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Title: 2-Oxoglutarate-dependent dioxygenases in cancer

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Abstract:

2-oxoglutarate-dependent dioxygenases (2OGDD) are a superfamily of enzymes that play diverse roles in many biological processes, including regulation of HIF-mediated adaptation to hypoxia, extracellular matrix formation, epigenetic regulation of gene transcription, and the reprogramming of cellular metabolism. 2OGDD all require oxygen, reduced iron, and 2-oxoglutarate to function, although their affinities for each of these co-substrates, and hence their sensitivity to depletion of specific co-substrates, varies widely. A number of 2OGDD are recurrently dysregulated in cancer. Moreover, cancer-specific metabolic changes, such as occur subsequent to *SDH*, *FH*, or *IDH* mutations, can dysregulate specific 2OGDD. This latter observation suggests that the role of 2OGDD in cancer extends beyond cancers that harbor 2OGDD mutations. Herein, we review the regulation of 2OGDD in normal cells and how that regulation is corrupted in cancer.

Introduction

It has been known for many decades that cancer cells display characteristic alterations in metabolism and epigenetics. Many cancers divert glucose carbons toward glycolysis (canonical anaerobic metabolism) and away from oxidative phosphorylation (canonical aerobic metabolism) even when oxygen is available (known as the "Warburg Effect")[1]; and cancer genomes often display global DNA hypomethylation as well as focal locus-specific increases in DNA and histone methylation[2]. The question of whether such changes in metabolism and epigenetics actually cause cancer was controversial until oncogenic driver mutations were identified in metabolic and epigenetic genes. It is now clear that some cancers are caused by mutations in *fumarate hydratase* (*FH*)[3], *succinate dehydrogenase* (*SDH*)[4], and *isocitrate dehydrogenase* (*IDH*)[5,6], which lead to the accumulation of the 2-oxoglutarate (2OG) analogues fumarate, succinate, and the R-enantiomer of 2-hydroxyglutarate (R-2HG), respectively. Similarly, some cancers are caused by mutations in epigenetic regulators such as the *EZH2* H3K27 methyltransferase[7], the *KMT2A* H3K4 methyltransferase[8], the *TET2* DNA hydroxylase[9,10], and the *KDM6A* H3K27 lysine demethylase[11].

2-oxoglutarate-dependent dioxygenases (2OGDD) are a superfamily of enzymes that sit at the nexus of cancer metabolism and cancer epigenetics (Box 1). These enzymes have the potential to sense oxygen, reactive oxygen species, iron availability, and specific metabolites, including 2-oxoglutarate (2OG) and its structurally related metabolites. For example, EGLN prolyl 4-hydroxylases (also known as PHDs) and the FIH1 asparaginyl hydroxylase are 2OGDD that act as cellular oxygen sensors by regulating the hypoxia-inducible transcription factor HIF[12]. HIF transcriptionally regulates hundreds of genes, including genes that contribute to the Warburg Effect and genes linked to DNA and histone methylation. Other 2OGDD play direct roles in the control of DNA (TET and ABH enzymes) and histone (KDM enzymes) methylation, as well as mRNA processing (FTO) and protein translation (OGFOD1, MINA53, and NO66)[13].

Some 2OGDD are directly dysregulated in cancer, by amplification, silencing, deletion and mutation (Table 1). Other 2OGDD appear to be indirectly dysregulated in cancer, by hypoxia and by the action of aberrantly accumulated metabolites that possess pro-oncogenic activities (*i.e.*, 'oncometabolites'). Several 2OGDD promote or suppress tumor growth in preclinical cancer models, further implicating the dysregulation of 2OGDD activity in oncogenesis. However, much remains unknown about the roles of specific 2OGDD in cancer. The study of these enzymes has been further motivated by the observation that their activities can be modulated by small molecules, suggesting that 2OGDD are potential therapeutic targets in cancer.

The 2OGDD reaction

2OGDD all share the same reaction mechanism but act on different substrates, including proteins, DNA, RNA, fatty acids and other small molecules (Box 1)[14]. 2OGDD all require the same co-substrates: dioxygen (O_2), which provides the oxygen atom for hydroxylation, divalent iron (Fe^{2+}), and 2OG, and yield a hydroxylated product, CO_2 and succinate (Figure 1a). Catalysis follows an ordered sequence. First, active site-bound Fe^{2+} coordinates 2OG binding in a bidentate manner. Next, substrate binding to the active site

displaces an Fe^{2+}-ligated water molecule, thereby allowing oxygen to bind[15,16]. Next, oxidative decarboxylation of 2OG forms succinate, CO_2 and a ferryl (Fe^{4+}) intermediate, which reacts with the substrate's C-H bond resulting in hydroxylation and reduction of Fe^{4+} to Fe^{2+}. Finally, the hydroxylated product is released from the catalytic site, followed by the release of succinate. The hydroxylated product can then undergo further non-enzymatic modifications such as demethylation. Although reducing agents (*e.g.* ascorbate, glutathione, cysteine) are not direct cofactors of the 2OGDD reaction, they support catalysis by preventing inadvertent iron oxidation and, in the case of EGLN, by preventing oxidation of intramolecular cysteine residues that are required for catalytic activity[17,18].

The affinity of specific 2OGDD for oxygen, iron and 2OG varies, providing a mechanism of regulation of subsets of 2OGDD at the level of co-substrate availability (Figure 2, Table 2). In addition, several endogenous 2OG analogues, including pyruvate, citrate, isocitrate, succinate, fumarate, malate, oxaloacetate, R-2HG and its enantiomer S-2HG can act as competitive inhibitors of 2OGDD[19-22], modulating 2OGDD activity in both physiologic and pathophysiologic states (Figure 1b, Figure 2, Table 2). Although other mechanisms of regulation of 2OGDD have been reported, including alternative splicing and translation initiation[23-25], post-translational modification (*e.g.*, sumoylation[26,27], phosphorylation[28-30]), and differential expression of binding partners[31,32], their contributions to the regulation of 2OGDD activity are less well-understood.

Herein, we review our current knowledge about the metabolic regulation of 2OGDD activity and discuss how dysregulation of these activities contribute to tumorigenesis. We also discuss some of the outstanding questions in the field that warrant further investigation.

Oxygen

The importance of dysregulated oxygen signaling in tumorigenesis is perhaps best exemplified by the role of HIF in tumors that harbor *von Hippel Lindau* (*VHL*) mutations, including clear cell renal cell carcinoma, hemangioblastoma, and paraganglioma[33]. In normal cells under normoxic conditions, HIFα is prolyl hydroxylated by EGLN enzymes. This hydroxylation promotes HIFα binding to the pVHL tumor suppressor protein, which is the substrate recognition subunit of an E3 ubiquitin ligase that ubiquitylates HIFα and targets it for degradation. Oxygen is an indispensable co-substrate for EGLN enzymes, and their catalytic activity is highly dependent on oxygen availability. Even a modest decline in cellular oxygen levels inhibits EGLN activity and HIFα hydroxylation. As a result, HIFα accumulates, dimerizes with its partner protein ARNT (also called HIF1β), and transcriptionally activates HIF-responsive genes. Loss-of-function *VHL* mutations likewise prevent the degradation of hydroxylated HIFα, resulting in hypoxia-independent stabilization of HIFα and constitutive expression of HIF-target genes. pVHL likely has functions unrelated to HIF and there is some evidence that loss of HIF-independent pVHL function can contribute to paraganglioma formation[34,35]. However, dysregulation of HIFα, and particularly HIF2α, appears to be a fundamental mechanism of pathogenesis in *VHL* mutant kidney cancers[36-42].

Interestingly, loss-of-function *EGLN1* mutations and gain-of-function *HIF2α* mutations, although rare, have been identified in paragangliomas (Table 1)[43-47]. This observation, and the finding that formation of paraganglioma-like lesions after inactivation of *EglN1* in rodents is Hif2-dependent[48], suggests that these tumors, like *VHL*-mutated paragangliomas, are driven by HIF. Whether loss-of-function *EGLN1* mutations also promote tumorigenesis by HIF-independent mechanisms is not known. EGLN enzymes have been reported to have many substrates other than HIF. However, a recent study assessing the hydroxylation of these putative non-HIFα substrates by recombinant EGLN enzymes using three independent methods under conditions in which HIFα is robustly hydroxylated did not detect hydroxylation of any non-HIFα proteins[49]. It should also be noted that roxadustat, the first-in-class clinical EGLN inhibitor, has been approved for the treatment of anemia secondary to renal failure[50,51], and several other EGLN inhibitors have advanced to the final phase of clinical development with no major side-effects. This suggests that EGLN enzymes are not promiscuous hydroxylases with pleiotropic functions.

The oncogenic consequences of constitutive HIF activation clearly demonstrate that the transcriptional response to hypoxia can promote tumorigenesis. However, it is important to keep in mind that *VHL* and *EGLN1* mutations create a state of *pseudo-hypoxia*. In *VHL*-mutant and *EGLN1*-mutant tumors, HIF activation is independent of cellular oxygen levels. The questions remain: can hypoxia *directly* contribute to tumorigenesis and, if so, does it do so by dysregulating 2OGDD? With respect to the former question, a number of conditions are associated with chronic hypoxemia, including life at high altitude, hemoglobinopathies, and certain cardiopulmonary diseases, and these conditions are not associated with a conspicuously increased risk of cancer.

