Abrogating GPT2 in triple negative breast cancer inhibits tumor growth and promotes autophagy

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Supplementary Methods

Analysis of Public Datasets

A dataset containing mRNA expression of 41 breast cancer cell lines[1] was used for mRNA expression analysis of *GPT2*. Cell lines were classified into luminal and basal according to their molecular characteristics[2]. Publicly available data from the Molecular Taxonomy of Breast Cancer International Consortium[3] (METABRIC) was analyzed with regard to gene expression and clinical characteristics of primary breast cancer tumors. Publicly available RNA-sequencing data and clinical annotations from The Cancer Genome Atlas[4] (TCGA) were analyzed from primary breast cancer tumors. Level 3 normalized gene expression data (TCGA_BRCA_exp_HiSeqV2-2015-02-24) was obtained from the cBioPortal website[5,6]. Gene expression data was log2 transformed and subset to the genes of interest. Patient data were subjected to Kaplan-Meier analysis of overall survival (OS) and recurrence-free survival (RFS). Differences of Kaplan-Meier curves were statistically tested using the log-rank test. Differences between subgroups depicted in the boxplots were analyzed with ANOVA and significance was calculated with Bonferroni's Multiple Comparison Test. Data was analyzed using GraphPad Prism 5 (La Jolla, CA, USA) or the R statistical computing environment[7] (version 3.2.2).

Alanine Assay

GPT2 inhibitor (BCLA) inhibitor titration was done by measuring the alanine levels in the supernatant using the alanine assay kit from Sigma. To analyze alanine levels supernatant was diluted 1:10 and 5μl of the diluted media was used in the assay according to manufacturer's instructions.

mTORC1 activity assay

Cells were seeded to 80% confluency. After overnight incubation cells were starved (with media containing 0.1% serum) for 24 h. The following day starvation media was replaced with amino acid free media (RPMI 1640 media w/o amino acids, US Biologicals, Salem, USA, and

10% Dialyzed Fetal Bovine serum, Thermo Fisher Scientific, Waltham, USA) for 50 min following by replacement with regular media. Protein lysates were harvested after 5, 10, 15, 30 and 45 min and analyzed by immunoblotting.

Hematoxylin/eosin staining and immunohistochemistry of mouse tumor tissues

Five μm sections of paraffin-embedded tumors were used for routine hematoxylin/eosin (HE) staining. p62 was detected in de-paraffinized tissue specimens after antigen retrieval (10 min and 20 min, respectively at boiling temperature in 10 mM sodium citrate pH 6.0), block of endogenous peroxidases in 3% H2O2 in PBS for 10 min, blocking in 100% goat serum for 1 h at room temperature (RT) and overnight incubation at 4° C in anti-p62 antibody (1:600; GP62-C Progen, Heidelberg, Germany). Slides, incubated with appropriate peroxidase-coupled secondary antibody (1:200) for 1h at RT, were incubated with HRP substrate solution (DAB/H2O2, Sigma Aldrich, Munich, Germany). Nuclei were counterstained with hematoxylin for 1 min and mounted with Eukitt (R. Langenbrinck, Teningen, Germany). Sections incubated without primary antibody were included as negative controls. Microscopy was performed using Axioskop 2 microscope and Axiovision software (Carl Zeiss, Gottingen, Germany).

Mouse tumor tissue metabolite measurements

Extraction

Mouse tumor tissues were ground with 7 mm steel bead in a 2 mL centrifuge tube with 20Hz for 60-90 sec in a Retsch mill (Retsch GmbH, NRW, Germany) and extracted in 180 μL of 100% methanol for 15 min at 70° C with vigorous shaking. As internal standard 5 μL Ribitol (0.2 mg/mL) were added to each sample. After the addition of 100 μL chloroform, samples were shaken at 37° C for 5 min. To separate polar and organic phases, 200 μL of water were added and samples were centrifuged for 10 min at 16,000 x g. For derivatization, 300 μL of the polar (upper) phase were transferred to a fresh tube and dried in a vacuum concentrator without

heating. Amino acids were measured in the polar phase using the UPLC analysis (method in section extracellular metabolite measurements).

