Cellular responses to metabolic stress

A study on the role of mitochondria in the crosstalk between cell metabolism and survival signaling

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Scientific environment

The work presented in this thesis was performed at the Department of Biomedicine part of the Faculty of Medicine and Dentistry at the University of Bergen. My PhD project has been conducted in the Cellnet group (Mitochondrial Research group) under the supervision of Assistant Professor Karl Johan Tronstad, with co-supervisor Professor Stein Ove Døskeland (Translational Research Group) and Professor Rolf K. Berge (Mitohealth).

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Abbreviations

4E-BP1	4E-binding protein 1
α-KG	α-ketogluterate
AMP;ADP;ATP	Adenosine mono;di;tri-phosphate
AMPK	amp kinase
CPT	carnitine palmitoyltransferase
eIF4G	eukaryotic initiation factor 4G
ETC	Electron transport chain
ETS	electron transport system (uncoupled respiration)
F1,6-BP	fructose 1,6-bisphosphate
FAO	fatty acid oxidation/acyl-CoA oxidase
FCS	foetal calf serum
G6-P	glucose-6-phosphate
GlcNAc	N-acetylglucosamine
Glut	glucose transporter
HK	hexokinase
IDH	isocitrate dehydrogenase
IGF	insulin growth factor
LDH	lactate dehydrogenase

mTORC	mammalian target of rapamycin complex
PDH	pyruvate dehydrogenase
PEP	phospho-enoylpyruvate
PFK	phospho-fructokinase
PK	pyruvate kinase
PPAR	peroxisome prolferator-activated receptor
RBC	red blood cells
ROS	reactive oxygen species
TCA	Tricarboxylic acid cycle
TEM	transmission electron microscopy
TTA	tetradecylthioacetic acid
UCP	uncoupling protein

Abstract

Metabolic imbalance is associated with increased risk of several diseases, including cancer. Metabolism affects or is affected by virtually all other cellular processes, which is not surprising considering their conserved role throughout evolution. Mitochondria are important sensors of the intracellular metabolic environment and play a major role in bioenergetics and survival signaling in mammalian cells. Accordingly, these cell organelles have been implicated in the development of cancer, which includes changes in cellular metabolism and cell death signaling.

Metabolic reprogramming in cancer cells increases cell growth and proliferation. This entails greater nutrient uptake which is metabolized to provide energy and intermediates for cell constituents. This reprogramming has generally been associated with increased therapeutic resistance, but may also prove to be utilized in metabolic targeted therapies. Major signaling networks that are involved in tumor growth and metabolic reprogramming are PI3K/Akt/mTOR signaling pathways often found mutated in cancer cells. Activation of this pathway results in high glucose dependency and aggressive tumors. On the other hand, the activation of a low-energy-responsive AMP-activated protein kinase (AMPK) signaling pathway leads to growth inhibition. Both of these signaling pathways may regulate or be regulated by mitochondrial functions, which has been the basis of this Ph.D study.

The aim of this work was to investigate interactions between cell metabolism and signalling that affect mitochondrial function under conditions of energetic and metabolic stress. In order to reflect different contexts of mitochondrial regulation, we studied these mechanisms in both metabolically restricted cancer cells, as well as metabolically flexible primary rat hepatocytes.

We found that leukemia cells that have mutations in the PI3K/Akt/mTOR signaling pathway (Jurkat) were more susceptible to glucose deprivation and agents that challenge this metabolism (such as palmitic acid). Alternatively, leukemia cells that

use more oxidative phosphorylation for their metabolism (HL-60) showed increased resistance to glucose deprivation, but higher susceptibility to agents interfering with their mitochondrial function (such as resazurin and AICAR). Metabolically flexible primary cells (hepatocytes) were found to adjust their metabolism in response to agents that induce increased metabolic stress (TTA). Interestingly, this involved the nutrient sensing mTOR signaling pathway, which may play a role in regulating cell size, whereas we found no indications of hyperplasia (no neoplastic growth of the liver).

These studies support the growing understanding that metabolic characterization of cancer cells and its effects on and by mutations in cell signalling pathways, not only gives us a better understanding of tumour biology, but may also provide additional treatment targets and strategies in cancer therapy. It is thus a possibility that treatments targeted to metabolism cause cell stress and death in metabolically compromised cancer cells, while more benign reversible stress responses may occur in normal cells.

Sammendrag

Metabolsk ubalanse er assosiert med økt risiko for en rekke sykdommer, inkludert kreft. Metabolisme påvirker eller blir påvirket av praktisk talt alle andre cellulære prosesser, noe som ikke er overraskende med tanke på deres konserverte rolle gjennom evolusjonen. Mitokondriene er viktige sensorer av det intracellulære metabolske miljøet og spiller en stor rolle i bioenergi og overlevelse signalisering i mammalske celler. Følgelig er disse organellene innblandet i utviklingen av kreft, som inkluderer endringer av cellenes stoffskifte og celledød signalisering.

Metabolsk omprogrammering i kreft celler øker celle vekst og proliferering. Dette innebærer høyere opptak av næringsstoffer som metaboliseres for å gi energi og mellomprodukter for celle bestanddeler. Denne omprogrammeringen har vært assosiert med økt terapeutisk resistens, men kan også vise seg å bli utnyttet i metabolsk målrettede behandlingsformer. Store signaliserings nettverk som er involvert i tumorvekst og metabolsk omprogrammering er PI3K/Akt/mTOR signal veien ofte funnet mutert i kreftceller. Aktivering av denne veien resulterer i høy glukose avhengighet og aggressive svulster. I forhold, fører en aktivering av en lav-energi responderende AMP-aktivert protein kinase (AMPK) signal vei til vekst-hemming. Begge disse signalveiene kan regulere eller bli regulert av mitokondrielle funksjoner, noe som har vært utgangspunktet for denne PhD studien.

Målet med denne avhandlingen var derfor å undersøke samspillet mellom celle-metabolisme og signalisering, og effekten på mitokondriell funksjon under forhold som involverer energi og metabolsk stress. For å belyse ulike sammenhenger ved mitokondriell regulering studerte vi disse mekanismene i både metabolsk rigide kreftceller og i metabolsk fleksible primære rottehepatocytter.

Vi fant at lekuemiceller som har mutasjoner i PI3K/Akt/mTOR signaliseringsveien (Jurkat) er mer utsatt for glukose deprivasjon og agenter som utfordrer denne energien veien (f.eks. palmitinsyre). Alternativt, viste mer metabolsk fleksible

leukemiceller (HL-60) bedre motstand mot glukose deprivasjon, men økt stress og død når de blir utsatt for agenter som forstyrrer deres mitokondrielle funksjon (f.eks. resazurin og AICAR). Metabolsk fleksible primære celler (hepatocytter) tilpasser metabolismen til agenter som induserer økt metabolsk stress (TTA). Et interessant funn var at dette involverte den mTOR signaliseringsveien som påvirkes av næringsforhold og deltar i reguleringen av cellestørrelse. Vi fant imidlertid ikke indikasjoner på hyperplasi (ingen neoplasi i lever).

Disse studiene støtter den økende forståelsen av at metabolsk karakterisering av kreftceller og dens virkninger på og av cellesignalisering mekanismer, ikke bare gir oss en bedre forståelse av tumorbiologi, men kan gi oss en pekepinn for en mengde potensielle kreftbehandlingsmål. Det er derfor en mulighet at behandling rettet mot metabolismen vil indusere cellulære stressreaksjoner og celledød i kreftceller, mens reversible stressresponser vil kunne forekomme i normale friske celler.

List of publications

I. Erikstein BS, Hagland HR, Nikolaisen J, Kulawiec M, Singh KK, Gjertsen BT, Tronstad KJ. *Cellular stress induced by resazurin leads to autophagy and cell death via production of reactive oxygen species and mitochondrial impairment.* J Cell Biochem. 2010 Oct 15;111(3):574-84.

II. Hagland HR, Nikolaisen J, Nilsson LIH, Pettersen IN, Omsland M, Strand I, Myklebust R, Gjertsen BT, Bruserud Ø, Lorens JB, Døskeland SO, Tronstad KJ. *Modulation of mitochondrial energy metabolism leads to respiratory dysfunction and metabolic stress via cell specific pathways in leukemia cells*. [Submitted]

III. Hagland HR, Nilsson LIH, Burri L, Nikolaisen J, Døskeland SO, Berge RK, Tronstad KJ. *The pan-PPAR activator tetradecylthioacetic acid (TTA) increases mitochondrial respiration and hypertrophy in rat hepatocytes involving the mTOR/4E-BP1 pathway*. [Manuscript]

IV. Hagland H, Nikolaisen J, Hodneland LI, Gjertsen BT, Bruserud O, Tronstad KJ. *Targeting mitochondria in the treatment of human cancer: a coordinated attack against cancer cell energy metabolism and signalling.* Expert Opin Ther Targets. [Review]. 2007 Aug;11(8):1055-69.

1. Introduction

1.1 Prefrace

The word “metabolism” derives from the Greek language and interprets to “change” or “out throw” and in the more practical sense it involves the biochemical reactions in an organism where substances are broken down to sustain life. Normally the phrase is divided into two sub categories namely catabolic metabolism and anabolic metabolism. The prior involves the breakdown of substrates to produce energy, whereas the latter is using energy to produce cell components. The mitochondria are key mediators in cellular metabolism and are small double membrane organelles found in almost all eukaryotic cells (Henze and Martin, 2003b). Most of the adenine triphosphate (ATP), which in essence is the fuel that sustains cell life, is produced here via ATP synthase coupled to oxidative phosphorylation and the tricarboxylic acid cycle (TCA). Our ATP turnover at rest is 28 g/min, which is equivalent to 1.4 kg/hour (Salway, 2006). During strenuous exercise this number increases to an incredible 0.5 kg/min (!), which underscores the important role the mitochondria have in maintaining metabolic homeostasis.

External and internal factors such as nutritional composition, energy status, oxygen tension and chemical compounds cause mitochondrial responses, either directly or indirectly. Furthermore, the mitochondrial tissue distribution is highly diverse in amount, composition and functionality, underscoring that they can take on pleiotropic roles in cells. Accordingly, the metabolic flexibility of a cell may be determined by the mitochondrial function or malfunction, which is why many diseases are closely linked to mutations in mitochondrial genes, which makes them interesting targets to investigate in a whole range of diseases. This thesis focuses on the metabolic flexibility of various cancer cell lines (metabolically compromised) as well as primary hepatocytes (metabolically flexible) with regards to mitochondrial function. Mitochondria are of special interest in cancer cell energy metabolism, as they are key regulators of maintaining a steady supply of precursors for maintaining high

proliferation rates. In addition, their central role in cell death makes these organelles a promising "dual hit target" for selectively eliminating cancer cells.

1.2 Cellular Energy Metabolism

1.2.1 Energy substrates

The main energy substrates used in metabolism are carbohydrates, lipids and proteins. How the body utilizes these substrates depends on the metabolic state (fed or starved), type of tissue, energetic demand (exercise or resting) and oxygen availability. Glucose is the only substrate for the red blood cells (RBC) as they lack mitochondria, whereas the brain cannot use fatty acids as fuel and rely on glucose or the fatty acid derivatives ketone bodies (β-hydroxybuterate and acetoacetate) during fed or fasted state (prolonged) respectively (Salway, 2006). Under aerobic conditions fatty acids are the preferred fuel by muscle cells and are metabolized by β-oxidation to make ATP in the mitochondria. The liver plays a vital role in glucose homeostasis and is the main organ (some from the kidneys), that make glucose during fasting. The substrates for glucose production are amino acids and glycerol, which help maintain glucose levels above 3.5 mmol/L (Salway, 2006). Thus, during fasting fatty acids enters the liver cells and are used to make ketone bodies and ATP to fuel gluconeogenesis. Initially the liver response to drop in blood glucose levels is to release its glycogen reserves (glucose stored in long chains), whilst starting the generation of glucose through the gluconeogenesis process.

1.2.2 Glucose homeostasis

Glucose transporters (GLUTs)

Glucose is a polar molecule which does not readily diffuse across the hydrophobic plasma membrane; therefore, specific carrier molecules exist to mediate its uptake. This transport is conducted via bidirectional proteins called glucose transporters (GLUTs) across the membrane without requiring energy (Joost and Thorens, 2001).

GLUTs consist of a family of 13 members, GLUT 1 to 12 plus the proton (H^+)-myoinositol cotransporter (HMIT) (Joost et al., 2002, Uldry and Thorens, 2004). These transporters belong to a family of proteins called solute carrier family 2 (gene symbol SLC2A). Structurally, the GLUTs can be divided into three classes: GLUT1 to -4 (class 1), GLUT5, -7, -9, and -11 (class 2), and GLUT6, -8, -10, and -12 and HMIT (class 3) (Joost et al., 2002, Uldry and Thorens, 2004). Class 1 is comprised of the most-well-characterized glucose transporters, GLUT1-4 (Table 1). GLUT1 is ubiquitously distributed in various tissues with different levels of expression in different cell types. GLUT2 is a low-affinity transporter for glucose and is found primarily in the intestines, pancreatic β-cells, kidney, and liver (Thorens et al., 1988). This low affinity is an important regulator of glucose homeostasis, which is absolutely crucial for normal mental capacity (Owen et al., 1998). GLUT3 mRNA expression is almost ubiquitous in human tissues, although the protein distribution is restricted to brain, testis (Haber et al., 1993) and placenta in early pregnancy (Brown et al., 2011). GLUT3 transports glucose with high affinity (it has the lowest K_m of the GLUTs), which allows for easy influx of glucose to the cells even when blood glucose levels drops. This underscores the importance of maintaining adequate nutrient supply to the brain during all types of physiological conditions. GLUT4 is the major glucose transporter in adipose tissue, as well as in skeletal and cardiac muscle. These tissues are insulin sensitive and insulin stimulation leads to activation of downstream pathways of the insulin receptor, which mediates the rapid translocation of GLUT4 from intracellular vesicles to the cell surface, resulting in an increase in cellular glucose transport activity (Birnbaum, 1989, Langfort et al., 2003, Lira et al., 2007). GLUT4 is consequently highly expressed at the cell plasma membrane in the fed state, whereas it is kept in intracellular vesicles upon fasting. Another important feature of GLUT 4 is that it has a 3-fold higher glucose transport capacity than the widely expressed GLUT1 (Yu et al., 2011).

Receptor	Tissue Distribution	Special properties
GLUT 1	Ubiquitly expressed	High capacity, low K_m (1-2 mmol/L)
GLUT 2	Intestines, pancreatic β-cells, kidney and liver	High capacity, low affinity (K_m 15-20 mmol/L)
GLUT 3	Brain, testis and placenta	High capacity, low K_m (1 mmol/L)
GLUT 4	Skeletal and cardiac muscle, adipose tissue	Activated by insulin, K_m (5 mmol/L)

Table 1. Overview of Glut receptor distribution and blood glucose consentrational activation. (adapted from www.medbio.info/, 2012)

Regulation of glycolysis

After glucose enters the cell it is phosphorylated by hexokinases or in the liver by glucokinase, which essentially traps the glucose inside the cell by adding a charged phosphate, creating glucose-6-phosphate (G6-P). Figure 1 illustrates the conversion of glucose to pyruvate through glycolysis. The G6-P is converted to fructose 6-phosphate (F 6-P) by the enzyme phosphoglucose isomerase, and subsequently phosphofructokinase (PFK-1) converts it to fructose 1,6-bisphosphate (F1,6-BP). PFK-1 uses one mole ATP to add on the second phosphate group on the fructose molecule. Several additional steps are needed before the irreversible step of pyruvate kinase makes pyruvate from phosphoenolpyruvate (PEP) (Salway, 2006). Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are considered the main controlling steps for glycolysis flux modulation as they catalyse the three irreversible reactions in the glycolytic sequence (Weber et al., 1966). During starvation these irreversible steps are overcome by specific enzymes for the gluconeogenic pathway (figure 1).

Since glycolysis requires energy and releases energy, it is most efficient when linked to oxidative phosphorylation which is performed in the mitochondria under aerobic conditions. In normal catabolic expenditure of one glucose, two pyruvate molecules are made and a net of 2 mole ATP. In the presence of oxygen and normal metabolic checkpoints, pyruvate enters the mitochondria via the pyruvate carrier (Hildyard and Halestrap, 2003) where pyruvate dehydrogenase complex (PDH) catalyze the conversion to acetyl-CoA, yielding NADH and CO_2 as byproducts.

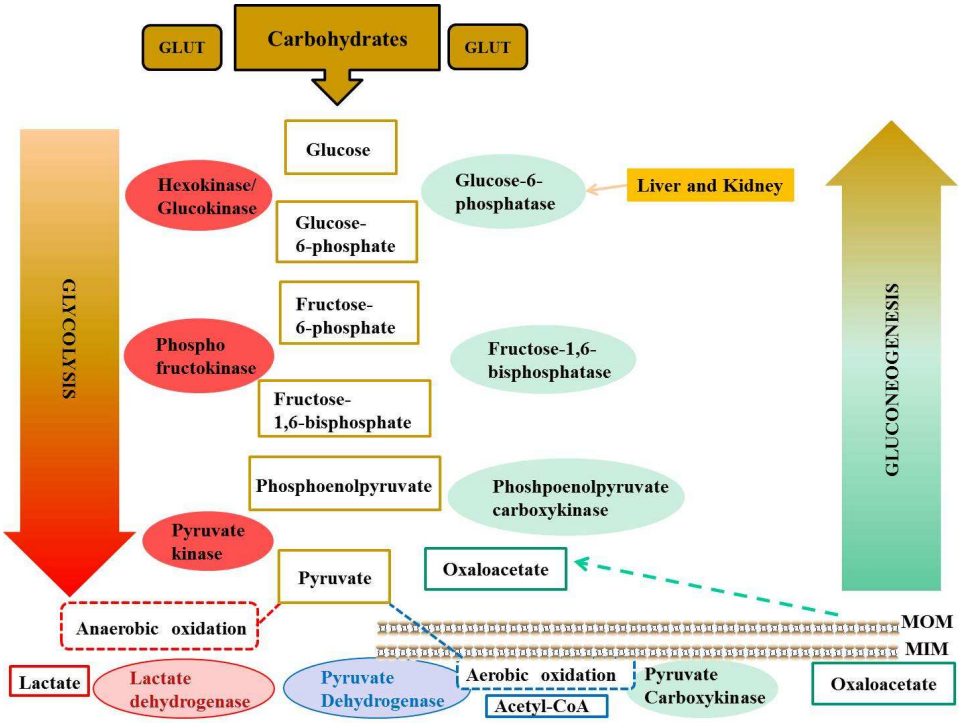


Figure 1. Oxidation of Glucose in glycolysis and production of glucose in gluconeogenesis. The three irreversible enzymatic steps in glycolysis are marked in red, whereas the gluconeogenesis specific enzymes are marked in green. Intermittent steps are excluded, but included in the text. Pyruvate generated through glycolysis is either transported into the mitochondria for complete oxidation via the tricarboxylic acid cycle (oxygen present), or converted to lactate by lactate dehydrogenase during anaerobic conditions. Pyruvate has to be actively imported into the mitochondria

through the mitochondrial outer membrane (MOM), and mitochondrial inner membrane (MIM), before it is converted to acetyl-CoA which combines with oxaloacetate to form citrate. During gluconeogenesis oxaloacetate is converted to malate and transported out of the mitochondria before returned to oxaloacetate which is further converted to phosphoenolpyruvate (PEP) by phosphoenolpyruvate carboxykinase (PEPCK).