Tissue oxygen concentrations are typically 1-10% whereas tumors can be profoundly hypoxic, with oxygen concentrations of less than 2% and, in some cases, less than 0.1%[52]. Under hypoxic conditions, specific 2OGDD with low affinity for oxygen (*i.e.,* with high O_2 K_m values) are inhibited while 2OGDD with high affinity for oxygen (*i.e.,* with low O_2 K_m values) remain active (Figure 2, Table 2). EGLN enzymes are the major cellular oxygen sensors, having O_2 K_m values above atmospheric oxygen concentrations[12]. On the opposite end of the spectrum are the collagen prolyl 4-hydroxylases (P4HA1-3), which have high affinities for oxygen and are therefore catalytically active even in profound hypoxia. The asparaginyl hydroxylase FIH1 has intermediate affinity for oxygen[53]. It remains active under moderately hypoxic conditions that are sufficient to affect EGLN activity but becomes inactive under more severe hypoxia. Hydroxylation of HIFα proteins by FIH1 blocks association of HIFα with the transcriptional co-activator CBP/p300, thereby inhibiting HIF-mediated transcription[54,55]. FIH1 also hydroxylates asparagine, aspartate and histidine residues on a large pool of ankyrin-repeat-domain-containing non-HIF substrates, and it is speculated that these alternative substrates function to sequester FIH1 from HIFα, thereby promoting HIF activity[56,57]. It should be noted that, although FIH1 can regulate the transcriptional activity of HIF *ex vivo*, it is less clear whether FIH1 plays a physiologic role in regulating the cellular response to hypoxia *in vivo*. Genetic deletion of *Fih1* in mice does not affect canonical HIF-regulated phenotypes such as angiogenesis or erythropoiesis.

Rather, mice lacking *Fih1* display a generalized metabolic dysregulation that appears to be neurogenically driven[58].

The extent to which other 2OGDD can act as direct oxygen sensors is an area of active investigation. Although hypoxia-associated histone hypermethylation is a well-described phenomenon[59-61], until recently it was not clear whether this hypermethylation is a direct consequence of inhibition of oxygen-sensitive JmjC-domain-containing histone lysine demethylases (KDM), which are 2OGDD, or an indirect effect of hypoxia on chromatin structure. We recently showed that KDM6A, but not its paralogue KDM6B, is highly oxygen-sensitive (Figure 2, Table 2) and that KDM6A regulates cellular differentiation in an oxygen-dependent, but HIF-independent manner[62]. Mechanistically, we identified structural differences in the JmjC catalytic domains of KDM6A and KDM6B that likely explain their differential oxygen sensing capacities (Figure 3). A companion study found that acute inactivation of KDM5A rapidly induces H3K4 methylation that phenocopies the effects of acute hypoxia[63]. This is consistent with the observation that KDM5A has a high O_2 K_m value (Figure 2, Table 2). Specific KDM4 paralogues have also been reported to have oxygen-sensing capabilities, although there are conflicting data as to their O_2 K_m values, especially in the case of KDM4A (Table 2)[62,64,65]. In the case of the TET family of DNA hydroxylases, two independent studies determined that the O_2 K_m values of TET1 and TET2 are very low (Figure 2, Table 2)[20,66], suggesting that these 2OGDD do not act as oxygen sensors[20,66]. However, tumor hypoxia has been reported to cause DNA hypermethylation via reduced TET activity[66]. The basis for these inconsistencies is not clear, although it should be noted that the latter study was performed at 0.5% oxygen and, as stated above, tumors can be even more profoundly hypoxic. It is also known that *in vitro* oxygen K_m measurements can be affected by a number of technical factors. This is well-exemplified by EGLN enzymes, for which longer, more 'natural,' substrates yield lower O_2 K_m values than do short synthetic peptides[16]. The differences in K_m values for oxygen that have been observed with different length substrates are likely due to the fact that substrate binding precedes oxygen binding and affects the structure of the oxygen binding site[15,16]. It is also possible that the oxygen-sensing capacities of specific 2OGDD *in vivo* are affected by post-translational modifications, accessory proteins, or metabolic factors that are typically absent when performing *in vitro* assays with recombinant proteins.

Interestingly, a number of oxygen-sensing KDM enzymes are either genetically inactivated or overexpressed in cancer (Table 1). This raises the possibility that, in hypoxic tumors, metabolic dysregulation of KDM enzymes impacts tumor behavior. For example, KDM5A has been shown to inhibit the migration and invasion of glioma cells and is transcriptionally silenced in aggressive gliomas[67,68]. Hypoxia-associated inhibition of KDM5A catalytic activity could be an alternative mechanism by which KDM5A is downregulated as brain tumors outgrow their blood supply. Similarly, *KDM6A* is mutated and inactivated in a number of tumor-types and has been shown to function as a tumor suppressor *in vitro* and *in vivo* (Table 1)[11,62,69-71]. It is possible that, in *KDM6A-wild type* tumors, the hypoxic tumor microenvironment directly downregulates KDM6A activity and thereby promotes tumor progression.

Profound intratumoral hypoxia should, theoretically, inhibit oxygen-sensing KDMs, including KDMs such as KDM5A, KDM6A and KDM4, that have tumor-promoting functions in some tumor contexts (Table 1). This raises the intriguing possibility that hypoxic tumors engage specific mechanisms to maintain the activity of tumor-promoting KDMs, such as through enhanced of 2OG availability (discussed in further detail below) or through increased KDM expression. Indeed, a number of 2OGDD are transcriptional targets of HIF[59]. Given that transcriptional programs induced by HIF vary across cell types[72], it is possible that induction of specific 2OGDD by HIF fine-tunes the 2OGDD response to hypoxia in a cell-type specific manner. Although it is certainly plausible that upregulated expression of hypoxia-sensitive 2OGDD represents a mechanism by which cancer cells compensate for the loss of tumor-promoting 2OGDD activities under hypoxic conditions, it is important to bear in mind that aggressive tumors often outgrow their blood supply, become hypoxic, and upregulate HIF. Caution must therefore be exercised when ascribing cause-and-effect relationships to correlations between HIF-responsive 2OGDD overexpression and patient outcomes.

It should be noted that the contribution of oxygen to 2OGDD regulation is not limited to those 2OGDD that are direct oxygen sensors (Figure 4). As discussed below, hypoxia can produce secondary effects on cellular metabolism that result in the accumulation of metabolites, including succinate[73,74] and S-2HG[75-78], that can inhibit 2OGDD that are relatively oxygen-insensitive (Figure 2, Table 2). It should also be noted that 2OGDD are not the only oxygen sensors in cells. ADO, a non-2OG-dependent cysteine oxidase, signals oxygen availability by oxidizing N-terminal cysteines in substrate proteins to cysteine sulfinic acid[79]. Cysteine sulfinic acid modifications are involved in regulating redox balance, circadian rhythms and protein stability[80].

Situated as they are at the interface between oxygen and diverse biological processes, 2OGDD directly link changes in oxygen availability to changes in chromatin structure, gene expression and other biological functions. In normal cells, the complex interplay between oxygen availability and 2OGDD activity provides a critical mechanism of cellular adaptation. We are just now starting to appreciate how these same mechanisms are co-opted by cancer cells to promote their survival as they outgrow their blood supply and become progressively more hypoxic.

Iron

Dietary intake is the body's only source of iron, and cellular iron availability is primarily regulated by recycling[81]. The hepcidin-ferroportin axis controls the in-flow of iron from the intestine, liver and iron-storing macrophages, and plasma transferrin distributes iron to tissues. Normal plasma iron concentrations are ~20 μM, whereas total iron binding capacity is ~3-fold higher. Conditions that dysregulate transferrin and hepcidin expression, such as inflammation, hinder normal iron recycling[82]. It is possible that functional iron deficiency inhibits 2OGDD that require high iron concentrations for catalysis, such as PHYH, BBOX1, KDM6B, TET1 and TET2 (Figure 2, Table 2)[83]. Whether this contributes to the well-established link between chronic inflammation and cancer is not known[84,85], although it is interesting to note that several highly iron-

dependent 2OGDD, including KDM6B, TET1 and TET2, suppress tumor growth in specific cellular contexts (Table 1)[86-92].

Most of the body's iron is utilized by erythrocytes and, as an integral component of hemoglobin, iron plays a central role in oxygen delivery. Iron deficiency can therefore indirectly lead to cellular hypoxia and, potentially, to inhibition of hypoxia-sensitive 2OGDD. Other divalent metals such as Zn^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+} and Co^{2+} can compete with iron and inhibit 2OGDD *in vitro* and in cells[93,94]. Other metals, such as Cr^{6+}, that do not compete with iron but do oxidase ascorbate (see below) can also inhibit 2OGDD[95]. Whether these divalent metals play a role *in vivo* in regulating or dysregulating 2OGDD activity is not known.

Ascorbate

Reactive oxygen species (ROS) are chemically reactive byproducts of normal cellular metabolism. The major sources of ROS in cells are the mitochondrial electron transport chain and plasma membrane NADPH oxidases (NOX). ROS are produced by the transfer of free electrons to molecular oxygen. This results in the production of superoxide, which is converted to hydrogen peroxide by superoxide dismutase (SOD). Hydrogen peroxide can react with redox-sensitive cysteine residues on proteins or can be metabolized to water by antioxidant proteins such as glutathione peroxidases (GPX). Hydrogen peroxide can also react with reduced iron, resulting in oxidation of iron molecules and production of highly reactive hydroxyl radicals that are damaging to cells.

2OGGD require a reducing agent, typically ascorbate, to prevent spurious iron oxidation and to prevent oxidation of intramolecular cysteine residues. Humans depend on dietary ascorbate because they do not express functional gulonolactone oxidase (GULO), the enzyme that catalyzes the terminal step in ascorbate biosynthesis. The importance of ascorbate for 2OGDD function is best exemplified by the disease scurvy, wherein ascorbate deficiency impairs collagen cross-linking due to decreased activity of the P4HA family of 2OG-dependent collagen prolyl hydroxylases. This leads to bleeding gums and poor wound healing. Although other reducing agents, such as reduced glutathione and dithiothreitol (DTT), can substitute for ascorbate *in vitro*, there appears to be significant variability in the capacity of different reducing agents to support the activities of specific 2OGDD[17]. This suggests that it may be possible to augment the function of specific 2OGGD with specific reductants.

