Cancer Drug Discovery and Development

Steven Kanner *Editor*

# Tumor Metabolome Targeting and Drug Development

Humana Press

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# Chapter 1
# Tumor Cell Metabolic Reprogramming and Drug Targeting

**Steven B. Kanner**

**Abstract** The metabolic network in cells promotes the generation of both energy and biomass needed for them to grow, divide, and differentiate. However, tumor cells exhibit significant differences from normal cells in their metabolism. Starting in the 1920s, Otto Warburg observed that tumors utilize glucose aerobically, leading predominantly to lactate generation at the expense of energy production, a phenomenon now known as the "Warburg effect." Since then, the scientific community has confirmed and extended these observations. Together, this knowledge has prompted the drive toward novel approaches in the discovery of therapeutic agents targeting the reprogrammed metabolism of tumor cells by focusing on critical vulnerabilities in the metabolome. In this volume, the interplay between the cellular metabolic network, oncogenes, tumor suppressors, hypoxia, autophagy, and biosynthetic anabolism is discussed, and former and emerging approaches to drug development in targeting cancer cell metabolism are explored.

**Keywords** Cancer · Metabolism · Drug discovery · Autophagy · Hypoxia · Warburg effect · Glycolysis · Tumor

An early concept based on observations that tumor cells exhibit significant differences from normal cells in their metabolism later became known as the "Warburg effect." In Otto Warburg's original studies, he observed that tumors utilize glucose aerobically, consuming this carbohydrate with an alternate outcome as compared to untransformed cells, prompting the production of lactate at the expense of energy production [1, 2]. Since then, the scientific community has demonstrated that not only is this confirmed and better understood, but that cancer cells also have other addictions, including one to glutamine for anaplerosis. Overall, evaluation of the metabolism of tumor cells has prompted a significant understanding of the interplay between cell metabolism, oncogenes, tumor suppressors, hypoxia, and biosynthetic anabolism [3–8]. This enhanced perception has helped to drive novel approaches in the discovery of therapeutic agents addressing the reprogrammed metabolism of tumor cells by targeting critical vulnerabilities in the metabolome.

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In this book, the chapter authors address different aspects of the altered metabolism of tumor cells, including (1) changes in adenosine triphosphate (ATP) production and consumption that modulate the ATP to ADP ratio, (2) hypoxia and the effects of reactive oxygen species (ROS) on glycolysis, (3) regulation of mitochondrial respiration, (4) induction and suppression of autophagy and the "reverse Warburg effect," (5) the identification of cancer targets within the metabolic network, and (6) specific emerging drugs disrupting cancer cell metabolism that will add to the armamentarium of therapies for various malignancies. Together, these chapters not only illustrate the complexities surrounding the remodeled metabolic status in the cancer cell but also elaborate on how not all cancer cells exhibit the same metabolic changes, and that targeting them through these differences compared to normal cells presents their own set of challenges.

Bingham, Stuart, and Zachar discuss the interplay between the 'matter/energy metabolism' of cancer cells and associated redox regulation. They review the observations of increased ROS in tumors and their influence on signal transduction, particularly mitochondrial redox signaling, and how this may be repurposing rather than resulting from the matter/energy metabolic changes that are associated with the transformed phenotype. They propose that redox regulation plays a central role in tumor cell metabolism and suggest alternate approaches to discovering novel therapeutics.

In their chapter, Serafim and Oliveira review regulation of mitochondrial respiration in the malignant state. Of interest, there are many molecular alterations that occur in tumor cells and their mitochondria, including activation of oncogenes, changes in gene expression, and mutations that lead to alterations in the respiratory chain. Together, these modulations promote altered mechanisms for the generation of ATP, and ultimately differentiate tumor cells from their normal counterparts leaving them potentially vulnerable to drug targeting. The authors provide a detailed review of the metabolic remodeling that occurs in mitochondria during cancer, they elaborate on drugs that target various elements in mitochondria as antitumor agents, and they provide a platform for the potential discovery of novel therapeutics that focus on various aspects of mitochondrial respiration.

In their review, Pulkoski-Gross, Evensen, and Cao describe hypoxia in its different presentations in tissues, including acute hypoxia, chronic hypoxia, tumor hypoxia, hypoxemic hypoxia, and other related conditions. The hypoxic microenvironment of tumors is complex, and the author's treatise provides an understanding of how the fundamental properties of the different conditions may support or impact the tumor. The hypoxic phenomenon influencing many solid tumors may relate to their resistance to anticancer treatments and impair the clinical outcome of the patient. The authors discuss hypoxia in the context of cancer cell metabolism and the role of targeting hypoxia or its environmental influences as novel approaches to antitumor therapy.

Bruchelt, Handgretinger, Weckenmann, and Hahn discuss the antioxidative defense system observed in tumor cells, and how anticancer drugs that target the oxidative pathways may be a viable option for future endeavors. Production of ROS occurs mainly in mitochondria and may promote cell proliferation, but multiple

mechanisms exist that protect cells from ROS-induced damage when levels are elevated. One example includes the nicotinamide adenine dinucleotide phosphate (NADPH)-associated pathways that drive peroxide detoxification. In addition, the microenvironment of the tumor, whether pro- or antioxidative, may influence the survival of individual cancer cells, further establishing the heterogeneity associated with the oxidative state in malignant cells. The authors discuss potential targeting of elements within the interplay between ROS and metabolic pathways and review targets for ROS-based therapies that could lead to refined antitumor agents in the future.

In their chapter on *Modulating Autophagy and the "Reverse Warburg Effect,"* Vaccaro, Gonzalez, Alvarez, and Ropolo review autophagy, a set of distinct cellular processes that are activated upon nutrient stress enabling cells to derive sustenance from their own organelles. Autophagy is highly regulated and may be induced or suppressed in tumor cells depending on environmental conditions or exposure to different drugs. Autophagy may be activated in epithelial cancer cells undergoing the Warburg effect within a tumor, but the "reverse Warburg effect" is induced when tumor stromal cells undergo autophagy, influenced by the cancer cell/stromal microenvironment. The authors discuss different molecular mechanisms involved in autophagic regulation, various drugs that modulate autophagy, and the interplay between autophagy and tumor cell metabolism.

In discussing *Metabolic Adaptation in Reprogrammed Cancer Cells*, Marks and Kung review genetic lesions in metabolic pathway components that lead to cancer cell vulnerabilities enabling unique targeting alternatives. For example, they describe the observed mutations in the isocitrate dehydrogenase (IDH) enzymes involved in production of α-ketoglutarate (α-KG). Mutant forms of the enzyme convert α-KG to 2-hydroxy-glutarate, which has functional consequences including epigenetic modifications that may contribute to the transformed state. Recently, investigators have demonstrated that an inhibitor of this mutant enzyme exhibits antitumor activity [9]. The authors also review loss-of-function mutations in fumarate hydratase and succinate dehydrogenase that occur in certain tumor types and also extensively discuss another metabolic enzyme that has been implicated in tumorigenesis, the muscle 2 isoform of pyruvate kinase (PKM2). Interestingly, the expression of this isoform is tightly associated with the increased levels of lactate that Warburg had observed. Evidence has been provided that either inhibition or activation of PKM2 may lead to reduced tumorigenesis, leading investigators to generate small molecule activators of PKM2 that exhibit antitumor activity in xenograft tumor models [10, 11]. Importantly, PKM2 is regulated by the nonessential amino acid serine and other factors, so further work will be needed to determine how to use putative drugs targeting PKM2 among the many different cancer types.

Färnegårdh, Shoshan, and Ährlund-Richter review the known biology of the bifunctional kinase/phosphatase enzyme family known as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB). Although four family members have been described, the enzyme best studied in relation to cancer cell metabolism is PFKFB3. This family member plays a key role in glycolysis by generating glycolytic intermediates, and the authors describe small molecule inhibitors of its kinase activity and how they may be used in combination with other targeted agents to reduce tumor cell growth.

Together, the observations reviewed in this book summarize many of the influences and outcomes of metabolic reprogramming that occur in malignant cells. Changes within the glycolytic/glutaminolytic pathways and the tricarboxylic acid (TCA) cycle underscore the plasticity evident within the metabolic network that enables cancer cells to adapt to varying microenvironmental cues, including hypoxia, nutrient deprivation, stromal factors and metabolites. One of the key opportunities of the metabolic alterations that occur in malignancies is that they provide a platform for therapeutic targeting [12–17]. Although by themselves these vulnerabilities may not represent major survival points for all tumors, or even all the malignant cells within a tumor, targeting these metabolic components together with more standard chemotherapies or newer drugs that modulate oncogenes or other genetic drivers may provide a means to counter resistance mechanisms [12–17]. Additional challenges remain, including which drug combinations are most appropriate, which pathways will affect cancer stem cells that continually drive tumor growth, and which patients should be selected for a given drug regimen. Genetic changes in the cancer cell metabolic network evident in some tumor types may provide a basis for identifying patients with an increased likelihood of benefitting from specific therapeutic targeting.

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# Chapter 2
# Cancer Metabolism: A Nexus of Matter, Energy, and Reactive Oxygen Species

**Paul M. Bingham, Shawn D. Stuart and Zuzana Zachar**

**Abstract** The last decade has witnessed the accumulation of a formidable body of evidence that matter/energy metabolism in tumor cells is quite distinct from that of most normal cells. At the same time, diverse data indicate that the redox metabolism (including generation of reactive oxygen species; ROS) of cancer cells is also strikingly distinct from most normal cells. Both of these insight streams have been well reviewed recently. Our ultimate objective is to explore the current state of our knowledge about the *relationship between* these two areas of metabolism. We will focus on the important working hypothesis that these metabolic reconfigurations might represent two facets of a single underlying redesign of all metabolism in cancer. It is widely recognized that *either* matter/energy metabolism *or* redox metabolism in tumors may contain effective therapeutic targets; the nexus *between* them may hold even greater clinical promise.

**Keywords** Tumor metabolism · Redox metabolism · Glycolysis · TCA cycle · Pyruvate dehydrogenase · Alpha-ketoglutarate dehydrogenase · Glyceraldehyde-phosphate dehydrogenase · Phosphoglycerate dehydrogenase · Pyruvate kinase · Hydrogen peroxide

## 2.1 Introduction

Oxidation/reduction (redox) reactions are central to both the extraction of energy from reduced hydrocarbon food molecules (catabolism) and the remodeling of these molecules for use as building blocks for biological structures (anabolism). Many of these same redox processes also inevitably produce a low level of secondary products

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resulting from the unavoidable donation of reducing potential to molecular oxygen to create reactive oxygen species (ROS; especially, superoxide and its dismutation product, hydrogen peroxide). The resulting ROS react with biomolecules in diverse ways, especially by the oxidation of specific, reactive cysteine sulfhydryls in proteins. These fundamental thermodynamic relationships have led to the view that ROS from core metabolism should be regarded simply as inevitable by-products, entities to be managed to avoid potentially catastrophic damage to the cell. However, it is clear that ROS are also sometimes produced deliberately for purposes of signaling, the transmission of information from one functional entity to others. Moreover, the targets of these reactions are often designed such that redox modification alters biological function in adaptive ways with presumptive regulatory function.

These considerations suggest the possibility that the inevitable production of ROS by redox metabolism might be modulated by adaptive design in catalysts and targets to generate a purposeful signal stream carrying information outward from key redox metabolic reactions to other elements of metabolism and cellular machinery. Indeed, in view of the properties of ROS as information-bearing molecules, such signals might be uniquely useful in the control and *integration* of metabolism, reflecting potentially powerful refinement of the metabolic regulation mediated by more well-characterized processes such as allosteric and second messenger modulation. Indeed, in view of the opportunity to create multiple reactive sulfhydryls with distinct reactivities, ROS signals can potentially have exceptionally subtle effects, *activating* an enzymatic activity at one signal intensity, *inactivating* that same activity at another signal amplitude, for example.

Such hypothetical relationships between matter/energy and redox metabolism are of particular interest to the cancer problem because current evidence indicates that both are profoundly altered in tumor cells. The hypothesis that these two domains are functionally integrated offers the opportunity for extensive new insight, including potentially substantive new approaches to therapeutic attack on cancer.

Our goal here is to explore selected elements of evidence that support the hypothesis for functional integration of matter/energy and redox metabolism in cancer cells. We will briefly review relevant elements of each area of metabolism, then explore their potential interrelationship. Though our understanding of this potential relationship remains extremely incomplete, we know enough to begin to explore its implications.

## 2.2 Some ROS are Uniquely Useful Informatonal Molecules in Metazoan Metabolism

Eukaryotic cells have a special coordination problem. On the one hand, these cells make use of intracellular membranes to delimit compartments with different functions. This has the highly beneficial effect of allowing distinct, specialized (even incompatible) processes to proceed efficiently and at high local reactant concentration in different compartments.

On the other hand, this very functional segregation creates a special problem of coordination between distinct compartments. For example, even small metabolic intermediates are generally too large and hydrophilic to efficiently cross lipid membranes. Moreover, introduction of transporters supporting inter-compartment equilibration of metabolites would often defeat one of the central purposes of compartment organization, selective concentration of reactants. Further, exchange of selected macromolecular surrogates is often likely to be too expensive and cumbersome when real-time, dynamic information flow on the scale of seconds is required (as for matter/energy metabolism, for example).

Thus, eukaryotic cells may need to arrange for real-time information to flow between membrane-bound compartments in the form of proxies that can rapidly cross lipid membranes. While this problem has some relevance to all compartments (nuclei, Golgi/ER, and endosomes, for example), it is convenient and particularly relevant here to conceptualize solutions to this challenge using the mitochondrial compartment where highly dynamic metabolic processes are central. Mitochondria usually import adenosine diphosphate (ADP) and phosphate from the cytosol and export adenosine triphosphate (ATP; [22]). They likewise use their inner membrane potential to help manage cytosolic calcium levels [69].

Conceivably, these two exchanges (ATP, $Ca^{++}$) are sufficient to mutually inform the mitochondrial matrix and cytosol about their common metabolic goals (coordinating cytosolic glycolysis and matrix tricarboxylic acid (TCA) cycle functions, for example). However, there is sound reason to doubt their sufficiency. ATP and $Ca^{++}$ levels are each a readout at the apex of a complex, anastomosing set of metabolic pathways. For example, both glycolysis and the TCA cycle also branch to fulfill anabolic functions beyond their role in ultimately burning reduced hydrocarbons to water and carbon dioxide to maintain ATP levels and mitochondrial membrane potential.

It is reasonable to anticipate that other informational channels between mitochondria and cytosol are required. Hydrogen peroxide ($H_2O_2$) is particularly promising in this informational role. $H_2O_2$ is generated by several processes in mitochondria (below). Thus, $H_2O_2$ production potentially carries substantial information about the moment-to-moment flux through various steps in mitochondrial metabolism.

Moreover, this water-like molecule is expected to be able to cross biological membranes at an appreciable rate by simple diffusion. In addition, $H_2O_2$ is likely to move efficiently through widely present water channels (aquaporins; reviewed in [57]) and other channels that discriminate against other, larger metabolic intermediates (the mitochondrial VDAC channel, for example, [16]).

Consistent with the importance of $H_2O_2$ as an inter-compartment information carrier, a number of enzymes involved in both cytosolic and mitochondrial matter/energy metabolism are regulated by redox modification potentially produced by $H_2O_2$ (below; Fig. 2.1).

Also consistent with this perspective, there is strong circumstantial evidence that the mitochondrial production of $H_2O_2$ is not merely a passive thermodynamic inevitability, but may be adaptively modulated to maximize the usefulness of the information conveyed by $H_2O_2$ production (below).

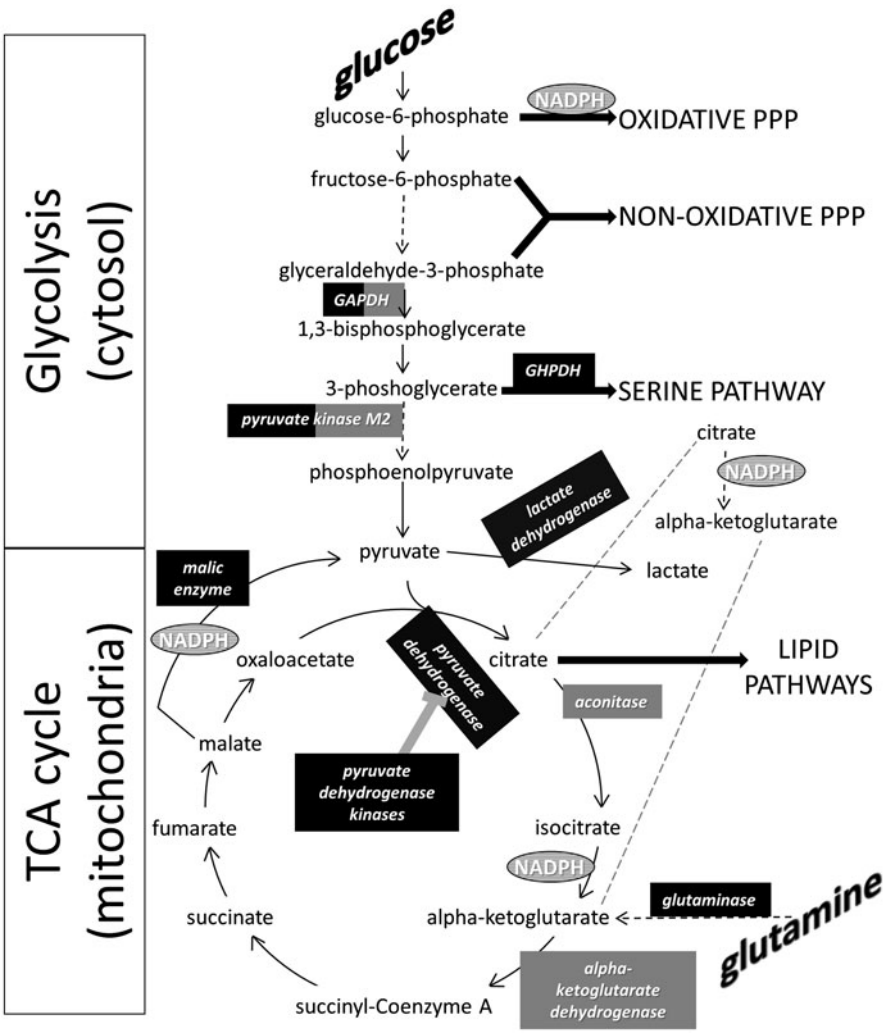


**Fig. 2.1** Flow of glucose and glutamine carbon through core metabolic pathways. The oxygen-independent, cytosolic glycolytic pathway and the oxygen-dependent, mitochondrial tricarboxylic acid (TCA) cycle are shown. Enzymes catalyzing selected steps are boxed in italics. [GAPDH is glyceraldehyde-3-phosphate dehydrogenase; GHPDH is 3-glycerophosphate dehydrogenase (text).] Selected enzymes whose levels or activities are *differentially regulated* in many cancers are blocked in *black* and enzymes subject to *redox regulation* are blocked in *gray*. *Solid line arrows* indicate single metabolic steps, while *dotted line arrows* indicate multiple steps. Several anabolic outputs are indicated in *capital letters* following block arrows. Major sources of nicotinamide adenine dinucleotide phosphate (NADPH) (*gray ellipses*) for anabolism and extinction of redox signals are indicated. Note that citrate (or isocitrate) can be exported from the mitochondrion to be oxidized to alpha-ketoglutarate (by isocitrate dehydrogenase 1; IDH1) providing cytosolic NADPH; one of two mitochondrial isocitrate dehydrogenase isoforms (IDH2) catalyzes this same reaction

Finally, these problems of coordination between subcellular compartments are analogous to a problem at the next larger organizational level faced by multicellular animals. Specifically, individual cells in such animals are units of ontogeny (birth, differentiation, and death), but they are frequently not units of metabolic function. More commonly, sets of cells must coordinate their metabolism, sometimes even actively exchanging metabolites in response to changing demand (see, for example, [14]). There is significant evidence that $H_2O_2$'s membrane transiting capabilities are also used for coordination at this scale. Though it is beyond the scope of this review, intercellular transmission and propagation of redox signals may also have important implications for this organizational scale as well as at the intracellular level where we will focus our attention (below).

## 2.3 Matter and Energy Metabolism in Cancer

Warburg and colleagues [86] observed remarkably large differences between the matter/energy metabolism of tumors and of normal tissues in intact animals. Though small groups of investigators continued to pursue the implications of this observation (see, for example, [5]), the potential implications of this work were largely overshadowed by the power of the analysis of the macromolecules involved in malignancy for decades. However, beginning about 10 years ago, cancer metabolism has received widespread interest. Over the last 5 years in particular, focus on cancer metabolism has grown exponentially.

Insights from this work have been wide ranging and diverse and have been extensively reviewed recently [11, 12, 28, 32, 52, 70, 81]. However, we emphasize that there is significant evidence that a complete picture of tumor metabolism will require the broadening of our current perspective to include both the tumor cell and its metabolic symbiosis with the stromal cells (including fibroblasts) making up a large portion of the tumor mass [13]. Though we expect more realistic *in vivo* perspectives to further revolutionize our understanding of tumor metabolism, our focus here will remain narrowly on what has been learned about the matter/energy metabolism of isolated tumor cells.

We begin by summarizing a few of the key aspects of work to date and to focus attention on several specific details that will help us explore the interface between matter/energy metabolism of cancers and their redox metabolism. Among the insights from this large body of work is that cancer cell metabolism shares some properties with rapidly growing normal cells (see, for example, [2]); however, there is also evidence that therapeutic attack on cancer cell metabolism can sometimes be achieved without unacceptable effects on normal cell metabolism. [We note that we will not comment on mutations in metabolic genes, such as the two isocitrate dehydrogenases, that apparently exert their cancer-producing effects secondarily through modification of epigenetic programming (reviewed in [92])].

Warburg's most fundamental and enlightening observation was the capacity of tumors in intact animals to withdraw much more glucose from and discharge much

more lactate into the blood flowing through the tumor-containing tissue than was the case for normal organs. This effect is extremely large (orders of magnitude; [86]). Though our current understanding of the teleological implications of this observation has greatly increased, it is important to recognize that our picture is still far from complete.

At present, what we believe we know is that the Warburg effect is one manifestation of a broader reconfiguration of matter/energy metabolism, whose adaptive goals include the shifting of more incoming resources to anabolic (cell/tissue-building) functions. These functions are central to growing tumor tissue and strongly attenuated in differentiated normal cells.

Among the features of this reorganization that are currently believed to be understood are as follows. We focus on specific cases that are both well documented and where there is evidence that the altered metabolic flows are important to the survival and/or transformed nature of the tumor cell. We begin with cytosolic matter/energy metabolism and follow with mitochondrial metabolism.

First, the enhanced tumor uptake of glucose is thought to have as a primary function the feeding of larger amounts of carbon into anabolic branches of glycolysis. For example, not only are core glycolytic enzymes upregulated in cancer cells (see, for example, [73]), but activities such as phosphoglycerate dehydrogenase (PHGDH) that diverts glycolytic three-carbon units into amino acid synthesis are also sometimes overexpressed [47, 64]. Specifically, PHGDH activity shunts a three-carbon glycolytic intermediate into the anabolic pathway most directly supporting serine synthesis. Serine is not only essential for protein synthesis but also required for specific steps in nucleotide and lipid biosynthesis. The PHGDH gene is commonly amplified in breast cancers and melanomas and this amplification correlates with poor prognosis (ibid.).

The amplified PHGDH gene is associated with elevated PHGDH enzymatic activity and flux of carbon through the serine biosynthetic pathway (ibid.). Moreover, RNA interference (RNAi) knockdown of PHGDH activity suppresses growth of tumor cell lines carrying PHGDH gene amplification. Most importantly from our perspective, such knockdowns compromise tumor growth both in in vitro 3-D culture assays [47] and in tumor xenograft models [64].

Finally, Possemato, et al. [64] looked at the global metabolomic effects of PHGDH knockdown in overexpressing cells. In spite of the resulting reduced flux through the serine pathway, steady-state serine levels were not reduced by PHGDH knockdown. Instead, rather dramatic reductions were found in the levels of alpha-ketoglutarate. [Alpha-ketoglutarate results from the transamination reaction producing phosphoserine from phosphohydroxypyruvate (the PHGDH product) on the way to serine production.] This result indicates rather subtle regulation of serine levels and also suggests that this transamination reaction is a major supplier of alpha-ketoglutarate in these tumor cell types.

As yet, no potentially clinically useful drugs targeting enhanced PHGDH expression have been developed.

Second, the importance of increasing anabolic diversion from the already elevated carbon flow through tumor cell glycolysis is further illustrated by the upregulation

of flux through the pentose phosphate pathway (PPP). The recent study of Ying et al. [91] is especially informative. These investigators constructed a conditionally expressed Kras experimental system in animals wherein Kras-dependent pancreatic tumors (ductal carcinomas) arise spontaneously, allowing tumors and explanted, cultured tumor-derived cells to be studied. Turning off Kras expression causes these tumors to regress *in vivo*.

Using this system, the authors ask about metabolic changes associated with the loss of elevated Kras expression in tumors and explanted tumor cells. They first recapitulate the longstanding observation that flux through glycolysis is upregulated by activated Kras expression, associated with increased expression of several of the major enzymes of glycolysis (see, for example, [1]). The authors then show that flux through the non-oxidative arm of the PPP (ultimately into ribose for nucleotide biosynthesis) shows strong Kras-dependent enhancement. Moreover, attenuating this flux (through RNAi knockdowns of rate-limiting enzymes) significantly attenuates tumor cell clonogenicity in soft agar, suggesting that this increased carbon flow is important to supporting the transformed phenotype.

These results are also noteworthy for the *lack* of activation of the oxidative branch (also sometimes called the reductive branch; Fig. 2.1) of the PPP. An attractive, simple view has been that the nicotinamide adenine dinucleotide phosphate (NADPH) generated by this oxidative branch was also important for anabolic processes. However, this traditional view has been criticized (reviewed in [84]), suggesting that other sources of cytosolic anabolism-essential NADPH might be more important (Fig. 2.1). The results of Ying et al. [91] support the hypothesis that tumor cell anabolic NADPH is likely derived from other sources in addition to the oxidative PPP at least in some tumor types.

As yet, no potentially clinically useful drugs targeting enhanced non-oxidative PPP have been developed.

Third, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes one of the steps in glycolysis . Moreover, GAPDH is substantially upregulated in a number of tumor types, including pancreatic and lung—especially in more advanced tumors (reviewed in [78]). This is particularly interesting as GAPDH is not only an essential component of upregulated tumor glycolysis, but it is also apparently a regulatory molecule with diverse functions (reviewed in [75]; also see below). Recently, Tang et al. [78] extended GAPDH expression level analysis to a large set of colorectal carcinomas (CRCs), including both primary tumor samples and liver metastases. GAPDH was substantially upregulated in primary tumor cells relative to surrounding noncancerous and stromal cells. GAPDH was also further upregulated in liver metastases compared to the matched primary tumor of origin, further strengthening the correlation between malignant potential and GAPDH expression levels.

The work of Tang et al. [78] concerns us here because these authors used the sensitivity of GAPDH to a site-specific alkylating agent (3-bromopyruvate, in its propyl ester form; abbreviated 3-BrOP) to explore the importance of GAPDH overexpression. Their results show that inhibition of GAPDH activity by 3-BrOP correlated very well with the capacity of this agent to kill GAPDH overexpressing tumor cells in culture. While this evidence for the importance of GAPDH is somewhat circumstantial, the importance of GAPDH to the larger picture of the relationship between matter/energy and redox metabolism warrants attention to this case.

It will be of interest to see if inhibitors of GAPDH function (perhaps analogous to 3-BrOP) can be used to achieve differential killing of tumor cells *in vivo*, in spite of the importance of GAPDH in both normal and tumor cells as suggested in preclinical models [38].

Fourth, the final step of glycolysis is the substrate-level phosphorylation event creating ATP and pyruvate from phosphoenolpyruvate and ADP. The enzyme catalyzing this step is pyruvate kinase (named for the reverse of the normal metabolic flow reaction), abbreviated PK. PK exists in two major isoforms produced by alternative splicing, PKM1 and PKM2, each with distinct regulatory properties including response to allosteric modifiers [33]. PKM2 is normally found only in embryonic tissues, with PKM1 predominating in adult tissues; however, PKM2 is also the form of PK in most tumors [15, 18, 53].

Substitution of PKM1 for PKM2 (through gene replacement) led to an alleviation of the Warburg pattern of elevated glycolysis in cell culture [15], suggesting an important function for this isoform in tumor-specific metabolism. Moreover, PKM2's regulatory properties and the fact that it is a relatively inactive enzyme (reviewed in [15]) led to the hypothesis that PKM2's functions included controlling exit from glycolysis so as to allow the piling up of glycolytic intermediates for anabolic use.

While gene replacement results do not demonstrate direct metabolic function (indirect effects can also be important; see below for the PKM2 case in particular), acute stimulation and inhibition of PKM2 through short-term treatment with small molecule modifiers strongly support this picture of PKM2 function in tumor metabolism ([3, 41]). Moreover, recent studies demonstrate that small molecule activators of PKM2 (producing activity more similar to PKM1) have significant *in vivo* antitumor activity, further supporting the cancer phenotypic relevance and clinical targeting potential of PKM2 [4]

Fifth, the terminal product of glycolysis, pyruvate, has three major fates. It can be diverted directly into anabolic use for amino acid biosynthesis [22]. Alternatively, it can have two catabolic fates, importation to the mitochondrion to supply the TCA cycle or reduction to lactate by lactate dehydrogenase (LDH), generally for export into the extracellular fluid (Fig. 2.1). The reduction of pyruvate to lactate is a thermodynamically reversible reaction; however, there are two isoforms of LDH each kinetically favoring one or the other of these two reactions. Specifically, the LDH-A aka LDH-1 homotetramer favors reduction of pyruvate while alternative isozyme homotetramer (LDH-B) favors oxidation of lactate (reviewed in [24]). The LDH-A isozymic form is often highly overexpressed in tumor cells in clinical biopsy samples [40].

Fantin, et al. [19], building on earlier results, constructed a relatively complete picture of the role of LDH-A in tumor cell metabolism. These authors showed that RNAi knockdown of the gene encoding the LDH-A monomer dramatically shifted the patterns of metabolism in several tumor cells, increasing mitochondrial oxidative phosphorylation substantially. This result strongly suggests that competition between LDH-A and mitochondria for pyruvate is one of the central determinants of the Warburg metabolic pattern. [A few cancer cell types were actually killed outright by LDH-A knockdown, indicating that some cancer cell mitochondrial

metabolism is sufficiently altered as to be incapable of supporting cell survival even in the luxuriantly oxygenated tissue culture environment.]

Fantin et al. [19] also showed that growth of LDH-A knockdown tumor cell lines was especially strongly inhibited in hypoxic culture conditions. This is consistent with the traditional expectation for the function of LDH in regeneration of $NAD^+$ from NADH to sustain glycolysis in the absence of molecular oxygen to support NADH oxidation (through cytosolic/mitochondrial shuttles; [22]).

Finally, knockdown of LDH-A quite dramatically inhibited tumor growth in *in vivo* xenograft models (ibid.).

The use of gene knockdown technology (rather than acutely acting small molecule inhibitors/activators) increases the ambiguity in interpreting these results. However, the suggestion that high LDH-A activity is central to the tumor metabolic pattern and crucial to malignancy *in vivo* is, nonetheless, very strong.

At present, there are no small molecule agents targeting LDH-A that would allow assessment of clinical impact of these insights; however, attention continues to be devoted to developing such agents (reviewed in [24]).

We now turn to mitochondrial metabolism in tumor cells.

First, the pyruvate dehydrogenase complex (PDH; Fig. 2.1) catalyzes the step that introduces cytosolically derived pyruvate into the mitochondrial TCA cycle, apparently in competition with LDH reduction. PDH catalyzes the oxidative decarboxylation of pyruvate, followed by the transfer of the resulting two-carbon acetyl unit to coenzyme A (CoA). This acetyl-CoA is a substrate for the subsequent transfer of the two-carbon unit to oxaloacetate to form citrate ([22]; Fig. 2.1).

PDH activity is regulated by phosphorylation by specialized kinases that inactivate the first step in PDH function by phosphorylation (PDK1–4; reviewed in [6, 70]). PDKs 1 and 3 are upregulated selectively in a number of tumors [36, 39, 49, 62], at least in part through hypoxia-inducible factor-1 (HIF-1) activation. Moreover, the overexpression of PDK1 and 3 is essential for various properties characteristic of malignancy [49, 55].

Thus, attacking PDK-dependent regulation of PDH is a potentially attractive chemotherapeutic target. One approach has been the use of dichloroacetate (DCA), a pyruvate analog which appears to partially inhibit a subset of PDKs. The inhibition of PDKs leads to higher PDH activity [8] and a so-called reversal of the Warburg effect, resulting in cell death presumably due to increased oxygen use by hypoxic cells. DCA has significant activity in *in vivo* tumor xenograft models (ibid.) suggesting possible clinical usefulness. At present, while there is some anecdotal evidence for clinical efficacy, DCA causes toxic neuropathy [34] which may ultimately limit its use.

An alternative approach is to attempt to exploit the sensitivity of PDKs to the oxidation/reduction/acylation state of the PDH cofactor lipoate [94]. Analogs of the lipoate catalytic intermediates (called "thioctoids" generically) selectively activate hyperphosphorylation of PDH in tumor cells, apparently mediated by thioctoid action on the extensively altered PDK spectrum in tumor cells. This PDK hyper-activation is accompanied by inactivation of PDH activity and cell death. Thus, rather than attempting to undo the downregulation of PDH characteristic of cancer cells (as DCA does), these agents apparently drive this downregulation much further, producing PDH activity too low to support viability. RNAi knockdown of tumor cell PDKs

substantially reduces sensitivity to thioctoid drugs, providing strong circumstantial evidence for a PDK target.

Initial clinical trials indicate an excellent safety profile for thioctoids and provide suggestive anecdotal evidence for efficacy against several cancers, including relapsed, refractory AML [63]. The lack of hematoxicity of thioctoids (ibid.) encourages the view that these agents may be able to target tumor cell metabolism without significant cross talk with metabolic regulation of growing normal cells.

Second, elevated levels of glutamine utilization have long been recognized to be characteristic of and essential to most tumor cells [68]. It was initially recognized [90, 20] that Myc activation indirectly stimulated glutaminase expression enhancing tumor glutamine use and that such Myc-transformed cells became glutamine dependent. Moreover, suppression of glutamine use (through glutaminase inhibition) is sufficient to kill such Myc-transformed cells [93]. Glutamate metabolism can also be activated through other pathways in tumor cells, including Kras activation [88] and activation of several rho guanosine triphosphatases (GTPases; [85]).

Notably, in both the Kras and rho GTPase cases, the investigators showed both that inhibition of flux through the glutamine metabolic pathway was sufficient to suppress transformed behavior (growth in soft agar) and that this suppression of cancer-like behavior could be overcome by supplementing with alpha-ketoglutarate ([85, 88], Fig. 2.1). These results indicate that the malignancy-essential function of increased glutamine flux is to feed alpha-ketoglutarate into the mitochondrial TCA cycle.

One simple supposition from some of these results would be that the anaplerotic support of mitochondrial anabolic metabolism, especially provision of intermediates for amino acid and lipid synthesis (Fig. 2.1; [22]), might be the key function of tumor-specific increased glutamine flux. Indeed, this is likely to be the case in at least some contexts. However, Weinberg et al.'s [88] analysis suggests a more complex possibility. Specifically, these authors observe that mitochondrial ROS are required for the transformed phenotype (growth in soft agar) (also see [30]). Moreover, this ROS signal could be produced by mitochondria that lacked cytochrome oxidase and that were, thus, incapable of net ATP generation and probably inefficient at large-scale anabolic substrate production. This result raises the important possibility that the malignancy-essential function of glutamine metabolism (and production of alpha-ketoglutarate) is to support mitochondrial ROS production.

Wang et al. [85] describe a small molecule targeting the specific glutaminase isoform overexpressed in the class of tumors they analyze. This molecule significantly attenuates tumorigenicity. It will be of interest to determine whether molecules of this general form might have clinical applicability.

## 2.4 Redox Metabolism in Cancer

There is now abundant, diverse evidence for the alteration of redox metabolism in cancer cells. However, it is not generally understood that this altered redox metabolism might be very intimately connected to the regulation of the widely appreciated cancer-specific alterations of matter/energy metabolism.

Our purpose in this section is to review several key pieces of evidence that indicate that this putatively intimate relationship is potentially central to cancer cell function and, thus, worthy of much more extensive attention than it has yet received. We will explore several specific examples of cases of known redox regulation of activities implicated in cancer metabolism. We will propose reinterpretation of these results to suggest that *redox regulation of matter/energy metabolism* is both central to and extensively redesigned in tumors. The tumor-stromal matter/energy metabolic symbiosis almost certainly also makes use of *inter*cellular redox signaling to achieve integrated transcellular metabolic performance [46]; however, for our purposes here we will remain focused on *intra*cellular redox signaling.

We begin by noting that there is now extensive, sound evidence that redox metabolism is substantially altered in tumor cells compared to most normal cells ([77, 81]; recently reviewed in [82]). Several recent, independent cases are especially illuminating. In both of these examples, an hypothesis-neutral screen was carried out searching for small molecules that would selectively kill tumor cells in culture. In each case, a different small molecule class attacking redox signaling was identified. For emphasis, note that these screens would have identified small molecules attacking *any* vulnerable target, yet both found a redox target. These data strongly suggest an especially rich opportunity to target redox metabolism selectively in cancer cells.

Specifically, Shaw, et al. [74] identified a class of piperidine derivatives with strong selectivity for killing Kras transformed mouse embryonic fibroblasts (MEFs) compared to their non-transformed parents. The lead member of this compound class, lanperisone, provoked elevated ROS production preferentially in Kras-transformed cells and antioxidants protected transformed cells from lanperisone killing. Lanperisone also produced significant tumor growth inhibition in *in vivo* mouse xenograft models.

In a second hypothesis-neutral screen of a large set of compounds, Raj et al. [65] identified piperlongumine, a natural product, capable of killing transformed cells in culture and significantly inhibiting tumor xenograft growth *in vivo*. Piperlongumine produces substantial elevation of ROS levels selectively in tumor cells. Especially importantly from our perspective, the authors developed substantial circumstantial evidence that piperlongumine acts by attacking glutathione-based ROS detoxification systems. This observation is consistent with elevated importance of redox signaling in tumor cells, making them more vulnerable to poisoning of the ROS detoxification systems necessary to control such signals. More specifically, redox signals are also involved in cell death control (reviewed in [25]) and these agents likely interfere with normal redox signal attenuation, elevating a metabolic regulatory signal into a cell death signal through enhancement of signal duration and/or amplitude.

Several other observations are suggestive in this context. Li et al. [44] produced compelling evidence that the tumor suppressor activity of p53 did not result from its well-known effects on cell cycle arrest, apoptosis, and senescence. These authors suggest the importance of less appreciated p53 functions, including regulation of metabolism with elements designed to produce antioxidant capability. Analogously, the tumor suppressor activity of the SIRT3 mitochondrial sirtuin is largely or entirely attributable to its regulation of mitochondrial redox metabolism through control of

the mitochondrial superoxide detoxifying enzyme, manganese superoxide dismutase (MnSOD; [35, 79]).

These results, those of Weinberg, et al. ([88] above), and related earlier studies (reviewed in [82]), strongly suggest that tumor cells show elevated levels of ROS generation inherently and with a central causal function, though the specific contexts and cellular compartments involved remain incompletely understood. We wish to focus attention on the potential implications of these observations for how the properties of tumor cells might be regulated differently than non-transformed cells through differential deployment of redox signaling. This putative nexus between matter/energy and redox metabolism may be a very rich new frontier for clinical exploitation.

Various cellular sources produce superoxide through the partial reduction of molecular oxygen. Superoxide, in turn, is rapidly dismutated into a second, biologically active ROS, hydrogen peroxide (see [59, 79], and references therein). Either of these two active compounds produces redox modifications of proteins, especially at sensitive cysteine sulfhydryl residues and these initial redox modifications also provoke secondary modification (for example, glutathione disulfide formation with the affected sulfhydryl) (reviewed in [17, 45, 77]). In a number of cases, such redox modification is known to alter the properties of the affected proteins and these phenomena form the basis of what is widely considered to be redox control of function (ibid.). Superoxide is highly reactive and, in general, will act only extremely locally. Hydrogen peroxide is both more stable and potentially membrane permeant, allowing activity over larger distances and, probably, between cellular compartments (ibid.). Our focus herein is on the potential roles of these molecules in the transformed state and especially in controlling aspects of tumor metabolism.

Cytosolic NADPH-dependent oxidases (NOXs) play a central role in local signaling in normal cells, through their controlled release of superoxide, generated using the potential in NADPH to reduce molecular oxygen [7]. Moreover, overexpression of one such NOX is sufficient to transform NIH 3T3 cells as assessed by loss-of-contact inhibition and formation of tumors in animals [76]. NOXs are generally cytosolic; however, there is evidence that ROS generated from mitochondrial sources is also essential to the malignant state.

The major sources of mitochondrial ROS under most conditions appear to include Complexes I and III of the electron transport chain (ETC) embedded in the inner mitochondrial membrane. In addition, another major mitochondrial source apparently exists which may consist of some combination of the dihydrolipoamide dehydrogenase (E3) component common to several dehydrogenase complexes (including alpha-ketoglutarate dehydrogenase, KGDH) and Complex II of the ETC (reviewed in [56, 66]). All of these sources use metabolic reducing potential to drive the reduction of oxygen to produce superoxide/hydrogen peroxide signals. As a result, it is reasonable to suppose that the level of ROS production is responsive to metabolic flux [59].

Given these insights, it is widely assumed that the dominant logic of cancer mitochondrial ROS production is that it increases in response to increased metabolic flux, with redesigned tumor metabolism producing increased mitochondrial ROS as

a by-product (see, for example, [11, 89]). On this plausible view, the primary issue to be understood is how tumor cells manage these troublesome, elevated mitochondrial ROS by-products.

However, there is considerable reason to believe that the mitochondrial ROS/tumor metabolism relationship is more complex, interesting, and useful than this simple perspective. For example, Kras transformation is dependent on mitochondrial ROS [88]. This observation suggests that mitochondrial redox signaling can be upstream of the transformed phenotype, rather than being a mere by-product thereof. The results Weinberg et al. [88] report also indicate that the flow of glutamine-derived carbon into the TCA cycle (rather than glycolytic carbon) is essential for the generation of this redox signal. This observation is especially evocative as others have shown that blockage of glutamine flux is sufficient to produce dramatic interference with the malignant phenotype ([93]).

Weinberg et al. [88] provide strong circumstantial evidence suggesting that mitochondrial ROS acts through the MEK/ERK pathway to produce cancer-related phenotypic effects in their specific experimental system. Moreover, it is now well recognized that mitochondrial ROS is intimately involved in a variety of signaling processes, ranging from receptor tyrosine kinase signaling to response to hypoxia (reviewed in [27]). It is especially noteworthy here that the PTEN phosphatase, upstream of Akt activation, is, itself, a target for redox inactivation (allowing redox activation of Akt) [42, 60]. Akt activation not only upregulates metabolic flow, potentially including mitochondrial carbon flux (ibid.), but this kinase also *downregulates* ROS detoxification (reviewed in [37]). These properties strongly suggest the possibility of a switch-like positive feedback loop defining a new state of elevated redox signaling in cancers.

Also striking is the observation that redox activation of HIF-1 expression is essential to efficient *in vivo* malignant function for several different tumor models [21]. Though HIF-1 is upstream of some of the metabolic changes in cancer [73], these observations place redox regulation upstream of HIF-1 activation, in turn. Moreover, several lines of evidence indicate that hypoxic upregulation of HIF-1 is produced by mitochondrial ROS signals [10, 26]. The results of Ma et al. [50] indicate an analogous result for Kaposi's sarcoma. More specifically, their results suggest that antioxidant treatment downregulated Akt activation, lending further support for a determinative role for Akt in establishing a state-like tumor-specific elevated redox signaling regime.

Thus, the "oxidative stress" common to cancer cells (reviewed in [82]) may reflect a deliberate repurposing of redox signaling rather than a by-product of the matter/energy metabolic changes associated with malignant transformation.

While various results suggest that the redesigned mitochondrial redox metabolism of tumor cells may serve a high-level signaling function (above), we wish to focus here on the possibility that tumor-specific redox metabolism might also play a role in managing the moment-to-moment flow of matter and energy in the tumor metabolome.

It is useful to begin this argument by looking more carefully at several details of the relationship between matter/energy metabolism and redox signaling. It is striking that

hypoxia (oxygen *deprivation*) results in *elevated* production of mitochondrial redox signals that mediate HIF activation and the hypoxic response [10, 26]. Likewise, the response to glucose *withdrawal* is *elevated* production of mitochondrial ROS signals [23, 71]. Collectively, these results are consistent with the view that mitochondrial ROS production is not driven by simple mass action, but rather results from strategic, deliberate, designed production of redox signals.

Though our knowledge is still extremely fragmentary, there are several specific cases of redox regulation of matter/energy metabolism that are highly suggestive of a potentially broader picture and we will focus our attention on several of these. We begin with two potentially illuminating cases involving cytosolic metabolism.

First, GAPDH is upregulated in tumor cells as part of the systematic reconfiguration of tumor cell matter/energy metabolism (above). Most important for our purposes here, however, is that GAPDH is well known to be regulated by redox modification of a cysteine sulhydryl in the enzyme active site ([9, 72], reviewed in [83]). This redox control is thought to have the function of blocking flux through glycolysis leading to overpopulation of the early intermediates that feed the oxidative PPP, in turn, allowing PPP-dependent production of NAPDH (reviewed in [83]).

NAPDH feeds glutathione and thioredoxin-based ROS detoxification in the cytosol and this redox inhibition of GAPDH is assumed to represent a feedback circuit producing homeostatic control of ROS levels. Such feedback control is, indeed, almost certainly a major function of this redox process; however, we note that in at least some tumor cells the oxidative PPP is underutilized. In contrast, the non-oxidative PPP (providing anabolic intermediates, especially for nucleic acid synthesis, but not NADPH) is activated (Fig. 2.1). Thus, it is likely that the redox regulation of GAPDH in tumor cells may also have a rather different function, the control of diversion of glycolytic carbon into the production of anabolic intermediates (Fig. 2.1).

It is also noteworthy that GAPDH interacts with other cellular proteins, including nuclear regulatory factors, and that some of these interactions are strongly affected by redox modification of GAPDH ([31], reviewed in [83]). This regulatory sophistication introduces the possibility of yet more extensive redox control of matter/energy metabolism through GAPDH in tumor cells, a possibility of great future interest.

