Metabolic and oncogenic adaptations to pyruvate dehydrogenase inactivation in fibroblasts

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Huabo Wang‡, Jie Lu,‡ Sucheta Kulkarni‡, Weiqi Zhang‡, Joanna E. Gorka‡, Jordan A. Mandel‡, Eric S. Goetzman§, and Edward V. Prochownik¶¶

From the ‡Section of Hematology/Oncology and ¶¶Division of Medical Genetics, Children’s Hospital of Pittsburgh of UPMC, Pittsburgh, Pennsylvania 15224, the ¶¶Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15219, and the ¶¶¶The Hillman Cancer Center of UPMC, Pittsburgh, Pennsylvania 15232

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Eukaryotic cell metabolism consists of processes that generate available energy, such as glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation (Oxphos), and those that consume it, including macromolecular synthesis, the maintenance of ionic gradients, and cellular detoxification. By converting pyruvate to acetyl-CoA (AcCoA), the pyruvate dehydrogenase (PDH) complex (PDC) links glycolysis and the TCA cycle. Surprisingly, disrupting the connection between glycolysis and the TCA cycle by inactivation of PDC has only minor effects on cell replication. However, the molecular basis for this metabolic re-equilibration is unclear. We report here that CRISPR/Cas9-generated PDH-knockout (PDH-KO) rat fibroblasts reprogrammed their metabolism and their response to short-term c-Myc (Myc) oncoprotein overexpression. PDH-KO cells replicated normally but produced surprisingly little lactate. They also exhibited higher rates of glycolysis and Oxphos. In addition, PDH-KO cells showed altered cytoplasmic and mitochondrial pH, redox states, and mitochondrial membrane potential (∆ΨM). Conditionally activated Myc expression affected some of these parameters in a PDH-dependent manner. PDH-KO cells had increased oxygen consumption rates in response to glutamate, but not to malate, and were depleted in all TCA cycle substrates between α-ketoglutarate and malate despite high rates of glutaminolysis, as determined by flux studies with isotopically labeled glutamine. Malate and pyruvate were diverted to produce aspartate, thereby potentially explaining the failure to accumulate lactate. We conclude that PDH-KO cells maintain proliferative capacity by utilizing glutamine to supply high rates of AcCoA-independent flux through the bottom portion of the TCA cycle while accumulating pyruvate and aspartate that rescue their redox defects.

Eukaryotic cell metabolism represents a delicate integration of energy-generating processes such as glycolysis and oxidative phosphorylation (Oxphos)² and energy-consuming ones such as macromolecular synthesis, ionic gradient maintenance, and xenobiotic detoxification (1–3). During quiescence, energy in the form of ATP is produced largely by the mitochondrial TCA cycle coupled with the electron transport chain (ETC) and Oxphos. The entry-level substrate for the TCA cycle, namely acetyl-CoA (AcCoA), is derived anaerobically from the step-wise conversion of glucose to pyruvate (glycolysis). Following transport into the mitochondria, pyruvate is converted to AcCoA by the multisubunit enzyme pyruvate dehydrogenase (PDH) complex (PDC) in a three-step coordinated reaction that also yields CO₂ and a molecule of NADH for subsequent use as an electron donor (4, 5). PDC is the critical link between glycolysis and the TCA cycle as evidenced by its complex regulation and, most tellingly, by individuals with genetic deficiencies in one of its component activities. PDC deficiency, the severity of which is determined by the underlying PDC mutations, presents in early childhood and is marked by variable degrees of metabolic acidosis resulting from the conversion of accumulated pyruvate to lactate (4–6). Importantly, no cases of complete PDC deficiency have ever been identified, strongly suggesting that some residual enzyme activity is necessary for survival. This is supported by the finding that total body inactivation of the gene encoding the α subunit of PDH (pdha1) in mice is embryonic lethal (7). The treatment of patients with PDC deficiency includes ketogenic diets which, in addition to providing an alternative AcCoA source via fatty acid β-oxidation (FAO), also down-regulate the glycolytic pathway and minimize pyruvate and lactate generation (8).

Recently, we showed that total inactivation of the pdha1 gene in hepatocytes does not affect their long-term regenerative

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² The abbreviations used are: Oxphos, oxidative phosphorylation; 4-OHT, 4-hydroxytamoxifen; AcCoA, acetyl coenzyme A; ETC, electron transport chain; FAO, fatty acid oxidation; HB, hepatoblastoma; OCR, oxygen consumption rate; PDC, pyruvate dehydrogenase complex; PDH, pyruvate dehydrogenase; ROS, reactive oxygen species; CM-H₂DCFDA, chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate; FBS, fetal bovine serum; 2-NBDG, 2-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose; NAO, acridine orange 10-nonyl bromide; ∆ΨM, mitochondrial membrane potential; CCCP, carbonyl cyanide p-chlorophenylhydrazonate; ANOVA, analysis of variance; DHAP, dihydroxyacetone phosphate; LDH, lactate dehydrogenase; TCA, tricarboxylic acid; MycER, Myc-estrogen receptor; FBS, fetal bovine serum; RP, reverse phase; ESI, electrospray ionization; MEF, murine embryob fibroblast.
capacity. Additionally, the growth of malignant hepatoblastomas (HBs) in pdha1−/− (KO) livers is only modestly impaired, although it is accompanied by significant metabolic acidosis as a result of excess lactate accumulation (9, 10). These results demonstrated unequivocally and somewhat surprisingly that even under the most demanding proliferative stresses, communication between glycolysis and the TCA cycle can be severed while only minimally affecting normal and transformed cell replication.

The above findings raised several important questions that are now explored here. Among these are whether the liver is unique in its ability to sustain high levels of normal and neoplastic proliferation in PDC’s absence. Others include how cells metabolically adapt following PDC loss and whether and to what extent they accumulate glycolytic intermediates. Answers to such questions could explain how cells normally achieve metabolic re-equilibration to accommodate nutritional and neoplastic challenges. We address these and additional questions by exploring the consequences of pdha1 inactivation in rat fibroblasts in which the c-Myc (Myc) oncoprotein, fused to mutations by exploring the consequences of pdha1 inactivation in rat fibroblasts in which c-Myc (Myc) oncoprotein, fused to the hormone-binding domain of the estrogen receptor, can be conditionally activated by 4-hydroxysteromifen (4OHT) (11, 12). We demonstrate that these so-called Rat1a–MycER cells undergo significant metabolic re-programming that compensates for the loss of PDC activity, restores normal levels of AcCoA, and allows them to proliferate as well as their wildtype (WT) counterparts. Short-term MycER activation also results in distinct metabolic responses in the two cell types. Together, these findings point to major similarities and differences in the ways that hepatocytes and fibroblasts cope with the loss of PDH and emphasize the flexibility that can be marshaled in response to what should be a devastating metabolic deficit.

Results

Inactivation of pdha1 reduces cell size but not growth rate

A CRISPR-Cas9–based approach was used to target the pdha1 gene in Rat1a–MycER fibroblasts (11, 12). Over half of the randomly selected, stably transfected clones showed absence of PDHα1 protein expression and are hereafter referred to as “knockout” (KO) cells (Fig. S1A). To minimize clonal variability, we combined five randomly selected clones from each group for all subsequent studies.

PDC activity is significantly up-regulated in two different forms of liver cancer in vivo and correlates with a loss of inhibitory phosphorylation on Ser293 of PDHα1 (9, 13, 14). This is associated with decreased expression of PDHα1’s inhibitory kinase PDK1 and increased expression of the stimulatory PDP2 phosphatase (6, 13–15). In keeping with this overall theme, the short-term (8 h) activation of MycER in WT cells was accompanied by an ~2.5-fold up-regulation of PDC activity that correlated with the loss of PDHα1 phosphorylation (Fig. S1, B and C). However, only a modest increase in PDP2 and no discernible change in PDK1 were observed (Fig. S1C). Because PDK1 and PDP2 are themselves regulated by metabolites such as ATP, ADP, NADH, and AcCoA (6, 15), it seems likely that the loss of PDHα1 phosphorylation in WT cells in response to MycER induction is more dependent on these enzymes’ activities than on their actual levels as occurs in hepatocytes (9, 13, 14).

PDH has been reported to localize to the nucleus under some conditions, where its regional generation of AcCoA may allow for the selective acetylation of histone and gene expression changes (16). Although we also found a significant amount of nuclear PDH in WT cells, neither its level nor its phosphorylation were significantly altered by MycER activation (Fig. S1D).

WT and KO cells grew at identical rates (Fig. S1E), although the latter cells were ~15% smaller (Fig. S1F). This may reflect a compensatory change by which cells conserve energy through the reduction of biomass (see below) (17).

WT and KO cells differ in their rates of glycolysis and Oxphos

The rates of glucose uptake by WT and KO cells were compared using the fluorescent glucose analog 2-NBDG as a surrogate. KO cells showed significantly higher time-dependent rates of 2-NBDG uptake than WT cells but no further increase in response to MycER activation (Fig. 1, A and B). In contrast, WT cells increased 2-NBDG uptake by ~20% following MycER activation (Fig. 1B).

