

Metformin decreases glucose oxidation and increases the dependency of prostate cancer cells on reductive glutamine metabolism

Sarah-Maria Fendt¹, Eric L. Bell², Mark A. Keibler¹, Shawn M. Davidson^{2,7}, Gregory J. Wirth³, Brian Fiske^{2,7,12}, Jared R. Mayers^{2,7,12}, Matthias Schwab^{11,12}, Gary Bellinger^{5,12}, Alfredo Csibi¹⁰, Akash Patnaik^{4,5}, Marie Jose Blouin⁹, Lewis C. Cantley^{5,6}, Leonard Guarente², John Blenis¹⁰, Michael N. Pollak⁹, Aria F. Olumi³, Matthew G. Vander Heiden^{2,7,8*} and Gregory Stephanopoulos^{1*}

¹Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

²Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

³Department of Urology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

⁴Division of Hematology/Oncology, Department of Medicine, Beth Israel Deaconess Cancer Center, Harvard Medical School, Boston, Massachusetts

⁵Division of Signal Transduction, Department of Medicine, Beth Israel Deaconess Cancer Center, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

⁶Department of Systems Biology, Harvard Medical School, Boston, MA 02115, USA

⁷Koch Institute for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

⁸Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA

⁹Department of Oncology, McGill University and Jewish General Hospital, Montreal, Quebec, Canada

¹⁰Department of Cell Biology, Harvard Medical School

¹¹Doctor Margarete Fischer-Bosch-Institute of Clinical Pharmacology, Stuttgart, and Department of Clinical Pharmacology, Institute of Experimental and Clinical Pharmacology and Toxicology, University Hospital, Tuebingen, Germany

¹²equal contribution

*corresponding author

Metformin inhibits cancer cell proliferation and epidemiology studies suggest an association with increased survival in cancer patients taking metformin, however, the mechanism by which metformin improves cancer outcomes remains controversial. To explore how metformin might directly affect cancer cells, we analyzed how metformin altered the metabolism of prostate cancer cells and tumors. We found that metformin decreased glucose oxidation and increased dependency on reductive glutamine metabolism in both cancer cell lines and in a mouse model of prostate cancer. Inhibition of glutamine anaplerosis in the presence of metformin further attenuated proliferation while increasing glutamine metabolism rescued the proliferative defect induced by metformin. These data suggest that interfering with glutamine may synergize with metformin to improve outcomes in patients with prostate cancer.

Introduction

Altered cellular metabolism is a hallmark of cancer cells (1-3). Tumor cells adapt their metabolism to provide the needed quantities of ATP, redox equivalents, and biosynthetic precursors necessary for cell growth and uncontrolled proliferation (4-6). Individual cancers appear to have distinct metabolic dependencies to sustain growth and proliferation. Addiction to glutamine (7-9), glucose (1, 10), and other amino acids (11) have been described, but how to best target these unique dependencies for therapeutic benefit remains unknown (12).

One potential approach has focused on targeting specific metabolic enzymes including pyruvate kinase (11, 13), lactate dehydrogenase (14) and glutaminase (15, 16), but the feasibility of targeting these enzymes in cancer patients remains unknown. Metabolism-altering drugs in general, however, have been used in the clinic and are well tolerated by humans. For instance, dichloroacetate, a compound used to treat lactic acidosis (17) can alter mitochondrial metabolism in human tumors (18), and can induce death of cancer cells in culture that fail to adapt to increased pyruvate oxidation (19). The anti-diabetic drug metformin is another widely prescribed compound that can directly impact cell metabolism. Metformin improves glycemic control in diabetes at least in part by modulating signaling pathways that regulate metabolism in the liver, a process thought to be mediated through activation of AMPK (20). How exactly metformin acts as an anti-diabetic agent remains controversial, but one target of metformin in cells is mitochondrial complex I (20). Inhibition of this complex by metformin can induce energy stress as one mechanism of AMPK activation, leading to reduced rates of hepatic

gluconeogenesis. Since the drug is effective and very well tolerated by patients, it is among the most widely prescribed anti-diabetic drugs.

Some reports provide evidence that diabetics treated with metformin have lower than expected cancer burden relative to diabetics taking other agents despite similar glucose control (21-24), but how metformin accomplishes this is not known (25). *In vitro* antineoplastic activity of ~5 mM metformin has been confirmed by many laboratories in a variety of in model systems. While some activity of biguanides *in vivo* may be attributable to systemic effects such as reduction of insulin levels, there is considerable interest in possible 'direct' effects of these compounds under conditions where sufficient intracellular drug concentrations are achieved (26). Indeed, the ability of cancer cells to take up metformin has been challenged (26). Nevertheless, numerous studies have demonstrated anti-proliferative effects of metformin in various cancer cell lines and in prostate cancer cells these metformin effects appear to be independent of AMPK. Instead metformin appears to activate p53, leading to subsequent-REDD1 mediated mTOR and cyclin D1 inhibition (27-29). Yet, in line with other tumor types, it remains possible that metformin induces antineoplastic activity via direct effects on metabolism. Whether metformin has a direct effect on prostate cancer cell metabolism is unexplored and consequently how best to use the anti-proliferative effect of metformin for cancer therapy might be missed.

To understand how to best use metformin in prostate cancer patients, we sought to understand if and how metformin affects the metabolism of prostate cancer cells. We found that both glucose and glutamine metabolism are affected by metformin via

complex I inhibition. Prostate cancer cells with higher glucose oxidation appear to be the most sensitive to metformin. Moreover, we identified reductive glutamine metabolism as essential for maintaining modest proliferation in the presence of metformin, thus suggesting that interruption of glutamine metabolism may have a synergistic effect with metformin. In agreement with this hypothesis, we found that metformin also increased reductive glutamine metabolism in tumors arising in a SV40 driven mouse model of prostate cancer.

Methods

Cell lines and cell culture conditions

All cell lines were obtained from ATCC. All experiments were performed in RPMI (Mediatech), with the exception of Huh7 cells which were cultured in DMEM, each supplemented with 10% fetal bovine serum (Invitrogen) and 1% pen/strep (Mediatech). For labeling experiments fetal bovine serum was replaced with dialyzed fetal bovine serum (Invitrogen), and glucose or glutamine was replaced with either U-13C glucose (CLM-1396 Cambridge Isotopes), U-13C glutamine (605166 Sigma) or 5-13C glutamine (CLM-1166 Cambridge Isotopes). Cell lines were treated with metformin or any other small molecule for 3 days in all experiments.

