ISCI, Volume *19*

Supplemental Information

Multi-Omic Approaches Identify Metabolic and Autophagy Regulators Important in Ovarian Cancer Dissemination

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Supplemental Figures and Figure Legends

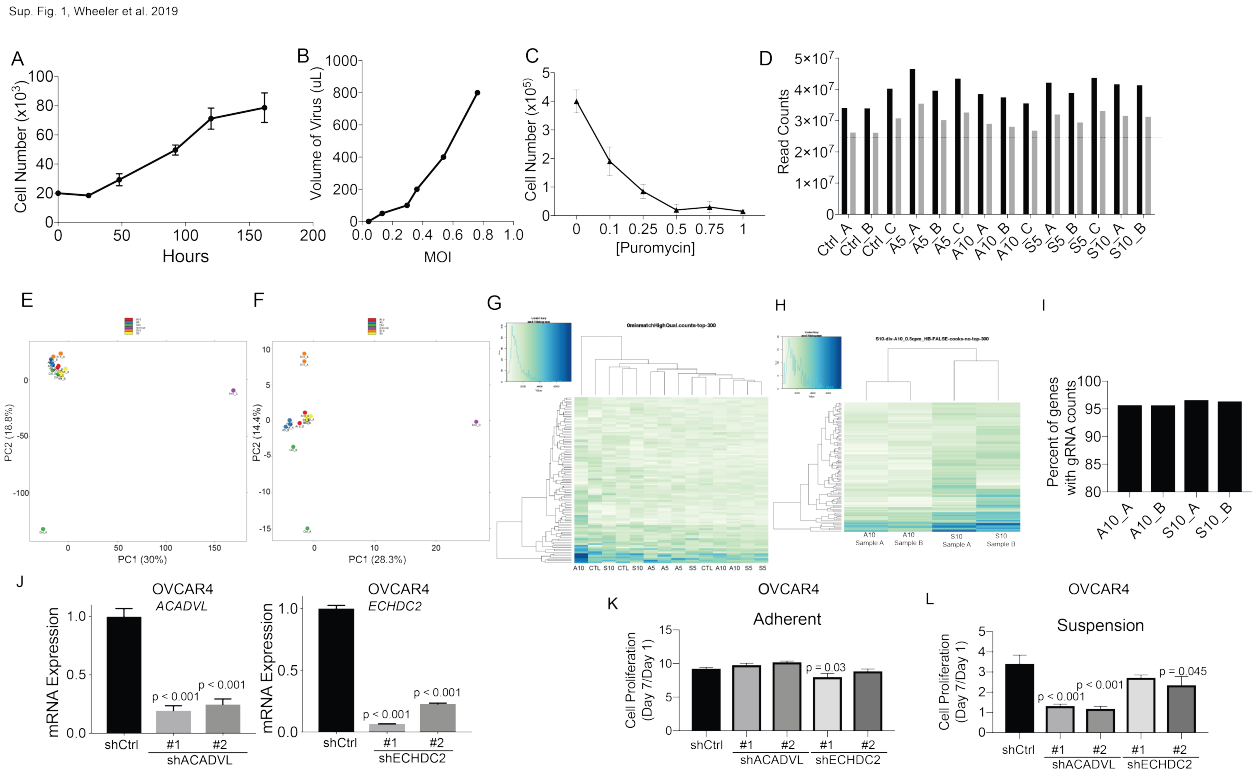

Figure S1. An HGSOC cell line grown in suspension and a CRISPR/Cas9 Screen. (Related to Figure 1) A) PEO1 cells were plated in a low attachment plate (poly-HEMA coated) and grown for 168 hours. **B**) Multiplicity of infection (MOI) of GeCKO library on PEO1 cells. **C**) Puromycin kill curve for PEO1 cells. **D**) Read counts for each sample - black bars = raw count and gray bars = counts of perfect match quality reads. **E**) Principal component analysis of all samples. **F**) Principal component analysis after removing A10_C (purple dot). **G**) Unsupervised hierarchal clustering of top 300gRNA from all samples. **H**) Unsupervised hierarchal clustering of top 300 gRNA comparing adherent day 10 (A and B) with suspension day 10 (A and B). **I)** Percent of genes with at least one gRNA detected in A10 (A and B) and S10 (A and B). **J)** OVCAR4 cells were transduced with shControl (shCtrl) or shACADVL (#1 and #2) or shECHDC2 (#1 and #2). RNA was extracted from cells and used for qRT-PCR against *ACADVL* and *ECHDC2*. Internal control = *18s*. **K)** OVCAR4 shCtrl, shACADVL, and shECDHC2 cells were plated on tissue culture treated plastic (adherent) on day 0. Double strand DNA content was used as a surrogate for cell number and was measured on day 1 and day 7. Y-axis represents the change in cell number from Day 1 to Day 7. **L)** OVCAR4 shCtrl, shACADVL, and shECDHC2 cells were plated in low adherent tissue culture plates (suspension) and double strand DNA content was measured on day 1 and day 7. Y-axis represents the change in cell number from Day 1 to Day 7. Statistical test = ANOVA. Error bars = S.E.M.

