Metabolic Flux Control of Mitochondrial Dynamics in Cancer Cells

This thesis was submitted to the School of Biochemistry and Immunology, Trinity College as part of a Ph.D. program.

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Declaration

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Ryan McGarrigle
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Abstract

The many functions of mitochondria have been intrinsically linked to their morphology, which is controlled by constant fusion and fission of outer and inner membranes. Mitochondrial dynamics controls the number of mitochondria and how much mitophagy takes place. This careful balance determines the energy output of the cell and controls cell death. Excessive fission leads to fragmented mitochondria, which is generally associated with metabolic dysfunction. Excessive fusion results in highly interconnected hyperfused mitochondrial networks and can serve to counteract metabolic insults and avoid autophagy. The processes that govern mitochondrial dynamics are often deregulated in cancer cells. Cancer cells alter mitochondrial dynamics to resist apoptosis and by adjusting their bioenergetic and biosynthetic needs can drive tumour initiation, proliferation and therapeutic resistance. The mechanisms by which mitochondria dynamically divide and fuse are well characterised, but the signal transduction pathways that modulate fission and fusion events are poorly described.

Using confocal microscopy, fluorescent reporter proteins and antibodies, mitochondrial fusion rates were quantified in HeLa cell. Using these techniques along with various biochemical assays it was shown that by changing the primary carbohydrate substrate from glucose to galactose caused a shift in metabolism from aerobic glycolysis to oxidative phosphorylation. The shift away from glycolysis did not affect mitochondrial fusion rates under basal conditions but when cells were treated with electron transport chain inhibitors mitochondrial fusion rates were reduced. It was observed that prolonged growth in galactose caused changed in mitochondrial supercomplex formation. Using pharmacological and molecular interventions, type II hexokinase (HKII) was identified as a potential control node of mitochondrial function in cancer cells. HKII is the predominant isoform expressed in many cancer cells has been shown to increase aerobic glycolysis and suppress apoptosis, thus ensuring cell survival. However, despite the reliance of many cancers on glycolysis, mitochondrial integrity must be maintained through the processes of mitochondrial fission and fusion, to facilitate bioenergetics plasticity, evade pro-death signalling and allow differentiation/dedifferentiation control of cell
populations. No direct link between bioenergetic control of glycolysis and mitochondrial dynamics has been shown before, although hexokinase II is known to localise to the outer mitochondrial membrane. Here we show that reducing hexokinase II protein expression using siRNA reduces mitochondrial fusion rates in HeLa cells. Detachment of HKII from the mitochondrial outer membrane caused excessive mitochondrial fragmentation and lead to cell death. Point mutations in both N- and C-terminal active sites of HKII also reduce fusion rates and increased ROS production.

These findings expand our understanding of cellular control over mitochondrial dynamics and reveal a new role of hexokinase II in promoting cancer cell survival though mitochondrial maintenance.
Abbreviations

2-DG  2-Deoxy-D-glucose
AA    Antimycin A
ADP   Adenosine diphosphate
AICAR 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside
AKT/PKA v-akt murine thymoma viral oncogene homolog/Protein Kinase B
AMPK  Adenosine monophosphate-activated kinase
ANOVA Analysis of variance
ANT   Adenine nucleotide translocase
Apaf-1 Apoptosis protease-activation factor-1
ATP   Adenosine triphosphate
BAD   Bcl-2-associated death promoter
BAX   Bcl-2-associated antagonist killer
Bcl-2  B-cell lymphoma-2
BSA   Bovine serum albumin
CAF4  CCR4 associated factor 4
CaMKII Ca2 / calmodulin-dependent protein kinase II a
CCCP  Carbonyl cyanide-m-chlorophenylhydrazone
CI-IV Complex I-IV
CoQ   Coenzyme Q
CoQH2 Coenzyme Q, reduced
DCA   Dichloroacetate
DMEM  Dulbecco's modified Eagle's medium
Dmfn  Drosophila mitofusion
dmn1  Yeast homologue of DRP1
DMSO  Dimethyl sulfoxide
DNA   Deoxyribonucleic acid
DRP1  Dynamin related protein 1
DsRed Discosoma sp. red protein
EDTA  Ethylenediaminetetraacetic acid
EGTA  ethylene glycol tetraacetic acid
ER    Endoplasmic reticulum
ETC   Electron transport chain
FAD(H2) Flavin adenine dinucleotide (reduced)
FCCP  Carbonyl-cyanide-trifluromethoxyphenylhydrazone
FDH   Fumarate dehydrogenase
FGFR  Fibroblast growth factor receptors
FMN   Flavin mononucleotide
FZO   Fuzzy onions
G6P   Glucose-6-phosphate
Gal   Galactose
GFP   Green fluorescent protein
Gln   Glutamine
Glu  Glucose
H2O2  Hydrogen peroxide
HeLa  Human cervical carcinoma
hFIS  Human fis1
HIF  Hypoxia inducible factor
HK  Hexokinase
IDH  Isocitrate dehydrogenase
IM  Inner membrane, mitochondrial
IMS  Intermembrane space
KCNC  Potassium Cyanide
LDH  Lactate dehydrogenase
LKB1  Serine–threonine kinase liver kinase B1
M  Malonate
MDV  Mitochondria division protein
MEF  Mouse Embryonic fibroblasts
MES  compound 2-(N-morpholino)ethanesulfonic acid
MFF  Mitochondrial fission factor
MFK  Mitofusin
MID49  Mitochondrial dynamics protein 49
MOPS  compound 3-(N-morpholino)propanesulfonic acid
mtDNA  Mitochondrial deoxyribonucleic acid
mTOR  Mechanistic/Mammalian target of rapamycin
NAD(H)  Nicotinamide adenine dinucleotide, reduced
NADPH  Nicotinamide adenine dinucleotide phosphate, reduced
O2  Superoxide anion
Oligo  Oligomycin
OMM  Outer membrane, mitochondrial
OPA1  Optic atrophy 1
OXPHOS  Oxidative phosphorylation
PA-GFP  Photo-activatable green fluorescent protein
PAGE  Polyacrylamide gel electrophoresis
PBS  Phosphate buffered Saline
PDH  Prolyl hydroxylase
PDK  Pyruvate Dehydrogenase
Pi  Inorganic phosphate
PI  Pixel intensity
PI3K  phosphatidylinositide 3-kinases
Pier A  Piericidin A
PINK1  PTEN-induced putative kinase 1
PMF  Proton-motive force
PPP  Pentose phosphate pathway
PTEN  Phosphatase and tensin homolog deleted on Chromosome 10
PTM  Post-translation modification
PTP  Permeability transition pore
RCR  Respiratory control ratio
<table>
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<tr>
<td>RHEB</td>
<td>Ras-homolog enriched in brain</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Rot</td>
<td>Rotenone</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate Dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethyl rhodamine methyl ester</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex proteins</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
<tr>
<td>ΔΨ</td>
<td>Membrane potential</td>
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Chapter 1: General Introduction
1.1 General Introduction

The regulation of functional and structural changes in mitochondria, one of the most essential organelles inside cells, has been studied for a relatively short period of time. Mitochondria are the site at which adenosine triphosphate (ATP), the molecular unit of free energy, is synthesised, without which most cellular processes would come to a halt. The process of ATP production and the generation of intermediate metabolites are the two salient functions of mitochondria; leading to the view that they are bioenergetic and biosynthetic organelles. However, not limited to these functions, mitochondria also control levels of intracellular calcium and programmed cell death, making them important signalling organelles as well. The structure of these integral organelles appears to be directly related to their function, giving rise to the field of “Mitochondrial Dynamics”. It has been proposed that structural changes can occur to meet the energy demands of the cell, conversely, it is emerging that cell bioenergetics can directly influence mitochondrial structure in a bi-directional cross talk. Recent advances within imaging technology have allowed proper assessment and quantification of changes in mitochondrial structure within the cell, in their fully functional state, for the first time. Mitochondrial studies in cancer cells are still incomplete despite the known link between cancer and altered states of metabolism. In this study, the influence of bioenergetics on the structure of the mitochondria are examined in cancer cells and what follows is a brief overview of the current knowledge of mitochondrial dynamics and tumour metabolism and mitochondrial respiration.

1.1.1 Mitochondria

The critical role of Mitochondria in the life of a cell is well established – they have been labelled ‘the power plant’ within cells. Their task, although key, has been simplified to carrying out a series of oxidative reactions to produce energy in the form of ATP. Mitochondrial ATP generation allows cells to maintain a high ATP/ADP ratio, which is necessary to thermodynamically drive many intracellular biochemical reactions. The importance of mitochondrial ATP can be demonstrated dramatically in vivo by the use of toxins such as cyanide, which inhibit respiration and thereby ultimately causes cell death (Berg 2002). In the past two decades, the research has increasingly shifted to the
mechanisms by which mitochondria communicate their biosynthetic and bioenergetics state and fitness to the rest of the cell. The mitochondrial signalling mechanisms have far reaching functions beyond metabolism. In a healthy cell, no biological process should be undertaken without first considering the fitness of mitochondria. This ensures no discrepancy between the metabolic demand of the cell and the ability of the mitochondria to meet that demand. Functions such as calcium-dependant signalling (Rizzuto, De Stefani et al. 2012), reactive oxygen species (ROS) generation to activate hypoxic gene expression (Chandel, Maltepe et al. 1998) and the control of programmed cell death, apoptosis, through the release of cytochrome c (Liu, Kim et al. 1996) ensure that the demands are met.

As mitochondria are associated with many diverse cellular processes and because their dysfunction is implicated in several diseases, it is important to have an understanding of how they are created, maintained and regulated. Mitochondria are unique organelles in that they contain their own genome, mitochondrial DNA (mtDNA). The endosymbiotic theory has now come to be accepted and postulates that genome-containing organelles such as mitochondria and chloroplasts are derived from ancient bacteria that formed a symbiotic relationship with an early proto-eukaryotic cell, approximately 1.45 billion years ago (Margulis 1970, Margulis 1981, Emelyanov 2001). Since their discovery 125 years ago, comparisons to bacteria have been expressed without experimental evidence to justify a serious link. With the discovery of mtDNA (Nass and Nass 1963) the theory now gain credence. The characteristics that mitochondria display that bear resemblance to their bacteria-like ancestors include double membranes, circular plasmid DNA and the ability to synthesize ATP across its membranes (Ernster and Schatz 1981). Due to these distinct characteristics of mitochondria and the maternal mechanism of inheritance, dividing cells have the extra challenge of segregating mitochondria effectively. This is achieved through remodelling where mitochondria can fuse with each other and divide within the cell. There is a constant cycle of fusion, where two mitochondria join to form a singular mitochondrion, and fission, in which singular mitochondrion divide into two mitochondria. The balance of this process allows for the maintenance of mtDNA within the cell, provides a good mechanism for mitochondrial distribution during cell division and also has very important roles in mitochondrial function and dysfunction (Section 1.2.4).
Mitochondrial structural changes, via fission and fusion, have become collectively known as mitochondrial dynamics. Through technological advances in microscopy and the use of receptor proteins, mitochondrial dynamics can be visualised and has become a key research subject with implications for many cellular functions and ultimately cell survival. To ensure survival, mitochondria must be able to adapt to meet cellular energy demands that are a result of physiological and environment changes (Jones and Thompson 2009). Cancer cells are bioenergetically highly active and robust cells, capable of surviving in many extreme environments whereas on the opposite of the spectrum neurons have fine metabolic limits where even small changes can cause catastrophic cell death (Ames 2000, Strausberg 2005). Mitochondrial dynamics are of high importance in these types of cells, but for differing reasons.

1.1.2 Mitochondrial Bioenergetics: Overview

Bioenergetics is the broad term given to the reactions that are concerned with energy flow through biological systems. Energy is usually utilized in the form of ATP, a molecule that when hydrolysed provides the free energy required for most cellular processes. ATP is produced in two ways in mammalian cells, either via glycolysis or oxidative phosphorylation (OXPHOS), yielding a net amount of two or thirty-six molecules of ATP, respectively. In non-proliferative healthy tissue, 80-90% of ATP is produced by the much more efficient OXPHOS system rather than glycolysis (Lodish 2000). Through the double membrane structure of mitochondria and its functionally specialized compartments, a number of pathways such as the β-oxidation of fatty acids, oxidation of amino acids, the tricarboxylic acid cycle (TCA cycle, also known as the Krebs Cycle) and the complexes of the electron transport chain (ETC) converge to produce ATP (Nelson 2008). Many of the ideas connected to mitochondria and energy such as the discovery of cytochromes, mitochondrial respiration, OXPHOS and recognition of the role of ATP, had already been established independently. Despite these insights it was not until the formulation of the “Chemiosmotic hypothesis” by Mitchell and colleagues that an understanding of how all these interconnected pathways oversee the conversion and storage of chemical energy in living organisms was realised (Mitchell 1961). The pathways mentioned above are utilized to produce reduced electron carriers such as nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH\textsubscript{2}), which then feed electrons into the
complexes of the ETC (Lodish 2000). The ETC is comprised of four complexes (CI - IV) located on the mitochondrial inner membrane (IM). Free energy provided by electrons from NADH and FADH$_2$ are used to create a proton (H$^+$) gradient between the IM and the intermembrane space (IMS). This is achieved by pumping H$^+$ from the mitochondrial matrix via the membrane spanning complexes of the ETC, by CI, CIII and CIV into the IMS (Figure 1.1). H$^+$ are pumped against the concentration gradient, creating a gradient across the membrane, effectively storing energy in the form of a transmembrane electrochemical gradient referred to as the proton motive force (PMF). Protons forced into the IMS can travel down the gradient from high concentration to low concentration back inside the matrix again through another transmembrane protein complex known as ATP synthase (also referred to as CV) (Scheffler 2007). While not part of the ETC, ATP synthase is coupled to the ETC (Figure 1.1). The energy generated by the PMF is enough to cause ATP synthase to rotate; this rotation is used to combine adenosine diphosphate (ADP) with inorganic phosphate (P$_i$) to form ATP. Newly formed ATP is translocated to the cytosol by the adenine nucleotide translocase (ANT), an IM antiporter that exchanges ATP for ADP (Scheffler 2007).
Figure 1.1: Pathways that converge to produce ATP in mitochondria. Multiple cellular pathways converge from glycolysis, fatty acid synthesis and glutaminosis at the TCA to generate the electron carriers NADH and FADH$_2$ that are then used at the ETC to create and electrochemical gradient by pumping protons across the IMM. Protons then travel through ATP synthase back into the matrix utilizing the potential energy to generate ATP.
1.1.3 The Electron Transport Chain

The electron transport chain is comprised of four protein complexes found within the mitochondrial IM (Figure 1.1) and they provide a way of converting the energy stored in carbon bonds provided from diet, to useful energy in the form of ATP. The catabolism of sugars, fats and proteins in the TCA cycle (Figure 1.1) are used to reduce three molecules of NAD$^+$ to NADH and one molecule of FAD into FADH$_2$ (Nelson 2008). When reduced, they transport the electrons to CI and CIII of the ETC. The electrons begin in a high-energy state, but as they are passed down to lower states through the ETC, the energy is used to pump protons out of the matrix (Lodish 2000). Thermodynamically and electrochemically each step in the ETC is associated with a free energy change and each component undergoes a cycle of oxidation and reduction reactions. The ETC can be considered from the point of view of an electron, finding itself at a high potential (in NADH or FADH$_2$), then dropping through a series of intermediate stages at CI or CII respectively, being passed along the via ubiquinone to CIII and CIV and to the final acceptor, oxygen. The drop in potential between each intermediate step is used for proton pumping; the drop is also so great that the reaction is virtually irreversible (Scheffler 2007). CII, also known as succinate dehydrogenase, is the only member of the chain that is not used for proton pumping. CI and II both pass their electrons to a mobile electron carrier called ubiquinone (also referred to as coenzyme Q) then onto CIII. CIII then passes its electron to CIV via another mobile electron carrier called cytochrome c (Scheffler 2007).

CI, properly referred to as NADH-ubiquinone oxidoreductase, is the largest of the four complexes and is comprised of 45 subunits (>900kDa) in mammalian mitochondria (Sazanov 2015). The overall reaction catalysed by this complex can be described as follows:

\[ \text{NADH} + \text{Q} + 5\text{H}^+_{\text{matrix}} \rightarrow \text{NAD}^+ + \text{QH}_2 + 4\text{H}^+_{\text{inter membrane}} \]

Q and QH$_2$ refer to the oxidized and reduced forms of ubiquinone, respectively. The reaction is accompanied by the net transfer of four protons from the matrix side to the inter membrane space. It is a membrane-spanning complex and the complex’s shape is often represented by a boot or L-shape, with the long arm extending into the inner membrane and the short arm facing into the matrix. The short arm of the L is the functional region of
the NADH dehydrogenase containing a flavin mononucleotide (FMN) cofactor as well as seven to nine iron-sulphur (Fe-S) clusters (species dependent) (Sazanov 2007). Initially NADH binds to FMN and transfers its two high-potential electrons to give the reduced form FMNH₂ and they are then further transferred to the series of Fe-S clusters. Electrons are then passed to ubiquinone and the reduction of ubiquinone leads to the pumping of the four protons from the matrix to inter membrane space (Scheffler 2007).

CII (succinate;ubiquinone oxidoreductase) is the simplest of all the complexes consisting of only four polypeptides and is found anchored to the inner membrane. CII also functions as succinate dehydrogenase in the Krebs cycle. Electrons from the oxidation of succinate to fumarate are channelled through this complex to ubiquinone; this provides a direct link between the ETC and the TCA cycle (Iverson 2013). The oxidation of succinate generates FADH₂, electrons are passed to Fe-S centres in CII and then on to ubiquinone for entry into the ETC. Summarised as:

\[
\text{Succinate} + Q \rightarrow \text{fumarate} \ QH_2
\]

Reduced ubiquinone, or ubiquinol, transfers electrons to the next proton pumping complex in the respiratory chain.

CIII (ubiquinone-cytochrome c oxidoreductase) is also referred to as cytochrome c reductive. Mitochondrial cytochromes are electron-transferring proteins that contain a heme prosthetic group that can alternate between a ferrous reduced state (+2) and an oxidized ferric state (+3). Electrons are transferred from ubiquinol to oxidized cytochrome c (cyt c) and in doing so this pumps H⁺ out of the matrix (Iverson 2013) (Scheffler 2007). The reaction is summarised as follows:

\[
\text{QH}_2 + 2 \text{Cyt c}_{\text{ox}} + 2 \text{H}^+_{\text{matrix}} \rightarrow Q + 2 \text{Cyt c}_{\text{red}} + 4 \text{H}^+_{\text{inter membrane}}
\]

The final stage in the ETC is the oxidation of the reduced cyt c generated from CIII, which is coupled to the reduction of O₂ to two molecules of H₂O. CIV (cytochrome c oxidase) pumps four more H⁺ from the matrix using molecular oxygen as the terminal electron acceptor (Lodish 2000). Mammalian CIV contains 13 subunits, it contains four redox centres: two heme groups, a and a₃, a copper atom called CuB and a copper atom pair
called the CuA centre (Lodish 2000). A molecule of reduced cytochrome c transfers an electron initially to the CuA pair and from there then the electrons moves through heme a to heme a3 which is reduced to the Fe^{2+} form. This reduction is important because that when in the Fe^{2+} form cytochrome c oxidase is now capable of binding oxygen. Reduced CuB and the heme a3-oxygen complex now allow the oxygen to be reduced forming a bridge between the heme a3 and the CuB^{2+}. With the addition of a H^+ and another electron the O=O bond is cleaved leaving CuB^2–OH and Fe^{4+}=O at the heme a3 site (Scheffler 2007). The addition of the final electron from cyt c and a second H^+ reduces the heme a3 to give Fe^{3+}-OH. Reaction with two additional protons allows the release of two molecules of water and resets the enzyme to its initial, fully oxidised form. For each reaction cycle where two molecules of water are produced four H^+ are pumped from the matrix, therefore in each complete cycle CIV removes eight H^+ from the matrix (Lodish 2000), fully summarised as follows:

\[
4 \text{Cyt c}_\text{red} + 4 \text{H}^+ (s) \text{matrix} + 4 \text{H}^+ (p) \text{matrix} + \text{O}_2 \rightarrow 4 \text{Cyt c}_\text{ox} + 2 \text{H}_2\text{O} + 4 \text{H}^+ \text{inter membrane}
\]

### 1.1.4 Oxidative Phosphorylation (OXPHOS)

It was first assumed that a high-energy intermediate formed following electron transfer, would act as a phosphoryl transfer or to the formation of an activated protein conformation then driving ATP synthesis. The search for these intermediates proved fruitless. Not until 1961 did Mitchell and colleagues propose the highly controversial theory, for the time, that electron transport and ATP synthesis are coupled by a H^+ gradient across the inner mitochondrial membrane (Mitchell 1961). The oxidation of NADH and FADH\_2 are coupled to the phosphorylation of ADP though this H^+ gradient (Nelson 2008).

This model, known as the “chemiosmotic hypothesis”, places the emphasis on H^+, and therefore on the pH gradient across the inner membrane. Since protons are also charged, such a gradient also creates a membrane potential, ΔΨ (Scheffler 2007). The driving force for ATP synthesis is not only the concentration gradient represented by ΔpH, but also an electrochemical contribution by moving a positive charge from a high to low potential. The PMF (or pH gradient) generated by the active pumping of protons across the IM creates a potential difference across the membranes; a net negative charge is then conferred to the matrix. The electrochemical potential is made up of the electric potential (ΔΨ) and
chemical potential ($\Delta \mu_{H^+}$) (Scheffler 2007). The chemiosmotic hypothesis has now become firmly established by direct experimental evidence (Nath and Villadsen 2015).

ATP synthase uses the potential energy stored in the proton gradient to drive the synthesis of ATP from ADP and $P_i$ (Figure 1.2). It is estimated that between 3 and 4 protons are required to synthesis one molecule of ATP, the ratio can change depending on cellular conditions (Lodish 2000). This reaction is reversible and protons can be pumped out of the matrix at the expense of ATP during times of decreased membrane potential, meaning that protons will be pumped into the IMS to maintain membrane potential (Berg 2002).

ATP synthase is divided into two subunits the $F_o$ and the $F_1$ subunit. The $F_1$ subunit consists of five types of protein; it is the site of the catalytic activity on the inside of the matrix. The $F_o$ subunit is the hydrophobic segment that spans the inner mitochondrial membrane and it contains the proton channel (Berg 2002). An oxygen molecule of ADP attacks the phosphorus atom of $P_i$ to form a pentavalent intermediate, which can then dissociate into ATP and $H_2O$. Enzyme bound ATP forms readily on the surface of the enzyme. ATP does not leave the catalytic site unless $H^+$ flow through the enzyme therefore the $H^+$ gradient does not form ATP but rather releases it from the synthase (Berg 2002).

Using a range of techniques including cryoelectron microscopy, NMR and fluorescence microscopy it was observed that ATP synthase is actually a molecular motor (Stock, Leslie et al. 1999). Protons enter the $F_o$ subunit and exit on the proton-poor matrix side, driving the motor. A component of the $F_o$ subunit known as the ‘c’ ring contains several aspartate residues that form a series of half-channels. When an aspartate residue is protonated to its neutral form, the c ring can rotate (Stock, Leslie et al. 1999). This rotation moves a newly protonated aspartic acid residue into contact with the membrane, this in turn moves the charged aspartate residue from contact with the matrix half-channel to the cytosolic half-channel. This further moves a different protonated aspartic acid residue from contact with the membrane to the matrix half-channel (Stock, Leslie et al. 1999) (Berg 2002). The protons can then dissociate from the aspartic acid and move through the half-channel thereby completing its journey back into the matrix thus, restoring the original state.
These processes all contribute to producing ATP in healthy cells. ETC defects have an immediate effect on ATP production and therefore critically affect cell survival. Inhibition of CI or CIII usually will increase the production ROS that can then go on to have a wide range of effects on a cell including: DNA damage, promotion of apoptosis, induces immune responses, and deactivate enzymes by oxidation of cofactors (McLennan and Degli Esposti 2000, Liu, Fiskum et al. 2002, Belikov, Schraven et al. 2015, Kim and Song 2016). Excessive ROS production has been linked to carcinogenesis (Ott, Gogvadze et al. 2007). What this study hopes to achieve is a further understanding of how mitochondrial function relates to structural changes and how inhibition of the ETC and other bioenergetics proteins affect the behaviour of mitochondria.
Figure 1.2: The function and molecular composition of ATP synthase. (A) Protons from the intermembrane space in high concentration enter the $F_0$ region of ATP synthase, travel through a half-channel where they then bind to an aspartate residue. The aspartic acid is now exposed to the membrane causing the c ring to rotate. The proton now will be exposed to the matrix side half-channel dissociates from the aspartic acid and move down the concentration gradient into the matrix. This rotation causes the $F_1$ subunit (B), the catalytic region to rotate releasing the newly formed ATP. Taken from (Lytovchenko, Naumenko et al. 2014).
1.2 Mitochondrial Dynamics

The dynamic nature of mitochondria was not fully realised until recently with the help of technological advances. The use of fluorescent microscopy combined with specific antibodies with conjugated fluorescent dyes, specifically to mitochondria and related proteins, allows for visualisation within cells. Rhodamine derivative dyes also allowed for visualisation of mitochondria in living cells. These dyes accumulate inside the organelle driven by membrane potential changes (Scaduto Jr and Grotyohann 1999). Other techniques such as expressing proteins with fluorescent tags also helped visualise mitochondria in living cells. These techniques allow us to visualise mitochondria without the restrictions associated with electron microscopy. When represented in dynamic manner with temporal resolution, it was observed - contrary to the model used in many texts - that mitochondria did not solely arrange as singular isolated organelles in the cytoplasm. Rather, they arranged as a highly dynamic interconnected tubular network within eukaryotic cells (Chen and Chan 2009).

The structural organisation, size, shape and movement of these organelles are regulated by frequent fission and fusion events along the cytoskeleton. Singular mitochondrion of 2 – 25 µm in length can be divided into two or more smaller organelles of ~0.5 µm or larger (Scheffler 2007). Through fusion, several mitochondria can combine with each other to form a larger singular mitochondrion. Though at first glance movements are seemingly random, these behaviours are critically important to mitochondrial function. Defects in either process of fusion or fission, usually result in embryonic lethality in mice (Ishihara, Nomura et al. 2009, Wallace and Fan 2009). At a cellular level mitochondrial dynamics are responsible for multiple functions including maintenance of mitochondrial morphology, mtDNA sharing and stability, respiratory capacity control, apoptosis and response to cellular stresses (Lee, Jeong et al. 2004, Frezza, Cipolat et al. 2006, Detmer and Chan 2007).

The control mechanisms of dynamics are still under investigation though some of the key proteins have been identified. Fission and fusion are mediated mainly by a group of dynamin-related guanosine triphosphatase (GTPases), often referred to as ‘mitodynamins’ (Scott and Logan 2011).
The key player in fission has been identified as dynamin-related protein 1 (DRP1). DRP1 assembles on mitochondrial tubules and is thought to mediate constriction and scission (Loson, Liu et al. 2014). DRP1 has been shown to be essential for most types of mitochondrial fission. Inhibition of DRP1 by expression of dominant negative variants or by RNA interference in mouse embryonic fibroblasts (MEF) result in elongated mitochondria that tangled and subsequently collapse (Wakabayashi, Zhang et al. 2009). DRP1 mutants also show resistance to mitochondrial fragmentation following dissipation of mitochondrial membrane potential after treatment with the ionophore carbonyl cyanide-p-trifluromethoxyphenylhydrazone (FCCP), which promotes mitochondrial fragmentation (Wakabayashi, Zhang et al. 2009). Other proteins are also thought to be involved in fission, such as FIS1, although so far, their roles are not as well understood as DRP1.

Dynamin-related proteins found in the mitochondrial OM and IM mediate fusion. Fusion occurs in two steps: at first outer fusion occurs, which is then followed by inner fusion. Mutations in the genes coding for these proteins give rise to fragmented mitochondria, because fission still occurs while fusion is blocked.

1.2.1 Mitochondrial Fusion

Fusion proteins collectively known as mitofusins were firstly discovered in mutant Drosophila where male sterility was observed (Hales and Fuller). For spermatids to mature, multiple mitochondria must fuse to form two elongated mitochondria that wrap around each other to form a spherical structure called the nebenkern (Hales and Fuller, Scheffler 2007). Cross-sectioned electron microscope images revealed the nebenkern to have an onion-slice like appearance due to the concentric membranes formed by the wrapped mitochondria. Mitofusin-deficient sterile mutants lacked the definition in slices and were therefore named fuzzy onions (FZO) due to the disruption of the nebenkern (Hales and Fuller, Hwa, Hiller et al. 2002). The two mammalian homologues, mitofusin 1 and mitofusin 2 (MFN1 and MFN2) (Santel and Fuller 2001, Legros, Lombes et al. 2002, Chen, Detmer et al. 2003), localise to the mitochondrial outer membrane. Knock out studies have shown that cells lacking either MFN1 or MFN2 have highly fragmented mitochondria in contrast to the large interconnected tubular networks observed in wild-
type cells (Santel and Fuller 2001). Another fusion protein identified through human genetic studies is optical atrophy 1 (OPA1), a mutation in the gene encoding for OPA1 leads to a disease in which retinal ganglion cells degenerate and cause atrophy of the optic nerve (Griparic, van der Wel et al. 2004). OPA1 is the mammalian ortholog of Mgm1 found in yeast, which was found to be essential for mitochondrial fusion in yeast (Zick, Duvezin-Caubet et al. 2009). Depletion of OPA1 results in severe mitochondrial fragmentation due to the loss of mitochondrial fusion (Griparic, van der Wel et al. 2004).

Mitochondrial fusion promotes exchange of mtDNA and other vital components, thus reinvigorating the mitochondrial network. It is distinct from other forms of intracellular fusion events such as vesicle docking as identical organelles must fuse together and exclude all other intracellular membranes. Successful mitochondrial fusion also requires coordination of the fusion of outer and inner membranes, which are biochemically distinct. Outer membrane fusion is controlled by the highly conserved GTPases, Fzo in yeast and MFN1 and MFN2 in humans (Chen, Detmer et al. 2003). Fzo is an integral outer membrane protein that acts in a similar way to SNARE proteins of the secretory pathway. MFN1 and MFN2 appear to have some functional differences, which would suggest that they fulfil partially redundant functions in the fusion process, although both proteins can support fusion by themselves (Chen and Chan 2010). The lack of functional mitofusin reveals its essential role in remodelling mitochondria. Work carried out by Hermann and colleagues on yeast strains lacking the mitofusin homologue showed that two separate population of mitochondria fluorescently labelled green and red could not merge with each other as you would expect to see in the wild-type cells (Hermann, Thatcher et al. 1998). This was also observed in mouse models as cells cultured from the knock out models displayed highly fragmented mitochondria (Chen, Detmer et al. 2003).

### 1.2.2 OM Fusion

The large GTPase mitofusins traverse the outer mitochondrial membrane twice with an amino and carboxy termini both facing into the cytoplasm (Figure 1.3, upper section). The mitofusins on separate adjacent mitochondrion bind to other copies of themselves (Koshiba, Detmer et al. 2004). Mitofusins are required on both mitochondria; since mitofusin-deficient mitochondria cannot fuse with wild-type mitochondria (Koshiba,
Detmer et al. 2004). MFN1 and MFN2 complex with each other to form homo-oligomeric and hetero-oligomeric complexes (Detmer and Chan 2007). This mediates the fusion event and the oligomerisation explains why there is a requirement for both mitochondria to have a functional mitofusin. The oligomer forms an extended antiparallel-coiled coil bringing the outer membranes to within approximately 100 Å of each other and thereby tethers mitochondria. Once tethered to one another, GTP hydrolysis then becomes crucial for fusion and GTPase domain defects lead to tethered but unfused mitochondria (Hales and Fuller 1997). The exact mechanism by which GTP hydrolysis triggers a mitofusin to merge the membranes still remains unknown.

1.2.3 IM Fusion

Another GTPase, OPA1 in humans or MGM1 in yeast, controls inner membrane fusion. OPA1 is located in the mitochondrial inner membrane facing into the intermembrane space (Olichon, Emorine et al. 2002), although there have been conflicting reports on the exact locations of OPA1 isoforms (Satoh, Hamamoto et al. 2003) (Figure 1.3, lower section). It contains an N-terminal mitochondrial targeting sequence, a transmembrane domain, a central dynamin-related GTPase domain and a C-terminal helical domain (Satoh, Hamamoto et al. 2003). Apart from perturbation of mitochondrial fusion, studies showed MEF cells carrying null alleles of OPA1 had other cellular defects including reduction and disorganization of cristae membranes, severely reduced respiratory capacity and sensitivity to apoptosis (Satoh, Hamamoto et al. 2003, Frezza, Cipolat et al. 2006).
Figure 1.3: Fusion of two mitochondria to form one mitochrondrion. Mitochondrial fusion required two types of proteins Mitofusin 1/2 and OPA1 for outer-membrane (OM) and Inner-membrane (IM) fusion, respectively.
1.2.4 Mitochondrial Fission

Mitochondrial fission facilitates the redistribution of mitochondria. Redistribution can occur for many reasons including the response to local changes in the demand for ATP and allowing for disposal of faulty mitochondrial fragments through mitophagy (Mao and Klionsky 2013).

Fission is required for cells to divide and in the case of neurons, transport mitochondria from the cell body, along the axon, to the synapse. Fission is the opposite of fusion; it is the process where one mitochondrion divides into two separate mitochondria. This is very important for cell division as de novo mitochondria cannot be created (Scott and Logan 2011). A deficit in fission leads to highly fused, elongated mitochondria (Chan 2012).

