Sybille Mazurek · Maria Shoshan
Editors

Tumor Cell Metabolism

Pathways, Regulation and Biology

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Preface

Almost all papers and reviews on the altered energy metabolism of tumor cells use the nearly century-old discovery of the Warburg effect as a starting point. One practical implementation of the Warburg effect, i.e., FDG-PET scan based on the increased glucose uptake of tumor cells, has already had an enormous impact on clinical routines and decisions. By contrast, the implications and consequences of the Warburg effect for the understanding of tumor biology and hence also for future treatment strategies are still in a state of development, intense research, and excitement.

About a decade ago, when interest in the Warburg effect was clearly rekindled and growing, there was considerable focus on the roles of energy and ATP metabolism in tumor biology. This has now changed to the understanding that tumor cell metabolism is fundamentally altered—indeed, to the point of being named a hallmark of cancer (Hanahan and Weinberg 2011)—and that these alterations develop in order to support not only energy production but also macromolecule synthesis required for rapid proliferation (Ward and Thompson 2012). A complementing understanding is that these alterations continue to develop with, and to influence, tumor progression. A key illustration of the progressive alterations is the adaptability of tumors to cellular stress (in particular, nutrient and oxygen restriction, and chemo- and radiation therapy). These responses involve a remarkable metabolic flexibility which in turn can involve schoolbook biochemistry as well as newly discovered metabolic pathways and complex processes such as glutaminolysis, novel signal transduction cascades, and autophagy.

Mitochondria have already since Warburg's time been in focus; however, while they were long believed to be "damaged" in tumor cells, it is now understood that, yes, their various functions may be altered, but these changes make mitochondria efficient contributors to the metabolic plasticity of the tumor cell. The rerouting, or alternative uses, in tumor cells of classical biochemical pathways—from the tricarboxylic acid cycle to nucleotide synthesis—is therefore now under scrutiny, as is the regulation of cellular mitochondrial content. The small but complex mitochondrial genome

(mtDNA) may harbor possibly cancer-specific mutations, the significance of which needs to be examined.

That cellular metabolism is an important determinant of tumor cell phenotype as well as tumor biology thus implies an extremely complex network of possible signaling pathways, enzymes, and metabolites. In addition, while some pathways are likely common to most cancer cells, many others are not always required or present in a given cancer cell. In short, research in this field seeks to answer such diverse questions as which oncogenes initiate altered metabolism, and how; moreover, what are the connections between metabolism and resistance to therapy, and between metabolism, differentiation states, and metastasis. It is also of interest to determine which pathways are most commonly altered, and conversely, which ones might be of diagnostic or predictive value or might be targeted in individualized therapy.

It is of course impossible for one single book to cover all aspects of the influence of cellular metabolism on tumor biology and tumor cell phenotypes, and we are more than regretfully aware that the present book leaves big gaps in terms of topics. What we do present here includes on the one hand specifics such as chapters on the roles and possible clinical value of specific oncogenes, enzymes and pathways, and an example of the use of metabolic tracers, and on the other hand also more general overviews of hypoxia, autophagy, and the microenvironment, and not least, overviews of metabolic wiring and the troubling flexibility of tumor cells.

Due to the sophistication and heterogeneity of the metabolic wiring and flexibility of tumors, research in this field is still in a state of mapping and charting. We hope that this book will contribute both to a general understanding of the complexity and to further mapping and interest in these intriguing questions. To then decipher what the metabolic profiles of tumors—whether in terms of proteomes, metabolomes, kinomes, mitochondrial functions, etc.—actually imply in terms of therapeutic targets, tumor progression, and prognosis is a major task for the future.

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Part I
On the Roles of Altered Tumor Cell Metabolism in Tumor Biology

Chapter 1
Metabolic Remodeling in Bioenergetic Disorders and Cancer

Emilie Obre and Rodrigue Rossignol

Contents

1.1 Energy Metabolism, Control, and Regulation

The basic principles of energy metabolism regulation were deciphered in the late 1950s with the work of Warburg, Lenhinger, Krebs, Chance, Petersen, Weinhouse, and Vaupel among several others (Scheffler 1999; Weinhouse 1956). The regulation of controlling enzymes belonging to glycolysis, PDH complex, and Krebs cycle, all involved in ATP synthesis, mostly occurs by metabolic intermediates as

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ATP itself, citrate, F1, 6BP, and Pi. Another level of regulation of mitochondrial energy fluxes, as ATP synthesis or respiration was identified by Chance and Williams in the 1950s with the so-called respiratory control by ADP (Chance and Williams 1956; Cogliati et al. 2013). Thereafter, a large number of additional molecular regulations of oxidative phosphorylation (OXPHOS) were identified, as the recently discovered OPA1-dependent stabilization of the respiratory supercomplexes (Cogliati et al. 2013), the ATP synthase-dependent assembly of complex III (Ostojić et al. 2013), and the energy state-dependent RHEB-induced control of mitochondrial turnover (Melser et al. 2013). Consideration of the numerous means to regulate ATP transduction in the cell led to the notion of a "multistep control" of energy metabolism (Benard et al. 2010). More recently, the regulation of energy metabolism was closely linked, in a mutual way, with the control of cell growth and division. For instance, a signaling pathway central to cell biology and governed by the HiF1α transcription factor was shown to mediate a shift in a subunit of respiratory chain complex IV (Fukuda et al. 2007). Conversely, succinate accumulation in the cytosol is capable of inhibiting HiF1α degradation and to promote its stabilization (Pollard et al. 2005). Likewise, the AMP-activated protein kinase (AMPK) pathway stimulates the expression of several OXPHOS proteins when ATP needs are increased, as testified by a higher ADP/ATP ratio in the cytosol (Hardie et al. 2003). Another central pathway is the control of energy metabolism is the PGC1α pathway, a transcription co-activator, which participates in the stimulation of oxidative phosphorylation in cooperation with ERR-α or to the induction of gluconeogenesis in cooperation with HNFAα (Lustig et al. 2011). The RAS protein, involved in the control of cell mitogenic activities, also controls oxidative phosphorylation, both in cancer and noncancer tissues (Wei et al. 2012; Palorini et al. 2013; Gough et al. 2009; Telang et al. 2007). A role in modulation of OXPHOS capacity was also discovered for MYC and for p53, both of which play central roles in the control of cell growth and division, leading to the emerging concept of oncobioenergetics (Jose and Rossignol 2013). Central to this review article, a new layer of upper- or meta-regulation of energy metabolism was identified with the discovery of rewiring of metabolic circuits, governed by genetic determinants connected or not with changes in cell microenvironment. In particular, this upper level of bioenergetic control makes the link between catabolism and anabolism, thereby providing a more integrated view of cell metabolism plasticity. Prior to discussing the molecular bases and the physiology of metabolic remodeling, we provide below a rapid overview on cellular bioenergetics.

In most human tissues, mitochondria provide the energy necessary for cell growth and biological activities. It has been estimated that about 90 % of mammalian oxygen consumption is mitochondrial, which primarily serves to synthesize ATP, although in variable levels according to the tissue considered and the organism's activity status. Mitochondria intervene in the ultimate phase of cellular catabolism, following the enzymatic reactions of intermediate metabolism that degrade carbohydrates, fats, and proteins into smaller molecules such as pyruvate, fatty acids, and amino acids, respectively (Fig. 1.1). Mitochondria further transform these energetic elements into NADH and/or $FADH_2$, through β-oxidation and the

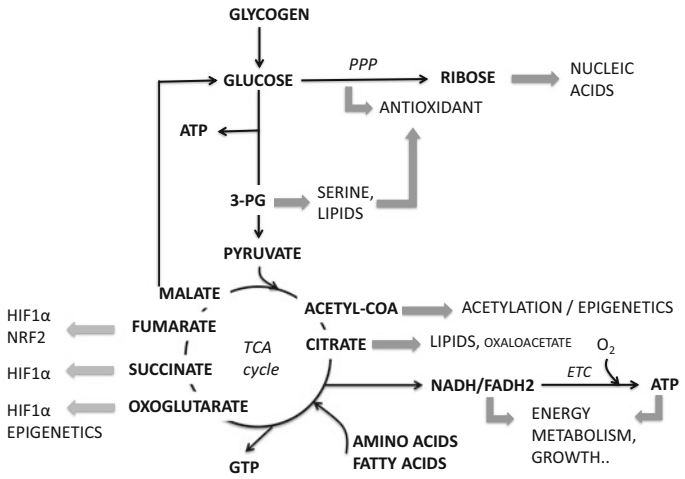

Fig. 1.1 Overview of energy metabolism pathways. In this chapter we refer to the production of biological energy in the form of adenosine triphosphate or ATP. This process occurs primarily through glycolysis, the end product of which is pyruvate and through subsequent oxidative phosphorylation. In most tissues, the pyruvate enters the mitochondrion and generates acetyl-CoA which is further oxidized at the level of the Krebs cycle to produce ATP, NADH, and $FADH_2$. The latter reduced equivalents are further oxidized by the respiratory chain to generate ATP via chemiosmosis, at the level of the F_1F_0 ATP synthase. This second mechanism of ATP production is referred to as oxidative phosphorylation. The Krebs cycle can also process alpha-ketoglutarate formed from glutamine, via glutaminolysis, or acetyl-CoA generated from fatty acids beta-oxidation. Those anaplerotic pathways are of particular importance in cancer cells. The citrate produced in the Krebs cycle can also escape this cycle (truncated Krebs cycle) and serve for lipid synthesis. As discussed in this chapter, this canonic description of energy metabolism does not apply to several cancers where the pathways are truncated (glycolysis and Krebs), rewired (anaplerotic entries from canonical or noncanonical glutaminolysis), and branched (lipid or serine synthesis from glycolysis). Therefore, while the pentose phosphate pathway and the Krebs cycle generate both reducing equivalents (NADH, NADPH, FADH2), ATP and GTP used for energy needs, these pathways also produce intermediates such as 3PG used for biosynthesis. Some metabolites such as fumarate can also modulate transcription factors as NRF2 while oxoglutarate can serve as substrate for HIF1α degradation and acetyl-CoA for histone acetylation. This figure illustrates the close link between catabolism, anabolism, and genetic/epigenetic regulations. *3-PG* 3-phosphoglyceric acid; *PPP* pentose phosphate pathway; *TCA* tricarboxylic acid cycle, i.e., Krebs cycle; *ETC* electron transport chain

Krebs cycle. Those reduced equivalents are then degraded by the mitochondrial respiratory chain in a global energy converting process called oxidative phosphorylation (OXPHOS) where the electrons liberated by the oxidation of NADH and $FADH_2$ are passed along a series of carriers regrouped under the name of "respiratory chain" or "electron transport chain" (ETC) and ultimately transferred to molecular oxygen (Fig. 1.2). ETC is located in the mitochondrial inner membrane, with an enrichment in the cristae. ETC consists of four enzyme complexes (complexes I–IV) and two mobile electron carriers (coenzyme Q and cytochrome c). These complexes are composed of numerous subunits encoded by both nuclear

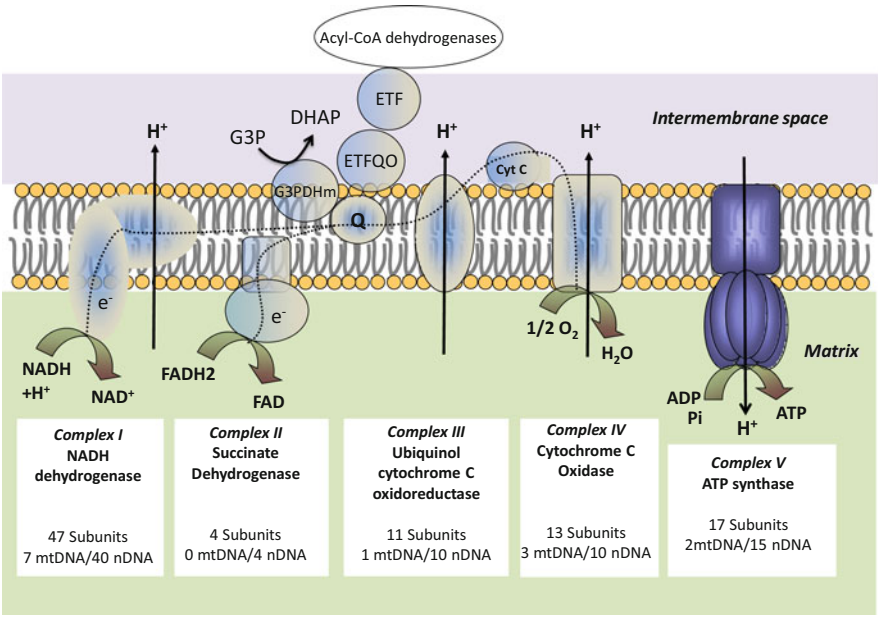

Fig. 1.2 The respiratory chain. For mammals, the respiratory chain consists of four enzyme complexes (complexes I–IV) and two intermediary substrates (coenzyme Q and cytochrome c). The NADH, H^+, and $FADH_2$ produced by the intermediate metabolism are oxidized further by the mitochondrial respiratory chain to establish an electrochemical gradient of protons, which is finally used by the F_1F_0-ATP synthase (complex V) to produce ATP, the only form of energy used by the cell. In this simple representation of the respiratory chain, the supramolecular organization (supercomplexes, dimers) is not shown. Of importance for this chapter, electrons can also be delivered to the respiration chain at the level of coenzyme Q by the ETF system or by the glycerol 3 phosphate dehydrogenase system. The respiratory chain can generate reactive oxygen species, and current research in the field of cancer metabolism indicates that such feature plays a role in metabolic remodeling, notably in metastasis. Uncoupling proteins can be expressed in the inner mitochondrial membrane to modulate ROS production. Also, different isoforms of complex IV subunits were found in cancer cells (COX4-1 and COX-2), depending on HIF1α stabilization. Lastly, mutations in mtDNA, impacting respiratory chain complexes activity, were found in a large number of tumors. *ETF* electron-transferring-flavoprotein dehydrogenase, *mtDNA* mitochondrial DNA, *nDNA* nuclear DNA

genes and mitochondrial DNA, with the exception of complex II (nuclear only). It was demonstrated that these complexes assemble into supramolecular assemblies called "supercomplexes" or respirasome (Schägger and Pfeiffer 2000; Schagger 2001). It is still debated whether some complexes, as complex I, can be found alone or if all are embedded in supercomplexes. In addition to the classic ETC components, other proteins are involved in the oxidation of nutrient-derived reduced equivalents and the subsequent reduction of coenzyme Q, used for ultimate ATP synthesis. This is the case for the electron-flavoprotein system, composed of the ETF and the ETF-QO, which connect fatty acid oxidation and coenzyme Q

reduction. The glycerol-3-phosphate dehydrogenase, which oxidizes cytosolic NADH to reduce mitochondrial FAD, also supports oxidative phosphorylation and participates to REDOX homeostasis. Lastly, the NADH-shuttling system, as the malate-aspartate shuttle, also supports OXPHOS and REDOX homeostasis via the delivery of cytosolic NADH to the mitochondrial matrix. The oxidation of NADH or $FADH_2$ by complex I or complex II, respectively, triggers the transfer of electrons from complex I (or II) to complex IV and mediates the extrusion of protons from the matrix to the intermembrane space, thus generating an electrochemical gradient of protons ($\Delta\hat{u}_H{}^+$) which is finally used by the F_1F_0 ATP synthase (i.e., complex V) to produce adenosine triphosphate (ATP), the main energetic currency of the cell. This gradient has two components: an electric potential ($\Delta\Psi$) and a chemical potential ($\Delta\mu_H{}^+$) that can also be expressed as a pH gradient (ΔpH). According to the chemiosmotic theory proposed by Peter Mitchell (1961), $\Delta\hat{u}_H{}^+ = \Delta\Psi - Z\Delta\text{pH}$, with $Z = -2.303$ RT/F.

Under physiological conditions, mitochondrial energy production can alternate between two energy steady states: basically, at state 4 (also denominated the "leak respiration state"), respiration is slow and ATP is not produced ($\Delta\Psi$ is high), while during state 3, respiration is faster and ATP is largely produced ($\Delta\Psi$ is lower). In particular conditions, such as mitochondrial inner membrane permeabilization or the use of a chemical uncoupler, $\Delta\Psi$ can be totally dispersed. As a consequence, respiration is accelerated and ATP production annihilated. The inhibition of respiratory chain complexes also generally decreases $\Delta\Psi$. Under physiological conditions, it is considered that mitochondria produce ATP in an intermediate state lying between state 3 and state 4. As shown by E. Gnaiger, respiration strongly depends on the availability of energy substrates which are multiple and can cooperate at the level of the Q-junction, thereby determining the value of the apparent maximal (uncoupled) respiration (Gnaiger 2009). ATP is the only form of energy used by the cell, and when produced in the mitochondrion, it is exported to the cytosol by the adenine nucleotide translocators (ANT1-4) in exchange for cytosolic ADP. Generally, the transport of energy metabolites, nucleotides, and cofactors in and out of the mitochondrial matrix is performed by specific transporters located in the inner membrane (Palmieri and Pierri 2010). These carriers can consume the membrane electrochemical gradient or not, depending on their mechanism of transport (electroneutral or electrogenic). A large part of OXPHOS regulation occurs at the level of these carriers, as shown by the control of the glutamate-aspartate shuttle (SLC25A12 also named Aralar or AGC1) by calcium (Fig. 1.3) and the "Gas pedal" model proposed by Frank Gellerich (Gellerich et al. 2013). Studies of metabolic control also showed that a large part of the control of mitochondrial respiration is located at the level of substrate carriers (Rossignol et al. 2000).

Therefore, the regulation of mitochondrial energy production at the level of ETC is concerted and multisite (Fig. 1.4) since modulations have been described at the level of the individual complexes, membrane leak, respirasome cohesion, or carrier activity. One should add to this molecular description the numerous signaling pathways that modulate OXPHOS properties (AMPK, HIF1α, PGC1α, RAS,

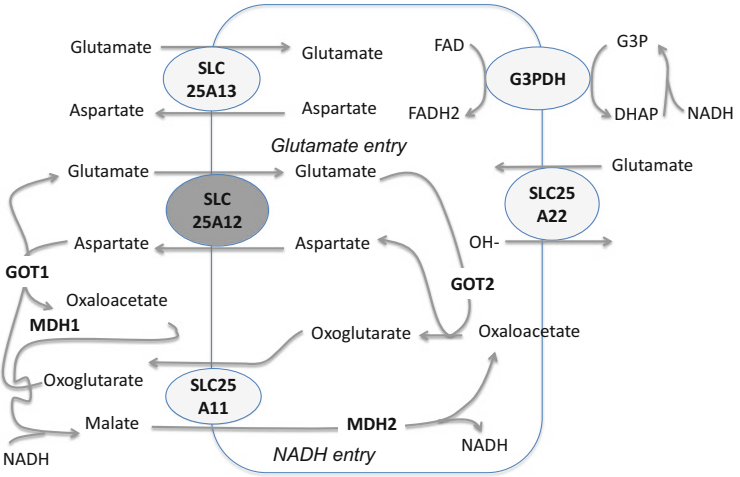

Fig. 1.3 Glutamine import in the mitochondrion. Mitochondria from mammals differ from those of yeast in numerous ways, one being the capacity to import NADH to fuel the respiratory chain. In yeast, a NADH transporter exists. In mammals, the NADH produced by glycolysis and other cytosolic reactions must enter the mitochondrion to be reoxidized by the respiratory chain, and this occurs by NADH-shuttle systems. The malate-aspartate shuttle is shown here. The cytosolic malate dehydrogenase consumes NADH to produce malate from oxaloacetate. This malate enters the mitochondrion (in exchange with alpha-ketoglutarate) where it is converted back into oxaloacetate and NADH. Oxaloacetate is transformed to aspartate by consuming glutamate, using the enzyme glutamate-aspartate aminotransferase. This glutamate is imported by the glutamate-aspartate antiporter (SLC25A12, Aralar, AGC1), so that aspartate is exported to the cytosol where it is converted to oxaloacetate. The net effect of this system in NADH entry in the mitochondrion. *GOT1 and -2*, cytoplasmic and mitochondrial aspartate aminotransferase, respectively; *MDH1 and -2*, cytosolic and mitochondrial malate dehydrogenase, respectively; *SLC* solute carrier family, *DHAP* dihydroxyacetone phosphate, *G3P* glyceraldehyde 3-phosphate

MYC, . . .); this has been reviewed elsewhere (Jose et al. 2013). To conclude, the different levels of OXPHOS regulation include (1) the direct modulation of respiratory chain kinetic parameters, (2) modulation of OXPHOS intrinsic efficiency by changes in the basal proton conductance or the induced proton conductance, (3) possible changes in the morphological state of the mitochondrial compartment, (4) modulation of mitochondrial biogenesis and degradation, and (5) in situ regulation of mitochondrial heterogeneity by the cellular and the mitochondrial microenvironment. Most of these regulatory mechanisms of mitochondrial energy transduction were discovered at the level of the respiratory chain and its surrounding lipidic environment. Below, we discuss a wider level of bioenergetic regulation (6) which considers a large-scale modification of the metabolic pathways involved in catabolism and anabolism.

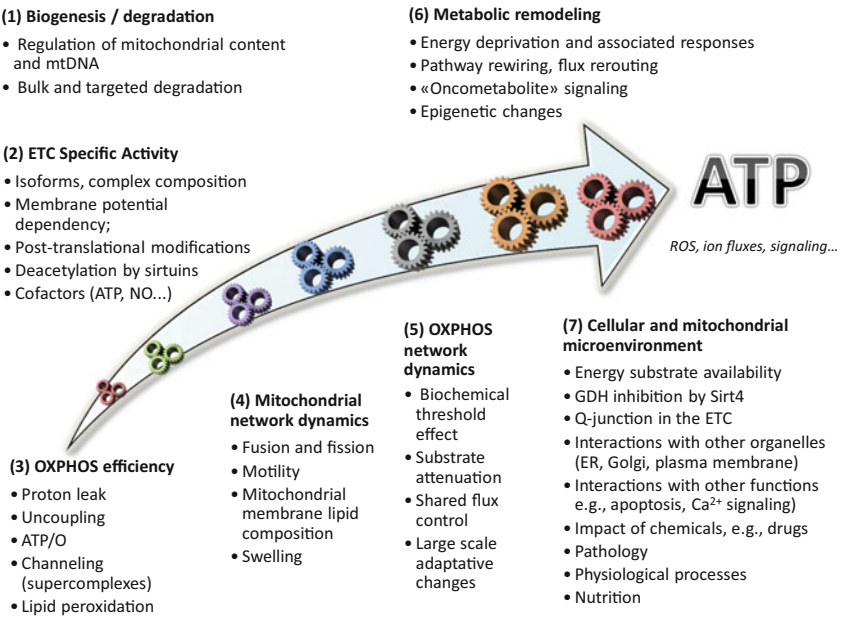

Fig. 1.4 Multisite regulation of OXPHOS. The regulation of ATP production occurs at several sites depicted along the arrow. (1) Regulation of mitobiogenesis (mitochondrial content and mtDNA levels) occurs according to energy needs by, among others, PGC1α, Sirt1, AMPK, and ERRα. Agents shown to affect these processes include AICAR, resveratrol, and bezafibrate. Downregulation occurs via RHEB-dependent bulk degradation or Parkin-dependent targeted degradation. (2) At the level of the electron transport chain (ETC), isoforms and expression levels can differ between tissues (the heart and liver isoforms of COX, for instance) and/or according to current conditions; moreover, supercomplex cohesion can vary, and complexes can harbor different posttranslational modifications, e.g., acetylation which is regulated by sirtuins. Sirtuin inhibitors, some vitamins, and cannabinoids may impact such modifications. (3) OXPHOS efficiency and the coupling of ATP synthesis to NADH and FADH2 oxidation further depend on the intrinsic properties of the proton pumps (slipping), on the ATP synthase, and on the membrane permeability to protons (uncoupling and decoupling can occur). Lipid peroxidation can hamper efficiency; this may be countered by antioxidants. (4) ATP synthesis is modulated also by mitochondrial network dynamics, involving fusion and fission of mitochondria and the overall shape and motility of the mitochondrial network, either fused or fragmented. Moreover, the mitochondrial membrane composition which impacts its fluidity and leakiness to protons can also modulate ATP synthesis; diet is suggested to affect these properties. The roles of fusion and fission proteins as bioenergetic modulators are not yet clear. Mdivi-1 is a small-molecule inhibitor of mitochondrial division. (5) OXPHOS networks or interactions: the principles of mitochondrial bioenergetics and pharmacology must be considered when trying to analyze or extrapolate genotype-phenotype relationships. For instance, the biochemical threshold effect defines a value of inhibition of individual ETC complexes above which the overall flux of respiration will collapse. This value is high (around 70 %) and varies between tissues. The control coefficient of ETC complexes is also different in different tissues, providing a biochemical basis for the tissue specificity of mitochondrial disorders. Another level of bioenergetics regulation concerns energy substrate delivery to the chain, with the phenomena of channeling, metabolic remodeling, and hormonal control of glucose and lipids catabolism. (6) A recent layer of bioenergetics control was found in the form of metabolic remodeling as extensively discussed in this chapter. Genetic or environmentally mediated toxic alterations of each of these levels have been found in human diseases, e.g., mitochondrial diseases, rare motoneuron disorders, metabolic syndrome, and neurodegenerative diseases. Drugs are also being developed to stimulate energy transduction at each of these levels. (7)

1.2 Metabolic Remodeling in Physiology and Metabolic Disorders

The different tissues of the human organism demonstrate a preference for particular energy substrates, dictated by their enzymatic equipment, linked with their physiological function. The adipocytes involved in fat storage do not contain the machinery for β-oxidation, so that fatty acids are not consumed. Likewise, the brain relies mostly on glucose and lactate, with a limited capacity to oxidize fat. Conversely, the heart favors fatty acid oxidation for ATP synthesis, while the skeletal muscle (depending on the type of fibers) consumes glucose, glycerol, fatty acids, ketones, and then amino acids as valine. The liver serves for energy storage in the form of glycerol and also fat. Therefore, tissue-specific differences in the molecular organization of the metabolic pathways determine the metabolic abilities of these tissues, as well as their storage capacity, both qualitatively and quantitatively (Fig. 1.5). Physiology can also adapt to variations in nutrient availability, as metabolic coupling exists between organs, as observed in conditions of fasting. In such situation, the liver can liberate ketone bodies which will be consumed by the brain. Intermediates of amino acids degradation, as 3-hydroxybutyrate, can also be used for gluconeogenesis in different tissues under such conditions. Studies performed in the 1970s revealed genetic mechanisms of metabolic control, as well as described the regulatory role of different hormones, as insulin, leptin, and ghrelin. Therefore, metabolic flexibility can be modulated at the level of the cell, the tissue, or the whole organism by interrelated mechanisms. As discussed below, such regulatory circuits involved in the control of metabolic plasticity could serve for therapeutic intervention in situations of excess food intake or genetic disorders, as suggested for the metabolic syndrome but also for cancer.

Metabolic syndrome is a multisystemic disease with a complex pathophysiology. One determinant is excess food intake which triggers increased (visceral) fat storage, insulin resistance, and metabolic alteration at the cellular level. Mitochondrial deficiency was observed in models of metabolic syndrome where a reduction of the respiratory rate and an increased production of reactive oxygen species were reported (Curtis et al. 2012). Accordingly, ROS-induced alterations of mitochondrial proteins were also described in the metabolic syndrome, as S-glutathionylations and carbonylations (Curtis et al. 2012). Interestingly, proteomic studies revealed that adaptative processes occurred in different diseased tissues to counter those ROS-induced damages (Peinado et al. 2014). The observed upregulation of enzymes involved in ROS and aldehydes detoxification strengthened the hypothesis of oxidative stress in the pathophysiology of the metabolic

Fig. 1.4 (continued) In particular in cancer research, the role of the microenvironment and pathological tissue in regulation of bioenergetics is attracting increasing attention, for instance, regarding the plasticity of the cancer cell in adapting to various conditions of substrate availability and hypoxia

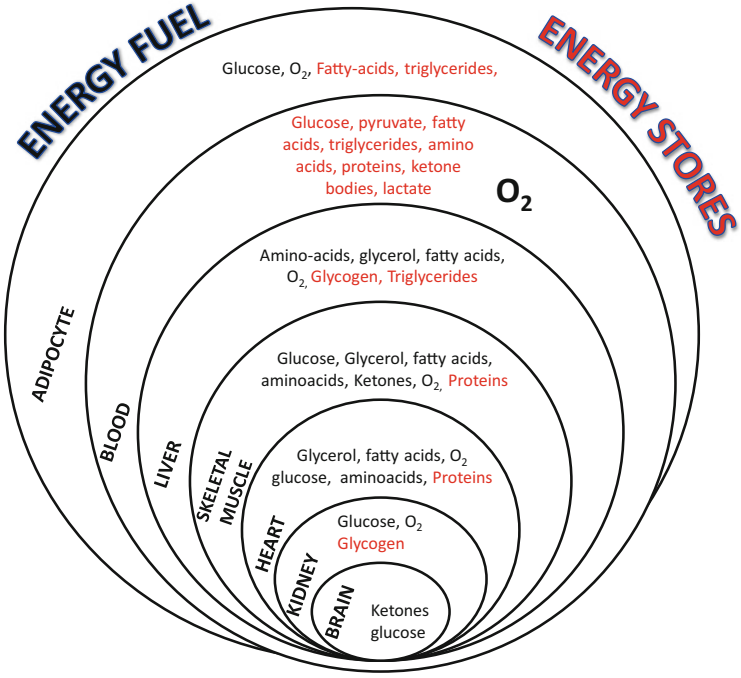

Fig. 1.5 Metabolic rigidity and metabolic flexibility. The different tissues present with specificities in the type of substrate primarily used for energy synthesis. A metabolic coupling between tissues exists to cope with this metabolic rigidity. The different tissues also differ in the form of energy they store. When a normal tissue undergoes cancer transformation, this metabolic rigidity is lost and the phenomenon of metabolic flexibility is observed. This is not a general rule as this feature also depends on the bioenergetics environment of the cancer cells

syndrome. Murphy and colleagues recently proposed that an excess of ROS could participate in the metabolic remodeling described in tissues from patients with the metabolic syndrome (James et al. 2012). Such remodeling includes the cleavage of aconitase, a TCA enzyme which normally transforms citrate in isocitrate. When aconitase is cleaved (Bulteau et al. 2003), citrate accumulates in the matrix and leaks out from the mitochondrion by a dedicated carrier. In the cytosol, the ATP citrate lyase (ACLY) cleaves citrate in acetyl-CoA and oxaloacetate, so that lipid synthesis can proceed from acetyl-CoA (Hatzivassiliou et al. 2005), while oxaloacetate can enter neoglucogenesis. This circuit of truncated Krebs cycle can accommodate constant overfueling of the mitochondrion by pyruvate generated from carbohydrates. The alteration of the Krebs cycle toward anabolism was also observed in some cancer cells, where truncation of the Krebs cycle allows lipid synthesis from citrate, produced either from pyruvate (as in metabolic syndrome) or from glutamine (as in cancer) (Fig. 1.6).

In the case of cancer, there are no excess food intake, but a strong demand for both ATP production and various biosyntheses as lipids. The role of ACLY goes

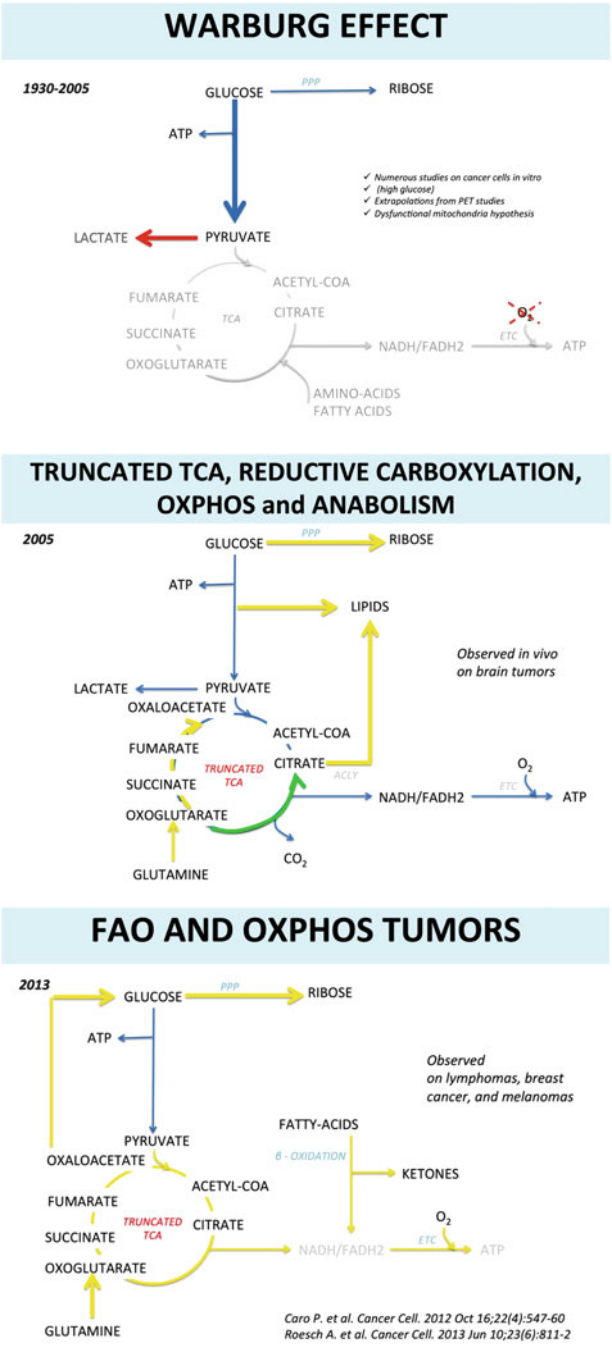

Fig. 1.6 Examples of metabolic remodeling. In addition to the classic Warburg effect (high glycolysis and poor OXPHOS), other types of metabolic remodeling were recently described in cancer cells. They include lipid synthesis from glutamine or oxidative tumors deriving ATP from fatty acid oxidation and from amino acid degradation. This is discussed in the text

beyond lipid synthesis, as the acetyl-CoA generated by this enzyme can serve for histone acetylation, and the subsequent regulation of bioenergetic genes expression, as LDHA (Wellen et al. 2009). This loop of bioenergetic control evidenced in cancer was not studied in metabolic syndrome. Another example of metabolic remodeling in metabolic disorders was described in genetic mitochondrial diseases (Saada 2014). The group of M.J. Falk recently investigated the common downstream effects of primary respiratory chain dysfunction on global gene expression and pathway regulation. To this end, a bioinformatic analysis of transcriptome datasets from all publicly available studies of respiratory dysfunction resulting from genetic disorders, acute pathophysiologic processes, or environmental toxins was developed.

This analysis revealed the cellular and tissue adaptative response to mitochondrial dysfunction, which identified several commonly dysregulated genes across diverse mitochondrial diseases etiologies, models, and tissue types (Zhang and Falk 2014; Zhang et al. 2013). In particular, the so-called integrated nutrient-sensing signaling network (NSSN) centered on the AKT/mTORC pathways appeared to be one central mediator of the cellular response to respiratory chain dysfunction. NSSN includes the AMPK (low energy sensor), mTORC1 (cell growth regulator by balancing cytosolic protein synthesis and autophagy), SREBP (lipid homeostasis), *FOXO1* (glucose homeostasis), and *PPAR* family transcription factors (lipid metabolism), as well as *YY1*/*PGC1α* (mitochondrial ribosome biogenesis) and *HIF1α* (hypoxia response) transcription factors. The metabolic remodeling suggested by such transcriptomic analyses and the associated GSEA and KEGG functional analyses revealed a modulation of the genes involved in fatty acid and amino acid metabolism, as a central feature of metabolic remodeling in mitochondrial diseases. This type of study indicates that cells or tissues carrying an ETC defect do not simply rely of the Pasteur effect to activate glycolysis to generate ATP, but that a more profound metabolic remodeling occurs to fulfill other needs that remain to be identified, in order to propose innovative therapeutic approaches. A recent study on resveratrol showed that fibroblasts from patients carrying a complex I or a complex IV defect can be rescued at the level of respiration and ATP synthesis by treatment with this drug (Lopes Costa et al. 2014). In this study, two types of patients were identified as responders or nonresponders. The differences between these patients are not well understood at the molecular level, and a thorough proteomic analysis of metabolic remodeling could provide such information.

1.3 Molecular Basis of the Metabolic Flexibility of Tumors

At the molecular level, metabolic flexibility relies on the rewiring of existing metabolic pathways and the synthesis/degradation of metabolic "pathway switching proteins" or "alternative pathway enhancing proteins" which allow an efficient rerouting of metabolites selected by cellular needs. A thorough

investigation of the molecular and signaling mechanisms underlying cancer cells (and other metabolically diseased cells) metabolic remodeling could allow to identify "pathway switching proteins" and "alternative pathway enhancing proteins," which could be considered as innovative targets for the metabolic therapy of cancer. One strategy to alter the metabolic flexibility of cancer cells resides in the ability to block the catalytic function of PSPs or APEPs. As shown in Fig. 1.6, different types of metabolic remodeling have been reported. The first observation by Otto Warburg revealed that some tumors consumed large amount of glucose without a parallel consumption of oxygen but increased production of lactate (Warburg 1930). Following these seminal findings, many studies addressed the molecular basis of the Warburg effect (Jose and Rossignol 2013). Several mechanisms were identified, as the stimulation of glycolysis by oncogenes (the so-called high glycolysis), notably via the expression of rapid fetal-like isoforms. The Warburg effect also raised the hypothesis of dysfunctional mitochondria, to explain why pyruvate was not degraded by the Krebs cycle. During his Nobel Prize Lecture, at Lindau, Germany, in 1966, Otto Warburg stated that "The prime cause of cancer is the replacement of the respiration of oxygen in normal body cells by a fermentation of sugar." From the early 1990s until now, molecular mechanisms were discovered at the level of the mitochondrion to explain the Warburg effect, with the inhibition of PDH by PDK1 overexpression (controlled by HIF1α), the reduction of mitochondrial biogenesis (notably triggered by p53 inactivation), or the inhibition of respiratory chain activity [also triggered by mutant p53 via SCO2 and by HIF1 α through a COX4-1/2 subunit isoform shift (Fukuda et al. 2007)].

Yet, it is very important to mention here that not all cancer cells conform to the Warburg effect and that some cancer cells represent an opposite phenotype, i.e., with enhancement of the OXPHOS system (Fig. 1.6). As we discussed in a previous article, a large body of evidence indicates the existence of oxidative cancer cells and tumors both in vitro and in vivo (Jose et al. 2011a). Already in 1976, Reitzer LJ reported that "in HeLa cells glutamine provides more than half of the cellular energy by aerobic oxidation from citric acid cycle metabolism when glucose is present." Likewise, the idea that glucose, glutamine, hydroxybutyrate, or palmitate can serve both for energy production and anabolism (lipid and cholesterol synthesis) was experimentally tested (Morton et al. 1976). This work demonstrated that freshly excised Morris hepatomas can oxidize palmitate and hydroxybutyrate to produce ATP. The molecular determinants of this oxidative phenotype include the activation of mitochondrial biogenesis, the stimulation of fatty acid oxidation, the stimulation of canonical or noncanonical glutaminolysis, and the activation of amino acid degradation pathways. The "oxidative phenotype" of cancer cells illustrated in Fig. 1.6 (bottom panel where ATP is produced by OXPHOS from fatty acid oxidation or glutamine oxidation) was found in lymphomas, melanomas, glioblastomas, and breast cancer. Between these two extreme phenotypes, i.e., the Warburg (glycolytic) and the "oxidative," other types of metabolic remodeling were recently described. Indeed, looking at the anabolic side, studies revealed the existence of Krebs cycle truncation aiming at the conversion of glutamine to lipids,

via citrate extrusion from the mitochondrion and production of acetyl-CoA using the enzyme ATP citrate lyase.

Two modes of glutamine utilization have been described, with the Krebs cycle running in the textbook direction "clockwise" or in the deviant mode "anticlockwise" (Mullen et al. 2012; Metallo et al. 2012; Fendt et al. 2013; DeBerardinis et al. 2007). The first mode requires anaplerotic entry of glutamine carbons in the TCA at the level of oxoglutarate and the normal route toward citrate, which require the use of acetyl-CoA derived from pyruvate. The second mode of glutaminolysis (anticlockwise) is made possible by the accumulation of oxoglutarate and the presence of high levels of NADH, as found in situations of defective oxoglutarate dehydrogenase and succinate dehydrogenase. Reversal of the truncated TCA is facilitated by the isoform shift of IDH from IDH2 to IDH3 which consumes NADH. Little is known on the metabolic remodeling which utilizes fatty acids as carbon substrates or amino acids as valine, since the acetyl-CoA produced by β-oxidation could serve for ketogenesis but also other means, as reentry in the TCA. It may be pointed out here that most of the metabolic deviations were discovered during attempts to understand the link between mutations in TCA cycle enzymes such as SDH, IDH1/2, and FH and a predisposition to tumors as diverse as hereditary paragangliomas (Niemann and Muller 2000), leiomyomas (Pollard et al. 2005), and glioblastomas multiforme (Parsons et al. 2008). The fate of branched-chain amino acids or ketone bodies is also poorly described, despite reports that indicate the use of such energy sources by different types of tumors (Martinez-Outschoorn et al. 2012). Strong advances in the field of metabolic remodeling were presented by the group of R. DeBerardinis who investigated the fate of glucose and glutamine in different types of cancers, both in vitro and in vivo, even in human subjects (Marin-Valencia et al. 2012a). In 2012, this group reported that glucose oxidation by the mitochondrion is active in glioblastomas, as measured in the mouse brain in vivo (Mullen et al. 2012). Interestingly, glucose was converted to CO_2 and glutamine. Analysis of human gliomas xenografts also showed that glutamine can regenerate glucose through neoglucogenesis, evidencing the complexity of tumor metabolic remodeling. As mentioned in the introduction, neoglucogenesis typically occurs in the liver, while here malignant brain cells are capable of doing so from glutamine. The study of brain tumors metabolism by NMR showed that ^{13}C glucose is converted to lactate, glycine, glutamate, and glutamine, indicating again the importance of glutamine synthesis in brain tumors (Marin-Valencia et al. 2012b).

1.4 The Signaling Pathways Involved in Metabolic Remodeling

The understanding of metabolic remodeling in cancer cells and other diseases also requires the investigation of the signaling mechanisms involved in pathways switching. So far, many determinants have been found to explain the Warburg

type of metabolic remodeling, with well-described roles for HIF1α, MYC, p53, PTEN, PI3K, Akt, LKB1, and AMPK on most glycolytic enzymes and LDH. At the level of the mitochondrion, PDH inhibition depends on HIF1α, and glutaminolysis is activated by MYC, as well as lipid synthesis from citrate (at the level of ACLY). A general negative regulator of the Warburg effect is AMPK, in agreement with the frequent alteration of the LKB1-AMPK axis in cancer cells (Faubert et al. 2013). So far, no cancer-related dysregulation has been discovered at the level of the newly identified pyruvate carrier (Herzig et al. 2012), which could participate in the onset of the Warburg effect.

Recently, some signals and genes involved in anabolic stimulation, and in connection with glycolysis, were discovered. It was found that when cancer cells are confronted with serine deprivation, they activate the mTOR pathway to stimulate PKM2 protein synthesis, which in turn shifts glycolysis to its anabolic mode, thereby providing serine through the phosphorylated pathway (Ye et al. 2012). It was further discovered that in addition to mTOR, the protein TP53, a target of p53 tumor suppressor, spares the available serine for glutathione synthesis and limits other utilizations (Maddocks et al. 2013). In addition to mTOR and P53, the HIF1α pathway was shown to control PKM2 and PDK1 expression under hypoxia. The former protein drives anabolism from glycolysis, while the latter blocks fueling of the Krebs cycle with acetyl-CoA derived from glucose. The oncogene N-MYC was recently shown to induce a large-scale remodeling of energy metabolism in human cancer cells, with an activation of fatty acids oxidation concomitant with a stimulation of glycolysis (Zirath et al. 2013). Analysis of C-MYC bioenergetics properties also showed that activation of this oncogene stimulates OXPHOS, redirects glutamine toward lipids synthesis, and gives the preference to glutamine instead of glucose to fuel the energetic machinery (Wise et al. 2008).

The inhibition of P53 tumor suppressor also triggers the stimulation of glycolysis (notably via TIGAR inhibition), along with the inhibition of oxidative phosphorylation via respiratory chain complex IV destabilization [through SCO2 (Matoba et al. 2006)]. Lastly, the discovery of oxidative tumors in lymphoma by the group of Nika Danial revealed a switch toward fatty acid utilization controlled by PPARγ alpha and a successful cancer-killing strategy (in vitro) using a PPARγ antagonist (T0070907) (Caro et al. 2012a). Of central importance for the regulation of oxidative phosphorylation, the RAS oncogene was shown to stimulate respiration, by molecular mechanisms which remain unclear (Wei et al. 2012; Gough et al. 2009; De Groof et al. 2009; Baracca et al. 2010; Weinberg et al. 2010). Cancer bioenergetics studies revealed that oxidative phosphorylation is required for K-RAS to promote tumor progression, notably through the activation of mitochondrial respiration and the subsequent production of reactive oxygen species. K-RAS also stimulates the cytosolic part of the glutamate-aspartate shuttle (GOT1, MDH1, ME1) (Son et al. 2013) used to reoxidize the cytosolic NADH.

Besides the metabolic remodeling triggered by oncogenes, variations in the microenvironment can also induce pathway rewiring or branching, as discussed above in conditions of serine deprivation or as found in situations of glutamine deprivation. Upon glutamine removal, cancer cells rely of the pyruvate carboxylase

to fuel the Krebs cycle and to generate citrate by a truncated TCA (Cheng et al. 2011). The metabolic remodeling that occurs in conditions of glucose deprivation is less documented despite the fact that aglycemia is encountered in tumors [we discussed this point in a recent review (Jose et al. 2013)]. In addition to these biochemical studies, recent findings suggested a link between the metabolic remodeling of tumors and chemoresistance: a subclass of resistant melanomas undergo a shift toward OXPHOS which opened a therapeutic window by using OXPHOS inhibitors as oligomycin or BZ-423 (Roesch et al. 2013). Likewise, metabolic remodeling has implication for epigenetics, cell anchorage, metastasis, and immune response, which demonstrate the importance and complexity of its investigation.

We described above a series of metabolic remodelings found in various types of cancer cells. So far, it cannot be said that only a limited number of profiles exists in tumors, albeit with variations in the pathways utilized. Yet, one can distinguish types of remodeling patterns, the objectives of which are (1) lipid synthesis from glutamine, (2) serine synthesis from glutamine or pyruvate, (3) ATP synthesis from fatty acids or amino acids, and (4) ROS generation by the ETC. The need for the tumor to consume building blocks such as arginine and asparagine inspired the therapeutic strategy to utilize arginase and asparaginase, which proved to be efficient. Likewise, we must understand the biological significance of the observed metabolic remodeling to efficiently target pathways of vital importance for tumor growth and progression. The inhibition of fatty acid oxidation by PPARγ blockers was efficient on selected lymphomas as shown in the study of Nika Danial's group (Caro et al. 2012b), and the inhibition of lipolysis and subsequent fatty acid oxidation by orlistat and etomoxir was efficient on a mice model of leukemia (Samudio et al. 2010). Again, these findings indicate the need for a better stratification of human cancer based on their bioenergetic and associated biosynthetic profile. Clearly, an exhaustive evaluation of tumor proteomics cannot be done for each patient, but relevant biomarkers could help to delineate the metabolic profile of a given tumor, taken as a whole, and to propose adapted metabolic therapies. Ideally, circulating biomarkers could be discovered, and the potential of metabolomics must be considered to reach that goal. We also need to better connect, if relevant, the oncogenetic signature with the metabolic remodeling pattern, to evidence potential links between a particular subgroup of tumors (e.g., RAS mutated, or EGFR mutated, or resistant to a certain therapy) and specific metabolic features. Are MYC-driven tumors more prone to glutaminase therapy? Are RAS-driven tumors more sensitive to OXPHOS-targeted approaches?

A recent study by the group of JE Sarry on the anticancer effect of the AMPK agonist metformin (Scotland et al. 2013) showed that not all leukemic cells are sensitive and that the cell capacity to perform the Pasteur effect was a good indicator of their sensitivity. Likewise, we tested the anticancer effect of AICAR on different cancer cell lines (Jose et al. 2011b, 2012) and showed that different sensitivities as well as different modes of action could be found in each cell line. This argues again in favor of requisite a metabolic profiling of tumors prior to consider a metabolic therapy.

1.5 Future Directions in the Field of Energy Metabolism

As argued in this review, cancer research on tumor energy metabolism partly aims at the identification of specific mechanisms used by cancer cells to transduce energy from different sources of carbons, as glucose, fatty acids, amino acids, or ketone bodies. In 2013, around 400 publications were reported in the PubMed database with the association of the terms "cancer" and "energy metabolism." Likewise, 90 clinical trials reported in the clinicaltrial.gov database also associated these two terms. How cancer cells transduce energy is determined by several factors which include the oncogenetic profile and the microenvironment, notably the type and concentration of available energy substrates, as well as the metabolic cross-talk with surrounding cells. The modalities of energy transduction are also closely determined by anabolic needs as amino acids, lipids, and nucleic acid syntheses, which consume carbon intermediates generated by the bioenergetic pathways, thereby impacting the modalities of energy transduction and the selection of appropriate "branched pathways" according to specific needs. Metabolic remodeling is also controlled, directly or indirectly, by numerous oncogenes and tumor suppressors. Hence, to determine the metabolic profile of a tumor requires the combination of series of investigations including bioenergetics, metabolomics, proteomics, and transcriptomics. Studies in vivo or on freshly excised tumors should be preferred to in vitro analyses on cancer cells which adapt to the artificial cell culture conditions and may not retain the particularities of human tumors that we need to decipher in order to propose innovative therapeutic strategies. A global analysis of catabolism and anabolism must be undertaken on those tumor samples to determine the biological objective of tumor metabolic remodeling. If serine is one endpoint, a strategy aiming at serine deprivation in tumor could be developed, as done for arginine and asparagine in leukemias. Undoubtedly, in silico reconstruction of metabolic pathways and their deviations will help to resolve such objectives and to test the validity of different bioenergetics targets.

Moreover, the transcriptomic study of large panels of human tumors, well clinically and genetically annotated, could allow stratification of tumors in bioenergetic groups, based on the expression level of different markers involved in metabolic switches, such as ACLY, PC, and IDH2/3. The direct assessment of tumor metabolic profile in vivo will give more accurate information on which pathway to target. However, this raises the problem of tumor internal heterogeneity. While genetic studies of tumors show the coexistence of cancer cell clones of different adaptative and resistance abilities, the biochemical studies performed on tumors do not yet take into account this heterogeneity.

Importantly, the problem of metabolic resistance will have to be considered, as suggested by the great metabolic flexibility of cancer cells. Therefore, a better definition of the link between the large-scale metabolic remodeling of tumor and their extended oncogenetic profile is required to adapt metabolic strategies that could be the more effective. In particular, to target "nodal enzymes" at the interface between catabolism and anabolism requires (1) their identification and

(2) assessment of their regulating power (flux control coefficients). However, the complexity of cell metabolism in terms of numbers of reactions and biochemical intermediates strongly impedes our capacity to perform in vitro metabolic and bioenergetic analyses and thereby also the discovery of novel targets for cancer metabolic therapy. The metabolic charts indicate the existence of several subpathways potentially involved both in energy transduction and anabolism, but only some of them have been explored so far in the context of cancer adaptation to metabolic stress. The power of in silico analyses of cancer metabolism is needed to (1) identify the possible routes of energy transduction linked with anabolism; (2) select the optimal ones, with the better performance and significance for cancer metabolism; and (3) designate the enzyme with the highest control of the identified pathways.

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Chapter 2
Tumor Cell Complexity and Metabolic Flexibility in Tumorigenesis and Metastasis

Michael V. Berridge and Patries M. Herst

Contents

2.1 Introduction

Reductionist approaches to the genetic drivers of carcinogenesis and the biochemical and cellular hallmarks of cancer have increasingly fueled efforts to understand and treat cancer. These approaches have largely involved aggressive transplantable

A previous version of this chapter included an incorrect figure (Fig. 2.1. was represented incorrectly). For this reason an erratum has been published, correcting the mistake in the previous version and showing the correct figure (see DOI 10.1007/978-3-7091-1824-5_16). The version readers currently see is the corrected version with the figure in chapter 2 shown correctly. The reader sees the chapter in its intended form. The Publisher would like to apologize for the earlier mistake.

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tumors or tumor cell lines cultured in the laboratory under conditions that are near optimal for cell growth and division. In patients, however, cancer cells are part of a developing tumor in a tissue that strives to maintain homeostasis through numerous local and systemic control systems. Developing cancer cells are exposed to a constantly changing microenvironment. They face selective pressures dictated by interactions with normal tissue cells of similar and unrelated origin including stromal, vascular, and neural cells and cells of the innate and adaptive immune system. Tumor spread is primarily mediated by the proximity of blood and lymphatic vessels although some tumors including prostate and colorectal cancers can spread by perineural invasion (Liebig et al. 2009; Magnon et al. 2013).

The vast majority of tumors are heterogeneous mixtures of cancer cells at different stages of development and normal cells of mixed origin, with cancer cell heterogeneity loosely related to the hierarchical structure of the tissue of origin. Thus, within a developing primary tumor, stem cell-like cells, early progenitor cells, and differentiating cells coexist and compete with normal cells for nutrients and other resources. Genetic and epigenetic changes are played out within the framework of tissue microenvironment. From this complex tissue biology, circulating tumor cells emerge and metastatic progression is initiated.

This review explores how genetic and epigenetic events drive complex changes in cancer cell energy metabolism that are associated with tumorigenesis and metastasis and discusses how the acquisition of metabolic flexibility advantages tumor cells over their normal counterparts.

2.2 Tumor Cell Complexity

Cancers originate in particular tissues of the body. Most are carcinomas of epithelial origin. Others are of endothelial, neural, and mesenchymal origin and leukemias and lymphomas of the blood-forming system. Thus, cancer initiation is tissue context dependent and occurs against a background of developmentally determined tissue modules that make up the various organ systems of the body. Normal tissue development, which occurs as part of organogenesis in the early embryo, involves epigenetic remodeling. In contrast, cancer development involves the sequential accumulation of DNA mutations, often in stem cell-like cells or early progenitor cells within a particular tissue (Herst and Berridge 2013), as well as epigenetic reprogramming. A good example is the process of epithelial-to-mesenchymal transition (EMT), a multistage process that is essential for embryonic development. In cancer, EMT drives the conversion of fully differentiated epithelial cells into poorly differentiated, migratory, and invasive mesenchymal cells through a combination of genetic and epigenetic changes. The essential features of EMT are disruption of intercellular contacts and enhanced cell motility leading to escape from parental epithelial tissue. The resulting mesenchyme-like phenotype is migratory and invasive leading to metastatic progression (Guarino et al. 2007; Tiwari et al. 2012; Wang and Shang 2013). Thus, cancer can be considered as a new disorganized tissue with a set of hallmarks that distinguish it from its tissue of origin (Hanahan and Weinberg 2011).

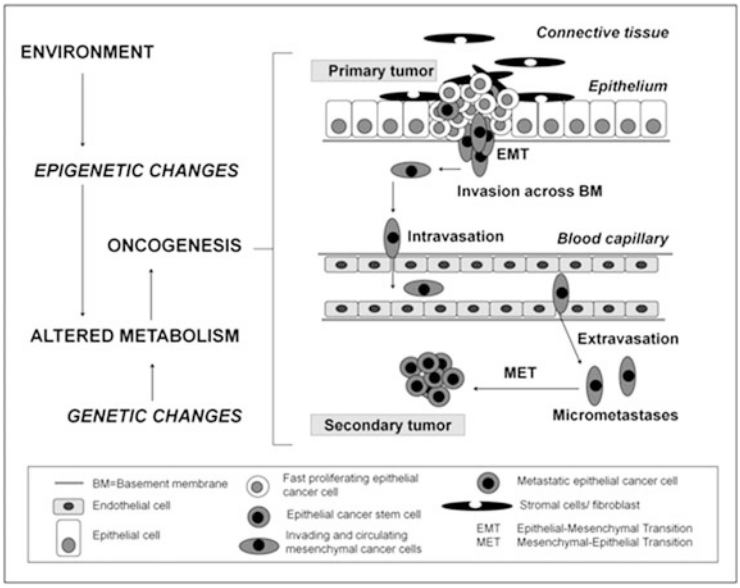

Fig. 2.1 Overview of oncogenesis of epithelial tumors. Oncogenesis takes place in context of the tumor microenvironment that includes oxygen tension, pH, nutrient supply, and interactions with other cell types in close proximity of the tumor, such as stromal cells and immune cells. Initiation begins with oncogenic mutations or epigenetic changes in expression of tumor suppressor genes and oncogenes of an epithelial cell, leading to alterations in cancer cell metabolism. Altered metabolism drives the establishment of a primary epithelial tumor which is initially contained within its tissue of origin. Some cancer stem cells undergo EMT, which makes them less sticky and more aggressive, breaking the basal membrane barrier and invading underlying tissues. Some of these mesenchymal tumor cells enter blood vessels (intravasation) and travel in the blood stream as circulating tumor cells. Once they leave the blood stream (extravasation), they form micrometastases in new tissues and organs. Once they undergo MET, they grow into macrometastases or secondary tumors of epithelial origin

Cells in tissues are hierarchically organized and constitute organ structures that are complex mixtures of cells that include mesenchymal, vascular, lymphatic, and neural cells as well as cells of the innate and adaptive immune system. Each of these cell populations plays a distinct role in facilitating or compromising tumor growth and spread. For example, mesenchymal cells generate the connective tissue that structurally supports the tumor as it grows first at its primary site and later during metastasis. Vascular endothelial cells and, in some cases, neural cells develop structures that connect the growing tumor with its wider environment providing nutrients and oxygen and coordinating pseudo-physiological responses. These structures are also centrally involved in tumor metastasis that involves breaking constraints on tissue boundaries, basement membrane penetration, intravasation, circulation, extravasation, and seeding in tissues of distant organs (see Fig. 2.1). In addition, infiltrating immune cells including T cells, monocytes, macrophages, and dendritic cells contribute to or hinder tumor progression by generating pro- and anti-inflammatory responses that

protect against or mediate immune attack to which tumor cells respond by mounting evasive responses (Croci et al. 2007; Stewart and Smyth 2011; Choi et al. 2013).

Context is paramount in the development of cancer. The tendency to develop cancer is inherited in only 5–10 % of all cancers. Not surprisingly, these familial mutations often occur in tumor suppressor genes such as *BRCA1* and *BRCA2* in breast and ovarian cancer, *TP53* in, for example, Li–Fraumeni syndrome, *APC* in familial adenomatous polyposis, and mismatch repair genes in colorectal cancer. Although these inherited mutations are present in all cells, tumors only develop in particular organs and thus are tissue context related.

2.3 Tumor Cell Hierarchy and Differentiation Therapy

Tumors are categorized in terms of location, size, cell type, state of differentiation, local invasion, and spread to lymph nodes and distant organs. This reflects the hierarchical nature of the tissue of origin. Normal tissue hierarchy is based on the presence of several distinct cell types, each with a specific role in tissue function and maintenance. Individual tissues have small populations of stem cells with self-renewing capacity, and these cells have the ability to differentiate into more than one cell type. Stem cells give rise to committed progenitors cells with limited ability to self-renew. These progenitor cells have the ability to respond rapidly to physiological demand by undergoing a number of divisions prior to differentiating into functional end cells. A good example of hierarchical tissue is the hemopoietic system that generates the different blood cell types. Pluripotent stem cells in the bone marrow produce committed myeloid and lymphoid progenitors that ultimately produce 8–10 terminally differentiated blood cells. Each lineage can be further subdivided into numerous functional and tissue-specific cell types. Most tumor-initiating mutations are thought to occur in stem cell populations or in committed progenitors, and it is the expansion of these mutant cells at the expense of cells with a more differentiated phenotype that characterizes individual cancers. This was first described in 1997 for acute myeloid leukemia (Bonnet and Dick 1997) and subsequently extended to solid tumors (Reya et al. 2001; Visvader and Lindeman 2008; Baccelli and Trumpp 2012). Differentiation plasticity has also been described in some tumors including melanoma, pancreatic cancer, and head and neck squamous cell carcinoma (HNSCC) with re-expression of germ line or early developmental markers a common characteristic (Caramel et al. 2013; Giudice et al. 2013; Ziv et al. 2013).

The stem cell nature of cancer is also reflected in the concept of differentiation therapy (Warrell et al. 1991) whereby cancer is treated by removing differentiation blocks. The best example is the treatment of acute promyelocytic leukemia (APL) patients with all-*trans* retinoic acid in combination with the cytotoxic drug arsenic trioxide. This is now standard therapy for APL and has raised 5-year survival rates from <40 % to >90 % (reviewed by Lallemand-Breitenbach et al. 2012). Recently, Aurora kinase A inhibitors were shown to terminally differentiate leukemic cells

responsible for acute megakaryocytic leukemia, primary myelofibrosis, and myelodysplastic syndrome by inducing polyploidy (Krause and Crispino 2013). Other studies have reported that salinomycin, a livestock antibiotic, induced cell death and differentiation in HNSCC stem cells (Kuo et al. 2012).

A better understanding of the cell of origin and the environmental constraints on developing tumors will enhance our ability to target tumor-initiating cells and limit their invasive and metastatic potential.

2.4 Bioenergetic Pathways of Proliferating, Self-Renewing, and Differentiating Cells

The two main energy-producing pathways are glycolysis and mitochondrial electron transport (mtET) combined with oxidative phosphorylation (OXPHOS) as depicted in Fig. 2.2. Under hypoxic conditions, HIF-1α stabilization results in the transcription of downstream hypoxia-inducible genes which mediate glycolysis. Degradation of HIF-1α under normoxic conditions results in a switch from glycolytic to mitochondrial energy metabolism (Brahimi-Horn and Pouyssegur 2006; Denko 2008; Majmundar et al. 2010).

Although bioenergetically more favorable than glycolysis, mtET generates reactive oxygen species (ROS) at respiratory complexes I, II, and III, compromising genome integrity (Herst and Berridge 2006). Interestingly, the choice of energy metabolism is not determined solely by oxygen tension. Differentiated cells primarily use OXPHOS for their energy requirements, whereas rapidly proliferating cells use aerobic glycolysis to fuel their anabolic metabolism (Vander Heiden et al. 2009; Berridge and Tan 2010; Schulze and Harris 2012). This cellular bioenergetic strategy has been rationalized in terms of balancing energy requirements and the production of metabolic intermediates with the need to maintain genomic integrity. The high-energy demands of physical movement and brain function are likely drivers behind the evolution of OXPHOS in complex organisms with the less energy-efficient glycolytic pathway downgraded to a minor component of aerobic respiration, albeit an essential one. Muscle and brain cells, that use over 80 % of the body's energy resources, are nondividing cells and are therefore largely impervious to increased ROS levels produced during mtET. In contrast, rapidly dividing cells need to maintain DNA integrity and therefore bias their metabolism towards glycolysis. Inefficient respiration due to environmental and nutrient stress also increases ROS production that would be unfavorable to proliferating cells.

Embryonic stem cells (ESC) and pluripotent stem cells (PSC) from blastocysts rely on glycolysis even under aerobic conditions and have little oxidative capacity (Prigione and Adjaye 2010; Zhang et al. 2012; Shyh-Chang et al. 2013). They have low numbers of mitochondria and few copies of mtDNA per cell (Facucho-Oliveira and St John 2009; St John 2012). Early embryonic developmental stages are

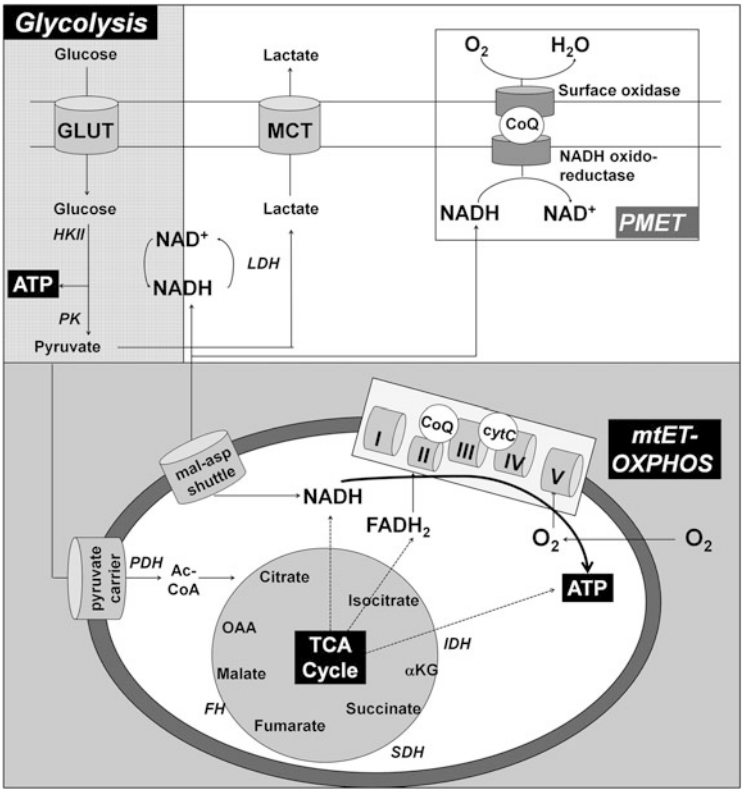

Fig. 2.2 Overview of energy-generating pathways in cells. Under hypoxic conditions, cells use glycolysis (*hatched gray box*) to generate energy. Glycolysis is the oxidation of glucose to pyruvate in the cytoplasm and is associated with production of lactate and PMET. In the presence of oxygen, most cells will use aerobic respiration to generate energy. This pathway consists of glycolysis plus mitochondrial electron transport (mtET) coupled with oxidative phosphorylation (OXPHOS) (*dark gray box*) that occurs in the mitochondria. Although mtET/OXPHOS is more efficient than glycolysis, it also produces more ROS that may compromise genomic integrity. Many cancer cells use glycolysis even in the presence of oxygen (aerobic glycolysis). *GLUT* glucose transporter, *HKII* hexokinase II, *PK* pyruvate kinase, *MCT* monocarboxylate transporter, *LDH* lactate dehydrogenase, *PMET* plasma membrane electron transport, *CoQ* coenzyme Q10, *I–V* respiratory complexes I to V, *PDH* pyruvate dehydrogenase, *PF* fumarate hydratase, *SDH* succinate dehydrogenase, *IDH* isocitrate dehydrogenase, *OAA* oxaloacetate, *αKG* α-ketoglutarate

characterized by elevated mitochondrial content, high mitochondrial DNA (mtDNA) copy number, and reliance on pyruvate as a metabolic fuel. Differentiation shifts metabolism towards OXPHOS as evidenced by reduced glycolytic flux and increased mitochondrial OXPHOS fueled by glucose, fatty acids, and glutamine in differentiated embryonic stem cells (Cho et al. 2006; Chung et al. 2007; Facucho-Oliveira and St John 2009; Shyh-Chang et al. 2013; Wang and Shang 2013). Not surprisingly, dedifferentiation of fibroblasts into induced pluripotent stem cells (iPSC) increased

glycolytic rates and decreased mitochondrial cristae compared with parental fibroblasts. These stem cells exhibited a metabolome and metaboproteome consistent with glycolytic nuclear remodeling (Armstrong et al. 2010; Prigione et al. 2010; Folmes et al. 2011; 2012; 2013).

Considerable evidence points to a population of quiescent hemopoietic stem cells in the bone marrow existing in an hypoxic niche employing a predominantly glycolytic metabolism maintained by HIF-1α stabilization (Suda et al. 2011). These cells are characterized by low mitochondrial mass and immature internal cristae (Chung et al. 2007). Nevertheless, other hemopoietic stem cell populations reside in well-oxygenated perivascular regions of the bone marrow and move between these two distinct niches and into the highly oxygenated peripheral circulation. Whether this movement between niches and the circulation is associated with changes in the balance between glycolytic and mitochondrial metabolism or is characterized by glycolysis regardless of oxygen tension (aerobic glycolysis) warrants further investigation. It would also be interesting to know whether transition from a quiescent to an activated state such as occurs during self-renewal of stem cells or in cells induced to rapid proliferation is associated with a switch from OXPHOS to glycolysis.

The effect of oxygen tension on energy metabolism is not necessarily bidirectional. Mouse embryonic fibroblasts reduce oxygen consumption when switched from 20 % O_2 to 1 % O_2 but continue low oxygen consumption when returned to 20 % O_2 indicating stable metabolic reprogramming (Suda et al. 2011). Neural stem cells in hypoxic regions of the brain and mesenchymal stem cells also show properties consistent with low levels of glycolytic metabolism (Chen et al. 2008; Renault et al. 2009; Shyh-Chang et al. 2013), whereas MSCs continue to consume O_2 at a high rate when transferred to normoxic conditions (Pattappa et al. 2011). Consequently, aerobic glycolysis in proliferating cells in mature organisms may be a consequence of sustained hypoxia during early development resulting in cellular bioenergetics being remodeled towards glycolytic metabolism regardless of whether oxygen is present or not.

In general, quiescent stem cells and differentiated cells employ OXPHOS, while non-quiescent pluripotent and embryonic stem cells, progenitor cells, and myoblasts are highly glycolytic and use OXPHOS to varying degrees. These considerations provide fundamental insight into bioenergetics changes that occur during normal tissue development. The extent to which they can be extrapolated to tumor development is of considerable interest and will be discussed next.

2.5 Bioenergetic Remodeling in Tumor Cells

Tumor cell metabolism is often, but not always, biased towards aerobic glycolysis particularly in highly aggressive metastatic tumors (Warburg 1956; Moreno-Sanchez et al. 2007; DeBerardinis et al. 2008; Vander Heiden et al. 2009; Berridge and Tan 2010). Nevertheless, the contribution of glycolytic ATP to the total energy

budget of most solid tumors does not exceed 50–60 % (Warburg 1956; Nakashima et al. 1984; Brand 1997; Zu and Guppy 2004; Berridge et al. 2010) with low OXPHOS activity generating comparable amounts of ATP to glycolysis. The preference of many tumor cells for glycolysis over OXPHOS is a characteristic not only of proliferating anabolic cells within a tumor but may also be true for quiescent and self-renewing populations if tumors follow the patterns observed in normal tissue development (see Sect. 2.3). Thus, aspects other than oxygen tension in the tumor microenvironment appear to influence the choice of metabolic energy pathways of developing and metastasizing tumors. For example, stromal cells can affect energy metabolism in cancer cells as was shown for cancer-associated fibroblasts which, when cocultured with cancer cells, lose mitochondrial mass, while cancer cells increase their mitochondrial mass (Martinez-Outschoorn et al. 2011). Aerobic glycolysis in fibroblasts is thought to release lactate and ketone bodies that provide an energy source for cancer cells, facilitating tumor progression and metastasis (Migneco et al. 2010; Martinez-Outschoorn et al. 2011, 2012).

Although most aggressive and metastatic cancers use glycolysis as their preferred energy pathway, purely glycolytic mitochondrial gene-knockout B16ρ° melanoma cells exhibit a long 20–30-day lag to tumor growth, grow more slowly as tumors than parental cells, and are unable to form metastases in C57BL/6 and NOD/scid mice (Berridge and Tan 2010; Tan et al. 2013). The difference between glycolytic tumors that form from ρ° cells and those that develop naturally could be due to the latter being able to switch more readily between glycolysis and OXPHOS when the environment dictates, whereas tumors that grow from ρ° cells have fewer choices.

The dynamic balance between glycolytic and mitochondrial ATP production in vivo and in vitro and the flexibility of the control mechanisms involved are now considered.

2.5.1 Consequences of a Glycolytic Metabolism: Plasma Membrane Electron Transport

Relying on glycolysis as the sole or primary energy source has significant metabolic implications (Herst et al. 2004; Herst and Berridge 2007; Vander Heiden et al. 2009). In order to provide high sustained levels of glycolysis necessary to fuel rapid cell proliferation, cells increase glucose transporter expression and activation to facilitate glucose uptake (Ahmed and Berridge 1998, 1999; Kunkel et al. 2003; Fiorentini et al. 2004; Migneco et al. 2010), increase glycolytic enzyme expression (Papandreou et al. 2006), and inhibit pyruvate dehydrogenase (PDH) activity by transactivating pyruvate dehydrogenase kinase 1 (PDK1) (Kim et al. 2006).

In addition, cells must find ways of recycling excess NADH produced during glycolysis and TCA cycle activity so as to maintain the intracellular NADH/NAD^+ balance. Traditionally this has been thought to occur through increased lactate dehydrogenase (LDH), causing acidification of the tumor microenvironment (Warburg 1956; Schornack and Gillies 2003; Fantin et al. 2006).

In addition to LDH activity, highly glycolytic cells recycle intracellular NADH through plasma membrane electron transport (PMET) (Crane et al. 1991; del Castillo-Olivares et al. 2000; Herst and Berridge 2006, 2013) (see Fig. 2.2). PMET may be an evolutionary remnant from pre-endosymbiotic times when the cell membrane was the only means of generating energy. A major PMET pathway in mammalian cells involves a multicomponent system that reduces the water-soluble tetrazolium dye, WST-1, to its formazan in the presence of an obligate intermediate electron acceptor (Berridge and Tan 2000; Herst et al. 2004; Berridge et al. 2005; Herst and Berridge 2006, 2007). PMET activity is closely linked with metabolic rates and intracellular NADH flux. Mitochondrial gene-knockout (ρ°) cells that are incapable of OXPHOS and OXPHOS-competent cancer cells under hypoxic conditions or in the presence of mitochondrial poisons increase their PMET activity three- to fourfold (Berridge and Tan 2000; Herst et al. 2004; Scarlett et al. 2004; Tan and Berridge 2004; Berridge et al. 2005; Herst and Berridge 2007).

Increased PMET activity combined with increased LDH activity ensures a favorable ratio of NADH/NAD^+ for sustained glycolysis regardless of oxygen tension. Therefore, inhibiting PMET may be another strategy to kill quiescent stem cells as well as highly glycolytic proliferating tumor cells (Herst and Berridge 2006).

2.5.2 Genetic and Epigenetic Changes

Tumorigenesis involves not only cumulative nuclear and mitochondrial DNA mutations but also changes in the epigenome as a consequence of the ever-changing microenvironment (see Fig. 2.3). Disentangling the complex interplay between genetic and epigenetic contributions to cancer development and metastasis is one of the major current challenges of tumor cell biology.

2.5.2.1 Nuclear Mutations Affecting Energy Metabolism

Most attention in the field of cancer cell metabolism has focused on rapidly proliferating cells in vitro and on fast-growing tumor models where a shift from OXPHOS towards glycolytic metabolism is a feature held in common with rapidly dividing non-tumor cells. Mutations that have predisposed cells towards a more glycolytic metabolism have been reported in several nuclear-encoded enzymes involved in energy metabolism (Pollard et al. 2003; Wallace 2012). These include mitochondrial fumarate hydratase (*FH*) (Toro et al. 2003; Isaacs et al. 2005), succinate dehydrogenase (*SDH*) subunits A–D (King et al. 2006; Bayley

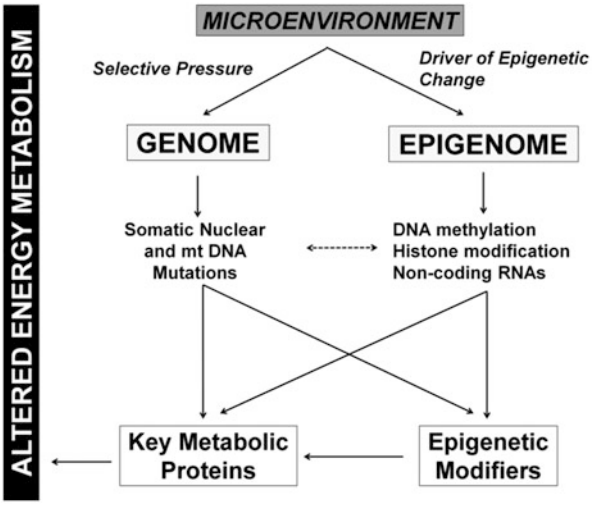

Fig. 2.3 Overview of the genetic and epigenetic changes that can alter energy metabolism that drives oncogenesis. The tumor microenvironment exerts selective pressure on the genome, favoring advantageous nuclear and mitochondrial mutations in genes encoding key metabolic proteins. The environment also drives changes in the epigenome that affect expression of these genes either directly or by changing the expression of epigenetic modifiers. See text for a full explanation

et al. 2010; Gill 2012) and isocitrate dehydrogenase 2 (*IDH2*) (Borodovsky et al. 2012), and cytosolic *IDH1* (Parsons et al. 2008; Mardis et al. 2009; Borodovsky et al. 2012) and phosphoglycerate dehydrogenase (*PDH*) (Locasale et al. 2011; Possemato et al. 2011). Many other classical oncogenes and tumor suppressors are indirectly involved in controlling metabolism through key regulatory nodes including mTOR and PI3K/AKT, MYC, and mutant RAS and RAF (Sun et al. 2011; Shaw and Cantley 2012; Nemazanyy et al. 2013), PDK and its phosphorylation regulators, PDK1 and PDP2 (Kaplon et al. 2013), hexokinase (HK) II relocation to mitochondria (Mathupala et al. 2009), and the pyruvate kinase (PK) splice variant, PKM2 (Christofk et al. 2008; Mazurek 2011; Gui et al. 2013), all involved to varying degrees in this reprogramming and with some more related to cell proliferation per se than to cancer.

Much less attention has been paid to quiescent cells or slowly self-renewing cells that are present in most if not all tumors and to the metabolic preferences of low-grade indolent tumors. Acquisition of certain mutations in key metabolic enzymes suggests that the metabolism of quiescent and self-renewing cells may also be biased towards glycolytic metabolism. Recent evidence from a variant human osteosarcoma cell line with cancer stem cell-like properties indicated greater dependence on glycolytic metabolism compared with parental cells (Palorini et al. 2013). In addition, brain tumor-initiating cells have been shown to preferentially express the high-affinity neuronal glucose transporter, Glut-3, a

prognostic marker in brain tumor patients (Flavahan et al. 2013), implying metabolic remodeling towards glycolytic metabolism.

2.5.2.2 Mitochondrial Mutations Affecting Energy Metabolism

The contribution of mtDNA mutations to tumor formation and to metastasis is often glossed over. This is in part due to few genetic tools being available to explore the role of these mutations in cancer, the presence of multiple mtDNA copies per mitochondrion and hundreds per cell, and mitochondrial dynamics. In addition, the phenomenon of heteroplasmy or mixed mitochondrial genotypes adds another layer of complexity to analytical approaches. The presence of mtDNA sequences in the nucleus of mammalian cells can also confound assessment of mtDNA mutations, and these artifacts need to be carefully controlled in such studies (Schon et al. 2012).

Nevertheless, mutations in mtDNA occur in almost all tumors although not all are directly linked to cancer (Wallace 2005; Schon et al. 2012). This is particularly relevant because the mitochondrial genome encodes 13 essential proteins of the mtET chain. Mutations in any of these subunit proteins have the potential to compromise mtET, thus altering the bioenergetic balance between mitochondrial respiration and glycolytic energy production.

The remaining 24 mitochondrial genes encode 22 tRNAs and 2 rRNAs essential for mitochondrial protein synthesis. Likewise, mutations in these genes have the potential to reduce protein synthesis below a threshold level required to maintain mtET and OXPHOS (Tan et al. 2013). The mitochondrial genome also contains a displacement loop (D-loop) and adjacent control regions that are involved in mtDNA replication and transcription. In a recent study, tumor-related somatic mtDNA mutations occurred at a frequency of 13–63 % across five tumor types (Larman et al. 2012), supporting previous evidence of a high somatic mtDNA mutation rate, mostly homoplastic, in colorectal cancers (Polyak et al. 1998). In another study, mtDNA mutations in 921 tumors were analyzed by whole genome sequencing and showed 56 % of all tumors contained a least one mtDNA mutation with 28 % being in complex I and 35 % being in the D-loop (Iommarini et al. 2013). The presence of mtDNA sequences in the nucleus of mammalian cells can complicate assessment of mitochondrial mutations, and these artifactual contributions need to be carefully controlled in such studies (Schon et al. 2012).

Mitochondrial DNA haplotypes contribute to metabolic diseases including cancer in ways that are poorly understood, and these natural variations constitute a variable genetic background on which somatic mtDNA mutations are superimposed (Wallace 2012). Mitochondrial fusion–fission processes have also been implicated in maintaining mitochondrial genome integrity and therefore the respiratory balance in cells (Vidoni et al. 2013), but the relevance of these control mechanisms to cancer is currently uncertain.

Mammalian mitochondrial respiratory complexes I–V are composed of 13 mitochondrially encoded subunits and about 77 nuclear-encoded proteins

(Schon et al. 2012) that are organized into supramolecular structures collectively referred to as the respirasome (Acin-Perez et al. 2008; Lapuente-Brun et al. 2013). Many hundreds of nuclear genes also affect mitochondrial integrity and metabolic function, but this subject is beyond the scope of this review. A brief summary of the contribution of mutations in complexes I–V subunits to cancer as well as the evidence for mitochondrial genome involvement in tumorigenesis and metastasis has been presented recently (Tan et al. 2013).

2.5.2.3 Epigenetic Changes Affecting Energy Metabolism

All cancers are characterized by tumorigenic mutations and epigenetic modifications that define the different stages of tumor progression and underpin tumor cell heterogeneity (Timp and Feinberg 2013). Although some mutations may be sufficient to bring about metabolic remodeling (Turcan et al. 2012), the local microenvironment is intimately involved in this remodeling process through epigenetic changes in tumor cells and their evolving progeny. In this context, considerable genetic heterogeneity is seen within a particular tumor and between tumors within an individual (Shah et al. 2009; Yancovitz et al. 2012; Burrell et al. 2013), and this heterogeneity would be expected to be associated with epigenetic heterogeneity. However, little attention has been paid to the epigenetic complexity of individual tumors.

Epigenetic changes are mediated through a range of epigenetic modifiers including DNA methyltransferases, histone methyltransferases, histone demethylases, histone acetyltransferases, and histone deacetylases. Mutations in these modifiers have been shown to affect mainly hematological cancers as well as some rare pediatric cancers and some highly aggressive variants of adult tumors. Epigenetic changes in expression of these modifiers have been shown to play an important role in the more common solid cancers (Timp and Feinberg 2013). The three main drivers of epigenetic change, DNA methylation, histone modifications, and noncoding microRNAs, are now discussed.

DNA Methylation

The methylation status of DNA at CpG islands can promote carcinogenesis by either silencing tumor suppressor genes through hypermethylation and/or activating oncogenes through hypomethylation (Timp and Feinberg 2013). Tissue-specific methylation patterns are found in regions adjacent to CpG islands, and these facilitate metabolic reprogramming and distinguish between normal and cancerous tissue (Doi et al. 2009).

Links between oncogenic mutations in three TCA cycle genes associated with epigenetic changes and altered cancer metabolism have been described recently. A mutation in *IDH1* generates the oncometabolite 2-hydroxyglutarate (D-2HG) instead of α-ketoglutarate (α-KG) in acute myeloid leukemia, stage II–III gliomas,

and secondary GBM and in metastatic colon cancer, prostate cancer, thyroid cancer, and sarcomas (Turcan et al. 2012; Ward and Thompson 2012; Rakheja et al. 2013). D-2HG inhibits α-KG-dependent dioxygenases and histone lysine demethylases leading to a global hypermethylation profile that promotes carcinogenesis. Thus, there is an emerging role of D-2HG as an oncometabolite in hematolymphoid and central nervous system neoplasms (Zhao et al. 2009; Teicher et al. 2012; Rakheja et al. 2013) and other cells.

Similar hypermethylation patterns have been observed in SDH mutants in paragangliomas, gastrointestinal stromal tumors, and pheochromocytomas. Here succinate accumulation mimics the effect of *IDH* mutations (Killian et al. 2013; Letouze et al. 2013; Mason and Hornick 2013). In addition, inactivating mutations in *FH* are associated with a similar hypermethylation profile in an individual paraganglioma (Letouze et al. 2013).

Mutations in these genes facilitate carcinogenesis by changing the methylation profile of the DNA. Global DNA hypermethylation facilitates carcinogenesis by silencing tumor suppressors, blocking differentiation, and pushing cancer cells towards aerobic glycolysis by stabilizing HIF-1α.

Histone Modifications

Modification of histone tails by altering their methylation or acetylation status can either activate or inhibit transcription. Although hypermethylation and silencing of tumor suppressor genes has been shown for many solid tumors, this tends to occur in regions that are already inactivated by histone modifications. This suggests that DNA methylation occurs secondary to histone modification. For instance, hypermethylated tumor suppressor regions in breast cancer cells were shown to occur at sites that are already repressed in normal cells of the same lineage (Sproul et al. 2011).

Increased acetylation by inhibition of histone deacetylases (HDAC) has been shown to initiate differentiation, reduce the number of cancer stem cells, and inhibit clonogenic sphere formation in HNSCC (Giudice et al. 2013). However, differentiation is not a uniform characteristic of HDAC inhibitors. The HDAC inhibitors, valproic acid, and suberoylanilide hydroxamic acid were shown to reprogram differentiated cancer cells towards a dedifferentiated more resistant stem cell-like state in two highly aggressive breast cancer cell lines in vitro. Expansion of breast cancer stem cells by valproic acid has important clinical implications for the use of HDAC inhibitors in the treatment of cancer (Debeb et al. 2012).

MicroRNAs

MicroRNAs (miRNAs) are nuclear-encoded 20–25 nucleotide noncoding RNAs that play an important role as negative regulators of translation and stability of mRNA of key genes in most fundamental cellular pathways (Yates et al. 2013)

including mitochondrial energy metabolism (Bienertova-Vasku et al. 2013; Tomasetti et al. 2013). Suppression of miRNA expression promotes tumorigenesis as most miRNAs are tumor suppressors (Garzon et al. 2006), although some have oncogenic activity (Zhang et al. 2007).

Similar to nuclear and mitochondrial mutations and changes in DNA and histone methylation patterns, miRNAs also alter the cell's preference for glycolytic or mitochondrial energy. They can directly inhibit expression of key proteins or inhibit key regulatory pathways that promote either glycolysis or OXPHOS. For example, miR-338 was shown to target cytochrome C oxidase IV, favoring glycolysis (Aschrafi et al. 2008). Other miRNAs have been shown to promote OXPHOS by decreased expression of glucose transporters, GLUTs 1–3 (miR-133) and HKII (miR-143) (Fang et al. 2012; Jiang et al. 2012). Aberrant expression of these miRNAs in bladder, colorectal, and pancreatic tumors increases GLUT1 and GLUT 3 expression and HKII expression and activity promoting aerobic glycolysis (Singh et al. 2011; Chen et al. 2012; Fei et al. 2012).

In contrast, expression of miR-210 under hypoxic conditions promotes aerobic glycolysis. Expression of miR-210 is induced by binding of HIF-α and NFkB p50 to its promoter (Chan et al. 2012). In the presence of oxygen, miR-210 represses MET, causing electron leakage and increased ROS production and leading to decreased ATP levels. Other targets of miR-210 include subunits of respiratory complexes I, II, and IV and glycerol-3-phosphate dehydrogenase. Inhibition of these target genes contributes to decreased mtET and OXPHOS and a shift towards glycolysis (Tomasetti et al. 2013).

The term epithelial-to-mesenchyme plasticity (EMP) has been used recently to describe the dynamic and reversible regulation of EMT (Thompson and Haviv 2011). For example, the miR200s regulates mtET and metastatic lung colonization in breast cancer (Korpal et al. 2011), suggesting that flexible transition between these two states was crucial for metastatic progression. It would be of considerable interest to investigate markers of energy metabolism in this model to determine whether EMP is associated with concurrent metabolic changes.

In addition to regulating translation of nuclear transcripts in the cytoplasm, miRNAs have also been found in mitochondria targeting both nuclear and mitochondrially-encoded genes. For example, miR-181c has been shown to enter mitochondria to remodel cytochrome oxidase (COX I) by inactivating mtET and promoting glycolysis (Das et al. 2012). Localization of mature miRNAs has been demonstrated for mitochondria isolated from human muscle cells (Barrey et al. 2011). These mito-miRNAs are nuclear encoded and targeted to mitochondrial tRNA and rRNA genes (Bandiera et al. 2011). Mito-miRNAs are likely to facilitate fast changes in mitochondrial gene expression to meet changes in metabolic demands of the cell.

Together this brief overview shows that altered miRNA expression patterns in tumors alter the balance between OXPHOS and glycolysis in favor of glycolysis.

Concluding Remarks

Metabolic flexibility is a consequence of epigenetic reprogramming events that are driven by a constantly changing tumor microenvironment imposed on progressive genetic change. While the nature of the constraints on metabolic flexibility is poorly understood, environmental conditions such as hypoxia and nutrient availability make a major contribution to metabolic flexibility in tumors with evolving cell populations. The complex interplay between genetic and epigenetic changes promotes glycolytic energy metabolism even in the presence of oxygen and appears to favor cancer cells competing for oxygen and nutrients.

What has become increasingly clear in the last couple of years is that anticancer treatment strategies that rely on single-target approaches will only produce short-term gains as cancer cells will adapt by changing their genetic and epigenetic makeup. Highly complex tumor biology demands strategic therapeutic approaches that address different aspects of this biology simultaneously to circumvent metabolic compensation. It is particularly important that these strategies are adapted to the ongoing metabolic changes that drive the different stages of tumorigenesis.

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Chapter 3
Autophagy and Tumor Cell Metabolism

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3.1 Introduction

The word autophagy, from the Greek for *self-eating*, refers to the catabolic processes through which the cell recycles its own constituents in the lysosome (Mizushima et al. 2008; Yang and Klionsky 2010). This chapter will focus on macroautophagy (hereafter referred to as autophagy), because the evidence that the other forms of autophagy play any role in tumor biology is relatively limited.

