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Title: Glyceraldehyde 3-phosphate dehydrogenase modulates non-oxidative pentose phosphate pathway to provide anabolic precursors in hypoxic tumor cells

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Abstract:

Cancer cells exhibit enhanced lactate production to satisfy biosynthetic ATP requirements and also supply ribose 5-phosphate (R5P) and NADPH via the pentose phosphate pathway (PPP). Yet, little is known about the mechanism by which glycolytic flux is diverted to PPP to fulfill the increased demand for anabolic precursors and reducing equivalents. Here we show, using a ^{13}C-labeling methodology quantifying glycolysis and the PPP metabolism, that hypoxic cancer cells not only increase net glycolytic flux but also activate the exchange fluxes catalyzed by aldolase and transaldolase. The increased carbon exchange in the upward direction promotes the supplementation of R5P through the non-oxidative PPP and essentially controls the anaplerosis of upper glycolytic metabolites consumed for biosynthesis. This cascade of events is regulated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which plays a critical role in diverting metabolites for the synthesis of nucleotide precursors and thus acts as a limiting enzyme under hypoxia.

Introduction

Most human cancer cells convert glucose to lactate regardless of oxygen availability in a process known as aerobic glycolysis or the "Warburg effect"[1]. The hypoxic microenvironment of cancer cells stabilizes hypoxia-inducible factors (HIFs)[2], which stimulate the overexpression of most glycolytic enzymes[3] for the generation of ATP and facilitate malignant progression[4]. The enhanced glycolysis is part of the reprogramming of cellular metabolism to satisfy the metabolic demands for cancer growth. By overexpressing pyruvate dehydrogenase kinase 1 (PDK1) that inactivates pyruvate dehydrogenase and leads to reduced oxidative phosphorylation, HIFs block the generation of reactive oxygen species (ROS)[5]. Another consequence of inactivated PDH, or attenuated oxidative phosphorylation by inhibition of cytochrome oxidase in electron transport chain, is reduced ATP synthesis, which is compensated by increased glycolytic flux. The enhanced glycolysis under hypoxia allows cancer cells to quickly generate the necessary ATP supply in the oxygen-limited environment. Concomitant with elevated energy production under hypoxia, cancer cells are also required to increase the rate of anabolic processes in response to such oxidative stress in order to fulfill the high demands for biosynthesis. Thus, enhanced anabolic processes are required for glycolytic intermediates such as R5P for nucleotide biosynthesis, NADPH through the PPP for supply of redox equivalents, and serine and glycine, via phosphoglycerate dehydrogenase, for amino acid and one carbon metabolism[6]. Although the molecular mechanism of HIF stabilization and-its physiological response, i.e. enhanced glycolysis, have been elucidated, it is still challenging to configure the landscape of the

molecular mechanism and the metabolic rearrangements by which glycolytic flux is diverted toward the synthesis of the key building block precursors with persistent glycolytic phenotype due to complex biochemical reactions of glycolysis and the PPP.

The bridge between glycolysis and R5P comprises irreversible (oxidative PPP) and reversible (non-oxidative PPP) branches. The oxidative PPP generates two NADPH and one R5P from the oxidation of one glucose-6-phosphate (G6P) by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). The oxidative PPP-derived NADPH is utilized for redox homeostasis and lipid biosynthesis[7]. In addition, the G6PD[8] and 6PGD[9] enzymes have been reported to play key roles in tumor growth. The non-oxidative PPP connects fructose-6-phoshphate (F6P) and glycerlaldehyde-3-phosphate (GAP) to R5P via the transketolase (TKT) and transaldolase (TAL) enzymes. In glycolysis, the reaction of aldolase (ALDO) is a parallel pathway to that of TAL between GAP to F6P. The connecting enzymes between glycolysis and the PPP have been known as key therapeutic targets for cancer[10]. However, the complexity of the metabolic network has prevented elucidation of the pathological rewiring of metabolism even when each reaction is targeted. Therefore, an integral study focusing on the synergy between glycolysis and the PPP can provide a more robust platform to formulate therapeutic strategies. To this end, we introduced isotope-tracing techniques targeting glycolysis and the PPP and used stable isotopic tracers and GC-MS in order to quantify metabolism using ^{13}C-metabolic flux analysis (MFA) methodology[11]. We discovered significant increase of exchange fluxes through ALDO and TAL under hypoxia, which increased the supply

of the nucleotide precursor R5P through the non-oxidative PPP. Most importantly, the activation of increased carbon exchange flux along the non-oxidative PPP was modulated by glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Result and Discussion