The precise balance between fusion and fission must be maintained to ensure that the mitochondria remain a dynamic population, thus ensuring metabolic stability and overall architecture and infrastructure of the mitochondria. Mitochondrial fission contributes to maintaining proper distribution and quality control of mitochondria.

The absence of fission in neurons results in mitochondrial accumulation in the cell body with no transport of mitochondria to the synapse. This leads to neurite death and as a result neurons would no longer be able to communicate with each other via the release of neurotransmitters (Chen and Chan 2009).

1.2.5 DRP1 and fission

The master mediator in fission is a large cytosolic dynamin related protein, GTPase DRP1 (DNM1 in yeast), another protein analogous to endocytic vesicle machinery. Like classical endocytic Dynamin, DRP1 can self-assemble into spirals and use GTP hydrolysis to undergo conformational change (Ingerman, Perkins et al. 2005, Mears, Lackner et al. 2011). DRP1 is recruited from the cytosol to the mitochondrial outer membrane (Figure 1.4) and is thought to mediate constriction and scission. Because DRP1 is mostly found in the cytosol it must be recruited to the mitochondria, then it must bind to a second class of receptor proteins located on the mitochondrial outer membrane. This second class of
fission proteins is much less understood and the list of proteins is currently incomplete, proteins such as mitochondria division protein (MDV1), CCR4 associated factor 4 (CAF4) and fission 1 (FIS1) have been discovered in yeast but their roles in higher eukaryotes is more confusing (van der Bliek, Shen et al. 2013). Receptors for DRP1 such as mitochondrial fission factor (MFF), mitochondrial dynamics protein 49 (MID49) and ganglioside-induced differentiation-associated protein 1 (GDAP1) have been discovered yet it is not clear whether they take part in redundant pathways or act at different steps in the same pathway (van der Bliek, Shen et al. 2013).
Figure 1.4: Fission of one mitochondrion to form two mitochondria. During fission, DRP1 is recruited from the cytosol to the outer mitochondrial membrane. It is then thought to constrict following oligomerisation leading to scission of mitochondrial tubules.
The physiological relevance of fission is most clearly demonstrated by Waterham and colleagues (Waterham, Koster et al. 2007). The authors reported a new born girl with a defect of fission in mitochondria and peroxisomes caused by a heterozygous, dominant-negative mutation in the DRP1 gene resulted in abnormal brain development, optic atrophy and hypoplasia, microcephaly, persistent lactic academia and a mildly elevated plasma concentration of very-long-chain fatty acids. The defect in DRP1 proved lethal after 37 days.

Other studies independently revealed that a DRP1 deficiency results in embryonic lethality, with death in utero at E10.5-12.5 in DRP1+/− knockout mice (Ishihara, Nomura et al. 2009, Wakabayashi, Zhang et al. 2009). The main developmental abnormalities found in the knockout mice were in forebrain development, liver development and in synapse development; reinforcing the concept that fission is important for mitochondrial transport along the axon (Ishihara, Nomura et al. 2009, Wakabayashi, Zhang et al. 2009). Interestingly, MEFs cultured from the DRP1−/− mice had relatively normal rates of respiration and remain viable. The mitochondria appeared to be highly elongated yet function remained. Differences between controls and knockouts became apparent during cell division where it was observed that mitochondria did not fragment during mitosis like in control cells (Ishihara, Nomura et al. 2009) resulting in all daughter cells inheriting mitochondria but in an uneven fashion. DRP1 does not appear to be essential for mitochondrial fission during cell division but acts to ensure even distribution of mitochondria. It was revealed that daughter cells with less mitochondria had reduced ATP production and had increased susceptibility to apoptosis (Ishihara, Nomura et al. 2009). Another study showed that overexpression of DRP1 in C.elegans causes excessive mitochondrial fragmentation and in the same study RNAi knock-downs blocked OM scission, although inner-membrane scission still occurs (Labrousse, Zappaterra et al. 1999).

1.2.6 Mehanisms of fission

The precise molecular mechanisms underlying mitochondrial fission are not entirely understood. Fission has been studied largely in yeast and Drosophila however many of the fission protein homologues have yet to be discovered in mammalian cells. At least in yeast
Dnm1 mediates fission via a mitochondrial outer membrane protein, FIS1, and a soluble protein, MDV1, or its parologue CAF4 [one study showed that MDV1 and CAF4 may not be functionally equivalent (Guo, Koirala et al. 2012)] (Griffin, Graumann et al. 2005, Naylor, Ingerman et al. 2006). During fission, FIS1 functions as the mitochondrial receptor for Dnm1 via MDV1/CAF4 and MDV1 facilitates a transient interaction between Dnm1 and FIS1. A helix-loop-helix motif on the N-terminal region of MDV1 interacts with FIS1. The C-terminal WD40 domain interacts with a variable domain of Dnm1 (Tieu, Okreglak et al. 2002, Bui, Karren et al. 2012). After Dnm1 is transported to the mitochondrial outer membrane, Fis1-MDV1 nucleates the Dnm1 assembly at scission sites, where Dnm1 oligomerises and subsequently constricts the mitochondrial membrane in a GTP-dependent manner (Ingerman, Perkins et al. 2005, Bhar, Karren et al. 2006).

One study demonstrating a role for DRP1 in mitochondria fission used DRP1 fused to GFP to show that DRP1 is concentrated in specific foci on mitochondria where scission eventually occurs (Figure 1.5). DRP1-GFP accumulated in small patches that were invariably localized to the parts of the mitochondrial outer membrane that were constricted (Labrousse, Zappaterra et al. 1999).
During mitochondrial fission DRP1, which normally exists as either cytosolic dimers or tetramers, assembles into large oligomers as foci at the mitochondrial fission sites controlled by GTP binding (Smirnova, Griparic et al. 2001). These oligomers then wrap around the mitochondrial tubule and constrict and sever the mitochondrial membrane following conformational change by GTP hydrolysis (Smirnova, Griparic et al. 2001).

MDV1 and CAF4 homologues have yet to be found in mammalian cells. There are several other fission-related proteins that have been identified, though their mechanism in mitochondrial fission remains elusive (Otera, Ishihara et al. 2013).

Human FIS1 (hFIS1) remains somewhat of an enigma and its role in the induction of mitochondrial fission in human cells remains controversial (Otera, Ishihara et al. 2013); although its role in yeast is well established. hFIS1 is evenly localised throughout the mitochondrial outer membrane as expected, though DRP1 recruitment to the membrane is not or is only marginally affected by hFIS1-knockdown or overexpression (Lee, Jeong et al. 2004). One study showed that hFIS1 is dispensable for fission in HCT116 cells.

Figure 1.5: DRP1 is enriched foci on mitochondria where mitochondria divide. DRP1 (green) can be seen localising at regions on mitochondria (red) that subsequently appears to separate. Taken from (Labrousse, Zappaterra et al. 1999).
suggesting that there must be other mechanisms that control fission in human cells, potentially other receptors for DRP1 to bind (Otera, Wang et al. 2010).

1.2.7 Role of MFF in fission

hFIS1 depletion does not affect mitochondrial recruitment of DRP1 and hFIS cannot rescue the phenotype in yeast cells lacking FIS1 (Lee, Jeong et al. 2004, Stojanovski, Koutsopoulos et al. 2004). This leaves the door open for other candidates that act as DRP1 receptor in mammalian cells. One promising protein is MFF, a C-tail anchored protein identified in a Drosophila RNA interference library search (Otera, Ishihara et al. 2013). RNAi of MFF resulted in mitochondrial elongation and overexpression induced mitochondrial fragmentation. These results taken together suggest that MFF plays a role in mitochondrial fission (Otera, Wang et al. 2010). Both in vitro and in vivo experiments demonstrated that MFF transiently interacts with DRP1 through its N-terminal cytoplasmic region (Otera and Mihara 2011).

1.2.8 Mito-ER tethering

Recent studies have highlighted the importance of mitochondria-endoplasmic reticulum contact sites in relation to mitochondrial fission, fusion, and apoptosis. The ER is the largest of the membrane bound organelles, it is the site of protein synthesis and controls protein trafficking. The ER is also an intracellular store of Ca\(^{2+}\) and holds most of the biosynthetic enzymes that are required for cellular lipid production. ER-mitochondrial contact sites have been well established as the site for lipid and calcium flux. Areas of contact are known as mitochondrial-associated membranes (MAMs), in the sites are located many proteins that allow for the transfer of proteins, lipids and calcium between the ER and mitochondria (Csordás, Renken et al. 2006, Flis and Daum 2013). Despite the close interaction at contact sites, mitochondria and ER do not fuse thus maintaining their own distinct structures. A number of tether proteins have been identified however the mechanisms are far from understood. It was proposed that MFN2 formed part of a tether complex (de Brito and Scorrano 2008) (Naon, Zaninello et al. 2016), further studies in
several other labs have cast doubts on this assumption as a loss of MFN2 leads to increased ER/mitochondrial contact sites (Cosson, Marchetti et al. 2012) (Filadi, Greotti et al. 2017). The mechanisms of which have been shown to regulate mitochondrial dynamics, particularly DRP1 mediated fission (Hoppins and Nunnari 2012). Currently, it is unknown how ER identifies sites for mitochondrial division by it is most likely to be regulated through various molecular tethers, reviewed in detail by Pailluson (2016).

The human mitofusin protein MFN2 appears to have additional roles beyond mediating mitochondrial fusion. In 2008, de Brito and Scorrano showed that MFN2 exists on the endoplasmic reticulum (ER) membranes and tethers ER to mitochondria. In cultured cells lacking functional MFN2, mitochondria were not held as closely against ER membranes as in wild-type cells. Close contact between mitochondria and ER is important for the transfer to mitochondria of calcium released from the ER during cell signalling events (de Brito and Scorrano 2008). It remains to be established how mitofusins enable fusion between outer mitochondrial membranes are only capable of tethering (with no fusion) mitochondria to ER membranes. Another study showed that defective MFN2 in neurons slows mitochondrial transport along axons and results in mitochondrial clustering around the nucleus instead of movement to synapses (Misko, Jiang et al. 2010). Further research will determine the relative importance of mitochondrial fusion, mitochondria-ER tethering and mitochondrial transport in the symptoms of MFN2-associated Charcot-Marie-Tooth disease type 2A.

1.2.9 DRP1 and actin at ER sites

Mechanisms that allow for the recruitment and assembly of Drp1 at scission points have been elusive in mitochondrial structure research. Ground-breaking studies into ER-mitochondria interactions have demonstrated that the ER is a functional platform for the coordinated polymerization of actin filaments (Hatch, Gurel et al. 2014). Actin filaments are essential to drive the assembly of Drp1 oligomers at constriction sites. Actin filaments accumulate between the mitochondria and ER aided by mitochondrial actin-nucleating protein Spire1C and ER-bound inverted-formin 2 (INF2) (Korobova, Ramabhadran et al. 2013, Manor, Bartholomew et al. 2015). The disruption of either Spire1C or INF2 activities reduces mitochondrial constriction (Manor, Bartholomew et al. 2015). It is
speculated that actin polymerization and/or the dimerization of myosin-II mediates the constriction of actin filaments (Korobova, Gauvin et al. 2014). This constriction exerts pressure on the mitochondrial membrane. This initial assembly forms the platform where DRP1 can oligomerize and carry out the next step in fission (Hatch, Gurel et al. 2014). The final step in complete fission following Drp1 oligomerization involves classic Dynamin 2 (Dyn2) (Lee, Westrate et al. 2016).

1.2.10 Post-translational modification of DRP1

It has been observed that in certain tumors Drp1 has undergone posttranslational modifications. Mitochondria in rapidly dividing cancer cells are kept in a fragmented state to ensure equal distribution to daughter cells. It has been shown that DRP1-mediated mitochondrial division is required for RAS-induced transformation. MEFs infected with the oncogenic form of RAS (RAS\textsuperscript{G12V}) undergo rapid immortalization and transformation, the mitochondrial network in these cells is highly fragmented (Serasinghe, Wieder et al.). Mutant RAS causes the Ras-Raf-ERK signal transduction cascade implicated in various proliferation, differentiation and survival pathway in cancer. DRP1 expression was increased following transformation. Once activated, ERK 1/2 was shown to phosphorylate DRP1 at Serine 616 thus activating DRP 1 (Kashatus, Nascimento et al. 2015). Inhibition of MAPK signalling rapidly mediated mitochondrial fusion via DRP1 (Serasinghe, Wieder et al.). Promotion of mitochondrial fission is thought to direct cell towards glycolysis as well are aiding cellular division. Metastatic breast cancer cells have been shown to 5-fold higher levels of pS616-DRP1 (Zhao, Zhang et al. 2013).

As mentioned previously DRP1 is cytosolic until recruited to the OMM through specific receptors to drive mitochondrial fission. When apoptosis is triggered DRP1 is recruited to receptors proteins such as MiD49 or MFF following the activation of BAX/BAK but before the release of cytochrome c (Youle and Strasser 2008). When DRP1 was removed in knockout studies the remodeling of cristae was blocked, this could explain why there was a delay in cytochrome c release when DRP1 was reduced (Clerc, Ge et al. 2014). However, linking DRP1 to cristae remodeling was still not fully understood. It was shown that during apoptosis DRP1 is no longer cycled on and off of the mitochondria as would be the case during steady state conditions, instead, it is stabilized and trapped at the scission
site (Frank, 2001). Small Ubiquitin-related Modifier (SUMO)-ylation protein were found to be located at the site of scission. Specifically, mitochondrial Anchored-Protein Ligase (MAPL) a SUMO E3 ligase was identified as a key molecule required for cell death (Prudent, Zunino et al. 2015). MAPL is inserted into the OMM and faces out into the cytosol where it can interact with Drp1 (Prudent, Zunino et al. 2015). Overexpression of FLAG-MAPL in HeLa cells showed that FLAG-MAPL accumulated at ER/mitochondria contact sites at points of mitochondrial division (Braschi, Zunino et al. 2009). During apoptosis, DRP1 was shown to be SUMOylated by MAPL at ER contact sites, visualised using YFP-SUMO. It was also demonstrated that loss of MAPL delayed cytochrome c release and no accumulation of YFP-SUMO at sites of mitochondrial constriction (Prudent, Zunino et al. 2015). Loss of MAPL also results in a change in cristae remodeling which is caused by a delay in OPA1-oligomer disassembly (Prudent, Zunino et al. 2015). This is a process shown to be dependent on calcium uptake from the ER.

1.2.11 mtDNA and ER sites

It has been shown that sites of mitochondrial fission are not random but rather tightly controlled, yet it is still unclear how scission sites are selected. During cellular division, mitochondrial fission must ensure that cells must evenly distribute the mitochondria evenly to daughter cells. New studies now have shown through a fluorescently labeled mitochondrial DNA polymerase holoenzyme (Figure 1.6), sites that are actively engaging mtDNA synthesis are also where contact points between ER/mitochondria occur (Lewis, Uchiyama et al. 2016). This adds weight to other studies that demonstrate the role of ER in mitochondrial fission.
Figure 1.6: mtDNA is found at mito-ER sites where division takes place. mtDNA (green) can be seen localising at regions where ER (red) and mitochondria (blue) come into contact, that subsequently appears to separate. Taken from (Labrousse, Zappaterra et al. 1999).
1.2.12 Mitochondrial dynamics and apoptosis

The Bcl-2 family proteins, which are normally associated with the control of apoptosis, also seem to have a role in the restructuring of mitochondria through fission and fusion. The intrinsic pathway of apoptosis is engaged by release of cytochrome c and other pro-apoptotic molecules from the mitochondrial IMS into the cytosol (Tait and Green 2010). Cytochrome c leaves the mitochondria following the permeabilization of the mitochondrial outer membrane by the BAX and BAK members of the Bcl-2 family. Cytochrome c binds to apoptotic protease-activating factor 1 to form a complex that cleaves and activates the initiator caspase 9. Caspase 9 then activates the executioner caspases 3 and 7, leading to the degradation of intracellular substrates (Chan 2012), rendering cells incompatible with life.

Once apoptosis is initialised, mitochondria undergo increased fission and fragment near the time of cytochrome c release. The inhibition of DRP1 can prevent this fission and reduce the amount of cytochrome c release, providing an anti-apoptotic mechanism (Frank, Gaume et al. 2001). Whether or not mitochondrial fission plays a central role in mitochondrial outer membrane permeabilisation remains controversial with some studies showing that inhibition of DRP1 only delays cytochrome c release (Parone, James et al. 2006). It has been suggested that DRP1 increases the ability of BAX to assemble on lipid membranes (Montessuit, Somasekharan et al. 2010) which has led to the hypothesis that mitochondrial membrane deformation, mediated by DRP1, can facilitate BAX oligomerisation during apoptosis. Cells with reduced MFF or FIS1 also display less apoptosis when treated with apoptosis inducers such as actinomycin D and staurosporine (Lee, Jeong et al. 2004, Gandre-Babbe and van der Bliek 2008). However, mitochondrial hyperfusion due to DRP1 depletion is sufficient to cause cytochrome c release due to the accumulation of damaged mitochondria (Qian, Wang et al. 2013), it cannot be assumed that cancer cells who evade apoptosis should have decreased DRP1 expression. DRP1 expression is increased in some cancers; it is required to facilitate rapid proliferation (Smirnova, Gripic et al. 2001, Rehman, Zhang et al. 2012). A balance between fission and fusion must be maintained for cell survival and proliferation.
1.2.13 Fission and mitophagy

Fission is also involved in the recycling of mitochondria in a process known as “mitophagy”. If mitochondria become damaged beyond repair they are labelled by ubiquitination for degradation via mitophagy (Kirkin, McEwan et al. 2009). The Youle laboratory has carried out experiments on HeLa cells involving Parkin and PINK, two proteins that appear to have decreased expression in familial Parkinson’s disease. They observed that when these two proteins were over expressed and mitochondria were damaged/uncoupled following treatment with FCCP, Parkin/PINK were recruited to the now highly divided mitochondria and were subsequently degraded within the cell (Youle and Narendra 2011, Youle and van der Bliek 2012). Without the maintenance role provided by fission, mitochondria would accumulate so much damage that they would be rendered inefficient at producing energy and most likely would lead to cell death due ATP insufficiency and apoptosis.
1.3 Mitochondria and Cancer

In 2000, Hanahan and Weinberg published the seminal review defining the hallmarks of cancerous cells. In this they proposed that there are six hallmarks for cancer that provide a logical framework for understanding the remarkable diversity of neoplastic diseases. The hallmarks are traits that enable tumour growth and metastatic dissemination, they include: Sustaining proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality inducing angiogenesis and resisting cell death (Figure 1.6) (Hanahan and Weinberg 2000). In 2011 they revisited the hallmarks and added two additional features, avoiding immune destruction and deregulation of cellular energetics (Hanahan and Weinberg 2011). This section will explore the concept of deregulated cellular energetics.

Cancer cells are characterised by excessive proliferation and increased resistance to apoptosis due to deregulation of multiple cellular signalling mechanisms (Evan and Vousden 2001). The metabolism that supports cancer cell proliferation is very different from that of a non-transformed cell. Cancer cells have a rapid uptake of glucose, converting the majority of it to lactate (Warburg 1956) This enduring and fundamental observation made by Warburg created the field devoted to the study of tumour metabolism. Is now widely accepted that the high rate of glucose metabolism is a metabolic hallmark of proliferating cells (Kim and Dang 2006).

1.3.1 Warburg effect

Normal animal and human tissues convert most of the sugar (glucose) they eat to the compound pyruvate by glycolysis. Post glycolysis the pyruvate enters the mitochondria where it is oxidized to carbon dioxide (CO$_2$) and water (H$_2$O). The mechanism by which ATP is generated I have explained in the preceding sections. When oxygen is limiting, cells will utilize glycolysis to generate ATP, since oxygen is not available to terminally accept the electrons in CIV of the ETC. This process anaerobic glycolysis, is much less efficient than OXPHOS, producing only a net of two ATP molecules for every glucose molecule and also generates “lactate” which can be harmful to most cells in high enough concentrations (Berg 2002). Although less efficient and potentially harmful, when oxygen
is limited it becomes paramount to the production of ATP and, therefore also, cell survival until oxygen again becomes available. In the 1920s Otto Warburg made the surprising discovery that tumour cells unlike normal non-proliferative tissue utilize glycolysis instead of oxidative phosphorylation even in oxygen-rich conditions, now termed the “Warburg effect” (Warburg, Wind et al. 1927, Warburg 1956). He discovered that the tumours consumed large amounts of glucose and instead of the pyruvate being metabolized to carbon dioxide in the mitochondria via the TCA cycle it is converted to lactic acid (Figure 1.7). The elevated consumption of glucose by certain cancers has been exploited clinically as a diagnostic tool via $^{18}$F-deoxyglucose positron emission tomography ($^{18}$FDG-PET) and as a prognostic marker (Plathow and Weber 2008).
Figure 1.6: The Original and Emerging Hallmarks and Enabling Characteristics of cancerous cells. Six hallmarks of cancer originally proposed by Hanahan and Weinberg (top panel) and the two new emerging hallmarks (bottom panel) that include the capability to reprogram cellular energy metabolism to promote and support proliferation by increased aerobic glycolysis and evasion of immune destruction Hanahan and Weinberg, 2011a).
To summarise, it can be said that cancer cells that adhere to the Warburg effect can remodel their glycolytic and mitochondrial machinery such that the former is upregulated (goes faster) and the latter is downregulated (goes slower) (Pedersen 2007). Due to this discovery it was suggested that cancer cells have defective mitochondria and that aerobic glycolysis provoked by this mitochondrial impairment was the origin of cancer cell transformation (Warburg 1956, Frezza and Gottlieb 2009).
Figure 1.7: Many tumour and highly proliferative cells switch from standard oxidative phosphorylation to glycolysis even under normoxic conditions. This glycolytic shift yields less ATP and produces lactate but allows for greater nucleotide synthesis and generation of biomass via the pentose phosphate shunt. Taken from (Vander Heiden, Cantley et al. 2009).
Mutations that affect the expression of mtDNA have been reported in a number of studies. They have been found to be involved in a variety of cancers such as increased breast cancer risk in African American women with a variant in the CI, subunit ND3 gene. Single nucleotide polymorphisms (SNPs) in the cytochrome c oxidase subunit 1 (T6777C) have been linked with epithelial ovarian cancer, along with variants in several nuclear deoxyribonucleic acid (nDNA) mitochondrial genes. mtDNA control region variant C150 has been associated with an increased risk of human papilloma virus (HPV) infection and cervical cancer in Chinese woman (Wallace 2012). Yet the requirement of mtDNA is essential for cancer cells to function, confirmed by the elimination of mtDNA from various cancers. Culturing cells in ethidium bromide removes mtDNA. Cancer cells lacking mtDNA showed reduced growth rates, decreased colony formation and reduced colony formation in nude mice (Desjardins, Frost et al. 1985, Morais, Zinkewich-Peotti et al. 1994, Cavalli, Varella-Garcia et al. 1997). mtDNA mutation alone is not sufficient to explain the shift from OXPHOS to glycolysis. Therefore, cancer cells still require mtDNA and need to sustain an adequate balance between fusion and fission for maintenance and sharing of mtDNA.

There is also evidence to support the existence of mitochondrial enzyme defects in some cancers. Succinate dehydrogenase (SDH or CII) mutations have been observed in paragangliomas and pheochromocytomas, rare tumours derived from paraganglia, neuroendocrine tissues distributed along the paravertebral axis from the base of the skull and neck to the pelvis (Bardella, Pollard et al. 2011). SDH defects account for 10-30% of sporadic cases and 10-70% in familial paragangliomas (Wallace 2012). The inhibition of CII increases mitochondrial and cytosolic levels of succinate, which in turn inhibits α-ketoglutarate-dependent prolyl hydroxylases (PDHs) causing the stabilization of hypoxia-inducible factor 1α (HIF1α). HIF1α then drives a glycolytic phenotype shifting energy metabolism away from OXPHOS (Marin-Hernandez, Gallardo-Perez et al. 2009). SDH cells also have increased ROS production generated by CIII (Muller, Liu et al. 2004), which can also cause PDH inactivation also resulting in HIF1α stabilization. Other examples (reviewed in detail by Wallace), include fumarate dehydrogenase (FDH) and isocitrate dehydrogenase (IDH) (Wallace 2012).
Uncoupling proteins (UCP) initially identified as a critical response to cold temperature and used to generate heat by uncoupling respiration have also been reported to have increased expression in several cancers including, colon, liver, breast, thyroid and acute myeloid leukaemia along with a positive correlation in tumour grade in breast cancers (Bensinger and Christofk 2012). Uncoupling proteins increase membrane permeability. Protons that usually travel from the intermembrane space back into the mitochondria via ATP synthase (as described in section 1.1.4) can re-enter into the matrix through the membrane. This generates heat at the expense of ATP in a process known as non-shivering thermogenesis (Palou, Picó et al. 1998). Uncoupling of respiration can lead to decreased ATP production resulting in increased reliance on glycolysis, decreased ROS production, decreased apoptotic sensitivity and increased chemotherapeutic resistance (Derdak 2008).

It is obvious from the literature that mitochondrial defects in cancer cells can cause a shift in energy metabolism. However, contrary to conventional wisdom; functional mitochondria are essential for the cancer cell. Defects although common in cancer cells, do not inactivate mitochondrial energy metabolism but alter bioenergetics to meet the cells needs and optimize growth in the cancer cell environment. Though mitochondrial defects are present in some cancers and generally increased, glycolysis is a hallmark of tumour progression, this does not necessarily imply that the mitochondrial defects are the root cause of cancer. Although the impact of the Warburg hypothesis has profoundly influenced the present perception of cancer metabolism and some ideas have stood the test of time, there have been many misconceptions about the underlying biochemical mechanisms of cancer cell transformation. Apart from a handful of cancers such as the previous examples (SDH, FDH and IDG mutations) where the deficit in metabolism results in the formation cancer, mitochondrial impairment observed in the vast majority of cancers is much more likely to be the consequence of complex metabolic shifts that allow cancer cells to proliferate and survive (Frezza and Gottlieb 2009), rather than a driving force for oncogenesis.

Despite the heavy reliance on glycolytic metabolism, cancer cells still have mitochondria and can be forced to produce ATP via OXPHOS under specific conditions, such as in HeLa cells, MDA- MB-453 cells (Reitzer, Wice et al. 1979, Mazurek, Michel et al. 1997, Rossignol, Gilkerson et al. 2004). Cancer cells are highly dynamic and can adapt their metabolism to survive in a range conditions. Recent studies have shown that restricting
glycolysis or diverting pyruvate into the mitochondria can induce respiration. By controlling the fate of pyruvate, either by inhibiting lactate dehydrogenase (LDH) or activating pyruvate dehydrogenase (PDH) by inhibiting pyruvate dehydrogenase kinase (PDK), tumour cells can be induced to oxidise pyruvate in the TCA cycle and stimulate mitochondrial respiration (Rossignol, Gilkerson et al. 2004, Fantin, St-Pierre et al. 2006, Bonnet, Archer et al. 2007). These experiments demonstrated that the mitochondria in tumour cells, by and large, are not defunct and retain the ability to carry out metabolism similarly to a non-tumour cell, when coerced into doing so.

1.3.2 Cancer cells rely on aerobic glycolysis despite viable mitochondria

Metabolic transformations have potential advantages for cancer cells. Although aerobic glycolysis is an inefficient way to generate ATP, it is not utilized because of damaged mitochondria. In fact the process actually confers an advantage to cancer cells and to other proliferating cells such as ES cells and lymphocytes (Fox, Hammerman et al. 2005) by facilitating the uptake and incorporation of nutrients (e.g., nucleotides, amino acids, and lipids) into the biomass needed to produce a new cell (Vander Heiden, Cantley et al. 2009). The rapid proliferation associated with tumour growth can only proceed when there is ample supply of the building blocks required to make DNA, RNA protein, and lipid and complex carbohydrate to prepare for mitosis (Wu and Le 2013).

1.3.3 Regulation of Warburg effect

The molecular basis for the initiation of the Warburg effect remains poorly understood. One signalling pathway that plays a crucial role in regulating cell growth, survival and metabolism is the phosphoinositide 3-kinase/Protein kinase B/mammalian/mechanistic target of rapamycin (PI3K/AKT/mTOR) pathway. Aberrations in this pathway can lead to signalling cascades that can drive a cancerous phenotype. This pathway is one of the most frequently deregulated in cancer cells and epidemiological data indicates that sporadic mutation or deregulation of PI3K, AKT and Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) are, together with p53, among the most prevalent alterations in human cancer (Yuan and Cantley, 2008). In many cancers, the pathway is over active, thus reducing apoptosis and allowing proliferation.
Once activated, the PI3K pathway not only provides strong growth and survival signals to tumour cells but also has profound effects on their metabolism (Pal and Mandal 2012). The pathway can be activated by tyrosine kinase growth factor receptors, such as ErbB family receptors, fibroblast growth factor receptors (FGFR), insulin-like growth factor 1 receptor (IGF-1R), and others (Pal and Mandal 2012). G protein-coupled receptors such as activated RAS can also stimulate PI3K via its catalytic subunit leading to a cascade that results in the phosphorylation of AKT (Castellano and Downward 2011). AKT drives the glycolytic phenotype and can stimulate ATP generation through multiple mechanisms thus ensuring that cell can adapt to meet the demands of excessive growth signals. AKT stimulates glycolysis by increasing the expression and membrane translocation of glucose transporters and by phosphorylating key glycolytic enzymes, such as hexokinase and phosphofructokinase 2 (Jang, Kim et al. 2013). The increased and prolonged AKT signalling that is associated with transformation inhibits forkhead box subfamily O (FOXO) transcription factors, resulting in a host of complex transcriptional changes that increase glycolytic capacity (Cairns, Harris et al. 2011).

Phosphorylated AKT also inhibits the tumour suppressor activity of tuberous sclerosis complex proteins 1 and 2 (TSC1 and TSC2) through direct phosphorylation of TSC2 (Khatri, Yepiskoposyan et al. 2010). These act as a GTPase activating protein for ras-homolog enriched in brain (RHEB), another important member of the pathway that controls growth and cell cycle progression through mTOR activation (Sato, Umetsu et al. 2008). In its steady state, the TSC1/TSC2 complex causes GTP hydrolysis by RHEB, which converts this protein from its active GTP-binding form to its inactive GDP-binding state this drives mTOR activation, thus allowing signal propagation.

mTOR is found in two structurally and functionally distinct complexes termed TOR complex 1 (TORC1) and TORC2 (Hall 2008). mTORC1 controls a broad range of cellular processes including the control of mRNA translation, ribosome biogenesis, autophagy and metabolism (Figure 1.8). mTORC1 controls the growth-related
Figure 1.8 mTOR pathways and relationships to the PI3K pathway. mTORC1 functions to regulate the accumulation of biomass in a cell by activating mRNA translation and ribosome biogenesis and limits autophagy. Ras driven activation of the PI3K/AKT pathway causes inactivation of TSC1/2. Rheb is the free to activate mTORC1 stimulating growth and proliferation. Loss of LKB1 decreases AMPK phosphorylation also resulting in activation of mTORC1 through TSC1/2 and Rheb. Activation of S6K1 leads to a positive feedback loop for this activation. This aberrant pathway leads to increased cell survival, changes to metabolism and proliferation. Taken from (Sabatini 2006)
processes mentioned above, the best characterized substrates of TORC1 are 4E-BP and S6K via which mTORC1 controls proteins synthesis (Hall 2008). mTORC2 is activated by growth factors alone, via PI3K-dependent ribosome association. mTORC2 substrates include AKT, SGK1 and PKCα. At the molecular level, mTOR directly stimulates mRNA translation and ribosome biogenesis, and indirectly causes other metabolic changes by activating transcription factors such as HIF 1α even under normoxic conditions (DeBerardinis, Lum et al. 2008). The subsequent HIF1-dependent metabolic changes are a major determinant of the glycolytic phenotype downstream of PI3K, AKT and mTOR (Sabatini 2006, DeBerardinis, Lum et al. 2008).

In addition, phosphorylated AKT increases cell survival by inhibiting the pro-apoptotic Bcl-2 family members, Bcl-2-associated antagonist killer (BAK) and Bcl-2-associated death promoter (BAD) (Cantley 2002, Engelman, Luo et al. 2006) or phosphorylation of mouse double minute 2 (MDM2) oncogene that antagonizes p53-mediated apoptosis (de Rozieres, Maya et al. 2000). Normal activation of p53 causes the oligomerisation of BAX, which then translocates to the mitochondrial membrane. The mitochondria then release apoptogenic proteins that ultimately lead to caspase activation and apoptosis (Amaral, Xavier et al. 2010). Deregulation and loss of tumour suppressors allows cancer cells to evade this process thus ensuring continued survival and proliferation.

HIF 1 and 2 are the main transcription factors that control gene expression changes when a cell is subjected to low oxygen conditions. Under normoxic conditions, HIF1α subunits undergo oxygen-dependent hydroxylation by prolyl hydroxylase enzymes, this subsequently to their degradation via an ubiquitin ligase. When oxygen concentrations are low HIF1α subunits are rapidly stabilised (Cairns, Harris et al. 2011). HIF1α can also be activated during normoxic conditions by oncogenic signalling pathways (Figure 1.9), including PI3K (Plas and Thompson 2005) and by mutations in tumour suppressors such as SDH and FDH, as mentioned before (Selak, Armour et al. 2005) (King, Selak et al. 2006). Once HIF1α is activated the transcription of glucose transporters and most glycolytic enzymes are amplified (Figure 1.9), thereby increasing the cell ability to perform glycolysis (Semenza 2010). HIF1 also impacts negatively on oxidative phosphorylation at this point by activating PDK (Kim, Tchernyshyov et al. 2006, Papandreou, Cairns et al. 2006). Activated PDK inactivates mitochondrial pyruvate dehydrogenase, which reduces
the flow of pyruvate flux into the TCA cycle. Pyruvate is mostly converted to lactic acid via LDHA. Lactate is transported out of the cell and in the process, generates NAD\(^+\) to further sustain glycolysis. The reduced pyruvate flux into the TCA leads to a decrease in OXPHOS and oxygen consumption, reinforcing the glycolytic phenotype and sparing oxygen under hypoxic conditions. HIF1\(\alpha\) also directly influences mitochondrial electron transport. It alters the composition of CIV (cytochrome c oxidase) by up-regulating a more efficient isoform (COX 4–2) and promotes proteasomal degradation of the COX 4–1 isoform that predominates during normoxia (Fukuda, Zhang et al. 2007).