Derivatization (Methoximation and Silylation)

Pellets were re-dissolved in 20 µL methoximation reagent containing 20 mg/mL methoxyamine hydrochloride (Sigma-Aldrich, Saint-Louis, USA, 226904) in pyridine (Sigma-Aldrich, Saint-Louis, USA, 270970) and incubated for 2 h at 37° C with shaking. For silylation, 32.2 µL N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma-Aldrich, Saint-Louis, USA M7891) and 2.8 µL Alkane Standard Mixture (50 mg/mL C10 - C40; Sigma-Aldrich, Saint-Louis, USA, 68281) were added to each sample. After incubation for 45 min at 50° C, samples were transferred to glass vials for GC/MS analysis.

Gas Chromatography/Mass Spectrometry (GC/MS) analysis

A GC/MS-QP2010 Plus (Shimadzu, Kyoto, Japan) fitted with a Zebron ZB 5MS column (Phenomenex, Torrence, USA; 30 meter x 0.25 mm x 0.25 µm) was used for GC/MS analysis. The GC was operated with an injection temperature of 230° C and 1 µL sample was injected with split mode (1:5). The GC temperature program started with a 1 min hold at 40° C followed by a 6° C/min ramp to 210° C, a 20° C/min ramp to 330° C and a bake-out for 5 min at 330° C, using Helium as carrier gas with constant linear velocity. The MS was operated with ion source and interface temperatures of 250° C, a solvent cut time of 8.0 min and a scan range (m/z) of 40 – 700 with an event time of 0.2 s. The "GCMS solution" software (Shimadzu, Kyoto, Japan) was used for data processing.

*Reference numbers correspond to references in the main manuscript file

Supplementary Tables

Supplementary Table 1: siRNA used in this study

siRNA	Annotation	Supplier	Catalogue Number	Target Sequence
siGENOME Non-Targeting siRNA Pool #2	NTC	Dharmacon	D-001206-14-05	UAAGGCUAUGAAGAGAUAC
				AUGUAUUGGCCUGUAUUAG
				AUGAACGUGAAUUGCUCAA
				UGGUUUACAUGUCGACUAA
ON-TARGETplus non-targeting Pool	siControl	Dharmacon	D-001810-10	UGGUUUACAUGUCGACUAA
				UGGUUUACAUGUUGUGUGA
				UGGUUUACAUGUUUUCUGA
				UGGUUUACAUGUUUUCCUA
siGenome Set of 4 Upgrade siRNA *GPT2*	siGPT2	Dharmacon	D-004173-01	UCAAAUGGCUCCAGACAUG
			D-004173-03	GUGAAAGACUUCCACAUCA
			D-004173-04	UCAAGAAGGUGCUGUACGA
			D-004173-18	GUGAAAAGGUUAAAUCGUA
siGenome Upgrade siRNA *PC*	siPC#1	Dharmacon	D-008950-18	GGAUAAUGCUUCCGCCUUC
	siPC#2	Dharmacon	D-008950-19	UCUCUGAGCGAGCGGACUU

Supplementary Table 2: sgRNAs used in this study

sgRNA	Supplier	Sequence	Target Exon	Target
sgRNA 1	Synthego Corporation	GUGCAUAGUGCCAUCACCUG	4	46900682
sgRNA2	Synthego Corporation	UGUCUUGUUCCCCAGGUGA	4	46900683

Supplementary Table 3: Primers and probes used in this study

Gene	Supplier	Primer Left	Primer Right	UPL Probe #
GPT1	Roche	gggaaggcacctaccacttc	ttggcatggaacctgctc	66
GPT2	Roche	ggatcttcattcctgccaaa	acatgtctggagccatttga	75
PC	Roche	agcaagctcttcagcatgg	aggggcactcatacaggaag	52
PUM1	Roche	tcacatggatcctcttcaagc	cctggagcagcagagatgtat	86

Supplementary Table 4: Primary antibodies used in this study

Protein name	Host	Product ID	Product ID (Company)
beta-Actin	Mouse	Actin (clone C4)	MP Biomedicals
beta-Actin	Rabbit	Actin 21-33	Sigma Aldrich
GPT2	Mouse	sc-398383	Santa Cruz Biotechnology
PC	Rabbit	HPA058765	Atlas Antibodies
P62	Guinea Pig	GP62_C	Progen
P-70S6K	Rabbit	CST2708	Cell signaling technology

|			
p-P70S6K (T389)	Rabbit	CST9234	Cell signaling technology
4EBP1	Rabbit	CST9644	Cell signaling technology
mTOR	Rabbit	CST2972	Cell signaling technology
p-mTOR (S2448)	Rabbit	CST2791	Cell signaling technology