Hypoxia enhances glycolysis and lactate secretion

To assess glycolysis of cancer cells in oxygen-limited environment, we measured glucose consumption and lactate production rates in mammary (MDA-MB-231), liver (HepG2), prostate (Du145) and lung (A549) cancer cells under normoxia (21% O_2) and hypoxia (1% O_2). For all cells, glucose consumption increased significantly from 1.2 to 2.0-fold under hypoxia (Figure 1A) and simultaneously lactate secretion increased from 1.3 to 2.3-fold (Figure 1B). It has been known that glycolytic enzyme overexpression under hypoxic conditions enhances glycolysis[12]. Interestingly, the average yield of secreted lactate to consumed glucose for the four cell lines examined under hypoxia (2.01 ± 0.20 mol lactate/mol glucose), was close to the theoretical maximum (2 mol lactate/mol glucose) regardless of oxygen conditions, indicating a tight redox balance for NADH produced by GAPDH and NAD^+ regenerated from lactate dehydrogenase (LDH) in cancer cells[13]. However, glutamine carbon may replenish lactate through phosphoenolpyruvate carboxykinase (PEPCK) and malic enzyme (ME) (Figure 1C).

We assessed glucose and glutamine contributions to lactate production using [U-$^{13}C_6$]glucose and [U-$^{13}C_5$]glutamine. In MDA-MB-231 cells, the glutamine tracer contributed only 2.3% and 1.5% of secreted lactate carbons under normoxia and hypoxia (Figure 1D), respectively. On the other hand, the glucose tracer labeled 97.4% and 98.2%, respectively, of lactate under normoxia and hypoxia (Figure 1E). HepG2 cells similarly showed little incorporation of glutamine carbon into lactate (Figure 1F). Thus, most lactate was derived from glucose under both normoxic and hypoxic conditions. In addition, some ^{13}C-atoms from the glucose tracer were metabolized in the tricarboxylic acid cycle (TCA) at citrate, malate, succinate and α-ketoglutarate and mixed with natural carbons from glutamine or other amino acids (Figures 1E and G). It is known that overexpression of PDK1[5] and PDK3[14] under hypoxia blocks the introduction of glucose to TCA intermediates by PDH inhibition. Interestingly, the ^{13}C-labeling of TCA metabolites, i.e. Cit, Mal, Suc and AKG decreased significantly under hypoxia from normoxia in HepG2 (Figure 1G) but showed little reduction in MDA-MB-231 (Figure 1E). So, in contrast to the similar overall enhancement of glycolysis under hypoxia (Figures 1A and B), the two cancer cells showed different susceptibilities of their TCA metabolism to oxygen limitation. It has been observed that MDA-MB-231 contains pathogenic mutations in mitochondrial DNA, e.g. complex I, that enhances the aggressiveness of breast cancer cells[15].

Activation of anabolism via the non-oxidative pentose phosphate pathway

The observed enhancement of glycolysis under hypoxia likely impacts the oxidative and non-oxidative arms of the PPP. Therefore, to evaluate the metabolic activity of the PPP relative to the glycolytic pathway, we cultured MDA-MB-231 and HepG2 cells under normoxia and hypoxia in the presence of [1,2-$^{13}C_2$]glucose[16] (Figure 1H). We analyzed ^{13}C-labeling of 3-phosphoglycerate (3PG), PEP and ribose extracted from RNA using GC-MS. The M1/M2 isotopomer ratio of 3PG and PEP provides a measure of the metabolic activity of the oxidative PPP relatively to glycolysis[17] (Figure 1H). Intriguingly, the oxidative PPP activities under hypoxia decreased significantly 2-fold and 3-fold in MDA-MB-231 and HepG2, respectively (Figure 1I). Consistent with a report using Imatinib-sensitive murine hematopoietic cells[18], hypoxia also appears to decrease the metabolic flux of the oxidative PPP and increase flux through the non-oxidative PPP.