Cancer progression has a direct impact on mitochondrial function, structural reorganisation of mitochondria may occur through mitochondrial dynamics to facilitate the shift away from OXPHOS to a glycolytic phenotype. Glycolytic enzymes may also have a role in mitochondrial remodelling either by direct interaction or through modulating metabolism.
Figure 1.9: The PI3K/AKT/mTOR pathway is a master regulator of aerobic glycolysis. Aberrations in some of the above pathways often associated with cancer can also promote a glycolytic phenotype associated with many tumour cells. The PI3K/AKT/mTOR pathway, HIF1α and MYC participate in many parts of this phenotype. Following mutation in tumour suppressors such as PTEN, TSC1 and TSC2 this pathway can be constitutively activated. Once activated this pathway can enhance glucose uptake and essential amino acid and protein translocation. Under normoxia HIF1α is degraded following modifications by propyl hydroxylases by the tumour suppressor VHL. Activation of PI3K/AKT/mTOR leads to stabilisation of HIF1α even under normoxia driving transcription of GLUT1, PDK1 and LDHA. The combined effect results in increased glucose uptake, glucose utilization and lactate production. Taken from (DeBerardinis, Lum et al. 2008).
1.3.4 Role of Hexokinase (HK) in cancer

Hexokinases catalyse the first rate limiting step in glucose metabolism; resulting in ATP-dependent phosphorylation of glucose to glucose-6-phosphate (G6P) (Wilson 2003). The ‘Warburg effect’ states that rapidly growing cancer cells have increased rates of glucose uptake, it stands to reason then that the enzymes responsible for the processing of glucose would be different in cancer cells as part of their metabolic reprogramming to ensure cancer cell proliferation (Pedersen 2007). Glucose entrapment by phosphorylation is facilitated through hexokinase. The phosphorylation of glucose to G6P also ensures that there is a constant concentration gradient that allows glucose to continue to enter into the cell (Nelson 2008). If cellular metabolism is altered in such a way that the cell can continually sustain glucose uptake, which is required for proliferation without being inhibited by the usual regulatory mechanisms then this change would be advantageous to a tumour. Hexokinases are the gatekeepers of glycolysis, hereby controlling all the major pathways of glucose utilization that occur downstream.

There are four isoforms of HK expressed in mammalian cells: HKI, HKII, HKIII and HKIV (also referred to a glucokinase) (Wilson 2003). The four isozymes have the same biochemical function but are distinguished by their distribution throughout tissues and their enzymatic activities. HKI-HKIII are high-affinity isoforms, while glucokinase is low affinity. HKI and HKII are associated with the mitochondria and are inhibited by high concentration of G6P, HKIII is inhibited by physiological concentrations of glucose, glucokinase is not inhibited by G6P and is mainly expressed in the liver and pancreas (Wilson 2003).

HKI is found in most adult mammalian tissues. HKII however is expressed in embryonic tissues and only highly expressed in a handful of adult tissues in healthy individuals including skeletal, cardiac muscle (Ritov and Kelley 2001, Calmettes, John et al. 2013). HKI and HKII are thought to have a role in cell survival since cancer cells express high levels of HKII, as do activated immune cells (Donnelly, Loftus et al. 2014). The high expression of HKII in proliferative tissue is thought to be partly responsible for the accelerated flux of glucose compared to non-proliferative tissue. HKII appears to be bound to the mitochondrial in cancer cells, attached to voltage dependent anion channel...
(VDAC), a transmembrane protein found in the mitochondrial membrane (Wilson 2003). Some studies have shown that HKII detachment from this channel protein can initiate apoptosis (Chiara, Castellaro et al. 2008) and conversely overexpression of mitochondrial bound HKII has been shown to be neuroprotective during hypoxia (Mergenthaler, Kahl et al. 2012). HKIII lacks the hydrophobic N-terminal sequence known to be crucial for mitochondrial binding (Gogvadze, Orrenius et al. 2008).

One study using HK2−/− MEFs showed that deletion of HKII results in resistance to oncogenic transformation after being subjected to oncogenic Ras, implicating HKII in the initiation of tumourogenesis by metabolic reprogramming (Patra, Wang et al. 2013). Oncogenic Ras elevates ribonucleotide synthesis largely through non-oxidative branch of the pentose phosphate pathway (PPP). In this study, the absence of HKII reduced the diversion of glucose to ribonucleotide synthesis through the non-oxidative branch of the PPP in KRas-driven non-small cell lung cancer (NSCLC) cells. Induction of HKII expression by oncogenic Ras is critical for the accelerated ribonucleotide synthesis (Patra, Wang et al. 2013). HKII silencing also led to a reduction in metabolic serine production, also, which has been shown to be critical for tumour cell growth and proliferation (Kalhan and Hanson 2012, Patra, Wang et al. 2013).

Liver and pancreatic cells that express the low-affinity HKIV switch to the high affinity forms HKII and to a lesser extent HKI during tumourgenesis. In the process HKIV is silenced (Rempel, Mathupala et al. 1996, Pedersen, Mathupala et al. 2002).

1.3.5 AMPK and cancer

AMP-activated protein kinase (AMPK) is a master regulator and crucial energy sensor. During times of energy depletion, AMPK is activated promoting ATP production by increasing the activity or expression of catalytic proteins while simultaneously conserving ATP by switching of biosynthetic pathways (Hardie 2011). AMPK functions as a metabolic checkpoint and regulates cellular processes directed by energy availability. During periods of energetic stresses AMPK becomes activated in response to an increased AMP/ATP ratio (Hardie 2011). Once activated it shifts cells to an oxidative metabolic
phenotype thereby inhibiting cell proliferation. AMPK opposes the effects of AKT and functions as a potent inhibitor of mTORC1 (Hahn-Windgassen, Nogueira et al. 2005). Oncogenic pathways that suppress AMPK signalling can drive transformation of cells. When AMPK is suppressed, fuel signals become uncoupled from growth signals, allowing tumours cells to divide under abnormal nutrient conditions. Cells are then free to respond to inappropriate growth signalling pathways that are activated by oncogenes and the loss of tumour suppressors (Cairns, Harris et al. 2011). Many cancers exhibit a loss of appropriate AMPK signalling that may also drive their glycolytic phenotype through the activation of HIF1α and mTOR. Serine–threonine kinase liver kinase B1 (LKB1) an upstream regulator of AMPK and tumour suppressor (Shackelford and Shaw 2009) is frequently mutated in sporadic cases of NSCLC (Ji, Ramsey et al. 2007) and cervical carcinoma (Wingo, Gallardo et al. 2009). In fact, the HeLa cell line is LKB1 deficient, which can then lead to AMPK suppression, thereby driving tumorigenesis (Hawley, Boudeau et al. 2003).

1.3.6 Cancer cells require mitochondria despite their apparent redundancy in ATP production

Although much research on mitochondrial dynamics is done in cancer cell lines, there is very little information published about how dynamics are specifically regulated in tumour cells in vivo, or compared to ‘normal cell’ line models.

Some cancer cells seem to have a fusion deficit, this can lead to increased mtDNA mutations (deceased copy number/increase in point mutations) and increased ROS production. mtDNA mutations have been reported in breast cancer (Parrella, Xiao et al. 2001). Increased ROS production has been found in prostate cancer (Khandrika, Kumar et al. 2009). Down regulation of the fusion proteins and inhibition of OXPHOS could lead to extensive fragmentation of mitochondria seen in these lines. Tumour progression, inhibition of OXPHOS and increased ROS production and increased cell division and proliferation could all contribute to increased fission.

The role of mitochondrial dynamics in cancer relates to the requirement for mitochondrial division during mitosis. This coordination between mitochondrial division and mitosis (mitochondrial segregation) ensures equitable distribution of mitochondria to daughter
cells. The molecular basis for the coordination of mitochondrial and nuclear division is emerging. At the transition from the G1 phase to S phase of the cell cycle, mitochondria fuse and increase ATP production. DRP1 inhibition induces mitochondrial hyperfusion and triggers DNA replication and cyclin E accumulation. The coordination of fission and mitosis is substantially regulated by cyclin B1–CDK1, which simultaneously initiates mitosis and activates DRP1 by phosphorylating serine 616 (Ser 616). Another mitotic kinase, aurora A kinase (AurAK), phosphorylates the Ras-like GTPase (RalA), leading to mitotic, mitochondrial accumulation of RalA and its effector, RalA binding protein 1 (RalBP1). RalBP1 serves as a scaffold for recruiting DRP1 and cyclin-CDK to mitochondria and inducing fission.

Increased fission in lung-cancer cells and tumours from patients who have not received treatment reflects post-translational DRP1 activation. In patients with lung cancer, increased DRP1 expression predicts a likelihood of recurrence that is increased more than 3-fold, as well as a greater likelihood of chemotherapeutic resistance to cisplatin. Impaired fusion, resulting from down-regulation of MFN2, also contributes to network fragmentation. Mitochondrial fragmentation contributes to the cancer phenotype in several ways. Fragmentation may accelerate mitotic fission and also interrupts intra-mitochondrial calcium waves, preventing calcium-mediated apoptosis. Inhibition of DRP1 or augmentation of MFN2 decreases proliferation and increases apoptosis in cancer cells and leads to regression of human lung tumours in a xenotransplantation model.

Contrary to the highly fragmented phenotype seen in these tumour cells, HeLa cells when grown in culture have a highly interconnected mitochondrial reticulum with extremely efficient fusion rates. This may be caused by prolonged ex vivo culture and possible elevated expression of fusion proteins compared to ‘normal cells’. Fusion has been implicated in a cells ability to survive in response to starvation or other stressors and staving off apoptosis and mitophagy (Tondera, Grandemange et al. 2009). When cells are subjected to modest levels of stress (well below levels needed to induce apoptosis), their mitochondria fuse to each other forming a closed network, similar to networks observed when mitochondrial fission is blocked. Stress-induced mitochondrial hyperfusion (SIMH), as this process was called, might counter stress by optimizing mitochondrial ATP production (Tondera, Grandemange et al. 2009).
The present study attempts to understand the interactions between mitochondrial dynamics and bioenergetic pathways such as glycolysis. The interactions between HKII and mitochondrial function are explored. Cancer cells can modulate mitochondrial respiration and glycolysis to maintain a careful balance of: ROS production and antioxidants, ATP production/consumption, mitophagy-autophagy and biosynthesis depending on their environment and nutrient availability. The control and regulation of each of these processes is underpinned by changes in mitochondrial structure and function through fission and fusion events. The main aims are to gain a better understanding of not only cancer cell metabolism but to understand mechanisms that allow for metabolic reprogramming that are common in other tissues such as stem cells and immune cells (Kishton, Barnes et al. 2016, Margineantu and Hockenbery 2016, O' Neill, Kishton et al. 2016).
Chapter 2: Materials and Methods
2.1 Methods and materials

All chemicals were obtained from Sigma Aldrich (Wicklow, Ireland), or Melford Laboratories Ltd (Suffolk, UK), unless otherwise indicated.

2.1.1 Buffers and solutions

DNA gel electrophoresis buffer – TAE buffer:
40 mM Tris Base, pH 8.0, 20 mM acetic acid, 2.5 mM EDTA.

LDS Protein Electrophoresis Sample Buffer (4x):
900 μL of 4 x LDS Sample Buffer (Invitrogen), 100 μL of 500 mM Dithiothreitol, (Invitrogen).

Low Salt Buffer:
50 mM Tris Base, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M Sucrose, 0.3 % (w/v) CHAPS, 0.1 % (v/v) β-mercaptoethanol.

Lysis Buffer:
50 mM Tris Base, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M Sucrose, 0.3 % (w/v) CHAPS. 0.1 % (v/v) β- mercaptoethanol and Complete Protease Inhibitor Cocktail (1 tablet per 50 mL of buffer, Roche) were added just before use.

MES SDS NuPAGE Protein Electrophoresis Buffer (20x):
1 M MES, pH 7.7, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA.
MOPS SDS NuPAGE Protein Electrophoresis Buffer (20x):
1 M MOPS, pH 7.7, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA.

Tris-Buffered Saline Solution (TBS):
20 mM Tris Base, pH 7.5, 150 mM sodium chloride.
TBS-Tween (TBS-T):
20 mM Tris Base, pH 7.5, 150 mM sodium chloride, 0.1 % (v/v) Tween 20.

Western blotting blocking solution:
5 % (w/v) non-fat dried milk solution in TBS-T.

Western Blot Transfer Buffer (20x):
500 mM Bicine, pH 7.2, 500 mM Bis Tris, 20.5 mM EDTA, 1 mM chlorobutanol.

WB Stripping Buffer:
1x TBS, pH 7.5, 2 % (w/v) SDS, 0.1 % (v/v) β-mercaptoethanol.

Krebs Buffer:
3 mM Potassium phosphate, 140 mM sodium chloride, 25 mM Tris-hydrochloride, 2 mM magnesium chloride, 2 mM calcium chloride, pH 7.4

Sucrose-Tris-EDTA (STE) buffer:
10 mM Tris, pH 7.4, 350 mM Sucrose, 1 mM EDTA.

Potassium phosphate buffer:
0.5 M Potassium phosphate, pH 7.5 by mixing equimolar mono and dibasic potassium phosphate.
2.1.2 Antibodies and Reagents

Mitochondria-targeted photo-activatable green fluorescent protein (PA-GFP-mito) and pDsRed2-mito plasmids were kind gifts from Dr. Richard Youle (NIH, Bethesda, Maryland, USA) and Dr. Seamus Martin (Trinity College, Dublin).

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Table 2.2.1.1 Primary antibodies
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<tr>
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Table 2.2.2.1 Secondary antibodies including horseradish peroxidase-conjugated secondary antibodies.

2.2 Cell Culture

2.2.1 Maintenance of cultured cell lines
The cervical cancer cell line, HeLa was obtained from ATCC (catalogue number CCL-2TM). Cells were maintained in either high glucose Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMax (Invitrogen) supplemented with 10 % foetal bovine serum and penicillin-streptomycin solution (100 units/ml penicillin G, 0.1 mg/ml streptomycin sulphate, (Invitrogen) or DMEM without glucose supplemented instead with 10 mM galactose, 2 mM L-glutamine, 10 % foetal bovine serum and penicillin-streptomycin solution (100 units/ml penicillin G, 0.1 mg/ml streptomycin sulphate, (Invitrogen). All cell culture work was done under sterile conditions in a NUAIRE laminar flow hood, and cells were maintained at 37 °C, in 95 % humidity and 7.5 % CO₂. Cell growth was monitored daily using a light microscope. An Invitrogen Countess Automated Cell Counter was used to determine cell number and cell viability was assessed by exclusion of trypan blue dye. Cells were maintained in T175 or T75 flasks until they reached 70 - 80 % confluency, then subcultured, usually every 2-3 days.
2.3 Molecular Biology

2.3.1 Transient transfection of plasmids

Cells were transfected by electroporation using Lonza Amaza Nucleofector Kit. Cells were regularly passaged when they reached 70-80 % confluency. Redundant media was removed and cells were washed once in PBS. Cells were incubated for 5 min at 37 ºC with trypsin. After >90 % of cells detached trypsinization was neutralized with fresh media. Cells were then counted; 1 x 10⁶ cells were pelleted at 200 x g for 10 min at room temperature and supernatant was completely removed. Cell pellets were resuspended in 100 µL room temperature Nucleofector Solution. To this suspension 2.0 µg DNA was added (1.0 µg each of DsRed and PA-GFP plasmids). Cell/DNA suspension was transferred into an electroporation cuvette. Program 0-005 on the Nucleofector I Device was employed. Once the program had finished the cuvette was removed and to it 900 µL of culture media was added. 150 µL of this suspension was then added to a 35-mm glass bottomed µ-dish (Ibidi). Media was added to the dish to bring the final volume 2.0 mL. Media was refreshed again after 24 h. Cells were imaged 48 h post transfection. Transfection efficiencies of >80 % where regularly achieved by this method with minimal toxicity to cells.

2.3.2 Transient transfection with siRNA

Transfection of plasmids was done using either RiboJuice transfection reagent or by electroporation. Cells were plated on 6-well plates or 35 mm confocal dishes to obtain confluency of ~60 %. Redundant media was removed and cells were washed with PBS then replaced with 1.25 mL of complete media. Transfection solution were made as follows; in a microcentrifuge tube, 6.0 µL (35 mm confocal dish or 6-well) of RiboJuice (Millipore) was mixed by gentle vortexing with 244 µL of Opti-MEM and incubated at room temperature for 5 min. To this, 7.5 µL of 1.0 µM siRNA was added to make a final concentration of 5.0 nM. This was mixed gently and incubated at room temperature for 10 min. Transfection complexes were added drop-wise to bring the final volume to 1.5 mL. Cells were incubated at 37 ºC for 24 h. Media was replaced the following day. Cells were either harvested at 48 h post-transfection and analysed or used for confocal imaging.
For electroporation, 1 x 10⁶ cells were collected per transfection. Cells were pelleted then resuspended in 100 μl of transfection buffer (5 mM KCl, 15 mM MgCl₂, 15 mM HEPES, 150 mM Na₂HPO₄/NaH₂PO₄ pH 7.2) with 2 μg of plasmid. The suspension is then transferred to an electroporation cuvette. Cells are then electroporated using the pre-loaded HeLa setting on a Lonza Nucleofector II machine. Cells were then resuspended in 900 μL of media. Cells were counted and plated at required density. Transfection was checked by epi-fluorescence microscopy.

2.3.3 Site-directed mutagenesis

Construction of human HK2 mutants. Hexokinase II mutants were generated by PCR according to the standard procedure of the QuickChange site directed mutagenesis kit (Stratagene) using FLHKII-pGFPN3 (Addgene) construct as a template. All amplification reactions (50 μL) contained PfuUltra HF polymerase buffer, 200 μM each of the four deoxynucleoside triphosphates, 10 ng of template DNA, 125 ng of primers forward and reverse, 2.5 U of PfuUltra HF polymerase (Agilent). The cycling parameters were 95 ºC for 2 min for initial denaturation, followed by 16 cycles at 95 ºC for 30 s, 60 ºC for 45 s, 68 ºC for 7 min, with a final elongation step 68 ºC for 10 min. Digestion of template DNA was performed in 40 μL of PCR reaction by adding 4 μL of Dpn buffer and 2 μL of DpnI (10U/μL). Sample was digested for 4 h at 37 °C. Reaction mixtures (2.5 μL) were transformed into high competent cells E. coli (XL10 gold cells) according to the provided protocol.
2.3.4 Cell viability
Cell viability was assessed using an Alamar Blue redox indicator (Invitrogen). The assay allows for quantification of cell viability by virtue of the ability of healthy, metabolically active cells to convert the dye to a colorimetric indicator. Cells were seeded in 96-well plates at a density of 5x10³ cell per well, 24 h before the experiment. Cells were treated with various concentrations of drug or vehicle control (DMSO) for 4 h with the addition 10ul of AlamarBlue dye per well (containing 100 μl of medium). After 4 h absorbance of each well was measured using a Spectramax PLUS Microplate Spectrophotometer at 570 and 600 nm (Abs 570/600). All values were corrected for blank and expressed as percentage of control.

2.4 Protein Analysis

2.4.1 Preparation of cell extracts
Following removal of redundant media cells were promptly placed on ice then washed twice with ice-cold PBS. Excess PBS was removed by aspiration. Cells were lysed in 100-500 μL of lysis buffer, depending on the size of culture dish to generate protein lysates between 1-2 mg/ml. Cells were scraped and removed from culture dishes to 1.5 mL microcentrifuge tubes. Lysates were mixed for 10 min at 4 ºC, then either used immediately or snap frozen in liquid nitrogen and stored at -20 ºC for later analysis.

2.4.2 Enriched mitochondrial fraction
Mitochondrial fractions were prepared using the Mitochondrial Isolation Kit for Cultured Cells (Thermo Scientific Pierce). Briefly > 2 x 10⁷ cells were trysinized and collected in a 15 mL falcon tube, pelleted then washed twice with PBS. Cells were resuspended in 800 μL of a hypotonic solution (Reagent A) and allowed to swell for 2 min. At this point one of two methods were used:

A) A homogenizer based method used for Complex I assays or
B) A detergent based method used for western blotting (advantage of detergent based method being that up to five samples could be processed simultaneously).

Method A involved placing the cells into a 1.0 mL Dounce homogenizer. 15 strokes of the tight pestle were used to lyse > 80 % of cells, checked by microscopy and by assaying
citrate synthase to verify mitochondrial release. Method B involved adding 10 μL of detergent (Reagent B) into the sample then vortexing for 5 seconds every minute for 5 min. After either step 800 μL of 320 mM sucrose buffer (Reagent C) was added to each tube. Lysates were then centrifuged at 700 x g for 10 min at 4 °C to removed unbroken cells and nuclear debris. It was necessary to repeat this centrifugation to ensure all unwanted debris was removed.

The supernatant was then centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant of this fraction was kept and used for cytosolic protein analysis. The pellet was then washed in Reagent C and spun again at 12,000 x g for 5 min. An additional wash step was also performed using Sucrose Tris EGTA buffer (STE). Pellet was then resuspended in either 75 μL STE or lysed for western blotting. Mitochondrial fractions were then either maintained on ice for further analysis or snap frozen in liquid nitrogen and stored at -80 °C.

2.4.3 Protein Estimation
The protein concentration was estimated as per the Bradford method, with modifications. BSA served as the protein standard, serial dilutions ranging from 25-1,500 μg/ml were made from a 2 mg/ml solution. 10 μl of each standard or unknown sample were pipetted into a 96-well microtitre plate and to this 300 μL of Coomassie Plus Regent (Bradford) was added. Samples were shaken for 30 sec followed by 10 min incubation in the dark at room temperature for 10 min. Absorbance was read at 595 nm in a SpectraMAX PLUS XS Microplate Spectrometer.

2.4.4 SDS-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)
For SDS-PAGE analysis, samples were added directly to 4X LDS Sample Buffer to give a final concentration of 1X LDS, Samples were heated at 70 °C for 10 min and sonicated using a MicroTip sonicator (20 % output, each sample was sonicated three times for 3 sec) to disrupt the genomic DNA.

Some fractions were pre-cleared prior to protein estimation, whole cells extracts were centrifuged at 10,000 x g for 15 min and the supernatant was transferred to a fresh microcentrifuge tube. Bradford Assay measured protein concentrations prior to the addition of LDS. 6 %, 8 % and 10 % Bis-Tris gels were used for all western blots.
depending on protein size to be analysed. Gels were prepared according to the recipes in Table 2.4.4.1.

<table>
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<th>Resolve</th>
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<tr>
<td>1.25 M Tris Bis, pH 6.6-6.8 [mL]</td>
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<td>2.14 2.14 2.14</td>
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<tr>
<td>Water [mL]</td>
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<td>3.787 3.296 2.769</td>
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<tr>
<td>10 % APS [µL]</td>
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<tr>
<td>TEMED [µL]</td>
<td>10</td>
<td>14 14 14</td>
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Table 2.4.4.1 Recipes for SDS-PAGE.

Resolving gel acrylamide polymerization was initialized by the addition of TEMED followed by APS, this was vortexed and allowed to degas before being poured into an empty gel cassette. To ensure a level boundary between the stack and resolve, the resolve was overlaid with butan-1-ol saturated against 1.25 M Tris-Bis solution. Once the gel had set the solvent overlay was rinsed off with distilled water and any excess was removed using 3MM filter paper.

Stacking gel was mixed thoroughly and poured on top of the resolving gel. A comb was inserted into the unset gel to form the wells for loading samples. After the stack had fully polymerized the well were washed with distilled water to remove any unset acrylamide. Gel cassettes were then placed into a vertical electrophoresis tanks immersed in either 1X MOPS or MES buffers, as appropriate. An Antioxidant solution was added to the cathode buffer chamber to maintain proteins in a reduced state during the electrophoresis.

Depending on the experiment and immunoblotting antibody, 5-30 µg of protein extract was loaded into each well and samples were flanked by Precision Plus pre-stained protein mass markers (BioRad), in order to determine the progress of protein migration. Gels were resolved at 80 V for stacking and 150 V for resolving until protein marker were sufficiently separated.
2.4.5 Western Immunoblotting

Proteins resolved by SDS-PAGE were transferred onto Immobilon-P PVDF membrane (Millipore) by wet electroblotting. Briefly, PVDF membrane was activated in methanol and then pre-soaked in Transfer buffer along with sheets of 3MM filter paper before use. A sandwich was constructed with a pre-soaked sheet of 3MM paper at the anode (+) followed by the gel, PVDF membrane and another pre-soaked sheet of 3MM paper at the cathode (-). Proteins were electroblotted in Transfer Buffer for 2 h at 40 V, using an XCell II Blot module (Invitrogen) placed in an ice-bath at RT or alternatively, for large or multiple gels, for 3 h at 50 V, using a Trans-Blot module (BioRad), at 4 °C.

Following transfer, the efficiency and quality of SDS-PAGE and electroblotting were confirmed by brief staining of membranes with PonceauS, which was then washed away in TBS-T. Next, membranes were blocked by incubation in 5 % non-fat dried milk (NFDM) solution in TBS-T (Blocking solution) for 1 hour at RT on a seesaw rocker to reduce non-specific binding of antibodies. When blocked, membranes were washed briefly in TBS-T and placed in a 50 ml falcon tube with the diluted primary antibody. Primary antibodies were diluted in Blocking solution or BSA according to Table 2.4.4.1. The 50 ml falcon tubes were placed on a roller. Depending on the primary antibody, the membrane was incubated for either 2 h at RT or overnight at 4 °C. Membranes were next washed three times for 10 min in TBS-T on a see-saw rocker and immediately put into horseradish peroxidase (HRP)-conjugated secondary antibody, generated specifically against the primary antibody species (Table 2.4.4.1). Membranes were incubated for 1 h at RT on a roller, followed by three 10 min washes in TBS-T. Proteins labelled with antibodies were detected using Enhanced Chemi-Luminescence (ECL; Millipore). Briefly, two ECL reagents were mixed in a 1:1 ratio, overlaid onto the membrane and incubated for 3 min at RT. Then membranes were dried briefly with paper towel and sealed between two transparent plastic sheets. Western immunoblots were either exposed to X-ray film or detected using a Chemi-Luminescent gel documentation system (BioRad). Images generated using the BioRad system where analysed using the ImageLab software (BioRad). X-ray film was scanned using an Epson Perfection 4990 transparency scanner. If required, the intensity of protein bands from x-ray film were quantified densitometrically, using ImageJ software.
2.5 Microscopy

2.5.1 Confocal Microscopy
Live cells were viewed using an Olympus FV1000 Point Scanning Confocal Microscope, FV10-ASQ Olympus Fluoview Ver.2 software and a 60X oil immersion objective. Sequential excitation at 405 nm, 488 nm and 546 nm were provided by near-violet laser diode, argon and helium-neon gas lasers, respectively. The imaging chamber was heated to 37 °C, humidified and contained 5 % CO₂ for live cell imaging. Immunofluorescence slides were imaged using Lecia SP8 gated STED microscope and analysed using Lecia Application Suite X software.

2.5.2 Quantification of Mitochondrial Fusion Rates
The method of quantification of mitochondrial fusion rates was based on that of Karbowski et al. (2004a), with modifications, and relied on the principle that mitochondria share matrix contents upon fusion. By conferring high fluorescence to a small group of mitochondria within the cellular mitochondrial matrix (photoconverting mitochondria-expressed GFP to a state of 100-fold increased fluorescence in a small region of interest, or ROI), the rate of fusion can be monitored by measuring the rate of decrease in fluorescence intensity in the ROI as matrix contents are shared by fusing organelles. All experiments were carried out using the Olympus FV1000 Point Scanning Confocal Microscope, FV10-ASW Olympus Fluoview Ver. 2 software and a 60X oil immersion objective with 1.7x zoom applied.

HeLa cells cultured in 35mm glass bottomed μ-dishes (Ibidi, Munich, Germany) were placed in the live imaging chamber and covered with the CO₂ regulator/humidifier apparatus. Sequential excitation at 488 nm and 543 nm allowed for visualisation of PA-GFP and DsRed protein expression in transfected cells, respectively. Using the Multiple Time Lapse facility within the software environment, six separate fields were pre-selected per group (control and inhibitor-treated) per dish, each containing 1 – 7 cells per field. Cells were imaged, with z-stacking (5 – 8 slices of 0.8 – 1.4 μm thickness), before and after photoactivation of discrete 7 μm² regions of interest (ROIs) of the mitochondrial network, with post-activation imaging intervals set at 1, 15 and 30 min. Photoactivation was achieved by 5-second irradiation of each ROI using the 405 nm laser set to 1 – 4 %
power output, depending on cell type. The photoactivated protein exhibited 85-95% increased fluorescence compared to its non-activated state. Regions exhibiting less than 85% increase in fluorescence post-activation were excluded from studies, as were cells that entered mitosis during the experimental period.

Using the Image Analysis function in the software package, the mean pixel intensities of the photoactivated ROIs were calculated in both the red and green channels at all time points and expressed as a percentage of pixel intensity 1 minute post-activation, deemed to be the point at which the pixel intensity was highest. Pixel intensities were also measured in three 7 μm² ROIs in cell-free areas and subtracted from the cellular ROIs as ‘background’ fluorescence. Each treatment was repeated on at least three separate dishes of cells on three separate occasions (n = 3); thus each data point on graphs depicting fusion rates is representative of data from 20 – 120 cells (average ~70 cells).

### 2.5.3 Immunocytochemistry
Cells cultured on 13mm glass coverslips were fixed in 4% paraformaldehyde in PBS for 15 minutes, permeabilised in 0.3% Triton in TBST for 10 min, rinsed in PBS and blocked in 1% FBS in TBST for 1 h. Cells were incubated in primary antibody in 1% BSA in TBST for 2 h and in secondary antibody (AlexaFluor 594/488/647, Invitrogen) in 1% BSA in TBST for 1 h. DAPI was added for 5 min prior to mounting on to glass slides using Hydromount. Slides were stored in the dark at 4°C until imaged.

### 2.6 Live Cell Metabolic Assay

#### 2.6.1 Measurement of oxygen consumption and lactic acid production
Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the XF24 (Seahorse Biosciences). Cells were seeded onto the XF24-well micro plate at a density of 20,000-40,000 cells/well. The following day cells were washed in buffered DMEM supplemented with either 25 mM Glucose or 10 mM Galactose for 1 hour at 37 °C in a CO₂-free incubator before transfer to the XF24 analyser. Experiments consisted of 3-min mixing, 2-min wait, and 3-min measurement cycle. Oxygen consumption was measured under basal conditions in the presence of the mitochondrial inhibitors 0.5 μM oligomycin (Calbiochem), which inhibits ATP synthase, or in the
presence of 0.5 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, Sigma), the mitochondrial uncoupler, to assess maximal oxidative capacity. Finally, 10 μM rotenone and 5.0 μM antimycin A were injected to give a blank rate. Blank rate for ECAR was determined by injection of 10 mM 2-deoxyglucose (2-DG). All experiments were performed at 37 ºC. Basal rates for OCR and ECAR were calculated by subtracting initial rates from blank rates. Data presented are the mean ± SEM from three independent experiments corrected to 1 x 10^6 cells.

2.7 Functional Mitochondrial Assays

2.7.1 CI assay
Mitochondria were isolated from cells as described above in section 2.4.2. Isolated mitochondria were subjected to three cycles of freeze thawing in hypotonic buffer before measuring CI activity in order to maximize substrate availability to enzyme. 10 μg of sample (100 μg/ml final concentration) was added a 100-μl microcell cuvette. Assay buffer contained 50 mM, pH 7.5 potassium phosphate buffer, 0.3 % (w/v) fatty acid–free BSA, 0.3 mM KCN and 0.1 mM NADH. Contents were then mixed and a baseline reading was taken at 340 nm for 2 min. The reaction was initiated by the addition of 50 μM decyl Q. The contents were again mixed and immediately read. Activity was measured by following the decrease of absorbance at 340 nm for 3 min. Inhibitor sensitive rate was followed after the addition of 1.25 μM rotenone. Specific activity was calculated by subtracting the rotenone rate from the initial rate following the addition decyl Q. Enzyme activity was calculate using the following formula:

\[
\text{Activity (nmol/min/mg)} = \Delta \text{Absorbance/min} \times 1,000\varepsilon \times \text{sample volume (ml)} \times [\text{protein}] \\
(\text{mg/ml})
\]

Extinction coefficient (ε) of NADH was taken to be 6.22 mM⁻¹ cm⁻¹.

2.7.2 In-gel Complex I activity assay
Following separation by Native Blue Page individual complex I and respirasome complex I activity was measured by developing the BN-PAGE gel in 1 mg/ml nitro blue tetrazolium
(NBT) and 0.14 mM NADH in a 0.1 M Tris-HCL pH 7.4 buffer. Complex activity was measured by colour change to purple/blue and quantified by densitometry.

