Drosophila larvae synthesize the putative oncometabolite L-2-hydroxyglutarate during normal developmental growth

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L-2-hydroxyglutarate (L-2HG) has emerged as a putative oncometabolite that is capable of inhibiting enzymes involved in metabolism, chromatin modification, and cell differentiation. However, despite the ability of L-2HG to interfere with a broad range of cellular processes, this molecule is often characterized as a metabolic waste product. Here, we demonstrate that *Drosophila* larvae use the metabolic conditions established by aerobic glycolysis to both synthesize and accumulate high concentrations of L-2HG during normal developmental growth. A majority of the larval L-2HG pool is derived from glucose and dependent on the *Drosophila* estrogen-related receptor (dERR), which promotes L-2HG synthesis by up-regulating expression of the *Drosophila* homolog of lactate dehydrogenase (*dLdh*). We also show that dLDH is both necessary and sufficient for directly synthesizing L-2HG and the *Drosophila* homolog of L-2-hydroxyglutarate dehydrogenase (*dL2HGDH*), which encodes the enzyme that breaks down L-2HG, is required for stage-specific degradation of the L-2HG pool. In addition, dLDH also indirectly promotes L-2HG accumulation via synthesis of lactate, which activates a metabolic feed-forward mechanism that inhibits dL2HGDH activity and stabilizes L-2HG levels. Finally, we use a genetic approach to demonstrate that dLDH and L-2HG influence position effect variegation and DNA methylation, suggesting that this compound serves to coordinate glycolytic flux with epigenetic modifications. Overall, our studies demonstrate that growing animal tissues synthesize L-2HG in a controlled manner, reveal a mechanism that coordinates glucose catabolism with L-2HG synthesis, and establish the fly as a unique model system for studying the endogenous functions of L-2HG during cell growth and proliferation.

2-hydroxyglutarate | LDH | lactate | Warburg effect | estrogen-related receptor

One of the hallmarks of cancer is a dramatic reprograming of cellular metabolism that results in enhanced biosynthesis (1). These metabolic changes are particularly apparent in tumors that use the Warburg effect, also referred to as aerobic glycolysis, a metabolic program characterized by elevated levels of glucose consumption and enhanced lactate production (1, 2). By activating aerobic glycolysis, tumors are able to synthesize macromolecules rapidly from glycolytic intermediates. In addition, elevated levels of lactate dehydrogenase (LDH) activity allow proliferating cells to synthesize lactate and maintain the NAD^+ levels required for high rates of glucose catabolism and biomass production (1).

The metabolic reprogramming of cancer cells, however, extends beyond biosynthesis, as many tumors also generate progrowth metabolites, or oncometabolites, that promote tumor formation via nonmetabolic means. Most notable among these compounds is D-2-hydroxyglutarate (D-2HG), which is associated with cancers such as gliomas and acute myelogenous leukemias (3). Although D-2HG is generated as a normal byproduct of γ-hydroxybutyrate metabolism (4), oncogenic D-2HG production is the result of neomorphic mutations in the active site of isocitrate dehydrogenase 1 or 2 (IDH1/2) (5). Tumors that harbor these IDH1/2 mutations inappropriately convert 2-oxoglutarate (2OG) to D-2HG, which acts as a competitive inhibitor of 2-oxoglutarate–dependent dioxygenases [2OGDs; e.g., Jmj histone lysine demethylases, ten-eleven translocation (TET) enzyme family] (3, 6–9). As a result, IDH1/2 mutant cells experience widespread chromatin remodeling and changes in gene expression and are unable to differentiate properly (8, 9).

Although most oncometabolite research is focused on D-2HG, the L-2-hydroxyglutarate (L-2HG) enantiomer is an even more potent 2OGD inhibitor, and high L-2HG levels are associated with renal cell carcinomas, gliomas, and a neurometabolic disorder known as L-2-hydroxyglutaric aciduria (10–12). Unlike D-2HG, however, L-2HG has no known role in metabolism and eukaryotes lack an enzyme dedicated to L-2HG synthesis. Instead, L-2HG is considered an aberrant metabolite produced by the nonspecific activity of enzymes such as malate dehydrogenase, lactate dehydrogenase A (LDHA), and lactate dehydrogenase C (12–18). Consistent with these observations, L-2HG accumulation in cancer cells does not result from ectopic synthesis; rather, it stems from decreased expression of the enzyme L-2-hydroxyglutarate dehydrogenase (L2HGDH), which is solely responsible for L-2HG degradation (10, 19). Therefore, most studies suggest that neither healthy tissues nor cancer cells appear capable of regulating L-2HG production. This model of L-2HG synthesis, however, has been challenged by a recent study of T lymphocytes,

Significance

Oncometabolites are small molecules that promote tumor formation and growth. L-2-hydroxyglutarate (L-2HG) is a putative oncometabolite that is associated with gliomas and renal cell carcinomas, as well as a severe neurometabolic disorder known as L-2-hydroxyglutaric aciduria. However, despite that L-2HG is commonly considered a metabolic waste product, this compound was recently discovered to control immune cell fate, thereby demonstrating that it has endogenous functions in healthy animal cells. Here, we find that the fruit fly, *Drosophila melanogaster*, also synthesizes high concentrations of L-2HG during normal larval growth. Our discovery establishes the fly as a genetic model for studying this putative oncometabolite in healthy animal tissues.

