the genetic stability/integrity of derived hiPSC clones is characterised and those with confirmed normal karyotypes prioritised for downstream use. Among the most common genetic variants which have been identified in hiPSC lines are aneuploidies including full trisomy of chromosome 8 and full or partial trisomy of chromosome 12 (Taapken et al., 2011). Giemsa-banding (G-banding) is the most common method utilised for assessing
the genomic integrity of hiPSC lines and permits detection of aneuploidy and large-scale chromosomal aberrations including duplications, translocations, inversions and deletions.

Although not considered to be a necessary step when establishing hiPSC models of diseases with nuclear associated Mendelian inheritance, the multicopy and heteroplasmonic nature of the mitochondrial genome necessitates mitochondrial genotyping for all hiPSC clones established. Determining the mutation loads of individual hiPSC clones as soon as possible following stable culture permits the strategic selection of lines most appropriate for disease modelling purposes such that complete characterisation of all established clones is not necessary.

### 3.2 Aims and Hypothesis

In this chapter the overall aim was to establish and characterise successfully reprogrammed hiPSC lines harbouring disease causing mtDNA mutations at suprathreshold heteroplasmy levels appropriate for disease modelling purposes. Particular focus was made towards reprogramming patient fibroblasts harbouring two of the most common mtDNA disease mutations: m.3243A>G \textit{MT-TL1} affecting mt-tRNA\textsubscript{Leu(UUR)} and m.8344A>G \textit{MT-TK} affecting mt-tRNA\textsubscript{Lys}, both of which are commonly associated with myopathic phenotypes. Given the importance of mitochondrial function during the acquisition and maintenance of pluripotency it was hypothesised that the efficiency of successful reprogramming of mitochondrial disease patient fibroblasts harbouring suprathreshold levels of pathogenic variant might show impairments. However, accounting for published literature (see Introduction section 1.4.6 for overview) it was anticipated that hiPSCs with a range of mutation loads ranging from undetectable levels (i.e. completely WT) to higher suprathreshold levels would be obtained from reprogramming of bulk fibroblast populations with heteroplastic mutation loads.

### 3.3 Results

#### 3.3.1 Sendai Virus Based Reprogramming Under Feeder-Dependant Conditions

During a first round of reprogramming, fibroblasts from: one control individual (designated WT), a MELAS patient with the m.3243A>G \textit{MT-TL1} pathogenic variant in mt-tRNA\textsubscript{Leu(UUR)} (designated MELAS1), a MERRF patient with the m.8344A>G \textit{MT-TK}
pathogenic variant in mt-tRNAlys (designated MERRF1), a MELAS patient with the m.13528A>G (p.T398A) and m.13565C>T (p.S410F) MT-ND5 pathogenic variants affecting mt-ND5 (designated MELAS2) and a CMT2 patient with the m.9185T>C (p.Leu220Pro) MT-ATP6 pathogenic variant affecting mt-ATP6 (designated CMT) were reprogrammed under feeder-dependent conditions. Available details on the clinical phenotypes of these patients is presented in Table 2-1 (Methods Section 2.1.1). Heteroplasmy levels of the initial bulk fibroblast populations, as assessed through measurement of dye-terminator sequencing electropherograms, were 35 % m.3243A>G for MELAS1, 55 % m.8344A>G for MERRF1, 100 % homoplasmy m.13528A>G and m.13565C>T for MELAS2, and 100 % homoplasmy m.9185T>C for CMT.

Approximately 3-4 weeks after reprogramming induction, >5 hiPSC colonies with well-defined borders, ~3-5 mm in diameter were identified in the majority of reprogrammed cultures. Cells within these colonies showed typical hiPSC morphology including a high nuclear to cytoplasmic ratio and pronounced nucleoli. In total, 3 colonies were picked from WT reprogramming, 8 colonies from MELAS1, 5 colonies from MELAS2 and 11 colonies from CMT. Although no quantitative measures were made, reprogramming efficiency and the morphology of cells within the reprogrammed cultures were comparable between WT, MELAS1, MELAS2 and CMT. By contrast, reprogramming of the MERRF1 line appeared much more inefficient with only three hiPSC colonies identified, all of which were picked. It was also noted there appeared to be a larger number of cells within the reprogramming MERRF1 culture that had a morphology not typical for hiPSCs, fibroblasts or feeder cells, some of which showed high proliferative activity and potentially resembled partially reprogrammed cells.

Following transfer from feeder-dependent to feeder-independent culture conditions, all picked clonal hiPSC lines maintained typical PSC-like morphology, but regions of spontaneous differentiation were observed which necessitated cleaning prior to passaging through aspiration. After 4-5 passages under feeder-free conditions, all hiPSC clones appeared to stabilise with regions of spontaneous differentiation being observed much less frequently. No obvious detriments in the growth kinetics of any of the established clones were noted.
3.3.1.1 Mitochondrial Genotyping and Mutation load Quantification of Clonal hiPSC Lines

Following stabilisation of the established clonal hiPSC lines under feeder-independent culture conditions, whole DNA (including mtDNA) was extracted and dye-terminator sequencing performed in order to determine the heteroplasmy level of the specific patient derived mtDNA mutations. As expected, hiPSC clones derived from reprogramming of MELAS2 and CMT patient fibroblast populations with homoplasmic levels of the m.13528A>G (p.T398A) and m.13565C>T (p.S410F) mutations in mt-ND5, and
m.9185T>C (p.Leu220Pro) pathogenic variant in mt-ATP6 respectively, also showed homoplasmic levels of the specific mutations (Figure 3-1).

Figure 3-1 Dye-terminator sequencing chromatograms showing the homoplasmic presence of both the m.13528A>G and m.1365C>T pathogenic variants in a representative hiPSC clone derived from MELAS2 fibroblasts, and homoplasmic presence of the m.9185T>C pathogenic variant in a representative hiPSC clone derived from CMT fibroblasts. The WT human mtDNA sequence (rCRS NC_012920) is shown above the respective chromatogram traces.
In contrast, individual hiPSC clones derived from reprogramming of the MELAS1 bulk fibroblast population with heteroplasmic levels of m.3243A>G in mt-tRNA\textsubscript{Leu(UUR)} showed a range of different mutation loads (Figure 3-2). Undetectable levels of m.3243A>G (WT homoplasm) were observed in 1 of the clonal MELAS1 lines, but the remaining 8 lines all showed mutation loads greater than that of the initial bulk fibroblasts, up to levels as high as 89% m.3243A>G.

![Dye-terminator sequencing chromatograms showing a range of different m.3243A>G heteroplasmy levels in hiPSC clones derived from the same heteroplasmic bulk MELAS1 fibroblast population. The WT human mtDNA sequence (rCRS NC_012920) is shown above the chromatogram traces.](image)

**Figure 3-2** Dye-terminator sequencing chromatograms showing a range of different m.3243A>G heteroplasmy levels in hiPSC clones derived from the same heteroplasmic bulk MELAS1 fibroblast population. The WT human mtDNA sequence (rCRS NC_012920) is shown above the chromatogram traces.
Interestingly, of the three hiPSC clones derived from reprogramming of MERRF1 bulk fibroblasts with heteroplasmic levels of m.8344A>G in mt-tRNA<sup>Lys</sup>, 2 showed undetectable levels of m.8344A>G, with the third line showing 52% heteroplasmy, similar to that of the bulk fibroblast population (Figure 3-3). Given the apparent inefficiency of MERRF1 reprogramming and the low heteroplasmy levels of the successfully derived lines when comparing to that of the initial starting fibroblast population, m.8344A>G appears detrimental to the reprogramming process.

![Dye-terminator sequencing chromatograms](image)

Figure 3-3 Dye-terminator sequencing chromatograms showing that 2/3 of the clonal MERRF1 hiPSC lines had undetectable levels of m.8344A>G, with the third line showing heteroplasmy levels comparable to that of the initial bulk fibroblast population. The WT human mtDNA sequence (rCRS NC_012920) is shown above the chromatogram traces.

The mutation loads of the various hiPSC lines picked and expanded from this first reprogramming induction have been summarised in Figure 3-4 below. Although a
directed assessment of the mtDNA mutation loads of the hiPSC clones across time and passage number was not conducted, repeated measurements of the mtDNA mutations randomly throughout general maintenance and prior to downstream experiments revealed no substantial changes in mutation load outside of that associated with the variability of the assessment method utilised.

Figure 3-4 The mutation loads of individual hiPSC clones (crossed markers) successfully picked and expanded during the first reprogramming induction under feeder-dependant conditions shown alongside that of the corresponding initial bulk fibroblast population (dashed line).

Whilst this research project utilised dye-terminator sequencing for the relative ease and availability of equipment for routine mutation load quantification, this method is not particularly accurate for heteroplasmy quantification, and is additionally insensitive to the presence of pathogenic variants at heteroplasmy levels approaching 1% (Yan et al., 2014). For this reason, m.3243A>G heteroplasmy of selected MELAS1 hiPSC lines which were a focus of much of the work presented in this thesis, were also measured using a second more sensitive technique.
NGS of the mitochondrial genome is particularly useful in a clinical diagnostic setting for patients with a mtDNA disease phenotype/history that do not harbour any of the most common mtDNA pathogenic variants, and additionally represents one of the most accurate measures of mtDNA heteroplasmy (Theunissen et al., 2018; Alston et al., 2017). Pyrosequencing permits proportional detection of WT or pathogenic sequence variants through bioluminescence and represents another widely utilised method for measuring m.3243A>G heteroplasmy with a sensitivity permissible for detection of pathogenic variants with less than 1% mutation loads (White et al., 2005; Yan et al., 2014).

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis is routinely employed by the diagnostic mitochondrial disorders service at the UCLH Neurogenetics Laboratory for detection of m.3243A>G and has been shown to be sensitive to mutation loads as low as 1% m.3243A>G. This method is reliant on the introduction of a HaeIII restriction site by the presence of the m.3243A>G variant and thus following PCR of a 199bp region spanning this site with fluorescent conjugated primers, and subsequent HaeIII digest results in differential sized products which can be measured via fluorescent based fragment analysis. Using this strategy 3 independent whole cell DNA preparations were subjected to this analysis for MELAS1 Cl1, MELAS1 Cl2, MELAS1 Cl3, MELAS1 Cl4, MELAS1 Cl5, alongside the WT Cl2 line (Table 3-1). Whilst the relative heteroplasmy differences between MELAS1 hiPSC lines as measured through dye-terminator sequencing were consistent with that observed through PCR-RFLP analysis, MELAS1 Cl1 and MELAS1 Cl2 hiPSC lines showed higher m.3243A>G mutation loads through the latter method, suggesting dye-terminator sequencing might incorrectly underestimate mid-range m.3243A>G heteroplasmy. Of particular note and importance, m.3243A>G was not detected in the MELAS1 Cl5 hiPSC line, thus downstream experiments can confidently rely on this particular line as an appropriate isogenic control.

Table 3-1 PCR-RFLP Analysis of m.3243A>G Heteroplasmy

<table>
<thead>
<tr>
<th>Clonal hiPSC Line</th>
<th>m.3243A&gt;G Heteroplasmy (Dye-terminator)</th>
<th>m.3243A&gt;G Heteroplasmy (PCR-RFLP) Mean ±StDev N=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MELAS1 Cl1</td>
<td>44%</td>
<td>57 ± 9%</td>
</tr>
<tr>
<td>MELAS1 Cl2</td>
<td>53%</td>
<td>74 ± 9%</td>
</tr>
<tr>
<td>MELAS1 Cl3</td>
<td>80%</td>
<td>86 ± 3%</td>
</tr>
<tr>
<td>MELAS1 Cl4</td>
<td>89%</td>
<td>86 ± 2%</td>
</tr>
</tbody>
</table>
3.3.1.2 Characterisation of Selected Subset of hiPSC Clones

Although identification of clones with cell morphologies and growth properties typical for PSCs via light microscopy is alone not sufficient to denote the derived cell line as a true hiPSC, full characterisation of all established lines was not necessary to meet the aims of this study. For this reason, a subset of the established clonal lines which displayed stable growth, and mutation loads most appropriate for downstream disease modelling purposes were selected. Considering the interesting range in mutation loads of MELAS1 hiPSC lines, all clones were subjected to further downstream characterisation. Further characterisation was also performed for all three MERRF1 hiPSC lines established. Given the homoplasmic levels of the respective mutations in all MELAS2 and CMT hiPSCs, only a single stably growing clone from each of these lines were selected for initial characterisation (MELAS2 Cl3 and CMT Cl7).

Clearance of Sendai Viral Transgenes

Although the Sendai virus delivery system utilised by the CytoTune 2.0 reprogramming kit is entirely RNA-based and thus does not integrate into the target cells genome, and additionally lacks all of the components required for replication of further infectious viral particles, long-term presence (>10 passages) of viral transgenes has been described in derived hiPSC lines (Schlaeger et al., 2015; Ye & Wang, 2018). For this reason, it is important to confirm that the viral based transgenes have been cleared from the expanded hiPSC lines prior to further downstream characterisation of pluripotency. RT-PCR was performed on cDNA preparations with primer pairs targeting sequences specific for the hKOS, hc−Myc and hKLF4 Sendai viral transgenes in addition to a primer pair specific for the Sendai virus backbone. No PCR amplicons were detected for any of these transgenes in any of the hiPSC lines assessed at early passages prior to or shortly after (1-2 passages) initial freezing down of cryopreserved stocks (Figure 3-5). All subsequent experiments were performed on hiPSC clones at a later passage than confirmation of this SeV transgene clearance. RT-PCR amplification with primer pairs targeting the housekeeping gene HMBS encoding porphobilinogen deaminase (PBGD) served as a positive control for the RT-PCR reaction and were successful in all hiPSC samples assessed.
Figure 3-5 Example images showing the absence of RT-PCR products targeting Sendai Virus transgenes (hKOS, hc-Myc, hKLF4) and vector backbone (Backbone). RT-PCR amplification of the HMBS housekeeping gene (HMBS+) served as a positive control. HMBS- present on some gels represents RT-PCR for HMBS of RT- treated RNA. 100bp ladder shown on each gel (100bp increment from 100bp). Expected RT-PCR product sizes were: HMBS 73bp, Backbone 181bp, hKOS 528bp, hc-Myc 532bp, hKLF4 410bp.
Positive and Homogenous Expression of Pluripotency Associated Genes

Following confirmation that Sendai virus-based transgenes are no longer enforcing the exogenous expression of pluripotency markers, mRNA expression levels for a panel of pluripotency associated genes were assessed by RT-qPCR. *POUSF1*, *SOX2* and *MYC* which encode OCT4, SOX2 and c-Myc respectively are not only important for driving the reprogramming of cells to pluripotency but also form part of the endogenous transcription factor network underlying maintenance of pluripotency self-renewal (Shi & Jin, 2010; Zhang et al., 2014; Wolff et al., 2018; Adachi et al., 2010; Fagnocchi & Zippo, 2017; Gu et al., 2016). Expression levels of all three of these genes were comparable to those of SHEF ESCs in all hiPSC clones assessed (Figure 3-6), which, given the Sendai virus-based transgenes were cleared at time of assessment, must be due to successful activation of endogenous expression.

OCT4 and SOX2 together with NANOG encoded by *NANOG* constitute the core pluripotency network considered sufficient to drive maintenance of the pluripotent state (Boyer et al., 2005). *NANOG* expression was detected in all hiPSC lines assessed, although variability in the relative expression between lines was observed (Figure 3-6). Of particular note, MERRF1 Cl1 (0% m.8344A>G) and MELAS2 Cl3 (100% m.13528A>G and m.1365C>T) showed ~5-fold higher *NANOG* expression levels relative to the SHEF ESC line, whereas all 8 MELAS1 clones showed comparably ~7-fold lower levels of expression. Nevertheless, positive NANOG expression within a 10-fold range to that of the SHEF ESC line was detected in all hiPSC lines assessed, showing successful activation of the core pluripotency network.

Although NANOG is indispensable for OSKM mediated reprogramming (Schwarz et al., 2014) it can together with Lin-28 Homolog A (LIN28A encoded by *LIN28A*), a pluripotency associated RNA-binding protein, replace KLF4 and c-Myc as part of the reprogramming factor cocktail for successful derivation of hiPSCs (Yu et al., 2007). Expression levels of *REXI* and *DNMT3B*, which are both genes typically expressed at high levels in hPSCs, are also commonly used for assessing hiPSC pluripotency. High levels of *LIN28A*, *REXI*, and *DNMT3B* expression within a 10-fold range to that of the SHEF ESC line were detected in all hiPSC lines assessed (Figure 3-6), further confirming successful activation of the endogenous core pluripotency network.
Figure 3-6 mRNA expression levels of endogenous pluripotency transcription factors (POU5F1, SOX2, NANOG, MYC) and several genes typically expressed at high levels in the pluripotent state (REX1, DNMT3, LIN28A) of selected hiPSC clones. mRNA expression levels are expressed relative to that of the HMBS house-keeping gene and have been shown alongside that of the SHEF human ESC line. Bars show the mean mRNA expression level ± StDev, n=3 technical replicates.

Although RT-qPCR assessment of pluripotency associated gene expression is informative, it only provides a snapshot of the global expression levels within all cells of a given population. For this reason, immunofluorescence assessment of pluripotent markers is often performed in order to obtain an overview of pluripotency markers within the population at a more single cell level. Immunofluorescence analysis also provides insight into the expression of pluripotency markers at the protein level. All of the derived hiPSC lines assessed showed a typical nuclear localised staining pattern for the pluripotency transcription factor NANOG and more diffuse cell surface staining pattern.
for the TRA-1-60 pluripotency associated cell surface antigen (Figure 3-7). Although high levels of cytosolic background were observed through immunofluorescence imaging of the OCT4 pluripotency transcription factor by epifluorescent microscopy, nuclear localisation was evident in all hiPSC lines assessed, and distinguishable from the more diffuse staining pattern of spontaneously differentiating cells within the culture (Figure 3-7). Confocal microscopy imaging of OCT4 immunofluorescence staining showed the expected nuclear localisation within all hiPSCs assessed (Figure 3-8). Importantly, the positive staining of these pluripotency markers was present in the large majority if not all cells within hiPSC colonies, and across all colonies within a given hiPSC line.
Figure 3-7 Representative epifluorescence images showing positive homogenous immunofluorescence staining for the pluripotency markers NANOG, TRA-1-60 and OCT4 across selected WT and MELAS1 hiPSC lines. Fields of view with regions of spontaneous differentiation in the WT hiPSC culture are shown to validate the specificity of the staining pattern for PSCs. Scale bars = 500µm
Figure 3-8 Representative maximum intensity projection confocal microscopy images showing positive homogenous immunofluorescence staining for the pluripotency markers NANOG, TRA-1-60 and OCT4 across selected WT, MERRF1, MELAS2 and CMT hiPSC lines. Staining of the SHEF ESC line is shown as a positive control. A field of view with regions of spontaneous differentiation in the WT hiPSC culture are shown to validate the specificity of the staining pattern for pluripotent stem cells. Scale bars = 100µm.
hiPSCs Show a Normal Cytogenetic Karyotype

Following successful pluripotency assessment, a number of the hiPSC lines were subjected to karyotype assessment: WT Cl2, MELAS1 Cl1 (44% m.3243A>G), MELAS1 Cl2 (53% m.3243A>G), MELAS1 Cl4 (89% m.3243A>G), MELAS1 Cl5 (0% m.3243A>G), MERRF1 Cl2 (0% m.8344A>G), MERRF1 Cl3 (52% m.8344A>G), MELAS2 Cl3 (100% m.13528A>G and m.1365C>T) and CMT Cl7 (100% m.9185T>C).

The lines chosen for karyotype characterisation were rationally based on previous assessments of pluripotency and mutation load outlined above. Cytogenetic karyotype analysis was performed by TDL Genetics (London) with at least 13 metaphase cells assessed for each cell line. All hiPSC lines analysed showed a modal complement of expected chromosomes with normal chromatid lengths and G-banding patterns (Figure 3-9). This instils confidence that any phenotypic observations made between hiPSCs with different mtDNA mutations and/or mutation loads are unlikely to be associated with any large scale nuclear genetic abnormalities.
Figure 3-9 Representative karyograms of hiPSC lines showing 23 pairs of chromosomes with normal large-scale structure and Giemsa banding patterns.

A summary of the 16 hiPSC lines subjected to more in depth characterisations from this first reprogramming experiment is presented in Table 3-2 below.
Table 3-2 A summary of the clonal hiPSC lines subjected to further downstream characterisation. hiPSC clones shown in **bold** were the focus of further downstream experiments due to their complete and positive characterisation.

<table>
<thead>
<tr>
<th>Patient Line</th>
<th>hiPSC Clone</th>
<th>mtDNA Mutation (Mutation Load)</th>
<th>SeV Transgenes</th>
<th>RT-qPCR for Pluripotency mRNA Expression</th>
<th>IF for Pluripotency Marker Expression</th>
<th>Giemsa Band Karyotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>C11</td>
<td>N/A</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td></td>
<td>C12</td>
<td>N/A</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>C13</td>
<td>N/A</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td><strong>MERRF1</strong></td>
<td>C11</td>
<td>m.8344A&gt;G (0%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td></td>
<td>C12</td>
<td>m.8344A&gt;G (0%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>C13</td>
<td>m.8344A&gt;G (52%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
<tr>
<td><strong>MELAS1</strong></td>
<td>C11</td>
<td>m.3243A&gt;G (44%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>C12</td>
<td>m.3243A&gt;G (53%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
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<tr>
<td></td>
<td>C13</td>
<td>m.3243A&gt;G (80%)</td>
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<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td></td>
<td>C14</td>
<td>m.3243A&gt;G (89%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>C15</td>
<td>m.3243A&gt;G (0%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
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<tr>
<td></td>
<td>C16</td>
<td>m.3243A&gt;G (64%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td></td>
<td>C17</td>
<td>m.3243A&gt;G (33%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td></td>
<td>C18</td>
<td>m.3243A&gt;G (50%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Not Performed</td>
</tr>
<tr>
<td><strong>MELAS2</strong></td>
<td>C13</td>
<td>m.13528A&gt;G and m.13565C&gt;T (100%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>C17</td>
<td>m.9185T&gt;C (100%)</td>
<td>Absent</td>
<td>POU5F1, SOX2, NANOG, MYC, REX1, DNMT3B, LIN28A</td>
<td>NANOG, TRA-1-60, OCT4</td>
<td>Normal</td>
</tr>
</tbody>
</table>

O’Callaghan, B.J.
3.3.2 Second Sendai Virus Based Reprogramming Under Feeder-Free Conditions

Following the relatively poor efficiency of reprogramming MERRF1 fibroblasts harbouring m.8344A>G, and unsuccessful derivation of a hiPSC clone harbouring high heteroplasmy levels of m.8344A>G appropriate for disease modelling purposes, a second reprogramming induction was performed under feeder-free conditions. In addition to MERRF1, fibroblasts from two unrelated MERRF patients harbouring m.8344A>G (MERRF2 and MERRF3) were also subjected to reprogramming. Available details on the clinical phenotypes of these patients is presented in Table 2-1 (Methods Section 2.1.1). Including additional MERRF fibroblast lines with the m.8344A>G was decided not only to improve the likelihood of obtaining hiPSC lines with suprathreshold m.8344A>G heteroplasmy, but also to explore whether the detriments observed in the previous reprogramming attempt were specifically associated with MERRF1 fibroblasts or might be more generally associated with m.8344A>G.

Interestingly, m.8344A>G heteroplasmy levels of the bulk fibroblast population expanded from a newly thawed vial of MERRF1 fibroblasts was considerably higher than that which was used in the initial reprogramming attempt (86% vs 55% previously). This highlights the importance of confirming heteroplasmy levels regularly during experimental procedures in order to ensure confidence in any genotype-phenotype correlations observed. m.8344A>G heteroplasmy levels of the bulk MERRF2 and MERRF3 fibroblasts reprogrammed were 68% and 30% respectively.

Approximately 4 weeks after reprogramming induction colonies of cells distinguishable from the remaining fibroblasts were observed in all three of the different MERRF lines, however the morphology of these colonies and the cells within them was atypical. Cells within the observed colonies generally appeared much flatter and less compacted than those typically observed within stable hiPSC cultures. Furthermore, surrounding regions of many of the colonies showed the presence of cells with variable morphologies presumably arising from spontaneous and random differentiation of fully/partially reprogrammed cells. Nevertheless, a large number of colonies were picked from each of the three cell lines 4-6 weeks after reprogramming induction: 25 from MERRF1, 15 from MERRF2 and 12 from MERRF3. The subsequent expansion of the picked hiPSC clones proved extremely difficult, with the majority of lines showing a high propensity for spontaneous differentiation in culture which necessitated prolonged passaging and cleaning through aspiration.
3.3.2.1 Mutation load Quantification of Clonal MERRF hiPSC Lines Suggest Selection Against m.8344A>G

Following approximately 4 weeks in culture after initial picking of colonies, the majority of hiPSC clones appeared to somewhat stabilise and DNA was extracted for mtDNA mutation load measurement. hiPSC clones from all three MERRF fibroblast lines showed a wide range of m.8344A>G heteroplasmy levels: 0-83% for MERRF1, 0-89% for MERRF2 and 0-78% for MERRF3 (Figure 3-10). All MERRF1 hiPSC clones had heteroplasmy levels lower than that of the initial bulk fibroblast population with only 1/25 MERRF1 hiPSC clones showing a mutation load greater than that obtained previously. The remaining 24 hiPSC clones appeared to distribute in two distinct populations, 7/25 clones at undetectable mutant heteroplasmy (0% m.8344A>G) and 17/25 clones at mid-range (34.0 ±8.5%, mean ±StDev m.8344A>G) heteroplasmy (Figure 3-11). In contrast, MERRF2 and MERRF3 hiPSC clones with heteroplasmy levels similar and slightly higher than that of the initial bulk fibroblast population were successfully obtained (Figure 3-10). Similar to MERRF1, a large population of hiPSC clones showed undetectable mutant heteroplasmy: 3/15 MERRF2 and 6/12 MERRF3 hiPSC clones (Figure 3-11). The remaining 12/15 MERRF2 hiPSC clones belonged to a population with mid-range heteroplasmy levels (69.0 ±13.3%, mean ±StDev m.8344A>G). The remaining 6/12 MERRF3 hiPSC clones did not appear to distribute into a specific population which might simply be a consequence of the limited number of hiPSC clones successfully obtained for this patient line, preventing patterned grouping from being observed.
Figure 3-10 The mutation loads of individual hiPSC clones (blue markers) successfully picked and expanded during the second reprogramming induction under feeder-free conditions is shown alongside that of clonal fibroblasts (red markers) expanded from the same bulk population reprogrammed. The mutation load of the bulk fibroblast population is shown as a dashed black line. Statistical differences in heteroplasmia levels between hiPSC clones and the corresponding fibroblast clones assessed through two-tailed Mann-Whitney test. ***p<0.0001, *p<0.01
Figure 3-11 Histograms showing the relative frequency distribution of hiPSC clones and fibroblast clones derived from the three different bulk MERRF fibroblast populations. Bars show the percentage of cell lines with a m.8344A>G mutation load ±5% the centre of the bin (i.e. 10% heteroplasmy bin widths).
3.3.2.2 *Heterogenous m.8344A>G Mutation loads in the Bulk MERRF Fibroblast Populations Underlie Clonal hiPSC Line Range*

Considering previous studies have shown the mutation load range of heteroplasmic hiPSC clones is representative of that present in individual cells of the bulk population reprogrammed (Yokota et al., 2015; Ma et al., 2015a), it is likely that the range of m.8344A>G mutation loads present in the MERRF hiPSC clones obtained here is accounted for by a variable range across the bulk MERRF fibroblast populations. In order to gain insight into the heteroplasmy of individual cells within the bulk MERRF fibroblast populations, MERRF fibroblasts from the same stock of cells reprogrammed were subjected to single cell clonal selection by FACS and cultured to sufficient density for DNA extraction and mtDNA mutation load measurement. A large number of fibroblast clones sufficient for DNA extraction were obtained from each of the three bulk fibroblast populations: 85 from MERRF1, 238 from MERRF2 and 31 from MERRF3. By the time cells had been grown to sufficient density for DNA extraction (near confluent 6-well vessel), the large majority of fibrobast lines showed limited proliferative activity and had presumably entered senescence, preventing further functional characterisations from being conducted.

Clonal fibroblasts from all three bulk MERRF populations showed a maximal range of m.8344A>G heteroplasmy, from undetectable 0% levels (WT homoplasmy) up to 100% (m.8344A>G homoplasmy) (Figure 3-10). Clonal fibroblasts from the bulk MERRF1 population tended towards very high >90% (57/85 MERRF1 fibroblast clones) or very low <10% (10/85 MERRF1 fibroblast clones) m.8344A>G mutation loads with comparably fewer showing heteroplasmy levels between 10% and 90% (18/85 MERRF1 fibroblast clones) (Figure 3-11). Clonal fibroblasts from the bulk MERRF2 population strongly clustered towards very high >90% (126/238 MERRF2 fibroblast clones) m.8344A>G mutation loads, and a relatively high number also showed very low <10% (25/238 MERRF2 fibroblast clones) heteroplasmy, but a much larger proportion of the clones showed heteroplasmy levels between 10% and 90% (87/238 MERRF2 fibroblast clones) than was observed for MERRF1. By comparison, the large majority of clonal fibroblasts from the MERRF3 population strongly clustered towards very low <10% (24/31 MERRF3 fibroblast clones), but there was also an indication for tendency towards very high >90% m.8344A>G mutation loads (4/31 MERRF3 fibroblast clones) with comparably fewer clones showing heteroplasmy levels between 10% and 90% (3/31 MERRF3 fibroblast clones).
M.8344A>G loads of the clonal fibroblast lines does not follow a normal distribution and therefore quoting the mean average heteroplasmy levels of these fibroblast clones is not statistically relevant. However, given the heteroplasmy level of the bulk fibroblast population is representative of the mean average mutation load of the individual cells within that population (assuming mtDNA copy number is comparable between cells), the mean average mutation load of the clonal fibroblasts can serve as a useful measure for comparison. The mean average clonal fibroblast m.8344A>G mutation loads were comparable to that of the bulk fibroblasts for all MERRF lines (Table 3-3) which suggests the clonal fibroblast populations are collectively representative of the bulk population. Further highlighting the apparent tendency for very high m.8344A>G heteroplasmy within individual fibroblast clones from MERRF1 and MERRF2, the median heteroplasmy is greater than the mean heteroplasmy for both of these patient lines. Similarly, the median heteroplasmy of the MERRF3 clonal fibroblast lines is lower than the mean heteroplasmy highlighting the tendency very low mutation loads in MERRF3 fibroblast clones.

**Table 3-3 Mutation loads of Clonal Cell Lines Established from Bulk MERRF Fibroblast Populations**

<table>
<thead>
<tr>
<th>Patient Line</th>
<th>Cell Type</th>
<th>m.8344A&gt;G Mutation load</th>
<th>Number of Clones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>MERRF1</td>
<td>Bulk Fibroblasts</td>
<td>86%</td>
<td>N/A</td>
</tr>
<tr>
<td>(m.8344A&gt;G)</td>
<td>Clonal Fibroblasts</td>
<td>79.7%</td>
<td>97.3%</td>
</tr>
<tr>
<td></td>
<td>Clonal hiPSCs</td>
<td>26.4%</td>
<td>30.0%</td>
</tr>
<tr>
<td>MERRF2</td>
<td>Bulk Fibroblasts</td>
<td>68%</td>
<td>N/A</td>
</tr>
<tr>
<td>(m.8344A&gt;G)</td>
<td>Clonal Fibroblasts</td>
<td>73.2%</td>
<td>91.9%</td>
</tr>
<tr>
<td></td>
<td>Clonal hiPSCs</td>
<td>55.2%</td>
<td>61.5%</td>
</tr>
<tr>
<td>MERRF3</td>
<td>Bulk Fibroblasts</td>
<td>30%</td>
<td>N/A</td>
</tr>
<tr>
<td>(m.8344A&gt;G)</td>
<td>Clonal Fibroblasts</td>
<td>17.9%</td>
<td>1.5%</td>
</tr>
<tr>
<td></td>
<td>Clonal hiPSCs</td>
<td>20.3%</td>
<td>1.9%</td>
</tr>
</tbody>
</table>

A number of interesting observations suggestive of a negative selection pressure against m.8344A>G can be made by comparing the heteroplasmy levels of the hiPSC and fibroblast clones obtained from the same bulk population. Both the mean and median heteroplasmy levels of MERRF1 and MERRF2 hiPSC clones were substantially lower than that of the corresponding clonal fibroblasts (Table 3-3), with this difference in
heteroplasmy distributions being significant when assessed through non-parametric analysis (Figure 3-10). In contrast, the mean and median heteroplasmy levels of MERRF3 hiPSC clones was comparable to that of the corresponding clonal fibroblasts and not significantly different. Interestingly, while a large proportion of fibroblasts within the MERRF1 and MERRF2 populations show very high \( >90\% \) m.8344A\( \rightarrow \)G mutation loads, no hiPSC clones with such high levels of heteroplasmy were obtained (Figure 3-11). While more limited numbers of fibroblast and hiPSC clones were successfully obtained for MERRF3, this also appeared true for this patient line. Considering the relative rarity of fibroblast clones with heteroplasmy levels between 10\% and 90\%, particularly for MERRF1, it is surprising that the majority of hiPSC clones obtained show such mid-range heteroplasmy levels.

### 3.3.2.3 Characterisation of selected MERRF hiPSC Clones

Although prolonged passaging did somewhat stabilise the hiPSC clones derived from the three different MERRF patient fibroblast lines, the large majority proved extremely difficult to maintain in a homogenous population of colonies with typical PSC-like morphology. Spontaneous differentiation of cell clumps seeded from apparently stable looking cultures with homogenous PSC morphology was a common problem which made downstream characterisation difficult. The severity of this spontaneous differentiation phenotype showed no obvious correlation with m.8344A\( \rightarrow \)G mutation load, with hiPSC lines both high and low in m.8344A\( \rightarrow \)G heteroplasmy from all three patients showing this propensity. Out of the three MERRF fibroblast lines reprogrammed, clonal hiPSC lines derived from the MERRF1 patient line (that also used during the first round of reprogramming) appeared most stable and a predominant effort was spent on the characterisation of lines from this patient. MERRF2 hiPSC clones were the most difficult to culture and minimal downstream characterisation was carried out with these lines. Taking into account the much larger number of hiPSC clones established during this second reprogramming induction it was decided that the majority of the established lines would be cryopreserved prior to any further downstream characterisation. Preliminary characterisations for a number of the most stable hiPSC clones with most interesting mutation loads for downstream experimental purposes were however conducted.
Sendai Virus Clearance

As before, determining the clearance of SeV transgenes was among one of the first characterisations performed on selected hiPSC clones. SeV transgene assessment was performed approximately 3-4 weeks after initial picking of colonies, a time point at which clearance would have been expected. 7 of the most stable hiPSC clones derived from MERRF1 reprogramming with a m.8344A>G heteroplasmy level ranging from undetectable to maximal (83%) were tested for SeV transgene clearance through RT-PCR analysis: Cl2 (47% m.8344A>G), Cl3 (26% m.8344A>G), Cl4 (38% m.8344A>G), Cl6 (0% m.8344A>G), Cl10 (0% m.8344A>G), Cl12 (0% m.8344A>G), Cl14 (20% m.8344A>G) and Cl23 (83% m.8344A>G). Two of the most stable hiPSC clones from MERRF3, which fortuitously covered the lowest and highest m.8344A>G mutation loads for this patient line, were also assessed: Cl2 (0% m.8344A>G) and Cl9 (78% m.8344A>G). While MERRF2 hiPSC clones were rarely a homogenous cultures of cells with pluripotent-like morphology, 7 of the most stable lines covering full heteroplasmy range were also assessed: Cl1 (61% m.8344A>G), Cl3 (85% m.8344A>G), Cl4 (50% m.8344A>G), Cl5 (89% m.8344A>G), Cl11 (80% m.8344A>G), Cl12 (0% m.8344A>G) and Cl14 (74% m.8344A>G). The majority of the assessed hiPSC clones showed no evidence for remaining SeV transgenes, however amplification products were observed in some of the lines (Figure 3-12). MERRF1 Cl2, MERRF2 Cl3, MERRF2 Cl4 and MERRF2 Cl11 showed amplification products for the SeV backbone, with MERRF2 Cl3, MERRF2 Cl4 and MERRF2 Cl11 also showing amplification products for the hc-Myc vector. Given alternative hiPSC clones with similar m.8344A>G heteroplasmy were obtained for each of these patient lines, it was decided that these lines would not be carried forward rather than attempts made to establish clearance of the remaining transgenes.
Figure 3-12 Example images showing the presence of RT-PCR products targeting Sendai Virus transgenes hc-Myc and vector backbone (Backbone) in some of the hiPSC clones assessed. RT-PCR amplification of the HMBS housekeeping gene (HMBS+) served as a positive control. HMBS present on some gels represents RT-PCR for HMBS of RT-treated RNA. 100bp ladder shown on each gel (100bp increment from 100bp). Expected RT-PCR product sizes were: HMBS 73bp, Backbone 181bp, hKOS 528bp, hc-Myc 532bp, hKLF4 410bp.
Failure to Initiate Expression of Full Pluripotency Network in hiPSC Clone with High m.8344A>G Mutation load

In view of the difficulties faced during the long-term culture of hiPSC clones derived from MERRF2 and MERRF3, it was difficult to generate a clean and homogenous culture of cells appropriate for RNA extraction and RT-qPCR analysis. For this reason, it was decided that further preliminary RT-qPCR analysis would be restricted to the most stable hiPSC clones derived from MERRF1. Focus was made on the expression levels of REX1 and DNMT3B in particular, as these have been shown to distinguish fully reprogrammed from partially reprogrammed hiPSCs (Chan et al., 2009). RT-qPCR analysis for MYC and DNMT3B pluripotency associated markers revealed comparable expression levels to that of the WT Cl2 hiPSC line previously characterised as part of the first reprogramming induction by all MERRF1 hiPSC clones assessed (Figure 3-13). The large majority of MERRF1 hiPSC clones also showed comparable expression levels for the REX1 gene, however there was one notable exception, with MERRF1 Cl23 harbouring highest 88% m.8344A>G heteroplasmy showing ~150-fold lower levels of REX1 expression (Figure 3-13).