Autophagy starts with the formation of a double-membrane bound vacuole, known as the autophagosome, that engulfs fractions of the cytoplasm in either an unselective or a selective manner via the activity of the autophagy adaptors (SQSTM1/p62, NBR1, NDP52, and optineurin) that form a bridge between the target and the growing autophagosome membrane (Mizushima and Komatsu 2011; Boya et al. 2013). After being formed, most autophagosomes receive input from the endocytic vesicles to form an amphisome before the autophagic cargo undergoes complete degradation in the lysosomal lumen (Fig. 3.1). Basal rate autophagy exercises quality control on the cytoplasm of most cells by removing damaged organelles and protein aggregates. Autophagy is a response to a range of stimuli and in most cases protects cells against stressful situations (Kroemer et al. 2010). In response to starvation, autophagy is important for the lysosomal recycling of metabolites into the cytoplasm, where they are reused either as a source of energy or to provide building blocks for the synthesis of new macromolecules.

The discovery of *ATG* (autophagy-related) genes in eukaryotic cells and that of the role of ATG proteins in the formation of autophagosomes were milestones in the understanding of the molecular aspects of autophagy (Mizushima et al. 2011). ATG proteins are recruited on a membrane known as the phagophore. Several cellular pools of membranes contribute to the formation of the phagophore (Hamasaki et al. 2013; Moreau et al. 2013). The hierarchical intervention of ATG with other proteins leads to the elongation and the closure of the membrane to form the autophagosome (Fig. 3.1). At a molecular level, the first step in the initiation of autophagy is the activation of a molecular complex containing the serine/threonine kinase ULK1 (the mammalian ortholog of Atg1 in yeast). The activation of this complex is downregulated by MTORC1, which integrates multiple signaling pathways that are sensitive to the availability of amino acids, ATP, growth factors, and the level of ROS. The expansion, curvation, and closure of the autophagosome are controlled by another molecular complex including phosphatidylinositol 3-kinase (PI3K) and Beclin 1 (the mammalian ortholog of Atg6 in yeast), which allows the production of phosphatidylinositol 3-phosphate (PI3P) to occur, and the subsequent recruitment of PI3P-binding proteins WIPI1/2 and two ubiquitin-like conjugation systems ATG12–ATG5-ATG16L and LC3–PE. The final fusion with lysosome requires small Rab GTPases and the transmembrane protein LAMP2. Acid hydrolases and the cathepsins present in the lysosomal lumen degrade the autophagosomal cargoes.

Advances in our understanding of the autophagic process paved the way for the discovery of the importance of autophagy in development, tissue homeostasis,

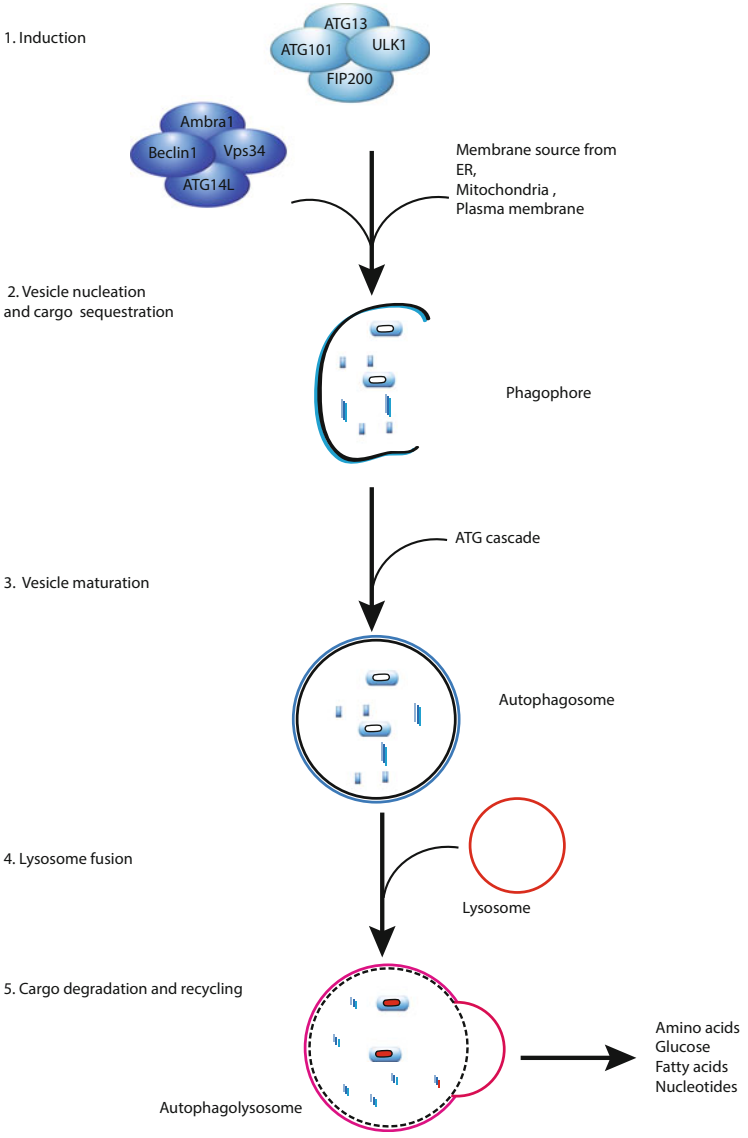

Fig. 3.1 Overview of the autophagy pathway**.** Autophagy is orchestrated by the coordinated action of Atg proteins to form the autophagosome from the pre-autophagosomal structure to which Atg proteins are hierarchically recruited to form the isolation membrane of the phagophore. Through a process of maturation and fusion, these membrane-bound structures become autolysosomes, degrading their contents and releasing amino acids, fatty acids, nucleotides, and other molecules required to maintain cell metabolism

metabolism, the immune response, and various diseases (Deretic and Levine 2009; Ravikumar et al. 2010; Rubinsztein et al. 2012; Choi et al. 2013). Interest in the role of autophagy in cancer stems from the discovery that *BECN1* (the gene that encodes Beclin 1, the human ortholog of the yeast Atg6) is a haplo-insufficient tumor suppressor gene (Rubinsztein et al. 2012; Choi et al. 2013). In fact, it appears that autophagy is under the control of a large panel of oncogenes and products of tumor suppressor genes (Botti et al. 2006; Maiuri et al. 2009). However, the role of autophagy in tumors is complex and ranges from a tumor-suppressive role to a role in helping cells to adapt to the environment. In cancer cells, autophagy fulfills a dual role, having both tumor-promoting and tumor-suppressing properties (Liu and Ryan 2012; White 2012; Lorin et al. 2013). By maintaining cellular homeostasis in healthy cells, autophagy prevents DNA damage and genomic instability, which can lead to tumoral transformation. Autophagy can also facilitate oncogene-induced senescence or protect tumors against necrosis and inflammation, thus limiting tumor growth. On the other hand, autophagy can contribute to tumor progression, by allowing tumor cells to survive stressful conditions and sustaining the deep metabolic reorganization that cancer cells undergo after oncogenic transformation. Autophagy also appears to be important in supporting tumor development by maintaining the survival and self-renewal of cancer stem cells (Gong et al. 2013; Guan et al. 2013; Pan et al. 2013).

In this chapter, we will discuss the interplay between autophagy and tumor cell metabolism, the relationship between cell metabolism and the regulation of autophagy by acetylation, and finally the emerging role of autophagy in cancer stem cells.

3.2 Autophagy and Metabolic Adaptation

One of the hallmarks of tumors is the upregulation of cytosolic glycolysis: the conversion of glucose into lactate under hypoxic or normoxic conditions by cancer cells. This "aerobic glycolysis," despite the fact that it reduces efficiency (thus increasing the rate of energy production), is associated with a reduction of the activity of mitochondrial electron chain transport (DeBerardinis 2008). This metabolic reprogramming, also known as the "Warburg effect," is induced by the oncogenic transformation of tumor cells (Fig. 3.2). This metabolic adaptation is associated with cell transformation and seems to require the activation of oncogenes, such as RAS (Dang and Semenza 1999; Manning and Cantley 2007), AKT (Manning and Cantley 2007), and MYC (Gordan et al. 2007), and the inhibition of tumor suppressors, such as p53 (Bensaad et al. 2006; Matoba et al. 2006; Kawauchi et al. 2008). MYC and RAS transformation impair acetyl-CoA production, an essential component of the mitochondrial tricarboxylic acid (TCA) cycle, by blocking its generation from the decarboxylation of pyruvate (White 2012). RAS transformation also impairs acetyl-CoA production by blocking the β-oxidation of fatty acids. In addition, MYC transformation stimulates glycolysis, glutaminolysis,

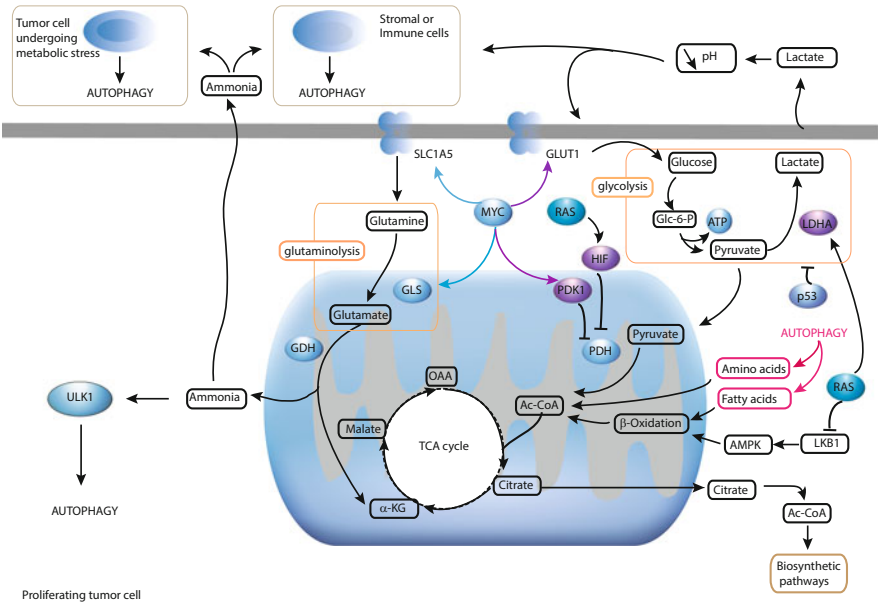

Fig. 3.2 The autophagy and metabolic coupling between tumor and stroma cells**.** In a context of metabolic stress, cancer cells catabolize glutamine to form α-ketoglutarate, which enters the tricarboxylic acid (TCA) cycle—also known as Krebs cycle—and increases mitochondrial activity, thus promoting survival and growth. The activation of oncogenes, such as RAS and MYC, seems to orchestrate the metabolic changes associated with cell transformation. RAS diminishes the pool of acetyl-CoA by three known mechanisms. First, RAS can activate lactate dehydrogenase (LDH), which converts pyruvate to lactate, which is excreted. Second, RAS can activate hypoxia-inducible factor (HIF), thus inhibiting pyruvate dehydrogenase (PDH) and the conversion of pyruvate to acetyl-CoA (Ac-CoA). Third, RAS inhibits liver kinase B1 (LKB1), blocking AMP kinase (AMPK) and β-oxidation. Defective autophagy results in reduced citrate levels, impaired TCA cycle function, and reduced mitochondrial respiration. Autophagy can potentially compensate for the metabolic reprogramming by RAS by degrading proteins and lipids to provide amino acid and fatty acid substrates that produce acetyl-CoA. Tumor cells might also compensate for autophagy impairment by upregulating glycolysis, glutaminolysis, or the reductive carboxylation of α-ketoglutarate (α-KG) from glutamine. The transcription factor Myc favors the "Warburg effect" by increasing the abundance of key glycolytic enzymes, including Glut1, LDHA, and PDK1. Myc stimulates glutamine metabolism by increasing the abundance of glutamine transporters (SLC1A5) and glutaminase (GLS). α-KG is produced via the double deamination of glutamine, a process known as glutaminolysis. Glutamine is first deaminated by GLS to produce glutamate. Glutamate is then converted to α-KG by glutamate dehydrogenase (GDH). Ammonia is generated as a by-product of glutamine deamination and induces autophagy in an autocrine or paracrine fashion through an unknown mechanism, although the kinase ULK1 (unc-51-like kinase 1) seems to be required. Ammonia is a diffusible factor that stimulates the autophagy program in the adjacent stroma cells (in particular, cancer-associated fibroblasts CAFs). Increased aerobic glycolysis occurs in stromal cells, leading to the generation and secretion of high levels of glutamine into the tumor microenvironment, which maintains the tumor cell metabolism. In contrast, ammonia does not interfere with the activity of mTOR, which is a key inhibitor of autophagy. Cancer cells are rendered less sensitive to ammonia by the upregulation of TIGAR, a p53-inducible regulator of glycolysis. The ability of TIGAR to limit autophagy is closely correlated to the suppression of ROS and is p53 independent. Interestingly, the levels of Acetyl-CoA and of NAD^+/NADH, which are both produced as a result of metabolic activity, can regulate the outcome of autophagy through acetylation-associated, posttranscriptional modifications of

and the uptake of both glucose and glutamine (Marino and Kroemer 2010). Activation of RAS is also able to induce autophagy in tumor cells (Guo et al. 2011; Lock et al. 2011; Yang and Kimmelman 2011). Tumor cells preferentially use aerobic glycolysis as an energy source, but cancer cells also depend on functional mitochondria for their growth and development. Autophagy might be essential to provide substrates for anaplerotic reactions, such as amino acids through protein degradation or lipids through the degradation of membrane organelles or of lipid droplets, in order to sustain mitochondrial metabolism (White 2012). As most of the glucose is consumed by glycolysis, glutamine becomes the main substrate for the mitochondrial TCA cycle and the generation of fatty acids and NADPH. Autophagy supports the profound metabolic rearrangements that cancer cells undergo, and this makes them highly dependent on autophagy for survival.

3.2.1 Autophagy and the Tumor Microenvironment

Tumor development (in particular that of solid tumors) depends on the exchanges that occur between cancer cells and their cellular and extracellular microenvironments [for reviews, see Mantovani et al. (2008), McAllister and Weinberg (2010)]. Various cell populations, including macrophages, lymphocytes, vascular cells, and carcinoma-associated fibroblasts, supplying growth factors, inflammatory cytokines, angiogenic factors, and elements of the extracellular matrix compose the tumoral stroma. The tumor cell microenvironment plays a major role in cancer progression by promoting neoangiogenesis, tissue remodeling, and the secretion of several factors (e.g., chemokines, cytokines, etc.) by immune cells. The role of the microenvironment in the regulation of autophagy in tumor cells in conjunction with the action of tumor cells on autophagy levels in cells in the surrounding stroma is of particular interest. The physiologically extreme conditions of the tumoral microenvironment (nutrient limitation/starvation, acidic pH, hypoxia, oxidative stress, immune responses) promote the autophagic response of cancer cells (i.e., survival and meeting the high energy demands of cancer cell metabolism). Tumor cells can also influence the autophagic activity of stromal cells. Understanding how autophagy regulates cancerous epithelial cells, fibroblasts, and immune cells and consequently the interactions between tumors and the stromal metabolism can be expected to provide new insights into the role of autophagy in the development and progression of tumors.

Several studies have shown that tumor cells release autophagy inducers into the microenvironment. These releases influence the autophagic activity of surrounding stromal cells, resulting in the secretion of high-energy metabolites (such as lactate

Fig. 3.2 (continued) autophagic key components that constitute the link between metabolic status and autophagy. *HATs* histone acetylases, *HDACs* histone deacetylases, *OAA* oxaloacetate, *PDK1* pyruvate dehydrogenase kinase 1, and the tricarboxylic acid (TCA) cycle

and ketones) as well as chemical building blocks such as amino acids (glutamine and nucleotides) that promote cancer development and progression (Marino and Kroemer 2010; Cheong et al. 2011). These catabolites stimulate the oxidative phosphorylation metabolism and mitochondrial biogenesis in epithelial cancer cells. A novel paradigm, known as the "autophagic tumor stroma model of cancer metabolism" or the "reverse Warburg effect," has recently been proposed to explain tumor metabolism (which is reprogrammed by oncogenic stress as mentioned above), in which the tumor stroma generates the fuel required for cancer growth (Pavlides et al. 2010). In this model, the induction of autophagy and the autophagic destruction of mitochondria force stroma cells to undergo glycolysis result in the production and transfer of energy-rich nutrients to anabolic tumor cells, which use them to fuel their mitochondrial metabolism (Eng et al. 2010; Martinez-Outschoorn et al. 2012). One autophagy inducer, ammonia, generated by amino acid catabolism including glutaminolysis, has been identified as a diffusible factor (Cheong et al. 2011); see Fig. 3.2. Ammonia stimulates autophagy in the neighboring stroma cells, leading to protein degradation, the generation of high glutamine levels, and the secretion of glutamine into the tumor microenvironment. Cancer cells convert glutamine into glutamate, thus releasing ammonia. Glutamate is further catabolized to α-ketoglutarate, a substrate of the tricarboxylic acid (TCA) cycle, which increases the mitochondrial activity of epithelial cancer cells. Epithelial cancer cells are less sensitive to ammonia, because TIGAR is upregulated (Ko et al. 2011). Several groups of researchers have demonstrated that autophagic cancer-associated fibroblasts (CAFs) produce a key source of energy-rich glutamine to "fuel" the mitochondrial activity of cancer cells. A vicious catabolic cycle is set up between the tumor stroma and anabolic tumor cell expansion; this highlights the metabolic coupling between epithelial and stroma cancer cells (in cancers of different histological types) (Kalluri and Zeisberg 2006). These studies show that glutamine differentially affects individual cell types within the tumor microenvironment. In tumor epithelial cells, glutamine increases mitochondrial biogenesis, providing protection against apoptosis and reducing autophagy. In contrast, glutamine decreases Caveolin-1 (Cav-1) expression in the stromal compartment of the tumor and increases autophagy. The loss of Cav-1 expression in cancer-associated fibroblasts is a marker of a lethal tumor-promoting microenvironment and is associated with poor prognosis in several types of cancers, such as advanced prostate cancer (Di Vizio et al. 2009), breast cancer (Sotgia et al. 2011; Pavlides et al. 2012), and metastatic melanoma (Wu et al. 2011). The use of autophagy inhibitors could not only promote tumor cell death by targeting tumor cells directly but also uncouple the epithelial and stromal compartments, leading to a decrease in epithelial mitochondrial activity.

3.2.2 Autophagy and Hypoxia

Limited access to oxygen owing to inadequate tissue perfusion, i.e., hypoxia, is a common feature of solid tumors. Hypoxia is a stimulus for inducing autophagy in order to promote tumor cell adaptation to anaerobic conditions (Martinez-Outschoorn et al. 2009; Mathew et al. 2007). The detection of markers of increased autophagic activity in hypoxic tumor tissues has been described (Rouschop et al. 2010). The transcriptional regulators that allow cells to adapt to hypoxic environments are the hypoxia-inducible factors (HIFs) HIF-1α and HIF-2α. They form a heterodimer with the constitutively expressed HIF-1β subunit. The oxygen-sensitive transcription factors HIF-1α and HIF-2α are tightly controlled through oxygen-dependent hydroxylation by prolyl hydroxylases. This hydroxylation leads to their degradation by the von Hippel-Lindau (VHL) ubiquitin-ligase under normoxic conditions. Under hypoxic conditions, the activity of prolyl hydroxylation is reduced, and both HIF-1α and HIF-2α are stabilized. These HIF complexes determine hypoxia-induced gene expression, including the production of the Bcl-2 homology-domain-3, which contains the proteins BNIP3 and BNIP3L (Semenza 2010). These BH3-only proapoptotic genes were initially described as promoters of cell death (Webster et al. 2005; Lee and Paik 2006), and now the BNIP3/BNIP3L proteins are known to destabilize inhibitory interactions between their antiapoptotic counterparts Bcl-xL/Bcl-2 and Beclin 1, leading to autophagy and promoting survival (Bellot et al. 2009). Autophagy induced by BNIP3 (known as mitophagy) results in the clearance of damaged mitochondria, which are a major source of cell-damaging reactive oxygen species (ROS), thus reducing ROS production (Xing et al. 2008). HIFs also regulate autophagy via TSC1/TSC2 activation and indirectly through a negative feedback mechanism, on MTORC1 activity (Rabinowitz and White 2010). Interestingly, activation of HIF by RAS impairs acetyl-CoA production by activating pyruvate dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH). It is worth noting that RAS also impairs acetyl-CoA production through other mechanisms, including lactate dehydrogenase (LDH) stimulation, which depletes pyruvate, and by inhibiting liver kinase B1 (LKB1) and blocking AMP-activated protein kinase (AMPK) activation and preventing the mobilization of lipid stores and β-oxidation. Thus, RAS potentially leaves cells dependent on autophagy to provide substrates, such as amino acids and fatty acids, for acetyl-CoA biosynthesis (Fig. 3.2). Stroma cells, which also inhabit the same environment (oxidative stress, hypoxia) as tumor cells, contribute to the survival and proliferation of these cells (McAllister and Weinberg 2010). Indeed, ROS which are produced during hypoxia also induce the stabilization of HIF1/2α and the activation of NF-κB (a master regulator of inflammation) in CAFs (Eng and Abraham 2011). This stabilization leads to the autophagic degradation of Cav-1, a shift from mitochondrial oxidative phosphorylation towards aerobic glycolysis, with a loss of mitochondrial activity, and the increased production and release of L-lactate and ketone (Chiavarina et al. 2010). Furthermore, the ammonia produced by tumor cells could diffuse into the oxygen-depleted regions and thus help to sustain the survival of tumor cells (Eng et al. 2010).

3.3 Metabolism and Posttranslational Modification Regulation of Autophagy

Autophagy involves the hierarchical assembly and coordinated actions of products of the *Atg* family of genes. At least 30 members of the Atg (autophagy-related) protein family and their binding partners that orchestrate this degradative process have been identified. Autophagy is strictly controlled by posttranslational modifications, such as tyrosine or serine/threonine phosphorylation, lysine/arginine methylation, SUMOylation, lipidation, and ubiquitination of keys components of autophagy [for review, see McEwan and Dikic (2011)]. Henceforth, evidence is accumulating that protein lysine acetylation, which results from the transfer of an acetyl-group from acetyl-CoA to the ε-amino group of the lysine residue, is an evolutionarily conserved metabolic regulatory mechanism involved in coordinating various different metabolic pathways and autophagy (Zhao et al. 2010a). In addition, these posttranslational modifications concern key proteins, other than the histone proteins classically located both in the cytoplasm (including the core autophagy proteins, cytoskeletal proteins) and in the nucleus (such as transcriptional factors, histones), and modulate, thus influencing the rate at which autophagy occurs, which is known as the autophagic flux. This reaction is catalyzed by histone acetylases (HATs), which are also known as lysine acetyltransferases (such as p300), and the reverse reaction is accomplished by histone deacetylases (HDACs) or lysine deacetylases, which are zinc-dependent or NAD^+-dependent enzymes (such as SIRT1/2 sirtuins). The use of HDAC inhibitors and the studies of HATs and HDACs gain- and loss-of-function mutants highlight the pivotal role played by HATs and HDACs in autophagy regulation, where they act at multiple levels.

3.3.1 Posttranscriptional Modification Regulation at the Cytoplasmic Level

Studies have shown that resveratrol, an activator of the deacetylase Sirt1, and spermidine, an inhibitor of histone acetylases, influence the acetylation-modified proteome, induce autophagy, and increase longevity in yeast, nematodes, and flies (Eisenberg et al. 2009; Morselli et al. 2011). Changes in the acetylation status of >100 proteins that form part of the central network of autophagic regulators or executors have been identified after treatment with resveratrol and spermidine (Morselli et al. 2011). Since then, reversible cytoplasmic acetylation of core autophagy components, such as Atg5, Atg7, Atg8, and Atg12, has been reported to regulate autophagosome formation both in yeast and mammalian cells (McEwan and Dikic 2011), and it has been shown that acetylation of Atg proteins can either promote or inhibit their function in autophagy. In mammalian cells under nutrient-rich conditions, the acetyltransferase p300 directly interacts with Atg7 and acetylates the autophagy proteins Atg5, Atg7, Atg8, and Atg12 to inhibit autophagy

(Lee and Finkel 2009). In contrast, during starvation, p300 dissociates from Atg7, and the NAD^+-dependent deacetylase Sirt1 removes acetyl groups from Atg7, Atg5, Atg12, and Atg8, which allows autophagy to proceed (Lee et al. 2008). Some aspects of the phenotype of Sirt1 knockout mice resemble those of Atg5-knockout mice, which suggest that Sirt1-dependent deacetylation could be important for basal autophagy and neonatal survival (Lee et al. 2008). Another histone acetylase, Esa1p in *Saccharomyces cerevisiae* (Yi et al. 2012) and its mammalian ortholog TIP60 (Lin et al. 2012) have recently been shown to act as evolutionarily conserved regulators of autophagy by enhancing another posttranslational modification of Atg3 or ULK1, respectively, and protein lipidation of Atg8/LC3 (in the yeast). Acetylation of Lys183 enhanced the lipid-conjugating activity of Atg3p, and acetylation of Lys19 and Lys48 promoted the interaction between Atg3p and Atg8p, which is necessary for the conjugation of Atg8p to PE in yeast (Yamaguchi et al. 2010). The reverse reaction is accomplished by the deacetylase Rpd3, which contributes to attenuating the formation of autophagosomes during starvation. More interestingly, it has been demonstrated that Atg3 acetylation is subject to both spatial and temporal regulation during nitrogen starvation in the yeast. Thus, the acetyltransferase Esa1p/TIP60 has been demonstrated to be a positive regulator of autophagy in response to nitrogen deprivation in yeast and growth factor or serum deprivation in mammalian cells, respectively (Lin et al. 2012; Yi et al. 2012). Consistent with an essential role of TIP60 in autophagic induction, *TIP60*−/− mouse blastocysts failed to undergo implantation and died around embryonic day 3.5 at a time when autophagic activity is high during normal implantation (Hu et al. 2009; Mizushima and Komatsu 2011).

In addition, the acetylation of autophagy substrates can promote their lysosomal degradation [such as that of the cytotoxic huntingtin (HTT) protein] (Jeong et al. 2009), and the acetylation of microtubules and of the actin cytoskeleton, which provide support for intracellular transport/movement and also influence the occurrence/outcome of autophagy (Kochl et al. 2006). Microtubule stability and function are regulated by the reversible acetylation of α-tubulin mediated by HDAC682 and ELP3/KAT9 acetylases (Creppe et al. 2009), which also regulate the dynamics of actin (Zhang et al. 2007) and of SIRT2 deacetylase (North et al. 2003). In response to nutrient deprivation, tubulin acetylation on Lys40 increases in both the labile and stable microtubule fractions and promotes autophagy by favoring the activation and the association of key components for initiating autophagosome formation and maturation. Indeed, whereas the markers of phagophore/autophagosome formation (BECN1, class III PtdIns3K, WIPI1, ATG12–ATG5 and LC3-II) are specifically recruited on labile microtubules, mature autophagosomes (marked with LC3-II) can move along stable microtubules (Geeraert et al. 2010). In addition, tubulin acetylation is also essential for the fusion of autophagosomes to lysosomes (Kochl et al. 2006; Xie et al. 2010).

It is worth noting that HATs and HDACs are also subjected to reversible acetylation posttranscriptional modifications that provide a mechanism of fine-tuning and control of the activity of HATs and HDACs. For example, SIRT2

controls the self-acetylation of p300, which may also acetylate SIRT2 and inhibit its enzymatic activity (Black et al. 2008; Han et al. 2008).

3.3.2 *Posttranscriptional Modification Regulation at the Nuclear Level*

In addition to being regulated by cytoplasmic acetylation reactions, autophagy can also be regulated by the acetylation of nuclear proteins, which can influence the expression of genes encoding proteins involved in autophagy. These nuclear targets of acetylation-mediated regulation include transcription factors, such as Foxo3 (Kume et al. 2010), and histones (Eisenberg et al. 2009; Morselli et al. 2011). An example of the expression regulation of autophagy genes by histone acetylation is the increased expression of *ATG7* gene, resulting in spermidine-mediated histone hyperacetylation of the promoter region of the gene (Eisenberg et al. 2009). More recently, it has been demonstrated that the induction of autophagy is coupled to the reduction of histone H4 lysine 16 acetylation (H4K16ac) via the downregulation of the histone acetyltransferase hMOF (also designated KAT8 or MYST1), which regulates the outcome of autophagy and initiates a regulatory feedback loop (Fullgrabe et al. 2013). At a genome-wide level, H4K16 deacetylation is associated predominantly with the downregulation of autophagy-related genes (including genes belonging to the autophagic core machinery, such as ATG9A, GABARAPL2, MAP1LC3B, ULK1, ULK3, VMP1). Antagonizing the down-regulation of H4K16ac when autophagy is induced results in the promotion of cell death (associated with an overstimulation of autophagic flux), indicating that H4K16ac is a key determinant of survival versus death responses to the induction of autophagy.

In the case of the transcription factors belonging to the FOXO family members in mammalian cells, FOXO1 and FOXO3 have been shown to play important roles in regulating autophagy in skeletal and cardiac muscles by activating genes that are involved in autophagosome formation (such as *MAP1LC3*, *PIK3C3*, *GABARAPL1*, *ATG12*, *ATG4*, *BECN1*, *ULK1,* and *BNIP3)* (Mammucari et al. 2007; Sengupta et al. 2009; Zhao et al. 2010b). The multiple, posttranslational modifications (including acetylation) undergone by the FOXO transcription factors control their subcellular localization, DNA binding, and transcriptional properties (Van Der Heide et al. 2004; Boccitto and Kalb 2011). The acetylation of FOXO1 and FOXO3 is mediated by p300 acetyltransferase; it impairs their transcriptional activities and inhibits autophagy (Matsuzaki et al. 2005; Hariharan et al. 2010). The acetylation of FOXO1/3 promotes their subsequent phosphorylation by AKT1, leading to their dissociation from DNA, and subsequent nucleocytoplasmic transport (Matsuzaki et al. 2005; Tzivion et al. 2011). Under low-energy conditions, deacetylation of FOXO1/3 is mediated by the sirtuin deacetylases, such as SIRT1 and SIRT2, inducing the expression of genes that are involved in autophagosome

formation and also the gene that encodes PNPLA2, which is a major lipase involved in mobilizing fat from lipid droplets in mammals (Zimmermann et al. 2004; Gronke et al. 2005; Wang and Tong 2009). In addition, it has been shown that deacetylation-activated FOXO1 and FOXO3 activate the autophagic process in a transcription-independent manner by direct protein–protein interaction with ATG7 in response to serum deprivation in the context of human cancer cells (Zhao et al. 2010b).

3.3.3 Cross Talk Between Metabolism and Autophagy

Lysine protein acetylation can regulate the activity of the core components of autophagy, thus making it possible to couple the regulation of autophagy and the metabolic status of the cell. As already mentioned, autophagy ensures the maintenance of cellular energy/metabolic homeostasis by regulating intracellular storage, for example, by means of lipid mobilization (macrolipophagy) (Singh et al. 2009; Dong and Czaja 2011; Singh and Cuervo 2012). In a context of starvation, β-oxidation of fatty acids in the mitochondria results in lipid mobilization, leading to the production of NADH and the coenzyme of p300, i.e., acetyl-CoA. Increased levels of acetyl-CoA can produce a negative feedback by inhibiting starvation-induced autophagy/macrolipophagy via p300-mediated acetylation of autophagic components, and the associated lower level of NAD^+/NADH can lead to the inactivation of sirtuins, which in turn induces acetylation-associated autophagy, which constitutes the link between metabolic status and autophagy (see as below). In the context of Ras-driven cancers, which are more dependent on autophagy than normal cells in order to survive nutrient starvation or metabolic stress, Atg7 deficiency in lung tumor causes a shift from the development of carcinoma to oncocytomas which are rare and benign tumors that accumulate respiration-defective mitochondria (Guo et al. 2013). This effect has been attributed to defective mitochondrial fatty acid oxidation, which confirms that mitochondrial function maintained by Atg7 is critical for the metabolism and growth of Ras-driven NSCLC. In addition, it was also demonstrated in this study that autophagy suppresses the progression of K-Ras-induced lung tumors to oncocytomas, promoting the carcinoma fate.

Thus, acetylation joins phosphorylation, ubiquitination, and lipidation in the complex regulatory network controlling autophagy and constitutes the connection between autophagy and cellular metabolism. However, the mechanisms responsible for the recruitment of the HATs and HDACs during starvation or metabolic stress—thus controlling the acetylation of key components of autophagy and the initiation, duration, and magnitude of autophagy—remain topics for further investigation.

3.4 Autophagy, Metabolism, and Cancer Stem Cells

Recent work has highlighted the importance of the role played by autophagy in cancer stem-cell maintenance and tumor development. Cancer stem cells are a subpopulation of cells within tumors that are responsible for tumor recurrence and metastasis and tumor resistance to anticancer therapies. Autophagy seems to play a critical role in maintaining and regulating all the basic properties of both stem cells and cancer stem cells including survival, self-renewal, quiescence, differentiation, and proliferation (Cufi et al. 2011; Mortensen et al. 2011; Oliver et al. 2012; Salemi et al. 2012; Guan et al. 2013; Pan et al. 2013; Phadwal et al. 2013). We recently showed that autophagy is also crucial for the maintenance and tumorigenicity of cancer stem cells in breast cancer (Gong et al. 2012). Primary breast cancer stem cells have been shown to exhibit a higher rate of autophagy than their non-stem counterparts. Moreover, Beclin 1 is critical for the maintenance and tumor development of enriched cancer stem-cell tumors, in a xenograft mouse model, whereas its expression limits the development of classical xenografts (Gong et al. 2012). These findings indicate the existence of two separate, context-dependent autophagic programs that are regulated or respond in opposite ways by or to Beclin 1 (Koukourakis et al. 2010; Gong et al. 2012). Starvation- and hypoxia-related autophagy is a cytoprotective adaptive mechanism used by CSC to resist microenvironmental stresses (Guan et al. 2013). Cytogenetically abnormal, spheroid-forming, tumorigenic, and invasive neoplastic epithelial cells preexist in human breast ductal carcinoma in situ and require cellular autophagy to survive (Espina and Liotta 2011).

The suppression of autophagy by chloroquine abolishes spheroid outgrowth and survival in culture. These findings indicate that autophagy is necessary for the survival, growth, and invasion of the cytogenetically abnormal, tumorigenic DCIS cells (Espina and Liotta 2011; Guan et al. 2013). Similar data were obtained when knockdown of an essential gene of autophagy or a pharmacological inhibitor of autophagy, such as salinomycin, was employed (Yue et al. 2013).

Another study suggests that autophagy could promote the survival of pancreatic cancer stem cells (Singh et al. 2012). Moreover, autophagy also plays an essential role in glioblastoma stem-cell migration and invasion by modulating ATP metabolism and remodulating the subcellular structure, for instance, by mitochondrial fusion (Galavotti et al. 2013).

What is the possible contribution of autophagy to the metabolic shift of cancer cells towards enhanced glycolysis (the "Warburg effect") during the acquisition of stemness by CSC-like cell populations? This question calls for further investigation. If a "Warburg effect" does indeed play a causal role in the gain of stemlike properties by protecting tumor-initiating cells from the pro-senescent effects of mitochondrial respiration-induced oxidative stress, the ability of autophagy to functionally engage the glycolytic metabolite may generate a cellular state that is metabolically endowed with immortalization.

Paradoxically, autophagy appears to play two opposing roles: it acts as a facilitator of the "Warburg effect," but it also acts as an antagonist of the "Warburg effect." Both the inhibition and promotion of autophagy appear to impair the occurrence of cancer cells with tumor-initiating capacities to a similar extent. In normal tissues, autophagy-mediated damage mitigation may efficiently suppress tumorigenesis; conversely, macromolecular recycling may support CSC survival by buffering bioenergetic demands under stressful metabolic and microenvironmental conditions. Therefore, the activation of autophagy in normal tissues operates as a *bona fide* tumor-suppressive mechanism, whereas the inhibition of autophagy may be extremely beneficial for anti-CSC therapy in established tumors. However, both autophagy promoters (e.g., MTOR inhibitors) and autophagy inhibitors (e.g., chloroquine) block tumorigenesis and cancer progression by eliminating CSCs. The explanation of this "autophagy paradox" may lie in the interaction between tumor cells and adjacent, autophagic stromal cells, as described in Sect. 3.2 (Fig. 3.2).

To conclude, autophagy appears to play two opposing roles in tumorigenesis. The current hypothetical model describes autophagy as suppressing tumor initiation, but promoting tumor development and progression (Mathew et al. 2007; Koukourakis et al. 2010).

Conclusion

As our understanding of the biological functions of autophagy increases, the involvement of autophagy in cancer becomes a critical point of concern. The molecular cross talk between autophagy and cell death was initially considered to be a major determinant in the balanced role of autophagy in tumor suppression and tumor progression (Scarlatti et al. 2009). However, this relationship probably represents only aspect of the role of autophagy in cancer. The roles played by autophagy in tumor immunogenicity, inflammatory response, metabolism, proliferation, and the behavior of tumor-initiating cells are key questions for present and future studies (Michaud et al. 2011; Cheong et al. 2012; White 2012; Leone and Amaravadi 2013; Maes et al. 2013; Pan et al. 2013). It is now clear that the cross talk between autophagy and metabolism is an important aspect of cancer that contributes to the metabolic demand of cancer cells and also to the posttranslational modifications of proteins, i.e., acetylation, to modulate autophagic activity (McEwan and Dikic 2011). As discussed in this chapter, it is clear that autophagy plays an important role in the self-renewal of cancer stem cells. Whether autophagy also supports metabolism in cancer stem cells calls for further investigation (Shyh-Chang et al. 2013). Another important aspect is how basic knowledge about the autophagic process can be translated into therapeutic interventions. It is reasonable to speculate that autophagy modulation should be viewed as a potential therapeutic approach in cancer. Several phase I/II clinical trials are in progress using lysosome-inhibitor drugs, such as chloroquine, rapamycin, or hydroxychloroquine, alone or in combination

(continued)

with chemotherapy, to treat a range of hematological and solid tumors (Rubinsztein et al. 2012; Choi et al. 2013). However, it is not possible to exclude the possibility that the beneficial effects of these drugs could be independent of their blocking effect on the autophagic pathway. The development of more specific autophagy modulators, both for therapeutic investigations and to allow acute modulation of this process for cell biology and physiological studies, is a major challenge for the future.

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Chapter 4
Tumour Hypoxia and the Hypoxia-Inducible Transcription Factors: Key Players in Cancer Progression and Metastasis

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Contents

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4.1 Introduction

Oxygen is a prerequisite for animal life and it is the electron acceptor in mitochondrial metabolism providing the organism with energy, ATP, from carbohydrates. In higher animals, circulatory systems have evolved to provide all cells in the body with sufficient oxygen. Gradients of oxygen availability occur naturally as oxygen diffuse from providing vessels into consuming tissues. During development and growth, low oxygen levels, or hypoxia, naturally arise in tissues and the response from hypoxic cells drives the co-development of the vasculatory system.

Hypoxia can arise by physiological and pathophysiological processes such as muscle exercise, tissue growth, wounding, fibrosis, inflammation and cancer. Inflammation, fibrosis and wound (healing) are frequently concurrent with malignancy and therapy. Smoking cigarettes leads to mild tissue hypoxia through the action of carbon monoxide (Sagone et al. 1973), and conditions associated with smoking such as chronic obstructive pulmonary disease (COPD) can further lower oxygenation locally as well as systemically.

Solid tumours are frequently hypoxic since maintaining oxygen homeostasis becomes increasingly difficult as the tumours grow. Thomlinsson and Gray reported already in 1955 that poor perfusion and hypoxia led to internal necrosis in solid tumours (Thomlinson and Gray 1955). Cell growth leads to compromised perfusion by pushing cells further away from existing vessels as well as adding to intra-tumour pressure that disturbs the function of these vessels. The tumour-induced vessels are malformed and leaky, which further contributes to poor perfusion and increased intra-tumour pressure (Fig. 4.1) (Chung et al. 2010). The net effect is regional hypoxia, shortage of nutrients and accumulation of metabolic waste products affecting the pH in the tumour. Para-malignant conditions such as increased susceptibility to venous embolism and anaemia may further contribute to low oxygenation of both cancerous and stromal tumour cells.

Hypoxic conditions elicit cellular responses that strive to preserve energy and monitor the generation of reactive oxygen species (ROS). Normal cells respond to low oxygen levels by repression of proliferation, decreased rate of oxidative phosphorylation, increased glycolysis and increased generation of angiogenic factors to restore perfusion. The hypoxic response in tumour cells deviates to varying degrees from that of normal cells. The Warburg effect, or increased utilisation of glycolysis for energy even when oxygen is available (Cairns et al. 2011; Koppenol et al. 2011), may prime tumour cells to better withstand hypoxic conditions. The occurrence and selection for tumour cells that can survive and grow under harsh intra-tumour conditions allow continued tumour growth. Low oxygenation in tumours is more or less concurrent with nutrient deprivation. Depending on the reason for hypoxia and the local demand for oxygen and glucose, glucose may diffuse over a wider tissue range than oxygen, and tumour cells can thereby have enough glucose to compensate for its less efficient utilisation in energy production via glycolysis. Thus, hypoxic and hypoglycaemic tumour cells are different, but partially overlapping, populations (Papadogiorgaki et al. 2013).

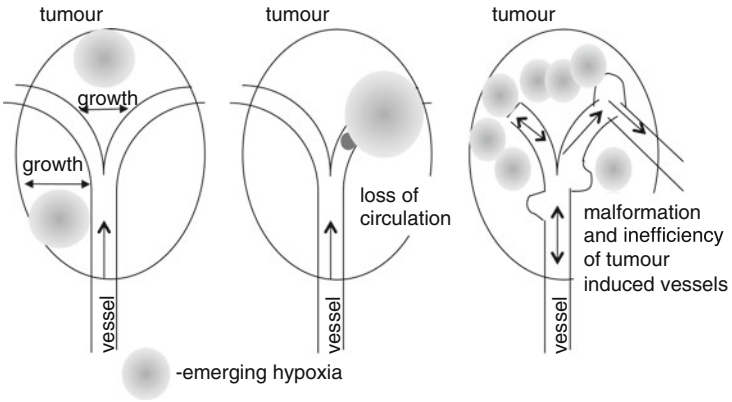

Fig. 4.1 Several conditions contribute to tumour hypoxia. Growth of the cancer cells pushes the cells further from the provisioning capillaries increasing the diffusion distance for oxygen and nutrients. Embolism or collapse of tumour vessels can cause loss of circulation. The tumour-induced vasculature is often malformed, instable and inefficient with dead-ends and shunts between arterial and venous flows. Republished with permission © Annika Jögi

This chapter will cover tumour hypoxia and the hypoxic response in relation to epithelial-to-mesenchymal transition (EMT) and cancer stem cells (CSC) pivotal for tumour progression/metastasis. I will discuss the impact of hypoxia on signalling pathways, which are well known to influence the EMT and CSC phenotypes such as MYC-, Notch- and Wnt/beta-catenin signalling.

4.1.1 Tumour Hypoxia

Tumour hypoxia has long been recognised to be associated with poor prognosis and resistance to therapy, including radiation therapy (Gray et al. 1953; Moulder and Rockwell 1987; Sorensen et al. 2013; Nordsmark et al. 2005; Hockel et al. 1996; Jubb et al. 2010; Teicher 1994). The physiological conditions causing hypoxia within the tumour have different duration and severity, which in turn leads to a dynamic oxygen environment. Cells can suffer mild to severe oxygen deprivation of short duration (acute), such as when a provisioning vessel is clogged by emboli and subsequently cleared, or long-term (chronic) lack of oxygen that may be slowly relieved by the outgrowth of new vessels (neoangiogenesis). Lasting severe hypoxia/anoxia is not compatible with survival of mammalian cells.

Physiologically, hypoxia is defined as a state when oxygen availability falls below the level necessary for the normal function of cells, tissues or organs. This is a definition of limited value in tumours, which lack a normal function. Normal end capillary oxygen partial pressure is in the range of 40–45 mmHg, corresponding to about 6 % of the atmospheric gas pressure at sea level, with considerable variation between and within organs. In an early study showing worse patient outcome in

hypoxic tumours of cervical cancer, 10 mmHg partial oxygen pressure (about 1.3 %) was used as a cut-off (Hockel et al. 1996; Hockel and Vaupel 2001), and later studies have often, but not uniformly, adopted 1 % as a default hypoxic condition. In in vitro cell culture studies, 1 % of oxygen conditions have likewise frequently been defined as hypoxic. Notably, incubators for culturing cells under regulated oxygen conditions have a slightly increased pressure making it difficult to convert in vitro oxygen percentage to partial pressure of oxygen. In addition, the oxygen percentage is usually measured in the atmosphere surrounding the cell culture dish, not in the medium or at the cell surface.

At oxygen levels around 1 %, the mitochondria and respiratory chain are still functional, but their activity may be downregulated in cancer cells in favour of aerobic glycolysis, i.e. the Warburg effect. The cellular response to hypoxia at this oxygen level is, as far as we know today, mainly regulated by the hypoxia-inducible transcription factors, HIFs. In experimental conditions, 0.1 % oxygen is often denoted severe hypoxia, and at such conditions, other stress responses in addition to HIF-induced transcription kick in, notably the unfolded protein response, UPR. The UPR is HIF independent and initially promotes survival, but will with prolonged stress lead to apoptosis.

Autophagy, another stress response induced by hypoxia, is partially regulated through the HIFs (Lorin et al. 2013). It can have tumour-suppressing as well as tumour-promoting effects by providing energy and metabolites (autophagy is covered in detail in Chap. 3). Mitochondria targeting autophagy (mitophagy), which can be induced by HIF1 (Zhang et al. 2008), decreases the oxygen demand and the generation of reactive oxygen species, helping cells survive under hypoxic conditions (Lorin et al. 2013).

Hypoxic conditions also activate the NF-kappaB transcription factors (Koong et al. 1994; Cummins et al. 2006). This can occur via the HIFs (Walmsley et al. 2005; Scortegagna et al. 2008; Culver et al. 2010), but NF-kappaB in turn also affects the activity of the HIFs (Nam et al. 2011). NF-kappaB is activated by ROS that accumulate under hypoxia and contributes to malignant processes such as angiogenesis, cell survival, invasion, metastasis and migration in addition to inflammation and is associated with poor patient outcome (Hoesel and Schmid 2013).

Hypoxic stress and reactive oxygen species normally lead to p53 accumulation and activation, inducing cell cycle arrest and DNA repair mechanisms (Lavin and Gueven 2006; Graeber et al. 1994; Danielsen et al. 1998; An et al. 1998) (via HIF1) (Chandel et al. 2000), although in the majority of cancer cells, p53 or the p53 pathway is altered (Vousden and Prives 2009).

4.2 Hypoxia-Inducible Factors

The HIFs are often referred to as the master regulators of the hypoxic response. These transcription factors belong to the large family of basic helix-loop-helix (bHLH) transcription factors also encompassing the MYC proto-oncogene, several

tissue-specific transcription factors pivotal in organ development and their ubiquitous hetero-dimerisation partners, the E-proteins. All these factors have an N-terminal basic DNA-binding domain followed by the helix-loop-helix domain mediating factor dimerisation. The HIFs belong to a subgroup of bHLH factors defined by the PAS (Per, ARNT (aryl hydrocarbon receptor nuclear translocator, aka HIF1-beta), Sim) domain that takes part in factor dimerisation. The transcription-activating complex consists of an alpha and a beta subunit, where the alternative beta subunits are also known as ARNT1 (Reyes et al. 1992), ARNT2 (Hirose et al. 1996), and ARNT3 (Takahata et al. 1998), respectively, and these are also part of the aryl hydrocarbon receptor complex. Three HIF-alpha subunits have been identified: HIF1-alpha (HIF1a) (Wang et al. 1995; Wang and Semenza 1993, 1995), HIF2-alpha (HIF2a) (encoded by the gene EPAS1) (Tian et al. 1997) (Ema et al. 1997; Flamme et al. 1997; Wiesener et al. 1998) and HIF3-alpha (HIF3a) (aka IPAS) (Makino et al. 2001). HIF1a and HIF2a both form transcriptionally active complexes with HIF-beta subunits, whereas HIF3a, a transcriptional target of HIF1a that comes in a number of splice variants, has mainly been attributed negative regulatory functions (Makino et al. 2002). HIF1a and HIF2a show a large degree of homology, and on the C-terminal side of the PAS domain, they both harbour regions involved in post-transcriptional regulation in response to oxygen availability overlapping with the two transactivating (N-TAD and C-TAD) domains (Fig. 4.2a).

HIF1a is widely expressed in most animal cells and tissues (including a *Drosophila* homologue) (Semenza 2011). HIF2a was first reported to have a more restricted expression mainly in endothelial cells (Tian et al. 1997), but later studies and new antibodies have shown HIF2a expression in numerous mammalian cell lines, tissues and tumour types (Wiesener et al. 1998, 2003; Jogi et al. 2002; Helczynska et al. 2008). Increased protein levels of HIF1a and HIF2a have been detected in several tumour forms and they have been ascribed oncogenic function (Semenza 2011). High HIF-alpha protein levels correlate to poor patient outcome in a number of tumour forms such as breast (Helczynska et al. 2008; Yamamoto et al. 2008; Bos et al. 2003), colorectal (Yoshimura et al. 2004) and prostate cancer (Nanni et al. 2009). However, in neuroblastoma, HIF2a was linked to worse patient outcome, while HIF1a did not correlate significantly to outcome (Holmquist-Mengelbier et al. 2006; Noguera et al. 2009). In glioma, expression of HIF2a correlated to poor patient survival (Li et al. 2009), which was also seen in colon cancer (Yoshimura et al. 2004). Both HIF1a and HIF2a benefit the tumour cells by enabling metabolic adaptation and induction of angiogenesis. It was reported early on that HIF1a-deficient transplanted experimental teratoma tumours grew faster, partially due to loss of hypoxia-induced apoptosis (Carmeliet et al. 1998), and more recently similar findings were made for HIF2a (Acker et al. 2005). Other transplantation studies, in contrast, find reduced tumour growth and angiogenesis in tumours lacking HIF1a (Ryan et al. 1998, 2000). Furthermore, lack of HIF1a led to cell death under hypoxic conditions due to inability to cope with increased ROS levels (Kim et al. 2006). In a genetically induced breast cancer model, loss of HIF1a

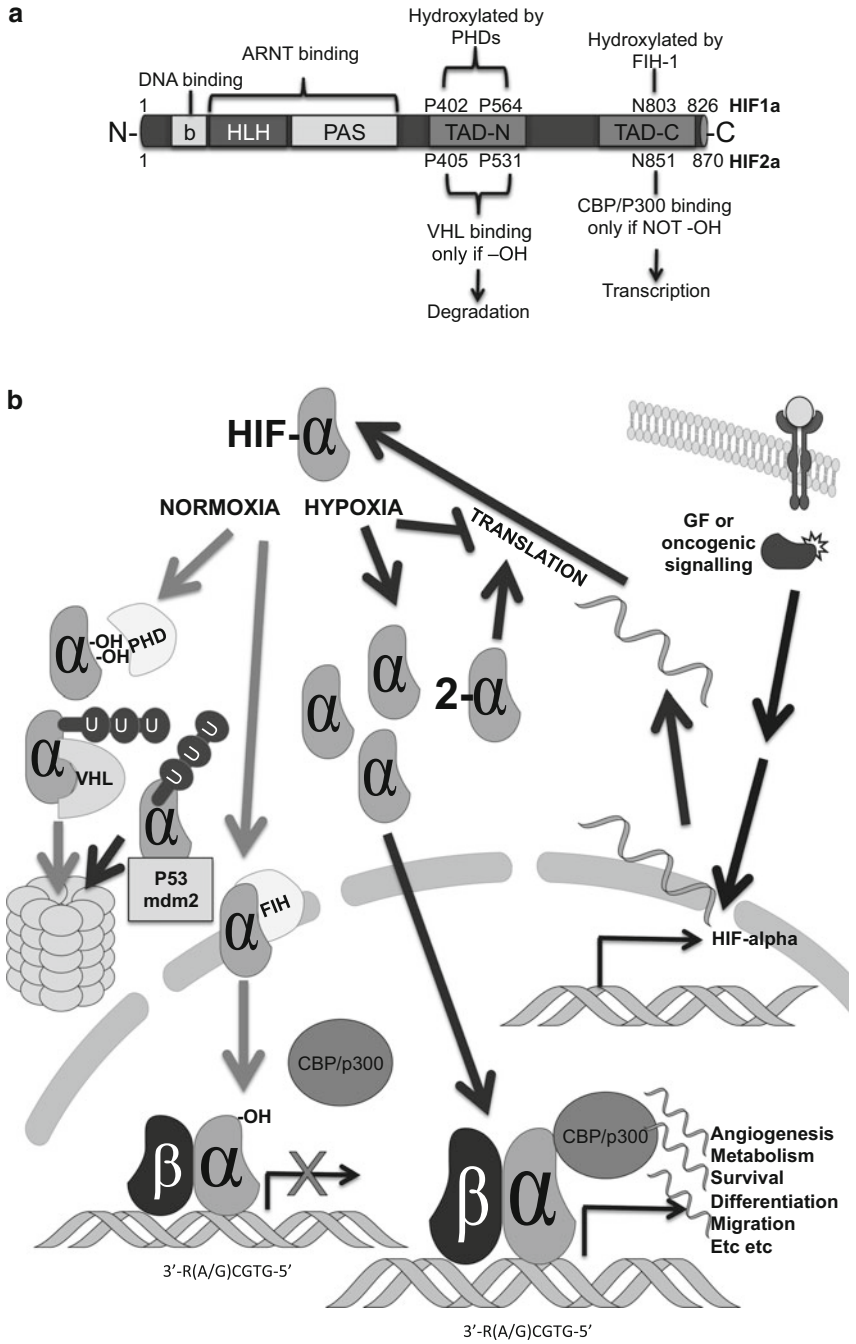

Fig. 4.2 The hypoxia-inducible transcription factors are the main mediators of the hypoxic response. (**a**) Schematic presentation of the HIF-alpha subunit. The basic (**b**) domain is DNA binding, and the helix-loop-helix (HLH) and PAS domains interact with the HIF-beta subunit. There are two transactivating domains, TAD-N and TAD-C. Hydroxylation of two proline

reduced metastasis (Liao et al. 2007), and recently, in a transplanted model, loss of HIF1a reduced metastasis and notably the CSC pool (Schwab et al. 2012).

There are few reports of mutations in the genes encoding HIF1a or HIF2a, despite directed efforts (Anastasiadis et al. 2002; Park et al. 2009), and presumably many non-published negative results. Polymorphisms in the HIF1a gene leading to more stable mRNA have been linked to increased risk of prostate cancer (Vainrib et al. 2012). Recently, mutations in *EPAS1*, encoding HIF2a, were detected in nonhereditary pheochromocytoma and paraganglioma (Zhuang et al. 2012; Yang et al. 2013; Comino-Mendez et al. 2013). The altered HIF2a protein showed increased stability leading to accumulation (Zhuang et al. 2012; Yang et al. 2013). Increased HIF protein levels due to oncogenic signalling are reported in several cancers (Semenza 2013).

Loss of function of the negative regulator of HIF1 and HIF2, the tumour suppressor protein von Hippel-Lindau (pVHL), is associated with the von Hippel-Lindau syndrome with increased susceptibility for kidney cancer, mainly clear cell renal carcinoma (ccRCC) (Shen and Kaelin 2013). These tumours have elevated protein levels of HIF-alpha, preferentially HIF2a, and the clear cell phenotype comes from extensive accumulation of fat in the tumour cells due to HIF-driven metabolic changes (Shen and Kaelin 2013; Kaelin 2008). In kidney cancer, HIF1a can have tumour suppressor function, and loss of HIF1a in pVHL-mutated tumours is associated with tumour progression and increased HIF2a activity (Shen and Kaelin 2013; Shen et al. 2011). Much of the studies on the role of HIFs in cancer has been performed in kidney cancers with loss of pVHL due to the high protein levels under normoxia in these cells (Shen and Kaelin 2013), but this situation may vary from other cancers with less prominent HIF dysregulation.

Fig. 4.2 (continued) residues within the TAD-N domain by pVHL destines HIF for degradation. Hydroxylation of an asparagine residue in the TAD-C domain by FIH-1 inhibits interaction with coactivators CBP/p300. B. Under normoxic conditions HIF-alpha are directly hydroxylated by the PHDs and thereby recognised by the pVHL E3 ligase and ubiquitinated and destined for proteasomal degradation. Binding to the p53/mdm2 complex also leads to HIF-alpha ubiquitination and degradation. When oxygen is lacking, or due to oncogenic events such as increased gene expression or loss of pVHL, HIF-alpha can accumulate and translocate to the nucleus. In the nucleus HIF-alpha and -beta associate, bind DNA and recruit coactivator CBP/p300 to initiate transcription. Hydroxylation of an asparagine residue in the TAD-C counteracts coactivator binding. When the alpha subunits are stabilised, they can bind and interact with numerous proteins. HIF2-alpha can bind mRNA in a complex with the RNA binding protein RBM4 and promote translation of specific mRNAs under hypoxic conditions. Generally hypoxia and HIF1a counteract protein translation. With elements from Motifolio©

4.2.1 Hypoxic Regulation of HIF-Alpha Subunits

The HIF-induced transcription is regulated by the abundance and activity of the alpha subunits, while the beta subunits are expressed in an oxygen-independent manner. Both HIF1a and HIF2a are mainly regulated by increased protein stability at low oxygen levels. However, increased mRNA levels have also been reported (Holmquist-Mengelbier et al. 2006; Jiang et al. 1997; Mohlin et al. 2013). At normoxia, HIF-alpha subunits are hydroxylated at two proline residues (P402 and 564 in HIF1a and P405 and 531 in HIF2a) by the enzymatic action of either of three HIF prolyl hydroxylase-domain proteins PHD1 (EGLN2), PHD2 (EGLN1) and PHD3 (EGLN3) (Bruick and McKnight 2001; Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001; Taylor 2001). The hydroxylated HIF-alpha subunits bind to pVHL, an E3 recognition component of the ubiquitin ligase complex, leading to HIF-alpha ubiquitination and rapid degradation by the proteasome (Cockman et al. 2000; Huang et al. 1998; Kallio et al. 1999; Maxwell et al. 1999; Ohh et al. 2000; Salceda and Caro 1997; Tanimoto et al. 2000) (Fig. 4.2). This regulatory process is directly regulated by oxygen since the hydroxylation requires dioxygen, 2-oxoglutarate/alpha-ketoglutarate and iron(II) (Epstein et al. 2001). In glioma, decreased levels of 2-oxoglutarate/alpha-ketoglutarate through mutation of the $NADP^+$-dependent isocitrate dehydrogenase 1 (IDH1) induced HIF accumulation in non-hypoxic cells (Zhao et al. 2009).

An additional (dioxygen, 2-oxoglutarate/alpha-ketoglutarate and iron (II) dependent) hydroxylation reaction influences the transcriptional activity of the HIF-alpha subunits. The factor inhibiting HIF1 (FIH-1) hydroxylates an asparagine residue in the HIF-alpha C-terminal transactivating domain (N803 in HIF1a and N851 in HIF2a) rendering HIF less able to recruit the transcriptional coactivator CBP/p300 (Lando et al. 2002a, b). Despite its name, FIH-1 hydroxylates both HIF1a and HIF2a, but has higher affinity for HIF1a (Bracken et al. 2006). In both proline and asparagine hydroxylation reactions, one oxygen atom is incorporated into the hydroxylated proline or asparagine residue of HIF-a and the other into 2-oxoglutarate forming carbon dioxide and succinate, which may enter the TCA cycle and act as an electron donor to the electron transport chain in addition to modulating pH. The tumour suppressor p53 can also mediate ubiquitination of the HIF-alpha subunits, independent of oxygen availability, thereby affecting HIF activity and tumour angiogenesis (Ravi et al. 2000).

4.2.2 Non-Hypoxia-Driven Regulation of the HIFs

In addition to the hypoxia-driven rescue from proteasomal degradation, the HIF-alpha subunits can also be regulated on transcriptional and translational levels. Physiologically induced growth factor signalling or oncogenic mechanisms such as receptor mutation or overexpression can via PI3 kinase and AKT induce HIF-alpha

transcription and translation (Semenza 2011; Laughner et al. 2001) or loss of the tumour suppressor p53 (Ravi et al. 2000). This would prepare cells both for increased oxygen and energy needs in growth factor-stimulated growth. Growth factors shown to induce HIF-alpha include insulin (Treins et al. 2002), insulin-like growth factor-I (Beppu et al. 2005) and vascular endothelial growth factor (VEGF) (Calvani et al. 2008). Epithelial growth factor receptor (EGFR/HER1/ErB1) signalling also induced HIF1a (Zhong et al. 2000). Most of these studies are on HIF1a regulation, but it is not unlikely that HIF2a can be induced by growth factor signalling as well, and HIF2a levels in neuroblastoma cells and neuroblasts correlate to IGF-2 levels (Jogi et al. 2002; Mohlin et al. 2013). With HIF1a being the preferred substrate for FIH-1, which would still act on the HIF-alpha subunits present at oxygenated conditions, HIF2a accumulation by other means than hypoxia may lead to significant HIF2 activity.

HIF-associated factor (HAF) is an E3 ubiquitin ligase that binds HIF1a independently of oxygen conditions and pVHL and destines HIF1a for proteasomal degradation (Koh et al. 2008). By contrast, HAF association to HIF2a instead reinforced its transcriptional activity (Koh et al. 2011).

4.2.3 Posttranslational Modifications of HIF1a and HIF2a

Both HIF1a and HIF2a are phosphorylated (Richard et al. 1999; Conrad et al. 1999) and differential phosphorylation patterns between the two factors lead to different ability to interact with other proteins (To et al. 2006).

Acetylation is an additional means whereby HIF transcriptional activity is regulated. The sirtuins (sirtuins are covered in the chapter by Marcia Haigis) are a family of proteins that acetylate the HIF-alpha subunits (Chen et al. 2012) in an HIF isoform-specific manner (Yoon et al. 2014). Sirtuins can also downregulate HIF activity in a non-catalytic manner (Hubbi et al. 2013). HIF1a and HIF2a are further subjected to sumoylation (van Hagen et al. 2010; Bae et al. 2004; Carbia-Nagashima et al. 2007), nitrosylation (Li et al. 2007a) and neddylation (Ryu et al. 2011). All these post-translational modifications may contribute to the complex tuning of HIF activity.

4.2.4 Transcriptional Regulation by HIF1a and HIF2a

Under hypoxic conditions, the stabilised HIF-alpha subunits bind to HIF-beta, translocate to the nucleus and bind to DNA sites with the core sequence 3′-R(A/G)CGTG-5′ known as the HIF-responsive element (HRE) in the promoter or enhancer region of regulated genes (Kimura et al. 2000, 2001). Transcription activation requires the recruitment of coactivators CBP/p300 and SRC/p160 proteins, and the ability of HIF-alpha to bind these is regulated by FIH-1 (Fig. 4.2).

At least HIF1a is also able to inhibit transcription of target genes by recruiting negative cofactor 2, thereby blocking the assembly of the transcription preinitiation complex (Denko et al. 2003).

The surrounding DNA landscape and chromatin state further influence HIF-recruitment to HRE sites and transcription initiation. The HIFs bind preferentially to genes expressed also in the normoxic state indicating that they are not themselves directing hypoxia-induced chromatin changes to their target genes (Xia and Kung 2009; Schodel et al. 2011) and histone demethylase activity promotes HIF transcriptional activity (Luo et al. 2012). That the HIFs bind HREs where the chromatin is already open has been suggested as one explanation for the cell-type-dependent differences between which genes are transcriptionally induced by the HIFs (Schodel et al. 2011). A number of chromatin modulators including histone demethylases are among the genes, which are transcriptionally induced by HIFs, suggesting a significant role for them in chromatin regulation (Xia et al. 2009; Krieg et al. 2010).

In concordance with the main purposes of the hypoxic response, i.e. to secure energy production and increase perfusion/angiogenesis to relieve the hypoxic stress, many HIF-induced genes are involved in metabolism, e.g. in glycolysis and glucose uptake (e.g. aldolases A and C, enolases 1 and 2, hexokinases 1 and 2, lactate dehydrogenase, pyruvate dehydrogenase kinases 1 and 3, glucose transporters 1 and 3) and, furthermore, in angiogenesis (e.g. vascular endothelial growth factor (VEGF), platelet-derived growth factor B (PDGFB), placental growth factor (PGF)) (Carmeliet et al. 1998; Iyer et al. 1998; Kelly et al. 2003). Growth factors, for example, VEGF, can also promote cell survival and proliferation through autocrine and paracrine signalling (Weigand et al. 2005; Perrot-Applanat and Di Benedetto 2012; Baek et al. 2000; Bachelder et al. 2002). Several hundred genes have been reported to be HIF induced, and the list is rapidly growing as new cell types are analysed by genome-wide methods. Notably, there are large variations between the genes induced in different cells and under different settings such as level of hypoxia and time frame.

HIF1a and HIF2a were initially seen as homologues acting in partially different cells and with different kinetics but inducing the same responses. However, lately the differential and sometimes even opposing effects of HIF1 and HIF2 have gained focus (Keith et al. 2012). Diverging ability to recruit cofactors, different affinity for regulating factors, varying affinity for specific HREs and diverse activities in addition to transcriptional regulation of HIF1a and HIF2a shape their effect on cell phenotype. HIF1 and HIF2 do bind to the same core DNA sequence, but there are plenty of reports on differential preference for one of the factors in regulation of specific genes, such as HIF1, preferentially inducing genes of the glycolytic pathway, e.g. hexokinases, phosphofructokinase and phosphoglycerate kinase 1 (Ryan et al. 1998; Iyer et al. 1998; Hu et al. 2003), and HIF2, e.g. the stemness-associated gene Oct-4 (Covello et al. 2006). However, it is likely an oversimplification to view certain genes as HIF1 and others as HIF2 driven since numerous factors influence HIF DNA binding and transcription activation. Furthermore, the picture is complicated by the fact that HIF1a and HIF2a have different kinetics in response to

hypoxia, with HIF1a being active early under hypoxic conditions and then declining, while HIF2a levels increase later and remain for a longer period (Helczynska et al. 2008; Holmquist-Mengelbier et al. 2006). HIF2a also has a higher oxygen threshold for activation and induces transcription at oxygen concentrations approaching physiological levels (Holmquist-Mengelbier et al. 2006). As one example, VEGF gene expression has been shown to be induced by (1) both HIF1a and HIF2a, (2) preferentially by HIF1a and (3) preferentially by HIF2a in different settings (Holmquist-Mengelbier et al. 2006; Ryan et al. 1998; Iyer et al. 1998; Hu et al. 2003; Raval et al. 2005; Tang et al. 2004; Rankin et al. 2008). Indeed, HIF1a was first identified through its binding to the erythropoietin (EPO) receptor, but later HIF2a has been shown to induce EPO expression (Yeo et al. 2008; Rankin et al. 2007).

4.2.5 Hypoxia and HIF Activity Affect Protein Translation

Mammalian target of rapamycin (mTOR) promotes mRNA translation into protein by phosphorylation of 4EBP1 leading to the release of the eukaryotic translation initiation factor 4E (eIF4E) allowing it to bind to the 5′ cap of messenger RNA and initiate translation (Gingras et al. 2001). mTOR complex I activity is up-regulated in many tumours through activation of oncogenes and/or loss of tumour suppressors. Energy depletion, lack of amino acids (Gingras et al. 2001; Sengupta et al. 2010) and hypoxia (Brugarolas et al. 2004; Koritzinsky et al. 2006; Liu et al. 2006; Braunstein et al. 2007) can all inhibit mTOR and induce a general repression of protein translation and thereby preservation of resources. Hypoxic suppression of mTOR complex I can be due to lowered ATP levels (Liu et al. 2006) or it can be HIF1a orchestrated. HIF1 induces the expression of REDD1, which is an inhibitor of mTOR activity. High REDD1 levels lead to activation of the mTOR inhibitor TSC2 (Brugarolas et al. 2004; DeYoung et al. 2008). In addition, induced expression of the HIF transcriptional target BNIP3 (B-cell lymphoma 2 (Bcl2)/adenovirus E1B 19 kDa protein-interacting protein 3) also lowers mTOR activity (Li et al. 2007b). On the other hand, HIF1a induces the expression of eIF4E1 allowing translation of selective transcripts under hypoxic conditions, which was shown to promote tumorigenic traits in breast cancer cells (Yi et al. 2013).

Contrary to HIF1a, HIF2a has an mTOR-activating effect that would promote hypoxic protein synthesis and proliferation. Assuming HIF2a is the predominant HIF at near-physiological and chronically low oxygen levels, allowing protein synthesis would benefit cell survival in these settings. HIF2a-induced growth factors promote activation of mTOR (Roberts et al. 2009), and HIF2a-induced expression of FIP200 has been reported to promote mTOR complex I activation (Gan et al. 2005; Chano et al. 2006).

4.2.6 Translational Regulation by HIF2a

HIF2a was recently shown to further counteract the general hypoxic repression of protein translation by associating to an RNA hypoxia-responsive element (rHRE) of specific target mRNAs by forming a complex with the RNA binding protein RBM4 and the alternative translation initiation factor eIF4E2. This complex captures the 5′ cap and locates the mRNA to polysomes for active translation under hypoxic conditions (Uniacke et al. 2012). This cytoplasmic activity is specific to HIF2a and is HIF-beta independent. The hypoxia-specific translation initiation by HIF2a was first shown for epithelial growth factor receptor (EGFR), a protein known to be translationally upregulated at hypoxia (Franovic et al. 2007), and was then seen for other growth factor receptors such as PDGF receptor alpha and IGF1 receptor (Uniacke et al. 2012). These are all examples of growth factor receptors important in human cancers, and upregulation by HIF2a activity, induced due to hypoxia or oncogenic events, could contribute to cell proliferation, cell survival and tumour progression.

4.2.7 HIF1a, HIF2a and p53

Another important non-transcriptional HIF activity is the interplay between HIF1a and the tumour suppressor p53. As mentioned above, p53 accumulates in response to hypoxic stress. In normal cells, MDM2 regulates p53 levels by binding and inducing p53 to be ubiquitinated. HIF1a binds the MDM2/p53 complex and skews the effect of MDM2 to instead promote p53 activity (An et al. 1998; Sanchez-Puig et al. 2005; Chen et al. 2003). However, the association of HIF1a with the MDM2/p53 complex provides a negative feedback on HIF1a by MDM2-dependent ubiquitination of HIF1a which targets it for proteasomal degradation (Ravi et al. 2000).

HIF2a does not associate with MDM2 or p53 but counteracts p53 activity by a number of pathways affecting p53 phosphorylation (Bertout et al. 2009) as well as via induced expression of growth factors like PDGF-beta and TGF-alpha. This activity of HIF2a would further contribute to proliferation under hypoxic conditions.

4.2.8 Effects of Hypoxia and the HIFs on the Oncogene MYC

The MYC oncogene, overexpressed to varying degrees in many cancers, is a transcription factor of the bHLH family belonging to the leucine zipper group and is transcriptionally active as a heterodimer with MAX (MYC is covered in detail in Chap. 5). MYCN is a MYC homologue identified through its

overexpression and amplification in neuroblastoma. Generally, MYC promotes cell proliferation, anabolic metabolism and protein synthesis.

HIF1a mediates hypoxia-induced proliferation suppression by inhibiting MYC activity (Koshiji et al. 2004). MYC activity is dependent on binding SP1, but under hypoxic conditions, HIF1a sequestration of SP1 leads to disruption of the MYC/MAX complex and displacement of MYC from numerous target gene promoters (Dang et al. 2008). Furthermore, HIF1a induces the transcription of MXI1, a MAX-binding transcriptional repressor and negative regulator of MYC (Corn et al. 2005). At hypoxia, the HIF1a-induced MYC repression counteracts protein synthesis and proliferation in favour of glycolysis providing ATP. HIF2a is not able to interact with SP1 due to specific phosphorylation at the HIF2a PAS domain (Mylonis et al. 2006). HIF2a can bind to MAX and this stabilises the MYC/MAX complex promoting proliferation under hypoxic conditions (Gordan et al. 2007).

The levels of HIF1a, HIF2a and MYC influence the effect of hypoxia on MYC activity. The increased MYC levels in tumours range widely from moderate upregulation to very high levels resulting from mutation or gene amplification. At very high MYC or MYCN levels, as a consequence of gene amplification, HIF1a cannot repress MYC activity (Kim et al. 2007; Qing et al. 2010), and under such conditions, MYC and HIF1a can both contribute to induced glycolysis by induced expression of glycolytic proteins (HK2, PDK1, PGK1) and also to VEGF expression.

4.2.9 Hypoxia, HIFs and microRNA

The expression of a number of small non-protein-coding RNAs, microRNAs (miR), is modified by hypoxia. The microRNAs influence cell phenotype and behaviour by post-transcriptional regulation of gene expression. Altered microRNA expression is reported in several malignancies and affects patient outcome. Expression of many microRNAs in cancer is linked to hypoxic conditions, HIF activity and angiogenesis (Kulshreshtha et al. 2007). The pro-oncogenic miR-21 (Moriyama et al. 2009) was induced in hypoxic breast cancer cells (Kulshreshtha et al. 2007), and miR-21 overexpression induced HIF1 and angiogenesis (Liu et al. 2011). Another hypoxia-induced microRNA is miR-210 (Kulshreshtha et al. 2007; Camps et al. 2008; Chan and Loscalzo 2010; Gee et al. 2010; Puissegur et al. 2011; Quero et al. 2011; Huang et al. 2009), which also contributes to angiogenesis by promoting VEGF expression in an HIF1-dependent manner (Quero et al. 2011; Devlin et al. 2011). The p53-regulated miR-107, which has tumour-suppressing effects, is induced under hypoxic conditions and has a suppressing effect on HIF1 and tumour angiogenesis (Yamakuchi et al. 2010).

4.2.10 Hypoxic Modulation of Wnt/Beta-Catenin Activity

The Wnt-signalling pathway is an important positive regulator of cell proliferation, growth, differentiation, (cancer) stem cells and the EMT phenotype (Reya and Clevers 2005; Anastas and Moon 2013). Wnt/beta-catenin signalling is distorted in several cancers (Anastas and Moon 2013). The interaction between HIF- and Wnt-signalling pathways is complex and not completely delineated. HIF1a can bind to beta-catenin, thereby competing for its transcriptional cofactor TCF4 and leading to hypoxic downregulation of beta-catenin activity (Kaidi et al. 2007). On the other hand, beta-catenin can augment HIF-induced transcription promoting cellular adaptation to hypoxic conditions (Kaidi et al. 2007; Lim et al. 2008). In a number of studies on embryonic stem cells and in cancer, Wnt/beta-catenin activity was augmented in an HIF1a-dependent manner (Mazumdar et al. 2010; Jiang et al. 2007; Genetos et al. 2010; Giles et al. 2006; Zhao et al. 2011).

4.2.11 HIF1a and Notch Signalling

Notch signalling is instrumental in embryogenesis and organ development. When Notch transmembrane receptors bind to Notch ligand expressed on adjacent cells, the Notch intracellular domain (NICD) is cleaved and translocates to the nucleus where it acts as a transcription modulator (Ranganathan et al. 2011). Among the first to link Notch stem cell pathway to HIF activity were Gustafsson and colleagues (Gustafsson et al. 2005) who showed the association between HIF1a and NICD stability and transcriptional activity. The positive effect of hypoxia on the Notch pathway is in line with the higher levels of Notch protein that we detected in hypoxic neuroblastoma cells with a stem cell-like phenotype (Jogi et al. 2002). Another mechanism whereby HIF1 and hypoxia promote Notch signalling is the induction of expression of APH1-A encoding a component of the gamma-secretase complex, which cleaves Notch during activation (Wang et al. 2006).

It was recently shown in breast cancer cells that the Notch ligand Jagged2 is induced by hypoxia in an HIF1a-dependent manner contributing to hypoxia-induced Notch signalling (Pietras et al. 2011). Watabe et al. showed that Jagged2 was upregulated by hypoxia in the invasive front of breast tumours promoting breast cancer CSCs, EMT and metastasis (Xing et al. 2011). FIH-1 interaction with Notch provides an additional cross-linking between Notch and hypoxic signalling (Zheng et al. 2008).

4.3 Tumour Hypoxia, HIF Signalling and Patient Outcome

Numerous publications support a link between low oxygenation of the primary tumour and poor patient outcome. High levels of HIF1a (Jubb et al. 2010) (a meta-analysis) (Bos et al. 2003; Kronblad et al. 2006) and HIF2a (Helczynska et al. 2008; Holmquist-Mengelbier et al. 2006; Li et al. 2009; Chen et al. 2011) have been linked to increased metastasis and poor survival as mentioned above. Furthermore, increased expression of the HIF target gene VEGF correlates to poor prognosis (Gasparini et al. 1997; Linderholm et al. 2000) through its ability to drive angiogenesis and induce vascular permeability and by acting as a cell growth and survival factor (Hamerlik et al. 2012; Goel and Mercurio 2013). Also the HIF target gene carbonic anhydrase IX (CAIX) has been linked to poor patient survival (Brennan et al. 2006; Chia et al. 2001), and the conjunct acidification of the tumour extracellular environment can contribute to inflammation and a pro-invasive tumour phenotype (see Chap. 9). BNIP3 is another HIF-induced protein linked to tumour recurrence and decreased patient survival (Giatromanolaki et al. 2004; Tan et al. 2007). Expression of this factor in tumour cells reflects HIF activity, but despite being generally viewed as pro-apoptotic, BNIP3 adjusts cell metabolism to hypoxia and contributes to cancer cell survival under hypoxic conditions by inducing mitophagy (Zhang et al. 2008). Moreover, HIF1a-induced autophagy promoted a CSC phenotype in pancreatic cancer cells (Zhu et al. 2013).

4.4 Tumour Hypoxia and Tumour Progression

Progressive tumour disease includes tumour growth, tumour recurrence after therapy, invasive growth and/or metastasis. Numerous processes are involved in tumour progression and metastasis. For tumour progression, vascularisation must be induced to provide the proliferating cells with nutrients and oxygen and remove waste products (Naumov et al. 2006a, b) as well as to provide disseminating cancer cells access to the circulation. When metastasis takes place, epithelial-derived cancer cells in the primary tumour acquire motility by the process of EMT (Polyak and Weinberg 2009; Kalluri and Weinberg 2009; Thiery et al. 2009). TGF-beta signalling is well established to induce EMT in epithelial and cancer cells (Katsuno et al. 2013; Morrison et al. 2013). EMT traits are loss of polarity and cell–cell contact between the epithelial cells, marked by loss of cell surface E-cadherin and gain of mesenchymal markers such as N-cadherin and vimentin. The motile mesenchymal phenotype allows the cancer cells to migrate and reach the vasculature and then intra-vasate into the circulatory system. Tumour establishment at the metastatic site involves the reversed process; mesenchymal-to-epithelial transition (MET) and the metastases reexpress E-cadherin (Hugo et al. 2007).

The CSC theory postulates that only a subpopulation of cancer cells has the ability to propagate the tumour and that these tumour cells possess properties

similar to stem cells (Visvader and Lindeman 2012). The embryonal and adult tissue stem cells reside in the so-called niches, locations where the microenvironment perfectly supports the needs of these cells to remain in a stem cell state (Spradling et al. 2001). Differentiation of daughter cells is accompanied by migration out of the specific niche. In many cases, these stem cell niches are locations of specific oxygen access; a well-studied example is the haematopoietic stem cells (HSCs) that reside in the most hypoxic regions of the bone marrow and have a glycolytic metabolism. Their differentiation is concurrent with leaving this niche and increased oxygenation (Cipolleschi et al. 1993; Parmar et al. 2007). Other HSC markers showed localisation of a stem cell niche close to endothelial cells that are likely to be well oxygenised (Kiel et al. 2005), suggesting that different stem cell populations reside in different niches with diverging oxygen microenvironments ranging from hypoxic to perivascular. With the similarities between normal stem cells and CSCs came the proposal that also the CSCs reside in niches and that oxygen levels are an important part of the microenvironment of the niches.

The properties that define a CSC are self-renewal, pluripotency and the ability to initiate tumour formation (after introduction in immune compromised mice). Because of the latter criteria, such cells are often referred to as tumour-initiating cells (TICs). This nomenclature also acknowledges that CSCs may arise from transformation of a stem or progenitor cell, but just as well, through dedifferentiation or by other means acquiring this phenotype in a more differentiated cell. CSCs were first identified in leukaemia (Lapidot et al. 1994) where HSC markers are well defined. Cancer cell subpopulations with CSC properties have since been identified in a number of solid tumours, e.g. brain (Singh et al. 2004), breast (Al-Hajj et al. 2003), colon (O'Brien et al. 2007; Ricci-Vitiani et al. 2007), lung (Eramo et al. 2008), melanoma (Schatton et al. 2008), neuroblastoma (Hansford et al. 2007), ovarian (Alvero et al. 2009), pancreas (Hermann et al. 2007) and prostate (Patrawala et al. 2006).

Importantly, cancer cells undergoing EMT can acquire CSC traits and increased tumour-initiating ability through this process (Mani et al. 2008; Morel et al. 2008). Moreover, CSCs express less adhesion molecules such as E-cadherin. There is thus overlap in the protein expression patterns associated with EMT and the CSC phenotype, respectively, i.e. in two central concepts in cancer progression.

4.4.1 Tumour Hypoxia Induces Angiogenesis and Metastasis

Hypoxia and HIF-induced pro-angiogenic molecules, primarily VEGF, are instrumental in the development of the tumour (-induced) vasculature (Semenza 2003). Hypoxic conditions contribute to the tissue remodelling necessary for angiogenic sprouting, e.g. by inducing expression of extracellular proteases such as the metalloproteinases MMP2 and MMP9 (Yang et al. 2010) and components of the plasminogen activating cascade (Buchler et al. 2009; Oszajca et al. 2008; Tacchini

et al. 2003). The remodelling of the extracellular matrix may furthermore release more pro-angiogenic components located there.

Hypoxic conditions in the tumour also affect the stromal cells. Infiltrating immune cells and fibroblasts react by producing pro-angiogenic factors as well as cytokines which modulate the immune response and ECM components contributing to fibrosis. The many and complex effects of hypoxia on immune cells will not be covered here. The endothelial cells are also influenced by hypoxia; VEGF induces angiogenic processes and promotes vascular permeability allowing serum with liquid and proteins to enter the tissue and tumour cells to enter circulation (Garcia-Roman and Zentella-Dehesa 2013). Recently, HIF1a in endothelial cells was shown to contribute to tumour metastasis by promoting tumour cell passage over the endothelial barrier in a mouse breast cancer model (Branco-Price et al. 2012).

The tumour vascularity, i.e. the endothelial cells, is instrumental in the establishment of niches providing the specific microenvironment including cell–cell interactions, soluble factors, ECM and oxygen conditions necessary for maintaining the pool of tumour propagating cells referred to as CSCs (Kiel et al. 2005; Ghajar et al. 2013; Infanger et al. 2013; Beck et al. 2011; Calabrese et al. 2007).

Breast cancer cells that have undergone experimentally induced EMT have a high expression of VEGF, which caused their increased ability to form tumours (Fantozzi et al. 2014). Such high VEGF expression was also found in patient-derived cancer cells with a CSC phenotype (Fantozzi et al. 2014), further linking expression of the HIF-target gene VEGF to EMT and CSC phenotype. Moreover, as mentioned above, VEGF promotes cancer cell survival and progression and glioma CSC viability (Hamerlik et al. 2012; Goel and Mercurio 2013).

4.4.2 Hypoxic Induction of EMT

Hypoxia can induce EMT in normal (Higgins et al. 2007) as well as cancer cells derived from various tumour types (Chen et al. 2010; Zhang et al. 2013; Chang et al. 2011; Sahlgren et al. 2008; Krishnamachary et al. 2003, 2006). Several pathways contributing to EMT are influenced by low oxygenation. Hypoxia promoted EMT can be achieved through the direct transcriptional action of HIF (Higgins et al. 2007), as the EMT-inducing bHLH transcription factor Twist is a direct transcriptional target of HIF1a (Yang et al. 2008; Sun et al. 2009). Twist represses E-cadherin and Twist overexpressing breast cancer cells are more motile and invasive (Mironchik et al. 2005). In a mouse lung cancer model, HIF2a was shown to induce EMT, with induced expression of Snail and vimentin, and cancer invasion (Kim et al. 2009). Hypoxia-induced EMT can also be mediated via induction of Notch signalling in cultured breast cancer cells (Sahlgren et al. 2008) including upregulation of Jagged proteins similar to what has been described for CSC regulation (see below). During EMT, the loss of cell surface-associated E-cadherin allows beta-catenin to translocate to the nucleus and involve

in transcriptional signalling that modulates cell growth (Bienz and Clevers 2003). Wnt/beta-catenin signalling is implicated in EMT as well as the CSC phenotype (Katoh and Katoh 2007; Takebe et al. 2011; Liu et al. 2005; de Sousa et al. 2011) and this can be induced through HIF1a (Jiang et al. 2007). In human prostate cancer cells, HIF1-induced EMT showed concurrent increased Wnt signalling (Jiang et al. 2007). Furthermore, knockdown of beta-catenin reversed HIF1-induced EMT and metastatic potential (Zhao et al. 2011). HIF-induced VEGF, discussed above, can in addition to contributing to angiogenesis and cell survival induce EMT (Gonzalez-Moreno et al. 2010; Yang et al. 2006).

4.4.3 Tumour Hypoxia and HIFs Promote Stem Cell Phenotype

Oxygen gradients and hypoxia arise naturally during embryogenesis and organ development and regeneration (Simon and Keith 2008). Both HIF1a and HIF2a are expressed during embryonal development with different but overlapping expression patterns (Jain et al. 1998). Gene targeting of either HIF1a or HIF2a results in mid-gestation lethality. Embryos lacking HIF1a show severe malformations affecting the vasculature, somite formation and the neural fold (Ryan et al. 1998). More than 30 years ago it was shown that low oxygen levels were necessary for proper development of the neural folds and neural crest in rat embryos in vitro (Morriss and New 1979). Furthermore, culture of bovine blastocysts (Harvey et al. 2004) and human embryonic stem (ES) cells (Ezashi et al. 2005) at physiological to mildly hypoxic conditions compared to air oxygen levels showed maintained undifferentiated morphology and marker expression at the lower oxygen levels (2–7 % oxygen). However, the comparison to hyperoxic conditions makes it difficult to ascertain if these effects are due to hypoxic or rather to near-physiologic oxygen conditions maintaining stem cell populations.

Loss of HIF2a has been reported to affect function of the sympathetic ganglia and paraganglia (Tian et al. 1997), the circulatory system (Peng et al. 2000), lung maturation (Compernolle et al. 2002) and pathology of multiple organs including retinopathy, hepatic steatosis, cardiac hypertrophy and skeletal myopathy (Scortegagna et al. 2003) in different mouse strains. We showed expression of HIF2a in human foetal paraganglia at 8.5-week gestation (Nilsson et al. 2005). Taken together, these reports suggest important developmental roles for the HIFs and presumably hypoxia in embryogenesis and organ development.

We showed that neuroblastoma cells under hypoxic conditions dedifferentiated and acquired stem cell-like traits both in vitro and in vivo (Jogi et al. 2002, 2004). In breast cancer, ductal carcinoma in situ, we found that cancer cells residing in the perinecrotic areas were more immature and expressed the early luminal keratin K19 (Helczynska et al. 2003). In oestrogen receptor (ER) positive lesions, the expression of ER was lost in hypoxic and HIF1a-expressing cells, further emphasising their

loss of differentiation (Helczynska et al. 2003). Furthermore, in in vitro studies of primary human breast cells and MCF10 cells in three-dimensional culture, we found that hypoxia increased the protein levels of HIF1a and HIF2a and hindered ECM-induced differentiation (Vaapil et al. 2012). Notably, the hypoxic cells remained proliferative, whereas the normoxic cells entered a differentiated post-mitotic state upon ECM stimulation (Vaapil et al. 2012).

Neuroblastoma cells with stem cell traits in patient tumour specimens were found to reside adjacent to vessels and have high protein levels of HIF2a, but not HIF1a (Pietras et al. 2008). Forced downregulation of HIF2a in patient-derived cells grown in vitro led to partial differentiation and loss of VEGF expression (Pietras et al. 2009). Rich and colleagues showed that glioblastoma stem cells express high levels of HIFs, especially HIF2a, and VEGF and that hypoxic conditions contribute to maintaining and expanding the glioblastoma CSC population as well as the tumour-inducing capacity of these cells (Li et al. 2009). In addition, hypoxia or induced expression of HIF2a induced stem cell phenotype including self-renewal, in non-stem glioblastoma cells, and this was accompanied by expression of the important stem cell transcription factors Oct-4 (Tai et al. 2005; Nichols et al. 1998; Hochedlinger et al. 2005), Nanog (Hart et al. 2004) and MYC (Heddleston et al. 2009). Of these, Oct-4 was shown to be a direct HIF2a transcriptional target (Covello et al. 2006). Indeed, induced HIF activity increased stem cell marker expression in cancer cells derived from prostate, brain, kidney, cervix, lung, colon, liver and breast tumours (Mathieu et al. 2011), further strengthening for the link between the HIFs and CSCs.

The Rich lab showed that glioma CSC proliferation and tumour growth are promoted by iNOS (Eyler et al. 2011) and iNOS is another transcriptional target of HIF2a (Yang et al. 2010). Also in glioma, HIFa-expressing cells with CSC phenotype were found in at least two different niches, perinecrotic and perivascular (Heddleston et al. 2010), suggesting that both hypoxic- and non-hypoxic HIFa expression contribute to the CSC phenotype. Landberg et al. report an increased CSC population in oestrogen receptor alpha (ER) positive breast cancer cells after exposure to hypoxia, but not in ER negative cells (Harrison et al. 2013). The hypoxic effect on CSC fraction was dependent on HIF1a and Notch (Harrison et al. 2013). Notch signalling maintains the Notch-expressing cells in a stem or progenitor state (Artavanis-Tsakonas et al. 1999; Hansson et al. 2004), and Notch-induced inhibition of differentiation is reported in a number of progenitor cell types: myogenic, haematopoietic and neuronal (Nofziger et al. 1999; Dahlqvist et al. 2003; Varnum-Finney et al. 2000; de la Pompa et al. 1997). As described above, HIF1a enhances Notch signalling and thus contribute to maintaining an undifferentiated state.

