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Supplemental Information

Reprogramming of Amino Acid Transporters to Support Aspartate and Glutamate Dependency Sustains Endocrine Resistance in Breast Cancer

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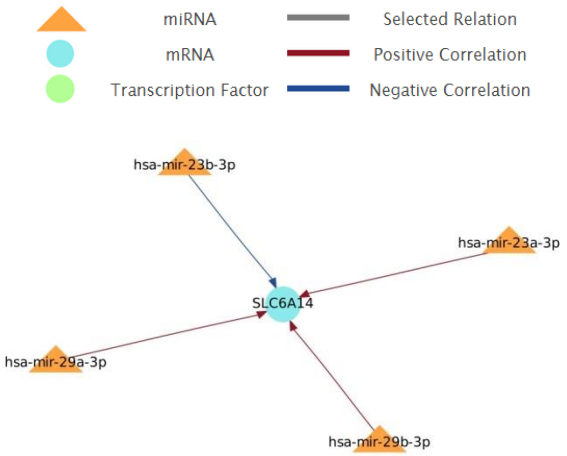
Figure S1 (related to Figure 1)

A



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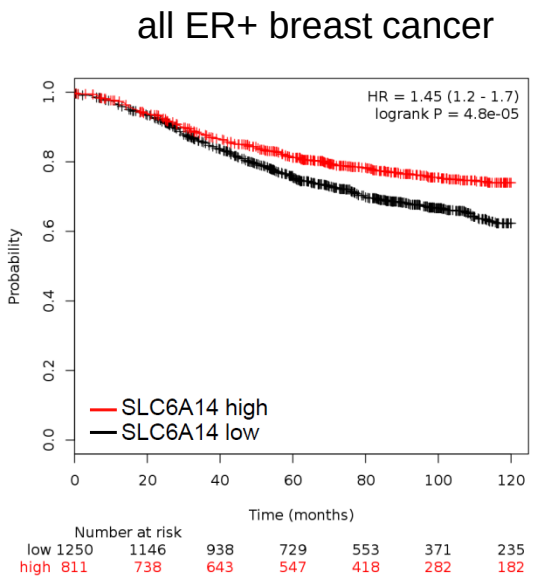

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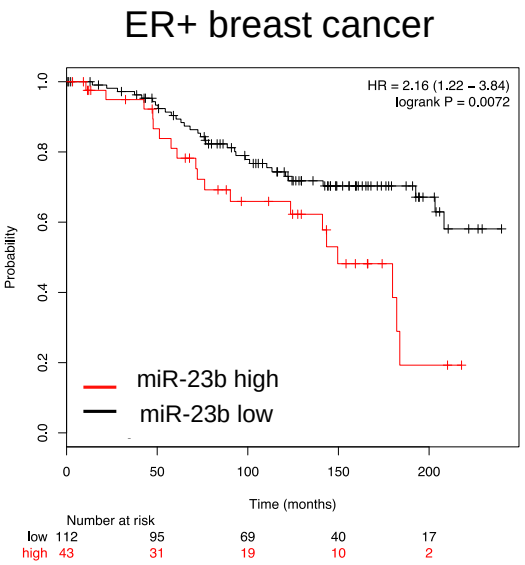

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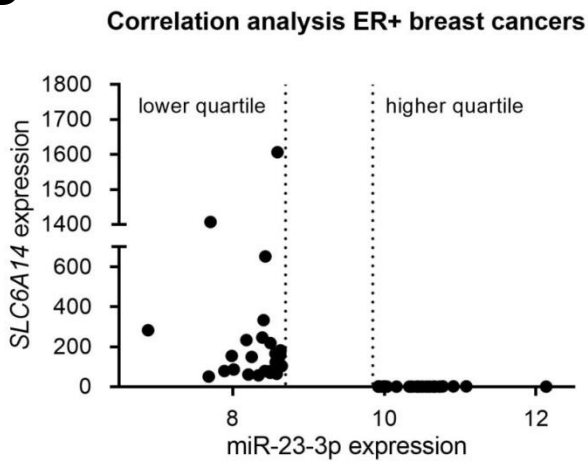

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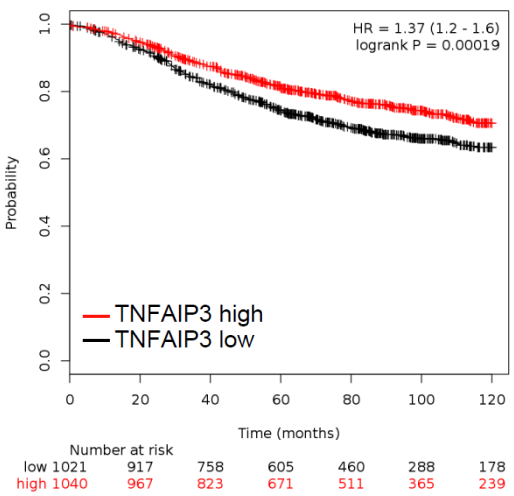

Figure S1 (related to Figure 1). SLC6A14 and miR-23b-3p network and clinical relevance.

