Figure S1

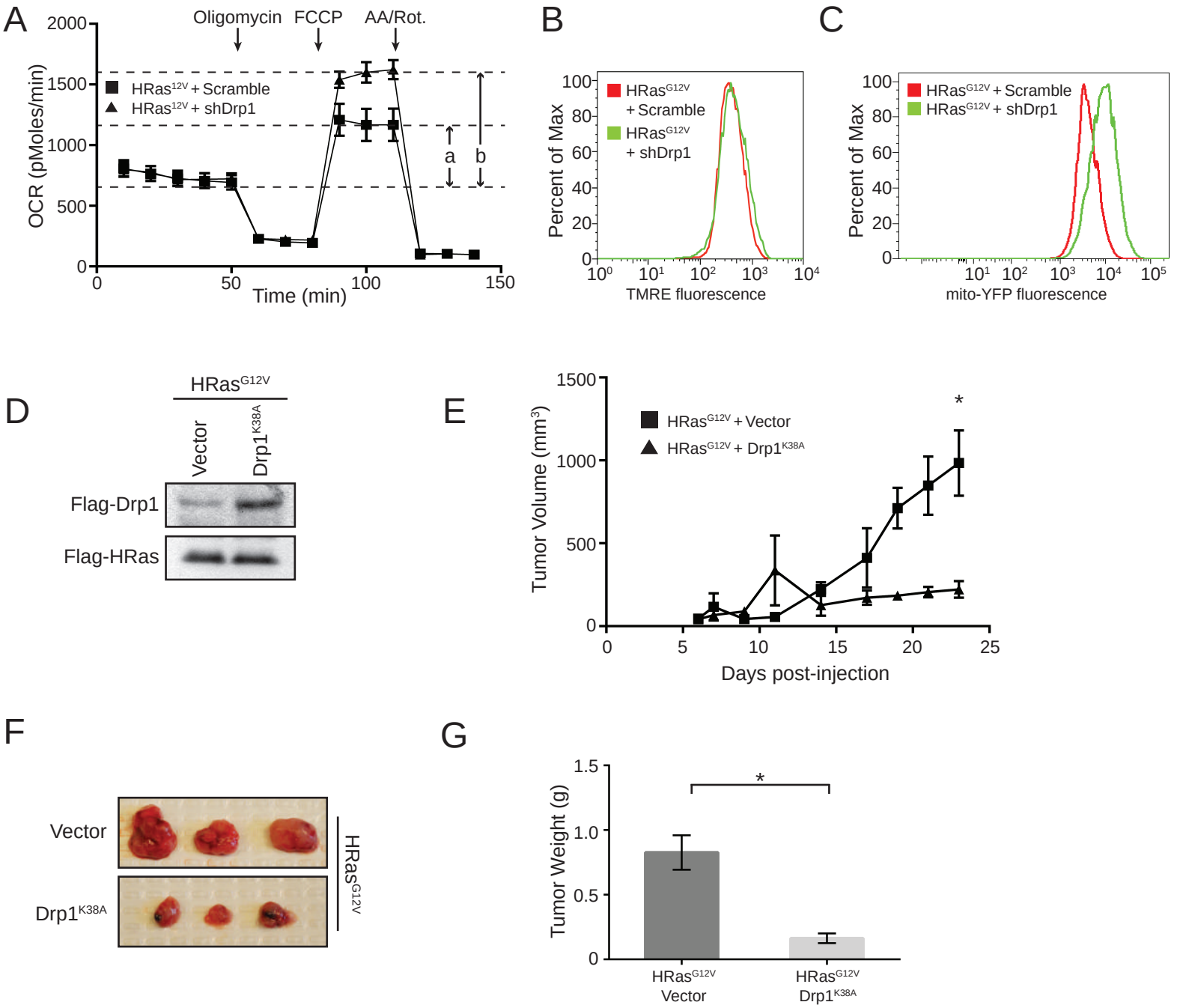

Figure S1. **Inhibition of Drp1 in Ras-expressing HEK-TtH Cells.** (Related to Figure 1) A. Oxygen consumption rate was measured using an XF24 extracellular flux analyzer in HEK-TtH cell expressing $HRas^{G12V}$ and an shRNA targeting either scramble control or Drp1. Oligomycin, FCCP, Rotenone and Antimycin A were added at the indicated time-points (arrows). Spare respiratory capacity is measured as the difference between basal oxygen consumption rate and the FCCP uncoupled oxygen consumption rate (a,b). B. TMRE was added to HEK-TtH cell expressing $HRas^{G12V}$ and an shRNA targeting either scramble control or Drp1 and fluorescence was measured by flow cytometry. C. The levels of fluorescence in HEK-TtH cells expressing mito-YFP, $HRas^{G12V}$, and an shRNA targeting either scramble control or Drp1 were analyzed by flow cytometry. D. HEK-TtH cells stably expressing $HRas^{G12V}$ and either empty vector or $Drp1^{K38A}$ were analyzed by immunoblot for expression of Flag-Drp1 and Flag-HRas. E. The cells were injected into SCID/Beige mice and tumor volume was measured over time. F-G. Tumors were removed at day 24 to be photographed and weighed. n=3 tumors per cell line; error bars: S.E.M. of mean tumor volume (E) or tumor weight (G). * Two-tailed t-test, p=0.0352 (E) or p=0.0280 (G).