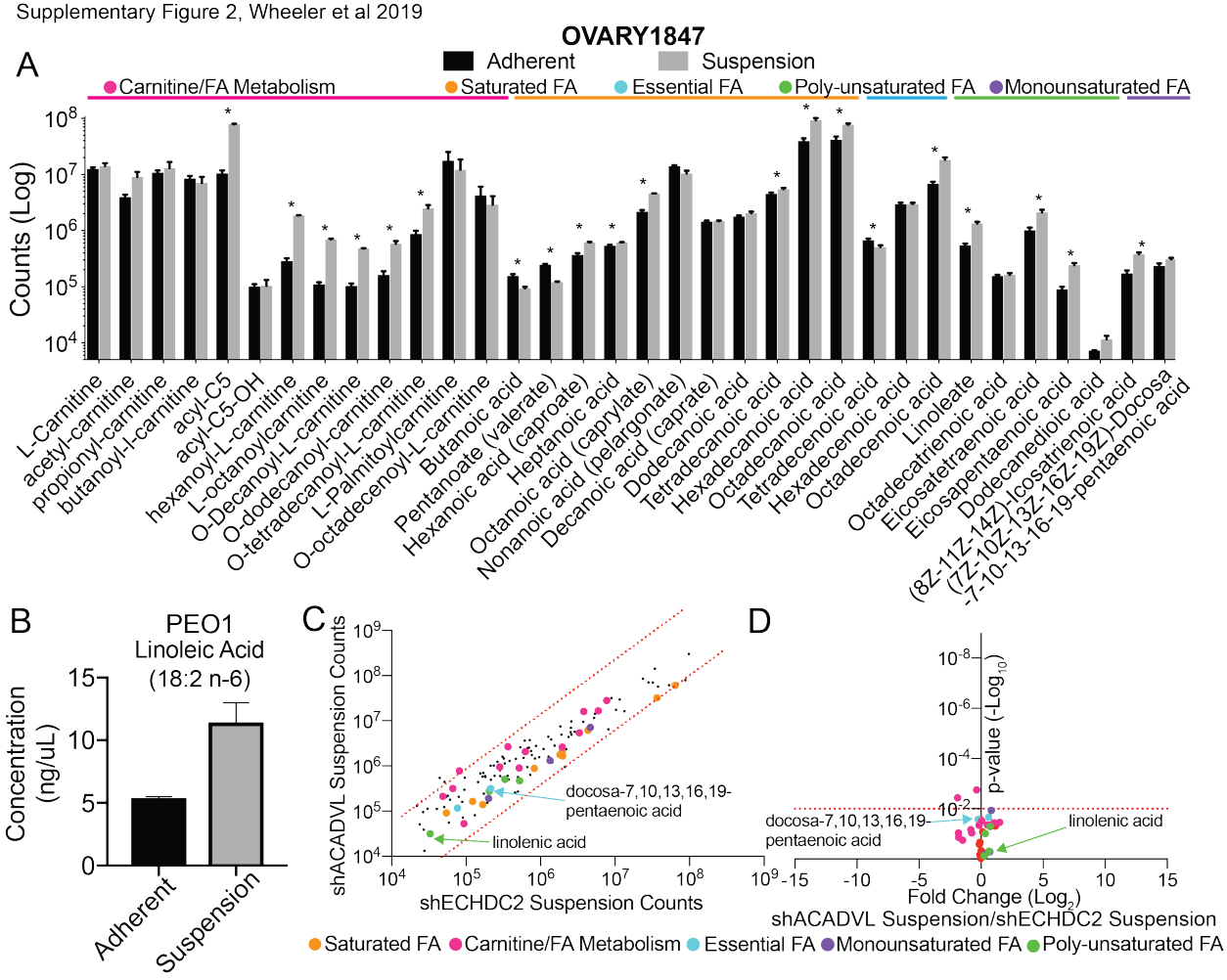

Figure S2. HGSOC cells cultured in suspension are enriched for fatty acids and fatty acid metabolites. (Related to Figure 2) A) HGSOC cells (OVARY1847) were cultured in adherent and suspension cultures for 48 hrs. Cells were collected and used for global non-targeted metabolomics. Fatty acids and fatty acids metabolites are shown for OVARY1847 cells grown in adherent (black bars) or suspension (gray bars) conditions. *p-value < 0.05. **B)** PEO1 cells were grown in adherent and suspension culture for seven days. Cells were collected, fatty acids were extracted, and the extracts were used for mass spec. **C**) Global non-targeted metabolomics analysis of shACADVL PEO1 cells cultured in suspension versus shECHDC2 PEO1 cells cultured in suspension. Metabolite counts