Supplemental Methods

Knockdown of Src and p27:

shRNA targeting Src (sh-Src; Clone IDs: TRCN0000023594, TRCN0000023595, TRCN0000023596, TRCN0000023597 or TRCN0000023598, Sigma) or scrambled shRNA (Open Biosystems) were prepared by individually transfecting the plasmids into 293FT cells (Life Technologies) using the Virapower lentiviral expression system according to the manufacturer's protocol. Target cells were infected in suspension while spinning at 1,000 rpm for 1hr. Puromycin was used to select for individual clones 48 hrs post-infection with the knock-down verified by western blot.

p27 knock-down: pSIREN retroviral vectors containing p27 or luc shRNA, and GFP (generously provided by Yukiko Gotoh, Institute of Molecular and Cellular Biosciences, University of Tokyo, Japan) were transfected into Phoenix Eco cells (Invivogen) using Lipofectamine 2000 (Life Technologies). Retroviruses were collected after 72hrs and concentrated by centrifugation. D2A1 and D2.0R cells were transduced in suspension while spinning at 1,000 rpm for 1hr and were flow sorted for GFP.

Caspase 3/7 activity

Caspase 3/7 activity was performed using the Caspase-Glo® 3/7 Assay (Promega). Cells were seeded in 3D cultures ± inhibitors as previously described and assayed after 48 hrs. Briefly, plates and reconstituted caspase 3/7 reagent were allowed to equilibrate to room temperature (RT), reagent was then added to wells at a 1:1 ratio and incubated for 30 minutes at RT. Fifty microliters were removed from the culture plates and transferred to a white 96 well plate and luminescence was measured using a Victor plate reader (PerkinElmer). Wells with media + caspase 3/7 reagent only served as blank controls.

Cytotox-Glo cytotoxicity assay

MCF10A (ATCC), human mammary epithelial cells (Lonza) and D2.0R cells were seeded on BME+Col-1 ± AZD0530, AZD6244 or their combination as previously described with the exception of using white 96-well plates. Forty-eight hrs later, cells were assayed according to the manufacturer's protocol. Briefly, 25ul of cytotox-Glo reagent was added/well, incubated for 10 min and luminescence was read to assess cytotoxicity followed by addition of 25 ul of lysis reagent, 10 min incubation and luminescence was read for assessment of total cell number.

Q-RT-PCR assays

PCR primers used: 5'-TCAGCGCAAGTGGAATTT-3' and 5'-GGGCCTGTAGTAGA ACTCG-3' for p27, 5'-CATGTTTGCAGGGAAGGATGCCA A-3' and 5'-AACCAGACAGTTGAGGCCACCTTA-3' for Src, 5'-CCACAAAGGGA GCATCAGTTA-3' and 5'-CACCACTGAGAATGAGGAAGAC-3' for c-Yes, 5'-TCT GAGACAGAAGCGTG TTATT-3' and 5'-GGCACAGGAGCAGCTATTTA-3' for Fyn, 5'-CATCTCTCCTCGCATCACTTT-3' and 5'-GGATCTCCCAGGCATCTTTATC-3' for Lyn, 5'-CATGAGTTGAGAGACCCAGAAA-3' and 5'-GGTTGGGAAGTAGGCT GAAA-3' for Fgr, 5'-T GTGTGTCAAGCTGGAGAAG-3' and 5'-CTGTATGGAGTT GCGGTCTATC-3' for Frk, 5'-GTCACCAGAGAGCCCATTTAC-3' and 5'-GGACAA CCTGCTACCTTCATC-3' for Blk and 5'-TGCTGGACCAAACACAAACG-3', 5'-CC ATCCAGCCATTCAGTCTTG-3'for cyclophyllin B. Relative gene expression was calculated by the delta-delta Ct method with cyclophyllin B as the reference gene. Average and standard error from 3 independent experiments were calculated.

Collagen quantification in fibrotic lungs

Masson trichrome staining was performed on 6um lung sections from CD1/NU/NU mice instilled with Ad-TGFb$^{223/225}$, aged for 3 weeks and then injected with 1x10^{6} D2.0R GFP and then gavaged 24hrs later with vehicle (n=4) or AZD0530 (50mg/kg) (n=8) once daily for 21 days. Sections were then scanned at 40X using the Aperio Scanscope XT scanner to generate digital images for quantification of the collagen using Definiens Tissue Studio™ software (v 3.0, Definiens), with the exclusion of the mainstem bronchi. An algorithm based on color separation was developed to identify and quantify collagen present in the tissue section. The data were expressed as the percentage of the area of collagen divided by the lung parenchymal tissue reference area.

