Supporting Information

Dunn et al. 10.1073/pnas.1311909111

SI Materials and Methods

Plasmids. All ORFs used in the screen were cloned into the pLX304 vector backbone and obtained from the Center for Cancer Systems Biology (CCSB)/Broad Institute ORF collection (1). The pLX304-GAB2-3YF mutant was generated by serial overlapping site-directed mutagenesis via a protocol similar to that described previously (2). Y452F, Y476F, and Y584F mutations were introduced using the following primers: Y452F-For (5′-CAG CAC CAA TTC TGA AGA CAA CTT TGT GCC CAT GAA-3′) and Y452F-Rev (5′-TTC ATG GGC ACA AAG TTG TCT TCA GAA TTG GTG CTG-3′), Y476F-For (5′-ATT CCC AGA GCG TCT TCA TCC CAA TGA GCC C-3′) and Y476F-Rev (5′-GGG CTC ATT GGG ATG AAG ACG CTC TGG GAA T-3′), Y584F-For (5′-CAG GAG ACA GCG AAG AGA ACT TTG TCC CTA TGC-3′) and Y584F-Rev (5′-GCA TAG GGA CAA AGT TCT CTT CGC TGT CTC CTG-3′), and pLX-for, (5′-CAC CAA AAT CAA CGG GAC TT-3′) and pLX-rev (5′-CAA CAC CAC GGA ATT GTC AG-3′). The pLX-LacZ, pLX-KRAS^{V12}, and pLX-HRAS^{V12} plasmids were obtained from the CCSB/Broad Institute ORF collection. The pLenti6.3-blasticidin-ID4 has been described previously (3). Retroviral plasmid pBabe-Puro-MEK^{DD} has been described previously (4). The pBabe-GFP-myristoylated AKT1 plasmid was generated by subcloning the myristoylated Flag epitope tagged AKT1 (myr-Flag-AKT1) cassette (4) into the pBabe-GFP vector (Addgene plasmid 10668). All short hairpin RNA (shRNA) constructs were obtained from The RNAi Consortium (Broad Institute, Cambridge, MA) and have the following clone reference numbers: shLacZ (TRCN0000231710), shGAB2 #6 (TRCN0000155921), shGAB2 #7 (TRCN0000154991), shGAB2 #9 (TRCN0000155271), and shGAB2 #10 (TRCN0000154706).

Cell Lines and Reagents. Immortalized human embryonic kidney epithelial cells overexpressing MEK^{DD} (HA1E-M) cells (4), immortalized fallopian tube secretory epithelial cells (FTSECs) (5), and immortalized ovarian surface epithelial (IOSE) cells (6) were described previously. NCI-H1435 and MDA-MB-468 cell lines were purchased from the American Type Culture Collection. NIH-OVCAR3, COV362, FUOV1, IGROV1, MDA-MB-453, BT20, A2780, HCC1187, OVCAR8, Kuramochi, EFO21, COV504, OVISE, SK-OV-3, TOV21G, and EFO27 were genotyped at the Broad Institute (7). All cell lines were maintained in DMEM (Mediatech) supplemented with 10% (vol/vol) FBS (Sigma-Aldrich) and 1% penicillin/streptomycin (Cellgro).

Pooled in Vivo ORF Screen. The 588 ORFs representing genes amplified in the ovarian cancer The Cancer Genome Atlas (TCGA) dataset as well as positive and negative controls were obtained from the CCSB/Broad Institute ORF collection. HA1E-M cells were plated at a density of 6,800 cells per well in 96-well flat-bottom plates (VWR) and infected in arrayed fashion with lentivirus generated from the lentiviral ORF plasmids. After 2 d, singly infected confluent wells were pooled into collections of 22–24 ORF-expressing HA1E-M cells, expanded into T-150 tissue culture flasks (VWR), and injected. Positive controls 1:24 and 15:24 mixtures of pLX304-AKT1–infected HA1E-M cells with uninfected cells as well as a mixture of HA1E-M cells infected with pLX304-BRAF^{V600E}, pLX304-KRAS^{V12}, or pLX304-HRAS^{V12}. Negative controls included a 1:24 mixture of pLX304-EGFP–infected HA1E-M cells with uninfected HA1E-M cells. A replicate of infected HA1E-M cells was treated with 8 μg/mL blasticidin and monitored for cell death. Each pool of HA1E-M cells was injected into three sites each in two NCr nude mice (Taconic) per pool. Cells were trypsinized, resuspended in PBS, and injected at a concentration of 10×10^6 cells per mL in 200 μL (2×10^6 cells) per site.

