MYC overrides HIF to regulate proliferating primary cell metabolism in hypoxia

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# Abstract

# Keywords

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# Introduction

Cellular responses to hypoxia propel many physiologic and pathologic activities from wound healing and angiogenesis to vascular remodeling and fibrosis ([Lee et al., 2019a](#ref-lee2019); [Semenza, 2012](#ref-semenza2012b)). These activities require cells to continue energetically demanding tasks, such as macromolecular biosynthesis and proliferation, despite limited oxygen availability. Since respiration is the most efficient way for cells to produce energy, cell metabolism must adapt to meet energetic needs when oxygen supply is limiting. Understanding how these metabolic adaptations sustain critical cellular processes in hypoxia is fundamentally important to our understanding of human health and disease.

Cells typically respond to hypoxia by shifting energy production away from respiration and toward glycolysis. This response is mediated primarily by stabilization of the hypoxia-inducible transcription factor 1α (HIF-1α). HIF-1α activates the transcription of glucose transporters, glycolytic enzymes, lactate dehydrogenase, and pyruvate dehydrogenase kinase, while decreasing the expression of tricarboxylic acid (TCA) cycle and electron transport chain enzymes ([Lee et al., 2020](#ref-lee2020b); [Semenza, 2012](#ref-semenza2012b)). Although HIF-1α is constitutively expressed, it is hydroxylated by prolyl hydroxylase enzymes (PHDs) in normoxia and targeted for proteasomal degradation. PHDs are the principal oxygen sensors in metazoan cells ([Kaelin and Ratcliffe, 2008](#ref-kaelin2008a)). PHDs are α-ketoglutarate-dependent dioxygenase enzymes that require molecular oxygen for their enzymatic activity. When oxygen tension falls, PHD activity decreases, leading to HIF-1α stabilization and activation of its associated transcriptional program. Overall, this transcriptional program should increase glycolytic capacity and divert glucose-derived pyruvate from oxidative phosphorylation toward lactate fermentation to maintain glycolytic ATP production.

In addition to metabolic changes designed to maintain energy supply, hypoxic cells also reduce energy demands through down-regulation of Na+/K+-ATPase, slowing protein translation, and attenuating cell proliferation ([Hubbi and Semenza, 2015](#ref-hubbi2015); [Wheaton and Chandel, 2011](#ref-wheaton2011)). In particular, HIF-1α decreases cell proliferation by activating cyclin-dependent kinase inhibitor expression, inhibiting cell cycle checkpoint progression ([Gardner et al., 2001](#ref-gardner2001)), and antagonizing pro-proliferative MYC signaling ([Koshiji et al., 2004](#ref-koshiji2004)). Despite these canonical effects of HIF-1α activation, there are many examples where cells continue to proliferate despite hypoxic stress, including cancer cells, stem cells, and lung vascular cells ([Hubbi and Semenza, 2015](#ref-hubbi2015)). How these cells meet the metabolic needs of sustained proliferation in hypoxia and how these adaptations are regulated are an active area of investigation ([Jain et al., 2020](#ref-jain2020); [Lee et al., 2020](#ref-lee2020b); [Oldham et al., 2015](#ref-oldham2015)). Since hypoxia is a prominent feature of cancer biology as tumor growth outstrips blood supply, most detailed metabolic studies of hypoxic cell metabolism have used tumor cell models, yielding important insights into the metabolic pathobiology of cancer ([Garcia-Bermudez et al., 2018](#ref-garcia-bermudez2018); [Jiang et al., 2016](#ref-jiang2016); [Lee et al., 2019b](#ref-lee2019a); [Melendez-Rodriguez et al., 2019](#ref-melendez-rodriguez2019); [Metallo et al., 2011](#ref-metallo2011); [Wise et al., 2011](#ref-wise2011)). For example, stable isotope tracing and metabolic flux analyses identified a critical role for reductive carboxylation of glutamine-derived α-ketoglutarate for lipid biosynthesis in supporting tumor growth ([Gameiro et al., 2013a](#ref-gameiro2013a); [Metallo et al., 2011](#ref-metallo2011); [Scott et al., 2011](#ref-scott2011); [Wise et al., 2011](#ref-wise2011)), and metabolomic studies identified aspartate as a limiting metabolite for cancer cell proliferation under hypoxia ([Garcia-Bermudez et al., 2018](#ref-garcia-bermudez2018)). By contrast, comparatively little is known about metabolic adaptations of primary cells to hypoxia. Indeed, the importance of reductive carboxylation or aspartate biosynthesis remains to be elucidated in these cells. A more complete understanding of primary cell metabolic adaptation to hypoxia would provide an important context for understanding how metabolic reprogramming supports normal cellular responses to hypoxia, how these responses may be (mal)adaptive in a variety of disease contexts, and how the hypoxia metabolic program in primary cells differs from that observed in cancer cells.

To address these questions, we have developed models of bioenergetic carbon flux in human lung fibroblasts (LFs) and pulmonary artery smooth muscle cells (PASMCs) cultured in 21% or 0.5% oxygen. These cells may be exposed to a wide range of oxygen concentrations *in vivo*, continue to proliferate despite hypoxic culture conditions *in vitro*, and play important roles in the pathology of non-cancerous diseases in which tissue hypoxia is a prominent feature. We found that hypoxia fails to increase glycolysis in these primary cells despite robust up-regulation of the HIF-1α transcriptional program. In normoxia, HIF-1α stabilization by the PHD inhibitor molidustat (BAY-85-3934, “BAY”) ([Flamme et al., 2014](#ref-flamme2014)) did increase glycolysis and lactate efflux; however, hypoxia blocked this response. These findings suggested the existence of important hypoxia-dependent regulatory mechanisms that override the metabolic consequences of HIF-1α-dependent up-regulation of glycolytic gene expression in human primary cells. Transcriptomic profiling suggested a critical role for the transcription factor MYC in the adaptive response to hypoxia. Using knockdown and overexpression approaches, we demonstrate that MYC attenuates HIF-driven glycolysis in hypoxia and following BAY treatment.