Ex-vivo PuMA assay

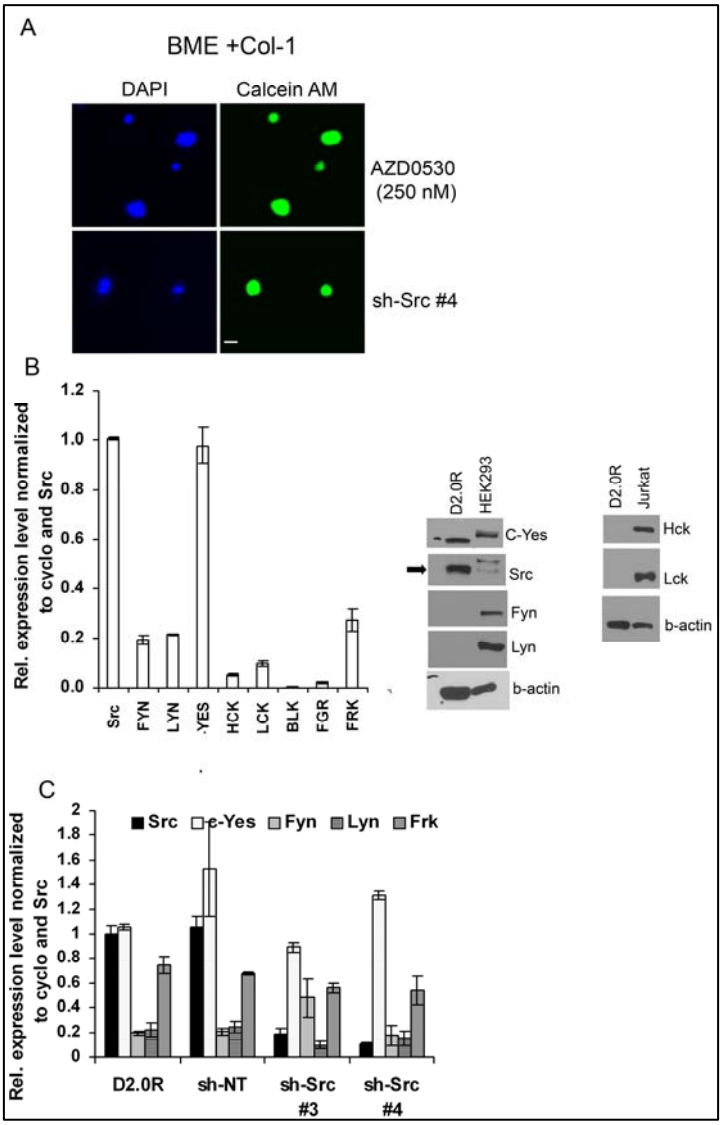
D2.0R-GFP cells (2x10^{5}) were tail vein-injected in CD1/NU/NU mice fibrotic or non-fibrotic lungs. Fifteen minutes post-injection, mice were euthanized by CO_2 inhalation. The exposed trachea was infused with 1.2 ml of culture medium/agarose solution. Inflated lungs were removed and placed in cold PBS at 4°C for 20 min to solidify the agarose. Sections (1–2 mm in thickness) were cut from each lobe. Four lung sections were placed on a 1.5 × 0.7-cm piece of sterile Gelfoam (Pfizer-Pharmacia & Upjohn Co.) that had been pre-incubated in culture medium. AZD0530, AZD6244 or both were immediately added to the culture media at day 0, 5 hours after tail-vein injections. Lung sections were incubated at 37°C in humidified conditions with 5% CO2. Fresh culture medium ± inhibitors was replaced every 48hrs. The experiments were performed in triplicates, using 12 lung slices per condition.