Simultaneous assessment of pluripotency marker protein via immunofluorescence analysis revealed all hiPSC lines assessed including MERRF1 Cl23 showed a typical staining pattern for the pluripotency transcription factor NANOG and pluripotency associated cell surface marker TRA-1-60 across all cells within pluripotent-like colonies throughout the culture dish (Figure 3-14).
Figure 3-13 mRNA expression levels of selected pluripotency markers in a stable subset of MERRF1 hiPSC clones. mRNA expression levels are expressed relative to that of the HMBS house-keeping gene and have been shown alongside that of the WT Cl2 line obtained during the first reprogramming induction. Bars show the mean mRNA expression level ± StDev, n=2 technical replicates.
Figure 3-14 Representative epifluorescence images showing positive homogenous immunofluorescence staining for the pluripotency markers NANOG and TRA-1-60 across selected MERRF1 hiPSC lines. Scale bars = 500µm
MERRF1 Cl14 and MERRF1 Cl23 Show a Normal Cytogenetic Karyotype

Although MERRF1 Cl23 showed detriments in the initiation of the full endogenous pluripotency associated transcriptional network, given its apparent stability in culture compared with the large majority of other hiPSC lines derived during this induction, and very high m.8344A>G heteroplasmy levels likely suitable for disease modelling purposes, it was decided to determine whether this line was karyotypically normal. Another of the most stable lines, MERRF1 Cl14, which was also picked alongside MERRF1 Cl23 (on the same day) and showed lower, likely subthreshold 20% levels of m.8344A>G which might be suitable as an isogenic control was also characterised. As before, cytogenetic karyotype analysis was performed by TDL Genetics (London) with at least 11 metaphase cells assessed for each cell line. All hiPSC lines analysed showed a modal complement of expected 46 chromosomes with normal chromatid lengths and G-banding patterns (Figure 3-15). The apparent pluripotency detriments associated with MERRF1 Cl23 do therefore not appear to be associated with any large-scale genetic aberrations.

m.8344A>G Heteroplasmy Shift in MERRF1 Cl23

Five passages after initial assessment of m.8344A>G load, MERRF1 Cl14 and MERRF1 Cl23 heteroplasmy levels were reassessed. Whereas MERRF1 Cl14 showed similar m.8344A>G heteroplasmy levels after five passages (22% vs 20% originally), m.8344A>G load of MERRF1 Cl23 showed a marked reduction from 83% to 73%. In order to gain insight into any segregation in m.8344A>G heteroplasmy that might account for this shift and additionally determine whether a subset of the bulk MERRF1 Cl23 population might show more complete initiation of the core pluripotency network, MERRF1 Cl23 hiPSCs were subjected to single cell clonal selection by FACS and expanded for further characterisation. A total of 37 stable clones with typical hiPSC morphology were successfully obtained. m.8344A>G mutation load assessment revealed a surprisingly large range in heteroplasmy levels ranging from that of the original MERRF1 Cl23 culture characterised (83% m.8344A>G) to as low as 41% m.8344A>G, which was broadly distributed about the heteroplasmy level of the bulk population (73% m.8344A>G) (Figure 3-16A-B). RT-qPCR analysis of MERRF1 Cl23 clonally selected lines with highest (83% m.8344A>G) and lowest (41% m.8344A>G) mutation loads revealed that similar to the bulk population, MYC and DNMT3B expression levels were comparable to that of the WT Cl2 hiPSC line, however both clones showed substantially
lower levels of $REXI$ expression as previously observed with the bulk MERRF1 Cl23 population (Figure 3-16C).

**Figure 3-15** Karyograms of MERRF1 Cl14 and Cl23 hiPSC lines showing 23 pairs of chromosomes with normal large-scale structure and Giemsa banding patterns
Figure 3-16 Characterisation of clonally selected clones from the bulk MERRF1 CI23 population. (A) M.8344A>G loads of individual hiPSC clones successfully expanded from single cell clonal selection of the MERRF1 CI23 hiPSC line (red crossed markers) is shown alongside that of the bulk population (black dashed line). (B) Histogram showing the relative frequency distribution of hiPSC derived from the bulk MERRF1 CI23 population. Bars show the percentage of cell lines with a m.8344A>G mutation load ±5% the centre of the bin (i.e. 10% heteroplasmy bin widths). (C) mRNA expression levels of selected pluripotency markers in MERRF1 CI23 clones with highest (83% m.8344A>G) and lowest (41% m.8344A>G) mutation loads. mRNA expression levels are expressed relative to that of the HMBS house-keeping gene and have been shown alongside that of the WT CI2 line obtained during the first reprogramming induction. Bars show the mean mRNA expression level ±StDev, n=2 technical replicates.
3.4 Discussion

The main aim of this chapter was to successfully derive hiPSC lines with high, suprathreshold heteroplasmy levels of disease associated mtDNA mutations appropriate for further downstream disease modelling purposes. While hiPSC clones with either homoplasmic or heteroplasmic levels of disease associated mtDNA mutations were successfully obtained from the reprogramming of all mitochondrial disease patient fibroblasts subjected in this study, mtDNA pathogenic variant specific detriments to the reprogramming process and/or maintenance of the pluripotency state were not noted.

3.4.1 M.3243A>G is not Overtly Detrimental to the Reprogramming Process

Similar to previous studies with fibroblast harbouring m.3243A>G (see Table 1-1, Introduction 1.4.6), hiPSC clones obtained from reprogramming of the MELAS1 patient fibroblast population showed a wide range of mutation loads from undetectable, to very high and presumably suprathreshold levels, fortuitously providing isogenic hiPSC clones suitable for both control and disease modelling purposes. While the heterogeneity of m.3243A>G within individual cells of the bulk MELAS1 fibroblast population used in this study is unknown, it is likely that the range of hiPSC m.3243A>G heteroplasmy levels obtained here is accounted for by individual fibroblasts with such underlying heteroplasmy. Indeed, clonal expansion of fibroblast lines from other MELAS patients harbouring m.3243A>G has revealed heterogenous mutation loads across individual fibroblast clones, which are generally representative of associated hiPSC lines derived from them (Yokota et al., 2015; Ma et al., 2015a). In keeping with previous reports of reprogramming MELAS fibroblasts, no obvious detriment in reprogramming efficiency was noted for the MELAS1 patient line used here. As the majority of MELAS1 hiPSC lines obtained showed higher heteroplasmy levels than that of the bulk fibroblast population, the m.3243A>G does not appear to be considerably detrimental to the reprogramming process. There have been a number of reports of successful derivation of hiPSC lines with homoplasmic m.3243A>G mutation loads (Introduction 1.4.6). Although it has been reported that homoplasmic m.3243A>G patient fibroblasts show similar reprogramming efficiencies to control cells (Mizuguchi et al., 2017), clonally expanded fibroblasts with >90% m.3243A>G show very low reprogramming efficiencies compared to corresponding clones with lower m.3243A>G mutation loads (Yokota et al., 2015). In line with this, the highest m.3243A>G level detected in any of the MELAS1 hiPSC clones derived here was 89%. However, this may simply be due to lack of
individual fibroblasts within the population reprogrammed showing >90% m.3243A>G mutation loads. Subjecting the MELAS1 fibroblast population to single cell sorting and clonal expansion in a similar way to that performed for the MERRF fibroblast populations would have been informative for delineating this possibility.

3.4.2 hiPSCs with Homoplasmic Levels of mtDNA Encoded OXPHOS Complex Subunits can be Successfully Derived

Successful derivation of hiPSCs which like the CMT hiPSC lines established here have homoplasmic levels of the m.9185T>C pathogenic variant affecting MT-ATP6 of OXPHOS CV have been previously reported (Lorenz et al., 2017). However, the work presented here shows for the first time the successful derivation of MELAS2 hiPSC lines with homoplasmic levels of the m.13528A>G and m.1365C>T mutations affecting MT-ND5 of OXPHOS CIV. No apparent detriments in either the reprogramming or subsequent characterisation of hiPSCs established from the clonal CMT and MELAS2 lines were noted, thus it would appear that any biochemical deficits associated with these mutations are not limiting for the acquisition and maintenance of pluripotency. It is important to highlight that only one hiPSC clone from each of these patient lines was fully characterised however, thus potentially masking overall detriments.

3.4.3 High m.8344A>G Heteroplasmy is Detrimental to Pluripotency Acquisition and/or Maintenance

By comparison, there was strong evidence for both a selection against m.8344A>G during pluripotency acquisition/maintenance, and a detrimental impact of m.8344A>G on reprogramming efficiency.

3.4.3.1 Reprogramming Inefficiency Under Feeder-Dependant Conditions

Under feeder-dependant conditions, reprogramming of MERRF1 fibroblasts resulted in the generation of only 3 hiPSC clones, 2 of which showed undetectable levels of m.8344A>G. As the reprogramming of the MELAS1, MELAS2, CMT and WT fibroblast lines performed in parallel with the same reprogramming reagents and cell culture media, this would suggest that this relative inefficiency associated with the MERRF1 fibroblasts is caused by some factor intrinsic to the MERRF1 cell line. Reprogramming efficiencies can vary for a wide variety of reasons with donor age, time in culture and associated cellular senescence being well described negative correlates with reprogramming efficiency (Banito et al., 2009; Trokovic et al., 2015). All hiPSC lines reprogrammed here
were subjected to reprogramming at a passage number lower than 10 and did not appear to show any signs of senescence (e.g. flattened morphology or limited proliferative potential). Although the exact age of the MERRF patient donors whose fibroblast lines were used in this study are unknown, I am aware from speaking with clinical colleagues at the MRC Centre for Neuromuscular Diseases that it is unlikely the skin biopsies were taken at greater than 60 years of age for these patients. Interestingly, it has been shown that hiPSCs and fibroblast populations reprogrammed show age-related accumulation of somatic mtDNA mutations (Kang et al., 2016), although a direct link between such mtDNA mutations and reprogramming efficiency has not been investigated in any detail.

3.4.3.2 Improved Reprogramming Efficiency Under Feeder-Free Conditions

Although a second reprogramming induction of the three MERRF fibroblasts lines under feeder-free conditions appeared to be much more efficient, a number of impairments not typically observed were still noted. Among these was the morphological heterogeneity of cells within the reprogramming culture and propensity towards spontaneous differentiation, both of which are observations highly suggestive of incomplete or partial reprogramming.

While most optimal culture conditions were enforced for the reprogramming process it is possible that a bad quality batch of mTeSR1, Cytotune-IPS 2.0 Sendai virus kit or other reagents used might have accounted for these impairments in the reprogramming process. Indeed, collaborators using the same batch of mTeSR1 did note difficulties in the routine culture of their hiPSC lines (J. Meng, personal communications). Due to limitations in the amount of Cytotune-IPS 2.0 Sendai virus kit I had available, it was decided that this second induction would be performed on patient lines only. With hindsight it would have been informative to have reprogrammed a control fibroblast line alongside, such that impairments associated with reagents and/or culture conditions could have been delineated.

Regardless, a number of additional observations indicative of m.8344A>G being detrimental to reprogramming and pluripotency were made during this second feeder-free induction. Among the most significant was the apparent selection against high levels of m.8344A>G in established clones with hiPSC characteristics, when comparing to both the bulk fibroblasts they are reprogrammed from and associated clonally expanded fibroblasts. These data showed that, while there are individual cells within the bulk
fibroblast population that have high m.8344A>G heteroplasmy, they either fail to successfully progress through at least one of the stochastic stages of pluripotency acquisition, are unstable as pluripotent cells or selectively eliminate mutant mitochondrial genomes.

3.4.3.3 Potential Impact of High m.8344A>G on Cell Reprogramming and Pluripotency Maintenance

Given the large number of individual fibroblasts within the bulk MERRF1 and MERRF2 populations that show apparently 100% m.8344A>G mutation load it would appear that at least at homoplasmic levels the pathogenic variant is preventative to reprogramming or endogenous maintenance of pluripotency. While it is possible these cells may have a small number of WT mitochondrial genomes below the detection limit of the measurement method used, it remains unlikely that any of the MERRF hiPSC clones successfully obtained in this study originate from apparently homoplasmic fibroblast cells undergoing such a drastic shift in m.8344A>G mutation load. As discussed in detail throughout sections 1.4.1-1.4.5 of the introduction, it has become increasingly clear that mitochondrial function serves an important role during the reprogramming process and a plethora of important functions in established hiPSCs. The underlying impairments observed may therefore be more directly associated with m.8344A>G and associated mitochondrial impairments.

ROS Signalling During Reprogramming

In relation to the reprogramming process, impairments in mitochondrial function caused by mtDNA mutations would be expected to attenuate the transient but essential burst in oxidative mitochondrial activity occurring shortly after reprogramming induction (Kida et al., 2015) that is responsible for the ROS-dependant activation of NFE2L2 transcriptional activity and subsequent HIF1α stabilization (Hawkins et al., 2016). Interestingly, studies with MEFs from mutator mice which contain multiple mtDNA mutations in fact show increased ROS production caused by dysfunctional mitochondria is detrimental to reprogramming (Hämäläinen et al., 2015). Application of the antioxidants N-acetyl-L-cysteine (NAC) and mitochondria-targeted ubiquinone (MitoQ) both increased the reprogramming efficiency of mutator MEFs. A very high dose of MitoQ did however, attenuate reprogramming efficiency. It would therefore appear that a fine “Goldilocks” balance in mitochondrial OXPHOS and ROS production is necessary during the reprogramming process. It is plausible that OXPHOS impairments associated
with high m.8344A>G heteroplasmy levels may therefore impair this fine balance in the cellular redox state, in addition to attenuating the larger transient increase which occurs. In line with this, elevations in ROS have been described in hiPSCs harbouring >50% m.8344A>G heteroplasmy levels (Chou et al., 2016).

**Metabolically Sensitive Epigenetic Modifications**

Another possible explanation for the impairments observed in MERRF fibroblast reprogramming and/or pluripotency maintenance could be related to alterations of metabolically sensitive chromatin modifications. As described in section 1.4.5.2 of the introduction, reprogramming necessitates a wide-spread alteration to the epigenetic landscape with a large number of the enzymes responsible for the deposition and removal of these chromatin modifications being dependant on intermediate metabolites as co-substrates. Considering mitochondria are important primary sources of αKG for histone and DNA demethylation (TeSlaa et al., 2016), citrate for acetyl-coA dependant histone acetylation (Moussaieff et al., 2015) and potentially NAD⁺ for histone deacetylation, it can be speculated that mitochondrial dysfunction might impair the efficiency and completeness of the epigenetic rewiring underlying pluripotency acquisition. The validity of this theory is further supported by the relatively high frequency of what were presumably partially reprogrammed cells within each of the reprogramed MERRF cultures. In addition to pluripotency acquisition, similar detriments in the maintenance of epigenetic marks necessary for pluripotency might also account for the observed impairments. Indeed, even following the selection of colonies with typical hiPSC morphology, the subsequent maintenance of the large majority of MERRF hiPSCs as a pure and homogenous culture proved extremely difficult, with rates of spontaneous differentiation being considerably higher than that typically observed. Of relevance, attenuating mitochondrial production of citrate has been shown to reduce histone acetylation modifications and increase the propensity of hESC spontaneous differentiation (Moussaieff et al., 2015).

**Association of REX1 with Partial Reprogramming and Mitochondrial Function**

Although this may just be a serendipitous observation, it was interesting to note that the MERRF1 Cl23 hiPSC line with highest and presumably suprathreshold m.8344A>G levels obtained from the second reprogramming failed to instigate expression of the *REX1* gene. While this is highly suggestive of this line being partially reprogrammed (Chan et al., 2009) it is worth noting that the functional importance of REX1 in hPSCs is strongly
associated with mitochondrial function and cell metabolism. In an interesting study by Son et al., it was shown that REX1 was crucial to maintenance of the pluripotent state, with REX1 knockdown substantially reducing the expression of pluripotency markers in hESCs, while simultaneously increasing differentiation markers and spontaneous differentiation propensity (Son et al., 2013). While the propensity for the MERRRF1 Cl23 hiPSC line to spontaneously differentiate in culture was high, it was no more than that of other hiPSC lines established from this reprogramming induction which did express normal levels of REX1 (e.g. MERRF1 Cl14). Son et al., linked the importance of REX1 to its function as a transcriptional regulator of cyclin B1 and B2 and further downstream of this, the requirement of cyclin B mediated enhancement of DRP1-dependant mitochondrial fission. This mitochondrial fission was shown to be important for maintaining the typical glycolysis favouring and low mitochondrial OXPHOS metabolism of hPSCs. It can be speculated that this REX1-dependant attenuation of mitochondrial function for pluripotency maintenance is not necessary in the context of hiPSCs with high m.8344A>G heteroplasmy and might in fact be detrimental to pluripotency maintenance and hiPSC survival. It is therefore possible that this chance observation highlights REX1 as a factor that typically limits successful maintenance of pluripotent cells with severe mitochondrial detriments, at least those associated with high levels of m.8344A>G.

3.4.3.4 Selection against m.8344A>G During Reprogramming and hiPSC Culture

A number of observations, particularly with MERRF1 hiPSC clones established in the second reprogramming induction, do suggest that, when at high but sub-homoplasmic levels some form of active selection against m.8344A>G might be occurring at a cellular or population level, during and/or shortly after pluripotency acquisition. Of note was the differential distribution of heteroplasmy levels between clonal fibroblast and hiPSC lines. While very few MERRF1 fibroblast clones showed mid-range m.8344A>G heteroplasmy levels, the large majority of MERRF1 hiPSCs reprogrammed from them had mutation loads of 20-50%. This could be caused by the random segregation of the m.8344A>G from the bimodally distributed fibroblast populations with low and high mutation loads. If m.8344A>G provides a proliferative advantage of some sort when present at mid-range heteroplasmy levels, increases in mutation load from lower levels might be occurring at a population level. Similarly, if m.8344A>G is detrimental to cell proliferation and/or survival, a shift from higher to lower mutation loads could also account for the observed distribution in hiPSC m.8344A>G heteroplasmy. A related possibility is that
mitochondria with greater proportions of the m.8344A>G mitochondrial genome might be actively selected against at the cellular level. Given the observation of the MERRF1 hiPSC clone with highest heteroplasmy (MERRF1 Cl23) showing a decline in m.8344A>G heteroplasmy over 5 passages in culture it seems more likely that decreases rather than increases in m.8344A>G heteroplasmy account for the observed hiPSC mutation load distributions. In line with this, decreases in mtDNA mutation loads have been described in the literature following extended culture of mitochondrial disease hiPSCs (see Table 1-1 in section 1.4.6.2 of the introduction), including a 70% to 50% reduction in m.8344A>G containing iPSCs over 20 passages (Chou et al., 2016). Clonal selection of the bulk MERRF1 Cl23 hiPSC population revealed that even after a relatively short duration in culture (13 passages since initial picking), m.8344A>G within individual cells had already started to become lower, more broadly distributed and heterogenous.

**Selection at a Population Level**

Selection at a population level through negative proliferation or survival pressure in cells with high heteroplasmy levels could account for the heteroplasmy shift observed in MERRF1 Cl23 during culture, and potentially the broad distribution of m.8344A>G heteroplasmy levels of the originally picked MERRF1 hiPSC clones. In line with this, oxidative mitochondrial glutaminolysis pathways are crucial to the survival and proliferation of hPSCs (Zhang et al., 2016; Tohyama et al., 2016). Similar negative selection pressures on the proliferation/survival of hematopoietic stem cells is thought to account for the decline in m.3243A>G heteroplasmy observed in patient blood over time (Rajasimha, Chinnery & Samuels, 2008).

**Selection at a Cellular Level**

Selection against the m8344A>G through active targeting of mutant mitochondria for degradation by mitophagy and/or preferential replication of WT over mutant mitochondrial genomes are however, also possible explanations. In line with this, fibroblasts harbouring m.8344A>G show increased mitophagic flux (De la Mata et al., 2012) and evidence for an increase in mitophagy has also been described in hiPSCs harbouring high levels of m.3243A>G (Lin et al., 2019a). Interestingly a necessary increase in mitophagic flux occurs shortly after reprogramming induction (Ma et al., 2015b). It could therefore be speculated that a particularly strong selection against mitochondrial organelles with more depolarised $\psi_m$ as a consequence of high m.8344A>G mutation load might occur early during the reprogramming process and account for the
lower mutation loads in derived hiPSC clones. It is important to note however that more recent evidence suggests this transient increase in mitophagic flux during reprogramming shows no selectivity towards mitochondria with more depolarised $\psi_m$ (Xiang et al., 2017).

### 3.4.4 Summary

In conclusion, hiPSCs harbouring high and presumably suprathreshold levels of m.3243A>G affecting mt-tRNA$^{\text{Leu(UUR)}}$, m.13528A>G (p.T398A) and m.13565C>T (p.S410F) mutations affecting mt-ND5, and m.9185T>C (p.Leu220Pro) pathogenic variant affecting mt-ATP6 were successfully established. M.8344A>G affecting mt-tRNA$^{\text{Lys}}$ appears particularly detrimental to pluripotency at high heteroplasmy levels which could potentially limit the use of hiPSC lines with suprathreshold levels of this pathogenic variant for downstream disease modelling purposes.
Chapter 4  Assessment of Mitochondrial Function and Cell Metabolism in hiPSCs with mtDNA Disease Mutations

4.1  Overview

Whilst guided differentiation of hiPSCs into disease relevant cell types serves as an excellent avenue for modelling mitochondrial disease in vitro, exploring mitochondrial dysfunction and associated metabolic impairments in hiPSCs may at first appear uninformative, given hiPSCs favour glycolytic over mitochondrially driven oxidative metabolic flux pathways to meet their energetic demands. However, the significance of mitochondrial function beyond ATP synthesis is well appreciated, with recent evidence highlighting crucial roles of mitochondrial function in hPSCs, among which includes the generation of metabolic intermediates required for lipid, nucleotide and protein biosynthetic pathways, and as co-substrates for enzymatic protein modifications such as those underlying epigenetics. In addition to providing insight into the biochemical and cellular deficits associated with these mtDNA mutations, assessment of mitochondrial function and associated cellular metabolic fluxes in hiPSCs can guide the selection of the most appropriate cell lines to carry forwards, for downstream differentiation into skeletal myogenic cells and disease relevant in vitro modelling purposes. Mutation loads which give rise to biochemical deficits in hiPSCs are likely to persist or worsen upon differentiation towards more oxidatively demanding disease relevant cell types.

4.1.1  OXPHOS Dysfunction Caused by mtDNA Mutations

The primary consequence of disease causing mtDNA mutations is associated with impairments in the function and/or expression of the OXPHOS complex subunit encoded by the gene affected, or in the case of mutations in mt-tRNA genes potentially all mtDNA encoded OXPHOS complex subunits. As mtDNA encodes subunits which contribute to all of the OXPHOS complexes except OXPHOS CII, mt-tRNA mutations have the potential to more widely impact ETC function and OXPHOS.
Previous assessments on the impact of the m.13528A>G (p.T398A) and m.13565C>T (p.S410F) MT-ND5 mutations affecting the MT-ND5 subunit of OXPHOS CI have been performed with the very same patient fibroblasts reprogrammed here, alongside cybrid cells harbouring homoplasmic mutant mitochondrial genomes derived from them (McKenzie et al., 2007; Kovac et al., 2019). In line with a specific impairment of OXPHOS CI, mutant cybrids with these mutations showed lower levels of CI-linked respiration (rotenone-sensitive) whereas CII-linked respiration (succinate-dependant) was largely unaffected (McKenzie et al., 2007). Specific impairments in CI-linked but not CII-linked respiration have also been described in an additional study using fibroblasts and cybrids derived from an unrelated patient harbouring homoplasmic levels of the m.13528A>G and m.13565C>T mutations (Petruzzella et al., 2012). Patient fibroblasts harbouring these ND5 mutations also showed a more depolarised $\psi_m$ (as measured through TMRM fluorescence) (McKenzie et al., 2007) and a smaller contribution of CI to maintenance of the $\psi_m$ (rotenone-sensitive) (Kovac et al., 2019), further evidence for a selective impairment of OXPHOS CI.

A number of previous assessments of the m.9185T>C (p.Leu220Pro) MT-ATP6 pathogenic variant affecting the MT-ATP6 subunit of OXPHOS CV have confirmed this variant has a detrimental impact on mitochondrial ATP synthase function. While ATP synthesis rates in isolated lymphoblast mitochondria from a patient with very high levels of m.9185T>C (>91%) were largely unaffected, CV ATPase activity was modestly reduced (Castagna et al., 2007). In line with this, blue-native PAGE analysis of OXPHOS CV in muscle tissues taken from patients with homoplasmic levels of m.9185T>C revealed impairments in both OXPHOS CV assembly and ATPase activity (Pitceathly et al., 2012). In fibroblasts with homoplasmic levels of m.9185T>C and homoplasmic cybrids derived from them, reductions in ATP synthesis rates have been shown alongside a knock-on reduction in overall cellular respiration rates (both CI and CII-dependant) (Auré et al., 2013). More recently, similar impairments in ATP synthesis rates have also been shown in hiPSC derived neural progenitor cells with homoplasmic levels of m.9185T>C (Lorenz et al., 2017). In addition, neural progenitor cells and neurons harbouring m.9185T>C also showed more hyperpolarised $\psi_m$ which is presumably caused by impairments in CV function and attenuation of the utilisation and associated dissipation of the mitochondrial H$^+$ electrochemical gradient for ATP synthesis.
As already alluded to, mutations in mt-tRNA genes can potentially impact the translation and consequentially the assembly and function of all OXPHOS complexes except the entirely nuclear encoded OXPHOS CII. However, significant impairments in the protein expression and function of all assembled OXPHOS complexes is not observed in patient derived cells or tissues, likely because such detriments preclude survival. In relation to the m.3243A>G MT-TL1 and m.8344A>G MT-TK mutations affecting mt-tRNA<sub>Leu(UUR)</sub> and mt-tRNA<sub>Lys</sub><sup>139</sup> respectively, detriments in OXPHOS CI expression levels and function are commonly reported, with OXPHOS CIV detriments caused by m.8344A>G also commonly observed. For example, CI activity was shown to be reduced in mitochondrial extracts from heteroplasmic fibroblasts with the m.3243A>G or m.8344A>G mutation, with CIV activity also being reduced in m8344A>G fibroblasts (James et al., 1996). Although steady-state levels of certain OXPHOS complexes might be maintained, assessment of mitochondrial protein synthesis rates in near homoplasmic m.3243A>G cybrids through radio-label tracing experiments do reveal more global detriments in mitochondrial translation products (Chomyn et al., 1992). Similar experiments on clonal myoblasts with high m.8344A>G heteroplasmy levels also reveal similar global detriments in mitochondrial translation (Hanna et al., 1995). It is interesting to note however, that particular mitochondrial encoded proteins are more vulnerable to specific mt-tRNA mutations with the studies discussed here suggesting CIV subunits (MT-COXI and MT-COXII/III) are severely affected by m.8344A>G (Hanna et al., 1995), whereas m.3243A>G severely affected CI subunits (particularly MT-ND2) in addition to impacting CIV (Chomyn et al., 1992). Although it remains unclear exactly why certain OXPHOS complexes are differentially vulnerable to specific mt-tRNA mutations, modest correlations have been made with the number of amino acids within a specific mitochondrial protein that are encoded by the mutated mt-tRNA (Antonio Enriquez et al., 1995; Chomyn et al., 1992).

4.1.2 Downstream Metabolic Impacts of OXPHOS Dysfunction

Due to the intrinsic link of mitochondrial function with key catabolic and anabolic biochemical pathways, OXPHOS dysfunction has the potential to impact a wide range of downstream cellular processes. Among these, the mitochondrial-localised TCA cycle is tightly co-ordinated with OXPHOS function through the reliance of several TCA cycle enzymes on oxidised NAD<sup>+</sup> and FAD co-enzymes, generated by OXPHOS Complexes I and II as part of a functional ETC. As discussed thoroughly in the context of hiPSCs (see Introduction 1.4.5 for in-depth overview), the TCA cycle serves as an important source
of numerous metabolic intermediates necessary for downstream biosynthetic and cell signalling pathways. Of particular significance, citrate derived from glucose flux through the TCA-cycle has been shown to be important as both a substrate for lipid biosynthetic pathways necessary for hPSC growth and proliferation (Zhang et al., 2016; Tohyama et al., 2016), and as a source of nucleocytoplasmic acetyl-CoA necessary for HAT-dependant histone acetylation modifications contributing to pluripotency (Moussaieff et al., 2015). Whilst it is possible chronic mitochondrial dysfunction might lead to detriments in TCA-cycle and other important mitochondrial metabolism derived products such as citrate, cells maintain effective mechanisms for maintaining such fluxes and/or alternative pathways for generation of these products. In relation to mitochondrial dysfunction, one of the most commonly described compensatory mechanisms is a shift towards aerobic glycolysis where increased conversion of pyruvate to lactate by lactate dehydrogenase (LDH) can serve to replenish NAD+ from NADH (Kami et al., 2012; Mullen et al., 2012). It is through this mechanism that pyruvate supplementation is supportive to the survival of cells with mitochondrial dysfunction, by maintaining NAD+-dependant enzymatic reactions such as those involved in aspartate biosynthesis and ATP generation through glycolysis (Wilkins, Carl & Swerdlow, 2014; Sullivan et al., 2015; Birsoy et al., 2015).

In addition to the regeneration of oxidised NAD+ coenzyme, cells also have effective compensatory flux pathways for regeneration of TCA cycle intermediates (process known as anaplerosis). In addition to the predominant source of TCA-cycle influx through acetyl-CoA reaction with oxaloacetate catalysed by citrate synthase, two of the other most important mechanisms are the conversion of pyruvate into oxaloacetate by pyruvate decarboxylase, and glutaminolysis conversion of glutamine into glutamate by glutaminase and subsequently α-KG by glutamate dehydrogenase. Indeed, glutaminolysis serves as the predominant source of late stage TCA cycle metabolites in hiPSCs and this flux is among the main reasons hiPSCs necessitate a functional mitochondrial ETC (Zhang et al., 2016; Tohyama et al., 2016). Interestingly, cells with both chronic and acute mitochondrial dysfunction initiate a metabolic switch towards glutamine-dependant reductive carboxylation in order to compensate for detriments in the mitochondrial generation of citrate from glucose (Mullen et al., 2012). In this study by Mullen et al., cybrid cells harbouring a homoplasmic m.14787_14790delTTAA MT-CYTB frameshift variant associated with severe CIII dysfunction displayed increased glucose utilisation that was concordant with a substantial increase in lactate production. A decreased flux of
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glucose derived carbons towards TCA-cycle derived citrate was observed however, suggesting the elevated glucose uptake was primarily used for anaerobic glycolysis purposes. Instead mitochondrial derived citrate was maintained through a compensatory flux of glutamine derived α-KG towards citrate via NADP+/NADPH dependant isocitrate dehydrogenase enzymes. Even though CII dependant oxidation of FADH$_2$ to FAD is very likely impacted by CIII dysfunction as well, an increase in the oxidative metabolism of glutamine derived α-KG to succinate (by succinyl-CoA synthetase) and subsequently malate (by succinate dehydrogenase, i.e. OXPHOS CII) was also observed. This is most likely accounted for by the majority of mitochondrial α-KG being glutamine derived rather than glucose-derived in these cells with impaired OXPHOS function. Indeed, cybrid cells with homoplasmic levels of the m.8993T>G (p.Leu156Arg) MT-ATP6 pathogenic variant or heteroplasmic (50%) levels of the m.6930G>A (p.G343*) MT-CO1 pathogenic variant that display more modest impairments in ETC function, show considerable increases in oxidative glutaminolysis pathways but comparably smaller increases in glutamine-dependant reductive carboxylation fluxes (Chen et al., 2018a). This more recent study did replicate the substantial increases in glutamine-dependant reductive carboxylation fluxes in cybrids with more severe ETF function however, including homoplasmic cybrids with the same frameshift MT-CYTB pathogenic variant used previously, in addition to cybrids homoplasmic for the m.6930G>A (p.G343*) MT-CO1 mutation.

4.1.3 Methods to Assess Biochemical and Metabolic Impact of mtDNA Mutations in hiPSCs

To study the impact of mtDNA mutations on the function of hiPSC mitochondria a number of different techniques can be employed. At the primary level, denaturing PAGE and western blot analysis can be used to delineate whether the mtDNA pathogenic variant at the specific heteroplasmy levels in question has any impact on the functional assembly and/or stability of specific OXPHOS complexes. In order to gain insight into any impact the mtDNA mutations have on ETC function, measurements of $\psi_m$ can be made through live-cell imaging of fluorescent dyes that accumulate in mitochondria in a $\psi_m$-dependant manner such as TMRM. Finally, to gain insight into any impact of the pathogenic variant on downstream metabolic pathways, metabolomic flux analysis of heavy-isotope labelled metabolic substrates can be used.
4.2 Aims and Hypothesis

In this chapter, the overall aim was to characterise the mitochondrial and cellular phenotypes of stable hiPSC lines harbouring mtDNA mutations described in the previous chapter. Particular focus was made on MELAS1 hiPSC lines due to the interesting range of m.3243A>G loads that were successfully obtained, including isogenic controls, and the stability of these cells during hiPSC culture. Functional characterisations of the most stable MERRF hiPSC line harbouring m.8344A>G at mid-range heteroplasmy levels derived during the first reprogramming induction, alongside an associated isogenic control were also made due to the apparent severity of this pathogenic variant on reprogramming and pluripotency maintenance. Characterisations of hiPSC lines with homoplasmic levels of the m.13528A>G and m.13565C>T (MELAS2) and m.9185T>C (CMT) mtDNA mutations specifically affecting individual subunits of particular OXPHOS complexes were also performed. It was predicted that the MELAS1 and MERRF1 hiPSC lines with high levels of m.3243A>G affecting mt-tRNA\textsuperscript{Leu(UUR)} and m.8344A>G affecting mt-tRNA\textsuperscript{Lys}, would show widespread impairments in mitochondrial OXPHOS. By comparison, specific impairments associated with OXPHOS CI were predicted for the MELAS2 hiPSCs with the m.13528A>G and m.13565C>T mutations affecting the ND5 subunit of OXPHOS CI. Similarly, specific impairments associated with OXPHOS CV were expected in CMT hiPSCs with m.9185T>C affecting the ATP6 subunit of OXPHOS CV. It was hypothesised that hiPSCs with high mtDNA mutation loads would show deficits in the expression of specific assembled OXPHOS complexes, and that this would be associated with downstream impairments in maintenance of the ψ\textsubscript{m} and cellular metabolic fluxes.
4.3 Results

4.3.1 Western Blot Assessment of Assembled OXPHOS Complexes

In order to gain insight into the primary consequence of the various mtDNA mutations within the specific mitochondrial disease hiPSC lines, western blot assessments were performed for levels of OXPHOS complex subunits which are among the last of the necessary subunits assembled as part of the five different functional OXPHOS complexes, thus reasonably good measures of overall steady-state levels of the 5 different OXPHOS complexes (Figure 4-1). Detriments in the transcription, translation and/or assembly of specific mitochondrial encoded proteins caused by the various mitochondrial mutations of interest would therefore be expected to result in reductions in the cellular levels of these associated protein subunits. As a downstream consequence of mitochondrial dysfunction, increases or decreases in mitochondrial mass might be expected due to enhanced mitophagic flux clearance of damaged mitochondria or through a compensatory increase in mitochondrial biogenesis respectively. Measurements of TOM20 protein which is a component of the translocase of the outer mitochondrial membrane complex were also performed as an indirect measure of mitochondrial mass (Figure 4-1).
Figure 4-1 Representative immunoblot showing the protein levels of OXPHOS complex protein subunits which are representative of overall steady-state levels of the five different OXPHOS complexes. Immunoblotting for TOM20 was used as an indirect measurement of mitochondrial mass, and β-actin was used as a protein loading control. The percentage in brackets indicated the mutation load of the respective mitochondrial disease hiPSC line (see text for details on the specific mtDNA mutations).
A high degree of variability in the levels of steady-state OXPHOS complex subunits were observed across blots (Figure 4-2), which is likely associated with both biological fluctuations in the levels of these proteins and/or associated assembled complexes, and technical variability of the immunoblotting detection method used. No consistent differences in the levels of steady-state protein subunits contributing to OXPHOS CII (SDHB), CIII (UQRC2), CIV (MT-CO1) or CV (ATP5A) were observed between any of the mitochondrial disease hiPSC lines assessed. A modest but consistent reduction in the level of the NDUFB8 subunit contributing to OXPHOS CI was observed in the MELAS1 Cl4 hiPSC line with highest heteroplasmic level (89%) of m.3243A>G affecting mt-tRNA<sub>Leu(URR)</sub> and MELAS2 Cl3 hiPSC line with homoplasmic levels of the m.13528A>G and m.13565C>T mutations affecting the MT-ND5 subunit of OXPHOS CI. No differences in the levels of assembled OXPHOS complexes or mitochondrial mass (TOM20) survived statistical significance following correction for multiple comparisons between lines (two-way ANOVA with Tukey post-hoc correction). However, rational comparison of control hiPSC lines (WT Cl1, MERRF1 Cl2, MELAS1 Cl5) with MELAS1 Cl4 (89% m.3243A>G) and MELAS2 Cl3 (100% m.13528A>G and m.1365C>T) showed significantly lower levels of assembled OXPHOS CI when multiple comparisons were not accounted for (p<0.05, two-tailed unpaired t-test).
Figure 4-2 Steady-state levels of OXPHOS complex subunits contributing to OXPHOS CI (NDUFB8), CII (SDHB), CIII (UQCRC2), CIV (MT-CO1) and CV (ATP5A), and levels of TOM20 in hiPSC lines. Bars represent the mean integrated immunoblot band intensity, normalised to that of β-actin, and expressed relative to that of the WT CI2 hiPSC line which was present on all blots quantified. Error bars represent the StDev, and numbers in the bars represent the number of independent protein lysates assessed.
4.3.2 MtDNA Copy Number is Unaltered in MELAS1 hiPSCs

In addition to assessment of mitochondrial mass via measurements of cellular levels of mitochondrial proteins such as TOM20, quantifications of mitochondrial genome copy number can also serve as an informative measure of mitochondrial content. An initial assessment of mtDNA copy number through RT-qPCR measurements of relative mtDNA vs nDNA copy number using primer pairs targeting the MT-CYB gene and APP gene respectively showed a trend towards a modest reduction in mtDNA copy number in hiPSCs with high m.3243A>G heteroplasmy level (MELAS1 Cl3 80% m.3243A>G, MELAS1 Cl4 89% m.3243A>G) (Figure 4-3A). While the rational comparison of the MELAS1 Cl4 hiPSC line with highest heteroplasmy (89% m.3243A>G) and corresponding isogenic control MELAS1 Cl5 hiPSC line (0% m.3243A>G) revealed this reduction in mtDNA copy number was statistically significant (p<0.05, two-tailed unpaired t-test), this significance did not survive statistical testing with correction for multiple comparisons (one-way ANOVA with Tukey post-hoc correction).