Mice bearing KO HB tumors manifest high levels of lactate production and metabolic acidosis that contribute to their eventual demise (9). This undoubtedly reflects the conversion of accumulated pyruvate to lactate rather than AcCoA. However, initial attempts to demonstrate higher lactate production by logarithmically growing KO fibroblasts were unsuccessful.

This could have been the result of the diversion of excess KO cell pyruvate into other pathways and/or intracellular conditions that inhibit lactate production or its consumption (18). To minimize these contributions, we grew both cell lines to confluence, progressively reduced the serum concentration to further inhibit cell proliferation, and measured lactate levels in culture supernatants 3 days later. Under these conditions, KO cells produced significantly more lactate than WT cells that correlated inversely with serum concentrations (Fig. 1C). Thus, KO cells do excrete more lactate but only during times of proliferative arrest and cellular stress.

In contrast, KO cells had lower oxygen consumption rates (OCRs) (Fig. 1D), implying that they were less able to maintain a mitochondrial proton gradient. Consistent with this idea, KO cells contained lower levels of NAD+/NADH (Fig. 1E), suggesting that their attenuated proton gradient was due to a deficiency of reducing equivalents. Unlike WT cells, which rapidly increased NADH levels in response to MycER activation, KO cells showed no such response, although both cell types decreased their NAD+/NADH ratios in response to Myc activation (Fig. 1F). Taken together, these findings suggested that the increased NADH content of WT cells in response to MycER activation originated with the PDH reaction, which generates NADH as a by-product of the intra-mitochondrial oxidation of pyruvate to AcCoA.

We performed additional respirometry studies using various substrates and inhibitors. At baseline, intact KO cells again showed lower OCRs than their WT counterparts, thus underscoring a pre-existing deficit in one or more TCA cycle substrates and/or a greater reliance on glycolysis as an energy source (Fig. S2A). Following digitonin permeabilization, the
KO cell response to malate was also markedly diminished (Fig. S2B). In keeping with the fact that mitochondria are impermeable to oxaloacetate (19), neither WT nor KO cells demonstrated any response to this substrate (data not shown). These findings suggested either that malate was inefficiently entering the mitochondria via the malate–
\( \alpha \)-ketoglutarate shuttle, that it was not being converted to oxaloacetate, that oxaloacetate was not being converted to citrate following condensation with AcCoA, or that malate and/or oxaloacetate were being diverted for non-TCA cycle functions. The equivalent citrate synthase activity of WT and KO cells argued that the latter’s attenuated response to exogenous malate was not due to reduced activity of this enzyme (Fig. S2C).

Despite their markedly reduced malate response, KO cells utilized exogenous palmitoylcarnitine and engaged in FAO as well as WT cells when glucose was absent, and glycolytic intermediates were depleted as a result of cell permeabilization (Fig. S2D). These results indicated that the metabolic defect of KO cells was partially attenuated via the utilization of alternative substrates such as fatty acids.

To better define how KO cells compensated for the absence of glycolytically-derived AcCoA to power the ETC, we quantified OCRs following the sequential addition of a broader range of TCA cycle and anaplerotic substrates. A reduced KO cell response to malate was again noted, and the expected absence of a pyruvate response was confirmed (Fig. 2, A and B). Unex-
expectedly, KO cells demonstrated an exaggerated response to glutamate (Fig. 2C), suggesting its preferential use as an anaplerotic substrate. Given that three NADH molecules and one FADH2 molecule are generated during glutamine’s stepwise conversion to oxaloacetate, whereas malate provides only a single NADH molecule, increased glutaminolysis might better address the presumed redox deficit arising from the loss of the NADH-generating PDH reaction. Finally, because the produc-

Figure 2. Differential TCA cycle substrate preferences of WT and KO cells. A–D, medium from cells plated the day before was replaced with fresh medium lacking or containing 4OHT for 8 additional h. Following permeabilization with digitonin and the addition of cytochrome c and ADP, malate, pyruvate, glutamate, and palmitoylcarnitine were sequentially added. E, following the addition of all the above substrates, the contribution of Complex I to overall oxygen consumption was calculated following the addition of the Complex I inhibitor rotenone. F, contribution of Complex II was then calculated following the addition of the Complex II substrate succinate. G, increased glutamine uptake by KO cells. [3H]Glutamine labeling was performed for the times indicated in fresh medium lacking exogenous glutamine with or without 4OHT (250 nM). H, WT cells are more sensitive than KO cells to glutamine deprivation. 2/4100 WT or KO cells were seeded into 24-well plates in standard medium containing the indicated concentrations of glutamine, and growth was monitored continuously for the next 4 days. Data from six biological replicas were combined and averaged. See Fig. S1D showing that WT and KO cells grow equally well in high-glutamine–supplemented medium (2 mM). I, WT cells undergo apoptosis in the presence of limiting concentrations of glutamine. Equal numbers of WT and KO cells were plated in standard medium and grown to ~50–70% confluence over the next 1–2 days. Fresh medium containing 6 μM glutamine was then added to triplicate wells for the ensuing 72 h, at which time the contents of each well was evaluated by propidium iodine staining of nuclear DNA to determine the fraction of apoptotic cells (numbers in parentheses, p = 0.01).
tion of glutamine-derived intermediates is AcCoA-independent, this could potentially reduce, if not entirely eliminate, the reliance of KO cells on this latter substrate. In neither WT nor KO cells was the glutamate response Myc-responsive.

Unlike the indistinguishable palmitoylcarnitine responses of WT and KO cells when TCA substrates were absent (Fig. S2D), KO cells were significantly more reliant on FAO when they were present (Fig. 2D). These results suggested that, unlike WT cells whose AcCoA supply could derive from both glycolysis and FAO, most if not all KO cell AcCoA was derived from FAO as recently demonstrated for KO hepatocytes and HBs (9). Addition of the Complex I inhibitor rotenone followed by succinate to drive Complex II indicated that both complexes contributed nearly equally to the production of mitochondrial-reducing equivalents and that neither was significantly altered in KO cells (Fig. 2, E and F). Thus, given the proper substrates, KO cells are capable of proceeding beyond the malate block (Fig. 2B).

Glutaminolysis supports cell growth by providing TCA cycle intermediates while also serving as a source of critical amino acids, nucleotides, and lipids (20–22). Moreover, the lactate generated by the Warburg effect can itself enhance glutaminolysis as can Myc in some instances (23, 24). Consistent with their increased utilization of glutamate as an energy source (Fig. 2C), KO cells had 2.5–3-fold higher glutamine uptake than WT cells and further increased this in response to MycER activation (Fig. 2G). These cells also grew faster and showed reduced apoptosis when cultured in limiting amounts of glutamine, most likely reflecting their more efficient uptake and utilization of this amino acid (Fig. 2, H and I).

Increased KO cell glutaminolysis (Fig. 2, C and G) could have two nonmutually exclusive explanations. First, glutamate could enter the TCA cycle following conversion to \( \alpha \)-ketoglutarate, eventually furnishing fumarate and reducing equivalents in the form of NADH and FADH2. Less likely, glutamate could be utilized more indirectly in a noncanonical pathway involving reductive carboxylation, in which \( \alpha \)-ketoglutarate is converted to citrate and then to AcCoA in a cytoplasmic reaction catalyzed by ATP citrate lyase. The AcCoA could then be used as a source of the \textit{de novo} synthesis of fatty acid synthesis (25–27), which could be used as FAO substrates. To distinguish between these alternatives, we first showed that isolated mitochondria from KO cells, while still failing to respond to malate and pyruvate, retained their exaggerated glutamate response (Fig. 3, A–D). This indicated that the cytoplasmically-localized enzymes needed for fatty acid synthesis and transport into the mitochondria were unlikely to be required for this process. This exaggerated KO cell response to glutamate was retained even when exogenous malate was withheld (Fig. 3, E–G). Second, the addition of the fatty-acid synthase inhibitor C75 to intact WT cells led only to a modest increase in OCR (Fig. 3H), which likely reflected the sparing and re-dedication of mitochondrial citrate into TCA cycle-directed energy generation rather than its utilization for \textit{de novo} lipid biosynthesis. In KO cells, virtually no response to C75 was seen indicating that no more and perhaps even less citrate was being used for lipogenesis than in WT cells. Finally, we radiolabeled cells with \([3H]\)glutamine for a more extended period of time and then extracted and quantified newly synthesized lipids. Despite their significantly higher rate of glutaminolysis (Fig. 2G) and lack of contribution from either malate or pyruvate (Fig. 2, E and F), KO cells consumed the same amount of glutamine for lipid biosynthesis as WT cells (Fig. 3I). However, both WT and KO cells increased their incorporation of \([3H]\)glutamine into lipid in response to MycER activation in accordance with previous findings that Myc promotes \textit{de novo} lipogenesis and glutaminolysis (24, 28, 29). From these studies and those showing a higher KO cell OCR in response to glutamate, we conclude that the exaggerated response of KO cells and isolated mitochondria to glutamate likely stems from its direct use to drive the TCA cycle in a “forward” direction. The lack of effect of C75 on KO cell OCR response may indicate that any sparring of citrate in the cells contributes little additional benefit because they already utilize large amounts of glutamine in the forward TCA reaction for this purpose.

