Supporting Information

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SI Materials and Methods

Cell culture. MCF7aro cells, LTEDaro cells and three AI-resistant cell lines EXE-R, ANA-R and LET-R were generated and maintained as previously described (1). HCC1428aro stable cell line was generated by transient transfection of HCC1428 cells with aromatase gene expression vector (pHβ-Aro) and selected by 800 μg/ml G418 for 3 months. HCC1428aro/LET-R cell line was generated by culture of HCC1428aro cells in phenol red-free IMDM medium with 10% charcoal:dextran-stripped (CD) FBS in the presence of 1 nmol/L testosterone (T) plus 200 nmol/L letrozole for more than 8 months. The resistant cells were maintained in the same medium. MCF7aro/pMG and MCF7aro/SGK3 cells were generated as previously described (2). The doxycycline-inducible SGK3 expression cell line MCF7aro/TO/SGK3 cells were generated using a retroviral expression system as previously described (3), except that pRetroX-Tet-On Advanced vector was modified by substitution of a hygromycin resistance gene for a neomycin resistance gene and that hygromycin B was used to replace G418 for selection since MCF7aro cells are G418-resistant. To generate MCF7aro/pTomo and MCF7aro/pTomo-SGK3 cells, MCF7aro were infected with lentiviral vectors pTomo and pTomo-SGK3, respectively. After cultured for 2~3 weeks, the stably infected cells were sorted by GFP using flow cytometry. LET-R/SERCA2b cells were generated by transient transfection of LET-R cells with pMG-SERCA2b-HA expression vector and selected by 150 μg/mL hygromycin B..

Antibodies. The rabbit monoclonal antibodies to SGK3 (D42C2), phospho-SGK3 (Thr-320) (D30E6), SERCA2 (D51B11), BiP (C50B12), ATF4 (D4B8), XBP-1s (D2C1F), Calreticulin (D3E6), Calnexin (C5C9), Akt, phospho-Akt (Ser-473), phospho-Akt (Thr-308) (C31E5E), cleaved PARP (D64E10) and cleaved Caspase-7 (D6H1), and mouse CHOP monoclonal antibody (L63F7) were purchased from Cell Signaling Technology. Rabbit polyclonal ERα (HC-20) antibody and mouse monoclonal LAMP2 antibody (H4B4) were purchased from Santa Cruz Biotechnology. Rabbit monoclonal ERα (phosphoSer118) antibody (E91) and rabbit polyclonal GREB1 antibody (ab72999) were purchased from Abcam. Anti-FLAG (M2), anti-HA, anti-FLAG M2 affinity gel, and EZview Red anti-HA affinity gel were purchased from Sigma.

Plasmid constructs. pMG-SGK3, pMG-FLAG-SGK3, pMG-FLAG-SGK3/R90A vectors were described previously (3, 4). To generate scramble shRNA and two individual SGK3 shRNA viral vectors (pLKO.1-SGK3shRNA-1 and pLKO.1-SGK3shRNA-2), the following sets of oligonucleotides were synthesized: scrambled shRNA sense, 5'-CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG-3'; scrambled shRNA antisense, 5'-AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG-3'; SGK3 shRNA1 sense, 5'-CCGGGCGAGACCCTAGTTAAGAGAACTCGAGTTCTCTTAACTAGGGTCTCGCTTTTTG-3'; SGK3 shRNA1 antisense, 5'-AATTCAAAAAGCGAGACCCTAGTTAAGAGAACTCGAGTTCTCTTAACTAGGGTCTCGC-3'; SGK3 shRNA2 sense, 5'-

CCGGGCAGGACTAAACGAATTCATTCTCGAGAATGAATTCGTTTAGTCCTGCTTTTT G-3'; SGK3 shRNA2 antisense, 5'-AATTCAAAAAGCAGGACTAAACGAATTCATTCTCGAGAATGAATTCGTTTAGTCCTG C-3'. The oligonucleotide sets were annealed and ligated into the vector pLKO.1 digested by *Age* Ⅰ and *EcoR*. pTomo vector was a gift from Inder Verma (Addgene plasmid # 26291) (5). To generate viral vector pTomo-SGK3, the coding region of SGK3 was generated by PCR and cloned into pTomo vector using *Xba* Ⅰ and *BamH* Ⅰ restriction sites (note: digestion of pTomo with *Xba* Ⅰ and *BamH* Ⅰ will remove RFP coding region from the vector). To generate pMG-SERCA2b-HA, a HA-tag encoding sequence was added by PCR to C-terminus of the coding regions of SERCA2b using pCS6-SERCA2b vector (purchased from transOMICS technologies) as the PCR template and with the following primers: sense, 5'-CTGAGATCTCACCATGGAGAACGCGCACACCAAGAC-3'; anti-sense: 5' –TGTGCTAGCTCAAGCGTAATCTGGAACATCGTATGGGTAAGACCAGAACATATCGCT AAAG-3'. The resulting DNA fragments were cloned into pMG-H2 vector using *Bgl* Ⅰ*–Nhe* Ⅰ restriction sites. All the inserts were verified by DNA sequencing.

Lentivirus production and transduction. For knockdown experiments, transductions were carried out according to the protocol from TRC (http://www.addgene.org/tools/protocols/plko/). Briefly, 293T cells in each 10 cm dish were transfected with 4 μg pLKO.1 shRNA plasmid, 3 μg pCMV-dR8.2dpvr and 1 μg pCMV-VSV-G using jetPRIME transfection reagent. Next day, medium was replaced. After 24 hours of virus production, supernatant was collected, filtered, and either immediately used for infection of the target cells or stored at -80°C. The target cells were supplemented with 5 μg/mL protamine sulfate (Sigma) before infection with virus. Medium was replaced after overnight infection, and 48 hours later, cells were selected in 5 μg/mL puromycin. For overexpression experiments, 293T cells in each 10 cm dish were transfected with 4 μg pTomo or pTomo-SGK3 plasmid, 4 μg pCHGP-2, 1.33 μg pCMV-Rev and 1.33 μg pCMV-G using jetPRIME transfection reagent. Next day, medium was replaced. After 24 hours of virus production, supernatant was collected, filtered, and used for infection of the target cells or stored at -80°C.