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which revealed that these immune cells generate L-2HG as a means of controlling cell fate and gene expression (20). Furthermore, both hypoxia and the disruption of mitochondrial metabolism cause human cells to generate high levels of L-2HG, indicating that synthesis of this compound might alleviate oxidative stress (17, 21–23). These observations hint at diverse endogenous roles for L-2HG and suggest that the function of this putative oncometabolite should be further studied in healthy tissues. To address this need, we have established the fruit fly *Drosophila melanogaster* as a genetic model system for studying L-2HG in the context of aerobic glycolysis and rapid tissue growth.

The fly is ideally suited to study the molecular mechanisms that link glycolytic flux to biosynthesis. Similar to cancer cells, growing *Drosophila* larvae up-regulate glycolysis, the pentose phosphate pathway, and lactate production as means of supporting the nearly 200-fold increase in body mass that occurs during this developmental stage (24). The resulting metabolic program exhibits the hallmark characteristics of aerobic glycolysis and establishes the fly as a powerful in vivo model for studying this metabolic state. Furthermore, studies in the fly were the first to demonstrate that the estrogen-related receptor (ERR) family of nuclear receptors acts as a conserved transcriptional regulator of aerobic glycolysis (24–26). Here, we extend the metabolic parallels between *Drosophila* development and tumor growth by demonstrating that the *Drosophila* estrogen-related receptor (dERR) also promotes L-2HG synthesis. Moreover, we determine that the larval L-2HG pool is largely derived from glucose oxidation and synthesized by the *Drosophila* ortholog of LDH (dLDH), thereby establishing a direct link between larval glycolytic metabolism and L-2HG production. Finally, we demonstrate that dLDH and L-2HG are key regulators of position effect variegation (PEV) and DNA methylation, supporting a model in which L-2HG acts as a metabolic signal that coordinates glycolytic flux with epigenetic modifications and the regulation of gene expression.

Results

***Drosophila* Larvae Synthesize High Concentrations of L-2HG.** In an effort to characterize the metabolic basis of *Drosophila* larval growth, we used a targeted GC-MS–based approach to identify compounds that are abundant in larvae but absent in other developmental stages. This approach identified 2HG, whose levels are elevated between threefold and 10-fold in larvae compared with embryos, wandering third-instar larvae, white prepupae, and adults (Fig. 1*A*). To characterize this larval 2HG pool further, we used a chiral derivatization method to individually measure the concentrations of D-2HG and L-2HG in w^{1118} second-instar larvae (Fig. S1 *A* and *B*). Although L2 larvae harbor relatively low quantities of D-2HG (~0.1 nmol/mg of body mass; Fig. 1*B*), the concentration of L-2HG surpasses 2 nmol/mg of body mass (Fig. 1*B* and Fig. S1*B*), which exceeds the IC_{50} of all analyzed 2OGDs and indicates that larvae accumulate physiologically relevant levels of this compound (6, 7). Intriguingly, we observed that the *Drosophila* late embryonic and L3 cell lines Kc167 and BG1-c1, respectively, also produce relatively high concentrations of L-2HG (~0.2–0.8 nmol/mg), but only possess a basal level of D-2HG (Fig. 1*C*), indicating L-2HG accumulation is a general feature of rapid cell growth and proliferation in flies.

The extent to which larvae accumulate L-2HG is striking, as the only other healthy tissues known to accumulate high concentrations of L-2HG are T lymphocytes and cultured human cells that experience hypoxia (20, 21). Since we raised larvae under normoxic conditions (*Materials and Methods*), our results suggested that larvae accumulate L-2HG via a novel metabolic mechanism. To pinpoint the metabolic origin of L-2HG, L2 larvae were fed semidefined media that were supplemented with either U-^{13}C–labeled glucose or U-^{13}C–labeled proline (an anaplerotic amino acid in insects). Larvae raised on labeled food incorporated ^{13}C into L-2HG, indicating that *Drosophila* metabolism is capable