Cancer cells have increased ROS relative to normal cells, and it has been suggested that redox stress inhibits 2OGDD that constrain tumor growth and thereby promotes tumorigenesis (Table 1)[96]. Although the precise mechanisms by which cancer-associated redox stress leads to 2OGDD dysfunction are unclear, studies have suggested that oxidation of Fe^{2+} plays a role[97]. There is a growing body of evidence that subclinical ascorbate deficiency can promote tumorigenesis by inhibiting 2OGDD activity. Although ascorbate does not appear to be required for the physiologic regulation of Egln enzymes in mice[98], ascorbate supplementation downregulates HIF levels in tumor cells *ex vivo*[99] and suppresses tumor growth

in vivo in a HIF-dependent manner[100-102]. In the case of TET enzymes, ascorbate deprivation in *Gulo-/-* mice impairs Tet function and expands the hematopoietic stem cell compartment whereas ascorbate supplementation suppresses mutant *Tet2*-mediated leukemogenesis by augmenting residual Tet2 function and activating other Tet paralogs[91,103]. Ascorbate supplementation also restores TET function and slows the growth of renal cell cancer cells *in vitro* and *in vivo*[104,105]. Interestingly, the anti-tumor effects of ascorbate supplementation have been observed even in mice that express wild-type Gulo[91], suggesting that ascorbate is limiting even in tumor cells that are able to synthesize ascorbate. It is possible that the increased requirement for ascorbate under 21% oxygen *ex vivo* and in tumors *in vivo* is due to an increased rate of ascorbate oxidation in these settings.

2-oxoglutarate

2OG is a tricarboxylic acid (TCA) cycle intermediate that is produced by oxidative decarboxylation of isocitrate by IDH enzymes (IDH1 in the cytoplasm and IDH2 and IDH3 in the mitochondria), and oxidative deamination of glutamate by glutamate dehydrogenase (Figure 4c). The concentration of 2OG in cells is estimated to be in the high micromolar to low millimolar range under physiological conditions. Given that the measured 2OG K_m values for 2OGDD are in the low micromolar range (Figure 2, Table 2), it is generally assumed that 2OG is not limiting for 2OGDD activity in cells. However, this assumption may be flawed. 2OG K_m values are typically measured in the absence of endogenous 2OG-competitive inhibitors (see below), and *in vivo* 2OG K_m values may therefore be quite different than those measured *in vitro*. Moreover, accurate measurement of metabolite concentrations in living cells is very challenging. Cellular volumes fluctuate dramatically and vary significantly from cell to cell, even in clonal cell populations, and metabolites, including 2OG, turn over very rapidly and exist at different concentrations in different subcellular compartments, with a considerable amount of intracellular 2OG being sequestered in mitochondria[106]. Finally, 2OG levels may themselves be subject to regulation by, for example, regulation of intracellular transport of 2OG or regulation of the shuttling of 2OG between subcellular compartments. This all begs the question, is 2OGDD activity sensitive to changes in intracellular concentrations of 2OG? We recently reported that acute inhibition of EGLN activity, either by genetic deletion of *EglN1* or by small molecule EGLN inhibition, increases total intracellular 2OG[107]. These results suggest that, at least under some conditions, 2OG levels in cells are limiting and are a function of 2OG utilization. Interestingly, another recent study found that physiologic changes in intracellular 2OG can indeed modulate the activity of specific 2OGDD[108]. Whether dysregulation of 2OG homeostasis directly contributes to tumorigenesis is not known, although one recent study found that the p53 tumor suppressor promotes the activities of specific 2OGDD tumor suppressors, including TET enzymes, by upregulating 2OG levels[109].

Fumarate and Succinate

The ability of 2OG to act as a co-substrate to support 2OGDD reactions is not merely a function of 2OG availability. It is also a function of the relative concentrations of 2OG and its endogenous analogues, including the TCA cycle intermediates fumarate and succinate. Fumarate and succinate are structurally and chemically similar to 2OG (Figure 1b) and can therefore act as competitive inhibitors of 2OG[110]. Interestingly, similar to their oxygen sensitivities, the sensitivities of 2OGDD to fumarate and succinate vary dramatically (Figure 2, Table 2).

The modulation of 2OGDD activity by fumarate appears to play an important role in cellular physiology. Radiation-induced DNA damage and subsequent activation of DNA-PK has been shown to recruit cytoplasmic FH to sites of double strand DNA damage, where FH is phosphorylated and inhibited[111,112]. This results in local accumulation of fumarate, which inhibits KDM2B and promotes H3K36 methylation and the subsequent recruitment and repair of DNA breaks by non-homologous end-joining (NHEJ) machinery[111]. Although these findings suggest that fumarate can suppress tumorigenesis by enhancing DNA repair, another study found, on the contrary, that fumarate and other 2OG-like oncometabolites inhibit DNA repair and enhance the sensitivity of tumor cells to inhibitors of poly (ADP-ribose) polymerase (PARP)[113]. This study found that oncometabolite inhibition of KDM4B results in hypermethylation of H3K9 loci at sites of DNA damage, which impairs the recruitment of factors that mediate homology-directed repair (HDR) of DNA double-strand breaks. Fumarate has also been implicated in promoting innate immune memory by inhibiting KDM5 enzymes and enhancing H3K4 methylation[114].

Physiologic 2OGDD activity is also susceptible to modulation by succinate. SDH, in addition to oxidizing succinate to fumarate in the TCA cycle (Figure 1b), also plays a critical role in the respiratory electron transport chain as Complex II (*i.e.*, succinate-ubiquinone reductase). Succinate levels are induced in hypoxic cardiomyocytes by aspartate and glutamate anaplerosis into the TCA cycle and by reversal of SDH/Complex II activity[73,74]. This accumulated succinate is transported out of the mitochondria to the cytosol, where it can inhibit the activity of succinate-sensitive 2OGDD and thereby amplify the direct effects of hypoxia on 2OGDD activity[20] (Figure 4). It has also been reported that maintenance of a high 2OG:succinate ratio stimulates Tet and Kdm activity[115-117] in mouse embryonic stem (ES) cells. However, the specific impact of 2OG and succinate on ES cell function is still somewhat unclear, with conflicting reports suggesting that high 2OG levels promote either pluripotency[115,117] or differentiation[116].

Loss-of-function mutations in *FH* and *SDH* have been identified in various cancers, including papillary renal cell carcinoma and paraganglioma[3,4]. These mutations result in the accumulation of high levels of intracellular fumarate and succinate, respectively, which act as oncometabolites, at least in part, by modulating the activities of 2OGDD. For example, fumarate and succinate inhibit EGLN enzymes and induce aberrant HIF stabilization and constitutive HIF activation[118-121]. As described above, kidney cancers and paragangliomas display constitutive activation of HIF secondary to loss of pVHL[33], and gain-of-function mutations in *HIF2α* have been identified in paragangliomas[45-47]. Based on these observations, it would be reasonable to conclude that HIF similarly functions as an oncogenic driver in *FH*- and *SDH*-mutant tumors

as in *VHL*-mutant tumors. However, a cautionary note is provided by studies of *Fh*-deficient mice, where the formation of pre-malignant renal cysts is unaffected by concurrent loss of *Epas1*, the gene that encodes Hif2α, and is worsened by concurrent loss of *Hif1α*[122]. Cyst formation in *Fh*-deficient mice appears to be driven by upregulation of antioxidant signaling as a consequence of the covalent attachment of fumarate to specific cysteine residues in Keap1, thereby preventing Keap1 from downregulating Nrf2. It remains unknown whether *FH*- and *SDH*-mutant tumors are dependent on HIF transcriptional activity.

Besides EGLN enzymes, a number of other 2OGDD are inhibited by fumarate and succinate *in vitro* and in FH- and SDH-deficient cells[123-125] (Figure 2, Table 2), and several of these 2OGDD, including TET1 and TET2, have tumor suppressor activity[89-91] (Table 1). However, it remains to be elucidated which, if any, of these 2OGDD are functionally relevant tumor suppressors in *FH*- and *SDH*-mutant tumors.

R-2-hydroxyglutarate

In addition to fumarate and succinate, another structural analogue of 2OG, R-2HG, is an oncometabolite. R-2HG is a reduced form of 2OG and is produced at very low levels in normal cells as a byproduct of cellular metabolism[126]. IDH1 and IDH2 are homodimeric enzymes that catalyze the reversible oxidative decarboxylation of isocitrate to 2OG (Figure 1b). Cancer-associated mutations in *IDH*, at Arg-132 of IDH1 and Arg-140 or Arg-172 of IDH2, alter the catalytic activity of IDH such that the mutant enzymes catalyze an irreversible reaction in which 2OG is reduced but not carboxylated, resulting in the production of R-2HG[127].