To estimate the relative contributions of the oxidative and non-oxidative PPP to R5P, we analyzed the ^{13}C-labeling of ribose in RNA. The M1 labeling (one ^{13}C atom) of the ribose fragment (*m/z* 287) derived from RNA reflects relative oxidative PPP flux to R5P due to the loss of the first ^{13}C atom as CO_2, while M2 labeling (two ^{13}C atoms) reflects flux through the non-oxidative PPP (Figure 1H). The M2/M1 ratio is thus correlated to the relative contributions to ribose synthesis of the non-oxidative PPP to the oxidative PPP. We found that the relative non-oxidative PPP contribution to ribose synthesis increased significantly by 1.5-fold in MDA-MB-231 and 1.6-fold in HepG2 under hypoxia (Figure 1J). Therefore, in addition to activating

glycolysis, hypoxic cancer cells reduced the flux through the oxidative PPP and, at the same time, they enhanced the non-oxidative PPP flux via TKT and TAL for nucleotide biosynthesis.

Increase of fluxes through ALDO and TAL into ribose synthesis under hypoxia

To better elucidate the metabolic distribution of glucose-carbon atoms between glycolysis and the non-oxidative PPP, we analyzed the reversibility of ALDO and TAL at the junction points of F6P and GAP. We measured mass ions of dephosphorylated fructose derived from phosphorylated fructose metabolites (denoted as F6(B)P) such as fructose 1,6-bisphosphate (FBP) and F6P (Figures 2A and B). The M1 isotopomer (one ^{13}C atom) of fructose fragment *m/z* 307 (C4-C5-C6) is transferred by ALDO and TAL exchange reactions when cells are grown in [1-^{13}C]glucose (Figure 2B); the M1 isotopomer abundance increased under hypoxia (Figure 2C). On the other hand, the abundance of the M1 isotopomer at the first four carbon atoms of fructose (*m/z* 364, C1-C2-C3-C4) decreased by dilution with ^{12}C atoms of the opposite side of fructose carbon atoms (C4-C5-C6) via the backward reaction of ALDO under hypoxia (Figure 2D). Simultaneously, the exchange flux between DHAP and GAP via TPI needs to increase to assemble the two intermediates. In addition, we demonstrated the biochemical reaction with [4,5,6-$^{13}C_3$]glucose since the M3 labeling of the tracer is conserved in the non-oxidative PPP (Figures 2E and F). In contrast to the [1-^{13}C]glucose experiment, the exchange flux of ALDO and TAL reduced the M3 labeling of fructose (*m/z* 307, C4-C5-C6) (Figure 2G) and enhanced the M3 labeling of fructose (*m/z* 364, C1-C2-C3-C4) (Figure 2H). As such, the M1/M3 ratio of

fructose (*m/z* 307) in the presence of a mixture tracer of [1-^{13}C]glucose+[4,5,6-$^{13}C_3$]glucose should indicate fluxes via ALDO and TAL, and this increased 2.4-fold in MDA-MB-231 and 1.4-fold in HepG2 under hypoxia (Figure S1A), mirroring our observations with [1,2-$^{13}C_2$]glucose (Figure S1B). These data indicated that the exchange flux of ALDO and TAL mediated the anabolic pathway of the non-oxidative PPP via TKT under hypoxia.

