**Supplemental Information**

# Metabolic Imaging Detects Resistance

# to PI3Kα Inhibition Mediated by Persistent

# FOXM1 Expression in ER[+] Breast Cancer

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**Supplemental figures**
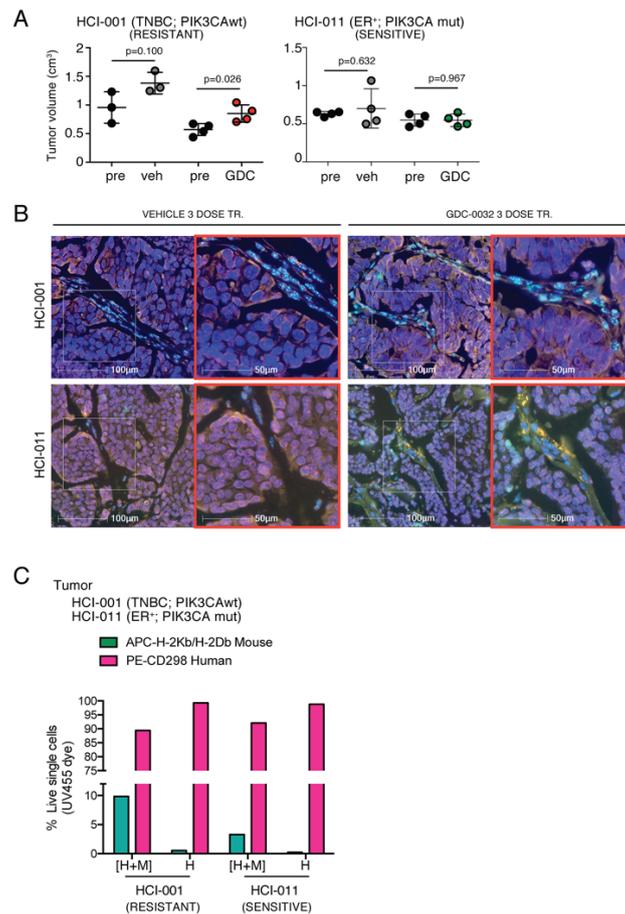


**Figure S1, related to Figure 1. Resistance to a PI3Kα inhibitor can be detected using metabolic imaging with hyperpolarized [1-$^{13}$C]pyruvate**

A) HCI-001 and HCI-011 tumor volumes (cm$^3$) before (pre) and after short-term treatment with GDC-0032 (HCI-001 n=4; HCI-011 n=4) or drug vehicle (HCI-001 n=3; HCI-011 n=4). P-values were calculated using two-sided Welch's T-tests.

B) FFPE sections of HCI-001 and HCI-011 tumors treated short-term with vehicle (left) or GDC-0032 (right) were stained for simultaneous detection of human (red) and mouse (green) centromeres (FISH analysis); with a DAPI counterstain (blue). Images were captured at 40x magnification.

