Chapter 2

# Role of Glutamate Dehydrogenase in Cancer Growth and Homeostasis

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Additional information is available at the end of the chapter

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## 1. Introduction

Glutamate Dehydrogenase (GDH) catalyzes the oxidative conversion of glutamate to alpha ketoglutarate and ammonium supplying the TCA cycle with intermediates in support of anaplerosis (**Figure 1 Rxn1**). Conversely GDH catalyzes the reductive amination of alpha ketoglutarate and ammonium producing glutamate when the TCA cycle pool is filled. The net GDH flux resulting from these bidirectional fluxes can be obtained from the conversion of either 15N labeled glutamate and analyzing 15N ammonium or from 15N labeled $NH_4$+ and monitoring 15N labeled glutamate. Besides deamination and 15N $NH_4^+$ production, 15N labeled glutamate can be converted to 15N labeled amino acids, most prominently alanine, via transamination reactions (**Figure 1, Rxn2**). In contrast to glutamate deamination which yields net keto acid production for anaplerosis [1], transamination does not yield net keto acid production (consuming a keto acid e.g. pyruvate in the process of generating alpha ketoglutarate). Under physiological conditions plasma glutamate concentration, 10-20uM, is limiting for GDH flux supplying TCA intermediates while plasma glutamine concentration, 600uM, is not [2]. The conversion of 15N amide labeled glutamine to 15N ammonium (**Figure1, Rxn1**) approximates the net glutaminase flux generating glutamate and ammonium, both potential substrates for GDH. Indeed GDH can also incorporate the amide derived 15N ammonium and alpha ketoglutarate into glutamate (**Figure 1, Rxn3**, reductive amination) which can subsequently transaminate with pyruvate generating 15N alanine [3]. Noteworthy this glutaminolytic anabolic pathway providing glutamate has been proposed as the primary metabolic transformation in tumor cells [4]. **Figure 1, Rxn 3** also illustrates how ammonium production from the 15N amide of glutamine may underestimate the true glutaminase flux; to the extent that this occurs, it contributes to differences in estimated net glutaminase fluxes between the chemically measured glutamine disappearance and 15N amide ammonium appearance. Glutamine labeled with 15N in the amino position provides

an assessment of net GDH flux (**Figure 1,Rxn1)** as 15N $NH_4^+$ and, or, ALT flux as 15N alanine produced(**Figure1,Rxn2**).