To explore further the basis for the differential responses of WT and KO cells to glutamate, palmitoylcarnitine, and malate and the relationships of these substrates to one another, we performed additional studies using both intact and permeabilized cells. As seen previously, KO cells again showed an exaggerated response to exogenous glutamate (Fig. 4A) as well as a lower baseline OCR following their permeabilization (Fig. 4B). An equivalent response of both cell types was also again seen following the addition of palmitoylcarnitine (Fig. 4C). Yet, when malate was added, KO cells showed a significantly blunted response (Fig. 4D). Along with the results presented in Fig. S2, these findings indicate that, even when KO cells were allowed to generate presumably equivalent amounts of fatty acid-derived AcCoA, they remained largely refractory to malate. This independently reinforced the idea that the malate unresponsiveness of KO cells is due to a defect in the conversion of malate to oxaloacetate and/or oxaloacetate to citrate rather than to a lack of AcCoA.

**PDH loss differentially affects mitochondrial mass, reactive oxygen species (ROS), acid-base and redox states, and membrane polarization in response to MycER activation**

The enforced expression of Myc and other oncoproteins can significantly impact mitochondrial mass, induce ROS, and perturb cytoplasmic and mitochondrial pH and redox states. These changes can be rapid and involve varying degrees of metabolic re-programming (12, 13, 28, 30, 31–35). WT and KO fibroblast lines provided the opportunity to investigate how these factors are affected as a result of glycology–TCA cycle dissociation.

Mitochondrial mass was assessed by staining with acridine orange 10-nonyl bromide (NAO), the intensity of which is independent of membrane potential (\( \Delta \Psi M \)) (36). The mitochondrial mass of KO cells was greater than that of WT cells, but in neither case did MycER activation affect this property (Fig. 5A).

ROS are products of normal cytoplasmic and mitochondrial metabolism, are carefully regulated, and contribute to cell signaling and proliferation (37–39). However, excessive and/or de-regulated ROS production, originating from either of these compartments is mutagenic and can promote neoplastic transformation and tumor evolution (40–48). Myc is a potent inducer of mitochondrial ROS, particularly \( \text{O}_2\text{-}\) and Myc and ROS cooperate to promote genomic instability and tumor evo-
Because the uncoupling of glycolysis and Oxphos in KO cells might alter ROS production and/or distribution, we measured these using chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), an indicator of total cellular ROS, particularly H2O2, and MitoSOX®TM, a sensitive and specific indicator of mitochondrial-generated superoxide (O2•−). KO cells contained higher levels of total ROS than WT cells; in both cases, these increased significantly in response to an 8-h induction of MycER as observed previously (Fig. 5B)(12, 34, 50).

In contrast, and consistent with their low basal rate of Oxphos, KO cells generated lower levels of mitochondrial ROS than WT cells; in both cases, these increased significantly in response to an 8-h induction of MycER as observed previously (Fig. 5B)(12, 34, 50).

Acid-base differences were next assessed following stable transfection of WT and KO cells with vectors encoding cytoplasmic- and mitochondrial matrix-localized versions of the pH-sensitive yellow fluorescent protein derivative pSypHer (32, 55). WT cell cytoplasm was significantly more alkaline than KO cell cytoplasm and became even more so following MycER activation. In contrast, KO cell cytoplasmic pH remained unchanged (Fig. 5D). The greater acidity of KO cells more than likely reflects their accumulation of pyruvate and its upstream precursors than it does their marginally greater accumulation of lactate (Fig. 1C and see below). In contrast, the KO cell intramitochondrial pH was more alkaline than that of WT cells and is consistent with a depletion of acidic TCA intermediates (see below). Finally, the mitochondria of both cell types acidified in response to MycER activation, although this was more pronounced in KO cells (Fig. 5E).

Differential NAD+ and NADH levels in WT and KO cells (Fig. 1E) did not account for other factors that can impact the cellular redox state such as the levels and ratios of NADP+/
Metabolic and oncogenic adaptations to PDH inactivation

PDH loss perturbs metabolite levels and glutamine flux

A nondirected, mass spectroscopic (MS) survey of >600 steady-state metabolites was performed on log-phase WT and KO cells prior to and following MycER activation. Of the 10 metabolites showing the highest relative abundance in KO versus WT cells, six were related to the hexose catabolic and pyrimidine nucleotide biosynthetic pathways (Fig. S3 and Table S1). Similarly, six of the 10 least abundant compounds were pyrimidine or purine metabolites.

KO cells contained significantly elevated levels of most glycolytic intermediates, most notably pyruvate (6.4-fold increase), glucose (6.0-fold increase), and fructose 1,6-diphosphate (4.6-fold increase) (Fig. 6A, Fig. S4, and Table S1). Perhaps as a consequence of their already high glycolytic intermediate content, KO cells did not further increase pyruvate levels following 4OHT exposure, whereas WT cells increased levels by 5.1-fold so as to now closely match those of 4OHT-treated KO cells. Consistent with previously determined measurements (Fig. 1, B and C), lactate was not elevated in KO cells; indeed, its basal levels were actually somewhat lower than those in WT cells both prior to and following 4OHT exposure. Thus, despite pyruvate’s accumulation to high basal levels in KO cells, virtually none of the excess was converted to lactate except under extraordinary culture conditions (Fig. 1C). This, together with the finding that glucose uptake was only modestly higher in KO cells (Fig. 1D), makes it highly likely that the accumulation of glycolytic intermediates is a direct result of impaired PDH activity, aided by an inability to convert accumulated pyruvate into lactate.

Unexpectedly high levels of fructose and mannose 6-phosphate, but not galactose 1-phosphate, were also noted in KO cells (5.4–4.4–, and 1.1-fold, respectively) (Fig. 6A and Fig. S4). Less pronounced increases (1.22-fold) of sorbitol/mannitol, which can be derived from both glucose and fructose, were also noted (63). This suggested that these hexose sugars were derived by the selective reversal of the normally catabolic pathways that channel them into glycolytic degradation. This was further supported by the finding that dihydroxyacetone phosphate (DHAP), a fructose precursor, was the least elevated of all glycolytic intermediates and nearly 3-fold less abundant than its immediate precursor, fructose 1,6-biphosphate. This suggested that DHAP was being utilized in other pathways, specifically the de novo synthesis of fructose.

Following DHAP’s conversion to glycerol 3-phosphate, the latter can be esterified with a variety of fatty acyl groups thereby generating triglycerides and membrane lipids, including phosphatidylincholines, phosphatidylethanolamines, ceramides, and sphingomyelins. The high levels of DHAP in KO cells might therefore favor the storage of triglycerides, the synthetic pathways for which are active in fibroblasts (64–66). Many of the 173 precursors comprising these pathways were significantly elevated in KO cells (mean up-regulation of all 173 = 1.31-fold, p = 9.9 × 10⁻⁸ as determined by a paired t test) thus suggesting that these lipids were being newly synthesized (Fig. 6B and Fig. S5). However, we noted two relevant exceptions. First, the levels of acylcarnitines, including those derived from palmitate, oleate, and stearate were, without exception, either unchanged.

Figure 4. Differential responses of WT and KO cells to glutamine, palmitoylcarnitine, and malate. A, changes in baseline OCRs of intact cells following the addition of glutamine. B, baseline respiration of intact cells. C and D, response of permeabilized cells following the sequential addition of palmitoylcarnitine and malate.

NADPH, FAD⁺/FADH2, numerous anti-oxidant proteins, or other small molecule oxidants and anti-oxidants (56, 57), nor did it consider the substantial compartmentalization of these factors, which accounts for disparities between absolute and relative NAD⁺ and NADH levels (58–59). Therefore, we utilized redox-sensitive variants of GFP (roGFP) that, analogous to pSyPher, are selectively targeted to the cytoplasm and mitochondrial matrix (59–61). These studies showed both the cytoplasmic and mitochondrial compartments of KO cells to be significantly more oxidized than the corresponding WT compartments (Fig. 5, F and G). In both cell lines, mitochondria were more oxidized than the cytoplasm, a finding that was consistent with our previous findings in nontransformed primary murine embryo fibroblasts (MEFs) (58). Although MycER activation in WT cells led to a more reduced cytoplasm and a more oxidized mitochondrial matrix, the cytoplasm of KO cells became more oxidized, and the redox state of the mitochondrial matrix was unchanged. PDH loss therefore significantly affected both the cytoplasmic and mitochondrial redox balance and its response to the short-term activation of MycER.

Finally, we evaluated ΔΨM of WT and KO cells using tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a cationic, mitochondria-localized dye that accumulates in a ΔΨM-dependent manner leading to aggregation and a fluorescent shift from green to red (62). In keeping with the idea that KO cells generate more protons by virtue of their increased rates of glutaminolysis (Fig. 2C) and that these are subject to more efficient pumping into the mitochondrial intermembrane space as a consequence of their higher NAD⁺/NADH ratio (Fig. 1E), KO cells were significantly more polarized than WT cells (Fig. 5H).
or less abundant in KO cells. It is tempting to speculate that these were being selectively utilized to support the higher rates of FAO by these cells (Fig. 2E, Fig. S5, and Table S1).

