MYC uncouples HIF target gene expression from glycolytic flux in hypoxic proliferating primary cells

Courtney A. Copeland1, Benjamin A. Olenchock1, David R. Ziehr1,2, Sarah McGarrity1,3, Kevin Leahy1, Jamey D. Young4, Joseph Loscalzo1, and William M. Oldham1,‡

1 Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA  
2 Department of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA  
3 Center for Systems Biology, School of Health Sciences, University of Iceland, Reykjavik, Iceland  
4 Departments of Chemical & Biomolecular Engineering and Molecular Physiology & Biophysics, Vanderbilt University, Nashville, TN

‡ Correspondence: [William M. Oldham <[woldham@bwh.harvard.edu](mailto:woldham@bwh.harvard.edu)>](mailto:woldham@bwh.harvard.edu)

# Abstract

Hypoxia requires metabolic adaptations to sustain cellular functions that underlie numerous physiologic and pathologic processes. While many studies have explored the metabolic consequences of hypoxia in cancer models, comparatively little is known about the metabolic response of primary cells to hypoxia. Here, we performed metabolic flux analyses of proliferating human lung fibroblasts and pulmonary artery smooth muscle cells in hypoxia. Unexpectedly, glycolytic flux was decreased in hypoxic cells despite activation of the hypoxia-inducible factor (HIF) transcriptional program and increased expression of glycolytic enzymes. Pharmacologic activation of HIF with the prolyl hydroxylase (PHD) inhibitor molidustat in normoxia did increase glycolytic flux, but hypoxia abrogated this effect. Multi-omic profiling of cells treated with hypoxia or molidustat, separately or together, revealed distinct molecular responses to hypoxia and pharmacologic PHD inhibition and suggested a critical role for MYC in modulating the HIF response in hypoxia. MYC knockdown in hypoxia increased lactate efflux while MYC overexpression in normoxia blunted the effects of molidustat treatment. Together, these data suggest that other factors, notably MYC, supersede the anticipated effects of HIF-dependent up-regulation of glycolytic gene expression on glycolytic flux in hypoxic proliferating primary cells.

# Keywords

hypoxia, metabolic flux analysis, hypoxia-inducible factor, prolyl hydroxylase, metabolism, MYC, pulmonary artery smooth muscle cell, lung fibroblast

# Introduction

Cellular responses to ambient oxygen levels drive numerous physiologic and pathologic processes from wound healing and angiogenesis to pulmonary vascular remodeling and fibrosis[1](#ref-RN1980). These responses require a sustained capacity for cell proliferation, migration, and protein synthesis, with their associated energetic and metabolic demands, even in the face of limited oxygen availability, or hypoxia. Metazoan cells depend on aerobic respiration to meet cellular energy needs. With an inadequate oxygen supply, cells must reduce energy consumption and shift energy production away from oxidative phosphorylation. Cells accomplish this goal through stabilization of the hypoxia-inducible transcription factor 1α (HIF-1α), which activates the transcription of glucose transporters, glycolytic enzymes, lactate dehydrogenase, and pyruvate dehydrogenase kinase, while decreasing the expression of enzymes in the tricarboxylic acid (TCA) cycle and electron transport chain[1](#ref-RN1980),[2](#ref-RN1198). 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[3](#ref-RN999). 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, the HIF-1 transcriptional program should increase glycolytic capacity and divert glucose-derived pyruvate from oxidative phosphorylation toward lactate fermentation to maintain ATP production and to minimize the formation of reactive oxygen species (ROS)[4](#ref-RN2525).

While a “glycolytic shift” of primary carbon metabolism in hypoxia is well-described, the effects of hypoxia on other metabolic pathways are an area of active investigation[2](#ref-RN1198),[5](#ref-RN953),[6](#ref-RN1603). Since hypoxia is a prominent feature of cancer biology as tumor growth outstrips blood supply, most detailed metabolic studies of cell metabolism in hypoxia have used tumor cell models, yielding important insights into the metabolic pathobiology of cancer[7](#ref-RN2395)–[12](#ref-RN975). 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[7](#ref-RN2395),[8](#ref-RN1447),[13](#ref-RN628),[14](#ref-RN1966), and metabolomic studies identified aspartate as a limiting metabolite for cancer cell proliferation under hypoxia[10](#ref-RN634). By contrast, comparatively little is known about metabolic adaptations of primary cells to hypoxia and how hypoxic metabolic reprogramming supports homeostasis or promotes pathobiology. Indeed, the importance of reductive carboxylation or aspartate biosynthesis remains to be elucidated in these cells. This and related information 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 were selected as they 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-1 transcriptional program. In normoxia, HIF-1α stabilization by the PHD inhibitor molidustat (BAY-85-3934, “BAY”)[15](#ref-RN580) 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-1-dependent up-regulation of glycolytic gene expression in human primary cells. Transcriptomic profiling suggested a critical role for the transcription factor c-MYC (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 in these primary cells.

# Results

The goal of this study was to identify the metabolic changes associated with hypoxia in proliferating primary LFs and PASMCs. 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. 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[2](#ref-RN1198),[16](#ref-wenger2015). From this starting point, we identified the optimal cell seeding density and time course to capture exponential cell growth (**Figure 1A**), thought to be an indicator of metabolic steady state. LFs cultured in 0.5% oxygen grew slower than LFs cultured in 21% oxygen (**Figure 1B**), but slower growth was not associated with decreased cell viability (**Figure S1A**). These 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) (**Figures 1K, E-H**). These changes persisted for the duration of the experimental time course.

## Extracellular flux analysis reveals little impact of hypoxia on glycolysis

Having established a model system, we next determined the extracellular fluxes of glucose (GLC), lactate (LAC), pyruvate (PYR), and amino acids (**Figures 1I-J**). Flux calculations incorporated changes in cell number, extracellular metabolite concentrations, and medium evaporation over time[17](#ref-RN1523) (**Figure S1**). Surprisingly, we observed no significant differences in glucose uptake or lactate efflux rates in 0.5% oxygen cultures (**Figure 1I**) despite activation of the HIF-1 transcriptional program as reflected by increased expression of GLUT1 and LDHA. No change in glucose or lactate fluxes were observed when the ambient oxygen level was decreased further to 0.2% (**Figure S2**). Similar results were also observed in PASMCs where lactate efflux was significantly *decreased* by 0.5% ambient oxygen (**Figure S3**).

In addition to glucose and lactate, we also determined the extracellular fluxes of pyruvate and amino acids. Overall, changes were modest, with hypoxia generally decreasing the fluxes of all measured metabolites. Notably, a substantial decrease in glutamine consumption was observed in LFs cultured in 0.5% oxygen. This observation is in contrast to previous studies in cancer cell metabolism demonstrating increased glutamine uptake as a key feature of the metabolic response to hypoxia[7](#ref-RN2395),[8](#ref-RN1447),[13](#ref-RN628). Similar patterns were observed in 0.2% oxygen (**Figure S2**). In PASMCs, glutamine uptake did increase, as did the uptake of branched-chain amino acids and arginine (**Figure S3**), highlighting differential responses of these mesenchymal cells to hypoxia.

Given that hypoxia did not increase glucose and lactate fluxes as expected based on the observed increases in glycolytic gene expression, we next assessed the capacity of HIF-1α to augment glycolysis in LFs. Cells were treated with the prolyl hydroxylase inhibitor BAY to stabilize HIF-1α under 21% oxygen conditions (**Figure 1**). Similar to hypoxia, BAY decreased cell growth rate (**Figures 1A-B**) and activated the HIF-1 transcriptional program (**Figures 1D-H**). Compared to hypoxia, BAY treatment resulted in a similar activation of HIF-1 target gene transcription and protein expression. In normoxia, this transcriptional program was associated with substantially increased glucose uptake and lactate efflux (**Figure 1I**). Comparatively modest effects of BAY on amino acid fluxes were observed as compared to 0.5% oxygen culture conditions (**Figure 1J**) with preservation of glutamine uptake, alanine efflux, and glutamate efflux rates.

## Stable isotope labeling suggests limited metabolic reprogramming in hypoxia

To validate the findings from our extracellular flux measurements, we next treated LFs with stable carbon isotopes of glucose and glutamine to measure the rate and trace the incorporation of 13C into key downstream carbon utilization pathways (**Figures 2, S4-S5**). Consistent with our extracellular flux measurements, we found that the rate of label incorporation from [U-13C6] glucose into pyruvate was similar across 21%, 0.5%, and DMSO-treated conditions, but markedly increased with BAY treatment (**Figures 2A-B**).

Several patterns of isotope labeling emerge across the treatment conditions (**Figures 2C, S4**). For the glycolytic intermediates fructose bisphosphate (FBP), 3-phosphoglycerate (3PG), and pyruvate (PYR), label incorporation from the glucose tracers is decreased with hypoxia and BAY treatments (*i.e.*, the unlabeled, or M0, fraction was greater). This is also true for the tricarboxylic acid cycle (TCA) metabolites citrate (CIT), α-ketoglutarate (AKG), and malate (MAL) labeled with [1,2- 13C2 glucose]. By contrast, these metabolites show increased labeling with [U-13C6] glucose. The citrate labeling pattern indicates increased label incorporation into M3 and M5 isotopes, reflecting carbon entry through pyruvate carboxylase, which catalyzes the carboxylation of pyruvate to oxaloacetate. The [U-13C5] glutamine label was not incorporated into pyruvate or upstream glycolytic intermediates. The labeling of TCA metabolites by glutamine was decreased in hypoxia and BAY treatment with a more pronounced effect of BAY treatment. Compared to previous studies of metabolic flux in cancer cells[7](#ref-RN2395),[8](#ref-RN1447), no marked increase in M5-labeled citrate from [U-13C5] glutamine was observed with hypoxia treatment, suggesting no change in reductive carboxylation for lipid synthesis in LFs. The overall fraction of M5-citrate in these cells was low (< 7%).

The stable isotope labeling patterns in PASMCs were similar to LFs (**Figure S5**) where labeling was attenuated in hypoxia. The most notable differences between LF and PASMC labeling were observed in citrate. Compared with LFs, PASMCs demonstrated a marked reduction of M2-citrate labeled by glucose and no change in the labeling of M3-citrate by [U-13C6] glucose. These data suggest decreased glucose entry into the TCA cycle *via* pyruvate dehydrogenase and no change in the activity of pyruvate carboxylase, respectively. Interestingly, unlike LFs, PASMCs demonstrated increased production of M5-citrate from [U-13C5] glutamine in hypoxia (7.91% ± 0.89% *v*. 18.24% ± 2.67%, p = 0.04) consistent with increased reductive carboxylation in support of lipid synthesis in these cells, similar to prior observations in cancer cells, but distinct from LFs studied here.

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

The mass isotopomer distribution for a given metabolite is determined based on a complicated 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 (**Figure S6**). Thus, we performed isotopically non-stationary metabolic flux analysis as implemented by Isotopomer Network Compartment Analysis (INCA)[18](#ref-RN1522)–[20](#ref-RN964) (**Figures 3, S7, Tables S1-S3**). Overall, proliferating LFs demonstrated high rates of glucose uptake and glycolysis. Approximately 10% of cytoplasmic pyruvate enters the TCA cycle with the balance converted to lactate (**Figure S7A**). 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. A significant increase in pentose phosphate pathway flux was also observed, although the absolute flux through this pathway is low (**Figure 3A**). By contrast, HIF-1 activation by BAY in 21% oxygen increased glycolysis and lactate fermentation by ~50% (**Figure 3B**), but had a similar effect as hypoxia in decreasing serine and glutamine incorporation. Metabolite fluxes in DMSO-treated cells were similar to 21% oxygen controls.

