

Revisiting the TCA cycle: signaling to tumor formation

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A role for mitochondria in tumor formation is suggested by mutations in enzymes of the TCA cycle: isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH) and fumarate hydratase (FH). Although they are all components of the TCA cycle, the resulting clinical presentations do not overlap. Activation of the hypoxia pathway can explain SDH phenotypes, but recent data suggest that FH and IDH mutations lead to tumor formation by repressing cellular differentiation. In this review, we discuss recent findings in the context of both mitochondrial and cytoplasmic components of the TCA cycle, and we propose that extrametabolic roles of TCA cycle metabolites result in reduced cellular differentiation. Furthermore, activation of the pseudohypoxia pathway likely promotes the growth of these neoplasias into tumors.

Fundamentals of the TCA cycle

The TCA cycle is a central pathway in the metabolism of sugars, lipids and amino acids [1]. The original investigations of the TCA cycle took place in the context of oxidative metabolism, and it is often presented in a simplistic perspective of a cyclic pathway constantly oxidizing the acetyl moiety of acetyl-CoA to CO₂, generating NADH and FADH₂, which feed electrons to the respiratory chain (Figure 1 and Box 1). The work that defined the TCA cycle was performed using whole tissue homogenates. When differential centrifugation techniques allowed the preparation of pure mitochondria, it became evident that although mitochondria were necessary and sufficient to perform the cycle, isoforms of some of the enzymes were also found in the cytoplasm not associated with mitochondria (Table 1).

The presence of these activities in the cytoplasm, as well as sophisticated shunts within the cycle, suggest another layer of complexity to this pathway. In addition, the cycle is not a closed pathway but rather integrates several metabolic pathways in the cell, such as the metabolism of amino acids, fatty acids and heme [1]. The cytoplasmic and mitochondrial pools of TCA cycle intermediates are in tight connection, and accumulation of a metabolite in one of these pools is reflected in the other. The metabolites can freely diffuse through channels in the outer mitochondrial membrane and can be transported across the inner mitochondrial membrane through active carriers (e.g. the tri-

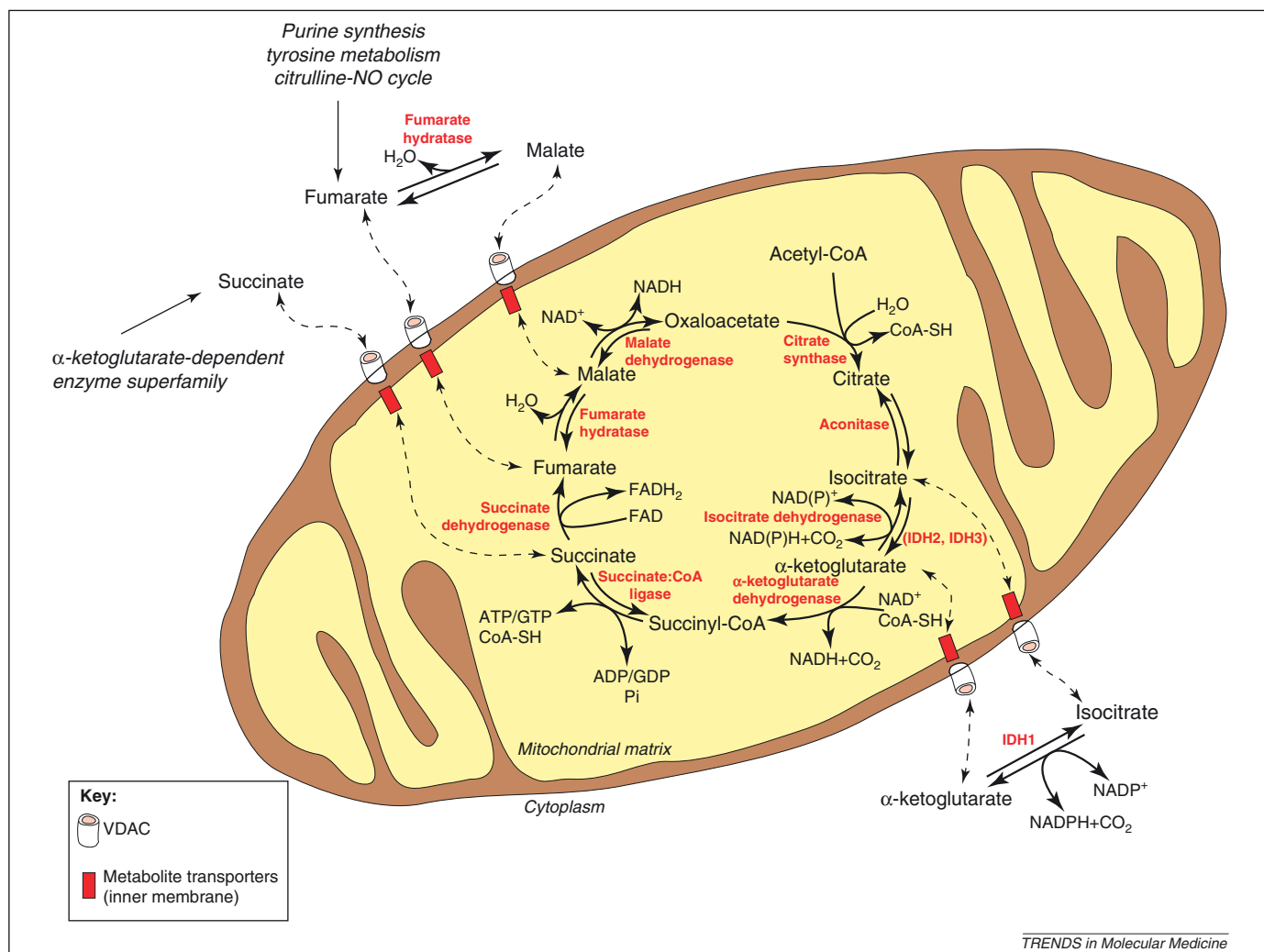
carboxylate carrier, the dicarboxylate carrier and several antiporters) [1].