IDH mutations are present in a wide range of cancers, including gliomas, chondrosarcomas, cholangiocarcinomas and acute myeloid leukemia (AML)[5,6]. In AML, R-2HG is necessary and sufficient to mediate the oncogenic effects of mutant IDH[128-130], and drugs that inhibit mutant IDH activity have been approved for the treatment of *IDH* mutant AML[131,132]. R-2HG is hypothesized to transform cells by competitively inhibiting the binding of 2OG to 2OGDD that function as tumor suppressors. The best-validated target of R-2HG in cancer is TET2, a DNA hydroxylase that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)[133,134]. TET enzymes can further oxidize 5hmC to generate 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)[135,136]. Several lines of evidence suggest that mutant IDH promotes leukemogenesis, at least in part, by inhibiting TET2. The catalytic activity of TET2 is inhibited by R-2HG *in vitro*[137,138], and primary *IDH* mutant AML cells display global loss of 5hmC[139,140]. Furthermore, loss of TET2, like accumulation of R-2HG, is sufficient to promote cytokine-independence and block the differentiation of hematopoietic cells[128,141]. Finally, *IDH* and *TET2* mutations are common, but are largely mutually exclusive, in *de novo* AML[10,141]. Taken together, these observations suggest that mutant IDH and TET2 loss activate similar oncogenic pathways and that TET2 is a functionally important target of R-2HG in *IDH* mutant AML. Interestingly, R-2HG is actually quite a poor TET inhibitor, with an IC50 value of ~5 mM (Figure 2, Table 2)[138,142]. Nonetheless, the observation that R-2HG levels in *IDH* mutant tumors can be as high as 10 mM[143-145], and the wealth of genetic evidence that *IDH* and *TET2* mutations activate similar leukemogenic pathways, strongly implicate TET2 as a functionally important R-2HG target in *IDH* mutant AML. But what

about other *IDH* mutant tumor-types, such as *IDH* mutant chondrosarcoma, that do not show evidence of TET inhibition and 5hmC loss[146], and tumors, such as glioma, that do not harbor loss-of-function mutations in TET2[147]? It is possible that R-2HG promotes transformation by targeting 2OGDD other than TET enzymes in these *IDH* mutant solid tumors.

A number of other 2OGDD besides TET2 have been proposed as potential tumor suppressor targets of R-2HG in *IDH* mutant tumors[21,137,148-150], with attention particularly focused on KDM enzymes. R-2HG can inhibit a number of KDM enzymes *in vitro* and *in vivo*[149,151-155] and many KDM enzymes are actually significantly more sensitive to inhibition by R-2HG than is TET2 (Figure 2, Table 2). Moreover, several KDM enzymes are recurrently mutated or otherwise downregulated in cancer (Table 1), suggesting that these enzymes can function as tumor suppressors and that their inhibition by R-2HG could contribute to mutant IDH-mediated transformation. However, little evidence currently exists to directly functionally implicate specific KDM enzymes in mutant IDH-mediated transformation.

EGLN enzymes were initially reported to be inhibited by R-2HG, implying that HIF acts as an oncogenic driver in *IDH* mutant tumors[148]. However, we found that, on the contrary, R-2HG can act as an alternative 2OG-like co-substrate to *potentiate* EGLN activity (Table 2), and that R-2HG *blunts* hypoxia-induced stabilization of HIFα[128,138]. In keeping with these observations, HIF levels are low in *IDH1* mutant gliomas[156,157] and in T cells exposed to R-2HG[158]. It should be noted that loss of *Hif1*α potentiates tumor growth in an *Idh-wild type* orthotopic brain tumor model[159], suggesting that HIF1α functions as a tumor suppressor in brain tumors more generally, not just in the context of *IDH* mutant glioma.

How R-2HG activates EGLN mechanistically has not been definitively established. Although our biochemical studies suggest that EGLN1 stimulates rapid oxidation of R-2HG to 2OG, which is then decarboxylated to succinate by canonical EGLN1 activity[138], others have reported that R-2HG can be converted to 2OG even in the absence of EGLN1, by prolonged incubation of R-2HG with high concentrations of iron and reducing agents[160]. Either mechanism would explain the unexpected finding that transformation by R-2HG is associated with enhanced EGLN activity[128,138].

S-2-hydroxyglutarate

2HG is a five-carbon dicarboxylic acid with a chiral center at the second carbon atom, resulting in two possible enantiomers of 2HG, R-2HG (*i.e.*, D-2HG) and S-2HG (*i.e.*, L-2HG) (Figure 1b). Like R-2HG, S-2HG is a byproduct of a normal cellular metabolism. It is produced by malate dehydrogenase (MDH) upon conversion of oxaloacetate to malate in the TCA cycle[161]. S-2HG levels are normally very low in cells. However, under hypoxic and acidic conditions, promiscuous substrate utilization by lactate dehydrogenase (LDH) promotes reduction of 2OG to S-2HG (Figure 1b, Figure 4)[75-77], resulting in the accumulation of low millimolar concentrations of S-2HG[76].

Hypoxia-induced S-2HG appears to play an important role in anti-tumor immunity[78]. In response to T-cell receptor triggering, $CD8^+$ T cells switch to a glycolytic metabolic program and produce S-2HG. S-2HG inhibits EGLN enzymes and induces HIF, which upregulates LDH expression, further amplifying S-2HG production. The accumulated S-2HG inhibits TET2 activity, which promotes $CD8^+$ T-lymphocyte effector function. Although this process does occur under normoxic conditions, S-2HG production is strongly potentiated by hypoxia (Figure 4). Given that tumors and inflammatory tissues are generally hypoxic, and given that TET enzymes do not appear to be direct oxygen sensors (Figure 2, Table 2), this hypoxia-S-2HG feedback loop could be a mechanism by which $CD8^+$ T-lymphocytes mitigate the immunosuppressive effects of intratumor hypoxia and enhance their anti-tumor activity[78,162,163].

There is evidence to suggest that S-2HG, like R-2HG, can function as an oncometabolite. In renal cell carcinoma, S-2HG levels are elevated and 5hmC levels are decreased due to downregulation of expression of L2HGDH (L-2HG dehydrogenase)[164], an enzyme that metabolizes S-2HG to 2OG[161]. Ectopic expression of L2HGDH results in decreased accumulation of S-2HG, restoration of 5hmC, and suppression of cell proliferation and colony formation.

Other 2OG-dependent cellular processes

2OGDD are not the only enzymes that require 2OG. A number of other enzymes (Box 1), including amino acid transaminases and components of the 2OG dehydrogenase complex, are involved in 2OG metabolism and can potentially be affected by 2OG availability and endogenous 2OG analogues. We recently reported that the 2OG-dependent transaminases BCAT1 and BCAT2 are potently inhibited by R-2HG[165]. BCAT enzymes catalyze the reversible transamination of branched chain amino acids to produce branched chain α-ketoacids in a reaction that interconverts 2OG and glutamate. BCAT enzymes and glutaminase are the two principal sources of glutamate in glial cells, and inhibition of BCAT by R-2HG induces a dependence on glutaminase in *IDH* mutant glioma. Consistent with these findings, a similar metabolic dependency can be induced by direct inhibition of BCAT1 in *IDH* wild-type glioma cells[166]. It is not known whether other 2OG-like oncometabolites affect non-2OGDD-mediated 2OG-dependent cellular processes. However, given the critical importance of 2OG in cells, perturbations in 2OG metabolism are likely to have wide-ranging biological effects.

Target validation and therapeutic intervention

2OGDD can be inhibited with drug-like small molecules, such as compounds that act as competitive inhibitors of 2OG. This has motivated efforts to therapeutically target oncogenic 2OGDD. For example, loss of the retinoblastoma tumor suppressor protein (pRB) dysregulates the H3K4 demethylase KDM5A, which blocks differentiation and cellular senescence[167], and genetic ablation of *Kdm5a* significantly retards the development of *Rb1-/-* murine tumors[168,169]. KDM5A and its paralogue KDM5B have also been implicated in the development of cancer drug resistance and cancer 'stemness' (Table 1)[170-175]. Studies suggest that

KDM5 inhibitors will not only have anti-tumor activity but will also augment the activity of other anti-cancer drugs. This has led to the development of a number of tool compounds that selectively inhibit KDM5 enzymes[176-178].

KDM6 H3K27 demethylases have also been proposed as therapeutic targets in cancer. A study found that KDM6B and KDM6A have opposing functions in T-cell acute lymphoblastic leukemia (T-ALL), with the former acting as an oncoprotein and the latter as a tumor suppressor[71,179]. A subsequent study argued that KDM6A is an oncoprotein in TAL-positive, but not TAL-negative, T-ALL[180]. These findings provide a rationale for targeting KDM6A in TAL-positive T-ALL and KDM6B in TAL-negative T-ALL. There is also emerging interest in targeting KDM6A in breast cancer[181] and in *histone 3.3 K27M*-positive pediatric brainstem glioma[182].

Several members of the KDM4 family of H3K9 demethylases are overexpressed and sometimes amplified in various cancers, including breast cancer (Table 1)[183-188]. Both gain-of-function and loss-of-function experiments suggest that KDM4C promotes mammary transformation, which is associated with increased expression of stem cell markers[185]. Tool compounds that inhibit KDM4 activity promote H3K9 methylation and inhibit the viability of breast cancer cells, although it is not yet known whether these anti-tumor effects are on-target[189]. KDM4 family members have also been linked to potentiation of ER and AR signaling in breast and prostate cancer, respectively[183,190], and KDM4 inhibition suppress AR signaling and prostate cancer cell proliferation in preclinical models[191,192].

In addition to 2OGDD 'onco-enzymes,' a number of other 2OGDD are potential therapeutic targets in cancer by virtue of the fact that their activities oppose the functions of tumor suppressors that are lost in cancer. For example, loss of the MEN1 tumor suppressor protein attenuates H3K4 methylation by KMT2A (MLL)[169]. We found that murine tumors driven by *Men1* loss are significantly retarded by concurrent inactivation of *Kdm5a*, perhaps by restoring H3K4 methylation homeostasis. Along similar lines, it might be possible to target the loss of tumor-suppressive 2OGDD by inhibiting the enzymes that oppose them. For example, preclinical and early clinical data support the use of DNA demethylating agents in *TET2* mutant myelodysplastic syndrome and AML[193,194].