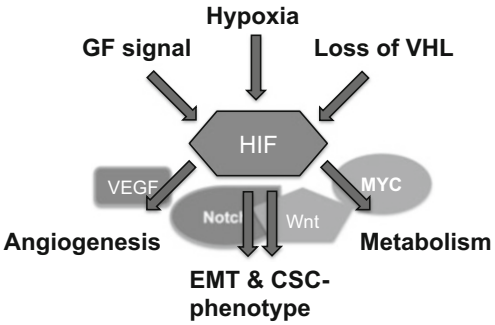

Fig. 4.3 Hypoxia, HIF1a and HIF2a are drivers behind tumour progression. HIF1a and HIF2a modulate the phenotype of cancer cells, including metabolism, angiogenesis and EMT and CSC phenotype directly as well as indirectly

Concluding Remarks

Hypoxia is common in solid tumours, and both HIF1a and HIF2a, the primary mediators of the hypoxic response, are widely expressed in human cancers. The HIFs are accumulated and activated by hypoxia, but can also be induced by oncogenic events. Tumour hypoxia and HIF1a and HIF2a activity are linked to tumour progression and poor prognosis. The HIFs play key roles in cancer progression and metastasis by inducing cell survival, adapted metabolism, angiogenesis, EMT and CSC phenotype (Fig. 4.3). This occurs in a complex interplay between HIF1a and HIF2a, in which these factors can join forces as well as oppose each other. Furthermore, the HIFs interact with other signalling pathways such as those of MYC, p53, Notch and Wnt/beta-catenin and can modulate their effects on tumour characteristics. The interplay between these important signalling pathways in cancer progression remains to be fully delineated.

Because hypoxic signalling correlates to and even drives core processes of tumour progression and is also confined to the tumour, it is an appealing target for tumour therapy. However, the partially diverging roles of HIF1 and HIF2 and the various responses to hypoxia in different cells and settings remain a challenge.

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Part II
Some Specific Regulators of Cellular Metabolism in Normal and Cancer Cells

Chapter 5
MYC Regulation of Metabolism and Cancer

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5.1 Introduction

The field of cancer metabolism has grown rapidly over the last decade as more links between oncogenes and metabolism are uncovered 90 years after Otto Warburg made his seminal observations that many cancers consume vast amounts of glucose and convert it to lactate (Vander Heiden et al. 2009; Koppenol et al. 2011; Cantor and Sabatini 2012). To grow, proliferate, and survive, cancer cells exhibit different metabolic programs including the Warburg effect. However, the Warburg effect per se is insufficient to provide cancer cells with all the substrates for growth and survival. In the past decade, many genetic changes in cancers have been linked to the Warburg effect, glutaminolysis, carbohydrate, and fatty acid metabolism. Among the genetic alterations, oncogenic MYC stood out as a canonical oncogene that was first to be linked mechanistically to altered glucose metabolism, when it was discovered to transactivate *LDHA*, encoding lactate dehydrogenase A (Shim et al. 1997). MYC is now known to amplify the expression of thousands of genes, with the bulk involved in various aspects of cell metabolism including glycolysis, glutaminolysis, nucleotide, protein, and fatty acid metabolism (Dang 2012). MYC is also involved in mitochondrial and ribosome biogenesis. ln this chapter, we will provide an overview of key roles of MYC in regulating metabolism and how they contribute to cancer development and progression.

5.2 MYC and Cancer

Cell growth or the increase in cell mass required for cell proliferation involves the import of nutrients and their conversion to cell mass and ATP. MYC and the mTOR pathway are critical for cell growth. Discovery of the retroviral v-MYC oncogene (Duesberg and Vogt 1979) led to the identification of its cellular homologue termed c-MYC (Vennstrom et al. 1982). Herein, the human gene will be termed *MYC* (italicized) and the protein termed MYC. The normal proto-oncogene *MYC* is downstream of many growth factor signaling pathways, including receptor tyrosine kinase pathway, T cell receptor pathway, and WNT signaling pathways that regulated its expression through sensing extracellular cues (Dang 2012). The *MYC* proto-oncogene is a member of the *MYC* family, which includes *MYCN* and *MYCL*. While *MYCN* is commonly amplified in neuroblastoma, an aggressive childhood cancer, *MYCL* is only occasionally amplified in some human small cell lung cancer (Brodeur et al. 1984; Nau et al. 1985).

The *MYC* proto-oncogene is activated by chromosomal translocations in human Burkitt's lymphoma (Dalla-Favera et al. 1982; Taub et al. 1982). The juxtaposition of *MYC* to one of three immunoglobulin enhancers via chromosomal translocations deregulates its expression. *MYC* is now found to be one of the most frequently amplified human oncogene among many different types of cancers, illustrating its central role in human cancer development (Beroukhim et al. 2010; Atlas 2012a, b;

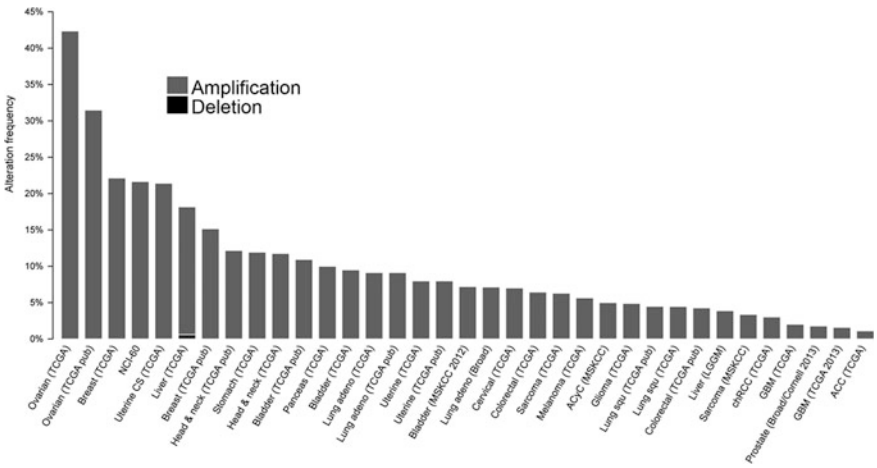

Fig. 5.1 MYC alterations in human cancer and cancer cell lines. TCGA data are displayed through cBioPortal. Note that MYC is largely amplified across multiple human cancers

Cancer Genome Atlas Research N 2012; Cancer Genome Atlas Research N et al. 2013) (Fig. 5.1).

Furthermore, loss of upstream regulators such as APC in the WNT pathway can also lead to deregulated *MYC* gene expression in cancers (Beroukhim et al. 2010; He et al. 1998). In addition to its important role in cancer, *MYC* plays a pivotal role in maintaining the pluripotency of stem cells (Varlakhanova et al. 2010). Notably, MYC is also one of the four transcription factors that can induce pluripotency in human skin fibroblast (Takahashi and Yamanaka 2006). These observations suggest that MYC's contribution to cellular dedifferentiation might be central to its neoplastic transforming activity.

Given its important role in growth, *MYC* expression is tightly regulated in non-transformed cells such that its acute overexpression results in activation of cell cycle checkpoints such as ARF and p53 (Dang 2012). Hence in normal cells, overexpressed MYC leads to cell growth arrest or apoptosis. Unlike their normal counterparts, many cancers with deregulated MYC lose these checkpoints such as loss of p53 in human Burkitt's lymphoma (Schmitz et al. 2012, 2014).

The role of MYC in tumorigenesis has been extensively studied in a number of human cell lines and transgenic mouse models. While overexpression of MYC in cells that have lost checkpoints results in tumorigenesis, loss of MYC can trigger cell death in a Burkitt's lymphoma model cell line with a tetracycline-regulated MYC transgene (Yustein et al. 2010). Activation of an MYC transgene specifically in the liver of a mouse model induced the formation of large liver tumors, which also exhibited the so-called oncogene addiction. Being addicted, these tumors regressed upon silencing of the MYC transgene (Felsher 2010). These findings suggest that MYC is important in tumor initiation and maintenance. Once these tumors are established, they can also be addicted to MYC, perhaps partially via the

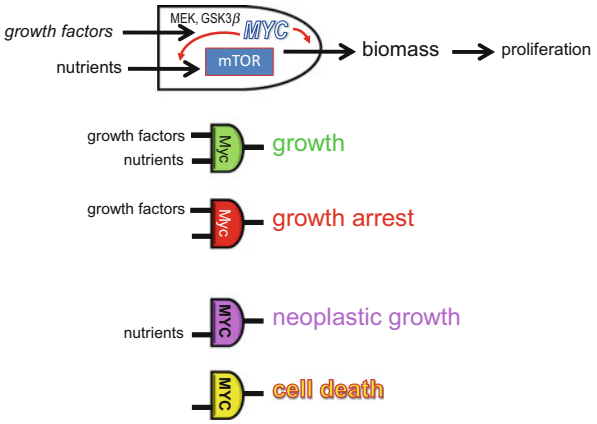

Fig. 5.2 MYC proto-oncogene mediates a transcriptional program that provides nutrients for the cell and stimulates it to grow. In normal cells, it is surmised that growth factors and nutrients are both required for cell proliferation, such that nutrient deprivation will result in diminished (lowercase MYC) MYC expression leading to growth arrest

metabolic pathways that MYC regulates. Indeed, we hypothesize that deregulated MYC results in deregulated cell growth signaling that requires a commensurate constitutive source of bioenergetic nutrients, such that the MYC-transformed cell becomes addicted to glucose, glutamine, and other nutrients (Fig. 5.2). In this regard, we will review in this chapter the connections between MYC, cell growth, proliferation, and metabolism as they are related to cancer biology and therapy.

5.3 Function of MYC

MYC protein is composed of a transactivation domain at N-terminal and a helix-loop-helix leucine zipper domain at C-terminal for DNA binding (Baudino and Cleveland 2001). Upon induction, MYC heterodimerizes with its partner MAX, which is also a helix-loop-helix leucine zipper protein, to bind the consensus DNA sequence (CACGTG or E-box) or its variants and alter gene expression (Dang 2012).

The oncoprotein MYC has many binding targets, perhaps up to 15 % of genes (Fernandez et al. 2003; Cawley et al. 2004). Global mapping of MYC binding sites in the human genome using human Burkitt's model lymphoma cells had demonstrated that approximately 3,000 genes are associated with MYC (Zeller et al. 2006). Among these 3,000 MYC-bound genes, 688 that were found to have altered are involved in protein synthesis and cell metabolism, suggesting that MYC activates metabolic reprogramming in cancer cells to fulfill the increased metabolic needs required for rapid growth. Subsequent global mapping of MYC binding sites has broadened the number of putative MYC targets, but expression analysis shows that many key metabolism genes are among the most upregulated genes.

Recent studies have suggested that MYC is a universal amplifier of gene expression through the release of RNA Pol II promoter pausing (Lin et al. 2012; Nie et al. 2012). However, MYC does not uniformly upregulate all genes to the

same degree, suggesting that there is a hierarchy to global MYC regulation. That is, MYC regulates specific genes to different degrees depending on their specific cellular function. For example, MYC would not amplify tumor suppressor genes to the same extent as it would amplify the expression of growth-promoting genes in a cell stimulated to grow. Further, these observations could not explain MYC-mediated suppression. For instance, a few studies showed that MYC suppresses cyclin-dependent kinase inhibitors p15 and p21 by recruiting Miz-1 (Seoane et al. 2001, 2002; Staller et al. 2001). For cancer cells to proliferate, the balance between the expression of growth-promoting genes and growth-arresting genes needs to be maintained.

5.4 Role of MYC in Cell Growth and Proliferation

MYC consistently alters specific groups of genes that are involved in metabolism, protein biosynthesis, cell cycle regulation, angiogenesis, and apoptosis (Prendergast 1999; Nilsson and Cleveland 2003; Baudino et al. 2002). We will not discuss all of MYC's target genes, but three functions relevant to cancer proliferation stand out: cell cycle, protein synthesis, and metabolism. While these roles of MYC have been studied separately, these processes are inextricably linked with metabolism, fueling MYC-driven cell growth and proliferation.

MYC has been shown to drive the cell cycle through E-box-dependent promoter regulation of cyclins D1 and D2, *CDK4*, and cyclin B1 (Fernandez et al. 2003; Bouchard et al. 2001; Menssen and Hermeking 2002; Hermeking et al. 2000). Via inhibiting Miz1, MYC can enhance the cell cycle through repression of CDK repressors p21 and p151NK4A (Wu et al. 2003; Seoane et al. 2001).

Cell cycle progression requires that cells attain a certain cell size before initiating DNA replication and the ensuing cell division to produce two daughter cells. In addition to accelerating the progression of the cell cycle, MYC also accelerates protein synthesis and increases cell size. Indeed, MYC overexpressing fibroblasts show protein synthesis that is about threefold higher than in *MYC* knockout fibroblasts (Mateyak et al. 1997). MYC plays a key role in regulating ribosome biogenesis (Kim et al. 2000; Schlosser et al. 2003; Poortinga et al. 2004). Studies have also shown that *Drosophila* mutants for these ribosomal protein genes have smaller body size which phenocopies a natural *Drosophila* MYC mutant fly, termed diminutive (Orian et al. 2003; Fernandez et al. 2003). Conversely, overexpressing MYC in *Drosophila* (which is called dMYC) increases their cell size, thus increasing the size of body parts (when dMYC is expressed in a tissue-specific fashion) (de la Cova et al. 2004; Moreno and Basler 2004; Secombe et al. 2004). Not only do dMYC-overexpressing cells grow bigger, but they competitively induce apoptosis on their surrounding cells which have lower levels of dMYC (de la Cova et al. 2004). In vertebrates, overexpression of MYC in the liver caused liver hypertrophy (Kim et al. 2000) and in B cells caused enlarged lymphocytes (Iritani et al. 2002). MYC is unique among transcription factors in that it can activate

transcription mediated by all three RNA polymerases I, II, and III to drive ribosomal biogenesis and protein synthesis (Gomez-Roman et al. 2003). MYC binds to pol III-transcribed tRNA and 5S rRNA promoter genes which all play a major role in protein synthesis. MYC also directly stimulates rRNA transcription (Arabi et al. 2005; Grandori et al. 2005; Grewal et al. 2005). All these studies suggest several ways by which MYC increases cell size via ribosome biogenesis and protein synthesis. Taken together these studies show that MYC increases cell size and protein synthesis by driving ribosome synthesis, necessitating the increased uptake or synthesis of nucleotides and amino acids. Below we will discuss how MYC reprograms metabolism to work hand in hand with cell cycle and ribosome biogenesis to drive cancer cell growth and proliferation.

5.5 MYC and Metabolism

Cancer cells exhibit profound metabolic changes, promoting the synthesis of cellular building blocks to support cellular growth, proliferation, and survival (Ward and Thompson 2012). Cancer cells must produce sufficient lipids and phospholipids to build cellular and organelle membranes and sufficient nucleotides to replicate DNA, increase mRNA and build ribosomes, and acquire and produce sufficient amino acids to fuel protein synthesis.

The most noted change in cancer metabolism has been aerobic glycolysis or the Warburg effect, first noted by Otto Warburg in his landmark studies beginning in the 1920s (Koppenol et al. 2011; Warburg et al. 1927). In contrast to non-cancer cells where pyruvate derived from glucose via glycolysis enters the mitochondria and is oxidatively metabolized to maximize ATP production, proliferating cancer cells primarily convert pyruvate to lactate even in the presence of oxygen. Although aerobic glycolysis sacrifices ATP production per molecule of glucose, the increase of glucose flux in aerobic glycolysis provides the opportunity for cancer cells to maximize cellular building blocks via shunting of glycolytic intermediates into biosynthetic pathways. Glycolytic intermediates provide fuel for the pentose phosphate pathway, which provides ribose or nucleotide synthesis and NADPH for cellular reducing power; the serine biosynthesis pathway, which plays a critical role in nucleotide synthesis; and glycerol, which plays a key role in triglyceride metabolism (Ward and Thompson 2012).

In addition to glucose metabolism, cancer cells show additional metabolic changes. Many cancer cells become markedly dependent on glutamine for glutathione, protein, and nucleotide synthesis. Cancer cells often boost nucleotide metabolism, upregulating the synthesis of purine and pyrimidines to support DNA and RNA synthesis. Additionally, cancer cells reprogram how they synthesize and take up nonessential and essential amino acids.

One of the great advances in our understanding of cancer metabolism over the last 15 years is relationship between the recurrent genetic changes observed in cancer and the metabolic phenotypes of the resultant cancers. The p53 protein, one

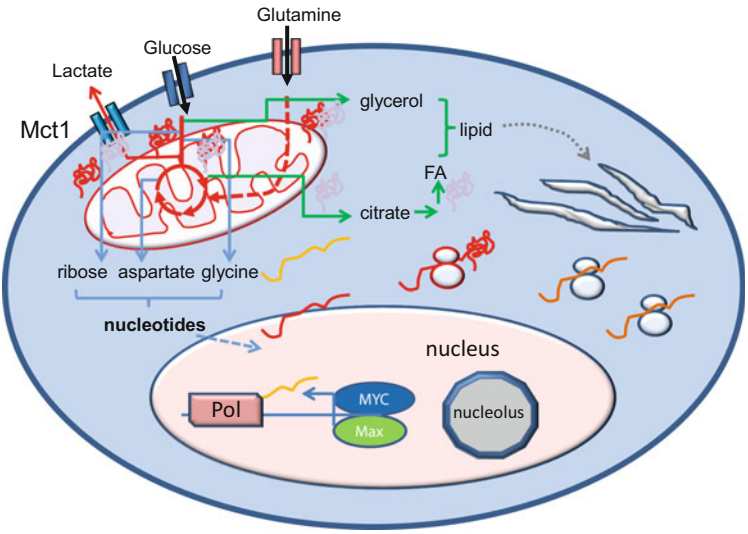

Fig. 5.3 MYC stimulates genes involved in glycolysis, glutaminolysis, lipid, and amino acid synthesis as well as mitochondrial and ribosomal biogenesis. Glucose and glutamine are depicted to be transported and catabolized through glycolysis and the mitochondrion to produce ATP and the building blocks for nucleotide, lipid, and protein synthesis. The mitochondrion is depicted as a central biosynthetic organelle in addition to its function in oxidative phosphorylation. *FA* fatty acid, *MCT1* monocarboxylate transporter 1

of the most frequently mutated or lost genes in cancer, has been shown to be a key regulator of glucose, glutamine, and amino acid metabolism (Bensaad et al. 2006; Hu et al. 2010; Jiang et al. 2011; Maddocks et al. 2013). The mammalian/mechanistic target of rapamycin (mTOR) complex 1, which serves as both a metabolic sensor and regulator, is recurrently activated in cancers via constitutive activation of growth factor/PI3K/AKT signaling pathways via activating mutations or loss of inhibitors such as PTEN, LKB1, or tuberous sclerosis complex proteins (Laplante and Sabatini 2012; Willems et al. 2012; Fresno Vara et al. 2004; Rodon et al. 2013; Atlas 2012a, b; Song et al. 2012; Sanchez-Cespedes 2011; Luo et al. 2010). Common KRAS mutations have been reported to activate glucose, glutamine, and nucleotide metabolism (Son et al. 2013; Gaglio et al. 2011; Ying et al. 2012). However, the oncogene that has perhaps the best studied role in metabolism is MYC (Dang 2012). Expression of MYC induces profound metabolic reprogramming in cancer, controlling glucose, glutamine, nucleotide, lipid, and amino acid metabolism (Fig. 5.3).

5.5.1 MYC, the Warburg Effect, and Mitochondria

Aerobic glycolysis or the Warburg effect relies on increased uptake and retention of glucose and increased glycolysis to convert this glucose to pyruvate and, consequently, to lactate. In cancer cells, MYC controls a transcriptional program to promote the Warburg effect. Glycolysis requires the uptake of glucose into the cell by glucose transporters. MYC can directly upregulate the glucose transporter GLUT1 (Osthus et al. 2000). After uptake by transporters, glucose is phosphorylated by hexokinases and becomes trapped in the cell. Hexokinase II has been shown to be overexpressed in cancer and can be induced by MYC (Kim et al. 2004). MYC also shows almost uniform upregulation of glycolytic genes, including phosphoglucose isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and enolase, binding to the promoters of these genes in upregulating them (Osthus et al. 2000; Kim et al. 2004). By upregulating almost the entire pathway, MYC is capable of enhancing the Warburg effect.

In certain experimental systems, MYC can induce the expression of PKM2 as well as influencing the splicing of the PKM transcript to PKM2 by upregulating the RNA binding splicing proteins HNRNPA1 and HNRNPA2 (David et al. 2010). PKM2 is an important enzyme in cancer metabolism, which differs from its alternative splice form PKM1 in its ability to promote the Warburg effect, regulate pentose phosphate pathway flux, regulate serine biosynthesis, and bind phosphotyrosines (Ye et al. 2012; Anastasiou et al. 2011; Christofk et al. 2008; Chaneton et al. 2012). Upon phosphorylation by ERK, PKM2 but not PKM1 appears to translocate to the nucleus and phosphorylates H3 tyrosine 11 at the MYC promoter, enhancing MYC expression (Yang et al. 2012).

Aerobic glycolysis requires the glycolytic cofactor NAD^+ for the activity of GAPDH. Cancer cells can regenerate NAD^+ from NADH by converting pyruvate to lactate via the enzyme lactate dehydrogenase. MYC directly upregulates the lactate dehydrogenase A (LDHA), promoting the conversion of pyruvate to lactate (Shim et al. 1997). Inhibiting LDHA genetically or pharmacologically diminishes the growth of MYC-dependent cancer cell lines (Le et al. 2010). The buildup of lactate can be toxic to cells, so cancer cells undergoing aerobic glycolysis need to excrete lactate. MYC upregulates the monocarboxylate transporter 1 (MCT1/SLC16A1), which transports lactate out of the cells. Inhibiting lactate export via MCT1 inhibition can result in cell death in MYC-dependent cells (Doherty et al. 2014).

Mitochondria, being the powerhouses of cellular metabolism, are also affected by MYC. Genes involved in mitochondrial biogenesis are upregulated by MYC in both mammals and *Drosophila* (Orian et al. 2003; O'Connell et al. 2003; Morrish et al. 2003; Wonsey et al. 2002; Li et al. 2005). MYC also targets ferritin, IRP1, IRP2, and transferrin receptor (TFRC1) which are all genes involved in iron metabolism largely involving the mitochondrion (O'Connell et al. 2003; Bowen et al. 2002; Wu et al. 1999; O'Donnell et al. 2006). In addition to iron metabolism, nucleotide synthesis genes are also upregulated by MYC, including carbamoyl

phosphate synthetase, aspartate transcarbamoylase, dihydroorotase (CAD), and ornithine decarboxylase (ODC) (Bello-Fernandez et al. 1993; Miltenberger et al. 1995; Liu et al. 2008). Specifically, DHODH, which is a target of MYC, requires a functional mitochondrial electron transport chain for its catalytic conversion of orotate to dihydroorotate in nucleotide synthesis. Hence many pathways influenced by MYC require the function of mitochondria to support cell growth and metabolism.

Although MYC plays an important role in inducing glycolysis and mitochondrial function, tumor cells are often hypoxic due to the imperfect neo-vasculature found in solid tumors. The hypoxia-inducible factor (HIF1) inhibits pyruvate conversion to acetyl-CoA, by shunting it to lactate via activation of lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase (PDK1) that inhibits pyruvate dehydrogenase (PDH). This activation of LDHA, and suppression of PDH by HIF1, stops glucose from supplying carbons to the TCA cycle (Kim et al. 2006). Surprisingly, glutamine's involvement in the TCA cycle persists under hypoxia (Le et al. 2012).

5.5.2 MYC and Glutamine Metabolism

Glutamine is another major bioenergetic source for tumor cells, especially as a source of nitrogen and carbon for nucleotide and amino acid synthesis. Glutamine is imported to the cells through glutamine transporter (e.g., ASCT2). Glutamine is then converted to glutamate by glutaminase. Glutamate can be further metabolized to α-ketoglutarate (αKG) through glutamine dehydrogenase, glutamine pyruvate transaminase (GPT), or glutamine oxaloacetate transaminase (GOT) to enter TCA cycle and be catabolized by the mitochondria.

Cancer cells are addicted to glutamine, which was documented few decades ago, for the following reasons (Reitzer et al. 1979). First, glutamine helps to feed TCA cycle and results in a truncated TCA cycle, which allows acetyl-CoA from glycolytic pathway to be used for de novo fatty acid synthesis and cholesterol synthesis instead of making citrate with oxaloacetate. Fatty acids and cholesterols are important for building new cell membrane. Other intermediates of TCA cycle can also be used for biosynthesis under the constant supply of αKG from glutamine. The role of glutamine to replenish the intermediates in TCA cycle is far more critical in cancer cells than normal cells due to increased biosynthesis. Second, glutamine and its derivatives glutamate and aspartate are source for nucleotide synthesis, which is also increased in proliferated cancer cells.

MYC drives glutamine metabolism by targeting a number of genes that are involved in the pathway. For instance, MYC directly binds to the promoter region of two high-affinity glutamine importers ASCT2 and SN2 and upregulated their mRNA expression to increase glutamine import (Wise et al. 2008). To also increase flux from glutamine to glutamate, MYC activates glutaminase (GLS) both transcriptionally and posttranscriptionally (Wise et al. 2008; Gao et al. 2009). In both

P493 Burkitt's lymphoma and PC3 prostate cancer cell line, MYC activation increases GLS protein at a much higher level compared to mRNA expression, suggesting that MYC regulates mitochondrial GLS indirectly (Gao et al. 2009). Further investigation showed that MYC suppresses miR-23a and miR-23b, resulting in a derepressed GLS protein translation from miR-23a/b (Gao et al. 2009). As a result, elevated GLS facilitates glutamine to enter TCA cycle in the mitochondria.

MYC-overexpressed cells often exhibit addiction to glutamine. High level of MYC appears to prime fibroblast to a glutamine-dependent state (Yuneva et al. 2007). Upon glutamine deprivation, cells underwent apoptosis. This observation, however, can be rescued by oxaloacetate and pyruvate, suggesting TCA cycle intermediate depletion leads to apoptosis (Yuneva et al. 2007). Similar observations were made in glioma cells. Using small-interference RNA (siRNA) targeting MYC helps glioma cells to develop resistance to glutamine deprivation (Wise et al. 2008).

MYC-dependent glutaminolysis is observed to be a critical alternative energy source pathway in nutrient-limiting environment, specifically under glucose and oxygen deprivation conditions (Le et al. 2012). C^{13}-labeled glutamine was used to track the flux of glutamine metabolism in P493 B cell in the presence and absence of glucose. Interestingly, under glucose-deprived condition, much higher levels of these labeled isotopologues of TCA cycle intermediates, such as fumarate, malate, and aspartate, were found compared to glucose-abundant condition (Le et al. 2012). This observation suggests that while glucose is still a preferable carbon source of TCA cycle, glutamine can virtually replace glucose in the absence of glucose. Nevertheless, under glucose-abundant condition, glutamine is used for glutathione synthesis to reduce oxidative stress (Le et al. 2012). Flux analysis using C^{13}, N^{15} glutamine as a tracer in another study has documented that glutamine can also contribute to proline biosynthesis (Liu et al. 2012). A recent study using hyperpolarized C^{13}-pyruvate magnetic resonance spectroscopic imaging (MRSI) to visualize tumor formation and regression in an *MYC*-inducible mouse hepatocellular carcinoma cancer model has observed that glutamine is converted to αKG through increased flux from pyruvate to alanine in premalignant stage (Hu et al. 2011).

A tissue-specific relationship and MYC and glutamine metabolism have been demonstrated in *MYC*-driven liver cancer versus lung cancer (Yuneva et al. 2012). Differential expression of glutamine synthetase determines the direction of glutamine metabolism (Yuneva et al. 2012). For instance, with low glutamine synthetase expression, *MYC*-induced liver tumors exhibit increased glutamine catabolism (Yuneva et al. 2012). In contrast, glutamine accumulation found in *MYC*-induced lung cancers is possibly associated with elevated glutamine synthetase (Yuneva et al. 2012). As tissue origins of tumors can dictate the expression of metabolic pathways, the fate of glutamine can be varied based on the tissue of origin even on the same oncogenic background.

Given that *MYC*-induced tumors rely on glutamine metabolism for cell growth and survival, targeting glutaminase appears to be a feasible way to treat *MYC*-overexpressed cancer. In fact, BPTES, a glutaminase inhibitor, has been shown to

effectively slow down tumor growth in P493 B cells as well as *MYC*-induced tumor xenografts (Le et al. 2012; Wang et al. 2010).

5.5.3 MYC and Amino Acid Transporters and Synthesis

MYC plays a key role in the acquisition and uptake of the amino acids required for cell growth. MYC stimulates the uptake and catabolism of glutamine by upregulating glutamine transporters and the enyzme glutaminase. As discussed above, MYC can drive the synthesis of serine and glycine from glucose via upregulation of key enzymes. Additionally, MYC-driven increase in glutamine metabolism can promote the synthesis of alanine and aspartate, as glutamine-derived glutamate is used to transaminate pyruvate to produce alanine and oxaloacetate to produce aspartate. MYC also promotes the synthesis of proline from glutamine by upregulating proline synthesis genes and indirectly downregulating genes that degrade proline (Liu et al. 2012).

Leucine plays a key role in cancer metabolism through its key role in the regulation of mTORC1 activity (Nicklin et al. 2009). Glutamine can be exchanged through the L-type amino acid transporter (LAT1 composed of SLC7A5 and SLC3A2) for leucine, with glutamine uptake being the rate-limiting step (Nicklin et al. 2009). MYC promote the uptake of glutamine through the regulation of SLC1A5 and then stimulate its exchange for leucine by upregulating SLC7A5 (Gao et al. 2009; Hayashi et al. 2012). While MYC activation has been shown to stimulate leucine uptake (Murphy et al. 2013), the crosstalk between SLC1A5, SLC7A5, MYC, and mTOR remains to be fully elucidated (Sinclair et al. 2013).

5.5.4 Fatty Acid Metabolism

Glucose first enters the cell via glucose transporters and is retained intracellularly once it is phosphorylated by hexokinases. This six-carbon phosphate then is phosphorylated again and split into two three-carbon structures that can then be converted to glycerol. This glycerol can either be used for lipogenesis or to make pyruvate. In the case of its transformation to pyruvate (the process of glycolysis), the pyruvate is then transaminated to alanine with glutamate derived from glutamine as the nitrogen donor or converted to acetyl-CoA in the Krebs cycle. Acetyl-CoA is then transformed into citrate and oxaloacetate, completing the Krebs cycle, generating ATP, carbon dioxide, and other carbon substrates for other pathways. For example, citrate could be exported into the cytoplasm and converted to acetyl-CoA by ATP citrate lyase (ACLY) for lipogenesis.

The pathway for fatty acid synthesis takes place in the cytoplasm. Citrate from the Krebs cycle in mitochondria is first released into the cytoplasm and converted into acetyl-CoA by ATP citrate lyase (ACLY). The production of malonyl-CoA by

acetyl-CoA carboxylase (ACACA) is the first committed step of fatty acid synthesis. Acetyl-CoA carboxylase (ACACA) is the major site of regulation of fatty acid synthesis. Fatty acid synthase (FASN) then converts the malonyl-CoA into a 16-carbon palmitate. Tracing glucose in MYC-induced cells has been shown to be incorporated into increased acetyl-CoA production and in turn increased palmitate synthesis, consistent with the metabolic pathway of glucose conversion to acetyl-CoA and then in turn to palmitate (Morrish et al. 2010). Though this shows the involvement of MYC in lipid metabolism, this also has epigenetic implications, because the traced acetyl-CoA is shown to be incorporated into the acetylation of H4K16.

This upregulation of the lipogenesis pathway by MYC was found in Burkitt's lymphoma, hepatocellular carcinoma, and osteocarcinoma cell lines. However, not all cancers induce lipogenesis. Many prostate cancers oxidize lipids to make ATP (Tennakoon et al. 2013). Others have also shown that pharmaceutical inhibition of lipid oxidation in N-*MYC*-amplified neuroblastoma cells leads to cell cycle arrest, apoptosis, and neuronal differentiation (Zirath et al. 2013). This differentiation is accompanied with lipid accumulation. Gene expression analysis shows inhibition of MYC's correlation with decreased expression of oxidative phosphorylation and fatty acid oxidation genes (Zirath et al. 2013). These observations suggest that cancer metabolic networks depend on the specific cell type, the driving oncogene (s), and the tumor microenvironment.

5.5.5 MYC and Nucleotide Biosynthesis

MYC drives glucose and glutamine metabolism to provide carbon sources for biosynthesis and continually generate ATP to support tumor cell growth and survival. However, for cancer cells to proliferate (increase cell numbers), sufficient supply of nucleotides is equally crucial. Global mapping of MYC target genes using ChIP-PET has indicated that many genes that are involved in nucleotide synthesis pathway are direct MYC targets (Zeller et al. 2006). For instance, the enzymes that are involved in dNTP metabolism, such as inosine monophosphate dehydrogenase (IMPDH), thymidylate synthase (TS), and phosphoribosyl pyrophosphate synthetase 2 (PRPS2), were found to be induced by MYC (Mannava et al. 2008; Liu et al. 2008). Targeting IMPDH by its specific inhibitor mycophenolic acid (MPA) results in apoptosis and S phase arrest in P493 B cells, which can be rescued by exogenous guanosine (Liu et al. 2008).

Despite direct regulation of nucleotide synthesis pathway, MYC also promotes the channeling of glycolytic intermediates to make amino acids that are required for nucleotide synthesis, such as serine and glycine (Vazquez et al. 2011). Glycolytic intermediate 3-phosphoglycerate is oxidized by phosphoglycerate dehydrogenase (PHGDH) and then converted to serine in a series of reactions that are catalyzed by phosphoserine aminotransferase 1 (PSAT1) and phosphoserine phosphatase (PSPH). Further, serine hydroxymethyltransferase (SHMT) can convert serine to

glycine while simultaneously converting tetrahydrofolate to 5,10 methylenetetrahydrofolate. Several studies have shown PHGDH, and PSPH can be induced by MYC (Vazquez et al. 2011). In addition, both mitochondrial and cytoplasmic SHMTs that were documented are direct MYC targets (Nikiforov et al. 2002). However, decreased tumor burden was not found when breed Burkitt's lymphoma mouse model expresses human MYC transgenes with inactivated alleles of PHGDH or SHMT, suggesting that target genes that are involved in serine and glycine pathway individually may not be sufficient (Nilsson et al. 2012).

5.5.6 MYC and Oncometabolites

While altered metabolism in cancers is often thought of as downstream of oncogenes, recent studies have shown that metabolites themselves can contribute to tumor formation most likely through alterations in the cancer epigenome. Mutations in TCA cycle enzymes can lead to the accumulation of succinate and fumarate, which are believed to promote cancer through alteration of epigenetic state and reactive oxygen species (Letouze et al. 2013; Sullivan et al. 2013). As MYC can increase the amount of glutamine entering the TCA cycle, it is possible that the MYC activation could stimulate succinate and fumarate accumulation in the TCA cycle mutant cell lines.

The most studied oncometabolite to date is 2-hydroxyglutarate. Isocitrate dehydrogenase (IDH) catalyzes the formation of alpha-ketoglutarate from isocitrate in the TCA cycle. Mutants IDH1 and IDH2, which are recurrently mutated in several types of cancer including leukemia and gliomas, produce 2-hydroxyglutarate. 2-Hyrdroxyglutarate can inhibit alpha-ketoglutarate-dependent histone demethylases (Chowdhury et al. 2011; Lu et al. 2012) and DNA demethylase Tet2, causing epigenetic changes which inhibit cellular differentiation (Figueroa et al. 2010). However, recent studies suggest that mutant IDH is not the only source of 2-hydroxyglutarate in cancer. A recent study showed the accumulation of 2-hydroxyglutarate in triple negative breast cancers lacking an IDH mutation (Terunuma et al. 2014). These high 2-hydroxyglutarate breast cancers, which exhibited a hypermethylation, showed strong overexpression of MYC and had an MYC expression signature. Knockdown of MYC in these breast cancer cell lines decreased levels of 2-hydroxyglutarate. MYC increased the metabolism of glutamine, which was the source of carbons used to produce the 2-hydroxyglutatarate. While this study suggests that MYC may upregulate 2-hydroxyglutarate, it is not yet known whether this applies to tissues beyond breast cancer.

5.6 MYC-Driven Metabolism and Cancer Therapy

Although MYC is an intriguing therapeutic target, for example, via bromodomain inhibitors, its downstream transcriptional targets and altered metabolism offer additional therapeutic opportunities (Loven et al. 2013; Delmore et al. 2011). Increased understanding of the reliance of cancer on altered metabolism and the mutations that underlie metabolic reprogramming have driven interest in using anti-metabolism therapies to treat cancer. However, once these therapies reach the clinic, it will be challenging to predict which tumors respond to which therapies. Although MYC status will likely not be sufficient to predict therapeutic response to antimetabolic therapies in all cases due to tissue- and tumor-specific effects, MYC-driven metabolic reprogramming provides intriguing therapeutic targets. MYC drives nucleotide metabolism, which is the target of some of the oldest and most successful chemotherapies. Blocking glucose metabolism by inhibiting glucose uptake and glycolysis is challenging due to the reliance of the vast majority of non-cancer cells on glucose. Targeting aerobic glycolysis through inhibition of lactate dehydrogenase or monocarboxylate transporter-dependent lactate export is a potentially viable target; the lack of high-quality inhibitors and the dependence of rapidly growing non-cancer cells on aerobic glycolysis present challenges in targeting aerobic glycolysis (Doherty et al. 2014; Le et al. 2010). Glutamine metabolism was first tried in the clinic using nonspecific amino acid analogues which alter the activity of a large variety of enzymes, leading to off-target effect of lack of efficacy (Rajagopalan and DeBerardinis 2011; Shapiro et al. 1979). However, the identification of allosteric inhibitors of glutaminase have opened the door to a less toxic inhibition of glutamine metabolism (Robinson et al. 2007; Le et al. 2012). A glutaminase inhibitor began clinical trials in early 2014 (Gross et al. 2014).

Conclusion

MYC has been studied over the years as a master oncogenic regulator, especially because it regulates many genes that are crucial for cancer cell growth and proliferation. Among many of its activities, three major functions of MYC were discussed: cell cycle, protein synthesis, and metabolism. In concert with MYC's ability to induce cell proliferation, MYC also upregulates many cellular metabolic pathways that are involved in nutrient import and macromolecular biosynthesis. In essence, MYC is an amplifier of gene expression that coordinates the import of nutrients and the bioenergetics demands of replicating a cell, shunting nutrients into cell biomass. Glucose, glutamine, and lipid metabolic pathways are regulated by MYC in various cells to support the increased demand for energy and raw building blocks of proliferating cells. Normal proliferating cells depend on similar metabolic pathways; however, normal *MYC* expression is dependent on external cues

(continued)

and growth factors, such that altered metabolism induced by MYC is dependent on these external cues. Normal *MYC* expression is attenuated by the absence of growth factors or nutrients. By contrast, cancer cells with deregulated *MYC* expression that no longer requires external cues are forced to undergo cell growth independent of nutrient sensing. In this regard, MYC-dependent cancer cells are addicted to nutrients, such as glucose and glutamine. Insights into these metabolic pathways and how MYC regulates them allow for the identification of new therapeutic targets and provide the hope that new therapies might emerge in the clinic for different types of cancers.

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Chapter 6
Pyruvate Kinase M2: A Metabolic Tuner

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Abbreviations

PK Pyruvate kinase
PKM2 Pyruvate kinase M2 isozyme

6.1 Introduction

Glycolysis pathway contains three irreversible and rate-limiting reactions; of these, the last step involves pyruvate kinase, which catalyzes the irreversible transphosphorylation from phosphoenolpyruvate (PEP) to ADP, producing pyruvate and ATP. The presence of pyruvate kinase (PK) in mammals as four isozymes depends upon the metabolic requirements of a tissue. The four isozymes are M1, M2, L, and R (encoded by two genes, PKM and PKLR, respectively). The L-type isozyme (PKL) has the lowest affinity for its substrate PEP and is expressed in tissues showing high rate of gluconeogenesis, such as the liver (parenchyma cells), kidney (proximal renal tubules), and intestine (Steinberg et al. 1999; Brinck et al. 1994). The R isozyme of pyruvate kinase (PKR) is expressed in red blood cells (RBCs) which lack mitochondria (Rodriguez-Horche et al. 1987). The non-allosteric, high-affinity M1 isozyme (PKM1) is expressed in energy-requiring tissues like muscle, heart, and brain (Yamada and Noguchi 1999b; Reinacher et al. 1979). The M2 isozyme of pyruvate kinase (PKM2) is typically present in normal proliferating cells, embryonic cells, and tumor cells. However, few differentiated tissues like the adipose, lung, distal renal tubules, Henle's loops, collecting tubules of the renal medulla, retina, and pancreatic islets also express M2 isozyme (Reinacher and Eigenbrodt 1981; Reinacher et al. 1979; Brinck et al. 1994; Steinberg et al. 1999; Yamada and Noguchi 1999a; Eigenbrodt et al. 1992) (Fig. 6.1).

The PKM gene is located on chromosome 15q22 position and has been largely conserved throughout evolution. M1 and M2 isozymes are the product of alternate splicing where mutually exclusive incorporation of exon 9 makes M1, while exon

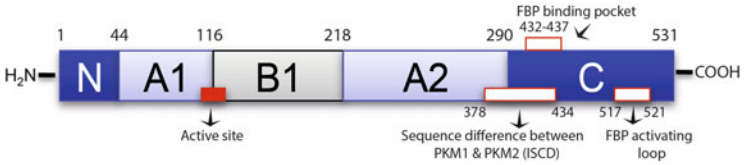

Fig. 6.1 Domain structure of PKM2 subunit

10 incorporation makes M2 isozyme. The exon that is exchanged because of alternative splicing encodes 56 amino acids, in which a total of 22 amino acids differ within a length of 45 residues between domain A2 and C, generally known as inter-subunit contact domain (ISCD) (Fig. 6.1). The enzyme is usually a homotetramer composed of four identical subunits, each containing four domains: the A-, B-, C-, and N-terminal domain. Domain A is subdivided into A1 and A2 and shares PEP binding site with B1 domain; however, FBP binding pocket is shared by A- and C-terminal domains as shown in Fig. 6.1. B is the most mobile domain of the enzyme and responds highly towards ligand binding (Larsen et al. 1998).

Enzyme	Isoforms	Km (mM)		Expression in cancer	Remarks	References
Pyruvate kinase	M1	PEP	0.11	Reduced	High affinity and non-cooperative binding with PEP. Expressed in muscle, heart, and brain	
		ADP	0.28			
	M2			Increased	Prototype, expressed in proliferating cells and tumor cells in particular	(Mazurek et al. 2005; Gupta and Bamezai 2010)
	Tetrameric	PEP	0.03			
		ADP	0.25			
	Dimeric	PEP	0.46			
		ADP	0.29			
	L	PEP	0.75	Reduced	Allosteric binding with PEP, expressed in the liver	
		ADP	0.56			
	R	PEP	1.10	Reduced	Allosteric binding with PEP, expressed in RBC	
		ADP	0.18			

Fundamental differences between M1 and M2 isozymes of PK enable these to perform isozyme-specific functions. X-ray crystallographic analyses have shown that the inter-subunit contact is actually responsible for the inter-subunit communication, required for allosteric cooperativity in M2 isoenzyme (Muirhead 1990; Muirhead et al. 1986); however, the sequence difference between M1 and M2 in ISCD region renders M1 isoenzyme totally noncooperative and non-allosteric. The structure of human PKM2 has been determined in complex with inhibitors where each subunit (monomer) has individual site for substrate (PEP), allosteric-activator (FBP), and ADP binding (Dombrauckas et al. 2005). In a typical allosteric protein binding of the substrate at one site increases its affinity to other sites in the same molecule, providing a typical sigmoidal curve; however, upon binding of activator like FBP, the enzyme loses its allosteric property, and all substrate binding sites show equal and noncooperative affinity towards the substrate. The same is true with PKM2, where a unique FBP activating loop (517–521) surrounds the FBP binding pocket, and hence the FBP molecule itself results in partial closed conformation of allosteric site; however, in case of rabbit muscle PK which does not respond to FBP, the loop is located away from FBP binding site. Binding of FBP alters the conformation of FBP activating loop, which in turn impacts the inter-subunit contacts. Hence, the unique orientation of FBP activating loop is perhaps the most significant

difference between M1 and M2 isoforms of PK, which possibly leads to the absence and presence of allostericity in the two isoforms, respectively.

6.2 Regulation of Pyruvate Kinase M2

Expression and functional behavior of PK is regulated at multiple levels (Fig. 6.2). As far as genetic mutations controlling PK expression and stability are concerned, PKLR gene on chromosome 1q21–q22 is known for frequent mutations (Tani et al. 1987; Satoh et al. 1988). These include missense, nonsense, deletion, and frame shift mutations (Lenzner et al. 1997; Kanno et al. 1997; Van Wijk et al. 2009), distributed throughout the worldwide population (Pissard et al. 2006) with diverse phenotypic implications (University et al.). Many of these mutations negatively regulate the expression level in PKLR, leading to PK deficiency in red blood cells, a source of hereditary nonspherocytic hemolytic anemia. A very frequent mutation within PKLR gene among the Northern European population is a single amino acid substitution (R510Q), which is associated with PKLR degradation and PK deficiency (Wang et al. 2001). However, unlike PKLR gene, with multiple documented widespread mutations, there is only one report about mutations within the M gene in the cell lines and a patient of Bloom syndrome (Anitha et al. 2004). These missense mutations (H391Y and K422R) were observed within the inter-subunit contact domain of the PKM2 protein (coded by exon 10) involved in allosteric signal transduction from one to another subunit (Fig. 6.2). Without affecting the stability or expression level of the PKM2 protein, the two mutations had differential impacts over the activity and allosteric behavior of PKM2 (Akhtar et al. 2009; Webb et al. 2011). In addition, the H391Y and K422R mutations have been shown to promote the subunit exchange between wild-type and mutant monomers of PKM2, thereby generating different kinds of hetero-oligomers which promoted cellular growth and polyploidy in an ex vivo model (Gupta et al. 2010).

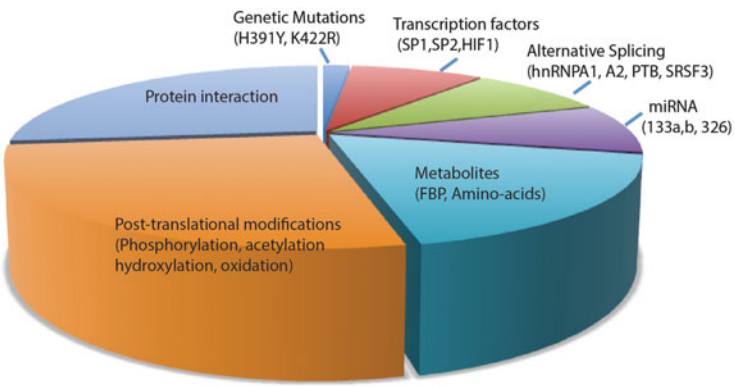

Fig. 6.2 Regulation of PKM2 expression and functions

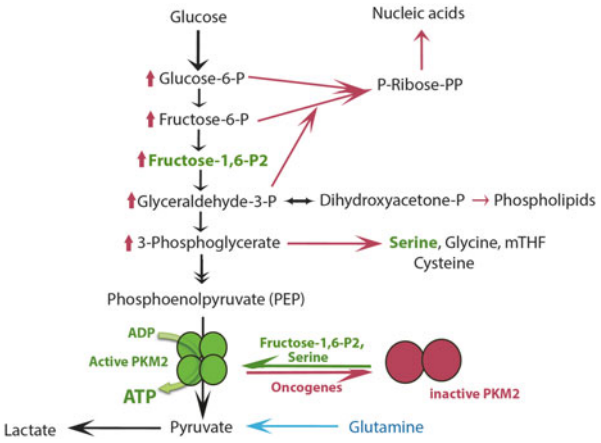

Fig. 6.3 Switching between tetrameric and dimeric PKM2; regulation of cell proliferation by controlling metabolism

The other but generalized level of regulation, which occurs in pyruvate kinase, is the alternate splicing that vastly affects the preferred expression of isozyme of choice, i.e., M1 or M2. The M1- and M2-type pyruvate kinase isozymes are different splicing products (exon 9 for PKM1 and exon 10 for PKM2) of the M gene (Noguchi et al. 1986). It has been shown that c-Myc favors exon 10 inclusion by controlling the expression of the splicing factors heterogeneous nuclear ribonucleoproteins A1 and A2 (hnRNPA1, hnRNPA2) and polypyrimidine tract protein (PTB) (David et al. 2010). A recent report also has suggested the involvement of another factor, SRSF3 (serine/arginine (SR)-rich family of pre-mRNA splicing factors), which triggers exon 10 expression and promotes cellular proliferation and aerobic glycolysis. Transcription factors, like SP1, SP3, and the hypoxic inducible factor 1 (HIF1), are also known to control PKM2 expression. SP1 has been described as a regulator of PKM expression modulated by cell state, with increased glucose and decreased reactive oxygen species-related PKM promoter activity (Schafer et al. 1996, 1997). HIF1α induces PKM2 expression by binding to hypoxia response element in PKM2 gene in response to the receptor tyrosine kinase/PI3K/AKT/mTOR signaling cascade, which also upregulates c-Myc expression (Fig. 6.3) (Sun et al. 2011; Iqbal and Bamezai 2012). MicroRNAs, a class of small noncoding RNAs, are also known to affect PKM expression, either through promoting RNA degradation or by repressing translation (Pillai 2005). Computational target gene prediction has revealed PKM2 as a target of microRNA326 (Kefas et al. 2010). In glioma cells, high levels of PKM2 correlated with low levels of microRNA326. Similarly, in tongue squamous cell carcinoma (SCC), downregulation of computationally predicted microRNA133a and 133b promoted PKM2 overexpression. These microRNAs were suppressed in tongue carcinoma cells as compared to tongue epithelial cells of the same patient.

Moreover, transfection of tongue SCC cell lines with microRNA133a and 133b led to an inhibition of cell proliferation (Wong et al. 2008). Once the PKM2 protein is synthesized in the cell, there are various levels of regulation. PKM2 is present in

the cells in multimeric forms: catalytically active tetrameric form and catalytically inactive dimeric, trimeric, and monomeric forms. For instance, dimeric form is present in relatively higher amount in proliferating cells as compared to the quiescent cells. There are many known methods triggered by exogenous signaling which modify the relative ration of dimer and tetramer, and hence the net PKM2 activity in cells decides the cell fate. For example, FBP is known to regulate the process of switching the multimeric forms of PKM2. It is known that in the presence of fructose-1,6-bisphosphate (FBP), the affinity for PEP for all subunits within the tetramer becomes independent of each other and increases tremendously (Dombrauckas et al. 2005), and the enzyme tends to stay in tetrameric form. Conversely, when FBP levels drop below a certain level, the tetrameric form dissociates to the dimeric form. Furthermore, some amino acids, such as L-serine, have also been shown to regulate PKM2 activity. L-Serine increases the affinity of PKM2 to its substrate PEP and reduces the amount of FBP necessary for tetramerization (Eigenbrodt et al. 1992). However, PKM2 activity is inhibited by L-alanine, L-cysteine, L-methionine, L-phenylalanine, L-valine, L-leucine, L-isoleucine, and L-proline as well as saturated and monounsaturated fatty acids (Marchut et al. 1986; Eigenbrodt et al. 1992). Nonetheless, there are varieties of posttranslational modifications, which PKM2 undergoes, resulting in modulated activity and subunit dissociation. This includes tyrosine, serine, and threonine phosphorylation, lysine acetylation, proline hydroxylation, as well as cysteine oxidation (Hitosugi et al. 2009; Presek et al. 1988; Eigenbrodt and Glossmann 1980; Eigenbrodt et al. 1998). The tyrosine-105 phosphorylation of PKM2 by oncogenic tyrosine kinases (e.g., FGFR1) has been shown to inhibit its activity by causing the release of its allosteric-activator FBP (Hitosugi et al. 2009). In another study by Lv et al., acetylation of PKM2 at lysine-305 was found responsible for downregulation of PKM2 activity. Both phosphorylation and acetylation induced inhibition of PKM2 activity resulted in enhanced Warburg effect and tumor growth (Luo et al. 2011). (Anastasiou et al. 2011) have shown remarkable role of oxidized PKM2 in enduring oxidative stress in cancer cells. In this paper, the authors report that intracellular reactive oxygen species (ROS)-induced oxidation of cysteine-358 decreases its activity to divert glucose flux into anabolic PPP, thereby generating sufficient reducing potential in the form of NADPH for detoxification of ROS (Jiang et al. 2010). Moreover, the authors show that oxidized PKM2, by virtue of its lower activity, promotes tumor growth, which was inhibited when mice were fed on *N*-acetyl-L-cysteine (NAC; scavenger of ROS). This report extended the relevance of PKM2 to cancer cells and also emphasized the critical role of this enzyme in cancer. Furthermore, low activity PKM2 has been reported to promote de novo serine biosynthesis by causing accumulation of glycolytic intermediate 3-phosphoglycerate (Ye et al. 2012). Recently, insulin has been shown to exert contrasting effects on PKM2 expression and activity through PI3K/mTOR/HIF1α- and ROS-dependent mechanisms, respectively (Iqbal et al. 2013).

6.3 Canonical and Noncanonical Functions of PKM2

The primary role of PKM2 is to produce net ATP from glycolysis; however, due to its unique positioning at the crossroads of glycolysis, TCA cycle makes it a potential metabolic tuner in dividing cells. When quiescent cells expressing other pyruvate kinase isozymes enter the cell cycle, the M2 type of pyruvate kinase is reexpressed (Mazurek et al. 2005; Yamada and Noguchi 1999b; Reinacher and Eigenbrodt 1981; Reinacher et al. 1979; Yamada and Noguchi 1999a; Bluemlein et al. 2010); Eigenbrodt et al. 1992). Recent discoveries of novel proteins interacting with PKM2 have confirmed its key function during cell division, tumor metabolism, and cancer growth.

6.3.1 Pyruvate Kinase M2, Warburg Effect, and Cancer Cell Metabolism

In the absence or scarce oxygen, normal cells convert glycolytic pyruvate into lactate (anaerobic glycolysis; e.g., in the skeletal muscle during strenuous exercise), thus preventing pyruvate to enter mitochondrial oxidative phosphorylation. As a result, rapid but limited ATP production takes place, generating only two moles of ATP per mole of glucose. When oxygen is abundant, normal cells perform mitochondrial oxidative phosphorylation and generate 36 moles of ATP per glucose mole. However, cancer and normal proliferating cells convert pyruvate into lactate irrespective of the presence of oxygen, a phenomenon termed as Warburg effect or aerobic glycolysis, bypassing mitochondrial oxidative phosphorylation (Vander Heiden et al. 2009a). Warburg postulated that high lactate production is due to mitochondrial dysfunction in cancer cells. However, it proved unfitting in later studies (Frezza and Gottlieb 2009). In fact, in some cases, mitochondria play a contributory role in cancer progression (Weinberg et al. 2010; Funes et al. 2007).

6.3.2 Why the Aerobic Glycolysis?

To explain why cancer cells switch to aerobic glycolysis, which is less efficient in terms of ATP production, several explanations have been proposed. Gatenby and Gillies in their review entitled *Why Do Cancer Cells Have High Aerobic Glycolysis?* argue that aerobic glycolysis is an adaptation to hypoxic (low-oxygen) conditions existing in premalignant phase of tumor development (Gatenby and Gillies 2004). As a result, cancer cells produce ATP even in the absence of oxygen. High lactate production causes acidification of microenvironment and eventually results in evolution of acid-resistant cell type, which grows uncontrollably. In addition, it has been proposed that aerobic glycolysis fulfills the need of cancer

cells to synthesize raw material (nucleotides, amino acids, and phospholipids) for their daughter cells (Lunt and Vander Heiden 2011). Even when it comes to ATP generation, glycolysis is known to produce ATP at a much faster rate than mitochondrial oxidative phosphorylation and therefore may be preferred to meet the energy demands of cancer cells (Pfeiffer et al. 2001). Due to anabolic obligations, cancer cells preferably utilize glucose for macromolecular synthesis (Vander Heiden et al. 2009a). Aerobic glycolysis provides the platform for macromolecular synthesis through two ways: first, high rates of glucose uptake (Hume and Weidemann 1979) and, second, prevention of entry of pyruvate into mitochondria by its conversion into lactate (Vander Heiden et al. 2009a). Moreover, high lactate production is critical in regulation of glucose flux into biosynthetic pathways, through faster incorporation of carbon into biomass which in turn facilitates rapid cell division (Lunt and Vander Heiden 2011) and also creates acidic environment required for invasiveness and metastasis (Robey and Martin 2011). Recent evidences demonstrate role of remarkable role of aerobic glycolysis in modulating immune response against cancer cells (Husain et al. 2013; Chang et al. 2013).

Warburg's observation of upregulation of glycolysis in cancer even in the presence of O_2 had highlighted the exclusive importance of glycolytic pathway in dividing cells (Warburg 1956; Ferguson and Rathmell 2008). A dividing cell has dual dependence on glycolysis for (1) energy and (2) the glycolytic intermediates (phosphometabolites) required as precursors for the synthesis of nucleic acids, amino acids, and lipids (Mazurek et al. 1997). This dual dependence ensures the activation of synthetic processes, only when the source of energy (glucose) is sufficient in microenvironment to maintain the "metabolic homeostasis" of the cell. Since accumulation of synthetic precursors and availability of energy from glycolysis are mutually exclusive (one at a time), the dividing cell efficiently coordinates both pathways in a cyclic manner, where PKM2 plays a major role because of its positioning at the last step of glycolysis (Mazurek et al. 2005). Hence, expression of PKM2 isoform in a dividing cell is a metabolic requirement, and its presence at the last step of glycolysis decides the fate of glucose carbons to channel either in synthetic pathway (nucleogenic) or for energy production (glycogenic). The destined ATP is produced by maintaining an active tetramer state of the enzyme (PKM2); however, when the cell senses the requirement of precursors, especially during cell division, the activity of this enzyme is downregulated (by subunit dissociation) in a reversible manner, to block the glycolytic flux towards pyruvate production. This allows accumulation of the glycolytic intermediates used further as synthetic precursors of nucleic acid, lipid, and amino acid synthesis (Eigenbrodt et al. 1992) (Fig. 6.3). Thus, understanding of nucleogenic and glycogenic cycles in cancer cells vis-à-vis fast dividing normal cells (e.g., stem cells and activated lymphocytes) and the stabilization of irreversible subunit dissociation of the PKM2 tetramer in a cancer cell require focused attention. Identifying the molecules and associated phenomenology would pave the way for clear understanding of aerobic glycolysis and specific therapeutic interventions.

The subunit dissociation (tetramer to dimer) is a well-known process for activity downregulation when the availability of FBP is low under physiological conditions

(Mazurek et al. 2005). Binding of FBP is known to tetramerize the enzyme, while its release causes dissociation to dimer. However, as in vitro the purified protein is a homo-tetramer even in the absence of FBP, the exact mechanism of dimerization/tetramerization under physiological condition is yet not known. The role of PKM2 in tumor development was earlier indicated by the fact that many oncogenic viral pathogens during evolution have chosen PKM2 for their phenotypic effect by inducing its dissociation into dimer after physical interaction (Zwerschke et al. 1999; Presek et al. 1988). Some proteins known for cellular growth and proliferation like A-Raf (Mazurek 2007; Mazurek et al. 2007) and PML (promyelocytic leukemia protein) (Shimada et al. 2008) are known to downregulate PKM2 activity by interacting with it. Interaction of PKM2 with growth factor receptor like FGFR-1 (fibroblast growth factor receptor-1), receptor tyrosine kinase like FlT3, JAK-2, and oncogenes like BCR-ABL further supports to the proposed potential (Hitosugi et al. 2009). Lysophosphatidic acid (LPA), a mitogenic factor, also interacts with PKM2 (Desmaret et al. 2005), and Oct4 (octamer-4), a homeodomain transcription factor expressed in normal embryonic stem cells, has been reported as PKM2 interacting partner. Oct4 is involved in stem cell self-renewal, and its knockdown is reported to induce cell differentiation (Niwa et al. 2000). A physical interaction of PKM2 with Oct4 probably indicates their auxiliary function to induce cell division and tumor sustenance under malfunctioning conditions, especially when PKM2 is already known to promote cancer of adult germ cells (Lee et al. 2008).

Change in the expression of pyruvate kinase isoform is a consistent observation, which is coupled to metabolic transformation in cancer cells. Tissue-specific isoenzymes like PKM1 in the brain and PKL in the liver are replaced by tumor-specific isoenzyme PKM2 (Reinacher and Eigenbrodt 1981; Eigenbrodt et al. 1992; Guminska et al. 1997; Hacker et al. 1998; Steinberg et al. 1999; Mazurek 2011b). Immunohistological, blood, and stool analyses of cancer patients have revealed the presence of dimeric PKM2, thus emphasizing the role of this enzyme in cancer (Schneider et al. 2002; Hugo et al. 1999; Tonus et al. 2012). However, PKM2 as a tumor-promoting enzyme gained enormous attention when two articles from Christofk et al. appeared in Nature. In these papers, the authors have elegantly shown that replacement of PKM2 with PKM1 isoform in cancer cells significantly reduced cancer metabolism. They further demonstrated that tumor growth diminished when PKM2 was replaced by its isoform PKM1, thereby concluding that switch to PKM2 is indeed critical for cancer metabolism and tumor growth (Christofk et al. 2008a). In another paper, the authors have shown that PKM2 is a phosphotyrosine-binding protein and binding of tyrosine phosphorylated protein decreases PKM2 activity (Christofk et al. 2008b). Interestingly, decrease in PKM2 activity benefits cancer cells as it results in pooling of the glycolytic intermediates, which are precursors for major biosynthetic pathways like pentose phosphate pathway (PPP) (Christofk et al. 2008b; Mazurek 2011b; Mazurek et al. 2005) (Fig. 6.4).

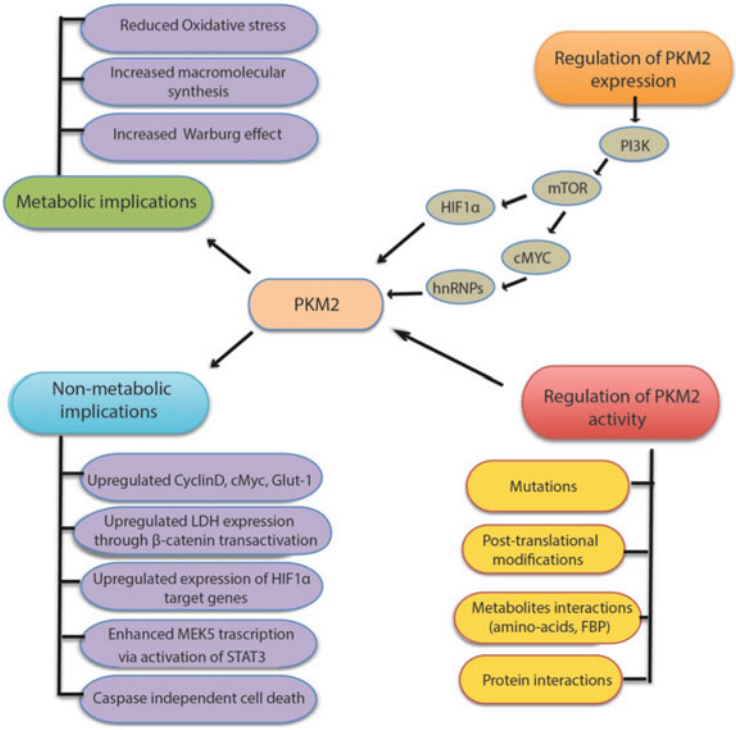

Fig. 6.4 Regulation of pyruvate kinase M2 expression and activity and its implications

6.3.3 Non-metabolic Functions

Besides its metabolic role, PKM2 is reported to support tumorigenesis through its critical "non-metabolic" attributes. In a noteworthy study published by Luo et al., PKM2 has been shown to interact inside the nucleus with its transcriptional activator HIF1α. This interaction increases transcriptional activity of HIF1α, which results in higher expression of its (HIF1α) target genes, e.g., GLUT1 (glucose transporter), LDHA (lactate dehydrogenase A), and PKM. The authors, thus, provided a possible explanation to previous observations of increased glucose uptake and lactate production (aerobic glycolysis) on switch to PKM2 expression and unraveled a "positive feedback loop" mechanism that reprograms the glucose metabolism (Luo et al. 2011). Yang et al. showed that activation of epidermal growth factor receptor (EGFR) resulted in translocation of PKM2 into nucleus where it associated with phosphorylated β-catenin to form a complex, which enhanced cyclin D1 and c-Myc expression (Yang et al. 2011). PKM2-dependent transactivation of β-catenin is critical for EGFR-promoted tumor cell proliferation and development. Upregulation of c-Myc by PKM2 forms a "positive feedback loop" as c-Myc regulates alternative splicing in favor of PKM2 (David et al. 2010).

Another recent article reported that PKM2 dimer is a protein kinase and that it regulates gene transcription by activating the transcription of MEK5 through Stat3 phosphorylation (Gao et al. 2012). The authors demonstrated that PKM2 dimer is an active protein kinase (with PEP as phosphate donor), while PKM2 tetramer is a pyruvate kinase. Further, they showed that expression of PKM2 mutant that existed as dimer promoted cellular proliferation, indicating that protein kinase ability is important in cellular proliferation. This study revealed an important link between metabolic transformation and gene expression. Recently, ERK2-dependent phosphorylation and nuclear translocation of PKM2 are reported to promote upregulation of glycolytic genes, such as GLUT1 and LDHA, and thus the Warburg effect (Yang et al. 2012b). Although PKM2 has been linked tightly with cancer metabolism and tumor growth, there are reports where the role of PKM2 has been challenged. Bluemlein et al. observed that PKM2 is also a predominant isoform in tissue-matched controls, besides being predominant in cancer tissues. This study challenged the notion of PKM1 to PKM2 switching during cancer progression and also questioned the image of PKM2 as cancer-specific enzyme (Bluemlein et al. 2011). Another recent article showed that PKM2 silencing did not completely inhibit tumor growth, suggesting the presence of other metabolic pathway that bypasses its function (Cortes-Cros et al. 2013).

Interaction of PKM2 with an analogue of growth inhibiting hormone (somatostatin) is known to cause caspase-independent cellular apoptosis by interacting and localizing the PKM2 in nucleus (Stetak et al. 2007), while some cytokines were found to enhance cellular proliferation involving PKM2 in a similar way (Hoshino et al. 2007). In a report, PKM2 showed immunomodulatory effects by interacting with SOCS3 (suppressor of cytokines signaling 3) resulting in disruption of antigen presenting ability of dendritic cells (Zhang et al. 2010).

6.3.4 PKM2 and Epigenetics

There are evidences showing how metabolism leads to epigenetic modulation affecting gene expression and subsequent pathways. A recent study showed PKM2 reportedly localizes into the nucleus and directly phosphorylates histone H3 at threonine 11, upon epidermal growth factor stimulation. This is required for the dissociation of HDAC3 from the cyclin D1 (CCND1) and Myc promoter regions and also subsequent acetylation of histone H3 at K9 (lysine 9) and transcriptional initiation. According to a recent report, a positive correlation was also found between PKM2 nuclear localization and histone H3 T11 phosphorylation in glioma malignancy grades (Yang et al. 2012a), and this function appears to be specific to the M2 isoform of PK. PKM2 has also been shown to interact with and modulate an array of transcription factors, including HIF1α, β-catenin, Oct4, and Stat3 (Lee et al. 2008; Luo et al. 2011; Yang et al. 2011; Gupta and Bamezai 2010), as mentioned earlier, indicating its potential role in epigenetic modulations.

6.4 PKM2 and Cancer Therapeutics

Due to indispensable reliance of cancer cells on their metabolic requirements, metabolism of cancer cells is now perceived as a crucial therapeutic target. Proliferation only proceeds when the metabolism of the cells is able to provide a budget of metabolic intermediates that is high enough to ensure both energy generation and synthesis of cell building blocks in sufficient amounts (Mazurek 2011a). As the last enzyme in glycolytic pathway, PKM2 plays a key role in determining whether glucose carbons are used for glycolytic energy generation or are channeled into synthetic processes. Therefore, a balanced regulation of PKM2 activity is crucial for proliferating cells to survive under varying nutrient supply, since any change in the activity of PKM2 above or below a required threshold may block cell proliferation. Hyperactivation of PKM2 inhibits the fueling of synthetic pathways generating cell building blocks, while complete inhibition could potentially cause ATP crises in a dividing cell especially under hypoxic conditions when glutaminolysis, the second main pillar of energy regeneration in proliferating cells is inhibited (Eigenbrodt et al. 1994; Mazurek 2011a; Mazurek et al. 2005). If cells are not limited for ATP, they may still require production of pyruvate for entry into the TCA cycle or for generation of NAD^+ by lactate dehydrogenase. Importantly, an alternative mechanism for pyruvate generation when PKM2 activity is low has recently been described. This mechanism involves direct transfer of phosphate from PEP onto the enzyme phosphoglycerate mutase (PGAM), thus allowing generation of pyruvate in the absence of ATP production (Vander Heiden et al. 2011). Together, this data suggests that PKM2 activity must remain flexible in order to optimally support proliferation and that fixation of PKM2 either in the nearly inactive dimeric form or in the highly active tetrameric form may inhibit cancer growth. Hence, it is possible that either inhibiting or activating PKM2 could be viable therapeutic strategies for cancer.

6.4.1 Therapeutic Modulation of PKM2 Activity

In the quest of identifying new lead structures that target PKM2, one of the first compounds described was A771726, which is the active metabolite of leflunomide, a well-known inhibitor of dihydroorotate dehydrogenase, a key enzyme in pyrimidine synthesis. Binding of A771626 to PKM2 is known to induce inactivation by dimerization of enzyme, resulting in inhibition of cell proliferation in rat Novikoff hepatoma cells (Muellner et al. 2006). Another inhibitor was identified in a high-throughput screen of 107,360 small molecules. These compounds were tested in a lactate dehydrogenase-coupled kinetic PK assay to identify inhibitors of PKM2 (Vander Heiden et al. 2009b). The compound which showed inhibition of PKM2 with the lowest IC50 value, termed compound 3 (N-(3-carboxy-4-hydroxy)phenyl-2,5-dimethylpyrrole), was tested in the human non-small cell lung carcinoma cell

line H1299, engineered to express either PKM1 or PKM2. Furthermore, shikonin, a component of zicao (dried root of *Lithospermum erythrorhizon*), a Chinese herbal medicine, was recently identified as a natural inhibitor of PKM2 with an IC50 value of 0.3 μM (Chen et al. 2011). Two other studies investigated the effect of PKM2 silencing in combination with docetaxel and cisplatin, respectively. Moreover, there are few cases of synthetic lethality where silencing of PKM2 in combination of docetaxel (2 μg/ml) treatment resulted in higher intracellular drug concentration, due to an inhibition of the ATP binding cassette transporters by the decrease of ATP levels (Shi et al. 2010). Reduction in xenograft tumor growth by docetaxel was significantly greater in PKM2 downregulated tumors in comparison to control tumors (Shi et al. 2010). Similarly, cisplatin also showed a greater inhibition of xenograft tumor growth when PKM2 was silenced, and no increase in toxicity was observed (Guo et al. 2011). Collectively, these studies showed that PKM2 inhibition could potentially be used in combination with chemotherapy drugs to enhance their antitumor activity.

Not even inhibition, but activation of PKM2 may also be a strategy for inhibition of tumor growth. High-throughput screening of ~300,000 small molecules of the NIH Molecular Libraries identified two types of lead structures including substituted thieno[3,2-b]pyrrole[3,2-d]pyridazinone1 (Jiang et al. 2010) and substituted *N*,*N*′-diarylsulfonamide 2 (Boxer et al. 2010) which activate PKM2. It has been shown that *N*,*N*′-diarylsulfonamide 2 mimics FBP action by reducing the K_m of PKM2 for PEP to 1/10 without affecting Vmax. Different analogues of the lead structure were synthesized which were found to be selective for PKM2 as compared to the other isozymes.

Another chemotype of PKM2 activators are 2-Oxo-*N*-aryl-1,2,3,4-tetrahydroquinoline-6-sulfonamides (Walsh et al. 2011). The compounds were also identified in a high-throughput screen of nearly 300,000 small molecules of the NIH Molecular Libraries using pyruvate kinase-luciferase-coupled assay. Most of the analogues studied showed a high selectivity for PKM2. Compound 66, which reduced Km of PKM2 for PEP to 1/5, had an AC50 of 90 nM and was cell permeable (Walsh et al. 2011). Effects of these activators on cell proliferation remain to be studied. In addition to a direct interaction with pyruvate kinase type M2 also, indirect targeting may affect the activity of the enzyme. For example, targeting of the tyrosine kinases BCR-ABL by imatinib, JAK2 by AG490, and FLT3 by TKI258 decreased phosphorylation of PKM2 on Y105, which resulted in an activation of PKM2 (Hitosugi et al. 2009). The approaches summarized above suggest that in principle both inhibition and an activation of PKM2 could be a viable strategy for cancer treatment. However, when targeting tumor metabolism, possible metabolic escape mechanisms have to be taken under consideration. In another piece of study, peptide aptamers known to specifically bind to PKM2 and induce a dimerization and inactivation of PKM2 have been shown to inhibit cell proliferation, in high glucose conditions. However, these peptide aptamers promoted cell proliferation and inhibited apoptosis when the cells were cultured in low glucose (Spoden et al. 2008, 2009). It was explained mechanistically that at high glucose concentrations glycolysis is the main energy source. Therefore, the peptide aptamer

induced fixation of PKM2 in the almost inactive dimeric form, inhibited glycolysis and the cell proliferation due to energy deficiency. At low glucose supply, when glutaminolysis ensures energy, fixation of PKM2 in the inactive dimeric form is an advantage in that way that the less glucose available is completely channeled into the synthetic pathways of cell building blocks (Spoden et al. 2008, 2009).

6.4.2 Non-metabolic Functions of PKM2

As described above, most of therapeutic strategies targeting PKM2 have been focused at the cytosolic enzyme; however, PKM2 is also known to be present in the nucleus where it has been shown to promote proliferation through phosphorylation of the ε-amino group of histone H1 or by serving as a coactivator of the transcription factors Oct4 and HIF1 (Guminska et al. 1988; Lee et al. 2008; Luo et al. 2011; Ignacak and Stachurska 2003). Also, a seven-amino acid somatostatin analogue, TT232 (or TNL232), was shown to trigger nuclear translocation of PKM2, where PKM2 induced caspase-independent apoptosis (Stetak et al. 2007). The latter was independent of PKM2 catalytic activity since a catalytic inactive mutant of PKM2 (Lys^{294} mutated) also produced a comparable result. TLN232 (NCT00735332) is under phase II clinical trials for treating renal cell carcinoma, metastatic melanoma, and pancreatic cancer (Thallion et al.). There is another known strategy, which is based on the interaction of tumor endothelial marker protein-8 (TEM8) with PKM2. TEM8 is located on the cell surface and plays a role in angiogenesis. It is shown that PKM2 released from tumors into the blood stream may stimulate angiogenesis by binding to TEM8. In that case, a synthetic antibody-like molecule which consists of the N-terminal 200 amino acids of human TEM8, linked to the 232 amino acids of the FC1 portion of human IgG1, was found to bind to amino acid residues 379–385 of PKM2 and to suppress the growth and metastasis of xenograft tumors in mice (Duan et al. 2007).

6.4.3 PKM2 and Cancer Diagnostics

Detection of dimeric PKM2 protein is also used for cancer diagnosis. It is known that the expression of dimeric form of PKM2 in cancer cell is so high that it gets released from tumors into the blood of the patients and can be quantified in plasma using an ELISA, which specifically recognizes the dimeric form of PKM2. Levels of dimeric PKM2 in plasma have been shown to positively correlate with tumor stage in multiple tumor types tested, including the thyroid, lung breast, esophageal, stomach, pancreatic, colorectal, ovarian, cervical, kidney, and skin (Ahmed et al. 2007; Kaura et al. 2004; Kumar et al. 2007; Luftner et al. 2000; Ugurel et al. 2005; Wechsel et al. 1999; Schneider et al. 2002). A major field of application of the plasma PKM2 test is the follow-up studies to carefully monitor the success or

failure of therapy. In a recent study, elevated circulating plasma PKM2 levels in human samples preceded breast cancer diagnosis (Ladd et al. 2013). Commercial kits (ELISA based) are also available to quantify dimeric PKM2 in stool of patients with colorectal cancer with a sensitivity of 80 % and specificity of more than 90 % (Tonus et al. 2012).

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Chapter 7
Role of the Pentose Phosphate Pathway in Tumour Metabolism

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7.1 The Pentose Phosphate Pathway in Cell Metabolism

The pentose phosphate pathway (PPP) was first described in 1926 and constitutes an essential metabolic pathway involved in the synthesis of nucleic acid precursors and in the generation of reducing power, both indispensable to the maintenance of cell integrity. It is classically divided into two parts, known as the oxidative (ox-PPP) and the nonoxidative (nonox-PPP) branches (see Fig. 7.1). The ox-PPP catalyses the irreversible transformation of glucose-6-phosphate into ribulose-5-phosphate with the subsequent production of important amounts of NADPH and CO_2. The nonox-PPP branch is a reversible pathway that interconverts pentose phosphate and other sugar phosphate, contributing to the synthesis of ribose-5-phosphate as well as to the redirection of the excess of pentose phosphate towards glycolysis. It has been estimated that the percentage of glucose metabolized through PPP ranges from 5 to 30 % depending on the tissue, with higher percentages in lipid-synthesizing tissues (such as the liver, white adipose tissue, lactating mammary glands, adrenal glands, and gonads) and in red blood cells (Luzzatto and Notaro 2001; Riganti et al. 2012). Throughout the next pages, a detailed view of the PPP and the function that this pathway plays in cancer will be provided, with particular emphasis in the role of the main enzymes of PPP in cancer cell biology.

7.1.1 The Oxidative Branch of the Pentose Phosphate Pathway

The oxidative branch of the pentose phosphate pathway is a major source of metabolic precursors for biosynthetic processes (i.e. for nucleic acid synthesis) and reducing power (i.e. for lipid synthesis, maintenance of reduced pool of glutathione, etc). It operates as an irreversible pathway which produces ribulose-5-phosphate, NADPH, and CO_2 by consuming glucose-6-phosphate and $NADP^+$. This pathway consists of three metabolic reactions which are considered to operate as depicted in Fig. 7.1.

The first enzyme of the ox-PPP is glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49). It catalyses the oxidation of glucose-6-phosphate to 6-phosphoglucono-δ-lactone, a cyclic and unstable lactone ester of phosphogluconic acid. This irreversible reaction produces NADPH from $NADP^+$ and is highly regulated, among others, by NADPH and palmitoyl-CoA both negatively modulating G6PD enzymatic activity (Fig. 7.2). G6PD can be active in human cells as a dimer or tetramer (formed by the association of two dimers), each inactive monomer being composed of 515 amino acids (Au et al. 2000; Riganti et al. 2012). The amount of $NADP^+$ is critical for the activity of this enzyme, since it is necessary for stabilizing the dimer (Au et al. 2000). On the contrary, NADPH lacks stabilizing effects (Kotaka et al. 2005) and its binding to G6PD instead of $NADP^+$ leads to a reduction of G6PD activity. Therefore, G6PD activity is directly modulated by the

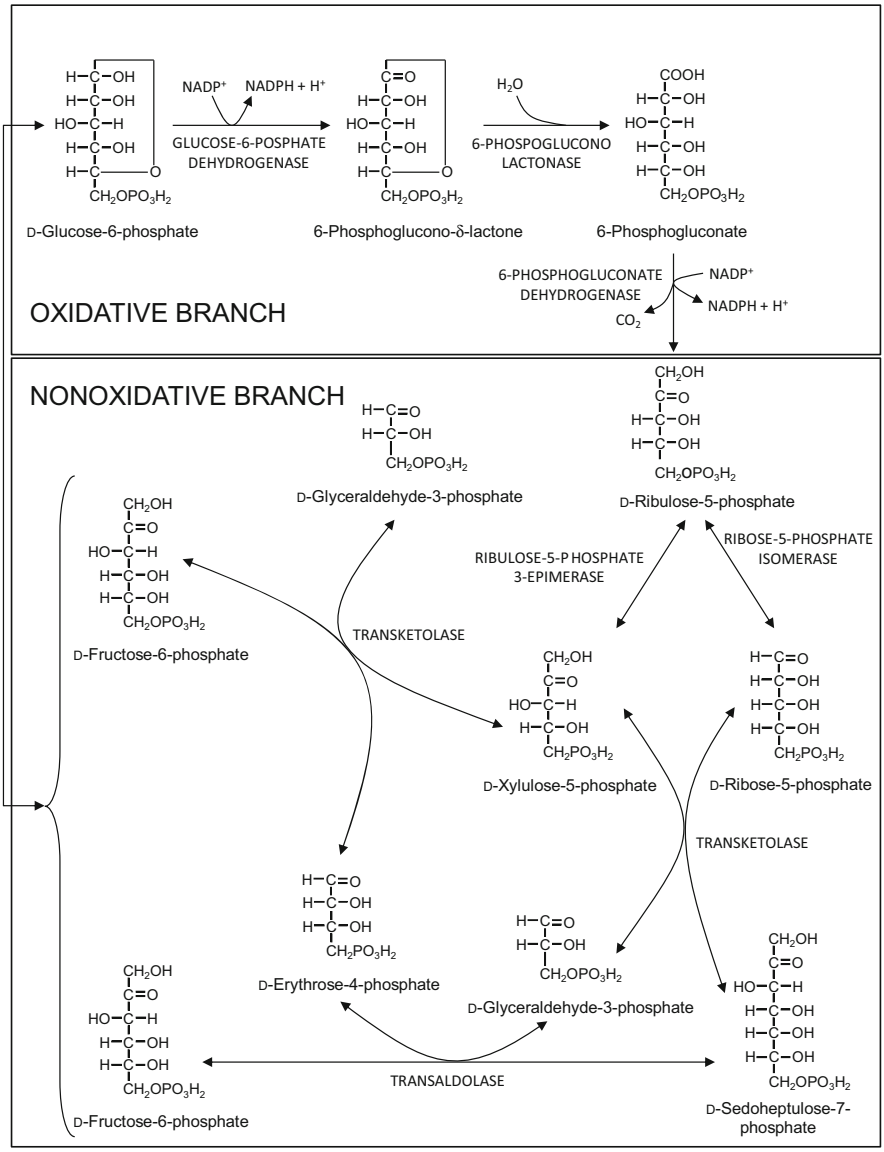

Fig. 7.1 Reaction scheme of the pentose phosphate pathway

$NADP^+$/NADPH ratio. Here, it is worth noting that G6PD usually works at 1–2 % of its maximal potential in healthy subjects, because of the high concentration of NADPH in resting conditions. Upon NADPH oxidation, $NADP^+$/NADPH ratio increases and G6PD shifts to the most active state, increasing ox-PPP flux (Eggleston and Krebs 1974). Consistent with the role of G6PD in the synthesis of NADPH for lipogenesis, a negative regulation of this enzyme by the lipid intermediate

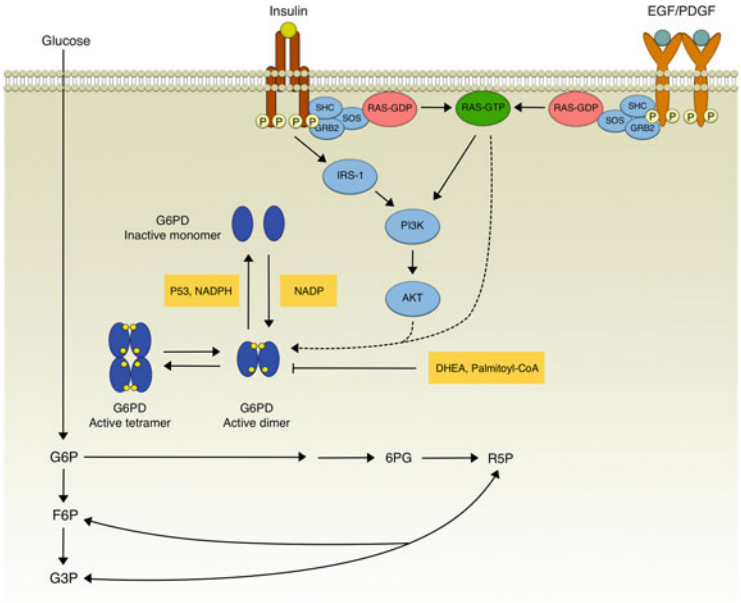

Fig. 7.2 Main regulatory mechanisms of G6PD. G6PD is positively and negatively modulated by different mechanisms. The figure shows simply the main positive and negative regulators of G6PD as well as the transitions between the monomeric-dimeric-tetrameric states of the enzyme. *Dotted lines* indicate indirect effects on the enzyme by the involvement of additional effectors. *G6P* glucose-6-phosphate, *6PG* 6-phosphogluconate, *R5P* ribose-5-phosphate, *F6P* fructose-6-phosphate, *G3P* glyceraldehyde-3-phosphate, *DHEA* dehydroepiandrosterone, *EGF* epidermal growth factor, *PDGF* platelet-derived growth factor

palmitoyl-CoA has also been described (Asensio et al. 2007; Kawaguchi and Bloch 1974; Taketa and Pogell 1966). Accordingly, G6PD deficiency, which is the most common enzyme deficiency in the world (Cappellini and Fiorelli 2008; Luzzatto and Notaro 2001), yields a diminution of NADPH and a subsequent decrease in cholesterol synthesis (Rawat et al. 2012) and protection under oxidative stress (Rajasekaran et al. 2007) due to a lower production of reduced glutathione from NADPH. Besides regulation by direct binding of cofactors, G6PD is also modulated by signalling pathways. Thus, G6PD activity is upregulated by the action of EGF and PDGF (Tian et al. 1994; Stanton et al. 1991), whereas G6PD mRNA levels are increased through the action of insulin via PI3K activation (Talukdar et al. 2005; Wagle et al. 1998).

The second enzyme of the ox-PPP is 6-phosphogluconolactonase (6PGL; EC 3.1.1.31), which accelerates the spontaneous ring-opening hydrolysis of 6-phosphoglucono-δ-lactone to produce 6-phosphogluconate.

Finally, 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) catalyses the oxidative decarboxylation of 6-phosphogluconate and yields ribulose-5-phosphate, CO_2, and NADPH. The resulting ribulose-5-phosphate can be then converted into

ribose-5-phosphate by a reversible reaction of the nonox-PPP (see next section) and used for the synthesis of nucleotides.

7.1.2 The Nonoxidative Branch of the Pentose Phosphate Pathway

The nonoxidative branch of the pentose phosphate pathway is a reversible pathway that interconverts different carbon length sugar phosphate by two- and three-carbon unit exchange. This pathway includes four reversible enzymatic reactions.

Ribose-5-phosphate isomerase (RPI, EC 5.3.1.6.) interconverts ribulose-5-phosphate and ribose-5-phosphate via production of an enediol intermediate. Ribose-5-phosphate produced in this reaction is used in the biosynthesis of coenzymes (including NADH, NADPH, FAD, and B12) and nucleic acids (DNA and RNA).

Ribulose-5-phosphate-3-epimerase (RPE, EC 5.1.3.1.) interconverts ribulose-5-phosphate to another ketose, xylulose-5-phosphate. This reaction, as the previously described one, proceeds also by an enediol intermediate.

Transketolase (TKT; EC 2.2.1.1.) is a thiamine pyrophosphate-dependent enzyme that acts in two steps of the nonox-PPP transferring in both steps two-carbon units from one sugar phosphate (donor) to another (acceptor). Its mechanism of action involves the binding of a ketose phosphate substrate (xylulose-5-phosphate), expulsion of the glyceraldehyde-3-phosphate product, and transfer of the two-carbon unit to an aldose phosphate (ribose-5-phosphate), yielding a molecule of sedoheptulose-7-phosphate. Transketolase can process a variety of 2-keto sugar phosphates in a similar manner.

Transaldolase (TA, EC 2.2.1.2) reaction is similar to the glycolytic aldolase reaction of glycolysis and catalyses the transference of three-carbon units. Its mechanism of action involves the binding of a ketose phosphate substrate (sedoheptulose-7-phosphate), expulsion of the erythrose-4-phosphate product, and transfer of the three-carbon unit to an aldose phosphate (glyceraldehyde-3-phosphate) to yield the product fructose-6-phosphate.

Despite the PPP was described almost a century ago, the precise reaction scheme of the nonox-PPP remains controversial and incompletely understood. ^{14}C-isotope labelling experiments demonstrated that the degree of ^{14}C-isotope incorporation in carbon atoms of fructose-6-phosphate and its distribution differs from what is predicted by the proposed scheme of individual reactions (Horecker et al. 1954). Indeed, Horecker, who originally discovered the reactions of the nonox-PPP, wrote: “From the results with labelled pentose phosphate, it is apparent that the transketolase-transaldolase sequence of reactions is by itself insufficient to account for the hexose monophosphate formation, since the distribution of isotope in the product differs from that predicted by these reactions”. The discrepancies between the widely accepted reaction scheme of the PPP and the observed experimental results suggest that this pathway has not been properly characterized yet.

Transketolase enzyme reaction is the rate-limiting step of the nonox-PPP (Comin-Anduix et al. 2001; Sabate et al. 1995). The characterization of the reaction modus and substrate specificity of TKT is of utmost interest to resolve the discrepancy between experimental results and PPP reaction schemes presented in textbooks. One possible explanation might lie on the existence of additional nonox-PPP enzymes not yet considered in the reaction schemes, such as TKT isoforms, which may carry out non-standard transketolase reactions. Throughout evolution of higher vertebrates, genome duplication led to duplication of the *TKT* gene giving rise to the transketolase-like 1 (*TKTL1*) precursor gene. This duplication was followed by an integration of the TKTL1 precursor mRNA into the genome leading to the intronless transketolase-like 2 gene (*TKTL2*). After this, the *TKTL1* precursor gene mutated creating the recent *TKTL1* gene (Coy et al. 2005). In comparison to known transketolase proteins, the *TKTL1* gene encodes for TKTL1 protein isoform harbouring a 38-amino acid deletion due to deletion of original *TKT* gene exon 3. This deletion results in the generation of a transketolase protein similar to His103 yeast mutant, which has been reported to be capable of catalysing one-substrate reaction as well as to display a reduced affinity to thiamine (Selivanov et al. 2004). This reaction transforms a five-carbon molecule into three- and two-carbon molecules (Coy et al. 2005). Therefore, it is speculated on the possibility that TKTL1 enzyme catalyses also this reaction, although this hypothesis requires further research. Thus, the existence of different TKT isoforms provides a greater complexity in the operating modus of the nonox-PPP.