RT-quantitative PCR. RT-quantitative PCR was performed as described previously (2). The primers used in real-time PCR were the following, for SGK3: 5'-CCAGGAGTGAGTCTTACAG-3' and 5'-CCAGCCACATTAGGATTA-3'; for SGK1: 5'-AAGCAGAGGAGGATGG-3' and 5'-GAGAAGGACTTGGTGGA-3'; for ERα: 5'-CGAGTCCTGGACAAGATCACAG-3' and 5'-TCTCCAGCAGCAGGTCATAGAG-3'; for β-actin: 5'-CACCAACTGGGACGACAT-3' and 5'-GCACAGCCTGGATAGCAAC-3'; for GAPDH: 5'-AGGTGAAGGTCGGAGTCAAC-3'and 5'-ATCTCGCTCCTGGAAGATGG-3'.

Western blotting. Cells were lysed in Nonidet P-40 buffer (50 mM Tris-Cl, pH 7.5, 1% Nonident P40, 150 mM NaCl, 0.5% sodium deoxycholate and complete protease inhibitor cocktail) on ice for 20 min, and then sonicated for 30s. After centrifugation, the supernatants were collected and mixed with 2×SDS sample buffer and boiled for 5 min. Protein concentration was quantified using the BioRad Protein Assay. Western blotting was performed as previously described (2).

Transmission electron microscopy. LET-R cells were transfected with siRNA negative control or SGK3 siRNA for 72 h, and then collected for transmission electron microscopy processed at Electron Microscopy Core Facility at Beckman Research Institute of City of Hope.

Immunofluorescence microscopy. For immunofluorescence microscopy, the cells grown in 35 mm glass bottom dishes were washed with warm PBS and fixed in cold methanol/acetone (1:1) at −20 °C for 10 min. The fixed cells were blocked in 5% BSA in 1× TBST for 1 hour at room temperature and incubated with primary antibodies (1:100) at 4 °C overnight. Then the cells were washed 3 times with 1× TBST and incubated with Alexa Fluor 568-conjugated anti-rabbit IgG secondary antibody and/or FITC-conjugated anti-mouse IgG secondary antibody (1:100) in the dark for 1 hour at room temperature. After washing three times, the cells were mounted in 4′,6-diamidino-2-phenylindole (DAPI) solution (Vector) and visualized under a confocal microscope.

References

1. Masri S, *et al.* (2008) Genome-wide analysis of aromatase inhibitor-resistant, tamoxifen-resistant, and long-term estrogen-deprived cells reveals a role for estrogen receptor. *Cancer research* 68(12):4910-4918.
2. Wang Y, *et al.* (2011) SGK3 is an estrogen-inducible kinase promoting estrogen-mediated survival of breast cancer cells. *Molecular endocrinology* 25(1):72-82.
3. Wang Y, Zhou D, & Chen S (2014) SGK3 is an androgen-inducible kinase promoting prostate cancer cell proliferation through activation of p70 S6 kinase and up-regulation of cyclin D1. *Molecular endocrinology* 28(6):935-948.
4. Wang Y, Xu W, Zhou D, Neckers L, & Chen S (2014) Coordinated regulation of serum- and glucocorticoid-inducible kinase 3 by a C-terminal hydrophobic motif and Hsp90-Cdc37 chaperone complex. *The Journal of biological chemistry* 289(8):4815-4826.
5. Marumoto T, *et al.* (2009) Development of a novel mouse glioma model using lentiviral vectors. *Nature medicine* 15(1):110-116.

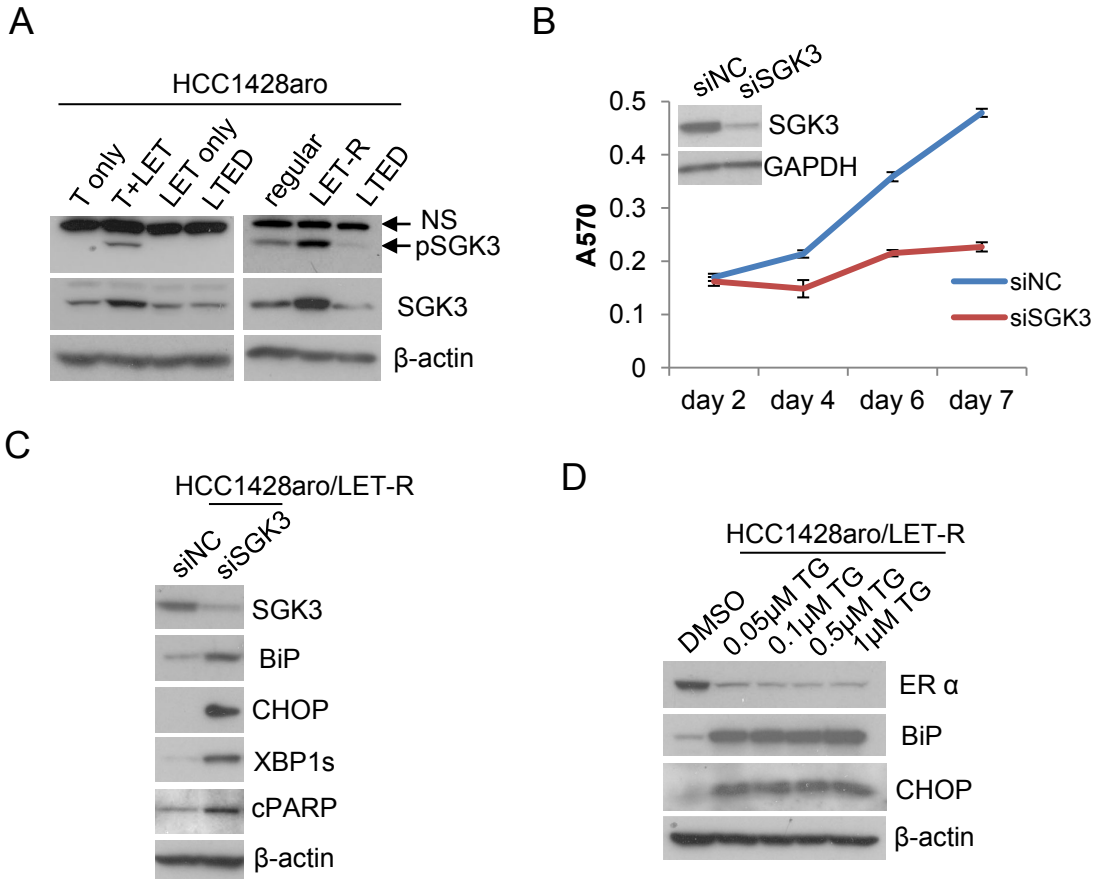
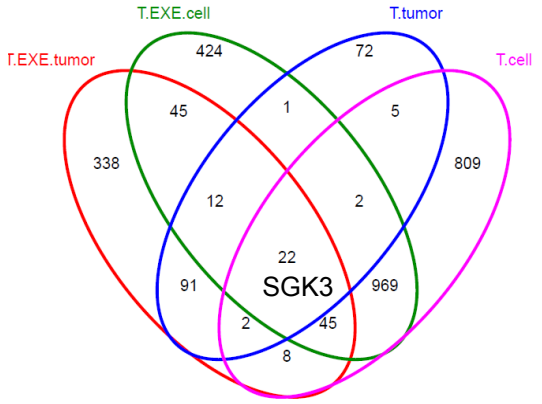