Physiology

Growth rates were determined using a hemocytometer or an automated cell counter (Nexcelom). To determine uptake and secretion rates, glucose and lactate concentrations were detected by a Waters Alliance 2695 HPLC (Waters) with a Waters

410 Differential Refractometer and a Bio-Rad HPX-87H column (Bio-Rad). The column was eluted at 50°C with 14 mM of sulfuric acid at a flow rate of 0.7 ml/min. Glutamine consumption and glutamate secretion was measured with an YSI 7100MBS (YSI Life Sciences) according to the manufacture's protocol.

Carbon contribution to tricarboxylic acid cycle and palmitate

Labeled tissue cultures were washed with saline and metabolism was quenched with -20°C cold 70% methanol. After cell scraping in 70% methanol (containing internal standard norvaline), -20°C cold chloroform was added and the samples were vortexed at 4°C to extract metabolites. Phase separation was achieved by centrifugation at 4°C. Methanol phase (polar metabolites) and chloroform phase (fatty acids) were separated and dried by applying constant air flow. Dried metabolite samples were stored at -80°C.

Polar metabolite samples were derivatized with methoxyamine (TS-45950 Thermo Scientific) for 90min at 40°C and subsequently with N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamid, with 1% tert-Butyldimethylchlorosilane (375934 Sigma) for 60min at 60°C. Fatty acids were esterified with 2 % sulfuric acid in methanol for 120min at 60°C and subsequently extracted with hexane. Isotopomer distributions of polar metabolites and fatty acids were measured with a 6890N GC system (Agilent Technologies) combined with a 5975B Inert XL MS system (Agilent Technologies).

Isotopomer distributions were analyzed using the Matlab-based software Metran (30-33). Total contribution of carbon was calculated using the following equation (34):

$$\text{total contribution of carbon} = \frac{\sum_{i=0}^n i * m_i}{n * \sum_{i=0}^n m_i}$$

Where n gives the number of C atoms in the metabolite, i the different mass isotopomers and m the abundance of a certain mass.

Isotopomer distributions of fatty acids were further fitted to an isotopic spectral analysis model assuming that the measured mass distribution vectors are a function of the rate of fatty acid synthesis and contribution of the labeled carbon source to fatty acids (35, 36).

Net glutamine flux was calculated from glutamine uptake rates and glutamate secretion rates.

Small molecules for in vitro experiments

Metformin (D5035 Sigma) was dissolved in milliQ water and applied at concentrations of 0.5-2.5mM. Rotenone (R8875 Sigma) was dissolved in DMSO and applied at concentrations of 0.17-0.85 μ M. BPTES was a gift from Agios Pharmaceuticals (Cambridge, USA). BPTES and 968 (AG-690 Specs) were dissolved in DMSO and applied at concentrations of 4 or 8 μ M and 5 or 10 μ M, respectively.

TSC2 knockdown and mRNA expression levels

Lenti virus with shRNA against TSC2 (TRCN0000040178, TRCN0000040181, Sigma) was applied. Control cells were infected with empty pLKO.1. For quantitative real time PCR, RNA was isolated using RNeasy Mini Kit (Qiagen) and complementary DNA was generated from 1 μ g RNA using High Capacity cDNA Reverse Transcription Kit (4368814 Qiagen) according to manufacturer's protocols. Complementary DNA was

analyzed using the Light Cycler 480 II (Roche) with SYBR Green master mix from Bio-Rad. Primers sequences were obtained from Primer Bank (37).

Metformin quantification

Tumor tissue samples (20 – 30 mg) were homogenized in 0.9% NaCl using a mortar and pestle and centrifuged (10 min, 13000 rpm). An aliquot of the supernatant was used for protein determination; in an additional aliquot, metformin was quantified. Metformin was quantified with an Agilent 6460 triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1200 HPLC system. Ionization mode was electrospray (ESI), polarity positive. Electrospray jetstream conditions were as follows: capillary voltage 3500 V, nozzle voltage 1000 V, drying gas flow 11 l/min nitrogen, drying gas temperature 350°C, nebulizer pressure 55 psi, sheath gas temperature 350 °C, sheath gas flow 11 l/min. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using m/z 130.1 and the product ion m/z 60.1. Dwell time was 100 ms, the fragmentor was set at 60, and the collision energy at 10. HPLC separation was achieved on a HILIC plus column (50×2.1 mm I.D., 3.5 µm particle size, Agilent, Waldbronn, Germany) using 0.1 % formic acid in water/acetonitrile 20:80 (v/v) as mobile phase at a flow rate of 0.65 ml/min.

'Transgenic Adenocarcinoma of Mouse Prostate' (TRAMP)

TRAMP mice were injected with three consecutive intraperitoneal injections (with a 24 hours interval) of vehicle or metformin (300 or 500mg/kg/day). Metformin was dissolved in phosphate buffered saline, and the vehicle was phosphate buffered saline. Mice were sacrificed two hours after the last injection. Prostate tumor tissue was removed and

quenched in liquid nitrogen. Pulverized tumor tissue was extracted and analyzed as described above. Metabolite pool sizes were normalized to analyzed tumor tissue weight. Mass isotopomer distributions of citrate were normalized to the glutamine enrichment of the tissue.

All animal experiments were approved by the Committee on Animal Care at MIT and/or Harvard.

Statistics

Error bars depict standard deviation. R and p values for the depicted correlations were calculated with the Matlab integrated function `corrcoef`: `[R,P]=corrcoef` returns P, a matrix of p-values for testing the hypothesis of no correlation. Each p-value is the probability of getting a correlation as large as the observed value by random chance, when the true correlation is zero. P values were also calculated for bar graphs using a student's T-test (two-tailed, unequal variance) to determine whether two samples are likely to have come from the same two underlying populations that have the same mean.

Results

Metformin decreases proliferation and promotes glucose fermentation in prostate cancer cell lines

To begin to characterize the effect of metformin on the metabolism of three different prostate cancer cell lines, we measured proliferation, glucose uptake rate, and lactate secretion rate in the absence or presence of increasing doses of metformin. For all

three cell lines, the decrease in proliferation observed with increasing metformin concentration was accompanied by an increase in the rate of glucose uptake and lactate secretion (Figure 1A). Interestingly, the magnitude of the decrease in proliferation along with the increase in glucose uptake / lactate secretion was different across all three cell lines. LNCaP cells exhibited the greatest response, PC3 cells showed an intermediate response, and DU145 hardly responded to metformin. In the presence of 2.5mM metformin compared to no-treatment, all cell lines displayed an increase in the amount of lactate secretion relative to glucose uptake suggesting that a larger percentage of the glucose is converted to lactate in these cells. However, the increase in lactate secretion relative to glucose uptake was different in different cell lines (increase in this ratio for DU145: $13\pm 3.9\%$, PC3: $23\pm 2.2\%$, and LNCaP: $49\pm 3.0\%$), suggesting that metformin differently affected carbon metabolism in these different cell lines. Furthermore, the degree to which metformin altered glucose metabolism correlated with the decrease in proliferation rate when cells were treated with the drug (Figure 1B).