1.2.3 The Mitochondria

The mitochondria are small double membrane organelles found in almost all eukaryotic cells. They are considered to be derivatives of ancient aerobic bacteria that merged with a primitive eukaryotic cell over two billion years ago (Wallace, 2005). A relic from this event is that they comprise their own DNA, which encodes 37 genes for 12S and 16S rRNAs, 22 tRNAs and 13 polypeptides belonging to the electron transport chain (ETC). The remaining mitochondrial proteins are encoded in the nucleus, estimated to around 1500 genes, and has to be imported into the mitochondria through various import systems (Wallace, 2005). Mitochondrial distribution throughout tissues is highly diverse in amount, composition and functionality, underscoring the fact that they can take on pleiotropic roles in cells (Hagland et al., 2007). Figure 2 illustrates the different processes in which the mitochondria are involved. This thesis will focus on some of these processes in connection to cellular metabolic response mechanisms.

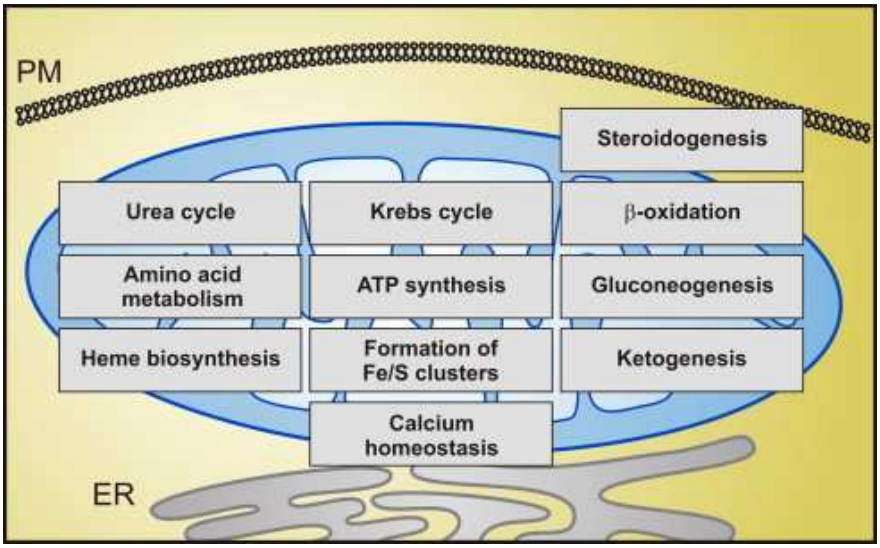


Figure 2. Mitochondria in cell life. Through oxidative phosphorylation mitochondria produce the bulk of intracellular ATP, and hence are considered the cells power plants. In addition, mitochondria regulate Ca^{2+} homeostasis and host some steps of several other metabolic circuitries including (but not limited to) the TCA cycle, the urea cycle, gluconeogenesis, ketogenesis, heme biosynthesis, fatty acid β-oxidation, steroidogenesis, the metabolism of certain amino acids and the formation of Fe/S clusters. ER, endoplasmic reticulum, PM, plasma membrane. (adapted in full from (Galluzzi et al., 2010))

Tricarboxylic acid cycle (TCA cycle)

The TCA cycle is involved in the metabolism of sugars, lipids and amino acids and takes place in the mitochondrial matrix (Salway, 2006). Originally it was thought of as an ongoing cycle whose main function was to oxidize acetyl-CoA, producing CO_2 and $NADH/FADH_2$ electrons for oxidative phosphorylation (Raimundo et al., 2011). Back then whole tissue homogenates were used and it was only after the technique of differential centrifugation, which resulted in appropriate separation of cellular components, that it became clear that many of the isoforms and enzymes of the TCA cycle were also present in the cytoplasm (Raimundo et al., 2011). These cytoplasmic

localized isoforms and enzymes involves almost all of the TCA cycle intermediates except for those involved in succinate turnover (Raimundo et al., 2011). The cytoplasmic and mitochondrial pool of TCA intermediates are connected to each other, which is reflected in a correspondingly increase or decrease of metabolites according to levels in each pool (Raimundo et al., 2011). The mitochondrial outer membrane (MOM) has channels where these metabolites freely diffuses into the inter-membrane space, whereas the mitochondrial inner membrane (MIM) actively transports these metabolites across the MIM in a controlled fashion (Salway, 2006).

The first step of the TCA cycle is the condensation of acetyl-CoA with oxaloacetate to form citrate. Acetyl-CoA can be generated either from pyruvate, amino acid catabolism or fatty acid β-oxidation. The full oxidation of acetyl-CoA requires an eight step process, involving different enzymes and co-enzymes, which eventually results in the production of 3 NADH, 1 $FADH_2$ and 1 GTP/ATP together with waste products such as H_2O from glycolysis, and CO_2 (Figure 3). The first step in the cycle is mediated by citrate synthase and generates citrate. Citrate is converted to isocitrate by aconitase, which is further converted to α-ketogluterate (α-KG) by isocitrate dehydrogenase (IDH) generating NADH. The amino acids glutamate, histidine, proline and ornithine enters the TCA cycle via α-KG during amino acid catabolism, and therefore belongs to the glucogenic amino acids (Salway, 2006). The enzyme α-ketogluterate dehydrogenase (α-kgDH) turns α-ketogluterate into succinyl-CoA generating NADH. The branched-chain amino acids valine and isoleucine together with methionine may also enter the TCA cycle by conversion to succinyl-CoA. The next step involves succinyl-CoA synthetase and is the only step where GTP or ATP is generated together with succinate. Succinate feeds electrons into the electron transport chain via succinate dehydrogenase (SDH) also referred to as Complex II. Otherwise the only complex of the respiratory chain which is exclusively encoded in the nucleus (Wallace, 2005). Succinate dehydrogenase converts succinate to fumarate, producing $FADH_2$ which donates its electrons to electron carriers of the ETC. Fumarate is converted to malate by fumarase, a reaction found in both the TCA

cycle and cytoplasm (Raimundo et al., 2011). Malate dehydrogenase completes the TCA cycle making oxaloacetate and NADH.

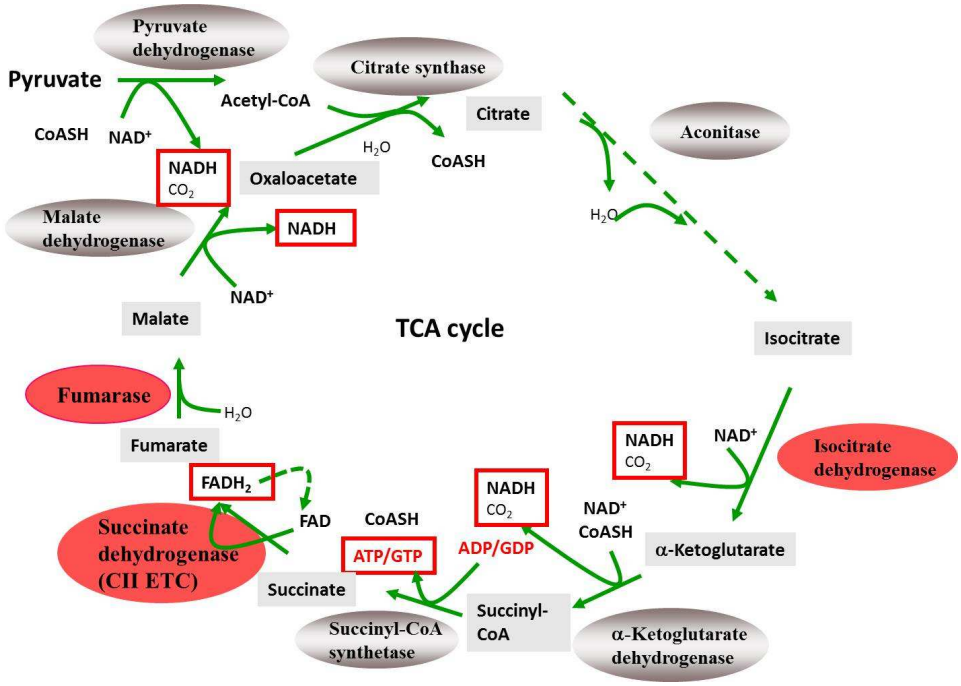


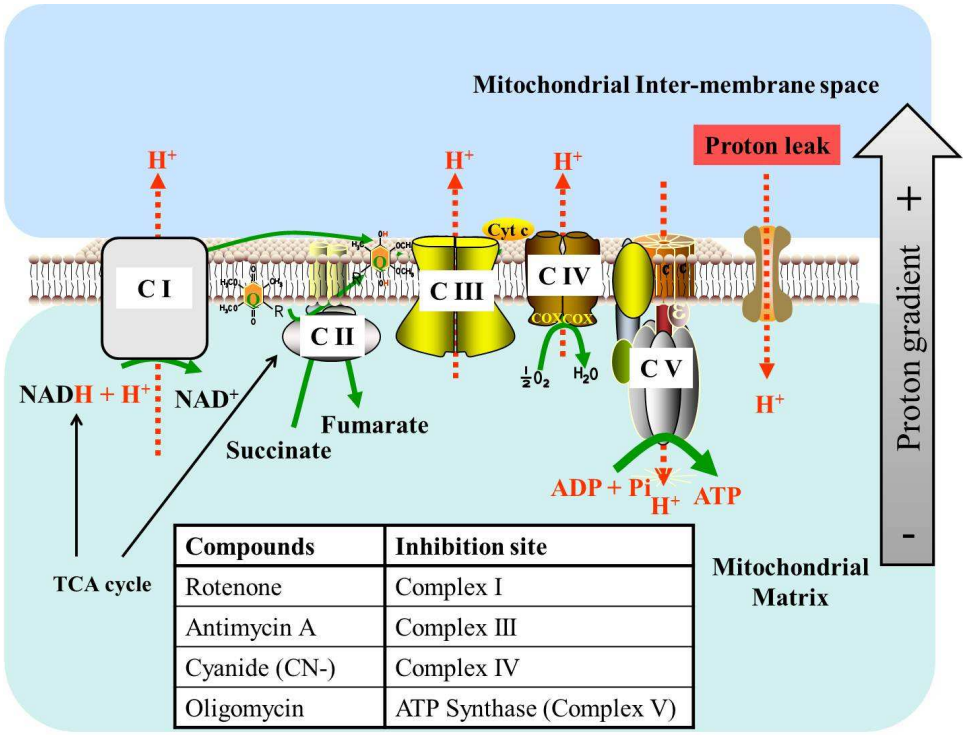
Figure 3. The TCA cycle. The first committed step is the combination of oxaloacetate with acetyl-CoA to make citrate. The source of acetyl-CoA depends on the cells metabolic situation, and can be generated from fatty acid β-oxidation, pyruvate from glycolysis or catabolism of amino acids. The enzymes shown in red have been found mutated in many cancers (described in later section). Succinate dehydrogenase is also part of the electron transport chain (ETC).

Oxidative phosphorylation

The NADH and $FADH_2$ made from oxidation/reduction reactions in the TCA cycle are used as electron donors in the electron transport chain, which is found in the inner membrane of the mitochondria. These co-factors cannot cross the mitochondrial membrane and as such must be recycled in the electron transport chain (ETC) (Salway, 2006), or via the exchange of TCA cycle intermediates between the

cytoplasm and matrix. The ETC is made up of four complexes, commonly referred to as Complex I-IV and ATP synthase (complex V). Electrons from electron donors NADH and $FADH_2$ are fed into the ETC via complex I and II respectively, and consequently transferred via ubiquinol (QH_2), the reduced form of ubiquinone, to cytochrome c oxidoreductase (complex III). Furthermore, Complex III transfers the electrons to cytrochrome *c* which diffuses to cytochrome c oxidase (complex IV). Complex IV is where O_2 is reduced to H_2O.

The flow of electrons drive a proton motive force where complex I, III and IV pumps protons (H^+) into the inter-membrane space. This results in a membrane potential, which has to be maintained to have a functional oxidative phosphorylation system, thus driving the ATP synthase (complex V) in the right direction where adenosine 5'diphosphate (ADP) is converted to adenosine 5'triphosphate (ATP). The protons in the inter-membrane space flow back into the matrix via the proton channel F_0 of the ATP synthase (figure 4). The net yield of ATP from one glucose molecule via glycolysis, TCA cycle and oxidative phosphorylation has been a point of debate for many decades. But, the general consensus is that around 32 ATP are produced by catabolizing one glucose molecule (Salway, 2006), however this relies on a tight membrane integrity of the mitochondria and no influence of uncoupling proteins (UCP) (section 1.2.4). As a byproduct of oxidative phosphorylation, about 2% of the cellular oxygen is partly reduced to superoxide O_2^- (Salway, 2006). These are harmful reactive oxygen species (ROS), to which our body has developed defense mechanisms in the form of antioxidants (further explained in section 1.2.4)



Compounds	Inhibition site
Rotenone	Complex I
Antimycin A	Complex III
Cyanide (CN-)	Complex IV
Oligomycin	ATP Synthase (Complex V)

Figure 4. The Electron transport chain (ETC). Electrons from the NADH/$FADH_2$ generated in the TCA cycle fuels the pumping of protons across the matrix to the inter-membrane space, whereas the electrons transferred reduce O_2 to H_2O at complex IV. The charge difference between the matrix side and the inter-membrane space is indicated by the proton gradient. This membrane potential is used to fuel the synthesis of ADP to ATP by ATP synthase by allowing the protons to re-enter into the matrix through the proton channel in the F0 particle. The ATP synthase is not the only escape route for the protons in the inter-membrane space, since the presence of uncoupling proteins may mediate proton leak. Common inhibitors of the ETC and their inhibition site are listed in the table.

Fatty acid biosynthesis and oxidation

The liver plays an important role in glucose homeostasis, but is also crucial in lipogenesis and fatty acid oxidation. After a meal, when the glycogen reserves are

full, the remaining glucose available is shuttled through the pentose phosphate pathway making NADPH which e.g. is needed for lipid synthesis and antioxidant defense reactions (glutathione reduction). Fatty acid biosynthesis is made possible by high levels of NADH and ATP made in the mitochondria, which inhibits isocitrate dehydrogenase leading to citrate accumulation. Citrate is consequently transported out of the mitochondria through the tricarboxylate carriers and converted to acetyl-CoA and oxaloacetate in the cytoplasm by citrate lyase. Acetyl-CoA is, in the presence of insulin, converted to malonyl-CoA by acetyl-CoA carboxylase. The carnitine palmitoyl transferase (CPT) I and II are involved in transporting fatty acids across the two mitochondrial membranes respectively, where malonyl-CoA is a potent inhibitor of CPTI (Salway, 2006, Skrede et al., 1997). This in effect inhibits fatty acid oxidation during lipogenesis as malonyl-CoA is also a precursor for fatty acid synthesis. Increased levels of citrate and ATP in the cytoplasm inhibit PFK-1, thus increasing the glucose-6 phosphate (G6P) levels when insulin is present. Since G6P is the precursor for the pentose phosphate pathway the NADPH is continuously produced during high glucose levels (Salway, 2006). Simplistically put, when blood glucose drops, the GLUT2 transporter with low glucose affinity does not transport glucose into the insulin producing pancreatic β-cells and consequently insulin is not secreted (Salway, 2006). The pancreatic α-cells produce glucagon which activates glycogen breakdown in the liver and glucose release into the blood stream, thus preventing hypoglycemia. During starvation cortisol is released from the adrenal cortex, and together with glucagon stimulates hormone-sensitive lipase which results in fatty acid release from adipocytes to be used for oxidative fuel in the liver. The fatty acids enters the mitochondria via CPTI and CPTII and are oxidized through a number of steps generating high levels of NADH and $FADH_2$, which are recycled to NAD^+ and FAD in the ETC, where electrons are used for oxidative phosphorylation generating ATP for gluconeogenesis as described previously.

1.2.4 Metabolic flexibility and Cellular Stress

The general understanding of metabolism is based on the old biochemistry work done in the 1920s to 1960s, which included the before mentioned glycolysis (Embden, Meyerhof and Parnas), respiration (Warburg), the TCA cycle (Krebs), glycogen catabolism (Cori and Cori), oxidative phosphorylation (Mitchell) and ATP in energy transfer reactions (Lipmann) (DeBerardinis and Thompson, 2012). The understanding of how substrates were utilized in complex enzymatic reactions, to maintain whole body energy homeostasis, was a tremendous break through and has since led to dietary treatment options to many metabolic diseases which prior had been untreatable. The discovery of deoxyribonucleic acid (DNA) in the 1950s and its huge implications in genetic control directed the research into a new field, that of genes and proteins, leaving the area of basic biochemistry. This resulted in a wealth of information on genetic mutations and affected proteins that were implicated in different diseases. However, the connections between these mutations, the aberrant signaling pathways and how this affected the metabolism have only the last decade become an area of great interest (DeBerardinis and Thompson, 2012). Cellular metabolism is now understood to be more than a self-regulating network that operates in the background of biological signaling, furthermore it clearly affects and is affected by protein signaling itself (DeBerardinis and Thompson, 2012).

