A study on features and markers of cellular metabolic rewiring

Deusdedit Tusubira

Thesis for the Degree of Philosophiae Doctor (PhD)
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Scientific environment

The work in this thesis was done in the laboratory of Professor Karl Johan Tronstad, Department of Biomedicine, University of Bergen, from August 2014 to June 2018 under the supervision of Professor Karl Johan Tronstad (main supervisor) and Dr. Gro Vatne Røsland (co-supervisor). Funding was provided by the Norwegian Government under loan Office for Education, Quota Scheme (*Statens lånekasse for utdanning, Kvoteordningen*). Supplementary laboratory costs were provided by the involved laboratories at the University of Bergen.

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My sincere gratitude also goes to everyone in 'Cellnet Research group', especially the group leader; Jim Lorens, lab manager; Sissel Vik Berge, and officemates; Maria K. Lie and Ina K. Pettersen and all members of Karl Johan's group; the pleasant-organiser, Sissel E. Dyrstad, and Fredrik Hoel. Professor Rolf K. Berge was so kind and allowed me to do some of my work with his group. I am also grateful to Carine Lindquist, and all co-authors not mentioned due to space limitations. Everyone was always present when protocols, data or other technical aspect of my project required a second opinion.

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Bergen, September 2018 Deusdedit Tusubira

Abbreviations

ACC	Acetyl-CoA carboxylase
ACOX	Acyl-CoA oxidase
ACS	Acyl-CoA synthase
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMA	Antimycin A
AMP	Adenosine monophosphate
AMPK	AMPK activated kinase
ATP	Adenosine triphosphate
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CPT I / II	Carnitine palmitoyl transferase I / II
DNA	Deoxyribonucleic acid
Drp1	Dynamin-related protein 1
ECAR	Extracellular acidification rate
EMT	Epithelial mesenchymal transition
ER	Endoplasmic reticulum
ETC	Electron transport chain
FAD/FADH	Oxidised/reduced flavin adenine dinucleotide
FDG	Fluorodeoxyglucose
FH	Fumarate hydratase
G6P	Glucose 6 phosphate
GDH	Glutamate dehydrogenase
GLUT	Glucose transport protein
GTP	Guanine triphosphate
HIF1α	Hypoxia inducible factor 1α
IDH	Isocitrate dehydrogenase
IMM	Inner mitochondrial membrane
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LKB1	Liver kinase B1

LPL	Lipoprotein lipase
MCT	Monocarboxylate transporter
MET	Mesenchymal epithelial transition
Mfn 1 / 2	Mitofusins 1/ 2
MPC	Mitochondrial pyruvate carriers
mRNA	messenger RNA
mTOR	Mechanistic target of rapamycin (mammalian target of rapamycin)
NAD/NADH	Oxidised/ reduced nicotinamide adenine dinucleotide
NRF1	Nuclear respiratory factor 1
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OPA	Optic atrophy
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PET	Positron emission tomography
PGC1α	PPAR γ coactivator 1α
Pmf	Proton motive force
PPARs	Peroxisome proliferator-activated receptors
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SDH	Succinate dehydrogenase
SIRT	Sirtuin (silent mating type information regulation 2 homolog) protein
TCA	Tricarboxylic acid cycle
TDG	Tetradecylglycine
TEM	Transmission electron microscope
TFAM	Mitochondrial transcription factor A
TG	Triacylglycerols
tRNA	Transfer RNA
TTA	Tetrathioacetic acid
UCP	Uncoupling protein

VHL	Von Hippel–Lindau syndrome protein
VLDL	Very low density lipoproteins
αKGDH	Alpha- ketoglutarate dehydrogenase
1-triple TTA	2-(tridec-12-yn-1-ylthio)acetic acid

Abstract

Mammalian cells regulate energy metabolism through molecular responses linked to the microenvironment and nutrient supply. Context dependent abnormalities of energy metabolism are important in disorders such as cancer, diabetes, cardiovascular diseases and neurodegeneration. However, these mechanisms are diverse and often not fully understood.

The aim of this study was to investigate cellular mechanisms of metabolic modulation and how it relates to cellular (patho) physiology. We particularly focused on the mitochondria, as these organelles have a critical role in processes of metabolic adaptation. This project investigated metabolic adaptation from two different angles. In one part, we explored metabolic reprogramming as an integral driving force in epithelial to mesenchymal transition (EMT). This is a physiological process that involves important changes in cellular traits and functions. In the other part, we investigated mechanisms and markers of metabolic adaptations occurring when cells and rats were treated with a hypolipidemic modified fatty acids. Although there are conceptual differences between these two contexts, the mechanisms of mitochondrial regulation may be connected. Therefore they may disclose new knowledge that could have applicative value in research on new therapeutic targets and treatment strategies.

In the first part, we analysed gene expression in human breast tumours and found that EMT, which facilitates cancer cell migration and possibly metastasis, was associated with reduced mRNA levels of succinate dehydrogenase subunit C (*SDHC*) and a consequent reduction in mitochondrial activity. Further, we showed that suppression of mitochondrial SDH activity through SDHC CRISPR/Cas9 knockdown or by using the enzymatic inhibitor malonate induced EMT in cell cultures. Similar mitochondrial effects occurred when we modified breast epithelial cells to overexpress EMT-linked transcription factors (TWIST, SNAI2). In these cells, we also observed changes in mitochondrial morphology consistent with loss of functional capacity. These findings describe how altered function of a single mitochondrial metabolic enzyme may have extensive impact on fundamental regulatory programs in a cell.

In part two of our studies, we focused on adaptability of fatty acid oxidation, which represent a central fuelling pathway for mitochondrial energy metabolism. Treatment of cells and rats with tetradecylthioacetate (TTA) was used as a strategy to increase the activity of fatty acid oxidation, in order to investigate consequent adaptations in the metabolic machinery. In this work, we also applied targeted modulation of nutrient- and energy sensitive signalling factors such as AMPK, PPARs and mTOR, to investigate mechanisms involved. Our results lead to the characterisation of pyruvate dehydrogenase kinase 4 (PDK4) as a sensitive and robust marker of increased fatty acid oxidation in various contexts of metabolic adaptation. In a separate study, rats were treated with 2-(tridec-12-yn-1-ylthio) acetic acid (1-triple TTA), a derivative of TTA with a carbon-carbon triple bond in omega-1 position. Similar to TTA, 1-triple TTA was found to increase hepatic mitochondrial fatty acid oxidation and reduce plasma lipid level in rats. Further studies are required to determine how the triple bond in 1-triple TTA affects mechanism of action, e.g. through altered bioavailability, stability and ligand-interactions.

Through the work of this project, we also validated the use of significantly smaller reaction volumes for analysis of gene expression using quantitative PCR. This was described in a short paper where we reduced the single reaction volume from 10 µl to 1 µl without compromising data quality. Such downscaling facilitates significant savings in the use of chemicals and sample material upon clinical and biomedical applications.

In conclusion, this study illuminates important aspects of context-dependent metabolic adaptations through mitochondrial regulation. Shifts in cell metabolism may reflect and interact with mechanisms decisive for cellular (patho) physiology. This may be partly through effects on cellular plasticity, such as the processes of EMT. Hence, implementation of pharmacological approaches to control metabolism may represent an attractive therapeutic strategy in various diseases, including cancer, metabolic diseases and neurodegeneration. This work provided new knowledge on features and markers of metabolic rewiring, and may eventually contribute to future developments of biomedical and clinical applications.

List of publications

Paper 1

Gro V. Røsland*, Sissel E. Dyrstad*, **Deusdedit Tusubira**, Reham Helwa, Tuan Zea Tan, Maria L. Lotsberg, Ina K.N. Pettersen, Anna Berg, Charlotte Kindt, Fredrik Hoel, Kirstine Jacobsen, Ari J. Arason, Agnete S.T. Engelsen Henrik J. Ditzel, Per E. Lønning, Camilla Krakstad , Jean Paul Thiery, James B. Lorens , Stian Knappskog, Karl J. Tronstad **Reduced succinate dehydrogenase (SDH) activity as a consequence of decreased SDHC expression promotes epithelial to mesenchymal transition (EMT) in breast cancer.** *Submitted to Genes and development*

*Equal contributors

Paper 2

Deusdedit Tusubira#, Ina Katrine Nitschke Pettersen#, Lena Hansen, Sissel Elisabeth Dyrstad, Xiaozheng Liu, Kjetil Berge, Hege Wergedahl, Bodil Bjørndal, Arild Rustan , Nils Halberg, Gro Vatne Røsland, Rolf Kristian Berge, Karl Johan Tronstad. **Increased PDK4 mRNA expression is a sensitive marker of upregulated fatty acid oxidation.** *Manuscript*

# Equal contribution

Paper 3

Lindquist, C., Bjørndal, B., Rossmann, C.R., **Tusubira, D.,** Svardal, A., Røsland, G.V., Tronstad, K.J., Hallström, S. and Berge, R.K., 2017. **Increased hepatic mitochondrial FA oxidation reduces plasma and liver TG levels and is associated with regulation of UCPs and APOC-III in rats**. Journal of lipid research, 58(7), pp.1362-1373.

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Paper 4

Sissel E. Dyrstad, **Deusdedit Tusubira**, Stian Knappskog, Karl J. Tronstad[1] & Gro V. Røsland, **Introducing nano-scale quantitative polymerase chain reaction.** *Manuscript accepted for publication in BBRC*

Contents

1. Introduction

1.1 Cell energy metabolism

Mammalian cells possess precise mechanisms that modulate energy supplies as a reaction to environmental changes (Seebacher et al., 2010). Adequate metabolic responses are essential in supporting production of exact ATP (adenosine triphosphate) amounts for physiological homeostasis. Defects in these responses may lead to pathological conditions of varying kinds and severity. These include; obesity-related-complications, cancer, cardiovascular diseases, metabolic syndrome, neurodegeneration and others. At the centre of most metabolic processes is the mitochondrion an essential organelle in mammalian cells.

1.2 The mitochondrion

Mitochondria derive their name from their thread-like nature when observed under light microscope (van der Giezen, 2011). Their characteristic structure, as observed under transmission electron microscope (TEM), consists of an outer and inner membrane (Figure 1). The outer membrane is smooth and porous, whereas the inner membrane is highly convoluted (forming cristae) and is impermeable to most ions (Mitchell and Moyle, 1967).

As the centre of cellular metabolism, the mitochondrion is involved in both catabolism and anabolism. It is estimated to generate more than 80 % of the cells' ATP (Rolfe and Brown, 1997). The inner membrane contains proteins and enzymes for electron transport chain (ETC) and oxidative phosphorylation (OXPHOS), whereas the enzymes of the tricarboxylic acid (TCA) cycle and fatty acid oxidation are located in the mitochondrial matrix, inside the inner mitochondrial membrane (Figure 1).

The mitochondria are key organelles involved in almost all aspects of cellular metabolism. They are involved in cellular signalling and adaptations as a response to environmental stimuli. Besides, they are crucial organelles in the production of reactive oxygen species (ROS), (Bohovych and Khalimonchuk, 2016, Ryan and

Hoogenraad, 2007) as well as in apoptosis (Sinha et al., 2013). They are also essential for intracellular calcium signalling (Fall and Keizer, 2001).

The function, biomass and quality of mitochondria are continuously maintained and adjusted within the cell through a combination of mitochondrial biogenesis, mitophagy, fission and fusion processes (Gottlieb and Bernstein, 2016). It is also important to note that the size and shape of cristae in each mitochondrion changes in response to cells' metabolic state. This is because cristae topology affects the ability of mitochondria to generate ATP (Mannella, 2006).

The mitochondrial genome is distinct from the nuclear genome as it contains multiple copies of circular DNA (mtDNA) which are maternally inherited (Birky, 2001). On the other hand, many of the proteins needed for its overall function are synthesised from the nuclear genes. In humans, the mitochondrial genome only encodes for 13 protein subunits of the ETC complexes, plus 22 tRNAs and 2 rRNAs (a total of 37 genes) (Ryan and Hoogenraad, 2007).