Past clinical trials have not found ascorbate supplementation to be efficacious for cancer prevention or treatment[195]. However, there is renewed interest in ascorbate as an anti-cancer agent due to recent advances in our understanding of the role of ascorbate in 2OGGD function[196]. A number of ascorbate clinical trials are currently underway in patients with diverse cancers based on the rationale that ascorbate can reactivate 2OGDD tumor suppressors. However, these trials are using high doses of intravenous ascorbate that achieve plasma levels that are orders of magnitude above the ascorbate K_m values for 2OGDD, which are in the 100-300 μM range[23]. It should be noted that such high levels of ascorbate can actually deplete reduced glutathione and paradoxically increase redox stress[197]. It is therefore possible that enhanced redox stress, independent of 2OGDD reactivation, could contribute to any observed antitumor activity of high-dose ascorbate, especially in tumors that are susceptible to oncogene-induced redox

stress[198,199]. It is likewise possible that high ascorbate levels could result in cardiotoxicity similar to that observed in clinical trials of high-dose vitamin E for the prevention of cardiovascular disease[200]. It will be important to define the patients most likely to benefit from ascorbate, such as those with tumors that harbor loss-of-function 2OGGD mutations (e.g. *TET2*), and to identify the optimal level of ascorbate repletion for those patients. Additionally, other approaches to augmenting 2OGDD function, for example with allosteric activators, may provide a way to specifically enhance the activities of 2OGDD tumor suppressors without incurring the risks of activation of oncogenic 2OGDD or the risks of indiscriminate dysregulation of redox homeostasis.

Conclusions and future directions

2OGDD are a superfamily of druggable enzymes linked to a variety of fundamental processes including the HIF-dependent transcriptional response to hypoxia, extracellular matrix formation, DNA methylation, histone methylation, RNA processing and protein translation. 2OGDD can potentially sense oxygen, redox stress, iron availability and 2OG. As such, they are poised to transduce a variety of signals into changes in gene expression and cellular behavior. Oncometabolites transform cells, at least in part, by modulating the activity of specific 2OGDD. In addition, many 2OGDD are recurrently amplified or mutated in cancer or are indirectly affected by mutations that dysregulate the enzymes they oppose.

Our knowledge of the biochemical and biological functions of 2OGDD and their roles in cancer is far from complete. For example, it is not clear how the same enzyme, such as KDM5A, can act as a tumor suppressor in one context and an oncoprotein in another[67,68,168,169,173,175,201]. It is also unclear to what extent the functions of various KDM paralogues are redundant with one another. The most striking evidence for non-redundancy comes from examples where paralogues seemingly oppose one another in the same cellular context, such as KDM6A and KDM6B in T-ALL and kidney cancer[71,202]. Conversely, KDM paralogues can also have a certain degree of functional redundancy[203,204]. Understanding such redundancies is especially important because sgRNA- and shRNA-based dependency studies, such as the Broad Institute DepMap (https://depmap.org/portal/), typically involve inactivation of single genes. In such settings, paralogue compensation can obscure cancer dependencies. First-generation 2OGDD inhibitors typically inhibit multiple paralogues. Whether it will be desirable or not to develop paralogue-specific inhibitors will depend on the answers to these questions. Hopefully, as we gain a greater understanding of the role of 2OGDD in oncogenesis, we will advance the development of clinical 2OGDD modulators to treat patients with cancer.

Table 1: 2OGDD implicated in cancer

Tumor suppressors	Cancer Types	Genetic/transcriptional evidence	Functional evidence	References
EGLN1	PG	mutated and inactivated	loss induces paraganglioma-like lesions	43,44
JARID2*	AML secondary to MDS and MPN	lost by chromosomal deletion	loss promotes blast transformation of MPN to AML in PDX and GEMM models	205
KDM2B*	aggressive brain tumors	Silenced	negatively regulates ribosomal RNA synthesis to suppress cell proliferation	206
KDM3B	MDS and AML	lost by chromosomal deletion	overexpression represses AML colony formation	142,207
KDM5A*	AMKL, GBM	translocation partner with NUP98 in pediatric AMKL; silenced in GBM	inhibits malignant phenotypes in glioma	67,68,208
KDM5B*	AML	**	loss promotes malignant phenotypes	209
KDM5C	many cancer types	mutated and inactivated	maintains genomic stability; negative regulator of enhancer activity	70,210-212
KDM6A*	many cancer types	mutated and inactivated	negatively regulates super-enhancers; loss promotes malignant phenotypes	11,62,69-71
KDM6B*	PAAD	loss of heterozygosity	relieves differentiation arrest, promotes C/EBP-dependent differentiation	86,87
TET1	many cancer types	mutated and inactivated, silenced	loss induces lymphoma, loss promoted malignant phenotypes	88-90,213,214
TET2	many cancer types	mutated and inactivated	promotes malignant phenotypes	9,10,91,92,215
Oncogenes	**Cancer Types**	**Genetic/transcriptional evidence**	**Functional evidence**	**References**
ASPH	HCC, PAAD, CHOL	overexpressed	promotes malignant phenotypes	216-218
FTO	MLL-rearranged AML, advanced CESC	Overexpressed	promotes proliferation and migration, and inhibits apoptosis	219,220
JARID2*	HCC, BLCA	Overexpressed	promotes EMT, promotes colony formation and invasion	221,222
JMJD6	BRCA, NBL	amplified and overexpressed	cooperates with c-MYC to enhance malignant phenotypes	223,224
KDM2A	BRCA, LUAD	amplified and overexpressed	promotes malignant phenotypes and enhances ERK signaling	225,226
KDM2B*	PAAD	Overexpressed	cooperates with KRAS; dependency in synovial sarcoma	227,228
KDM3C	AML	**	dependency in AML1-ETO-positive and MLL-AF9-positive AML	229,230
KDM4A	many cancer types	amplified and overexpressed	promotes chromosomal copy gain and activation of hormone receptors	183,190,231,232
KDM4B	OV, EGC	Overexpressed	promotes malignant phenotypes; overexpression promotes c-Jun function	233,234
KDM4C	BRCA, ESCA, lymphoma, AML	amplified (solid tumors); translocation partner with IGH locus (lymphoma)	promotes malignant phenotypes; dependency in AML	183-188
KDM5A*	many cancer types	amplified and overexpressed	promotes malignant phenotypes and chemotherapy resistance; dependency in Rb-mutated and MEN-mutated tumors	168-173,175,201
KDM5B*	many cancer types	amplified and overexpressed	promotes malignant phenotypes and chemotherapy resistance	82,235
KDM6A*	aggressive BRCA	Overexpressed	promotes malignant phenotypes	181,236,237
KDM6B*	hematologic cancers	Overexpressed	promotes hematopoietic stem cell self-renewal; dependency in T-ALL	71,238,239
KDM7B	LUAD, PRAD	Overexpressed	promotes malignant phenotypes	240-242
MINA53	many cancer types	Overexpressed	promotes malignant phenotypes by activating cyclins and cyclin-dependent kinases	243,244

* evidence for tumor suppressive and oncogenic functions
** none reported

Abbreviations: AML – acute myeloid leukemia; BLCA – bladder urothelial carcinoma; BRCA – breast cancer; CESC – cervical cancer; CHOL – cholangiocarcinoma; EGC – gastric cancer; ESCA – esophageal cancer; GBM – glioblastoma multiforme; HCC – hepatocellular carcinoma; LUAD – lung adenocarcinoma; MDS – myelodysplastic syndrome; MPN – myeloproliferative neoplasm; NBL – neuroblastoma; OV – ovarian cancer; PAAD – pancreatic cancer; PG – paraganglioma; PRAD – prostate cancer

Table 2: K_m/IC50 values of 2OGDD for co-substrates and 2OG analogues

	K_m (μM)			IC50 (μM)			
2OGDD	Fe^{2+}	2OG	O_2	Fumarate	Succinate	R-2HG	S-2HG
ABH2	–	4[148]	–	–	–	420-500[148,245]	150[148]
ABH3	–	–	–	–	–	500[245]	–
BBOX1	10[246]	100-300[246,247]	55[246]	–	–	13200[148]	140[148]
EGLN1	0.05[93]	1-270[22,248,249]	65-250[16,23,249], >450[248]	80[22]	510[22]	7300[148] / K_m 300* [138]	420-1150** [138,148]
EGLN2	0.05[93]	2[22]	230[23]	120[22]	830[22]	– / K_m 210* [138]	630** [138]
EGLN3	0.1[93]	10[22]	230[23]	60[22]	570[22]	–	90** [138]
FIH1	0.5[250]	25-150[249,250]	90-240[249-251]	>10,000[22]	>10,000[22]	1100-1500[138,148]	190-300[138,148]
KDM2A	–	6[148]	–	–	–	110[148]	50[148]
KDM4A	<0.1[21]	6-25[21,64,65,148]	55-170[62,64,65]	1500-2300[21,124]	800[21,124]	2-160[21,148,252]	25-290[21,148]
KDM4B	<0.1[21]	6[21]	150[62]	>5000[21]	2300[21]	150[21]	450[21]
KDM4C	–	4-10[64,148]	160[64]	–	–	80[148]	95[148]
KDM5A	–	–	90[62]	–	–	–	–
KDM5B	<0.1[21]	10[21]	40[62]	>5000[21]	1400[21]	3600-10870[21,137]	630-1600[21,137]
KDM5C	–	5[253]	35[62]	–	–	–	–
KDM5D	–	–	25[62]	–	–	–	–
KDM6A	<0.1[21]	8-10[21,254]	200[62]	3000[21]	270[21]	180[21]	180[21]
KDM6B	6[21]	8-50[21,254]	25[62]	>5000[21]	550[21]	350[21]	750[21]
P4HA1	2[255]	20[255]	40[255]	190** [22]	400** [256]	1800[138]	310[138]
PHYH	20*** [257]	50-190[249,257,258]	95[249]	–	–	–	–
PLOD1	2[259]	100[259]	45[259]	–	–	–	–
TET1	5[20]	55[20]	0.3-30[20,66]	390[20]	540[20]	4000[138]	1000[138]
TET2	4[20]	60[20]	0.5-30[20,66]	400[20]	570[20]	5000[138]	1600[138]