The second class of noticeably depleted lipids in KO cells were the plasmalogens, a subgroup of peroxisome-derived ether glycerophospholipids with the ether moiety bonded in position sn-1 to an alkenyl group (Fig. 6B) (67, 68). Although these may constitute as much as 20% of all phospholipid mass in some cells, their function(s) remains enigmatic. The disparities in abundance between these compounds and those comprising the other lipid pathways discussed above suggest distinct modes of regulation among compounds derived from peroxisomal and nonperoxisomal sources.

Our results indicated that WT and KO cells might utilize different pathways for the de novo synthesis of lipids. Because our results in KO cells indicated that such lipids were not originating from glycolytically-derived AcCoA or from reverse carboxylation (Fig. 3I), we asked whether they could be synthesized from exogenous acetate or from pre-existing fatty acids such as palmitate. As seen in Fig. 6, C and D, KO cells incorporated larger amounts of both precursors, particularly acetate, into membrane lipids. Staining with the neutral lipid-specific
dye BODIPY-493/503 indicated that proliferating KO cells contained more neutral lipid than WT cells, suggesting that the uptake of exogenous lipids exceeded their ability to be incorporated into new membranes and that the excess was stored. However, as serum concentrations were reduced, and proliferation slowed, the uptake of BODIPY-493/503 equalized in both cell types (Fig. 6E). Collectively, these results suggest that WT and KO cells differentially modulate their rates of exogenous fatty acid uptake, storage, and utilization into distinct anabolic and catabolic pathways.

Additional MS-based showed AcCoA levels to be surprisingly similar in WT and KO fibroblasts with both declining by 40–50% following exposure to 4OHT (Fig. 7A and Fig. S6A). These results were confirmed by an independent colorimetric assay (BioVision, Inc., data not shown) and differ from our previous documentation of an 80% reduction in steady-state AcCoA levels in PDH KO livers and HB tumors (9). Thus, unlike the case of slowly proliferating hepatocytes, or more proliferative HB tumors, rapidly growing KO fibroblasts maintain relatively normal AcCoA levels that most likely derive from the increased reliance on FAO and/or acetate (Figs. 2E and 6C).

In contrast to their normal AcCoA levels, KO cells contained lower levels of several TCA intermediates, including α-ketoglutarate, succinate, fumarate, and malate (Fig. 7, A and B, and Fig. S6A). Because our MS analysis did not include oxaloacetate, we measured this intermediate on total cell lysates and found its levels to be ~5-fold higher in KO cells, despite the equivalent activity of citrate synthase (Fig. 7, B–D, and Fig. S2C). Thus, KO cells are depleted of multiple sequentially-generated TCA cycle intermediates downstream of and including α-ketoglutarate and appear unable to generate citrate from what are otherwise excessive levels of oxaloacetate, normal levels of AcCoA, and normal citrate synthase activity. These studies suggested that the excess oxaloacetate in KO cells was being diverted away from its normal use as a precursor of citrate.

Further analysis showed that 14 amino acids were depleted in KO cells with glutamine and glutamate being the most severely affected (7.7- and 2-fold, respectively) (Fig. 7D and Fig. S6B). Coupled with our finding that KO cells showed a higher rate of glutamine uptake (Fig. 2G), these results suggest that intracellular glutamine was being rapidly consumed following its transport into the cell. Because intracellular glutamine depletion was not occurring via reductive carboxylation pathways (Fig. 3I), these findings strongly supported the idea that forward glutamine flux through the “bottom” portion of the TCA cycle was accelerated, thus at least partly accounting for the accumulation of oxaloacetate and the depletion of TCA substrates between oxaloacetate and glutamine. Of note is that of the five nonessential amino acids whose levels were unchanged in KO cells, two (arginine and proline) are derived from glutamate, and the remaining three (alanine, glycine, and serine) are derived directly from pyruvate or 3-phosphoglycerate, which are elevated in KO cells (Fig. 6A and Fig. S4). This suggested that exogenous glutamine was being utilized to drive high levels of TCA cycle function and, along with pyruvate, the synthesis of amino acids for which it is a direct precursor.

Aspartate, the only amino acid whose basal level was higher in KO cells (1.5-fold increase), is synthesized intramitochondri-
ally from oxaloacetate and glutamate in a reaction catalyzed by glutamic oxaloacetic transaminase 2 (GOT2). Oxaloacetate may also be derived anaplerotically from pyruvate via the ATP-consuming pyruvate carboxylase (PC) reaction ([69, 70]). Because of the large excess of pyruvate in KO cells and evidence that virtually none of it was being converted to lactate or alanine ([Fig. 1C and Fig. S4]), we speculated that these cells were more actively converting pyruvate to oxaloacetate as a means of limiting the accumulation of the former substrate. Indeed, PC activity either in the absence or presence of exogenously-added pyruvate was 2-fold higher in KO ([Fig. 7E]). KO cells therefore appear to accumulate high levels of oxaloacetate and aspartate.

**Figure 7. Perturbation of TCA cycle substrates and amino acids in KO cells.** A, MS quantification of steady-state levels of TCA cycle substrates in WT and KO cells prior to and after an 8-h exposure to 4OHT. See Fig. S6A for actual values. B, relative levels of TCA cycle substrates and the anaplerotic contribution of glutamine to KO cell metabolism. Red and green substrates indicate those that were more or less abundant, respectively, in KO cells. The red arrow indicates the presumed exaggerated conversion of pyruvate to oxaloacetate in KO cells based on their elevated levels of PC enzymatic activity and higher levels of oxaloacetate (C and E). C, oxaloacetate levels in WT and KO cells. D, MS quantification of amino acids in WT and KO cells. E, PC activity in WT and KO cells. Whole cells were assayed for total PC activity either prior to or after the addition of excess pyruvate. F, OCRs of WT and KO cells provided with only palmitoylcarnitine as a source of AcCoA along with glutamate as a source of TCA cycle substrates. OCR was then measured following the addition of malate. G, cartoon depicting the possible immediate fates of uniformly-labeled glutamine. H, incorporation of [15N, 13C]-uniformly-labeled glutamine into the indicated intermediates in WT and KO cells. Each comparison was performed upon five replicas with the mean values being shown in the figure. I, p values between WT and KO cells for the results shown in H. N/A = not applicable.
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via the up-regulation of two distinct but inter-dependent pathways. The first relies on the rapid influx and utilization of glutamine and proceeds via successive steps of the TCA cycle (Fig. 7B), whereas the second pathway relies on the more direct conversion of pyruvate to oxaloacetate while simultaneously minimizing pyruvate’s conversion to lactate.

The blunted OCR of KO cells in response to malate, even when supplied with an AcCoA source in the form of palmitoyl carnitine (Fig. 4D), suggested that the conversion of oxaloacetate to aspartate mediated by GOT2 (22) prevented the former substrate’s efficient conversion into citrate via the citrate synthase reaction. If so, then an excess of glutamate might further suppress KO cell OCRs because glutamate contributes the amine moiety for the GOT2 reaction. High levels of glutamate should accelerate this reaction and further divert any remaining oxaloacetate away from the citrate synthase reaction. We confirmed this by showing that the addition of glutamate did indeed reduce the OCR in both WT and KO cells when previously supplied with palmitate (Fig. 7F).

To further confirm and extend the above-reported results, we next performed metabolic flux experiments using MS to trace the fate of uniformly labeled [15N,13C]glutamine in WT and KO cells. Following a 60-min labeling period, we quantified isotope incorporation into three TCA cycle intermediates (citrate, succinate, and malate) as well as glutamate and aspartate (Fig. 7G). Several results of this experiment supported our fore-going findings and the notion that overall flux through the bottom portion of the TCA cycle was significantly accelerated in KO cells. First, KO cells contained proportionately more uniformly-labeled glutamate (M + 6) and less unlabeled glutamate (M + 0) than WT cells (Fig. 7H), consistent with the former having higher uptake and smaller endogenous glutamine pools and a more rapid conversion into glutamate. Second, the proportional amounts of isotopically-labeled citrate, succinate, and malate were all higher in KO cells, indicating their more rapid derivation from glutamate. This, coupled with the overall lower steady-state levels of the latter two substrates (Fig. 7, A and B, and Table S1), was consistent with their rapid derivation from glutamine carbons and their subsequent rapid depletion. Third, glutamine-derived aspartate accumulated more rapidly in KO cells, suggesting that this was the result of a higher rate of its synthesis rather than a slower rate of subsequent utilization. Finally, the presence of a disproportionately higher level of M + 3 citrate in KO cells strongly supported the idea that it derived from the malic enzyme–mediated conversion of (M + 4) malate to pyruvate, which was in turn converted to oxaloacetate by the high-level activity of PC and then to (M + 3) citrate.

Discussion

This study expands upon our previous work with hepatocyte-specific pdha1−/− mice (7, 9). Although many similarities were noted, we also observed exceptions that likely reflect cell type, proliferation, and environmental differences as well as differential responses to Myc de-regulation. These studies reveal the different strategies that may be mobilized for neo-metabolic repurposing in lieu of the compromise of more standard pathways. While recognizing that other cell types may employ variations on the themes reported here or different strategies entirely for overcoming these metabolic hurdles, we have chosen to investigate one cell line in particular depth to define its metabolic plasticity as comprehensively as possible.