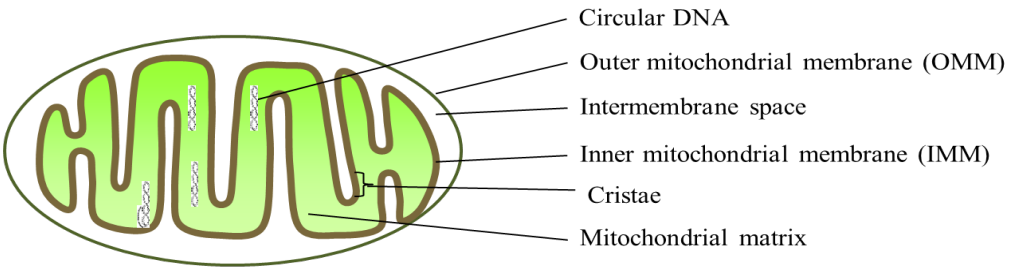

Figure 1: Simplified illustration of the mitochondrial organelle. Note the multiple copies of mtDNA, and the two membranes; an outer membrane enclosing a highly convoluted inner membrane. Some reactions take place in the mitochondrial matrix and others on the inner membranes.

1.3 Glycolysis

Cells generate ATP both in the cytosol and mitochondria. In the cytosol, glucose is converted to pyruvate through glycolysis (Figure 2). Cytosolic glucose can be mobilised from intracellular glycogen storages or taken up from circulation.

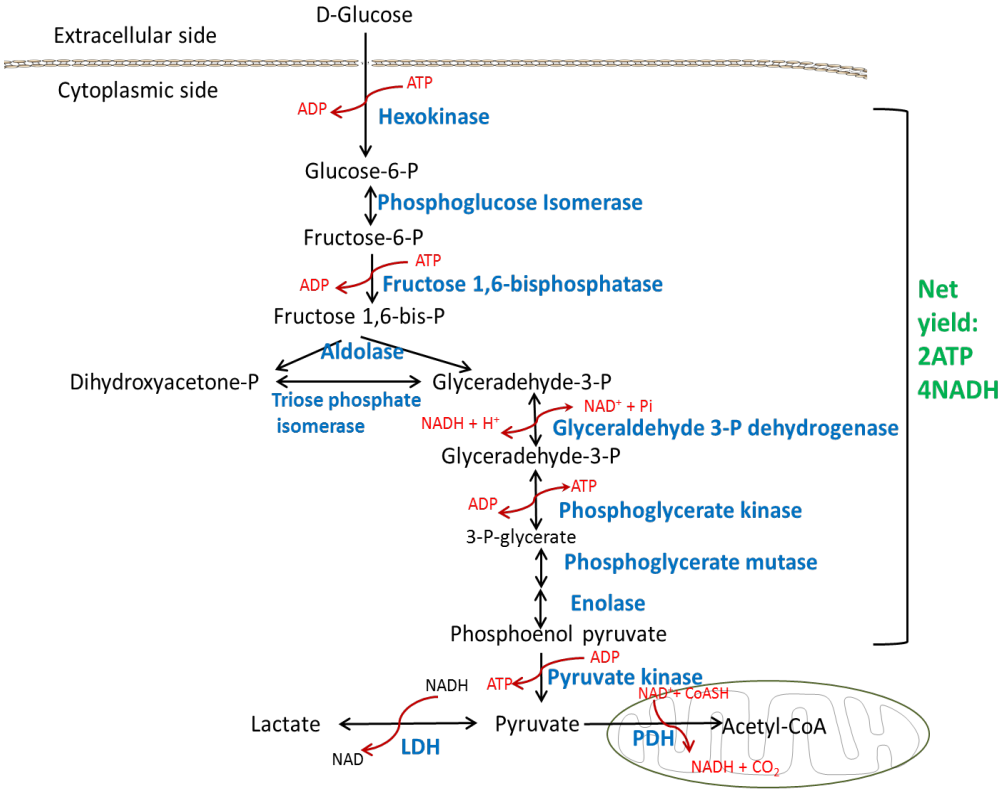

Figure 2: Glycolytic pathway. Glucose is transported from the extracellular side of the cell to the cytosol by glucose transporters (not shown). Glucose 6 phosphate (glucose-6-p) is broken down in a series of 10 reactions to pyruvate. The enzymes involved are shown in **blue** and major regulatory enzymes are underlined. Pyruvate is transported to mitochondrial matrix where it is converted to acetyl-CoA by pyruvate dehydrogenase enzyme (PDH). Pyruvate can also be reduced to lactate by lactate dehydrogenase (LDH). Lactate efflux from the cell takes place via monocarboxylate transporters (not shown). Abbreviations: fructose-6-p, fructose 6 phosphate, fructose-1, 6-bis-P, fructose 1,6 bisphosphate, dihydroacetone-P, dihydroacetone phosphate, glyceraldehyde -3-P, glyceraldehyde 3 phosphate, 3-p-glycerate, 3 phosphoglycerate, LDH and PDH.

Extracellular glucose molecules are transported to the cytosol by glucose transporters (GLUTs). In the cytosol, glucose is phosphorylated to glucose-6-phosphate (G6P) by hexokinase. G6P is broken down to pyruvate in a series of 10 reactions (Figure 2). These reactions are tightly regulated, and the fate of glucose is determined by many factors some of which are context dependent as shall be discussed later. For every glucose molecule, there is a net production of 2 ATP molecules. In the presence of oxygen (aerobic conditions), pyruvate is transported to mitochondrial matrix where it is converted to acetyl-CoA by pyruvate dehydrogenase (PDH). In the absence of oxygen (anaerobic conditions), pyruvate is primarily converted to lactate by lactate dehydrogenase (LDH). Lactate is transported out of the cell by monocarboxylate transporters (MCTs).

Glycolysis is tightly regulated to meet cellular demand for ATP. In general phosphofructokinase (PFK) is the major regulatory enzyme of this pathway (Berg JM, 2002, Mor et al., 2011). PFK is allosterically controlled by other metabolites such as citrate ATP and AMP (Abuelgassim et al., 1992).

1.4 Tricarboxylic acid (TCA) cycle

The TCA cycle is composed of a series of reactions fundamental to aerobic energy metabolism (Figure 3) (Krebs, 1970). These reactions oxidise acetyl-CoA, within the mitochondrial matrix to generate cellular energy, water and carbon dioxide.

As a major source of acetyl-CoA for the TCA cycle, pyruvate enters the mitochondrial matrix through mitochondrial pyruvate carrier protein (MPC), located in the inner mitochondrial membrane. Functional MPC is a highly conserved a heterocomplex composed of MPC1 and 2 (Bricker et al., 2012, Herzig et al., 2012, McCommis and Finck, 2015). Upon entering the mitochondrial matrix, pyruvate is converted to acetyl-CoA by PDH. PDH is part of the multienzyme, pyruvate dehydrogenase complex, (often termed PDC), a major regulator of cellular bioenergetics (Patel et al., 2014, Park et al., 2018). PDH is important in the maintenance of glucose homeostasis through its role in the reciprocal regulation of fatty acid and glucose oxidation (Holness and Sugden, 2003). The activity of PDH is

inhibited through phosphorylation by isoenzymes of the pyruvate dehydrogenase kinase family (PDK 1-4) (Jeong et al., 2012) (section 1.9). Other sources of acetyl-CoA include fatty acids and amino acid catabolism (Krebs, 1970). In our studies we show that mRNA expression of PDK4 represents a sensitive marker of fatty acid oxidation. This exemplifies how linking of metabolic pathways are vital for metabolic reprogramming to maintain homeostasis.

The TCA cycle takes place in a series of 8 reactions that start by the condensation of acetyl-CoA with a four-carbon substrate, oxaloacetate, forming a six-carbon tricarboxylic acid; citrate (Figure 3). This reaction is catalysed by citrate synthase. The functional groups in citrate are reorganised by the enzyme aconitase, forming isocitrate. In the subsequent reactions, two carbon atoms are released as CO_2. Isocitrate dehydrogenase (IDH) oxidises isocitrate to form alpha-ketoglutarate followed by alpha-ketoglutarate dehydrogenase (αKDH) to form succinyl-CoA. These two reactions are also accompanied by release of 2 NADH electron carriers, (Figure 3). In the subsequent reaction, succinyl-COA is converted to succinate by succinyl-CoA synthetase. This reaction is accompanied by generation of GTP through substrate level phosphorylation. Succinate is converted to fumarate by succinate dehydrogenase (SDH). As SDH is also part of the ETC, it links ETC with the TCA cycle. This is another important enzyme which we found to be involved in mechanisms of cell plasticity in breast cancer cells, as shall be discussed later. The SDH reaction generates 1 $FADH_2$. Fumarate is hydrolysed to malate by fumarase. The cycle is completed when malate is oxidised by malate dehydrogenase to regenerate oxaloacetate. This reaction produces the last electron carrier, NADH. The 3 NADH and $FADH_2$ electron carriers are used in ATP generation through OXPHOS (section 1.5). For each acetyl-CoA oxidised through this cycle, 12 ATPs are potentially generated.

The TCA cycle is a hub for anabolic and catabolic reactions. TCA cycle intermediates are replenished through anaplerotic reactions thus providing important means of responding to varying metabolic needs by replacing particular metabolites (Gibala et al., 2000). It also provides a means by which the carbon skeletons of amino

acids such as glutamine, glutamate and others are fed into the cycle (Brunengraber and Roe, 2006).

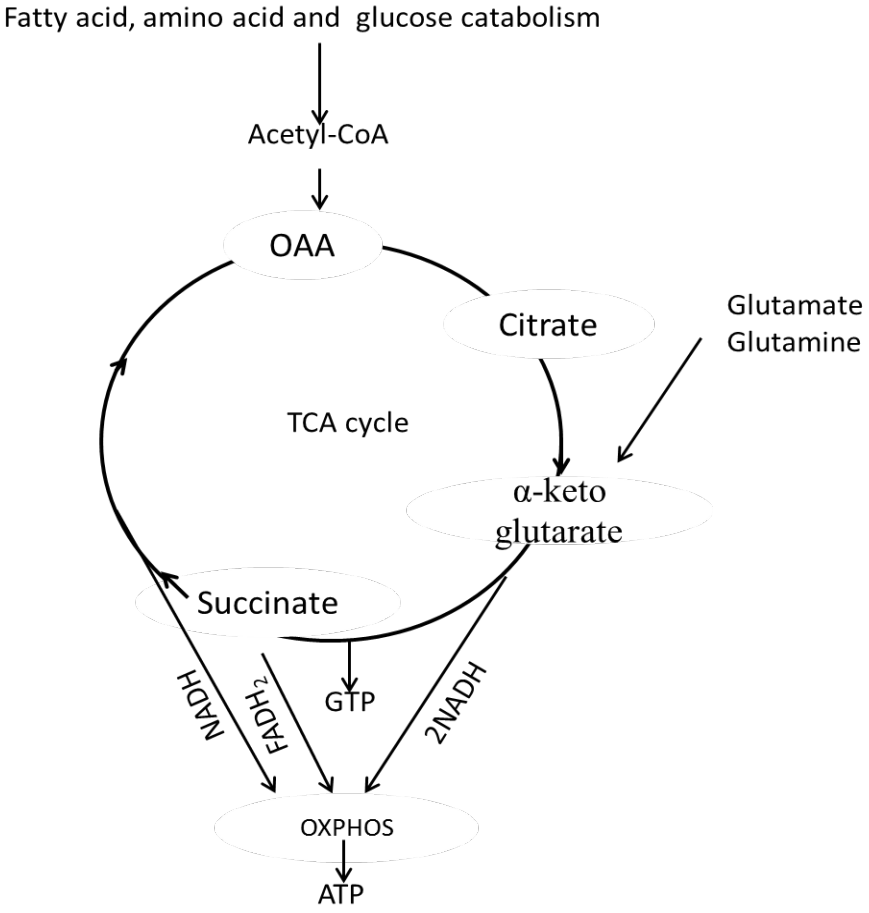

Figure 3: A simplified diagram of the TCA cycle. The TCA cycle is composed of reactions important in catabolism of acetyl-CoA. Acetyl-CoA condenses with oxaloacetate (OAA) to form citrate which is eventually oxidised in a series of 8 reactions (see text). Intermediates in the TCA cycle may be replenished through anaplerosis. Glutaminase (GLS) and mitochondrial glutamate dehydrogenase (GDH) are important enzymes in anaplerosis. They replenish alpha-ketoglutarate. The enzyme SDH used for oxidation of succinate also compose complex II of the ETC. The electron carriers, NADH and $FADH_2$, transfer electrons to ETC and OXPHOS for ATP generation (Krebs, 1970).