* K_m values for R-2HG as a co-substrate
** K_i value
*** K_a value

Figure Legend

Figure 1: Schematic of 2OGDD reaction and structure of the 2OG analogues

a. The substrate becomes hydroxylated in a reaction utilizing three co-substrates: (1) divalent iron (Fe^{2+}), which is coordinated to the catalytic site by two conserved histidine residues and a positively-charged arginine or lysine residue; (2) 2-oxoglutarte (2OG); and (3) molecular oxygen (O_2), which provides the oxygen atom for the hydroxyl group. During catalysis, 2OG becomes decarboxylated to succinate and CO_2. The hydroxylated substrate can undergo further non-enzymatic modification, such as demethylation. Structural 2OG analogues (fumarate, succinate, R-2-hydroxyglutarate, and S-2-hydroxyglutarate) have the potential to act as competitive inhibitors. Vitamin C is not a direct co-substrate of the 2OGDD reaction but supports the reaction by preventing inadvertent iron oxidation.

b. 2OG and its structurally similar analogues. Abbreviations: ACO, aconitase; CS, citrate synthase; FH, fumarate hydratase; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; OGDC, oxoglutarate dehydrogenase complex; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; SCS, succinyl-CoA synthase; SDH, succinate dehydrogenase

Figure Legend

Figure 2: Kinetic and inhibitory values of 2OGDD for co-substrates and 2OG analogues.

a. μM K_m values of oxygen, iron and 2-oxoglutarate. *K_a value.

b. μM IC50 values of fumarate, succinate, R-2-hydroxyglutarate and S-2-hydroxyglutarate. **K_i value.

High K_m or low IC50 values that provide potential for regulation are in the outer perimeter. In the case of 2OGDD for which divergent K_m/IC50 values have been reported, the values presented are those determined by our laboratories or are an average of all reported values. For full details see Table 2.

Figure Legend

Figure 3: Structural basis for the differential oxygen-sensing capacities of KDM6A and KDM6B JmjC domains.

a. Alignment of KDM6A isoform 3 (NP_066963.2) and KDM6B isoform 1 (NP_001073893.1) residues with ClustalW shows a high degree (79.2%) of sequence identity.

b. The JmjC domain crystal structures for KDM6A (PDB: 3avr)[260] and KDM6B (PDB: 5oy3)[261] were obtained from the Protein Data Bank and composite images of the active sites, with added oxygen molecules (red) to illustrate scale, were created using PyMOL. These structures show that the size of the oxygen-binding cavity opening is smaller in KDM6A than KDM6B, offering a potential explanation for the differential O_2 sensing by the two closely-related enzymes. The bound substrate is indicated in yellow (KDM6A) or orange (KDM6B).

Figure Legend

Figure 4: Dysregulation of 2OGDD activity by hypoxia.

The extent to which a given 2OGDD is inhibited by hypoxia is a function of multiple factors, including their level of expression, their oxygen affinity and their sensitivity to inhibition by succinate and S-2HG.

a. Under normoxic conditions, hypoxia-sensitive (high O_2 Km) and hypoxia-resistant (low O_2 Km) 2OGDD are active (●).

b. Moderate hypoxia results in decreased activity (◉) of only hypoxia-sensitive 2OGDD, including EGLN enzymes. EGLN inhibition results in induction of HIF transcriptional activity.

c. More profound hypoxia results in more profound inhibition (○) of hypoxia-sensitive 2OGDD and increased expression HIF target genes, including LDH and a subset of 2OGDD. Production of S-2HG by LDH is potentiated by hypoxia and by cellular acidosis, resulting in accumulation of high concentrations of S-2HG. In some cell types, severe hypoxia also dysregulates TCA cycle function, which results in enhanced production of succinate. The S-2HG and succinate that accumulate under hypoxia promote the inhibition of 2OGDD that are both hypoxia-sensitive and hypoxia-resistant.

Abbreviations: GDH, glutamate dehydrogenase; FH, fumarate hydratase; HRE, HIF-responsive element; IDH, isocitrate dehydrogenase; LDH, lactate dehydrogenase; OAA, oxaloacetate; SDH, succinate dehydrogenase

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Disclosures:

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William G. Kaelin, Jr.
Lilly Pharmaceuticals (Board of Directors)
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Cedilla Therapeutics (Founder)
Fibrogen (Scientific Advisory Board, Royalties)
Nextech Invest (Scientific Advisory Board, Carried Interest)
Tango Therapeutics (Founder)
Tracon Therapeutics (Scientific Advisory Board)

