Metabolism, Activity, and Targeting of D-and L-2-Hydroxyglutarates

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Abstract

Isocitrate dehydrogenases (IDH1/2) are frequently mutated in multiple types of human cancer, resulting in neomorphic enzymes that convert α-ketoglutarate (α-KG) to 2-hydroxyglutarate (2-HG). The current view on the mechanism of IDH mutation holds that 2-HG acts as an antagonist of α-KG to competitively inhibit the activity of α-KG-dependent dioxygenases, including those involved in histone and DNA demethylation. Recent studies have implicated 2-HG in activities beyond epigenetic modification. Multiple enzymes have been discovered that lack mutations but that can nevertheless produce 2-HG promiscuously under hypoxic or acidic conditions. Therapies are being developed to treat IDH-mutant cancers by targeting either the mutant IDH enzymes directly or the pathways sensitized by 2-HG.

Production of Oncometabolite 2-HG by Cancer-Associated Mutations in IDH1 and IDH2

The metabolic genes encoding IDH1/2 were found to be mutated by two genomic studies in human glioma and acute myeloid leukemia (AML) in 2008 and 2009 [1,2]. These seminal, and surprising, findings set off intense efforts to determine the biochemical mechanisms and clinical implications of IDH mutations. We now know that IDH1 and IDH2 are the most frequently mutated metabolic genes in human cancer [3,4]. IDH1 and IDH2 mutations occur frequently in low-grade glioma (~80%), AML (~12%), cartilaginous tumors (~75%), intrahepatic cholangiocarcinoma (ICC) (15–20%), and angioimmunoblastic T cell lymphoma (AITL) (30–40%), sporadically in melanoma (6%), prostate cancer (3%), hepatocellular carcinoma (HCC) (1%), and medulloblastoma (1%), and infrequently in thyroid, pituitary, stomach, breast, and pancreatic cancers. Genomic studies have also

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established that IDH mutations are early events, perhaps the first genetic lesions that occur during tumorigenesis [5–7] (see Outstanding Questions). Importantly, IDH mutations define distinct subtypes of tumors within otherwise heterogeneous glioma [7–9], AML [10], ICC, and HCC cancers [11], and these mutations exhibit unique ages of onset, clinical behaviors, and responses to therapy. Thus, IDH mutations appear to initiate pathogenesis by a common mechanism.

### Outstanding Questions

- What enzymes produce 2-HG in cells lacking IDH mutations?
- What metabolic and stress conditions affect 2-HG production in cells lacking IDH mutations?
- Does 2-HG bind to and affect the activities of proteins besides α-KG-dependent dioxygenases?
- Does 2-HG function in any normal cellular process?

Mutations targeting IDH1 and IDH2 in different types of tumors share four features, providing initial insights into the mechanism of IDH mutations. First, IDH1 and IDH2 mutations are somatic, not germline. Second, all tumors with IDH1/2 mutations are heterozygous, suggesting a gain-of-function and dominant effect over the remaining wild-type allele. Third, nearly all IDH1/2 mutations occur in a few hotspots in the enzymes’ active sites – Arg132 in IDH1 and correspondingly Arg172 in IDH2, plus Arg140 in IDH2—suggesting a direct impact on the catalytic properties of the enzymes. Fourth, IDH1 and IDH2 mutations occur in a mutually exclusive manner, indicating a common biochemical mechanism targeting the same pathway by either mutant protein.

During the past 8 years, we have gained extensive mechanistic understanding of how IDH mutations contribute to tumorigenesis. Soon after their discovery, it was demonstrated that all tumor-derived mutations in IDH1 and IDH2 disrupt their normal catalytic activity; that is, converting isocitrate to α-KG [also known 2-oxoglutarate (2OG)] [12–14]. However, the most remarkable feature of IDH mutations is the neomorphic enzymatic (see Glossary) activity acquired by the mutant enzymes, which can convert α-KG to a previously little-known metabolite, D-2-hydroxyglutarate (D-2-HG), now referred to as an oncometabolite [14,15]. Although long recognized as a key nexus for multiple metabolic pathways, α-KG is also a co-substrate for α-KG/Fe(II)-dependent dioxygenases [16,17]. This non-metabolic function of α-KG and the fact that D-2-HG differs from α-KG by only an oxygen in place of a hydroxyl group (Figure 1) have led to the discovery that D-2-HG is an antagonist of α-KG, competitively inhibiting α-KG/Fe(II)-dependent dioxygenases, including the JmjC-domain family of histone demethylases and the TET family of DNA dioxygenases [18,19]. This antagonist property provides a biochemical basis for, and is supported by, the genetic observations that IDH1/2 mutations are associated with the CpG island methylator phenotype (G-CIMP) in glioma [20] and ICC [21]. It is further supported by the observation that G-CIMP can be established in primary astrocytes when mutant IDH1 is ectopically expressed [22]. This property is also consistent with the fact that IDH1/2 mutation occurs in

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a mutually exclusive manner with TET2 mutations in AML [23]. Co-crystal structural studies reveal that 2-HG occupies the same space as α-KG in the active site of histone demethylases [19]. High 2-HG concentration is associated with increased histone methylation in primary glioma and induces cell differentiation in vitro [19,24]. Thus, by impairing histone and DNA methylation, thereby altering gene expression, IDH mutations block or skew progenitor cell differentiation, promoting tumorigenesis in conjunction with subsequent oncogenic mutations.

In this review we recap early investigations on 2-HG before the discovery of its production by mutant IDH enzymes. We then discuss recent developments regarding the metabolism, biochemical targets, and cellular functions of 2-HG and therapeutic strategies to treat mutant IDH-associated tumors.