In order to provide greater sensitivity and exclude any effect of unaccounted SNP variations within the sequences targeted by the primers, a subsequent assessment of mtDNA copy number between MELAS1 hiPSC lines with varying m.3243A>G heteroplasmy was performed using primer pairs targeting three different regions of the mitochondrial genome (MT-CYB, MT-ND1, MT-ND4) and normalisation performed against two nuclear genomic regions (APP and B2M) (Figure 4-3B). Unlike the initial experiment no consistent reductions in mtDNA copy number were observed in the MELAS1 Cl4 hiPSC line with highest 89% m.3243A>G heteroplasmy using this more sensitive method (note this experiment was also performed using different DNA extractions from that of the initial experiment).
Figure 4-3 RT-qPCR assessment of mtDNA copy number in MELAS1 hiPSCs harbouring m.3243A>G. (A) mtDNA content quantified through measurement of the levels of MT-CYB mtDNA genomic region relative to the APP nDNA genomic region. Bars represent the mean mtDNA copy number relative to that of WT CI2 with error bars ±StDev (n=3 independent DNA extractions). (B) mtDNA content quantified through measurement of the levels of MT-CYB, MT-ND1 and MT-ND4 mtDNA genomic regions relative to the geometric mean of the APP and B2M nDNA genomic regions. Bars represent the mean mtDNA copy number relative to that of MELAS1 CI5 with error bars ±StDev (n=3 independent DNA extractions, different from that used in the initial experiment described for A).
4.3.3 Basal $\psi_m$ and Relative Contribution of OXPHOS Complexes to $\psi_m$ Maintenance in hiPSCs is Unaffected by mtDNA Mutations

In order to gain insight into any downstream consequences the mtDNA mutations might have on the biochemical function of hiPSC mitochondria, measurements of $\psi_m$ were performed through live-cell imaging of TMRM.

4.3.3.1 Assessments of Basal $\psi_m$ and Mitochondrial Morphology

hiPSC mitochondria showed a globular and punctate TMRM staining pattern which was densely compacted within the small cytoplasmic areas surrounding the nuclear compartment (Figure 4-4A). While the compact nature of hiPSCs makes it difficult make conclusive observations of mitochondrial morphology, no obvious differences were observed, indicating the mtDNA mutations assessed have no substantial impact on mitochondrial network dynamics.

TMRM staining intensity appeared most pronounced at the edges of hiPSC colonies which potentially indicates cells at the periphery of colonies have a more polarised $\psi_m$. One potential explanation of this might be associated with better nutrient availability to cells at the periphery, with the cells more compacted towards the centre of the hiPSC colonies having reduced surface area for nutrient uptake. This observation might also be associated more technically with loading of he cells with TMRM. Just as the greater compactness might restrict nutrient uptake, accumulation of the TMRM dye might also be negatively impacted. Whilst comparable colony sizes and seeding densities were achieved between lines compared within an individual experiment, and no notable differences in proliferation of the various cell lines used noted, $\psi_m$ measurements made from these cells here may be confounded by any slight differences unaccounted for. Future experiments might benefit from seeding hiPSCs supplemented with ROCKi for imaging the next day, in order to achieve more comparable cell seeding distributions limiting the potential of such confounding factors.

Measurements of mean mitochondrial basal TMRM fluorescence intensity from thresholded maximum intensity z-projections of confocal microscopy images revealed no obvious differences between hiPSC lines (Figure 4-4B) suggesting the mtDNA mutations assessed also do not appear to impact hiPSC basal $\psi_m$. 
Figure 4-4 Assessment of $\psi_m$ through live-cell imaging of hiPSCs stained with 25nM TMRM. (A) Representative maximum intensity projections of hiPSCs stained with 25nM TMRM. Images shown were all taken during one experimental imaging session and the same manipulations to brightness and contrast made to each image using ImageJ FIJI software. Scale bars = 100µm. (B) Basal mitochondrial TMRM intensities quantified from thresholded maximum intensity projections as a measure of basal $\psi_m$. Bars display the mean mitochondrial TMRM fluorescence intensity normalised to that of the WT C12 hiPSC line imaged during the same experimental imaging session with error bars ±StDev. Numbers in the bars display the number of replicate images used for analysis, which were taken across at least two independent imaging sessions for each hiPSC line.
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To provide greater sensitivity for detection of any subtle differences in basal $\psi_m$ and better account for the variation in mitochondrial TMRM staining intensities observed between individual microscopy fields of view, further experiments taking advantage of the high-throughput Opera Phenix spinning-disc confocal imaging platform were performed. This permitted large numbers of individual z-stack confocal images to be taken from different hiPSC lines within a short duration of time, limiting any variations in TMRM measurements which might be associated with slight differences in TMRM dye loading durations and/or microscopy imaging conditions. During a first experiment aiming to distinguish any potential differences between hiPSCs lines representing a range of different mtDNA mutations, >12 independent 323x323 µm fields of view were sequentially imaged for each hiPSC line (Figure 4-5). This experiment was replicated across 2-3 wells for each hiPSC line with normalisation performed to the mean basal TMRM intensity of the WT Cl2 hiPSC line in each instance. This experiment highlighted a relatively large variation in mean basal TMRM intensity between individual images analysed from the same hiPSC line. While there appeared to be consistent but subtle differences between hiPSC lines, there was no biologically relevant associations between TMRM intensity and any of the mtDNA mutations which was beyond the variability observed between the various control hiPSC lines used in this experiment (WT Cl2, MELAS1 Cl5 and MERRF1 Cl2). This would further indicate the mtDNA mutations assessed do not substantially impact basal $\psi_m$ maintenance in hiPSCs.
Figure 4-5 High-throughput measurements of hiPSC basal $\psi_m$ assessed through confocal imaging of TMRM fluorescence using the Opera Phenix spinning disc confocal microscopy platform. (A) Column scatter graph showing the variability in mean mitochondrial TMRM fluorescence pixel intensity from thresholded maximum intensity projections of multiple 323x323µm fields of view normalised to the mean of WT Cl2 (cross markers, >12 fields of view per well, 2-3 wells per cell line). Lines show the mean TMRM fluorescence intensity of analysed images ±StDev. Numbers below the scatter show the total number of fields of view analysed for each hiPSC line. (B) TMRM intensity measurements from multiple fields of view within an individual experimental well were averaged and normalised to that of the WT Cl2 hiPSC line. Bars show the mean average well TMRM intensity with error bars ±StDev. Numbers in the bars show the number of individual experimental wells analysed for each hiPSC line. Statistical significance assessed by one-way ANOVA with Tukey post-hoc correction for multiple comparisons indicated by *p<0.05.
It was decided that a final experiment focused on comparing basal TMRM intensities of MELAS1 hiPSC lines would be performed, given the interesting range of m.3243A>G mutation loads of these lines, and the consistent reduction in OXPHOS CI that was observed in the MELAS1 Cl4 hiPSC line through western blot assessment. In this experiment 25 independent 323x323 µm fields of view were sequentially imaged for each hiPSC line, which as before were normalised to the mean TMRM intensity of the WT Cl2 control hiPSC line. This experiment was replicated across 4 wells for each hiPSC line to give a total of 100 independent fields of view for analysis (Figure 4-6A). Calculation of the mean TMRM intensity per well revealed all hiPSC lines assessed showed comparable basal TMRM fluorescence (Figure 4-6B). This experiment, together with those performed previously, strongly suggest m.3243A>G does not significantly alter \( \psi_m \) maintenance in hiPSCs.

### 4.3.3.2 Sensitivity of hiPSC \( \psi_m \) to Mitochondrial Toxins

While none of the mtDNA mutations assessed appeared to substantially alter basal \( \psi_m \), compensatory increases or decreases in the activity of specific OXPHOS complexes might occur in order to maintain the \( \psi_m \). Electron flow into the mitochondrial ETC occurs through NADH oxidation at OXPHOS CI and FADH\(_2\) oxidation at OXPHOS CII. Given OXPHOS CII is constituted by subunits entirely encoded by nDNA it might be expected that CII function is spared and might in fact show an increased contribution to \( \psi_m \) maintenance in the setting of mtDNA mutations. On the other hand, contribution of OXPHOS CI which includes mtDNA encoded subunits might show reduced contribution to \( \psi_m \) maintenance in hiPSCs harbouring particular mtDNA mutations. Indeed, western blot analysis suggests high levels of m.3243A>G affecting mt-tRNA\(^{\text{Leu(URR)}}\) and homoplasmic levels of the m.13528A>G and m.13565T>C mutations affecting MT-ND5 subunit negatively impact the levels of assembled OXPHOS CI. For this reason, the sensitivity of hiPSC \( \psi_m \) maintenance to inhibition of OXPHOS CI (and associated electron flux from CI) was investigated through addition of the CI inhibitor rotenone. Another potential compensatory mechanism for \( \psi_m \) maintenance is reversal of OXPHOS CV activity such that ATP is hydrolysed in order to pump H\(^+\) out of the mitochondrial and contribute to \( \psi_m \) polarisation, rather than dissipating the electrochemical H\(^+\) gradient for ATP synthesis. For this reason, the sensitivity of \( \psi_m \) maintenance to the CV inhibitor Oligomycin-A was also investigated. This also permitted further insight into the impact of m.9185T>C affecting the MT-ATP6 subunit of OXPHOS CV.
Figure 4-6 Focused high-throughput measurements of MELAS1 hiPSC basal \( \psi_m \) assessed through confocal imaging of TMRM fluorescence using the Opera Phenix spinning disc confocal microscopy platform. (A) Column scatter graph showing the variability in mean mitochondrial TMRM fluorescence pixel intensity from thresholded maximum intensity projections of multiple 323x323µm fields of view normalised to the mean of WT CI2 (cross markers, 25 fields of view per well, 4 wells per cell line). Lines show the mean TMRM fluorescence intensity of analysed images ±StDev. Numbers below the scatter show the total number of fields of view analysed for each hiPSC line. (B) TMRM intensity measurements from multiple fields of view within an individual experimental well were averaged and normalised to that of the WT CI2 hiPSC line. Bars show the mean average well TMRM intensity with error bars ±StDev. Numbers in the bars show the number of individual experimental wells analysed for each hiPSC line.
Live imaging of hiPSC mitochondrial TMRM fluorescence was performed whilst sequentially adding: 10µg/ml oligomycin-A to inhibit CV, followed by 5µM rotenone to inhibit CI and finally 1µM FCCP to completely dissipate mitochondrial electrochemical gradients (Figure 4-7A). Following addition of oligomycin-A a modest increase in mitochondrial TMRM fluorescence was generally observed, indicative of \( \psi_m \) polarisation following prevention of H\(^+\) flux through CV, as normally occurring in healthy cells during CV dependant ATP synthesis. Following rotenone addition, a reduction in mitochondrial TMRM fluorescence was observed, indicative of \( \psi_m \) depolarisation caused by inhibition of CI-dependant H\(^+\) transport from the mitochondrial matrix, and associated reduction in CIII and CIV H\(^+\) pumping activity due to prevention of CI derived electron entry into the ETC. FCCP addition caused a substantial reduction in mitochondrial TMRM fluorescence to background levels due to complete dissipation of mitochondrial electrochemical gradients. Live TMRM fluorescence time-course analysis of the different hiPSC lines revealed no obvious differences in the kinetics of the \( \psi_m \) response to addition of any of the toxins (Figure 4-7B). Furthermore, assessment of the relative mitochondrial TMRM fluorescence intensity following stabilisation after oligomycin-A and rotenone addition were comparable between all hiPSC lines (Figure 4-8). The relative contribution of OXPHOS CI and associated CI-dependant electron transfer therefore appears unaffected by the mtDNA mutations assessed. In addition, none of the mtDNA mutations assessed cause a compensatory reversal of OXPHOS CV.
B (continued)

MERRF1 CI3 (52% m.8344A>G)

MELAS2 CI3 (100% m.13528A>G and m.13565C>T)

CMT CI7 (100% m.9185T>C)

MELAS1 CI5 (0% m.3243A>G)

MELAS1 CI2 (53% m.3243A>G)

MELAS1 CI4 (89% m.3243A>G)
Figure 4-7 Live imaging of hiPSCs incubated with 25nM TMRM during the sequential addition of mitochondrial toxins as indicated by the black bars: 10µg/ml oligomycin-A (Olig), 5µM rotenone (Rot) and 1µM FCCP. (A) Representative confocal images of a single hiPSC field of view showing TMRM fluorescence following the sequential addition of oligomycin-A, rotenone and FCCP. Scale bars = 50µm. (B) Representative traces showing the kinetic changes in mitochondrial TMRM fluorescence following the sequential addition of mitochondrial toxins. TMRM fluorescence is shown plotted relative to the basal fluorescence intensity prior to toxin addition (100%) and following addition of FCCP (0%).
Figure 4-8 Relative TMRM fluorescence intensity in the presence of 10µg/ml oligomycin-A and 5µM rotenone. Bars display the mean mitochondrial TMRM fluorescence intensity normalised to that of the same field of view imaged prior to mitochondrial toxin addition with error bars ±StDev. Numbers in the bars display the number of replicate images used for analysis, which were taken across at least two independent imaging sessions for each hiPSC line.
4.3.4  **NAD*/NADH Redox Index is Unaffected in hiPSCs with mtDNA Mutations**

Taking into consideration the plethora of mitochondrial metabolic fluxes reliant on NAD*/NADH co-enzymes and importance of the mitochondrial ETC for maintenance of the NAD*:NADH ratio, bioluminescent based measurements of NAD*/NADH were made using the NAD/NADH-Glo™ Assay from Promega. By taking advantage of the specific lability of NAD* and NADH to heating under basic and acidic conditions respectively, it was possible to obtain independent measurements of NAD* and NADH which could then be used to calculate the NAD*:NADH ratio.

While significant differences in cellular NAD* were observed between the different hiPSC lines, the magnitude of these differences was generally modest and within the variability observed between control hiPSC lines (compare WT Cl2 vs MELAS1 Cl5), thus potentially of minimal biological relevance (Figure 4-9). There was one exception however, with MELAS1 Cl2 harbouring 53% m.3243A>G heteroplasmy showing approximately 2x higher cellular NAD* levels than that observed in the other hiPSC lines assessed. A similar pattern was also observed for cellular NADH levels, with much higher levels observed in MELAS1 Cl2 whereas the other hiPSC lines assessed showed comparable levels not beyond the variability observed between control lines (Figure 4-9).

These data would suggest that the overall NAD*/NADH pool is substantially higher in the MELAS1 Cl2 hiPSC line, potentially due to a compensatory upregulation in de novo NAD biosynthesis pathways. Indeed, calculation of the total NAD cofactor pool (sum of cellular NAD* and NADH) exemplified this potential increase in de novo biosynthetic flux (Figure 4-9).

Interestingly, while a substantial increase in the NAD cofactor pool was observed in the MELAS1 Cl2 hiPSC line, the NAD*:NADH ratio was maintained at comparable levels to that of the other hiPSC lines (Figure 4-9). While independent measurements of the NAD*:NADH ratio remained consistently between 5 and 8 for WT Cl2, MELAS1 Cl2 and MELAS1 Cl4, that of MELAS1 Cl5, MELAS2 Cl3 and ATP6 Cl7 were highly variable and often showed higher (>10) ratios, mostly associated with the lower cellular NADH levels observed in these hiPSC lines. Together these data suggest that there are no substantial differences in the levels of NAD cofactors and/or the NAD*/NADH redox state between hiPSCs which exceeds that of experimental variability, with the exception of MELAS1 Cl2 in which elevated NAD cofactor levels were observed.
Figure 4-9 Cellular levels of NAD coenzymes in hiPSCs harbouring mtDNA mutations. Cellular levels of NAD$^+$ and NADH were measured from aliquots of cell lysates heated under acidic and basic conditions respectively. NAD$^+$:NADH pool calculated from the sum of NAD and NADH levels measured from aliquots of the same cell lysate. NAD$^+$:NADH ratio calculated as the relative ratio between cellular levels of NAD$^+$ and NADH measured from the same cell lysate. Bars show the mean amount/ratio with error bars ± StDev, n = 4 independent cell lysates. Statistical significance assessed by two-way ANOVA with Tukey post-hoc correction for multiple comparisons indicated by ***p<0.001, **p<0.01, *p<0.05, ###p<0.001 vs all other hiPSC lines.

4.3.5 MELAS1 hiPSCs with high m.3243A>G Mutation load Show Impairments in Glucose Utilisation

Whilst detriments in $\psi_m$ maintenance might be expected as a primary consequence of mitochondrial dysfunction associated with mtDNA mutations, surprisingly no substantial impairments were observed through TMRM fluorescence measurements. It is possible however that any mitochondrial dysfunctions caused by the mtDNA mutations investigated here are somehow masked through compensatory mitochondrial or more global cellular metabolic flux changes. While no obvious alterations in relative OXPHOS
CV utilisation of the $\psi_m$ (measured through oligomycin sensitivity) were observed between the different hiPSC lines, it is possible that other biochemical pathways not explored in this study that also utilise the $\psi_m$ may have been decreased in order to compensate for mtDNA associated mitochondrial impairments. It is also possible that cellular metabolic fluxes may have been rewired in order to be less reliant on mitochondrial function. To this end it was decided to conduct glucose flux analysis of isogenic MELAS1 hiPSCs with undetectable (Cl5: control) mid-range (CI2: 53% m.3243A$>$G) and high presumably suprathreshold heteroplasmy (CI4: 89% m.3243A$>$G) in order to explore the potential possibility of masked impairments in glucose-dependant metabolic flux pathways (Figure 4-10).
Figure 4-10 Schematic showing the formation of 13C heavy isotope labelled isotopomers from metabolic flux of [U-13C]Glucose through glycolysis and a single oxidative TCA cycle. 13C labelled carbons are indicated by blue circles and unlabelled carbons by black circles. Abbreviations: F16P, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate.
4.3.5.1 m.3243A>G Mutation load Dependant Changes in Intracellular Levels of Cellular Metabolites

Although the absolute number of hiPSCs present on the culture vessel at the time of incubation with heavy-isotope labelled glucose and metabolite extraction were not measured, no obvious detriments in cell proliferation were noted at the time of this experiment nor during routine hiPSC culture. Assuming comparable numbers of cells successfully attached during seeding, and proliferation rates were not substantially different between the plates of hiPSC lines, measurements of relative intracellular metabolite abundance on a plate basis can be informative measures for identifying potential differences in cellular metabolic flux pathways. In total, 22 different intermediate metabolites and biosynthetic products belonging to glycolytic, TCA cycle and amino acid metabolic flux pathways at sufficient levels for detection were analysed (Figure 4-11). Through this analysis a number of interesting observations were made. Cellular metabolite levels described in this section are quoted as a percentage of that observed in the isogenic control MELAS1 Cl5 hiPSC line with undetectable levels of m.3243A>G.

While relative cellular glucose levels of MELAS1 Cl5 (100 ± 16%, n=4) and MELAS Cl2 (107 ± 29%, n=5) were comparable, MELAS1 Cl4 with highest 89% m.3243A>G levels showed significantly higher cellular glucose concentrations (167 ± 21%, n=4, p<0.05, two-way ANOVA with Tukey post-hoc correction) suggestive of either an increase in glucose uptake and/or decrease in subsequent glucose-dependant metabolic fluxes. While levels of the glycolytic intermediate DHAP were also trending towards higher levels in MELAS1 Cl4 hiPSCs (Cl5 100 ± 16%; Cl4 152 ± 4%; n=4), glycolytic intermediates further downstream including 3PG (Cl5 100 ± 14%; Cl4 95 ± 9%; n=4), PEP (Cl5 100 ± 5% Cl4 97 ± 15%, n=4) and pyruvate (Cl5 100 ± 28%; Cl4 113 ± 21%; n=4) were comparable. It is interesting to note that while the glycolytic reactions upstream of DHAP are not reliant on oxidized coenzymes (e.g. NAD+ or FAD), downstream of DHAP the catalytic conversion of GA3P to 3PG by the GAPDH dehydrogenase necessitates NAD+ for its catalytic function. Unlike the elevated levels of DHAP observed in MELAS1 Cl4 with highest m.3243A>G heteroplasmity, MELAS2 Cl2 hiPSCs with mid-range 53% m.3243A>G heteroplasmity showed slightly lower levels of DHAP (Cl5 100 ± 16%; Cl2 65 ± 7%; n≥4) and modest reduction in downstream 3PG (Cl5 100 ± 14%; Cl2 69% ± 14%; n≥4), although this difference was not significantly different. This might suggest that the different m.3243A>G heteroplasmity levels and associated severity
of mitochondrial dysfunction may initiate alternate glycolytic fluxes. Cellular levels of glucose-6-phosphate derivatives including glycerol-3-phosphate, myo-inositol 3-phosphate and downstream myo-inositol were lower in MELAS1 hiPSCs with mid-range and high m.3243A>G heteroplasmy levels, which might be suggestive of a compensatory downregulation in these alternative glucose metabolic pathways. Overall increases in glycolytic flux and concomitant production of lactate have been described in fibroblasts with high m.3243A>G mutation loads (Yokota et al., 2015), presumably as a mechanism through which NAD$^+$ can be regenerated to maintain reductive NAD$^+$-dependant enzymatic reactions and cellular redox state. In line with this, lactate is also a well described clinical biomarker of mitochondrial diseases including MELAS (Gorman et al., 2016). Intracellular levels of lactate in MELAS1 Cl2 and Cl4 hiPSCs with midrange and high m.3243A>G heteroplasmy respectively were in fact slightly decreased compared to that of MELAS1 Cl5 (Cl5 100 ± 28%; Cl2 61 ± 11%; Cl4 83 ± 8%; n≥4). M.3243A>G and associated mitochondrial impairments therefore do not appear to cause a compensatory upregulation of pyruvate fermentation to lactate by lactate dehydrogenase (LDH) in hiPSCs.

While levels of intracellular pyruvate, the terminal product of glycolysis, appeared to be largely maintained in MELAS1 Cl2 and Cl4 hiPSCs (Cl5 100 ± 28%; Cl2 78 ± 13%; Cl4 83 ± 8%; n≥4), levels of early stage TCA cycle intermediates were generally lower, in line with predicted impairments in TCA cycle fluxes due to attenuations in mitochondrial OXPHOS activity. While no differences in the intracellular levels of TCA cycle intermediates survived statistical testing following correction for the comparison of multiple metabolites (two-way ANOVA with Tukey post-hoc correction), rational testing of differences in levels of citrate (Cl5 100 ± 16%; Cl2 54 ± 8%; Cl4 65 ± 2%; n≥4) and α-KG (Cl5 100 ± 21%; Cl2 50 ± 8%; Cl4 65 ± 2%; n≥4) suggest these reductions are significant (p<0.001 for both citrate and α-KG, Cl5 vs Cl2 and Cl5 vs Cl4, one-way ANOVA with Tukey post-hoc correction). In hiPSCs, TCA cycle intermediates upstream of α-KG are predominantly generated from glucose derived carbons whereas those downstream from and including α-KG are generated from glutamine derived carbons (Tohyama et al., 2016). These data would therefore suggest that the mitochondrial dysfunction associated with m.3243A>G in MELAS1 Cl2 and Cl4 is affecting both glutamine and glucose oxidative mitochondrial metabolism. Unlike the levels of early TCA cycle intermediates, levels of latter stage TCA cycle metabolites including succinate (Cl5 100 ± 39 %; Cl2 76 ± 15 %; Cl4 75 ± 2%; n≥4), fumarate (Cl5 100 ± 20%; Cl2 80
± 7%; Cl4 94 ± 4%; n≥4) and malate (Cl5 100 ± 16%; Cl2 78 ± 10%; Cl4 94 ± 4%) were largely unaffected. While the catalytic conversion of α-KG to succinate is dependent on mitochondrial NAD⁺, the subsequent reactions of the TCA cycle up to oxaloacetate (undetectable through the gas chromatography mass spectrophotometry method used here) do not require NAD⁺.

As already alluded to, mitochondrial metabolism and the generation of TCA cycle intermediates are not only important for the generation of ATP through OXPHOS, but also serve as necessary substrates for a number of further catabolic and anabolic cellular pathways (see Introduction 1.4.5). Alanine is a non-essential amino acid and another downstream product of pyruvate metabolism that is often elevated in the blood of mitochondrial disease patients (Gorman et al., 2016) presumably as a consequence of reduced mitochondrial pyruvate oxidation. In line with the normal pyruvate and lactate levels observed, MELAS1 hiPSC lines with m.3243A>G also showed comparable levels of alanine to that of the isogenic MELAS1 control line absent of m.3243A>G (Cl5 100 ± 12%; Cl2 135 ± 24%; Cl4 106 ± 12%). A trend towards reduced levels of several other amino acids and amino acid derivatives were observed in MELAS1 hiPSCs with both mid-range and higher levels of m.3243A>G including: β-alanine (Cl5 100 ± 25%; Cl2 67 ± 9%; Cl4 69 ± 7%), asparagine (Cl5 100 ± 16%; Cl2 54 ± 8%; Cl4 62 ± 9%), 5-oxoproline (Cl5 100 ± 9%; Cl2 68 ± 8%; Cl4 74 ± 13%), GABA (Cl5 100 ± 34%; Cl2 48 ± 8%; Cl4 45 ± 6%) and glutamate (Cl5 100 ± 14%; Cl2 65 ±29; Cl4 47 ± 28%), supporting the important role of mitochondria in amino acid catabolism and generation of intermediates for amino acid anabolism. Reductions in cellular levels of aspartate were also observed in the MELAS1 hiPSC line with highest m.3243A>G heteroplasmy (Cl5 100 ± 11%; Cl2 96 ± 39%; Cl4 53 ± 23%), in agreement with previous studies highlighting the importance of mitochondrial ETC function for aspartate biosynthesis (Birsoy et al., 2015; Sullivan et al., 2015).
Figure 4-11 Relative abundance of glycolysis, TCA cycle, amino acid and G6P derivative metabolites in MELAS1 hiPSCs with different m.3243A>G mutation loads. Measurements made 3 days post seeding of 1x10⁶ cells. Bars show the mean cellular metabolite abundance expressed relative to that of MELAS1 CI5, from n≥4 replicate experiments, with error bars ± StDev. Statistical significance assessed by two-way ANOVA with Tukey post-hoc correction for multiple comparisons indicated by *p<0.05. Abbreviations: dihydroxyacetone phosphate, DHAP; 3-phosphoglyceric acid, 3PG; phosphoenolpyruvate, PEP.
4.3.5.2 Heavy-Isotope \([^{13}\text{C}]\)-Glucose Tracing Experiments Reveal Impairments in Glucose-Metabolic Fluxes in hiPSCs with High \(m.3243\text{A}\>\text{G} \) Heteroplasmy

In order to gain insight into the impact of \(m.3243\text{A}\>\text{G} \) specifically on glucose metabolic flux pathways of hiPSCs, heavy-isotope \([^{13}\text{C}]\)-glucose tracing experiments were performed (Figure 4-12). Heavy-isotope-glucose derived carbon incorporation and metabolic flux analysis are also independent of total cells within the measured culture and thus unaffected by any slight variations in hiPSC seeding densities and/or proliferation rates which could otherwise confound the measurements of intracellular metabolites described above. Heavy-isotope label incorporations described in this section are expressed as a percentage of the cellular metabolite which included heavy-isotope glucose-derived \(^{13}\text{C}\)-carbons.

Whilst levels of cellular glucose were higher in MELAS1 Cl4 hiPSCs with 89% \(m.3243\text{A}\>\text{G} \) mutation load, the proportion of intracellular glucose containing the heavy-isotope label after two-hour incubation was comparable between each of the hiPSC lines assessed (CI5 36 ± 4%; CI2 35 ± 6%; CI4 37 ± 3%). This would suggest that an increase in glucose-uptake at least partially accounts for the higher levels of intracellular glucose observed in MELAS1 Cl4 hiPSCs described above. Whilst MELAS1 Cl4 showed higher cellular levels of DHAP, the proportion of cellular DHAP labelled with heavy-isotope glucose-derived carbons was significantly lower in this hiPSC line, whereas that of MELAS1 Cl2 with mid-range \(m.3243\text{A}\>\text{G} \) mutation load was comparable to that of the MELAS1 Cl5 control (CI5 68 ± 3%; CI2 68 ± 2%; CI4 51 ± 2%; p<0.001 CI5 vs CI4 and CI2 vs Cl4, two-way ANOVA with Tukey post-hoc correction). This suggests that there is a reduction in the rate of early glycolytic steps in the hiPSC line with highest \(m.3243\text{A}\>\text{G} \) heteroplasmy level, thus reduced glycolytic flux is also contributing to the elevated intracellular glucose levels observed in MELAS1 Cl4. In line with this reduction in glycolytic flux of MELAS1 Cl4, the relative incorporation of glucose-derived heavy-isotope carbons was significantly lower in all downstream glycolytic metabolites assessed including: 3PG, PEP and pyruvate (p<0.001 CI5 vs CI4 and CI2 vs Cl4, two-way ANOVA with Tukey post-hoc correction; n≥4). A significant reduction in heavy-isotope label incorporation into lactate was also observed in MELAS1 Cl4 (p<0.001 CI5 vs CI4 and CI2 vs Cl4, two-way ANOVA with Tukey post-hoc correction; n≥4), with the magnitude of this reduction being comparable to that observed for pyruvate. Thus, no compensatory increase in pyruvate fermentation towards lactate is observed in MELAS1 Cl4, in line with measurements of total cellular lactate levels described above. Furthermore
calculation of the $^{13}$C-labelled pyruvate to lactate ratio (calculated from the product of intracellular metabolite levels and percentage $^{13}$C-labelled), which is a crude measure of pyruvate to lactate fermentation flux, revealed comparable or potentially lower ratios for the MELAS1 CL2 and CL4 hiPSC lines compared with the MELAS1 CL5 isogenic control (CL5 0.92 ± 0.35; CL2 0.67 ± 0.10; CL4 0.61 ± 0.11; n≥4). Interestingly, similar measurements of the $^{13}$C-labelled pyruvate to alanine ratio revealed flux rates for MELAS1 CL4 were comparable to that of the isogenic MELAS1 CL5 line, but this flux pathway was potentially elevated in MELAS1 CL2 with mid-range m.3243A>G heteroplasmy (CL5 0.65 ± 0.21; CL2 1.30 ± 0.45; CL4 0.41 ± 0.07; n≥4).

As a consequence of attenuated glycolytic flux and associated reduction in glucose derived heavy-isotope incorporation into pyruvate, levels of heavy-isotope incorporation into all downstream TCA cycle intermediates were also significantly lower including: citrate, α-KG, succinate, fumarate and malate (p<0.001 CL5 vs CL4 and p<0.05 CL2 vs CL4, two-way ANOVA with Tukey post-hoc correction; n≥4). Other metabolites for which glucose-derived carbons contribute at notable levels within the two-hour labelling time frame showed similarly reduced incorporation in MELAS1 CL4 compared to MELAS1 CL2 and CL5 including: alanine, aspartate and glutamate (p<0.001 CL5 vs CL4 and CL2 vs CL4, two-way ANOVA with Tukey post-hoc correction; n≥4). In line with the uncoupling of TCA cycle oxidation of glucose-derived carbons from citrate to α-KG in hiPSCs described previously (Tohyama et al., 2016), the proportion of citrate with heavy-isotopes incorporated was almost double that of α-KG in all three hiPSC lines assessed. Unfortunately, the data obtained here do not allow causation to be confidently determined: i.e. whether TCA cycle impairments feedback to impair overall glucose utilisation or whether reduced glycolytic rates feedforward to account for reduced heavy-isotope incorporation into TCA cycle intermediates. Calculations of $^{13}$C-labelled TCA cycle metabolite ratios, like that performed above for lactate and alanine, are inappropriate due to the cyclic intermediate rather than end-point nature of these metabolites (i.e. levels of these metabolites are also dependant on their catabolic fluxes).
Figure 4-12 Percentage incorporation of heavy isotope [U-13C]-glucose derived carbons into various glycolytic, TCA cycle, amino acid and G6P-derivative intermediate metabolites. Measurements made from MELAS1 hiPSCs incubated with [U-13C]-glucose for 2 hrs. Bars show the mean percentage of metabolite pools incorporating heavy-isotope 13C, as a proportion of the cellular total, from n≥4 replicate experiments, with error bars ± StDev. Statistical significance assessed by two-way ANOVA with Tukey post-hoc correction for multiple comparisons indicated by ***p<0.001, **p<0.01, *p<0.05. Abbreviations: dihydroxyacetone phosphate, DHAP; 3-phosphoglyceric acid, 3PG; phosphoenolpyruvate, PEP.
4.3.5.3 [U-13C]-Glucose Derived Isotopologue Analysis Hints Towards Impaired TCA Cycle Fluxes in hiPSCs with High m.3243A>G Heteroplasmy

The elevated levels of intracellular glucose and depleted incorporation of heavy-isotope glucose derived 13C-carbons into downstream glycolytic intermediates provide strong evidence for attenuated glycolytic fluxes in MELAS1 Cl4 hiPSCs with high m.3243A>G heteroplasmy. As already mentioned, both of these observations could be associated with impairments in one or many of the downstream metabolic reactions. Assessment of specific [U-13C]-Glucose derived metabolite isotopologues not only provides further information about the specific metabolic flux pathways active but can also potentially reveal downstream impairments otherwise masked by the substantial reduction in overall glucose utilisation observed for MELAS1 Cl4 (Figure 4-13).

As expected, the large majority of glucose-derived carbons enter the TCA cycle through oxidation into acetyl-CoA and subsequently citrate (which gives m+2 isotopologues) in all three hiPSC lines assessed, rather than reductive carboxylation into oxaloacetate (which gives m+3 isotopologues). The fractional abundance of m+2 citrate isotopologues (which arise from the condensation of a [U-13C]-glucose derived acetyl-CoA molecule with unlabelled oxaloacetate) were comparable between the three MELAS1 hiPSC lines, however a much larger proportion of citrate remained unlabelled (i.e m0) in MELAS1 Cl4. This potentially points towards a reduction in the cyclic oxidative TCA flux in the MELAS1 Cl4 hiPSC line with highest m.3243A>G heteroplasmy. Indeed, the fractional abundance of m+4 citrate isotopologues which arise from the sequential incorporation of two [U-13C]-glucose derived acetyl-CoA molecule through two rotations of the TCA cycle were lower in MELAS1 Cl4. This reduction in TCA cycle flux is further supported by the reduced fractional abundance of m+2 and m+4 isotopologues for downstream intermediates including α-KG, succinate and malate.

Together with previous observations these data would suggest that hiPSCs harbouring high levels of m.3243A>G show reduced metabolic fluxes which utilise glucose, potentially caused by a compensatory decrease in oxidative TCA cycle fluxes normally contributing to the metabolism of glucose-derived substrates.
Figure 4-13 Mass isotopomer distributions for selected glycolytic and TCA cycle metabolic intermediates. Measurements made from MELAS1 hiPSCs incubated with [U-13C]-glucose for 2 hrs. Typical pyruvate and lactate [U-13C]-glucose derived isotopomers incorporate three glucose-derived $^{13}$C (i.e. m+3), and one rotation of [U-13C]-glucose through the TCA cycle results in intermediate metabolite isotopomers incorporating two glucose-derived $^{13}$C (i.e. m+2). Bars show the mean isotopomer abundance expressed as a fraction of all isotopomers, from n≥4 replicate experiments, with error bars ± StDev.
4.3.6 MELAS1 hiPSCs Show Normal Expression Levels and Phosphorylation Status of Proteins Involved in Mitochondrial Metabolism and Biogenesis

In order to gain further insight into any potential compensatory changes in mitochondrial function which might have initiated in order to maintain metabolic fluxes in the presence of m.3243A>G, a number of different proteins involved in mitochondrial metabolism and/or signalling mitochondrial biogenesis were rationally chosen for assessment by western blot analysis (Figure 4-14). AMPKα is an essential kinase involved in maintaining cellular energy homeostasis, and phosphorylation of its Thr-172 residue by upstream kinases is essential for its activation. AMPKα is allosterically regulated via the ATP to ADP/AMP ratio and Thr-172 phosphorylation appears to be facilitated when cellular AMP levels rise (i.e. the ATP:ADP/AMP ratio falls) (STEIN et al., 2000). ACLY is responsible for the conversion of mitochondria derived citrate into nucleocytoplasmic Acetyl-CoA available for de novo lipid synthesis pathways and protein acetylation reactions. Phosphorylation of ACLY’s Ser-455 residue by PKB among other upstream kinases enhances greatly its activity and is often associated with elevated glycolytic and de novo lipid biosynthetic pathways (Berwick et al., 2002). The tricarboxylate transporter protein (citrate carrier: CIC) expressed on the IMM is responsible for the transport of citrate and malate between the mitochondria and cytoplasm (generally citrate from the mitochondrial matrix to the cytoplasm, and malate into the mitochondria from the cytoplasm), and is essential for downstream generation of nucleocytoplasmic acetyl-CoA. Finally, TFAM is important for both the compaction of mtDNA into nucleoids and mtDNA transcription. TFAM has been shown to be directly involved in regulating mtDNA copy number (Kanki et al., 2004) and its overexpression ameliorates mitochondrial deficits associated with mtDNA mutations (Iyer et al., 2009; Filograna et al., 2019).

