Enzymatic activation of pyruvate kinase increases cytosolic oxaloacetate to inhibit the Warburg effect

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Pharmacological activation of the glycolytic enzyme PKM2 or expression of the constitutively active PKM1 isoform in cancer cells results in decreased lactate production, a phenomenon known as the PKM2 paradox in the Warburg effect. Here we show that oxaloacetate (OAA) is a competitive inhibitor of human lactate dehydrogenase A (LDHA) and that elevated PKM2 activity increases de novo synthesis of OAA through glutaminolysis, thereby inhibiting LDHA in cancer cells. We also show that replacement of human LDHA with rabbit LDHA, which is relatively resistant to OAA inhibition, eliminated the paradoxical correlation between the elevated PKM2 activity and the decreased lactate concentration in cancer cells treated with a PKM2 activator. Furthermore, rabbit LDHA-expressing tumours, compared to human LDHA-expressing tumours in mice, displayed resistance to the PKM2 activator. These findings describe a mechanistic explanation for the PKM2 paradox by showing that OAA accumulates and inhibits LDHA following PKM2 activation.

One of the most common metabolic phenotypes in cancer, the Warburg effect, is characterized by increased glucose consumption and lactate production in the presence of oxygen. The Warburg effect supports increased growth and proliferation by increasing glycolytic intermediates required for biosynthesis as well as increasing lactate production that acidifies the tumour microenvironment to aid in metastasis and immune evasion. The Warburg effect is regulated by aberrant oncogenes, which upregulate gene expression, increase enzymatic activity and alter the subcellular localization of glycolytic enzymes to enhance glucose uptake and glycolytic activity. One notable exception to this observation is pyruvate kinase, which catalyses the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP and is downregulated in many types of cancer cells. This is due to the expression of PKM2, the isoform primarily expressed in rapidly proliferating tissues including cancer. Unlike PKM1, the constitutively active splice variant of pyruvate kinase that is often expressed in differentiated tissues, PKM2 exists in a low activity dimer conformation until binding of the coactivator fructose 1,6-bisphosphate, which promotes the formation of the active tetramer. The expression of PKM1 and PKM2 is regulated by the oncogene c-myc, which favours PKM2 expression. Additionally, oncogenic tyrosine kinases, including FGFR1, BCR-Abl and FTL3-ITD, phosphorylate PKM2 and disrupt the formation of the high activity tetramer conformation. Thus, the aberrant oncogene expression that promotes the Warburg effect downregulates pyruvate kinase activity.

The effects of PKM2 expression on tumourigenesis and tumour growth have been extensively studied and have been shown to be highly context dependent. Although human cell line studies consistently showed that decreased PKM2 activity promotes cancer cell proliferation, mouse studies found conflicting results. On the one hand, in breast and hepatocellular carcinoma mouse models, decreased PKM2 activity (via deletion of PKM2) is advantageous for tumour growth. PKM2 knockout induces PKM1 expression in some tumour cells in mice; however, the PKM1-expressing tumour cells were less proliferative than the tumour cells with complete knockout of both PKM1 and PKM2 (ref. ). This is consistent with the hypothesis that decreased pyruvate kinase activity promotes cancer cell proliferation. On the other hand, deletion of PKM2 in bone marrow haematopoietic cells delayed leukaemia initiation, and deletion of PKM2 in skeletal muscle tissue delayed soft tissue sarcoma initiation and yet had no effect on sarcoma growth. Deletion of PKM2 in haematopoietic cells reduced lactate production, while PKM2 deletion in skeletal muscle tissue had no effect on lactate production. These results indicate that the role of PKM2 in tumour growth may be dependent on its effects on lactate production. Therefore, it is important to identify the mechanistic link between PKM2 activity and lactate production to better understand the context-specific role of PKM2 in tumour growth. Although numerous previous studies have observed decreased PKM2 activity and increased lactate production and tumour growth, the mechanism responsible for this paradoxical correlation remains unclear.

To examine the PKM2 paradox in the Warburg effect, we can either (1) increase pyruvate kinase activity in cancer cells that display low pyruvate kinase activity or (2) inhibit pyruvate kinase activity in normal cells that display high pyruvate kinase activity. However, immortalized normal cells in culture also display the Warburg effect phenotype with high glycolytic activity, and thus mechanistic studies of the effects of decreased PKM2 activity in normal culture cells are limited. Therefore, we examined the PKM2...
paradox by increasing pyruvate kinase activity with the small molecule PKM2 activators DASA and TEPP-46 or by expression of the constitutively active PKM1 isoform in cancer cells.

Here, we report that high pyruvate kinase activity reduces LDHA activity by increasing cytosolic oxaloacetate (OAA), which we show is a competitive inhibitor of LDHA, to concentrations sufficient to inhibit LDHA in cancer cells. We also show that PKM2 activation increases OAA concentrations by upregulating the de novo synthesis of OAA. In this pathway, glutamate pyruvate transaminase 2 (GPT2) converts pyruvate and glutamate to alanine and α-ketoglutarate (α-KG). Increased α-KG flux through the tricarboxylic acid (TCA) cycle by PKM2 activation then leads to increased intracellular OAA, which inhibits LDHA. Finally, we demonstrate that the inhibition of LDHA by OAA increases the response of tumours to the PKM2 activator in mice, thus demonstrating that OAA is a regulator of the Warburg effect and an important metabolite linking glycolysis and glutamine metabolism in cancer.

**Results**

Pyruvate kinase activity decreases LDH activity and lactate. Previous studies have described the paradoxical correlation between PKM2 activity and lactate production in non-small cell lung carcinoma H1299 cells that predominantly express PKM2 (refs. 8,9,19). To analyse this correlation, we used two approaches to increase pyruvate kinase activity in H1299 cells: treatment of the cells with the PKM2 activator DASA5 and stable expression of PKM1, the constitutively active isoform of pyruvate kinase5,6, in place of PKM2. Treatment of H1299 cells with DASA resulted in a concentration-dependent increase in PKM2 activity (Fig. 1a), consistent with previously published results5. Similarly, PKM1-expressing H1299 cells with short hairpin RNA (shRNA) knockdown of endogenous PKM2 displayed greater pyruvate kinase activity than cells rescued with PKM2 (Extended Data Fig. 1a). Consistent with the PKM2 paradox, treatment of cells with 40 μM DASA or expression of PKM1 decreased intracellular lactate concentrations and reduced lactate production (Fig. 1b and Extended Data Fig. 1b.c). Lactate production was also reduced in RPMI 8226 cells treated with DASA (Extended Data Fig. 1d). Pyruvate concentrations were not altered by pyruvate kinase activation in H1299 cells (Fig. 1d and Extended Data Fig. 1e).

Two possible mechanisms that could explain the reduced lactate production following PKM2 activation are (1) reduced glucose uptake or (2) decreased LDH activity. Because 40 μM DASA treatment did not alter glucose uptake in H1299 and RPMI 8226 cells (Extended Data Fig. 1f.g), we hypothesized that decreased lactate production is due to reduced LDH activity. We examined the effects of pyruvate kinase activation on cellular LDH activity using two approaches. In the first approach, we performed time-series measurements of [13C] pyruvate and [13C] lactate labelling to obtain a kinetic measurement of LDH activity in cells, which we refer to as in situ LDH activity. We first pretreated the cells with vehicle or DASA overnight and then replaced the media with media containing [13C]6 glucose in the presence of vehicle or DASA. We then determined the fractions of pyruvate and lactate in which all three carbons were labelled with [13C] at 5, 10 and 20 min after the addition of [13C]6 glucose and calculated the percentage change per minute in [13C] labelling for both pyruvate and lactate in vehicle and DASA treated cells. In situ LDH activity was defined as the [13C] lactate labelling rate (percentage increase per minute in [13C] lactate labelling) divided by the [13C] pyruvate labelling rate (percentage increase per minute in [13C] pyruvate labelling) (Fig. 1e). Using this approach, we observed a significant decrease in the [13C] lactate labelling rate in cells treated with DASA (Fig. 1f), while we observed no significant change in [13C] pyruvate labelling rate following DASA treatment in H1299 cells (Fig. 1g). As a result, in situ LDH activity was decreased in DASA treated H1299 cells (Fig. 1h). A decrease in situ LDH activity was also observed in DASA treated RPMI 8226 cells as well as in H1299 cells expressing PKM1 in place of PKM2 (Extended Data Fig. 1h–m).

In the second approach to analyse LDH activity, we measured LDH activity in cell lysates using an assay in which diluted cell lysates were supplemented with exogenous pyruvate and NADH, and the decrease in NADH fluorescence was used as a measure of LDH activity. Using this approach, there was no difference in LDH activity between vehicle and DASA treated H1299 and RPMI 8226 cells, nor was there a difference in LDH activity between PKM1 and PKM2 expressing H1299 cells (Fig. 1i and Extended Data Fig. 1i,a,o). Taken together, these results indicate that (1) pyruvate might be metabolized to metabolites other than lactate, which we will address later in this study and (2) inhibition of in situ LDH activity following pyruvate kinase activation is lost in the lysates possibly due to dilution, leading to the hypothesis that the decrease in LDH activity following pyruvate kinase activation is dependent on a metabolite.