# Results

## Hypoxia uncouples HIF-dependent glycolytic gene expression from glycolytic metabolic flux

The goal of this study was to characterize hypoxia-induced metabolic changes in proliferating primary LFs and PASMCs. To accomplish this goal, we adopted a metabolic flux analysis technique that enabled us to link intracellular metabolic fluxes to cell proliferation rates. Metabolic flux analysis fits cell proliferation rate, extracellular flux measurements, and 13C intracellular isotope labeling patterns to a computational model of cell metabolism ([Antoniewicz, 2018](#ref-antoniewicz2018)). This analysis reconstructs comprehensive flux maps that depict the flow of carbon from extracellular substrates, through intracellular metabolic pathways, and into cell biomass and metabolic by-products ([Young, 2014](#ref-young2014)). These models assume that cells are at a metabolic pseudo-steady state over the experimental time course ([Buescher et al., 2015](#ref-buescher2015)). Exponential growth phase is thought to reflect metabolic pseudo-steady state as cells in the culture steadily divide at their maximal condition-specific rate, provided nutrient supply does not become limiting ([Ahn and Antoniewicz, 2011](#ref-ahn2011); [Buescher et al., 2015](#ref-buescher2015)). Thus, we first set out to define experimental conditions to capture exponential growth phase in normoxic and hypoxic cultures.

Cells were seeded and placed into hypoxia for 24 h prior to sample collection to provide adequate time for activation of the hypoxia-dependent transcriptional program (**Fig ??A**). We selected 0.5% oxygen for hypoxia as this level yielded the most reproducible phenotypic differences compared to 21% oxygen culture while being physiologically relevant and above the KM of cytochrome *c* oxidase (electron transport chain complex IV) for oxygen ([Lee et al., 2020](#ref-lee2020b); [Wenger et al., 2015](#ref-wenger2015)). From this starting point, we identified the optimal cell seeding density and time course to capture exponential cell growth (**Fig ??B**). LFs cultured in 0.5% oxygen grew more slowly than LFs cultured in 21% oxygen (**Fig ??C**), but slower growth was not associated with decreased cell viability (**Fig S1A**). As anticipated, hypoxic cells demonstrated robust stabilization of HIF-1α protein associated with up-regulation of downstream targets, such as glucose transporter 1 (GLUT1) and lactate dehydrogenase A (LDHA) (**Figs ??D-H**). These changes persisted for the duration of the experimental time course.

Having identified experimental conditions for exponential growth, we next determined extracellular fluxes of glucose (GLC), lactate (LAC), pyruvate (PYR), and amino acids (**Figs ??I-J**). Flux calculations incorporated changes in cell number, extracellular metabolite concentrations, metabolite degradation rates, and medium evaporation over time ([Murphy and Young, 2013](#ref-murphy2013a)) (**Figure S1**). Interestingly, while we observed a modest increase in glucose uptake, we found that hypoxia actually decreased lactate efflux (**Figs ??I**). This decrease in lactate efflux occurred despite activation of the HIF-1α transcriptional program as reflected by increased expression of GLUT1 and LDHA. To test if more severe hypoxia would augment glycolysis, we culture cells in 0.2% ambient oxygen (**Fig S2**). These results were similar to 0.5% oxygen culture. We observed no change in glucose or lactate fluxes. To test if this unexpected response was unique to LFs, we next studied PASMCs under 0.5% oxygen conditions (**Fig S3**). PASMCs grew faster than LFs, and so samples were collected every 12 h for 48 h for these cells. Again, similar to LFs at 0.5% and 0.2% oxygen, we observed no change in glucose uptake and reduced lactate efflux in PASMCs regardless of HIF-1α stabilization. Together, these data suggest that hypoxia uncouples HIF target gene expression and glycolytic flux in proliferating primary cells.

Given that hypoxia exposure did not increase glycolysis in LFs, we next wanted to determine how these cells responded to HIF-1α stabilization in normoxia. To accomplish this, LFs were treated with the PHD inhibitor molidustat (BAY, 10 μM) using a similar time course as our hypoxia experiments. Cells were treated with BAY for 24 h to activate the HIF transcriptional program prior to sample collection (**Fig ??A**). As with hypoxia, BAY decreased cell growth rate (**Figs ??B-C**) and activated the HIF-1α transcriptional program (**Figs ??D-H**). Compared to hypoxia, BAY treatment resulted in a similar activation of HIF-1α target gene transcription and protein expression. Unlike hypoxia, however, HIF-1α stabilization in normoxia markedly increased glucose uptake and lactate efflux (**Fig ??I**), as expected based on the pattern of glycolytic gene expression that we observed. Interestingly, although hypoxia and BAY treatments resulted in similar increases in HIF-1α, GLUT1, and LDHA, the glycolytic response was markedly different.

## Extracellular fluxes are treatment and cell-type dependent

In addition to glucose and lactate, we also determined the extracellular fluxes of pyruvate and amino acids (**Figs ??J, ??J**). To our knowledge, this is the first comprehensive extracellular flux profiling of key metabolic substrates in these primary cells. In LFs, overall, changes in these fluxes were modest, with hypoxia generally decreasing the fluxes of all measured metabolites. These findings were similar with 0.2% oxygen exposure (**Fig S2J**).

