1 **Title:** EMT-derived alterations in glutamine metabolism sensitize mesenchymal breast cells
2 to mTOR inhibition

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4 **Running title:** EMT-induced drug sensitivity in breast tissue

5

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30 The authors declare no conflicts of interest.

Abstract

Epithelial-to-mesenchymal transition (EMT) is a fundamental developmental process with strong implications in cancer progression. Understanding the metabolic alterations associated with EMT may open new avenues of treatment and prevention. Here, we used ^{13}C carbon analogs of glucose and glutamine to examine differences in their utilization within central carbon and lipid metabolism following EMT in breast epithelial cell lines. We found that there are inherent differences in metabolic profiles before and after EMT. We observed EMT-dependent re-routing of the TCA-cycle, characterized by increased mitochondrial IDH2 - mediated reductive carboxylation of glutamine to lipid biosynthesis with a concomitant lowering of glycolytic rates and glutamine-dependent glutathione (GSH) generation. Using weighted correlation network analysis, we identified cancer drugs whose efficacy against the NCI-60 Human Tumor Cell Line panel is significantly associated with GSH abundance and confirmed these *in vitro*. We report that EMT-linked alterations in GSH synthesis modulate the sensitivity of breast epithelial cells to mTOR inhibitors.

Implications: EMT in breast cells causes an increased demand for glutamine for fatty acid biosynthesis, altering its contribution to glutathione biosynthesis which sensitizes the cells to mTOR inhibitors.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) is a fundamental developmental process where tightly bound epithelial cells differentiate into migratory mesenchymal cells that can relocate into adjacent or distant tissues. This process is vital for tissue restructuring during embryonic development and is also necessary for proper wound healing in adult tissue. EMT has strong implications in cancer progression and metastasis where primary tumor cells of epithelial origin can take on a motile phenotype with the ability to migrate through the body and establish secondary tumors at distant locations [1].

Metabolic reprogramming is recognized as one of the ten cancer hallmarks as proposed by Hanahan and Weinberg [2]. In contrast to rapidly dividing cancer cells, a mesenchymal phenotype faces a different set of metabolic requirements whose relation to malignant

60   transformation has been intensely studied and associated with enhanced glycolysis,

61   increased glutaminolysis, nucleotide metabolism and abnormal choline metabolism [3–5].

62   Quantitative understanding of the metabolic requirements of mesenchymal cells is however

63   lacking, particularly the changes to the turnover and quantity of metabolites involved in

64   xenobiotic clearance, *i.e.* the drug response of cells. Cancer cells that undergo EMT have

65   increased resistance to various drugs [6–8], which indicates that the xenobiotic clearance of the

66   cells is altered. There are three phases involved in the metabolism of xenobiotics: I)

67   Modification, II) Conjugation and III) Excretion. Conjugation involves the binding of particular

68   metabolites (*e.g.* glutathione, UDP-glucuronate, PAPS, S-adenosylmethionine) to a

69   xenobiotic compound [9], which leads to the assumption that the availability of these

70   metabolites within cells influences the activity of the drugs. Therefore, accurate metabolic

71   measurements of EMT may contribute to better understanding of the drug resistance of

72   cancer cells and lead to novel therapeutic approaches aimed at eliminating metastatic

73   cancer cells.

74   We have previously used both ultra-performance liquid chromatography coupled mass

75   spectrometry (UPLC-MS) and NMR to study EMT and cancer metabolism [10–12]. Integrated

76   analyses of these metabolomics data with transcriptomic and proteomic data within

77   genome-scale metabolic models predicted metabolic differences that occur following EMT in

78   breast epithelium [12]. These included alterations to glycolysis, the pentose phosphate

79   pathway, TCA cycle and fatty acid synthesis. Although these models provided useful insights

80   into metabolic alterations associated with EMT, they lacked accuracy in predicting internal

81   fluxes in a quantitative manner in the compartmentalized central carbon metabolism.

82   In order to better understand the metabolic changes that accompany EMT we characterized

83   the internal flow of metabolites in D492 breast epithelial cells and their mesenchymal

84   variant, D492M, to determine metabolic changes within central carbon metabolism

85   following EMT in breast epithelial cells. We performed stable isotope tracing of ^{13}C labeled

86   glucose and two separate ^{13}C labeled glutamine analogs. UPLC-MS and NMR were used to

87   measure label incorporation into metabolites associated with central carbon metabolism

88   and lipid biosynthesis. We subsequently performed shRNA lentiviral silencing of key genes to

89   further elucidate their role in EMT metabolic re-programming. Finally, using an integrated

90   network analysis of the NCI-60 Human Tumor Cell Line panel and an untargeted

91 metabolomic analysis, we investigate how the EMT-dependent re-routing of central carbon

92 metabolism affects drug responsiveness in D492 and D492M cells.

93

2. Materials and methods

94

2.1 Cell culture

95

96 D492 and D492M cells were kindly provided by the Stem Cell Research Unit, University of

97 Iceland, and were cultured in DMEM/F12-based medium H14 at 37°C in 5% CO2 as

98 previously described [13]. All experiments were performed within 4 passages from thawing,

99 within the range of 30-40 passages in total. For the labeling experiments, the cells were fed

100 with medium containing 100% ^{13}C-labeled glutamine at the 1 or 5 position (Cambridge

101 Isotope Laboratories, Inc., MA, USA) or ^{13}C-labeled glucose at the 1 and 2 positions

102 (Cambridge Isotope Laboratories, Inc.). Cells were screened for *Mycoplasma* infections every

103 month using PCR-based tests at the Biomedical Center, University of Iceland.

104

2.2 Lentiviral shRNA production and transduction

105

106 HEK293T cells were transfected using TurboFect transfection reagent (Thermo Fisher

107 Scientific, MA, USA) at 80% confluency in T25 cell culture vessels. The cells were then

108 incubated at 37°C and 5% CO2. Viral supernatant was collected at two timepoints, the first

109 being after 48 hours in culture, and the second 72 hours after changing medium at the first

110 timepoint. The viral supernatant was filtered through a 0.45 μm filter using a syringe and

111 stored at -20°C until usage. The lentiviral vectors were acquired from GeneCopoeia. They

112 contained an shRNA construct for the selective targeting of IDH2. The construct was based

113 on a psi-LVRH1MH vector with an mCherry fluorescent reporter, resistance against

114 hygromycin B and the identical hairpin sequence TCAAGAG. The target sequence was IDH2

115 5'GTACAAGGCCACAGACTTTGT-3'. The D492 and D492M cell lines were transduced using 1

116 mL of filtered viral supernatant at 70% confluency and incubated at 37°C and 5% CO2 for 24

117 hours, at which timepoint the medium was changed to fresh H14 medium. After further 48

118 hours, the cells were grown in medium containing hygromycin B (200 μg/mL) for three

119 weeks to selectively grow cells containing the shRNA construct.

120

2.3 Real-Time PCR

Whole-cell RNA was extracted using Tri-Reagent (Thermo Fisher Scientific, AM9738). Reverse

transcription was performed using High-Capacity cDNA Reverse Transcription kit (Thermo

Fisher, 4368814). The expression of the genes *IDH1* and *IDH2* was quantified, where *ACTB*

(Beta-actin) and was used as an endogenous reference gene. The primers for *IDH1*, *IDH2* and

ACTB were designed using the Primer3 software in the Benchling website

(https://benchling.com). Primers were designed to span exon junctions and have a melting

temperature above 55°C. The expression of IDH2 and IDH1 was assessed using real-time PCR

(qPCR). Real-time quantitative PCR reactions were carried out using Luna® Universal qPCR

Master Mix (New England Biolabs, MA, USA) according to manufacturer's instructions on a

Bio-Rad CFX384 Touch™ Real Time System (Bio-Rad, CA, USA). Gene expression levels were

determined using CFX Manager Software (Bio-Rad) and differences in relative expression

were estimated with the $2^{\Delta\Delta Ct}$ method. The primer sequences used for quantifying the gene

expression were: IDH1-forward 5'- CGACATGGTGGCCCAAGCTATG-3', IDH1-reverse 5'-

TCATGCCGAGAGAGCCATACCC-3', IDH2-forward 5'-ATGAGGCCCGTGTGGAAGAGTT-3', IDH2-

reverse 5'-CAGATGATGGGCTCCCGGAAGA-3', ACTB-forward 5'-

CTTCCTGGGTGAGTGGAGACTG-3' and ACTB-reverse 5'-GAGGGAAATGAGGGCAGGACTT-3'.