The connection of cell metabolism to major signaling pathways such as the Akt/mTOR/AMPK network (section 1.2.5), has given us a new insight into metabolic flexibility or lack thereof. Mitochondrial dysfunction has been reported in many metabolic diseases, such as diabetes 2, insulin resistance, cardiovascular diseases, obesity and cancer (Henze and Martin, 2003a, Kim et al., 2008, Lesnefsky et al., 2001, McBride et al., 2006, Modica-Napolitano et al., 2007, Wallace, 2005). Obesity has thus been linked to an increased risk of developing cancer (Calle and Kaaks, 2004), where several mechanisms have been proposed to explain their interconnectivity. Mutations caused by cellular stress reactions may affect important

signaling pathways involved in metabolic control and consequently lead to disease. The cells capability to adapt to increased stress, such as hypoxia, nutrient excess/deprivation or ROS seem to depend on the metabolic flexibility of the cell, which again is founded in its active signaling pathways.

Hypertrophy and hyperplasia

Hypertrophy is defined as an increase in cell size which subsequently results increased organ size *in vivo*. In pure hypertrophy the cells do not proliferate, but merely increases in size due to more structural proteins and organelles (Kumar, 2007). This is usually a stress related response and is most commonly found in terminally differentiated cells, while cells capable of dividing might respond by hyperplasia. Hypertrophy has been linked to major signaling pathways such as Akt and mTOR (Distefano et al., 2009, Haga et al., 2009), which regulate cell size and initiate cell division upon growth factor activation (Gibbons et al., 2009, Shaw and Cantley, 2006, Zoncu et al., 2011). Hyperplasia is an increase in cell number, which again leads to an enlargement of the organ (Kumar, 2007). These processes may act in coherence with each other with the same net result of an increased organ size. One example of normally occurring hypertrophy and hyperplasia is during pregnancy with the growing uterus by both growing and dividing smooth muscle cells. Hyperplasia as a consequence of increased hormonal production, which can be seen in wound healing, is a controlled process. However, the pathological hyperplasia response can be a nestling ground for loss of growth control resulting in cancer growth as seen in livers expressing constitutively active PPARα (Huang et al., 2011).

Reactive oxygen species (ROS)

As mentioned earlier, about 2% of the oxygen used in the ETC is released as ROS. This level of ROS plays an important role in the regulation of cell signaling, differentiation and proliferation (Wellen and Thompson, 2010). ROS, such as the superoxide ·O2-, H_2O_2 and ·OH, are very unstable, short lived molecules which react rapidly and spontaneously with adjacent molecules causing cellular damage.

To combat the effect of ROS the cells have evolved their own anti-oxidant defense. Superoxide dismutase (SOD) dismutes superoxide anions (·O2-) to hydrogen peroxide (H_2O_2), which can move through membranes and act as a mitochondrial signaling molecule (Mailloux and Harper, 2011). The H_2O_2 can oxidize thiol groups in phosphatases, kinases, transcription factors and metabolic enzymes (Mailloux and Harper, 2011). One low molecular weight thiolating agent is glutathione peroxidase, which has a high affinity to H_2O_2 converting it to water. The recycling of glutathione relies on NADPH, which illustrates the importance of maintaining a steady turnover of NADPH producing reactions in the antioxidant defense (Mailloux et al., 2007). Catalase is another cell antioxidant defense mechanism with a much lower affinity to H_2O_2 and predominantly found in the peroxisomes (Wanders and Waterham, 2006). These are the enzymatic cell defense, whereas there are also free radical scavengers such as vitamins A, C, and E, together with phytochemicals such as phenols, polyphenols and flavonoids found in foods. However in regards to effectiveness in radical scavenging the cellular antioxidant defense exceeds the free scavengers significantly (Halliwell, 2012). The cellular antioxidant system is energetically costly and requires ATP to produce glutathione and SOD at high levels (Diaz Vivancos et al., 2010). Moreover, it is not a fast acting system since it relies on the biosynthesis of new antioxidant acting molecules in response to increased ROS. Therefore, it would be beneficial for the cell to have an alternative antioxidant defense which may act more rapidly to changes in ROS.

Uncoupling proteins (UCP)

Endogenous proteins called uncoupling proteins (UCPs) may act by uncoupling the ETC from ATP production, by giving protons an alternative route back through the inner mitochondrial membrane thereby relieving the membrane potential (Ricquier, 2005). This phenomenon was first discovered in brown adipose tissue with the uncoupling protein UCP1, which was involved in non-shivering thermogenesis (Cannon and Nedergaard, 1985, Nicholls and Locke, 1984). UCP1 allows for protons to leak back into the matrix without passing through the ATP synthase complex,

which results in generation of heat, maximum electron shuttling, increased oxygen consumption but diminished ATP production (Wallace, 2005). Since UCP1 was identified, four more homologous anion transporters have been discovered (UCP1-5) (Ricquier and Bouillaud, 2000). However, only UCP2 and UCP3 have been found to mitigate ROS production in the mitochondria (Echtay et al., 2002, Negre-Salvayre et al., 1997, Yonezawa et al., 2009). Since UCP1 is exclusively found in brown adipocyte tissue, it was of great interest to see that UCP2 was more widely expressed and found in the macrophages, spleen, thymus, hypothalamus, stomach and the pancreatic cells, whereas UCP3 is mostly expressed in skeletal muscle and some in the heart and brown adipose tissue (Mailloux and Harper, 2011).

The general notion that a high membrane potential leads to increased generation of ROS is the basis of the antioxidant capacity of the uncoupling proteins, which alleviates the high membrane potential (Mailloux and Harper, 2011). For the uncoupling proteins to act as a first line of defense against ROS, they have to be expressed and localized to the mitochondria, and either held in check by inhibitors or be induced by activators. Indeed, various activating allosteric regulators with association to the mitochondria have been identified such as fatty acids, glutamine, nucleotide and superoxide (Echtay et al., 2002, Hurtaud et al., 2006, Hurtaud et al., 2007, Negre-Salvayre et al., 1997, Ricquier, 2005, Yonezawa et al., 2009, Mailloux and Harper, 2011).

Cell death – Necrosis and Apoptosis

Necrosis is cell death caused by hypoxia, trauma, toxins or infections. It has generally been considered to be an uncontrolled cell death pathway. However, recent updates on cell death have proposed that necrosis is not a random process. Terms such as caspase-independent cell death, programmed necrosis or necroptosis have been used to describe this alternative cell death program (Galluzzi and Kroemer, 2008, Galluzzi and Kroemer, 2009). Necrosis has different morphological features than the other cell death pathway, termed apoptosis. Apoptosis is a non-

inflammatory cell death, which cells use in the response to extrinsic or intrinsic cell death signals. The apoptotic cascade is amplified by the release of apoptotic factors from the mitochondria (Jacobson et al., 1997). This activates the caspases, which are the executioners of the apoptotic pathway. They are named for their cysteine-aspartate protease activity where they cut the bond between a cysteine amino acid and an aspartate amino acid (Jacobson et al., 1997).

The essential notion is that the cells energy level plays a fundamental role in the death pathway chosen (figure 5). Apoptosis requires ATP for successful execution, while necrosis is not dependent on energy per se (Kroemer et al., 1998). Evidence show that programmed necrosis plays an important part in our immune defence against viruses (Cho et al., 2009). In cancers, programmed necrosis may lead to harmful pathological cell loss which activates local inflammation responses and promotes tumour growth. However, necrotic cell death may be exploited to eliminate cancer cells therapeutically. The use of alkylating DNA damaging agents (Zong et al., 2004), tumour-targeted photosensitizing molecules (Agostinis et al., 2004) or modulating energy metabolism in response to cell death stimuli (Zhang et al., 2009) may induce necrosis through increased ROS production and can be exploited in therapeutic treatment. However, many cancer cells tolerate high levels of ROS as they have additional mutations in their cell cycle repair regulators such as p53, which would otherwise halt cell cycle progression to repair any DNA damage caused by increased ROS (Zhang et al., 2011, Levine, 1997). In addition the strengthening of the cancer cells antioxidant defence has also been connected with ROS inducing chemoresistance (Derdak et al., 2008).

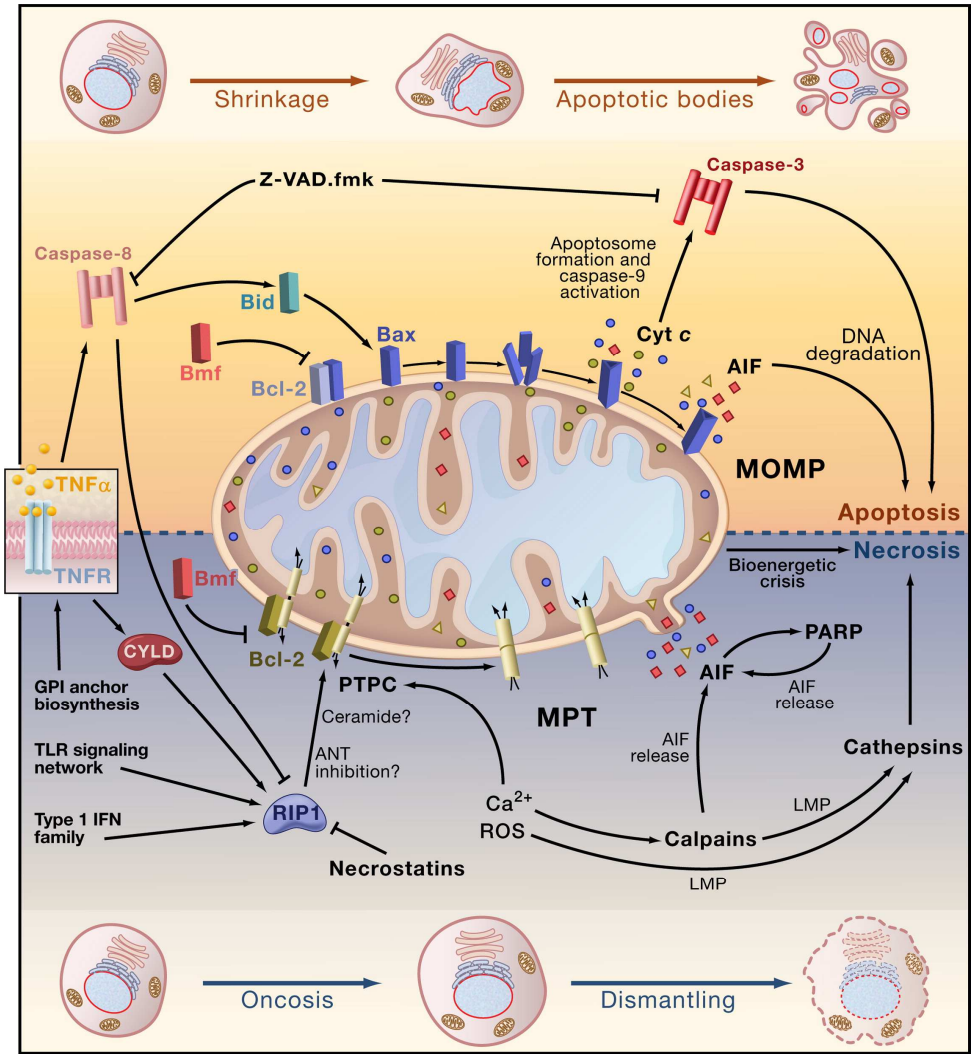


Figure 5. The interface between Apoptosis and Programmed necrosis. The programmed necrosis pathway depend on the activation of the serine/threonine kinase RIP1 (receptor-interacting protein kinase 1), which can be triggered by ligation of the tumor necrosis factor receptor (TNFR) or inhibition of caspases (Hitomi et al., 2008). The programmed necrosis pathway depend on the activation of the serine/threonine kinase RIP1 (receptor-interacting protein kinase 1), which can be triggered by ligation of the tumor necrosis factor receptor (TNFR) or inhibition of caspases

(Hitomi et al., 2008). In NIH 3T3 murine fibroblasts, TNFR activation ignites the extrinsic apoptotic pathway, which depends on caspase-8. Caspase-8 mediated degradation of RIP1 may represent one of the major molecular switches between apoptosis and necroptosis. Apoptosis and necroptosis may preferentially involve mitochondrial outer membrane permeabilization (MOMP) and mitochondrial permeability transition (MPT) of both membranes, respectively. For the sake of clarity, multiple intermediate regulators of apoptosis have not been depicted. Abbreviations: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; Cyt *c*, cytochrome *c*; GPI, glycosylphosphatidylinositol; IFN, interferon; LMP, lysosomal membrane permeabilization; PTPC, permeability transition pore complex; ROS, reactive oxygen species; TLR, Toll-like receptor; Z-VAD.fmk, Z-Val-Ala-Asp(OMe).fluoromethylketone. This figure is adapted from (Galluzzi and Kroemer, 2008).

Autophagy

Autophagy means "self-eating", and is a cells rescue mission in response to nutrient depletion, hypoxia or starvation. One characteristic of autophagy is the presence of autophagosomes, which are double membrane organelles containing cellular components which are being "re-cycled", during starvation (Jaboin et al., 2009). The process of autophagy is regulated differently to apoptosis, but excessive autophagy will ultimately lead to cell death (Wong et al., 2010). As many cancer cells have defective apoptotic machinery and thus are not responding to apoptotic agents, autophagy has emerged as interesting targets for cancer treatment.

Autophagy is primarily a starvation or damage prevention response, which is activated when challenging the cells energy status (Amaravadi and Thompson, 2007). *In vitro* this is done by inducing cellular stress leading to ROS, removing essential nutrients from the growth medium, or depriving the cells of oxygen for limited periods (Frezza et al., 2011). *In vivo*, the induction of ROS by chemical agents can lead to autophagy and more precisely mitophagy if the cell perceives the

mitochondria as potentially damaging organelles (Kim et al., 2007, Erikstein et al., 2010). Paradoxically, basal level of autophagy induced by stresses such as hypoxia, nutrient restriction or growth factor withdrawal, tips the cellular fate towards survival (Wong et al., 2010). This recycling of cellular components to support survival implicates many of the key signaling pathways involved in maintaining cellular energy homeostasis.

1.2.5 Cellular energy sensors and major signaling pathways

AMP-activated protein kinase (AMPK)

Free adenine nucleotides in mammalian cells lie in the range of 3,000–8,000 mM for ATP, 50–200 mM for ADP and 0.5–5 mM for AMP (Xiao, 2011). AMP is made by the conversion of 2 ADP to ATP and AMP by adenylate kinase, an enzyme widely and highly expressed in eukaryotic cells (Hardie et al., 2012). The reaction is reversible and the levels of nucleotides determine if the reaction is displaced towards ATP and AMP production or vice versa. Consequently, an increase in the ADP/ATP ratio would indicate falling energy status of the cell and AMP levels would increase accordingly. Several enzymes involved in glucose homeostasis are AMP/ATP sensors, however the most famous AMP sensor of the cell is the AMP-kinase (Hardie et al., 2012). Upon activation AMPK suppresses anabolic pathways, while activating catabolic pathways that generate ATP (reviewed in (Hagland et al., 2007, Hardie et al., 2012)).

AMPK is an energy sensor and metabolic modulator. The kinase is activated by high levels of AMP, which is indicative of cellular stress. Genes encoding AMPK subunits are found in essentially all eukaryotes with a conserved phosphorylation activation site at Thr172 (Hardie et al., 2012). Upstream activators of AMPK involves the Liver Kinase B1 (LKB1), which provides a high basal level of phosphorylation of Thr172 that is further modulated by the binding of AMP (Hardie et al., 2012). Another activator of AMPK is the $Ca^{2+/}$calmodulin-activated protein

kinase kinase (CAMKKβ), consequently triggering AMPK activation in response to increased cellular Ca^{2+} levels without necessarily requiring a change in ADP or AMP levels (Hardie et al., 2012). However, the effects of ADP and AMP on AMPK involves direct binding on the regulatory subunit which is unaffected by the upstream phosphorylation kinases or phosphatases (Hardie et al., 2012). Since the levels of ADP and ATP is so much higher in cells there have been speculations as to whether the AMP levels would ever become high enough *in vivo* to compete with the allosteric site by ATP and ADP (Oakhill et al., 2012). However, the recent identification that ADP also moderately activate AMPK, is likely to represent an important physiological mechanism for regulating the activity of the enzyme (Xiao, 2011). Moreover, the activation can inhibit cell proliferation of both non-malignant as well as cancer cells (Motoshima et al., 2006). This has led to the search of AMPK activators to be used in cancer treatment. There are at least two interesting AMPK activators which have shown to have promising effect at inhibiting cancer growth *in vitro*. One is the 5-aminoimidazole-4-carboxamide riboside (AICAR), which is an AMP analog and directly activates AMPK (Woodard et al., 2010), while the other more indirect agent is metformin, which is a complex I inhibitor and thus causes an increase of intracellular AMP (Foretz, 2010, Viollet et al., 2012). In addition, the activation leads to upregulation of several transcription- and cofactors involved in mitochondrial biogenesis, including nuclear transcription factor 1 and 2 (NRF1/2), mitochondrial DNA transcription factor and peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) (Kukidome et al., 2006). Another important cellular metabolic sensor to which AMPK negatively regulates is the mammalian target of rapamycin (mTOR) (Motoshima et al., 2006) (section 1.2.5).

Peroxisome proliferator activated receptors (PPARs)

The PPAR family of ligand-activated transcription factors includes the PPARα, PPARδ and PPARγ subtypes, which are essential lipid and nutritional sensors with individual agonist specificity and tissue dependent expression. These factors heterodimerize with RXR in order to activate gene transcription of target genes, and

are involved in the regulation of multiple cellular functions including metabolism, proliferation and differentiation (Escher and Wahli, 2000). Various roles of the PPARs have been discussed with respect to tumour development and progression. PPARα activation by peroxisome proliferators has for instance been associated with hepatic carcinogenesis in rodents (Reddy et al., 1980), but humans seem to be more refractory to such effects (Cattley et al., 1998). Some fatty acids and derivatives are potent agonists for PPARs, and activate transcription of target genes (Gottlicher et al., 1993). Several activating agents, both natural and synthetic, exert anti-tumour effects (Tronstad et al., 2003). Especially, PPARγ has emerged as a potential therapeutic target for the treatment of solid tumours and haematological malignancies (Wang et al., 2006). PPARγ has many activities and are involved in glucose as well as lipid homeostasis and cell differentiation. It interacts with mitochondrial biogenesis and AMPK via its cofactor PGC-1α. These factors also work together with other signalling pathways that are linked to cell growth, such as the JAK-STAT pathway that is prominent in haematological malignancies (Rajasingh and Bright, 2006).

The complicated mechanisms and actions of the PPARs and their ligands can possibly explain the diverging results on the therapeutic potential that has been reported. Hopefully, future research will reveal therapeutic modalities that allow selective modulation of this system in order to treat malignant disorders.