Figure S2

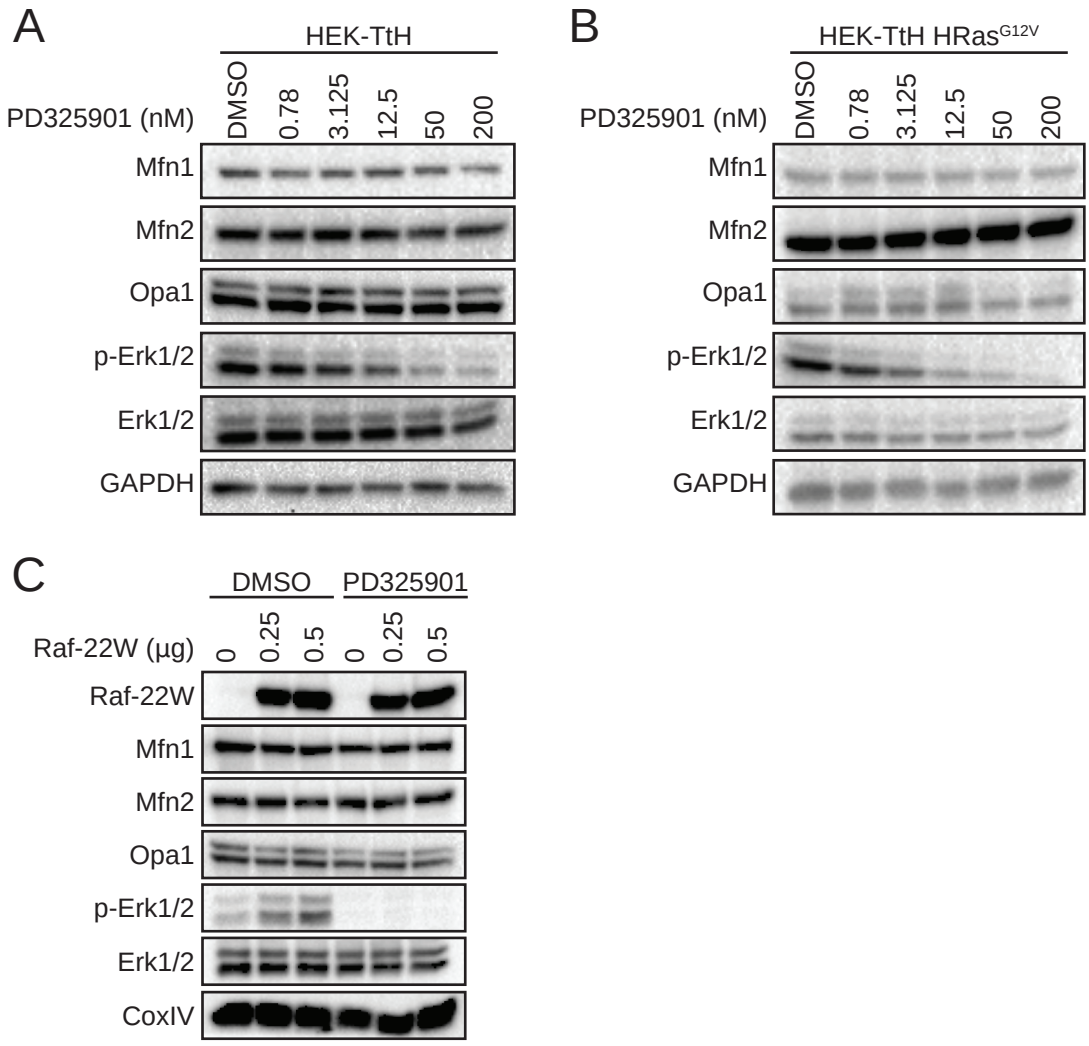

Figure S2. **Inhibition of Mek/Erk signaling does not affect levels of fusion proteins.** (Related to Figure 2) A. HEK-TtH cells, supplemented with 10% FBS, were treated with 0.78-200nM of the MEK inhibitor PD325901 and the levels of Mfn1 and Mfn2, the processing of Opa1 and the phosphorylation of Erk1/2 (Y202/T204) were analyzed by immunoblot. GAPDH: Loading Control. B. HEK-TtH cells stably expressing HRas^{G12V} were treated with 0.78-200nM of the MEK inhibitor PD325901 and the levels of Mfn1 and Mfn2, the processing of Opa1 and the phosphorylation of Erk1/2 (Y202/T204) were analyzed by immunoblot. GAPDH: Loading Control. C. HeLa cells were transfected with increasing amounts of active Raf-22W in the presence of DMSO or MEK inhibitor PD325901 and the levels of Mfn1 and Mfn2, the processing of Opa1 and the phosphorylation of Erk1/2 (Y202/T204) were analyzed by immunoblot. GAPDH: Loading Control.