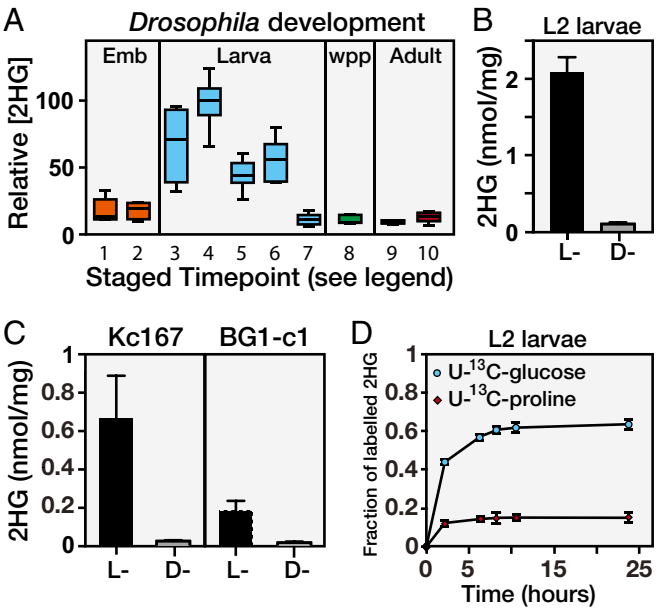


Fig. 1. *Drosophila* larvae accumulate high concentrations of L-2HG. (*A*) Relative abundance of 2HG in a w^{1118} background. Staged time points are as follows: (1) embryos (Emb; 0–12 h after egg laying), (2) Emb (12–24 h after egg laying), (3) L1 larvae, (4) L2 larvae, (5) early-L3 larvae (0–12 h after L2-L3 molt), (6) mid-L3 larvae (24–36 h after L2-L3 molt), (7) wandering L3 larvae, (8) white prepupae (wpp), (9) adult females (3 d posteclosion), and (10) adult males (3 d posteclosion). Data are represented as box plots ($n = 6$). The concentrations of D-2HG and L-2HG in midsecond-instar w^{1118} larvae (*B*) and the *Drosophila* cell lines Kc167 and BG1-c1 (*C*) are shown. Data are shown as mean ± SD ($n \geq 3$). (*D*) The w^{1118} midsecond-instar larvae were fed either U-^{13}C-glucose or U-^{13}C-proline, and the incorporation of ^{13}C isotopes into 2HG was monitored over a 24-h period.

of synthesizing this compound (Fig. 1*D*). Moreover, ~60% of the L-2HG pool was labeled with ^{13}C after 24 h of U-^{13}C-glucose consumption, but only ~15% of this pool contained ^{13}C as the result of feeding labeled proline (Fig. 1*D*). Because animal cells synthesize L-2HG from 2OG, our results suggested that larvae ultimately generate this compound by shuttling glucose-derived pyruvate into the TCA cycle. Indeed, we found that m + 2 was the most abundant L-2HG isotopologue following U-^{13}C-glucose feeding, an observation that is consistent with fully labeled pyruvate entering the TCA cycle via the pyruvate dehydrogenase complex (Fig. S1*C*). In contrast, feeding of U-^{13}C-proline primarily produced the m + 5 isotopologue (Fig. S1*C*). Although there are likely additional anaplerotic compounds that are used to generate L-2HG, our observations indicate that a majority of the larval L-2HG pool is generated from glucose oxidation.

dERR Promotes L-2HG Accumulation. Rapidly growing *Drosophila* larvae rely on elevated levels of glucose catabolism to generate biomass (24); therefore, our ^{13}C tracer analysis suggests that larvae generated L-2HG as a byproduct of this metabolic program. We tested this hypothesis by measuring L-2HG levels in *dERR* mutants, which fail to up-regulate carbohydrate metabolism before the onset of larval development (24). Not only were L-2HG levels dramatically lower in *dERR* mutants compared with w^{1118} controls but this L-2HG phenotype was also rescued by the expression of a *dERR-GFP* transgene in the *dERR* mutant background (Fig. 2*A*). These studies indicated that L-2HG production is dependent on the metabolic program that is established by dERR at the onset of larval growth.

In an effort to identify the enzyme that synthesizes L-2HG, we searched for enzymes that are both regulated by dERR and only expressed at a high level in larvae, mimicking the L-2HG

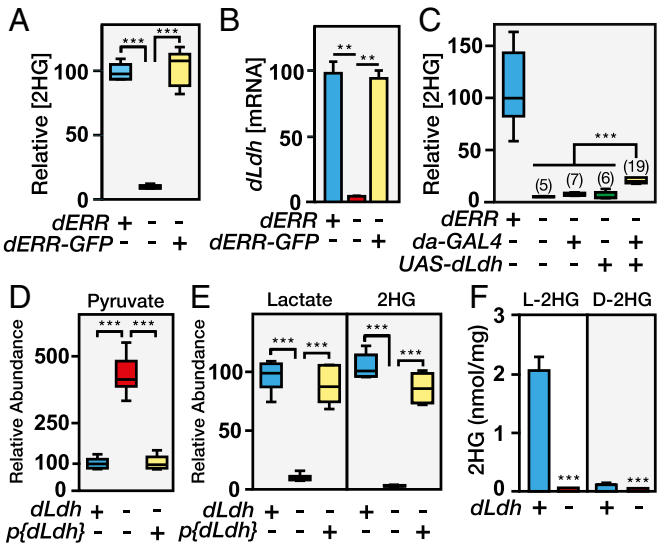


Fig. 2. L-2HG is generated by the aerobic glycolytic program. (*A*) Relative abundance of 2HG in mid-L2 larvae from w^{1118} controls, $dERR^1/dERR^2$ mutants, and $dERR^1/dERR^2$ mutants that express a *dERR-GFP* transgene. (*B*) Relative *dLdh* mRNA levels were measured in the same genotypes described in *A*. Transcript levels were normalized to the abundance of *rp49* mRNA. Data are presented as mean ± SEM ($n = 3$). (*C*) Relative abundance of 2HG in mid-L2 larvae of the following genotypes: w^{1118}, $dERR^1/dERR^2$, $dERR^1$ + /$dERR^2$ *da-GAL4*, *UAS-dLdh*/$^+$; $dERR^1/dERR^2$, and *UAS-dLdh*/$^+$; $dERR^1$ + /$dERR^2$ *da-GAL4*. The numbers in parentheses refer to the mean value for the sample set. The relative abundance of pyruvate (*D*) and lactate (*E*) as well as 2HG was measured in mid-L2 larvae from $dLdh^{prec}$ controls, $dLdh^{16}/dLdh^{17}$ mutants, and a *p{genomic dLdh}*$^+$; $dLdh^{16}/dLdh^{17}$ rescue strain. (*F*) Concentration of L-2HG and D-2HG in $dLdh^{prec}$ controls (+) and $dLdh^{16}/dLdh^{17}$(–) mid-L2 larvae. Data are presented as mean ± SD. In *A* and *C*–*E*, data are represented as box plots ($n = 6$). $^{**}P < 0.01$; $^{***}P < 0.001$.