1.5 Oxidative phosphorylation

Glycolysis, the TCA cycle and other catabolic pathways produce reduced electron carriers in form of $FADH_2$ and NADH. Oxidation of these is coupled to ATP generation by OXPHOS. OXPHOS was originally described in the 60's by Peter Mitchell (Mitchell, 1972, Mitchell, 1961). It involves transport of electrons through ETC to molecular oxygen (final electron acceptor). This process is coupled to phosphorylation of ADP, thus forming the universal high energy fuel molecule, ATP (Figure 4).

The ETC is composed of four protein complexes numbered I to IV coupled to a phosphorylating enzyme complex, ATP synthase (F_1F_0 ATP synthase). ATP synthase is also referred to as respiratory complex V. All complexes are embedded in the IMM. Unlike complex II (SDH), complex I, III and IV also function as proton pumps. As mentioned, SDH is also part of the TCA cycle and is located on the matrix side of the IMM (Figure 4).

Electrons from NADH enter the ETC through complex I. They are transported by difference in reduction potentials through complex I cofactors to ubiquinone, (UQ). For every pair of electrons transferred from NADH, two protons are transported from the mitochondrial matrix to the intermembrane space.

Electrons from UQ are transferred to complex III bound cofactors and later delivered to cytochrome *c* (Cyt *c*). Complex III is the second proton pump and transfers four protons for every pair of electrons. Cyt *c* is located on the cytosolic side of the IMM (Figure 4). Electrons from Cyt *c* are transferred to complex IV (cytochrome oxidase). Cytochrome oxidase is the last enzyme of the respiratory chain. It transfers a pair of electrons to molecular oxygen. Every pair of electrons transported through this complex is coupled to pumping of a pair of protons out of the mitochondrial matrix. Another pair of protons reacts with molecular oxygen to generate water.

As protons accumulate on the cytosolic side of the IMM, an electrochemical gradient is created (membrane potential), representing a proton motive force (pmf). The pmf

leads to spontaneous flux of protons from the cytosolic side of the IMM through ATP synthase back to the matrix side. The energy released in this process is used to fuel its synthase activity, thus generating ATP from ADP and Pi (Boyer, 1997).

Some of the protons 'leak' spontaneously through the IMM outside the ATP synthase complex. This is termed 'uncoupling'. It results in decreased efficiency of OXPHOS as the leaking protons are not coupled to ATP synthesis (Kadenbach, 2003), and the energy is rather released as heat. Uncoupling is important in heat generation in brown adipocytes. In mammals, it has been estimated that about 20 % of mitochondrial respiration is uncoupled for heat generation (Rolfe and Brown, 1997).

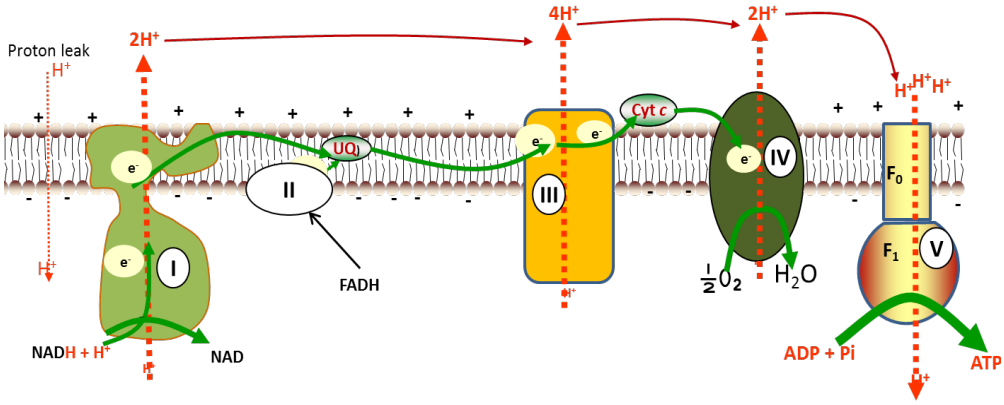

Figure 4: Overview of oxidative phosphorylation, electron transport chain (ETC). Electrons are donated by NADH at complex I and succinate at complex II and shuttled to complex III through UQ. From complex III the electrons are transported to complex IV where they reduce molecular oxygen to water. Electron transport is accompanied with the pumping of protons to the cytosolic side of the IMM through complex V. This makes it positively charged relative to matrix side.

1.6 Fatty acids and fatty acid oxidation

Fatty acids have a plethora of functions ranging from cellular energy storage (Nakamura et al., 2014) to signalling (Papackova and Cahova, 2015). Intracellular fatty acids may act as ligands for peroxisome proliferator nuclear receptors (PPARs), thus regulating countless metabolic pathways (Jump and Clarke, 1999, Besseiche et

al., 2015). PPARs are key regulators of oxidative pathways and are important in energy mobilisation and transduction (Besseiche et al., 2015) (section 1.10).

Due to their hydrophobic nature, plasma fatty acids are mostly bound to proteins such as albumin. On release by lipoprotein lipases, free fatty acids enter cell cytoplasm in alternative ways (Figure 5). They may passively cross the plasma membrane or be actively transported by specific transport proteins; mostly fatty acid transport proteins (FATPs) and fatty acid translocases (FAT/ CD36) (Doege and Stahl, 2006). Intracellular fatty acids are bound to fatty acid binding proteins (FABPs). During activation, fatty acids are esterified to coenzyme A (CoA) in the cytosol by a family of acyl-CoA synthetases (ACS) to form acyl-CoAs (Ellis et al., 2010, Hiltunen and Qin, 2000). Fatty acid acylation is coupled to hydrolysis of an ATP molecule and release of pyrophosphate (PPi). Fatty acyl-CoAs are bound to acyl-CoA binding proteins (ACBPs) hence protecting them from hydrolysis by acyl-CoA hydrolases.

Catabolism of fatty acids for energy purposes generally occurs through mitochondrial beta-oxidation. Long-chain fatty acyl-CoAs (LCFAs) are transported to the mitochondrial matrix by the carnitine shuttle (Stephens et al., 2007). In the carnitine shuttle, the CoA of acyl-CoA is exchanged with a carnitine moiety by carnitine palmitoyl transferase I (CPT I) to form acyl-carnitine. CPTI exists in three tissue-specific isoforms: CPT1a-hepatic, CPT1b-muscular and CPT1c-neural (Lopes-Marques et al., 2015, Virmani et al., 2015). Acyl-carnitine is transported through mitochondrial membrane by carnitine acyl transferase (CAT); in IMM, on the matrix carnitine palmitoyl transferase II (CPT II) exchanges the acyl group on carnitine with CoA to regenerate acyl-CoA.

Intramitochondrial acyl-CoA is oxidised through a four-step cycle involving dehydrogenation, hydration, dehydrogenation and thiolytic cleavage. This results into release of NADH, $FADH_2$, acetyl-CoA and an acyl-CoA shortened by 2 carbon atoms (Hiltunen and Qin, 2000). Acetyl-CoA is further oxidised in the TCA, whereas the electron carriers $FADH_2$ and NADH fuel mitochondrial respiration. Complete

oxidation of a single palmitic acid (16C) is estimated to yield up to 120 ATP molecules.

Mitochondrial beta-oxidation is regulated depending on activity of acetyl-CoA carboxylase (ACC) producing malonyl-CoA, an intermediate of fatty acid biosynthesis. High concentrations of malonyl-CoA inhibit CPT I, thus decreasing mitochondria uptake of LCFAs (Bastin, 2014, Houten and Wanders, 2010).

Fatty acids may also be oxidised in other cellular compartments; in mammalian cells beta-oxidation takes place both in mitochondria and peroxisomes (Poirier et al., 2006). Peroxisomes may use additional mechanisms of fatty acid oxidation that include omega-oxidation (Poirier et al., 2006, Miura, 2013). In omega-oxidation, fatty acid catabolism starts with oxidation of omega carbon atom. It involves use of mixed function oxidases. Omega-oxidation is a significant subsidiary pathway for fatty acid catabolism when beta-oxidation is blocked (Miura, 2013).

Very long and branched chain fatty acids, prostaglandins and leukotrienes are primarily oxidised through beta-oxidation in the peroxisomes. The first step in peroxisomal beta oxidation is catalysed by acyl-CoA oxidase (ACOX) (Poirier et al., 2006), followed by a series of reactions that produce short / medium chain fatty acids and hydrogen peroxide. The short and medium chain fatty acids are eventually oxidised through beta-oxidation in the mitochondria.

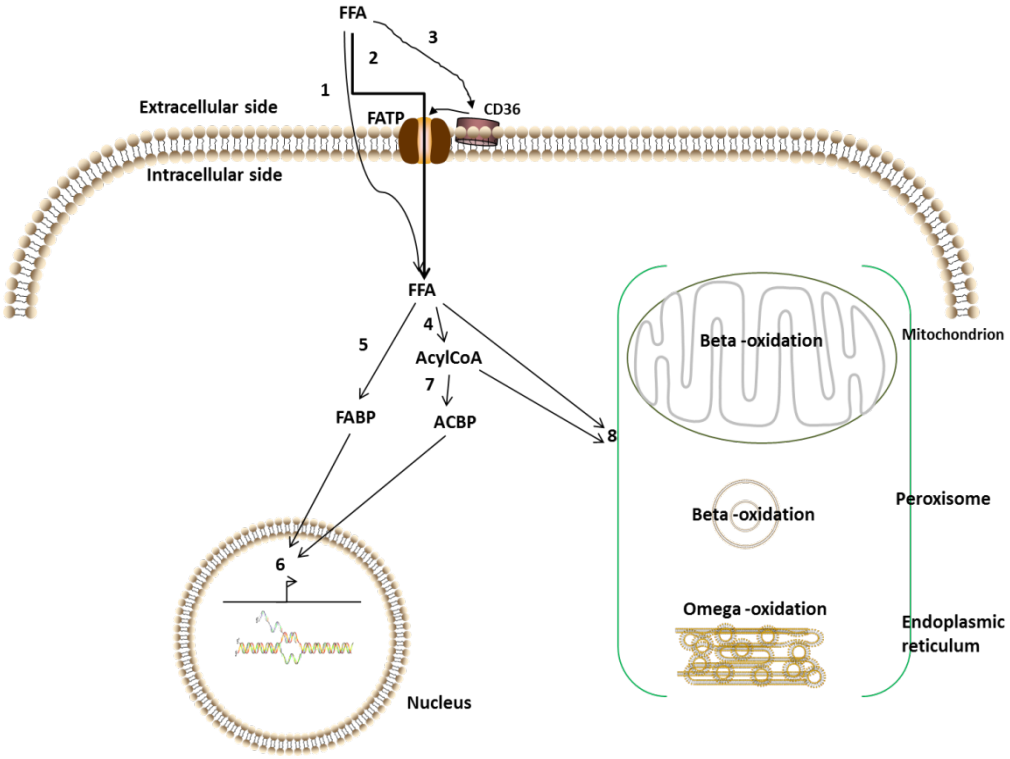

Figure 5: Fatty acid utilisation. Exogenous free fatty acids are released from transport proteins by lipases and transported through cell membrane by; passive diffusion **(1)**, fatty acid transport proteins FATPs (**2**), or bound to CD36 surface receptor (**3**) followed by FATP facilitated transport. Intracellular fatty acids are either acylated by fatty acyl-CoA synthetases (**4**), or bound to fatty acid binding protein, FABP (**5**). Acyl-CoAs are bound to Acyl-CoA binding proteins ACBPs (**7**). In the nucleus fatty acid may regulate nuclear receptors such as peroxisome proliferator activated receptors PPARs (**6**). Acyl-CoAs and free fatty acids (FFAs) are oxidised **(8)** by beta- and omega-oxidation in the mitochondria, peroxisomes and endoplasmic reticulum (Figure adapted from (Doege and Stahl, 2006)).

1.7 Contextual regulation of mitochondrial function

Mitochondrial adaptations may occur in response to changes in cellular energy demand (Yu and Pekkurnaz, 2018). A good example is the observation that during muscle exercise, cellular signals lead to an increase in the number of mitochondria (Ryan and Hoogenraad, 2007, Jose et al., 2013). Such adaptations occur to support energy homeostasis, and are specific to the cellular context (Tronstad et al., 2014).

Optimal mitochondrial health involve processes of biogenesis, fusion, fission and mitophagy (Gottlieb and Bernstein, 2016). Below, important aspects of mitochondrial biogenesis will be introduced.