Supplementary Figures and Figure Legends

Supplementary Figure 1
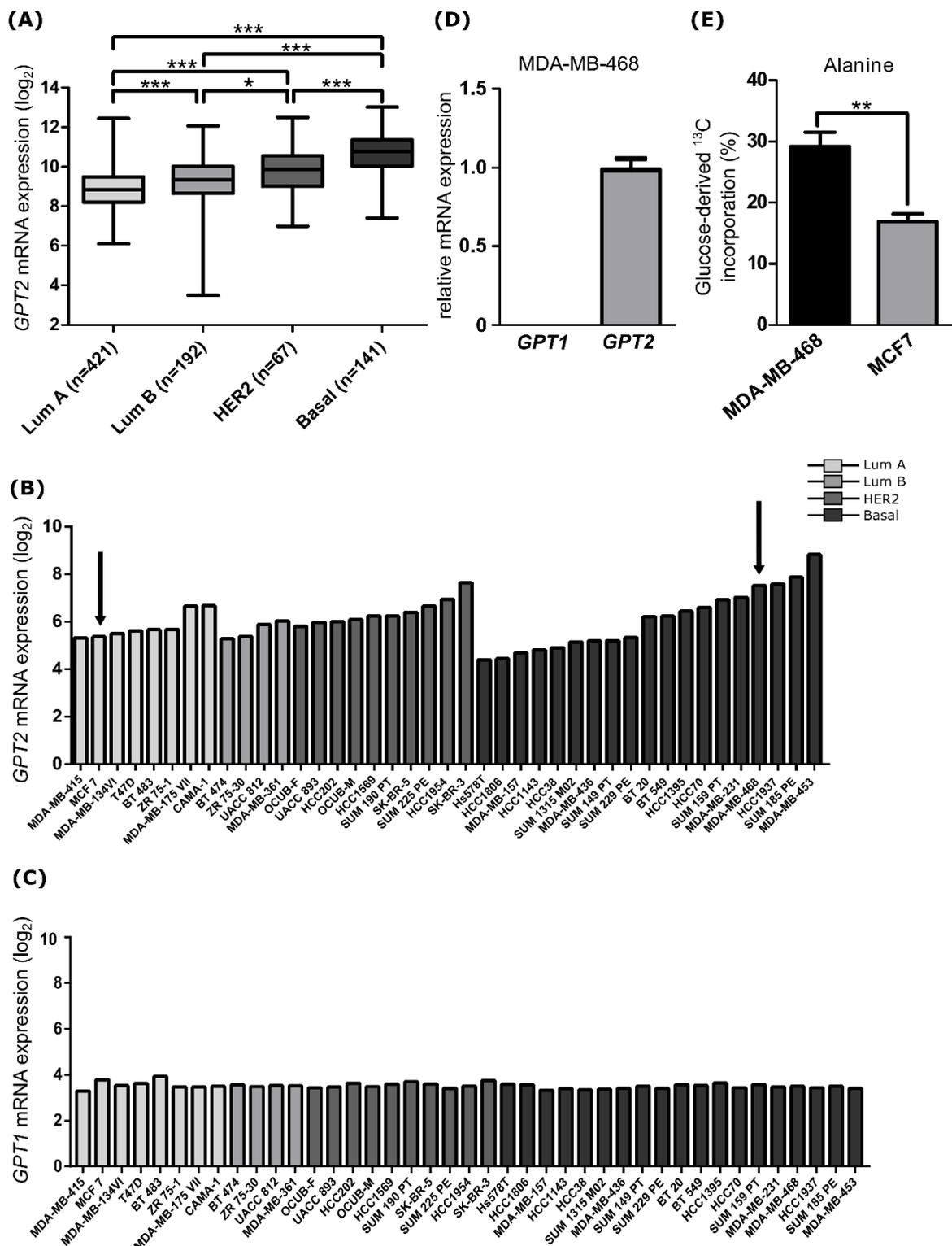
Supplementary Figure 1: GPT2 is upregulated in basal breast cancer. A, Box plots showing *GPT2* mRNA expression analysis of the TCGA dataset separated by molecular subtypes. Histograms showing *GPT2* (B) and *GPT1* (C) mRNA expression analysis of 41 breast