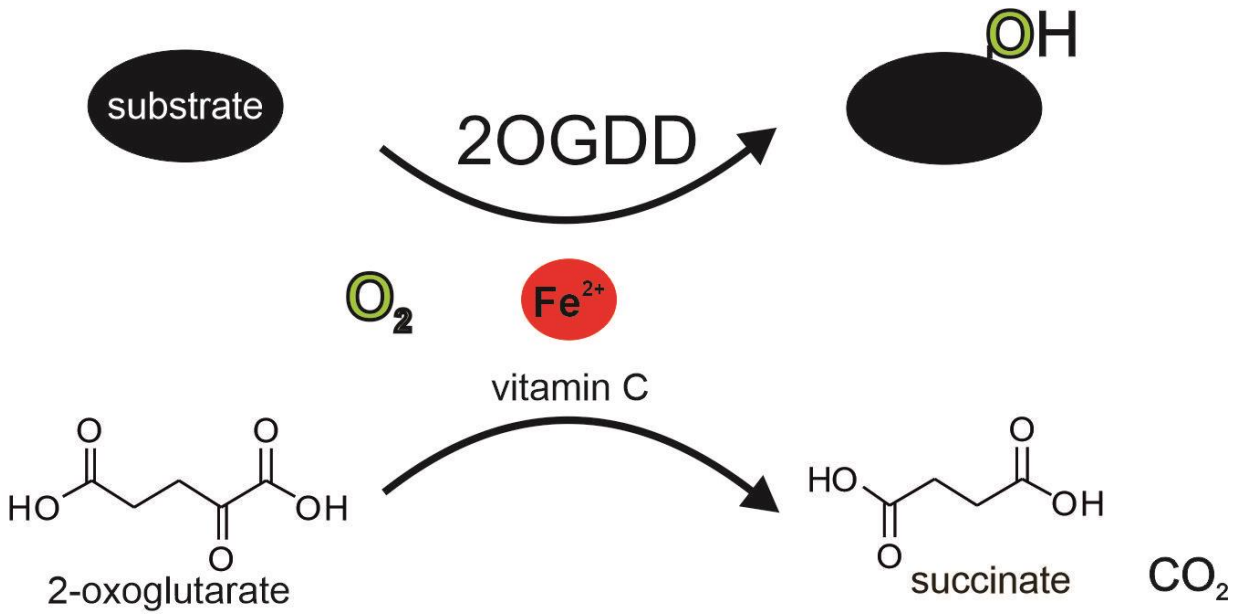


Figure 1: Schematic of 2OGDD reaction

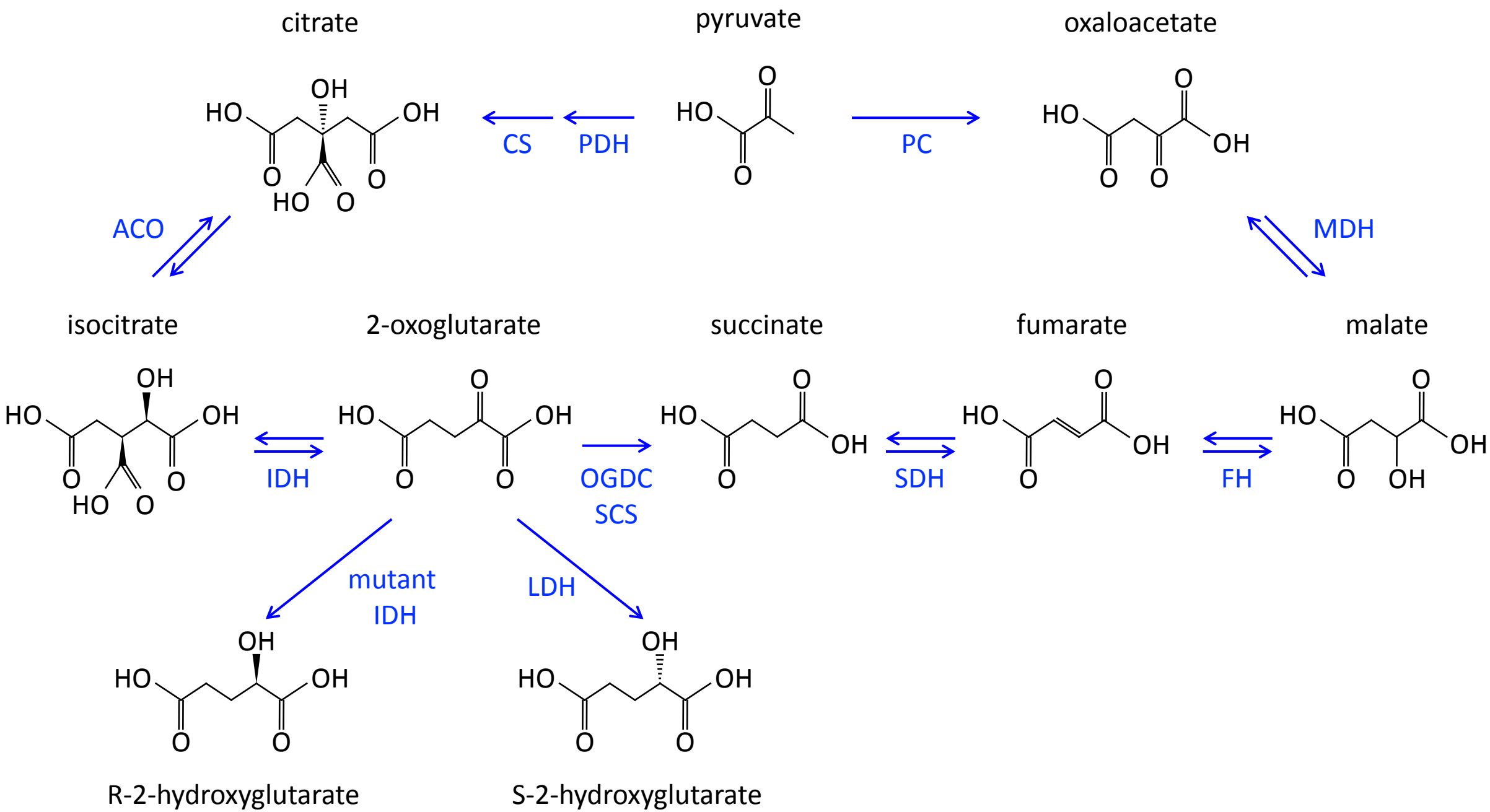
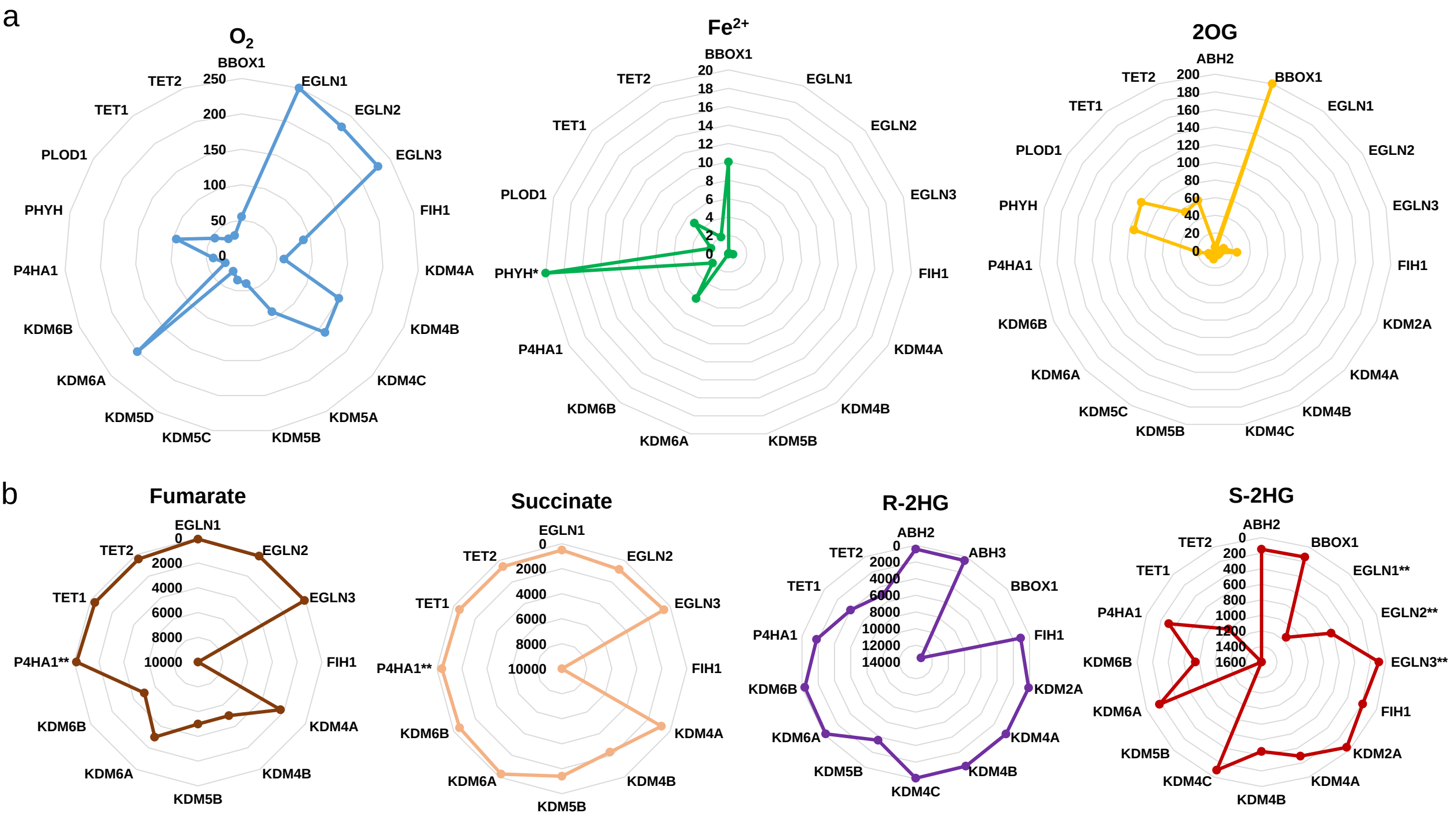


Figure 1b: 2OG and its structurally similar analogues.


a
O_2
BBOX1
EGLN1
EGLN2
EGLN3
FIH1
KDM4A
KDM4B
KDM4C
KDM5A
KDM5B
KDM5C
KDM5D
KDM6A
KDM6B
P4HA1
PHYH
PLOD1
TET1
TET2
250
200
150
100
50
0
Fe^{2+}
BBOX1
EGLN1
EGLN2
EGLN3
FIH1
KDM4A
KDM4B
KDM5B
KDM6A
KDM6B
P4HA1
PHYH*
PLOD1
TET1
TET2
20
18
16
14
12
10
8
6
4
2
0
2OG
ABH2
BBOX1
EGLN1
EGLN2
EGLN3
FIH1
KDM2A
KDM4A
KDM4B
KDM4C
KDM5B
KDM5C
KDM6A
KDM6B
P4HA1
PHYH
PLOD1
TET1
TET2
200
180
160
140
120
100
80
60
40
20
0
b
Fumarate
EGLN1
EGLN2
EGLN3
FIH1
KDM4A
KDM4B
KDM5B
KDM6A
KDM6B
P4HA1**
TET1
TET2
0
2000
4000
6000
8000
10000
Succinate
EGLN1
EGLN2
EGLN3
FIH1
KDM4A
KDM4B
KDM5B
KDM6A
KDM6B
P4HA1**
TET1
TET2
0
2000
4000
6000
8000
10000
R-2HG
ABH2
ABH3
BBOX1
FIH1
KDM2A
KDM4A
KDM4B
KDM4C
KDM5B
KDM6A
KDM6B
P4HA1
TET1
TET2
0
2000
4000
6000
8000
10000
12000
14000
S-2HG
ABH2
BBOX1
EGLN1**
EGLN2**
EGLN3**
FIH1
KDM2A
KDM4A
KDM4B
KDM4C
KDM5B
KDM6A
KDM6B
P4HA1
TET1
TET2
0
200
400
600
800
1000
1200
1400
1600

a

```
KDM6A 1099 QLHELTKLPAFVRVVSAGNLLSHVGHTILGMNTVQLYMKVPGSRTPGHQENNNFCSVNIN
KDM6B 1343 QLQELLKLPAFMRVTSTGNMLSHVGHTILGMNTVQLYMKVPGSRTPGHQENNNFCSVNIN
           **:** *****:**.*:**:****************************************

KDM6A      IGPGDCEWFVVPEGYWGVLNDFCEKNNLNFLMGSWWPNLEDLYEANVPVYRFIQRPGDLV
KDM6B      IGPGDCEWFAVHEHYWETISAFCDRHGVDYLTGSWWPILDDLYASNIPVYRFVQRPGDLV
           *********.* * ** .:. **:::.:::* ***** *:*** :*:*****:*******

KDM6A      WINAGTVHWVQAIGWCNNIAWNV 1241
KDM6B      WINAGTVHWVQATGWCNNIAWNV 1485
           ************ **********
```