In normoxia, the magnitude of intracellular metabolite fluxes was generally similar in LFs and PASMCs (**Figures S7A, S7B, Tables S1, S3**). LFs had greater consumption of serine while PASMCs had greater efflux of alanine. In hypoxia, PASMCs exhibited similar decreases in glycolytic flux as LFs but also a marked, and unexpected, increase in TCA flux (**Figure S7C**). The increased TCA flux in PASMCs was driven by increased glutamine consumption in these cells. This finding is similar to a prior report of glutamine-driven oxidative phosphorylation in hypoxic cancer cells[21](#ref-RN528), although glycolysis was also up-regulated in the hypoxia-exposed cancer cells studied in this report.

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 S7D**). 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-1 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 1B**), 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, 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 these cells to hypoxia.

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. Since the net lactate transport flux is secretion, this observation suggests increased lactate uptake with hypoxia or BAY treatment. This finding may be consistent with the HIF-driven increased expression of the reversible lactate transporter MCT4[22](#ref-RN366). 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 (**Figures 4, S5**). Notably, 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[23](#ref-RN2600) 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 in areas of high glucose utilization. Subsequently, investigators have demonstrated the importance of lactate as a metabolic fuel *in vivo*[24](#ref-RN2756),[25](#ref-RN2718). 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 (**Figure 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 glycogen synthesis[26](#ref-RN1678)–[28](#ref-RN1668). Together, these data suggest that lactate also makes a small (~5% carbon) contribution to glycogen precursors.

## 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 (**Figures 5A-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. Interestingly, when combined with 0.5% oxygen, BAY treatment was unable to enhance lactate efflux. These data suggest that hypoxia antagonizes the effects of HIF-1 activation on glycolytic flux in these primary 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 (**Figures 5D-F**). Both 0.5% oxygen and BAY treatment induced marked changes in intracellular metabolite levels (**Figure S8**). Of 133 metabolites, 98 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 (**Figure S8C**). Metabolite set enrichment analysis of KEGG biochemical pathways was consistent with the results of the metabolic models demonstrating significant enrichment of the pentose phosphate pathway with 0.5% oxygen or BAY treatment; decreased alanine, aspartate, and glutamate metabolism with hypoxia; and increased glycolysis/gluconeogenesis with BAY (**Figures S8D, S8E**). Indeed, 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[9](#ref-RN1438),[10](#ref-RN634).

Principal component analysis revealed greater class similarity among both treatment groups cultured in 0.5% oxygen than among the BAY-treatment groups (**Figure 5D**). Moreover, these hypoxia-treated cells were well-segregated from BAY-treated cells. These observations are, again, consistent with the results of the metabolic flux models demonstrating an overriding effect of hypoxia *per se* on metabolic flux and highlighting important differences between hypoxic and pharmacologic PHD inhibition. To identify the key differences among these treatments, we performed linear modeling focusing on those metabolites with a significant interaction term in the model described by treatment × oxygen (**Figure 5E**) (*i.e.*, metabolites where the change with hypoxia treatment was different from the change with BAY treatment). Of 133 metabolites, 77 were significantly differentially regulated by hypoxia and BAY treatments. Several patterns emerged from this analysis (**Figure 5F**). Interestingly, the hypoxia-regulated metabolite, 2-hydroxyglutarate[6](#ref-RN1603), increased following BAY treatment, but was unaffected by hypoxia treatment in proliferating LFs. The tricarboxylic acid (TCA) cycle metabolite aconitate was decreased in hypoxia, but increased by BAY treatment, while the opposite effect was observed for hydroxyproline and taurine. The glycolytic intermediate glyceraldehyde 3-phosphate (GAP) was decreased by both hypoxia and BAY, while hypoxia reversed the BAY-mediated decrease in γ-aminobutyric acid (GABA). A metabolite set enrichment analysis of these differentially regulated metabolites revealed the TCA cycle to be the most enriched KEGG metabolite set (**Figure 5G**). Leading edge analysis demonstrated negative enrichment scores associated with all of the TCA metabolites detected by our platform (**Figure 5H**). This result indicates a more modest impact of BAY treatment on the TCA cycle than hypoxia, 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 in normoxia (**Figure 3**).

In addition to these differential effects on polar metabolite levels, perhaps the most significant contrast between hypoxia and BAY treatment is the impact of hypoxia on cellular redox state. As oxygen deprivation causes reductive stress[29](#ref-RN2409), we next measured the impact of these treatments on intracellular NAD(H) as a surrogate for cellular redox state (**Figures 5I-K**). 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[30](#ref-RN2210), 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 transcriptomic analysis of LFs treated with hypoxia or BAY, separately or together (**Figure 6, S9**). As anticipated, both hypoxia and BAY treatment induced substantial changes in gene expression (**Figure S9A, S9B**). Of the 7,789 differentially expressed genes across both conditions, 891 (11%) were unique to BAY treatment in normoxia, 1,649 (21%) were shared between BAY and hypoxia, while 5,249 (67%) were unique to 0.5% hypoxia culture (**Figure S9C**). Gene set enrichment analysis of these differentially regulated metabolites was performed using Molecular Signatures Database “Hallmark” gene sets[31](#ref-RN3112) (**Figures 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 demonstrates clear separation among the four treatment groups (**Figure 6A**). 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 uncouples the effects of BAY treatment. To identify those transcripts that were differentially affected by hypoxia compared to BAY treatment alone, we performed linear modeling to identify transcripts with a significant interaction term in the model described by treatment × oxygen (**Figure 6B**). With this analysis, we found several patterns of transcriptional changes (**Figure 6C**). For example, EPAS1, the gene encoding the HIF-2α protein, was markedly decreased by hypoxia, compared to its modest reduction following BAY treatment. A similar, but opposite, effect on RBM3 (RNA-binding protein 3) expression was observed. RBM3 supports the viability and proliferation of neural stem cells in hypoxia[32](#ref-RN3111). Histone deacetylase 9 (HDAC9) and prolyl 4-hydroxylase subunit α2 (P4HA2) demonstrated opposing effects following hypoxia and BAY treatment, where BAY decreased HDAC9 expression and increased P4HA2 expression. Together, this subset of transcriptional changes illustrates important differences between hypoxia and HIF stabilization in normoxia.

Interestingly, an enrichment analysis of transcripts differentially regulated by 0.5% oxygen and BAY treatment actually demonstrated greater enrichment of the “hypoxia” gene set following BAY treatment alone than with hypoxia itself (**Figure 6D**). Similarly, the gene set for glycolysis was also more enriched following BAY treatment than hypoxia treatment. The most enriched gene sets associated with hypoxia included pro-proliferative “E2F targets” and “G2/M checkpoint” proteins as well as “MYC targets”. These findings were further supported by a transcription factor enrichment analysis identifying enrichment of MYC target proteins associated with hypoxia (**Figure 6E**).

## MYC antagonizes HIF-dependent glycolytic fluxes

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 7A-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 7C-E**). As expected, MYC-deficient cells proliferated more slowly in normoxia and MYC was absolutely essential for sustaining cell proliferation in hypoxia (**Figure 7D**). Consistent with our hypothesis, MYC knockdown cells demonstrated increased lactate efflux upon hypoxia treatment, unlike control siRNA-treated cells (**Figure 7E**). We next performed the complementary experiment to determine whether MYC overexpression could attenuate the increase in glycolysis observed with BAY treatment (**Figure 7F-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

In this work, we used 13C metabolic flux analysis to identify hypoxia-mediated metabolic changes in proliferating human primary cells. Our principal finding was that hypoxia reduced, rather than increased, carbon flux through glycolysis and lactate fermentation pathways despite robust activation of the HIF transcriptional program and up-regulation of glycolytic genes. Certainly, the LFs studied here are capable of augmenting glycolysis in response to HIF stabilization, as demonstrated by experiments with the PHD inhibitor BAY; however, these effects are completely attenuated when BAY-treated cells are cultured in hypoxia. Together, these findings suggest that changes in enzyme levels alone are insufficient to alter metabolic flux in hypoxia and point to the importance of regulatory mechanisms that supersede the effects of HIF-dependent gene transcription in primary cells.

Our data indicate that hypoxia-induced MYC expression is one such regulatory mechanism. MYC is a transcription factor that regulates the expression of numerous genes involved in many biological processes, including metabolism, proliferation, apoptosis, and differentiation[33](#ref-dang2006)–[35](#ref-stine2015). As deregulated MYC activity has been associated with the majority of human cancers[36](#ref-vita2006), much of our understanding of MYC regulation comes from studies using cancer cell models[34](#ref-li2020d),[35](#ref-stine2015),[37](#ref-dang2012a),[38](#ref-madden2021), while the role of MYC in the biology of untransformed cells is less well understood. The literature describes a complex and reciprocal relationship between HIF and MYC that depends on both environmental (*e.g.*, hypoxia) and cellular context[34](#ref-li2020d). Generally, HIF-1 has been observed to inhibit MYC through multiple mechanisms [koshiji2004; koshiji2005;[39](#ref-gordan2007);[40](#ref-zhang2007)], and this previous work is consistent with our observations that HIF stabilization following BAY treatment decreased MYC protein and target gene expression. Conversely, MYC has been implicated in increased HIF activity through transcriptional and post-transcriptional mechanisms[41](#ref-zhang2009a)–[43](#ref-doe2012), primarily in the context of malignant transformation. The observation that MYC may antagonize the transcriptional effects of HIF to sustain primarily cell proliferation and metabolism in hypoxia suggests a substantially different regulatory relationship than has been previously described.

Understanding how MYC transcriptional activity affects hypoxic primary cell metabolism is imperative to our understanding of cellular adaptation to hypoxia. MYC stimulates the expression of nuclear-encoded mitochondrial genes and promotes mitochondrial biogenesis, both directly and through activation of mitochondrial transcriptional factor A (TFAM)[44](#ref-li2005a). Indeed, we found that the oxidative phosphorylation gene set was relatively enriched with hypoxia treatment compared to BAY treatment (**Figure 6D**). In this way, hypoxic MYC activation may sustain energy production by oxidative phosphorylation, thereby decreasing the energetic demands driving increased glycolytic flux. Beyond oxidative phosphorylation, MYC targets genes involved in many other intermediary metabolic pathways, including amino acids, nucleotides, and lipids[35](#ref-stine2015), that may also impact the central pathways of carbon metabolism studied in this work.

Beyond MYC, the identification of other HIF-independent mechanisms regulating primary cell adaption to hypoxia is of critical importance. Cells express several oxygen-dependent enzymes in addition to PHD whose activities may be altered in hypoxia but not by PHD inhibition. For example, PHD is one of many α-ketoglutarate-dependent dioxygenase enzymes that rely on molecular oxygen for their catalytic activity[45](#ref-RN2611). Jumonji-C (JmjC) domain-containing histone demethylases are other prominent members of this family whose inhibition by hypoxia has been shown to cause rapid and HIF-independent induction of histone methylation[46](#ref-RN3117). Indeed, our transcription factor enrichment analysis (**Figure 6E**) demonstrates that this family of lysine demethylases (KDMs) was the most enriched with 0.5% oxygen treatment as compared to BAY treatment. Similarly, a recently described cysteamine dioxygenase has been shown to mediate the oxygen-dependent degradation of Regulators of G protein Signaling 4 and 5 and IL-32[47](#ref-RN1384). In addition to dioxygenase enzymes, electron transport chain dysfunction resulting from impaired Complex IV activity leads to increased ROS production in hypoxia[48](#ref-RN295). Mitochondrial ROS increase the half-lives of several mRNAs in hypoxia, including MYC, as we observed in this work, independent of HIF stabilization[49](#ref-RN742). Finally, hypoxia imposes a reductive stress on cells associated with an increase in the NADH/NAD+ ratio secondary to impaired electron transport (**Figure S5K**)[50](#ref-RN2618),[51](#ref-RN2619). NADH accumulation may slow glycolysis *via* feedback inhibition of GAPDH[30](#ref-RN2210). Any or all of these molecular mechanisms may also contribute to uncoupling glycolytic enzyme expression from glycolytic flux as observed in the experiments described here.