138

2.4 Proliferation assay

Cells were seeded in quadruplicates in 48-well plates (10.000 cells/well). They were grown in

a large chamber incubation system (PeCon GmbH, Erbach, Germany) at 37°C in 5% CO2 and

imaged for 12-72 hours using Leica DMI6000B. Images of cells were opened with Fiji [14],

where the cells were counted with the help of an in-house script.

144

2.5 Detection of intracellular NADP$^+$ and NADPH

NADP+ and NADPH were measured using NADP/NADPH-Glo™ Assay (G9081, Promega,

Madison, WI). Cells were seeded in triplicates in opaque 96-well plates (10.000 cells/well)

and incubated at 37°C in 5% CO2. After 24 hours, the medium was removed, cells were

149   washed with cold PBS and then supplemented with 50 µL PBS and 50 µL 1% DTAB in 0.2N

150   NaOH solution to induce cell lysis. Next steps were according to manufacturer's protocol.

151   The luminescence was measured 50 minutes after addition of the NADP-NADPH-Glo™

152   Detection reagent in SpectraMax®M3 Microplate Reader (Molecular Devices, CA, USA).

153

154   **2.6 Nuclear magnetic resonance (NMR)**

155   For NMR analysis, D492 and D492M cells were cultured in T225 flasks in supplemented

156   DMEM/F12 until they reached approximately 70% confluency. Cells were then fed with

157   either 1,2-^{13}C glucose or 1-^{13}C glutamine for 6 hours. Parallels without ^{13}C tracers were also

158   cultured. Culture medium was collected after incubation. Methanol extracts from glucose-

159   and glutamine-labeled cells were prepared as described previously [15]. The cell extracts were

160   freeze dried prior to NMR analysis. For NMR, freeze-dried cell extracts were dissolved in 600

161   µL D_2O in PBS while culture medium (500 µL) was diluted with in D_2O-based PBS (100 µL).

162   NMR analysis was performed using a 600 MHz Bruker Avance III NMR spectrometer (Bruker

163   Biospin GmbH, Germany), equipped with a 5 mm QCI Cryoprobe with integrated, cooled

164   preamplifiers for ^{1}H, ^{2}H and ^{13}C. Proton spectra were acquired at 300 K using 1D NOESY

165   (Bruker: noesygppr1d) with presaturation and spoiler gradients as previously described [16].

166   The spectra were collected with 32 scans and 4 dummy scans. The acquisition time was 2.73

167   s and relaxation delay 4 s, measuring the FID via collection of 64 K complex data points. The

168   ^{1}H spectra were Fourier transformed with a 0.3 Hz exponential line broadening and the

169   chemical shift was calibrated to alanine at 1.48 ppm. ^{1}H spectra from D492 (n=5) and D492M

170   (n=6) cells were transferred to MATLAB R2017a for multivariate data analysis. The spectra

171   were baseline corrected using asymmetric least squares method [17] and peak aligned using

172   icoshift [18]. The water peak and areas in the spectra with contamination and noise only were

173   removed. All spectra were mean normalized and mean centered. Principal component

174   analysis (PCA) was performed using PLS toolbox v8.2.1 (Eigenvector Research, WA, USA).

175   Proton decoupled ^{13}C spectra (Bruker: zgpg30) were acquired using a power gated

176   decoupling sequence with a 30° pulse angle as described in Bettum *et al.* [19]. The spectra

177   were collected with either 4 K (for 1,2-^{13}C-glucose) or 16 K (for 1-^{13}C-glutamine) scans and 16

178   dummy scans. The acquisition time was 1.65 s, relaxation delay 0.5 s, measuring the FID via

179   collection of 96 K complex data points over a sweep width of 197.175 ppm. The ^{13}C spectra

180 were Fourier transformed with a 3.0 Hz exponential line broadening and the chemical shift

181 was calibrated to the 3-^{13}C-alanine peak at 19.0 ppm or 1-^{13}C-glutamine peak at 176.4 ppm.

182 ^{13}C-labeled metabolites downstream from the tracers were identified by comparing [13]C

183 spectra with natural abundance spectra acquired under the same conditions. Levels of

184 selected metabolites in the extracts were semi-quantitatively assessed by integration of

185 resonance signals using TopSpin 4.0.8 (Bruker Biospin GmbH) after correcting for natural

186 abundance levels. The ^{13}C spectra were normalized to the total area under the curve (AUC)

187 in the ^{1}H spectra acquired from the same sample.

188

189 **2.7 Metabolomics**

190 *2.7.1 Sample extraction*

191 Polar and non-polar metabolites were extracted from cell cultures by

192 methanol/chloroform/water extraction. Cells were harvested in ice-cold methanol, vortexed

193 vigorously and let stand on ice for 10 minutes. Equal amounts of water and chloroform were

194 added to a final composition of 1:1:1 ($CH_3OH:H_2O:CHCl_3$), vortexed and left to stand

195 overnight at 4°C. The organic phase (lipids) was collected into a glass vial and dried in a

196 stream of N_2 and stored under N_2 at -80°C until analysis. The aqueous phase (polar

197 metabolites) was stored at -80°C and evaporated in a miVac concentrator (SP Scientific,

198 Warminster PA, USA) before analysis.

199 *2.7.2 UPLC-MS*

200 Before UPLC-MS analysis, the organic phase was reconstituted in MTBE before a methanol

201 solution containing 1M NaOH was added (10:1 v/v, respectively). This was incubated for 3.5

202 hours at 37°C, when 1 μL formic acid was added (to neutralize the solution), the samples

203 were dried in a stream of N_2 and then resuspended in isopropanol:ACN:H_2O (6:9:1, v/v/v).

204 The aqueous phase (metabolites) were reconstituted in isopropanol:ACN:H_2O (2:1:1, v/v/v).

205 Ultra performance liquid chromatography (UPLC) (Acquity, Waters, Manchester, UK) was

206 coupled with a quadrupole-time of flight mass spectrometer (Synapt G2, Waters,

207 Manchester, UK). For the lipid samples, chromatographic separation was achieved as

208 previously described [20]. For the metabolomic samples, chromatographic separation was

209 achieved by hydrophilic interaction liquid chromatography (HILIC) using an Acquity amide

column, 1.7 μm (2.1 x 150 mm) (Waters, Manchester, UK). All samples were analyzed in positive ionization and negative ionization mode using acidic and basic chromatographic conditions. In positive mode and in negative acidic conditions, mobile phase A was 100% ACN and B was 100% H2O both containing 0.1% formic acid. The following elution gradient was used: 0 min 99% A; 7 min 30% A; 7.1 min 99% A; 10 min 99% A. In negative mode basic conditions, mobile phase A contained ACN:sodium bicarbonate 10 mM (95:5) and mobile phase B contained ACN:sodium bicarbonate 10 mM (5:95). The following elution gradient was used: 0 min 99% A; 6 min 30% A; 6.5 min 99% A; 10 min 99 % A. In all conditions, the flow rate was 0.4 mL/min, the column temperature was 45°C, and the injection volume was 3.5 μL. The mass spectrometer was operated using a capillary voltage of 1.5 kV, the sampling cone and the extraction cone were of 30 V and 5 V. The cone and the desolvation gas flow were 50 L/h and 800 L/h, while the source and desolvation gas temperature were 120°C and 500°C. MS spectra were acquired in centroid mode from m/z 50 to 1000 using scan time of 0.3 s. Leucine enkephalin (2 ng/μL) was used as lock mass (m/z 556.2771 and 554.2615 in positive and negative experiments respectively). A typical analytical block consisted of: 1) pooled QC samples to equilibrate the system, 2) calibrators, 3) samples and spiked pooled QC samples and 4) calibrators.

2.7.3 Data analysis

TargetLynx (v4.1; Waters) was used to integrate chromatograms of all isotopologues of the metabolites of interest. Ion chromatograms were extracted using a window of 0.02 mDa which was centered on the expected m/z for each targeted isotopologue. The output was a mass distribution vector (MDV) describing the relative amount of each detected isotopologue of the metabolite. Ion chromatograms of isotopologues of interest extracted and corrected for abundance of naturally occuring isotopes using the IsoCor software [21]. When calculating the total contribution (TC) of carbon sources to metabolites, we used the following equation [22] :

$$TC = \frac{\sum_{i=0}^{n} i \cdot m_i}{n} \tag{1}$$

Where n is the number of C atoms in the metabolite, i represents the isotopologues and m is the relative fraction of the isotopologues.