Figure S3

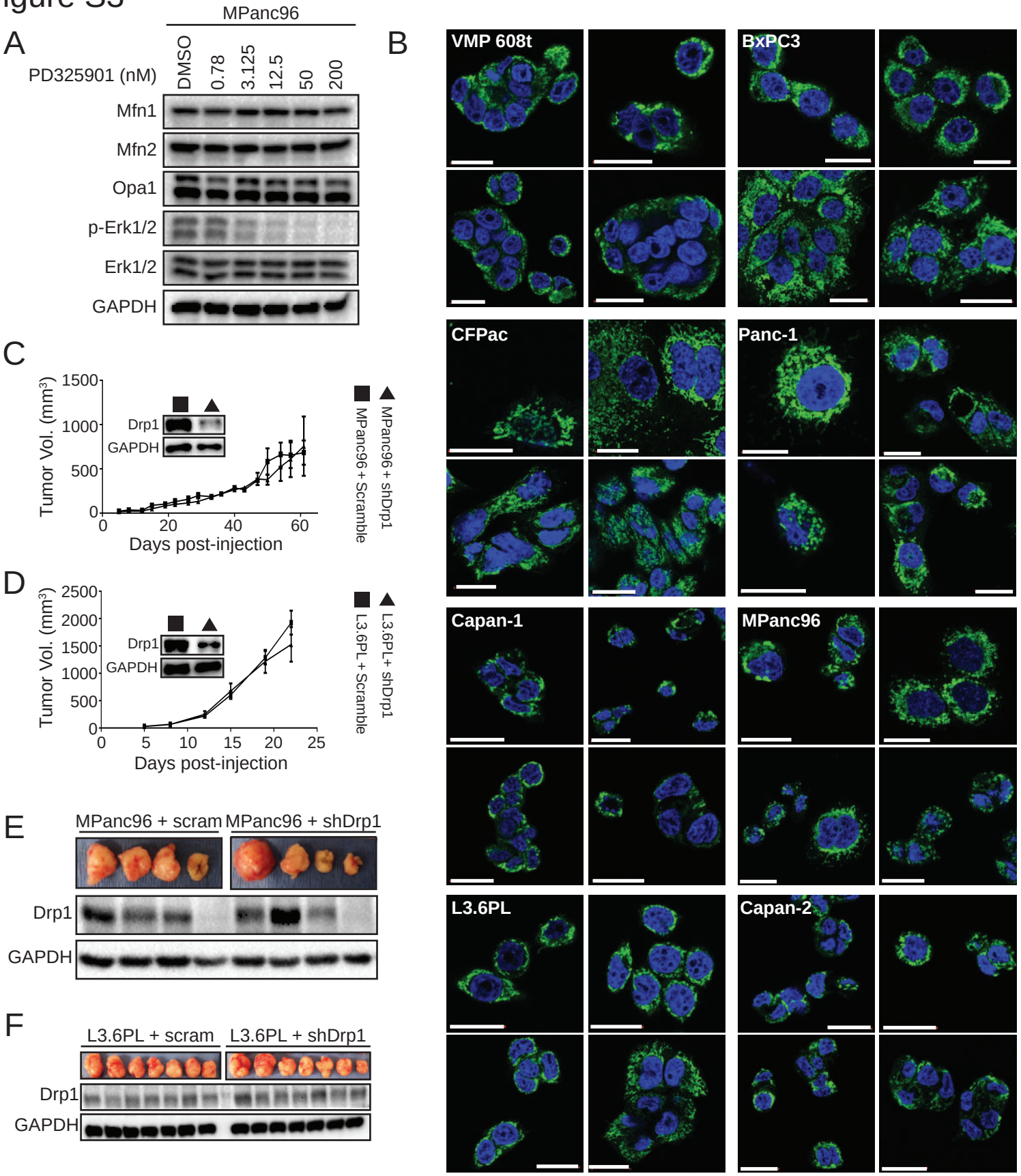

Figure S3. **Analysis of Pancreatic Cancer Cell lines.** (Related to Figure 4) A. MPanc96 cells were treated with 0.78-200nM PD325901 for 8 hours and the levels of the indicated proteins were analyzed by immunoblot. GAPDH: Loading Control. B. Confocal microscopy of the mitochondrial morphology of 8 patient-derived pancreatic cancer cell lines. Green: anti-Tom20; Blue: DAPI. 4 representative images of each cell line are shown. Scale Bar = 20µm. C-F. Xenograft growth of pancreatic cancer cell lines. MPanc96 (C & E) and L3.6PL (D & F) cells expressing an shRNA targeting either scramble control or Drp1 were analyzed by immunoblot then injected into Nude mice and tumor volume was measured over time. GAPDH: Loading controls. Error bars: S.E.M. of mean tumor volume.