To quantify the rates of glycolytic and PPP reactions (Figure S2), we also carried out ^{13}C-metabolic flux analysis (^{13}C-MFA) by parallel labeling experiments (Table S1)[19, 20]. In the resulting flux distribution (Tables S2 and S3), the oxidative PPP fluxes reached 9.6 nmol/10^6 cells/h in MDA-MB-231 and 38.9 nmol/10^6 cells/h in HepG2 under normoxia, but decreased significantly to 6.2 nmol/10^6 cells/h in MDA-MB-231 and 15.5 nmol/10^6 cells/h in HepG2 under hypoxia (Figure 3A). The oxidative PPP is one of the primary pathways used to produce NADPH needed for reductive biosynthesis and redox defense[7]. We analyzed cellular lipid content to examine possible correlation between the oxidative PPP flux and accumulation of lipids (Figure 3B). However, lipid content of MDA-MB-231 or HepG2 showed small difference between normoxia and hypoxia. Furthermore, the fraction of newly synthesized biomass palmitate, as determined by isotopomer spectral analysis[21], decreased by only 7% at MDA-MB-231 and 10% at HepG2 under hypoxia (Figure 3C). In addition, the ratio of GSH to GSSG, an indicator of redox defense, showed little correlation between normoxia and hypoxia (Figure 3D). Thus, it appears that the reduction of the oxidative PPP flux is not related to the supply of

biosynthetic precursors such as NADPH but occurs in response to changes that happen in another pathway, e.g. the non-oxidative PPP.

To evaluate the relative contribution on R5P of the oxidative and the non-oxidative PPP, we calculated, by ^{13}C-MFA, the ratio of the non-oxidative PPP flux to the oxidative PPP flux in supplying R5P. The relative non-oxidative PPP flux increased 1.4-fold in MDA-MB-231 and 2.0-fold in HepG2 under hypoxia (Figure S3), which was well correlated with Figure 1J showing enhancement of the anabolic reaction via TKT. This result is in line with the critical role of transketolase in diverting carbon substrates into nucleotide biosynthesis pathway [22]. Furthermore, the exchange flux of ALDO under hypoxia increased significantly, 5.7-fold in MDA-MB-231 and 4.2-fold in HepG2 (Figure 3E). The other parallel pathway of exchange TAL flux under hypoxia showed an 8.3-fold increase in MDA-MB-231 and 7.3-fold in HepG2 (Figure 3F). ALDO and TAL bridge the junction between glycolysis and non-oxidative PPP via TKT. Thus, the activated exchange flux through ALDO and TAL may elevate the pool sizes of upper glycolytic metabolites, which enhances ribose synthesis through TKT.

GAPDH modulates the non-oxidative PPP fluxes under hypoxia

To obtain a more mechanistic understanding of the described phenomena and also identify what specific genes are involved in the activation of exchange ALDO and TAL flux under hypoxia, we tested MDA-MB-231 cells stably infected with lentivirus containing specific shRNA genes under hypoxia (Figure 4A). In the case of ALDO- and TAL-knockdown cells,

glycolytic flux to phosphorylated fructose was reduced compared to control cells under hypoxia. In contrast, GAPDH-knockdown cells enhanced the exchange flux. This suggests that glycolytic flux to phosphorylated fructose is mediated by the ALDO and TAL reactions while it is negatively affected, under hypoxia, by the enzymes of lower glycolysis, e.g. GAPDH. To test this hypothesis, we measured the expression of lower glycolytic genes to identify possible bottlenecks between DHAP and lactate. Interestingly, HepG2 (Figure 4B) cells expressed most glycolytic enzymes highly except GAPDH under hypoxia. As demonstrated by Western blotting, hypoxia enhanced HIF1α stabilization while GAPDH expression increased moderately (Figure 4C).

We next employed LC-MS to obtain metabolite profiles of glycolysis intermediates and found that the concentrations of GAPDH substrates, DHAP and GAP, increased significantly compared to other upstream and downstream metabolites under hypoxia (Figure 4D). GAPDH flux can be regulated by the redox balance of NAD^+ to NADH ratio since NAD^+ is a substrate for the glycolytic reaction of GAPDH. We, therefore, measured the NAD^+/NADH ratio, which was found to be reduced under hypoxia for the four cancer cell lines (Figure 4E). This observation is in agreement with previous reports that, under oxygen-limited conditions, NADH was accumulated due to incomplete oxidative phosphorylation[15]. Therefore, the altered redox status reduced the enzymatic activity of GAPDH, relatively to the other glycolytic enzymes activated by HIFs, and maintained higher concentrations of upper glycolytic metabolites that boosted the non-oxidative PPP for biosynthesis of DNA and RNA.