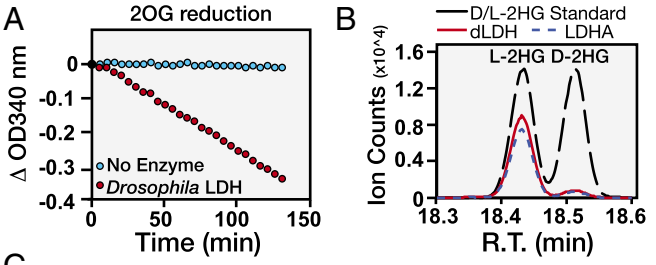
accumulation pattern. One gene that fulfilled these criteria was the *Drosophila* homolog of LDH (*dLdh*; also known as *ImpL3*). The expression of *dLdh* is dependent on dERR activity and is restricted to late-stage embryos and growing larvae, and lactate levels decrease at the onset of metamorphosis, mirroring the temporal changes we observe in L-2HG abundance (24, 27, 28) (Fig. 2*B* and Fig. S2 *A* and *B*). To determine if *dERR* mutants lack L-2HG due to loss of dLDH activity, we used the *da-GAL4* driver to express a *UAS-dLdh* transgene ubiquitously in this mutant background (Fig. S2*C*). Even though most L-2HG is derived from glucose and glycolytic capacity is severely impaired in *dERR* mutants (24), *UAS-dLdh* expression increased L-2HG levels by threefold in *dERR*-mutant larvae compared with the negative control strains (Fig. 2*C*), indicating that dLDH is sufficient to drive L-2HG accumulation.

To determine if dLDH is also necessary for larval L-2HG synthesis, we generated two mutations that eliminate *dLdh* gene expression (Fig. S3 *A* and *B*). Animals that harbor a *trans*-heterozygous combination of these null alleles, $dLdh^{16}/dLdh^{17}$, grow at a normal rate until the midthird instar, at which point 50% of the larvae die and the remainder develop into morphologically normal adults (Fig. S3 *C* and *D*). However, despite the normal growth rate, GC-MS analysis of the $dLdh^{16}/dLdh^{17}$ midsecond-instar larvae revealed dramatic metabolic phenotypes. These mutants not only displayed a fivefold increase in pyruvate and a 20-fold decrease in lactate but 2HG levels were almost undetectable compared with a precise excision control strain ($dLdh^{prec}$) (Fig. 2 *D* and *E*). Furthermore, these metabolic defects were specifically due to the loss of dLDH activity, as the aberrant pyruvate, lactate, and 2HG levels observed in $dLdh^{16}/dLdh^{17}$ mutants were completely rescued by a *dLdh* transgene (Fig. 2 *D* and *E*). We obtained a similar phenotype by expressing a *UAS-dLdh-RNAi* transgene, which depleted *dLdh* mRNA levels and induced a significant reduction in the abundance of lactate and 2HG (Fig. S4). Finally, we confirmed that loss of dLDH activity predominantly affects the L-2HG pool. While $dLdh^{16}/dLdh^{17}$ larvae exhibited a modest decrease in D-2HG levels, the abundance of L-2HG decreased by 98% in these mutants (2.1 nmol/mg [$dLdh^{prec}$] vs. 0.04 nmol/mg [$dLdh^{16}/dLdh^{17}$]; Fig. 2*F*). Overall, these results demonstrate that dERR promotes L-2HG synthesis by up-regulating *dLdh* gene expression.

dLDH Directly Synthesizes L-2HG from 2OG. Our findings that dLDH is both necessary and sufficient for L-2HG accumulation suggests that this enzyme could be directly responsible for synthesizing L-2HG. We tested this possibility by incubating purified dLDH with NADH and either pyruvate or 2OG, which serves as the precursor for L-2HG in mammalian cells (17, 21). Based on changes in both NADH concentration (340-nm absorbance) and end-point GC-MS analysis, dLDH not only converts pyruvate to lactate but also synthesizes L-2HG from 2OG (Fig. 3 *A* and *B* and Fig. S5*A*). In agreement with earlier studies (16, 21), we also observed that human LDHA catalyzed the reaction of 2OG to L-2HG, thereby demonstrating that LDH generates L-2HG via an evolutionarily conserved enzymatic mechanism (Fig. 3*B* and Fig. S5*B*).

During the course of these kinetic analyses, we discovered an unexpected relationship between lactate and L-2HG synthesis. Although dLDH converted pyruvate to lactate with normal Michaelis–Menten kinetics ($K_{m,\ pyruvate} = 3.6$ mM at pH 7.5; Fig. 3*C*), dLDH catalyzed the reduction of 2OG with a much lower efficiency and displayed non-Michaelis–Menten kinetics ($K_{0.5,\ 2OG} = 57.5$ mM; Hill coefficient = 3; Fig. 3*C*). However, despite the ability of dLDH to synthesize lactate at a much faster rate than L-2HG, both of these compounds are found at nearly identical concentrations in midsecond-instar larvae (Fig. 3*C*), indicating that lactate and L-2HG levels are coordinately regulated.

