Supporting Information

GSTP1 is a Driver of Triple-Negative Breast Cancer Cell Metabolism and Pathogenicity

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Supplemental Methods

Cellular Phenotype Studies

Migration assays were performed using Transwell chambers (Corning) with 8μm pore-sized membranes coated with collagen. 50,000 cells were seeded into the top chamber and allowed to migrate for 24 hours. Chambers were fixed with Diff-Quik (Dade Behring) solutions.

Cell survival and proliferation assays were performed using the WST-1 reagent (Roche) for the MCF7 cell line. Cells were seeded in a 96-well plate with a volume of 200 μl serum-free media for survival and serum-containing media for proliferation (20,000 cells for survival and 10,000 cells for proliferation). WST-1 reagent was added to each well and incubated at 37° for 30 minutes before measuring absorbance. Cell survival measures cell death under serum-free and non-proliferative conditions, whereas cell proliferation assays are performed in the presence of serum and measures proliferation of cells.

Cell survival and proliferation assays for the 231MFP cells were performed using Hoechst 33342 dye (Invitrogen) according to manufacturer's protocol. Briefly, cells were seeded into 96-well plates (40,000 for survival and 20,000 for proliferation) in a volume of 200 μl and allowed to adhere overnight. Medium was removed from each well and 100 μl of staining solution containing 10% formalin and Hoechst 33342 dye was added to each well and incubated for 15 minutes in the dark at room temperature. After incubation, staining solution was removed and wells were washed twice with PBS before imaging.

Cell survival assays were also performed using Hoechst 33342 dye for the HCC38, HCC70, and HCC1143 cell lines with the exception of HCC38 shGSTP1 cells that were performed using the WST-1 reagent. Cell survival assays using Hoechst

33342 were performed as described above. Cell survival assay for HCC38 shGSTP1 cells was performed with WST-1 reagent as described above.

Metabolomic Profiling of Cancer Cells

Metabolomic data and SRM transitions are in **Table S2**. Briefly, 2 million cells were plated overnight, serum starved for 2 hours prior to harvesting, after which cells were washed twice with PBS, harvested by scraping, and flash frozen.

For nonpolar metabolomic analyses, flash frozen cell pellets were extracted in 4mL of 2:1:1 chloroform/methanol/PBS with internal standards dodecylglycerol (10 nmoles) and pentadecanoic acid (10 nmoles). Organic and aqueous layers were separated by centrifugation, and organic layer was extracted. Aqueous layer was acidified with 0.1% formic acid followed by re-extraction with 2 mL chloroform. The second organic layer was combined with the first extract and dried under nitrogen, after which lipids were resuspended in chloroform (120 µl). A 10 µl aliquot was then analyzed by both single-reaction monitoring (SRM)-based LC-MS/MS or untargeted LC-MS.

For polar metabolomic analyses, frozen cell pellets were extracted in 180 µl of 40:40:20 acetonitrile/methanol/water with internal standard d_3N^{15}- serine (1 nmole). Following vortexing and bath sonication, the polar metabolite fraction (supernatant) was isolated by centrifugation. A 20 µl aliquot was then analyzed by both single-reaction monitoring (SRM)-based LC-MS/MS or untargeted LC-MS.

For the SRM transitions where we monitor the transition of parent masses to the loss of the headgroup (e.g. loss of phosphocholine from phosphatidylcholine), we have ascertained the acyl chain specificities from previously described procedures (Long et al., 2011). For phospholipids such as PCs and PEs, we ascertained fatty acid acyl chain composition from phospholipids using a mobile phase containing both ammonium hydroxide and formic acid and monitored the fatty acid fragmentations from [M-

H+HCO_2H] m/z at 40 V collision energy in negative ionization mode. For other phospholipids such as PAs and PIs, we monitored the fatty acid fragmentations from [M-H] m/z at 40 V collision energy in negative ionization mode in mobile phase containing just ammonium hydroxide. For the lipids that we have measured in this study, the designated acyl chains represent the primary fatty acids that were on the lipid backbone **(**examples provided in **Fig. S4A)**. However, this method is less sensitive than monitoring the loss of headgroup from the phospholipid, and thus we used SRM transitions for many phospholipids where we monitored for loss of headgroups (e.g. PCs, PEs, PSs, PAs, PIs).