(A) The integration analysis of deregulated miRNA and mRNA in MCF7-LTED cells compared to parental cells was obtained using the web tool MAGIA[2] (Bisognin et al. 2012). The network centered on the SLC6A14 gene revealed that, among the deregulated miRNAs in LTED cells, four show predictive interaction with the SLC6A14 transcript. Of the four miRNAs, only the miR-23b-3p has a negative correlation, as you would expect for a miRNA and its target gene (i.e. miR-23b-3p enhanced expression and SLC6A14 reduced expression in MCF7-LTED cells). (**B,C**) Lower SLC6A14 and higher miR-23b expression identifies a subset of ER+ breast cancer patients characterized by worse prognosis**.** Kaplan-Meier analysis of relapse-free (B) or overall (C) survival of ER+ breast cancer patients based on (B) SLC6A14 or (C) miR-23b expression. The curated cohort of patients is generated as detailed in (Lánczky et al. 2016). The miRNA cohort of patients is the METABRIC (untreated samples) as detailed in KM plotter. Hazard ratio (HR) and log-rank Mantel-Cox P-value are shown. (**D**) miR-23b-3p and SLC6A14 expression levels are inversely correlated in the TCGA dataset. Correlation analysis between miR-23b-3p and SLC6A14 was performed on the patients selected based on higher (upper quartile) and lower (lower quartile) expression levels of miR-23b-3p (n=104). The correlation is significant and negative (Spearman r = -0.7369, (CI: -0.8159—0.6309), p-value (two-tailed)<0.0001). Importantly, it is worth noticing that *SLC6A14* expression is completely lost in the high miR-23b-3p tumors. This support our hypothesis that SLC6A14 and miR-23b-3p expression are tightly linked in breast cancer specimens. (**E**) Lower TNFAIP3 expression identifies a subset of ER+ breast cancer patients characterized by worse prognosis. Kaplan-Meier analysis of relapse-free survival of ER+ breast cancer patients based on TNFAIP3 expression. The curated cohort of patients is generated as detailed in (Lánczky et al. 2016).

Figure S2 (related to Figure 2)