***Drosophila* L-2-Hydroxyglutarate Dehydrogenase Regulates L-2HG Accumulation in a Lactate-Sensitive Manner.** Considering that dLDH acts on 2OG with a relatively low efficiency, we hypothesized that



C

Drosophila LDH enzyme kinetics

pyruvate → lactate (pH 7.5)		2-oxoglutarate → L-2-hydroxyglutarate (pH 7.5)	
K_{cat} (1/s)	105.4 ± 7.5	K_{cat} (1/s)	2.2 ± 0.1
$K_{m,\ pyruvate}$ (mM)	3.6 ± 0.7	$K_{0.5,\ 2\text{-oxoglutarate}}$ (mM)	57.5 ± 1.8
		Hill coefficient	3
mid-L2 Metabolite Concentration (mM)			
[pyruvate]	1.0 ± 0.1	[2-oxoglutarate]	0.4 ± 0.1
[lactate]	2.1 ± 0.2	[L-2-hydroxyglutarate]	2.1 ± 0.2

Fig. 3. dLDH directly catalyzes the formation of L-2HG from 2OG. (*A*) Purified dLDH was incubated with NADH and 2OG. The ability of dLDH to reduce 2OG was monitored by changes in NADH concentration (absorbance at 340 nm). (*B*) GC-MS was used to conduct an end-point analysis of D-2HG and L-2HG accumulation in the reactions catalyzed by dLDH and human LDHA. R.T., retention time. (*C*) Kinetic parameters for the conversion of pyruvate to lactate and 2OG to 2HG by purified dLDH (pH 7.5). Note that dLDH exhibits non-Michaelis–Menten kinetics for the 2OG-to-2HG reaction. The $K_{m,\ pyruvate}$ value deviates from previous reports due to differences in pH of the in vitro reactions.

the larval L-2HG pool represents a combination of increased synthesis and decreased degradation. In mammals, the mitochondrial enzyme L2HGDH controls L-2HG levels by converting this compound to 2OG (13, 19). To determine if *Drosophila* L-2HG levels are controlled by a similar mechanism, we generated two mutations in the sole fly *L2HGDH* ortholog (*dL2HGDH*; also known as *CG10639*; Fig. S6*A*). Animals that carry a *trans*-heterozygous combination of these mutations, $dL2HGDH^{12}$ and $dL2HGDH^{14}$, are viable and display significantly higher L-2HG levels throughout the fly life cycle (Fig. 4*A* and Fig. S6*B*). Furthermore, ubiquitous overexpression of *UAS-dL2HGDH* in a $dL2HGDH^{12}/dL2HGDH^{14}$ mutant background completely rescued this metabolic phenotype (Fig. 4*B*), demonstrating that dL2HGDH is responsible for degrading L-2HG. The most dramatic aspect of the *dL2HGDH* mutant phenotype, however, was found in early prepupae, where L-2HG levels were elevated nearly 20-fold in $dL2HGDH^{12}/dL2HGDH^{14}$ mutants (Fig. 4*A*), a difference that reflects the stage-specific regulation of L-2HG metabolism. Although control larvae experience a 90% decrease in L-2HG immediately before metamorphosis (Fig. 1*A*), this metabolic switch fails in *dL2HGDH* mutants and L-2HG levels remain at levels normally associated with larval stages (Fig. 4*A*).

The manner by which L-2HG levels drop at the onset of metamorphosis suggests that dL2HGDH enzymatic activity is relatively low in larvae compared with pupae. This stage-specific regulation, however, does not occur at a transcriptional level, as *dL2HGDH* mRNA levels peak during larval development and actually drop before puparium formation (27) (Fig. S6*C*), indicating that larval L-2HG degradation is controlled at a post-transcriptional level. Considering that both lactate and L-2HG are structurally similar α-hydroxy acids, we hypothesized that lactate might regulate L-2HG levels by acting as a competitive inhibitor of dL2HGDH. Therefore, we raised larvae on food supplemented with increasing lactate concentrations and measured the relative abundance of both lactate and L-2HG. Although both control and *dLdh* mutant larvae exhibited significantly elevated levels of L-2HG when raised on high-lactate food (Fig. 5 *A* and *B* and Fig. S7), L-2HG levels in *dL2HGDH* mutants were resistant to this dietary treatment (Fig. 5*C*), suggesting that lactate-mediated L-2HG accumulation requires dL2HGDH. Consistent with this hypothesis, lactate inhibited the activity of partially purified dL2HGDH in vitro. A concentration of lactate similar to the concentration found in midsecond-instar larvae, 2 mM, induced an ∼50% decrease in dL2HGDH activity, whereas the addition of 10 mM lactate nearly eliminated L-2HG degradation (Fig. 5*D*).

These findings hint at an elegant model for how dLDH can generate such high levels of L-2HG accumulation despite its low affinity for 2OG. If lactate inhibits dL2HGDH activity, then the stage-specific *dLdh* expression could both increase L-2HG synthesis and inhibit dL2HGDH activity via lactate production, thereby stabilizing the larval L-2HG pool. Such a model would also explain why both *dERR* and *dLdh* mutant larvae exhibit such low L-2HG levels, because the loss of dLDH activity would result in both decreased synthesis and increased degradation. Indeed, when we measured L-2HG abundance in *dL2HGDH; dLdh* double mutants, where loss of dL2HGDH renders larvae unable to degrade L-2HG, we found that L-2HG levels were increased 20-fold compared with the *dLdh* single mutant (Fig. 5 *E* and *F*). Overall, these results reveal a metabolic feed-forward mechanism, wherein dLDH both synthesizes L-2HG and indirectly inhibits L-2HG degradation via the production of lactate.