In addition to its effects on cellular metabolism, another canonical role of HIF-1 activation is slowing of cellular proliferation rate in the face of limited oxygen availability[52](#ref-RN615), which is precisely what we observed here (**Figures 1B, 5A**). The effects of HIF-1 on cell proliferation rate are mediated, in part, by increased expression of cyclin-dependent kinase inhibitor p21 (*CDKN1A*), inhibition of E2F targets[53](#ref-RN3152), and inhibition of pro-proliferative MYC signaling[54](#ref-RN3151). These transcriptional effects are precisely what is observed in BAY treated LFs in normoxia. By contrast, hypoxia culture was associated with decreased expression of p21, consistent with a previous report[55](#ref-RN3150), as well as increased expression of MYC protein and enrichment of MYC target genes. Indeed, the most marked differences between hypoxia and BAY treatment on LF gene transcription were the up-regulation of pro-proliferative gene sets containing E2F targets and G2/M checkpoint proteins. Much of this transcriptional response may be mediated by hypoxia-induced up-regulation of MYC, which is known to stimulate cell cycle progression through its effects on the expression and activity of cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors[56](#ref-RN3154). Clarifying the complex interactions among HIFs, MYC, and cell proliferation will be important for understanding the cellular response of these mesenchymal cells to tissue injury.

Taken together, these findings raise important questions regarding the cell-autonomous role of HIFs in the hypoxia response. On an organismal level, HIFs drive expression of angiogenic and erythropoietic factors to increase oxygen delivery to hypoxic tissues. Within individual cells, HIF-1α seems to be important for mitigating the adverse effects of ROS formation by dysfunctional electron transport in the mitochondria. Indeed, hypoxia increased oxygen consumption and ROS production in HIF-1α-null mouse embryonic fibroblasts (MEFs), which was associated with increased cell death[4](#ref-RN2525). Interestingly, these cells also had increased ATP levels compared to wild type, suggesting that mitochondrial function was adequate under 1% oxygen culture conditions to support oxidative phosphorylation and meet the energy needs of the cells. Given the prominence of HIFs in mediating the transcriptional response to hypoxia, it is somewhat surprising that neither PHD, HIFs, nor their downstream targets were found to be selectively essential as a function of oxygen tension in a genome-wide CRISPR growth screen of K562 human lymphoblasts cultured in normoxia or hypoxia[5](#ref-RN953). Similarly, knockout of HIF signaling did not affect growth, internal metabolite concentrations, glucose consumption, or lactate production under hypoxia by human acute myeloid leukemia cells[57](#ref-wierenga2019). Together with our results, these studies highlight the need for additional research linking hypoxia-induced metabolic changes to their transcriptional and post-transcriptional regulatory mechanisms, particularly in primary cells.

This work also highlights two specific metabolic features that appear to be important in the metabolic response of these primary cells to hypoxia. First, both LFs and PASMCs demonstrated notable incorporation of lactate-derived carbon into intracellular metabolic pathways that increased with hypoxia and BAY treatments (**Figures 4, S5**). This finding is consistent with increasing evidence suggesting an important role for lactate as a metabolic fuel in several organ systems[23](#ref-RN2600),[58](#ref-RN1157). Although typically considered a metabolic waste product[59](#ref-RN3081), an important contribution of lactate *import* in supporting metabolic homeostasis in the face of an ischemic insult, which is associated with increased extracellular lactate, is an evolutionarily attractive hypothesis that merits further investigation. Second, PASMCs, but not LFs, demonstrated significant rates of reductive carboxylation that increased in 0.5% oxygen (**Figure S5**). Reductive carboxylation was first identified in hypoxic tumor cells where stable isotope tracing revealed 13C incorporation from labeled glutamine into lipids[7](#ref-RN2395),[8](#ref-RN1447),[13](#ref-RN628),[14](#ref-RN1966). Hypoxia drives PASMC proliferation *in vivo* contributing to the development pulmonary hypertension in humans and animal models. Isocitrate dehydrogenase has previously been implicated in the pathobiology of this disease[60](#ref-RN1259), and our findings suggest that reductive carboxylation catalyzed by isocitrate dehydrogenase may be a metabolic vulnerability of hypoxic PASMCs associated with pulmonary vascular disease.

Our finding that hypoxia was associated with decreased glycolysis and lactate fermentation was unexpected. Several aspects of our experimental design may have contributed to this finding. First, our goal was to understand how metabolic reprogramming may support cell proliferation in hypoxia. Thus, we measured metabolite fluxes in cells during the exponential growth phase accounting for cell growth rate, metabolite degradation rates, and medium evaporation with multiple measurements over a 72 h time course. Often, cells are studied near confluence, where metabolic contributions to biomass production are less and the rate of glycolysis in hypoxia may be higher. Second, we began our experimental treatments 24 h prior to collecting samples to ensure that the hypoxia metabolic program was established prior to labeling. Similar studies[8](#ref-RN1447),[61](#ref-RN713) typically placed cells into hypoxia at the time of labeling. Third, and perhaps most importantly, these flux determinations were performed in human primary cell cultures rather than immortalized cell lines. Although both cell types used in this study were derived from lung, we anticipate that many of our findings will be generalizable to primary cells from different tissues.

In summary, in this metabolic flux analysis of proliferating human primary cells *in vitro*, we have demonstrated that MYC uncouples an increase in HIF-dependent glycolytic gene transcription from glycolytic flux in hypoxia. Indeed, the degree of metabolic reprogramming in hypoxia was modest and suggests close coupling between proliferation and metabolism. In light of our findings, additional studies are warranted to clarify the role of HIFs in mediating the metabolic response to hypoxia, to determine how MYC activity is regulated by hypoxia, and to identify other key regulators of hypoxic metabolic reprogramming in primary cells. Moreover, these data strongly caution investigators against drawing conclusions about metabolite flux from measures of gene transcription alone.

# Acknowledgements

This work was supported by grants from the NIH (K08HL128802), American Lung Association, Pulmonary Hypertension Association, and the American Thoracic Society Foundation to W.M.O and from the NIH (U01HG007690, U54HL119145, R01HL155107, R01HL155096) and the American Heart Association (D700382, CV-19) to J.L.

# Author Contributions

W.M.O. conceived and designed the analysis. C.A.C., B.A.O., D.R.Z., S.M., K.L., and W.M.O. collected the data. J.D.Y. and W.M.O. contributed data or analysis tools. W.M.O. performed the analysis. W.M.O. drafted the manuscript. All authors participated in interpreting the results and revising the manuscript. All authors approve the final submission.

# Declaration of Interests

The authors declare no competing interests.

# Figure Legends

Figure 1: **Effects of prolyl hydroxylase inhibition on extracellular metabolite fluxes in lung fibroblasts.** Lung fibroblasts (LFs) were cultured with 21% oxygen (*red*), 0.5% oxygen (*blue*), DMSO (0.1% v/v, *green*), or molidustat (BAY, 10 μM, *purple*) beginning 24 h prior to time 0. (**A**) Growth curves of LFs in each experimental condition (n = 8). (**B**) Growth rates from (A) were determined by robust linear modeling of log-transformed growth curves. (**C**, **D**) Representative immunoblots of LF protein lysates cultured as in (A). (**E**, **F**) Relative change in HIF-1α (E) and LDHA (F) protein levels normalized to 21% oxygen or DMSO treatment time 0 (n = 4). (**G**, **H**) Relative changes in GLUT1 (G) and LDHA (H) mRNA levels normalized to 21% oxygen or DMSO treatment time 0 (n = 4). (**I**, **J**) Extracellular fluxes of the indicated metabolites (n = 8). Data are mean ± SEM. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. Tukey’s *post hoc* test was applied to determine differences between 21% and 0.5% oxygen (\*), between DMSO and BAY treatment (†), or between 0.5% oxygen and BAY treatment (‡) with adjusted p-values < 0.05 considered significant.

Figure 2: **Stable isotope tracing of lung fibroblasts.** (**A**) Fraction of pyruvate labeling following treatment of lung fibroblasts (LFs) with [U-13C6] glucose. (**B**) Rate values determined from asymptotic regression fit of the data from (A). (**C**) Isotopic labeling of key intracellular metabolites after 72 h of treatment with the indicated tracers. Experimentally determined mass isotope distributions were corrected for natural isotope abundance. Data are the mean ± SEM of 4 biological replicates. Significant differences in labeling patterns between 21% and 0.5% oxygen (\*), DMSO and BAY treatment (†), and 0.5% oxygen and BAY treatment (‡) for each combination of metabolite and tracer are highlighted. FBP, fructose bisphosphate; 3PG, 3-phosphoglycerate; AKG, α-ketoglutarate.

Figure 3: **Metabolic flux maps of lung fibroblasts.** (**A**) Ratio of metabolic fluxes in 0.5% oxygen compared to 21% oxygen. (**B**) Ratio of metabolic fluxes in cells treated with molidustat (BAY) compared to DMSO vehicle control. Fluxes with non-overlapping confidence intervals are highlighted with colored arrows to indicate significant changes. Arrow thickness corresponds to absolute flux in hypoxia- or BAY-treated cells.

Figure 4: **PHD inhibition increases lactate uptake and oxidation.** Mass isotopomer distributions of key metabolites following labeling with [U-13C3] lactate (2 mM) for 72 h indicates increased lactate uptake and oxidation in hypoxia or with molidustat (BAY) treatment. Data are mean ± SEM of n = 4 biological replicates. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. Tukey’s *post hoc* test was applied to determine differences between 21% and 0.5% oxygen (\*), between DMSO and BAY treatment (†), or between 0.5% oxygen and BAY treatment (‡) with adjusted p-values < 0.05 considered significant.

Figure 5: **Metabolomic analysis of molidustat treatment in normoxia and hypoxia.** (**A**-**C**) Hypoxia inhibits the effects of HIF-1α stabilization on glycolysis. Lung fibroblasts (LFs) were cultured in standard growth medium and treated with molidustat (BAY, 10 μM) or vehicle (DMSO, 0.1%) in 21% or 0.5% oxygen conditions (n = 4). (A) Growth rates were determined by linear fitting of log-transformed growth curves. (**B**-**C**) Extracellular fluxes of glucose (note reversed *y*-axis) (B) and lactate (C). (**D**) Principal components analysis of intracellular metabolites following 72 h of treatment described above suggests a dominant effect of hypoxia over PHD inhibition on the metabolome (n = 4). (**E**) Volcano plot of the differential effects of hypoxia and molidustat (BAY) treatment on intracellular metabolites. *Blue* circles indicate the fold change with hypoxia was greater than the fold change with BAY treatment while *purple* circles indicate the opposite. (**F**) Representative metabolites highlighted in (E) reveal different patterns of metabolic effects. (**G**) Metabolite set enrichment analysis of metabolites from (E). KEGG pathways with p-values < 0.05 are shown. (**H**) Leading edge analysis of the most enriched KEGG metabolite set from (G). Negative values indicate relative enrichment associated with BAY treatment compared to hypoxia treatment (as in (E)). Abbreviations: PYR, pyruvate; SUC, succinate; PEP, phosphoenolpyruvate; CIT, citrate; AKG, α-ketoglutarate; MAL, malate; ACO, aconitate; FUM, fumarate. (**I**-**K**) Intracellular NAD+, NADH, and their ratio were determined by enzymatic assay (n = 5). Black \* indicate a significant effect of hypoxia, colored \* indicate a significant effect of treatment within a given oxygen tension as indicated by the color. Comparisons for fluxes (A-C) and NAD(H) measurements (I-K) were performed using a mixed-effects linear model with replicate as a random effect. Adjusted p-values for the indicated comparisons were determined using Tukey’s *post hoc* test.

