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THE EMERGING HALLMARKS OF CANCER METABOLISM

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Abstract

Tumorigenesis is dependent on the reprogramming of cellular metabolism as both direct and indirect consequence of oncogenic mutations. A common feature of cancer cell metabolism is the ability to acquire necessary nutrients from a frequently nutrient-poor environment and utilize these nutrients to both maintain viability and build new biomass. The alterations in intracellular and extracellular metabolites that can accompany cancer-associated metabolic reprogramming have profound effects on gene expression, cellular differentiation and the tumor microenvironment. In this Review, we have organized known cancer-associated metabolic changes into six hallmarks: (1) deregulated uptake of glucose and amino acids, (2) use of opportunistic modes of nutrient acquisition, (3) use of glycolysis/TCA cycle intermediates for biosynthesis and NADPH production, (4) increased demand for nitrogen, (5) alterations in metabolite-driven gene regulation, and (6) metabolic interactions with the microenvironment. While few tumors display all six hallmarks, most display several. The specific hallmarks exhibited by an individual tumor may ultimately contribute to better tumor classification and aid in directing treatment.

INTRODUCTION

While the first observations on metabolic alterations that are characteristic for tumors were first made nearly a century ago, the field of cancer metabolism has become a topic of a renewed interest in the past decade. Aided by new biochemical and molecular biological tools, studies in cancer cell metabolism have expanded our understanding of the mechanisms and functional consequences of tumor-associated metabolic alterations at various stages of tumorigenesis. In particular, it has become evident that tumorigenesis-associated metabolic alterations encompass all stages of cell-metabolite interaction, (a) affecting the metabolite influx through conferring an increased ability to acquire the necessary nutrients; (b) shaping the way the nutrients are preferentially assigned to metabolic pathways that contribute to cellular tumorigenic properties, as well as (c) exerting long-ranging effects on cellular fate, among which are alterations in differentiation of cancer cells themselves as well as of the components of the tumor microenvironment (Figure 1). In this Perspective, we take a

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detailed look at distinct hallmarks of tumorigenesis-associated metabolic reprogramming, and examine the functional contribution of these hallmarks to the establishment and maintenance of the tumorigenic state.

DEREGULATED UPTAKE OF GLUCOSE AND AMINO ACIDS

In order to fulfill the biosynthetic demands associated with proliferation, a cell must increase the import of nutrients from the environment. Two principal nutrients that support survival and biosynthesis in mammalian cells are glucose and glutamine. Through the catabolism of glucose and glutamine, a cell maintains pools of diverse carbon intermediates, which are utilized as building blocks for the assembly of various macromolecules. In addition, controlled oxidation of carbon skeletons of glucose and glutamine allows a cell to capture their reducing power either in the form of NADH and $FADH_2$, which mediate the transfer of electrons to the electron transport chain to fuel ATP generation, or in the form of a related cofactor NADPH, which provides reducing power for a wide variety of biosynthetic reactions, as well as helps maintain cellular redox capacity.

A markedly increased consumption of glucose by tumors in comparison to the non-proliferating normal tissues was first described more than 90 years ago by the German physiologist Otto Warburg (Warburg O, 1924; Warburg et al., 1927). This observation has been confirmed in a variety of tumor contexts and shown to correlate with poor tumor prognosis (Som et al., 1980). Positron emission tomography (PET)-based imaging of the uptake of a radioactive fluorine-labeled glucose analog, ^{18}F-fluorodeoxyglucose (^{18}F-FDG) has been successfully used in the clinic for tumor diagnosis and staging, as well as for monitoring responsiveness to treatment (Almuhaideb et al., 2011).

Glutamine, a second principal growth-supporting substrate contributes not only carbon, but also reduced nitrogen for the *de novo* biosynthesis of a number of diverse nitrogen-containing compounds. Thus, glutamine provides the nitrogen required for the biosynthesis of purine and pyrimidine nucleotides, glucosamine-6-phosphate, as well as non-essential amino acids. Glutamine also has been reported to play a role in the uptake of essential amino acids. While non-essential amino acids can be produced by mammalian cells *de novo*, essential amino acids must be acquired from external sources. Interestingly, the import of an essential amino acid leucine through the plasma membrane-localized neutral amino acid antiporter LAT1 was shown to be coupled to a simultaneous efflux of glutamine (Nicklin et al., 2009). In such a manner, intracellular glutamine may facilitate the import of a broad range of LAT1 substrates, including leucine, isoleucine, valine, methionine, tyrosine, tryptophan and phenylalanine (Yanagida et al., 2001).

The high demand of proliferating tumor cells for glutamine was first described by the American physiologist Harry Eagle in 1950s, who demonstrated that the optimal growth of cultured HeLa cells requires a 10- to a 100-fold molar excess of glutamine in culture medium relative to other amino acids (Eagle, 1955). Furthermore, glutamine was found to be the most rapidly consumed amino acid by Ehrlich ascites carcinomas as well as by a number of hepatomas and carcinosarcomas proliferating *in vivo* (Marquez et al., 1989; Sauer et al., 1982). In fact, numerous tumorigenic contexts are associated with the depletion of

glutamine from the tumor environment as compared to the corresponding normal tissue (Marquez et al., 1989; Rivera et al., 1988; Roberts and Frankel, 1949; Yuneva et al., 2012). Following the success of the ^{18}F -FDG imaging paradigm, ^{18}F-labeled glutamine tracers have recently shown promise in preclinical and early clinical studies (Lieberman et al., 2011; Venneti et al., 2015). Use of ^{18}F-labeled glutamine as a tracer appears to provide potentially useful tumor information where the use of ^{18}F-fluorodeoxyglucose is not feasible – for instance, in imaging of tumors that are localized to sites of heavy glucose utilization, such as the brain.

What causes tumor cells to internalize high quantities of glucose and glutamine? Normally, despite being surrounded by nutrient-rich plasma and the extracellular fluid, metazoan cells do not import nutrients in a constitutive manner. On the contrary, nutrient uptake is strictly regulated by growth factor signaling (Thompson, 2011) (Figure 2). For instance, when deprived of growth factors, hematopoietic and neuronal cells fail to consume glucose in quantities sufficient to even maintain cellular bioenergetics (Lindsten et al., 2003; Rathmell et al., 2000). This, in turn, negatively affects cell size, mitochondrial potential and ATP generation – all despite the abundance of glucose in the culture medium (Rathmell et al., 2000). However, survival of growth-factor deprived cells can be readily restored by a combined expression of a plasma membrane glucose transporter GLUT1 and the first enzyme of the glycolytic pathway hexokinase (HK) (Rathmell et al., 2003).

In addition to soluble growth factors, interactions of cells with the extracellular matrix play a role in regulating glucose uptake. Culturing mammary epithelial cells under conditions where the cells are detached from the extracellular matrix compromises glucose uptake and results in a depressed mitochondrial potential and decreased ATP levels (Grassian et al., 2011; Schafer et al., 2009). Taken together, these observations suggest that the influx of glucose into cells is not driven by the immediate bioenergetic needs of a cell, but on the contrary, is rationed strictly as a result of extracellular stimuli.

In contrast to their normal counterparts, which require adhesion- and growth-factor-driven signaling inputs to maintain survival and proliferation, cancer cells accumulate oncogenic alterations that convey to them a significant degree of independence from these external requirements (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 2004). In particular, genetic alterations that target PI-3 kinase, its negative regulators PTEN and INPP4B, as well as activating mutations and gene amplifications in a variety of upstream receptor tyrosine kinases, result in constitutive glucose uptake and metabolism in diverse cancer types.

A point of convergence of signals from receptor tyrosine kinases as well as from the extracellular matrix, PI3K/Akt signaling acts as a master regulator of glucose uptake. PI3K/Akt signaling promotes both the expression of glucose transporter *GLUT1* mRNA and the translocation of GLUT1 protein from the endomembranes to the cell surface (Barthel et al., 1999; Wieman et al., 2007). In addition, Akt potentiates the activity of the hexokinase (HK), which phosphorylates glucose molecules, thus preventing their efflux back into the extracellular space, as well as of the phosphofructokinase (PFK) enzyme, which catalyzes the key irreversible step of glycolysis (Deprez et al., 1997; Gottlob et al., 2001). In fact, the ^{18}F-FDG-PET signal intensity in tumors correlates closely with the level of PI3K/Akt

pathway activity, and is attenuated by PI-3 kinase and receptor tyrosine kinase inhibitors (Benz et al., 2011; Lheureux et al., 2013; Ma et al., 2009). Furthermore, exogenous expression of a constitutively active form of Akt alone can stimulate glycolysis and is sufficient to restore cell size, viability, mitochondrial potential and ATP levels in growth factor-deprived cells in a manner that is dependent upon the presence of glucose in the culture medium (Plas et al., 2001; Rathmell et al., 2003). Constitutively active form of Akt also prevents a decrease in ATP levels triggered by the loss of cellular attachment (Schafer et al., 2009). In addition, Akt signaling is central to facilitating glucose uptake in physiological settings of increased biosynthetic demand. For instance, targeted deletion of Akt1 in the mouse mammary gland abrogates a lactation-induced increase in glucose uptake, resulting in insufficient milk production (Boxer et al., 2006).

However, the PI3K/Akt signaling module is not the only oncogenic stimulus that plays a role in facilitating glucose uptake. Other oncogenic signaling proteins - notably, Ras, have been found to upregulate *GLUT1* mRNA expression and increase cellular glucose consumption (Murakami et al., 1992). Taken together, multiple growth signaling nodes that become aberrantly activated in cancer share an ability to facilitate cellular access to glucose, a principal metabolic substrate.

The signaling pathways that regulate glutamine uptake are still being elucidated. The transcription factor *c-myc*, which is upregulated in proliferating cells and is frequently targeted by amplification in various tumor types, is a principal driver of glutamine utilization by proliferating cells (Wang et al., 2011b; Wise et al., 2008). To this end, *c-myc* induces the transcription of glutamine transporters ASCT2 and SN2, and in addition, promotes expression of glutamine-utilizing enzymes glutaminase (GLS1), phosphoribosyl pyrophosphate synthetase (PRPS2) and carbamoyl-phosphate synthetase 2 (CAD), which support transporter-facilitated glutamine uptake by converting glutamine to glutamate (Eberhardy and Farnham, 2001; Gao et al., 2009; Mannava et al., 2008). The resulting glutamate cannot exit the cell through glutamine transport and as it accumulates, promotes TCA cycle anaplerosis and stimulates uptake of cysteine by acting as an exchange substrate for the cysteine antiporter, xCT (Conrad and Sato, 2012).

In addition to a positive regulatory input from *c-myc*, glutamine uptake is also subject to negative regulation by the *Rb* tumor suppressor family of proteins. The deletion of *Rb* family proteins has been shown to upregulate the uptake and utilization of glutamine via the E2F-dependent upregulation of ASCT2 and GLS1 (Reynolds et al., 2014). Taken together, *c-myc* and E2F, two critical facilitators of cell division, exert their effects, in part, via enabling the access of cells to glutamine - a metabolic substrate critical for the biosynthetic demands that accompany DNA replication.

For any given cell, an attempt to proliferate in the absence of proper metabolic resources could end in a disaster. To avoid this, aberrantly activated oncogenes and/or loss of tumor suppressors can lock cancer cells in a state where they constitutively scavenge available glucose, glutamine and essential amino acids from the extracellular environment, which, in turn, enables their uncontrolled proliferation.