We utilized a highly tractable pdha1 KO fibroblast model that allowed a deeper appreciation of how cells lacking direct communication between glycolysis and the TCA cycle adapt to maintain highly-proliferative rates while still allowing a balanced synthesis of ATP and anabolic precursors. The ability to precisely time MycER activation provided additional important insights into how this potent oncogene differentially programs metabolism in WT and KO cells. The short-term duration of the oncogenic stimulus also maximized the likelihood that the measured responses were direct ones while minimizing longer-term outcomes such as apoptosis that are usually encountered with chronic Myc overexpression (71–73). Yet, even with this brief stimulus, we discerned significant effects on multiple metabolic parameters.

Among the distinguishing features of KO cells were enhanced glycolysis, reduced baseline OCRs, and substantial accumulation of glycolytic intermediates (Figs. 1, A and D, and 6A, Table S1, and Fig. S4). This suggested that the inability to provide pyruvate-derived AcCoA is the limiting factor for glycolytically-linked Oxphos. Evidence for respiratory chain dysfunction was provided by the finding that KO cells were deficient in both NAD+ and NADH (Fig. 1E), possibly reflecting in part their switch from Oxphos to glycolysis. Given that PDC generates one-quarter of the TCA cycle’s NADH, its absence probably further contributes to the redox imbalance of KO cells as does their higher rate of glutaminolysis-linked FADH2 generation and their propensity to utilize FAO as an alternative energy source (Fig. 2D) (74). The lower levels of mitochondrially-derived ROS (Fig. 5C) are also consistent with their reduced mitochondrial activity and likely represent less electron leakage by Complexes I and III during the normal course of electron transfer (75–78). However, this may be a somewhat simplified interpretation given that ROS production can be significantly influenced by the choice of TCA cycle substrates (79). Numerous human tumors, as well as two different types of murine liver cancers studied by our group, have significantly reduced mitochondrial mass that presumably explains much of the Warburg effect (9, 13, 14, 31). The increased KO cell mitochondrial mass (Fig. 5A) may therefore represent a means of coping with compromised mitochondrial function.

Given that KO cells excreted only marginally more lactate than WT cells, their increased cytoplasmic acidity more than likely reflects both their increased glycolytic rate and their accumulation of other acidic glycolytic intermediates, particularly pyruvate, phosphoenolpyruvate, and 2- and 3-phosphoglycerate (Figs. 1C and 6A and Fig. S4). An additional proton source may be the respiratory production of CO2, which accounts for approximately one-third of intracellular acidification when glucose is the primary energy source but as much as 100% when FAO is the source (80).

There are several nonmutually exclusive explanations for why higher lactate levels in KO cells were documented only under extreme conditions despite their >6-fold higher levels of pyruvate (Fig. 1C and Fig. S4). First, the rapidity of cell growth (Fig. S1D) may impose increased anabolic demands on pyru-
of this substrate (Fig. 3G), allowing them to maintain continuous supplementation of the TCA cycle beyond that achievable by WT cells.

The finding that glutamine, glutamate, and all TCA cycle substrates between α-ketoglutarate and oxaloacetate were depleted in KO cells (Fig. 7, A and B) suggested that the overall flux between these points was high, as confirmed by experiments performed with uniformly-labeled $^{15}$N, $^{13}$C-glutamine (Fig. 7, H and I). Thus, more so than the metabolism of WT cells, that of KO cells is proportionately largely unidirectional and culminates with the accumulation of oxaloacetate and its conversion to aspartate (Fig. 8). The rapidity of this directionality coupled with higher efficiency of glutamine uptake and the larger aspartate pools may also allow KO cells to proliferate and survive under glutamine-deprived conditions (Fig. 2H). The pyruvate carboxylase-mediated conversion of pyruvate to oxaloacetate, particularly with regard to the biosynthesis of alanine and aspartate, thus minimizing lactate production. Although the synthesis of alanine from pyruvate is direct, that of aspartate is indirect and requires that pyruvate first be converted to oxaloacetate via the action of pyruvate carboxylase (69, 70). The higher oxaloacetate and aspartate levels in KO cells along with 2-fold higher pyruvate carboxylase activity are entirely consistent with the idea that lactate production is minimized (Fig. 7, B, D, and E). Similarly consistent was the finding that the assay we employed for PDH activity (i.e. the release of $^{14}$CO$_2$ from $[^{14}$C]pyruvate) should have indirectly detected pyruvate carboxylase activity as $[^{14}$C]oxaloacetate was converted to citrate and $^{13}$CO$_2$ was released as the substrate entered the TCA cycle. Rather, we detected no $^{14}$CO$_2$ above background in KO cells (data not shown). The markedly reduced KO cell utilization of malate as an oxidative substrate (Figs. 2A, 3B, and Fig. S2B) is further consistent with this idea and suggests that malate-derived oxaloacetate originating from the TCA cycle is also being converted to aspartate. An alternative use of oxaloacetate, namely in gluconeogenesis, seems unlikely given the high rates of cell growth, the high glucose content of the cell culture medium used in these studies, and the fact that fibroblast gluconeogenesis is typically low (69, 70). In addition to feedback inhibition of lactate dehydrogenase (LDH) by pyruvate and lactate (81), pyruvate carboxylase’s 10–30-fold lower $K_m$ value for pyruvate may also play a role (82–85).

KO cell acetate production may also be suppressed as a consequence of their low NADH levels given that LDH requires NADH as a cofactor. Although energetically wasteful, the LDH reaction is nonetheless necessary to maintain high levels of glycolysis as the NAD$^+$ generated by LDH serves as an electron acceptor during the more proximal steps of glycolysis (86, 87). Glycolysis and the LDH reaction thus reinforce one another by contributing to cytoplasmic redox balance. The lower levels of KO cell NADH (Fig. 1E) plus their lower cytoplasmic pH (Fig. S5D) might be expected to inhibit LDH and lactate generation (81). Finally, WT cell lactate production is already quite high (Fig. 1D) due to rapid growth and reliance on Warburg-type respiration. Thus, the ultimate reason for the unexpectedly low KO cell lactate content may well be multifactorial.

Seemingly paradoxically, KO cells were more resistant to glutamine deprivation than WT cells despite their greater reliance on glutamine as an Oxphos substrate (Fig. 2, C and G). Many cancer cells, and even some rapidly growing normal cells, are “addicted” to glutamine, and the overexpression of oncogenes such as Myc can drive much of this increased demand (14, 20–22, 88). Glutamine and glutamate are converted to α-ketoglutarate and are important anaplerotic TCA cycle substrates due to their ability to replenish downstream substrates and sustain the generation of reducing equivalents and anabolic precursors (20, 89–92). In the latter case, reductive carboxylation may be used for de novo lipidogenesis (25, 26, 90–92). Finally, glutamine serves as the amide donor during the GOT2-mediated conversion of oxaloacetate to aspartate, high levels of which are necessary to maintain viability and proliferation during times of ETC compromise (93–96). The growth and survival of KO cells in the presence of limiting amounts of exogenous glutamine (Fig. 2H) may reflect their more efficient uptake
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L-a-ketoglutarate (Fig. 7E and Fig. S4) also not only provides a way to dispose of excess pyruvate but ensures an additional source of critical aspartate (Fig. 8). Indeed, the up-regulation of pyruvate carboxylase can rescue cells from the cell cycle arrest and apoptosis imposed by glutamine deprivation (97). High KO cell pyruvate levels may also correct redox deficits by serving as an electron acceptor (93, 95, 96).

Also, initially puzzling was the markedly attenuated OCR response of KO cells to malate (Figs. 2A and 3B and Fig. S2B). Although conceivably attributable to a lack of glucose-derived AcCoA, we noted that this blunted response persisted even when KO cells were supplied with palmitoylcarnitine (Fig. 4D). Although this could be partially explained by a redirecting of oxaloacetate into the synthesis of aspartate, this reasoning is probably incomplete given the large excess of oxaloacetate. Thus, the failure of KO cells to respond to exogenous malate may also be a result of their being depleted of TCA cycle substrates between a-ketoglutarate and oxaloacetate (Figs. 7B and 8). The most immediate consequence of this would be a failure to generate reducing equivalents at levels commensurate with those provided by WT cells and thus a reduction in OCR. Support for this idea was provided by showing that a partial malate response could be evoked in KO cells supplemented with glutamate and palmitate (Fig. 7F). The incomplete correction can be attributed to the continued diversion of oxaloacetate into aspartate even when KO cells were supplied with sufficient glutamate to generate a continuous source of reducing equivalents and TCA cycle intermediates (Fig. 8).

The greater mitochondrial mass of KO cells (Fig. 5A) might represent a compensatory response to the Oxphos deficiencies caused by altered intra-mitochondrial pH, redox state, and levels of TCA cycle substrates. We have proposed such responses as a way of restoring deficits ATP and/or AcCoA levels (9). The slightly smaller size of KO cells (Fig. S1E) may represent an additional compensatory mechanism that minimizes biomass accumulation without compromising proliferation (12).