These results lead to the conclusion that GAPDH is a key modulator of exchange fluxes via ALDO and TAL. Reduction of the flux through GAPDH will result in the accumulation of upper glycolytic metabolites between hexokinase (HK) and GAPDH under hypoxia. We further demonstrated correlation between the exchange flux of ALDO and TAL and the non-oxidative PPP flux using a GAPDH inhibitor (iodoacetate, IAA), HK inhibitor (2-deoxy-D-glucose, 2DG), and GAPDH shRNA knockdown and hexokinase 2 (HK2) knockout (ko) cells. Treatment of cells with IAA and GAPDH shRNA enhanced the exchange flux significantly (Figure 4F) and simultaneously increased the non-oxidative PPP flux to R5P (Figure 4G). These results indicate that GAPDH, under hypoxia, functions as a bottleneck enzyme to redirect metabolites to the non-oxidative PPP via the exchange fluxes of ALDO and TAL. Interestingly, HK2 inhibition by 2DG and/or HK2 knockout cells (HK2-ko) yielded the opposite result in terms of the exchange flux of ALDO and TAL in the GAPDH inhibition experiments. Here, limiting supply of glycolytic metabolites by HK inhibition or HK2 knockout decreased the accumulated metabolites in upper glycolysis and reduced the exchange flux of ALDO and TAL (Figure 4F), and then the non-oxidative PPP fluxes by removal of the effect of GAPDH limitation (Figure 4G).

Discussion and Conclusion

In this article, several metabolic phenotypes of most cancer cells have been elucidated and found to be consistent with literature results. Most cell lines exhibit enhanced glycolysis and lactate secretion under hypoxia, featuring the well-known Warburg effect. The oxidative PPP activity is reduced at low oxygen availability whereas the non-oxidative PPP branch is enhanced

for R5P synthesis. Enzymatically, ALDO and TAL may control the exchange fluxes into ribose synthesis, diverting anabolic precursors from glycolysis. The above metabolic rewiring is triggered by the key enzyme GAPDH. The relative expression of GAPDH is reduced compared to other glycolytic enzymes under hypoxia. In addition, the attenuated NAD^+ to NADH ratio further decreases the activity of the enzyme. GAPDH thus functions as the limiting enzyme that redistributes metabolic precursors toward nucleotide biosynthesis.

The primary analytical tool of our work was the isotope-tracing technique using labeled metabolites and mass isotopomer analysis, which has been applied to dissect many complex biochemical reaction systems[23, 16, 24]. The novelty of our work is the systematic calculation of integrative fluxes covering most enzymatic reactions in glycolysis and the PPP. To further decipher the complex nature of the network, the absolute values of both the overall net reactions and the reverse pathways were determined. This is accomplished by ^{13}C-metabolic flux analysis (^{13}C-MFA) using multiple stable isotopic tracers and GC-MS[11, 19]. It turns out that the estimation of exchange fluxes plays a critical role in understanding the system. On a relative basis, the net flux of GAP to 3PG is constant but fluxes of ALDO, TAL, TPI and the oxidative PPP exhibit a significant change as elucidated in Table S4. These flux results indicate that most cancer cells in our study are able to maintain high glycolytic fluxes, and concomitantly divert anabolic precursors through ALDO and TAL, resulting in sustained glycolytic phenotype and enhanced supply of precursors toward R5P.