**Evolutionary Conservation and Functional Importance of 2-HG Removal**

2-HG was first described in 1868 by the German biochemist Karl Heinrich Ritthausen [25], who was better known for his discovery of glutamic and aspartic acids. In 1872, Ritthausen determined the optical rotation of 2-HG [26]. In 1920, Swedish physiologist Torsten Thunberg described 2-HG as a substrate of intermediary metabolism in frog muscle, and he also demonstrated that 2-HG can be oxidized to α-KG [27]. Not until 17 years later did Weil-Malherbe characterize an enantiomer-specific L-2-HG dehydrogenase that oxidizes L-2-HG to α-KG in many animal tissues [28]. However, 2-HG did not attract much interest until recently because no physiological function of 2-HG was described in any organism in which it was studied, including bacteria, yeast, plants, Drosophila, other animals, and humans. This lack of interest changed in 1980 with the discovery that 2-HG accumulation is linked to so-called 2-hydroxyglutaric aciduria (2HGA) diseases. 2-HG can be detected in the urine of healthy humans at a concentration of 20 mg/g (23 μmol/mmol creatinine) [29,30]. In 1980, D-2-HG and L-2-HG were found at 60- to 100-fold and 11- to 25-fold higher concentrations in two unrelated children [31,32]; these phenotypes define two clinically related, biochemically distinct diseases, D- and L-2-hydroxyglutaric aciduria (D2HGA and L2HGA) [33]. 2-HG aciduria begins during infancy or early childhood and worsens over time, usually leading to severe disability by early adulthood. Both diseases are rare and they share many clinical features, including delayed development, seizures, and, most notably, abnormalities in the cerebrum that affect muscle function, speech, vision, thinking, emotion, and memory. In addition, brain malignancies have been reported in some individuals with L2HGA, but not with D2HGA [34].

The causes and mechanisms of 2HGA diseases have been identified in 2004. Separate biochemical purification of the enzymes acting on L-2-HG and mapping of L2HGA DNA led to the simultaneous discovery of L-2-hydroxyglutarate dehydrogenase (L2HGDH) [35,36]. The same purification revealed a separate enzyme, D-2-hydroxyglutarate dehydrogenase (D2HGDH), that acts specifically on D-2-HG [37]. The D2HGDH gene was found to be mutated in about half of individuals with D2HGA [38,39] and, recently, in a small fraction of diffuse large B cell lymphomas [40]. These studies established that L2HGDH and D2HGDH localize in the mitochondrion and, when homozygously mutated in the germline, cause L2HGA and D2HGA, respectively (Figure 1). Subsequently, sequence
comparisons and biochemical studies have characterized a single FAD-dependent L2HGDH enzyme in Escherichia coli (YgaF) [41] and Drosophila (CG10639) [42] and two yeast D2HGDH enzymes (Dld2 and Dld3) [43]. Thus, removal of 2-HG is an evolutionarily conserved, and likely to be functionally important, activity. However, only recently has the source of 2-HG production being identified.

In 2010, the puzzle presented by the half of individuals with D2HGA that lack D2HGDH mutations was explained by the finding of IDH2 mutations in these individuals (now referred to as type II D2HGA) [44]. A third type of 2HGA, combined D,L-hydroxyglutaric aciduria, was reported in neonates with encephalopathy and accumulation of both D- and L-2-HG [45]. This phenotype is caused by mutations in the mitochondrial citrate carrier SLC25A1 [46]. It is currently unclear how SLC25A1 deficiency causes elevated D- and L-2-HG. Possibly, the phenomenon may be explained by mitochondrial accumulation of citrate and other TCA intermediates, including \( \alpha \)-KG, the precursor of both 2-HG enantiomers whose production is catalyzed promiscuously by various enzymes (see below).

### 2-HG Production by Promiscuous Enzymatic Reactions

In contrast to the long history of discovery of 2-HG and enzymes for its metabolic removal, only recently have we understood how 2-HG is produced. The first reported 2-HG-producing activity in mammalian cells was an oxidoreductase in rat kidney, liver, and brain mitochondria that catalyzes the interconversion of several oxoacids, including \( \alpha \)-KG, and hydroxy-acids, including D-2-HG [41]. As determined by mass isotopomer distribution analysis, cultured lymphoblasts from individuals with D2HGA or L2HGA rapidly interconverted \( \alpha \)-KG to either D-2-HG or L-2-HG by an activity in the mitochondrion [47,48]. Thus, \( \alpha \)-KG is the apparent direct precursor of D- and L-2-HG in mammals and other organisms. In 2007, Schaftingen and colleagues identified the elusive mitochondrial ‘\( \alpha \)-KG reductase’. Surprisingly, a previously well-characterized enzyme, mitochondrial malate dehydrogenase (MDH2/mMDH) and its cytoplasmic counterpart (MDH1/cMDH) can catalyze NAD\(^+\)-dependent \( \alpha \)-KG reduction to L-2-HG [49]. MDH normally catalyzes interconversion of malate and oxaloacetate. Thermodynamically, MDH favors oxaloacetate-to-malate reduction, but the reaction may be driven in reverse by oxaloacetate and NADH (Figure 2). MDH-catalyzed \( \alpha \)-KG-to-D- or –L-2-HG reduction would be coupled with NADH oxidation to NAD\(^+\) as in the oxaloacetate-to-malate reduction reaction, but MDH conversion of \( \alpha \)-KG to 2-HG was \( 10^7 \)–\( 10^8 \) less efficient catalytically [49]. Thus, the investigators proposed that L-2-HG is formed by a ‘side reaction’ of MDH, L-2-HG serves no particular physiological purpose, and L-2-HG is removed by L2HGDH during ‘metabolite repair’.

Although enzymes are remarkably efficient and specific, many enzymes can exhibit enzyme promiscuity in catalyzing a fortuitous side reaction in addition to their primary reactions [50]. While normally physiologically irrelevant, under certain environmental conditions these promiscuous activities may affect enzyme evolution and may benefit or harm cell function. Besides MDH, at least two additional metabolic enzymes, 3-phosphoglycerate dehydrogenase (PHGDH) and lactate dehydrogenase A (LDHA), have been reported to promiscuously produce 2-HG. PHGDH catalyzes the first step of serine biosynthesis,
converting 3-phosphoglycerate (3PG) to 3-phosphohydroxy pyruvate (3PHP) coupled with NAD\(^+\) reduction, a pathway that is conserved in all organisms (Figure 2). Unexpectedly, \textit{E. coli} PHGDH (SerA) was found to be capable of catalyzing the NADH-dependent reduction of \(\alpha\)-KG to both L- and D-2-HG [51]. A similar promiscuous reductase activity was detected for PHGDH, which converts \(\alpha\)-KG to D-2-HG in human breast cancer cell lines [52] and in yeast [43]. The affinity of \textit{E. coli} and human PHGDH for \(\alpha\)-KG is 30- to 40-fold lower than the affinity for its primary substrate 3PG, an effect that may explain why the \(\alpha\)-KG reductase activity of PHGDH was not detected in a different study [53].