graphed as a scatter plot. x-axis – shECHDC2 suspension and y-axis – shACADVL1 suspension. Red dotted line indicated p-value threshold. **D)** Same as C, but scatter plot of log2 fold change – x-axis and p-value -axis. Red dotted line indicates p-value threshold. Error bars = S.E.M.

Supplementary Figure 3, Wheeler et al 2018

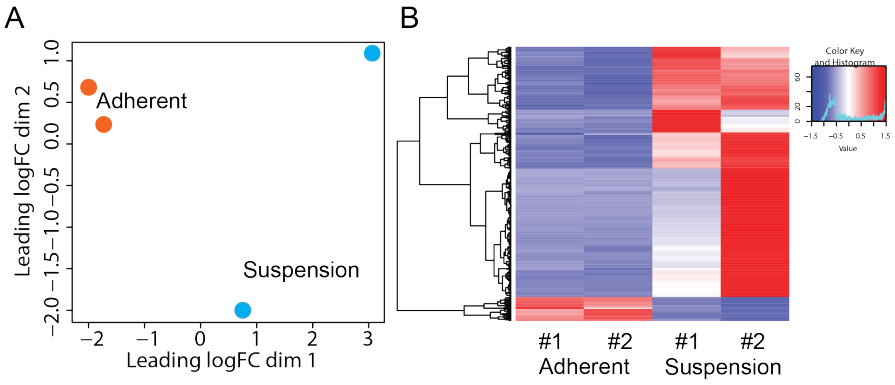

Figure S3. Principal component analysis (A) and hierarchical clustering (B) of RNA-sequencing data. (Related to Figure 3) A) PEO1 cells were grown in adherent or suspension cultures for 10 days. RNA was extracted from cells and utilized for RNA-sequencing. Principal component analysis of replicates for Adherent (red) and Suspension (blue) samples. **B**) Unsupervised hierarchical clustering of 300 top differentially regulated genes ($p<0.0001$).