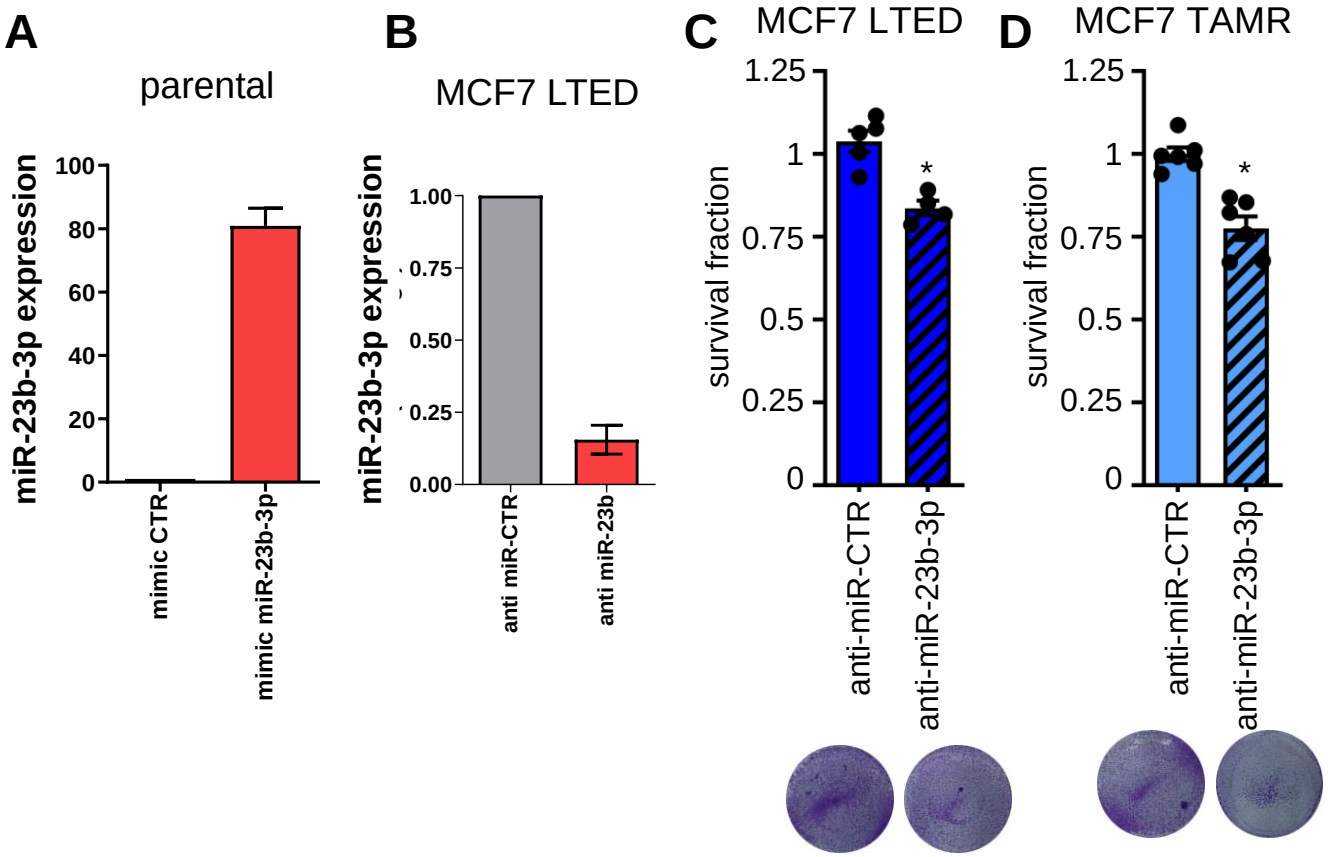

Figure S2 (related to Figure 2). Transfection efficiency of miR-23b-3p inhibitor and miR mimic and cell survival after miR-23b-3p silencing.

(**A,B**) Cells were transfected with either mimic CTR and mimic miR-23b-3p (for parental MCF7 cells, A) or anti-CTR and anti-miR-23b-3p (for MCF7 LTED cells, B) and subjected to qRT-PCR to assess the expression of miR23b-3p as described in the Methods section. (**C,D**) LTED and TAMR MCF7 cell derivatives transfected for 72 hours with the oligos described in figure were subjected to crystal violet cell viability assay and subsequent quantification as detailed in Methods. Student t-test was used for statistical analysis. n=3 biological replicates. * $P < 0.05$.

Figure S3 (related to Figure 3)