C) Percentage of human (PE-CD298 positive) and mouse (APC-H-2Kb/H-2Db positive) live cells isolated from untreated disaggregated tumors determined by flow cytometry using a BD FACSymphony analyzer. Percentages of human breast cancer epithelial and mouse stomal cells (H+M), or human breast cancer epithelial cells (H) are shown. Mean of 1 biological experiment. Live cells were determined by dye-staining, eBioscience™ Fixable Viability Dye eFluor™ 455 nm negative.
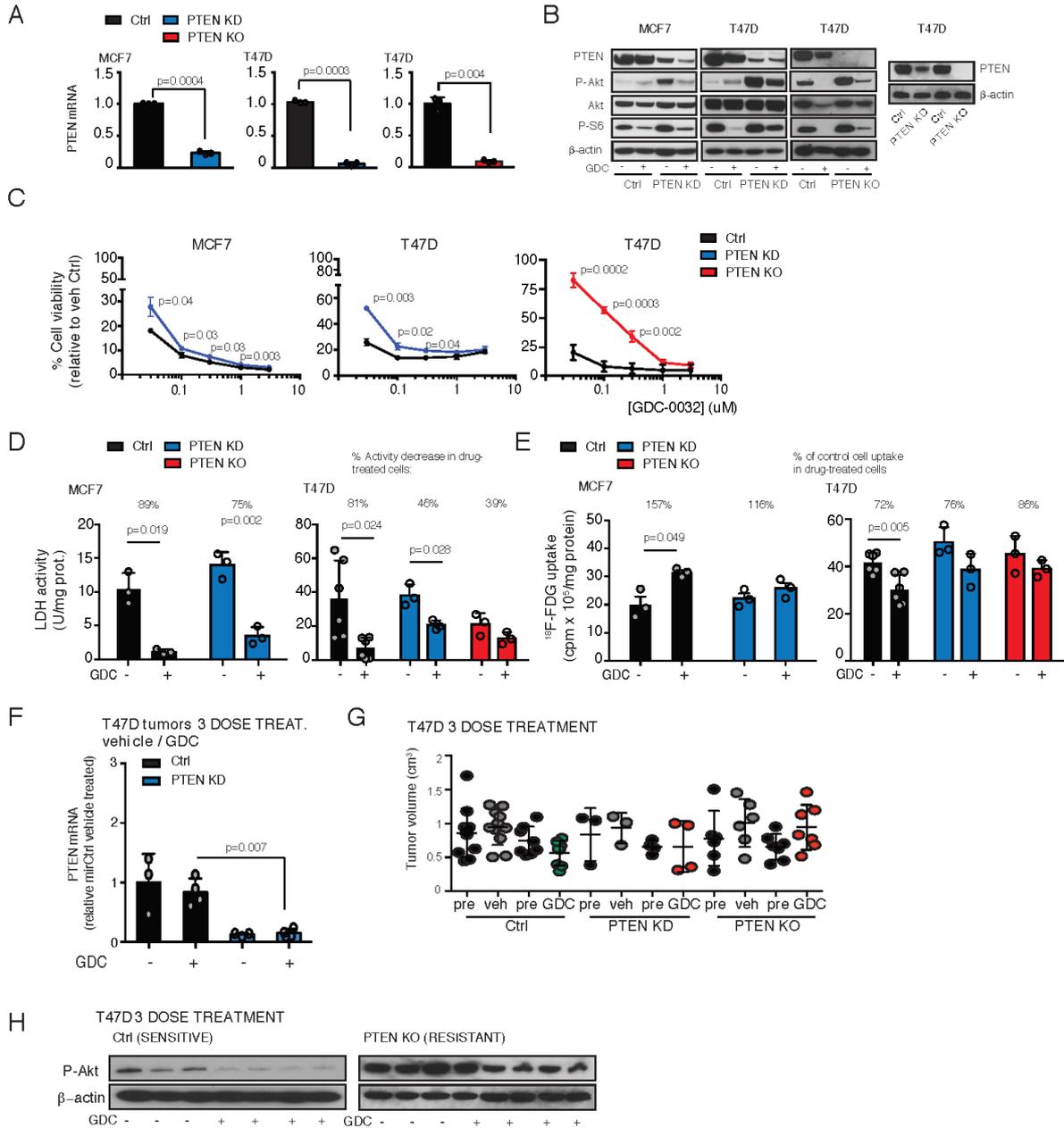
**Figure S2, related to Figure 2. Imaging with hyperpolarized [1-$^{13}$C]pyruvate detects sensitivity and induced resistance to PI3Kα inhibition**

We engineered MCF7 or T47D cells to knockdown or knock out PTEN expression using a synthetic shRNA embedded in a miR30 backbone (PTEN KD) or by using CRISPR-Cas9 (PTEN KO).

A) Cells were seeded at low density and expression of *PTEN* was determined by qPCR and normalized by the geomean of the housekeeping genes *β-ACTIN* and *B2M*. Figure shows the mean ± SD per condition (n=3). P-values were calculated using one-sided T-tests on the averages of technical replicates.