Identification of ORF Sequences in Tumors. Tumors were resected when they reached a minimum largest diameter of 1 cm. Tumors were minced to homogeneity in culture medium, and genomic DNA was isolated from two independent tissue sections using the DNeasy Blood & Tissue Kit (Qiagen). ORF sequences were amplified by PCR from 150 ng genomic DNA by amplifying across the ORF sequence using pLX304-specific flanking primers pLX-for and pLX-rev for 35 cycles using KOD polymerase (EMD Millipore). PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen), and 200 ng of purified amplicon DNA from each single reaction were cloned into 150 ng of the pDONR223 vector (Invitrogen) using the BP reaction (Invitrogen) for 4 h at room temperature. DH5α *Escherichia coli* cells were transformed with the entire reaction and plated on LB agar plates containing 50 μg/mL spectinomycin. After 12 h, bacterial cells were harvested by scraping, and pDONR223 plasmids harboring recombined ORF sequences were purified using the QIAprep Spin Miniprep Kit (Qiagen). The pDONR223 ORF plasmids from each tumor were sheared, barcoded, pooled, and sequenced on a HiSeq system (Illumina). ORF incidence per tumor was calculated by dividing the number of duplicated ORF reads by the total number of nonvector duplicated ORF reads in a given pool. ORFs were scored as present if they comprised at least 0.1% of the nonvector sequences.

Chemicals. GDC-0941 and AZD-6244 were purchased from Selleck Chemicals. Torin 2 was obtained from Tocris Bioscience.

Immunoblotting. Immunoblotting was performed as described (8). Antibodies to the following proteins used for immunoblotting were purchased from Cell Signaling: GAB2 (no. 3239), AKT (no. 9272), phospho-S473 AKT (no. 4060L), mTOR (no. 2972), S6 kinase (no. 9202), phospho-S6 kinase (no. 9205), ERK1/2 (no. 9102), and phospho-ERK1/2 (no. 9101). Additional antibodies for immuneprecipitation and immunblotting GAB2 included an anti–C-terminal antibody (no. sc-9313, Santa Cruz) and an anti–N-terminal antibody (no. AP6908a, Abgent). Antibody to β-actin (sc-47778) was purchased from Santa Cruz Biotechnology.

Cell Proliferation Assay. The 1,700 IGROV1, FUOV1, and OV56 cells; 1,500 NIH:OVCAR3; 2,000 OV7 and OVK18; and 3,000 MDA-MB-453 cells were seeded into each well of 96-well plates 24 h before infection. Six replicate infections were performed for control shRNAs targeting LacZ (shLacZ) and GAB2-specific shRNAs in the presence of 4 μg/mL polybrene for 24 h followed by selection with 2 μg/mL puromycin. The ATP content was measured at 6 d postinfection by using the CellTiter-Glo luminescent cell viability assay (Promega).

Anchorage-Independent Growth Assay. Growth in soft agar was determined by plating 1×10^4 cells in triplicate in 5 mL medium containing 0.4% Noble agar (BD Biosciences) which was placed on top of 4 mL solidified 0.6% agar. Colonies greater than 100 μm in diameter were counted 4 wk after plating.

High-Density Tissue Microarray and Immunohistochemistry. A tissue microarray (TMA) comprised 134 cases of high-grade, late-stage ovarian serous carcinoma as well as immunohistochemistry methods

were described previously (9, 10). For immunohistochemistry, antibody to human GAB2 (sc-9313, Santa Cruz Biotechnology) was used at a dilution of 1:500. GAB2 staining was scored on a scale of 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) intensity. All tissue review was performed in a blinded manner by pathologists.

Analysis of Primary Human TCGA Genomics and Reverse Phase Protein Array Data. We used the Broad Institute Firehose analysis run from August 25, 2012, which may be found in the TCGA Data Coordination Center (https://confluence.broadinstitute.org/display/GDAC/Home), for all primary cancer data files (copy number, expression, protein levels) and analysis results [Genomic Identification of Significant Targets in Cancer (GISTIC) result files]. Screenshots of the segmented primary breast and ovarian cancer copy number data (from Affymetrix SNP6 arrays) with scores from TCGA ovarian specific GISTIC analysis were taken using the Integrative Genome Viewer (www.broadinstitute.org/igv/).

A total of 326 primary ovarian cancer samples from TCGA had reverse phase protein array (RPPA), mRNA expression and copy number data available. GAB2 mRNA levels were pulled from mRNA expression data, using Agilent G4502A arrays. High expression was defined as 1 SD above the mean of GAB2. Protein levels of GAB2 were determined from RPPA arrays. Amplification of GAB2 was pulled from the ovarian cancer GISTIC results; only those amplifications larger than the arm level amplifications observed for the sample were considered highly amplified.