The extraordinary complexity in terms of shunts, anaplerotic reactions (reactions that replenish the TCA cycle metabolite pools, such as generation of oxaloacetate from pyruvate) and the dual compartmentalization of reactions and metabolites suggests that the TCA cycle can operate in a cell-specific manner and respond to environmental and developmental cues within the same cell type, depending, for example on the expression levels of the different enzyme isoforms and whether circumstances require anabolism or catabolism. The TCA cycle occurs in multiple flavors beyond the formulation by Hans Krebs; there are many different 'TCA cycles' ongoing in the human body at any given moment.

In this review, the molecular mechanisms linking TCA cycle defects and tumor formation will be discussed. In particular, the dual compartmentalization of the TCA cycle between mitochondria and the cytoplasm, how mutations in TCA cycle enzymes have implications for tumor promotion by activating the hypoxia response pathway and the role of TCA cycle metabolites in the regulation of cellular differentiation will be reviewed in detail.

Genetic defects in the TCA cycle

Genetic defects affecting TCA cycle proteins have been known for more than 20 years. Until recently, only recessive defects were known, and their clinical outcomes were generically similar to defects in the respiratory chain and oxidative phosphorylation, consisting of severe multisystem disorders but no tumor predisposition [2]. Mutations in mitochondrial DNA (mtDNA) cause defects in the respiratory chain and oxidative phosphorylation, but not in the TCA cycle. Several mtDNA mutations have been observed in association with both primary tumors and metastasis. However, it remains unclear if those mtDNA mutations were the primary cause of the tumors or if they conferred growth advantage to tumors already formed. Importantly, over 250 known pathological germline mtDNA mutations in patients with metabolic diseases do not appear to predispose to tumor formation. The only respiratory chain defect that is known to lead to the formation of tumors is MERRF (myoclonic epilepsy and ragged-red fibers); patients with MERRF have presented with lipomas [3]. In contrast to the germline mtDNA mutations, a vast literature supports the role of somatic mtDNA mutations in increasing the metastatic potential and aggressiveness



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Figure 1. The TCA cycle. Shown here are the core mitochondrial components and some of the cytoplasmic enzymes that catalyze steps in the TCA cycle. The cytoplasmic and mitochondrial transamination reactions involving TCA cycle metabolites were omitted for simplicity. The transport of the metabolites across the inner membrane is catalyzed by a number of carriers and antiporters, and the metabolites cross the outer membrane by diffusing through channels such as VDAC (voltage-dependent anion channel). The metabolites are shown in black, the enzymes are shown in red and the pathways in italic font. Full arrows represent the direction of a reaction and intermittent arrows represent the translocation of metabolites between mitochondria and cytoplasm. Abbreviations: IDH, isocitrate dehydrogenase; VDAC, voltage-dependent anion channel.

of tumors [4], highlighting that defects in mitochondrial metabolism are probably lethal in early stages of development, whereas developed tissues are able to cope and adapt.

Recently, dominant defects associated with tumor formation were described in three enzymes involved in the TCA cycle: isocitrate dehydrogenase (IDH), succinate dehydrogenase (SDH) and fumarate hydratase (FH) [5–7]. This review focuses on the mechanisms that link these defects with tumor formation.

Isocitrate dehydrogenase

NAD⁺-IDH is monomeric in the cytoplasm and dimeric in mitochondria, whereas NAD⁺-IDH occurs in a complex of subunits of three different types, α , β and γ , in the stoichiometry 2 α :1 β :1 γ [1]. Mutations in the NAD⁺-dependent IDH subunits IDH1 and IDH2 have been found in benign as well as advanced stage astrocytomas and oligodendromas [7]. These mutations were also present in a small fraction of acute myeloid leukemia patients [8]. In contrast to the heritable tumor suppressor nature of the dominant SDH and FH defects, the

IDH somatic mutations do not involve a loss of heterozygosity and behave like oncogenic gain-of-function mutations [9,10]. Interestingly, identical germline mutations in IDH have been found in patients with D-2-hydroxyglutaric aciduria, for which no evidence of tumor formation has been reported [11]. This suggests that the IDH mutations confer a growth advantage not in the initial stages of tumorigenesis but later during tumor progression.