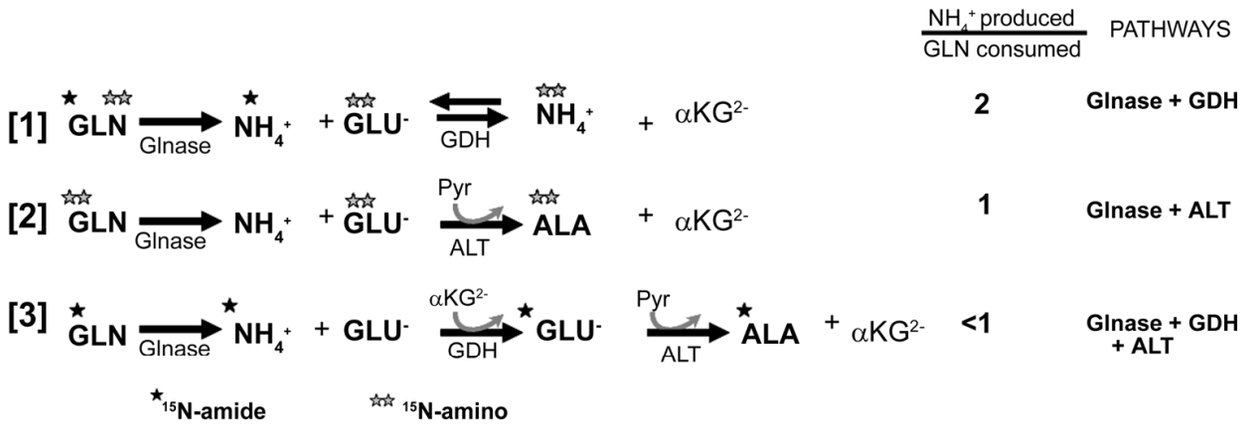


**Figure 1. GDH determines the fate of 15N glutamine**. Pathways of glutaminolysis, net keto acid production and $NH_4^+$ produced per glutamine consumed ratio. [1] Deamidation coupled to GDH deamination yielding 2NH4+/Gln and net keto acid (αKG). [2] Deamination coupled to ALT-mediated transamination yielding 1 NH4+/Gln and no net acid production. [3] Deamidation coupled to GDH reductive amination and transamination yielding < 1 NH4+/Gln and net keto acid

## 2. Glutamine is the major source of ammonium produced in cultured cells

Because the bulk (≈90%),[1,3-5] of the ammonium produced by cells in culture derives from glutamine's 2 nitrogen moieties (preformed DMEM media glutamate is <50uM) chemical measures of ammonium produced (after subtracting any ammonium produced in the absence of glutamine) and glutamine consumed offers an index of the GDH pathway activity. A ratio of 1, for example, would be consistent with glutamine metabolized by glutaminase with $NH_4^+$ released to the media and glutamate either released to the media or transaminated to amino acids e.g. alanine (**Figure 1, Rxn2**). In either case there is no net GDH flux and therefore no net generation of keto acids. In contrast a ratio of 2 $NH_4^+$/glutamine (**Figure 1, Rxn1**) is consistent with glutamine metabolized by glutaminase and the glutamate produced metabolized by GDH yielding $NH_4^+$ and net keto acid (anaplerosis) for the TCA cycle(running in either the normal forward or reverse direction,[6]). On the other hand, an $NH_4^+$ produced per glutamine consumed less than 1 (**Figure 1, Rxn3**) is consistent with glutaminase generating $NH_4^+$ from the amide nitrogen with reductive amination [3,4] catalyzed by GDH reducing the $NH_4^+$/Gln ratio to less than 1 producing glutamate and consuming net keto acid (alpha ketoglutarate); subsequent transamination yields amino acids(e.g. alanine, aspartate) consuming keto acids (cataplerosis) containing the amide nitrogen of glutamine[3,4]. Normal breast cell line exhibits an $NH_4^+$/glutamine ratio less than 1[7] whereas cancerous breast cell lines exhibit a ratio greater than 1[7] consistent with a quantitative difference in bidirectional GDH fluxes between normal (reductive amination) and tumorigenic cells (oxidative deamination). In

addition, in vivo administered [2-(14)C] labeled glutamate was taken up by tumors with 14C distributed more into protein and lactate than in normal tissues [ 8 ]. In line with this early finding, a recent study[9] showed[U-(13)C] glucose contributed less than 50% of the acetyl COA pool in human brain tumors consistent with glutaminolysis and GDH's role in maintaining TCA pool homeostasis (anaplerosis).

## 3. Intracellular glutamate and alpha ketoglutarate are in near equilibrium

In most cells intracellular glutamate and alpha ketoglutarate are in near equilibrium[4,10], and changes in TCA cycle intermediates($\alpha$KG) as well as the redox state(NADPH/NADP), energy charge(ADP,GTP) and cell pH shift the GDH catalyzed flux to net production or consumption of $\alpha$KG (**Figure 2**). Normally pyruvate (glucose) provides the TCA cycle with pool intermediates while generated glutamate is transaminated ($NH_4^+$/GLN ratio<1, **Figure 1 Rxn3**). In cancer cells, glucose is shunted into aerobic glycolysis (Warburg effect, [11]) and the TCA cycle intermediates are reduced as the result of cataplerosis as evidenced by lower intracellular glutamate [7]. This reduction in TCA cycle intermediates "pulls" glutamate through GDH generating $\alpha$KG as evidenced by the higher steady state $NH_4^+$/GLN ratio>1, **Figure 1, Rxn1** and consistent with glutamate (glutaminolysis) supporting anaplerosis (**Figure 2**). As a corollary, the ammonium to alanine produced ratio increases [7] reflecting the increased GDH and decreased ALT flux as the result of reduced intramitochondrial pyruvate(metabolized in cytosol to lactate, **Figure 2**). Thus the increased glutamate flux through GDH generates $\alpha$KG while sparing keto acid consumption (reduced transamination).