USE OF OPPORTUNISTIC MODES OF NUTRIENT ACQUISITION

Despite the avidity with which they take up glucose and amino acids, cancer cells *in vivo* often encounter conditions of nutrient scarcity as a result of the increased rates of nutrient consumption and the inadequacies of the tumor vascular supply (Vaupel et al., 1989). To deal with depleted supplies of the normal anabolic precursors, certain cancers acquire mutations that activate the cellular ability to utilize alternative ways of obtaining necessary nutrients. For instance, plasma and interstitial fluid of tissues is rich in soluble protein, yet extracellular protein is not generally utilized as a source of amino acids. Remarkably, expression of mutant Ras or c-Src alleles provides cells with a mechanism that allows them to recover free amino acids through the lysosomal degradation of extracellular proteins (Commisso et al., 2013) (Figure 3A). In contrast to the uptake of low molecular weight nutrients, which enter the cell through dedicated transporters, capture of extracellular macromolecules occurs through macropinocytosis, a process in which bulk extracellular fluid is taken up into giant vesicles (Kerr and Teasdale, 2009). Macropinocytosis is stimulated by Ras- and c-Src-driven actin cytoskeleton remodeling. Fluid-filled macropinosomes are trafficked into the interior of the cell, where they fuse with lysosomes and the engulfed proteins are subjected to proteolytic degradation, liberating free amino acids. Indeed, supplementation of culture medium with physiological levels (20–30 mg/mL) of serum albumin enables proliferation of $KRAS^{G12D}$-transformed MEFs in the absence of an essential amino acid leucine (Palm et al., 2015). Albumin supplementation has even been shown to restore the proliferation of a $KRAS^{G12D}$-driven mouse pancreatic cancer line in the culture medium that was devoid of all free amino acids (Kamphorst et al., 2015). In contrast to the effect of $KRAS^{G12D}$, PI3K/Akt signaling does not promote cellular utilization of extracellular protein, despite the ability of this pathway to stimulate the uptake of low molecular weight nutrients (Palm et al., 2015).

Remarkably, mTORC1 inhibitors greatly increase the cellular ability to recover amino acids from captured extracellular protein and enhance their growth in the absence of essential amino acids (Palm et al., 2015). Thus, the utilization of extracellular protein as nutrients is repressed by mTORC1 under amino acid-replete conditions and is only utilized as an emergency source in conditions when free amino acids are insufficient. A spatial difference in the effect of rapamycin treatment in a $KRAS^{G12D}$-driven mouse model of pancreatic cancer provides a striking illustration of this paradigm. While rapamycin suppresses cancer cell proliferation at the tumor margin where the vascular delivery of nutrients is effective, rapamycin enhances cell proliferation within the poorly-vascularized tumor core by promoting enhanced lysosomal degradation of extracellular proteins (Palm et al., 2015).

In addition to soluble extracellular proteins, free amino acids can be recovered from the engulfment and digestion of entire living cells via a process known as entosis (Figure 3B), as well as from phagocytosis of apoptotic cellular corpses (Figure 3C) (Krajcovic et al., 2013; Stolzing and Grune, 2004). In a manner similar to macropinocytosis, cargo engulfed via entosis or phagocytosis becomes digested within lysosomes, providing a supply of amino acids to support cell survival and proliferation during conditions of amino acid deficit (Krajcovic et al., 2013). Interestingly, cells that harbor a mutant KRAS allele are more likely to perpetrate entosis than to be consumed in this process (Sun et al., 2014). Thus, KRAS-

mutant cells may not only have a nutritional advantage over their non-mutant neighbors, but also actively eliminate the latter. Such a property may contribute to cell-cell competition within tumors, leading to the emergence of more aggressive cell populations.

A second consequence of a lack of vascular nutrient delivery in growing tumors is an emergence of hypoxic areas, which leads to a suppression of a number of biosynthetic reactions that require molecular oxygen as an electron acceptor. For example, hypoxia compromises stearoyl-CoA desaturase 1 (SCD1)-catalyzed introduction of double bonds into the *de novo* produced fatty acids, creating a deficit of unsaturated fatty acid species. To supplement for the missing fatty acids, hypoxic cells import "ready-made" unsaturated fatty acids from the surrounding environment in a form of single acyl chain-containing lysophospholipids (Kamphorst et al., 2013) (Figure 3D). Accordingly, removal of serum lipids triggers ER stress and apoptosis in certain cancer cells, when cultured under hypoxic conditions (Young et al., 2013).

In other cancer cells, the extracellular liberation of free fatty acids from more complex lipid species is increased. An elevated expression of lipoprotein lipase (LPL) and monoacylglycerol lipase (MAGL) across diverse cancer subtypes and correlates with invasiveness (Kuemmerle et al., 2011; Nomura et al., 2010). Finally, while some tumors develop adaptations that allow them to recover fatty acids directly from plasma, others induce the release of stored lipids from the neighboring normal cells. For instance, upregulation of a long chain fatty acid-binding protein FABP4 on the surface of metastatic ovarian cancer cells allows them to acquire fatty acids directly from the adipocytes of the omental fat (Nieman et al., 2011).

Taken together, tumor cells utilize opportunistic modes of nutrient acquisition, which allow them to survive and proliferate in metabolically unfavorable conditions. These adaptations involve an ability to access normally inaccessible nutrient sources, as well as to recover premade molecules when their synthesis within the cell has been compromised.

Even in the absence of available extracellular nutrients, cancer cells have been shown to utilize adaptive metabolic pathways to sustain their viability. In particular, cancer cells can withstand long periods of nutrient deprivation via the self-catabolic process of macroautophagy (Boya et al., 2013). During macroautophagy, intracellular macromolecules and whole organelles are enveloped by double membrane structures and fuse with lysosomes. Once inside a lysosome, the engulfed cargo is degraded by resident proteases and lipases to liberate free amino and fatty acids. Indeed, autophagy allows nutrient- or growth factor-deprived cells to maintain their viability in culture for weeks (Lum et al., 2005). In addition, deletion of a core component of the autophagy machinery, *Atg7*, was shown to dramatically change the nature of lung tumors driven by $Kras^{G12D}$ and $Braf^{V600E}$ oncogenes from malignant adenocarcinomas to benign oncocytomas (Guo et al., 2013; Strohecker et al., 2013). However, autophagy cannot supply cells with new biomass and thus cannot support proliferation in nutrient-poor conditions.

USE OF GLYCOLYSIS/TCA CYCLE INTERMEDIATES FOR BIOSYNTHESIS AND NADPH PRODUCTION

Cell proliferation not only increases the amount of nutrients a cell requires as outlined above, but also actively changes the way nutrients are used. Indeed, when a tumor cell is quiescent, glucose is preferentially utilized for mitochondrial acetyl-CoA generation, which is then subjected to oxidation in the tricarboxylic (TCA) cycle. The electrons extracted from the oxidative reactions of the TCA cycle are shuttled through NAD^+/NADH and FAD/$FADH_2$ to the electron transport chain, creating an electrochemical gradient that fuels ATP production.

The carbon economy of a proliferating cell differs dramatically from that of a cell that is quiescent (Vander Heiden et al., 2009) (Figure 4A). The major use of reduced carbon in proliferating cells is for the biosynthesis of a diverse array of biomolecules – among which are fatty acids and cholesterol, pentose and hexose sugar derivatives, glycerol, nucleotides and non-essential amino acids. To achieve this, a proliferating cell must first transform acquired nutrients into diverse pools of structural intermediates (Figure 4B). These molecules include cytosolic acetyl-CoA, one-carbon-carrying folate cycle units and S-adenosylmethionine (SAM), as well as a battery of glycolytic and TCA cycle intermediates. In addition, many biosynthetic reactions are reductive by nature and thus require a source of reducing power. For instance, generation of palmitic acid consumes 14 reducing equivalents, while building a cholesterol molecule requires 26 (Lunt and Vander Heiden, 2011). A designated donor of reducing equivalents that is used in cellular biosynthetic reactions is NADPH. Generation of NADPH from $NADP^+$ is enabled by the controlled oxidation of carbon substrates in pathways distinct from those that generate NADH to support mitochondrial electron transport. Thus, a proliferating cell must allocate a portion of its carbon substrates to be used in NADPH production.

The reprogramming of carbon metabolism by proliferating cells provides part of the explanation for Warburg's original observations concerning cancer metabolism. Experimenting with tumor slices incubated with glucose *ex vivo*, Warburg demonstrated that despite consuming massive amounts of glucose and being exposed to ambient oxygen, cancer cells did not take advantage of the bioenergetic benefits offered by the coupling of glycolysis to the TCA cycle. On the contrary, when grown in glucose-rich medium, any cells converted the surplus pyruvate to lactate, which, in turn, was secreted into the extracellular environment. Working under assumption that the major use of glucose in cells was to generate ATP, Warburg and many subsequent investigators misinterpreted this phenomenon, attributing it to the "irreversible injuring of respiration", which leaves tumor cells with a considerably less efficient way of deriving ATP from substrate-level phosphorylation reactions of glycolysis (Warburg, 1956).

However, subsequent investigations into the mitochondrial status of tumor cells have shown that by and large, tumor cells possess functional mitochondria and retain the ability to conduct oxidative phosphorylation. In fact, targeted depletion of mitochondrial DNA reduces the tumorigenic potential of cancer cell lines *in vitro* and *in vivo* (Cavalli et al., 1997; Morais et al., 1994; Tan et al., 2015). Moreover, preferential conversion of glucose

into lactate has been later demonstrated in genetically normal proliferating cells, as well as in cells infected by viruses (Brand et al., 1986; Chambers et al., 2010; Noch and Khalili, 2012). These observations suggest that, rather than being an adaptation to a defect in respiration, the Warburg effect is a regulated metabolic state and may, in fact, be beneficial during a time of increased biosynthetic demand.

Why then do proliferating cells convert excess pyruvate to lactate rather than transport the excess pyruvate into the mitochondria to maintain oxidative phosphorylation? The answer is just now coming into focus. Surprisingly, proliferating cells have only a modest increase in their consumption of ATP relative to their need for precursor molecules and reducing equivalents in the form of NADPH. Glucose catabolism is a robust provider of these precursors and reducing equivalents. In contrast, TCA cycle activity which generates NADH and ATP is the major negative regulator of glucose metabolism. By converting excess pyruvate to lactate, proliferating cells prevent accumulation of cytosolic NADH and reduce ATP production promoting continued cytosolic glucose metabolism free from feedback repression by excess mitochondrial ATP generation.

This realization has spurred further exploration of advantages that the decoupling of glycolysis from oxidative phosphorylation, might offer to a proliferating cell. Though glycolysis is classically depicted as a single chain of molecular events that leads to the generation of pyruvate, a number of glycolytic intermediates can be diverted into branching pathways, generating diverse biosynthetic precursors. First in the series of these branching pathways is the pentose phosphate pathway (PPP), in which glucose-6-phosphate becomes partially oxidized to generate NADPH and ribose-5-phosphate – a structural component of nucleotides. Utilization of PPP is frequently elevated in tumorigenesis. The key enzymes in the pathway, transketolase-like 1 (TKTL1) and transaldolase (TALDO) are frequently overexpressed in cancer (Wang et al., 2011a; Xu et al., 2009b). Both oncogenes and tumor suppressors have been shown to regulate PPP activity. For instance, Ras-driven transformation induces transcriptional upregulation of enzymes that mediate ribose-5-phosphate biosynthesis (Ying et al., 2012). Furthermore, wild-type, but not the mutant, p53 inhibits PPP by directly binding and inactivating the rate-limiting PPP enzyme glucose-6-phosphate dehydrogenase (G6PD) (Jiang et al., 2011).

Fructose-6-phosphate, which follows glucose-6-phosphate in the glycolytic pathway, can also leave glycolysis and become utilized as a substrate for hexosamine biosynthesis. A first committed step of hexosamine biosynthesis is a glutamine fructose-6-phospate aminotransferase 1 (GFPT1)-catalyzed production of glucosamine-6-phosphate from fructose-6-phosphate and glutamine. Through the generation of N-acetylglucosamine (GlcNAc), a substrate for N- and O-linked glycosylation, the hexosamine pathway provides substrates for cellular glycosylation reactions as well as heparan sulfate and hyaluronic acid biosynthesis, potentiates receptor-mediated signaling and regulates the stability of select proteins, including *c-myc* (Itkonen et al., 2013; Spiro, 2002; Wellen et al., 2010).

The next glycolytic intermediate that is utilized in macromolecular synthesis is dihydroxyacetone phosphate (DHAP). DHAP is converted by glycerol-3-phosphate

dehydrogenase 1 (GPD1) into glycerol-3-phosphate, which is utilized in the biosynthesis of diverse phospholipids, a major structural component of cellular membranes.