The IGF/PI3K/Akt network

Obesity, insulin insensitivity and diabetes 2 are closely connected. They are a consequence of increased metabolic stress, which results in hyperlipidemia and hyperinsulinemia. High insulin levels leads to increased levels of insulin-like growth factor I (IGF-I), as well as decreased levels of IGF binding proteins 1 and 2 which mediates inhibition of the bioavailability of IGF (Gallagher and LeRoith, 2010). Insulin responsive tissues such as the liver, muscle and adipocytes respond to the binding of insulin growth factor, and subsequently activates its downstream effector

proteins which involves the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway (Gallagher and LeRoith, 2010).

IGF signaling has been linked to a number of cancers where the proposed beneficial proliferative effect is activated through the PI3K-Akt pathway (Gallagher and LeRoith, 2010). Cancers which are sensitive to insulin can be growth inhibited by calorie restriction (reviewed in (Speakman and Mitchell, 2011)). This is based on the fact that increased Akt signaling correlates with increased glycolysis and proliferation in many tumors (Plas and Thompson, 2005). Akt is a signaling hub thought to interact with as many as 9000 proteins (Lawlor and Alessi, 2001). Figure 6 show the PI3K-Akt signaling pathway involved in metabolic control, where Akt mediates translocation of GLUT4 (Yamada et al., 2005), inhibits the tuberous sclerosis complex 2 (TSC2), which in effect activates the mTOR pathway involved in protein synthesis and cell growth (Zoncu et al., 2011). Furthermore, the activation of mTOR prevents autophagy, signaling to the cell that energy and substrates are in an abundance and anabolic reactions may proceed (Zoncu et al., 2011).

The Akt pathway is one of the most commonly altered pathways (due to gain of function mutations) in transformed cells (Elstrom et al., 2004). It is not merely regulated by insulin signaling, but a whole range of growth factor receptors which mediates their signal through PI3Kinase (Plas and Thompson, 2005). The PI3K phosphorylates the phosphatidylinositol diphosphate (PIP2) to PIP3, which again can be dephosphorylated by the tumor suppressor phosphatase and tensin homolog (PTEN) (Plas and Thompson, 2005). Full Akt activation is achieved by the phosphorylation on separate sites by the upstream kinase phosphatidylinositol-dependent kinase-1 (PDK1) (Alessi et al., 1997, Cohen et al., 1997). The increased glycolytic flux seen in cancers, which was first documented by Otto Warburg in the 1920s (Warburg et al., 1927), involves a whole range of enzymes which can be linked to the Akt pathway to maintain normal metabolic homeostasis (described below). Figure 6 illustrates the Akt involvement of several key metabolic enzymes, where hexokinase is the first enzyme in glycolysis and, upon Akt signaling, translocate to

the voltage dependent anion channel (VDAC) on the mitochondrial outer membrane (Mathupala et al., 2001). Hexokinase plays a critical role in maintaining the high catabolic rates of rapidly growing tumors, and the binding to VDAC is thought to prevent release of the pro-apoptotic proteins cytochrome c and reactive oxygen species (ROS) (da-Silva et al., 2004, Kroemer et al., 2007, Pastorino et al., 2002).

Chronic exposure to high nutrient concentration can lead to cellular stress reactions, one being overexpression of the IGF1 receptor in response to constantly high insulin levels. Consequently, abnormal signaling adaptations may occur where the cell becomes autonomic and unresponsive to extracellular growth inhibitions or nutrient depletion. The constitutive activation of pathways involved in nutrient control is therefore found in many types of cancer, where the activation of the PI3K-Akt pathway has been found to render cells dependent on glucose for their survival (Elstrom et al., 2004).

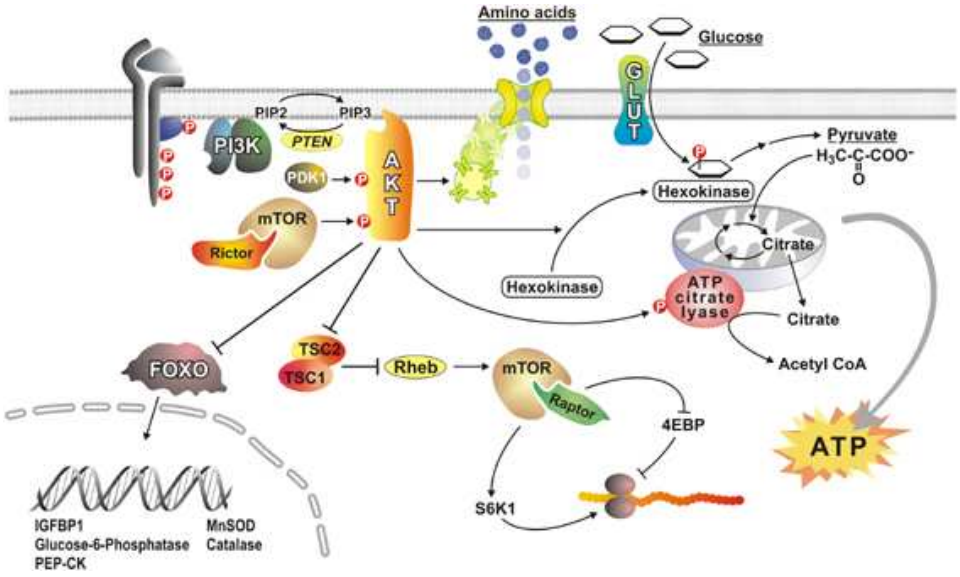


Figure 6. Akt signalling controls cellular metabolism. Akt regulation of nutrient transporters, FOXO transcriptional regulation of cellular metabolism, and TSC2 regulation of mRNA translation are highlighted. Adapted in full from (Plas and Thompson, 2005).

Mammalian target of Rapamycin (mTOR)

The intermediary mechanisms between nutrient sensing and protein synthesis *in vivo* are only partly understood (Sengupta et al., 2010). However, recent findings suggest that mTOR lies at the heart of intracellular nutrient sensing and control major metabolic pathways (Howell and Manning, 2011, Hsieh et al., 2012). The mTOR protein is highly conserved among eukaryotic cells, and can form two distinct protein complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), where mTORC1 is the primary nutrient sensor (Howell and Manning, 2011).

Extracellular signalling from growth hormones, cytokines, nutrients or stress all converge on the mTORC1 pathway, which in effect initiates protein translation when nutrients and growth hormones are high, while shutting down these responses when energy levels are low (Shaw and Cantley, 2006). Upstream regulators of mTORC1, which are also induced or repressed by nutritional status, involve the PI3K-Akt pathway (Cho, 2001, Cho et al., 2001, Hirashima, 2003, Jiang, 2003, Wan et al.,

2011), MAP-Kinase-extracellular regulated kinase (ERK) pathway (Bergan et al., 2011, Bost, 2005, Reindl et al., 2011) and AMPK pathway (Speakman and Mitchell, 2011). Recently, it was shown that mTORC1 was involved in regulation of fasting-induced ketogenesis via the peroxisome proliferation activating receptor (PPARα) transcriptional regulator (Sengupta et al., 2010).

Downstream targets of mTORC1 includes the eukaryotic translation initiation factor 4E-binding (eIF4E binding) proteins (4E-BPs) which act by repressing translation by binding to eIF4E (Le Bacquer et al., 2007). Phosphorylation of 4E-BP1 by mTORC1 releases its inhibitory binding of eIF4E, which is free to bind to a scaffolding protein eIF4G and initiate 5' mRNA translation (Le Bacquer et al., 2007). 4E-BP1s have been linked to glucose and lipid homeostasis and are highly expressed in liver (Tsukiyama-Kohara et al., 2001).

1.2.6 Cancer Metabolism

The Warburg effect – increased glycolysis

Tumors consist of a heterogeneous distribution of cancer cells, which gene and protein expression may also be highly diverse. However, cancer cells do have some commonalities. One being increased aerobic glycolysis, also known as the Warburg effect, named by its discoverer Otto Warburg (Warburg et al., 1927). Warburg postulated that the increased glycolysis was a response to defective mitochondrial machinery (Warburg, 1956). This has later been disproven, as many highly proliferating tumor cells do not have defects in their oxidative metabolism capacity (Moreno-Sanchez et al., 2007). Instead, resent research have demonstrated important links between cancer cell metabolism involving the mitochondria and its tumor growth abilities (DeBerardinis et al., 2008, Galluzzi et al., 2010, Raimundo et al., 2011).

The infamous Cell paper by Hanahan and Weinberg in 2000, called "Hallmarks of Cancer", proposes six molecular, biochemical and cellular traits that characterize the development and progression of malignant tumors (Hanahan and Weinberg, 2000).

However, back then none of these traits included metabolic reprogramming, although this phenomenon was well accepted and widely used in clinical assessment of tumors (^{18}Fluoro-2-deoxyglucose positron emission tomography, FDG-PET). For a long time the fundamental biochemistry of cell metabolism was something regarded as autonomous and not worthy of further studies, where in fact none of the six hallmarks would exist without the metabolic reprogramming supporting their effect. However, during the last decade the interest in the "old" biochemistry has regained its position in science as many of the oncogenes and tumor suppressor genes found mutated in cancer are involved in metabolic control (DeBerardinis et al., 2008, DeBerardinis and Thompson, 2012, Elstrom et al., 2004, Erickson and Cerione, 2010, Gallagher and LeRoith, 2010, Galluzzi et al., 2010, Goel et al., 2003). Since it is now generally accepted that cancer cells must have a changed metabolism to sustain the rapid uncontrolled cell growth, the "Hallmarks of Cancer: the next generation" was recently published including metabolic reprogramming as one of the main traits needed for cancer progression (Hanahan and Weinberg, 2011).

The Hallmarks of Cancer connected to metabolism

Growth Signal autonomy

Cancer cells can acquire the capability to sustain proliferative signaling by increasing the growth factor expression on the cell surface, thereby sensitizing the cell to lower amounts of growth signals. This has been linked to the N-acetylglucosamine (GlcNAc) regulation which is nutrient-responsive (Wellen and Thompson, 2010).

Evading growth suppressors

Many tumor suppressors have shown to be important metabolic check points. These are often involved in turning off anabolic processes in response to cellular stress. A well-known tumor suppressor is the phosphatase and tensin homolog (PTEN), which when inactivated leads to glucose dependent metabolism via constitutive activation of the PI3K-Akt pathway (Antico Arciuch et al., 2011, Elstrom et al., 2004).

Escaping apoptosis

Apoptosis is a programmed cell death initiated upon cellular damage, which cannot be repaired. One mediator of apoptosis is the p53 tumor suppressor which is a cell stress sensor and has been connected to oxidative phosphorylation (Matoba et al., 2006) via the transcriptional regulation of Cytochrome C Oxidase 2 (SCO2) Complex IV. In addition p53 can also be translocated directly to the mitochondria (Marchenko et al., 2000) where it interacts with apoptotic regulators such as B cell lymphoma (Bcl-2) (Tomita et al., 2006). Recently, mutated p53 was also connected to the deregulation of autophagy and resistance to apoptosis in response to the omega-3 fatty acid, docosahexaenoic acid (DHA) treatment (Jing et al., 2011).

Unlimited replicative potential

The reactivation of telomerase to inhibit chromosome shortening and thereby avoid cell senescence has been connected to the oncogene c-Myc (Kim and Chen, 2007). Glutaminolysis which is a common metabolic response in cancer cells depend on c-Myc activation (Wise and Thompson, 2010).

Angiogenesis, invasion and metastasis

The matrix metalloproteinase (MMP) found in the extracellular matrix (ECM) aid in the increased vascularization of the tumor (Campbell et al., 2010). In addition to stimulating vascular endothelial growth factor (VEGF) release from heparan sulphate proteoglycan (Campbell et al., 2010), the MMPs have shown to be more active in glycolytic cells inducing break down of the ECM and increase invasiveness. This was linked to the PI3K pathway through PDK1 (Xie et al., 2006).

Glycolysis, glutaminolysis and oxidative phosphorylation in cancer cells

For cancer cells to survive and have a high proliferation rate, they must adapt to the harsh extracellular environments they may encounter by outgrowing nutrient and oxygen transporting vasculature. The obstacles to overcome include hypoxia, higher acidity due to lactate secretion and lack of nutrients and growth factors. The hypoxia inducible factor 1 (HIF-1), is induced under hypoxic conditions via ROS generation

from the mitochondria, or mutations in the TCA cycle enzymes (DeBerardinis et al., 2008, Raimundo et al., 2011). When oxygen is present the HIF-1α subunit is hydroxylated by prolyl hydroxylases (PHDs) thereby recruiting the von Hippel-Lindau (VHL) tumor suppressor which targets the complex for proteasomal degradation (Raimundo et al., 2011). HIF-1 targets genes encoding glucose transporters, glycolytic enzymes and lactate dehydrogenase- A (LDH-A) (Dang and Semenza, 1999). Consequently, increased HIF-1α expression is correlated with increased angiogenesis, aggressive tumor growth and poor patient prognosis (Powis and Kirkpatrick, 2004). HIF-1α may also reduce the mitochondrial oxygen consumption by restricting the TCA cycle from its acetyl-CoA precursor pyruvate (Selak et al., 2005).

Mutations in succinate dehydrogenase (SDH), fumarase and isocitrate dehydrogenase (IDH1/2) have been found in a number of tumors and are thought to have a role in cancer formation (Raimundo et al., 2011, King et al., 2006, Selak et al., 2005). The accumulation of fumarate, succinate and citrate caused by such mutations have been implicated in an inhibitory effect of the PHDs thus making HIF-1α free to associate with the HIF-1β subunit in the nucleus and initiate transcription (Raimundo et al., 2011). Additionally an overexpression and/or over-activation of the tree irreversible enzyme reactions involved in glycolysis has been described for a number of tumour cells (Hagland et al., 2007, Moreno-Sanchez et al., 2007, Pelicano et al., 2006, Moon et al., 2011).

Many tumors express the embryonic pyruvate kinase isoform PKM2 (Christofk et al., 2008). This isoform has a lower affinity to its substrate PEP, and consequently stalls glycolysis at this step. This leads to accumulation of glucose metabolites at their irreversible steps (Figure 1), which can be channeled into other pathways such as the pentose phosphate pathway (PPP), the hexoamine pathway and nucleotide synthesis, to support anabolic growth (Christofk et al., 2008, Wellen and Thompson, 2010). Since pyruvate may be limited due to the PKM2 expression, the cell has to use other

means of maintaining substrates for the TCA cycle and thus precursors and co-factors for lipid, amino acid and nucleotide synthesis. This is achieved by increasing glutamine import, which is converted to glutamate via glutaminase, before transaminated by α-ketoacid transaminases to α-KG that feeds into the TCA cycle (Erickson and Cerione, 2010, Smolkova et al., 2011, Wise and Thompson, 2010). Since very little glutamine is fully oxidized to acetyl-CoA, this reaction is thought to be incomplete and has therefore been given a similar name as the incomplete oxidation of glucose in glycolysis, namely glutaminolysis (Newsholme et al., 1985). Increased uptake of glutamine and its consequent conversion to glutamate, yields high amounts of nitrogen needed to maintain a steady state production of new amino acids. The α-KG in the mitochondria can then be diverted into two alternative pathways depending on redox status and the ETC. An ETC independent pathway directly converts α-KG to citrate by reductive carboxylation via IDH2 (Wise et al., 2011). This reaction depends on NADPH which is produced in the mitochondria by the conversion of malate to pyruvate via the mitochondrial malic enzyme. If oxygen is present and ETC is functioning, α-KG may proceed in its normal direction through α-KG dehydrogenase (Smolkova et al., 2011). This process requires steady supply of co-factors such as NAD^+ and regeneration of $FADH_2$ to FAD. Since these co-factors cannot cross the mitochondrial membranes, they have to be synthesized and/or recycled in the matrix in order for the TCA cycle to run efficiently (Belenky et al., 2007). The recycling of NADH and $FADH_2$ is achieved by complex I and II respectively. The NAD synthesis is conducted by the NAD enzyme NMNAT3 in the mitochondria (Nikiforov et al., 2011).

Normally the TCA cycle is linked to the oxidative respiration pathway and NAD^+ is recycled from NADH. During hypoxia this cannot be completed, NADH would not be recycled and enzymatic reactions requiring NAD^+ as co-factors would stall. As described above this could result in reversal of TCA at α-KG, or it may be bypassed by upregulation of uncoupling proteins allowing protons to leak back into the matrix independent of oxygen availability or ATP synthesis (Robbins and Zhao, 2011). This

would support high TCA cycle flux even during hypoxic conditions. These mitochondria linked effects thus creates the nestling ground for increased glycolytic flux even in the presence of oxygen, which has been known as the Warburg effect (figure 7).

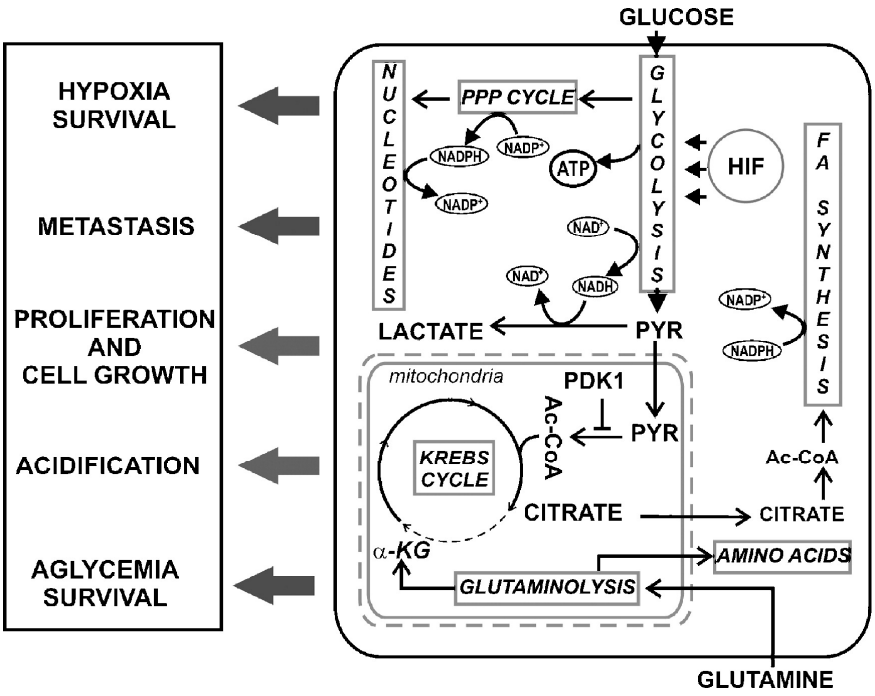


Figure 7. Major features of cancer cell metabolism. The major features of cancer cell energy metabolism, specifically, glycolysis as a key source of ATP and metabolic precursors via concomitant output from the pentose phosphate pathway ("PPP CYCLE") are summarized. The pentose phosphate pathway is also an important source of NADPH and ribose. Enhanced lactate production contributes to extracellular acidification, which supports invasion. Citrate from the TCA cycle is extruded from mitochondria and supports fatty acid synthesis, especially when also enforced by glutaminolysis. Glutaminolysis may also provide alanine and other amino acids for use in protein biosynthesis. Glycolysis allows survival during hypoxia, and, in turn, upon re-established sufficient oxygen levels oxidative

glutaminolysis may allow for ATP production and biosynthesis at when glucose levels are low (aglycemia). Anoxic glutaminolysis may also contribute to biosynthesis at sufficient ATP levels. Collectively, these metabolic changes support proliferation and cell growth (adapted from (Smolkova et al., 2011)).