The metabolic response to metformin is consistent with mitochondrial complex I inhibition

In order to understand how metformin influences glucose metabolism, we first asked whether the known action of metformin as an inhibitor of mitochondrial complex I is also observed in these cells. Complex I is a component of the electron transport chain that transfers electrons derived from nutrient oxidation to oxygen. To determine if metformin affected this process in prostate cancer cells, we measured oxygen consumption in the absence or presence of increasing concentrations of metformin. Metformin inhibited

oxygen consumption in a dose-dependent manner in all three cell lines (Supplementary Figure 1), consistent with complex I inhibition. To determine if complex I inhibition could account for the differential metformin sensitivity across the three cell lines, we tested the effect of increasing doses of the established complex I inhibitor rotenone on cell proliferation (Figure 2A). Rotenone inhibited cell proliferation in a dose-dependent manner that varied across cell lines. Consistent with complex I being involved in metformin toxicity, the sensitivity of all three cell lines to rotenone was the same as that observed with metformin (*i.e.* DU145 cells were the least sensitive, PC3 cell had an intermediate phenotype, and LNCaP cells were the most sensitive). Moreover, addition of metformin did not have any further effect on cells treated with the highest dose of rotenone, supporting the hypothesis that the anti-proliferative effect of metformin is mediated by complex I inhibition in these cells (Figure 2A). Furthermore, we tested if the correlation between decreased proliferation and increased glucose uptake was also observed with rotenone treatment. Consistent with a role for complex I inhibition in altering glucose metabolism in response to metformin, proliferation and glucose uptake rates in the presence of rotenone were highly correlated (Figure 2B). Finally, we tested whether hypoxia affects metformin sensitivity. Hypoxic cells are more dependent on glycolysis as an adaptation to low oxygen (38), raising the possibility that hypoxic cells could be less sensitive to metformin. Indeed we found that PC3 cells in hypoxia are unaffected by metformin and that LNCaP cells were much less affected in hypoxia compared to normoxia (Figure 2C). Together, these data suggest that complex I inhibition by metformin was responsible for both altered glucose metabolism and the inhibition of cell proliferation in prostate cancer cell lines, and that the magnitude of the

effect on glucose metabolism is associated with the ability of metformin to suppress cell proliferation.

Metformin decreases entry of glucose carbon into the tricarboxylic acid cycle and increases glutamine anaplerosis

To investigate the metabolic effects of metformin beyond the macroscopic parameters of glucose uptake and lactate secretion, we cultured all three cell lines in the presence of uniformly labeled ^{13}C -glucose or uniformly labeled ^{13}C -glutamine and measured the incorporation of labeled carbon from either nutrient into the tricarboxylic acid cycle metabolite α -ketoglutarate by gas chromatography - mass spectrometry. In all cell lines tested, metformin decreased glucose contribution to α -ketoglutarate in a dose-dependent manner, while glutamine contribution to α -ketoglutarate increased in a reciprocal pattern (Figure 3A). As in most cultured cells, glutamine accounted for the majority of tricarboxylic acid cycle carbon in all three lines tested (39). However, variability in the degree of glucose contribution was observed. Interestingly, the magnitude of glucose carbon contribution to the tricarboxylic acid cycle was also correlated with the sensitivity of the cell lines to metformin; DU145 were the least sensitive to metformin and showed the least amount of glucose oxidation, PC3 cells had an intermediate phenotype, and the LNCaP cells displayed the most glucose oxidation and were most sensitive to metformin (Figure 3A).

Inhibition of electron transport has been reported to switch glutamine metabolism from an oxidative to a reductive pathway (40). Consistent with the inhibition of complex I by metformin in these cells, metformin treatment led to an increase in reductive glutamine metabolism. This was demonstrated by an increase in the M+5 mass isotopomer of citrate (Figure 3B) and further confirmed by measuring the contribution of glutamine labeled with ^{13}C only at the C5 position, to palmitate, which is lost if glutamine contributes oxidatively to fatty acids. Metformin increased the reductive contribution of glutamine to palmitate in a dose-dependent manner (Supplementary Figure 2), as well as the overall contribution of carbon to the tricarboxylic acid cycle. These data suggest that complex I inhibition by metformin decreased glucose entry into the tricarboxylic acid cycle and subsequently increased glutamine anaplerosis via reductive carboxylation to citrate. Furthermore, these phenomena track with sensitivity to metformin.

To further confirm that complex I inhibition by metformin results in the switch from glucose towards additional glutamine for fueling the tricarboxylic acid cycle, we measured glutamine contribution to α -ketoglutarate and the reductive glutamine contribution to palmitate in the presence of rotenone (Supplementary Figure 3). Similar to metformin, rotenone increased the contribution of glutamine carbon to the tricarboxylic acid cycle and reductive glutamine contribution to palmitate, providing further support for the role of complex I inhibition in the anti-proliferative effect for metformin in prostate cancer cells.

Decreased glutamine flux increases sensitivity to metformin

We next questioned whether increased glutamine metabolism is an adaptive response to complex I inhibition that might attenuate the anti-proliferative effect of metformin. To test this hypothesis, we took advantage of the small molecular inhibitors 968 and BPTES, both of which target glutaminase (15, 16) (Figure 4A). Glutaminase catalyzes the conversion of glutamine to glutamate, and the increased activity of this enzyme is at least partially responsible for elevated glutamine metabolism in cancer (16, 41). Consistent with increased glutamine metabolism being an adaptive response following metformin treatment, we detected larger decrease in cell number with the simultaneous administration of metformin and a glutaminase inhibitor (968 or BPTES) than observed with either metformin or glutaminase inhibitor acting alone for all three cell lines (Figure 4B, Supplementary Figure 4A). The combination of rotenone and 968 produced a similar effect (Figure 4C). Finally, we tested if withdrawal of glutamine from the medium yields increased metformin sensitivity. Since none of the prostate cancer cell lines proliferate in the absence of glutamine, we used Huh7 liver cancer cells, which have been previously reported to grow without glutamine (42). Indeed, Huh7 cells cultured without glutamine displayed a significantly increased metformin sensitivity (Supplementary Figure 4B).