OAA inhibits human LDHA in vitro and in cells. To evaluate the hypothesis that the decrease in LDH activity following pyruvate kinase activation is dependent on a metabolite, we examined whether pyruvate kinase activation regulates the concentration of a metabolite that alters LDH activity. There are two main LDH isoforms in mammalian cells, LDHA and LDHB. While LDHB is ubiquitously expressed, LDHA is upregulated in cancer cells through the activities of the oncoproteins MYC and HIF1α (refs. 20,21). LDHA has previously been shown to have multiple substrates, including pyruvate and α-KG2, indicating flexibility of the substrate binding pocket and suggesting that other metabolites might influence its activity. OAA, a keto acid that shares structural similarity with pyruvate and α-KG, has been shown to inhibit both bacterial and rabbit LDH; however, the strength of this inhibition varies greatly among organisms22–24, and whether OAA inhibits human LDHA (hLDHA) has not been assessed. Thus, we tested whether OAA inhibits purified recombinant hLDHA in vitro. We observed that...
OAA inhibits hLDHA with a half-maximum inhibitory concentration (IC₅₀) of 1.1 mM (Fig. 2a). Additionally, OAA is not a substrate for hLDHA (Extended Data Fig. 2a), and the structurally similar metabolites malate, aspartate and α-KG did not inhibit hLDHA activity (Extended Data Fig. 2b). We sought to further characterize the OAA inhibition of hLDHA by determining the inhibition type, the inhibition constant (Kᵢ) and the dissociation constant (Kₛ). These analyses show that OAA is a competitive inhibitor (Fig. 2b) with a Kᵢ of 290 µM (Fig. 2c) and a Kₛ of 642 µM (Fig. 2d and Extended Data Fig. 2c).

To determine whether intracellular OAA concentration is high enough to inhibit LDH activity, we sought to measure intracellular OAA concentrations using the GC–MS method that we developed for the simultaneous detection of α-KG and glutamate in our...
recent study\textsuperscript{27}, the details of which are described in the Methods section (Extended Data Fig. 3a). Using the isotope-ratio-based approach (Extended Data Fig. 3b)\textsuperscript{28}, we determined whole cell OAA concentrations in H1299 and RPMI 8226 cells to be 941 ± 259 and 2,650 ± 480 μM, respectively. These concentrations are near or higher than the IC\textsubscript{50} and K\textsubscript{i} of OAA against hLDHA.

If OAA inhibits hLDHA, we hypothesized that the addition of exogenous OAA to cells would further decrease LDH activity. We treated H1299 cells with cell membrane-permeable diethyl-ester OAA and found that 1 mM diethyl-ester OAA increased intracellular OAA concentrations to levels twice as high as endogenous OAA concentrations (Fig. 3a) and decreased in situ LDH activity (Fig. 3b–d), intracellular lactate concentrations (Fig. 3c) and lactate production (Fig. 3f) without altering LDH expression (Fig. 3g). To address whether the decrease in LDH activity is due to reduced glucose uptake, we analysed glucose uptake in vehicle and diethyl-ester OAA treated cells and observed that diethyl-ester OAA increased glucose uptake (Extended Data Fig. 3c). We further examined if aspartate, which can be converted from OAA, reduces glucose uptake in cells, as demand for TCA cycle activity might be reduced by exogenous OAA. However, we observed increased glucose uptake by 10 mM exogenous aspartate to a greater extent than diethyl-ester OAA treatment (Extended Data Fig. 3d). Thus, the decrease in \textsuperscript{13}C lactate labelling rate, LDH activity and lactate production following diethyl-ester OAA treatment is not due to decreased glucose uptake. The results in Figs. 2 and 3 show that (1) OAA inhibits hLDHA in vitro, (2) the OAA concentration in cancer cells is sufficient to inhibit hLDHA and (3) increasing OAA levels with a membrane-permeable analogue further suppresses LDH activity. Taken together, these results indicate that OAA inhibits LDH in cancer cells.

Activation of PKM2 increases cellular OAA concentrations. Given that PKM2 activation appeared to inhibit LDH through a metabolite (Fig. 1), and that OAA can inhibit hLDHA, we next asked whether increasing pyruvate kinase activity alters OAA levels. In H1299 and RPMI 8226 cells, OAA concentrations increased 1.5- to twofold following treatment with DASA (Fig. 4a and Extended Data Fig. 4a), an increase that was similar to the increase observed in H1299 cells treated with 1 mM diethyl-ester OAA and that was inversely correlated with LDH activity and lactate concentrations in cells (Figs. 1b,c,f,h, 3 and 4a). Similarly, replacement of PKM2 with PKM1 also increased OAA concentrations in H1299 cells (Extended Data Fig. 4b). Thus, pyruvate kinase activation increases intracellular OAA concentration, which correlates with decreased LDH activity and intracellular lactate concentration.

Quantification of cytosolic OAA. Because LDH is a cytosolic protein, we next sought to address whether cytosolic OAA concentrations are sufficient to inhibit LDHA. OAA is often considered a mitochondrial metabolite because it is part of the TCA cycle, and it is not known to be directly transported across the mitochondrial

Fig. 2 | OAA is a competitive inhibitor of hLDHA. a, The IC\textsubscript{50} of OAA against hLDHA determined using purified recombinant hLDHA and analysis of \textsuperscript{13}C\textsubscript{1} lactate derived from \textsuperscript{13}C\textsubscript{1} pyruvate by GC–MS. The IC\textsubscript{50} was identified as 1,140 ± 146 μM. b, Lineweaver–Burk analysis shows that OAA functions as a competitive inhibitor of hLDHA. c, Dixon analysis shows that OAA inhibits human LDHA and the K\textsubscript{i} was determined. d, The K\textsubscript{i} was determined by incubating purified human LDHA with increasing concentrations of OAA. The fluorescence intensity (excitation (ex.) 280 nm, emission (em.) 340 nm) fluorescence intensity without OAA treatment; f, fluorescence intensity with OAA treatment at the indicated concentrations. Data are presented as mean ± s.d. from n = 3 for a of independent replicates in vitro. Results in b–d are representative experiments of three independent replicates in vitro.
Fig. 3 | Diethyl-ester OAA treatment increases OAA concentration in cells. a, Relative whole cell OAA concentrations in H1299 cells treated with increasing concentrations of diethyl-ester OAA or vehicle in addition to 13C6 glucose. Whole cell metabolites were extracted with 1:1 methanol:water containing ninhydrin, with or without 13C OAA spike, derivatized and analysed using GC–MS. b, 13C lactate labelling rate (percentage increase per minute in 13C lactate labelling) of H1299 cells incubated with 13C6 glucose and vehicle or 1 mM diethyl-ester OAA. c, 13C pyruvate labelling rate (percentage increase per minute in 13C pyruvate labelling) of H1299 cells incubated with 13C6 glucose and vehicle or 1 mM diethyl-ester OAA. d, 13C lactate labelling rate in b divided by 13C pyruvate labelling rate in c as a surrogate to in situ LDH activity in H1299 cells treated with vehicle or 1 mM diethyl-ester OAA. In situ LDH activities are normalized to the vehicle control. e, Relative whole cell lactate concentrations in H1299 cells treated with increasing concentrations of diethyl-ester OAA or vehicle overnight. f, Relative lactate production in H1299 cells incubated with vehicle or 1 mM diethyl-ester OAA. g, Western blot results of H1299 whole cell lysates from cells treated with vehicle or increasing concentrations of diethyl-ester OAA with antibodies against LDHA and β-actin. Western blot results are representative experiments of three biologically independent replicates. Data are represented as the mean and error bars represent the standard deviation from n=3 for a,e, n=4 for b–d,f of biologically independent replicates. Western blot results in g are representative experiments of three biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
**Fig. 4** Fractionation of H1299 cells demonstrates that cytosolic OAA concentrations are sufficient to inhibit hLDHA. **a**, Relative whole cell OAA concentrations in H1299 cells treated with 40 μM DASA or vehicle in addition to 13C6 glucose overnight. Whole cell metabolites were extracted with 1:1 methanol-water containing ninhydrin, with or without 12C OAA spike, derivatized and analysed using GC–MS. **b**, Schematic representation of 13C6 glucose–derived carbon tracing of metabolites in cells. **c**, Western blot results of fractionated H1299 cells treated with 40 μM DASA or vehicle. Antibodies against LDHA, p70 S6 kinase (cytosolic marker), calreticulin (endoplasmic reticulum marker), golgin (Golgi marker) and COX IV (mitochondrial marker). **d,e**, Isotopomer analysis of 13C6 glucose–derived carbon incorporation of cytosolic OAA (**d**) and small organelles OAA (**e**) from fractionated H1299 cells treated with either 40 μM DASA or vehicle in addition to 13C6 glucose overnight. **f**, Ion counts, normalized to the 12C OAA spike, of OAA isotopomers from the cytosolic fraction of H1299 cells treated with either 40 μM DASA or vehicle in addition to 13C6 glucose overnight. **g**, Top, table of H1299 whole cell volume as well as small organelle volumes, determined using SBFSEM. Bottom, percentage of small organelles or cytosol and nucleus in H1299 cells. **h**, Cytosolic OAA concentrations in H1299 cells treated with 40 μM DASA or vehicle overnight. Data are represented as the mean and error bars represent the standard deviation from n = 4 for **a–f**, n = 12 for **g**, n = 3 for **h** of biologically independent replicates. Western blot results in **c** are representative experiments of three biologically independent replicates. P values were determined by a two-tailed Student’s t-test.

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**NATuRE METABoLISM** | VOL 3 | JULY 2021 | 954–968 | www.nature.com/natmetab

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**Table:**

| Cytosol + nucleus (μm³) | Organelles (μm³) | Whole cell volume (μm³) |
|------------------------|------------------|------------------------|
| 1,565.6 ± 438.1        | 341.4 ± 86.6     | 1,907.0 ± 466.4        |

**Percentage cytosol + nucleus** | **Percentage organelles**
---|---
82.6 ± 3.5 | 17.4 ± 3.5
membrane through transporters. However, OAA is an intermediate in the malate–aspartate shuttle system, where it can be produced by glutamate OAA transaminase (GOT1) and malate dehydrogenase (MDH1) from aspartate and malate in the cytosol (Fig. 4b)\(^5\). A previous study has shown whole cell malate and aspartate concentrations are greater than 1 mM, while mitochondrial concentrations are much lower (less than 200 µM)\(^5\). These data indicate that a considerable amount of malate and aspartate are probably present within the cytosol, suggesting that OAA may also be present within the cytosol.