Relative levels of metabolites were quantified by integrating the area under the curve for each metabolite, normalizing to internal standard values, and then normalizing to the average values of the control groups.

Cysteine and Lysine Reactivity Profiling

Cells were washed with PBS and harvested by scraping. 1 mg of protein was incubated with 10 μM iodoacetamide-alkyne or dichlorotriazine-alkyne for 30 min at room temperature. Following incubation, biotin-azide, TCEP, TBTA ligand, and Cu(II)SO_4 were added and incubated for an additional hour at room temperature. Following incubation, the mixture was subject to centrifugation at 6500 x *g* for 4 minutes. The supernatant was removed and 500 μl of ice cold methanol was added to each tube. Solubilized protein was centrifuged at 6500 x *g* for 4 minutes at 4°C and supernatant was removed. Pellet was washed again with 500 μl of ice cold methanol and subject to centrifugation once more. The supernatant was removed and 1 mL of 1.2% SDS/PBS (w/v) was added until pellet was resolubilized. Mixture was heated at 90°C for 5 minutes, then centrifuged for 5 minutes at 6500 x *g*. The 1 mL sample was then added to 5 mL of PBS. Avidin-agarose beads (Thermo Scientific) were washed with PBS and 170 μl of the bead slurry was

added to each sample that was subsequently rotated overnight at 4°C. Samples were then warmed on a rotator at room temperature the following day and subject to centrifugation at 1400 x *g* for 3 minutes. The supernatant was removed and the beads were washed with 5 mL of 0.2% SDS/PBS (w/v), then subject to centrifugation at 1400 x *g* for 3 minutes. Beads were then transferred to Micro Bio-Spin columns (BioRad) and washed three times with PBS, followed by three times with water. The washed beads were then transferred to 500 µl of 6 M urea. 25 µL of 195 mM DTT was added to each sample, which was then incubated at 65°C for 20 minutes. Tubes were cooled to room temperature before adding 25 µL of 400mM iodoacetamide solution, then incubated at 37°C for 30 minutes. The reaction was diluted by adding 950 µL of PBS, then centrifuged at 1400 x *g* for 2 minutes, after which the supernatant was removed. 200 µL of 2 M urea and 0.5 µg/µl of Sequencing Grade Trypsin (Promega) were added and samples were incubated overnight at 37°C. Beads were centrifuged at 1400 x *g* for 3 minutes. Supernatant was added into a Micro Bio-Spin column to elute tryptic peptides while filtering out beads. Samples were acidified and stored at -80°C until they were ready for proteomic analysis. Tryptic digests were analyzed using a Thermo LTQ-XL and quantified by spectral counting.

Table S1. Cysteine and lysine-reactivity profiling of non-TNBC/TNBC breast cancer cell lines and CDH1 knockdown MCF7 breast cancer cells. Proteomes from MCF10A, non-TNBC, and TNBC cell lines were labeled with dichlorotriazine-alkyne and shControl and shCDH1 from MCF7 cells were labeled with iodoacetamide-alkyne (10 μM) for 30 min prior to a click reaction with biotin-azide. Probe labeled proteins were subsequently avidin bead-enriched and enriched proteins were trypsinized and analyzed by LC-MS/MS and quantified by spectral counting.

Tab 1. Raw proteomic data from MCF7 shControl and shCDH1 cell proteomes enriched by iodoacetamide-alkyne labeling

Tab 2. Proteomic data from MCF7 shControl and shCDH1 cells from iodoacetamide-alkyne enriched proteomes that show >2 average spectral counts and >2-fold above no-probe controls in either shControl or shCDH1 cells.

Tab 3. Data in Tab 2 were further filtered for metabolic enzymes.

Tab 4. Metabolic enzyme targets in MCF7 cells that were significantly ($p<0.01$) elevated in shCDH1 cells >5-fold compared to shControl cells.

Tab 5. Raw proteomic data from MCF10A, non-TNBC, and TNBC cell lines enriched by dichlorotriazine-alkyne labeling

Tab 6. Proteomic data from MCF10A, non-TNBC, and TNBC cell lines from dichlorotriazine-alkyne enriched proteomes that show >2 average spectral counts.