Fig.S1. SGK3 is upregulated and important for cell proliferation/survival in HCC1428aro/LET-R cells. (**A**) Western blotting analysis of phosphorylated and total SGK3 levels. **Left panel**: HCC1428aro cells grown in hormone-depleted medium and treated with or without 1 nM T alone or plus 200 nM letrozole for 8.5 months; **right panel**: HCC1428aro, HCC1428aro/LET-R and HCC1428aro/LTED cells were cultured in their normal growth media as described in SI Materials and Methods. (**B**) Effect of SGK3 silencing on HCC1428aro/LET-R cell proliferation/survival. HCC1428aro/LET-R cell were transfected with siRNA negative control or SGK3 siRNA, and were harvested for Western blotting analysis (insert) at 72 h posttreatment, or measured for cell proliferation/survival using MTT assay at the indicated time. (**C**) Western blotting analysis of EnR stress markers in HCC1428aro/LET-R cells transfected with siRNA negative control or SGK3 siRNA for 72 h. (**D**) Western blotting analysis of HCC1428aro/LET-R cells treated with different concentrations of thapsigargin (TG) as indicated for 48 h.

A



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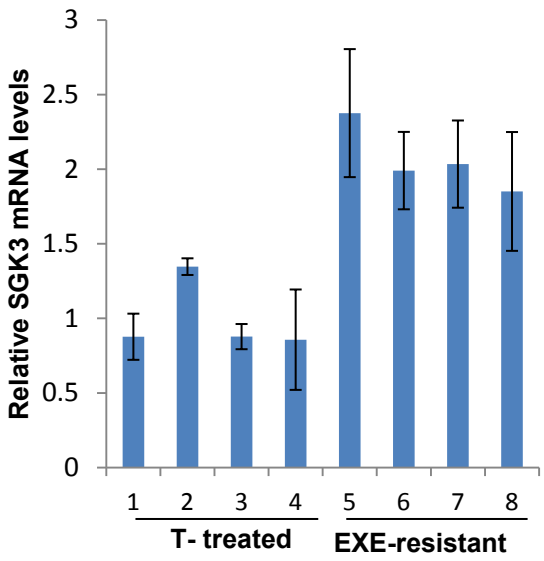


B

Study	Comparison (SGK3 mRNA levels)	Fold change	*p*-value	FDR
Xenograft	Pos (T) vs. Neg Ctrl	2.5	0.0022	0.30
	EXE-resistant vs. Neg Ctrl	3.9	9.8×10^{-5}	0.048
Cell line	T vs. DMSO	5.6	8.2×10^{-8}	3.5×10^{-6}
	T + EXE vs. DMSO	19.4	2.1×10^{-13}	1.3×10^{-10}

Fig.S2. Upregulation of SGK3 expression in T-treated and EXE-resistant MCF7aro cells and –derived xenografts. Xenografts were generated in ovariectomized nude mice through subcutaneous injections of MCF-7aro cells (4 mice/group). EXE-resistant xenografts were established through daily injection of exemestane (EXE) (250 µg/day) to mice with T tablets. The negative and positive (T-treated) controls were those mice inserted with placebo and T tablets, respectively. In cell lines, MCF7aro cells were cultured in hormone-depleted medium and treated with DMSO or 1 nM T for 48 h. T+EXE: MCF7aro cells were long-term cultured in hormone-depleted medium with T plus EXE and became resistant to EXE. (**A**) Comparison of upregulated gene expression profiles of MCF7aro cells and MCF7aro-derived xenografts treated with T or T plus EXE. Genes upregulated in the microarray analysis (>1.5-fold increase in T or T+EXE vs. negative control, $p<0.05$) were analyzed using IPA. Negative controls were placebo for xenograft and DMSO for cell line. SGK3 was among 22 genes that were upregulated in both xenografts and cells treated with T or T plus EXE. (**B**) Relative *SGK3* mRNA levels in MCF7aro cells and MCF7aro-derived xenografts treated with T or T plus EXE determined by microarray analysis. (**C**) RT-qPCR analysis of *SGK3* mRNA levels in MCF7aro-derived xenografts treated with T or T plus EXE. Four samples from each group were analyzed. Relative expression level was calculated by dividing *SGK3* mRNA level by *β-actin* level. qPCRs were performed in triplicate, and data were expressed as means ± SD.