Figure 6: **Transcriptomic analysis of molidustat treamtent in normoxia and hypoxia.** (**A**) Principal components analysis of transcriptional changes following 72 h of treatment with 0.5% oxygen or molidustat (BAY), separately or together (n = 4). (**B**) Volcano plot of the differential effects of hypoxia and molidustat (BAY) treatment on intracellular metabolites. (**C**) Representative transcripts from (B). (**D**) Gene set enrichment analysis of transcripts from (B). (**E**) Transcription factor enrichment analysis suggests mechanisms for differential regulation of gene expression following hypoxia or BAY treatment.

Figure 7: **MYC regulates HIF-dependent glycolytic flux.** (**A**) Representative immunoblot of MYC protein expression in lung fibroblsts following 72 h of treatment with 0.5% oxygen or molidustat (BAY, B). (**B**) Quantification of immunoblots shown in (A). (**C**) Representative immunoblot of lung fibroblasts (LFs) treated with siRNA targeting MYC (M) demonstrating adequate protein knockdown. (**D**) Growth rates of MYC-knockdown cells cultured in hypoxia. (**E**) Lactate efflux rates of MYC-knockdown cells cultured in hypoxia. (**F**) Representative immunoblot of LFs treated with MYC adenovirus. (**G**) Growth rates of MYC overexpressing cells cultured with BAY. (**H**) Lactate efflux rates of MYC overexpressing cells cultured with BAY. Data are mean Comparisons were performed using a mixed-effects linear model with replicate as a random effect. Adjusted p-values for the indicated comparisons were determined using Tukey’s *post hoc* test. Black \* indicates a significant effect of treatment, colored \* indicate a significant effect of oxygen within a given treatment as indicated by the color. Data are mean ± SEM of n = 4-5 biological replicates. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. The multivariate *t* distribution was used to determine significant differences as indicated by black \* for difference within groups defined by the x-axis and colored \* for differences between groups defined by the x-axis as indicated by the color with adjusted p-values < 0.05 considered significant.

# Figures

![Figure 1: Effects of prolyl hydroxylase inhibition on extracellular metabolite fluxes in lung fibroblasts. Lung fibroblasts (LFs) were cultured with 21% oxygen (red), 0.5% oxygen (blue), DMSO (0.1% v/v, green), or molidustat (BAY, 10 μM, purple) beginning 24 h prior to time 0. (A) Growth curves of LFs in each experimental condition (n = 8). (B) Growth rates from (A) were determined by robust linear modeling of log-transformed growth curves. (C, D) Representative immunoblots of LF protein lysates cultured as in (A). (E, F) Relative change in HIF-1α (E) and LDHA (F) protein levels normalized to 21% oxygen or DMSO treatment time 0 (n = 4). (G, H) Relative changes in GLUT1 (G) and LDHA (H) mRNA levels normalized to 21% oxygen or DMSO treatment time 0 (n = 4). (I, J) Extracellular fluxes of the indicated metabolites (n = 8). Data are mean ± SEM. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. Tukey’s post hoc test was applied to determine differences between 21% and 0.5% oxygen (*), between DMSO and BAY treatment (†), or between 0.5% oxygen and BAY treatment (‡) with adjusted p-values < 0.05 considered significant.](data:application/pdf;base64,)

Figure 1: **Effects of prolyl hydroxylase inhibition on extracellular metabolite fluxes in lung fibroblasts.** Lung fibroblasts (LFs) were cultured with 21% oxygen (*red*), 0.5% oxygen (*blue*), DMSO (0.1% v/v, *green*), or molidustat (BAY, 10 μM, *purple*) beginning 24 h prior to time 0. (**A**) Growth curves of LFs in each experimental condition (n = 8). (**B**) Growth rates from (A) were determined by robust linear modeling of log-transformed growth curves. (**C**, **D**) Representative immunoblots of LF protein lysates cultured as in (A). (**E**, **F**) Relative change in HIF-1α (E) and LDHA (F) protein levels normalized to 21% oxygen or DMSO treatment time 0 (n = 4). (**G**, **H**) Relative changes in GLUT1 (G) and LDHA (H) mRNA levels normalized to 21% oxygen or DMSO treatment time 0 (n = 4). (**I**, **J**) Extracellular fluxes of the indicated metabolites (n = 8). Data are mean ± SEM. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. Tukey’s *post hoc* test was applied to determine differences between 21% and 0.5% oxygen (\*), between DMSO and BAY treatment (†), or between 0.5% oxygen and BAY treatment (‡) with adjusted p-values < 0.05 considered significant.

![Figure 2: Stable isotope tracing of lung fibroblasts. (A) Fraction of pyruvate labeling following treatment of lung fibroblasts (LFs) with [U-13C6] glucose. (B) Rate values determined from asymptotic regression fit of the data from (A). (C) Isotopic labeling of key intracellular metabolites after 72 h of treatment with the indicated tracers. Experimentally determined mass isotope distributions were corrected for natural isotope abundance. Data are the mean ± SEM of 4 biological replicates. Significant differences in labeling patterns between 21% and 0.5% oxygen (*), DMSO and BAY treatment (†), and 0.5% oxygen and BAY treatment (‡) for each combination of metabolite and tracer are highlighted. FBP, fructose bisphosphate; 3PG, 3-phosphoglycerate; AKG, α-ketoglutarate.](data:application/pdf;base64,)

Figure 2: **Stable isotope tracing of lung fibroblasts.** (**A**) Fraction of pyruvate labeling following treatment of lung fibroblasts (LFs) with [U-13C6] glucose. (**B**) Rate values determined from asymptotic regression fit of the data from (A). (**C**) Isotopic labeling of key intracellular metabolites after 72 h of treatment with the indicated tracers. Experimentally determined mass isotope distributions were corrected for natural isotope abundance. Data are the mean ± SEM of 4 biological replicates. Significant differences in labeling patterns between 21% and 0.5% oxygen (\*), DMSO and BAY treatment (†), and 0.5% oxygen and BAY treatment (‡) for each combination of metabolite and tracer are highlighted. FBP, fructose bisphosphate; 3PG, 3-phosphoglycerate; AKG, α-ketoglutarate.

![Figure 3: Metabolic flux maps of lung fibroblasts. (A) Ratio of metabolic fluxes in 0.5% oxygen compared to 21% oxygen. (B) Ratio of metabolic fluxes in cells treated with molidustat (BAY) compared to DMSO vehicle control. Fluxes with non-overlapping confidence intervals are highlighted with colored arrows to indicate significant changes. Arrow thickness corresponds to absolute flux in hypoxia- or BAY-treated cells.](data:application/pdf;base64,)

Figure 3: **Metabolic flux maps of lung fibroblasts.** (**A**) Ratio of metabolic fluxes in 0.5% oxygen compared to 21% oxygen. (**B**) Ratio of metabolic fluxes in cells treated with molidustat (BAY) compared to DMSO vehicle control. Fluxes with non-overlapping confidence intervals are highlighted with colored arrows to indicate significant changes. Arrow thickness corresponds to absolute flux in hypoxia- or BAY-treated cells.

![Figure 4: PHD inhibition increases lactate uptake and oxidation. Mass isotopomer distributions of key metabolites following labeling with [U-13C3] lactate (2 mM) for 72 h indicates increased lactate uptake and oxidation in hypoxia or with molidustat (BAY) treatment. Data are mean ± SEM of n = 4 biological replicates. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. Tukey’s post hoc test was applied to determine differences between 21% and 0.5% oxygen (*), between DMSO and BAY treatment (†), or between 0.5% oxygen and BAY treatment (‡) with adjusted p-values < 0.05 considered significant.](data:application/pdf;base64,)

Figure 4: **PHD inhibition increases lactate uptake and oxidation.** Mass isotopomer distributions of key metabolites following labeling with [U-13C3] lactate (2 mM) for 72 h indicates increased lactate uptake and oxidation in hypoxia or with molidustat (BAY) treatment. Data are mean ± SEM of n = 4 biological replicates. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. Tukey’s *post hoc* test was applied to determine differences between 21% and 0.5% oxygen (\*), between DMSO and BAY treatment (†), or between 0.5% oxygen and BAY treatment (‡) with adjusted p-values < 0.05 considered significant.

![Figure 5: Metabolomic analysis of molidustat treatment in normoxia and hypoxia. (A-C) Hypoxia inhibits the effects of HIF-1α stabilization on glycolysis. Lung fibroblasts (LFs) were cultured in standard growth medium and treated with molidustat (BAY, 10 μM) or vehicle (DMSO, 0.1%) in 21% or 0.5% oxygen conditions (n = 4). (A) Growth rates were determined by linear fitting of log-transformed growth curves. (B-C) Extracellular fluxes of glucose (note reversed y-axis) (B) and lactate (C). (D) Principal components analysis of intracellular metabolites following 72 h of treatment described above suggests a dominant effect of hypoxia over PHD inhibition on the metabolome (n = 4). (E) Volcano plot of the differential effects of hypoxia and molidustat (BAY) treatment on intracellular metabolites. Blue circles indicate the fold change with hypoxia was greater than the fold change with BAY treatment while purple circles indicate the opposite. (F) Representative metabolites highlighted in (E) reveal different patterns of metabolic effects. (G) Metabolite set enrichment analysis of metabolites from (E). KEGG pathways with p-values < 0.05 are shown. (H) Leading edge analysis of the most enriched KEGG metabolite set from (G). Negative values indicate relative enrichment associated with BAY treatment compared to hypoxia treatment (as in (E)). Abbreviations: PYR, pyruvate; SUC, succinate; PEP, phosphoenolpyruvate; CIT, citrate; AKG, α-ketoglutarate; MAL, malate; ACO, aconitate; FUM, fumarate. (I-K) Intracellular NAD+, NADH, and their ratio were determined by enzymatic assay (n = 5). Black * indicate a significant effect of hypoxia, colored * indicate a significant effect of treatment within a given oxygen tension as indicated by the color. Comparisons for fluxes (A-C) and NAD(H) measurements (I-K) were performed using a mixed-effects linear model with replicate as a random effect. Adjusted p-values for the indicated comparisons were determined using Tukey’s post hoc test.](data:application/pdf;base64,)

Figure 5: **Metabolomic analysis of molidustat treatment in normoxia and hypoxia.** (**A**-**C**) Hypoxia inhibits the effects of HIF-1α stabilization on glycolysis. Lung fibroblasts (LFs) were cultured in standard growth medium and treated with molidustat (BAY, 10 μM) or vehicle (DMSO, 0.1%) in 21% or 0.5% oxygen conditions (n = 4). (A) Growth rates were determined by linear fitting of log-transformed growth curves. (**B**-**C**) Extracellular fluxes of glucose (note reversed *y*-axis) (B) and lactate (C). (**D**) Principal components analysis of intracellular metabolites following 72 h of treatment described above suggests a dominant effect of hypoxia over PHD inhibition on the metabolome (n = 4). (**E**) Volcano plot of the differential effects of hypoxia and molidustat (BAY) treatment on intracellular metabolites. *Blue* circles indicate the fold change with hypoxia was greater than the fold change with BAY treatment while *purple* circles indicate the opposite. (**F**) Representative metabolites highlighted in (E) reveal different patterns of metabolic effects. (**G**) Metabolite set enrichment analysis of metabolites from (E). KEGG pathways with p-values < 0.05 are shown. (**H**) Leading edge analysis of the most enriched KEGG metabolite set from (G). Negative values indicate relative enrichment associated with BAY treatment compared to hypoxia treatment (as in (E)). Abbreviations: PYR, pyruvate; SUC, succinate; PEP, phosphoenolpyruvate; CIT, citrate; AKG, α-ketoglutarate; MAL, malate; ACO, aconitate; FUM, fumarate. (**I**-**K**) Intracellular NAD+, NADH, and their ratio were determined by enzymatic assay (n = 5). Black \* indicate a significant effect of hypoxia, colored \* indicate a significant effect of treatment within a given oxygen tension as indicated by the color. Comparisons for fluxes (A-C) and NAD(H) measurements (I-K) were performed using a mixed-effects linear model with replicate as a random effect. Adjusted p-values for the indicated comparisons were determined using Tukey’s *post hoc* test.