Tab 7. Data in Tab 6 were further filtered for metabolic enzymes.

Tab 8. Metabolic enzyme targets in TNBC cells that were significantly ($p<0.01$) elevated in TNBC cells >5-fold compared to non-TNBC cells.

This table is related to **Fig. 1** and **Fig. S1.**

Table S2. Metabolomic profiling of pharmacological and genetic inactivation of GSTP1.

Tab 1. SRM-based metabolomic profiling data of shControl and shGSTP1 231MFP cells

Tab 2. SRM-based metabolomic profiling data of vehicle-treated versus LAS17-treated (10 μM, 20 h) 231MFP cells.

Tab 3. Abbreviations

Tab 4. SRM transitions

This table is related to **Fig. 3** and **Fig. S4.**

Table S3. Proteomic profiling of GSTP1-Interacting Proteins. Proteomic profiling of anti-flag pulldown of 231MFP breast cancer cells expressing GFP or GSTP-Flag.

Tab 1. Total proteomic data from pulldown experiment

Tab 2. List of proteins possessing >3 average spectral counts in GSTP1-Flag pulldown group.

Tab 3. List of proteins that were significantly ($p<0.05$) pulled down with >2-fold enrichment in GSTP1-Flag compared to GFP-expressing controls.

This table is related to **Fig. 5** and **Fig. S5.**

Figure S1.