cancer cell line dataset[1], arrows indicating MCF7 and MDA-MB-468. D, qRT-PCR analysis of *GPT1* and *GPT2* expression in the MDA-MB-468 cell line. Graph shows expression of *GPT1* relative to *GPT2*. E, Percentage of labelled alanine pools in MDA-MB-468 and MCF7 cells 24h post treatment with treated with 75 μM and 100 μM of GPT2 inhibitor β-chloro-L-alanine respectively with 30 min incubation with media supplemented with 13C-glucose, measured by GC-MS. Data are represented as mean ± SD, n=3 biological replicates unless stated otherwise. ***$P<0.001$, ** $P<0.01$, * $P<0.05$, t-test.
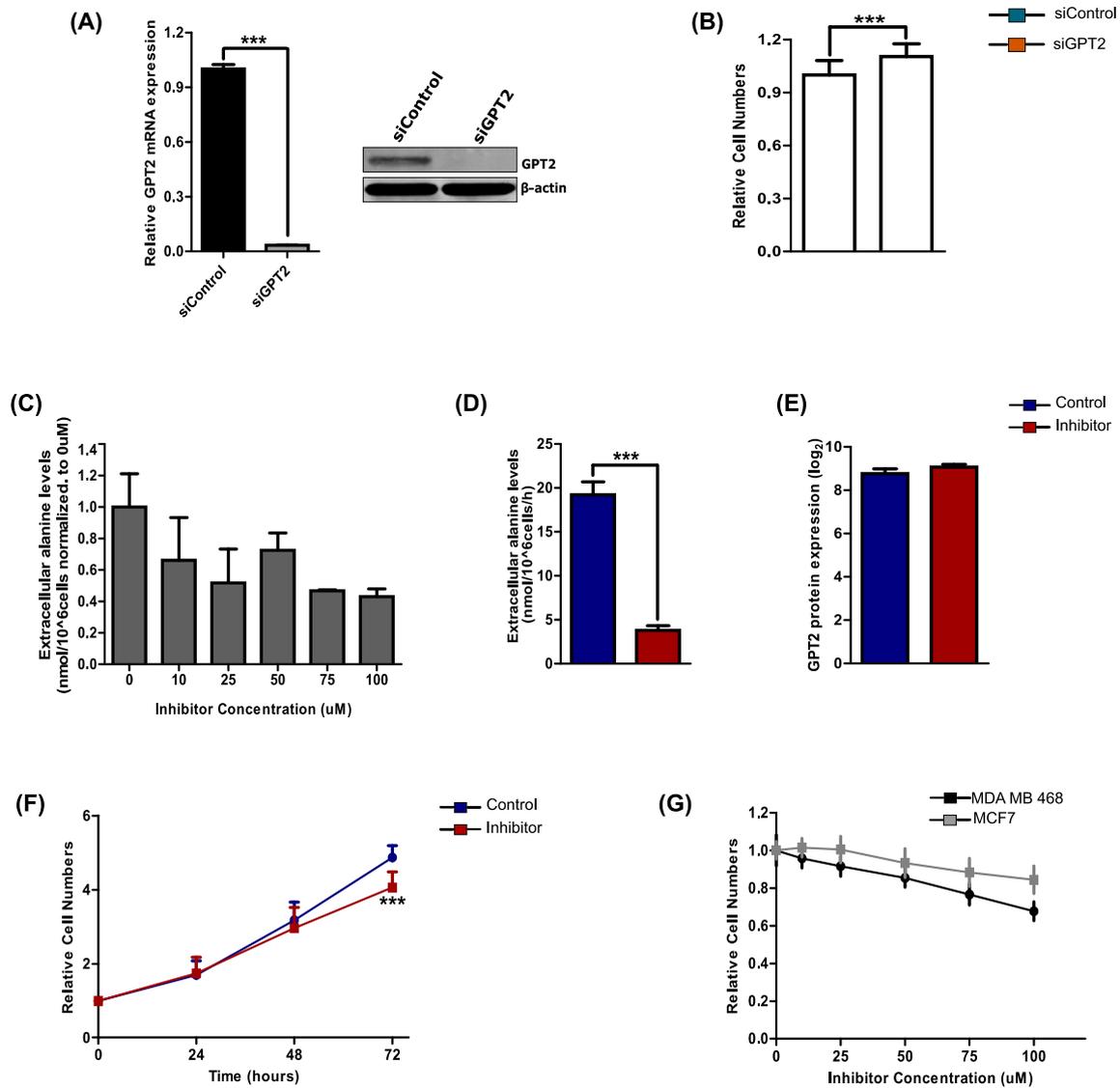
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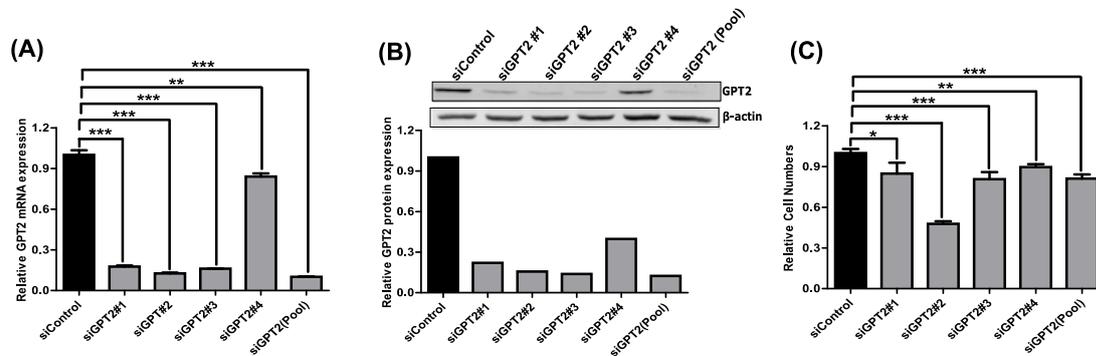
Supplementary Figure 2: **Abrogation of GPT2 inhibits MDA-MB-468 cell growth.** Analysis of *GPT2* mRNA levels (A), GPT2 protein expression (B) and relative cell numbers (C) upon siRNA transfection of individual siRNAs or a pool (and corresponding control). Data are represented as mean ± SD, n=3 biological replicates unless stated otherwise. ***$P<0.001$, ** $P<0.01$, * $P<0.05$, t-test.