238 In order to evaluate the percentage of glucose that enters the oxidative part of the pentose

239 phoshate pathway, and re-enters glycolysis, we utilized a formula from Lee *et al.* [23]:

$$PPP_{Cycle} = \frac{m1/m2}{3 + m1/m2} \qquad (2)$$

240 In equation 2, m1 and m2 are the fractional abundances of M+1 and M+2 lactate

241 isotopologues, respectively, (*e.g.* from **Figure S2**).

242

243 **2.8 RNA sequencing**

244 Quantified transcript pseudocounts from kallisto [24] were obtained for D492 and D492M in

245 triplicates from Briem *et al.* (from the authors) [25]. These data were imported into R and

246 simultaneously log2-transformed and variance-stabilized using DESeq2's rlog function [26].

247

248 **2.9 Proteomic analysis**

249 A proteomic dataset for the D492 and D492M cells was obtained from the ProteomeXchange

250 Consortium via the PRIDE [27] partner repository with the dataset identifier PXD024164. The

251 raw data were processed using MaxQuant [28] for both the protein identification and

252 quantification.

253

254 **2.10 Western blot analysis**

255 D492 and D492M cells were grown to 80-90% confluent as described above followed by lysis

256 in RIPA buffer. The lysates were subjected to five freeze-thaw cycles, centrifuged at 14000

257 RCF for 20 minutes at 4°C. Protein concentration was quantified using a Pierce BCA Protein

258 Assay kit (Thermo).

259 10-20 µg of protein were loaded onto pre-cast 4-12% NuPAGE Bis-Tris gels (Thermo) and

260 transferred to a 0.45 µm nitrocellulose membrane (Thermo). The membrane was blocked in

261 5% BSA (Thermo) for 60 minutes, followed by overnight primary antibody incubation at 4°C.

262 The primary antibody was anti-IDH2 monoclonal rabbit (12652, Cell Signaling Technologies,

263   MA, USA) in 1:1000 dilution. IDH2 levels were normalized against β-actin (MA5-15739,

264   Thermo) in 1:10000 dilution.

265   Bands were detected by secondary antibody incubation for two hours at room temperature

266   using anti-rabbit IgG (H+L) DyLight™ 800 4x PEG Conjugate and anti-mouse IgG (H+L)

267   DyLight™ 680 Conjugate (Cell Signaling Technologies) in 1:15000 dilution. Imaging was

268   performed using Odyssey® CLx (LI-COR Biosciences, NE, USA) to scan the films at 169 μm

269   resolution. The results were analysed in the Image Lab™ software (Bio-Rad).

270

271   **2.11 Measurement of metabolite exchange rates**

272   Glucose and lactate measurements were performed in an ABL 90 blood gas analyzer

273   (Radiometer, Brønshøj, Denmark). Glutamine uptake was measured using L-

274   Glutamine/Ammonia assay kit (K-GLNAM, Megazyme, Bray, Ireland). The following formula

275   was used to quantify the exchange rates of metabolites in the cells:

$$v_k = \frac{V\left([M_k]_f - [M_k]_i\right)}{A} \tag{3}$$

276   Where v_k is the exchange rate for metabolite k, $[M_k]_{48}$ and $[M_k]_0$ are the concentrations of

277   metabolite k in the culture media after 48 and 0 hours, respectively, and A is the area under

278   the growth curve.

279

280   **2.12 Weighted correlation network analysis**

281   Drug sensitivity data were gathered from the NCI-60 Human Tumor Cell Lines [29] using the

282   rcellminer R-package [30]. We focused specifically on FDA-approved drugs so after filtering the

283   data (using WGCNA's goodSamplesGenes function [31]), the final size of the drug sensitivity

284   matrix was 214 drugs x 59 cell lines (one cell line, MDA-N, was removed due to missing

285   data). To construct a network of drug-sensitivities, a weighted correlation network analysis

286   was employed using the WGCNA R-package [31]. A soft threshold value of 9 was used to obtain

287   a scale-free network topology (R^2 = 0.85). Highly correlated modules that had an average

288   distance < 0.65 were merged. To associate the modules to a specific mechanism of action

289   (MOA), the MOA terms for each drug were obtained (from rcellminer) and tested for

290 overrepresentation in the modules. MOA with Bonferroni-adjusted p-value < 0.05 were

291 labeled as overrepresented and used for the functional annotation of the drug modules.

292 The eigenvalues (first principal components) for each module were identified and used to

293 test association of drug-modules with metabolite levels. The correlation of the drug modules

294 to metabolite levels of the cells within the NCI-60 (from Ortmayr *et al.* [32]) was calculated.

295 The R-package igraph [33] was used to visualize the drug correlation network using the

296 Fruchterman-Reingold [34] force-directed layout. An R-script for the network analysis is in

297 **Supplementary file 1.**

298

299 **2.13 Drug treatment assays**

300 Cells were seeded (3000 cells/well) in white Costar® 96-well plates (Corning, NY, USA) and

301 maintained at 37°C in 5% CO_2. After 24 hours, drugs were added with or without buthionine

302 sulphoximine (BSO; B2515, Sigma Aldrich) where DMSO was used as a control. Then, 72

303 hours later cell viability was evaluated using the CellTiter-Glo® (CTG) assay (Promega,

304 Madison, WI, USA), by adding CTG assay mix directly into the wells in a 1:1 ratio. After 10

305 minutes, luminescence was measured by Victor™ X3 Multiplate reader (Perkin Elmer, MA,

306 USA). The drugs tested were mTOR inhibitor everolimus (Sigma Aldrich) and taxane drug

307 paclitaxel (Fresenius Kabi, Halden, Norway).

308

309 **2.14 Detection of intracellular glutathione abundance**

310 Cells were seeded (2000 cells/well) in white 384-well plates (Greiner Bio-One,

311 Kremsmünster, Austria) and maintained at 37°C in 5% CO_2. After 24 hours, BSO (treated) or

312 medium (non-treated) was added. After additional 24 (and 48 for non-treated) hours,

313 glutathione measurement was performed by using GSH-Glo Glutathione Assay kit (V6911,

314 Promega) in accordance with the manufacturer's protocol.

315

316 **2.15 Statistical analysis**

317   Student's t-test was employed for comparison of two treatments. Benjamini-Hochberg

318   adjustment for multiple comparisons was performed when appropriate. Analysis of variance

319   (ANOVA) was used to compare data from three or more treatments or the simultaneous

320   evaluation of the effect of two grouping variables. The asterisks in each figure represent the

321   p-values (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, ns = not significant). Data were

322   assumed to be normally distributed. Statistical analysis and image generation was carried

323   out in the R environment [35] using the *ggplot2* [36] and *ggpubr* [37] packages. In our graphs, all

324   data points are plotted and summarized using mean and standard error.

325

326   3. Results

327   **3.1 Glycolysis rates determine the pentose phosphate shunt in the D492 EMT cell model**

328   A clear difference in the overall metabolic profiles of D492 and D492M cells was confirmed

329   by principal component analysis (PCA) of their 1H NMR spectra. The score and loading plots

330   from PCA (**Figures S1A** and **S1B**) indicated that D492 cells had more intracellular isoleucine,

331   leucine, valine, alanine, arginine, glutathione, myo-inositol, asparagine, proline, AMP, ADP,

332   ATP, tyrosine, phenylalanine, and NAD+, and less glutamine, glutamate, phosphocholine,

333   glycine, threonine, glucose, fumarate, NADP and NADH. The rate of glucose uptake and

334   lactate secretion was also higher in the epithelial phenotype of D492 cells compared to the

335   mesenchymal phenotype (**Figure 1B**), indicative of enhanced glycolysis in D492. To

336   determine pentose phosphate pathway (PPP) split ratios in the cell lines we used 1,2-^{13}C

337   glucose (**Figure 1A**) as previously described [38]. Label contribution of 1,2-^{13}C-glucose to

338   lactate after 6 hours was higher in D492 epithelial cells than in D492M mesenchymal cells

339   (**Figure 1C**) confirming enhanced glycolysis. In order to determine the differences in PPP

340   activity we calculated the percentage of glucose diverted into the PPP using measured

341   lactate isotopologue abundances (**Figure S2**) and equation 2. Roughly 2% of glucose was

342   found to enter the PPP cycle in both cell lines. Therefore, because of the overall higher

343   glucose uptake, the flux into oxidative PPP is higher in D492. As a result, reduction of NADP

344   to NADPH via the oxidative phase of the PPP in D492M cells is dampened.