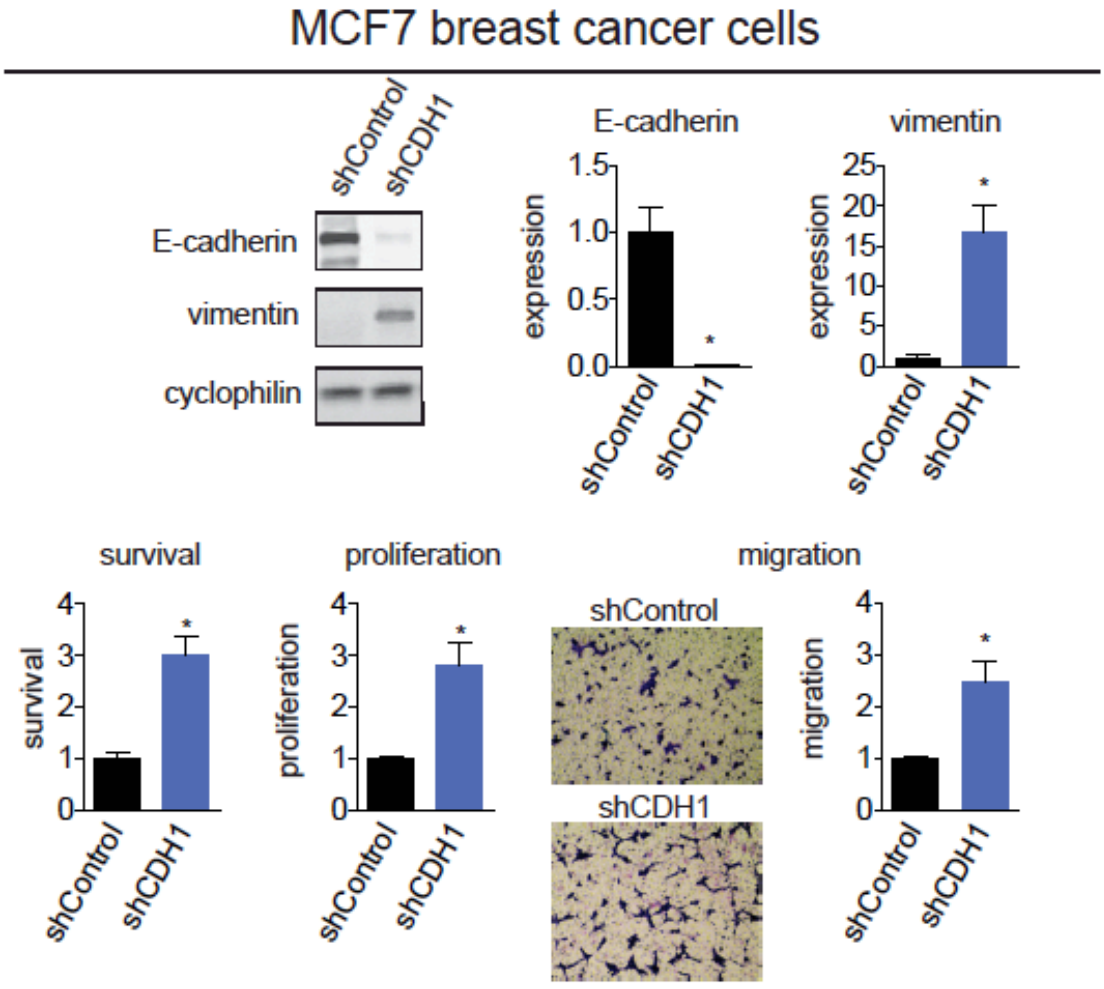

Fig. S1. CDH1 knockdown in MCF7 breast cancer cells. CDH1 was stably knocked down in MCF7 breast cancer cells using a short-hairpin oligonucleotide targeting CDH1. Knockdown was confirmed by Western blotting of E-cadherin. The EMT marker vimentin is heightened in shCDH1 cells as determined by Western blotting. Western blotting images shown are represented images from n=3/group and CDH1 and vimentin expression were normalized to the cyclophilin loading control and quantified by densitometry. Serum-free survival, proliferation, and migration were significantly enhanced upon CDH1 knockdown in MCF7 cells. Survival and proliferation were assessed 48 h post-seeding of cells by the WST-1 cell viability assay. Cell migration assays were performed by transferring cancer cells to serum-free media prior to seeding

cells into inserts with 8 μm pore size containing membranes coated with collagen (10 μg/ml). Migrated cells were subsequently fixed, stained, and counted. Data is presented as mean ± SEM, n=3-5/group. Significance is presented as *$p<0.05$ compared to shControl cells. This figure complements **Fig. 1**.

Figure S2

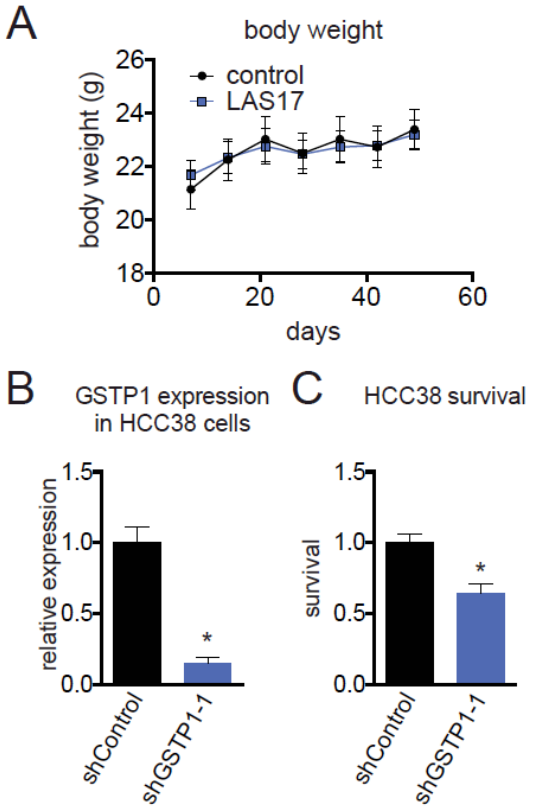

Fig. S2. Effect of GSTP1 inhibition on mouse body weight and in breast cancer cells. (A) Body weight in SCID mice treated once per day with LAS17 (20 mg/kg ip). **(B)** GSTP1 expression in HCC38 shControl and shGSTP1-1 cells, determined by qPCR. **(C)** Serum-free cell survival 48 h after seeding in shControl and shGSTP1-1 HCC38 cells determined by WST1 assay. Data are presented as mean ± SEM, n=5-6/group. Significance is presented as *p<0.05 compared to vehicle-treated or shControl cells. This figure is related to **Fig. 2**.