2. Aims of study

The overall aim of this thesis was to investigate crosstalk between cell metabolism and signalling in the regulation of mitochondrial function during conditions of energetic and metabolic stress. The mechanisms were studied in metabolically restricted cancer cells and metabolically flexible primary rat hepatocytes to reflect different contexts of mitochondrial regulation.

2.1 Specific Aims

- Identify mitochondrial and cellular responses in cancer cell lines after treatment with agents causing metabolic stress
- Identify protective or combined effects of glucose depletion together with pathway-selective agents in two metabolically different leukemia cell lines
- Identify mechanisms of mitochondrial stimulation in hepatocytes of rats treated with a known PPAR-activator.

3. Material and Methodology considerations

3.1 Pitfalls when using cell cultures

3.1.1 Cell lines and Growth medium

The number of cell lines and cell culture medium available these days is overwhelming. Most cell lines which can be bought commercially have been made immortal either by inducing expression of genes to keep them proliferating indefinitely or they have been selected for such characteristics after patient biopsies. The ability to grow cell lines in culture was a tremendous breakthrough in modern medicine, and has since been used for studies not only involving cancer but also HIV, vaccine development, toxicity and gene mapping (Lucey et al., 2009).

In the early days of cell culturing it was found that the cell lines thrived in a nutrient rich and growth factor supplemented medium. This usually involves growing cells in medium containing 11.2-25mmol/L Glucose, which is a hyperglycemic condition hard to achieve physiologically unless one has diabetes (normal blood glucose levels ranges from 4-11mmol/L). These hyperglycemic conditions are often the nutrient background to many of the cancer metabolism studies that have been published. In our experiments we also used the recommended culture conditions for our cell lines, which meant keeping them in high glucose environments (RPMI 1640 > 11mmol/L), supplemented by excess glutamine (4 mmol/L) and foetal calf serum (FCS). To test their glucose dependency we grew them in glucose deprived medium, but which still contained glutamine and FCS. In retrospect we should have used dialyzed FCS as this contains less glucose than normal FCS. Therefore, we cannot rule out the presence of glucose, although at very low concentration, during the first hours of our glucose deprived experiments. Additionally, cancer cells thrive on both glucose and glutamine in parallel as substrates to maintain high proliferation (DeBerardinis et al., 2008). Consequently, with the added glutamine in excess we were still giving them one of their preferred substrates during the glucose deprived conditions. However,

glutamine has a short half-life in medium (Ozturk and Palsson, 1990), and even if cells were able to utilise this amino acid to support growth I believe the growth rate would be significantly reduced.

This being said, the glucose deprived conditions were used as a model to look for extreme responses, as the cells would have to rely on their metabolic flexibility to use other substrates to maintain growth and survival.

3.1.2 Physiological relevance

The extrapolation of these experiments to the physiological state is hard as the whole body energy homeostasis is kept in rigorous control, where blood glucose levels cannot drop below 3.5mmol/L and glutamine levels are kept at 0.4-0.5mmol/L (Owen et al., 1998). Another consideration is that the lactate secreted from glycolytic cancer cells in culture would not accumulate in extreme concentrations in the body, as they do in our closed flask environment, but would be cleared away either by using the Cori cycle or by cells in close proximity to the tumor (Samudio et al., 2008). However, rapidly growing tumors *in vivo* are exposed to conditions where oxygen and nutrient availability are restricted due to poor vascularisation, which may mimic the glucose deprivation conditions we used. Although these experimental setting most likely do not reflect the exact conditions to which spontaneously arisen cancers are exposed to *in vivo*, they may give us useful leads as to cellular stress responses involved under extreme conditions.

3.2 Respiration

3.2.1 The Oxygraph 2K

The Oxygraph-2K provides a high-resolution approach to the monitoring of cellular and mitochondrial respiratory function. Its sensitivity is especially important in monitoring low respiratory activities found in metabolically compromised cells or tissues. The quality of the experimental results is based on a good understanding of

the machine, where background flux corrections, as well as careful and meticulously cleaning of all the parts are done prior to each run. The background correction was performed to avoid false readings, as a high experimental background of oxygen would give us an overestimation of respiration data, and consequently incorrect respiratory coupling ratios. In addition, remnants of inhibitors or substrates used from previous runs could interfere with the next experiment if not cleaned properly. All in all, a single run (duplicates as it has two chambers) usually takes over 2 hours, of which we have to be present and monitor the oxygen flux. It was therefore of great importance to plan our primary cell experiment well (paper III), since these cells were could not be used for oxygen flux measurements the following day.

3.2.2 Intact or permeabilised cells

Most of the initially published respiratory data were done on mitochondrial fractions, as the sensors used then had low resolution and required allot of material. Consequently, much of the respiring data which has formed the basis of our oxidative phosphorylation understanding was done on liver mitochondria (more mitochondria in liver cells). Now we are able to measure respiration in intact cells or tissue and in very little material as such. Measurements in intact cells give information about the balanced metabolic mechanisms under viable conditions, whereas analysis of permeabilised cells and isolated mitochondria address specific processes of mitochondrial respiration. For those reasons the choice of respiration medium is important. For intact cells we used the corresponding cell culture medium (without FCS addition), and MIRO5 (Oroboros), which is a mitochondrial respiration medium, for the permeabilised cells. The use of culture medium gives us an accurate reading as to how the cells are respiring while grown in culture. The MIRO5 medium is made to simulate intracellular environment for the mitochondria, although without any substrates available. This is why we have to add the different substrates to this medium to run the TCA cycle (glutamate, malate and succinate) in addition to ADP, which is the substrate for the ATP Synthase to make ATP.

4 Summary of papers

4.1 Paper I

Cellular stress induced by resazurin leads to autophagy and cell death via production of reactive oxygen species and mitochondrial impairment.

Background

Resazurin (Alamar Blue) is widely used as a cell proliferation marker and is a redox reactive compound. Based on earlier findings that chemical reactions involving resazurin generates reactive oxygen species (ROS) (Prutz et al., 1996), we wanted to use resazurin as an oxidative stress inducer in different cancer cell lines and primary AML cells.

Results

We found that the HL-60 cell line was much more sensitive to resazurin treatment than the Jurkat cell line. Transmission electron microscopy confirmed cellular stress with autophagosomes in HL-60 after 48 hours of treatment, while signs of mitophagy were seen in both cell lines after 24 hours. Resazurin treatment caused a dramatic increase in ROS production, especially in the HL-60 cells. Although HL-60 cells were found to have higher routine respiratory rates than Jurkat cells, there was no correlation between the effects seen by resazurin treatment and oxygen consumption. The anti-proliferative effects were confirmed in native acute myelogenous leukemia (AML) blasts.

Conclusions

The results suggest that resazurin triggers cellular ROS production and initiates cell-specific stress responses such as mitochondrial dysfunction, reduced proliferation, autophagy and cell degradation.

4.2 Paper II

Modulation of mitochondrial energy metabolism leads to respiratory dysfunction and metabolic stress via cell specific pathways in leukemia cells.

Background

Mutations in specific pathways affecting cellular metabolism determines the metabolic flexibility of cancer cells. Here we used the two cell lines HL-60 and Jurkat, which had previously been found to have different metabolism to address the importance of mitochondrial respiration in glucose dependency and metabolic stress.

Results

In Jurkat cells, a low respiratory rate correlated with glucose dependence and Akt activation, whereas HL-60 cells had a higher tolerance for glucose deprivation. Of the metabolic modulators Jurkat were selectively sensitive to Palmitic acid, while HL-60 cells did not tolerate AICAR treatment. Glucose deprivation protected the mitochondrial respiration effects seen when treating cells with high glucose and modulating agent. Moreover, Jurkat cells selectively downregulated complex II activity upon glucose deprivation. However, HL-60 cells showed signs of autophagy after AICAR treatment when glucose was present, whereas during glucose deprivation also necrosis was identified (flow cytometry). Constitutive activation of the Akt pathway has previously been linked to glucose dependence, which correlated with expression levels seen in our Jurkat model. The introduction of myr-Akt in HL-60 cells did not change the metabolic preference or mitochondrial respiratory rates in these cells. The uncoupling protein 2 (UCP2) was found at higher expression levels in Jurkat cells than in HL-60 cells, additionally glucose deprivation led to a profound increase in UCP3 expression in Jurkat cells.

Conclusions

Mitochondrial respiration represents a vital determinant of metabolic flexibility, and respiratory dysfunction is a common effect of metabolic stress irrespective of glucose dependency and Akt activation.

4.3 Paper III

The pan-PPAR activator tetradecylthioacetic acid (TTA) increases mitochondrial respiration and hypertrophy in rat hepatocytes involving the mTOR/4E-BP1 pathway

Background

Mitochondria are crucial whole cell energy homeostasis both in normal cells as well as cancer cells. We wanted to investigate mechanisms of mitochondrial regulation in hepatocytes of rats treated with the PPAR-activator TTA, which is known to mediate potent induction of mitochondrial fatty acid oxidation. This represents a very flexible metabolic system where we aimed to identify new mechanisms of crosstalk between cell signalling and mitochondrial function.

Results

The TTA treated rats had lower cholesterol, induction of PPARα responsive genes in the liver and increased mitochondrial biogenesis. The mitochondria were functional as assessed by TCA cycle enzymes and oxidative phosphorylation. However a much higher LEAK rate was observed in TTA treated rats than in control. This could be connected to a profound increase in UCP3 gene expression. Furthermore, protein expression showed a marked upregulation of the mTORC1/4EBP1 pathway, in addition to upstream signalling proteins Akt and ERK1/2.

Conclusions

Hepatocytes are metabolically flexible and respond to TTA induced metabolic stress by upregulating catabolic and anabolic pathways resulting in cell hypertrophy. As the increased catabolism may exceed the cells energetic need, the oxidative phosphorylation system becomes less coupled (higher LEAK respiration). The cellular response seems to involve upregulation of stress response pathway ERK1/2 and the intracellular nutrient-sensitive mTOR pathway.

5 Discussion

5.1 Mitochondria in health and disease

The mitochondria are vital for cellular survival. Their compartmentalized structure makes an optimal environment for many bioenergetic and biosynthetic pathways crucial for cell energy homeostasis (Galluzzi et al., 2010). In addition many pro- and anti-apoptotic signalling pathways converge at the mitochondria, where mitochondrial proteins play an important part. Their diverse distribution and consequently pleiotropic roles in tissue metabolism, results in a multitude of mitochondrial linked diseases (Kim et al., 2008, Lesnefsky et al., 2001, Modica-Napolitano et al., 2007).

Mitochondrial ROS generation in response to cellular stress, may lead to advanced mutagenesis and promote tumorigenesis (Galluzzi et al., 2010). Damaged or dysfunctional mitochondria are normally cleared by the specialized autophagic response of mitophagy (Kim et al., 2007, Egan, 2011), however if the cell beholds additional mutations in cell survival pathways, this response might be obscured (Maiuri et al., 2010). Total cell ATP depletion leads to necrotic cell death, which is associated with high ROS levels (Amaravadi and Thompson, 2007, Hitomi et al., 2008). The necrotic cell death pathway is a non-reversible pathway as opposed to apoptosis, which is mediated through several signalling cascades and may up to a point be reversed (Kroemer et al., 1998). Together with autophagy, they give us three possible ways of inducing cell stress and death by challenging mitochondrial metabolism. It has therefore been important to find mechanisms, or conditions which impact the mitochondrial ROS production (paper I), causing increased cell stress (paper II and III) and consequently cell death (paper I and II).

5.2 Cellular stress reactions

The link between increased ROS and cancer is interesting, based on the fact that change in nutrient or oxygen availability can induce stress and lead to ROS, which may harm the normal metabolism and cause mutations in important cell cycle check points (Ralph et al., 2010, Sung et al., 2011). The exact mutations and consequent protein expression changes will determine whether the cell commits to a specific metabolism (i.e. glycolytic) or if it has the capability to make energy from different catabolic processes (i.e. mitochondria). This is important as highly glycolytic tumors have shown considerable treatment resistance (Hagland et al., 2007, Mailloux et al., 2010). Resazurin is a ROS inducing agent that specifically affected cancer cells with a less glycolytic phenotype (paper I), whereas metabolic substrate such as palmitic acid was a powerful cell stress inducer in glycolytic cells (paper II). TTA caused mitochondrial proliferation, higher oxygen flux and hypertrophic response in metabolically flexible primary cells (paper III). This response was associated with higher uncoupling effect, which would allow the liver to metabolize mitochondrial substrates and recycle NADH and $FADH_2$ irrespective of its own ATP synthesis needs.

During fasting the recycling of NADH to NAD^+ is a rate limiting step, which is partly aided by β-hydroxybuterate dehydrogenase recycling of NADH producing NAD^+ and β-hydroxybuterate in the liver (Salway, 2006). This is crucial as the brain need ketones and glucose made from gluconeogenesis at levels which may exceed the ATP requirement of the liver. Parallels can be drawn to the cancer cell energy homeostasis, where substrate turnover rate has to match the energy and biomolecule requirements of cell proliferation. If the ATP requirement is met, but the TCA flux generating precursors for cell biosynthesis is limited, the cell may respond by uncoupling the ETC to maintain a high turnover of NADH/$FADH_2$ to NAD^+/FAD (Ayyasamy et al., 2011, Mailloux et al., 2011, Robbins and Zhao, 2011, Samudio et al., 2008). Consequently, the limitation of the system would be the rate at which the

TCA cycle can produce its intermediary compounds (Ralph et al., 2010). The relative conversion of NADH to NAD^+ is also a determinant in the enzyme kinetics of the TCA cycle (Salway, 2006).

During prolonged starvation, high mitochondrial NADH/NAD^+ from fatty acid oxidation in the liver, turns oxaloacetate to malate, which is subsequently exported out of the mitochondria and used for gluconeogenesis during fasting (Owen et al., 1998). This halts the conversion of lipid derived acetyl-CoA to citrate, where it instead is converted to ketone bodies through HMGCoA synthase and β-hydroxybuterate dehydrogenase as described above. However, in the presence of glucose and continued fatty acid oxidation, which results in high NADH levels inside the mitochondria, the uncoupling proteins may come in to play (paper III). The high NADH build up has previously been linked to increased ROS (Ralph et al., 2010), and ROS induction may activate the uncoupling proteins as an antioxidant defence mechanism (Echtay et al., 2002, Hurtaud et al., 2006, Mailloux and Harper, 2011).

5.3 Increased ROS may lead to cell death

In paper I we found that resazurin caused high levels of ROS in HL-60 cells compared to the Jurkat cell line. This response was connected to induction of autophagy in HL-60 cells after 48 hours, whereas both cell lines showed signs of mitophagy after 24 hours of treatment (paper I, figure 2 B-C). It would therefore seem that both cell lines were able to initiate mitophagy, whereas only HL-60 cells invoked additional autophagy upon resazurin treatment. The HL-60 cells derive from a different type of leukemia (acute myeloid leukemia, AML), and have a higher respiratory rate but lower routine control ratio (Routine/Maximal or Routine/ETS, paper I and paper II, respectively) than Jurkat cells (paper I, figure 4C). The lower the ratio, the higher the membrane potential, which has been linked to increased ROS production (Mailloux and Harper, 2011). Furthermore, the HL-60 cells are more reliant on oxidative respiration, which subsequently make them susceptible to

treatments obscuring their respiratory function (Netto et al., 2009). Jurkat cells had a lower respiratory rate than HL-60 cells, but utilized as much as 70% of their respiratory capacity under normal conditions (paper I, figure 4C). They had a higher resazurin to resorufin conversion rate but showed less ROS production upon treatment. The high routine control ratio suggests a low membrane potential, which correlates with reduced ROS production. However, there seemed to be fewer mitochondria per Jurkat cell compared to HL-60 (TEM pictures in paper I and II), which would affect the ROS level as well as routine respiration data, but not coupling efficiency (paper I and II).

Jurkat cells have a mutation in the tumor suppressor PTEN, which consequently leads to increased Akt activation (Freeley et al., 2007, Shan et al., 2000). Activation of the Akt pathway is a commonly found mutation in cancer cells, and is related to increased glucose dependence (Elstrom et al., 2004, Lawlor and Alessi, 2001). We found that Jurkat cells were more sensitive to glucose deprivation than HL-60 cells (paper II), which correlates with glucose dependence. However, high glycolytic flux does not support cell proliferation by itself and must involve some of the biochemical pathways in the mitochondria for precursors for lipid, amino acid, and nucleotide synthesis (Matés et al., 2009, Wise and Thompson, 2010, Erickson and Cerione, 2010).

5.4 The role of TCA and UCP in cancer growth

Increased import of glutamine has been identified in many cancers, connected to maintaining a high TCA flux by providing the substrate α-KG (Erickson and Cerione, 2010, Matés et al., 2009, Wise and Thompson, 2010). In the mitochondria glutamine is converted to glutamate through glutaminase, an enzyme found upregulated in cancer (Erickson and Cerione, 2010), and subsequently α-ketoacid transaminase turns glutamate to α-KG, which feeds into the TCA cycle.

