Supplemental figure 1. Dose-dependent inhibition of mTORC1 and mTORC2 signaling pathways mediated by OXA-01, OSI-027, and rapamycin. Ovcar-3 ovarian carcinoma cells (PTEN, PI3K wildtype) were treated with varying doses of drug as shown. **A.** Serum-deprived cells were treated for 2 hours then stimulated with insulin immediately prior to lysis. The effects of drug treatment on phosphorylation of mTOR pathway effectors, as analyzed by western blot. **B.** Quantification of the band density from (A) for phospho-Akt (closed circles), phospho-S6 (open diamonds), and phospho-4E-BP1 (closed triangles) is shown as a percent of DMSO-treated controls and graphed as a function of drug concentration.

Supplemental figure 2. Effects of mTOR inhibition in RIP-Tag2 tumors on phospho-S6K signaling. Confocal images showing tumor blood vessels (CD31; green) and phosphorylated S6-kinase (red) in RIP-Tag2 after 7 days of treatment. Phosphorylated S6-kinase appeared less after both OXA-01 and rapamycin treatment compared to vehicle. Scale bar represents 25 μm.

Supplemental Methods:

Protein kinase assays

Assays of a panel of 40 other recombinant kinases including both protein and lipid kinases were performed at 100 mM ATP concentration by SelectScreen profiling service (Invitrogen, CA, USA). A broad panel of kinases was tested at a single concentration of OSI-027 or OXA-01 (3μM) to evaluate percent inhibition of each kinase or mutant variant, using the Ambit KinomeScan platform.

In vivo tumor growth inhibition studies:

Tumor volumes were determined from caliper measurements using the formula V = (length x width2)/2. Tumor sizes and body weights were measured twice weekly. Tumor growth inhibition (TGI) was determined at different time points by the following formula: %TGI = {1 – [(Tt/T0) / (Ct/C0)] / 1 – [C0/Ct]} x 100, where Tt = median tumor volume of treated at time t, T0 = median tumor volume of treated at time 0, Ct = median tumor volume of control at time t, and C0 = median tumor volume of control at time 0. Median TGI was calculated for the entire dosing period. Percent regression represents the maximum regression observed on any day during the dosing period. % Regression = 100(V0-Vi)/ V0; where V0 is the mean tumor volume for treated group at the initiation of treatment and Vi is the mean tumor volume for that group at time x.

Cell Culture:

To study the effect of drug treatment on cellular signaling, Ovcar-3 cells were plated in normal growth medium. After 24 hours, serum was removed and cells were serum-starved overnight. Rapamycin, OSI-027 and OXA-01 were solubilized in DMSO and added to cells at varying concentrations. After a two-hour incubation cells were growth factor stimulated with 10ng/ml insulin for 3 to 5 minutes, then rinsed with cold PBS and lysed.

Western Blot analysis

For analysis of cells cultured in vitro, cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail, 1 mM sodium orthovanadate and 10 mM sodium fluoride (all reagents purchased from Sigma, St. Louis, MO). Lysates were cleared by centrifugation and equal protein was loaded per well. Lysates were fractionated on 4-12% tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes using a semidry apparatus. Membranes were blocked with 5% nonfat dry milk in tris-buffered saline, and incubated overnight with primary antibody in 3% bovine serum albumin. HRP-conjugated

secondary antibodies were incubated in nonfat dry milk for one hour. SuperSignal chemiluminescent reagent (Pierce Biotechnology, Rockford, IL) was used according to the directions and blots were imaged using the Alpha Innotech image analyzer. Alpha Innotech software was used to quantify band density values. All antibodies used for Western blot analysis, unless otherwise noted, were purchased from Cell Signaling Technologies (Danvers, MA). The specific phospho-epitopes are described in the results section. The antibody against β-actin was purchased from Sigma. Analysis of VEGF in RIP-Tag tumors was performed using the mouse angiogenesis antibody array from R&D Systems (Minneapolis, MN), according to the manufacturer instructions.

Immunohistochemistry

Endothelial cells were labeled with hamster anti-CD31 (PECAM-1, Clone 2H8, 1:500, Thermo Scientific, Hudson, NH) or rat anti-CD31 (PECAM-1, clone MEC 13.3, 1:500, Pharmingen, San Diego, CA). VEGF-A was labeled with goat anti-VEGF-A (1:500, R&D Systems); basement membrane was identified with rabbit anti-type IV collagen (1:8000, Cosmo Bio USA, Carlsbad, CA); erythrocytes with rat anti-TER-119 (Ly-76, 1:500, BD Biosciences, San Jose, CA); proliferating cells with rabbit anti-phosphohistone-H3 (PHH3, 1:1000; Millipore/Upstate Biotechnology, Billerica, MA); and apoptotic cells with rabbit anti-activated caspase-3 (1:1000; R&D Systems). Targets of the mTOR pathway were stained with rabbit anti-phospho-Akt (Ser473, D9E, 4060, Cell Signaling) and phospho-4E-BP1 (Thr37/46, 236B4, 2855, Cell Signaling) according to the manufacturer's protocols.

VEGF fluorescence image analysis

VEGF fluorescence intensity was determined as the percentage of pixels with fluorescence brighter than baseline fluorescence of exocrine pancreas. VEGF fluorescence within the

compartment of blood vessels was determined in 4 steps. (1) Images of RIP-Tag2 tumors were acquired with the 10X objective. (2) Images of PECAM-positive staining within the same field were also acquired. (3) Regions of interest that were PECAM-positive were identified in ImageJ. (4) Fluorescence intensity of the corresponding VEGF image in the PECAM-positive region of interest was then determined as described above. Five PECAM positive regions in each tumor and 5 tumors per mouse were quantified. VEGF fluorescence intensity in the tumor cell compartment was determined with the same 4 steps, but the regions of interest were defined as PECAM-negative areas.