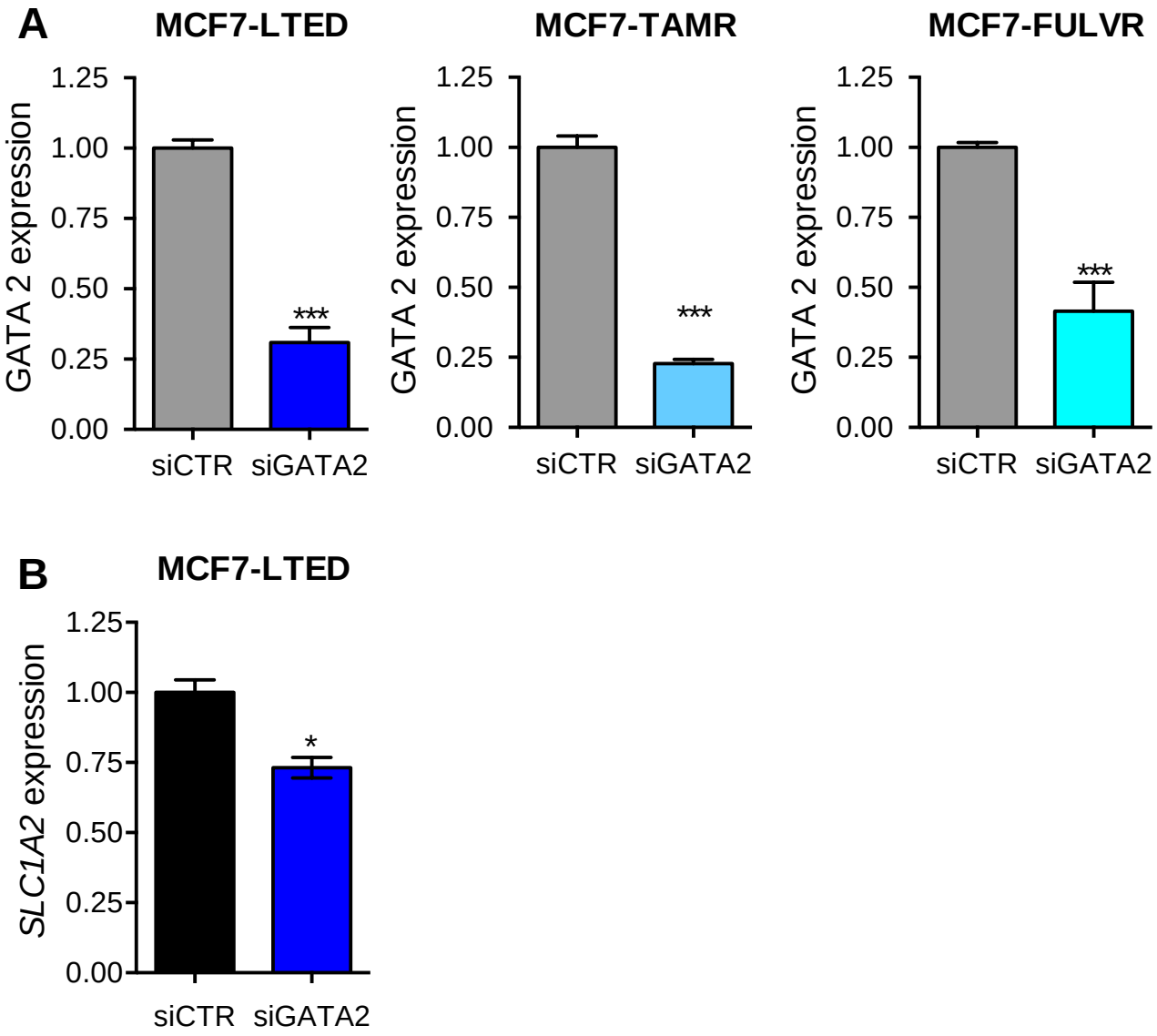

Figure S3 (related to Figure 3). GATA2 and SLC1A2 expression levels after GATA2 silencing.
Transfection efficacy of siRNA against GATA2 was demonstrated in ETR cells. LTED, TAMR and FULVR MCF7 cells were transfected with siCTR or siGATA2 oligos as described in Methods. 72 hours post transfection cells were lysed, RNA extracted and subjected to qRT-PCR for GATA2 (**A**) or SLC1A2 (**B**). Student t-test was used for statistical analysis. n=3 biological replicates. * $P < 0.05$ *** $P < 0.001$.

Figure S4 (related to Figure 4)