1.7.1 Mitochondrial biogenesis

Change in cellular energy status, usually indicated by AMP/ADP ratio, is an important stimulus for mitochondrial biogenesis. An increase in AMP/ADP ratio that accompanies a low energy status is detected by AMP-activated protein kinase, AMPK (Hardie and Carling, 1997, Qi and Young, 2015) (Figure 6). High concentration of AMP activates AMPK by promoting phosphorylation of threonine 172 (T172) within the regulatory subunit (α) by liver kinase B1 (LKB1) (Sanders et al., 2007, Hawley et al., 1996). Activated AMPK uses its kinase activity to activate pathways that replenish cellular ATP (catabolic reactions), while at the same time inhibiting those that consumes it (anabolic reactions) (Carling, 2017).

Increase in mitochondrial mass through biogenesis is an important adaptive mechanism for high cellular energy needs. Among the proteins regulated by activated AMPK is peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC1α) (Jager et al., 2007). PGC1α is a co-activator for the main transcription factor of mitochondrial biogenesis; nuclear respiratory factor-1 (NRF-1). NRF-1 activates transcription of mitochondrial transcription factor A (TFAM) and other vital components of the mitochondria (Tronstad et al., 2014, Scarpulla, 2002). Consequently, mitochondrial biogenesis is stimulated.

Mitochondrial health is also controlled through retrograde signalling (Ryan and Hoogenraad, 2007). Dysfunctional mitochondria generate signalling molecules that induce nuclear responses for its biogenesis (Figure 6). These include; Ca^{2+}, ROS which activates JNK, NAD+ and AMP. All these signalling molecules converge at PGC1α (da Cunha et al., 2015).

Low nutrient levels induce mitochondrial biogenesis through sirtuin 1 (SIRT1) which in turn activates PGC1α through NAD+ mediated deacetylation of a specific lysine

residue (Rodgers et al., 2005, Lagouge et al., 2006). Energy state also influences mitochondrial biogenesis through intracellular calcium signalling, e.g. associated with high muscle activity (Morgan et al., 1997).

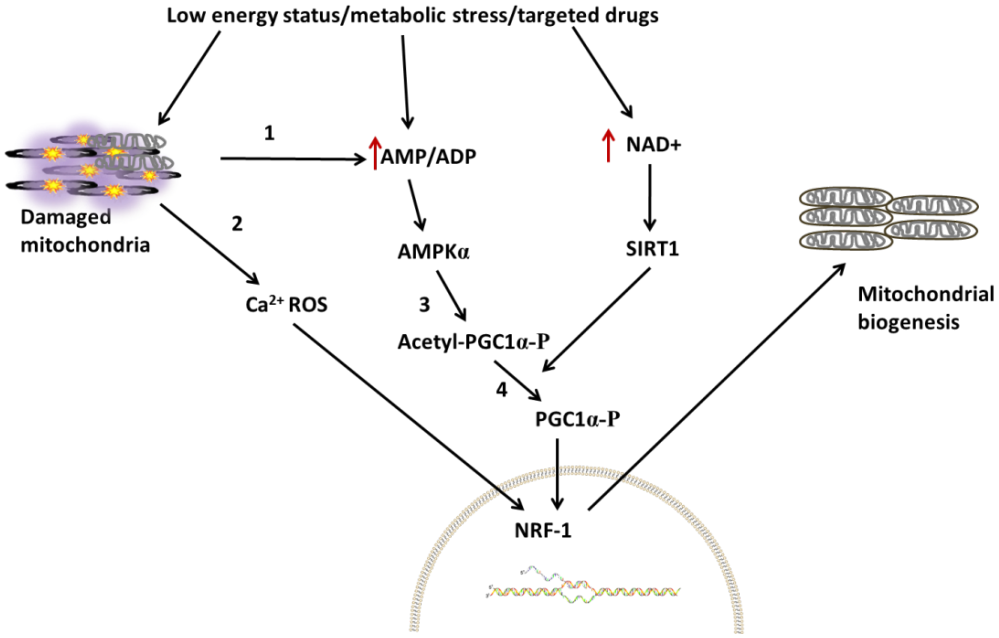

Figure 6: Regulation of mitochondrial biogenesis through NRF-1. Metabolic stress in cells increases amount of dysfunctional and damaged mitochondria. It also increases the content of AMP (**1**) and signalling molecules; Ca^{2+} and ROS (**2**). High AMP activates AMPK which in turn phosphorylates PGC1α (**3**). Increase in NAD+ promotes SIRT1 deacetylation of PGC1α (**4**), a co-activator for transcription of NRF-1. NRF-1 promotes mitochondrial biogenesis.

1.7.2 Mitochondrial dynamics

The processes of mitochondrial life cycle include fusion, fission, biogenesis and mitophagy, thus mitochondria change in shape, size and number. Mitochondrial dynamics determine both morphology and physiological functions of a cell as response to nutrient supply and demand for ATP. Other cellular factors such as metabolic stress, mitochondrial health and cell plasticity are important in determining mitochondrial phenotype (paper 1) (Figure 7) (Gottlieb and Bernstein, 2016, Wai and Langer, 2016, Twig and Shirihai, 2011, Chan, 2012). Mitochondrial breakdown

serves to maintain organelle quality by eradicating dysfunctional components (Berman and Hollenbeck, 2013).

Proteins that participate in mitochondrial dynamics include mitofusins (Mfn 1\2) which orchestrate fusion of the outer membrane and optic atrophy 1 (OPA1) for inner membrane (Zorzano et al., 2010). Fused mitochondria form filaments and networks. Mitochondrial fusion leads to formation of long networks that expedite mixing of mtDNA, proteins and membrane lipids (Wai and Langer, 2016). The opposing processes (fission) entail assembly dynamin-related protein (Drp1) on the mitochondrial surface. Drp1 proteins constrict and fragment mitochondria into separate units (Zorzano et al., 2010).

The balance between fragmented and fused mitochondria provides a mechanism for supporting mitochondrial quality, and the process is often referred to as the mitochondrial lifecycle (Figure 7). Mitochondrial stress may promote compensatory mechanisms that lead to biogenesis to support respiratory capacity and ATP generation. Fragmentation is usually associated with metabolic dysfunction (e.g. due to disease) (Tronstad et al., 2014, Wai and Langer, 2016). Mitochondrial damage signals such as ROS, Ca^{2+}, loss of membrane potential (ψ_m), release of Cyt *c* and others are associated with mitophagy and apoptosis (Picard and Turnbull, 2013, Cai and Tammineni, 2016).

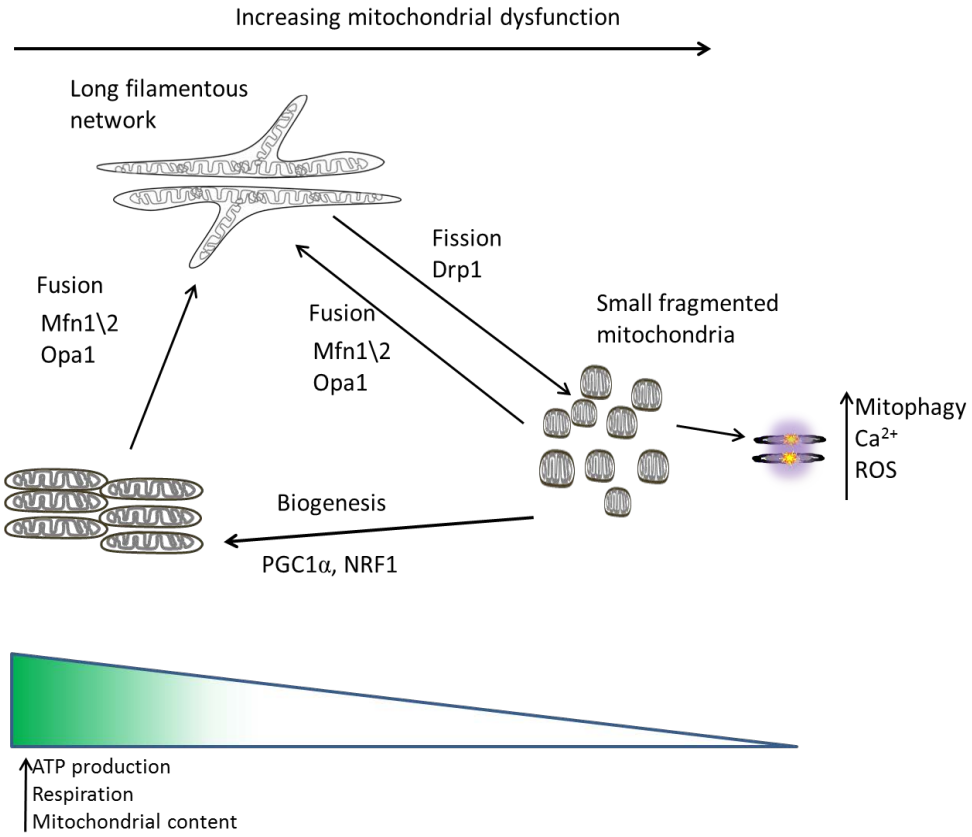

Figure 7: Mitochondrial lifecycle. Mitochondrial fusion and fission are linked to metabolic adaptation for cell survival and proliferation. Cellular insults may promote biogenesis thus increasing cell mitochondria content. At the same time mitochondrial fusion promotes respiration and ATP generation due to increase in mitochondrial content. Mitochondrial dynamics maintain mitochondrial quality, through a health balance between mitochondrial fusion and fission. This is effect through the fusion proteins; mitofusins Mfn1 /2 and OPA1 that lead to formation of long filaments and fission protein Drp1 that lead to fragmentation. Damaged mitochondria release signalling molecules like Ca^{2+} and ROS that promote mitophagy and apoptosis.

1.8 Cellular metabolic remodeling

Metabolic remodelling has been extensively studied in cancer (Costa and Frezza, 2017); it involves change in cell fuel molecule preference as response to microenvironment. This phenomenon is also sometimes referred to as metabolic shift,

metabolic reprogramming or metabolic rewiring. These terms are used synonymously in this thesis. In metabolic remodelling cells could change their preferred fuel molecule. Some cells may present context-dependent changes in their preferences for fatty acids, glucose or amino acids as biosynthetic or fuel molecules. This is common in many physiological and pathological processes including formation of stem cells (Simsek et al., 2010, Cuyas et al., 2018), EMT (Paper 1), and in immune responses (van der Windt and Pearce, 2012).

1.8.1 Cancer cell metabolism

Metabolic reprograming is one of the major functional capabilities that allow cancer cells to survive, proliferate and disseminate (Hanahan and Weinberg, 2011). It comprises redirecting fuel molecules from what normally happens in most tissues to pathways that support anomalous metabolic requirements (Vander Heiden et al., 2009, Wenner, 2012).

Abnormal energy metabolism in cancer cells was originally reported by Otto Warburg in the 1950s. He proposed that the origin of cancer was due to defective mitochondria (Warburg, 1956) but now we know that several factors factors such as mutations and environmental many carcinogens are involved (Hanselmann and Welter, 2016). According to the 'Warburg effect', cancer cells reprogram their glucose metabolism in such a way that it is essentially limited to glycolysis (Vander Heiden et al., 2009). This is also commonly referred to as 'aerobic' glycolysis. Aerobic glycolysis is associated with low mitochondrial ATP production and excessive generation of lactate due to extreme reliance on glycolysis. However, it is important to mention that aerobic glycolysis is not found in all cancer cells (Mayers and Vander Heiden, 2017) and may be found in other proliferating cells (Lunt and Vander Heiden, 2011).

Cancer cells may compensate for the low mitochondrial ATP production by increasing the rates of glucose uptake through upregulation of GLUTs (Labak et al., 2016), which subsequently increase glycolytic flux (Dai et al., 2016). Relative increase in glucose uptake by cancer cells is used in diagnosis by a method of

positron emission tomography (PET) imaging. In PET imaging, tumor cells take up radio-labelled fluorodeoxyglucose (^{18}FDG) at a faster rate compared to normal cells. These can be seen on scan (Shen et al., 2017).