345   To examine the contribution of glucose to the TCA cycle, we measured the contribution of

346   the labeled 1,2-^{13}C-glucose to citrate. Citrate is either oxidized for energy production in the

347 TCA cycle or used as a precursor for lipid biosynthesis [39] through ATP-citrate lyase (ACLY) in

348 the cytosol (**Figure 1A**). No difference in glucose-dependent citrate generation (**Figure 1D**)

349 was observed which was reflected by the contribution of glucose to palmitate (**Figure 1E**).

350 Furthermore, the contribution of glucose to other TCA cycle components and downstream

351 metabolites (malate, aspartate and glutamate) after 6 hours in culture was minimal (**Figure**

352 **S2**). Thus, the only difference in glucose utilization within central carbon metabolism

353 between D492 and D492M was the increased glycolytic activity of the former.

354

355 **3.2 Glutamine fuels citrate and lipogenic acetyl-CoA production via reductive carboxylation**

356 **following EMT in D492 cells**

357 Glutamine is a major contributor of carbons into the TCA cycle through anaplerosis (*i.e.*

358 glutaminolysis), particularly in cancer cells [40–42]. Glutamine is the second-highest consumed

359 carbon source in D492 and D492M (after glucose [12]) with an average uptake rate of around

360 60 fmoles/cell/hour in both cell lines (**Figure 2B**). We found that both glucose and glutamine

361 are essential for the proliferation of D492 and D492M (**Figure S3**). Glutamine can replenish

362 the TCA cycle via glutamate and α-ketoglutarate which can be metabolized within the TCA

363 cycle both oxidatively and reductively (**Figure 2A**). In order to discriminate oxidative and

364 reductive TCA cycle carbon flow, D492 and D492M cells were cultured with isotopic

365 glutamine analogs labeled at either the 1 or 5 positions. These isotopomers of glutamine

366 allow the specific quantification of glutamine to citrate either reductively (1-^{13}C-glutamine)

367 or oxidatively (5-^{13}C-glutamine) (**Figure 2A**). The 5-^{13}C-glutamine analog can additionally

368 quantify the contribution of glutamine to fatty acids solely through reductive carboxylation

369 (**Figure 2A**). Importantly, intracellular glutamate label from a labeled glutamine source is a

370 direct measurement of the contribution of glutamine to the glutamate pool. Compared to

371 D492, the glutamate pool in the D492M cells was not as dependent on glutamine as

372 observed from lower labeling incorporation from both 1- and 5-^{13}C glutamine (**Figures S4A**

373 and **B**). This was supported by NMR measurements (**Figure S1C**) and is likely due to higher

374 amounts of glutamate being derived from elsewhere (*e.g.* transamination reactions and

375 protein catabolism) in D492M. As we were specifically interested in the contribution of

376 glutamine to metabolites beyond glutamate (*e.g.* citrate), we accounted for the differences

377 in glutamine-to-glutamate labeling by dividing the total contribution from glutamine to the

13

378   target metabolites with the total contribution of glutamine to glutamate in each cell line.

379   The results therefore represent the relative contribution of glutamate to metabolites.

380   Approximately 60% of the citrate pool was derived from glutamate in both D492 and D492M

381   as shown by the isotope enrichment from 5-^{13}C glutamine (**Figure 2C**). Enrichment of ^{13}C in

382   citrate derived from 1-^{13}C glutamine was however higher in D492M (**Figure 2C**), supporting

383   increased reductive carboxylation. These results were mirrored in the 5-^{13}C-glutamine-

384   dependent labeling profiles of palmitate, where there was a 6-fold increase in reductive

385   contribution of glutamine in D492M cells (**Figure 2D**).

386

387   **3.3 Metabolic re-routing following EMT affects redox metabolism in D492 cells**

388   The reductive pathway of glutamine to citrate is typically activated in hypoxia or following

389   changes in electron transport chain activity [41,43,44]. Reductive glutaminolysis affects the redox

390   status of cells through NADPH which serves as a cofactor for the reversible isocitrate

391   dehydrogenase enzymes, IDH1 and IDH2 [45]. Interestingly, we observed an increased

392   NADPH/NADP ratio in D492M over D492 (**Figure 3A**) that is not compatible with the PPP

393   fluxes in the cells. However, even though reductive carboxylation is a NADPH-requiring

394   process (**Figure 3B**), studies have shown that its heightened activity may lead to increased

395   cytosolic or mitochondrial NADPH levels based on the coordination of different isoforms of

396   isocitrate dehydrogenase [45,46]. NADPH is known for its role in defenses against reactive

397   oxygen species (ROS), where it is used to reduce the oxidized form of glutathione (GSSG) to

398   generate the reduced form of glutathione (GSH). Due to the observed alterations in

399   glycolysis, glutamine metabolism and NADPH/NADP$^+$ ratios, we hypothesized that this would

400   be reflected in ROS generation within the cells. This in turn would translate to alterations in

401   glutathione metabolism.

402   Label incorporation from glutamine to glutathione was a significantly lower following EMT in

403   D492 cells as measured by UPLC-MS (**Figure 3C**) and NMR (**Figure S1C**), further supporting

404   EMT-associated alterations in redox metabolism. In addition, intracellular GSH concentration

405   was 33% lower in D492M cells on average (**Figure 3D**).

406   Proline has been shown to be important to maintain redox homeostasis, by recycling NADPH

407   to NADP$^+$ [47], coupling it to NADPH-generating pathways. Proline is synthesized from

14

glutamate in two reactions, both of which oxidize NADPH (**Figure 3E**). We traced the proline

synthesis from glutamate in D492 and D492M cells and observed a 2-fold increase in proline

synthesis from glutamate following EMT (**Figure 3F**). In summary, differences in the redox

state of the D492 EMT model can be related to decreased glycolytic/PPP flux and

concomitant changes to glutaminolysis that is defined by decreased GSH synthesis, increased

proline synthesis and increased reliance on reductive carboxylation for citrate synthesis.

**3.4 Mitochondrial isocitrate dehydrogenase 2 (IDH2) is essential for EMT-linked reductive
glutamine metabolism**

Based on the difference in reductive carboxylation and NADPH/NADP$^+$ ratio (**Figure 2C** and

3A, respectively) and recent literature [45,48], we hypothesized that isocitrate dehydrogenase

(IDH) would contribute to the discrimination between the D492 and D492M metabolic

phenotypes (*i.e.* metabotypes) through glutamine consumption and influence redox balance.

Two isoforms of IDH are known to use NADP$^+$ as a cofactor: Cytosolic IDH1 and

mitochondrial IDH2. Quantification of IDH mRNA (by RNA sequencing) and protein (by MS

proteomics) in D492 and D492M cells revealed increased IDH2 levels in D492M as compared

to D492 (**Figure 4A**). These findings were confirmed by Western blot (**Figure S5**). No

difference was observed in IDH1 levels (**Figure 4A**)**.** Using shRNA lentiviral transduction, we

knocked down IDH2 expression in both cell lines and investigated the metabolic and

morphological effects.

Following a significant reduction in IDH2 expression levels, the mRNA levels of the cytosolic

isoform IDH1 did not change in the D492M-IDH2 cell line (**Figure 4B**). There were no

differences observed in neither morphology nor growth rate upon IDH2 knockdown in

D492M **(Figure 4C** and **D)**. A significant reduction in reductive carboxylation activity was

observed, as indicated by the 1-^{13}C-glutamine contribution to citrate and 5-^{13}C-glutamine

contribution to palmitate (**Figure 4E** and **F**, respectively). No significant difference was

observed in the overall contribution of glutamine to citrate or glucose to citrate (**Figure 4E**).