Figure S4

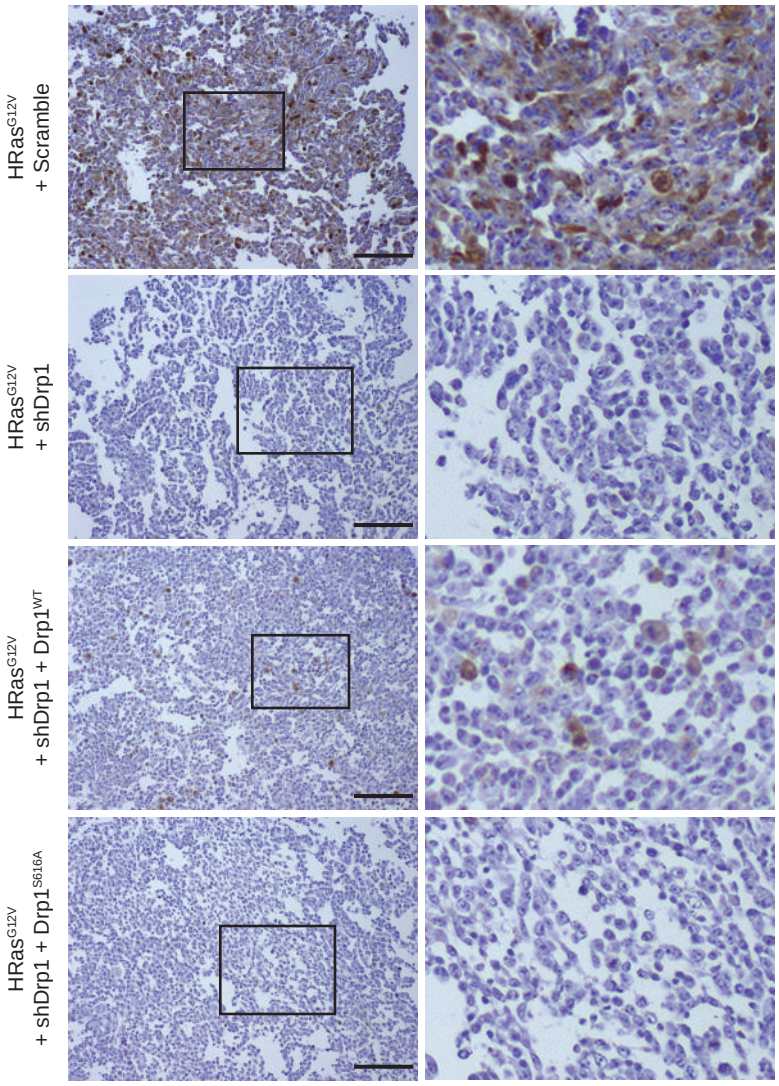

Figure S4. **Validation of the use of a phospho-specific S616 Drp1 antibody for immunohistochemistry.** (Related to Figure 5). HEK-TtH cells were engineered to express Flag-HRas^{G12V} plus an shRNA targeting either scramble control or Drp1 and then rescued with either vector, Flag-Drp1^{WT} or Flag-Drp1^{S616A}. Cell pellets were fixed in formalin, embedded in paraffin and sections were cut and stained using an antibody specific for p-Drp1 (S616). Left image: Scale bar = 100μm; Right image: enlargement of boxed area.