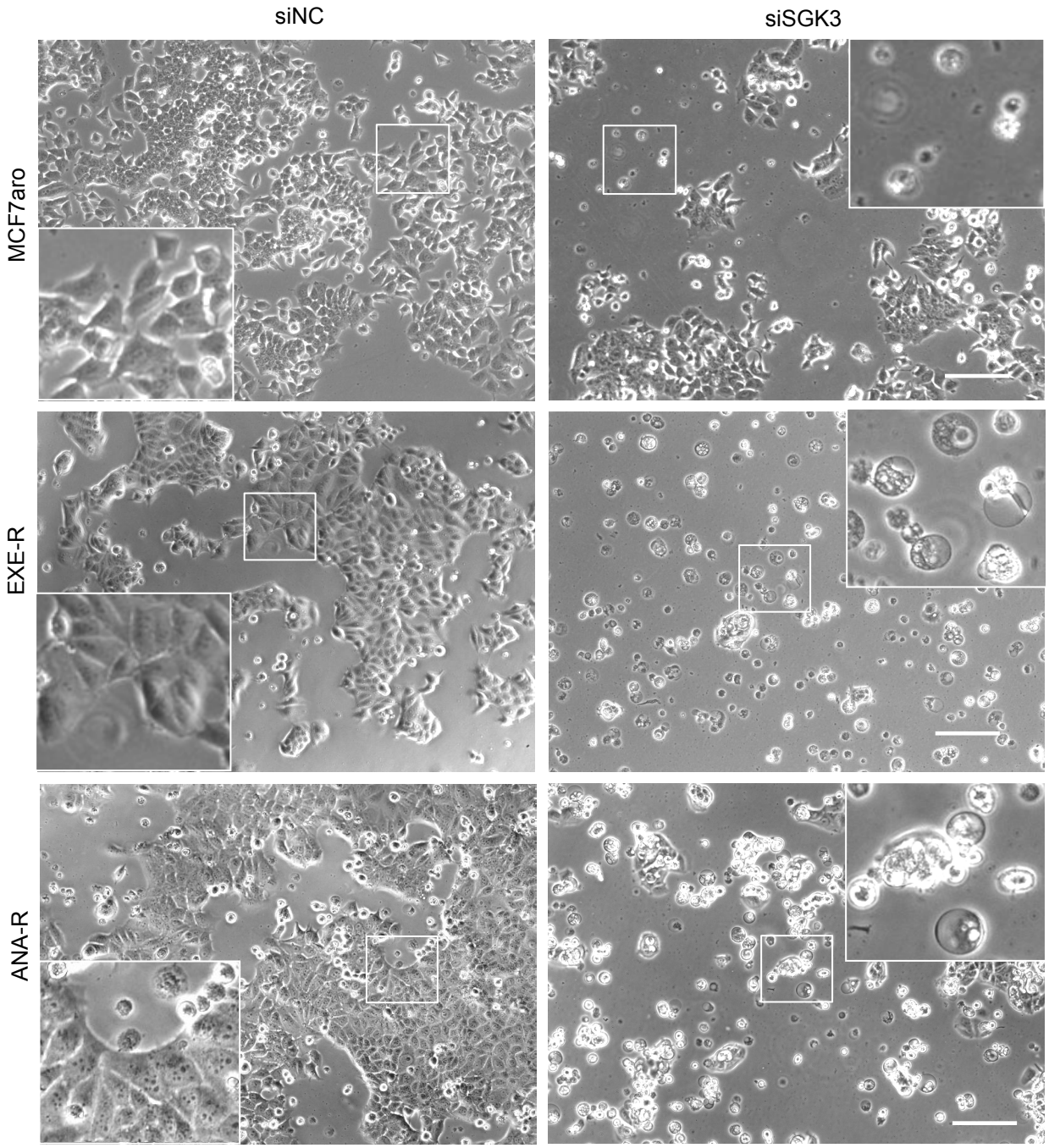


Fig.S3. Light microscopy of MCF7aro and AI-resistant cells after transfected with siRNA negative control or SGK3 siRNA. Cells were transfected with siRNA negative control or SGK3 siRNA for 4 days and imaged under a light microscope. Representative images are presented. Magnification: 100×. Scale bar: 50 μm.