While protein bands of correct molecular weight were detected for all other proteins discussed above, none of them showed substantial deviation from the levels expressed by the WT Cl2 hiPSC line (Figure 4-15). The Thr-172 phosphorylation status of AMPKα showed very high intra line variability, with this being very apparent by looking at the blot presented in Figure 4-14, which shows high relative AMPKα Thr-172 phosphorylation in WT Cl2, MELAS1 Cl3 and MELAS1 Cl4 compared to MELAS1 Cl5 and MELAS1 Cl2 in one simultaneously prepared batch of cell lysates, whereas the opposite appeared true from a second batch of cell lysates. Nevertheless, there did not appear to be any association with m.3243A>G given lysates from the two control lines.
(WT Cl2 and MELAS1 Cl5) simultaneously prepared together showed opposing AMPKα phosphorylation status. This variability is unlikely to be associated with lysate preparation errors given ACL phosphorylation was maintained in the same lysates lacking AMPKα phosphorylation.
Figure 4-14 Representative immunoblot showing the levels of proteins associated with mitochondrial function and cellular metabolism in MELAS1 hiPSCs. Two individual lysate preparations have been shown alongside one another to highlight the consistent expression of most proteins assessed, with the exception of AMPKα phosphorylation (Pi-AMPKα). Immunoblotting for TOM20 was performed used as an indirect measurement of mitochondrial mass, and β-actin was used as a protein loading control. The percentage in brackets indicates m.3243A>G load of the respective MELAS1 hiPSC line. Pi-AMPKα immunoblot specific for Thr-172 phosphorylation and Pi-ACL immunoblot specific for Ser-455 phosphorylation.
Figure 4-15 Levels of proteins associated with mitochondrial function and/or cellular metabolism in MELAS1 hiPSCs. Bars represent the mean integrated immunoblot band intensity, normalised to that of β-actin, and expressed relative to that of the WT CI2 hiPSC line which was present on all blots quantified. Also shown are the levels of Thr171 phosphorylated AMPKα and Ser455 phosphorylated ACL which are modifications associated with activation of the respective proteins. The ratio of phosphorylated to non-phosphorylated AMPKα and ACL is expressed relative to that of WT CI2. Error bars represent the StDev, and numbers in the bars represent the number of independent protein lysates assessed.
4.4 Discussion

In this chapter, the main aim was to probe the downstream consequences of mtDNA mutations on the mitochondrial function and cellular metabolism of hiPSCs using selected lines successfully established in Chapter 3. This was not only to gain further insight into the biochemical consequences of the specific mtDNA mutations and vulnerability of hiPSCs to mitochondrial dysfunction, but also in order to guide rational selection of specific lines for targeted differentiation into disease relevant cell types for downstream in vitro disease modelling purposes.

4.4.1 mtDNA Pathogenic Variant Associated OXPHOS Complex Deficits Masked in Glycolytic Favouring hiPSCs

4.4.1.1 OXPHOS Subunit Translation Largely Unaffected

On one hand, it might be expected that a cell type, such as hiPSCs, less reliant on mitochondrial function might be more likely to show impairments in OXPHOS subunit translation and/or assembly. Assessments of hiPSC lines derived from mtDNA disease patients revealed minimal differences however, in the levels of assembled OXPHOS complex expression. This suggests that at least under basal conditions, the mtDNA mutations investigated here have little impact on the translation and/or subsequent assembly of OXPHOS complexes in hiPSCs. In line with a specific impairment towards OXPHOS CI, the MELAS2 CI3 hiPSC line harbouring homoplasmic m.13528A>G (p.T398A) and m.13565C>T (p.S410F) missense mutations affecting the OXPHOS CI subunit MT-ND5, showed a modest reduction in levels of assembled OXPHOS CI. Modest reductions in levels of assembled OXPHOS CI were also noted in the MELAS1 CI4 hiPSC line harbouring high 88% m.3243A>G heteroplasmy, which is a common observation for cells harbouring this specific mtDNA pathogenic variant at sub homoplasmic levels (Dunbar et al., 1996), and in line with previous reports of hiPSCs with 85% m.3243A>G heteroplasmy (Lin et al., 2019a). Impairments in the assembly and activity of OXPHOS CV have been previously described in muscle tissue samples from CMT2 patients with homoplasmic levels of m.9185T>C (Pitceathly et al., 2012). While assessment of the ATP5A subunit of OXPHOS CV did not appear to differ in any of the lines assessed, it is possible that levels of the ATP5A subunit might not be readily impacted in this setting, given levels of ATP5A are maintained in rho0 cells lacking mtDNA encoded OXPHOS subunits entirely (Appleby et al., 1999).
No obvious alterations were observed in the MERRF1 Cl3 hiPSC line with 52% m.8344A>G heteroplasmy, for which impairments in OXPHOS CI and CIV might be expected at suprathreshold levels (Hanna et al., 1995; James et al., 1996). In light of the severe impact of this pathogenic variant on reprogramming efficiency at higher heteroplasmy levels, as explored in detail in Chapter 3, it would appear that m.8344A>G heteroplasmy levels sufficiently low to maintain levels of mitochondrial protein translation have been selected for during the reprogramming process. Of note, the OXPHOS CIV subunit probed for here (MT-CO1) is encoded by the mitochondrial genome, and has previously been shown to be a particularly vulnerable subunit in cells with suprathreshold levels of m.8344A>G (Hanna et al., 1995; Pereira et al., 2018).

4.4.1.2 Normal $\psi_m$ Maintenance

While only slight reductions in the levels of assembled OXPHOS complexes were observed, downstream mitochondrial function could potentially be impacted more severely. It is possible that instead of affecting the levels of assembled OXPHOS complexes through impairments in translation, stability and/or assembly of OXPHOS complex subunits, the mtDNA mutations instead alter OXPHOS complex subunits conformational structures and consequently enzymatic function. Although $\psi_m$ is not a direct measure of OXPHOS complex activity and additionally affected by non-OXPHOS dependant dissipators and generators of the $H^+$ gradient, it can nevertheless serve as a useful indicator of OXPHOS function and overall mitochondrial health. No impairments in $\psi_m$ maintenance were observed in any of the hiPSC lines assessed however, including the MELAS1 Cl4 and MELAS2 Cl3 lines which showed reductions in assembled OXPHOS CI. This is in contrast to a previous report of hiPSCs with 85% m.3243A>G which showed more depolarised $\psi_m$ (Lin et al., 2019a), and well described $\psi_m$ depolarisations in other cell types harbouring high levels of many of the mtDNA mutations investigated here including: m.3243A>G, m.8344A>G and m.13528A>G and m.13565C>T (McKenzie et al., 2007; Kovac et al., 2019).

This would suggest that even though levels of CI are reduced in MELAS1 Cl4 and MELAS2 Cl3, the conserved CI activity is sufficient for $\psi_m$ maintenance. As hiPSCs favour glycolytic over oxidative metabolism in order to meet the majority of cellular ATP demand (Varum et al., 2011), it is likely that molecular mechanisms which typically utilise the $\psi_m$ (most notably OXPHOS CV) are less active, reducing reliance on ETC (and CI) for $\psi_m$ maintenance. In line with this redundancy in OXPHOS CI contribution to $\psi_m$,
all hiPSC lines assessed showed a similar sensitivity to inhibition with rotenone whereas a smaller depolarisation would be expected if OXPHOS CI dysfunction was sufficiently severe such that compensatory changes in $\psi_m$ maintenance fluxes were required. In a similar manner, there was no evidence for a compensatory reversal of OXPHOS CV hydrolysing ATP in order to maintain the $\psi_m$ (as tested through oligomycin-A application). This is in contrast to observations made in patient fibroblasts with the m.3243A>G, m.8344A>G and m.13528A>G and m.13565C>T mutations, where CV reversal and reduced CI contribution to $\psi_m$ maintenance were both observed (Kovac et al., 2019).

Patient lymphoblasts harbouring the m.8993T>G pathogenic variant in MT-ATP6 (Sgarbi et al., 2006; Baracca et al., 2007) and more recently, hiPSC derived neural progenitors and terminally differentiated neurons harbouring m.9185T>C (Lorenz et al., 2017), both which affect the MT-ATP6 subunit of OXPHOS CV, have been shown to have a slightly hyperpolarised $\psi_m$, presumably due to impaired utilisation of the $\text{H}^+$ gradient for ATP synthesis. No such hyperpolarisation of the $\psi_m$ was observed in CMT Cl7 hiPSCs homoplasmic for m.9185T>C however, which is likely due to the limited generation of ATP through OXPHOS in this cell type. Supporting this, addition of oligomycin only caused a very subtle hyperpolarisation of the $\psi_m$ in all hiPSC lines assessed.

### 4.4.1.3 Are hiPSC Culture Conditions Contributing to the Masking of OXPHOS Deficits

Whilst cell-type specific differences in mitochondrial activity likely explain why no $\psi_m$ impairments were observed in this study, specifically the glycolytic favouring metabolism of hiPSCs, differences with a previous report investigating hiPSCs with comparable m.3243A>G mutation loads (Lin et al., 2019a) are more difficult to explain. It has become clear that hiPSC metabolism is particularly sensitive to cell culture conditions and nutrient availability, with the presence of endogenous lipids in cell culture media being a particularly important factor (Zhang et al., 2016). Both mTeSR1 (used in this study) and Essential 8 (E8; used by Lin et al., 2019a) media formulations contain limiting amounts of lipids which promotes fatty-acid synthesis pathways that are less dependent on oxidative mitochondrial function (Zhang et al., 2016). mTeSR1 contains more lipid supplements compared to E8 and thus promotes higher oxidative mitochondrial fluxes. It is therefore possible that the different media formulations used
here account for the different observations of mitochondrial function observed. In the Lin et al., 2019a study, they showed increased mitophagic flux in hiPSCs with high m.3243A>G and thus it is also possible that the apparent reduction in $\psi_m$ they described is instead accounted for by a reduction in hiPSC mitochondrial content. Of note, the analysis method performed on confocal imaging stacks here accounts for variations in mitochondrial content, and no obvious changes in mitochondrial content were noted by imaging, western blot or mtDNA content analysis.

Although prolonged investigations were not performed, early comparisons shortly after hiPSC line establishment were made between the growth characteristics of MERRF1 (Cl2 and Cl3 from first reprogramming) and MELAS1 hiPSCs in E8 and mTeSR1. Whilst no substantial detriments were noted in cells cultured in E8, hiPSCs grown in mTeSR1 appeared more stable in our hands (i.e. propensity for spontaneous differentiation was less). While no substantial change in mtDNA mutation load was observed during routine culture in mTeSR1, a possible but very modest reduction in m.3243A>G mutation load was observed after culturing MELAS1 Cl4 for two passages in E8, however this 2% fall from 89% to 87% was within the range of mutation load fluctuations observed across the three years of this research project. As hiPSC culture ‘stability’ is likely associated with overall cellular fitness, it is possible the rationally chosen mTeSR1 formulation is in fact masking mtDNA pathogenic variant associated phenotypes in these cell types. This might also explain why no drops in mtDNA mutation loads were observed, unlike that which has been described in previous studies (see Introduction section 1.4.6.5). It would be interesting to explore further the potential impact of different culture conditions and media formulations on hiPSCs with mtDNA pathogenic variant associated impairments.

In relation to previous observations made in Chapter 3, this lipid-dependant alteration of cell metabolism might at least partially explain impaired reprogramming efficiencies of cells harbouring high levels of m.8344A>G. Whilst initial reprogramming of MERRF1 fibroblasts gave rise to stable hiPSC lines which passed pluripotency assessments, the highest m.8344A>G mutation load successfully obtained in a clonal line was only 52% (MERRF1 Cl3), and overall reprogramming efficiency was very low. By contrast much higher mutation loads were successfully obtained in a subsequent reprogramming under feeder-independent conditions using mTeSR1 culture media, and overall reprogramming efficiencies were much higher. Considering lipid availability is much more limited under feeder-independent conditions it can be speculated that a reduced reliance on oxidative
metabolism facilitated the reprogramming of MERRF fibroblasts under these culture conditions.

4.4.2 MELAS1 hiPSCs with m.3243A>G Show a Complex Heteroplasmym-Phenotype Relationship

MELAS1 Cl2 hiPSCs with mid-range 53% m.3243A>G heteroplasmym levels showed comparable measures of mitochondrial health to that of control (WT and isogenic) hiPSC lines in most of the assessments performed. This included an apparently negligible impact of the pathogenic variant at such mid-range mutation loads on levels of assembled OXPHOS CI, whereas reductions of this OXPHOS complex were observed in the MELAS1 Cl4 hiPSC line with higher 89% m.3243A>G heteroplasmym level. Sensitive measures of hiPSC metabolites via mass spectrometry did reveal a number of downstream impacts of m.3243A>G at mid-range m.3243A>G heteroplasmym levels, which in some instances differed from those observed in hiPSCs with higher mutation loads.

Whilst heavy-isotope glucose flux tracing suggests MELAS1 Cl2 hiPSCs show normal flux of glucose carbons as a proportion of the cellular total, several differences in levels of cellular metabolite pools were noted. Among these, MELAS1 Cl2 hiPSCs showed reduced levels of the glycolytic intermediates DHAP and 3PG which could indicate reductions in the activity of metabolic fluxes upstream of these products and/or increased activity of metabolic fluxes downstream of these products. Of note upstream catalysis of glucose to these products necessitates ATP and oxidised NAD\(^+\), both of which might be potentially reduced in hiPSCs with mitochondrial impairments, whereas downstream conversion to pyruvate in fact results in the net generation of ATP. By comparison, MELAS1 Cl4 hiPSCs showed elevated levels of both cellular glucose and DHAP whereas levels of 3PG were comparable to that of the control line. This might suggest that MELAS1 Cl4 hiPSCs have more severe reductions in cytosolic ATP available for catalysing the sequential conversion to DHAP but sufficient oxidised NAD\(^+\) available for the subsequent conversion to 3PG. Indeed, heavy isotope glucose flux tracing analysis highlights early impairments in transient glycolytic metabolic fluxes in MELAS1 Cl4 hiPSCs whereas those of MELAS1 Cl2 hiPSCs were highly comparable to that of the MELAS1 Cl5 isogenic control line. In relation to this, MELAS1 Cl2 hiPSCs showed an elevated cellular pool of NAD/NADH which might be caused by compensatory feedback mechanisms being initiated in response to limited cytosolic NAD\(^+\) availability. By comparison, the NAD/NADH pool of MELAS1 Cl4 hiPSCs was comparable to that of
the isogenic control line. Interestingly, similar increases in \textit{de novo} NAD synthesis pathways has been described in cybrid cells harbouring heteroplasmic levels of m.3243A$>$G, whereas cybrids homoplasmic for m.3243A$>$G showed comparable levels to that of the isogenic control line (Kopinski \textit{et al.}, 2019). Downstream of glycolysis, MELAS1 Cl2 and MELAS1 Cl4 hiPSC lines both showed comparable reductions in total cellular levels of the early TCA cycle metabolites citrate, $\alpha$-KG and succinate. This is in line with predicted impairments in mitochondrial NAD$^+/\text{NADH}$ recycling necessary for TCA cycle flux associated with the mtDNA pathogenic variant and OXPHOS dysfunction.

\subsection*{4.4.3 Metabolic Perturbations Cause Distinct Compensatory Cellular Phenotypes}

Together, these data show that while impairments in glycolytic and downstream mitochondrial metabolic pathways are observed in MELAS1 hiPSCs with both mid-range and higher m.3243A$>$G heteroplasmy levels, the severity of the biochemical deficit associated with each of these mutation loads differs, leading to alternate compensatory pathways being activated. In line with this, cybrid cells with severe mitochondrial impairments associated with a homoplasmic m.14787_14790delTTAA \textit{MT-CYTB} frameshift pathogenic variant or homoplasmic m.6930G$>$A (p.G343*) \textit{MT-CO1} pathogenic variant have been shown to switch towards reductive and oxidative glutamine rather than oxidative glucose mitochondrial metabolic flux pathways (Mullen \textit{et al.}, 2012; Chen \textit{et al.}, 2018a). Cybrids with less severe mitochondrial dysfunction associated with homoplasmic levels of the m.8993T$>$G (p.Leu156Arg) \textit{MT-ATP6} pathogenic variant or heteroplasmic (50%) levels of the m.6930G$>$A (p.G343*) \textit{MT-CO1} pathogenic variant also showed a switch towards glutaminolysis favouring rather than glucose utilising mitochondrial metabolism, but without initiating reductive glutamine metabolic fluxes (Chen \textit{et al.}, 2018a).

It would therefore appear that at the cellular level, a number of distinct compensatory metabolic and phenotypic states can be entered, each of which has its own biochemical threshold for initiation. In line with this, isogenic cybrid cells harbouring increasing levels of m.3243A$>$G show abrupt rather than staggered or continuous changes in gene expression profiles (Picard \textit{et al.}, 2014). This additional layer of complexity might help explain a number of the heterogenous clinical phenotypes associated with mitochondrial disease. Among these, cell type specific differences in mtDNA mutation load biochemical thresholds are likely accompanied by distinct transcriptional profiles, each of which has
itself a specific biochemical threshold. These discrete and independent cellular phenotypes could partially explain the distinct clinical phenotypes that are observed in mitochondrial disease patients harbouring sometimes highly comparable m.3243A>G (and other mtDNA mutations) heteroplasmic levels (Boggan et al., 2019; Altmann et al., 2016). The additional layer of complexity associated with these metabolically sensitive transcriptional states might facilitate better correlation between the specific vulnerability of certain tissues and organs to particular mitochondrial mutations and associated metabolic perturbations. Mitochondrial diseases commonly present and/or irreversibly exacerbate following exposure to environmental stressors (e.g. infection). It is possible that increased metabolic demands during these periods of environmental stress cause initiation of these detrimental transcriptional states which are subsequently maintained, underlying the chronic mitochondrial disease state.

4.4.4 Summary

To summarise this chapter, even at high or homoplasmic levels of a variety of mtDNA mutations, biochemical mitochondrial deficits assessed through numerous methods were relatively modest if at all detectable. Downstream impairments in cellular metabolism were observed however, following more in-depth and focused investigations of MELAS1 hiPSCs harbouring m.3243A>G. As heteroplasmic level dependant mitochondrial and cellular impairments were observed in MELAS1 hiPSCs, these lines appear most appropriate for downstream differentiation for in vitro disease modelling purposes. As hiPSCs are a glycolytic favouring cell type and culture conditions potentially mask mitochondrial impairments, other hiPSC lines harbouring mtDNA mutations might also be useful, upon differentiation into more disease relevant and oxidative metabolising myogenic cell types.
Chapter 5  Myogenic Differentiation of hiPSCs Harbouring mtDNA Mutations

5.1 Overview

Skeletal muscle is among the most commonly affected organ systems in mitochondrial disease patients, with patients commonly reporting musculoskeletal symptoms including exercise intolerance, muscle weakness and fatigue, and respiratory difficulties (Boggan et al., 2019; Ng & Turnbull, 2016). Myopathy and the presence of ragged red fibres in patient muscle biopsies remain key diagnostic indications of a mitochondrial disease phenotype (DiMauro & Paradas, 2014).

Despite this, skeletal myogenic cells have been largely overlooked as a target cell type for differentiation of hiPSCs harbouring mtDNA disease associated mutations, with investigators instead focusing largely on differentiation into cardiomyocytes and cortical neurons (see Introduction section 1.4.6). This is understandable taking into account myogenic differentiation protocols replicating developmental signalling cues are in their infancy compared to those giving rise to these other mitochondrial disease relevant cell types.

Upon the onset of this research project, publications from the Stanford/Skerjanc and Pourquié research groups described significant advancements in myogenic differentiation protocols utilising small molecules and growth factors to recapitulate developmental signalling gradients occurring during myogenesis, that appeared to efficiently give rise to skeletal myogenic cell progeny (Shelton et al., 2014; Chal et al., 2015). In view of this progress, and the currently unexplored effect of mtDNA mutations in hiPSC derived skeletal muscle cell types, it was decided that this research project would focus on differentiation into skeletal myogenic cells. Evidence supporting a role of cellular metabolism driving myogenic cell identity transitions also began accumulating throughout the duration of this project (Ryall et al., 2015; Das et al., 2017; Yucel et al., 2019), highlighting skeletal myogenic cell types as a particularly relevant model for exploring the impact of mitochondrial dysfunction on metabolically sensitive epigenetic modifications and cell identity transitions.
5.1.1 Myogenic Differentiation Strategy

As already mentioned, the protocols described in Shelton et al., 2014 and Chal et al., 2015 were used as initial starting points for the differentiation protocol used here during the development of the in vitro myogenic model of mitochondrial disease. These protocols both built on previous work surrounding patterning of PSCs towards a paraxial mesoderm lineage through Wnt signalling activation via inhibition of GSK3β, and FGF2 mediated signalling activation (Borchin, Chen & Barberi, 2013; see also Introduction section 1.6.3.2 for in-depth overview). The protocol by Chal et al. 2015 also included BMP4R antagonists during initial mesoderm patterning, in order to prevent lateral over paraxial transitions of mesoderm precursors, and additional promyogenic growth factors (HGF, IGF, FGF) at latter stages in order to enhance the survival/proliferation of myogenic cell types. Both rational additions likely facilitate overall differentiation efficiency and expansion of the desired myogenic cells, reducing contamination of end-point cultures with undesired cell types, and were therefore included in the differentiation protocol optimised here.

5.1.2 Transgene-Free Myogenic Differentiation Permits Exploration of Impairments in Metabolically Sensitive Cell Identity Transitions

Whilst overexpression of myogenic regulatory factors such as MyoD might at first appear to be the most technically feasible and efficient way for driving differentiation of hiPSCs towards myogenic cell types, given its expression efficiently transdifferentiates somatic cells, there are a number of reasons why pushing hiPSCs through sequential cell identity transitions occurring during the developmental myogenic programme is the preferred strategy for this project. Some of these are technical and include the difficulty of transfecting hiPSCs, and the resultant requirement of more complex inducible viral vectors and adjuvants for efficient myogenic conversion (Goudenege et al., 2012; Pawlowski et al., 2017).

As explored in Introduction section 1.5, cell metabolism and cell-fate decisions are bidirectionally linked, with changes in cell metabolism being important for driving both the early differentiation of hiPSCs, and downstream myogenic cell-identity changes. Of note, a switch towards more oxidative cellular metabolism is crucial for early mesoderm differentiation (Cliff et al., 2017; Lu et al., 2019) and transient changes in oxidative metabolic fluxes are essential for satellite cell activation, myoblast differentiation and myotube formation (Ryall et al., 2015; Das et al., 2017; Yucel et al., 2019).
Overexpression of myogenic regulatory factors, even if transient, will bypass many of the metabolic and epigenetic transitions underlying these cell identity changes occurring during the myogenic differentiation process, potentially masking disease relevant impairments in the metaboloepigenetic axis which is the primary focus of this research project. In contrast, recapitulating the developmental myogenesis programme sequentially pushes the differentiating cells through each of these metabolic and epigenetic barriers, and thus serves as a much better model for exploring the impact of mitochondrial dysfunction on metabolically sensitive epigenetic transitions.

In addition, hiPSC derived myogenic cells differentiated in this way can be maintained in a proliferative myoblast like state by culturing in serum containing media, with serum withdrawal promoting terminal differentiation into multinucleated myotubes (Chal et al., 2015, 2016), comparable to the culture sensitivities of primary human and murine myoblasts, and the commonly used C2C12 immortalised murine myoblast cell line (Owens, Moreira & Bain, 2013; Saini, Al-Shanti & Stewart, 2010). This robust myogenic cell-identity change not only permits the exploration of mitochondrial and cellular phenotypes in two distinct myogenic cell types, but also the metabolic and epigenetic rewiring underscoring this cellular transition (Yucel et al., 2019). The fusion of myoblasts into myotubes is part of the physiological process underlying muscle repair/regeneration, and this transition is therefore of great relevance to the mitochondrial disease setting, given the strong myopathic phenotypes which are often observed in these patients.

5.1.3 Characterisation of Myogenicity in Differentiated Cultures

As with the hiPSC lines harbouring mtDNA mutations established in Chapter 3, characterisation of the differentiated progeny is crucial for ensuring phenotypic observations made are associated with the correct and desired cell type. This can be conducted in much the same way as done previously for the hiPSCs, namely through visual assessments of cell morphologies, mRNA expression through RT-qPCR and protein expression through immunofluorescence. Taking into consideration previous evidence highlighting cell identity changes being dependent on changes in cellular metabolism, and the detrimental impact of mtDNA disease associated dysfunction observed during reprogramming of patient fibroblasts harbouring m.8344A>G (see Chapter 3), such characterisation can also reveal impairments in the differentiation of target cell types.
5.1.3.1 Core Transcription Factors for Assessment of Myogenic Differentiation and Maturation

With the sizeable amount of research which has been conducted towards understanding developmental myogenesis, a wide range of key myogenic markers have been well defined.

The expression of core regulatory transcription factors is among some of the most useful markers for defining differentiating cell populations and this remains true of myogenic cells and their precursors. As the master regulator of myogenesis, MyoD is a defining marker of skeletal myogenic cells including activated satellite cells, myoblasts and myocytes (Hernández-Hernández et al., 2017), and thus serves as an important marker to show positive expression within a true myogenic cell population. In addition to expression in myogenic precursors during development, PAX7 is also a defining transcription factor expressed by the resident skeletal muscle stem cell niche (satellite cells) (Pourquié, Al Tanoury & Chal, 2018), and can therefore serve as a useful marker of skeletal myogenic precursors and potentially provide insight into the maintenance of satellite-like cell population in the differentiating myogenic cultures, as has been previously described (Shelton et al., 2015; Chal et al., 2015). Myogenin (MyoG) is a transcription factor expressed later in the skeletal myogenic programme, that is crucial to the differentiation of proliferating myoblasts into myocytes and subsequently myotubes (Chal & Pourquié, 2017) thus representing another useful transcriptional marker of myogenesis. As these transcription factors are expressed at different stages during developmental myogenesis, assessment of these markers can provide insight into the relative maturity of the differentiated myogenic cell culture.

5.1.3.2 Myosin Heavy Chain Isoforms as a Marker of Muscle Fibre Type and Maturity

In addition to expression of myogenic transcription factors, assessment of functionally important skeletal muscle genes/proteins can also provide information on myogenicity and maturity of differentiated cultures. Desmin is a muscle-specific intermediate filament protein important for the structural organisation of myofibrils, which although not specific for skeletal muscle (also expressed in cardiac muscle) can serve as a useful marker of both skeletal myoblasts and myotubes (Hnia et al., 2015). In a similar manner, assessments of proteins such as titin and MyHC which contribute to the sarcomeric contractile apparatus (Granzier & Labeit, 2005) can additionally be used to identify...
myotubes. In relation to this, assessments of spontaneous cellular contractions within the culture dish would also be highly indicative of functionally active skeletal myotubes.

Interestingly, mammals including humans express a number of distinct MyHC isoforms in skeletal muscle, each of which can show different temporal expression during skeletal muscle development and/or a specific defining expression in different muscle fibre subtypes (Schiaffino et al., 2015). The MYH3 gene encoding embryonic MyHC is expressed in early developing muscle fibres including those during primary myogenesis, and is downregulated postnatally (Cho, Webster & Blau, 1993; Schiaffino et al., 2015). The MYH8 gene encoding neonatal MyHC is associated with secondary myogenesis and thus shows a similar but slightly delayed temporal expression compared to MYH3 (Cho, Webster & Blau, 1993; Schiaffino et al., 2015). The MYH7 gene encoding slow MyHC is also expressed in embryonic and fetal muscle (Cho, Webster & Blau, 1993; Schiaffino et al., 2015), but in adults is specifically expressed by slow skeletal muscle fibre subtypes (note, it is also expressed in cardiac muscle) (Pette & Staron, 2000). By comparison, embryonic and fetal skeletal muscle express limited amounts of MYH1 encoding fast 2X MyHC and MYH2 encoding fast 2A MyHC (Schiaffino et al., 2015). Instead MYH1 and MYH2 are preferentially expressed in adult fast 2X and fast 2A muscle fibres respectively (Pette & Staron, 2000).

Muscle fibre types not only differ in their MyHC expression profile but also show distinct cellular metabolisms and contractile properties. As their names would suggest, fast muscle fibre types show much quicker shortening velocities compared to slow muscle fibre types. In association with the differing energy demands of the fibre types, fast 2X fibre types predominantly favour glycolytic metabolic fluxes, fast 2A fibre types being more oxidative but also showing glycolytic metabolic activity, and slow fibre types predominantly favouring oxidative metabolic fluxes (Schiaffino & Reggiani, 2011).

Considering slow muscle fibre types are more reliant on mitochondrial function, it might be expected that slow rather than fast muscle fibres of mtDNA disease patients might be more vulnerable to associated mitochondrial deficits. While studies investigating fibre type predominance in mitochondrial disease are limited, muscle biopsies from patients with suspected mitochondrial disease and MELAS patients harbouring m.3243A>G generally show an increased proportion of type 1 muscle fibres (Koo et al., 1993; Enns et al., 2005). Reduced proportions of type 1 muscle fibres have been reported in a rat model of mitochondrial myopathy (zidovudine induced mtDNA depletion) (Venhoff et al.,
Muscle fibre type is not static and can dynamically change in response to a number of different stimuli including stimulation (neuromuscular activity), hormones and aging (Schiaffino & Reggiani, 2011). In line with the more oxidative metabolism of slow fibre types, PGC1α, the master regulator of biogenesis is expressed at high levels in muscles with high proportions of slow fibre types (Lin et al., 2002). Interestingly, this PGC1α expression appears to be not only responsible for promoting oxidative metabolism in slow muscle fibres, but also drives the a switch in expression of functionally important proteins (including MyHC isoforms) from fast to slow fibre defining isoforms (Lin et al., 2002; Mortensen et al., 2006). Additionally, more recent evidence has shown that nucleocytoplasmic acetyl-CoA availability might also be a contributing factor to fibre type identity, with impairments in the generation of nucleocytoplasmic acetyl-coA associated with an increased expression of slow muscle fibre type proteins and a decrease in fast fibre type proteins (Yucel et al., 2019). Mitochondrial dysfunction caused by mtDNA mutations could therefore be potentially impacting downstream myogenic differentiation pathways associated with fibre type switching, representing an additional cell identity transition warranting exploration.

5.2 Aims and Hypothesis

The first aim of this chapter was to optimise a skeletal myogenic differentiation protocol that would permit the efficient and reproducible differentiation of hiPSCs harbouring mtDNA mutations into disease relevant skeletal muscle cell types.

The differentiated cells were then characterised for myogenicity through immunofluorescence and qPCR analysis in order to gain insight into any detrimental impacts on myogenic differentiation efficiencies and/or subsequent cellular maturation. Mitochondrial function of the myogenic cell cultures were assessed through western blot analysis, live cell imaging of TMRM fluorescence and cellular respiration analysis, in order to gain insight into disease-relevant mitochondrial deficits, and permit correlations to be made with any myogenicity impairments observed.

As more oxidatively demanding cell types than hiPSCs, mitochondrial impairments were predicted to be more severe in the differentiated myogenic cultures. In light of the importance of mitochondrial function for cell fate identity transitions, particularly those
of myogenic cells, impairments in overall myogenicity and cell maturation were predicted in lines with suprathreshold levels of mtDNA mutations.

5.3 Results

5.3.1 Optimisation of Myogenic Differentiation

The directed differentiation of hiPSCs towards myogenic cells necessitated a considerable amount of optimisation in order to efficiently obtain myogenic cultures with minimal amounts of contaminating cell types. The optimisation phase was restricted to use of a 6-well plate format with 10cm² surface areas in order to avoid variations associated with scaling up/down of differentiation inductions and was largely maintained throughout the duration of this project. hiPSC seeding media was supplemented at all times with 10µM Y-27632 ROCKi, in order to enhance reproducible cell survival and attachment. Optimisation of the differentiation process was iteratively continued throughout the entirety of this research project, and thus the observations made below towards optimal conditions were based off all of the differentiation inductions performed. Figure 5-1 shows a schematic of the optimised differentiation protocol alongside representative images of a differentiating WT Cl2 hiPSC line using these optimised conditions.
Figure 5-1 Schematic of the optimised skeletal myogenic differentiation protocol. See Methods section 2.1.4 for CL, CLF, HIFL, KI, KIH AND IST media formulations. Also shown are representative phase contrast images during the initial stages of a successful myogenic differentiation induction performed of the WT C12 hiPSC line. * highlights the dense colonies surviving ICLF stimulation, from which cells proliferate and migrate away from. Arrows on the d28 phase contrast image highlight dense colonies of cells with small somas which can give rise to neural-like contaminants in end stage cultures. Scale bars = 500µm
5.3.1.1  *hiPSC Line Specific Impairments in Myogenic Differentiation*

A number of different hiPSC lines were subjected to the myogenic differentiation protocol with varying success. As already mentioned, several MERRF1 hiPSC lines established as part of the second reprogramming failed to differentiate into desired myogenic cell types on multiple occasions, including lines which had both high 73% m.8344A>G heteroplasmy (MERRF1 Cl23), lower 20% m.8344A>G heteroplasmy (MERRF1 Cl14) and undetectable levels of m.8344A>G (MERRF1 Cl6). Cultures instead tended towards a very heterogenous population of cell types unsuitable for further downstream use. Taking into account the difficulties faced during the establishment, maintenance and downstream characterisation of these and other MERRF lines established during the second reprogramming induction, it is likely this is associated with the quality of these apparent hiPSC lines. Indeed, the differentiation impairment observed with MERRF1 Cl6, which lacks m.8344A>G entirely highlights that this is not an associated phenotype of m.8344A>G.

Several unsuccessful attempts were also made to differentiate the MELAS2 Cl3 hiPSC line with homoplasmic levels of the m.13528A>G (p.T398A) and m.13565C>T (p.S410F) missense mutations affecting MT-ND5. Cells typically failed to survive following ICLF stimulation, even upon increases in initial cell seeding densities. Any cells which did remain following ICLF stimulation showed limited proliferation activity which preceded their use in downstream assays. Considering the impact these mutations had on levels of OXPHOS CI in hiPSCs, it is possible this could be a phenotype associated with the MT-ND5 mutations. It is important to note however, that similar difficulties were faced differentiating the WT Cl2 hiPSC line on several occasions. Whilst preliminary tests differentiating the CMT Cl7 hiPSC line with homoplasmic levels of the m.9185T>C (p.Leu220Pro) pathogenic variant were reasonably successful, cultures typically maintained a high contamination of neural-like cells, even after passaging, which preceded their suitability for downstream analysis.

Given the successful inductions performed on MERRF1 hiPSCs with/without midrange levels of m.8344A>G and MELAS1 hiPSCs with a range of different m.3243A>G mutations established as part of the first reprogramming induction, as will be described below, it was decided that these lines would be focused on for downstream *in vitro* mitochondrial disease modelling purposes.
5.3.2 Differentiation and Characterisation of Myogenic Cells Harbouring m.8344A>G

Whilst MERRF1 hiPSC lines obtained from the second reprogramming induction failed to differentiate into myogenic cell types, MERRF1 Cl2 and MERRF1 Cl3 hiPSC lines from the first reprogramming induction with undetectable and mid-range levels of m.8344A>G respectively could be successfully differentiated. The kinetics of myogenic differentiation was comparable between the two MERRF1 hiPSC lines and did not differ from that of the WT Cl2 hiPSC line. In all instances, m.8344A>G heteroplasmy of the starting MERRF1 Cl3 hiPSC population was 50-55%, and maintained in the terminally differentiated myogenic cultures, as assessed through dye terminator sequencing.

5.3.2.1 MERRF1 hiPSCs Can Be Terminally Differentiated into Multinucleated Myotubes

After 35 days in culture, WT Cl2, MERRF1 Cl2 (0% m.8344A>G) and MERRF1 Cl3 (52% m.8344A>G) were seeded for serum withdrawal and terminal differentiation. Characterisation of the terminally differentiated cultures after 1 week of serum withdrawal was performed through immunofluorescence staining and confocal microscopy for several key myogenic markers (Figure 5-2). No obvious differences in overall myogenicity were observed between the WT and two MERRF1 lines, with this being evident in the lower power magnification images taken in Figure 5-2E.

Terminally differentiated, multinucleated myotubes were observed in cultures differentiated from all three hiPSC lines. A typical staining pattern was observed for desmin (Figure 5-2A), with longitudinal fibre structures being evident along the length of myotubes. Myotubes also showed positive staining for MyHC (note MF20 stains all MyHC isoforms), which upon close inspection showed a striated staining pattern, expected for this sarcomeric protein (Figure 5-2B, C and E). A similar, but more obvious striated staining pattern was also observed for the sarcomeric protein titin (Figure 5-2A and D). In line with the formation of functional sarcomeric structures, spontaneous contractions within regions of high cell density were observed within the terminally differentiated MERRF1 Cl3 and WT Cl2 myogenic cultures prior to assessment for assays. Spontaneous contractions were uncommon events and the lack of such observations in MERRF1 Cl2 did not preclude the possibility that they also showed spontaneous contractile activity which was simply not detected.
Assessment of the essential myogenic regulatory factor, MyoD, revealed an expected nuclear localised staining pattern within the nuclei of myotubes (Figure 5-2B). Mononucleated cells interspersed between the myotubes also showed positive MyoD staining, in line with the presence of myoblasts and/or myocytes within the differentiating culture (Figure 5-2B). Nuclei of myotubes also showed a strong and expected nuclear localised staining pattern for the myogenic differentiation factor, myogenin, which similar to MyoD, was also observed in surrounding mononucleated cells, further supporting the presence of non-terminally differentiated myocytes within the myogenic cultures (Figure 5-2C). Of particular interest, a proportion of mononucleated cells expressing the satellite cell marker PAX7 were also observed, interspersed and sometimes in extremely close proximity to the differentiated myotubes (Figure 5-2A, C, D and E). Co-staining with the proliferation associated marker Ki67 revealed a subset of these PAX7+ cells showed low/no expression of Ki67 which could indicate they are in a quiescent state, not dissimilar from the resident satellite cell population of adult muscle (Figure 5-2D).
Figure 5-2 Representative immunofluorescence images of MERRF1 Cl2 and Cl3 with/without 52% m.8344A>G mutation load, and WT Cl2 terminally differentiated myogenic cultures after 7 days of serum withdrawal. All hiPSC lines successfully formed multinucleated myotubes which stained positive for desmin, MyHC, and titin. Positive nuclear staining for the crucial myogenic regulatory factors, MyoD and myogenin was also observed. A population of mononucleated cell staining positive for PAX7 was also observed interspersed between the fused myotubes, a proportion of which showed minimal staining for the proliferative marker Ki67, indicative of a quiescent state. Arrows mark PAX7+ nuclei in order to better observe the localisation of these cells in relation to the differentiated myotubes, and other nuclear costains. Scale bars = 100µm.
5.3.2.2 **MERRF1 Myotubes harbouring Mid-Range Levels of m.8344A>G Show Modest Mitochondrial Impairments**

Whilst no substantial impairments in mitochondrial function were observed in MERRF1 Cl3 hiPSCs with 52% m.8344A>G heteroplasmy, the potential impact of this pathogenic variant in the more oxidatively demanding differentiated myogenic cultures was explored.