Higher rates of glycolysis in cancer cells may be a consequence of oncogenes (e.g. Ras oncogenes (Lv et al., 2016) or activation of hypoxia response system (HIF1α/HIF2α (Lu et al., 2002, Semenza, 2010)). HIFs may be activated by oncogenes or local hypoxia. Local hypoxia may result from insufficient supply of oxygen in the growing tumour mass (Gao et al., 2016) or induction of a pseudo hypoxic state caused by tumorigenic mutations in SDH (Selak et al., 2005). Results from our studies (paper 1) and others support an association between altered SDH function and cancer (Mahdi et al., 2015, Aspuria et al., 2014). Mutations in other TCA cycle enzymes and OXPHOS have also been associated with emergence of a cancer phenotype. For example, overexpression of mutant IDH promotes aerobic glycolysis and cell proliferation in glioma cells (Nie et al., 2015).

The reasons for aerobic glycolysis are diverse. One of the hypotheses is that increased glycolytic flux provides biomass for cell division and proliferation (Vander Heiden et al., 2009, Lunt and Vander Heiden, 2011). In this hypothesis, intermediates of the glycolytic pathway are used for biosynthesis of nucleotides, NADPH, amino acids and lipids (Li et al., 2016, Bentaib et al., 2014, Lunt and Vander Heiden, 2011).

Altered metabolic landscape in cancer cells surge their dependence on glutamine, as it serves as an anaplerotic feeder to the TCA cycle (Li et al., 2016, Altman et al., 2016). This pathway involves conversion of glutamine to glutamate by glutaminase followed by glutamate dehydrogenase based oxidative deamination to alpha-ketoglutarate, a TCA cycle intermediate (Figure 8) (Hensley et al., 2013, Mullen et al., 2011). The TCA cycle in cancer cells provides both ATP and other biosynthetic products. The TCA cycle intermediates, citrate and malate, are also used in the biosynthesis of fatty acids in these cells (Locasale and Cantley, 2011). This metabolic phenotype furnishes the cell with metabolic flexibility typical of cancer, characterised by cell survival, proliferation and dissemination. Metabolic flexibility is the ability to

respond and adapt to changes in tumour microenvironment (Goodpaster and Sparks, 2017). A metabolically flexible cell can switch between glucose, fatty acids and amino acids as fuel molecules. Metabolic inflexibility is a potential therapeutic target in metabolic disorders (Muoio, 2014).

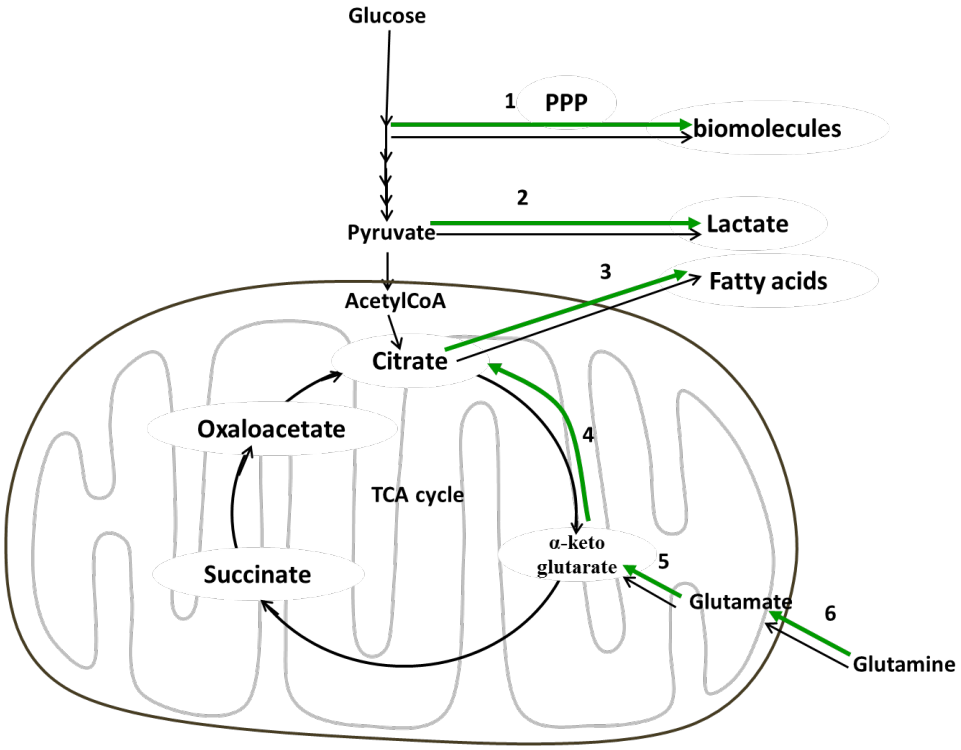

Figure 8: Metabolic reprogramming in cancer cells. Processes of both glycolysis and TCA cycle have been reported upregulated in cancer cells. There is increased activity of the pathways marked green; the pentose phosphate pathway (PPP) **(1)** produces NADPH and ribose used in biosynthesis of many biomolecules. Lactate production is increased (**2**). Citrate lyase produces NADPH and an acyl-CoA for fatty acid synthesis (**3**). Isocitrate dehydrogenase (IDH) **(4)**, glutamate dehydrogenase (GDH) (**5**) and glutaminase (**6**) feeds the TCA cycle through glutamine (Locasale and Cantley, 2011).

1.8.2 Metabolism and cell plasticity

Cell plasticity is the ability of some cells to take on new phenotypic characteristics as they change positions or move between tissues and organs. These new phenotypic

characteristics may encompass both metabolic and morphological features. Cell plasticity has also been implicated in physiological and pathophysiological processes where it contributes to wound healing and tissue repair (Jessen et al., 2015), fibrosis and cancer metastases in adults (Lopez-Novoa and Nieto, 2009, Kalluri, 2009). During embryonic development, it is crucial for laying of body plan and differentiation of tissues and organs (Thiery et al., 2009),

This type of cellular transformation may involve dynamic phenotype transitions such as when an epithelial (differentiated) cell converts to a mesenchymal (stem-like) phenotype, which is termed epithelial to mesenchymal transition (EMT) and back to the epithelial character (MET) on differentiation at new site (Nieto et al., 2016).

EMT represents a continuum of processes where epithelial cells, originally anchored to the basal lamina, sequentially lose their cell-cell adhesion and regular apical-basal polarity thus allowing them to break free and drift toward the stroma (Nieto et al., 2016). At the same time, they gradually gain mesenchymal properties i.e. increased cell-matrix interactions, survival, stemness, and enhanced motility.

EMT is orchestrated by an adaptive response to stimuli from the microenvironment. The stimuli may be due to hypoxia (Lehmann et al., 2017), inflammatory cytokines (TGF-β) (Lopez-Novoa and Nieto, 2009, Feldkoren et al., 2017) and energy supply (Thomson et al., 2011). EMT-stimuli indirectly or directly induce expression of EMT-linked transcription factors (EMT-TFs) such as TWIST, SNAIL, and ZEB1/2. These elicit loss of epithelial markers; cytokeratins, E-cadherins, and gain of mesenchymal markers; N-cadherin, vimentin (Scanlon et al., 2013).

The transition of an epithelial to a mesenchymal phenotype is a complex cellular process whose mechanisms are not entirely understood. EMT-TFs activate many genes including those related to energy metabolism (Thomson et al., 2011). The change in metabolic program that adapts the cells to the mesenchymal phenotype may involve the mitochondria (paper 1). Mitochondrial bioenergetics has been implicated in cell differentiation and fate decisions plus EMT (Sciacovelli and Frezza, 2017, Ito

and Ito, 2016, Ryall et al., 2015), however the molecular mechanisms in this process are not well understood.

Hypoxia has been associated with stemness (Xie et al., 2016a), EMT and metastases in many cancers including breast cancer (Gao et al., 2016). Under hypoxia, HIF1 is stabilised, which indirectly inhibit activity of PDH through upregulation of PDK (Kim et al., 2006). PDH inhibition activates glycolytic lactate production, which is also an enabler of tumour growth (Choi et al., 2013). The rate of glycolysis is also increased in EMT by the nutrient sensor, mammalian target of rapamycin (mTOR) through indirect inhibition of PDH (Cheng and Hao, 2017, Gulhati et al., 2011). As previously mentioned, mutations in the TCA cycle enzymes are also associated with EMT in cancer (Sajnani et al., 2017). We and others have observed a correlation between mutations and inhibition of SDH and EMT (paper 1) (Aspuria et al., 2014).

Another example underscoring the relationship between cell plasticity and metabolic reprogramming is found in activated T-cells. As a response from external stimuli, metabolic reprogramming is involved in cell transformation from quiescence, through differentiation, activation and proliferation (Buck et al., 2015).

Cancer cell metabolic reprogramming and the process of EMT illustrate the importance of context-dependent impact of cellular metabolism. Namely, cells rewire their metabolism in response to physiological context defined by both extracellular and intracellular factors.

1.9 PDKs are key modulators of energy metabolism

PDKs are key modulators in the regulation of PDH (see section 1.4) (Holness and Sugden, 2003), thus they function as nodes in rewiring of energy metabolism (Park et al., 2018, Zhang et al., 2014). They regulate both glucose and fatty acid flux in tissues with high energy requirements (Visiedo et al., 2013). They are also central to gluconeogenesis and lactate production as part of mechanisms for contextual metabolic remodelling. A high activity of PDH breaks down glucose thus providing acetyl-CoA for fatty acid synthesis (Sugden and Holness, 2002, Hwang et al., 2009,

Randle et al., 1994). PDH is inhibited through phosphorylation by PDK and activated through dephosphorylation by pyruvate dehydrogenase phosphatase (PDP). A change in PDK expression represents an important mechanism that regulates PDH activity under varying cellular metabolic states. PDK has four isoforms (PDK 1-4) which are expressed in specific tissues in a context dependent manner. Of the four isoforms, PDK4 is important in long term metabolic regulation under pathophysiological conditions including starvation and metabolic syndrome (Sugden and Holness, 2002, Randle et al., 1994).

PDK4 has been linked to changes in fatty acid oxidation, partly as a direct or indirect target of PPARs. The mechanisms that link PDK4 to PPARs may be complex, since upregulation of PDK4 is normal under conditions of raised free fatty acids levels, which are in turn ligands of PPARs (Sugden and Holness, 2003). In one of the studies, PDK4 knockout mice exhibited lower body weight and higher levels of both PGC1α and PPARα (Hwang et al., 2009).

Many compounds modulate metabolism by targeting fatty acid utilisation through interactions with energy and nutrient-sensitive signalling factors. Below we discuss a class of potent activators of fatty acid oxidation, which has been used in our study (Papers 2 and 3).

1.10 Modified fatty acids as modulators of energy metabolism

Synthetic fatty acid analogues may be used to modulate lipid metabolism in experimental models. This strategy has been investigated by Berge and other research groups for several decades (Willumsen et al., 1997, Bremer, 2001, Berge et al., 2002). Thia (thioether) fatty acids represent a class of molecules that have demonstrated biological activities useful for mechanistic studies and with potential application in treatment of metabolism related disorders (Dyroy et al., 2006, Tronstad et al., 2003a). Tetradecylthioacetic acid (TTA) is the most well-studied 3-thia synthetic fatty acid analogue (Berge et al., 2002). It is synthesised by introducing a

sulphur atom in the beta-position of the 16-carbon fatty acid (palmitic acid) to form a new compound also classified as a thioether.

Inside the cell, TTA is activated through acylation to form TTA-CoA by acyl-CoA synthetases, just like all natural fatty acids (Skorve et al., 1995); however, it does not undergo normal mitochondrial beta-oxidation (Berge et al., 1989). Under these circumstances it is catabolised through omega- and sulphur oxidation in the endoplasmic reticulum and beta-oxidation in peroxisomes (Berge and Hvattum, 1994, Skrede et al., 1997). Changes in gene transcription related to fatty acid oxidation are observed early after TTA administration to rats (Vaagenes et al., 1998) and on longer treatment, distinct hypolipidaemic effects are observed in rat models (Berge et al., 1999). Feeding male rats with TTA increases hepatic beta-oxidation and decreases circulating lipids (Berge et al., 2002, Skrede et al., 1997). TTA was also found to counteract dyslipidaemia in patients with type II diabetes (Lovas et al., 2009).