Increased glutamine flux decreases metformin sensitivity

To test whether increased glutamine flux is sufficient to limit the anti-proliferative effect of metformin, we took advantage of the recently reported ability of mTOR to positively regulate net glutamine flux into the tricarboxylic acid cycle (43). mTOR was activated in these cells by a knockdown of its negative regulator *TSC2* (Supplementary Figure 5A). Net glutamine flux into the tricarboxylic acid cycle increased in the *TSC2* knockdown

cell lines by approximately twofold (Figure 5A). In line with the hypothesis that increased glutamine metabolism mitigated metformin toxicity, cells with activated mTOR were approximately threefold less sensitive to metformin when compared with control cells (Figure 5B). To further test the importance of glutamine conversion to α -ketoglutarate as an adaptive response to metformin, we used a cell-permeable form of α -ketoglutarate, dimethyl-ketoglutarate, in LNCaP cells following drug treatment. In line with our previous findings, dimethyl-ketoglutarate supplementation in the presence of metformin increased cell number by 30% (Supplementary Figure 5B). These results suggest that augmenting glutamine metabolism limited the anti-proliferative effect of metformin.

Metabolic alteration triggered by metformin in vivo match the in vitro response

To test whether our findings are relevant to tumors *in vivo*, we first established that metformin reaches prostate tumors in mice. Using the TRAMP prostate cancer mouse model, we determined that tumors of mice dosed three times with 500 mg/kg(body weight) metformin had taken up between 23-103 ng/mg(tissue protein) metformin into the prostate tumors. Metabolite analysis of both prostate cancer cell lines and prostate tumor tissue derived from TRAMP mice treated with vehicle or metformin showed that the most pronounced alteration in metabolite concentration as a result of metformin treatment was a fall in the citrate pool, which is associated with an activation of reductive glutamine metabolism (Fendt *et al.*, submitted) (Figure 6A). This effect of metformin was observed both in cells and in tissues (Figure 6A,B). This finding demonstrates that at least some of the metabolic effects of metformin *in vitro* are also observed in tumors *in vivo*.

Finally, we assessed the extent to which reductive glutamine metabolism was impacted along with glucose metabolism by the presence of metformin *in vivo*. To trace glutamine metabolism in tumors, three consecutive intra-peritoneal injections of uniformly labeled ^{13}C -glutamine were administered to TRAMP mice treated with metformin or vehicle. Following tumor harvest the enrichment of M+5 citrate was measured in prostate tumors as a surrogate for reductive glutamine metabolism. We found a significant increased enrichment of M+5 citrate in different parts of the prostate from metformin treated animals (Figure 6C). This increase in M+5 citrate enrichment was accompanied by a decrease in the relative citrate pool size (Figure 6D). In conclusion these data show that the metabolic effects of metformin in tumor cells *in vitro* extend to similar responses in tumors *in vivo*.

Discussion

The oral anti-diabetic drug metformin is beneficial for at least a subset of cancer patients, but whether metformin can directly affect metabolism in tumor cells was not known. We systematically dissected the metabolic alterations metformin triggers in prostate cancer cell lines through complex I inhibition and found that this results in an adaptive increase in glutamine metabolism (Figure 7). It is possible that in addition to the adaptive response in glutamine metabolism described here in response to metformin, energy homeostasis regulation by AMPK following metformin treatment further diminishes the effect of the drug as shown recently for non small lung carcinoma (44). Our finding of an adaptive response in glutamine metabolism also complements a recent study showing that cell lines grown on glutamine as the major carbon source are sensitive to metformin (45). The finding of this study can be explained by our data since

we show that metformin leads to reductive glutamine metabolism and consequently leads to a dramatic decrease in ATP production by the tricarboxylic acid cycle. Importantly, we additionally show that inhibition of glutamine metabolism can promote metformin toxicity, suggesting that therapy with an agent that limits glutamine metabolism may potentiate the effect of metformin.

Our *in vivo* model demonstrated that mice tolerate metformin at a dose of 500 mg/kg/day, considerably higher than the conventional antidiabetic dose in humans of ~20 mg/kg/day, which led to accumulation of the drug to ~ 60 ng/mg (tissue protein) in cancer tissue. This exposure level was observed to have *in vivo* metabolic effects on prostate tissues that matched our *in vitro* data. We demonstrate for the first time that this is correlated with effects on cellular carbon metabolism, specifically a dependency on reductive glutamine metabolism. This finding suggests that high-dose biguanide exposure might be a method to therapeutically induce metabolic stress in a subset of tumors, either alone or in rational combinations. It is important to recognize, however, that the effect of conventional antidiabetic metformin doses currently being examined for antineoplastic activity in clinical trials may have a smaller effect on metabolism. Our work suggests that a dose escalation study of biguanides may be warranted to determine if the dose range with more profound direct effects on cancer metabolism can safely be administered to humans.

Because the anti-proliferative effect of metformin tracks with its ability to inhibit complex I, and this effect is mitigated by increased glutamine metabolism, it is possible that cells with a high dependence on oxidative glucose metabolism and low dependence on

glutamine metabolism may be most sensitive to this drug. Whether these findings will extend to non-prostate cancer cases remains to be determined. Importantly, these results do not rule out non-cancer cell autonomous effects in patients. However, a direct effect on prostate cancer cells may be beneficial. Prostate cancer cells, compared to non-transformed prostate epithelium *i*) reactivate aconitase activity leading to increased citrate oxidation (46), and, *ii*) display high rates of *de novo* lipid synthesis (47, 48). These features may predict beneficial therapeutic effects from combining metformin and glutaminase inhibitors as we found cell lines displaying an oxidative metabolism were most sensitive to metformin. Furthermore, cells adapt to metformin treatment by increasing reductive glutamine metabolism, which directly effects the carbon source selection for fatty acid synthesis. Thus, glutaminase inhibitors might potential combination treatments that specifically enhance the beneficial effect of metformin in cancer treatment.

Acknowledgements

We are grateful to Agios Pharmaceuticals for providing BPTES. SMF is supported by the German Research Foundation (DFG), grant FE1185. LG is supported by the NIH and the Glenn Foundation for Medical Research. LCC is a cofounder of Agios Pharmaceuticals, a company that seeks to develop novel therapeutics that target cancer metabolism. MGVH is a consultant and SAB member for Agios. AC is supported by a fellowship from the LAM Foundation. MGVH acknowledges NIH grant support from 5-P50-090381-09 and 5-P30-CA14051-39 and support from the Burrough's Wellcome

Fund, the Smith Family, and the Damon Runyon Cancer Research Foundation. We also acknowledge NIH grant 1R01DK075850-01 and 1R01CA160458-01A1 to GS.