To determine cytosolic OAA concentrations, we used a modified version of a previously described fractionation method to rapidly isolate small organelles (endoplasmic reticulum, Golgi and mitochondria) from the cytosol\(^4\). Cells were labelled with \(^{13}\)C-glucose overnight, homogenized and separated into cytosol and small organelle fractions using differential centrifugation (Extended Data Fig. 4c). We confirmed that LDHA is predominantly present in the cytosolic fraction by western blot as described in other studies (Fig. 4c)\(^5\). We then performed \(^{13}\)C-glucose–derived carbon tracing and quantified OAA using the isotope-ratio-based method in the cytosol and small organelle fractions.

One concern with this approach is that metabolites might leak from subcellular compartments into the cytosolic fraction during homogenization. To assess this possibility, we examined the isotopomer distributions of OAA (Fig. 4b). We observed a significant difference in incorporation of \(^{13}\)C-glucose–derived carbon into OAA between the cytosolic and small organelle fractions, indicating distinct metabolite populations in each compartment and showing that there is minimal leakage between compartments during homogenization (Fig. 4d,e). DASA decreased incorporation of \(^{13}\)C-glucose–derived carbon into cytosolic OAA, as shown by increased non-labelled M+0 OAA and decreased M+4 OAA, which contains four \(^{13}\)C isotopes, following DASA treatment (Fig. 4d), a surprising observation given that whole cell OAA concentrations are increased with DASA treatment (Fig. 4a). To investigate whether the overall abundance of cytosolic OAA was changed, we analysed total ion counts for \(^{13}\)C carbon labelled cytosolic OAA following vehicle or DASA treatment. We observed a threefold increase in total ion counts, mainly due to the increase in M+0 OAA ion counts, following DASA treatment, suggesting that pyruvate kinase activation induces de novo synthesis of cytosolic OAA from carbon sources other than glucose (Fig. 4d,f).

Next, to determine the concentration of cytosolic OAA, we assessed the volume of the cytosol in H1299 cells. First, we analysed whether there was a difference in endoplasmic reticulum, Golgi or mitochondrial content between DASA and vehicle treated cells. Using flow cytometry analysis with ER-BODIPY, Golgi tracker red and mitotracker red stains, we determined that DASA treatment does not alter organelle abundance (Extended Data Fig. 4d). We therefore determined cytosolic volume in untreated cells only. To determine cytosolic volume, we used serial block-face scanning electron microscopy (SBFSEM). We generated block-face images of embedded H1299 cells with 50 nm sections, such that each cell was captured in approximately 250–350 serial images. We determined the absolute and relative areas of the cytosol, nucleus and small organelles fractions in each section, and integrated across all sections to determine the volume for the whole cell and each subcellular compartment (Extended Data Fig. 4e). The nuclear volume was included with the cytosolic volume, assuming that the nuclear pores allow for passive metabolite transport between cytosol and nucleus\(^3,4\). Overall, the average volume of cytosol and nucleus in 12 cells is 82.6 ± 3.5% of total cell volume (Fig. 4g and Extended Data Fig. 4f). While each cell type and cell line varies in organelle content, H1299 cells display roughly similar organelle content to what has been previously reported\(^4,5\). Using the cytosolic OAA amounts shown in Fig. 4f and the cytosolic volume determined using SBFSEM, we calculated cytosolic OAA concentrations to be approximately 2 mM and to increase to approximately 7 mM following PKM2 activation with DASA (Fig. 4h).

PKM2 activation increases glutamine-derived OAA. We observed that glucose-derived OAA in the cytosolic fraction (Fig. 4d) was decreased even though OAA concentrations were increased by PKM2 activation. This suggests that OAA is synthesized from a non-glucose carbon source in a PKM2-activity-dependent manner. Because glutamine is a main carbon source for TCA cycle metabolites\(^6\) (Fig. 5b), we examined whether OAA is synthesized from glutamine when PKM2 is activated. Indeed, DASA increased \(^{15}\)C-glutamine–derived carbon incorporation into OAA in each cell line tested (Fig. 5c and Extended Data Fig. 5b), suggesting that glutamine is a precursor for de novo OAA synthesis following PKM2 activation with DASA.

We next investigated the pathway by which PKM2 activation increases glutamine-derived OAA to decrease LDH activity. Pyruvate

Fig. 5 | PKM2 activity increases glutamine-derived OAA. a, Isotopomer analysis of \(^{13}\)C6 glucose–derived carbon incorporation to OAA in H1299 cells treated with 40 µM DASA or vehicle in addition to \(^{13}\)C6 glucose overnight. b, Schematic representation of \(^{13}\)C6 glucose or \(^{15}\)C5 glutamine–derived carbon tracing of TCA cycle metabolites along with GPT2. c, Isotopomer analysis of \(^{13}\)C5 glutamine–derived carbon incorporation to OAA in H1299 cells treated with 40 µM DASA or vehicle in addition to \(^{13}\)C5 glutamine overnight. d, Isotopomer analysis of \(^{13}\)C6 glucose–derived carbon incorporation to alanine in H1299 cells treated with 40 µM DASA or vehicle in addition to \(^{13}\)C6 glucose overnight. e, Isotopomer analysis of \(^{13}\)C5 glutamine–derived carbon incorporation to α-KG in H1299 cells treated with 40 µM DASA or vehicle in addition to \(^{13}\)C5 glutamine overnight. f, \(^{13}\)C lactate labelling rate (percentage increase per minute in \(^{13}\)C lactate labelling) of H1299 vector and GPT2 shRNA knockdown cells incubated with \(^{13}\)C6 glucose and vehicle or 40 µM DASA. Western blot results of H1299 vector and GPT2 shRNA knockdown cells with antibodies against GPT2 and β-actin are also shown. g, \(^{13}\)C pyruvate labelling rate (percentage increase per minute in \(^{13}\)C pyruvate labelling) of H1299 vector and GPT2 shRNA knockdown cells incubated with \(^{13}\)C6 glucose and vehicle or 40 µM DASA. h, \(^{13}\)C lactate labelling rate in f divided by \(^{13}\)C pyruvate labelling rate in g as a surrogate to in situ LDH activity in H1299 vector and GPT2 shRNA knockdown cells treated with vehicle or 40 µM DASA. In situ LDH activities are normalized to the vehicle control. i, Relative lactate production in H1299 vector and GPT2 shRNA knockdown cells incubated with vehicle or 40 µM DASA. Data are represented as the mean and error bars represent the standard deviation from n = 5 for a,e, n = 3 for c,d,f–h,i of biologically independent replicates. Western blot results in f are representative experiments of three biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
can be metabolized to OAA through the TCA cycle via GPTs, which perform a coupled reaction of converting pyruvate and glutamate to alanine and α-KG (Fig. 5b)\(^{37,38}\). The α-KG can then be converted to malate and ultimately OAA in the TCA cycle (Fig. 5b). Consistent with this possible pathway, we observed increased \(^{13}\)C\(^6\) glucose–derived carbon incorporation into alanine (Fig. 5d) and increased incorporation of \(^{13}\)C\(^5\) glutamine–derived carbon to α-KG following PKM2 activation with no change in GPT2 protein expression (Fig. 5e and Extended Data Fig. 5c). To further assess the role of this pathway, we knocked down GPT2 in H1299 cells. These studies

![Diagram](image-url)
showed that GPT2 depletion blocked the decrease in 13C lactate labelling rate, the decrease in situ LDH activity (Fig. 5f–h), the reduction in lactate production (Fig. 5i) and the increase in OAA concentrations (Extended Data Fig. 5d) following PKM2 activation. These results indicate that GPT2 metabolically links PKM2 activation and LDH inhibition, possibly through glutaminolysis, in cancer cells.

Additional potential mechanisms do not affect LDH activity. Given that PKM2 has been shown to broadly affect cellular metabolism43,44, we examined whether metabolic enzymes other than GPT2 affect LDH activity upon PKM2 activation. As altered serine metabolism is one of the well-known effects of PKM2 activation45,46, we asked whether changes in serine metabolism alter LDH activity in response to PKM2 activation. We performed shRNA knockdown of phosphoglycerate dehydrogenase (PHGDH), the enzyme that catalyses the first step of the serine synthesis pathway, in H1299 cells and examined the rate of 13C lactate labelling, 13C pyruvate labelling and in situ LDH activity. We observed that H1299 cells with stable knockdown of PHGDH (Extended Data Fig. 5e) displayed similar 13C lactate labelling rate, 13C pyruvate labelling rate and LDH activity to those in vector control cells with or without DASA treatment (Extended Data Fig. 5f–h). This suggests that while upstream changes in flux through PHGDH may occur following PKM2 activation, the consequences of these changes do not alter LDH activity downstream.

We also asked whether changes in OAA synthesis from pyruvate directly through the activity of pyruvate carboxylase (PC) altered LDH activity in response to PKM2 activation. OAA directly generated from pyruvate would have three carbons labelled with 13C following incubation with 13C6 glucose. We did not see a significant difference in M + 3 labelling of OAA between vehicle or DASA treated H1299 or RPMI 8226 cells, suggesting pyruvate incorporation to OAA through PC is not increased following PKM2 activation (Figs. 4d and 5a and Extended Data Fig. 5a). To further examine whether PC affects LDH activity following PKM2 activation, we generated H1299 cells with shRNA knockdown of PC and performed time-series measurements to assess rate of 13C6 glucose incorporation to pyruvate and lactate. We observed that despite knockdown of PC, DASA treatment decreased the 13C lactate labelling rate and in situ LDH activity, suggesting PC is not required for the paradoxical correlation between PKM2 activation and LDH inhibition (Extended Data Fig. 5i–l).