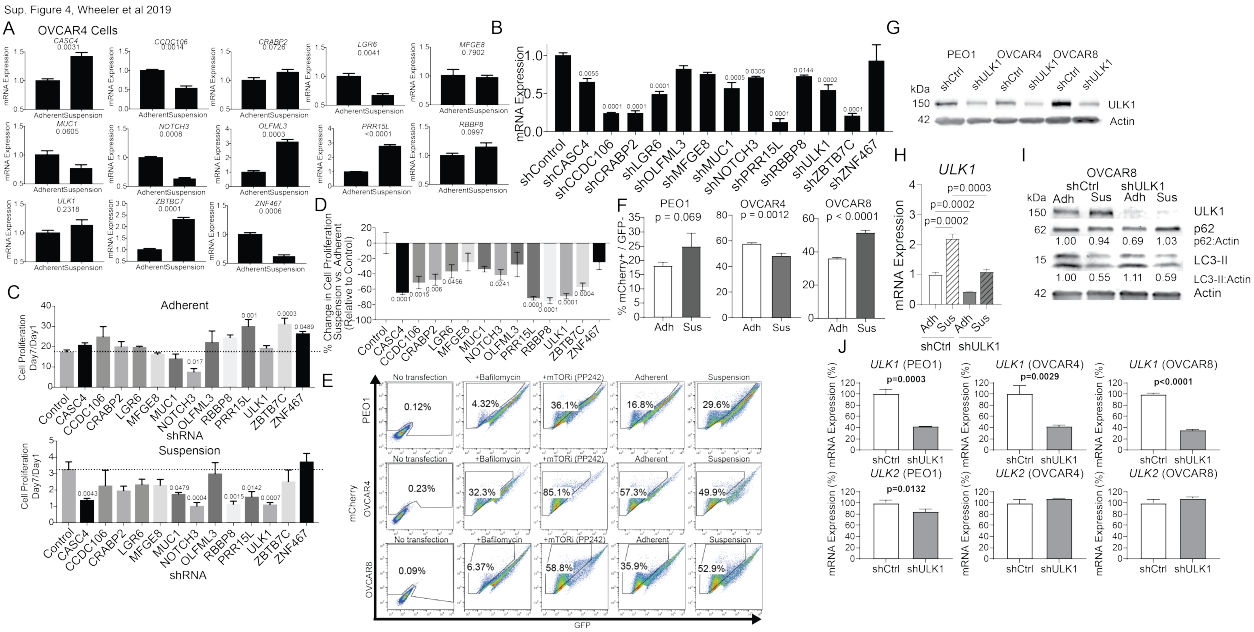

Figure S4. Confirmation of CRISPR/Cas9 and RNA-seq in HGSOC OVCAR4 cells. (Related to Figure 4) A) OVCAR4 cells were grown in adherent and suspension settings for 7 days. RNA was extracted from cells and used for RT-qPCR against indicated genes. 18S = internal control. Statistical test = two-sided unpaired t-test. **B)** Control shRNA and pooled shRNA against the 13 selected genes were transduced into OVCAR4 cells. RNA was extracted and used for RT-qPCR against indicated small hairpin targeted gene. 18S = internal control. Statistical test = ANOVA. **C**) shControl (Control) and pooled shRNA cells were cultured in adherent conditions for 1 and 7 days. On days 1 and 7 a CyQuant assay examined double stranded DNA content as a surrogate for cell number. Intensity of CyQuant at Day 7/Day 1 graphed. **D**) Percent change between Adherent and Suspension growth shown in C. Statistical test = ANOVA. Error bars = S.E.M. **E)** PEO1, OVCAR4, and OVCAR8 cells were transfected with plasmid ptfLC3 encoding a tandem fluorescent mCherry/GFP-tagged LC3. After 24 hrs, cells were seeded into adherent and suspension conditions for 48 hrs, and then analyzed by flow cytometry. Loss of GFP indicates lysosomal fusion with autophagosomes. Representative gating strategy of mCherry positive and GFP negative cells. Non-transfected cells are negative for mCherry and GFP and were gated out in subsequent analyses. Negative control: Transfected cells were treated with 100 nM bafilomycin A1 for 16 hrs. Positive control: Transfected cells were treated with 10 μM mTOR inhibitor (mTORi) PP242 for 16 hrs. **F)** Percentage of cells that are mCherry+/GFP-. Data are plotted as mean. Error bars = S.D. N=3. Statistical test = two-sided t-test. **G)** PEO1, OVCAR4, OVCAR8 cells were transduced with shCtrl or shULK1. Protein was collected and used for immunoblot against ULK1. Loading Control = Actin. **H)** shCtrl and shULK1 OVCAR8 cells cultured adherent (Adh) and suspension (Sus) for 48 hrs. RNA was collected and used for qRT-PCR against *ULK1*. Internal Control = *GAPDH*. Statistical test = two-sided unpaired t-test. Error bars = S.D. **I)** Same as I, but protein was also collected and used for immunoblot against ULK1, p62, and LC3-II. Loading Control = Actin. Values underneath blots are densitometry analysis of p62 (top) and LC3-II (bottom) expression normalized to Actin and shCtrl Adh. **J**) RT-qPCR for *ULK1* and *ULK2* in PEO1, OVCAR4, and OVCAR8 shCtrl and shULK1 cells. Internal control = *GAPDH*. Statistical test = two-sided unpaired t-test. Error bars = S.D.