Several studies examined the effect of TKTL1 protein suppression on the total transketolase enzyme activity of different cell lines and tissues (Hu et al. 2007; Zhang et al. 2007; Yuan et al. 2010; Xu et al. 2009). In these studies, expression of human TKTL1 protein was inhibited by RNA interference in different cell models and a significant inhibition of transketolase reaction and/or associated glucose metabolism was found. Therefore, these findings demonstrate that TKTL1 is an active enzyme that clearly contributes to total transketolase activity in mammalian cells. Moreover, several in vivo studies using RNA inhibitory experiments and TKTL1 knockout mice clearly demonstrated at the highest level of evidence that TKTL1 has an important function in colon mucosal repair (Bentz et al. 2011). Thus, knockout of TKTL1 in mice led to suppression of mucosal repair and aggravates murine experimental colitis (Bentz et al. 2011). Also, it has been reported that TKTL1 plays an important and protective role against ROS allowing the maintenance of redox homeostasis in vitro (Xu et al. 2009; Wanka et al. 2012) and in vivo (Bentz et al. 2011). Recently, it has been demonstrated that downregulation of the Werner syndrome protein (WRN) induced an increase in oxidative stress accompanied by the downregulation of TKTL1, as well as of G6PD, and isocitrate dehydrogenase 1 (IDH1), further supporting the role of TKTL1 in redox homeostasis (Baomin et al. 2014; Li et al. 2009). This data is also in line with the strong expression of TKTL1 in germ cells (Rolland et al. 2013), the protective role of TKTL1 in the brain (Wanka et al. 2012; Coy et al. 2005), and the evolution of cognitive functions during transition from Neanderthals to *Homo sapiens* (Green et al. 2010; Prüfer et al. 2014; Pääbo 2014).

Depending on the cellular needs, the PPP can operate in different modus, and its two branches (ox-PPP and nonox-PPP) can be partially decoupled. When both ribose-5-phosphate and NADPH are needed, the main flux of carbons is driven through the ox-PPP. However, when the need of ribose-5-phosphate is greater than the need of NADPH, the carbon flux can be driven in a greater extent through the nonox-PPP. On the contrary, when there is a high demand of NADPH but not that of ribose-5-phosphate, the excess of this metabolite is directed towards glycolysis through the nonox-PPP. In the same line, when both NADPH and ATP are needed, but ribose-5-phosphate is not, glycolytic carbons are shunted into the ox-PPP and subsequently into the nonox-PPP and glycolysis.

7.2 The Role of the Pentose Phosphate Pathway in Cancer Metabolism

The widely recognized as the main characteristic of tumour cells is its accelerated and uncontrolled proliferation. Accordingly, the requirements of nutrients are particularly high in proliferating and tumour cells, since they need not only to preserve their integrity and perform their physiological functions but also to generate a new daughter cell. Then, as cancer is a tissue-proliferation disorder, it is expected that cancer cells rewire metabolism at the service of proliferation to provide themselves with energy and precursors of macromolecules (Ward and Thompson 2012; Schulze and Harris 2012). Furthermore, development of malignancy involves a metabolic reprogramming closely related to the acquisition of the well-known cancer hallmarks, extending thus the role of metabolism beyond growth and proliferation (Kroemer and Pouyssegur 2008; Hanahan and Weinberg 2011).

The metabolic feature associated to cell malignant transformation that has been known for the longest time is the enhanced aerobic glycolysis, consisting in an increased metabolism of glucose to lactate even in the presence of oxygen. This phenomenon is commonly referred to as the "Warburg effect" due to Otto Warburg, who first described it in the 1920s (Warburg et al. 1924, 1927). Although this adaptation accounts for one of the essential metabolic requirements of cancer cells, the high production of energy, two additional metabolic requirements must be fullfilled by cancer cells to survive and proliferate: biosynthesis of macromolecules and maintenance of redox homeostasis (Cantor and Sabatini 2012), two metabolic processes in which PPP importantly participates in. In brief, cell division requires high amounts of nucleic acids for DNA replication and significant synthesis of lipids for membrane duplication. The former are produced from the pentose phosphate generated through the PPP, and the latter are synthesized from acetyl-CoA and NADPH partly produced by the ox-PPP. Thus, the PPP promotes nucleotide and lipids synthesis but also allows carbon recirculation through nonox-PPP to glycolysis in order to preserve the formation of other molecules with a significant

role in tumour physiology such as ATP, amino acids, or lactate. Moreover, the capacity of ox-PPP of producing NADPH allows the maintenance of the redox balance, which is widely accepted to be altered in cancer cells and requires additional mechanisms to be maintained (Trachootham et al. 2009; Sosa et al. 2013).

7.2.1 Oxidative Branch of the Pentose Phosphate Pathway in Cancer

As described earlier, the ox-PPP is an irreversible metabolic pathway driven by GPGD, 6PGL, and 6PGD. Given that this pathway is involved in two cellular essential processes related to anabolism and redox homeostasis (synthesis of ribose and NADPH), the role of the ox-PPP and its constituent enzymes in tumour biology has been mainly studied in the context of cell proliferation, transformation, and maintenance of redox state of cancer cells.

7.2.1.1 Glucose-6-Phosphate Dehydrogenase

G6PD usually works at a low basal rate in non-transformed cells (Riganti et al. 2012). Nevertheless, it can exert a strong proliferative role when it becomes deregulated. The key role of G6PD in tumorigenesis is supported by the fact that *G6PD* gene overexpression transforms NIH3T3 cells and induces tumours in nude mice (Kuo et al. 2000). In accordance, it has also been described that cells overexpressing G6PD proliferate more than wild-type cells, suggesting that G6PD levels correlates with cell proliferation rate (Tian et al. 1999; Leopold et al. 2003).

As described earlier, the ox-PPP branch is one of the main metabolic pathways involved in the production of NADPH, which is essential to the maintenance of the reduced antioxidant pool, such as reduced glutathione. In this sense, the essential role of the enzyme G6PD in protection against oxidative stress is sturdily documented (Gao et al. 2009; Ho et al. 2007; Cheng et al. 2004). Cells lacking G6PD show increased propensity for oxidant-induced senescence and increased sensitivity to diamide-induced oxidative damage. In fact, in an attempt to separately evaluate the role of G6PD in ribose synthesis and redox homeostasis, it has been concluded that G6PD is dispensable for pentose synthesis but essential to defence against oxidative stress (Pandolfi et al. 1995). In this regard, it is widely accepted that most tumours deal with increased levels of reactive oxygen species (ROS), leading to conditions of high oxidative stress. Compared to normal cells, malignant cells display higher levels of endogenous oxidative stress in vitro and in vivo (Szatrowski and Nathan 1991; Kawanishi et al. 2006). Breast tumours are a paradigmatic example, since they are characterized by persistent ROS generation

(Brown and Bicknell 2001; Kang 2002) and reliance on ox-PPP branch to modulate oxidative stress. In these tumours, markers of constitutive oxidative stress have been detected in samples from in vivo breast carcinomas (Toyokuni et al. 1995; Portakal et al. 2000) as well as elevated levels of 8-hydroxy-2′-deoxyguanosine, one of the major oxidatively modified DNA base products, compared with normal control samples from the same patient (Toyokuni et al. 1995). Consequently, breast tumours also display greater reliance on ROS detoxification systems, which increases gradually as tumour progresses. Advanced breast tumours display an increased need to detoxify ROS as demonstrated by the higher expression of the ox-PPP enzymes detected in a genome-scale study based on the gene expression analysis of a large cohort of clinical samples (Jerby et al. 2012). Also, metastases of breast cancer display an increased expression of enzymes of the PPP such as G6PD and 6PGL (Chen et al. 2007), and breast cancer cells MCF7 (derived from metastatic pleural effusion) have an increased expression of G6PD compared with the near-normal breast cancer cells MCF10 (Drabovich et al. 2012).

Given the important role of G6PD in healthy and cancer cell physiology, it is not surprising that G6PD expression and activity is regulated by some of the most important oncogenes and tumour suppressor genes. G6PD upregulation has been reported in NIH3T3 fibroblast transfected with a mutated copy of *K-RAS* gene (de Atauri et al. 2011; Vizan et al. 2005), indicating that K-RAS regulates G6PD expression by a yet not described mechanism. Also, as mentioned above, G6PD is positively regulated by PI3K, one of the most frequently activated oncogenes in various types of cancer (Samuels et al. 2004; Luo et al. 2003). On the contrary, G6PD activity is negatively regulated by tumour suppressor gene *P53*, which impairs dimer formation by direct binding to the enzyme, therefore decreasing G6PD activity (Jiang et al. 2011). However, tumour-associated *P53* mutants lack the G6PD-inhibitory activity, enhancing ox-PPP flux. Therefore, enhanced PPP glucose flux due to *P53* inactivation increases glucose consumption and direct glucose towards biosynthesis in tumour cells. According to the role of G6PD in oxidative stress, G6PD is also regulated by transcription factors involved in response to cellular stress, such as NRF2, which has been recently described to play a key role in tumorigenesis (DeNicola et al. 2011; Mitsuishi et al. 2012). This transcription factor is frequently upregulated in various types of human cancers, resulting in an overactivation of its target genes and providing cells with additional capabilities of malignance (Singh et al. 2006; Solis et al. 2010; Tsai et al. 2008). A significant portion of the NRF2 target genes are metabolic genes involved in PPP and NADPH production, such as *G6PD*, *PGD* (phosphoglycerate dehydrogenase), *TKT*, *TALDO1* (transaldolase), *ME1* (malic enzyme 1), and *IDH1*, all of them containing antioxidant response element (ARE) sequences in their promoters. This provides additional evidence of the relevant function that PPP and NADPH production have in tumorigenesis.

7.2.1.2 6-Phosphogluconate Dehydrogenase

The role of 6PGD in cancer was initially related to the detection and prognosis of tumours. High 6GPD activity in primary breast tumours was associated to poor relapse-free survival times when compared with those with low 6GPD activity (Brocklehurst et al. 1986; Kolstad et al. 1967). More recently, several reports have described a functional role of this enzyme in cancer pathogenesis. 6PGD inhibition in lung cancer cell lines resulted in tumour growth inhibition by senescence induction both in vitro and in vivo, what may be partly due to accumulation of growth-inhibitory metabolic intermediates (Sukhatme and Chan 2012). Furthermore, it has been also described that 6PGD inhibition downregulate c-Met receptor activation by inhibiting the phosphorylation of activating tyrosine residues. This downregulation of c-Met receptor subsequently inhibited cell migration in vitro, providing a functional role of 6PGD in cancer cell migration and c-Met signalling (Chan et al. 2013).

7.2.2 Nonoxidative Branch of the Pentose Phosphate Pathway in Cancer

As described above, the nonox-PPP is a metabolic pathway that consists of two reversible enzymatic reactions: transketolase (TKT) and transaldolase (TA). It has been reported that the TKT family includes genes encoding two other TKT-like proteins (TKTL1 and TKTL2) in addition of TKT. Among them, TKTL1 has been reported to be overexpressed in several cancer cell lines and tissues such as the colon, lung bladder, thyroid, breast, liver larynx and brain (Langbein et al. 2006; Zerilli et al. 2008; Zhang et al. 2007; Foldi et al. 2007; Völker et al. 2007) and its expression correlates with poor prognosis of patients (Langbein et al. 2006; Schwaab et al. 2011; Lange et al. 2012; Kayser et al. 2011; Völker et al. 2007; Grimm et al. 2013) and resistance to radio- and chemotherapy (Schwaab et al. 2011). The reversibility of this branch confers great versatility to the pathway, allowing the cell to activate ribose-5-phosphate synthesis or glycolytic recirculation of the PPP intermediates depending on its metabolic requirements. Thereby, if ox-PPP is active and nucleotide precursors are synthesized efficiently, nonox-PPP is able to reincorporate the excess of pentose phosphate into the glycolytic pathway, guaranteeing energy obtaining and the supply of many metabolic precursors (glycerol, amino acids, acetyl-CoA, ...) essential to cell proliferation. On the other hand, if the ox-PPP is not active, the nonox-PPP can produce the required amount of ribose-5-phosphate. However, if the activation of the ox-PPP is not accompanied by the activation of the nonox-PPP, pentose phosphate may accumulate, and tumour requirements would not be fulfilled. Therefore, nonox-PPP, apart from playing its own role, is important to enable the activation of the ox-PPP during tumorigenesis.

Given the reversibility of the reactions involved in the nonox-PPP, the Warburg effect can enhance the use of this branch (Pelicano et al. 2006). In experiments in vitro using pancreatic adenocarcinoma cells, around 85 % of the ribose has been reported to be synthesized through the nonox-PPP (Boros et al. 1997). Accordingly, in experiments in vivo using pancreatic ductal adenocarcinoma mouse model, ribose biogenesis is mainly carried out through the nonox-PPP, being this pathway essential to tumour progression in vivo (Ying et al. 2012). Furthermore, the function of the nonox-PPP in cancer is also demonstrated by additional studies reporting the involvement of this pathway in oncogenic transformation (Smith et al. 2009; Xu et al. 2009; Sun et al. 2010) and several processes accompanying it, such as metabolic reprogramming, uncontrolled tumour cell proliferation invasiveness and metastasis (Frederiks et al. 2008; Kohrenhagen et al. 2008; Krockenberger et al. 2007; Langbein et al. 2006, 2008; Schwaab et al. 2011; Kayser et al. 2011; Zerilli et al. 2008). The specific role of each enzyme is described below.

7.2.2.1 Transaldolase

The role of TA in cancer cells remains unclear, since available data supports both pro- and anti-tumorigenic role of this enzyme. Higher rates of TA expression have been reported in specific groups of head and neck squamous cell carcinoma (HNSCC) (Chung et al. 2004) and in lung epithelium of smokers in comparison with non-smokers (Hackett et al. 2003). A role of TA, but not necessarily its overexpression, has also been described in many other tumours (Samland and Sprenger 2009). Furthermore, it has been reported that in vivo, TA forms a complex with two enzymes of the ox-PPP, G6PD and 6PGD, being this complex upregulated in cancer (Huang et al. 2005). This finding led some researchers to propose the overexpression of TA as a biomarker for cancer development (Riganti et al. 2012). However, the overexpression of TA in the above-mentioned tumours is frequently related to better prognosis and recurrence-free survival rate (Chung et al. 2004). Thus, despite the enzymatic complex in which TA has been identified might be involved in carcinogenesis, the presence of G6PD in the complex might largely explain the role of this complex in carcinogenesis, while TA might be a mere bystander. In accordance with this reasoning, TA overexpression led to contrary metabolic effects than those induced by G6PD overexpression, i.e. it accelerated the turnover of NADPH, decreased the amount of reduced glutathione, and increased the cell sensitivity to ROS-induced apoptosis (Banki et al. 1996). According to this data, a tumour suppression role of TA is more likely than an oncogenic role. In fact, *TALDO1* is located in chromosome 11 (11p15.5–p15.4) (Banki et al. 1997), a region containing tumour suppressor genes that is frequently deleted in cancers, such as oesophageal cancer (Lam et al. 2002), and its expression has been reported in differentiating and maturating processes that are totally opposed to malignant transformation (Grossman et al. 2004). In conclusion, there are indications that may

lead to suggest a possible role of TA as tumour suppressor gene; however, further studies are required in order to validate this hypothesis.

7.2.2.2 Transketolases

The role of transketolases in cancer development has been more clearly described than TA's. TKT activity has been reported as the PPP enzymatic activity with the highest control coefficient of tumour growth in mice with Ehrlich's ascites tumour (Comin-Anduix et al. 2001) as well as to be increased in (pre)neoplastic lesions in rat liver (Frederiks et al. 2008). Also, inhibition of TKT by oxythiamine led to a decrease of 90 % in final tumour mass in mice hosting Ehrlich's ascites tumour (Boros et al. 1997). These findings clearly prove the essential role that TKT plays in tumorigenesis.

From the two active transketolases described in tumours, TKT and TKTL1, the latter has been suggested as novel candidate *oncogene* (Smith et al. 2009) and a putative drug target for those tumours in which it is overexpressed (Riganti et al. 2012; Sun et al. 2010). Moreover, increased TKTL1 is activated by hypomethylation (Sun et al. 2010), and its levels correlate with activated proliferation and tumour progression (Diaz-Moralli et al. 2011; Krockenberger et al. 2010; Zerilli et al. 2008; Langbein et al. 2008), whereas TKTL1 silencing leads to inhibition of glucose metabolism, cell proliferation, and tumour growth (Hu et al. 2007; Zhang et al. 2007; Xu et al. 2009; Sun et al. 2010). Also a protective role for TKTL1 under starvation conditions has been reported. Recently, Wanka et al. demonstrated that Tp53-induced glycolysis and apoptosis regulator (TIGAR) protect glioma cells from starvation-induced cell death only when TKTL1 protein is present (Wanka et al. 2012).

The role of transketolases in cancer cells has been related to the hypoxia-inducible factor 1α (HIF-1α), the master regulator of the response to hypoxia that is deeply involved in tumour physiology (Sun et al. 2010; Zhao et al. 2010). It has been reported that TKT participates in a component of HIF-1α-dependent imatinib (Gleevec) resistance in chronic myeloid leukaemia cells (Zhao et al. 2010). The inhibition of TKT expression by shRNA in imatinib-resistant cells led to their resensitization, whereas the same treatment in cells overexpressing TKTL1 was ineffective. These results showed that both TKT and TKTL1 play a similar role in conferring resistance to imatinib (Zhao et al. 2010). Moreover, it has been also reported a collaborative role of HIF-1α and TKTL1 in the metabolic reprogramming of TKTL1-mediated head and neck squamous cell carcinoma tumorigenesis, where TKTL1 contributes to carcinogenesis through increased aerobic glycolysis and HIF-1α stabilization (Sun et al. 2010), reinforcing the idea of a physiological connection between transketolases and HIF-1α in cancer. Also, hypoxia (Bentz et al. 2013) as well as chemotherapy (Wanka et al. 2012) induce the expression of TKTL1.

7.3 The Pentose Phosphate Pathway as Potential Cancer Therapeutic Target

The significance of cancer and its worldwide impact have revealed the need of developing effective therapies to fight against this fatal disease. As mentioned above, one of the first and most obvious cancer features is the accelerated and uncontrolled proliferation of tumour cells. Given that cell division requires genome replication, researchers have developed pharmacologic strategies targeted to hinder the synthesis of nucleotides in order to impair tumour growth (Farber and Diamond 1948; Heidelberger et al. 1957). Molecules such as aminopterin, methotrexate, and 5-fluorouracil have been long and widely used in cancer therapy due to the capability of these molecules of inhibiting either nucleotide synthesis or DNA replication, thus impairing primarily cancer cell proliferation (Farber and Diamond 1948; Heidelberger et al. 1957). Their effectiveness opened the door to study other potential therapeutic targets in the pathway of synthesis of the sugar component of nucleotides, as is the PPP. This pathway has been considered a rational therapeutic target because it meets two essential requirements of cancer cells: ribose synthesis for nucleotides production and NADPH synthesis for redox homeostasis maintenance and lipogenesis (Butler et al. 2013; Vander Heiden 2011). Accordingly, the main enzymes of the PPP, G6PD, and TKT have been proposed as potential therapeutic targets in cancer (Boros et al. 1997; de Atauri et al. 2011; Rais et al. 1999; Ramos-Montoya et al. 2006), and inhibitors of the two enzymes have been designed and assessed. Therefore, the inhibitor of G6PD, dehydroepiandrosterone (DHEA), and the inhibitor of TKT, oxythiamine (OT), have been described as potential antitumoral agents (Rais et al. 1999; Ramos-Montoya et al. 2006; Cascante et al. 2002).

Deficiency in G6PD is found in approximately 400 million people worldwide, with patients suffering mild anaemia but no other serious health issues, which opens a potential therapeutic window for inhibition of this enzyme in cancer treatment. The reduction of G6PD levels seems to exert different effects on cell proliferation. In cancer cells, inhibition of G6PD leads to a clear decrease in proliferation (Li et al. 2009). Also, G6PD inhibition in human foreskin fibroblast reduces cell growth and induces cellular senescence (Ho et al. 2007). Nevertheless, the complete absence of the enzyme in G6PD-deleted embryonic stem cells does not reduce proliferation, but makes cells more sensitive to strong antioxidants (Fico et al. 2004). In accordance to that, the inhibition of the ox-PPP is especially attractive since it not only targets the production of nucleotide precursors but also the ROS protection system of cancer cells. Targeting cancer cells with ROS-mediated mechanisms has been proposed as an interesting therapeutic approach. As mentioned earlier, cancer cells usually work with increased levels of ROS and acquire protective and compensating mechanisms by activation of ROS detoxification mechanisms. Moderate levels of ROS can promote many aspects of tumour biology (Cairns et al. 2011). However, a delicate balance exists between ROS-producing and ROS-removing reactions. Since this equilibrium is forced in

cancer cells, they are more sensitive to further external insults affecting this balance, promoting ROS formation, inhibiting ROS removal reactions, or both actions simultaneously (Trachootham et al. 2009). Thus, given the role of G6PD in ROS detoxification, inhibition of this enzyme is likely to break this equilibrium, dismantling the ROS adaptive response and causing cell death. This hypothesis is fully supported by the fact that G6PD-deficient cells show enhanced oxidative stress and increased sensitivity to oxidative damage, indicating that G6PD plays a fundamental role in controlling ROS levels, and that it might be a potential target in a ROS-based cancer therapy approach (Cheng et al. 2004; Gao et al. 2009; Ho et al. 2007).

The above-mentioned crucial roles of G6PD in cell physiology have led researchers to assess the efficacy of the G6PD inhibitor DHEA against breast cancer by performing several clinical trials (search for clinical trial identifier NCT00972023 and NCT02000375 at www.clinicaltrials.gov/ct2/search).

Also, TKT has been explored as potential therapeutic target. It has been observed that inhibition of the nonox-PPP provokes a greater decrease in tumour proliferation than the inhibition of the ox-PPP. In vivo testing of OT and DHEA in C57BL/6 mice hosting Ehrlich's ascites tumour cells revealed a 90.4 and a 46 % decrease in the final tumour mass, respectively, after 3 days of treatment (Boros et al. 1997). Likewise, the administration of OT and DHEA resulted in cell cycle arrest in Ehrlich's ascites tumour in vivo, and the combined administration of both drugs displayed a synergic effect (Rais et al. 1999).

However, several limitations complicate the interpretation of OT-mediated TKT inhibition experiments as well as hinder the efficacy of OT in cancer treatment: first, the lack of specificity caused by the fact that OT is an antimetabolite of thiamine that potentially affects all thiamine-dependent enzymatic activities, such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase; second, the versatility of the nonox-PPP and the bidirectional activity catalysed by TKT, which means that the inhibition of the enzyme does not necessarily lead to an inhibition of the synthesis of pentose phosphates; and third, the above-mentioned evidence that the transketolase mainly overexpressed in cancer cells might be TKTL1, whose activity is not clearly reported to be thiamine dependent and, therefore, might be insensitive to OT-mediated inhibition. To overcome the lack of specificity of OT, recently, diphenyl urea derivatives have also been reported as transketolase inhibitors by likely interfering with the enzyme dimerization. This new family of inhibitors represent a new avenue for the design of more selective inhibitors of TKT/TKTL1 with a novel binding mode, which is not based on mimicking the thiamine pyrophosphate cofactor binding (Obiol-Pardo et al. 2012).

On the other hand, clinical and experimental data support the requirement of thiamine to sustain enhanced nonox-PPP flux in tumours, being this fact further supported by the signs of thiamine deficiency in cancer patients. Thus, it has been hypothesized that thiamine supplementation in cancer patients may promote tumour growth (Boros et al. 1998). Certainly, evidence of significant stimulatory effect on tumour proliferation by thiamine supplementation has been provided using an Ehrlich's ascites tumour mouse model (Comin-Anduix et al. 2001).

Interestingly, it has also been reported in this study that overdoses of thiamine (2,500 times the recommended dietary intake (RDI)) slightly decreases tumour cell proliferation, suggesting that cancer patients requiring thiamine to treat thiamine deficiency should receive overdoses of thiamine to avoid the range of thiamine concentrations that supports proliferation. Other studies in breast cancer also have shown that tumour latency was significatively longer in animals fed with a low-thiamine diet compared with animals with normal-thiamine diet (Daily et al. 2012). The importance of the thiamine availability in cancer and its relation with tumour growth through promoting TKT and other thiamine-dependent pathways is an open area of research that might bring new opportunities for therapeutic intervention and dietary modification to reduce disease progression in cancer patients (Zastre et al. 2013).

Conclusion

In summary, an evolving body of evidence indicates that PPP plays a fundamental role both in healthy and cancer cells physiology. However, given the particular metabolic requirements and biochemical architecture of cancer cells, ox- and nonox-PPP are likely to play a critical role in some types of tumours. A better knowledge of the biochemistry and regulation of PPP in cancer cells as well as the identification of those tumours largely reliant on PPP will surely culminate in novel and interesting findings with clinical relevance. Also, further research on the promising role of TKTL1 in cancer biology will likely provide new and interesting knowledge of the mechanisms underlying tumour metabolic reprogramming.

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Chapter 8
Enzymes of the Tumour Metabolome in Diagnostic Applications

Philip D. Hardt

Contents

8.1 6-Phosphofructo-1-Kinase

6-Phosphofructo-1-kinase (PFK, EC 2.7.1.11) catalyses the ATP-dependent phosphorylation of fructose 6-P to fructose 1,6-P2 within the glycolytic pathway (Fig. 8.1). In differentiated cells, PFK is the bottleneck within the glycolytic sequence, which is regulated by the so-called Pasteur effect. High rates of mitochondrial ATP regeneration inhibit PFK and glucose consumption rates. Hypoxia-induced inhibition of mitochondrial ATP production corresponds to an increase in AMP levels, which in turn activates PFK and increases the conversion rates of glucose. PFK is a tetrameric protein which may consist of three different subunits: subunit M, which is mainly expressed in the muscle; subunit L, which is characteristic for the liver; and subunit P, which is mainly found in platelets. The different

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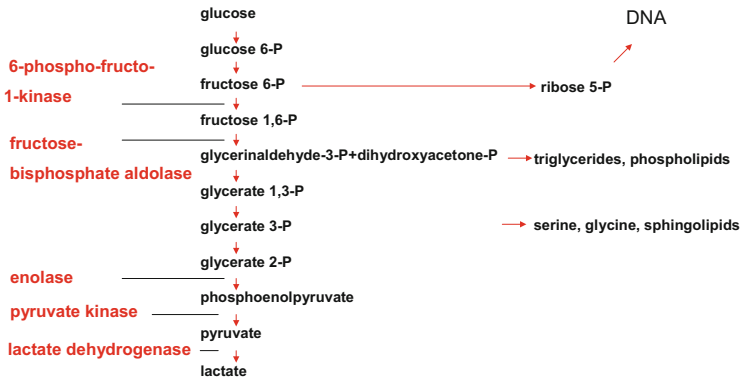

Fig. 8.1 Glycolytic enzymes with specific changes in tumour metabolism

isoenzymes of PFK differ in the composition of the three subunits, whereby homomeres but also heteromeres are described. In tumours, an upregulation of the subunits L and P is described. Based on the observation that plasma samples of cancer patients inhibit PFK activity, a PFK inhibition test has been developed and evaluated by the same research group in patients with gastric cancer, pancreatic cancer and lung cancer several years ago (Nakamura et al. 1987). Although there were promising results and there is still a lack of routine markers in some of these entities today, there are no established commercial tests of PFK inhibition used in clinical routine cancer management to date.

8.2 Fructose-Bisphosphate Aldolase

Fructose-bisphosphate aldolase (EC 4.1.2.13) catalyses the cleavage of fructose 1,6-P2 into glyceraldehyde 3-P dehydrogenase and dihydroxyacetone P within the glycolytic sequence (Fig. 8.1). There are three different isoforms of fructose-bisphosphate aldolase: type A is found in muscle cells and red blood cells, type B is expressed in liver cells, and type C is mainly found in neuronal cells. In serum samples of patients with hepatocellular carcinoma or stomach cancer, increased levels of the aldolase isoenzyme type A are described. In addition, preliminary results showed that the quantification of aldolase type A mRNA may allow discrimination between hepatocellular carcinoma and liver cirrhosis (Castaldo et al. 2000). However, neither strategy has been used in clinical routine in the following years, and they do not play a relevant role in cancer management at present.

8.3 Enolase

Enolase (EC 4.2.1.11), which catalyses the conversion of glycerate 2-P to phosphoenolpyruvate, consists of two subunits (Fig. 8.1). These subunits are encoded by three different genes and are termed enolase subunit type α, type β and type γ. The combination of the different subunits results in different isoforms of enolase. The isoform that consists of two α subunits is called non-neuronal enolase, while the enolase isoenzymes type αγ and γγ are termed neuron-specific enolase (NSE). NSE is normally found in neuronal cells of the central, as well as the peripheral, nervous system. Furthermore, it is present in neuroendocrine tissues, e.g. within APUD cells. The measurement of plasma NSE levels has been established in clinical practice years ago as a marker of neurologic outcome after cardiac arrest (Shinozaki et al. 2009).

In addition, NSE was found to be elevated in small cell lung cancer. The quantification of NSE in serum became a valuable standard marker in the treatment and follow-up of small cell lung cancer. The application of NSE quantification in serum has also been discussed in the context of neuroblastoma, melanoma and seminoma (Cooper 1994). In some cases of colorectal cancer and liver cancer, the plasma levels were reduced (Paus and Myklebust 1996). However, there is no role for enolase measurements in CRC.

8.4 Pyruvate Kinase (M2-PK, PKM2)

Pyruvate kinase (EC 2.7.1.40) is a key enzyme within glycolysis which catalyses the ATP-producing conversion of phosphoenolpyruvate (PEP) to pyruvate (Fig. 8.1). Specific isoenzymes of pyruvate kinase are expressed in different tissues according to their metabolic function (type L, M1, M2 and R). In normal cells, these isoenzymes consist of four subunits. In tumour formation, the tissue-specific isoenzymes disappear, and the pyruvate kinase isoenzyme type M2 (M2-PK, PKM2) is expressed (Mazurek 2011). M2-PK may occur in a highly active tetrameric form, which is responsible for glycolytic energy production, as well as in a dimeric form with a low activity which favours the channelling of glucose carbons into synthetic processes (i.e. nucleic acids, amino acids and fatty acids) (Fig. 8.2). In tumours, direct interaction with different oncoproteins, including pp60v-src-kinase, oncogenic fibroblast growth factor 1 and human papilloma virus 16 E7, induces a dimerisation of M2-PK. Consequently, in tumours the dimeric form is predominant (Mazurek 2011). Tumours may release the dimeric form of M2-PK into the blood (Wechsel et al. 1999; Lüftner et al. 2000; Schneider et al. 2000; Kaura et al. 2004; Ugurel et al. 2005; Ahmed et al. 2007; Kumar et al. 2007; Hardt et al. 2000); additionally, dimeric M2-PK excreted by mucosal tumours can also be detected in bile (Dhar et al. 2013), faeces (Mazurek et al. 2000; Moreadith and Lehninger 1984) and urine (Hardt, unpublished data).

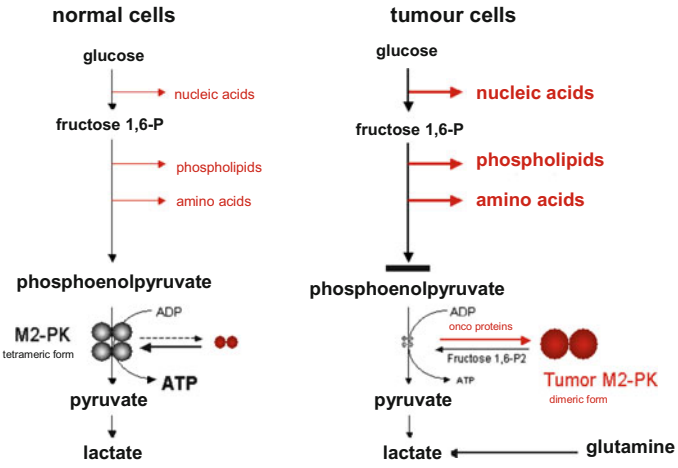

Fig. 8.2 M2-PK in normal cells and tumour cells [modified from Mazurek (2011)]

An enzyme-linked immunosorbent assay (ELISA) which allows the specific quantification of the dimeric form of M2-PK was developed several years ago (ScheBo Biotech AG, Giessen, Germany). The initial studies concentrated on the plasma levels of M2-PK and since 1997 more than 40 studies have been published for this application. These studies include melanoma, thyroid cancer, breast cancer, lung cancer, kidney cancer, oesophageal cancer, gastric cancer, pancreatic cancer, colorectal cancer (CRC), ovarian cancer, cervical cancer and renal cell cancer. In all these cancers, a significant increase in M2-PK blood levels has been shown, frequently in correlation with tumour staging (Wechsel et al. 1999; Lüftner et al. 2000; Schneider et al. 2000; Kaura et al. 2004; Ugurel et al. 2005; Ahmed et al. 2007; Kumar et al. 2007; Hardt et al. 2000). The quantification of M2-PK in EDTA-plasma has been proposed as a relevant follow-up tool for various cancer therapies (Wechsel et al. 1999; Schneider et al. 2000). The use of the plasma M2-PK test as a general screening marker is not recommended since plasma M2-PK levels may also increase in cases of severe inflammation (Hardt et al. 2000). Measurement of M2-PK in bile revealed a sensitivity of 90.3 % for biliary tract cancer with a specificity of 84.3 U/ml using a cut-off value of 24.4 U/ml (Dhar et al. 2013).

Very recently, there have been two new studies that suggested M2-PK measurement in serum as a primary screening tool for CRC screening (Demir et al. 2013, Meng et al. 2012). Despite the fact that measurements in serum have a lower specificity when compared to EDTA-plasma (Oremek et al. 2003), the papers deserve some attention as the concept of measurement in blood samples might be of interest as a screening concept in certain regions of the world. One study carried out in a limited number of CRC patients, adenoma patients and controls reported a very good overall performance and suggested that the plasma test might be suitable for mass screening (Meng et al. 2012). If population-based prospective studies

would reproduce these results, M2-PK measurement in the plasma might serve as a tool for mass screening in populations or countries were colonoscopy is not easily available. Potentially greater patient acceptance of a blood test (rather than a stool test) might also be worthy of consideration to increase participation rates in some screening programmes.

In CRC and adenoma, M2-PK is also released into the patients' faeces (Hardt et al. 2003). A sandwich ELISA based upon two monoclonal antibodies, which specifically recognise the dimeric form of M2-PK, is commercially available for the quantification of M2-PK in stool. More recently, a point of care rapid test based on the same specific antibodies has also been developed by the same company. The faecal M2-PK test has now been evaluated for CRC screening in more than fifteen different studies. A meta-analysis of seventeen published studies on this topic was published by Tonus et al. in 2012 (Tonus et al. 2012). The authors re-analysed data from 704 CRC patients and 11,407 healthy controls. The mean sensitivity and specificity of faecal M2-PK was 80.3 % and 95.2 %, respectively. In head-to-head comparison with the guaiac-based faecal occult blood test (gFOBT) (four studies), the mean sensitivity of faecal M2-PK was 81.1 % compared to 36.9 % for the gFOBT. The sensitivity of faecal M2-PK for adenoma depends on the tumour size (eight studies): adenoma <1 cm in diameter, 25 %; adenoma >1 cm, 44 %; and adenoma of unspecified diameter, 51 %. The authors conclude that faecal M2-PK should be recommended as a routine test for CRC screening because it detects bleeding as well as non-bleeding tumours and adenoma with high sensitivity and specificity, whereas tests for the detection of blood (gFOBT and iFOBT/FIT) are restricted to detecting bleeding lesions.

8.5 Lactate Dehydrogenase

Lactate dehydrogenase (LDH) (EC 1.1.1.27) catalyses the reduction of pyruvate to lactate (Fig. 8.1). The tetrameric protein may consist of the following subunits: type H (heart muscle type, also termed type B), type M (skeletal muscle type, also termed type A) and type C (exclusively expressed in the testes). Depending upon the oxygen supply and metabolic functions of the tissues, different hybrid forms appear: isoenzyme 1, H4; isoenzyme 2, MH3; isoenzyme 3, M2H2; and isoenzyme 4, M4. In tumours, an increased expression of the M type is described, which is characterised by a high affinity to pyruvate and an optimal adaption for survival in hypoxic conditions.

The measurement of LDH in serum serves as an indicator of cell damage and is widely used in clinical routine. In cases of increased serum LDH levels, an analysis of the different isoforms allows the localisation of the cell damage: LDH-1 and LDH-2 are elevated in myocardial infarction or haemolysis. Damage to liver cells results in an elevation of LDH-5.

Besides being established as a marker of cell damage, serum LDH has been studied in breast cancer, myeloma, melanoma, adenocarcinoma of the lung,

testicular cancer and lymphoma. Especially for lymphoma, serum LDH became an important part of the "IPI score" (international prognostic score) that is used in classification and patient follow-up. However, serum LDH does not have a relevant role in screening or differential diagnosis of cancer entities because of its very low specificity.

8.6 Malic Enzyme

NADP-dependent malic enzyme (EC 1.1.1.40) is a glutaminolytic enzyme involved in the degradation of glutamine to lactate. It has been shown to be over-expressed in human colon tumours when compared to normal colon tissue (Mazurek et al. 2000), and it has also been purified from breast cancer cells (Moreadith and Lehninger 1984). Along with NADP-dependent isocitrate dehydrogenase, which is also upregulated in cancer cells, it plays an important role in the detoxification of drugs used in cancer therapy, and it appears to be involved in the prevention of apoptosis in tumour cells [21]. Malic enzyme might therefore be of interest not only as a classical tumour marker but also as a parameter to help guide personalised cancer chemotherapy. However, the determination of malic enzyme has not yet gained a role in clinical routine.

Conclusion
A number of relevant laboratory tests for the screening and/or follow-up of cancer patients have been derived from metabolic research. Some of the most promising markers originate from enzymes involved in the glycolytic pathway. In cancer patients, specific enzyme isoforms may be elevated in samples of blood, bile or faeces and may serve as laboratory markers in clinical routine practice. LDH was established decades ago in clinical practice as a marker of cellular damage and tumour burden. Enolase has been used in the follow-up of small cell lung cancer. M2-PK in plasma can be applied for follow-up studies, and it might play a role in future screening concepts. Faecal M2-PK has proven a very good performance in CRC screening when compared to established alternatives. In future, more specific studies will show whether metabolic markers in body fluids and stool may serve as a basis for individualised tumour therapeutic approaches.

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Chapter 9
Contribution of pH Alterations to the Tumor Microenvironment

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Contents

9.1 Introduction

Deregulated pH in tumor cells and in the tumor microenvironment is an established feature of most cancers, a common characteristic shared by solid tumors with different genetic signatures and different tissue origins. Generally, the intracellular

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pH (pHi) in normal differentiated adult cells is about 7.2, so slightly lower than the extracellular pH (pHe) of body fluids and tissues which is 7.4. In contrast, cancer cells are characterized by a reversed pHi–pHe gradient since the pHi is higher than pHe, with pHi values ranging from 7.2 to 7.7 and pHe values ranging from 6.0–6.2 to 6.8–7.1 (Gillies et al. 2002; Gallagher et al. 2008; Chiche et al. 2010). The intracellular alkalization of tumor cells is triggered by the overexpression and/or the increased activity of membrane-bound ion pumps and transporters able to enhance H^+ removal from cytoplasm: a process that, overall, has to be extraordinarily efficient considering that high proliferative and metabolic rates generate increased metabolic acids. The maintenance of a more alkaline pHi is important for supporting cellular growth (Pouysségur et al. 1985; Webb et al. 2011) and increasing resistance to apoptosis (Matsuyama et al. 2000; Lagadic-Gossmann et al. 2004); moreover, controlled and spatially organized regulation of pHi and pHe is essential for efficient cell migration and invasive growth (Stock and Schwab 2009; Webb et al. 2011). The acidic pH of the interstitial space is maintained by a combination of causes: high proliferative rate, increased efflux of intracellular H^+, and low perfusion, leading to both oxygen shortage and limited capacity to disperse the acidic byproducts of metabolism. Cancer entrenchment and progression is overall greatly influenced by the decreased pHe of tumor tissues, due to its impact on all the cellular components of the microenvironment (i.e., cancer cells, immune cells, endothelial cells, cancer-associated fibroblasts, mesenchymal stem cells, extracellular matrix, secreted factors).

In this chapter, we will discuss what causes tumor acidosis and the main molecular players related to pH regulation in cancer. The biological effects triggered by low pH will be outlined in the context of the complexity of tumor microenvironment.

9.2 Metabolic Sources of Acidity in Tumors

The phenomenon most commonly associated with the onset of acidosis during cancer progression is hypoxia due to its impact on energy metabolism (Gatenby and Gillies 2004; Chiche et al. 2010): the switch to a glycolytic O_2-independent production of energy leads to the final formation of high amount of lactate and protons (see below). A significant part of the metabolic reprogramming that cells undergo to cope with oxygen shortage is mediated, at the molecular level, by hypoxia-inducible factor 1 (HIF-1) (Semenza 2013). Moreover, as observed by Otto Warburg for the first time over 80 years ago, cancer cells produce excessive lactate also in the presence of oxygen, a state termed “aerobic glycolysis” or “Warburg effect” (Warburg 1956; Vander Heiden et al. 2009). Highly proliferating normal cells also show such enhancement in the non-oxidative/glycolytic energy production, irrespective of oxygen tension and despite the much lower efficiency in terms of ATP produced per single molecule of glucose consumed (Newsholme et al. 1985). High glycolytic flux, together with increased glutamine metabolism, provides an effective strategy to direct available nutrients into the synthesis of new

biomolecules (Newsholme et al. 1985; Helmlinger et al. 2002). The advantage of this phenotype in relation to a high rate of cell division is supported by its conservation through evolution (Vander Heiden et al. 2009). Conversely, the oxidative metabolism represents the pathway of choice in nonproliferating/differentiated tissues and in conditions of nutrient limitation (Vander Heiden et al. 2009). Besides HIF-1, important oncogenes like c-Myc, AKT, RAS, and p53 have key roles in the metabolic reprogramming that confer on cancer cells the crucial plasticity needed to survive and proliferate in a hypoxic, harsh, and quickly changing microenvironment (Fan et al. 2010; Chen and Russo 2012). Independently of the mechanisms and the advantage of upregulated glycolysis, the "lactic acid" produced by hypoxic/high glycolytic cells is widely assumed to be the major cause of acidosis in the tumor microenvironment (Chiche et al. 2010). The involved glucose metabolism steps will be briefly summarized below.

In normoxic conditions, the cytosolic glycolytic pathway transforms glucose into pyruvate. None of the glycolytic enzymatic reactions requires oxygen. The global equation can be written as follows:

$$\begin{aligned}&\text{Glucose} + 2\text{ADP}^{3-} + 2\text{HPO}_4{}^{2-} + 2\text{NAD}^+ \\ &\quad \rightarrow 2\text{Pyruvate}^- + 2\text{ATP}^{4-} + 2\text{NADH} + 2\text{H}^+ + 2\text{H}_2\text{O}\end{aligned} \tag{9.1}$$

Pyruvate fuels the mitochondrial process of the tricarboxylic acid cycle (TCA cycle or Krebs cycle) that oxidizes pyruvate to CO_2 and water through the reduction of NAD^+ and FAD coenzymes. These reduced coenzymes will be re-oxidized in the coupled mitochondrial process of oxidative phosphorylation (OXPHOS) through an electron transfer chain (or respiratory chain) that requires O_2 as final electron acceptor, eventually producing water. The proton gradient that the respiratory chain creates in the intermembrane mitochondrial space during OXPHOS is exploited by the ATP synthase to produce ATP molecules.

In hypoxic conditions, the mitochondrial oxidative process is impaired or strongly decreased, with a consequent accumulation of pyruvate from glycolysis. Nonetheless, the glycolytic pathway is upregulated in order to supply the cell with the required ATP, which cannot be provided by OXPHOS. Pyruvate accumulates and, instead of entering the TCA cycle, is reduced to lactate by the enzyme lactate dehydrogenase (LDH), which entails the oxidation of NADH to NAD^+ that allows glycolysis to be refueled:

$$2\text{pyruvate}^- + 2\text{NADH} + 2\text{H}^+ \text{ ----- } \overset{\text{LDH}}{\text{----->}} 2\text{lactate}^- + 2\text{NAD}^+ \tag{9.2}$$

Lactate can be removed from the cell through (a class of proteins called) monocarboxylate transporters (MCTs, see later). This transport is carried out in a coordinate manner with a proton. Such cotransport of $1H^+$ per lactate molecule that crosses the plasma membrane has led to the wide use of the term lactic acid. The net effect of the lactate removal is the concurrent removal of one proton, which will eventually contribute to tissue acidification.

What we have described so far is the traditional interpretation of acidosis in the tumor microenvironment, where the upregulation of the glycolytic pathway (to different extents due to hypoxic conditions or to the Warburg effect) leads to a high LDH activity and considerable export of lactic acid into the extracellular milieu. Hence, a more pronounced glycolytic phenotype is what is usually referred to as the source of acidity.

However, we would like to question this concept and show that metabolic acids are produced abundantly by "highly proliferating" cells and that glycolytically produced "lactic acid" is not the only source of acidosis in tumors. The two moles of glycolysis-derived H^+ [see (9.1)], when not fully consumed in the LDH reaction, are usually imported into mitochondria and/or in general do not represent a challenge for the buffering capacity of a cell. The stoichiometry of the LDH enzymatic reaction (9.2) clearly shows that protons are consumed (in the same amount that is produced through the glycolytic pathway) and that lactic acid is never produced as an acid inside the cell. In fact, the reaction catalyzed by LDH does not affect the carboxylic group of pyruvate (always deprotonated itself) but only the keto group –C=O in position two, which is reduced to a –CHOH (Quaytman and Schwartz 2007). Moreover, the pKa value of lactic acid is 3.87 at 37 °C, so even at a physiologically "extreme" pH 6.0, the acid form would represent less than 1 % of the total (Lane et al. 2009). The co-removal via MCTs of one H^+ with one lactate molecule from the cytoplasm does contribute to extracellular acidification, but it does not imply that the source of acidity is intracellularly produced lactic acid or that the "proton-producing pathway" has to be strictly glycolysis. While lactate overproduction is clearly a direct indication that glycolytic flux is enhanced and that pyruvate is transformed into lactate, the classical biochemical equations as reported above would question the "message" that, in the presence of a high LDH activity, the majority of protons responsible for acidification of the extracellular compartment come from glycolysis (Robergs et al. 2004).

Then, where do the protons come from? In our opinion, they are produced inside the cell as a result of the "normal" multiple metabolic processes, not only glycolysis, ending with CO_2 production. Due also to the presence of intracellular isoforms of carbonic anhydrases (Supuran 2008), CO_2 contributes to the shift of the equilibrium of the main buffering system according to the reaction $CO_2 + H_2O \rightarrow HCO_3^- + H^+$ (a spontaneous reaction that would occur, at a slower rate, also without any enzymatic catalysis). CO_2 is overall the final product of human metabolism, so it is reasonable to think that in high-proliferating cancer cells, protons arise from such "physiological," though enhanced and deregulated, metabolism. Assuming a proportional expression of MCTs to export lactate, a higher or lower Warburg phenotype would mainly affect the overall efficiency of H^+ removal from the cytosol, not the H^+ formation. In this perspective, the reaction (9.2) would be beneficial not only for the restoration of NAD^+ equivalents immediately available to the upstream glycolytic enzymes but also for the clearance of metabolically produced H^+. At the tumor site, however, when the mass has grown and vascularization is scarce and malfunctioning, the decreased O_2 delivery also implies an inefficient removal of the locally produced CO_2 and H^+, which normally

would be buffered in the bloodstream. Thus, both the higher activity of proton extruders and CA isoforms expressed in cancer cells (see below) and the lack of efficient removal of metabolic acids from the tumor tissue contribute to a more acidic pHe in comparison to normal tissues.

9.3 Role of Glycolysis and Oxidative Phosphorylation In Vivo

In vivo investigations aimed at understanding acidosis within solid tumors have not focused on the "lactic acid" interpretation, which, as such (and to the best of our knowledge), has not been extensively challenged. It is not trivial to experimentally distinguish the fact that the tumor microenvironment is acidic and with a high concentration of lactate from the fact that "lactic acid" produced via glycolysis is not responsible for the development of tumor acidity, especially if expression of lactate transporters and other proton transporters is not closely regulated and comparable. In this perspective, CCL39 cells (*ras*-transfected Chinese hamster lung fibroblasts) deficient in glucose transport and phosphoglucose isomerase have been used to analyze changes in tumor pHe in nude mice in comparison to parental cells (Newell et al. 1993; Chiche et al. 2012). Tumors derived from parental and glycolysis-deficient cells showed minimal differences in growth rate, no differences in the measured pHe, and no correlation between "lactic acid" content and acidosis. In a study with *ras*-transfected CHO (Chinese hamster ovarian) cells, an LDH-deficient subline was found to give rise to tumors with mean pHe values comparable to those measured in tumors originated from the parental cell line (and in both cases lower than that of normal tissue) (Yamagata et al. 1998). With the same approach, a CCL39 model was used to show that CO_2 is a significant factor contributing to acidification in the glycolysis-impaired tumors (Helmlinger et al. 2002). These results show that the glycolysis-produced "lactic acid" interpretation does not explain tumor acidosis. Nonetheless, such experimental observations, despite the limitation of the model used, challenge an assumption too often taken for granted. Thus, it becomes reasonable to question whether other murine or human models would show the same behavior. For instance, a study carried out on rat gliomas (Provent et al. 2007) also pointed out that lactate distribution does not match the H^+ distribution in vivo. Generally, proton extrusion seems to represent the most important factor regulating acidosis to an extent far more significant than glycolytic phenotype per se.

The moment it was established that lactate is produced in normal skeletal muscle cells also in "resting conditions" and under normal values of O_2 tension (Miller et al. 2002; Brooks 2009), a new way of thinking about lactate started. Nowadays, lactate is widely considered not simply an unwanted byproduct of anaerobiosis but an important player as a both metabolic fuel and signaling molecule. This holds true in different areas of physiology (brain, cardiac and skeletal muscle, and liver) and is

strongly emerging also in the context of cancer (Passarella et al. 2008; Dhup et al. 2012). The value of lactate as biomarker associated with high risk of metastasis and tumor recurrence and with chemo- and radioresistance has been well known for many years (Walenta et al. 2000; Brizel et al. 2001; Quennet et al. 2006). Moreover, exogenous lactate was demonstrated to affect the immune response at the tumor site (Fischer et al. 2007; Husain et al. 2013) and cellular motility (Walenta and Mueller-Klieser 2004) and to stimulate revascularization (Hunt et al. 2007).

More importantly, new aspects of this ongoing "lactate paradigm shift" have been recently proposed by two independent groups (Dhup et al. 2012; Pavlides et al. 2012). The work from Sonveaux's lab supports the idea of a "cooperative model" between glycolytic and oxidative tumor cells (Sonveaux et al. 2008). This is a model in which oxidative and glycolytic tumor cells are "metabolic symbionts," and the glucose consumption of a subset of glycolytic cancer cells produces the lactate that becomes the fuel for another subset of oxidative tumor cells. Lisanti's lab, on the other hand, identifies lactate as a mediator of the so-called reverse Warburg effect (Pavlides et al. 2009). According to this model, the tumor-associated fibroblasts of the stroma are highly glycolytic, and their production of metabolites including lactate represents the fuel for the oxidative cancer cells.

An emerging aspect of the importance of lactate in cancer metabolism concerns the compelling evidence that mitochondria have a role in lactate metabolism due to the existence of a mitochondrial form of LDH (mLDH). Included in the mitochondrial proteome database MitoCarta (Pagliarini et al. 2008), mLDH has been proven to exist in a variety of mammalian mitochondria and recently also in cancer cell lines from different tissues (De Bari et al. 2010; Hussien and Brooks 2011; Pizzuto et al. 2012). Briefly, it was shown that lactate was entering (in a carrier-mediated fashion) mitochondria of both normal prostate cells (PNT1A) and tumor cells (PC3). The reports provided evidence for mLDH localization in the inner membrane and for specific kinetic features compared to the cytoplasmic isoenzymes and, furthermore, also for different activity in normal cells and cancer cells. More recently, the presence of mLDH in HepG2 cells was confirmed (by immunoblotting, confocal microscopy, and enzymatic assays) and the inner mitochondrial membrane localization confirmed as well (Pizzuto et al. 2012). However, other studies have questioned the existence of mLDH (Gladden 2004) and the debate will likely continue. The data on a direct movement of lactate into mitochondria are fairly in line with the "intracellular lactate shuttle hypothesis" (ILS) proposed for skeletal muscle cells (Brooks 1998). ILS posits that lactate produced as the result of glycolysis and glycogenolysis in the cytosol is balanced by oxidation in mitochondria of the same cell (Brooks et al. 1999).

All these studies clearly need to be expanded to different models, considering the variability of cancer types, so to test the existence of a "general" mitochondrial lactate metabolism and a lactate-mediated cooperation between tumor cells and between tumor cells and stromal cells. Such evidence would open up new scenarios to the understanding of energy metabolism especially in the context of the tumor "Warburg phenotype."

9.4 Regulation of pH in Tumor Cells

Because of the importance of pH in cellular processes like protein structure and function, enzymatic activities, cell signaling, and intracellular organelle function and traffic, cells have developed several systems to finely regulate intracellular pH in response to changes in pHe or intracellular accumulation of acidic metabolites. The pHe in blood and tissues under physiological conditions is maintained around 7.3–7.4, while pHi is slightly more acidic being in the range 7.1–7.3. The more acidic pHi relies on passive transport of protons through the plasma membrane (negative electrical potential inside) and the production of acid equivalents through glycolysis, oxidative phosphorylation, glutaminolysis, and ATP hydrolysis. In physiological conditions, the pH buffering capacity of cells depends on the concentration of weak acids or bases and the spontaneous hydration of CO_2 (physicochemical buffers) (Roos 1978; Roos and Boron 1981). However, adaptation or response to prolonged stress affecting pHi homeostasis requires more advanced and diversified pH-regulating cellular systems. We describe below the role of such systems and their function and contribution to tumor pH modulation and disease progression.

9.4.1 Carbonic Anhydrases

Carbonic anhydrases (CA) belong to a family of metalloenzymes that reversibly catalyze the hydration of carbon dioxide into bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), thus mediating acid-base balance in the cells and tissues. The 16 mammalian α-CA isoenzymes show different subcellular localization and tissue distribution. Since the CA system is involved in homeostatic regulation of pH and carbon dioxide, it has fundamental implications in tissue physiology and in several pathological conditions (Supuran 2008). The expression of the transmembrane isoenzyme CA9 is increased in many tumors and functions as marker of disease progression and response to therapy (Generali et al. 2006; Tan et al. 2009; Zheng et al. 2010). CA9 is normally expressed in the stomach but its expression is very strongly upregulated by HIF-1 in many types of tumors, and it is also constitutively expressed in tumors defective for the von Hippel-Lindau (VHL) protein, such as renal cell carcinoma. CA12 is also localized on the plasma membrane and overexpressed in tumors. CA9 and CA12 are crucial proteins regulating pHi and display a fundamental role in extracellular acidification of tumor tissues, contributing to confer a survival advantage to cancer cells exposed to hypoxic and acidic environment (Wykoff et al. 2000; Hussain et al. 2007; Chiche et al. 2009).

9.4.2 MCT Transporters

The monocarboxylate transporters (MCTs) more relevant for the topic of pH regulation are the MCT1–4 of the SLC16A family. They catalyze the H^+-linked cotransport of monocarboxylates such as lactate, pyruvate, and ketone bodies across the plasma membrane. Of the naturally occurring MCT substrates (lactate, pyruvate, hydroxybutyrate, and acetoacetate), lactate is quantitatively the most important. Among the four members of the group, MCT2 has the highest affinity for most of the substrates (Halestrap 2013) but it has a low expression level in most human tissues. MCT4 has a lower affinity for monocarboxylate species (Dimmer et al. 2000) and is strongly expressed only in glycolytic tissues that must export large amounts of lactate and protons. MCT3, less well characterized, is mainly expressed in the retinal pigment epithelium. Conversely, MCT1 is almost ubiquitously expressed (Halestrap and Meredith 2004). MCT1 and MCT4 in particular have an increased expression and/or activity in cancer. The preferential overexpression of MCT4 in malignant cells is a characteristic shared with activated lymphocytes, astrocytes, and white muscle fibers that are highly dependent on glycolysis and need to handle large amounts of lactate (Halestrap 2012). It is well established that HIF-1 regulates the expression of MCT4 but not of MCT1 (Ullah et al. 2006).

MCT1 and MCT4 have been considered markers of poor prognosis in colorectal cancer (Nakayama et al. 2012), and they have been correlated to invasiveness and aggressiveness of different types of malignancies (Pinheiro et al. 2010a; Izumi et al. 2011; Miranda-Gonçalves et al. 2013); moreover, MCT4 expression in stromal cells predicts poor outcome in triple-negative breast cancer (Witkiewicz et al. 2012). In a study on melanoma, MCT1 and MCT4 were shown to increase in expression during progression from nevi to advanced melanoma (Ho et al. 2012).

A further interesting question to be addressed about MCTs is related to the previously described ILS hypothesis. Originally proposed in skeletal muscle cells (Brooks 1998), the possible role of MCTs in the mitochondrial translocation of lactate in cancer cells is an issue that needs to be further investigated. In a recent study, MCT2 and MCT4 localization in mitochondria (as well as on the plasma membrane) has been observed in MCF7 and MDA-MB-231 breast carcinoma cell lines (Hussien and Brooks 2011).

The "spatial organization" of proton efflux and consequently of MCTs (together with the other H^+ transporters) is another active area of investigation whose findings are expected to have profound implications for the understanding of cell migration, invasiveness, and the overall development of a metastatic malignancy (Cardone et al. 2005; Grillon et al. 2011).

Lastly, the association of MCT with chaperone proteins in order to obtain a fully active efflux/influx of substrates is now well established. CD147 (or basigin) association with MCT1 and MCT4 is better characterized and many recent reports are available (Muramatsu and Miyauchi 2003; Hashimoto et al. 2006; Gallagher et al. 2007; Le Floch et al. 2011; Walters et al. 2013). Some reports also include

CD44 (or hyaluronan receptor) as a chaperone protein associated to MCT (Slomiany et al. 2009; Pinheiro et al. 2010b).

9.4.3 Sodium–Hydrogen Exchanger

Sodium–hydrogen exchanger (NHE-1) is a transmembrane protein with ubiquitous expression and which exchanges intracellular protons for extracellular sodium. At physiological pH, NHE-1 is quiescent but becomes activated during cytosolic acidification via increased affinity of the intracellular allosteric proton-binding site (Cardone et al. 2005). During mitogenic stimulation and oncogenic transformation, the protein is hyperactive and contributes to extracellular acidification and maintenance of a more alkaline pHi, normally conducive for growth. NHE-1 expression is often polarized in the leading edge of invadopodia providing localized pH regulation: increased pHi triggers changes in the cytoskeleton aiding cell extension and migration, while acidification of pHe facilitates ECM degradation and local invasion (Stock and Schwab 2009). NHE-1-dependent cellular alkalinization during neoplastic transformation was reported to drive aerobic glycolysis, proliferation, and serum- and anchorage-independent growth (Reshkin et al. 2000).

9.4.4 Vacuolar-ATPase

Vacuolar-ATPase (V-ATPase) is a transmembrane proton ATPase expressed in vesicles from the endo-lysosomal compartment and on the plasma membrane of osteoclasts and renal intercalated cells (Nishi and Forgac 2002; Forgac 2007). ATP hydrolysis is mediated by the catalytic complex (V1) facing the cytosolic site, while proton translocation occurs via the transmembrane complex (V0), providing intraluminal acidification. The activity and expression of V-ATPase are important for regulation of intracellular vesicular functions and traffic, both in normal and tumor cells (Nishi and Forgac 2002; Marshansky and Futai 2008). V-ATPase can be functionally expressed on the plasma membrane of human tumor cells where they provide protons extrusion leading to extracellular acidification, thus contributing to tumor invasion and growth (Martinez-Zaguilan et al. 1993, 1999; Hinton et al. 2009; Avnet et al. 2013). Furthermore, V-ATPase has been involved in the acquisition of the multidrug resistance phenotype, and molecular or pharmacological inhibition of the V-ATPases may reverse resistance to chemotherapy (Raghunand et al. 1999b; You et al. 2009; Fais 2010; Fan et al. 2013) and retard tumor growth in vivo (Lu et al. 2005; Xu et al. 2012a). This protein is also a crucial regulator of autophagy since by providing lysosomal acidification, it regulates the degradation rate of the autophagic cargo (Codogno and Meijer 2005; Meijer and Codogno 2009).

Additional systems like the HCO_3- transporters, the anion exchangers, and the aquaporins may have a role in pH regulation in normal cells, but their contribution to regulation of tumor cell pHi has not yet been fully established (Parks et al. 2011).

9.5 Tumor-Supportive Effects of Altered pH

The effects of acidosis on several aspects of tumor biology have been investigated since the early 1980s. An acidic microenvironment is a common feature of solid and some hematological tumors (Vaupel et al. 1989; Gillies et al. 1994; Mortensen et al. 1998). The acidic environment of tumors contributes to several features of malignancy which include migration and invasive capacity, angiogenesis, resistance to cell death, anchorage-independent growth, genetic instability, and immune escape (Morita et al. 1992; Yuan et al. 2000; Orive et al. 2003; Gatenby and Gillies 2008; Calcinotto et al. 2012). An interesting and growing research area focuses on identifying pH sensors that mediate the response and the metabolic adaptation of cancer cells to acidic conditions. Some interest was generated by the finding that G-protein-coupled receptors (GPR) are involved in pH homeostasis by acting as proton sensors and may be important for promoting tumor growth (Ludwig et al. 2003; Ihara et al. 2010; Ryder et al. 2012). We will now discuss several biological processes involved in malignant progression and regulated by tumor pH alterations (Fig. 9.1).

9.5.1 Invasion/Migration

Invasion of the extracellular matrix (ECM) is the first step of the complex metastatic process. Among the factors regulating tumor cell migration and invasion are integrins and ECM-degrading proteolytic enzymes like cathepsins, matrix metalloproteases (MMPs), and serine proteases (Mohamed and Sloane 2006; Friedl and Wolf 2009). The acidic extracellular environment actively promoted by tumor cells significantly contributes to degradation and remodelling of the ECM (Gatenby et al. 2006). Acidic pH stimulates the secretion and activity of several proteases like cathepsins B, L, and D and MMP-2 and MMP-9 (Kato et al. 1992; Bourguignon et al. 2004; Lu et al. 2005; Rofstad et al. 2006; Giusti et al. 2008). As a consequence, tumor cells exposed to acidic conditions acquire a more aggressive phenotype characterized by increased migratory and invasive capacity in vitro (Martinez-Zaguilan et al. 1996; Lu et al. 2005; Rofstad et al. 2006; Moellering et al. 2008) and increased metastatic behavior in vivo (Rofstad et al. 2006; Moellering et al. 2008). The acid-mediated invasion hypothesis has also been supported by observations made in a model of colon carcinoma, indicating that peritumoral regions characterized by an acidic pH represented also sites of local invasion (Estrella et al. 2013). Notably, tumor acidosis has often been considered to be a direct consequence of the glycolytic switch promoted by HIF-1α, leading to the assumption that hypoxic

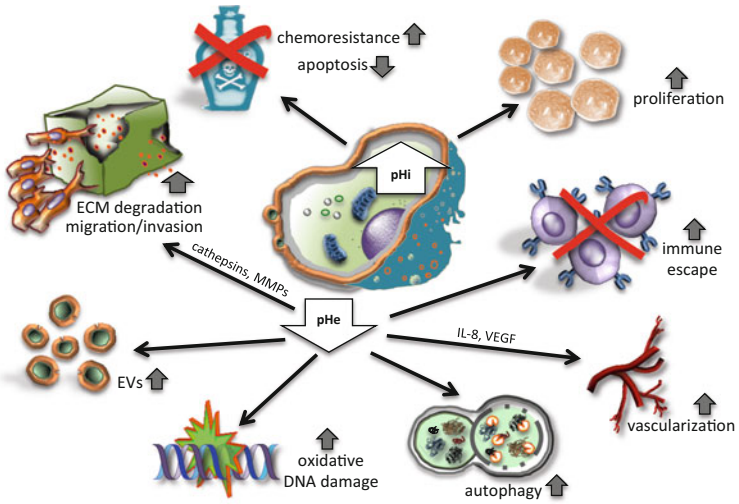

Fig. 9.1 The reported tumor-supportive effects of disrupted proton dynamics. References for the described mechanisms are reported within the text

areas and acidic areas are colocalized within a tumor. Although a high glycolytic rate is considered as a major source of acidity, other metabolic pathways (like glutaminolysis and the TCA cycle) contribute to proton production and may explain why the distribution of oxygen pressure and pH in vivo is not always correlated (Helmlinger et al. 1997, 2002; Yamagata et al. 1998; Provent et al. 2007).

As mentioned above, several pH-regulating proteins are upregulated in tumors exposed to acidosis and control the pH-dependent invasive phenotype. The most important proteins mediating such effect are the NHE-1 (Stock et al. 2005; Stock and Schwab 2009; Lee et al. 2010; Steffan et al. 2010), the V-ATPase (Chung et al. 2011; Wiedmann et al. 2012; Xu et al. 2012b; Hendrix et al. 2013), and the CA-IX (Svastova et al. 2012). Interestingly, pseudopodia of invasive cancer cells were found to be enriched in beta-actin and in glycolytic enzymes, suggesting the close association of a localized acid-producing mechanism with the extracellular acidification provided by NHE-1 or other systems (Nguyen et al. 2000; Beckner et al. 2005; Brisson et al. 2012).

Besides the role in ECM degradation, an acidic environment also stimulates angiogenesis by inducing the release of pro-angiogenic factors (VEGF and IL-8), thus contributing to tumor cell dissemination (Xu and Fidler 2000; Fukumura et al. 2001; Xu et al. 2002; Rofstad et al. 2006; Taraboletti et al. 2006; Giusti et al. 2008). Therefore, inhibition of tumor acidic pHe regulation may represent a tool in limiting dissemination of cancer cells and formation of metastases, either by directly targeting the pH-regulating systems (Fais et al. 2007; Supuran 2008; Neri and Supuran 2011; Porporato et al. 2011) or by chemical buffering of the tumor pH, as also recently suggested by studies using sodium bicarbonate (Robey et al. 2009; Silva et al. 2009).

9.5.2 Resistance to Cell Death

While normal cells are sensitive to acidosis-induced cell death, cancer cells have adapted to the acidic environment and exploit this condition as a mechanism for resistance to various cytotoxic insults including nutrient deprivation, radiotherapy, and chemotherapy (Okada et al. 2001; Reichert et al. 2002; Thews et al. 2006; Parks et al. 2011; De Milito et al. 2012). Different signaling and metabolic pathways contribute to adaptation to acidosis (Parks et al. 2011; Wojtkowiak et al. 2011). For example, lymphoma cells cultured under acidic conditions (pH 6.5) were resistant to apoptosis induced by deprivation of glucose or glutamine, a process mediated by the upregulation of Bcl-2 and the activity of GPR65 (Ryder et al. 2012). As mentioned above, an important consequence of the accumulation of acids in cancer cells is the increased proton efflux through the plasma membrane, which causes a shift in intracellular pH towards more alkaline values. This has been reported for several human tumors in vitro including breast carcinoma (Raghunand et al. 1999a), B- and T-cell leukemia (Rich et al. 2000), and melanoma (Wahl et al. 2002; De Milito et al. 2010) and further confirmed by magnetic resonance imaging (MRI) studies in vivo in several animal models (Gillies et al. 1994, 2002, 2008; De Milito et al. 2010; Hjelmeland et al. 2011).

Changes in the pH gradient between the extracellular environment and the cell cytoplasm and/or in the pH gradient between the cell cytoplasm and the lysosomal compartments are common in many tumors (Simon et al. 1994; Mahoney et al. 2003). Since the entry of drugs into the cell may be dependent on both concentration gradients and pH gradients, the reversed pH gradients of tumors may affect drug distribution, uptake, and activity (Altan et al. 1998; Gerweck 1998; Raghunand and Gillies 2000; De Milito and Fais 2005b; Gerweck et al. 2006; Tredan et al. 2007). Drugs that behave as weak bases (including doxorubicin, mitoxantrone, chloroquine, vincristine, vinblastine) are protonated in the acidic tumor environment, and in their charged form, their membrane permeability is strongly reduced, with decreased cellular uptake as a consequence (Jensen et al. 1994; Raghunand et al. 2003; Tredan et al. 2007; Pellegrini et al. 2014). Similarly, weakly basic drugs crossing the plasma membrane can accumulate within acidic organelles (lysosomes, endosomes, secretory vesicles) (Ouar et al. 1999; Raghunand et al. 1999b; De Milito and Fais 2005a), where they become sequestered and/or secreted. Indeed, alkalinization of tumor pHe and increase of endosomal pH augment the uptake, retention, and cytotoxic activity of several weakly basic chemotherapeutics (Raghunand et al. 1999a, 2003; Luciani et al. 2004; Patel et al. 2013). Acidic pH was also shown to increase P-glycoprotein activity in human and rat prostate carcinoma cells, which may in part contribute to drug resistance in hypoxic/acidic tumor regions (Thews et al. 2006; Sauvant et al. 2008).

Malignant and drug-resistant cancer cell lines are characterized by a slightly more alkaline intracellular pH than drug-sensitive cells (Simon et al. 1994; Belhoussine et al. 1999; Miraglia et al. 2005). Besides the role of alkaline pHi in

determining the fate of weakly basic drugs, such conditions also affect the activity of proapoptotic compounds. An early event during apoptosis induced by several agents is the rapid acidification of intracellular pH (Gottlieb et al. 1996; Goossens et al. 2000; Marches et al. 2001; Nilsson et al. 2006). Acidification of the intracellular environment may also enhance the effect of proapoptotic drugs (Li and Eastman 1995; Park et al. 1996). Drug-induced hydrogen peroxide production was reported to induce intracellular acidification and release of cytochrome C, thereby creating an intracellular environment permissive for caspase activation (Hirpara et al. 2001). It is important to mention that a more alkaline pHi confers cancer cells with an increased proliferative capacity, rendering them more responsive to growth factors (L'Allemain et al. 1984; Paris and Pouyssegur 1984). Moreover, pHi regulation is also important for cell cycle control (Pouysségur et al. 1985; Lagadic-Gossmann et al. 2004; Webb et al. 2011), whereby acidification of pHi may inhibit mTOR and protein synthesis (Pouysségur et al. 1985; Balgi et al. 2011).

9.5.3 Immune Escape

Metabolic acidosis and other forms of clinical acidemia are often associated with immunodeficiency status. Immune cells represent a major cell population within a tumor tissue and include macrophages, dendritic cells, neutrophils, NK cells, B and T lymphocytes. Tumor metabolic adaptation and reprogramming is thought to impact on the antitumor immune response (Lardner 2001; Kareva and Hahnfeldt 2013), with acidity and lactate accumulation being major consequences of the metabolic alterations affecting immune cells within tumors. Moreover, inflammatory sites are normally characterized by local acidosis contributed by anaerobic glycolysis of infiltrated immune cells and by bacterial metabolism. Recently, it was suggested that extracellular acidosis also functions as danger signal for the innate immune system, thus contributing to chronic inflammatory diseases (Rajamaki et al. 2013). Acidic environment was reported to inhibit human lymphokine-activated killer (LAK) cells (Severin et al. 1994), mouse NK cells (Loeffler et al. 1991), and IL-2-stimulated lymphocytes (Loeffler et al. 1992). The activity of mouse cytotoxic T lymphocytes (CTL) is significantly hampered at low pH conditions (Redegeld et al. 1991), and culturing tumor-specific mouse and human T lymphocytes at pH 6.0–6.5 induces anergy (Calcinotto et al. 2012), with impaired cytolytic activity and cytokine secretion and reduced expression of IL-2Ra (CD25) and T-cell receptors (TCR). Normalization of tumor pH using the proton pump inhibitor esomeprazole not only normalized T-cell functions but also increased the efficacy of active and adoptive immunotherapy (Calcinotto et al. 2012). Moreover, tumor-derived lactic acid inhibits proliferation, cytokine production, and cytotoxicity of human T lymphocytes (Fischer et al. 2007) and also inhibits dendritic cell functions (Gottfried et al. 2006). Thus, both acidosis and lactic acidosis are

considered important immune escape mechanisms (Lardner 2001; Bellone et al. 2013; Kareva and Hahnfeldt 2013).

9.5.4 Modulation of Autophagy

The cellular homeostasis pathway known as autophagy (we refer to macroautophagy) normally provides a survival advantage to cells subjected to nutrient limitation, oxygen shortage, or chemotherapy insult (Meijer and Codogno 2009). Autophagy involves the degradation of cytosolic material and organelles via the lysosomal digestion system, and the role of autophagy in cancer is extensively described in a chapter of this book. A sustained autophagy plays a relevant role in cancer progression, and it has been reported that the ability of lactic acidosis to confer resistance to glucose deprivation was associated with elevated autophagy in a model of breast carcinoma (Wu et al. 2012). In line with this, chronic autophagy was observed in breast cancer cell lines adapted to grow in acidic conditions (Wojtkowiak et al. 2012) and also in several human melanoma cell lines exposed to chronic or transient acidic stress (Marino et al. 2012), indicating that autophagy may contribute to cancer cells' adaptation to chronic acidosis. However, inhibition of autophagy by the use of chloroquine is not achieved in low pH-adapted cancer cells because of the low chloroquine uptake in acidic conditions (Pellegrini et al. 2014).

9.5.5 Extracellular Vesicles

Extracellular vesicles (EVs) are different types of membrane vesicles that can be released from both healthy and tumor cells. Exosomes and microvesicles (endosomal and plasma membrane origin, respectively) carry proteins, DNAs, mRNAs, and microRNAs able to affect cell functions in autocrine and paracrine modes, ultimately playing a role also in tumor progression and response to micro-environment stressors (Kharaziha et al. 2012; Kucharzewska and Belting 2013). Interest in their roles in mediating cell–cell communication and tumor progression is on the increase (Al-Nedawi et al. 2009; Hendrix et al. 2010; Kucharzewska and Belting 2013). Interestingly, acidic pH has been identified as a key factor for exosome traffic and release in tumor cells (Parolini et al. 2009). Moreover, in EVs from ovarian cancer cells, low pH has been suggested to trigger VEGF release (Taraboletti et al. 2006) and to enhance their cathepsin B-mediated invasiveness (Giusti et al. 2008). Collectively, these data indicate that the signaling/mediator action exerted by EVs in relation to the acidic tumor microenvironment requires further studies, possibly expanding the investigation to EVs of different origin (i.e., malignant cells and different stromal cells populating the tumor microenvironment).

9.6 Targeting pH Modulation in Cancer Therapy

Targeting the unique reversed pH gradient observed in cancer represents an important and tantalizing strategy in cancer therapy. Although the therapeutic aspects are not the focus of this book, we find it interesting that therapeutic efforts to reduce tumor growth and metastasis by pH modulation are ongoing in preclinical and clinical settings. We refer the interested readers to a series of updated review articles focusing on such approach (Fais 2010; Neri and Supuran 2011; Webb et al. 2011; De Milito et al. 2012; Parks et al. 2013).

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Part III
On the Role of Mitochondria in Tumor Metabolism

Chapter 10
Mitochondrial Mutations in Cancer Progression: Causative, Bystanders, or Modifiers of Tumorigenesis?

Ivana Kurelac, Michele Vidone, Giulia Girolimetti, Claudia Calabrese, and Giuseppe Gasparre

Contents

10.1 Introduction

Mitochondria are semiautonomous organelles in the way that they possess their own small double-stranded circular chromosome, which in humans is on average 16,565 bp long. With a compact structure, the mitochondrial DNA (mtDNA) encodes 13 subunits of the respiratory chain complexes I, III, IV, and V, 22 transfer

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RNAs, and 2 ribosomal RNAs for *in organello* translation. The latter is a necessary process since mtDNA does not follow the universal code for protein translation and codon usage is different from that occurring within the cytosol. The remaining subunits of the respiratory complexes, especially those forming the large complex I holoenzyme, are encoded by nucleus-residing genes and subsequently imported within mitochondria with the aid of chaperones. The mtDNA also contains a 1Kb promoter-like region called displacement loop (D-loop) where replication and transcription starting sites are mapped.

Mitochondria constitute a network that harbors a number of mtDNA molecules ranging from several hundreds to nearly 100,000. Therefore, the mitochondrial genome of an organism is polyploid, which gives rise to the possibility of the coexistence of different genetic variants within a cell or a tissue, a condition known as heteroplasmy. Homoplasmy, on the other hand, is referred to as a genetically homogeneous mtDNA content. As a consequence of mitochondrial polyplasmy, the phenotypic effect of a variant depends on the mutant load. Usually, a certain critical portion of mutated molecules needs to be reached before functional consequences begin to arise, which depends on the type of variant and on the context in which it occurs (Rossignol et al. 2003). Threshold values have been determined for some of the most common mtDNA mutations (Carelli et al. 2002; Laloi-Michelin et al. 2009), but the lack of appropriate and standardized methods for heteroplasmy investigation has held back a complete understanding of the mtDNA genotype–phenotype correlation (Wong and Boles 2005).

The mitochondrial genome is maternally inherited. Oocyte mitochondria exclusively contribute to gamete development, whereas sperm mtDNA is eliminated after fertilization (St John et al. 2010). As a consequence, the human lineage carries mtDNA molecules derived by descent from the same ancestral mitochondrial genome (Torroni et al. 2006). Since mtDNA displays high genetic variability due to an elevated mutagenesis rate (see below), the ancestral mitochondrial chromosome has over time acquired diverse polymorphisms which currently define more than 30 mitochondrial haplogroups (van Oven and Kayser 2009). On the other hand, non-polymorphic mtDNA variants, which cause defects in the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS), are related to mitochondrial diseases, such as Leber's hereditary optic neuropathy (LHON); neuropathy, ataxia, and retinitis pigmentosa (NARP); myoclonic epilepsy with ragged red fibers (MERRF); chronic progressive external ophthalmoplegia (CPEO); and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Wallace 1999). Moreover, with the emerging roles of mitochondria in many vital cellular processes, it is now recognized that OXPHOS damage may also contribute to the development of other pathologies, such as diabetes, neurodegenerative diseases, and cancer (Wallace 2005).

The first large study reporting mtDNA variants in cancer was performed in 1998 on colorectal cancer cell lines (Polyak et al. 1998). Ever since, a plethora of reports have described the occurrence of mtDNA mutations in virtually all cancer types (Brandon et al. 2006; Chatterjee et al. 2006; Yu 2012). Nevertheless, no consensus has been reached to date to standardize approaches for mtDNA mutations

recognition, often resulting in erroneous interpretations (Yao et al. 2009; Liu et al. 2012). A good practice would be to evaluate the actual population frequency of a specific mtDNA mutation through the use of carefully curated and comprehensive databases, such as the Human Mitochondrial Database (HmtDB) (Lascaro et al. 2008; Rubino et al. 2012), when attempting to infer a role in disease. Another recommendable approach to reduce misleading analyses would be to assess if the mtDNA alleles observed in a sample are coherent with the corresponding haplogroup (Salas et al. 2005; Rubino et al. 2012). Furthermore, during mtDNA sequencing, it is important to avoid the co-amplification of nuclear mitochondrial sequences (NumtS), i.e., noncoding mitochondrial pseudogenes abundantly incorporated in the nuclear chromosomes (Hazkani-Covo et al. 2010; Simone et al. 2011; Lang et al. 2012; Petruzzella et al. 2012).

mtDNA mutations in cancer have been initially associated only with highly glycolytic neoplastic cells, which were observed in 1924 by German biochemist Otto Warburg, who suggested such a phenotype to be a consequence of mitochondrial damage (Warburg 1956; Warburg et al. 1924). However, considering the vast spectrum of mitochondrial roles in metabolism, as well as in apoptosis and hypoxic adaptation, it is not surprising that mtDNA mutations may have a greater impact on tumor development and progression (Galluzzi et al. 2010).

A complete functional effect of mtDNA mutations in cancer is difficult to assess, mainly due to the peculiarities of mitochondrial genetics and to the heterogeneous and ever-changing tumor microenvironment. The most widely used approach for analysis of mtDNA functional effects involves the generation of *trans*-mitochondrial hybrids (cybrids), which allow to distinguish a mitochondria-specific contribution from that of the nuclear genome (Moraes et al. 2001). On the other hand, introduction of allotopically expressed mtDNA genes recoded for translation in the cytoplasm is used to investigate the complementation of damaging mtDNA mutations effects (Bonnet et al. 2007). It is important to note that functional studies on mtDNA mutations in molecular oncology ought to be preferentially performed in vivo, in order to take into account the dynamic cancer microenvironment and selective pressures such as aglycemia and hypoxia, conditions that are difficult to reproduce in vitro.

In this review, the central role of the mitochondrion within cancer progression will be revised, with particular focus on the consequences of genetic lesions and functional impairment occurring in oxidative metabolism enzymes, both encoded by the mitochondrial chromosome and by the nucleus. Reference will be made to the pleiotropic effects of diverse mtDNA mutations and to the triggering of the Warburg effect, especially in hypoxic conditions. Finally, an excursus on the peculiarities of oncocytic tumors, a subset of neoplasms well characterized in terms of mitochondrial aberrations, will provide ground for several general considerations on the importance of these organelles in determining a cancer cell fate.

10.2 Sources of mtDNA Mutations in Cancer

It has been estimated that mtDNA has a 10- to 17-fold higher mutation rate than the nuclear DNA (Tuppen et al. 2010), a feature that has been attributed to elevated oxidative damage to which the mitochondrial chromosomes are exposed due to their proximity to the electron transport chain (ETC) (Chatterjee et al. 2006). Moreover, mitochondria do not contain the full range of DNA repair mechanisms that operate in the nucleus (Boesch et al. 2010). The currently known repertoire of mtDNA repair includes single-nucleotide base excision repair (BER), long-patch BER, single-strand break repair, YB-1-mediated mismatch repair, removal of adenine opposite 8-oxo-dG, and MTH1 removal of 8-oxo-dGTP and 8-oxo-2-′-dATP from the mitochondrial nucleotide pool (Cline 2012).

Damage to mtDNA bases in both normal population and during cancer progression may be caused either by endogenously produced metabolic products or by exogenous sources.

The main endogenous determinants are represented by reactive oxygen species (ROS), which are generated subsequently to the leakage of electrons during OXPHOS, and may physiologically serve as signaling molecules in the cell (Ray et al. 2012). Anomalous high ROS levels are known to augment the mutation rate of both mitochondrial and nuclear DNA through mechanisms that include base modifications, sugar breakdown products, base-free sites, and strand breaks (Cline 2012). The type of ROS-derived damage usually regards 8-OH-GTP, which may cause a transversion of G:C to T:A (Wallace 2005). Furthermore, ROS may be responsible for changes in the cellular dGTP pool and 8-OH-dGTP generation, which in turn leads to transversion from A:T to C:G (Wiseman and Halliwell 1996). It is important to note that, on these bases, the expected spectrum of mutations should provide an excess of A:T to C:G and G:C to T:A transversions in the mtDNA sequence. However, the current spectrum of mtDNA mutations in human populations and in cancer does not correspond to the view of a damage induced prevalently by ROS, as observed, for instance, in colorectal cancer (Skonieczna et al. 2012), suggesting the existence of different predominant mutagenic mechanisms during tumor progression.

In fact, other endogenous cell metabolites such as estrogens may cause formation of adducts such as S-adenosylmethionine, which are able to methylate DNA in a nonenzymatic manner (alkylation damage) (Alexeyev et al. 2013). The mtDNA may also react with the products of endogenous fatty acid peroxidation, resulting in adduct formation (Nair et al. 2005). Moreover, reactive nitrogen species (RNS) can promote mutational events since they react with the superoxide anion $O_2^{\bullet -}$ and cause DNA base oxidation and sugar fragmentation, which may induce mtDNA strand breaks (Burney et al. 1999).

Random mtDNA mutations may also arise as a result of an erroneous DNA replication caused by polymerase errors, particularly plausible at the homopolymeric stretches that frequently occur in mtDNA regions (Denver et al. 2000). Mutations in either the polymerase or the exonuclease domain of POL-γ, the

functional DNA-dependent DNA polymerase in mitochondria, have been in fact associated with increased occurrence of mtDNA mutations (Copeland et al. 2003). In cancer, elevated mtDNA mutagenesis and frequent mtDNA copy number alterations have often been attributed to dysfunction of proteins involved in mtDNA integrity maintenance. For example, *POLG*, the gene encoding POL-γ, has been found mutated in 63 % of breast tumors, with a consequent mtDNA depletion and increased tumorigenicity (Singh et al. 2009). The knockdown of nuclear-encoded RNA helicase, SUV3, results in reduced mtDNA copy number and elevated somatic mtDNA mutation frequency in murine models (Chen et al. 2012). Similar consequences have been observed in colorectal cancers carrying mutations in the mitochondrial transcription factor (TFAM), which also plays a histone-like function within the nucleoids (Campbell et al. 2012).

Moreover, given that tumor suppressor p53 is known to enter mitochondria and play a role in mtDNA maintenance, it is not surprising that p53 disruption has been associated with occurrence of mtDNA alterations (Lebedeva et al. 2009). In this context, it is tempting to speculate that mutations in other genes involved in the mitochondrial genome organization and maintenance may contribute to the development of cancer-associated mtDNA alterations. Among these, mutations in *OPA1*, responsible for a dominant form of optic atrophy, have been shown to induce the accumulation of mtDNA deletions in the skeletal muscle of patients (Amati-Bonneau et al. 2008; Elachouri et al. 2011). Although OPA1 has not yet been linked to cancer, it is possible that it might contribute to mitochondrial alterations in certain contexts of tumorigenesis.

Furthermore, it is likely that the inherited genetic background of mtDNA variants may also contribute to the spectrum of mtDNA mutations in cancer. For instance, the co-occurrence of germline and somatic mtDNA mutations in cancer has led to the hypothesis that inherited mutations may predispose to a facilitated acquisition of additional mutations (Petros et al. 2005; Gochhait et al. 2008). This aspect is still largely unexplored in cancer, since the scenario of "universal" variability in the form of inherited low heteroplasmy mtDNA variants has only recently been discovered in healthy individuals (He et al. 2010; Payne et al. 2013).

Exogenous sources of mtDNA mutations include ultraviolet light, ozone, ionizing radiations, metals, pesticides, air pollutants or pharmaceutical drugs, asbestos, and arsenic (Partridge et al. 2009; Boesch et al. 2010). The benzo[a]pyrene and acrolein components of cigarette smoke, the fungal toxin aflatoxin B1, platinum-based chemotherapy agents, and antiviral nucleoside analogs all induce DNA adducts that may interfere with the POL-γ function (Cline 2012). These mutagens are also exogenous sources of ROS; therefore, it is difficult to assess whether mutations are a direct consequence of exogenous agents or if the latter increase of free radicals generation leads to oxidative damage and mtDNA mutagenesis. It is quite possible that both scenarios occur in vivo (Chatterjee et al. 2011).

It is important to note that certain chemotherapeutic approaches employed in cancer therapies may have off-target effects on mitochondria, causing both direct and indirect damage to mtDNA. These include cisplatinum (Chatterjee et al. 2011), bleomycin, and neocarzinostatin (Cline 2012). For example, platinum-based

chemotherapy drugs, such as cisplatin, carboplatin, and oxaliplatin, bind directly to DNA to form single base adducts and intra- and interstrand cross-links between guanine bases (Cline 2012).

In summary, the high occurrence of mtDNA mutations in cancer is thought to be a result of (1) proximity of mtDNA to ROS production sites, (2) an inadequate set of mtDNA repair mechanisms, (3) potential dysfunction of proteins involved in mtDNA integrity maintenance, (4) diverse exogenous mutagens including chemotherapics, and (5) accumulation of inherited germline mutations.

10.3 Selection of mtDNA Mutations in Cancer

Whether an mtDNA mutation will be expanded and stabilized in a tissue mainly depends on putative selective pressures which in cancer are heterogeneous and change over time (Aanen and Maas 2012). By analyzing mtDNA mutational hot spots, their mutant load, and the type of damage they induce, it is possible to characterize the selective forces which drive their accumulation in cancer, providing at the same time information about the complex mechanisms which operate during tumor progression in general.

In healthy individuals, inherited mitochondrial mutations in protein-coding genes are subjected to negative selection and are preferentially eliminated within a few generations (Stafford and Chen-Quin 2010; Freyer et al. 2012). Inheritance of tRNA and rRNA mutations, whose functional effects are more difficult to ascertain, is considered to be either subjected to random genetic drift or to negative selection (Schon et al. 2012). The regulatory D-loop region particularly displays a high variability rate (Pereira et al. 2009). Among the mtDNA coding regions, the most evolutionary conserved genes are *MT-COI*, *MT-COII*, *MT-ND4*, and *MT-ND4L*, whereas the most polymorphic regions include *MT-ATP6*, *MT-ND6*, and *MT-CYTB* (Pereira et al. 2009).

In cancer tissues, the D-loop appears to be the region that is more prone to acquire somatic mutations, but it seems that there are no preferential hot spots among rRNA, tRNA, or coding genes, since cancer-specific mutations are distributed uniformly across the genome (Iommarini et al. 2012; Liu et al. 2012). However, in colon cancer, most somatic mtDNA mutations were shown to accumulate in *MT-ND4L*, while genes encoding subunits of complex V remained mutation free (Skonieczna et al. 2012), indicating that a selective bias might exist, at least in some types of cancer. Whatever the case, it is certain that selection of mtDNA mutations in cancer varies from the purifying forces observed in mitochondrial genome evolution of healthy individuals.