The NADPH/NADP$^+$ ratio was lowered upon IDH2 knockdown and proline synthesis from

glutamate was reduced (**Figure 4G** and **H**, respectively), suggesting that the redox

homeostasis of these cells is coupled to IDH2-mediated reductive carboxylation. The

glutamine-dependent synthesis of glutathione, however, was not affected by the IDH2
knockdown (**Figure 4I**). In contrast to D492M cells, D492 cells increased the expression of
the cytosolic isoform IDH1 upon IDH2 knockdown (**Figure S6A**). The upregulation of IDH1
was accompanied by increased glutamate-to-lipid contribution via reductive carboxylation
(**Figure S6B**) and decreased growth rate (**Figure S6C**). In addition, we observed a shift in
NADPH-to-NADP$^+$ ratio, increased proline synthesis from glutamate and reduced glutathione
synthesis in the D492-IDH2 knock-down cells compared to the wildtype D492 cells (**Figure
S6D-F**). These results indicate that the epithelial phenotype, but not the mesenchymal
phenotype of D492 cells, can compensate for the IDH2 knockdown by increasing IDH1
expression.

3.5 Alteration in glutathione biosynthesis drives sensitivity to mTOR inhibition

The different metabotypes of the cell lines, characterized by different glycolytic rates,
altered carbon source preference for TCA cycle activity and changes to the synthesis of
proline and glutathione are reminiscent of cancer stem cell metabotypes [49,50]. Due to
glutathione's role in drug resistance in various cancer cell types [51–53], we focused our
attention on the differences in glutathione metabolism between D492 and D492M cells
(**Figure 3**). We hypothesized that metabolic rerouting of glutamine-derived glutamate for
glutathione synthesis would affect drug sensitivity in these cells.

To identify drugs that are selectively affected by glutathione concentrations within cells, we
performed an integrated network analysis of 1) drug sensitivity profiles within the NCI-60
Human Tumor Cell Line database [29] and 2) untargeted metabolomic analysis of NCI-60 cell
lines from Ortmayr *et al.* [32]. The network analysis revealed that 214 FDA-approved drugs in
the NCI-60 database were grouped into 8 intra-correlated drug modules (**Figure 5A**), whose
functional annotation showed that drugs grouped together according to their mechanism of
action. The modules were comprised of DNA-damaging agents and cell cycle arresting
compounds (*e.g.* alkylating agents, nucleotide analogs, and paclitaxel, n = 122), hormones (n
= 7), tyrosine kinase inhibitors targeting EGFR and ERBB2 (*e.g.* erlotinib and lapatinib, n =
21), mTOR and serine/threonine kinase inhibitors (*e.g.* everolimus, rapamycin, temsirolimus,

467　n = 8), ALK/CDK inhibitors (n = 17), tyrosine kinases targeting VEGFR, PDGFR and FGFR (n =

468　7), MAP kinase inhibitors n = 10) and non-specific drugs (n = 6).

469　Interestingly, glutathione levels were negatively correlated with the mTOR inhibitor module,

470　represented in **Figure 5A** as the size of the nodes. When the modules were represented

471　collectively as a single unit using singular value decomposition, mTOR inhibitors were

472　significantly correlated with both reduced and oxidized glutathione (**Figure 5B**). This was

473　further supported by the Spearman correlation p-value for mTOR inhibitors and intracellular

474　GSH and GSSG levels (**Figure S7**). These results imply that high glutathione availability is

475　associated with low sensitivity to mTOR inhibitors and vice versa. Other conjugation

476　metabolites (*i.e.* UDP-glucuronate and S-adenosylmethionine) did not display this type of

477　relationship with mTOR inhibitors.

478　Following this we examined the effects of the mTOR inhibitor everolimus (strong negative

479　correlation with GSH levels) and paclitaxel (no correlation with GSH levels) on D492 and

480　D492M cells. D492M cells were more sensitive to both everolimus and paclitaxel than D492

481　(**Figure 5C**). In order to establish a functional link between glutathione abundance and mTOR

482　inhibitors, we co-treated D492 and D492M cells with buthionine sulphoximine (BSO), an

483　inhibitor of the rate-limiting enzyme glutamate-cysteine ligase (GCL) in glutathione synthesis

484　(**Figure 5D** and **E**), and either everolimus (**Figure 5F**) or paclitaxel (**Figure 5G**). The sensitivity

485　of both D492 and D492M cells to everolimus was enhanced by co-treatment with BSO,

486　whereas these effects were not observed when the cells were co-treated with paclitaxel and

487　BSO. Together, these data suggest that glutathione availability directly affects sensitivity to

488　drugs that specifically affect the mTOR pathway.

489

4. Discussion

491　D492 and D492M cells represent only two of the numerous phenotypes within the spectrum

492　of EMT [54]. Herein, we have thoroughly characterized the central carbon metabolic activity of

493　these cell types using ^{13}C isotope tracers, specialized metabolic assays, and shRNA-mediated

494　knockdown of gene expression. Furthermore, we have evaluated the functional

495　consequences of EMT-mediated differences in redox metabolism using both *in silico* and *in*

496　*vitro* drug-sensitivity analyses.

497

4.1 IDH2 plays a key role in EMT in breast epithelium

499   The data presented here support previous findings where genome-scale metabolic models [12]

500   and signal-network models constrained with cell type-specific transcriptomic data [53]

501   predicted lower glycolytic activity of D492M cells compared to D492 cells. In addition, the

502   data indicate that D492M cells increasingly rely on reductive carboxylation of glutamine to

503   citrate via isocitrate dehydrogenase activity. Transcriptomic and proteomic data from D492

504   and D492M cells confirm that the predominant EMT-associated form of isocitrate

505   dehydrogenase is the mitochondrial $NADP^+$-dependent IDH2, with D492M cells showing

506   significantly higher levels on both the transcript and protein levels (**Figure 4A**). Knockdown

507   of IDH2 by lentiviral shRNA induction caused a marked reduction of reductive carboxylation

508   of glutamine to citrate in D492M cells but did not affect the overall contribution of either

509   glucose or glutamine to citrate (**Figure 4E**). These results were reflected in the label

510   incorporation of 5-^{13}C-glutamine and 1,2-^{13}C-glucose to palmitate (**Figure 4F**). The decrease

511   in citrate labeling from 1-^{13}C-glutamine indicates that in these cells, reductive carboxylation

512   primarily takes place within the mitochondria, as opposed to in the cytosol via IDH1 activity.

513   The knockdown of IDH2 and the subsequent re-routing of glutamine metabolism

514   significantly diminished the ratio of NADPH to $NADP^+$ and the synthesis of proline from

515   glutamate (**Figure 4G** and **H**), consistent with the relationship of reductive carboxylation and

516   proline synthesis to redox homeostasis [45,55]. However, there was no clear connection of IDH2

517   to glutathione synthesis (**Figure 4I**).

518   Our findings highlight the importance of IDH2 in the increased reductive carboxylation

519   following EMT in breast epithelium. However, we cannot exclude the importance of IDH1 in

520   this context. It is reasonable to assume that when reductive carboxylation takes place in the

521   cytosol via IDH1, the resulting citrate is transported into the mitochondria where IDH2 takes

522   part in its ultimate oxidation as previously proposed [45]. When IDH2 levels are diminished, the

523   activity of this pathway would inevitably be halted. Nevertheless, our results demonstrate

524   that IDH2 knockdown significantly affects the reductive carboxylation of glutamine to citrate

525   and ultimately fatty acids which establishes a functional role of IDH2 in this process. Thus,

526 not only are the D492 and D492M cells different in their the overall lipid composition [56], but

527 also the origin of lipid carbons.

528

529 **4.2 Alterations in reductive carboxylation and redox metabolism follow EMT in breast**

530 Our results show that glutamine-derived citrate is utilized for fatty acid synthesis in the D492

531 cell model, but the reliance on this pathway is enhanced following EMT. We show that there

532 is a concurrent increase in NADPH/NADP$^+$ ratio and proline synthesis along with a decrease

533 in glutathione synthesis (**Figure 3C-F**). It has previously been shown that anchorage-

534 independent growth relies on increased reductive carboxylation and subsequent mitigation

535 of mitochondrial ROS [45]. The role of proline in anchorage-independent growth has also been

536 demonstrated by showing that its degradation and cycling is higher in breast cancer cells

537 grown in 3D culture than in 2D [55], which ultimately altered the NADPH/NADP$^+$ balance.