Although the mechanisms behind the hypolipidaemic effects are not fully understood, they may involve hepatic drainage of circulating fatty acids (Berge et al., 2005) (Figure 9). In this hypothesis, it is proposed that TTA increases general transport, uptake and oxidation of the fatty acids by the liver from extrahepatic tissues. This is coupled to increased generation of ketone bodies. Fatty acid uptake is a result of increased activity of lipoprotein lipase and decreased activity of ApoC-III. TTA and related compounds activate PPARs, especially PPARα (Bhurruth-Alcor et al., 2010, Guerre-Millo et al., 2000, Gottlicher et al., 1993). PPARs are important regulators of genes involved in lipid metabolism and are indirectly associated with mitochondrial and peroxisomal proliferation as previously mentioned (Raspe et al., 1999) (Figure 9 below). As a result, the mitochondrial beta-oxidation of oxidisable fatty acids is amplified. This is because PPARs also activate hydroxyglutaryl-CoA synthase (HMGCoA synthase) (Berge et al., 1999).

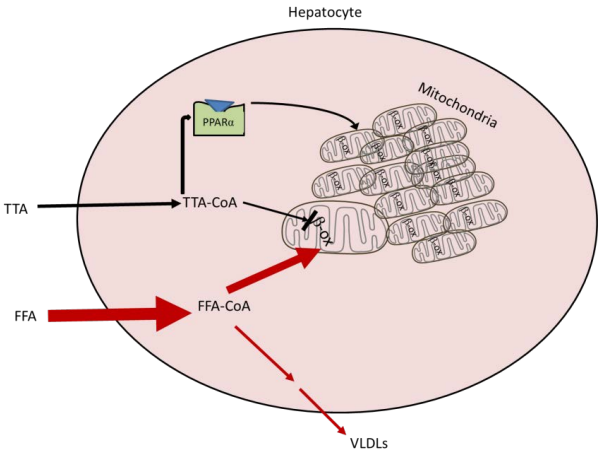

Figure 9: Triacylglycerol lowering effect of Tetradecylthioacetic acid. Uptake of TTA in liver cells activates PPARα targeted genes which promotes mitochondria biogenesis. Total mitochondrial beta-oxidation is increased. TTA inhibits mitochondrial beta-oxidation. There is increased flux of normal free fatty acids (FFA) into the cells. Fatty acyl-CoAs for triacylglycerol synthesis and VLDL formation is decreased. B-ox, β-oxidation (Berge et al., 2002))

Additional effects of 3-thia fatty acids include improvement in insulin-sensitivity (Madsen et al., 2002), reduced proliferation of rapidly proliferating cells (Berge et al., 2003), cell differentiation/anti-inflammatory effects (Aukrust et al., 2003) and modification of hepatic energy status (Grav et al., 2003).

In another branch of research, TTA has demonstrated anticancer properties in cell and animal models. It reduces glioma cell proliferation in culture (Tronstad et al., 2001a, Tronstad et al., 2001b) and the mechanisms may have a connection with mitochondrial beta-oxidation (Berge et al., 2003). In a glioma animal study, TTA was found to act through PPAR-dependent and independent mechanisms to mediate antitumor effects (Berge et al., 2001). In another study, TTA was found to reduce the growth of cultured native human acute myelogenous leukaemia blasts (Tronstad et al., 2002). Antileukaemic effects of TTA were subsequently observed in two independent animal studies (Iversen et al., 2006, Erikstein et al., 2010). Data supporting antitumor effects of TTA has also been obtained from in vitro and in vivo studies in colon cancer models (Jensen et al., 2007). The mechanisms behind these

antitumor effects of TTA are complex, but cell culture studies suggest that they may involve direct interactions with mitochondria and subsequent apoptosis (Tronstad et al., 2003b) and elements of ER stress (Lundemo et al., 2011).

Other thia fatty acids have been synthesised and investigated, where the position of the sulphur atom is changed, and triple bounds have been inserted in the acyl-chain (Dyroy et al., 2006, Jorgensen et al., 2009). Novel glycerolipids and phospholipids containing such fatty acids have also been made (Bhurruth-Alcor et al., 2010, Jorgensen et al., 2009). These studies show that the position of the sulphur atom in the acyl-chain has profound implications on the biological activities of the new compounds. For instance, when male wistar rats were fed on thioethers with sulphur atom at even positions, their hepatic and cardiac mitochondrial beta-oxidation was decreased. As a result, their blood lipids were raised, and this had consequences for hepatic lipid accumulation (Gudbrandsen et al., 2005, Dyroy et al., 2006). An example of a thia fatty acid thioether with carbon atom at even position is tetradecylthiopropionic acid (TTP).

1.11 Assessment of mitochondrial bioenergetic function

Assessing mitochondrial respiration through measurements of mitochondrial oxygen consumption is an important method to evaluate the functional activity and integrity of the organelles. In our studies, we extensively measured oxygen consumption rate (OCR) to characterise effects on cell metabolism and mitochondrial function (Divakaruni et al., 2014). Oxygen flux is directly coupled to mitochondrial respiration and provides useful information on mitochondrial function and overall cellular metabolic status (Zhang et al., 2012, Pesta and Gnaiger, 2012, Divakaruni et al., 2014). Similarly, measurements of the extracellular acidification rate (ECAR) can be used to monitor lactate production resulting from glycolysis.

There are well-established protocols involving sequential additions of specific metabolic modulators to assess parameters of mitochondrial function. A trace from a

typical test on mitochondrial respiratory function is shown in Figure 10. Below is a short summary of the most central descriptors we used for this purpose.

Basal respiration; Basal respiration represents oxygen consumption for cellular energy demands under normal cell culture conditions, in presence of the relevant substrate(s). It is comprised of oxygen consumption for ATP synthesis and proton leak (**leak respiration**).

Leak respiration: Leak respiration is measured after addition of oligomycin. Oligomycin inhibits ATP synthase, thus lowering the rate of respiration leaving only residual OXPHOS independent respiration. Leak respiration represents any oxygen consumption not coupled to ATP synthesis. ATP-linked respiration may be calculated as the difference between basal and leak respiration. Increased leak respiration may indicate loss of mitochondrial integrity and uncoupling (Kroemer et al., 1998, Izyumov et al., 2004). This is an important marker of cellular stress associated with cellular bioenergetic collapse (Izyumov et al., 2004).

Respiratory capacity: The capacity of the electron transport system is measured after addition of an uncoupler (such as CCCP). This allows spontaneous flow of protons across the IMM back to the mitochondria matrix. This raises the rate of respiration due to dissipation of the membrane potential. The resulting OCR represents the maximal cell respiratory rate. For each cell type we performed a titration to obtain optimal concentration of CCCP. Spare respiratory capacity represents the capacity to respond to a sudden increase in energy demand. It's calculated by subtracting basal respiratory rate from maximum rate.

Non-mitochondrial oxygen consumption: This background activity was determined following inhibition of Complex I with rotenone and Complex III with antimycin A. Any remaining oxygen consumption constitutes non-mitochondrial respiration.

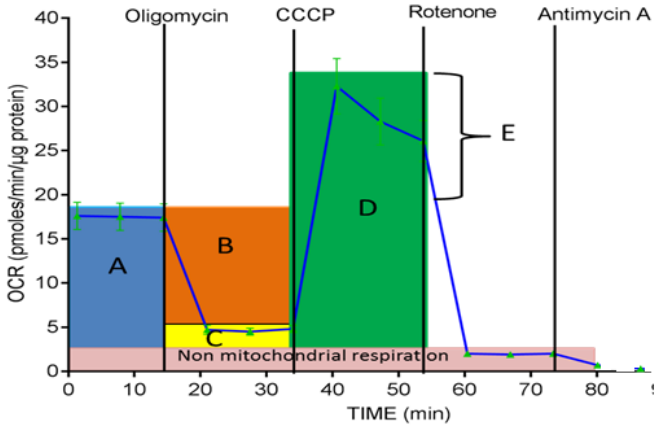

Figure 10: Mitochondrial function descriptors. A. Basal respiration; this is the oxygen consumption under normal cell culture conditions. **B.** ATP-linked respiration; this is the difference between basal respiration and leak respiration. **C.** leak respiration; it is the residual respiration due to oxygen consumption not coupled to ATP synthesis. **D.** Respiratory capacity; it gives the maximum oxygen consumption rate that can be achieved by the cell. **E.** Spare respiratory capacity; is the difference between basal respiration and maximal respiratory capacity. Non-mitochondrial respiration is due to background oxygen consuming reactions (Dott et al., 2014).

1.12 Assessment of gene expression by realtime quantitative polymerase chain reaction

Quantitative PCR (qPCR) remains a traditional method to assess gene expression in various fields of life sciences. First, cDNA (complementary DNA) is produced using RNA isolated from the biological sample as template, in a reaction catalysed by the enzyme reverse transcriptase. Then, qPCR is performed to measure the level of the gene of interest, involving real-time detection of double stranded DNA during a controlled amplification process (Bustin et al., 2005, Arya et al., 2005). For a specific gene, the number of cycles required to produce a detectable fluorescence signal (Ct) depends on the initial quantity of cDNA, which is determined by the original number of mRNA copies in the sample. This method combines nucleic amplification and detection in a single reaction (Kubista et al., 2006). A common method for qPCR data analysis is the double delta Ct (ΔΔCt) method, which calculates the expression level

of the gene of interest in the sample relative to a control sample by the use of an internal house-keeping control gene (Livak and Schmittgen, 2001). Though this is a very sensitive method for quantifying gene expression (Saunders, 2004) the costs of reagents are significant. As part of our studies, we validated the use of significantly reduced qPCR reaction volume, thus providing an opportunity for saving resources (paper 4).

2. Aim of the study

The overall aim of this project was to study how context-dependent modulation of metabolism may contribute to changes in cell physiology. The mechanisms were investigated in the context of EMT which represents a physiological program shift in cell phenotype, and in cells and rats treated with mitochondrial-targeting PPAR-activating modified fatty acids.

We hypothesized that under these conditions, modulation of cellular energy metabolism involves specific mitochondria targeted cross-talk between signaling, gene expression and metabolism.

2.1 Objectives

1. Determine if metabolic reprogramming occurs during epithelial to mesenchymal transition (EMT) and investigate the mechanisms involved.

2. To explore mechanisms and markers of metabolic adaption associated with increased fatty acid oxidation mediated by the modified fatty acid TTA.

3. Investigate the role of mitochondria in the hypolidaemic effects of the thioether fatty acid, 1-triple TTA, in a rat model.

4. To contribute with method developments for improving cost-efficiency of quantitative PCR analysis.

3. Summary of papers

3.1 Paper 1

Reduced succinate dehydrogenase (SDH) activity as a consequence of decreased *SDHC* expression promotes epithelial to mesenchymal transition (EMT) in breast cancer

The reversible cell transition between epithelial and mesenchymal phenotypes (EMT-MET) is an important physiological process, representing cellular plasticity. Mutations in the mitochondrial enzyme, succinate dehydrogenase SDH, have been associated with EMT and cancer. However, specific contribution of the mitochondria and metabolic reprogramming in the initiation of EMT program is not well characterised. We hypothesized that remodeling of mitochondrial metabolism constitutes a central part of the EMT program, and that this may be mediated through interaction with SDH. We extensively analysed microarray datasets from breast cancer patient cohorts to search for associations between SDH and EMT markers. This was followed by analysis of cell models of EMT for phenotypic changes in metabolism.

Low level of *SDHC* expression correlated with increased EMT status in breast cancer patient samples. Interestingly, low SDH expression was associated with poor survival in patients with basal-like molecular subtype. Moreover, both *SDHC* gene knockdown through CRISPR/cas9 and enzymatic inhibition of SDH using malonate caused induced EMT in cultured breast cells. To further explore the role of SDH, we overexpressed EMT-promoting transcription factors (TWIST, SNAI2) in epithelial cells. This suppressed SDH and SDH-linked mitochondrial respiration. To understand how EMT affected the content and shape of mitochondria, we performed confocal microscopy and 3D quantitative image analysis. TWIST overexpression led to a reduction in mitochondrial volume; they were more dispersed with fragmented network of thinner tubules. More to this, there was reduction in the ratio of mitochondrial to nuclear DNA and expression of mitochondrial metabolic enzymes. This study highlights contextual modulation where cells undergoing phenotypic

changes of cell plasticity such as EMT reprogram metabolism. This may serve to adapt to harsh tumor microenvironments and thus confer increased tolerance to oxidative stress and hypoxia.

3.2 Paper 2

Increased PDK4 mRNA expression is a sensitive marker of upregulated fatty acid oxidation.