Second, the M2 variant of pyruvate kinase (PKM2) is one of the central elements of the tumor-specific redesign of tumor cell matter/energy metabolism (Fig. 2.1 above). Remarkably, the PKM2 variant (but not the adult normal cell PKM1 form) is sensitive to redox modification of a specific cysteine residue and this modification substantially downregulates PKM2 activity ([3], Fig. 2.1). These authors also provide compelling circumstantial evidence that one function of this redox modification is to direct the pile-up of glycolytic glucose-6-phosphate to support the oxidative PPP for purposes of generation of NADPH for ROS detoxification.

This proposed autoregulation of ROS levels through PKM2 may, indeed, be a function of this process; however, as with GAPDH above, it is an important possibility that this process also directs glycolytic carbon into the anabolic non-oxidative PPP. Indeed, given how many metabolic steps there are separating pyruvate kinase and the oxidative PPP (Fig. 2.1), this alternative hypothesis is especially attractive for future investigation. Especially compelling evidence for this possibility comes

from the observation that defeating the possibility of this redox repression of PKM2 (using a constitutive small molecule activator) severely reduces the diversion of glycolytic carbon from the steps just above the PK step into serine biosynthesis ([41], Fig. 2.1).

Luo et al. [43] have recently shown that the PKM2 is a target of HIF-1 activation while also directly interacting with HIF-1, including in the nuclear compartment, to stimulate transcription of HIF-1 targets. The authors propose plausibly that this relationship constitutes a positive feedback loop contributing to the stabilization of the tumor-specific pattern of glucose metabolism. It will be of great interest to understand whether redox modification influences this transcription cofactor function of PKM2. For example, if redox modification of PKM2 enhanced HIF-1 coactivator function, this positive feedback loop might synergize with the putative Akt redox positive feedback loop discussed above, further stabilizing the tumor cell elevated redox metabolic state.

The two cases immediately above represent redox regulation of cytosolic matter/energy flows. There is also strong evidence for redox control of *mitochondrial* matter/energy metabolism. Some of these include redox modification of ETC functions (reviewed in [29]). However, we will focus here on several matrix functions which suggest additional examples of potentially adaptive function for redox modification.

First, one of the important functions of *tumor* mitochondrial matrix matter/energy metabolism is the production of citrate for export to the cytosol in support of lipid synthesis and/or cytosolic production of NAPDH (Fig. 2.1). Moreover, the mitochondrial step apparently competing directly with citrate export is the aconitase isomerization of citrate to isocitrate (Fig. 2.1). Aconitase is well known to be one of the mitochondrial enzymes most sensitive to redox inhibition [61], suggesting that redox regulation may control the export of citrate to the cytosol, either for anabolic or for ROS attenuation purposes.

Second, alpha-ketoglurate dehydrogenase (KGDH) is the entry point to mitochondrial metabolism for carbon derived from glutamine, typically the other major matter/energy source for tumor cells in addition to glucose (Fig. 2.1). The KGDH substrate, alpha-ketoglutarate (KG), also serves vital additional functions. For example, KG can serve as a precursor for glutathione synthesis. Further, under hypoxic conditions KG serves as a precursor for reductive isocitrate/citrate synthesis, the crucial pathway for production of citrate for lipid synthesis under severely oxygen-deprived conditions often arising in solid tumors [56, 58]. In addition, KG is an essential cofactor for series of dioxygenases catalyzing steps crucial to important regulatory functions, including prolyl hydroxylases, histone demethylases and methylcytosine hydroxylases (reviewed in [48, 89]). Indeed, recent insights into the impact of the oncometabolite 2-hydroxyglutarate (a KG competitor) indicates that effective KG levels are crucial for the proper functioning of these processes (ibid.).

Thus, control of KG levels is expected to be an extremely high priority. Control of KGDH activity is likely to be central to KG homeostasis and KGDH activity is highly sensitive to redox inhibition [54, 58]. Thus, redox signals are likely to be central to the crucial management of KG levels. It will be of great interest to understand the precise source and physiological logic of the redox signals controlling KGDH activity.

## 2.5 Concluding Remarks

While our understanding of genetic/epigenetic causation of cancer continues to grow dramatically, this improved understanding has been less helpful clinically than we might have hoped. Gain-of-function driver mutations are apparently less common than loss-of-function changes in tumor suppressors (B. Vogelstein, quoted in [51]). Moreover, gain-of-function drug targets can be expensive to diagnose on a patient-by-patient basis (depending on tumor type). Finally and most importantly, the genetic regulatory circuitry altered in genetic/epigenetic ontogeny of cancer is so extensively redundant that even highly potent drugs targeting individual changes tend to provide only relatively transient (if sometimes profound) benefit to patients (see, for example, [67]).

These results indicate that therapeutic attack on features of the transformed *state*, rather than its genetic/epigenetic drivers, will almost certainly prove to be a vital component of improved cancer therapy in the future. Our recently improving understanding of matter/energy metabolism in cancers increasingly supports the view that metabolism is not only profoundly altered in tumor cells, but the altered metabolism of diverse tumors shows extensive and fundamental similarity from tumor to tumor. In addition, diverse evidence indicates that this characteristic, pervasive, near-universal tumor metabolic pattern is crucial to the persistence of the transformed state and/or to the survival of individual tumor cells in the challenging environment of poorly vascularized solid tumors. Thus, the altered metabolism of tumor cells may provide targets for clinical attack that are not only powerful, but also potentially available in many or even most tumor types.

Finally, we have argued herein that the systematically altered redox behavior of tumor cells should not be regarded as epiphenomenal, a by-product of altered matter/energy metabolism, for example. Rather, we suggest redox and matter/energy metabolism, at least in tumor cells, may well prove to be just two facets of a unified functional metabolic whole. By better understanding the role of redox processes as a central feature of the control of altered metabolism in cancer, we may open a large new door to improve chemotherapeutic attack on universal properties of the transformed state.

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# Chapter 3
# Regulating Mitochondrial Respiration in Cancer

**Teresa L. Serafim and Paulo J. Oliveira**

**Abstract** Mitochondria are a major focus of research in cancer due to their critical role in tumor physiology and metabolism. Metabolic remodeling is observed in tumor cells, often resulting in increased glycolytic activity, which serves for the generation of adenosine triphosphate (ATP), and as hubs for biosynthesis of key metabolites essential for cancer cell growth and proliferation. Mitochondria, thus, appear as a critical nexus in cancer metabolic alterations. Not only increased overexpression of oncogenes leads to altered mitochondrial respiration due to remodeling of mitochondrial gene expression and substrate channeling, but also particular mutations in components of the respiratory chain trigger an upstream feedback mechanism which also leads to metabolic reshaping in cancer cells. Mitochondrial respiration can thus be controlled by intrinsic and extrinsic mechanisms in cancer cells, which ultimately translates into different abilities to generate mitochondrial ATP. Altered mitochondrial structures and processes can be a target for chemotherapeutics, which are increasingly being developed to specifically target mitochondria in tumors. The present chapter reviews current knowledge on regulation of mitochondrial respiration and overall metabolism and how these specific alterations in the cell powerhouse can be used to eliminate tumors.

**Keywords** Cancer metabolism · Mitochondria · Oxidative phosphorylation · Respiration · Chemotherapy

## 3.1 Cancer Metabolism

### *3.1.1 Overview*

Under normal conditions, cells have controlled programs for maintaining homeostasis in tissues, relying normally on aerobic respiration, using cytosolic and mitochondrial metabolisms to produce adenosine triphosphate (ATP) and for the

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biosynthesis of cellular building molecules [311]. Any deviation from these programs may result in an anomalous situation. Tumors, deviations from normal cell homeostasis, contain a mixed cell population, with some showing fast proliferation. Together with unregulated cell growth, tumor cells display loss of contact inhibition, which is necessary for normal tissue formation. Interestingly, both uncontrolled growth and loss of contact inhibition appear to be linked with altered cellular metabolism [187]. The progressive growth of a tumor greatly increases the demand for oxygen and nutrients, resulting in the inability of tumor cells that are distant from blood vessels to be steadily supplied [129]. As a consequence, hypoxic regions are formed within the tumor. Therefore, one of the main mechanisms for the metabolic remodeling observed in cancer cells is an adaptation to a novel environment, where oxygen can be limiting [183]. Malignant cells will survive under hypoxic conditions due to the activity of distinct oncogenic proteins, which induce the expression of specific encoding genes for metabolic proteins and consequently modulate their function in cancer cells [137]. The molecular mechanism behind this adaptation and energy metabolic adjustment is not completely understood and this phenomenon is not general to all cancer cells.

One important characteristic of tumors is the induction of angiogenesis [311]. New vessels are formed in the tumor microenvironment providing oxygen, which, although not as well distributed as in a normal tissue, favors ATP production through oxidative phosphorylation (OXPHOS). Still, most cancer cell types will continue to use glycolysis, which not only provides a survival advantage over non-transformed cells but also ensures the persistence of the most successful cancer cells [128]. Cancer cells manage to adapt from aerobic to anaerobic glycolysis to survive in a new microenvironment, upregulating transporter proteins that extrude lactic acid from the cell into the surrounding extracellular medium, as well as undergoing many other alterations [286]. This phenomenon is widely explored in cancer biology and was termed the Warburg effect [309].

Glycolysis accounts for most of ATP generation in a majority of cancer cell types [203]; however, mitochondrial ATP production in other tumors may be entirely similar to a non-tumor cell. It has been proposed that this switch may be related to specific cell or tissue types, with this metabolic flexibility being important for certain tumors to grow and metastasize [45]. Moreover, a large number of mitochondrial alterations exist in most cancer cells. In fact, tumor cells that show negative mitochondrial alterations are particularly aggressive, showing a rapid growth rate [279]. The downregulation of some mitochondrial proteins in cancer cells, including the OXPHOS machinery, is achieved by distinct mechanisms, specifically activated by the profound hypoxic environment, the loss of tumor-suppressor genes and/or activation of oncogenes, and the direct inhibition of mitochondrial complex subunits [112]. Tumor microenvironment can also dictate the type of metabolic pathway to be predominantly used in cells, which, in turn, gives self-renewal ability to the tumor [20].

Hanahan and Weinberg reformulated their six hallmark signatures of cancer [150], adding the reprogramming of energy metabolism plus the evasion from immune destruction as new cancer features. The “Hallmarks of Cancer” appear now as a signature of the disease which can help in stratification, diagnosis, prognosis, and

treatment: limitless replication potential, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion, metastasis, metabolic remodeling, and evading immune destruction [151].

In fact, more and more evidence enhances the importance of cancer metabolism research. It is our objective to understand the mitochondrial alterations in tumorigenesis, namely those altering mitochondrial respiration, and evidence the most promising therapies that target these alterations.

### *3.1.2 Mitochondrial OXPHOS*

Mitochondria are essential organelles for cell survival and growth and are the main producers of cellular ATP via OXPHOS, which provides 15 times more ATP than glycolysis [4]. These organelles are also involved in calcium signaling [148], heme and steroid synthesis [260], and redox homeostasis [149]. The actual mechanism of OXPHOS was mechanistically explained by Peter Mitchell's chemiosmotic hypothesis [217, 218], elucidating the biochemical mechanism of ATP synthesis in mitochondria. Under normal conditions, electrons are transferred from carbohydrates and lipids via nicotinamide adenine dinucleotide (NAD; reduced form) to complex I (NADH dehydrogenase), the major entrance point of electrons in the respiratory chain (or electron transport chain (ETC)), or from succinate to complex II (succinate dehydrogenate), that directly connects the tricarboxylic acid cycle (TCA) to the system [104]. Other components involved in electron entry to ETC are the electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) [327] and glycerol-3-phosphate dehydrogenase (G3PDH) [180]. Coenzyme $Q_{10}$ accepts the electrons from different sources and channels them to complex III (subunit for ubiquinol: cytochrome *c* oxidoreductase) [119]. Electrons then flow through complex III to complex IV (cytochrome *c* oxidase, COX), where oxygen is reduced to water. Protons are pumped from the matrix to the intermembrane space, coupled to electron transport at complexes I, III, and IV, creating an electrochemical gradient, composed of an electric component ($\Delta\Psi m$), being negative inside, and of a pH component ($\Delta pH$), alkaline in the matrix [53]. The proton motive force is then used by complex V (ATP synthase) to produce ATP from adenosine diphosphate (ADP) and phosphate [171]. The ETC is coupled with the phosphorylation system, in order to maximize mitochondrial ATP production and minimize heat production [30]. All these processes must follow strict regulated conditions, otherwise cell death or malignancy can occur. Therefore, under normal conditions, different mechanisms of regulation of mitochondrial respiration exist. One crucial factor is not only the modulation of complex IV isoforms [43], but also the activation of four mitochondrial dehydrogenases, namely flavin adenine dinucleotide (FAD)-glycerol-3-phosphate dehydrogenase [152], pyruvate dehydrogenase phosphatase [83], NAD-isocitrate dehydrogenase [84], and oxoglutarate dehydrogenase [213] by calcium ions, which leads to their stimulation. Mitochondrial respiration regulation depends as well on fusion and fission proteins that are responsible for mitochondrial morphology [15]. Moreover, there are other proteins that are responsible for mitochondrial biogenesis

and degradation, which have a role in regulating mitochondrial respiration [40]. Besides the direct regulation of OXPHOS by proteins, the availability of substrates (NADH, H + /NAD + , ADP/ATP, oxygen gradients, glucose, and glutamine) [135], as well as the interaction with other cellular organelles [77] or even chemicals and drugs, can also impact mitochondrial respiration [16] .

### *3.1.3 Cancer Metabolism*

Metabolism is the sum of all chemical reactions that occur in cells or organisms [116]. In this particular section, energy metabolism in cancer is discussed. The analysis of mitochondrial metabolic alterations is important to better approach the regulatory adaptations that occur in mitochondrial respiration of cancer cells.

Cells exposed to low oxygen availability (hypoxia) upregulate glycolysis, resulting in increased lactic acid production. Cancer cells can preferentially use this pathway, once it generates ATP more rapidly than OXPHOS, even if in far lower amounts [262]. Glycolytic genes are regulated by the hypoxia-inducible factor-1 (HIF-1) ([72]; Fig. 3.1). Within any cell type, HIF-1 controls the expression of a very large number of genes. In particular, HIF-1 modulates the expression of aldolase, phosphoglycerate kinase, phosphofructokinase, lactate dehydrogenase A (LDHA), and lactate-extruding enzyme monocarboxylate transporter 4 (MCT4), as well as hexokinases (Hk1 and Hk2) [57]. At the same time, HIF-1 indirectly inhibits pyruvate conversion to acetyl-coenzyme A (CoA) by leading to an overexpression of pyruvate dehydrogenase kinase 1 (PDK1), which inhibits pyruvate dehydrogenase (PDH) [71]. In mitochondria, HIF inhibits the respiratory chain by targeting a Bcl-2 family member (BNiP3) and by reducing COX activity by upregulating microRNA-210 [257]. In several tumors, impairment of the TCA cycle leads to succinate accumulation, which acts as a signaling molecule and triggers the reactivation of HIF-1 [269]. Due to the lower energy efficacy of aerobic glycolysis, glucose uptake verified in most tumors is higher than in normal tissues [304], with increased expression of glucose transporters (Glut1, Glut3, and other isoforms) [270]. However, when elevated intracellular glucose is available, cells redirect pyruvate towards lipid synthesis, which is necessary for membrane assembly. While in non-tumor cells pyruvate is mostly imported into mitochondria to produce NADH and succinate, which will fuel the ETC in two different sites [6], pyruvate can also be converted to lactate by LDH in the cytosol and extruded, causing extracellular acidification, which is also advantageous to cancer cells as it decreases immune detection and facilitates invasion [320]. Contributing to cancer success, the downregulation of oxidative metabolism can favor malignant cells to evade apoptosis [159].

The Warburg effect can be observed even after re-oxygenation of tumors due to the formation of new blood vessels. Warburg initially observed that cancer cells would rather use glycolysis than OXPHOS to obtain most of their energy [309]. The original observation was based on the fact that tumors have elevated levels of glucose consumption and lactate production (Pasteur effect) while in the presence of oxygen [184]. The Warburg effect was observed in vitro and in vivo and is well documented

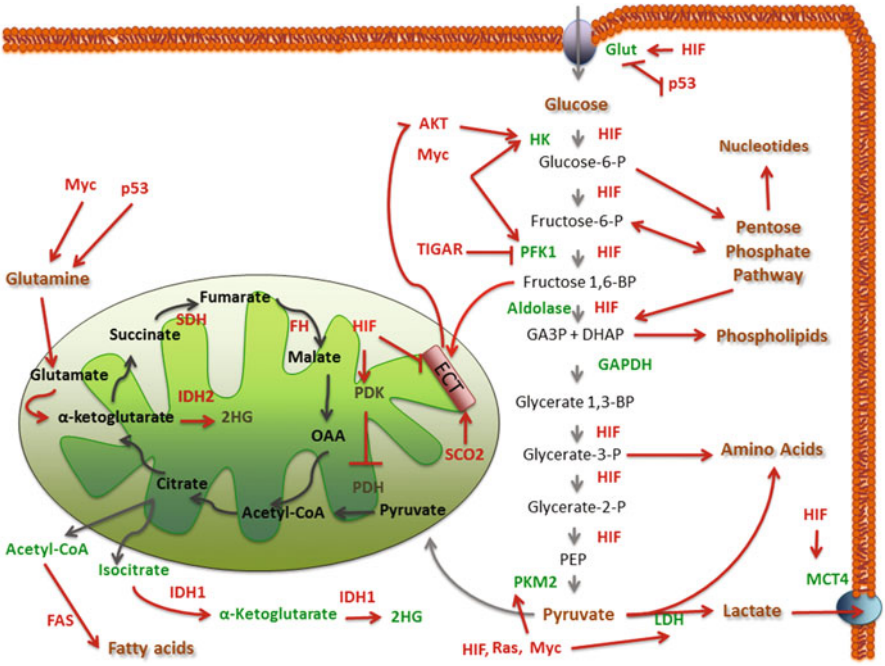


**Fig. 3.1** Cancer metabolism. Proliferating cancer cells show upregulation of glucose transporters (Glut) in order to import a large amount of glucose to be processed in glycolysis. Glycolysis is entirely regulated by HIF; however, oncogenes (e.g., Myc and Ras) and suppressor genes (e.g., TP53-induced glycolysis and apoptosis regulator (TIGAR)) ultimately control the flux. The ultimate product of glycolysis is pyruvate, which is normally converted to lactate in cancer cells. Pyruvate can also originate from non-essential amino acids or be converted to acetyl-coenzyme A and enter mitochondria to generate citrate. Due to altered mitochondrial function observed in cancer cells, citrate will mostly leave these organelles to promote lipid synthesis. Other pathways that are altered and important for the survival of cancer cells are the pentose phosphate pathway, which supplies RNA and DNA, but especially the glutamine pathway which fuels cells with other amino acids and proteins. *MCT4* monocarboxylate transporter; *Glucose-6-P* Glucose-6-phosphate; *Fructose-6-P* Fructose-6-phosphate; *Fructose 1,6-BP* Fructose 1,6-biphosphate; *GA3P* Glyceraldehyde-3-phosphate; *DHAP* Dihydroxyacetone phosphate; *Glycerate 1,3-BP* Glycerate 1,3-biphosphate; *Glycerate-3-P* Glycerate 3-phosphate; *Glycerate-2-P* Glycerate-2-phosphate; *PEP* Phosphoenolpyruvate; *2HG* 2-hydroxy-glutarate; *HK* Hexokinase; *PFK1* Phosphofructokinase; *GAPDH* Glyceraldehyde 3-phosphate dehydrogenase; *PKM2* pyruvate kinase isoform 2; *LDH* Lactate dehydrogenase; *IDH1* Isocitrate dehydrogenase isoform 1; *IDH2* Isocitrate dehydrogenase isoform 2; *SCO2* synthesis of cytochrome *c* oxidase deficient homolog 2; *PDK* pyruvate dehydrogenase kinase; *PDH* pyruvate dehydrogenase; *SDH* Succinate dehydrogenase; *FH* Fumarate hydratase; *FAS* Fatty acid synthase; *HIF* Hypoxia inducible factor; *ETC* Electron transport chain

for several tumor types, where the overproduction of lactate leads to the acidification of the tumor microenvironment, being recognized as a major metabolic hallmark of cancer, although many tumors do not have this effect [294]. Therefore, the Warburg effect can originate from an increase in glucose consumption and glycolysis activity and/or downregulation of mitochondrial metabolism [90].

Another phenomenon similar to the Warburg effect but caused by a different event is the Crabtree effect [91]. Fast-growing cells, including tumors, display inhibition of respiration due to an excessive increase of intracellular glucose. The Crabtree effect is considered a short-term and reversible event. The possible advantage of this phenomenon would be the adaptation of cancer cell metabolism to the heterogeneous microenvironment found in tumors [91].

Even if both the Warburg and Crabtree effects were common to all cancer cells, one must take into account that both metabolic effects, as well as other metabolic alterations, are not exclusive to cancer cells, since they can also be observed in activated T lymphocytes and some proliferating normal cells [141]. Moreover, each type of cancer carries its own mutation load and different tissues of origin differently prime tumors to metabolic alterations. In addition, an increase in the glycolytic flux may not directly result from increased expression of glycolytic enzymes, but instead result from altered proteins that co-regulate glycolysis [227].

Within the tumor, some cancer cells quickly interchange the metabolism between fermentation and oxidative metabolism, according to the presence or absence of nutrients and environmental conditions, thus showing a large plasticity [259]. Therefore, tumor cells can behave differently depending on many intrinsic and/or extrinsic factors, which limits the use of metabolic remodeling per se to distinguish a particular type of tumor.

More research must be performed to identify differences between normal and cancer cells and to identify the best therapeutic approaches. In particular, the central role of mitochondria, by modulating several key functions in the cell, deserves special attention. Mitochondria can serve both as a hub for metabolic alterations and as a target for chemotherapeutics.

## 3.2 Mitochondrial Metabolism Remodeling in Cancer

### *3.2.1 Biosynthesis and Energy Production*

The proliferation of cancer cells is supported not only by altered energy production but also by increased biosynthesis and maintenance of specific redox balance [18]. The remodeling of mitochondrial metabolism is evidenced by the preferential use of glycolysis and the increased usage of biosynthetic pathways, such as those of amino acids and fatty acids [120].

As described earlier, ATP production by mitochondria in most tumor types is diminished. One possible explanation for the disruption of the normal flux of the Krebs cycle may be the channeling of cycle intermediates, including malate and citrate, for other biosynthetic pathways. Both molecules can leave mitochondria, thus deviating the carbon flux. Malate can be used to provide the cytoplasm with NADPH, and citrate is used to support fatty acid and cholesterol synthesis [225]. Moreover, citrate is a crucial sensor of energy level, exerting a negative feedback on the Krebs cycle and glycolysis, slowing or even arresting the two pathways [161].

Another observation to support a low mitochondrial activity in cancer is a decrease of ADP transport to the mitochondrial matrix, as well as the inhibition of ATP synthase [64], decreasing ATP production in mitochondria. In the Krebs cycle, carbon can be dissipated as $CO_2$, while carbons originating from glycolysis supply precursors for biosynthesis. In truth, there is a waste of carbon by lactate export, although there are several advantages in the process, including evasion from the immune system [80].

Several groups [76, 123, 324] have provided evidence of the importance of amino acid metabolism in tumor proliferation, demonstrating that cancer cells have increased glutamine consumption by glutaminolysis when compared with their normal counterparts, although others suggest that this may be an in vitro artifact [223]. Glutamine is the most abundant amino acid in mammals [185] and a major factor in anaplerosis [76]. Oxidation of glutamine was observed to be essential not only for cancer systems but also for normal proliferating cells, such as lymphocytes, enterocytes, and fibroblasts [76]. Glutamine metabolism can be roughly divided into α-nitrogen (Krebs cycle) and γ-nitrogen (nucleotide and hexosamine synthesis) [57]. In the latter reactions, glutamine is converted to glutamate by cytoplasmic or mitochondrial glutaminase. From here, glutamate can follow one of two pathways: as a source of oxaloacetate (OAA) for the Krebs cycle or via transaminase by consuming OAA and generating aspartate, which then leaves mitochondria [223]. OAA is an essential substrate because it leads to citrate production when condensed with acetyl-CoA. After being exported to the cytosol, citrate can be used by ATP citrate lyase (ACL) to produce OAA and acetyl-CoA, essential for cholesterol and fatty acid synthesis and also for modification of chromatin structure [153, 315]. α-Ketoglutarate can also be originated from isocitrate by the action of isocitrate dehydrogenases (IDH1 and IDH2). The two enzymes exist in the cytoplasm and mitochondria, respectively, and, when mutated, convert α-ketoglutarate to 2-hydroxy-glutarate, which is recognized as an oncometabolite [252].

Glutamine metabolism can also provide precursors for the synthesis of glutathione (GSH), which serves as a redox buffer against increased oxidative stress, being important for tumors with rapid growth, thus presenting a high production of reactive oxygen species (ROS) [109]. Finally, glutamine is required as a nitrogen donor to produce purine and pyrimidine nucleotides during cell proliferation [123].

Interestingly, the serine pathway, another amino acid biosynthetic flux, has an important role in most estrogen-negative breast cancers [248]. In fact, some tumors showing overexpression of phosphoglycerate dehydrogenase (PHGDH) redirect glycolytic intermediates into serine and glycine metabolism [202].

Besides amino acid metabolism, other metabolic pathways can be altered, including fatty acid β-oxidation. The contribution of β-oxidation to metabolism in cancers was suggested as providing an important source of acetyl-CoA, NADH, H +,and ATP, to sustain energy production and proliferation. However, there is still a large unknown to be investigated [161]. Fatty acid synthesis in normal cells occurs at a low rate, since fatty acids can be easily obtained via blood circulation. However, proliferation of some tumors was still observed even when mitochondrial catabolism of fatty acids originating from the blood stream was not occurring, forcing de novo fatty acid synthesis at very high rates [212] or export of citrate from mitochondria to produce

acetyl-CoA [275]. To support this hypothesis, citrate transport is increased in tumor cells and also associated with glutamine uptake [237]. Moreover, increased lipogenesis in cancer is closely associated with the overexpression and hyperactivity of ACL, acetyl-CoA carboxylase (ACC), or fatty acid synthase (FAS) [186]. Among these proteins, FAS was the most consistently increased in cancer cells, being expressed at low levels in normal cells and tissues [36]. In malignant cells, FAS is involved in lipid production for membrane incorporation, as well as synthesis of lipids for cell signaling, such as phosphatidylinositol-3,4,5-trisphosphate, which activates protein kinase B/Akt leading to cell proliferation and survival [323], lysophosphatidic acid, which stimulates tumor aggressiveness by signaling a family of G-protein-coupled receptors [256], and prostaglandins formed by cyclooxygenases, which support migration and tumor–host interactions [143]. Moreover, fatty acid synthesis participates in the activation of oncogenic pathways, such as Ras, Src, or Wnt [247]. Lipid metabolism also involves important mitochondrial proteins, such as uncoupling protein 2 (UCP2), normally expressed in central and peripheral tissues [88]. Uncoupling proteins have multiple roles, which are tissue-dependent, including heat generation [303], fatty acid derivatives transport [105], and control of oxidative stress [23]. In some tumor models, high expression of UCP2 was observed to be associated with malignancy, increased aerobic glycolysis, and resistance to apoptosis [265].

Mitochondria are responsible for a significant part of ROS as well as reactive nitrogen species (RNS) generation in cells [38]. Both ROS and RNS act as biological mediators by regulating mitogen-activated protein kinases (MAPKs) essential in signaling pathways involved in cell survival, proliferation, and differentiation [222]. ROS are mostly produced by mitochondrial complexes I and III [51]. Complex II has also been shown to be another source, possibly at the FAD coenzyme present in SDHA [145] or in a mutated SDHC subunit [281].

In malignant cells, ROS promote mitogenic signaling, cell survival, disruption of cell death signaling, epithelial–mesenchymal transition (EMT), metastasis, and chemoresistance [54]. In fact, increased uncontrolled mitochondrial ROS production affects HIF-1 by stabilizing HIF-1α, the oxygen-sensitive subunit, allowing the dimerization with HIF-1β to form an active molecule [85].

The transcription factor p53 regulates ROS production and induces cell death when damage is extensive [253]. Excessive ROS production can damage proteins, lipids, and DNA, leading, in extreme situations, to cell death [299], once ROS production exceeds the capacity of cell antioxidant defenses [54]. In fact, some findings suggest that the mitochondrial antioxidant defenses do not provide efficient removal of ROS, especially $H_2O_2$, in most tumor tissues [48]. Another mitochondrial source of ROS, which has been associated with carcinogenesis, is p66Sch. This adaptor protein seems to promote increased oxidative stress by inhibiting the mitochondrial enzyme manganese superoxide dismutase (SOD2) activity [233]. On the other hand, SOD2 is an effective antioxidant enzyme with antitumor activity, since its overexpression results in inhibition of tumor growth [14]. In melanoma and some cancer cell types, SOD2 expression was found to be decreased, more likely due to epigenetic silencing [158]. However, other studies are contradictory, showing that SOD2 overexpression in cancers of the gastrointestinal tract is correlated with an invasive and

metastatic profile, resulting in poor prognosis for the patients [169, 250]. Similarly to other proteins, SOD2 has heterogenic expression, probably due to cancer type or developmental stage.

Without having an intrinsic antioxidant activity, the overexpression of the anti-apoptotic Bcl-2 protects against ROS-induced apoptosis by promoting overexpression of antioxidants such as reduced GSH, catalases, and NAD(P)H [194]. At the same time, other studies showed that Bcl-2 induces increased generation of mitochondrial ROS [49]. Even though cancer cells are often shown to have higher ROS production, coupled with high expression of cell antioxidants, the opposite can occur. For instance, a lower than normal generation of mitochondrial ROS was recently correlated to intrinsic chemotherapy resistance of cancer stem cells [92].

Due to the proximity to ROS sources, mitochondrial DNA (mtDNA) is continuously at risk for suffering oxidative damage. In fact, a correlation between altered mitochondrial gene expression and cellular metabolism alteration has been observed in some tumor types. Whereas mtDNA-encoded subunits correspond to catalytic enzymes, nDNA-encoded subunits have functional and structural activities [53]. Thus, the coordination of the expression of nDNA-and mtDNA-encoded genes is essential for normal mitochondrial physiology [53]. In different systems, loss of mtDNA is associated with a decrease in oxygen consumption and increased oxygen tension inside cells [62]. In fact, mutations and altered mtDNA copy number were observed in diverse types of tumors and cancer cell lines (see also Sect. 3.2.3), leading to altered mitochondrial protein expression, morphology, and general physiology [11, 193, 204]. However, since these mutations result in a large range of tissue-dependent phenotypic variation, this complicates the identification of OXPHOS alterations as a unique pathogenic factor [216]. Importantly, mtDNA alterations can even lead to the activation of oncogenes including Ras and a downstream increase in Akt and Erk pathway signaling, besides several metabolic modifications [62].

### *3.2.2 Oncogenes Vs. Suppressor Genes and Mitochondria*

Oncogenes such as Myc, Ras, or Src induce the expression of glucose transporters (Glut), which are associated with tumor invasiveness and metastasis, but also are implicated in the regulation of mitochondrial activity [72]. The Myc gene is essentially engaged in conserved core target genes, which are involved in ribosomal and mitochondrial biogenesis, energy metabolism, and cell cycle regulation [103]. Under normal conditions, Myc stimulates glucose oxidation and lactate production, while under hypoxia, Myc and HIF-1 cooperate to increase pyruvate dehydrogenase kinase 1 (PDK1) activity, leading to OXPHOS inhibition [176]. In addition, Myc can regulate the alternative splicing of the pyruvate kinase (PK) transcript, in favor of isoform M2 (PKM2) [74], which is one of the most regulated enzymes in glycolysis [206] . Pyruvate kinase converts phosphoenolpyruvate to pyruvate and produces ATP in the final step of glycolysis. Pyruvate kinase isoform M2 is the predominant form in many cancer cells [61]. This protein can promote glucose metabolism in cancer cells by increasing lactate production and reducing oxygen consumption [302],

also directly binding to HIF-1, promoting its transcriptional activity [205]. Pyruvate kinase isoform 2 interacts with a specific cell surface marker in cancer stem cells, CD44, whose ablation leads to depletion of GSH and increased generation of intracellular ROS in glycolytic cancer cells [292]. In fact, PKM2 confers cancer cells with resistance to oxidative stress [7]. Regulation of glycolysis by Myc involves several other glycolysis-associated target proteins, including hexokinase 2 (HK2), phosphofructokinase (PFKM), and enolase1 (ENO1) [177]. Loss of Myc results in a profound decrease in the expression of genes involved in metabolism [308], while the activation of Myc and consequent upregulation of glycolysis can direct cells to use other substrates to fuel mitochondria; this allows cancer cells to easily adapt to different environments, including hypoxia and nutrient deprivation [300]. In fact, tumors in which Myc is upregulated are particularly sensitive to the amount of glutamine present, which suggests that Myc is regulated by glutamine metabolism as well [301]. Moreover, Myc induces lipogenic genes contributing to lipid membrane synthesis for fast-growing cells rather than used for fat storage [70]. Therefore, the ability of Myc to induce mitochondrial biogenesis despite glycolysis upregulation makes sense, since cells need a constant supply of amino acids and fatty acids to proliferate, and these are supplied by mitochondria [68]. Interestingly, inhibition of tumorigenesis is obtained after a brief suppression of Myc [164], while in other tumors this is not observed [29]. The evidence suggests that tissue specificity or even mutagenic or epigenetic alterations influence tumor regression following Myc suppression [318, 330].

Another oncogenic protein is Ras, which is mutated in one quarter of all cancers, leading to increased aggressiveness [255]. Ras is associated with metabolic alterations, increased lactic acid accumulation, altered expression of mitochondrial genes, increased ROS production, and significantly decreased OXPHOS activity [124]. Specifically, mitochondrial dysfunction was associated with mitochondrial localization of STAT3, which is regulated by oncogenic Ras, and at the same time promotes mitochondrial respiration and an increase in glycolytic activity [139, 254].

Ras is activated by growth factors to transduce proliferation signals, mediating important pathways such as PI3K/Akt and MAPK [3, 255]. Similarly to Myc, the PI3K/Akt pathway can lead to glycolytic upregulation by diverse ways, including by increasing Glut1 expression [13], stimulating phosphofructokinase activity and increasing the association of hexokinase with mitochondria [258]. Both PI3K/Akt/mTOR and MAPK pathways were shown to be involved in lipogenesis [319]. Increased glycolytic activity is intrinsically associated with the activation of Akt for cell survival [107]. This protein can stimulate glycolysis in a dose-dependent manner, which is correlated with tumor aggressiveness in vivo [107]. Together with a high activity of the PI3K/Akt pathway, the inactivation of phosphatase and tensin homolog (PTEN), a negative regulator of PI3K pathway is often also found [108]. Moreover, the hyperactivity of Akt can also lead to the increase of mammalian target of rapamycin (mTOR) activity, which in turn increases nutrient uptake during tumor cell proliferation [106]. Furthermore, Akt is important in lipid metabolism, activating enzymes involved in cholesterol synthesis, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase and HMG-CoA reductase, and in fatty acids biosynthesis, namely FAS and stearoyl-CoA desaturase [246].

Low intracellular glucose or glutamine levels often result in lower ATP production and increased AMP levels [215]. AMP-activated protein kinase (AMPK) is an ATP sensor that is activated during metabolic stress, promoting cell survival by blocking the cell cycle progression or by inducing biosynthetic pathways for proliferation under harsh conditions. AMPK also participates in the inactivation of mTOR, through phosphorylation of tuberous sclerosis complex subunit 2 (TSC2) [69]. In a regular cell environment, when nutrients are not limiting, cells accumulate biomass and, in some cases, proliferate [333]. Several proteins are involved in this process, including insulin growth factor 1 (IGF-1), epidermal growth factor (EGF), or platelet-derived growth factor (PDGF), which are often absent in cancer [311]. In fact, some cancer cells can proliferate without external growth stimuli, altering the normal function of their downstream targets, Akt and mTOR [311]. Therefore, the mTORC1 complex senses the nutritional status of the cell, linking nutrient availability with proliferative activity [60]. On the other hand, mTORC2 activates Akt, which in turn promotes glycolytic activity, through phosphorylation of several proteins including hexokinase II, and also inhibits apoptosis by activating FoxO3A [114]. FoxO3A can also be activated downstream of HIF-1 during hypoxia, inhibiting a set of nuclear-encoded mitochondrial genes and consequently decreasing mitochondrial mass, oxygen consumption, and ROS production and promoting cell survival [167].

The switch to glycolysis in cancer cells is also associated with the inactivation of the tumor suppressor p53 [140], occurring via defective trans-activation of TP53-induced glycolysis and apoptosis regulator (TIGAR), which is an isoform of 6-phosphofructo-2-kinase with the ability to inhibit glycolysis and ROS generation [103]. Similarly to TIGAR, the mitochondrial protein SCO2, which promotes mitochondrial respiration by inducing the correct assembly of COX complex, is induced by p53, favoring mitochondrial respiration [17]. Moreover, PGC-1α can bind to p53 and modulate the transactivation of pro-arrest and metabolic genes [271]. Silencing or alteration of p53 activity can occur during the development of some types of tumors, especially during hypoxia, impacting the response of cells to DNA damage [274]. Interestingly, a p53-responsive gene, Lpin1, induced following DNA damage and glucose deprivation, is involved in the regulation of fatty acid oxidation in mouse C2C12 myoblasts [10]. On the other hand, p53 can accelerate the development of nearby capillary networks and contribute to minimizing hypoxia, through the consequent inactivation of thrombospondin (Tsp-1), a potent anti-angiogenic molecule [188]. Similarly to Myc, p53 promotes glutamine utilization by upregulating glutaminase 2 [157], but as opposed to the former, it can have an inhibitory effect on the expression of Glut1 and Glut4 [267]. Interestingly, the overexpression of Glut1 was shown to inhibit p53 and Puma activities during growth factor induction [329].

Some TCA cycle enzymes can act as tumor suppressors, including succinate dehydrogenase (SDH) and fumarate hydratase (FH), which convert succinate to fumarate and fumarate to malate, respectively [138]. Interestingly, oncogenic mutations in SDH and FH can result in hypoxia-like response and glycolysis activation due to substrate accumulation, resulting in the development of paragangliomas (PGLs) as well as leiomyomatosis and renal cell carcinoma, respectively [32].

Sirtuins, proteins with de-acetylase activity, also modulate metabolism in cancer. Sirtuin 1 (Sirt1) was found altered in some cancer types, although the data are controversial whether this protein works as a tumor suppressor or as a promoter [82]. Sirtuin 1 acts as tumor promoter when inhibiting the activity of p53 through deacetylation at the C-terminal K382 residue [305]. Interestingly, Sirt1 and PGC-1α can activate HIF2α, and consequently reprogram the metabolism of cancer cells by inhibiting the supply of fatty acids and pyruvate to mitochondrial metabolism, besides the upregulation of angiogenesis via expression of vascular endothelial growth factor (VEGF) [181]. On the other hand, Sirt1 can act as a tumor suppressor by regulating c-Myc, decreasing its activity [27]. Interestingly, both Sirt1 and fatty acid oxidation can be controlled by β-adrenergic/cAMP signaling [47].

Other sirtuins were also pointed out as having a role in tumorigenesis, namely Sirt3 and Sirt5, mitochondrially located sirtuins. Sirtuin 5 (Sirt5) overexpression was identified in pancreatic cancer [231], while a decrease in Sirt3 expression/activity leads to increased ROS production, a shift towards glycolysis metabolism, and tumor growth [117]. Furthermore, a number of studies showed that Sirt3 can control mitochondrial ATP production, possibly through regulating complex I activity [132]. In addition, Sirt3 decreases cyclophilin D (cypD) activity, promoting its dissociation from the adenine nucleotide translocator 1 (ANT1). Sirtuin 3 can also promote the separation of hexokinase II from the outer membrane voltage-dependent anion channel (VDAC), resulting in increased OXPHOS [277]. Sirtuin 3 can prevent oxidative stress through IDH2 activation and decrease chromosomal instability caused by ROS generation through increasing the activity of SOD2 [295, 322]. Both effects may be considered tumor-suppressant activities.

### *3.2.3 Mitochondrial OXPHOS in Cancer*

Mitochondrial OXPHOS complexes are organized in large supermolecular structures, constituted by a diverse number of subunits. Defects in specific complex subunits can alter electron flux through the chain ([208]; Fig. 3.2). Some studies demonstrated the relationship between mitochondrial structure and metabolic state when cells were forced to use OXPHOS to synthesize ATP. In the absence of glucose, some cancer cell lines rapidly show morphological adaptations to the new substrate availability, namely by increasing the synthesis of OXPHOS components, cristae content, and elongation and ramification of mitochondrial network [261]. When cancer cells are made to rely more on glycolysis, the mitochondrial structure appears to become more fragmented [147]. Interestingly, a correlation between decreased levels of fusion proteins MFN2, MFN1, or OPA1 and inhibition of Krebs cycle, decrease of OXPHOS, and stimulation of glycolysis and lactic fermentation was previously observed [52].

One characteristic of some cancer cells is higher $\Delta\Psi m$ when compared with normal counterparts [175]. Mechanistically, this can be explained by mitochondrial membrane composition alterations, decreased proton influx, or a decreased activity of ATP synthase, among other causes [283]. In addition, cells usually regulate their

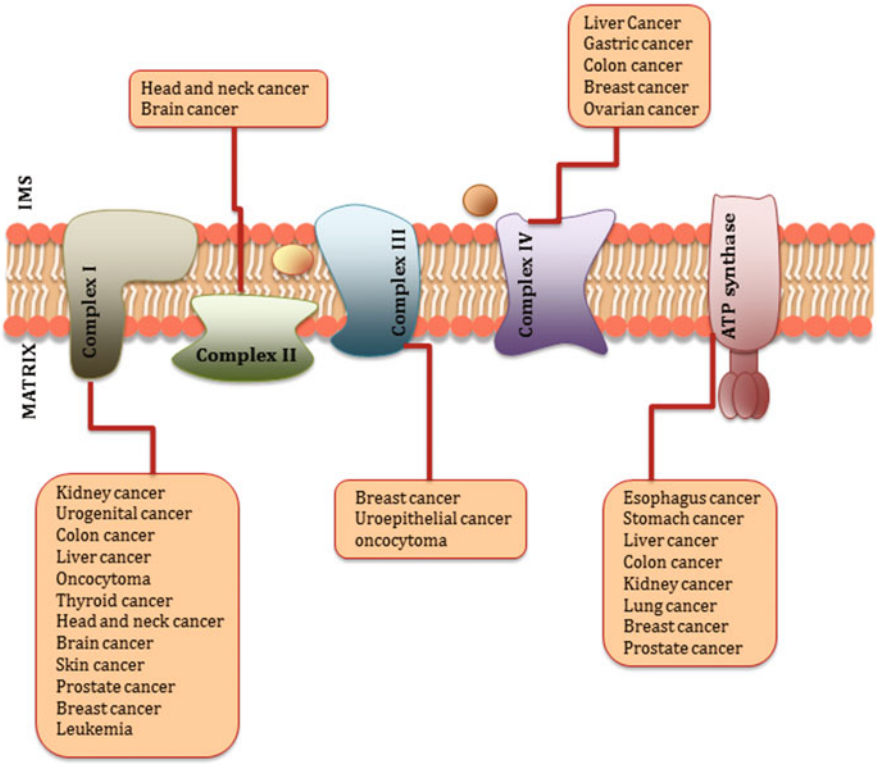


**Fig. 3.2** Different cancer types associated with specific mitochondrial respiratory chain complex alterations. *IMS* mitochondrial intermembrane space

$\Delta\Psi m$ under a certain threshold to avoid the formation of ROS by the respiratory chain, while in cancer cells, an incomplete OXPHOS may lead to higher $\Delta\Psi m$ and increased ROS production [306]. Moreover, the expression of mitochondrial proteins involved in OXPHOS appears to be decreased. Besides the inhibition of OXPHOS by intrinsic cellular signaling, mtDNA and/or nuclear gene mutations or damaged enzymes can also result in lower respiration [48]. Downregulation of mitochondrial proteins leads to general reduction of OXPHOS activity, especially complex I, suggesting that at least in some cases, defective mitochondrial activity is associated with altered cellular metabolism [126].

Mitochondrial complex I is a major site of oxygen superoxide anion production, being also involved in apoptosis and age-related diseases [235]. Moreover, complex I can be regulated by hormones, growth factors, and neurotransmitters [235]. Complex I subunits have been shown to have more significant mutations than any other complex in mitochondria, leading to the development of several diseases, including cancer. Mutations in nuclear or mtDNA genes encoding complex I subunits may result in deficient complex I activity, with ROS overproduction and, consequently, upregulation of nuclear genes such as Mcl-1, HIF-1α, and VEGF [57]. As already

described, these three genes regulate alterations in cell metabolism and metastatic potential [162]. Loss or reduced expression of GRIM-19 and NDUFS3 complex I subunits are present in primary renal cell carcinomas and urogenital tumors [154] and in highly invasive breast carcinoma [288]. Mutations in mitochondrial NADH dehydrogenase (ND) subunit 1 gene are present in patients with renal adenocarcinoma [48], colorectal carcinoma [325], hepatocellular carcinoma [195], and thyroid carcinoma, contributing to a decrease in enzymatic activity [25]. Mutations in the subunits ND2 and ND4–6 are present in thyroid cancer cell lines and renal oncocytomas [127], which also show low oxygen consumption, increased ROS production, and glucose dependency, besides fast tumor growth [236]. Particularly, the demethylation of the D-loop regulates ND2 expression in colorectal cancer [113], while mutations in ND subunit 4 have been identified not only in acute myeloid leukemia, but also in head and neck squamous cell carcinoma [67]. Finally, complex I subunit ND6 was described to be decreased in prostate cancer [66].

Complex I is also a caspase-3 and Calpain 10 substrate. Caspase-3 cleaves the largest subunit of the complex (p75), inhibiting its activity leading to mitochondrial membrane potential disruption and ROS production [174]. Upon increased calcium accumulation, Calpain10 inhibits complex I [9]. Complex I dysfunction can also promote fibroblast activation, through increased ROS generation, and melanoma cell invasiveness [291]. In extreme situations, where complex I is lost, oxiphilic tumors and oncocytomas can be originated, showing upregulation of the other mitochondrial complexes [331]. Mitochondrial complex I is, in fact, considered a sensible pacemaker of mitochondrial respiration [235].