Certain somatic mtDNA mutations may positively contribute to cancer cell development, as suggested from the observation of mitochondrial genotypes pattern in some tumor tissues. For example, in hepatocellular carcinoma, renal, breast, gastric, rectal, and ovarian cancer, variable heteroplasmic levels of an ND5-truncating mitochondrial mutation were recurrently found (Larman

et al. 2012), suggesting a role in conferring a selective advantage to neoplastic cells. It is interesting to note that the same mutation was found in the germline of a patient presenting a nasopharyngeal oncocytoma (Gasparre et al. 2009). Albeit heteroplasmic in all the tissues analyzed, the mutation shifted to homoplasmy only in the oncocytic areas of the tumor (Gasparre et al. 2009). Along this line, since the majority of mtDNA mutations in cancer have long been reported only as homoplasmic, a positive selection during tumor progression was one of the most recognized hypotheses. However, mathematical modelings suggest that a shift to homoplasmy might be the result of a random event, i.e., achieved without the action of selective forces (Coller et al. 2001). In fact, studies that have more accurately taken into account the load of mtDNA mutations, such as the most recent next-generation sequencing (NGS) approaches (He et al. 2010; Larman et al. 2012; Guha and Avadhani 2013), suggest that mutant load in cancer tissues may vary and include also an extremely low-level heteroplasmy, which was up to date neglected due to technical limitations (Kurelac et al. 2011). Therefore, the exclusively positive selection of mtDNA mutations in cancer has recently become questioned: a large number of studies, together with advances in mitochondrial genome analyses and a better understanding of pathogenic effects, suggested that they are not only positively selected during tumor progression as initially recognized but that a relaxed negative selection is the most plausible mechanism that shapes mtDNA variation in cancer cells (Stafford and Chen-Quin 2010; Liu et al. 2012). Overall, mtDNA mutations in cancer may be likely subjected to the negative selection observed also in the evolution of healthy individuals, albeit at certain points of cancer progression, purifying effects may be lost due to metabolic reprogramming and to the onset of diverse microenvironment conditions, leading to a shift to homoplasmy (Fig. 10.1).

Prediction of functional effects is particularly important in this context to understand selective forces in cancer. It has been shown that non-synonymous mutations occur with a higher frequency in cancer than in the general population and that these changes are usually predicted to influence protein function (Larman et al. 2012; Liu et al. 2012). However, mtDNA mutations with substantially high pathogenicity scores are not often found in human neoplasms and are mainly restricted to oncocytomas, a peculiar type of less aggressive tumors (Pereira et al. 2012). In fact, highly severe mutations, such as stop/frameshift, which may completely inhibit OXPHOS, are usually heteroplasmic or purified in cancers (Brandon et al. 2006; Larman et al. 2012) (Fig. 10.2a). Similarly, the analysis of mutations affecting complex I genes implied a strong negative selection of stop and frameshift mutations in most human tumors (Iommarini et al. 2012). Investigation of the same dataset showed that this holds true also for mutations affecting other respiratory chain complexes whose subunits are encoded by the mtDNA (Fig. 10.2b). Moreover, an analysis of colon cancer datasets indicated only 7 % of stop-inducing mutations (Skonieczna et al. 2012).

The degree of mitochondrial function necessary for cell survival may depend on the tumor stage and microenvironment and may range from completely attenuated OXPHOS activity during early glycolytic cancer metabolism to even enhanced

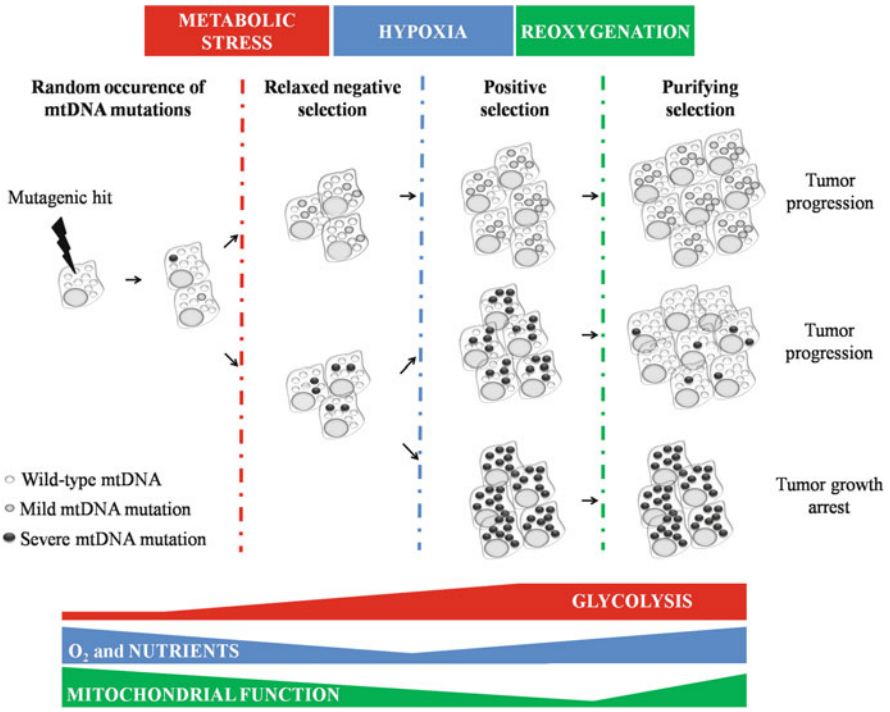

Fig. 10.1 Selection of mtDNA mutations in cancer. At the initial stages of tumorigenesis, uncontrolled cellular proliferation causes metabolic stress and promotes glycolysis, during which mtDNA mutations may accumulate randomly under a relaxed negative selection, since mitochondrial function is not essential. In hypoxic conditions, downregulation of mitochondrial function lowers cell requirements for oxygen and thus mtDNA mutations may be positively selected. On the other hand, in a reoxygenized environment, mtDNA mutations may become subjected to a negative selection, since mitochondrial function may regain importance, i.e., due to higher energy requirements. In this context, if a severe mtDNA mutation shifts to homoplasmy during the hypoxic stage, this irreversible mitochondrial damage may lead to tumor growth arrest in reoxygenized conditions, conversely from mild mtDNA mutations which may instead escape negative selection

OXPHOS activity observed in several tumor types (Smolkova et al. 2011). For example, in hypoxic conditions, where lack of oxygen is the main selective pressure, cancer cells carrying damaging mtDNA mutations may be preferentially selected since downregulation of mitochondrial function becomes advantageous. On the other hand, in tumor areas that have gone through hypoxic adaptation, the elevated anabolic metabolism of macromolecule biosynthesis requires functional mitochondria, such as during the process of non-anoxic glutaminolysis (Wise et al. 2011). Therefore, it is most probable that the early shift to glycolysis, shared by most solid tumors, allows for a relaxed negative selection of pathogenic mtDNA mutations in cancer (Liu et al. 2012), while the same mutations may become disadvantageous, for instance, in reoxygenated metastatic cancer tissue (Horton

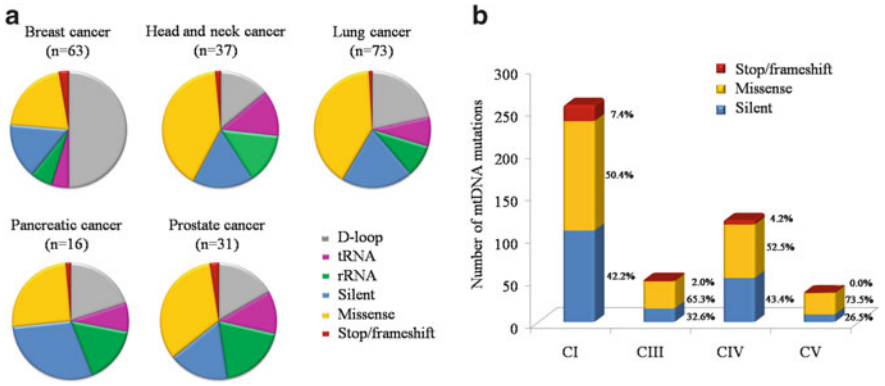

Fig. 10.2 Somatic mtDNA mutations in cancer. (**a**) Somatic mtDNA mutation frequency in different cancers, with distinction between mtDNA regions and mutation types for protein-coding genes. (**b**) Distribution of protein-coding mtDNA mutations in cancers of different origin, with distinction between mitochondrial respiratory complexes. Data for this analyses were extracted from reports published between 1998 and 2011, in which the entire mtDNA was sequenced and for which the somatic status of the mutations was indicated (Iommarini et al. 2012). Oncocytic tumors were excluded from the dataset

et al. 1996; Brandon et al. 2006) (Fig. 10.1). In the event of accumulation of disadvantageous mutations to homoplasmy during the initial permissive glycolytic phases, it is plausible that the consequence would be the inability for tumors to progress to malignancy, as deduced from the study of oncocytomas, which remain mostly confined as low proliferative lesions (Iommarini et al. 2012; Pereira et al. 2012).

Taken together, mtDNA mutations in cancer are subjected to ever-changing selective pressures which may be neutral, positive, or purifying, depending on the mutation type and on the specific phases of tumor progression in which they occur (Fig. 10.1). As a consequence, mtDNA mutations may be impartial bystanders, but also positively selected or eliminated, depending on whether their overall functional effect is advantageous during the waves of tumor reprogramming and adaptation (Smolkova et al. 2011).

It is important to note that cancer-specific changes in mtDNA copy number have also been reported as means of regulating mitochondrial function, which may be altered by defects in mitochondrial biogenesis or may be a consequence of mutations within the D-loop origin of replication. In particular, increase in mtDNA copy number has been observed in endometrial and esophageal squamous cell carcinoma, prostate cancer, ovarian cancer, papillary thyroid carcinoma, and in oncocytic tumors, while advanced gastric cancer, breast cancer, Ewing's sarcoma, and renal cell cancer appear to display a low number of mtDNA copies (Yu 2012). It is most likely that similarly to mtDNA selection, mtDNA copy number in cancer also depends on the tumor stage and metabolic requirements (Cook and Higuchi 2012).

10.4 Consequences of mtDNA Mutations on Tumorigenesis and Tumor Progression

Mitochondrial dysfunction has been associated with promotion of tumor growth ever since Warburg observed a higher glucose uptake and increased lactate production in cancer cells (Warburg et al. 1924). The first genetic evidence for mitochondria-induced cancer development was found in two nuclear metabolic genes encoding tricarboxylic acid (TCA) cycle enzymes, namely, succinate dehydrogenase (SDH) and fumarate hydratase (FH), in which loss of function mutations has been reported to promote tumorigenesis by stabilizing the hypoxia-inducible factor 1-alpha (HIF1α) in normoxic conditions, in patients with hereditary paragangliomas (HPGL) (Baysal et al. 2000; Astuti et al. 2001) and hereditary leiomyomatosis and renal cell cancer (HLRCC) (Tomlinson et al. 2002). Along this line, loss of function mtDNA mutations have initially been considered as pro-tumorigenic events (Polyak et al. 1998; Petros et al. 2005) and even sometimes as the initiators of tumorigenesis (Shay and Werbin 1987). However, considering the complexity of mitochondrial genetics, the vastness of cellular and metabolic reactions in which mitochondria are involved, and the extremely high tumor heterogeneity, this definition has been corrected, particularly in the context of a recent revision of the Warburg hypothesis, by which now it is recognized that a certain, at least minimal, degree of mitochondrial function is mandatory for cancer cell survival (Wallace 2012).

A classification of mtDNA mutations based on their effect on tumor progression may be complex, since it ought to depend both on the type of damage they induce and on the context in which they arise. For example, mtDNA variants causing mild complex I dysfunctions, such as missense mutations, are indeed usually associated with pro-tumorigenic effects, whereas mutations leading to complete mitochondrial complex I disassembly have been shown to reduce tumorigenic potential (Gasparre et al. 2011; Iommarini et al. 2014). Furthermore, a 21 bp deletion in *MT-CYTB* was appointed to cause an increase in the tumorigenic potential of bladder cancer cells (Dasgupta et al. 2008), whereas a similar *MT-CYTB* mutation decreased tumor growth of *KRAS*-mutated osteosarcoma cells (Weinberg et al. 2010), indicating that *KRAS*-induced tumorigenicity may be more reliant on mitochondrial function. Despite both mutations causing increased ROS production, the nuclear background presumably set the frame for the final outcome of the mtDNA mutation, just as the tumor cell microenvironment sets pressures for mtDNA mutation selection. It is important to note that even the same mutation may exhibit different, even opposite, effects on tumorigenesis, depending on the mutant load.

Nowadays, mtDNA mutations in cancer are generally rather considered as modifiers of tumor progression, and three groups of mtDNA mutations may be distinguished with respect to their effects on tumorigenesis: neutral, pro-tumorigenic/pro-metastatic, and antitumorigenic. A difficult task concerns the positioning of D-loop mutations within these categories. Although frequently described in cancer, a clear indication on their functional consequences has been

lacking to date. In fact, the D-loop appears to be hostile for functional investigation, and only a few works indicate an indirect positive contribution of D-loop mutations to mitochondrial dysfunction and cancer progression (Gasparre et al. 2008; Li et al. 2008), suggesting that the bulk of data on D-loop cancer variants ought to be interpreted with caution, especially since this is a highly polymorphic region.

mtDNA mutations exhibiting a neutral effect on tumorigenesis are the most common somatic changes observed in cancer, as suggested by several revisions on the currently available analyses on cancer-specific mtDNA mutations (Lu et al. 2009; Iommarini et al. 2012; Yu 2012). It has been estimated that synonymous changes represent 75 % of all cancer-related coding mtDNA mutations (Yu 2012), indicating that many of such randomly occurring genetic events are mere bystanders that may not affect tumor progression. It is important, however, not to neglect the potential effects of such variants since a few have been shown to alter protein structure (Kimchi-Sarfaty et al. 2007; Komar 2007). Moreover, some studies implicated a haplogroup-related predisposition to cancer development, indicating that mtDNA polymorphisms may have a role in cancer progression, a hypothesis that warrants further investigation in larger cohorts (Liu et al. 2003; Canter et al. 2005). It has been indeed hypothesized that haplogroups may favor a looser coupling between oxidation and phosphorylation, thereby generating a surplus of heat at the expenses of ATP and in turn a better survival of human populations in colder climates (Ruiz-Pesini et al. 2004). Although controversial, this hypothesis suggests that polymorphic germline mtDNA variants may exert a phenotypic effect, when working synergistically. On the other hand, even functionally active (pathogenic/phenotype changing) mtDNA mutations may be neutral, as it has been observed for the m.6930G > A/*MT-COI*, which causes decrease in ATP synthesis and reduces respiration of osteosarcoma-derived cybrids, but shows no influence on tumor growth (Sharma et al. 2011). Similarly, the heteroplasmic m.3571insC/*MT-ND1* induces no modifying effects on tumorigenic potential compared to wild-type mitochondria with completely functional complex I (Gasparre et al. 2011). However, the same mutation in mutant loads higher than 83 % exhibits antitumorigenic properties in the same cells.

Pro-tumorigenic mtDNA mutations are also commonly found in cancer tissues and mostly include point mutations inducing amino acid changes. The first functional studies on the role of mtDNA mutations in cancer demonstrated that an *MT-ATP6* mutation increased tumorigenesis in vivo by causing elevated ROS production and downregulation of apoptotic mechanisms in prostate and cervix cancer cells (Petros et al. 2005; Shidara et al. 2005). Although this mutation is not usually found in cancer, but is etiological for the NARP syndrome, these experiments confirmed the Warburg hypothesis and were in line with the suggested bioenergetic profile of decreased ATP synthase expression described in most solid tumors (Cuezva et al. 2009). It is important to note that such a mutation may not be an initiator of tumorigenesis since otherwise NARP patients would be more prone to cancer development. A subsequent series of studies aimed at identifying diverse mechanisms by which mtDNA mutations may exhibit pro-tumorigenic properties. Taken together, a dozen pro-tumorigenic mtDNA

mutations were functionally characterized up to date, which include different mtDNA loci, including the D-loop (Li et al. 2008), and affect function of diverse respiratory chain complexes. For instance, the m.6124T > C/*MT-COI* induced a decrease in cytochrome c oxidase (COX) activity and elevated tumorigenic potential of prostate cancer cells (Arnold et al. 2013); reduced complex III activity caused by a deletion in *MT-CYTB* increased bladder cancer growth (Dasgupta et al. 2008) and mtDNA mutations in genes encoding different complex I subunits led to increased tumorigenic potentials of head-and-neck (Zhou et al. 2007; Sun et al. 2009), colon (Park et al. 2009), lung (Dasgupta et al. 2008; Ishikawa et al. 2008), and breast (Kulawiec et al. 2009b) cancer cells. Particularly interesting are the mtDNA mutations that have been shown to increase metastatic potential of cancer cells in vivo. These were mainly associated with increased ROS production (Ishikawa et al. 2008; Kulawiec et al. 2009a), although mtDNA mutations that promote metastatic events through ROS-independent mechanisms have been described (Imanishi et al. 2011).

Certain mutations may induce neutral effects on tumorigenesis and yet confer resistance to therapeutic approaches, which substantially renders them pro-tumorigenic. Association of mtDNA mutations with therapy resistance is a particularly interesting field of research since their identification may be helpful in the prediction of therapy response in patients. Pancreatic cancer-specific mtDNA mutations showed to confer resistance to staurosporine-induced apoptosis (Mizutani et al. 2009); mtDNA-depleted HeLa (Shidara et al. 2005) and osteosarcoma (Yen et al. 2005) cells showed resistance to cisplatin, and chronic lymphocytic leukemia patients refractory to conventional therapeutic agents tended to have higher mtDNA mutation rates than patients who responded to treatment (Carew et al. 2003). Moreover, a case of ovarian carcinoma harboring a pathogenic mtDNA mutation, probably induced by carboplatin chemotherapy, displayed resistance to paclitaxel combined treatment (Guerra et al. 2012). Albeit in a small number of studies, the functional status of mtDNA mutations has been shown to play an essential role also in radiation induced cytotoxicity, in the way that radiosensitivity of cells bearing such mutations was reduced (Alsbeih et al. 2009; Cloos et al. 2009). Interestingly, gamma radiation commonly used in anticancer therapy was shown to increase mtDNA copy number, which may further prompt accumulation of mitochondrial mutations (Bartoletti-Stella et al. 2013).

Finally, mtDNA mutations that lead to a reduced tumorigenic potential have only recently been recognized, along with a thorough revision of the essential function of mitochondria in cancer and of the Warburg hypothesis (Wallace 2012). The first evidence came from experiments with mtDNA-depleted cancer cells (Rho zero), characterized by a severe mitochondrial respiration damage and displaying low proliferative and low invasive potentials, often failing to form tumors in vivo, unlike their nuclearly isogenic counterparts carrying normal mtDNA pool (Morais et al. 1994; Cavalli et al. 1997; Shidara et al. 2005; Imanishi et al. 2011). Likewise, several functional studies demonstrated how clearly pathogenic mtDNA mutations reduce or inhibit tumor growth. These mainly include mutations affecting complex I subunits (Park et al. 2009; Gasparre et al. 2011) and

tRNA loci (Arnould et al. 2002; Iommarini et al. 2014), which lead to such an extent of complex I deficiency and respiratory chain defects to cause lethality. In fact, highly severe mutations are negatively selected in cancer tissue and are usually found heteroplasmic (Brandon et al. 2006; Larman et al. 2012). In case such mutations do cross the threshold value for their disruptive phenotypic effect, the concordant low aggressive phenotype is developed, as observed in oncocytomas (Iommarini et al. 2012; Pereira et al. 2012) (see Sect. 10.6).

10.5 Mitochondrial Mutations and Cancer Metabolism

Apart from being genetically determined, cancer is also a metabolic disease. Metabolic reprogramming has been recently introduced as one of the hallmarks of cancer cells (Hanahan and Weinberg 2011; Ward and Thompson 2012), since a high proliferation rate requires rapid energy production, a shift toward biosynthetic reactions and parallel maintenance of the redox homeostasis (Cairns et al. 2011). It has been extensively demonstrated that oncogene-driven tumorigenesis is always accompanied by adequate metabolic adaptations. Oncogenes promoting phosphatidylinositide 3-kinases (PI3K) signaling was shown to lead to increased glucose uptake and glycolysis (Elstrom et al. 2004). Myc-induced transformation promotes glutaminolysis, the second major carbon source in cancer metabolism necessary for TCA cycle maintenance and anaplerosis (DeBerardinis et al. 2007; Wise et al. 2008). On the other hand, loss of p53 function is associated with downregulation of mitochondrial metabolism (Matoba et al. 2006; Wang et al. 2013) and redirection of glucose to the pentose phosphate pathway, which ensures NADPH-dependent redox maintenance (Bensaad et al. 2006).

In the context of genetically regulated metabolic reprogramming (DeBerardinis et al. 2008; Ward and Thompson 2012), mtDNA mutations represent a powerful tool for modulation, since mitochondria play a substantial role in a number of metabolic reactions. Moreover, due to heteroplasmy and threshold effect, the reversibility and the array of mtDNA mutation-induced phenotypes, ranging from mild to severe functional consequences, allow an efficient fine-tuning of cancer cell metabolism depending on the ever-changing selective pressures during tumor progression. The following key elements of cellular metabolism have been shown to be altered in response to the occurrence of mtDNA mutations in cancer cells:

ROS The most investigated metabolic consequence of cancer mtDNA mutations is related to ROS signaling, since complex I and complex III are two main sources of ROS, and they are both partly encoded by mtDNA genes. Increased ROS levels have been mainly associated with promotion of tumor progression and metastases (Ishikawa et al. 2008). Their mitogenic properties are exhibited through the interaction with various regulatory factors such as MAP kinases, PI3Ks, PTEN, and protein tyrosine phosphatases (Ray et al. 2012), and their pro-metastatic capacity has been suggested to be related to HIF1α stabilization (Chandel et al. 2000;

Ishikawa et al. 2008). However, although ROS appear to be required for anchorage-independent growth, chronically increased levels severely damage mitochondria. At high levels, they are toxic, and, depending on their type and concentration, they may mediate both pro- and antiapoptotic events (Shen et al. 1998). It has been shown that functionally mild mtDNA mutations, such as the heteroplasmic m.12417insA/*MT-ND5*, lead to pro-tumorigenic signaling by increasing superoxide in mitochondria. Elevated peroxide levels in cytosol, which are induced by the homoplasmic version of the same mutation, may instead lead to apoptosis (Park et al. 2009). In that study, an increased expression of anti-oxidative enzymes levels was detected in heteroplasmic cells. Similar compensatory effect in the form of upregulation of detoxifying enzymes was observed in osteosarcoma and thyroid cancer cells carrying the nearly homoplasmic m.3571insC/*MT-ND1* mutation (Porcelli et al. 2010), suggesting that the effect of a mutation on tumor progression will depend also on the activity of detoxifying mechanisms which may protect from harmful ROS effects. It is interesting to note that it remains to be explained how such truncative mutations that likely abolish function of the main ROS-generating sites may lead to an increased ROS production.

ATP Production of energy in the form of ATP is the main task of OXPHOS and thus mtDNA mutations are very often associated with a decrease in OXPHOS-derived energy production (Park et al. 2009; Gasparre et al. 2011; Sharma et al. 2011; Jandova et al. 2012a). However, this is recuperated through increased glycolysis rate, and thus an alteration in ATP concentrations has rarely been brought into a direct association with a modification of hyperproliferative processes. Nevertheless, it has been demonstrated that despite the reduced OXPHOS in cancer, a significant amount of cancer cell energy still derives from at least partially functional ETC (Zu and Guppy 2004). In fact, severe mtDNA mutations, such as the m.3243A > G/*MT-TL1*, were shown to induce energetic crisis, alteration in AMP/ADP/ATP levels, and subsequent decrease of tumor growth of cybrids in vivo (Iommarini et al. 2014), indicating that substantial damage to OXPHOS-mediated energy production may be fatal for the cancer cell. In fact, such mutations are typical of neuromuscular mitochondrial diseases and rarely found in human cancers (Iommarini et al. 2012). The mechanism by which these severe mutations modulate tumor progression involves activation of AMP-activated protein kinase (AMPK) (Iommarini et al. 2014), the main cellular energy sensor which promotes catabolic ATP-generating reactions, while downregulating ATP-consuming biosynthetic pathways (Hardie 2011), for which a tumor suppressor role has been inferred (Faubert et al. 2013). It is interesting to note that AMPK also upregulates mitochondrial biogenesis and regulates disposal of damaged mitochondria (mitophagy) to preserve the overall cellular ATP-generating capacity (Hardie 2011), suggesting that AMPK status may contribute to mtDNA selection in cancer.

NAD^+/NADH NAD homeostasis is vital for cellular signaling reactions, such as protein ribosylation and deacetylation reactions, and generation of Ca^{2+}-mobilizing messenger molecules (Chiarugi et al. 2012). In comparison to normal cells, the NAD^+/NADH ratio is somewhat reduced in cancer due to the high glycolysis rate

accompanied with lower ETC reducing activity and due to elevated biosynthetic reactions for which NAD^+-derived NADP is required (Chiarugi et al. 2012). An additional drop in this ratio would be fatal for a cell and thus targeting NAD^+ synthesis has been proposed as a cancer therapy (Hasmann and Schemainda 2003). Along this line, since mitochondrial complex I is the main regenerator of cellular NAD^+, mtDNA mutations causing defects in NADH dehydrogenase activity may compromise NAD^+ homeostasis and prevent tumor growth. In fact, severe mtDNA mutations leading to substantial reduction in the NAD^+/NADH ratio were shown to decrease the tumorigenic potential of cancer cells (Calabrese et al. 2013; Iommarini et al. 2014). It must be noted, however, that non-lethal reduction in NAD^+ levels induced a more aggressive, pro-metastatic phenotype in breast cancer models (Santidrian et al. 2013) and some studies indicate that the decrease in NAD^+/NADH ratio promotes tumor growth through NADH-regulated PTEN inactivation and promotion of AKT survival signaling (Pelicano et al. 2006; Sharma et al. 2011). Again, the specific tumor microenvironment, the genetic background, and the degree of imbalance in NAD^+ homeostasis probably explain the pleiotropic effects of mtDNA mutations on cancer progression.

Lactate High rate of aerobic glycolysis in cancer leads to elevated lactate production which compromises intracellular pH homeostasis but promotes invasiveness by acidification of tumor stroma (Warburg et al. 1924; Chiche et al. 2010). HIF1α-signaling contributes to lactate increase in cancer cells by inhibiting pyruvate dehydrogenase and redirecting pyruvate from the TCA cycle to fermentation and, at the same time, activating pH maintenance pathways which ensure that cell survival is not jeopardized by lactate-induced acidosis. Several complex I mtDNA mutations have been shown to contribute to elevated lactate levels in cancer cells and to promote tumor growth by inducing HIF1α-dependent mechanisms (Zhou et al. 2007; Sun et al. 2009). On the other hand, the increase in lactate concentration described in breast cancer cell lines carrying the m.12084C > T/*MT-ND4* and the m.13966A > G/*MT-ND5* mutations was not associated with elevated HIF1α expression but was nonetheless sufficient to confer a higher metastatic potential (Imanishi et al. 2011). The latter study suggests that mtDNA mutations may influence tumor progression by regulating cancer cell pH homeostasis, also in an HIF1-α-independent manner. Interestingly, the same breast cancer cells deprived of their mtDNA produced extremely high lactate levels, which were not associated with metastasis development, indicating that cells that have not gone through HIF1α-mediated adaptation may not sustain the extreme acidosis caused by severe mitochondrial defects (Imanishi et al. 2011).

Although lactate acidosis is mainly associated with hypoxia, it has also been observed in normoxic cancer cell environment (Icard and Lincet 2012). It has been suggested that lactate produced by hypoxic cells is not merely a waste product, but may be used as an energy fuel for ATP production through OXPHOS in normoxic cells (Feron 2009). Such metabolic symbiosis spares glucose which may then reach hypoxic and anoxic cells where it is used for anaerobic glycolysis. It is important to

note that in this context, different selective pressures would affect accumulation of mtDNA mutations between normoxic and anoxic cancer cell.

TCA Cycle Intermediates The TCA or Krebs cycle is a central pathway in the metabolism of sugar, lipids, and amino acids. Far from being a closed pathway, the TCA cycle rather integrates several metabolic reactions in a cell, such as the metabolism of amino acids, fatty acids, and heme, via anaplerotic reactions. The cytoplasmic and mitochondrial pools of TCA cycle intermediates are in tight connection, and metabolite accumulation in one of the pools is immediately reflected in the other.

Alteration of TCA cycle as a mechanism of tumorigenesis has been initially associated to mutations in two key enzymes that catalyze essential steps within the cycle, namely, SDH and FH, whose loss of function leads to the accumulation of fumarate and succinate, causing abnormal normoxic stabilization of HIF1α, since high levels of these metabolites inhibit the prolyl-hydroxylase reaction which mediates HIF1α degradation (Isaacs et al. 2005; Selak et al. 2005). Moreover, high concentrations of fumarate and succinate have been shown to induce aberrant patterns of gene expression by inhibiting enzymes involved in DNA and histone demethylation (Cervera et al. 2009) or by causing protein impairment via succinylation (Alderson et al. 2006).

Imbalance in TCA cycle intermediates concentrations has also been observed as a consequence of mtDNA mutations. Cancer cells harboring nearly homoplasmic levels of the m.3571insC/*MT-ND1* mutation, which leads to a dysfunctional complex I, display an increased α-ketoglutarate/succinate ratio as a direct consequence of NADH accumulation. In this case, conversely from what is observed in SDH and FH mutated tumors, HIF1α is chronically destabilized even in hypoxic conditions (pseudonormoxia), since α-ketoglutarate feeds and promotes the prolyl-hydroxylase reaction (Gasparre et al. 2011). Therefore, the m.3571insC/*MT-ND1* may hamper tumor growth by introducing imbalance in TCA cycle metabolites. It is very likely that other mtDNA mutations may modify tumor progression by similarly changing the cellular metabolite concentrations.

The stalling of the TCA cycle due to FH or SDH deficiency may prevent cells from generating TCA cycle intermediates such as malate, oxaloacetate, and citrate by conventional oxidative metabolism. It has been shown that in such conditions, a cell turns to alternative pathways. For instance, in vitro studies on cancer cell lines with FH mutations or defective complex I or III have demonstrated a switch to anoxic glutaminolysis as a dominant mode of metabolism for supporting cell proliferation and generating citrate (Mullen et al. 2011). However, according to the model suggested by Smolkova and colleagues (Smolkova et al. 2011), this process would not be sufficient to support proliferation for a long period due to energetic deficiency, and increase in glycolysis or oxidative carboxylation would have to be established. In this context, it is important to note that because of its essential dependence on complex II (Smolkova and Jezek 2012), oxidative glutaminolysis may be sustained only in cancer cells with at least partially functional ETC. For instance, severely dysfunctional complex I cells should be able to

sustain oxidative glutaminolysis, whereas cancer cells with a downstream damage in ETC would compromise this metabolic pathway and possibly hold back tumor progression.

10.6 Cell-Systemic Consequences of mtDNA Mutations That Modify Tumor Progression

Apart from their role in metabolic reactions, mitochondria are involved in many other pivotal cellular processes, such as apoptosis, hypoxic adaptation, or autophagy. The ways in which mtDNA mutations may influence tumor progression by altering proper functions of such pathways are here described.

Apoptosis The most common form of cell death in mammalian cells involves the mitochondrial apoptotic pathway, in which the outer mitochondrial membrane is permeabilized, releasing caspase-activating molecules and caspase-independent death effectors (Green and Kroemer 2004). This process is regulated by cytosolic p53 and Bcl-2 family of pro- and antiapoptotic proteins, but may also be a consequence of mitochondrial damage, such as dissipation of mitochondrial membrane potential (Green and Kroemer 2009). Evasion of apoptosis and, in particular, a related resistance to mitochondrial outer membrane permeabilization is a hallmark of cancer cells. In this context, mtDNA mutations are often associated with modulation of apoptotic mechanisms in cancer. Decrease in apoptosis was associated with elevated ROS production in m.6124T > C/*MT-COI* mutant prostate cancer cells (Arnold et al. 2013), m.10398G > A/*MT-ND3* mutant breast cancer cells (Kulawiec et al. 2009b), and colorectal cancer cell heteroplasmic for the m.12417insA/*MT-ND5* (Park et al. 2009). On the other hand, certain mutations have been shown to boost apoptotic mechanisms and prevent tumor growth, such as the homoplasmic version of the m.12417insA/*MT-ND5* (Park et al. 2009).

Interestingly, mtDNA mutations appear to have the ability to influence expression of apoptotic genes. Depending on the degree of respiratory chain damage induced by different mtDNA mutations, osteosarcoma-derived cybrids displayed diverse levels of Bcl-2 family protein expression and exhibited either pro- or antiapoptotic signaling, despite their isogenic nuclear background (Kwong et al. 2007). In particular, COX- and CYTB-deficient cells showed lower expression of Bcl-XL and Bcl-2, respectively, resulting in resistance to staurosporine-induced apoptosis. On the other hand, NARP mutants that maintain all respiratory chain complexes, albeit at reduced amounts and activities, displayed staurosporine sensitivity followed by low Bcl-2 and high Bcl-XL expression levels. Moreover, thapsigargin, which mimics stress derived from the endoplasmic reticulum, was shown to induce apoptosis in all of these cells, while Rho zero cells and MERRF mutants, which completely lack functional and structural integrity of respiratory chain complexes, were resistant to this type of programmed death (Kwong et al. 2007).

Interestingly, it has been shown that mitochondrial damage may cause alterations in pyrimidine synthesis and folate metabolism and thus result in less efficient DNA repair and chromosomal instability (Desler et al. 2007; Minocherhomji et al. 2012). In this context, since p53 responds to DNA damage, it is interesting to speculate that mtDNA mutations might influence p53 activity by regulating the nucleotide pool of a cancer cell (Naviaux 2008).

Taken together, the apoptotic responses in mtDNA-mutated cancer cells may depend on the type of cellular stress, their effective consequence on mitochondrial membrane potential, and on the expression of pro- and antiapoptotic proteins.

Hypoxic Adaptation The ability to stabilize HIF1α is considered a feature of malignant tumors. During the oxygen deprivation that follows the increase in size of a cancer mass before neovascularization sets in, HIF1α stabilization is pivotal to adapt to hypoxic conditions, mainly by upregulating the expression of glycolytic genes (Semenza et al. 1994). Moreover, it has been shown that hypoxic adaptation also requires HIF1α-mediated suppression of TCA cycle and OXPHOS (Kim et al. 2006; Papandreou et al. 2006). As mentioned earlier, *FH* and *SDH* mutations have been associated with tumorigenesis due to an aberrant HIF1α stabilization. Indeed, by slowing down oxidative metabolism during hypoxia, tumor cells decrease their oxygen requirements, and in this context, many mtDNA mutations have been shown to contribute to hypoxic adaptation. For instance, cells harboring the m.4776G > A/*MT-ND2* missense mutation showed an increased tumorigenicity and HIF1α accumulation (Zhou et al. 2007). These effects were due to the ROS-mediated upregulation of pyruvate dehydrogenase kinase 2, with a subsequent inhibition of pyruvate dehydrogenase and finally block of pyruvate entrance in the TCA cycle (Sun et al. 2009). Moreover, it has been shown that ROS-mediated HIF1α accumulation may lead to enhanced metastatic potential of cells harboring the m.13997G > A/*MT-ND6* missense mutation (Ishikawa et al. 2008). However, conversely from the hypoxic and anoxic areas of quickly growing primary tumors, it is interesting to note that metastatic cells ought to be well oxygenated. Consequently, revival of mitochondrial function may be profitable in such cells. Indeed, there are single reports of mtDNA mutations present in primary tumor, but purified in putative metastases (Horton et al. 1996). Therefore, it remains to be explained why mitochondrial damage should be an advantageous feature of pre-metastatic clones.

Besides the well-known negative regulation of mitochondrial function by HIF1α, through activation of pyruvate dehydrogenase kinase 1 (Kim et al. 2006; Papandreou et al. 2006), there are indications that HIF1α stabilization actually depends on functional mitochondria. For instance, several studies have reported that loss of respiratory complex III may contribute to ROS-mediated destabilization of HIF1α (Guzy et al. 2005; Mansfield et al. 2005). Moreover, it has been shown that dysfunction in complex I caused by the severe m.3571insC/*MT-ND1* mutation strongly contributes to HIF1α destabilization by alteration of TCA cycle metabolite balance (Gasparre et al. 2011). Allotopic expression of functional ND1 in *MT-ND1* osteosarcoma mutant cells demonstrated that a certain portion of functional

complex I is required for the establishment of the Warburg effect during response to hypoxia (Calabrese et al. 2013). In this context, it is interesting to note that, conversely from *SDH* and *FH* mutated tumors, a novel class of mutations in isocitrate dehydrogenase (*IDH*) 1 and 2 genes was recently reported in gliomas and acute myeloid leukemia (AML) (Koivunen et al. 2012; Losman et al. 2013). These mutations have been shown to induce the accumulation of (R)-2-hydroxyglutarate (R-2HG), an activator of prolyl-hydroxylases, thus causing consequent destabilization of HIF1α. This characteristic has been suggested to be responsible for less malignant behavior in glioma patients (Koivunen et al. 2012).

Therefore, it seems that a two-way relationship between hypoxic adaptation and mitochondrial function exists, in which mtDNA mutations may result as positive or negative modifiers of hypoxic adaptation, since the ETC is de facto a fundamental oxygen sensor as is the HIF1α pathway.

Signaling Pathways In order to maintain cellular homeostasis, mitochondria are capable of communicating with the nucleus through so-called retrograde signaling (Guha and Avadhani 2013). Among others, mitochondria-to-nucleus crosstalk includes Ca^{2+} (Biswas et al. 1999) and a redox-related flow of information (Ray et al. 2012), both of which may be altered due to a mitochondrial damage (Amuthan et al. 2002; Ishikawa et al. 2008). For instance, mtDNA depletion has been shown to induce a drop in membrane potential, which decreases Ca^{2+} uptake into mitochondria, increasing the levels of cytoplasmic Ca^{2+} concentrations, activating retrograde signaling, and promoting invasiveness of lung cancer cells (Amuthan et al. 2002). On the other hand, ROS signaling has been associated with AKT survival pathway. In particular, in cancer cells carrying heteroplasmic complex I damaging *MT-ND5* mutation, ROS- and NAD^+-/NADH-mediated phosphorylation of AKT led to upregulation of HIF1α activity (Sharma et al. 2011). These effects on AKT signaling pathway were specific to complex I damage, since mutations in COX only displayed a decrease in ATP and respiration defects, without affecting the ROS-AKT-HIF axis (Sharma et al. 2011). Similarly, AKT activation was induced by mtDNA depletion in a number of cancer cell lines, in which increases in NADH concentrations and subsequent decreases in NADPH were associated with PTEN inactivation and promotion of AKT signaling (Pelicano et al. 2006). Moreover, a CPEO-associated mtDNA mutation in *MT-TRNA* for leucine was shown to cause ROS-independent AKT activation, resulting in apoptosis resistance and increase in metastatic potential of breast cancer cells (Kulawiec et al. 2009a).

Phosphorylation of AKT leads to mTOR kinase activation, one of the best known pathways regulating cell metabolism (Memmott and Dennis 2009) and the main cellular sensor of nutrients promoting protein and lipid synthesis. Alternatively, mTOR is negatively regulated by AMPK, which switches off biosynthetic reactions in low energy conditions, in order to maintain cellular energy homeostasis (Hardie 2011). In the study by Sharma et al, complex I mutant cybrids displayed dephosphorylation of AMPK, in comparison to wild-type mtDNA cybrids. Conversely, severe mtDNA mutants appear to induce AMPK activation, which ultimately results in suppression of tumor growth, in agreement with the inferred role

of tumor suppressor for this crucial kinase (Iommarini et al. 2014). Therefore, depending on their type and mutant load, mtDNA mutations may influence mTOR signaling through both AKT and AMPK pathways.

Furthermore, it has recently been demonstrated that mtDNA depletion may lead to increased expression of HMGR, a rate-limiting enzyme in the mevalonate pathway, which is an important source of farnesyl moieties (Cook and Higuchi 2012). The latter are used for K-Ras prenylation, a posttranslational modification that allows K-Ras/Raf complex formation and downstream AKT activation. That study provides an interesting example on how mitochondrial function is connected to the cellular signaling pathways and suggests that targeting such metabolic reactions may be potentially exploited in cancer therapy development.

Apart from mTOR, a number of other signaling pathways have been shown to be influenced by mitochondrial damage, such as ERK1/2-MAP kinase (Weinberg et al. 2010) and NFKB2 (Higuchi et al. 2002; Dasgupta et al. 2008; Jandova et al. 2012b).

Taken together, it is emerging that oncogenic signaling pathways may depend on mitochondrial function, among others, through alteration of cellular ROS and Ca^{2+} concentrations and by modulating the availability of metabolites involved in pro-oncogenic signaling.

Epigenetics Oncogenes and tumor suppressors are known to regulate metabolic reprogramming during cancer progression (Ward and Thompson 2012) and thus create selective pressures under which mtDNA mutations may be accumulated or purified. In turn, mitochondrial damage induced by mtDNA mutations may have consequences on oncogenes and tumor suppressors function by regulating their gene expression, indicating that oncogenic properties may be transmitted from mitochondria to the nucleus (Ma et al. 2010; Jandova et al. 2012b). It was shown that certain mtDNA haplogroups may influence nuclear methylation patterns (Bellizzi et al. 2012) and that mtDNA depletion leads to general hypomethylation of nuclear genes, potentially promoting activation of oncogenes (Smiraglia et al. 2008).

Since epigenetic control of gene expression involves a series of enzymatic reactions, including DNA and histone methylation, acetylation, hydroxylation, and phosphorylation, mechanisms through which mitochondrial alterations may regulate nuclear gene expression mainly concern the availability of metabolic substrates or cofactors required for these reactions (Naviaux 2008). For instance, the NAD^+/NADH ratio is important for the activities of sirtuin histone deacetylases (Imai et al. 2000); TCA cycle intermediates have been shown to regulate histones and DNA demethylation reactions by Jumonji C-domain-containing histone lysine demethylases (JHDM) and ten-eleven translocation family of 5-methylcytosine hydroxylases (TET) (Lu and Thompson 2012), and cytosolic citrate is converted to acetyl-CoA required as a donor of acetyl groups for HAT-mediated histone acetylation (Wellen et al. 2009). Moreover AMPK, activated by a low ATP/AMP ratio, is able to phosphorylate histones (Bungard et al. 2010). The abundance of metabolic substrates and cofactors in epigenetic mechanisms is highly dependent

on the metabolic state of a cell and may be regulated by a mitochondrial damage induced both by mtDNA mutations and mutations in nuclear genes encoding mitochondrial proteins. The latter have been particularly often associated with epigenetic reprogramming in cancer, since TCA cycle intermediates may inhibit α-ketoglutarate-dependent dioxygenases involved in DNA and histone demethylation, as observed in tumors harboring dysfunctional FH and SDH where fumarate and succinate have been shown to inhibit JHDM and TET protein function by competitive inhibition (Xiao et al. 2012). Along this line, increase in R-2HG concentrations, caused by *IDH* mutations, has been shown to induce epigenetic alterations that affect cell differentiation in gliomas and AML, eventually leading to cell transformation (Koivunen et al. 2012; Losman et al. 2013). Interestingly, the same metabolite has been associated with inability to stabilize HIF1α, which may at least partly explain the more favorable prognosis in glioma patients carrying *IDH* mutations (Koivunen et al. 2012; Losman et al. 2013).

Furthermore, it was recently discovered that DNA methyltransferase 1 may translocate into mitochondria (Shock et al. 2011) and methylate mtDNA CpG islands. Interestingly, the distribution of CpG islands in mtDNA reveals that they are more common in the regulatory D-loop region, where the control of the whole polycistron resides, and less frequent than expected by chance in mitochondrial tRNA and coding genes (Chinnery et al. 2012). Along this line, it has been shown that mutations in mtDNA may influence its own methylation causing metabolic alterations potentially important for cancer progression (Raimundo et al. 2012).

Autophagy Autophagy is the main cellular route for degradation of bulk cytoplasm and organelles, which may lead either to cell death (Gozuacik and Kimchi 2004) or to the recycling of cellular building blocks for macromolecule synthesis in stress conditions such as hypoxia (Lozy and Karantza 2012). Generally, it is inhibited by high nutrient availability and growth factors, whereas ROS, hypoxia, and starvation promote autophagy (Lozy and Karantza 2012). In normal conditions, autophagy accounts for removal of damaged and permeabilized mitochondria (mitophagy) and thus counteracts proapoptotic effects of mitochondrial outer membrane permeabilization (Hill et al. 2012). In cancer, autophagy seems to be context dependent (Lozy and Karantza 2012). On one hand, the decrease in autophagy often observed in cancer may permit the relaxation of negative selection and promote accumulation of proapoptotic mtDNA mutations. Along this line, complementation of mitochondrial damage caused downregulation of mTOR signaling pathway and subsequent increase in autophagy, inhibiting the metastatic activity of breast cancer cells (Santidrian et al. 2013). Conversely, *KRAS*-driven tumorigenesis was shown to upregulate autophagy in order to maintain functional mitochondrial metabolism, necessary for propagation of such tumors (Guo et al. 2011). In such *KRAS*-driven models, defects in autophagy were shown to mimic the antitumorigenic effects of severe mtDNA mutations by redirecting cancer progression from carcinoma to benign oncocytoma (Guo et al. 2013). The relationship between mitochondria, autophagy, and tumor progression is therefore not straightforward, and further studies are warranted to resolve their connections.

10.7 Oncocytic Tumors

Cells of certain tumor types are characterized by abnormal accumulation of mitochondria in their cytoplasm, a phenotype that in pathology is referred to as oncocytic change (Tallini 1998). Oncocytic tumors are mainly observed in lesions of endocrine tissues such as the thyroid (Gasparre et al. 2010a), kidney (Akhtar and Kott 1979), parotid, or pituitary gland (Silbergeld et al. 1993), but have also been described in cases of breast, colon, lung, glioblastoma, nasopharynx, and endometrial cancers (Damiani et al. 1998; Gasparre et al. 2010b; Guerra et al. 2011; Marucci et al. 2013). Mitochondria of oncocytic cells are often damaged, as observed from electron micrographs showing their swollen morphology and deranged cristae. This has been partly explained by the high frequency of pathogenic mtDNA mutations that is found in oncocytic lesions. Depending on the tissue type, 45–100 % of such neoplasms carry some kind of mtDNA mutations, which is significantly more frequent than what is observed in their non-oncocytic counterparts (Gasparre et al. 2007, 2010b; Porcelli et al. 2010; Pereira et al. 2012; Kurelac et al. 2013). In particular, frameshift and nonsense mtDNA mutations are far more common and are often homoplasmic in oncocytomas, resulting in substantial defects in mitochondrial function.

It has been hypothesized that the mitochondrial hyperplasia in oncocytic tumors is a direct consequence of mtDNA mutations, since the resulting mitochondrial damage may provoke increased organelle biogenesis in order to compensate for the bioenergetic defect (Savagner et al. 2001). In fact, introduction of a severe complex I disruptive homoplasmic mtDNA mutation in cybrid cells was shown to lead to the development of osteosarcoma with an oncocytic phenotype, followed by a less efficient tumor settlement and decreased tumor growth, compared to their wild-type or heteroplasmic counterparts (Gasparre et al. 2011). This is in line with the recent revision of the Warburg hypothesis, which suggests that mitochondrial function must not be completely abolished, since it would lead to lethality. In fact, oncocytic tumors are most often low aggressive and are usually considered benign quiescent neoplasias (Gasparre et al. 2010b; Rossi et al. 2013), as supported by a lower genomic instability in pituitary oncocytomas (Kurelac et al. 2013). Functional studies in athymic mice demonstrate that the most common mtDNA mutation found in oncocytomas, namely, the m.3571insC/*MT-ND1*, is capable of abolishing osteosarcoma growth in vivo and that compensation with a functional ND1 reverts the phenotype and reestablishes tumorigenic potential (Gasparre et al. 2009; Calabrese et al. 2013). This mutation, when homoplasmic, leads to abolishment of complex I function and affects efficiency of complex I assembly. In particular, it results in a truncated ND1 subunit which most likely hampers the formation of the binding site between different assembly modules of the complex, potentially affecting the whole respirasome (Vogel et al. 2007; Baradaran et al. 2013). In agreement with the main role of complex I in sustaining mitochondrial membrane potential and cellular redox homeostasis by oxidation of NADH, in the m.3571insC/*MT-ND1* homoplasmic cancer models, both ATP production and

NAD^+/NADH ratio were significantly reduced when compared to the heteroplasmic counterparts and wild-type cells, leading to activation of AMPK (Iommarini et al. 2014) and destabilization of HIF1α (Calabrese et al. 2013), respectively. These studies demonstrate that functional complex I is necessary for the activation of the HIF1α pathway and for the establishment of the Warburg effect (Calabrese et al. 2013), explaining at least in part why oncocytic tumors carrying such damage often do not progress to the malignant state. In addition, the inability to stabilize HIF1α may contribute to increased mitochondrial hyperplasia in oncocytic tumors, since this transcription factor is a known negative regulator of mitochondrial biogenesis (Papandreou et al. 2006; Bartoletti-Stella et al. 2013).

It is interesting to note that an oncocytic change, followed by tumor growth arrest, was recently observed to be triggered during *KRAS*-driven cancer transformation when the pivotal autophagy-regulating gene was knocked out (Guo et al. 2013). This suggests that mtDNA mutations causing inadequate function of autophagic machinery may also contribute to the mechanism of oncocytic phenotype development.

Despite being a relatively rare entity, oncocytic tumors have provided many insights on the role of mtDNA mutations and mitochondrial metabolism in cancer. Most importantly, analyses of mtDNA mutations in oncocytic tumors allowed to identify a novel class of cancer-associated genes, namely, the oncojanus, that may behave in a twofold manner. Depending on the mtDNA type and mutant load, the same gene may behave as both tumor suppressor and as a lethality gene, underlining the importance of the application of appropriate measures when analyzing mtDNA mutations in cancer.

Conclusions

The consequences of mtDNA mutations are numerous and primarily depend on the type of damage, i.e., which part of the ETC is involved, to what extent, and during which phase of tumorigenesis. It is unlikely that mtDNA mutations on their own are sufficient to initiate tumorigenesis, but they may provide a fertile ground for adaptive mechanisms and may modify tumor progression. The general rule of thumb seems to be that mutations which cause severe mitochondrial damage leading to extreme metabolic conditions usually compromise tumor growth, whereas mild mutations which allow fine-tuning of tumor metabolism may be advantageous since they represent a pool of adaptive tools in cancer cell evolution. The most intriguing point is that an mtDNA mutation may in some contexts act as a neutral bystander, whereas in others it becomes advantageous for tumor growth, and thus appropriate analyses must be performed in order to assign a role for mtDNA mutations in tumor progression, especially in the context of using mtDNA mutations as diagnostic and prognostic markers in oncology.

(continued)

In this context, the advent of NGS, which has already revolutionized the field of genomics, is expected to further elucidate the mechanisms of mitochondrial genetics in cancer (He et al. 2010; Tang and Huang 2010; Zaragoza et al. 2010; Payne et al. 2013). It is peculiar to observe that the mtDNA does not represent the direct target in any of the currently used high-throughput sequencing methods, but is paradoxically retained as off-target sequence because of the overlapping nuclear probes designed against NumtS (Pesole et al. 2012; Samuels et al. 2013). As a consequence of this unintended contamination with mtDNA in NGS, a series of bioinformatics tools have been proposed to recover the lost mtDNA information from high-throughput sequencing (Picardi and Pesole 2012; Calabrese et al. 2014). Therefore, large-scale mtDNA genotyping in public cancer studies is actually possible and will inevitably lead to even better understanding of mtDNA selection in cancer and thus to a further elucidation of the roles of mitochondria and metabolic reprogramming in this complex disease.

Overall, the combination of next generation genetic approaches in mitochondrial genomics, sustained by a deeper understanding of the metabolic consequences that revolve around this pivotal organelle, is likely to provide oncology not only with powerful prognostic tools but also with a novel array of strategies and candidates for the development of anticancer therapies.

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Chapter 11
The Relevance of the Mitochondrial H^+-ATP Synthase in Cancer Biology

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11.1 Introduction

During the last decade, energy metabolism has become a central issue of investigation in cancer studies. Indeed, energy metabolism regulates the complex network of cellular processes that allow adaptation of the cell to different normal and pathological situations. Uncontrolled proliferation is not an exception because it is very much influenced by changes in the activity of the metabolic pathways of the cell. The dependency of cancer cells on aerobic glycolysis was basically neglected for many years due in part to the prevailing idea that cancer was considered only a genetic disease. Nowadays, thanks to the effort of many laboratories, cancer can also be regarded as a metabolic disease since its onset and progression cannot be understood without the reprogramming of energy metabolism of the cells.

One of the most powerful techniques available in oncology for the diagnosis, staging, and follow-up of most cancer patients is tumor imaging using ^{18}F-deoxyglucose positron emission tomography (^{18}FDG-PET) (Rigo et al. 1996). This technique is based on the high glucose avidity of cancer cells (Ortega et al. 2009), and it represents the translation to the clinics of the original Warburg (Warburg 1930) observation: "cancer cells have an enhanced aerobic glycolysis when compared to normal cells." In addition to the differential uptake of FDG, some human cancers also display a differential uptake of choline, acetate, and some amino acids, suggesting a heterogeneous alteration of the metabolic pathways as solution to cover the energy and building blocks demands of proliferating cancer cells (Cairns et al. 2011). In the present chapter, we will review (1) the overall changes of the metabolic pathways that support proliferation and (2) the findings that implicate the bioenergetic alteration of mitochondria in carcinogenesis. Other related topics that integrate mitochondrial bioenergetics with the cell death machinery and the mechanisms that trigger the silencing of oxidative phosphorylation (OXPHOS) in prevalent human carcinomas have been recently summarized elsewhere (Willers and Cuezva 2011; Sanchez-Arago et al. 2013a).

11.2 Overview of the Changes in Metabolic Pathways During Proliferation

Glycolysis and mitochondrial OXPHOS are the two main pathways of energy provision that are tightly and inversely regulated in the cell (Fig. 11.1). The control of the flux through these pathways is primarily exerted by regulation of the activity of key enzymes by the availability of metabolic intermediates such as ATP and NADH and/or by genetic regulation of the expression of the genes involved (Formentini et al. 2010). In normal differentiated aerobic cells, glucose is partially oxidized to pyruvate by glycolysis in the cytoplasm (Fig. 11.1). Pyruvate can be further oxidized in mitochondria to form the acetyl-CoA that enters the tricarboxylic acid cycle (TCA) for its complete oxidation to CO_2, or it is reduced and

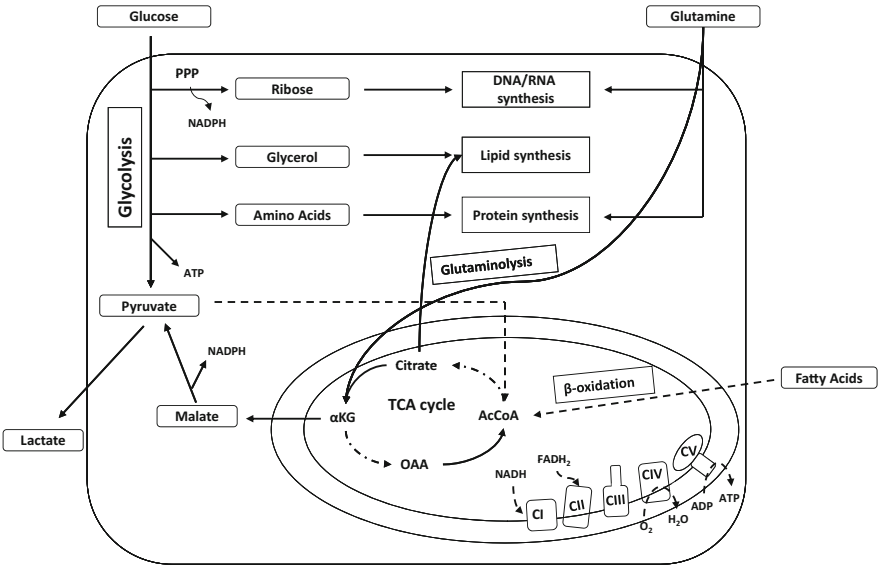

Fig. 11.1 Metabolic pathways in tumor cells. The scheme shows some of the relevant aspects of the metabolism of cancer cells. After entering the cell by specific transporters, glucose enters the glycolytic pathway, and it can be (1) catabolized by the pentose phosphate pathway (PPP) to obtain ribose required for the synthesis of nucleotides and reducing power in the form of NADPH, (2) used for the synthesis of glycerol and amino acid precursors that are needed for the synthesis of lipid and proteins, respectively, or (3) utilized to generate pyruvate. In the cytoplasm, the generated pyruvate can be reduced to lactate and further excreted from the cell or oxidized in the mitochondria by pyruvate dehydrogenase to generate acetyl-CoA. The operation of the tricarboxylic acid cycle (TCA cycle) and the activity of oxidative phosphorylation (OXPHOS) complete the oxidation of mitochondrial pyruvate. The transfer of electrons obtained in biological oxidations ($NADH/FADH_2$) to molecular oxygen by respiratory complexes (CI to CIV) of the inner mitochondrial membrane and the synthesis of ATP by the H^+-ATP synthase (CV) are also represented. Different pathways that drain intermediates of the TCA cycle (oxaloacetate (OAA), α-ketoglutarate (αKG), malate, and citrate), for biosynthetic purposes are shown. The incorporation of glutamine carbon skeletons into the TCA cycle by glutaminolysis and its diversion to other biosynthetic pathways is depicted as well as the conversion of fatty acids into acetyl-CoA by β-oxidation. Continuous arrows represent activated pathways in cancer cells, whereas discontinuous lines represent repressed pathways in cancer cells

excreted from the cell as lactate if oxygen availability is limited and/or mitochondria have an impaired function (Fig. 11.1). The oxidation of fatty acids in mitochondrial β-oxidation is an additional pathway that supplies energy and acetyl-CoA in many normal aerobic cells (Fig. 11.1). Under normal oxygenated conditions, the electrons obtained in the oxidation of acetyl-CoA are collected in the *redox* coenzymes NADH and $FADH_2$. These electrons are funneled into the respiratory chain which is placed in the inner membrane of mitochondria to reduced O_2 and generate the water of respiration (Fig. 11.1). Electron transfer in the respiratory chain promotes proton pumping from the matrix interior to the intermembrane space to generate the proton gradient that is used as intermediate for the synthesis of

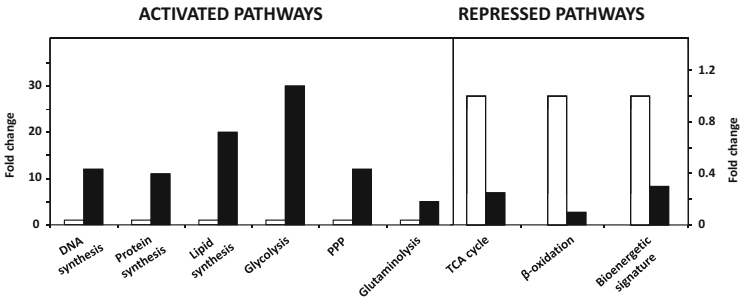

Fig. 11.2 Metabolic reprogramming in proliferating cells. *Plots* represent changes in the activity of metabolic pathways when comparing proliferating and nonproliferating cells. Increased rates of DNA, lipid, and protein synthesis support high rates of cellular proliferation. Highly proliferative cells are dependent on enhanced activities of glycolysis, glutaminolysis, and the pentose phosphate pathway (PPP). The activities of the TCA cycle and β-oxidation are repressed in proliferative cells when compared to quiescent cells. A change in the bioenergetic signature between normal (quiescent) and tumor (proliferative) tissues is also shown. Data taken from (Wang and Green 2012b)

ATP in OXPHOS. The reentrance of H^+ through the H^+-ATP synthase (CV in Fig. 11.1) harnesses most of the ATP that is utilized by normal aerobic differentiated cells.

Proliferative cells exhibit different metabolic requirements when compared to normal differentiated cells (Fig. 11.2) (Vander Heiden et al. 2013; DeBerardinis et al. 2008; Wang and Green 2012a, b; Brand and Hermfisse 1997). Highly proliferative cells have an increased demand of carbon skeletons, nitrogen, and reducing equivalents to duplicate the mass of the cell that supports cell division in addition to the requirements needed to maintain housekeeping functions. This situation makes necessary the consumption of more nutrients and the diversion of metabolic precursors into the pathways of macromolecular synthesis (Figs. 11.1 and 11.2). Thus, proliferating cancer cells experience the reprogramming of metabolism to balance biosynthetic pathways with those that supply ATP to assure cellular growth and survival (Sanchez-Arago et al. 2013a; Cuezva et al. 2009). However, it has been reported that ATP demand is not significantly different between proliferating and differentiated cells (Vander Heiden et al. 2013; Kilburn et al. 1969; Locasale and Cantley 2010; Sanchez-Arago et al. 2013b), suggesting that maintenance of the differentiated state is also a very energy demanding process.

As mentioned above, a fundamental feature of proliferating cancer cells is the increased uptake of glucose and its conversion to lactate in the presence of oxygen (Fig. 11.1), the so-called enhanced aerobic glycolysis of carcinomas. The abnormal rate of glycolysis of cancer cells was ascribed to an altered mitochondrial function as a compensatory mechanism to supply the energy required for proliferation (Warburg 1956a, b). It is well established that the rate of ATP production by OXPHOS determines the rate of glucose consumption by the cell (Sanchez-Arago et al. 2010). However, Warburg's hypothesis was taken with suspicion by the

scientific community because it appeared paradoxical that cancer cells that theoretically have an increased need for ATP relied on the less efficient glycolytic pathway for ATP provision. It is now clear that the catabolism of glucose through glycolysis provides cancer cells with other metabolic needs in addition to ATP (Fig. 11.1). In fact, glucose carbon skeletons provide ribose and NADPH for nucleotide biosynthesis through the pentose phosphate pathway (Fig. 11.1), glycerol and citrate for the synthesis of lipids and cholesterol (Fig. 11.1), and other intermediates for the synthesis of nonessential amino acids (Fig. 11.1). Many cancer cells have an increased flux from glucose into serine and glycine (de Koning et al. 2003) that represent important intermediates in the building of proteins, lipids, and nucleic acids (Possemato et al. 2011; Locasale et al. 2011) and also help cells to mitigate oxidative stress (Mullen and DeBerardinis 2012). These amino acids are generated by the glycolytic enzyme phosphoglycerate dehydrogenase (PHGDH) that has been found to be activated in tumors (Locasale et al. 2011; Pollari et al. 2011) and by an alternative glycolytic pathway that seems to be controlled by the inhibition of PKM2 (Vander Heiden et al. 2013). It has been suggested that aerobic glycolysis could provide an additional advantage to proliferative cells because it allows cells to use glucose at a faster rate when compared to OXPHOS (Pfeiffer et al. 2001; Koppenol et al. 2011).

Anabolic processes also require a source of reducing power in the form of NADPH that, similar to ATP, must be constantly generated to support the biosynthetic processes. The catabolism of glucose by the pentose phosphate pathway (PPP) is a major source of NADPH production in cancer cells (Fig. 11.1). It has been reported that the flux through the oxidative part of the PPP is not activated in some proliferating cells (Boros et al. 2000), suggesting that other pathways might also participate in the replenishment of the NADPH required for synthetic purposes (Fig. 11.1). The generation of NADPH also contributes to the maintenance of the cellular *redox* state by keeping glutathione in the reduced state.

The activity and purpose of the TCA cycle is an important aspect that differentiates quiescent and proliferative cells (Fig. 11.2). Whereas in quiescent cells the TCA cycle is mainly used to maximize the production of ATP, in proliferative cells the TCA cycle plays a role as a source of intermediates for biosynthetic purposes in processes that consume ATP. For example, mitochondrial citrate is exported out to the cytoplasm (Fig. 11.1) where it is converted to oxaloacetate (OAA) and acetyl-CoA. This process is essential for de novo synthesis of fatty acids and cholesterol required for the building up of membranes of the proliferating cells (Kannan et al. 1980; Ookhtens et al. 1984). Tumor cells and hematopoietic cells display an upegulated expression of the lipogenic enzymes ATP citrate lyase and fatty acid synthase which are both important to stimulate proliferation (Bauer et al. 2005; Hatzivassiliou et al. 2005). OAA and α-KG are also involved in the biosynthesis of macromolecules as they constitute an intracellular pool of precursors required for the synthesis of nonessential amino acids and nucleotides.

The catabolism of glutamine, the most abundant amino acid in mammals, is also relevant during proliferation (Figs. 11.1 and 11.2) (DeBerardinis et al. 2008; Cuezva et al. 2009). In proliferating cells, glutamine is metabolized by multiple

pathways for different purposes (Fig. 11.1) (Eagle et al. 1956; Kovacevic and McGivan 1983). Glutamine is the nitrogen donor for several metabolic enzymes and for de novo synthesis of purines and pyrimidines (Fig. 11.1). In addition, glutamine can be partially oxidized by glutaminolysis providing NADPH and lactate to the cell (Fig. 11.1) (Reitzer et al. 1979). Glutamine is also essential to reload the TCA cycle with carbon skeletons as a result of citrate being exported from mitochondria for lipid synthesis (Fig. 11.1). Glutamine anaplerosis of the TCA cycle favors the biosynthesis of many precursors that derive from the intermediates of the cycle (DeBerardinis et al. 2008; Mullen and DeBerardinis 2012). The noncanonical conversion of glutamine into α-ketoglutarate (α-KG) that is directed to the synthesis of isocitrate and finally citrate is especially important for de novo lipogenesis to support tumor growth when the mitochondrial function is impaired or in hypoxic conditions (Mullen et al. 2011; Wise et al. 2011; Metallo et al. 2011). The reductive glutamine metabolic pathway has been found in adipose cells, in T cells, and in transformed cancer cells (Metallo et al. 2011; Des Rosiers et al. 1994; Yoo et al. 2008; Le et al. 2012). Recently, glutamine has been shown to be essential to support pancreatic cancer growth by a series of metabolic reactions that are signaled by the oncogene KRAS (Son et al. 2013). Alanine is another important amino acid involved in transaminations and in the synthesis of new proteins (Moreadith and Lehninger 1984). Alanine has been shown to be more abundant in prostate cancer tissue providing a biomarker of the disease (Costello and Franklin 2005; Tessem et al. 2008). The increase in alanine concentration of cancer cells could be a consequence of an increase in the catabolism of cellular proteins and/or of the increased protein synthesis required.

Rapidly proliferating cells also rely on an altered lipid metabolism (Fig. 11.2). The bulk of cell membrane lipids are phospholipids, sterols, and sphingolipids. The production of these lipids requires fatty acids as building blocks that come from either exogenous sources or from de novo fatty acid synthesis. While most normal human cells prefer exogenous sources, tumor cells have developed the capability to easily synthesize fatty acids de novo (Ookhtens et al. 1984; Medes et al. 1956). Consistent with these findings, the expression of CD36 which is a widely expressed transmembrane protein involved in the uptake of fatty acids is implicated in breast cancer and significantly decreases its expression in malignant and stromal tissue as an early step in tumorigenesis (DeFilippis et al. 2012). As we have discussed, de novo synthesis of fatty acids for the formation of membranes often derives from glucose and glutamine catabolism (Currie et al. 2013). However, some tumors also consume lipids from their environment rendering fatty acid uptake as a potential therapeutic target. For example, fatty acid binding protein 4 (FABP4), a lipid chaperone, is involved in providing fatty acids from surrounding adipocytes to ovarian tumors increasing metastasis and tumor growth (Nieman et al. 2011). The treatment of prostate cancer cells with fatty acid synthase (C75) or ATP citrate lyase (SB-204990) inhibitors showed reduced viability of the cells only when cultured in the absence of lipoproteins, an exogenous lipid source (Ros et al. 2012). Figure 11.2 provides a summary of the changes and relative activities in metabolic pathways observed in proliferating cells.

11.3 The Bioenergetic Function of Mitochondria in Cancer Cells

If the induction of glycolysis in cancer cells is nowadays no longer questioned, the impairment of mitochondrial function is still a subject of debate (Koppenol et al. 2011; Krebs 1981; Warburg 1966; Weinhouse 1976; Dang 2010; Schulze and Harris 2012). A key player in regulating the bioenergetic function of mitochondria is the H^+-ATP synthase (Fig. 11.1), a reversible engine located in the inner mitochondrial membrane that synthesizes or hydrolyzes ATP upon changes in cellular conditions. The mammalian H^+-ATP synthase is a multiprotein complex of 650 kDa that consists of two main domains: F0, which is a hydrophobic subcomplex embedded in the inner membrane and contains the proton channel, and the hydrophilic F1 subcomplex exposed to the matrix that is responsible for the catalytic function (Boyer 1997). The F1 portion consists of three catalytic subunits (β-F1-ATPase) and three regulatory subunits (α-F1-ATPase) that are arranged around a central stalk (the γ subunit) as segments of an orange (Abrahams et al. 1994). The F0 domain is a rotary motor that uses the proton flux to generate the conformational changes in the F1 domain needed for the synthesis of ATP (Boyer 1993; Capaldi and Aggeler 2002; Yoshida et al. 2001). The ATPase inhibitory factor 1 (IF1) is a physiological inhibitor of the H^+-ATP synthase that exerts a very important regulation of its activity in cancer (Sanchez-Cenizo et al. 2010; Sanchez-Arago et al. 2012).

Despite recent claims that cancer cells retain a functional bioenergetic activity of mitochondria (Koppenol et al. 2011; Dang 2010; Schulze and Harris 2012), a large number of studies established the impaired bioenergetic function of the organelle in the cancer cell [for a detailed review, see Pedersen (1978)]. To list some, it was shown that pyruvate dehydrogenase of tumor mitochondria kinetically resembles that of embryonic tissues (Lazo and Sols 1980). As indicated for proliferating cells (Fig. 11.2), the Krebs cycle in tumor mitochondria was shown to be truncated at the level of the conversion to citrate (Parlo and Coleman 1984), later on confirmed by a proteomic approach (Bi et al. 2006). In this situation, glutamine primes the TCA cycle with carbon skeletons (DeBerardinis et al. 2008; Baggetto 1992). Deficiencies in enzyme complexes of the respiratory chain and oxidative phosphorylation have also been described in cancer (Krieg et al. 2004). Consistent with some of the molecular and functional alterations described in cancer mitochondria, the organelles have less *cristae* than in normal cells (Pedersen 1978; Cuezva et al. 2002). Likewise, carcinomas have a diminished mitochondrial content when compared to noncancerous cells (Pedersen 1978; Cuezva et al. 2002; de Heredia et al. 2000). Mutations in mitochondrial DNA of human carcinomas (Carew and Huang 2002) and in nuclear genes involved in energy transduction are known to predispose to some types of inherited neoplasia syndromes (Eng et al. 2003). Conversely, a recent study has demonstrated that the introduction of noncancerous mitochondria into highly metastatic cells (cybrid) reverses the oncogenic characteristics by inhibiting tumor promoting pathways of the cells (Kaipparettu et al. 2013), thus supporting

that rectifying mitochondrial function is a promising target in cancer therapy. All these findings ultimately suggest that by one mechanism or another mitochondrial activity of the cancer cell is compromised.

Transcriptomic, proteomic, functional, and structural studies of human carcinomas strongly emphasize that a repressed bioenergetic activity of mitochondria is concomitant with the enhanced glycolytic flux required for tumor progression (Cuezva et al. 2009; Martinez-Reyes et al. 2012). To list few examples, it has been documented that the transformed state is accompanied by an increased flux of glycolysis due to aberrant mitochondrial respiration (Ramanathan et al. 2005). In H-RASV12/E1A-transformed cells, the tumorigenic potential of the cells is directly proportional to the enhancement of glycolysis and the suppression of mitochondrial respiration (de Groof et al. 2009). Other findings demonstrate that the previous selection of cancer cells with a diminished bioenergetic activity of mitochondria is needed for in vivo tumor progression (Sanchez-Arago et al. 2010). Importantly, the acquisition of the metabolic phenotype that is compatible with tumor progression is a reversible trait acquired by adaptation of the cells to the milieu where tumors develop in vivo (Sanchez-Arago et al. 2010). In gliomas, it has been documented that the enhanced infiltration in the brain parenchyma parallels the repression of mitochondrial biogenesis and an increased glycolysis (Keunen et al. 2011). A recent study has reported that inhibition of complex I of the mitochondrial electron transport chain promotes breast cancer cell migration and invasion by the upregulation of HIF1α and VEGF as a consequence of an increased production of ROS (Ma et al. 2013).

Hence, to understand the role of mitochondria in cancer biology, it is necessary to take into account other aspects different from the impact of cancer genes in energy metabolism. Specifically, we have to consider the inverse correlation that exists between the activity of OXPHOS and glycolysis (Sanchez-Arago et al. 2010; Isidoro et al. 2005; Aldea et al. 2011). As it has been extensively discussed, the activation of several oncogenes and the repression of tumor suppressors do have an impact on the metabolic switch of the cells. However, the overwhelming list of different mutations described so far in cancers cannot explain why most prevalent carcinomas, independently of the tissue of origin, histological type, and the diversity of the genetic alterations that they contain, converge on the same protein signature of energy metabolism (Acebo et al. 2009). The simplest explanation is that cancer cells just reproduce the bioenergetic phenotype of the metabolism of proliferating cells (Fig. 11.2). In fact, the rewiring of energy metabolism to an enhanced glycolysis or to an increased dependence on OXPHOS is a two-way path through which cells can transit in any direction without the need of genetic alterations. In other words, it is a reversible trait that can be taken in either of the two directions depending upon environmental, developmental, and/or pathological cues. Some relevant examples to understand the bioenergetic phenotype of carcinomas are the metabolic changes experienced by cells during adaptation to hypoxic milieus (Semenza 2008, 2011) and the dedifferentiation of adult somatic cells into iPS cells (Folmes et al. 2011), where the activation of glycolysis is concurrent with the repression of OXPHOS. Two other examples walking down the opposite

direction of the trail are the metabolic rewiring experienced by hepatocytes from the fetal to the neonatal stage (Cuezva et al. 2007) and the differentiation of stem cells into different cellular lineages (Sanchez-Arago et al. 2013b; Rehman 2010; Chen et al. 2008). In these latter cases, it involves the repression of glycolysis with the further concurrent development of the bioenergetic function of mitochondria. Development further affords the example of the switch in the expression of protein isoforms typical of cancer cells and embryonic tissues to adult-type proteins (Cuezva et al. 1997, 2007; Mazurek 2011). Moreover, the parallelism between cancer and embryonic cells not only impinges on the content and dedifferentiated state of mitochondria (de Heredia et al. 2000; Cuezva et al. 2007) but also in the structure of the organelle since they have less *cristae* when compared to mitochondria in normal cells (Cuezva et al. 2002; Arismendi-Morillo 2011). It is well established that dimers of the H^+-ATP synthase promote the high local curvature of the inner mitochondrial membrane at *cristae* ridges (Paumard et al. 2002; Strauss et al. 2008; Davies et al. 2011, 2012). Apparently, aging seems to meltdown the inner membrane *cristae* of mitochondria by age-dependent dissociation of ATP synthase dimers (Daum et al. 2013). We suggest that the lower relative expression of the H^+-ATP synthase in cancer cells as we will see in the next section explains the aberrant structure of mitochondria observed in carcinomas.

11.4 The Clinical Relevance of the Bioenergetic Signature of Cancer

As mentioned above, studies in development highlighted the inverse correlation that exists between the expression of a key component of OXPHOS such as the catalytic subunit of the H^+-ATP synthase (β-F1-ATPase) with the expression and activity of the enzymes of glycolysis (Cuezva et al. 1997, 2007). These observations prompted us to design a simple test to determine the protein signature of energy metabolism (Cuezva et al. 2002) that could be translated into the cancer field with the purpose of contrasting the feasibility of Warburg's hypothesis (Warburg 1956a, b). With this aim, we studied in normal and tumor biopsies derived from the same cancer patients the expression level of two mitochondrial proteins: β-F1-ATPase and Hsp60, respectively, representing the bioenergetic and structural function of mitochondria. We also determine the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as representative of the glycolytic pathway (Cuezva et al. 2002; Isidoro et al. 2004). Whereas the β-F1-ATPase/Hsp60 ratio provides an index of the bioenergetic competence of the organelle, the β-F1-ATPase/Hsp60/GAPDH or the β-F1-ATPase/GAPDH ratio provides indexes of the overall mitochondrial potential of the cell, which are two levels that could compromise the bioenergetic activity of mitochondria in carcinomas (Cuezva et al. 2002). The ratio that estimated the overall mitochondrial potential of the

cell was defined as the bioenergetic cellular (BEC) index (Cuezva et al. 2002), nowadays simplified to the β-F1-ATPase/GAPDH ratio (Lopez-Rios et al. 2007).

Consistent with Warburg's postulates, a significant reduction of the β-F1-ATPase/Hsp60 ratio was observed in colon, lung, breast, esophageal, gastric, and renal carcinomas when compared to paired normal tissues (Cuezva et al. 2002; Isidoro et al. 2004), suggesting a deficit in the bioenergetic competence of the mitochondrion. Remarkably, these changes in the mitochondrial proteome of the tumor were accompanied by the concurrent sharp increase of the glycolytic GAPDH marker (Cuezva et al. 2002; Isidoro et al. 2004) as well as by several other glycolytic proteins such as lactate dehydrogenase (Isidoro et al. 2005) and pyruvate kinase M2 (Isidoro et al. 2004, 2005; Aldea et al. 2011). Consistently, the estimation of the overall mitochondrial activity of the cell relative to its glycolytic potential (BEC index) revealed larger differences between normal and tumor tissues, thus supporting a bioenergetic deficit of mitochondria in the carcinomas (Cuezva et al. 2002). This proteomic feature was defined as the "bioenergetic signature of cancer" (Cuezva et al. 2002, 2009) and its downregulation has been confirmed and extended to different carcinomas (He et al. 2004; Hervouet et al. 2005; Meierhofer et al. 2004; Unwin et al. 2003; Yin et al. 2004; Lin et al. 2008) (see (Cuezva et al. 2009) for a recent review). More recently, we have functionally supported the relevance of Warburg's postulates after the demonstration that the rates of glucose capture assessed by FDG-PET imaging inversely correlate with the bioenergetic signature in lung carcinomas (Lopez-Rios et al. 2007). Moreover, the generation of isogenic HCT116 colon cancer cell lines expressing different levels of β-F1-ATPase, to assess the contribution of mitochondrial bioenergetics in cancer progression (Sanchez-Arago et al. 2010), allowed us to demonstrate that the activity of glycolysis and OXPHOS is inversely correlated, being the overall activity of OXPHOS the one that defines the rate of glucose utilization by aerobic glycolysis (Sanchez-Arago et al. 2010). Hence, interference with OXPHOS is part of the mechanism that triggers the increased glucose avidity of the cancer cell. Quantitative assays, using high-affinity monoclonal antibodies against proteins of the "bioenergetic signature" (Acebo et al. 2009), have revealed an unanticipated finding: tumors from different tissues and/or histological types have the same proteomic signature of energy metabolism, indicating that cancer abolishes the tissue-specific differences in the bioenergetic phenotype of the cell (Acebo et al. 2009). In other words, energy metabolism represents an additional hallmark of the cancer phenotype (Ortega et al. 2009; Hanahan and Weinberg 2000) and a promising target for the treatment of diverse neoplasias (Cuezva et al. 2009; Acebo et al. 2009).

Many of the potential cancer biomarkers that are being discovered at the bench-side are rarely translated in benefit of the management of the patients because they lack support from data obtained in clinically oriented studies. To bridge this gap, we have studied the bioenergetic signature of tumors in large cohorts of different cancer patients (Cuezva et al. 2002, 2004; Isidoro et al. 2005; Aldea et al. 2011; Lopez-Rios et al. 2007; Ortega et al. 2008) to explore its relevance as predictor of overall survival (OS) and/or of disease recurrence (DFS). Figure 11.3 provides

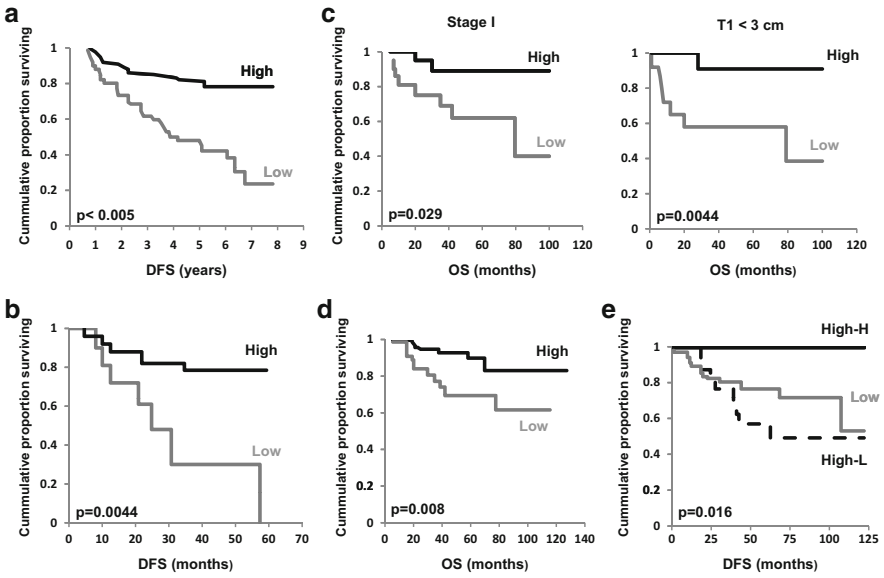

Fig. 11.3 Bioenergetic signature is a biomarker of cancer prognosis. Kaplan–Meier survival analysis show the association of markers of the bioenergetic signature with overall (OS) and/or disease-free (DFS) survival in different cohorts of cancer patients. (**a**) and (**b**). Significant association of the high expression level of β-F1-ATPase (**a**) and of the β-F1-ATPase/GAPDH ratio (**b**) (*black curves*) with better prognosis in two independent cohorts of colon cancer patients (Cuezva et al. 2002; Aldea et al. 2011). (**c**) A high BEC index (*black curve*) in early stage disease of lung cancer patients predicts better outcome (Cuezva et al. 2004). (**d**) A high β-F1-ATPase/Hsp60 ratio in breast carcinomas is a significant marker of better prognosis (Isidoro et al. 2005). (**e**) The combination of markers of the bioenergetic signature (high and low, *black and gray curves*, respectively) with the expression level of HuR [high (-H) and low (-L)] allows the discrimination of breast cancer patients that within the group of higher bioenergetic signature have higher risk of disease recurrence (*dotted curve*) because they have low expression of HuR (high-L) (Ortega et al. 2008). High and low expression levels are represented in *black and gray curves*, respectively. The log-rank test *p* value is shown. The curves have been redrawn from the indicated references

representative examples of this sort of studies in large cohorts of colon (Fig. 11.3a, b), lung (Fig. 11.3c), and breast (Fig. 11.3d, e) cancer patients. Immunohistochemical determination of β-F1-ATPase in a large cohort of stage II colon cancer patients using colon-tissue microarrays (Fig. 11.3a) indicated that a low tumor expression of β-F1-ATPase afforded an excellent marker of both the overall (not shown) and disease-free survival (Fig. 11.3a) of colon cancer patients (Cuezva et al. 2002). The clinical utility of β-F1-ATPase as a marker of colon cancer prognosis has been independently confirmed in a different large cohort of colon cancer patients (Lin et al. 2008). A more recent study in a more heterogeneous cohort of colon cancer patients using reverse phase protein microarrays, a technique that affords a quantitative approach to assess the bioenergetic signature (β-F1-ATPase/GAPDH ratio), provided essentially the same findings (Fig. 11.3b) (Aldea et al. 2011). It is worth mentioning here that a study in a large cohort of colon cancer patients investigating

the genetic alterations that impact on metabolic genes has concluded that the impairment of OXPHOS is the only metabolic pathway that correlates with patient prognosis (Sheffer et al. 2009). The bioenergetic signature also predicts the overall survival of lung cancer patients at early stages of the disease (stage I or tumor size less than 3 cm) (Fig. 11.3c), and the lower the bioenergetic signature (β-F1-ATPase/Hsp60/GAPDH ratio) of the tumor, as assessed by protein expression on 2D gels (Cuezva et al. 2004), the worse the prognosis for lung cancer patients (Fig. 11.3c). Similar results have been obtained in a different cohort of lung cancer patients using immunohistochemistry to assess β-F1-ATPase expression in the tumors (Lopez-Rios et al. 2007). In that study (Lopez-Rios et al. 2007), a low expression of the protein also correlated with a worse prognosis for lung cancer patients. Interestingly, multivariate Cox regression analysis indicated that tumor β-F1-ATPase expression in lung and colon carcinomas is an independent marker of survival (Aldea et al. 2011; Lopez-Rios et al. 2007). Similarly, the bioenergetic signature has also been shown to provide relevant markers of disease progression in breast cancer patients (Fig. 11.3d) (Isidoro et al. 2005). The robustness of markers of the bioenergetic signature for cancer diagnosis has been illustrated by Fisher linear discriminant analysis (Isidoro et al. 2005; Aldea et al. 2011; Cuezva et al. 2004). Using cross-validation, a classification sensitivity of lung, colon, and breast tumor biopsies >95 % has been reported, strongly supporting that alteration of the bioenergetic function of mitochondria is a hallmark of cancer. Overall, these studies emphasized that an impaired bioenergetic function of mitochondria compromises survival by favoring recurrence of the disease and cancer progression, strongly supporting the original Warburg hypothesis.

A translation repression mechanism regulates the expression of β-F1-ATPase in fetal rat liver (Izquierdo and Cuezva 1997) and in rat hepatomas (de Heredia et al. 2000). Consistently, the downregulation of β-F1-ATPase expression in prevalent human carcinomas (lung, breast, colon) is also exerted at posttranscriptional levels by a specific translation masking event of the β-F1-ATPase mRNA (Willers et al. 2010). Mechanistically, translational regulation could be explained by differences in the affinity of the mRNA to the translational machinery as well as by the action of regulatory proteins and miRNAs that bind sequence elements within the mRNA for controlling its translation (Willers and Cuezva 2011). In this regard, we have described that the 3′UTR of rat β-F1-ATPase mRNA is essential for translation of the transcript due to its ability to interact with components of the translational machinery (Izquierdo and Cuezva 1997). That is, the 3′UTR behaves as a translational enhancer both in vitro (Izquierdo and Cuezva 1997, 2000) and in transfected cells (Di Liegro et al. 2000). This activity of the 3′UTR is essential for conferring the appropriate bioenergetic phenotype to daughter cells during cellular proliferation because it drives the synthesis of β-F1-ATPase at the G2/M phase of the cycle (Martinez-Diez et al. 2006) when *cap*-dependant translation is partially inhibited (Pyronnet and Sonenberg 2001). More recently, we have demonstrated that the 3′UTR of human β-F1-ATPase mRNA is also required for efficient translation of the transcript (Willers et al. 2010).

The control of the translation of β-F1-ATPase mRNA during development (Izquierdo and Cuezva 1997) and in rat hepatomas (de Heredia et al. 2000) involves specific proteins that bind the rat transcript. The binding of these proteins is regulated by the energy and *redox* state of the cell (Izquierdo and Cuezva 2005). It is assumed that the binding of proteins to the 3′UTR of β-F1-ATPase mRNA sterically hinders the initiation of translation (Cuezva et al. 2007; Izquierdo and Cuezva 1997). With these findings in mind, we have pursued the molecular and functional characterization of the human β-F1-ATPase mRNA interacting proteins (Ortega et al. 2008, 2010). We have identified that the AU-rich element-binding protein HuR, which is a central regulator of posttranscriptional gene expression (Levy et al. 1998), interacts with the human 3′UTR of β-F1-ATPase mRNA (Ortega et al. 2008). However, functional studies demonstrated that HuR plays an ancillary role in β-F1-ATPase expression in human cells (Ortega et al. 2008). However, the analysis of the expression of HuR in a large cohort of breast carcinomas has pointed out its relevance as an independent marker of breast cancer prognosis (Ortega et al. 2008). In fact, when HuR expression is studied in combination with the bioenergetic signature of the tumor, it allows the identification of breast cancer patients at higher risk of disease recurrence (Fig. 11.3e) (Ortega et al. 2008). These results strongly encourage the incorporation of HuR as an additional protein marker of the bioenergetic signature for the follow-up of breast cancer patients.

In addition, we have identified nine RNA binding proteins that interact in vitro with β-F1-ATPase mRNA (Ortega et al. 2010) and found the in vivo association of G3BP1 (Ras-GAP SH3 binding protein 1) with the 3′UTR of human β-F1-ATPase mRNA (Ortega et al. 2010). This interaction is functionally relevant because it represses β-F1-ATPase mRNA translation by preventing its recruitment into active polysomes (Ortega et al. 2010). Since G3BP1 is overexpressed in several tumors and cancer cell lines (Barnes et al. 2002; Guitard et al. 2001; Zhang et al. 2007) and β-F1-ATPase expression in human cancer is exerted at the level of translation (Willers et al. 2010), the findings suggest that G3BP1 could play an essential role in the glycolytic switch that occurs in cellular transformation, contributing to define the bioenergetic phenotype of cancer (Ortega et al. 2010). Ongoing studies are aimed at establishing the potential role of G3BP1 as biomarker in cancer prognosis.

In contrast to these findings, the downregulation of β-F1-ATPase expression in chronic myeloid leukemia is mediated by hypermethylation of the promoter of the gene (Li et al. 2010). In addition, in colorectal cancer, the deregulation of the activity of the H^+-ATP synthase due to chromosomal instability of the ATP5A1 gene has been described (Bacolod and Barany 2010). Moreover, the decreased expression of this gene also affords a marker of unfavorable clinical outcome (Bacolod and Barany 2010). It has been recently described that miRNAs that are deregulated in various cancers have an important impact on signaling pathways in mitochondria (Bienertova-Vasku et al. 2013). Interestingly, translation silencing of β-F1-ATPase mRNA during development of the human fetal liver seems to be exerted by the action of miRNA-127-5p (Willers et al. 2012), a mechanism that is not operative in prevalent human carcinomas because this miRNA is not expressed in carcinomas.