We found that Jurkat cells seem to utilize more of their complex II capacity during glucose rich conditions, whereas glucose deprivation showed a specific drop in complex II activity (paper II). If pyruvate derived from glycolysis was feeding into the TCA cycle of Jurkat cells during glucose rich conditions this may together with glutamine, or on its own provide enough substrates for running the TCA cycle in the normal direction, thus fuelling complex II with electrons. However, during glucose deprived conditions the lack of pyruvate, may force the cells to rely on glutamine as their TCA cycle substrate, and thus reverse the cycle generating citrate directly from reductive carboxylation of α-KG (Wise et al., 2011), circumventing complex II (paper II). Indeed, this situations may mimic what was seen under normoxic and hypoxic conditions, where citrate was made from pyruvate during normoxia, but during hypoxia glutamine was found to be the precursor by reductive carboxylation (Metallo et al., 2012). Consequently, the reverse TCA cycle may be connected to increased ROS, which is a well-known phenomenon during hypoxia (Ralph et al., 2010). This connection would further support the observed effect during anaerobic glycolysis, where pyruvate is not transported to the mitochondria for oxidation, but converted to lactate and secreted.

Glutamine may also regulate translation of UCP2 (Hurtaud et al., 2007), which could function as a feed forward reaction to speed up the TCA cycle when there is no substrate limitations. We found that UCP2 levels dropped upon glucose deprivation (paper II), maybe related to the reversal of the TCA cycle. Interestingly the expression of UCP3 was significantly increased during glucose deprivation in Jurkat cells, which could be a response to increased ROS as UCP3 has been shown to regulate ROS levels (Mailloux and Harper, 2011). However this combination with glucose deprivation and upregulation of UCP3, could lead to ATP depletion, thus activating the AMP sensitive AMPK (paper II). A similar response was observed in the hepatocytes from TTA-treated rats, which was connected to increased LEAK activity (paper III). Although HL-60 cells showed a modest upregulation in UCP3

transcription in response to glucose deprivation, these cells presented higher endogenous levels of the mitochondrial antioxidant SOD2 (paper II).

5.5 Metabolic reprogramming support cell growth

The fact that highly proliferating lymphocytes are glucose dependent may not be surprising as this was identified and even connected to increased glutaminolysis in the early eighties (Newsholme et al., 1985). The rationale was that lymphocytes need to be able to proliferate to great numbers upon activation, and as such would need substrates to sustain this proliferation, which involved glucose and glutamine utilization (Newsholme et al., 1985). In support of this observation, two independent groups recently published that quiescent human primary T and B lymphocytes use fatty acid oxidation (FAO) to maintain energy homeostasis, whereas upon activation they switch to glycolysis and glutaminolysis (Le et al., 2012, Wang et al., 2011). Consequently the high glucose and glutamine turnover in resting lymphocytes were converted to glutamate, aspartate, lactate and ammonia and hardly contributed to energy generation (Newsholme et al., 1985). Thus the excess glutamine utilized was not based on energy requirements of the cell, but rather served as a metabolic primer for rapid cell growth in response to signals initiating cell proliferation.

In our Jurkat model Akt was constitutively phosphorylated at its active site (paper II), which is due to the mutation in PTEN (Freeley et al., 2007). Moreover the cells were highly susceptible to palmitic acid treatment which caused cell death (paper II). The activation of the Akt pathway in leukemia cells have previously been reported to inhibit the carnitine palmitoyltransferase 1A (CPT1A) found in the outer mitochondrial membrane and involved in fatty acid β-oxidation (Deberardinis et al., 2006). Thus, inability to import and utilize palmitic acid may explain why the cells did not tolerate this treatment. This would further imply that normal T lymphocytes that have been activated by an extracellular agent may be susceptible to fatty acid treatment. Extrapolation of this to our cancer cell models, suggests that there are two

modes of action that are specifically required for tumorigenesis, a theory which is also supported by others (Jose et al., 2011, Zoncu et al., 2011). One is the activation of an autonomous cell growth signal through mutations in growth receptors or their downstream pathways, and two is the mutation in genes involved in increased metabolic turnover examples being Akt for glycolysis, and c-Myc for glutaminolysis.

Stress and growth-associated crosstalk between metabolism and signaling are further elucidated in our hepatocyte model (paper III). Here, we accelerated metabolic flux by treatment of a fatty acid which could not be oxidized. Our data strongly suggest that cell stress responses are triggered by this modified fatty acid (paper III), and similar observations have recently been identified in cultured cancer cells (Lundemo et al., 2011). In our studies, these stress responses included increased mitochondrial biogenesis, oxidative capacity and increased substrate utilization. This was further connected to upregulation of the mTORC1/4EB-P1 pathway (paper III). The mTORC1 upregulation has been implicated in cancer progression (Gibbons et al., 2009, Zoncu et al., 2011), but may not be the instigator of tumorigenesis as we only found hypertrophic cells (paper III). All in all, it seems that the metabolic reprogramming in hepatocytes is well tolerated as these cells are highly metabolically flexible. However in less flexible cells, it may be hypothesized that this metabolic reprogramming might act as a priming condition, which together with growth receptor activation could lead to rapid cell proliferation. This further suggests that an activating mutation, in pathways causing metabolic reprogramming, would in combination with an activating mutation in a growth receptor, possibly lead to uncontrolled cell growth.

In this perspective, recent publications show that cells in nutrient rich environments (glucose + glutamine), increase their glycolytic flux, which generates UDP-N-acetylglucoseamine (UDP-GlcNacs), produced in the hexoamine pathway (Wellen et al., 2010). Moreover, the GlcNAcs cause N-glycosylation of tyrosine kinase receptors which has been connected to cellular transformation (Vander Heiden et al.,

2012, Wellen and Thompson, 2010). The hexoamine pathway diverges from glycolysis at fructose-6-phosphate, one of the irreversible steps in glycolysis, and is only active when substrates such as glutamine and acetyl-CoA are also present in excess (Wellen and Thompson, 2010). Additionally, high levels of acetyl-CoA, from increased ATP-citrate lyase (ACL) activity, can cause histone acetylation leading to enhanced transcription in nutrient rich environment (Wellen et al., 2009). Therefore, the true metabolic reprogramming caused by mutations, would render cells unable to cope with nutrient deprivation or metabolic agents interfering with their preferred metabolism (Jurkat + palmitic acid, paper II). However, cancer cells that maintains metabolic flexibility and thus are able to utilize the full spectra of metabolic substrates could prove to be more resistant to such treatments (HL-60 cells + palmitic acid, paper II). This underscores the possible potential of proper metabolic profiling of cancers before commencing treatment.

5.6 Lifestyle and Cancer

The increasing evidence linking overfeeding, obesity and diabetes 2 with cancer clearly shows that we are not *preventing* cancer with our high-carbohydrate, high-fat western-type diet (Calle and Kaaks, 2004). Hyperglycemia and insulin insensitivity are traits of diabetes 2, and interestingly of cancer cells themselves (Elstrom et al., 2004, Sung et al., 2011, Yamada et al., 2005). Constant nutrient availability, which exceeds the energy requirements of the cell result in increased cell stress and consequently aberrant signalling. The work done on glycosylations (GlcNAcs) and acetylations (acetyl-CoA) all confirm that both unicellular and multicellular organisms use metabolite-mediated posttranslational modifications to match nutrient abundance throughout the metabolic network (DeBerardinis and Thompson, 2012). Since, GlcNAcs are mediators of growth factor receptors, they may lead to a growth advantage of a cell just by increasing the number of growth receptors on the cell surface (Wellen et al., 2009, Wellen and Thompson, 2010). The constant over-

exposure to nutrients, may lead to cellular metabolic adaptations which provide the nestling ground for tumorigenesis. Conversely, the opposite might be true where caloric restriction inhibits tumorigenesis. Indeed, there are numerous studies supporting this notion, however they seem to converge on the insulin growth factor (IGF-1) responsive tumors, whereas cancers with mutations downstream of this receptor do not respond to calorie restriction (Speakman and Mitchell, 2011).

Another emerging target for caloric restriction is brain tumors (Maurer et al., 2011, Zhou et al., 2007). This is based on basic physiology, which show that during prolonged starvation the ketones are the primary fuel (>70%) for brain cells (Cahill, 2006) and can only be utilised as such by functional mitochondria. Thus their metabolic flexibility must be intact to survive pro-longed starvation. The commonly found mutation in IDH1/2 in various gliomas have been linked to increased dependence of glutamine and glucose at the expense of functioning TCA cycle (Reitman et al., 2011). This suggest that these cancers are not metabolically flexible, and that the use of ketogenic diet as treatment may be a promising alternative or supplementary treatment to the much more invasive tumor resection treatment offered the last fifty years (Maurer et al., 2011, Scheck et al., Zhou et al., 2007, Zuccoli et al., 2010).

6 Conclusions and Future Perspectives

The retained capacity of metabolic flexibility, seen in some cancers, may render them more resistant to substrate deprivation, albeit more sensitive to treatments inducing ROS. This was identified in the paper I where resazurin caused metabolic stress predominantly in the metabolically flexible HL-60 cells, which involved responses such as increased ROS and autophagy. Furthermore the glucose dependency seen in many cancers could be exploited by treating with a substrate that would interfere with this preferred metabolism. This was identified in paper II, where Jurkat cells were more glucose dependent, and more susceptible to treatment with the fatty acid palmitic acid. The HL-60 cells on the other hand showed high sensitivity to AICAR treatment, which was further augmented when combined with glucose deprivation. These responses seemed to rely on the signalling pathways that were upregulated, as constitutive Akt signalling was found in Jurkat, while HL-60 cells did not have this activating mutation. The ability of HL-60 to induce or inhibit the mTOR pathway seemed to play a role in the autophagy response seen both in paper I and II. High cellular metabolic flexibility, which is not constrained by activating or deactivating mutations, may trigger many of the same pathways when exposed to a metabolic stress inducer (TTA). This was confirmed in paper III where highly metabolically flexible primary rat hepatocytes showed enhanced mTOR activity upon increased metabolic stress, which involved mitochondrial biogenesis, increased oxidative capacity and higher uncoupled respiration. Interestingly, the treatment led to cell hypertrophy, but we found no indications of hyperplasia (neoplasia in the liver).

In conclusion, increased functional load, either by nutrient excess (i.e. high glucose and glutamine) or specific metabolic modulators (palmitic acid, AICAR, TTA), can cause cellular stress reactions that threaten cell survival if the limits of metabolic flexibility are challenged. In cancer, metabolic rigidity involving mitochondrial alterations seem to be promoted by common growth inducing oncogenic mutations. In contrast, metabolically flexible hepatocytes not having dysregulated growth

demonstrated high adaptability and tolerance to stressful conditions, in a response also involving hypertrophy. In agreement with recent literature, this study underscores the importance of metabolic rigidity and associated stress mechanisms in cancer biology, and further emphasizes the potential of utilizing associated treatment strategies in future cancer therapy.

To pursue these findings further, it would be interesting to co-treat rats with both TTA and mTORC1 inhibitors, to see if this led to a different cell response than hypertrophy. In addition, it would be interesting to treat rats with TTA before subsequently giving them a TTA-free normal chow diet, to see whether the mitochondrial proliferation response and hypertrophy were reversed. If this was reversed, the exact reversal time would have to be investigated. This may be important as clinical trial treatments with mTORC1 inhibitors against cancer have shown to induce hyperlipidemia, high cholesterol, hypertension and generally symptoms associated with obesity (Reardon et al., 2006, Riely et al., 2007). Thus we could postulate that a pre-treatment with TTA may lead to a hypolipidemic response that could protect the patients during mTORC1 inhibitory treatment.

Altogether it seems that the ability of cancer cells to modulate their metabolism depends on the flexibility of their signalling pathways. The therapeutic window for targeting cancer metabolism does, therefore, rely on their inflexibility in nutrient utilisation, which can be distinguished from normal healthy cells. There are several considerations to be made if one is to actively pursue using inhibitors of cancer cell metabolism *in vivo*. For instance inhibitors of the glutamine receptor SLC1A5, which is upregulated in many cancers (Wise and Thompson, 2010) results in deactivation of mTOR *in vitro* (Nicklin et al., 2009). As mentioned above, the use of such inhibitors *in vivo* may lead to metabolic effects that are not desirable. Another consideration to be made if using the glutamine receptor inhibitor, is that the drug would have to be constructed as to not cross the blood-brain barrier, as glutamine turnover is essential for neuronal signalling (Yudkoff et al., 2008). Furthermore, it may disturb blood

ammonia homeostasis, as well as gluconeogenesis during fasting, which both involve glutamine. Although, the glutamine addiction, seen in many cancers but not all, provides an exciting new therapeutic target, the patient's metabolic state has to be closely monitored during such treatment.

Great advances have been made the last years in trying to assess the metabolic profiling of tumors *in vivo*, both with radiolabelling glutamine and glutamate for the use in PET (Qu et al., 2012, Koglin et al., 2011), as well as advances in higher-field magnetic resonance spectroscopy (MRS) which has drastically improved and made it possible to detect many metabolites *in vivo* (Choi et al., 2012). Understanding the metabolic phenotype of cancer cells and cell signalling involvement, not only gives us a better understanding of tumour biology, but may present a wide range of potential targets for advanced cancer therapies. More studies involving diet manipulations with regards to cancer growth are called for, and can hopefully lead to a more effective combinational treatment together with today's standard care.

References

AGOSTINIS, P., BUYTAERT, E., BREYSSENS, H. & HENDRICKX, N. 2004. Regulatory pathways in photodynamic therapy induced apoptosis. *Photochemical & photobiological sciences : Official journal of the European Photochemistry Association and the European Society for Photobiology,* 3**,** 721-9.

ALESSI, D. R., JAMES, S. R., DOWNES, C. P., HOLMES, A. B., GAFFNEY, P. R., REESE, C. B. & COHEN, P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current biology : CB,* 7**,** 261-9.

AMARAVADI, R. K. & THOMPSON, C. B. 2007. The roles of therapy-induced autophagy and necrosis in cancer treatment. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 13**,** 7271-9.

ANTICO ARCIUCH, V. G., RUSSO, M. A., DIMA, M., KANG, K. S., DASRATH, F., LIAO, X. H., REFETOFF, S., MONTAGNA, C. & DI CRISTOFANO, A. 2011. Thyrocyte-specific inactivation of p53 and Pten results in anaplastic thyroid carcinomas faithfully recapitulating human tumors. *Oncotarget,* 2**,** 1109-26.

AYYASAMY, V., OWENS, K. M., DESOUKI, M. M., LIANG, P., BAKIN, A., THANGARAJ, K., BUCHSBAUM, D. J., LOBUGLIO, A. F. & SINGH, K. K. 2011. Cellular model of Warburg effect identifies tumor promoting function of UCP2 in breast cancer and its suppression by genipin. *PloS one,* 6**,** e24792.

BELENKY, P., BOGAN, K. L. & BRENNER, C. 2007. NAD+ metabolism in health and disease. *Trends in biochemical sciences,* 32**,** 12-19.

BERGAN, H. E., KITTILSON, J. D. & SHERIDAN, M. A. 2011. Nutrition-regulated lipolysis in rainbow trout (Oncorhynchus mykiss) is associated with alterations in the ERK, PI3K-Akt, JAK-STAT, and PKC signaling pathways. *General and comparative endocrinology*.

BIRNBAUM, M. J. 1989. Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell,* 57**,** 305-15.

BOST, F. 2005. The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. *Diabetes,* 54**,** 402-411.

BROWN, K., HELLER, D. S., ZAMUDIO, S. & ILLSLEY, N. P. 2011. Glucose transporter 3 (GLUT3) protein expression in human placenta across gestation. *Placenta,* 32**,** 1041-9.

CAHILL, G. F., JR. 2006. Fuel metabolism in starvation. *Annual review of nutrition,* 26**,** 1-22.

CALLE, E. E. & KAAKS, R. 2004. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. *Nat Rev Cancer,* 4**,** 579-591.

CAMPBELL, N. E., KELLENBERGER, L., GREENAWAY, J., MOOREHEAD, R. A., LINNERTH-PETRIK, N. M. & PETRIK, J. 2010. Extracellular matrix proteins and tumor angiogenesis. *Journal of oncology,* 2010**,** 586905.

CANNON, B. & NEDERGAARD, J. 1985. The biochemistry of an inefficient tissue: brown adipose tissue. *Essays in biochemistry,* 20**,** 110-64.

CATTLEY, R. C., DELUCA, J., ELCOMBE, C., FENNER-CRISP, P., LAKE, B. G., MARSMAN, D. S., PASTOOR, T. A., POPP, J. A., ROBINSON, D. E., SCHWETZ, B., TUGWOOD, J. & WAHLI, W. 1998. Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? *Regul Toxicol Pharmacol,* 27**,** 47-60.

CHO, H. 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB[beta]). *Science,* 292**,** 1728-1731.

CHO, H., THORVALDSEN, J. L., CHU, Q., FENG, F. & BIRNBAUM, M. J. 2001. Akt1/PKB[alpha] is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J. Biol. Chem.,* 276**,** 38349-38352.

CHO, Y. S., CHALLA, S., MOQUIN, D., GENGA, R., RAY, T. D., GUILDFORD, M. & CHAN, F. K. 2009. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell,* 137**,** 1112-23.

CHOI, C., GANJI, S. K., DEBERARDINIS, R. J., HATANPAA, K. J., RAKHEJA, D., KOVACS, Z., YANG, X. L., MASHIMO, T., RAISANEN, J. M., MARIN-VALENCIA, I., PASCUAL, J. M., MADDEN, C. J., MICKEY, B. E., MALLOY, C. R., BACHOO, R. M. & MAHER, E. A. 2012. 2-hydroxyglutarate detection by magnetic resonance spectroscopy in IDH-mutated patients with gliomas. *Nature medicine,* 18**,** 624-9.

CHRISTOFK, H. R., VANDER HEIDEN, M. G., HARRIS, M. H., RAMANATHAN, A., GERSZTEN, R. E., WEI, R., FLEMING, M. D., SCHREIBER, S. L. & CANTLEY, L. C. 2008. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature,* 452**,** 230-3.

COHEN, P., ALESSI, D. R. & CROSS, D. A. 1997. PDK1, one of the missing links in insulin signal transduction? *FEBS letters,* 410**,** 3-10.

DA-SILVA, W. S., GOMEZ-PUYOU, A., DE GOMEZ-PUYOU, M. T., MORENO-SANCHEZ, R., DE FELICE, F. G., DE MEIS, L., OLIVEIRA, M. F. & GALINA, A. 2004. Mitochondrial bound hexokinase activity as a preventive antioxidant defense: steady-state ADP formation as a regulatory mechanism of membrane potential and reactive oxygen species generation in mitochondria. *The Journal of biological chemistry,* 279**,** 39846-55.

DANG, C. V. & SEMENZA, G. L. 1999. Oncogenic alterations of metabolism. *Trends in biochemical sciences,* 24**,** 68-72.

DEBERARDINIS, R. J., LUM, J. J., HATZIVASSILIOU, G. & THOMPSON, C. B. 2008. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell metabolism,* 7**,** 11-20.