Figure S3

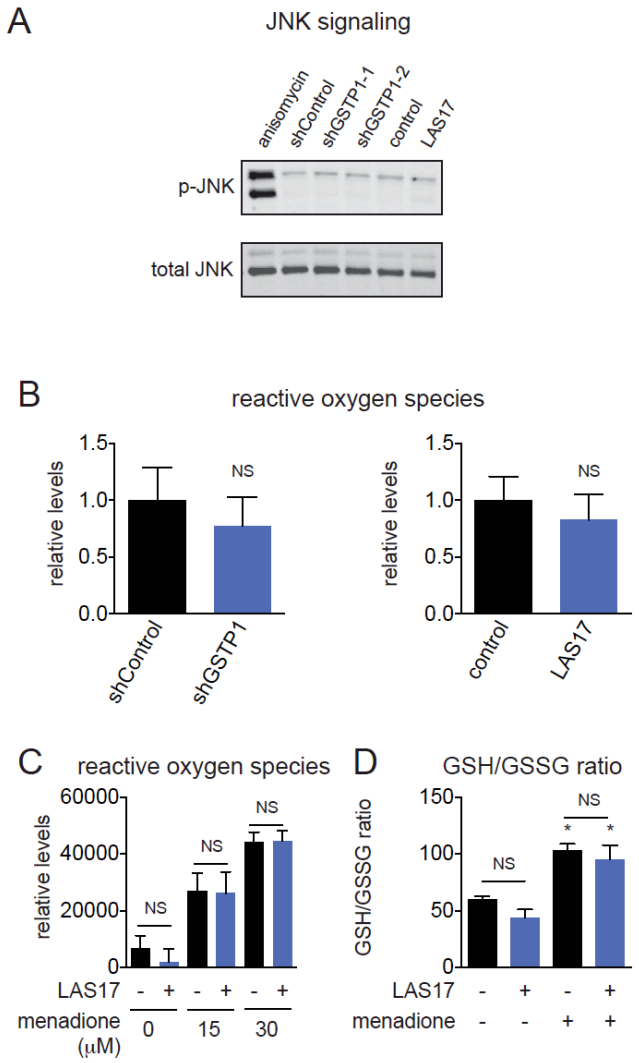

Fig. S3. Effect of GSTP1 inactivation on JNK signaling, reactive oxygen species, or GSH/GSSG ratios. (A) shGSTP1 and LAS17-treated 231MFP cells do not show any changes in phosphorylated JNK levels compared to shControl or DMSO vehicle-treated control cells. Treatment of 231MFP cells with anisomycin (1 μM, 1 h), a JNK activator, dramatically elevated p-JNK levels. **(B)** Reactive oxygen species in shGSTP1 and LAS17-treated 231MFP cells, compared to shControl or DMSO vehicle-treated control cells, as measured by CellROX green reagent. **(C)** Reactive oxygen species in DMSO vehicle-treated or LAS17-treated 231MFP cells treated with DMSO vehicle or the oxidant menadione. **(D)** Ratio of reduced (GSH) or oxidized glutathione (GSSG) levels in

231MFP cells treated with DMSO or LAS17 and also treated with DMSO or menadione. Data are presented as mean ± SEM, n=3/group. NS refers to not significantly changing compared to control groups ($p>0.05$). This figure is related to **Fig. 3.**