Perhaps the most intensely studied growth-promoting mechanism that shunts metabolites out of the glycolytic pathway is the use of 3-phosphoglycerate as a precursor for the biosynthesis of serine, glycine, and as a means to generate methyl donor groups and NADPH. As metabolic flux studies reveal, cancer cells may utilize as much as 50% of glucose-derived carbon in serine biosynthesis and its subsequent catabolism (Locasale et al., 2011). A rate-limiting serine biosynthesis enzyme, 3-phosphoglycerate dehydrogenase (PHGDH), is frequently focally amplified in breast cancer and melanoma and is required for the growth of PHGDH-amplified cells *in vitro* as well as *in vivo* (Locasale et al., 2011; Possemato et al., 2011).

Serine has a unique metabolic role in the cell as a major substrate for the so-called one-carbon, or folate cycle. To this end, its γ-carbon can be transferred to a carrier molecule, tetrahydrofolate (THF), in a reaction catalyzed by serine hydroxylmethyltransferase 2 (SHMT2) in the mitochondria, and SHMT1 in the cytosol, generating 5, 10-methylene-THF and glycine. 5, 10-methylene-THF then undergoes a series of oxidative-reductive transformations, creating a battery of one-carbon-THF species (Tibbetts and Appling, 2010). One-carbon-THF species are utilized as substrates for the biosynthesis of purines, thymidine, as well as in the production of S-adenosylmethionine, a principal substrate for cellular methylation reactions. In addition, stepwise oxidation of one-carbon-THF species was recently shown to produce up to 50% of all cellular NADPH (Fan et al., 2014). Accordingly, hypoxia-driven induction of SHMT2 expression was shown to protect cells from hypoxia-associated oxidative stress (Ye et al., 2014). Thus, one-carbon pathway metabolites contribute to a number of cellular biosynthetic and regulatory processes. Finally, methylene tetrahydrofolate dehydrogenase 2 (MTHFD2), a component of the mitochondrial arm of the one-carbon pathway, was found to be among the top three most frequently overexpressed metabolic enzymes in cancer, suggesting that alterations in the one-carbon pathway may be universally selected for in tumorigenesis (Nilsson et al., 2014).

In conclusion, glycolytic intermediates leave glycolysis to take part in diverse biosynthetic reactions; accordingly, the rate-limiting enzymes within branching pathways of glycolysis are frequently upregulated in tumors. To balance biosynthetic outputs of glycolysis with its role in providing pyruvate that supports TCA cycle activity, proliferating cells have evolved a novel mechanism by which to regulate the last step of glycolysis. This step is regulated by pyruvate kinase (PK), an enzyme that converts phosphoenolpyruvate to the final product of glycolysis, pyruvate. With the exception of liver and kidney, which express tissue-specific PK isoforms PKL and PKR, most tissues express the PKM (muscle) form of PK. PKM exists in two splice variants (Noguchi et al., 1986). While PKM1 is more efficient at producing pyruvate, the majority of proliferating cells and essentially all cancer cells express primarily the PKM2 variant (Christofk et al., 2008a).

Unlike PKM1, PKM2's activity is highly regulated. Thus, PKM2 is inhibited by tyrosine phosphorylation and activated by serine (Chaneton et al., 2012; Christofk et al., 2008b; Ye et al., 2012). In such a manner, growth factor-dependent signal transduction inhibits PKM2,

leading to accumulation of glycolytic intermediates until the growing cell's need for a catabolizable pool of free serine is saturated. In support of this paradigm, a reintroduction of the PKM1 isoform compromises cancer cell proliferation and tumorigenesis *in vivo*, in part by negatively affecting serine-dependent biosynthesis (Ye et al., 2012). In addition to serine, PKM2 can also be allosterically activated by excess accumulation of other byproducts of glucose metabolism including succinylaminoimidazolecarboxamide (SAICAR), an intermediate product of ribose-5-phosphate biosynthesis (Keller et al., 2012).

The emerging evidence favors the hypothesis that glycolysis is utilized by proliferating cells as a versatile production line that generates metabolic intermediates for numerous biosynthetic processes. Any excess glycolytic flux not utilized for biosynthesis is preferentially converted to lactate to help preserve a sufficient pool of NAD^+ to sustain glycolysis and avoid flooding the mitochondria with a supply of NADH that would suppress the TCA cycle. Excess NADH generated by glycolysis is not imported into mitochondria of proliferating cells solely through chemical shuttles but is also utilized to convert the glycolytic intermediate DHAP into glycerol-3-phosphate that donates electrons directly into the electron transport chain utilizing glycerol-3-phosphate dehydrogenase (mGPDH) localized to the outer mitochondrial membrane. By using FAD as an electron acceptor, the mGPDH-catalyzed reaction reduces net mitochondrial electrical chemical potential from NADH generated via glycolysis. In fact, increased activity of GPDH has been detected in prostate cancer cells, insulinomas and carcinoid tumors (Chowdhury et al., 2005; MacDonald et al., 1990).

Mitochondria are protected further from excess glycolysis because any pyruvate that does enter the TCA cycle is channeled into citrate that is secreted into the cytosol through the mitochondrial tricarboxylate carrier and broken down into acetyl-CoA and oxaloacetate. Oxaloacetate is converted to malate and re-imported into mitochondria to maintain anaplerosis, while the acetyl-CoA serves as a precursor for lipid biosynthesis and protein acetylation. The electrons entering the mitochondrial electron transport chain through glycerol phosphate dehydrogenase activity and Complex I as a result of mitochondrial conversion of malate to oxaloacetate are sufficient to maintain both mitochondrial integrity and ATP production despite reduced catabolic TCA cycle activity.

A variety of oncogenes have been shown to contribute to the metabolic adaptations of proliferating cells outlined above. For instance, *c-myc* coordinately increases the expression of PDK1, lactate dehydrogenase A (LDH-A), an enzyme which catalyzes the reductive conversion of pyruvate to lactate, and monocarboxylate transporter (MCT1), which facilitates the efflux of lactate into the extracellular space (Wahlstrom and Arsenian Henriksson, 2015). In addition to *c-myc*, TCF/β-catenin signaling has also been shown to upregulate MCT1 and PDK1 transcription (Pate et al., 2014). Finally, stabilization of HIF1α by hypoxia or in various oncogenic contexts also triggers coordinated transcriptional upregulation of LDH-A and PDK1 (Kim et al., 2006; Papandreou et al., 2006).

Even with these adaptations, proliferating cells often accumulate electron transport flux that exceeds the capacity of the ATP synthase, resulting in the formation of excess reactive oxygen species (ROS). In fact, the damaging consequences of the overproduction of ROS

from the overloaded electron transport chain may underlie the phenomenon of oncogene-induced cellular senescence (OIS). Triggered by the introduction of a mutant BRAF or RAS alleles, OIS is associated with a profound oxidative damage and irreversible growth arrest (Courtois-Cox et al., 2008). Interestingly, attenuation of the activity of pyruvate dehydrogenase (PDH), a gatekeeper enzyme that converts pyruvate into acetyl-CoA, via the ectopic expression of its negative regulator pyruvate dehydrogenase kinase 1 (PDK1) or an RNAi-mediated depletion of a PDH phosphatase PDP2 was found to bypass mutant $BRAF^{V600E}$-induced OIS and potentiate tumorigenesis (Kaplon et al., 2013).

Whereas overproduction of ROS is detrimental to cell growth and survival, moderate levels of ROS, which are generated either during respiration or in a targeted manner by a class of NADPH oxidases, constitute an important signaling input that contributes to the maintenance of the tumorigenic state (Sullivan and Chandel, 2014). To this end, ROS act as inhibitors of protein phosphatases such as PTEN and PTP1B, as well as activators of Src family kinases and MAPK (Denu and Tanner, 1998; Kamata et al., 2005; Lee et al., 2002; Yan and Berton, 1996). Furthermore, increased ROS levels facilitate the activation of HIF1α and NRF2 transcription factors, thus promoting transcriptional programs that further contribute to tumorigenesis (Bertout et al., 2008; Sporn and Liby, 2012).

In contrast to the proliferating bulk of a tumor, accumulating evidence suggests that quiescent subpopulations of tumor cells are markedly less glycolytic and exhibit higher dependence on oxidative phosphorylation, as well as elevated expression of mitochondrial respiratory components. Among such populations are stem-like subclones that emerge following the oncogene ablation *in vivo*, as well as circulating tumor cells (CTCs) (LeBleu et al., 2014; Viale et al., 2014). Such apparent dichotomy in utilization of carbon for either predominantly biosynthetic or predominantly bioenergetic purposes may explain why tumors retain the capacity for oxidative phosphorylation, despite the original hypothesis put forth by Otto Warburg.

In addition to glycolytic intermediates, growth factor signaling promotes the use of select TCA cycle intermediates in the generation of biosynthetic precursors as well. In particular, PI3K/Akt activation allows a cell to expand its cytosolic pool of acetyl-CoA, a substrate for the *de novo* biosynthesis of fatty acids. To this end, Akt activates the ATP-citrate lyase (ACLY) enzyme, which catalyzes cytosol-localized cleavage of the TCA cycle-derived citrate into acetyl-CoA and oxaloacetate (Bauer et al., 2005; Berwick et al., 2002).

While the *de novo* biosynthesis of fatty acids is low in normal adult tissues – with the exception of lipogenic tissues such as liver, adipose tissue or mammary epithelium in the period of lactation, tumorigenesis is associated with a dramatic increase in lipid production (Li and Cheng, 2014; Menendez and Lupu, 2007). An increased capacity for the producing lipids *de novo* not only facilitates the formation of lipid bilayers, but also enables a cell to alter its membrane composition in favor of oxidative damage-resistant saturated fatty acids as means of adapting to oxidative stress (Rysman et al., 2010). The biosynthesis of fatty acid chains begins with the carboxylation of cytosolic acetyl-CoA by acetyl-CoA carboxylase (ACC) to produce malonyl-CoA, which is further assembled into long fatty acid chains by fatty acid synthase (FASN). In fact, all three major components involved in fatty acyl chain

biosynthesis – ACLY, ACC and FASN – are frequently upregulated in transformed cells; furthermore, their inhibition reduces tumor cell growth *in vitro* and *in vivo* (Chajes et al., 2006; Flavin et al., 2010; Migita et al., 2008; Milgraum et al., 1997). In addition to fatty acid production, the *de novo* biosynthesis of cholesterol from malonyl-CoA plays a role in tumorigenesis as well. Thus, interference with cholesterol biosynthesis by inhibiting a rate-limiting enzyme, hydroxymethylglutaryl coenzyme A reductase (HMGCR) restores the normal acinar morphology of breast cancer cells, suggesting that changes in membrane composition and fluidity may have an effect on tissue architecture and anchorage-independent growth (Freed-Pastor et al., 2012).

In addition to supplying citrate, the TCA cycle also provides metabolic precursors for the biosynthesis of non-essential amino acids including aspartate and asparagine. In fact, aspartate biosynthesis has recently been shown to be critically dependent upon a cell's ability to conduct oxidative phosphorylation (Birsoy et al., 2015; Sullivan et al., 2015). To maintain the ability to synthesize these amino acids, their production must be carefully balanced with an anaplerotic influx into the cycle. The major anaplerotic substrate in growing cells is glutamine. Glutamine can be captured in mitochondria by glutaminase and the resulting glutamate converted into α-ketoglutarate by either glutamate dehydrogenase or amino acid transaminases. Most proliferating cells depend on a continuous supply of glutamine to maintain the integrity of TCA cycle intermediates (DeBerardinis et al., 2007). In *c-myc*-transformed cells in particular, glutamine deprivation leads to the collapse of the TCA cycle and triggers cell death, which can be rescued by the addition of oxaloacetate or a membrane-permeable form of α-ketoglutarate (Wise et al., 2008; Yuneva et al., 2007). Oxidation of glutamine-derived α-ketoglutarate into oxaloacetate not only helps to maintain the ability of cells to synthesize citrate. Thus, the resulting oxaloacetate can be converted to malate, which, in turn, can be further oxidized by the malic enzyme (ME1) to pyruvate in a reaction that produces NADPH in a manner independent of glucose (Son et al., 2013). Finally, α-ketoglutarate may provide a source of citrate for the *de novo* lipogenesis in a setting in which hypoxia or certain oncogenic contexts create a deficit of glucose-derived acetyl-CoA. In a process referred to as reductive carboxylation, a section of the TCA cycle reverses itself, converting α-ketoglutarate into citrate, which can be then used to generate a source of cytosolic acetyl-CoA (Metallo et al., 2012; Wise et al., 2011).