2.7.3 CIII assay
Assay buffer containing 25 μM potassium phosphate, pH 7.5, 62.5 μg ml⁻¹ oxidized cytochrome c, 0.5 mM KCN, 0.1 mM EDTA and 2.5 % (v/v) Tween-20 was prepared. To each 1-ml cuvette, 50 μg/ml of whole cell lysate was added for each sample. In parallel a separate cuvette containing the same quantity of reagents and sample with the addition of 10 μM antimycin A was made up. The reaction was initiated by the addition of 5 μM deculuniquinol. The contents were mixed by inversion, sealed with parafilm. The increase in absorbance at 550 nm for 3 min was then observed. Specific CIII activity is the antimycin-A sensitive activity. Activity in nmol min⁻¹ mg⁻¹ was calculated using the equation above. Extinction coefficient of reduced cytochrome c was taken to be 18.5 mM⁻¹ cm⁻¹.

2.7.4 CII/III assay
Assay buffer containing 50 mM potassium phosphate buffer, pH 7.5 and 10 mM succinate were added to each cuvette along with WCL samples (80 μg/ml). The cuvette was mixed by inversion with parafilm then incubated at 37 °C for 10 min. The reaction was initiated by the addition of 62.5 μg ml⁻¹ oxidized cytochrome c. The cuvette was mixed by inversion then the increase in absorbance at 550 nm was followed for 3 min. 10 μM Antimycin A was added to inhibit the reaction. Specific activity was calculated by subtracting the inhibitor rate form the initial rate of reaction. Extinction coefficient for reduced cytochrome c was taken to be 18.5 mM⁻¹ cm⁻¹.

2.7.5 CI/III assay
Samples (50 μg) were incubated in 700 μl of distilled water in a 1-ml cuvette for 2 min to induce an osmotic shock before buffer was added to obtain a final concentration of 50 mM potassium phosphate buffer, pH 7.5, 0.3 % (w/v) BSA, 0.3 mM KCN and oxidised 62.5 μg ml⁻¹ cytochrome c. The cuvette was mixed by inversion. A baseline measurement was made for 2 min then the reaction was initiated by the addition of 0.2 mM NADH. The increase in absorbance at 550 nm was then followed for 3 min. The specific activity was
determined after the addition of 1.25 µM rotenone. Extinction coefficient for reduced cytochrome c was taken to be 18.5 mM⁻¹ cm⁻¹.

### 2.7.6 CIV assay
Assay buffer containing 125 mM potassium phosphate, pH 7.0 and 62.5 µg ml⁻¹ reduced cytochrome c was added to a 1-ml cuvette. A baseline reading was then taken for 2 min and the reaction was initiated by the addition of sample (50 µg ml⁻¹). The cuvette was mixed by inversion and a decrease at 550 nm was observed for 3 min. Specificity was checked by adding 0.3 mM KCN into a parallel cuvette. Extinction coefficient for reduced cytochrome c was taken to be 18.5 mM⁻¹ cm⁻¹.

### 2.7.7 Citrate Synthase (CS) assay
Assay buffer containing 100 mM Tris base, pH 8.0 with 0.2 % (v/v) Triton X-100, 100 µM DTNB, 0.3 mM Acetyl CoA and 40 µg of sample were added to a to a 1-ml cuvette. The cuvette was mixed by inversion with parafilm. A baseline was measured at 412 nm for 2 min. The reaction was initiated by the addition of 0.5 mM oxaloacetic acid and mixed by inversion. The increase in absorbance was measured for 4 min. CS rate was expressed as nmol min⁻¹ mg⁻¹ was calculated using the equation above. Extinction coefficient was taken to as 13.6 mM⁻¹ cm⁻¹.

### 2.7.8 Measurement of Reactive Oxygen Species
Two methods were used to determine ROS production:

Method A (H₂DCFDA): Cells were seeded on to a clear-bottomed black-walled 96-well plate, 5000 cells/well. After cells have adhered to the plate, cells were loaded with 20 µM H₂DCFDA (Invitrogen) in KREBS buffer. Increase in fluorescence using 488/535 nm was measured for 45 min. To induce ROS production 2 µM Antimycin A was added as positive control.

Method B (MitoSOX): Mitochondrial ROS was measured by confocal microscopy using the mitochondrial superoxide indicator (MitoSOX Red, ThermoFisher). Cells were seeded on to confocal dished at 150,000 cells per dish. MitoSOX was added for 30 min. Cells were
then washed 3 times in KREBS buffer. Using a confocal microscope with a 60X objective the increase in fluorescence at 535/580 nm was measured for 20 min. To induce ROS production cells were treated with 2 μM antimycin A. Images were quantified using LASX software. Regions of interest were quantified from > 100 cells per experiment.

2.7.9 Quantification of ΔΨm
Mitochondrial membrane potential was measured by confocal microscopy using the ΔΨm-dependent dye tetramethylrhodamine, methyl ester (TMRM, Invitrogen, CA, USA). This cationic, red-orange fluorescence-emitting dye is sequestered by mitochondria in a ΔΨm-dependent manner and at non-quenching concentrations (below 50 nM), indicates mitochondrial depolarisation upon loss of signal (and, conversely, hyperpolarisation upon increase in signal). Cells were loaded with the dye in normal culture medium containing 20 nM TMRM for 30 min. Excitation with the 535 nm laser set to 1.4 % power output allowed for visualisation of red-emitting TMRM within mitochondria of cells, viewed through a 60x oil objective. The imaging settings were adjusted such that the background fluorescence was negligible and the signal from cells was below saturation level. Six randomly chosen fields were pre-selected using the software’s Multiple Time Lapse facility and imaged before and after incubation with FCCP. Using the software’s Image Analysis facility, mean pixel intensities of each field were calculated and represented over time.

2.8 Statistics
Statistical analysis was carried out using GraphPad Prism (Version 6) and Microsoft Excel (2011). Data represented as mean ± SEM, n=3 unless otherwise stated in figure legend. Statistical significance was indicted when p<0.05. Unpaired t-test were used to compare two-conditions. One-way ANOVA with uncorrected Fisher’s LSD analysis was used to compare more than two groups of individual treatments.
Chapter 3: Effects of glucose and galactose on mitochondrial fusion rates in HeLa cells
3.1 Introduction

Recent developments in cancer research have demonstrated the critical roles that mitochondria play in the initiation and progression of cancer. Changes in morphology and structure influence how mitochondria control cellular energetics, apoptosis, calcium signalling and ROS production; all of which have been implicated in cancer cell biology. The links between mitochondrial dynamics, energy production and tumorigenesis remain to be firmly established. The metabolic flexibility conferred to cancer cells by their mitochondria allows them to proliferate and overcome intracellular processes that initiate cell death. Cancer cells alter mitochondrial dynamics, bioenergetic and biosynthetic processes to resist apoptosis during oncogenesis, to sustain proliferation and infer therapeutic resistance. The downstream pathways and signals that coordinate adaptations in mitochondria and bestow cancer cells with robustness are poorly understood.

3.1.1 Metabolic reprogramming of cancer

The reprogramming of metabolism is a key hallmark of cancer (Hanahan and Weinberg 2011). The shift to aerobic glycolysis is the preferred bioenergetic pathway for many cancer cells. ATP production is around 18-fold less efficient by glycolysis compared to OXPHOS. While this may seem counterintuitive, this pathway has advantages for proliferation. Cancer cells make up for low ATP productivity, in part, by upregulating key transporters and glycolytic enzymes that promote glucose uptake and processing. Hexokinase II is upregulated in many cancer cells. HKII the predominate isoform in cancer cells due to its high affinity for glucose and dual catalytic sites. Several other proteins work synergistically with HKII to sustain a malignant state. These include GLUT1, which promotes glucose entry into the cell (the primary substrate); VDAC, which binds HKII to the mitochondria; ATP synthase, which provides ATP (the second substrate); and finally, the ANT, which transports ATP to the VDAC-HKII complex. The net result of this setup is highly glycolytic phenotype that produces large amounts of G6P. The G6P can be channelled in the PPP to generate ribose-6-phosphate and NADPH for nucleotide synthesis (Patra and Hay 2014). Excess NADPH can be used to reduce glutathione, an important antioxidant.
Aerobic glycolysis may aid the initial formation of tumours. Rapid proliferation of cells with a poor vascular system may be subjected to hypoxic conditions. Through glycolysis, ATP can still be produced; there is no longer a reliance on oxygen availability. In order for this shift to occur, an increase in specific isoforms of glycolytic enzymes and glucose transporters would be expected, followed by the conversion of pyruvate to lactate. In order for this initial transformation mitochondrial respiration appears to be suppressed but the mitochondria still continue to be maintained.

3.1.2 Transformation of cells affects mitochondria

There is now a considerable body of research that suggests that targeting the aberrant pathways that control metabolism will be of benefit for improved cancer therapeutics. In normal mammalian cells, energy regulation requires constant balance of cellular activity, nutrient availability and energy production. This is maintained by a complex interplay of signalling and feedback pathways that link energy and nutrient sensors to effector molecules, which can modulate cellular processes. Effector molecules such as kinases and transcription factors activate or inhibit pathways leading to increased flux through one system or attenuation of a pathway if it is deemed energetically unfavourable. If energy demand is high and ATP/AMP ratios are low, AMPK is activated by phosphorylation. Activated AMPK initiates a cascade of phosphorylation events that activate several catabolic pathways such as glycolysis and OXPHOS, subsequently generating ATP while simultaneously inhibiting anabolic pathways such as protein synthesis and fatty acid synthesis (Hardie, Ross et al. 2012). Cancer cells on the other hand do not have this regulation. The loss of the tumour suppressor LKB1 (as seen in HeLa cells) results in the inactivation of AMPK (Nguyen, Babcock et al. 2013). Loss of AMPK activation results in cells without the ability to enforce metabolic checkpoints that would prevent cancer cell progression; pathways affected include mTORC1 and p53 (Luo, Zang et al. 2010). AMPK has also been shown to influence mitochondrial function through sirtuin 1 (Sirt1) (Price, Gomes et al. 2012), a histone deacetylase that has been implicated in mitochondrial biogenesis and regulation of mitophagy. (reviewed by Tang 2016). Mitophagy is controlled by fission events. Many studies that investigate the regulatory mechanisms of mitochondrial function do so at the level of the respiratory chain and their surrounding membranes. Mitochondrial fusion/fission dynamics interact with bioenergetics through a
process of bi-directional crosstalk and changes in mitochondrial fusion and fission influence regulation of bioenergetic status.

Many studies have uncovered the pathways that influence transformation from normal to cancerous cells. The enhancement of glycolytic systems has been well described in various cancer cell lines, however the changes associated with mitochondrial oxidative phosphorylation during oncogenesis and on-going cancer survival are less well known. It is known that the tumour suppressor p53 promotes OXPHOS by upregulating proteins required for respiration such as CIV and cytochrome c oxidase (Matoba, Kang et al. 2006). The oncogene MYC encodes the transcription factor c-Myc, which increases glycolytic enzymes such as lactate dehydrogenase A and HKII (Dang, Kim et al. 2008). The lack of LKB1 in HeLa cells means that they are unable to correctly maintain homeostasis and prevent uncontrolled growth (Nguyen, Babcock et al. 2013). Oncogenesis and suppression of mitochondrial functions are closely linked; it would appear however that these processes are reversible and highly plastic. While many cancers favour glycolysis over OXPHOS, it would appear that mitochondrial respiration can be re-started when required by the cell (Birsoy, Possemato et al. 2014).

3.1.3 Mitochondrial Dynamics allows cancer cells to shift to OXPHOS

As far back as 1979 there was evidence that “glutamine, not sugar, is the major energy source for cultured HeLa cells”, which demonstrated that under certain conditions HeLa cells could utilise OXPHOS to preferentially produce ATP over aerobic glycolysis (Reitzer, Wice et al. 1979). Several cancer cells can switch their metabolism from glycolysis to OXPHOS; indeed several glioma cell lines are highly dependent on mitochondrial respiration (Griguer, Oliva et al. 2005). Several cells that include breast carcinoma cells, hepatoma cells, pancreatic cancer cells and cervical cancer cells have the ability to switch from glycolysis to OXPHOS under limiting glucose conditions (Rossignol, Gilkerson et al. 2004, Plecitá-Hlavatá, Lessard et al. 2008, Smolková, Bellance et al. 2010). These results appear to contradict the common held orthodoxy that oncogenesis triggers a shift from OXPHOS to glycolysis but other regulators of carcinogenesis such as the tumour microenvironment constantly cause re-shaping the metabolic profile of cancer cells. Constant changing of metabolism in vivo requires certain
plasticity in mitochondria in order for cells to survive; this is controlled by mitochondrial dynamics (Vyas, Zaganjor et al. 2016).

Glucose deprivation in osteosarcoma and hepatocellular carcinoma cells leads to a shift to OXPHOS. This is accompanied by a restructuring of the mitochondrial network; it becomes highly branched and interconnected. This change is complimented by upregulation of ETC complexes (Rossignol, Gilkerson et al. 2004, Plecitá-Hlavatá, Lessard et al. 2008). When glucose becomes limiting in HTB-126 cancer cells mitochondrial biogenesis is actually increased, a result that was not seen in corresponding non-cancer cells (Smolková, Bellance et al. 2010). Glucose deprivation also leads to an important adaption, increased glutamine metabolism. Glutamate dehydrogenase activity (GDH) increases in cancer cells that have limiting glucose, and glutaminosis provides substrates for OXPHOS (Yang, Suddarth et al. 2009). GDH activity is linked to oncogenic Akt signalling. When glucose is unavailable Akt is decreased (Graham, Tahmasian et al. 2012). Akt suppresses GDH in cancer cells when glucose is available. Akt is central to cancer bioenergetics as it causes extensive remodelling. It increases GLUT1 recruitment to plasma membrane, inhibits fatty acid oxidation and importantly for the present study promotes the mitochondrial localisation of hexokinase (Wieman, Wofford et al. 2007, Neary and Pastorino 2013). Akt is also activated when mitochondria respiration is impaired (Pelicanò, Xu et al. 2006). Negative regulation of mitochondrial complexes in cancer cells activates Akt through PTEN.

Surprisingly few studies have investigated the roles of how cancer metabolism affects mitochondrial dynamics. Several studies have reported on the anti-apoptotic role that HKII has when bound to mitochondria but there is little research available on how this interaction affects mitochondrial dynamics. Research into shape and structure of mitochondria is still in its infancy and how cancer related pathways such as MAPK, PI3K/AKT control mitochondria dynamics is very poorly studied.

The plastic and fluid nature of mitochondria and its internal structures plays a central role in initiation and progression in cancer. Better understanding of processes that are engaged in mitochondrial dynamics and their roles in cancer cell initiation, maintenance and proliferation offer potential novel therapeutic avenues at various stages of cancer progression. Therapeutics should also be tailored to cancer cells within their own
microenvironments. Research has demonstrated that different environments will yield different metabolic profiles that are sensitive to different compounds/drugs.
3.1.4 Aims of chapter

Cancer cells can survive in various environments by altering their metabolism. Fusion and fission dynamics are critical in the maintenance of mitochondrial function. It is hypothesised that changes in bioenergetics will cause changes in mitochondria dynamics. Disturbances in the balance between fusion and fission can result in loss of ATP production, increased ROS production, increased mtDNA damage and apoptosis. This chapter focuses on mitochondrial fusion when cells are in glycolytic and OXPHOS states. It is hypothesised that altering carbohydrate substrates that force metabolic shifts will have direct modification on the respiratory complexes that control mitochondrial ATP production. This study focuses on how the complexes of the electron transport chain respond to metabolic shifts in cancer cells and how key molecules control bioenergetics. The main aims are as follows:

1. **To quantify the effects of glucose and galactose on mitochondrial function in HeLa cells:** Using Seahorse XF24 analyser changes in oxygen consumption and extracellular acidification rates were quantified when sugar substrate was changed from glucose to galactose. Rates were also quantified for cells given a glucose bolus following prolonged growth in galactose medium.

2. **To quantify the effects of glucose and galactose on mitochondrial fusion rates in HeLa cells:** Using live cell microscopy with fluorescently labelled mitochondria fusion rates were quantified in glycolytic and OXPHOS. Fusion rates and cell viability was measured when these conditions groups were treated with ETC inhibitors.

3. **To investigate DRP1 localisation and quantify mitochondrial fragmentation:** Using immunofluorescence confocal microscopy, the subcellular location of the main mitochondria fission protein DRP1 was examined when cells were treated with ETC inhibitors.

4. **To quantify ETC complex activities in supercomplex assemblies in Hela cells:** Using non-denaturing gel techniques, mitochondrial complex enzymes were examined in their native states. Specific activities were quantified spectrophotometrically for ETC complexes individually and as in gel-complexes.

5. **To investigate the role of AMPK in controlling mitochondrial fusion:** AMPK is activated in response to depleted ATP. The changes in AMPK phosphorylation
between glucose-fed and galactose-fed cells were measured. Mitochondrial fusion rates were quantified following AMPK.
3.2 Results

3.2.1 Rapid reversal of a glycolysis to OXPHOS switch induced by galactose in HeLa cells

Cancer cells that produce ATP in accordance to the Warburg Effect would be expected to take up glucose and under aerobic conditions convert it into lactate, whereas normal non-proliferative cell counterparts would convert glucose into pyruvate which is then utilized by mitochondria (Lunt and Vander Heiden 2011). HeLa cells are cervical carcinoma cell lines that whilst glycolytic in nature, are capable of utilizing mitochondrial respiration to produce ATP. Cells were cultured in glucose-free galactose media for 14 days.

Using the Seahorse XF analyser (Seahorse Biosciences) a series of simultaneous real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were taken for HeLa cells in each culture medium. HeLa cells cultured in the presence of glucose showed classic Warburg Effect respiration (aerobic glycolysis) (Figure 3.1). When the carbon substrate was changed from glucose to galactose there was a metabolic shift away from glycolysis towards oxidative phosphorylation (Figure 3.1).

Unpublished data from the Davey laboratory (Dr. Stephen Quinn, PhD Thesis) showed that mitochondrial fusion rates in glucose HeLa cells were unaffected by inhibition of ETC complexes using classical inhibitors such as antimycin A, piericidin A and azide. Conversely, when the carbon substrate was changed from glucose to galactose fusion rates became sensitive to inhibition. The fusion rates in treated cells could be rescued by giving them a glucose bolus. It was demonstrated in the current experiment that galactose fed cells undergo regular OXPHOS respiration that can be rapidly reversed by the addition of glucose (Figure 3.1). The basal rates of oxygen consumption (rotenone/antimycin-sensitive rate) were significantly increased in galactose-fed HeLa with a rate 118.6 ± 10.10 nmol/min compared to 24.3 ± 2.56 nmol/min glucose-fed HeLa (Figure 3.1, panel A). The shift from glycolysis to OXPHOS was also shown by the ECAR, a measure of lactate production. There was a highly significant decrease in ECAR in the Galactose-fed HeLa. 4.57 ± 1.16 mpH/min compared to 23.07± 5.02 mpH/min in the glucose-fed HeLa (Figure 3.1, panel B). When the media was changed back from galactose to glucose again for 1
hour, the effect was reversed, with a reversion back to the highly glycolytic state, similar to the glucose-fed HeLa cells. From the time-course trace it can be seen that when mitochondria were uncoupled using FCCP, there was little to no spare respiratory capacity (Figure 3.2, panel A). Mitochondria in these cells were operating at maximal respiration, suggesting a high level of basal depolarisation. Glucose-fed HeLa cells have a slight spare glycolytic capacity that was not present at all in galactose-fed cells (Figure 3.2, panel B). The blue line shows that growth in galactose media for 14 days appears to attenuate this glycolytic spare capacity even when glucose becomes available. This suggests a possible reduction in expression in glycolytic enzymes.

The shift from aerobic glycolysis to OXPHOS increased the doubling rate of galactose-HeLa cells, approximately 2-fold longer than glucose HeLa cells (Figure 3.3).
Figure 3.1: Galactose increased oxidative phosphorylation, parameters in HeLa cells. OCR and ECAR were measured using XF24 analyser (Seahorse Bioscience). Cells were seeded at 30,000 per well 24 h before the assay. An average of 6 wells per group were measured. Cells were assayed in DMEM supplemented with 10 mM glucose, 10 mM galactose or galactose cells given a 1 h bolus of glucose (Gal + Glu). (a) OCR was calculated as basal oxygen consumption – rotenone/antimycin rate. (b) ECAR was calculated as basal rates-2-DG treated rates. Data was normalised to 10^6 cells. Data shown n=3 ± SEM. One-way ANOVA with Dunnetts multiple comparison. *p<0.05, **p<0.01, ***p<0.001.
Figure 3.2: Glucose-deprived cells switch to oxidative phosphorylation in galactose-mediated cell survival. (a) Oxygen consumption rate measurements of HeLa cells in glucose-DMEM (red), galactose-DMEM (blue) and galactose-fed cells give a 1 h bolus of glucose (green). (b) Extra cellular acidification rate measurements in DMEM medium supplemented as indicated.
Figure 3.3: Galactose media reduced proliferation in HeLa cells. Cells were seeded into a 96-well plate at 5000 cells/per. After 24 h Alamar Blue was added. Change in fluorescence was measured at 570/610 nm. Data shown n=3 ± SEM. One-way ANOVA with Dunnetts multiple comparison. **p<0.01.
3.2.2 Mitochondrial fusion rates are similar when cells express a glycolytic or OXPHOS phenotype

Mitochondrial fusion rates in HeLa cells were assessed using live cell confocal microscopy. Cells were co-transfected with two mitochondrial specific fluorescent reporter tags. Firstly, a mito-DsRed (DsRed-mito) plasmid that conferred red fluorescence to all mitochondria, allowed visualisation of mitochondrial networks and also provided a suitable background control to monitor mitochondria movement for glucose-fed cells (Figure 3.4, red panel) and galactose-fed cells (Figure 3.6, red panel) and to check of photo bleaching. The second plasmid, a photo-activatable green fluorescent protein (PA-GFP-mito), undergoes photo conversion upon irradiation with 405 nm light, exhibiting a 100-fold increase in fluorescence emission at 488 nm. This property allowed “activation” of discreet regions of mitochondria, termed regions of interest (ROI). In a ROI, pixel intensity should decrease as mitochondria actively fuse and share the photo-activated protein, monitored by the loss of the 488 nm emission spectra in the ROI (Figures 3.4 and 3.6, green panel). A decrease in pixel intensity (PI) indicates active mitochondrial fusion. Conversely, when the pixel intensity remains high, it can be inferred that fusion was not taking place.

Resting HeLa cells exhibited highly active and efficient mitochondrial fusion rates in both glucose and galactose fed cells (Figures 3.5 and 3.7, respectively). The PI in the ROI dropped to 36.0 ± 1.20% after 30 min for glucose HeLa (Figure 3.5). The PI of the DsRed protein in the same ROI showed statistically insignificant fluctuations during this time period, indicated that the PI decrease observed in relation to the PA-GFP was not due to a movement en masse of mitochondria away from the ROI. Galactose HeLa cells show comparable rates of fusion with PI values decreasing 36.6 ± 5.20% after 30 min (Figure 3.7).
Figure 3.4: Active mitochondrial fusion in glucose-fed HeLa cells. Glucose-fed HeLa cells co-expressing mitochondrially targeted fluorescent proteins, mito-DsRED (left panels) and mito-PA-GFP (middle panels), were imaged using time-resolved live cell confocal microscopy. Photo-activation of discrete ROIs increased green fluorescence intensity 10-fold. Pixel intensity of the ROI decreased over time as fusion events occur. Images were taken at 1, 15 and 30 min post-activation. Scale = 20 μm.
Figure 3.5: Quantification of changes of mean pixel intensity over time inside activated ROIs. Mean PI of photo-activated ROIs were calculated using Olympus FV10-ASW Olympus Fluorview software and expressed as a percentage of fluorescence at t=1, the maximum intensity.
Figure 3.6: Galactose cells have similar dissipation in the ROI seen in glucose cells. Cells were deprived of glucose and cultured in galactose-supplemented media for at least 14 days. Mitochondrially targeted fluorescent marker where then transfected. Scale= 20μm.
Figure 3.7: Galactose-fed cells have similar rates of fusion as glucose HeLa cells. Images from cells cultured on galactose for at least 14 days were analysed as before.
3.2.3 Galactose-fed cells are sensitive to ETC inhibitors

Previous work carried out in the lab showed that inhibitors of the electron transport chain compromised mitochondrial fusion rates in galactose-fed HeLa cells. This was confirmed in a second strain of HeLa cells from a different source. Cells were transfected with fluorescent mitochondrial reporters then pre-treated with compounds for 10 min before mitochondrial fusion assays.

Complex I was inhibited by piericidin A. Piericidin A was used in place of rotenone as the latter was found to inhibit microtubule function (Srivastava and Panda 2007). Even as early as 30 min mitochondria appear to be punctate in galactose-fed cells (Figure 3.8) but not in glucose-fed cells following treatment by ETC inhibitors (Figure 3.8). Fusion was halted in galactose-fed cells with PIs remaining >90%. Treatment with CI, III, IV inhibitors in glucose-fed cells had little effect on fusion rate as the cells maintained high rates with PIs dissipating to around 30% (Figure 3.9). Oligomycin and FCCP decreased fusion rates in both cells suggesting ATP synthase and a mitochondrial membrane potential are required for mitochondrial fusion to occur. Glucose-fed cells tolerated ATP synthase inhibition better than galactose-fed cells but rates were still significantly reduced (not indicated on graph) from 21.3 ± 3.23% to 55.7 ± 2.42% (Figure 3.9).
Figure 3.8: Inhibition of ETC complexes compromises fusion in galactose-fed but not glucose-fed cells. Cells were transfected with the mitochondrial markers. After a 24 h to allow for expression, cells were pre-treated with 4 μM piericidin A and 2.4 μM antimycin A for 10 min before ROI were photo-activated. There is significantly less dilution of ROI in the galactose cells compared to the glucose. Scale = 20 μm.
Figure 3.9: ETC inhibitors impair fusion rates in galactose cells but not in glucose cells. Cells pre-treated with compounds for 10 min before assay. 4 μM piericidin A (pA), 2.4 μM antimycin A (Anti A), 10 mM sodium azide (Azide), 5 μM FCCP and 1 μM oligomycin (Oligo). Bar represents percentage PI at 30 min post-activation. t=1 was taken to be 100 %. Student t-test between each pair. **p<0.01, ***p<0.001.
3.2.4 Cell viability is reduced in galactose but not glucose cells with piericidin A, rotenone, antimycin A and metformin.

Mitochondrial fusion is required to maintain healthy mitochondria, once halted it was hypothesised that cell death would follow. In this experiment cells were treated with various compounds for 24 h. In galactose cells fusion was impaired almost immediately following treatment, it was not known if this was reversible with time. Glucose cells appeared to be insensitive to the compounds after 30 min, it was unclear if they eventually succumbed to effects of prolonged inhibition.

Only the glucose-fed cells survive when the proton pumping complexes were inhibited (Figure 3.10, panel B). Cell viability of galactose when treated with complex inhibitors was comparable to a positive control of triton treated cells, equivalent to total cell death (Figure 3.11, panel B). Rotenone, piericidin A, antimycin A and metformin have no effect on glucose-fed cells (Figure 3.10, panel B) but were toxic to galactose-fed cells (Figure 3.11, panel B). Glucose-fed cells were still susceptible to oligomycin and FCCP suggesting that mitochondria membrane potential must be maintained for cell survival (Figure 3.10, panel B). Galactose-fed cells fared slightly better with oligomycin, FCCP and 2-DG, this could be due their ability to continue to maintain membrane potential through proton pumping via the ETC. Glucose-fed cells were more sensitive to 2-DG than galactose cells with ~100 % cell death compared to ~50 %, respectively (Figure 3.10, panel B).
Figure 3.10: ETC inhibitors do not affect Glucose cell viability. Glucose HeLa cells were treated with compounds for 24 h (same concentrations as before). (a) Phase images show cell density/morphology after 1 h treatment. (b) Cell viability following 24 h treatment. Alamar Blue was added for the last 4 h. Following incubation with Alamar blue the change in fluorescence was then measured. Cells were blanked using a triton treated sample. Data shown mean ± SEM, ***p<0.001.
Figure 3.11: Galactose cells are highly susceptible to cell death following treatment with ETC inhibitors. Galactose-fed HeLa cells were treated with compounds for 24 h (same concentrations as before). (a) Phase images show cell density/morphology after 1 h treatment. (b) Cell viability following 24 h treatment. Alamar Blue was added for the last 4 h. Following incubation with Alamar blue the change in fluorescence was then measured. Cells were blanked using a triton treated sample. Data shown mean ± SEM. ***p<0.001
3.2.5 Glucose-HeLa cells mitochondrial morphology was not affected by complex I and complex III inhibitors

Based on previous experiments on mitochondrial fusion rates it was known that fusion rates in glucose-HeLa cell were unchanged when treated with piericidin A and antimycin A. The effect of inhibitors on DRP1 and mitochondrial structure was investigated by IF microscopy. It was hypothesised that mitochondrial structure would remain similar to that of the untreated control. Glucose-fed HeLa cells were treated with rotenone, piericidin A and antimycin A for 30 min. Cells were immunostained with anti-TOM 20 and anti-DRP1. Previous blots had shown that glucose-fed cells did not localise DRP1 to the mitochondria when treated with ETC, the exact subcellular location remained unknown (Dr. Stephen Quinn, PhD Thesis).

In the control group around 18.8 ± 6.76% of cells have fragmented mitochondria (Figure 3.14). This was probably due to normal turnover of cells and damage caused by cell culture techniques.

When the electron transport complexes were inhibited, DRP1 was found throughout the cytoplasm but has a predominately perinuclear localisation similar to the control (Figure 3.12), this was an unexpected result, potentially due to poor staining. Glucose-fed cells have a large hyperfused mitochondrial network, approximately 2 nm in width. There was no significant change in mitochondrial fragmentation with pA or AA with fragmentation percentages of 28.6 ± 3.95% and 25.4 ± 3.20% (Figure 3.12). Rotenone treated cells caused the mitochondria to fragment slightly more than the control and mitochondria move to the nucleus, DRP1. Treatment with oligomycin did not cause the DRP1 to disperse from the nucleus (Figure 3.13). Mitochondrial fragmentation also increased with mitochondria concentrating more around the nucleus (Figure 3.13). FCCP caused the majority of the DRP1 to relocate away from nucleus to throughout the cytosol (Figure 3.13). Mitochondrial fragmentation increased to 86.0 ± 7.10% of cells had punctate mitochondria (Figure 3.14). Treatment with 2-DG for 30 min caused the mitochondria to become condensed and less fibrous (Figure 3.13). DRP1 fluorescence increased expanding away from the nucleus. Fragmentation increased to 81.9 ± 6.32% cells containing fragmented mitochondria (Figure 3.14). Metformin had little effect on DRP1 or mitochondrial fragmentation (Figure 3.14).
Figure 3.12: Complex I and III inhibitors have no effect of mitochondrial morphology and DRP1 expression in glucose fed HeLa cells. Cells were treated with piericidin A (4 µM), rotenone (1 µM) or antimycin A (2.4 µM) for 30 min then prepared for immunostaining. Cells were incubated with anti-TOM 20 for mitochondria (red), anti-DRP1 (cyan) and DAPI (blue). Scale 20 µm.
Figure 3.13: 2-Deoxyglucose and FCCP altered DRP1 location and expression. Cells were treated with oligomycin (1 μM), FCCP (0.5 μM), 2-DG (30 mM) and metformin (10 mM) for 30 min. Cells were fixed and permeabilised for immunostaining as previously described. Scale 20 μm
Figure 3.14: Oligomycin, FCCP and 2-DG cause excessive mitochondrial fragmentation in glucose cells. Quantification of mitochondrial fragmentation taken from confocal images. Organelles were classified as fragmented when >50% of the total cellular mitochondria displayed a major axis <5 μm. 60-80 cells were counted per experiment. Data show mean ± SEM. Student t-test was used to compare each group to the control. ***p<0.001.
3.2.6 DRP1 localization was altered in galactose-fed HeLa cells treated with ETC inhibitors and 2-DG

Galactose cells were highly sensitive to ETC inhibitors and as little as 5 min treatment can result loss of mitochondrial fusion. To assess the role of DRP1, galactose cells were treated with each compound for 30 min then immunostained with anti-TOM 20 and anti-DRP1. then imaged by confocal microscopy.

Inhibition of complex I and III with piericidin A, rotenone and antimycin A leads to extensive fragmentation of mitochondria (Figure 3.15). In contrast to glucose HeLa cells DRP1 expression does increase throughout the cell. Rotenone and antimycin A cause the greatest increase. DRP1 concentrates in various regions around the cell following inhibition, possibly at sites of the greatest fission. Complete depolarisation with FCCP results in a substantial increase in DRP1 expression throughout the cell body along with the extensive mitochondria fragmentation (Figure 3.16). Oligomycin and 2-DG caused increases in DRP1. 81.9 ± 1.90% of cells have fragmented mitochondria in 2-DG treated cells (Figure 3.17). Oligomycin caused similar translocation of DRP1 that was seen using rotenone and antimycin A (Figure 3.15, top panels). Metformin caused mitochondria to condense and DRP1 expression to increase although there was still a significant amount of DRP1 that remains surrounding the nucleus (Figure 3.16)
Figure 3.15: ETC inhibitor cause mitochondria fragmentation and cause DRP1 to move throughout the cell. Cells were grown on glass coverslips in galactose media. Cells were treated with EtOh (control), piericidin A (4 μ), rotenone (1 μM) and antimycin A (2.4 μM) for 30 min. Cells were then prepared for immunostaining. Scale 20 μm
Figure 3.16: Galactose cell mitochondria fragment when treated with 2-DG and metformin along with FCCP and Oligomycin. Cells were treated with oligomycin (1 µM), FCCP (0.5 µM), 2-DG (30 mM) and metformin (10 mM) for 30 min. Cells were then prepared for immunostaining and imaged using a Leica SP8 confocal. Scale = 20 µm.
Figure 3.17: Extensive mitochondria fragmentation with all inhibitors in galactose cells. Organelles were classified as fragmented when >50% of the total cellular mitochondria displayed a major axis <5 μm. 60-80 cells were counted per experiment.
3.2.7 Electron transport complex activities in glucose- and galactose-fed Hela cells

Since increased oxygen consumption and increased expression levels of glycolytic enzymes previously reported (Rossignol, Gilkerson et al. 2004) the possibility that CI activity increased in Galactose-fed HeLa cells was investigated. A CI assay was performed on isolated mitochondria from HeLa cells. Assays were normalized to citrate synthase and expressed as percentage of the glucose control group. There was no significant difference in CI activity between the three media groups (Figure 3.18, panel A). All groups had similar initial rates and sensitivities to rotenone. CIII activity was also analysed in whole cell lysates. Again, there was no significant difference in the specific activity of CIII following and all three groups had similar initial rates and sensitivities to antimycin A (Figure 3.18, panel B).