Notably, we observed a significant decrease in glutamine consumption in hypoxic LFs. This finding contrasts with previous studies of cancer cell metabolism demonstrating increased glutamine uptake as a key feature of the metabolic response to hypoxia ([Gameiro et al., 2013b](#ref-gameiro2013); [Metallo et al., 2011](#ref-metallo2011); [Wise et al., 2011](#ref-wise2011)). In these systems, glutamine-derived α-ketoglutarate was reductively carboxylated by isocitrate dehydrogenase enzymes to generate citrate for lipogenesis. In addition, glutamine has been shown to support TCA cycling in hypoxia in a Burkitt lymphoma model ([Le et al., 2012](#ref-le2012)). Unlike LFs, PASMCs did exhibit a trend toward increased glutamine uptake (**Figure S3J**), suggesting a greater reliance on these metabolic pathways in their adaptive response to hypoxia.

In LFs, among all of the measured amino acid fluxes, proline consumption uniquely increased (**Fig ??J**). Hypoxia increases collagen expression in these cells ([Liu et al., 2013](#ref-liu2013a)) and proline constitutes ~ 10% of the total amino acid content of collagens. Together, these data suggest an important contribution of extracellular proline to collagen production in hypoxic LFs as has been observed in other fibroblast cell lineages ([Szoka et al., 2017](#ref-szoka2017)).

In PASMCs, we observed increased consumption of the branched-chain amino acids (BCAAs) leucine and valine as well as arginine (**Figure S3J**), which was not observed in LFs. BCAAs are transaminated by branch chain amino transferase enzymes to branched chain α-keto acids (BCKAs). BCKAs are further metabolized to yield acyl-CoA derivatives for lipogenesis or oxidation ([Crown et al., 2015](#ref-crown2015); [Mann et al., 2021](#ref-mann2021)). Previous studies have shown that hypoxia up-regulates arginase expression in hypoxic PASMCs ([Chen et al., 2009](#ref-chen2009); [Xue et al., 2017](#ref-xue2017)) to support polyamine and proline synthesis required for cell proliferation ([Li et al., 2001](#ref-li2001)). Interestingly, activation of these metabolic pathways in hypoxia was not observed in LFs and suggests distinct metabolic vulnerabilities of these different cell types.

Compared to hypoxia treatment, BAY demonstrated more modest effects on amino acid fluxes generally (**Figure ??J**). In particular, glutamate efflux was not affected by BAY treatment, while it was reduced by hypoxia. Alanine efflux was increased by BAY treatment, but decreased by hypoxia. In addition to the glucose and lactate fluxes noted above, these findings further highlight fundamental differences in the metabolic consequences of HIF activation in normoxia and hypoxia.

## Isotope tracing reveals altered substrate utilization in hypoxia

To investigate intracellular metabolic reprogramming in hypoxic cells, we performed 13C stable isotope tracing with [U-13C6]-glucose, [1,2-13C2]-glucose, and [U-13C5]-glutamine. Isotopic enrichment of downstream metabolites in glycolysis and the TCA cycle were determined by LC-MS (**Figs S3, S4**). Overall, relatively small changes in the patterns of isotope incorporation were observed following hypoxia or BAY treatment. The most substantial differences were observed in pyruvate (PYR), the terminal product of glycolysis, and citrate (CIT), a central metabolic node in TCA and fatty acid metabolism (**Figs ??A-C**). Both hypoxia and BAY treatments decreased incorporation of glucose-derived carbon into pyruvate (**Fig ??A**) (*i.e.,* the unlabeled, or M0, fraction was greater). This suggests a greater contribution from an unlabeled carbon source, such as extracellular pyruvate, lactate, or alanine, than from glucose to the intracellular pyruvate pool following PHD inhibition.

Total citrate labeling from [U-13C6]-glucose was unchanged across the treatment conditions (**Fig ??B**). As expected, we observed decreased M2 and M4 citrate isotopes, consistent with decreased pyruvate dehydrogenase activity in hypoxia. Interestingly, we observed increased M3 and M5 citrate isotopes. Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate after which all three pyruvate carbons are incorporated into citrate by citrate synthase. Thus, this labeling pattern suggests a more prominent contribution of pyruvate carboxylase to sustain TCA cycle anaplerosis despite pyruvate dehydrogenase inhibition following HIF-1α activation. By contrast to glucose labeling, much less citrate was labeled by glutamine with hypoxia or BAY with a more pronounced effect of BAY treatment (**Fig ??C**), suggesting a less important contribution of glutamine to TCA anaplerosis under these conditions. In addition, the overall fraction of M5 citrate resulting from reductive carboxylation of glutamine-derived α-ketoglutarate was low (< 7%) (**Fig ??D**). Although a hypoxia-mediated increase in M5 citrate was observed, the overall fraction was much less than the 10-20% levels previously reported in cancer cells ([Metallo et al., 2011](#ref-metallo2011); [Wise et al., 2011](#ref-wise2011)).

The stable isotope labeling patterns in PASMCs were generally similar to LFs (**Figure S5**). The most notable differences between LF and PASMC labeling were observed in citrate. Compared with LFs, a much lower fraction of total citrate was labeled by glucose in PASMCs. Less activity of pyruvate carboxylase in these cells was suggested by decreased M3 and M5 citrate isotopes after glucose labeling. Interestingly, the M5 citrate fraction in PASMCs was more consistent with previous reports from the cancer literature (**Fig ??D**), suggesting a more important role for glutamine metabolism for biomass synthesis in these cells.

## Glycolytic flux in hypoxia is closely coupled to cell growth rate

The mass isotopomer distribution for a given metabolite is determined by the complex relationship among the rate of isotope incorporation into the metabolic network, the contributions of unlabeled substrates, and fluxes through related pathways. To clarify how these labeling patterns reflect changes in intracellular metabolite fluxes, we next generated metabolic flux models incorporating the extracellular flux measurements and stable isotope tracing data described above. Preliminary labeling time courses indicated that, even after 72 h of labeling, intracellular metabolites did not reach isotopic steady state (**Fig S6**). Thus, we performed isotopically non-stationary metabolic flux analysis as implemented by Isotopomer Network Compartment Analysis (INCA) ([Jazmin and Young, 2013](#ref-jazmin2013); [Murphy and Young, 2013](#ref-murphy2013a); [Young et al., 2014](#ref-young2014a)) (**Figs ??E-F, S7, Tables S1-S3**).