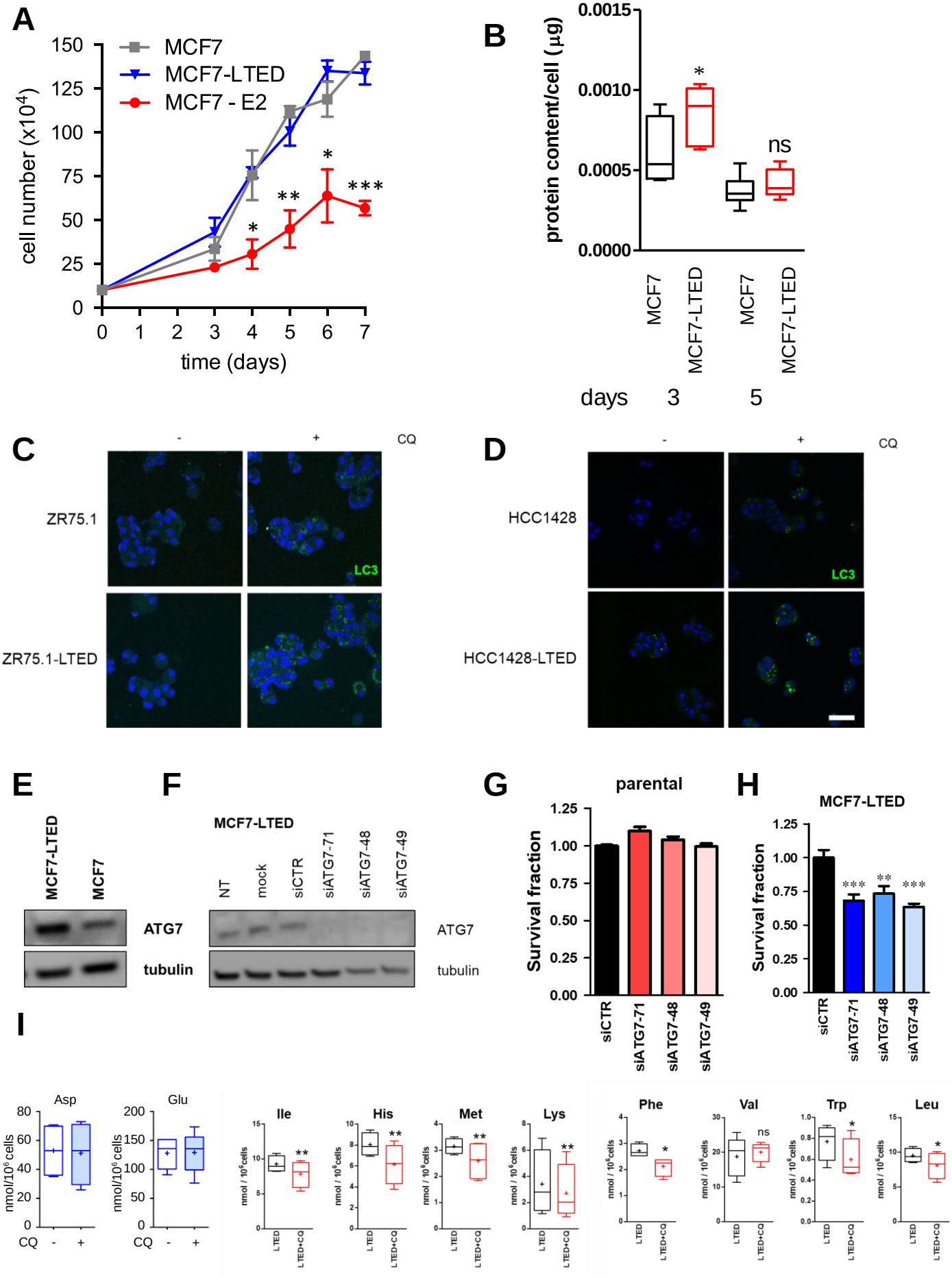

Figure S4 (related to Figure 4). Autophagy activation together with selective import of aspartate and glutamate compensates for reduction in amino acid upload in ETR cells.

(**A,B**) Comparison of MCF7 and MCF7-LTED cells survival and protein content. (A) No significant reduction in cell survival between LTED and parental MCF7 cells was observed by Trypan Blue dye exclusion test within the (7 days) time range. However, estrogen (E2) deprivation resulted in a significant (versus MCF7) cell growth reduction starting at day 4. (B) Cells were lysed and protein quantification was measured using Bradford (Bio-Rad Protein Assay). Total protein content was divided by the number of cells lysed to obtain an estimation of the protein content per cell. n were at least 3 biological replicates per condition performed in duplicate. Two-way ANOVA Bonferroni's corrected test was used, *P<0.05, ** $P < 0.01$ and *** $P < 0.001$. (**C,D**) Representative images of LTED and parental cells (ZR75.1 -c- and HCC1428 -d-) that were cultured in basal medium (-) or treated (+) with 25 µmol/L of CQ for 16 hours and subjected to confocal analysis (green: LC3; blue: TO-PRO-3, nuclei). LC3 puncta were quantified as described in Methods and the quantification analysis is shown in Figure 4. Scale bar: 20 µM. (**E-H**) ATG7 silencing impairs cell survival of MCF7-LTED cells. LTED and parental MCF7 cells were transfected as described in Methods using 3 different siRNA designed to target the ATG7 (Autophagy Related 7) transcript (siATG7, Sigma) and a non targeting control (siCTR). Mock transfected cells were incubated in the lipo-cationic agent used for transfection. E, ATG7 is significantly overexpressed in LTED cells when compared to parental MCF7 cells. F, ATG7 knock-down efficiency was monitored by Western blot analysis (Santa Cruz Biotechnology antibody, sc-376212). (**G,H**) Parental and MCF7-LTED cells were transfected and allowed to grow for 48 hours prior to subject the cells to the colorimetric non-radioactive quantification of cell proliferation and viability by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay (Sigma) using the manufacturer's instruction. siCTR transfected cells were used as comparator. n=3 biological replicates. One-way ANOVA Dunnett's corrected test was used. ** $P<0.01$; *** $P<0.001$. (**I**) CQ treatment impairs essential amino acids intracellular levels in MCF7-LTED cells but not aspartate (Asp) and glutamate (Glu). Intracellular amino acids were extracted from LTED cells maintained in culture medium or in medium containing 25 µmol/L chloroquine (CQ) for 16 hours. Amino acids derivatization was performed using 4-N, N-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS). DABS-amino acids were detected at visible light wavelengths using HPLC as described in Methods. The quantification of essential amino acids from 4 biological replicates is represented in a box and whiskers graph (mean, 25- and 75-percentile are shown and + indicates the median of the values). n=4 biological replicates. Student paired t-test * $P<0.1$; ** $P<0.5$. The essential amino acids quantified are phenylalanine (Phe), valine (Val), tryptophan (Trp), methionine (Met), leucine (Leu), isoleucine (Ile), lysine (Lys), and histidine (His).

Figure S5 (related to Figure 5)