**MERRF1 Myotubes with 52% m.8344A>G Heteroplasmy Maintain mitochondrial OXPHOS Complex Translation and Assembly**

Assessment of assembled OXPHOS complexes from protein lysates extracted from the d42 terminally differentiated myogenic cultures characterised above revealed no substantial alterations in levels of any of the assembled OXPHOS complexes however, including OXPHOS CI and CIV for which impairments associated with m.8344A>G were expected (Figure 5-3). Whilst the WT Cl2 line was lost during a subsequent differentiation induction, levels of assembled OXPHOS complexes were comparable between d49 terminally differentiated myogenic cultures of the MERRF1 Cl2 (0% m.8344A>G) and MERRF1 Cl3 (52% m.8344A>G) lines (data not shown). These data therefore suggest mitochondrial protein translation is largely maintained in myogenic cultures harbouring 54% m.8344A>G heteroplasmy.
Figure 5-3 Representative immunoblot showing the protein levels of subunits contributing to all five OXPHOS complexes in terminally differentiated myogenic cultures after 7 days of serum withdrawal revealed no substantial impairments in the MERRF1 Cl3 line harbouring 52% m.8344A>G mutation load. Immunoblotting for TOM20 was used as an indirect measurement of mitochondrial mass, and β-actin was used as a protein loading control. The percentage in brackets indicated the mutation load of the respective mitochondrial disease hiPSC line (see text for details on the specific mtDNA mutations).
Potential Impact of 54% m.8344A>G Heteroplasmy on Myotube $\psi_m$

In order to gain insight into any downstream consequences the mtDNA mutations might have on the biochemical function of mitochondria within myotubes, measurements of $\psi_m$ were performed through live-cell imaging of TMRM.

5.3.2.2.1.1 Assessment of Basal $\psi_m$ and Mitochondria Morphology

Myotubes showed a dense and complex structured TMRM staining pattern in line with these cell types being more oxidatively demanding than the hiPSCs they were differentiated from (Figure 5-4A). Variability in the density of the mitochondrial network were observed between individual myotubes within the same culture, which appeared to be at least partly associated with the relative tension of the contractile myotubes in the culture dish. Whilst no substantial differences in the structure of the mitochondrial network were noted, WT Cl2 myotubes generally showed the most interconnected mitochondrial network, with that of MERRF1 Cl2 (0% m.8344A>G) showing slightly more fragmented or globular network, and MERRF1 Cl3 (52% m.8344A>G) showing an even more fragmented structure.

Measurements of mean mitochondrial basal TMRM fluorescence intensity from thresholded maximum intensity z-projections of confocal microscopy images revealed the MERRF1 Cl2 myotubes absent of m.8344A>G showed comparable basal $\psi_m$ to the WT Cl2 line (106 ± 11% of WT Cl2) (Figure 5-4B). In contrast, MERRF1 Cl3 with mid-range m.8344A>G heteroplasmy showed much lower basal $\psi_m$ (81 ± 1% of WT Cl2) (Figure 5-4B). Whilst the difference between MERRF1 Cl2 and MERRF1 Cl3 $\psi_m$ was not significantly difference when repeated measures from the same induction were pooled (N=3 separate inductions), treating each of the 3 repeated experiments from each induction as separate measures (i.e. n=9) showed this difference to be significant (p<0.05, two-tailed Mann-Whitney test). These data therefore suggest that MERRF1 Cl3 myotubes have impairments in $\psi_m$ maintenance associated with the 52% m.8344A>G heteroplasmy.
Figure 5-4 Assessment of $\psi_m$ through live-cell imaging of terminally differentiated myotube cultures stained with 25nM TMRM. (A) Representative maximum intensity projections of myotubes stained with 25nM TMRM. Images shown were all taken during one experimental imaging session and the same manipulations to brightness and contrast made to each image using ImageJ FIJI software. Scale bars = 50µm. (B) Basal mitochondrial TMRM intensities quantified from thresholded maximum intensity projections as a measure of basal $\psi_m$. Bars display the mean average mitochondrial TMRM fluorescence intensity of 3 different differentiation inductions with error bars ±StDev. 3 replicate measures of TMRM intensity for each induction were performed, with normalisation to the intensity of the WT Cl2 line imaged during the same experimental imaging session.
5.3.2.2.1.2 Sensitivity of Myotube $\psi_m$ to Mitochondrial Toxins

In order to better understand the relative contribution of different OXPHOS complexes to $\psi_m$ maintenance in the hiPSC derived myotubes, and gain further insight into the underlying OXPHOS impairment(s) responsible for the potential reduction in basal $\psi_m$ observed, the sensitivity of $\psi_m$ maintenance to treatment with mitochondrial toxins was performed through live cell imaging of TMRM fluorescence. As performed previously on hiPSC lines, oligomycin-A (CV inhibitor), rotenone (CI inhibitor) and FCCP (protonophore that dissipates mitochondrial $\psi_m$ entirely) were sequentially applied (Figure 5-5A). No substantial changes in $\psi_m$ were observed following addition of oligomycin-A in any of the myotube cultures highlighting no compensatory reversal of OXPHOS CV contributing to $\psi_m$ maintenance (Figure 5-5B). Following rotenone addition, a substantial and rapid decline in TMRM fluorescence was observed, highlighting a considerable contribution of OXPHOS CI activity and downstream flux of CI electrons through the ETC, to $\psi_m$ maintenance (Figure 5-5B). Following addition of FCCP, a further reduction in TMRM fluorescence was observed, associated with electron flux into the ETC through OXPHOS CII (Figure 5-5B). Unlike hiPSCs, the contribution of OXPHOS CI to $\psi_m$ maintenance in myotubes appears to be considerably more substantial. Triplicate measurements of relative $\psi_m$ following the addition of the mitochondrial toxins was performed for two of the myogenic differentiation inductions which revealed no substantial differences in the relative contribution of OXPHOS complexes to $\psi_m$ maintenance in these cell types (Figure 5-6). Unfortunately, the third induction was lost during rotenone addition because of poor attachment of these cultures on the glass coverslip, which contracted and detached, presumably as a consequence of mitochondrial Ca\(^{2+}\) release following rotenone induced depolarisation of the $\psi_m$. Nevertheless, these data highlights no compensatory changes in the relative contribution of OXPHOS complexes to $\psi_m$ in myotubes with/without m.8344A>G, thus the potential reductions in basal $\psi_m$ observed are associated with global dysfunction of the ETC.
Figure 5-5 Live imaging of hiPSC-derived myotubes incubated with 25nM TMRM during the sequential addition of mitochondrial toxins as indicated by the black bars: 10µg/ml oligomycin-A (Olig), 5µM rotenone (Rot) and 1µM FCCP. (A) Representative confocal images of a single myotube culture field of view showing TMRM fluorescence following the sequential addition of oligomycin-A, rotenone and FCCP. Scale bars = 50µm. (B) Representative traces showing the kinetic changes in mitochondrial TMRM fluorescence following the sequential addition of mitochondrial toxins. TMRM fluorescence is shown plotted relative to the basal fluorescence intensity prior to toxin addition (100%) and following addition of FCCP (0%).
Figure 5-6 Relative TMRM fluorescence intensity in the presence of 10µg/ml oligomycin A and 5µM rotenone. Mitochondrial TMRM fluorescence intensity was normalised to that of the same field of view imaged prior to mitochondrial toxin addition. Bars display the mean average of two different myogenic differentiation inductions with error bars ±StDev. Triplicate measurements were performed for each differentiation induction.

Whilst potential impairments in mitochondrial function were observed in myotubes differentiated from the MERRF1 Cl3 hiPSC line with mid-range levels of m.8344A>G heteroplasmy, no substantial impairments were observed in the myogenic differentiation efficiency of these cells. In parallel, myogenic differentiation inductions were being performed on MELAS1 hiPSCs, and differentiation impairments were observed in lines harbouring m.3243A>G (as will be described below). Taking into consideration the focus of this project was not only to investigate mitochondrial dysfunction in hiPSC-derived myotubes, but also to explore downstream impacts on cellular identity changes and metabolically sensitive epigenetic modifications, focus was turned towards the use of MELAS1 cells for the remainder of this project.
5.3.3 Differentiation and Characterisation of Myogenic Cells Harbouring m.3243A>G

Selected MELAS1 hiPSC lines harbouring various levels of m.3243A>G heteroplasmy were successfully differentiated into myogenic cells on multiple occasions. Whilst one or more of the lines were often lost early during the differentiation process (most commonly following ICLF stimulation), or did not differentiate efficiently into myogenic cell types with low levels of undesired cell contamination, a number of successful inductions were performed such that insight into the impact of m.3243A>G could be explored. Inductions were typically performed simultaneously on WT Cl2, MELAS1 Cl5 (0% m.3243A>G), MELAS1 Cl2 (53% m.3243A>G), MELAS1 Cl3 (80% m.3243A>G) and MELAS1 Cl4 (89% m.3243A>G). Unfortunately, differentiation of the WT Cl2 line performed in the inductions alongside the MELAS1 lines was repeatedly unsuccessful, with loss of the culture occurring early in the differentiation process. Nevertheless, the isogenic MELAS1 lines which include MELAS1 Cl5 lacking m.3243A>G acted as an excellent control. Mutation loads were routinely assessed from pelleted hiPSC cells used before differentiation induction, and on several occasions post terminal differentiation with deviations beyond that of the measurement variability not observed. Due to noticeable correlations in differentiation efficiencies between inductions (i.e. if a differentiation induction was comparably poor in one line, it was generally poor in all other lines subjected simultaneously), comparisons between cell lines within one induction were always performed. Wherever possible, attempts were made to measure any interesting phenotypic observations in additional differentiation inductions.

5.3.3.1 MELAS1 hiPSCs with Mid-Range and High m.3243A>G Heteroplasmy Levels Display Consistent Impairments in Myogenic Differentiation Efficiency

In order to determine whether m.3243A>G impacts myogenic differentiation and/or maturation, a detailed characterisation of differentiated cultures was performed through immunofluorescence (Figure 5-7) and RT-qPCR analysis (Figure 5-9). In order to quantitate variations in myogenicity in an unbiased manner, macros were developed using ImageJ FIJI in order to assess the proportions of nuclei within each culture showing colocalisation with the various myogenic markers stained for (Figure 5-8). Whilst MELAS1 hiPSC lines with a range of different m.3243A>G mutation loads were successfully differentiated into multinucleated myotubes on several occasions by both
myself and colleague Dr Monika Madej, it became clear that the myogenic differentiation efficiency was negatively impacted in MELAS1 hiPSCs harbouring m.3243A>G. Firstly, the MELAS1 Cl4 hiPSC necessitated an initial seeding density two times greater than that of the other cell lines (i.e. $2 \times 10^4$ cells/cm$^2$), due to poorer proliferation during early stages of the differentiation protocol, such that upon ICLF stimulation complete loss of the culture typically occurred. In addition to this a number of other notable impairments in myogenic cell identity transitions were noted, which will be discussed in more detail below.

Following on from observations of previous myogenic differentiation inductions it became clear that the myogenic cultures underwent a number of morphological changes upon serum withdrawal for subsequent terminal differentiation into multinucleated myotubes. For this reason, myogenic markers were assessed through immunofluorescence analysis at three different stages of terminal myogenic differentiation: those maintained in the proliferation promoting KIH media, cultures 3 days after serum withdrawal by which time increased myotube formation was evident, and 7 days after serum withdrawal by which time myotubes had continued to mature. In line with this, mRNA expression of the genes encoding desmin and titin increased following serum withdrawal (Figure 5-9), and the proportion of cells expressing desmin (myoblasts, myocytes and myotubes) which also expressed the myotube marker titin increased following serum withdrawal (Figure 5-8). Furthermore, assessments of the myogenic precursor/satellite cell marker PAX7, by both RT-qPCR (Figure 5-9) and immunofluorescence (Figure 5-8), showed substantial reductions upon serum withdrawal. A population of PAX7$^+$ cells was maintained alongside terminally differentiated myotubes in all MELAS1 cultures however, a proportion of which were negative for Ki67 staining and thus in a quiescent state (Figure 5-7).
Figure 5-7 Representative confocal images of terminally differentiated myogenic cultures from MELAS1 hiPSCs following 7 days of serum withdrawal. Numbers in brackets refer to m.3243A>G load of the specific MELAS1 line. Maximum intensity projections prepared from confocal z-stack images showing immunofluorescence staining for myogenic markers: desmin, titin, MyoD, myogenin and PAX7. Scale bars = 100um.
Figure 5-8 Immunofluorescence characterisation of terminally differentiating myogenic cultures from MELAS1 hiPSCs harbouring a range of different m.3243A>G mutation loads. 3 different time points have been characterised following seeding for terminal differentiation: cells maintained in proliferation promoting KIH media (Proliferation), cells after 3 days of serum withdrawal (d3 Fusion) and cells after 7 days of serum withdrawal (d7 Fusion). Quantification of % of nuclei colocalised with (MyoD, myogenin, PAX7) or found within cells positive for (desmin, titin) various myogenic markers. Bars represent the mean % of total nuclei colocalised with the particular myogenic mark from n≥6 1434x1071μm fields of view with error bars ±StDev. Statistical significance to MELAS1 C15 under the same culture conditions assessed by two-way ANOVA with Dunnett’s post-hoc correction for multiple comparisons indicated by *p<0.05.
Whilst cultures differentiated from MELAS1 hiPSC lines with a range of different m.3243A>G mutation loads (MELAS1 C15, 0%; MELAS1 C12, 53%; MELAS1 C13, 80%; MELAS1 C14, 89%) did show positive staining for key myogenic markers including desmin, titin, MyoD, myogenin, and PAX7, those differentiated from the MELAS1 C15 hiPSC line lacking m.3243A>G showed much greater proportions of cells expressing such markers, suggestive of overall better myogenicity.

Figure 5-9 RT-qPCR for the myogenic marker genes DES encoding desmin, TTN encoding titin, and PAX7 encoding PAX7. Bars show the mean mRNA expression level relative to that of the MELAS1 C15 line in proliferation media, with error bars ±StDev, n=3 technical replicates.
Differentiated MELAS1 Cl5 myogenic cultures showed a high proportion of titin+ myotubes, which increased across the culture dish upon serum withdrawal, and continued to increase in size and diameter over 7 days of terminal differentiation Figure 5-7. Adjacent myotubes appeared to self-arrange into parallel structures across the culture dish, not dissimilar from the arrangement of myofibres in vivo (Figure 5-10). By contrast, myogenic cultures differentiated from MELAS1 Cl2 harbouring mid-range 53% m.3243A>G heteroplasmy, MELAS1 Cl3 with higher 80% m.3243A>G and MELAS Cl4 with highest 89% m.3243A>G mutation load, showed much lower proportions of titin+ myotubes, which although increased during the first 3 days of serum withdrawal, did not mature into large calibre myotubes such as those seen in MELAS1 Cl5 cultures. Whilst myotubes in MELAS1 myogenic cultures harbouring m.3243A>G, did form parallel structures across the culture dish, myotube formation appeared generally more disorganised in comparison to that of MELAS1 Cl5 (Figure 5-10). In fact, the overall health of myogenic cultures from the MELAS1 lines harbouring both mid-range and higher m.3243A>G heteroplasmy appeared to decline during this terminal differentiation, with contaminating cells negative for myogenic markers tending to proliferate and become noticeable proportions of the overall cell cultures. In line with this, the proportion of cells expressing myogenic markers in MELAS1 Cl5 cultures was largely maintained across the terminal differentiation process, whereas that of the MELAS1 lines harbouring mid-range and higher m.3243A>G heteroplasmy declined substantially during extended serum withdrawal for 7 days.
Figure 5-10 Epifluorescence image of cultures after 7 days of serum withdrawal (d7 Fusion) taken at lower magnification to better highlight the overall myogenicity of each of the cell lines. Scale bars = 100um

5.3.3.2 M.3243A>G Prevents or Delays Myotube Maturation In Vitro

Whilst impairments in overall myogenicity associated with m.3243A>G were consistently observed, even MELAS1 Cl4 hiPSCs with high 89% m.3243A>G heteroplasmy could successfully form terminally differentiated myotubes. Despite this, the myotubes which formed appeared to poorly mature during extended culture. For this reason, it was decided to further investigate the developmental stage of the differentiated myotubes, by assessing the expression of MyHC forms which show distinct developmental and fibre type expression patterns.

Assessment of the mRNA expression for all skeletal muscle relevant MyHC genes was performed on the same myogenic cultures characterised above: which included MYH3 (Embryonic MyHC), MYH8 (Neonatal MyHC), MYH7 (Slow MyHC), MYH1 (Fast
MyHC-2X) and MYH2 (Fast MyHC-2A) (Figure 5-11). Whilst the expression of all the MyHC genes expressed appeared to increase over time following serum withdrawal, in line with terminal differentiation towards myotubes, differences in the relative increase in expression compared to that observed in cultures maintained in proliferation media were observed with: MYH2>MYH1>MYH7>MYH8>MYH3. As MYH2 and MYH1 are both associated with fast adult myosin heavy chain isoforms, and MYH7 with slow adult myosin heavy chains (but also expressed earlier during development), this might suggest the cultures are recapitulating latter stages of muscle development. Of note, the induction of the MyHC genes was poorer in MELAS1 myogenic cultures harbouring both mid-range and higher levels of m.3243A>G. Furthermore, whilst a decrease in expression of the neonatal MyHC encoding MYH8 gene was observed from 3d to 7d post serum withdrawal in the MELAS1 Cl5 myogenic cultures lacking m.3243A>G, the other three lines continued increase over this duration. This might therefore suggest that in addition to negatively impacting overall myogenicity, m.3243A>G might also be causing a delay/impairment in the subsequent maturation of terminally differentiated myotubes.

Terminally differentiated MELAS1 Cl5 (0% m.3243A>G) and MELAS1 Cl4 (89% m.3243A>G) myogenic cultures after 7 days of serum withdrawal, were stained with antibodies targeting specific MyHC isoforms including embryonic MyHC, neonatal MyHC, fast MyHC (note the particular fast isoform targeted is not known) and slow MyHC. Whilst no detectable specific staining of embryonic MyHC was observed in titin+ fibres from both cultures, neonatal MyHC was detected in all titin+ myotubes from both cultures (Figure 5-12). Interestingly, whilst strong positive staining for fast MyHC and modest for slow MyHC were observed in MELAS1 Cl5 terminally differentiated cultures, staining for both these MyHC isoforms in terminally differentiated MELAS1 Cl4 myogenic cultures revealed much lower levels, if at all detectable above background (Figure 5-12). Comparable staining of an additional end-point myogenic differentiation induction of the MELAS1 Cl5 and MELAS1 Cl4 performed by Dr Monika Madej, revealed this result to be consistent. These data therefore further suggest m.3243A>G negatively impacts the developmental maturation of hiPSC derived myotubes.
Figure 5-11 RT-qPCR assessment of MYH genes encoding MyHC isoforms differentially expressed during development and across different muscle fibre types. Bars show the mean mRNA expression level relative to that of the MELAS1 C15 line in proliferation media, with error bars ±StDev, n=3 technical replicates.
Immunofluorescence staining of terminally differentiated myogenic cultures after 7 days of serum withdrawal for the MELAS1 Cl5 line absent of m.3243A>G and MELAS1 Cl4 hiPSC with 89% m.3243A>G heteroplasmy. Costaining of titin alongside neonatal MyHC, fast MyHC and slow MyHC revealed that while all isoforms are present in myotubes from MELAS1 Cl5, only neonatal MyHC is expressed in MELAS1 Cl4. For each staining condition, imaging parameters, and manipulations to brightness and contrast of the displayed images were kept consistent. Scale bars = 100um. Percentages in brackets indicates m.3243A>G load of the specific MELAS1 line.
Bearing in mind the impairments in mitochondrial function observed in MELAS1 Cl4 hiPSCs with high m.3243A>G mutation load, it was predicted that myogenic cells differentiated from them would also show signs of mitochondrial dysfunction. As more oxidatively demanding cell types, mitochondrial impairments might be more severe, and additionally present at lower m.3243>G heteroplasmy levels in the differentiated myogenic cultures.

MELAS1 Myotubes with mid-range and high m.3243A>G heteroplasmy show global reductions in OXPHOS Complexes and Mitochondrial Content

Assessment of assembled OXPHOS complexes in protein lysates extracted from three different terminally differentiated myogenic cultures of MELAS1 Cl5, MELAS1 Cl2, MELAS1 Cl3 and MELAS1 Cl4 was performed. Whilst there was considerable variability in the relative levels of assembled OXPHOS complexes between lines from a single induction (see the blot with side by side comparison of two inductions in Figure 5-13A), a consistent reduction in the levels of all OXPHOS complexes was generally observed in MELAS1 myogenic cultures harbouring both mid-range and higher m.3243A>G heteroplasmy levels (Figure 5-13B). A reduction in the mitochondrial marker TOMM20 was also consistently observed, suggestive of a reduction in overall mitochondrial mass (Figure 5-13B). Normalisation of OXPHOS complex levels to TOMM20, revealed that the observed reduction in levels of assembled OXPHOS complexes appears to be largely associated with an overall reduction in mitochondrial content (Figure 5-13B). In line with the selective vulnerability of OXPHOS CI to m.3243A>G, a consistent reduction in the levels of OXPHOS CI was observed in the MELAS1 Cl4 with highest 89% m.3243A>G heteroplasmy, when normalised to TOMM20 rather than β-actin (Figure 5-13B).
Figure 5-13 Assessment of OXPHOS complexes in terminally differentiated myogenic cells harbouring m.3243A>G. (A) Representative immunoblot of OXPHOS protein subunits which are representative of overall steady-state levels of the five different OXPHOS complexes. Lysates from two different differentiation inductions have been shown alongside one another to highlight the variable levels of OXPHOS complexes. The percentage in brackets indicated m.3243A>G load of the respective mitochondrial MELAS1 line. (B) Quantification of assembled OXPHOS complexes from 3 different myogenic differentiation inductions. Band intensities were normalised to both β-actin and TOMM20 as loading controls for cells and mitochondria respectively. Bars represent the mean integrated immunoblot band intensity, normalised to that of β-actin or TOMM20, and expressed relative to that of the MELAS1 CI5 line differentiated alongside, with error bars = ±StDev (N=3). The protein levels of OXPHOS complex
In order to delineate the impact of m.3243A>G on the mitochondrial mass specifically within myotubes, immunofluorescence costaining of titin and TOMM20 was performed and quantitative image analysis conducted on maximum intensity projections of confocal z-stack images (Figure 5-14A). Whilst the proportion of titin+ myotube area which showed colocalisation with mitochondria (TOMM20+) was largely comparable between MELAS1 myotubes with varying m.3243A>G heteroplasmy, a trend towards modestly lower levels of mitochondrial content was observed in MELAS1 Cl4 myogenic cells with highest m.3243A>G heteroplasmy, with the difference to MELAS1 Cl5 after 7 days of serum withdrawal being statistically significant (p<0.05, two-way ANOVA with Dunnett's post-hoc correction for multiple comparisons) (Figure 5-14A).
Figure 5-14 Immunofluorescence analysis of mitochondrial content in MELAS1 titin+ myotubes. (A) Representative maximum intensity projections prepared from z-stack confocal images showing staining for mitochondria (TOMM20) and myotubes (titin) within MELAS1 myogenic cultures during terminal differentiation. Scale bars = 100µm. (B) Measurement of the mitochondrial TOMM20+ area within titin+ myotubes as a proportion of total myotube area. Bars show the mean TOMM20 area as a percentage of titin from measurements across n=6 randomly chosen 323x323µm fields of view, with error bars ±StDev. Statistical significance to MELAS1 C15 under the same culture conditions assessed by two-way ANOVA with Dunnett’s post-hoc correction for multiple comparisons indicated by *p<0.05.
MtDNA Content is Maintained in MELAS1 Myogenic Cultures

In order to provide additional insight into the impact of m.3243A>G on the mitochondrial content of terminally differentiated myogenic cultures, DNA including mtDNA extracted from two independent terminally differentiated MELAS1 myogenic cultures after 7 days of serum withdrawal was used to measure mtDNA copy number through RT-qPCR analysis (Figure 5-15). No substantial difference in mtDNA content was observed however, between terminally differentiated MELAS1 Cl2 myogenic cultures with 53% m.3243A>G or MELAS1 Cl4 myogenic cultures with 89% m.3243A>G heteroplasmy when compared with MELAS1 Cl5 myogenic cells lacking m.3243A>G which were differentiated alongside.

![mtDNA Content](image)

**Figure 5-15** Assessments of mtDNA content in terminally differentiating MELAS1 myogenic cultures. mtDNA content in terminally differentiated myogenic cultures, 7 days after serum withdrawal, quantified through measurement of the levels of MT-CYB, MT-ND1 and MT-ND4 mtDNA genomic regions relative to the geometric mean of the APP and B2M nDNA genomic regions. Bars represent the mean mtDNA copy number relative to that of MELAS1 Cl5 line with error bars ±StDev (N=2 independent terminally differentiated cultures).
MELAS1 Myotubes Show Normal Expression Levels and Phosphorylation Status of Proteins Involved in Mitochondrial Metabolism and Biogenesis

As reductions in mitochondrial mass were observed by protein assessment of myogenic cultures, and immunofluorescence characterisation of myotubes, harbouring m.3243A>G, yet mtDNA copy number was largely maintained, this might suggest a compensatory increase in mtDNA replication has been evoked. However, mRNA expression levels of the genes encoding PGC1α (PPARGC1A), the master regulator of mitochondrial biogenesis; and TFAM (TFAM), associated with mtDNA maintenance, replication and transcription, both showed slightly lower levels in MELAS1 myogenic cultures (Figure 5-16A), although TFAM protein levels did not appear to differ (Figure 5-16B). In line with terminal differentiation towards myotubes being associated with an increased reliance on mitochondria, increases in mRNA expression of both TFAM and PPARGC1A (Figure 5-16A) were observed following serum withdrawal.

Assessment of the AMPKα and its active phosphorylated isoform which is sensitive to rises in cellular AMP (i.e. falls in ATP:ADP/AMP and therefore energy status) revealed no consistent alterations between terminally differentiating MELAS1 myotubes with mid-range 53% (Cl2) or higher 89% (Cl4) m.3243A>G heteroplasmy, compared to terminally differentiating myotubes absent of m.3243A>G (Cl5) (Figure 5-16B). Whilst the blot shown in Figure 5-16B appears to show higher phosphorylation of AMPKα in terminally differentiated MELAS1 Cl4 myotubes after serum withdrawal, this result was not consistent, with comparisons between MELAS1 Cl5 and MELAS1 Cl4 from another differentiation induction revealing the opposite to be true (data not shown). It is also important to note, that the intense immunoblot bands present in samples of myogenic cells cultured in serum at the apparently correct MW for AMPKα and pi-AMPKα were also observed during probing for other protein epitopes, and thus this signal is likely not associated with correct detection of AMPKα and pi-AMPKα epitopes.

In line with increased oxidative demands of terminally differentiated myotubes, increases in the levels of PDHE1α protein, an essential catalytic subunit of the pyruvate dehydrogenase complex which catalyses the conversion of mitochondrial pyruvate into acetyl-CoA for TCA cycle metabolism; PDK4, the kinase which phosphorylates and activates the pyruvate dehydrogenase complex; CIC, the tricarboxylate transporter expressed on the IMM, and TFAM, were all observed following serum withdrawal (Figure 5-16B). No consistent differences in the levels of these proteins were observed...
between the different terminally differentiating MELAS1 myogenic cultures however (Figure 5-16B) (repeated blots of second terminal myogenic differentiation induction of MELAS1 Cl5 and MELAS1 Cl4, data not shown).

ACLY and its active phosphorylated isoform are important for the maintenance of nucleocytoplasmic acetyl-CoA, and are often associated with elevated glycolytic and lipid biosynthesis pathways. No substantial differences in the levels of ACLY or its phosphorylation status were observed between the lines (Figure 5-16B).
Figure 5-16 Assessments of proteins associated with mitochondrial metabolism and biogenesis, in terminally differentiating MELAS1 myogenic cultures. (A) RT-qPCR assessment of PPARGC1A and TFAM genes associated with mitochondrial biogenesis and mtDNA maintenance/replication. Bars show the mean mRNA expression level relative to that of the MELAS1 C15 line in proliferation media, with error bars ±StDev, n=3 technical replicates. (B) Immunoblot showing the levels of proteins associated with mitochondrial function and cellular metabolism. The percentage in brackets indicates m.3243A>G load of the respective MELAS1 myogenic culture. Pi-AMPKα immunoblot specific for Thr-172 phosphorylation and Pi-ACL immunoblot specific for Ser-455 phosphorylation.
MELAS1 Myotubes Harbouring m.3243A>G Show Impairments in $\psi_m$ Maintenance

In the light of impairments in mitochondrial content of terminally differentiation myotubes harbouring m.3243A>G observed above, further investigations measuring the $\psi_m$ of terminally differentiated myotubes were performed through live-cell imaging of TMRM.

5.3.3.3.1.1 Measurements of Basal $\psi_m$

As previously utilised for hiPSCs, I took advantage of the high-throughput Opera Phenix spinning-disc confocal imaging platform to acquire a high number of replicate images for analysis, in order to better account for variability in TMRM fluorescence staining across the cultures.

As observed previously with the terminally differentiated myogenic cultures from the WT Cl2 line, myotubes in MELAS1 derived cultures showed a dense and complex structured TMRM staining pattern, comparable with observations made from TOMM20 staining above. MELAS1 Cl5 myogenic cultures showed long mitochondrial lengths that persisted throughout significant lengths of an individual myotube (Figure 5-17). Whilst MELAS1 Cl2 and MELAS1 Cl3 myogenic cultures with 53% and 80% m.3243A>G heteroplasmy respectively, also showed long mitochondrial lengths within myotubes, the network appeared less complex, with an overall reduction in connectivity throughout individual myotubes observed (Figure 5-17). By comparison, MELAS1 Cl4 myotubes generally showed a much more fragmented and less densely packed mitochondrial network, in line with reduced mitochondrial content as quantified above (Figure 5-14).
Figure 5-17 Representative maximum intensity projections of MELAS1 myotubes stained with 25nM TMRM. Images shown for each time point were all taken during one experimental imaging session and the same manipulations to brightness and contrast made to each image using ImageJ FIJI software for presentation purposes. Note each time point of differentiation induction was imaged on a different experimental imaging session thus comparisons between cell lines at the same time point can be made, but not between different myogenic differentiation stages. Scale bars = 100µm.
Measurements of mean mitochondrial basal TMRM fluorescence intensity from two independent terminally differentiated myogenic differentiation inductions revealed that whilst MELAS1 Cl4 with highest 89% m.3243A>G heteroplasmy only showed modest, if any reduction in basal $\psi_m$ compared to MELAS1 Cl5 lacking m.3243A>G (Figure 5-18). By comparison, MELAS1 Cl2 and MELAS1 Cl3 lines with 53% and 80% m.3243A>G mutation load respectively, showed consistent and significant reductions in basal $\psi_m$ ($p<0.001$, two-way ANOVA with Dunnett's post-hoc correction for multiple comparisons) (Figure 5-17B and C). Whilst a more depolarised $\psi_m$ was observed in MELAS1 Cl2 and MELAS1 Cl3 compared to MELAS1 Cl5 under serum containing culture conditions, this difference increased upon serum withdrawal/lowering promoting myotube formation. It is important to note however, that the analysis performed here does not specifically measure myotubes. Characterisations of terminally differentiated MELAS1 cultures show declines in the overall proportion of cells contributing to myotubes, thus it is possible this difference observed is merely associated with the proportion of other non-myotube cell types measured. Even so, MELAS1 Cl4 myogenic cultures were morphologically similar to MELAS1 Cl2 and MELAS1 Cl3, thus suggesting mid-range 53-80% m.3243A>G heteroplasmy is indeed impacting $\psi_m$, whereas at higher 89% m.3243A>G heteroplasmy, $\psi_m$ is somehow maintained.
Figure 5-18 Basal mitochondrial TMRM intensities quantified from thresholded maximum intensity projections as a measure of basal ψm from two independent terminally differentiating MELAS1 myogenic cultures. Column scatter graphs show the variability in mean mitochondrial TMRM fluorescence pixel intensity from thresholded maximum intensity projections of multiple 323x323µm fields of view normalised to the mean of MELAS1 Cl5 (cross markers, >6 fields of view per well, 3-5 wells per cell line). Lines on the scatter graph show the mean TMRM fluorescence intensity of analysed images ±StDev. Numbers below the scatter show the total number of fields of view analysed for each hiPSC line. Bar charts show the mean average TMRM intensity measured across each of the experimental wells, normalised to that of the MELAS1 Cl5 myogenic line with error bars ±StDev. Numbers in the bars show the number of individual experimental wells analysed for each line. Statistical significance to the MELAS1 Cl5 line under the same culture conditions as assessed through two-way ANOVA with Dunnett’s post-hoc correction for multiple comparisons indicated by *p<0.01, **p<0.001.
5.3.3.3.1.2 Sensitivity of MELAS1 Myotube $\psi_m$ Maintenance to Mitochondrial Toxins

In order to better understand the relative contribution of different OXPHOS complexes to $\psi_m$ maintenance in the MELAS1 myotubes with differing m.3243A>G mutation load, and gain further insight into the underlying OXPHOS impairment(s) responsible for the reduction in basal $\psi_m$ observed in MELAS1 Cl2 (53% m.3243A>G) and MELAS1 Cl3 (80% m.3243A>G), the sensitivity of $\psi_m$ maintenance to treatment with mitochondrial toxins was measured through live cell imaging of TMRM fluorescence (Figure 5-19A), as conducted previously on MERRF1 myotubes. As MELAS1 Cl4 myotubes with highest 89% m.3243A>G mutation load showed apparently normal basal $\psi_m$, this would also permit insight into changes in OXPHOS complex function which might be contributing to compensatory maintenance of $\psi_m$ in these differentiated cultures.

No substantial changes in $\psi_m$ were observed following addition of oligomycin-A in any of the myotube cultures highlighting no compensatory reversal of OXPHOS CV contributing to $\psi_m$ maintenance (Figure 5-19B). Rotenone addition provoked a substantial and rapid decline in TMRM fluorescence (Figure 5-19B), similar to that observed in MERRF1 myotubes, indicative of substantial CI contribution to $\psi_m$ maintenance. Triplicate measurements of relative $\psi_m$ following the addition of the mitochondrial toxins was performed for two of the MELAS1 myogenic differentiation inductions which revealed no substantial differences in the relative contribution of OXPHOS complexes to $\psi_m$ maintenance in these cell types (Figure 5-19C).

Similar to MERRF1 myotubes harbouring mid-range levels of m.8344A>G, the more depolarised $\psi_m$ observed in MELAS1 myotubes harbouring 52-80% m.3243A>G heteroplasmy appears to be associated with global impairments to the ETC, rather than specific OXPHOS complexes. In addition, any compensatory changes associated with maintenance of the $\psi_m$ in MELAS1 Cl4 with high 89% m.3243A>G appear to be equally affecting the relative contribution of all OXPHOS complexes.
Figure 5-19 Live-cell imaging of terminally differentiated MELAS1 myotubes, incubated with 25nM TMRM during the sequential addition of mitochondrial toxins as indicated by the black bars: 10µg/ml oligomycin-A (Olig), 5µM rotenone (Rot) and 1µM FCCP. (A) Representative confocal images of a single MELAS1 Cl5 myotube culture field of view showing TMRM fluorescence following the sequential addition of oligomycin-A, rotenone and FCCP. Scale bars = 50µm. (B) Representative traces showing the kinetic changes in mitochondrial TMRM fluorescence following the sequential addition of mitochondrial toxins. TMRM fluorescence is shown plotted relative to the basal fluorescence intensity prior to toxin addition (100%) and following addition of FCCP (0%). (C) Relative TMRM fluorescence intensity in the presence of 10µg/ml oligomycin-A and 5µM rotenone. Mitochondrial TMRM fluorescence intensity was normalised to that of the same field of view imaged prior to mitochondrial toxin addition. Bars display the mean average of two different myogenic differentiation inductions with error bars ±StDev. n≥2 measurements contribute to the mean for each differentiation induction.
Mitochondrial Dysfunction in Terminally Differentiated Myogenic Cells is Not Associated with Alterations in Cellular Respiration

As terminally differentiated myogenic cultures harbouring mid-range and high levels of m.3243A>G showed evidence for reductions in mitochondrial mass, and MELAS1 C12 and MELAS1 C13 lines with 53% and 80% m.3243A>G heteroplasmy respectively, additionally showed impairments in basal $\psi_m$ maintenance, the impact of mitochondrial dysfunctions on cellular respiration was explored through measurements of OCR using the Seahorse Bioscience XF24 Extracellular Flux Analyser. Measurements of ECAR, which is an indicative measure of glycolysis flux associated with lactic acid release, were also conducted. By sequentially adding 1µM oligomycin-A to inhibit OXPHOS CV associated respiration, followed by 1µM FCCP to uncouple the $\psi_m$ and cause maximal mitochondrial associated respiration, and finally 1µM antimycin-A inhibitor of OXPHOS CIII and overall mitochondrial respiration, various measures of cellular OCR can be calculated (Figure 5-20). The difference in OCR before and after oligomycin-A addition provides a measure of cellular respiration contributing to ATP production (ATP-linked respiration). OCR after $\psi_m$ uncoupling with FCCP provides a measure of maximal cellular OCR. Measurements of OCR after inhibition of mitochondrial respiration with antimycin-A permits non-mitochondrial related OCR to be accounted for and therefore calculations of basal mitochondrial respiration and maximal mitochondrial respiration to be calculated.

Figure 5-20 Schematic showing change in cellular OCR after the sequential addition of 1.2µM Oligomycin-A (CV inhibitor), FCCP ($\psi_m$ uncoupler) and Antimycin-A. Also shown is the respective measures which can be calculated from the OCR after toxin addition. Figure adapted from Seahorses Biosciences, Agilent.
Analysis of terminally differentiating MELAS1 Cl5 (0% m.3243A>G), MELAS1 Cl2 (53% m.3243A>G), MELAS1 Cl3 (80% m.3243A>G) and MELAS1 Cl4 (89% m.3243A>G) lines cultured in proliferation promoting KIH media, and terminally differentiated myotubes in reduced serum (3% KSR) and no serum (IST media) media for 7 days were performed, with >4 replicate wells measured for each experimental condition. Whilst a relatively large well to well variability was observed, MELAS1 myogenic cultures harbouring m.3243A>G showed no substantial changes in basal OCR (Figure 5-21) or ECAR (Figure 5-22), suggestive of no considerable impact of m.3243A>G on oxidative vs glycolytic flux in these cells under basal conditions (Figure 5-23).