B) Expression of PI3K/Akt/mTOR pathway proteins in cells used in A) treated with drug vehicle or GDC-0032.

C) Viability of cells used in A) treated with different concentrations of GDC-0032 for 120 h, starting 24 h after plating. Figure shows the mean ± SD per group and drug concentration (n=3, 5 technical replicates). P-values were calculated using two-sided Welch's T-tests on the averages of technical replicates.

D) LDH activity measured in cells used in A) treated with drug vehicle or GDC-0032 for 72 h, starting at 24 h after plating. Figure shows the mean ± SD (n=3 to 6 experiments). The figures show the % decrease in LDH activity in the drug-treated cells. P-values were calculated using two-sided Welch's T-tests on the averages of replicates.

E) [$^{18}$F]FDG uptake by the cells used in A) following treatment with drug vehicle or GDC-0032 for 72 h. Figure shows the % of control cell [$^{18}$F]-FDG uptake in the drug-treated cells ± SD for each condition (n=3 to 6 experiments). P-values were calculated using two-sided Welch's T-tests.

F) Expression of *PTEN* determined by qPCR and normalized by the geomean of *β-ACTIN* and *B2M* in GDC-0032 sensitive (Ctrl; PTEN wt) and PTEN-knockdown drug-resistant tumors (PTEN KD) recovered at the end of the imaging protocol. Figure shows the mean ± SD per condition (triplicates). P-values were calculated using two-sided Welch's T-tests.

G) Volumes (cm$^3$) of T47D PTEN wt (Ctrl), PTEN-knockdown (PTEN KD) and PTEN-knockout (PTEN KO) tumors were determined using caliper measurements before (pre) and after 3 doses of GDC-0032 or drug vehicle. For vehicle-treated tumors, n=10 for Ctrl, n=3 for PTEN KD and n=6 for PTEN KO and for GDC-0032-treated tumors n=7 for Ctrl, n=4 for PTEN KD and n=7 for PTEN KO. P-values were calculated using two-sided Welch's T-tests.

