Supplementary Methods

Flow cytometry
Cells were plated and allowed to adhere to culture plates for 24 hrs and then treated as specified. Subsequently, cells were harvested in 1x PBS containing 0.1% BSA. For cell cycle analysis, cells were washed 2 x in ice-cold PBS, and fixed in ice-cold 70% ethanol. Each sample was divided into two groups. Half of each sample was stained with propidium iodide (Invitrogen, Carlsbad, CA), while the remainder was left unstained to be used as negative control. RNase A was added to each sample to a final concentration of 0.5 µg/ml and incubated overnight at 4°C. All samples were subsequently analyzed using a BD LSRFortessa flow cytometer. Data were analyzed using FlowJo software, and the DNA content of the various cell cycle phases was quantified.

Apoptosis measurement using Annexin V/PI double staining method
Apoptotic UM cells were quantified using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Pharmingen) according to the manufacturer’s protocol. Briefly, cells were seeded in 6-well plates and allowed to adhere for 24 hrs, and then treated with either ICG-001 or DMSO for the designated time and dosage. At the end of the noted time, both culture medium containing any floating cells, as well as, lifted adherent cells were collected by centrifugation at 1000 rpm at 4°C for 5 minutes, followed by two cold PBS washes. Cells were then resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 1 x 10⁶ cells/ml. 100 µl of the cell suspension was transferred to a 5 ml culture tube, and 5 µl of FITC Annexin V and 5 µl of PI was added. Controls used to set up compensation and quadrants included unstained cells, cells stained with FITC Annexin V (no PI) and cells stained with PI (no FITC Annexin V). The cells were gently vortexed and incubated in the dark for 15 minutes at room temperature. An additional 200 µl of binding buffer was added to the cell suspension and analysis using a BD LSRFortessa flow cytometer to quantify percentages
of apoptotic and necrotic cells was performed within 60 minutes. Cell populations were plotted with Annexin V conjugated FITC intensity on the logarithmic X-axis and PI on the Y-axis. Cells that stained positive for FITC and negative for PI were determined to be undergoing apoptosis, while cells that stained positive for both FITC Annexin V and PI were either in the end stage of apoptosis or undergoing necrosis.

**Western Blotting:** Cells were treated with various concentrations of ICG-001 for a designated amount of time. For whole-cell extractions, cells were harvested, washed 2x with ice-cold PBS, and lysed in NP-40 buffer with phosphatase and protease inhibitor. Protein concentrations were measured using Pierce BCA-protein assay (ThermoFisher Scientific) and 30 µg of protein lysates were loaded in each well of 4-12% SDS-PAGE gradient gel. Proteins were transferred onto nitrocellulose membrane and blocked with 5% BSA for 1hr. Primary and secondary antibodies were reacted in blocking solution at 1:1000 dilution. Proteins were detected using Pierce ECL blotting substrate (ThermoFisher Scientific). Proteins were detected with the following antibodies: Anti-β-actin (MA1-140) from ThermoFisher Scientific (Waltham, MA) and anti-p-YAP Ser127 (#4911), anti-YAP (#4912), anti-p-mTOR Ser2448 (#5536), anti-mTOR (#2983), anti-p-p70S6 Thr421/Ser424 (#9204), anti-p70S6 (#9202), anti-β-catenin (#9564), anti-cleaved caspase 3 (#9664), anti-cleaved PARP (#5625), anti-p-MAPK Thr202/Tyr204 (#4370), anti-MAPK (#4695S), anti-p-Akt Ser473 (#4060), anti-Akt (#4691), and anti-KLF4 (#4038) from Cell Signaling (Denvers, MA).

**Wound-healing Assay**

For in vitro wound-healing assays, cells were grown in automated 96-well cell-migration plates and imaged with IncuCyte live-cell imaging instruments (Essen BioScience). Briefly, cells were plated as a confluent monolayer, and a scratch wound was made using a 96-pin wound-making tool (WoundMaker; Essen BioScience, Ann Arbor, MI). Next, cells were treated with 5 µM of ICG-001 for 24hrs. Cells were
imaged every 30 minutes. Relative Wound Density (cell density in the wound area expressed relative to the cell density outside of the wound area) was calculated by the IncuCyte image analysis software.