Authors Contribution

SMF conceived and designed the study, performed *in vitro* labeling and proliferation experiments, helped with mouse experiments, measured cell lines and tumor tissue mass distributions and/or metabolite levels, analyzed all data and drafted the manuscript. ELB helped designing the study, helped with mouse experiments, generated TSC2 knockdown cell lines and helped drafting the manuscript. MAK performed HPLC and extracellular metabolite measurements and helped with proliferation experiments. SMD performed mouse labeling experiments. GJW had the initial idea to investigate prostate cancer and metformin. BF and JRM helped with mouse experiments. MS performed metformin measurements. GB helped with mouse experiments. AP provided initial TRAMP mice and helped with mouse experiments. AC, MJB, JB, LCC, LG, and MNP provided advice and support. AFO contributed to the initial idea to investigate prostate cancer and metformin and provided support. MGVH and GS provided conceptual advice and helped drafting the manuscript.

References

1. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer cell*. 2008;13:472-82.
2. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell*. 2008;134:703-7.
3. Hanahan D, Weinberg Ra. Hallmarks of cancer: the next generation. *Cell*. 2011;144:646-74.
4. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell metabolism*. 2008;7:11-20.
5. Lunt SY, Vander Heiden MG. Aerobic Glycolysis: Meeting the Metabolic Requirements of Cell Proliferation. *Annual Review of Cell and Developmental Biology*. 2011;27:441-64.

6. Cairns Ra, Harris IS, Mak TW. Regulation of cancer cell metabolism. *Nature Reviews Cancer*. 2011;11:85-95.
7. DeBerardinis RJ, Cheng T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene*. 2010;29:313-24.
8. Lu W, Pelicano H, Huang P. Cancer metabolism: is glutamine sweeter than glucose? *Cancer cell*. 2010;18:199-200.
9. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends in biochemical sciences*. 2010;35:427-33.
10. Kaelin WJ, Thompson CB. Q & A Clues from cell metabolism. *Nature*. 2010;465:3-5.
11. Kung C, Hixon J, Choe S, Marks K, Gross S, Murphy E, et al. Small Molecule Activation of PKM2 in Cancer Cells Induces Serine Auxotrophy. *Chemistry & biology*. 2012;19:1187-98.
12. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. *Nature Reviews Drug Discovery*. 2011;10:671-84.
13. Anastasiou D, Yu Y, Israelsen WJ, Jiang J-K, Boxer MB, Hong BS, et al. Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. *Nature Chemical Biology*. 2012;1-9.
14. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:2037-42.
15. Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, et al. Glucose-Independent Glutamine Metabolism via TCA Cycling for Proliferation and Survival in B Cells. *Cell Metabolism*. 2012;15:110-21.
16. Wang J-B, Erickson JW, Fuji R, Ramachandran S, Gao P, Dinavahi R, et al. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer cell*. 2010;18:207-19.
17. Michelakis ED, Webster L, Mackey JR. Dichloroacetate (DCA) as a potential metabolic-targeting therapy for cancer. *British journal of cancer*. 2008;99:989-94.
18. Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy a, Niven E, et al. Metabolic modulation of glioblastoma with dichloroacetate. *Science translational medicine*. 2010;2:31ra4.
19. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, et al. A mitochondria-K⁺ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer cell*. 2007;11:37-51.
20. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. *Clinical science (London, England : 1979)*. 2012;122:253-70.
21. Bodmer M, Meier C, Krahenbuhl S, Jick SS, Meier CR. Long-Term Metformin Use Is Associated With Decreased Risk of Breast Cancer. *Diabetes Care*. 2010;33.
22. Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO, Bilo HJ. Metformin Associated With Lower Cancer Mortality in Type 2 Diabetes. *Diabetes Care*. 2010;33:322-6.
23. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris A, D, Evans J, MM. New Users of Metformin Are at Low Risk of. *Diabetes Care*. 2009;32.
24. Wright JL, Stanford JL. Metformin use and prostate cancer in Caucasian men: results from a population-based case-control study. *Cancer causes & control : CCC*. 2009;20:1617-22.
25. Zoulay LAA. Metformin and the Risk of Cancer. *Diabetes Care*. 2012;35:2665-73.
26. Pollak MN. Investigating Metformin for Cancer Prevention and Treatment: The End of the Beginning. *Cancer discovery*. 2012.
27. Ben Sahra I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, et al. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer research*. 2010;70:2465-75.
28. Ben Sahra I, Laurent K, Loubat a, Giorgetti-Peraldi S, Colosetti P, Auburger P, et al. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. *Oncogene*. 2008;27:3576-86.

29. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberge P, et al. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer research*. 2011;71:4366-72.
30. Antoniewicz MR, Kelleher JK, Stephanopoulos G. Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. *Metabolic engineering*. 2007;9:68-86.
31. Antoniewicz MR, Kelleher JK, Stephanopoulos G. Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements. *Metabolic engineering*. 2006;8:324-37.
32. Young JD, Walther JL, Antoniewicz MR, Yoo H. An Elementary Metabolite Unit (EMU) Based Method of Isotopically Nonstationary Flux Analysis. *Biotechnology*. 2008;99:686-99.
33. Noguchi Y, Young JD, Aleman JO, Hansen ME, Kelleher JK, Stephanopoulos G. Effect of anaplerotic fluxes and amino acid availability on hepatic lipooptosis. *The Journal of biological chemistry*. 2009;284:33425-36.
34. Nanchen A, Fuhrer T, Sauer U. Determination of metabolic flux ratios from ¹³C-experiments and gas chromatography-mass spectrometry data: protocol and principles. *Methods in molecular biology* (Clifton, NJ). 2007;358:177-97.
35. Kharroubi AT, Masterson TM, Aldaghlis TA, Kennedy KA, Kelleher JK. Isotopomer spectral analysis of triglyceride fatty acid synthesis in 3T3-L1 cells. *The American journal of physiology*. 1992;263:E667-75.
36. Yoo H, Antoniewicz MR, Stephanopoulos G, Kelleher JK. Quantifying reductive carboxylation flux of glutamine to lipid in a brown adipocyte cell line. *The Journal of biological chemistry*. 2008;283:20621-7.
37. <http://pga.mgh.harvard.edu/primerbank/citation.html>.
38. Mucaj V, Shay JE, Simon MC. Effects of hypoxia and HIFs on cancer metabolism. *International journal of hematology*. 2012;95:464-70.
39. Deberardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. *Current opinion in genetics & development*. 2008;18:54-61.
40. Mullen AR, Wheaton WW, Jin ES, Chen P-H, Sullivan LB, Cheng T, et al. Reductive carboxylation supports growth in tumour cells with defective mitochondria. *Nature*. 2011;481:385-8.
41. Tennant DA, Durán RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. *Nature reviews Cancer*. 2010;10:267-77.
42. Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature*. 2011;481:380-4.
43. Csibi A, Fendt S-M, Li C, Pouligiannis G, Choo AJ, Chapski DJ, et al. The mTORC1 Pathway Stimulates Glutamine Metabolism and Cell Proliferation by Repressing SIRT4. *Cell*. 2013;153:840-54.
44. Shackelford DB, Abt E, Gerken L, Vasquez DS, Seki A, Leblanc M, et al. LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. *Cancer cell*. 2013;23:143-58.
45. Javeshghani S, Zakikhani M, Austin S, Bazile M, Blouin M-J, Topisirovic I, et al. Carbon source and myc expression influence the antiproliferative actions of metformin. *Cancer research*. 2012.
46. Mycielska ME, Patel A, Rizaner N, Mazurek MP, Keun H, Patel A, et al. Citrate transport and metabolism in mammalian cells: prostate epithelial cells and prostate cancer. *BioEssays : news and reviews in molecular, cellular and developmental biology*. 2009;31:10-20.
47. Beckers A, Organe S, Timmermans L, Scheys K, Peeters A, Brusselmans K, et al. Chemical inhibition of acetyl-CoA carboxylase induces growth arrest and cytotoxicity selectively in cancer cells. *Cancer research*. 2007;67:8180-7.
48. Schrijver ED, Brusselmans K, Heyns W, Cells C. RNA Interference-mediated Silencing of the Fatty Acid Synthase Gene Attenuates Growth and Induces Morphological Changes and Apoptosis of LNCaP Prostate Cancer Cells RNA Interference-mediated Silencing of the Fatty Acid Synthase Gene Attenuates

Figure Legend

Figure 1: Metformin decreases proliferation and promotes glucose fermentation in prostate cancer cell lines. (A) Proliferation, glucose uptake rate and lactate secretion rate of cell lines treated with metformin. (B) Correlation between absolute alteration in proliferation and glucose uptake or lactate secretion rate for different metformin concentrations compared to the condition with no metformin added. All error bars indicate the standard deviation. p-values lower 0.1 are depicted in the figure. Standard deviation, correlation coefficient R, and p-values were calculated from three biological replicates.

Figure 2: The metabolic response to metformin is consistent with mitochondrial complex I inhibition. (A) Proliferation of cell lines treated with the complex I inhibitor rotenone. (B) Correlation between absolute alteration in proliferation and glucose uptake rate metformin (light blue) or rotenone (dark blue) treated cells compared to the condition with no drug added. (C) Sensitivity of cell lines towards metformin (2.5mM) in normoxia compared to hypoxia. All error bars indicate the standard deviation. Standard deviation, correlation coefficient R, and p-values were calculated from three biological replicates.

Figure 3: Metformin decreases entry of glucose carbon into the tricarboxylic acid cycle and increases glutamine anaplerosis. (A) Glucose (upper panel) and glutamine (lower panel) contribution to the tricarboxylic acid cycle in prostate cancer cell lines measured

by the incorporation of [U-¹³C] labeled glucose or [U-¹³C] labeled glutamine into α -ketoglutarate. (B) The increase in glutamine contribution leads to reductive instead of oxidative fueling as shown by the increase in M+5 citrate from [U-¹³C] labeled glutamine. All error bars indicate the standard deviation. Standard deviation and p-values were calculated from four replicates derived from two independent experiments.

Figure 4: Decreased glutamine flux increases metformin sensitivity. (A) Enzymatic conversion steps from glutamine to α -ketoglutarate including known inhibitors of the pathway. Relative cell count of cell lines treated with a combination of metformin (B) or rotenone (C) with the glutaminase inhibitor 968. Cell counts were normalized to the condition with no metformin and no 968 added. Standard deviation and p-values were calculated from three biological replicates.

Figure 5: Increased glutamine flux decreases metformin sensitivity. (A) Glutamine flux of TSC2 knockdown cell lines relative to control cell line. Control cells for knockdown were infected with an empty plasmid. (B) Metformin sensitivity of TSC2 knockdown cell lines, which exhibit increased glutamine flux based on cell counts. All error bars indicate the standard deviation. Standard deviation and p-values were calculated from at least three biological replicates.

Figure 6: Metabolic alteration triggered by metformin *in vivo* match the *in vitro* response. (A) Relative citrate pool size in prostate cancer cell lines normalized to the condition with no metformin added. Standard deviation and p-values are calculated from four biological replicates. (B) Citrate pool size in prostate tissue from TRAMP mice treated with (four animals) or without (three animals) metformin (left panel). One to two

different parts of tissue were analyzed per animal. Average of citrate pool size for TRAMP mice treated with or without metformin (right panel) Enrichment of M+5 citrate (C), which is indicative for reductively utilized glutamine, and relative citrate pool size (D) of prostate tissue from TRAMP mice treated with (one animal) or without (two animals) metformin. Three to four different parts of the prostate were analyzed per animal. All error bars indicate the standard deviation.

Figure 7: Metformin attenuates proliferation in prostate cancer cells by inhibiting Complex I of the respiratory chain. This effect of metformin increases the dependency of the prostate cancer cell lines on reductive glutamine anaplerosis of the tricarboxylic acid cycle. Consequently, a combination therapy between metformin and inhibitors of the glutamine pathway might have a therapeutic benefit.