Figure S5

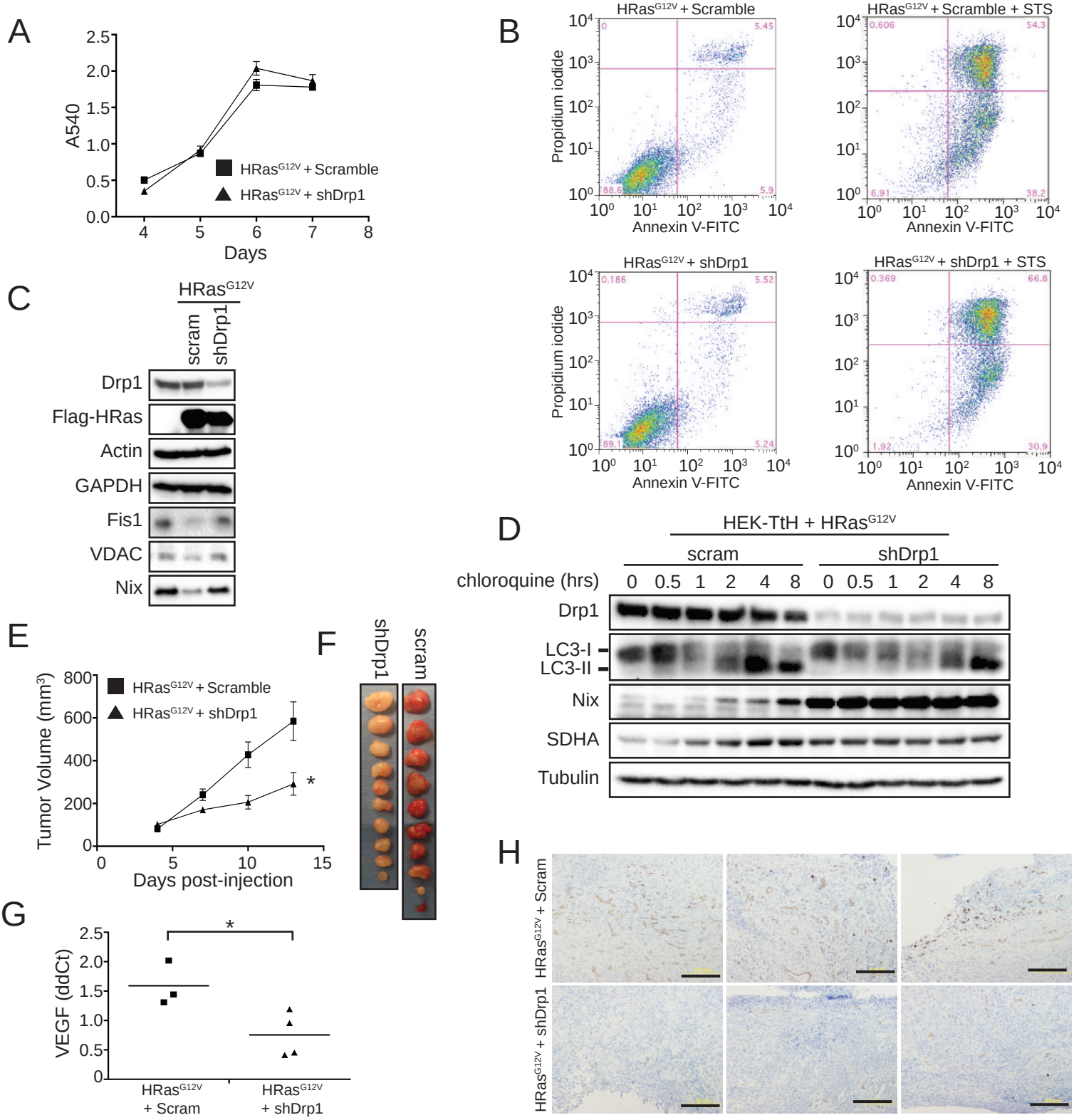

Figure S5. **Drp1 knockdown has no effect on proliferation or apoptosis, but decreases angiogenesis.** (Related to Figure 6) A. MTT assays were performed 4, 5, 6 and 7 days after seeding HEK-TtH cells expressing Flag-HRas^{G12V} plus an shRNA targeting either scramble control or Drp1. B. The same cell lines were left untreated or treated overnight with 1μM staurosporine (STS) then stained with FITC-conjugated Annexin V and propidium iodide and analyzed by flow cytometry. C. Immunoblot analysis of the indicated proteins from HEK-TtH cells with or without HRas^{G12V} and an shRNA targeting either scramble control or Drp1. D. HEK-TtH cells expressing HRas^{G12V} and an shRNA targeting either scramble control or Drp1 were treated with 100μM Chloroquine, harvested at the indicated timepoints and analyzed by immunoblot analysis of the indicated proteins. E. The indicated HEK-TtH cells were injected into Nude mice and tumor volume was measured over time *p<0.05. F. Tumors from were removed and photographed. G. RNA was isolated from 3 or 4 size-matched tumors and subjected to quantitative RT-PCR to determine the levels of VEGF. H. Portions of 3 size-matched tumors were formalin fixed and embedded in paraffin then sections were cut and stained for murine CD31. Scale bar = 200μm.

Supplemental Experimental Procedures

Plasmids