To analyze *GAB2* and additional components of the phosphatidylinositol 3-kinase (PI3K) pathway, copy number pipeline methodology developed at the Broad Institute was applied to the copy number data obtained on the Affymetrix platform across 481 OV samples from the TCGA study deposited at Broad Genome Data Analysis Center (GDAC) Firehose database. We called high-level somatic copy number alternations based on GISTIC 2.0 (11) for *GAB2* and the PI3K pathway genes *PIK3CA*, *PIK3CB*, *AKT1*, *AKT2*, *AKT3*, *PTEN*, *PIK3R1*, and *PDK1*.

Animal Injections/Tumorigenicity. Cell line xenograft experiments were performed as described (4, 8). Animal protocols were approved by the Dana–Farber Cancer Institute Institutional Care and Use Committee. HA1E-M cells were grown as described above, trypsinized, resuspended in PBS, and injected s.c. at an inoculum of 2×10^6 cells per site in 6-wk-old Ncr-nude mice (Taconic). IOSE-derived cells were grown as described above, resuspended in 400 μL 1× PBS mixed with 400 μL of Matrigel Basement Membrane Matrix (BD Biosciences), and injected s.c. at an inoculum of 2×10^6 cells per site in 6-wk-old *NOD/IL2Rγc/scid* mice (Taconic) and monitored for tumor formation.

1. Yang X, et al. (2011) A public genome-scale lentiviral expression library of human ORFs. *Nat Methods* 8(8):659–661.
2. Atanassov II, Atanassov II, Etchells JP, Turner SR (2009) A simple, flexible and efficient PCR-fusion/Gateway cloning procedure for gene fusion, site-directed mutagenesis, short sequence insertion and domain deletions and swaps. *Plant Methods* 5:14.
3. Ren Y, et al. (2012) Targeted tumor-penetrating siRNA nanocomplexes for credentialing the ovarian cancer oncogene ID4. *Sci Transl Med* 4(147):ra112.
4. Boehm JS, et al. (2007) Integrative genomic approaches identify IKBKE as a breast cancer oncogene. *Cell* 129(6):1065–1079.
5. Karst AM, Levanon K, Drapkin R (2011) Modeling high-grade serous ovarian carcinogenesis from the fallopian tube. *Proc Natl Acad Sci USA* 108(18):7547–7552.
6. Liu J, et al. (2004) A genetically defined model for human ovarian cancer. *Cancer Res* 64(5):1655–1663.
7. Cheung HW, et al. (2011) Systematic investigation of genetic vulnerabilities across cancer cell lines reveals lineage-specific dependencies in ovarian cancer. *Proc Natl Acad Sci USA* 108(30):12372–12377.
8. Cheung HW, et al. (2011) Amplification of CRKL induces transformation and epidermal growth factor receptor inhibitor resistance in human non-small cell lung cancers. *Cancer Discov* 1(7):608–625.
9. Clauss A, et al. (2010) Overexpression of elafin in ovarian carcinoma is driven by genomic gains and activation of the nuclear factor kappaB pathway and is associated with poor overall survival. *Neoplasia* 12(2):161–172.
10. Liu JF, Hirsch MS, Lee H, Matulonis UA (2009) Prognosis and hormone receptor status in older and younger patients with advanced-stage papillary serous ovarian carcinoma. *Gynecol Oncol* 115(3):401–406.
11. Mermel CH, et al. (2011) GISTIC2.0 facilitates sensitive and confident localization of the targets of focal somatic copy-number alteration in human cancers. *Genome Biol* 12(4):R41.

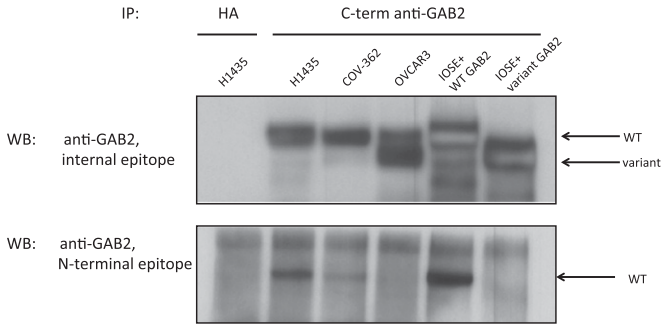

Fig. S1. GAB2 isoform expression. GAB2 immune complexes were isolated from NCI-H1435, COV-362, and OVCAR3 cells as well as IOSE cells overexpressing the wild-type (WT) full-length isoform of GAB2 or the variant isoform of GAB2 using an antibody specific for the C terminus of GAB2 or HA as a negative control. Immune complexes were immunoblotted with either the same antibody (*Upper*) or an antibody recognizing the first N-terminal 39 aa (*Lower*).