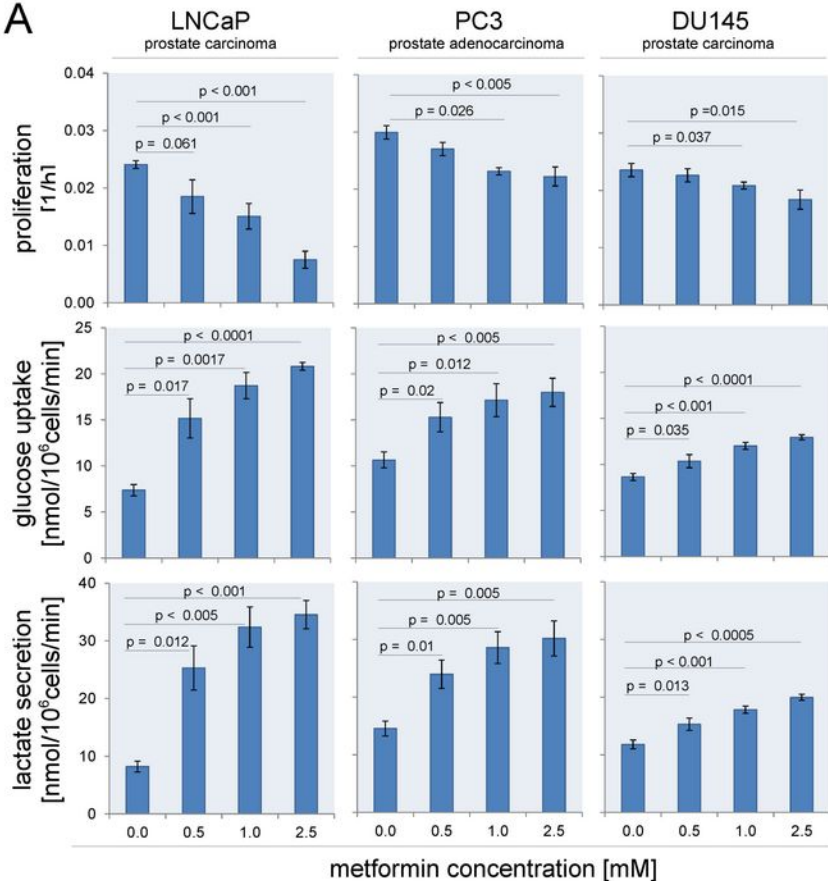
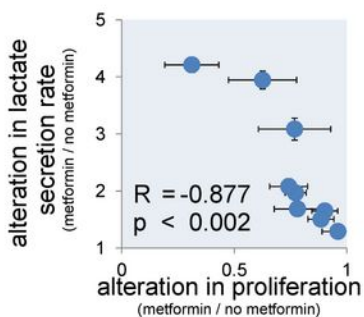
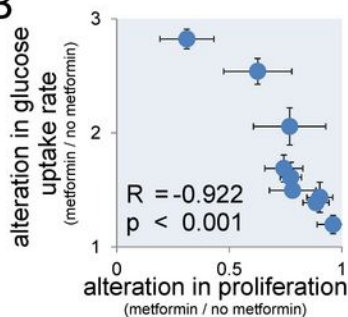
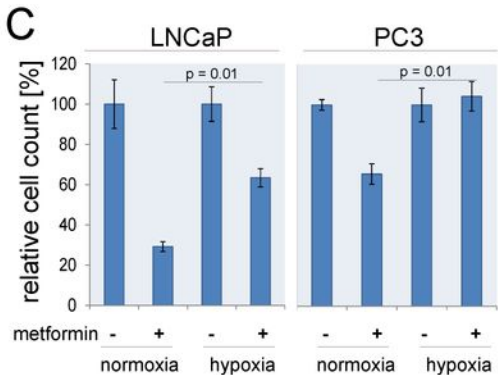
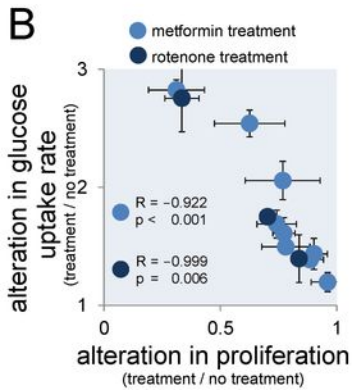
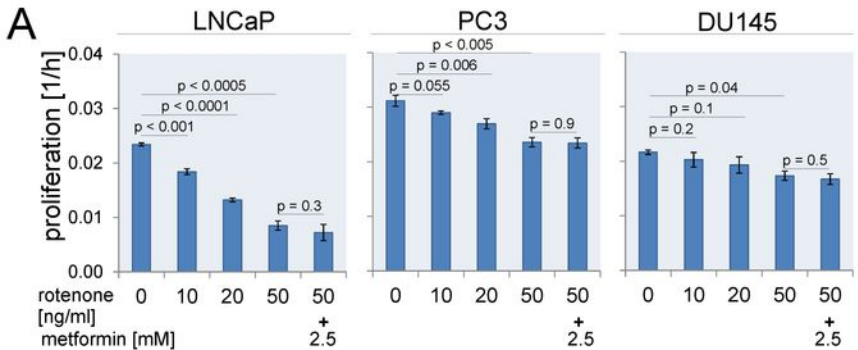
A**B**

Figure 2



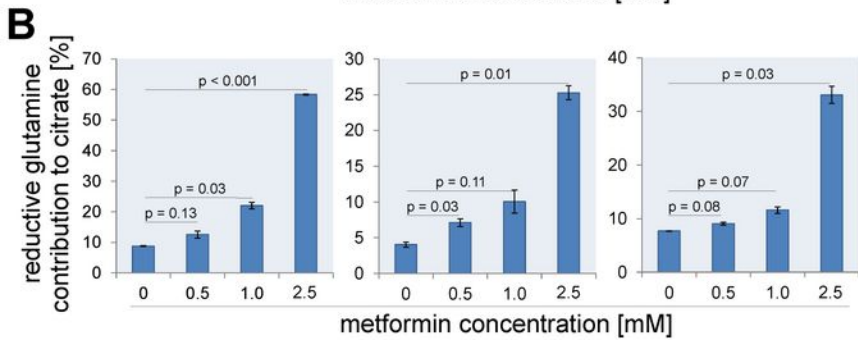
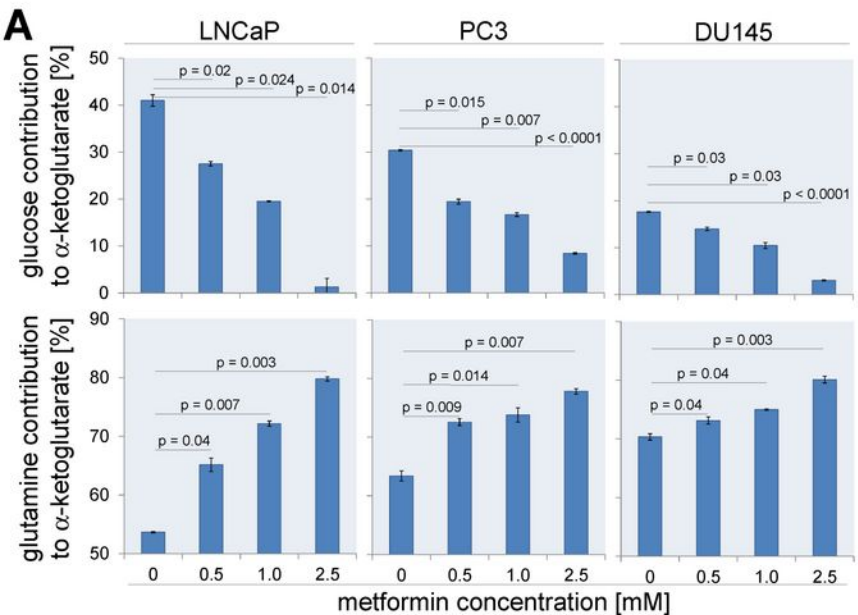