Glutamine-derived α-ketoglutarate is not the only anaplerotic substrate that can be exploited by proliferating cells. In fact, tracing the fate of glucose-derived carbons in human patients and in mouse models of glioblastoma and non-small cell lung carcinoma (NSCLC) reveals that at least in these tumorigenic contexts, the principal source for the TCA cycle intermediates is not glutamine, but glucose (Maher et al., 2012; Marin-Valencia et al., 2012; Sellers et al., 2015). Anaplerotic entry of glucose-derived carbons into the TCA cycle takes place via carboxylation of pyruvate into the TCA cycle intermediate oxaloacetate, catalyzed by pyruvate carboxylase (PC). Indeed, survival of glutamine-deprivation-resistant derivatives of a glioblastoma cell line in culture was found to be dependent on pyruvate carboxylase (Cheng et al., 2011). Moreover, PC inhibition in NSCLC-derived cell lines suppresses fatty acid biosynthesis and dramatically reduces proliferation even in the presence of ample glutamine (Sellers et al., 2015). These reports highlight that the cell

lineage context, as well as potential secondary effects associated with the adaptation of cells to tissue culture conditions may play an important role in the choice of an anaplerotic substrate.

Finally, extracellular acetate has recently been shown to be utilized by some cancers as a source of acetyl-CoA for biosynthesis. Specifically, a variety of tumor types localized to the brain, including primary glioblastomas as well as brain metastases that originated from other tissues, have been shown to assimilate exogenous acetate and incorporate acetate-derived carbons into *de novo* synthesized fatty acids (Comerford et al., 2014; Mashimo et al., 2014; Schug et al., 2015). Imported acetate is converted to acetyl-CoA in a reaction catalyzed by acetyl-CoA synthetase 2 (ACSS2), which is a target for amplification in breast cancer. Thus, hypoxia-associated deficit of acetyl-CoA can be augmented not only through the reductive carboxylation of α-ketoglutarate, but also by increasing the utilization of free acetate from plasma and interstitial fluid. Furthermore, upregulation of ACSS2 may potentially allow cells to recycle acetate released from deacetylation of histones and other cellular proteins as well.

INCREASED DEMAND FOR NITROGEN

In addition to increasing the consumption of carbon in biosynthetic pathways, growth signaling concomitantly elevates the cellular demand for reduced nitrogen. Indeed, a proliferating cell must synthesize a number of nitrogen-containing molecules *de novo*, including nucleotides, non-essential amino acids and polyamines. As discussed above, the transcriptional effects of *c-myc* and E2F result in enhanced cellular uptake of glutamine. A non-essential amino acid containing two atoms of reduced nitrogen, glutamine serves as the principal way in which reduced nitrogen is transported between cells in metazoan organisms. The amide group of glutamine is an indispensable donor of nitrogen for the biosynthesis of purine and pyrimidine bases (Figure 5). Thus, one glutamine molecule is used in the production of uracil and thymine, cytosine and adenine each require two, and building a guanine base costs a cell three molecules of glutamine. In addition, the assembly of both pyrimidine and purine rings utilizes aspartate, which is derived from the transamination of the TCA cycle metabolite oxaloacetate and glutamic acid, both of which are catabolites of glutamine. Thus, glutamine is a key structural building block in the biosynthesis of nucleotides. Accordingly, glutamine levels have been shown to be rate-limiting for cell cycle progression, and glutamine deprivation leads to cell cycle arrest in the S-phase in certain cellular contexts (Fontenelle and Henderson, 1969; Gaglio et al., 2009).

The oncogene *c-myc* orchestrates nucleotide biosynthesis by upregulating the expression of a number of nucleotide biosynthesis enzymes. Among the *c-myc*-regulated targets are phosphoribosyl pyrophosphate synthetase 2 (PRPS2), which catalyzes a first step of purine biosynthesis, as well as carbamoyl phosphate synthetase II (CAD), which initiates the pyrimidine ring-building cascade (Cunningham et al., 2014; Eberhardy and Farnham, 2001; Mannava et al., 2008). Other targets of *c-myc* that are involved in nucleotide biosynthesis are thymidylate synthase (TS) and inosine monophosphate dehydrogenase 1 (IMPDH1) and 2 (IMPDH2) (Liu et al., 2008; Mannava et al., 2008). Thus, *c-myc* not only facilitates glutamine uptake, but also promotes its utilization in the biogenesis of purine and

pyrimidine bases. Besides the regulatory inputs from *c-myc*, mutant p53 alleles have also been shown to facilitate expression of nucleotide biosynthesis genes, among which are IMPDH1 and 2, GMP synthetase (GMPS) and nucleoside salvage enzymes deoxycytidine kinase (DCK) and thymidine kinase (TK1) (Kollareddy et al., 2015). Furthermore, activity of CAD enzyme is regulated via phosphorylation by MAPK and by mTORC1-dependent S6 kinase (Ben-Sahra et al., 2013; Graves et al., 2000; Robitaille et al., 2013). In this manner, mTORC1-driven CAD activation may enable the cell to regulate pyrimidine biogenesis in response to the levels of intracellular glutamine, as the latter contributes to mTORC1 activation (Duran et al., 2012; Jewell et al., 2015).

In addition to being utilized for nucleotide biosynthesis, glutamine can be directly deamidated to glutamate by glutaminase which is frequently upregulated in cancer cells in a *c-myc*-dependent manner. Glutamine-derived glutamate serves as a donor of nitrogen for the production of a number of non-essential amino acids via transamination. In contrast, biosynthesis of asparagine from aspartate, catalyzed by asparagine synthetase (ASNS), utilizes the amide nitrogen of glutamine. Though structurally similar to glutamine, asparagine is the only amino acid that mammalian cells do not catabolize. Yet despite this, asparagine plays a crucial regulatory role in conditions of glutamine deprivation (Zhang et al., 2014). Exactly how asparagine supports cell survival and adaptation to glutamine depletion remains to be fully elucidated. ASNS is frequently upregulated in tumors and is associated with poor prognosis (Sircar et al., 2012; Zhang et al., 2014). In contrast, acute lymphoblastic leukemia cells lack ASNS expression, leading to asparagine auxotrophy. Depletion of plasma asparagine by a recombinant form of bacterial L-Asparaginase is an effective anticancer intervention for this cancer type (Hill et al., 1967; Pieters et al., 2011).

Despite the existence of a glutamine biosynthetic pathway in mammalian cells, the majority of proliferating cells in culture require an exogenous supply of glutamine. Interestingly, some cell types, such as embryonic stem cells and luminal breast cancer cells, are capable of proliferating in the absence of glutamine in the culture medium, indicating that in some cellular contexts, glutamine can be produced *de novo* (Carey et al., 2015; Kung et al., 2011). In addition, human glioblastoma samples implanted directly into the brains of immunocompromised mice were found to accumulate glutamine relative to the normal brain tissue (Marin-Valencia et al., 2012); furthermore, a similar accumulation of glucose-derived glutamine within the tumor tissue was observed in a mouse model of c-Met-driven liver cancer (Yuneva et al., 2012). Glutamine synthetase (GS) has been found to be overexpressed in some cancers (Osada et al., 2000); yet its role and its mode of activation remain to be fully understood.

Arginine, despite being a non-essential amino acid, also becomes conditionally essential in some tumorigenic contexts (Figure 5). A carrier of four nitrogen atoms, arginine serves as a precursor for a wide variety of nitrogenous compounds, among which are polyamines, creatine, agmatine and pyrroline-5-carboxylate, a precursor for the biosynthesis of proline. The *de novo* biosynthesis of arginine is an integral part of the urea cycle, where it is derived from argininosuccinate in a cleavage reaction catalyzed by argininosuccinate lyase (ASL). Argininosuccinate, in turn, is produced by the enzyme argininosuccinate synthase (ASS1) from citrulline and aspartate. Intriguingly, both ASS1 and ASL enzymes are frequently

epigenetically silenced in melanoma, renal cell carcinoma and hepatocellular carcinoma (Bowles et al., 2008; Ensor et al., 2002; Yoon et al., 2007). Reduced expression of ASS1 and ASL are associated with poor prognosis and resistance to chemotherapy (McAlpine et al., 2014; Nicholson et al., 2009). Accordingly, the auxotrophy of tumors for arginine is being exploited in the development of anticancer therapies (Delage et al., 2010).

Why do tumors opt out of the *de novo* production of arginine and instead begin relying on its exogenous influx? One possibility is that the inactivation of ASL and ASS1 allows cancer cells to accumulate ornithine, which is then utilized in the production of polyamines, a class of nitrogen-containing polycationic aliphatic carbon molecules. Polyamine levels increase in proliferating cells; moreover, polyamines have been shown to inhibit apoptosis and promote tumor growth and invasion (Casero and Marton, 2007; Gerner and Meyskens, 2004). Another possible reason for arginine auxotrophy in cancer may be related to the fact that the suppression of ASS1-driven argininosuccinate production leads to an accumulation of its substrate aspartate, which can then be applied toward nucleotide biosynthesis.

In addition to its effects on glutamine metabolism, *c-myc*-orchestrated remodeling of non-essential amino acid metabolism involves the metabolism of proline (Phang et al., 2008). Proline can be produced either from glutamate or from arginine-derived ornithine via a common intermediate, pyrroline-5-carboxylate. The expression of the principal enzyme in proline biosynthesis, pyrroline-5-carboxylate reductase (PYCR1), is upregulated by *c-myc* (Liu et al., 2012). In fact, a meta-analysis of metabolic enzyme expression across diverse tumor types identified PYCR1 as one of the most commonly overexpressed genes in tumors (Nilsson et al., 2014). Reciprocally, proline oxidase (POX), which mediates proline degradation, is negatively controlled by *c-myc* via *miR-23b*, and is a p53 target as well (Donald et al., 2001; Liu et al., 2012). Expression of POX has been shown to inhibit tumor cell growth by triggering cell cycle arrest in the G2 phase (Liu et al., 2009). The contribution of altered proline metabolism to tumorigenesis remains to be elucidated. One explanation suggests that the elevated proline pool may facilitate production of collagen and new extracellular matrix deposition, facilitating tumor invasion. Taken together, while this area of cancer metabolism remains under active investigation, it is clear that similarly to carbon, the metabolism of nitrogen undergoes complex reprogramming in tumorigenesis.

ALTERATIONS IN METABOLITE-DRIVEN GENE REGULATION

Aberrantly activated growth and survival signals that drive tumorigenesis facilitate the reprogramming of cancer cell metabolism to enable increased nutrient acquisition and biosynthesis. However, metabolic networks themselves are not merely passive recipients of growth signals, but quite the contrary, directly transmit the information about the cellular metabolic state to a diverse array of regulatory enzymes, among which are those that mediate the deposition and removal of epigenetic marks from chromatin (Katada et al., 2012)(Figure 6).

A key metabolite that builds up when cells metabolize more glucose than needed for bioenergetic support is cytosolic acetyl-CoA. Cytosolic acetyl-CoA is the obligate substrate for enzymes that acetylate histones and other proteins. The deposition of acetyl marks on

histones is associated with the increased accessibility of the genomic DNA for the assembly of transcriptional complexes, and has a rapid turnover rate. Histone acetylation is exquisitely sensitive to alterations in the cellular nutritional and signaling status (Cai et al., 2011; Shi and Tu, 2013). Indeed, withdrawal and readdition of glucose, as well as activation of oncogenic signaling via introduction of an oncogenic KRAS mutant or a constitutively active form of Akt, increase total histone acetylation, which, in turn, promotes the enhanced and broader gene expression (Lee et al., 2014). Immunohistochemical analysis of glioblastoma and prostate tumor samples has shown that Akt activation levels closely correlate with global histone acetylation status (Lee et al., 2014). Activated Akt expands the extramitochondrial pool of acetyl-CoA by activating ACLY, which converts cytosolic citrate into acetyl-CoA and oxaloacetate. Furthermore, studies have shown that both the glucose- and oncogene-driven increases in global histone acetylation are blocked by ACLY inhibition (Lee et al., 2014; Wellen et al., 2009).