**Global gene expression profiling**

ICG-001 was purchased from Selleckchem (Houston, TX). \( GNAQ^{MT} \) Mel202 UM cells were treated with 3 \( \mu \)M of ICG-001, dissolved in DMSO for 24h or 48h in 6-well plates. For controls, cells were treated with DMSO alone for the respective times. Following treatment, cells were harvested in RNAlater (Qiagen; Germantown, MD) and RNA was isolated using the RNeasy Micro Kit (Qiagen). Briefly, an equal number of cells were centrifuged at high speeds to remove RNAlater. Buffer RLT with 1% beta-mercaptoethanol was utilized for cell lysis followed by homogenization using a QIAshredder spin column. RNA was precipitated with 70% EtOH and transferred to the RNeasy MinElute spin column. Samples were treated with DNase, washed, and eluted as per the manufacturer’s instructions where the average sample yield was 16.5 \( \mu \)g. The RNA integrity for each sample was assessed with an RNA 6000 Nano chip on a 2100 Bioanalyzer (Agilent; Santa Clara, CA). The average RNA integrity score (RIN) for the sample set was 9.98.

**aRNA Isolation**

RNA samples were put through the Illumina TotalPrep RNA Amplification Kit (Ambion by Life Technologies; Carlsbad, CA) to generate biotinylated, amplified RNA (aRNA) for downstream processing. In summary, a reverse transcriptase with an oligo(dT) primer was utilized in first strand synthesis to produce cDNA containing a T7 promoter sequence. Second strand synthesis followed, whereby the product became the template for in vitro transcription with T7 RNA polymerase along with biotin-UTP. After purification of the aRNA, the samples were subjected to quantitation utilizing the spectrometric Quant-iT RiboGreen assay (Invitrogen, Ltd.; Eugene, OR) where the average aRNA yield per sample was 46.1 \( \mu \)g.
Direct Hybridization & Imaging

The aRNA was prepared for direct hybridization by diluting to the appropriate concentration following the Whole-Genome Gene Expression Direct Hybridization (Illumina, Inc.; San Diego, CA) assay guide. The biotin-labeled samples were loaded onto Beadchip arrays which contain complementary gene-specific sequences and incubated at 58°C for approximately 18 hrs. The arrays were removed from overnight incubation and washed as per the manufacturer’s recommendation. Cy3-Streptavidin was added to introduce fluorescent and differential detection of the signal to the analytical probes that have been hybridized to the array by the use of the Illumina iScan (Illumina, Inc.; San Diego, CA). An internal laser was utilized to excite the fluorophore product on the array allowing light emissions to be recorded in high-resolution imagery. The GenomeStudio (V2011.1) Gene Expression Analysis Module (v1.9.0) was applied to the scanned microarray images where preliminary quality control data was assessed.

Gene expression differences were derived utilizing the t-test and imposing a fold change exceeding 4/3x (p<0.05), using the R statistical system. Enriched pathways were inferred using the Gene Set Enrichment Analysis (GSEA) method¹, and the Molecular Signature Database (MSigDB) pathway collection². Gene sets with a nominal P value <0.05 and FDR < 0.25 were defined as significantly enriched.

Application of the ICG-001 gene signature to patient specimens

Gene expression profiles were obtained from UM cells treated with ICG-001 for 24 and 48 hrs: 2,413 genes were differentially expressed in the 24 hrs signature, compared to the 2,493 genes that were differentially expressed in the 48 hrs signature. The two signatures were intersected and 1,510 genes were found to be common between the two signatures and their expression leveled changed in the same direction (i.e. suppressed or induced in both signature). This core (shared) transcriptomic signature of ICG-001 was then applied to the TCGA-UM patient dataset and prognostic significance was evaluated.
To do so, we first computed the z-scores for all the genes altered in the ICG-001 treated samples with respect to vehicle-treated samples. The z-scores of genes repressed after ICG-001 treatment were subtracted from the z-scores of genes that were induced after ICG-001 treatment, resulting in a corresponding activity score for each specimen. Within each dataset, specimens were ranked according to their gene signature activity score, and association with overall survival was evaluated using the log-rank test between the bottom 20% of the specimens and the rest (top 80%) of the specimens.

References

1. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005; 102: 15545-15550.

2. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdottir H, Tamayo P, Mesirov JP. Molecular signatures database (MSigDB) 3.0. Bioinformatics 2011; 27: 1739-1740.