H) Western blots of P-Akt in T47D PTEN wt (Ctrl), or PTEN-knockout (PTEN KO) tumors recovered at the end of treatment with 3 doses of GDC-0032 or drug vehicle.
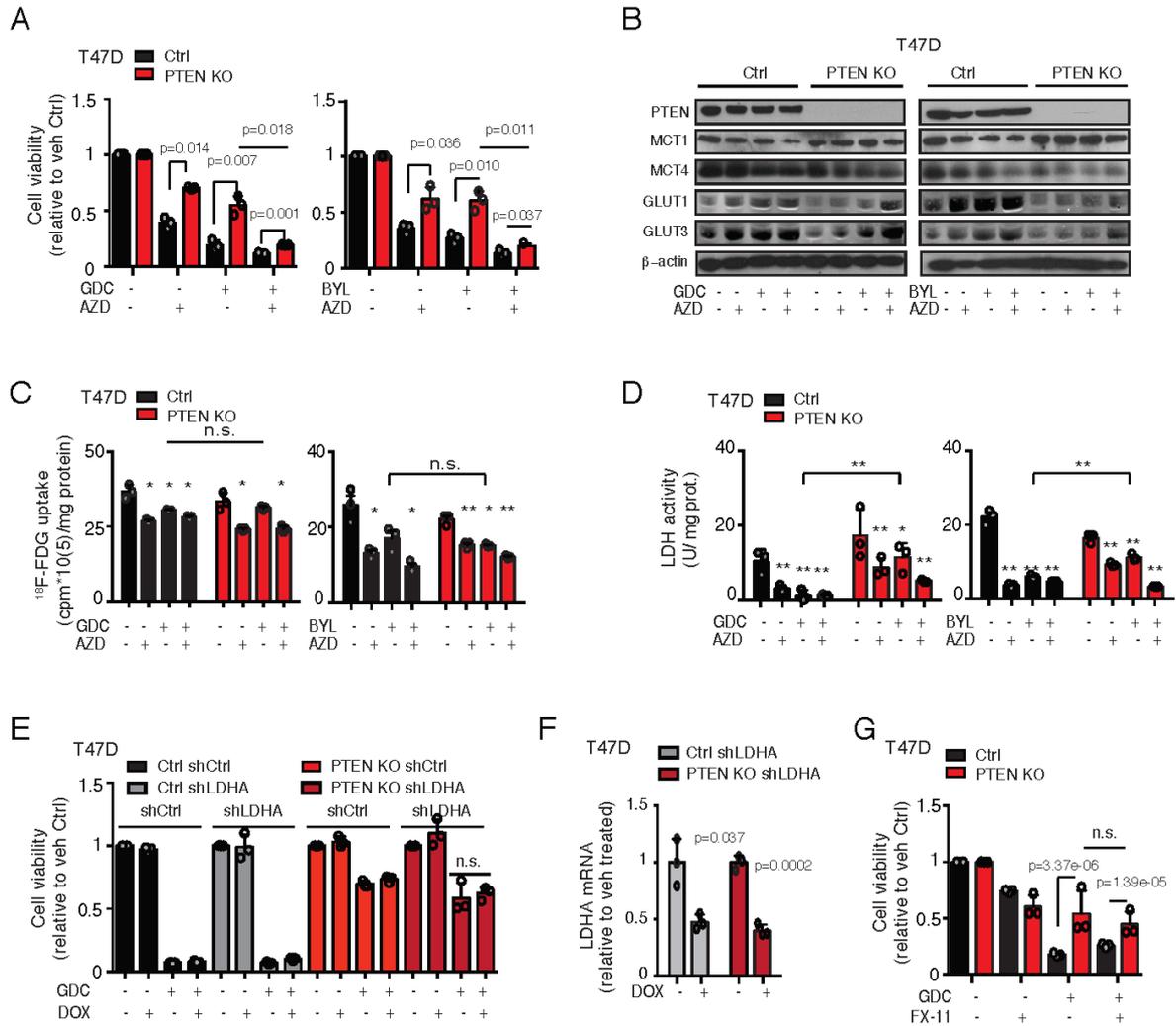
**Figure S3, related to Figure 3. Combination treatments that overcome resistance to PI3Kα-specific inhibition result in decreased LDHA expression**

A) Viabilities of T47D PTEN wt (Ctrl) and PTEN-knockout (PTEN KO) cells treated with the indicated drug combinations for 120 h, starting 24 h after plating. Figure shows the mean ± SD per condition (n=3, 5 technical replicates).  P-values were calculated using two-sided Welch's T-tests on the averages of technical replicates.

B) [$^{18}$F]FDG uptake in PTEN wt (Ctrl) and PTEN KO T47D cells treated with the indicated drug combinations for 72 h. Figure shows the mean ± SD per condition (n=3). P-values were calculated using two-sided Welch's T-tests on technical replicates, * p $\leq$ 0.05 and ** p $\leq$ 0.005.

C) LDH activity in PTEN wt (Ctrl) and PTEN KO T47D cells treated with the indicated drug combinations for 72 h. Figure shows the mean ± SD per condition (n=3).  P-

values were calculated using two-sided Welch's T-tests on technical replicates, * p ≤ 0.05 and ** p ≤ 0.005.

D) Expression of PTEN, HK-II, LDHA, monocarboxylate (MCT1 and MCT4) and glucose transporters (GLUT1 and GLUT3) in PTEN wt (Ctrl) and PTEN KO T47D cells treated with the indicated drug combinations for 72 h.

E) Viabilities of PTEN wt (Ctrl) and PTEN KO T47D cells expressing doxycycline-inducible shRNA sequences targeting LDHA (shLDHA) or control sequences (shCtrl) and treated with the indicated drug combinations for 120 h. Figure shows the mean ± SD per condition (n=3, 5 technical replicates). P- values were calculated using two-sided Welch's T-tests on the averages of technical replicates.