Overall, LF and PASMC metabolic fluxes were dominated by high rates of glucose uptake and glycolysis (**Figs S7A-B**). Approximately 10% of cytoplasmic pyruvate enters the TCA cycle with the balance converted to lactate. Consistent with extracellular flux measurements and isotope labeling patterns described above, significant reductions in glycolysis, the TCA cycle, and amino acid metabolism were observed in the metabolic flux models of LFs cultured in hypoxia (**Fig ??E**). A significant increase in pentose phosphate pathway flux was also observed, although the absolute flux through this pathway is low. By contrast, HIF-1α activation by BAY in 21% oxygen increased glycolysis and lactate fermentation by nearly 50% (**Figure ??F**), but had a similar effect on decreasing serine and glutamine uptake as hypoxia. Metabolite fluxes in DMSO-treated cells were similar to 21% oxygen controls (**Table S1-S2**).

In normoxia, the magnitude of intracellular metabolite fluxes was generally similar in LFs and PASMCs (**Figures S7A-C, Tables S1, S3**). Compared to LFs, PASMCs had slower rates of glycolysis and faster rates of TCA metabolism driven, in part, by increased glutamine uptake. In hypoxia, PASMCs exhibited similar decreases in glycolytic flux as LFs but also a marked, and unexpected, increase in TCA flux (**Figure S7D**). The increased TCA flux in PASMCs was driven by increased glutamine consumption. This finding is similar to a prior report of glutamine-driven oxidative phosphorylation in hypoxic cancer cells ([Fan et al., 2013](#ref-fan2013)), where oxidative phosphorylation continued to provide the majority of cellular ATP even in 1% oxygen.

Given the global decrease in bioenergetic metabolic flux in hypoxic LFs, we hypothesized that these differences may be a consequence of decreased growth rate. After normalizing metabolite fluxes in normoxia and hypoxia to the cell growth rate, modest increases (~10%) in glycolytic flux were observed (**Figure S7E**). This finding suggests that, while glycolysis increases relative to growth rate in hypoxic cells, the regulators of cell proliferation rate override the consequences of the HIF-1α transcriptional program. Indeed, even after adjusting for cell growth rate, the relative increase in glycolytic flux is modest compared to the marked up-regulation of glycolytic protein levels. BAY treatment decreased cell proliferation rate (**Figure ??B**), indicating that, unlike hypoxia, BAY treatment in normoxia uncouples cell proliferation and metabolic flux.

## Hypoxia and BAY treatment increase lactate oxidation

Although the metabolite exchange fluxes for bidirectional reactions tend to be poorly resolved by metabolic flux analysis, two observations are worth highlighting (**Tables S1-S3**). First, consistent with the stable isotope tracing results, the modeled rate of reductive carboxylation through reverse flux by isocitrate dehydrogenase in LFs is low (~4 fmol/cell/h), unchanged by hypoxia, and modestly increased by BAY treatment. By contrast, the rate of reductive carboxylation increases 6-fold in PASMCs in hypoxia, highlighting an important role for this pathway in the metabolic response of PASMCs to hypoxia (**Fig ??A**).

Second, PHD inhibition is associated with a marked increase in the lactate transport exchange flux in LFs from ~ 0 to 1,500 and 700 fmol/cell/h in 0.5% oxygen and BAY treatment conditions, respectively, with similar results in PASMCs (**Fig ??B**). Since the net lactate transport flux is secretion, this observation suggests increased lactate uptake with hypoxia or BAY treatment, a finding that may be consistent with the HIF-driven increased expression of the reversible lactate transporter MCT4 ([Contreras-Baeza et al., 2019](#ref-contreras-baeza2019)). To investigate this hypothesis, LFs and PASMCs were treated with [U-13C3] lactate (2 mM) and 13C incorporation into intracellular metabolites was analyzed by LC-MS (**Figs ??, S4, S5**). Lactate labeled ~50% of citrate and ~20% of downstream TCA cycle metabolites in both LFs and PASMCs, indicating that lactate may be an important respiratory fuel source in these cells even though lactate efflux is high. Although lactate has been used less commonly than glucose and glutamine in stable isotope tracing studies, Faubert and colleagues ([2017](#ref-faubert2017)) demonstrated lactate incorporation in human lung adenocarcinoma *in vivo*. In this study, lactate incorporation corresponded to regions of high glucose uptake as determined by [ 18F ]-fluorodeoxyglucose positron emission tomography, suggesting that lactate consumption can occur even in areas of high glucose utilization. Subsequently, investigators have demonstrated the importance of lactate as a metabolic fuel *in vivo* ([Hui et al., 2017](#ref-hui2017); [Hui et al., 2020](#ref-hui2020)). As predicated from our metabolic flux analysis, with hypoxia or BAY treatment, we observed increased labeling of the TCA metabolites citrate (CIT), α-ketoglutarate (AKG), malate (MAL), and aspartate (ASP) in LFs. Interestingly, although increased labeling of pyruvate was observed in hypoxic PASMCs, the label was not incorporated into the TCA cycle as observed in LFs (**Fig S5**).

In addition to downstream metabolites, we also observed hypoxia- and BAY-dependent increases in lactate incorporation in fructose bisphosphate (FBP) and 3-phosphoglycerate (3PG). This observation is consistent with prior reports describing hypoxia-mediated increases in gluconeogenesis and glycogen synthesis ([Favaro et al., 2012](#ref-favaro2012); [Owczarek et al., 2020](#ref-owczarek2020); [Pelletier et al., 2012](#ref-pelletier2012)). These data suggest that lactate also makes a small (~5% carbon) contribution to glycogen precursors. Together, these findings from stable isotope tracing of lactate reveal its important contribution to primary cell metabolism under standard culture conditions, but also reveal increased utilization of this substrate in hypoxia.