Following oligomycin addition, a rise in the ECAR was observed to maximal levels (Figure 5-22), presumably as a consequence of a switch in the flux of glucose derived carbons from mitochondrial metabolism to glycolysis. Whilst not significant, there was a trend towards higher maximal ECAR following oligomycin addition in MELAS1 Cl4 myogenic cells compared to MELAS1 Cl5, particularly those cultured under serum containing proliferation media (Figure 5-23). This might suggest MELAS1 Cl4 myogenic cells show a compensatory increase in their glycolytic capacity. In contrast to the ECAR, OCR falls after oligomycin addition due to reduced electron flow through the ETC necessitating oxygen. Following oligomycin addition, a fall in OCR was observed as a consequence of reduced mitochondrial respiration rate contributing to OXPHOS CV dependent ATP synthesis (Figure 5-21). Calculation of OCR contributing to ATP synthesis revealed no significant differences between the differentiating myogenic cultures with/without m.3243A>G, under any of the culture condition investigated (Figure 5-23).

Following FCCP addition, an increase in OCR to maximal levels was observed (Figure 5-21), due to uncoupling of the $\psi_m$ such that resistance to electron flow through the ETC was dissipated. Calculation of the spare respiratory capacity (i.e. the difference between maximal and basal OCR) revealed a trend towards reduced spare respiratory capacity as a measure of OCR in MELAS1 Cl4, particularly in myotubes under fusion promoting low/no serum conditions (Figure 5-23). Calculation of the spare respiratory capacity as a percentage of the basal respiration rate revealed this reduction to be significantly different to MELAS1 Cl5 in myotubes cultured in, low 3% serum conditions (p<0.05, two-way
ANOVA with Dunnett’s post-hoc correction for multiple comparisons) (Figure 5-23). These data therefore suggests that MELAS1 Cl4 myotubes with high m.3243A>G mutation load might show impairments in overall mitochondrial function, when under more energy demanding conditions.

In line with impairments in mitochondrial respiration capacity and compensatory elevation in glycolytic capacity, the maximal OCR:maximal ECAR ratio, trended towards lower levels in MELAS1 myogenic cultures harbouring m.3243A>G, particularly in myotube cultures under fusion promoting low/no serum conditions (Figure 5-23). The maximal OCR:ECAR ratios of MELAS1 Cl2 and MELAS1 Cl4 myotubes cultured in 3% serum containing media, was significantly lower compared to MELAS1 Cl5 myogenic cells under the same conditions (p<0.05, two-way ANOVA with Dunnett's post-hoc correction for multiple comparisons) (Figure 5-23).

Together these data suggest that at high and potentially mid-range mutation loads m.3243A>G might be causing impairments in myotube respiration, and initiating a compensatory increase in the glycolytic capacity of myogenic cells in order to maintain cellular ATP requirements.
Figure 5.21 Assessment of cellular respiration in terminally differentiating MELAS1 myotubes cultured under proliferation promoting and myotube fusion (low 3% serum and no serum) conditions through extracellular flux analysis. Traces showing change in OCR following the sequential addition of 1.2µM oligomycin-A (O) to inhibit OXPHOS CV-dependant ATP synthesis, 1 µM FCCP to uncouple the ψm and permit maximal cellular respiration, and 1 µM antimycin-A (A) to inhibit CIII and mitochondrial respiration. Markers represent the mean OCR normalised to protein content of n≥4 replicate wells at each time point with error bars ±StDev.
Figure 5-22 Assessment of glycolytic flux in terminally differentiating MELAS1 myotubes cultured under proliferation promoting and myotube fusion (low 3% serum and no serum) conditions through extracellular flux analysis. Traces showing the change in ECAR following the sequential addition of 1.2µM oligomycin-A (O), 1 µM FCCP and 1 µM antimycin-A (A). Markers represent the mean ECAR normalised to protein content of n≥4 replicate wells at each time point with error bars ±StDev.
Figure 5-23 Measurements of OCR and ECAR parameters under basal and maximal stimulating conditions. Bars in C-E show the mean average from n≥4 replicate wells, with error bars ±StDev. Statistical significance to the MELAS1 C15 line under the same culture conditions as assessed through two-way ANOVA with Dunnett's post-hoc correction for multiple comparisons indicated by *p<0.01, **p<0.001.
Mitochondrial Ultrastructure is Largely Maintained in Myotubes Harbouring m.3243A>G

In order to understand the potential impact of m.3243A>G associated mitochondrial dysfunction, on the ultrastructure of mitochondria within terminally differentiated myotubes, TEM imaging was performed. This also permitted insight into subcellular ultrastructures including sarcomere units. TEM imaging of terminally differentiated MELAS1 myotubes after 7 days of serum withdrawal was performed for the MELAS1 Cl5 (0% m.3243A>G), MELAS1 Cl2 (53% m.3243A>G) and MELAS1 Cl4 (m.3243A>G) lines. Mitochondrial structures were easily distinguishable within myotubes of all cells by their electron dense double membrane and cristae invaginations (see “M” in Figure 5-24A).

A large range of different mitochondrial structures were observed in myotubes from all three lines, from globular almost circular mitochondria, to very long elongated mitochondria which extended parallel through the myotube (Figure 5-24). Whilst mitochondria of all morphologies were observed in myotubes from all three MELAS1 lines with differing m.3243A>G heteroplasmy, mitochondria tended to show a more globular and less elongated structure in MELAS1 Cl2 and MELAS1 Cl4 myotubes with 53% and 89% m.3243A>G heteroplasmy respectively.

In order to provide some quantitative measures of mitochondrial ultrastructure, mitochondria were manually segmented and calculations of mitochondrial: area, perimeter, length (maximum Feret length), diameter (minimum Feret length) (Figure 5-24B). Measures of circularity ($= \frac{4\times\text{Area}}{\text{Perimeter}^2}$), and roundness ($= \frac{4\times\text{Area}}{\pi \times \text{Length}^2}$), were also performed in order to describe ultrastructure morphology, with values closer to one being indicative of more globular mitochondria, whilst numbers closer to 0 being indicative of more elongated mitochondria (Figure 5-24B). Whilst great variability within myotubes from the same MELAS1 line were observed, no consistent differences in mitochondrial perimeter, area or length were observed. MELAS1 Cl2 and MELAS1 Cl4 harbouring 53% and 89% m.3243A>G mutation load, showed a tendency towards higher mitochondrial diameters compared to MELAS1 Cl5 without m.3243A>G, however this result was not significant (two-way ANOVA, with Dunnett’s post-hoc correction for multiple comparisons) (Figure 5-24B). A large variability in measures of mitochondrial circularity and roundness were observed within individual MELAS1 myotube lines, underscoring the range of mitochondrial morphologies in these cell types (Figure 5-24B).
In line with MELAS1 lines harbouring m.3243A>G showing a tendency towards more globular rather than elongated mitochondria, measures of roundness and circularity tended closer to 1 in MELAS1 Cl2 and MELAS1 Cl4 myotubes, compared to MELAS1 Cl5, although this result was again not significant (two-way ANOVA, with Dunnett’s post-hoc correction for multiple comparisons) (Figure 5-24B).

In relation to myotube associated cellular ultrastructure, MELAS1 myotubes showed parallel bundles of fibres indicative of sarcomeric units, which showed intermittent very electron dense regions, likely corresponding to actin rich regions associated with z-lines (see arrows in Figure 5-24A). Whilst such sarcomeric structures were observed in myotubes of all three MELAS1 lines, they were much more common in the MELAS1 Cl5 line absent of m.3243A>G, in line with this line showing better myotube maturation.

In relation to other notable ultrastructural observations, MELAS1 Cl4 myotubes showed the presence of organelle structures with less electron dense matrix than that of mitochondria, which were rarely observed in MELAS1 Cl2 or MELAS1 Cl5 myotubes (see “*” in Figure 5-24A). These structures were often closely juxtaposed with elongated MELAS1 Cl4 mitochondria, and could potentially represent endoplasmic reticulum (ER) structures with expanded lumens. Expansions of ER have been previously associated with ER stress (Akiyama et al., 2009), and thus could potentially suggest ER stress pathways are being initiated in MELAS1 Cl4 myotubes, particularly those closely juxtaposed with mitochondria.

These data therefore suggest that whilst mitochondria within myotubes harbouring mid-range and higher m.3243A>G heteroplasmy maintain an apparently normal cristae structure, the mitochondrial network is slightly more fragmented and globular in myotubes harbouring m.3243A>G. In relation to mitochondrial dysfunctions at high m.3243A>G heteroplasy, expanded ER structures were observed in MELAS1 Cl4 myotubes which is potentially associated with ER stress responses being evoked in these cell types.
Figure 5-24 Transmission electron microscope (TEM) analysis of cellular and mitochondria ultrastructure in terminally differentiated MELAS1 myotubes after 7 days of serum withdrawal. (A) Representative TEM micrographs showing the presence of both globular and more elongated mitochondria within terminally differentiated myotubes from MELAS1 lines with differing m.3243A>G heteroplasmy. The M in each micrograph highlights a mitochondrial structure. The arrows point towards bundles of electron dense fibres contributing to sarcomeric structures. Structures corresponding to expanded endoplasmic reticulum organelles in MELAS1 Cl4 myotubes indicated by *. (B) Quantitative measures of mitochondrial ultrastructure imaged through TEM. Each marker in the scatter plot shows the measurement from an individual mitochondrion (Cl5 = 95, Cl2 = 58, Cl4 = 86), with lines showing the mean±StDev.
5.4 Discussion

The main aim of this chapter was to successfully differentiate hiPSC lines, including those with suprathreshold heteroplasmy levels of disease associated mtDNA mutations, into myogenic cell types and terminally differentiated multinucleated myotubes. This was not only in order to permit exploration of mitochondrial and cellular phenotypes associated with the mtDNA disease mutations in a more disease-relevant setting, but also to explore the potential impact of the mtDNA mutations and associated mitochondrial dysfunction on myogenic cell identity changes occurring during differentiation and subsequent culture maturation.

5.4.1 Oxidatively Demanding Myotubes are More Sensitive to Mid-Range m.8344A>G Mutation loads than hiPSCs

Whilst hiPSCs harbouring mid-range 52% levels of m.8344A>G showed no evidence for mitochondrial or downstream cellular dysfunctions (see results presented in Chapter 4), terminally differentiated myotubes derived from these hiPSCs presented with mitochondrial associated impairments. This is in line with terminally differentiated myotubes being much more oxidatively demanding cell types, more reliant on mitochondrial function, with an associated lower m.8344A>G threshold for exerting detectable biochemical deficits. Although translation and assembly of OXPHOS complexes, including those encoded by the mitochondrial genome were largely maintained, myotubes harbouring 52% m.8344A>G consistently showed a more depolarised $\psi_m$. This basal $\psi_m$ depolarisation conforms with previous observations that have been made in primary patient fibroblasts (Kovac et al., 2019) and cybrids (Chang et al., 2013b) harbouring m.8344A>G. Unlike previous observations made in fibroblasts however, there did not appear to be any compensatory contribution of OXPHOS CV working in reverse to $\psi_m$ maintenance (oligomycin sensitivity), or any differences in the relative contribution of OXPHOS CI (rotenone sensitivity) (Kovac et al., 2019). Whilst cell-type specific differences might account for this discrepancy, the m.8344A>G heteroplasmy level of the fibroblasts and cybrids used in these studies were both higher (82% and 85% m.8344A>G respectively) (Kovac et al., 2019; Chang et al., 2013b) than that of the differentiated myotubes investigated here (52% m.8344A>G). Furthermore, fibroblasts with lower 46% m.8344A>G heteroplasmy showed comparable measures of mitochondrial function (including $\psi_m$ polarisation) to that of fibroblasts from control individuals (Kovac et al., 2019). These data therefore suggest that while mid-range
m.8344A>G mutation loads do impact mitochondrial function, particularly in highly oxidative cell types such as myotubes, only at higher heteroplasmy levels are more severe detriments and associated compensatory changes in the contributions of OXPHOS complexes to $\psi_m$ maintenance observed.

Whilst there have been previous reports showing comparable mitochondrial deficits in hiPSCs with mtDNA disease mutations and more oxidatively demanding progeny differentiated from them (Yang et al., 2018; Chou et al., 2016), differences in mtDNA mutation load threshold between hiPSCs and differentiated cell types have also been described (Russell et al., 2018; Perales-Clemente et al., 2016). For example, hiPSCs with 63% but not 53% heteroplasmy levels of a large scale mtDNA deletion (m.Δ7777:13794) show more depolarised $\psi_m$, but in post-mitotic neurons differentiated from them, reductions in $\psi_m$ are observed at lower 50% m.Δ7777:13794 mutation loads (Russell et al., 2018). Furthermore, hiPSCs with 80% m.3243A>G mutation load show no impairments in basal or maximal OCR, yet following differentiation into cardiomyocytes, reductions in both basal and maximal OCR are observed (Perales-Clemente et al., 2016).

In line with undetectable impairments in the levels of assembled OXPHOS complexes in myotubes with 52% m.8344A>G heteroplasmy, individual muscle fibres from MERRF patients harbouring m.8344A>G are rarely COX- (OXPHOS CIV) at heteroplasmy levels lower than 95% (Moslemi et al., 1998). Deficits in mitochondrial translation have been described in primary clonal myoblasts with m.8344A>G mutation loads as low as 35% (Hanna et al., 1995), however differentiation of primary myoblasts into myotubes revealed myotubes might in fact show a >80% m.8344A>G threshold for such impairments (Boulet, Karpati & Shoubridge, 1992). Nevertheless, the observed impairment in $\psi_m$ maintenance observed in myotubes with 52% m.8344A>G heteroplasmy shows, that while the levels of assembled OXPHOS complexes are apparently unaffected, the functional activity of the ETC is negatively impacted at these mid-range m.8344A>G heteroplasmy levels.

Similar to observations in hiPSC-derived cardiomyocytes harbouring 40% m.8344A>G heteroplasmy, myotubes differentiated here with 52% m.8344A>G mutation load showed a slightly more fragmented and globular network in comparison to both isogenic myotubes absent of m.8344A>G and control myotubes differentiated in parallel (Chou et al., 2016). As discussed in detail in Introduction section 1.1.7.4, mitochondrial fission, fusion and targeted degradation are all dependant on both $\psi_m$ and overall ATP energy production.
status, with mitochondrial fusion being promoted by ATP and more polarised $\psi_m$, whereas mitochondrial fission is promoted by ADP and indirectly enhanced by $\psi_m$ depolarisation. The more fragmented mitochondrial network observed in myotubes harbouring 52% levels of m.8344A>G is therefore further suggestive of deficits in the biochemical function of mitochondria in these cell types.

Together these data show that as predicted, more energy demanding, and oxidative metabolising myogenic cell types have a lower m.8344A>G threshold for exerting a detrimental impact on mitochondrial biochemistry, in line with the particular vulnerability of muscle tissue in mtDNA disease patients. This highlights hiPSC derived myogenic cells as a useful phenotypical in vitro system for mitochondrial disease modelling purposes.

5.4.2 M.3243A>G Negatively Impacts Myogenicity and Developmental Maturity of Differentiating Myogenic Cultures

Unlike hiPSCs harbouring mid-range 52% m.8344A>G mutation loads, hiPSCs harbouring mid-range (53%) and higher (80-89%) m.3243A>G heteroplasmy levels showed consistent impairments in both overall myogenic differentiation efficiency, but also the subsequent maturation of myotubes. A number of recent reports systematically assessing various hiPSC traits including gene expression levels, DNA methylation, cell morphologies and differentiation efficiencies, have provided great insight into the underlying origins of such hiPSC variabilities. Whilst factors including origin cell-type reprogrammed (Burrows et al., 2016; Kyttälä et al., 2016; Rouhani et al., 2014) and culture conditions (Kilpinen et al., 2017; Schwartzentruber et al., 2018) do appear to influence hiPSC and differentiation phenotypes, the large majority of hiPSC variability arises from inter-donor specific genetic differences (Burrows et al., 2016; Kyttälä et al., 2016; Rouhani et al., 2014; Kilpinen et al., 2017). By taking advantage of the natural heterogeneity of heteroplasmic mtDNA mutations in primary patient cells to derive hiPSC lines on the same nuclear background with varying m.3243A>G heteroplasmy (including those absent of m.3243A>G), and by making cautious comparisons between cells differentiated in parallel, much of this variability has been accounted for in this study. Thus, the impaired myogenicity and subsequent myogenic maturation observed in this study can be confidently correlated with m.3243A>G and associated mitochondrial impairments.
5.4.2.1 Impairments in Oxidative Mitochondrial Metabolism May Underly Impaired Myogenic Differentiation Efficiencies

As explored in detail in introduction section 1.5, metabolic flux changes are crucial for driving both the early differentiation of hiPSCs, and downstream myogenic cell-identity changes. Whilst hiPSC differentiation towards early ectoderm cell types is associated with a necessary maintenance of high glycolytic activity, differentiation towards endoderm and particularly mesoderm lineages is associated with a necessary shift towards more oxidative metabolic fluxes (Cliff et al., 2017; Lu et al., 2019). Taking this into account, it is relatively unsurprising that mitochondrial detriments associated with m.3243A>G will negatively impact the targeted differentiation of hiPSCs to skeletal myogenic cells of the mesoderm lineage.

Whilst a number of studies have reported the successful differentiation of many mtDNA disease hiPSCs into oxidatively demanding cell types (including the data described in this chapter for the myogenic differentiation of hiPSCs harbouring 52% m.8344A>G), impairments in differentiation efficiencies have been described (see introduction section 1.4.6.6).

Whilst downstream cardiomyocyte differentiation/development differs from that of skeletal myocytes (lateral plate rather than paraxial mesoderm), both cell types originate from meso-endoderm cells which first pass through an epithelial to mesenchymal transition (Tirosh-Finkel et al., 2006). Indeed, the majority of efficient cardiomyocyte differentiation protocols first promote mesodermal acquisition through Wnt signalling activation by GSK3β inhibition, comparable to that performed here and elsewhere for skeletal myogenic differentiation (Machiraju & Greenway, 2019; Burridge et al., 2014). In line with the importance of oxidative metabolic fluxes for mesoderm cell identity acquisition, impairments in cardiomyocyte differentiation efficiencies have been repeatedly reported for mtDNA disease hiPSCs (Yokota et al., 2017; Ma et al., 2015a; Galera-Monge et al., 2019), and selection against m.3243A>G during differentiation, resulting in falls in mutation loads of differentiated cardiomyocyte progeny have also been described (Yokota et al., 2017). In agreement with differentiation towards mesoderm lineages being particularly reliant on acquisition of increased oxidative metabolic fluxes, impairments associated with differentiation towards neural stem cells (NSCs) and neural crest cells (NCCs) are more rarely described, and in fact the very same hiPSC lines harbouring >90% m.3243A>G which poorly differentiate into...
cardiomyocytes, can be efficiently differentiated into NSCs (Yokota et al., 2017). Whilst high glycolytic flux is maintained in hiPSC derived NSCs and NCCs, the subsequent terminal differentiation of these cells into mature neurons is associated with a switch towards more oxidative metabolic fluxes (Zheng et al., 2016; Cliff et al., 2017). Accordingly, hiPSC lines with >90% m.3243A>G or homoplasmic m.5541C>T which can be efficiently differentiated into NSCs or NCCs, show substantial impairments in the subsequent terminal differentiation towards more oxidatively demanding mature neurons (Yokota et al., 2017; Hatakeyama et al., 2015).

Many of these previous reports associated the reduced cardiomyocyte and mature neuron differentiation efficiencies with overall reduction in cell viabilities occurring upon these driven cell-identity transitions. Although cell viability impairments were not investigated in any great detail here, no substantial differences were noted between hiPSC lines with or without m.3243A>G, during early stages of the differentiation process, although higher initial seeding densities of the MELAS1 Cl4 line with highest 89% m.3243A>G were typically required for successful differentiation. Interestingly, a recent publication has shown that high levels of the m.13513G>A (p.Asp393Asn) missense pathogenic variant affecting MT-ND5 of OXPHOS CI, not only impairs cardiomyocyte differentiation efficiency through reductions in cell viabilities early during the differentiation process, but also impairs the epithelial-mesenchymal transition underlying initial patterning towards the mesodermal lineage (Galera-Monge et al., 2019). Instead, hiPSC lines with mtDNA mutations favoured differentiation towards the neuroectoderm lineage, both during spontaneous differentiation promoting conditions, and those used to drive cardiomyocyte identity acquirement (Galera-Monge et al., 2019). Neural-like cells were a common contaminant cell type in the myogenic differentiations performed here, but purposefully selected against during passing procedures. Whilst no quantitative measures were performed, such neural-like contaminants were particularly prevalent in pre-passaged myogenic differentiation inductions from the MELAS1 Cl2 and MELAS1 CL3 lines with 53% and 80% m.3243A>G heteroplasmy respectively, but comparably less in MELAS1 Cl5 without m.3243A>G and MELAS1 Cl4 with highest 89% m.3243A>G heteroplasmy. Whilst no thorough exploration of these early stages of mesodermal acquisition were performed, it should be noted that homogenous expression of brachyury, a key marker of early mesoderm, was observed across all differentiating MELAS1 hiPSCs lines after 3 days of ICL stimulation, through immunofluorescence analysis, and comparable induction of CDX1, another important regulator of paraxial mesoderm.
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patterning, was observed in MELAS1 Cl5 and MELAS1 Cl4 lines stimulated with CHIR99021 for 2 days by western blot analysis (data not shown). Similar immunofluorescence assessment of MELAS2 Cl3, harbouring homoplasmic m.13528A>G (p.T398A) and m.13565C>T (p.S410F) affecting MT-ND5, which failed to differentiate into myogenic cell types on several occasions, also showed positive activation of brachyury early during differentiation induction (data not shown). Thus, it would appear that the myogenicity impairments observed here are associated with latter cell-identity changes than the epithelial-mesenchymal transition. Nevertheless, these previously reported data, collectively with the observations made here in this project, suggest that mtDNA disease mutations have the potential to impact crucial cell-identity transitions during differentiation towards oxidatively demanding cell types.

5.4.2.2 m.3243A>G Related Impairments in Myotube Maturation Might be Associated with Alterations in Mitochondrial Ca\(^{2+}\) Buffering and/or Changes in Histone Acetylation

In addition to impairments in the overall differentiation toward cells with myogenic identity, the subsequent maturation of titin\(^+\) myotubes which successfully passed through all the necessary early myogenic cell-identity transitions was also impaired. Whilst MELAS1 myotubes lacking m.3243A>G successfully transited into a latter developmental stage associated with the expression of adult slow and particularly fast MyHC isoforms, no such initiation of adult MyHC expression was observed in MELAS1 Cl4 myotubes harbouring 89% m.3243A>G. Unlike overall impairments in myogenic differentiation efficiency which are confounded by not only a reduced propensity to differentiate to the desired cell type, but also potentially elevated propensities for differentiating into cells of different lineages and subtypes, this maturation phenotype can be more directly associated with the underlying m.3243A>G mutation.

Muscle-fibre type switching and associated expression of these well-defined MyHC isoforms is controlled by a wide-range of different mechanisms, with thyroid hormone stimulation being particularly important for initiating the switching from neonatal to fast muscle fibre types, and slow motor neuron innervation and activity being important for initiating the switch towards slow muscle fibre types (Schiaffino et al., 2015). As the \textit{in vitro} myogenic model established here lacks motor neurons and thyroid hormone releasing cells, the detriments in myotube maturation appear to be somehow related with the downstream, cell-intrinsic molecular mechanisms responsible for initiating these
transitions. Molecular mechanisms governing fibre identity transition are often associated with Ca\(^{2+}\) dependant signalling pathways and include, calcineurin dependant activation of nuclear factor of activated T-cells (NFAT) and MEF2 transcription factors (Schiaffino & Serrano, 2002), and Ca\(^{2+}\)/calmodulin-dependent protein kinase II activation of MEF2 through enhancement of nuclear export of class II HDACs (Lu et al., 2000). This also brings to attention an interesting association between histone acetylation and regulation of fibre type identity, which recent evidence suggests can be sensitive to changes in nucleocytoplasmic availability of acetyl-CoA for histone acetylation deposition (Das et al., 2017). Taking into account the importance of mitochondria for buffering intracellular Ca\(^{2+}\) and maintaining levels of metabolic derived enzyme co-substrates for histone acetylation and deacetylation reactions, it is possible that the mitochondrial dysfunction associated with m.3243A>G results in alterations in the activity of these crucial signalling pathways for myotube maturation.

In line with the overall importance of mitochondria for myotube maturation, overexpression of PGC1\(\alpha\) and associated increase in mitochondrial mass/function, accelerates myotube maturation in vitro including the downregulation of embryonic/neonatal MyHC isoforms, and upregulation of slow MyHC (Mortensen et al., 2006). Accordingly, MELAS1 myotubes harbouring m.3243A>G not only showed lower induction of adult MyHC genes and protein, but also did not appear to downregulate the expression developmental MyHC isoforms during the 7 days of terminal differentiation, whereas falls in the expression of genes encoding the embryonic (MYH3) and neonatal (MYH8) MyHC isoforms were observed in the MELAS1 Cl5 line lacking m.3243A>G from 3 to 7 days post serum withdrawal.

Together, these data highlight the importance of mitochondria for both the differentiation and subsequent maturation of myotubes and show the detrimental impact of m.3243A>G on these important cell-identity transitions. Considering the importance of these cell-identity changes for muscle repair and growth, it is possible that the myopathic phenotypes often observed in mitochondrial disease patients are not only associated with detriments in overall muscle function and health, but also impaired muscle repair and/or growth capacities.
5.4.2.3 MELAS1 Myotubes with Different m.3243A>G Mutation loads Show Distinct Mitochondrial Deficits

In order to more confidently correlate impairments in myogenic differentiation and subsequent maturation of myotubes with m.3243A>G, it was important to explore the impact of this pathogenic variant on mitochondrial function. Taking into account the comparable differentiation impairments observed in hiPSC-derived myogenic cultures with both mid-range and higher m.3243A>G mutation loads, similar or mutation load dependant deficits in mitochondrial function might also be expected. Similar to hiPSCs, differences in mitochondrial impairments were noted between MELAS1 myogenic cultures with mid-range and higher levels of m.3243A>G however, more in line with threshold dependant initiation of distinct compensatory mechanisms.

Compensatory Pathways Initiated to Maintain $\psi_m$ in MELAS1 Myotubes with High but not Mid-Range m.3243A>G Heteroplasmy

Unlike the more glycolytic favouring hiPSCs they were derived from, terminally differentiating MELAS1 myotubes with greater oxidative metabolic fluxes showed a consistent reduction in basal $\psi_m$ at 53% m.3243A>G heteroplasmy (MELAS1 Cl2), with this reduction in $\psi_m$ being even more severe at 80% m.3243A>G mutation loads (MELAS1 Cl3). In MELAS1 Cl4 myotubes harbouring higher 89% m.3243A>G heteroplasmy, this reduction was however much less severe, in some instances undetectable, which might indicate a threshold-dependant activation of compensatory signalling or biochemical pathways which act to maintain the $\psi_m$. No differences in the contribution of OXPHOS CI (rotenone sensitive) or compensatory reversal of OXPHOS CV (oligomycin sensitive) were observed, suggestive of either a global reduction in pathways utilising the $\psi_m$ and/or overall increase in ETC activity. As OCR and ECAR measurements showed comparable trends toward decreased oxidative activity and increased glycolytic activity in MELAS1 myogenic cultures harbouring both mid-range and higher m.3243A>G mutation loads, it appears more likely that the maintenance of $\psi_m$ in the MELAS1 Cl4 myotubes with high 89% m.3243A>G is associated with reductions in $\psi_m$ dissipators rather than increase in oxidative ETC activity.

Do Alterations in Mitophagy Activity and/or Mitochondrial Biogenesis Pathways Underlie the Impairments Observed in MELAS1 Myotubes with m.3243A>G?

Whilst western blot analysis showed reductions in the mitochondrial content of MELAS1 myotubes with 53% and 80% m.3243A>G mutation loads, only at highest 89%
m.3243A>G mutation loads was a specific reduction in OXPHOS CI, typical for this pathogenic variant observed. In addition, only MELAS1 Cl4 myotubes with highest m.3243A>G showed apparent reductions in the mitochondrial content of titin+ myotubes as measured through immunofluorescence analysis. Taking into account the particularly notable reductions in mitochondrial mass that were observed in MELAS1 Cl4 myotubes, it is possible that whilst mid-range m.3243A>G heteroplasmy only cause slight $\psi_m$ depolarisation and reductions in oxidative metabolic pathways, at higher 89% m.3243A>G levels associated with more severe reductions in OXPHOS CI, the $\psi_m$ becomes sufficiently depolarised to strongly initiate mitophagy clearance pathways. This could at least partially explain the more modest basal $\psi_m$ reduction in MELAS1 Cl4 myotubes, as mitochondria with severely reduced $\psi_m$ are potentially removed through selective mitophagy mechanisms. This theory would necessitate individual mitochondrial units having different basal $\psi_m$ spanning across the threshold for mitophagy initiation. Whilst inter-mitochondrial differences in m.3243A>G heteroplasmy could potentially account for this, reductions in m.3243A>G mutation load would be expected if mitochondrial clearance was preferentially targeting mitochondrial units with high proportions of m.3243A>G, which was never observed. Variation in $\psi_m$ across mitochondrial units was also observed in myogenic cultures lacking mtDNA mutations, thus it can be speculated that only in MELAS1 Cl4 myogenic cells with highest m.3243A>G mutation load, do the mitochondria with most depolarised $\psi_m$ drop below the threshold for mitophagy activation and clearance. Indeed, mitophagy flux is typically low for cells under in vitro culture conditions, and generally requires stressors promoting oxidative metabolism for mitophagy activation to be observed, such as culturing in galactose-containing glucose-free media (MacVicar & Lane, 2014; Diot et al., 2015). Evidence for increased mitophagy activity has been previously shown under basal culture conditions, in glycolytic favouring cell types harbouring mtDNA mutations however, including hiPSCs harbouring 85% m.3243A>G (Lin et al., 2019a) and fibroblasts harbouring 57% m.8344A>G (De la Mata et al., 2012).

HiPSC-derived neurons harbouring 80% m.3243A>G have been shown to have a specific reduction in levels of OXPHOS CI, comparable to that observed here in MELAS1 Cl4 myogenic cultures with highest 89% m.3243A>G (Hämäläinen et al., 2013). Interestingly, this study by Hämäläinen et al., also showed that whilst OXPHOS CII and OXPHOS CIV subunits display a normal distribution throughout the mitochondrial network of neurons, OXPHOS CI subunits instead showed a much more punctated
staining pattern with colocalisation observed between mitophagy markers and these OXPHOS CI puncta. Similar to differentiated myotubes in the study, no substantial changes in m.3243A>G heteroplasmy were observed following differentiation into such neuron cell types. Thus, similar mitophagy activation might also be occurring in the oxidatively favouring myogenic cultures with 89% m.3243A>G differentiated in this study, accounting for both a selective reduction in OXPHOS CI, and selection against mitochondria with lower basal $\psi_m$.

It is important to note that data presented here do not directly show evidence for an increase in mitophagy activity, however, and decreases in the activity of mitochondrial biogenesis pathways might also be contributing to, or in fact be solely responsible for, the reductions in mitochondrial mass observed. Indeed, mRNA expression levels of the genes encoding TFAM and PGC1α did appear to be modestly lower in myogenic cultures harbouring m.3243A>G, although TFAM protein levels and mtDNA content were largely maintained. An increase in subsarcolemma mitochondria is often observed in muscle fibres of mitochondrial disease patients (the defining ragged red fibres) however, and it is thought that this is largely associated with a compensatory activation of mitochondrial biogenesis pathways in an attempt to maintain sufficient mitochondrial function to meet the energetic requirements of the muscle fibre (Alston et al., 2017; Uittenbogaard & Chiaramello, 2014).

Interestingly, it has been shown that during the terminal differentiation of C2C12 myoblasts to myotubes, an initial increase in mitophagic flux is initiated in order to clear the cells of myoblast mitochondria preferentially set up for more glycolytic favouring cellular metabolism, which is then followed by a subsequent PGC1α-mediated increase in mitochondrial biogenesis giving rise to more oxidative coupled mitochondria in the differentiated myotube progeny (Sin et al., 2016). In line with this, an increase in the mRNA expression levels of PPARGC1A was observed in the hiPSC derived myogenic cultures following serum withdrawal and associated terminal differentiation into myotubes. Inhibition of autophagy (and therefore mitophagy) not only impairs this important mitochondrial remodelling, but also attenuates C2C12 myoblast differentiation into myotubes (Sin et al., 2016). In line with this, more recent evidence has shown that an increase in mitochondrial fission (a precursory requirement for mitophagy) occurs during the terminal differentiation of primary murine myoblasts, and inhibition of mitochondrial fission similarly impairs myoblast differentiation (Yucel et al., 2019).
Alterations to this essential mitochondrial network remodelling might therefore be an additional factor contributing to the differentiation and maturation impairments observed in hiPSC-derived myotubes harbouring m.3243A>G shown here.

5.4.3 Evidence of ER Stress in MELAS1 Myotubes with High m.3243A>G Mutation load

In line with mitochondria in MELAS1 myotubes harbouring the m.3243A>G having a more depolarised $\psi_m$, TEM analysis revealed myotubes harbouring both mid-range (53%) and higher (89%) m.3243A>G heteroplasmy show trends towards mitochondria with a more globular and rounded morphology, but with largely maintained cristate ultrastructure. In addition to providing insight into the cellular ultrastructure of hiPSC-derived myotubes, including the presence of sarcomeric-like structures, an unexpected increase in what appeared to be ER-like structures was observed closely juxtaposed to mitochondria within MELAS1 Cl4 derived myotubes harbouring 89% m.3243A>G mutation load. Expansions of the ER have been previously associated with ER-stress (Akiyama et al., 2009), potentially highlighting an initiation of the ER-stress response in MELAS1 myotubes with highest m.3243A>G heteroplasmy. Interestingly, it has been shown that in *pink1* and *parkin* Drosophila models of Parkinson’s disease, and Parkinson’s disease patient fibroblasts, mitochondrial dysfunction provokes an ER-stress response, and is associated with an increase in mitochondria-ER contact points (Celardo et al., 2016). More recently, inhibitors of the ER-stress response were shown to rescue galactose-induced cell death of cybrids with OXPHOS CI dysfunction (including those with m.3243A>G) (Soustek et al., 2018), thus highlighting ER-stress as a potentially universal pathomechanism contributing to cellular detriments associated with chronic impairments of mitochondrial function, caused by disease associated genetic mutations.

Considering the importance of mitochondrial-ER contacts for initial pre-constriction of the OMM prior to fission (Elgass et al., 2015), the increased association between mitochondria and ER observed in MELAS1 Cl4 myotubes might also be associated with enhanced mitochondrial fission, in line with potential increases in fission and mitophagic fluxes described above. Mitochondrial-ER contacts serve a number of important functions under normal physiological conditions including: the transfer of key ER-derived phospholipid constituents of mitochondrial membranes such as phosphatidylcholine and Ca$^{2+}$-induced activation of mitochondrial OXPHOS (Filadi, Theurey & Pizzo, 2017). The increased ER volume and identification of close associations with presumably unhealthy mitochondria might therefore be serving...
compensatory protective roles (e.g. Ca\textsuperscript{2+}-buffering, mitochondrial protein folding) rather than being a detrimental response of mitochondrial dysfunction.

### 5.4.4 Potential Masking of Mitochondrial Deficits By In Vitro Culture Conditions

It is well appreciated that in many aspects, in vitro cell culture poorly recapitulates the in vivo setting, with important differences including the lack of other tissue relevant cell types (e.g. motor neurons and pericytes in relation to skeletal muscle) and 2 dimensional rather than 3 dimensional architecture of adherent cells in monolayer culture. In relation to mitochondrial function, the supraphysiological levels of components within cell culture media are arguably among the most important factors which might otherwise conceal more subtle but disease relevant mtDNA disease phenotypes.

As mitochondria represent the major source of cellular ROS under normal physiological conditions, elevations in ROS (i.e. oxidative stress) as a result of mitochondrial dysfunction, has been a thoroughly studied pathomechanism explored in the context of mtDNA pathogenic variants including m.3243A>G and m.8344A>G (Rusanen, Majamaa & Hassinen, 2000; Wu et al., 2010; Voets et al., 2012; Chou et al., 2016). Inclusion of various antioxidant molecules (e.g. enzymes, precursors, substrates) is common under normal routine cell culture practice, and such molecules were indeed included in the various medias utilised throughout this research project. This includes molecules such as ascorbic acid (Vitamin C) as part of FBS/KSR supplementation in fibroblast, hiPSC and myogenic cell culture media, and selenium as part of the IST supplement utilised in terminal myogenic differentiation medium. Consequently, elevations in mitochondrial derived ROS in the context of mitochondrial dysfunction might be masked by the exogenous presence of such molecules. For this reason, future experiments might benefit from culturing mtDNA disease cells in media lacking such antioxidant supplements in order to reveal or otherwise hidden oxidative stress associated cellular phenotypes.

Whilst the data presented in this chapter supports differentiation into oxidatively metabolising myogenic cell types as a useful model for exploring mtDNA disease pathomechanisms, for a number of different reasons the composition of the cell culture media utilised here might be facilitating cellular metabolic rewiring in order to bypass metabolic pathways reliant on mitochondrial function.

Among the most obvious, supplementation of cell culture media with exogenous uridine was performed throughout the duration of this research project in order to support
pyrimidine biosynthesis pathways which are expected to be impaired as a consequence of inefficient ETC driven uridine biosynthesis (King & Attardi, 1989). Whilst uridine is not considered to be an “essential nutrient” due to the ability of mammalian cells to catalyse its biosynthesis as described above, mammals can utilise exogenous dietary sources, and thus uridine/pyridine deficiency is unlikely to be contributing to mitochondrial disease patient phenotypes. The rational supplementation of uridine to cell culture media in order to remove any negative selection bias against mtDNA genomes with pathogenic variants is therefore unlikely to be masking any disease relevant phenotypes of interest.