In a CII/III assay, succinate was used as the substrate (CII substrate) and cytochrome c was used as the electron acceptor instead of ubiquinone, thereby measuring the overall activity of CII and CIII together. Galactose cultured cells have a 27.3 ± 5.93% increase in specific activity as shown in Figure 3.19, panel A. This increase was reversed when the cells were re-fed glucose media for 1 hour, and there was no significant difference between glucose or galactose fed cells that had been re-fed a glucose bolus for 1 hour. CI/III uses NADH as the electron donor and cytochrome C as the acceptor, thereby allowing for combined measurement of CI and CIII. Figure 3.19, panel B shows an increased activity in the galactose-cultured cells, although experimental noise prevented obtaining statistically significant data.

Using reduced cytochrome c as the substrate CIV activity was assayed. Rates of activity for CIV were nonlinear but rather followed first order kinetics, calculated using the equation:

\[
K = \frac{\ln\left(\frac{A_t}{A}\right)}{t} \times n_t
\]

Where \(A_t\) = initial absorbance and \(A\) = absorbance at regular time intervals. No significant difference in CIV activity between the three groups was observed (Figure 3.20, panel A).
Citrate synthase assay were done on whole cell lysates to determine mitochondrial populations, but there was again no significant difference in citrate synthase activities between groups (Figure 3.20, panel B).
Figure 3.18: Alternating carbohydrate substrate had no effect on complex I or complex III activities in HeLa cells. Cells were sub-cultured 24 h before experiment and maintained in either glucose (Glu) or galactose (Gal) media. Gal+ have been re-fed glucose 1 h before assay. Enzymatic ETC activities were measured spectrophotometrically. (a) Mitochondria were isolated from >20 x 10⁶ cells. Mitochondria were freeze-fractured 3 times. Samples (50 µg) were loaded into a microcell cuvette containing assay buffer, KCN, BSA and NADH. Rates were measured ΔAb₃₄₀nm. A blank rate was observed for 3 min. Assay was initiated by addition of decylQ and monitored for 5 min. Blank rates were obtained following the addition of 10 µM rotenone. (b) Whole cell lysate (WCL) samples were freeze-fractured 3 times. Samples (50 µg) were loaded into 1 ml cuvettes assay buffer, KCN, oxidized cytochrome c, EDTA and Tween-20. Rates were taken ΔAb₅₅₀nm. Initial rates were measured for 3 min. Inhibitor insensitive rate was obtained by the addition of 5 µM antimycin A. Student t-test, ns = not significant.
Figure 3.19: Galactose had no effect of CIV and Citrate synthase activities. WCL samples were freeze-fractured 3 times. (A) Samples (50 μg) were loaded into 1 ml cuvettes containing assay buffer and reduced cytochrome c. Assay was initiated by addition of sample. Rates were measured as $\Delta A_{550nm}$. (B) Samples (40 μg) were added into 1 ml cuvette containing Tris-buffer with Triton X-100, DTNB, Ac CoA. Rates were measured as $\Delta A_{412nm}$ No significant difference in citrate synthase activity between groups. Student t-test. * p<0.05, ns = not significant.
Figure 3.20: Galactose increased complex II/III activity in HeLa cells. WCL samples were freeze-fractured 3 times. (A) Samples (100 μg) were loaded into 1 ml cuvettes containing assay buffer, KCN, succinate. Rates were taken \( \Delta A_{550nm} \). Blank rate of observed for 3 min. Assay was initiated by addition of oxidised cytochrome c. Initial rates were measured for 3 min. Inhibitor insensitive rate was obtained by the addition of 5 μM antimycin A. (B) Samples (100 μg) were loaded into 1 ml cuvettes assay buffer, KCN, oxidised cytochrome c and BSA. Rates were taken \( \Delta A_{550nm} \). Reaction was initiated by the addition of NADH. Inhibitor insensitive rate was obtained following addition of 10 μM rotenone. Student t-test. *p<0.05, ns = non-significant.
3.2.8 Mitochondrial supercomplexes were detected in galactose-fed HeLa cells

The increased OXPHOS seen in the galactose-fed cells in the absence of increased individual enzyme activity suggested that while the overall amount of mitochondrial protein was similar between glucose and galactose-fed cells, galactose-fed cells may arrange their ETC enzymes differently within the mitochondrial membrane. Supercomplex formation has been previously reported to exist as a dynamic and plastic model (Porras and Bai 2015). This is where individual complexes associated with each other within the mitochondrial membrane in order to transport electrons more efficiently. The increase in CII/III and the slight increase seen in CI/III in galactose cells suggest that there was more of an interaction between these complexes. To further investigate these complex interactions a non-denaturing Blue Native gel system was used to examine proteins within their membranes. The previous experiment required the fracturing of membranes through freeze-thaw cycles to ensure maximum activity of free-floating enzymes, but non-denaturing Blue Native gels avoided this step. This is more reflective of interactions in living cells.

There were significantly more complex I+III+IV and complex III+IV interactions in Gal and Gal+ cells (Figure 3.21). Although we were unable to detect CI amounts by immunoblotting on their own, an in-gel activity assay measured by colorimetric change showed that CI activity one its own was unchanged between the groups but within the supercomplex bands (Figure 3.22), galactose-fed and glucose re-fed cells had significantly darker bands, indicating greater supercomplex activity.
Figure 3.21: Supercomplex assemblies were depressed in glucose-fed cells but CI+III+IV and III+IV assemblies increased upon switching to galactose. Western blot analysis using anti-Core 1 revealed supercomplexes containing complex III. >40 x 10^6 cells per condition were collected and mitochondria isolated by centrifugation in a sucrose buffer. Mitochondria were solubilized with digitonin then centrifuged at 100,000 g for 10 min. 150 µg of protein was added to each lane. Glucose (Glu), galactose (Gal) and galactose with glucose refeed (Gal+).
Figure 3.22: Increased activity of monomeric complex I and complex I containing supercomplexes in galactose-fed cells. Following separation of isolated solubilised mitochondria on a 4-12 % BN-PAGE gel, complex I activity was measured by colorimetric change. Gel was incubated in 0.1 M Tris-HCL pH 7.4 buffer containing 1 mg/ml nitro blue tetrazolium and 0.14 mM NADH at room temperature. Non-synaptic mitochondria (NSM) was added as a positive control. Glucose (Glu), galactose (Gal) and galactose with glucose reefed (Gal+).
3.2.9 AMPK activation was increased in galactose-fed HeLa cells

AMPK is a master regulator of metabolism, functioning as a sensor of the ration of AMP to ATP in cells. AMPK is activated by phosphorylation to pAMPK. This usually happens when there is more AMP compared to ATP, indicating that the cell needs to change its energy consumption and output to favour ATP production. A range of proteins including LKB1 can phosphorylate AMPK (Shackelford and Shaw 2009). LKB1 is missing in HeLa cells (Hawley, Boudeau et al. 2003). The lack of LKB1 is an oncogenic factor allowing for glycolytic metabolism to be the preferred method of ATP production. Activated AMPK has also been implicated in cells that are susceptible to increased mitochondrial fission.

In cells cultured in galactose, we would have expected to see a decrease in pAMPK signalling due to the fact that they produce more ATP and HeLa cells are LKB1 deficient. However, galactose-fed HeLa cells had a 3-fold higher expression of activated AMPK (Figure 3.23). In Figure 3.23, the three left-most lanes were loaded with cells treated with 10 mM 2-DG to fully activate AMPK, this control was necessary to rule activation by stress due to the preparation. Cells were also treated with 20 μM iULK1 to inhibit pAMPK and still galactose-fed HeLa cells had increased pAMPK expression.

AMPK phosphorylation was increased by 2-DG in glucose-fed HeLa cells. It was expected that piericidin A and antimycin A would active pAMPK in galactose-fed cells but not in glucose-fed cells. Glucose-fed cells had a slight increase in AMPK phosphorylation when treated with piericidin A and antimycin A (Figure 3.24). It was difficult to measure increases in pAMPK in galactose-fed cells as the basal expression was already significantly higher compared to glucose-fed cells. However, there was an increased in pACC, the downstream target of pAMPK when galactose-fed cells were treated with piericidin A and antimycin A (Figure 3.24). 2-DG slightly increased pACC expression in glucose-fed cells but had little effect in galactose-fed cells.
Figure 3.23: Increased pAMPK expression in galactose-fed HeLa cells.
Cells were pre-treated with 10 mM 2-DG or 10 μM Rotenone for 1 h in the activated control and 20 μM iULK1 for the negative control.
Figure 3.24: 2-DG activates AMPK in glucose-fed HeLa cells. ACC is phosphorylated in galactose-fed cells when treated with piericidin A and antimycin A. Cells were incubated with compounds for 1 h then prepared for western blot. Cells were incubated with compounds for 1 h then prepared for western blot. 20 μg of samples were added per lane.
3.2.10 Activation of AMPK reduced fusion rates in glucose-fed HeLa cells.

In order to examine the effect of increasing AMPK phosphorylation without inhibiting glycolysis or the ETC, AICAR was used to treat glucose-fed cells. AICAR generates an AMP analogue that drives AMPK activation. Glucose cells were treated with AICAR to mimic the activated state that was seen in the galactose-fed cells (Figure 3.23). It was hypothesised that if AMPK was phosphorylated to the same extent as galactose-fed cells then maybe a sensitivity to ETC inhibitors would be gained. Following 48 h incubation with AICAR, cells were harvested and analysed by western blot (Figure 3.25). pACC and pAMPK levels were increased at least 2 and 3-fold without excessive cell death. Mitochondrial fusion rates were then measured by time-resolved confocal microscopy. There was a decrease in the fusion rates from 36.6 ± 5.18 % to 66.5 ± 5.50% at 30 min post-activation (Figure 3.26). No further effect was measured with co-treatment with ETC inhibitors (Figure 3.27).
Figure 3.25: AICAR increased pAMPK and its downstream target pACC in glucose-fed cells. Cells were incubated with 1 mM AICAR for 24 h then cells were scraped and prepared for western blot. 20 μg of sample were loaded per well. (a) Representative blot of treatments. Membranes were incubated overnight at 4°C in anti-pACC (1:1000), anti-pAMPK and anti-Tubulin (1:10,000). (b) Densitometry for pACC and (c) densitometry for pAMPK normalised to Tubulin controls. Student t-test. *p<0.05.
Figure 3.26: Activation of AMPK by AICAR reduces mitochondrial fusion rates. Cells were transfected with mitochondrial markers then incubated with 1 mM AICAR for 24 h before fusion assay. Mitochondrial fusion rates were measured as before. (solid line) Sham-treated control rates and (broken line) AICAR treated cell rates.
Figure 3.27: ETC inhibition does not further reduce fusion rates. Cells were treated with AICAR for 24 h then either (a) 4 μM piericidin A (pA) or (b) 1 μM antimycin A was added for 10 min before fusion assay.
3.2.11 Dichloroacetate increased oxygen consumption in glucose cells and increased fusion sensitivity to piericidin A.

Galactose-fed cells have increased sensitivity to ETC inhibitor and also generate ATP through OXPHOS. In order to investigate the impact of shifting metabolic flux from glycolysis into OXPHOS on mitochondrial fusion rates, glucose cells were transiently treated with dichloroacetate (DCA). DCA is a compound that inhibits pyruvate dehydrogenase kinase (PDHK) thus keeping PDH in an active state and driving metabolic flux from glycolysis into oxidative phosphorylation.

Glucose-fed HeLa cells treated with 8 mM DCA for 1 hour increased oxygen consumption rate 2-fold compared to the control (Figure 3.27, panel A). There was also a significant decrease in the extracellular acidification rate from 32.9 ± 2.50 to 21.8 ± 0.84 mpH/min (Figure 3.27, panel B). This demonstrates that there was a metabolic shift from the glycolytic phenotype into OXPHOS. Since it was observed that oxygen consumption rate increased it was likely that fusion rates will be sensitive to electron transport chain inhibitors. Fusion rates in glucose-fed HeLa cells were insensitive to inhibition by ETC inhibitors (Figures 3.28 and 3.29). DCA treatment alone results in fusion rates comparable to untreated cells.

However, cells pre-treated with DCA (8 mM) for 1 h and piericidin A (4 μM) for 10 min before the assay (times and concentrations based on galactose experiments) had slightly reduced fusion rates. PI remained at 55.0 ± 2.65% compared to 35.8 ± 1.23% in the control 30 min post activation of the PA-GFP (Figure 3.29). Antimycin A treatment reduced fusion rates less effectively with PI decreasing to 45.4 ± 2.50% after 30 min (Figure 3.29).
Figure 3.27: DCA treatment caused an increase in metabolic flux from Glycolysis into. Cells were seeded into the seahorse plate at 30,000 cells/well. 24 h later 8 mM DCA was added to the wells for 1 h before assay. Student t-test. *p<0.05.
Figure 3.28: Confocal imaging of mitochondrial fusion rates in glucose-fed HeLa when treated with DCA and combined DCA and ETC inhibitors. DsRed and PA-GFP expressing cells were pre-treated with 8 mM DCA for 1 h before experiments. Cells treated with 10 μM piericidin A and 2.4 μM antimycin were also incubated with inhibitors for 10 min before assay. Scale 20 μm
Figure 3.29: DCA had no direct effect on fusion rates but increased sensitivity to piericidin A. (a) Cells pre-treated with 8 mM DCA for 1 h have comparable rates to untreated Glucose-fed HeLa cells. (b,c) Comparison of DCA pre-treated glucose-fed HeLa cells with either 4 μM piericidin A (pA) or 2.4 μM antimycin A (AA). Student t-test *p<0.05.
3.3 Discussion

Previous studies and experiments revealed that altering the carbohydrate substrate from glucose to galactose caused a shift from glycolysis to OXPHOS (Rossignol, Gilkerson et al. 2004).

It was shown that after 14 days in galactose media ECAR rates are reduced and oxygen consumption is increased, suggesting increased mitochondrial respiration and reduced dependence on glycolysis (Figure 3.1). Increased OXPHOS in galactose-fed cells is accompanied with an extended doubling time. Proliferation is 2-fold lower in galactose-fed cells compared to glucose-fed cells (Figure 3.3). Decreased proliferation is most likely a result of there being less substrate available for the synthesis of biomass via the PPP under glucose deprivation (Vander Heiden, Cantley et al. 2009).

Despite the differences in ATP production and proliferation, both glycolytic and OXPHOS HeLa cells have similar rates of mitochondrial fusion (Figures 3.5 and 3.7). It is known that fusion is partially inhibited when ATP levels are lowered and continued fusion is an energetic process. (Legros, Lombes et al. 2002). It is thought that glucose and galactose-fed cells expend some of their ATP to maintain fusion, possibly through GTP production (Boissan, Montagnac et al. 2014). Silencing of mitochondrial nucleoside diphosphate kinases, which associates with OPA 1 to convert ATP to GTP caused mitochondrial fragmentation (Boissan, Montagnac et al. 2014). When fusion is disrupted for prolonged periods cell viability decreases, as seen in galactose-fed cells with ETC inhibitors and glucose-fed cells with 2-DG (Figures 3.9, 3.10 and 3.11). The key difference between glucose and galactose-fed HeLa cells was found to be the latter sensitivity to ETC inhibitors. When complex activity was inhibited, mitochondrial fusion was halted. With ETC treatment galactose-fed cells did not have the ability to generate ATP through OXPHOS and subsequently failed to maintain mitochondria fusion leading to cell death (Figure 3.11).

Fusion was found to be reliant on mitochondrial membrane potential (Legros, Lombes et al. 2002) and when cells were depolarised by FCCP there was a loss in cell viability after 24 h in both glucose and galactose cells (Figure 3.9, 3.10 and 3.11). Galactose-fed cells
also had a loss of membrane potential when treated with ETC inhibitors (Dr. Stephen Quinn, PhD Thesis).

Glucose cells were mostly insensitive to ETC inhibition both in terms of fusion and viability (Figure 3.9 and 3.10). Following treatment with ETC inhibitors at concentrations more than sufficient to generally inhibit ETC activity, fusion rates remained high (Figure 3.9). Only disruption of microtubule activity as an off-target effect of rotenone caused a reduction in fusion (Srivastava and Panda 2007, Wu, Kalyanasundaram et al. 2013). Both media conditions were sensitive to complete loss of membrane potential, shown by FCCP. Complete depolarization by FCCP halted fusion rates but that this is reversible (Dr. Stephen Quinn, PhD Thesis and Legros, 2002). Inhibition of ETC caused a partial depolarisation in glucose cells. When cells were treated with both rotenone and oligomycin, cells were depolarized (Dr. Stephen Quinn, PhD Thesis). A further experiment would be to inhibit all proton-pumping complexes and then monitor over an extended period to see if repolarization occurs.

Inhibition of ATP synthase by oligomycin reduced fusion rates in both conditions. Oligomycin causes mitochondria to hyperpolarize. However, hyperpolarization alone does not seem sufficient to inhibit fusion, as glucose cells are relatively hyperpolarized to begin with. Inhibition of ATP synthase could cause a reduction in glycolysis over time. In the standard model of cancer cell metabolism, Hexokinases I and II may translocate onto the MOM (Mathupala, Ko et al. 2009). Hexokinase is the rate-limiting enzyme in glycolysis and requires ATP to phosphorylate glucose (Berg 2002). Proximity to mitochondria gains HK preferential access to ATP produced by OXPHOS (Roberts and Miyamoto 2015). In this model, oligomycin reduces fusion rates by decreasing mitochondrial ATP output that is required to sustain glycolysis.

Conversely it is possible that ATP synthase works to maintain membrane potential differently under each condition. In the galactose-fed cells, ATP synthase is the main source of ATP. As ATP is required to facilitate fusion, inhibition of ATP synthesis may cause a reduction in fusion rates. In glucose treated HeLa cells, inhibition of ATP synthase actually increased glycolysis (Figure 3.2, panel B) and therefore increased the glycolytic ATP output. When the ETC is inhibited in glucose cells, ATP synthase maybe working in reverse: instead of producing ATP, it may use ATP to pump protons into the mitochondrial
Intermembrane space thus preserving mitochondrial membrane potential. This phenomenon has been observed in activated macrophages and isolated mitochondria within synaptosomes (Scott and Nicholls 1980, Garedew, Henderson et al. 2010). Glycolytic flux could be increased when complexes I-IV were inhibited in cancer cells as well (Kauppinen and Nicholls 1986), therefore, this may explain why only the glucose cells were able to maintain fusion rates with complex I-IV treatment but not with oligomycin. Oligomycin treatment accelerates glycolysis in glucose-fed cells (Figure 3.2, panel B) but fusion is partially inhibited (Figure 3.9) due to the inability to pump protons via ATP synthase in order to maintain membrane potential. Galactose cells did not have the glycolytic capacity to produce ATP (Figure 3.2, panel B) and may not have the ability to accelerate glycolysis when treated with ETC inhibitors.

From Stephen Quinn’s work, it is known that a glucose-bolus was sufficient to recover fusion rate in galactose-fed cells that have been treated with ETC inhibitors. In this study, it was shown that a glucose bolus increased the rate of glycolysis and decreases OXPHOS in untreated cells (Figure 3.1 and 3.2). The increased glycolytic output could be used to generate more ATP that in turn restored fusion rates. This experiment also reveals that prolonged culture in galactose media had reduced the maximum glycolytic output of the cells when glucose is reintroduced. Basal ECAR level in glucose-bolus cells are still ~2-fold lower than normal glucose-fed cells but significantly greater than with only galactose media. The reduction in glycolytic spare capacity that was seen in glucose-bolus cells (Figure 3.2, green, panel B) suggests that prolonged glucose deprivation or increased dependence on OXPHOS may have caused a reduced expression of glycolytic enzymes.

When treated with piericidin A, rotenone or antimycin A, the mitochondria of glucose HeLa cells maintained a consistent diameter and network (Figure 3.12). The size remained mostly elongated as part of a larger network and mitochondria were spread throughout most of the cell. Rotenone caused some mitochondria to locate closer to the nucleus; this could be due in part to dysfunctional microtubule dynamics. In contrast, galactose-fed HeLa cells when treated with the above compounds for 1 h had a different appearance. Piericidin A induced a swollen shape in mitochondria around the nucleus and fragmentation in the distal regions of the cell (Figure 3.15). Rotenone and antimycin A treatment caused the mitochondria to over-fragment (Figure 3.15). Mitochondrial size was very heterogeneous throughout the cell. Oligomycin caused a similar appearance in both
glucose and galactose-fed cells creating small punctate mitochondria (Figures 3.13 and 3.16, respectively). It has been observed that when apoptosis is induced in HeLa cells the mitochondrial network collapses with subsequent formation of punctiform mitochondria followed by perinuclear clustering of fragmented organelles (Karbowski and Youle 0000).

Glucose-fed HeLa cells were more robust when mitochondria were damaged, possible due to increased glycolytic output under inhibition of mitochondrial function (Figures 3.10 and 3.1 respectively). It has been have demonstrated that 143B-p0 cells have similar fusion rates as HeLa cells despite the fact they have no functional respiratory chain (Legros, Lombes et al. 2002). Cells that have respiration-incompetent mitochondria require pyruvate supplementation to survive (Wilkins, Carl et al. 2014). It has been hypothesized that extra pyruvate is required for the maintenance of cellular redox status (King and Attardi 1996). These cells rely solely on glycolysis for ATP, in doing so they generate high concentration of NADH. In order to sustain glycolysis, there needs be constant replenishment of NAD\(^+\), under normal conditions this can be produced by mitochondrial respiration. The supplemented pyruvate is converted to lactate by lactate dehydrogenase thus generating the NAD\(^+\) required for glycolysis and proliferation (Wilkins, Carl et al. 2014). In the present model, the glucose cells do this by inhibited PDH and increasing glycolysis as demonstrated by the ECAR traces (Figure 3.2, panel B). Even when treated with DCA, lactate production in glucose cells remained relatively high compared to galactose cells. This would explain why the DCA cells were still able to maintain fusion with ETC inhibition. The galactose-fed cells seem to be unable to increase their glycolysis sufficiently to maintain fusion rates. An interesting experiment would be to supplement the galactose media with pyruvate and treat with ETC inhibitors to see if galactose can raise lactate production bypassing the need to rapidly phosphorylate glucose for glycolysis. Further experiments using mass spectrometry with carbon-labelled glucose, galactose and glutamine would be required to determine what pathways the substrates concentrate in and which are essential for maintenance of fusion.

It has been shown in previous work that DRP1 may be used differently in glucose and galactose cells when under respiratory inhibition. Glucose-fed HeLa cells did not recruit DRP1 to the mitochondrial outer membrane when treated with ETC inhibitors (Dr. Stephen Quinn, PhD Thesis). Galactose-fed HeLa cells did however recruit DRP1 to the MOM under similar conditions. Galactose cells had some differences in DRP1 localization when
treated with piericidin A and antimycin A (Figure 3.15). However, the resolution of the microscopy was not powerful enough to determine if it co-localised to scission sites on mitochondria. Treatment of ETC inhibitors for 1 h was not sufficient to observe these changes. Further study with longer incubation may shed light on DRP1 localisation. Studies have identified Mff as a target for pAMPK (Toyama, Herzig et al. 2016). Once Mff is phosphorylated it causes DRP1 to localise to the mitochondria and initial fission (Otera, Wang et al. 2010). Increased pAMPK levels in galactose cells may explain why they have such a low threshold of mitochondrial disruption before mitochondrial fragmentation takes place. Any dip in ATP production may move AMPK activation over a threshold to DRP1 recruitment. Overexpression of mutant Mff or knockdown experiments in galactose HeLa cells may elucidate further the roles DRP1, Mff and AMPK fission events. Activation of AMPK by AICAR induced a reduction in fusion in glucose-fed HeLa cells (Figure 3.26) A 2-fold increase in AMPK activation (Figure 3.25) resulted in a proportional decrease in fusion rates, although the decrease was not significantly exacerbated by piericidin A or antimycin A.

In order to explain the apparent increase in OXPHOS levels observed in galactose-fed cells it was hypothesised that an increase in complex activities could potentially account for the increased metabolism. Modification to the electron transport chain may have taken place as a result of oncogenesis. Studies in several diseases such as Parkinson’s disease have revealed that deficiencies in complex I activity can lead to a range of pathologies (Schapira, Cooper et al. 1990, Fassone and Rahman 2012). Complex I deficiency may also generate ROS further driving oncogenesis (Murphy 2009, Ogrunc, Di Micco et al. 2014). Loss of complex activity may drive the Warburg effect further and increase proliferation (Santidrian, Matsuno-Yagi et al. 2013). Contrary to this hypothesis, it was found that when assayed individually complexes I, III and IV have the same specific activity between treatments (Figure 3.18 and 3.19). This reinforces current understanding that in many cancers, cells do not have defective mitochondria but rather direct substrates away from the ETC in order to drive proliferation (Vander Heiden, Cantley et al. 2009, Zong, Rabinowitz et al. 2016). That being said it was interesting to find that galactose-fed HeLa cells had an increase in CII/III activity and a slight increase in CI/III activity when assayed together (Figure 3.20). Increased electron flow between these complexes, suggests the possible formation of supercomplexes. Where the respiratory complexes are organized in bigger structures within the inner mitochondrial membrane to perform a quick and efficient
transport of electrons (Dudkina, Kouril et al. 2010).

Although there is some evidence for complex II to participate in the respirasome (Acin-Perez, Fernandez-Silva et al. 2008), it does not usually form part of any respiratory chain supercomplexes. This is possibly due to its role in the TCA (Dudkina, Kouril et al. 2010). The method used to assay enzyme activities included steps to maximise activities of enzymes by disrupting their membranes (Barrientos, Fontanesi et al. 2001). The drawback is that the freeze-fracturing process may have attenuated the protein-protein interactions found in supercomplexes. Increased activity is facilitated by close proximity within the membrane. To qualify protein interaction in a native state within the membrane, proteins were separated using non-denaturing BN-PAGE gels. Section 3.2.8 has revealed that supercomplex formation was much more likely to occur in galactose-fed HeLa cells (Figure 3.21). Enzyme activity for complex I could also be performed with supercomplexes still intact. Here it was shown that CI activity was similar in glucose and galactose when assayed as an individual enzyme, however more of the CI was bound in a supercomplex with CIII and CIV (Figure 3.22). It is difficult to see if the CI/III activity is increased due the proximity to CI/III/IV supercomplex and the intense colour change produced. The activity here was noticeably greater when compared to the glucose. This result helps explain how the galactose-fed cells have a 3-fold increase in oxygen consumption. Shifting metabolism back to OXPHOS not only reduced proliferation but increased sensitivity to several more known compounds including metformin, which is known to be a CI inhibitor as well as an AMPK activator.

Previous studies have reported anti-tumour activity for DCA (Kankotia and Stacpoole 2014). It is either used alone or in combination with already established treatments for cancer such as cisplatin. The mechanism for DCA-induced antiproliferative and pro-apoptotic effects is yet unresolved although it has be speculated that by increasing flux through PDH and accelerating mitochondrial respiration hinders cells ability to sustain aerobic glycolysis (Kankotia and Stacpoole 2014). Here it was found that DCA increased OXPHOS transiently and did not appear to alter mitochondria fusion (Figure 3.27). The shift to OXPHOS did not fully sensitise mitochondrial fusion to ETC inhibitors in glucose-fed HeLa cells. Piericidin A reduced fusion rates to 55% while antimycin A only reduced fusion rates to 45%. Cells treated with DCA for 24 h did not show the same increase in OXPHOS that 1 h treatment did. This experiment showed that expressional change must
take place in cancer cells before their mitochondria become sensitive to inhibition. The data reinforces the supercomplex observations in that 1 h treatment was probably not sufficient to induce the formation of supercomplexes. Further experiments are required to confirm if DCA has any effect on supercomplex formation.

These studies have shown that inhibition of OXPHOS by ETC inhibitors causes a significant decrease in cell viability in galactose-fed HeLa cells mediated by a loss in mitochondrial fusion. Glucose-fed HeLa cells that predominately produce ATP through aerobic glycolysis were unaffected by ETC inhibitors. The shift to OXPHOS in galactose-fed HeLa cells did not change the activity of mitochondrial enzymes in the ETC. Overall mitochondrial content was also unchanged and mitochondria of HeLa cells in both media appeared as long interconnected filaments, as previously reported (Rizzuto 1998, De Giorgi, Lartigue et al. 2000). The data suggest that a change in supercomplex assembly facilitates the increased capacity for non-glycolytic metabolism. It was shown that galactose cells also have activated AMPK. Pharmacological activation of AMPK using AICAR reduces mitochondrial fusion rates in glucose cells. This supports emerging evidence that AMPK drives mitochondrial fission by phosphorylating MFF (Zhang and Lin 2016). It was demonstrated that expression and structural change over an extended time period is required to cause glucose HeLa cells to become sensitive to ETC inhibitors. Glucose HeLa can be forced to shift to OXPHOS by pharmacologically activating PDH (Xie, Wang et al. 2011). It has been shown that glucose cells undergoing OXPHOS under these conditions were still insensitive to ETC inhibitors.

It was unknown how the shift from glycolysis to OXPHOS might subsequently affect the glycolytic pathway and mitochondrial dynamics. Hexokinase is at the interface of both mitochondria and glycolysis. Previous experiments indicated that HKII was decreased during glucose deprivation (PhD Thesis, Dr. Stephen Quinn). Due to the importance of HKII in cancer cells and its mitochondrial location it was hypothesised that HKII would be a node of interest. Hexokinase II is at the interface of both mitochondria and glycolysis. Galactose-fed cells bypass the hexokinases when entering glycolysis. The following chapter builds on fusion and cell viability data discovered in the present chapter with emphasis on mitochondria-bound HKII.
Chapter 4: Effects of Hexokinase II on Mitochondrial Dynamics
4.1 Introduction

Results in chapter 3 suggest that the glycolytic phenotype of HeLa cells was not pre-programmed, but remained responsive to carbon source. Following the switch from glucose to galactose, mitochondria arranged their proteins in order to facilitate increased mitochondrial respiration. Fusion and cell viability were affected when cells were treated with ETC inhibitors in the absence of glucose. Interestingly, cells were not resensitised to ETC inhibitors during 1 h of DCA-treatment. In fact, mitochondrial respiration was increased under these conditions. When glucose supply is limited, mitochondria display sensitivity to ETC inhibitors. The key difference regarding glycolytic flux under glucose or galactose substrates is where they enter the pathway. Both glucose and galactose are routed through glucose 6-phosphate; however, glucose is first directly phosphorylated by hexokinase, whereas galactose is first processed via the Leloir pathway. This key biochemical difference along with the mitochondrial localisation of hexokinase II (HKII) (Polakis and Wilson 1985, Rossignol, Gilkerson et al. 2004), suggested HKII as a possible regulatory node.

4.1.1 Hexokinase II is bound to mitochondria and is thought to be protective

HKII is a key regulator in glucose metabolism and of the four isoforms it has the highest affinity for glucose (Wilson 2003). HKII distinguishes itself from other isoforms in that it has two catalytic domains in its N- and T- terminal (Tsai and Wilson 1996). Thus, it is the preferred isoform in proliferative tissues. High rates of glucose phosphorylation ensure adequate glucose is sequestered inside cells to sustain aerobic glycolysis. It has been speculated that the mitochondrial location of HKII gives the cell preferential access to ATP generated from mitochondrial respiration (Arora and Pedersen 1988). The MOM location of HKII has also been implicated in the prevention of opening the mPTP (Shulga, Wilson-Smith et al. 2010). The mechanism by which HKII prevents apoptosis in this manner is highly debated and very speculative. What is known is that there is an interaction between the N-terminal of HKII and VDAC (Xie and Wilson 1988). When glucose is available, HKII is phosphorylated by Akt, which promotes mitochondrial binding (Doerks, Copley et al. 2002, Majewski, Nogueira et al. 2003). Dissociation of HKII from VDAC initiates apoptosis (Shoshan-Barmatz, Zakar et al. 2009).
In a highly glycolytic environment HKII is predominantly located on the MOM (Wolf, Agnihotri et al. 2011). HKII is bound to VDAC by the N-terminal-binding domain (Nakashima, Paggi et al. 1988). HKII/VDAC interaction is regulated by several factors including glucose/glucose-6-phosphate, ATP/ADP ratios, intracellular lactate, pH and phosphorylation by Akt (Sun, Shukair et al. 2008, McCommis and Baines 2012, Roberts, Tan-Sah et al. 2013). When Akt is activated by PI3K, it modulates bioenergetic pathways such as increasing glycolysis. Akt works by phosphorylation of proteins causing increased protein-protein interactions (McCommis and Baines 2012). The anti-apoptotic effects of Akt involve VDAC, it increases HKII binding and causes localisation of anti-apoptotic Bcl2 member Bcl-Xₐ to the VDAC complex (Majewski, Nogueira et al. 2003). Apoptosis is multifactorial process one aspect of this is how VDAC/HKII complex inhibits the opening of the mPTP. It is speculated that HKII reduces the amount of free VDAC sites that can interact with pro-apoptotic molecules such as Bax (Pastorino, Shulga et al. 2002). The complex also attenuates the localisation and oligomerisation of Bad to the MOM, which is required for the activation of mPTP. Bad and Bax are activated by their upstream regulator tBid (Westphal, Dewson et al. 2011). It is not clear how these proteins activate the mPTP but it is known that HKII prevents the activation/oligomerisation of Bad and Bax causing a reduction in apoptosis (Majewski, Nogueira et al. 2003).