In addition to acetyl-CoA, a p300 histone acetyltransferase has recently been shown to utilize crotonyl-CoA as a substrate as well. Notably, deposition of crotonyl marks on select amino acid residues within histone tails was found to activate gene expression even more potently than acetyl marks (Sabari et al., 2015). Crotonyl-CoA can be produced from the catabolism of lysine and tryptophan, as well as of a short-chain fatty acid butyrate. In fact, a large variety of novel histone marks, such as formylation, propionylation, butyrylation, malonylation and succinylation have recently been identified by tandem mass spectrometry (Chen et al., 2007; Wisniewski et al., 2008; Xie et al., 2012). Further investigation of these marks has potential to dramatically expand the number of metabolic inputs into the regulation of global patterns of gene expression.

Numerous methylation reactions in the cell, including the deposition of methyl marks on histone tails, cytosine methylation on DNA and adenosine methylation on mRNA utilize S-adenosylmethionine (SAM) as a donor of methyl groups. SAM is a product of the one-carbon metabolic pathway and is fueled by serine catabolism as described above. A number of recent reports demonstrate that histone and DNA methylation is sensitive to alterations in SAM levels (Chiang et al., 2009; Shyh-Chang et al., 2013; Towbin et al., 2012).

The removal of acetyl and methyl marks is also guided by the cellular metabolic state. For instance, sirtuins, a class of deacetylases that catalyze the removal of acetyl marks from histone and non-histone proteins, utilize NAD^+ as a cofactor, while FAD serves as a cofactor for a lysine-specific demethylase LSD1 (Imai et al., 2000; Shi et al., 2004). Sensitive to changes in NAD^+ or FAD availability, these enzymes orchestrate global post-translational and epigenetic changes that promote energy conservation (Hino et al., 2012; Schwer and Verdin, 2008).

A wide variety of post-translational modifications in the cell are carried out by the members of a large class of α-ketoglutarate-dependent dioxygenases. Among α-ketoglutarate-dependent dioxygenases are a TET family of DNA demethylases, Jumonji C family of histone demethylases, mRNA demethylases FTO and ALKBH5, and a family of prolyl hydroxylase (PHD) enzymes, which, among other processes, regulate HIF1α levels in response to oxygen availability and oxidative stress. The reaction mechanism of α-

ketoglutarate-dependent dioxygenases involves a concurrent oxidation of a co-substrate α-ketoglutarate into succinate. Accordingly, intracellular levels of α-ketoglutarate can directly influence the activity of these enzymes. In addition, α-ketoglutarate-dependent dioxygenases are prone to the inhibition by their reaction product, succinate, as well as by fumarate, the downstream product of succinate degradation in the TCA cycle. For instance, a biallelic loss of succinate dehydrogenase (SDH), an alteration found in familial paragangliomas and pheochromocytomas, as well as in a subset of sporadic gastrointestinal stromal tumors, creates a build-up of succinate (Astuti et al., 2001; Janeway et al., 2011). Similarly, loss of a fumarate-metabolizing enzyme fumarate hydratase (FH) and accumulation of fumarate is found in the familial cancer syndrome HLRCC (hereditary leiomyomatosis and renal cell cancer) and in a subset of paragangliomas and pheochromocytomas as well (Tomlinson et al., 2002). Tumors that exhibit SDH- and FH loss share a number of phenotypic features that are consistent with dioxygenase inhibition, among which is a characteristic global increase in DNA methylation (Killian et al., 2013; Letouze et al., 2013; Xiao et al., 2012), as well as elevated levels of HIF1α (Selak et al., 2005). In fact, the latter, at least in the context of SDH loss, can be reversed by the addition of a membrane-permeable form of α-ketoglutarate (MacKenzie et al., 2007).

Another prominent class of tumor-associated genetic alterations that modulate the activity of α-ketoglutarate-dependent dioxygenases are gain-of-function mutations of isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2). IDH1 and IDH2 mutations have been identified in low-grade glioma, where they represent a likely initiator lesion, as well as in chondrosarcoma, cholangiocarcinoma, and acute myeloid leukemia (AML) (Balss et al., 2008; Borger et al., 2012; Cohen et al., 2013; Mardis et al., 2009; Parsons et al., 2008; Paschka et al., 2010). Mutant alleles of IDH1 and IDH2 exhibit an unusual neomorphic enzymatic function. In contrast to wild-type isocitrate dehydrogenases, which convert the TCA cycle metabolite isocitrate to α-ketoglutarate, a mutant form of IDH prefers to use α-ketoglutarate as a substrate, catalyzing its conversion to the D-enantiomer of 2-hydroxyglutarate (2-HG) (Dang et al., 2010; Ward et al., 2010). Owing to its structural similarity to α-ketoglutarate, 2-HG acts as a competitive inhibitor of α-ketoglutarate-dependent dioxygenases (Xu et al., 2011). Indeed, IDH-driven gliomas, leukemias and chondrosarcomas display a prominent CpG island hypermethylation, reminiscent of the hypermethylator phenotype seen in SDH- and FH-deficient cancers (Figueroa et al., 2010; Lu et al., 2013; Turcan et al., 2012). IDH mutations in AML are mutually exclusive with inactivating mutations in TET2 methylcytosine hydroxylases, further implicating mutant IDH as a *bona fide* driver of epigenetic remodeling (Figueroa et al., 2010). Furthermore, a knock-in of a mutant IDH1 allele into normal hematopoietic cells and into chondrocytes *in vivo* triggers an aberrant expansion of targeted cell lineages (Hirata et al., 2015; Sasaki et al., 2012). In various cellular settings, ectopic expression of a mutant IDH allele or treatment with exogenous D-2-HG is sufficient to induce DNA and histone hypermethylation and block cellular differentiation (Losman et al., 2013; Lu et al., 2012). Conversely, small molecule-mediated inhibition of mutant IDH1 in glioma and IDH2 in leukemia markedly promoted differentiation in these contexts (Rohle et al., 2013; Wang et al., 2013). The abundance of 2-HG has been utilized for ^{1}H magnetic resonance spectroscopy-based imaging of IDH-mutant gliomas (Choi et al., 2012). Moreover, small molecule inhibitors of

IDH1 and IDH2 have been reported to induce remissions in phase I clinical trials for treatment of AML patients with the respective mutations.

Notably, some tumor types display elevations in 2-HG even in the absence of IDH mutations. For instance, elevated 2-HG levels have been detected in triple-negative breast cancer (Terunuma et al., 2014). In addition, a specific increase in the L enantiomer of 2-HG, which was shown to exert similar, but not identical, inhibitory effects on α-ketoglutarate-dependent dioxygenases was found in clear cell renal cell carcinoma, where it correlates with increased methylcytosine levels (Shim et al., 2014). Furthermore, L-2-HG levels were found to increase in response to hypoxia as a result of promiscuous substrate use by LDH-A and malate dehydrogenase (MDH) (Intlekofer et al., 2015), and two recent studies implicate L-2-HG as a metabolic mediator of the cellular stress response to hypoxia (Intlekofer et al., 2015; Oldham et al., 2015).

METABOLIC INTERACTIONS WITH THE MICROENVIRONMENT

The information about a metabolic state of a cell affects not only its own long-term decision-making, but also has the potential to influence the fate of other cells in its vicinity. Indeed, a variety of genetically stable cell types, among which are tumor-associated fibroblasts, endothelial cells, as well as innate and adaptive immune system components, are known to undergo characteristic phenotypic changes as a consequence of residing in the vicinity of a growing tumor (Hanahan and Coussens, 2012). How cancer cells reprogram their microenvironment to assist tumor growth and dissemination is an area of intense investigation, but it is clear that such reprogramming encompasses multiple strategies, among which are secreted growth factors and alterations to the extracellular matrix and cell-cell interactions.

In addition to these inputs, proliferating cancer cells alter the metabolic composition of the extracellular milieu around them as well (Figure 7). The high utilization of extracellular glucose and glutamine by cancer cells results in the accumulation of extracellular lactate, which was shown to affect a number of cell types within the tumor microenvironment. Increased lactate levels promote the emergence of an immune-permissive microenvironment by attenuating dendritic and T cell activation and monocyte migration (Fischer et al., 2007; Goetze et al., 2011; Gottfried et al., 2006). In addition, lactate stimulates the polarization of resident macrophages to a so-called M2 state, which plays a role in immunosuppression and wound healing (Carmona-Fontaine et al., 2013; Colegio et al., 2014). Furthermore, lactate accumulation is instrumental for the promotion of angiogenesis. Thus, lactate promotes the stabilization of HIF1α and activates NF-κB and PI-3 kinase signaling in endothelial cells, as well as induces secretion of a pro-angiogenic factor VEGF from tumor-associated stromal cells (Constant et al., 2000; Schmid et al., 2007; Sonveaux et al., 2012; Vegran et al., 2011). Increased levels of lactate also stimulate hyaluronic acid production by fibroblasts, which may contribute to tumor invasiveness (Stern et al., 2002).

The secretion of lactate into the extracellular space via the monocarboxylate transporter MCT1 is coupled to the co-transport of H^+, which causes acidification of the cellular microenvironment. In addition, the surplus of CO_2 generated in mitochondrial

decarboxylation reactions contributes to the extracellular acidification as well. To this end, CO_2 diffuses into the extracellular space, where it becomes converted to H^+ and HCO_3^- by a class of extracellular carbonic anhydrases (Swietach et al., 2007). Expression of carbonic anhydrases, in particular the CAIX isoform, is elevated during hypoxia (Svastova et al., 2004). Increased extracellular acidification stimulates the proteolytic activity of matrix metalloproteinases (MMPs) and cathepsins, promoting the degradation of the extracellular matrix components and enhancing tumor invasion (Martinez-Zaguilan et al., 1996; Rothberg et al., 2013).

Whereas lactate accumulation and concomitant acidification of the extracellular space may be seen as collateral effects of cancer-specific metabolic reprogramming, some tumors employ a distinct strategy as means of promoting the emergence of an immune-permissive microenvironment around them. Specifically, numerous solid tumor types overexpress tryptophan-degrading dioxygenases indoleamine-2, 3-dioxygenase (IDO1) and tryptophan-2, 3-dioxygenase (TDO2), which catalyze the conversion of an essential amino acid, tryptophan, into its derivative, kynurenine (Munn and Mellor, 2007). As a consequence, tryptophan depletion triggers amino acid deprivation-associated apoptosis of effector T cells (Fallarino et al., 2002). Furthermore, accumulated kynurenine acts as a ligand for aryl hydrocarbon receptor (AhR) (Opitz et al., 2011). In a manner that is dependent on AhR, kynurenine promotes regulatory T-cell phenotype, further contributing to the suppression of antitumor immune responses (Fallarino et al., 2006). Finally, kynurenine potentiates autocrine signaling through AhR on cancer cells themselves, promoting the degradation of the extracellular matrix and invasion (Opitz et al., 2011). Small molecule inhibitors of IDO1 are currently being tested in clinical trials (Vacchelli et al., 2014).

Reciprocally, the conditions within the tumor microenvironment have profound effects on the metabolism of a cancer cell. As discussed earlier, tumors are often faced with nutrient- and oxygen-poor surroundings and develop various nutrient-scavenging strategies to bypass these limitations. Furthermore, hypoxia impedes the ability of cells to carry out oxidative phosphorylation and other reactions that require oxygen and disrupts the redox balance, affecting cellular signaling and transcriptional programs. Taken together, reciprocal interactions between cancer cells and their microenvironment impose a selective pressure that further shapes cancer cell metabolism and actively contributes to the emergence of a more aggressive state.