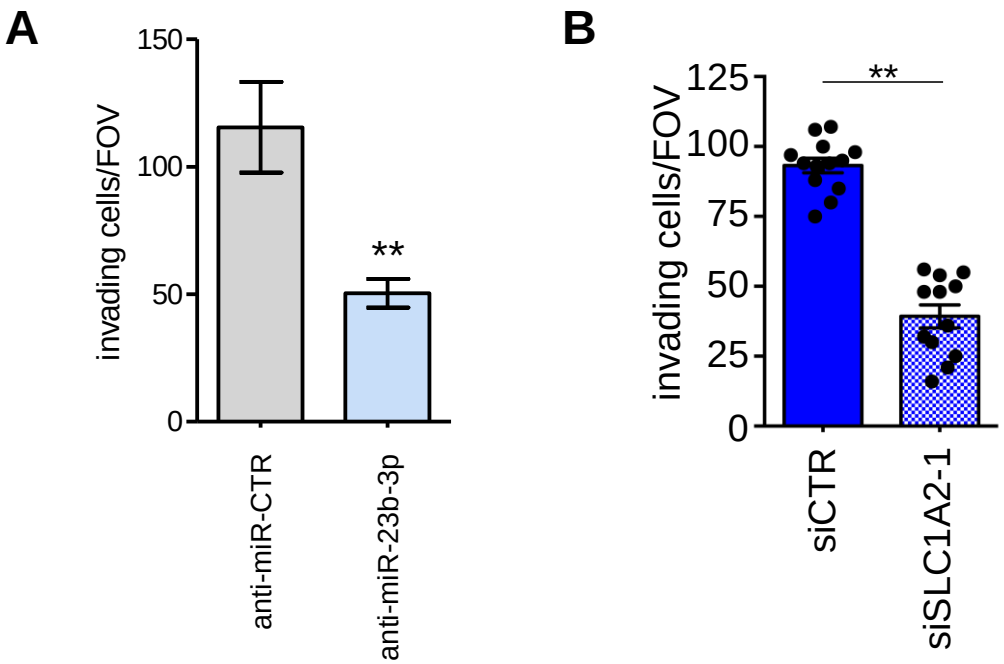

Figure S5 (related to Figure 5). miR-23b-3p and SLC1A2 targeting reduces *in vitro* MCF7-LTED cells invasion ability. (**A**) MCF7-LTED cells were transfected with either siCTR or siSLC1A2 or (**B**) anti-miR-23b-3p or a non-targeting control (anti-miR-CTR) and subjected to invasion assay overnight as described in Methods. Data represent mean ± SEM. n=3 biological replicates. Each dot represents one of the 4 fields of view analyzed per biological experiment. Student t test; ** $P < 0.01$.

Figure S6 (related to Figure 6)