## Hypoxia abrogates the effects of BAY on increasing glycolysis

To reconcile the differential effects of prolyl hydroxylase inhibition by hypoxia and BAY, we next addressed whether hypoxia could suppress the effects of BAY on glucose and lactate fluxes (**Figs ??A-C**). LFs cultured in standard growth medium were treated with BAY and placed in either 21% or 0.5% oxygen. Similar to previous experiments, BAY treatment decreased cell growth rate, increased glucose uptake, and increased lactate efflux in 21% oxygen. However, when combined with 0.5% oxygen, BAY treatment was unable to enhance lactate efflux. These data indicate that hypoxia antagonizes the effects of HIF-1α activation on glycolytic flux in these cells.

To investigate these metabolic differences further, we performed metabolomic profiling of LFs treated for 72 h with hypoxia or BAY separately or in combination. Both 0.5% oxygen and BAY treatment induced marked changes in intracellular metabolite levels (**Fig S8**). Of 133 total metabolites, 99 were differentially regulated by hypoxia and 54 were differentially regulated by BAY. Of the differentially regulated metabolites, 44 were affected by both 0.5% oxygen and BAY treatment. Metabolite set enrichment analysis of KEGG biochemical pathways identified increased enrichment of arginine and proline metabolism and fatty acid biosynthesis pathways with hypoxia (**Fig S8D**). By contrast, BAY treatment was enriched for metabolites involved in pentose/glucuronate interconversions and glycolysis (**Fig S8E**). Aspartate was the most significantly decreased metabolite with both treatments, consistent with prior reports demonstrating an important role for HIF-1 regulation of aspartate biosynthesis in cancer cells ([Garcia-Bermudez et al., 2018](#ref-garcia-bermudez2018); [Melendez-Rodriguez et al., 2019](#ref-melendez-rodriguez2019)).

Principal component analysis revealed greater similarity among both treatment groups cultured in 0.5% oxygen than among the BAY-treatment groups (**Fig ??D**). Moreover, these hypoxia-treated cells were well-segregated from cells treated with BAY alone. These observations are, again, consistent with the results of the metabolic flux models demonstrating an overriding effect of hypoxia *per se* on cell metabolism and highlighting important differences between hypoxic and pharmacologic PHD inhibition. To identify the metabolic changes that depend on hypoxia rather than BAY inhibition, we identified differentially regulated metabolites in BAY treated cells cultured in normoxia and hypoxia (**Fig ??E**). Of 133 metabolites, 83 were significantly differentially regulated by hypoxia in BAY-treated cells treatments. An enrichment analysis of these differentially regulated metabolites demonstrated up-regulation of arginine and proline metabolism and down-regulation of the TCA cycle as the most impacted by hypoxia in BAY treated cells (**Fig ??F**). Indeed, leading edge analysis highlights negative enrichment scores associated with all of the TCA metabolites detected by our platform (**Fig ??G**). This result indicates better preservation of TCA cycle flux in normoxic BAY-treated cells than in hypoxic cells, as suggested by our metabolic flux models where hypoxia resulted in a 1.5-2-fold reduction of TCA flux compared to a 1.1-1.5-fold reduction with BAY treatment (**Fig ??**).

In addition to these differential effects on polar metabolite levels, we reasoned that another critical difference between hypoxia and BAY treatment is the impact of hypoxia on cellular redox state. As oxygen deprivation causes reductive stress ([Xiao and Loscalzo, 2020](#ref-xiao2020)), we next measured the impact of these treatments on intracellular NAD(H) (**Figs ??H-J**). As expected, hypoxia increased the NADH/NAD+ ratio, driven primarily by a decrease in intracellular NAD+. Interestingly, while BAY treatment increased the levels of NADH, a concomitant increase in NAD+ resulted in preservation of the NADH/NAD+ ratio. As NADH accumulation is a putative inhibitor of glycolytic flux ([Tilton et al., 1991](#ref-tilton1991)), this may be one mechanism by which glycolytic flux is decreased in hypoxia but not following BAY treatment.

## Transcriptomic analysis identifies regulators of metabolism in hypoxia

To identify the upstream regulators of the observed metabolic changes, we next performed RNA-seq transcriptomic analysis of LFs treated with hypoxia or BAY, separately or together (**Fig ??, S9**). As anticipated, both hypoxia and BAY treatment induced substantial changes in gene expression (**Fig S9A, S9B**). Of the 9,923 differentially expressed genes across both conditions, 869 (9%) were unique to BAY treatment in normoxia, 4,002 (40%) were shared by BAY and hypoxia, while 5,052 (51%) were unique to 0.5% hypoxia culture (**Fig S9C**). This distribution of transcriptional changes was nearly identical to that observed for the metabolic changes (**Fig S8C**) where half of the hypoxia-mediated changes overlapped with BAY treatment and half were unique to hypoxia. Gene set enrichment analysis of these differentially regulated metabolites was performed using Molecular Signatures Database “Hallmark” gene sets ([Liberzon et al., 2015](#ref-liberzon2015)) (**Figs S9D, S9E, S9F**). As expected, both treatments were associated with enrichment of the “hypoxia” and “glycolysis” gene sets.