In a study to determine how hypoxic cells maintain the synthesis of citrate, it was found that mitochondrial IDH2 can catalyze the reverse reaction (reductive carboxylation) in hypoxic cells, converting \(\alpha\)-KG to isocitrate and then citrate and, surprisingly, with a concomitant increase in D-2-HG [54]. It is unknown whether wild-type IDH2 can directly produce 2-HG. Subsequent metabolomics studies discovered that hypoxia increased \(\alpha\)-KG and L-2-HG in a broad range of transformed human cell lines [55,56]. Three different mechanisms were proposed for the hypoxia-induced L-2-HG elevation: reduced malate and increased NADH favoring the promiscuous reaction of MDH2, reduced \textit{D2HGDH} gene expression [56], and promiscuous reaction by LDHA [55] (Figure 2). L-2-HG accumulation in hypoxic cells can be efficiently reduced by the single knockdown of LDHA and L-2-HG accumulation was modestly decreased further by the double knockdown of LDHA and \textit{MDH2}, suggesting that LDHA is the major producer of L-2-HG in hypoxic cells [55]. Similarly, knockdown of \textit{LdhA}, but not \textit{Mdh1} or \textit{Mdh2} alone, decreased L-2-HG in hypoxic primary mouse T cells in response to T cell receptor stimulation [57]. This finding is consistent with a \textit{Drosophila} genetic study showing that dLDH is necessary and sufficient to produce L-2-HG from \(\alpha\)-KG in rapidly growing larvae [42] (see Outstanding Questions).

Metabolic acidosis is a common feature of hypoxia and this has prompted investigators to test the effect of pH on 2-HG production by different enzymes. It was found that acidity stimulates purified recombinant LDHA, and to a lesser extent MDH, to produce L-2-HG by several orders of magnitude \textit{in vitro} and accumulates L-2-HG in cultured cells by several-fold in a manner correlating with pH decrease [58,59]. \(\alpha\)-KG is a weak acid that equilibrates between protonated and deprotonated forms. Low pH drives the equilibrium toward protonation. According to molecular modeling, LDHA prefers the protonated form of \(\alpha\)-KG. When the pH was decreased from 7.4 to 6.0, a pH near that in hypoxic cells, the Michaelis–Menten constant (\(K_m\); an inverse measure of affinity) of LDHA for \(\alpha\)-KG was reduced by about fourfold [58].

### Pathological Significance of 2-HG Accumulation in Cells Lacking IDH Mutation

Elevated 2-HG has now been observed \textit{in vitro} in many tumor cells lacking an \textit{IDH} mutation. These include DLD1 and RKO colorectal cancer cells [60], papillary thyroid carcinoma [61], renal cell carcinoma [62], Myc-driven ER-negative breast cancer [63], and AML [64]. Elevated 2-HG is observed even in non-tumorigenic heart during ischemic preconditioning [65] and hematopoietic stem cells after disruption of the respiratory chain.

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[66]. Are there additional enzymes, besides PHDGH, MDH1/2, and LDHA, and other conditions besides hypoxia and acidity, that can promiscuously produce or stimulate 2-HG production? In human cells both D2HGDH and L2HGDH are localized in the mitochondrion. Thus, the mitochondrion is probably the primary site that accumulates toxic concentrations of 2-HG, and this organelle is likely to have needed to evolve a mechanism for 2-HG removal (see Outstanding Questions). However, PHDGH, LDHA, and MDH1 are all localized in the cytoplasm. Is MDH2 the only enzyme producing 2-HG in mitochondria or are other enzymes (e.g., IDH2) involved? Enzyme promiscuity can be affected by reaction conditions (e.g., solvent, pH, temperature), substrate similarity (coenzyme, enantiomers), the structure of the substrate-binding pocket, and other conditions that relax active site stringency [67]. An oxidase or reductase whose active site can accommodate α-KG could theoretically produce 2-HG. The neomorphic activity acquired by IDH1/2 mutations is an illuminating example of enzyme promiscuity: a single point mutation at R132 in IDH1 or R172 in IDH2 reorganizes the active site, causing reduced affinity for its principal substrate (isocitrate) and increased affinity for NADPH to support α-KG reduction [14,68].

Can cells lacking IDH mutations acquire pathological concentrations of 2-HG? Hypoxia and an acidic extracellular environment are common features of tumor tissues, suggesting that 2-HG may accumulate to a pathologically significant concentration in cells lacking IDH mutations. 2-HG has been reported to reach millimolar concentrations comparable with concentrations found in IDH1-mutated cells [57,60]. Because L-2-HG is five- to tenfold more potent than D-2-HG in inhibition of some α-KG-dependent dioxygenases [18,19], it seems plausible that 2-HG elevation in IDH wild-type cells would have a pathological effect. The human PHGDH gene is genomically amplified in a subset of breast cancer [69] and melanoma [70] and its knockdown decreases cell proliferation but not intracellular serine concentration; thus, increased D-2-HG, but not altered serine biosynthesis, may contribute to tumorigenesis in these tumors. More broadly, hypoxia and an acidic environment resulting from lactate accumulation (the Warburg effect) are commonly associated with tumorigenesis. Because only a limited number of tumor samples has been analyzed for 2-HG accumulation, we do not know whether moderate 2-HG accumulation contributes to tumor development. A recent study found that tumor hypoxia broadly reduced the activity of TET dioxygenases and caused DNA hypermethylation [71]. Reduced TET activity resulted from a deficient supply of oxygen, a co-substrate of α-KG-dependent dioxygenases, but it remains to be determined whether 2-HG was elevated in these tumors to a concentration sufficiently high to have inhibited TET activity. An intriguing, still untested possibility is whether 2-HG elevation broadly impairs the DNA methylation landscape and thereby contributes to tumorigenesis.