The neomorphic activity of mutant IDH1 and IDH2 converts α -ketoglutarate to 2-hydroxyglutarate, thus depleting α -ketoglutarate. The decrease in α -ketoglutarate depletes the rest of the cycle (succinate, fumarate, malate), regardless of whether the mutation is in the cytoplasmic or the mitochondrial enzyme [12]. The perturbation of TCA cycle function by mutant IDH1/2 also underlies major increases in amino acid levels, enabling anabolism [12]. Interestingly, recessive mutations in the β subunit of IDH3, the mitochondrial NAD⁺-dependent enzyme that functions in the traditional formulation of the TCA cycle, are loss-of-function mutations that but do not predispose to tumor formation (Table 1).

Box 1. Traditional formulation of the TCA cycle

The TCA cycle [1] can be divided into two stages: decarboxylating, in which citrate (six carbon atoms) is converted to succinyl-CoA (four carbons) releasing two CO₂ molecules; and reductive, the successive oxidations of succinate to fumarate, fumarate to malate, and malate to oxaloacetate (Figure 1).

The 'first' reaction of the cycle is the condensation of acetyl-CoA with oxaloacetate to form citrate, catalyzed by citrate synthase. Citrate can be exported to the cytoplasm and serve as a precursor for lipid synthesis or remain in the mitochondria, where it is converted to isocitrate by aconitase. Aconitase contains a nonheme 4Fe-4S cluster and has one cytoplasmic isoform, which also functions as an iron regulatory protein.

The conversion of isocitrate in α -ketoglutarate is the first oxidative decarboxylation of the cycle. This reaction is catalyzed by isocitrate dehydrogenase, which occurs in three forms: NAD⁺-dependent and localized to mitochondria (IDH3), as well as NADP⁺-dependent and localized to either mitochondria (IDH2) or the cytoplasm (IDH1).

The α -ketoglutarate dehydrogenase complex (α -kgDHC) catalyzes the conversion of α -ketoglutarate to succinyl-CoA and CO₂. α -Ketoglutarate is a substrate for the α -ketoglutarate-dependent dioxygenase superfamily, which includes the PHDs that regulate the α subunits of hypoxia-inducible factors.

Succinyl-CoA is the precursor for heme synthesis in animals. If metabolized within the citrate cycle, succinyl-CoA generates succinate and GTP or ATP. The reaction is catalyzed by succinate:CoA ligase (SUCL), which is a dimer of the α subunit (SUCLG1) and one of the β subunits, either ATP-forming (SUCLA2) or GTP-forming (SUCLG2).

Succinate is oxidized to fumarate by succinate dehydrogenase (SDH), composed of four subunits termed A–D (SDHA–D). The SDH reaction is part of both the citrate cycle and the respiratory chain, where SDH is referred as complex II. All other oxidative steps of the cycle generate NADH to feed complex I of the respiratory chain, whereas the electrons removed from succinate are channeled through FAD to ubiquinone. Succinate is also product of the reactions catalyzed by the α -ketoglutarate-dependent dioxygenase superfamily and can inhibit those reactions.

Fumarate hydratase (FH) catalyzes the hydration of the double bond in fumarate, generating malate. In most tissues, FH is found both in the cytoplasm and in mitochondria.

The 'last' reaction of the cycle recycles oxaloacetate from malate. This reaction is catalyzed by malate dehydrogenase (MDH) and couples the oxidation of malate with the reduction of NAD⁺. MDH is localized to both mitochondria and the cytoplasm.

Succinate dehydrogenase

The SDH complex is a heterotetramer of the SDHA, SDHB, SDHC and SDHD subunits. The recently discovered SDH5 is a factor required for the flavination of SDHA and assembly of the tetramer [13]. Dominant mutations in the SDH subunits SDHB, SDHC and SDHD predispose carriers to carotid body paragangliomas and adrenal gland pheochromocytomas [5,14–16]. More recently, dominant defects in SDH5 were also found to predispose individuals to paragangliomas [13]. A germline SDHA mutation has also been found in a patient presenting with paraganglioma [17]. Finally, mutations in SDHB are associated with renal cell carcinoma and T cell acute leukemia [18,19], and mutations in SDHB, SDHC and SDHD are associated with gastrointestinal stromal tumors [20]. It is presently unclear why certain individuals with SDH mutations develop these secondary tumors.

The majority of SDHD defects are nonsense mutations, whereas in SDHB there is a similar proportion of missense and nonsense mutations [21]. Interestingly, SDH mutations often cause carotid body paraganglioma, which has an increased incidence in high-altitude dwellers, suggesting that SDH mutations mimic the biological pathways triggered by environmental hypoxia. Although SDHB, SDHC and SDHD are physically interacting structural subunits of SDH, there are certain differences among the phenotypes arising from germline subunit mutations. Whereas germline mutations in SDHB associate with extra-adrenal and malignant paragangliomas, those in SDHD cause mostly benign head and neck paragangliomas [21]. Notably, SDHD and SDH5 (SDHAF2) mutations show a puzzling pattern of predisposition: maternally inherited mutations are at no risk of developing tumors, whereas the paternally inherited ones carry an age-dependent risk [5,13]. The mechanism underlying this parent-of-origin effect remains unclear, but probably involves epigenetic regulation (i.e. imprinting) that might be tissue-specific.