Given the disparate effects of hypoxic and pharmacologic PHD inhibition on cellular metabolism described above, we focused our transcriptomics analyses on the differences between hypoxia and BAY treatments. Principal component analysis again demonstrated clear separation among the four treatment groups (**Fig ??A**). The first and second principal components correspond to 0.5% oxygen and BAY treatments, respectively. Consistent with our prior observations, the combination of 0.5% oxygen plus BAY was more similar to 0.5% oxygen alone with decreased distance between both hypoxia-treated groups along the axis of the second principal component, again, consistent with the hypothesis that hypoxia overrides the effects of BAY treatment. To identify the transcripts driving these differences, we identified genes differentially expressed following hypoxia in BAY-treated cells (**Fig ??B**). Interestingly, an enrichment analysis of these differentially expressed transcripts identified pro-proliferative gene sets like “E2F targets”, “G2/M checkpoint”, and “MYC targets” associated with hypoxia (**Fig ??C-E**). These findings were further supported by a transcription factor enrichment analysis identifying enrichment of MYC transcription factor activity associated with hypoxia, but not BAY treatment (**Fig ??F**). Classically, hypoxia and HIF activation are thought to inhibit cell proliferation by inhibiting pro-proliferative MYC signaling ([Koshiji et al., 2004](#ref-koshiji2004)). These results indicate that hypoxia-induced MYC activation may be sustaining proliferation in these LFs. We reasoned that these pro-proliferative signals may also account for the unexpected effects of hypoxia on glycolysis that we observed.

## MYC antagonizes HIF-dependent glycolytic fluxes

To test the role of hypoxia-induced MYC activation in the metabolic response to hypoxia in proliferating primary cells, we first measured MYC protein levels by immunoblot.

Consistent with our bioinformatic results, immunoblotting demonstrated increased MYC protein levels in hypoxia-treated cells, but not with BAY-treatment alone, where MYC was decreased (**Figure ??A-B**). To test the hypothesis that hypoxia-induced MYC expression inhibits glycolysis in hypoxic primary cells, we first combined MYC knockdown with hypoxia treatment (**Figure ??C-E**). As expected, MYC-deficient cells proliferated more slowly in normoxia and MYC was absolutely essential for sustaining cell proliferation in hypoxia (**Figure ??D**). Consistent with our hypothesis, MYC knockdown cells demonstrated increased lactate efflux upon hypoxia treatment, unlike control siRNA-treated cells (**Figure ??E**). We next performed the complementary experiment to determine whether MYC overexpression could attenuate the increase in glycolysis observed with BAY treatment (**Figure ??F-H**). MYC increased the proliferation rate of DMSO-treated cells, although it did not augment the proliferation rate of BAY-treated cells. As expected, MYC overexpression blocked the BAY-stimulated increase in lactate efflux. Together, these data suggest that hypoxia-induced MYC expression may be one factor that uncouples the HIF transcriptional program from glycolytic flux in proliferating primary cells.

# Discussion

# Materials and Methods

## Data Availability

# Acknowledgements

# Author Contributions

# Disclosures and competing interests

# References

Ahn, W.S., and Antoniewicz, M.R. (2011). Metabolic flux analysis of CHO cells at growth and non-growth phases using isotopic tracers and mass spectrometry. Metab Eng *13*, 598–609. <https://doi.org/10.1016/j.ymben.2011.07.002>.

Antoniewicz, M.R. (2018). A guide to 13C metabolic flux analysis for the cancer biologist. Exp Mol Med *50*, 1–13. <https://doi.org/10.1038/s12276-018-0060-y>.

Buescher, J.M., Antoniewicz, M.R., Boros, L.G., Burgess, S.C., Brunengraber, H., Clish, C.B., DeBerardinis, R.J., Feron, O., Frezza, C., Ghesquiere, B., et al. (2015). A roadmap for interpreting (13)C metabolite labeling patterns from cells. Curr Opin Biotechnol *34*, 189–201. <https://doi.org/10.1016/j.copbio.2015.02.003>.

Chen, B., Calvert, A.E., Cui, H., and Nelin, L.D. (2009). Hypoxia promotes human pulmonary artery smooth muscle cell proliferation through induction of arginase. American Journal of Physiology-Lung Cellular and Molecular Physiology *297*, L1151–L1159. <https://doi.org/10.1152/ajplung.00183.2009>.

Contreras-Baeza, Y., Sandoval, P.Y., Alarcon, R., Galaz, A., Cortes-Molina, F., Alegria, K., Baeza-Lehnert, F., Arce-Molina, R., Guequen, A., Flores, C.A., et al. (2019). Monocarboxylate transporter 4 (MCT4) is a high affinity transporter capable of exporting lactate in high-lactate microenvironments. J Biol Chem *294*, 20135–20147. <https://doi.org/10.1074/jbc.RA119.009093>.

Crown, S.B., Marze, N., and Antoniewicz, M.R. (2015). Catabolism of Branched Chain Amino Acids Contributes Significantly to Synthesis of Odd-Chain and Even-Chain Fatty Acids in 3T3-L1 Adipocytes. PLoS One *10*, e0145850. <https://doi.org/10.1371/journal.pone.0145850>.

Fan, J., Kamphorst, J.J., Mathew, R., Chung, M.K., White, E., Shlomi, T., and Rabinowitz, J.D. (2013). Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia. Mol Syst Biol *9*, 712. <https://doi.org/10.1038/msb.2013.65>.

Faubert, B., Li, K.Y., Cai, L., Hensley, C.T., Kim, J., Zacharias, L.G., Yang, C., Do, Q.N., Doucette, S., Burguete, D., et al. (2017). Lactate Metabolism in Human Lung Tumors. Cell *171*, 358–371 e9. <https://doi.org/10.1016/j.cell.2017.09.019>.

Favaro, E., Bensaad, K., Chong, M.G., Tennant, D.A., Ferguson, D.J., Snell, C., Steers, G., Turley, H., Li, J.L., Gunther, U.L., et al. (2012). Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells. Cell Metab *16*, 751–764. <https://doi.org/10.1016/j.cmet.2012.10.017>.