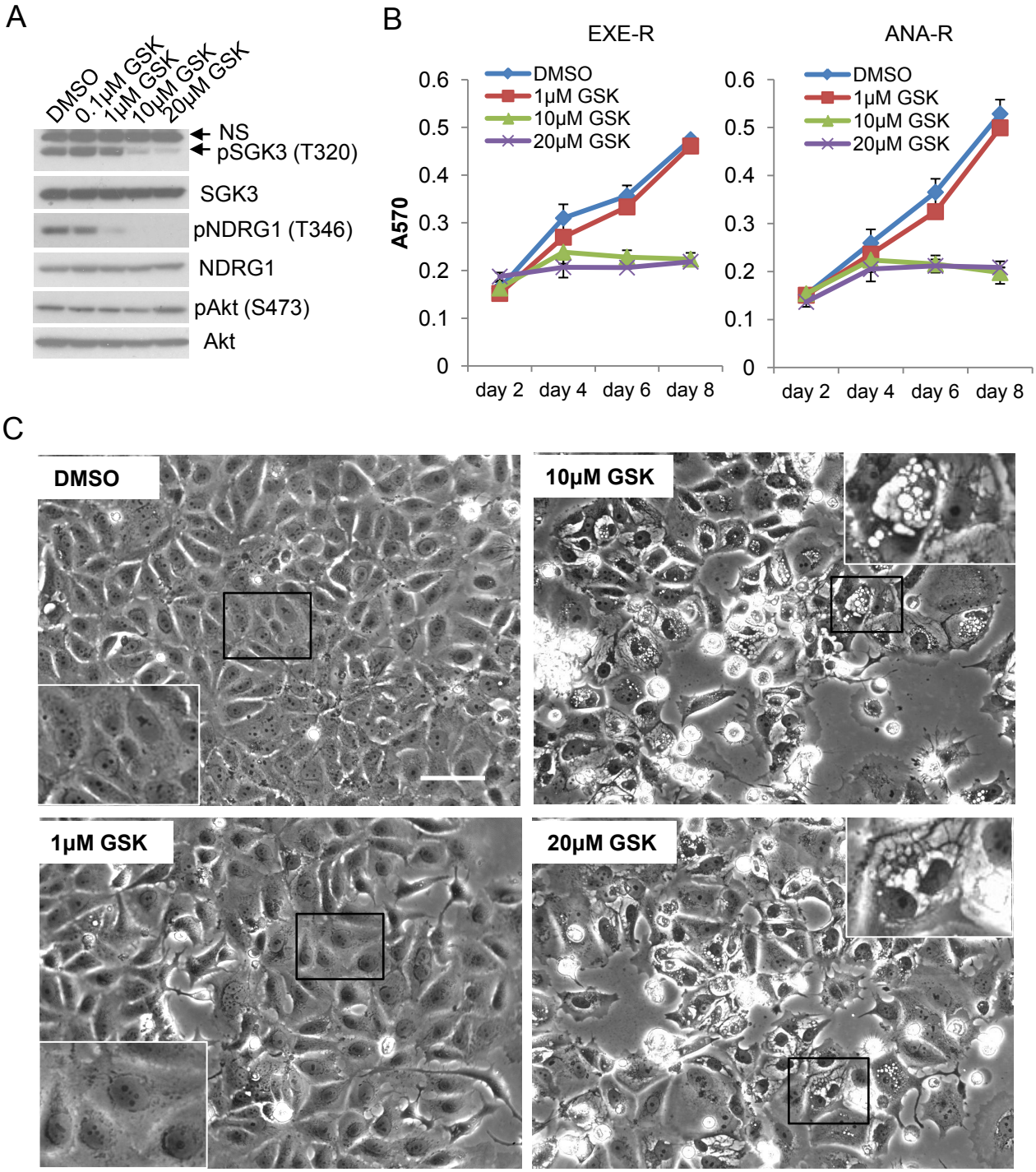


Fig.S4. The effects of GSK650394 on AI-resistant cell proliferation/survival. **(A)** Western blotting analysis of EXE-R cells treated with the indicated concentrations of GSK650394 for 1 h. **(B)** The effect of GSK650394 on cell proliferation of AI-resistant cells. EXE-R and ANA-R cells were treated with the indicated concentrations of GSK650394 and measured for cell proliferation using MTT assay at the indicated time. **(C)** Light microscopy of EXE-R cells treated with the indicated concentrations of GSK650394 for 48 h. Magnification: 200×. Scale bar: 20 μm.

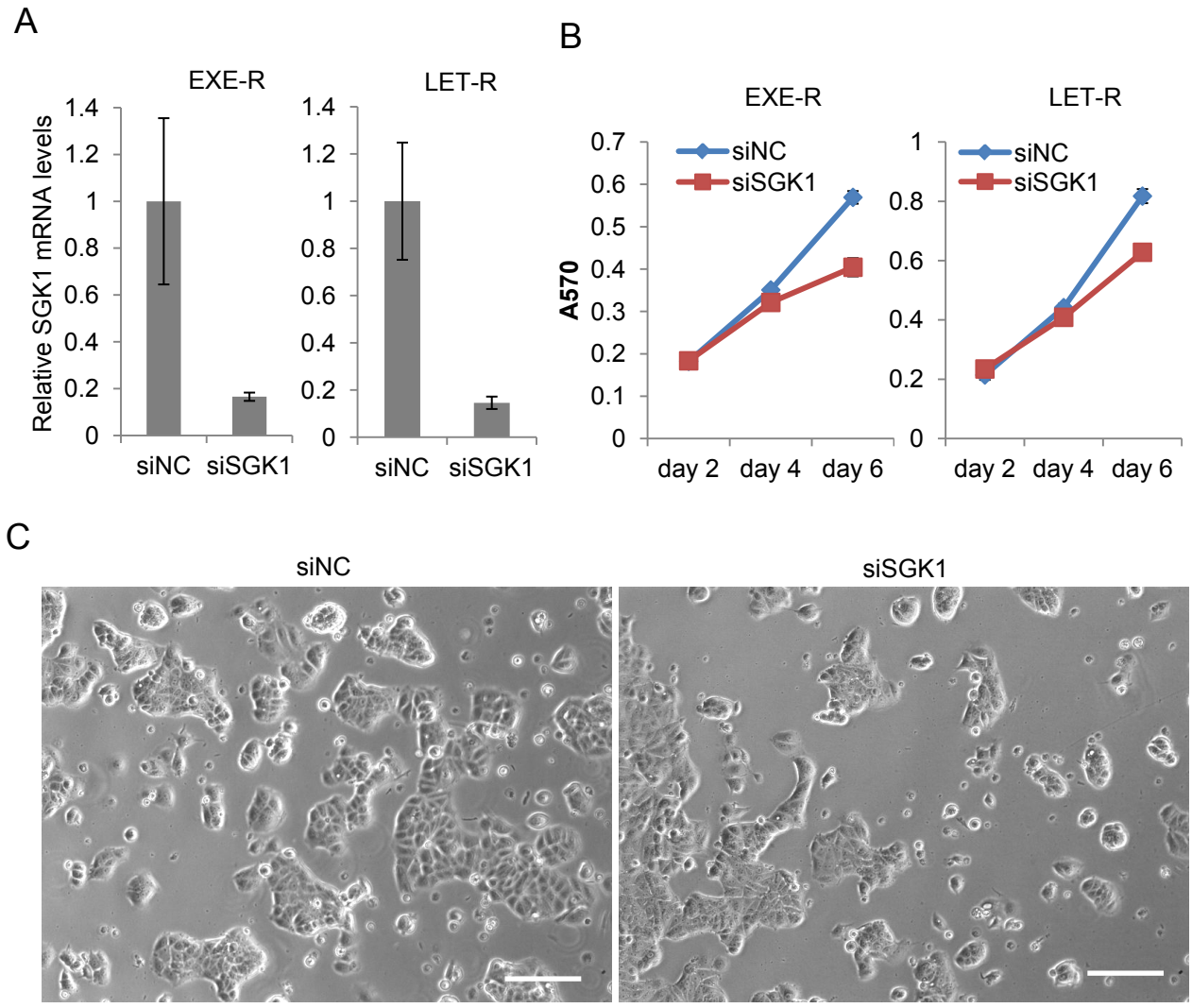


Fig.S5. The effects of silencing SGK1 on AI-resistant cells. (A) RT-qPCR analysis of SGK1 expression levels in EXE-R and LET-R cells after transfected with SGK1 siRNA or siRNA negative control. Relative expression level was calculated by dividing *SGK1* mRNA level by *β-actin* level. **(B)** The effect of SGK1 knockdown on cell proliferation of EXE-R cells and LET-R cells. Cell proliferation was measured using MTT. **(C)** Light microscopy of the EXE-R transfected with siRNA negative control or SGK1 siRNA for 4 days. Representative images were shown. Magnification: 100×. Scale bar: 50 μm.