DEBERARDINIS, R. J., LUM, J. J. & THOMPSON, C. B. 2006. Phosphatidylinositol 3-kinase-dependent modulation of carnitine palmitoyltransferase 1A expression regulates lipid metabolism during hematopoietic cell growth. *The Journal of biological chemistry,* 281**,** 37372-80.

DEBERARDINIS, R. J. & THOMPSON, C. B. 2012. Cellular metabolism and disease: what do metabolic outliers teach us? *Cell,* 148**,** 1132-44.

DERDAK, Z., MARK, N. M., BELDI, G., ROBSON, S. C., WANDS, J. R. & BAFFY, G. 2008. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. *Cancer research,* 68**,** 2813-9.

DIAZ VIVANCOS, P., WOLFF, T., MARKOVIC, J., PALLARDO, F. V. & FOYER, C. H. 2010. A nuclear glutathione cycle within the cell cycle. *The Biochemical journal,* 431**,** 169-78.

DISTEFANO, G., BOCA, M., ROWE, I., WODARCZYK, C., MA, L., PIONTEK, K. B., GERMINO, G. G., PANDOLFI, P. P. & BOLETTA, A. 2009. Polycystin-1 regulates extracellular signal-regulated kinase-dependent phosphorylation of tuberin to control cell size through mTOR and its downstream effectors S6K and 4EBP1. *Molecular and cellular biology,* 29**,** 2359-71.

ECHTAY, K. S., ROUSSEL, D., ST-PIERRE, J., JEKABSONS, M. B., CADENAS, S., STUART, J. A., HARPER, J. A., ROEBUCK, S. J., MORRISON, A., PICKERING, S., CLAPHAM, J. C. & BRAND, M. D. 2002. Superoxide activates mitochondrial uncoupling proteins. *Nature,* 415**,** 96-9.

EGAN, D. F. 2011. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. *Science,* 331**,** 456-461.

ELSTROM, R. L., BAUER, D. E., BUZZAI, M., KARNAUSKAS, R., HARRIS, M. H., PLAS, D. R., ZHUANG, H., CINALLI, R. M., ALAVI, A., RUDIN, C. M. & THOMPSON, C. B. 2004. Akt stimulates aerobic glycolysis in cancer cells. *Cancer research,* 64**,** 3892-9.

ERICKSON, J. W. & CERIONE, R. A. 2010. Glutaminase: a hot spot for regulation of cancer cell metabolism? *Oncotarget,* 1**,** 734-40.

ERIKSTEIN, B. S., HAGLAND, H. R., NIKOLAISEN, J., KULAWIEC, M., SINGH, K. K., GJERTSEN, B. T. & TRONSTAD, K. J. 2010. Cellular stress induced by resazurin leads to autophagy and cell death via production of reactive oxygen species and mitochondrial impairment. *Journal of cellular biochemistry,* 111**,** 574-84.

ESCHER, P. & WAHLI, W. 2000. Peroxisome proliferator-activated receptors: insight into multiple cellular functions. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 448**,** 121-138.

FORETZ, M. 2010. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J. Clin. Invest.,* 120**,** 2355-2369.

FREELEY, M., PARK, J., YANG, K.-J., WANGE, R. L., VOLKOV, Y., KELLEHER, D. & LONG, A. 2007. Loss of PTEN expression does not

contribute to PDK-1 activity and PKC activation-loop phosphorylation in Jurkat leukaemic T cells. *Cellular Signalling,* 19**,** 2444-2457.

FREZZA, C., ZHENG, L., TENNANT, D. A., PAPKOVSKY, D. B., HEDLEY, B. A., KALNA, G., WATSON, D. G. & GOTTLIEB, E. 2011. Metabolic profiling of hypoxic cells revealed a catabolic signature required for cell survival. *PloS one,* 6**,** e24411.

GALLAGHER, E. J. & LEROITH, D. 2010. The proliferating role of insulin and insulin-like growth factors in cancer. *Trends in endocrinology and metabolism: TEM,* 21**,** 610-8.

GALLUZZI, L. & KROEMER, G. 2008. Necroptosis: a specialized pathway of programmed necrosis. *Cell,* 135**,** 1161-3.

GALLUZZI, L. & KROEMER, G. 2009. Shigella targets the mitochondrial checkpoint of programmed necrosis. *Cell host & microbe,* 5**,** 107-9.

GALLUZZI, L., MORSELLI, E., KEPP, O., VITALE, I., RIGONI, A., VACCHELLI, E., MICHAUD, M., ZISCHKA, H., CASTEDO, M. & KROEMER, G. 2010. Mitochondrial gateways to cancer. *Molecular Aspects of Medicine,* 31**,** 1-20.

GIBBONS, J. J., ABRAHAM, R. T. & YU, K. 2009. Mammalian target of rapamycin: discovery of rapamycin reveals a signaling pathway important for normal and cancer cell growth. *Seminars in oncology,* 36 Suppl 3**,** S3-S17.

GOEL, A., MATHUPALA, S. P. & PEDERSEN, P. L. 2003. Glucose metabolism in cancer. Evidence that demethylation events play a role in activating type II hexokinase gene expression. *The Journal of biological chemistry,* 278**,** 15333-40.

GOTTLICHER, M., DEMOZ, A., SVENSSON, D., TOLLET, P., BERGE, R. K. & GUSTAFSSON, J. A. 1993. Structural and metabolic requirements for activators of the peroxisome proliferator-activated receptor. *Biochemical pharmacology,* 46**,** 2177-84.

HABER, R. S., WEINSTEIN, S. P., O'BOYLE, E. & MORGELLO, S. 1993. Tissue distribution of the human GLUT3 glucose transporter. *Endocrinology,* 132**,** 2538-43.

HAGA, S., OZAKI, M., INOUE, H., OKAMOTO, Y., OGAWA, W., TAKEDA, K., AKIRA, S. & TODO, S. 2009. The survival pathways phosphatidylinositol-3 kinase (PI3-K)/phosphoinositide-dependent protein kinase 1 (PDK1)/Akt modulate liver regeneration through hepatocyte size rather than proliferation. *Hepatology,* 49**,** 204-14.

HAGLAND, H., NIKOLAISEN, J., HODNELAND, L. I., GJERTSEN, B. T., BRUSERUD, O. & TRONSTAD, K. J. 2007. Targeting mitochondria in the treatment of human cancer: a coordinated attack against cancer cell energy metabolism and signalling. *Expert opinion on therapeutic targets,* 11**,** 1055-69.

HALLIWELL, B. 2012. Free radicals and antioxidants: updating a personal view. *Nutrition reviews,* 70**,** 257-65.

HANAHAN, D. & WEINBERG, R. A. 2000. The hallmarks of cancer. *Cell,* 100**,** 57-70.

HANAHAN, D. & WEINBERG, ROBERT A. 2011. Hallmarks of Cancer: The Next Generation. *Cell,* 144**,** 646-674.

HARDIE, D. G., ROSS, F. A. & HAWLEY, S. A. 2012. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature reviews. Molecular cell biology,* 13**,** 251-262.

HENZE, K. & MARTIN, W. 2003a. Evolutionary biology: essence of mitochondria. *Nature,* 426**,** 127-8.

HENZE, K. & MARTIN, W. 2003b. Evolutionary biology: Essence of mitochondria. *Nature,* 426**,** 127-128.

HILDYARD, J. C. & HALESTRAP, A. P. 2003. Identification of the mitochondrial pyruvate carrier in Saccharomyces cerevisiae. *The Biochemical journal,* 374**,** 607-11.

HIRASHIMA, Y. 2003. Insulin down-regulates insulin receptor substrate-2 expression through the phosphatidylinositol 3-kinase/Akt pathway. *J. Endocrinol.,* 179**,** 253-266.

HITOMI, J., CHRISTOFFERSON, D. E., NG, A., YAO, J., DEGTEREV, A., XAVIER, R. J. & YUAN, J. 2008. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell,* 135**,** 1311-23.

HOWELL, J. J. & MANNING, B. D. 2011. mTOR couples cellular nutrient sensing to organismal metabolic homeostasis. *Trends in endocrinology and metabolism: TEM,* 22**,** 94-102.

HSIEH, A. C., LIU, Y., EDLIND, M. P., INGOLIA, N. T., JANES, M. R., SHER, A., SHI, E. Y., STUMPF, C. R., CHRISTENSEN, C., BONHAM, M. J., WANG, S., REN, P., MARTIN, M., JESSEN, K., FELDMAN, M. E., WEISSMAN, J. S., SHOKAT, K. M., ROMMEL, C. & RUGGERO, D. 2012. The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature,* 485**,** 55-61.

HUANG, J., JIA, Y., FU, T., VISWAKARMA, N., BAI, L., RAO, M. S., ZHU, Y., BORENSZTAJN, J. & REDDY, J. K. 2011. Sustained activation of PPARα by endogenous ligands increases hepatic fatty acid oxidation and prevents obesity in ob/ob mice. *The FASEB Journal*.

HURTAUD, C., GELLY, C., BOUILLAUD, F. & LEVI-MEYRUEIS, C. 2006. Translation control of UCP2 synthesis by the upstream open reading frame. *Cellular and molecular life sciences : CMLS,* 63**,** 1780-9.

HURTAUD, C., GELLY, C., CHEN, Z., LEVI-MEYRUEIS, C. & BOUILLAUD, F. 2007. Glutamine stimulates translation of uncoupling protein 2mRNA. *Cellular and molecular life sciences : CMLS,* 64**,** 1853-60.

JABOIN, J. J., HWANG, M. & LU, B. 2009. Autophagy in lung cancer. *Methods in enzymology,* 453**,** 287-304.

JACOBSON, M. D., WEIL, M. & RAFF, M. C. 1997. Programmed cell death in animal development. *Cell,* 88**,** 347-54.

JIANG, Z. Y. 2003. Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc. Natl Acad. Sci. USA,* 100**,** 7569-7574.

JING, K., SONG, K. S., SHIN, S., KIM, N., JEONG, S., OH, H. R., PARK, J. H., SEO, K. S., HEO, J. Y., HAN, J., PARK, J. I., HAN, C., WU, T., KWEON, G. R., PARK, S. K., YOON, W. H., HWANG, B. D. & LIM, K. 2011. Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53. *Autophagy,* 7**,** 1348-58.

JOOST, H. G., BELL, G. I., BEST, J. D., BIRNBAUM, M. J., CHARRON, M. J., CHEN, Y. T., DOEGE, H., JAMES, D. E., LODISH, H. F., MOLEY, K. H., MOLEY, J. F., MUECKLER, M., ROGERS, S., SCHURMANN, A., SEINO, S. & THORENS, B. 2002. Nomenclature of the GLUT/SLC2A family of sugar/polyol transport facilitators. *American journal of physiology. Endocrinology and metabolism,* 282**,** E974-6.

JOOST, H. G. & THORENS, B. 2001. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). *Molecular membrane biology,* 18**,** 247-56.

JOSE, C., BELLANCE, N. & ROSSIGNOL, R. 2011. Choosing between glycolysis and oxidative phosphorylation: a tumor's dilemma? *Biochimica et biophysica acta,* 1807**,** 552-61.

KIM, H. & CHEN, J. 2007. c-Myc interacts with TRF1/PIN2 and regulates telomere length. *Biochemical and Biophysical Research Communications,* 362**,** 842-847.

KIM, I., RODRIGUEZ-ENRIQUEZ, S. & LEMASTERS, J. J. 2007. Selective degradation of mitochondria by mitophagy. *Archives of biochemistry and biophysics,* 462**,** 245-53.

KIM, J. A., WEI, Y. & SOWERS, J. R. 2008. Role of mitochondrial dysfunction in insulin resistance. *Circulation research,* 102**,** 401-14.

KING, A., SELAK, M. A. & GOTTLIEB, E. 2006. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene,* 25**,** 4675-4682.

KOGLIN, N., MUELLER, A., BERNDT, M., SCHMITT-WILLICH, H., TOSCHI, L., STEPHENS, A. W., GEKELER, V., FRIEBE, M. & DINKELBORG, L. M. 2011. Specific PET imaging of xC- transporter activity using a (1)(8)F-labeled glutamate derivative reveals a dominant pathway in tumor metabolism. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 17**,** 6000-11.

KROEMER, G., DALLAPORTA, B. & RESCHE-RIGON, M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis. *Annual review of physiology,* 60**,** 619-42.

KROEMER, G., GALLUZZI, L. & BRENNER, C. 2007. Mitochondrial membrane permeabilization in cell death. *Physiological reviews,* 87**,** 99-163.

KUKIDOME, D., NISHIKAWA, T., SONODA, K., IMOTO, K., FUJISAWA, K., YANO, M., MOTOSHIMA, H., TAGUCHI, T., MATSUMURA, T. & ARAKI, E. 2006. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes,* 55**,** 120-7.

KUMAR, V., ABBAS, A.K., FAUSTO, N., MITCHELL, R.N. 2007. *Robbins Basic Pathology, 8th Edition,* Philadelphia, USA, Saunders Elsevier.

LANGFORT, J., VIESE, M., PLOUG, T. & DELA, F. 2003. Time course of GLUT4 and AMPK protein expression in human skeletal muscle during one month of physical training. *Scandinavian journal of medicine & science in sports,* 13**,** 169-74.

LAWLOR, M. A. & ALESSI, D. R. 2001. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *Journal of cell science,* 114**,** 2903-10.

LE, A., LANE, A. N., HAMAKER, M., BOSE, S., GOUW, A., BARBI, J., TSUKAMOTO, T., ROJAS, C. J., SLUSHER, B. S., ZHANG, H., ZIMMERMAN, L. J., LIEBLER, D. C., SLEBOS, R. J., LORKIEWICZ, P. K., HIGASHI, R. M., FAN, T. W. & DANG, C. V. 2012. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell metabolism,* 15**,** 110-21.

LE BACQUER, O., PETROULAKIS, E., PAGLIALUNGA, S., POULIN, F., RICHARD, D., CIANFLONE, K. & SONENBERG, N. 2007. Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. *The Journal of clinical investigation,* 117**,** 387-96.

LESNEFSKY, E. J., MOGHADDAS, S., TANDLER, B., KERNER, J. & HOPPEL, C. L. 2001. Mitochondrial dysfunction in cardiac disease: ischemia--reperfusion, aging, and heart failure. *Journal of molecular and cellular cardiology,* 33**,** 1065-89.

LEVINE, A. J. 1997. p53, the cellular gatekeeper for growth and division. *Cell,* 88**,** 323-31.

LIRA, V. A., SOLTOW, Q. A., LONG, J. H., BETTERS, J. L., SELLMAN, J. E. & CRISWELL, D. S. 2007. Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle. *American journal of physiology. Endocrinology and metabolism,* 293**,** E1062-8.

LUCEY, B. P., NELSON-REES, W. A. & HUTCHINS, G. M. 2009. Henrietta Lacks, HeLa cells, and cell culture contamination. *Archives of pathology & laboratory medicine,* 133**,** 1463-7.

LUNDEMO, A. G., PETTERSEN, C. H., BERGE, K., BERGE, R. K. & SCHONBERG, S. A. 2011. Tetradecylthioacetic acid inhibits proliferation of human SW620 colon cancer cells--gene expression profiling implies endoplasmic reticulum stress. *Lipids in health and disease,* 10**,** 190.

MAILLOUX, R. J., ADJEITEY, C. N. & HARPER, M. E. 2010. Genipin-induced inhibition of uncoupling protein-2 sensitizes drug-resistant cancer cells to cytotoxic agents. *PloS one,* 5**,** e13289.

MAILLOUX, R. J., BERIAULT, R., LEMIRE, J., SINGH, R., CHENIER, D. R., HAMEL, R. D. & APPANNA, V. D. 2007. The tricarboxylic acid cycle, an ancient metabolic network with a novel twist. *PloS one,* 2**,** e690.

MAILLOUX, R. J., DUMOUCHEL, T., AGUER, C., DEKEMP, R., BEANLANDS, R. & HARPER, M. E. 2011. Hexokinase II acts through UCP3 to suppress mitochondrial reactive oxygen species production and maintain aerobic respiration. *The Biochemical journal,* 437**,** 301-11.

MAILLOUX, R. J. & HARPER, M. E. 2011. Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free radical biology & medicine,* 51**,** 1106-15.

MAIURI, M. C., GALLUZZI, L., MORSELLI, E., KEPP, O., MALIK, S. A. & KROEMER, G. 2010. Autophagy regulation by p53. *Current opinion in cell biology,* 22**,** 181-5.

MARCHENKO, N. D., ZAIKA, A. & MOLL, U. M. 2000. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *The Journal of biological chemistry,* 275**,** 16202-12.

MATÉS, J. M., SEGURA, J. A., CAMPOS-SANDOVAL, J. A., LOBO, C., ALONSO, L., ALONSO, F. J. & MÁRQUEZ, J. 2009. Glutamine homeostasis and mitochondrial dynamics. *The International Journal of Biochemistry & Cell Biology,* 41**,** 2051-2061.

MATHUPALA, S. P., REMPEL, A. & PEDERSEN, P. L. 2001. Glucose catabolism in cancer cells: identification and characterization of a marked activation response of the type II hexokinase gene to hypoxic conditions. *The Journal of biological chemistry,* 276**,** 43407-12.

MATOBA, S., KANG, J. G., PATINO, W. D., WRAGG, A., BOEHM, M., GAVRILOVA, O., HURLEY, P. J., BUNZ, F. & HWANG, P. M. 2006. p53 regulates mitochondrial respiration. *Science,* 312**,** 1650-3.

MAURER, G. D., BRUCKER, D. P., BAHR, O., HARTER, P. N., HATTINGEN, E., WALENTA, S., MUELLER-KLIESER, W., STEINBACH, J. P. & RIEGER, J. 2011. Differential utilization of ketone bodies by neurons and glioma cell lines: a rationale for ketogenic diet as experimental glioma therapy. *BMC cancer,* 11**,** 315.

MCBRIDE, H. M., NEUSPIEL, M. & WASIAK, S. 2006. Mitochondria: more than just a powerhouse. *Current biology : CB,* 16**,** R551-60.

METALLO, C. M., GAMEIRO, P. A., BELL, E. L., MATTAINI, K. R., YANG, J., HILLER, K., JEWELL, C. M., JOHNSON, Z. R., IRVINE, D. J., GUARENTE, L., KELLEHER, J. K., VANDER HEIDEN, M. G., ILIOPOULOS, O. & STEPHANOPOULOS, G. 2012. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature,* 481**,** 380-4.