In addition to uridine, cells in culture harbouring pathogenic mtDNA variants or absent of mtDNA are also auxotrophic for pyruvate as a consequence of deficiencies in maintenance of cellular NAD+/NADH redox status for non-oxidative metabolism (King & Attardi, 1989). Whilst pyruvate supplementation, together with the supraphysiological concentrations of glucose in cell culture media formulations used (e.g. 17mM in DMEM/F12 base media vs ~5mM physiological blood glucose concentration) helped support maintenance of heteroplasmic mtDNA mutation loads throughout this project, mechanistically this is likely through facilitating non-oxidative glycolytic metabolism and fermentation to lactate, thus permitting a reduced reliance on mitochondrial function for cellular energy requirements and redox reactions. Indeed, increases in the anaerobic fermentation flux of pyruvate to lactate by LDH is a well described compensatory mechanism in cells with dysfunctional mitochondria which can serve to replenish ATP and NAD+ available for crucial cellular oxidative processes (Kami et al., 2012; Mullen et al., 2012; Wilkins, Carl & Swerdlow, 2014; Sullivan et al., 2015; Birsoy et al., 2015). Given the supraphysiological nature of these components within cell culture media, which are potentially bypassing quite broadly mitochondrial detriments associated with mtDNA pathogenic variants, future experiments assessing mitochondrial dysfunction and downstream cellular phenotypes might benefit from prior culture of cells under more glucose limiting conditions absent of pyruvate supplementation. Other culture conditions which serve to promote oxidative mitochondrial vs non-mitochondrial metabolic flux pathways could also be utilised. For example, culturing in galactose rather than glucose containing media formulations results in no net ATP production through glycolysis, due to an initial ATP-dependant metabolic reaction catalysed by galactokinase. Through such galactose vs glucose media replacement, impairments in oxidative cellular energy requirements could be teased from other downstream pathomechanisms associated with
mtDNA pathogenic variants in the in vitro model system described here. Previous studies have shown the utility of galactose vs glucose media in revealing proliferation/viability impairments in cells with mitochondrial dysfunction, including cells harbouring mtDNA pathogenic variants (Martínez-Reyes et al., 2016; Chin et al., 2018; Iannetti et al., 2018; Gaude et al., 2018; Soustek et al., 2018). Galactose replacement has also been shown to initiate an increase in mitophagic flux and concordant reduction in m.3243A>G heteroplasm in primary fibroblasts from MELAS patients (Diot et al., 2015), and it would also be interesting to see whether similar reductions in m.3243A>G heteroplasm also occur in more disease relevant post-mitotic cell types such as the terminally differentiated myotubes described here.

In addition to restriction of metabolic substrates, increasing oxidative metabolic demands is also likely to facilitate revealing otherwise hidden mitochondrial dysfunction. As an electrically active cell type, electrical or chemical stimulation of the myotube cultures described here represents one strategy by which physiological energy demands might be partly recapitulated.

5.4.5 Summary

To summarise the results of this chapter, myogenic cells, including terminally differentiated multinucleated myotubes, can be successfully differentiated from hiPSCs harbouring the m.8344A>G or m.3243A>G mutation. In line with the higher oxidative demands of differentiated myogenic cell cultures, mitochondria deficits not previously observed in hiPSCs were revealed following myogenic differentiation, particularly in terminally differentiated myotubes. Mitochondrial impairments associated with m.3243A>G appear detrimental to overall myogenicity, and delay or prevent the subsequent maturation of terminally differentiated myotubes.
Chapter 6 Impact of mtDNA Mutations on Histone Acetylation Modifications

6.1 Overview

Whilst all nucleated cells of the human body contain the entire nuclear genome, the specialised and distinguished functions of individual cell types require the specific expression of only a small subset of the genes encoded by the nDNA. The non-genetic regulation of transcription (i.e. epigenetics) has a number of complex layers which operate in parallel to drive what are normally unidirectional changes in cell-identity, in addition to the maintenance of gene expression profiles which define different cell-types (Li, Liu & Belmonte, 2012). As discussed in detail in introduction sections 1.4.5.2 and 1.5.2, cell metabolism and changes in metabolic fluxes that accompany cell-identity transitions, are not only important for meeting the differential energy and biosynthetic demands of distinct cell types, but also drive maintenance and rewiring of the transcriptional network, at least partly through alterations in levels of metabolic intermediates which serve as co-substrates for enzymatic chromatin modifications (Gut & Verdin, 2013). Both the deposition and removal of a surprising majority of posttranslational histone modifications, and DNA methylation/hydroxymethylation (Gut & Verdin, 2013) are reliant on intermediate metabolic co-substrates, and evidence has accumulated that a large number of these chromatin modifications are sensitive to changes in cellular metabolism (see introduction sections 1.4.5.2 and 1.5.2). Histone acetylation appears to be one of the epigenetic marks most sensitive to changes in cellular metabolic fluxes and is important for both maintenance of the pluripotent state (Moussaieff et al., 2015) and the driving of several key myogenic cell-identity transitions (Ryall et al., 2015; Das et al., 2017; Yucel et al., 2019).

6.1.1 Histone Acetylation Regulates Chromatin Structure and Gene Expression

In order to understand how histone acetylation modifications contribute to the dynamic regulation of gene expression, it is first important to introduce the nucleoprotein composition and structure of the nuclear genome. Chromatin refers to the macromolecular nucleoprotein complex consisting of nuclear DNA together with associated proteins
involved in the packaging of the genome within the eukaryotic nucleus. Among the most abundant proteins associated with the nuclear genome are the histones, which together organise the nuclear DNA into nucleosome structures which represent the fundamental repeating unit of chromatin. Each nucleosome consists of 145-147 DNA base pairs, wound around an octameric nucleosome particle complex made up of two of each of the core histones: H2A, H2B, H3 and H4 (McGinty & Tan, 2015). Each of the core histones consists of a main globular domain that forms the central core of each nucleosome unit through heterodimeric associations between complementary histone domains, and a more disorganized lysine-rich N-terminal domain which protrudes outward from the central core. Amino acid residues on the N-terminal tail are exposed for potential interactions with other proteins including histone modifying enzymes (Strahl & Allis, 2000). Due to the high abundance of lysine residues within the N-terminal tails of the core histones, the N-terminal protrusions have an overall positive charge which facilitates interactions with negatively charged DNA, as well as negatively charged regions of neighbouring nucleosomes (Pepenella, Murphy & Hayes, 2014), both of which promote the more tight compaction of chromatin (heterochromatin). Acetylation of lysine residues neutralises the associated positive charge and thus reduces the strength of such nucleosomal-nucleosomal and nucleosomal-DNA electrochemical interactions, promoting the opening of chromatin (euchromatin) into a more transcriptionally permissible structure (Pepenella, Murphy & Hayes, 2014).

In addition to this direct effect on chromatin structure and enhancement of gene expression, histone acetylation modifications also serve as specific recognition sites (or histone code) for bromodomain (BRD) containing proteins (so called “readers” of histone acetylation). In line with histone acetylation being a mark strongly associated with active transcription, BRD containing proteins generally show pro-transcriptional activity. For example, many histone acetyltransferase enzymes either form a complex with a protein which contains a bromodomain (e.g. bromodomain- and PHD finger-containing protein 1, BRPF1; which recruits KAT6A and KAT6B) (Ullah et al., 2008), or in fact include a bromodomain within their own structure (e.g. KAT2A, KAT2B, KAT3A and KAT3B) (Wapenaar & Dekker, 2016), thus providing a positive feedback loop mechanism enhancing pro-transcriptional acetylation deposition on histones within the same and neighbouring nucleosomes across a gene body. BRD containing proteins also act as transcriptional coactivators, through direct recruitment of the transcriptional machinery (e.g. Transcription initiation factor TFIID subunit 1, TAF1; which is part of the
Transcription factor II D complex) (Jacobson et al., 2000), and recruitment of transcriptional elongation factors (e.g. bromodomain-containing protein 4, BRD4; recruitment of core positive transcription elongation factor b) (Moon et al., 2005; Yang et al., 2005). BRD-containing proteins are also found in several ATP-dependant chromatin remodelling complexes responsible for the movement, ejection or replacement of nucleosome components, which can also facilitate pro-transcriptional opening of chromatin (e.g. Protein polybromo-1, PB1; which is part of the Poly-bromo associated BRM-associated factor complex) (Clapier et al., 2017).

Whilst histone acetylation is a mark predominantly associated with positive regulation of gene expression, with marks such as H3K9ac and H3K27ac being abundant at histones associated with promoter regions of actively transcribed genes (Igolkina et al., 2019), more recent evidence has shown that H4K20ac is in fact enriched at histones associated with promoter regions of genes lowly expressed, and associated with the RE1-Silencing Transcription factor (REST) transcriptional repressor (Kaimori et al., 2016). Whilst the exact molecular mechanism by which H4K20ac mediates such transcriptional repression remains to be determined, monomethylation of this same residue (i.e. H4K20me1) has been previously associated with active transcription elongation (Schwartz, Meshorer & Ast, 2009), and thus H4K20ac might competitively prevent deposition of this pro-transcriptional mono-methylation modification.

6.1.2 Importance of Histone Acetylation for Maintenance of the Pluripotent State and During Cellular Differentiation

The pluripotent nature of hiPSCs is defined by the capacity to generate differentiated cell types belonging to all three germ layers (mesoderm, ectoderm and endoderm). This ability is largely governed by the highly open, euchromatic structure of the genome, that is transcriptionally permissible and poised for gene expression activation upon receiving differentiation cues (Gaspar-Maia et al., 2011).

H3K9ac, a pro-transcriptional modification is present at high levels on actively transcribed genes underlying the pluripotent state (e.g. OCT4 and SOX2), alongside high levels of H3K4me2/3, another pro-transcriptional marker, but with much lower levels of H3K27me3 which is a marker typically associated with silencing of gene expression (Azuara et al., 2006). Interestingly, certain genes associated with early-lineage commitment also show high levels of H3K9ac (and H3K4me2/3) but remain
transcriptionally silent due to the copresence of H3K27me3, with this combination of both transcriptionally active and inactive marks thought to poise such genes for rapid expression induction upon appropriate differentiation cues (Azuara et al., 2006). Inhibition of Class I/II and IV HDACs (NAD$^+$ independent, non-sirtuin) responsible for H3K9ac deacetylation (amongst other targets) not only promotes maintenance of the pluripotent state (Qiao et al., 2015), but also enhances pluripotency following reprogramming factor overexpression (Mali et al., 2010), in line with PSCs having more open, transcriptionally permissible and acetylation rich euchromatin.

KAT8 is a histone acetyltransferase that is largely responsible for deposition of H4K16ac (Smith et al., 2005), a histone acetylation mark which attenuates inter-nucleosome interactions and facilitates euchromatin formation (Shogren-Knaak et al., 2006). hPSCs show a high abundance of H4K16ac, and express high levels of KAT8, with spontaneous differentiation being associated with reductions in both KAT8 protein and H4K16ac levels (Mu et al., 2015; Mujoo et al., 2017). Highlighting the particular importance of H4K16ac for maintenance of the pluripotent state, knockdown of KAT8 in mouse ESCs (mESCs), and resultant reduction in H4K16ac levels, is associated with attenuated expression of pluripotency genes and aberrant expression of lineage specific genes, i.e. an increased propensity for spontaneous differentiation (Li et al., 2012).

In line with histone acetylation deposition being important for initiating the expression of genes associated with alternate cell identities, inhibition of Class I/II and IV HDACs later in neural cell identity acquisition enhances neural differentiation efficiency (Qiao et al., 2015). Therefore, enhancing histone acetylation in the absence of cues promoting cell-identity changes facilitates maintenance of the same cell-identity (particularly pluripotency), whilst enhancing histone acetylation soon after the presence of differentiation cues enhances the acquisition of pro-transcriptional histone acetylation at lineage-specific genes associated with the target cell-identity.

Of particular relevance to this research project, histone acetylation in hPSCs is sensitive to changes in cellular metabolic fluxes, with chemical inhibitors of metabolic pathways contributing to nucleocytoplasmic acetyl-CoA generation resulting in a reduction in both histone acetylation levels and reduced expression of pluripotency related genes, with resultant increase in the spontaneous differentiation propensity (Moussaieff et al., 2015). Mitochondrial dysfunctions associated with disease causing mtDNA mutations therefore have the potential to impact levels of histone acetylation in hPSCs, and could potentially...
6.1.3 Histone Acetylation Changes Drive Cell Identity Changes Underlying Myogenesis

As already alluded to, changes in the histone acetylation profile are important for driving defining changes in gene expression underlying cell identity transitions. During cellular differentiation, the opposing activity of HATs responsible for histone acetylation deposition, and HDACs responsible for histone deacetylation, alter the histone acetylation landscape such that pro-transcriptional acetylation marks are removed from genes defining precursor cell types whilst being deposited at genes contributing to the defining function of the differentiated progeny.

Similar to PSCs, proliferative myogenic precursor cells show a higher abundance of acetylated histones within their chromatin, compared with their terminally differentiating progeny. For example, primary murine myoblasts show a drastic reduction in H3 pan-acetylation following differentiation into myotubes, with similar reductions in H3K9ac and H4K12ac also observed during the terminal differentiation of C2C12 myoblasts to myotubes (Asp et al., 2011). More recent analysis has confirmed that a large number of specific histone acetylation marks decrease during the terminal differentiation of primary murine myoblasts into myotubes in vitro, including: H3K9/14ac, H3K18ac, H3K27ac, H3K56ac and H4K16ac (Yucel et al., 2019). Similar declines in these marks are also observed between proliferating myoblasts and terminally differentiating myocytes in vivo (Yucel et al., 2019). Quiescent murine satellite cells show overall lower levels of the majority of these specific histone acetylation marks, with a substantial increase occurring upon satellite cell activation and subsequent differentiation into myoblasts (Yucel et al., 2019), thus histone acetylation status appears to be correlated with myogenic proliferation potential (i.e. is higher in proliferative activated satellite cells and myoblasts, whilst lower in non-proliferative quiescent satellite cells and terminally differentiated myotubes).

Whilst the large majority of histone acetylation marks appear to decline during the terminal differentiation of myoblasts to myotubes, maintenance and/or increases in histone acetylation occur at genes expressed by terminally differentiating myotubes. For example, whilst H3K18ac levels decrease at genes which become silenced in C2C12
myotubes compared to myoblasts, H3K18ac is maintained at gene sites which show upregulated expression in myotubes (Asp et al., 2011). KAT3B appears to be a particularly important HAT for myogenesis, with KAT3B knockout mice showing in vivo myogenesis impairments and ESCs derived from them failing to express MYOD1 and MYF5 during embryoid body differentiation (Roth et al., 2003). In line with this, increases in KAT3B, alongside concomitant elevations in H3K9ac, H3K18ac and H3K27ac are observed at the MYOD locus during terminal differentiation of C2C12 myoblasts to myotubes, and inhibition of histone acetylation deposition attenuates C2C12 myoblast to myotube differentiation (Hamed et al., 2013). Similar increases in H3K9/K14ac and H3K27ac are also observed at the MYOD locus during the terminal differentiation of primary human myoblasts (Das et al., 2017). Unlike murine myoblast differentiation, global levels of H3K9/14ac appear to increase rather than decrease during the differentiation of primary human myoblasts to myotubes (see supplementary material of Das et al., 2017), highlighting potential interspecies differences in the histone acetylation mechanisms governing myogenic cell identity transitions.

In addition to the important role of histone acetylation modifications for driving the expression of MYOD, histone acetylation deposition also serves as one of the mechanisms by with MyoD exerts its promyogenic transcriptional activity. MyoD directly interacts with KAT3B, and this association with KAT3B potentiates MyoD-dependant transcriptional activation (Yuan et al., 1996). Interestingly, this role of KAT3B as a MyoD coactivator is independent of its acetyltransferase activity, with mutant KAT3B proteins lacking acetyltransferase activity still potentiating MyoD dependant transcriptional activation (Puri et al., 1997). In addition to KAT3B, KAT2B also potentiates MyoD-dependent transcriptional activation which unlike KAT3B co-activation does require its histone acetyltransferase activity (Puri et al., 1997). In line with MyoD-dependant recruitment of HATs for histone acetylation deposition, levels of H4 pan-acetylation are substantially increased in MEFs transdifferentiated into skeletal myocytes through MyoD overexpression, specifically at sites which also showed concomitant MyoD-dependant transcriptional activation (Cao et al., 2010; Bergstrom et al., 2002), including the important late stage myogenic transcription factor myogenin (de la Serna et al., 2005). Furthermore, primary myoblasts from MYOD1KO mice show reduced levels of pro-transcriptional protein complexes which include KAT3B and RNA polymerase II, at gene sites which are normally transcriptionally activated by MyoD (Blum et al., 2012). In line with the role of MyoD for recruitment of HATs, these gene
sites also showed lower levels of H3K27ac in both MYOD1KO myoblasts and myotubes (Blum et al., 2012). Whilst re-expression of MyoD in MYOD1KO myoblasts did not rescue reduced H3K27ac at these myoblast-associated gene sites, H3K27ac levels were rescued in MYOD1KO myotubes, potentially highlighting time-dependant requirements of MYOD, during or prior to changes in myogenic cell-identity. More recently, Myf5 has been shown to bind a highly comparable set of gene sites to MyoD and facilitate the deposition of histone H4 acetylation at comparable levels (Conerly et al., 2016). Unlike MyoD however, Myf5 does not efficiently recruit the transcriptional machinery (Conerly et al., 2016), thus it sets up the chromatin landscape for latter myogenic gene induction, in line with its role earlier in the myogenic differentiation process.

As already discussed in Introduction section 1.5.2.2, both the deposition and removal of histone acetylation modifications which underly myogenic transcriptional profiles are sensitive to, and in fact driven by, changes in cell metabolism. These include the transition from oxidative to glycolytic favouring metabolism accompanying satellite cell activation which drives both, an increase in nucleocytoplasmic acetyl-CoA available for histone acetylation deposition at genes associated with activated satellite cells (including MYOD) (Yucel et al., 2019), in addition to reductions in nucleocytoplasmic NAD⁺ available for sirtuin dependant deacetylation of such genes (Ryall et al., 2015). Whilst an opposing metabolic flux change accompanies the differentiation of myoblasts into myotubes, mitochondrial derived citrate is still necessary for providing nucleocytoplasmic acetyl-CoA for histone acetylation deposition at target myotube genes, particularly those defining fast muscle fibre types (Das et al., 2017).

Evidence has begun accumulating that impairments in mitochondrial function, associated with mtDNA depletion (Martínez-Reyes et al., 2016; Lozoya et al., 2019) and m.3243A>G (Kopinski et al., 2019) both result in falls histone acetylation, at least partly due to reductions in mitochondrial derived citrate, nucleocytoplasmic acetyl-CoA availability and consequently HAT activity. Whilst NAD⁺ levels appear to be largely maintained in proliferative cybrid cells harbouring high levels of m.3243A>G (Kopinski et al., 2019), it is possible that in more oxidatively demanding myogenic cell types, alterations in the cellular redox state might alter nucleocytoplasmic NAD⁺ availability for class III sirtuin HDACs, which might also impact the histone acetylation profile.
6.2 Aims and Hypothesis

The primary aim of this chapter was made towards understanding the impact of mtDNA associated perturbations of cellular metabolism on histone acetylation epigenetic modifications in hiPSCs and differentiated myogenic progeny. Given the interesting range of m.3243A>G mutation loads in MELAS1 hiPSCs derived in Chapter 3, and the myogenic differentiation impairments associated with m.3243A>G focus was made on exploring the levels of histone acetylation modifications in these MELAS1 cell lines.

Taking into account the importance of mitochondrial metabolism for the generation of nucleocytoplasmic acetyl-CoA, it was predicted that impairments in mitochondrial metabolism associated with mtDNA mutations, particularly high loads of m.3243A>G, would reduce nucleocytoplasmic acetyl-CoA availability, HAT activity and consequently global levels of histone acetylation modifications. Given the importance of histone acetylation modifications contributing to maintenance of the euchromatic pluripotent state, and metabolically driven changes in levels of histone acetylation which drive mesodermic and myogenic differentiation, reductions in histone acetylation were predicted to be particularly severe during such cell identity transitions.

6.3 Results

In order to explore the potential impact of mtDNA mutations on maintenance of hiPSC histone acetylation modifications and/or changes in histone acetylation which accompany specific cell-identity transitions, isolated histones were acid extracted form nuclei pellets using the EpiQuik Total Histone Extraction Kit. Equal amounts of histone protein were then separated through PAGE and transferred to nitrocellulose membranes for western blot analysis. Membranes were immunoblotted with a panel of different antibodies which specifically bind epitopes of histones with particular histone acetylation modifications including H3K9ac, H3K12ac, H3K14ac, H3K27ac, H4K5ac, H4K8ac, H4K12ac and H4K16ac. Immunoblot for an antibody targeting the N-terminal of histone H3 (pan) served as a control for discrepancies in histone content loaded.
6.3.1 hiPSCs Harbouring mtDNA Mutations Show Modest Changes in Global Levels of Histone Acetylation Modifications.

Whilst differences in the levels of various histone acetylation modifications were observed between histone extracts from hiPSCs with different mtDNA mutations and mutation loads (see blot in Figure 6-3), none of these differences appeared consistent between different histone preparations assessed from the same lines (Figure 6-2 and Figure 6-3). Lower levels of various histone acetylation modifications including H3K9ac, H3K14ac, H3K27ac and H4K8ac, were more often observed in MELAS1 Cl2 (53% m.3243A>G) and particularly MELAS1 Cl4 (89% m.3243A>G), compared to MELAS1 Cl5 (Figure 6-2), however the variability between repeated measures, and higher histone acetylation levels observed in some histone preparations assessed limits confidence in this observation. Similar but more subtle reductions in H3K27ac and H4K8ac acetylation marks were also observed in MELAS2 Cl3 (100% m.13528A>G and m.1365C>T) and CMT Cl7 (100% m.9185T>C) (Figure 6-3).
Figure 6-1 Assessment of global histone acetylation modifications in histone extracts prepared from hiPSC lines with/without mtDNA mutations. (A) Immunoblots of hiPSC histone extracts, for a range of specific histone acetylation modifications. Immunoblot for histone H3 (pan) served as a control for discrepancies in histone content loaded. Also shown is an image of Ponceau S staining, highlighting the presence of isolated histones in the prepared extracts.
Figure 6-2 Quantification of global levels of histone acetylation modifications in MELAS1 hIPSCs. Bars represent the mean integrated immunoblot band intensity, normalised to that of pan-H3, and expressed relative to that of the MELAS1 C15 (n=4). Error bars represent the StDev, and numbers in the bars represent the number of independent histone extracts assessed.
Figure 6-3 Quantification of global levels of histone acetylation modifications in MELAS2 and CMT hiPSC. Bars represent the mean integrated immunoblot band intensity, normalised to that of pan-H3, and expressed relative to that of the WT CI2 (n=2). Error bars represent the StDev, and numbers in the bars represent the number of independent histone extracts assessed.
6.3.2 Potential Impact of m.3243A>G on Histone Acetylation Changes Occurring During the Transition of hiPSCs to Paraxial Mesoderm

Whilst hiPSC proliferation and maintenance of the pluripotent state necessitates de novo histone acetylation deposition, paraxial mesoderm lineage commitment also necessitates the removal of histone acetylation modifications at genes defining the pluripotent state (and overall euchromatic structure of hiPSC chromatin) in addition to the simultaneous deposition of histone acetylation modifications at lineage commitment genes. In order to better understand global changes in histone acetylation modifications occurring during this transition from pluripotency to paraxial mesoderm, and gain insight into the potential impact of mitochondrial dysfunction on such changes, MELAS1 hiPSC lines with different m.3243A>G mutation loads were subjected to myogenic differentiation induction, and cells collected for histone extraction and assessments by western blot at three defining stages of early differentiation: d3 (after ICL stimulation), d6 (after ICLF stimulation) and d8 (after HIFL stimulation) (Figure 6-4). Surprisingly, increases in a large number of the histone acetylation modifications were observed in control MELAS1 Cl5 (0% m.3243A>G) hiPSCs, over the first three days of stimulation with ICL containing media (d3), including: H3K9ac, H3K14ac, H3K18ac, H4K5ac and H4K16ac (Figure 6-5). Levels of the other three histone acetylation modifications assessed (H3K27ac, H4K8ac and H4K12ac), appeared to be largely maintained by comparison. Following an additional three days of differentiation induction in ICLF containing media (d6), further increases in the relative levels of H4K16ac were observed. By comparison most other histone acetylation modifications showed modest to substantial declines during this period including H3K14ac, H4K5ac, H4K8ac and H4K12ac. By comparison, H3K9ac, H3K18ac and H3K27ac showed comparable levels between d3 and d6 of differentiation induction. From d6 to d8 (HIFL media), H3K18ac and H3K27ac levels were maintained at comparable levels, but levels of all other histone acetylation modifications assessed showed modest to large declines during this 2 day period.

MELAS1 Cl2 (53% m.3243A>G) and MELAS1 Cl4 (89% m.3243A>G) showed comparable levels of histone acetylation modifications to MELAS1 Cl5 on day 0 (i.e. as hiPSCs) (Figure 6-5), in line with observations made above comparing MELAS1 hiPSC lines (Figure 6-2). Whilst the temporal pattern of histone acetylation changes during these initial 8 days of differentiation induction was similar between the MELAS1 lines, histone acetylation increases were often much smaller and in some instances, the subsequent fall in levels of histone acetylation modifications appeared to occur earlier and/or with greater
magnitude in MELAS1 lines harbouring m.3243A>G, particularly in MELAS1 Cl4 with highest 89% m.3243A>G heteroplasmy (Figure 6-5). This was particularly evident when looking at H3K9ac and H4K16ac status, for which increases were observed in MELAS1 Cl5 (0% m.3243A>G) whereas maintenance/decreases from levels of the starting hiPSC population were observed for MELAS1 Cl2 (53% m.3243A>G) and MELAS1 Cl4 (89% m.3243A>G).

Figure 6-4 Assessment of global histone acetylation modifications in histone extracts prepared from MELAS1 lines with/without m.3243A>G during the early stages of the myogenic differentiation process. (A) Immunoblots of hiPSC histone extracts before differentiation induction (d0), after 3 days of ICL stimulation (d3), after subsequent 3 days of ICLF (d6) and after additional two days of HIFL stimulation (d8). Immunoblot for histone H3 (unmodified) served as a control for discrepancies in histone content loaded. Also shown is an image of Ponceau S staining, highlighting the presence of isolated histones in the prepared extracts.
Figure 6-5 Quantification of global levels of histone acetylation modifications throughout the early stages of differentiation (from blot presented in Figure 6-4). Markers show the integrated immunoblot band intensity, normalised to that of pan-H3, and expressed relative to that of MELAS1 Cl5 on d0.
6.3.3 Terminally Differentiating hiPSC-Derived Myogenic Cultures Harbouring m.3243A>G Show Reductions in Histone Acetylation Levels

6.3.3.1 Western Blot Assessment of Global Histone Acetylation Status

In order to gain insight into changes in histone acetylation modifications occurring during the transition of proliferative myogenic cells, to more oxidatively metabolising terminally differentiated myotubes, histones were collected from myogenic cells maintained in serum containing proliferation media, 3 days after serum withdrawal, promoting fusion into myotubes, and 7 days after serum withdrawal after which myotube cultures had began to mature (Figure 6-6).

During the 3 days following serum withdrawal, MELAS1 Cl5 cultures showed modest elevations in most histone acetylation marks assessed including: H3K9ac, H3K14ac, H3K18ac, H3K27ac, H4K8ac and H4K12ac, with the remaining two acetylation marks H4K5ac and H4K16ac showing no substantial changes during this same period (Figure 6-7). During the following 4 days of serum withdrawal, falls in almost every histone acetylation mark were observed which by d7 had typically declined to levels below that originally observed in myogenic cells maintained in proliferation media (Figure 6-7). H3K18ac was the only exception to this, with levels continuing to increase during this later 4 day period.

Whilst MELAS1 Cl2 and MELAS1 Cl4 myogenic cultures harbouring mid-range and higher m.3243A>G heteroplasmy showed similar patterns of histone acetylation changes during this period, overall levels of most histone acetylation modifications (with the exception of H3K18ac) were typically lower for cells within proliferation media and 3 days after serum withdrawal, with this being particularly evident in the MELAS1 Cl4 myogenic culture with highest m.3243A>G heteroplasmy. After 7 days of serum withdrawal during which reductions in overall levels of histone acetylation were observed, differences between MELAS1 myogenic cultures were much less evident, with one notable exception being a continued increase in levels of H3K9ac for MELAS1 Cl2 with mid-range m.3243A>G heteroplasmy. In fact, declines in the levels of histone acetylation modifications in MELAS1 Cl2 myogenic cultures from d3 to d7 post serum withdrawal were generally much less substantial and in some instances maintenance of comparable or higher levels of histone acetylation were observed between these time points.
It should be noted that this experiment was only performed on a single myogenic differentiation induction, and histone content loaded for each line (adjudged by pan-H3 levels) did show reasonably large variability between cells lines, particularly for the MELAS1 Cl4 line with highest m.3243A>G mutation load, which could potentially confound the observations made as a consequence of immunoblot intensities falling outside of the linear range of the chemiluminescence detection method used. Nevertheless, comparable histone loading was achieved for myogenic cultures in proliferation media, a time point during which these impairments in histone acetylation status were most evident.
Figure 6-6 Assessment of global histone acetylation modifications in histone extracts prepared from MELAS1 myogenic cultures with/without m.3243A>G following serum withdrawal and terminal differentiation induction. Immunoblots of myogenic culture histone extracts maintained in proliferation media (proliferation), after 3 days of serum withdrawal (d3 fusion) and after additional 4 days of serum withdrawal (d7 fusion). Immunoblot for histone H3 (unmodified) served as a control for discrepancies in histone content loaded. Also shown is an image of Ponceau S staining, highlighting the presence of isolated histones in the prepared extracts.
Figure 6-7 Quantification of global levels of histone acetylation modifications in proliferation media (d0) and following 3 days (d3) and 7 days (d7) of serum withdrawal and terminal myogenic differentiation (from blot presented in Figure 6-6). Markers show the integrated immunoblot band intensity, normalised to that of pan-H3, and expressed relative to that of MELAS1 C15 maintained in proliferation media.
6.3.3.2  m.3243A>G Does not Impact H4K16ac Status in Specific Myogenic Cell Types

Taking into account the myogenic differentiation efficiency and maturation impairments of MELAS1 Cl2 and MELAS1 Cl4 cell lines, it is possible that differences in the global levels of histone acetylation modifications observed through western blot analysis above are merely associated with differences in the proportion of specific myogenic cell types contributing to the overall culture. As H4K16ac appears particularly important for driving metabolically sensitive changes in myogenic cell identity and specific myogenic cell types show different levels of the histone acetylation modification (Ryall et al., 2015; Yucel et al., 2019), focus was made towards understanding the relative levels of H4K16ac in specific myogenic cells within the differentiating myogenic cultures of MELAS1 lines with/without m.3243A>G, through immunofluorescence analysis. Co-staining with titin and MyoD permitted terminally differentiating myotubes to be distinguished from myogenic precursors including activated satellite cells, myoblasts and myocytes (Figure 6-8). Similar co-staining for titin and myogenin permitted myotubes to be distinguished from latter stage myogenic precursors including myoblasts and myocytes (Figure 6-8).

Nuclei showed a reasonably large variability in H4K16ac, myogenin and MyoD staining intensities which appeared to be largely associated with the relative compactness of the nuclear compartment: i.e. nuclei with smaller xy area showed more concentrated and intense staining whilst nuclei with larger xy area showed lower intensity staining. In order to account for this effect of nuclear compactness, and provide some semiquantitative insight into potential differences in the levels/concentrations of these particular myogenic transcription factors and the H4K16ac modification, between MELAS1 myogenic cultures, intensity measurements were measured from confocal immunofluorescence images using ImageJ FIJI software. To this end, fluorescent intensities were assessed for each MyoD+ and myogenin+ nuclei within/outside of titin+ myotubes across ≥8 225x225µm confocal fields of view. For each nuclei, a single 0.48µm thick z-section focal plane for which the nuclei was in focus throughout was chosen, and a nuclear region of interest defined by DAPI stain. Fluorescence intensity measurements were then measured for the Alexa Fluor 488 channel (MyoD or myogenin) and Alexa Fluor 647 channel (H4K16ac), the signals of which were normalised to chromatin concentration via DAPI staining intensity. All coverslips assessed were stained simultaneously, and imaging parameters kept constant for all channels across the different coverslips, permitting semi-quantitative comparisons of relative staining intensities and associated
MyoD, myogenin and H4K16ac quantities. Through this analysis a number of interesting observations were noted.

In line with MyoD expression declining upon the fusion of myocytes into titin+ myotubes, MyoD staining intensities tended towards lower levels in titin+ myotubes compared to titin- counterparts for all MELAS1 myogenic cultures, with this being particularly evident in cultures after 7 days of serum withdrawal (Figure 6-9). Whilst caution must be made comparing between coverslips, MyoD intensities tended towards lower levels in MELAS1 myogenic cultures harbouring mid-range (MELAS1 Cl2) and higher (MELAS1 Cl4) m.3243A>G heteroplasmy levels, particularly after 7 days of serum withdrawal, which could potentially indicate alterations in MyoD content within individual myogenic cells of these cultures (Figure 6-9). In line with the importance of myogenin for both driving the differentiation of myocytes into myotubes and subsequent maintenance of myotube transcriptional states, levels were not considerably different between cells pre/post serum withdrawal, or between nuclei within titin- or titin+ myotubes, although levels did appear to be lower in MELAS1 Cl5 titin+ myotubes following serum withdrawal (Figure 6-9). Whilst differences in myogenin content between the different MELAS1 lines was much less evident than that observed for MyoD, myogenin intensities appeared to tend towards lower levels in MELAS1 Cl2 and MELAS1 Cl4 lines harbouring m.3243A>G, although this was not consistent across all cell-identities/culture conditions (Figure 6-9).

In line with terminal differentiation into myotubes being associated with declines in levels of H4K16ac, levels of H4K16ac tended towards lower levels in nuclei found within titin+ myotubes, compared with MyoD+ titin- or myogenin+ titin- myotube precursors (Figure 6-9). In addition, nuclear H4K16ac intensities within titin+ nuclei declined following serum withdrawal facilitating myotube formation and maturation (Figure 6-9). MELAS1 Cl2 myogenic cells harbouring mid-range m.3243A>G heteroplasmy showed comparable levels of H4K16ac to MELAS1 Cl5 lacking m.3243A>G, with the exception of MyoD+ titin- myogenic precursors which showed lower levels (Figure 6-9). Whilst H4K16ac levels were generally comparable between MELAS1 Cl5 and MELAS1 Cl4 myogenic cells, higher levels were also observed, particularly in nuclei within myogenin+ and/or titin+ cells. This might potentially highlight specific impairments in H4K16ac deacetylation dynamics and/or increases in H4K16ac acetylation dynamics, rather than the predicted impairments in histone acetylation deposition that were expected.
Overall, these data suggest that whilst subtle differences are observed, successfully differentiated myogenic cells within MELAS1 myogenic cultures appear to maintain normal H4K16ac dynamics, largely unaffected by the presence of m.3243A>G. More quantitative measures of the levels/concentrations of MyoD and myogenin regulatory factors suggest that in addition to overall impairments in the formation of myogenic cell types expressing these specific factors (see results presented in section 5.3.3), myogenic cells in MELAS1 C2 and Cl4 cultures harbouring the m.3243A>G might also show reductions/alterations in the expression levels of these specific factors.
Figure 6-8 Assessment of myogenic transcription factor and H4K16ac levels within the nuclei of specific cell types within the differentiating myogenic cultures. Representative maximum intensity projection confocal microscopy images showing immunofluorescence staining of MELAS1 myogenic cultures 7 days after serum withdrawal promoting myotube formation, for H4K16ac alongside MyoD and titin, and H4K16ac alongside myogenin and titin. Scale bars = 50µm.
Figure 6-9 DAPI normalised immunofluorescence staining intensities of nuclear localised MyoD, myogenin and H4K16ac within the differentiating myogenic cultures. Bars show the mean fluorescence intensity for the indicated mark, normalised to nuclear DAPI fluorescence intensity, from a 0.48 µm confocal z-section, with error bars ±StDev. The numbers in the bars show the number of independent nuclei assessed for each condition from ≥8 individual 225x225 µm fields of view. Statistical significance assessed by three-way or two-way ANOVA with Tukey post-hoc correction for multiple comparisons. Statistical significance between MELAS1 Cl2 or MELAS1 Cl4 nuclei harbouring m.3243A>G, compared with the MELAS1 Cl5 isogenic control line indicated by #p<0.05, ##p<0.01, ###p<0.001. Statistical significance between different myogenic cell identities of the same MELAS1 cell line indicated by *p<0.05, **p<0.01, ***p<0.001. In all instances, comparisons have been limited to differences in only one of the three experimental parameters: cell line, titin+ vs titin− or cell culture conditions (proliferation vs 7 days post serum withdrawal).
6.4 Discussion

The main aim of this chapter was to gain insight into the impact of mtDNA pathogenic variant associated mitochondrial dysfunction on the levels of histone acetylation modifications with a particular focus on the dynamic changes occurring during cell-identity transitions of MELAS1 lines with/without m.3243A>G. This also permitted insight into global histone acetylation changes occurring during the myogenic differentiation of hiPSCs, which because of the relative infancy of efficient myogenic differentiation protocols remains largely unexplored at present.

6.4.1 Variability in hIPSC Histone Acetylation Status Prevents Confident Identification of Impairments Associated with mtDNA Mutations

Whilst hiPSCs favour glycolytic metabolism, oxidative mitochondrial metabolism is still necessary for maintaining nucleocytoplasmic levels of citrate and therefore acetyl-coA for histone acetylation (Moussaieff et al., 2015). Whilst MELAS1 hiPSCs harbouring m.3243A>G did show reductions in cellular citrate, overall levels of the various histone acetylation marks of hiPSCs assessed, appeared largely unaffected by mtDNA mutations. Whilst levels of specific histone acetylation modifications did differ between histone extracts simultaneously prepared from hiPSC lines with/without mtDNA mutations, inconsistent variability was observed. Subtle reductions in various histone acetylation marks including H3K9ac, H3K14ac, H3K27ac and H4K8ac were common, particularly in histone extracts from MELAS1 Cl4 hiPSCs with 89% m.3243A>G heteroplasmy, however higher levels of these particular modifications in some histone extracts simultaneously prepared alongside MELAS1 Cl5 hiPSCs lacking m.3243A>G, limits confidence in this observation.