Flamme, I., Oehme, F., Ellinghaus, P., Jeske, M., Keldenich, J., and Thuss, U. (2014). Mimicking hypoxia to treat anemia: HIF-stabilizer BAY 85-3934 (Molidustat) stimulates erythropoietin production without hypertensive effects. PLoS One *9*, e111838. <https://doi.org/10.1371/journal.pone.0111838>.

Gameiro, P.A., Laviolette, L.A., Kelleher, J.K., Iliopoulos, O., and Stephanopoulos, G. (2013b). Cofactor balance by nicotinamide nucleotide transhydrogenase (NNT) coordinates reductive carboxylation and glucose catabolism in the tricarboxylic acid (TCA) cycle. J Biol Chem *288*, 12967–12977. <https://doi.org/10.1074/jbc.M112.396796>.

Gameiro, P.A., Yang, J., Metelo, A.M., Perez-Carro, R., Baker, R., Wang, Z., Arreola, A., Rathmell, W.K., Olumi, A., Lopez-Larrubia, P., et al. (2013a). In vivo HIF-mediated reductive carboxylation is regulated by citrate levels and sensitizes VHL-deficient cells to glutamine deprivation. Cell Metab *17*, 372–385. <https://doi.org/10.1016/j.cmet.2013.02.002>.

Garcia-Bermudez, J., Baudrier, L., La, K., Zhu, X.G., Fidelin, J., Sviderskiy, V.O., Papagiannakopoulos, T., Molina, H., Snuderl, M., Lewis, C.A., et al. (2018). Aspartate is a limiting metabolite for cancer cell proliferation under hypoxia and in tumours. Nat Cell Biol *20*, 775–781. <https://doi.org/10.1038/s41556-018-0118-z>.

Gardner, L.B., Li, Q., Park, M.S., Flanagan, W.M., Semenza, G.L., and Dang, C.V. (2001). Hypoxia inhibits G1/S transition through regulation of p27 expression. J Biol Chem *276*, 7919–7926. <https://doi.org/10.1074/jbc.M010189200>.

Hubbi, M.E., and Semenza, G.L. (2015). Regulation of cell proliferation by hypoxia-inducible factors. Am J Physiol Cell Physiol *309*, C775–82. <https://doi.org/10.1152/ajpcell.00279.2015>.

Hui, S., Ghergurovich, J.M., Morscher, R.J., Jang, C., Teng, X., Lu, W., Esparza, L.A., Reya, T., Le, Z., Yanxiang Guo, J., et al. (2017). Glucose feeds the TCA cycle via circulating lactate. Nature *551*, 115–118. <https://doi.org/10.1038/nature24057>.

Hui, S., Cowan, A.J., Zeng, X., Yang, L., TeSlaa, T., Li, X., Bartman, C., Zhang, Z., Jang, C., Wang, L., et al. (2020). Quantitative Fluxomics of Circulating Metabolites. Cell Metab <https://doi.org/10.1016/j.cmet.2020.07.013>.

Jain, I.H., Calvo, S.E., Markhard, A.L., Skinner, O.S., To, T.L., Ast, T., and Mootha, V.K. (2020). Genetic Screen for Cell Fitness in High or Low Oxygen Highlights Mitochondrial and Lipid Metabolism. Cell *181*, 716–727 e11. <https://doi.org/10.1016/j.cell.2020.03.029>.

Jazmin, L.J., and Young, J.D. (2013). Isotopically nonstationary 13C metabolic flux analysis. Methods Mol Biol *985*, 367–390. <https://doi.org/10.1007/978-1-62703-299-5_18>.

Jiang, L., Shestov, A.A., Swain, P., Yang, C., Parker, S.J., Wang, Q.A., Terada, L.S., Adams, N.D., McCabe, M.T., Pietrak, B., et al. (2016). Reductive carboxylation supports redox homeostasis during anchorage-independent growth. Nature *532*, 255–258. <https://doi.org/10.1038/nature17393>.

Kaelin, W.G., and Ratcliffe, P.J. (2008). Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell *30*, 393–402. <https://doi.org/10.1016/j.molcel.2008.04.009>.

Koshiji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C., and Huang, L.E. (2004). HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. EMBO J *23*, 1949–1956. <https://doi.org/10.1038/sj.emboj.7600196>.

Le, A., Lane, A.N., Hamaker, M., Bose, S., Gouw, A., Barbi, J., Tsukamoto, T., Rojas, C.J., Slusher, B.S., Zhang, H., et al. (2012). Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab *15*, 110–121. <https://doi.org/10.1016/j.cmet.2011.12.009>.

Lee, J.W., Ko, J., Ju, C., and Eltzschig, H.K. (2019a). Hypoxia signaling in human diseases and therapeutic targets. Exp Mol Med *51*, 1–13. <https://doi.org/10.1038/s12276-019-0235-1>.

Lee, P., Chandel, N.S., and Simon, M.C. (2020). Cellular adaptation to hypoxia through hypoxia inducible factors and beyond. Nat Rev Mol Cell Biol *21*, 268–283. <https://doi.org/10.1038/s41580-020-0227-y>.

Lee, W.D., Mukha, D., Aizenshtein, E., and Shlomi, T. (2019b). Spatial-fluxomics provides a subcellular-compartmentalized view of reductive glutamine metabolism in cancer cells. Nat Commun *10*, 1351. <https://doi.org/10.1038/s41467-019-09352-1>.

Li, H., Meininger, C.J., Hawker, J.R., Haynes, T.E., Kepka-Lenhart, D., Mistry, S.K., Morris, S.M., and Wu, G. (2001). Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells. American Journal of Physiology-Endocrinology and Metabolism *280*, E75–E82. <https://doi.org/10.1152/ajpendo.2001.280.1.E75>.

Liberzon, A., Birger, C., Thorvaldsdottir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst *1*, 417–425. <https://doi.org/10.1016/j.cels.2015.12.004>.