F) Expression of *LDHA* in the cells used in (D) was determined by qPCR and normalized by the geomean of *β-ACTIN* and *B2M*. Figure shows the mean ± SD per condition (n=3). P values were calculated using two-sided Welch's T-tests on the averages of technical replicates.

G) Viabilities of PTEN wt (Ctrl) and PTEN KO T47D cells treated with the indicated drug combinations for 120 h. Figure shows the mean ± SD per condition (n=3, 5 technical replicates). P-values were calculated using two-sided Welch's T-tests on the averages of technical replicates.

A

Input Ctrl FOXM1 Input Ctrl FOXM1 Input Ctrl FOXM1 Input Ctrl FOXM1
LDHA #1+2

vehicle    GDC         vehicle    GDC
T47D Ctrl (SENSITIVE)    T47D PTEN-KO (RESISTANT)

Input Ctrl FOXM1 Input Ctrl FOXM1 Input Ctrl FOXM1 Input Ctrl FOXM1
LDHA #3+4

vehicle    GDC         vehicle    GDC
T47D Ctrl (SENSITIVE)    T47D PTEN-KO (RESISTANT)

LDHA (1+2)
Fold enrichment
p=0.0001

LDHA (3+4)
p=0.004    p=0.003

CCNB1
p=0.0005

■ Ctrl vehicle
■ Ctrl GDC-0032
■ PTEN KO vehicle
■ PTEN KO GDC-0032

B

T47D
■ Ctrl  ■ PTEN KO
FOXM1 mRNA

GDC  -  -  +  +  -  +    -  -  +  +  -  +
BYL  -  -  -  -  +  +    -  -  -  -  +  +
AZD  -  +  -  +  -  +    -  +  -  +  -  +

C

MCF7
cell viability (relative to veh control)
p=0.005
GDC  -  +  -  +
RESISTANT
CONT. TREATMENT

T47D
p=0.007
BYL  -  +  -  +
RESISTANT
CONT. TREATMENT

D

MCF7
Ctrl (SENSITIVE)  CONT. TREAT. (RESISTANT)
FOXM1
1  0.13  1  1.13
β-actin
GDC  -  +  -  +

T47D
Ctrl (SENSITIVE)  CONT. TREAT. (RESISTANT)
FOXM1
1  0.26  1  1.22
β-actin
BYL  -  +  -  +

MCF7  T47D
PTEN
1  0.87  1  0.93
β-actin
C  R  C  R

E

MCF7
LDH activity (U/mg prot)
p=0.002
GDC  -  +  -  +
RESISTANT
CONT. TREATMENT

T47D
p=0.010
BYL  -  +  -  +
RESISTANT
CONT. TREATMENT

F

MCF7
Ctrl (SENSITIVE)  CONT. TREAT. (RESISTANT)
LDHA
P-S6
β-actin
GDC  -  +  -  +

T47D
Ctrl (SENSITIVE)  CONT. TREAT. (RESISTANT)
LDHA
P-S6
β-actin
BYL  -  +  -  +

G

MDAMB231
Cell viability (relative to veh Ctrl)
n.s.
GDC  -  +

MDAMB468
n.s.
GDC  -  +

H

MDAMB231  MDAMB468
FOXM1
HK-II
LDHA
P-S6
β-actin
GDC  -  +    GDC  -  +

I

T47D shFOXM1
VEHICLE         DOX
51μm            51μm
FOXM1

6

**Figure S4, related to Figure 4. PI3Kα inhibition decreases FOXM1 expression whereas drug-resistant tumors show sustained expression**

A) T47D Ctrl and PTEN KO cells treated with vehicle or GDC-0032 for 72 h were subjected to chromatin immunoprecipitation assays using a FOXM1-specific antibody (FOXM1 Ab), a control antibody (IgG) and a positive control H3-antibody (H3 Ab). Promoter regions corresponding to 2 FOXM1-sites (oligos 1+2 or oligos3+4) in the *LDHA* promoter were amplified using qPCR, in three technical replicates. A FOXM1-site in the *CCNB1* promoter was used as a positive control. Figure shows the mean ± SD per condition (n=3). P-values were calculated using two-sided Welch's T-tests.