Mutations in nuclear-encoded complex II subunits were associated with the occurrence of specific tumors [156]. Complex II, or SDH, is composed of four distinct subunits (SDHA, SDHB, SDHC, and SDHD) and is the only complex totally encoded by nuclear DNA. Loss of function or mutations in SDHB, SDHD, and SDHC (although in a lesser degree) can result in head and neck PGLs, extra-adrenal PGLs, and pheochromocytomas [35]. Tumors appear to be more aggressive when mutated SDHB is present, having a poor prognosis and metastatic potential [35]. Many mutations in complex II that are associated with cancer development occur in an iron–sulfur (Fe–S)-containing subunit. These tumors exhibit high levels of HIF-1α expression, promoting the downregulation of SDHB expression [46]. Hypoxia can further inhibit complex II activity, promoting an increase in ROS [201]. Mutations in SDHC can result in increased superoxide anion production and consequent oxidative stress, increased glucose consumption and genomic instability [281]. Interestingly, the downregulation of complex II subunits does not promote cell death; however, specific inhibition promotes it [197, 198].

Complex III has also been implicated in carcinogenesis, by being involved in generating ROS that is required for HIF hypoxic activation [179]. Complex III mutations in cytochrome *b* are found in human breast cancer cells [293] and murine and human uroepithelial carcinoma, which have in common increased ROS and lactate production, high oxygen consumption and induction of tumor growth, invasiveness, and immune system detection escape [73]. Although complex III is present in low amounts in oncocytoma [25] and breast cancer [251], UQCRFS1 (encoding RISP

protein) and UQCRH (encoding Hinge protein) complex III subunits were found to be overexpressed in human breast cancer cell lines and primary tumors [232].

Complex IV (COX) is the terminal step in the ETC, responsible for the conversion of $O_2$ to $H_2O$ [160]. In fact, the expression of COX subunits is regulated by oxygen [121]. Therefore, it was suggested that reduced oxygen levels lead to isoform rearrangement, where COXIV-1 is degraded by mitochondrial protease LON and COXIV-2 is increased, resulting in optimization of COX activity for the new hypoxic condition with minimal ROS production [34, 121]. However, virtually all oxygen is consumed and the decrease of hydroxylase activity would result in activation of the HIF pathway [297]. Consequently, differential expression of COX subunits, namely low expression of COXII and high expression of COXI and COXIII, was detected in hepatoma, colon, and prostate cancer [1, 155, 289]. High expression of COXI is also associated with gastric tumorigenesis and ex vivo de-differentiation [207], while mutations in COXI are associated with prostate cancer [241]. In 40 % of breast and ovarian tumors, a decrease in COX subunit II expression was identified [86]. The COXVa subunit has a role in migration and invasion of non-small-cell lung carcinoma cells [55]. A metastasis-associated mechanism, involving Wnt/Snail signaling, suppresses mitochondrial respiration and COX activity, inducing a metabolic switch to glycolysis and pyruvate carboxylase expression [196]. Interestingly, expression of COX levels varies significantly between tissues, being higher in the liver [115]. Whether this impact regulates COX activity/role in cancers in the liver versus other tissues remains to be known.

The downregulation of β-F1-ATPase is considered a feature of liver, kidney, colon, breast, and many other human carcinomas, where its reduction was correlated with increased expression of some glycolytic markers [63, 163]. Specifically, alterations of ATP6 subunit were found in prostate cancer [1], as well as in in vitro tumor models with decreased respiration rates, high proliferation, and significant resistance to apoptosis [276]. The natural inhibitor Factor 1 (IF1) of ATP synthase is also overexpressed in human cancer cells [264]. Altogether, overexpression of IF1, the limited expression of the catalytic β subunit, and upregulation of glycolytic proteins lead to inhibition of ATP synthase activity [96].

Interestingly, the most aggressive cancers have little or no mtDNA content [211]. Indeed, although $\rho^0$ cells, which lack mtDNA, have similar mitochondrial membrane potential to cancer cells [211], the former have increased capacity to invade neighboring tissues and promote metastasis [211].

### *3.2.4 Tumor Oxygen Gradients and Mitochondrial Respiration*

Evidence suggests that cancer cells and the other microenvironment constituents co-evolve during the process of carcinogenesis [245]. The expression of metabolic biomarkers is altered according to the distance from the nearest vessels [280]. In fact, increased glucose uptake, hypoxia, and acidosis are not always fairly distributed in the tumor [56]. The microenvironment of tumors is heterogeneous due to inefficient blood supply, creating nutritional as well as metabolic gradients inside the tumor

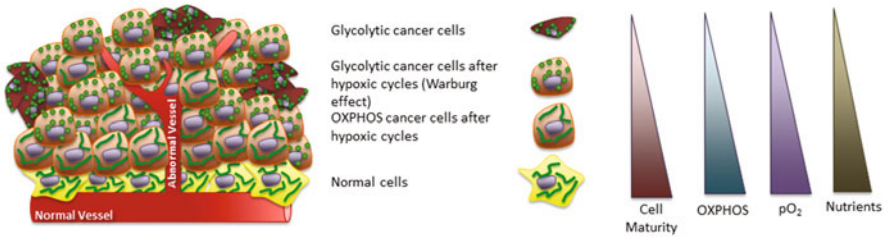


**Fig. 3.3** Mitochondrial metabolism and dependence on oxygen and nutrient gradients within the tumor. The cycles of hypoxia or lack of nutrients can result in different cell metabolism used for adenosine triphosphate (ATP) production. Cancer cells under higher stress preferentially use glycolysis instead of oxidative phosphorylation (OXPHOS), while others with mild strain can maintain their mitochondrial ATP production. Moreover, such mitochondrial metabolic changes can also influence the maturity of the cell, if it is more or less differentiated. Mitochondria are represented as *round* or *filamentous green* bodies

[287]. Oxygen gradient in tumors can be created from both passive physical diffusion and oxygen consumption resulting from cellular activity (Fig. 3.3; [75]). Another possible reason for the differences in oxygen distribution and consequent acidosis in tumors has to do with malformed vasculature [39]. Peripheral cells present high proliferative capacity with full nutritional capacity supplied by blood, while cells with low blood supply present a less active mitochondrial metabolism [102]. In fact, the most aggressive tumors are those found under hypoxic conditions, where they suffer cycles of hypoxia and re-oxygenation [87]. Metabolic demand, vessel morphology, hemoglobin oxygen saturation, and blood flow rate can lead to differential hypoxia cycling in tumors [280]. An increasing distance from the source of nutrients will first promote decreased cell proliferation and later result in its stimulation [119]. The hypoxic core is also the site where cancer stem cells are thought to be maintained in an undifferentiated state [242], thus restraining their oxidative metabolism, again suggesting a close relationship between tumor hypoxic cores and cell immaturity.

As described previously, HIF-1 is activated and modulates the mitochondrial respiratory chain by regulating COX. Therefore, at low oxygen availability, the COXIV-2 isoform is more active and more efficient in using oxygen [121]. These observations explain mitochondrial activity and ATP production even under hypoxic conditions. However, a negative correlation between oxygen gradients and ROS generation is often found in the tumor microenvironment. In fact, cells under a high ROS-prone environment must upregulate antioxidant defenses in order to modulate the malignant phenotype, allowing them at the same time to escape from cell death induction [234]. A signaling gradient of declining transforming growth factor beta-1 (TGF-β1) concentration, which is important during development, is also often deregulated in human tumors. Mitochondrial ATP synthesis can be modulated by TGF-β1, stimulated through ANT1 and ANT2 regulation [191, 200], or inhibited via cyclooxygenase-2 (COX-2) and prostaglandin (PG) E2 [50]. The latter signaling pathway is connected with increased inflammation, ROS generation, altered cytokine/chemokine expression, and enhanced signaling via nuclear factor kappa B (NFkB), which combined results in increased risk factors for carcinogenesis [170].

Besides the variability of oxygen tension within the tumor microenvironment, cancer-associated fibroblasts (CAFs) are able to mimic hypoxia, expressing HIF-1 without real oxygen deprivation [298]. Interestingly, TGF-β signaling and consequent metabolic reprogramming of CAFs are activated due to the loss of caveolin-1 (Cav-1) [44]. In CAFs, glycolytic enzymes are upregulated, while OXPHOS pathway is downregulated leading to overproduction of pyruvate and lactate that will fuel the surrounding cancer cells' metabolism, a phenomenon called "reverse Warburg effect" [26]. Moreover, Cav-1 seems to contribute to glucose uptake and ATP generation, through HMGA1-mediated Glut3 transcription [146]. Therefore, these results can help to explain the existence of cancer cells showing increased aerobic glycolysis in oxygenated tumor regions. Indeed, CAFs can even mediate EMT and enhance the motility response of cancer cells [131].

Unfortunately, much needs to be done to confirm the present ideas, especially the reverse Warburg effect in vivo. Measuring oxygen gradients in intact tumors has also been hard, making the identification of gradients in mitochondrial respiration difficult. Some techniques to measure oxygen gradients are available, including measuring oxygen supply at the microvessel level by using microelectrodes and phosphorescent lifetime imaging with *p*O2-calibrated dyes [280]. Immunohistochemistry aimed at evaluating hypoxia gradients by detecting hypoxic markers is another possible technical approach [263].

From the previous sections, it is evident that mitochondria and the process of carcinogenesis are interconnected. Whether mitochondrial alterations are causally linked with cancer or are merely a small component of a larger metabolic remodeling is still under debate, although it appears that mitochondrial alterations are a piece of a more complex puzzle. Whatever the mechanism is, it is clear that mitochondria are important targets in cancer therapy. Therefore, the design and synthesis of effective pharmaceutical agents that would directly target mitochondrial alterations and decrease tumor size can be achieved. In addition, the differential metabolism used by normal and cancer cells can provide knowledge to discover new drugs with little or no side effects on normal cells.

## 3.3 Targeting Tumor Mitochondria—Closing Down the Factory

Distinct approaches to control cancer are available such as surgery, radiotherapy, and hormone and biological therapies. However, in many cases, those methods are clearly not fully effective, so chemotherapy is usually another tool to eradicate cancer. Unfortunately, the low specificity and the fact that the drugs currently in use have uncomfortable side effects drive the search for more effective and selective drugs.

Guchelaar et al. [142] and Decaudin et al. [78] were the first to point out mitochondria as a potential target for anticancer drugs, proposing the modulation of extrinsic and intrinsic regulators and finding developing chemotherapeutics that would act on mitochondria. Later, a new term, mitocan, was coined to refer to all compounds that exert their action by targeting mitochondria.

The first goal in chemotherapy administration is reached when the drug is selectively accumulated by the tumor. Furthermore, the drug needs to get in the tumor cell and reach mitochondria. The selective accumulation of promising anticancer molecules inside mitochondria of tumor cells, thus sparing normal cells, is a key point in the design of novel molecules [220]. The design of mitochondrial-directed agents, by either chemical conjugation or targeting transporters, has demonstrated promising efficacy; however, their specificity is still discussed. New agents specifically target cancer cells when fused with peptides that recognize cancer-cell-specific surface receptors or internalized through the plasma membrane due to the biological activity of the molecule. Furthermore, if the agent contains a lipophilic cationic moiety, its accumulation by polarized mitochondria, which are negatively charged in the matrix, increases several fold [122]. Thus, the extent to which a drug may interact or even bind to subcellular components, such as membranes and cell organelles, depends on the physicochemical properties of the drug. In order to reduce undesirable side effects, which may result from the drug being accumulated in wrong tissues or in normal cells, or even in wrong organelles, efficient mitochondria-specific delivery systems have been proposed.

To specifically target mitochondria, distinct approaches can be found, including delocalized lipophilic cations (DLCs), mitochondrial targeting sequence (MTs)-containing polypeptides, synthetic peptides and amino-based transporters, and vesicle-based carriers, as reviewed by Weissig and Souza [314]. Unfortunately, many of these strategies can fail if the compound does not reach tumor cells. In fact, several potent anticancer candidates have been shelved due to low solubility and low membrane permeability. It is not easy to design a drug that would combine all essential properties regarding bioavailability and high pharmacological activity [314]. The mechanism by which mitochondrial drugs trigger apoptosis depends on the molecular mitochondrial target site. Nowadays, the vast majority of conventional anticancer drugs activate death pathways, using multiple activation routes (e.g., p53 or death receptors) in order to exert their cytotoxic action [89]. Many of these agents fail due to disruption of endogenous apoptosis-inducing pathways in tumor cells. Newer and more specific therapies have become more prevalent in the treatment of specific cancers as the molecular mechanisms of carcinogenesis become better characterized.

### *3.3.1 Targeting Mitochondrial Feeding*

Although not technically mitocans, some compounds will target different steps of the glycolytic pathway, preferentially affecting those tumors that rely on glycolysis. Inhibition of glycolysis can lead to increased tumor susceptibility to common anticancer agents with minimal effects on normal cells [136]. For example, ATP depletion and consequent death by dephosphorylation of pro-apoptotic BAD protein as well as BAX-induced outer mitochondrial membrane permeabilization were observed when the energy-depleting agent 3-bromopyruvate (3BrPA) and glucose analog 2-deoxy-D-glucose (2DG) were used together [79, 328].

3BrPA is a lactic acid analog known for its alkylating activity, selectively targeting hepatocellular carcinoma cells in vitro [182]. In vivo,3BrPA suppresses metastatic lung tumors with no apparent side effects [130]. This compound suppresses glycolysis by inhibiting the activity of hexokinase and by interfering with VDAC–hexokinase interaction. 3BrPA is believed to enter the cancer cell via lactic acid transporters that are overexpressed in these cells [238], and inhibits SDH activity and mitochondrial respiration [182]. 3BrPA alone promotes cell death of AS-30D hepatocellular carcinoma cells which exhibit the "Warburg effect," while in combination with other chemotherapeutics, such as [Cu(isaepy)2], a DLC-like molecule, inhibits mitochondrial oxygen consumption and produces ROS leading to cell death [118, 237]. Moreover, an in vivo antitumor effect in hepatic and pancreatic cancer was observed in combination with the 90-kDa heat-shock protein (HSP90) inhibitor geldanamycin [42]. 2DG, in turn, is a non-metabolizable glucose analog used in human lymphoma cells to inhibit glucose metabolism and which, in combination with tumor necrosis factors (TNF), induces apoptosis [134]. 2DG suppresses intracellular ATP and potentiates phosphatidylserine exposure induced by Fas [134]. Certain pancreatic tumors, with specific Glut-1 expression profiles, were shown to be susceptible to 2DG, due to greater accumulation of this drug [209]. 2DGwas also used as adjuvant in combination with ETC blockers, which were particularly effective against colon cancer cells [28].

Dichloroacetate (DCA), structurally similar to pyruvate, stimulates OXPHOS through inhibition of pyruvate dehydrogenase kinase (PDK), hence activating pyruvate dehydrogenase (PDH) and shifting metabolism from glycolysis to glucose oxidation. Michelakis et al. [214] observed that DCA leads to mitochondrial depolarization and increased mitochondrial ROS generation, leading to death of glioblastoma multiforme cells, both in vitro and in vivo. The mechanism of action involves targeting PDK II, highly expressed in this type of cancer. When associated with irradiation or etoposide, DCA induces apoptosis of glioma cancer stem cells in vitro, inducing the overexpression of BH3-only proteins (Bad, Noxa, and Puma), while reducing their growth in vivo [226]. Interestingly, DCA has higher activity in cells with defective mitochondria, presenting an effective synergistic effect with other mitocans [285]. Unfortunately, DCA does not have a selective activity, acting on both cancer and normal cells, although DCA has also been used to treat mitochondrial diseases [285]. Therefore, this compound is not a good solution in cancer cells with functional mitochondria, suggesting that DCA may benefit only a selected subset of patients. Another strategy to control glycolysis is through the suppression of glucose transports. Sensitizing tumor cells with phloretin, a glucose transporter inhibitor, enhanced the activity of daunorubicin [41].

Lipid metabolism has been a potential target for antitumor therapy with enzymes such as FAS, ACC, or ACL being good targets. Their downregulation was shown to decrease the proliferation of tumors [290]. Moreover, statins, the cholesterol-lowering agents, were shown to reduce the incidence of some cancers, and also to improve chemotherapy efficacy [33]. Palmitoylcarnitine and carnitine can induce apoptosis in transformed cells by increasing the synthesis of ceramide, a pro-apoptotic lipid, as well as by inducing glucose and fatty acid oxidation, leading to mitochondrial ROS production [316].

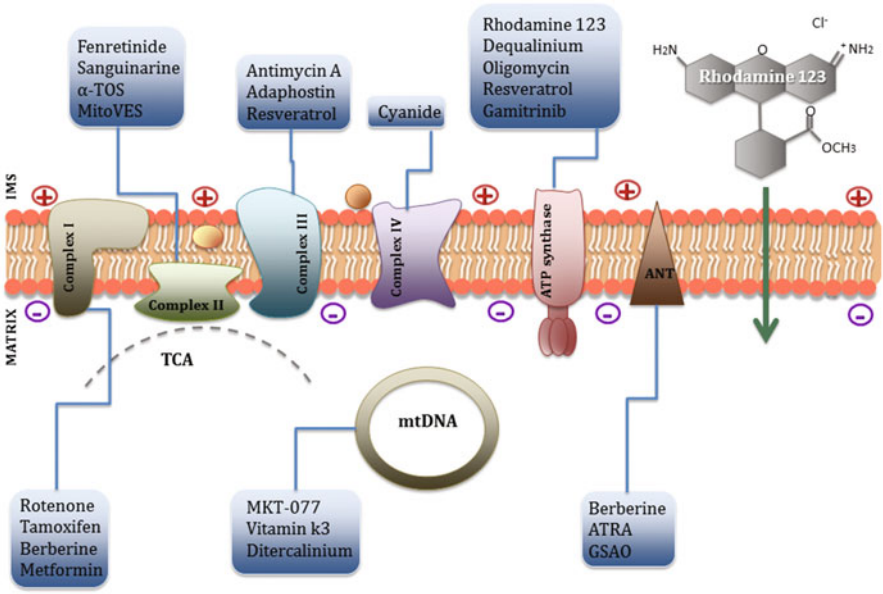


**Fig. 3.4** Mitochondria-targeting agents. Cancer cells have altered metabolism, conferring benefits for cell survival and chemotherapy resistance. Several agents are currently under clinical trial to selectively target mitochondria in tumor cells and alter their physiology. One strategy is by using the higher mitochondrial membrane potential ($\Delta\Psi m$) normally found in several tumors (e.g., Rhodamine 123). Several agents target components of the respiratory chain, the adenine nucleotide translocator (ANT), or mitochondrial DNA (mtDNA). Disturbance of mitochondrial function in cancer cells can result in the induction of apoptotic cell death. *TCA* Tricarboxylic acid cycle, *ATRA* All-trans retinoic acid, *GSAO* glutathione-coupled trivalent arsenical, *α-TOS* α-Tocopheryl succinate, *IMS* mitochondrial intermembrane space

For some cancer types, the inhibition of glycolysis per se is not enough, since cancer cells can adapt by remodeling their metabolism with tumor recurrence likely to occur . In those cases, targeting different metabolic pathways may be the solution.

### *3.3.2 Targeting Mitochondria*

By taking advantage of mitochondrial alterations in several cancer types, specific mitochondrially targeted agents can be designed (Fig. 3.4). For example, some cancer cells present higher $\Delta\Psi m$ when compared with non-tumor counterparts [8]. Thus, positively charged lipophilic molecules can be designed to accumulate inside mitochondria, disrupting the organelle and causing cell death. For example, the positively charged Rhodamine-123 is preferentially accumulated in mitochondria of cancer cells, showing a higher degree of toxicity towards them [221]. Rhodamine-123 and analogs are a clear example of using a biophysical characteristic of mitochondria in cancer cells (i.e., higher $\Delta\Psi m$) to undergo selective toxicity and accumulation [190]. Once accumulated by mitochondria in cancer cells, $\Delta\Psi m$ is disturbed and

Rhodamine-123 inhibits the $F_oF_1$-ATPase [219]. Rhodamine-123 has also been used in conjugation with other compounds, such as 2DG, in the treatment of human breast carcinoma. The two compounds jointly inhibit the growth of cancer cells, whereas no toxicity was observed in normal cells [24]. A similar effect was observed during in vivo studies, suggesting that the disturbance of OXPHOS and glycolytic pathways in tumor cells can be an effective treatment [19]. Cyanine analogs, including MKT-077, are also preferentially accumulated in tumors with higher $\Delta\Psi m$ [312]. Although tested during phase I clinical trials, further trials with MKT-077 were stopped due to renal toxicity in some patients [31, 312].

Berberine, a phytoalkaloid presenting a positive charge in its structure, is accumulated in tumor cells at low concentrations [273]. Berberine targets the respiratory chain by inhibiting mitochondrial complex I and interferes as well with the mitochondrial phosphorylative system [239], especially with the ANT [240]. Berberine also induces apoptosis by increasing ROS production, leading to overexpression of p53 and downstream apoptotic proteins [166]. Another phytochemical, sanguinarine, disrupts mitochondrial calcium loading capacity and increases p53 expression [272]. Sanguinarine interferes with the mitochondrial respiratory chain, namely at complex II [12], and causes ROS-induced DNA damage [58], GSH depletion, and cleavage of poly (ADP-ribose) polymerase and beta-catenin [59]. Dequalinium and F16 are other lipophilic cations with mitochondrial disruptive effects [111, 313]. However, there are no current clinical trials with any of these molecules.

Agents that interfere with mitochondrial respiration, including OXPHOS uncouplers cause cell death due to bioenergetic disruption . Numerous inhibitors of the mitochondrial respiratory chain are used as tools to better understand mitochondrial respiration; however, in general, these mitochondrial poisons are toxic in vivo, due to their nonspecific activity. Classic mitochondrial poisons include rotenone (complex I), antimycin A (complex III), cyanide (complex IV), and oligomycin (complex V, or ATP-synthase), besides protonophores such as carbonylcyanide trifluoromethoxyphenylhydrazone (FCCP) [97]. These and other mitochondrial inhibitors decrease the capacity to stimulate ROS production and apoptosis of cancer cells. For example, tamoxifen targets complex I [224], fenretinide inhibits complex II [65], and complex III is predominantly inhibited by adaphostin [192]. Alternative molecules presenting lower toxicity have been developed: α-Tocopheryl succinate (α-TOS) is a vitamin E analog capable of preferentially targeting mitochondria in cancer cells, inducing proliferation arrest [249]. α-Tocopheryl succinate is tumor-selective due to its ester structure, since the hydrolysis of α-TOS to α-tocopherol occurs in normal cells but not in tumor cells [168]. Moreover, α-TOS induces cell death by targeting the ubiquinone-binding site at complex II, causing electron leakage, stimulating ROS generation and killing malignant cells at nontoxic concentrations for normal cells [100, 230]. α-Tocopheryl succinate facilitates the translocation of Bax from the cytosol to mitochondria and subsequent cytochrome *c* release [321]. α-Tocopheryl succinate also induces apoptosis in proliferating endothelial cells by causing oxidative damage and suppressing angiogenesis in vitro and in vivo in different breast cancer models [99]. Another compound with a similar activity to α-TOS is mitoVES [101].

Resveratrol is polyphenolic phytoalexin, found in the skin of red grapes, berries, and peanuts, and which presents with chemotherapeutic and chemopreventive properties [165]. Resveratrol induces the redistribution of Fas/CD95 and TRAIL receptors in lipid rafts in colon carcinoma cells [81]. Resveratrol also decreases ROS production by competing with coenzyme Q and decreasing complex III activity [332]. Nitric oxide production, caspase activation, and p53 are also necessary for the mechanism of action of resveratrol in tumor cells [178]. In normal cells, resveratrol increases mitochondrial capacity by activation of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), which in turn stimulates sirtuin 1 (SIRT1) [189]. Nevertheless, structure–activity studies showed that resveratrol can interfere with mitochondrial ATP synthesis by binding to F1-ATPase, which may contribute to cell death induction [133]. Resveratrol has a low bioavailability [307]; hence, structural modifications may increase its clinical usefulness. In fact, a complex between triphenylphosphonium and resveratrol leads to mitochondrial accumulation of this compound [21]. Resveratrol is currently under clinical evaluation for colon cancer and multiple myeloma treatments [144, 282]. Moreover, resveratrol and other polyphenols are claimed to activate Sirt 3 [132]. Upregulation of this mitochondrial sirtuin may have a similar effect to that of DCA, which increases mitochondrial metabolism and disturbs cancer cell homeostasis.

Both hormones, insulin and insulin-like growth factor, are associated with a range of cancers [244]. Evidence shows that obese and diabetic individuals are a risk group for the development of cancer, and also have a worse prognosis in the event of the disease. Metformin is an anti-glycemic agent used in type 2 diabetes, thought to decrease cancer incidence [296]. Metformin is an AMPK activator and inhibits complex I in human breast cancer in situ [317], also increasing tumor cell sensitivity to chemotherapy [125]. However, caution is required in patients with diabetes since the use of metformin as adjuvant may not be as effective, because these patients may already have a long-term prescription [199]. Metformin also compromises the growth of breast cancer tumors in mice, by modulating endoribonuclease Dicer (DICER), through mir33a upregulation and by targeting c-Myc [22].

Other drugs can target other mitochondrial structures. Lonidamine is an inhibitor of aerobic glucose utilization and can also directly interact with hexokinase [110]. Arsenic trioxide ($As_2O_3$) triggers cancer cell death by inhibiting thioredoxin reductase and promoting oxidative stress [93], which has been shown to be effective against acute promyelocytic leukemia (APL) [5]. Arsenic trioxide has also been used in combination with all-trans retinoic acid (ATRA) showing a synergistic effect against APL mouse models [210]. ATRA is a natural derivative of vitamin A, which stimulates the expression of retinoic acid receptor-responsive genes [210]. This compound suppresses mitochondrial respiration, decreases $\Delta\Psi m$, and triggers ANT-dependent MPT and cell death independent from nuclear receptor binding, suggesting another potential mechanism of action is involved [228]. The potential of $As_2O_3$ and ATRA in the treatment of other cancer types is also being explored [326]. A GSH-coupled trivalent arsenical compound (GSAO) causes apoptosis in angiogenic endothelial cells both in vitro and in vivo, although it was initially suggested that proliferating cancer cells would be targeted as well [98]. However,

low toxicity towards the latter was observed [98]. GSAO can inhibit ATP/ADP transport by cross-linking two of the three matrix-facing cysteine thiols in the ANT. This will lead to ATP depletion, ROS generation, and ultimately mitochondrial depolarization and apoptosis [98]. Angiogenic cells can often circumvent many therapies; however, these cells have a decreased capacity to buffer the arsenical moiety by expressing low MRP1/2 [37]. GSAO is currently in clinical trials in cancer patients and promising results are anticipated [37, 94] .

HSP90 is not normally present in mitochondria of normal cells; however, this chaperone is upregulated in mitochondria in cancer cells, due to a possible induction by Ras and Akt oncogenes [243]. HSP-90 is an ATPase-directed molecular chaperone that supervises protein folding during cellular stress responses, with the protein complexes involved in cell proliferation and cell survival [243]. The molecular chaperone Hsp90 provides an attractive target for therapeutic interventions in cancer. Shepherdin is a peptidomimetic that is easily accumulated in mitochondria, and which is an antagonist of the complex between Hsp90 and survivin (cell cycle-regulating protein), plus other additional client proteins such as TRAP-1 [278]. Shepherdin inhibits Hsp90 chaperone activity via an ATP competition mechanism and kills cancer cells by inducing the mitochondrial permeability transition (MPT) [278]. Shepherdin showed no toxicity for brain and liver mitochondria in several human cancers [172, 243]. Gamitrinib was conceived by coupling an HSP90 inhibitor to lipophilic cationic moieties. Gamitrinib specifically targets mitochondria in cancer cells, and antagonizes the ATPase activity of HSP90. Gamitrinib causes the death of cancer cells and suppresses tumor growth in vivo, with no apparent effect on normal counterparts [173].

Some test compounds specifically target mtDNA. A vitamin K sub-type, vitamin k3, is a synthetic compound that has been described to inhibit DNA polymerase $\Upsilon$, thus disturbing mtDNA replication and promoting ROS generation leading to apoptosis [266]. However, vitamin k3 can interfere with calcium homeostasis and decrease GSH levels as well [95]. In vitro studies demonstrated that vitamin k3 displayed antitumor activity against pancreatic and breast cancer cells [2]. Ditercalinium is another agent which is preferentially accumulated in mitochondria, and that targets mtDNA, inhibiting replication [229]. After treatment with ditercalinium, ultrastructural studies showed a depletion of mtDNA and loss of mitochondrial cristae [268]. Agents that disturb mtDNA are predicted to affect mitochondrial respiration by leading to loss of OXPHOS subunits encoded by the mitochondrial genome.

## 3.4 Concluding Remarks

The present chapter demonstrates that the profound metabolic remodeling of cancer cells, including mitochondrial rearrangement, not only is an indirect response to cell survival or proliferation but also can be controlled by specific cell signaling [310]. Nevertheless, there are no specific mitochondrial or metabolic alterations common to all cancer types, although the activation of different metabolic pathways results in similar phenotypes. There are no doubts that mitochondrial deregulation and

metabolism remodeling are important hallmarks of cancer cells; however, as pointed out, there are other cases where an altered metabolic pattern is not observed. Besides, many proteins involved in carcinogenesis have dual and opposite functions even inside the same tumor. It is also important to take into account the model that is being used to evaluate the protein activity, since many of them vary their behavior between in vitro and in vivo situations [334]. The large number of functions mitochondria have in cells implies that many of those may be altered during cancer, some of which will contribute to carcinogenesis while others will act as tumor suppressors. The mitochondrial respiratory chain has an important function not only in the context of ATP production, but also in maintaining a determined redox balance. A specific tumor signature requires that each one of these functions is altered somehow to respond to metabolic and survival cues. In the traditional model, a decrease in mitochondrial ATP production, resulting from different factors such as a hypoxic environment or low glucose, will drive the generation of malignant mitochondrial ROS production and trigger mitochondrial biogenesis [253]. Mitochondrial respiration can then be regulated by differential expression of OXPHOS subunits or by upstream signaling and/or metabolic pathways. By its turn, inhibition or stimulation of mitochondrial respiration can feed back onto other cancer cell pathways or even increase genomic instability, thus contributing to higher aggressiveness.

Targeting mitochondria in tumors based on specific respiratory alterations or components implies a type of knowledge that we may not have at the moment. Even inside the same tumor mass, mitochondrial respiration is different according to the oxygen gradient. In the absence of oxygen, mitochondria can still maintain $\Delta\Psi m$ by the reverse action of ATP synthase [284]. This means that compounds targeting the respiratory chain will not work; instead, the inhibition of the ATP synthase in a selective manner in tumor cells is a solution in the future.

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# Chapter 4
# Regulation of Cancer Cell Metabolism by Hypoxia

**Ashleigh Pulkoski-Gross, Nikki A. Evensen and Jian Cao**

**Abstract** The growth of a tumor usually results in the development of hypoxia, which is primarily a consequence of the tumor outgrowing existing vasculature and the disorganized nature of vascular growth induced by the tumor itself. The low oxygen tension at the site of neoplastic growth has a significant effect on the metabolic status of the cells involved. In order for the cells to survive the harsh conditions of low oxygen and nutrition, the metabolism of the cell switches from an aerobic type of metabolism to an anaerobic one, relying primarily on glycolysis for the production of energy and metabolic intermediates that feed various biosynthetic pathways. Because this phenotype is associated with increased cell survival, drug resistance, and ultimately poor patient prognosis, the metabolic components and the mediators of the hypoxic response are viable targets in the war on cancer. Currently, a variety of drugs are being explored that influence the mediators of the hypoxic response, such as hypoxia-inducible factor-1 (HIF-1), and those that target metabolic enzymes directly. These agents show promise in improving the current standard of care by acting in a synergistic manner with current cancer therapies.

**Keywords** Tumor hypoxia · Metabolism · Hypoxia-inducible factors · Glycolytic switch · Metabolic targeting · HIF inhibition

## 4.1 Introduction

The term "hypoxia" refers to conditions of low oxygen and, in the medical field, relates to a lack of sufficient delivery of molecular oxygen to a target tissue. While hypoxia is a complication of a variety of diseases, this chapter endeavors to define the classes of hypoxia and its consequence at a tumor site. Tumor vasculature and the phenomenon of angiogenesis has been regarded by the medical community for

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quite some time, beginning with Virchow's observations of abnormal vessel growth published in 1863, followed up by the postulation by Goldmann that tumor growth is related to vessel growth [1, 2]. Thomlinson and Gray in 1955 reported their observations that lung carcinomas greater than 200 μm in size have necrotic centers and tumor cells cannot grow greater than 180 μm away from the stroma, leading them to suggest that a lack of oxygen may be the cause and associated the possible lack of oxygen with cancer progression [3]. Volumes of work on the angiogenic nature of tumors and the consequences of it on both cancer cell behavior and clinical outcome have followed. It is estimated that approximately 60 % of tumors develop pockets of hypoxia [4]. Overall, tumor hypoxia is associated with poor clinical prognosis [4]; no matter the mechanism by which tumor hypoxia develops, the end result is a mass that consists of treatment-resistant, aggressive cancer cells [4]. This phenotype allows for local invasion and metastasis, which is ultimately the reason for failure of treatment in a majority of patients suffering from neoplastic diseases. Therefore, it is imperative to define and understand all facets of this phenomenon in order to modify current treatment strategies and develop novel, effective therapies. Herein, we will demonstrate the current understanding of hypoxia and its effects on cancer cell metabolism, leading to an exploration of the current and future hypoxia-targeting strategies.

## 4.2 Tumor Hypoxia

I. What is tumor hypoxia?

Typically, normal tissues maintain a partial pressure of oxygen ($pO_2$) that is > 20 mmHg, with a few exceptions of specialized tissues [5–7]. $PO_2$ in tumors is usually less than that of its normal counterpart [8]. In general, 10 mmHg $pO_2$ is regarded as hypoxic; values of < 2.5 mmHg have been reported in experimental tumor models as severe hypoxia with anoxia reported in some cases [9–12]. Multiple studies involving human tumor tissue have revealed hypoxic patterns, some of which specifically reveal that hypoxic pockets develop as tumor size increases [8, 13–17]. While hypoxia is a broad term that refers to a status of sub-oxygenated tissue, it is naive and potentially misleading to consider tumor hypoxia as a single, static phenomenon. The most accurate way in which to contemplate the tumor context is as an area that has constantly fluctuating oxygen levels that vary throughout the tumor volume, depending upon the size of the tumor [17–19]. Two broad classifications of hypoxia include chronic and acute, also referred to as sustained or long-term hypoxia and intermittent, cyclic, fluctuating, transient, or repetitive hypoxia, respectively [4, 20]. Acute hypoxia is generally considered to be a perfusion limitation on the tissue, while chronic hypoxia is a diffusion limitation issue. These general groupings are not sufficient, however, as acute and chronic hypoxia can be further classified based on the mechanism by which oxygen delivery is impeded [20].

II. Acute Hypoxia

Acute hypoxia generates in a tumor based on brief blood flow stops and hypoxemia and has been experimentally associated with increased metastases [21]. This particular type of hypoxia was directly observed as a separate event from

chronic hypoxia in 1987 [22]. Since then, it has been recognized that acute hypoxia can generate in a variety of ways. Ischemic acute hypoxia is related to blood vessel obstruction, whether by tumor cells, blood cells, abnormal microclotting, or the inability of the local vasculature to perform its normal vasomotor activities [20], all of which result in a reduction in tissue perfusion. Both structural and functional abnormalities of the vasculature contribute to the development of hypoxia (review Ref. [23]). Hypoxemic hypoxia refers to an impaired delivery of oxygen as a result of reduced numbers of red blood cells or flow reversal among other mechanisms [20]. The collective result of these types of oxygen delivery impediments is a short, repetitive withdrawal of oxygen from the tumor tissue, classically regarded as lasting less than 2 hours per hypoxic event [20]. Acute hypoxia presents its own challenges to treatment strategies, as the hypoxia–reoxygenation cycle should dictate treatment regimens, especially regarding radiotherapies [24].

III. Chronic Hypoxia

Conversely, chronic hypoxia is a condition of extended periods of hypoxia, typically on the scale of hours to days. It falls into three distinct categories: diffusion-limited hypoxia, hypoxemic hypoxia, and hypoxia related to poor microvasculature perfusion [20]. A neoplastic mass is able to outgrow the existing vascular structures, resulting in a game of 'catch-up' for the microvessels. The inability of the vessels to supply a rapidly growing tumor causes pockets of hypoxia, as the diffusion limitation of oxygen dictates that only regions within that limit will be properly oxygenated. Furthermore, despite the ability of a tumor to produce angiogenic signals and encourage growth of new vessels, they tend to lack the proper organization to efficiently deliver blood to the tissue [25, 26]. Chronic hypoxemic hypoxia is an overall reduction of oxygen in the blood, whether it be related to anemia [13, 27, 28], being supplied with poorly oxygenated blood, or having poor oxygen gradients established in the tumor area [13, 20]. Poor microvasculature perfusion is usually caused by leaking vessels that derive from the disorganized nature of the neovasculature [29, 30]. Chronic hypoxia is associated with the development of necrotic centers of larger tumors, as the diffusion limitation of oxygen is approximately 150 μm from a blood vessel [3, 31]. A cell's distance from capillaries determines its mitotic index; the further the cell is from a capillary, the less it will replicate [32, 32]. This type of hypoxia is thought to induce adaptation of the vasculature that can cause reoxygenation to some extent [33]. While this phenomenon may seem advantageous to improving treatment of tumors, restoring oxygen to hypoxic regions of cells has been experimentally shown to support an aggressive phenotype that includes the ability to avoid apoptosis and efficiently form secondary metastases [35].

IV. What is the Consequence of a Hypoxic Tumor Milieu?

The consequences that derive from a hypoxic tumor environment vary depending on the type of hypoxia [20]. Hypoxia is known to induce a multitude of changes in a cell, most of which are mediated through hypoxia-responsive proteins and radical species signaling. The overall outcome of these changes is an invasive, aggressive cancer cell capable of surviving and thriving under the harsh conditions created by the tumor. Not only does hypoxia result in an aggressive population of cells, but it also influences clinical outcomes, in some cases regardless of the primary

treatment [36]. Ischemic hypoxia completely blocks blood flow, thus, not only is the delivery of oxygen impaired, but so is the delivery of nutrition and chemotherapeutics. Hypoxemic hypoxia results in reduced oxygen delivery, but since blood flow is not reduced in any way, delivery of drugs and glucose to the tissue is not compromised [20]. For the most part, subtypes of chronic hypoxia do not have completely abolished blood flow, but delivery of chemotherapeutics and nutrition are limited even though some perfusion of the tissue is intact. Furthermore, hypoxic tissues tend to be more radioresistant because these therapies exert the most damage to the cancer cell's DNA when in the presence of oxygen, meaning the outcome of radiation therapy is less than optimal in a hypoxic milieu [3, 17, 24, 37, 38]. For these reasons, the hypoxic phenomenon has repeatedly been linked to disease progression, metastases, and poor clinical outcome [23, 39–41]. The documented poor patient prognosis is related to a metabolic switch that occurs under hypoxia, as tumor cell survival is dependent on adapting to the altered microenvironment.

## 4.3 Altered Cancer Cell Metabolism

I. Glucose Metabolism

Tumor hypoxia has been shown repeatedly to be associated with negative clinical outcomes; these outcomes are ultimately related to the cell response at the molecular level. Oxygen is a crucial molecule in aerobic cell metabolism, as it acts as a major participant in the reduction–oxidation reactions required for eukaryotic metabolism [42]. Metabolism of glucose in the eukaryotic cell begins with glycolysis, a nine-step process that requires an input of two high-energy adenosine triphosphate (ATP) molecules, but the net of the reactions is two ATP molecules. Further, the process yields two three-carbon molecules of pyruvate, which is a critical molecule for normal respiration. Pyruvate is shuttled to the mitochondrial matrix to participate in the tricarboxylic acid (TCA) cycle, also known as the Krebs cycle or the citric acid cycle. It is here that pyruvate is decarboxylated by pyruvate dehydrogenase to acetyl coenzyme A (acetyl-CoA). These acetyl groups are oxidized; however, this process is not necessarily dependent upon oxygen; the oxygen required for the TCA cycle is provided by molecules of water. The oxidative process that occurs during this cycle provides energy to the electron transport chain (ETC) in the form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), which in turn provides a chemiosmotic gradient for the oxidative phosphorylation (OXPHOS) pathway. Under normal oxygen conditions, eukaryotic cells utilize the ETC coupled with OXPHOS to generate the required ATP to meet the energy needs of the cell via the mitochondria [43]. Oxygen is a key part of this process, as it is ubiquitous and able to diffuse through the cell and act as the terminal electron acceptor of the ETC [44].

Since oxygen is vital to efficiently generating the necessary ATP supply, hypoxia (such as occurs in the case of malignant tumors) can wreak havoc on the normal metabolic processes and phenotype of a cell. A variety of mechanisms exist in a cell that sense oxygen and regulate the response to that stressor [43]. Most mammalian

cells are capable of altering gene expression and protein synthesis in response to hypoxia [45]; these gene products tend to increase the likelihood of survival under hypoxia by contributing to a switch in metabolism from an aerobic process to what is traditionally considered an anaerobic one [46]. Up to 60 % of ATP generated in cancer cells is derived from the anaerobic glycolytic pathway (47–49). This phenomenon was documented and postulated on as early as the 1920s by Otto Warburg. The so-called Warburg effect describes a switch from OXPHOS to glycolysis as the primary mechanism by which ATP is generated in cancer cells, which leads to a tumor's signature, increased lactic acid production [50–52]. Warburg hypothesized that a dysfunction of the mitochondria was the cause of the glycolytic switch, but more recent evidence has shown that this is not necessarily the case [47, 53, 54]. While this may seem to be a wasteful form of energy production for highly proliferative cells since glycolysis nets only 2 ATP while the process of oxygen-dependent energy generation nets 36 ATP molecules, the upregulation of glucose transporters (e.g., GLUT-1) that allows for an increase in glucose uptake, combined with an increase in enzymes that contribute to glycolysis such as phosphoglycerate kinase-1 (PGK-1), hexokinase (HK), and phosphofructokinase L (PFK-L) [55, 56], may allow the glycolytic flux to be high enough for the ATP production to match the seemingly more efficient ETC and coupled OXPHOS [57]. This upregulation of glycolytic metabolism participants has been correlated to poor prognosis in a variety of cancer types [58–61].

Interestingly, it is still unclear why this glycolytic switch occurs not only in hypoxic cells, but also among cells that have access to oxygen with no damage to their oxidative metabolic pathways [57, 62]. It has been proposed that the metabolic switch not only allows for production of ATP in the absence of oxygen, but also may mitigate apoptotic death (which also relates to metastasis in that cells able to survive periodic hypoxia will likely be able to colonize distant organs) [63, 64]. In 2004, Gatenby and Gillies suggested that the initial hypoxic insult at a tumor site results in a switch to primarily glycolytic metabolism and the survival properties conferred to the cells perpetuates it, even in the presence of oxygen [64]. However, whether the glycolytic switch results solely from hypoxic insult or via another mechanism [65], the dependence on this type of metabolism appears to offer a growth and survival advantage. Despite the idea that glycolysis is a seemingly inefficient method of generating energy, that is not necessarily the primary concern of the proliferating cancer cell. The glycolytic switch provides an advantage to the rapidly proliferating tumor cells by providing materials for cell biosynthesis and allows control over those metabolic pathways that can branch off from the glycolytic cycle [57]. A multitude of anabolic processes are required for cell proliferation, such as triglyceride (TG) and phospholipid synthesis, ribose 5-phosphate (R-5-P) synthesis, and amino acid synthesis and these products derive their precursors from the glycolytic pathway [66]. Therefore, the increase in glycolytic metabolism in the rapidly proliferating cells of a tumor may not be solely related to a demand for ATP under low oxygen conditions, but also to the demand for the precursors of lipids, nucleic acids, and proteins for the expanding cell population [67].

II. Lipids

Cancer cells tend to be rapidly proliferating cells that require an array of macromolecules to sustain clonal expansion, which includes lipids. The increased rate of lipid production supports the ability of the cell to synthesize new membrane for the daughter cells, as well as fatty acids that participate in protein modification and, therefore, signaling [68]. Under normal conditions, the enzymes that are involved in the process of lipid synthesis are expressed at low levels, with the exception of certain tissues such as the liver and adipocytes, because cells will utilize exogenous lipids after import from the extracellular space to construct membrane [69]. However, in the case of malignant cells, most of the fatty acids that are synthesized derive from glucose in a de novo fashion, rather than from being derived from the tumor host [68, 70, 71]. The de novo synthesis of the long-chain fatty acids provides a modifiable unit to form unsaturated fatty acids for membrane construction and TGs for energy storage [72]. Interference with lipid synthesis results in growth arrest and apoptosis, indicating that this is a critical process for cancer cell survival [73].

The de novo synthesis of fatty acids and lipids normally begins with entry of the pyruvate that is garnered from glycolysis into the TCA cycle, generating intermediates in the mitochondria that play a role in lipid metabolism. Citrate is the primary metabolite that is transported out of the mitochondria to the cytosol for processing into fatty acids, which are used to build structural lipids and protein modifications [74, 75]. Once citrate has been modified by the ATP-dependent citrate lyase (ACLY) into acetyl-CoA and oxaloacetate, the acetyl-CoA carboxylase (ACC) processes the molecule of acetyl-CoA to form malonyl-CoA. Fatty acid synthase (FASN), along with an additional acetyl-CoA, is responsible for coupling these moieties repeatedly to create long-chain fatty acids [72]. These three enzymes, ACLY, ACC, and FASN, are essential to the process of fatty acid generation [69]. A commitment to lipid biosynthesis is only made once ACC acts on the acetyl-CoA, however [72]. The expression of all of these enzymes has been shown to increase in the case of tumor cells and has been correlated to an increased risk of recurrence in some cases [73, 76–82].

Ischemic tissues have long been documented to contain an increased accumulation of fatty acids [83]. Under conditions of hypoxia, FASN is upregulated to support fatty acid synthesis, which not only provides the cell with lipids for energy, but also potentially balances some of the redox imbalance in the hypoxic cancer cell [84, 85]. In addition to the upregulation of FASN, hypoxia supports conversion of acetyl-CoA from acetate in the cytoplasm via acetyl-CoA synthetase 2 (ACS-2) to sustain fatty acid synthesis and cell survival [72, 86]. Furthermore, hypoxia induces a switch to reductive carboxylation of α-ketoglutarate derived from abundant glutamine for citrate production via isocitrate dehydrogenase-1 (IDH-1) which can potentially be utilized for fatty acid synthesis [72, 87–89]. The reduction of β-oxidation under low oxygen tension also contributes to the ability of the malignant cell to accumulate lipids, as β-oxidation normally serves to catabolize fatty acids to regenerate acetyl-CoA, NADH, and $FADH_2$ (72, 83). Not only does hypoxia cause changes in lipid metabolism, but the induction of hypoxia-inducible protein-2 (HIG-2) can also contribute to the ability of a cell to form lipid droplets for storage of excess lipids, although phospholipid generation is the primary use for the extra lipids [90, 91].