Transparent Methods

Cell Culture. PEO1, OVCAR4, OVCAR8, and OVARY1847 human high grade serous ovarian cancer cell lines were authenticated using small tandem repeat (STR) analysis (The University of Arizona Genetics Core). HGSOC cells were cultured in RMPI-1640 medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. 293FT cells were cultured in high-glucose DMEM supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. All cells were maintained in 5% CO_2 at 37 °C and were monthly tested for mycoplasma using LookOut Mycoplasma PCR Detection Kit (Sigma, MP0035). PEO1 cells utilized for the GeCKO screen had been thawed within the previous three weeks.

Immunoblotting and Densitometry of Autophagy Proteins. Cells were lysed and briefly sonicated in RIPA buffer (150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) supplemented with cOmplete EDTA-free protease inhibitors (Roche #11873580001). Protein was separated by SDS-PAGE and transferred to PVDF membrane using the TransBlot Turbo (BioRad). Membranes were blocked for 1 hour at room temperature in LI-COR Odyssey buffer (LI-COR #927-50000). Primary antibody was incubated overnight at 4 °C in Odyssey buffer. Primary antibodies and concentrations were: rabbit-anti-ULK1 (Cell Signaling #8054, 1:1000), mouse-anti-p62/SQSTM1 (Cell Signaling #88588, 1:1000), mouse-anti-β-actin (Abcam ab6276, 1:10,000), and rabbit-anti-LC3A/B (Cell Signaling #4108, 1:1000). Membranes were washed 3 times for 5 minutes each in TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20), then incubated in LI-COR IRDye 800CW secondary antibodies (goat-anti-mouse #925-32210 or goat-anti-rabbit #925-32211) at 1:20,000 dilution in Odyssey buffer for one hour at room temperature. Following an additional 3 washes in TBST, blots were scanned using the LI-COR Odyssey Imaging System. Protein band intensity was quantified using LI-COR Image Studio v4.0 software. LC3-II and p62 were normalized to Actin for each sample, with shCtrl Adherent condition set as 1.00.