Fumarate hydratase

Active FH is a homotetramer [1]. Recessive mutations in FH cause severe encephalopathies and early death, whereas dominant FH mutations predispose to tumor formation [6]. The recessive and dominant mutations are equally distributed along the FH gene [22]. FH behaves as a tumor suppressor, with the normal allele being lost in the tumors due to loss of heterozygosity [23]: the parents of FH recessive patients (defect in both alleles, either homozygous or compound heterozygous) have a dominant defect (heterozygous mutation; tumors develop when the normal FH allele is lost) and often present with tumors [22]. Dominant FH defects predispose to multiple cutaneous and uterine leiomyomas (MCUL), as well as to hereditary leiomyomatosis and renal cell cancer (HLRCC) [6]. Whereas the skin and uterine leiomyomas are benign, the kidney tumors in HLRCC are particularly aggressive [24]. The morphologic spectrum of kidney tumors in HLRCC patients is broad, including papillary type II, tubulo-papillary, tubular, collecting duct and clear cell carcinoma [25–28]. Clinical presentations with minor penetrance in patients with dominant FH mutations include Leydig cell tumors, ovary cystadenomas, cerebral cavernomas, uterine leiomyosarcomas and breast cancer [29].

The majority of defects in FH are missense mutations, with the next most frequent being frameshift and nonsense mutations [22]. Large-scale FH deletions also occur [6].

The cytoplasmic and mitochondrial FH isoforms are encoded by the same gene [30]. Cytoplasmic FH exists in all tissues except the brain [30]. In cells from patients with FH mutations, mitochondrial FH is present at normal levels, although with markedly reduced activity, whereas no FH is detected in the cytoplasm [30]. FH mutant cells are thus null for cytoplasmic FH activity, implying that the tumor suppressor role is associated with the cytoplasmic FH isoform. Accordingly, overexpression of cytoplasmic FH in FH^{−/−} mouse embryonic fibroblasts (MEFs) rescued most of the fumarate accumulation [31]. Because fumarate is generated in the cytoplasm by several pathways, it is

Table 1. Compartmentalization of the TCA cycle enzymes and diseases arising from their malfunction

Activity		Enzymes	Localization	Mutation	Diseases	Refs.
Citrate synthase		CS	Mitochondria			
Citrate lyase		ACLY	Cytoplasm			
Aconitase		ACO1	Cytoplasm			
		ACO2	Mitochondria			
Isocitrate dehydrogenase		IDH1 (NADP ⁺ -dependent)	Cytoplasm	Dominant (neomorphic activity)	Gliomas, acute myeloid leukemia	[7,9]
		IDH2 (NADP ⁺ -dependent)	Mitochondria	Dominant (neomorphic activity)	Gliomas, acute myeloid leukemia	[7,9]
		IDH3 α subunit (NAD ⁺ -dependent)	Mitochondria			
		IDH3 β subunit (NAD ⁺ -dependent)	Mitochondria			
		IDH3 γ subunit (NAD ⁺ -dependent)	Mitochondria	Recessive	Retinitis pigmentosa	[60]
α -Ketoglutarate dehydrogenase	α -Ketoglutarate decarboxylase	OGDH	Mitochondria			
	Dihydrolipoyl succinyltransferase	DLST	Mitochondria			
	Dihydrolipoyl dehydrogenase	DLD	Mitochondria	Recessive	Encephalopathy	[61]
Succinate:CoA ligase		SUCLG1	Mitochondria	Recessive	Hepatoencephalomyopathy	[62]
		SUCLA2	Mitochondria	Recessive	Hepatoencephalomyopathy	[62]
		SUCLG2	Mitochondria			
Succinate dehydrogenase		SDHA	Mitochondria	Recessive	Encephalomyopathy	[63]
				Dominant	Gastrointestinal stromal tumors, pheochromocytomas, paragangliomas	[5,16,64]
		SDHB	Mitochondria	Dominant	Gastrointestinal stromal tumors, pheochromocytomas, paragangliomas	[5,16,64]
		SDHC	Mitochondria	Dominant	Gastrointestinal stromal tumors, pheochromocytomas, paragangliomas	[5,16,64]
		SDHD	Mitochondria	Dominant	Pheochromocytomas, paragangliomas	[5,16]
Succinyl-CoA-3-oxoacid-CoA transferase		SCOT	Mitochondria	Recessive	Ketoacidosis	[65]
Fumarate hydratase		FH	Mitochondria cytoplasm	Recessive	Encephalopathy	[66]
				Dominant	Leiomyomas, renal cell cancer, Leydig cell tumors, ovary cystadenomas, cerebral cavernomas, uterine leiomyosarcomas and breast cancer	[21]
Malate dehydrogenase		MDH1 (NAD ⁺ -dependent)	Cytoplasm			
		MDH2 (NAD ⁺ -dependent)	Mitochondria			
Malic enzyme		ME1 (NADP ⁺ -dependent)	Cytoplasm			
		ME2 (NAD ⁺ -dependent)	Mitochondria	Recessive	Idiopathic generalized epilepsy	[67]
		ME3 (NADP ⁺ -dependent)	Mitochondria			
Glutamate-oxaloacetate transaminase		GOT1	Cytoplasm			
		GOT2	Mitochondria			
Glutamate-pyruvate transaminase		GPT1	Cytoplasm			
		GPT2	Mitochondria			