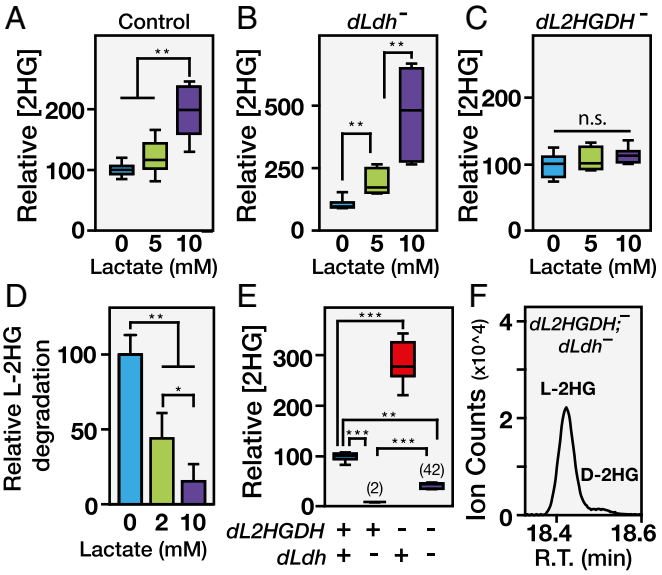


Fig. 5. dL2HGDH activity is inhibited by lactate. (*A*–*C*) 2HG levels in mid-L2 larvae raised on semidefined media containing 0, 5, or 10 mM lactate. Dietary lactate induced elevated 2HG levels in both w^{1118} controls and $dLdh^{16}/dLdh^{17}$ ($dLdh^{-}$) mutants, but not $dL2HGDH^{12}/dL2HGDH^{14}$ ($dL2HGDH^{-}$) mutants. (*D*) Ability of lactate to inhibit L-2HG degradation was assessed by incubating partially purified dL2HGDH with 2 mM L-2HG and 0, 2, or 10 mM lactate ($n = 6$, data presented as mean ± SD). (*E*) Relative 2HG levels in mid-L2s of w^{1118} controls, $dL2HGDH^{12/14}$; $dLdh^{16/17}$ single mutants, and $dL2HGDH^{12/14}$; $dLdh^{16/17}$ double mutants. (*F*) Relative abundance of D-2HG and L-2HG levels in *dL2HGDH; dLdh* mutant midsecond-instar larvae was measured using GC-MS. In *A*–*C* and *E*, data are represented as box plots ($n = 6$). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

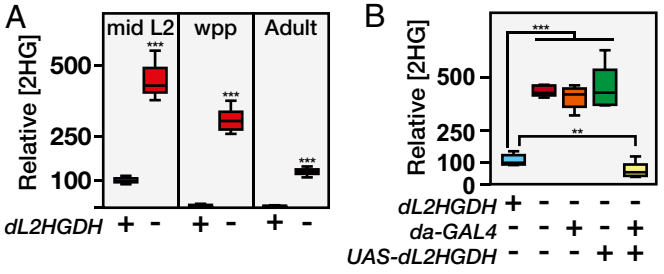


Fig. 4. dL2HGDH controls the stage-specific accumulation of L-2HG. (*A*) Relative 2HG levels in staged samples of mid-L2, white prepupae (wpp), or adult males (3 d posteclosion) between w^{1118} controls (+) and $dL2HGDH^{12}/dL2HGDH^{14}$ (–) mutants. (*B*) Relative 2HG levels in mid-L2 larvae for the following five genotypes: w^{1118}, $dL2HGDH^{12}/dL2HGDH^{14}$, $dL2HGDH^{12}/dL2HGDH^{14}$; *da-GAL4*, $dL2HGDH^{12}/dL2HGDH^{14}$; *UAS-dL2HGDH*, and $dL2HGDH^{12}/dL2HGDH^{14}$; *da-GAL4 UAS-dL2HGDH*. Data are represented as box plots ($n = 6$). $^{**}P < 0.01$; $^{***}P < 0.001$.

L-2HG Regulates PEV. The 2HG accumulation in mammalian cells is associated with changes in epigenetic modifications and chromatin architecture (6, 7), suggesting that the larval L-2HG pools function in a similar capacity. We tested this possibility using the $In(1)\ y^{1}\ w^{m4}$ inversion of the X chromosome as a readout of heterochromatin formation. In flies that harbor this chromosome, the *white* (*w*) locus is positioned near the centromere, and as a result, *w* expression is silenced by the pericentric heterochromatin. This phenomenon is known as PEV, and the resulting changes in *w* gene expression are primarily influenced by heterochromatin formation (reviewed in ref. 29). Our analysis revealed that *dLdh* mutations are very strong recessive suppressors of PEV (Fig. 6 *A* and *B*), demonstrating that dLDH activity normally promotes heterochromatin formation. In contrast, the *dL2HGDH* mutations do not consistently alter PEV under these conditions (Fig. 6 *A* and *B*), suggesting that this phenomenon is sensitive to loss of L-2HG, but not excess amounts of this compound. Such a result is not unexpected because the larval concentration of L-2HG exceeds the reported IC_{50} values of most 2OGDs, and increased levels