538 Phang [57] hypothesized that in cancer cells, proline is directed away from protein synthesis

539 and towards redox regulation, a pathway that proline has previously been shown to

540 participate in within mammalian cells [58]. More recently, it was shown that NADPH-

541 dependent proline synthesis and reductive carboxylation act as alternative bins for electrons

542 under hypoxic conditions when the electron transport chain is disabled. This way, electron

543 transfer may continue functioning in cancer cells to maintain their viability [50]. These

544 observations fit well with the decreased oxygen consumption rate in D492M mesenchymal

545 cells [12], their shift towards higher overall NADPH (*c.f.* **Figure 3B**), increased reductive

546 carboxylation and higher proline synthesis. Furthermore, D492M cells display a concomitant

547 decrease in glutathione synthesis and intracellular abundance. Interestingly, Snail mediated

548 EMT induction in MCF7 breast cancer cells has previously been shown to result in

549 intracellular glutathione reduction and elevation of ROS [59].

550

551 **4.3 Diminished glutathione abundance potentiates sensitivity to PI3K/Akt/mTOR inhibitors**

552 We have previously reported a reduction in oxidative phosphorylation following EMT in

553 D492 cells [12]. This causes a metabolic shift towards anaplerosis, an upregulation of pathways

554 receiving otherwise ETC-directed electrons (*i.e.* proline synthesis and reductive

555 carboxylation) [50] and decreased glutamine-derived glutathione synthesis. Glutathione is the

556 most abundant nonprotein thiol in animal cells and it plays a crucial role in the conjugation

557 phase of xenobiotic metabolism. This leads to increased water-solubility of foreign

558 compounds (*e.g.* drugs or toxins) and their reduced efficacy [60,61].

559 Due to the reduction in glutathione synthesis and overall abundance in the mesenchymal

560 D492M cells compared to epithelial D492 cells (**Figure 3C** and **D**), we hypothesized that this

561 would result in altered drug sensitivity of the mesenchymal phenotype. Integrated network

562 analysis suggested that D492M cells are more sensitive to drugs that specifically target

563 mTOR (**Figure 5A** and **B**). Furthermore, the lack of a significant relationship between UDP-

564 glucuronate and S-adenosylmethionine, compounds known to partake in the conjugation to

565 xenobiotic compounds [9], suggests that mTOR inhibitors are specifically affected by

566 glutathione availability. We tested the efficacy of everolimus, a well-known mTOR inhibitor,

567 and found that D492M cells were more sensitive than D492 cells (**Figure 5C**). Furthermore,

568 we showed that depleting intracellular glutathione levels via BSO treatment (**Figure 5E**)

569 increased the sensitivity to everolimus (**Figure 5F**). In contrast, BSO treatment did not affect

570 sensitivity to paclitaxel, a microtubule stabilizer and mitotic inhibitor (**Figure 5G**). These

571 results indicate that glutathione availability primarily affects the sensitivity to drugs that

572 target the mTOR pathway.

573 In recent years, studies have shown a direct relationship between the mTOR signaling

574 pathway and oxidative stress response [62,63]. Furthermore, mTOR signaling has been shown to

575 be highly relevant in the EMT process and chemoresistance of ovarian cancer cells and

576 melanoma [64,65]. Collectively, our results introduce a valuable mechanistic insight into the

577 altered drug sensitivity following EMT in breast epithelium and support previous findings

578 that glutathione depletion in combination with mTOR inhibitors may specifically target the

579 metastatic potential and/or stemness of cancer cells.

580

581 **4.4 Conclusions**

582 In summary, we have defined alterations in central carbon metabolism of a breast epithelial

583 cell model of EMT using ^{13}C carbon tracing. We show that glutamine metabolism is re-routed

584 towards reductive carboxylation to fuel fatty acid synthesis following EMT due to activity of

585 the mitochondrial NADP$^+$-dependent isocitrate dehydrogenase (IDH2). This leads to

586 decreased glutathione production and disrupted redox homeostasis within the cells.

587 Integrated network analysis of the NCI-60 Human Tumor Cell Line database revealed a

588 negative correlation between intracellular glutathione levels and sensitivity to mTOR

589 inhibitors, and by depleting intracellular glutathione levels in D492 and D492M cells, we

590 sensitized them to mTOR pathway inhibitors. Our results highlight a potential metabolic

591 weakness of low-glutathione, EMT-derived cells that may be exploited in anti-metastatic

592 treatment.

593

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734

Figure legends

736   **Figure 1. Glucose metabolism of D492 and D492M.** A) A schematic overview of label distribution

737   from 1,2-^{13}C glucose into central carbon metabolism. An atom transition map of glucose metabolism

738   showing a part of the metabolic fates of 1,2-^{13}C glucose within mammalian cells, where the ^{13}C-

739   isotopes are shown in black. Dashed lines indicate more than one reaction between metabolites. B)

740   Measured glucose uptake and lactate secretion in D492 and D492M. Total contribution of glucose to

741   C) lactate, D) citrate and E) palmitate was measured after culturing of D492 and D492M with 1,2-^{13}C-

742   glucose for 6 hours. Metabolites: f6p – fructose 6-phosphate, g3p – glyceraldehyde 3-phosphate, pyr

743   – pyruvate, co2 – carbon dioxide, accoa – acetyl CoA, oac – oxaloacetate, αkg – α-ketoglutarate, suc –

744   succinate, fum – fumarate, mal – malate, asp – aspartate. Enzymes: CS – citrate synthase, ACLY –

745   ATP-citrate lyase. Pathways: PPP – Pentose phosphate pathway.

746

747   **Figure 2. Glutamine metabolism of D492 and D492M.** A) Atom transition map of glutamine

748   metabolism showing the different metabolic fates of 1-^{13}C glutamine (grey) and 5-^{13}C glutamine

749   (black) within mammalian cells. Dashed lines indicate more than one reaction between metabolites.

750   B) Measured glutamine uptake in D492 and D492M. C) Contribution of 1- and 5-^{13}C-glutamine to

751   citrate in D492 and D492M cells after 6 hours in culture. The total contribution of the glutamine

752   analogs to citrate was normalized to the different origins of glutamate in the cells (total contribution

753   of glutamine to glutamate). D) Total contribution of 5-13C-glutamine to palmitate after 6 hours in

754   culture, normalized to glutamate origin. Metabolites: co2 – carbon dioxide, accoa – acetyl CoA, oac –

755   oxaloacetate, αkg – α-ketoglutarate, suc – succinate, mal – malate, asp – aspartate. Enzymes: ME –

756   malic enzyme.

757

758   **Figure 3. Redox metabolism is altered following EMT of D492**. A) NADPH-to-NADP ratio in D492 and

759   D492M cells. B) A schematic showing the connection of mitochondrial and cytosolic reductive

760   carboxylation, and NADP$^+$/NADPH balance. C) Total contribution of glutamate to oxidized glutathione

761   (GSSG) in D492 and D492M cells after 6 hours in culture. D) Measured abundance of glutathione

762   (GSH) in D492 and D492M after 24 and 48 hours in culture. A two-way ANOVA test revealed a

763   significant difference in GSH levels between the cells, independent of time. E) The two reactions

764   needed to convert glutamate into proline. Both reactions oxidize NADPH. P5CS: Delta-1-pyrroline-5-

765   carboxylate synthase, PYCR: Pyrroline-5-carboxylate reductase. F) Total contribution of glutamate to

766   proline in D492 and D492M cells after 6 hours in culture. The results from B and D are from the

767   combined analysis of 1- and 5-^{13}C-glutamine results, since both 13C carbons are detected in the

768   proline and GSSG carbon skeletons.

769

770   **Figure 4. IDH2 mediates reductive carboxylation activity and is coupled to redox metabolism of**

771   **D492M.** A) Relative differences in gene expression and protein translation of IDH1 and IDH2 in D492

772   and D492M. The results are displayed as log-fold D492M/D492 ratio of abundance of IDH1/2

773 transcripts from RNA sequencing, and IDH1/2 protein from a proteomic analysis of D492 and D492M.
774 Results are depicted as mean + standard error (n=3). B) Real-Time PCR from D492, D492M and an
775 IDH2 -silenced D492M cell line showing the gene expression levels of IDH2 and IDH1. C) Phase-
776 contrast images of D492M-WT and D492M-IDH2 cells. D) Proliferation of D492M-WT and D492M-
777 IDH2 cell lines (mean + standard error, n=8). E) Effect of IDH2 silencing on the contribution of 1-^{13}C-
778 glutamine, 5-^{13}C-glutamine and 1,2-^{13}C-glucose to citrate in D492M, where the former two were
779 normalized to glutamate origin. F) Effect of IDH2 silencing on the contribution of 5-^{13}C-glutamine
780 (normalized to glutamate origin) and 1,2-^{13}C-glucose to palmitate in D492M. G) Effect of IDH2
781 silencing on the NADPH/NADP$^+$ ratio in D492M. H) Effect of IDH2 silencing on the contribution of 1-
782 ^{13}C-glutamine to proline (normalized to glutamate origin). I) Effect of IDH2 silencing on the
783 contribution of 1-^{13}C-glutamine and 5-^{13}C-glutamine to oxidized glutathione (normalized to glutamate
784 origin). E,F,H and I) are from cells cultured with ^{13}C-labeled carbon sources for 6 hours. Student's
785 two-tailed t-test (with Benjamini-Hochberg adjustment for multiple comparisons) was used to
786 estimate significance of results.