Figure S4

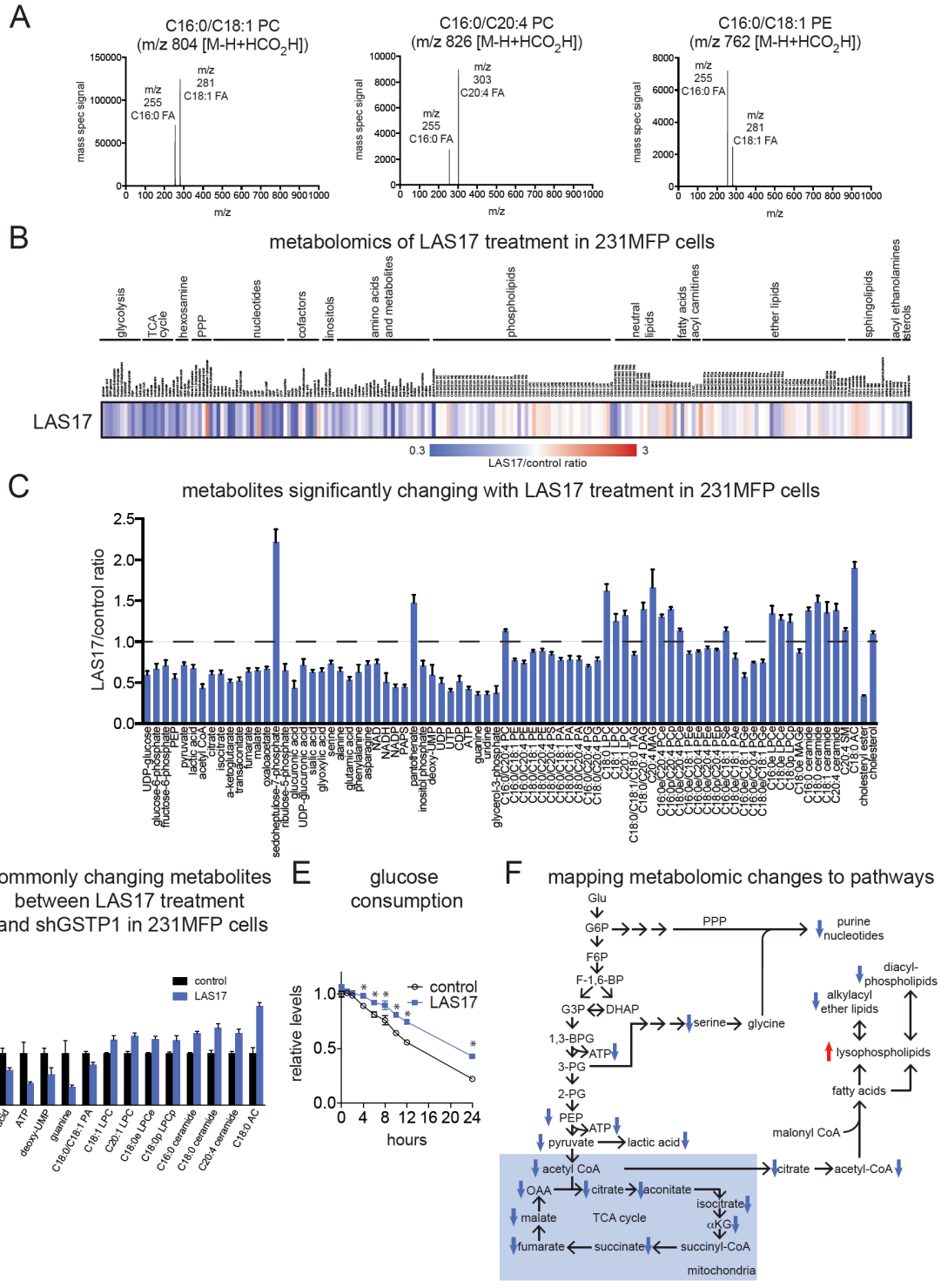
A
C16:0/C18:1 PC
(m/z 804 [M-H+HCO2H])
m/z 255 C16:0 FA
m/z 281 C18:1 FA
C16:0/C20:4 PC
(m/z 826 [M-H+HCO2H])
m/z 255 C16:0 FA
m/z 303 C20:4 FA
C16:0/C18:1 PE
(m/z 762 [M-H+HCO2H])
m/z 255 C16:0 FA
m/z 281 C18:1 FA
mass spec signal
m/z
B
metabolomics of LAS17 treatment in 231MFP cells
glycolysis
TCA cycle
hexosamine
PPP
nucleotides
cofactors
inositols
amino acids and metabolites
phospholipids
neutral lipids
fatty acids acyl carnitines
ether lipids
sphingolipids
acyl ethanolamines
sterols
LAS17
0.3
3
LAS17/control ratio
C
metabolites significantly changing with LAS17 treatment in 231MFP cells
LAS17/control ratio
D
commonly changing metabolites between LAS17 treatment and shGSTP1 in 231MFP cells
control
LAS17
relative levels
lactic acid
ATP
deoxy-UMP
guanine
C18:0/C18:1 PA
C18:1 LPC
C20:1 LPC
C18:0e LPCe
C18:0p LPCp
C16:0 ceramide
C18:0 ceramide
C20:4 ceramide
C18:0 AC
E
glucose consumption
control
LAS17
relative levels
hours
F
mapping metabolomic changes to pathways
Glu
G6P
PPP
purine nucleotides
F6P
F-1,6-BP
G3P
DHAP
serine
glycine
diacyl-phospholipids
alkylacyl ether lipids
1,3-BPG
ATP
lysophospholipids
3-PG
2-PG
fatty acids
PEP
ATP
malonyl CoA
pyruvate
lactic acid
acetyl CoA
citrate
acetyl-CoA
OAA
citrate
aconitate
isocitrate
malate
TCA cycle
αKG
fumarate
succinate
succinyl-CoA
mitochondria