This is a potential mechanism to prevent toxicity from a buildup of free fatty acids or act as energy storage [90].

In addition to the fatty acid synthesis, neoplastic tissues have been documented to accumulate cholesterol through the mevalonate pathway [72, 92]. Cholesterol is a key component of the cell's lipid bilayer and composes the lipid rafts that organize signal transduction pathways [72]. It is highly regulated through 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (HMGCR), whose expression is increased under hypoxia [72, 93].

III. Amino Acids and Proteins

To meet the demand for macromolecules caused by the proliferation rate of malignant cells, alternative sources of carbon are used, including some amino acids [69]. Anaplerosis refers to the replenishment of metabolites and glutamine metabolism is a key player in this phenomenon. Glutamine is a nonessential, free amino acid that is found in high amounts in the human body and is used as another carbon source [94]. Glutaminase expression and activity are increased during rapid proliferation and tracking labeled carbon via nuclear magnetic resonance (NMR) spectroscopy has shown that glutamine can contribute oxaloacetate to replenish the TCA cycle [69, 95]. Glutaminolysis contributes NADPH to the cellular pool, which is a cofactor that can play a role in multiple metabolic processes and redox balance [69, 95]. Glutamine breakdown primarily produces lactate and alanine, which play roles in preserving amino acid pools [69, 95]. Furthermore, glutaminolysis provides some of the carbon required for fatty acid synthesis and for aspartate, which is required for synthesis of other amino acids. Glutaminases that play a key role in the breakdown of glutamine for these processes have been shown to be upregulated under conditions of hypoxia [96]. To support the cancer cell metabolism, the uptake of amino acids is enhanced under hypoxic conditions, similar to glucose transport in that the transporters are upregulated [97].

In addition to changes in amino acid synthesis, the overall metabolic switch that occurs in response to hypoxic stress, cancer cells also undergo a global shut down of protein synthesis in an effort to decrease energy use since translation accounts for much of the energy consumption of a cell [98]. There is a marked reduction in translation of messenger RNA (mRNA) under conditions of hypoxia compared to normoxic cells within a rather rapid time frame [99, 100]. Inhibition of protein synthesis is a common consequence in response to various other stress stimuli, including endoplasmic reticulum (ER) stress and amino acid starvation. The ER plays a major role in protein production as all proteins destined for the secretory pathway are translated into the ER where they are then further processed by formation of disulfide bonds or addition of sugar moieties [101]. Hypoxic stress has been shown to lead to ER stress by negatively impacting protein processing within the ER. In particular, the formation of disulfide bonds involves the activity of foldases, which are enzymes that catalyze the formation of these bonds by acting as donors and acceptors of passing electrons, with oxygen being required as the last acceptor in this process. The lack of oxygen as an acceptor during this process that occurs under hypoxic conditions ultimately leads to proteins that cannot be properly folded, which leads to ER stress (for a review of ER stress and hypoxia, see [102]).

For these reasons, it is no surprise that many proteins upregulated during ER stress are also induced in response to hypoxia. A key factor involved in the inhibition of protein synthesis is eukaryotic translation initiation factor 2α (eIF2α). One of the known mechanisms of eIF2α phosphorylation is via protein kinase R (PKR)-like ER kinase (PERK) activation. Upon phosphorylation, eIF2α becomes inactive and slows the rate of general protein translation. The phosphorylation of eIF2α is a consequence of not only ER stress, but also hypoxic stress, presence of oxidants or reactive metals, and amino acid deprivation. PERK activation is also a known consequence of ER stress but recently has been correlated with hypoxic conditions as well. Studies by Koumenis et al. have linked the increased phosphorylation of eIF2α and subsequent decreased rate of protein synthesis under hypoxic stress to the activation of PERK [103]. Whether or not this is indirectly through the induction of ER stress is not clear. Hypophosphorylation of the 4E binding protein 1 (4E-BP1) plays a role in decreased translation by sequestering the eukaryotic translation initiation factor 4E (eIF4E) to prevent complexing mRNA and ribosome under hypoxia [99].

In addition to inhibiting the overall rate of translation, phosphorylation of eIF2α also allows translation of a specific subset of genes that harbor upstream open reading frames, including activating transcription factor 4 (ATF4) [104]. ATF4 plays a role in changing amino acid metabolism, as evidenced by impaired activation of genes involved in importing amino acids and the requirement for amino acid supplementation for growth of ATF4 (–/–) cells. These genes include the heavy chain of X-c cystine/glutamate exchanger (SLC3A2), asparagine synthase (ASNS), and glycine transporter 1 (GLYT1) (105). Together, slowing down translation and increasing the import of amino acids allow for the maintenance of a pool of amino acids within cells under stress.

IV. Nucleic Acids

Proliferating cells require the R-5-P precursor for DNA and RNA synthesis. This can occur by conducting intermediates from the glycolytic pathway to the pentose phosphate pathway (PPP) [68].

R-5-P can be generated by either the oxidative or the non-oxidative arm of the PPP. The oxidative branch involves glucose 6-phosphate dehydrogenase and 6-phosphate-gluconate dehydrogenase while the non-oxidative branch requires transketolase (TKT) and transaldolase [106]. In malignant cells, the non-oxidative branch is the primary source of R-5-P; hypoxia induces increased expression of TKT to drive this R-5-P synthesis [106]. Since the entirety of the non-oxidative PPP is reversible, substrate concentrations are the driving force behind high R-5-P production. Under hypoxic conditions, glycolytic rates are increased and substrates are freely available for use in additional anabolic pathways, including the PPP. Moreover, the glycolytic intermediates contribute to serine synthesis which ultimately results in the glycine necessary for purine synthesis [106, 107].

Overall, the hypoxic condition results in a switch to glycolysis by the cancer cell that not only provides energy, but also the necessary components for biosynthesis of lipids, amino acids, and nucleic acids. This phenomenon is briefly summarized in Fig. 4.1.

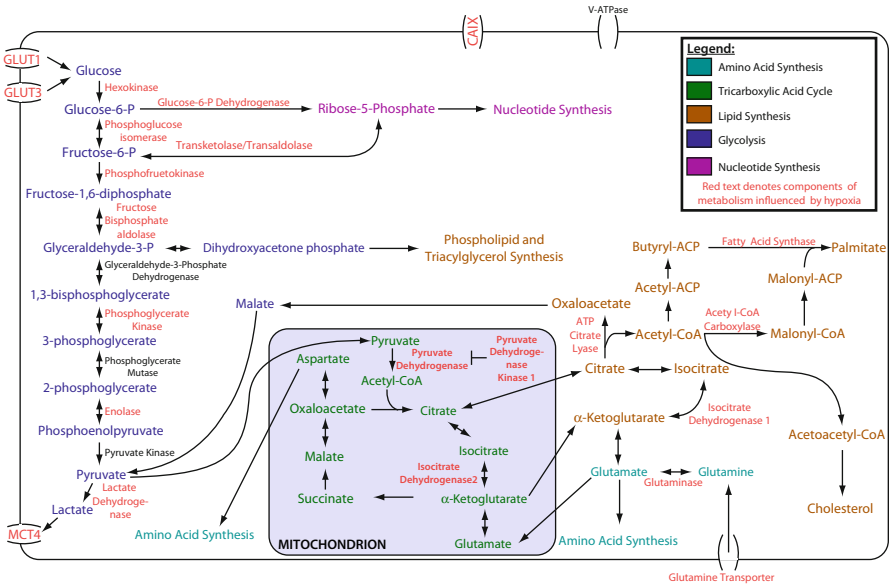


**Fig. 4.1** Select components of cancer cell metabolism affected by hypoxic conditions. Cancer cells in vivo are often exposed to hypoxic or anoxic conditions, which result in a metabolic switch. Glycolysis becomes the primary mechanism by which the cells catabolize glucose and the intermediates and byproducts participate in anabolic processes for lipids, amino acids, and nucleotides. Further, the tricarboxylic acid (TCA) cycle is supplied by the increased glycolysis and glutamine catabolism. Hypoxia contributes to this switch by influencing the expression of key enzymes in glycolysis, glutamine catabolism, and fatty acid synthesis. The components listed in *red* text are those that have been documented to be upregulated under conditions of low oxygen tension

## 4.4 Regulators of the Hypoxic Response

In the case of tumor pathology, a large percentage of cancer cells are at least transiently hypoxic, whether by acute or chronic hypoxia [108]. Because of this, there is a heterogeneous environment of oxygen, pH, growth factors, glucose, and other nutrients [109]. The milieu that is created by blood flow obstructions and lack of sufficient oxygen selects for the cells that are most adaptable to hypoxia [109]. Collectively, these adaptations result in cancerous cells able to thrive under extremely harsh conditions created by the tumor mass. This section seeks to outline the mechanisms by which the cancer cell is able to sense and respond to hypoxia, resulting in the aforementioned altered metabolism.

I. Reactive Oxygen Species

Reactive oxygen species (ROS) are those molecules of partially reduced $O_2$, including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), and the hydroxyl radical (HO·) [44]. ROS are generated under normal circumstances due to electron leaking from the mitochondrial ETC. The ETC is composed of large protein complexes that function to carry electrons along the inner mitochondrial membrane

and this action creates a proton gradient across the membrane. Complex I passes an electron to the ubiquinone molecule and then along to complex II, succinate dehydrogenase. The succinate dehydrogenase then transfers the electron to ubiquinone. From there, the electron moves through complex III, from ubiquinol to cytochrome C. Cytochrome oxidase (complex IV) passes the electrons to oxygen resulting in water as a by-product [44]. According to the chemiosmotic coupling hypothesis, energy produced by this process in the form of a proton gradient is used to rotate complex V, or ATP synthase, which generates ATP from adenosine diphosphate (ADP) and inorganic phosphate [110, 111]. As electrons pass from protein complex to protein complex, there are two major steps that can result in electron leaking resulting in ROS formation. The passage of electrons from complex I to the ubiquinone molecule and the passage from ubiquinol to cytochrome C can allow for electron leaking, with complex III being the major source [46, 112]; between 0.1 and 4 % of the oxygen used in normal respiration is converted to ROS [111, 113]. Small increases in ROS can push a cell to proliferate or differentiate and they can act as second messengers, but surplus species can cause stress and significant cellular damage [114, 115]. Unless these ROS are neutralized by detoxification enzymes such as superoxide dismutase, catalase, etc. or antioxidants, they can cause damage to macromolecules of the cell, including DNA, lipids, and proteins [116]. Cancer cells have persistent oxidative stress despite their upregulation of the endogenous antioxidant systems [117]. The persistent stress of ROS may contribute to potentiation of disease, especially a disease like cancer [118].

Malignant cells have been documented to have high levels of ROS [119, 120]; indeed, hypoxia can be a source of ROS production via the mitochondria [121]. The reactive oxygen that is generated under hypoxia is capable of damaging key enzymes that regulate the TCA cycle, which is a mechanism by which hypoxia can exert a force on metabolism in the cancer cell [122, 123]. ROS damage the enzymes involved in the TCA cycle, further reducing any potential ETC activity and OXPHOS, resulting in an increased dependence on glycolysis. The TCA cycle is crucial in providing the necessary components for the ETC and OXPHOS, which under normal conditions would be the primary mechanism by which a eukaryotic cell would generate ATP.

ROS are capable of influencing cell behavior, not only by directly damaging macromolecules, but also by influencing signaling pathways. A variety of pathways have been shown to be influenced by ROS, including the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3OH kinase pathway (PI3K), PI3K-NFE2-like 2 (Nrf2) pathway, redox factor-1 (Ref-1), and nuclear factor-κB (NF-κB), among others [124–126].

Hypoxia inducible factor (HIF)-1α has been demonstrated to be influenced by ROS [127]; ROS are capable of influencing HIF-1 DNA binding, which can directly increase the expression of glycolytic enzymes among other hypoxia-responsive genes, amplifying the effects of hypoxia. It has been found that ROS are required and sufficient for HIF-1 binding to DNA, some of which generates from respiring mitochondria in cancer cells [46, 128]. Furthermore, ROS influence HIF-1α stabilization in an iron-dependent manner, oxidizing Fe(II) to Fe(III), which reduces the available pool of Fe(II) for the prolyl hydroxylases required for HIF-1α degradation [129, 130]. The HIF system is a major source of metabolic reprogramming in hypoxic cells.

II. Hypoxia-Inducible Factors

In order for cells to survive under the harsh conditions of hypoxia, numerous cellular changes must occur. Cells need to adapt to the changing environment and compensate for the lack of oxygen as a cofactor and electron acceptor by initiating a transcriptional reprogramming to alter the expression of key genes involved in metabolic pathways. This reprogramming is mainly orchestrated by the HIFs, including HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) and HIF-α subunits, of which there are three forms encoded by different genes (HIF-1α, -2α, and −3α) [131, 132]. Under hypoxic conditions, the α and β subunits dimerize to form the transcription factor HIF-1, which can then bind to a consensus sequence (RCGTC) known as the hypoxia response element (HRE) within promoter regions of various target genes [133], regardless of the individual alpha subunit that interacts with HIF-1β. It is believed that the alpha subunits are differentially expressed depending on the tissue type and length of exposure time to hypoxic stress, and that they have different roles in regulating gene transcription in response to this stress [134–136]. HIF-1β is constitutively and ubiquitously expressed and is not sensitive to oxygen levels within the cell. Each of the alpha subunits, however, is quickly inactivated and degraded in the presence of oxygen, allowing for the tight control of the cellular response to hypoxic stress [132].

The HIF-α subunits are not oxygen sensing but are instead directly regulated by proteins that require oxygen for their functions. The proteins responsible for this regulation of HIF-α are the prolyl hydroxylase (PHD) proteins and factor inhibiting HIF-1 (FIH-1) protein, both of which function by hydroxylating their target proteins. Hydroxylation of –CH groups within proteins to form –COH requires oxygen as the substrate and, therefore, is limited by cellular oxygen levels. Inhibition of the transcriptional activation activity of HIF-1 under normoxic conditions is dependent on FIH-1-mediated hydroxylation of an asparagine residue within the C-terminal transactivation domain of HIF-α. This modification prevents the interaction of coactivators, such as p300/CBP, with HIF-1 [137, 138].

In addition to the functional regulation of HIF-α under normoxia, the stability of the alpha subunit is also negatively controlled by hydroxylation. HIF PHD hydroxylates HIF-α on two proline residues within the oxygen-dependent degradation domain (ODD), which allows for the interaction of the ubiquitin E3-containing ligase von Hippel–Lindau protein (pVHL). The polyubiquitination of HIF-α targets it for destruction via the proteasome and, therefore, inhibits its accumulation under normal oxygen conditions [139]. There are other pathological conditions, such as von Hippel–Lindau disease, characterized by inactivation of one allele of the VHL gene, that leads to increased HIF-α proteins and expression of their target genes [140], which ultimately mimics hypoxic stress. However, these conditions that are not directly associated with hypoxia are beyond the scope of this chapter. These two levels of control allow HIF-α to act as a master regulator of the cellular response to hypoxia. Upon exposure to hypoxic conditions, the PHDs and FIH-1 no longer have oxygen as an available substrate and, therefore, HIF-α can accumulate, dimerize with HIF-1β forming HIF-1, and interact with other coactivators to initiate transcription of more than 60 different genes that ultimately alter metabolism and cell behavior to

allow for survival in these harsh conditions. Due to the occurrence of hypoxic stress within tumors, the HIF-α is commonly present and active in cancer cells. The transcriptional changes initiated by HIF-1 affect a wide of range of metabolic pathways in order to maintain homeostasis with limited oxygen availability, including glucose metabolism, cellular respiration, lipid production, and nucleic acid synthesis.

Due to the vital role of oxygen as the last electron acceptor in OXPHOS, it is known that under low oxygen tension cancer cells rely heavily on glycolysis. With increased research on the HIF-1-dependent changes, it is now understood that this switch in glucose metabolism is due to the induction of key regulatory genes by HIF-1. A common feature of cancer cells is the increased uptake of glucose, which would allow for the reliance on glycolysis for energy production. Hypoxic stress specifically leads to increased mRNA and protein levels of GLUT-1, and a putative HRE was found within the promoter region [141]. Furthermore, the increase in GLUT-1 and GLUT-3 that increases the influx of glucose into the cells has been demonstrated to be dependent on HIF-1 [142]. This upregulation of glucose transporters ultimately leads to more available glucose for eventual breakdown via glycolysis.

In addition to the effects on GLUT expression, HIF-1 has been shown to directly regulate a number of key enzymes involved in the glycolytic switch and overall energy metabolism in response to hypoxia. One of the key steps in the breakdown of glucose is the conversion to pyruvate, which requires multiple enzymes and leads to the net production of two molecules of ATP. The transcriptional activities of many of those genes in this metabolic cascade are responsive to HIF-1, including PFK-L, aldolase (ALDA), PGK-1, enolase (ENO), and HK [55, 143, 144]. Once pyruvate is generated, the cell can convert it either into acetyl-CoA for eventual entry into the TCA cycle or into lactate as part of the glycolytic process. HIF-1 mediates the conversion to lactate in favor of acetyl-CoA by directly upregulating pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA) [55, 145, 146]. The direct binding of HIF-1 to the HRE within the PDK1 promoter leads to increased PDK1 expression. PDK1 can then phosphorylate and inactivate pyruvate dehydrogenase (PDH), which is the enzyme responsible for converting pyruvate to acetyl-CoA [147], and instead LDHA catalyzes the conversion of the pyruvate to lactate. The induction of these two proteins by HIF-1 has multiple effects on cellular metabolism and the cell's use of oxygen in limiting conditions. The decreased amount of acetyl-CoA for entry into the TCA cycle results in less OXPHOS via the ETC and, therefore, lowers mitochondrial oxygen consumption and ROS production [147, 148].

Another consequence of the conversion of glucose to pyruvate is the reduction of $NAD^+$ to NADH. Normally, NADH is reoxidized via the ETC within the mitochondria replenishing the pool of $NAD^+$. However, with limited OXPHOS in response to hypoxic stress NADH needs to be oxidized by formation and secretion of lactate to the extracellular space [145]. The formation of lactate from pyruvate regenerates $NAD^+$ to allow for continued glycolysis. The secretion of lactate is then mediated by monocarboxylate transporter 4 (MCT-4), which is also a target of HIF-1 [149].

A major negative effect of hypoxia is the increased production of ROS. ROS are generated because of inefficient transfer of electrons during the ETC. Therefore, the transcriptional changes orchestrated by HIF-1 to inhibit the amount of OXPHOS

serve to minimize the deleterious effects of the hypoxic condition, while maintaining sufficient energy production. However, there are other entry points into the TCA cycle that do not rely on pyruvate conversion to acetyl-CoA. HIF-1 regulates various components of the complexes responsible for the transfer of electrons during the ETC to exert an additional level of control of cellular respiration and ROS production. For example, NDUFA4L2, a subunit of complex 1, is induced by hypoxia via the binding of HIF-1 to the promoter. This increased expression of NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2 (NDUFA4L2) results in decreased complex I activity and lower oxygen utilization by the mitochondria [150]). Subunits of cytochrome C oxidase (COX), which is involved in the final transfer of electrons to oxygen, are also differentially regulated by HIF-1. The COX4-2 subunit protein is directly upregulated by HIF-1 in response to hypoxic stress while the degradation of COX4-2 is induced by HIF-1's effect on the mitochondrial protease Lon. This switch under hypoxic conditions leads to optimization of COX activity and reduced production of ROS [151]).

The effects of HIF-1 on increasing glycolysis while reducing the available pool of acetyl-CoA for the TCA cycle also have profound effects on lipids due to the fact that many of the metabolites of glycolysis and the TCA cycle are substrates for lipid metabolism. Additionally, HIF-1 directly regulates a few key enzymes required for lipid accumulation, inhibition of β-oxidation, and increased TGs. De novo fatty acid synthesis for lipid production under normal conditions requires a pool of citrate that can be converted into acetyl-CoA which is then used for fatty acid production. Under hypoxic conditions, cancer cells adapt to limited production of citrate by using glutamine as the major carbon source (for a review of lipid metabolism, see [72]). It has been shown that hypoxic cells rely on the reductive carboxylation of glutamine-derived α-ketoglutarate to form isocitrate via IDH1 and IDH2, which, through isomerization, replenishes the pool of available citrate for fatty acid synthesis [152, 153]. This pool of citrate was also found to feed into the production of aspartate, malate, and fumarate [152, 153], which affects amino acid production and nucleotide synthesis [67]. This switch to reductive carboxylation was observed in renal cell carcinoma (RCC) cell lines that lack pVHL as well, implicating the requirement for HIF proteins. Additionally, silencing of HIF-2α or HIF-1α reversed this switch in the carbon source utilized for lipid synthesis [152, 153].

In addition to increasing acetyl-CoA for fatty acid synthesis, cells exposed to hypoxia have shown increased expression of FASN [72, 154]. Furuta et al. found that FASN was upregulated in cancer cell lines exposed to hypoxic stress by the direct binding of sterol regulatory-element binding protein-1 (SREBP-1), a transcription factor known to regulate various lipogenic genes involved in fatty acid, phospholipid, and TG synthesis. This transcriptional activation of FASN via SREBP-1 required HIF-1 and Akt as shown by decreased SREBP-1 phosphorylation and FASN protein expression when HIF-1 and/or Akt were inhibited [85]. In livers from mice lacking pVHL or lacking pVHL and HIF-1α (therefore, only expressing HIF-2α), FASN and ACC, two targets of SREBP-1c, were downregulated, thus, suggesting negative regulation of lipid synthesis by HIF-2α [155]. These results together suggest that our understanding of the complex transcriptional changes initiated by the HIF proteins

is far from complete, including, but not limited to, the regulation of lipid synthesis in response to hypoxia.

The studies by Rankin et al. also found that livers lacking pVHL alone or in combination with loss of HIF-1α had increased TGs and free fatty acids, an accumulation of neutral lipids, and increased cholesterol levels. Fatty acids present within cells can be broken down by β-oxidation for energy and also to produce acetyl-CoA for entry into the TCA cycle. Rankin et al. found that genes required for β-oxidation, including acyl-CoA synthase long-chain family member 1 (Acsl1) and carnitine-palmitoyltransferase I (Cpt-1), are downregulated in an HIF-2α-dependent fashion [155]. A role for HIF-1α in decreased β-oxidation has also been suggested via regulation of peroxisome proliferator-activated receptor α (PPARα) expression and function. HIF-1α had been shown to affect PPARα by directly repressing PPARα promoter activity [156] as well as decreasing the ability of PPARα to bind to its target gene muscle carnitine palmitoyltransferase I (M-CPT I), resulting in decreased expression of M-CPT I. M-CPT I is a key enzyme required for the transport of fatty acids into the mitochondria for breakdown via the β-oxidation pathway [157]. Whether these effects seen in specific cell types and with certain isoforms of key enzymes are more broadly applicable will require further investigation.

In agreement with the decreased breakdown of TG and fatty acids, there is also an increase in storage of lipids in the form of TGs and/or lipid droplets under hypoxia. In the same study by Rankin et al., HIF-2α was found to be required for increased expression of adipose differentiation-related protein (Adfp), which is a lipid droplet-binding protein required for lipid accumulation in various model systems [155]. To further support the formation of lipid droplets in hypoxic cells via HIF regulation, HIF-1 was shown to directly induce the expression of HIG-2, which was upregulated in cells exposed to hypoxic conditions. HIG-2 was found to be associated with lipid droplets in these cells and expression of HIG-2 under normoxia was enough to induce lipid droplet accumulation [158]. HIF-1α has also been shown to directly activate transcription of PPARγ in mouse cardiomyocytes as part of a stress-induced hypertrophy model, which is thought to present with a similar metabolic switch seen in hypoxic cells. PPARγ can then activate the transcription of genes required for TG synthesis, including glycerol-3-phosphate dehydrogenase (GPD1) that generates glycerol-3-phosphate from glycolysis metabolites, as well as glycerol phosphate acyltransferase (GPAT) that catalyzes the rate-limiting step in the formation of TGs [159]. These pathways activated by HIF-1 can also relate back to increased glycolysis in hypoxic cells, which would increase the intermediates, including glycerol-3-phosphate.

Another way to increase the amount of stored lipids, as well as the level of cholesterol, within a cell is to increase the uptake of lipids via low-density lipoproteins (LDLs) and very LDLs (VLDLs). It has been demonstrated that HIF-1 directly binds to the promoter of the VLDL receptor (VLDLR). Furthermore, silencing of HIF-1α has been demonstrated to lead to a decrease in VLDLR and a subsequent decrease in LDL and VLDL uptake in response to hypoxia [160, 161]. These effects of HIF-1 on VLDLR regulation help explain the higher levels of cholesterol observed in clear-cell RCC [161]. Lipoprotein receptor-related protein 1 (LRP1), which is also involved

in the internalization of LDL, has also been shown to be a direct target of HIF-1 in vascular smooth muscle cells. LRP1 expression in these cells leads to increased cholesterol esters [162]. Together, these transcriptional changes orchestrated by the HIFs allow for increased lipid storage and decreased break down of fatty acids.

Another level of metabolism that is altered in response to hypoxic stress via HIF-1 regulation is the biosynthesis of nucleotides. Cancer cells utilize the non-oxidative branch of the PPP to generate R-5-P, the sugar backbone of nucleic acids. HIF-1 contributes to this switch in cancer cells indirectly by affecting the rate of glycolysis and directly by regulating the transcription of key enzymes involved in this switch to the non-oxidative PPP (for review see [106]). For example, the increased mRNA expression level of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase isozyme 3 (PFKFB3) under hypoxia is dependent on HIF-1α expression. PFKFB3 gene codes for the enzyme responsible for maintaining levels of fructose-2,6-bisphosphate (F-2,6-P2), which in turn activates PFK-1 while inhibiting fructose-1,6-bisphosphatase [163]. PFK-1 is a key mediator of glycolysis and its activity results in the production of glycolytic intermediates that can directly feed into ribose-5-phosphate via TKT, while fructose-1,6-bisphosphatase is involved in gluconeogenesis [67]. The level of PFKFB has in fact been shown to affect the amount of F-2,6-P2 and 5-phosphoribosyl-1-pyrophosphate (PRPP), a major precursor for nucleotides [164]. Later studies verified direct interaction of HIF-1α with the PFKFB3 promoter [165]. Additionally, Zhao et al. provide evidence to suggest that HIF-1 positively regulates the expression level of TKT and negatively regulates glucose-6-phosphate dehydrogenase (G6PD), which results in induction of the non-oxidative PPP while inhibiting the oxidative pathway of nucleotide synthesis, respectively [166]. Collectively these results provide evidence that the HIF system is increasing both the glycolytic pathway and the flux of glycolytic intermediates into the non-oxidative PPP required for continued nucleotide synthesis under hypoxic conditions.

In contrast to the data described earlier, pyrimidine nucleotide synthesis has been shown to be negatively influenced by HIF-1 activity. The carbamoyl phosphate synthetase-aspartate carbamoyltransferase-dihydroorotase (CAD) enzyme, which catalyzes the rate-limiting step of the pyrimidine synthesis cascade, is repressed due to the direct binding of HIF-1 to the HRE within the CAD promoter leading to lower CAD mRNA levels [167]. Whether these differences are tissue specific or in response to various levels of oxygen tension need further clarification.

III. AMP-activated protein kinase and mechanistic target of rapamycin

Mechanistic target of rapamycin (mTOR) has been shown to be responsive to changes in the oxygen tension of a cell, unrelated to changes in cellular ATP levels [100]. Under hypoxia, mTOR inhibition can occur, thus, inhibiting phosphorylation of its targets, which includes eIF2α and 4E-BPs. This results in a decrease in global translation, which is one of the more rapid cellular responses to hypoxia; however, there is still selective translation of particular mRNAs, such as vascular endothelial growth factor-A (VEGF-A) [100]. This mTOR inhibition requires the TSC1/TSC2 (tuberous sclerosis complexes) complex along with Redd1 [168]. Redd1 is transcribed by the HIF dimer under hypoxia, but mTOR inhibition may also

occur through the energy sensing AMP-activated protein kinase (AMPK)/TSC2/Ras-homolog enriched in the brain (Rheb) pathway independent of the HIF response [169].

## 4.5 Current and Potential Therapies Addressing Hypoxia and Resultant Metabolic Change

Ideal cancer treatment strategies are those that are effective against malignant cells while sparing normal tissue. Exploiting unique aspects of cancer pathologies has been successful in certain circumstances and hypoxia has been pursued as another targetable attribute of cancer. Since hypoxia is an abnormal tissue status, several strategies have been investigated to combat hypoxia including inducing oxygenation, developing hypoxia-activated cytotoxins, sensitizing hypoxic cells to treatments (e.g., radiosensitizing), and targeting molecules that have upregulated activity under hypoxic conditions. This section will explore hypoxia-combating therapy strategies and any effects on ameliorating the abnormal metabolic status of tumor tissue under low oxygen tension, as well as any potential future therapies to interfere with altered metabolism.

I. Reversing Hypoxia

A rather obvious approach to combating tumor hypoxia is to reoxygenate malignant tissue. Indeed, increasing oxygen concentrations at the site of tumors has been shown to improve patient outcome when combined with radiotherapy [170]. In the patient, chronic hypoxia can be remediated with breathing carbogen (95 % oxygen, 5 % carbon dioxide) while acute hypoxia can be alleviated by using nicotinamide to improve tumor perfusion, leading to improved radiation therapy response [171, 172]. Unfortunately, this may not be an effective mode by which to target cancer cell metabolism, as aerobic glycolysis has long been documented in cancer cells [52]. It has been shown that the HIF-1α component of the HIF-1 complex responsible for inducing transcription of hypoxia-responsive genes, including glycolytic enzymes and glucose transporters, is stabilized by by-products of glycolysis such as pyruvate [173, 174]. This suggests a feed-forward mechanism that will not be ameliorated once glycolysis has begun by reoxygenating the tumor tissue.

II. Hypoxia-activated Cytotoxins

The idea of hijacking the malignant program to activate prodrugs is not a novel concept; however, the success of prodrugs has been compromised to a certain extent because of a lack of consistently high expression of any activating enzymes among cancer cell types [175]. For this reason, hypoxia is an attractive target as it is a very common feature among progressing tumors and results in upregulation of the required reductases, as some are under control of the HIF system [175–178]. The general mechanism of these drugs includes an initial reduction of the prodrug, potentially followed by a reoxidation by oxygen if present. In this way, well-oxygenated tissues are spared the negative effects of the activated prodrug because the drug easily reverts to its inactive state. One caveat with this approach is that the redox cycling of the

drug and any oxygen present results in ROS generation. Therefore, the prodrug is solely effective against hypoxic cells only if it is more toxic than the inactive prodrug or ROS [175]. Furthermore, metabolism of the drug before it reaches its ultimate target is another issue. These drugs are designed to be metabolized under hypoxic conditions, and if the drugs are processed as they diffuse through areas of hypoxia, the more hypoxic cell population will be left untreated [175]. Work is being done with some existing prodrugs and their derivatives to study the properties of the compounds to determine tissue diffusion and, therefore, effectiveness of the drug [175, 179]. The class of drugs introduced here do not necessarily act to influence metabolism, but rather act in a cytotoxic manner (for review, see Ref. [175]).

III. Sensitizing Hypoxic Cells

Not only do some of the bioreductive agents used to target hypoxic cell populations act as cytotoxins, but they also act to sensitize those cells to ionizing radiation. The compound tirapazamine is capable of enhancing the toxic effects of ionizing radiation, as the drug has been found to induce breaks in DNA that are more difficult for the cell to repair than those of γ-rays (even if the irradiated cells are under aerobic conditions) [180], however similar to other bioreductive agents, it is not directly effective against aberrant metabolism in malignant cells.

IV. Targeting Hypoxia-Specific Molecules

Since the HIF system is a major regulator of the metabolic response, targeting HIF-1 is a viable mechanism by which to reduce expression of the enzymes and other gene products that contribute to a sustained switch in metabolism, survival of malignant cells, and ultimately poor prognosis in the patient. Administering antisense coding for HIF-1α to animals bearing T-cell lymphoma caused a reduction in tumor load and, more specifically, a reduction in neovasculature in the developing tumor [181]. This implies that the targeting of the HIF-1 system downregulates its target genes, as some of the gene products play a role in angiogenesis. Prevention of HIF-1α expression can also be achieved using EZN-2968, a locked nucleic acid-oligonucleotide [182, 183]. It resulted in a decrease in secretion of some HIF-1 target genes as well as decreased tumor weight in an animal model [182]. This drug is currently in phase I clinical trials. Further, aminoflavone is the active form of the prodrug AFP464 that can inhibit HIF-1α expression [184]. This drug is currently in phase I clinical trials as well. Preventing the expression of the HIF-1α proteins is also possible; traditional topoisomerase inhibitors (as well as modified topoisomerase inhibitors [185]) have been associated with decreased protein expression of HIF-1α, but not necessarily mRNA expression [186, 187]. Similarly, cardiac glycosides (digoxin, etc.) have been shown to decrease the HIF-1α protein levels in both cell lines and tumor xenografts [188]. PX-478 is a novel compound that has completed phase I clinical trials after in vitro and in vivo findings that HIF-1α is inhibited with PX-478 treatment [189, 190].

Further, disrupting the ability of HIF-1 to interact with p300/CBP by overexpressing the fragment of HIF-1 that binds to p300/CBP (CH1) to compete with fully functional HIF-1 at transcription sites has been efficacious in reducing transcription of target genes and, therefore, the tumor volumes in affected animals [191]. Relatedly, natural inhibitors of HIF-1 DNA binding exist, as demonstrated first by Bhattacharya et al. regarding p35srj [192]. P35srj serves to inhibit the binding of

HIF-1 to p300/CBP as well. Exogenous compounds have also been found to interfere with HIF-1 DNA binding, specifically diphenylene iodonium which has been shown to decrease HIF-1 interaction with target sequences and reduce expression of target genes [193, 194]. Chetomin, a compound derived from *Chaetomium* species of fungus, is capable of interrupting the interaction between p300/CBP and HIF-1 and HIF-2 [195]. Bortezomib, traditionally regarded as a proteasome inhibitor, has been proposed as a specific HIF-1 inhibitor [196]. In previous clinical trials, bortezomib was documented to influence HIF-1 activity [197], and later it was proposed that bortezomib impacts FIH inhibition of HIF-1/p300 binding [196]. Anthracyclines, such as daunorubicin and doxorubicin, have also been found to prevent HIF-1 from binding DNA, resulting in a decrease in expression of HIF-1 target genes and abrogation of tumor progression [198]. The use of a dominant negative mutant of HIF-1α (dnHIF-1α) has been shown to hinder HIF-1-mediated transcription and reduce the amount of target gene expression, including ALDA and GLUT-1, key players in the abnormal metabolism found in malignant cells; this action led to a decrease in tumor growth in vivo [199]. The strategy of targeting the HIF system can be combative against abnormal metabolism, as a multitude of enzymes involved in cancer cell metabolism are controlled by the HIFs, including LDH-A, PDK, and GLUT-1. Reducing the ability of the cancer cell to produce the proteins required to meet the biosynthetic and energetic needs of the rapidly proliferating cells can shift the cell toward apoptosis, the ultimate goal of cancer treatment.

Disrupting the stability of the HIF-1α protein may also be a viable treatment mechanism. Heat shock protein 90 (Hsp90) is a chaperone protein that influences the stability of a wide variety of proteins, including HIF-1α. Hsp90 inhibitors have been shown to induce degradation of HIF-1α and Hsp90 inhibitors have been used in clinical trials [183, 200]. However, because Hsp90 is a rather promiscuous binding partner, the ability of Hsp90 inhibitors to act in an antitumorigenic fashion may not be completely related to HIF-1α inhibition [183]. Deacetylases also influence HIF activity and small molecule inhibitors for these proteins have also been pursued as potential therapies [201, 202]. A summary of these drugs is shown in Fig. 4.2.

In a related vein, the HRE that HIF-1 binds to in order to support transcription can be used to drive the expression of select antitumoral genes if the promoter-gene construct is delivered to the site of hypoxic tissues [203, 204]. A variety of methods have been examined as vehicles for gene delivery, including macrophages and anaerobic bacteria, which tend to colonize areas of hypoxia [205, 206].

V. Targeting Metabolic Enzymes

Targeting hypoxia and the resultant stabilization of the HIF-1 complex is a viable approach to ameliorating the abnormal metabolism of cancer cells, but another approach is to directly influence the enzymes involved in glycolysis and anabolism that are the product of the hypoxic response [207, 208]. Inhibition of GLUT-1 by the small molecule WBZ117 results in a decrease in cancer cell survival both in vitro and in vivo [209]. Phloretin is an additional inhibitor of glucose transport and treatment induces apoptosis and reduced tumorigenesis in an animal model [210]. Silybin is another flavanoid that is capable of inhibiting glucose transport, specifically through inhibition of GLUT-4 [211]. The pyruvate mimetic dichloroacetate (DCA) is capable

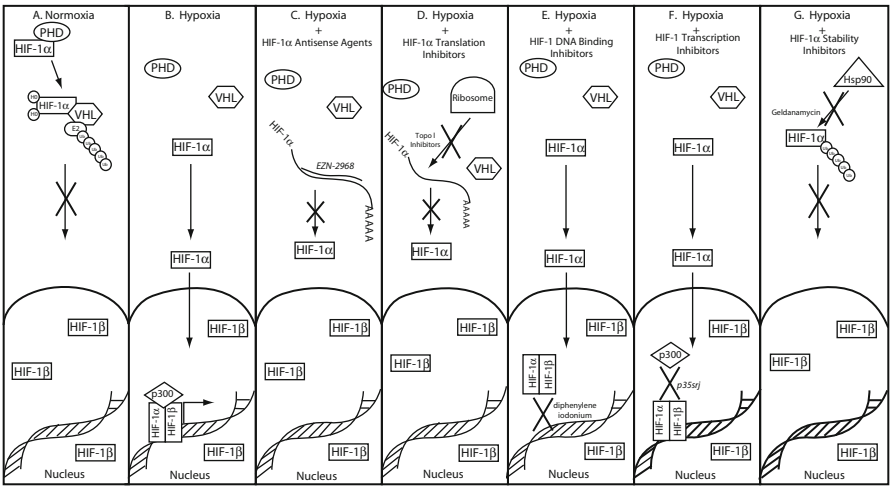


**Fig. 4.2** Inhibitors of hypoxia-inducible factor-1 (HIF-1) activity. **a** Under normoxic conditions, the HIF-1α subunit is degraded by the proteasome after von Hippel–Lindau (VHL)-mediated ubiquitination that is preceded by hydroxylation by the oxygen-sensitive prolyl hydroxylase (PHD). **b** Hypoxic conditions inhibit PHD hydroxylation of HIF-1α, thus stabilizing the protein for translocation to the nucleus. Once in the nucleus HIF-1α dimerizes with the oxygen-insensitive HIF-1β subunit. This dimer is capable of binding the hypoxia response element (HRE) in the promoter of select genes and recruitment of p300/CBP allows for transcription of the hypoxia responsive genes. **c** Antisense agents are available against HIF-1α. EZN-2698, a locked nucleic acid-oligomer, is capable of binding HIF-1α mRNA and ultimately results in a decrease in the HIF-1α messenger RNA (mRNA) pool. **d** Inhibition of translation of HIF-1α decreases accumulation of the subunit under hypoxia. Agents associated with translation inhibition includes topoisomerase I inhibitors, cardiac glycosides, and PX-478. **e** Compounds have been identified that interfere with HIF-1 complex binding to DNA. While this strategy does not directly influence either subunit level, transcription of the target genes is abrogated. Such compounds include diphenylene iodonium and anthracyclines. **f** p35srj and chetomin have been shown to inhibit the recruitment of p300/CBP to DNA bound HIF-1 complex that is critical for HIF-1 target gene expression. **g** Influencing HIF-1α stability has been demonstrated to prevent HIF-1 target gene expression. Use of Hsp90 and HDAC inhibitors result in a VHL-independent ubiquitination of HIF-1α and ultimately results in a decrease in HIF-1 activity

of inhibiting PDK, barring the activity of the pyruvate dehydrogenase complex. This small molecule has shown some promise in in vitro and in vivo studies, but importantly has been shown to improve disease response when combined with current therapies in glioblastoma patients [212, 213]. Pyruvate kinase M2 (PKM2) isozyme has also been studied as a viable target in cancer metabolism, as it is the predominant form in neoplastic tissues [66]. Inhibition of PKM2 by somatostatin and its analog TT-232 result in nuclear translocation of the enzyme and subsequent cell death; TT-232 has passed phase I clinical trials [214, 215]. Despite that, it is currently under debate as to whether or not PKM2 is truly an acceptable drug target for the cancer arsenal, as it has recently been shown that PKM2 inhibition can skew glycolysis and cause the accumulation of phosphoenolpyruvate, but it does not influence xenograft

tumor growth [216, 217]. PFKFB3 is capable of being inhibited by 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO), which has shown the ability in both in vitro and in vivo studies to reduce glycolytic flux and result in decreased disease progression in an animal model [218]. 3-bromopyruvate (3-BrPA) is a small molecule that was initially characterized as able to inhibit glycolysis, but it is currently unclear whether this is its only action [207]. Recent evidence indicates that MCT-1 is required for cellular uptake of 3-BrPA, but beyond that it is unclear that this compound only targets metabolic enzymes [219]. HK-2, the enzyme that commits glucose in the cell to glycolysis, can be inhibited using lonidamine and 2-deoxy-glucose among other compounds [94, 220]. *S*-*p*-bromobenzylglutathione cyclopentyl diester (BBGC) has been shown to interfere with the function of glyoxalase-1, preventing detoxification of normal metabolic by-products and allowing them to accumulate in the cell [221]. A series of N-hydroxyindole compounds have been tested for inhibition of LDHA and have shown a reduction in lactate production and cancer cell proliferation [222]. 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic acid (FX11) is a small molecule that has been shown to interfere with LDHA activity and induce antitumoral effects in xenograft models [223].

Taking advantage of the glycolytic phenotype, it is possible to induce acidosis of the malignant cells (decreasing intracellular pH [$pH_i$]) and prevent acidification of the tumor tissue (increasing extracellular pH [$pH_e$]) [207]. Frequently in hypoxic environments, cells adapt by upregulating glycolytic processes and concomitantly upregulating transporters that aid in detoxifying the cell of lactate and other acidic products of metabolism. Transporters such as MCT-1, carbonic anhydrase IX (CAIX), and V-ATPases are major players in maintaining cellular pH. Targeting CAIX with small molecule inhibitors, including sulfonamides, results in apoptosis in vitro and a reduction in disease progression in in vivo models [224–226]. Not only have small molecule inhibitors of CAIX been discovered, but also antibodies effective against the transporter activity have been developed and brought through phase I and II clinical trials. Phase III clinical trials revealed that those patients that received the most benefit from the adjuvant treatment were those with high CAIX expression, but the primary endpoint of the study was not met [227]. Similarly, inhibition of the vacuolar $H^+$-ATPases (V-ATPases) has shown to be effective in reducing cancer cell growth and overall distant metastasis [228, 229]. In those tumor cells and xenografts that express MCT-1, inhibition of the lactate transporter inhibits growth and induces radiosensitivity [230]. It is hypothesized in the case of heterogeneously hypoxic tumors, there is a metabolic symbiosis that occurs where oxygen-exposed cells can use the lactate by-products from glycolytic cells to fuel their energy requirements and allowing the glucose to be used primarily by these hypoxic, glycolytic cell populations [230]. Therefore, targeting MCT-1 not only may target hypoxic cells but also well-oxygenated cells.

Lipid synthesis is another target for antimetabolic cancer therapies because of its contribution to cell survival and proliferation and was recognized as a potential therapy for cancer over 20 years ago [231]. FASN structure has been determined [232] and there are a variety of pharmacological agents available for inhibition of this enzyme. FASN activity can be inhibited by the compound C75, as demonstrated

by Kuhajda, et al., which is a derivative of cerulenin [233]. The parent compound cerulenin is isolated from *Cephalosporium caerulens* and functions to inhibit FASN by covalently modifying the active site of FASN, but has limited bioavailability [234, 235]. FASN activity can also be impeded by tetrahydrolipstatin (orlistat), a compound originally found to inhibit pancreatic lipase and is now a Food and Drug Administration (FDA)-approved antiobesity drug [236–239]. Another naturally occurring compound found in green tea, epigallocatechin-3-gallate (EGCG), has been found to reduce FASN expression and activity, among other flavanoid compounds [240–243]. Triclosan is an antibiotic capable of reducing FASN activity and has been shown in vitro to reduce carcinogenesis [241]. Inhibition of FASN has been associated with decreased cancer cell proliferation, increased apoptosis, and an overall decrease in disease progression in animal models [235, 244–246]. FASN correlation with poor patient prognosis and its overexpression in cancer (conferring a large therapeutic index) makes it a viable target for treatment of cancer [247].

Additional enzymes in lipid synthesis pathways may serve as targets in cancer metabolism. It has been documented that inhibiting HMGCR by use of statins results in an antiproliferative effect in tumors [248]. Furthermore, the enzyme ACL that is responsible for the conversion of citrate to acetyl-CoA can be targeted with a small molecule inhibitor (SB-204990), resulting in *in vitro* and *in vivo* reduction in cancer cell proliferation and xenograft progression, respectively [249, 250]. ACCs catalyze the reaction that converts acetyl-CoA to malonyl Co-A for fatty acid synthesis. Soraphen A, a naturally occurring macrocyclic polyketide, is an inhibitor of ACC and its use results in a decrease in fatty acid synthesis that can be reversed by palmitate supplementation, confirming the need for fatty acid synthesis in cancer cell viability [251]. Further, inhibition of ACC resulted in growth arrest followed by cell death [251]; however, this compound is primarily being used in preclinical work at this time.

Glutamine metabolism is another aspect of aberrant cancer metabolism that is capable of being targeted. It has been demonstrated that tumor cell survival under hypoxia depends upon glutamine metabolism [252]. It has been shown in vitro and in vivo bis-2-(5- phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) inhibits glutaminase activity, but does cause an increase in glycolytic intermediates, indicating the usefulness of a treatment of this nature would be in combination with targeting of related metabolic pathways to avoid compensatory action by the cancer cell [223, 253].

Several of these compounds for metabolic inhibition are in early clinical trials, usually in combination with more traditional chemotherapeutics. Evidence points to synergistic effects of metabolic interference with the standard chemotherapeutics, as the dysregulation of metabolism is a key component of drug resistance (for review, see [94]).