## 4. Glutamate is generated by extra- and intracellular glutaminases

Glutaminolysis as illustrated in **Figure 2** is associated with the increased expression of both the extrinsic cell membrane phosphate independent glutaminase/gamma glutamyltransferase/gamma glutamyltranspeptidase (PIG, GGT, GGTP) which generates extracellular glutamate [2,12] and intracellular phosphate dependent glutaminases, Phosphate dependent glutaminases (PDG,GLS1 and GAC, [13,14]) which generates glutamate cytosolically [2,13]; extracellular glutamate can be transported(GLAST, **Figure2**) into the cytosol functioning as an inhibitor of the intracellular glutaminases[2]. Noteworthy, c-myc signaling up-regulates both the cell membrane glutamine transporter (ASC, **Figure 2**) and the intracellular glutaminases in cancer cells [15]. On the other hand, increased expression of the extracellular PIG is also a hallmark of cancer cells [16] and PIG hydrolysis of ϒ-glutamyl-tagged fluorescent markers can be used to delineate tumor boundaries [16]. However, in contrast to glutamine uptake, cell membrane glutamate transport (GLAST1) is shifted from the cell membrane to an intracellular location in breast cancer cells as shown in **Figure 3**, effectively uncoupling extracellular glutamate from inhibiting the intracellular glutaminases; this allows full blown expression of intracellular glutamate generation(**Figure 1RXI**) and, if the relocated glutamate transporter, GLAST1 transports glutamate from the outer surface of inner mitochondrial membrane into the into the mitochondria matrix [17],