Lower levels of mitochondrial-generated ROS (Fig. 5C) may reflect several of the unique characteristics of KO cells. Among these are reduced basal OCRs and electron flow across the ETC where mitochondrial ROS originate as a result of leakage from Complexes I and III (76). The fact that short-term MycER induction increased total and mitochondrial ROS in both cell types indicates that Myc’s effect is mediated via processes that do not require PDC. However, the alkalinity of KO cell mitochondrial matrix (Fig. 5E), which probably reflects their low ATP production and relatively hyperpolarized state (Fig. 5H), is conducive to ROS production (98–103), although the underlying causes are almost certainly complex and influenced by other factors such as Ca2+ flux (104).

In both WT and KO cells, the more oxidized environment of the mitochondrial matrix relative to that of the cytoplasm (Fig. 5, F and G) was reminiscent of our previous findings in MEFs (59). However, MycER induction in WT cells further increased mitochondrial oxidation, which is the opposite of what is observed in MEFs (59). The possibilities to explain this include the vastly different growth rates and origins of the cell types, the much longer times of MycER activation in MEFs (24 h versus 8 h), different metabolic consequences reflecting these different induction times and the degree to which prolonged MycER activation leads to eventual apoptosis. Finally, because oxaloacetate is a potent inhibitor of Complex II (succinate dehydrogenase), its high concentrations in KO cells (Fig. 7, B and C) could promote FAD2+ accumulation and an even more oxidized environment as was clearly seen (99, 105).

KO cells are more likely to derive a-ketoglutarate in an AcCoA-independent manner via glutaminolysis that also generates NADH during the glutamine dehydrogenase-mediated conversion of glutamine to a-ketoglutarate (106). This might partially compensate for the loss of PDC-mediated NADH generation. Glutaminolysis provides a means by which a-ketoglutarate, at least half the NADH pool, and all FADH2 can potentially be generated via AcCoA-independent reactions. Although the absolute amounts of these electron donors in KO cells are significantly lower than they are in WT cells (Fig. 1D), glutaminolysis nonetheless provides a stopgap means by which they can potentially be sustained at levels that are disproportionately to the available AcCoA pool. This might permit low levels of FAO-, acetate-, and branched-chain amino acid–derived mitochondrial AcCoAs (9) to continue to be utilized for anabolic purposes such as lipid biosynthesis rather than for Oxphos. The acidic pH of KO cell cytoplasm (Fig. 5D) may also contribute to their more efficient uptake and utilization of glutamine (Fig. 2H) as would glutamine dehydrogenizes negative regulation by NADH and ATP and positive regulation by ADP (107).

KO cells’ more efficient use of glutamine to sustain proliferation and survival (Fig. 2, H and I) strengthens the idea that metabolic reprogramming of the “bottom” half of the TCA cycle (Fig. 7B) has true biological repercussions. It points to a potential AcCoA-independent alternative pathway that glucose- or fatty acid-starved tumors might mobilize to maintain redox and energy homeostasis.

The more robust OCR response of KO cells to exogenous palmitoylcarnitine (Fig. 2D) is consistent with previous in vivo findings in KO livers and HBs (9) and supports the idea that, in PDC’s absence, AcCoA may be provided by FAO and acetate. A reduced reliance on these alternatives may well be explained by the above-discussed re-programming of mitochondria to a partially AcCoA-independent form of energy production as well as to the well-known tendency of FAO to be inhibited by high levels of glycolytic intermediates (108). A dividend of this only partial reliance on FAO would be a more focused utilization of stored cellular lipids for de novo membrane biosynthesis (109). Our results show that the top portion of the TCA cycle, which relies on AcCoA to provide reducing equivalents (Fig. 7B), can be compromised without impairing proliferation and viability so long as glutaminolysis remains operative and fully supplied.

High levels of fructose, mannose 6-phosphate, and sorbitol/mannitol are generated by KO cells as a consequence of the extremely high levels of glycolytic intermediates (Figs. S2–S4) in an apparent reversal of their normal disposal via the glycolytic pathway. For example, fructose, via the sequential action of fructokinase and aldolase B, is normally converted to fructose 1-phosphate and then to DHAP and glyceraldehyde. Similarly, mannose, a C2 epimer of glucose, is converted to mannose 6-phosphate by hexokinase and then to fructose 6-phosphate.
by the action of phosphomannose isomerase. Finally, high-glucose levels in KO cells (Fig. 6A and Fig. S4) might activate the polypol pathway and drive sorbitol synthesis via aldose reductase, using NADPH as a co-factor. In turn, sorbitol can be catalyzed to fructose by sorbitol dehydrogenase (62, 109), which requires NADH and could perhaps contribute to its depletion in KO cells (Fig. 1E). Although galactose 1-phosphate was not elevated in KO cells, we did note higher levels of the nucleotide sugars UDP-galactose and UDP glucose (Table S1). The accumulation of these different hexoses and their derivatives is thus a likely consequence of glycolytic intermediate buildup.

There are several possible fates for the aspartate generated by KO cells. First, it may be used for de novo protein synthesis. Second, its cytoplasmic conversion to oxaloacetate and then to malate, in conjunction with the rapid intra-mitochondrial production of α-ketoglutarate, may allow high rates of α-ketoglutarate–malate antiporter activity, thereby helping to maintain as normal a redox balance as possible in otherwise metabolically compromised KO cells. Finally, aspartate may be used for other key biosynthetic purposes. In support of this idea was our finding that the levels of adenine, adenosine, and 2-deoxyadenosine, whose C6 amino groups are donated by aspartate, were increased 6.8-, 9.3-, and 84-fold, respectively, relative to WT cells, whereas the levels of guanine and guanosine, whose C2 amino groups are donated by glutamine, were reduced by 9.1- and 16.7-fold, respectively (Fig. 5E). These latter findings are consonant with the low KO cell concentrations of glutamine and its apparent dedicated use by the TCA cycle.

Collectively, our results recall recent studies showing that cells unable to proliferate due to defective ETC function and redox imbalance (i.e. low NAD+/NADH levels) can be rescued with exogenous pyruvate, which serves as an electron acceptor, thereby allowing the regeneration of NAD+ to support glycolysis and the resumption of normal proliferation (93, 95, 96). Such cells contain extremely low levels of aspartate, and although their proliferative defect can be rescued with exogenous aspartate, they retain their redox imbalance. This defect is fairly mild, and it is unclear whether it varies among different cellular compartments.

KO cells bear no apparent ETC structural defects but do have significant functional ones, as evidenced by reduced levels of NAD+ and NADH, a higher NAD+/NADH ratio, depleted TCA intermediates, and redox and acid-base alterations (Figs. 1E, 5, D–G, and 7B and Fig. S6A). The redox imbalance, coupled with lower levels of malate, might be expected to create an aspartate deficit similar to that described in cells with ETC dysfunction (93, 95, 96). In fact, the opposite of this was found, with aspartate being the only amino acid whose levels were actually increased (Fig. 7B and Fig. S6B). We propose that this finding, seemingly contradictory to those of Birsoy et al. (93) and Sullivan et al. (95), is actually entirely consistent with their models and results from the high levels of pyruvate accumulation, which serves as a de facto form of pyruvate supplementation (94, 96).

Pyruvate normally has five fates. First, in the PDC reaction, it is converted to αCoA and generates NADH, neither of which occurs in KO cells. Second, it may be converted to lactate and regenerate NAD+ for subsequent use as an electron acceptor. This too does not seem to occur to any greater extent in KO cells except under exceptional circumstances (Figs. 1C and Fig. S4). Third, pyruvate can be converted to malate in the cytoplasm and subsequently to oxaloacetate by the action of cytoplasmic NADP+-malate dehydrogenase 1 (93, 95, 96). Aspartate can be subsequently generated by the action of cytoplasmic GOT1. Because these reactions generate NAD+ or NADP+, they explain why exogenous pyruvate complements both the redox dysfunction and proliferative arrest of ETC-defective cells, whereas aspartate supplementation complements only the latter. Fourth, intramitochondrial pyruvate can furnish oxaloacetate via the action of pyruvate carboxylase. Finally, cytoplasmic pyruvate can be converted to alanine. Thus, we propose that the excess KO cell pyruvate, rather than being catalyzed into lactate, is largely converted to oxaloacetate or perhaps first into malate and then into oxaloacetate and finally into aspartate. An alternative, albeit untested, source for aspartate could be from the reversal of the phosphoenolpyruvate carboxykinase reaction. Although generally considered as catalyzing the first step of gluconeogenesis, the reaction is highly reversible (110) and might conceivably be favored by the high levels of phosphoenolpyruvate that also characterize KO cells (Fig. S4).

An additional pathway that has been proposed as a source of aspartate in ETC-defective cells involves the reductive carboxylation of α-ketoglutarate to citrate. The subsequent conversion of citrate to oxaloacetate via ATP-citrate lyase could provide substrate for the GOT1-mediated generation of aspartate as well as αCoA for lipid biosynthesis (93, 95, 96). Given the excess of pyruvate in KO cells, it is conceivable that this pathway plays a less prominent role than it does in cells with defective ETC function but otherwise normal PDC activity. This is supported by our finding that WT cells are more reliant than KO cells on exogenous glutamine for their survival, take up and catabolize larger amounts of exogenous glutamine (Fig. 2H), and do not engage in higher rates of reductive carboxylation (Fig. 3F).