## 4.6 Conclusions and Future Directions

It has become increasingly clear that the metabolic status of a malignancy can dictate patient outcome. The switch from normal aerobic metabolism to produce energy and precursor material for the cell's survival and maintenance to a more glycolytic phenotype has been repeatedly correlated with more aggressive cancer cell behavior and ultimately poor patient survival rates. Targeting the metabolome is a viable mechanism by which to combat neoplastic disease. However, it is unlikely that a single agent will effectively target all aberrant cancer cells; inhibiting the variety of ways the ever-evolving cancer cell deals with metabolic stress will be the most efficacious. The ultimate goal of any cancer treatment is to have an effective, highly specific agent that negatively affects cancer cells' survival. In the case of modulating cancer cell metabolism, the hypoxic environment that often develops at a tumor site can be exploited to deliver specific therapies that target metabolism to neoplastic tissues. A major player in the hypoxic response is the HIF system and targeting this particular component of the cancer cell response may abrogate cell survival, as HIF influences glucose metabolism, lipid metabolism, and ROS protection, all of which play an important role in allowing for cancer cells to survive. Cutting the hypoxic response of the cell down may weaken the ability of the cell to adapt to the changing microenvironment, as well as improve response to therapies targeting other aspects of cancer cell biology. Therefore, it will be prudent to develop effective combination therapies that not only take into consideration the genetic abnormalities that cancer cells often harbor, but also their microenvironment and metabolic status as a way of improving the current standard of care.

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# Chapter 5
# Glucose Metabolism and the Antioxidative Defense System in Cancer Cells: Options for the Application of ROS-based Anticancer Drugs

**Gernot Bruchelt, Rupert Handgretinger, Mirjam Weckenmann and Talia Hahn**

**Abstract** Cancer cell metabolic pathways (aerobic glycolysis, Warburg effect) may be used as targets for the development of new drugs with more specific therapeutic strategies. Reactive oxygen species (ROS) are often involved in these metabolic pathways. Their generation, as well as the defensive reactions against them, present attractive targets. In this chapter, the major aspects of aerobic glycolysis in cancer cells are summarized first, while presenting the principles of ROS biochemistry. ROS formation, and the defense mechanisms against them, are rather heterogeneous in various cancer cell types. The basic mechanisms, therefore, are described first in two well-defined non-malignant cell types, erythrocytes and neutrophils. This is followed by a description of the more complex situation in cancer cells, where the influence of anti-/pro-oxidative microenvironments on cellular proliferation and survival is discussed. In the second part, potential targets for ROS-based therapeutics are presented and the mechanisms of some of them (dichloroacetate, iron dependency, arsenic trioxide, and high-dose intravenous (i.v.) ascorbic acid) are described in more detail.

**Keywords** Warburg effect · Aerobic glycolysis · Reactive oxygen species (ROS) · Antioxidative defense · Cancer cell metabolism · Erythrocytes · Neutrophils · Dichloroacetate (DCA) · Arsenic trioxide (ATO) · Ascorbic acid

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## 5.1 The Warburg Effect and Current Explanations of the Aerobic Glycolysis

Otto Warburg first described how certain cancer cells metabolize glucose preferentially to lactate instead of $CO_2$ and $H_2O$ ($C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$) even in the presence of oxygen (aerobic glycolysis) [120]. This was an unexpected observation since proliferating cancer cells require a lot of energy (adenosine triphosphate (ATP)). Aerobic glycolysis of 1 mol glucose to 2 mol lactate produces only about 2 mol ATP in contrast to ~ 32 mol ATP by metabolizing glucose in the mitochondria via oxidative phosphorylation (OXPHOS). Warburg supposed that mitochondrial defects may be responsible for this phenomenon [72, 119]. This was, however, a controversial supposition since mitochondrial defects are apparently not an obligatory requirement for aerobic glycolysis [33, 77]. It was soon realized that cancer cells, and generally rapidly proliferating cells, compensate for their less effective ATP production by a higher glycolytic flux. The increased glycolysis in malignant cells is clinically used for diagnosis and detection of tumors by positron emission tomography (PET): In contrast to normal cells, the glucose analogue 2 Fluorodeoxyglucose ([$^{18}$F] 2FDG) is taken up in large amounts by many tumor cells via the glucose transporter 1 (GLUT1) [44, 45]. Identification of the hypoxia-inducible factor (HIF), a transcription factor which is activated in the absence of oxygen, may provide a molecular explanation for increased glucose uptake and high glycolytic flux [37, 110, 123]. Under hypoxic conditions (e.g., in muscle cells), glucose metabolism switches from OXPHOS to enhanced glycolysis. HIF consists of an α-unit and a β-unit. Under normoxic conditions, the α-unit is modified by hydroxylation of proline residues by prolyl hydroxylases. Proline hydroxylation labels HIF-1α for von Hippel–Lindau(pVHL)/proteasomal degradation [32]. In cancer cells, degradation of HIF-1α may be prevented, even in the presence of oxygen, by inhibitors of prolyl hydroxylases, e.g., succinate, fumarate, or reactive oxygen species (ROS) [68]. Furthermore, HIF activates pyruvate dehydrogenase kinase (PDK), which inactivates pyruvate dehydrogenase (PDH) by phosphorylation (Fig. 5.3). As a consequence, pyruvate is preferably metabolized to lactate instead of acetyl-coenzyme A (acetyl-CoA) [74, 101]. A major breakthrough for elucidating the enhanced aerobic glycolysis in cancer cells were observations, during the 1990s, that many oncogenes like Myc, phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB/Akt)/mammalian target of rapamycin (mTOR), and Ras/Raf/mitogen-activated protein kinase (MAPK) pathways are able to enhance glycolysis and/or block the tricarboxylic acid (TCA) cycle and OXPHOS. Defective tumor suppressor genes like p53, and mutations in enzymes of the TCA cycle, such as succinate dehydrogenase (SDH) and fumarate hydratase (FH) [75] or the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzymes isocitrate dehydrogenases (IDH2 and IDH1; [133]), can also enhance glycolysis or block TCA/OXPHOS. These aspects are summarized in several excellent articles and reviews [5, 67, 69, 80, 77].

Besides energy metabolism, another very important consequence of accelerated aerobic glycolysis is enhanced production of the metabolites needed for cell proliferation in cancer cells [36, 87, 116]. Glucose metabolism is especially involved in lipid synthesis for cell membrane formation by (a) enhanced citrate production and its transfer from mitochondria to cytosol with subsequent splitting of acetyl-CoA (and oxaloacetate) by citrate lyase [4, 135] and (b) enhanced NADPH supply needed for fatty acid and sterol (cholesterol, etc.) synthesis which originates with acetyl-CoA (see Fig. 5.3). Furthermore, transketolase-like protein 1 (TKTL-1), present in the pentose phosphate pathway (PPP) of many cancer cells, may also contribute to fatty acid synthesis [130]. Other than glucose, glutamine is another essential substrate for cancer cell proliferation, although glutamine is not an essential amino acid for normal cells [129]. Glutamine and glucose metabolism work together in several processes, e.g., nucleic acid synthesis: Ribose-5-phosphate is derived from glucose via the PPP and it serves as a substrate for nucleoside synthesis. Glutamine is the main nitrogen source for purine (A and G) and pyrimidine (U, T, and C) bases. Under hypoxic conditions, some cells are able to metabolize glutamine to citrate via α-ketoglutarate (2-oxoglutarate) by reductive carboxylation with NADPH-dependent isocitrate dehydrogenase (IDH2) in mitochondria [128].

The third aspect of aerobic glycolysis involves the relationship between deregulated glucose (and glutamine) metabolism in cancer cells and the generation of/defense against ROS. A summary of some principal features of ROS and the defense systems against them follows.

## 5.2 The Dual Role of Oxygen for Life

In their standard textbook "Free Radicals in Biology and Medicine," [57] start with a citation of Neil Young: "The same thing (i.e., oxygen) which makes you live will kill you in the end." The ~21 % of oxygen present in air [ground-state oxygen, ($^3\Sigma g^- O_2$)] can easily be transformed to ROSs via several chemical and biochemical reactions that are able to destroy lipids, DNA, proteins, and other compounds [9, 14, 29]. ROSs include free radicals, such as superoxide ($O_2^{\bullet -}$), $^\bullet$OH-radicals, singlet oxygen ($^1\Sigma g^+ O_2$) (and nitric oxide radicals), as well as other non-radical ROSs such as $H_2O_2$. The most important sources of ROS within a cell are the electron transport chains (ETCs) [42] and the NADPH oxidases (NOXs) [10]. Formation of ROS by ETC and NOXs starts with the generation of $O_2^{\bullet -}$. Additionally, $O_2^{\bullet -}$ and $H_2O_2$ can also be directly generated in some enzymatic and non-enzymatic reactions. In peroxisomes, for example, $H_2O_2$ is produced by long-chain fatty acid oxidation.

Under normal conditions, different antioxidative defense systems, mainly enzymes (superoxide dismutase (SOD), peroxiredoxines (Prx), glutathione peroxidases (GPs), and catalase), vitamins (tocopherol and vitamins A and C), lipoic acid, and other small molecules like uric acid protect cells against "oxidative stress." Oxidative stress reflects an imbalance between ROS generation and degradation. At low physiological concentrations, ROSs serve as substrates for signal transduction [40], and even at high concentrations they are useful for the bactericidal activity of neutrophils (see Fig. 5.2). In cancer cells, the interaction of glucose metabolism, ROS formation,

and antioxidative defense is highly complex and varies significantly in different cancer types. The basic processes in malignant and non-malignant cells are similar and, therefore, are first comparatively outlined in two non-malignant cells, erythrocytes (Fig. 5.1), and neutrophil granulocytes (Fig. 5.2). In contrast to cancer cells, these are homogeneous cell populations and some of the most important features of ROS production and ROS defense as well as the role of glucose metabolism in these processes can be satisfactorily described. Generally, glucose may be metabolized along different routes. The main pathways are (a) glycolysis (glucose→pyruvate→lactate), (b) complete oxidation to $CO_2$ and $H_2O$ (glucose→pyruvate→TCA cycle→OXPHOS), (c) the PPP, and (d) metabolism to/of glycogen (storage form).

Figure 5.1 shows glucose metabolism, the endogenous generation of ROS during hemoglobin (Hb) oxidation, and the antioxidative defense mechanism in erythrocytes. In these cells, the endogenously generated ROSs are dangerous and must be rapidly destroyed for survival of functional erythrocytes. In contrast, the generation of ROS in neutrophils is necessary for killing microbes (Fig. 5.2). If granulocytes are defective in NOX (i.e., NOX2), patients suffer from severe and often life-threatening infections due to their inability to produce $O_2^{\bullet -}$ (chronic granulomatous disease (CGD)) [66].

## 5.3 Glucose and Glutamine Metabolism in Cancer Cells and its Relationship to Oxidative Stress and Antioxidative Defense

In 1981, Oberley et al. [96], in the free radical theory of cancer, first postulated that ROSs play an important role in the initial formation of cancer cells [96]. Later on, cancer cells were reported to generate more ROSs than normal cells leading to genetic instability and progression to more aggressively malignant cells [114]. Besides the mitochondrial respiratory chain (see further), an additional major source of ROS production is the NOX system ($O_2 + NADPH \rightarrow O_2^{\bullet -}$) which plays more than an essential role in neutrophil function (NOX2) (Fig. 5.2) [47]. Members of the NOX family (NOX1-5, and dual oxidases DUOX1 and 2) which are involved in redox-sensitive signal pathways, under the control of oncogenes and tumor suppressors, play an important role in the initiation and progression of cancer cells [10]. As a consequence of increased ROS production, however, cancer cells are more vulnerable to self-destruction by processes such as ROS-induced apoptosis. One strategy cancer cells use for self-protection, therefore, is enhancement of their antioxidative defense system, usually, by shifting glucose metabolism to the PPP. Figure 5.3 summarizes the different pathways of glucose (and glutamine) metabolism used for ROS generation and protection in cancer cells.

The main source of ROS production in cancer and non-cancer cells are mitochondria, especially in the generation of superoxide during the electron flux in the respiratory chain [2]. ROS generation can occur at different complexes of the respiratory chain during oxidative phosphorylation, especially at complex I when the mitochondria are not producing ATP (high$\Delta$ p: proton motive force) and at a reduced coenzyme Q-concentration, as well as at a high NADH/$NAD^+$ ratio in the mitochondrial matrix [94]. Production of ROS may be proportional to the

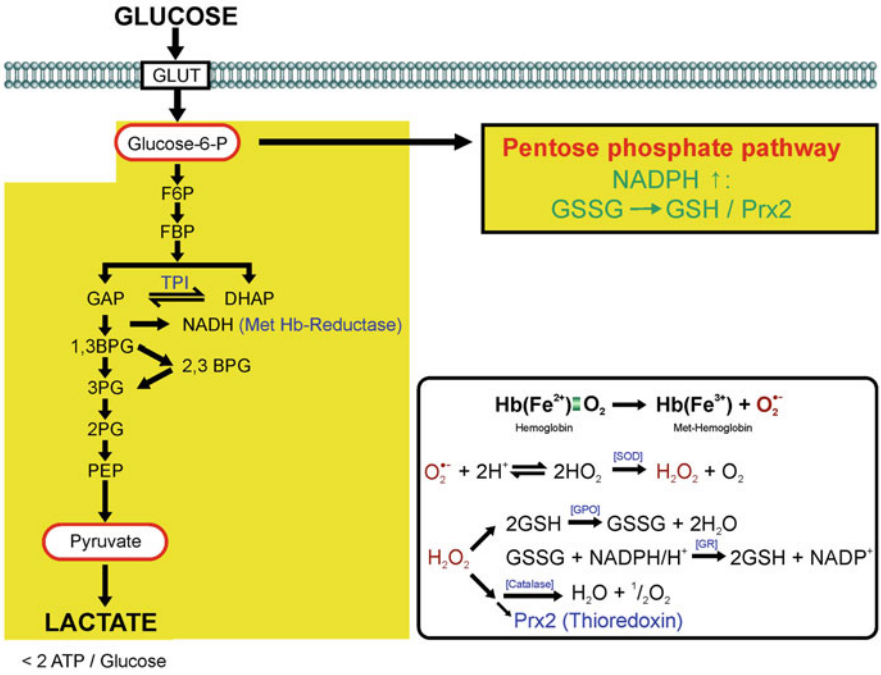


**Fig. 5.1** Glucose metabolism, generation of reactive oxygen species (ROS), and the antioxidative defense system in erythrocytes. Energy (adenosine triphosphate (ATP)) production in erythrocytes is completely dependent on glycolysis (glucose→lactate) due to the lack of mitochondria and other organelles. Erythrocytes are exposed to much higher oxygen concentrations than any other cell by binding it to hemoglobin (Hb) and, as a consequence, a significant generation of ROS is a non-avoidable side effect: During the attachment of oxygen to $Fe^{2+}$ of Hb, electrons are shifted to a slight extent from iron to oxygen, leading to Met ($Fe^{3+}$) Hb and superoxide anion ($O_2^{\bullet -}$) which is rapidly dismutated to $H_2O_2$ and $O_2$ by superoxide dismutase (CuZnSOD). The cells cope with ROS production by an intense shift of glucose-6-phosphate (glucose-6-P) to the pentose phosphate pathway (PPP): $H_2O_2$ is detoxified by glutathione peroxidase (GPO; GPx1): $H_2O_2 + 2\,GSH \rightarrow H_2O + GSSG$ and the produced glutathione disulfide (GSSG) is reduced back to glutathione (GSH) by glutathione reductase (GR) using nicotinamide adenine dinucleotide phosphate (NADPH) formed in the PPP (glucose-6-P + $NADP^+ \rightarrow$ 6-phospho-glucolactone + NADPH). A shift of glucose-6-P to the PPP is a preferred strategy of cancer cells to cope with oxidative stress (Fig. 5.3). Additionally, $H_2O_2$ can also be detoxified by NADPH-dependent peroxiredoxins, Prx2 (a thioredoxin-containing defense system) [84], whereas catalase, although present in high amounts, seems to be of minor importance for the detoxification of the continuously generated, small amounts of $H_2O_2$. Catalase may be important as a sink only under high influx of $H_2O_2$ from outside [127]. This effective antioxidative defense system, in combination with an NADH-dependent metHb reductase, allows erythrocytes to survive in the blood circulation for more than 100 days. The erythrocyte model shows the important role of iron for generation of ROS even when it is properly stored (as in Hb). For contrast, see Sect. 1.5.3

amount of oxygen consumed during normal respiration ($O_2 + 2H_2 \rightarrow 2H_2O$). Theoretically, cancer cells which metabolize glucose preferentially to lactate should, therefore, produce less ROSs. This, however, is obviously not automatically the case,

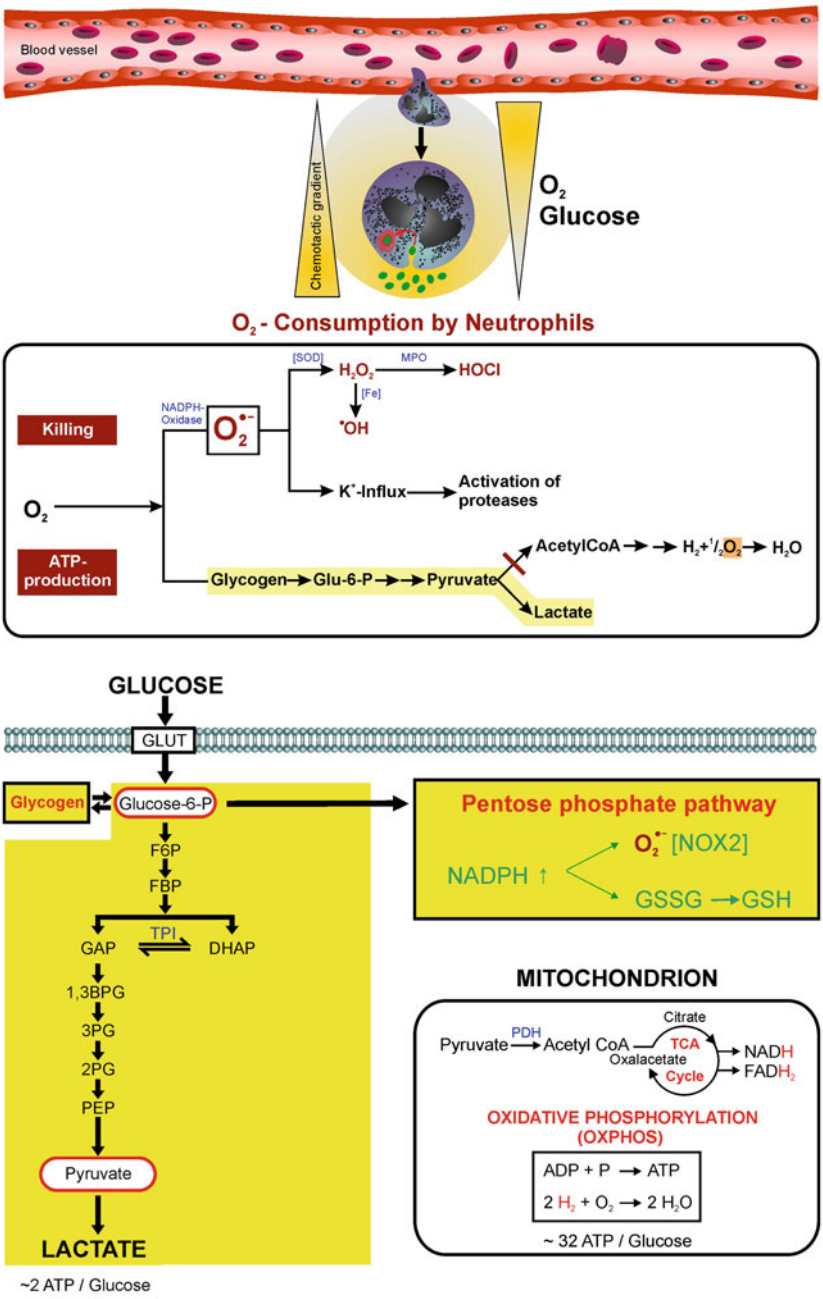


**Fig. 5.2** Glucose metabolism, generation of reactive oxygen species (ROS), and the antioxidative defense system in neutrophil granulocytes. The situation of neutrophils is, in many respects, analogous to that of cancer cells: In contrast to erythrocytes, neutrophils operate outside the blood

since under hypoxic conditions, surprisingly, even more ROSs are generated [18, 54]. Production of ROS during OXPHOS seems to depend on tight mitochondrial coupling [97, 98]. Moreover, it was shown that overexpression of frataxin, a mitochondrial protein of iron metabolism, led to enhanced OXPHOS production, but to a lower production of ROS [109].

The actual concentration of ROS depends on the production rate and on the efficacy of the defense system which, in mitochondria, is composed mainly of MnSOD ($O_2^{\bullet -} \rightarrow H_2O_2$) as well as the glutathione (GSH) [89] and the peroxiredoxin/thioredoxin systems [24] which detoxify $H_2O_2$. The tripeptide, GSH, is synthesized in the cytosol and then transported into the mitochondria [51]. Its central role for the antioxidative protection of cancer cells is outlined in more detail later. Mitochondria contain only low concentrations of catalase [49]. In contrast, catalase is abundant at high concentrations in peroxisomes. The enzyme consists of a peroxisomal targeting signal (PTS) for import into peroxisomes. Peroxisomal biogenesis factor 5 (Pex5), as a soluble receptor for proteins containing a PTS, mediates the import of proteins in peroxisomes. [41] showed that peroxisomal protein import is impaired under oxidative stress conditions. Pex5 may act as a redox sensor, and thereby oxidative stress leads to localization of peroxisomal proteins (e.g., catalase) to the cytosol. These mechanisms grant the cell a rapid response to oxidative stress in the cytosol, thereby preventing apoptosis and maintaining a reduced environment.

### 5.3.1 *The Central Role of NADPH for Protection From ROS in Cancer Cells*

Although the generation of small amounts of ROS in cancer cells may be beneficial for proliferation, elevated concentrations may lead to cell death [15]. The maintenance of an effective antioxidative defense system against ROS often results from cancer

vessels (uptake and killing of microbes indicated as small green ellipses in the upper part of the figure). They are attracted to microbes by a chemotactic gradient and after phagocytosis, generation of ROS ($O_2^{\bullet -}$, $H_2O_2$, HOCl,$^{\bullet}$OH radicals) is necessary for killing. The neutrophils attack microbes in an environment with low oxygen tension and low glucose concentration when they are distant from blood vessels. This situation is similar to those cancer cells which grow at a distance from blood vessel supply [7]. Although neutrophils contain mitochondria and require a lot of energy for evasion, crawling, and phagocytosis, they metabolize glucose completely by glycolysis (glucose→lactate production under the influence of hypoxia inducible factor (HIF)) instead of oxidative phosphorylation, in order to save the limited amount of oxygen for generation of ROS [25]. Neutrophils are almost independent of glucose supply from outside due to huge amounts of glycogen stored before extravasation from blood vessels. The shift of glucose-6-phosphate to the pentose phosphate pathway (PPP) (generation of nicotinamide adenine dinucleotide phosphate (NADPH)) fulfills two opposite functions: NADPH is necessary, first, for the generation of superoxide by NADPH-oxidase (NOX2) within the microbe-engulfed phagolysosome, and secondly, for the regeneration of glutathione (GSH) which is used for the detoxification of $H_2O_2$ escaped from phagolysosomes (red area within the neutrophils in the upper part of the figure) and could, therefore, damage neutrophils prematurely during bactericidal activity. When GSH is consumed in the end phase of phagocytosis and killing, ROS concentration increases and activates a membrane-bound acid sphingomyelinase by oxidation of cysteine residues, leading to ceramide formation from sphingomyelin and subsequent apoptosis [108]

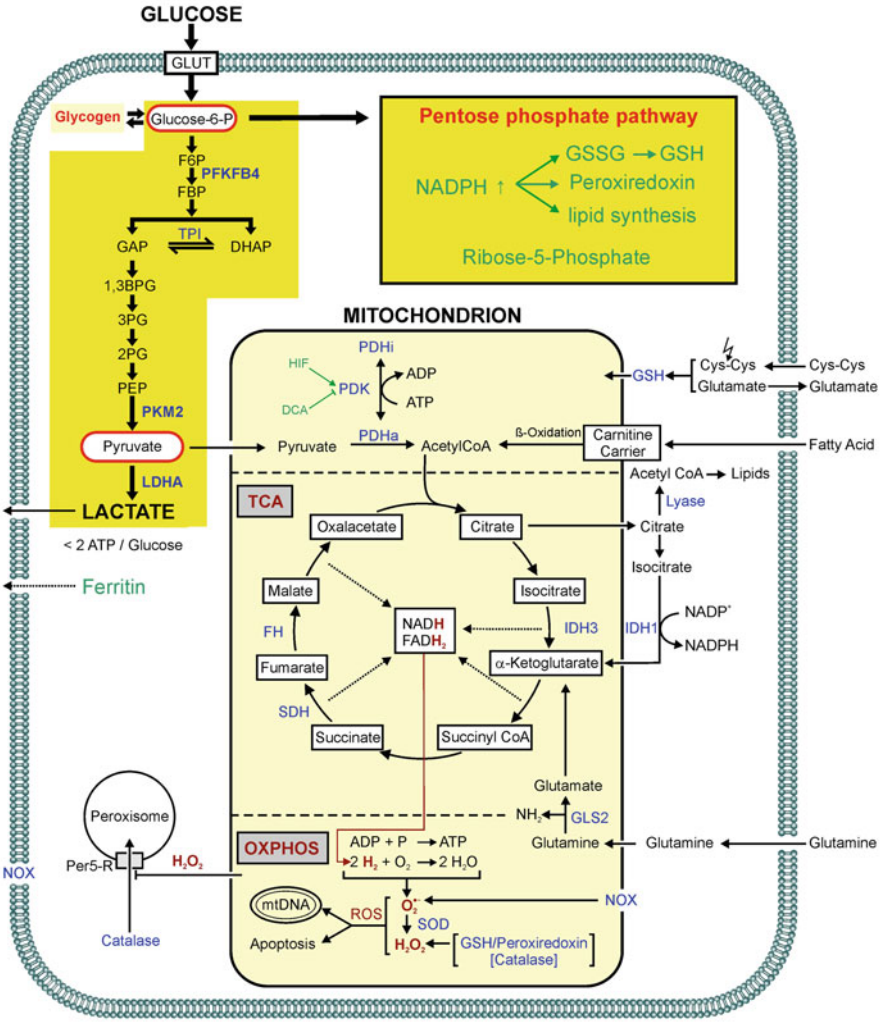


**Fig. 5.3** Overview of glucose/glutamine metabolism in cancer cells with respect to oxidative stress and antioxidative defense. The different shades of yellow indicate the relative importance of the respective pathways. For additional explanation, see text

cell-specific changes in normal glucose (and glutamine) metabolism. The most important change is a shift of glucose-6-phosphate (glucose-6-P) into the pentose phosphate pathway [105] leading to enhanced NADPH generation which is used for the regeneration of the GSH and peroxiredoxin/thioredoxin systems in the detoxification of $H_2O_2$ (see Fig. 5.1). There are many potential initiators for this shift in different cancer cells: Some tumor cells are characterized by a specific isoenzyme pattern of glycolytic enzymes. Not only in some cancer cells [52, 121] but also in non-malignant cells [12], an isoform of pyruvate kinase, PKM2, is expressed which

has a weaker enzymatic activity in the conversion of phosphoenolpyruvate (PEP) to pyruvate. Oxidation of PKM2 keeps its activity low [1], leading to enhanced PEP concentrations which inhibit triosephosphate isomerase (TPI). As a consequence, the metabolism from glucose-6-P to pyruvate is partially inhibited, leading to an enhanced turnover of glucose-6-P by glucose-6-P dehydrogenase, the "start" enzyme of the PPP [53]. Similarly, expression of PFKFB4, which inhibits the conversion of fructose-6-phosphate (F6P) to 1,6 biphosphofructose (FBP) by elevation of fructose 2,6 biphosphate, also governs glucose-6-P into the PPP in prostate cancer cells [106].

Another significant player influencing glycolysis and PPP is the tumor suppressor gene p53, coding for a nuclear transcription factor which regulates the transcription of many target genes. It suppresses glycolysis in different ways, including reduction of GLUT 1 and GLUT 4 as well as reduction of phosphofructokinase (PFK-1) (F6P → FBP) and phosphoglycerate mutase (PGM) (3PG→2PG) activities [82]. Furthermore, in mitochondria, p53 transactivates glutaminase (GLS2) (glutamine→glutamate;→ α-ketoglutarate) leading to enhanced turnover of the TCA cycle [62] and increases synthesis of cytochrome *c* oxidase (SCO2) [90]. Moreover, p53 prevents the entrance of glucose-6-P into the PPP by blocking glucose-6-phosphate dehydrogenase [64, 111]. If p53 is defective, as in many cancer cells, then these processes are reversed. Another source for enhanced NADPH generation in the PPP begins with the degradation of glycogen, under hypoxic conditions, by glycogen phosphorylase (PYGL). This leads, via generation of glucose-1-phosphate and further to glucose-6-phosphate, to enhanced glycolysis but particularly to enhanced generation of NADPH in the PPP [38].

Besides the PPP, NADPH can be generated by isocitrate dehydrogenase (IDH2) which regenerates GSH disulfide (GSSG) to GSH (and oxidized thioredoxin) in mitochondria [65]. In some tumor cells, additional possibilities to enhance NADPH for a more effective regeneration of GSH from GSSG include expression of IDH1 which converts isocitrate to α-ketoglutarate [104] (Fig. 5.3) or the enhanced conversion of malate to pyruvate [31].

Glutamine, whose uptake is under the control of the Myc oncogene [28], plays an important role in the synthesis of γ-glutamyl-cysteinyl-glycine (GSH), both by glutamate delivery and by cystine uptake as an antiport with glutamate (Fig. 5.3) [31].

Taken together, cancer cells are engaged in enhancing antioxidative defense systems in many ways, in order to improve their self-protection against oxidative damage. The shift of glucose metabolism to the PPP by various mechanisms seems to be the most important strategy of cancer cells and offers, in principle, several possibilities for specific targeted therapies.

The aspects described above characterize some possible mechanisms for ROS generation and ROS protection in different cancer cells (glioblastoma, prostate cancer, neuroblastoma, melanoma, and others) which are highly heterogeneous indeed. Based on the progress achieved in metabolic profile analysis of a certain tumor entity, concepts for more therapeutically specific interventions will probably be developed in the near future. However, the situation that exists in a cancer patient's tumor is actually more complex than what occurs in a single cell:

- Not only cells of different tumors but also cells obtained from the same tumor entity can be rather heterogeneous and metabolize compounds, such as glucose/glutamine, differently. Furthermore, the behavior of the same cancer cells changes with alterations in their environmental oxygen and nutritional supplies (e.g., as a function of proximity to blood vessels) [46].
- Cancer stem cells (generally characterized by low ROS levels) compared to more differentiated cancer cells (elevated ROS levels) significantly differ regarding their redox status [76]. Therapeutic destruction of cancer stem cells, using various therapeutic strategies, is of utmost importance in order to achieve a sustained effect. A basic problem, however, is that cancer stem cells behave in many respects similarly to certain normal stem cells, such as hematopoietic stem cells (HSCs). HSCs reside in their resting state in hypoxic niches and use glycolysis (for which the presence of HIF-1α for expression of pyruvate dehydrogenase kinase (Pdk2/4) is essential), rather than OXPHOS, for ATP production. In this way, OXPHOS-related generation of ROS is limited and stem cells are better protected from oxidative stress. They switch rapidly, however, to oxidative phosphorylation when they begin to differentiate [115, 122, 134]. Surprisingly, Vlashi et al. [118] showed that cancer stem cells (glioma) prefer OXPHOS instead of aerobic glycolysis, associated with low ROS production [118].
- An additional and important aspect of cancer cell growth is the microenvironment which greatly influences therapeutic success. Its role in the interaction of cancer cells with ROSs will, therefore, be outlined in more detail.

## 5.4 Influence of Tumor Microenvironment with Respect to Oxidative Stress for Cancer Cell Survival

Bone marrow (BM) metastases are a frequent complication of cancer, occurring in up to 70 % of patients with advanced neuroblastoma (NB), breast carcinoma (BC) or prostate carcinoma, and in approximately 15–30 % of patients with carcinoma of the lung, colon, stomach, bladder, uterus, rectum, thyroid, or kidney. Once tumors metastasize to the bone, they are usually incurable.

In patients with NB or BC, metastatic disease in the BM is observed more frequently than at any other site [100], and a high incidence of BM metastases in these patients is associated with advanced disease and poor prognosis. These observations suggest the presence of BM microenvironmental elements that are favorable for NB and BC tumor cell survival and growth.

Recent studies support a role for tumor-stromal environment as a leading player, and not just a supporting extra, in the initiation of carcinomas and the control of metabolism by oncogenes and tumor suppressors [6]. The last decade has witnessed an increased interest in the influence of the supporting stromal tissue on tumor cell metabolism. The metabolic interactions involved, however, have been poorly characterized.


**Fig. 5.4** Colony formation by MDA435 breast carcinoma (BC) cells in the absence and presence of bone marrow (BM) cell conditioned medium (CM) and commercial human erythrocyte catalase. BM CM was prepared from myelomonocytic BM cell cultures (prepared by Ficoll-gradient cell separation followed by collection of adherent cells) and incubated in serum-free RPMI 1640 medium for 4 days. CM was rendered cell free by centrifugation and Millipore filtration. CM was diluted to achieve a final concentration of 10 %, and human erythrocyte catalase solution (Sigma) was diluted in RPMI 1640 medium to achieve a final concentration of 20 U/ml. MDA435 BC cells were suspended at $10^3$ cells/ml in serum-free RPMI 1640 medium containing 0.3 % agar into 35-mm Petri dishes and layered over the dilutions. Phase contrast photographs of cells at magnification $\times$ 40 were taken after 10 days of incubation

A recent report [136] demonstrates BM stromal cell support of chronic lymphocytic leukemia (CLL) cells by providing them with cysteine, a nutrient essential for resistance to oxidative stress. Cysteine is required to produce GSH. Most cells, including cancer cells, take up extracellular cystine and reduce it to cysteine inside the cell (see Fig. 5.3). CLL cells lack the transporter for cystine import while their neighboring stromal cells are able to take up cystine, convert it to cysteine, and then secrete it. CLL cells avidly import cysteine and use it to produce GSH, resulting in increased ROS resistance and survival. Blocking of the putative BM stromal cell function suppressed CLL cell viability and, moreover, improved the efficacy of chemotherapeutic agents against CLL.

We have shown that serum-free conditioned medium (CM) from human BM myelomonocytic cell cultures strongly supports the survival and growth of different human cell lines derived from BC and NB tumors, while other human tumor cell lines failed to respond to the same CM. This rescue activity, demonstrated in vitro in serum- and adhesion-deprived, semi-solid cultures either by colony formation or by colorimetric measurement of cell viability could not be replicated by a variety of known growth factors and cytokines [48, 55]. Fractionation of the CM from a myelomonocytic cell line HL-60, with activity identical to BM cell CM, resulted in the isolation of a $\sim$60-kDa protein capable of sustaining NB and BC cell growth in serum- and adhesion-deprived cultures. Using mass spectrometry, the active protein was identified as human catalase and was subsequently shown to be replaceable by commercial human erythrocyte catalase or bovine liver catalase. The growth-promoting activity of BM cell and of HL-60 cell CM was absorbed with rabbit antibody against human catalase and was inactivated by an irreversible catalase inhibitor, aminotriazole. Figure 5.4 shows that the formation of colonies of MDA435 breast cancer cells is significantly improved by the presence of CM or catalase.

These studies identify possible metabolic exchanges between tumor cells and their microenvironmental stromal cells. These stromal–tumor interactions may be critical for survival of certain cancer cells and thereby may represent biochemical pathways for effectively targeting drug resistance of tumor cells in vivo.

An additional study considers the involvement of oxidative stress-associated compounds in immune suppression of tumor cells [86]. Much effort has been invested in enhancing antigen presentation for the purpose of activating antitumor immune cells. Once tumors are established, however, these processes have relatively limited efficacy, and the rate of clinical responses in cancer patients, although encouraging, remains relatively low. Investigation into the cause of this relative failure of anticancer immune therapy led to the conclusion that oxidative stress-associated compounds induce modifications of certain proteins involved in immune system recognition and elimination of tumor cells. These compounds, and the cells that release them, probably act as important factors limiting the effect of immune therapeutics. These investigators suggest that elimination of tumor-infiltrating myeloid cells could also contribute to a reduction in ROSs (and nitrogen) and substantially improve the effect of anticancer immune therapy.

In an attempt to advance the important success of anticancer immune therapy, these investigators suggest solutions in marked contrast to the studies described above which demonstrate the antitumor action of stromal myeloid cell-derived ROS. It may be most beneficial to equally consider each of these antitumor strategies, and others which will probably arise, and not succumb to the strict compartmentalization occurring in the field of anticancer research.

## 5.5 Therapeutic Options for Affecting the Redox Status of Cancer Cells

In the face of many factors that influence cancer cell metabolism, it is not easy to present a common concept of ROS and cancer therapy. Nevertheless, despite the individual differences between the various cancer types, some basic features exist which can be used as potential favorite target structures. Generally, it would be helpful to clarify whether a distinct cancer entity is more or less sensitive to ROS compared to normal cells. Use of ROS-based therapies has led to an often scientifically unfounded conclusion that cancer cells are more sensitive. Considering the many protective mechanisms against ROS in cancer cells, however, this is not completely clear-cut. On the other hand, characterization of some specific ROS-protective metabolic pathways, used by a certain cancer type, may enable the development of improved specific treatment strategies [22, 42, 126]. One strategy for cancer destruction by ROS is to enhance ROS concentration, either by ROS-generating drugs or endogenously shifting metabolism toward ROS producing pathways, e.g., from glycolysis to OXPHOS. Another possibility is to impair the cellular defense system against ROS, especially the NADPH-dependent GSH and peroxiredoxin/thioredoxin systems. The variety of processes by which cancer cells shift glucose-6-P to the PPP

(Sect. 1.3.1) may facilitate their specific impairment. Examination of the effects of various compounds in cell cultures that have been described in the literature may help to elucidate the mechanisms involved although they often lack specificity. In the following section, some examples of ROS-based therapies and their relationship with the glucose/glutamine metabolism are presented.

### *5.5.1 Shift of Glucose Metabolism from Glycolysis to OXPHOS*

The most characteristic metabolic feature of cancer cells is the preferred metabolism of pyruvate to lactate (using LDHA) instead of acetyl-CoA (using PDH, followed by oxidation to $CO_2$ and $H_2O$ in the TCA and OXPHOS), even in the presence of oxygen. The advantages of this pathway for cancer cells are the rapid generation of energy (ATP) due to increased glucose uptake and accelerated glycolysis, combined with the enhanced generation of metabolites which make cancer cells less dependent on nutritional supply. Another important aspect is the lower amount of ROS produced in the course of mitochondrial respiration and improved protection against them by an enhanced shift of glucose-6-P to the PPP. A shift of pyruvate→lactate to pyruvate→acetyl-CoA could, therefore, significantly disturb this preferred glucose metabolism of cancer cells and should lead to increased concentrations of cytotoxic ROS. Inhibition of LDHA was shown to increase the generation of ROS and, thereby, cause cell death [79]. Bonnet et al. [13] reported that dichloroacetate (DCA) could induce apoptosis and inhibit growth of several cancer cells. This process was associated with increased mitochondrial production of $H_2O_2$. The report attracted much interest because DCA is a very cheap drug probably without severe toxicity during short-term treatment [71]. DCA has been used clinically for treatment of congenital lactic acidosis, an inborn error of mitochondrial metabolism affecting the PDH system [112]. PDH is a complex enzyme containing thiamine pyrophosphate, lipoamide, $NAD^+$, and FAD as cofactors, which converts pyruvate to acetyl-CoA:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{Acetyl} - \text{CoA} + \text{CO}_2 + \text{NADH (net reaction)}$$

It exists in an active, non-phosphorylated form and in an inactive phosphorylated form, which is generated by PDH kinase (Fig. 5.3). DCA inhibits PDH kinase, thereby shifting the equilibrium between the active, unphosporylated PDH and the inactive, phosphorylated PDH to the active form. As a consequence, pyruvate can be more easily metabolized to acetyl-CoA instead of to lactate. This is in contrast to HIF-1α which activates PDH kinase, thereby favoring the pyruvate metabolism to lactate. The DCA-induced shift may promote pyruvate metabolism to the TCA cycle and oxidative phosphorylation, associated with an increase in the generation of ROS resulting in apoptosis. In this respect, it is interesting to note that lipoic acid, a cofactor of PDH, was reported to induce ROS-mediated apoptosis in colon carcinoma cells, but not in normal colon cells [124]. Moreover, the same group published that carnitine, which promotes mitochondrial acetyl-CoA formation by increased uptake of

fatty acid and subsequent β-oxidation, also enhances ROS generation and apoptosis [125]. Therefore, increased turnover of acetyl-CoA in the TCA cycle and OXPHOS seems indeed to be able to enhance ROS production and to induce cell death in cancer cells. Augmentation of ROS-generating reactions presents an attractive possibility specifically for cancer therapy since normal cells preferentially use oxidative phosphorylation. Their mitochondria, therefore, are probably better equipped for rescue from the accompanying ROS formation. It could be speculated that in mitochondria of cancer cells, the antioxidative system is downregulated due to the preferred use of glycolysis and that it is overcharged during a sudden ROS attack from outside or by a shift from glycolysis to OXPHOS. DCA could induce ROS-related effects in glioblastoma and other cancer cells [88, 91, 92, 131] but is not effective in all cancer cell types. DCA was reported to be selectively toxic to cells with defects in the ETC but only at rather high concentrations [113]. In neuroblastoma cells, for example, 1–2 mM DCA could not significantly enhance oxygen consumption nor kill the cells in vitro [95]. Many neuroblastoma cells contain only a low copy number of mitochondrial DNA (mtDNA) which is associated with reduced activities of some of the respiration complexes (I, II, and IV) and, consequently, with a low oxygen consumption rate [39]. In contrast to Niewisch et al., it was published that growth of undifferentiated neuroblastoma cells could be inhibited by DCA, however, at much higher concentrations [117].

### 5.5.2 *Cancer Treatment with ATO and its Relation to ROS Formation*

Inorganic arsenic trioxide (ATO, $As_2O_3$), already clinically approved for the treatment of several hematological malignancies, is currently under investigation for other malignancies, e.g., NB [102], BC [103], small cell lung carcinoma [35], and others. Recent studies show that $As_2O_3$ induces apoptosis in NB cells [102]. The mechanisms by which $As_2O_3$ induces apoptosis involve the generation of ROS which lead to the decrease in mitochondrial membrane potential and activation of caspases. Furthermore, the ROS-based cytotoxic effects of ATO are supported by the inhibitory effect of ATO on compounds which contain vincinal thiol-groups and are engaged in antioxidative defense systems like thioredoxins/thioredoxin reductase [85] and by its interactions with GSH-related enzymes [30].

On the other hand, we have recently shown that BM cells and HL-60 cell CM reproducibly and substantially reverse the cytotoxic action of $As_2O_3$-treated NB cell lines in a dose-dependent manner [48]. We and others have observed that catalase, at very low concentrations, equivalent to that of cytokines and growth factors, supports the survival and growth [48, 107] of many cell types especially in cultures with low cell density. We have compared the response of NB cells with different malignancy potentials to this rescue activity. Less malignant cells are unable to survive in sparse, serum- and adhesion-deprived cultures, perhaps due to their higher sensitivity to toxic oxygen substrates resulting from peroxisomal catalase deficiency. Addition

of catalase to those cultures was substantially advantageous to their viability and growth. Interestingly, growth of more highly malignant cells was not affected by deprived conditions and was not enhanced by the additional catalase. The rapid growth of those malignant cells, under nutrient and adhesion deprivation, probably leads to increased ROS with which they are apparently able to cope. Despite their resistance, those cells succumbed to $As_2O_3$-induced cytotoxicity; however, they were rescued by catalase. These data show again the different sensitivities of cancer cells and the important role microenvironment plays for therapeutic success. We conceive that the anticellular action of $H_2O_2$ and ROS, which may be released by $As_2O_3$-treated tumor cells, as well as by their microenvironmental stroma, is inhibited due to their enzymatic decomposition by intercellular catalase. This action of intercellular or extracellular catalase [107, 60] may, thereby, overcome the apparent failure of intracellular peroxisomal catalase to protect tumor cells from $As_2O_3$-induced ROS. There is, however, no documentation of such a failure. Alternatively, ATO may inhibit the GSH/peroxiredoxin/thioredoxin system which rescues cells from $H_2O_2$ and catalase may compensate for this inhibition.

### 5.5.3 *Iron-dependent ROS Generation in Cancer Cells as Potential Target Structure*

Iron is known to induce ROS generation, among them the highly cytotoxic OH radicals generated in the Fenton reaction ($Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + {}^{\bullet}OH + {}^{-}OH$) [56]. This occurs especially in situations of iron overload if it is not properly stored in proteins ("loosely bound iron"/labile iron pool (LIP)). Kiessling et al. [73] reported that in a cutaneous T-cell lymphoma cell line, inhibition of NFκB signaling led to ROS-induced cell death**.**This inhibition led to a downregulation of ferritin heavy chain (FHC) in this T-cell lymphoma cell line, but not in normal T-cells, causing an increase of free intracellular iron. Cell death was effectively blocked by the iron chelator desferrioxamine, by N-acetylcystein and GSH as well as by downregulation of FHC by small interfering RNA (siRNA). The authors suggest that FHC could be a target structure for therapy in lymphoma with aberrant signaling. Additionally, impairment of the GSH system should support this therapy (and similar ROS-based therapies): There are different possibilities to inhibit the GSH system, such as GSH concentration can be lowered by BSO (L-buthionine-*S*-sulfoximine), an inhibitor of GSH synthesis [43]. Asparaginase, used in the treatment of ALL in childhood, is able to deplete glutamine in blood plasma [132] and can reduce GSH synthesis, too. Furthermore, ascorbic acid was also reported to lower GSH concentrations [27]. With respect to the treatment of cutaneous T-cell lymphoma, inclusion of ascorbic acid could be of additional benefit due to its pro-oxidative effects in the presence of "free" (labile) iron (see Sect. 1.5.4).

Many cancer cells contain and release elevated amounts of ferritin which may be used even as a tumor marker, as in neuroblastoma [58]. Ferritin is able to store up to 4,500 iron ions which can be reductively released, most effectively by superoxide

anion [8]. Besides superoxide, ascorbic acid may also liberate iron ($Fe^{2+}$) from ferritin (but to a much lesser extent), leading to the formation of cytotoxic ROS [3]. This process could be exploited for cancer therapy with high-dose ascorbic acid.