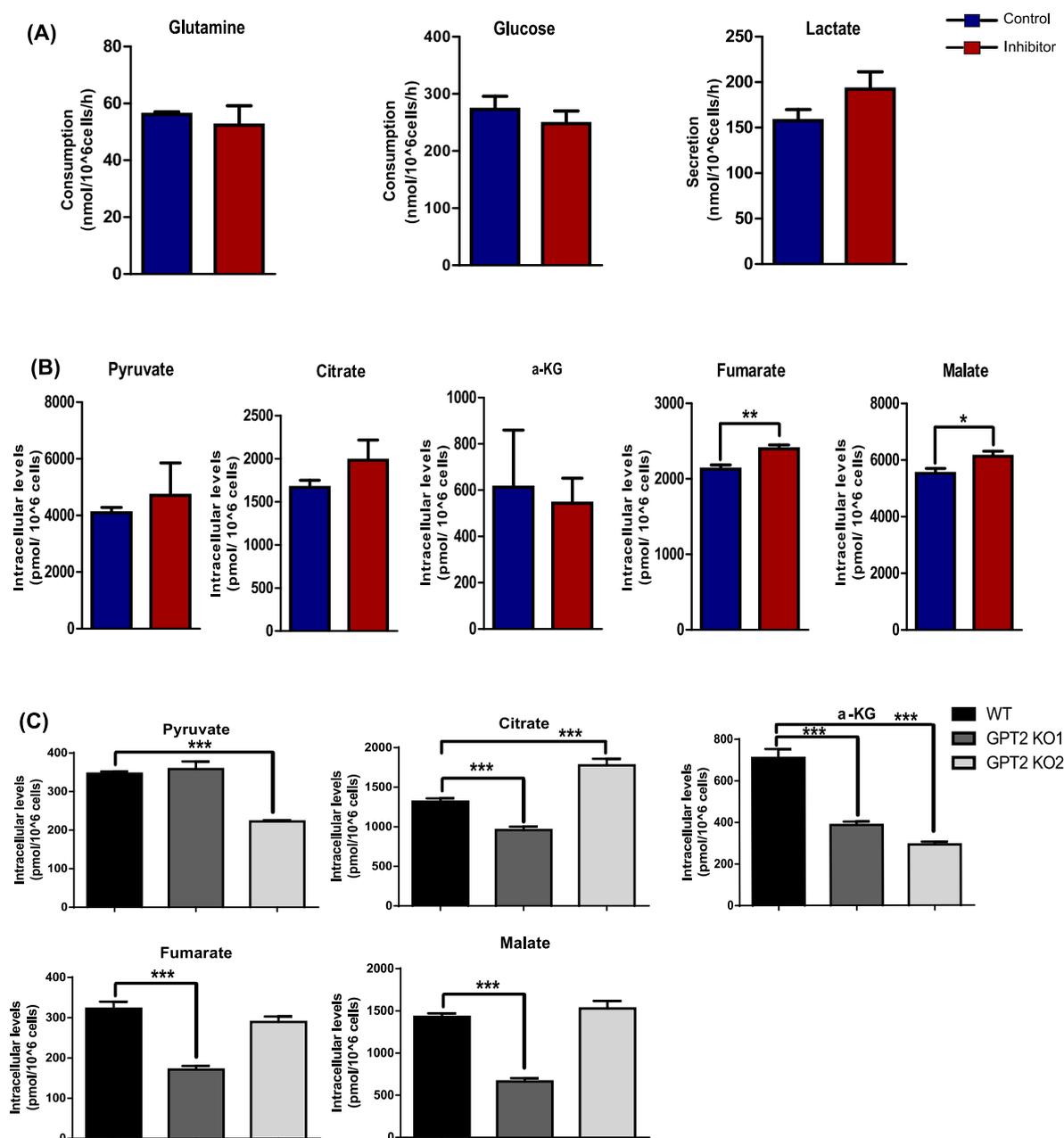
Supplementary Figure 3: **Abrogation of GPT2 mildly affects MCF-7 cell growth.** A, qRT-PCR analysis of *GPT2* mRNA expression and western blot analysis of GPT2 protein expression 72h after transfection with a pooled siRNA targeting *GPT2* (or corresponding non-targeting control). B, Relative cell numbers upon *GPT2* knock-down. Relative extracellular alanine levels at different GPT2 inhibitor concentrations (C) or 72 h after treatment with 100 μM inhibitor (D). E, RPPA analysis of GPT2 protein expression 24h after treatment with 100 μM of GPT2 inhibitor β-chloro-L-alanine (or corresponding water control). F, Relative cell numbers treated with 100 μM of the GPT2 inhibitor β-chloro-L-alanine. G, Relative cell numbers of MDA-MB-468 and MCF7 72h after treatment with different concentrations of