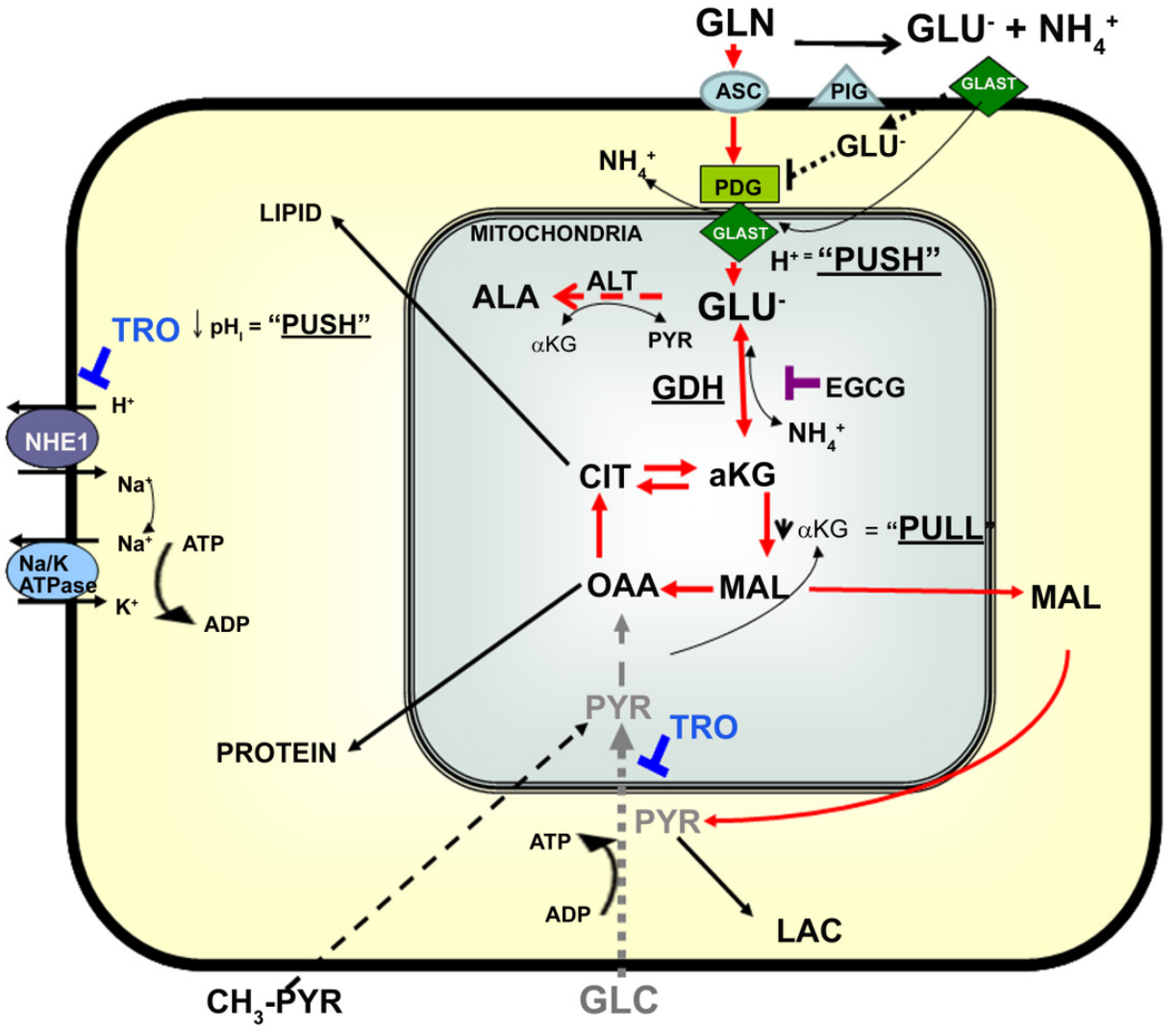


**Figure 2. Central role of GDH flux in cancer cells**. Glucose **(GLC**) derived pyruvate**(PYR**) is metabolized(Warburg effect) to lactic acid(LAC) at the expense of the **TCA** (tricarboxylic acid) cycle intermediate pool(*α*KG) "pulling" glutamate through GDH to supply anaplerosis. **NHE(sodium hydrogen ion exchanger 1)** mediated acid extrusion is up-regulated coupled to anaerobic glycolysis acidifying extracellular milieu while cell membrane glutamate transporter(**GLAST1**) relocates to mitochondria with **PIG** produced $GLU^-$ accumulating extracellularly and contributing with reduced pHe to host defense barrier. Glutamine(**GLN**) transported into cell by **ASC** and hydrolyzed to glutamate on outer surface of inner mitochondrial membrane by **GAC [13]** coupled with **GLAST1.** GLC removal (dashed line) accentuates GDH flux by "pull" mechanism while NHE mediated acid extrusion supported by GDH and accelerated *α*KG input with cytosolic **malate**(MAL) conversion to **PYR** supplying anaplerosis. TRO blocks PYR entry into mitochondria and accelerates GDH flux by exaggerated pull mechanism in conjunction with reduced pHi as result of NHE1 inhibition ("push" mechanism). **EGCG** inhibits GDH and induces cell death that can be partly rescued with methyl pyruvate(**CH3-PYR**) and restored TCA cycle pool while correction of cellular acidosis requires GDH flux pointing to the dual role for GDH in anaplerotic and acid base homeostasis.