Fig. S4. Functional metabolomic profiling and pathway mapping of GSTP1 inhibition in 231MFP TNBC cells. (A) Representative ms2 spectra of phospholipid formate adducts in 231MFP cell metabolomes fragmented with 40 V collision energy under LC/MS solvent conditions containing both ammonium hydroxide and formic acid to ascertain acyl chain lengths of phospholipids targeted by SRM analyses. **(B)** Targeted and untargeted SRM-based metabolomic profiling of DMSO vehicle and LAS17 (10 μM, 20 h)-treated 231MFP cells. Shown is a heatmap of the LAS17/control ratios of all the metabolites that were measured by SRM analysis. Detailed data and explanation of abbreviations and SRM transitions are in **Table S2. (C)** Shown are metabolites that were significantly ($p<0.05$) changed in levels upon LAS17 treatment (10 μM, 20 h) compared to DMSO-vehicle-treated controls. Detailed data and explanation of abbreviations are in **Table S2. (D)** Metabolites that were commonly changed between LAS17 treatment and shGSTP1 in 231MFP breast cancer cells ($p<0.05$). **(E)** Relative media glucose levels over time in 231MFP cells treated with DMSO vehicle or LAS17 (10 μM). **(F)** Pathway mapping of many of the metabolic changes observed in LAS17-treated 231MFP cells indicates a primary impairment in glycolysis with resulting secondary impairments in nucleotide and lipid metabolism. Data in **(C** and **D)** are presented as mean ± SEM, n=4-5/group. Significance in **(E)** is presented as *$p<0.05$ compared to vehicle-treated controls. This figure is related to **Fig. 3.**