REMAINING QUESTIONS

Oncogene-driven metabolic reprogramming allows cancer cells to maintain deregulated proliferation, withstand metabolic challenges that are associated with oxygen and nutrient limitations, maintain a dedifferentiated state through alterations in global patterns of gene expression, and corrupt the surrounding microenvironment into actively assisting tumor growth and dissemination. While most of the studies to this point have been focused on alterations in the metabolism of glucose and glutamine, cancer cells utilize a great variety of other nutrients, among which are sulfur–containing amino acids cysteine and methionine,

essential fatty acids, choline, trace metals, and vitamins. We are only beginning to understand the extent to which these nutrients contribute to tumorigenesis.

For instance, multiple lines of evidence indicate that the metabolism of sulfur may undergo reprogramming in cancer. Thus, cancer cells upregulate the uptake of cysteine via the transcriptional upregulation of the xCT transporter, as well as secondary to the increased rate of glutamine utilization by cells (Lo et al., 2008). In fact, no less than 30% of imported glutamine was shown to leave the cell through the xCT transporter as glutamate, while being exchanged for cysteine (Collins et al., 1998). Consistent with its heavy utilization by cancer cells, cysteine was found to be the second most depleted amino acid in pancreatic tumors in comparison to normal pancreatic tissue (Kamphorst et al., 2015). Cysteine has several metabolic fates in proliferating cells, among which are the biosynthesis of glutathione and iron-sulfur clusters, as well as of hydrogen sulfide (H_2S), a gasotransmitter with complex and not yet fully defined functions in cellular physiology, among which are protection from oxidative stress, increased mitochondrial respiration, protection from apoptosis and facilitation of angiogenesis (Wu et al., 2015).

Alterations in serine metabolism of tumors have received considerable attention in the past four years. Notably, in addition to the *de novo*-produced serine, the inputs from the exogenous one-carbon donors may be affecting one-carbon metabolism and SAM levels as well. Indeed, dietary serine and glycine deficiency has been shown to impair xenograft growth of colon cancer cells in mice (Maddocks et al., 2013). Two other dietary compounds that can be utilized as donors of methyl groups for SAM production are choline and its derivative betaine. Increased choline uptake is observed in breast and prostate cancer; in fact, radioactive ^{18}F- and ^{11}C-choline is used in the clinic for PET-based tumor imaging (Krause et al., 2013; Kwee et al., 2007). High dietary intake of choline has been linked to a significantly increased risk of lethal prostate cancer (Richman et al., 2012). In contrast, a diet high in choline and betaine was associated with a decreased breast and lung cancer risk (Xu et al., 2009a; Ying et al., 2013; Zhang et al., 2013). Furthermore, diets deficient in choline and betaine were shown to directly affect histone and DNA methylation in a number animal models (Du et al., 2009; Mehedint et al., 2010; Niculescu et al., 2006), suggesting that alterations in exogenous methyl donor availability may have strong effects on cellular epigenetic state and thus contribute to tumorigenesis.

The effect of various micronutrients – namely, vitamins and trace metals – on tumor growth is a promising area of research. Micronutrients are utilized as cofactors for various enzymes and play complex and not yet fully defined roles in regulating cellular metabolic circuits, as well as signal transduction. For instance, ascorbic acid acts as a cofactor for the α-ketoglutarate-dependent dioxygenases. Notably, ascorbic acid treatment was shown to affect DNA methylation in embryonic stem cells and mouse embryonic fibroblasts in a manner that is dependent on TET2 (Blaschke et al., 2013; Minor et al., 2013). As many cancers are characterized by increased methylation of CpG islands, alterations in ascorbic acid import may thus affect their epigenetic state as well. Furthermore, levels of select trace metals, such as zinc and copper, are altered in cancer cells compared to the normal tissue. Copper levels in particular can be elevated as much as 2–3 fold in breast and ovarian tumors, as well as in leukemia (Gupte and Mumper, 2009). Interestingly, copper was recently shown to be

required for $BRAF^{V600E}$-driven ERK activation in melanoma cells, and the effect of copper depletion could be rescued by the constitutive activation of ERK (Brady et al., 2014).

Finally, the contribution of a broad spectrum of metabolites produced by the body's "forgotten organ", i.e., microbiota, to tumor initiation and progression is only beginning to be elucidated (Garrett, 2015). Taken together, the concept of metabolic alterations of cancer cells, first called to attention nearly a century ago, continues to uncover new connections between nutrient utilization and tumorigenic state. Furthermore, the study of cancer metabolism is not only shedding light on carcinogenesis but is also revealing new principles of how the biochemistry of anabolic metabolism is orchestrated to support normal cell growth, proliferation, and differentiation.

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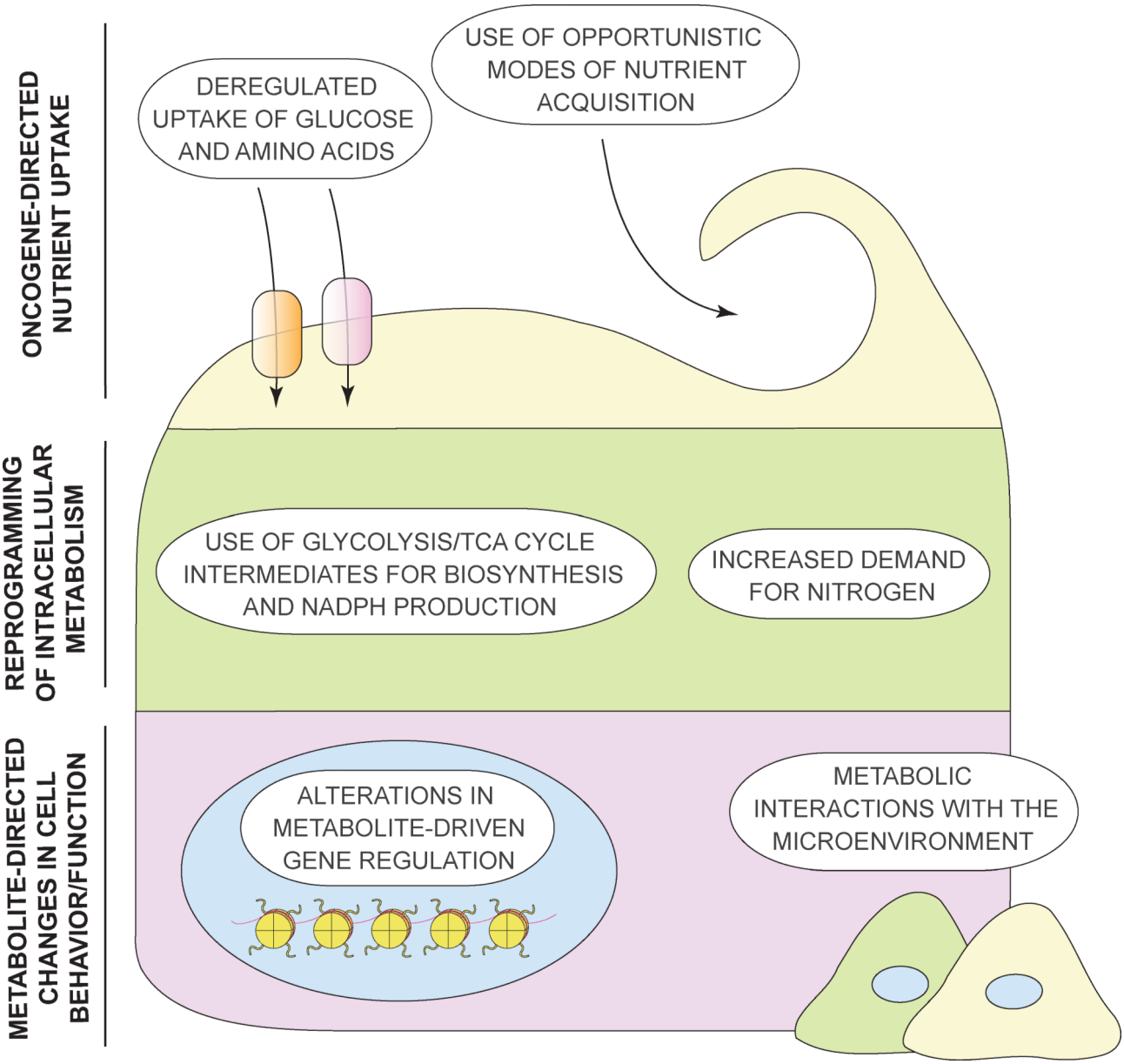

Figure 1. The emerging hallmarks of cancer metabolism

Cancer cells accumulate metabolic alterations that allow them to gain access to conventional nutrient sources as well as to unconventional nutrient sources, utilize these nutrients towards the creation of new biomass to sustain deregulated proliferation, and take advantage of the ability of select metabolites to affect the fate of cancer cells themselves as well as a variety of normal cell types within the tumor microenvironment. Three layers of cell-metabolite interaction are depicted, all of which become reprogrammed in cancer. On top are the adaptations that involve nutrient uptake (Hallmarks 1 and 2), followed by alterations to intracellular metabolic pathways (Hallmarks 3 and 4) in the middle. Finally, long-ranging effects of metabolic reprogramming on the cancer cell itself (Hallmark 5), as well as on other cells within its microenvironment (Hallmark 6) are depicted at the bottom.

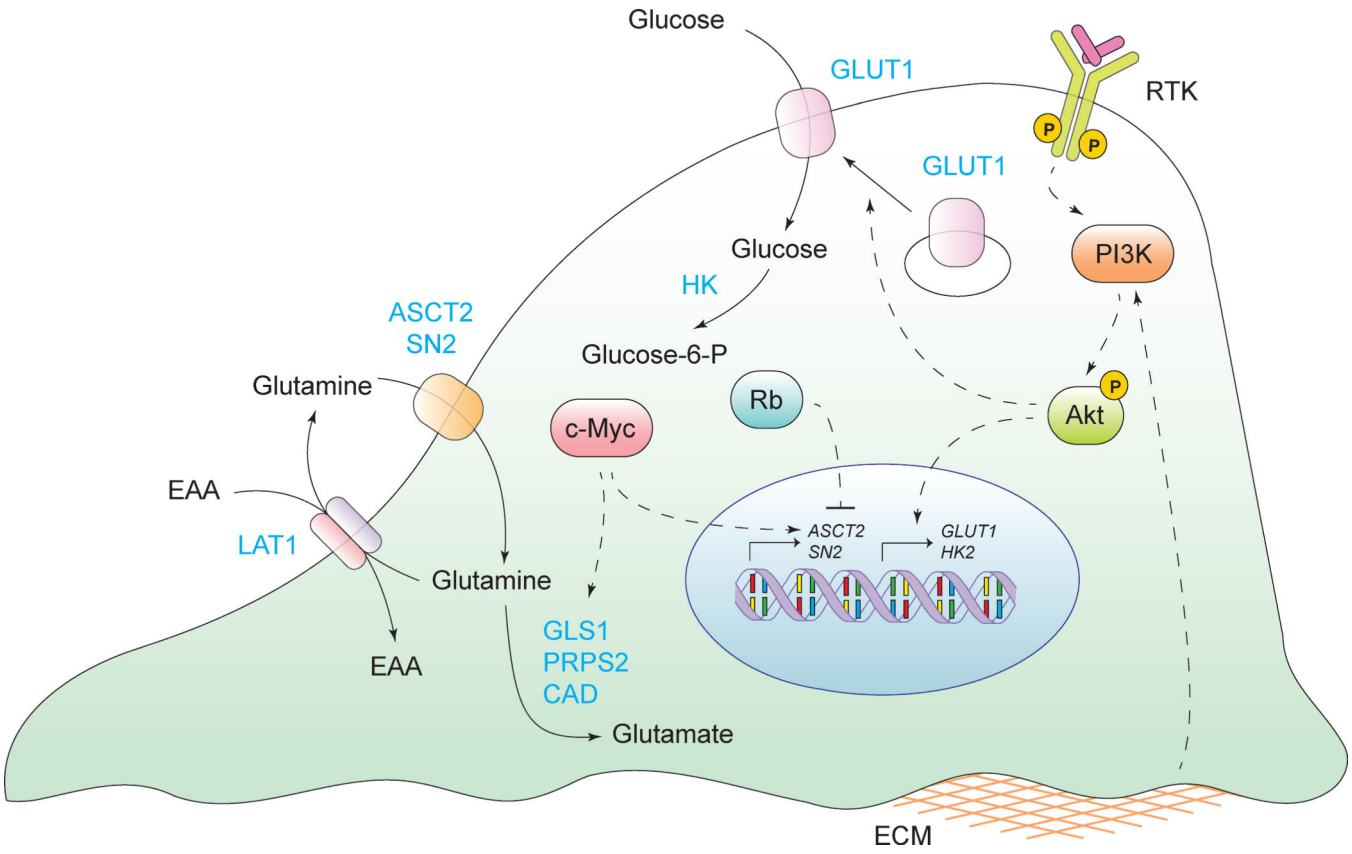

Figure 2. Deregulated uptake of glucose and amino acids

Aberrantly activated oncogenes and loss of tumor suppressors deregulates the import of glucose and amino acids into cancer cells. Solid arrows depict the movement of metabolites or proteins and metabolic reactions. Dashed arrows depict positive and negative regulatory effects of signal transduction components. RTK, receptor tyrosine kinase; GLUT1, glucose transporter 1; ASCT2/SN2, glutamine transporter; LAT1, neutral amino acid transporter; EAA, essential amino acids; GLS1, glutaminase 1; PRPS2, phosphoribosyl pyrophosphate synthetase 2; CAD, carbamoyl-phosphate synthetase 2; HK, hexokinase; ECM, extracellular matrix.