MODICA-NAPOLITANO, J. S., KULAWIEC, M. & SINGH, K. K. 2007. Mitochondria and human cancer. *Current molecular medicine,* 7**,** 121-31.

MOON, J. S., JIN, W. J., KWAK, J. H., KIM, H. J., YUN, M. J., KIM, J. W., PARK, S. W. & KIM, K. S. 2011. Androgen stimulates glycolysis for de novo lipid synthesis by increasing the activities of hexokinase 2 and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 in prostate cancer cells. *The Biochemical journal,* 433**,** 225-33.

MORENO-SANCHEZ, R., RODRIGUEZ-ENRIQUEZ, S., MARIN-HERNANDEZ, A. & SAAVEDRA, E. 2007. Energy metabolism in tumor cells. *The FEBS journal,* 274**,** 1393-418.

MOTOSHIMA, H., GOLDSTEIN, B. J., IGATA, M. & ARAKI, E. 2006. AMPK and cell proliferation--AMPK as a therapeutic target for atherosclerosis and cancer. *The Journal of physiology,* 574**,** 63-71.

NEGRE-SALVAYRE, A., HIRTZ, C., CARRERA, G., CAZENAVE, R., TROLY, M., SALVAYRE, R., PENICAUD, L. & CASTEILLA, L. 1997. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 11**,** 809-15.

NETTO, C. D., SANTOS, E. S., CASTRO, C. P., DA SILVA, A. J., RUMJANEK, V. M. & COSTA, P. R. 2009. (+/-)-3,4-Dihydroxy-8,9-methylenedioxypterocarpan and derivatives: cytotoxic effect on human leukemia cell lines. *European journal of medicinal chemistry,* 44**,** 920-5.

NEWSHOLME, E. A., CRABTREE, B. & ARDAWI, M. S. 1985. Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. *Quarterly journal of experimental physiology,* 70**,** 473-89.

NICHOLLS, D. G. & LOCKE, R. M. 1984. Thermogenic mechanisms in brown fat. *Physiological reviews,* 64**,** 1-64.

NICKLIN, P., BERGMAN, P., ZHANG, B., TRIANTAFELLOW, E., WANG, H., NYFELER, B., YANG, H., HILD, M., KUNG, C., WILSON, C., MYER, V. E., MACKEIGAN, J. P., PORTER, J. A., WANG, Y. K., CANTLEY, L. C., FINAN, P. M. & MURPHY, L. O. 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell,* 136**,** 521-34.

NIKIFOROV, A., DOLLE, C., NIERE, M. & ZIEGLER, M. 2011. Pathways and subcellular compartmentation of NAD biosynthesis in human cells: from entry of extracellular precursors to mitochondrial NAD generation. *The Journal of biological chemistry,* 286**,** 21767-78.

OAKHILL, J. S., SCOTT, J. W. & KEMP, B. E. 2012. AMPK functions as an adenylate charge-regulated protein kinase. *Trends Endocrinol. Metab.,* 23**,** 125-132.

OWEN, O. E., SMALLEY, K. J., D'ALESSIO, D. A., MOZZOLI, M. A. & DAWSON, E. K. 1998. Protein, fat, and carbohydrate requirements during starvation: anaplerosis and cataplerosis. *The American journal of clinical nutrition,* 68**,** 12-34.

OZTURK, S. S. & PALSSON, B. O. 1990. Chemical decomposition of glutamine in cell culture media: effect of media type, pH, and serum concentration. *Biotechnology progress,* 6**,** 121-8.

PASTORINO, J. G., SHULGA, N. & HOEK, J. B. 2002. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. *The Journal of biological chemistry,* 277**,** 7610-8.

PELICANO, H., MARTIN, D. S., XU, R. H. & HUANG, P. 2006. Glycolysis inhibition for anticancer treatment. *Oncogene,* 25**,** 4633-46.

PLAS, D. R. & THOMPSON, C. B. 2005. Akt-dependent transformation: there is more to growth than just surviving. *Oncogene,* 24**,** 7435-7442.

POWIS, G. & KIRKPATRICK, L. 2004. Hypoxia inducible factor-1alpha as a cancer drug target. *Molecular cancer therapeutics,* 3**,** 647-54.

PRUTZ, W. A., BUTLER, J. & LAND, E. J. 1996. Photocatalytic and free radical interactions of the heterocyclic N-oxide resazurin with NADH, GSH, and Dopa. *Archives of biochemistry and biophysics,* 327**,** 239-48.

QU, W., OYA, S., LIEBERMAN, B. P., PLOESSL, K., WANG, L., WISE, D. R., DIVGI, C. R., CHODOSH, L. P., THOMPSON, C. B. & KUNG, H. F. 2012. Preparation and characterization of L-[5-11C]-glutamine for metabolic imaging of tumors. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine,* 53**,** 98-105.

RAIMUNDO, N., BAYSAL, B. E. & SHADEL, G. S. 2011. Revisiting the TCA cycle: signaling to tumor formation. *Trends in Molecular Medicine,* 17**,** 641-649.

RAJASINGH, J. & BRIGHT, J. J. 2006. 15-Deoxy-delta12,14-prostaglandin J2 regulates leukemia inhibitory factor signaling through JAK-STAT pathway in mouse embryonic stem cells. *Exp Cell Res,* 312**,** 2538-46.

RALPH, S. J., RODRÍGUEZ-ENRÍQUEZ, S., NEUZIL, J., SAAVEDRA, E. & MORENO-SÁNCHEZ, R. 2010. The causes of cancer revisited: "Mitochondrial malignancy" and ROS-induced oncogenic transformation – Why mitochondria are targets for cancer therapy. *Molecular Aspects of Medicine,* 31**,** 145-170.

REARDON, D. A., QUINN, J. A., VREDENBURGH, J. J., GURURANGAN, S., FRIEDMAN, A. H., DESJARDINS, A., SATHORNSUMETEE, S., HERNDON, J. E., 2ND, DOWELL, J. M., MCLENDON, R. E., PROVENZALE, J. M., SAMPSON, J. H., SMITH, R. P., SWAISLAND, A. J., OCHS, J. S., LYONS, P., TOURT-UHLIG, S., BIGNER, D. D., FRIEDMAN, H. S. & RICH, J. N. 2006. Phase 1 trial of gefitinib plus sirolimus in adults with recurrent malignant glioma. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 12**,** 860-8.

REDDY, J. K., AZARNOFF, D. L. & HIGNITE, C. E. 1980. Hypolipidaemic hepatic peroxisome proliferators form a novel class of chemical carcinogens. *Nature,* 283**,** 397-8.

REINDL, K. M., KITTILSON, J. D., BERGAN, H. E. & SHERIDAN, M. A. 2011. Growth hormone-stimulated insulin-like growth factor-1 expression in rainbow trout (Oncorhynchus mykiss) hepatocytes is mediated by ERK, PI3K-

AKT, and JAK-STAT. *American journal of physiology. Regulatory, integrative and comparative physiology,* 301**,** R236-43.

REITMAN, Z. J., JIN, G., KAROLY, E. D., SPASOJEVIC, I., YANG, J., KINZLER, K. W., HE, Y., BIGNER, D. D., VOGELSTEIN, B. & YAN, H. 2011. Profiling the effects of isocitrate dehydrogenase 1 and 2 mutations on the cellular metabolome. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 3270-5.

RICQUIER, D. 2005. Respiration uncoupling and metabolism in the control of energy expenditure. *The Proceedings of the Nutrition Society,* 64**,** 47-52.

RICQUIER, D. & BOUILLAUD, F. 2000. The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *The Biochemical journal,* 345 Pt 2**,** 161-79.

RIELY, G. J., KRIS, M. G., ZHAO, B., AKHURST, T., MILTON, D. T., MOORE, E., TYSON, L., PAO, W., RIZVI, N. A., SCHWARTZ, L. H. & MILLER, V. A. 2007. Prospective assessment of discontinuation and reinitiation of erlotinib or gefitinib in patients with acquired resistance to erlotinib or gefitinib followed by the addition of everolimus. *Clinical cancer research : an official journal of the American Association for Cancer Research,* 13**,** 5150-5.

ROBBINS, D. & ZHAO, Y. 2011. New Aspects of Mitochondrial Uncoupling Proteins (UCPs) and Their Roles in Tumorigenesis. *International journal of molecular sciences,* 12**,** 5285-93.

SALWAY, J. G. 2006. *Medical Biochemistry at a glance 2nd Ed.,* Malden, Massachusetts, Blackwell Publishing Ltd.

SAMUDIO, I., FIEGL, M., MCQUEEN, T., CLISE-DWYER, K. & ANDREEFF, M. 2008. The warburg effect in leukemia-stroma cocultures is mediated by mitochondrial uncoupling associated with uncoupling protein 2 activation. *Cancer research,* 68**,** 5198-205.

SCHECK, A. C., ABDELWAHAB, M. G., FENTON, K. E. & STAFFORD, P. The ketogenic diet for the treatment of glioma: Insights from genetic profiling. *Epilepsy Research*.

SELAK, M. A., ARMOUR, S. M., MACKENZIE, E. D., BOULAHBEL, H., WATSON, D. G., MANSFIELD, K. D., PAN, Y., SIMON, M. C., THOMPSON, C. B. & GOTTLIEB, E. 2005. Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase. *Cancer cell,* 7**,** 77-85.

SENGUPTA, S., PETERSON, T. R., LAPLANTE, M., OH, S. & SABATINI, D. M. 2010. mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature,* 468**,** 1100-4.

SHAN, X., CZAR, M. J., BUNNELL, S. C., LIU, P., LIU, Y., SCHWARTZBERG, P. L. & WANGE, R. L. 2000. Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Molecular and cellular biology,* 20**,** 6945-57.

SHAW, R. J. & CANTLEY, L. C. 2006. Ras, PI(3)K and mTOR signalling controls tumour cell growth. *Nature,* 441**,** 424-30.

SKREDE, S., SORENSEN, H. N., LARSEN, L. N., STEINEGER, H. H., HOVIK, K., SPYDEVOLD, O. S., HORN, R. & BREMER, J. 1997. Thia fatty acids, metabolism and metabolic effects. *Biochimica et biophysica acta,* 1344**,** 115-31.

SMOLKOVA, K., PLECITA-HLAVATA, L., BELLANCE, N., BENARD, G., ROSSIGNOL, R. & JEZEK, P. 2011. Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. *The international journal of biochemistry & cell biology,* 43**,** 950-68.

SPEAKMAN, J. R. & MITCHELL, S. E. 2011. Caloric restriction. *Molecular aspects of medicine,* 32**,** 159-221.

SUNG, M. K., YEON, J. Y., PARK, S. Y., PARK, J. H. & CHOI, M. S. 2011. Obesity-induced metabolic stresses in breast and colon cancer. *Annals of the New York Academy of Sciences,* 1229**,** 61-8.

THORENS, B., SARKAR, H. K., KABACK, H. R. & LODISH, H. F. 1988. Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney, and beta-pancreatic islet cells. *Cell,* 55**,** 281-90.

TOMITA, Y., MARCHENKO, N., ERSTER, S., NEMAJEROVA, A., DEHNER, A., KLEIN, C., PAN, H., KESSLER, H., PANCOSKA, P. & MOLL, U. M. 2006. WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. *The Journal of biological chemistry,* 281**,** 8600-6.

TRONSTAD, K. J., BERGE, K., BERGE, R. K. & BRUSERUD, O. 2003. Modified fatty acids and their possible therapeutic targets in malignant diseases. *Expert Opin Ther Targets,* 7**,** 663-77.

TSUKIYAMA-KOHARA, K., POULIN, F., KOHARA, M., DEMARIA, C. T., CHENG, A., WU, Z., GINGRAS, A. C., KATSUME, A., ELCHEBLY, M., SPIEGELMAN, B. M., HARPER, M. E., TREMBLAY, M. L. & SONENBERG, N. 2001. Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. *Nature medicine,* 7**,** 1128-32.

ULDRY, M. & THORENS, B. 2004. The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Archiv : European journal of physiology,* 447**,** 480-9.

VANDER HEIDEN, M. G., LUNT, S. Y., DAYTON, T. L., FISKE, B. P., ISRAELSEN, W. J., MATTAINI, K. R., VOKES, N. I., STEPHANOPOULOS, G., CANTLEY, L. C., METALLO, C. M. & LOCASALE, J. W. 2012. Metabolic Pathway Alterations that Support Cell Proliferation. *Cold Spring Harbor symposia on quantitative biology*.

VIOLLET, B., GUIGAS, B., SANZ GARCIA, N., LECLERC, J., FORETZ, M. & ANDREELLI, F. 2012. Cellular and molecular mechanisms of metformin: an overview. *Clinical science,* 122**,** 253-70.

WALLACE, D. C. 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annual review of genetics,* 39**,** 359-407.

WAN, M., LEAVENS, KARLA F., SALEH, D., EASTON, RACHAEL M., GUERTIN, DAVID A., PETERSON, TIMOTHY R., KAESTNER, KLAUS H., SABATINI, DAVID M. & BIRNBAUM, MORRIS J. 2011. Postprandial Hepatic Lipid Metabolism Requires Signaling through Akt2 Independent of the Transcription Factors FoxA2, FoxO1, and SREBP1c. *Cell Metabolism,* 14**,** 516-527.

WANDERS, R. J. & WATERHAM, H. R. 2006. Biochemistry of mammalian peroxisomes revisited. *Annual review of biochemistry,* 75**,** 295-332.

WANG, R., DILLON, C. P., SHI, L. Z., MILASTA, S., CARTER, R., FINKELSTEIN, D., MCCORMICK, L. L., FITZGERALD, P., CHI, H., MUNGER, J. & GREEN, D. R. 2011. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity,* 35**,** 871-82.

WANG, T., XU, J., YU, X., YANG, R. & HAN, Z. C. 2006. Peroxisome proliferator-activated receptor gamma in malignant diseases. *Crit Rev Oncol Hematol,* 58**,** 1-14.

WARBURG, O. 1956. On respiratory impairment in cancer cells. *Science,* 124**,** 269-70.

WARBURG, O., WIND, F. & NEGELEIN, E. 1927. The Metabolism of Tumors in the Body. *The Journal of general physiology,* 8**,** 519-30.

WEBER, G., SINGHAL, R. L., STAMM, N. B., LEA, M. A. & FISHER, E. A. 1966. Synchronous behavior pattern of key glycolytic enzymes: glucokinase, phosphofructokinase, and pyruvate kinase. *Advances in enzyme regulation,* 4**,** 59-81.

WELLEN, K. E., HATZIVASSILIOU, G., SACHDEVA, U. M., BUI, T. V., CROSS, J. R. & THOMPSON, C. B. 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. *Science,* 324**,** 1076-80.

WELLEN, K. E., LU, C., MANCUSO, A., LEMONS, J. M., RYCZKO, M., DENNIS, J. W., RABINOWITZ, J. D., COLLER, H. A. & THOMPSON, C. B. 2010. The hexosamine biosynthetic pathway couples growth factor-induced glutamine uptake to glucose metabolism. *Genes & development,* 24**,** 2784-99.

WELLEN, K. E. & THOMPSON, C. B. 2010. Cellular metabolic stress: considering how cells respond to nutrient excess. *Molecular cell,* 40**,** 323-32.

WISE, D. R. & THOMPSON, C. B. 2010. Glutamine addiction: a new therapeutic target in cancer. *Trends in biochemical sciences,* 35**,** 427-33.

WISE, D. R., WARD, P. S., SHAY, J. E., CROSS, J. R., GRUBER, J. J., SACHDEVA, U. M., PLATT, J. M., DEMATTEO, R. G., SIMON, M. C. & THOMPSON, C. B. 2011. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. *Proceedings of the National Academy of Sciences of the United States of America,* 108**,** 19611-6.

WONG, C. H., ISKANDAR, K. B., YADAV, S. K., HIRPARA, J. L., LOH, T. & PERVAIZ, S. 2010. Simultaneous induction of non-canonical autophagy and apoptosis in cancer cells by ROS-dependent ERK and JNK activation. *PloS one,* 5**,** e9996.

XIAO, B. 2011. Structure of mammalian AMPK and its regulation by ADP. *Nature,* 472**,** 230-233.

YAMADA, E., OKADA, S., SAITO, T., OHSHIMA, K., SATO, M., TSUCHIYA, T., UEHARA, Y., SHIMIZU, H. & MORI, M. 2005. Akt2 phosphorylates Synip to regulate docking and fusion of GLUT4-containing vesicles. *The Journal of cell biology,* 168**,** 921-8.

YONEZAWA, T., KURATA, R., HOSOMICHI, K., KONO, A., KIMURA, M. & INOKO, H. 2009. Nutritional and hormonal regulation of uncoupling protein 2. *IUBMB life,* 61**,** 1123-31.

YU, Y., MAGUIRE, T. G. & ALWINE, J. C. 2011. Human cytomegalovirus activates glucose transporter 4 expression to increase glucose uptake during infection. *Journal of virology,* 85**,** 1573-80.

YUDKOFF, M., DAIKHIN, Y., HORYN, O. & NISSIM, I. 2008. Ketosis and brain handling of glutamate, glutamine, and GABA. *Epilepsia,* 49 Suppl 8**,** 73-5.

ZHANG, D. W., SHAO, J., LIN, J., ZHANG, N., LU, B. J., LIN, S. C., DONG, M. Q. & HAN, J. 2009. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science,* 325**,** 332-6.

ZHANG, Y., DU, Y., LE, W., WANG, K., KIEFFER, N. & ZHANG, J. 2011. Redox control of the survival of healthy and diseased cells. *Antioxidants & redox signaling,* 15**,** 2867-908.

ZHOU, W., MUKHERJEE, P., KIEBISH, M. A., MARKIS, W. T., MANTIS, J. G. & SEYFRIED, T. N. 2007. The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutrition & metabolism,* 4**,** 5.

ZONCU, R., EFEYAN, A. & SABATINI, D. M. 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology,* 12**,** 21-35.

ZONG, W.-X., DITSWORTH, D., BAUER, D. E., WANG, Z.-Q. & THOMPSON, C. B. 2004. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes & Development,* 18**,** 1272-1282.

ZUCCOLI, G., MARCELLO, N., PISANELLO, A., SERVADEI, F., VACCARO, S., MUKHERJEE, P. & SEYFRIED, T. N. 2010. Metabolic management of glioblastoma multiforme using standard therapy together with a restricted ketogenic diet: Case Report. *Nutrition & metabolism,* 7**,** 33.