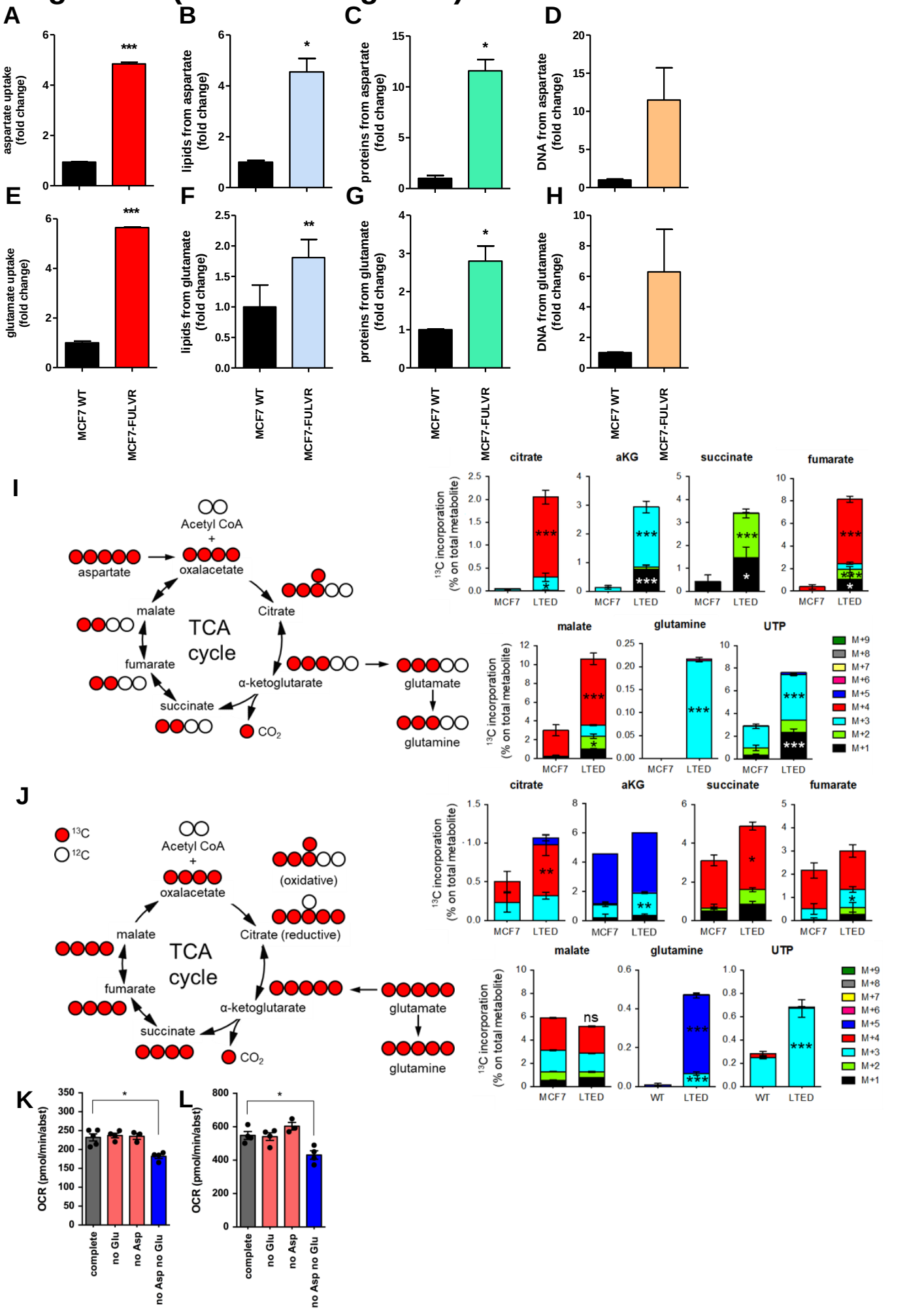

Figure S6 (related to Figure 6). Metabolic plasticity of ETR cells is sustained by aspartate and glutamate. (**A-D**), FULVR and MCF7 cells were subjected to ^{14}C radioactive aspartate uptake (A) or cultured for 24 hours in a medium containing radioactive aspartate. Lipids, proteins or DNA were extracted in parallel and radioactive signal measured to monitor the amount of aspartate that is incorporated into lipids (B), proteins (C) and DNA (D) as detailed in Methods. Each value was normalized on protein content. (**E-H**), FULVR and MCF7 cells were subjected to ^{14}C radioactive glutamate uptake (E) or cultured for 24 hours in a medium containing radioactive glutamate. Lipids, proteins or DNA were extracted in parallel and radioactive signal measured to monitor the amount of glutamate that is incorporated into lipids (F), proteins (G) and DNA (H) as detailed in Methods. Each value was normalized on protein content. Data represent mean ± SEM, n = 3. Student t test; *, P < 0.05; ** P < 0.01; *** P < 0.001; ns = not significant. (**I**) Schematic overview of metabolism of downstream ^{13}C-labeled aspartate and fluxes and relative incorporation of ^{13}C carbons derived from aspartate in the metabolites reported in figure. U-^{13}C-Asp was administrated together with unlabeled Glu to evaluate whether the addition of Glu may impact on the metabolic fluxes as described in main Figure 6 when a single amino acid was used for the flux analysis; (**J**) Schematic overview of metabolism of downstream ^{13}C-labeled glutamate (Glu) and fluxes and relative incorporation of ^{13}C carbons derived from glutamate in the metabolites reported in figure. U-^{13}C-glutamate was administrated together with unlabeled aspartate (Asp) to evaluate whether the addition of Asp may impact on the metabolic fluxes as described in main Figure 6 when a single amino acid was used for the flux analysis; n=3 biological replicates. Two-way ANOVA, Sidak's corrected. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns = not significant. (**K,L**). FULVR MCF7 cells were subjected to Seahorse XFe96 Mito Stress Test analysis and Oxygen Consumption Rate (OCR) was measured in real time in the presence (complete) or absence of the indicated amino acids. Basal (K) and maximal (L) respiration was calculated as detailed in the Methods. Data represent mean ± SEM. n=3 biological replicates. One-way ANOVA Dunnett's corrected test; *P<0.05, ** P < 0.01 and *** P < 0.001.

Figure S7 (related to Figure 7)