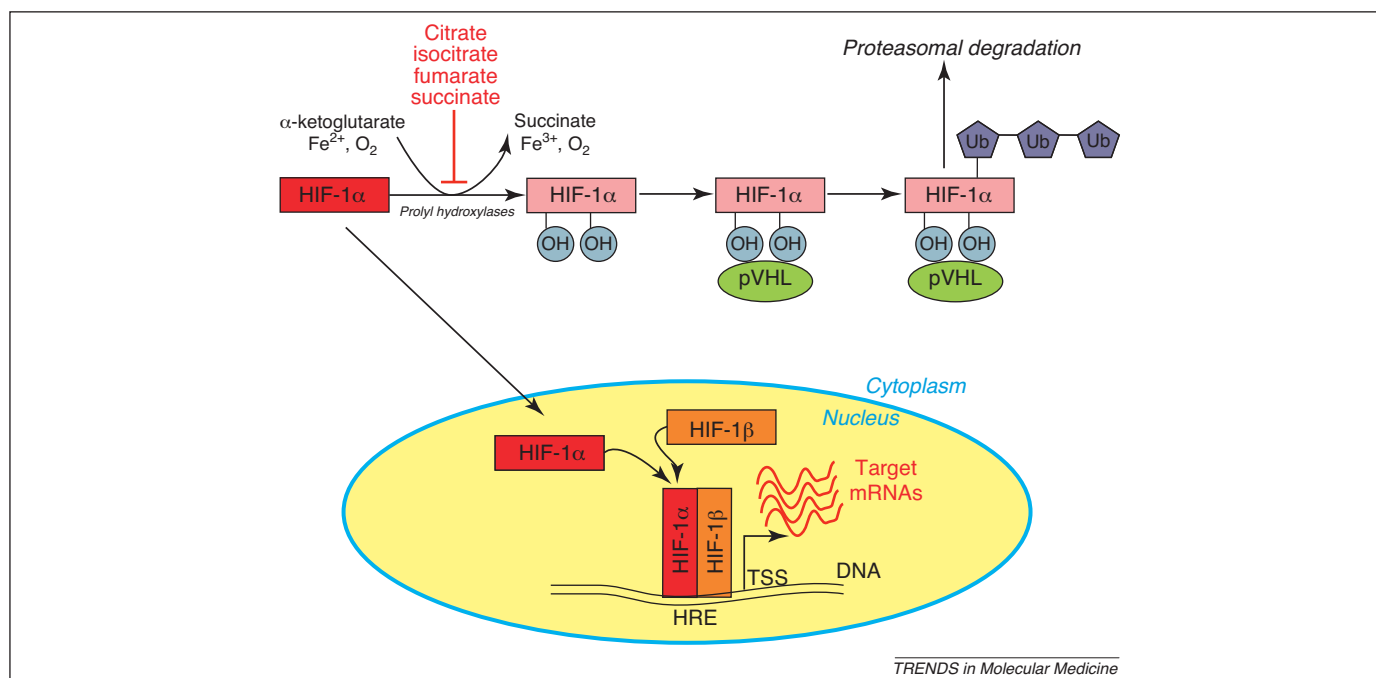


Figure 2. Role of TCA cycle metabolites in the regulation of hypoxia inducible factor (HIF) α subunits in hypoxic, pseudohypoxic and nonhypoxic conditions. The regulation of the three α subunits (HIF-1 α , HIF-2 α and HIF-3 α) by hydroxylation is similar, but for simplicity only HIF-1 α is represented in this figure. The asparaginyl hydroxylase reaction, the phosphorylation-dependent nuclear import, and the coactivators and polymerase complex were omitted for simplicity. The HIF-1 β subunit is expressed constitutively in the nucleus and is available for dimerization with α subunits that reach the nucleus. Abbreviations: Ub, ubiquitin; HRE, hypoxia response element; TSS, transcription start site.

probable that the primary function of cytoplasmic FH is to maintain low fumarate levels in the cytoplasm by conversion to malate, thus providing increased substrate for the generation of cytoplasmic NADPH through the malic enzyme reaction. This suggests that cytoplasmic FH is important for antioxidant defense and anabolism. Along these lines, a role for cytoplasmic FH in the DNA damage response was reported [32], indicating that the absence of cytoplasmic FH would result in increased genomic instability.

The TCA cycle in control of the hypoxia response

Cells and tissues react to lower O_2 availability by triggering a stress pathway designated as the hypoxia response. The term pseudohypoxia is often used to refer to the activation of this pathway under nonhypoxic conditions. The hypoxia response is a common feature of solid tumors and is characterized by increased glycolytic metabolism and promotion of angiogenesis [33]. The induction of the hypoxia pathway in tumors therefore underlies the so-called glycolytic shift, which is a typical feature of tumors [33]. This glycolytic shift was first observed in the 1920s by Otto Warburg, who noted that tumor cells rely on anaerobic ATP production through glycolysis, even in the presence of oxygen (i.e. aerobic glycolysis).