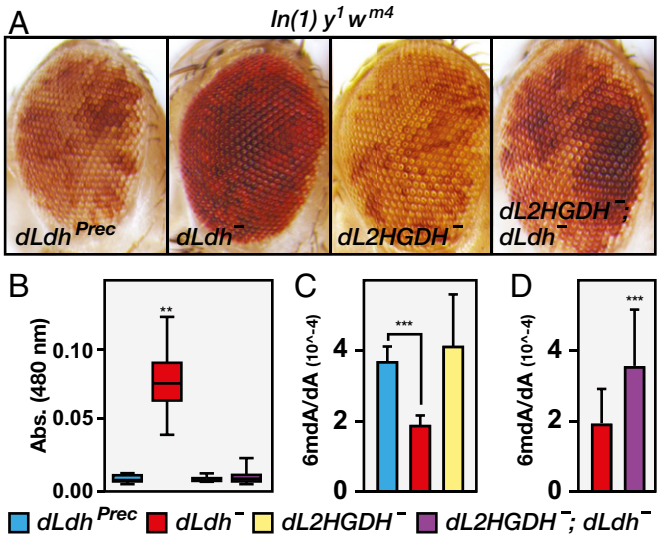


Fig. 6. L-2HG influences PEV and DNA methylation. (*A*) Control and mutant flies harboring the *In(1) y*^{1} *w*^{m4} inversion were grown on semidefined media, and adult males were aged for 3 d before imaging the eye pigment distribution. (*B*) PEV phenotype of adult male flies from was quantified based on the concentration of red eye pigment (480-nm absorbance). Data are graphically represented as box plots ($n > 20$ adult male heads per genotype). (*C* and *D*) Ratios of 6mdA/dA in adult genomic DNA were detected by LC-tandem MS (MS/MS). Data are presented as mean ± SD ($n \geq 7$). $^{**}P < 0.01$; $^{***}P < 0.001$.

would likely have a minimal effect on the activity of target enzymes (6). Because *Drosophila* larvae absorb minimal amounts of L-2HG from their food (Fig. S8), we assessed the PEV phenotype in *dL2HGDH; dLdh* double mutants, which accumulate much higher L-2HG levels than the *dLdh* single mutant (Fig. 5 *E* and *F*). We found that this double-mutant strain exhibits the same level of eye pigmentation as wild-type controls (Fig. 6 *A* and *B*), indicating that the ability of *dLdh* mutations to suppress the PEV phenotype is due to loss of L-2HG.

Intriguingly, we found a similar correlation between L-2HG production and DNA methylation. Although the *Drosophila* genome contains very low levels of 5-methylcytosine, the *Drosophila* Tet-family homolog was recently found to regulate *N*-6-methyldeoxyadenosine (6mdA) abundance in embryos (30). Therefore, we used LC-tandem MS to measure 6mdA quantitatively in larval and adult genomic DNA. Although *dLdh* mutants maintained normal 6mdA levels during larval development (Fig. S9), the abundance of 6mdA in adult genomic DNA was decreased in the absence of dLDH activity (Fig. 6*C*), suggesting that L-2HG production during larval development influences 6mdA levels in cells that will form adult tissue. In contrast, the *dL2HGDH* mutations did not consistently alter 6mdA levels (Fig. 6*C*), suggesting that similar to the PEV result, DNA methylation is sensitive to loss of L-2HG, but not to elevated amounts of this compound. Finally, the *dL2HGDH; dLdh* double-mutant strain exhibited significantly higher 6mdA levels than the *dLdh* single mutant (Fig. 6*D*), indicating that the *dLdh* mutant genomes contain fewer 6mdA residues as the result of decreased L-2HG accumulation, and revealing a direct link between synthesis of this compound and the regulation of epigenetic modifications.

Discussion

L-2HG was long considered a metabolic waste product, as eukaryotic genomes lack enzymes devoted to synthesizing this molecule, most cell types quickly degrade L-2HG, and L-2HG accumulation in humans is primarily associated with disease states (3, 12, 15). Recent studies, however, indicate that L-2HG can function as a metabolic signaling molecule and demonstrate a need to better understand the normal cellular functions of this compound (17, 20, 21). Our findings establish *Drosophila* as a model for studying L-2HG and provide a genetic framework for conducting in vivo studies of this putative oncometabolite.

Unlike previous reports of L-2HG production in cancer cells and diseased tissues (10, 14), flies generate L-2HG in a controlled fashion, demonstrating that studies of larval metabolism can be used to elucidate the endogenous molecular mechanisms that regulate L-2HG accumulation. Intriguingly, although our analyses reveal that a majority of the larval L-2HG pool is derived from glucose oxidation, L-2HG accumulation is dependent on the normoxic production of lactate, which is a hallmark of aerobic glycolysis. These observations indicate that rapid tissue growth in flies relies on the complex integration of glucose-dependent biosynthesis, lactate production, and mitochondrial metabolism. Furthermore, our findings highlight the importance of recent stable isotope tracer studies of in vivo cancer metabolism, which revealed that tumors not only generate lactate but are also reliant on significant levels of glucose oxidation (31, 32). Because endogenous tumor metabolism appears to strike a balance between lactate production and the shuttling of pyruvate into the mitochondria, our results suggest the metabolic mechanism that generates L-2HG in flies could also function in cancer cells.