### 5.5.4 *High Dosage of i.v.-Administered Ascorbic Acid as a Pro-drug for Cancer Therapy*

The concept of cancer therapy with high-dose ascorbic acid (oral uptake of 10 g vitamin C/day or more) was originally proposed by Cameron and Pauling [16, 17]. Satisfactory proof of success could not, however, be achieved [26, 93]. One reason is that plasma ascorbate concentrations never exceeded ~ 250 μmol/l after oral uptake of even such large amounts [99]. On the other hand, it is well known that ascorbic acid, at high concentrations (> 1 mmol/l), is cytotoxic for tumor cells in cell culture due to its pro-oxidative effects, possibly induced by traces of transition metals like iron and copper in the cell culture medium. This effect, therefore, was considered an artifact with no relevance for therapy [23, 70]. The concept of tumor therapy, however, with high-dose ascorbic acid was re-evaluated by the group of Levine et al., who proposed intravenous (i.v.) application of high doses of vitamin C. This dosage is well tolerated and serum ascorbate concentrations of 20 mmol/l and more can be obtained for a certain time period during and after infusion [81]. In cell culture experiments and later in animal experiments, Chen et al. showed that ascorbic acid is cytotoxic in the millimolar range for many different cancer cells but to a much lesser extent for normal cells [19, 20, 21]. The authors could demonstrate the generation of $H_2O_2$ in the extracellular environment of tumors in amounts sufficient for cell killing and concluded that it was generated in a transition metal-dependent process outside the cells. From there, $H_2O_2$ can easily penetrate every cell membrane and attack DNA and other cell components. The source of the transition metal may be ferritin, released from cancer cells [34], where it is present in high amounts [63]. Ascorbic acid could liberate reductively small amounts of iron:

$$(\text{Ferritin } (Fe^{3+}) + AA \rightarrow Fe^{2+} + {}^{\bullet}AA;$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2{}^{\bullet-} \left[{}^{\bullet}AA\text{: Ascorbyl Radical}\right]$$

Once initiated, the reaction is self-accelerated by increased iron release in response to superoxide and by the regeneration of $Fe^{3+}$ to $Fe^{2+}$ by ascorbic acid. The acidic environment due to released lactate [61] in cancer cells helps to keep iron in a non-precipitated form. I.V. administered vitamin C has been widely used in cancer therapy, mainly in alternative medicine; it is usually well tolerated; however, until now, proof of its effects in controlled studies is still needed.

Vitamin C, moreover, is often used in cancer therapy in combination with chemotherapeutics [11] mostly with the aim of protecting normal tissues. This should be carefully considered since vitamin C could reduce the antitumor effects of some drugs [59]. However, opposite effects were also described [78]. This, for example,

could play a role in ATO therapy if ascorbic acid is administered i.v. at high concentrations. Vitamin C (ascorbic acid) was shown to enhance ATO toxicity in multiple myeloma cells [50], in acute promyelocytic leukemia-derived NB4 cells [27] and in hepatocellular carcinoma cells [83], but in its oxidized form, dehydroascorbate, the cytotoxicity against HL60 and U266 cells was decreased [70].

In summary, there are many interactions between glucose metabolism, the generation of ROS, and rescue from them in cancer cells, especially due to NADPH-dependent pathways which are influenced manifold in cancer cells. Advancement in the metabolic profile characterization of different tumors will probably provide more specific therapeutic opportunities in the near future.

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# Chapter 6
# Modulating Autophagy and the "Reverse Warburg Effect"

**Maria I. Vaccaro, Claudio D. Gonzalez, Silvia Alvarez and Alejandro Ropolo**

**Abstract** Autophagy is a highly regulated cellular pathway for degrading long-lived proteins and is the only known pathway for clearing cytoplasmic organelles. Autophagy is a major contributor to maintain cellular homeostasis and metabolism. The quality control of mitochondria is essential to maintain cell energy and this process appears to be achieved via autophagy. Warburg hypothesized that cancer growth is caused by the fact that tumor cells mainly generate energy by the non-oxidative breakdown of glucose. This cellular behavior relies on a respiratory impairment, characterized by a mitochondrial dysfunction, which results in a switch to glycolysis. Moreover, epithelial cancer cells may induce the Warburg effect in neighboring stromal fibroblasts in which autophagy was activated. Here, we introduce the autophagy process, its regulation, the selective pathways, and its role in cancer cell metabolism. We define the Warburg effect and the "reverse" hypothesis and we discuss the potential value of modulating autophagy. The association of the Warburg effect in tumor and stromal cells to cancer-related autophagy is of significant relevance in experimental therapeutics.

**Keywords** Autophagy · Cancer · Warburg effect · Mitochondria · Caveolin · Mitophagy · Cancer cell metabolism · Metformin · Rapamycin · Chloroquine · mTOR · PI3K · VMP1 · BECN1 · ROS · RAGE

## 6.1 Autophagy, a Self-Eating Cellular Process

Autophagy is an evolutionarily conserved and highly regulated lysosomal pathway that degrades macromolecules (e.g., proteins, glycogen, lipids, and nucleotides) and cytoplasmic organelles [1–3]. This catabolic process is involved in the turnover of

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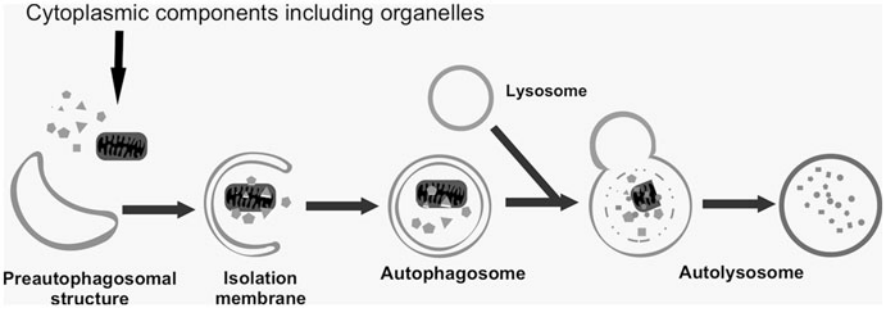


**Fig. 6.1** During autophagy, an isolation membrane forms as a pre-autophagosomal structure, invaginates and sequesters cytoplasmic constituents. The edges of the membrane fuse to form a double-membrane vesicle known as the autophagosome. The outer membrane of the autophagosome fuses with the lysosome to deliver the inner vesicle with the contents to the lumen of the degradative compartment, the autolysosome

long-lived proteins and other cellular macromolecules, and it might play a protective role in development, aging, cell death, and defense against intracellular pathogens [4, 5]. By morphological studies, autophagy has been linked to a variety of pathological processes, such as neurodegenerative diseases and tumorigenesis, which highlights its biological and medical importance [6, 7].

Autophagy consists of several sequential steps, which are: induction, autophagosome formation, and autophagosome–lysosome fusion and degradation. Although autophagy was first identified in mammalian liver upon glucagon treatment approximately 50 years ago, its molecular understanding started only in the past decade, largely based on the discovery of the autophagy-related genes (ATGs) by genetic analyses in yeast.

Depending on the delivery route of the cytoplasmic material to the lysosome, there are three major types of autophagy in eukaryotes: (1) chaperone-mediated autophagy (CMA), (2) microautophagy, and (3) macroautophagy, hereafter referred to as autophagy [8]. CMA allows the direct lysosomal import of unfolded, soluble proteins that contain a particular pentapeptide motif. In microautophagy, cytoplasmic material is directly engulfed into the lysosome at the surface of the lysosome by membrane rearrangement. Finally, autophagy involves the sequestration of cytoplasm into a double-membrane cytosolic vesicle, referred to as an autophagosome that subsequently fuses with a lysosome to form an autolysosome for the degradation by lysosomal hydrolases [9].

### *6.1.1 The Process of Autophagy*

Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double-membrane vesicles called autophagosomes, which eventually acquire lysosomal-like features [9, 10]. The autophagic process is described in Fig. 6.1. An isolation membrane forms, invaginates, and sequesters cytoplasmic components. The edges of the membrane fuse to form the autophagosome. The outer membrane

of the autophagosome fuses with the lysosome to deliver the inner membrane vesicle to the lumen of the digestive compartment forming the autolysosome.

Autophagy is mediated by a set of evolutionarily conserved gene products (termed the ATG proteins) originally discovered in yeast [11]. In mammalian cells, BECN1 [2, 12–14] promotes autophagosome formation when it functions as part of a complex with the class III phosphatidylinositol 3-kinase (PI3K) mediating the localization of other autophagic proteins to the autophagosomal membrane [15]. However, despite the advances in understanding autophagy, autophagosome formation in mammalian cells is a complex process, and neither the molecular mechanisms nor all the implicated genes involved in its formation are fully elucidated.

Although autophagy has been studied in mammals since the 1960s, only since 2000 has yeast genetics allowed us to understand this process at a molecular level. More than 30 highly conserved genes that are involved in autophagy have been identified so far [16]. Among these, a core molecular machinery has defined and is composed of four subgroups: first, the ATG1/unc−51-like kinase (ULK) complex; second, the class III phosphatidylinositol 3 kinase (PtdIns3K)/Vps34 complex I; third, two ubiquitin-like proteins ATG12 and ATG8 (LC3) conjugation systems; and four, two transmembrane proteins, ATG9/mATG9 (and associated proteins involved in its movement such as ATG18/WIPI-1) and VMP1 (whose expression triggers autophagy) [17–19]. Basal autophagy in unstressed cells is kept down by the action of the mammalian target of rapamycin complex 1 (mTORC1). Key upstream regulators of mTORC1 include the class I phosphoinositide 3-kinase (PI3K)-Akt pathway, which keeps mTORC1 active in cells with sufficient growth factors, and the adenosine monophosphate (AMP)-activated protein kinase (AMPK) pathway that inhibits mTORC1 upon starvation and calcium signals [20, 21].

### *6.1.2 Regulation of Autophagy Induction Through mTOR*

Under stress conditions such as amino acid starvation, autophagy is strongly induced in many types of cultured cells. The effects of individual amino acids differ in their abilities to regulate autophagy. Amino acids including Leu, Tyr, Phe, Gln, Pro, His, Trp, Met, and Ala suppress autophagy in an ex vivo-perfused liver [22]. However, such profiles depend on cell types showing their different amino acid metabolisms in tissues. The questions on how cells sense amino acid concentration and physiological significance of autophagy regulation by amino acid starvation are not fully understood yet. It has been demonstrated that amino acid signaling pathways exist, which involve the activation of serine/threonine kinase mTOR and the subsequent regulation of the class III PI3K. The mTOR is involved in the control of multiple cell processes in response to changes in nutrient conditions [23]. Especially, mTORC1 requires Rag GTPase, Rheb, and Vps34 for its activation and subsequent inhibition of autophagy in response to amino acids [[24], 25]. Energy levels are primarily sensed by AMP-activated protein kinase (AMPK), a key factor for cellular energy homeostasis. In low energy states, AMPK is activated and the activated AMPK then inactivates mTORC1 through TSC1/TSC2 and Rheb protein [26].

Thus, the inactivation of mTORC1 is essential for the induction of autophagy and plays a central role in autophagy. In addition to amino acid signaling, hormones, growth factors, and many other factors, including bcl-2 [27], reactive oxygen species (ROS) [28], calcium [29], BNIP3 [30], p19ARF [31], DRAM [32], calpain [33], TRAIL [34], FADD [35] and myo-inositol-1,4,5-triphosphate (IP3) [36], have also been reported to regulate autophagy. But, not all autophagy signals are transduced through mTOR signaling. A recent study showed that small-molecule enhancers of the cytostatic effects of rapamycin (called SMERs) induce autophagy independently of mTOR [37]. Activities of the ULK1 kinase complex are regulated by mTOR, depending on nutrient conditions. Under growing and high-nutrient conditions, the active mTORC1 interacts with the ULK1 kinase complex (ULK1–mATG13–FIP200–ATG101) and phosphorylates ULK1 and mATG13, and thus inhibits the membrane targeting of the ULK1 kinase complex. During starvation conditions, on the other hand, the inactivated mTORC1 dissociates from the ULK1 kinase complex and results in the ULK1 kinase complex, free to phosphorylate components, such as mATG13 and FIP200, in the ULK1 kinase complex, leading to autophagy induction [38].

The pancreatitis-associated protein named vacuole membrane protein 1 (VMP1) is a transmembrane protein with no known homologs in yeast. VMP1 expression induces autophagosome formation, even under nutrient-replete conditions while remaining an integrated autophagosomal membrane protein in mammalian cells [39]. VMP1 expression is induced by hyperstimulation of Gq-coupled cholecystokinin (CCK) receptor in pancreatic acinar cells during acute pancreatitis [40] and by mutated KRas in pancreatic cancer cells [41]. VMP1 interacts with Beclin 1/ATG6 through its hydrophilic C-terminal region (VMP1-ATG domain), which is necessary for early steps of autophagosome formation [39, 42]. Besides, EPG-3/VMP1 is one of three essential autophagy genes conserved from worms to mammals, which regulates early steps of the autophagic pathway in Caenorhabditis elegans [43]. VMP1 along with ULK1 and ATG14 localizes in the endoplasmic reticulum-associated autophagosome formation sites in a PI3K activity-independent manner, confirming the key role ofexpression is induced by hyperstimulat VMP1 in the formation of autophagosomes [18]. Interestingly, Dictyostelium cells lacking the VMP1 gene showed accumulation of huge ubiquitin-positive protein aggregates containing the autophagy marker ATG8/LC3 and p. 62 homolog [44]. Moreover, the knockdown of VMP1 expression abolishes starvation and rapamycin- induced autophagosome formation [39], as well as autophagy induced by hyperstimulation of the Gq-coupled CCK receptor in pancreatic acinar cells [40] or by chemotherapy in pancreatic tumor cells [45]. Furthermore, VMP1 is the only human disease-inducible ATG protein described so far.

### *6.1.3 The Class III PI3K Complex in Autophagosome Nucleation*

The autophagosome formation process is composed of isolation membrane nucleation, elongation, and completion steps. In mammals, the class III PI3K complex plays an essential role in isolation membrane nucleation during autophagy [46], while

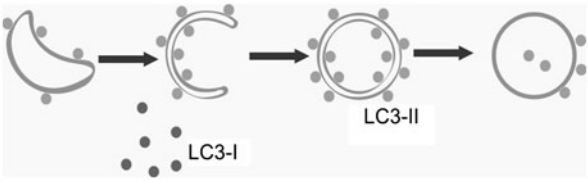


**Fig. 6.2** During autophagy the cytosolic form of LC3 (LC3-I) undergoes C-terminal proteolytic and lipid modifications (LC3-II) and translocates to the autophagosomal membrane. LC3 is currently used as a specific marker of autophagy

the class I PI3K pathway is also involved in autophagy regulation through the insulin signaling cascade to activate mTOR and PKB [3]. The class III PI3K (Vps34) is associated with Beclin1 (ATG6) and p150, the homolog of Vps15 (phosphoinositide-3-kinase, regulatory subunit 4), to form the class III PI3K core complex.

As the first step of autophagosome formation, the autophagosome nucleation system includes ATG12–ATG5–ATG16 L, which is essential for the formation of pre-autophagosomes. ATG12 is a 186-amino acid protein and is conjugated to ATG5 [47]. The carboxy-terminal glycine residue of ATG12 is activated by E1-like ATG7 through a high-energy thioester bond in an ATP-dependent manner [48–51]. ATG12 is then transferred to E2-like ATG10 [52] and finally attached to lysine 149 of ATG5 via an isopeptide bond [48]. The ATG12–ATG5 conjugate further interacts with ATG16L1 to form a ~ 350 kDa multimeric ATG12–ATG5–ATG16 protein complex through the homo-oligomerization of ATG16 [53].

Once the autophagosome formation is completed, ATG proteins are released back to the cytoplasm by an uncharacterized mechanism. The second ubiquitin-like protein conjugation system is the modification of LC3 (a mammalian homolog of ATG8) by the phospholipid phosphatidylethanolamine (PE) [54], an essential process for the formation of autophagosomes. LC3 is cleaved by cysteine protease ATG4 and then conjugated with PE by ATG7 and ATG3, a second E2-like enzyme. This lipidated LC3-II then associates with newly forming autophagosome membranes. LC3-II remains on mature autophagosomes until its fusion with lysosomes [55]. The conversion of LC3 to LC3-II is thus well known as a marker of autophagy induction (Fig. 6.2). However, the increase of LC3-II alone is not enough to show autophagy activation because the inhibition of LC3-II degradation in the lysosome by the impaired autophagy flux can also cause its accumulation.

While the origin of autophagic vacuoles remains disputable, several hypotheses have been proposed for the source of autophagosomal membrane during autophagosome formation. The first hypothesis is the "de novo" formation of autophagosome by ATG9 reservoirs [56]. In the second hypothesis, various organelles such as endoplasmic reticulum (ER) [57], mitochondria [58], and plasma membrane [59] are used as an origin for the formation of the phagophore. Recently, cup-shaped structures called omegasome, a discrete region of the ER, were identified as a platform for autophagosome formation [60]. The ATG5 complex, LC3, and ULK1 have been shown to recruit into the omegasome after starvation, and ATG5- and LC3-positive membranes seem to emerge from the omegasome. It was also observed that

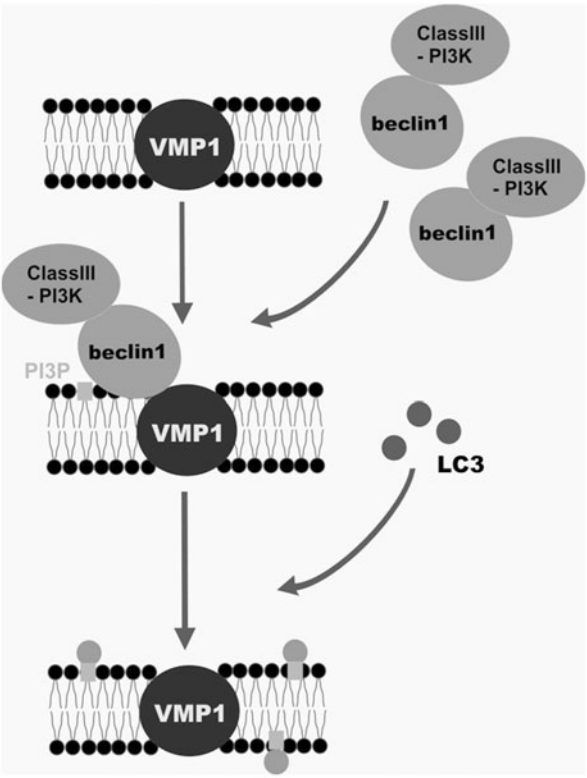


**Fig. 6.3** VMP1 is a transmembrane protein whose expression triggers autophagy interacting with BECN1 and regulating the autophagy-specific PI3K complex in mammalian cells

omegasomes form in close proximity to the Vps34-containing vesicles which may synthesize phosphatidylinositol 3-phosphate (PI(3)P). This hypothesis is also supported by the notion of a physical association between the ER and early autophagic membranes [57].

Recent studies have identified new regulators of autophagosome maturation and degradation, including activating molecule in Beclin 1-regulated autophagy (AMBRA1) [61], ultraviolet radiation resistance-associated gene (UVRAG) [62], Rubicon [63], and VMP1 [42]. VMP1, along with Ulk1 and ATG14, localizes to the sites where autophagosomes are formed independently of the other ATG proteins [18]. The 20 amino acids of the C-terminal hydrophilic domain of VMP1, the VMP1 autophagy-related domain (VMP1-ATGD) [39], bind with the BH3 domain of Beclin 1 promoting the displacement of Bcl-2, a negative regulator of autophagy, and driving BECN1 to the autophagic pathway. This interaction leads the formation of a VMP1-BECN1-hVps34 complex and the subsequent association of ATG16L1 to the autophagosomal membranes, providing a model describing one of the key steps in the peripheral anterior synechia (PAS) formation and autophagy regulation in mammalian cells (Fig. 6.3) [42, 64].

### 6.1.4 *Vesicle Completion, Autophagosome—Lysosome Fusion, and Degradation*

Autophagosome then fuses with lysosomes/vacuoles, which is an essential process for completion of the autophagy pathway. The sequestration of cytoplasm into a double-membrane cytosolic vesicle is followed by the fusion of the vesicle with a late endosome or lysosome to form an autophagolysosome (or autolysosome). Then, the inner membrane of the autophagosome and autophagosome-containing cytoplasm-derived materials are degraded by lysosomal/vacuolar hydrolases inside the autophagosome. The molecular mechanisms underlying the transport and fusion of autophagosomes are just beginning to be understood, and through active investigations, several major events involved in the process have recently been clarified. In mammalian cells, autophagosome maturation is a prior step for the fusion between autophagosomes and lysosomes. The degradation products, including macromolecules, are then exported to the cytosol for reutilization by the cell. This process is poorly understood.

## 6.2 Selective Types of Autophagy

Early studies suggested that autophagy was a nonselective process in which cytoplasmic structures were randomly sequestered into autophagosomes before being delivered to the mammalian lysosome or the plant and yeast vacuole for degradation. Now there is growing evidence that unwanted cellular structures can be selectively recognized and exclusively eliminated within cells. This is achieved through the action of specific autophagy receptors, such as p62 and Nbr1. Thus, excess or damaged organelles including mitochondria, peroxisomes, lipid droplets, endoplasmic reticulum, and ribosomes can be specifically sequestered by autophagosomes and targeted to the lysosome for degradation. Importantly, there is growing evidence that selective autophagy subtypes also have a wide range of physiological functions. In yeast, the cytosol-to-vacuole (Cvt) pathway transports hydrolases into the vacuole. In eukaryotes, autophagy plays a central role in both innate and acquired immunity [65]. In pancreatic cells autophagy has recently been shown to specifically turn over secretory granules damaged by acute pancreatitis as a protective cellular response [40].

### 6.2.1 *Mitophagy, the Selective Autophagic Degradation of Damaged Mitochondria*

Three major pathways of mitochondrial quality control have been described so far. Two AAA protease complexes can degrade misfolded mitochondrial membrane proteins with catalytic sites facing both sides of the inner membrane. Mitochondrial

proteins can also be degraded by translocation to lysosomes; vesicles budding from mitochondrial tubules sequester selected mitochondrial cargos, and deliver those mitochondrial components to the lysosome for degradation. The third pathway, known as mitophagy, involves sequestration of an entire mitochondrion within the autophagosome, followed by fusion with a lysosome.

Concomitant with the energy production through oxidative phosphorylation, mitochondria also generate ROS, which in excess cause damage through the oxidation of proteins, lipids, and DNA often inducing cell death. Therefore, the quality control of mitochondria is essential to maintain cellular homeostasis and this process appears to be achieved via autophagy. It has been postulated that mitophagy contributes to differentiation and development by participating in the intracellular remodeling that occurs, for example, during hematopoiesis and adipogenesis. In mammalian red blood cells, the expulsion of the nucleus followed by the removal of other organelles, such as mitochondria, are necessary differentiation steps. Nix/Bnip3L, an autophagy receptor whose structure resembles that of ATG32, is also an outer mitochondrial membrane protein that interacts with gamma-aminobutyric acid receptor-associated protein (GABARAP) [66, 67] and plays an important role in mitophagy during erythroid differentiation [68, 69]. Although autophagosome formation probably still occurs in Nix/Bnip3L-deficient reticulocytes, mitochondrial elimination is severely impaired. Consequently, mutant reticulocytes are exposed to increased levels of ROS and die, and Nix/Bnip3L knockout mice suffer severe anemia. Depolarization of the mitochondrial membrane potential of mutant reticulocytes by treatment with an uncoupling agent results in restoration of mitophagy [69], emphasizing the importance of Nix/Binp3L for the mitochondrial depolarization and implying that mitophagy targets uncoupled mitochondria. Hematopoietic-specific ATG7 knockout mice also exhibited severe anemia as well as lymphopenia, and the mutant erythrocytes markedly accumulated degenerated mitochondria but not other organelles [70]. The mitochondrial content is regulated during the development of T cells as well; that is, the high mitochondrial content in thymocytes is shifted to low mitochondrial contents in mature T cells. ATG5- or ATG7-deleted T cells fail to reduce their mitochondrial content resulting in increased ROS production as well as an imbalance in pro- and anti-apoptotic protein expression [71–73]. All together, this evidence demonstrates the essential role of mitophagy in hematopoiesis.

Recent studies have described the molecular mechanism by which damaged mitochondria are selectively targeted for autophagy, and have suggested that the defect is implicated in familial Parkinson's disease (PD) [74]. PINK1, a mitochondrial kinase, and Parkin, an E3 ubiquitin ligase, have been genetically linked to both PD and a pathway that prevents progressive mitochondrial damage and dysfunction. When mitochondria are damaged and depolarized, PINK1 becomes stabilized and recruits Parkin to the damaged mitochondria. Various mitochondrial outer membrane proteins are ubiquitinated by Parkin and mitophagy is then induced. Of note, PD-related mutations in PINK1 and Parkin impair mitophagy [75–78], suggesting that there is a link between defective mitophagy and PD. How these ubiquitinated mitochondria are recognized by the autophagosome remains unknown. Although p. 62 has

been implicated in the recognition of ubiquitinated mitochondria, elimination of the mitochondria occurs normally in p62-deficient cells [79, 80].

Mitochondrial function is essential for cancer cells. However, different cancer cell types undergo different bioenergy alterations, some to more glycolytic and others to more oxidative, depending in part on the developmental state of the cell undergoing neoplastic transformation. Therefore, different alterations in bioenergy metabolism or mitochondrial ROS production and redox biology can be found depending on the specific environment of the cancer cells promoting the cell survival [81]. In this context, for example, mitophagy is an important mechanism to promote cell survival by the clearance of damaged mitochondria that are potential sources of ROS [82].

## 6.3 Autophagy in Cancer Cell Metabolism

Both downregulated and excessive autophagy have been implicated into the pathogenesis of diverse diseases, such as certain type of neuronal degeneration, diabetes and its complications, and cancer [83]. Autophagy has also been implicated in cell death called autophagic or type II programmed cell death, which was originally described on the basis of morphological studies detecting autophagic vesicles during tissue involution [84].

Cancer cells in general tend to undergo less autophagy than their normal counterparts, at least for some tumors [85, 86]. The Beclin1 autophagy gene is monoallelically deleted in 40–74 % of cases of human sporadic breast, ovarian, and prostate cancer [86]. Heterozygous disruption of Beclin1 increases the frequency of spontaneous malignancies and accelerates the development of virus-induced premalignant lesions [86] suggesting that defective regulation of autophagy promotes tumorigenesis. It has been proposed that autophagy suppresses carcinogenesis by a cell-autonomous mechanism involving the protection of genome integrity and stability, and a nonautonomous mechanism involving suppression of inflammation and necrosis. On the other hand, autophagy may support the survival of rapidly growing cancer cells that have outgrown their vascular supply and are exposed to an inadequate oxygen supply or metabolic stress. By contrast, excessive levels of autophagy promote cell death [87]. Accordingly, it has been proposed that autophagy plays an important role both in tumor progression and in promotion of cancer cell death [88], although the molecular mechanisms responsible for this dual action of autophagy in cancer have not been elucidated.

It has been suggested that autophagy may be a cancer cell survival response to tumor-associated hypoxia. Tumor hypoxia has been used as a marker of poor prognosis [89]; however, how cancer cells become more malignant or survive with an extremely poor blood supply is poorly understood. When cancer cells are exposed to hypoxia, anaerobic glycolysis increases and provides energy for cell survival, but as the glucose supply is also insufficient because of the poor blood supply, there must be an alternative metabolic pathway that provides energy when both oxygen and glucose are depleted [90, 91]. Hypoxia in pancreatic cancer has been reported to

increase its malignant potential [89]. Proliferating cancer cells require more nutrients than surrounding noncancerous cells do, though nutrition is supplied via functionally and structurally immature neo-vessels. Because autophagy-specific genes promote the survival of normal cells during nutrient starvation in all eukaryotic organisms, autophagy may react to the cancer microenvironment to favor the survival of rapidly growing cancer cells. LC3 expression in surgically resected pancreatic cancer tissue, showed activated autophagy in the peripheral area, which included the invasive border and concomitantly shows enhanced expression of carbonic anhydrase [92]. This observation suggests that autophagy may promote cell viability in hypo-vascularized cancer tissue.

It has also been proposed that autophagy is a cancer cell survival response to tumor-associated inflammation [93]. Cancer-associated inflammation results in promotion of carcinogenesis and resistance to therapy. Several phenotypic alterations observed in cancer cells are a result of inflammatory signals found within the tumor microenvironment [93]. The receptor for advanced glycation end products (RAGE) is an induced inflammatory receptor constitutively expressed on many murine and human epithelial tumor cell lines [94, 95] and the highest levels of RAGE expression were observed in murine and human pancreatic adenocarcinoma tumors. Genotoxic and/or metabolic stress lead to modest but reproducible increases in overall expression of RAGE on epithelial cell lines. RAGE expression correlates directly with the ability of both murine and human pancreatic tumor cell lines to survive cytotoxic insult. Targeted knockdown of RAGE significantly increases cell death, whereas forced overexpression promotes survival. Recently, it was reported that the enhanced sensitivity to cell death in the setting of RAGE knockdown is associated with increased apoptosis and decreased autophagy. In contrast, overexpression of RAGE is associated with enhanced autophagy, diminished apoptosis, and enhanced cancer cell viability. Knockdown of RAGE enhances mTOR phosphorylation in response to chemotherapy, thus preventing induction of a survival response. Inhibition of autophagy by means of silencing Beclin1 expression in pancreatic cancer cells enhances apoptosis and cell death [96]. These observations suggest that RAGE expression in cancer cells has a role in tumor cell response to environmental stress through the enhancement of autophagy. However, increased sensitivity to chemotherapeutic agents in RAGE-knockdown pancreatic cancer cells is dependent on ATG5 expression but independent of BECN1 expression [96]. These last findings suggested that the role of autophagy in the resistance to microenvironment insult or in the sensitivity to chemotherapeutic agent is the result of complex molecular pathways in the tumor cell.

On the other hand, repression of autophagy has been suggested as a cancer cell response to prolonged hypoxic conditions. Pancreatic cancer cell response to prolonged hypoxia may consist of inhibition of autophagic cell death. The short isoform of single-minded 2 (SIM2s) is a member of the basic helix–loop–helix family of transcriptional regulators [97] and is upregulated in pancreatic cancer. Microarray studies identified the pro-cell death gene BNIP3 as a target of SIM2s repression. Prolonged hypoxia induces cell death via an autophagic pathway involving the

hypoxia-inducible factor 1 (HIF1)-mediated upregulation of BNIP3 [30, 98]. The deregulation of both SIM2s and BNIP3 is associated with poor prognostic outcomes [99]. Decreased BNIP3 levels and poor prognosis clearly correlate with elevated SIM2s expression in pancreatic cancer. The loss of BNIP3, either by hypermethylation or by transcriptional repression, is correlated with inhibition of cell death [100, 101], whereas upregulation of BNIP3 sensitizes pancreatic carcinoma cells to hypoxia-induced cell death [102]. SIM2s expression, concomitant with its repression of BNIP3, enhances tumor cell survival under prolonged hypoxic conditions. Recent data link increased SIM2s expression with enhanced cell survival during hypoxic stress concomitantly with BNIP3 repression and the attenuation of hypoxia-induced autophagic processes. Thus, the inhibition of autophagic cell death by BNIP3 repression enhances tumor cell survival under prolonged hypoxic conditions.

Decreased autophagy in some cancer cells has been related to malignant stages of the disease. Cancer cells, in general, tend to undergo less autophagy than their normal counterparts, supporting the contention that defective autophagic cell death plays a role in tumor progression. Studies of carcinogen-induced pancreatic cancer in animal models have shown that pancreatic adenocarcinoma cells have lower autophagic capacity than premalignant cells [103]. The WIPI protein family, which includes ATG18, the WIPI-1 homolog in *Saccharomyces cerevisiae*, was genetically identified as a gene contributing to autophagy [103]. Human WIPI-1a is a member of a highly conserved WD-repeats protein family**.** hWIPI-1 is linked to starvation-induced autophagy in the mammalian system. Amino acid deprivation triggers an accumulation of endogenous hWIPI-1 protein to large vesicular and cup-shaped structures, where it colocalizes with LC3. Starvation-induced hWIPI-1 formation is blocked by wortmannin, a principal inhibitor of PI-3 kinase-induced autophagosome formation [104]. Interestingly, WIPI proteins are linked pathologically to cellular transformation because all human WIPI genes are reportedly expressed aberrantly in a variety of matched human cancer samples. Strikingly, hWIPI-2 and hWIPI-4 messenger RNA (mRNA) expression is substantially decreased in 70 % of matched kidney (ten patients) and 100 % of pancreatic (seven patients) tumor samples. The majority of these samples were derived from advanced stage tumors, such as pancreatic adenocarcinomas stages I–IV. Hence, cancer-associated downregulation of hWIPI-2 and hWIPI-4 supports the possibility that decreased autophagic activity is necessary for the malignant stages of pancreatic cancer.

## 6.4 Otto Warburg and the "Warburg Effect"

Born in Freiburg, Germany, in 1883, Otto Warburg was one of the leading chemists of the first half of the twentieth century. The son of a very famous physicist, student of the eminent chemist Emil Fisher, and Nobel Prize laureate, Warburg devoted several years of his life to elucidate the mechanisms by which cancer cells obtain energy especially under fast-growing conditions [105].

By 1920, measuring lactate production and oxygen consumption on rat liver carcinoma tissue, Otto Warburg and colleagues proposed that cancer cells display some very relevant differences when compared with normal tissues with regard to their glucose metabolism by favoring glycolysis despite oxygen availability. Warburg hypothesized that cancer growth is caused by the fact that tumor cells mainly generate energy (in the form of ATP) by the non-oxidative breakdown of glucose. This view contrasts with the observation that normal cells produce ATP through oxidative phosphorylation obtaining "fuel" by the oxidative breakdown of glucose [105].

The ATP yield from glycolysis under anaerobic conditions (2 ATPs per molecule of glucose) is much smaller than the yield from the complete oxidation of glucose to $CO_2$ under aerobic conditions (30 or 32 ATPs per molecule of glucose) [106]. About 15 times more glucose is consumed anaerobically in contrast to the aerobic pathway to yield the same amount of ATP. As a consequence, glucose uptake proceeds about ten times faster in most solid tumors than in normal tissues [107]. Tumor cells commonly experience hypoxia (limited $O_2$ supply), and as a result, cancer cells depend on anaerobic glycolysis for their ATP production.

This phenomenon of preferred *aerobic glycolysis* was denominated the "Warburg effect" resulting in increased lactate production, even in the presence of adequate oxygen partial pressures. It was suggested that this cellular behavior relies on a respiratory impairment, characterized by a mitochondrial dysfunction, which results in a switch to glycolysis. It was proposed that the high glycolytic rate might also result from a decreased number of mitochondria in tumor cells [108].

This effect, first described in cancer tissues, was further identified in many other rapidly dividing normal cells [109]. Several mechanisms have been proposed to explain the Warburg effect in cancer tissues. These mechanisms may be involved in transcriptional and posttranslational related metabolic changes. Transcriptional upregulation of glycolytic enzymes was extensively studied for decades. Some well-characterized transcriptional regulators have been associated with the molecular basis of the Warburg effect. The HIF1 transcription factor increases the glycolytic enzymes and glucose transmembrane transport, and upregulates pyruvate dehydrogenase kinases (which results into a reduction of the pyruvate flux to the tricarboxylic acid cycle) [110]. The degradation of this transcriptional regulator involves some mediators, like the Von Hippel–Lindau tumor suppressor ubiquitin ligase, which seems to be consistently altered in some cancer cells [110]. In these tumors, even in normoxic conditions, HIF1 seems to increase the glycolytic rate, to elevate lactate production and to activate the PI3K/AKT/mTOR pathway [110].

In cancer cells, the reduced expression of the tumor suppressor protein, p53, might be also linked to the Warburg effect. In fact, p53 reduces the glycolysis rate by increasing the activity of fructose-2,6-bisphosphatase, a mechanism also involved in the regulatory pathways of apoptosis [111]. It also seems to increase the oxidative phosphorylation process. Other transcriptional regulators might be linked to the Warburg effect, such as the alpha estrogen-related receptor (of potential relevance in breast cancer); in the same direction, increased expression of oncogenes such as MYC

also seems to be associated with an increased glycolytic rate and might be involved in the pathophysiology of the metabolic modifications found in tumors [112]. Besides, glycolytic enzymes and glucose transmembrane transport are activated by MYC overexpression.

As mentioned before, the posttranslational regulation of the Warburg effect was also under scrutiny. As a relevant example, activation of the PI3K/AKT downstream pathway leads to an increased glucose influx and the phosphorylation of hexokinase and phosphofructokinase-2 with a concomitant upregulation of the glycolytic pathway [110]. Several posttranslational modifications of the M2 isoform of pyruvate kinase result in a change in its activity, modulating the glycolytic pathway in several tissue types. The K305 acetylation of this M2 isoform reduces its enzymatic activity and increases the enzyme degradation via CMA [110]. By oxidation, acetylation, phosphorylation, etc., the posttranscriptional modification of the M2 isoform of the pyruvate kinase influences glycolysis in various models and experimental conditions.

Tumor overexpression of endogenous microRNA (miRNA) was recently linked with metabolic regulation of cancer cells and the "Warburg effect" [110]. Although attractive, the biological impact of this association remains to be clarified.

All of these mechanisms, heterogeneous by nature, were proposed as possible explanations of this phenomenon in cancer cells. Often these mechanisms were extrapolated from isolated cancer cell experiments *in vitro* not including other components of the neoplastic tissue. Other cellular components apart from the cancer cells, vascular growth rates, and oxygen partial pressure in different tumor segments, as well as differential concentrations of distinct transcriptional factors across the tumor volume were not considered in many of these experiments. Nevertheless, some findings collected from the first decade of the twenty-first century changed our form of understanding of this metabolic behavior in cancer tissues. These findings will be summarized in the following section.

## 6.5 The "Reverse Warburg Effect"

### *6.5.1 The Concept*

The Warburg effect was thought to occur only in cancer cells until recently. In 2008, Vincent et al. [113] showed that human skin keloid fibroblasts display similar bioenergetic mechanisms as cancer cells in generating ATP mainly from glycolysis. This observation may be explained by the similarity in the hypoxic microenvironment in solid tumors and keloids [113]. In line with this previous study, Lisanti et al. proposed in 2010, a new hypothesis for understanding the Warburg effect in tumors [114]. They suggested that epithelial cancer cells induce the Warburg effect in neighboring stromal fibroblasts.

As a first step, these cancer-associated fibroblasts undergo myofibroblastic differentiation and secrete lactate and pyruvate through the glycolytic pathway. This process is induced, as previously stated, by cancer cells by a mechanism involving

oxidative stress with overproduction of reactive oxygen species, loss of Caveolin-1, mitophagy and/or mitochondrial dysfunction, and increased production of NO [115].

In a second step, epithelial cancer cells take up the energy-rich metabolites that enter in the tricarboxylic acid (TCA) pathway and, in consequence, ATP is generated by oxidative phosphorylation. These cells expand their mitochondrial mass to satisfy an increased metabolic demand, upregulate enzymes involved in antioxidant defense to cope with oxidative stress, and increase the tumor aggressive behavior [116].

This interesting hypothesis was based in studies performed in a co-culture system mimicking tumor–stroma co-evolution, where stromal fibroblasts (human telomerase reverse transcriptase (hTERT)-BJ1 cells) interact with human breast cancer cells (MCF-7) [117]. The conclusions are consistent with Warburg's original view, but one must take into consideration that the phenomenon is occurring in the tumor stroma.

For a deeper analysis of the phenomenon, a division into two general steps can be made as follows.

### *6.5.2 Step 1: Cancer-Associated Fibroblasts Undergo Aerobic Glycolysis to Produce Energy-Rich Nutrients*

Epithelial cancer cells firstly induce the Warburg effect in adjacent cancer-associated fibroblasts through the downregulation of Caveolin-1 [114]. The loss of Cav-1 expression may be sufficient to induce this constitutive fibroblastic phenotype, although this mechanism needs further investigation. The absence of stromal Cav-1 is associated with a high rate of tumor recurrence, metastasis, and poor clinical outcome [118].

Cav-1 is a structural component of caveolae. These structures are flask-shaped invaginations of the plasma membrane occupying up to 30 % of the cell surface and represent a predominant location of endothelial nitric oxide synthase (eNOS). Among other functions, Cav-1 can regulate eNOS activity and NO release [119]. NO plays an important signaling role in vascular function and a regulatory role in mitochondrial function [120, 121]. However, if overproduced, mitochondrial dysfunction accompanied by increased production of ROS may develop. Oxidative stress is inseparably linked to mitochondrial dysfunction and mitochondrial turnover is dependent on autophagy [122].

ROS is a term that actually groups a range of oxygen-derived molecules formed by the incomplete reduction of $O_2$ during oxidative metabolism that have both specific mechanisms of production and intracellular targets [123]. The biologically important species of this group are superoxide anion ($O_2\bullet^-$) and hydrogen peroxide ($H_2O_2$), as they are formed by controlled mechanisms, and $H_2O_2$ is a signaling molecule. A major endogenous source of both $O_2\bullet^-$ and $H_2O_2$ is the mitochondrial electron-transport chain, where continuous electron leakage occurs during aerobic respiration

[124]. In addition, low levels of these two species are produced by the membrane-localized nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. The low-level steady state of these species in mitochondria ($10^{-8}$M $H_2O_2$ and $10^{-10}$ M $O_2\bullet^-$) [125] is accomplished by a group of antioxidants species that includes compounds of nonenzymatic (as glutathione) and enzymatic nature (as superoxide dismutase or catalase). Oxidative stress is an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage [123].

It is imperative to briefly discuss the importance of the $H_2O_2$steady-state concentrations related to biological effects. At concentrations lower than 0.7 μM, $H_2O_2$ mainly acts as a signaling molecule redox-regulating several physiological processes. Apoptosis may be triggered at the 1–3 μM range, and necrosis may develop at concentrations higher than 3 μM [126, 127]. This brief analysis indicates the relevance of taking into account steady-state levels when addressing fundamental questions on biological effects of $H_2O_2$ and the existence of a cellular fine regulation of the subcellular concentrations of this species.

Not surprisingly, mitochondria and ROS are emerging as important players in autophagy. The cross talk between autophagy, redox signaling, and mitochondrial dysfunction is not well understood. Recently, it was suggested that chronic expression of RCAN1-1 L (stress-inducible protein) induces mitochondrial autophagy and metabolic shift from oxidative phosphorylation to glycolysis [128]. Moreover, mitophagy may also be important in attenuating apoptosis or necrosis, by clearance of damaged mitochondria [122].

The occurrence of mitophagy (including a decrease in mitochondrial mass) is not only favored by the occurrence of oxidative stress, but by the activation of HIF1 (a key factor involved and activated in hypoxic conditions) as well [115]. The overproduction of ROS is sufficient to induce HIF1 through its stabilization under normoxic conditions [129]. In this scenario, the cancer-associated fibroblasts are obliged to produce ATP through aerobic glycolysis with an increased glucose consumption rate (due to a low-energy yield, as previously explained) and the concomitant production of a high amount of lactate and pyruvate (energy-rich nutrients).

### *6.5.3 Step 2: Cancer Cells Uptake Nutrients and Produce ATP by Oxidative Phosphorylation*

Stromal metabolism produces high-energy nutrients (for example, lactate) and recycled chemical building blocks (as nucleotides, amino acids, and fatty acids obtained through the process of mitophagy) that are taken up by cancer cells to power their own growth [117]. Lactate is taken up by a monocarboxylate transporter situated in the cancer cell membrane, converted into acetyl-coenzyme A (CoA), which subsequently condenses with oxaloacetate to initiate the TCA cycle for energy production [130]. NADH is formed as a product of these cyclic reactions and channeled into the mitochondrial respiratory chain where it is oxidized to NAD+, a process coupled to

the synthesis of ATP [131]. This results in a unilateral and net energy transfer from the catabolic tumor stroma to the anabolic cancer cells [116]. As a consequence, cancer cells synthetize their own ATP and increase mitochondrial mass through biogenesis.

In normal mammalian cells, mitochondria have an average half-life of 4–20 days, depending on the organ and age. The respiratory chain is located in the mitochondrial inner membrane and consists of four complexes: Complex I (NADH dehydrogenase), Complex II (Succinate dehydrogenase), Complex III (ubiquinone-cytochrome c oxidoreductase), and Complex IV (cytochrome oxidase). These complexes may interact to form multicomplexes with defined stoichiometry [132]. Due to the reducing power of NADH (utilized to reduce $O_2$ to water), an electrochemical gradient produced by the respiratory chain is used by ATP synthase (sometimes called Complex V) as a driving force to phosphorylate adenosine diphosphate (ADP) to ATP. This important bioenergetic process occurs in organelles that are not static. The increase in mitochondrial size, number and mass, a process known as mitochondrial biogenesis, is triggered by a variety of stimuli and involves a complex network connecting different regulatory pathways that are tightly coordinated [133, 134].

Therefore, it is obvious that the increased production of additional energy in the form of ATP occurring in cancer cells will be sensed, and adaptive changes in mitochondrial content will be triggered culminating in increased and coordinated biogenesis of new mitochondria. For example, it has been shown that the number of mitochondria in co-cultured MCF7 cells is greatly increased, as compared to homotypic cultures of MCF7 cells [115, 135].

It is important to point out that, in addition to increasing mitochondrial mass, cancer cells escape oxidative mitochondrial damage by upregulating enzymes involved in antioxidant pathways, including catalase and peroxiredoxin-1 [115]. The pathways and regulation of the "reverse Warburg effect" need further analysis, opening and interesting area of investigation. Understanding the mechanisms involved in each of the steps will lead to the development of new therapeutic strategies for cancer prevention.

A scheme of the two steps described above is shown in Fig. 6.4.

## 6.6 Drugs, Warburg Effect, and Reverse Warburg Effect

Several lines of evidence suggest that both inhibitors and activators of autophagy may have utility in the treatment of patients with chemotherapy-resistant cancers, since strong overactivation as well as strong inhibition of autophagy induces death in highly aggressive cancer cells, such as pancreatic cancer cells, and sensitizes them to hypoxia starvation [136]. Such autophagy activating (e.g., rapamycin derivatives, sirolimus and temsirolimus or sulforaphane—a naturally occurring dietary substance enriched in broccoli) or inhibiting drugs (e.g., antibiotic monensin, antimalarial drug chloroquine) are available and generally are well tolerated by patients.