Viral Transduction Preparation. PEO1 cells transduced with GeCKO lentivirus were selected with 0.5 μg/mL puromycin. The Functional Genomics Facility (FGF) at the University of Colorado packaged the GeCKO library into lentiviral particles using pΔ8.9 and pCMV-VSV-G packaging vectors. Viral titers were performed on PEO1 cells to determine multiplicity of infection (MOI). Dilutions ranging from 50 to 800 μl of lentivirus in 1mL of media were used to transduce PEO1 cells and incubated for 24 hours. Puromycin selection was performed and the cells were counted at 48 hours when all non-transduced cells had died. The MOI was calculated, and given a goal MOI of 0.5, the optimal dilution of lentivirus was deemed to be 35-40%.

Viral Transduction. GeCKO library lentivirus particles were packaged at the FGF. PEO1 cells were plated on 19 - 15 cm dishes and allowed to recover for 24 hours. Transduction of the GeCKO library was performed at an MOI of 0.5 with polybrene (8 μg/mL). A dish was maintained as a non-transduced control. The transduction reaction incubated for 16 hours. Two 5 million cell aliquots were collected, snap frozen, and stored in -80 °C freezer, as “Post-Transduction Collection.” Cells were selected with puromycin every 24 hours for a total of 72 hours. Puromycin containing media was removed from all plates and cells were allowed to recover for 7 days in the adherent setting with media change and split as needed. Three post-selection samples of 5 million cell aliquots were collected, snap frozen, and stored in -80 °C freezer.

CRISPR/Cas9 Genome-Wide Screen. Following post-selection recovery period, cells were plated on tissue culture dishes treated without (adherent) or with (suspension) poly-HEMA (6 mg/mL). Given findings of slower growth in suspension setting, each adherent dish was plated with 5 million cells, while each suspension dish was plated with 10 million cells. For the adherent dishes, media was changed every 48 hours and cells were split at 90% confluence. For the suspension plates, fresh media was added at the same intervals as the adherent cells. Cells were checked daily for media exhaustion and optimal confluence (<90%). At days 5 and 10 a minimum of 5 million cells was collected for both adherent and suspension conditions. Cell pellets were snap frozen and stored at -80 °C until DNA extraction. DNA extraction was performed using the Quick DNA Mini-Prep protocol (Zymo Research). Nuclease-free water was used for elution and heated to 65 °C to increase yield. Total elution volume was 50 μL. DNA concentration was measured using the NanoDrop.

gRNA Library Preparation. Before initiation of the screen, as part of quality assurance, barcoding and sequencing primers (Table S8) were confirmed to amplify the product of interest. We utilized Q5 polymerase for all PCR (New England BioLabs). As previously described [1], we followed a nested PCR protocol, where the product of PCR1 served as the template for PCR2. To standardize PCR conditions all DNA samples were resuspended to 100 ng/μl. PCR1 was performed in a 60 μl reaction with 1 μg of sample template. PCR1 and 2 were run with the following program: initial 98 °C for 5 min, followed by 98 °C for 30 sec, 60 °C for 30 sec, 72 °C for 30 sec for 15 cycles, and 72 °C for 7 min. Ten microliters of PCR1 was run on gel electrophoresis to confirm product size. In PCR2, the product is 195 base pairs, and the reverse primer included a 16bp barcode. The remaining PCR1 product was used as a starting template for PCR2. A small proportion of samples did not have a 500 base pair band on the initial run. For these samples, the concentration was recalculated using the NanoDrop and purified using the DNA Clean Up kit (Zymo Research). These samples were then run through PCR1 for a second time, and a 500 base pair band was noted on the subsequent gel. All purified PCR products underwent spectrophotometry using the NanoDrop. Each PCR2 product was run on a 2% agarose gel and a band was noted at 200 base pairs. Amplification product was cut out and purified using a Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. The samples were quantified in duplicate using the QuBit (Thermo Scientific).