Two separate Drp1 shRNA sequences (5'-CAGGAGCCAGCTAGATATTAA) (Friedman et al., 2011) or (5' GGACTCTAAACAGGTTACTGA) were cloned into pSuperior-Retro-Neo/GFP or pSuperior-Retro-Puro plasmids (Oligoengine). pSuperior-Retro-Puro-scramble control, pBabe-Bleo HRas^{G12V}, pGEX-5X2-Drp1$^{518-736}$ and pBabe-Neo-Drp1^{K38A} were described previously (Hamad et al., 2002; Kashatus et al., 2011). pGEX-5X2-Drp1$^{518-736,\ S616A}$ and pGEX-4T3-hErk2^{R67S} were generated by site-directed mutagenesis, shRNA-resistant pBabe-Neo-Drp1-flag and pBabe-Neo-Drp1^{S616A}-flag were generated by cloning flag-tagged Drp1 (Frank et al., 2001) into pBabe-Neo then introducing silent mutations at the shRNA recognition sequence and the S616A mutation by site-directed mutagenesis. Mek-DD was generated from human Mek1 (Turski et al., 2012) by mutagenesis and Mek-DD and Raf-22W (Hamad et al., 2002) were cloned into pcDNA3.1. mito-PAGFP was received from Addgene (Plasmid 23348). pDsRed2-Mito was received from Clontech (Plasmid 632421). Transgenes and shRNAs were stably introduced into human HEK-TtH or HeLa cells by retroviral infection as previously described (O'Hayer and Counter, 2006). Transient transfections were performed using FuGENE 6 transfection reagent (Promega) according to the manufacturer's instructions. PD325901 (TSZ chem) was resuspended in DMSO (AMRESCO) at a stock concentration of 10mM and diluted with DMEM to reach the indicated final concentrations.