LDHA variants with different sensitivity to OAA inhibition. We next asked whether the inhibitory effects of OAA on LDHA are sufficient to alter the Warburg effect and affect tumour growth by using an LDHA that is more resistant to the inhibitory effects of OAA. A previous study found that OAA had a higher IC50 of 290 ± 48 μM compared to the Kᵢ for hLDHA determined in this study (290 ± 100 μM). Consistent with this, we found that the IC50 of OAA was approximately threefold higher for rbLDHA versus hLDHA (3.3 ± 0.48 and 1.1 ± 0.14 mM, respectively) (Figs. 2a and 6a). In addition, our Lineweaver–Burk plot analysis indicates that OAA is a competitive inhibitor against rbLDHA similar to hLDHA (Fig. 6b), leading to the assumption that OAA binds to the substrate binding pockets of both rbLDHA and hLDHA. On the basis of this assumption, we obtained the three-dimensional models of rbLDHA and hLDHA with OAA docked at the substrate binding pocket by performing molecular dynamics simulations of the two LDHA complexes. These models suggest that the interaction of the di-anion OAA with rbLDHA is weakened, relative to that of hLDHA, by the mutations of charged residues (K222, E229 and R315) in hLDHA to neutral residues (A222, Q229 and H315) in rbLDHA and the mutations of hydrophobic residues (V189 and V273) in hLDHA to bulkier residues (I189 and I273) in rbLDHA (Fig. 6c). These results may explain the difference in IC50 of OAA against hLDHA and rbLDHA.

OAA inhibition of LDH increases efficacy of PKM2 activator. Because rbLDHA is less sensitive to OAA inhibition than hLDHA, we hypothesized that expression of rbLDHA would make cancer cells resistant to treatment with a PKM2 activator with respect to lactate production and tumour growth. We generated H1299 cells in which endogenous LDHA and LDHB were knocked down and rescued with rbLDHA or with hLDHA as a control. While LDHB is also inhibited by OAA (Extended Data Fig. 6a), its cellular concentration (0.077 mg ml⁻¹) is tenfold less than that of LDH (0.73 mg ml⁻¹) in H1299 cells (Extended Data Fig. 6b) and LDHB contributes to only 20% of total LDH activity in cells (Extended Data Fig. 6c). Therefore, we focussed our analyses on LDHA and only rescued LDH activity with hLDHA or rbLDHA. LDHA protein and activity levels were restored to similar levels in these two cell lines (Fig. 6d). Using these hLDHA- and rbLDHA-expressing cell lines, we first examined whether DASA differentially affected in situ LDHA activity and lactate concentrations. In cells expressing hLDHA, DASA decreased the 13C lactate labelling rate, in situ LDHA activity, lactate concentrations and lactate production (Fig. 6c–i), similar to what was observed in parental H1299 cells (Fig. 1b,c,f–h). In contrast, DASA did not alter these metabolic effects in rbLDHA-expressing cells (Fig. 6c–i). Thus, expression of rbLDHA blocks the paradoxical correlation between elevated PKM2 activity and decreased intracellular lactate concentrations in the Warburg effect.

Next, we assessed the proliferation of hLDHA and rbLDHA-expressing H1299 cells as well as the effects of pharmacological PKM2 activation when these cells were grown as mouse xenografts. As the PKM2 activator DASA displays poor bioavailability in mice, we used the PKM2 activator TEPP-46, which is structurally similar to DASA, increases PKM2 activity via a similar mechanism and produces similar metabolic changes with respect to glutamine-derived OAA synthesis in H1299 cells (Extended Data Fig. 6a).
Data Fig. 7a,b). Using TEPP-46, the mouse xenograft study yielded several important observations. First, there was no difference in the growth of hLDHA expressing and vector control H1299 tumours in mice treated with vehicle or TEPP-46, indicating the hLDHA rescue is sufficient to generate a similar growth phenotype as the parental H1299 tumours (Fig. 7a). Second, there was no difference in the growth of hLDHA and rbLDHA-expressing H1299 tumours in mice treated with vehicle (Fig. 7a). Third,
TEPP-46 reduced the growth rate of hLDHA expressing tumours as well as and the levels of Ki67, a marker of proliferating cells, whereas it had no effect on rbLDHA-expressing tumours (Fig. 7a and Extended Data Fig. 7c).

OAA and lactate are inversely correlated in human samples. We next sought to examine whether the above-described inverse correlation between lactate and OAA concentrations in cancer cell lines is observed in human primary cells. Myeloma plasma cells that clonally expand in bone marrow are expected to display a glycolytic phenotype as compared to that of normal plasma cells that are terminally differentiated and less proliferative. Consistent with this, we observed higher lactate concentration in CD138\(^+\) plasma cells from the bone marrow aspirates of patients with multiple myeloma as compared to those from healthy individuals (Fig. 7b, left). In addition, CD138\(^+\) myeloma plasma cells have decreased OAA concentrations relative to CD138\(^+\) normal plasma cells (Fig. 7b, right), indicating an inverse correlation between lactate and OAA concentration in human primary samples.

Discussion
A long-standing question in the field of cancer metabolism is how decreased pyruvate kinase activity regulates pathways downstream of pyruvate kinase to increase lactate levels\(^4\). This study shows that PKM2 activation increases the production of OAA, which then accumulates to levels that inhibit hLDHA and therefore reduce lactate production in cancer cells (Fig. 7c). Accordingly, our studies demonstrate that OAA is a key intermediate in the paradoxical correlation between elevated PKM2 activity and decreased intracellular lactate concentrations in the Warburg effect, where enzymatic activation of pyruvate kinase increases OAA concentrations to inhibit LDHA activity and tumour growth.

In addition to its canonical role in glucose metabolism, PKM2 has been shown to regulate gene transcription through its nuclear
protein kinase activity. In this capacity, the low activity dimer conformation of PKM2 undergoes nuclear translocation where it phosphorilates signal transducer and activator of transcription (STAT) proteins44. Additionally, nuclear PKM2 has been shown to interact with HIF1α and β-catenin, which are known to regulate expression of glycolytic enzymes to support the Warburg effect45–47. In the current study, we did not investigate whether these non-canonical activities of PKM2 contribute to the paradoxical correlation between PKM2 activity and lactate levels in the Warburg effect. However, our data show that expression of rbLDHA in place of hLDHA is sufficient to block the decrease in LDH activity, intracellular lactate concentration and tumour growth following pharmacological activation of PKM2. In addition, we showed that inhibition of LDH following PKM2 activation is dependent on glutamine metabolism through GPT2 that is regulated by the canonical enzymatic activity of PKM2. These results indicate that cellular OAA concentration is probably dependent on the canonical enzymatic activity of PKM2, which further regulates downstream events including intracellular lactate concentration and tumour growth following PKM2 activation.

We have shown that tumour growth in a mouse xenograft model is unaffected by rbLDHA expression. However, hLDHA-expressing tumours display decreased growth on treatment with the PKM2 activator, while rbLDHA-expressing tumours did not. These data indicate that the antiproliferative effects of PKM2 activation depend on OAA-mediated inhibition of LDH. However, the downstream mechanisms by which the decreased LDH activity decreases tumour growth remains to be investigated. It is possible that the decrease in hLDHα activity following TEPP-46 treatment alters NAD+/NADH, which may affect the activity of numerous downstream enzymes that use NAD+ as a cofactor. Detailed investigation of these potential mechanisms will require further investigation.

Finally, we investigated the correlation of lactate and OAA concentrations in primary CD138⁺ cells from patients with myeloma and normal healthy individuals. We observed an inverse correlation with HIF1α and β-catenin, while rbLDHA-expressing tumours did not. These data indicate the relevance of OAA as a regulator of the Warburg effect in rapidly proliferating cells in humans.

Methods

Cell lines. Non-small cell lung carcinoma H1299 cells were obtained from J. Chen at U. Chicago. Multiple myeloma RPMI 8226 cells were provided by the laboratory of W.I.G. Female human embryonic kidney 293T cells were obtained from Y. Machida at Mayo Clinic. H1299, RPMI 8226 and 293T cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). H1299 derivatives stably expressing PKM2 or PKM1 with knockdown of endogenous PKM2 were cultured in RPMI 1640 supplemented with 10% FBS, 1% P/S, 300 μg/ml hygromycin and 2 μg/ml puromycin. H1299 derivatives stably expressing human LDHA or rabbit LDHA with knockdown of endogenous human LDHA and LDHB were also cultured in RPMI 1640 supplemented with 10% FBS, 1% P/S, 300 μg/ml hygromycin and 2 μg/ml puromycin. H1299 derivatives with shRNA knockdown of GPT2 were cultured in RPMI 1640 supplemented with 10% FBS, 1% P/S and 2 μg/ml puromycin. H1299 derivatives with shRNA knockdown of PHGDH or PC were cultured in RPMI 1640 supplemented with 10% FBS, 1% P/S and 1 μg/ml puromycin. All cells were cultured in 37 °C humidified incubators with 5% CO2.

Xenograft model. Male 4–6-week old athymic nude mice (Athymic Nude-Foxn1nu) were purchased from Envigo. All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee (IACUC), and conformed to the federal guidelines for the care and maintenance of laboratory animals. Mice were housed under a 12 h light-dark cycle in plastic cages and fed an irradiated chow diet. Housing temperatures and humidity were kept within a range of 21.7–22.8 °C (71–73 °F) and 40–60%, respectively. Water and cages were autoclaved. Cages were changed once weekly, and the health status of the mice was monitored using a dirty bedding sentinel program. For the human and rabbit LDHA xenograft study, mice were subcutaneously inoculated with 1 × 10⁶ cells in PBS. After tumours reached a volume of about 50 mm³, mice were randomized into two groups (control and treatment). For treatment, 50 mg/kg -1 TEPP-46 in 0.5% hydroxypropylmethylcellulose/0.1% tween 80 solution was administered orally twice daily. Control animals received 0.5% hydroxypropylmethylcellulose/0.1% tween 80 solution orally twice daily. Tumour growth was recorded by measurement of two perpendicular diameters of the tumour over the 18-day treatment course using the formula 4π/3 × (W/2)² × (L/2), where L represents length of the longest dimension and W represents the width at a right angle to the longest dimension for the calculation of tumour volume. Tumour volumes were monitored not to exceed the maximum allowable tumour volume (1,500 mm³).

Humane endpoints to euthanize mice were as follows: weight loss greater than or equal to 20% of body weight, inability to ambulate, inability to reach food and/or water, tumours greater than or equal to 10% body weight, tumours that have ulcerated and a body condition score of one or less using the IACUC approved scoring system. P values were determined by a two-tailed Student’s t-test.