![Figure 6: Transcriptomic analysis of molidustat treamtent in normoxia and hypoxia. (A) Principal components analysis of transcriptional changes following 72 h of treatment with 0.5% oxygen or molidustat (BAY), separately or together (n = 4). (B) Volcano plot of the differential effects of hypoxia and molidustat (BAY) treatment on intracellular metabolites. (C) Representative transcripts from (B). (D) Gene set enrichment analysis of transcripts from (B). (E) Transcription factor enrichment analysis suggests mechanisms for differential regulation of gene expression following hypoxia or BAY treatment.](data:application/pdf;base64,)

Figure 6: **Transcriptomic analysis of molidustat treamtent in normoxia and hypoxia.** (**A**) Principal components analysis of transcriptional changes following 72 h of treatment with 0.5% oxygen or molidustat (BAY), separately or together (n = 4). (**B**) Volcano plot of the differential effects of hypoxia and molidustat (BAY) treatment on intracellular metabolites. (**C**) Representative transcripts from (B). (**D**) Gene set enrichment analysis of transcripts from (B). (**E**) Transcription factor enrichment analysis suggests mechanisms for differential regulation of gene expression following hypoxia or BAY treatment.

![Figure 7: MYC regulates HIF-dependent glycolytic flux. (A) Representative immunoblot of MYC protein expression in lung fibroblsts following 72 h of treatment with 0.5% oxygen or molidustat (BAY, B). (B) Quantification of immunoblots shown in (A). (C) Representative immunoblot of lung fibroblasts (LFs) treated with siRNA targeting MYC (M) demonstrating adequate protein knockdown. (D) Growth rates of MYC-knockdown cells cultured in hypoxia. (E) Lactate efflux rates of MYC-knockdown cells cultured in hypoxia. (F) Representative immunoblot of LFs treated with MYC adenovirus. (G) Growth rates of MYC overexpressing cells cultured with BAY. (H) Lactate efflux rates of MYC overexpressing cells cultured with BAY. Data are mean Comparisons were performed using a mixed-effects linear model with replicate as a random effect. Adjusted p-values for the indicated comparisons were determined using Tukey’s post hoc test. Black * indicates a significant effect of treatment, colored * indicate a significant effect of oxygen within a given treatment as indicated by the color. Data are mean ± SEM of n = 4-5 biological replicates. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. The multivariate t distribution was used to determine significant differences as indicated by black * for difference within groups defined by the x-axis and colored * for differences between groups defined by the x-axis as indicated by the color with adjusted p-values < 0.05 considered significant.](data:application/pdf;base64,)

Figure 7: **MYC regulates HIF-dependent glycolytic flux.** (**A**) Representative immunoblot of MYC protein expression in lung fibroblsts following 72 h of treatment with 0.5% oxygen or molidustat (BAY, B). (**B**) Quantification of immunoblots shown in (A). (**C**) Representative immunoblot of lung fibroblasts (LFs) treated with siRNA targeting MYC (M) demonstrating adequate protein knockdown. (**D**) Growth rates of MYC-knockdown cells cultured in hypoxia. (**E**) Lactate efflux rates of MYC-knockdown cells cultured in hypoxia. (**F**) Representative immunoblot of LFs treated with MYC adenovirus. (**G**) Growth rates of MYC overexpressing cells cultured with BAY. (**H**) Lactate efflux rates of MYC overexpressing cells cultured with BAY. Data are mean Comparisons were performed using a mixed-effects linear model with replicate as a random effect. Adjusted p-values for the indicated comparisons were determined using Tukey’s *post hoc* test. Black \* indicates a significant effect of treatment, colored \* indicate a significant effect of oxygen within a given treatment as indicated by the color. Data are mean ± SEM of n = 4-5 biological replicates. Comparisons were made using linear mixed effects models with treatment group as a fixed effect and biological replicate as a random effect. The multivariate *t* distribution was used to determine significant differences as indicated by black \* for difference within groups defined by the x-axis and colored \* for differences between groups defined by the x-axis as indicated by the color with adjusted p-values < 0.05 considered significant.

# Methods

## Resource Availability

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, William Oldham ([woldham@bwh.harvard.edu](mailto:woldham@bwh.harvard.edu)).

### Materials Availability

This study did not generate new unique reagents.

### Data and code availability

The original data and analysis code are available as a reproducible research compendium formatted as an R package available at OldhamLab/Copeland.2021.hypoxia.flux (<https://github.com/oldhamlab/Copeland.2021.hypoxia.flux>). RNA-seq data has been deposited in the NIH Short Read Archive (PRJNA721596).

## Experimental Model Details

### Lung fibroblasts

Primary normal human lung fibroblasts (LFs) were purchased from Lonza (CC-2512) and cultured in FGM-2 (Lonza CC-3132) in a standard tissue culture incubator in 5% CO2 at 37 °C. Cells from two donors were used in these studies: #33652 (56 y.o., male) and #29132 (19 y.o., female). Cell authentication was performed by the vendor.

### Pulmonary artery smooth muscle cells

Primary human pulmonary artery smooth muscle cells were purchased from Lonza (CC-2581) and cultured in SmGM-2 (Lonza CC-3182) in a standard tissue culture incubator in 5% CO2 at 37 °C. Cells from multiple donors were used in these studies: #30020 (64 y.o., male), #27662 (35 y.o., male), #26698 (51 y.o., male), and #19828 (51 y.o., male). Cell authentication was performed by the vendor.

## Method Details

### Metabolic flux protocol

For extracellular flux measurements, cells were seeded in either standard growth medium or MCDB131 medium without glucose, glutamine, or phenol red (genDEPOT) supplemented with 2% dialyzed fetal bovine serum (Mediatech) and naturally labeled glucose and glutamine (“light” labeling medium). For LFs, glucose was supplemented at 8 mM and glutamine was supplemented at 1 mM. For PASMCs, glucose was supplemented at 5.55 mM and glutamine was supplemented at 10 mM. These concentrations match the concentrations of these substrates determined in standard growth medium. Preliminary experiments were performed to identify the optimal cell seeding density, exponential growth phase, and labeling duration consistent with metabolic and isotopic steady state. On Day -1, 25,000 cells were seeded in a 35 mm dish in “light” labeling medium. Hypoxia-treated cells were transferred to a tissue culture glovebox set to 0.5% oxygen and 5% CO2 (Coy Lab Products). Medium was supplemented with DMSO 0.1% or BAY (10 μM) for DMSO and BAY treatment conditions. On Day 0, cells were washed with PBS and the medium was changed to either “light” medium for flux measurements or “heavy” medium for tracer experiments. For LFs, samples were collected on Day 0 and every 24 h for 72 h. For PASMCs, samples were collected on Day 0 and every 12 h for 48 h. Medium and cell lysates were collected at each time point for intra- and extracellular metabolite measurements and total DNA quantification. Dishes without cells were weighed daily to correct for evaporative medium losses and to empirically determine degradation and accumulation rates of metabolites. Medium samples and cell lysates for DNA measurement were stored at -80 °C until analysis. Each individual experiment included triplicate wells for each treatment and time point, and each experiment was repeated 4-8 times.

### Cell count

Direct cell counts of trypsizined cell suspensions in PBS were obtained following staining with propidium iodide and acridine orange using a LUNA-FL fluorescence cell counter (Logos Biosystems). Indirect cell counts for flux measurements were interpolated from total DNA quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo). Cells were washed once with two volumes of PBS, lysed with Tris-EDTA buffer containing 2% Triton X-100, and collected by scraping. Total DNA in 10 μL of lysate was determined by adding 100 μL of 1X PicoGreen dye in Tris-EDTA buffer and interpolating the fluorescence intensity with a standard curve generated using the λ DNA standard. Cell counts were interpolated from a standard curve of DNA obtained from known cell numbers seeded in basal medium (**Figure S1A**). No difference in total cellular DNA was identified between normoxia and hypoxia cultures (**Figure S1B**).

### Immunoblots

Cells were washed with one volume of PBS and collected by scraping in PBS. Cell suspensions were centrifuged at 5,000 ×*g* for 5 min at 4 °C. Pellets were lysed in buffer containing Tris 10 mM, pH 7.4, NaCl 150 mM, EDTA 1 mM, EGTA 1 mM, Triton X-100 1% v/v, NP-40 0.5% v/v, and Halt Protease Inhibitor Cocktail (Thermo). Protein concentrations were determined by BCA Protein Assay (Thermo). Lysates were normalized for protein concentration and subjected to SDS-PAGE separation on stain-free tris-glycine gels (Bio-Rad), cross-linked and imaged with the Chemidoc system (Bio-Rad), transferred to PVDF membranes with the Trans-Blot Turbo transfer system (Bio-Rad), imaged, blocked in 5% blocking buffer (Bio-Rad), blotted in primary and secondary antibodies, and developed using WesternBright ECL (Advansta). Band signal intensity was normalized to total protein per lane as determined from the stain-free gel or membrane images.

### RT-qPCR

Total RNA was isolated from cells with the RNeasy Mini Kit (Qiagen). cDNA was synthesized from 0.25-1.00 ng RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-qPCR analysis was performed with an Applied Biosystems 7500 Fast Real Time PCR System with TaqMan Universal PCR Master Mix and pre-designed TaqMan gene expression assays (Life Technologies). Relative expression levels were calculated using the comparative cycle threshold method referenced to *ACTB*.

### Glucose assay

Medium samples were diluted 10-fold in PBS. Glucose concentration was determined using the Glucose Colorimetric Assay Kit (Cayman) according to the manufacturer’s protocol. Standards were prepared in PBS.

### Lactate assay

Medium samples were diluted 10-fold in PBS. Glucose concentration was determined using the ʟ-Lactate Assay Kit (Cayman). Medium samples did not require deproteinization, otherwise the samples were analyzed according to the manufacturer’s protocol. Standards were prepared in PBS.