Recipes

RIPA buffer: 1% NP-40, 20mM Tris pH 8.0, 137mM NaCl, 10% glycerol, 2mM EDTA

SDS-Page sample loading buffer: 4% SDS, 100mM Tris pH 6.8, 2% 2-mercaptoethanol, 20% glycerol, 10µg/ml Bromophenol Blue

GST Elution Buffer: 100mM Tris pH 8.0, 120mM NaCl

Kinase Buffer: 20mM HEPES, 10mM MgCl2, 25mM β-glycerophosphate, 1mM Sodium orthovanadate, 100µM Dithiothreitol (DTT) and 100µM Adenosine triphosphate (ATP) with (Hot) or without (Cold) 1µCi γ-^{32}P-ATP

Antibodies

List of antibodies used in this study: α-Drp1, α-Opa1 (BD Transduction Laboratories), α-pS616-Drp1, α-β-tubulin, α-Erk1/2, α-pT202/Y204 Erk1/2, α-Mek, α-CoxIV, α-actin, α-GAPDH, α-BNIP3L/Nix, α-LC3A/B, α-SDHA, α-Mfn1, α-Mfn2, (Cell Signaling Technologies), α-Tom20, α-Raf, α-Fis1 (Santa Cruz Biotechnology), α-Flag (SIGMA), or α-VDAC/Porin (Calbiochem, EMD Millipore).

Real-time PCR

Total RNA was extracted from a small portion of size-matched frozen xenograft tumors using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. RNA concentration and quality was measured (BioTek Synergy 2 spectrophotometer) and samples were treated with DNase I (New England Biolabs) then inactivated. 1µg RNA per sample was reversed-transcribed (iScript cDNA synthesis kit, BioRad). Real-time PCR was performed on the Applied Biosystems StepOnePlus Real-Time PCR System employing SensiFast SYBR (Bioline) green detection chemistry. ΔΔCt was

calculated for each sample reaction using hβ2M gene as an internal control. The following primers were used: β2M forward (5'-CTATCCAGCGTACTCCAAAG), β2M reverse (5'-ACAAGTCTGAATGCTCCACT); VEGF forward (5'-AGGAGGAGGGCAGAATCATCA), VEGF reverse (5'-CTCGATTGGATGGCAGTAGCT). The real-time PCR conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec and 65°C for 30 sec. After the last PCR cycle, each sample was subject to thermal melting curve analysis to check for non-specific product formation.

Flow Cytometry

For apoptosis measurement the indicated HEK-TtH cells were harvested by trypsinization, washed with cold PBS and resuspended in 1X Binding Buffer (10mM HEPES, pH 7.4; 140mM NaCl; 2.5mM $CaCl_2$) at 10^6 cells/ml. 100µl of each cell suspension was transferred to a 5ml flow tube to which Annexin V-FITC (BD Pharmingen) and 15µg/ml Propidium Iodide (PI) (Sigma) were added. Cells incubated 15 minutes at RT in the dark, then 400µl of 1X Binding Buffer was added to each tube and cells were analyzed immediately on a FACSCalibur Benchtop Analyzer (Becton Dickinson/Cytek). For mitochondrial mass measurement the indicated cell lines were engineered to stably express mitochondrially-targeted YFP. Cells were then harvested by trypsinization, washed with cold PBS, resuspended in PBS at $5x10^6$ cells/ml and analyzed immediately on a FACSCalibur Benchtop Analyzer (Becton Dickinson/Cytek). For membrane potential measurement the indicated cells lines were harvested by trypsinization, counted and adjusted to a density of 10^6 cells/ml in full culture media. Cells were incubated with 50nM TMRE (Biotium) for 30 minutes at 37°C, 5% CO_2, then washed once with PBS, resuspended in PBS/0.2% BSA and analyzed immediately on a FACSCalibur Benchtop Analyzer (Becton Dickinson/Cytek).