Figure S5

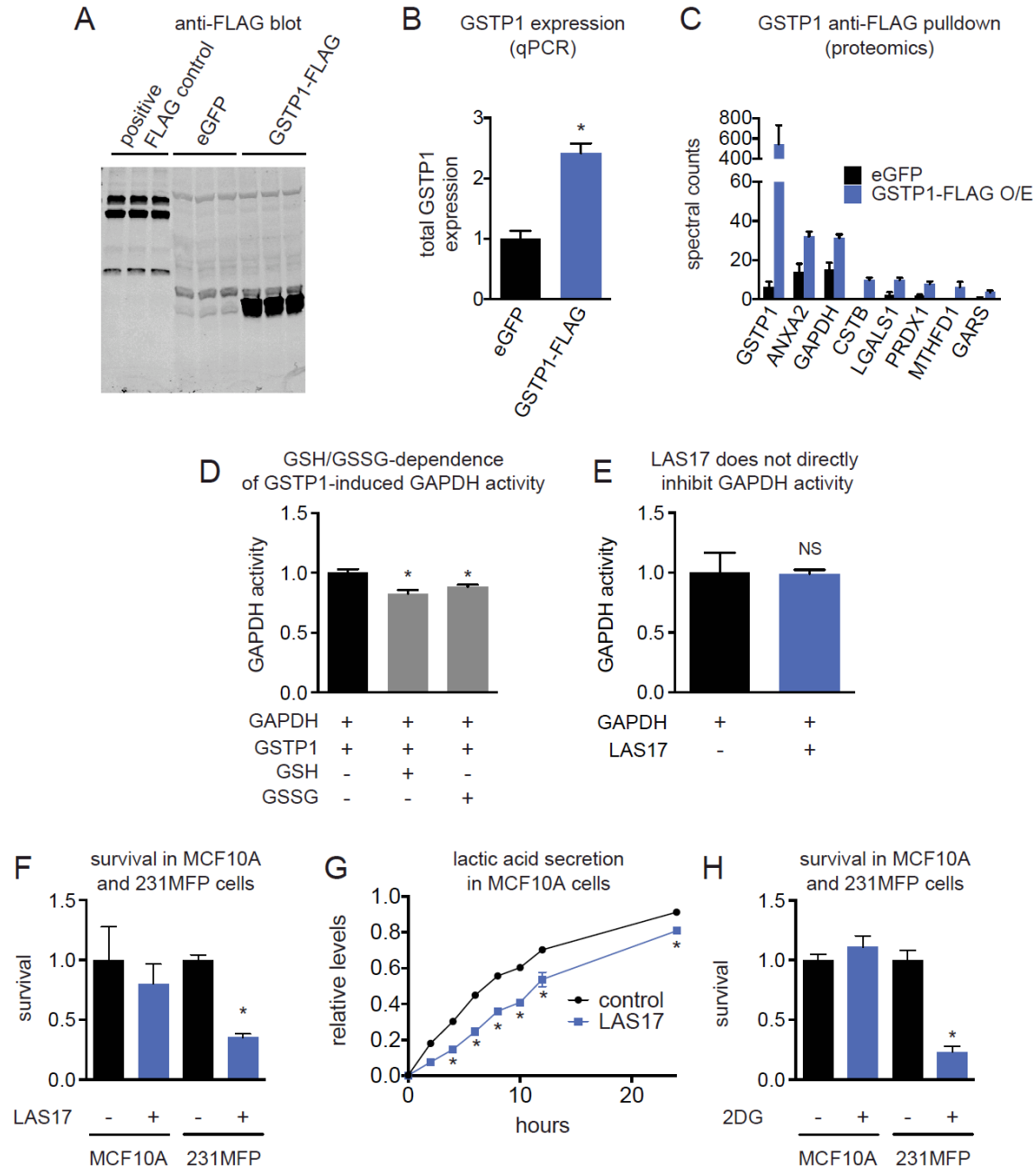

Fig. S5. **Characterizing the role of GSTP1 in regulating GAPDH activity and glycolysis. (A)** We generated stable control GFP-infected (eGFP) and GSTP1-FLAG overexpressing 231MFP cells to identify GSTP1 interaction partners. GSTP1-FLAG expression was confirmed by an anti-FLAG Western blot that includes a positive FLAG-labeled protein control. **(B)** qPCR analysis of total GSTP1 expression in eGFP versus

GSTP1-FLAG overexpressing 231MFP cells. **(C)** Proteins significantly enriched ($p<0.05$ compared to eGFP controls) by anti-FLAG pulldown of eGFP or GSTP1-FLAG 231MFP cell lysates and subsequent proteomic analysis of pulled-down proteins. **(D)** the effect of GSH or GSSG treatment on GSTP1-induced GAPDH activity. GSH or GSSG (1 mM) were co-incubated with pure and active GAPDH and GSTP1 for 1 h. **(E)** LAS17 does not inhibit GAPDH activity. LAS17 (10 μM) was incubated with pure GAPDH for 30 min before initiation of the GAPDH assay. GAPDH assay was performed using the GAPDH activity assay kit, which measures the rate of production of NADH, a byproduct of the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate.
(F) Serum-free cell survival of MCF10A and 231MFP cells treated with vehicle DMSO or LAS17 (10 μM) 48 h after seeding. **(G)** Lactic acid secretion in MCF10A cells treated with vehicle DMSO or LAS17 (10 μM) over a 24 h period. **(H)** Serum-free cell survival of MCF10A and 231MFP cells treated with vehicle DMSO or 2-deoxyglucose (20 mM) 48 h after seeding. Data are presented as mean ± SEM, n=3-5/group. Significance is expressed as *$p<0.05$ compared to mock **(B)**, GAPDH+GSTP1 **(D)**, vehicle-treated DMSO control in each respective cell line **(F, G, H)**. NS denotes no significant change ($p>0.05$). This figure is related to **Fig. 5**.