HKII has also been implicated in altering the VDAC/ANT interactions by preventing conformational changes in the ANT that lead to mPTP opening. It is unclear how ADP/ATP flux through the ANT and VDAC prevents mPTP, it has been shown that isoforms of ANT maintain mitochondrial membrane potential in different ways (Chevrollier, Loiseau et al. 2011). It is thought that the ANT1 and ANT3 export mitochondrial ATP in exchange for ADP. ANT2 appears to be key to cancer metabolism as it is thought to transport glycolytic ATP into the mitochondria. The F1F0-ATPase complex then hydrolyses the ATP, thus pumping protons into the intermembrane space preserving mitochondrial membrane potential (Chinopoulos and Adam-Vizi 2010), ensuring cell survival and continued proliferation. Unlike ANT1 and 3, ANT2 is not pro-apoptotic (Bauer, Schubert et al. 1999, Zamora, Granell et al. 2004).
4.1.2 Hexokinase II as an antioxidant

HKII has been shown to have some antioxidant properties. The main mechanism of antioxidant ability is increased G6P production generated by glycolysis. NADPH produced by the PPP is used to reduce glutathione, which quenches reactive oxygen species (ROS) and acts as a cofactor for multiple peroxidase enzymes (Kerksick and Willoughby 2005). Reduction of HKII expression in cardiac cells accelerates transition to heart failure mediated by an increase in ROS (Wu, Wyatt et al. 2012). It has been shown that cells exposed to ROS for extended periods will increase HKII expression; this could be a feedback system to prevent increased ROS or a driver of proliferation (Silveira, Pilegaard et al. 2006). When cells were exposed to ROS with additional antioxidants, HKII expression remained the same as controls. Although ROS production in cancers cells is a controversial topic, almost all cancers have elevated ROS levels alongside increased levels of antioxidant proteins (Liou and Storz 2010). A delicate balance of intracellular ROS is required for cancer cell function (Liou and Storz 2010). Low doses of ROS stimulate cellular proliferation in a wide variety of cancers (Storz 2005). ROS production can be pro-cancerous and also detrimental to continued growth at high concentrations, reviewed by Ogrunc (2012). Some chemotherapies actually generate intracellular ROS as a way of killing cancer cells (Trachootham, Alexandre et al. 2009).

Apoptosis is usually measured through cleavage of caspases or by cytochrome c release (Luo, Budihardjo et al. 1998, Namura, Zhu et al. 1998). These events are usually preceded by a depolarisation of mitochondria (Heiskanen, Bhat et al. 1999). In controlled cell death, the degradation of mitochondria is controlled through fission processes. DRP1 is required for apoptosis, the loss of which results in cell survival and swollen mitochondria (Qian, Wang et al. 2013). There are few studies reporting on how mitochondria dynamics are affected following loss of HKII, locally and globally. It remains to be shown that DRP1 is recruited to the mitochondria to initiate apoptosis through fission. It is unknown if HKII is required by mitochondria to maintain fusion and if this process is glucose dependant. Due to its role in glycolysis and anti-apoptotic functions it is hypothesised that HKII may influence mitochondrial function. In the previous chapter it was stated that mitochondrial function is closely related to its structure and how mitochondrial networks are organised throughout the cell. If HKII has a role in mitochondrial dynamics, it is predicted that mitochondrial fusion and fission rates will be affected.
Cancer cells tend to have higher expression of DRP1; this is thought to facilitate increased cellular division (Inoue-Yamauchi and Oda 2012, Liesa, Ferreira-da-Silva et al. 2015). Thus, many cancer cells are reported to have highly fragmented mitochondria. DRP1 processing of mitochondria in mitophagy reduces the number of damaged mitochondria that accumulate within a cell.

4.1.3 Hexokinase II removal from MOM increases ROS production and reduces cell viability

Various studies on HKII removal from the mitochondria have focused on ROS and apoptosis (Sun, Shukair et al. 2008). Findings have clearly demonstrated that HKII is essential for reduced cytotoxic levels of ROS in a variety of cell models (Wu, Wyatt et al. 2012, Nederlof, Guerel et al. 2013, Roberts, Tan-Sah et al. 2013). Surprisingly there has been little research directly measuring mitochondrial function. It is also not known how detachment of HKII from the OMM affects glycolysis. It is not known if HKII requires VDAC interaction to efficiently make use of mitochondrial ATP. Glucose deprivation studies have concluded that HKII requires glucose for ROS protection have been quite limited due to experiments being carried out at low glucose concentrations with no alternate carbohydrate supplement (Sun, Shukair et al. 2008). Using galactose to sustain cells the role of HKII in mitochondrial health uncoupled from its glycolytic function is an interesting area to study.

Greater understanding of how bioenergetic pathways control mitochondria and *vice versa* may lead to an improved understanding of cellular survival not only in cancer cells but in brain tissues and cardiac muscle. Mitochondrial-bound HK is a gatekeeper of metabolism, promoting sustained ATP production while maintaining a careful balance of ROS. The damage incurred during ischemia is often exacerbated following reperfusion due to the generation of ROS. It is known that dysfunctional mitochondria produce ROS, and HKII has been shown to be protective in such conditions. HKII interactions with mitochondria could provide insights that may have diverse many therapeutic applications.
4.1.4 Aims of chapter

Cancer cells upregulate HKII to increase glucose uptake; this allows for increased glycolysis and provides glucose 6 phosphate for the pentose phosphate pathway. Increased HKII is speculated to allow cancer cells to avoid apoptosis through its interaction with mitochondria. In this chapter, the role of HKII in mitochondrial maintenance was assessed in glucose-fed and galactose-fed HeLa cells. The objectives were:

1. **To assess the role of Hexokinase II in HeLa cell protection.** HKII translocation to the mitochondria was assessed when both glucose-fed and galactose-fed cells were exposed to ETC inhibitors.

2. **To determine how mitochondrial bound HKII affects mitochondrial fusion.** Using a peptide consisting of the N-terminal region of HKII and a TAT sequence to allow entry into the cell, HKII was selectively removed from the MOM. Following removal of HKII, mitochondrial fusion rates were measured alone with quantification of mitochondrial fragmentation, oxygen consumption and lactic acid production.

3. **To quantify mitochondrial fusion rates when HKII is silenced.** HeLa cells were transfected with HKII-siRNA to silence protein expression. Mitochondrial fusion rates, oxygen consumption rates and lactic acid production were then assayed to determine how knockdowns affect mitochondrial function.

4. **To quantify mitochondrial membrane potential and ROS production when HKII is silenced.** Using the fluorescent mitochondrial dyes TMRM and JC-1, mitochondrial membrane potential and reactive oxygen species production, respectively, was measured following HKII silencing.
4.2 Results

4.2.1 Galactose-fed cells express significantly less HKII

The change in metabolism from glycolysis to OXPHOS did not result in a significant change in mitochondrial protein activity level or in overall mitochondrial content therefore it was concluded that expressional change must occur further up the pathway. The DCA experiment in Chapter 3 revealed that glucose-fed HeLa cells were capable of increasing OXPHOS rates over basal conditions. This suggests that mitochondrial respiration can be increased however increasing flux through mitochondria alone was no sufficient to sensitise fusion rate to ETC inhibitors to the same extent as galactose-fed cells. It was hypothesised that expressional change in glycolytic enzymes would need to take place before glucose-fed cells would become susceptible to ETC inhibition. Previous work (Dr Stephen Quinn, PhD Thesis) carried out in the lab showed that there were decreases in hexokinase II expression levels when cells were cultured in galactose. To elaborate upon this finding, HKII expression was examined in whole cell lysates and in mitochondrial sub-cellular fractions.

HKII expression was reduced in galactose-fed HeLa whole cell extracts and in mitochondria isolates prepared by hypotonic lysis and differential centrifugation (Figure 4.1) In cells given a glucose bolus, HKII further dissociated from the MOM but not completely, presumably in response to increased concentrations of cytosolic glucose.
Figure 4.1: Decreased expression of HKII in galactose-fed HeLa cells and reduced localisation to the MOM. HeLa cells were grown on DMEM supplemented with either 25 mM glucose (Glu) or 10 mM galactose (Gal) for 14 days. Cells were then scraped and mitochondria were isolated by differential centrifugation in a sucrose buffer. 20 μg protein was loaded per lane for WCL (left) and 5 μg per lane for mitochondrial fractions (right).
4.2.2 Hexokinase localizes to mitochondria in glucose-fed HeLa cells when treated with mitochondrial inhibitors

HKII binds to VDAC on the MOM, this location is thought to provide a protective mechanism against apoptosis. It has been reported to prevent the release of mitochondrial intermembrane space proteins such as cytochrome c that activate the execution phase of apoptosis, controlled by Bcl-2 family of proteins (Pastorino and Hoek 2003) (Pastorino, Shulga et al. 2002). It was initially hypothesised that under stressful conditions HKII may dislocate from the MOM in treatments that were known to cause cell death.

To investigate the potential protective effects of HKII against apoptosis cells were treated with compounds that inhibit mitochondrial and glycolytic pathways. Cells were treated with each compound for 30 min then analysed by immunoblots. Inhibition of the ETC inhibitors caused increased enrichment of HKII in the mitochondrial fraction. HKII amounts increased 4.33-fold with piericidin A, 3-fold with rotenone, 4.60-fold with antimycin A and 3.64-fold with oligomycin A compared to the control (Figure 4.2, panels A and C). 2-DG treatment caused a slight decrease. FCCP and Metformin had no significant effects on HKII localisation (Figure 4.2, panels A and C).

Contrary to the initial hypothesis, HKII located in the mitochondrial fraction was increased when mitochondrial complexes were inhibited. 2-DG which caused a build-up of 2-deoxyglucose-6-phosphate caused a slight detachment probably due to product inhibition (Figure 4.2). Depolarisation of mitochondria did not result in a significant change in HKII localisation.

Galactose-fed cells that are known to be susceptible to cell death when treated with these compounds surprisingly had increased HKII located in the mitochondrial fraction (Figure 4.2, panel B and D). Interestingly ATP synthase inhibition by oligomycin did not cause HKII enrichment in the mitochondrial fraction, which was observed in glucose cells (Figure 4.2, panels B and D). Metformin caused a 4-fold enrichment in galactose-fed cells but not in glucose-fed cells (Figure 4.2).
Figure 4.2: Hexokinase II localises to mitochondria when respiration is inhibited. Cells were plated seeded on to 15 cm dishes and grown to 80% confluency. Cells were treated with compounds for 30 min before scraping and mitochondria isolation. Figure shows western blot of glucose (a and b) and galactose HeLa cell lysates (c and d). Cells were treated with 4 μM piericidin A (pA), 1 μM rotenone (Rot), 2.4 μM antimycin A (Anti A), 1 μM oligomycin (Oligo), 0.5 μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 30 mM 2-deoxyglucose (2-DG) and 10 mM metformin.
4.2.3 2-Deoxyglucose reduces fusion rates in glucose and galactose-fed HeLa cells.

2-Deoxyglucose competes with glucose for phosphorylation by hexokinase. 2-deoxyglucose-6-phosphate is not processed any by glycolytic enzymes. This results in a build-up followed by product inhibition of hexokinase. It is not known how 2-DG affects mitochondrial fusion rates. Cells were first transfected with cDNA encoding subunit VIII COX (mitochondrial marked) targeted DsRed (mtDsRed) and photoactivatable GFP (mtPA-GFP) (Figure 4.3). The cells were then pre-treated with 10 mM 2-DG for 20 min before the assay. Glucose-fed HeLa cells showed decreased rates of fusion when treated with 10mM 2-deoxyglucose (Figure 4.4, panel A). Despite galactose-fed HeLa not requiring any of the hexokinase isozymes for ATP production, it was surprising that when glycolysis was inhibited by 2-DG there was also a decrease in fusion (Figure 4.4, panel B). Following 20 min pre-incubation with 2-DG the rate of fusion was significantly decreased at 15 min post photoactivation with PI values of 76.2 ± 5.20% compared to the control 54.7 ± 2.70% (p<0.05) and at 30 min post photoactivation with PI values of 69.7 ± 0.27% when compared to the control at 32.2 ± 1.80% (p<0.001) in galactose-fed HeLa cells (Figure 4.4, panel B)
Figure 4.3: Assay of mitochondrial fusion dynamics in 2-Deoxyglucose treated glucose-fed and galactose-fed HeLa cells. Cells were either treated with 10 mM 2-DG (right side panels) or water (left side panels) for 10 min before assay. Fusion rates were analysed by a time course fluorescence assay as describe before. Treated cells had significantly reduced fusion rates. Scale 20 μm.
Figure 4.4: 2-DG reduces fusion rates in glucose- and galactose-fed HeLa cells. PIs for ROI were measured for each activated cell, obtained from confocal images. Solid green and red lines represent PA-GFP and DsRed for vehicle treated cells. Broken green and red lines represent PA-GFP and DsRed for 2-DG treated cells. Comparison was made for each time point post activation. 2-DG perturbs fusion rates in glucose and galactose cultured HeLa cells. (a) Glucose-fed HeLa (b) galactose-fed HeLa. Student t-test at each time point. *p<0.05, ***p<0.001.
4.2.4 HeLa cells cultured in glucose have decreased fusion rates when treated with clotrimazole

Clotrimazole (CTZ), an antifungal medication that has been reported to have anti-tumour properties. It was used in several studies as a convenient way to detach HK from the outer mitochondrial membrane (Penso and Beitner 1998, Furtado, Marcondes et al. 2012). In this regard CTZ can be used to study the effect of detaching HKII from the mitochondria.

Western immunoblot of isolated mitochondria from cells treated with clotrimazole (CTZ) showed that 75 µM was sufficient to detach HKII from the MOM (Figure 4.6, panel B). When glucose-fed HeLa cells were treated with CTZ, there was a significant decrease in the rate of fusion (Figure 4.5 and 4.6, panel A). After 15 min, the PI remained high at 88.2 ± 1.30% compared to 54.7 ± 2.70% in the control group. After 30 min, the PI was 69.8 ± 1.50% compared to that of the control at 41.3 ± 2.90% (Figure 4.6, panel A). This shows that at 75 µM CTZ had inhibited mitochondrial fusion rates in glucose-fed HeLa cells.
Figure 4.5: Mitochondrial fusion dynamics was perturbed by clotrimazole treatment. Cells expressing mitochondrial markers were pretreated with (a) DMSO or (b) 100 μM Clotrimazole for 1 h to ensure removal of HKII from the OMM, confirmed by western blot. Scale 20 μm.
Figure 4.6: Clotrimazole detached HKII from the mitochondria and significantly perturbed fusion rates in glucose-fed HeLa cells. (a) Cells were pre-treated with CTZ (75 μM) for 1 h then fusion rates were assayed and quantified. (b) To ensure detachment of HKII from OMM cells were treated with either CTZ or a water control in 15 cm² plates for 1 h, mitochondria were isolated, following collection. Samples were analysed by western blot in duplicate. Cells treated with CTZ had significantly reduced fusion rates and detached HKII from OMM. Scale 20 μm. Student t-test at each time point, *p<0.05, **p<0.01.
4.2.5 Off target effects and non-specific effects of clotrimazole

Cell viability following CTZ treatment was analysed by an Alamar blue assay. Both glucose and galactose cultured HeLa cells were incubated in CTZ for 4 h with the Alamar Blue reagent. The concentration required to detach the HK from the outer mitochondria membrane, 75 μM (Figure 4.7, panel A), was toxic to cells. IC₅₀ values of 28.38 μM and 17.59 μM were determined for glucose and galactose-fed cells respectively (Figure 4.7, panel A).

CTZ was a dose dependent inhibitor of CI (Figure 4.7, panel B). CI activity was measured polarographically; oxygen consumption rates were determined for over a range of concentrations (Oroboros, Oxygraph). Fresh, tightly coupled liver mitochondria were used as a source. Thus, CTZ would not be an appropriate compound for further fusion experiments due to interactions with ETC enzymes.
Figure 4.7: Clotrimazole reduced HeLa cell viability and inhibited complex I activity. Both glucose and galactose-fed cells were seeded at 5000 cells per well in 96-well plates (A). A range of concentrations of CTZ was incubated with the cells for 4 h with Alamar Blue reagent. Cell viability was calculated and plotted against Log_{10} CTZ concentrations. CI activity was measuring using a Clark electrode containing isolated liver mitochondria from Wister rats. Glutamate/Malate was used as a substrate for CI. Data expressed as a percentage of the untreated control. CTZ was cytotoxic to both glucose and galactose-fed HeLa cells and CI activity is also reduced, in a dose dependent manner. ***p<0.001
4.2.6 Mitochondrial localization of HKII to the MOM can be disrupted by transduction of a TAT-tagged N-terminal HKII peptide

Due to the toxic off target effects of clotrimazole a 15 amino acid sequence of the N-terminus region of HKII was synthesised. This is the region that is thought to bind directly to VDAC and thereby anchoring HKII to the mitochondria (Polakis and Wilson 1985, Nakashima, Mangan et al. 1986). HKI also can do this but not with the same affinity, earlier experiments (Dr. Stephen Quinn, PhD Thesis) showed no difference in HKI expression in whole cell extracts or at the mitochondrial surface. The HIV-1 TAT sequence was added to promote uptake into the cells.

In both the glucose and galactose-fed cells the HK-TAT peptide caused the mitochondrial bound HKII to dissociate. No detachment was observed in a control peptide (Ctl-TAT) obtained by linking a non-sense sequence of similar length to the same TAT motif (Figure 4.8).

HK-TAT caused significant cell death after 1 h at concentrations greater than ~60 μM in glucose and galactose-fed cells, IC50 were 51.67 μM and 37.65 μM respectively (Figure 4.9, panels A and B). Caspase 3 and 7 were detected after 1 h treatment with the peptide indicating detachment of HKII induces apoptosis in HeLa cell (Figure 4.10, panels C and D).
Figure 4.8: HK-TAT selectively removed HKII from mitochondria. Cells were treated with HK-TAT (30 µM) or a control peptide (Ctl-TAT) for 30 min. Cells were then scraped and lysed, then mitochondria fractions were isolated by centrifugation. 5 µg of protein was added per lane.
Figure 4.9: HKII detachment from mitochondria caused cell death after an hour. Cells were seeded into a 96-well plate and cultured for 24 h. Cells were treated with a range of concentrations of HK-TAT or Ctl-TAT peptides for 1 h then assayed using ApoGlo multiplex assay kit. Cell viability assay measuring a peptide substrate that becomes fluorescent upon cleavage by live-cell proteases in (a) glucose-fed and (b) galactose-fed cells.
Figure 4.10: HKII detachment from mitochondria induced apoptosis after an hour. Cells were loaded into a white-96 well plate. A luminescence assay measuring caspase 3/7 release determined induction of apoptosis. To induce apoptosis, staurosporine was used as positive control (green square). (a) Glucose-fed cells and (b) galactose-fed cells.
4.2.7 HK-TAT peptide caused significant reduction in mitochondrial fusion rates

To specifically assess the effects of HKII localisation on mitochondrial fusion rates, the HK-TAT peptide was used to displace bound HKII from the MOM. By competitively binding to the VDAC site, functional HKII enzyme is released into the cytosol. HKII localising to the MOM was important for protection from apoptosis and the release of cytochrome c as well as to utilize ATP coming from the mitochondria to facilitate glycolysis. Disruption of this could result in a loss of function of mitochondria due loss of protection and a reduction in glycolysis that seems to support fusion in glucose fed cells.

Treatment of glucose-fed HeLa cells with HK-TAT (30 µM) decreased fusion ~2-fold with PI remaining high at 78.0 ± 3.84% (Figure 4.12 and 4.13, Panel B) at 30 min compared to 39.3 ± 2.03% (Figure 4.11 and4.13, panel A) in the control peptide (Ctl-TAT). Ctl-TAT had no effect on fusion rates.
Figure 4.11: Fusion Rates were unchanged in Ctl-TAT treated glucose HeLa cells. Glucose HeLa cells expressing DsRED (left panels) and PA-GFP (centre panel) were treated with 30 μM of a non-specific control peptide linked to a TAT sequence. The rate of fusion was measured over 30 min by measuring PI of a green ROI. >50 cells imaged. Scale 20 μm.
Figure 4.12: HK-TAT induced detachment of HKII decreased mitochondrial fusion rates in glucose fed HeLa cells. Cells expressing DsRED (left panels) and PA-GFP (centre panels) were treated with 30 μM HK-TAT peptide and imaged immediately. Images were taken at 1, 15 and 30 min. Detachment of HKII was confirmed by western blot. >50 cells imaged. Scale 20 μm.
Figure 4.13: HKII detachment using HK-TAT peptide significantly reduced mitochondrial fusion rates in glucose-fed HeLa cells. Quantification of fusion rates from images was calculated by measuring PI of the ROI. PIs of green regions in Ctl-TAT (a) decrease at regular rates. HK-TAT (b) treated cells do not have regular dissipation of green signal.
4.2.8 HK-TAT peptide caused complete halt of mitochondrial fusion in galactose-fed HeLa cells

Treatment of galactose-fed cells caused a complete abrogation in fusion rates. PI remained at 92.4 ± 4.03% and 97.3 ± 7.45% (Figure 4.15 and 4.16, panel B) indicating no change in intensity of the green ROI. Ctl-TAT did not affect fusion rates (Figure 4.14 and 4.16, panel A).

Prolonged incubation with HK-TAT induces apoptosis due to the opening of permeability transition pore (PTP) following the abrogation of HK/VDAC binding. Here it was shown that mitochondrial fusion occurs prior to induction of apoptosis. Concentration and incubation of HK-TAT in fusion experiments were sub-optimal for induction of apoptosis. These results suggest that HKII has an essential role in maintaining mitochondrial health through fusion, independent of its role in glycolysis. Cancer cells with high levels of HKII have increased levels of mitochondrial protection as they can maintain mitochondrial fusion under extreme stress that healthy cells cannot. Galactose-fed HeLa cells with their down regulated HKII expression appear to be more sensitive to HKII detachment as 30 μM caused complete halt to fusion.
Figure 4.14: Fusion rates were unchanged in galactose-fed HeLa cells treated with Ctl-TAT. Galactose HeLa cells expressing DsRED (left panels) and PA-GFP (centre panel) were treated with 30 μM of a non-specific control peptide linked to a TAT sequence. The rate of fusion was measured over 30 min by measuring PI of a green ROI. >50 cells imaged. Scale 20 μm.
Figure 4.15: Green region of interest remained intense following detachment of HKII from OMM. Galactose-fed cells expressing DsRED (left panels) and PA-GFP (centre panels) were treated with 30 μM HK-TAT peptide and imaged immediately. Images were taken at 1, 15 and 30 min. Detachment of HKII was confirmed by western blot. >50 cells imaged. Scale 20 μm.
Figure 4.16: HKII detachment using HK-TAT peptide halted mitochondrial fusion rates in galactose fed cells. Quantification of fusion rates from images was calculated by measuring PI of the ROI. PIs of green regions in Ctl-TAT (a) decrease at regular rates. HK-TAT (b) treated cells do not have regular decrease in green signal.
4.2.9 Detachment of HKII from mitochondrial membrane decreases oxygen consumption and extracellular acidification

HKII detachment resulted in an immediate loss of mitochondrial fusion (Figures 4.11-4.16) demonstrating the requirement of HeLa cells to retain it on the MOM for normal function. Following detachment by HK-TAT, unbound HKII should retain functionality to phosphorylate glucose and continue glycolysis. Oxygen consumption and lactate production were measured to determine the effects of HKII from the mitochondria on mitochondrial respiration and glycolysis. It was hypothesised that OXPHOS would be severely decreased as fusion was compromised.

Oxygen consumption rate decreased from $28.1 \pm 4.37$ pMole O$_2$/min to $11.3 \pm 1.70$ pMole O$_2$/min when treated with HK-TAT (Figure 4.17, panel A). Surprisingly the ECAR also decreased significantly from $11.6 \pm 3.3$ mpH/min in Ctl-TAT treated cells to $2.31 \pm 0.96$ mpH/min (Figure 4.17 and 4.19, panel B). Spare glycolytic capacity was also reduced in HK-TAT treated cells. Following oligomycin treatment there was no further increase in lactate production similar to that seen in the control (Figure 4.17, Panel B). This demonstrates that HKII is required on the mitochondria in order to support mitochondrial respiration and to properly function as a glycolytic enzyme.

Similarly, impaired fusion was recorded in galactose-fed cells when HKII was detached from the mitochondria (Figure 4.18) and this was also reflected in respiration rates. Oxygen consumption decreases from $131.7 \pm 8.6$ pMole O$_2$/min to $11.5 \pm 1.70$ pMole O$_2$/min (Figure 4.18, panel A). Glycolysis was also reduced from $4.28 \pm 0.59$ mpH/min to $1.22 \pm 0.56$ mpH/min (Figure 4.18, panel B).
Figure 4.17: Detachment of HKII from mitochondria reduced oxygen consumption rates and extracellular acidification rates in glucose-HeLa cells. (a) Oxygen consumption rates in cells treated with either 30 μM HK-TAT peptide or control Ctl-TAT peptide. Peptides were added just before the plate was inserted into the Seahorse XF-24. (b) Simultaneous measurement of extracellular acidification rates in both treatment groups. Normalised to protein content of each well.
Figure 4.18: Detachment of HKII from mitochondria reduced oxygen consumption rates and extracellular acidification rates in galactose-HeLa cells. (a) Oxygen consumption rates in cells treated with either 30 μM HK-TAT peptide or control Ctl-TAT peptide. Peptides were added just before the plate was inserted into the Seahorse XF-24. (b) Simultaneous measurement of extracellular acidification rates in both treatment groups. Normalised to protein content of each well.
Figure 4.19: Oxygen consumption and lactate production rates were reduced in glucose and galactose-fed HeLa cells. (a, c) Mitochondrial oxygen consumption rates in glucose and galactose cells. (b,d) Extracellular acidification rates minus 2-DG rate in glucose and galactose cells. Student t-test to determine significance. *p<0.05, **p<0.01, ***p<0.001.
4.2.10 HK-TAT causes extensive mitochondrial fragmentation

Live cell fusion assays revealed that cells were beginning to fragment as the assay progressed more rapidly than observed with ETC inhibitors or 2-DG. It was unknown if the rapid onset of fission was accompanied by localisation of DRP1 to the mitochondria. To further quantify this question, cells were treated with HK-TAT or Ctl-TAT peptides for 1 h and the mitochondrial morphology observed.

HK-TAT caused extensive fragmentation of the mitochondria. Most cells had fragmented mitochondria when HKII was removed from the MOM, this was the same in both glucose and galactose-fed cells (Figure 4.20 and 4.21, respectively). Fission was initiated in both media conditions. Almost all cells had punctate mitochondria throughout the entire cell body.

DRP1 spread throughout the cell body. The detachment of HKII from the MOM increased mitochondrial fragmentation and caused DRP1 to localise to the mitochondria, probably to facilitate scission processes. Any protective effects of mitochondrially bound HKII was independent of glucose, as galactose-fed HeLa underwent similar extensive fragmentation (Figure 4.22).
Figure 4.20: Mitochondrial fragmentation in glucose-fed HeLa cells following HKII detachment from MOM. Cells were seeded and grown on glass cover slips for 24 h. Cells were treated with either 30 μM Ctl-TAT or HK-TAT for 1 h then prepared for immunostaining with anti-TOM-20, anti-DRP11, and DAPI. Scale=20 μm
Figure 4.21: Mitochondrial fragmentated following HKII detachment from MOM is independent of glucose. Cells growing on galactose for 14 days were seeded and grown on glass cover slips for 24 h. Cells were treated with either 30 µM Ctl-TAT or HK-TAT for 1 h then prepared for immunostaining with anti-TOM-20, anti-DRPI1 and DAPI. Scale =20 µm
Figure 4.22: Mitochondria fragment following HKII detachment from the OMM. Percentage of cells with fragmented mitochondria was calculated. (a) Glucose cells and (b) galactose cells were treated with peptides for 1 h. Between 60-80 cells were used per experiment. Student t-test to determine significance, ***p<0.001. Organelles were classified as fragmented when >50% of the total cellular mitochondria displayed a major axis <5 μm.
4.2.11 Silencing HKII reduced glycolytic and OXPHOS rates in glucose HeLa cells

The silencing of HKII has led to perturbations in mitochondrial fusion rates. An accumulation of damaged organelles caused by improper maintenance of mitochondria could have deleterious effects on mitochondrial respiration. Conversely it was not known if the reduction in fusion was the result of cells switching to an OXPHOS phenotype brought on by the reduced glycolytic capacity. Using the Seahorse XF 24 analyser mitochondrial and glycolytic metabolism was measured by way of oxygen consumption and lactic acid production. Basal rates were calculated by subtracting the rotenone + antimycin A/2-DG rates from the initial rates. Coupling efficiency and spare respiratory capacity was calculated by subtracting oligomycin and FCCP rates from initial rates. Spare glycolytic capacity was calculated by subtracting oligomycin rates from initial rates.

Following transfection with siRNA glycolysis was reduced ~2-fold in glucose fed HeLa cells. The scrambled control treated cells had a rate of 18.7 ± 1.13 mpH/min compared to the HKII knock down 10.3 ± 2.46 mpH/min (Figure 4.24, panel A). Spare glycolytic capacity following the knock down was reduced from 19.8 ± 2.97 mpH/min to -0.89 ±1.22 mpH/min (Figure 4.24, panel C). This suggests that glucose-HeLa cells when expressing endogenous levels of HKII have an excess of enzyme that not saturated with substrate. Reducing HKII levels not only decreases glycolytic rates but also removes the ability to increase glycolysis following mitochondrial inhibition.

Oxidative phosphorylation was reduced from 35.6 ± 2.22 pMoles O₂/min to 18.6 ± 4.73 pMoles O₂/min although this was not significant (p value = 0.051) (Figure 4.24, panel B). Glucose HeLa cells tend to have such low rates of oxygen consumption it was difficult to measure small changes at this seeding density. No significant change in spare respiratory capacity or coupling efficiency (Figure 4.24, panel D).
Figure 4.23: HKII knock down caused a decrease in glycolysis and OXPHOS. Glucose HeLa cells were transfected with either a scramble control (Scr-siRNA) or HKII-siRNA and plated at 20,000 cells/well into a Seahorse culture plate. After 48h the plate was run according to the program described. ECAR (a) was measured as the rate of change of pH. OCR (b) was determined by rate of change of oxygen consumption. Spare glycolytic capacity and mitochondria coupling efficiency was obtained by injection of 0.5 μM Oligomycin (Oligo). Maximum respiration was obtained via 0.5 μM FCCP. Blank rates for glycolysis and OXPHOS were measured after the injection of 30 mM 2-DG and 1 uM rotenone (Rot) and 1 uM antimycin A respectively. Results are mean ± SEM for at least three independent transfections.
Figure 4.24: HKII knock down decreased glycolysis and spare glycolytic capacity. Basal rate of glycolysis (a) calculated by subtracting the average 2-DG rate from initial rates on shown on previous figure. Basal oxygen consumption rates (b) calculated by subtracting average rotenone/antimycin A treated rates. Spare glycolytic capacity (c) from increased ECAR following inhibition of mitochondrial respiration by oligomycin. Oxygen consumption rate indicative of ATP production (d) was calculated by subtracting oligomycin rate from initial rate. Results are mean ± SEM for at least three independent transfections. *p<0.05.
4.2.12 Silencing HKII reduced glycolytic and respiration rates in galactose-fed HeLa cells

HKII role in galactose-fed cells remained unknown. HKII was down regulated in galactose-fed cells yet significant levels remain on the mitochondria and in the cytosol. To evaluate whether this remaining HKII had a role in respiration, galactose-fed cells were transfected and assayed similarly to glucose cells.

HKII silencing caused a reduction in oxygen consumption rates from 103.1 ± 9.66 pmol O₂/min in controls to 34.1 ± 11.16 pmol O₂/min in HKII knock down cells (Figure 4.26, panel B). There was also a significant decrease in the ATP producing efficiency from 69.6 ± 14.49 to 10.1 ± 7.68 pmol O₂/min in control and HKII knock down cells respectively (Figure 4.26, panel D). There was no change in the spare respiratory capacity (Figure 4.26, panel B). Galactose-fed HeLa cells both in control and HKII silenced cells have little to no spare respiratory capacity.