### Pyruvate assay

Pyruvate was measured using either an enzymatic assay (most samples) or an HPLC-based assay (medium from 0.2% oxygen experiments). For the enzymatic assay, medium samples were diluted 20-fold in PBS. Pyruvate concentration was determined using the Pyruvate Assay Kit (Cayman). Medium samples did not require deproteinization, otherwise the samples were analyzed according to the manufacturer’s protocol. Standards were prepared in PBS. For the HPLC assay, 2-oxovaleric acid was added to medium samples as an internal standard. Samples were subsequently deproteinized with 2 volumes of ice-cold acetone. Supernatants were evaporated to < 50% of the starting volume at 43 °C in a SpeedVac concentrator (Thermo Savant) and reconstituted to the starting volume with HPLC-grade water prior to derivatization. Samples were derivatized 1:1 by volume with *o*-phenylenediamine (25 mM in 2 M HCl) for 30 min at 80 °C. Derivatized pyruvate was separated with a Poroshell HPH C-18 column (2.1 × 100 mm, 2.7 μm) on an Infinity II high-performance liquid chromatography system with fluorescence detection of OPD-derivatized α-keto acids as described previously[62](#ref-RN730).

### Amino acid assay

Medium amino acid concentrations were determined following the addition of norvaline and sarcosine internal standards and deproteinization with 2 volumes of ice-cold acetone. Supernatants were evaporated to < 50% of the starting volume at 43 °C in a SpeedVac concentrator (Thermo Savant) and reconstituted to the starting volume with HPLC-grade water prior to analysis. Amino acids in deproteinized medium were derivatized with *o*-phthalaldehyde (OPA) and 9-fluorenylmethylchloroformate (FMOC) immediately prior to separation with a Poroshell HPH-C18 column (4.6 × 100 mm, 2.7 μm) on an Infinity II high-performance liquid chromatography system with ultraviolet and fluorescence detection of OPA- and FMOC-derivatized amino acids, respectively, according to the manufacturer’s protocol (Agilent)[63](#ref-RN1294).

### MYC knockdown

Approximately 1.25 M lung fibroblasts were reverse transfected in 6-cm dishes with 40 pmol siMYC or non-targeting siCTL pools (Dharmacon) in 20 uL RNAiMAX (Thermo). After 24 h, cells were collected by trypsinization and re-seeded as described in *Metabolic flux protocol* above for growth rate and lactate efflux measurements.

### MYC overexpression

Lung fibroblasts were seeded at 25,000 cells per 35 mm dish on Day -2. On Day -1, cells were transduced with adenovirus for MYC (Vector Biolabs) or YFP overexpression[6](#ref-RN1603). After 24 h, the medium was changed and samples were collected as described in *Metabolic flux protocol* above.

### Flux calculations

The growth rate () and flux (*v*) for each measured metabolite were defined as follows[17](#ref-RN1523):

where *X* is the cell density, *k* is the first-order degradation or accumulation rate, and *M* is the mass of the metabolite. These equations are solved as follows:

Growth rate () and cell count at time 0 () were determined by robust linear modeling of the logarithm of cell count as a function of time (). Metabolite mass was calculated from the measured metabolite concentrations and predicted well volume accounting for evaporative losses (**Figure S1C**). First-order degradation and accumulation rates were obtained from robust linear modeling of metabolite mass *v*. time in unconditioned culture medium. Rates that significantly differed from 0 using Student’s *t*-test were incorporated into the flux calculations. Final fluxes were obtained by robust linear modeling of *versus* to determine the slope from which was calculated using equation (4).

### Metabolomics

#### Metabolite extraction

Intracellular metabolites were obtained after washing cells with 2 volumes of ice-cold PBS and floating on liquid nitrogen. Plates were stored at -80 °C until extraction. Metabolites were extracted with 1 mL 80% MeOH pre-cooled to -80 °C containing 10 nmol [D8]-valine as an internal standard (Cambridge Isotope Labs). Insoluble material was removed by centrifugation at 21,000 ×*g* for 15 min at 4 °C. The supernatant was evaporated to dryness at 42 °C using a SpeedVac concentrator (Thermo Savant). Samples were resuspended in 35 μL LC-MS-grade water prior to analysis.

#### Acquisition parameters

LC-MS analysis was performed on a Vanquish ultra-high-performance liquid chromatography system coupled to a Q Exactive orbitrap mass spectrometer by a HESI-II electrospray ionization probe (Thermo). External mass calibration was performed weekly. Metabolite samples (2.5 μL) were separated using a ZIC-pHILIC stationary phase (2.1 × 150 mm, 5 μm) (Merck). The autosampler temperature was 4 °C and the column compartment was maintained at 25 °C. Mobile phase A was 20 mM ammonium carbonate and 0.1% ammonium hydroxide. Mobile phase B was acetonitrile. The flow rate was 0.1 mL/min. Solvent was introduced to the mass spectrometer *via* electrospray ionization with the following source parameters: sheath gas 40, auxiliary gas 15, sweep gas 1, spray voltage +3.0 kV for positive mode and -3.1 kV for negative mode, capillary temperature 275 °C, S-lens RF level 40, and probe temperature 350 °C. Data were acquired and peaks integrated using TraceFinder 4.1 (Thermo).

#### Stable isotope quantification

All metabolites except fructose 2,6-bisphosphate (FBP) and 3-phosphoglycerate (3PG) were measured using the following mobile phase gradient: 0 min, 80% B; 5 min, 80% B; 30 min, 20% B; 31 min, 80% B; 42 min, 80% B. The mass spectrometer was operated in selected ion monitoring mode with an m/*z* window width of 9.0 centered 1.003355-times half the number of carbon atoms in the target metabolite. The resolution was set at 70,000 and AGC target was 1 × 105 ions. Peak areas were corrected for quadrupole bias as previously described[64](#ref-RN1063). Mass isotope distributions for FBP and 3PG were calculated from full scan chromatograms as described below. Raw mass isotopomer distributions were corrected for natural isotope abundance using a custom R package (mzrtools, <https://github.com/oldhamlab/mzrtools>) employing the method of Fernandez, *et al.*[65](#ref-RN557).

#### Metabolomic profiling

For metabolomic profiling and quantification of isotopic enrichment for FBP and 3PG, the following mobile phase gradient was used: 0 min, 80% B; 20 min, 20% B; 20.5 min, 80% B; 28 min, 80% B; 42 min, 80% B. The mass spectrometer was operated in polarity switching full scan mode from 70-1000 m/*z*. Resolution was set to 70,000 and the AGC target was 1x106 ions. Peak identifications were based on an in-house library of authentic metabolite standards previously analyzed utilizing this method. For metabolomics studies, pooled quality control (QC) samples were injected at the beginning, end, and between every four samples of the run. Raw peak areas for each metabolite were corrected for instrument drift using a cubic spline model of QC peak areas. Low quality features were removed on the basis of a relative standard deviation greater than 0.2 in the QC samples and a dispersion ratio greater than 0.4[66](#ref-RN3077). Missing values were imputed using random forest. Samples peak areas were normalized using probabilistic quotient normalization[67](#ref-RN3094). Differentially regulated metabolites were identified using limma[68](#ref-limma). Metabolite set enrichment analysis was performed using the fgsea package with metabolite pathways downloaded using multiGSEA[69](#ref-fgsea),[70](#ref-multiGSEA).

### Biomass determination

The dry weight of LFs was determined to be 493 pg/cell. The dry weight of PASMCs was determined to be 396 pg/cell. These values were estimated by washing 3 × 106 cells twice in PBS and thrice in ice-cold acetone prior to drying overnight in a SpeedVac. The composition of the dry cell mass was estimated from the literature[71](#ref-RN1741),[72](#ref-RN2005), and stoichiometric coefficients were determined as described[18](#ref-RN1522),[73](#ref-RN2517).

### Metabolic flux analysis

Metabolic flux analysis was performed using the elementary metabolite unit-based software package INCA[19](#ref-RN2501). Inputs to the model include the chemical reactions and atom transitions of central carbon metabolism, extracellular fluxes, the identity and composition of 13C-labeled tracers, and the MIDs of labeled intracellular metabolites. The metabolic network was adapted from previously published networks[18](#ref-RN1522),[74](#ref-RN2266) and comprises 48 reactions representing glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, anaplerotic pathways, serine metabolism, and biomass synthesis. The network includes seven extracellular substrates (aspartate, cystine, glucose, glutamine, glycine, pyruvate, serine) and five metabolic products (alanine, biomass, glutamate, lactate, lipid). Models were fit using three 13C-labeled tracers, [1,2-13C2] glucose, [U-13C6] glucose, and [U-13C5] glutamine. The MIDs of twelve metabolites (2-oxoglutarate, 3-phosphoglycerate, alanine, aspartate, citrate, fructose bisphosphate, glutamate, glutamine, lactate, malate, pyruvate, serine) were used to constrain intracellular fluxes. The following assumptions were made:

1. Metabolism was at steady state.
2. Labeled CO2 produced during decarboxylation reactions left the system and did not re-incorporate during carboxylation reactions.
3. Protein turnover occurred at a negligible rate compared to glucose and glutamine consumption.
4. Acetyl-CoA, aspartate, fumarate, malate, oxaloacetate, and pyruvate existed in cytosolic and mitochondrial pools. Aspartate and malate were allowed to exchange freely between the compartments.
5. The per cell biomass requirements of proliferating lung fibroblasts were similar to published estimated in other cells[71](#ref-RN1741),[72](#ref-RN2005).
6. Dilution of alanine, aspartate, glutamate, glutamine, lactate, and pyruvate was allowed to occur through reversible exchange with unlabeled substrates in the medium as these metabolites were present in unconditioned medium or serum.
7. Succinate and fumarate are symmetric molecules that have interchangeable orientations when metabolized by TCA cycle enzymes.

Flux estimation was repeated a minimum of 50 times from random initial values. Results were subjected to a χ2 statistical test to assess goodness-of-fit. Accurate 95% confidence intervals were computed for estimated parameters by evaluating the sensitivity of the sum-of-square residuals to parameter variations[18](#ref-RN1522),[75](#ref-RN60).

### NAD(H) assay

Cellular NAD+ and NADH were measured using an enzymatic fluorimetric cycling assay based on the reduction of NAD+ to NADH by alcohol dehydrogenase (ADH) and subsequent electron transfer to generate the fluorescent molecule resorufin[6](#ref-RN1603). Briefly, cells were washed twice with one volume PBS. Pyridine nucleotides were extracted on ice with buffer containing 50% by volume PBS and 50% lysis buffer (100 mM sodium carbonate, 20 mM sodium bicarbonate, 10 mM nicotinamide, 0.05% by volume Triton-X-100, 1% by mass dodecyltrimethylammonium bromide) and collected by scraping. Extracts were divided equally and 0.5 volume of 0.4 N HCl was added to one sample. Both extracts were heated at 65 °C for 15 min to degrade selectively either the oxidized (buffer) or reduced (HCl) nucleotides. The reaction was cooled on ice and quenched by adding 0.5 M Tris-OH to the acid-treated samples or 0.2 N HCl plus 0.25 M Tris-OH to the buffer samples. Samples were then diluted in reaction buffer (50 mM EDTA and 10 mM Tris, pH 7.06). Cell debris was pelleted by centrifugation, and 50 μL was incubated for 2 h with 100 μL reaction buffer containing 0.6 M EtOH, 0.5 mM phenazine methosulfate, 0.05 mM resazurin, and 0.1 mg/mL ADH. Fluorescence intensities were measured with a Spectramax Gemini XPS (Moelcular Devices) with excitation 540 nm, emission 588 nm, and 550 nm excitation cut-off filter. Sample intensities were compared to a standard curve generated from known concentrations of NADH. The ratio of fluorescence in buffer-extracted to acid-extracted samples corresponds to the NADH/NAD+ ratio. Absolute NADH and NAD+ were normalized to cell count from cells estimated from total DNA quantification as described above.