The major regulator of the hypoxia response is the transcription factor hypoxia inducible factor (HIF) [33], whereby activity is regulated by TCA cycle metabolites. HIF is a heterodimer, with one α subunit and one β subunit. There are three known α subunits (HIF-1 α , HIF-2 α and HIF-3 α) and two β subunits, ARNT1 (aryl hydrocarbon receptor nuclear translocator 1, often designated HIF-1 β) and ARNT2. HIF-3 α functions as a dominant negative regulator of HIF-1 α and HIF-2 α by binding

to the β subunits but not to DNA [33]. Whereas the β subunits are constitutively expressed, the α subunits are normally degraded in the presence of O_2 . This process involves the hydroxylation of proline residues in the α subunits, which is catalyzed by prolyl hydroxylases (PHDs) (Figure 2). The PHDs are members of the superfamily of α -ketoglutarate-dependent dehydrogenases, which couple the hydroxylation of the substrates with the oxidation of α -ketoglutarate to succinate in reactions that are dependent on O_2 and Fe^{2+} [33]. Importantly, the PHDs can be inhibited by several TCA cycle metabolites, as discussed below. The hydroxylated proline residues are recognized by an E3-ubiquitin ligase, pVHL (the product of the von Hippel-Lindau gene), and targeted to the proteasome. When the levels of O_2 , Fe^{2+} or α -ketoglutarate are low, the PHD reaction stops or slows, allowing the α subunits to accumulate. These translocate to the nucleus, dimerize with the β subunits and drive the hypoxia response transcriptional program (Box 2). In addition to the proline residues, asparagine residues of HIF α subunits are also subject to hydroxylation, catalyzed by Factor Inhibiting HIF (FIH), inhibiting the binding of HIF α subunits to transcriptional coactivators [33].

Because α -ketoglutarate and succinate are directly involved in the PHD reaction that stabilizes HIF α subunits, the TCA cycle stands to have a major role in the regulation of the hypoxia response. Whereas α -ketoglutarate is necessary for the reaction to occur, succinate, fumarate and oxaloacetate inhibit the PHDs, leading to pseudohypoxia in tumor and embryonic cells [31,32,34–37] but not in nontransformed cells [30,36,38]. Pyruvate, citrate, isocitrate, fumarate and succinate inhibit PHDs in tumor cells, with fumarate showing the strongest effect [36,39–41]. Citrate inhibits FIH, whereas neither succinate nor fumarate are

Box 2. The hypoxia response, the TCA cycle and tumor formation

The relationships between hypoxia, the TCA cycle and tumor formation are complex. Although the general notion is that tumors are inherently hypoxic and non-respiring, multiple evidence shows that only the core of solid tumors is hypoxic [33]. A hallmark of hypoxia is the induction of pyruvate dehydrogenase kinase (PDK), which inactivates pyruvate dehydrogenase (PDH) by phosphorylation [33]. PDH converts pyruvate to acetyl-CoA, and its inactivation results in an inability to feed pyruvate into the TCA cycle. Although this results in lower TCA cycle flow, lower electron supply to the respiratory chain and therefore less aerobic ATP production, this effect can be counteracted by the hypoxia-driven increase in glycolysis [42]. However, tumors have heightened anabolic needs, to meet their growth rate, particularly building blocks used in the synthesis of proteins, lipids and nucleic acids. The hypoxia response addresses these anabolic needs of tumors by upregulating the oncogenic transcription factor Myc, which induces the enzyme machinery allowing mitochondrial import of glutamine, its conversion to glutamate, and deamination of glutamate to α -ketoglutarate, thus replenishing the TCA cycle [68]. The α -ketoglutarate can be reduced to isocitrate, through isocitrate dehydrogenase, and isocitrate

is then converted to citrate by aconitase. Citrate can be exported from mitochondria to cytoplasm, where it is cleaved by citrate lyase to generate oxaloacetate and acetyl-CoA, which is the source for lipid synthesis [42]. Hypoxia also induces the expression of transketolase, an enzyme of the pentose phosphates pathway, resulting in increased production of ribose-5-phosphate, the building block for nucleotide synthesis [69]. Therefore, the hypoxia response addresses the fundamental adaptive steps that the cells need to take to gain growth advantage.

Mutations in the TCA cycle cause perturbations of the metabolite pools which can result in pseudohypoxia. Although recessive germline mutations causing loss-of-function of the enzymes lead to serious neuromuscular disorders and infantile death, dominant mutations in the same nucleotides with loss of heterozygosity predispose to tumor formation [30]. One possible explanation for this discrepancy is that tissues that are still developing, in particular the brain, are not able to cope with the perturbation in the TCA cycle in the long term and thus fail to perform and eventually degenerate. A fully developed tissue, however, may adapt to the TCA cycle defect, and that adaptation might be so 'successful' that it results in overgrowth and tumor formation.

FIH inhibitors [31,36]. The decrease in α -ketoglutarate levels resulting from the neomorphic activities of mutated IDH1 and IDH2 also has the potential to affect the PHD reaction and thus the accumulation of HIF α subunits. Although these observations clearly place the TCA cycle upstream of the hypoxia response, it is worth highlighting that one of the consequences of the hypoxia/pseudohypoxia response is a decreased supply of pyruvate flow to the TCA cycle and an increase in glutamine catabolism that feeds the α -ketoglutarate pool [42]. The TCA cycle and the hypoxia response therefore form a complex regulatory network in which each of the pathways reciprocally affects the other (Box 2).