In addition to describing a metabolic feed-forward mechanism that promotes L-2HG accumulation, our findings reveal that the nuclear receptor dERR is capable of promoting L-2HG accumulation. Although recent studies in both T lymphocytes and mammalian cell culture have focused on the role of HIF1α in promoting L-2HG metabolism (17, 20, 21), siRNA targeting of HIF1α in human lung fibroblasts is not sufficient to prevent hypoxia-induced L-2HG production, hinting at an alternative mechanism that promotes synthesis of this compound. Our findings indicate that the ERR family could serve this role and suggest that future studies of L-2HG in mammalian systems should examine this conserved family of nuclear receptors.

Considering that many aspects of glucose metabolism are conserved between flies and mammals, the amount of L-2HG present in larvae is striking. Cultured mammalian cells maintain very low levels of L-2HG (17, 21), and although certain organs seem predisposed to accumulating this molecule (e.g., testis, brain) (13, 16), L-2HG is efficiently degraded in most tissues. There is a clear link, however, between disruption of mitochondrial metabolism and L-2HG production, as hypoxia, activation of HIFα signaling (21), disruption of the electron transport chain, and defects in citrate transport result in elevated L-2HG synthesis (17, 20–23). Although our study demonstrates that flies can generate L-2HG under normoxic conditions, larvae likely experience regular bouts of hypoxia, both while immersed in their food and as a direct result of rapidly increasing body size (33). Furthermore, dERR is both required for the hypoxia response in larvae and physically interacts with HIF1α (34), indicating that larval metabolism is acutely prepared to deal with a low-oxygen environment. Therefore, we propose a model wherein larvae constitutively express *dLdh* and generate L-2HG both as a means of supporting biosynthesis and preempting the need to frequently mount a hypoxia response. This model is supported by our findings that larval L-2HG is generated from glucose oxidation. By converting 2OG into L-2HG, larvae can couple glucose catabolism with biosynthetic processes that rely on the mitochondria (e.g., synthesis of fatty acids) regardless of oxygen availability.

L-2HG can likely inhibit dozens of enzymes, indicating that this compound could act as a means of coordinating metabolic flux with a wide range of cellular processes. Consistent with this possibility, our preliminary studies suggest that this compound regulates epigenetic modifications. Intriguingly, the manner by which dLDH activity modifies the PEV phenotype indicates that L-2HG can influence heterochromatin formation in cells that

will form the adult body. Therefore, future studies of dLDH and L-2HG could serve as a model for understanding how dietary cues can influence gene expression and metabolic disease symptoms over extended periods of time. In conclusion, our findings suggest that L-2HG is more than a metabolic waste product; rather, it serves to coordinate glycolytic flux with 2OG-dependent processes, such as heterochromatin formation and gene expression.

Materials and Methods

***Drosophila* Husbandry and Strain Creation.** Fly stocks were maintained on Bloomington Stock Center food. Unless noted, all strains were constructed in a w^{1118} background and all larvae were raised on yeast paste spread over molasses agar and incubated at 25 °C. L-2HG and lactate feeding experiments were conducted using previously described semidefined media (*SI Materials and Methods*). The *dLdh* mutations were generated using standard techniques to excise the p-element $\{EPgy2\}^{EY07426}$. All experiments used a *trans*-heterozygous combination of $dLdh^{16}$ and $dLdh^{17}$ alleles, and a precise excision allele ($dLdh^{prec}$) was used as a control. The *dL2HGDH* mutations were generated using CRISPR/Cas9 (*SI Materials and Methods*). All experiments used flies that harbored a *trans*-heterozygous combination of $dL2HGDH^{12}$ and $dL2HGDH^{14}$, which was generated by crossing homozygous $dL2HGDH^{12}$ males with $dL2HGDH^{14}$ virgin females.

Metabolite Analysis. The initial detection of metabolites was conducted using previously described GC-MS protocols (35). The resulting data are presented as box plots with $n = 6$ samples per data point. For the enantiomer-specific analysis and 2HG quantification, each sample tube containing 25 larvae was supplemented with 8 μg of disodium (*R,S*)-[2,3,3-$^2H_3$]-2-hydroxyglutarate ([$^2H_3$]-2HG; C/D/N ISOTOPES) and metabolite extraction was performed as described (35). Dried samples were derivatized with *R*-2-butanol and acetic anhydride according to a previous report (36).

Quantification of LDH Enzyme Activity. The *Drosophila* LDH cDNA was amplified from Drosophila Genomics Resource Center clone LD20346 and inserted into pGEX-4T1 (Amersham). Drosophila LDH was expressed in and purified from BL21-competent *Escherichia coli* using standard procedures (*SI Materials and Methods*). The reactions catalyzed by the purified *Drosophila* LDH and purchased recombinant human LDHA (BioVision) were monitored by measuring NADH consumption (OD_{340} using a plate reader; BioTek) at 25 °C in 200 mM Tris buffer (pH 7.5). The reaction products were confirmed by GC-MS using the methods described above.

L-2HG Degradation Assay. *Drosophila* lysate was prepared according to the reported method with a little modification (19). Details are provided in *SI Materials and Methods*. The levels of L-2HG were detected by GC-MS as described above.

Additional details and methods are included in *SI Materials and Methods*.

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