then it would supply GDH glutamate in support of anaplerosis . Noteworthy overexpression of PIG promotes tumorigenesis [16] presumably by building up extracellular glutamate and suppressing local immune responses [18] . In addition NHE mediated acid extrusion is up-regulated in cancer cells [19,20] importing a $Na^+$ load requiring Na+/K+ ATPase - ATP expenditure and ATP regeneration associated with acidogenic aerobic glycolysis(Warburg effect) and by substrate level phosphorylation. Because PIG (GGT/GGTP), NHE, glutamine transporter and glutaminase activities are all up-regulated in rapidly growing tumors, tagging molecular target inhibitors [21-24] with a ϒ-glutamyl moiety offers a tumor specific vehicle specific for limiting anaplerosis and preventing elevated cell pH, prerequisites for rapid tumor growth.

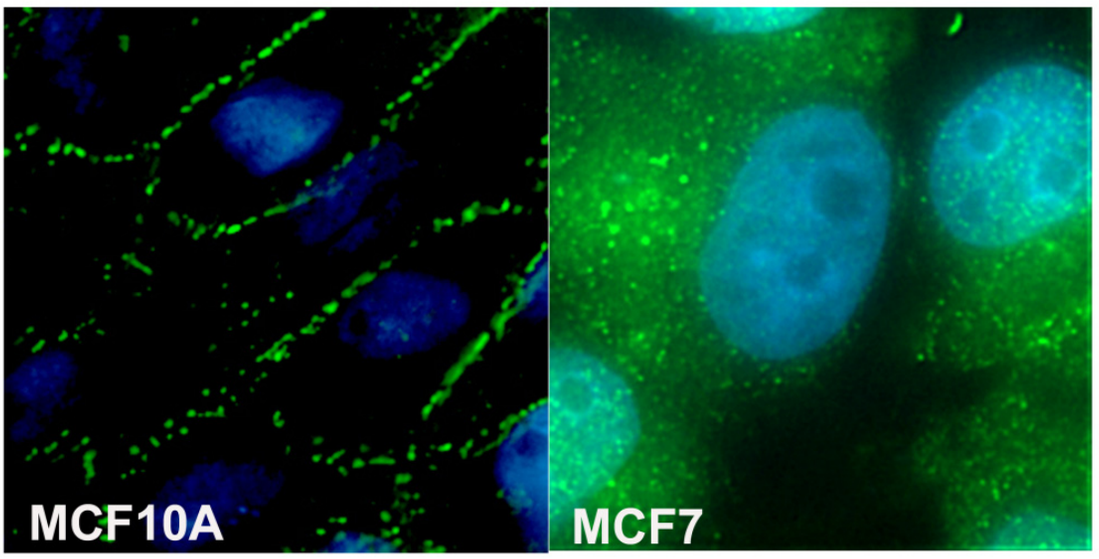


**Figure 3. GLAST localization in normal versus cancer cells**. MCF10A and MCF7 cells cultured on coverglass were stained with monoclonal antibodies to GLAST-1 . MCF10A demonstrate almost complete membrane localization of the transporter, while MCF7 have a cytoplasmic distribution pattern.

## 5. Glucose removal lowers TCA cycle intermediates and "pulls" glutamate through GDH

Removal of glucose from the media (Figure 2, dotted gray line from GLC) deprives cells of pyruvate input into the TCA cycle and a fall in the intermediate ($\alpha$KG) pool level[5] as reflected by a drop in glutamate [7]. As a consequence, GDH flux (**Figure 1, Rxn1**) increases [5] supplying anaplerosis as malate exits the cycle forming pyruvate which in turn supports citrate formation (**Figure 2**). Noteworthy this increased glutamate flux through GDH("pulling" effect) is maintained by 2 responses:1] by a small increase in glutaminase flux [5,7] and, 2] a large fall in glutamate transamination [5,7,25].Under glucose deprivation cell survival is dependent on GDH flux at least in part to supply anaplerosis [5,26]. Surprisingly cell number actually increase in the glutamine (1.3mM) alone compared to the glucose(5mM) plus glutamine media (**Figure 4A)** because of reduced cell death; this increased survival is attributed to the increased GDH flux [26]