Immunohistochemistry

For analysis of angiogenesis, FFPE blocks of size-matched HEK-TtH; $HRas^{G12V}$ and HEK-TtH; $HRas^{G12V}$; shDrp1 tumors were sectioned by the University of Virginia Research Histology Core for IHC as mentioned above, stained for mouse CD31-mouse (Abcam) and visualized on an Olympus BX51 microscope with a 20X objective.

Mitochondrial Stress Test

Oxygen consumption rate (OCR) was measured using a Seahorse XF24 Extracellular Flux Analyzer with the XF Cell Mito Stress Test Kit. Cells were seeded at $8X10^4$ cells per well in 100µl DMEM (Invitrogen 11965) containing 10% FBS and allowed to attach for 2 hours. 150µl DMEM-10% FBS was added per well and cells incubated overnight in 5% CO_2 humidified incubator. Prior to assay run, cells were changed into assay media, unbuffered DMEM (Invitrogen 12800) pH 7.4 and subjected to sequential injections of Oligomycin (1µM), FCCP (0.3µM), rotenone (1µM) and antimycin A (0.75µM). Spare respiratory capacity was calculated by dividing the OCR response to FCCP by the basal respiration, having subtracted the non-mitochondrial respiration previously. All values were normalized to cell number per wells setup in parallel.

Drug Treatments

PD325901 (TSZ chem) was resuspended in DMSO (AMRESCO) at a stock concentration of 10mM and diluted with DMEM to reach the indicated final concentrations. Staurosporine (ENZO) was resuspended in DMSO at a stock concentration of 1mM. Chloroquine diphosphate salt (MP Biomedicals) was resuspended in water at a stock concentration of 100mM.

MTT Assay

The described HEK-TtH cells were plated at 400 cells/well in 5 X 96-well plates and incubated for 72 hours. One plate was assayed every 24 hours by adding 15μl of 5mg/ml 3-(4,5-Dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma). After 4 hours at 37°C, medium was removed and cells were resuspended in 100μl DMSO. Absorbencies were recorded at 540nm (Martin and Clynes, 1993).

Supplemental References

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. (2001). The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev. Cell *1*, 515–525.

Friedman, J.R., Lackner, L.L., West, M., DiBenedetto, J.R., Nunnari, J., and Voeltz, G.K. (2011). ER tubules mark sites of mitochondrial division. Science *334*, 358–362.

Hamad, N.M., Elconin, J.H., Karnoub, A.E., Bai, W., Rich, J.N., Abraham, R.T., Der, C.J., and Counter, C.M. (2002). Distinct requirements for Ras oncogenesis in human versus mouse cells. Gene Dev. *16*, 2045–2057.

Kashatus, D.F., Lim, K.-H., Brady, D.C., Pershing, N.L.K., Cox, A.D., and Counter, C.M. (2011). RALA and RALBP1 regulate mitochondrial fission at mitosis. Nat. Cell Biol. *13*, 1108–1115.

Martin, A., and Clynes, M. (1993). Comparison of 5 microplate colorimetric assays for in vitro cytotoxicity testing and cell proliferation assays. Cytotechnology *11*, 49–58.

O'Hayer, K., and Counter, C. (2006). A genetically defined normal human somatic cell system to study ras oncogenesis in vivo and in vitro. Method Enzymol. *407*, 637–647.

Turski, M.L., Brady, D.C., Kim, H.J., Kim, B.E., Nose, Y., Counter, C.M., Winge, D.R., and Thiele, D.J. (2012). A novel role for copper in Ras/mitogen-activated protein kinase signaling. Mol. Cell. Biol. *32*, 1284–1295.