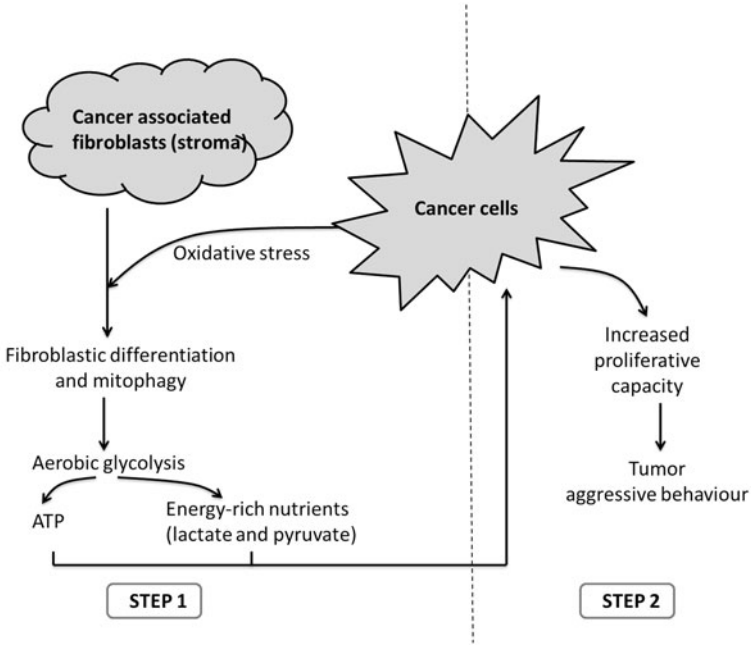


**Fig. 6.4** Schematic representation of the reverse Warburg effect. STEP 1: Cancer-associated fibroblasts (stroma) undergo fibroblastic differentiation triggered by oxidative stress generated by cancer cells. The occurrence of mitophagy obliges differentiated fibroblasts to produce ATP and energy-rich nutrients by aerobic glycolysis. STEP 2: Cancer cells take up ATP and nutrients, increasing their proliferative capacity and aggressive behavior

Metformin, a well-known antidiabetic agent, is one of the most studied agents in this area. This drug has been proposed as a potentially multi-faceted agent for cancer prevention. Meformin acts as an indirect activator of AMPK and is able to reduce mitochondrial respiratory chain Complex I activity. These have been proposed as mechanisms for reducing hepatic glucose output in patients with type 2 diabetes. In p53-deficient cancer cells, metformin treatment was associated with increased cell death. In normal cells, metformin treatment is followed by an increase in glycolytic rates as an alternative ATP-producing mechanism. In fact, one very rare but still possible adverse event of metformin is lactic acidosis. P53-deficient cells seem to experience problems in switching their metabolic pattern, which is followed by an enhanced cell death rate. By reducing the activity of the respiratory chain Complex I, metformin diminishes ROS generation in mitochondria [137]. The role of ROS in tumorigenesis and on cancer growth has been widely recognized. Metformin, as well as thrombospondin and endostatin, exhibits a mild to moderate antiangiogenic effect. This effect on angiogenesis may be the basis for its potential actions on cancer cells and/or its stroma [137].

As mentioned before, metformin activates the ATM/LKB1/AMPK axis. The tumor suppressor LKB1, well characterized in the pathophysiology of melanoma,

pancreatic, and lung cancer, might participate in the mechanism of action of metformin. Part of the preventative effects of metformin might be mediated by this suppressing factor. By activating AMPK, metformin may inhibit the mTOR pathway; this effect has been proposed as an explanation for the potential antineoplastic effects of metformin in breast and renal tumors [138]. Many of the mentioned mechanisms may explain the effects of metformin on the Warburg effect. Metformin has been suggested to reduce glycolysis and to increase mitochondrial respiration in tumors, associated with growth arrest [138]. It has been proposed that pyruvate kinase expression in the fibroblasts of tumor stroma is linked to cancer growth. ROS produced by cancer cells promotes oxidative stress in fibroblasts, which results in activation of HIF1 and nuclear factor kappa B (NFkB). NFkB increases proinflammatory cytokines and HIF1 alpha promotes autophagy and anaerobic glycolysis. Pyruvate kinase activity results in an increase in ketones and lactate. These nutrients are transferred to cancer cells and used for mitochondrial oxidative metabolism. Conversely, metformin reduces the mitochondrial chain activity by inhibiting Complex I. In this manner, metformin may alter some of the mechanisms involved into the reverse Warburg effect [139]. Metformin may also affect cell reprogramming by modifying the lipogenic enzymes acetyl-CoA carboxylase and fatty acid synthase [140]. These changes may also affect the metabolic behavior of both stroma and tumor cells. As mentioned before, the clinical impact of these modifications is still uncertain.

Other drugs exhibit a potential for the modification of the Warburg effect and autophagy rates. Mild autophagy induction such as hypoxia or starvation seems to protect the cells, but rapamycin or sulforaphane lead to elimination [136]. By contrast, an excessive autophagy rate may induce cell death. Inhibition of autophagy by nonensin or 3-methyladenine is able to eliminate highly aggressive pancreatic adenocarcinoma cells [136], as these drugs may totally block continuous recycling of cellular components necessary for new synthesis and survival.

In advanced cancer, autophagy may be necessary for the maintenance of the tumor and multiple clinical trials are underway to test this as a therapeutic approach in patients using hydroxychloroquine (HCQ) [141, 142]. Standard cancer chemotherapies may affect autophagy in different ways. Gemcitabine monotherapy or its combination with other agents has become the standard chemotherapy for the treatment of advanced pancreatic cancer. Gemcitabine is a relatively effective chemotherapeutic agent acting by competition with deoxycytidine triphosphate (dCTP) for the incorporation into DNA causing chain termination; on the other hand, gemcitabine serves as an inhibitory alternative substrate for ribonucleotide reductase and leads to a reduction of deoxynucleotide pools [143, 144]. This molecule inhibits cells that are insensitive to classic anticancer drugs, including other nucleoside analogs with similar structures. Although gemcitabine seems to exert its toxicity at least in part by activation of apoptosis [143], it was recently suggested that gemcitabine also induces autophagy in pancreatic cancer cells [45]. It has been proposed that the early induction of autophagy with gemcitabine may be mediated by an increased expression of VMP1 [145]. Capecitabine, a pyrimidine analog, induces apoptosis in several

cancer lines and is of modest efficacy in locally advanced PDAC when associated with limited field radiotherapy [144]. By displaying a Src kinase modulatory effect, capecitabine has been proposed to modulate autophagy [146]. The results in this area are still contradictory. Irinotecan is a topoisomerase I inhibitor which prevents DNA from unwinding. In a phase III trial, the combination of 5-fluouracil, leucovorin, oxaliplatin, and irinotecan resulted in better responses, progression free survival, and overall survival when compared with the standard single drug therapy with gemcitabine for metastatic PDAC [147]. In small cell prostatic carcinoma, irinotecan promoted an increase in autophagy of treated tumors as indicated by an increase in LC3B expression [148]. Even though authors of this research state that the role of autophagy is complex, there is evidence that autophagy supports both promotion and suppression of cancer growth. In general, as mentioned before, a considerable amount of caution should be exercised for the interpretation of the consequences of cancer chemotherapy on autophagy. Other chemotherapeutic agents like the glycoside oleandrin, some platinum compounds, the multikinase inhibitor sorafenib, and some histone deacetylase inhibitors have demonstrated effects on the autophagy rate in pancreatic carcinoma cell lines [149]. As proposed, autophagy may be involved in carcinogenesis, tumor progression, and dissemination, as well as may be associated at least in part with the actions of some chemotherapy for PDAC. All these modifications may alter both the Warburg and reverse Warburg effects. Nevertheless, the real contribution of these metabolic changes on tumor cell survival and clinical prognosis remains unclear.

## 6.7 Perspectives

The dysregulation of autophagic function has been implicated in a growing list of disease processes and has underscored the selective or substrate-specific versions of the pathway. In terms of cancer biology, autophagy has been viewed as having dual roles in both tumor suppression and progression, and the activation of autophagy selective forms can be used as a potential therapeutic approach for the treatment of specific cancers [150]. Autophagy is a major contributor to maintain cellular homeostasis and metabolism, and continued studies are required to identify key molecules regulating autophagy and a better understanding of the process at a molecular level.

Recently, two interesting approaches have been employed to identify new autophagy regulators: small molecule screening [151, 152] and studies on structural information of Atg proteins. These screens allowed the identification of compounds that can induce autophagy and promote long-lived protein degradation. Interestingly, some compounds are FDA-approved drugs for the treatment of human diseases [151]. The search for new autophagy regulators is a good way to explore the mechanism of autophagy and identify new molecules that may be useful for the treatment of human diseases.

Future research on the mechanism and regulation of selective autophagy and the physiological importance of this pathway in human disease may reveal new

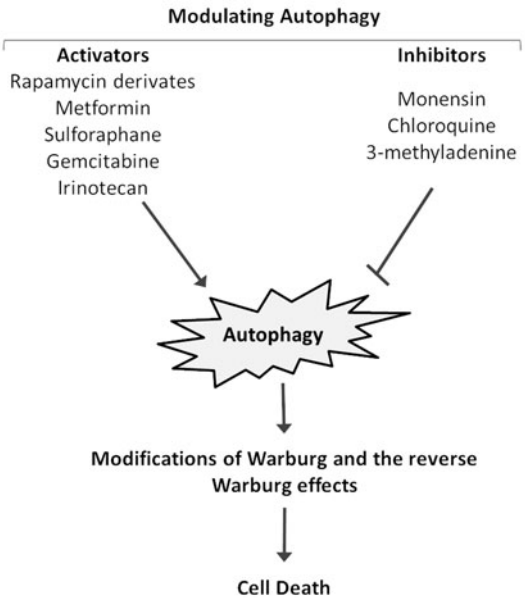


**Fig. 6.5** Pharmacological interventions that may modulate autophagy and the Warburg and reverse Warburg effects

therapeutic strategies. Some pharmacological interventions may modify the Warburg and the reverse Warburg effects. Several mechanisms for such actions were reported, but in general, the clinical relevance of these findings is still being clarified. Potential pharmacological interventions modulating autophagy and the Warburg and reverse Warburg effects are shown in Fig. 6.5.

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# Chapter 7
# Metabolic Adaptation in Reprogrammed Cancer Cells

**Kevin Marks and Charles Kung**

**Abstract** Cancer metabolism is an emerging field that offers promise in revealing new targets and strategies to treat tumors. Here, we describe several of the major advances in the understanding of how cancer cells rewire their metabolic circuitry and how these insights might be used to develop new drugs. We contrast how gain-of-function mutations in tyrosine kinases and metabolic enzymes such as isocitrate dehydrogenase promote tumorigenesis. While such recurrent mutations have not been described for the vast majority of metabolic enzymes, these enzymes are still potential targets in their own right, or are used by cancer cells in ways that can expose other vulnerabilities. We illustrate how the ability to study metabolism in molecular detail through sophisticated nutrient labeling studies has allowed connections to be made between cancer metabolism and cell signaling, epigenetics, as well as pathways targeted by standard-of-care chemotherapeutics.

**Keywords** Cancer metabolism · Metabolic adaptation · Isocitrate dehydrogenase · Pyruvate kinase M2 · Synthetic lethality · Epigenetics

## 7.1 Introduction

Cancer metabolism has emerged as a promising area for the discovery of new cancer drug targets. The approximately 2,000 proteins that comprise the metabolome offer a tantalizing landscape in which to discover new targets, and to develop anti-tumor agents that act through completely new mechanisms of action. Most of these enzymes, at first glance, appear to be well studied; indeed, the bewildering array of diagrams in biochemistry textbooks has not changed substantially in more than 50 years. However, closer scrutiny reveals that, in fact, many enzymes have not been closely studied in human cells, and even fewer in the context of cancer cells. Modern insights from genomics and metabolomics technology offer the opportunity

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to truly "rewrite the textbook." Nevertheless, the widespread acceptance of cancer metabolism as a fruitful area for drug discovery is unlikely to occur until the clinical success of the first modern drugs targeting enzymes in this biological space. As we will discuss in this chapter, the first wave of such molecules is fast approaching.

It is worth noting, at the outset, that the current era of enthusiastic study of cancer metabolism is not the birth of a new field, but rather a "renaissance." Cancer was considered by many to be a disease of metabolism until being overshadowed by advances in cell signaling, most notably the discovery of posttranslational modification of proteins and lipids by phosphorylation and their frequent dysregulation in cancer. Indeed, the classic Warburg effect [1], whereby cancer cells prefer to metabolize glucose and excrete it as lactate even under conditions of normal oxygen tension, was first noted in the 1920s. However, the work over the last decade or so, rather than simply revisiting old territory, has opened up wide swaths of new ground by harnessing the discoveries of the post-genome era coupled with modern improvements to classic experimental techniques such as carbon isotopomer labeling. These studies have revealed, in molecular detail, how cancer cells rewire their cellular metabolism and, in some cases, have linked specific adaptations to genetic lesions in metabolic enzymes. It has become clear that cancer cells adapt their metabolism both to fuel the abnormal metabolic demands of rapid proliferation and to deal with the nutrient and redox stress that this entails.

In this chapter, we will focus on the intertwined questions of how do cancer cells demonstrate metabolic flexibility or inflexibility, and how might these qualities be used to target them therapeutically. We will first tackle the question from the angle of "oncometabolites," metabolites that appear to directly drive tumorigenesis. Next, we will address the question of whether specific metabolic adaptations result in metabolites or pathways becoming essential or limiting in cancer cells, akin to the genetic concept of synthetic lethality (Fig. 7.1).

In considering how to think about these two types of adaptations in cancer metabolism, it is useful, to a certain extent, to draw analogies and contrasts with successful paradigms in cancer cell signaling. Changes in cell signaling, often driven by aberrant activity of protein tyrosine kinases, are a profound adaptation by cancer cells and represent well-established hallmarks of cancer biology [2]. Numerous tyrosine kinases with activating mutations or chromosomal translocations have been identified, and "validated in the clinic," with resistance to targeted therapies arising at the site of drug action. In contrast, deep sequencing of cancer genomes has thus far revealed very few recurring genetic lesions in metabolic enzymes. As we will discuss, even the best-known examples, mutations found in the isocitrate dehydrogenase family of enzymes, exert their tumorigenic effects through mechanisms that are quite different from the classic oncogene addiction model exploited by tyrosine kinase inhibitors. In the case of "non-druggable" oncogenes such as KRAS and p53, significant effort has been exerted to identify "bystander pathways" to which these tumors have become selectively addicted to as a consequence of reprogrammed cell signaling [3, 4]. Analogously, we will describe examples of metabolic adaptations in cancer cells that are not always directly targetable (e.g., loss-of-function mutations in putative tumor suppressor proteins such as fumarate hydratase (FH)) but, due to a

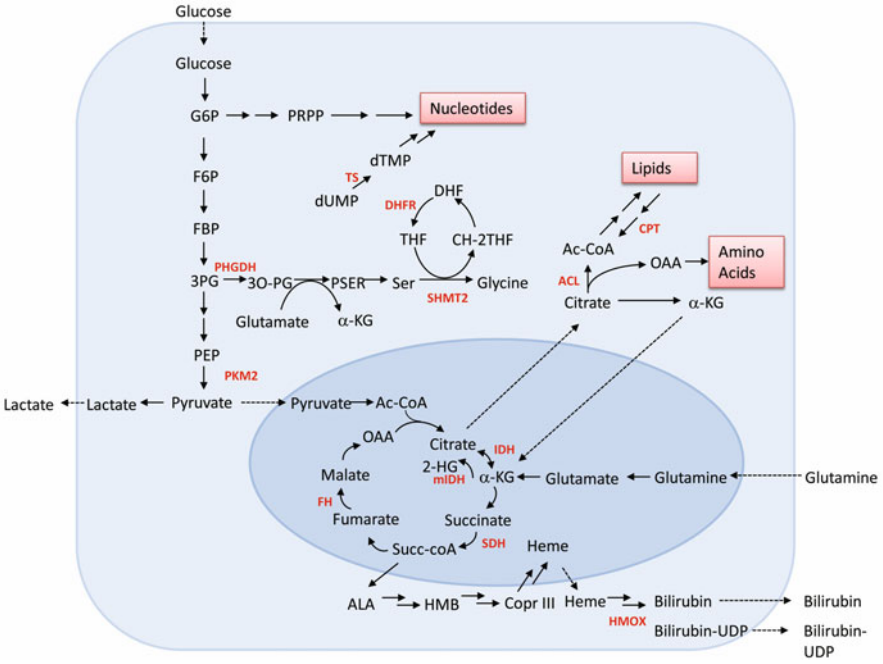


**Fig. 7.1** Schematic representation of most of the metabolic pathways and enzymes discussed in this chapter. Diagram is not meant to be comprehensive. *TS* thymidylate synthase, *DHFR* dihydrofolate reductase, *PHGDH* phosphoglycerate dehydrogenase, *CPT* carnitine palmitoyltransferase, *SHMT2* serine hydroxymethyltransferase, *ACL* ATP citrate lyase, *PKM2* pyruvate kinase M2; *IDH* isocitrate dehydrogenase, *mIDH* mutant isocitrate dehydrogenase, *FH* fumarate hydratase, *SDH* succinate dehydrogenase, *HMOX* heme oxygenase

loss of metabolic flexibility, result in unanticipated vulnerabilities that may be viable pathways for therapeutic intervention.

## 7.2 The Oncometabolite 2-HG Drives Epigenetic Reprogramming in Cancer Cells With Mutations in Isocitrate Dehydrogenase

One of the most exciting areas of research in cancer metabolism surrounds the isocitrate dehydrogenase family, comprised of three family members (IDH1, IDH2, and IDH3). In 2008, it was discovered that a significant fraction of glioblastoma tumors harbor a mutation in their IDH1 protein, with the most frequent mutation converting arginine residue 132 to a histidine (R132H) [5]. This mutation and analogous mutations in the IDH2 protein [6] remain one of the very few recurring mutations described in metabolic genes.

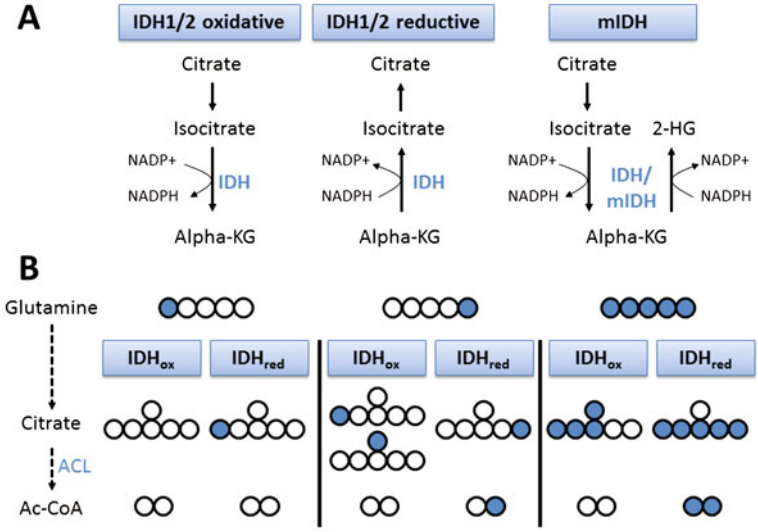


**Fig. 7.2** **a** IDH enzymes can act in both the forward oxidative and reverse reductive directions depending on the specific cellular context. Mutant IDH enzymes produce 2-hydroxy-glutarate (2-HG) via a neomorphic enzyme activity in concert with the unmutated enzyme. **b** Mass isotopomer tracing distinguishes between glutamine-derived citrate and acetyl-coA formed via isocitrate dehydrogenase (IDH) oxidative and reductive directions. *Circles* represent the carbon backbone of indicated metabolites. *Filled circles* represent labeled atoms (e.g., $^{13}C$)

The IDH enzymes catalyze the conversion of isocitrate to alpha-ketoglutarate (alpha-KG) (Fig. 7.2a) in an NAD(P)H-dependent manner in the oxidative "forward" reaction in the canonical Krebs cycle. In the initial report identifying the mutation in IDH1, it was demonstrated that the mutant enzyme has a severe catalytic defect in carrying out this reaction. Thus, it was initially assumed that the R132H mutation represented a loss-of-function mutation. It was subsequently proposed that the R132H allele acts as a dominant negative and promoted tumorigenesis through a decrease in alpha-KG levels and subsequent increase in hypoxia-inducible factor 1 (HIF-1) protein levels [7].

A key breakthrough in the field came when a neomorphic activity for the R132H enzyme was discovered [8]. The R132H IDH1 enzyme was shown to be much more efficient at using alpha-KG as a substrate in the "reverse" direction; remarkably, the product of the reaction was not isocitrate, but instead the metabolite 2-hydroxy-glutarate (2-HG). Broad metabolite profiling studies demonstrated that cells expressing IDH1 R132H had few changes in metabolite levels including alpha-KG and other tricarboxylic acid (TCA) cycle intermediates, but instead accumulated very high (> 10 mM) concentrations of 2-HG. The same was true in a panel of tissue samples from glioblastoma patients, where only tumors harboring IDH1 mutations had significant levels of 2-HG. From these observations, 2-HG was termed an "oncometabolite," although, as it had no known function, at the time it was still not clear exactly how elevated 2-HG levels would promote tumorigenesis.

Strong confirmatory evidence that the ability of IDH1 R132H to produce 2-HG was indeed the tumorigenic-relevant activity of the enzyme came from observations that the IDH2 enzyme was frequently mutated at arginine residues corresponding to IDH1 R132H in a significant portion of acute myelogenous leukemia patients. Several groups demonstrated that 2-HG was also produced by the IDH2 mutant enzymes, also resulting in accumulation of high concentrations of 2-HG [9, 10]. Furthermore, a spectrum of more rare mutant alleles of IDH2 all shared the common ability to produce 2-HG [11]. Importantly, the 2-HG-producing neomorphic activity of these mutated IDH (mIDH) enzymes is strongly consistent with the genetic observation that the mutant alleles are nearly always found in the heterozygous context. The wild-type and mutant enzymes work in concert to produce alpha-KG that can then be channeled to 2-HG [12].

The question of how 2-HG promotes tumorigenesis is not fully resolved, but the weight of evidence points at a surprising answer, providing one of the first links between cancer metabolism and epigenetics. One key consideration for how 2-HG might work is the fact that cells expressing mIDH enzymes accumulate extraordinarily high concentrations ($> 10$ mM) of this metabolite. This contrasts with the concentration of alpha-KG in the cell, which is thought to be approximately 0.4 mM [13, 14]. 2-HG and alpha-KG are structurally quite similar, differing only by the substitution of a hydroxyl group in place of a carbonyl group in alpha-KG. One important consideration, as discussed later, is that this substitution results in the introduction of a chiral center in the 2-HG molecule, with IDH mutant enzymes being demonstrated to produce the (R)-2-HG enantiomer almost exclusively [8].

Alpha-KG is utilized as a substrate by numerous dioxygenases within the cell, including histone demethylases and the TET family of 5-methylcytosine hydroxylases. 2-HG could potentially competitively inhibit the activity of these enzymes through its structural similarity to alpha-KG. Several groups have now independently demonstrated that this is the case. Xiong and colleagues first evaluated the ability of 2-HG to inhibit the human histone demethylase JHDM1A/KDM2A in an *in vitro* biochemical setting with purified enzyme [15]. Importantly, the inhibitory activity of 2-HG could be titrated out with increasing concentrations of alpha-KG, consistent with 2-HG being a direct competitive inhibitor. Further work using X-ray crystallography confirmed that 2-HG bound to a highly homologous *Caenorhabditis elegans* histone demethylase CeKDM7A in the same site as the alpha-KG binding site. Using octyl esters of 2-HG that could cross the cell membrane, the authors demonstrated that bulk cellular histone methylation could be increased by addition of 2-HG to U-87MG cells, consistent with inhibition of alpha-KG-dependent histone demethylases in the cell. Conversely, cells engineered to express IDH mutant enzymes had hypermethylation of histones that could be blocked by the cell-permeable octyl ester of alpha-KG. Increased levels of 2-HG in these cells were also associated with an induction in HIF-1 protein levels that could be blocked by knockdown of the alpha-KG-dependent prolylhydroxylase PHD2, suggesting that this could be one mechanism by which 2-HG exerted its tumorigenic effects. Interestingly, the authors report that R-2-HG is, in fact, consistently a weaker inhibitor of alpha-KG-dependent enzymes than S-2-HG.

Kawamura and colleagues also examined the effects of R-2-HG and S-2-HG on a panel of alpha-KG-dependent oxygenases and concluded that of the two enantiomers, the R-2-HG form is the weaker inhibitor [16]. Crystal structure analyses of both forms of 2-HG bound to the human FIH and JMJD2A enzymes demonstrated that 2-HG could bind to the active site of the enzyme and form a bidendate interaction with the active site metal species. When the authors conducted experiments with cell-permeable analogs of R/S-2-HG (in this case, the dimethyl esters), they observed some inhibition of hydroxylation of HIF on proline and asparagine residues in RCC4 cells, however ultimately no upregulation of HIF-1 expression. The authors concluded that 2-HG, and R-2-HG produced by mIDH in particular, is unlikely to regulate HIF expression *in vivo* due to considerations of potency, although they acknowledge that the extraordinarily high concentrations of 2-HG found in primary glioma and leukemia samples may, in fact, overcome this issue. However, R-2-HG is, in fact, a potent inhibitor of JMJD2 histone demethylase, and the authors propose that many of the pro-tumorigenic effects of 2-HG are likely to proceed through that activity.

Several groups have now evaluated the functional cellular consequences of 2-HG-induced epigenetic changes. In a key study, Melnick and colleagues observed that cancers harboring 2-HG-producing mIDH enzymes are mutually exclusive with loss-of-function mutations in the TET2 enzyme, implying that 2-HG and TET2 inactivation may share common mechanistic consequences for cancer cells [17]. The authors first profiled a cohort of nearly 400 primary de novo AML samples, examining mutation status of a number of genes including IDH and TET2, gene expression microarray profiling, and DNA methylation microarray profiling. They observed that IDH1 and IDH2 mutations were mutually exclusive within this cohort, but both were associated with a distinct cytosine methylation profile and general DNA hypermethylation. Profiling of the hypermethylation signature induced in TET2 mutant samples revealed a significant overlap between the two. TET2 mutation also induced a similar increase in 5-methylcytosine levels. Strikingly, both mIDH expression as well as TET2 knockdown in myeloid cells or primary mouse bone marrow cells resulted in an increase in c-Kit protein expression, a marker for hematopoietic progenitor cells. Together, these data suggest that 2-HG production in mIDH tumors can impair cellular differentiation at least partially by regulating DNA methylation through inhibition of enzymes such as TET2.

Further support for the ability of 2-HG to block cellular differentiation came from a study that examined the effect of mIDH on cellular differentiation in both tumor and non-transformed cell settings [18]. Thompson and coworkers introduced mIDH into 3T3-L1 cells and monitored cellular differentiation to adipocytes using Oil Red-O staining as well as evaluation of lineage-specific transcriptional markers and histone methylation. Expression of mIDH but not WT IDH enzyme resulted in a block in cellular differentiation, an effect that was also observed by directly treating 3T3-L1 cells with the octyl ester of R-2-HG. Similar effects were also observed in immortalized human astrocyte cells passaged over time in the presence of mIDH. Through further experimentation, the authors conclude that H3K9 methylation targeted by the histone demethylase JMJD2C (KDM4C) is sufficient to block differentiation

to adipocytes, and this demethylation reaction is inhibited by 2-HG generated by mIDH. Mak and coworkers generated IDH1 R132H conditional knock-in mice, in which the mIDH enzyme was expressed in either all hematopoietic cells or only the myeloid lineage cells [19]. Analysis of the distribution of hematopoietic progenitor cells in the bone marrow of older animals with the mutation revealed the presence of fewer mature cells and a corresponding increase in progenitor cells, in accordance with the changes in DNA and histone methylation in these cells.

Taken together, these *in vitro* and *in vivo* studies lend strong biochemical, cell biological, and genetic evidence that 2-HG production from mIDH lesions drive tumorigenesis and tumor maintenance by inhibiting alpha-KG-dependent enzymes, thus altering the cellular epigenetic state and engaging a metabolic block in differentiation. The exact targets of 2-HG are likely to be context dependent and perhaps pleiotropic, with further studies necessary to unravel the relative contributions of histone and DNA modification, regulation of HIF, or other alpha-KG-dependent enzymes. In an elegant illustration of how complicated the story is likely to become, Kaelin and colleagues have reported an alternative mechanism by which 2-HG may act on cells [20]. They showed that R-2-HG, but not S-2-HG, could actually activate the prolylhydroxylase enzymes EGLN1 (PHD2) and EGLN2, resulting in destabilization of HIF proteins and enhanced proliferation of human astrocytes. Activation of EGLN enzymes may promote tumorigenesis in contexts such as brain tumors where HIF proteins act as tumor suppressors, and the authors suggest that PHD2 inhibitors could be effective cancer therapies in mIDH settings.

Regardless of the exact mechanism by which 2-HG promotes tumorigenesis, mIDH enzyme inhibitors that suppress 2-HG formation may be effective cancer therapeutics, perhaps by inducing differentiation. There is already precedent for such differentiation therapy, as all-*trans* retinoic acid is highly effective in certain leukemias [21]. Recently, a potent inhibitor of IDH1 R132H was described by Su and colleagues [22]. The authors describe the synthesis and characterization of a series of molecules based on a phenyl-glycine chemical scaffold. Using human cell lines expressing mIDH and xenograft models in mice, the inhibitor was shown to reduce 2-HG levels by over 90 % upon repeated dosing of the compound. This study was the first to demonstrate that mIDH-specific inhibitors could be used to block 2-HG production *in vivo*. The same group has now demonstrated that an inhibitor of IDH2 R140Q can induce differentiation in primary AML cells carrying that mutation [23]. Heuser and colleagues observed enhanced apoptosis in primary AML cells carrying mutated IDH1 alleles treated with an independently identified mIDH inhibitor [24]. In a complementary study, Kaelin and colleagues were able use a small molecule inhibitor of mIDH1 to reverse the cytokine-independent growth conferred by mIDH1 expression in the TF-1 erythroleukemia cell line [25]. It will be critical to see, as these and other inhibitors progress into the clinic, whether the cellular effects of mIDH inhibition observed in these pre-clinical studies translate to clinical benefit for patients.

## 7.3 The Krebs Cycle Intermediates Succinate and Fumarate can also Serve as Oncometabolites in Cancer Cells that have Lesions in Succinate Dehydrogenase or Fumarate Hydratase

The focus on elucidating the mechanistic basis for 2-HG's oncometabolite activity is understandable given the attractiveness of mIDH enzymes for therapeutic drug intervention. However, the appreciation that 2-HG acts as an 'oncometabolite' was, in fact, predated by studies that elucidated a similar molecular mechanism downstream of cancer-associated mutations in the citric acid cycle genes encoding FH and succinate dehydrogenase (SDH) [26]. In contrast to IDH, these mutations have been shown to be loss-of-function lesions, and no neomorphic enzyme activities have been described. Thus, these mutations do not lead to production of a novel metabolite, but rather lead to the build-up of the substrates of these enzymes, fumarate and succinate. FH or SDH is mutated in a number of tumor types, including hereditary paragangliomas and pheochromocytomas, leiomyomas, and renal cell carcinoma. Interestingly, while these mutations lead to disparate tumor types, in each case, the tumors are highly vascularized and display signs of activated hypoxia signaling [27].

In fact, succinate and fumarate play a direct causal role in activation of HIF signaling. Selak and colleagues demonstrated that RNAi-mediated depletion of SDH led to stabilization of HIF1α and enhanced expression of HIF1α-dependent genes such as vascular endothelial growth factor (VEGF). Critically, they then demonstrated that succinate can directly inhibit prolyl hydroxylase activity at plausible levels (~500 μM) [28]. Additionally, fumarate can also inhibit prolyl hydroxylase activity, with a reasonable Ki (~50 μM) [29]. Inhibition of prolyl hydroxylase activity should directly activate hypoxia signaling, since prolyl hydroxylation of HIF typically targets that transcription factor for proteasome-mediated degradation. Further, several groups have extended these cellular and biochemical findings, including the generation of X-ray crystal structures that directly demonstrate the binding of succinate and fumarate to prolyl hydroxylase 2 [30]. However, while it is clear that succinate and fumarate can inhibit prolyl hydroxylases, it remains unclear whether inhibition of prolyl hydroxylases is the critical mediator of the transformative effects of SDH or FH loss.

For instance, mice with genetic knockout of all three prolyl hydroxylase genes fail to develop the renal cysts that are observed in $FH^{-/-}$ mice, indicating that loss of prolyl hydroxylase activity is not sufficient to phenocopy the effects of FH loss [31]. Instead, the authors noted that FH loss leads to a strong upregulation of genes regulated by the antioxidant sensing Nrf2 pathway. This transcriptional signature was shown to be independent of HIF, as it still occurred in FH-null/HIF1a-null cells. The authors propose that this HIF-independent phenotype arises via an inhibitory posttranslational succinylation of cysteine residues on Keap1, a redox sensor protein which typically binds to Nrf2 and mediates its degradation. Protein succinylation occurs via covalent conjugation of fumarate to cysteine residues, and in fact, FH-null tumors display a marked increase in protein succinylation [32]. Thus, elevated succinylation provides an alternate mechanistic hypothesis for FH-mediated tumorigenesis.

Loss of SDH or FH leads to very clear metabolic rewiring (dramatically elevated levels of substrate and truncated TCA cycle) and cancer-relevant sequelae (HIF and NRF2 signaling). This raises the question: Are there rational, targeted approaches to treat tumors with loss of SDH or FH? A reasonable first hypothesis would be to treat these tumors by inhibiting pathways downstream of HIF, since HIF signaling is hyperactivated in SDH- and FH-null tumors. HIF signaling leads to overexpression of epidermal growth factor receptor (EGFR), and the EGFR inhibitor erlotinib has so far shown some modest efficacy in clinical trials of hereditary leiomyomatosis and renal cell cancer (HLRCC), the familial cancer syndrome marked by FH deficiency [33]. VEGF inhibitors may also be viable in SDH- and FH-null tumors, if HIF stabilization proves to be a critical mediator of the molecular pathogenesis of these cancers. While these rational approaches may prove efficacious, unbiased searches for candidate targets may also be valuable, given the uncertainty regarding the relative importance of the events downstream of SDH and FH loss.

Gottlieb and colleagues recently applied such an unbiased search, and identified an unanticipated metabolic pathway that displays synthetic lethality with FH deficiency [34]. The authors established FH-deficient cell lines, and performed transcript profiling to assess the expression of metabolic genes in the FH-deficient cells versus the parental, FH-wild-type cells. This catalog of metabolic genes was then used to create an in silico model of the metabolic networks of FH-null and FH-wild-type cells. They then utilized flux balance analysis to predict enzymes that would be essential in the FH-null cells, but not their FH-wild-type counterparts. Flux balance analysis is a computational modeling technique that predicts the importance of each single enzyme in the metabolic network to fulfill the overall biosynthetic needs of metabolism. Strikingly, of the 24 enzymes that were predicted to be synthetically lethal with FH loss, 18 were components of the heme biosynthesis pathway. The authors validated this finding, showing that shRNAs that knock down the pathway component heme oxygenase 1 (HMOX1) prevented proliferation of the FH-null cells, without impact on the FH-wild-type cells. Similar results were seen with the heme oxygenase (HMOX) inhibitor zinc protoporphyrin, supporting the robustness of the finding and suggesting that drugs that target heme biosynthesis may prove useful in treating FH-null tumors. Further work will be required to validate HMOX as a target for FH-null tumors, but nonetheless this work is an elegant demonstration that computational modeling can predict synthetic lethality of metabolic targets, and provides a case study that genetic lesions in metabolic genes can lead to specific vulnerabilities in the metabolic network.

## 7.4 Cancer Cells Use Reductive Carboxylation as a Metabolic Adaptation to Hypoxia

Remarkably, the evolving elucidation of the role of mIDH enzymes in tumorigenesis is not the only well-described metabolic adaptation by cancer cells through this enzyme family. In the canonical view of the Krebs cycle, citrate is derived from the condensation of acetyl-CoA with oxaloacetate by the citrate synthase enzyme. When

considering carbon entering the Krebs cycle via glutaminolysis, citrate produced in this manner would be derived from alpha-KG that underwent six subsequent enzymatic conversions. In an alternative method to generate citrate, IDH can catalyze the reductive reverse reaction, converting alpha-KG back to isocitrate, which can isomerize to citrate. Citrate, in turn, can be broken back down to oxaloacetate and acetyl-coA by the action of ATP citrate lyase enzyme, with the acetyl-coA contributing 2-carbon units used for lipid synthesis. Clever use of isotopomer tracers can elucidate the origin of the acetyl-coA formed from citrate (Fig. 7.2b); for instance, glutamine labeled at the 5-carbon position will result in labeled acetyl-coA only when the reductive pathway is used. Similarly, the ratio of citrate itself derived from either pathway can be measured by taking advantage of the fact that the 1-carbon of alpha-KG (derived from glutaminolysis) is cleaved in the oxidative direction while being retained in the reductive direction, resulting in a one mass unit difference. While the IDH reductive pathway has been known for years, its contribution in mammalian cells has only recently begun to be fully appreciated. In recent years, it was discovered that reductive carboxylation was responsible for a significant percentage of glutamine-derived acetyl-coA in a brown adipocyte cell line [35].

Stephanopoulos and colleagues measured the contribution of the reductive pathway to total acetyl-coA pools in a number of cancer cell lines [36]. They observed that cancer cells under normal oxygen tension made approximately 20 % of the total acetyl-coA through glutamine, and nearly all of that came from the reductive pathway and not the oxidative pathway. Strikingly, when cells were incubated under hypoxia, they shifted their metabolism to generate roughly 80 % of their acetyl-coA from glutamine-derived reductive carboxylation. Furthermore, the authors observed that cell lines with loss of the Von Hippel–Lindau (VHL) tumor suppressor gene and, thus, dysregulation of the HIF pathway relied heavily on the reductive pathway for acetyl-coA synthesis even under normal oxygen tension.

In a complementary study, DeBerardinis and colleagues made similar observations of the heavy use of the reductive carboxylation pathway in cell lines with defective mitochondrial function [37]. The authors profiled isogenic human osteosarcoma cells with normal or defective mitochondrial complex III activity and found that the reductive carboxylation pathway was the primary means of generating citrate from glutamine in the mitochondrial-defective but not wild-type cells. A similar observation was made in the UOK262 cell line, derived from a renal tumor with loss of FH activity. In fact, chemical impairment of TCA cycle activity, using either complex I or complex III inhibitors, was sufficient to stimulate the activity of this pathway. The authors conclude that this specific adaptation in cancer cells allows cells to continue using glutamine as an anaplerotic substrate even when TCA activity is compromised.

Thompson and colleagues also observed the switch to reductive carboxylation in a number of cell lines [38]. Glioblastoma-derived SF188 cells subject to hypoxia generated more of their citrate from the reductive carboxylation pathway. The authors demonstrate that induction of the HIF-1 response is linked to the use of reductive carboxylation. Knockdown of HIF-1 in RCC4 cells, a VHL-null renal cell carcinoma

line with constitutive activation of HIF even under normal oxygen tension, suppresses reductive carboxylation and induces a switch back to oxidative metabolism to generate citrate.

Collectively, these results suggest that there may be some advantages, with respect to either tumor cell proliferation or possibly adaptation to cellular stress induced by hypoxic conditions, to using the reductive carboxylation pathway to generate citrate and lipids. These observations raise the question of whether targeting non mutant IDH enzymes with small molecule inhibitors could be an effective therapeutic strategy. The three studies described above, which largely agree on key points regarding the condition-dependent regulation of reductive carboxylation activity, come to somewhat different conclusions when it comes to the key question of which IDH isoform is responsible for the reductive carboxylation activity. Through RNAi knockdown studies, Stephanopoulos and colleagues identified IDH1 as being responsible, while DeBerardinis implicated both IDH1 and IDH2, and Thompson ascribed the activity primarily to IDH2. Interestingly, both the Stephanopoulos and Thompson studies showed that knockdown of the IDH enzyme responsible for reductive carboxylation activity in their respective cell systems results in impaired cell proliferation. Clearly, more work remains to be done on elucidating the contexts in which this metabolic adaptation supports tumor growth.

## 7.5 The M2 Isoform of Pyruvate Kinase Exemplifies Cancer Cell Emphasis on Metabolic Flexibility

A fascinating and complex enzyme that powerfully illustrates the importance that cancer cells place in maximizing metabolic flexibility is the pyruvate kinase M2 isoform (PKM2). Whereby many differentiated tissues express the PKM1 isoform, the switch to aerobic glycolysis in cancer cells is often concomitant with expression of PKM2, the isoform of pyruvate kinase expressed in embryonic and proliferative tissues [39].

One paradoxical observation between PKM1 and PKM2 is that the PKM2 isoform exhibits lower activity, despite the fact that tumor cells consume high amounts of glucose and convert most of that glucose to lactate, the classic Warburg effect. To understand why that might be, it is worth considering two of the major functions of glycolysis. The process of glycolysis results in the net generation of two molecules of adenosine triphosphate (ATP) per molecule of glucose, and this represents the fastest method for cells to generate energy. However, intermediates in the glycolytic pathway also serve as branch points for biosynthetic pathways that eventually lead to critical macromolecules important for cellular proliferation such as nucleotides for DNA synthesis and lipids for membrane biogenesis. Thus, compared with the constitutively active PKM1 enzyme, it has been proposed that PKM2 grants proliferating cells the metabolic flexibility to access both the energy-producing and macromolecular biosynthesis functions of glycolysis [40] (Fig. 7.3).

Given the metabolic flexibility conferred by using the PKM2 enzyme, it is important to understand the mechanisms by which PKM2 activity is regulated. When

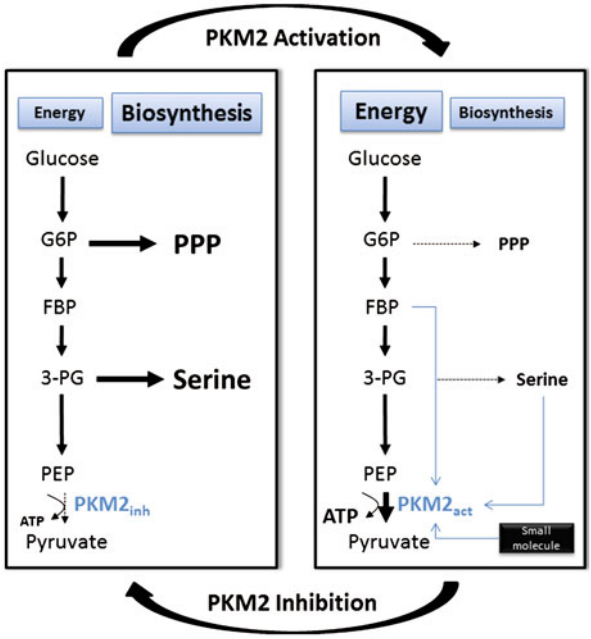


**Fig. 7.3** Schematic representation of oscillatory behavior of pyruvate kinase M2 (PKM2) enzyme in its active ($PKM2_{act}$) and inhibited ($PKM2_{inh}$) states. Activation of PKM2 by native ligands such as FBP and serine or by synthetic small molecules results in increased ATP energy production that may be important to counter energetic stress, but at the expense of carbon flow to branching pathways important for macromolecular biosynthesis. Inhibition of PKM2 by multiple mechanisms described in the text enables carbon flow to these pathways. Width of arrows qualitatively delineates degree of carbon flow

PKM2 is in its low-activity dimer state, it restricts glycolytic carbon flow. The upstream glycolytic metabolite fructose 1,6-bisphosphate (FBP) activates PKM2 in a feed-forward manner by binding to the enzyme and inducing a shift to the active tetrameric conformation. The activation of PKM2 results in increased carbon flow through lower glycolysis and lowering of FBP levels, which should relieve the activation of PKM2. Thus, normal proliferating cells have a built-in control mechanism by which they can oscillate between the energy-producing and macromolecular synthesis functions of glycolysis.

A key breakthrough in the understanding of how PKM2's regulation of cell metabolism could influence its role in cancer cell proliferation came from the observation that PKM2 is a phosphotyrosine-binding protein [41]. Remarkably, in the presence of phosphotyrosine-containing peptides, FBP is rapidly dissociated from PKM2, leading to inhibition of PKM2 enzyme activity. Given the critical importance of aberrantly activated tyrosine kinases in many human cancers, this observation suggested that PKM2 might represent a critical link between oncogenic growth signals and cancer cell metabolism. In fact, cancer cells that have exogenous PKM1 expression and, thus, the inability to "turn off" pyruvate kinase activity show reduced tumorigenicity in vivo [42, 43].

The observation that cancer cells might use tyrosine kinase phosphorylation as a means to enforce constitutive repression of PKM2 activity gained further traction when several groups independently identified site-specific posttranslational modifications on the PKM2 protein that all share the common feature of inhibiting PKM2 activity [44–46]. Phosphorylation on the PKM2 protein itself on Tyr308, oxidation of Cys358, and acetylation on Lys305 all result in reduced enzyme activity. Importantly, cancer cells where the PKM2 protein has been replaced with an enzyme with site mutations in any of these residues largely recapitulate the reduced tumorigenicity of cells that express PKM1. Collectively, these data indicate that cancer cells use multiple mechanisms to reduce pyruvate kinase activity, suggesting that a crucial role for PKM2 is, in fact, to promote macromolecular biosynthesis from glycolytic pathway intermediates.

Several groups have now published chemical structures of PKM2 activators that should prove useful as cellular probes to study cellular metabolism, and to test these specific hypotheses surrounding PKM2 biology [47–50]. The published molecules all bind to the same allosteric pocket at the dimer–dimer interface in PKM2, a pocket whose physiological function is not clear. These molecules, despite their distinctive binding pocket, have been shown to activate PKM2 in cancer cells analogously to the endogenous activator FBP, by increasing the affinity of the enzyme for its substrate phosphenolpyruvate (PEP). Importantly, unlike FBP-activated PKM2, PKM2 that is activated by these small molecules is recalcitrant to negative regulation by phosphotyrosine peptides, inhibitory amino acids, and protected from oxidation on Cys358 [42, 46, 47].

The first question that can be directly addressed by these activators is the model of how PKM2 regulates the dual functions of glycolysis. Genetic experiments to replace PKM2 with the constitutively active PKM1 isoform provide only indirect evidence for this model, as it cannot be ruled out that regulation of enzyme activity is not the only functional difference between the two isoforms. Dang and colleagues used a series of quinolone-based PKM2 activators to assess the effects of constitutive PKM2 activation in A549 cancer cells [47]. They observed that activation of PKM2 did not result in significant changes in metabolite steady-state pool levels. However, carbon flow to the serine biosynthetic pathway, branching off the glycolytic intermediate 3-phosphoglycerate (Fig. 7.1), was significantly attenuated. These data confirm the prediction that PKM2 serves as an important control point for the fate of glucose-derived carbon in cancer cells. Interestingly, PKM2 activation by itself had little effect on cellular proliferation, consistent with *in vitro* experiments where PKM2 was replaced with PKM1 [43]. In probing the molecular explanation for why this was the case, it was demonstrated that the PKM2 activation leads to the upregulation of proteins involved in serine pathway metabolism, including both the biosynthetic enzymes and high-affinity serine transporters. The significance of these observations was illustrated by the fact that specifically upon the withdrawal of serine, an amino acid that is not normally essential for cell proliferation, cancer cells became exquisitely sensitive to PKM2 activators and did not continue to proliferate. Thus, PKM2 activation induced an unanticipated state of serine auxotrophy, highlighting the importance of this normally non-essential amino acid in cancer metabolism.