which besides supporting anaplerosis also enhances NHE mediated acid extrusion (**Figure 4D**) although proliferation rate decreases (**Figure 4B**). Noteworthy is the increase in cell biomass(protein, nucleic acids and lipid) dependent upon glutaminolysis supported anaplerosis as shown by the increased incorporation of 14C-U-glutamine into cell biomass (**Figure 4C**).The critical role of GDH flux in cell survival is evident from the massive cell death induced by GDH inhibition under glucose depleted conditions with 100uM EGCG [5,26], an inhibitor of GDH [5]. Although supplying TCA cycle intermediates e.g. methyl pyruvate (10mM,**Figure 2**) rescued cells with GDH inhibited [5,26], a significant fraction of the population succumbed associated with a reduced cell pH [26]. Parenthetically, methylpyruvate is a strong acid constituting a large acid load which requires supplementing the media with equal moles of bicarbonate (10mM). Nevertheless, even after the above base compensation, supplying anaplerotic substrates does not restore NHE activity [26] pointing to an important dual role for GDH in maintaining both anaplerosis and pH homeostasis [22] for cell survival.

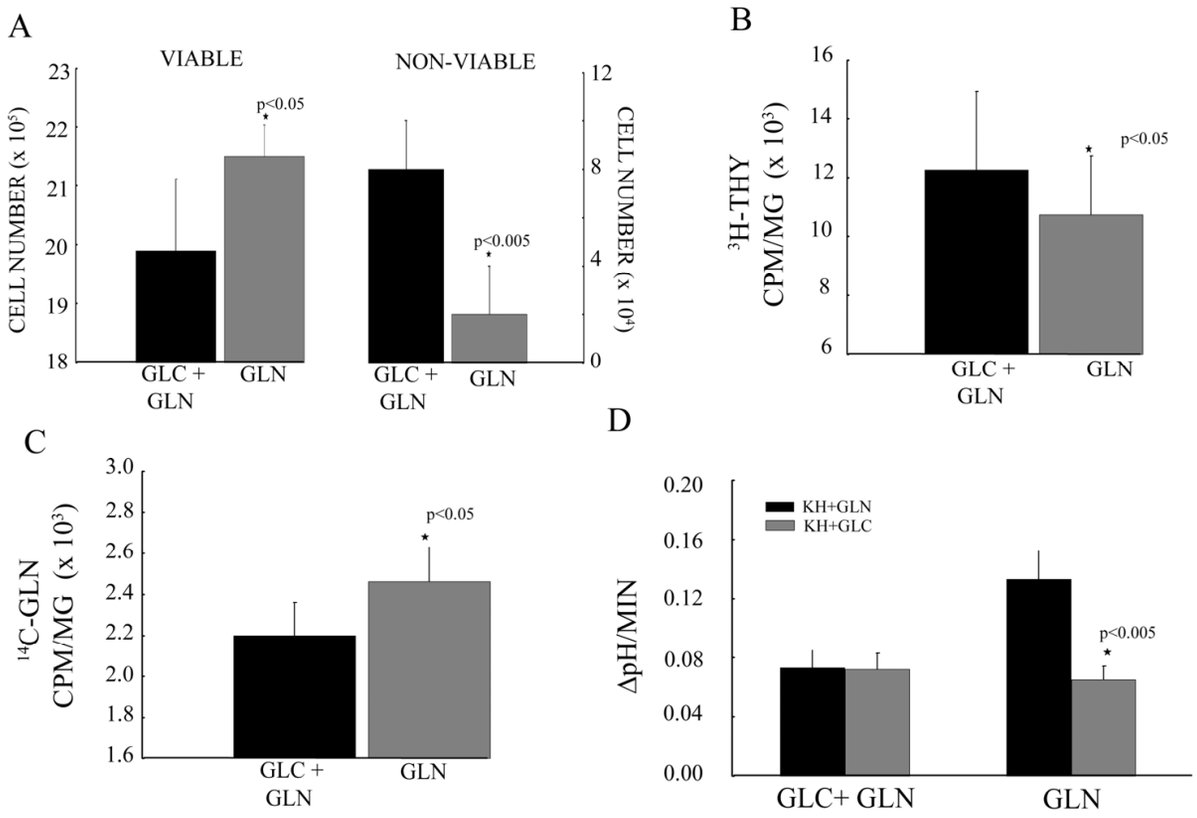


**Figure 4.** Physiological(1.3mM) glutamine concentration alone supports breast cancer cell survival associated with increased anaplerotic and acid extrusion function. 4A GLN increased cell number and decreased cell death compared to GLN plus 5mM glucose; 4B Gln slows cell proliferation compared with GLC+GLN; 4C GLN supports anaplerotic function[$^{14}$C-U-L-glutamine incorporation into TCA precipitated cellular protein and lipid 4D GLN supports accelerated NHE activity when assayed with K 10mM GLN as opposed to 10mM GLC

## 6. Cellular acidosis "pushes" glutamate through GDH

Glutamate flux through GDH can be also be "pushed" by a fall in intracellular pH [27]. Whether this reflects a shift from GHD1 to GDH2 isoform [28] is not known but, if so, this "pushing effect" of reduced pH effect could be additive with the above "pulling effect" of a reduced TCA pool (**Figure 2**). Indeed in metabolic acidosis, the ambient condition surrounding cancer cells in vivo, kidney cells' glutaminolysis is both "pushed"(reduced cell pHi, [27]) and "pulled"(inhibition of TCA, [29]) as a result of reduced TCA cycle pool size associated with true renal growth [30]. Interestingly enough, the in vivo kidney switches fuels from lactate to glutamine oxidation in metabolic acidosis[31] so that the anaplerotic glutaminolysis-GDH reactions matches [32] the cataplerotic reactions($CO_2$, biomass formation, [30,31] as does acid excretion ($2NH_4^+$/glutamine) and base($2HCO_3^-$/glutamine) generation. Furthermore the pH-dependent enlistment of GDH2 isoform alone (push mechanism) or accompanying GDH 1 flux (anaplerosis driven pull mechanism) would provide regulatory options in responding to anaplerotic/cataplerotic and, or, acid /base demands in tumors.

## 7. Glitazones accelerate GDH flux via the push/pull mechanism: A strategy for therapeutic intervention

Fortuitously there are agents that can be employed to impose this push/pull mechanism on the GDH flux in cancer cells and thereby present a window of vulnerability (targeted inhibitors). The antihyperglycemic agents, troglitazone (Rezulin) and rosiglitazone (Avandia) block pyruvate entrance into the TCA cycle(25,33] lowering $\alpha$KG(glutamate,7,25] and accelerating GDH flux via this "pull" mechanism (**Figure 2**). Simultaneously, both troglitazone and rosiglitazone directly inhibit NHE [25,34] lowering pHi and driving GDH via the "push" mechanism (**Figure 2**). Noteworthy the glutaminase flux (glutamine disappearance) remains unchanged while the $NH_4^+$ production increases as the result of the increase in deamination flux (**GDH Figure 1, Rxn1**). Although resembling glucose deprivation ("pull" mechanism), troglitazone further increases the $NH_4^+$ production (additive "push+pull" mechanism) exceeding the fall in alanine production ("pull" mechanism alone). More specifically the accelerated GDH flux ("push+pull") induced by troglitazone can be demonstrated using 15N amino labeled glutamine as shown in **Figure 5**; in contrast, another glitazone pioglitazone(Actos) activates GDH flux [34] solely by reducing cell pH("push" mechanism) and consequently does not reduce alanine production [34]. Noteworthy troglitazone acutely inhibits GDH flux (0-3hrs) as the result of a fall in mitochondrial membrane potential( $\Psi$m) requiring accelerated GDH flux(3-24hrs) to fully restore the $\Psi$m [7], a response that is PPAR$\gamma$ independent [7,25] and possibly mitoNEET [35] dependent. Little recognized is the direct inhibition of NHE[20,34] by both troglitazone and rosiglitazone as well as indirect inhibition mediated through PPAR$\gamma$ suppression of NHE gene expression[20,36]; in contrast, pioglitazone does not inhibit NHE directly[34] rather acts indirectly through PPAR$\gamma$[36]lowering cell pH[34] and accelerating GDH flux("push" mechanism). In combination, TRO +PIO together exert an additive effect on GDH flux