Plasmids and shRNA constructs. Mouse PKM2 complementary DNA (NM0110999, described previously), human PMK1 (NM182470) and human PKM2 shRNA (RHS3979-9605020) were purchased from Open Biosystems. Human LDHA cDNA (accession no. BC067223) was purchased from GE Dharmacon and rabbit LDHA cDNA (accession no. NM010182277) was purchased from Genscript. The lentiviral human LDHα shRNA (TRCN0000026537) and LDHB shRNA (TRCN0000028488) in plKO.1-puro vectors, lentiviral human GPT2 shRNA (TRCN0000053024) and TRCN0000030258 in plKO.1-puro vectors, lentiviral human PHGDH shRNA (TRCN0000028520) and (TRCN0000028532) in plKO.1-puro vectors and lentiviral PC shRNA (TRCN000078457) and (TRCN000078453) in plKO.1-puro vectors were all purchased from GE Dharmacon.

Chemicals. DASA was purchased from EMD Millipore (550602) and TEPP-46 was purchased from Asbous (AOB1123). Beta-nicotinamide adenine dinucleotide, reduced (N1829), oxaloacetic acid (O4126), l-aspartic acid (A9256), methoxamine (M6524), pyridine (270970) and adenosine 5-diphosphate (A2754) were purchased from Sigma. Phosphoenolpyruvic acid monopotassium salt (B20358) was purchased from Alfa Aesar. 13C6 glucose (CLM-1396), 13C5 glutamine (CLM-1822), 3,3-D3 lactate (DLM-9071), 3-¹3C pyruvate (CLM-1585), 13C6 or KG (CLM-4442) and 13C1 pyruvate (CLM-2440) were purchased from Cambridge Isotope Laboratories. D-glucose (50164244), dimethylformamide (PI20673), MSTFA + 1% TMCS (TS-48915) and MBTBSA + 1% TBDMS (TS-48927) were purchased from Fisher. Purified recombinant hLDHA (6374) and purified recombinant hLDHB (6375) were purchased from Biovieton. Purified rbLDHA was purchased from VWR EMID (93061).

Western blotting. Cell lysates were prepared and subjected to western blot analysis as performed previously41,42. The following commercial antibodies were used: anti-β-actin (Sigma A1978), anti-PKM2 (Cell Signaling Technology 31985), anti-PKM1 (Cell Signaling Technology 7067), anti-PKM (Abcam AB118499), anti-human specific LDHA (Cell Signaling Technology 20125), anti-LDH-B (Abcam AB75167), anti-human and rabbit LDHA (Abcam AB135396), anti-p70 S6 kinase (Cell Signaling Technology 27087T), anti-calreticulin (Cell Signaling Technology 122387T), anti-golgin (Cell Signaling Technology 131912T), anti-COX IV (Abcam AB118499) and anti-human specific LDHA (Cell Signaling Technology 20125), anti-LDH-B (Abcam AB75167), anti-human and rabbit LDHA (Abcam AB135396), anti-p70 S6 kinase (Cell Signaling Technology 27087T), anti-calreticulin (Cell Signaling Technology 122387T), anti-golgin (Cell Signaling Technology 131912T), anti-COX IV (Abcam AB118499) and anti-human specific LDHA (Cell Signaling Technology 20125), anti-LDH-B (Abcam AB75167), anti-human and rabbit LDHA (Abcam AB135396), anti-p70 S6 kinase (Cell Signaling Technology 27087T). For 13C glucose or 13C glutamine tracing experiments, cells were incubated with glucose or glutamine free RPMI 1640 medium supplemented with 10% FBS, 1% P/S, 300 μg/ml hygromycin and 2 μg/ml puromycin. 13C6 glucose or 13C5 glutamine were treated with 110 μM DASA or vehicle overnight. Collected cells were washed once with PBS, then metabolites were extracted using 1:1 ice-cold methanol:water, with or without 5 mM ninhydrin as described previously41. Ninhydrin was prepared fresh daily. Lysates were spun at 15,000 r.p.m. for 5 min at 4 °C to remove protein debris and metabolites were dried under N2 gas as previously described41.

Metabolite extraction for GC–MS analysis. For 13C glucose or 13C glutamine tracing experiments, cells were incubated with glucose or glutamine free RPMI 1640 medium supplemented with 10% FBS, 1% P/S and 2 mg/ml U-13C6 glucose or U-13C5 glutamine and treated with 110 μM DASA or vehicle overnight. Collected cells were washed once with PBS, then metabolites were extracted using 1:1 ice-cold methanol:water, with or without 5 mM ninhydrin as described previously41. Ninhydrin was prepared fresh daily. Lysates were spun at 15,000 r.p.m. for 5 min at 4 °C to remove protein debris and metabolites were dried under N2 gas as previously described41.
For OAA quantification in culture cells, cells were grown with 13C6 glucose containing RPMI 1640 medium and treated with 40 μM DASA or vehicle overnight. Collected cells were split into two equal samples, washed with PBS and extracted using 1:1 ice-cold methanol-water containing 5 mM ninhydrin as described previously35, with or without a 13C OAA spike. 13C OAA was prepared fresh daily. Lysates were spun at 15,000 g for 5 min at 4 °C to remove protein debris and metabolites were dried under N2 gas. For OAA quantification in human primary CD138+ cells, cells were extracted with 1:1 methanol:water containing 5 mM ninhydrin as described above. Lysates were spun to remove protein debris and dried as described above.

For lactate and pyruvate quantification, cells were grown in RPMI 1640 medium and treated with 40 μM DASA or vehicle. Collected cells were washed with PBS and extracted using 1:1 ice-cold methanol-water with D3 lactate or 13C3 pyruvate. Lysates were spun to remove protein debris and dried as described above.

DMF-MTBSTFA derivatization and GC–MS analysis. Dried metabolite samples were dissolved in 75 μl of DMF, derivatized with 75 μl of MTBSTFA + 1% TMBDMS and then analysed by GC–MS as performed previously26. Data were analysed using Agilent MassHunter Workstation Analysis and Agilent MSD ChemStation Data Analysis softwares. IsoPat2 software was used to adjust for natural abundance as previously described64,65.

Pure 13C OAA was detected as the OAA 3-TBDMS derivative as described previously with a main ion fragment of m/z 417 (Extended Data Fig. 3a)45. OAA was also detectable in cell metabolite extracts following extraction with ninhydrin containing extraction buffer and extraction buffer alone (Extended Data Fig. 3b). Mox-MSTFA derivatization of malate. Dried metabolite samples were dissolved in 417 μl methanol, vortexed and spiked with 13C4 malate (5 mM) and incubated at 70 °C for 30 min. Aliquots of the samples were derivatized using DMF-MTBSTFA as described previously26. Samples were derivatized with 5 mM ninhydrin and spiked with 13C4 lactate (5 mM) and incubated at 70 °C for 30 min. Aliquots of the samples were derivatized using DMF-MTBSTFA as described above.

Mox-MSTFA derivatization of malate. Dried metabolite samples were dissolved in 75 μl methoxamine (20 mg ml−1 in pyridine) and incubated at 70 °C for 30 min. Samples were then extracted with 1:1 methanol:water and analysed under GC–MS as described above. Concentrations were determined using the isotope-ratio-based approach described previously26,45. Lactate and pyruvate concentrations were then determined by analysing the M+3 peaks for both metabolites (m/z 262 for pyruvate and m/z 264 for lactate) compared to the M+0 peaks (m/z 259 for pyruvate and m/z 261 for lactate).

Human primary samples and isolation of CD138+ cells. The freshly obtained bone marrow aspirates from patients with multiple myeloma and healthy individuals underwent Ficoll–Paque gradient separation, red cell lysis and CD138+ selection as performed previously36. Purity of the sorted clonal PCIs was confirmed via light chain restriction using a slide-based immunofluorescence method. This study was approved by the Internal Review Board at the Mayo Clinic. All patients and healthy individuals included in this study had provided written informed consent to allow their bone marrow and peripheral blood samples to be used for research purposes in this study. Age, sex, treatment history and genotype of the samples were blinded for this study. OAA and lactate in CD138+ cells were quantified by GC–MS as described above with the use of 13C4 α-KG as an internal standard.

Glucose uptake assay. H1299 and RPMI 8226 cells (approximately 50% confluent) were incubated in phenol red free RPMI 8226 media containing 1% PBS and 1% P/S and treated with vehicle, 40 μM DASA, 1 mM diethyl-ester OAA or 10 mM aspartate. Media glucose amounts were quantified by the MBL Glucose Assay Kit (JM-K606-100).

Cell fractionation. Approximately 25 × 10^6 H1299 cells per sample labelled with 13C glucose and treated with 40 μM DASA or vehicle were gathered and washed with LC–MS/GC–MS compatible mitochondrial isolation buffer KPB (136 mM KCl, 10 mM K2HPO4, pH 7.25 adjusted with KOH)46. Cells were resuspended in KPB buffer and homogenized with a Dounce homogenizer. Degree of homogenization was assessed under microscope with trypan blue staining. Once homogenized, cell lysates were spun at 1,000g for 10 min to remove nuclear debris and unlysed cells. The supernatant was spun again at 17,000g for 10 min to separate the small organelles from the cytosolic fraction. Small aliquots were saved for western blot analysis to assess the quality of the protein fractionation. The small organelle pellet was then extracted with 1:1 methanol:water containing 5 mM AOAA spike, while the supernatant was extracted with 100% methanol containing 5 mM ninhydrin, with or without 13C OAA spike. Extracted metabolites were spun and dried under N2 as described above. The dried samples were derivatized using DMF-MTBSTFA and analysed via GC–MS as described above.

SBFSEM imaging. Samples were fixed, stained and prepared for serial block-face microscopy as described previously67,68. Embedded samples were prepared for SEM imaging, inserted into a VolumeScope SBFSEM (Thermo Fisher) and high-resolution block-face images were obtained69. The image stack was aligned, filtered and rendered using Amira software (Thermo Fisher) as previously described68.