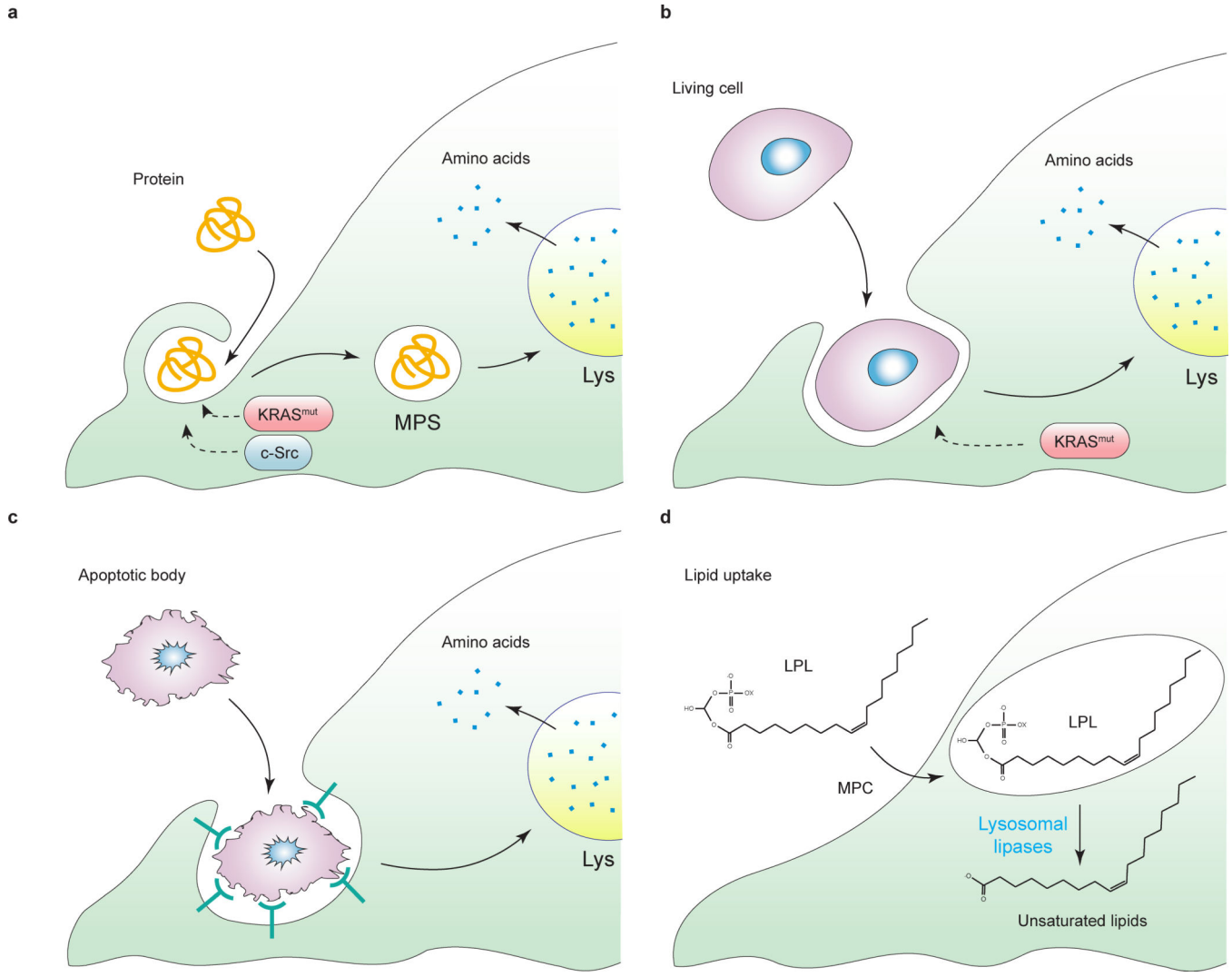

Figure 3. Use of opportunistic modes of nutrient acquisition

When free amino acids are unavailable, cancer cells may recover amino acids from **(a)** extracellular proteins via macropinocytosis. MPS, macropinosome, Lys, lysosome, **(b)** entosis of living cells, or **(c)** phagocytosis of apoptotic bodies. **d,** in conditions of oxygen deficiency, which prevents the *de novo* desaturation of stearate (C18:0) into oleate (C18:1), monounsaturated fatty acids such as oleate (C18:1) can be recovered from extracellular lysophospholipids (LPL). MPC, macropinocytosis; SCD1, stearoyl-CoA desaturase 1. X represents a lipid head group.

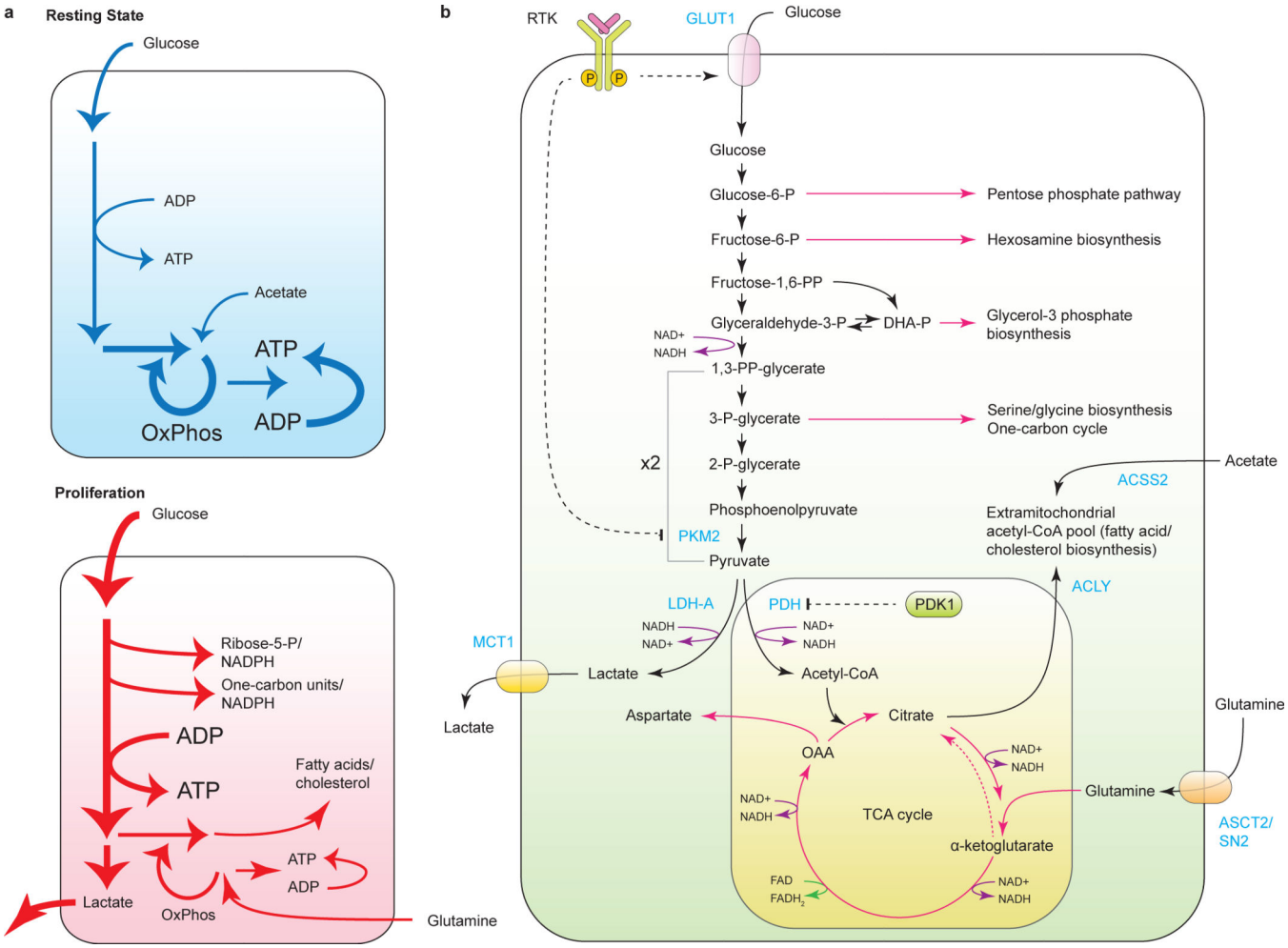

Figure 4. Use of glycolysis/TCA cycle intermediates for biosynthesis and NADH production

a, differences in the central carbon metabolism of a cell in a quiescent state compared to a proliferating cell; **b,** diverse biosynthetic outputs of central carbon metabolism. RTK, receptor tyrosine kinase; GLUT1, glucose transporter 1; PKM2, pyruvate kinase M2; ACSS2, acetyl-CoA synthetase 2; LDH-A, lactate dehydrogenase A; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1; ACLY, ATP-citrate lyase; MCT1, monocarboxylate transporter 1; ASCT2/SN2, glutamine transporter.