RNA-sequencing. As previously described [2]. RNA was isolated from PEO1 adherent (n=2) and suspension (n=2) following 7 days of growth using RNeasy columns with on-column DNase digest (Qiagen). RNA quality was confirmed using an Agilent Tapestation and all RNA used for library preparation had a RIN>9. Libraries were created using Illumina TruSEQ stranded mRNA library prep (#RS-122-2102) at the Genomics Core (The University of Colorado). HISAT2 [3] was used for alignment against GRCh37 version of the human genome. Samples were normalized using TPM (Transcripts per Million) measurement and gene expression using the GRCh37 gene annotation was calculated using home-made scripts. The analysis was performed by the Division of Translational Bioinformatics and Cancer Systems Biology at the University of Colorado School of Medicine.

Next Generation Sequencing. During the pre-sequencing quality assurance was performed on an Agilent Tapestation. PCR2 amplicons were sequenced at the Genomics Core (The University of Colorado) or Novogene Corporation. The Genomics Core pooled the libraries for sequencing. Libraries were sequenced on the HiSeq4000 and HiSeq2500 (Illumina). Due to sequence homology between libraries the pooled library sample was spiked with PhiX (15%).

CRISPR/Cas9 Sequencing Analysis. gRNA sequencing and differential gRNA analysis were conducted as previously described [1]. Principal component analysis and hierarchical clustering of the gRNA reads counts found that one Adherent Day 10 sample was different compared to the other collection points (Fig. S1E-G). This sample was removed from our analysis.

Global non-targeted metabolomics. As previously described [4]. PEO1 and OVARY1847 cells were cultured in adherent and suspension for 48 hrs. One million adherent and suspension cells were analyzed. Ultra-high performance liquid chromatography-mass spectrometry metabolomics was performed by University of Colorado School of Medicine Biological Mass Spectrometry Facility.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). RNA was isolated using RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol. NanoDrop spectrophotometry was performed to confirm the concentration of extracted RNA. RT-qPCR was performed using the Luna Universal One-step Reaction kit (New England BioLabs) on a BioRad CFX96 thermocycler with primers for each of the top genes of interest; 18S rRNA were utilized as internal controls (Table S9).

shRNA Knockdown. As previously described [5]. Gene specific shRNAs were obtained from the University of Colorado Functional Genomics Facility (Table S10). Plasmid isolation was performed using the Plasmid Midi-Prep Kit (Qiagen). Twenty-four hours after seeding, cells were transfected with a total of 12 μg of DNA, including lentiviral packaging plasmids and the shRNA, in addition to 36 μg of polyethyenimine (PEI), for a ratio of 1:3, DNA to PEI. Cells were incubated overnight and transitioned to Dulbecco's Modified Eagle Media (DMEM) the following morning. Forty-eight hours after medium change,

lentivirus was harvested. PEO1 cells were seeded into six-well plates. When cells reached 80% confluence, they were transduced with lentivirus encoding gene-specific shRNAs or a scrambled shRNA control. A control well was maintained without virus to confirm puromycin selection. A 48-hour puromycin selection was performed immediately following transduction. After medium change cells were allowed to recover then subjected to functional assays.

CyQuant Viability Assay. HGSOC cells were seeded in a 96-well plate and treated for 72 hours. Cells were incubated with CyQuant Direct (Thermo Fisher, Cat # 35011) for 2 hours. Fluorescence was measured on a Molecular Devices SpectraMax M2 microplate reader. Excitation = 508nm/Emission = 527nm.

Autophagy Assay. PEO1 or OVCAR8 cells were transfected using a 3:1 ratio of FuGENE 6 transfection reagent (Promega) and plasmid ptfLC3 (Addgene plasmid #21074, [6]). ptfLC3 encodes a tandem fluorescent mCherry/GFP-tagged LC3. After 24 h, cells were seeded into adherent and suspension conditions, then incubated for 48 h prior to collection for flow cytometry. For negative control, cells were treated with 100 nM bafilomycin A1 (Cell Signaling, # 54645S) for 16 h prior to collection. For positive control, cells were treated with 10 μM mTOR inhibitor PP242 (Torkinib, SelleckChem #S2218) for 16 h prior to collection. At collection, cells were trypsinized and pipetted into a single-cell suspension and finally resuspended in 4% FBS in PBS. Samples were run on a Beckman Coulter Gallios 561 flow cytometer. Data were analyzed in FlowJo 10. Gating strategy included removal of non-transfected (i.e. GFP-/mCherry- cells) prior to analysis. Adherent and suspension conditions were performed in triplicate and the percentage of mCherry+/GFP- cells was analyzed in GraphPad Prism 8.