Immunohistochemical staining (Ki67). IHC staining was performed at the Pathology Research Core (Mayo Clinic) using the Leica Bond RX stainer (Leica) as previously described70.

Purification of recombinant LDHA. Human LDHA cDNA was subcloned into pET53 Gateway destination vector from pENTR shuttle vector, which appends an N-terminal His-tag as described previously47. LDHA recombinant protein was purified with ProBond Resin His-tag beads (Life Technologies) as performed previously47. Purified proteins were stored at −80 °C in 20% glycerol until further analysis.

Determination of LDHA and LDHB protein concentrations. Western blot lysate of a known number of untreated H1299 cells was prepared as described above. The protein concentration of the sample was determined using a Bradford assay. The H1299 lysate was then run alongside known amounts of recombinant hLDHA or hLDHB protein. Following blotting with either anti-LDHA or anti-LDHB antibody, the blot was developed using a Protein Simple FluorChemE developer. The blot was further analysed in AlphaView SA software, where average pixel intensity of each band was determined. A standard curve was then constructed and used for the determination of LDHA or LDHB protein in the H1299 lysate.

In vitro PKM2 enzyme activity assay. Pyruvate kinase activity was measured by an LDH coupled enzyme assay, carried out with cell lysate in a buffer containing 50 mM tris-HCL, 100 mM KCl, 5 mM MgCl2, 1 mM ADP, 0.5 mM PEP, 0.2 mM NADH and 8 μl of LDH. The decrease in fluorescence (excitation 430 nm, emission 460 nm) from the oxidation of NADH to NAD+ was recorded by a spectrophotometer. The assay was performed at room temperature.

LDH enzyme activity assay and IC50 analysis. LDH activity was measured in PBS containing 0.2 mM NADH, 0.5 mM 13C1 pyruvate and varying concentrations of 13C OAA were added to start the reaction. The decrease in fluorescence (excitation 340 nm, emission 660 nm) from the oxidation of NADH to NAD+ was recorded by a spectrophotometer. When the reaction was approximately 50% complete, the reaction was quenched with excess 100% methanol. Samples were vortexed and spun. Metabolite extracts were then spiked with D3 lactate, dried under N2, and derivatized using DMF-MTBSTFA as described above. The amount of 13C1 lactate, which corresponds to lactate derived from 13C1 pyruvate, was then determined by comparing to the known amount of D3 lactate spike.

In vitro LDHA assay recapitulating cellular conditions. Purified recombinant hLDHA (0.73 mg ml−1) was incubated with 13C1 pyruvate and 13C OAA at concentrations that mimic those observed in vehicle or 40 μM DASA treated H1299 cells. The reaction was allowed to proceed for 5 s, and was then quenched...
with excess 100% methanol, vortexed and centrifuged to obtain metabolite extracts. The metabolite extracts were then spiked with D3 lactate, dried under N2, and derivatized using DNM-FTBTF as described above. The amount of [13C]lactate, which corresponds to lactate derived from [13C]pyruvate, was then determined by comparing to the known amount of D3 lactate spike.

**Dixon analysis for Kd determination.** Purified recombinant hLDHA was incubated in PBS containing 0.2 mM NADH. Then 100, 200 or 400 μM [13C]pyruvate and varying concentrations of [13C]OAA were added to start the reaction. The decrease in fluorescence (excitation 340 nm, emission 460 nm) due to the oxidation of NADH to NAD+ was recorded by a spectrophotometer. The assay was performed at room temperature. The reaction was quenched with 100% MeOH at a set time point before complete oxidation of NADH. Samples were then spiked with D3 lactate, dried under N2 gas, and derivatized with DNM-FTBTF as described above. The amount of [13C]lactate, the lactate derived from [13C]pyruvate, was determined. A Dixon plot was constructed by plotting OAA concentration against the inverse of lactate amounts. The lines for the Dixon plot converge above the x axis, and the value of OAA where they intersect is equal to Kd.

**Tryptophan fluorescence assay.** For this, 2 μM purified recombinant human LDHA was incubated with different OAA concentrations (0–2 mM), and tryptophan fluorescence at excitation of 280 nm and emission 340 nm was measured. The Kd was calculated by plotting the log(fluorescence change/fluorescence intensity) versus log(OAA concentration). Kd was calculated by the modified form of a Stern–Volmer plot in which the vertical intercept is log(Kd/Kx).

The assay was performed at room temperature.

**Flow cytometry.** H1299 cells were treated with 40 μM DASA or vehicle overnight. Cells were then stained with 500 nM MitoTracker (Life Technologies) in media for 30 min at 37 °C, 1 μM ER-BODPY (Life Technologies) in Hank's balanced salt solution for 30 min at 37 °C or 1 μM Golgi Stain (Life Technologies) in Hank’s balanced salt solution/HEPES buffer for 30 min at 4 °C followed by 30 min at 37 °C in media. Following incubation with stains, cells were gathered, washed and resuspended in PBS. Samples were analysed in the FL2 channel on a Becton Dickinson FACS Cantoll using FACS Diva analysis software.

**Theoretical models of OAA complexes with hLDHA and rbLDHA.** The three-dimensional models of hLDHA and rbLDHA structures were generated using SwissModel with (1) the sequences obtained from NCBI (BC067223 and NM_001082277.1 for hLDHA and rbLDHA, respectively) and (2) the template crystal structures with PDB IDs of 6MV8 (Chain A for hLDHA) and 5NQ8 (Chain A for rbLDHA). The OAA complex with hLDHA or rbLDHA was obtained from 20 distinct, independent, unrestricted, unbiased, isotropic-isothermal and 316-ns molecular dynamics simulations using a published protocol. Figure 6c was generated with PyMOL v.1.7.0.3.

**Statistical analysis.** Statistical significance was tested using unpaired two-tailed t-tests, assuming independent variables, normal distribution and equal variance of samples. Data are presented as mean ± standard deviation (mean ± standard error for xenograft studies). The statistical parameters can be found within the figure legends. A P value <0.05 was considered to indicate statistical significance. Statistical analyses were performed using Excel.

**Reporting Summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The accession codes used in this study are 6MV8 (hLDHA) and 5NQ8 (rbLDHA) from the PDB, Supplementary Information including Supplementary Fig. 1 exemplifying the gating strategy for Extended Data Fig. 4d are provided with this paper. Source data are provided with this paper.

Received: 20 May 2020; Accepted: 4 June 2021; Published online: 5 July 2021

**References**

1. Warburg, O. On the origin of cancer cells. *Science* 123, 309–314 (1956).
2. Lunt, S. Y. & Vander Heiden, M. G. Aerobic glycolysis: meeting the demand for ATP in cancer. *Sem. Cell Dev. Biol.* 1963, 364–368 (2010).
3. Hittosugi, T. et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* 2, ra73 (2009).
4. Christofk, H. R. et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452, 230–233 (2008).
5. Anastassiou, D. et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* 334, 1276–1283 (2011).
6. Wiese, E. K. & Hitosugi, T. Tyrosine kinase signaling in cancer metabolism: PKM2 paradox in the Warburg effect. *Front Cell Dev. Biol.* 6, 79 (2018).
7. David, C. J., Chen, M., Assanah, M., Canoll, P. & Manley, J. L. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463, 364–368 (2010).
8. Hitosugi, T. et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* 2, ra73 (2009).
9. Christofk, H. R. et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452, 230–233 (2008).
10. Anastassiou, D. et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* 334, 1276–1283 (2011).
11. Wiese, E. K. & Hitosugi, T. Tyrosine kinase signaling in cancer metabolism: PKM2 paradox in the Warburg effect. *Front Cell Dev. Biol.* 6, 79 (2018).
12. Jurica, M. S. et al. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* 6, 195–210 (1998).
13. David, C. J., Chen, M., Assanah, M., Canoll, P. & Manley, J. L. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* 463, 364–368 (2010).
14. Hittosugi, T. et al. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. *Sci. Signal.* 2, ra73 (2009).
15. Christofk, H. R. et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 452, 230–233 (2008).
16. Anastassiou, D. et al. Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. *Science* 334, 1276–1283 (2011).
17. Wiese, E. K. & Hitosugi, T. Tyrosine kinase signaling in cancer metabolism: PKM2 paradox in the Warburg effect. *Front Cell Dev. Biol.* 6, 79 (2018).
18. Jurica, M. S. et al. The allosteric regulation of pyruvate kinase by fructose-1,6-bisphosphate. *Structure* 6, 195–210 (1998).
42. Kung, C. et al. Small molecule activation of PKM2 in cancer cells induces serine auxotrophy. *Chem. Biol.* **19**, 1187–1198 (2012).

43. Chaneton, B. et al. Serum is a natural ligand and allosteric activator of pyruvate kinase M2. *Nature* **491**, 458–462 (2012).

44. Gao, X., Wang, H., Yang, J. J., Liu, X. & Liu, Z. R. Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase. *Mol. Cell* **45**, 598–609 (2012).

45. Luo, W. et al. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. *Cell* **145**, 732–744 (2011).

46. Wang, H. J. et al. JMJD5 regulates PKM2 nuclear translocation and reprograms HIF-1alpha-mediated glucose metabolism. *Proc. Natl Acad. Sci. USA* **111**, 279–284 (2014).

47. Yang, W. et al. Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. *Nature* **480**, 118–122 (2011).

48. Gruber, C. C. et al. An algorithm for the deconvolution of mass spectroscopic patterns in isotope labeling studies. evaluation for the hydrogen-deuterium exchange reaction in ketones. *J. Org. Chem.* **72**, 5778–5783 (2007).

49. Mazlaghaninia, M., Atri, M. S. & Seyedalipour, B. Scopoletin and morin inhibit lactate dehydrogenase enzyme activity is critical for cancer metabolism. *Hormozgan Med. J.* **23**, e8269 (2019).