GPT2 inhibitor. Data are represented as mean ± SD, unless otherwise mentioned n=3 biological replicates. ***P<0.001, t-test.
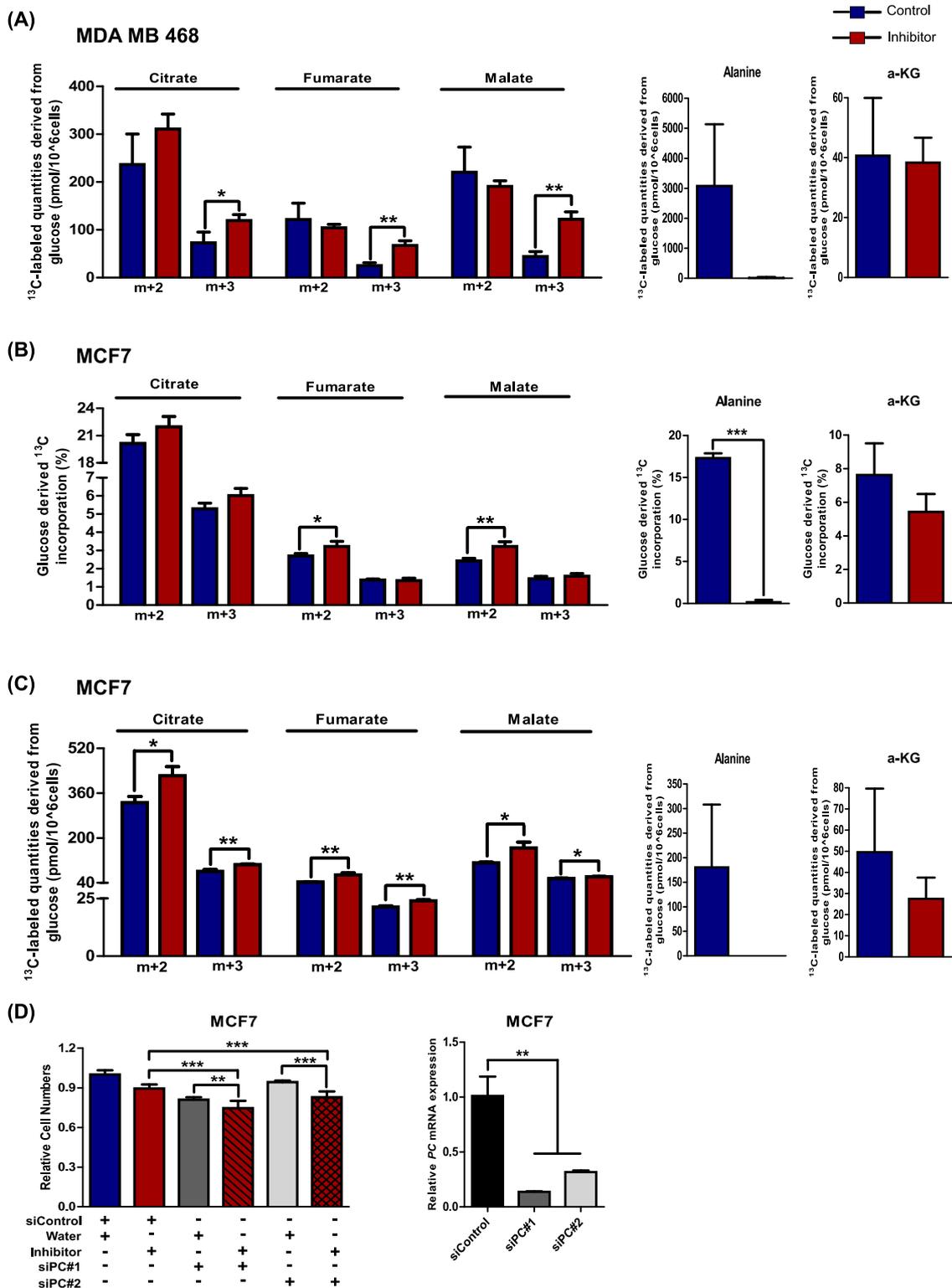
Supplementary Figure 4:

Supplementary Figure 4: Chemical or genetic inhibition of GPT2 does not affect glutamine uptake and TCA cycle intermediates in MCF7 cells. (a) Analysis of glutamine (UPLC) and glucose (GC-MS) consumption and lactate (GC-MS) secretion in MCF7 cells, 24h post treatment with GPT2 inhibitor (100 μM) (or corresponding water control), (b) GC-MS analysis of intracellular metabolites, 24h post treatment of MCF7 cells with GPT2 inhibitor (100 μM) (or corresponding water control), (c) GC-MS analysis of intracellular metabolites on

the MDA-MB-468 WT or *GPT2*-KO clones. Data are represented as mean ± SD, n=3 biological replicates. ** *P*<0.01, * *P*<0.05, t-test.

Supplementary Figure 5:

Supplementary Figure 5: **GPT2 inhibition rewires glucose metabolism.** A, Total labelled metabolite pools in MDA-MB-468 cells 24h post treatment with GPT2 inhibitor (75 μM) and 30 min incubation with media supplemented with 13C-glucose, measured by GC-MS. B, Percentage of labelled metabolite pools in MCF7 cells 24h post treatment with GPT2 inhibitor β-chloro-L-alanine (100 μM) and 30 min incubation with media supplemented with 13C-glucose, measured by GC-MS. C, Total labelled metabolite pools in MCF7 cells 24h post treatment with GPT2 inhibitor β-chloro-L-alanine (100 μM) and 30 min incubation with media supplemented with 13C-glucose, measured by GC-MS. D, Relative MCF7 cell numbers 72h after transfection with siPC (or corresponding control) and treatment with GPT2 inhibitor β-chloro-L-alanine (75 μM) (or corresponding water control) (left) qRT-PCR analysis of *PC* mRNA (n=1, 3 technical replicates) 72h after transfection in MCF7 cells (right). Data are represented as mean ± SD, n=3 biological replicates. ***$P<0.001$, ** $P<0.01$, * $P<0.05$ t-test.