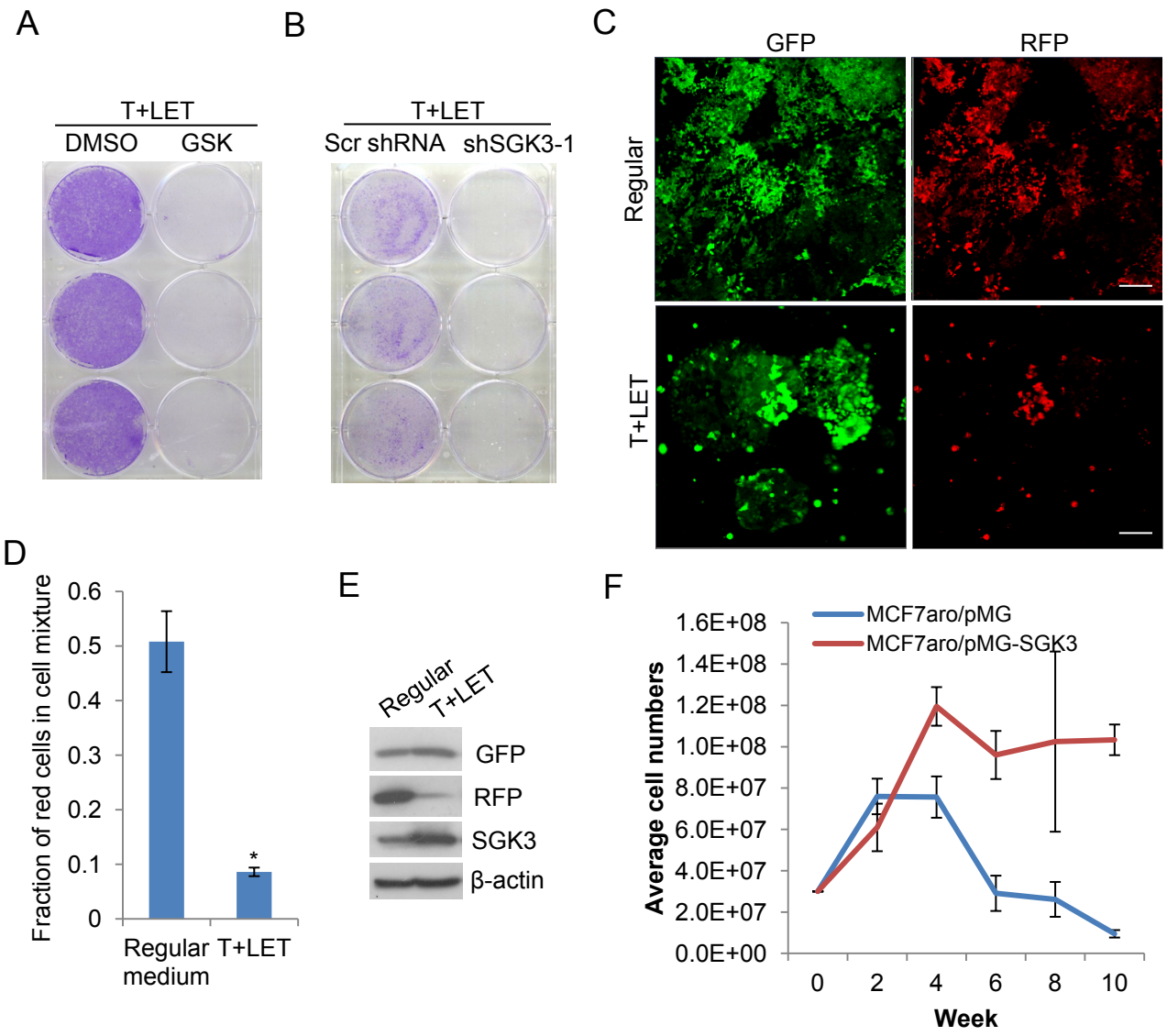
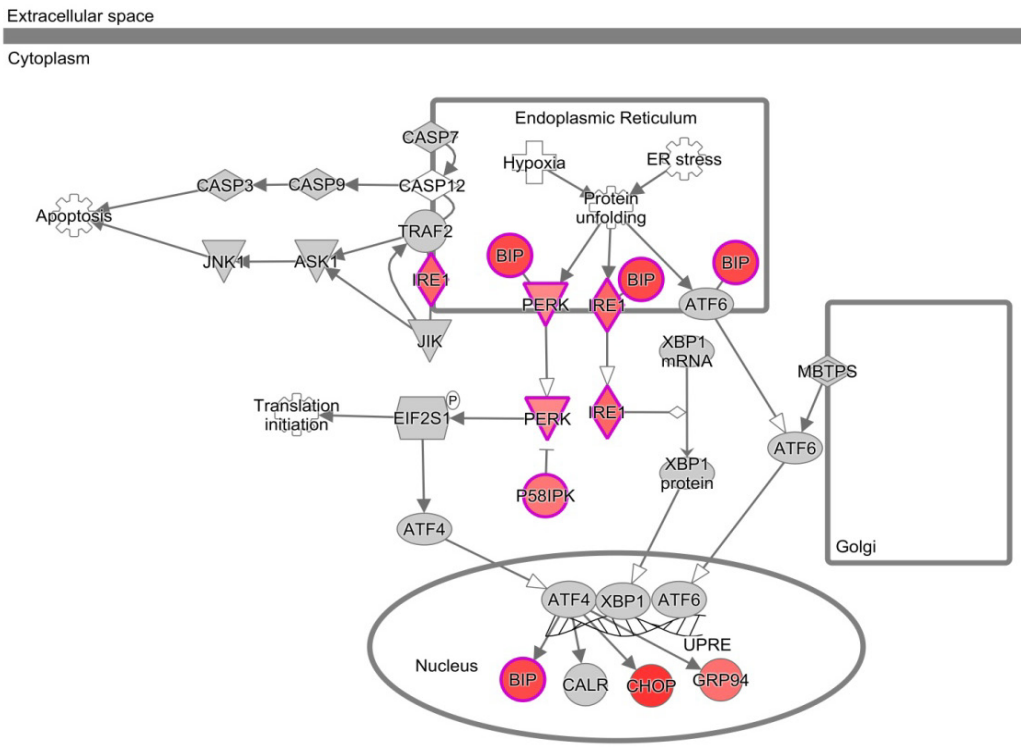