787

788 **Figure 5. Glutathione levels regulate sensitivity to mTOR inhibitors.** A) Network analysis of NCI-60
789 cell lines treated with various FDA-approved drugs (n =214) suggests the presence of 8 modules of
790 intra-correlated drugs. The efficacy of each individual drug was correlated with glutathione levels,
791 represented by node size. B) The correlation of drug modules' eigenvalues to reduced glutathione
792 (GSH), oxidized glutathione (GSSG), UDP-glucuronate and S-adenosylmethionine (SAM). Upper
793 numbers in table represent Pearson's correlation coefficient and the lower numbers (in brackets)
794 represent the correlation p-value. C) D492 and D492M cells treated with increasing concentrations of
795 the mTOR inhibitors everolimus and rapamycin. Results are presented as mean + standard error
796 (n≥4). D) Mechanism of glutathione synthesis inhibition by buthionine sulphoximine (BSO). GGC –
797 Gamma-L-glutamyl-L-cysteine. GCL – Glutamate-cysteine ligase, GSS – Glutathione synthetase. E)
798 Effect of 24-hour BSO-treatment on glutathione (GSH) concentration in D492 and D492M cells. F)
799 D492 and D492M cells treated with everolimus (0.005 µM) with and without BSO (50 µM). G) D492
800 and D492M cells treated with paclitaxel (0.005 µM) with and without BSO (50 µM). For F,G) the Y-
801 axis represents percentage of viability compared to non-treated cells after 72 hours of treatment
802 (mean + standard error, n≥4). Two-way analysis of variance (ANOVA) and Student's two-tailed t-test
803 (with Benjamini-Hochberg adjustment for multiple comparisons) were used to measure significance
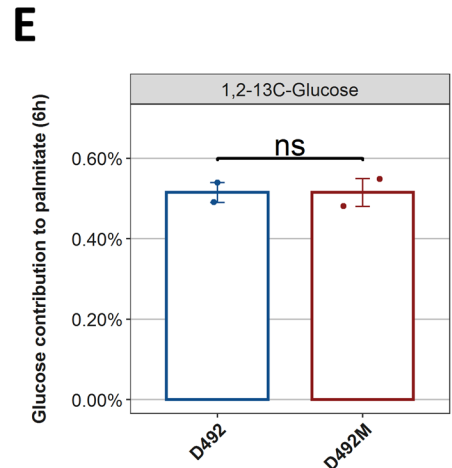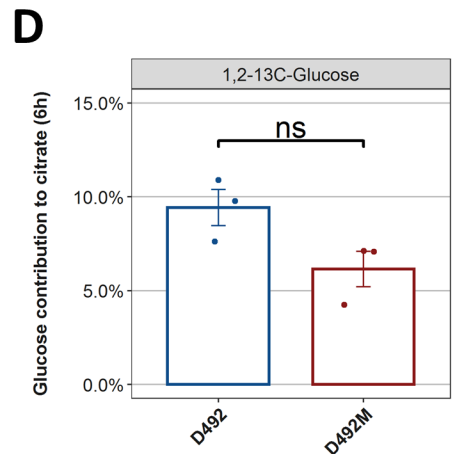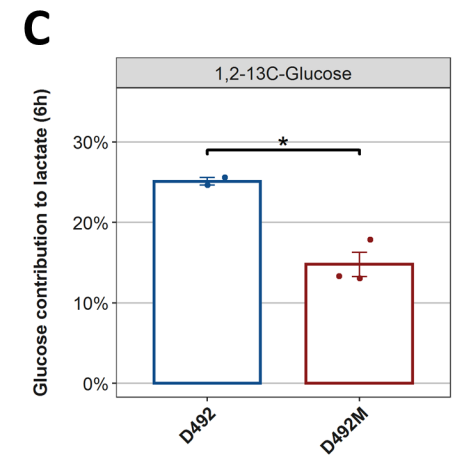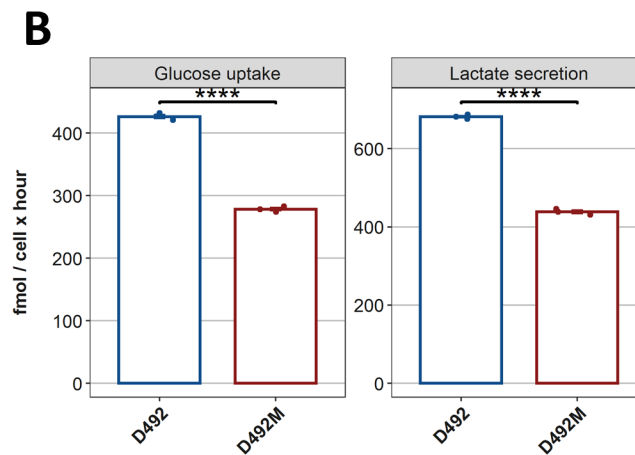804 of results in C) and E-G), respectively.

805
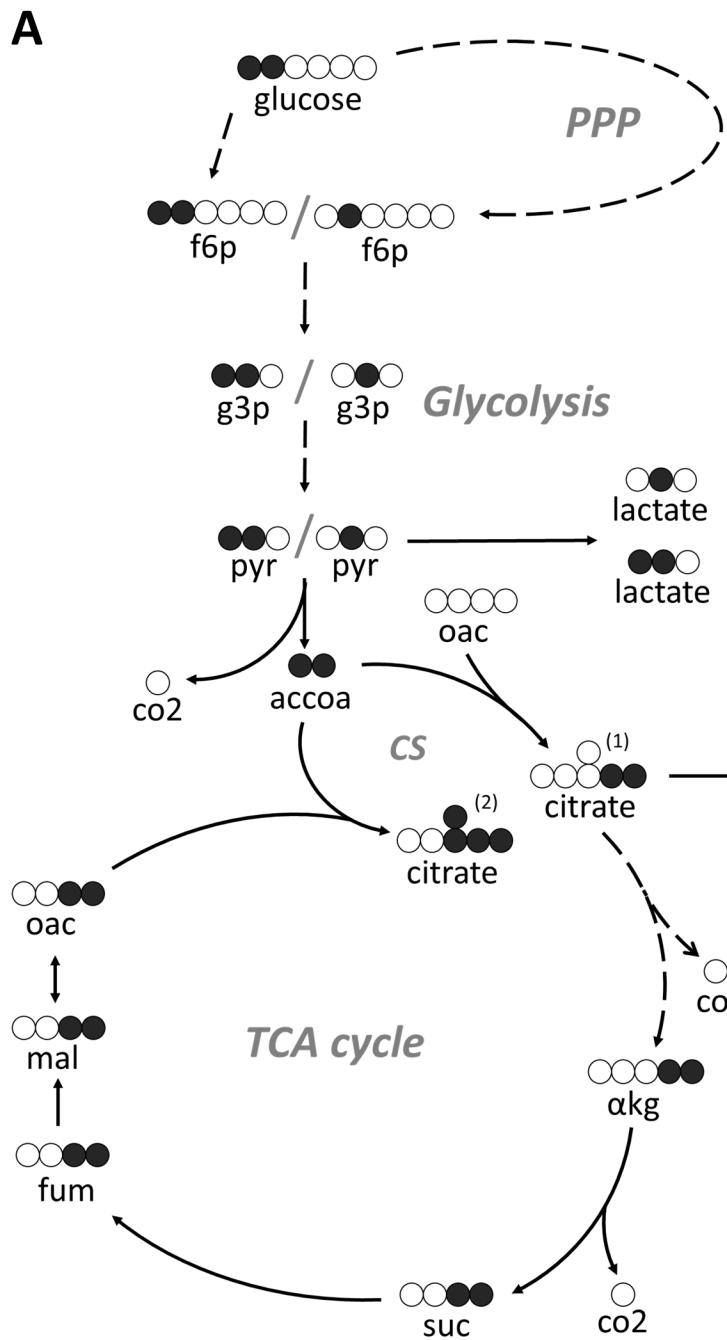
Figure 1
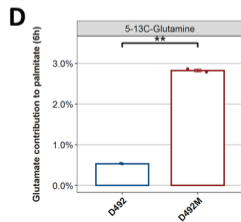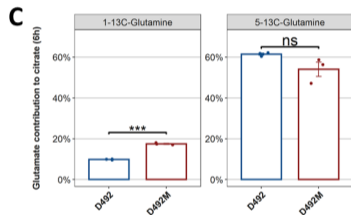
Figure 2

Figure 3

Figure 4

A

Legend:
- DNA-damaging agents
- Hormones
- Various
- Tyrosine kinase inh. 2
- mTOR/STK inh.
- ALK/CDK inh.
- Tyrosine kinase inh. 1
- MAPK inh.