Supplementary Figure 6:
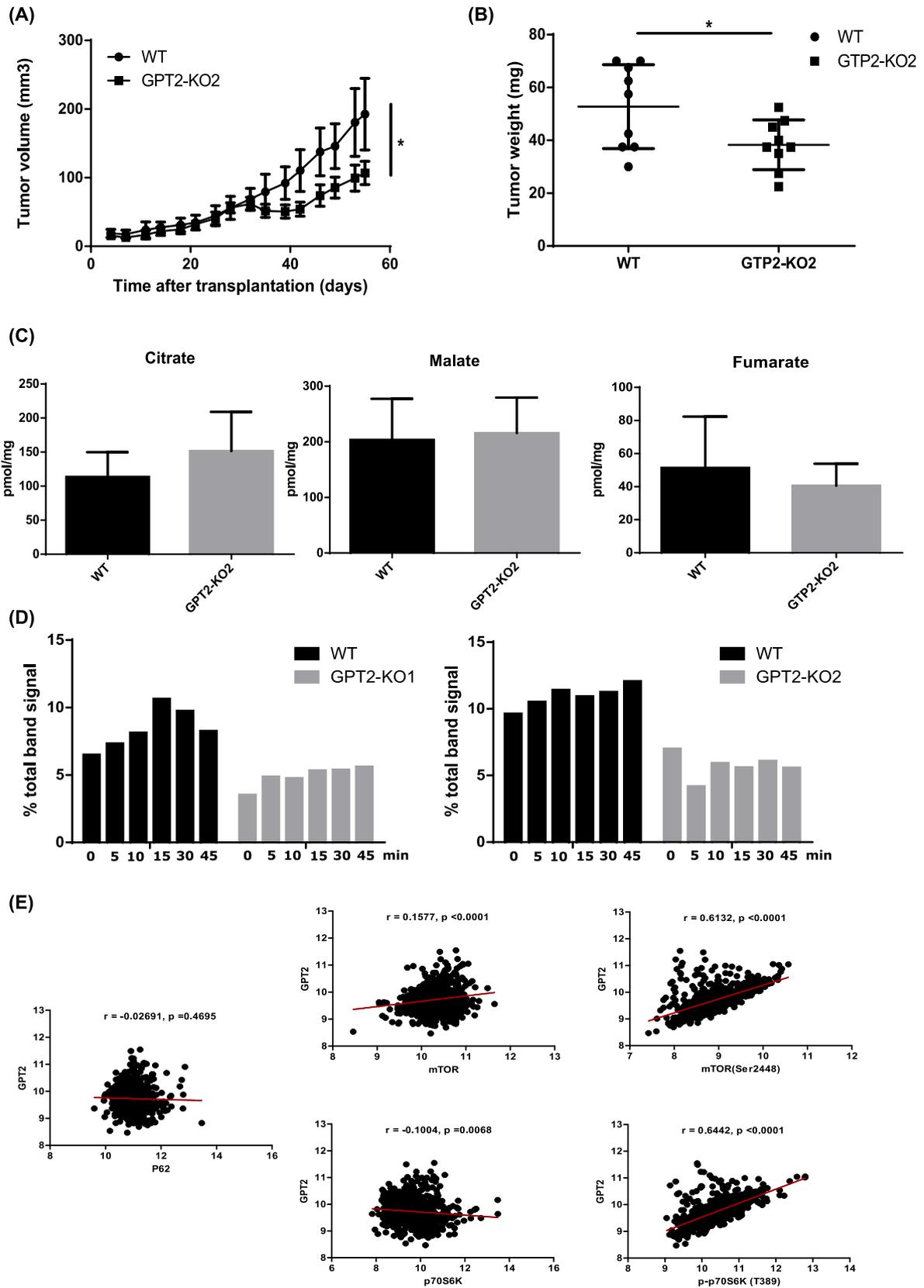
Supplementary Figure 6: **GPT2 protein expression correlates with high mTORC1 activity and low p62 levels in patients.** Xenograph growth (A) and tumor weight (B) of MDA-MB-468 WT and clone *GPT2* KO2. C, GC-MS analysis of metabolite levels in WT and *GPT2* KO2 tumors. D, 4EBP1 hyper phosphorylated band quantification of experiment shown in Figure 5b and 5c. E, Correlation between protein expression of GPT2 and autophagy related proteins in all patients in the PiA cohort. Correlation coefficient (r) and significance were calculated using Pearsons' correlation test. *$P<0.05$, ***$P<0.001$ t-test.

Supplementary References

1. Riaz M, van Jaarsveld MT, Hollestelle A, Prager-van der Smissen WJ, Heine AA, Boersma AW, Liu J, Helmijr J, Ozturk B, Smid M, Wiemer EA, Foekens JA, et al. miRNA expression profiling of 51 human breast cancer cell lines reveals subtype and driver mutation-specific miRNAs. *Breast Cancer Res* 2013;**15**: R33.

2. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe J-P, Tong F, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 2006;**10**: 515–527.

3. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiwa S, Yuan Y, Gräf S, Ha G, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012;**486**: 346-52.

4. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012;**490**: 61-70.

5. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 2013;**6**, pl1.

6. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, et al. The cBio Cancer Genomics Portal: An Open Platform for Exploring Multidimensional Cancer Genomics Data. *Cancer Discov* 2012;**2**: 401–404.

7. R Core Team R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. 2012 http://www.R-project.org/