Although it is clear that the pseudohypoxia response confers a growth advantage to the cells with IDH, FH and SDH defects, it remains to be determined if it is sufficient to initiate a tumor, or if the pseudohypoxia response confers an advantage in a later stage of tumor progression. The following section addresses this question.

Mechanisms beyond hypoxia

The molecular mechanisms linking TCA cycle defects to tumor formation remain elusive. The pseudohypoxia response has been a major focus of research in the field, and several lines of evidence have accumulated supporting a role for the hypoxia response in tumors of patients with FH or SDH defects [21]. The changed levels of α -ketoglutarate, succinate and fumarate in cells with IDH, SDH or FH mutations support the concept of perturbed PHD function. Therefore, defects in IDH, SDH and FH seem to decrease hydroxylation of HIF α subunits, thus triggering the pseudohypoxia response. However, the PHDs are part of a superfamily of proteins, α -ketoglutarate-dependent dioxygenases, converting α -ketoglutarate to succinate, while hydroxylating a wide range of substrates much broader than the HIF α subunits [43]. Furthermore, although the clinical presentations for SDH dominant defects are similar to other hypoxic syndromes, this is not the case for the IDH and FH phenotypes. Hypoxia has been presented as the driving mechanism of the formation of the tumors in TCA cycle defects, but it is fundamental to distinguish if

hypoxia is indeed the initiator mechanism or if it is instead a growth promotion mechanism that confers advantage to these tumors.

Uterine myomata are benign uterine smooth muscle tumors and constitute the major clinical presentation in patients with dominant FH mutations [6]. These myomata accumulate fumarate as well as succinate [44]. The uterine myomata in patients with FH mutations have increased HIF-1 α protein levels, HIF target expression and vascular density [44,45]. Myomata from HLRCC patients were shown, by transcriptional profiling, to have increased glycolytic potential compared with sporadic myomas, although with no evidence for increased hypoxia response under the same conditions [46]. Both fumarate and succinate inhibit the PHDs, particularly PHD2, and trigger the pseudohypoxia response in tumor cells *in vitro* [35–37,39]. Silencing of FH in cultured tumor cells also lead to increased glycolytic metabolism and accumulation of HIF α subunits [35]. However, no accumulation of HIF-1 α was detected in primary fibroblasts with recessive FH defects and fumarate accumulation [30,38]. It seems thus that intracellular fumarate accumulation triggers the pseudohypoxia response in tumor or embryonic cells, but not in primary fibroblasts. Mice with a kidney-specific FH knockout have renal cysts which display hypoxia response [47]. It should, however, be noted that the kidney FH knockout is not a model for FH defects arising in normal adult kidney, but for FH defects occurring in the kidney from the embryonic stage to adulthood [48]. In patients, the kidney tumors arise in normal kidney having one mutated FH allele, upon loss of the normal allele, but the conditional knockout mouse lost both FH alleles in embryonic kidney [6]. Therefore, the kidney-specific FH knockout is not a good model to study the role of FH in kidney tumors. Furthermore, the penetrance of FH mutations in kidney tumors is very low, suggesting that other factors could be involved [49].

The paradigm for the hypoxia pathway is the von Hippel-Lindau (VHL) disease, a dominant familial cancer syndrome caused by mutations in the *VHL* gene [33]. pVHL targets the HIF α subunits to the proteasome, resulting in accumulation of HIF-1 α and HIF-2 α in VHL

disease [33]. The clinical presentations of VHL syndrome are clear cell renal carcinoma, pheochromocytoma, pancreatic tumors and hemangioblastomas [50]. There is no overlap between the clinical presentations of VHL disease and of IDH mutations. VHL and SDH syndromes both have high penetrance in pheochromocytomas. The only phenotype shared between VHL syndrome and dominantly inherited FH defects is kidney cancer, but although VHL leads to clear cell renal carcinoma, FH leads to papillary type II, collecting duct morphology, or mixed [24,25,28].

The absence of overlap between the clinical presentations for VHL and IDH or FH suggests that the pseudohypoxia response is not an initiator event but rather a growth-promoting mechanism. In the case of SDH, which leads to phenotypes similar to VHL disease and to clinical presentations of patients living in chronic hypoxia due to high altitude, it remains possible that the pseudohypoxia response could contribute to the growth of the tumors from an earlier stage. The events that initiate the tumors in IDH and FH mutations remain unclear, and are discussed in the next section.