Cells may rewire their metabolic pathways to satisfy their demand for energy and biomolecules. In contexts of limited glucose supply, often there may be a metabolic switch towards increased fatty acid oxidation. This preserves glucose for other critical functions. Metabolic rewiring partly occurs through suppression of PDH function as a result of increased expression of PDH kinases (PDKs). Of the PDK isomers, PDK4 expression appears to be of particular importance in circumstances mentioned. Changes in PDK4 expression has been linked to major energy and nutrient-sensitive regulators such as PPARs, AMPK and mTOR, especially under conditions of increased fatty acid oxidation. In our study, we explored the association between increased fatty acid oxidation and PDK4 expression under targeted modulation of these regulators, and after treatment with the hypolipidemic modified fatty acid TTA.

In rats treated with TTA for 50 weeks, significant transcriptional upregulation of enzymes involved in fatty acid oxidation were found in liver and to some extent in heart, which is in agreement with previous reports also assessing enzymatic activities. We did not find fatty acid oxidation significantly upregulated in muscle and white adipose tissue (WAT). In the same tissues, increased PDK4 expression paralleled the activation profile of fatty acid oxidation, in TTA-treated rats compared to controls. The association between activation of fatty acid oxidation and increased PDK4 expression was further investigated in MDA-MB-231 cells after targeted metabolic modulation. Overexpression of PPARα significantly increased gene expression and activity of fatty acid oxidation, and this was accompanied by 25 fold increase in PDK4 expression. In accordance, overexpression of PDK4 caused reduced pyruvate oxidation and increased fatty acid oxidation. Selective activators of PPARα (WY

14,643) and AMPK (AICAR) did not give similar effects; however, TTA-treatment caused upregulation of fatty acid oxidation and PDK4 expression to a higher level than observed after PPARα overexpression. HeLa cells presented similar effects, although the magnitude of upregulation was generally lower in those cells. Interestingly, the rates of mitochondrial respiration remained relatively unchanged under the different conditions of metabolic modulation, but unlike the other modulators, TTA was found to act as a mild uncoupler. Inhibition of mTOR with rapamycin accentuated the effects of TTA on fatty acid oxidation and PDK4. Correlation analysis between fatty acid oxidation and PDK4 expression under different conditions of metabolic adaptation supported the close relationship between these two factors.

In conclusion, we suggest PDK4 expression to be a robust marker for fatty acid oxidation under metabolic adaptations driven by context-dependent regulatory programs. Analysis of PDK4 expression may be of broad value for investigations of (patho) physiological mechanisms involving metabolic adaptation.

3.3 Paper 3

Increased hepatic mitochondrial fatty acid oxidation leads to lower triacylglycerol levels in rat liver and plasma, and is associated with upregulation of uncoupling proteins and downregulation of apolipoprotein C-III

High blood triacylglycerols (TGs) are associated with increased risk of cardiovascular disease (CVD). Blood TGs are lowered through uptake mechanisms that involve inhibition of apolipoprotein C-III (APOC-III) and upregulation of lipoprotein receptors followed by intracellular fatty acid utilisation. In previous studies, the 3-thia modified fatty acid thioether, TTA was shown to target hepatic mitochondrial function and lead to hypolidaemic effects. In this study, a new TTA analogue, 2-(tridec-12 -yn-1-ylthio) acetic acid (1-triple TTA) was investigated, where the omega-1 position was modified with a triple bond. Due to its modifications, 1-triple TTA represents an LCFA unable to be catabolised by beta- or omega-oxidation.

To investigate the effects of lipid metabolism, male wistar rats were fed on 1-triple TTA, for three weeks followed by analysis of their mitochondrial fatty acid metabolism relative to control fed rats.

The expression of hepatic APOC-III gene was significantly lowered whereas that of LPL and the VLDL receptor was upregulated. Liver mitochondrial fatty acid oxidation was increased by more than 5 times. This was accompanied by lowering of hepatic (60 %) and plasma (53 %) TGs. Mitochondrial biomarkers such as mitochondrial DNA, citrate synthase activity, and expression of cytochrome c and TFAM were specifically elevated in liver. Hepatic energy status of treated rats was lowered without affecting muscle and heart. This is reflected by increased AMP/ATP and decreased ATP/ADP ratio. This was accompanied by upregulation of genes for hepatic uncoupling proteins (UCP) 2 and 3. Plasma concentration of the ketone body, D-β-hydroxybutyrate was also increased. In summary, administration of 1-triple TTA caused clearance of blood TGs possibly as a result of lowered APOC-III expression, increased hepatic TG uptake and higher rates of mitochondrial fatty acid oxidation. This mechanism may facilitate drainage of fatty acids from the blood to the liver for catabolic purposes and production of ketone bodies as extrahepatic fuel.

3.4 Paper 4

Introducing nano-scale quantitative polymerase chain reaction

One of the most commonly used methods in quantifying gene expression is the qPCR. Due to its convenience in terms of efficacy and accuracy, the method is widely applied in research and molecular diagnosis. The limitations include high cost of brand-specific reagents including fluorescent dyes or the fluorophore-labelled probes, as well as the usually small amount of available sample. In the present study, we investigated whether the qPCR reaction volume could be reduced without compromising the quality of results when common laboratory equipment are used.

We performed TaqMan® qPCR analysis at low (1 µl) and standard (10 µl) reaction volumes. To obtain accurate and reproducible results, we used a pipetting robot for transferring the reaction volumes to the 384-well qPCR plate. cDNA obtained from MCF7 and HCC827 cell lines was diluted in a ten-fold dilution series ranging from 10^{-1} to 10^{-7}. For the purpose of representing differently expressed targets, the RPL30, RPS29 and 18S were carefully chosen as detection targets. The results were analysed in three steps that involved: comparing shapes of amplification curves, followed by linear regression analysis and finally, calculating curve specific reaction efficiency coefficient E ($E = 10^{(-1/slope)}$)

Every step of sample cDNA dilution series resulted in a corresponding shift of the amplification curve to the right by three additional PCR cycles. Further, the slope of the regression line at low reaction volume (1 µl) was comparable to standard reaction volume (10 µl) for the samples from both cell lines. As expected, the expression levels among the three detection targets were observed in terms of cycle number as follows: 18s<RPS29<RPL30. The targets were detectable in dilutions up to 10^{-7} for 18s, 10^{-4} for RPS29 and 10^{-3} for RPL30, in both low (1 µl) and standard (10 µl) reaction volumes. For each obtained curve, the curve-specific reaction efficiency coefficient, E, was also within the accepted qPCR range (1.9-2.1). By considering the linear section of the curves we found the average intra-experimental coefficient of variation (% CV) to be < 0.5% for 10 µl, and <1.0% for 1 µl reaction volumes for all genes. In conclusion, we show that downscaled qPCR is indeed feasible without compromising the analytical outputs.

4. Discussion

To develop new effective interventions for treating diseases, knowledge about the underlying mechanisms is important. Although dysregulated metabolism and metabolic reprogramming have been linked to many diseases, it often remains elusive how this contributes to the molecular mechanisms driving pathogenic processes. In the present study, we used two different strategies to study context-dependent metabolic rearrangements, and how this may be connected to mitochondrial responses. In the first part, we found specific changes in mitochondrial energy metabolism that may contribute to EMT, a cellular program shift of significant interest e.g. in cancer biology. Furthermore, our studies suggest SDH as a prognostic marker in basal-like breast cancer. In the next part, we investigated how specific changes in metabolism may be elicited by using modified fatty acids to target mitochondrial function.

Changes in the microenvironment may lead to cellular effects ranging from mild adjustment in certain features to extensive adaptations of cellular functions, or even a complete change in cell phenotype. These effects are context-dependent and determined by both intra- and extracellular factors. Absence or presence of specific nutrients may induce perturbations within pathways that provide necessary metabolites and energy for cell survival and multiplication. Associated mechanisms of metabolic rewiring may form the basis for changes in cellular features under phenotypic plasticity (paper 1), and upon interventions of pathways involved in pathophysiological conditions such as diabetes, starvation, or diet (paper 2 and 3). Targeting specific driver mechanisms of metabolic rewiring such as SDH (Mills et al., 2016) or fatty acid oxidation (Monsenego et al., 2012, Chowdhury et al., 2018) could potentially be used in therapy or augmentation of existing therapies (Elia et al., 2016, Corbet and Feron, 2017). This could also be used in understanding metabolic regulations in conditions when low energy foods are consumed (Fowler, 2016).

Quantitative PCR was used to measure mRNA expression of genes of interest in all studies on metabolic rewiring included this thesis. As part of the work with this

project we validated the use of smaller PCR reaction volumes to save expenses and sample material. We found that significant downscaling could be performed without loss of data quality. This work is summarized in paper 4.

4.1 Purposes of metabolic rewiring

The multi-step process of EMT represents a good example of cellular phenotypic plasticity where original morphology is lost due to disintegration of cell-cell contacts followed by movement and re-establishment at alternative locations (Nieto et al., 2016). Mutations in the enzymes of central metabolism (TCA cycle) have been linked to EMT and cancer (Mills et al., 2016, Du et al., 2014). Interaction between these pathways is extensive. Thus, cellular metabolism requires synchronisation with physiological needs and this could be achieved through crosstalk with specific signalling networks (Bohovych and Khalimonchuk, 2016, Ryan and Hoogenraad, 2007). This balances both the use of biomolecules for energy and biosynthetic purposes.

Our studies show that remodelling of mitochondria associated with supressed activity of respiration to be linked to SDH in cell models. SDH suppression may be indirectly aimed at rewiring metabolism. This could be a protective mechanism for cell survival in a harsher environment as cells become mobile and nutrient deprived (Khan et al., 2015). Mitochondrial remodelling may be an inherent feature linked to conversion of epithelial cells to mesenchymal phenotype (paper 1). Interestingly, both inhibition of SDH by enzymatic inhibition using malonate as well as through Crispr/Cas9 *SDHC* knockdown, promoted EMT. These data are consistent with previous studies showing interactions of EMT in breast cancer metastases, stemness (Shen et al., 2015) and hypoxia (Gao et al., 2016).

The importance of mitochondria as a centre of cellular regulation under conditions of low energy status has been highlighted by our studies and those of many others (Cannino et al., 2018, Chiche et al., 2010, Weber et al., 2018). The effects on utilization of glucose and fatty acids as oxidative fuels is of particular interest in this

context, and PDK4 is a mitochondrial enzyme found to be important in this aspect. Furthermore, induced fatty acid oxidation in contexts of low energy status, have been reported to promote EMT phenotype through PDK4 (Sun et al., 2014). In SDH deficient cells, TCA cycle is truncated and this may affect anaplerosis. Anaplerosis is a very important feature of metabolic adaptation as it provides intermediates that help in energy provision (Brunengraber and Roe, 2006). A decrease in SDH activity may decrease the cell's dependency on oxidative OXPHOS for ATP generation, yet the cell remains with the ability to derive required biomolecules

In summary, metabolic rewiring may constitute both a driving force for changes in cellular phenotype and a physiological response to accommodate changes in the microenvironment. The eventual purpose is to support homeostasis at the cellular and systemic level, by preventing potential harmful effects and uphold vital functions in an ever-changing (patho)physiological context. The logical consequence is that targeted modulation of metabolism can be utilized as a biomedical strategy to control important cellular traits. This conclusion was repeatedly supported throughout the experiments our projects.

4.2 Mechanisms of metabolic rewiring

Although EMT is associated with cell plasticity and altered metabolic profile (Lussey-Lepoutre et al., 2015), the molecular mechanisms remain largely unexplored. Evidence support that molecular interactions between metabolism and cell plasticity in EMT may involve the metabolic sensors AMPK, mTOR and PPARs as well as HIF1α. Recent studies show that silencing of SDHB upregulates HIF1α and p-AMPKα (Chen et al., 2014). At the same time, other *in vitro* studies show that activation of PPARα inhibits HIF1α in breast carcinoma cells (Zhou et al., 2012) and inhibition of mTOR upregulates HIF1α in renal carcinoma cells (Liu et al., 2016). Interestingly, renal cell carcinoma is also associated with germline mutations in SDHB (Msaouel et al., 2017). SDH dysfunction may lead to stabilisation of HIF1α by accumulation of succinate, and increased expression of hypoxia related genes, similar to what is observed in cells exposed to hypoxia (Mohlin et al., 2017, Merlo et al.,

2017, Selak et al., 2006). This phenomenon is defined as pseudohypoxia. Hypoxia and pseudohypoxia leads to a similar shift of cellular energy metabolism (Mohlin et al., 2017). These mechanisms may also affect biosynthesis of nonessential amino acids from pyruvate through OAA, and dependence on glutamine as an anaplerotic feeder to the TCA cycle (Brunengraber and Roe, 2006, Cardaci et al., 2015).