50. Pang, Y. P. FF12MC: a revised AMBER forcefield and new protein simulation protocol. *Proteins* **84**, 1490–1516 (2016).

51. Hitosugi, T. et al. Phosphoglycerate mutase 1 coordinates glycolysis and biosynthesis to promote tumor growth. *Cancer Cell* **22**, 585–600 (2012).

52. Kurmi, K. et al. Tyrosine phosphorylation of mitochondrial creatine kinase 1 enhances a druggable tumor energy shuttle pathway. *Cell Metab.* **28**, https://doi.org/10.1016/j.cmet.2018.08.008 (2018).

53. Cooper, G., Reed, C., Nguyen, D., Carter, M. & Wang, Y. Detection and formation scenario of citric acid, pyruvic acid, and other possible metabolism precursors in carbonaceous meteorites. *Proc. Natl Acad. Sci. USA* **108**, 14015–14020 (2011).

54. Gonsalves, W. I. et al. Glutamine-derived 2-hydroxyglutarate is associated with disease progression in plasma cell malignancies. *JCI Insight* **3**, https://doi.org/10.1172/jci.insight.94543 (2018).

55. Hua, Y., Lasserstein, P. & Helmstaedter, M. Large-volume en-bloc staining for electron microscopy-based connectomics. *Nat. Commun.* **6**, 7923 (2015).

56. Mereuta, O. M. et al. High-resolution scanning electron microscopy for the analysis of three-dimensional ultrastructure of clots in acute ischemic stroke. *J. NeuroIntervent Surg.* **6**, 1–7 (2020).

57. Hurley, R. M. et al. S3BP1 as a potential predictor of response in PARP inhibitor-treated homologous recombination-deficient ovarian cancer. *Gynecol. Oncol.* **153**, 127–134 (2019).

Acknowledgements
We thank the Mayo Microscopy and Cell Analysis Core and the Mayo Pathology Research Core at Mayo Clinic Rochester for experimental and technical support. We thank J. Maher and S. Kaufmann for their critical reading of the paper. This research was supported in part by National Institutes of Health (NIH) grant no. R01 CA225680 (T.H.), Research Scholar grant (no. RSG-19-076-01-TBE) from the American Cancer Society (T.H.), the Eagles Cancer Research Fund (T.H.), a Team Science Platform Award from the Mayo Clinic Center for Biomedical Discovery (T.H.), the Developmental Therapeutics Program from the Mayo Clinic Cancer Center (T.H.) and the Mayo Clinic Breast SPORE grant no. P50 CA116201 (T.H.). W.L.G. was supported by the National Cancer Institute of the NIH under award no. K23 CA218742. S.T.L. was supported by NIH grant no. R25 GM075148-14. E.K.W. was supported by NIH grant no. T32 GM072474 and a predoctoral fellowship from the Mayo Foundation for Education and Research.

Author contributions
E.K.W., S.H., S.T.L., A.S. and T.H. performed the experiments and analysed the data with input from K.K. and L.M.K. L.G.A.-B. assisted with SBFSEM data analysis. Y.-P.P. performed the computational work. W.L.G. provided critical reagents and patient samples. E.K.W. and T.H. wrote the paper. All authors contributed to discussion about the data.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s42255-021-00424-5.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42255-021-00424-5.

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Peer review information *Nature Metabolism* thanks Chi Dang, Heather Christofk and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Primary Handling Editor: George Caputa.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Increased PKM2 activity decreases LDH activity and lactate concentration. Related to Fig. 1. a. Pyruvate kinase activity from lysates of PKM2 or PKM1-expressing H1299 cells. Corresponding western blots of H1299 vector, PKM2, and PKM1 cell lysates with antibodies against PKM, PKM2, PKM1, LDHA, and β-actin. b. Whole cell lactate concentrations of PKM2 and PKM1 expressing H1299 cells. c. Relative lactate production rate in PKM2 and PKM1 expressing H1299 cells. d. Relative lactate production rate in RPMI 8226 cells treated with vehicle or DASA. e. Pyruvate concentrations in PKM2 or PKM1 expressing H1299 cells. f-g. Relative glucose uptake of H1299 (f) and RPMI 8226 (g) cells with vehicle or DASA treatment. h. 13C lactate labelling rate of RPMI 8226 cells incubated with 13C6 glucose and vehicle or 40 μM DASA. i. 13C pyruvate labeling rate of RPMI 8226 cells incubated with 13C6 glucose and vehicle or 40 μM DASA. j. In situ LDH activity in RPMI 8226 cells with vehicle or DASA treatment. k. 13C lactate labeling rate of PKM2 and PKM1 expressing H1299 cells incubated with 13C6 glucose. l. 13C pyruvate labeling rate of PKM2 and PKM1 expressing H1299 cells incubated with 13C6 glucose. m. In situ LDH activity in PKM2 and PKM1 expressing H1299 cells. n. LDH activity from lysates of RPMI 8226 cells treated with 40 μM DASA or vehicle overnight. Below, the corresponding western blots of the RPMI 8226 lysates with antibodies against LDHA, LDHB, and β-actin. o. LDH activity from lysates of H1299 cells expressing PKM2 or PKM1. Below, the corresponding western blots of the PKM2 and PKM1 expressing H1299 lysates with antibodies against LDHA, LDHB, and β-actin. Data is represented as the mean and error bars represent the standard deviation from n = 3 for (a), (k), (l), (m), n = 4 for (b), (c), (d), (e), (f), (g), (h), (i), (j), n = 6 for (n), n = 7 for (o) of biologically independent replicates. Western blot results in (a), (n), (o) are representative experiments of 3 biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
Extended Data Fig. 2 | OAA is a competitive inhibitor of hLDHA. Related to Fig. 2. a. Gas chromatogram of malate 3-TMS derivative (m/z 335) of pure malate standard (top). Gas chromatogram of malate 3-TMS derivative (m/z 335) of an in vitro LDH activity assay sample in which purified recombinant hLDHA was incubated with OAA (bottom). No malate was detected. b. Relative LDHA activity in the presence of various metabolites, determined using purified recombinant human LDHA and a fluorescence based LDH activity assay. c. Fluorescence intensity scan at ex. 280 nm of purified human LDHA incubated with increasing concentrations of OAA. Data is represented as the mean and error bars represent the standard deviation from n = 4 for (b) of independent replicates in vitro. Chromatograms in (a) and fluorescence curves in (c) are representative experiments of three independent replicates in vitro and the P values were determined by a two-tailed Student’s t-test.
Extended Data Fig. 3 | GC–MS detection of OAA 3-TBDMS and analysis of glucose uptake. a. (left) Scan of m/z 417 of pure 12C OAA standard derivatized with DMF-MTBSTFA. (middle) Mass spectrum of pure 12C OAA standard, derivatized with DMF-MTBSTFA. m/z 417 corresponds to OAA 3-TBDMS M-57 fragment. (right) Standard curve showing a positive, linear relationship between OAA amount and m/z 417 peak intensity of pure OAA standard. b. (left) Scan of m/z 417 of H1299 cellular metabolites labelled with 13C glucose, extracted with 1:1 methanol:water containing 5 mM ninhydrin, with or without 12C OAA spike. (middle) Isotopomer distribution of OAA (m/z 417) from H1299 cellular metabolites labeled with 13C glucose, extracted with 1:1 methanol:water containing 5 mM ninhydrin, with or without 12C OAA spike. (right) Ion abundance, normalized using IsoPat2, of isotopomer distribution data from (middle). c. Relative glucose uptake of H1299 (left) and RPMI 8226 (right) cells treated with vehicle or 1 mM diethyl-ester OAA. d. Relative glucose uptake of H1299 (left) and RPMI 8226 (right) cells treated with vehicle or 10 mM aspartate. Data is represented as the mean and error bars represent the standard deviation from n = 4 for (c), (d) of biologically independent replicates. Results in (a) are representative experiments of three independent replicates in vitro. Results in (b) are representative experiments of 3 biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Activation of PKM2 increases cellular OAA concentrations to inhibit LDH activity. Related to Fig. 4. a. Relative whole cell OAA concentrations of RPMI 8226 cells treated with 40 μM DASA or vehicle overnight. Whole cell metabolites were extracted with 1:1 methanol:water containing 5 mM ninhydrin, with or without 13C OAA spike, derivatized, and analysed with GC-MS. b. Relative whole cell OAA concentrations of PKM2 and PKM1 expressing H1299 cells. c. Schematic of fractionation method. d. Relative mean signal intensity, determined by flow cytometry, of (left) ER-BODIPY red, (middle) Golgi tracker red, and (right) Mito tracker red stained H1299 cells, treated with 40 μM DASA or vehicle. e. Representative serial block face scanning electron microscopy (SBFSEM) image of an H1299 cell. Small organelles included in the small organelle fraction are colored blue. Representative golgi, endoplasmic reticulum (ER), and mitochondria are circled in white. Image is one slice from one representative cell. f. Whole cell volumes as well as fractionated volumes of 12 cells analysed using SBFSEM. g. Western blot of H1299 lysate and increasing concentrations of purified recombinant LDHA. Corresponding standard curve constructed from the average pixel density of each LDHA purified recombinant protein band and used to determine the amount of LDHA protein in H1299 lysate. h. In vitro LDHA activity assay, using purified recombinant LDHA and pyruvate and cytosolic OAA concentrations determined under 40 μM DASA or vehicle treatment in H1299 cells. LDHA activity was determined by analysing 13C1 lactate produced from 13C1 pyruvate by GC-MS. Western blot results are representative experiments of three independent replicates. Data is represented as the mean and error bars represent the standard deviation from n = 4 for (a), (b), (h), n = 3 for (d) of biologically independent replicates. Micrograph in (e) is a representative experiment of 12 biologically independent replicates. Standard curve in (g) is a representative experiment of 3 independent replicates in vitro. The western blot in (g) is a representative experiment of 3 biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | PKM2 activity increases glutamine derived OAA. Related to Fig. 5. a. Isotopomer analysis of $^{13}$C6 glucose-derived carbon incorporation to OAA in RPMI 8226 cells treated with $^{13}$C6 glucose and 40 μM DASA or vehicle overnight. b. Isotopomer analysis of $^{13}$C5 glutamine-derived carbon incorporation to OAA in RPMI 8226 cells treated with $^{13}$C5 glutamine and 40 μM DASA or vehicle overnight. c. Western blot analysis with antibodies against GPT2 and β-actin of H1299 lysates from cells treated with vehicle or 40 μM DASA overnight. d. Relative OAA concentrations in H1299 GPT2 knockdown cells treated with vehicle or 40 μM DASA overnight. e. Western blot analysis of H1299 vector and PHGDH knockdown cell lysates with antibodies against PHGDH and β-actin. f. $^{13}$C lactate labeling rate of H1299 vector and PHGDH knockdown cells incubated with $^{13}$C6 glucose and vehicle or 40 μM DASA. g. $^{13}$C pyruvate labeling rate of H1299 vector and PHGDH knockdown cells incubated with $^{13}$C6 glucose and vehicle or 40 μM DASA. h. In situ LDH activity in H1299 vector and PHGDH knockdown cells treated with vehicle or 40 μM DASA. i. Western blot analysis of H1299 vector and PC knockdown cell lysates with antibodies against PC and β-actin. j. $^{13}$C lactate labeling rate of H1299 vector and PC knockdown cells incubated with $^{13}$C6 glucose and vehicle or 40 μM DASA. k. $^{13}$C pyruvate labeling rate of H1299 vector and PC knockdown cells incubated with $^{13}$C6 glucose and vehicle or 40 μM DASA. l. In situ LDH activity in H1299 vector and PC knockdown cells treated with vehicle or 40 μM DASA. Data is represented as the mean and error bars represent the standard deviation from n = 4 for (a), (d), (f), (h), (j), (l), n = 3 for (b) of biologically independent replicates. Western blot results in (c), (e), (i) are representative experiments of 3 biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
Extended Data Fig. 6 | LDHB has a small effect on the total LDH activity in H1299 cells. Related to Fig. 6. a. The IC₅₀ of OAA against human LDHB determined using purified recombinant human LDHB and analysis of ¹³C lactate derived from ¹³C pyruvate. The IC₅₀ was identified as 1,021 ± 86 μM. 
b. Western blot of H1299 lysate and increasing concentrations of purified recombinant LDHB. Corresponding standard curve constructed from the average pixel density of LDHB purified recombinant protein band and used to determine the amount of LDHB protein in H1299 lysate. c. LDH activity, determined using a fluorescence based LDH activity assay using diluted lysates from H1299 vector and LDHB knockdown cells. The corresponding western blots of the H1299 lysates used in the above activity assay with antibodies against LDHB and β-actin. Data is represented as the mean and error bars represent the standard deviation from n = 4 for (a), n = 3 for (c) of independent replicates in vitro. Standard curve in (b) is a representative experiment of 3 independent replicates in vitro. Western blots in (b) and (c) are representative experiments of 3 biologically independent replicates. P values were determined by a two-tailed Student’s t-test.
Extended Data Fig. 7 | OAA inhibition of LDHA increases the response to TEPP-46 in vivo. a. Pyruvate kinase enzyme activity assay in lysates from H1299 cells treated with vehicle or 40 μM TEPP-46 overnight. The corresponding western blots of H1299 cell lysates with antibodies against PKM2 and β-actin. b. Isotopomer analysis of 13C5 glutamine-derived carbon incorporation to OAA in H1299 cells treated with 13C5 glutamine and 40 μM TEPP-46 or vehicle overnight. c. Ki67 staining of hLDHA- and rbLDHA-expressing H1299 tumours from mice treated with vehicle or TEPP-46. Ki67 staining results are representative experiments of 3 biologically independent replicates. Western blot results are representative experiments of 3 independent replicates. Data is represented as the mean and error bars represent the standard deviation from n = 3 for (a), (b) of biologically independent measurements. P values were determined by a two-tailed Student’s t-test.
Reporting Summary