B) Expression of *FOXM1* in Ctrl (PTEN wt) and PTEN KO T47D cells treated with the indicated drug combinations for 120 h. FOXM1 mRNA levels were determined by qPCR and normalized by the geomean of *β-ACTIN* and *B2M*. Figure shows the mean ± SD per condition (n=3). P-values were calculated using two-sided Welch's T-tests on the averages of technical replicates, * $p \leq 0.05$ and ** $p \leq 0.005$.

C) Viabilities of MCF7, T47D, MCF7 cells rendered resistant to GDC-0032 and T47D cells rendered resistant to BYL-719 by prolonged drug treatment, following treatment for 120 h. Figure shows the mean ± SD per condition (n=3, 5 technical replicates). P-values were calculated using two-sided Welch's T-tests on the averages of technical replicates.

D) Upper panel; FOXM1 expression in PTEN wt (Ctrl) and PTEN KD MCF7 and T47D cells treated with indicated drug combinations for 72 h. Lower panel; Immunoblot of PTEN in MCF7 and T47D cells that had been rendered resistant to GDC-0032 or BYL-719 (R) respectively by prolonged exposure to the drugs compared to the parental lines (C) following treatment.

E) LDH activity in the cells used in C treated with GDC-0032 or BYL-719 for 96 h. Mean ± SD per condition (n=3). P-values were calculated using two-sided Welch's T-tests.

F) Expression of P-S6 and LDHA in the cells used in C treated with GDC-0032, or BYL-719 for 96 h.  P values were calculated using two-sided Welch's T-tests.

G) Viabilities of MDAMB231 and MDAMB468 cells treated with GDC-0032 for 120 h. Mean ± SD per condition (n=3, 5 technical replicates). P-values were calculated using one-sample student T-tests on average the of technical replicates.

H) Immunoblot of cell lysates used in G) treated with GDC-0032, or BYL-719 for 96 h.

I) Immunohistochemical staining for human FOXM1 expression in formalin-fixed paraffin-embedded sections of pellets of T47D cells that had been transduced with a lentiviral vector expressing a doxycycline-inducible shRNA against human FOXM1, after treatment with drug vehicle (left) or doxycycline (right) for 120 h.

**A**

| PATHWAY | N Genes | Direction | pValue | FDR |
|---|---|---|---|---|
| HALLMARK_G2M_CHECKPOINT | 198 | Up | 9.64E-20 | 4.82E-18 |
| HALLMARK_E2F_TARGETS | 200 | Up | 4.00E-18 | 1.00E-16 |
| HALLMARK_MITOTIC_SPINDLE | 191 | Up | 7.47E-14 | 1.24E-12 |
| HALLMARK_OXIDATIVE_PHOSPHORYLATION | 197 | Down | 8.47E-04 | 8.61E-03 |
| HALLMARK_PROTEIN_SECRETION | 93 | Up | 8.61E-04 | 8.61E-03 |
| HALLMARK_UV_RESPONSE_DN | 123 | Up | 1.21E-03 | 1.01E-02 |
| HALLMARK_TNFA_SIGNALING_VIA_NFKB | 167 | Down | 1.43E-03 | 1.02E-02 |
| HALLMARK_MTORC1_SIGNALING | 195 | Up | 2.13E-03 | 1.33E-02 |
| HALLMARK_SPERMATOGENESIS | 73 | Up | 3.87E-03 | 2.15E-02 |
| HALLMARK_INTERFERON_ALPHA_RESPONSE | 89 | Down | 7.49E-03 | 3.47E-02 |
| HALLMARK_INTERFERON_GAMMA_RESPONSE | 155 | Down | 1.05E-02 | 4.48E-02 |
| HALLMARK_ANDROGEN_RESPONSE | 92 | Up | 1.07E-02 | 4.48E-02 |