2-HG Activity beyond Epigenetic Alteration

Most efforts to understand the mechanisms of IDH mutations and 2-HG in tumorigenesis have thus far focused on the KDM and TET enzymes and alterations of histone and DNA methylation, which have been extensively reviewed recently (e.g. [4,72]). Recent studies have revealed that D-2-HG can also antagonize other α-KG-dependent dioxygenases that are not involved in epigenetic alteration (Table 1).
2-HG Can Inhibit EGLN/PHD and Collagen Hydroxylases

An early study found that D-2-HG has the lowest half-maximal inhibitory concentration (IC$_{50}$ = 24–106 μM) toward KDM members, followed by ABH2 (424 μM), FIH (1500 μM), PHD2 (7300 μM), and BBOX1 (13 200 μM) [18]. Because D-2-HG accumulates to as high as 5–35 μmol/g (5–35 mM) in IDH-mutated glioma [68], D-2-HG in IDH-mutant tumors could inhibit other α-KG-dependent dioxygenases and thereby affect cellular pathways besides epigenetic control. The first reported instance of an IDH mutation was a non-epigenetic α-KG-dependent dioxygenase – the EGLN/PHD hydroxylase that catalyzes proline hydroxylation of hypoxia-inducible transcription factor 1α (HIF-1α). Hydroxylation of HIF-1α promotes its binding to the cullin2-VHL E3 ubiquitin ligase and subsequent degradation. In cultured cells, expression of mutant IDH1 increased HIF-1α, an effect that was reversed by the addition of cell-permeable α-KG [12]. HIF-1α accumulation was found in the brain tissues of mice expressing a brain-specific Idh1 R132H conditional allele [73]. Whether PHD is a major target of mutant IDH is uncertain given the finding that D-2HG has a lower IC$_{50}$ toward PHD than to other α-KG-dependent dioxygenases and that HIF could also be stabilized by other conditions, such as oxygen deprivation. A surprising finding was that D- but not L-2-HG stimulates PHD activity and reduces HIF levels, leading to enhanced proliferation of astrocytes [74]. Later, a separate study found that both enantiomers of 2-HG can be non-enzymatically oxidized to α-KG to concentrations that support in vitro catalysis by α-KG-dependent dioxygenases, including PHD2 [75]. L-2-HG is more potent than D-2HG in inhibiting α-KG-dependent dioxygenases and was found to also inhibit PHD and stabilize HIF-1α in vitro and in cells [58,59,75]. In addition to EGLN/PHD hydroxylase, the activity of collagen hydroxylase was inhibited in Idh1-mutant brain tissues, as seen by the perturbation of collagen maturation and basement membrane function [73]. Thus, D-2-HG, when accumulated to a high level, may inhibit both groups of prolyl hydroxylases and impairs HIF-1α signaling and collagen biogenesis.

2-HG Causes Genetic Instability

Disruptions in DNA repair pathways cause cells to accumulate DNA damage and become predisposed to oncogenic transformation. An ancient and critical DNA repair enzyme is E. coli AlkB, which was discovered by its activity to confer resistance to alkylating agents. AlkB repairs the cytotoxic lesions 1-methyladenine (1 mA) and 3-methylcytosine (3mC) in DNA, in a reaction requiring oxygen, α-KG, and Fe(II) [76,77]. Nine AlkB homolog (ALKBH) genes exist in mammalian cells (ALKBH1–8, FTO) and ALKBH2 and ALKBH3 repair alkylating DNA damage by a mechanism similar to that of E. coli AlkB [78]. D-2-HG has been demonstrated to directly inhibit ALKBH2 and ALKBH3 in vitro [18,79]. Cells expressing mutant IDH display reduced repair kinetics and accumulate double-strand DNA breaks (DSBs). These cells are sensitized to alkylating agents that depend on the catalytic activity of mutant IDH to produce D-2-HG, thus linking D-2-HG accumulation to DNA damage [79]. A more detailed kinetic analysis showed that when the ratio of D-2-HG to α-KG was set to mimic their average concentrations in IDH-mutated glioma (3.73 mM vs 100 μM), the repair efficiencies of ALKBH2 and ALKBH3 were 73–88% inhibited and were restored by addition of α-KG [80]. These findings suggested that the inhibition of ALKBH observed in vitro and in cultured cells can occur in IDH-mutated tumors.

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Besides ALKBH inhibition, D-2-HG can also impair DNA repair and cause genetic instability indirectly, by altering the expression of genes involved in DNA repair. In HCT116 colon cancer cells and HeLa cervical cancer cells engineered to express R132H mutant IDH1 in the endogenous locus, there was a significant increase in DSBs and sensitivity to inhibitors of poly(adenosine 5′-diphosphate-ribose) polymerase (PARP) [81]. These increases were attributed to a marked deficiency in homologous recombination (HR), a major pathway of DSB repair. An siRNA screen targeting 64 known α-KG-dependent dioxygenases identified two closely related H3K9 histone demethylases, KDM4A and KDM4B, that link DSB repair to the recruitment of repair factors to sites of DNA damage [81]. A possible link between KDM inhibition and increased DNA damage was suggested in a study of myeloid lineage-specific Idh1 R132H conditional knock-in mice, which exhibited diminished DNA repair, age-dependent accumulation of DNA damage, and impaired self-renewal of long-term hematopoietic stem cells [82]. These defects were linked to an increase in repressive histone methylation (e.g., H3K9, H3K27), both globally and in the promoter of the DNA damage sensor ATM, and to decreased ATM expression in IDH1-mutated mouse hematopoietic stem cells and human AML samples [82].

In sum, these studies suggest that IDH mutation and D-2-HG accumulation cause genetic instability and contribute to tumorigenesis, either directly by inhibiting ALKBH repair enzymes or indirectly by altering the expression of DNA repair genes. These studies also provide a molecular explanation for the finding that chemotherapy with a combination of DNA alkylating agents benefits glioma patients with IDH1 mutations [83,84]. Similarly, daunorubicin, an inhibitor of topoisomerase II, sensitizes Idh1 hematopoietic stem cells [82] and is more effective in treating IDH1-mutated AML [85]. These findings may be relevant for the design of treatment strategies for IDH-mutated tumors with regard to whether the use of inhibitors of mutant IDH might reduce the efficacy of DNA-damaging chemotherapy agents (see discussion below).