Serine has long been known to be an activator of pyruvate kinase [51], and this property was demonstrated to be shared by the PKM2 isoform. Gottlieb and colleagues used studies monitoring labeled carbon flow from PEP to pyruvate, and proposed that serine is an important endogenous regulator of PKM2 activity [52]. Knockdown of PKM2 resulted in an increase in the labeled PEP/pyruvate ratio and increased carbon flow to the serine biosynthetic pathway, as did withdrawal of serine and glycine from the media. The authors also used X-ray crystallography to show that serine bound to the same site as that previously shown to be the binding site of the inhibitory amino acid phenylalanine in rabbit PKM2 enzyme [53].

If serine is, indeed, an important regulator of PKM2 activator in vivo, it is intriguing to speculate that its role is to act as a feed-forward regulator of PKM2 activity analogously to FBP. In this model, when serine levels built up to a sufficient level, PKM2 would be activated to divert glucose carbon flow back to production of energy. PKM2 would thus be an integrator of signals from both glucose and amino acid metabolic pathways, and its oscillatory activity could be critical for optimal metabolic balance for cellular proliferation (Fig. 7.3).

In work that provided further direct experimental support for this general model, Lee and colleagues reported that phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR), an intermediate in the de novo purine nucleotide synthesis pathway, can bind to and activate PKM2 and does so specifically under the nutrient stress condition of glucose deprivation [54]. Remarkably, in cells deprived of glucose, measured SAICAR levels became elevated in an oscillatory manner over time, which corresponded to similar oscillatory changes in the concentration of metabolites such as pyruvate and lactate. The authors go on to show that knockdown of the enzymes responsible for SAICAR biosynthesis under these stress conditions results in impaired cell survival, thus implicating SAICAR and its regulation of pyruvate kinase activity as being crucial for cellular adaptation to these conditions. Taken together, these studies suggest that cancer cells take advantage of PKM2 expression to maximize cellular proliferation and respond to cellular stress under varying nutrient conditions.

Remarkably, PKM2 has recently been shown to have an ever-increasing number of non-glycolytic functions and even non-pyruvate-kinase enzymatic activities [55]. Semenza and colleagues demonstrated that prolyl-hydroxylated PKM2 could serve as a co-activator of HIF to induce glycolytic gene expression [56]. Translocation of PKM2 into the nucleus where it can regulate both EGFR and beta-catenin signaling has been described [57]. PKM2 has also been shown to act as a protein kinase, regulating both STAT signaling as well as phosphorylation of histone H3 [58, 59]. In assessing how PKM2 activation or inhibition could affect these functions of PKM2, and possible effects on tumorigenesis, it will be important to understand whether it is the monomer, dimer, or tetramer of PKM2 that is carrying out these activities. Vander Heiden and colleagues showed that small molecule activation of PKM2 *in vivo* increased PKM2 tetramer formation, and could suppress the formation of tumor xenografts [42]. While these results were similar to the genetic data showing reduced tumorigenesis of PKM1-expressing cells, it will be interesting to tease out whether this activity is due solely to diversion of glycolytic intermediates from macromolecular biosynthesis or whether PKM2 tetramerization negatively affects some of the other postulated non-pyruvate kinase tumor-promoting activities of PKM2.

In considering PKM2 as a potential therapeutic target in cancer metabolism, it has been proposed that both inhibitors and activators of the enzyme might be attractive strategies. The case for inhibition for PKM2 shares many common threads with strategies that seek to target other enzymes in the glycolytic pathway, with the therapeutic window for such agents being partially derived as a consequence of the "Warburg effect" observed in many cancer cells. A recent study by Hofmann and colleagues used RNAi to knock down PKM2 *in vitro* and in tumor xenografts [60]. They concluded that PKM2 was dispensable for *in vivo* tumor growth, despite a reduction in glucose conversion to lactate, perhaps by virtue of a metabolic bypass of the PKM2 reaction [61]. Although these data suggest that in fact PKM2 activity is not important for tumor growth, it is important to keep in mind that the oscillation model predicts that perturbing PKM2 activity in the absence of another cellular stress (e.g., serine deprivation) may be insufficient to reveal the effects of PKM2-conferred metabolic flexibility.

The studies described above are suggestive of a general strategy for identifying responders to PKM2 activators involving critical evaluation of the interplay between glycolytic flux and serine metabolism pathways. Key questions that remain to be addressed include whether cellular supplies of serine, or perhaps a serine-derived metabolite, are particularly constrained in specific tumor subtypes and whether this would confer sensitivity to PKM2 activation. Part of the challenge to translate these findings into a robust responder context in vivo is that serine is available to cells by multiple pathways including de novo synthesis, conversion from glycine, catabolism from proteins and serine-containing phospholipids, and by transporter-mediated uptake from the extracellular environment [62]. On the other hand, several studies have shown that cancer patients often have significant changes in plasma amino acid levels [63] and it is plausible that further study will reveal patients whose tumors are particularly dependent on serine (see the following section).

## 7.6 The Serine and Glycine Biosynthesis Axis is Important in Cancer Cells and Provides an Indirect Link to Alpha-Ketoglutarate Metabolism

The interplay between PKM2 and serine provides important clues that this typically non-essential amino acid is a key metabolite in cancer cell metabolism. The key role of serine in cancer metabolism is further highlighted by a number of recent studies that suggest that serine is a critical "hub" in cancer metabolism. The serine biosynthesis pathway branches off glycolysis at 3-phosphoglycerate (3-PG) and leads to production of serine from glucose via a three-step pathway (catalyzed by phosphoglycerate dehydrogenase (PHGDH), phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH)) (Fig. 7.1). PHGDH and PSAT1 are upregulated in breast malignancies and their expression correlates with poor prognosis [64]. In addition, it was recently discovered that the PHGDH gene is found in a region that is amplified in several cancer types [65, 66]. The core amplified region is

small, containing only five genes, and lacks other known oncogenes. This suggests that PHGDH itself may be a "driver" whose overexpression promotes tumorigenesis. Further, amplification of the PHGDH gene leads to high PHGDH expression, and PHGDH-amplified cancers divert a substantial fraction of glucose-derived carbon into serine biosynthesis. Thus PHGDH amplification causes a metabolic adaptation leading to diversion of glucose carbon into biosynthetic pathways [65, 66].

Proliferative cells utilize serine for multiple purposes, including assembly of protein and lipid, and also as a substrate for metabolic reactions that create additional biosynthetic intermediates. Most widely appreciated among these downstream reactions is serine cleavage by the serine hydroxymethyltransferases SHMT1 and SHMT2. SHMTs transfer a methyl group from serine to tetrahydrofolate, producing glycine while also transferring a one-carbon unit to tetrahydrofolate (Fig. 7.1). Thus, this reaction provides two critical biosynthetic building blocks. The folate cycle enables transfer of one-carbon units into multiple biosynthetic pathways, and most notably, into critical steps in the production of purine and pyrimidine nucleotides. This activity is critical for cancer cell proliferation. In fact, the earliest successful chemotherapeutic, aminopterin, targets the activity of the folate cycle enzyme dihydrofolate reductase [67, 68]. Glycine, the other product of serine cleavage, also serves as an input into important biosynthetic pathways and, in fact, has been recently implicated as a critical node in the cancer metabolic network.

Vamsi Mootha and colleagues recently performed a systematic assessment of nutrient consumption for each of the cell lines in the NCI-60 cell line panel [69]. They observed that glycine was the only nutrient for which consumption rate correlated significantly with proliferation rate. They then demonstrated that rapidly proliferating cell lines are conditional glycine auxotrophs, requiring either extracellular glycine or SHMT2 for their proliferation. This work demonstrates that glycine is a critical metabolic node in highly proliferative cancers. In addition, glycine has been shown to play an important role in tumor initiation [70]. Transcript profiling experiments in non-small-cell lung cancer tumor-initiating cells (TICs) revealed that TICs overexpress components of the serine/glycine/one-carbon pathway. Strikingly, several pathway components were each sufficient to transform NIH3T3 cells upon overexpression. Of these, glycine decarboxylase (GLDC) was the most potent transforming agent, and its depletion via RNAi led to reduced proliferation in an established lung cancer cell line. GLDC participates in the glycine cleavage system, which extracts a methyl group from glycine and transfers that methyl group to tetrahydrofolate, providing an additional source of one-carbon units for the folate cycle.

Collectively, these recent studies provide strong, convergent evidence that metabolic adaptation of the serine/glycine/one-carbon pathway is a consistent component of rewired metabolism in cancer. These recent observations provide several leads toward therapies that may improve significantly on the antifolates first discovered by Sidney Farber [67, 68]. However, several important questions must first be addressed. While multiple studies have implicated this metabolic axis in cancer, further work is needed to understand why this pathway is upregulated in cancer. For instance, why do cancer cells produce serine even when there is ample serine in culture media (and in human plasma)? As noted above, multiple cancer types display

marked elevation in activity of the serine biosynthetic pathway, including cancers with chromosomal amplification of PHGDH. Investigation of PHGDH-amplified cancers revealed that these cancers still take up serine from their environment and, notably, that serine produced intracellularly is freely exchanged into the environment [66]. Cancers seem to care about making serine, yet not about retaining serine. There are several viable hypotheses to explain this phenomenon. First, it is possible that serine that is produced intracellularly is channeled into specific metabolic pathways and, thus, is utilized differently than nutrient-derived serine. Second, it is possible that upregulation of the pathway accomplishes a metabolic goal beyond serine production. In favor of this hypothesis is the observation that PHGDH amplification increases pathway activity so much that PSAT1's production of alpha-KG comprises a significant source of cellular alpha-KG. Alpha-KG has typically been seen as a "side product" of PSAT1, yet PSAT1 is a primary source of alpha-KG in PHGDH-amplified cells [66]. Alpha-KG is a critical "hub" metabolite in its own right; it is a critical entry point into the TCA cycle and, as described above in the context of mIDH, also a necessary substrate for alpha-KG-dependent dioxygenases that function in HIF signaling and chromatin remodeling.

## 7.7 More Examples of Metabolic Adaptations Resulting in Exploitable Vulnerabilities

While the studies above heavily implicate metabolites in the TCA cycle and the serine/glycine axis as important mediators of cancer metabolism, additional studies using metabolomics or genomics approaches have recently drawn therapeutic attention toward additional unanticipated targets. For instance, transcript profiling studies of diffuse large B-cell lymphomas (DLBCLs) defined three subtypes of DLBCL. One of these, dubbed OxPhos-DLBCL, is characterized by high expression of genes in oxidative phosphorylation. Notably, this subtype is expected to be resistant to the current investigational agents for DLBCL, which target components of B-cell receptor signaling that are not present in the OxPhos-DLBCL subtype. Danial and colleagues hypothesized that this subset may instead be susceptible to metabolic targeting, since it has clear alterations in metabolic gene expression [71]. After identifying cell lines that faithfully model the OxPhos-DLBCL phenotype, the authors performed a phenotypic screen where they characterized each cell line for its ability to utilize several different carbon sources. This screen led to the unexpected observation that OxPhos-DLBCL cells can oxidize palmitate at rates far greater than other DLBCL subtypes. The authors then demonstrated that OxPhos-DLBCL cells can proliferate using palmitate as a carbon source and that an inhibitor of mitochondrial fatty acid oxidation was preferentially toxic to the cells of the OxPhos-DLBCL subtype. These cells also proved to be selectively sensitive to antagonists of peroxisome proliferator-activated receptor gamma (PPAR-gamma), a key transcriptional activator for fatty acid oxidation genes, and to RNAi targeting of glutamyl cysteine

synthase (GCS), an enzyme that is required to produce glutathione which can scavenge reactive oxygen species generated during oxidative phosphorylation. This study suggests novel therapeutic strategies for this subset of non-Hodgkins' lymphoma and demonstrates that metabolic characterization of cancer subtypes can provide a viable hypotheses for selectively targeting essential metabolic enzymes.

While research to identify cancer targets typically focuses on oncogenic events, loss-of-function lesions may also create therapeutic opportunities. One example of this paradigm has been noted earlier, with the case of FH and HMOX. DePinho and colleagues have recently approached this same concept from a novel, and potentially broadly applicable, route [72]. Chromosomal deletions that mediate loss of tumor suppressors typically co-delete adjacent genes, and the authors hypothesized that such "passenger deletions" might create metabolic vulnerabilities. They identified homozygously deleted loci in glioblastoma multiforme (GBM), and noted that several such loci include metabolic genes that encode essential enzymes. It is likely that tumors can survive loss of these nominally essential genes due to redundancy, i.e., a second, functionally redundant gene can provide the equivalent function. It was hypothesized that these passenger deletions might create tumor-specific dependence on the functionally redundant gene and that these functionally redundant genes would be suitable cancer targets. The authors demonstrated this concept of collateral vulnerability by targeting enolase 2 (ENO2) in tumors with deletion of enolase 1 (ENO1). ENO1 typically accounts for 90 % of total cellular enolase activity and, thus, ENO2 would be expected to be non-essential for proliferation [73]. Deletion of ENO1 was, therefore, hypothesized to reduce cellular enolase activity and sensitize cells to knockdown or inhibition of ENO2. ENO2 depletion by shRNA proved to be inconsequential for proliferation of ENO1 wild-type cells, but dramatically inhibited growth of ENO1-null cell lines. This effect was fully rescued by enforced expression of an shRNA-resistant ENO2 cDNA, demonstrating that the growth phenotype was due to on-target reduction of ENO2. Perhaps most interestingly, it was also noted that ENO1-null cell lines were highly sensitized to the effects of a pan-enolase transition state inhibitor, phonoacetohydroxamate (PHAH). PHAH can inhibit both ENO1 and ENO2, and the authors hypothesize that its dramatic growth inhibition in ENO1-deleted cells arises because the deletion lowers basal levels of enolase activity, bringing it closer to a toxicity threshold. Thus, inhibition of the "housekeeping enzyme" enolase may be a viable therapeutic strategy for a subset of GBM, and additionally, this concept of passenger deletion-mediated vulnerability could be extended to a wide number of tumor suppressor loci.

## 7.8 Conclusion

It is an exciting time for drug discovery in cancer metabolism. The emerging landscape of new targets, with the detailed pathway studies illustrating how these targets are regulating cancer cell metabolic adaptation, promises to deliver new therapeutic agents with completely novel mechanisms of action into the clinic. However, numerous challenges remain. As we have discussed in this chapter, while some metabolic

phenotypes, such as the Warburg effect, are shared by the vast majority of tumors, other metabolic signatures are confined to very discrete cancer subsets. It will be important to define these metabolic signatures in a robust manner to efficiently treat these patients. The therapeutic window of chemical agents that target cellular metabolism remains unclear, especially in light of the fact that few "genetically validated" targets exist. There is a clear need to understand the connections between cellular signaling and metabolic adaptation, and how the two influence each other, especially in light of the high likelihood of combination therapies. We look forward to continued rapid advances in our understanding of how metabolic adaptation promotes tumor progression and the promise of helping current and future patients in need.

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# Chapter 8
# Targeting of PFKFB3

**Katarina Färnegårdh, Maria Shoshan and Lars Ährlund-Richter**

**Abstract** More than half a century has passed since Otto Warburg enthusiastically acknowledged transplantable ascites tumor cells as a novel model for tumor cell metabolism. With this approach, Warburg had the tools to validate his 30-year-old hypothesis, and for the first time quantitatively determine that cancer cells tend to convert most glucose to lactate, regardless of oxygen conditions. Later, this effect could be identified as a general phenomenon in fast-proliferating cells and to represent a fundamentally altered intermediary metabolism in the cancer cell. The cellular rheostat controlling glycolysis involves the bifunctional kinase/phosphatase enzyme PFKFB3 and its product fructose-2,6-bisphosphate (F-2,6-$P_2$), a potent activator of phosphofructo-1-kinase (PFK1)—the enzyme that mediates the rate-limiting step of glycolysis. In this chapter, we summarize known roles of PFKFB3 in the cell. PFKFB3 is overexpressed in many types of cancer and is for several reasons considered to be instrumental for setting the enhanced intracellular levels of F-2,6-$P_2$. We elaborate on the rationale for reducing the high rate of glycolysis in the cancer cells by specific targeting of the 6-phosphofructo-2-kinase (PFK2) activity of the PFKFB3 enzyme. For this, we map the present status in the literature on links between expression of PFKFB family members and tumor proliferation.

**Keywords** Phosphofructokinase 1 (PFK1) · PFKFB3 · PFKFB4 · 6-phosphofructo-2-kinase (PFK2) · Fructose 2,6-bisphosphate · Glycolysis · Tumor · Drug development · Small molecule inhibitors

*Otto Warburg noted here a special acknowledgment: The transplantable ascites cancer was discovered by H. Loewenthal and G. Jahn [Z. Krebs- forsch. 37, 439 (1932)]. G. Klein (Stockholm) expanded our knowledge about the physiology and morphology of the ascites tumors and showed their great advantages as experimental material. [Exp. Cell Res. 2, 518 (1951).]*

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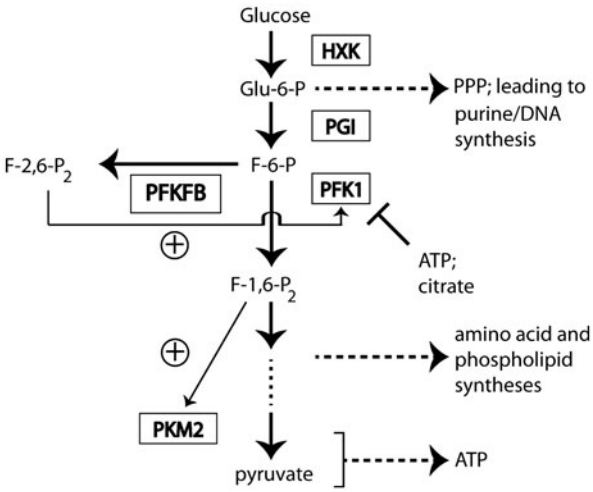


**Fig. 8.1** Overview of glycolysis and the regulatory role of phosphofructokinase/fructose bisphosphatase (PFKFB). Enzymes (*boldface*, *boxed*): *HXK* hexokinase, *PGI* phosphoglucose isomerase, *PFK1* phosphofructokinase, *PFKFB* phosphofructokinase/fructose bisphosphatase, *PKM2* pyruvate kinase, M2 and *PPP* pentose phosphate pathway. âĽ¨Metabolites: *Glu* glucose; *F*, fructose; and *P*, phosphate. See text for further details

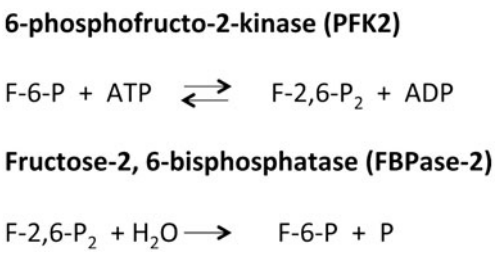


**Fig. 8.2** Phosphofructokinase (PFK2) and fructose-2, 6-bisphosphatase (FBPase-2) enzymatic reactions. (Modified from Sakakibara et al. 1984 [54])

## 8.1 PFKFB Gene Family

In glycolysis, the conversion of fructose 6-phosphate (F-6-P) into fructose 1,6-bisphosphate (F-1,6-$P_2$) is mediated by the enzyme phosphofructokinase 1 (PFK1; Fig. 8.1). This step is rate limiting [59] and being key in glycolytic flux, it is subject to complex regulation [41]. Notably, PFK1 is allosterically inhibited by high levels of adenosine triphosphate (ATP) [17], but this product inhibition can be bypassed by a potent allosteric activation by fructose-2,6-bisphosphate (F-2,6-$P_2$).

F-2,6-$P_2$ has been found in virtually every eukaryotic tissue or cell examined [46]. The steady-state concentration of F-2,6-$P_2$ is determined by a family of enzymes denoted PFKFB, exhibiting bifunctional 6-phosphofructo-2-kinase (PFK2) and fructose-2,6-bisphosphatase (FBPase-2) activities (Fig. 8.2). Four genes have been described to code for the PFKFB enzyme family and were originally cloned from liver (PFKFB1; [1, 28]), heart (PFKFB2; [18]), placenta (PFKFB3; [52]), and testis (PFKFB4; [30]), respectively.

The four PFKFB isozymes share the same overall domain organization and are all homodimers [53]. Each polypeptide chain contains two separate catalytic domains responsible for the kinase and phosphatase reactions, with the kinase domain located

in the N-terminal half of the enzyme and the phosphatase at the carboxyl half. Both catalytic cores are highly conserved (85 % [10]). The four isozymes are located on separate chromosomes, exhibit differences in tissue distribution, as well as relative ratio and kinetics of the PFKFB bifunctional kinase/phosphatase activities [10].

The antagonistic kinase/phosphatase enzymatic activities, provide the means for the generation as well as dephosphorylation of F-2,6-$P_2$. In the cell, the net outcome is determined not only by which isoforms are present at a specific time point, but also by a range of signals affecting the bifunctional enzyme, e.g. glucose uptake rate, levels of glycolytic intermediates, and posttranslational events (see, e.g. [47]).

A detailed understanding of the different PFKFB enzymes on the molecular level has been made possible via several published crystal structures of the different isozymes. For example, insights regarding the F-6-P pocket of the kinase active site have been obtained based on the X-ray structures of PFKFB3 in complex with F-6-P, phosphoenolpyruvate, and ethylenediaminetetraacetic acid (EDTA) [24, 25]. Detailed information about the FBPase-2 reaction on a molecular level was also published recently [26].

## 8.2 PFKFB3 Regulation

Among the PFKFB enzyme family members, the PFKFB3 isozyme has so far been studied in greatest detail. The human PFKFB3 gene spans a region of 32.5 kb and has a single chromosomal locus that was first cloned from highly proliferative placental tissue [52]. The gene contains 19 exons [31]. Six alternatively spliced variants of messenger RNA (mRNA) are known (UBI2K1–6), originally detected in human brain [22]. A ubiquitous isoform (UBI2K5) is expressed in all tissues [47, 48], but alternative splicing of exon 15 [44] results in an inducible isoform (UBI2K4) expressed in rapidly proliferating cells as well as in tumors [11, 31]. Compared to the ubiquitous form, this inducible PFKFB3 contains an additional 23-bp fragment at the carboxyl end [22].

In line with the key regulatory role of the PFKFB family of enzymes, the PFKFB3 gene and its products are subject to tight and complex regulation (Table 8.1).

Induction of PFKFB3 mRNA and protein has been shown to occur via, e.g., proinflammatory stimuli and hypoxia (reviewed in [12]). Furthermore, low pH has been shown to induce the metabolic stress sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK), which in turn is known to induce PFKFB3 gene expression [36]. The enzymatic activity of inducible PFKFB3 may also be regulated in response to oncogenic Ras [58], oncogenic JAK2/STAT5 signaling [49], or IL-6 and the STAT3 pathway [3]. PFKFB activity is further subject to hormonal regulation notably by insulin signaling which leads to elevated F-2,6-$P_2$ levels and increased glycolytic rate [4, 48]. Adrenalin and androgen enhance the PFK2 activity of PFKFB2 and, thereby, increase cellular F-2,6-$P_2$ levels [42].

Posttranslational regulation is mainly directed via phosphorylation which affects the enzymatic activities of the PFKFB proteins and is done by several kinases,

**Table 8.1** Characteristics of phosphofructokinase/fructose bisphosphatase PFKFB3 and PFKFB4

| Names[a] | PFKFB3 | PFKFB4 |
|---|---|---|
| | RP11–298K24.3<br>IPFK2<br>PFK2<br>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase<br>6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3<br>6PF-2-K/Fru-2,6-P2ase 3<br>6PF-2-K/Fru-2,6-P2ase brain/placenta-type isozyme<br>PFK/FBPase 3<br>Fructose-6-phosphate,2-kinase/fructose-2, 6-bisphosphatase<br>iPFK-2<br>Inducible 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase<br>Renal carcinoma antigen NY-REN-56 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4<br>PFK/FBPase 4<br>6PF-2-K/Fru-2,6-P2ase 4<br>6PF-2-K/Fru-2,6-P2ase testis-type isozyme<br>6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-4 |
| Gene chromosome location[a] | 10p15.1 | 3p22-p21 |
| Gene conserved in[a] | Chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, and *Caenorhabditis elegans* | Rhesus monkey, chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, and rice |
| Overexpression in clinical tumors | Refs. [6, 23, 40] | Refs.[15, 40, 60] |
| Overexpressed in tumor cell lines | Refs. [5, 6] | Refs. [15, 50] |
| Induced by hypoxia | Refs. [5, 8, 21, 37, 38, 40] | Refs. [5, 8, 21, 37, 40] |
| Induced by mitogens/inflammation | Refs. [11, 53] | |
| Induced by low pH | Ref. [36] | |
| Effect from gene deletion | Ref. [58] | |
| Effects from antisense | Ref. [11] | |
| Effects from siRNA | Ref. [9] | Ref. [50] |
| Effects from small molecules | Refs. [13, 14, 20, 27, 54, 56]<br>Patent applications:<br>Tapolsky et al., US2012/0177749<br>Chand et al.; WO2011/103557A1<br>J. Angbrant et al.: WO 2011161201 A1<br>S. Byström et al.: WO 2012035171 A2 | Ref. [56] |

[a]Data from Genecards.org

including AMPK, protein kinase B (AKT), protein kinase A, and protein kinase C [32, 34, 42, 43, 48].

## 8.3 PFKFB and Links to Cancer

With regard to cancer, the PFKFB1 and 2 isozymes are the least studied. To our knowledge, so far no reports have implied a role specifically for PFKFB1 in cancer, but two recent reports indicate a role for androgen-regulated PFKFB2 activity in prostate cancer [35, 42].

In contrast, the PFKFB3 isozyme and, to a somewhat less extent, also the PFKFB4 isozyme have been avidly discussed in the literature over the last 10–15 years regarding their role in inducing a "Warburg effect" in tumors of several origins (Table 8.1). The reasons for ascribing the PFKFB3 and PFKFB4 isozymes central roles in tumor metabolism are discussed further.

### *8.3.1 PFKFB3 is Considered to be Responsible for Setting the Intracellular Levels of F-2,6-$P_2$*

Differential tissue distribution of the various PFKFB isozymes has been observed. However, it is important to recognize that several tissues express more than one PFKFB member, which supports the notion that different isozymes play key roles under different physiological conditions. Notably, all four PFKFB isozymes are induced by low $O_2$ [37] and are, thus, present under hypoxic tumor conditions. However, regulation of this induction is assumed to be cell specific [37].

Among the PFKFB isozymes, PFKFB3 has been suggested to be decisive for setting the intracellular levels of F-2,6-$P_2$ [58, reviewed in 59]. A main argument is that the PFKFB3 isozyme stands out with a more than 700-fold higher PFK2 over FBPase-2 activity [53], thus selectively promoting the production of F-2,6-$P_2$. In contrast, the PFKFB1, 2, and 4 isozymes all exhibit more balanced enzymatic activities [47, 53].

Further support for a dominant role of PFKFB3 in regulating F-2,6-$P_2$ levels comes from studies of Telang et al., showing that fibroblasts derived from PFKFB3+/− mice maintain markedly decreased intracellular levels of F-2,6-$P_2$ [58]. Moreover, in Ras-transformed mouse lung fibroblasts, despite co-expression of all four PFKFB mRNAs, heterozygotic genomic deletion of the inducible PFKFB3 gene suppressed F-2,6-$P_2$ production [58].

### *8.3.2 Effects of PFKFB3 on Proliferation*

As a glycolytic enzyme, PFKFB3 is cytosolic. However, using a monoclonal antibody specific for the ubiquitous PFKFB3 (UBI2K5), Yalcin et al. showed a predominant

nuclear localization in HeLa, HCT116, and MDA-MB-231 cells, with also an ability to produce F-2,6-$P_2$ in the nucleus [59]. Interestingly, transfection and overexpression of UBI2K5 in the nucleus did not significantly alter glucose metabolism, but rather led to a marked increase in cell proliferation. This unexpected result indicates an intriguing link of PFKFB3 to proliferation via nuclear signaling [59].

In postmitotic neurons, the inducible form of PFKFB3 (UBI2K4) is constantly degraded via the E3 ubiquitin ligase APC/C-Cdh1 pathway [2, 19]. This suggests a rational explanation for the inability of postmitotic cells such as these to increase glycolysis [19].

Inactivation of APC/C-Cdh1 is necessary for the initiation of the S-phase. This inactivation alleviates degradation of PFKFB3 and, thus, allows for increased levels of F-2,6-BP levels and enhanced glycolysis during the proliferative phase [2]. From this, it follows that both adequate timing of PFKFB3 activity and elevated glycolysis may be important to the control of cell cycle progression [41].

### *8.3.3 Overexpression of PFKFB3 in Clinical Tumors*

Studies on patient tumors have provided strong arguments for a central role of PFKFB3 in the development and progression of human cancer.

Enhanced mRNA and protein levels of PFKFB3 were observed in a large panel of human tumors when compared to corresponding normal tissues [5]. Immunohistochemistry revealed a high ubiquitous distribution in the majority of the tumor specimens with the highest levels of PFKFB3 enhancement (two- to threefold) found in tumors originating from ovary, colon, pancreas, breast, and thyroid gland.

Presence of phosphorylated PFKFB3 protein has been linked to an invasive phenotype. Using antibodies specific for a consensus sequence for phosphorylation by AMPK, Bando et al. could show that the PFKFB3 protein was highly phosphorylated in colon and breast carcinoma compared to epithelial cells in the corresponding, normal tissue types. Interestingly, invasive areas of the tumors were most prominently stained [6].

Two PFKFB3 splice forms, UBI2K5 and UBI2K6, were detected as the predominant variants in patient tumors of rapidly proliferating high-grade astrocytoma, whereas two other splice forms, UBI2K3 and UBI2K4, were mainly restricted to low-grade astrocytomas and nonneoplastic brain tissue [61].

### *8.3.4 PFKFB3 Studies in Tumor Cell Lines and Animal Models*

Hypoxia-inducible factor 1 (HIF1) plays a role in tumor progression and is frequently upregulated in tumor cell lines where it induces a concomitant upregulation of transcription of the PFKFB3 and PFKFB4 genes [8, 38, 45].

Experimental overexpression of PFKFB3 in cultured cells has been shown to induce a phenotype similar to tumor cells, and enhanced growth *in vivo*. For example, forced expression of PFKFB3 protein in COS-7 cells activates glycolysis and promotes cell proliferation [6]. Presence of the inducible form of PFKFB3 was found to be critical for Ras-induced tumorigenesis, while genomic deletion of the PFKFB3 gene suppressed growth seen as soft-agar colonies or tumors in mice [58]. A recent study by Reddy et al. showed that knockdown of PFKFB3 led to reduced *in vitro* and *in vivo* tumor growth of cells transformed with mutant JAK2 [49]. Of interest, PFKFB3 was required for JAK2V617F-dependent lactate production, oxidative metabolic activity, and glucose uptake [49].

Perturbation of enhanced PFKFB3 expression, via antisense, small interfering RNA (siRNA) or small molecules, has been shown to lead to the reduction of tumor characteristics, such as growth *in vitro* and *in vivo* (literature Table 8.1). Antisense treatment slowed down tumor growth [11] and siRNA treatment induced cell cycle delay and inhibited anchorage-dependent growth of HeLa cells [9].

### *8.3.5 PFKFB4 in Clinical Tumors*

Fewer results are available from patient tumors regarding expression of PFKFB4.

Yun et al. reported that PFKFB4 mRNA was found to be overexpressed in non-muscle- invasive bladder cancer compared to corresponding nonmalignant tissue. The study also indicated a prognostic value of mRNA expression levels for recurrence and progression, i.e., the levels of PFKFB4 could be an independent predictor of bladder tumor progression [60].

Using previously published microarray data Goidts et al. found a statistical correlation between high levels of PFKFB4, but not PFKFB3, to poor survival in patients with primary glioblastoma [15].

### *8.3.6 PFKFB4 Studies in Tumor Cell Lines and Animal Models*

Similar to all the members of the PFKFB family, the PFKFB4 isozyme is induced by hypoxic conditions [8, 38, 39], but unlike PFKFB3 it has not been found regulated by inflammation or oncogenes.

A specific silencing of PFKFB4 was found to induce cell death in glioma cell lines [15]. This cell death was preceded by reduced lactate and ATP production, which led the authors to suggest that in these cells PFKFB4 supported glycolysis rather than the pentose phosphate pathway (PPP; see Fig. 8.1).

In a series of experiments Ros et al. recently demonstrated that a specific depletion of PFKFB4 caused a significant increase of F-2,6-$P_2$ in three prostate cell lines, indicating that in these cell lines a PFKFB4 phosphatase (FBPase-2) activity set the levels of F-2,6-$P_2$. The same study also demonstrated that depletion of PFKFB4 led to

a strong increase in reactive oxygen species (ROS) levels and apoptosis. Interestingly, and somewhat unexpectedly, they also showed that specific depletion of PFKFB3 did not reduce the F-2,6-$P_2$ levels and did not impair cell survival. However, a co-silencing of PFKFB3 after PFKFB4 significantly reduced apoptosis and blocked the induction of ROS levels. The authors suggested that PFKFB4 is essential for prostate cancer cell survival by maintaining the balance between the use of glucose for energy generation and the synthesis of antioxidants [50].

## 8.4 Small Molecules Reported to Inhibit PFK2 Activity

With the premise that enhanced glycolysis requires the enhanced presence of F-2,6-$P_2$ and the fact that all four members of the PFKFB gene family code for PFK2 enzymatic activity, it follows that the PFK2 activity of all four PFKFB isozymes are potential targets in pharmacological strategies to modulate the glycolytic phenotype of cancer cells.

Several attempts to generate small molecule inhibitors specific for PFK2 enzyme activity have been reported. Most of the small molecules described further exhibit competitive inhibition of F-6-P and are noncompetitive with ATP. Selectivity between effects on PFKFB3 and the other members of the PFKFB family of isozymes has not been described for most small molecule inhibitors with the exception of the compounds N4A and YN1 [56].

An alkylating molecule, N-bromoacetylethanolamine phosphate (Fig. 8.3a), was reported to compete with the F-6-P binding to the kinase site *in vitro* [27, 54]. Treatment of different tumor cell lines with compound **a** resulted in reduced levels of F-2,6-$P_2$ as well as reduced lactate production and inhibition of cell growth [20]. Several analogues of N-bromoacetylethanolamine phosphate were synthesized, among them *N*-(3-methoxypropyl)-bromoacetamide (Fig. 8.3b) which was shown to be five- to tenfold more potent when tested against the growth of white blood cell tumor cell lines. A structure–activity-relationship (SAR) analysis revealed that steric hindrance close to the leaving group (bromo) reduced the inhibitory effects, as did replacement of the bromine with the less reactive chlorine [20].

The compound 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) (Fig. 8.3c) was identified as a PFKFB3 inhibitor by computational methods, where a homology model of the PFKFB3 enzyme was generated using a published structure of the PFKFB4 isozyme as the input structure. Administration of 3PO to cells resulted in reduced intracellular levels of F-2,6-$P_2$, reduced lactate secretion and reduced glucose uptake *in vitro.* Moreover, in several carcinoma cell lines, reduced cell growth *in vitro* was observed after treatment with low micromolar concentrations, as were growth inhibitory effects on established tumors *in vivo*[13].

More potent analogues of 3PO were subsequently developed, for example ACT-PFK141 (Fig. 8.3d), and were reported to reduce tumor growth in animal models representing lung cancer, glioblastoma, and colon cancer (Patent application: Tapolsky et al., US2012/0177749). Recently, substituted benzindoles were described as a

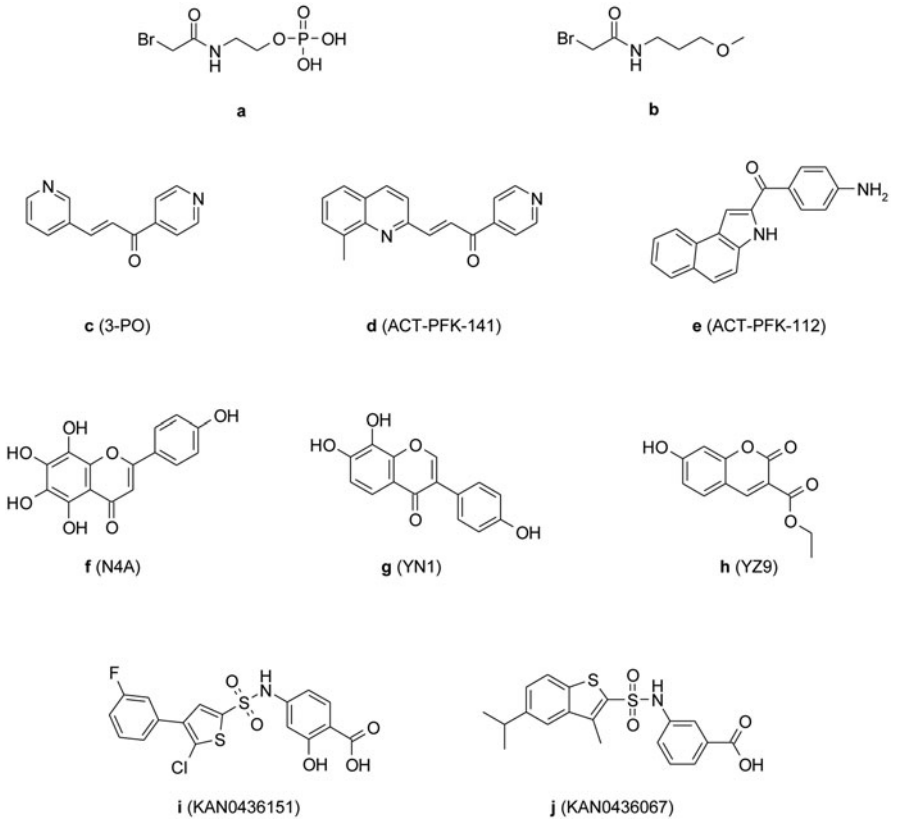


**Fig. 8.3** Small-molecule kinase inhibitors of phosphofructokinase/fructose bisphosphatase (PFKFB). **a)** N-Bromoacetylethanolamine phosphate, IC50 19μM (growth inhibition, U-937 cells). **b)** N-(3-Methoxypropyl)-bromoacetamide, IC50 2.4μM (growth inhibition, U-937 cells). **c)** 3-PO, Ki 25μM. **d)** ACT-PFK-141, IC50 0.21μM. **e)** ACT-PFK-112, 82% inhibition of PFKFB3 at an inhibitor concentration of 250nM. **f)** N4A, IC50 2.97μM and Ki 1.28μM. **g)** YN1, IC50 0.67μM and Ki 0.24μM. **h)** YZ9, IC50 0.18μM and Ki 0.094μM. **i)** KAN0436151, IC50 0.22μM. **j)** and KAN0436067, IC50 0.03μM. IC50 and Ki values from biochemical assays unless otherwise stated.

new class of PFKFB3 inhibitors, for example ACT-PFK112 (Fig. 8.3e) which was reported to inhibit lactate secretion and glucose uptake in Jurkat cells *in vitro,* as well as reduced growth of several tumor cell lines as xenografts (Patent application: Chand et al. 2011; WO2011/103557A1).

Additional small molecules targeting the F-6-P site of PFKFB3 have been developed by the company Advanced Cancer Therapeutics, with *in vivo* effects demonstrated either alone or as part of combination therapy with paclitaxel or gemcitabine (Tapolsky, G. Personal communication).[1]

[1] Tapolsky: Presentation at the World Cancer Metabolism Summit, Washington 2012; Characterization of Novel Antagonists of 6-Phosphofructo-2

A pharmacophore model based on the small molecules F-6-P, EDTA, and phosphoenol pyruvate, all known to bind in the F-6-P pocket, was used to identify six small molecules among 1,364 compounds in the NCI collection, using a combined virtual screening/biochemical screening approach [14]. As expected based on the model used, these compounds, e.g., N4A (Fig. 8.3f) were shown to be F-6-P competitive. The structural insights obtained by studying the ligand–protein complex between PFKFB3 and N4A were used to guide the identification of the more potent analogues YN1 (Fig. 8.3g) and YZ9 (Fig. 8.3h). The identified PFKFB3 inhibitors were shown to reduce the levels of F-2,6-$P_2$ in HeLa cells, resulting in decreased tumor growth and increased cell death *in vitro*[56].

Recently, a series of compounds with a salicylate group as a common structural motif, e.g., KAN0436151 (Fig. 8.3i), were demonstrated to be inhibitors of PFKFB3 and PFKFB4 (Patent application: J. Angbrant et al.: WO 2011161201 A1). Such compounds were shown to inhibit the PFK2 activity of PFKFB3 and PFKFB4 in biochemical assays and to decrease levels of F-2,6-$P_2$ in the human pancreatic carcinoma cell line PANC-1. Structurally somewhat related compounds, e.g., KAN0436067 (Fig. 8.3j), based on benzoic acid derivatives and analogues thereof were shown to similarly inhibit PFK2 activity of PFKFB3 and PFKFB4 in biochemical and various cellular assays. An inhibitory effect on growth of NUGC-3 cells *in vitro* was also shown (Patent application: S. Byström et al.: WO 2012035171 A2).

## 8.5 Scenarios for Therapeutic Effects by PFKFB3/PFK2 Inhibition, Alone or in Combination with Other Anticancer Drugs

The therapeutic potential of PFKFB3 inhibition is not restricted to a "mere" rewiring of metabolism. Combinations of PFKFB3 inhibitors with other pharmacological agents suggest several conceivable scenarios. Combinations with agents that in some way mimic a hypoxic condition might be efficient, since they would increase cellular dependence on upregulation of glycolysis and PFKFB3. Along these lines, oligomycin, an inhibitor of mitochondrial ATP production, was found to induce phosphorylation of PFKFB3 [6]. Although oligomycin cannot be used therapeutically, it would be interesting to examine cell death induced by oligomycin and an inhibitor of PFKFB3.

In combination with a DNA-damaging agent, such as one of the many anticancer drugs in current clinical use, a PFKFB3 inhibitor might prevent ATP-dependent chromatin remodeling in the DNA damage response [29].

Furthermore, compared to normal cells, cancer cells have higher levels of misfolded proteins which need to be degraded via the proteasome system. This process requires ATP [55], wherefore PFKFB3 inhibitors might, to some extent, mimic proteasome inhibitors such as bortezomib and the recently launched carfilzomib and prevent the cell from coping with toxic levels of misfolded proteins. Bortezomib is in clinical use but has undesired side effects, e.g., neuropathies. As a strategy for

increased tumor specificity, agents that are efficient in combination with bortezomib or carfilzomib might, thus, be tested for combination effects with PFKFB3 inhibitors.

As discussed earlier, in relation to the findings of Reddy 2012 [49], therapies that specifically block PFKFB3 activity or expression would be expected to inhibit JAK2/STAT5-dependent tumors.

PFKFB3 inhibition could moreover be expected to be more efficient in hypoxic tumors that are unable to switch to $O_2$ consumption for their energy requirements. On the other hand, highly glutaminolytic tumor cells would not be greatly affected by PFKFB3 inhibition as glutamine is converted to pyruvate/lactate without the involvement of glycolysis. However, tumors are believed to undergo cycles of glycolytic and glutaminolytic dependence [57], suggesting that repeated treatment with an inhibitor could be beneficial.

Faced with one type of nutrient deficiency or pathway inhibition, tumor cells are able to switch to another fuel or pathway, and can even degrade and recycle some of their own macromolecules and organelles in order to survive energy stress; this response is termed autophagy [33]. There are currently no reports on the role of PFKFB3 in autophagy.

## 8.6 Scenarios of Possible Side Effects

In drug discovery, preclinical development routinely includes efforts to mechanistically evaluate possible side effects, which are then further evaluated in clinical phase 1 studies. For PFKFB enzymes specifically, it can be noted that this family of enzymes is involved in normal processes in nonmalignant cells throughout the body. For instance, F-2,6-$P_2$ plays a crucial role in the control of the opposing hepatic glycolytic and gluconeogenic pathways. A therapeutic targeting may, thus, entail side effects also in normal cells, to be evaluated together with the benefits of targeting the cancer disease.

Concomitant expression of more than one PFKFB family member indicates the need of using pan-PFKFB kinase inhibitors for most efficient reduction of the F-2,6-$P_2$ levels. In this respect, a concomitant targeting of both PFKFB3 and PFKFB4 appears advantageous due to their high levels in cancer cells. A cross-reactivity inhibiting also PFKFB2 may potentially raise more concern. For instance, after an acute ischemic event, AMPK activates PFKFB2 in cardiac cells which leads to stimulation of glycolysis and chain events [32]. This indicates the need for selective inhibitors, and small molecules with isozyme selectivity have already been described [56]. Whether inhibition of PFKFB2 is contraindicated during ischemic events needs to be evaluated.

Side effects due to the links to metabolic dysregulation are, however, not necessarily troublesome. Targeting of the PFK2 activity of PFKFB enzymes may help to understand inflammatory diseases and diabetes, or might even be part of therapy. Indeed, PFKFB3 is a target gene of peroxisome proliferator-activated receptor gamma (PPARγ) [16], and using gene knockout mice, it has been shown that PFKFB3 is critically involved in the antidiabetic effect of PPARγ activation by suppressing excessive fatty acid oxidation-related ROS production and inflammatory responses in

adipose tissue/adipocytes [16]. A decrease in F-2,6-$P_2$ is associated with ketosis and risk for type II diabetes as it lifts F-2,6-$P_2$ inhibition of gluconeogenesis, resulting in the generation of glucose and high blood glucose levels. A similar process occurs during periods of fasting, starvation, low-carbohydrate diets, or intense exercise.

## 8.7 Conclusions

The dynamic increase in the understanding of cancer-promoting events and the development of specific tools in the form of small molecule inhibitors provide a basis for improved efficiency and tumor specificity of cancer treatment. We have here summarized present knowledge on the associations between PFKFB family members and cancer, and on the possibility of reducing enhanced glycolysis in cancer cells by impeding the kinase activity of PFKFB3. Going beyond the almost century-old initial focus on changed energy metabolism, recent research has pointed toward intriguing, but not fully understood, links between cell cycle control and mediators of glycolysis, herein represented by PFKFB3 splice variants. In view of the adaptability and plasticity of tumor cells, these findings lead us to suggest that small-molecule inhibition of PFKFB3 in combination with other drugs is likely more efficient than single-drug treatment.

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