### RNA-seq

RNA was collected from LFs treated for three days ± hypoxia ± BAY as described above. Four biological replicates were analyzed. Library construction and sequencing was performed by BGI Genomics using 100 bp paired end analysis and a read depth of 50M reads per sample. Sequences were deposited in the NIH SRA (PRJNA721596). Sequences were mapped to the human GRCh38 primary assembly and counts summarized using Rsubread[76](#ref-Rsubread). This data is available from the Oldham Lab GitHub repository (<https://github.com/oldhamlab/rnaseq.lf.hypoxia.molidustat>). Differentially expressed transcripts were identified using DESeq2[77](#ref-DESeq2). Gene set enrichment and transcription factor enrichment was performed using the fgsea and TFEA.ChIP R packages, respectively[69](#ref-fgsea),[78](#ref-TFEA.ChIP).

## Quantification and Statistical Analysis

The raw data and annotated analysis code necessary to reproduce this manuscript are contained in an R package research compendium available from the Oldham Lab GitHub repository (<https://github.com/oldhamlab/Copeland.2021.hypoxia.flux>). Data analysis, statistical comparisons, and visualization were performed in R[79](#ref-R-base) using the packages referenced in the data supplement. Experiments included technical and biological replicates as noted above. The number of biological replicates (n) is indicated in the figure legends. Summary data show the mean ± SEM. Outliers were identified using twice the median absolute deviation as a cutoff threshold. Two group comparisons (*e.g.*, 21% *v.* 0.5% oxygen) were performed using Student’s *t*-test on log-transformed values with pairing among biological replicates. Multifactor comparisons (*e.g.*, BAY and oxygen) were performed using linear mixed-effects models with Tukey’s *post hoc* testing. Metabolomics and RNA-seq data were analyzed as described above. Probability values less than 0.05 were considered significant.

## Key Resources Table

| **REAGENT or RESOURCE** | **SOURCE** | **IDENTIFIER** |
| --- | --- | --- |
| **Antibodies** | | |
| HIF-1α | BD Biosciences | 610958 |
| c-MYC | Cell Signaling Technologies | D84C12 |
| LDHA | Cell Signaling Technologies | 2012 |
| HRP-α-Rabbit IgG | Cell Signaling Technologies | 7074 |
| HRP-α-Mouse IgG | Cell Signaling Technologies | 7076 |
| **Bacterial and virus strains** | | |
| [1,2-13C2] glucose | Vector Biolabs | 1285 |
| [U-13C6] glucose | Oldham et al., 2015 |  |
| **[U-13C5] glutamine** | | |
| [U-13C3] lactate | Cambridge Isotope Labs | CLM-504-PK |
| [U-^13^C~6~] glucose | Cambridge Isotope Labs | CLM-1396-PK |
| [U-^13^C~5~] glutamine | Cambridge Isotope Labs | CLM-1822-H-PK |
| [U-^13^C~3~] lactate | Sigma | 485926 |
| Molidustat (BAY-85-3934) | Cayman | 15297 |
| **Critical commercial assays** | | |
| Glucose colorimetric assay kit | Cayman | 10009582 |
| ʟ-Lactate assay kit | Cayman | 700510 |
| Pyruvate assay kit | Cayman | 700470 |
| **Depositied data** | | |
| Raw and analyzed data | This paper | https://github.com/oldhamlab/Copeland.2021.hypoxia.flux |
| RNA-seq reads | This paper | SRA: PRJNA721596 |
| Summarized RNA-seq data | This paper | https://github.com/oldhamlab/rnaseq.lf.hypoxia.molidustat |
| **Experimental models: Cell lines** | | |
| Normal human lung fibroblasts | Lonza | CC-2512 |
| Pulmonary artery smooth muscle cells | Lonza | CC-2581 |
| **Oligonucleotides** | | |
| ACTB (Hs03023943\_g1) | Life Technologies | 4351370 |
| GLUT1 (Hs00892681\_m1) | Life Technologies | 4351370 |
| LDHA (Hs00855332\_g1) | Life Technologies | 4351370 |
| MYC ON-TARGETplus siRNA | Dharmacon | L-003282-02-0005 |
| ON-TARGETplus non-targeting control pool | Dharmacon | D-001810-10-05 |

# Supplemental Items Titles

Table S1: **Lung fibroblast fluxes in 21% and 0.5% oxygen.**

Table S2: **Lung fibroblast fluxes following DMSO and BAY treatment.**

Table S3: **PASMC fluxes in 21% and 0.5% oxygen.**

Figure S1: **Supporting data for extracellular flux calculations.**

Figure S2: **Extracellular flux measurements in 0.2% oxygen.**

Figure S3: **Extracellular flux measurements in pulmonary artery smooth muscle cells in 0.5% oxygen.**

Figure S4: **Mass isotopomer distributions after 72 h of labeling in lung fibroblasts.**

Figure S5: **Mass isotopomer distributions after 72 h of labeling in pulmonary artery smooth muscle cells.**

Figure S6: **Isotope incorporation in key metabolites over the experimental time course.**

Figure S7: **Isotopically non-stationary metabolic flux analysis.**

Figure S8: **Metabolomic profiling of hypoxia and BAY treated lung fibroblasts.**

Figure S9: **Transcriptomic profiling of hypoxia and BAY treated lung fibroblasts.**

# References

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

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

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

4. Zhang, H. *et al.* [Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia](https://doi.org/10.1074/jbc.M800102200). *J Biol Chem* **283**, 10892–903 (2008).

5. Jain, I. H. *et al.* [Genetic screen for cell fitness in high or low oxygen highlights mitochondrial and lipid metabolism](https://doi.org/10.1016/j.cell.2020.03.029). *Cell* **181**, 716–727 e11 (2020).

6. Oldham, W. M., Clish, C. B., Yang, Y. & Loscalzo, J. [Hypoxia-mediated increases in l-2-hydroxyglutarate coordinate the metabolic response to reductive stress](https://doi.org/10.1016/j.cmet.2015.06.021). *Cell Metab* **22**, 291–303 (2015).

7. Wise, D. R. *et al.* [Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability](https://doi.org/10.1073/pnas.1117773108). *Proc Natl Acad Sci U S A* **108**, 19611–6 (2011).

8. Metallo, C. M. *et al.* [Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia](https://doi.org/10.1038/nature10602). *Nature* **481**, 380–4 (2011).

9. Melendez-Rodriguez, F. *et al.* [HIF1alpha suppresses tumor cell proliferation through inhibition of aspartate biosynthesis](https://doi.org/10.1016/j.celrep.2019.01.106). *Cell Rep* **26**, 2257–2265 e4 (2019).

10. Garcia-Bermudez, J. *et al.* [Aspartate is a limiting metabolite for cancer cell proliferation under hypoxia and in tumours](https://doi.org/10.1038/s41556-018-0118-z). *Nat Cell Biol* **20**, 775–781 (2018).

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

12. Jiang, L. *et al.* [Reductive carboxylation supports redox homeostasis during anchorage-independent growth](https://doi.org/10.1038/nature17393). *Nature* **532**, 255–8 (2016).

13. Gameiro, P. A. *et al.* [In vivo HIF-mediated reductive carboxylation is regulated by citrate levels and sensitizes VHL-deficient cells to glutamine deprivation](https://doi.org/10.1016/j.cmet.2013.02.002). *Cell Metab* **17**, 372–85 (2013).

14. Scott, D. A. *et al.* [Comparative metabolic flux profiling of melanoma cell lines: Beyond the warburg effect](https://doi.org/10.1074/jbc.M111.282046). *J Biol Chem* **286**, 42626–34 (2011).

15. Flamme, I. *et al.* [Mimicking hypoxia to treat anemia: HIF-stabilizer BAY 85-3934 (molidustat) stimulates erythropoietin production without hypertensive effects](https://doi.org/10.1371/journal.pone.0111838). *PLoS One* **9**, e111838 (2014).

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

17. Murphy, T. A. & Young, J. D. [ETA: Robust software for determination of cell specific rates from extracellular time courses](https://doi.org/10.1002/bit.24836). *Biotechnol Bioeng* **110**, 1748–58 (2013).

18. Murphy, T. A., Dang, C. V. & Young, J. D. [Isotopically nonstationary 13C flux analysis of myc-induced metabolic reprogramming in b-cells](https://doi.org/10.1016/j.ymben.2012.07.008). *Metab Eng* **15**, 206–17 (2013).

19. Young, J. D. [INCA: A computational platform for isotopically non-stationary metabolic flux analysis](https://doi.org/10.1093/bioinformatics/btu015). *Bioinformatics* **30**, 1333–5 (2014).

20. Jazmin, L. J. & Young, J. D. [Isotopically nonstationary 13C metabolic flux analysis](https://doi.org/10.1007/978-1-62703-299-5_18). *Methods Mol Biol* **985**, 367–90 (2013).

21. Fan, J. *et al.* [Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia](https://doi.org/10.1038/msb.2013.65). *Mol Syst Biol* **9**, 712 (2013).

22. Contreras-Baeza, Y. *et al.* [Monocarboxylate transporter 4 (MCT4) is a high affinity transporter capable of exporting lactate in high-lactate microenvironments](https://doi.org/10.1074/jbc.RA119.009093). *J Biol Chem* **294**, 20135–20147 (2019).

23. Faubert, B. *et al.* [AMPK is a negative regulator of the warburg effect and suppresses tumor growth in vivo](https://doi.org/10.1016/j.cmet.2012.12.001). *Cell Metab* **17**, 113–24 (2013).

24. Hui, S. *et al.* [Glucose feeds the TCA cycle via circulating lactate](https://doi.org/10.1038/nature24057). *Nature* **551**, 115–118 (2017).

25. Hui, S. *et al.* Quantitative fluxomics of circulating metabolites. *Cell Metab* (2020) doi:[10.1016/j.cmet.2020.07.013](https://doi.org/10.1016/j.cmet.2020.07.013).

26. Pescador, N. *et al.* [Hypoxia promotes glycogen accumulation through hypoxia inducible factor (HIF)-mediated induction of glycogen synthase 1](https://doi.org/10.1371/journal.pone.0009644). *PLoS One* **5**, e9644 (2010).

27. Favaro, E. *et al.* [Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells](https://doi.org/10.1016/j.cmet.2012.10.017). *Cell Metab* **16**, 751–64 (2012).

28. Pelletier, J. *et al.* [Glycogen synthesis is induced in hypoxia by the hypoxia-inducible factor and promotes cancer cell survival](https://doi.org/10.3389/fonc.2012.00018). *Front Oncol* **2**, 18 (2012).

29. Xiao, W. & Loscalzo, J. [Metabolic responses to reductive stress](https://doi.org/10.1089/ars.2019.7803). *Antioxid Redox Signal* **32**, 1330–1347 (2020).

30. Tilton, W. M., Seaman, C., Carriero, D. & Piomelli, S. [Regulation of glycolysis in the erythrocyte: Role of the lactate/pyruvate and NAD/NADH ratios](https://www.ncbi.nlm.nih.gov/pubmed/1856577). *J Lab Clin Med* **118**, 146–52 (1991).