B

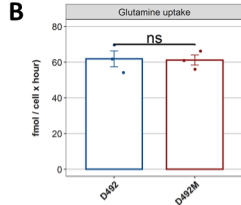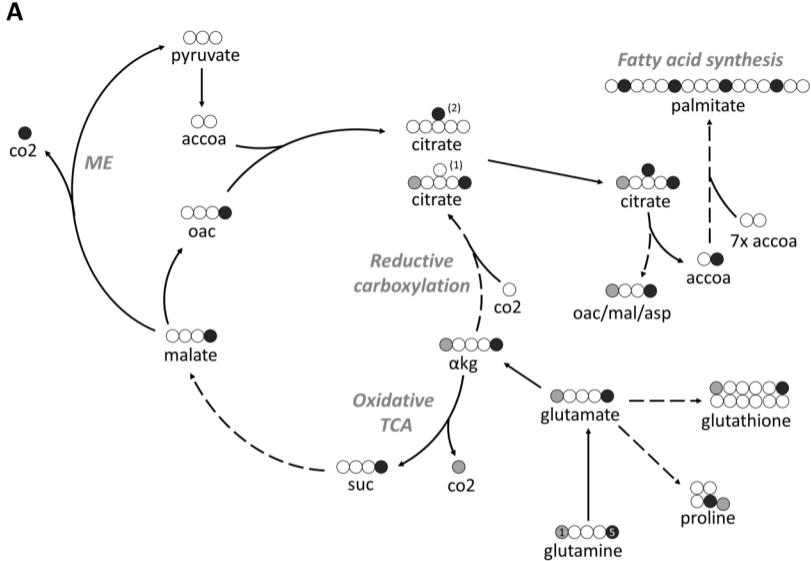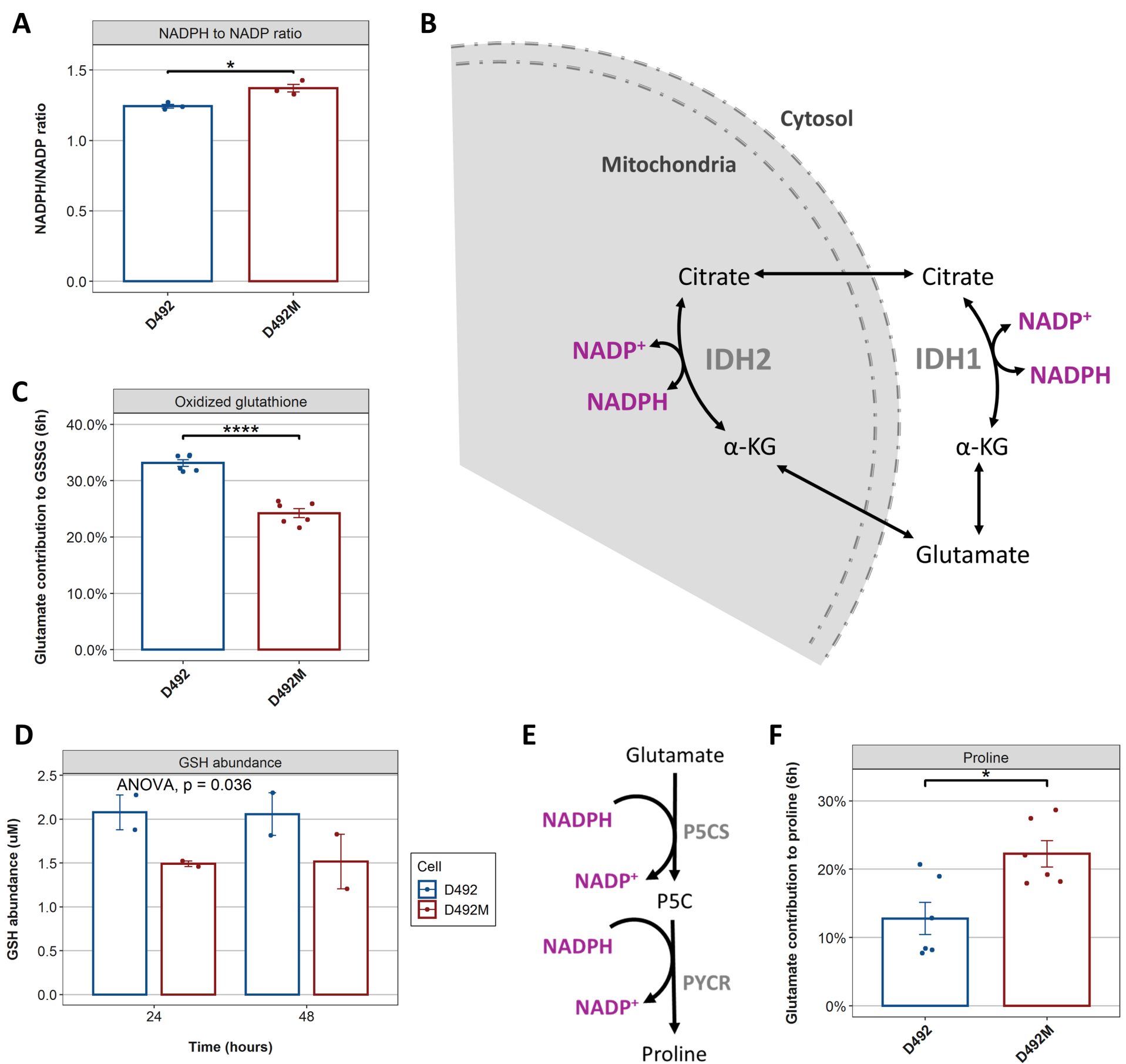|	GSH	GSSG	UDP-Glucuronate	SAM
Tyrosine kinase inh. 1	-0.27 (0.04)	-0.4 (0.002)	-0.34 (0.008)	0.32 (0.01)
Tyrosine kinase inh. 2	-0.034 (0.8)	-0.13 (0.3)	-0.014 (0.9)	-0.23 (0.07)
ALK/CDK inh.	-0.012 (0.9)	-0.01 (0.9)	0.064 (0.6)	0.17 (0.2)
mTOR/STK inh.	-0.27 (0.04)	-0.38 (0.003)	-0.18 (0.2)	0.089 (0.5)
Various	-0.28 (0.03)	-0.25 (0.05)	-0.3 (0.02)	0.21 (0.1)
Hormones	-0.27 (0.04)	-0.18 (0.2)	-0.17 (0.2)	0.15 (0.2)
DNA-damaging agents	0.15 (0.3)	0.073 (0.6)	0.16 (0.2)	0.18 (0.2)
MAPK inh.	0.063 (0.6)	0.16 (0.2)	-0.033 (0.8)	0.21 (0.1)

C

Everolimus — ANOVA, p = 2.63e-04

Paclitaxel — ANOVA, p = 1e-03

Survival (relative to control); Concentration (nM)

- D492
- D492M

D

Glutamate   Cysteine

GCL ⊣ BSO

GGC

Glycine

GSS

GSH

E

D492   D492M

Glutathione (uM)

Control, BSO (50 uM), BSO (150 uM)

D492: * , *
D492M: **, **

F

D492   D492M

Survival (relative to control)

Control, BSO, Everolimus, Everolimus + BSO

D492: ***, ****, *
D492M: ***, ****, *

G

D492   D492M

Survival (relative to control)

Control, BSO, Paclitaxel, Paclitaxel + BSO

D492: ***, ****, ns
D492M: ***, ****, ns

Figure 5