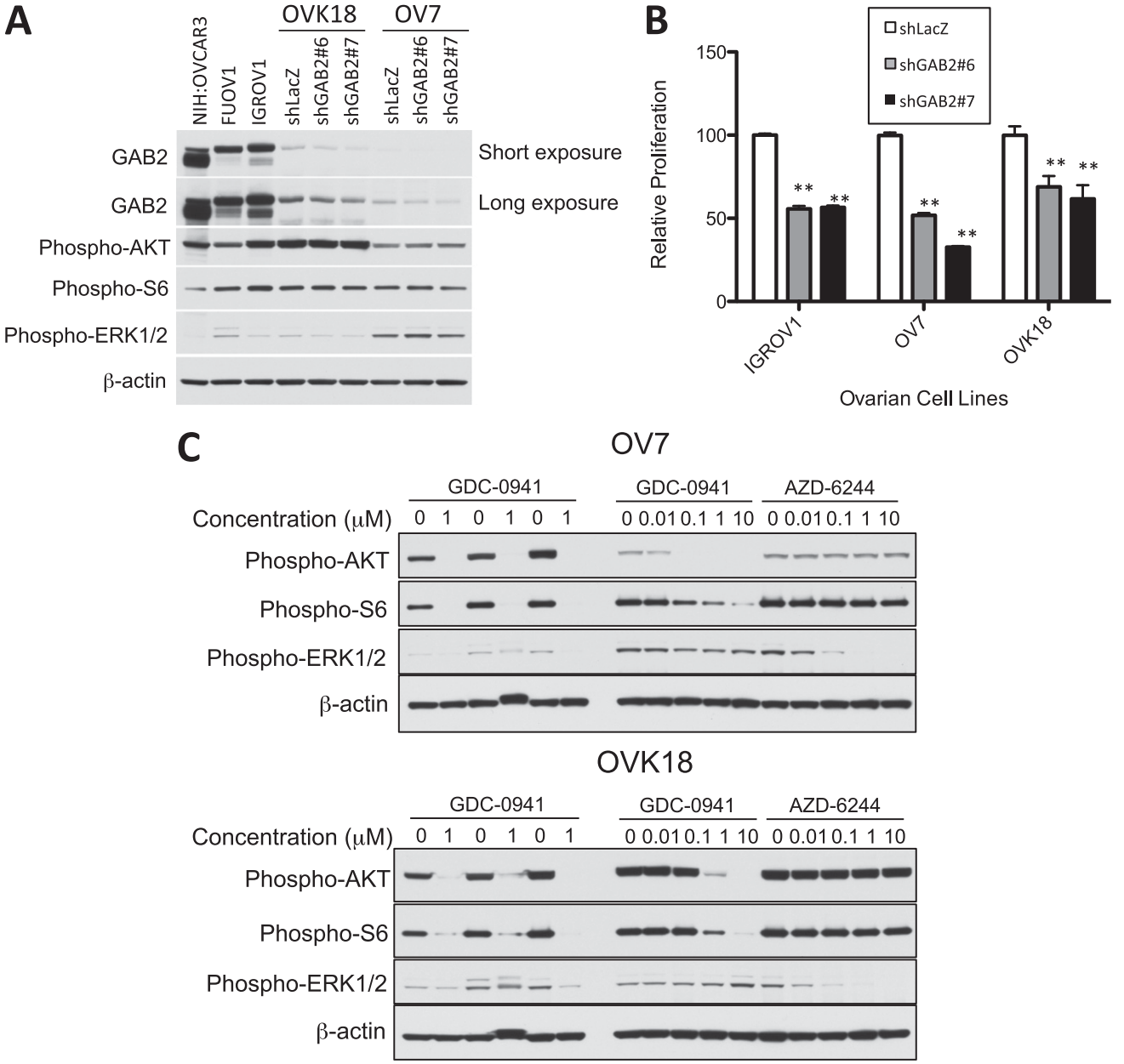

Fig. S2. Effects of GAB2 suppression in cells expressing low levels of GAB2. (*A*) OV7 and OVK18 cells were transduced with shLacZ, shGAB2 #6, and shGAB2 #7 and then monitored for levels of GAB2 protein as well as phosphorylated levels of AKT1, S6, and ERK1/2. (*B*) Effects of suppressing GAB2 expression on cell proliferation in a GAB2-overexpressing IGROV1 cell line and two cell lines with low GAB2 expression. $^{**}P < 0.0001$. Error bars reflect standard deviation. (*C*) Effect of increasing doses of the PI3K inhibitor GDC-0941 or the MEK inhibitor AZD-6244 on AKT1, ERK1/2, and S6 phosphorylation in GAB2 low-expressing OV7 and OVK18 cell lines.

Other Supporting Information Files

Dataset S1 (XLS)