presumably reflecting both TRO's "pull" action and PIO's PPARγ- mediated down-regulation of NHE gene expression. Significantly, the dual effect of glitazones to increase GDH flux while reducing NHE activity decreases proliferation(NHE) but increases cell survival(GDH) resulting in only a slight decrease in cell number[26]. Nor does adding troglitazone to glucose deleted cells induce massive cell death since the effect on GDH flux is additive (further reducing TCA intermediates and cell pH, **Figure 2**) and although proliferative rates are decreased, survival is enhanced [26]. Under these conditions, e.g. cell survival mechanisms, inhibition of GDH is most effective in causing massive cell death as occurs with the GDH inhibitor EGCG [5] combined with troglitazone [26]. Although rescue of cells is partly possible by restoring anaplerosis with methyl pyruvate, failure to restore NHE activity and the cellular acidosis preclude full recovery underlining the importance of both GDH and NHE in cell survival[26].

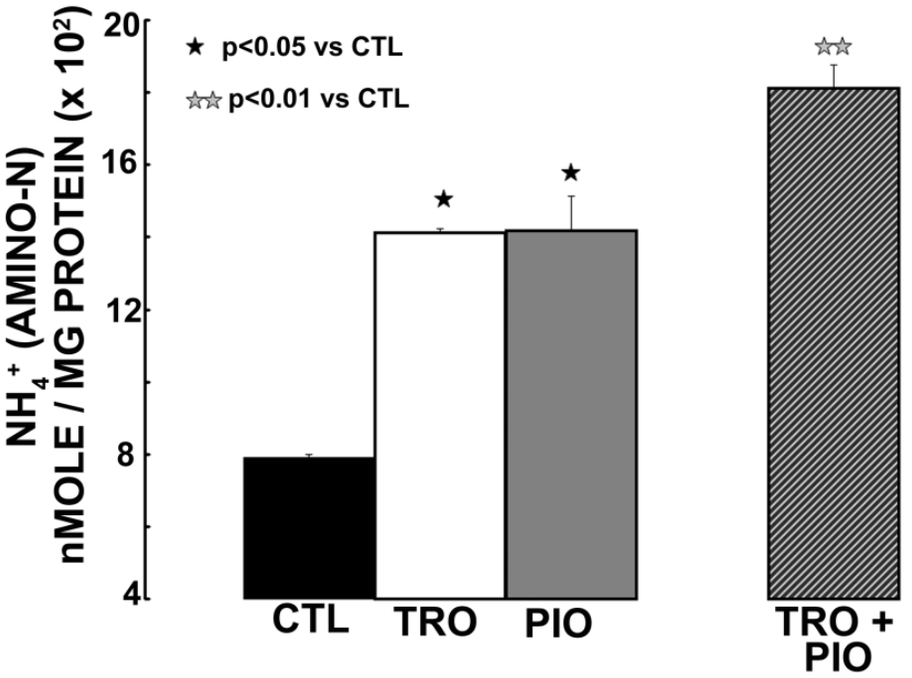


**Figure 5. Ammonium production from amino nitrogen of glutamine.** Cells were incubated for 18 hours in [2-15N] glutamine. TRO was used at 20 uM, PIO 10 uM. Results are from 3 experiments.

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