As differentiated cell types generally show lower levels of histone acetylation modifications relative to PSC counterparts (Krejčí et al., 2009; Moussaieff et al., 2015; Bhanu, Sidoli & Garcia, 2016), one possible explanation for the variable differences in histone acetylation status observed between the hiPSC lines could be associated with differences in the overall pluripotency of the cell populations assessed. HiPSC lines were carefully maintained under pluripotency promoting conditions prior to histone extraction, and histone extracts were prepared from cultures with normal hiPSC colony morphology, thus limiting this as a potential confounding factor. Future experiments might benefit from simultaneously preparing whole-cell protein or RNA lysates in order to determine
any confounding differences in pluripotency, through assessments of pluripotency marker expression via western blot or qPCR analysis respectively.

6.4.2 De Novo Increases in Histone Acetylation During Paraxial Mesoderm Patterning are Impaired by m.3243A>G

Given hiPSC chromatin is highly enriched with acetylation rich euchromatin, it might be expected that global decreases in histone acetylation modifications would occur upon pluripotency exit and cellular differentiation. Indeed, levels of histone H3 and H4 acetylation decline during spontaneous hESC differentiation (Moussaieff et al., 2015) and H3K9ac declines during targeted endoderm differentiation (Krejčí et al., 2009). Whilst initial declines in H3K9ac, among other histone acetylation marks, also occur shortly after neuroectoderm differentiation induction (Qiao et al., 2015; Gonzales-Cope et al., 2016), H3K14ac and H4K16ac do in fact increase (Gonzales-Cope et al., 2016), and subsequent increases in H3K9ac occur at latter stages of hESC neural commitment (Qiao et al., 2015). In comparison, changes in histone acetylation occurring during initial mesoderm differentiation of PSCs remain relatively unexplored at present.

In this study, most histone acetylation marks assessed did appear to decline to lower levels than hiPSCs 8 days after myogenic differentiation induction, however initial increases in 4/8 of the histone acetylation marks assessed did occur: H3K9ac, H3K14ac, H4K5ac and H4K16ac. These data might suggest that early mesoderm patterning might show alternate changes in histone acetylation status than that associated with early endoderm and ectoderm patterning. In line with this, pharmacological inhibition of Class I and II HDACs, and knockdown of HDAC1 or HDAC3 all promote mesoderm patterning during spontaneous mESC differentiation (Lv et al., 2014), suggesting increases in histone acetylation are indeed specifically important for early mesoderm differentiation. Furthermore, pharmacological inhibition of KAT3A and KAT3B in mESCs leads to a reduction in H3K27ac and H3K56ac marks, and facilitates spontaneous differentiation towards endoderm and ectoderm lineages whilst impairing mesoderm commitment (Wu, Kamikawa & Donohoe, 2018). In addition, pharmacological inhibition of KAT3A/3B during mesoderm differentiation induction (through CHIR99021 inhibition of GSK3β, as used here), impaired the transcriptional upregulation of genes encoding master regulators of mesoderm commitment including Brachyury and Nodal (Wu, Kamikawa & Donohoe, 2018).
It would therefore appear that unlike endoderm and ectoderm lineage commitment, early mesoderm patterning is associated with a necessary increase in the levels of various histone acetylation modifications. It is interesting to note that hPSC-derived early mesoderm cells are more reliant on oxidative glutamine metabolism compared with hPSCs and hPSC-derived early endoderm and ectoderm (Lu et al., 2019), thus it can be speculated that this alternate metabolic flux change is contributing to elevations/maintenance of nucleocytoplasmic acetyl-CoA available for histone acetylation deposition and/or causing a reduction in NAD⁺ available for sirtuin-dependant deacetylation.

Considering the important role histone acetylation plays in the initiation and maintenance of gene expression, it is possible that histone acetylation deficits result in impairments in the sequential expression of genes involved in the cell identity transitions underlying paraxial mesoderm and latter myogenic differentiation. Thus, impairments in myogenic differentiation observed for the MELAS1 Cl2 and Cl4 lines harbouring m.3243A>G, might originate from very early stages of the differentiation programme.

The early data presented here suggests early mesoderm differentiation of hPSCs is associated with an initial increase in histone acetylation modifications, and that H3K9ac, H3K14ac, H4K5ac and H4K16ac might be sensitive to mitochondrial dysfunction caused by mid-range and higher levels of m.3243A>G. These data might therefore suggest, that whilst the more glycolytic favouring metabolism of hiPSCs is sufficient to maintain nucleocytoplasmic acetyl-CoA levels for histone acetylation modifications, upon paraxial mesoderm differentiation induction and switch towards more oxidative favouring mitochondrial metabolism, cells with mitochondrial dysfunction caused by m.3243A>G have more limited nucleocytoplasmic acetyl-CoA availability which consequently impacts histone acetylation deposition.

6.4.3 Do Impairments in Histone Acetylation at MRF Genes and MRF Targets Underlie Myogenic Differentiation Impairments Associated with m.3243A>G?

Locus specific increases in H3K9ac, H3K9/14ac, H3K18ac and H4K12ac are observed during terminal differentiation of C2C12 myoblasts, specifically on gene sites upregulated in myotubes vs myoblasts, and associated with myotube formation and skeletal muscle function (Asp et al., 2011; Yucel et al., 2019). Elevations in Myod1 transcripts accompany the early differentiation of C2C12 myoblasts, and concordant
increases in H3K9ac, H3K18ac and H3K27ac modifications deposited on proximal and distal promoters of the Myod1 locus also occur (Hamed et al., 2013). Thus, increases in histone acetylation at genes upregulated in myotubes vs myoblasts appear consistent between murine and human myoblasts following serum withdrawal. In can be speculated that whilst simultaneous declines in histone acetylation modifications at myoblast specific gene sites (and euchromatic enriched regions) of murine myogenic models during terminal differentiation mask such locus-specific elevations, this deacetylation phase is slightly more delayed in human myoblasts, permitting acetylation deposition increases at myotube associated genes to be observed on a more global level. These data further highlight species dependant differences in global histone acetylation changes occurring during myogenesis, and it is therefore recommended that future investigations looking to explore histone acetylation modifications or other factors associated with myotube formation, should look towards human relevant models including primary human myoblasts, human myoblast cell lines (e.g. hTERT/cdk4 transformed human myoblasts) (Thorley et al., 2016) or hiPSC-derived myogenic cells such as those described here.

MELAS1 myogenic cultures harbouring mid-range and higher m.3243A>G mutation loads showed comparable temporal patterns for changes in histone acetylation throughout terminal myogenic differentiation, however MELAS1 CI2 harbouring mid-range m.3243A>G heteroplasmy and particularly MELAS1 CI4 with higher m.3243A>G mutation loads showed lower levels of most histone acetylation modifications assessed. Similar to observations made in early mesoderm patterning, H3K18ac appeared to be maintained in MELAS1 lines harbouring m.3243A>G, further suggesting deposition/maintenance of this particular mark is relatively resistant to mitochondrial dysfunction. H3K9ac, H3K14ac and H3K27ac are marks which are enriched at the promoter regions of the Myod1 locus of C2C12 myoblasts and positively correlated with MyoD1 transcript expression (Hamed et al., 2013; Das et al., 2017). The reduction in levels of these marks, observed in the myogenic cultures harbouring m.3243A>G, might therefore attenuate expression of this crucial myogenic regulatory factor and account at least partikey for impairments in overall myogenicity observed. Unfortunately, several sets of primer pairs targeting the MYOD transcript did not show target specific amplification for qPCR analysis (data not shown), which prevented correlations from been made with MYOD transcript levels. Quantitative analysis of MyoD immunofluorescence did show a trend towards reduced MyoD quantity/concentration within nuclei of MELAS1 CI2 and MELAS1 CI4 myogenic cultures harbouring m.3243A>G, however, which potentially
supports this hypothesis. In a similar manner, expression of the first MRF, Myf5, during mESC embryoid differentiation is largely governed by KAT3B deposition of H3K27ac at enhancer regions of the Myf5 gene (Francetic & May, 2012), thus reductions in H3K27ac deposition at genes associated with earlier stages of the myogenic differentiation programme might also be contributing to the myogenic differentiation impairments observed.

6.4.4 Summary

In summary of the key findings presented in this chapter, histone acetylation levels of hiPSCs appears largely resistant to mitochondrial dysfunction associated with disease causing mtDNA mutations. Upon mesoderm differentiation induction, associated with both an increased demand in changes in the histone acetylation landscape, and switch from glycolytic to more oxidative favouring metabolic fluxes, impairments in histone acetylation are observed in cultures harbouring m.3243A>G, most likely associated with reductions in histone acetylation deposition. Similar reductions in global histone acetylation levels are also observed in differentiating myogenic cultures harbouring m.3243A>G. These data might therefore highlight impairments in maintenance of nucleocytoplasmic levels for histone acetylation, and associated dysregulation of the mesoderm-myogenic transcriptome, as a potential mechanism underscoring the observed myogenicity impairments of these MELAS1 lines harbouring m.3243A>G. Impairments in the deposition and/or maintenance of metabolically sensitive histone acetylation modifications might therefore be contributing toward the detrimental skeletal muscle phenotypes observed in mitochondrial disease patients and be a contributing factor to the particular vulnerability of this tissue to mitochondrial dysfunction.
Chapter 7  Summary of Key Findings and Future Directions

7.1 Successful Establishment of mtDNA Disease hiPSCs for Disease Modelling, and m.8344A>G Dependant Impairments in hiPSC Reprogramming/Maintenance

Whilst there has been an ever-increasing number of reports highlighting the successful establishment of hiPSC lines with mtDNA mutations, parallel reprogramming of patient cells harbouring a wider variety of mtDNA mutations have been more rarely described. In Chapter 3, I show that in line with previous publications, most disease associated mtDNA mutations do not appear overtly detrimental to the reprogramming process, including the m.13528A>G and m.1365C>T mutations affecting MT-ND5 which have not been previously reported. Taking into account the breadth of literature highlighting the reliance of hiPSCs on glycolytic rather than oxidative metabolism, this observation was expected, and in fact among one of the rational reasons hiPSC technology was chosen as an initial starting point for development of the in vitro mitochondrial disease model established here. For this reason, it was initially surprising to observe severe impairments in overall reprogramming efficiency of several patient fibroblast lines harbouring m.8344A>G, and the subsequent difficulties faced in maintaining suprathreshold heteroplasmy levels and general pluripotency.

The hPSC field is now beginning to appreciate the necessary roles of mitochondrial metabolism during both the reprogramming process and pluripotency maintenance in routine culture, and the data presented here provides further evidence in support of this. Among some of the most notable observations, initial reprogramming of the MERRF1 patient fibroblast line harbouring heteroplasmic levels of m.8344A>G performed under feeder-dependant conditions, gave rise to a limited number of hiPSC colonies, the majority of which showed undetectable levels of m.8344A>G. In a subsequent feeder-independent reprogramming induction of fibroblast lines from three unrelated patients harbouring m.8344A>G, apparent improvements in reprogramming efficiency were observed, and individual clones with high and presumably suprathreshold levels of m.8344A>G were successfully obtained. Assessments of clonal fibroblast lines expanded from the bulk populations revealed however, that a significant selection against
m.8344A>G occurs during cell reprogramming. In addition, a 10% fall in m.8344A>G heteroplasmy level from 83% to 73% was observed in the MERRF1 Cl23 hiPSC clone with highest m.8344A>G heteroplasmy successfully obtained from the second reprogramming of MERRF1 fibroblasts, highlighting negative selection against such mutation loads, not only during the reprogramming process but also during subsequent maintenance of pluripotent cells in culture. More in-depth characterisations of the MERRF1 Cl23 hiPSC clone with high m.8344A>G, revealed that this line failed to initiate the expression of REX1, and is potentially only partially reprogrammed (Chan et al., 2009). Given the importance of REX1 for maintaining the glycolytic vs oxidative metabolism underlying hPSC pluripotency, it is suggested that assessments of REX1 expression should be included in routine pluripotency characterisation, particularly in hiPSC derived cell lines with mutations affecting metabolism associated genes.

### 7.2 Detectable Detriments in Mitochondrial Function of hiPSCs Caused by m.3243A>G

In the work presented in Chapter 3, I explored the impact of different mtDNA mutations on hiPSC mitochondrial physiology, which in most instances revealed relatively minor if any evidence of mitochondrial dysfunction. In line with previous reports, MELAS1 hiPSCs harbouring the m.3243A>G did show a number of subtle impairments in mitochondrial function however, most notably detriments in levels of assembled OXPHOS CI at high 89% m.3243A>G heteroplasmy. Through LC-MS metabolomic analysis and heavy-isotope glucose labelling experiments, I show for the first time that hiPSCs harbouring high 89% m.3243A>G heteroplasmy have reductions in overall glucose metabolic fluxes, and also show that MELAS1 Cl4 hiPSCs with 89% m.3243A>G heteroplasmy, and in MELAS1 hiPSC lines with both mid-range (MELAS1 Cl2, 53% m.3243A>G) and higher (MELAS1 Cl4, 89% m.3243A>G) mutation loads have reductions in levels of several mitochondrial derived metabolites including citrate. These data not only revealed hiPSC metabolic fluxes are indeed impacted by m.3243A>G associated mitochondrial dysfunction, but also provided me with the first evidence that mid-range m.3243A>G mutation loads are sufficient to exert a detectable biochemical deficit, and that mid-range and higher m.3243A>G mutation loads differentially impact cellular physiology. Elevations in the NAD/NADH pool were also observed specifically in the MELAS1 Cl2 but not MELAS1 Cl4 hiPSC line, further highlighting the divergent metabolic changes provoked by mid-range vs higher m.3243A>G mutation loads. In
relation to this, cybrid cell lines with mid-range and higher levels of m.3243A>G have also display differential cellular phenotypes (Picard et al., 2014). Interestingly, one of the differential phenotypes noted in these cells was substantial upregulation of \textit{de novo} NAD synthesis pathways and nuclear NAD content, in lines with 70-90\% m.3243A>G heteroplasmy, but not homoplasmic levels of the pathogenic variant (Kopinski et al., 2019), not dissimilar from the observations made between MELAS1 hiPSC lines with mid-range and higher m.3243A>G heteroplasmy.

### 7.3 Myogenicity Impairments Associated with the m.3243A>G in a Novel in vitro Model of Mitochondrial Diseased Muscle

Whilst hiPSCs can provide some useful insights into the detrimental impact of mtDNA mutations, they do not represent an improvement on commonly utilised \textit{in vitro} models of mtDNA disease such as cell cybrid cell lines, which are also primarily reliant on glycolytic metabolic fluxes. Although the data presented in this thesis support the important role of mitochondrial function in glycolytic favouring hiPSCs, the relevance of this to the human disease state is limited, considering oxidatively metabolising tissues are disproportionally affected in mtDNA disease patients. To this end, the focus of the work presented in Chapter 5 of this thesis was towards development of a more appropriate mitochondrial disease model through optimisation and implementation of a targeted myogenic differentiation protocol. The results presented in this chapter show for the first time that hiPSC lines harbouring mtDNA mutations, can be successfully differentiated into myogenic cell types, including terminally differentiated myotubes, with assessments of mitochondrial function revealing typical detriments associated with the m.8344A>G and m.3243A>G mutations.

Impairments in maintenance of the $\psi_m$ were seen in differentiated myotubes harbouring mid-range levels of the m.8344A>G or m.3243A>G mutations, which were otherwise not observed in the parental hiPSCs. This is in line with the increased reliance on oxidative metabolism and specific vulnerability of skeletal muscle cell types to mitochondrial dysfunction, thus highlighting the accurate phenocopying of this \textit{in vitro} disease model. Similar to observations of alternate metabolic impairments of MELAS1 hiPSCs with mid-range vs higher m.3243A>G heteroplasmy in Chapter 4, myotubes with mid-range 53\% vs higher 89\% m.3243A>G mutation loads showed differential impairments in maintenance of basal $\psi_m$. Whilst myotubes harbouring 53\% and 80\% m.3243A>G heteroplasmy showed a mutation load dependant decline in basal $\psi_m$, MELAS1 C14 with
higher 89% m.3243A>G heteroplasmy showed basal $\psi_m$ comparable to that of the isogenic MELAS1 Cl5 control line. In addition, there was evidence for global reductions in mitochondrial content of MELAS1 myogenic cultures with mid-range and higher m.3243A>G heteroplasmy, however, only in MELAS1 Cl4 with highest 89% m.3243A>G were specific reductions in levels of OXPHOS CI, and reductions in myotube mitochondrial content observed. It would therefore appear that quite abrupt, mutation load-dependant thresholds exist, which result in the presentation of distinct and alternate cellular phenotypes, rather than gradual mutation load dependant changes in phenotype severity. Such thresholds might help better explain the heterogenous nature of mtDNA disease presentations associated with different, but sometimes comparable loads, of the same mtDNA pathogenic variant (Boggan et al., 2019; Altmann et al., 2016).

M.8344A>G at mid-range 52% heteroplasmy levels did not appear to overtly impact the myogenic differentiation process however, impairments in both myogenic differentiation and subsequent maturation of myogenic cultures were observed in MELAS1 lines with both mid-range and higher m.3243A>G mutation loads. Thus, whilst m.3243A>G is not overtly detrimental to cell-identity changes associated with cellular reprogramming to pluripotency, subsequent differentiation through oxidatively demanding early mesoderm and latter myogenic cell-identity acquisitions appear negatively impacted by this mutation. In relation to this, specific impairments in the subsequent expression of adult MyHC isoforms in myotubes harbouring m.3243A>G points towards consistent impairments in myogenic cell-identity changes throughout the entire differentiation programme.

This brings to attention an interesting conundrum. Why is it m.8344A>G is selected against during the reprogramming process, yet m.3243A>G is not? Why does the m.3243A>G but not m.8344A>G pathogenic variant at comparable mutation loads impair mesoderm/myogenic differentiation efficiency? It is well appreciated that OXPHOS CI is specifically vulnerable in cells harbouring m.3243A>G (Matsubara et al., 2018; Yokota et al., 2015; Häimaläinen et al., 2013), with observations made in both MELAS1 hiPSCs and differentiated myotubes as part of the work in this thesis supporting this. By comparison, both OXPHOS CI and CIV are negatively impacted by m.8344A>G (Hanna et al., 1995; Hashimoto et al., 2015; Pereira et al., 2018). Whilst impairments in OXPHOS CIV will attenuate overall mitochondrial ETC function as a consequence of impairments to the terminal transfer of e\(^{-}\) to O\(_2\), specific impairments in OXPHOS CI still permit
electron entry through OXPHOS CII (entirely nuclear encoded) and downstream maintenance of ETC activity. It is possible that although mesoderm and myogenic cells, and associated cell-identity transitions, might be more reliant on OXPHOS CI activity, the reprogramming process and maintenance of the pluripotent state is instead more resistant to dysfunctions of OXPHOS CI but not more global ETC detriment. In line with this, myotube $\psi_m$ was much more sensitive to the OXPHOS CI inhibitor rotenone with reductions to 20-30% of basal levels observed, whereas rotenone instead resulted in much more modest reductions in $\psi_m$ of hiPSCs to 40-60% of basal. It can therefore be speculated that during the reprogramming process and/or maintenance of the pluripotent state, suprathreshold levels of m.8344A>G but not m.3243A>G mutation loads are selected against due to the more global impairments in mitochondrial ETC function. Following differentiation into mesoderm/myogenic cells more reliant on OXPHOS CI, detriments associated with the OXPHOS CI impairments of MELAS1 lines harbouring m.3243A>G are revealed. By comparison, the mitochondrial deficits associated with 52% m.8344A>G heteroplasmy of MERRF1 CI2 remain below thresholds associated with mitochondrial dysfunctions sufficient to provoke this differentiation phenotype. In relation to this, it is interesting to note that hiPSCs harbouring mtDNA disease mutations associated with severe OXPHOS CIII or OXPHOS CIV dysfunction and consequential global impact on ETC function have not yet been described in the literature.

7.4 Histone Acetylation Detriments Might Underly the Mesoderm/Myogenic Differentiation Impairments and be an Important mtDNA Disease Pathomechanism

This research project begun with a rational hypothesis based on previous reports spanning across the fields of PSCs, epigenetics, skeletal muscle and mitochondrial biology. Whilst associations between each of these individual areas has been accumulating in the literature throughout the duration of this research project, links spanning across all these fields remained unexplored. Connections between mtDNA disease and histone acetylation have been previously reported (Martínez-Reyes et al., 2016; Lozoya et al., 2019; Kopinski et al., 2019), however the relevance of these links in a disease-relevant cellular setting remain unexplored. The results described in Chapter 6 of this thesis show for the first time that mitochondrial impairments associated with mtDNA disease mutations do indeed impact global levels of histone acetylation modifications in disease-relevant mesoderm/myogenic cell types, and potentially represent the link between
mtDNA pathogenic variant associated mitochondrial dysfunctions and impairments in mesoderm/myogenic cell identity transitions described in Chapter 5.

Although reductions in cellular citrate levels were seen in MELAS1 hiPSC lines harbouring m.3243A>G, these impairments did not appear to have a substantial impact on the histone acetylation profile of these cells. Whilst this might at first seem surprising, given mitochondrial citrate is necessary for maintenance of the acetylated histone rich euchromatin of hiPSCs, the important relationship between histone acetylation and maintenance of the pluripotent state means that such impairments likely prevent stable hiPSC maintenance (Moussaieff et al., 2015). Deficits in histone acetylation deposition maintenance might therefore be one of the underlying factors which contributes to selection against m.8344A>G during reprogramming and/or pluripotency maintenance.

In parallel to the detrimental impact of mid-range and higher m.3243A>G mutation loads on myogenic differentiation and maturation, reductions in histone acetylation levels were observed in MELAS1 Cl2 and MELAS1 Cl4 lines throughout the mesoderm and myogenic differentiation processes. This further supports the hypothesis that the alternate mitochondrial requirements of hiPSCs and differentiating mesoderm/myogenic cells renders the accompanying cell-identity transitions alternatively sensitive to mitochondrial dysfunctions associated with m.3243A>G, the most notable of which are OXPHOS CI impairments. These data bring attention toward an interesting question: are the detriments in histone acetylation a consequence of alternate proportions of cell types within the myogenic cultures, or in fact responsible for the differentiation impairments observed? As discussed in section 7.6.4 of future directions below, experiments aiming to rescue these cellular phenotypes would be required in order to better delineate this important question. Taking into account the ever-strengthening link between mitochondrial function, histone acetylation and mesoderm/myogenic cell identity changes, it is my personal opinion that the detriments in histone acetylation are at least partly accountable for the differentiation impairments observed.

7.5 How Relevant are Differentiation Impairments to Adult-Onset mtDNA Disease Myopathic Phenotypes?

Mitochondrial diseases associated with nuclear genetic mutations often present early in childhood (Lightowlers, Taylor & Turnbull, 2015), and neurodevelopmental delay is a common phenotype of patients with mitochondrial disease caused by nDNA mutations
(Witters et al., 2018). By comparison, mitochondrial diseases associated with mtDNA mutations typically present later in life (Lightowlers, Taylor & Turnbull, 2015), and impairments in muscle development are rarely described, with myopathic phenotypes instead associated with progressive detriments in muscle function (weakness, exercise intolerance) or muscle mass (atrophy) (Ahmed et al., 2018; Ahuja, 2018).

A question that researchers in the field of hiPSC-disease modelling often receive is, how can hiPSC derived cells which are more closely related to more immature cells of early development, recapitulate cellular pathomechanisms occurring in an adult-onset disease? Whilst this may be considered a fair criticism towards the use of such hiPSC models, this does not precede the biological relevance of mechanistic observations made. Indeed, in many ways hiPSC modelling offers insight into otherwise inaccessible early stages of disease which might offer better targets for therapeutic intervention, prior to irreversible changes occurring at latter disease states. In addition, the myogenic cell-identity transitions recapitulated in the hiPSC-model established here, not only occur during developmental myogenesis, but also occur during post-developmental muscle growth/repair. In relation to the observations made here, the detriments in histone acetylation deposition observed might precede the subsequent declines in muscle cell function and/or viability which underly the myopathic mitochondrial disease state. In addition, impairments in muscle growth/repair pathways might also be contributing to the myopathic phenotypes. These data might therefore point towards modulation of histone acetylation and/or mechanisms governing muscle repair as novel strategies for improving muscle function in mitochondrial diseases with myopathic phenotypes.

### 7.6 Future Directions

Whilst the data presented in this thesis provides considerable advancements in the field of in vitro mitochondrial disease modelling, and provided initial evidence supporting a role of impairments in histone acetylation and myogenic cell-identity changes towards the myopathic phenotypes of mitochondria disease patients, as with any study, there remains a number of limitations and areas for future exploration which I discuss below.
7.6.1 Further Improvements in Reproducibility of the Myogenic Differentiation Protocol to Facilitate Biological Replication

Whilst interesting observations were reproduced across different cell line passage numbers or separate myogenic inductions wherever possible, and direct comparisons made across cell lines from individual experiments/differentiation inductions, there remains a number of observations for which biological replicates are limiting, particularly those associated with the differentiated myogenic cultures. This was in a large part associated with the difficulties faced in the myogenic differentiation process and focus of the project toward exploring multiple novel areas rather than biological replication. Whilst the hiPSC field faces difficulties associated with differentiation efficiency variabilities, much of this variability arises from inter-donor specific genetic differences (Burrows et al., 2016; Kyttälä et al., 2016; Rouhani et al., 2014; Kilpinen et al., 2017). In relation to the most interesting phenotypes observed in the MERRF1 and MELAS1 lines, this confounding factor is largely accounted for, by taking advantage of the natural heterogeneity of mtDNA mutations within a patient fibroblast population to simultaneously obtain hiPSC lines both with and without the mtDNA pathogenic variant of interest. Nevertheless, variability in differentiation efficiencies were noted between inductions (successful vs unsuccessful), with the sudden inability to successfully drive differentiation of the WT Cl2 line being particularly concerning, thus other confounding factors not accounted for are also at play. A recent report has shown that even the day a differentiation induction is started can introduce undesired variability in resultant cellular phenotypes (Volpato et al., 2018; Volpato & Webber, 2020). These observations therefore highlight the particular importance for biological replication of observations made in hiPSC-derived cell types, across several different differentiation inductions.

Whilst great progress was made in improving myogenic differentiation efficiency throughout this project, their remains room for improving the homogeneity of the endpoint cultures. More recently, inhibition of TGFβ signalling using small molecule antagonists of the ALK family of receptors such as SB-431542 or A83-01 can facilitate the formation and maturation of myotubes from hiPSC-derived myogenic progenitors (Xi et al., 2017; Sakai-Takemura et al., 2018; Hicks et al., 2018). Addition of such TGFβ inhibitors might represent a way by which myogenic cultures could be pushed towards myotube formation, reducing the amount of myogenic precursors which remain. In addition to facilitating latter myogenic cell-identity acquisition, strategies to select for or enhance a more homogenous starting population of myogenic precursors would also
prove useful. In relation to this, a number of cell-surface markers have been identified, which can be used to efficiently sort committed myogenic precursors from other contaminating cell types in the cultures through FACS (Magli et al., 2017; Sakai-Takemura et al., 2018). FACS sorting for such markers might therefore be a way by which confounding differences in overall myogenicity of different hiPSC lines can be accounted for. In addition, lentivirus encoding PAX7 under a doxycycline inducible promoter system have been used to establish cell lines which following initial mesoderm patterning through CHIR99021 inhibition of GSK3β could be efficiently differentiated into a relatively pure population of myogenic precursors (Magli et al., 2017; Rao et al., 2018). Whilst these strategies do not permit exploration of earlier stages of the myogenic differentiation programme, the improvements in purity of more terminally differentiated myogenic cell types might be better suited for assessments of these latter stages of the myogenic differentiation programme.

7.6.2 More In-Depth Explorations of hiPSC and Myotube Metabolic Fluxes

LC-MS metabolomic analysis including heavy isotope glucose flux experiments provided useful insight into downstream metabolic impairments of m.3243A>G in hiPSCs. In light of the reductions in histone acetylation observed in differentiating mesoderm and myogenic cells harbouring m.3243A>G, complementary metabolomic analysis of these differentiating cells with a particular focus on mitochondrial derived citrate would be informative for correlating the mitochondrial impairments of these lines with the reductions in histone acetylation deposition observed. In relation to this, through heavy-isotope labelling of metabolic substrates such as glucose and mass-spectrometry measurements of histone N-terminal tails, the relative contribution of carbons derived from specific metabolic substrates, to de novo histone acetylation deposition can be assessed (Kopinski et al., 2019). This could represent a useful strategy by which impairments in metabolic fluxes could be more directly correlated with the detriments in histone acetylation observed.

Given the importance of mitochondrial glutaminolysis metabolic pathways for hiPSC proliferation and viability (Tohyama et al., 2016; Zhang et al., 2016), the relatively specific reliance of early mesoderm on elevated oxidative glutaminolysis fluxes (Lu et al., 2019), and evidence of compensatory activation of reductive decarboxylative glutamine metabolism initiated in cells with mitochondrial dysfunction (Mullen et al.,
Among the many benefits of hiPSC technology for in vitro disease modelling purposes, is the ability to obtain different disease-relevant cell types by subjecting the hiPSC lines to alternate differentiation protocols. As summarised in Table 1-2 and discussed in section 1.4.6.7 of the introduction, hiPSCs harbouring a variety of different mtDNA disease mutations have been successfully differentiated into a number of different mitochondrial disease relevant cell types, most commonly neural progenitor cells, cortical neurons and cardiomyocytes. It would therefore be interesting to differentiate the hiPSC lines established as part of the work presented in this thesis towards alternate cell identities such that phenotypic comparisons can be made between different disease associated cell types. Whilst early mesoderm commitment is associated with elevated metabolic fluxes, early neuroectoderm differentiation towards NPCs is instead associated with maintenance of the glycolytic state (Cliff et al., 2017), thus it would be particularly interesting to explore the alternate effects of mtDNA mutations in these cell identity changes associated with different metabolic flux changes. In relation to this it would also interesting to explore the subsequent cell identity transition from NPCs towards more mature neurons, given impairments in this cell identity transition but not early neuroectoderm commitment have been previously described in hiPSCs harbouring mtDNA mutations (Yokota et al., 2017; Hatakeyama et al., 2015).

In addition to differentiation towards different cell types, it would also be interesting to explore further differences in downstream mitochondrial function, and resultant cellular phenotypes associated with different mtDNA mutations in the same hiPSC derived cell type. Further investigations with the MELAS2 hiPSC lines harbouring homoplasmic levels of the \textit{MT-ND5} m.13528A>G (p.T398A) and m.13565C>T (p.S410F) missense mutations affecting MT-ND5 would be particularly interesting, given the several unsuccessful attempts to differentiate the MELAS2 Cl3 cell line, and selective impairment of OXPHOS CI provoked by these mutations, for which it is postulated the mesoderm/myogenic differentiation process is particularly susceptible. In relation to this, it might be informative to explore the differential sensitivity of WT hiPSC lines during
the differentiation process, to pharmacological inhibition of the various OXPHOS complexes with rotenone (CI inhibitor), antimycin-A (CIII inhibitor), cyanide (CIV inhibitor) and oligomycin-A (CV inhibitor) for example. This would not only allow the selective importance of each of the OXPHOS complexes to the differentiation process to be explored but additionally permit better exploration of the transient importance of each of the OXPHOS complexes during the differentiation process (note however, cyanide is an irreversible inhibitor). Such inhibitors might also serve as useful controls for delineating the importance of mitochondrial function for histone acetylation maintenance/deposition in the different cell types of interest.

7.6.4 Pharmacological Rescue of the Cellular Phenotypes to Better Understand Molecular Pathomechanisms and Novel Therapeutic Strategies

Having established a disease-relevant in vitro model which potentially informs on novel pathomechanisms associated with myogenicity and histone acetylation impairments, further investigations attempting to rescue the observed phenotypes would not only provide better confidence towards the importance of the novel observations made but also guide potential strategies which might represent targets for novel therapeutic interventions.

In relation to the phenotypes observed in this project, rescue of the mitochondrial deficits represents the most upstream target for rescue. A number of different strategies have been explored for rescuing mitochondrial detriments associated with mtDNA mutations in vitro. Among these reducing mutation loads of heteroplasmic mtDNA mutations through mtDNA variant specific nucleases (e.g. restriction endonucleases, ZFNs and TALENs) (Srivastava & Moraes, 2001; Bacman et al., 2013; Gammage et al., 2014) or galactose enhancement of mitophagy (Diot et al., 2015) have shown some promising results. However, these strategies are not viable for therapeutic intervention in disease states associated with very high or homoplasmic levels of mtDNA mutations nor nDNA mutations. Other avenues explored include supplementation of metabolic co-substrates which often decline in mitochondrial disease states, including CoQ10 (Cotán et al., 2011; De la Mata et al., 2012) and riboflavin (Garrido-Maraver et al., 2012). Pyruvate and other electron acceptors such as α-ketobutyrate have also received attention, with studies showing such electron acceptors can rescue deficits in the NAD/NADH⁺ ratio and consequently OXPHOS function (Sullivan et al., 2015; Martínez-Reyes et al., 2016). Given the reductions in histone acetylation observed in this study, supplementation of
citrate or acetyl-CoA might be thought to be a useful strategy for rescue of this phenotype, bypassing mitochondrial requirements for generation of nucleocytoplasmic citrate and therefore acetyl-CoA. However, cells do not express plasma membrane transporters that permit acetyl-CoA uptake, and expression of the plasma membrane citrate carrier encoded by SLC13A5 is restricted to the liver and digestive tract (Uhlén et al., 2015). Several studies have shown that acetyl-CoA levels available for histone acetylation can be enhanced by supplementation with acetate, which is subsequently catalysed into acetyl-coA by ACSS2 (Mullen et al., 2012; Moussaieff et al., 2015). Interestingly, compensatory increases in nuclear localised ACSS2 occur when mitochondrial oxidative metabolism is impaired through restrictions in oxygen availability (Bulusu et al., 2017), and increases in overall levels of ACSS2 occur when ACLY mediated production of acetyl-CoA is inhibited (Zhao et al., 2016), both resulting in maintenance of histone acetylation levels when acetate is present in culture media. Acetate supplementation might therefore be a strategy by which histone acetylation deficits could be rescued, allowing better correlations to be made with the mesoderm/myogenic differentiation impairments observed. Supplementation of culture media during fibroblast reprogramming might also improve the reprogramming efficiency of lines harbouring mtDNA disease mutations, and reveal whether deficits in histone acetylation deposition might be among the reasons behind reprogramming impairments associated with high m.8344A>G mutation loads. These experiments could potentially reveal acetate supplementation as a novel therapeutic intervention for mitochondrial disease.

In relation more specifically to pharmacological modulators of histone acetylation, it would be interesting to explore the impact of different inhibitors and activators of specific HDAC and HAT enzymes during the myogenic differentiation process. Given the data presented in this thesis suggests mtDNA disease lines show detriments in histone acetylation modifications, inhibitors of HDACs and activators of HATs are of greatest interest. A number of HDAC inhibitors have received FDA approval for treatment of various cancers including, the HDAC class I, II and IV inhibitors Vorinostat, Romidepsin, Panobinostat and Belinostat (Suraweera, O’Byrne & Richard, 2018; Cengiz Seval & Beksac, 2019), and the specific SIRT1 (HDAC Class III) inhibitor Ex-527 has shown a promising safety profile in initial Phase I clinical trials (Westerberg et al., 2015) and subsequent Phase II clinical trials in patients with Huntington’s disease (Süssmuth et al., 2015). These compounds might therefore represent good candidates for initial testing in the in vitro model designed here, and potentially represent an accelerated route toward
the clinical use of such compounds for therapeutic intervention in mitochondrial disease patients. As discussed in section 1.1.7.4 of the introduction, SIRT1 also plays an important role in the activation of the master regulator of mitochondrial biogenesis PGC1α, and activation of PGC1α with resveratrol has been shown to improve measures of mitochondrial health in mtDNA disease patient fibroblasts *in vitro* (Mizuguchi *et al.*, 2017). It is therefore clear that further investigations are required in order to better understand the more global impact of HDAC modulators in the mitochondrial disease setting. Although pharmacological activators of HAT enzymes are much less well described/explored, a number of different salicylic-acid derived small molecules have been described in the scientific literature including, the KAT3B activator CTPB (Balasubramanyam *et al.*, 2003), the KAT3A/3B activator TTK21 (Chatterjee *et al.*, 2013) and the KAT2B activator pentadecylidenemalonate-1b which also inhibits KAT3A/3B activity (Sbardella *et al.*, 2008). Treatment with such compounds during mesoderm/myogenic differentiation induction might provide insight into the roles particular HATs play throughout the myogenic/mesoderm differentiation programme. Taking into account the hypothesis that impairments in mitochondrial derived acetyl-coA generation underly the histone acetylation and differentiation impairments observed, it is possible that such compounds may however show minimal benefit towards rescue of these cellular phenotypes.

### 7.6.5 ChIP-Seq to Gain Insight into Locus-Specific Changes in Histone Acetylation, and Correlation with Gene Expression Through RNA-Seq

Whilst the data presented in this thesis point towards global reductions in histone acetylation levels, in line with the universal requirement of acetyl-coA for the deposition of such marks, more detailed assessments of locus-specific changes in histone acetylation marks through chromatin IP followed by NGS would be informative. Indeed, locus specific changes in histone acetylation would allow better correlations to be made with the specific impairments in mesoderm/myogenic differentiation observed. Whilst global changes in histone acetylation modifications that accompany and/or drive such mesoderm and myogenic cell identity changes are important, the importance of locus specific changes for silencing of genes defining the parental cell lineage and induction of genes defining the differentiated progeny remain unknown. As part of the work carried out in this PhD project, a protocol was optimised that permits extraction of native chromatin from both hiPSCs and differentiated myogenic cultures, the subsequent digestion of extracted chromatin into 1-3 nucleosome fragments (147-441bp) suitable for
immunoprecipitation with antibodies targeting specific histone acetylation modifications and NGS library preparation (see methods section 2.2.13 for optimised protocol).

Parallel preparation of cDNA libraries for RNA-Seq would allow correlations between gene expression and locus specific histone acetylation modifications to be made, which would provide a useful link between the histone acetylation landscape and associated cellular phenotypes observed. Such analysis could also provide insight into other unexplored transcriptional changes which might reveal additional novel pathomechanisms for further exploration, and also serve as an informative data set to better characterise the maturity of the differentiated myogenic cultures.

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