2-HG Affects T Cell Differentiation and Tumor Immunity

Leukemic IDH mutations result in 2-HG accumulation and disrupt TET2 function, effects that have been shown to promote reversible leukemogenesis in cultured hematopoietic cells [23,86,87]. An intriguing new pathway linked to 2-HG is T cell differentiation and tumor immunity. T cell activation is associated with dramatic metabolic reprogramming, notably the switch from oxidative phosphorylation to glycolysis [88]. These metabolic changes support T cell proliferation, differentiation, and function. T-helper 17 (T$_{H17}$) and T regulatory (T$_{reg}$) cells are T cell subtypes that share similar differentiation precursors from naive CD4$^+$ T cells but perform different functions in immune homeostasis (i.e., promotion and suppression of inflammation, respectively). 2-HG was found to be five- to tenfold higher in T$_{H17}$ cells compared with T$_{reg}$ cells and elevated 2-HG was associated with increased promoter methylation and transcriptional inhibition of Foxp3, the master regulatory transcription factor of T$_{reg}$ cells [89]. Treatment of naive CD4$^+$ T cells with 2-HG or knockdown of Tet genes stimulated T$_{H17}$ cell differentiation. The origin of 2-HG in T$_{H17}$ cells was traced to glutamate, which can be converted to α-KG by glutamic-oxaloacetic transaminase (GOT). In mice, inhibition of GOT by a pan-transaminase inhibitor, aminooxyacetic acid (AOA), increased Foxp3 expression, reprogrammed T$_{H17}$ to T$_{reg}$ cells.
in vitro, and caused autoimmune encephalomyelitis. We do not yet know which enzyme produces 2-HG in TH17 cells.

CD8+ T cells, the cytotoxic T lymphocytes that kill cancer cells, traffic to hypoxic areas in tumors and inflamed tissues, and HIF-1α mediates this migration [90,91]. Activation of HIF-1α in CD8+ T cells increased LDHA, which converts glutamine-derived α-KG to L-2-HG and causes L-2-HG concentrations to be as high as 1.5 mM [57]. Increased L-2-HG altered the gene expression and differentiation pathway of CD8+ T cells, leading to enhanced antitumor capacity in mice when adaptively transferred with tumor cells. This enhanced antitumor immunity function could be recapitulated by simply soaking CD8+ T cells with cell-permeable L-2-HG. Both KDM and TET have been implicated as targets of L-2-HG [57]. In contrast to enhancing the function of immune cells, accumulation of 2-HG in tumors has been linked to resistance to tumor immunity. Human IDH1-mutant gliomas have reduced expression of chemokines and less immune infiltration compared with wild-type IDH1 gliomas [92,93]. Analysis in syngeneic mice showed that, compared with glioma with wild-type IDH1, IDH1-mutated gliomas have fewer immune cells, reduced expression of chemokine genes, neutrophil chemotaxis, and accumulation of T cells in tumors. Treatment of IDH1 wild-type cells in vitro with cell-permeable D-2-HG reduced chemokine production and, conversely, treatment of IDH1-mutated cells with an inhibitor of mutant IDH1 blocks D-2-HG-increased chemokine production and T cell infiltration [93]. It will be important to establish the target enzymes of 2-HG and downstream genes responsible for the enhancement and resistance to immunity in immune and tumor cells (see Outstanding Questions).

Does 2-HG Target Other Proteins Besides α-KG-Dependent Dioxygenases?

2-HG accumulation has also been linked to several additional cellular pathways. Prompted by the mutually exclusive mutation between IDH1 and PETN in low-grade glioma, it was found that D-2-HG activates mechanistic target of rapamycin (mTOR) signaling partly by inhibiting the activity of KDM4A, which promotes the stability of DEPTOR, a negative regulator of mTORC1/2 [94]. Accumulation D-2-HG in colorectal cancer cells lacking IDH mutation was associated with increased H3K4 trimethylation in the promoter of ZEB1, a regulator of epithelial–mesenchymal transition (EMT), and increased ZEB1 expression and EMT [60]. In addition, D-2-HG has been linked to the activation of nuclear factor kappa B (NF-κB) signaling [95] and altered metabolism [96–99], although we do not know the direct targets of 2-HG in these processes.

In addition to α-KG, 2-HG is structurally similar to other TCA metabolites, including succinate. This has led to studies to determine the effect of D-2-HG toward the enzyme that uses succinate as a substrate, succinate dehydrogenase (SDH). D-2-HG was found to be able to competitively inhibit SDH resulting in the accumulation of succinate, increased protein hypersuccinylation, and impairment of mitochondrial respiration [100]. In addition, 2-HG has been reported to bind the DNA methyltransferase DNMT1 and the small GTPase Cdc42, although neither enzyme binds α-KG [101,102]. 2-HG stimulated DNMT1 to bind the promoter and repress the expression of receptor-interacting protein kinase 3 (RIP3), a regulator of programmed necrosis/necroptosis, suggesting that impaired RIP3-dependent
necroptosis may contribute to tumorigenesis driven by \textit{IDH} mutation [102]. Binding of 2-HG to Cdc42 disrupted Cdc42’s association with the effector mixed lineage kinase 3 (MLK3), leading to suppression of MLK3 activation and MLK3-mediated apoptosis in serum-deprived mutant IDH1 cells [101]. Future genetic and biophysical characterizations are necessary to understand the significance and affinities of 2-HG binding to DNMT1 and Cdc42.

**Therapeutic Targeting of \textit{IDH} Mutant Tumors**

Two unique aspects make mutant IDH1 and IDH2 attractive targets for the treatment of \textit{IDH}-mutated tumors. First, mutant IDH1/2 acquires a neomorphic activity distinct from the wild-type IDH1/2. An inhibitor specific to a mutant enzyme should not interfere with wild-type enzyme activity. Second, the product of mutant IDH1, D-2-HG, apparently lacks any physiological function. Complete inhibition of D-2-HG synthesis, unlike inhibition of other gain-of-function oncogenes, should have no or minimal toxicity. There is great interest in developing small-molecule inhibitors of mutant IDH enzymes. However, we do not yet have definitive evidence of 2-HG addiction by IDH-mutant tumors. Although several investigators have reported that inhibiting 2-HG production resulted in reduced tumor growth [103–105], one study reported that, in some contexts, cell transformation by mutant IDH1 was not easily reversed by 2-HG depletion [106]. Presently, there are 19 registered clinical trials (see ClinicalTrials.gov) testing the responses of advanced hematological malignancies (AML, MDS/MPN, and AITL) and advanced solid tumors (glioma, cholangiocarcinoma, and chondrosarcoma) to six inhibitors of mutant IDH enzymes, four targeting mutant IDH1 (AG-120, IDH-305, FT-2102, and BAY-1436032), one targeting mutant IDH2 (AG-221), and one pan-inhibitor targeting both mutant IDH1 and mutant IDH2 (AG-881).