Our work provides new insights in the design of cancer therapeutics targeting the interplay between glycolysis and the PPP[9, 10, 25, 26, 27]. It was reported in literature that direct drug targeting of the non-oxidative PPP could be problematic[28]. Yet, we demonstrated that it is possible to modulate the non-oxidative PPP flux by controlling the activity of the limiting enzyme GAPDH. This offers an effective way of reducing glycolytic flux in cancer cells to a level commensurate to that of healthy cells. Importantly, tuning of GAPDH activity should be executed with caution, as there is a trade-off between energy production and the supply of anabolic building blocks. Inhibition of GAPDH could be beneficial for cancer treatment since the resulting reduction of glycolytic flux is selectively detrimental to human colorectal cancers harboring KRAS and BRAF mutations[29]. Our work also suggests that the reduction of GAPDH activity could also induce hold-up of glycolytic flux and accumulation of upper glycolytic metabolites, diverting fluxes toward nucleotide biosynthesis via non-oxidative PPP. Therefore, the extent of GAPDH modulation by drug targeting needs to be optimized so that the overall desired therapeutic effect can be achieved. We hereby stress the significance of therapeutic designs which balance targets for energy generation and anabolic metabolism simultaneously.

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manuscript and formulated conceptual ideas presented in the draft. ZZ, JRC and DMS constructed and provided HK2 knockout cells. OI provided conceptual advice and supported all procedures as a principal investigator helped design of experiments. GS supported all procedures as a principal investigator.

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Figure 1. Hypoxia increases glycolysis and non-oxidative PPP metabolism.

(A and B) Specific glucose consumption rates (A) and lactate production rates (B) under normoxia or hypoxia. MDA-MB-231, MDA231. Data are presented as mean ± SEM and *p < 0.05 (two-tailed t test) comparing normoxia and hypoxia.

(C) Schematic of carbon flow under hypoxia. Gln, Glutamine; PEP, phosphoenolpyruvate; Pyr, pyruvate; DHAP, dihydroxyacetone phosphate; Mal, malate; Cit, citrate; Suc, succinate; AKG, α-ketoglutarate.

(D and E) ^{13}C-labeling of intracellular metabolites in the presence of 80% [U-$^{13}C_5$]glutamine (D) and 100% [U-$^{13}C_6$]glucose (E) with MDA231.

(F and G) ^{13}C-labeling of intracellular metabolites in the presence of 80% [U-$^{13}C_5$]glutamine (F) and 100% [U-$^{13}C_6$]glucose (G) with HepG2. Data from (D) to (G) are presented as mean ± SD.

(H) Schematic of carbon atom transitions (^{12}C, open circles; ^{13}C, filled; non-oxidative PPP, red; oxidative PPP, blue; glycolysis, grey) in the presence of [1,2-$^{13}C_2$]glucose. F6(B)P, combined metabolites of F6P and FBP.

(I) The oxidative PPP flux versus glycolytic flux using the M1/M2 labeling ratio of 3PG (m/z 585, C1-C2-C3) and PEP (m/z 453, C1-C2-C3) from MDA231 and HepG2 cultures under normoxia and hypoxia.

(J) The non-oxidative PPP flux versus the oxidative PPP flux to R5P using the M2/M1 labeling ratio of ribose (m/z 287, C1-C2-C3-C4-C5) from MDA231 and HepG2 cultures under normoxia and hypoxia. Data of (I) and (J) are presented as mean ± SD and **P < 0.0001 (two-tailed t test) comparing normoxia and hypoxia.

Figure 2. Hypoxia activates exchange flux via ALDO and TAL.

(A and B) Schematic of carbon atom transitions by forward (A) and exchange fluxes (B) of ALDO and TAL via triosephosphate isomerase (TPI) in the presence of [1-^{13}C]glucose (74% composition). TAL-C_3, dihydroxyacetone moiety at TAL reactions.

(C and D) Fractional abundances of mass isotopomers from ion fragments (*m/z* 307, C4-C5-C6) (C) and (*m/z* 364, C1-C2-C3-C4) (D) ion fragments of dephosphorylated fructose from HepG2 in the presence of [1-^{13}C]glucose under normoxia and hypoxia.

(E and F) Schematic of carbon atom transitions by forward (E) and exchange fluxes (F) of ALDO and TAL in the presence of [4,5,6-$^{13}C_3$]glucose.

(G and H) Fractional abundance of fructose mass isotopomers from ion fragments (*m/z* 307) (G) and (*m/z* 364) (H) from dephosphorylated fructose in the culture of HepG2 with [4,5,6-

$^{13}C_3$]glucose (61% composition) under normoxia and hypoxia. Data are presented as mean ± SD and *P < 0.001 (two-tailed t test) comparing normoxia and hypoxia.