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- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Software and code

Policy information about: availability of computer code

Data collection
Agilent GCMS Acquisition B.07.00.1413, BD FACSDiva v8.0.2

Data analysis
Microsoft Excel 2010, Agilent MassHunter Workstation Analysis B.06.00, Agilent MSD ChemStation Data Analysis F.01.00.1903, IsoPat2 Mod21, Amira for EM Systems 6.7.0, PyMol v1.7.0.3, BD FACSDiva v8.0.2, AlphaView SA software v3.4, FlowJo v10.7.0

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Policy information about: availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

PDB accession numbers for the crystal structures of human and rabbit LDHA are described in the data availability section. No data sets were generated during the current study.
Field-specific reporting

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**
All of the data in vitro and cell line experiments were generated from at least 3 independent measurements as described in figure legend, which is based on standard protocols in the field. We performed power analysis to calculate the sample size of in vivo tumor xenograft study as performed in the recently published literature (PMID: 33833463). In brief, we estimate that the probability to obtain a difference between vehicle and drug treated tumors is 95% and at least 50% of tumors are decreased in the treatment group. Under such estimation, minimum sample size would be log(0.05/log (0.5)=4.32. Based on this calculation, we performed xenograft experiments with at least 5 tumors in one group.

**Data exclusions**
No data was excluded from the analyses.

**Replication**
Each experiment was independently performed for at least three times to ensure the reproducibility of the data.

**Randomization**
Patient samples were allocated into experimental groups based on cell characteristics and randomization is not relevant to data from cell line models. Xenograft tumors were randomized to either control or TEPP-46 treatment group once tumor volume reached to approximately 40 mm3. No specific randomization protocol was used for western blotting as the experiments were performed by a single person and the randomization disrupt the interpretation of the data.

**Blinding**
For cell-based experiments such as western blotting and FACS, blinding was not possible because the experiments were performed by a single researcher. Sample names were blinded from investigators during in vitro enzyme activity assays and a secondary person analyzed mass-spec data.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a | Involved in the study |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology and archaeology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |
| ☑ | Clinical data |
| ☑ | Dual use research of concern |
| n/a | Involved in the study |
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

**Antibodies**

The following commercial antibodies were used: anti-β-actin (Sigma A1978), anti-PKM2 (Cell Signaling Technology 3198S), anti-PKM1 (Cell Signaling Technology 7067), anti-ΔMK (Abcam AB118409), anti-human specific LDHA (Cell Signaling Technology 2012S), anti-LDHβ (Abcam AB75167), anti-human and rabbit LDHA (Abcam AB135396), anti-p70 S6 kinase (Cell Signaling Technology 2701S), anti-calcineurin (Cell Signaling Technology 12238T), anti-golgin (Cell Signaling Technology 13192), anti-COX IV (Proteintech 11242-1-AP), and anti-CPT2 (Santa Cruz sc-398383), anti-PHGDH (Sigma HPA021241), and anti-PC (Santa Cruz sc-271493), horseradish peroxidase-conjugated secondary antibodies anti-mouse (Fisher PI31430) and anti-rabbit (Fisher PI31460). All the primary antibodies were used at 1:500 dilution in 5% non-fat milk in TBST. Secondary antibodies were used at 1:3000 dilution in 5% non-fat milk in TBST.

**Validation**

All antibodies used in our study have been validated and detailed information could be found on the manufacturers’ websites or published literatures as listed below. Some of them (anti-LDHA, LDHB, PC, PHGDH antibodies) have also been validated by our experiments as shown in this manuscript using shRNA-based gene knockdown.

- anti-β-actin (Sigma A1978);
- https://www.sigmaaldrich.com/catalog/product/sigma/a1978?lang=en&region=US
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)
Non-small cell lung carcinoma H1299 cells were obtained from Dr. Jing Chen at U. Chicago. Multiple myeloma RPMI 8226 cells were provided by the laboratory of W.I.G. Female human embryonic kidney 293T cells were obtained from Dr. Yuichi Machido at Mayo Clinic.

Authentication
H1299 and RPMI 8226 were tested by short tandem repeat profiling through Mayo facility. 293T cells are not authenticated.

Mycoplasma contamination
All cell lines were tested for and contain no mycoplasma.

Commonly misidentified lines
None any commonly misidentified cell lines used in the study and provide a rationale for their use.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Male 4-6 week old athymic nude mice (Athymic Nude-Foxn1nu) mice

Wild animals
This study did not include wild animals.

Field-collected samples
This study did not involve samples collected from the field.

Ethics oversight
Animal experiments were conducted according to the protocols approved by Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic Rochester (protocol#: A00002705-16).

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Human research participants

Policy information about studies involving human research participants

Population characteristics
Clinical information such as age, gender, treatment history and genotype were blinded for this study.

Recruitment
All the multiple myeloma patients and healthy individuals included in this study had provided written informed consent to allow their bone marrow and peripheral blood samples to be utilized for research purposes in this study. CD138 positive cell isolation and purity check were done by hematology core at Mayo Clinic Rochester so that the potential bias is unlikely introduced into the human primary samples.

Ethics oversight
Mayo Clinic IRB (IRB#: 16-008291)

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
H1299 cells were treated with 40 μM DASA or vehicle overnight. Cells were then stained with 500 nM MitoTracker (Life Technologies) in media for 30 min at 37°C, 1 μM ER-BODIPY (Life Technologies) in HBSS for 30 min at 37°C, or 5 μM Golgi Stain (Life Technologies) in HBSS/HEPES buffer for 30 min at 4°C followed by 30 min at 37°C in media. Following incubation with stains, cells were harvested, washed, and resuspended in PBS.

Instrument
Becton Dickinson FACS Canto II

Software
BD FACSDiva v8.0.2

Cell population abundance
There was no sorting of cells prior to the analysis.

Gating strategy
Forward versus side scatter (FSC vs SSC) gating was used to identify live and single cells. More than 84% of total cells were gated. The gated cells were further analyzed to determine fluorescence positive vs negative cells with use of stained vs non-stained cells.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.