Fig.S6. SGK3 mediates AI resistance. (A) Effect of GSK650394 on colony formation of MCF7aro cells grown in hormone-depleted medium with T plus letrozole. MCF7aro cells were cultured in hormone-depleted medium added with T plus letrozole, and treated with DMSO or 20 μM GSK650394. **(B)** Effect of SGK3 knockdown on colony formation of MCF7aro cells grown in hormone-depleted medium with T plus letrozole. MCF7aro cells were transduced with scramble shRNA or SGK3 shRNA-1 viral vectors, and then cultured in hormone-depleted medium with T plus letrozole for about 2 months. Five μg/ml puromycin was added in the medium to eliminate the untransduced cells. (**C**) Fluorescence microscopy of the mixed cells after cultured in regular growth medium or hormone-depleted medium with T plus letrozole. MCF7aro/pTomo cells (which express both GFP and RFP) and MCF7aro/pTomo-SGK3 cells (which express GFP but not RFP) were mixed at 1:1 ratio and cultured in either regular growth medium or hormone-depleted medium with 1 nM T plus 200 nM letrozole. After 3 weeks, cells were imaged under a confocal microscope. The mixed cells cultured in regular growth medium were split when became confluency. Representative images were shown. Magnification: 40×. Scale bar: 200 μm. (**D**) Quantization of the fraction of MCFaro/pTomo control cells (red cells) in the cell mixtures cultured in regular medium or hormone-depleted medium with T plus letrozole. Five images were chose for analysis with Image-Pro Premier9.1 software. *, $p<0.05$, by the Student *t* test. (**E**) Western blotting analysis of the mixed cells after grown in either regular medium or hormone-depleted medium with T plus letrozole for 3 weeks. **(F)** MCF7aro cells stably transfected with empty vector pMG or pMG-SGK3 were cultured in hormone-depleted medium with 1nM T plus 1μM anastrozole. Cells were counted every other week.

A



B

Gene	Fold Change (siSGK3 vs. siNC)	*p*-Value
HSPA5 (BiP)	2.704	2.56E-08
DDIT3 (CHOP)	4.136	1.03E-07
ERN1 (IRE1)	2.280	9.61E-08
EIF2AK3 (PERK)	1.786	7.62E-07
ERO1LB	3.443	2.56E-07
HERPUD1	4.734	1.43E-09
GADD45A	2.202	1.59E-08
GADD45G	1.576	1.53E-03
TRIB3	2.240	8.82E-08
LAMP3	3.415	2.89E-05

Fig.S7. SGK3 maintains EnR homeostasis in AI-resistant cells. (A) IPA analysis of RNA-seq data reveals EnR stress pathway is activated in LET-R cells after knockdown of SGK3. The genes shown in red were upregulated (>1.5-fold increase in siSGK3 vs. siNC cells, $p<0.05$). (**B**) List of the EnR stress-associated genes that were upregulated in LET-R cells after knockdown of SGK3. The relative expression levels were determined by RNA-seq analysis.

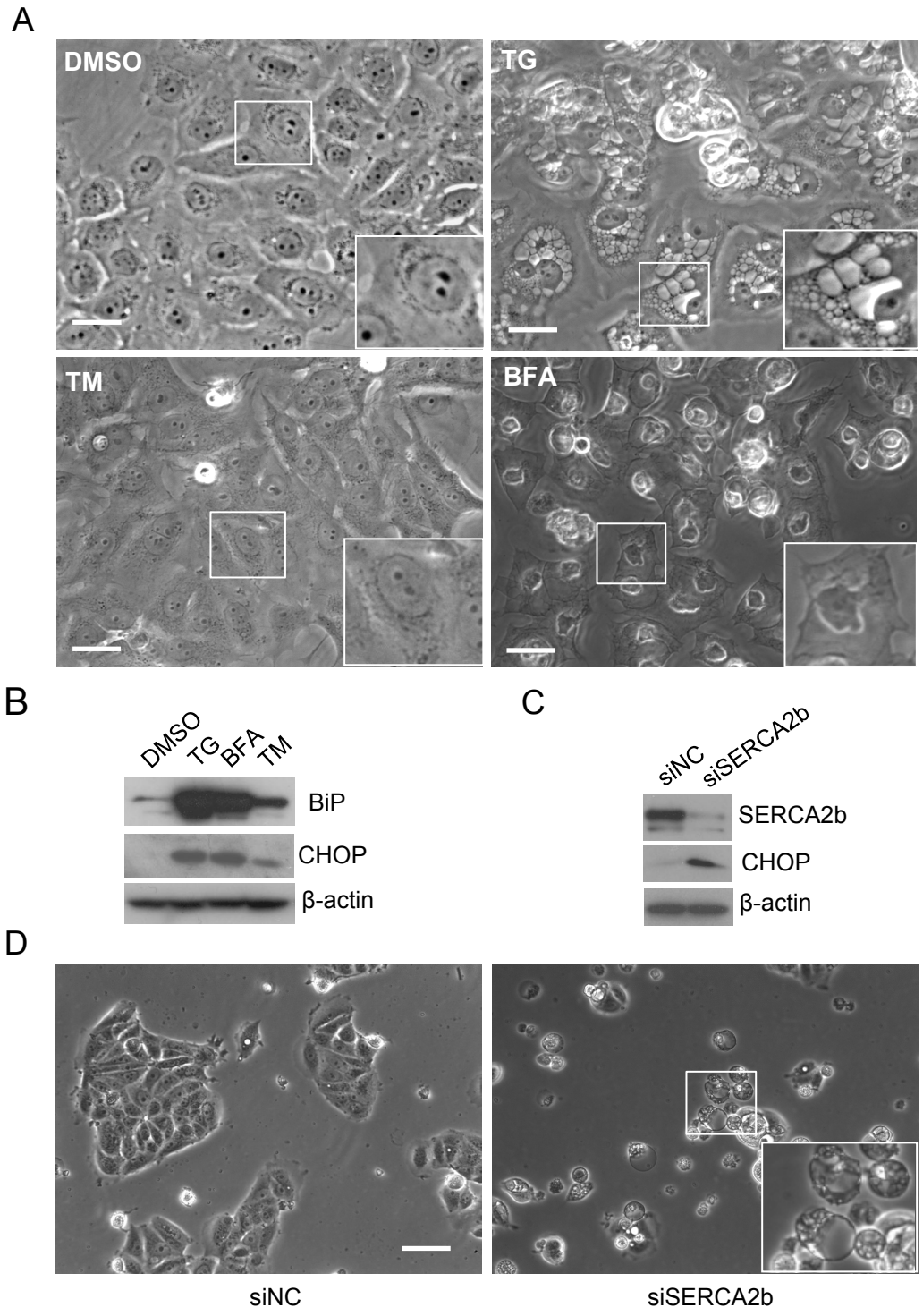


Fig.S8. Inhibition of SERCA2b induces massive EnR vacuolization and cell death in AI-resistant cells. (A) Light microscopy of EXE-R cells treated with DMSO, 1 μM TG, 5 μg/ml BFA or 4 μg/ml TM for 48 h. Magnification: ×400. Scale bar: 10 μm. **(B)** Western blotting analysis of EXE-R cells treated with DMSO, 1 μM TG, 5 μg/ml BFA or 4 μg/ml TM for 72 h. **(C)** Western blotting analysis of levels of SERCA2b and CHOP in EXE-R cells transfected with SERCA2b siRNA or siRNA negative control for 72 h. **(D)** Light microscopy of EXE-R cells transfected with SERCA2b siRNA or siRNA negative control for 72 h. Magnification: 200×. Scale bar: 20μm.