ECAR rates in galactose-fed cells were reduced from 2.03 ± 0.62 pmol O₂/min to 0.52 ± 0.21 pmol O₂/min (Figure 4.26, panel A). ECAR was so low that due to a poor signal to noise ratio no significance can be determined. There was essentially no spare glycolytic capacity in galactose-fed HeLa cells due to low flux through the glycolytic pathway.
Figure 4.25: HKII knock down caused a decrease in glycolysis and OXPHOS.
HeLa cells cultured on galactose for > 14 days were transfected with either a scramble control (Scr-siRNA) or HKII-siRNA and plated at 20,000 cells/well into a Seahorse culture plate. After 48h the plate was run according to the program described. ECAR (a) was measured as the rate of change of pH. OCR (b) was determined by rate of change of oxygen consumption. Spare glycolytic capacity and mitochondria coupling efficiency was obtained by injection of 0.5 μM Oligomycin (Oligo). Maximum respiration was obtained via 0.5 μM FCCP. Blank rates for glycolysis and OXPHOS were measured after the injection of 30 mM 2-DG and 1 μM rotenone (Rot) and 1 μM antimycin A respectively. Results are mean ± SEM for at least three independent transfections.
Figure 4.26: HKII knock down decreased glycolysis and spare glycolytic capacity. Basal rate of glycolysis (a) calculated by subtracting the average 2-DG rate from initial rates on shown on previous figure. Basal oxygen consumption rates (b) calculated by subtracting average rotenone/antimycin A treated rates. Spare glycolytic capacity (c) from increased ECAR following inhibition of mitochondrial respiration by oligomycin. Oxygen consumption rate indicative of ATP production (d) was calculated by subtracting oligomycin rate from initial rate. Results are mean ± SEM for at least three independent transfections. *p<0.05, **p<0.01.
4.2.13 Silencing of HKII expression leads to perturbation of mitochondrial fusion in glucose-fed HeLa cells

Inhibition of HK with 2-DG dramatically reduces fusion rates in glucose-fed and galactose-fed HeLa cells. Therefore, to specifically assess whether HKII is required for mitochondrial fusion, small interfering RNA (siRNA) against HKII (HKII-siRNA) or scrambled (control siRNA) were transfected into cells. Silencing of HKII expression in glucose-fed HeLa was verified by western blot (Figure 4.28, panels A and B) and galactose-fed HeLa (Figure 4.28, panels A and B). Single-cell targeting was visualised by using a far-red 647nm tag to label transfected cells (Figure 4.29, blue panel). Expression of HKII was been reduced to 37.1 ± 10.13% (Figure 4.28, panel B). When inspected using bright field microscopy, the number of cells in the field were reduced by ~50% in both glucose and galactose-fed cells (Figure 4.27).

Using the same time-lapse method as before images were taken at 5 time points. In HKII knock down HeLa cells there was significantly reduced fusion rates. The control groups showed complete dissipation of the photo-activated region, where by 30 min the pixel intensity of the activated ROI had been decreased to 36.6 ± 5.2% (Figure 4.29 and 4.30). In contrast, the HKII knock down cells showed significantly less dissipation in the activated ROI (Figure 4.29 and 4.30) even after 30 min with a PI value of 62.9 ± 2.60%. It was very clear from the confocal image that the green region was still visible in the t=30 image showing that there had been little mixing of neighbouring mitochondria showing that fusion has been perturbed in this experimental group.
Figure 4.27: HKII knock down decreased cell proliferation. Doubling time is increased post HKII knock down. Media appears to be less acidic after one day. Cells were seeded into 6-well plate post transfection with either a scrambled control (Scr-siRNA) or HKII targeted siRNA (HKII-siRNA). Scale = 100 μm
Figure 4.28: HKII expression was reduced in HeLa cells following transfection with HKII-siRNA. (a) Cells were transfected as before then scraped and lysed. 20 µg of lysate was loaded per well and separated by SDS-PAGE. Representative blot showing HKII expression and tubulin loading control. (b) Densitometry was normalised to loading control and shown as a percentage of the Scr-siRNA. HKII-siRNA transfection shows 62.92% knockdown. * p<0.05.
Figure 4.29: Assay of mitochondria fusion rates in HKII silenced glucose-fed HeLa cells. Cells were firstly transfected with a DsRed and PA-GFP plasmid. The following day in a 35 mm confocal dish they were transfected with either Scr–siRNA or HKII-siRNA for a further 48 h before assay. Transfected cells were identified by 647nm, far red-tagged siRNA, shown on left panel as pseudo-colour blue. HKII knock down cells (right hand side) show reduced rates of fusion compared to Scr siRNA treated control group (left hand side). Scale 20µm.
Figure 4.30: Fusion rates were reduced in HKII silenced glucose-fed HeLa cells. HKII knock down cells have reduced rates of fusion (b) compared to control Scr-siRNA (a). Cells were treated with siRNA for 48 h before assay. PI of ROI were measured, background control (red line) and photoactivated region (green line).
4.2.14 Silencing HKII decreased mitochondrial fusion rates in galactose-fed HeLa cells.

To further assess the role of HKII in mitochondrial fusion rates independently of its role in glycolysis, galactose-fed HeLa cells were transfected with either Scr-siRNA or HKII-siRNA.

Galactose-fed HeLa cells had been cultured on glucose-free, galactose supplemented media for 14 days, these cells derive their ATP from oxidative phosphorylation as opposed to the classic Warburg effect of aerobic glycolysis is usually associated with cancer. Galactose is metabolised via the Leloir pathway and enters glycolysis as glucose-6-phosphate down stream of HK.

Galactose-fed HeLa cells with decreased HKII expression had significantly decreased rates of fusion. Activated ROIs remained prominent in HK-siRNA images compared to Scr-siRNA controls (Figure 4.31) At 30 min there were significantly higher PI values in the knock down group with 59.1 ± 8.00% (Figure 4.32, panel B) compared to 33.8 ± 1.50% in the control (Figure 4.32, panel A).
Figure 4.31: Assay of fusion rates in HKII silenced galactose-fed HeLa cells. HKII knock down cells (right hand side) show reduced rates of fusion compared to Scr-siRNA treated control group (left hand side). Cells treated with RNAi constructs for 48 h before experiment. Pseudo-colour blue fluorescence shows cells successfully transfected with HKII-siRNA. Scale 20 μm.
Figure 4.32: Reduced fusion rates in HKII silenced galactose-fed HeLa cells. Cells were cultured on glucose-free galactose DMEM for at least 2 weeks, then cell were transfected with siRNA constructs for 48 h. (a) Scr-siRNA and (b) HKII-siRNA.
4.2.15 Mitochondrial inhibition had no additional effect on fusion rates in Glucose-HeLa cells.

Glucose-fed HeLa cells that derive ATP via glycolysis were pre-treated with inhibitors of the electron transport chain, but there was no additional reduction in the rate of fusion when CI was inhibited using 4 µM Piericidin A (Figure 4.32 and 4.33, panel B). Piericidin A was used instead of rotenone because rotenone can alter microtubule formation which is required for fusion to occur (Stephen Quinn, PhD Thesis). Similarly, there was no further decrease in fusion rates when CIII was inhibited with 2.4 µM Antimycin A (Figure 4.32 and 4.33, panel C).

4.2.16 Mitochondrial fusion was completely inhibited in HKII knock down galactose-fed HeLa cells following pre-incubation with ETC inhibitors.

Galactose-fed HeLa cell’s fusion rates were greatly reduced when treated with ETC inhibitors, but the contribution made by HKII to this is not known. In contrast to glucose-fed HeLa cells Following HKII silencing pre-incubation for 10 min with ETC inhibited (Figure 4.35). CI inhibition with 4 µM piericidin A resulted in complete inhibition of mitochondrial fusion, with PIs remaining at 87.8 ± 2.30% and 99.1 ± 1.20% at 15 min and 30 min post-activation (Figure 4.35 and 4.36, panel A). CIII inhibition with 2.4 µM antimycin A resulted in the complete inhibition of fusion with PI values remaining high at 91.9 ± 0.61% and 94.6 ± 1.42% at 15 min and 30 min respectively (Figure 4.35 and 4.36, panel B).
Figure 4.33: Assay of fusion rates in HKII silenced Glu-HeLa cells and ETC inhibition. HKII knock down was achieved using siRNA 48 h before assay. Cells were then pre-treated with antimycin A (2.4 μM) or piericidin A (2.4 μM) for 10 min before assay. Merged images of DsRED control (red), PA-GFP (green) and siRNA (blue) at each time-point. Scale 20 μm.
Figure 4.34: HKII knock down and ETC inhibition had no additional affect in glucose-fed HeLa cells. HKII knock down cells were pre-treated with (a) EtOH control, (b) 4 μM piericidin A or (c) 2.4 μM antimycin A before images were taken over the course of 30 min. >50 cells per experiment.
Figure 4.35: Assay of fusion rates in HKII silenced galactose-fed HeLa cells treated with ECT inhibitors. HKII was first knocked down using siRNA then treated with 2.4 μM antimycin or 4 μM piericidin A for 10 min before assay. Images show perturbation in fusion. Scale 20 μm.
Figure 4.36: HKII silenced galactose-fed HeLa cells treated with ETC inhibitors had highly perturbed fusion rates. HKII knock down cells were pre treated with (b) 4 μM piericidin A and with (c) 2.4 μM antimycin A.
4.2.17 Intracellular ROS production was increased in HKII knockdown cells

HKII is known to be an effective antioxidant, the exact mechanism for this this role is unknown. It has been speculated that increased G6P can be shunted off to the PPP that generates NADPH. NADPH functions as a cofactor to provide reducing power in many enzymatic reactions including the regeneration of glutathione, which can rescue cells from excessive ROS produced during rapid proliferation. Galactose cells should not be able to avail of this, as they cannot generate G6P rapidly enough. The increased sensitivity to complex inhibitors seen in galactose cells could be exacerbated by a low tolerance to ROS. However, an increase in mitochondrial respiration should lower the overall amount of ROS produced by cells due to constant flux through the ETC.

Knockdown experiments showed that when HKII was reduced, ROS production increased 2-fold in glucose-fed cells (Figure 4.37, panel A). There was a significant increase in ROS production when cells were treated with antimycin A and this was significantly exacerbated when HKII was removed suggesting that HKII is providing some protection from ROS generation.

Despite increased oxygen consumption galactose cells had significantly more basal ROS production compared to glucose-fed cells. ROS production was increased 2-fold in HKII knockdown cells (Figure 4.37, panel B). HKII knockdowns had around the same ROS production as antimycin A treated galactose control cells. Antimycin A and HKII knockdown cells had 3-fold increase compared to controls.

HKII knockdowns in both media types increased ROS production significantly. Galactose cells have less HKII to begin with; this could explain their high basal levels of ROS production. HKII silencing in glucose-fed cells increased ROS production to around the basal level of galactose cells.
**Figure 4.37: HKII silencing increases ROS production in glucose and galactose-fed cells.** (a) Glucose and (b) galactose cells were seeded at 5000 cell/well into black-walled clear 96-well plates. Cells were loaded with 20 μM H$_2$DCFDA. Media was replaced with KREBS supplemented with either glucose or galactose. 2 μM antimycin A was added as a positive control. Assays were ran for 45 min at 37°C using 488 nm excitation. Cell number was normalized by crystal violet assay following the run. One-way ANOVA with Bonferroni’s multiple comparisons. * p<0.05, ** p<0.01, *** p<0.001.
4.2.18 Mitochondria membrane potential depolarised in glucose-fed HeLa cells and hyperpolarised in galactose-fed HeLa cells

Mitochondrial membrane potential was assayed to investigate the difference in membrane potential between glucose-fed and galactose-fed cells. It was hypothesised that HKII could be influencing mitochondria by rapidly producing ATP through glycolysis. Glycolytic ATP could in theory be used by ATP synthase to pump protons into the intermembrane space preserving mitochondrial membrane potential. It was unclear as to how HKII may influence mitochondrial membrane potential in the absence of glucose. JC-1 dye was used to assay mitochondrial membrane potential. JC-1 accumulates in the mitochondria as either an orange emitting aggregate or in the cytoplasm as a green emitting monomer. A higher orange:green ratio indicates a higher mitochondria membrane potential. The ratio of orange to green can be determined fluorescently.

Glucose-fed HeLa cells were hyperpolarised compared to galactose HeLa cells with a ratio of ~2.5 compared to galactose at ~1.5 (Figure 4.38). Interestingly when HKII was silenced in glucose-fed cells, the mitochondria were depolarised (Figure 4.38, panel A) however in galactose-fed cells HKII knockdown cells hyperpolarised (Figure 4.38, panel B) the mitochondrial membrane potential.
Figure 4.38: HKII silencing affects membrane potential differentially in glucose-fed and galactose-fed HeLa cells. Cells were trypsinised, pelleted then washed twice in PBS then once in KREBS buffer containing (a) 10 mM glucose or (b) 10 mM galactose. Cells were loaded with 5 μM JC-1 for 20 min then washed in KREBS buffer. 150,000 cells in suspension were added to opaque black 96-well microplate and assayed in a plate reader. Data shown is the ratio of orange hyperpolarised aggregates 600 nm to green depolarised monomers at 535 nm. Mitochondria were depolarised by addition of 5 μM FCCP.
4.3 Discussion

Hexokinase plays an essential role in ATP production by phosphorylating glucose, creating a gradient for continued influx of glucose. Hexokinase catalyses the first step of glycolysis which can subsequently provide substrates for the TCA cycle or generate more ATP for cellular homeostasis when OXPHOS is not favourable, such as under hypoxia or during proliferation. Several studies have reported that HKI and HKII are implicated in cell survival (Ahmad, Ahmad et al. 2002, Bryson, Coy et al. 2002, Sun, Shukair et al. 2008). Hexokinases I and II are unique in that they can bind to the MOM. It has been hypothesised that the binding of HK to the mitochondrion prevents the release of pro-apoptotic proteins through the mPTP allowing cells to evade cell death (Pastorino and Hoek 2003, AZOULAY-ZOHAR, ISRAELSON et al. 2004). Based on the results of chapter 3, it was concluded that a shift away from glycolysis to OXPHOS in HeLa cells must be accompanied by expressional change in proteins that control metabolism. HKII was identified as a potential regulator of mitochondria due to its association with cancer and close integrations with mitochondrial proteins. HKII levels were significantly reduced both in whole cell and mitochondrial fractions of galactose-fed HeLa cells. The mechanism for the protective effects of HKII that glucose-fed cells appear to have over galactose-fed cells was studied. Glucose-fed cell mitochondria are much more resilient compared to galactose cells under stress; to assess the role of HKII in both cell types the hexokinase inhibitor, 2-Deoxyglucose was used. HKII was also removed from the MOM using a competitive oligopeptide and knocked down protein expression using siRNA.

When both glucose and galactose-fed cells were treated with ETC inhibitors, HKII was enriched on the MOM (Figure 4.2). Previous studies on lymphoma cells found an enrichment of HKII on the mitochondria when cells were treated with rotenone (Chen, Zhang et al. 2009). The same study found that cells with a mitochondria defect had similar enrichment of HKII. It was hypothesised that in the galactose-fed cells the HKII would dissociate from the mitochondria, which would in turn cause the opening of the mPTP, explaining the sudden loss of mitochondrial fusion, the increase in fission and subsequent cell death. However, galactose-fed cells also enriched HKII movement to the MOM (Figure 4.2, panels C and D). In chapter 3 it was shown that ETC inhibitors were toxic to galactose-fed cells (Figure 3.12). When combined with this result it can be inferred that
enrichment of HKII to the MOM alone is insufficient to prevent cell death. HKII bound to the mitochondria may be protective against apoptosis but only in the presence of glucose. An increase in HKII expression in response to ETC inhibition is a reasonable feedback method to ensure ATP production. Oligomycin is routinely used to reveal maximum glycolytic capacity; it is known that cells can rapidly increase glycolysis when ATP from OXPHOS becomes limiting. Galactose-fed cells produced little to no lactate (Figure 3.2, panel B), suggesting that the majority of pyruvate is used in the TCA or there is very little flux through the glycolytic pathway. If the latter, then substrates for OXPHOS would be generated from glutaminolysis. In both scenarios HK is bypassed by galactose. This suggests there may be a feedback mechanism in place that is independent of how ATP is being produced to cause an increase in HKII translocation. One explanation is that galactose cells enrich HKII on the mitochondria to prevent apoptosis until glucose becomes available again thereby priming the cell for survival. HKII translocation could be explained by a decrease in ATP synthesised in the mitochondrion. The cellular response could be a misinterpretation of the problem. Cells “think” that ADP is limiting for ATP synthase and translocate HKII to the MOM to provide more ADP. ADP concentrations around the ANT increase allowing ATP to exit the mitochondria (through VDAC). The ANT is an important constituent of the mPTP along with VDAC and cyclophilin-D (CyPD) (Halestrap 2009). HKII could be preventing the induction of the mPTP by keeping concentrations of ADP artificially high. When nucleotide concentrations are high they inhibit the opening of the mPTP by decreasing the affinity of Ca$^{2+}$ binding (Halestrap and Brenner 2003). In galactose-fed cells this requirement is met by the generation of ATP from ATP synthase. In glucose, it is met by low levels of OXPHOS and high glucose phosphorylation that keeps the ANT saturated with ADP from glycolysis. Galactose-fed cells cannot produce ADP from HK and therefore, when the ETC is inhibited the cells die.

2-Deoxyglucose directly competes with glucose for phosphorylation by HK. The subsequent increase in 2-deoxyglucose-6-phosphate causes product inhibition of HK. The resultant reduction in mitochondrial fusion rates in glucose-fed cells was predicted as this limits the amount of ATP that can be generated through glycolysis (Figure 4.4, panel A). The decrease in ATP could deregulate the processes that control mitochondrial fusion. Galactose-fed cells also had compromised mitochondrial fusion rates suggesting that when HK is inhibited it cannot perform any secondary function that may be controlling mitochondrial fusion (Figure 4.4, panel B). The phosphorylating ability of HK could be
compromised. 2-DG did not appear to dissociate HK from the MOM (Figure 4.2), which is consistent with the literature (Chen, Zhang et al. 2009, Mathupala, Ko et al. 2009). The mechanism by which 2-DG causes a reduction in fusion remains unknown. Off-target and additional effects of 2-DG such as inhibiting protein glycosylation, activating AKT and cell growth inhibition independently of catabolic blockage have been documented in the literature (Kang and Hwang 2006, Ralser, Wamelink et al. 2008, Zhong, Xiong et al. 2009).

VDAC provides exchange of metabolites, anions and cations across the MOM. The mechanisms that have been proposed to explain its role in cell survival remain controversial. VDAC is not an essential component of the mPTP (Baines, Kaiser et al. 2007) although several studies report that disruption of the HKII-VDAC bond can initiate apoptosis in cancer cells (Mathupala, Ko et al. 2006, Chiara, Castellaro et al. 2008). Overexpression of HKII in HeLa cells has been shown to inhibit the translocation of Bax and the release of cytochrome c (Pastorino, Shulga et al. 2002). Exploiting the ability of HeLa cells to generate ATP using either glucose or galactose allowed the examination of HKII’s role independent of its glycolytic function. Initially disruption of the HK-VDAC bond was attempted pharmacologically using Clotrimazole (CTZ) (Figure 4.6) (Penso and Beitner 1998, Chen, Zhang et al. 2009, Furtado, Marcondes et al. 2012). The removal of HK from the MOM caused a large reduction in fusion. Seahorse data also showed that oxygen consumption rates decreased when cells were treated with CTZ. Unfortunately, it was also discovered that CTZ was an inhibitor of complex I activity at the concentration required to remove HK from the outer mitochondrial membrane (Figure 4.7, panel B). This meant that it could not be ruled out that the effect was solely due to the removal of HK or an inhibitory effect on the ETC. To investigate this matter specifically, a peptide that consisted of the N-terminal region of HKII was used to competitively bind to the mitochondria in the place of HKII. This demonstrated that when HKII was removed from the MOM, cells growing on both sugars could no longer maintain mitochondrial fusion rates (Figures 4.8 and 4.13). Oxygen consumption and lactate production were both decreased suggesting that HKII has a preference for intra-mitochondrial ATP (Figure 4.19). Galactose-fed cells were more sensitive to the peptide compared to glucose-fed cells. Lower concentrations initiated apoptosis in galactose-fed cells (Figure 4.9 and 4.10). This is further evidence that a basal level of HK is required on the MOM for cell survival; this HKII may not participate in glycolysis. It could be argued that molecules like Bcl-XL.
and HK are anti-apoptotic by modulating VDAC to remain in an open state (Vander Heiden, Li et al. 2001, McCommis and Baines 2012). This open state maintains adenine nucleotide flux, thus maintaining outer membrane permeability (Vander Heiden, Chandel et al. 2000). When VDAC is closed the outer membrane becomes permeable and releases proteins from the intermembrane that then initiate a pro-apoptotic cascade (McCommis and Baines 2012). It is unclear how permeabilization of the membrane occurs. It has been speculated that an unknown pathway is responsible through swelling and rupturing the outer membrane. What is known is that when a pro-apoptotic protein, Bid, is cleaved by caspase-8 it induces VDAC closure resulting in apoptosis (Rostovtseva, Antonsson et al. 2004).

When HKII dissociates from VDAC, VDAC changes to a closed state (Figure 4.39). This prevents the flux of adenine nucleotides and other molecules such as ROS. When adenine nucleotides are limiting this can cause Ca\(^{2+}\) dependant activation of the mPTP and lead to apoptosis. Our results are consistent with previous observations in other cell types that demonstrated the importance of mitochondrial binding of HKII for protection from cell death (Majewski, Nogueira et al. 2004, Miyamoto, Murphy et al. 2007).

In addition to pharmacological inhibition and molecular dissociation from the MOM, RNA interference was also employed to specifically reduce HKII amounts. HKII expression was decreased by ~63% (Figure 4.28). Cell proliferation was decreased in both cell types, more so in glucose-fed cells (Figure 4.27). Mouse models of tumour growth have found that HKII is required to generate G6P and drives PPP: this would explain the reduction in proliferation (Patra, Wang et al. 2013). This study has demonstrated for the first time that in HKII knock down cells, mitochondrial fusion rates were impaired (Figure 4.30). The mechanism for this is still unclear. It is possible that fusion rates decreased as a result of decreasing concentrations of ATP evidenced by a 2-fold decrease in lactate production in glucose-fed cells (Figure 4.24). Previous studies have reported that HKII overexpression in lung and breast tissues reduces oxygen consumption (Patra, Wang et al. 2013). It was been hypothesised that reducing HKII expression would drive glucose-fed cells to respire like galactose cells; similar results have been observed in human glioblastoma cells (Wolf, Agnihotri et al. 2011). HKII knock downs did not increase OXPHOS. Oxygen consumption rates actually decreased slightly (Figure 4.23, panel B). HKII knockdowns reduced proliferation, mitochondrial fusion rates, glycolysis and OXPHOS. Four days after
knock down glucose-fed HeLa cells began to die.

Figure 4.39: HK-TAT was used to displace HKII from the MOM. HK-TAT (blue) preferentially binds to VDAC causing HKII (green) to dissociate from VDAC and the mitochondria. Following release from the mitochondria oxygen consumption, lactate production and fusion rates decreased in both glucose and galactose-fed cells. Increased fission and cleavage of pro-apoptotic were also measured.
To a lesser extent galactose-fed cells also had impaired fusion rates when HKII expression was reduced by siRNA (Figure 4.31). Compared to the scramble control there was a 33% reduction in fusion rates. Since galactose-fed cells do not require HKII for ATP production, any reduction in fusion rates, therefore, had to stem from the interaction of HKII either on the surface of the mitochondria or through interactions with other downstream targets that impact on mitochondria dynamics. Further study is required to investigate the possibility that HKII interacts with the ETC complexes. Perhaps silencing HKII reduces enzyme activities of ETC complexes thereby causing the reduction in mitochondrial fusion rates. Experimental limitations in the present study prevented the knocking down of sufficient cell number to perform mitochondrial complex assays.

Glucose-fed HeLa cells did not become re-sensitised to ETC inhibitors following HKII knockdown (Figure 4.34). Unlike other unregulated glycolytic enzymes, HKII is not a control node that regulates switching from a glycolytic phenotype to one of OXPHOS, such as PDK1 or LDH1 (Koukourakis, Giatromanolaki et al. 2005, Koukourakis, Giatromanolaki et al. 2006, Porporato, Dhup et al. 2011).

Since the antioxidant properties of HKII are studied extensively in several cell types (da-Silva, Gomez-Puyou et al. 2004, Sun, Shukair et al. 2008, McCommis, Douglas et al. 2013) it was, therefore, not surprising to find that ROS were increased in both cell types when HKII was knocked-down (Figure 4.37). It has been proposed that HKII attenuates ROS production by increasing metabolic flux through the PPP (Sun, Shukair et al. 2008). Increased availability of G6P for PPP produces NADPH, which reduces glutathione. Glutathione is a key survival antioxidant; it is used to convert H$_2$O$_2$ into H$_2$O (Marí, Morales et al. 2009). Cells overexpressing HKII had a greater survival rate when treated with H$_2$O$_2$. Preventing flux through the PPP by inhibiting G6P-dehydrogenase reversed the antioxidant effects of HKII overexpression (Sun, Shukair et al. 2008, Wu, Wyatt et al. 2012). In the present model, galactose cells have increased flux through the ETC; this should decrease ROS production but galactose-fed cells are also deficient in HKII (Murphy 2009, Liemburg-Apers, Willems et al. 2015). The reduction in HKII leads to a reduction in antioxidants, which could explain the increased amount of ROS compared to the glucose-fed cells. When HKII is knocked down we see even greater ROS production in the galactose-fed cells. The combination of poor OXPHOS support by the reduced HKII expression and their lack of tolerance of ROS could explain why galactose cells are so
sensitive to ETC inhibitors. Both of which could directly cause a reduction in mitochondrial fusion.

Mitochondrial membrane potential was decreased when HKII was knocked down in glucose-fed cells (Figure 4.38); these results support the hypothesis that cancer cells use glycolytic ATP to maintain mitochondrial membrane potential through the reverse action of ATP synthase described in chapter 3 (Chevrollier, Loiseau et al. 2011). When HKII is knocked down the overall amount of ATP that can be produced through glycolysis is reduced, extracellular acidification rates were decreased following HKII silencing. In this model, the HKII knockdown glucose-fed cells do not have the excess glycolytic ATP available to maintain mitochondrial membrane potential. The depolarisation brought on through reduction of glycolytic ATP could then explain the mitochondrial fusion impairment (Legros, Lombes et al. 2002). The response to HKII silencing in galactose-fed cells (Figure 4.38, panel B) is slightly more speculative. The hyperpolarisation seen in galactose-fed cells could possibly be due to reduced export of mitochondrial ATP to the cytosol. The mitochondrial localisation of HKII is thought to allow HKII to gain preferential access to mitochondrially generated ATP. In support of this view, HKII is bound to VDAC, and VDAC-HKII and ANT form a complex that allows for the rapid exchange of nucleotides (McComis and Baines 2012). It has been speculated that when HKII is removed from this complex, the VDAC channel closes (Rostovtseva and Bezrukov 2008). If HKII is removed it could cause VDAC to switch from an open conformation to a closed conformation thus preventing increased release to the cytosol. If the ETC is tightly coupled to ATP synthase then this could drive hyperpolarisation similarly to oligomycin inhibition of ATP synthase. This could explain the overall reduction in oxygen consumption seen in HKII knockdown galactose-fed cells.
Chapter 5: Functional Hexokinase is required for mitochondrial fusion
5.1 Introduction

From the previous chapter the data suggested that HKII has a role in controlling mitochondrial fusion rates and in reducing ROS. The mitochondrial binding of HKII is essential for apoptotic resistance and for continued mitochondrial and glycolytic metabolism. There is little research on how HKII has influence over mitochondria morphology, most research is centred on apoptosis how HKII controls apoptosis and ROS production. Further research on this area is required.

5.1.1 Hexokinase II has two catalytic sites

Early studies on the hexokinases showed that HKII had two catalytic sites on the N- and C-terminal, this is in contrast to HKI, which only has the active catalytic region on the C-terminal (Tsai and Wilson 1996). One study demonstrated that glucose phosphorylation and mitochondrial binding were required in order for HKII to exert its protective effects (Sun, Shukair et al. 2008). In this study, they used catalytically inactive HKII with both sites mutated. In order to assess the mitochondrial bonding, they generated N-terminal truncated HKII. This rendered it unable to form a complex with VDAC but remained catalytically functional. This is conceptually similarly to the HK-TAT displacement assay carried out in the previous chapter. The study did not investigate the impact that each individual half of HKII has on cell survival and ROS production. Cells were also cultured with glucose or in glucose deprivation without providing an alternative substrate. The present study attempts to address these issues.

Another study that attempted to investigate how the two functional halves of HKII affect the overall functionality of the enzyme. The study investigated why HKII could continue to phosphorylate glucose despite increasing concentrations of G6P. HKI becomes inhibited by similar concentrations of G6P. In the study they generated S155A mutants of both HKI and HKII and chimeric hybrids of HKI/HKII (Ardehali, Printz et al. 1999). These chimeric proteins consisted of the catalytically inactive N-terminal of HKI which was grafted to the C-terminal of HKII. The resulting protein had a similar K_i for G6P as HKI. The N-terminal HKI region does not phosphorylate glucose but it can still bind glucose. When the C-HKII domain was expressed without any N-terminal section, the K_i for G6P was significantly
higher than the chimeric protein and full-length HKII. When a mutation was introduced into the chimeric protein to prevent glucose binding on the N-terminal the $K_i$ for G6P increased significantly. Similarly, when a D209A kinase inactive mutation was inserted into WT-HKII, the $K_i$ for G6P was increased dramatically. The C-terminal catalytic domain in HKII is regulated tightly by its N-terminal. In HKII, the N-terminal not only serves to phosphorylate glucose but also to regulate production inhibition. Interestingly the study found that an S155A mutant did not cause the increase in the $K_i$G6P. S155A mutations reduce the overall catalytic ability of the N-terminal but did not cause changes in product inhibition. This can be partially explained by the fact that Ser 155 residue does not directly bind with glucose. Glucose binding on the N-terminal is required for regulation of product inhibition by G6P. It was also speculated that mitochondrial binding or other allosteric modifications also contributes to HKII increased resistance to product inhibition.

Apart from the studies referenced here there is little known about how the two regions of HKII work to control glycolysis and influence cellular metabolism. Most of these studies have been carried out in purified enzyme; little is known how this impacts on the cellular processes. One of the aims of the present study was to elucidate the role that these two active sites have in controlling mitochondrial dynamics both in the presence and absence of glucose. It was unknown which domain is responsible for antioxidant properties or if both are required. It was unknown if glucose is required for full function of HKII.
5.1.2 Aims of Chapter

Previous chapters highlighted the role that HKII has in maintaining cancer cell mitochondria in both glucose-fed and galactose-fed cells. To further elucidate how HKII maintains mitochondrial fusion and promotes cell survival mutant forms that are fully and partially catalytically compromised were generated. It was unknown if mitochondrial binding alone of HKII with no catalytic activity was sufficient to maintain mitochondrial fusion or if HKII required both its active sites intact to promote mitochondria fusion and cell survival. To address these questions the following objectives were carried out:

1. **Quantify mitochondrial fusion rates in glucose-fed and galactose-fed cells overexpressing WT-HKII and catalytically compromised mutants.** HKII mutants were generated using site-directed mutagenesis of a WT-HKII construct. Cells were transfected with single and double mutants of HKII catalytic regions. Mitochondrial fusion rates were then quantified for each treatment.

2. **Assess cell viability following ETC inhibition in cells overexpressing HKII mutants.** Using a cell viability assay, glucose-fed cells overexpressing HKII constructs were treated with ETC inhibitors. Sensitivity to inhibitors was measured.

3. **Investigate DRP1 localisation in HKII mutant cells.** Using immunofluorescence confocal microscopy, the subcellular location of the main mitochondria fission protein DRP1 was examined when cells were overexpressing HKII mutants.

4. **Measure mitochondrial membrane potential and ROS production in HKII mutant cells.** Using the mitochondrial indicator dyes, TMRM and MitoSOX mitochondrial membrane potential and mitochondrial ROS were quantified in cells overexpressing HKII mutants.

5. **Determine if overexpression WT-HKII can restore mitochondrial fusion in a cell line that is sensitive to ETC inhibitors.** Glucose-fed HEK cells were found to be sensitive to ETC inhibitors. Mitochondrial fusion rates were quantified in HEK cells overexpressing WT-HKII in the presence of ETC inhibitors.
5.2 Results

5.2.1 Mutant HKII reduced mitochondrial fusion rates in glucose-fed HeLa cells

A reduction in HKII expression compromised mitochondrial fusion rates therefore to further investigate the relevance of certain loci on the HKII protein structure, substitutions at specific resides of interest were made using site directed mutagenesis. As HKII has two repeated domains, single mutations were introduced into each of the active sites and also a functionally inactive double mutant was created. It was hypothesised that a deletion in the phosphorylation site of HKII would reduce its ability to facilitate glycolysis and to localise to the mitochondria and thus unable fulfil its anti-apoptotic role. It was hypothesised that this would result in a reduction of fusion.

Overexpression of WT-HKII had no significant change to fusion in glucose-fed HeLa cells with PI dissipating to 39.3 ± 3.08% compared to the empty vector control 38.1 ± 2.60% after 30 min post-activation (Figures 5.1 and 5.2). However, a mutation in the phosphorylation site at S155A located in the N-terminus of HKII caused a significant reduction in fusion 86.2 ± 4.10% at 15 min and only a further reduction to 84.3 ± 3.37% after 30 min (Figure 5.3, panel A). HKII with a mutant on the C-terminus phosphorylation site S603A was less significant but still caused fusion rates to decrease with PI at 75.2 ± 4.34% and 36.8 ± 3.98% after 15 and 30 min respectively (Figure 5.3, panel B). A double mutation on both active sites reduced fusion rates to 89.4 ± 1.79% and 72.2 ± 3.58% after 15 and 30 min (Figure 5.3, panel C).
Figure 5.1: Mutations in HKII phosphorylation sites compromised fusion in glucose-fed HeLa cells. Cells were co-transfected with mito-DsRED and mito-PA-GFP and either an empty vector control, WT-HKII, S155A, S603A or double mutant plasmids. Fusion assays were carried out as usual. Sample set of 20-40 cells. Scale = 20μm.
Figure 5.2: WT-HKII did not significantly change fusion rates in glucose-fed HeLa cells. Glucose-fed cells were co-transfected with mito-DsRED, mito-PA-GFP and either (a) an empty vector control or (b) WT-HKII plasmids. Cells were allowed to recover for 24 h before assay.
Figure 5.3: Fusion is reduced when catalytic sites are mutated in glucose-fed cells. Glucose-fed cells were transfected as before. WT-HKII plasmid was mutated on each of the two catalytic sites. Two single mutations at (a) S155A, (b) S603A and (c) a catalytically inactive double mutant.
5.2.2 Mutant HKII reduced fusion rates in galactose-fed HeLa cells

Glucose-fed HeLa cells reliance on glycolysis and thus hexokinases for ATP production makes them inherently vulnerable to mutants that affect HKII, in order to uncouple HKII role in ATP production from direct affects on mitochondria galactose was substituted for the standard glucose media. The same mutations as previously described were transfected into galactose-fed HeLa cells. In this case any effects from the mutation would not be directly related to glycolytic function.