31. Liberzon, A. *et al.* [The molecular signatures database (MSigDB) hallmark gene set collection](https://doi.org/10.1016/j.cels.2015.12.004). *Cell Syst* **1**, 417–425 (2015).

32. Yan, J. *et al.* [The RNA-binding protein RBM3 promotes neural stem cell (NSC) proliferation under hypoxia](https://doi.org/10.3389/fcell.2019.00288). *Front Cell Dev Biol* **7**, 288 (2019).

33. Dang, C. V. *et al.* [The c-Myc target gene network](https://doi.org/10.1016/j.semcancer.2006.07.014). *Semin Cancer Biol* **16**, 253–264 (2006).

34. Li, Y., Sun, X.-X., Qian, D. Z. & Dai, M.-S. [Molecular Crosstalk Between MYC and HIF in Cancer](https://doi.org/10.3389/fcell.2020.590576). *Front Cell Dev Biol* **8**, 590576 (2020).

35. Stine, Z. E., Walton, Z. E., Altman, B. J., Hsieh, A. L. & Dang, C. V. [MYC, Metabolism, and Cancer](https://doi.org/10.1158/2159-8290.CD-15-0507). *Cancer Discov* **5**, 1024–1039 (2015).

36. Vita, M. & Henriksson, M. [The Myc oncoprotein as a therapeutic target for human cancer](https://doi.org/10.1016/j.semcancer.2006.07.015). *Semin Cancer Biol* **16**, 318–330 (2006).

37. Dang, C. V. [MYC on the path to cancer](https://doi.org/10.1016/j.cell.2012.03.003). *Cell* **149**, 22–35 (2012).

38. Madden, S. K., de Araujo, A. D., Gerhardt, M., Fairlie, D. P. & Mason, J. M. [Taking the Myc out of cancer: Toward therapeutic strategies to directly inhibit c-Myc](https://doi.org/10.1186/s12943-020-01291-6). *Molecular Cancer* **20**, 3 (2021).

39. Gordan, J. D., Bertout, J. A., Hu, C. J., Diehl, J. A. & Simon, M. C. [HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity](https://doi.org/10.1016/j.ccr.2007.02.006). *Cancer Cell* **11**, 335–47 (2007).

40. Zhang, H. *et al.* [HIF-1 Inhibits Mitochondrial Biogenesis and Cellular Respiration in VHL-Deficient Renal Cell Carcinoma by Repression of C-MYC Activity](https://doi.org/10.1016/j.ccr.2007.04.001). *Cancer Cell* **11**, 407–420 (2007).

41. Zhang, J. *et al.* [Targeting angiogenesis via a c-Myc/hypoxia-inducible factor-1alpha-dependent pathway in multiple myeloma](https://doi.org/10.1158/0008-5472.CAN-08-4603). *Cancer Res* **69**, 5082–5090 (2009).

42. Chen, C. *et al.* [C-Myc enhances colon cancer cell-mediated angiogenesis through the regulation of HIF-1](https://doi.org/10.1016/j.bbrc.2012.12.006). *Biochem Biophys Res Commun* **430**, 505–511 (2013).

43. Doe, M. R., Ascano, J. M., Kaur, M. & Cole, M. D. [Myc posttranscriptionally induces HIF1 protein and target gene expression in normal and cancer cells](https://doi.org/10.1158/0008-5472.CAN-11-2371). *Cancer Res* **72**, 949–957 (2012).

44. Li, F. *et al.* [Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis](https://doi.org/10.1128/MCB.25.14.6225-6234.2005). *Mol Cell Biol* **25**, 6225–6234 (2005).

45. Islam, M. S., Leissing, T. M., Chowdhury, R., Hopkinson, R. J. & Schofield, C. J. [2-oxoglutarate-dependent oxygenases](https://doi.org/10.1146/annurev-biochem-061516-044724). *Annu Rev Biochem* **87**, 585–620 (2018).

46. Batie, M. *et al.* [Hypoxia induces rapid changes to histone methylation and reprograms chromatin](https://doi.org/10.1126/science.aau5870). *Science* **363**, 1222–1226 (2019).

47. Masson, N. *et al.* [Conserved n-terminal cysteine dioxygenases transduce responses to hypoxia in animals and plants](https://doi.org/10.1126/science.aaw0112). *Science* **365**, 65–69 (2019).

48. Chandel, N. S. *et al.* [Mitochondrial reactive oxygen species trigger hypoxia-induced transcription](https://doi.org/10.1073/pnas.95.20.11715). *Proc Natl Acad Sci U S A* **95**, 11715–20 (1998).

49. Guzy, R. D. *et al.* [Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing](https://doi.org/10.1016/j.cmet.2005.05.001). *Cell Metab* **1**, 401–8 (2005).

50. Chance, B. & Williams, G. R. [Respiratory enzymes in oxidative phosphorylation. III. The steady state](https://www.ncbi.nlm.nih.gov/pubmed/13271404). *J Biol Chem* **217**, 409–27 (1955).

51. Garofalo, O., Cox, D. W. & Bachelard, H. S. [Brain levels of NADH and NAD+ under hypoxic and hypoglycaemic conditions in vitro](https://doi.org/10.1111/j.1471-4159.1988.tb04851.x). *J Neurochem* **51**, 172–6 (1988).

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

53. Gardner, L. B. *et al.* [Hypoxia inhibits G1/s transition through regulation of p27 expression](https://doi.org/10.1074/jbc.M010189200). *J Biol Chem* **276**, 7919–26 (2001).

54. Koshiji, M. *et al.* [HIF-1alpha induces cell cycle arrest by functionally counteracting myc](https://doi.org/10.1038/sj.emboj.7600196). *EMBO J* **23**, 1949–56 (2004).

55. Mizuno, S. *et al.* [Hypoxia regulates human lung fibroblast proliferation via p53-dependent and -independent pathways](https://doi.org/10.1186/1465-9921-10-17). *Respir Res* **10**, 17 (2009).

56. Hydbring, P., Castell, A. & Larsson, L. G. [MYC modulation around the CDK2/p27/SKP2 axis](https://doi.org/10.3390/genes8070174). *Genes (Basel)* **8**, (2017).

57. Wierenga, A. T. J. *et al.* [HIF1/2-exerted control over glycolytic gene expression is not functionally relevant for glycolysis in human leukemic stem/progenitor cells](https://doi.org/10.1186/s40170-019-0206-y). *Cancer Metab* **7**, 11 (2019).

58. Hui, S. *et al.* [Glucose feeds the TCA cycle via circulating lactate](https://doi.org/10.1038/nature24057). *Nature* **551**, 115–118 (2017).

59. Rabinowitz, J. D. & Enerback, S. [Lactate: The ugly duckling of energy metabolism](https://doi.org/10.1038/s42255-020-0243-4). *Nat Metab* **2**, 566–571 (2020).

60. Fessel, J. P. *et al.* [Metabolomic analysis of bone morphogenetic protein receptor type 2 mutations in human pulmonary endothelium reveals widespread metabolic reprogramming](https://doi.org/10.4103/2045-8932.97606). *Pulm Circ* **2**, 201–13 (2012).

61. Grassian, A. R. *et al.* [IDH1 mutations alter citric acid cycle metabolism and increase dependence on oxidative mitochondrial metabolism](https://doi.org/10.1158/0008-5472.CAN-14-0772-T). *Cancer Res* **74**, 3317–31 (2014).

62. Guarino, V. A., Oldham, W. M., Loscalzo, J. & Zhang, Y. Y. [Reaction rate of pyruvate and hydrogen peroxide: Assessing antioxidant capacity of pyruvate under biological conditions](https://doi.org/10.1038/s41598-019-55951-9). *Sci Rep* **9**, 19568 (2019).

63. Long, W. Automated amino acid analysis using an agilent poroshell HPH-C18 column. *Application Note, Agilent Technologies, Inc.* **Publication Number 5991-5571EN**, 1–10 (2017).

64. Kim, D. *et al.* [SHMT2 drives glioma cell survival in ischaemia but imposes a dependence on glycine clearance](https://doi.org/10.1038/nature14363). *Nature* **520**, 363–7 (2015).

65. Fernandez, C. A., Des Rosiers, C., Previs, S. F., David, F. & Brunengraber, H. [Correction of 13C mass isotopomer distributions for natural stable isotope abundance](https://doi.org/10.1002/(SICI)1096-9888(199603)31:3<255::AID-JMS290>3.0.CO;2-3). *J Mass Spectrom* **31**, 255–62 (1996).

66. Broadhurst, D. *et al.* [Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies](https://doi.org/10.1007/s11306-018-1367-3). *Metabolomics* **14**, 72 (2018).

67. Dieterle, F., Ross, A., Schlotterbeck, G. & Senn, H. [Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics](https://doi.org/10.1021/ac051632c). *Anal Chem* **78**, 4281–90 (2006).

68. Ritchie, M. E. *et al.* [limma powers differential expression analyses for RNA-sequencing and microarray studies](https://doi.org/10.1093/nar/gkv007). *Nucleic Acids Research* **43**, e47 (2015).

69. Korotkevich, G., Sukhov, V. & Sergushichev, A. Fast gene set enrichment analysis. *bioRxiv* (2019) doi:[10.1101/060012](https://doi.org/10.1101/060012).

70. Sebastian, C. & Hackermüller, J. [multiGSEA: A GSEA-based pathway enrichment analysis for multi-omics data](https://doi.org/10.1186/s12859-020-03910-x). *BMC Bioinformatics* **21**, (2020).

71. Quek, L. E., Dietmair, S., Kromer, J. O. & Nielsen, L. K. [Metabolic flux analysis in mammalian cell culture](https://doi.org/10.1016/j.ymben.2009.09.002). *Metab Eng* **12**, 161–71 (2010).

72. Sheikh, K., Forster, J. & Nielsen, L. K. [Modeling hybridoma cell metabolism using a generic genome-scale metabolic model of mus musculus](https://doi.org/10.1021/bp0498138). *Biotechnol Prog* **21**, 112–21 (2005).

73. Zamorano, F., Wouwer, A. V. & Bastin, G. [A detailed metabolic flux analysis of an underdetermined network of CHO cells](https://doi.org/10.1016/j.jbiotec.2010.09.944). *J Biotechnol* **150**, 497–508 (2010).

74. Vacanti, N. M. *et al.* [Regulation of substrate utilization by the mitochondrial pyruvate carrier](https://doi.org/10.1016/j.molcel.2014.09.024). *Mol Cell* **56**, 425–35 (2014).

75. Antoniewicz, M. R., Kelleher, J. K. & Stephanopoulos, G. [Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements](https://doi.org/10.1016/j.ymben.2006.01.004). *Metab Eng* **8**, 324–37 (2006).

76. Liao, Y., Smyth, G. K. & Shi, W. [The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads](https://doi.org/10.1093/nar/gkz114). *Nucleic Acids Research* **47**, e47 (2019).

77. Love, M. I., Huber, W. & Anders, S. [Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2](https://doi.org/10.1186/s13059-014-0550-8). *Genome Biology* **15**, 550 (2014).

78. Puente-Santamaria, L., Wasserman, W. & del Peso, L. TFEA.ChIP: A tool kit for transcription factor binding site enrichment analysis capitalizing on ChIP-seq datasets. *Bioinformatics* (2019) doi:[10.1093/bioinformatics/btz573](https://doi.org/10.1093/bioinformatics/btz573).

79. R Core Team. [*R: A language and environment for statistical computing*](https://www.R-project.org/). (R Foundation for Statistical Computing, 2020).