A

Gene	Exp Fold Change	*p*-Value
ESR1 (estrogen receptor 1)	-1.673	1.84E-06
CCND1 (Cyclin D1)	-1.593	9.51E-07
IGFBP3	-2.186	6.18E-05
PGR (progesterone receptor)	-1.891	5.67E-05
GREB1L	-1.776	1.05E-05
MYBL1	-1.549	2.37E-05
KIAA0101	-1.805	2.94E-06

B

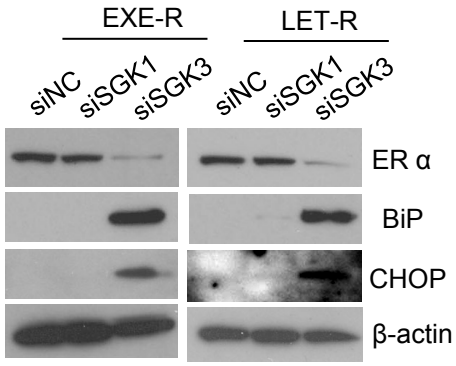


Fig.S9. SGK3 retains ERα expression and signaling in AI-resistant cells. (**A**) List of *ESR1* gene and some ERα-regulated genes whose expression was downregulated in LET-R cells after knockdown of SGK3. Relative expression was measured by RNA-seq analysis. **(B)** Western blotting analysis of EXE-R and LET-R cells after transfected with siRNA negative control, SGK1 siRNA, or SGK3 siRNA for 72 h.

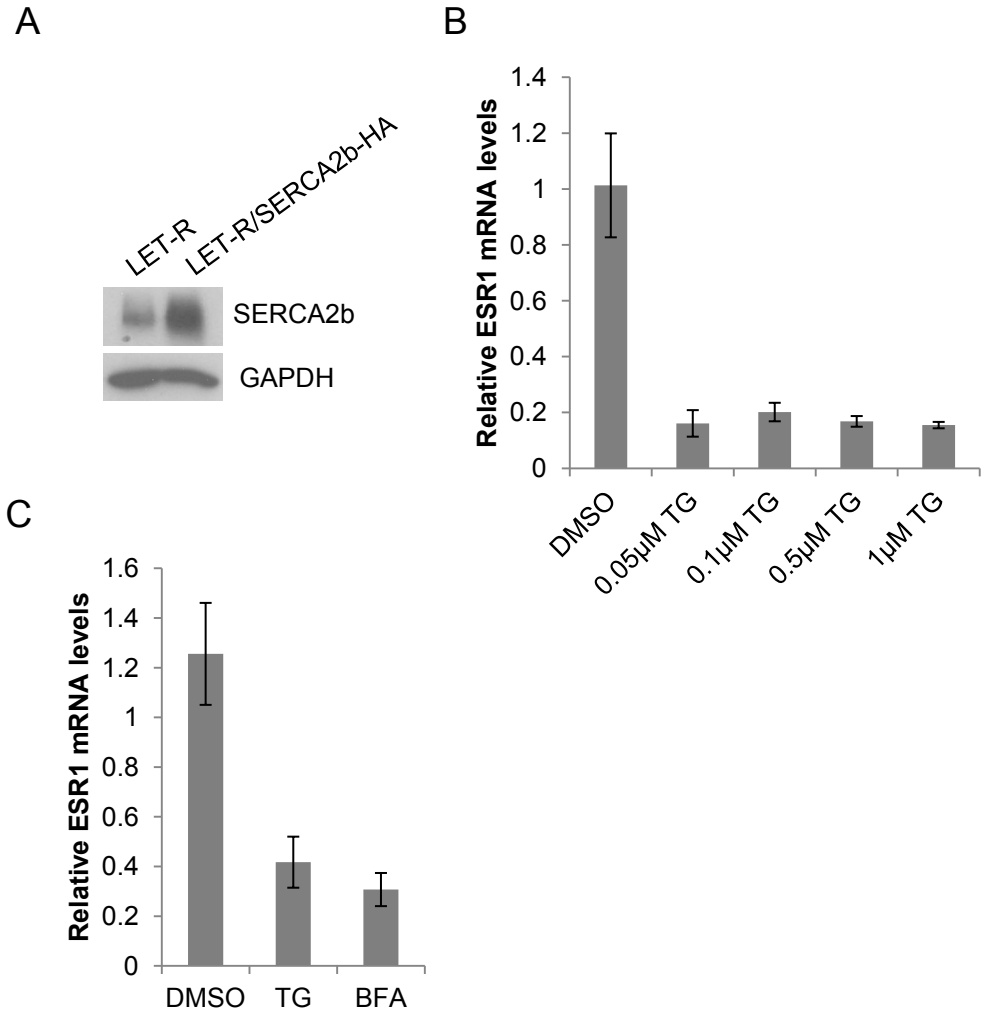


Fig.S10. SGK3 retains ERα expression through maintaining EnR homeostasis via SERCA2b. (**A**) Western blotting analysis of SERCA2b levels in LET-R and LTE-R/SERCA2b-HA cells. (**B**) RT-qPCR analysis of *ESR1* mRNA levels in LET-R cells treated with different concentrations of TG for 24 h. Relative expression level was calculated by dividing *ESR1* mRNA level by GAPDH level. qPCRs were performed in triplicate, and data were expressed as means ± SD. (**C**) RT-qPCR analysis of *ESR1* mRNA levels in MCF7aro cells treated with DMSO, 0.1 μM TG or 2.5 μg/ml BFA for 24 h.

SI table

Tab.S1. Top 5 hits of cMap analysis using 372 differentially expressed genes in microarray data of EXE-R cells transfected with siSGK3 vs. siNC

Rank	cMap name and cell line	mean	n	enrichment	*p*
1	thapsigargin - MCF7	0.948	2	1	0
2	pyrvinium - MCF7	0.569	4	0.954	0
3	thioridazine - MCF7	0.402	11	0.664	0
4	monensin - PC3	0.763	2	0.998	0.00002
5	fluspirilene - MCF7	0.707	2	0.993	0.00004