Further, our studies underscored the relationship between cell plasticity, energy status and mitochondrial morphofunction. The decrease in mitochondrial respiration may be explained by the many changes in mitochondria that include; reduced biomass (mtDNA and organelle volume), reduced biogenesis (PGC1α) and altered structure (dispersed, thinner tubule network). These results agree with previous studies that show a correlation between EMT and mitochondrial recycling (Gugnoni et al., 2016). Mitochondria may fuse to increase efficiency in response to energy demand due to cellular insult or stage in cell cycle. However, under some conditions, fusion may also be involved in cell plasticity and stemness (Menendez et al., 2011). These observations support our findings that changes in mitochondrial morphofunction may be important in cellular phenotype transitions such as EMT.

Compared to the metabolic effects we observed in EMT, we found conceptually opposite effects after treating cells and rats with TTA. TTA has been found to act through various mechanisms to induce fatty acid oxidation, including activation of PPARs and changed energy state (Berge et al., 2001, Grav et al., 2003). TTA is a well characterised 3-thia thioether fatty acid which induces mitochondrial biogenesis in rat liver (Bremer, 2001, Skrede et al., 1997, Berge et al., 2002). In the present work, we found that increased fatty acid oxidation was associated with significantly increased expression of PDK4. PDK4 is known to promote aerobic glycolysis and metabolic reprogramming by inhibiting PDH (Kim et al., 2015, Xie et al., 2016b, Zhang et al., 2014, Sun et al., 2014). Thus, upregulation of PDK4 is linked to a metabolic switch towards increased fatty acid oxidation under metabolic adaptation both *in vitro* and *in vivo*. Inhibition of mTOR by use of rapamycin augmented the effects of TTA, suggesting that these mechanisms may act synergistically to modulate cell metabolism.

TTA also has PPAR independent activities, and our studies showed that mitochondrial uncoupling may represent one of these, supporting previous findings (Grav et al., 2003, Berge et al., 2001). Our data demonstrated increased cellular leak respiration as an indicator of uncoupling, immediately after administration of TTA, but not palmitic acid (paper 2). The uncoupling effect was far lower than for CCCP, which is a commonly used experimental uncoupler. Uncoupling may constitute one of the mechanisms potentially leading to metabolic adaptation in cells exposed to TTA, as it leads to reduced energy yield (ATP) from OXPHOS, due to dissipation of the mitochondrial membrane potential. However, since the effect appeared immediately after administration to cells, TTA probably mediates increased transmembrane proton flux through direct interactions with the membrane, a mechanism that will be independent of the increased expression of uncoupling proteins (UCPs), which has been observed after longer treatment (Grav et al., 2003, Wensaas et al., 2009). It is likely that 1-tripple TTA act through similar mechanisms as TTA, but aspects of bioavailability and stability, ligand properties, and protein and membrane interactions may be affected by the terminal triple bond (Lund et al., 2016).

We also observed an increase in the expression of malic enzyme (ME-1) after treatment with TTA. ME-1 is involved in supporting mitochondrial oxidation pathways through TCA anaplerosis (Zelle et al., 2011). Hence, increased TCA anaplerosis is anticipated to accompany increased fatty acid oxidation. Cataplerosis of TCA cycle intermediates may lead to lowering of their concentration thus the need for their replenishment from alternative sources (Brunengraber and Roe, 2006).

Generally, there were only minor changes in mitochondrial oxygen consumption and glycolysis in cells presenting contextual metabolic adaptations involving increased fatty acid oxidation. Thus, the changes in metabolic phenotype did not lead to a switch in the balance between mitochondrial oxidation and glycolysis, but rather to the use of alternative substrates to fuel mitochondrial oxidative metabolism. We interpret the steady rates of mitochondrial oxygen consumption as a sign of energy

homeostasis, yet involving a different metabolic state, maintained through contextual regulation of essential mechanisms of metabolic rewiring and flexibility.

4.3 Interventions to promote or target metabolic rewiring

Treatments modulating energy metabolism could potentially be applied in management of various disorders (Ostojic, 2017). Nutrient restriction or agents modulating metabolism, may threaten cell survival if the threshold of metabolic flexibility is exceeded. Nutrient-sensitized screening strategies have shown promising leads in targeting specific metabolic traits, e.g. related to mitochondrial functions based on selective effect on cell growth and viability (Gohil et al., 2010). Many initiatives have aimed to utilize the clinical potential caused by metabolic reprogramming in cancer. Several strategies to target tumor energy metabolism and associated signaling have been studied (Tennant et al., 2010, Hagland et al., 2007). This includes the use of agonists and antagonists of the metabolic machinery, modulators of signaling programs, and direct targeting of the mitochondria. For instance metformin, a widely prescribed anti-diabetic drug, has emerged as a potential anticancer therapeutic based on epidemiological, preclinical and clinical studies (Gonzalez-Angulo and Meric-Bernstam, 2010, Lord et al., 2018). Metformin reduces levels of circulating glucose, and activates AMPK signaling pathways. Such effects may itself lead to reduced tumor growth, but it may also sensitize therapy-resistant tumor cells to other forms of anticancer treatment. Our group has previously reported antitumor effects of several treatments, namely: a modified fatty acid, hypoxia treatment, and khat extracts, in leukemia and other cell and animal models, where metabolic modulation seems to explain some of the observed effects (Bredholt et al., 2009, Iversen et al., 2006, Tronstad et al., 2003b, Moen et al., 2009).

The energy sensors PPARs, AMPK and mTOR could be targeted as adjuncts to conventional therapy in cancer and diabetes treatment (Troncone et al., 2017). However, the mechanistic effect of modulators of fatty acid oxidation is not always clear, since it partly depends on cell-specific as well as tissue-specific effects. In paper 2 we show that activation of fatty acid oxidation by panPPAR activator TTA,

correlates with expression of PDK4, a marker of cellular aerobic energy generation. TTA has previously been shown to possess hypolidaemic effects *in vivo* (Skrede et al., 2012). In paper 3 we show that a similar compound, 1-triple TTA, has similar hypolipidaemic effects in rats.

All tissues in an organism are optimised to carry out specific metabolic processes (Shlomi et al., 2008), and may thus be differently affected by pharmacological modulators of metabolism. Treatment with 1-triple TTA was found to cause tissue-specific physiological modulation of mitochondrial fatty acid oxidation in rats (paper 3). It promoted liver-specific lowering of intracellular energy charge along with increased beta-oxidation. This was accompanied by increases in mitochondrial markers such as mtDNA and citrate synthase activity, which designated mitochondrial biogenesis, similar what was previously observed with TTA (Hagland et al., 2013).

Thioethers (e.g. TTA) and other fatty acid analogues may lower liver cell energy status, thus inducing specific adaptive responses to increase energy supply (Grav et al., 2003). Increased fatty acid oxidation was associated with increased plasma level of the ketone bodies, β-hydroxybutyrate, and liver expression of HMG-CoA synthase. Besides, the decrease in plasma TGs may be explained by increased uptake through clearance of plasma lipoproteins including chylomicrons and re-uptake of VLDLs. Indeed, we observed increased expression of LDL receptors coupled to decrease in ApoC-III expression. According to our findings, 1-triple TTA mediates a metabolic shift similar to TTA in rats, partially caused by increased mitochondrial fatty acid oxidation in the liver. Through this mechanism, the fatty acids are drained from the blood, resulting in hypolidaemic effects and an altered metabolic state (Berge et al., 2005) .

In summary, treatments with both TTA and 1-triple TTA appear to induce a physiological low energy state and a consequent starving-type response. This involves pleiotropic mechanisms, including fuel switching manifested as induction of mitochondrial fatty acid oxidation, and mild uncoupling of respiration. Tissue-

specific responses of these modified fatty acids may be explained by its activity through PPARs, but there are most likely other mechanisms involved too.

5. Conclusions

It is evident from the presented data that metabolic rewiring is a complex context-driven biological activity. Moreover, it is coordinated by cross-talk between mitochondrial and nuclear signalling that encompasses transcriptional regulation, most of which are dependent on the microenvironment. Consequently, modulation of mitochondrial functions is likely to contribute to the context-dependent changes in cell physiology.

In paper 1 we show that EMT involves cellular metabolic rewiring linked to the repression of mitochondrial respiration and that reduced SDH function may be a mandatory early step in cell phenotype plasticity exemplified by the EMT process. An inverse relationship was found between EMT signature and SDH expression in breast cancer cohorts, supporting the clinical relevance of our findings. We speculate that the observed metabolic shift associated with EMT may contribute to increased cancer cell stemness, therapy resistance and metastasis. The mechanisms involved may also be relevant in patho-mechanisms of other disorders where cellular plasticity plays a role including neurodegeneration. Further, as we find low levels of *SDHC* to be associated with survival of patients with the particularly EMT-related breast cancer subtype basal-like breast carcinoma, we suggest SDH as a prognostic marker in breast cancer diagnostics.

In paper 2 and 3 we investigated mechanisms of metabolic adaption associated with increased mitochondrial fatty acid oxidation, mediated by the modified fatty acids TTA and 1-tripple TTA, respectively. Both these compounds appeared to mediate effects consistent with a starvation-like metabolic response. The changes in mitochondrial fuel utilization appear to involve multiple mechanisms, including energy- and nutrient-sensitive regulation of gene transcription and direct interactions with the mitochondrial inner membrane (uncoupling). Although the (bio)chemical interactions of TTA and 1-tripple TTA may differ, current knowledge suggests that they have significantly overlapping mechanisms of action.

Upregulated expression of PDK4 was found to be a sensitive marker for metabolic rewiring leading to increased mitochondrial fatty acid oxidation in cells and rats. This effect indicates an overarching shift from utilization of glucose to fatty acids as oxidative fuel. PDK4 expression was found to be a consistent and sensitive in this regard, compared to the expression of enzymes more directly involved in mitochondrial fatty acid oxidation. Measurement of PDK4 expression therefore constitutes a new useful tool to study (patho)physiological adaptations of metabolism *in vitro* and *in vivo*.

Although the role of metabolism in EMT and the metabolic effects of TTA or 1-tripple TTA represent conceptually different contexts, they both involved rewiring of nutrient- and energy-dependent mitochondrial pathways in a reciprocal manner. Further studies are necessary to reveal if metabolic modulation, e.g. by compounds such as TTA or 1-triple TTA, may be used for therapeutic purposes. For instance, it should be explored if targeting cellular plasticity and EMT through intervention of mitochondrial metabolism may have anti-tumor effects.

As a very useful methodical achievement resulting from this work, we established that significant downscaling of qPCR reaction volume is feasible without compromising the analytical outputs.

6. Future perspectives

In paper 1 we studied metabolic adaptations in EMT. This is a complex stepwise process of transient cell phenotypes. It would be very informative to study the stepwise contribution of energy changes by assessing the reverse process of EMT, namely MET (Nieto et al., 2016). The molecular mechanisms and how they relate to the distant microenvironment are not well understood despite the ascribed importance in cancer metastasis (Somarelli et al., 2016). Further, it would be interesting to use targeted metabolic modulators, such as TTA or 1-tripple TTA, to investigate if a pressure to maintain high rates of mitochondrial energy metabolism may prevent EMT in cancer models, and thereby prevent or reduce malignancy.

Our data suggest that TTA and its derivatives induce starving-related metabolism and this may be responsible for hypolidaemic effects observed in rats. There is a possibility that these modified fatty acids regulate respiration through ubiquinone and SDH or that they promote non-specific leak-respiration. It would be interesting to investigate how these effects may be associated with increased ROS production and related signalling. Moreover, we could correlate this metabolic phenotype by studying metabolic profile of treated cells and rats, using large-spectrum metabolomics platforms to achieve a broader view on the metabolic landscape.

It would also be interesting to further explore the different signals of low cellular energy status e.g. associated caloric restriction, ketogenic diets, low carbohydrate, anaplerotic diet and others. These could be important in design of therapies for metabolism related diseases such as metabolic syndrome (Mandecka and Regulska-Ilow, 2018).

Finally, it would be interesting to build on the presented work to establish cellular screening models and approaches to search for new drugs that may be utilized for targeted intervention of cell metabolism in a context-dependent manner.

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