11.5 The H^+-ATP Synthase and Tumor Suppression

A dysfunctional OXPHOS promotes cellular proliferation and invasion (Sanchez-Arago et al. 2013a; Amuthan et al. 2001), whereas an increase in oxidative metabolism halts cellular proliferation and tumor progression (Sanchez-Arago et al. 2013a; Cuezva et al. 2009; McFate et al. 2008; Bonnet et al. 2007). In this regard, the activity of OXPHOS has been demonstrated to be specifically required for the execution of cell death (Sanchez-Arago et al. 2013a; Dey and Moraes 2000; Kim et al. 2002; Tomiyama et al. 2006). In particular, molecular components that participate in OXPHOS including subunits of the H^+-ATP synthase are needed for the execution of cell death (Wang 2001; Matsuyama et al. 1998; Plas and Thompson 2002; Santamaria et al. 2006; Vahsen et al. 2004). Hence, bioenergetics and cell death are two master tasks of mitochondria that are molecularly and functionally integrated (Sanchez-Arago et al. 2013a). The inhibition of the activity of the H^+-ATP synthase with oligomycin blunts mitochondrial hyperpolarization and ROS production, prevents the oxidation and modification of mitochondrial proteins, and delays the release of *cyt c* and the execution of cell death (Santamaria et al. 2006). Consistent with these findings, the cell death response to different chemotherapeutic agents varies largely depending upon the relative activity of the two pathways that sustain energy metabolism (Santamaria et al. 2006; Sanchez-Arago and Cuezva 2011). Indeed, highly glycolytic cells with negligible contribution of OXPHOS for ATP provision have a cell death-resistant phenotype because mitochondrial ROS signaling after chemotherapeutic targeting is blunted (Santamaria et al. 2006; Sanchez-Arago and Cuezva 2011). Consistent with the tumor suppressor function of mitochondrial activity, there is a large body of data supporting that OXPHOS, both under basal conditions and in response to chemotherapeutic agents, abolishes tumorigenicity [see Sanchez-Arago et al. (2013a) for updated review]. A likely mechanism that explains the preferential death of cancer cells when forced to oxidize mitochondrial substrates is the overproduction of superoxide radical as a result of the stimulation of mitochondrial metabolism (Sanchez-Arago et al. 2013a; Santamaria et al. 2006; Michelakis et al. 2010). Both genetic (D'Errico et al. 2011) and pharmacological (Wang and Moraes 2011) studies have shown that the PGC1-α-mediated improvement of mitochondrial activity and metabolism restrains cancer progression by increasing ROS-mediated apoptosis in cancer cells (D'Errico et al. 2011).

Permeabilization of the inner mitochondrial membrane to low molecular weight solutes, the so-called permeability transition pore (PTP) opening, is the point of no return in cell death (Galluzzi et al. 2009; Bernardi 2013). Downregulation of the bioenergetic signature is also functionally linked to the resistance to chemotherapy in many different cancer cells (Sanchez-Arago et al. 2013a; Li et al. 2010; Shin et al. 2005; Hernlund et al. 2009) and in colon cancer patients (Lin et al. 2008), emphasizing that a low bioenergetic activity of mitochondria predisposes to cancer onset and progression, highlighting the emerging role that the H^+-ATP synthase plays in cell death (Matsuyama et al. 1998; Santamaria et al. 2006; Alavian

et al. 2011; Chivasa et al. 2011; Giorgio et al. 2013). In this regard, and although the molecular composition of the PTP remains unknown, recent findings support that a critical component of the PTP is subunit c of the H^+-ATP synthase (Bonora et al. 2013). Moreover, it has been shown that dimers of the H^+-ATP synthase form a channel with electrophysiological properties identical to those of the PTP (Giorgio et al. 2013). Overall, the downregulation of the H^+-ATP synthase, and thus of OXPHOS, is part of the molecular strategy adapted by cancer cells to prevent cell death. Consistently, cancer progression requires the silencing of the bioenergetic activity of mitochondria not only by downregulating the content of the H^+-ATP synthase as above discussed but also by overexpressing the natural physiological inhibitor of the enzyme, the so-called ATPase inhibitory factor 1 (IF1) (Sanchez-Cenizo et al. 2010; Sanchez-Arago et al. 2013c; Formentini et al. 2012).

Despite the relevance of OXPHOS as a tumor suppression pathway in different cancer cells, we have to take into consideration the diversity of mitochondrial functions in different cellular lineages. In this regard, it should be noted the case of melanomas. The common activating mutations in the BRAF oncogene drive dysfunctional OXPHOS that increases the tumorigenic properties of malignant melanomas making cells addicted to the glycolytic and the pentose phosphate pathways (Hall et al. 2013). The regulation of oxidative metabolism by BRAF is mediated by PGC1α and by the melanocyte lineage factor (MITF) (Haq et al. 2013). The use of inhibitors of the BRAF oncogene to treat melanomas seems to have a limited efficacy because cells put in place an adaptive metabolic program to induce OXPHOS (Haq et al. 2013). Apparently, the mechanism that mediates drug resistance is the increase of the expression of enzymes of OXPHOS in a slow-cycling subpopulation of cells that survive upon multidrug treatment (Roesch et al. 2013). The increased mitochondrial capacity and resistance to oxidative stress of these survivor cells is mediated by the action of PGC1α (Vazquez et al. 2013). Interestingly, the inhibition of mitochondrial respiration eliminates the emergence of these survivor cells sensitizing melanoma cells to therapy (Roesch et al. 2013). This is an example of how important is to understand mitochondrial function in particular cell types to prevent cancer onset and progression and its treatment.

11.6 IF1-Mediated Inhibition of the H^+-ATP Synthase Also Propitiates Cancer Progression

In addition to the down regulation of the H^+-ATP synthase, most prevalent human carcinomas also inhibit the activity of the complex by the overexpression of the ATPase inhibitory factor 1 (IF1). This small protein is overexpressed in lung, colon, breast, and ovarian carcinomas being its expression negligible in normal tissues (Sanchez-Cenizo et al. 2010; Sanchez-Arago et al. 2013c; Formentini et al. 2012). The overexpression of IF1 triggers the inhibition of the H^+-ATP synthase, the metabolic switch to an enhanced aerobic glycolysis, and the concurrent increase

in the mitochondrial membrane potential due to the prevention of the backflow of protons into the mitochondrial matrix (Sanchez-Cenizo et al. 2010; Formentini et al. 2012). Conversely, the silencing of IF1 has the opposite metabolic effects (Sanchez-Cenizo et al. 2010). These findings emphasize the relevance that the inhibition of the activity of the H^+-ATP synthase has for metabolic adaptation of cancer cells and tumor development. In fact, and in addition to promoting the metabolic switch observed in cancer cells, IF1 simultaneously triggers a ROS signaling cascade that mediates a nuclear response via NFkB to promote cell proliferation, invasion, and cell survival (Formentini et al. 2012). Remarkably, the regulation of the expression of this short-lived protein is exerted at posttranscriptional levels in colon, lung, breast, and ovarian carcinomas, further emphasizing the importance of translation and/or protein stabilization in favoring the metabolic reprogramming of cancer and stem cells (Sanchez-Arago et al. 2013b, c). In fact, the degradation of IF1 is essential in promoting the metabolic changes that support stem cell differentiation (Sanchez-Arago et al. 2013b). Moreover, IF1 is also a relevant predictive marker for clinical outcome of breast and colon cancer patients, suggesting the high potential of IF1 as a therapeutic target (Sanchez-Arago et al. 2013c).

Concluding Remarks

The study of metabolism in cancer has experienced a renaissance in the last decade. Moreover, the focus of cancer research in the twenty-first century has moved from the exclusive genetic interest to the broad study of the signaling pathways that regulate cell metabolism. The knowledge that cancer cells become addicted to certain metabolic pathways has opened up new and promising therapeutic approaches. The identification of the mechanisms that control the cell type-specific bioenergetic phenotype of tumor cells is a needed step to combat the disease. In this regard, basic studies are required to better characterize the alterations of the mitochondrial proteome especially of those that affect the content/activity of the H^+-ATP synthase and that are responsible for mediating cancer progression. Unveiling these mechanisms would make possible the development of effective therapies against cancer.

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Chapter 12
Canceromics Studies Unravel Tumor's Glutamine Addiction After Metabolic Reprogramming

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Contents

Abbreviations

α-KG	2-Oxoglutarate
APC/C	Anaphase-promoting complex/cyclosome

Dedicatory: This work is dedicated to Professor Dr. Ignacio Núñez de Castro on occasion of his 77th birthday. His early vision on the relevance of glutamine and glutaminases in cancer growth and proliferation, along with his seminal metabolic works in experimental tumors, paved the way for fertile research lines now being developed for many of us who had the privilege of being inspired by his example and master guidance.

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CE-MS	Capillary electrophoresis coupled to mass spectrometry
EAA	Essential amino acids
EATC	Ehrlich ascites tumor cells
EGF	Epidermal growth factor
FMNL3	Formin-like protein 3
GA	Glutaminase
GDH	Glutamate dehydrogenase
GFAT	Glutamine:fructose-6-P amidotransferase
GIP	Glutaminase-interacting protein
GlcN-6-P	Glucosamine-6-phosphate
GS	Glutamine synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HBP/HEX	Hexosamine biosynthetic pathway
HDAC	Histone deacetylase
H3K4me3	Histone H3 trimethyl Lys4
HMGA2	High-mobility-group AT-hook protein 2
IDH1	Isocitrate dehydrogenase 1
IMM	Inner mitochondrial membrane
LDH	Lactate dehydrogenase
MCA	Methylcholanthrene induced
mTORC1	Mammalian target of rapamycin complex 1
MUC-1	Mucin 1
NEDD-4	Neural precursor cell-expressed developmentally downregulated gene 4
NF-κB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance
O-GlcNAc	O-Linked *N*-acetyl-glucosamine
OXPHOS	Oxidative phosphorylation
PFKFB3	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase, isoform 3
PK	Pyruvate kinase
PPP	Pentose phosphate pathway
ROS	Reactive oxygen species
SCF	Skp1/cullin/F-box
SMP	Submitochondrial particles
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor beta
UDP-GlcNAc	UDP-*N*-acetylglucosamine
USP-15	Ubiquitin carboxyl-terminal hydrolase 15

12.1 Introduction

In the last decade, we have witnessed a renaissance of cancer metabolic studies after years of being largely ignored in favor of powerful large-scale—and trendy—functional genomic and proteomic studies. Nowadays, the study of cancer metabolism is regaining center stage and becoming a hot issue in tumor biology and clinical research, after a period where such kind of experimental approaches were somehow forgotten or disregarded. Paradoxically, this renewed interest has been largely induced by sound results from cancer genomic, proteomic, and metabolomic (canceromics) studies which are now identifying key players and revealing novel molecular mechanisms underlying cell proliferation in tumors. Thus, this canceromics work is bringing back the focus to metabolic studies which are recovering their original high-profile status in cancer research.

Cancer cells develop and succeed by shifting to different metabolic programs compared with their normal cell counterparts. One of the classical hallmarks of cancer cells was first noticed by Otto Warburg in his seminal studies of tumor metabolism: high glycolytic fluxes even in the presence of abundant O_2 and heightened levels of lactate produced (Fig. 12.1). Another common metabolic feature of cancer cells is a high rate of glutamine (Gln) consumption normally exceeding their biosynthetic and energetic needs. This anomalous Gln uptake was early recognized in pioneer studies of tumor metabolism in animal models (Kvamme and Svenneby 1961; Kovacevic and Morris 1972). The term Gln addiction is now widely used to reflect the strong dependence shown by most cancer cells for this essential nitrogen substrate after metabolic reprogramming (Wise et al. 2008).

Therefore, cancer cell metabolic reprogramming includes a shift in energy production from oxidative phosphorylation (OXPHOS) to less efficient glycolysis, even in the presence of oxygen (Warburg effect), and enhanced use of Gln for increased biosynthetic needs. This necessitates greatly increased glucose and glutamine uptake, both of which enter the hexosamine biosynthetic pathway (HBP) (Fig. 12.1). The HBP is also a sensor of the nutrient state of the cell, and its end product, UDP-*N*-acetylglucosamine (UDP-GlcNAc), is used in many enzymatic posttranslational modifications of cytosolic and nuclear proteins by O-linked β-*N*-acetylglucosamine (O-GlcNAc), with strong repercussions on key metabolic pathways (Zachara and Hart 2004; Ma and Vosseller 2013).

Glutamine is the most abundant amino acid in blood plasma and in optimal culture media (Ham and McKeehan 1979). The vast number of reactions where Gln is involved underscores the importance of this "conditionally essential" amino acid, because it becomes essential under shortage of nutrients supply and stress situations (Lacey and Wilmore 1990). Excellent reviews have previously detailed the multifaceted roles of Gln in cancer and its interaction with other main catabolic routes like glycolysis (Medina et al. 1992; Matés et al. 2002; Yuneva 2008; deBerardinis and Cheng 2010; Daye and Wellen 2012). In this chapter, we review glutaminolysis in tumor cells by focusing on glutaminase proteins with special emphasis on the

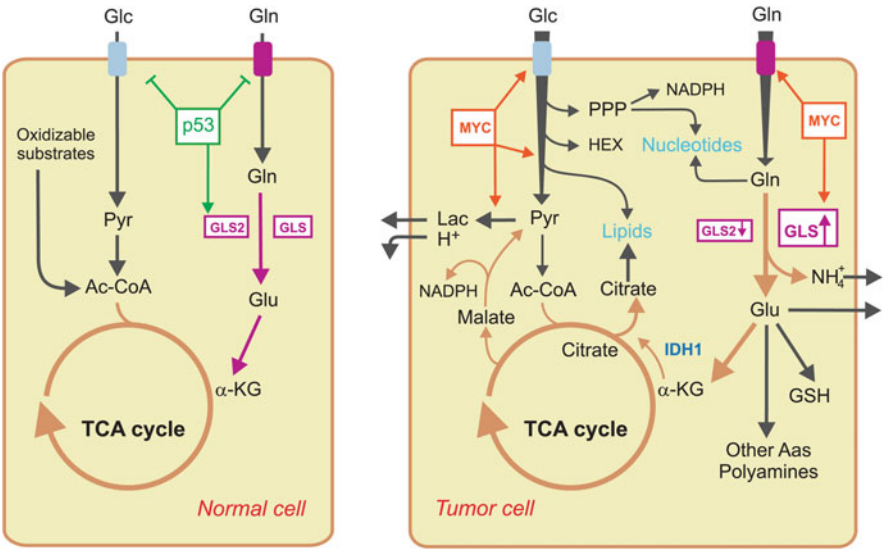

Fig. 12.1 *Main changes in Gln-related metabolic pathways after malignant transformation*. In normal, nonproliferating cells, a balanced metabolism of glucose (Glc) and Gln, along with a functional tumor suppressor p53 gene, allows a complete TCA cycle coupled to pyruvate (Pyr) production from glycolysis. Expression of GLS and GLS2 isoforms is kept under normal tissue-specific homeostatic values and mitochondrial Glu used for energy generation through α-KG incorporation into the TCA cycle. The metabolic reprogramming of cancer cells in Myc-dysregulated tumors includes increased cellular uptakes of Glc and Gln, as well as GLS overexpression. In contrast, GLS2 is repressed in many tumors. Glucose is metabolized into Pyr through the reactions of glycolysis, yielding two molecules of ATP. Pyruvate can be further oxidized in the Krebs cycle or converted into lactate when oxygen levels are low or during "aerobic" glycolysis. Partial oxidation of Gln into Pyr and further into lactate (glutaminolysis) serves as an alternative source of ATP production and to replenish intermediates in the TCA cycle. Excess of Glu and ammonia from enhanced Gln catabolism through GA is transported out of the cells (with relevance in the import of other nutrients like essential amino acids). Glu is also used for GSH, polyamines, and amino acids synthesis. Glutamine can be the main source for lipid synthesis being converted into citrate by reductive carboxylation of α-KG by isocitrate dehydrogenase 1 (IDH1). Malic enzyme reaction, one of the reactions of glutaminolysis, produces NADPH, which is also required for lipid synthesis. In addition, amido and amino nitrogen of Gln are used for nucleotide synthesis, together with phosphoribosyl pyrophosphate (PPP) produced from glucose, through the reactions of the pentose phosphate pathway. Finally, Glc and Gln also feed the hexosamine biosynthetic pathway (HEX/HBP) regulating the cellular nutrient status

host/tumor interaction and its relevance on the metabolic rewiring of proliferating cells. The mechanistic basis for this altered metabolic phenotype and how these changes are connected to oncogenic pathways is becoming increasingly understood. Based on these advances, new avenues of research have been initiated to find novel therapeutic targets and to explore strategies that interfere with metabolic idiosyncrasies of tumors as anticancer therapies.

12.2 Tumors as Metabolic Traps for Glucose and Glutamine

Cancer cells have metabolic requirements that separate them from normal cells and render them vulnerable to drugs that target these processes. The altered metabolism exhibited by most tumor cells is a hallmark of cancer (Hanahan and Weinberg 2011). Pioneer works studying differential metabolic traits of cancer cells firstly classified tumors as glucose and nitrogen traps, thus reflecting the ability of tumors to avidly consume both nutrients. The notion of the tumor as a trap for glucose in the host (Shapot 1979) reflects an extremely high rate of glucose uptake by cancer cells, which yields undetectable levels of glucose in the tumor itself and, as a result, induction of a progressive hypoglycemia and host hepatic glycogen depletion (Shapot 1980; Argilés and Azcón-Bieto 1988; Rivera et al. 1988). Even though aerobic glycolysis is not energetically as efficient as OXPHOS, it can support increased proliferation of tumor cells by allowing high rate of anabolism through fast delivery of energy and biosynthetic building blocks necessary to drive tumor growth (Vander Heiden et al. 2009) (Fig. 12.1). In this sense, tumor cells set before efficacy than efficiency in their proliferative program (Medina and Núñez de Castro 1990), although this apparently dissipative behavior may be a need to maximize growth and proliferation, as we will discuss later at the light of the coordinated regulation of glycolysis and glutaminolysis.

On the other hand, a century ago Müller reported a negative nitrogen balance in tumor-bearing patients (Müller 1889). Actually, Mischenko first noticed in 1940 the capacity of tumors to take up nitrogen of the host's tissue protein (reviewed in Shapot 1979). In 1951, Mider classified tumors as "nitrogen traps" indicating their ability to compete with advantage for host nitrogen compounds (Mider 1951). This process produces in the host a negative nitrogen balance and a characteristic weight loss and, in the tumor, a reciprocal nitrogen increase. Tumors generally enhance protein degradation and reduce protein synthesis in the host tissues, particularly in skeletal muscle (Lundholm et al. 1976; Kawamura et al. 1982; Souba 1993). The released amino acids are then taken by tumors for both oxidation and protein synthesis (Shapot 1979). High Gln consumption is a key feature of tumor cells but also of normal proliferating cells (Fig. 12.1). Thus, Gln is also a primary energy source for cultured cells like human fibroblasts (Zielke et al. 1980), cells of the immune system (Ardawi and Newsholme 1983; Brand et al. 1984), and other rapid proliferating cells like colonocytes (Ardawi and Newsholme 1985) and adipocytes (Kowalchuk et al. 1988).

A considerable number of sound evidences early confirmed that Gln is a major respiratory fuel for most human and experimental tumors (Kvamme and Svenneby 1960; Coles and Johnstone 1962; Kovacevic and Morris 1972; Abou-Khalil et al. 1983; Matsuno et al. 1986). The in vivo utilization of amino acids, glucose, and lactic acid was measured in a series of Morris hepatomas and Walker 256 carcinosarcomas: Gln was the most extensively consumed amino acid, especially in the fast-growing tumors, and its rate of uptake was proportional to its supply to the

tumor (Sauer et al. 1982; Sauer and Dauchy 1983). In agreement with these studies, comparison of Gln metabolism between tumor cells and non-transformed cells of the same origin demonstrated a considerably faster rate of Gln utilization by tumors, which usually behave as avid Gln consumers; for example, in human hepatocytes vs. hepatoma cells (Souba 1993), and in rat kidney fibroblasts vs. rat fibrosarcoma cells (Fischer et al. 1998).

In general, the rate of Gln consumption is proportional to the rate of tumor cell proliferation (Fischer and Chance 1990). Results using a great variety of tumor cells from different origins strongly support the view that Gln breakdown is intimately linked to cellular proliferation. In addition, the intracellular Gln concentration of cancer cells during their exponential growth phase is undetectable (Shapot 1979; Sauer et al. 1982; Carrascosa et al. 1984; Márquez et al. 1989) and has been negatively correlated with proliferation rates (Sebolt and Weber 1984) and with metastatic capacity in rat and mouse tumors (Pine et al. 1982; Coles and Johnstone 1962) (Fig. 12.1). Of note, in cultured cancer cells, high extracellular Gln levels stimulate proliferation (Turowski et al. 1994), while reduction of Gln availability induced differentiation (Spittler et al. 1997). This was the rationale behind traditional cancer therapies based on asparaginase/glutaminase treatment of patients to deplete circulating Gln levels, but, unfortunately, they also induce severe and multiple side effects in other tissues and organs (Medina et al. 1992).

12.3 Tumors Induce a Net Flux of Glutamine and Essential Amino Acids from Host Tissues

Tumors compete with the host for nitrogen compounds needed for the synthesis of nucleic acids, proteins, and lipids. Glutamine appears to be the principal of several amino acids involved in nitrogen transport from host to tumor (Shapot 1979). Preferential utilization of Gln by tumor cells has consequences for other tissues (Fig. 12.2). Actually, growing tumors can modulate Gln metabolism in host tissues by using some yet unidentified signals or mechanisms. Interestingly, most tumors behave as "Gln traps," and the source of Gln is mainly the host tissues because tumors usually show repression of their glutamine synthetase (GS; EC 6.3.1.2) activity, even though there are also tumors with active GS supporting their requirements for Gln and make them resistance to shortage or depletion of Gln supply (Marin-Valencia et al. 2012).

A nitrogen interchange takes place between host tissues and tumor cells, with Gln as the main nontoxic vehicle of nitrogen (Carrascosa et al. 1984). In experiments with animals infested with a highly malignant strain of Ehrlich ascites tumor cells (EATC), serial concentrations of amino acids were determined for host plasma, ascitic fluid, and tumor cells, throughout tumor development (life span 16 $\pm$ 1 days) (Márquez et al. 1989). Concentration gradients of Gln, Asn, and essential

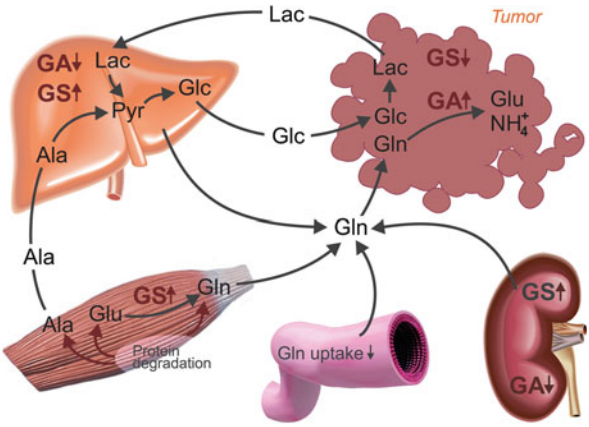

Fig. 12.2 *Nitrogen interchange between host tissues and tumor cells highlighting Gln as the main nontoxic vehicle of nitrogen*. Cancer induces major changes in interorgan Gln trafficking. A Gln/Glu cycle occurs between host tissues and the tumor. For liver and kidneys, evidence from model systems and/or human studies suggests that there is a change in net Gln flux during tumorigenesis by the simultaneous modulation of GS and GA expression leading to a net flux of Gln toward the tumor. The high glutaminolytic rate in tumor cells provokes a massive efflux of Glu and ammonia. In advanced stages of tumor development, intramuscular Gln pools are depleted in association with loss of lean muscle mass, mimicking the cachectic phenotype of humans in advanced stages of cancer. Simultaneously, in some experimental tumors, muscle GS activity is increased and Gln utilization by the gut is reduced; hence, they can also contribute to heightened circulating Gln levels. The excess of ammonia can be disposed through the urea cycle, and part of it may also be incorporated in muscle Gln through GS activity. On the other hand, the output of lactate coming from tumor aerobic glycolysis could be taken by the liver as gluconeogenic substrate, in a Cori-like cycle between the tumor and the liver

amino acids (EAA) (with the exception of Thr) from the host plasma toward the ascitic liquid were established, while on the other hand, concentration gradients from the ascitic liquid toward the plasma were established for Glu, Asp, Gly, Ala, Pro, and Thr. With the exception of Asp, the concentrations of these last amino acids were highest inside the cells. Arginine, Gln, and Leu were the only amino acids not detected in tumor cells during the exponential growth phase. In vitro incubations of tumor cells in the presence of Gln and/or glucose, as the nitrogen and energy sources, confirmed the amino acid fluxes previously deduced from the observed relative concentrations of amino acids in plasma, ascitic liquid, and tumor cells, suggesting that Glu, Ala, Asp, Gly, and Ser can be produced by tumors (Márquez et al. 1989). Similar in vivo amino acid concentration gradients were found in mice bearing the Lewis lung carcinoma (Rivera et al. 1988). The concentration gradients found for Gln, Glu, Asn, Asp, and EAA between the tumor and the host cannot be explained by a diminished food intake derived from cancer cachexia at least during the exponential phase of tumor growth (Márquez et al. 1989). However, in the final days of life, the concentration of Gln in plasma was lower than in control animals, although interpretation of this result is complicated by the interaction of numerous complex variables which lead to the death of the tumor-

bearing host. Many cancer patients develop Gln depletion in advanced stages which ultimately leads to cachexia (Fischer and Chance 1990; Souba 1993).

12.4 Enzymatic Imbalance in Host Glutamine Metabolism

The findings just mentioned support that changes in amino acid patterns occurring in the host system are related to tumor development (Medina et al. 1992). Furthermore, measurements of amino acids in host tissues during tumor growth have revealed a net flux of Gln from host tissues toward tumor cells along with a reverse flux of Glu and Asp from tumor to plasma (Carrascosa et al. 1984; Quesada et al. 1988; Rivera et al. 1988; Márquez and Núñez de Castro 1991). Therefore, the tumor can be viewed as a parasite organ which is inserted into the host metabolism and alter host homeostasis to satisfy its own requirements for nutrients. Thus, the metabolic reprogramming occurring in cancer is twofold: one is suffered by the tumor cell during transformation to cope with high demands for rapid growth and proliferation; the second one is induced by the tumor in the host tissues which are forced to satisfy tumor demands for nitrogen compounds and glucose (Fig. 12.2).

A Gln/Glu cycle occurs between host tissues and the tumor in order to maximize its growth and proliferation rates (Fig. 12.2). Support for the existence of this cycle in vivo has come also from studies on enzymatic activities of GS and glutaminase (GA; EC 3.5.1.2) in host tissues during tumor development. Thus, great increases were observed in muscle GS activity in rats bearing methylcholanthrene-induced (MCA) fibrosarcoma (Chen et al. 1993), in avian host bearing transplanted hepatomas (Matsuno and Satoh 1986), and in mice bearing Lewis lung carcinoma (Rivera et al. 1988) and EATC (Quesada et al. 1988). Furthermore, a simultaneous modulation of GS and GA activities in the liver and the kidney of mice bearing EATC was found shortly after tumor transplantation allowing a net increase in both tissue-free Gln and circulating Gln (Quesada et al. 1988). In addition, the liver GS/GA ratio in mice bearing EATC, obtained from their relative mRNA levels, significantly increased during tumor growth, mainly due to a strong decrease in liver GA mRNA whereas GS levels remained almost unaffected (Aledo et al. 2000a). Of note, renal GS activity was also increased in fibrosarcoma-bearing rats, a model of fast-growing Gln-consuming tumor where both the liver and the kidney become net Gln exporters (Chen et al. 1993; Souba et al. 1988). Simultaneously, Gln utilization by the gut was reduced (Fig. 12.2) and detrimental changes in mucosal architecture were also detected during progression of the malignant disease (Souba et al. 1988).

Similar dynamic studies on interorgan Gln trafficking during tumor development are scarce in humans, but they will be highly valuable not only to characterize the mechanisms elicited by the tumor to successfully grow and proliferate at the host expenses, but also to better understand the pathophysiology of cancer cachexia, a major source of morbidity and mortality (Hensley et al. 2013). The conclusion from

studies in animal models is that an enzymatic imbalance for key Gln-related enzymes occurs in host tissues—mainly the skeletal muscle, liver, kidney, and gut—leading to an increased supply of this amino acid toward the tumor (Fig. 12.2). The adaptative changes in gene expression for those enzymes would explain the high Gln uptake showed by tumor cells. The results suggest a long-term regulation of host enzymes in order to increase the circulating Gln levels needed for tumor growth. Thus, the tumor elicits a specific response in the host nitrogen metabolism so that the whole organism is mobilized to augment circulating Gln levels.

However, some of these effects in the host are seen well before, even when the tumor still remains in the lag phase and, consequently, the cell number is quite low. Why do the host tissues regulate their GA and GS enzymes to increase circulating Gln levels from the first few days after tumor transplantation? It is tempting to speculate that such early response could be, at least partly, related to increased Gln demands elicited by the host immune system. It is well known that Gln is essential for cell of the immune system (Castell et al. 1994), although sound works at the molecular level are lacking to address the role of Gln in the modulation of the immune response. In splenocytes of mice bearing EATC, a very early upregulation of Gls expression was detected: there was a 2.3-fold stimulation of spleen GA activity just 48 h after tumor implantation, along with enhanced mRNA levels of two different Gls transcripts (Aledo et al. 1998). However, this early immune response, characterized by a drastically reduced number of splenic Th lymphocytes and increased population of macrophages in the spleen of tumor-bearing mice, did not negatively affect the tumor (Segura et al. 1997). Furthermore, precursors of TGF-β—a cytokine known for its ability to suppress immune response—were detected in both tumor cells and ascitic fluid suggesting that tumor actively interacts with host immune system by means of tumor secreted factors (Segura et al. 1997).

Many analogies have been found between the energy metabolism of tumors and that of other rapidly growing cells such as small intestinal epithelia and reticulocytes (Krebs 1980). Taking into account the striking similarity in the pattern of substrate utilization between tumors and intestinal mucosa, as well as tumors' ability to utilize in vivo whatever substrate is in most abundant supply (glucose, Gln, and lactate utilizations were directly correlated with rate of supply in rat experimental tumors), it was early hypothesized that the biochemical strategy of tumor cells was essentially based on their integration with the energy supplies and needs of the host (Sauer et al. 1982; Weber 1977). Interestingly, similar metabolic changes to those occurring in cancer can also be seen in noncancerous states such as in embryonic tissues, where the rate of conversion of glycolytically generated pyruvate to lactate is also very high (Argilés and Azcón-Bieto 1988). In addition, a Gln/Glu cycle similar to that described between tumors and their hosts appears to be operative between fetus and placenta: the placenta supplies fetus with very large amounts of Gln while concurrently removing Glu from fetal circulation (Battaglia 2000). Therefore, intense aerobic glycolysis to lactate and partial oxidation of avidly consumed Gln seems to appear as hallmarks in the metabolism of poorly differentiated and highly proliferative tissues and cells. These facts have puzzled biochemists for many years, and we'll next review several hypotheses to explain

it. Nevertheless, recent findings from metabolomic studies have shed new lights into the relationships and interconnections between glycolysis and glutaminolysis that may help to explain these characteristic traits in cancer bioenergetics.

12.5 Tumor Glutamine Catabolism: Glutaminolysis

Glutaminolysis was the term first used by McKeehan to describe mitochondrial Gln catabolism to pyruvate/lactate (McKeehan 1982) and its importance in cell proliferation. The process can occur completely inside the mitochondria, though it can also take place as a cytosol/mitochondria compartmentalized process (Moreadith and Lehninger 1984; McKeehan 1986). Before being catabolized, Gln has to be transported through both plasma and inner mitochondrial membranes. These processes are not the objectives of this review, but interested readers may find excellent reviews on the topic (see, e.g., McGivan and Bungard 2007). Tumor cells can transport Gln through the plasma membrane quiet efficiently (Carrascosa et al. 1984) and at a faster rate than do their normal counterparts (Bode et al. 2002; Dudrick et al. 1993). The ASCT2 transporter is responsible for the accumulation of Gln in rapidly growing cells in cultures, especially epithelial cells and a number of tumor cell lines (Bungard and McGivan 2004; Bode et al. 2002).

Before glutaminolysis can take place, cells need to transport cytosolic Gln across their inner mitochondrial membrane (IMM) to reach the GA enzyme (Molina et al. 1995). Mitochondrial transport of Gln is also of importance, but mitochondrial Gln transporters are poorly characterized at the molecular level: data concerning the structure and function of the transport systems are scarce, and only one mitochondrial Gln carrier has been purified so far (Indiveri et al. 1998), which can give us an idea of the difficulty of the task. The crowded protein environment of the IMM and mitochondrial matrix, along with a very active Gln metabolism which overlaps with the transport process, has posed severe methodological shortcomings when measuring transport rates.

The Gln transporter from EATC mitochondria was studied using submitochondrial particles (SMP) without any interference by the mitochondrial Gln metabolism (Molina et al. 1995). Conveniently, the absence of any GA activity in these vesicles was critical, since this process interferes notably with Gln transport studies (La Noue and Schoolwerth 1979). Mitochondrial Gln transport in EATC showed allosteric kinetics with a Hill coefficient of 2.2 and a transport capacity one order of magnitude greater than the value reported for SMP isolated from rat kidney (Sastrasinh and Sastrasinh 1989). The comparison of the normalized tumor data with rat kidney data shows that the ratio $V_{max}/S_{0.5}$, a criterion of the transport efficiency, was about 16-fold greater in tumor mitochondria. Compared with hyperbolic kinetics, a protein following sigmoidal kinetics would allow a more sensitive control of the Gln transport rates. This mechanism could avoid a rapid depletion of cytosolic Gln essential for biosynthetic purposes. The pH optimum of the tumor mitochondrial carrier was about 8.0, in excellent agreement with the

value reported for GA (Campos et al. 1998). Therefore, when the mitochondrial electron transport is activated, the matrix pH becomes slightly alkaline and both the Gln carrier and GA can be fully operative.

There has been much controversy about the relative importance of the various steps in Gln degradation. In general, there is little direct and convincing evidence that plasma membrane transport of Gln is a rate-limiting step in Gln metabolism (McGivan and Bungard 2007). On the other hand, the rate-limiting step of the mitochondrial glutaminolytic process has not been fully elucidated. Some authors concluded that mitochondrial transport is not rate limiting for Gln hydrolysis, whereas others have suggested that transport could be the major site of regulation of the pathway (Matés et al. 2009 and references therein). Comparison of the normalized $V_{max}/S_{0.5}$ values for the carrier and GA in tumor cells points out that GA has a 15-fold higher catalytic efficiency than the carrier (Molina et al. 1995). This strongly suggests a regulatory role for the carrier in the glutaminolytic process, characteristic of the neoplastic transformation. In agreement with this result, no significant correlation between GA activity and Gln disappearance rate was observed in seven human breast cancer cell lines, suggesting that GA did not limit Gln utilization in these cell lines (Collins et al. 1998). A similar result was reported in rat kidney (Sastrasinh and Sastrasinh 1989); in contrast, GA was identified as the major control site of mitochondrial Gln metabolism in rat liver cells and several cultured mammalian cell lines (Low et al. 1993; Sri-Pathmanathan et al. 1990; Neermann and Wagner 1996). The regulation of glutaminolysis may vary between species, tissues, and cells, which may help to explain these discrepancies about its main control point. However, the few data available for tumor cells point toward the mitochondrial transport as the rate-limiting step of glutaminolysis.

Although Gln addiction can differ considerably between cell lines, it can be stated that cell proliferation rate is consistently dependent on Gln availability (Collins et al. 1998). The major degradative pathway for Gln and the first step in glutaminolysis is carried out in mitochondria and initiated by the enzyme GA (Kovacevic and McGivan 1983). Previous studies have shown that GA activity correlates with tumor growth rate and malignancy in rat and human hepatomas (Knox et al. 1969; Linder-Horowitz et al. 1969; Kovacevic and Morris 1972; Matsuno and Hirai 1989). Glutaminase was absent from normal mammary glands but was present in rat mammary tumors (Knox et al. 1970). In EATC, GA activity and mRNA levels increased in parallel reaching maximal values during the exponential phase of growth (Aledo et al. 1994), when the intracellular Gln concentration was undetectable (Márquez et al. 1989). However, this behavior has not been always found; for instance, cell lines of non-tumor origin also show relatively high GA activities, and there was no significant correlation between GA activity and cell growth rate in several cultured human cancer cell lines (Collins et al. 1998; Turner and McGivan 2003).

Tumor can behave as powerful energy dissipative systems; that is, neither energy nor biosynthetic requirements can totally justify the very high glucose and Gln consumptions (Medina and Núñez de Castro 1990; Souba 1993; Tennant et al. 2009). A clear example was obtained from perifused EATC at steady state:

they continuously wasted supplemented glucose and Gln, excreting into the medium two moles of lactate per mol of glucose and one mol of Glu and ammonia per mol of Gln consumed (Segura et al. 1989). However, when cancer cells are actively dividing, their high glycolytic and glutaminolytic fluxes seem to be essential parts of their proliferative program. Thus, the enhanced glucose uptake and glycolytic catabolism have been justified because of the gain of key metabolic advantages essential to drive tumor growth, namely, increased adaptation to live under reduced oxygen concentrations, generation of an acidic microenvironment (lactate) optimal for invasion and survival, enhanced pentose phosphate pathway (PPP) which generate enough reducing power (NAPDH) to reinforce antioxidant defenses, and, most importantly, the use of glycolytic intermediates for anabolic and anaplerotic purposes (reviewed by Kroemer and Pouyssegur 2008) (Fig. 12.1). In this regard, the expression of tumor-specific isoforms for key metabolic enzymes allows some of these alterations. For example, the M2 splice variant of pyruvate kinase (PK) is an enzyme recently identified as important for the Warburg phenomenon in cancer (Christofk et al. 2008). By using the low-active dimeric form of PKM2, cancer cells may deviate some energy-rich glycolytic intermediates upstream of pyruvate toward anabolic reactions instead of being processed to lactate (Mazurek et al. 2005).

In glioblastoma cells, the high rate of Gln metabolism exceeded its use as precursor for protein and nucleotide biosynthesis and was accompanied by secretion of lactate, alanine, and ammonia in an apparent wasteful behavior (DeBerardinis et al. 2007). Nevertheless, the high rate of glutaminolysis in these cells was justified because it provided NAPDH, to fuel lipid and nucleotide biosynthesis, and anaplerotic oxaloacetate as basic biosynthetic precursor (DeBerardinis et al. 2007). In EATC cells, the main products of glutaminolysis are ammonia, CO_2, Glu, and Asp (Coles and Johnstone 1962; Carrascosa et al. 1984; Márquez et al. 1989). The oxidative metabolism of Gln is the main source of energy with preference to glucose for some cultured tumor cells (Reitzer et al. 1979; Goossens et al. 1996). However, in rapidly dividing cells, the flux through GA greatly exceeds the maximal velocity of Glu oxidation (Moreadith and Lehninger 1984), which may partly explain its huge excretion as an end product.

On the other hand, this dissipative behavior shown by many transformed cells has been justified as an essential requisite to sustain high rates of cellular proliferation (Aledo 2004). Hence, plasma Gln can be considered as an energy transducing device moving between the host and the tumor; in fact, thermodynamic analysis of the global process of Gln uptake by the tumor cell, hydrolysis, and release of main products to the plasma yielded an actual Gibbs free-energy value of −49.3 kJ/mol, similar to the hydrolysis of one mole of ATP (Aledo 2004). Therefore, an appealing hypothesis was put forward: cancer cells receive this free-energy gift from host tissues and use part of it by coupling the exit of Asp and Glu (or even Gln) to the entry of other amino acids through ASC (SLC1A5), L (SLC7A5), and X_c^- (SLC7A11) transport systems. Hence, this Gln cycle can be coupled through GA and amino acids carriers to the entry of other essential nutrients required for cancer cell growth, proliferation, and invasive properties. Interestingly, ASC and X_c^-

transport agencies are overexpressed in many tumors; for instance, X_c^- is particularly active in glioma allowing these cells to secrete large amounts of Glu. Excess Glu acts as an excitotoxic weapon killing surrounding neurons and vacating space needed for glioma proliferation in the cranial cavity (De Groot and Sontheimer 2011). It is also noteworthy that Gln facilitates the uptake of EAA like Leu, leading to activation of mammalian target of rapamycin complex 1 (mTORC1); for example, a mechanism has been recently described by which Gln efflux through the antiporter SLC7A5 is coupled to Leu intake (Nicklin et al. 2009).

A second benefit derived from high glutaminolytic fluxes and partial oxidation of Gln is that ATP can be supplied at faster rates to cope with the extremely rapid proliferation required by cancer cells, which is an important kinetic advantage to maximize division rates. The rate of ATP production by glycolysis can be up to 100 times faster than that of OXPHOS (Voet and Voet 1995). In payment for this bonus of free energy and versatility, cancer cells return to the host part of the energy received in the form of nitrogen intermediates and products (Glu, Asp, Ala, ammonia); thus, they sacrifice thermodynamic efficiency for the sake of efficacy (Aledo 2004).

12.6 Metabolic Versatility of Cancer Cells: Interrelationship Between Glucose Metabolism and Glutaminolysis

Glucose and Gln serve as the basic nutrients for tumors, and recent studies are now supporting key facts about the molecular basis for their differential use in cell proliferation. Tumor decisions on the type of preferred metabolite to satisfy its energy and biosynthetic needs seem to be dictated by mutations affecting certain oncogenic signaling pathways. Activating mutations in the phosphatidylinositol 3-kinase (PI3K) and its downstream effector AKT make the cells take up glucose in excess of its bioenergetics needs and lead mitochondria to support glucose-dependent lipid synthesis and non-essential amino acid production (Elstrom et al. 2004; Wise et al. 2008). The PI3K/AKT pathway does not appear to be involved in the uptake and catabolism of Gln in Myc-transformed cells (Wise et al. 2008); however, oncogenic Myc can coordinate glucose and Gln utilization allowing simultaneous increases in the uptake and metabolism of both substrates (for review see Dang 2010; Daye and Wellen 2012).

The importance of Gln in cell survival pathways was stressed by Yuneva and coworkers in a carefully designed set of experiments demonstrating that deficiency in Gln, but not glucose, induced Myc-dependent apoptosis in cultured human fibroblast cells. Proteomic analysis by capillary electrophoresis coupled to mass spectrometry (CE-MS) was used to elucidate the Gln-derived function whose impairment allows apoptosis: an unexpected depletion of most Krebs cycle intermediates was found, clearly pointing toward a deficiency in the tricarboxylic acid

(TCA) cycle as the main cause of Myc-induced apoptosis by Gln deprivation (Yuneva et al. 2007). Furthermore, metabolomic studies using nuclear magnetic resonance (NMR) spectroscopy and ^{13}C-labeled substrates revealed the use of Gln as the major anaplerotic precursor in human glioma cells (DeBerardinis et al. 2007). Sound experimental evidences were later found supporting the role of Myc in metabolic reprogramming of tumor cells leading to Gln addiction (Wise et al. 2008). Even more, Myc overexpression in mouse embryonic fibroblasts induced key genes of glutaminolysis such as Gln transporters (ASCT2 and SN2), GA, and lactate dehydrogenase A (LDH-A) which converts Gln-derived pyruvate into lactate (Wise et al. 2008). The enhanced cellular dependence on Gln and glutaminolysis induced by Myc was justified by anaplerotic requirement in sustaining the TCA cycle and cell viability (Wise et al. 2008; Yuneva et al. 2007), and by stimulation of NADPH needed to support biosynthetic purposes for oncogenic growth (deBerardinis et al. 2007).

A connection has been recently discovered between glutaminolysis and mTORC1 signaling. Activation of mTORC1, a key regulator of nutrient uptake and cellular proliferation (Cornu et al. 2013), has been linked to Gln addiction in cancer cells: mTORC1 stimulates Gln metabolism by inducing glutamate dehydrogenase (GDH) (Csibi et al. 2013), while glutaminolysis and cellular level of αKG also activates mTORC1 thereby promoting cell growth and inhibiting autophagy (Duran et al. 2012).

As mentioned before, glutaminolysis and glycolysis can be regulated coordinately by the HBP, which may serve as a sensor for the nutrient state and has been proposed to act as a checkpoint in the regulation of glucose-dependent Gln uptake (Wellen et al. 2010). This route provides UDP-GlcNAc, the substrate for O-glycosylation of proteins. The enzyme glutamine:fructose-6-P amidotransferase (GFAT; EC 2.6.1.16) catalyzes the formation of glucosamine-6-P (GlcN-6-P) from fructose-6-P and Gln and represents the first and rate-limiting step in de novo biosynthesis of hexosamines. The role of Gln, a substrate of GFAT, in the modulation of HBP has been poorly characterized. We studied the effect of a reduced Gln hydrolysis rate on the HBP in MCF-7 breast cancer cells, which causes a shortage of Gln supply for its metabolization through Glu, but not as a substrate for others Gln-utilizing enzymes like GFAT (Donadio et al. 2008). The results show that GA inhibition greatly reduces GFAT activity. Impairment of GlcN-6-P production is predicted to reduce cell growth and to interfere with cell signaling. This resulted in disturbances of O-linked glycosylation pathways, altering the glycosylation status of the transcription factor Sp-1 and increasing its transcriptional activity (Donadio et al. 2008). The mechanism by which loss of GA activity influences glycosylation is unclear, but recent findings suggest that GFAT, and perhaps other components of the glycosylation machinery, is responsive to intracellular Gln availability which, in turn, is determined largely by GA activity. We performed a proteomic analysis of the global O-glycosylated protein pools in wild-type and GA-KO breast cancer cells: the results revealed marked alterations in the O-glycosylation pattern of key proteins controlling cell proliferation and differentiation, supporting O-GlcNAc as a sensor of the nutritional and energetic state of the cell (Donadio et al. 2008).

Thus, GA knockdown has dramatic consequences on cell growth, proliferation, and survival.

Studies of the metabolic requirements for cell proliferation are now revealing new insights into the molecular basis for the differential use of glucose and Gln in rapidly dividing cells. In this regard, the regulation of cell cycle by two ubiquitin ligase complexes, APC/C (anaphase-promoting complex/cyclosome) and SCF (Skp1/cullin/F-box), is essential to understand glucose and Gln requirements for cell cycle progression (see Moncada et al. 2012 for a review). APC/C degrades two key enzymes in the metabolic pathways of glycolysis and glutaminolysis: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3 (PFKFB3) (Almeida et al. 2010) and GLS (Colombo et al. 2010), respectively. The first enzyme is widely expressed in different organs and tumor cells, and its main role is the generation of fructose-2,6-bisphosphate, the most potent allosteric activator of the key glycolytic enzyme phosphofructokinase-1. PFKFB3 protein levels are maximal in mid-to-late G_1 phase; then, it disappears after the transition from G_1 to S. Thus, the presence of PFKFB3 is tightly controlled to ensure the upregulation of glycolysis at a specific point in G_1 (Moncada et al. 2012). In sharp contrast, GLS protein increased at mid-to-late G_1, but also remained elevated and persisted throughout S phase, unlike PFKFB3, indicating that glutaminolysis is required beyond G_1/S transition. These results were validated in human T lymphocytes, kidney HEK293 cells, human SHSY-5Y neuroblastoma cells, and synchronized HeLa cells (Colombo et al. 2011). In summary, both glucose and Gln are required for progression through the restriction point in mid-to-late G_1, but Gln is the only substrate essential for the progression through S phase into cell division (Colombo et al. 2011).

12.7 Glutaminase Isoenzymes and Their Opposing Roles in Tumorigenesis

Four different GA isoenzymes have been described so far in mammalian tissues which are encoded by separate genes in different chromosomes (Márquez et al. 2006; De la Rosa et al. 2009; Martín-Rufián et al. 2012). In humans, the *GLS* gene is located in chromosome 2 and encodes isozymes termed KGA and GAC, while the *GLS2* gene on chromosome 12 codes for isozymes called GAB and LGA (Aledo et al. 2000b). The KGA mRNA arising from the mammalian *Gls* gene is formed by joining exons 1–14 and 16–19, while the alternative spliced transcript GAC uses only the first 15 exons, omitting exons 16–19 (Elgadi et al. 1999; Porter et al. 2002). The existence of two transcript variants for the *Gls2* gene, GAB and LGA, has been also recently demonstrated (Martín-Rufián et al. 2012). The canonical long transcript, named GAB, is formed by joining the full 18 exons of the *Gls2* gene, whereas the short transcript LGA lacks exon 1. The LGA variant arises by alternative transcription initiation and possesses an alternative promoter located in

the first intron of the *Gls2* gene (Martín-Rufián et al. 2012). The distinct GA isozymes differ in their pattern of expression in mammalian tissues and cells, as well as in their molecular, kinetic, and regulatory properties (Márquez et al. 2006; Campos-Sandoval et al. 2007).

12.8 GLS Isoforms: KGA and GAC

As mentioned previously, GA also plays a key role in tumorigenesis. Thus, it is well documented that many tumors show an increased GA activity, which is positively correlated with their malignancy. The pattern of expression of GA isozymes in tumor cells has been investigated to clarify its role in the malignant transformation and the prospect of its use as a clinically relevant factor. We first reported that inhibition by antisense technology of Gls (KGA isoform), an enzyme linked to neoplastic transformation, allowed reversion of tumor cells to a more differentiated and less malignant phenotype. Thus, EATC transfected with antisense KGA cDNA constructs (0.28AS-2 cell line) were markedly impaired in their growth and proliferation capacity, showed marked changes in their morphology, and lost their tumorigenic capacity in vivo (Fig. 12.3) (Lobo et al. 2000). Moreover, knocking down Gls induced apoptosis in 0.28AS-2 cells, caused oxidative stress, and sensitized the cells to methotrexate (Lora et al. 2004).

Functional genomic analysis of 0.28AS-2 cells and wild-type EATC by differential display revealed significant changes at the level of their transcriptome (Martín-Rufián et al. 2006). Four genes were significantly downregulated in KGA-inhibited cells: high-mobility group HMGA2 protein, FMNL3 or formin-like protein 3, NEDD-4 ubiquitin protein ligase, and ubiquitin carboxyl-terminal hydrolase USP-15 (Fig. 12.3). It is worth mentioning that HMGA2 is a member of the *Hmga* gene family of non-histone chromosomal proteins, often referred to as architectural transcription factors, which has been clearly implicated in cell proliferation and tumorigenesis: overexpression of HMGA2 has been reported in various malignant tumors, including breast cancer. Further phenotypic changes were observed in KGA(–) 0.28AS-2 cells, including diminished expressions of mucin MUC-1 and cytokine TGF-β and reduced activity of *N*-acetyl-α-D-galactosaminidase (Fig. 12.3), that were in part responsible of the activation of the host immune system that rejected in vivo growth of 0.28AS-2 cells (Fig. 12.4) (Segura et al. 2001). Interestingly, mice inoculated with 0.28AS-2 cells kept immunologic memory and rejected a second inoculation with parental wild-type EATC line. The study of the immune system response in these mice revealed an increase in splenic CD18 cells and the presence of a large number of activated $F4/80^+$ macrophages in the ascites cavity (Fig. 12.4). These features were not observed in mice inoculated with the parental EATC line, indicating that a distinctive strong immune response was taking place in the animals inoculated with 0.28AS-2 cells. Therefore, inhibition of Gls expression induced phenotypic changes allowing an effective antitumor

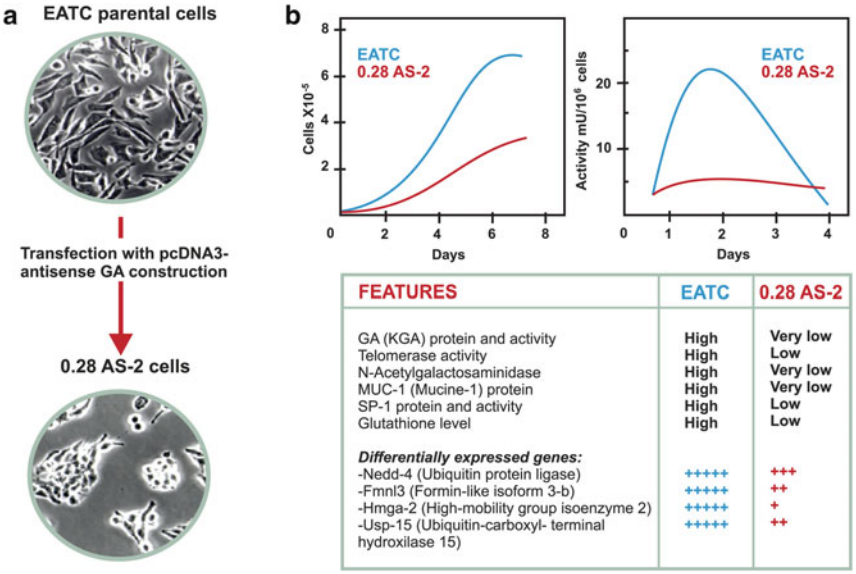

Fig. 12.3 In vitro *behavior of Ehrlich ascites tumor cells (EATC) after downregulation of GA by transfection with an antisense-Gls expressing construct*. (**a**) EATC transfected with the pcDNA3 vector containing a 0.28-kb antisense segment of rat Gls (KGA isoform), named 0.28AS-2, displays remarkable changes in their morphology and a clear impairment in growth rate and plating efficiency when they are compared with the parental cell line. (**b**) 0.28AS-2 cells show significant shortage in both KGA protein and GA activity. The 0.28AS-2 cells also display distinctive genetic and protein expression patterns, which could indicate that a reversion of their malignant phenotype is achieved after Gls inhibition. In this sense, 0.28AS-2 cells lose their capability to escape from animal immune system, and they are more sensitive to cytotoxic drugs than EATC parental cell line. The ability to revert the transformed phenotype of antisense GA transfected cells confirms the relevance of Gls in tumorigenesis process

immune response; thus, 0.28AS.2 cells were unable to develop in vivo tumors (Segura et al. 2001).

Recently, it has been reported that oncogenic transcription factor c-Myc, frequently dysregulated in human cancers, also regulates the expression of the *GLS* gene in human Burkitt lymphoma and prostate cancer cells (Gao et al. 2009). Mitochondrial GLS protein was induced tenfold in response to c-Myc, although its mRNA levels did not vary significantly, which suggested a regulation at the posttranscriptional level. Hence, the authors demonstrated an indirect mechanism of regulation through effects on the miRNAs miR23a and miR23b. Normally, these miRNAs bind to the 3′-untranslated region (3′-UTR) of the *GLS* gene and prevent translation of the message. However, c-Myc suppresses miR-23a/b expression and thus derepressed Gls translation, facilitating Gln oxidation in the mitochondria (Gao et al. 2009). Of note, another c-Myc-mediated posttranscriptional mechanism was found in the same human cancer cells involving key enzymes of proline metabolism: miR23b* (processed from the same transcript as miR-23b) downregulates proline oxidase, a tumor suppressor, and enhances proline

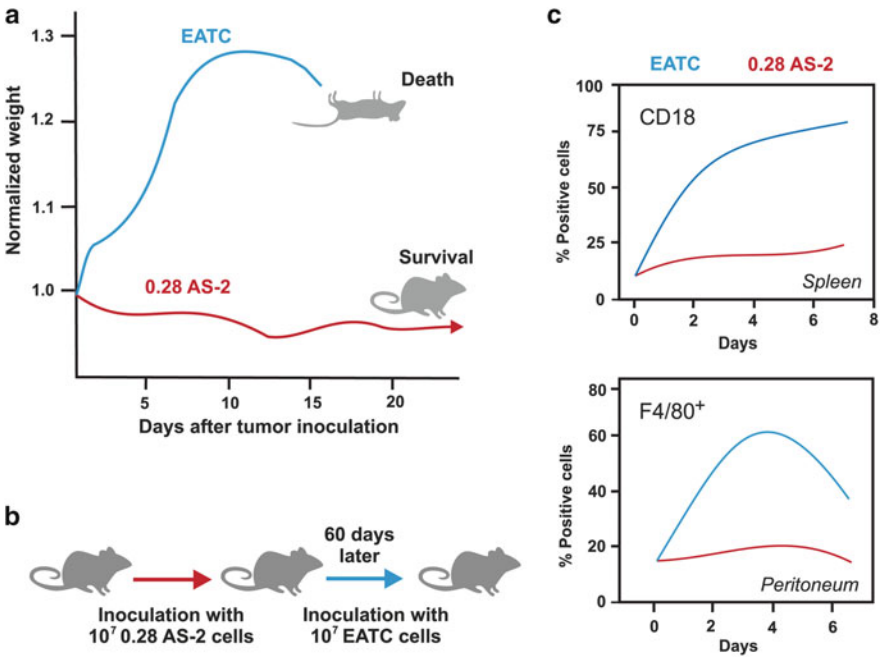

Fig. 12.4 In vivo *growth characteristics of the 0.28AS-2 cells*. (**a**) When 10^7 parental EATC were injected i.p. into normal Swiss albino mice, animals died around day 16. On the contrary, when 10^7 0.28AS-2 cells were injected, tumors were unable to grow and animals survived. (**b**) Mice inoculated with 0.28AS-2 cells kept immunologic memory and rejected a second inoculation with parental EATC. (**c**) Study of the immune system response in mice inoculated with 0.28AS-2 cells revealed, among other changes, an increase in splenic CD18 cells and the presence of a large number of activated F4/80$^+$ macrophages in the ascites cavity. These features, not observed in mice inoculated with parental EATC, indicate that a distinctive strong immune response occurred in animals inoculated with 0.28AS-2 cells. These data are consistent with the almost negligible expression observed in vitro in 0.28AS-2 cells of both MUC-1 and *N*-acetyl-α-D-galactosaminidase, proteins involved in the escape from host immune system

biosynthetic enzymes (Liu et al. 2012). In this way, c-Myc markedly increased Gln-derived proline biosynthesis; however, the relevance of this novel link between Gln and proline in cancer metabolic reprogramming remains to be established.

GLS isoforms are also upregulated by other oncogenic signaling pathways such as the small Rho GTPases (Wang et al. 2010). While screening for small molecule inhibitors that block oncogenic transformation induced by Rho GTPases in fibroblasts, the authors discovered that compound 968 (a bromo-phenanthridinone) was a very effective inhibitor of cellular transformation, but showed no effect on the growth of normal fibroblasts. Implementation of proteomic technologies allowed identification of the target for 968: the GLS isoform GAC (Wang et al. 2010). In agreement with this finding, knocking down both GLS isoforms, KGA and GAC, in Rho-transformed cells mimicked the effects of compound 968, while knocking down Rho proteins disrupted mitochondrial GA activity. The Rho GTPase-

mediated activation of GAC was shown to be dependent on nuclear factor-kappa B (NF-κB), which probably induces posttranslational modifications in the GAC protein and, thus, maximizes its enzyme activity in cancer cells (Wilson et al. 2013). It is well known that NF-κB is an important regulator of cell survival, proliferation, and differentiation and is frequently involved in malignant transformation. Of interest, activation of the NF-κB member p65 inhibits miR-23a expression in human leukemic Jurkat cells growing in Gln medium (without glucose) by recruiting histone deacetylase (HDAC) to miR-23a promoter (Rathore et al. 2012). Impaired miR-23a expression allowed a higher expression of GLS, higher consumption of Gln, and further protection (through enhanced GSH generation) against the damaging effects of high ROS levels usually found in leukemic cells, in a similar fashion to that previously reported for c-Myc regulation of miR-23a/b expression (Gao et al. 2009). Therefore, GLS upregulation through miRNAs seems to be a mechanism by which cancer cells gain selective advantages for using alternative sources of carbon favoring their adaptation to changing metabolic environments.

The GLS isoform GAC seems to be predominantly expressed in certain types of tumor with preference to KGA, the other GLS-encoded isozyme. In fact, GAC was first cloned and characterized from human colon cancer cells (Elgadi et al. 1999), while normal kidney and brain tissues were the sources of most mammalian KGA cDNA clones (Porter et al. 2002). The molecular pattern of GA isoform expression in cancer cells is currently being elucidated, and expression of concrete GLS isoforms might be related to the type of tumor. Hence, such tumor-specific pattern of GA expression can be of clinical interest. Furthermore, as GAC and KGA differ only in their C-terminal region, expression data previously obtained with molecular probes (oligonucleotides and antibodies) that do not discriminate between both isoforms should be revisited. A recent analysis of Gln dependency in non-small cell lung cancer identified GLS as the key gene associated with this dependence. In addition, the ratio GAC/KGA increased in lung tumors compared with matched normal lung tissue, mostly due to a significant downregulation of KGA because GAC expression levels were similar in tumor and normal tissue (van den Heuvel et al. 2012). Furthermore, transient knockdown of GAC had a greater effect on tumor cell growth than silencing of KGA, reinforcing the view of GAC playing a more relevant role than KGA in cancer.

Histone acetylation, and therefore gene expression, has been linked to glucose utilization through the actions of ATP citrate lyase (Wellen et al. 2009). Nowadays, novel experimental evidences are also linking Gln metabolism to the epigenome of cancer cells, concretely to changes in histone-modifying enzymes like HDAC and histone demethylases. The GLS inhibitor compound 968 was cytotoxic for noninvasive epithelial (T-47D and MDA-MB-361) and invasive mesenchymal (MDA-MB-231 and Hs-578 T) breast cancer cell lines, with the greatest effect being observed in MDA-MB-231 breast cancer cells (Simpson et al. 2012). A transcriptomic analysis found that compound 968 treatment induced significant downregulation of 20 critical cancer-related genes, the majority of which are anti-apoptotic and/or promote metastasis, including AKT, BCL2, BCL2L1, CCND1,

CDKN3, ERBB2, ETS1, E2F1, JUN, KITLG, MYB, and MYC. Histone H3K4me3, a mark of transcriptional activation, was reduced at the promoters of all but one of these critical cancer genes. Besides, the expression of other epigenetic regulatory genes, known to be repressed during apoptosis (e.g., DNMT1, DNMT3B, SETD1, and SIRT1), was also downregulated by this inhibitor of GLS isoforms. These changes in gene expression and histone modifications were accompanied by the activation of apoptosis and decreased invasiveness and resistance of MDA-MB-231 cells to chemotherapeutic drug doxorubicin (Simpson et al. 2012). Based on these and previous results, the authors consider that it is plausible that Glu production influences histone modifications to reprogram cells. Actually, Glu was chosen as one of 11 potential serum biomarkers selected as predictors of recurrent breast cancer in a metabolomics study combining NMR and GC–MS (Asiago et al. 2010).

Finally, in relation with signaling pathways controlling GLS isoforms, a synergistic cross-talk between KGA-mediated glutaminolysis and epidermal growth factor (EGF)-activated Raf-Mek-Erk signaling has been recently reported in human 293 T cells (Thangavelu et al. 2012). The increase in KGA activity mediated by the Raf-Mek-Erk module was found to be phosphorylation dependent, because specific inhibition of kinases or co-expression of protein phosphatase PP2A completely abrogates the enhanced KGA activity. Whether this regulation of *GLS*-encoded KGA isoform by Raf-Mek-Erk signaling could be relevant in cancer cells awaits further elucidation.

12.9 GLS2 Isoforms: GAB and LGA

In contrast to GLS isoforms, the role of GLS2 isoenzymes in tumor cells is greatly unknown. In leukemia cells from medullar blood of human patients and several established breast cancer cell lines, co-expression of both GLS and GLS2 transcripts was always found using a competitive RT-PCR assay (Pérez-Gómez et al. 2005). Co-expression was also confirmed at the protein level using isoform-specific antibodies, although the protein data suggest that GLS isoforms would account for the majority of GA activity in these human tumor cells (Pérez-Gómez et al. 2005). These results suggest that simultaneous expression of both GLS and GLS2 isozymes in the same cell type could be more frequent than previously thought: it has been found in human colorectal tumor cells (Turner and McGivan 2003), human hepatoma HepG2 cells, medullar blood mononuclear cells from patients suffering from leukemia, KU812F human myeloid cells, and human breast cancer cells MCF7 and ZR-75 (Pérez-Gómez et al. 2005).

Although co-expression of GLS and GLS2 may occur in some tumors, the relative abundances of GLS transcripts and proteins are considerably higher than those of GLS2 in a wide variety of cancer cells. For instance, in human colorectal tumor cells, most GA activity was GLS type although these cells expressed both GLS and GLS2 transcripts (Turner and McGivan 2003). These authors also show that GLS2 mRNA was consistently higher in slow-growing adenomas than in

rapidly proliferating carcinomas. On the other hand, mature lymphocytes from the medullar blood of a patient suffering aplasia did not express GLS transcripts but showed a 15-fold increase of GLS2 transcripts (Pérez-Gómez et al. 2005). Furthermore, GLS2 expression is downregulated in hepatocellular carcinomas (Hu et al. 2010; Suzuki et al. 2010) and highly malignant glioblastoma (Szeliga et al. 2005). Taken together, the data suggest that upregulation of GLS isoforms correlates with increased rates of proliferation, whereas prevalence of the GLS2 isoforms seems to be related with differentiated and quiescent cell states.

We postulated a completely different role for GLS and GLS2 isoforms in cancer based on their relative expression patterns in human leukemia, breast cancer cells, and hepatocellular transformation (Pérez-Gómez et al. 2005). The main facts taken into consideration were: (i) rat hepatoma cells express the KGA isoform; (ii) fetal hepatocytes express the KGA isoform and, shortly after the child is born, there is a loss of expression of KGA whilst LGA becomes turned on; (iii) normal nonproliferating hepatocytes express the LGA isoenzyme; and (iv) human hepatoma cells express both isoforms, although KGA seems prevalent and account for most of the GA activity. Therefore, we proposed that the process of malignant transformation shifts the pattern of GA expression in such way that GLS becomes upregulated while GLS2 is frequently repressed. For instance, transformed liver cells, like HepG2, return to a fetal-like phenotype, characterized by a higher rate of cell proliferation and prevalence of GLS expression.

Accordingly, we hypothesize that tumor could be inhibited by GLS silencing or, alternatively, by GLS2 overexpression. In glioblastomas (WHO grade IV), the most malignant brain tumors, high levels of GLS, and only traces or lack of GLS2 transcripts were found (Szeliga et al. 2009). Likewise, human glioblastoma T98G cell line expresses high amounts of GLS transcripts, while GLS2 transcripts are hardly detectable in these cells. Furthermore, in view of the presumed role of GLS2 in modulation of gene transcription (Olalla et al. 2002), we hypothesized that its deficit has implications for the physiology of glia-derived tumors, perhaps driving them toward a malignant phenotype. To address this question, human glioblastoma T98G cells were stably transfected with the full GAB cDNA coding sequence, and the effects of transfection on proliferation, migration, and survival were assessed. The transfected cells (T98-GAB) showed a 40 % decrease of cell survival were assessed, a 45 % reduction of cell migration, and a 47 % decrease in the proliferation index. Microarray analysis revealed a significantly altered expression of 85 genes in T98-GAB, but not in sham-transfected or control cells ($p < 0.005$). Microarray data, which included over 47,000 transcripts, were confirmed by qRT-PCR analysis for eight genes potentially relevant to glioma malignancy: S100A16, CAPN2, FNDC3B, DYNC1LI1, TIMP4, MGMT, ADM, and TIMP1 (Szeliga et al. 2009).

The molecular basis for this different behavior of GA isoforms in tumorigenesis is now starting to be uncovered. Two research groups identified GLS2 as a target for p53 tumor suppressor gene (Hu et al. 2010; Suzuki et al. 2010). Although traditionally considered as a gene which triggers apoptosis or senescence to prevent tumor progression, recently described roles for p53 protein also include the maintenance of normal metabolism and survival pathways allowing cells to adapt to

various types of metabolic stress (Vousden 2010). Both groups found that p53 is the first tumor suppressor gene involved in the regulation of Gln metabolism by activation of GLS2 expression. Although increase in GLS2 abundance was triggered by specific stresses that activate p53, basal GLS2 levels were also regulated by p53 under nonstressed conditions (Hu et al. 2010). Cells with heightened GLS2 levels showed enhanced Glu concentrations, increased OXPHOS activity, and higher GHS/GSSG ratios. Furthermore, overexpression of GLS2 afforded protection from ROS-induced apoptosis (Hu et al. 2010) or from DNA damage caused by daunorubicin (Suzuki et al. 2010). GLS2 was confirmed to be a p53 target gene in both non-tumor and tumor cells. With regard to cancer cells, it is remarkable that GLS2 transcripts were almost absent or significantly decreased in hepatocellular carcinomas compared to normal liver tissue, where GLS2 is abundantly expressed (Suzuki et al. 2010). These findings further support the hypothesis mentioned above and show that repression of GLS2 is a frequent trait associated with tumorigenesis. Of note, overexpression of GLS2 in human H1299 cells (non-small cell lung carcinoma) induced significant reductions in growth and colony formation ability, in agreement with results found in human glioblastoma T98G cells transfected with GLS2 cDNA (Szeliga et al. 2009). Hence, the authors suggested a potential tumor suppressor role for GLS2 (Suzuki et al. 2010).

Recent experimental evidences obtained from tumor and non-tumor cells have shed light on the seemingly opposing role play by GLS and GLS2 in tumorigenesis. Isoforms encoded by *GLS* are upregulated in parallel with the proliferation rate, whereas isoforms encoded by *GLS2* are related to quiescent, nonproliferating, and differentiated cell states. It is noteworthy that, GLS overexpression induced by oncogene c-Myc is related to enhanced cell proliferation by promoting glutaminolysis. Accordingly, silencing of GLS significantly decreased proliferation of prostate cancer cells in vitro (Gao et al. 2009), EATC in vitro and in vivo (Lobo et al. 2000), and T98G glioblastoma cells (Cheng et al. 2011). Indeed, inhibition of GA activity in non-tumor cells induced senescence, which is considered an important mechanism of tumor suppression (Unterluggauer et al. 2008). On the other hand, we demonstrated a similar tumor regression (as that obtained by blocking GLS with antisense or RNAi strategies) by overexpressing the *GLS2*-encoded GAB isozyme in human glioblastoma (Szeliga et al. 2009). Of interest, it has been demonstrated that GLS2 can be transcriptionally regulated by TAp63, a transcription factor belonging to the p53 family (Giacobbe et al. 2013). Specifically, these authors showed that GLS2 and TAp63 expression increase during differentiation of primary human keratinocytes whilst depletion of GLS2 inhibits skin differentiation.

An intriguing question arose whether or not combination of GLS silencing and GLS2 overexpression would increase the inhibition of cell proliferation and survival of glioblastoma cells elicited by individual manipulations. To answer this question, the expression of KGA and GAC isoforms was knocked down with siRNA in a human glioblastoma cell line that was (T98-GAB) or was not (T98G) previously transfected with GAB cDNA, respectively (Szeliga et al. 2009). Then cell viability and proliferation were investigated in so treated cells with a graded inhibition of KGA and GAC, in order to analyze the correlation between the

phenotypic changes and the Gln content as a marker of the intensity of its consumption. In both T98G and T98-GAB cell lines, silencing of GLS decreased cell viability and proliferation in a different, sequence-dependent degree, and the observed decreases were in either cell line highly correlated with increase of intracellular Gln ($r > 0.9$), a parameter manifesting decreased Gln degradation (Szeliga et al. 2013). The results show that combination of negative modulation of GA isoforms arising from GLS gene with the introduction of the GLS2 gene product, GAB, may in the future provide a useful means to curb glioblastoma growth in situ. At the same time, the results underscore the critical role of Gln degradation mediated by KGA in the manifestations of aggressive glial tumor phenotype.

In another recently published study, SFxL and LN229 glioma cells with silenced GLS expression (Cheng et al. 2011) were employed, along with T98-GAB glioma cell line, to ascertain whether modulations of GA expression may synergize with oxidative stress against proliferation of cancer cells (Martín-Rufián et al. 2014). GLS-silenced glioma cells showed lower survival ratios and a reduced GSH-dependent antioxidant capacity. Silencing *GLS* or overexpressing *GLS2* genes decreased glioma cell survival. This effect was increased by an oxidative insult. Furthermore, ROS generation by treatment with oxidizing agents synergized with either GLS silencing or GLS2 overexpression to suppress malignant properties of glioma cells, including the reduction of cellular mobility. Of note, blocking GLS or overexpressing GLS2 evoked lower c-Myc and Bcl-2 expression, as well as higher proapoptotic Bid expression (Martín-Rufián et al. 2014). In conclusion, the combination of modulation of GA expression and treatment with oxidizing agents may become a therapeutic strategy for gliomas and other intractable cancers.

Although repression of GLS2 is a frequent trait observed in tumors, this behavior is not universal and there are also some cancer cells where GLS2 is overexpressed. For example, the expression of GLS2 was significantly enhanced in cervical carcinoma; even more, this upregulation was related to therapeutic resistance (Xiang et al. 2013). However, when GLS2 was silenced in radioresistant cell (HeLaR), they showed substantially enhanced radiosensitivity with lower colony survival and higher apoptosis in response to radiation. In vivo, xenografts with GLS2-silenced HeLaR were more sensitive to radiation. At the molecular level, knockdown of GLS2 increased the intracellular ROS levels of HeLaR exposed to irradiation by decreasing the productions of antioxidant GSH, NADH, and NADPH. The authors conclude that GLS2 may have an important role in radioresistance in cervical cancer patients (Xiang et al. 2013). The heterogeneity of tumor-induced changes in the expression of key metabolic genes—like GLS and GLS2—suggests that different tumors might have differential requirements of glutaminolysis. Accordingly, increased Gln catabolism in mouse liver tumors was associated with decreased levels of GS and the switch from Gls2 to Gls (Yuneva et al. 2012). In contrast to liver tumors, MYC-induced lung tumors display increased expression of both GS and Gls and accumulate Gln. These results suggest that the metabolic profiles of tumors are likely to depend on both the genotype and

tissue of origin and have implications regarding the design of therapies targeting tumor metabolism (Yuneva et al. 2012).

In summary, GA is essential to the metabolic phenotype of growing tumors. Cancer cells may stop their proliferative program either by knocking down Gls or by upregulating Gls2 isoforms, in agreement with their seemingly opposing roles in cancer. Although it is presently unknown how GA isozymes may undergo such different roles in tumor biology, the control of GA isozyme expression may prove to be a key tool to alter both metabolic and oxidative stress in cancer therapy. At first glance, these contrasting roles of GA isozymes may appear inconsistent, as well as the fact that glutaminolysis in cancer can be activated by c-Myc for tumorigenesis and also by p53 for tumor suppression. However, there are some hints that may help to explain this apparently puzzling behavior. Recently, novel GA isoforms and extramitochondrial locations for these proteins have been discovered: identifying the function of each isozyme is essential for understanding the role of GA in tumors. In addition, the interactome of GA isoforms is starting to be uncovered adding a new level of regulatory complexity with important functional consequences, including selective and regulated targeting to specific cellular locations. Clearly, GLS and GLS2 show distinct kinetics, molecular and immunological properties that make the consequences of their enhanced expression quite different and strongly dependent on factors that include signals, environment, and cell/tissue type.

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Chapter 13
Essential Role of Mitochondria in Pyrimidine Metabolism

Monika Löffler, Elizabeth A. Carrey, and Elke Zameitat

Contents

In Memoriam Prof Dr. Hartmut Follmann, PhD *1936 † 2013
'The existence of DNA, its replication, and nowadays, deliberate modification and manipulation of its genetic message in vitro appear to be of such overwhelming scientific and public interest that the origin and nature of deoxyribonucleotides, its building blocks, are simply neglected by most geneticists and other life scientists. It is considered that these monomers are available intracellularily at all times, and for in vitro experiments they come in the convenient deoxyribonucleotide kits in any molecular biology laboratory.' (Follmann 2004)

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13.1 Mitochondria Are Self-Contained Sections in the Network of Metabolic Pathways

The mitochondrion forms a structural, functional and regulatory compartment within the cell. Its four structural regions—the outer membrane, inner membrane, intermembrane space and matrix—allow a sub-compartmentation in order to carry out its various metabolic actions, essential to the life of most eukaryotic cells. Each of the regions contains a unique collection of enzymes. Most of these are coded in the nucleus and imported into the mitochondrion from the cytosol by specialised protein translocases of the outer and inner membrane.

Each mitochondrion contains multiple copies of a circular double-stranded DNA located close to the inner membrane. Thirteen proteins encoded in the mitochondrion's own genome serve central functions in the respiratory chain and oxidative phosphorylation of the inner membrane: seven subunits out of 40 of complex I, one subunit out of 11 of complex III, three subunits out of 13 of cytochrome oxidase and two subunits out of 12 of ATP synthase. In contrast, all enzymes for the oxidation of substrates—e.g. enzymes of the tricarboxylic acid (TCA) cycle, pyruvate dehydrogenase and ß-oxidation—are encoded in the nucleus. Most of these enzymes are located in the matrix which is enclosed by the folded inner membrane. Hence, the enzymes of the matrix and the inner membrane are the major energy-producing team of the mitochondrion (Alberts et al. 2008; Voet and Voet 2011).

Mitochondria are also involved in pathways in intermediary metabolism: a series of enzyme-catalysed sequential reactions, in which the product of one reaction is the substrate of the next. The internal environment of the mitochondrion is very different from the traditional concept of enzymes and substrates in free solution in the cytosol. For example, it was concluded that the enzymes of the tricarboxylic acid cycle (TCA) form a *metabolon* in the matrix which allows more efficient passing of the intermediary product from one enzyme to the next, thus facilitating the catabolic function of the TCA cycle (Robinson et al. 1987). The association does not restrict the supply of important precursors for biosynthetic pathways and metabolic processes which proceed in the cytosol or other organelles.

Intermediates, such as citrate—a precursor for the cytosolic synthesis of fatty acids and isoprenoids from acetyl-CoA—must be translocated by means of more or less substrate-specific carriers in the inner mitochondrial membrane, and equally, transporters for metabolites trafficking from cytosol to matrix are obligatory. Due to its focal status in cellular energetics, the ADP-ATP translocator of the inner mitochondrial membrane is probably one of the best-known and most thoroughly researched systems (Voet and Voet 2011). In marked contrast, the manner of transport of pyrimidine ribo- and deoxyribonucleosides and nucleotides (precursors for mitochondrial metabolism and proliferation) is not fully understood to date.

The synthetic pathway for heme benefits from free diffusion through the outer mitochondrial membrane since the first step occurs in the mitochondrion through the addition of succinyl-CoA to glycine by 5-aminolevulinic acid synthase, and, after a few intermediate steps in cytosol, metabolites return to the organelle where

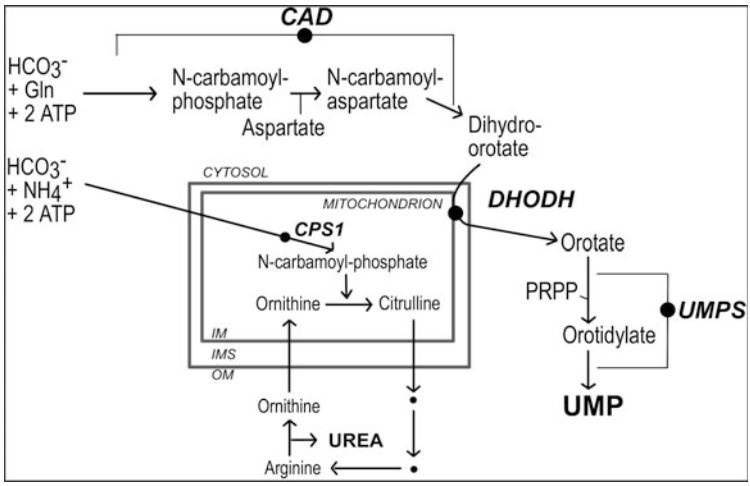

Fig. 13.1 Compartmentation of pyrimidine de novo synthesis and urea cycle. Carbamoyl phosphate synthetase I (CPS I) in mitochondria initiates the urea cycle (in liver only). Carbamoyl phosphate synthetase II (CPS II) in cytosol initiates the de novo synthesis of UMP. The CAD enzyme (CPS II + aspartate transcarbamoylase + dihydroorotase) and the UMP synthase (orotate phosphoribosyltransferase + orotidine 5'-monophosphate (orotidylate) decarboxylase) are located in cytosol. The formation of orotate from dihydroorotate is catalysed by the mitochondrial flavoenzyme dihydroorotate dehydrogenase (DHODH). *OM* outer membrane, *IM* inner membrane, *IMS* intermembrane space

the protoporphyrins take up the iron using ferrochelatase, which is bound to the inner membrane (overview Gattermann 1999; Wallace 2012). Similarly, the urea cycle starts with the synthesis of carbamoyl phosphate in the matrix (Fig. 13.1) and proceeds as a tightly linked pathway in which the three cytoplasmic enzymes are organised near the mitochondrion for efficiency of channelling of substrates (Cheung et al. 1989). Transporters convey ornithine and citrulline across the inner mitochondrial membrane. Finally, the substrates and enzymes for de novo synthesis of UMP are found in the cytosol except for one enzyme reaction, dihydroorotate dehydrogenase (DHODH), which is fixed in the mitochondrion (Fig. 13.1).

13.2 Intracellular Location and Regulation of the Enzymes of Pyrimidine De Novo Synthesis

Uracil, cytosine and thymine are the pyrimidine bases of ribose-containing nucleosides (uridine, thymidine and cytidine) or deoxyribose-containing deoxynucleosides and their corresponding (deoxy)ribonucleotides. The diverse functions of pyrimidines—building blocks for DNA and RNAs; prerequisites for the synthesis of glycogen, glycoproteins, glycolipids and phospholipids in membranes; and signalling molecules for cell surface receptors in the sympathetic

nervous system—lead to the need for permanent and well-balanced pools of these compounds in cells (review Keppler and Holstege 1982; review Connolly and Duley 1999).

In animal cells, pyrimidine nucleotides can be formed either by de novo synthesis of uridine monophosphate (UMP) from CO_2, glutamine and aspartate or by salvage/recycling of uridine (Fig. 13.2) (textbooks: Voet and Voet 2011; Grisham et al. 2008). Enzymes catalysing five reaction steps to UMP are found in the cytosol and one, DHODH, in the mitochondrion (Fig. 13.1). In contrast to purine synthesis, the pyrimidine ring is assembled first and is then linked to ribose-5-phosphate obtained from phosphoribosyl diphosphate (PRPP). As a result of exon shuffling and gene fusion, only three genes (in humans located on chromosomes 2p21, 16q22 and 3q13, respectively) encode the six enzymes for the synthesis of UMP (Barnes et al. 1993). The first three enzymes form a single trifunctional polypeptide, CAD: glutamine-dependent carbamoyl phosphate synthetase II (CPSII) + aspartate transcarbamoylase (ATCase) + dihydroorotase (DHOase) (Fig. 13.1). The fifth and sixth steps—orotate phosphoribosyltransferase (OTCase) and orotidine monophosphate (OMP) decarboxylase—are combined in the bifunctional uridine monophosphate synthase (UMPS) (review Jones 1980). In higher eukaryotes as well as in most unicellular organisms, the fourth enzyme, dihydroorotate dehydrogenase (DHODH), is located in the inner membrane of the mitochondrion with tight connection to the electron transport chain (Miller et al. 1968; Chen and Jones 1976; Löffler and Zameitat 2013). The other enzymes for de novo biosynthesis of cytidine and thymidine nucleotides (CTP synthase, ribonucleotide reductase (RNR) and thymidylate synthase) (Fig. 13.2) are located in the cytosol.

The very thorough investigations by Tatibana and Shigesada (1972) identified the relationship between proliferation and pyrimidine de novo synthesis in rat liver, and Ito and Uchino (1976) studied phytohaemagglutinin-stimulated lymphocytes as a model for leukaemia. A rapid ‘upregulation’ of the first steps of the de novo pathway was identified, but at the time, it was not possible to say whether this occurred through activation of existing enzyme or through production of new enzyme molecules. Classically, pyrimidine de novo synthesis is moderated through the rate-limiting activity of the CPSII in the CAD enzyme. This reaction is allosterically activated by PRPP and feedback inhibited by UTP, the most abundant pyrimidine ribonucleotide in cells. Additional growth-related regulation of the pyrimidine de novo pathway is achieved by phosphorylation reactions. Since the first demonstration that purified CAD is phosphorylated and CPS II is activated by protein kinase A (Carrey et al. 1985), it has become apparent that several protein kinases act upon this enzyme in vivo and enhance the production of pyrimidine precursors for growth and proliferation (Graves et al. 2000; Sigoillot et al. 2002, 2005; Ben-Sahra et al. 2013; Robitaille et al. 2013). On the other hand, two caspase-3 cleavage sites in the CPSII domain of CAD could explain the loss of CPSII activity during apoptosis (Huang et al. 2002).

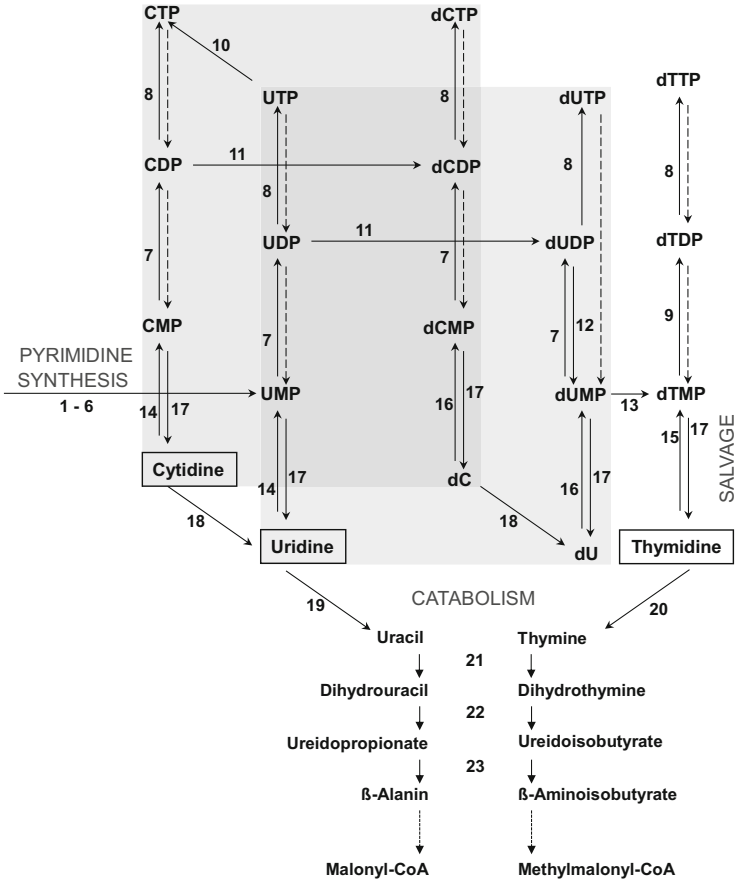

Fig. 13.2 Overview of paths to and from pyrimidines. The enzymes—given by numbers in the scheme—perform the biosynthesis (de novo enzymes 1–6), interconversion of UMP to other ribonucleotides and deoxyribonucleotides (7–13), the salvage of preformed metabolites from dietary products or catabolism of nucleic acids (14–18), the first steps of catabolism (19–20) and further catabolism (21–23). Note that most of the enzymes are in the cytosol, a few are in mitochondria and some isoforms are in cytosol and mitochondria (see text): 1, carbamoyl phosphate synthetase; 2, aspartate transcarbamoylase; 3, dihydroorotase; 4, dihydroorotate dehydrogenase; 5, orotate phosphoribosyltransferase; 6, orotidine 5′-monophosphate decarboxylase; 7, pyrimidine monophosphate kinases; 8, pyrimidine diphosphate kinases; 9, thymidine monophosphate kinase; 10, cytidine triphosphate synthetase; 11, ribonucleoside diphosphate reductase; 12, dUDP phosphohydrolase and dUTP pyrophosphohydrolase; 13, thymidylate synthase; 14, uridine/cytidine kinase; 15, thymidine kinase; 16, deoxycytidine/deoxyuridine kinase; 17, pyrimidine 5′-nucleotidases; 18, (deoxy)cytidine deaminase; 19, uridine phosphorylase; 20, thymidine phosphorylase; 21, dihydropyrimidine dehydrogenase; 22, dihydropyrimidinase; and 23, ureidopropionase

13.3 Metabolic Channelling in UMP De Novo Synthesis: A Paradox?

Multienzyme complexes and multifunctional enzymes, like CAD, UMPS and other enzymes in the parallel purine de novo pathway, are considered to be of physiological benefit for cells with a high demand for nucleic acid precursors (Zhang et al. 2008). It was usually assumed that such a benefit is kinetic: close proximity of enzyme active sites decreases diffusion time and raises local concentration of substrate while lowering local product concentration (Agius and Sherratt 1997). A more precise control of several enzyme activities through a single regulatory region seems most attractive from the perspective of intermediary metabolism. Further feasible advantages are in minimising side reactions, e.g. removal or decomposition of intermediates. In view of the low stability of carbamoyl phosphate (Irvine et al. 1997) and putative affinity of OMP for 5′-nucleotidase and orotidine for pyrimidine phosphorylase (Fig. 13.2, enzymes 17, 19) (see Sect. 7), this assumption seems reasonable. However, the location of the fourth enzyme DHODH in the mitochondrion suggests a problem if dihydroorotate synthesised in the cytosol must diffuse across the outer mitochondrial membrane to be oxidised to orotate, which in turn has to diffuse back into the cytosol to be a substrate for the bifunctional UMP synthase (Fig. 13.1).

The paradox was resolved following a study undertaken to locate the enzymes of UMP de novo synthesis (Carrey et al. 2002) using immunofluorescence and immunoelectron microscopy of spermatozoa and BHK fibroblasts which revealed the position of CAD and UMPS just around and outside mitochondria. Close association of the CAD protein with the mitochondrion would contribute to ‘channelling’ dihydroorotate more efficiently as a substrate for DHODH, especially since the equilibrium of the DHOase-catalysed reaction favours the reverse conversion of dihydroorotate to carbamoyl aspartate (Christopherson and Jones 1980). Aspartate may be supplied from the mitochondria for the ATCase reaction. The analogy with the urea cycle is compelling.

There has been controversy about the location of CAD since an earlier study that indicated some CAD molecules were cytosolic, possibly associated with the cytoskeleton. It was concluded from these findings that CAD could bind to and translocate along the filaments to mitochondria, which are known to be anchored to the cytoskeleton network (Evans and Guy 2004). Interestingly, CAD was also described in the nucleus (Angeletti and Engler 1998; Carrey et al. 2002). It is now known that relocation of the CAD protein is linked to its phosphorylation. Modification in vitro by protein kinase A was shown to activate the carbamoyl phosphate synthetase reaction, rendering it insensitive to feedback inhibition by UTP (Carrey et al. 1985). In vivo, after activation by MAP kinase, some CAD molecules transfer to the nucleus in parallel with increased pyrimidine biosynthesis in the cell (Sigoillot et al. 2005). Similarly, activation by S6 kinase appears to encourage polymerisation of CAD and causes a ‘punctate distribution’ in the cytoplasm (Robitaille et al. 2013): increased pyrimidine biosynthesis is accompanied by

increased carbamoyl aspartate in the cytosol (Ben-Sahra et al. 2013). Nevertheless, the need for the DHODH step means that the exterior of the mitochondrion remains the only feasible location for pyrimidine biosynthesis: it seems more likely that CAD in the nucleus may act as a monitor of external conditions, perhaps through the known affinity for its allosteric effectors.