Caspase-Glo Assay. As previously described [7]. OVCAR4 cells grown in adherent and suspension conditions were used for a Caspase-Glo 3/7 assay (Promega). Luminescence was measured on a Promega GloMax Multi-detection System.

Gene signature TCGA Datasets: Gene-level expression data in the form of raw read counts were downloaded and merged from the TCGA database. Kidney cancer – KIRC and KIRP, colorectal cancer – COAD, breast cancer – BRCA, brain cancer – LGG, Skin cancer – SKCM, and ovarian cancer – OV. For each cohort, expression data were downloaded at the sample level and then merged by Ensembl gene ID. For quality purposes, genes in each of the data sets were filtered based on expression levels. For all data sets genes were retained if they had an average of at least 10 reads across all samples. Original data sets contained ~30,000 unique Ensembl gene IDs, and after filtering, all contained ~15,000 unique Ensembl gene IDs. For each cohort, to extract expression values for each of the candidate genes expected to predict survival, expression data was extracted based on matching gene names from the hg38 Ensembl annotation database. Expression data was further sub-setted to those genes within our candidate list based on Ensembl ID.

Modeling survival and generating Kaplan-Meier curves were done in 3 steps: 1) The first step was to fit a Cox proportional hazard model for each gene and extract the coefficient. 2) A gene signature score was calculated by taking the weighted sum of the candidate genes using the coefficient from step 1 as the respective weights. We then used the median of the gene signature distribution to divide the scores into high (above median) and low (below median) scores. 3) The dichotomous description of gene signature score (high or low gene signature score) was then used as a predictor to fit new Cox proportional hazard models and generate a Kaplan-Meier curve. P-values were taken from the log-likelihood statistic from the Cox proportional hazard models. With our gene signature we tested two-hypotheses: 1) do the expression levels of the top 108 genes predict survival? and 2) do the expression levels of 108 random genes predict survival better than the top 108 genes? The p-value used to determine significance in our survival analysis was set at $p < 0.05$. In addition, we randomly selected the same number of genes used in each original gene signature analysis and used this random gene set as our gene signature, permuted the expression and outcome and predicted survival. This was done 1000 times, and for each permuted data set we modeled survival as described for the original analysis, and generated a distribution of log-likelihood statistics. We then calculated p-values for each cohort's permutation analysis, which describes the proportion of times the permuted likelihood test was more extreme (i.e. more significant) than the log-likelihood generated from the original analysis. Our gene signature was significantly better at predicting survival than a random selection of 108 genes for those datasets with a permuted p-value < 0.05. CoxPH

models and Kaplan-Meier curves were generated using the survival [8] and *simPH* [9] packages. All analyses for the gene signature scores were performed in R (v3.5.1).

Publicly Available Data. We examined significant genes associated with metastatic disease in Tothill Ovarian Cohort and Bittner Ovarian Cohort. Differentially regulated genes ($p<0.05$) were identified from both dataset and cross-referenced the genes identified in the CRISPR/Cas9/RNA-seq comparison (Oncomine, ThermoFisher).

Statistical Analysis. Prism Graphpad (version 8) software was utilized to generate graphs. All statistical tests are two-sided unpaired t-test unless noted. Hypergeometric distribution was calculated using the following website: http://systems.crump.ucla.edu/hypergeometric/. A significance threshold was set at $p<0.05$.

Data and Software Availability. The accession number for the CRISPR/Cas9 screen and RNA-seq data reported is GSE123290.

Supplemental References

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