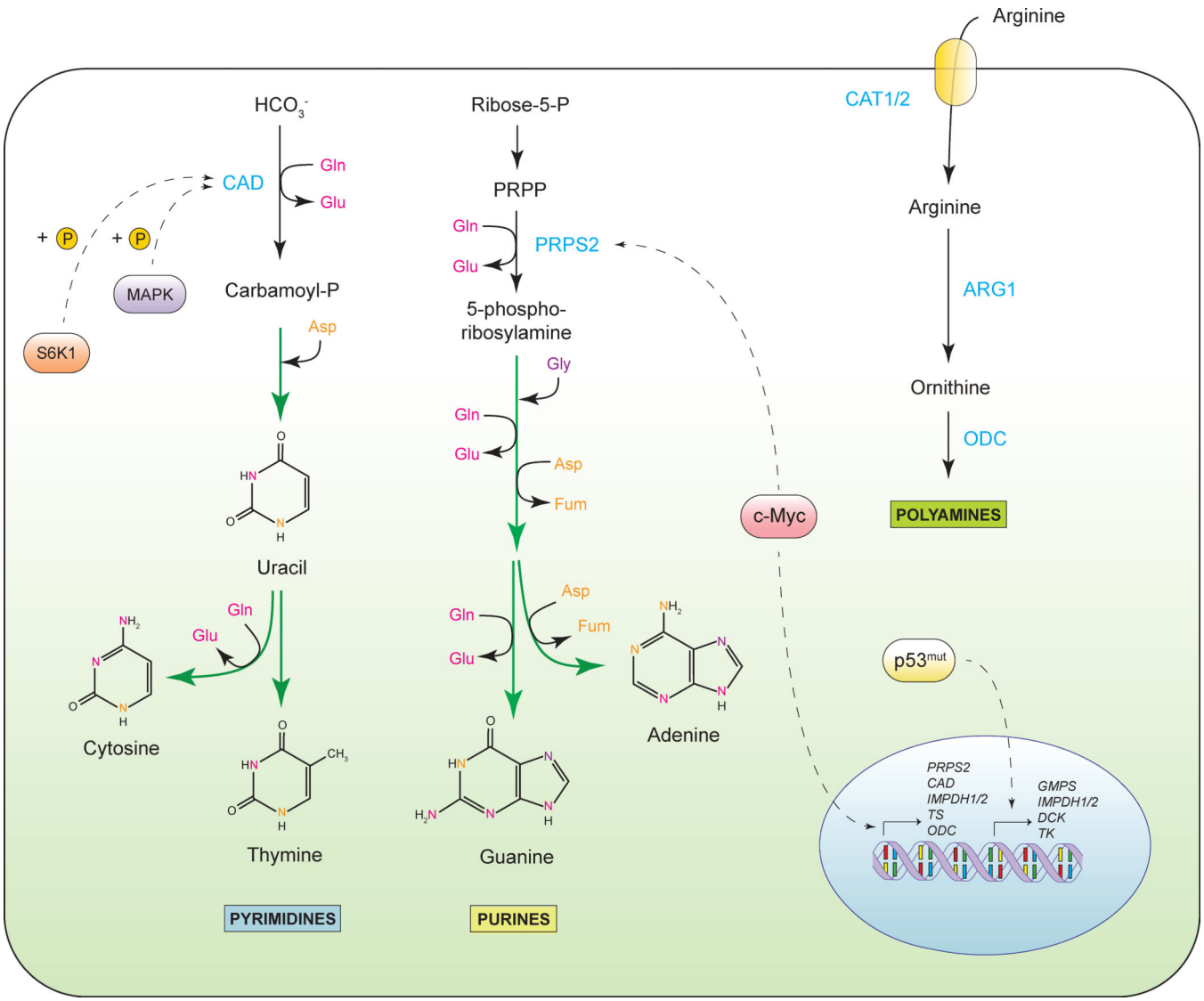

Figure 5. Increased demand for nitrogen

Sources of nitrogen atoms and regulation of nucleotide and polyamine biosynthesis. PRPP, phosphoribosyl pyrophosphate; PRPS2, phosphoribosyl pyrophosphate synthetase 2; CAD, carbamoyl-phosphate synthetase 2; Gln, glutamine, Glu, glutamate; Gly, glycine; Asp, aspartate; Fum, fumarate; IMPDH1/2, inosine-5-monophosphate dehydrogenase; TS, thymidylate synthase; GMPS, guanosine monophosphate synthetase; DCK, deoxycytidine kinase; TK, thymidine kinase, ARG1, arginase 1; ODC, ornithine decarboxylase; CAT1/2, cationic amino acid transporter.

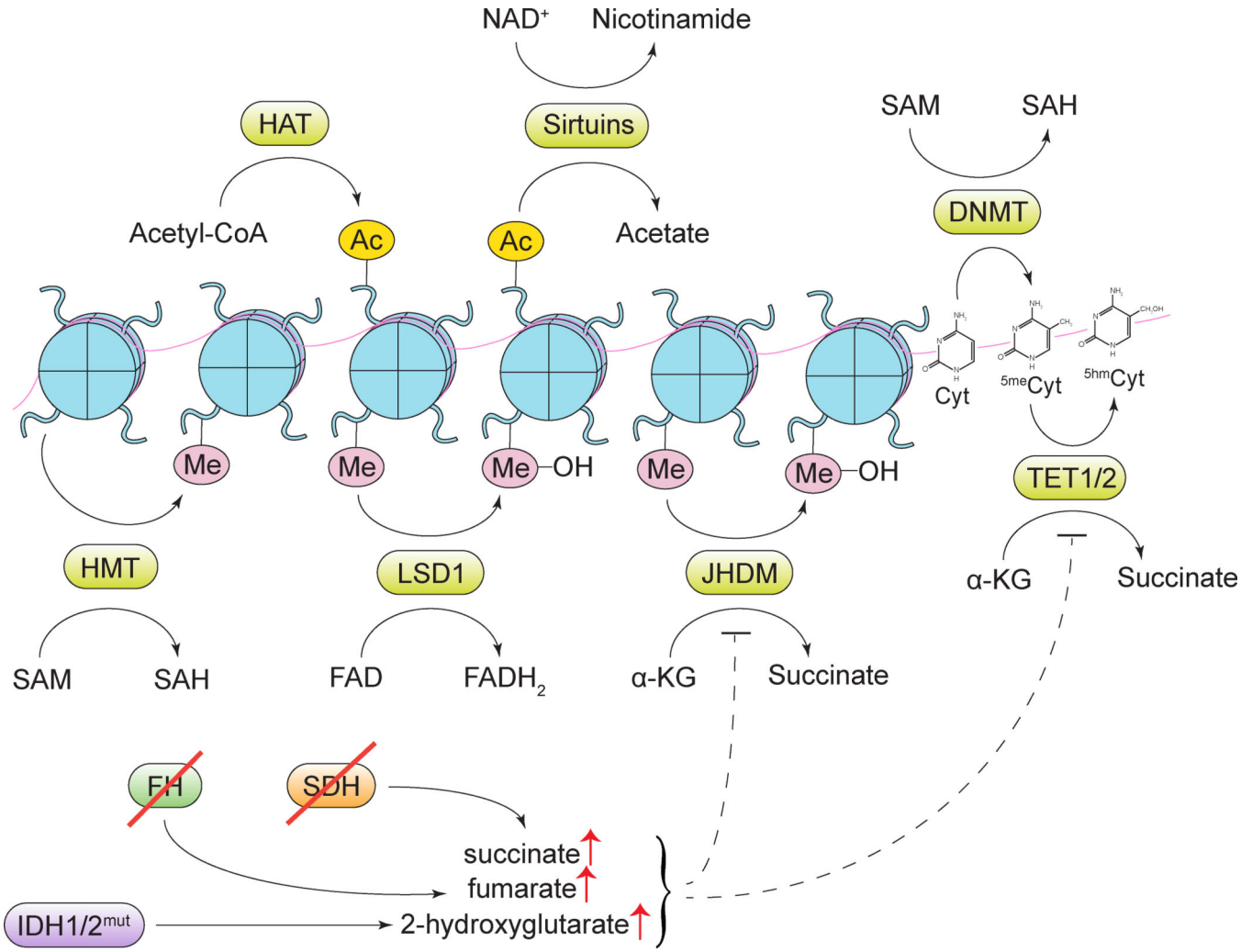

Figure 6. Alterations in metabolite-driven gene regulation

Diverse metabolites serve as cofactors or substrates for enzymes involved in deposition and removal of epigenetic marks. HAT, histone acetyltransferase enzymes; Ac, an acetyl mark; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DNMT, DNA methyltransferase enzymes; HMT, histone methyltransferase enzymes; Me, a methyl mark; LSD1, lysine-specific histone demethylase 1; JHDM, Jumonji domain-containing histone demethylase enzymes; Cyt, cytosine; ^{5me}Cyt, 5-methylcytosine; ^{5hm}Cyt, 5-hydroxymethylcytosine; TET1/2, ten-eleven translocation methylcytosine dioxygenase 1/2; α-KG, α-ketoglutarate; SDH, succinate dehydrogenase; FH, fumarate hydratase; IDH1/2, isocitrate dehydrogenase 1/2.

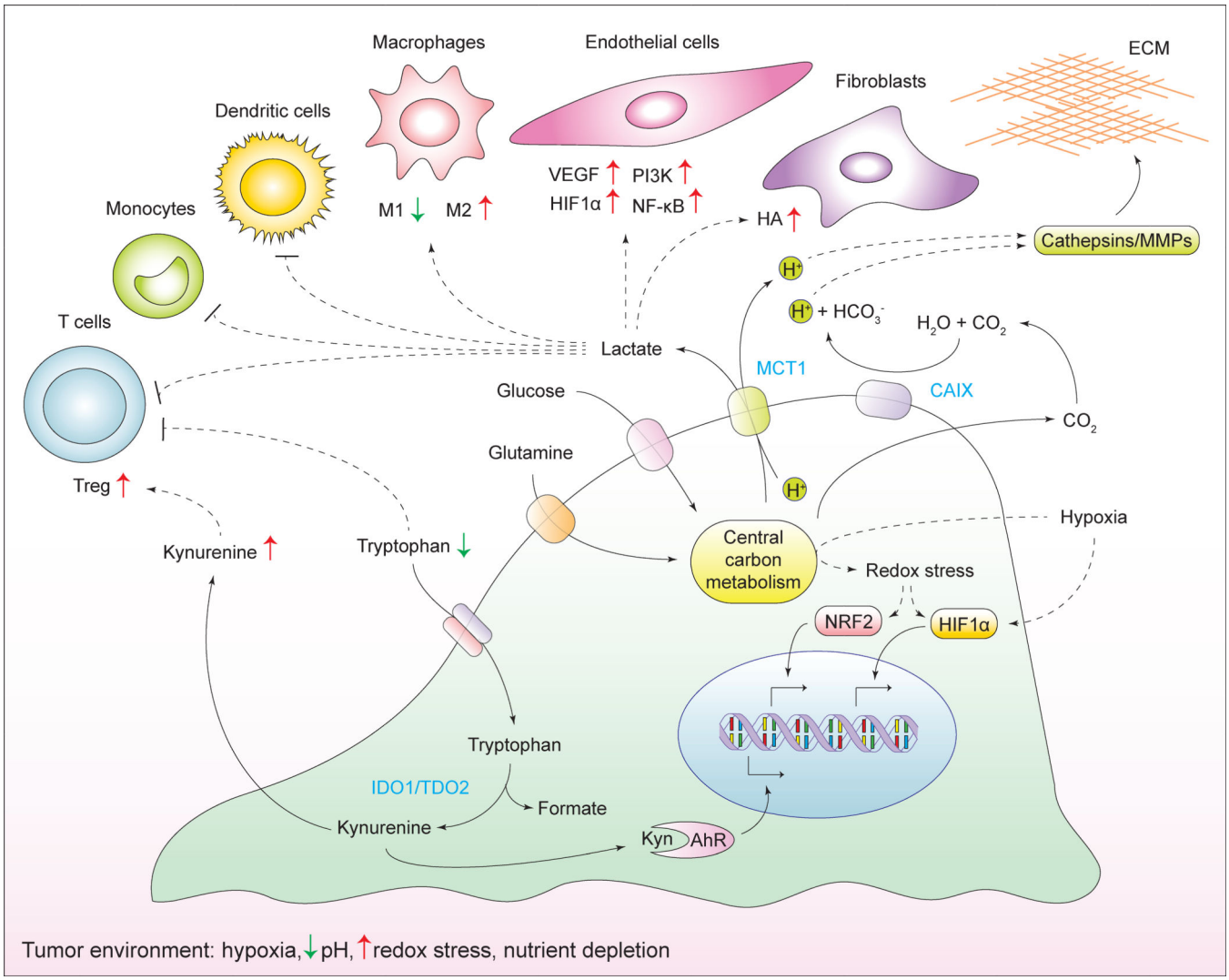

Figure 7. Metabolic interactions with the microenvironment

Cancer cells alter the chemical composition of the extracellular milieu, which exerts pleiotropic effects on the phenotypes of normal cells that reside in the vicinity of the tumor, as well as the extracellular matrix. Reciprocally, the microenvironment affects the metabolism and signaling responses of cancer cells themselves. ECM, extracellular matrix; Treg, regulatory T cells; HA, hyaluronic acid; MMPs, matrix metalloproteinases; MCT1, monocarboxylate transporter 1; CAIX, carbonic anhydrase IX; IDO1, indoleamine-2, 3-dioxygenase 1; TDO2, tryptophan-2, 3-dioxygenase 2; Kyn, kynurenine; AhR, aryl -hydrocarbon receptor.