b

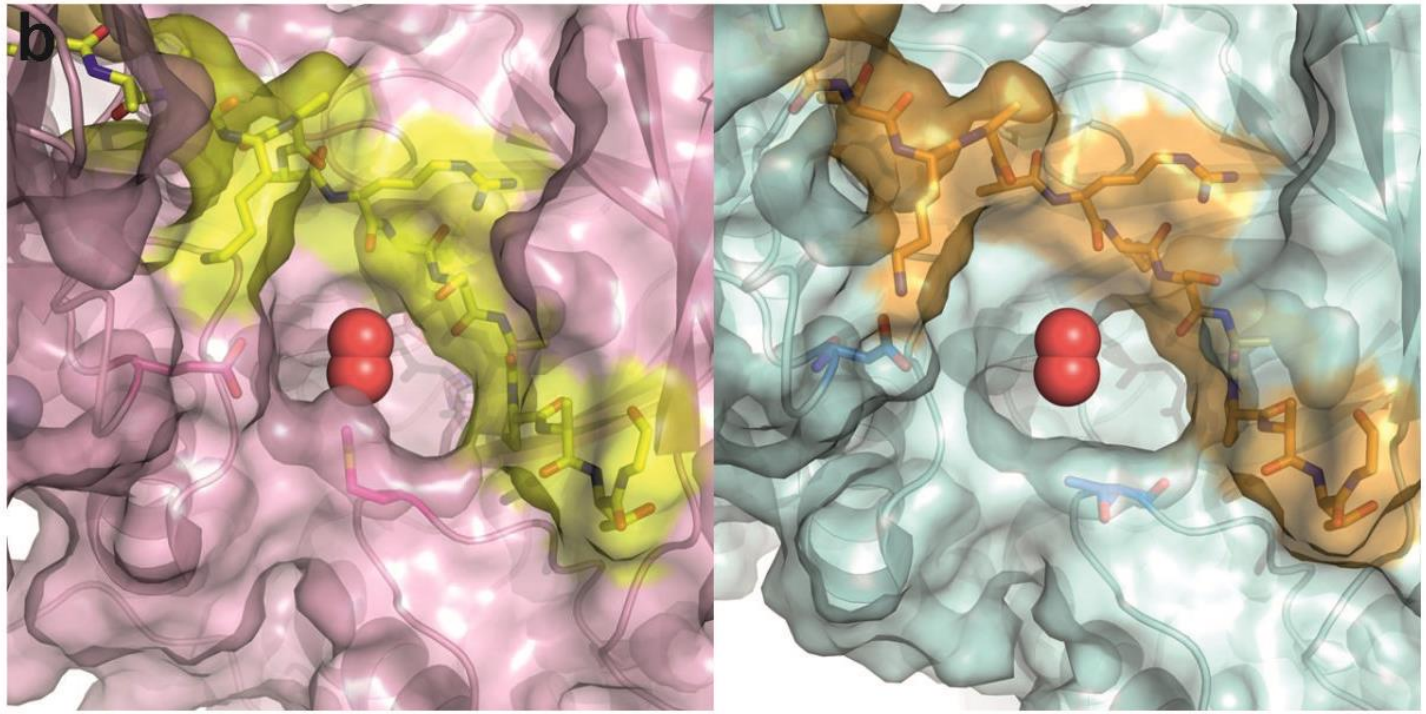


Figure 3: Structural basis for the differential oxygen sensing capacities of KDM6A and KDM6B JmjC domains.

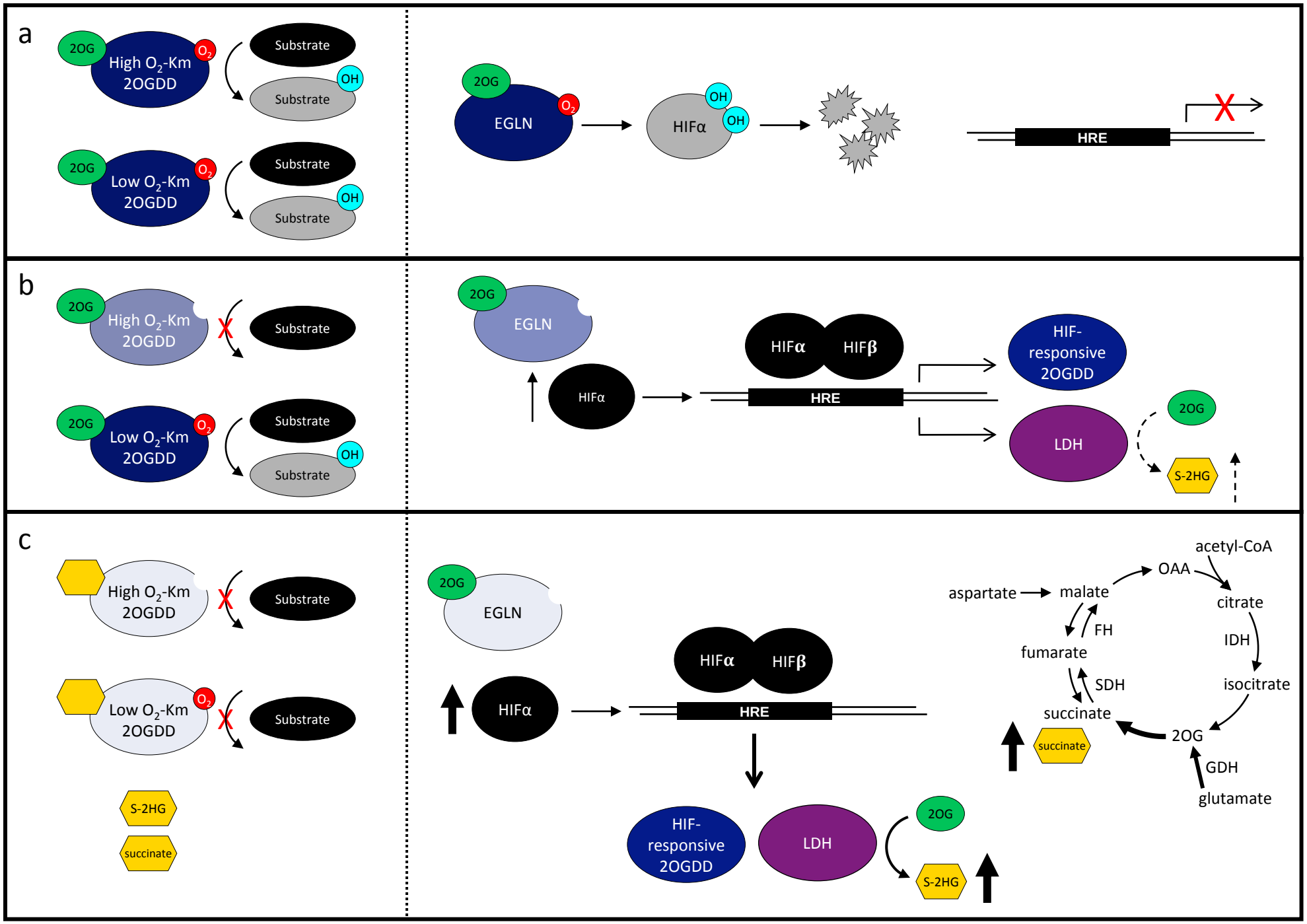


Figure 4: Dysregulation of 2OGDD activity by hypoxia.

Box 1: List of 2OGDD and other proteins that utilize and regulate 2OG

2OGDD

Protein hydroxylases
ASPH, EGLN1-3, FIH1, JMJD4-7, LEPRE1, LEPREL1, LEPREL2, MINA53, NO66, OGFOD1, P4HA1-3, P4HTM, PLOD1-3

Histone demethylases
KDM2A, KDM2B, KDM3A, KDM3B, KDM4A, KDM4B, KDM4C, KDM4D, KDM5A, KDM5B, KDM5C, KDM5D, KDM6A, KDM6B, KDM6C, KDM7A, KDM7B, KDM9

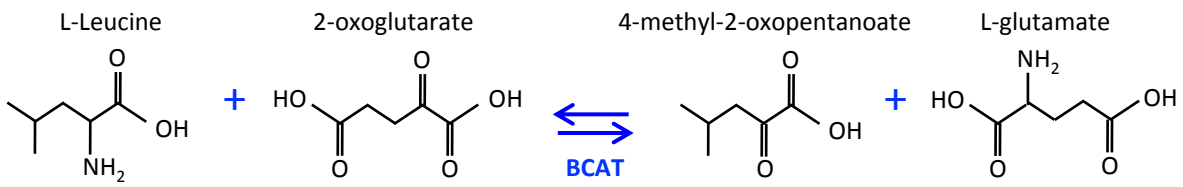
Nucleic acid oxygenases
ABH1, ABH2, ABH3, ABH5, ABH8, FTO, TET1-3, TYW5

Fatty acid & small molecule oxygenases
BBOX1, PHYH, TMLHE

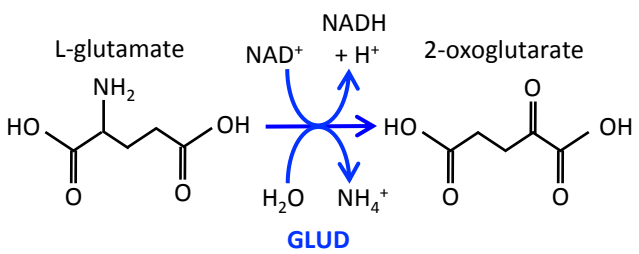
Unassigned catalytic function
ABH4, ABH6, ABH7, ASPHD1, ASPHD2, HSPBAP1, JARID2, JMJD8, KDM3C, OGFOD2, OGFOD3, PHF2, PHYHD1

Other proteins involved in 2OG metabolism

Transaminases
AADAT, ABAT, AGXT, AGXT2, BCAT1, BCAT2, CCBL1, CCBL2, GFPT1, GFPT2, GOT1, GOT2, GPT, GPT2, OAT, PSAT1, TAT



Dehydrogenases
AASS, ADHFE1, ALDH1B1, ALDH2, ALDH3A2, ALDH7A1, ALDH9A1, DHTKD1, DLD, GLUD1, GLUD2, IDH1, IDH2, IDH3A, IDH3B, IDH3G, OGDH, OGDHL, PHGDH, (D/R)-2HGDH, (L/S)-2HGDH



Transporters
SLC22A6, SLC22A7, SLC22A8, SLC22A11, SLC22A20, SLC22A25, SLC22A13, SLC22A12

Other
DLST, NIT2