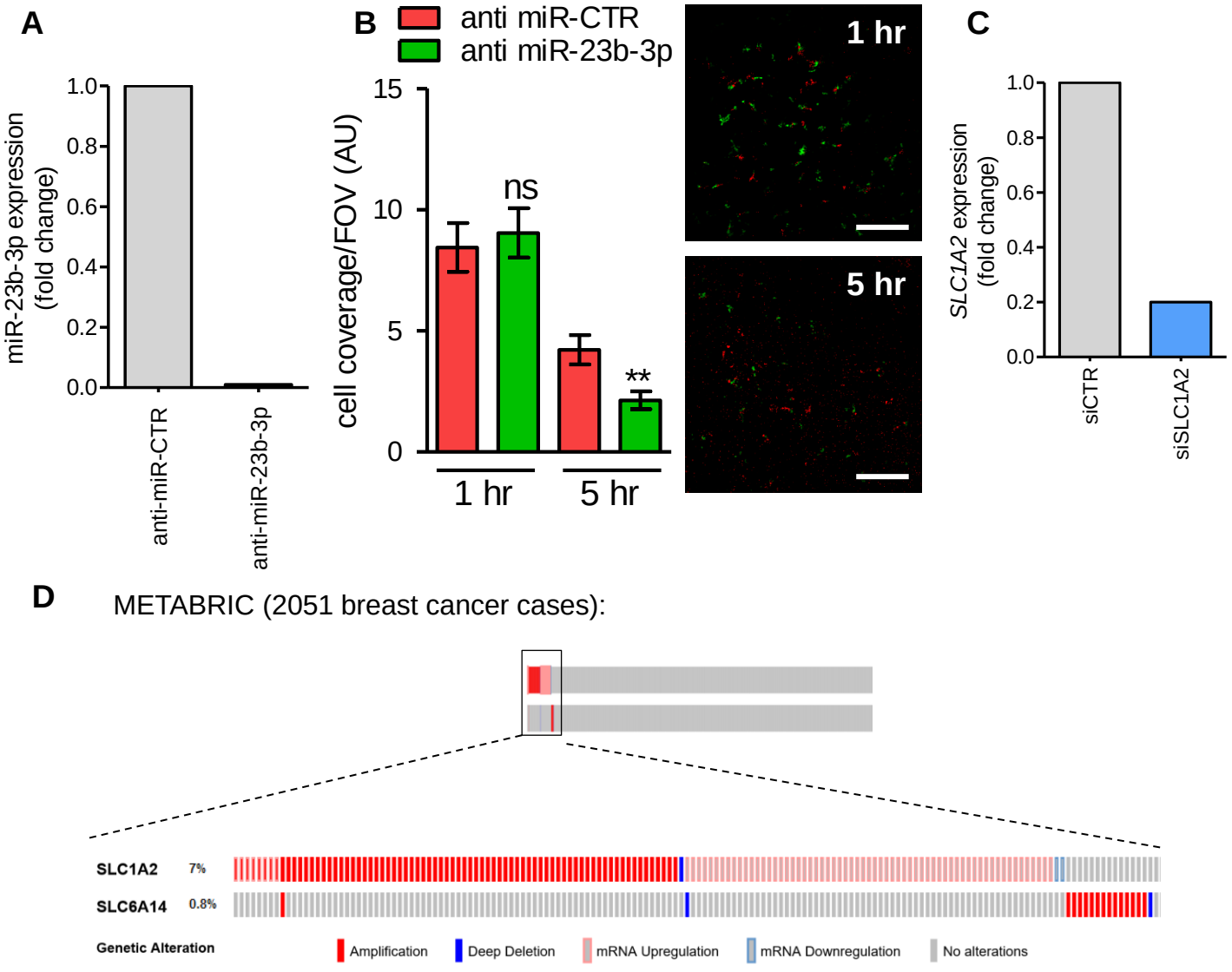

Figure S7 (related to Figure 7). Tail vein injection and *in vivo* "dye swap" lung retention assay. (**A-C**) MCF7-LTED cells were transfected with either anti-miR-23b-3p (A) or siSLC1A2 (C) and/or with the respective non-targeting control (anti-miR-CTR and siCTR). qRT-PCR analyses were performed on the cells that were used in the lung retention assays shown in Figure 7 (as detailed in Methods) to control that the injected cells were silenced for the gene of interest. Only a silencing efficiency control was done prior to injection. (B) anti-miR-CTR and anti-miR-23b-3p transfected MCF7-LTED cells were labelled with CellTracker dyes (Red for anti-miR-CTR and Green for anti-miR-23b-3p) before injecting a mixture of anti-miR-23b-3p and anti-miR-CTR transfected cells in a 1:1 ratio into the tail vein of SCID mice (n=3 per group) as described in Methods. The lungs were recovered at the time indicated and imaged to analyze the number of fluorescent cells that colonize the lungs. Data shown are mean for tumor cells coverage per field of view (FOV); n=3 mice per group per time point ± SEM. Representative images are shown, scale bar, 200 µm. ns= not significant, ** $P < 0.01$. (**D**) SLC6A14 and SLC1A2 genetic alteration in breast cancer clinical specimens. METABRIC data were retrieved using cBioPortal (http://www.cbioportal.org). SLC1A2 and SLC6A14 were altered in 157 (8%) of 2051 sequenced cases/patients. Of these 157 patients, 137 show amplification or overexpression of SLC1A2. Only 2 cases show concomitant alterations of both genes: one shows concordant upregulation of both genes and another shows the upregulation of *SLC1A2* and downregulation of *SLC6A14*.

Table S3 (related to Figure 7)

parameters	P value	Hazard Ratio (HR)
MKI67	0.135	1.29 (0.92 – 1.79)
ESR1	0.520	1.91 (0.26 – 13.81)
HER2 (ERBB2)	0.007	1.83 (1.18 – 2.85)
SLC6A14	**0.002**	**0.57 (0.4 – 0.81)**

parameters	P value	Hazard Ratio (HR)
MKI67	0.099	1.33 (0.95 – 1.85)
ESR1	0.468	2.08 (0.29 – 14.99)
HER2 (ERBB2)	0.008	1.83 (1.17 – 2.82)
TNFAIP3	**0.005**	**0.57 (0.39 – 0.85)**

Table S3 (related to Figure 7). Multivariate Cox Regression Analysis of the analysis of tamoxifen-treated samples for SLC6A14 and TNFAIP3.