**Figure S5, related to Figure 6: Persistent FOXM1 expression in breast cancer PDXs that have acquired resistance to PI3Kα inhibition**

A) RNAseq analysis of *FOXM1*, *HK-II*, *LDHA* and *PTEN* gene expression in GDC-0032-treated drug-sensitive PDX model (HCI-011, vehicle n=4 and GDC-0032 n=4) and in the drug-resistant model derived from a tumor that relapsed following prolonged drug treatment (HCI-011R, vehicle n=5 and GDC-0032 n=8). Mean values are marked by red lines. FDR adjusted p-values of significant differentially expressed pairs are shown.

B) RNAseq data was used to perform a gene set enrichment analysis (GSEA) of hallmark pathways in HCI-011 (vehicle n=4 and GDC-0032 n=4) versus HCI-011R (vehicle n=5 and GDC-0032 n=8) tumors treated with GDC-0032, as in (A).

C) Heat map derived from analysis of the RNAseq data for HCI-011 vs HCI-011R PDX models for the genes listed as driving resistance to PI3K inhibition (Hanker et al., 2019),  p value is < 0.01 or less.
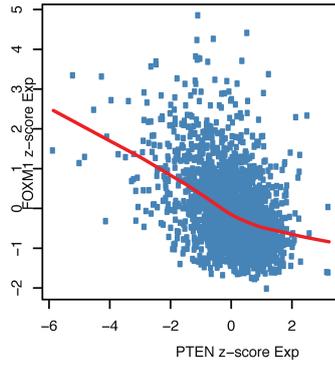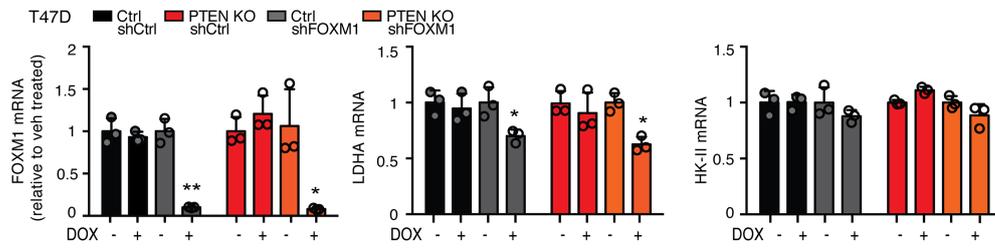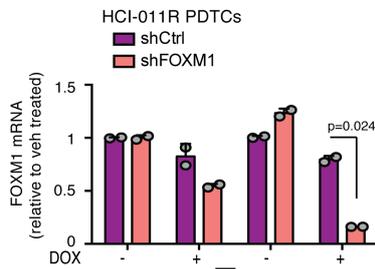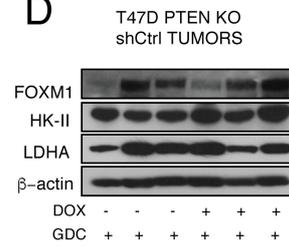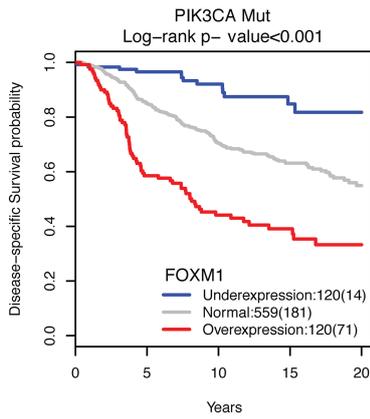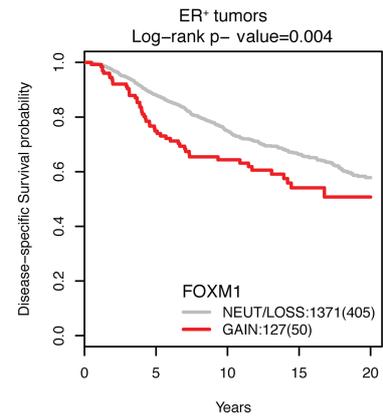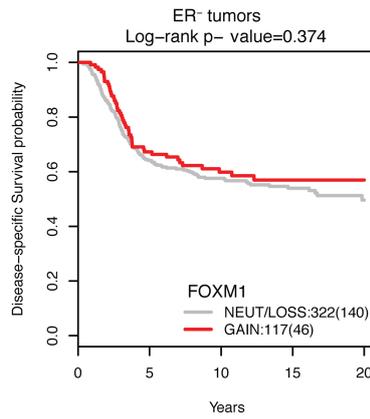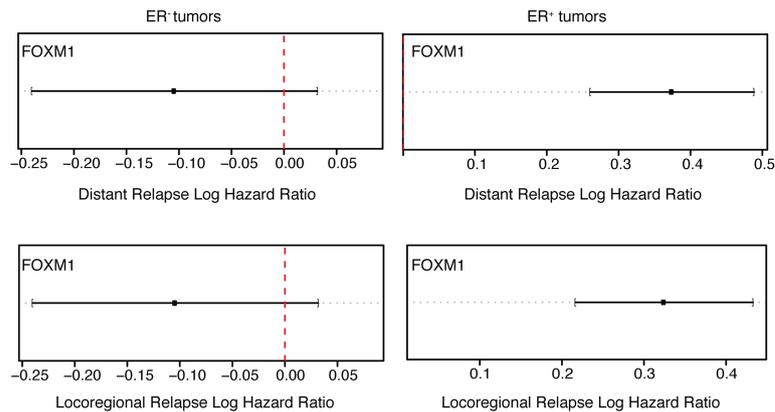
**Figure S6, related to Figure 7: Persistent FOXM1 expression contributes to drug resistance and can be explained by cytoplasmic localization of FOXO3a**