There was no significant difference between empty vector control and WT-HKII overexpression in galactose-fed HeLa cells (Figure 5.4 and 5.5). S155A and S603A mutations had similar effects that were observed in glucose-fed HeLa cells. PI dissipation was reduced to 87.8 ± 2.83% and 78.9 ± 3.56% in S155A (Figure 5.6, panel A) and again less significantly to 61.2 ± 9.6 % and 50.2 ± 9.98 % in the S603A mutant (Figure 5.6, panel B). A double mutation caused a reduction to 79.8 ± 4.15% and 57.6 ± 6.82% (Figure 5.6, panel C). This again suggested that functional HKII is required to maintain mitochondrial fusion despite energetic independence from glycolysis in cells treated with the galactose substrate.
Figure 5.4: Mutations in HKII phosphorylation sites compromise fusion in galactose-fed HeLa cells. Cells were co-transfected with mito-DsRED and mito-PA-GFP and either an empty vector control, WT-HKII, S155A, S603A or double mutant plasmids. Fusion assays were carried out as usual. Sample set of 20-40 cells over. Scale = 20μm
Figure 5.5: WT-HKII overexpression did not significantly reduce fusion in galactose-fed cells. Galactose-fed cells were transfected with mitochondrial markers and either (a) empty vector control or (b) WT-HKII plasmids. Following 24 h recovery period cells were assayed using confocal microscopy.
Figure 5.6: S155A and double mutation caused greatest decreases in mitochondrial fusion in galactose cells. Galactose-fed cells were transfected as before. Using site-directed mutagenesis two single mutations at (a) S155A and (b) S603A were introduced to the HKII plasmid. (c) A catalytically inactive double mutant on both sites.
Figure 5.7: Fusion rates at 30 min were highly compromised in S155A mutants in both glucose-fed and galactose-fed HeLa cells. Fusion rates were quantified for each plasmid in (a) glucose-fed cells and (b) galactose-fed cells after 24 h incubation. One-way ANOVA with Tukey’s multiple comparisons test. **p<0.01, ***p<0.001
5.2.3 Overexpression of HKII increased proliferation in glucose-fed HeLa cells

To assay the effects of HKII on viability cells were transfected with an empty vector plasmid, WT-HKII plasmid or mutated plasmids. Comparisons of the plasmids alone would show if they are lethal to cells. Treatments with various inhibitors would allow for the assessment of important to mitochondrial health and their role in glycolysis.

Overexpression of WT-HKII lead to a 29% increase in cell proliferation compared to an empty vector control (Figure 5.8, panel A). Expression of plasmids for 24 h did not change over all cell viability significantly. Mutant versions of HKII caused viability to decrease to 92% in S603A and 89% in S155A. A double mutation had the greatest effect reducing viability to 71% (Figure 5.8, panel A). Transfection efficiencies of <40% maybe the cause of such high viability despite mutation. Antimycin A treatment also appears to cause a slight increase proliferation in treated cells (Figure 5.8, panels B-F). Oligomycin was toxic to cells causing a 50% decrease in proliferation compared to the ethanol control. However, overexpression of HKII lessens the toxic effect of oligomycin, resulting in a viability increase of 37.5% compared to empty vector control (Figure 5.8, panels B and C). Double mutant and S155A mutants had an increased sensitivity to rotenone with a 2-fold decrease in viability (Figure 5.8, panels D and F). The double mutant was sensitive to metformin but S603A remained insensitive despite metformin being a complex I inhibitor. Oligomycin treatment had a similar effect on the S603A mutant as in the WT (Figure 5.8, panels B and E). S155A and the double mutant resembled that of the empty vector control (Figure 5.8, panels B, D and F), this suggests that any relief to toxicity that is provided by overexpression of WT-HKII requires a functional S155A site.
Figure 5.8: Viability assay of glucose-fed cells with HKII mutants and inhibitors. (a) Cells were transfected with empty vector control, WT-HKII, S155A single mutant, S603A single mutant and a combined S155A and S603A double mutant. Cells were seeded into a 96-well plate at 5000 cells per well and grown for 24 h. Each plasmid was also treated with inhibitors (b) empty vector, (c) WT-HKII, (d) S155A, (e) S603A and (f) double mutant. Treatments of each inhibitor were then added for a further 24 h with Alamar Blue being added for the final 4 h. One-way ANOVA compared to control **p<0.01, ***p<0.001.
5.2.4 Mutations in HKII caused mitochondrial fragmentation

Previous experiments in chapter 4 have shown that when HKII mutants were expressed mitochondrial fusion is compromised. In order to measure changes in mitochondrial morphology and DRP1 expression were measured by immunofluorescence. Mitochondria were imaged using anti-TOM-20 as a marker protein, as it did not appear to change between treatments.

Overexpression of WT-HKII had similar network like mitochondrial morphology that is common in glucose-fed HeLa cells (Figure 5.9, first row). DRP1 is mostly situated around the nucleus. Mitochondria are elongated and tubular (Figure 5.9, top row). HKII localises to various region of the cytoplasm and appear to concentrate around the mitochondria. Transfection efficiency for WT-HKII was ~60%. S155A show more fragmented mitochondria with most localising round the nucleus indicative of damage (Figure 5.9, second row). HKII did not show any specific localisation with most of it spreading throughout the cell body (Figure 5.9, top row- green). Transfection efficiency was less than 30% for the S155A mutant, possibly due to decreased cell viability. The perinuclear location of DRP1 in the control and WT seems to have been spread throughout the cell, an overall increase and localisation to the mitochondria was not observed here, probably due to poor staining. S603A resulted in slightly more fragmented mitochondria but not to the same extent as S155A (Figure 5.9, third row). DRP1 localised to around the nucleus. HKII did not show any specific localisation. Transfection efficiency was also very poor less than 30%. Double mutants had the most fragmented mitochondria with over 80% of cells with damaged mitochondria (Figure 5.9, forth row). Mitochondria appeared punctate and with majority of cells rounding up and showing signs of apoptosis. DRP1 spread away from the nucleus to the entire cell body, we did not see increases in DRP1 as expected. HKII also did not show signs of localisation under immunofluorescence. Data suggests that a functional S155A site is required by glucose-fed HeLa cells to maintain healthy mitochondria. This is one of the sites required for glucose-fed phosphorylation therefore removing its function could have knock on effects for the mitochondria.
Figure 5.9: HKII with a mutation on S155A caused mitochondria to fragment. Glucose-fed HeLa cells were transfected with GFP labelled WT-HKII or S155A, S603A and double mutant plasmids and seeded on to 12 mm glass coverslips. After 24 h cells were fixed and prepared for immunostaining and visualised using confocal microscopy. Anti-TOM-20 (red) was used as a mitochondrial marker with an anti-mouse Alexa 595 secondary antibody. Left-middle panels represent DRP1 (cyan) with an anti-rabbit Alexa 647 secondary, right-middle panel shows HKII/DAPI merge (green/blue) and right panel represent a merged image of all 4. Percentage fragmentation was assessed by counting cells containing fragmented or perinuclear mitochondria, ~60 cells per experiment.
5.2.5 Mutations in HKII cause disruption to mitochondrial morphology in galactose-fed HeLa cells

Galactose-fed HeLa cells had a similar decrease in mitochondrial fusion rates that was present in the glucose-fed cells. Therefore, we investigated how WT-HKII and mutant versions affect cells with already low levels of endogenous HKII. HKII is a master regulator of glycolysis and we have shown that under normal circumstances galactose-fed cells will express significantly less HKII. Whether or not HKII is downregulated due to facilitate OXPHOS or as a result of more OXPHOS remains to be seen. Here we investigated morphology and DRP1 changes.

WT-HKII in galactose-fed cells had an extensive mitochondrial network not dissimilar to the empty vector control. Mitochondria did appear slightly more condensed than they would with endogenous HKII levels (Figure 5.10, first row). DRP1 levels were elevated compared to the glucose counterpart. It remained mostly concentrated around the nucleus. HKII did not localise to any particular region of the cell. Transfection efficiency was around 60-70%. S155A mutant cells had highly fragmented mitochondria with the majority localised around the nucleus (Figure 5.10, second row). DRP1 was increased and spread around mitochondria. Very poor transfection efficiency of the mutant HKII, with around 20-30% of cells expressing the GFP tagged hexokinase. S603A mutant cells had more fragment mitochondria compared to WT-HKII but not to the same extent as S155A (Figure 5.10, third row). DRP1 expression was highly elevated compared to the control and was spread throughout the cell although there were still concentrated regions around the nucleus. S155A and S603A double mutant’s mitochondria were concentrated around the nucleus (Figure 5.10, forth row). The majority of cells had fragmented mitochondria. DRP1 expression was increased throughout the cell.
Figure 5.10: HKII with a mutation on S155A and S603A caused mitochondria to fragment and caused some increases in DRP1 expression. Galactose-fed HeLa cells were transfected with GFP labelled WT-HKII or S063A, S155A and double mutant plasmids and seeded on to 12 mm glass coverslips. After 24 h cells were fixed and prepared for immunostaining and visualised using confocal microscopy. Anti-TOM-20 (red) was used as a mitochondrial marker with an anti-mouse Alexa 595 secondary antibody. Left-middle panels represent DRP1 (cyan) with an anti-rabbit Alexa 647 secondary, right-middle panel shows HKII/DAPI merge (green/blue) and right panel represent a merged image of all 4. Percentage fragmentation was assessed by counting cells containing fragmented or perinuclear mitochondria, ~60 cells per experiment.
5.2.6 Overexpression of S155A and Double mutants caused depolarization in glucose-fed HeLa cells

The data from chapter 4 indicated that mitochondrial fusion is influenced by changes in mitochondrial membrane potential and HKII has a role in controlling fusion. Given that HKII has 2 phosphorylation sites it was hypothesised that deleterious mutations would affect mitochondrial function. To investigate this, we overexpressed a HKII plasmid along with 3 mutant versions created using site-specific mutagenesis. Two ADP phosphorylation sites were targeted individually and together as a double mutant. HKII was labelled with a GFP-tag in order to identify cells expressing plasmids.

Mitochondrial membrane potential was measured using time resolved confocal microscopy with TMRM staining. Overexpression of HKII did not change membrane potential compared to empty vector control (Figure 5.11 panel B). A similar result was measured in cells expressing the S603A mutation (Figure 5.11, panel D), possibly suggesting there is a redundancy in that phosphorylation site. S155A and the double mutation caused a ~2-fold decrease in membrane potential (Figure 5.11, panels C and E). There was a significant depolarisation in S155A and double mutants compared to empty vector, WT-HKII and S603A mutants (Figure 5.11, panel F).
Figure 5.11: Overexpression of WT-HKII and S603A mutants caused hyperpolarisation in glucose-fed HeLa cells. Cells were transfected with either (a) Empty vector, (b) WT-HKII, (c) S155A, (d) S603A or (e) double mutant. 24 h post transfection cells were loaded with 25 nM TMRM for 30 min @ room temperature. Cells were washed 3 times with KREBS buffer supplemented with 25 mM glucose. Change in fluorescence at 535 nm was measured by confocal microscopy for 10 min. FCCP was added to fully depolarise mitochondria. Images were then analysed by Olympus FluorView. (f) Percentage change in fluorescence compared to empty vector control shown. Student t-test compared to control. ***p<0.001
5.2.7 Overexpression of HKII depolarised mitochondria in galactose-fed HeLa cells

Galactose-fed HeLa cells overexpressing HKII had significantly depolarised mitochondria (49.04 ± 1.86%) compared to the empty vector control (Figure 5.12, panels F). The mutant forms S603A and S155A (38.1 ± 2.97% and 37.4 ± 0.51%) also had decreased TMRM fluorescence (Figure 5.12, panel C and D). The double mutant had an additive decrease on membrane potential (28.6 ± 0.67%) (Figure 5.12, panel E). Any additional HKII expression in OXPHOS dependant cells in glucose-free media depolarises mitochondria. Mitochondrial membrane potential was not directly comparable between glucose-fed and galactose-fed cells in this experiment. Galactose-fed cells membrane potential were undetectable using glucose settings on confocal. Due to galactose-fed cells high rates of OXPHOS they tend to be depolarised compared to glucose-fed cells, which tend to be hyperpolarised. Expression of mutant HKII may depolarises galactose-fed cells mitochondria to the point where apoptosis is being induced.
Figure 5.12: Overexpression of any HKII caused depolarisation in galactose–fed HeLa cells. Cells were transfected with (a) Empty vector, (b) WT-HKII, (c) S155A, (d) S603A or (e) double mutant. 24 h post transfection cells were loaded with 25 nM TMRM for 30 min @ room temperature. Cells were washed 3 times with KREBS buffer supplemented with 25 mM glucose. Change in fluorescence at 535 nm was measured by confocal microscopy for 10 min. FCCP was added to fully depolarise mitochondria. Images were then analysed by Olympus FluorView. (f) Percentage change in fluorescence compared to empty vector control shown. Student t-test compared to control. ***p<0.001
5.2.8 ROS production increased in S155A mutants

It has been reported that HKII overexpression can be protective against the apoptotic effects of hydrogen peroxide in cells (Sun, Shukair et al. 2008). Previously experiments have shown that HKII knockout cells tend to generate more endogenous ROS (Wu, Wyatt et al. 2012). It is not fully understood how HKII provides an antioxidant mechanism. It is unclear if HKII needs to be catalytically active to be able to protect cells from excessive ROS production. The present study has shown that when HKII is knocked down there is an increase in ROS production and when galactose is the main substrate there tends to be more ROS production. This may be because galactose-fed cells have less HKII or because galactose cannot be used by HKII to generate NADPH.

In this experiment HKII is overexpressed so we can assume that both glucose-fed and galactose-fed cells that are expressing high levels of HKII. Antioxidant properties inferred by the two catalytic sites were also determined.

Glucose-fed cells overexpressing WT-HKII and S603A mutants had the greatest reduction in ROS production (Figure 5.13, panels A and C). After cells were treated with antimycin A ROS production barely increased. This result compliments the knockdown studies in that they have the opposite effect on ROS production. S155A mutations caused ROS levels to increase 2-fold in the basal levels compared to the WT-HKII (Figure 5.13, panels B and D). ROS production forced by antimycin A was also increased and this trend continued throughout the assay. Double mutants had the same trend but not to the same extent, possibly due to low expression of double mutants in cells (Figure 5.13, panel D). Galactose-fed cells did not have the same protection as glucose-fed cells (Figure 5.14). WT-HKII overexpression had slightly higher levels of basal ROS production as glucose-fed cells but had no extra protection when the ETC was inhibited by antimycin A (Figure 5.14, panel A). It would seem that glucose is required for the antioxidant role of HKII. S603A mutants had a similar trend in glucose as in galactose. Double mutants had abnormally high levels of ROS production but generated more ROS when treated with antimycin A (Figure 5.14, panel D). Perhaps mitochondria were so damaged in these cells that the no longer could sustain respiration.
Figure 5.13: Overexpression of WT-HKII and S155A reduced ROS production in glucose-fed cells. Cells were transfected with either (a) WT-HKII, (b) S155A, (c) S603A or (d) double mutant plasmids. 24 h post transfection cells were loaded with 5 nM MitoSOX. Change in fluorescence at 520 nm was measured for 20 min then antimycin A was added to induce ROS production.
Figure 5.14: Overexpression of S155A HKII increases ROS production in galactose-fed cells. Galactose-fed cells were transfected with either (a) WT-HKII, (b) S155A, (c) S603A or (d) double mutant plasmids. 24 h post transfection cells were loaded with 5 nM MitoSOX. Change in fluorescence at 520 nm was measured for 20 min then antimycin A was added to induce ROS production.
5.2.9 WT-HKII overexpression rescued mitochondrial fusion in HEK cells with ETC inhibitors

To investigate if HKII overexpression could promote mitochondrial fusion, a cell type that was sensitive to ETC inhibitors in the presence of glucose was used. Several cell lines were screened for ETC sensitivity that were also able to overexpress HKII. HEK cells were similar to galactose-fed HeLa cells in basal fusion rates (Figure 5.15).

A mitochondrial fusion assay was carried out on glucose-fed HEK cells. Sensitivity to piericidin A and antimycin A was confirmed. WT-HKII was then transfected as before. Following WT-HKII overexpression cells were pre-treated with piericidin A and antimycin A. WT-HKII restored fusion in piericidin A cells from 72.2 ± 7.41% to 40.0 ± 5.66% after 30 min (Figure 5.16). Again, with antimycin A treatment fusion was restored from 82.4 ± 4.33% to 46.6 ± 1.82% at 30 post-activation (Figure 5.17).
Figure 5.15: Basal mitochondrial fusion rates in HEK cells are similar to HeLa cells. Glucose-fed HEK cells were transfected with mito-DsRED and mito-PA-GFP. Fusion was measured as before. Data shown n=2.
Figure 5.16: Overexpression of WT-HKII restored mitochondrial fusion following complex I inhibition. Glucose-fed HEK cells treated with 4 μM piericidin A and transfected with (a) empty vector control and (b) WT-HKII. Data shown n=2
Figure 5.17: Overexpression of WT-HKII restored mitochondrial fusion following complex III inhibition. Glucose-fed HEK cells treated with 2.4 μM piericidin A and transfected with (a) empty vector control and (b) WT-HKII. Data shown n=2
5.3 Discussion

Site-directed mutagenesis enabled the generation of full-length HKII plasmids with either one or two of the ATP binding residues removed. Combined with a fluorescent marker it was possible to identify and assay cells that were expressing mutant plasmids. Because HKII has been shown to have an enigmatic role in: lowering ROS induced damage (da-Silva, Gomez-Puyou et al. 2004, McCommis, Douglas et al. 2013), cell survival (Majewski, Nogueira et al. 2004, Robey and Hay 2006) and proliferation (Mathupala, Ko et al. 2006), experiments were designed to elucidate which components of HKII are essential to facilitate fusion and how they might be linked. The residues selected are analogues and are catalytically important, they have similar $K_m$ for glucose but Ser 155A has a higher affinity for ATP (Tsai and Wilson 1996). These data revealed that in glucose cells both residue residues are required to facilitate normal mitochondrial fusion rates.

It was shown for the first time that mitochondrial fusion was reduced in both glucose and galactose cells when mutations were introduced into HKII (Figure 5.7). S603A mutations caused the reduction in fusion rates compared to empty vector controls but not significantly different from WT-HKII overexpression in both media conditions (Figure 5.7). Ser 603 residue is located in the same region where you would find the singular catalytic region on the C-half of HKI (Tsai and Wilson 1996). Decreases in fusion rates in glucose cells could be attributed to the loss of around half of their catalytic ability. HKII is less efficient at phosphorylating glucose therefore less efficient ATP production by glycolysis could account for the small reduction in fusion. Reduction in fusion rates in S603A galactose-fed mutants is still unclear. These results would indicate that Ser 603 residue on HKII maybe dispensable for mitochondrial fusion. According to crystal-structural studies, Ser 603 has initially no interaction with glucose but it is thought to play a key transient role in phosphorylation once conformational change occurs (Mulichak, Wilson et al. 1998). This region is located in the C-terminal away from mitochondria. Compensation from the N-terminal and HKI may be able to sustain a basal level of fusion. Therefore Ser 603 may be dispensable in regards to facilitating fusion in both conditions. S603A HKII mutants maybe are acting like HKI but with enhanced mitochondrial binding ability.
In both media conditions S155A mutants reduced fusion to a greater extent than S603A mutants. Mutations in Ser 155 significantly decreased fusion compared to empty vector controls and WT-HKII. Ser 155’s location in the regulatory domain could mean that it has greater influence over the protein as a whole. Mutations in S155A reduce the \( V_{\text{max}} \) but do not affect glucose binding (Tsai and Wilson 1996). The N-terminal domain in HKII not only catalyses a reaction but also stabilises the entire enzyme (Rabeh 2016).

Mutations did not have excessive adverse reaction to cell viability in glucose cells (Figure 5.8, panel A). Overexpression of WT-HKII caused an increase in proliferation in glucose cells, in agreement with previous studies (Ahn, Hwang et al. 2009, Hamanaka and Chandel 2012). Proliferation was dependant on glucose availability, as similar results were not seen in galactose cells. S155A and S603A mutants did not reduce cell viability in Alamar blue assays; one possible explanation for this could be poor transfection efficiency. The assay requires at least 5000 cells/well and judging by epi-fluorescence analysis only around 20-40% of cells expressed the mutant plasmids (Figure 5.9). Increasing concentration of plasmids or changing transfection method did not solve this problem. Due to time constraints antimicrobial selection was not an option.

Interestingly even with poor transfections S155A and double mutants acquired a sensitivity to rotenone and a slight sensitivity to oligomycin (compared to WT-HKII overexpression) (Figure 5.8). WT-HKII and empty vector control are insensitive to rotenone and antimycin A. The Ser 155 site appears to be required to protect cells against complex I inhibition, as S603A mutants remained insensitive. In the present study (chapter 4) it was shown that when cells are treated with rotenone, HKII translocated to the mitochondria. It is not clear if this translocation still occurs in S155A mutants. Previous studies in HEK cells reported that when catalytically inactive, HKII mutants still maintained mitochondrial localisation, under normal conditions (Sun, Shukair et al. 2008). The mutation could influence translocation when the cells are under metabolic stress. Rotenone has been known to affect microtubule formation and activate GSK3\( \beta \) (Srivastava and Panda 2007, Gimenez-Cassina, Lim et al. 2008, Han, Casson et al. 2014). GSK3\( \beta \) is known to detach HKII from the mitochondrial membrane when it is activated (Pastorino, Hoek et al. 2005, Gimenez-Cassina, Lim et al. 2008). Complex I inhibition coupled with poor catalytic activity could
lead to a decrease in ATP could trigger cell death. This effect is not found when only S603A of HKII is mutated. It is unclear why antimycin A did not have a similar effect.

It is not clear what role the Ser 155 site has in galactose-fed cells. It decreased fusion and increased ROS production seemingly independent of glucose phosphorylation (Figures 5.2 and 5.12, respectively). It is also unclear how mutations affect G6P and ATP binding and if this is relevant to mitochondrial maintenance.

Previous studies have reported that the N-terminal not only is a catalytic site but also acts as a regulatory site stabilising the entire protein, therefore disruption of the N-terminal active site causes a high reduction in the overall stability of the protein (Rabeh 2016). A destabilised HKII may have erratic mitochondrial binding.

Overexpression of WT-HKII hyperpolarised the mitochondria of glucose-fed cells (Figure 5.11). Increased glycolysis might generate more ATP, leading to an underutilization of mitochondria for ATP generation. Excessive concentrations of glycolytic ATP could inhibit ATP synthase but ETC complexes will continue to pump protons into the mitochondrial inter membrane space, which would lead to hyperpolarisation (Bonnet, Archer et al. 2007). Several studies have shown that S155A and double mutants do not have the catalytic ability to generate sufficient amounts of ATP forcing mitochondrial respiration thus depolarising the mitochondria. In galactose-fed cells S155A and double mutants could not retain the TMRM dye for the course of the basal measurements, suggesting a “leaky” mitochondria (Figure 5.12). It is unclear why WT-HKII and S603A mutants depolarised galactose-fed cells. Reductions in fusion would suggest that mitochondria are unable to function properly; depolarisation could be due to increased oxygen consumption through uncoupled mitochondria.

Overexpression of WT-HKII in glucose-fed HEK cells restored fusion rate in the presence of complex I and III inhibitors (Figure 5.17). This experiment has shown for the first time the HKII can support fusion when the ETC is inhibited when glucose is readily available. Further study is required to investigate if there is an increase in glycolysis and if ROS production is attenuated when generated through complex I and III. It is unknown if mutant HKII proteins would have the same effect.
Chapter 6: General Discussion
6.1 General Discussion

Mitochondria are central to cell survival even when they are not the primary source of ATP. Constant rearranging of the molecular components within mitochondria finely tunes overall cellular bioenergetics. The pathways that cancer hijacks to ensure continued proliferation and sustain growth all converge at the mitochondria. The key enzyme that allows for increased aerobic glycolysis HKII actually anchors itself to mitochondrial membranes. Despite intense study of HKII and mitochondria there have been very few studies that actually examine how they influence each other. The field of mitochondrial dynamics is still in its infancy and the metabolic aspects of cancer have only been rediscovered in the past 10 years. Many questions regarding the regulation of mitochondrial function by various oncogenes, tumour microenvironments and emerging therapies remain to be answered. Using cancer cell models to study mitochondria can also provide insights into how other cells work. Cells that have high-energy demand and increased HKII expression can survive under extreme stress and in harmful environments. Tissues such as: cardiac muscle, immune cells and stem cells. This research may also provide an understanding into how tissues with low tolerance to metabolic change are so susceptible to mitochondrial dysfunction and oxidative stress.

Developments in imaging and metabolic assay techniques have advanced our current understanding of how important mitochondria are within cells. Mitochondrial plasticity controlled through fission and fusion shapes the fate of cells. Cancer cells exploit pathways that have evolved to ensure proliferation and avoid cell death. The ability to constantly rewire bioenergetics makes cells adaptable when facing challenges that arise in tumour microenvironments such as nutrient deprivation, apoptotic stimuli and hypoxia. Hexokinase II has been studied extensively from a glycolytic centric view despite its mitochondrial location. The present study has for the first time examined HKII’s role in mitochondrial dynamics. HKII is closely linked with cancer but emerging evidence has shown that it has protective antioxidant qualities in cardiac tissues and neurons (da-Silva, Gomez-Puyou et al. 2004, Wu, Wyatt et al. 2012). The present study has shown HKII reduced ROS in both glycolytic and OXPHOS environments (Figures 4.37, 5.13 and 5.12).
One of the main highlights of the present study is that HKII is required by HeLa cells to efficiently use their mitochondria (Section 4.2.12). Switching carbohydrate source from glucose to galactose to bypass HKII in the glycolytic pathway leads to an increase in OXPHOS metabolism, subsequently HKII is downregulated. Galactose-fed HeLa cells grew slower; they produced more ROS and became highly sensitive to ETC inhibitors. It was shown that transiently increasing OXPHOS by activating PDH in a glucose rich environment did not bring about this sensitivity. Galactose-fed cells had long-term glucose deprivation that in turn caused a reduction of HKII expression. This has implications for cancer therapies, depending on how cancer cells generate their energy, therapies would need to be targeted to a specific metabolic profile.

In chapter 3 it was found that glucose-fed and galactose-fed cells had similar basal rates of mitochondrial fusion and mitochondrial content was similar between both cells. When assayed extracellularly, the individual complexes of the ETC had little change in their specific activities. This could be to facilitate shifts back from glycolysis to OXPHOS when required during glucose deprivation. Interestingly galactose-fed cells had increased amounts of supercomplex assembles. Increased supercomplexes could help explain the large difference in oxygen consumption between the groups; glucose-fed cells consuming less oxygen through respiration than galactose-fed cells. When glucose is abundant the cells adopt a classic Warburg shift to maximise proliferation through glycolysis. Presumably glucose-fed cells are supressing mitochondrial respiration and supercomplex formation to drive increased glycolysis. When the substrate was changed from glucose to galactose, the cells rewired their metabolism in order to adapt to slower aerobic glycolysis. In the absence of glucose, the cells were unable to rapidly produce G6P that is required to maintain elevated glycolysis. Galactose-fed cells are able feed into the glycolysis, through the Leloir pathway, without the need for HKII. This process is several extra steps and slows down the rate of glycolysis. When the galactose was used as a substrate, cells decreased HKII expression and became susceptible to apoptosis.

These data and several previous studies demonstrated that the mitochondrial location of HKII is essential to cell survival (Woldetsadik and Magzoub 2016). Glucose-fed and galactose-fed cells with mitochondria-bound HKII had increased OXPHOS. When glucose was available, mitochondria-bound HKII also promoted greater glycolytic function. This finding reinforces the model that mitochondrial-bound HKII makes use of outgoing ATP
produced from the ETC to promote glycolysis. It was assumed that the removal of HKII from the glucose-fed cells would cause a major disruption in many cellular processes as ATP production was greatly reduced. However, galactose-fed HeLa cells were more sensitive to removal of HKII from the mitochondria compared to glucose-fed cells. The IC₅₀ for HK-TAT and clotrimazole were lower, probably due to there being less HKII on galactose-fed mitochondria to begin with. The compelling argument for the importance of HKII-mitochondrial binding is that galactose-fed cells were energetically independent of HKII, even still when it was removed from their mitochondria apoptosis was swiftly initiated.

It is still unclear how HKII regulates mitochondrial dynamics. Complete loss of HKII from the mitochondria causes recruitment of DRP1 and initiates fission. It has been speculated that this due to opening of the mPTP caused by a change in VDAC however the exact mechanism is unknown. HKII-VDAC complex is thought to keep VDAC in an open configuration allowing exchange of nucleotides through to the ANT. This action maintains mitochondrial membrane potential. When HKII is detached VDAC reverts to a closed conformation resulting in a loss of membrane potential and subsequently leads to cell death (McCommis and Baines 2012). Silencing HKII could be reducing fusion by causing deregulation of membrane potential through this mechanism. Cells have the ability to reverse the action of ATP synthase to support membrane potential, the VDAC-HKII complex keeps VDAC open for ATP to enter the mitochondria in glucose-fed cells but also could keep the channel open in galactose cells to allow for ATP to leave the mitochondria. In both cases fusion is decreased when HKII is removed, silenced or mutated. Membrane potential was decreased in glucose-fed HeLa and hyperpolarised in galactose-fed HeLa cells. Decreases in membrane potential in glucose-fed HeLa cells could be attributed to less reverse action of ATP synthase i.e. the pumping of protons back into the intermembrane space (Nesci, Trombetti et al. 2015). Hyperpolarisation in galactose-fed HeLa cells could be due to a build-up of ATP inside the mitochondria. A closed conformation VDAC would prevent ATP from leaving the mitochondria through VDAC. Unfortunately, there were experimental limitations prevented the measurement of intramitochondrial ATP and cytosolic ATP. It could not be ensured that during mitochondrial isolation membranes would not rupture thus contaminating the cytosolic fraction with mitochondrial ATP.
Glucose-fed and galactose-fed cells generate ATP through two very different mechanisms; both represent extremes that cancer cells may use to survive in a tumour environment.

**HKII**

**6.2 Future work**

The silencing of HKII lead to an increase in ROS production, it is unknown what effect the extra ROS has on cancer cell metabolism. Increased ROS production correlates with increased autophagy and mitophagy (Filomeni, Desideri et al. 2014, Filomeni, De Zio et al. 2015). Treatment with H$_2$O$_2$ has been demonstrated to cause mitochondrial fragmentation in skeletal muscle cells (Iqbal and Hood 2014). Following inhibition with antimycin A ROS increased, glucose-fed HeLa cells could have controlled the increased ROS through reduction of glutathione. Conversely, galactose-fed cells do not have the ability to increase glucose phosphorylation to generate the NADPH required for this mechanism and therefore succumb to toxic ROS production. Previous work has shown that re-introduction of glucose restores mitochondrial fusion (Stephen Quinn), the bolus of glucose for 1 h could allow for NADPH production and thus reduction of glutathione. To further investigate this hypothesis ETC inhibition experiments could be repeated with a PPP inhibitor such as 6-Aminonicotinamide (6AN), epiandrosterone (EPI), or dehydroepiandrosterone (DHEA) (Gupte, Li et al. 2002). If fusion rates decrease in glucose-fed cells following the dual treatment of PPP inhibitors and ETC inhibitors then ROS would be implicated in reducing fusion.

It is unknown if prolonged treatment of DCA would sensitise glucose-fed cells to ETC inhibitors. Due to DCA’s effect at the bottom of the glycolytic pathway, it seems unlikely that DCA would cause glucose-fed cells mitochondria to become sensitised to ETC inhibitors. HKII still would be active in phosphorylating glucose, unlike galactose-fed cells where HKII is bypassed and subsequently downregulated.

The toxicity of HK-TAT peptide on healthy cells remains to be examined. It is unclear why HeLa cells need HKII on the mitochondria to survive. The majority of tissues do not express HKII and can survive. Perhaps a side effect of oncogenesis is an over reliance on HKII to provide protection. Clinical trials of anticancer agents that target HKII and mitochondrial binding such as lonidamide, 2-DG and 3-bromopyruvate (3-BrPA) have to
be halted due to liver toxicity and non-selectivity of the drugs (Gill, Fernandes et al. 2016). More research into how to properly exploit this target is required.

6.3 Conclusions of Thesis

These studies have quantified for the first time the regulation of mitochondrial fusion and fission dynamics by glycolysis in cancer cells. The most significant conclusions that can be drawn from this work are as follows:

1. Mitochondrial fusion rates are linked to the bioenergetic state of the cell.
2. Prolonged shift to OXPHOS from glycolysis reduces HKII expression in HeLa cells.
3. Availability to glucose determines if mitochondrial fusion can be maintained when OXPHOS is inhibited.
4. Hexokinase II has a role in cell survival and mitochondrial maintenance.
5. Disruption of HKII from the MOM results in mitochondrial fission and cell death.
6. HKII must be catalytically active to maintain mitochondrial fusion rates.
Chapter 7: Bibliography
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