Figure 4

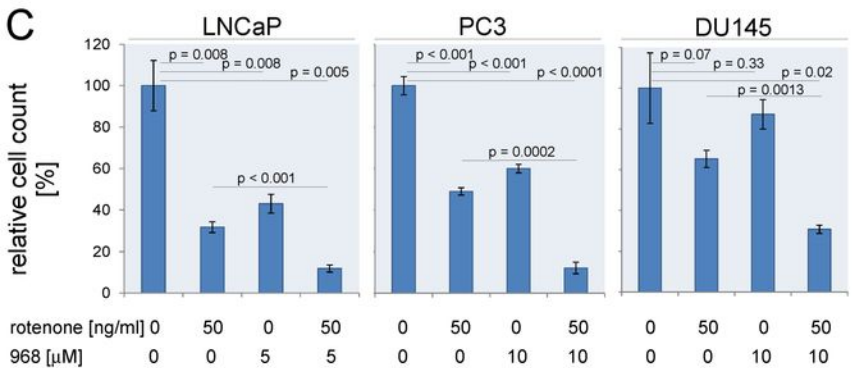
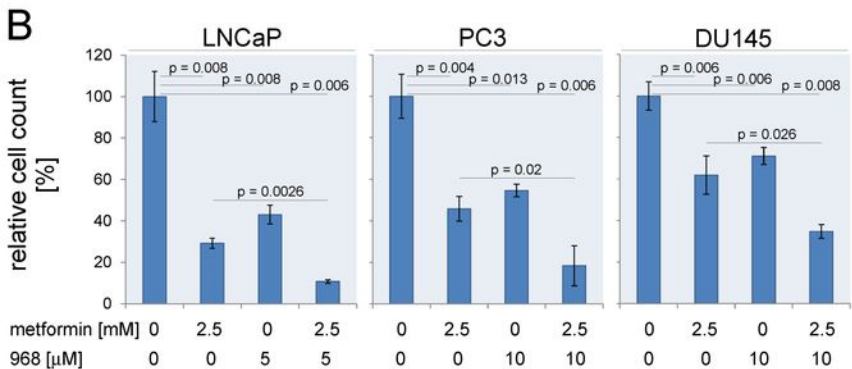
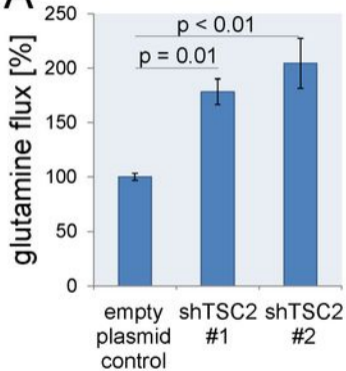


Figure 5

A



B

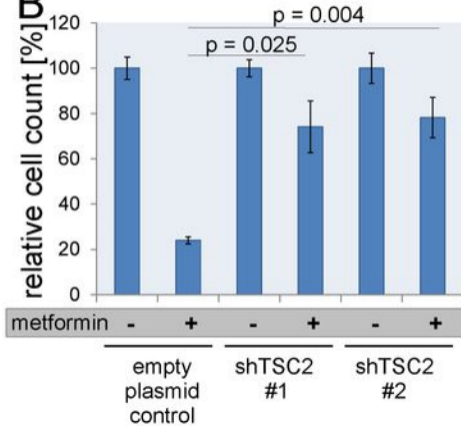


Figure 6

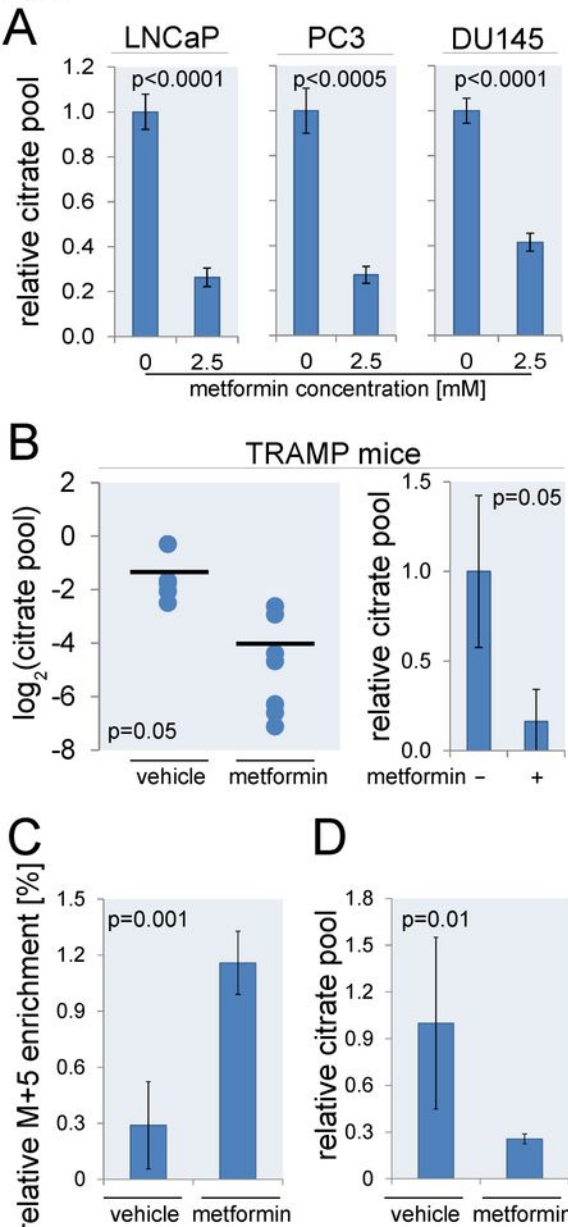


Figure 7