Still unresolved is the purpose of the high aspartate levels generated in KO cells as well as those with ETC dysfunction. Although exogenous aspartate does not correct the redox abnormalities in cells with ETC dysfunction (93, 95), it may partially do so in KO cells, most likely by contributing to the aspartate–malate shuttle, which transfers glycolytically-generated electrons into mitochondria. It may also simply allow for normal rates of protein synthesis to occur by serving as a source for asparagine. Finally, it may support nucleotide biosynthesis. Interestingly, with the exception of uracil, the levels of which were reduced by ~80% in KO cells, those of the remaining common pyrimidine bases and nucleosides remained unchanged or were altered by no more than about 2-fold. In contrast, the baseline levels of purines were profoundly affected, albeit in opposite ways for the two families as discussed above.

In conclusion, our results demonstrate that the seemingly benign consequences of PDC loss on Rat1α–MyCER cell proliferation, size, and survival are in fact the result of a profound and wide-ranging metabolic re-programming affecting the components and/or the directionality of glycolysis, FAO, and the TCA cycle. The production, accumulation, and utilization of several
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key anaplerotic substrates, including glutamine and oxaloacetate, are also markedly altered. The metabolic changes in response to short-term Myc overexpression build upon this altered metabolic foundation. The work reveals heretofore unappreciated strategies that can be evoked in response to changes in the availability of key metabolites that might accompany transformation and/or extracellular environments but that are otherwise ill-suited to supporting maximal cellular proliferation.

Experimental procedures
Cell lines and plasmids

Unless otherwise stated, all cell lines were maintained in Dulbecco’s modified high-glucose–containing minimal essential medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and penicillin plus streptomycin as described previously (50, 59, 111). Rat1a–MycER cells have been previously described and were established following stable expression of a human MycER fusion protein that is rapidly activated in response to the synthetic estrogen 4OHT (11, 12). CRISPR-Cas9–mediated inactivation of the endogenous rat pdha1 gene was achieved by stable transfection of Rat1a–MycER cells with the CRISPR plasmid pSpCas9(BB)(PX330) (Addgene, Inc., Cambridge, MA) (112) encoding the guide RNA sequence 5′-CACCGGGCGACGCTGCAAACG-3′. This sequence corresponded to the negative strand of nucleotides 36222–36244 of the rat genomic sequence corresponding to the negative strand of nucleotides codons 10–17 of the coding sequence (GenBankTM accession number AABR07000000). The plasmid was co-transfected into Rat1a–MycER cells using Superfect (Qiagen, Inc., Valencia, CA) along with an empty vector encoding blasticidin resistance. Individual blasticidin-resistant clones were expanded, and mutation of the targeted locus was confirmed by PCR amplification of a 496-bp region encompassing the pdha1 target site using the flanking primers forward 5′-GGGTGTAGCGCATCGCGGGCAATC-3′ and reverse 5′-GCCGGTTCTCAAGGGCGCTCAA-3′. Automated Sanger sequencing of the amplified product was performed with the internal sequencing primer 5′-GGCGCTCAAGGAGACTTG-3′ to confirm that an inactivating mutation had been generated. The absence of PDHα1 protein expression in each of the identified targeted clones was then confirmed by Western blotting as described previously (Fig. S1A) (9). Five such clones, either expressing or not expressing PDHα1, were combined for all subsequent studies and are referred to as WT or KO cells, respectively (Fig. S1A).

Cell growth and sizing

WT and KO cells were seeded into 12-well tissue culture plates at a density of 2000 cells/well. They were then placed into an IncuCyte S3 live cell imaging system and visualized using IncuCyte Zoom software according to the directions of the manufacturer (Essen BioSystems, Ann Arbor, MI). Cell density was determined from images obtained every 2 hours in six replica samples in each group. Cell size was determined on separate log-phase cultures by trypsinizing cells, staining with trypan blue, and then quantifying the diameters of at least 2500 viable cells from each group with a ViCell Viability Analyzer (BD Biosciences). p values were determined using a t test with Welch’s correction for unequal variance on Graphpad Prism 6 Software (San Diego). For studies under limiting glutamine concentrations, cells were seeded the day before in standard medium. The following day, fresh medium containing dialyzed FBS and the indicated concentrations of glutamine was added, and the cells were immediately placed under IncuCyte S3 monitoring as described above.

Measurements of intracellular pH and redox states

To measure cytoplasmic or mitochondrial pH and redox states, WT and KO cells were transfected with the pH-sensitive pSypHer-cyto or pSypHer-mito vectors or the redox-sensitive roGFP-cyto or roGFP-mito vectors (55, 59, 60). The latter two were generated in the pEGFP or pDsRED-mito vector backbones (Clontech) from which enhanced GFP or dsRED sequences had been deleted. Transfections into WT or KO Rat1a–MycER cells were performed using Superfect as described above followed by the selection of stably transfected clones in G-418. From among the stable transfectants, the brightest population of cells was selected by fluorescence-activated cell sorting (FACS) and expanded for further study.

Measurements of mitochondrial mass and membrane potential, ROS, redox state, and pH

Cells were seeded into 6-well plates at numbers that allowed them to achieve ~80% confluency by the next day. Fresh medium, either lacking or containing 250 nM 4OHT, was then added with subsequent measurements being performed following an 8-h period of MycER activation.

 Mitochondrial mass was determined by incubating cells for 45 min at 37 °C in fresh complete medium containing 20 mM of the mitochondrial-specific dye NAO (ThermoFisher Scientific, Waltham MA). Cell monolayers were then washed twice in PBS, trypsinized, and pelleted in ice-cold PBS before being analyzed immediately by flow cytometry as described previously (12, 16). All determinations were performed on six biological replicates with at least 20,000 events/sample recorded. ΔΨM was assessed by incubating cells for 15 min at 37 °C in fresh medium containing 0.2 μM JC-1 dye (Invitrogen). Cells were then trypsinized and prepared for flow cytometry as described above. The fraction of cells with the highest levels of JC-1 aggregates was then determined for each population by flow cytometry as described previously (12).

ROS were measured by two methods as described previously (12, 59). Total ROS were measured by incubating cells for 45 min at 37 °C in complete medium containing 5 μM CM-H2DCFDA (Molecular Probes, Inc. Eugene, OR). Mitochondrial ROS (specifically superoxide (O2•−)) were measured by incubating cells for 45 min in medium containing 5 μM MitoSox™ Red (ThermoFisher Scientific).

To determine the intracellular redox state, WT and KO cells expressing the redox-sensitive GFP variants roGFP-cyto and roGFP-mito (59, 60) were plated as described above and allowed to achieve ~80% confluency. The ratio of emission signal intensities at 488/405 nm was used as a surrogate measurement for the redox state of each cellular compartment.
Cytoplasmic and mitochondrial pH values were determined in cells stably expressing pSynHer-cyto and pSynHer-mito (55). As with roGFP determinations, the 488/405 nm emission ratios were used to determine the relative pH of each intracellular compartment.

**Measurements of glycolysis, Oxphos, and FAO**

Glucose uptake was quantified using the fluorescent glucose analog 2-NBDG (ThermoFisher Scientific) (113). Cells were seeded the day before in 6-well plates to achieve ~80% confluency the following day. Monolayers were then washed twice with PBS and incubated for the times indicated in glucose-free Dulbecco’s modified Eagle’s medium + 10% dialyzed FBS, containing 300 μM 2-NBDG. Cells were then washed in ice-cold PBS, trypsinized, and resuspended in PBS + 1% FBS before being subjected to flow cytometric analysis using excitation/ emission wavelengths of 485 and 535 nm, respectively. Mean fluorescence from three biological replicas was used for all time points.

Lactate levels in cell culture fluid were quantified as described previously using a Lactate Scout+ meter (SensLab EKF Diagnostics, Leipzig, Germany) (9) and were then adjusted to total protein content. OCRs were quantified with an Oroboros Oxygraph 2k respirometer (Oroboros Instruments, Innsbruck, Austria) using either intact cells, digitonin-permeabilized cells, or isolated mitochondria as indicated (59). In the former two cases, cells were seeded the day before into 100-mm tissue culture plates as described above. The medium was then changed, and where appropriate, 4OHT was added for 8 h to activate MycER. Respirometry experiments were then performed largely as described previously using ~3 × 10⁶ cells/chamber (9, 13, 14). All results were normalized to total protein content determined on an aliquot of the input sample. Baseline measurements on nonpermeabilized cells were performed following resuspension in MiR05 buffer (110 mM sucrose, 0.5 mM EGTA, 3 mM MgCl₂, 60 mM potassium lactobionate, 20 mM taunine, 10 mM KH₂PO₄, 20 mM HEPES, pH 7.2, and 1 mg/ml fatty acid–free BSA, Oroboros). Cells were permeabilized and stabilized by the addition of digitonin (final concentration 16.6 μM) and cytochrome c (final concentration 10 μM). Other substrates, added in the order indicated in each figure, included malate (final concentration 2.0 mM), pyruvate (final concentration 5 mM), succinate (final concentration 10 mM), palmitoylcarnitine (final concentration 12.5 μM), glutamate (final concentration 10 mM), and rotenone (final concentration 0.5 μM). All substrates were purchased from Sigma.