**Monotherapy Targeting Mutant IDH Enzymes**

The first reported small-molecule inhibitor of mutant IDH, compound 35 (later referred to as AGI-5198), was identified in a high-throughput screen against the IDH1(R132H) homodimer. AGI-5198 inhibited both R132H- and R132C-mutant IDH1 with similar potencies (IC\textsubscript{50} = 70 and 160 nM, respectively) and AGI-5198 did not inhibit wild-type IDH1 or mutant IDH2 (IC\textsubscript{50} > 100 μM) [104,107]. Using patient-derived IDH1 (R132H) TS603 glioma cells, this compound was shown to inhibit D-2-HG production and induce histone methylation and gliogenic differentiation. Also, AGI-5198 reduced colony formation in soft agar and tumor growth in a mouse xenograft model [104]. When tested in \textit{IDH1}-mutated cholangiocarcinoma cells, this compound exhibited greater potency, it inhibited colony formation and cell migration and induced apoptosis [108]. Several additional inhibitors have since been reported to potently inhibit mutant IDH1 with no apparent effect on wild-type IDH1 or IDH2 [109–113]. These latter inhibitors are structurally different and inhibit mutant IDH1 enzymes by different mechanisms, such as competing with \(\alpha\)-KG for binding to the active site [107], blocking magnesium binding [112], and locking the enzyme in a catalytically inactive conformation [111]. There are only two reports on the effect of mutant IDH1 inhibitors in patient-derived \textit{IDH1}-mutant glioma cells in an orthotopic mouse model and presently the results are inconclusive [105,106].
Inhibitors of mutant IDH2 have also been developed and advanced to clinical trials. The first inhibitor, AGI-6780, was a slow tight binder (causing inhibition at a concentration comparable with that of the enzyme) discovered in high-throughput screening against IDH2(R140Q) [114]. AGI-6780 binds allosterically to the dimer interface and induces the differentiation of leukemia cells expressing mutant IDH2. An optimized inhibitor, AG-221, displayed nanomolar potency against the IDH2(R140Q) homodimer (IC\textsubscript{50} = 0.1 μM), the IDH2(R140Q/WT) heterodimer (IC\textsubscript{50} = 0.03 μM), and the IDH2(R172 K/WT) heterodimer (IC\textsubscript{50} = 0.01 μM) and is much less inhibitory toward the wild-type IDH2 homodimer (IC\textsubscript{50} = 1.8 μM). AG-221 inhibited D-2-HG production and induced the differentiation of IDH2-mutated primary leukemia cells; it suppressed D-2-HG production and extended survival in tumor xenograft models [113]. An inhibitor of mutant IDH2, AGI-026, has also been shown to provide survival benefit in a D2HGA mouse model with an R140Q knock-in mutation in the \textit{Idh2} locus [115]. The only reported human clinical trials targeting mutant IDH thus far are two Phase I/II studies of relapsed or refractory AML patients treated with AG-221 (enasidenib) [116,117]. In August 2017, AG-221 was approved by the FDA as the first drug targeting a mutant IDH enzyme. Continuous daily treatment with enasidenib was generally well tolerated and induced hemato-logical responses with an overall response rate of 40.3% and a median response duration of 5.8 months in patients in whom prior AML therapy had failed. Induction of differentiation, not cytotoxicity, appears to drive the clinical efficacy. Suppression of 2-HG alone, however, did not predict response, as most non-responding patients also exhibited 2-HG suppression. Co-occurring mutations in NRAS and other MAPK pathway effectors were enriched in non-responding patients, suggesting that RAS signaling contributes to therapeutic resistance.

\textbf{Combination Therapy and Novel Strategies Targeting IDH-Mutant Tumors}

Combination therapy, a treatment using more than one medication or modality, is a traditional cornerstone of cancer therapy. The design of combination therapies is being significantly facilitated by cancer genomics, which has identified specific driver mutations and addicted oncogenes in various types and subtypes of tumors, revealing tumor vulnerabilities and novel targets. The first such vulnerability of IDH-mutant tumors was recognized retrospectively from the treatment of low-grade glioma patients with a chemotherapy known as PCV that includes a combination of two DNA alkylating agents, procarbazine and CCNU/lomustine, and a micro-tubule poison, vincristine. Following successful chemotherapy for recurrent malignant oligodendroglioma [118], randomized controlled trials confirmed the benefits of PCV for patients with low-grade glioma. PCV benefit was subsequently linked to \textit{IDH1} mutations with an overall survival of 9.4 years for patients with \textit{IDH} mutation versus 5.7 years for patients with wild-type \textit{IDH} [83,84]. One probable mechanism is that the D-2-HG accumulated in \textit{IDH1}-mutated glioma inhibited the activity of ALKBH2 and ALKBH3 to repair the alkylating lesions, sensitizing \textit{IDH1}-mutant cells to PCV [79]. Temozolomide (TMZ), another alkylating agent, is a more easily administered, less toxic alternative to PCV. It is still being debated whether TMZ confers survival benefit on patients with \textit{IDH}-mutant glioma [43].
IDH-mutant tumors are also vulnerable to inhibitors of nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the NAD⁺ salvage pathway [106]. This vulnerability was discovered because mutant IDH1 reduces the NAD⁺ concentration and NAD⁺ is a critical coenzyme for many enzymes. Depletion of NAD⁺ in IDH1-mutated cells was attributed to increased promoter methylation and decreased expression of NAPRT1, the rate-limiting enzyme in NAD⁺ biosynthesis, and NAD⁺ depletion rendered cells sensitive to inhibition of the NAMPT-catalyzed NAD⁺ salvage pathway [106]. Tateishi et al. showed that TMZ caused a burst of consumption of NAD⁺ and further sensitized IDH1-mutant cells to NAMPT inhibitors [119]. In addition to sensitizing cells to alkylating agents and NAMPT inhibitors, 2-HG can sensitize mutant IDH1-engineered cells to inhibitors of PARP [81,120], a family of proteins that catalyze polymerization of ADP-ribose derived from NAD⁺ and that is involved in the repair of various types of DNA damage. We currently do not know the molecular pathway underlying 2-HG-induced sensitivity to PARP1 inhibitors. There are conflicting reports on whether a decreased concentration of NAD⁺ sensitizes IDH1-mutated cells to PARP inhibitors [81,119,121]. Therefore, it appears that 2-HG accumulation can cause genetic instability by inhibiting the activity of ALKBH2/3 [18,79], decreasing the expression of ATM [82], and impairing the function of PARP, sensitizing IDH-mutant cells to multiple DNA-damaging agents and inhibitors of DNA repair pathways.