A) Pearson correlation of FOXM1 and PTEN expression in the METABRIC cohort (Curtis et al., 2012). A loess fit has been added to the plot.

B) Expression of *FOXM1, LDHA and HK-II* in PTEN wt (Ctrl) and PTEN KO T47D cells expressing a doxycycline-inducible shRNA sequence targeting FOXM1 (shFOXM1_seq1) treated with drug vehicle or doxycycline for 120 h determined by qPCR and normalized by the geomean of *β-ACTIN* and *B2M*. Figure shows the mean ± SD (n=3, 3 technical replicates).  P-values were calculated using two-sided Welch's T-tests on the average of technical replicates, * $p \leq 0.05$ and ** $p \leq 0.005$.

C) Expression of *FOXM1* in HCI-011R PDTCs treated with indicated drug combinations for 120 h determined by qPCR and normalized by the geomean of *β-ACTIN* and *B2M*. Mean ± SD per condition (n=2).  P-values were calculated using two-sided Welch's T-tests.

D) Immunoblot of FOXM1, HK-II and LDHA in PTEN KO T47D tumors expressing doxycycline-inducible shRNA control sequences (shCtrl) from mice treated with GDC-0032 (long-term treatment) and fed with doxycyline or a standard diet.

E) Disease specific Kaplan-Meier survival plots of breast cancer patients from METABRIC with low, medium and high FOXM1 expression (blue, grey and red lines, respectively). Under and over expression were defined by the 15% and the 85% percentiles of the distribution of expression respectively.

F) Disease specific Kaplan-Meier survival plots of ER$^-$ and ER$^+$ breast cancer patients from METABRIC with FOXM1 copy number gain (red) or neutral/loss (grey).

G) Relapse Log-hazard ratio for FOXM1 expression in a multiple Cox regression model, for ER+ and ER- breast cancer patients from METABRIC.