A plausible mechanism could involve the feedback inhibitor UTP, which causes conformational changes in the CAD molecule that tend to prevent access of kinases (Carrey, 1989). Thus a fall in intracellular UTP concentrations, as RNA synthesis and other demands accelerate, may allow CAD to be phosphorylated and to exert its effect by binding to the androgen receptor (Morin et al. 2012) and other sites in the nucleus or ribosomes (Robitaille et al. 2013, Ben-Sahra et al. 2013) to stimulate transcription and translation of the CAD gene and others. This model is supported by similar findings on new roles of enzymes of the cytosolic purine de novo synthesis in the nucleus—GMP synthetase is involved in transcriptional regulation of ecdysteroid target genes, and IMP dehydrogenase of *D. melanogaster* is a DNA-binding transcription repressor (Kozhevnikova et al. 2012)—hence, it is reasonable to speculate that the CAD molecules in the nuclei of mammalian cells could accelerate pyrimidine de novo synthesis but are not directly involved as catalysts.

The discovery that UMP synthase is a bifunctional protein began with the identification of orotic aciduria, the first known human genetic disorder of pyrimidine biosynthesis. Anaemia, bone marrow defect and immunodeficiency have been described as clinical manifestations. UMPS deficiency is treatable with lifelong oral uridine treatment applying several grams per day (review Traut and Jones 1996; review Webster et al. 2001). Mutations in the DHODH gene were found only recently in patients with the Miller syndrome and suggested as a putative cause for this malformation disorder (Ng et al. 2010), perhaps through a link with transcriptional elongation of crucial genes (White et al. 2011). Hereditary defects of the CAD enzyme have to date not been found in humans, but zebrafish mutants in CAD are deficient in cell proliferation and differentiation during growth and morphogenesis of retina, jaw and pectoral fins (Willer et al. 2005), confirming the importance of pyrimidine synthesis in de novo DNA synthesis and UDP-dependent protein glycosylation.

An efficient channelling of steps 5 and 6 in the pyrimidines de novo synthesis was deduced from experiments on the preferential use by UMPS of orotidylate (OMP) produced from orotate and PRPP over exogenously added OMP even at a 100-fold excess (review Keppler and Holstege 1982; review Traut and Jones 1996). The cytosolic location of mammalian UMPS was confirmed by immunofluorescent microscopy and immunoelectron microscopy which detected the enzyme in close proximity around and outside the mitochondria (Carrey et al. 2002).

The fourth step of pyrimidine de novo synthesis apparently disrupts the channelling along the complete pathway to UMP. We propose that the persistence of DHODH in mitochondria during evolution could have a very positive attribute: the removal of redox equivalent is much more efficient when directly connected to the constitutively active respiratory chain, rather than transferring hydrogen to any

other acceptor in the cytosol, e.g. oxygen, fumarate and NAD. These have been described as co-substrates for soluble non-membrane-bound DHODHs in some unicellular organisms and gram-positive bacteria (see 1.4). The resulting coproduct succinate or NADH, respectively, must then be reoxidised by a second enzyme whose activity could interfere with the DHODH-catalysed reaction and thus risk the impairment or even inhibition of pyrimidine de novo synthesis.

13.4 The Prominent Position of Dihydroorotate Dehydrogenase in Mitochondria

Carbamoyl phosphate synthetase II produces the 'high energy phosphate'-bound carbamoyl phosphate, using glutamine as donor for the first nitrogen atom of the pyrimidine ring and requiring the cleavage of two ATP molecules. This input of energy in the biosynthetic pathway is balanced by the DHODH-catalysed oxidation of dihydroorotate to orotate in mitochondria, through the intermediate electron acceptor flavin mononucleotide (FMN) of the enzyme and subsequent reduction of the second enzyme substrate ubiquinone (CoQ) to ubiquinol (QH_2). From QH_2, the electrons continue—via complex III—through the electron transport chain to the final acceptor molecular oxygen (O_2) (Fig. 13.3). The electrochemical gradient obtained from the chain finally enables ATP synthase to provide the energy for ATP synthesis, irrespective of the electron donor, e.g. succinate or dihydroorotate (Voet and Voet 2011; Grisham et al. 2008).

The mitochondrially associated oxidation of dihydroorotate was described in early work (Miller et al. 1968), and the location of the DHODH at the outer surface of the inner mitochondrial membrane was confirmed by Chen and Jones (1976) using differential solubilisation procedures.[1] In default of reliable antibodies, our localisation approach to the DHODH activity by electron microscopy using cerium substrate techniques pointed to a matrix-side release of redox equivalents (Angermüller and Löffler 1995). Later studies revealed that DHODH of animals is an integral protein of the inner mitochondrial membrane but exposed to the intermembrane space (Rawls et al. 2000). These studies showed that the enzyme is not proteolytically altered during import, neither in vitro nor in vivo, and its uptake required inner membrane potential and matrix ATP. The adjacent hydrophobic segment of the mitochondrial DHODH protein acts as a membrane anchor sequence that holds the protein in the outer surface of the inner membrane. Similarly, DHODH of gram-negative bacteria—from which mitochondria are presumably

[1] Some textbooks of biochemistry ignore the ubiquinone-dependent mitochondrial DHODH from higher eukaryotes, instead referring to the enzyme from gram-positive bacteria, e.g. *Clostridium oroticum* (soluble, inducible, NAD-dependent, catabolic). For correct information on the mitochondrial DHODH in the biosynthetic pathway, relevant textbooks may be consulted, e.g. Voet and Voet 'Biochemistry' and Grisham, Graham and Graham 'Biochemistry'.

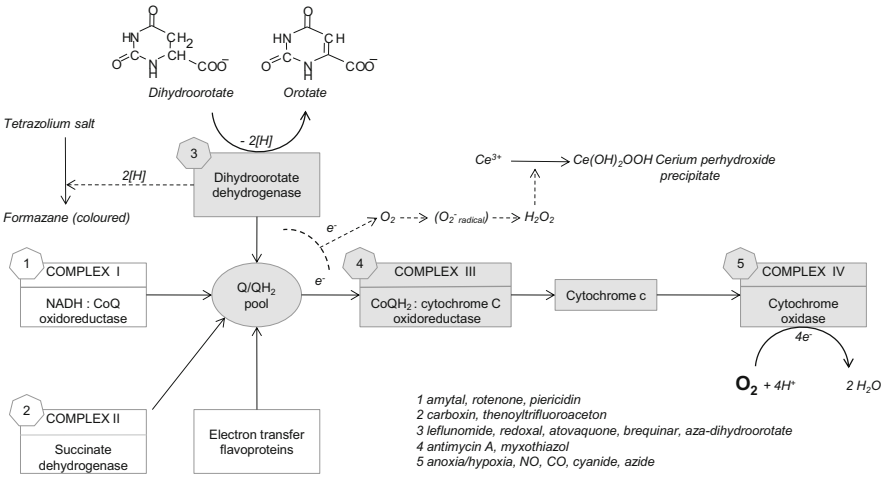

Fig. 13.3 Overview of mitochondrial dihydroorotate oxidation, linked to the functional electron transport chain via ubiquinone. Sites (1, 2, 3, 4 and 5) are indicated where specific inhibitors bind and block the electron flow. Hence, inhibition of complex III or IV could result in impairing the transfer of hydrogen from dihydroorotate to ubiquinone with concomitant decrease of UMP biosynthesis. Experimentally, the presence of complex III or IV inhibitors allowed the transfer of hydrogen to artificial electron acceptors, e.g. tetrazolium salts, to give coloured precipitates (Löffler et al. 1996) and also induced the formation of reactive oxygen species (ROS), which were captured by cerium ions to give an electron-dense precipitate (Angermüller and Löffler 1995). In both cases, specific inhibitors of DHODH (site 3) prevented the histochemical visualisation and localisation of the enzyme activity. CoQ, ubiquinone; QH_2, ubiquinol

descendants—is firmly attached to the cell membrane. The N-terminal sequences that direct DHODH import and location in mitochondria of higher eukaryotes are absent from the enzyme protein occurring in a few fungi, e.g. *S. cerevisiae*, and gram-positive bacteria, in which DHODH is a cytosolic protein (Jensen and Björnberg 1998).

13.5 Dihydroorotate Dehydrogenase and Hypoxia

Since activities of mammalian DHODH with substrates other than dihydroorotate and ubiquinone have not been reported to date, the DHODH-catalysed reaction is evidently part of the oxidative metabolism of mitochondria.

The oxygen consumption related to specific activity of DHODH was measured with a Clark-type oxygen electrode in isolated mitochondria of rat liver, kidney and heart, allowing direct comparison with the specific activity of the electron transport chain enzymes (Fig. 13.3) and revealing a tissue-specific capacity and stoichiometry (Jöckel et al. 1998). The specific activities evaluated from oxygen consumption

measurements were 35 nmol NADH/min/mg protein, 17 nmol succinate/min/mg protein and 2,5 nmol dihydroorotate/min/mg protein. In heart mitochondria, the difference in activity between respiratory enzymes and DHODH was much more pronounced, demonstrating the great functional importance of the electron transport chain for cardiac bioenergetics and the moderate importance of pyrimidine de novo synthesis for the specialised function of the myocardium (Löffler et al. 1997). A more recent study on tumour cells (premalignant prostate epithelial cells and malignant cutaneous keratinocytes) showed a reduction of about 30 % in oxygen consumption when the cells were treated with the DHODH inhibitor teriflunomide leading to an approximately 50 % reduction of uridine pools (Hail et al. 2010).

Earlier studies on tumour cells (EAT) cultured under graduated oxygen tension (20 % to 0.001 %) in a protective atmosphere were undertaken to elucidate whether the limited proliferative activity observed under hypoxia could result from an inadequate supply of nucleic acid precursors (Löffler 1980; Löffler 1989). Rapid cessation of DNA synthesis through inhibition of replicon initiation (Probst et al. 1988), insensitivity of G_2 cells to oxygen shortage and accumulation of cells before the G1/S transition of the cell cycle as well as dislocation in pyrimidine deoxynucleotide pools were observed. When this tumour cell line was cultured under 1 % O_2, supplementation with exogenous uridine improved all growth parameters, whereas the addition of deoxycytidine was necessary for cells kept under 0.1 % O_2. This may be due to the dependence on oxygen for generating the tyrosyl radical of ribonucleotide reductase (review Follmann 2004). From these and other experiments, it could be concluded the dihydroorotate dehydrogenase step is the likely focal point for the hypoxic failure in pyrimidine biosynthesis (Fig. 13.3). It has recently been shown that transcription of the *cad* gene is sensitive to hypoxia (Chen et al. 2005).

Studies with other tumour cell lines, NHIK3025 (Amellem et al. 1994), suggested that the biosynthetic pathway to pyrimidine(deoxy)nucleotides—through mitochondrion-coupled DHODH—is a potential link between environmental oxygen tension and the proliferative capacity of cells. Many observations support this concept: cells defective in the electron transport chain at the stage of complex III or IV (Fig. 13.3)—through physical limitations, absence of mitochondrion-coded subunits, mutants and inhibitors, respectively—express impaired DHODH activity and decreased UMP de novo synthesis (Grégoire et al. 1984; Löffler 1987; Gattermann et al. 2004; Beuneu et al. 2000; Hail et al. 2010). In permeabilised cells—L1210, CEM-SS, Raw264, HL60, K562 and U937—a dramatic decrease in dihydroorotate-dependent oxygen consumption was measured on addition of the complex IV inhibitor nitric oxide (NO) in parallel with a decrease in orotate formation. Application of cyanide and the complex III inhibitor antimycin (Fig. 13.3) as well as the DHODH-specific inhibitor leflunomide caused a comparable effect. In the course of culture experiments, impairments of the electron transport chain are relieved by uptake and salvage of the nucleoside uridine or by its ‘hidden’ presence in growth medium components, e.g. serum. In a similar way, direct inhibition of DHODH in the presence of antiproliferative drugs, such as leflunomide derivatives, brequinar or aza-dihydroorotate (Löffler 1980; Peters

et al. 1990; Rückemann et al. 1995; Herrmann et al. 2000; Gattermann et al. 2004), was neutralised by uridine.

Accordingly, all enzymes of the pyrimidine de novo synthesis pathway have been considered as promising targets for the development of antiproliferative drugs (Sect. 6). DHODH is of increasing interest lately as a target for new drugs to reduce aberrant immunological reactions and to interfere in the multiplication of parasites and parasitic protozoa in malaria-infected patients and also for new anticancer strategies (overview Christopherson et al. 2002; Knecht et al. 2000; Boa et al. 2005; Baumgarten et al. 2006; Zameitat et al. 2007; Hortua et al. 2012).

Although we have described uridine auxotrophy in cells with electron transport chain defects in vitro, evidence for pyrimidine deficiency in brain on infusion of mice with azide—which, comparable to cyanide, is an inhibitor of complex IV (Fig. 13.3) and often used in cell culture experiments to mimic 'bioenergetic hypoxia'—could not be shown (Garcia et al. 2005). Likewise, a reduction in pyrimidine levels and a p53 upregulation were observed when RKO (colonCA) were treated with the complex III inhibitor myxothiazole or with the DHODH inhibitor leflunomide but not in the presence of cyanide (Khutornenko et al. 2010). On the other hand, the visualisation of DHODH activity—like that of succinate dehydrogenase—in tissue sections, tumour cell smears and cultured cells by application of the nitro-blue tetrazolium technique was possible only in the presence of cyanide (Löffler et al. 1996). As a potent inhibitor of cytochrome oxidase (Fig. 13.3), cyanide can prevent the flow of electrons—originating from the substrate dihydroorotate or succinate, respectively—along the chain to complex IV and oxygen.

A direct link between hypoxia-induced cell cycle arrest in G_1 and early S-phase in cultured cells and a shortage of pyrimidine nucleotides with concomitant lack of deoxynucleotide precursors for DNA synthesis may be only part of the story. Activation of the retinoblastoma protein by dephosphorylation under moderate hypoxia may also be responsible for the hypoxia-induced cell cycle arrest in S-phase of human NHIK cells (Amellem et al. 1998). In fibroblasts under chronic hypoxia, a downregulated CAD expression in response to induced HIF-1α accumulation could be of significance (Chen et al. 2005). Hypoxia-inducible factor 1 was shown to mediate adaptive responses to reduced oxygen availability (chronic hypoxia) by regulating gene expression (Semenza 2011).

It has always been assumed that the DHODH-catalysed reaction—due to the involvement of flavin and ubiquinone—could give rise to formation of reactive oxygen species (ROS) in mitochondria (Forman and Kennedy 1975). The major intracellular source of oxygen radicals is the electron transport chain, where superoxide can be produced, e.g. by transfer of one electron to O_2 from the stable semiquinone produced during reduction of ubiquinone (coenzyme Q) to ubiquinol (QH_2) by the appropriate enzymes (Dröse and Brandt 2008). The study described above on the synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (Hail et al. 2010) implicated DHODH activity in the 4-HPR-induced generation of ROS and apoptosis in malignant cells. The drug was suspected to redox cycle within the mitochondrial electron transport chain by reacting at coenzyme Q-binding sites. A recent

study on rat skeletal muscle mitochondria demonstrated that DHODH itself can generate superoxide/H_2O_2 at a small rate compared to the rates from other sites in the electron transport chain. When oxidation of ubiquinol was prevented at the stage of complex III, e.g. by antimycin (Fig. 13.3), the dihydroorotate oxidation led to ROS production at 10-fold higher rates (Hey-Mogensen et al. 2014). The in situ DHODH activity in rat kidney and heart was located visually by the 'cerium capture technique', in which the electron-dense precipitate cerium perhydroxide identifies the location of oxidase activities in tissues and cells by electron microscopy: when azide was used on complex IV to prevent the flow of electrons along the electron transport chain, hydrogen peroxide (Fig. 13.3) was produced in an oxidase-like side reaction on addition of the specific substrate dihydroorotate, but could be switched off by the DHODH-specific inhibitor brequinar (Angermüller and Löffler 1995), which originally was developed as anticancer agent in the 1980s (Peters et al. 1990).

13.6 The Importance of Mitochondria in the Salvage of Pyrimidines

Replication of DNA in mitochondria (mt DNA) is independent of the cell cycle. Since mtDNA is exposed to reactive oxygen species, deoxynucleotides are constantly required for its repair in dividing and nondividing cells. There are two ways to fulfil this requirement: intramitochondrial salvage of deoxynucleosides or import of deoxynucleotides from the cytosol. In general, pyrimidine de novo synthesis and salvage pathways as well as the enzymes for interconversion of (deoxy)nucleoside mono-, di- and tri- phosphate (Fig. 13.2) ensure all pyrimidine nucleotide and deoxynucleotide pools in the cytosol of cells. Uridine, (deoxy)cytidine and thymidine originating from daily nutrients or intracellular turnover and breakdown of nucleic acids are generally rescued from circulation by (deoxy)ribonucleoside kinases which are located in the cytosol and also in mitochondria. These salvage kinases for pyrimidine nucleosides (Fig. 13.2, enzymes 14, 15, 16, 17) use ATP as the major phosphate donor and UTP as a minor donor. The broad substrate specificity of the cytosolic deoxycytidine kinase (dCK) (Fig. 13.3, enzyme 16), e.g. phosphorylation of deoxycytidine and deoxyuridine and also deoxyadenosine and deoxyguanosine (Csapó et al. 2001), enables this enzyme to activate a great number of clinically important nucleoside analogues in humans under therapy (review Parker 2009). As can be seen from Table 13.1, the compounds can be modified in the base or the sugar part of the molecule. Analogues of pyrimidine bases, purine bases and of appropriate (deoxy)nucleosides have been widely investigated and developed as chemotherapy. Fluorodeoxyuridine—as 5-FUMP after phosphorylation—can interfere with thymidylate synthase to restrict DNA synthesis, and 5'-fluorouracil is activated by orotate phosphoribosyl transferase to be finally incorporated into RNA (review Traut and Jones 1996). The anti HIV drug

Table 13.1 FDA-approved purine and pyrimidine antimetabolites (Parker 2009)

Drug	Generic name	Date approved
5-Aza-2′-deoxycytidine	Decitabine	2006
O^6-Methylarabinofuranosyl guanine	Nelarabine	2005
2′-Fluoro-2′-deoxyarabinofuranosyl-2-chloroadenine	Clofarabine	2004
5-Azacytidine	Vidaza™	2004
N^4-Pentyloxycarbonyl-5′-deoxy-5-fluorocytidine	Capecitabine	1998
2,2-Difluoro-2′-deoxycytidine	Gemcitabine	1996
2-Chloro-2′-deoxyadenosine	Cladribine	1991
Arabinofuranosyl-2-fluoroadenine	Fludarabine	1991
2′-Deoxycoformycin	Pentostatin	1991
5-Fluoro-2′-deoxyuridine	Floxuridine	1970
Arabinofuranosylcytosine	Cytarabine	1969
6-Thioguanine		1966
5-Fluorouracil		1962
6-Mercaptopurine		1953

3'-azido-3'deoxythymidine (AZT) is one of several similar compounds which after phosphorylation by human thymidine kinase interferes with reverse transcriptases of virus (Shaw and Locarini 1995).

In mouse L cells, the mitochondrial dCTP pool was labelled more efficiently by incorporation of tritiated uridine than was the whole-cell dCTP pool (Mathews and Song 2007). This and other experiments suggested that salvage pathways to dNTPs in mitochondria are most productive. In addition to the well-known cytosolic TK1 enzyme, cells express a mitochondrial thymidine kinase, TK2, with mitochondrial targeting and sorting signals. The TK1 and the TK2 are encoded by two separate genes located on chromosome 17 and 16, respectively (review Eriksson et al. 2002), and they differ in tissue distribution, kinetics and substrate specificities as well as in subcellular location. As a closely S-phase correlated enzyme, TK1 is present in proliferating cells, in malignant as well as normally growing cells and tissues. The level of a TK1 in serum of patients has been determined clinically for prognostics and monitoring of different haematological malignancies and is currently under further development as biomarker for solid tumours (Chen et al. 2010). In contrast to TK1, the mitochondrial TK2 is expressed in most tissues—very likely in close correlation to the number of mitochondria—and it is not cell cycle-regulated. The mitochondrial TK2 of cells lacking TK1 can replace the cytosolic enzyme, exporting thymidine nucleotides from mitochondria when incubated with exogenous labelled thymidine (Mathews and Song 2007; Rampazzo et al. 2007). In mitochondria, deoxycytidine and deoxyuridine likewise are phosphorylated by TK2 which is under strict feedback control by dTTP and dCTP (Fig. 13.4) (Wang et al. 2011).

The existence of a deoxyguanosine kinase in mitochondria (Fig. 13.4) was known early on (Gower et al. 1979). The mitochondrial enzyme also phosphorylates deoxyadenosine, but pyrimidine deoxynucleosides only to a minimal extent.

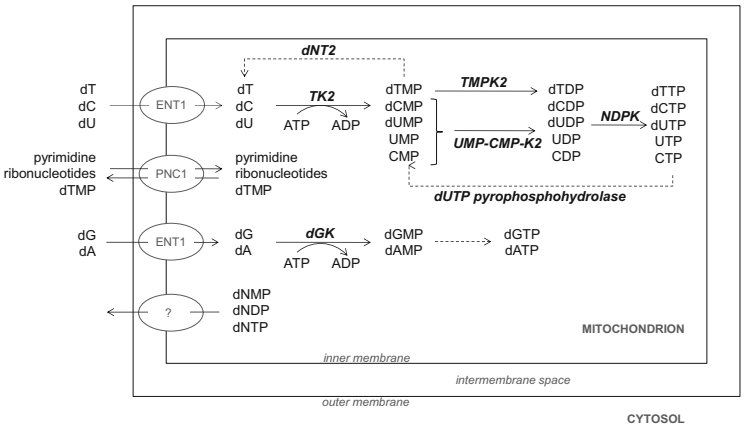

Fig. 13.4 Overview of salvage of pyrimidine and purine (deoxy)nucleosides and (deoxy)nucleotides in mitochondria. *ENT1* equilibrative nucleoside transporter, *PNC1* pyrimidine nucleotide transporter, *TK2* mitochondrial thymidine kinase, *dGK* deoxyguanosine kinase, *TMPK2* mitochondrial thymidine monophosphate kinase, *UMP-CMP-K2* mitochondrial UMP-CMP kinase, *NDPK* nucleoside diphosphate kinase, mitochondrial isoform

Like TK2, it is not correlated to cell cycle phases and was found in most cells including neoplastic tissues (Eriksson et al. 2002). Patients with genetic deficiencies in TK2 or dGK activity suffer from severe mitochondrial disorders, skeletal myopathy and in the case of dGK defects, fatal liver failure and often multiple organ dysfunction (Saada-Reisch 2004; Wang et al. 2011). In many of these patients, depletions of mtDNA and quantitative reduction in mtDNA copy number were diagnosed.

It remains to be clarified whether the salvage enzymes are adequately supplied with deoxynucleosides via equilibrative nucleoside transporters in the inner mitochondrial membrane (Baldwin et al. 2004; Lai et al. 2004) or by a translocation of phosphorylated deoxynucleosides (dNMPs, dNDPs and dNTPs) from the cytosol to the mitochondrion as described for uridine nucleotides (Fig. 13.4). Evidence for involvement of the carrier PNC1 in the mitochondrial trafficking of thymidine monophosphate was presented for human tumour cell lines (Franzolin et al. 2012), but in nonproliferating cells and tissues such as the heart, liver and brain, the deoxynucleosides thymidine and deoxycytidine enter the mitochondria (review McCann et al. 2012). At present, it is assumed that the deoxynucleoside salvage pathway to mitochondrial dNTPs is the chief process operating in quiescent and differentiating cells, while carrier-mediated transport of deoxynucleotides may be primarily active in proliferating normal and malignant cells (Chen et al. 2008; Mathews and Song 2007).

13.7 Pyrimidine Interconversion and Catabolism in Relation to Mitochondrial Diseases

Several diseases are known to arise from alteration or depletion of mtDNA, in turn caused by defects in the interconversion of pyrimidine nucleosides and nucleotides. Cytosolic kinases catalyse the phosphorylation of pyrimidine monophosphates (Fig. 13.2, enzymes 7, 8, 9) to form the di- and triphosphates. A mitochondrial thymidine monophosphate kinase (TMPK2) (Fig. 13.4) was characterised in HeLa cells, and TMPK2 mRNA was detected in erythroblastoma cells and differentiating macrophages (Chen et al. 2008). Likewise, a human mitochondrial UMP-CMP kinase (UMP-CMPK2) was shown to phosphorylate dUMP, dCMP, CMP and UMP (Fig. 13.4) (Xu et al. 2008). These enzymes and a mitochondrial nucleoside diphosphate kinase with a broad substrate specificity are guarantors for the local supply of deoxynucleotides as essential precursors for mtDNA replication and transcription. On the other hand, 5'nucleotidases (Fig. 13.2, enzyme 17) could oppose the formation of nucleotides. In mitochondria, the only known enzyme of this group is the mitochondrial 5'-deoxynucleotidase (dNT2) which seems to be specific for deoxyribonucleotides, predominantly TMP (Rampazzo et al. 2007). A distinct mitochondrial enzyme, dUTP pyrophosphohydrolase, removes two phosphates from dUTP and thus can reduce the risk of deoxyuracil incorporated in mtDNA (Fig. 13.2) (Ladner and Caradonna 1997; McCann et al. 2012). The prevention of this kind of DNA damage seems to be of such great importance that uracil DNA glycosylase is a component of the mitochondrial base excision repair pathway (Sect. 9) which enables efficient removal of sugar and base lesions in mtDNA (review Shaw and Locarini 1995). Other effective mechanisms, e.g. mismatch repair, seem not to play a role in mitochondria (review Stuart and Brown 2006).

Dysfunction with multiple depletion, deletion and point mutations of mtDNA were shown in patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (Nishigaki et al. 2013). This disease is characterised by progressive dysmotility, peripheral neuropathy and extramuscular muscle weakness as a result of inherited loss of thymidine phosphorylase (TP) activity (Fig. 13.2, enzyme 20). Dramatically elevated thymidine and deoxyuridine levels in cells and body fluids cause expansion of the dTTP and dUTP pool and contraction of the dCTP pool in cytosol and also in mitochondria.

Normally, the cleavage of uridine and thymidine by phosphorylases (Fig. 13.2 enzymes 19 and 20) to the appropriate base and ribose-1-phosphate or deoxyribose-1-phosphate, respectively, is the key reaction to the catabolic pathway. Deoxyribose-1-phosphate is cleaved by deoxyribose-phosphate aldolase to glyceraldehyde-3-phosphate and acetaldehyde (review Follmann 2004). Ribose-1-phosphate is converted to ribose-5-phosphate which can be recycled for synthesis of PRPP or via the pentose-phosphate pathway and glycolysis to provide energy. It should be mentioned that thymidine phosphorylase is also known as the platelet-derived endothelial cell growth factor (PD-ECGF). High TPase expression has been related

to angiogenesis (Bijnsdorp et al. 2011); human uridine phosphorylase was also surmised to have an additional function as regulator in growth and differentiation (Zhou et al. 1996).

The final three reactions (Fig. 13.2, enzymes 21, 22, 23) in pyrimidine catabolism begin with the reduction of the uracil and thymine ring by dihydropyrimidine dehydrogenase (DPD). The complete degradation of the pyrimidine ring, which for the most part occurs in liver and kidney, generally does not produce problems for the human body, since the intermediary metabolites ß-aminoisobutyric acid or ß-alanine shown in Fig. 13.2 can be excreted in urine or converted to malonyl-CoA and methylmalonyl-CoA which, by catabolic pathways of amino acid and fatty acid metabolism, contribute to ATP production in mitochondria. However, genetic defects in the three catabolic enzymes are associated with fatal neurological deficits (review Webster et al. 2001), for which no explanation can be given at present. Since DPD is of fundamental importance for the inactivation of 5-fluorouracil and other pyrimidine analogues prescribed in cancer treatment (Sect. 6, Table 13.1), even heterozygous patients experienced severe toxicity from these drugs.

13.8 Modulation of Pyrimidine Pools for Cell Proliferation

Physiological concentrations of all purines and pyrimidines in human and rodent cells and tissues were reviewed by Traut (1994). Normal (quiescent, resting) cells typically have a lower concentration of the four nucleotides than mitotic cells. However, tumour cells have a distinctly greater increase of pyrimidines than of purines: 686 ± 542 µM UTP and 402 ± 252 µM CTP in tumour cells versus 227 ± 230 µM UTP and 83 ± 133 µM CTP in normal cells and $3{,}134 \pm 2{,}135$ µM ATP and 473 ± 214 GTP in tumour cells versus $2{,}537 \pm 1{,}217$ µM ATP and GTP 232 ± 202 GTP in normal cells. A similar disproportionate nucleotide pool expansion was measured by means of HPLC analyses on mitogen-activated T-cells: twofold for purines and up to eightfold for pyrimidine pools including UDP-sugars (Fairbanks et al. 1995). Uptake and distribution analyses of radiolabelled precursors of the biosynthetic and the salvage pathway confirmed the importance of pyrimidine de novo synthesis for rapidly dividing lymphoblasts and the less significant elevation of salvage by which resting T-lymphocytes meet their metabolic requirements. The importance of pentoses as components of precursors for nucleic acids has been emphasised by studies on glycolytic pyruvate kinase type M2. Dimer-tetramer conversion of M2-PKwas shown to determine the relative amount of glucose directed either to synthetic processes or for glycolytic ATP production (Mazurek et al. 1997).

The marked increase in pyrimidine nucleotides of tumour cells and activated lymphocytes may reflect the need to attain a balance between pools of purine and pyrimidine nucleotides for DNA synthesis and proliferation, since pyrimidine nucleotides are less abundant in quiescent cells (Reichard 1988). In addition,

extra pyrimidine ribonucleotides are required for extensive synthesis of membranes for cell reproduction (Sect. 2).

Elevated activities of de novo pyrimidine biosynthetic enzymes were noted in early reports of rapidly growing cells such as ascites tumour, hepatoma nodules and regenerating liver (Calva and Cohen 1959), but an increase in the activities of salvage pathways (uptake of uridine) also occurs (overview Weber 1983). Intriguingly, the degradative enzymes uridine and thymidine phosphorylase and also dihydrouracil dehydrogenase were found to be more abundant in many solid tumours than in their normal counterparts (Naguib et al. 1985; Watanabe et al. 1995). In conclusion, a subtle interaction of all paths to and from pyrimidine pools seems to be a prerequisite for sustaining cell cycle progression of proliferating cells, whether normal or malignant.

13.9 Deoxynucleotide Pools in Mitochondria: Crucial for mtDNA

Mitochondrial DNA has neither introns nor protective histones and lacks the effective repair systems of the nuclear DNA. In close vicinity to the inner membrane with enzymes of electron transport chain and iron metabolism (Sect. 1), mtDNA is exposed to nearby reactive oxygen species (ROS) (Sect. 4). Unsurprisingly, mutations in mtDNA are about tenfold greater than in nuclear DNA, leading to mitochondrial diseases or neoplasia (review Naviaux 2000).

Common oxidative mtDNA lesions such as 8-oxo-deoxyguanosine were assumed to stem from intracellular oxidative stress with increased ROS production through the electron transport chain (review Stuart and Brown 2006; review Pieczenik and Neustadt 2007). The search for other mechanisms to impair mtDNA maintenance stimulated studies on the nature and sources of mitochondrial DNA precursor pools and also identified uridine uptake as a promising protective mechanism in neurodegenerative diseases (Cansev et al. 2013; Klivenyi et al. 2004). Also, in several human disorders, mitochondrial genome instability or low levels of mtDNA could be correlated with defective enzymes involved in nucleotide metabolism (review Mathews and Song 2007; review Desler and Rasmussen 2012). A recent investigation in *C. elegans* of the *rad-6* gene encoding UMPS has demonstrated that the reduced lifespan and radiation sensitivity of mutants arise from defective de novo pyrimidine synthesis and hence a restricted nuclear DNA repair capacity, even though uridine uptake appears to be upregulated, possibly as a response to orotate released from DHODH activity (Merry et al. 2013).

Mutations of mtDNA as well as depletion of mtDNA due to decreased fidelity of polymerase γ were explained as consequences of imbalances between purine and pyrimidine mitochondrial dNTP pools. The marked elevation of RNR activity in S-phase of proliferating cells is emphasised by a minimal expression of

deoxynucleotide triphosphohydrolase (Franzolin et al 2013). This enzyme has been described as a hitherto unknown regulatory element in keeping the deoxynucleotide pools low when cells are not replicating their DNA. Unbalanced precursor pools would decrease the fidelity of DNA polymerase and elevate mutation events. In the case of damaged nuclear DNA, it was shown that base excision repair (BER) could not proceed to completion if the synthesis of dNTPs was compromised. Critical depletion of dNTP pools resulted in the production of long-lived DNA strand breaks at repairing sites and reduction in the number of sites initiating repair, whereas elevation of dNTP pools to 10- to 50-fold normal levels did not inhibit repair of nuclear DNA (Snyder 1988).

Aberrations in dNTP pools in mitochondria arise in patients with TK2, dGK and TP deficiencies (Sects. 6 and 7). Studies in heterozygous patients with skeletal muscle involvement revealed that decreased ratios of pyrimidine dNTPs and deletions of mtDNA precede functional defects in the respiratory chain (Saada-Reisch 2004). TK2 knockin mice show the essential role of balanced deoxynucleotide pools for mitochondrial DNA maintenance (Akman et al. 2008). Cardiovascular complications, hepatic dysfunction, hyperlactatemia and neurotoxicity were stated in several patients under therapy with AZT (Sect. 6). The prominent inhibition of mitochondrial TK2 by this analogue with concomitant unbalanced mitochondrial deoxynucleotide pools was proposed to underlie mtDNA replication errors and deletions (McCann et al. 2012; Wang et al. 2011). Patients with deleterious mutations of the dGK gene expressed a marked phenotype/genotype correlation. Brain and liver were clinically involved and the mitochondrial electron transport chain was decreased in heart and muscle (Saada-Reisch 2004). From analyses of cells from patients with thymidine phosphorylase deficiency (Sect. 7), it was concluded that increased levels of thymidine and deoxyuridine could cause an abnormally high mitochondrial concentration of dUTP that can substitute for dTTP in mtDNA replication, in turn leading to mtDNA abnormalities including multiple sequence-specific point mutations (Nishigaki et al. 2013).

Using DNA polymerase assays and modern HPLC methods, only a few laboratories have measured the very small pools of deoxynucleotides in mitochondria, which have been reported to be less than 10 % of whole-cell deoxynucleotide pools, e.g. 3 % in quiescent fibroblasts (Ferraro et al. 2005; Mathews and Song 2007; Desler et al. 2007).

Since changes in the ratio of mitochondrial genome to nuclear genome (Mt/N) have been reported in many human diseases—cancer, ageing, diabetes and HIV complications (Malik and Czajka 2013)—the evaluation of mtDNA content in body fluids and tissue samples was proposed as a biomarker of mitochondrial dysfunction. Comparison of mitochondria in metastatic cells with those belonging to nontransformed cells revealed several differences in the structure and function of these organelles (review Verschoor et al. 2013; Wallace 2012).

The origin of replication and transcriptional promoters are found in a noncoding region of mtDNA comprising two hypervariable regions within a displacement loop (D-loop). Mutations in this region of mtDNA, which have been reported in tumours

on examination, can be expected to result in altered binding affinities of the nuclear proteins involved in mtDNA replication and transcription (Smiraglia et al. 2008). A decreased amount of both mtDNA and the mtDNA-encoded subunits of cytochrome c-oxidase were seen in many breast and ovarian tumours.

It would be of interest to find out whether acquired defects in the electron transport chain might change the activity of dihydroorotate dehydrogenase in pyrimidine de novo synthesis (Sect. 3) and possibly contribute to tumorigenesis. DHODH activity might be reduced, with cells surviving through increased uridine salvage activities, or could be pushed to contribute to superoxide production due to aberration of the functional electron transport chain (Sect. 4). Alternatively, gene multiplication and protein overexpression might overcome the putative restrictions. Enzyme overexpression was observed in DHODH of drug-resistant B-lymphocytes (Löffler et al. 2004) and in CAD when treated with the transition-state analogue PALA (Kempe et al. 1976).

The link between mutations in the mitochondrial genome, chromosomal stability and tumorigenesis has been experimentally approached using model organisms such as yeast or by generating cell lines depleted of mtDNA (rho0 cells) either chemically by ethidium bromide or genetically by transfection with the herpes simplex virus 12.5 gene (Rasmussen et al. 2003; Smiraglia et al. 2008). Cell lines depleted of mtDNA are auxotroph for pyrimidines and receive uridine from supplementation of the culture medium. Human cervical and breast cancer cells depleted of mtDNA were shown to contain unbalanced whole-cell dNTP pools, with great reduction of dTTP and dCTP but not dGTP and dATP (Desler et al. 2007). The overall repair activities in the nucleus were not substantially reduced, but chromosomal translocation and rearrangement were characteristically induced in rho0 cell lines. The tumorigenic phenotype of such cells is reversed after transfer of wild-type mitochondria (Singh et al. 2005). From these and other observations, it was concluded that mitochondrial function is fundamental for maintaining the integrity of the genome. This topical theme deserves specific attention and is a separate subject of this book (Chap. 10).

Concluding Remarks

This review discusses the dependence of cellular pyrimidine biosynthesis on functional mitochondria, as well as the contribution of cellular pyrimidine salvage to the function of mitochondria. The obligate contribution of molecular oxygen to the de novo formation of UMP which is the precursor compound of all pyrimidine ribo- and deoxyribonucleotides has been highlighted. Since dNTP pool size is a critical factor to sustain mtDNA copy number, de novo nucleotide synthesis and salvage pathways in mitochondria are required to maintain mtDNA fidelity.

The mitochondrion-bound dihydroorotate dehydrogenase has been postulated to hold a pivotal role during adaptation of the proliferative capacity of

(continued)

cells to different conditions of oxygenation, such as the hypoxia that would be found in growing tumours. We conclude that mitochondria have essential roles in the salvage and biosynthesis of pyrimidines respectively for quiescent cells and during proliferation.

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Part IV
On the New Omics of Metabolism

Chapter 14
Metabolic Fluxes in Cancer Metabolism

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14.1 Introduction

Malignant cells undergo a metabolic transformation to satisfy the demands of growth and proliferation. This was first recognized by Otto Warburg who discovered that rapidly proliferating ascite tumors produce large amounts of lactate through fermentation, even in the presence of oxygen (Warburg et al. 1926a; Warburg 1956a; Koppenol et al. 2011). He postulated injury of respiration as the cause of cancer. This phenomenon, termed the Warburg effect, has been observed for many tumors in conjunction with high glucose uptake.

The Warburg effect and cancer metabolism were largely neglected, as cancer biology was almost entirely focused on aspects of molecular biology responsible for the typical hallmarks of cancer (Weinberg 2011). However, cancer metabolism has experienced a renaissance in recent years, leading to the discovery of a large array of pathways and regulators affecting tumor metabolism [recent reviews: (Ward and Thompson 2012; Benjamin et al. 2012; Duckwall et al. 2013; Cantor and Sabatini 2012)]. Regulation of metabolism reaches already beyond the confines of cancer, with recent work highlighting the importance of metabolism for epigenetics and cellular differentiation (Folmes et al. 2012; Lu and Thompson 2012; Yun et al. 2012; Shyh-Chang et al. 2013; Yanes et al. 2010).

Overall, cancer cells seem to optimize metabolism toward biomass rather than ATP production. Recent work has helped to identify links between signaling pathways, cell cycle control, and metabolism, identifying roles for proteins such as PI3K/AKT, p53, or Myc in driving glycolysis or activating glutaminolysis. In many cases HIF (hypoxia-inducible factor) is involved as a master regulator. However, an increasing number of other proteins are found to influence energy metabolism, and a series of mutations in proteins, e.g., in succinate dehydrogenase (SDH) or fumarate hydratase (FH), drive altered metabolism. These findings have opened a new field of research in oncology, affecting diagnostic methods and drug discovery.

Metabolomics and Cancer Metabolism The expression refers to the complete set of small-molecule metabolites (such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites) to be found within a biological sample, such as a single organism (Oliver et al. 1998). Metabolomics studies the small-molecule composition of samples, often derived from whole organisms, including body fluids, e.g., blood or urine, but also tissue or even purified cells.

Metabolomics in cancer has developed almost in parallel with research in cancer metabolism, although metabolomics always had its own life, with little overlap between these two scientific communities. This arises from the fact that renewed interest in cancer metabolism was largely driven by cancer biologists, whereas metabolomics is more or less a discipline of analytical chemistry that has evolved independently driven by technological developments in mass spectrometry (MS) and nuclear magnetic resonance (NMR).

At this stage there is a significant gap between our view of cancer metabolism and what we see in typical metabolomics analyses. This is in part because metabolomics often looks for a diagnostic signature in body fluids, whereas metabolic flux analysis (MFA) or stable isotope-resolved metabolomics (SIRM) takes a targeted view at the passage of metabolites through specific metabolic pathways in cellular samples. Body fluids often do not directly reflect the metabolism of a tumor but rather represent the complete organism's response to cancer. This includes immunological responses, tumor-niche interactions, and responses of the body to cope with loss of function in organs affected by tumors. Nevertheless, the two are closely correlated.

One of the essential questions of cancer metabolomics is how the metabolic response observed in blood reflects the state of a cancer, especially with respect to biomarker discovery. Interestingly, small tumors (often <0.01 % of the body mass) can produce a significant metabolomics signature in blood or urine. For example, a blood plasma metabolomics study of head and neck tumors showed a strong signature for cancer vs controls, with the potential to distinguish late-stage from early-stage tumors (Tiziani et al. 2009a). Such early stage tumors have a diameter of up to 2 cm and a mass of a few grams. We therefore have to assume that the effects observed in blood are secondary to the primary tumor and reflect system-level changes. We also need to be careful to exclude trivial secondary effects such as malnutrition.

If we want to understand the effects of metabolism in cancer, we need a systematic approach, moving from model systems such as cultured cancer cell lines to primary cells from patients. As a final step, we need to understand how cancer can influence metabolic profiles in the whole body.

A powerful tool to study metabolism is the use of isotopically labeled metabolic precursors as tracers that allow us to monitor metabolic fluxes and map out metabolic pathways rather than just metabolite concentrations. By using different labeled tracer molecules, metabolic flux analysis (MFA)[1] models "where metabolites go" and can detect fluxes even for steady-state concentrations that barely change. MFA can also be used in the context of computational biology, using flux balance analysis (FBA) or constraint-based modeling (CBM) where metabolic pathways are modeled across a network of biochemical reactions on a genome scale, often combining transcriptomics and metabolomics, or better "fluxomics" data (Orth et al. 2010a).

This review provides an overview of the developments in the context of metabolism in cancer with a specific emphasis on the technologies used in metabolic flux analysis.

[1] In this review the expression metabolic flux analysis (MFA) is consistently used for metabolic analyses using isotopically labeled metabolites as tracers, equivalent to stable isotope resolved metabolomics (SIRM). MFA in this context does not refer to computational algorithms commonly used to decipher metabolic fluxes and does not imply that flux time-dependent data is employed.

14.2 Metabolic Pathways and Cancer

14.2.1 Aspects of Altered Metabolism in Cancer

Despite using the same nutrients, cancer cells exhibit a fundamentally differently tuned metabolism compared to healthy cells (summarized in Fig. 14.1), finding ways to proliferate. Otto Warburg showed that cancer cells are addicted to glycolysis; they ferment glucose into lactate rather than committing into oxidative phosphorylation, regardless of oxygen tension (Warburg et al. 1926b; Warburg 1956b). Warburg assumed that cancer cells have defective mitochondria causing a reduction of respiration, a hypothesis that was heavily disputed by Weinhouse who saw "essentially fallacious reasoning" in Warburg's arguments (Weinhouse 1956).

How glycolysis, which is an inefficient way of producing ATP, can maintain the energy requirements of cells remains unclear. Flux balance models of ATP production suggest that the Warburg effect represents a favorable catabolic state for all rapidly proliferating mammalian cells with high glucose uptake capacity (Vazquez et al. 2010). However, anaplerotic[2] mechanisms utilizing carbon sources other than glucose to replenish the TCA cycle may well be equally important for ATP production.

Cancer cells seem instead optimized toward biosynthesis and biomass production. As will be shown later, the high glycolytic flux of cancer cells feeds essential intermediates into branching biosynthesis pathways (Locasale and Cantley 2011). These include (i) glucose-6-phosphate which feeds into the pentose phosphate pathway (PPP) to make ribose-5-phosphate, an essential component for nucleotide synthesis; (ii) dihydroxyacetone phosphate (DHAP) as a precursor for triglycerides and phospholipid synthesis; (iii) pyruvate for alanine and acetyl-CoA synthesis; and (iv) serine which branches off from 3-phosphoglycerate (3PG), as a precursor for one carbon metabolism.

Mitochondria were initially regarded as universally defective in cancer cells. We now know that this is not the case; they do however adapt their function to the needs of cell proliferation. Besides acting as a hub for ATP production, mitochondria play a central role in amino acid, lipid, and nucleotide synthesis through the TCA cycle. As will be shown later, MFA has shown that the TCA cycle can feed on glutamine rather than acetyl-CoA produced from pyruvate, and this anaplerotic pathway can become dominant in cancer cells (DeBerardinis and Cheng 2010; Wise and Thompson 2010). This is often a consequence of the appearance of pyruvate kinase M2 (PKM2) in cancer cells (Mazurek et al. 2005), which becomes rate limiting and causes a decoupling of glycolysis and the TCA cycle. It can also be caused by a deactivation of pyruvate dehydrogenase (PDH) by pyruvate dehydrogenase kinases (PDK), thus preventing it from catalyzing the acetylation of coenzyme A (coA) and therefore blocking this entry point into the TCA cycle. Glutaminolysis can therefore

[2] Anaplerotic, from Greek ἀνά, "up," and πληρόω, "to fill," i.e., for the TCA cycle a mechanism that fills the cycle with nutrients.

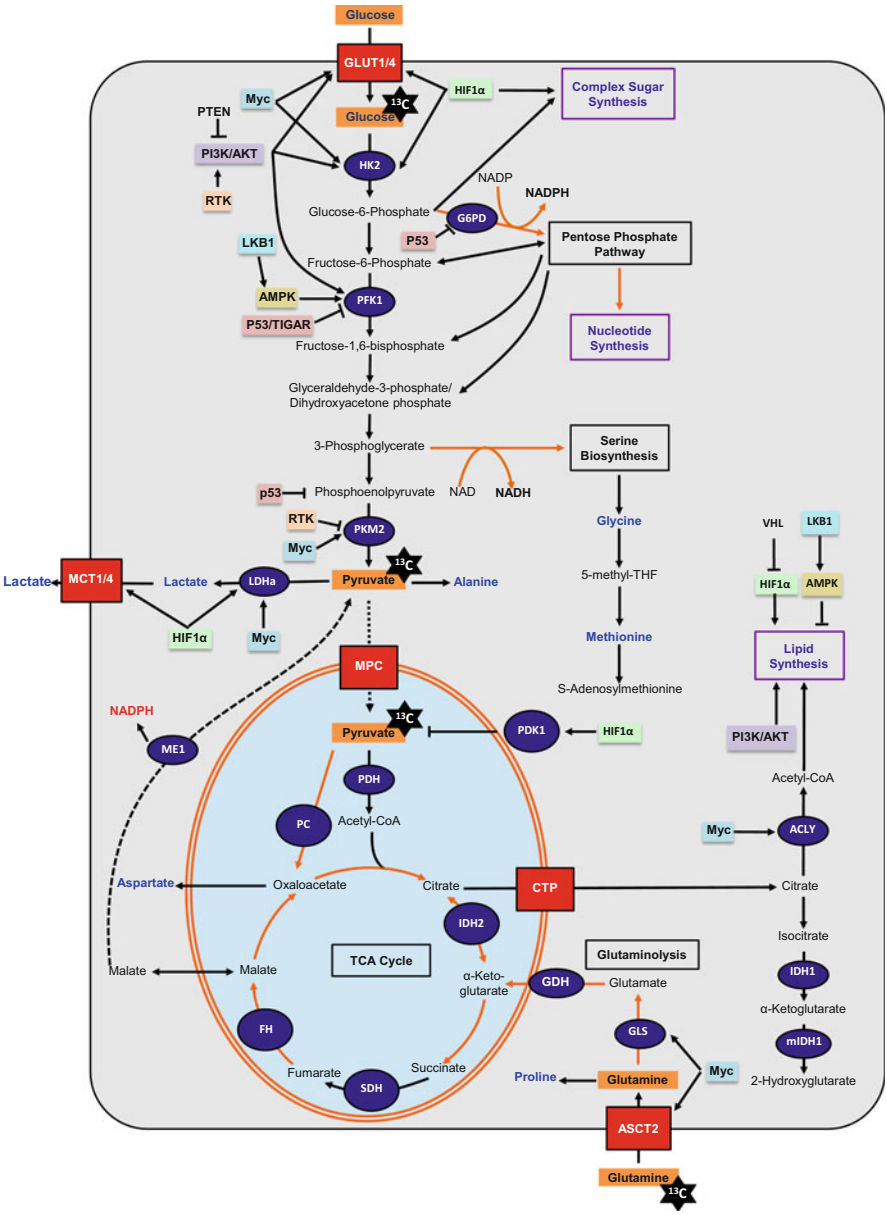

Fig. 14.1 Metabolic pathways in cancer metabolism. Abbreviations: *ACLY* ATP citrate lyase, *ASCT2* alanine–serine–cysteine transporter 2, *AKT* v-akt murine thymoma viral oncogene, *AMPK* AMP-activated protein kinase, *CTP* citrate transport protein, *DHAP*, dihydroxyacetone phosphate; *FH* fumarate hydratase, *GLS1* glutaminase, *GDH* glutamate dehydrogenase, *GLUT* glucose transporter, *HIF* hypoxia-inducible factor, *HK2* hexokinase-2, *IDH* isocitrate dehydrogenase, *LDHa* lactate dehydrogenase A, *LKB1* liver kinase B1, *MCT* monocarboxylic acid transporter, *ME1* cytosolic malic enzyme 1, *mIDH* mutant isocitrate dehydrogenase, *MPC* mitochondrial pyruvate carrier, *Myc* v-myc myelocytomatosis viral oncogene homologue, *NADPH* nicotinamide adenine dinucleotide phosphate, *p53* tumor protein p53, *PDH* pyruvate dehydrogenase, *PC* pyruvate carboxylase, *PDK1* pyruvate dehydrogenase kinase isoenzyme 1, *PFK1* 6-phosphofructose-1-kinase, *PFK2*

maintain the generation of essential building blocks for nucleic acid synthesis, such as glutamate and aspartate.

14.2.2 Adaptation of Cancer Metabolism to a Hypoxic Tumor Environment

Cancer metabolism is heavily influenced by adaptation to the tumor microenvironment which is highly hypoxic for most solid tumors. As the number of proliferating tumor cells surpasses the supply of nutrients, lactate levels build up causing acidosis along with high reactive oxygen species (ROS) levels. In solid tumors vascularization and angiogenesis lagging behind tumor growth add to the overall hypoxic environment. In hematological tumors, bone marrow and lymph nodes represent hypoxic environments. The hostile metabolic microenvironment of tumors is often heterogeneous both spatially and temporally and adds significant stress on tumor cells through factors such as hypoxia, acidosis, and high ROS. Cancer cells adapt to this hostile environment by changing their phenotype through development of resistance to hypoxia, acidic pH, and high ROS.

The master regulator for hypoxia is hypoxia-inducible factor 1 (HIF-1), a transcription factor which activates the transcription of hundreds of target genes [recently reviewed in Semenza (2013)]. HIF-1 is a heterodimer comprised of HIF-1α and HIF-1β (Wang et al. 1995; Wang and Semenza 1995), of which the HIF-1α subunit is O_2 regulated. Under normoxic conditions HIF-1α is hydroxylated by the O_2-dependent prolyl hydroxylase domains (PHD, specifically PHD2), which enables binding of von Hippel–Lindau tumor suppressor protein (VHL), which recruits an E3 ubiquitin ligase leading to the ubiquitination of HIF-1α and proteasomal degradation (Kaelin and Ratcliffe 2008; Tennant et al. 2009). Under hypoxic conditions HIF-1α gets stabilized by inhibition of the prolyl hydroxylation or loss of VHL. Inhibition of PHD2 can be achieved by increased levels of succinate or reduced levels of α-ketoglutarate.

Stabilization of HIF-1α causes a transcriptional program which promotes the expression of genes supporting fermentative glucose metabolism, including genes encoding for glucose transporters, glycolytic enzymes, lactate dehydrogenase A (LDHA), pyruvate dehydrogenase kinase-1 (PDK1), and others [HIF-1α target genes are reviewed in Semenza (2004)]. Through these metabolic shifts, hypoxic tumor cells take up more glucose and metabolize pyruvate into lactate.

Fig. 14.1 (continued) 6-phospho-2-kinase/fructose-2,6-bisphosphatase, *PHD* HIF prolyl 4-hydroxylase, *PI3K* phosphoinositide 3-kinase, *PKM2* pyruvate kinase muscle-2, *PTEN* phosphatase and tensin homologue, *SDH* succinate dehydrogenase, *TCA cycle* tricarboxylic acid cycle, *VHL* von Hippel–Lindau tumor suppressor

Hypoxia modulates mitochondrial respiration in an HIF-1α-dependent manner. HIF promotes the expression of pyruvate dehydrogenase kinase-1 (PDK1),[3] which inhibits pyruvate dehydrogenase (PDH). PDH is responsible for converting pyruvate into acetyl-CoA in the mitochondrion—the step that commits carbons from glucose into mitochondrial metabolism (Kim et al. 2006). By simultaneously diverting glycolytic flux into lactate by promoting LDHA expression, HIF-1α blocks glucose carbon incorporation into the TCA cycle. The activation of PDK1 seems to be particularly important in induced pluripotent stem cells. As shown by Prigione et al., the transduction of the four Yamanaka factors (4 F: OCT4, SOX2, KLF4, and c-MYC) is sufficient to upregulate PDK1, thereby initiating a Warburg-like metabolic restructuring associated with the conversion to pluripotency (Prigione et al. 2014).

Studies in acute lymphoblastic leukemia (ALL) show that they are dependent on glycolysis in aerobic conditions (Warburg 1956a). Levels of HIF-1α, GLUT1, GLUT3, CA4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were significantly greater in leukemic cells compared to healthy blood cells. Moreover, leukemias with higher glycolytic rates showed stronger resistance to chemotherapeutics, e.g., glucocorticoids. It was shown that inhibition of glycolysis using 2-deoxyglucose (2DG) rendered otherwise resistant leukemia cells susceptible to glucocorticoid treatment (Koppenol et al. 2011). 2DG also affects the pentose phosphate pathway and alters protein glycosylation. As a result 2DG induces cellular stress and kills leukemia cells in normoxia (Weinberg 2011).

14.2.3 Oncogenes and Tumor Suppressors Coupled to Metabolic Reprogramming

The cancer phenotype is represented by an accumulation of mutations and alterations of multiple signaling pathways. It is increasingly well understood that a large number of point mutations, translocations, amplifications, and deletions contribute to cancer development. It is also increasingly clear that there are several oncogenes and tumor suppressors, which influence cancer metabolism.

The PI3K/AKT/mTOR axis is one of the most commonly altered signaling networks in human cancer. AKT, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase that is negatively regulated by the tumor suppressor phosphatase and tensin homologue (PTEN) (Vivanco and Sawyers 2002); HIF-1-mediated gene expression is facilitated by loss of the *PTEN* tumor suppressor gene (Zundel et al. 2000). Activation of the PI3K pathway not only provides strong growth signaling but also promotes survival signaling. One of the well-studied downstream effectors of PI3K is AKT. Signaling through AKT has been

[3] It should be noted that PDK1 is unfortunately also used as an abbreviation for phosphoinositide-dependent kinase-1.

shown to enhance glycolysis through several mechanisms. AKT has been found to upregulate glucose uptake via cell surface expression of the glucose transporter 1 (GLUT1) and stimulates the phosphorylation of hexokinase and phosphofructokinase (Deprez et al. 1997; Gottlob 2001; Rathmell et al. 2003; Elstrom et al. 2004). Downstream of AKT, the mechanistic target of rapamycin (mTOR) stimulates numerous metabolic pathways (Zoncu et al. 2010; Laplante and Sabatini 2012). Notably, PI3K/AKT/mTOR together with the MAPK pathway has been associated with lipid biosynthesis; mTOR is thought to stimulate de novo lipid synthesis partly due to the increase of nuclear localized sterol regulatory element-binding protein (SREBP) (Peterson et al. 2011).

Considering that responses to cellular stress and the mTOR pathway play a role in nutrient uptake, regulation of energy metabolism, and cellular survival, combined inhibition of glycolysis and mTOR has been suggested as an avenue of treatment in leukemic cancers. It has been shown that in leukemia and lymphoma cells, the combination of the mTOR inhibitor rapamycin and 3-bromopyruvate effectively depleted ATP and limited nutrient uptake, cell proliferation, and cell survival (Xu et al. 2005).

The Myc family of genes encodes transcription factors that regulate growth and cell cycle entry. In normal cells, mitogen-induced signaling through growth factor receptors stimulates c-Myc (hereafter referred to as Myc), leading to increased S-phase entry and therefore proliferation. As for other oncogenic transcription factors, the targets of c-Myc include many genes encoding glycolytic enzymes. Myc promotes aerobic glycolysis by enhancing the expression of GLUT1 and lactate dehydrogenase A (LDHA) (Osthus 2000), which converts pyruvate to lactate. On the other hand, c-Myc induces the expression of enzymes involved in nucleotide metabolism. These include inosine 5′-monophosphate dehydrogenase, serine hydroxymethyltransferase, adenosine kinase, and adenylate kinase 2 (O'Connell 2003).

Myc also regulates glutaminolysis, one of the anaplerotic pathways of the TCA cycle. Much of this work involved MFA, which will be discussed later. Myc indirectly regulates the expression of mitochondrial glutaminase (GLS1) by suppressing miR-23a and miR-23b, as shown in human P-493 B lymphoma cells and PC3 prostate cancer cells (Gao et al. 2009). This has a major impact on the TCA cycle; growth of both cell lines was diminished significantly by glutamine withdrawal and moderately with glucose withdrawal (Gao et al. 2009), in support of previous results showing Myc-dependent apoptosis upon glutamine withdrawal (Yuneva et al. 2012). Myc has also been held responsible for the regulation of a form of glutaminolysis, where glutamine consumption exceeds the cellular requirement for protein and nucleotide biosynthesis (Wise and Thompson 2010; Wise et al. 2008). Moreover, Myc regulates proline metabolism via proline oxidase, as shown in Myc-inducible human Burkitt lymphoma cell lines and in human prostate cancer cells lines (Liu et al. 2012). Metabolic fluxes showed the conversion of glutamine into proline via GC–MS and NMR analyses. This has important effects on cell proliferation and survival. In human Burkitt lymphoma cell lines, Myc induces glutaminolysis that persists in hypoxia. Metabolic fluxes demonstrate that

in these cells, the TCA cycle can be solely supported by glutamine (Le et al. 2012). The effect of Myc on metabolism has also been the subject of in vivo MRS studies. ^{13}C-pyruvate imaging in mice reveals that the progression of Myc-driven liver cancers is modulated via LDHA but also that metabolic changes in alanine metabolism (GPT1/ALT transaminase) precede both tumor formation and regression (Hu et al. 2011).

The p53 tumor suppressor is encoded by the TP53 gene, which is the most frequently mutated gene in human tumors (Berg et al. 2002). p53 functions as a tumor suppressor and responds to various genotoxic stresses such as DNA damage, oncogenic stimulation, nutrient depletion, and hypoxia (Meek 2009). While the role of p53 in apoptosis, genomic stability, and inhibition of angiogenesis is well understood, its role in cellular metabolism has been the subject of recent discoveries [reviewed in Lee et al. (2014)]. The prevalent inactivation of p53 in tumor cells accelerates glycolysis and increases metabolic flux into the PPP. This is mediated via at least two different mechanisms.

Firstly, p53 binds glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme of PPP, inducing a conformational conversion of G6PD which inhibits PPP. Tumor-associated p53 mutants lack the G6PD-inhibitory activity and thereby do not inhibit PPP and associated NADPH production (Gao et al. 2011). NADPH contributes to the cellular defense against oxidative stress and is required for fatty acid synthesis.

An alternative pathway is via a p53-inducible gene product named TIGAR (TP53-induced glycolysis and apoptosis regulator). TIGAR is a negative regulator of the glycolytic enzyme phosphofructokinase-1 (PFK1) at the third step in glycolysis, which converts fructose-6-phosphate to fructose-1,6-bisphosphate. TIGAR causes a decline in fructose-1,6-bisphosphate levels and thereby blocks glycolysis at this step, thus driving glucose flux through the oxidative PPP for production of ROS-titrating NADPH (Green and Chipuk 2006).

p53 also regulates the synthesis of the protein SCO2 (synthesis of cytochrome c oxidase 2), which is required for the correct assembly of the cytochrome c oxidase (COX) complex in the electron transport chain to increase mitochondrial respiration (Matoba 2006). In addition, p53 promotes glutaminolysis by activating the expression of glutaminase 2 (GLS2) and thus increases the level of glutathione, a key antioxidant (Hu et al. 2010; Suzuki et al. 2010).

14.2.4 Cancer Mutations Promoting the Warburg Effect

It is not surprising that mutations in metabolic genes have a major effect on metabolic fluxes. Loss-of-function mutations in succinate dehydrogenase (SDH) are known in pheochromocytoma and paraganglioma; such mutations in fumarate hydratase (FH) were found in hereditary leiomyomatosis and renal cell carcinoma (HLRCC) [see Gottlieb and Tomlinson (2005) for a review]. Mutations in SDH and FH cause the accumulation of succinate and fumarate in cytosol, which inhibit

prolyl hydroxylases PHD2 and PHD3, and the stabilization of HIF-1α, thus causing the typical Warburg effect of high glycolytic flux and lactate production (Isaacs et al. 2005; Lee et al. 2005).

Isocitrate dehydrogenase (IDH) mutations can be seen as a case where a single point mutation (R132) affecting cellular metabolism is selected in cancer cells. IDH1 mutations were identified in a subset of gliomas and acute myeloid leukemias (AML) (Mardis et al. 2009; Parsons et al. 2008). Wild-type IDH1 produces α-ketoglutarate (α-KG) and NADPH by oxidative decarboxylation of isocitrate. Dang et al. showed by ^{13}C isotope labeling of glutamine that the R132 mutant IDH1 *consumes* NADPH and reduces α-KG to 2-hydroxyglutarate (2-HG) (Dang et al. 2009). In AML both the cytosolic IDH1 and the mitochondrial analogue IDH2 are commonly mutated (Ward et al. 2010). As α-KG is a necessary substrate for PHD2, reduced levels of α-KG cause reduced PDH2 activity and therefore HIF-1α stabilization causing again a Warburg-type metabolism (Semenza 2010).

The mechanism by which 2-HG functions as an "onco-metabolite" is multifactorial. 2-HG was shown to act as a competitive inhibitor of α-KG-dependent demethylases, including histone demethylases and the TET family of 5-methylcytosine hydroxylases, affecting CpG island hypermethylation (Dang et al. 2009). This links the oncogenic effect of IDH1 mutations to epigenetic regulation. In fact, 2-HG-producing IDH mutants can block cell differentiation by preventing histone demethylation (Dang et al. 2009). 2-HG not only identifies new mechanisms for epigenetic regulation but can also be used as a biomarker, which can be detected noninvasively by magnetic resonance spectroscopy in patients with glioma brain tumors (Pope et al. 2011; Andronesi et al. 2012).

IDH inhibitors tested in cell lines were found to reduce the amount of 2-HG and inhibit the growth of leukemia or glioma cells in a mutant-specific manner. Moreover, IDH inhibition changed the epigenetic state and induced hematopoietic or neural differentiation, suggesting that IDH inhibition might in fact open new avenues for the treatment of cancers (McKenney and Levine 2013). There may be additional opportunities if inhibitors can be made that specifically target cancers with IDH mutations.

Beyond oncogenic mutations, there are several *splicing isoforms* known to promote tumorigenesis. For example, the splice variant PKM2 of pyruvate kinase M becomes predominant in proliferating cells (Mazurek et al. 2005; Mazurek 2011). Pyruvate kinase is a glycolytic enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate in the last step of glycolysis. The PKM2 isoform has intrinsically lower enzymatic activity than PKM1 and is sensitive to tyrosine kinase signaling (Christofk et al. 2008a, 2008b). PKM2 can therefore sensitively regulate the Warburg effect, as the more active tetrameric form favors degradation of glucose to lactate (Warburg effect) whereas the nearly inactive dimeric form favors pathways branching of glycolysis, favoring the production of precursors for cellular building blocks (Mazurek 2011).

Other splice variants important for metabolic regulation include the phosphofructokinase/fructose-2,6-bisphosphatase B3 gene (*PFKFB3*), which has six splice variants with different metabolic activity (Bando 2005). Phosphofructokinase

catalyzes the rate-limiting conversion of fructose-1-phosphate to fructose-1,6-bisphosphate and is subject to positive allosteric regulation by the glycolytic metabolite fructose-2,6-bisphosphate. Intracellular levels of fructose-2,6-bisphosphate are modulated by PFK2. An alternatively spliced isoform of *GLS*, known as glutaminase C (GAC), was shown to be important for the mitochondrial glutamine metabolism of tumor cells.

14.3 Metabolic Flux Analysis in Studying Cancer Metabolism

While metabolomics measures static metabolite concentrations at a certain time point, MFA utilizes isotopically labeled metabolic precursors such as glucose and glutamine as tracers and observes the flux of individual-labeled atoms across metabolic networks. MFA overcomes an inherent limitation of end-point metabolomics, arising from the fact that metabolic fluxes may be reflected by constant steady-state metabolite levels in high-throughput conditions when influx and efflux are balanced. This may be a rare case, especially in the light of a large body of work in metabolomics showing significant signatures for cancer. However, in order to understand the usage of different metabolic processes and how they may be disrupted in disease, it is important to know where carbons or other atoms go, especially as the same metabolites are found in many different mechanisms. Metabolic tracers can be used to trace fluxes on various timescales. Typically, cells are fed labeled metabolic precursors for 1–24 h. However, the recent advancement of metabolic magnetic resonance imaging (MRI) using substrates such as [1-^{13}C]pyruvate shows that altered metabolic turnover on a shorter timescale may be relevant (Day et al. 2007).

14.3.1 Historical Aspects

Tracer-based studies use nuclear magnetic resonance (NMR) or mass spectrometry (MS) to identify stable isotope incorporation into different molecules. Such experiments were pioneered by Ugurbil and Shulman in 1978 (Ugurbil et al. 1978) who used ^{13}C NMR spectra to trace label distributions arising from [1-^{13}C]glucose in *Escherichia coli* under anaerobic and aerobic conditions. In a time course over 15 min, they showed label incorporation into lactate, succinate, acetate, alanine, valine, and fructose bisphosphonate. Under aerobic conditions they were also able to observe glutamate, labeled in various positions. In a subsequent study, they used metabolic flux analyses on isolated mitochondria and mammalian cells, studying altered metabolism in hyperthyroid rats (Shulman et al. 1979). By comparing flux from two different labeled precursors [1,3-^{13}C]glycerol and [2-^{13}C]glycerol, they

were able to calculate the percentage of hexoses cycled through PPP. Shulman's remarkable early work on metabolic fluxes in human cells has largely been overlooked in the more recent literature.

Groundbreaking work on using ^{13}C NMR to study cellular metabolism came from Malloy, who looked at metabolic flux in the TCA cycle in rat hearts (Malloy et al. 1988). By simulating spectra he could explain many of the complex coupling patterns that arise from spin coupling of adjacent ^{13}C-atoms in NMR spectra. For glutamate C3 he determined fractional label incorporation in neighbor carbons arising from [2-^{13}C]acetate as a metabolic precursor. NMR offers multiple experimental options to determine label incorporation levels and position; detailed experimental protocols were described by Szyperski and Wüthrich (Szyperski 1995; 1996).

GC–MS for MFA was introduced by Katz et al. (1989) in an analysis studying Krebs cycle activity in rats using [U-^{13}C]glucose which produces a vast amount of isotopomers and is therefore difficult to use in mammalian cells which feed on different precursors. Modern GC–MS can produce high-throughput metabolic flux profiles (fluxomes) and is usually used in a computational context of analytical metabolic flux ratio analysis and iterative isotopomer balancing, and ^{13}C-constrained flux analysis (Sauer 2004).

14.3.2 Technologies Used for MFA

From a technological point of view, NMR and MS remain the two dominating analytical technologies. In some cases scintillation counting has been used with ^{14}C or ^{2}H to study lipid label incorporation [see, e.g., Mullen et al. (2011)]. NMR has traditionally been carried out using ^{13}C-spectra to detect and quantify site-specific label incorporation into many metabolites. For ^{13}C-observed spectra the sensitivity is relatively low, but spectra have few background signals and there is no need for solvent suppression in aqueous samples. However, proton decoupling during acquisition is required to collapse the ^{1}H-^{13}C couplings, and this is associated with an NOE enhancement which affects signal intensities. For quantification care needs to be taken that spectra are sufficiently relaxed between scans.

When multiple adjacent carbon atoms get labeled, complex multiplet patterns of NMR signals arise (Fig. 14.2), which can be interpreted in terms of degrees of label incorporation into neighboring carbons. In the case of one-dimensional ^{13}C-observed spectra, these multiplets are directly observed in the spectra. For this the ^{1}H-^{13}C coupling must be purged using a decoupling sequence.

The disadvantage of directly observed ^{13}C NMR is the low sensitivity of the method. Alternatively, ^{1}H-NMR spectra can be used, in which ^{13}C label incorporation is observed from ^{1}H-^{13}C couplings. In this case the $^{1}J_{1H\text{-}13C}$ coupling introduces a splitting of ca 130Hz, and the label incorporation can be determined from the ratio of the center peak to the ^{13}C-coupled ^{1}H-signals. Using ^{1}H spectra is limited to proton-bound ^{13}C atoms. This analysis is much more sensitive than ^{13}C

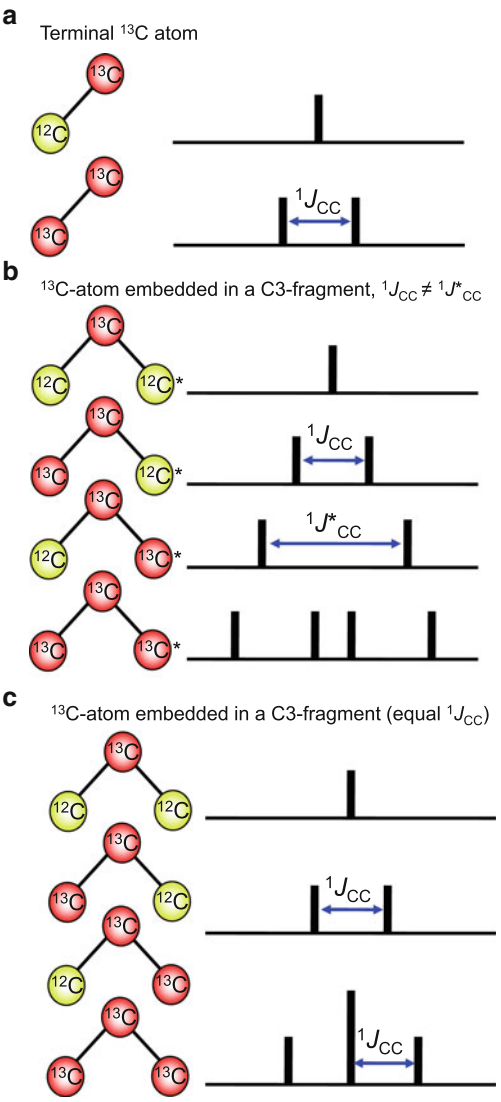

Fig. 14.2 ^{13}C-labeling patterns and corresponding multiplet structures [after McKenney and Levine (2013)]. Typically observed scalar coupling constants for metabolites are 42–48 Hz for CH_x-CH_x couplings and 50–60 Hz for CH_x–COOH couplings

NMR but suffers from significant signal overlap which can obscure results for complex systems. This can be partially overcome when using ^{1}H-^{1}H-TOCSY spectra.

Two-dimensional ^{1}H-^{13}C-HSQC spectra combine the two, offer significantly improved sensitivity over ^{13}C-observed spectra by observing ^{1}H, but require large numbers of increments (at least 2048, better 4096) and therefore moderately long acquisition times of 1–4 h to observe the ^{13}C–^{13}C scalar couplings. These provide valuable information about adjacent label incorporation. The second dimension (ω_1) in ^{1}H-^{13}C-HSQC spectra matches that of ^{13}C-observed spectra for ^{13}C atoms

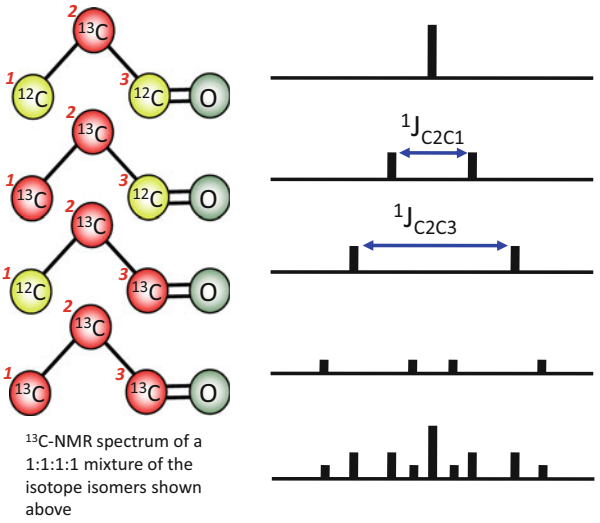

Fig. 14.3 ^{13}C NMR multiplet structures in metabolites with label incorporation in various adjacent atoms, with different coupling constants [after Mullen et al. (2011)]

bound to protons, whereas the observed dimension (ω_2) represents a ^{1}H spectrum showing only resonances of protons bound to ^{13}C. The disadvantage of HSQC spectra lies in the dependence of signal intensities on various relaxation times and on the size of the ^{1}H–^{13}C coupling constant which may vary between molecules.

While ^{1}H-observed methods cannot directly detect quaternary carbons, this becomes possible in highly resolved ^{1}H-^{13}C-HSQC spectra, where couplings to adjacent quaternary ^{13}C-atoms can be observed via ^{13}C–^{13}C spin couplings. Carboxylic acid groups, found in several Krebs cycle intermediates, have distinctly large values for scalar couplings to CH_x groups (typically 55–58Hz) (Szyperski et al. 1996). This may lead to complex multiplet patterns (Fig. 14.3), which can often be interpreted by line fitting. Examples include the analysis of glutamate C3 or C4 patterns in order to assess specific pathways [e.g., to estimate the amount of the TCA cycle used in citrate production (DeBerardinis et al. 2007)], to assess for pyruvate carboxylase activity (Cheng et al. 2011), or to study lipid label incorporation (Mullen et al. 2011).

The overall advantage of the NMR method is the ability to determine site-specific label incorporation in complex systems and has been used for several MFA studies looking at cancer metabolism.

In MS, label incorporations are simply reflected in small mass differences in metabolites. The general advantage of MS is in much higher sensitivity and the simplicity of the analysis. The key disadvantage is that site-specific label incorporations are much more difficult to identify, although this has often been attempted by fragmentation. A recent report explores tandem mass spectrometry using collision-induced fragmentation for this purpose (Antoniewicz 2013).

MS is almost always used in conjunction with compatible separation techniques including gas chromatography (GC) or liquid chromatography (LC). GC–MS requires derivatization of molecules, which is not easily possible for all metabolites.

LC–MS provides access to a larger range of metabolites and does not require derivatization (Antoniewicz 2013). For this GC–MS has the advantage that fragmentation patterns can be guided by the choice of the derivatization method and by the ability to use fragmentation.

The history of MFA and associated computational concepts has been summarized in an excellent review by Wiechert (Wiechert 2001). A series of excellent reviews summarize the experimental and computational methods around MFA (Sauer 2004; Wiechert 2001; Zamboni 2011).

The main driver for MFA has been the metabolic engineering of microbial processes, usually in order to optimize the production of a metabolic end product (Sauer 2006). It should be noted that the expression MFA, within this context, is often used to describe the entire process of measuring metabolic fluxes and interpreting them within a computational model. Here the term MFA is used more broadly to describe the process of label incorporation of downstream metabolites.

Recent interest in cancer metabolism has fueled the development of optimized methods to study metabolic fluxes in mammalian cells. The complexity of MFA rises quickly in complex systems, where metabolic processes occur in different compartments. Zamboni recently discussed these challenges and suggests several options for a rigorous analysis of metabolic fluxes (Zamboni 2011). One option is to use nonstationary methods, i.e., to measure fluxes at different time points after exposure to labeled metabolic tracers. For most cancer cell lines, metabolic fluxes are sufficiently slow to allow for flux times of several hours without massive scrambling of label signatures, and fluxes can often be quantified by using various ^{13}C-labeled metabolic precursors, usually with site-specific labels, rather than the cheaper uniformly labeled species.

In the context of biomedical applications, flux balance analysis (FBA) (Orth et al. 2010b; Thiele et al. 2013; Shlomi et al. 2007) now allows for genome-scale modeling, linking metabolic fluxes with gene expression and other omics data. FBA models can provide significant new insights into cellular mechanisms by combining transcriptomics and metabolic flux data. In a recent analysis of FH-deficient cells, FBA linked FH activity to heme oxygenase 1 (Hmox1, Hmox2), which catabolizes heme to biliverdin, and biliverdin reductase, which catabolizes biliverdin to bilirubin (Frezza et al. 2011). Another study of metabolic regulation linked to p53 was examined by constraint-based modeling (CBM), suggesting a role for p53 in enhancing gluconeogenesis (de novo synthesis of glucose) (Goldstein et al. 2013). Ruppin and coworkers developed algorithms for the systematic identification of new metabolic drug targets (Folger et al. 2011; Oberhardt et al. 2013; Yizhak et al. 2013). Genome-scale models of cancer metabolism showed correctly identified genes essential for cancer cell proliferation, confirmed known cytostatic drug targets, and predicted new ones, including the combination of drugs (Folger et al. 2011). This approach could provide essential information for drug redeployment, using combinations of known drugs [see, e.g., Khanim et al. (2009), Tiziani et al. (2009b), Lodi et al. (2011)].

An important issue for MFA is the choice of isotopically labeled precursors. Proper choice of labeled precursors can help to resolve ambiguities in metabolic mechanisms. Metallo et al. addressed this problem by testing 18 different ^{13}C-labeled tracers (11 glucose, 7 glutamine) in a lung cancer cell line (Metallo et al. 2009) for GC–MS analysis. They found that [1,2-$^{13}C_2$]glucose provided the highest level of precision for the overall network, glycolysis, and PPP. Net fluxes in the TCA cycle (succinate to fumarate and oxaloacetate to fumarate) were best characterized when using glutamine tracers with two or more carbons, specifically [1,2-^{13}C]Gln, [3,4-^{13}C]Gln, and [U-^{13}C]Gln.

For NMR, other considerations are important for choosing the optimal labeling strategy. In ^{13}C NMR or ^{1}H–^{13}C-HSQC spectra where ^{13}C–^{13}C spin–spin couplings can be resolved, coupling patterns become quite complicated for more than 2 spins (Szyperski 1995; Szyperski et al. 1996). It is therefore desirable to use metabolic precursors with no more than two adjacent spins. For [1,2-^{13}C]glucose as a metabolic precursor, many of the carboxylic acid groups in TCA cycle intermediates get labeled, providing additional valuable information, in particular in conjunction with ^{1}H–^{13}C-HSQC spectra.

Moreover, NMR does not benefit from a second label just to increase the mass difference. For example, a double-labeled glutamine would be of little value as it would not add any significant information. However, comparing spectra with precursors from different labeling partners can be of significant value for NMR analysis. This will be shown in the next section (Fig. 14.4) where the utility of [1-^{13}C]glucose, [1,2-^{13}C]glucose, and [3-^{13}C]glutamine to decipher flux pathways is demonstrated. Other examples have used joint labeling from different metabolic precursors. For example, DeBerardinis labeled [1,6-^{13}C]glucose together with [3-^{13}C]glutamine to identify the contribution of glutaminolysis and glycolysis for fatty acid synthesis (DeBerardinis et al. 2007).

14.3.3 Recent Approaches for MFA Using NMR Methods

NMR spectroscopy offers various routes to maximize the information content of MFA. Following earlier metabolomics work in AML cell lines (Khanim et al. 2009; Tiziani et al. 2009b; Lodi et al. 2011), we have recently started MFA using these systems. As an example Fig. 14.4 shows spectra obtained for ^{13}C-MFA experiments with various labeled metabolic precursors. There are clear advantages to using ^{1}H–^{13}C-HSQC spectra acquired with high resolution (4096 points) in the ω_1-dimension. For AML cells lines (K562) the sensitivity is sufficient to obtain natural abundance HSQC spectra with ca 10^6 cells (at 600 MHz). Label incorporation can be derived from (a) signal intensity ratios between labeled and unlabeled samples and from (b), the ratio of split and unsplit signals. This yields the amount of labeled vs unlabeled for a neighboring atom (C2) but only for those atoms where the observed atom is ^{13}C-labeled by itself.

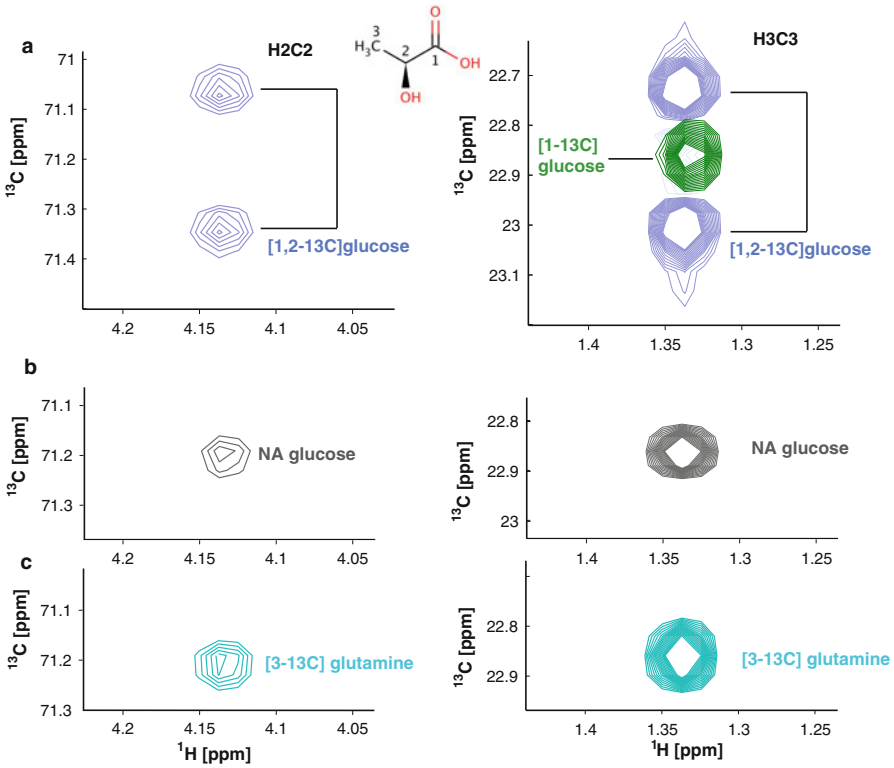

Fig. 14.4 Lactate signals in ^{1}H–^{13}C-HSQC NMR spectra of K562 AML cells grown for 24 h in the presence of various ^{13}C-labeled metabolic precursors. (**a**) [1,2-^{13}C]glucose (*blue*) and [1-^{13}C] glucose (*green*). (**b**) Natural abundance (NA) glucose, and **C:** [3-^{13}C]glutamine (*cyan*). (**a**) When cells are grown in [1,2-^{13}C]glucose (*blue*), lactate gets labeled in the C2 and C3 positions, causing a significant increase in signal intensity and mutual ^{13}C–^{13}C spin coupling ($^{1}J_{CC} = 47.5$Hz). For [1-^{13}C]glucose (*green*) label is incorporated into C3; the C2 signal is below the joint contour level, although clearly identifiable when plotted at however contour levels. The C3 resonance does not show splitting as very little of C2 is ^{13}C (natural abundance ca 1 %). (**b**) Natural abundance (NA) reference spectrum; the H3C3 signal shows a ~3-fold higher intensity than the H2C2 signal arising from 3 protons of the methyl group. The C3 signal shows no splitting in the ^{13}C-dimension (ω_1) because neither ^{13}C neighbor is ^{13}C-labeled. (**c**) For [3-^{13}C]glutamine spectra are almost identical to reference spectra without isotope labels, showing no label incorporation after similar intensities after a flux time of 24 h. All spectra shown in this section were processed and assigned using the MetaboLab software (Günther and Ludwig 2011)

Another opportunity in such spectra arises from the fact that the coupling constant is different for CHx vs COOH neighbor carbons, as shown in Fig. 14.5. In some cases such changes in coupling constant can be used to assign metabolic mechanisms. For malic acid a C2–C1 coupling is a strong indication of pyruvate carboxylase activity and requires that there is no subsequent scrambling of label. The spectra for malate show the same change in coupling constant for the two CH3 cross peaks, indicative of a C3–C4 coupling.

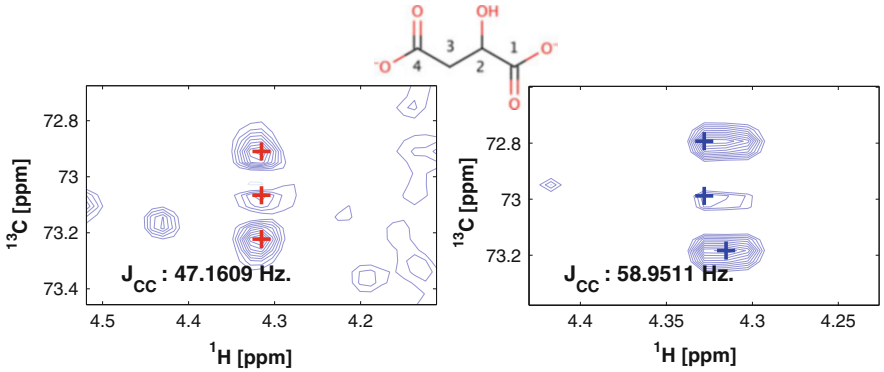

Fig. 14.5 Malate C2H2 signals in ^{1}H–^{13}C HSQC NMR spectra of K562 AML cells grown with [1,2-^{13}C]glucose as a metabolic precursor and two different conditions. The two spectra show a different coupling constant and partial labeling of the neighbor atom. In the spectrum on the left, the coupling constant is 47Hz, consistent with a C2–C3 coupling. In the spectrum on the right, the coupling constant is 58Hz, indicative of C2–C1 coupling to the carboxylic acid carbon. C2–C3 labeling arises from pyruvate carboxylase activity, whereas C2–C1 coupling can either come from [1,2-^{13}C]glucose or from scrambling of label in the C2 or C2 position after several passages through the TCA cycle

One difficulty for the interpretation of NMR signals is the lack of an internal reference. Whereas MS will yield separate signals for different mass increments (m + 1, m + 2 ...) within one mass spectrum, NMR spectra are more difficult to quantify. Comparing labeled and unlabeled samples requires careful preparation of cells under identical conditions with and without the isotope-labeled tracer. While the use of coupling patterns provides a lot of information, the coupling is only observed on ^{13}C-labeled carbons and does not allow for any conclusion on the neighbors of ^{12}C atoms. This problem can often be overcome by careful experiment design, where two situations are compared, yielding label incorporation ratios rather than absolute values.

14.4 Applications of MFA in Cancer Cell Lines

In a 2002 review, Boros, Cascante, and Lee showed the potential of metabolic profiling in cancer using stable isotope tracers (Boros et al. 2002). This landmark paper shows the fate of [1,2-^{13}C]glucose in various metabolic pathways, including glycolysis, formation of [2,3-^{13}C]lactate through the Embden–Meyerhof–Parnas pathway, further metabolism in the PPP, and processing of the glycolysis product [2,3-^{13}C]pyruvate in the TCA cycle. This work followed an earlier theoretical study (Boros et al. 1998) leading to the hypothesis that ribose, not lactate, is the major product of PPP in tumor cells, based on data from hepatoma HepG2 cells (Lee

et al. 1996) and in glioma cells (Portais et al. 1993). The latter by Portais et al. probably represents the first metabolic isotope tracer study in cancer cells.

Metabolic flux measurements have contributed considerably to recent discoveries in cancer metabolism. Key discoveries showed not only altered expression of metabolically active enzymes but also changes in metabolic fluxes. In the following section literature using flux analyses to study metabolic mechanisms in cancer cells will be reviewed.

The Pentose Phosphate Cycle The role of the PPP is predominantly to produce ribose-5-phosphate, which is needed in nucleotide synthesis. Moreover, the oxidative arm of the PPP allows for the production of NADPH, which supports antioxidant function and is required for anabolic pathways such as fatty acid synthesis. In cancer it is well possible that the main role of PPP is to counteract oxidative stress in the cytoplasm by producing NADPH. This is well supported by the observation that GAPDH, which catalyzes the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate, is inactivated in response to oxidant treatments (Ralser et al. 2007).

Boros, Cascante, and Lee studied PPP in human hepatoma cells (HepG2) using mass isotopomer analysis and observed label incorporations arising from [1,2-^{13}C2] glucose into riboses ([1-^{13}C]-, [5-^{13}C]-, [1,2-^{13}C2]-, and [4,5-^{13}C2]ribose). Only C5 of ribose was labeled from [6-^{13}C]glucose and [1-^{13}C]- and [5-^{13}C]ribose were found after incubation with [1-^{13}C]glucose (Lee et al. 1998). They later applied this work in several cancer models. In pancreatic ductal adenocarcinoma cell lines, they showed that inhibition of glycogen breakdown also inhibits cancer cell proliferation, induces apoptosis, and causes glucose carbon redistribution among major metabolic pathways for macromolecule synthesis, specifically affecting pentose synthesis and de novo fatty acid synthesis (Lee et al. 2004). In K562 human myeloid leukemia cells, Gleevec was reported to decrease ^{13}C-label incorporation into RNA ribose, accompanied by a significant fall in hexokinase and glucose-6-phosphate 1-dehydrogenase activities (Boren et al. 2001). Stable isotope-based dynamic metabolic profile arrays were then used to further differentiate the effect of Gleevec in sensitive and resistant K562 tumor cell lines (Boros et al. 2004). This analysis showed that metabolic pathways rigorously controlled by Gleevec include the oxidative decarboxylation of glucose in the pentose cycle and de novo palmitate and stearate synthesis.