Concluding Remarks

Both D- and L-2-HG enantiomers were considered dead-end metabolites without physiological functions and remained largely unnoticed for more than a century. The origin of their production was a mystery and the mechanism of their toxicity at elevated concentrations was unknown (see Outstanding Questions). The discovery of 2-HG production and accumulation in IDH-mutated tumors transformed 2-HG into the ‘poster child’ for oncometabolites. The major targets of 2-HG, α-KG-dependent dioxygenases, have been identified, including the KDM family of histone demethylases and the TET family of DNA demethylases. Recent studies have revealed activities of 2-HG beyond epigenetic control and have even linked 2-HG to the normal process of T cell regulation. Investigators have also identified additional sources of 2-HG from promiscuous enzyme reactions in cells lacking IDH mutations. These studies have raised many questions regarding the metabolism and activity of 2-HG. IDH mutation is one of the earliest genetic alterations during gliomagenesis, suggesting a function for D-2-HG in tumor initiation. What role does D-2-HG play in tumor progression and maintenance? Does 2-HG play different roles in tumors and T cells during tumorigenesis in vivo? Are there other enzymes that promiscuously produce 2-HG? What metabolic and stress conditions affect the promiscuity of these enzymes? 2-HG cannot diffuse across the cell membrane. Do specific transporters move 2-HG between different subcellular compartments or across the plasma membrane? D2HGDH and L2HGDH are localized in the mitochondrion. Do D-2-HG and L-2-HG actively accumulate in this compartment and affect mitochondrial respiration?

Answers to these and other questions will lead to new mechanistic and clinical understanding of 2-HG metabolism and activity and how 2-HG metabolism affects gene expression and other nuclear activities.

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Glossary

2-Hydroxyglutaric aciduria (2HGA)
a rare neurometabolic disorder (only several hundred cases reported worldwide) characterized by significantly elevated concentrations of 2-HG in plasma, cerebrospinal fluid, and urine. There are three types of 2HGA – D2HGA, L2HGA, and combined D,L-2HGA – depending on whether the excess 2-HG is the D or L isomer or both. A detailed description of 2HGA, including clinical diagnosis and management, patient support, and resources can be found at [https://ghr.nlm.nih.gov/condition/2-hydroxyglutaric-aciduria](https://ghr.nlm.nih.gov/condition/2-hydroxyglutaric-aciduria).

α-Ketoglutarate (α-KG)/Fe(II)-dependent dioxygenases
enzymes that catalyze the transfer of both oxygen atoms from molecular oxygen (O_2) to a substrate (hydroxylation). Dioxygenases differ from monooxygenases (e.g., cytochrome P450 oxidases), which transfer one oxygen atom from O_2 to a substrate and reduce the other oxygen atom to water. Dioxygenases are distributed widely in all kingdoms of life and more than 60 dioxygenases occur in humans [122]. Dioxygenases catalyze the hydroxylation of lysine, proline, and asparagine in proteins and of nucleotides in DNA and RNA. Members of this enzyme family regulate multiple cellular pathways, including collagen maturation, fatty acid metabolism, oxygen sensing, histone and DNA demethylation, DNA repair, and demethylation of tRNA and mRNA [16,17].

ALKBH
the bacterial family of α-KG/Fe(II)-dependent dioxygenases. The prototypical member of the family is the *Escherichia coli* AlkB, which was discovered by its activity in affording resistance to alkylating agents [126]. Nine ALKBH enzymes exist in mammalian cells (ALKBH1–8, FTO). Originally thought to be limited to repairing DNA alkylation, ALKBH has recently been found to function in RNA metabolism [127,128].

Collagen hydroxylase
also known as prolyl 4-hydroxylase; the first α-KG/Fe(II)-dependent dioxygenase to be identified [130], controlling the biogenesis and stability of collagen. There are three types of collagen hydroxylase, catalyzing the hydroxylation of C-3 of proline (C-P3H), C-4 of proline (C-P4H), and C-5 of lysine (PLOD1, 2, and 3) [17].

EGLN/PHD hydroxylase
refers to the Egl-9 family of hypoxia-inducible factors or prolyl hydroxylase domain-containing proteins. The family includes three members in human cells: EGLN1 (PHD2), EGLN2 (PHD1), and EGLN3 (PHD3). EGLN/PHD catalyzes proline hydroxylation of HIF-1α under normoxia conditions (10–21% oxygen); hydroxylation of HIF-1α promotes
its degradation by the ubiquitin proteasome pathway. A decrease in oxygen concentration to 1–5% (hypoxia) diminishes EGLN/PHD activity, thereby limiting the hydroxylation and degradation of HIF-1α and promoting HIF-1α activation of hypoxia adaptation genes. EGLN/PHD-mediated regulation of HIF-1α is the core of ancient oxygen-monitoring machinery used by metazoans from the nematode *Caenorhabditis elegans* to humans [129].

**Enantiomers**

two molecules that have the same chemical formula but are structurally mirror images (like the left and right hands). D and L isomers are determined by the configuration about an asymmetric carbon atom (a carbon having four different substituents), using the enantiomeric glyceraldehydes as a reference. D and L come from the Latin words *dexter* (on the right) and *laevus* (on the left). This old and more commonly used D/L nomenclature accidently, but not always, correlates with the modern R/S system of nomenclature, wherein (R) comes from rectus (right-handed) and (S) from sinister (left-handed). Enantiomers often have different chemical reactions with other enantiomer substances; enantiomers sometimes display biochemical and biological differences, such as the different inhibitory potencies of D- and L-2-HG toward α-KG/Fe(II)-dependent dioxygenases.