Mitochondria were isolated from cells grown in 150-mm tissue culture plates for 1–2 days until achieving ~80% confluency. Trypsinized cells were pelleted and washed twice in ice-cold PBS and resuspended in 0.5 ml of ice-cold SET buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.25 mM sucrose) containing protease inhibitor mixture (Sigma). Cells were then disrupted on ice with 10 strokes of an Isobiotec homogenizer (Heidelberg, Germany). The homogenate was clarified at 400 × g for 10 min at 4 °C, and the pellet was discarded. The mitochondria-rich supernatant fraction was further centrifuged at 12,000 × g for 15 min. The pellet was washed twice with ice-cold SET buffer and resuspended in MiR05 buffer. Samples were assayed immediately, and an aliquot was used to determine protein content at the end of the experiment (9, 13, 14).

To measure FAO, cells were seeded into 6-well plates as described above and grown overnight. They were harvested, washed twice in PBS, and resuspended in 195 μl of FAO reaction buffer containing 1 μCi of BSA-conjugated ³H-labeled palmitate (PerkinElmer Life Sciences) at 37 °C as described previously (9, 13, 14). Reactions were terminated with 40 μl of 1 M KOH, and acylcarnitine esters were hydrolyzed for 1 h at 60 °C. Perchloric acid (40 μl) was then added for an additional hour on ice. Following organic extraction in a 1:1 methanol/chloroform mix, the water-soluble ³H-labeled FAO products were quantified by scintillation counting.

**Measurements of NAD⁺ and NADH**

NAD⁺ and NADH levels were quantified using a NAD⁺/NADH Glo™ assay kit (Promega, Inc. Madison, WI). Cells were first seeded into 96-well plates at 10⁴ cells/well and incubated overnight. The following day, fresh medium either lacking or containing 250 nm 4OHT was added for 8 h before proceeding with the assay using the directions provided by the supplier. Each group was assayed in eight replicas.

**Measurement of glutamine, acetate, and palmitate uptake and conversion into membrane lipids**

Cells were seeded into 12-well plates in standard medium and grown overnight to 80–90% confluency. To quantify glutamine uptake, medium was replaced with fresh medium containing or lacking 4OHT, and the incubation was continued for 7 h. The cells were then washed twice with PBS, and 1 ml of fresh glutamine-free medium containing 10% dialyzed FBS and 1 μCi of l-[3,4-³H]glutamine (specific activity = 43.8 Ci/mmol, PerkinElmer Life Sciences) with or without 4OHT was added. Incubations were continued for an additional 30 or 60 min. The medium was then aspirated, and the cells were washed three times with ice-cold PBS, lysed directly on plates in scintillation fluid, and counted. To study the incorporation of radiolabeled glutamine into membrane lipids, cells were plated as described above. The next day, fresh glutamine-free medium with 10% dialyzed FBS and 1 μCi of l-[3,4-³H]glutamine was added along with 4OHT where appropriate for 8 h. The medium was then aspirated, and the cells were washed three times with ice-cold PBS and then immediately frozen at ~80 °C. At a later time, they were solubilized in 250 μl of a solution containing 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% SDS, 10% glycerol, 12.5 mM EDTA, 1% Triton X-100, and 0.5% Nonidet P-40. Lipids were extracted with 750 μl of a chloroform/methanol mix (1:2) with vigorous vortexing. The organic phase was then removed. An additional 250 μl each of chloroform and water was added with additional vortexing. The organic phase containing lipids was collected, combined with the original organic extract, and used for scintillation counting. All results were normalized to the total protein content that was determined on separate wells.

BODIPY-493/504 staining and quantification were performed upon cells that had been seeded into 6-well plates and allowed to achieve 30–50% confluency by the next day.
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Medium was then changed to fresh medium containing the indicate FBS concentrations, and the cells were incubated overnight before staining with BODIPY-493/504 as described previously and quantifying uptake by flow cytometry (12).

Apoptosis determinations

These studies were performed as described previously (53, 54). Briefly, the entire contents of cell monolayers, including detached cells, were harvested, pelleted, washed twice in ice-cold PBS, and then resuspended in 1 ml of 10 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1% Nonidet P-40, 10 μg/ml RNase A, and 15 μg/ml propidium iodide (Sigma). After incubating on ice for at least 20 min, the DNA content was determined by flow cytometry. The sub-G0/G1 fraction was used as the measure of the apoptotic population. Quantification was performed on at least 20,000 cells using single histogram statistics.

Western blotting

Total lysates from WT and KO Rat1a–MycER cells were prepared in the presence of protease and phosphatase inhibitors as described previously (50, 59, 83). After SDS-PAGE, proteins were transformed to polyvinylidene difluoride membranes (Merck Millipore, Burlington, MA) and then probed with the antibodies listed in Table S2. Chemiluminescent imaging was performed with a SuperSignal West Femto Maximum Sensitivity Substrate Kit (ThermoFisher Scientific) according to the directions of the supplier. For some studies, WT cells were first separated into nuclear and cytoplasmic fractions using a subcellular protein fractionation kit according to the directions of the supplier (ThermoFisher Scientific).

Oxaloacetate, citrate synthase, and pyruvate carboxylase assays

Oxaloacetate assays were performed with an oxaloacetate assay kit (Sigma, catalog no. MAK070) according to the directions of the supplier.

For citrate synthase assays, cells from a nearly confluent 100-mm plate seeded the day before were harvested by trypsinization, resuspended in 200 μl of ice-cold MiR05 Buffer containing 0.24 mg/ml digitonin and lysed in a Bullet Blender (speed 3 for 2 min). After clarification by centrifugation (13,000 rpm for 20 min at 4 °C), 10 μl was added to 185 μl of a solution containing 0.25% Triton X-100, 0.3 mM AcCoA, 0.1 mM DNTB. All reactions were performed in 96-well plates and initiated by the addition of 10 μl of 10 mM oxaloacetate. In parallel, a standard curve was constructed using serial 2-fold concentrations of purified citrate synthase (Sigma). Reactions were incubated at room temperature while monitoring absorbance at 410 nm on a SpectraMax-Plus plate reader (Molecular Devices, Inc., San Jose, CA) at 10-s intervals over a period of 2 min. All values were then normalized to the total protein content of each sample.

Pyruvate carboxylase activity was determined in a two-step assay coupled to citrate synthase activity. In the initial step, pyruvate carboxylase converts pyruvate to oxaloacetate in the presence of excess bicarbonate and ATP. In the second step, an excess of AcCoA and citrate synthase ensures the conversion of oxaloacetate to citrate. Free CoA generated in the second step reacts with DTNB and produces a colored product, the absorbance of which is measurable at 412 nm and is indicative of pyruvate carboxylase activity. WT and KO cells were washed in cold PBS and pelleted. Cell pellets were resuspended in 100 mM Tris-HCl, pH 8.0, with protease and phosphatase inhibitors. Lysates were prepared by passing the cell suspensions through a 27-gauge syringe several times followed by centrifugation at 13,000 rpm, 4 °C for 10 min. Cleared lysates were used in 15-min reactions in 90 mM Tris-HCl, pH 8.0, 50 mM NaHCO3, 5 mM MgCl2, 0.1 mM AcCoA, 5 mM ATP, 0.01% DTNB, and 5 units of citrate synthase in the presence or absence of 5 mM pyruvate. The activity indicated by difference between absorbance at the start and end point was normalized to protein levels. The absence of pyruvate allowed an assessment of the endogenous pyruvate carboxylase activity, whereas the pyruvate inclusion captured the difference in enzyme levels between WT and KO cells.

Metabolomic profiling

All steady-state determinations of cellular metabolites were performed by Metabolon (Durham, NC). Cells were seeded into 150-mm tissue culture plates at concentrations that allowed them to achieve ~80% confluency by the following day. Fresh medium either containing or lacking 250 nM OHT was then added, and incubation was continued for an additional 8 h. Cell monolayers were washed twice with ice-cold PBS, trypsinized, and sedimented at 500 × g followed by two additional washes in ice-cold PBS. Pellets were then snap-frozen in liquid nitrogen and stored at −80 °C until being analyzed.

Samples were prepared using the automated MicroLab STAR system (Hamilton, Reno, NV) with several recovery standards being added prior to the first step in the extraction process for quality control purposes (114). Proteins were precipitated and removed with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000, Clifton, NJ) followed by centrifugation. The resulting extract was divided into five fractions: two were used for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), and one was used for analysis by RP/UPLC-MS/MS with negative ion mode ESI, and one was used for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark, Inc., Raleigh, NC) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

The dataset comprised 641 metabolites of known identity. Following normalization to protein content, log transformation, and imputation of missing values, ANOVA contrasts were used to identify metabolites that differed significantly between experimental groups. Analysis by two-way ANOVA identified metabolites exhibiting significant interaction and main effects for experimental parameters of cell type and treatment. An estimate of the false discovery rate (q value) was also calculated, and a q value of <0.10 was taken as an indication of high confidence in a result.
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