Richardson and coworkers examined fluxes in MCF10 cells, using NMR and GC–MS (Richardson et al. 2008). They confirmed increased PPP activity, along with increased TCA cycle activity and increased synthesis of glutamate, glutathione, and fatty acids (including elongation and desaturation), and showed increased associated de novo synthesis of glycine.

Altered Metabolic Fluxes and PKM2 It has recently been suggested that the upregulation of PPP may be caused by a change in the isoform of pyruvate kinase found in cancer cells. The less active PKM2 isoform is predominant in all proliferating cells and is found in an inactive dimeric form, as shown by Mazurek

et al. (Mazurek et al. 2005), who suggested mechanisms where metabolites upstream of PK accumulate, and become available as precursors for pathways that branch of glycolysis, such as nucleic acid, amino acid, and phospholipid synthesis. This blockage in glycolysis may also affect PPP (Lunt and Vander Heiden 2011) and is in excellent agreement with Boros' and Cascante's flux experiments showing increased PPP in proliferating cells.

Serine Biosynthesis Another pathway that branches off from glycolysis is serine biosynthesis. Serine is formed from 3-phosphoglycerate, which is converted into serine via phosphohydroxypyruvate and phosphoserine, and is further converted into glycine. Serine feeds into one carbon metabolism which subsequently feeds into many other pathways and is linked to the epigenetic control via the production of S-adenosyl methionine (SAM). It is also required for histone methylation and is linked to mechanisms of action of methotrexate and 5-fluorouracil.

Serine biosynthesis is upregulated in a number of tumors. Flux experiments (using [U-$^{13}C_6$]glucose) showed that serine biosynthesis is upregulated in PKM2 vs PKM1 cells. Interestingly, serine was shown to be an allosteric activator of PKM2 thus conferring resistance to serine starvation (Ye et al. 2012). This process was shown to depend on the GCN2–ATF4 pathway which regulates the expression of genes involved in amino acid metabolism. Moreover, cell lines in which phosphoglycerate dehydrogenase (PHGDH), which catalyzes the first step of glycolysis, is amplified displayed increased flux of glucose carbons through the serine biosynthesis pathway branching from glycolysis (Possemato et al. 2011; Locasale 2013). PHGDH itself is upregulated by GCN2–ATF4 (Ye et al. 2012). PKM2 also binds tyrosine-phosphorylated peptides, causing the release of the allosteric activator fructose-1,6-bisphosphate (FBP), which inhibits PKM2 enzymatic activity (Christofk et al. 2008a, 2008b).

An alternate glycolytic pathway that depends on phosphorylation of the glycolytic enzyme phosphoglycerate mutase (PGAM1) in PKM2-expressing cells has been suggested. This pathway decouples ATP production from the conversion rate of phosphoenolpyruvate (PEP) to pyruvate, and the conversion of [^{13}C]PEP to [^{13}C] pyruvate was measured using HSQC NMR spectra (Vander Heiden et al. 2010).

When flux from glycolysis to the TCA cycle is reduced, cancer cells can only maintain an active mitochondrial metabolism if anaplerotic mechanisms are active to feed metabolites into the TCA cycle. There are two such mechanisms, glutaminolysis and pyruvate entry in to the TCA cycle via pyruvate carboxylase.

While it has long been known that cancer cells have a high glutamine consumption (Eagle 1955), far exceeding the consumption of any other amino acids, the importance of this in cancer has been realized only recently [see DeBerardinis and Cheng (2010), Wise and Thompson (2010) for detailed reviews]. This form of glutamine utilization is often called glutaminolysis, where glutamine enters the TCA cycle to form α-KG and is converted to oxaloacetate via succinate, fumarate, and malate and exits as pyruvate or aspartate.

Glutaminolysis has been examined extensively using flux analysis employing labeled glutamine and glucose as precursors. The leading work in this context by

DeBerardinis et al. in proliferating glioblastoma cells shows that glutamine maintains TCA cycle activity producing sufficient amounts of NADPH for fatty acid production (DeBerardinis et al. 2007). These cells lacked pyruvate carboxylase activity, producing anaplerotic oxaloacetate from glutamine. A detailed ^{13}C NMR analysis of positional labeling using [3-^{13}C]glutamine provides strong evidence for the conversion of [2-^{13}C]malate and [3-^{13}C]malate by malic enzyme into pyruvate, in turn producing NADPH. The same study used [2-^{13}C]glucose to measure flux into PPP, which was found to be ~6 % of the overall glycolytic flux, based on lactate C2/C3 signal ratios. More detailed flux experiments with two-stage glucose and glutamine labeling showed that the contribution of cycled glutamate is minimal at a flux time of 4.5 h.

Glutaminases (GLS) catalyze the first step of glutaminolysis, converting glutamine into glutamate. In the next step α-KG is formed from glutamate via glutamate dehydrogenase (GDH) or by various transaminases (e.g., alanine transaminase, ALT). It has recently been shown that Myc stimulates expression of glutaminase (GLS) (Gao et al. 2009; Wise et al. 2008). Moreover, experiments using metabolic flux analysis and an shRNA approach to knock out GLS showed that pyruvate carboxylase takes over as the alternative anaplerotic mechanism when glutaminolysis is silenced (Cheng et al. 2011). This is seen in the labeling pattern obtained for glutamate C2. Interestingly, GLS knockout does not prevent glutamine-derived ^{13}C-flux into malate and citrate pools, suggesting that aminotransferases play a significant role, at least in the absence of GLS.

Liu et al. also used flux experiments to show in Myc-inducible human Burkitt lymphoma and prostate cancer cell lines that Myc induces proline biosynthesis from glutamine (Liu et al. 2012). In further flux experiments using the same Burkitt lymphoma cell line, it has been shown that glutamine contributes significantly to the production of citrate carbons but also to fumarate and malate (Le et al. 2012). The labeling patterns in these experiments were interpreted toward an alternative Myc-induced glutaminolysis pathway, with higher alanine (but not lactate) production.

Glutaminolysis has furthermore been shown to be K-ras dependent. K-ras was shown to decouple glucose and glutamine metabolism by enhancing glycolysis, decreasing oxidative flux through the TCA cycle, and increasing the utilization of glutamine for anabolic synthesis (Gaglio et al. 2011).

Further flux analyses demonstrate that glutaminolysis can undergo two distinctly different pathways. In the normal oxidative pathway, α-KG is converted into succinyl-CoA to form succinate, fumarate, malate, and from there oxaloacetate, which forms citrate after condensation with acetyl-CoA. Flux analyses also demonstrate a reductive glutaminolytic pathway involving addition of an unlabeled carbon (from CO_2) by isocitrate dehydrogenase (IDH). There are 3 IDH enzymes: the $NADP^+$-dependent cytosolic IDH1, the mitochondrial IDH2, and the NAD^+-dependent mitochondrial IDH3.

Thompson and coworkers reported further experiments with glioblastoma cell lines, using [U-^{13}C]glutamine as a metabolic precursor for flux studies, employing GC–MS to trace citrate isotopomers (Wise et al. 2011). Citrate m + 5 arises from

reductive carboxylation, whereas oxidative metabolism produces citrate m + 4. For glioblastoma cells a significant increase of reductive carboxylation was observed under hypoxia, and glutamine was required for cells to proliferate under hypoxia (Wise et al. 2011). siRNA knockouts clearly proved a role for mitochondrial IDH2 in the increase of reductive carboxylation. Moreover, not only citrate but also other TCA cycle intermediates (oxaloacetate, malate, and fumarate) showed labeling patterns indicating their formation via reductive carboxylation rather than the oxidative TCA cycle.

Three concurrent studies reported similar observations for other cancer cell types. A detailed metabolic flux balance analysis was reported for various melanoma cell lines showing an increase of m + 5 citrate from [U-^{13}C]glutamine from 12 % under normoxia to 38 % under hypoxia (Scott et al. 2011). This study also quantified label incorporation into fatty acids. In contrast to glioblastoma cells, no appreciable flux of glutamine into lactate or alanine was observed.

Metallo et al. examined metabolic fluxes into lipids from ^{13}C-labeled glucose and glutamine tracers. While these cells produce palmitate mainly from glucose under normoxic conditions, they switch to 80 % IDH1-mediated cytosolic reductive carboxylation of α-KG (Metallo et al. 2012). The observation of reductive carboxylation was supported by label incorporation into acetyl-CoA from [5-^{13}C]glutamine. This is only possible via reductive carboxylation as the C5 gets abstracted in the conversion from α-KG to succinate in oxidative glutaminolysis. Interestingly, in these cells reductive carboxylation did not depend on mitochondrial IDH2, suggesting that IDH2 is an oxidative TCA cycle enzyme. This seems to be not consistent with other cell lines [see, e.g., Mullen et al. (2011)].

The same study (Metallo et al. 2012) showed that von Hippel–Lindau (VHL)-deficient renal cell carcinoma cells preferentially use reductive carboxylation for lipogenesis, even under normoxic conditions. As mentioned before, VHL mediates the oxygen-dependent degradation of HIF-1α and HIF-2α, and loss of VHL causes HIF-1α stabilization causing the Warburg effect of pseudo-hypoxia.

DeBerardinis and coworkers produced human osteosarcoma cells with a loss-of-function mutation in complex III, in which they observed a shift toward aerobic glycolysis associated with reductive carboxylation (Mullen et al. 2011). In these cells, several metabolites downstream of citrate were derived from reductive carboxylation, including acetyl-CoA, oxaloacetate, malate, and fumarate. Interestingly succinate was formed through both reductive and oxidative glutamine metabolism. These effects were shown to be IDH1 and IDH2 associated. This study used an elegant ^{13}C NMR approach to demonstrate fluxes of glutamine into fatty acids, utilizing ^{13}C–^{13}C scalar couplings between ω_1 and ω_2 carbons that arise from reductive carboxylation. The ω_1 carbon showed a triplet for two adjacent ^{13}C atoms and a doublet for only one adjacent ^{13}C. In mutant complex II cells, the triplet signal was the dominant species, showing that 67 % of glutamine-derived carbon arose from reductive carboxylation compared to 15 % for wild-type complex II cells.

The same study (Mullen et al. 2011) also examined cells derived from a renal tumor with hereditary leiomyomatosis and renal cell carcinoma (HLRCC)

syndrome, which harbor mutations in fumarate hydratase (FH). These cells represent an interesting model as a lack of FH precludes the use of oxidative glutaminolysis in the TCA cycle. Interestingly, these cells were shown to use two distinct pathways to produce TCA cycle intermediates from glutamine. Whereas the oxidative metabolism produces fumarate, reductive carboxylation produces citrate and acetyl-CoA for lipogenesis and malate.

Interestingly, reductive carboxylation was associated with an increase in the production of 2-hydroxyglutarate (2-HG), the non-carboxylating reduction product of α-KG (Wise et al. 2011). While 2-HG becomes the dominant product in cancers with IDH mutations (Dang et al. 2009; Ward et al. 2010), the enhancement observed in SF188 glioblastoma cells with wild-type IDH1/2 was much more modest (Dang et al. 2009; Ward et al. 2010).

For cancers with IDH mutations, 2-HG can even be observed by in vivo magnetic resonance spectroscopy (Pope et al. 2011; Andronesi et al. 2012). Nevertheless, increased 2-HG levels in blood may well represent a suitable biomarker for cancers associated with a significant level of reductive carboxylation.

Despite considerable new insights into the metabolism of cancer, many questions remain unanswered. While it is increasingly clear that cancer cells optimize their metabolism toward biomass production and maintain mitochondrial metabolism through anaplerotic mechanisms, the huge production of surplus lactate remains enigmatic. Why should cancer cells produce huge amounts of waste product? It has been suggested that oxygenated cancer cells can form a symbiosis with lactate-producing cells, using lactate as a substrate for their metabolism (Sonveaux et al. 2008). While this provides some explanation, the enigma is not fully resolved as only a small fraction of the surplus lactate is consumed.

14.5 Metabolic Fluxes In Vivo

Although the lion's share of metabolic flux analyses have utilized in vitro cellular models, a general effort has been made to move toward in vivo approaches. Despite in vitro studies allowing for a fine control of experimental conditions, they will never recapitulate the real complexity of biological systems, in which cells are highly influenced by the surrounding environment.

Animal models instead represent a valid compromise between cell cultures and humans, mimicking real tissue complexity. Most of the in vivo studies have been performed in mice, and there are only few reports using isotopic tracers in humans. These experiments are sometimes referred to as SIRM (stable isotope resolved metabolomics) experiments.

Fan and coworkers have carried out tracer-based metabolomics experiments using human non-small cell lung cancers (NSCLC) in xenograft mouse models. For this purpose mice received a bolus injection of [U-^{13}C]glucose, and NMR and GC–MS were employed to measure label incorporation into metabolites in extracts from tissue. The analysis revealed specific metabolic traits such as higher glycolytic

rate, PPP, and pyruvate carboxylase (PC) activity in the lung tumor compared to the normal lung used as internal control (Fan et al. 2010).

Other groups have applied in vivo MFA approaches on different types of cancers. Yuneva et al. investigated the metabolic phenotype of Myc- or MET-induced liver cancers in mice by injecting [U-^{13}C]glucose and [U-^{13}C]glutamine into tumor-bearing animals. In Myc-induced tumors increased glucose and glutamine catabolism was observed, whereas MET-induced tumors showed an increased glucose-derived glutamine synthesis (Yuneva et al. 2012).

Marin-Valencia et al. performed an infusion of [1,6-^{13}C]glucose, [U-^{13}C]glucose, [3,4-^{13}C]glucose, and [U-^{13}C]glutamine into mice bearing human glioblastoma (GBM) cells in order to investigate the fate of these tracer compounds in an in vivo setting. Surprisingly, these tumors metabolized a substantial fraction of glucose through mitochondrial oxidative phosphorylation and displayed PC-activity-derived anaplerosis, while glutamine was mainly used for gluconeogenesis (Marin-Valencia et al. 2012).

As ^{13}C-labeled metabolites are neither radioactive nor toxic, experiments are also feasible in humans. The limitation is most likely to be the cost of labeled metabolites, especially for site-specific labeled metabolic precursors, because large amounts will be needed. While such experiments are commonly carried out in conjunction with magnetic resonance imaging (MRI), there are only few reports of tracer-based in vivo or ex vivo human studies, where labeled precursors are injected into patients in order to study label incorporation into tissue samples. Fan and coworkers infused lung cancer (NSCLC) patients with 10 g [U-^{13}C]glucose prior to surgical resection of the lung tumor (Lane et al. 2008, 2009, 2011). These studies are facilitated by the availability of tumor along with healthy lung tissue. The results, obtained through NMR and GC–MS, suggested increased glycolysis, TCA cycle, and pyruvate carboxylase activity (^{13}C-enrichment in aspartate) in lung tumor tissues compared to normal tissue. Moreover, the lipidomics analysis by FT-ICR MS showed changes in lipid isotopomers derived from lipid vesicles-derived, but the nature of these lipids was not assigned (Lane et al. 2011; Fan and Lane 2008).

Maher et al. infused [U-^{13}C]glucose into a patient's glioblastoma tumors and brain metastases during surgical resection and analyzed label distributions in tumor tissue. The analysis using ^{13}C NMR revealed glycolytic metabolism of glucose to lactate together with its oxidation through TCA cycle. ^{13}C incorporation was also found in glycine, suggesting a contribution of glucose to nucleotide and protein synthesis. The authors also showed the existence of glucose-independent anaplerotic replenishment of the TCA cycle (significant unlabeled acetyl-CoA fraction) (Maher et al. 2012).

There are other reports of using in vivo metabolic fluxes to study non-cancer-related metabolism, for example, in diabetes (Bandsma et al. 2004; Meissner et al. 2011) or cardiac metabolism (Ashrafian et al. 2012; Vincent et al. 2003a). Ex vivo heart perfusion models have frequently been used with isotopically labeled tracers. Des Rosiers and colleagues determined the fate of three distinct possible heart substrates for energy production: [U-^{13}C]lactate, [U-^{13}C]pyruvate and [U-^{13}C]glucose (Khairallah 2003; Vincent et al. 2003b). Similarly, Ashrafian

et al. used a similar perfusion model in fumarate hydratase (FH1) cardiac knockout (KO) mice and showed increased reductive carboxylation and fumarate accumulation arising from the FH1 deletion (Ashrafian et al. 2012).

Using fluxes in vivo (or ex vivo) builds bridges to metabolomics. The latter represents a high-throughput approach, creating a snapshot of metabolite concentrations at one time. The outcome can usually not be attributed to one particular pathway, not even when tissue samples are analyzed. For blood samples even less can be attributed to specific pathways, because metabolite levels in blood arise from responses in many different organs.

However, if it was possible to measure isotopomer distributions after the administration of a labeled precursor in high-throughput mode, it might become possible to add a significant level of information to metabolomics. Stephanopoulos and coworkers proposed such an approach, termed nontargeted tracer fate detection (NTFD) as a method to elucidate metabolic pathways coupled to the applied tracer (Hiller et al. 2010). The NTFD methodology uses GC–MS to evaluate the labeling of *all* compounds, thereby detecting all compound fragments labeled by the externally supplied tracer. This can of course be applied to any type of biological system, whether a cell culture or an animal system. In principle it may even serve as a method applicable for diagnostic purposes in humans, similar to the isotope-based breath test used to detect the presence of *Helicobacter pylori* (Chey et al. 2007; Blashenkov et al. 2013). The advantage of such an approach is that it eliminates noise and that it generates data with some degree of mechanistic information. NTFD applied to a lung cancer cell line using [α-^{15}N]glutamine as a tracer showed label in various amino acids and nucleotides (Hiller et al. 2010). Curiously branched chain amino acids were labeled, suggesting transamination between different amino acids, and label incorporation in serine but not glycine, suggesting that this cancer cell lines produce glycine from an alternative source.

14.6 Summary and Outlook

In hindsight, how could we ever have believed that metabolism is a circumstantial side effect of cellular regulation rather than at the heart of a cell oncogenic transformation? The body of work now available on cancer cell metabolism is substantial and continuously increasing. Despite this immense level of progress, it is not obvious yet how these mechanisms can be easily translated into new drugs affecting cellular metabolism. A recent review summarizes the possibilities that are currently pursued (Jones and Schulze 2012).

In many cases we may find drugs that efficiently affect energy metabolism as being too toxic. An obvious target would be glutaminolysis, especially as glutaminolysis has been linked to cachexia, the progressive loss of muscle and adipose tissue mass that affects many cancer patients. Some of these efforts have been reviewed in (DeBerardinis and Cheng 2010). At this stage there is no consensus whether proteins involved in glutaminolysis are viable drug targets.

Immediate benefits from these modern aspects of the Warburg effect are in cancer diagnostics. There are several reviews that compare metabolomics studies and the biomarkers that were reported (Abbassi-Ghadi et al. 2013; Armitage and Barbas 2014; Chong and Cunningham 2005; Spratlin et al. 2009). One difficulty is that metabolomics has been applied to tissue, blood samples, urine, cerebrospinal fluid, and even saliva. Except for studies in tissue, the observed effects are difficult to correlate to specific mechanisms, as the response of the entire organism is observed.

In this context in vivo applications of tracer-based methods may open new avenues (Lane et al. 2009). Isotopically labeled metabolites are already successfully used for metabolic imaging using MRI, usually using pyruvate as a metabolic precursor (Golman et al. 2006; Kurhanewicz et al. 2011). However, recent work shows that this is also feasible for glucose (Rodrigues et al. 2013). The analysis of in vivo metabolism may however also benefit from short-term metabolization of isotopically labeled precursors followed by the analysis of biopsies, tumor samples, or even blood.

From a scientific point of view, it is desirable to identify proof of mechanism for biomarkers that are used for patient stratification. Metabolic profiles of patient-derived cancer cells are likely to differentiate between subtypes of cancers, especially in combination with genetic and epigenetic assignments. If these can be linked to outcomes of different treatment options, we may see a new era of patient stratification. Tracer-based methods are likely to enhance the opportunities arising from this context.

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Chapter 15
Targeted ^{13}C-Labeled Tracer Fate Associations for Drug Efficacy Testing in Cancer

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Contents

A significantly lower risk of liver cancer incidence was reported for the use of rosiglitazone (odds ratio: 0.73, 95 % confidence interval: 0.65–0.81) in a large population study in patients with type 2 diabetes mellitus (Chang et al. 2012). The protective effects were stronger for higher cumulative dosage and longer duration. In an in vitro efficacy study, we reported that in primary human hepatocellular cells (epithelial; HepG2), the selective peroxisome proliferator-activated receptor (PPARγ-)binding ligand, rosiglitazone, increases fatty acid oxidation (β carbon) and thus ^{13}C-stearate-derived acetyl-CoA exchange (^{13}C labeling) between the extracellular [U-$^{13}C_{18}$]-stearic acid and intracellular carbohydrate [3,4-$^{13}C_2$]-D-ribose pool (RNA). This rosiglitazone efficacy marker demonstrating the flow of carbons from the tracer substrate to product is depicted in Fig. 15.1, while additional details of methods and results are described in a dose-escalating thiazolidinedione efficacy study (Harrigan et al. 2006).

Close examination of the [U-$^{13}C_{18}$]-stearate-derived metabolome (Fig. 15.2; *EZ*Top[U-$^{13}C_{18}$]-stearate) shows a decrease in intracellular [U-$^{13}C_{18}$]-stearate (tracer) content in response to increased (10 μM) rosiglitazone treatment in HepG2 cells. In other words, rosiglitazone undesirably decreases ^{13}C-stearate uptake in HepG2 cells from the media at the expense of mobilizing and synthesizing an unlabeled intracellular pool. This is indicated by the significant >5 % decrease in ^{13}C-labeled intracellular stearate fractions (Fig. 15.2, pellet ^{13}C-stearate panel 107 and 108) after rosiglitazone treatment (see also Table 15.1, pellet stearate panel 107, 108, significant decrease in comparison with a 1 μM dose).

Nevertheless, as expected, rosiglitazone readily increases tracer stearate oxidation (β carbon) as seen by glutamate, lactate, and ribose labeling on the respective $M_2/\Sigma M$ positions (isobolome-wide acetate-dependent ($n = 9$) labeling after [U-$^{13}C_{18}$]-stearic acid's oxidation (supplied in media), Fig. 15.3; for enzyme and metabolite identifiers, please see Tables 15.3 and 15.4, respectively). However, the negative correlation coefficients between rosiglitazone dosing and ^{13}C-labeled stearate indicate the mobilization and synthesis of stearic acid from an unlabeled substrate pool, which necessitate the evaluation of additional tracer approaches for rosiglitazone efficacy testing in HepG2 cells.

Advancements in stable ^{13}C isotope biological chemistry demonstrate that [1,2-$^{13}C_2$]-D-glucose provides the most precise estimates for glycolysis, the pentose phosphate pathway, and the overall metabolic network (Metallo et al. 2009).

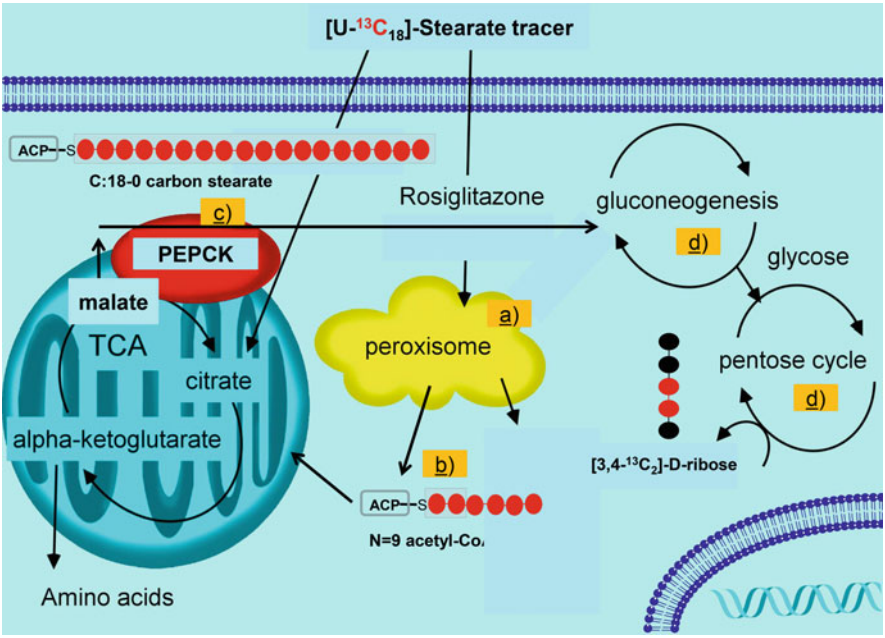

Fig. 15.1 Extracellular [U-$^{13}C_{18}$]-stearic acid tracer substrate entry and its metabolic hubs by rosiglitazone action in HepG2 cells. (**a**) Rosiglitazone increases peroxisomal long-chain fatty acid degradation while shifting, (**b**) acetyl-CoA flux via, (**c**) malate shuttling towards, (**d**) triose, as well as [3,4-$^{13}C_2$]-D-pentose and hexose labeling. Rosiglitazone also forces long-chain fatty acid oxidation to occur in the mitochondria and acetyl-CoA disposal via glutamate synthesis as well as malate shuttling to label RNA ribose and lactate. Please note that acetyl-CoA used by citrate synthase is indistinguishable, if it was generated after peroxisomal chain shortening and mitochondrial fatty acid β carbon oxidation. Increased ribose, lactate, and glutamate labeling after a stepwise rosiglitazone dosing are used as efficacy markers to visualize, via regression analysis, ^{13}C tracer fate associations in HepG2 cells

Because diverse ^{13}C tracer substrate methods are increasingly used in phenotype and drug development (Walther et al. 2012; Mullen et al. 2011; Son et al. 2013; Holleran et al. 1995), our observation of decreased ^{13}C-stearate uptake in a thiazolidinedione efficacy study generates the hypothesis that metabolic tracers, or their mixtures, should only be used with the utmost care as interchangeable efficacy tools, particularly in drug studies. We herein demonstrate a single ^{13}C-glucose tracer as the widely distributed and more precise substrate for cellular carbon metabolism for efficacy testing using the drug-responsive tracer fate regression statistics method among multiple products (Beger et al. 2009). The single [1,2-$^{13}C_2$]-D-glucose tracer generates the standard isotope-dependent targeted cross-labeled ^{13}C-stearate tracer fate metabolomics, which may be the desired approach for drug efficacy testing. Rosiglitazone-induced cross-labeled stearic acid oxidation can label RNA ribose on the recycled acetate-hosting carbon positions, as shown in Fig. 15.4.

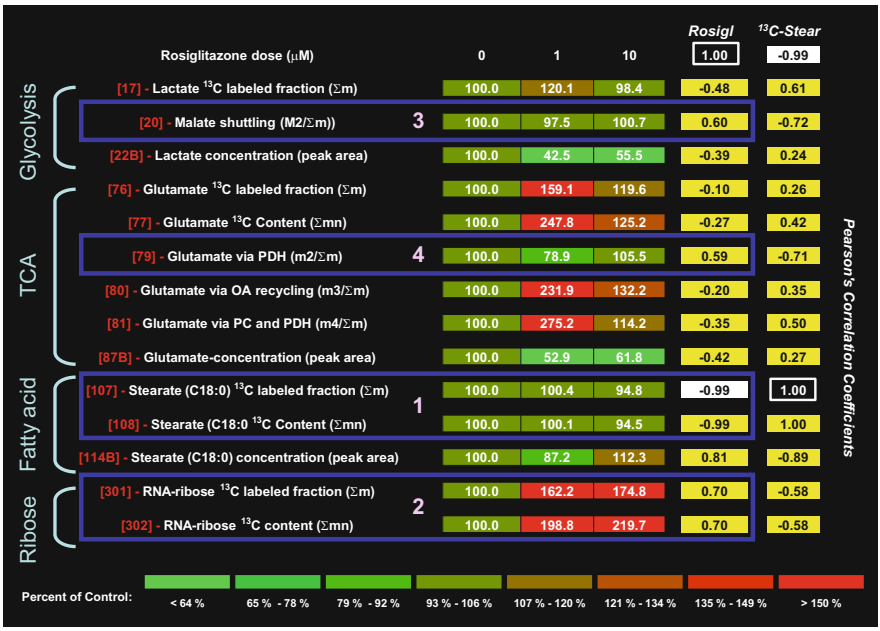

Fig. 15.2 EZTopolome (EZTop([U-$^{13}C_{18}$]-stearate) of in vitro grown HepG2 liver cells, which serves as the color-assisted visual isotopolome matrix screening tool. The EZTop shows decreased stearate tracer uptake (shown in *blue box* 1—line 107, line 108), while stearate concentration increases (line 114B) from unlabeled intracellular pools. Despite decreased tracer uptake, rosiglitazone increases ^{13}C-stearate carbon oxidation and labeling in RNA-derived ribose (shown in *blue box* 2—line 301, line 302) via exclusive malate shuttling as shown in *blue box* 3—^{13}C M_2-labeled lactate; line 20—or glutamate within the cycle by labeling glutamate on C4–C5 positions (shown in *blue box* 4—line 79). The ^{13}C-stearate *EZ*Topolome returns correlation coefficients between rosiglitazone dosing (*Rosigl* column, *yellow*) or ^{13}C-labeled stearate (*^{13}C-Stear* column, *yellow*) as reference variables (*white boxes* = 1) and the rest of the [U-$^{13}C_{18}$]-stearate isotopolome. One can determine the relationship (associations) between rosiglitazone dosing (Rosigl column) and that with ^{13}C-stearate uptake (^{13}C-Stear column), followed by its oxidation in the isotopolome, respectively. Reference ranges are shown with the *white-squared boxes* and regression coefficient of 1.00 with the *black background*

To this effect, [1,2-$^{13}C_2$]-D-glucose EZTopolome (Fig. 15.5; see also Table 15.2) reveals a dose-dependent increase by rosiglitazone on stearate oxidation. The key event in the drug-treated metabolome is the well-maintained glucose tracer uptake with unaltered glycolysis and intracellular stearic acid labeling from glucose, which replaces external stearic acid transport. Glutamate ^{13}C labeling shows much improved correlation coefficients with cross-labeled stearate fractions, and, as expected, rosiglitazone increases RNA-derived ribose labeling, where correlation coefficients are close to one with cross-labeled intracellular stearate as the reference range.

Regression analysis syntax ranges investigate the dependence (dependent or response variables) of all product isotopomers in a heat map format (EZTopolome;

Table 15.1 Targeted ^{13}C isotopolome (^{13}C-labeled metabolome) matrix of rosiglitazone-treated HepG2 (primary hepatocellular carcinoma) cells using [U-$^{13}C_{18}$]-stearate as the single tracer and GC-MS in 72 h cultures. Controls are HepG2 cells treated with 10 % DMSO. N = 3, average (±SD)

Metabolite (source-data-matrix-file-log)	Isotopomer fragment dimension	HepG2 (10 % DMSO)	HepG2 (1 μM Rosiglit)	HepG2 (10 μM Rosiglit)
Lactate (media-CAS: 50-21-5; 17)	^{13}C-labeled fraction (Σm)	0.68 (±0.0388)	0.82 (±0.0741)	0.67 (±0.0368)
Lactate (media-CAS: 50-21-5; 20)	Malate shuttling towards lactate ($m_2/\Sigma m$)	55.1 (±6.42)	53.7 (±5.1)	55.5 (±1.3)
Lactate (media-CAS: 50-21-5; 22B)	Peak-area (abundance)	54,795 (±41,284)	23,286 (±6,449)	30,396 (±5,440)
Glutamate (media-CAS: 617-65-2; 76)	^{13}C-labeled fraction (C_2–C_5 fragment) (Σm)	1.37 (±0.0284)	2.18 (±0.768)	1.64 (±0.0817)*
Glutamate (media-CAS: 617-65-2; 77)	^{13}C content (C_2–C_5 fragment) (Σm_n)	0.02 (±0.0007)	0.06 (±0.0329)	0.03 (±0.002)*
Glutamate (media-CAS: 617-65-2; 79)	^{13}C-m_2 (m/z 200) ($m_2/\Sigma m$)	35.4 (±0.374)	28 (±6.87)	37.4 (±0.615)*
Glutamate (media-CAS: 617-65-2; 80)	^{13}C-m_3 (m/z 201) ($m_3/\Sigma m$)	2.51 (±0.616)	5.81 (±2.48)	3.31 (±0.792)
Glutamate (media-CAS: 617-65-2; 81)	^{13}C-m_4 (m/z 202) ($m_4/\Sigma m$)	9.37 (±0.387)	25.8 (±15.5)	10.7 (±0.792)
Glutamate (media-CAS: 617-65-2; 87B)	Peak-area (abundance)	159,534 (±18,546)	84,404 (±28,071)*	98,584 (±12,619)*
Stearic acid (pellet-CAS: 57-11-4; 107)	^{13}C-labeled fraction (m/z 298) (Σm)	52.6 (±2.18)	52.8 (±0.642)	49.8 (±0.904)[a]
Stearic acid (pellet-CAS: 57-11-4; 108)	^{13}C content (m/z 298) (Σm_n)	4.43 (±0.19)	4.43 (±0.0553)	4.18 (±0.0766)[a]
Stearic acid (pellet-CAS: 57-11-4; 114B)	Peak-area (abundance)	28,695 (±1,969)	25,010 (±3,332)	32,231 (±3,850)
RNA ribose (pellet-CAS: 50-69-1; 301)	^{13}C-labeled fraction (C_3-C_5) (Σm)	1.76 (±0.188)	2.85 (±0.38)	3.07 (±0.552)

(continued)

Table 15.1 (continued)

Metabolite (source-data-matrix-file-log)	Isotopomer fragment dimension	HepG2 (10 % DMSO)	HepG2 (1 μM Rosiglit)	HepG2 (10 μM Rosiglit)
RNA ribose (pellet-CAS: 50-69-1; 302)	^{13}C content (C_3–C_5) (Σm_n)	0.03 (±0.0051)	0.06 (±0.0091)*	0.06 (±0.0145)

&source-data-matrix-file-log: source of metabolite, i.e., culture media or pellets with raw data locator file number
$M_n/\Sigma m$: isotopomer/^{13}C-labeled fraction as SUM($m_1 + m_2 + \ldots + m_n$) is shown as percent of total ion current (TIC) instead of fraction of 1
Σm_n: molar enrichment (ME) ^{13}C content as SUM($1\times m_1 + 2\times m_2 + \ldots + n\times m_n$) (Lee et al. 1992)
Number of observations per group: $n = 3$ (±SD)
CAS chemical abstracts service registry number
*$P < 0.05$ vs. 10 % DMSO control
[a]$P < 0.05$ vs. 1 μM rosiglitazone (escalating rosiglitazone dosing comparison between 1 μM and 10 μM rosiglitazone in HepG2 cells, where cells have been cultured in the same type and tracer-containing media as described in Harrigan et al.)

^{13}C isotopomer associations) either from rosiglitazone dosing or its ^{13}C-stearic acid, cross-labeled from [1,2-$^{13}C_2$]-D-glucose, as the independent variable (explanatory variable). We observed partial high correlations between ^{13}C glucose tracer uptake and lactate's ^{13}C-labeling patterns, which mark the Warburg phenotype (Yang et al. 2013; Boros et al. 2013). On the other hand, there were strong associations among ^{13}C-glutamate, RNA ribose, and lactate labeling, when the effect of glucose uptake as a controlling random variable was replaced with that of the cross-labeled ^{13}C-stearate fraction from the glucose tracer.

Both studies showed an inhibition of lactate production in response to rosiglitazone treatment, which is consistent with decreasing the Warburg effect in tumor cells, one of rosiglitazone's known biological effects. While decreased lactate production is a reliable efficacy response, increased ^{13}C labeling from both tracers is more consistent with toxicity response demonstrating RNA synthesis/turnover-dependent peroxide scavenging by NADPH availability via increased pentose cycle flux. We believe that the isotopolome can be tied to clinical and preclinical outcomes and current surrogate biomarkers that will yield "new mechanistic" markers as measures of drug efficacy (Vamecq et al. 2012) and toxicity (Sonko et al. 2011). These new mechanistic tracer efficacy or toxicity markers could potentially be further qualified and defined for specific "concept (s) of use" during in vitro studies (Beger and Colatsky 2011).

In conclusion, there are significant improvements in associations between [1,2-$^{13}C_2$]-D-glucose tracer deriving intracellular stearate to lactate, glutamate, and ribose ^{13}C labeling (Fig. 15.6) and with that obtained with an extracellular stearic acid tracer in response to rosiglitazone treatment with the central mechanism of increasing fatty acid beta-oxidation and turnover.

Table 15.2 Targeted ^{13}C isotopolome (^{13}C-labeled metabolome) matrix of rosiglitazone-treated HepG2 (primary hepatocellular carcinoma) cells using [1,2-$^{13}C_2$]-D-glucose as the single tracer and GC-MS in 72 h cultures

Metabolite (source-data-matrix-file-log)	Isotopomer fragment dimension	HepG2 (10 % DMSO)	HepG2 (1 μM Rosiglit)	HepG2 (10 μM Rosiglit)
Lactate (media-CAS: 50-21-5; 17)	^{13}C-labeled fraction (Σm)	21.5 (±0.629)	22.8 (±0.687)	23.6 (±0.0471)
Lactate (media-CAS: 50-21-5; 20)	Malate shuttling towards lactate ($m_2/\Sigma m$)	70.9 (±0.0925)	69.2 (±0.548)	70.5 (±0.682)
Lactate (media-CAS: 50-21-5; 22B)	Peak-area (abundance)	148,615 (±116,074)	35,950 (±7,661)	35,674 (±12,175)
Glutamate (media-CAS: 617-65-2; 76)	^{13}C-labeled fraction (C_2–C_5 fragment) (Σm)	17 (±0.231)	18.8 (±0.408)*	19.2 (±0.59)*
Glutamate (media-CAS: 617-65-2; 77)	^{13}C content (C_2–C_5 fragment) (Σm_n)	0.29 (±0.004)	0.33 (±0.0062)*	0.34 (±0.0098)*
Glutamate (media-CAS: 617-65-2; 79)	^{13}C-m_2 (m/z 200) ($m_2/\Sigma m$)	49.8 (±0.184)	49.7 (±0.336)	50.5 (±0.412)
Glutamate (media-CAS: 617-65-2; 80)	^{13}C-m_3 (m/z 201) ($m_3/\Sigma m$)	8.18 (±0.0171)	8.32 (±0.0682)	8.3 (±0.0323)*
Glutamate (media-CAS: 617-65-2; 81)	^{13}C-m_4 (m/z 202) ($m_4/\Sigma m$)	2.45 (±0.0581)	2.88 (±0.0158)*	3 (±0.0214)*,a
Glutamate (media-CAS: 617-65-2; 87B)	Peak-area (abundance)	33,445 (±4,507)	42,381 (±28,935)	47,175 (±3,164)
Stearic acid (pellet-CAS: 57-11-4; 107)	^{13}C-labeled fraction (m/z 298) (Σm)	12.9 (±2.33)	18.1 (±4.04)	22.4 (±2.33)*
Stearic acid (pellet-CAS: 57-11-4; 108)	^{13}C content (m/z 298) (Σm_n)	0.36 (±0.0681)	0.51 (±0.119)	0.64 (±0.0679)*
Stearic acid (pellet-CAS: 57-11-4; 114B)	Peak-area (abundance)	448,116 (±184,694)	369,434 (±155,377)	170,589 (±24,930)
RNA ribose (pellet-CAS: 50-69-1; 301)	^{13}C-labeled fraction (C_3–C_5) (Σm)	33.9 (±1.75)	36.4 (±0.231)	37.4 (±0.596)

(continued)

Table 15.2 (continued)

Metabolite (source-data-matrix-file-log)	Isotopomer fragment dimension	HepG2 (10 % DMSO)	HepG2 (1 μM Rosiglit)	HepG2 (10 μM Rosiglit)
RNA ribose (pellet-CAS: 50-69-1; 302)	^{13}C content (C_3–C_5) (Σm_n)	0.58 (±0.0294)	0.61 (±0.0043)	0.63 (±0.0101)

Controls are HepG2 cells treated with 10 % DMSO. $N = 3$, average (±SD)
&source-data-matrix-file-log: source of metabolite, i.e., culture media or pellets with raw data locator file number
$M_n/\Sigma m$: isotopomer/^{13}C-labeled fraction as SUM($m_1 + m_2 + \cdots + m_n$) is shown as percent of total ion current (TIC) instead of fraction of 1
Σm_n: molar enrichment (ME) ^{13}C content as SUM($1 \times m_1 + 2 \times m_2 + \cdots + n \times m_n$) (Lee et al. 1992)
Number of observations per group: $n = 3$ (±SD)
CAS chemical abstracts service registry number
*$P < 0.05$ vs. 10 % DMSO control
[a]$P < 0.05$ vs. 1 μM rosiglitazone (escalating rosiglitazone dosing comparison between 1 μM and 10 μM rosiglitazone in HepG2 cells, where cells have been cultured in the same type and tracer-containing media as described in Harrigan et al.)

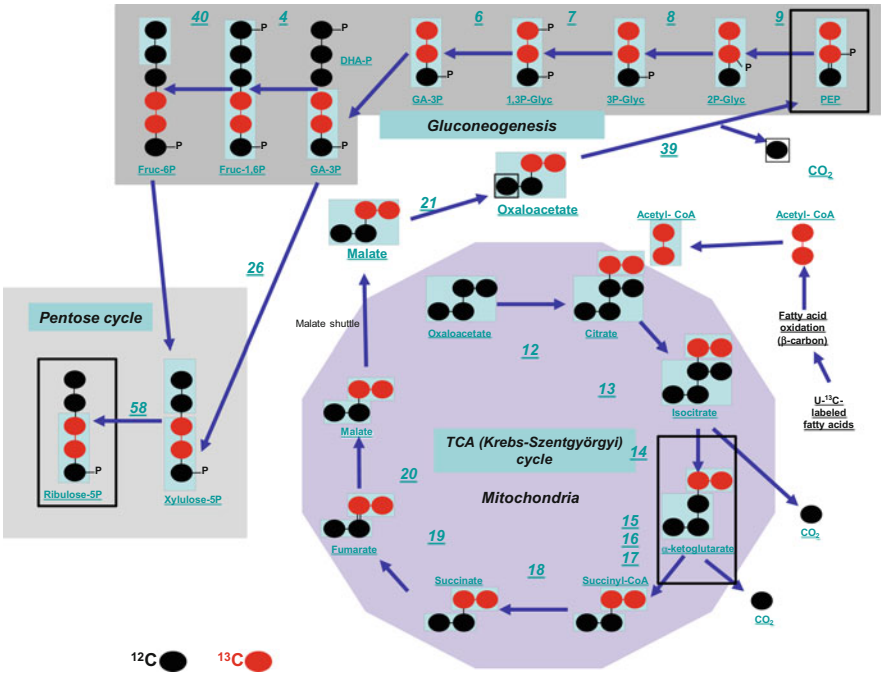

Fig. 15.3 Overview of RNA ribose, lactate, and glutamate labeling from [U-$^{13}C_{18}$]-stearate deriving acetyl-CoA and beta-carbon oxidation. For identifying enzymes and metabolite, please see Tables 15.3 and 15.4, respectively

Table 15.3 Enzyme identifiers by Arabic numbers that refer to article panels (Fig. 15.3)

001	Glucokinase	EC 2.7.1.1	en.wikipedia.org/wiki/Glucokinase
002	Glucose-6-phosphate isomerase	EC 5.3.1.9	en.wikipedia.org/wiki/Glucose_phosphate_isomerase
003	Phosphofructokinase 1	EC 2.7.1.11	en.wikipedia.org/wiki/Phosphofructokinase_1
004	Aldolase A	EC 4.1.2.13	en.wikipedia.org/wiki/Aldolase_A
005	Triosephosphate isomerase	EC 5.3.1.1	en.wikipedia.org/wiki/Triosephosphateisomerase
006	Glyceraldehyde-3-phosphate dehydrogenase	EC 1.2.1.12	en.wikipedia.org/wiki/Glyceraldehyde_3-phosphate_dehydrogenase
007	Phosphoglycerate kinase	EC 2.7.2.3	en.wikipedia.org/wiki/Phosphoglycerate_kinase
008	Phosphoglycerate mutase	EC 5.4.2.1	en.wikipedia.org/wiki/Phosphoglycerate_mutase
009	Enolase	EC 4.2.1.11	en.wikipedia.org/wiki/Enolase
010	Pyruvate kinase	EC 2.7.1.40	en.wikipedia.org/wiki/Pyruvate_kinase
011	Pyruvate dehydrogenase	EC 1.2.4.1	en.wikipedia.org/wiki/Pyruvate_dehydrogenase
012	Citrate synthase	EC 2.3.3.1	en.wikipedia.org/wiki/Citrate_synthase
013	Aconitase	EC 4.2.1.3	en.wikipedia.org/wiki/Aconitase
014	Isocitrate dehydrogenase	EC 1.1.1.42	en.wikipedia.org/wiki/Isocitrate_dehydrogenase
015	Oxoglutarate dehydrogenase	EC 1.2.4.2	en.wikipedia.org/wiki/Isocitrate_dehydrogenase
016	Dihydrolipoyl succinyltransferase	EC 2.3.1.61	en.wikipedia.org/wiki/Alpha-ketoglutarate_dehydrogenase
017	Dihydrolipoyl dehydrogenase	EC 1.8.1.4	en.wikipedia.org/wiki/Alpha-ketoglutarate_dehydrogenase
018	Succinyl coenzyme A synthetase	EC 6.2.1.5	en.wikipedia.org/wiki/Succinyl_coenzyme_A_synthetase
019	Succinate dehydrogenase	EC 1.3.5.1	en.wikipedia.org/wiki/Succinate_dehydrogenase
020	Fumarate hydratase	EC 4.2.1.2	en.wikipedia.org/wiki/Fumarase
021	Malate dehydrogenase	EC 1.1.1.37	en.wikipedia.org/wiki/Malate_dehydrogenase
022	Glucose-6-phosphate dehydrogenase	EC 1.1.1.49	en.wikipedia.org/wiki/Glucose-6-phosphate_dehydrogenase
023	Phosphogluconate 2-dehydrogenase	EC 1.1.1.43	en.wikipedia.org/wiki/Phosphogluconate_2-dehydrogenase

(continued)

Table 15.3 (continued)

024	Ribose-5-phosphate isomerase	EC 5.3.1.6	en.wikipedia.org/wiki/Ribose-5-phosphate_isomerase
025	Ribose-phosphate diphosphokinase: phosphoribosyl pyrophosphate synthetase	EC 2.7.6.1	en.wikipedia.org/wiki/Ribose-phosphate_diphosphokinase
026	Transketolase	EC 2.2.1.1	en.wikipedia.org/wiki/Transketolase
027	Transaldolase	EC 2.2.1.2	en.wikipedia.org/wiki/Transaldolase
028	Glucose-6-phosphatase: Glc-6-Pase	EC 3.1.3.9	en.wikipedia.org/wiki/Glucose_6-phosphatase
031	Lactate dehydrogenase	EC 1.1.1.27	en.wikipedia.org/wiki/Lactate_dehydrogenase
032	Pyruvate carboxylase	EC 6.4.1.1	en.wikipedia.org/wiki/Pyruvate_carboxylase
033	Fatty acid synthase	EC 2.3.1.85	en.wikipedia.org/wiki/Fatty_acid_synthase
034	Acyl-CoA dehydrogenase	EC 1.3.99.3	en.wikipedia.org/wiki/Acyl_CoA_dehydrogenase
035	Enoyl-CoA hydratase	EC 4.2.1.17	en.wikipedia.org/wiki/Enoyl_CoA_hydratase
036	L-β-Hydroxyacyl-CoA dehydrogenase	EC 1.1.1.35	en.wikipedia.org/wiki/Beta_oxidation
037	Acetyl-CoA C-acyltransferase	EC 2.3.1.16	en.wikipedia.org/wiki/%CE%92-ketothiolase
039	Phosphoenolpyruvate carboxykinase	EC 4.1.1.32	en.wikipedia.org/wiki/Phosphoenolpyruvate_carboxykinase
040	Fructose 1,6-bisphosphatase	EC 3.1.3.11	en.wikipedia.org/wiki/Fructose_1,6-bisphosphatase
041	Beta-ketoacyl-ACP synthase	EC 2.3.1.41	en.wikipedia.org/wiki/Beta-ketoacyl-ACP_synthase
042	Beta-ketoacyl-ACP reductase	EC 1.1.1.100	en.wikipedia.org/wiki/%CE%92-Ketoacyl_ACP_reductase
043	3-Hydroxyacyl ACP	EC 4.2.1.134	en.wikipedia.org/wiki/3-Hydroxyacyl_ACP_dehydratase
044	Enoyl-acyl carrier protein reductase	EC 1.3.1.9	en.wikipedia.org/wiki/Enoyl_ACP_reductase
045	Acetyl-coenzyme A acetyltransferase	EC 2.3.1.9	en.wikipedia.org/wiki/Acetyl-Coenzyme_A_acetyltransferase
046	HMG-CoA synthase	EC 2.3.3.10	en.wikipedia.org/wiki/HMG-CoA_synthase
047	HMG-CoA reductase	EC 1.1.1.34	en.wikipedia.org/wiki/HMG-CoA_reductase
048	Mevalonate kinase	EC 2.7.1.36	en.wikipedia.org/wiki/Mevalonate_kinase

(continued)

Table 15.3 (continued)

049	Phosphomevalonate kinase	EC 2.7.4.2	en.wikipedia.org/wiki/Phosphomevalonate_kinase
050	Pyrophosphomevalonate decarboxylase	EC 4.1.1.33	en.wikipedia.org/wiki/Pyrophosphomevalonate_decarboxylase
051	Isopentenyl-diphosphate delta-isomerase	EC 5.3.3.2	en.wikipedia.org/wiki/Isopentenyl-diphosphate_delta_isomerase
052	Phosphoglucomutase	EC 5.4.2.2	en.wikipedia.org/wiki/Phosphoglucomutase
053	Glycogen synthase	EC 2.4.1.11	en.wikipedia.org/wiki/Glycogen_synthase
054	Glutamate dehydrogenase	EC 1.4.1.3	en.wikipedia.org/wiki/Glutamate_dehydrogenase
055	ATP citrate lyase	EC 2.3.3.8	en.wikipedia.org/wiki/ATP_citrate_lyase
056	Acetyl-CoA carboxylase	EC 6.4.1.2	en.wikipedia.org/wiki/Acetyl-CoA_carboxylase
057	Glycogen phosphorylase	EC 2.4.1.1	en.wikipedia.org/wiki/Glycogen_phosphorylase
058	Ribulose-phosphate 3-epimerase	EC 5.1.3.1	en.wikipedia.org/wiki/Ribulose-phosphate_3-epimerase
059	Xylulokinase	EC 2.7.1.17	en.wikipedia.org/wiki/Xylulokinase
060	D-Xylose aldose-ketose-isomerase	EC 5.3.1.5	en.wikipedia.org/wiki/Xylose_isomerase
061	Dimethylallyltranstransferase	EC 2.5.1.1	en.wikipedia.org/wiki/Farnesyl_pyrophosphate_synthase
062	Farnesyl-diphosphate farnesyltransferase	EC 2.5.1.21	en.wikipedia.org/wiki/Squalene_synthase
063	Squalene monooxygenase	EC 1.14.99.7	en.wikipedia.org/wiki/Squalene_monooxygenase
064	2,3-Oxidosqualene-(cycloartenol) cyclase	EC 5.4.99.7	en.wikipedia.org/wiki/Cycloartenol_synthase
065	Sterol-carrier protein		en.wikipedia.org/wiki/Sterol_carrier_protein
066	DOXP synthase	EC 2.2.1.7	en.wikipedia.org/wiki/DOXP_synthase
067	DXP reductoisomerase	EC 1.1.1.267	en.wikipedia.org/wiki/DOXP_reductase
068	2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	EC 4.6.1.12	en.wikipedia.org/wiki/4-diphosphocytidyl-2-C-methyl-D-erythritol_synthase
069	4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase	EC 2.7.1.148	en.wikipedia.org/wiki/4-diphosphocytidyl-2-C-methyl-D-erythritol_kinase

(continued)

Table 15.3 (continued)

070	2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase	EC 4.6.1.12	en.wikipedia.org/wiki/2-C-methyl-D-erythritol_2,4-cyclodiphosphate_synthase
071	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	EC 1.17.4.3	en.wikipedia.org/wiki/HMB-PP_synthase
072	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase	EC 1.17.1.2	en.wikipedia.org/wiki/HMB-PP_reductase
073	Palmitoyl-(protein) hydrolase	EC 3.1.2.22	en.wikipedia.org/wiki/Palmitoyl%28protein%29_hydrolase
074	Acyl-CoA:acetyl-CoA C-acyltransferase	EC 2.3.1.16	en.wikipedia.org/wiki/Acetyl-CoA_C-acyltransferase
075	Long-chain-(S)-3-hydroxyacyl-CoA: NAD + oxidoreductase	EC 1.1.1.211	en.wikipedia.org/wiki/3-hydroxyacyl-CoA_dehydrogenase
076	Long-chain-(3S)-3-hydroxyacyl-CoA hydro-lyase	EC 4.2.1.74	en.wikipedia.org/wiki/Long-chain-enoyl-CoA_hydratase
077	Acyl-CoA:NADP + 2-oxidoreductase	EC 1.3.1.8	en.wikipedia.org/wiki/Acyl-CoA_dehydrogenase_%28NADP%2B%29
078	Stearoyl-CoA 9-desaturase	EC 1.14.19.1	en.wikipedia.org/wiki/Stearoyl-CoA_9-desaturase

While fatty acid oxidation can readily be measured using mass isotopomer distribution analysis (MIDA) in ^{13}C-labeled CO_2 and numerous TCA and pentose cycle ribose products in HepG2 cells with [U-$^{13}C_{18}$]-stearic acid (Fig. 15.7a), the Warburg effect, glycolysis, and pentose cycling are most efficiently determined with ^{13}C-glucose in a wide range of in vitro cell systems under various growth conditions (Fig. 15.7b).

Alternatively, targeted [1,2-$^{13}C_2$]-D-glucose fate associations, as the single metabolic tracer method in the same cell system, can reveal cross ^{13}C-labeled stearate breakdown and the Warburg effect, glycolysis, as well as TCA and pentose cycle metabolism as the single tracer substrate in the metabolome of tumor cells (Fig. 15.8).

Therefore, we recommend reevaluation of drug efficacy and mechanism of action studies for contemporary metabolomics investigations using multiple parallel or mixed tracer approaches. Herein, we recommend the use of a single glucose tracer and targeting its fate via cross-labeled multiple substrate-product patterns as independent explanatory variables for determining efficacy and phenotype-related associations in single experiments. The single glucose tracer approach, which inherently corrects for ^{13}C-labeled fractions and positional ^{13}C labeling in all targeted products as an internal standard throughout the ^{13}C-labeled metabolome (isobolome), minimizes the unforeseen complications of altered extra- and intracellular metabolome cross talk during efficacy testing.

Table 15.4 Metabolite identifiers in alphabetic order that refer to article panels (Fig. 15.3)

(α)Ketoglutarate	Alpha-ketoglutarate	en.wikipedia.org/wiki/Alpha-ketoglutaric_acid
1,3P-Glyc	Glycerol-1,3-bisphosphate	en.wikipedia.org/wiki/Glycerol
2,3-O-Squalene	2,3-Oxidosqualene	en.wikipedia.org/wiki/Squalene_oxide
2P-Glyc	Glycerol 2-phosphate	en.wikipedia.org/wiki/Glycerol
3P-Glyc	Glycerol 3-phosphate	en.wikipedia.org/wiki/Glycerol_3-phosphate
6-Phosphogluc	6-Phosphogluconate	en.wikipedia.org/wiki/6-Phosphogluconate
Acetoacetyl-CoA	Acetoacetyl-CoA	en.wikipedia.org/wiki/Acetoacetyl-CoA
Acetyl-CoA	Acetyl-CoA	en.wikipedia.org/wiki/Acetyl-CoA
ATP	Adenosine triphosphate	en.wikipedia.org/wiki/Adenosine_triphosphate
CMP	Cytidine monophosphate	en.wikipedia.org/wiki/Cytidine_monophosphate
CDP-ME	4-Diphosphocytidyl-2-C-methylerythritol	en.wikipedia.org/wiki/4-diphosphocytidyl-2-C-methylerythritol
CDP-MEP	4-Diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate	en.wikipedia.org/wiki/4-diphosphocytidyl-2-C-methyl-D-erythritol_2-phosphate
Chol	Cholesterol	en.wikipedia.org/wiki/Cholesterol
Cit	Citrate	en.wikipedia.org/wiki/Citrate
CO_2	Carbon dioxide	en.wikipedia.org/wiki/Carbon_dioxide
CTP	Cytidine triphosphate	en.wikipedia.org/wiki/Cytidine_triphosphate
DA-5-PP	Dimethylallyl-5-pyrophosphate	en.wikipedia.org/wiki/Dimethylallyl_pyrophosphate
DOXP	1-Deoxy-D-xylulose 5 phosphate	en.wikipedia.org/wiki/1-Deoxy-D-xylulose_5-phosphate
DHA-P	Dihydroxyacetone-phosphate	en.wikipedia.org/wiki/Dihydroxyacetone
Farnesyl-PP	Farnesyl pyrophosphate	en.wikipedia.org/wiki/Farnesyl_pyrophosphate
Fruc-1,6P	Fructose-1,6-bisphosphate	en.wikipedia.org/wiki/Fructose
Fruc-6P	Fructose-6-phosphate	en.wikipedia.org/wiki/Fructose_6-phosphate
Fum	Fumarate	en.wikipedia.org/wiki/Fumaric_acid
GA-3P	Glyceraldehyde 3-phosphate	en.wikipedia.org/wiki/Glyceraldehyde_3-phosphate
Geranyl-PP	Geranyl pyrophosphate	en.wikipedia.org/wiki/Geranyl_pyrophosphate
Gluc	Glucose (dextrose)	en.wikipedia.org/wiki/Glucose
Gluc-1P	Glucose-1-phosphate	en.wikipedia.org/wiki/Glucose_1-phosphate
Gluc-6P	Glucose-6-phosphate	en.wikipedia.org/wiki/Glucose-6-phosphate
Glutam	Glutamate	en.wikipedia.org/wiki/Glutamate
Gly	Glycogen	en.wikipedia.org/wiki/Glycogen

(continued)

Table 15.4 (continued)

(α)Ketoglutarate	Alpha-ketoglutarate	en.wikipedia.org/wiki/Alpha-ketoglutaric_acid
HMB-PP	(E)-4-Hydroxy-3-methylbut-2-enyl pyrophosphate	en.wikipedia.org/wiki/%28E%29-4-Hydroxy-3-methyl-but-2-enyl_pyrophosphate
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A	en.wikipedia.org/wiki/HMG-CoA
Isocit	Isocitrate	en.wikipedia.org/wiki/Isocitric_acid
Isopent-5-PP	Isopentenyl-5-pyrophosphate	en.wikipedia.org/wiki/Isopentenyl_pyrophosphate
Lact	Lactic acid	en.wikipedia.org/wiki/Lactic_acid
Lanost	Lanosterol	en.wikipedia.org/wiki/Lanosterol
Malate	Malic acid	en.wikipedia.org/wiki/Malate
Malonyl-CoA	Malonyl-CoA	en.wikipedia.org/wiki/Malonyl-CoA
MEP	2-C-Methylerythritol 4-phosphate	en.wikipedia.org/wiki/2-C-methylerythritol_4-phosphate
MEcPP	2-C-Methyl-D-erythritol-2,4-cyclopyrophosphate	en.wikipedia.org/wiki/2-C-methyl-D-erythritol_2,4-cyclopyrophosphate
Meval acid	Mevalonic acid	en.wikipedia.org/wiki/Mevalonic_acid
Meval-5-P	Phosphomevalonic acid	en.wikipedia.org/wiki/Phosphomevalonic_acid
Meval-5-PP	Mevalonate-5-pyrophosphate	en.wikipedia.org/wiki/Phosphomevalonic_acid
NADH	Nicotinamide adenine dinucleotide	en.wikipedia.org/wiki/NADH
NADPH	Nicotinamide adenine dinucleotide phosphate	en.wikipedia.org/wiki/NADPH
Oleate	Oleic acid	en.wikipedia.org/wiki/Oleic_acid
OAA	Oxaloacetic acid	en.wikipedia.org/wiki/Oxaloacetic_acid
Palmitate	Palmitatic acid	en.wikipedia.org/wiki/Palmitate
PEP	Phosphoenolpyruvate	en.wikipedia.org/wiki/Phosphoenolpyruvate
Pyruvate	Pyruvic acid	en.wikipedia.org/wiki/Pyruvic_acid
Ribose-5P	Ribose 5-phosphate	en.wikipedia.org/wiki/Ribose_5-phosphate
R-ulose-5P	Ribulose 5-phosphate	en.wikipedia.org/wiki/Ribulose_5-phosphate
Stearate	Stearic acid	en.wikipedia.org/wiki/Stearic_acid
Squal	Squalene	en.wikipedia.org/wiki/Squalene
Succin	Succinate	en.wikipedia.org/wiki/Succinate
Succinyl-CoA	Succinyl-CoA	en.wikipedia.org/wiki/Succinyl-CoA
UDP	Uridine diphosphate	en.wikipedia.org/wiki/Uridine_diphosphate
UDP-gluc	UDP-glucose	en.wikipedia.org/wiki/UDP-glucose
Xyl	Xylose	en.wikipedia.org/wiki/Xylose
Xylul	Xylulose	en.wikipedia.org/wiki/Xylulose
Xylulose-5P	Xylulose-5P	en.wikipedia.org/wiki/Xylulose-5-phosphate

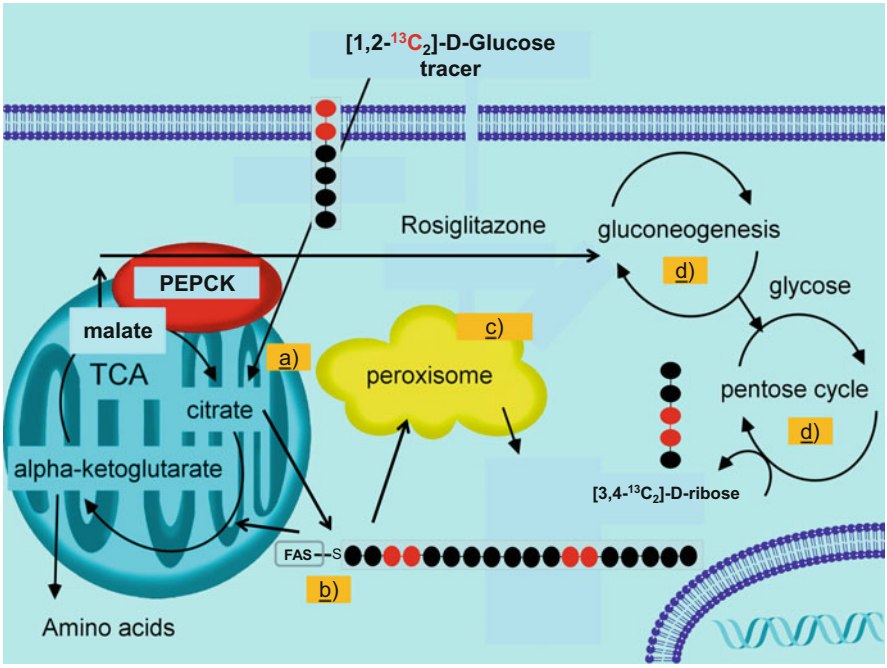

Fig. 15.4 Extracellular [1,2-$^{13}C_2$]-D-glucose tracer substrate entry and its metabolic hubs after rosiglitazone action in HepG2 cells. (**a**) Rosiglitazone increases lipogenic citrate labeling from [1,2-$^{13}C_2$]-D-glucose after increasing long-chain fatty acid degradation while routing, (**b**) newly labeled malonyl-CoA flux towards stearic acid synthesis/turnover, followed by (**c**) peroxisomal remodeling and intracellular fatty acid mobilization towards, (**d**) [3,4-$^{13}C_2$]-D-pentose and hexose labeling. Please note that acetyl-CoA can also yield [4,5-$^{13}C_2$]-D-pentose, based on labeled oxaloacetate recycling in the cycle

15.1 Materials and Methods

Two non-radiating stable isotope tracer carbon-labeled substrates were used in this metabolic flux analysis study: ***a)*** [1,2-$^{13}C_2$]-D-Glucose was purchased with >99 % purity and 99 % isotope enrichment for each position from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), and ***b)*** [U-$^{13}C_{18}$]-stearic acid (Spectra Stable Isotopes, Spectra Gases Inc., Branchburg, NJ). Rosiglitazone was provided by Pfizer, Inc., under a material transfer agreement.

15.1.1 Cells and Cell Culture

Human liver hepatocellular (epithelial) carcinoma HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). HepG2

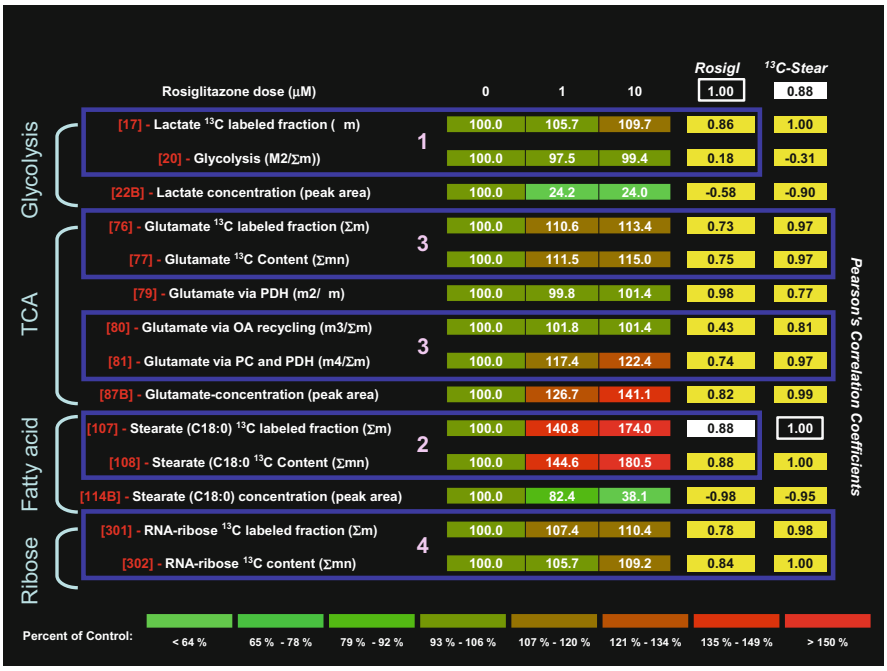

Fig. 15.5 EZTopolome (EZTop[1,2-$^{13}C_2$]-D-glucose) of in vitro grown HepG2 liver cells, which serves as the color-assisted visual isotopolome-wide matrix screening tool. There is maintained lactate labeling (shown in *blue box* 1—line 17 and glucose oxidation—please also see reference[1] SiDMAParray for glucose uptake/oxidation data with rosiglitazone dosing), with expected high and unaltered glycolysis (line 20). Importantly, the EZTop shows increased stearate labeling (shown in *blue box* 2—line 107, line 108) from glucose, which is responsive to rosiglitazone treatment, shown by the relatively high (0.88) positive correlation coefficient between rosiglitazone dosing and intracellular stearate ^{13}C-labeled fractions (and ^{13}C content). Glutamate's ^{13}C meta-labeling from the glucose tracer shows much improved correlation coefficients with that of cross-labeled stearate fractions as the independent explanatory variable (shown in *blue box* 3—lines 76, 77, 80, 81). Rosiglitazone, as expected, increases glucose-derived cross-labeled ^{13}C-stearate carbon labeling in RNA-derived ribose (shown in *blue box* 4—line 301, line 302), where correlation coefficients are close to one with cross-labeled stearate as the reference range. The [1,2-$^{13}C_2$]-D-glucose *EZ*Topolome returns correlation coefficients between rosiglitazone dosing (*Rosigl* column, *yellow*) or cross-^{13}C-labeled stearate (*^{13}C-Stear* column, *yellow*) as reference variables (*white boxes* = 1) and the rest of the [1,2-$^{13}C_2$]-D-glucose isotopolome

cells have an average doubling time of 34 h in DMEM with 10 % fetal bovine serum and 2.5 % horse serum (Gibco/BRL, Gaithersburg, MD) in the presence of antibiotics. The cells were incubated at 37 °C, 5 % CO_2, and 95 % humidity and passed by using trypsin 0.25 % (Gibco/BRL) no more than three times after receipt from the ATCC and prior to use in this study. HepG2 cells have previously been used in both in vitro and in vivo experiments and responded to treatment with troglitazone and other compounds with characteristic metabolic profile changes showing altered macromolecule synthesis and fatty acid cycling (Lee et al. 1997). Tracer-labeled

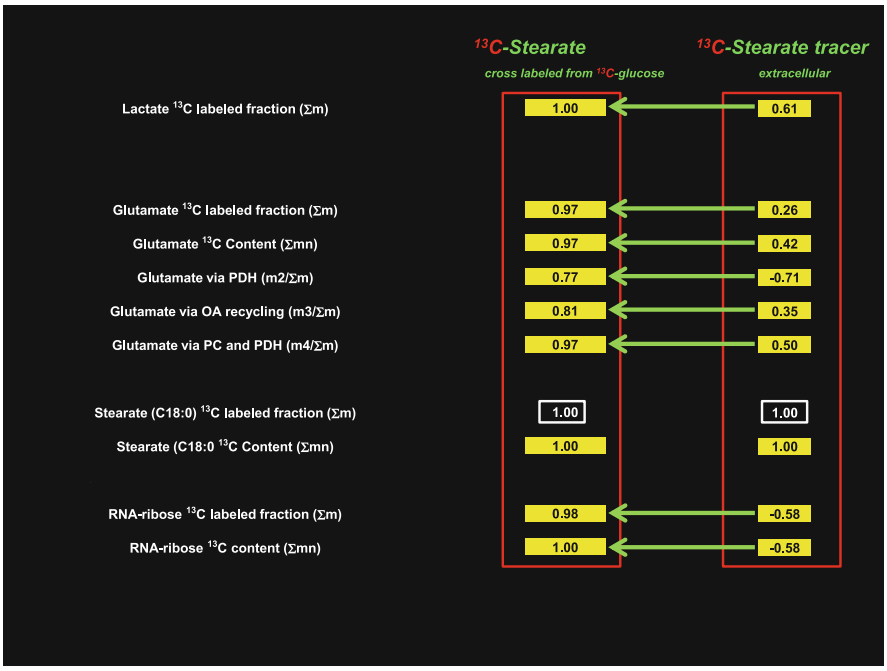

Fig. 15.6 Improvements in correlations between intracellular ^{13}C-labeled stearic acid fractions as the explanatory variable (shown in *white squares* with default correlation coefficient = 1), cross-labeled from the [1,2-$^{13}C_2$]-D-glucose tracer (column labeled as such in the *left red square*), in comparison with extracellular [U-$^{13}C_{18}$]-stearic acid (column labeled as such in the *right red square*) as the metabolic tracer. Correlation coefficients show the dependence (dependent or response variables) of selected multiple product isotopomers. We observed much improved correlations (shown by *green arrows*), i.e., closer associations with rosiglitazone's efficacy markers, reflecting this drug's central mechanism of stearate degrading action, from ^{13}C glucose-derived stearate in comparison with that of the extracellular stearate tracer

parallel cultures of HepG2 cells were used in this study as controls to compare vehicle (DMSO)-treated cell cultures to rosiglitazone treatment in an escalating regime of drug treatment.

Seventy-five percent confluent cultures of HepG2 cells were incubated in either [1,2-$^{13}C_2$]-D-glucose or [U-$^{13}C_{18}$]-stearate-containing media (100 mg/dl total glucose concentration = 5 mM; 50 % isotope enrichment; or 0.5 mM uniformly labeled stearate). Cells were plated at a density of 10^6 per T75 culture flask, and rosiglitazone was added in two concentrations, 1 μM or 10 μM dissolved in 10 % DMSO and 90 % culture media. Control cultures were treated with vehicle (10 % DMSO) only. The doses of rosiglitazone for the present study were selected based on in vitro experiments demonstrating that these drugs effectively control glucose levels via PPARγ activation in various human cell culture systems in the 1 μM to a 10 μM dose range [for review, see Otto et al. (2002)]. Drug treatments were carried out for 72 h in the presence of each tracer. Glucose and U-stearate ^{13}C tracer levels

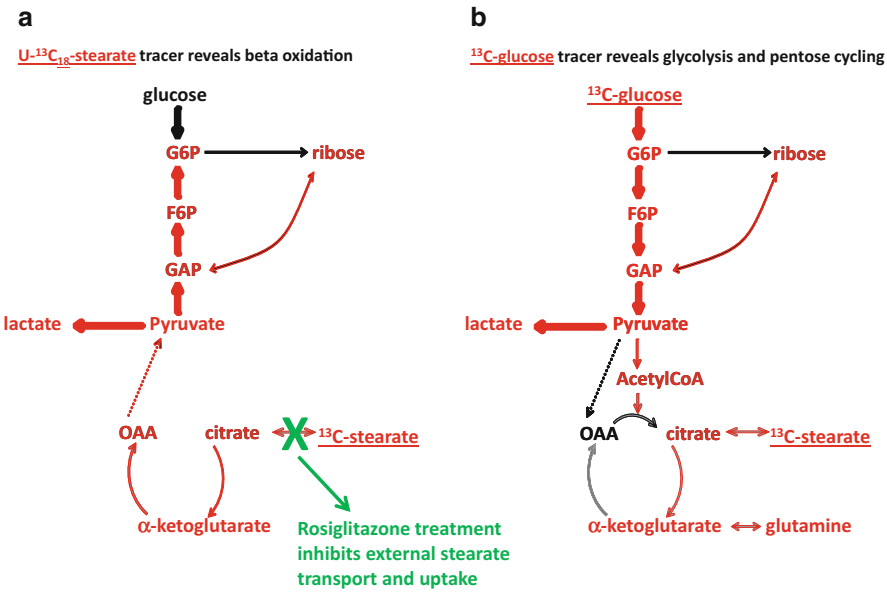

Fig. 15.7 Multiple ^{13}C-tracer substrates are used in metabolic profiling studies to reveal diverse metabolic phenotypes of tumor cells, where (**a**) uniformly ^{13}C-labeled stearic acid is used to study beta-oxidation and gluconeogenesis, ketogenic citrate labeling, and its recycling in the cytosol towards ribose labeling (*red arrows*) via mitochondria, while (**b**) ^{13}C-glucose is used to reveal the Warburg effect, glycolysis, pentose cycling and de novo (new) fatty acid synthesis in the cytoplasm. The *green cross* and *arrow* show limited stearate uptake after thiazolidinedione treatment, which is a limiting factor in multiple tracer studies by drug- and dose-related changes in tracer uptake (*G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *GAP* glyceraldehyde-3-phosphate, *OAA* oxaloacetate)

in the medium were monitored using a Cobas Mira chemistry analyzer (Roche Diagnostics, Pleasanton, CA, USA). Fresh, tracer-enriched culture medium and drug treatments were applied every 24 h, three times during the experiment, in order to provide for the cells with steady tracer-enriched medium and drug doses throughout the 72 h experimental period. After 72 h the cells were scraped, spun, and frozen at −80 °C until further processing as described below.

15.2 RNA Ribose Stable Isotope Studies

For mass isotopomer analysis RNA ribose was isolated by acid hydrolysis of cellular RNA after Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) extraction from the cell pellets. Ribose was derivatized to its aldonitrile acetate form using hydroxylamine in pyridine with acetic anhydride (Supelco, Bellefonte,

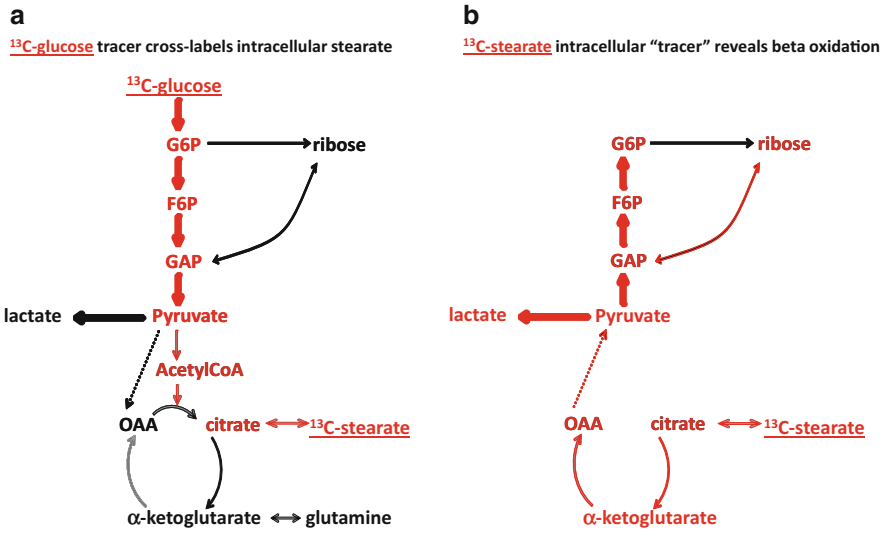

Fig. 15.8 Single [1,2-$^{13}C_2$]-D-glucose fate association study reveals diverse metabolic phenotypes of cultured tumor cells, where (**a**) [1,2-$^{13}C_2$]-D-glucose associations are used to reveal the Warburg effect, glycolysis, and pentose cycling in the cytoplasm, while (**b**) ^{13}C-stearate associations, cross-labeled from the glucose tracer, are used to study fatty acid oxidation and back labeling of products on specific carbon positions, lipogenic citrate synthesis, and its shuttling towards ketoglutarate(α) (*red arrows*) in mitochondria. Please note that cross-labeled stearate also labels lactate via gluconeogenesis and RNA ribose via the non-oxidative branch of the pentose cycle. There is no thiazolidinedione-related limiting action on internal stearate labeling from glucose (*G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *GAP* glyceraldehyde-3-phosphate, *OAA* oxaloacetate)

PA, USA) before mass spectral analyses. We monitored the ion cluster around the *m*/*z* 256 (carbons 1–5 of ribose) (chemical ionization, CI) and *m*/*z* 217 (carbons 3–5 of ribose) and *m*/*z* 242 (carbons 1–4 of ribose) (electron impact ionization, EI) to determine molar enrichment and the positional distribution of ^{13}C in ribose. By convention, the base mass of ^{12}C compounds (with their derivatization agents) is given as m_0 as measured by mass spectrometry as described elsewhere (Katz et al. 1993). Ribose molecules labeled with a single ^{13}C atom on the first carbon position (m_1) recovered from RNA were used to gauge the ribose fraction produced by direct oxidation of glucose through the G6PD pathway. Ribose molecules labeled with ^{13}C on the first two carbon positions (m_2) were used to measure the fraction produced by transketolase. Doubly labeled ribose molecules (m_2 and m_4) on the fourth and fifth carbon positions were used to measure molar fraction produced by triosephosphate isomerase and transketolase (Boros et al. 2002). Because transketolase has the highest metabolic control coefficient in the non-oxidative branch of the pentose cycle (Sabate et al. 1995), we use the term *transketolase* throughout the paper. It should be noted, though, that transketolase and transaldolase, besides other enzymes, all are participants in non-oxidative pentose cycle metabolism in human cells.

15.2.1 Lactate

Lactate from the cell culture media (0.2 ml) was extracted by ethylene chloride after acidification with HCl. Lactate was derivatized to its propylamine-heptafluorobutyrate ester form, and the *m/z* 328 (carbons 1–3 of lactate) (chemical ionization, CI) was monitored for the detection of m_1 (recycled lactate through the PC) and m_2 (lactate produced by the Embden-Meyerhof-Parnas pathway) for the estimation of pentose cycle activity (Lee et al. 1998b). In this study we recorded the m_1/m_2 ratios in lactate produced and released by HepG2 cells in order to determine pentose cycle activity and pentose cycling versus anaerobic glycolysis in response to 1 μM and 10 μM rosiglitazone treatments.

15.2.2 Glutamate

^{13}C-label distribution in glutamate (glutamic acid), which is synthesized from α-ketoglutaric acid of the TCA cycle, from tracer-labeled glucose or stearate is suitable for determining mitochondrial glucose and fatty acid disposal via anabolic substrate use within the cycle, also known as anaplerotic flux. For glutamate extraction tissue culture medium (1 ml) was first treated with 1 ml of 6 % perchloric acid, and the supernatant was passed through a 3 cm^3 Dowex-50 (H+) column. Amino acids were eluted with 15 ml 2 N ammonium hydroxide. In order to separate glutamate from glutamine, the amino acid mixture was passed through a 3 cm^3 Dowex-1 (acetate) column and then collected with 15 ml 0.5 N acetic acid. The glutamate fraction from the culture medium was converted to its trifluoroacetyl butyl ester (TAB) (Leimer et al. 1977). Under EI conditions, ionization of TAB-glutamate produces two fragments, *m/z* 198 and *m/z* 152, corresponding to C2–C5 and C2–C4 of glutamate. Glutamate labeled on the 4–5 carbon positions indicates pyruvate dehydrogenase activity, while glutamate labeled on the 2–3 carbon positions indicates pyruvate carboxylase activity for the entry of glucose carbons to the TCA cycle. TCA cycle anabolic glucose utilization is calculated based on the m_1/m_2 ratios of glutamate (Lee et al. 1996).

15.3 Lipid Extraction and Analysis

Lipid extractions were performed using methods described elsewhere (Lowenstein et al. 1975). In brief, the Trizol extracts (bottom layer) of cell pellets after RNA extraction were saponified with 500 μl of 30 % KOH-ethanol (1:1 v/v) at 70 °C overnight. The aqueous phase was acidified, and fatty acids were extracted in petroleum ether and dried under a stream of nitrogen. Fatty acids were methylated with 0.5 ml 0.5 N HCl in methanol for GC/MS analysis. Palmitate, stearate, and

oleate were monitored at *m/z* 270, *m/z* 298, and *m/z* 264, respectively, with the enrichment of ^{13}C-labeled acetyl units either from tracer-labeled glucose or stearate, which reflect synthesis, elongation, and desaturation of the new lipid fraction as determined by mass isotopomer distribution analysis (MIDA) of different isotopomers (Lee et al. 1991). Mass isotopomer distribution was determined using the method of Lee et al., which corrects for the contribution of derivatizing agent and ^{13}C natural abundance to the mass isotopomer distribution of the compound of interest. The calculated mass isotopomer distribution is expressed as molar fractions (m_0, m_1, m_2, m_3, etc.), which are the fractions of molecules containing 0, 1, 2, 3, etc. ^{13}C substitutions, respectively. Data reduction and regression analyses were performed using the computer software Microsoft Excel® version 5.0.

Determination of Precursor Enrichment β-oxidation of [U-$^{13}C_{18}$]-stearate generates [1,2-$^{13}C_2$]acetyl-CoA, which is subsequently used as a precursor for de novo lipogenesis, chain elongation, energy production, and anaplerosis via the tricarboxylic acid cycle. Thus, β-oxidation can be assessed through the determination of [1,2-$^{13}C_2$]acetyl-CoA enrichment in the different synthetic products.

Acetyl-CoA Enrichment for De Novo Synthesis from Glucose The enrichment of the cytosolic acetyl-CoA pool used in de novo fatty acid synthesis was determined from the mass isotopomer distribution in palmitate and stearate using the [1,2-$^{13}C_2$]-D-glucose as the tracer. De novo synthesis produces these long-chain saturated fatty acids with 2, 4, or 6 ^{13}C atoms (m_2, m_4, and m_6). The distribution of these mass isotopomers has been previously shown to be a binomial distribution (Lee et al. 1992; Hellerstein 1991). Thus, the acetyl-CoA enrichment may be obtained from the consecutive mass isotopomer ratio $m_4/m_2 = (n-1)p/q = 3.5p/q$, where n is the number of acetyl units in palmitate = 8, p is the enrichment of [1,2-$^{13}C_2$]acetyl-CoA, q is the unenriched acetyl-CoA, and $p + q = 1$.

Acetyl-CoA Enrichment from Mitochondrial Oxidation: [1,2-$^{13}C_2$]acetyl-CoA produced either from [1,2-$^{13}C_2$]-D-glucose or [U-$^{13}C_{18}$]-stearate from β-oxidation combines with oxaloacetic acid and forms citric acid in the mitochondria. Citrate participates in the tricarboxylic acid cycle, eventually forming [α-4,5-$^{13}C_2$] ketoglutarate and glutamate (Haber et al. 2001). The enrichment of the m_2 component of the C2–C5 fragment generally reflects tricarboxylic acid cycle activity. However, it is well known that contribution from unlabeled glutamate from the cells in culture with glutamine-containing medium can substantially dilute the glutamate enrichment (Darmaun et al. 1988). Therefore, the enrichment of the [4,5-$^{13}C_2$]glutamate reflects the lower limit of mitochondrial acetyl-CoA cycling and anaplerosis.

Acetyl-CoA Enrichment for Chain Elongation Enzymes involved in chain elongation from [U-$^{13}C_{18}$]-stearate to longer-chain fatty acids are located in the endoplasmic reticulum, mitochondria, and peroxisomes (Vance et al. 2002). Chain elongation of uniformly labeled stearate with [1,2-$^{13}C_2$]acetyl-CoA produces characteristic m+18 clusters that can be used to determine the acetyl-CoA enrichment

according to the rules of combination of two labeled precursors. The ratio of m+2 to m+0 acetyl-CoA for chain elongation is given by the ratio of m+20 to m+18 in arachidate using the [U-$^{13}C_{18}$]-stearate tracer. The enrichment of [1,2-$^{13}C_2$]acetyl-CoA for each step of chain elongation was determined using the method described elsewhere using HepG2 cells as the in vitro model of fatty acid metabolism in the presence of [U-$^{13}C_{18}$]-stearic acid (Wong et al. 2004).

15.4 Gas Chromatography/Mass Spectrometry (GC/MS)

Mass spectral data were obtained on the HP5973 mass-selective detector connected to an HP6890 gas chromatograph. The settings were as follows: GC inlet 250 °C, transfer line 280 °C, MS source 230 °C, and MS Quad 150 °C. An HP-5 capillary column (30 m length, 250 μm diameter, 0.25 μm film thickness) was used for glucose, ribose, deoxyribose, glutamate, and lactate analyses. Alternatively, a Bpx70 column (25 m length, 220 μm diameter, 0.25 μm film thickness, SGE Incorporated, Austin, TX) was used for fatty acid analysis with specific temperature programming for myristate (C:14), palmitate (C:16), stearate (C:18), oleate (C:18-1), as well as C:20, C:22, and C:24 fatty acid analyses.

15.5 Data Analysis and Statistical Methods

Each experiment was carried out using triplicate cell cultures for each condition within each experiment, and experiments were repeated once. Mass spectroscopic analyses were carried out by three independent automatic injections of 1 μl samples by the automatic sampler and accepted only if the standard sample deviation was less than 1 % of the normalized peak intensity. Statistical analysis was performed using the Student's t-test for unpaired samples. With two-tailed significance at the 99 % confidence interval ($\mu \pm 2.58\sigma$), $P < 0.01$ indicated significant differences in glucose carbon metabolism in control and rosiglitazone-treated HepG2 cells. SIDMAParray™ was produced by MeTa-Informatics (San Diego, CA). Please see additional method references in doi: 10.1007/s11306-006-0015-5.

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Conflict of Interest The authors declare no conflict of financial interest.

Disclaimer The views presented in this article do not necessarily reflect those of the Food and Drug Administration (FDA) of the United States of America.

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Erratum to Chapter 2: Tumor Cell Complexity and Metabolic Flexibility in Tumorigenesis and Metastasis

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In a previous version of this chapter an incorrect figure was shown for Fig. 2.1. The correct Fig. 2.1 is shown below. The Publisher would like to apologize for this mistake. The original version of the chapter has been corrected accordingly online and in print (see DOI 10.1007/978-3-7091-1824-5_2).

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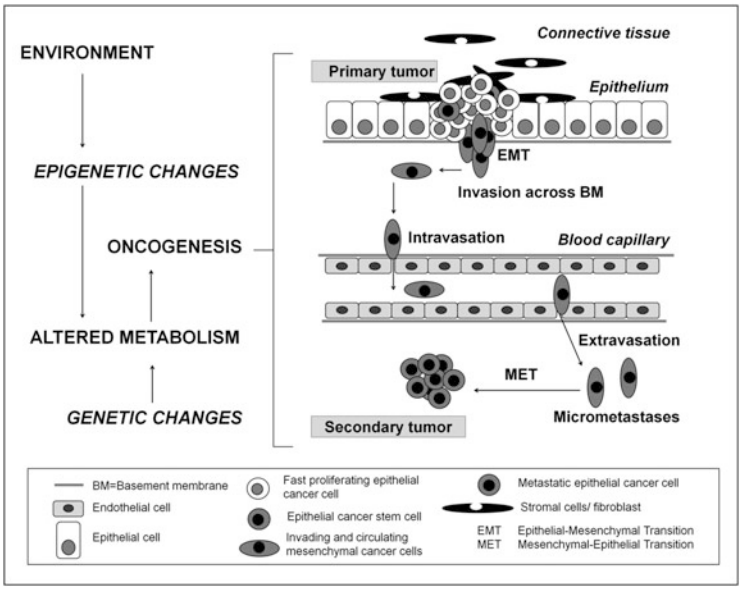

Fig. 2.1 Overview of oncogenesis of epithelial tumors. Oncogenesis takes place in context of the tumor microenvironment that includes oxygen tension, pH, nutrient supply, and interactions with other cell types in close proximity of the tumor, such as stromal cells and immune cells. Initiation begins with oncogenic mutations or epigenetic changes in expression of tumor suppressor genes and oncogenes of an epithelial cell, leading to alterations in cancer cell metabolism. Altered metabolism drives the establishment of a primary epithelial tumor which is initially contained within its tissue of origin. Some cancer stem cells undergo EMT, which makes them less sticky and more aggressive, breaking the basal membrane barrier and invading underlying tissues. Some of these mesenchymal tumor cells enter blood vessels (intravasation) and travel in the blood stream as circulating tumor cells. Once they leave the blood stream (extravasation), they form micrometastases in new tissues and organs. Once they undergo MET, they grow into macrometastases or secondary tumors of epithelial origin