**Enzyme promiscuity**

activity of an enzyme that is distinguished from, and unrelated to, the primary activity and that is not involved in an organism’s physiology. The catalytic efficiency of an enzyme’s promiscuity is often several orders of magnitude lower than the efficiency of its primary activity. Enzyme promiscuity should not be confused with enzymes that have multiple substrates. It is believed that primordial enzymes possessed broad specificity and converted multiple substrates with similar efficiencies. Although contemporary enzymes have become specialized in catalyzing one reaction, many enzymes can promiscuously catalyze reactions other than those for which they have evolved.

**KDM**
refers to the Jmjc-domain family of histone lysine (K) demethylases. These demethylases are evolutionarily conserved in eukaryotes and include 18 genes in humans. Except KDM1/LSD1, all KDMs belong to the α-KG/Fe(II)-dependent dioxygenase family, and they use α-KG as a co-substrate to oxidize the methyl group of lysine residues in histones, leading to eventual demethylation [123].

**Neomorphic enzyme**
an enzyme that has gained a novel function different from the wild-type gene by mutation.

**TET**
refers to the ten-eleven translocation family of DNA methylcytosine dioxygenases. Single TET enzymes are found in insects and there are three such enzymes in humans: TET1, TET2, and TET3. TET enzymes belong to the α-KG/Fe (II)-dependent dioxygenase family; they oxidize the methyl group on carbon 5 (C-5) of cytosine (5mC) in DNA. However, instead of a single demethylation reaction, TET enzymes catalyze three stepwise oxidations: (i) 5mC to 5-hydroxymethylcytosine (5hmC); (ii) 5hmC to 5-formylcytosine (5fC); and (iii)
5fC to 5-carboxylycitosine (5caC). DNA repair enzymes such as glycosylases remove 5caC, which is then replaced by unmethylated cytosine [124,125].

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Many enzymes can promiscuously produce 2-HG by side reactions, causing pathologically significant concentrations of 2-HG in cells lacking IDH mutations.

Metabolic and stress conditions such as T cell activation or hypoxia can promote enzyme promiscuity to produce 2-HG.

In addition to histone and DNA demethylases, 2-HG can impair the activity of other α-KG-dependent dioxygenases and their associated cellular pathways, such as DNA repair.
Figure 1. Metabolism and Targets of 2-Hydroxyglutarate (2-HG)

The thick and thin arrows represent the primary and promiscuous reactions, respectively. FAD, flavin adenine dinucleotide; FADH2, reduced form of FAD; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of NADP. Additional abbreviations are listed in Table 1.
Figure 2. Production of 2-Hydroxyglutarate (2-HG) by Promiscuous Enzymatic Reactions

The thick and thin arrows represent the primary and promiscuous reactions, respectively. PHDGH, phosphoglycerate dehydrogenase; 3PG, 3-phosphoglycerate; 3PHP, 3-phosphohydroxypyruvate. Additional abbreviations are listed in Figure 1.
## Table 1

Abnormal Accumulation of D- and L-2-HG Impairs Multiple Cellular Pathways

| 2-HG enantiomer | Producing enzyme | Molecular target | Affected cellular pathway | Associated disease | Refs |
|-----------------|------------------|------------------|---------------------------|--------------------|------|
| D-2-HG          | Mutant IDH1, 2   | PHD/EGLN         | HIF-1α                    | Glioma             | [12,73,74] |
| D-2-HG          | Mutant IDH1, 2   | TET              | DNA demethylation         | Glioma, AML        | [19,23] |
| D-2-HG          | Mutant IDH1, 2   | KDM              | Histone demethylation     | Glioma, AML        | [18,19] |
| D-2-HG          | Mutant IDH1      | ALKBH1, 2        | DNA repair                | Glioma             | [18,79,80] |
| D-2-HG          | Mutant IDH2      | FTO              | RNA demethylation         | AML                | [131] |
| D-2-HG          | Mutant IDH2      | N.D.             | ?                          | D-2HG aciduria type II | [44] |
| D-2-HG          | Mutant IDH1, 2   | N.D.             | STAT1 pathway; T cell function and infiltration | Tumor growth | [92,93] |
| D-2-HG          | Mutant IDH2      | N.D.             | N.D.                      | Cardiomyopathy     | [115,132] |
| D-2-HG          | Mutant IDH1, 2   | KDM4A DEPTOR     | mTOR pathway              | N.D.               | [94] |
| D-2-HG          | D2HGDH mutation  | N.D.             | N.D.                      | D-2HG aciduria type I | [38,123,133] |
| D-2-HG          | *In vitro* addition | PIN1, NF-κB pathway and stromal cells | AML | [95] |
| D-2-HG          | *In vitro* addition | Cytochrome c oxidase | Cell respiration | AML | [134] |
| L-2-HG          | LDHA*            | KDM              | Hypoxia                   | L-2HG aciduria     | [49,55,56,58,59] |
| L-2-HG          | MDH*             | KDM              | Hypoxia                   | L-2HG aciduria     | [49,55] |
| L-2-HG          | L2HGDH mutation  | AASS             | Hypoxia                   | L-2HG aciduria     | [103,135] |
| L-2-HG          | LDHA             | KDM              | T cell function and infiltration | Tumor suppression | [57] |
| L-2-HG          | L2HGDH low expression | N.D.        | N.D.                      | Kidney cancer      | [136] |

AASS, aminoadipate-semialdehyde synthase (lysine-α-KG reductase); DEPTOR, DEP domain containing mTOR-interacting protein; FTO, fat mass and obesity-associated, α-KG-dependent dioxygenase; KDM, lysine demethylase; PHD, prolyl hydroxylase domain-containing (also EGLN, Egl-9-family hypoxia inducible factor); PIN1, peptidylprolyl cis/trans isomerase, NIMA-interacting 1; TET, ten-eleven translocation family of methylcytosine dioxygenase.

*Under acidic or hypoxia conditions.