Liu, S.-S., Wang, H.-Y., Tang, J.-M., and Zhou, X.-M. (2013). Hypoxia-induced collagen synthesis of human lung fibroblasts by activating the angiotensin system. Int J Mol Sci *14*, 24029–24045. <https://doi.org/10.3390/ijms141224029>.

Mann, G., Mora, S., Madu, G., and Adegoke, O.A.J. (2021). Branched-chain Amino Acids: Catabolism in Skeletal Muscle and Implications for Muscle and Whole-body Metabolism. Front Physiol *12*, 702826. <https://doi.org/10.3389/fphys.2021.702826>.

Melendez-Rodriguez, F., Urrutia, A.A., Lorendeau, D., Rinaldi, G., Roche, O., Bogurcu-Seidel, N., Ortega Muelas, M., Mesa-Ciller, C., Turiel, G., Bouthelier, A., et al. (2019). HIF1alpha Suppresses Tumor Cell Proliferation through Inhibition of Aspartate Biosynthesis. Cell Rep *26*, 2257–2265 e4. <https://doi.org/10.1016/j.celrep.2019.01.106>.

Metallo, C.M., Gameiro, P.A., Bell, E.L., Mattaini, K.R., Yang, J., Hiller, K., Jewell, C.M., Johnson, Z.R., Irvine, D.J., Guarente, L., et al. (2011). Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature *481*, 380–384. <https://doi.org/10.1038/nature10602>.

Murphy, T.A., and Young, J.D. (2013). ETA: robust software for determination of cell specific rates from extracellular time courses. Biotechnol Bioeng *110*, 1748–1758. <https://doi.org/10.1002/bit.24836>.

Oldham, W.M., Clish, C.B., Yang, Y., and Loscalzo, J. (2015). Hypoxia-Mediated Increases in L-2-hydroxyglutarate Coordinate the Metabolic Response to Reductive Stress. Cell Metab *22*, 291–303. <https://doi.org/10.1016/j.cmet.2015.06.021>.

Owczarek, A., Gieczewska, K., Jarzyna, R., Jagielski, A.K., Kiersztan, A., Gruza, A., and Winiarska, K. (2020). Hypoxia increases the rate of renal gluconeogenesis via hypoxia-inducible factor-1-dependent activation of phosphoenolpyruvate carboxykinase expression. Biochimie *171-172*, 31–37. <https://doi.org/10.1016/j.biochi.2020.02.002>.

Pelletier, J., Bellot, G., Gounon, P., Lacas-Gervais, S., Pouyssegur, J., and Mazure, N.M. (2012). Glycogen Synthesis is Induced in Hypoxia by the Hypoxia-Inducible Factor and Promotes Cancer Cell Survival. Front Oncol *2*, 18. <https://doi.org/10.3389/fonc.2012.00018>.

Scott, D.A., Richardson, A.D., Filipp, F.V., Knutzen, C.A., Chiang, G.G., Ronai, Z.A., Osterman, A.L., and Smith, J.W. (2011). Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. J Biol Chem *286*, 42626–42634. <https://doi.org/10.1074/jbc.M111.282046>.

Semenza, G.L. (2012). Hypoxia-inducible factors in physiology and medicine. Cell *148*, 399–408. <https://doi.org/10.1016/j.cell.2012.01.021>.

Szoka, L., Karna, E., Hlebowicz-Sarat, K., Karaszewski, J., and Palka, J.A. (2017). Exogenous proline stimulates type I collagen and HIF-1α expression and the process is attenuated by glutamine in human skin fibroblasts. Mol Cell Biochem *435*, 197–206. <https://doi.org/10.1007/s11010-017-3069-y>.

Tilton, W.M., Seaman, C., Carriero, D., and Piomelli, S. (1991). Regulation of glycolysis in the erythrocyte: role of the lactate/pyruvate and NAD/NADH ratios. J Lab Clin Med *118*, 146–152.

Wenger, R.H., Kurtcuoglu, V., Scholz, C.C., Marti, H.H., and Hoogewijs, D. (2015). Frequently asked questions in hypoxia research. Hypoxia (Auckl) *3*, 35–43. <https://doi.org/10.2147/HP.S92198>.

Wheaton, W.W., and Chandel, N.S. (2011). Hypoxia. 2. Hypoxia regulates cellular metabolism. Am J Physiol Cell Physiol *300*, C385–93. <https://doi.org/10.1152/ajpcell.00485.2010>.

Wise, D.R., Ward, P.S., Shay, J.E., Cross, J.R., Gruber, J.J., Sachdeva, U.M., Platt, J.M., DeMatteo, R.G., Simon, M.C., and Thompson, C.B. (2011). Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. Proc Natl Acad Sci U S A *108*, 19611–19616. <https://doi.org/10.1073/pnas.1117773108>.

Xiao, W., and Loscalzo, J. (2020). Metabolic Responses to Reductive Stress. Antioxid Redox Signal *32*, 1330–1347. <https://doi.org/10.1089/ars.2019.7803>.

Xue, J., Nelin, L.D., and Chen, B. (2017). Hypoxia induces arginase II expression and increases viable human pulmonary artery smooth muscle cell numbers via AMPKα1 signaling. American Journal of Physiology-Lung Cellular and Molecular Physiology *312*, L568–L578. <https://doi.org/10.1152/ajplung.00117.2016>.

Young, J.D. (2014). INCA: a computational platform for isotopically non-stationary metabolic flux analysis. Bioinformatics *30*, 1333–1335. <https://doi.org/10.1093/bioinformatics/btu015>.

Young, J.D., Allen, D.K., and Morgan, J.A. (2014). Isotopomer measurement techniques in metabolic flux analysis II: mass spectrometry. Methods Mol Biol *1083*, 85–108. <https://doi.org/10.1007/978-1-62703-661-0_7>.

# Tables

# Figure Legends

# Figures