Figure 3. Exchange fluxes of ALDO and TAL are regulated by the rate-limiting enzyme GAPDH under hypoxia.

(A) The metabolic flux of the oxidative PPP, G6P → R5P + 2 NADPH + CO_2.

(B) Biomass lipid content of MDA-MB-231 (MDA231) and HepG2 under normoxia and hypoxia.

(C) The fraction of newly synthesized palmitate, g(t) of MDA231 and HepG2 cells under normoxia and hypoxia.

(D) The ratio of reduced (GSH) to oxidized (GSSG) glutathione in MDA231 and HepG2 under normoxia and hypoxia. The ratio was normalized to the normoxic value. Data (B), (C) and (D) are presented as mean ± SD.

(E) The exchange flux of ALDO, DHAP + GAP → F6(B)P.

(F) The exchange flux of TAL, GAP + TAL-C_3 → F6(B)P. Data (A), (E) and (F) were estimated by ^{13}C-metabolic flux analysis with [1,2-$^{13}C_2$]glucose and [1-^{13}C]glucose+[4,5,6-$^{13}C_3$]glucose tracers. Nor, normoxia; Hyp, hypoxia. Data (A), (E) and (F) are presented as 68% and 95% confidence intervals and *p < 0.05 (two-tailed t test) comparing normoxia and hypoxia.

Figure 4. Exchange fluxes of ALDO and TAL enhance the non-oxidative PPP flux.

(A) MDA231 was stably transfected by lentivirus containing random shRNA (Control), aldolase A (ALDOA) shRNA (shALDOA), transaldolase 1 (TALDO) shRNA (shTAL) and GAPDH shRNA (shGAPDH) with two different versions of shRNA. The exchange flux of ALDO and TAL was measured by dephosphorylated fructose (*m/z* 307) extracted from cells cultured in the presence of [1,2-$^{13}C_2$]glucose under hypoxia. Data are presented as mean ± SD and *p < 0.001 (two-tailed t test) comparing control and knockdown cells.

(B) Relative gene expression levels in the lower glycolytic pathway were analyzed by RT-qPCR in HepG2. Data are presented as mean ± SEM and *p < 0.05 (two-tailed t test) comparing normoxia and hypoxia.

(C) Western blotting with antibodies of Hif1α, GAPDH and Tubulin. N, Normoxia; H, hypoxia.

(D) Metabolic profiling of glycolytic intermediates by LC-MS. Data are presented as mean ± SD and *p < 0.05 (two-tailed t test) comparing normoxia and hypoxia.

(E) NAD^+/NADH ratio was measured in whole-cell extracts under normoxia and hypoxia. Data are presented as mean ± SD and *p < 0.05 (two-tailed t test) comparing normoxia and hypoxia.

(F and G) Exchange glycolytic flux (F) and the non-oxidative PPP flux to R5P (G) were measured under normoxia with wild-type MDA231 cells by addition of iodoacetate (IAA) and 2-deoxy-D-glucose (2DG) and no addition (control) (the first panel), with wild-type HepG2 (the second panel), with MDA231 cells stably transfected by GAPDH knock-down (shGAPDH) and random shRNA (control) (the third panel) and with HEK293T cells genetically engineered by hexokinase 2 knock-out (HK2-ko) and mock vector (control) (the fourth panel). Exchange flux

via ALDO and TAL and the non-oxidative PPP flux values were measured with ^{13}C-labeling of dephosphorylated fructose and RNA-derived ribose. The values were normalized with control. Data are presented as mean ± SD and *$p < 0.001$ (two-tailed t test) comparing control and each condition. *TPI1*, triosephosphate isomerase 1; *PGK1*, phosphoglycerate kinase 1; *PGAM 1*, phosphoglycerate mutase 1; *ENO1*, enolase 1; *PKM2*, pyruvate kinase muscle 2; *LDHA*, lactate dehydrogenase A.