Regulation of cellular differentiation by TCA cycle metabolites

A comparison of the expression profiles of FH defects in diploid primary fibroblasts and in uterine myomata revealed that the transcriptional activity of Serum Response Factor (SRF), a major regulator of smooth muscle differentiation, was downregulated in both samples [51]. This implies a loss of SRF signaling before the tumors form, raising the possibility that SRF might have a role in the initial events of tumor formation. SRF signaling is essential for smooth muscle differentiation [52]. The homeostasis of smooth muscle is maintained by progenitor cells, which have stem cell-like characteristics, and SRF triggers the differentiation of these cells to terminally differentiated myocytes [52]. A downregulation of SRF signaling in FH deficiency would, in principle, result in inhibition of smooth muscle progenitor cell differentiation, and consequently in the accumulation of cells with stem cell-like behavior. This scenario could explain the formation of myomata, benign smooth muscle tumors which are the major clinical presentation in patients with dominant FH defects. The proliferation, differentiation and death of stem cell-like populations are regulated by the redox environment [53]. The redox environment in primary cells with FH mutations is highly reduced, a condition which favors proliferation of stem cell-like populations, while inhibiting their differentiation or death [30]. Therefore, SRF downregulation and a reduced redox environment would contribute to the same effect: accumulation of smooth muscle progenitor cells culminating in the formation of myomata. Interestingly, SRF signaling is also downregulated in sporadic myomata, without FH mutations, implying that perturbation of SRF function is necessary for the formation of myomata [51]. Although it is tempting to speculate that FH defects 'hijack' this myomata-causing perturbation of SRF signaling, the question of how mutations in FH result in the downregulation of SRF signaling remains open. Given that SRF is regulated by phosphorylation [52], it is probable that some of the signaling cascades upstream of

SRF are affected by fumarate intracellular accumulation. Fumarate can modulate the activity of several enzymes (e.g. dopamine β -mono-oxygenase, malic enzyme, PHDs) [33,54]. In addition, fumarate can react with exposed protein thiols and lysines, forming adducts, a process designated succinylation (because the adduct formed resembles succinate) [55]. Succinylation is likely to affect the activity of the modified proteins, analogous to other post-translational modifications such as phosphorylation and acetylation. Interestingly, acetylation of cytoplasmic proteins is regulated by acetyl-CoA generated by the cleavage of citrate in cytoplasm [56]. Perturbations of the TCA cycle metabolite pools caused by IDH, SDH and FH mutations affect the cytoplasmic level of acetyl-CoA [12] and could therefore regulate the activity of key proteins involved in tumorigenesis by acetylation.

The effect of fumarate in cellular differentiation described above could represent a novel paradigm for the extrametabolic roles of TCA cycle metabolites. Another example of a metabolite regulating cellular differentiation is 2-hydroxyglutarate, generated from α -ketoglutarate by the neomorphic activities of mutated IDH1 and IDH2. The metabolite 2-hydroxyglutarate is structurally similar to α -ketoglutarate and inhibits α -ketoglutarate-dependent enzymes, particularly telomerase and histone demethylases [57–59]. Interestingly, the effect of 2-hydroxyglutarate on telomerase prevents differentiation of stem cells, elucidating the leukemogenic effect of IDH mutations [57]. Furthermore, a reductive shift in the redox environment is associated with IDH defects, similar to what is observed in FH defects and consistent with undifferentiated stem cells [12,30].

Therefore, we propose a model in which mutations in FH and IDH cause perturbations in the cytoplasmic TCA cycle metabolite pools resulting in the inhibition of proteins (e.g. SRF, telomerase) necessary for cellular differentiation, leading to the accumulation of undifferentiated cells.

Box 3. Outstanding questions

- The role of TCA cycle metabolites in tumor formation is only beginning to be unraveled. A fundamental question that remains unanswered is the tissue-specificity of the phenotypes. Are certain tissues affected because their 'normal' TCA cycle is less able to buffer fluctuations in specific metabolites? The answer is likely to bring us a new level of understanding of how the TCA cycle integrates with other pathways in different tissues and different conditions. The current state of metabolomic methodologies makes accessible the study of the 'TCA cycles' occurring in the human body, both physiological and pathological.
- What are the extrametabolic roles for the TCA cycle metabolites? Although some of these roles have been identified, it is certainly possible that the TCA cycle metabolites have the ability to modulate the activity of signaling cascades or transcription factors, thus effecting profound regulatory effects on several cellular decisions.
- How are α -ketoglutarate-dependent dioxygenases inhibited by TCA cycle metabolites? Although the prolyl hydroxylases and telomerase are already known to be inhibited by TCA cycle metabolites, it is probable that other proteins of the same family also have this regulatory potential. Identification of the tissue distribution and functions of proteins in that family would certainly contribute to the understanding of the role of TCA cycle metabolites in tumor formation.

The activation of the pseudohypoxia response at a later stage probably provides these cells with necessary adaptations to efficiently proliferate under varying conditions, also explaining the association of this response with TCA-related mutations that cause cancer.

Concluding remarks

The direct involvement of TCA cycle enzymes and metabolites in tumor formation brought a pathway long resigned to dusty biochemistry textbooks back to the limelight. The extrametabolic functions of the TCA cycle metabolites are now a major focus of research. Although some issues have been resolved, many questions remain. First and foremost, how the 'TCA cycles' operate in different cells, different tissues and even under different conditions in the same cell (Box 3). The heroic efforts of the chemists and biochemists that originally defined this key metabolic pathway, relying on a vast and solid knowledge of chemistry, can now be joined by researchers with contemporary genomic and metabolomic approaches to bring about a systematic understanding of TCA cycle metabolism and the vast implications this holds for biology and medicine.

Acknowledgments

This work was supported by the National Institutes of Health grants CA112364 and ES011163 to Gerald S. Shadel.

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