Statistical analysis of SCOM experiments

Pixel counts of GFP-positive metastatic lung lesions were quantified for the entire surface area of each lung. Lesions with >10 and <1,000 pixels represented individual tumor cells; lesions with >1,000 pixels represented multicellular metastatic lesions. Total tumor burden/lung was represented by the total number of pixels detected on the entire surface of each lung. Average tumor burden/lung was represented as the mean size of the metastatic lesions. Single-cell lesions (<1,000 pixels) and multicellular clusters (>1,000 pixels) were quantified, and the distribution of the sizes of metastatic lesions (represented by $pixels^2$ per lesion) per lung was calculated. Statistical analyses of differences in the distribution of tumor burden and metastatic lesion size between groups used unpaired Student's t-test for two-group comparisons, with $p<0.05$ considered significant.

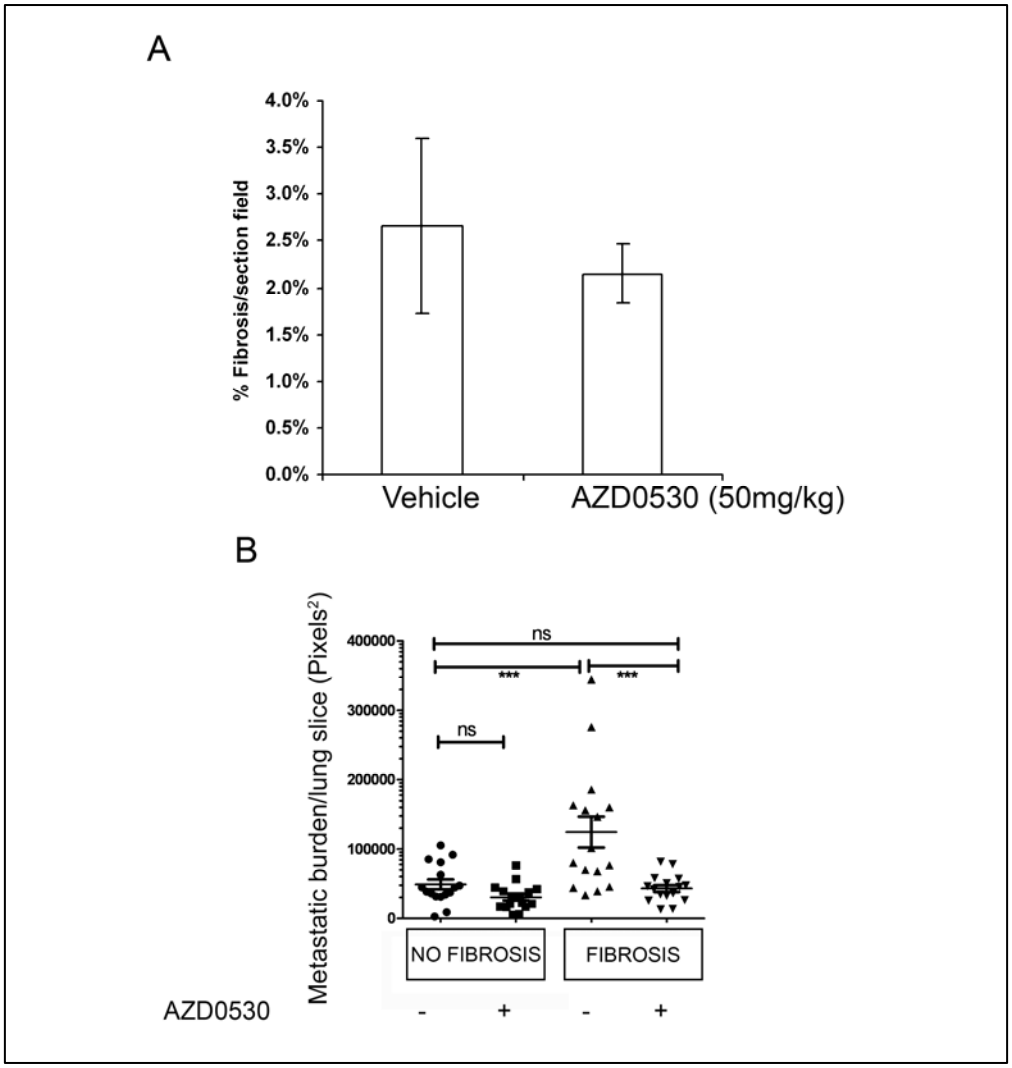
Supplemental Figures

Supplemental Figure 1



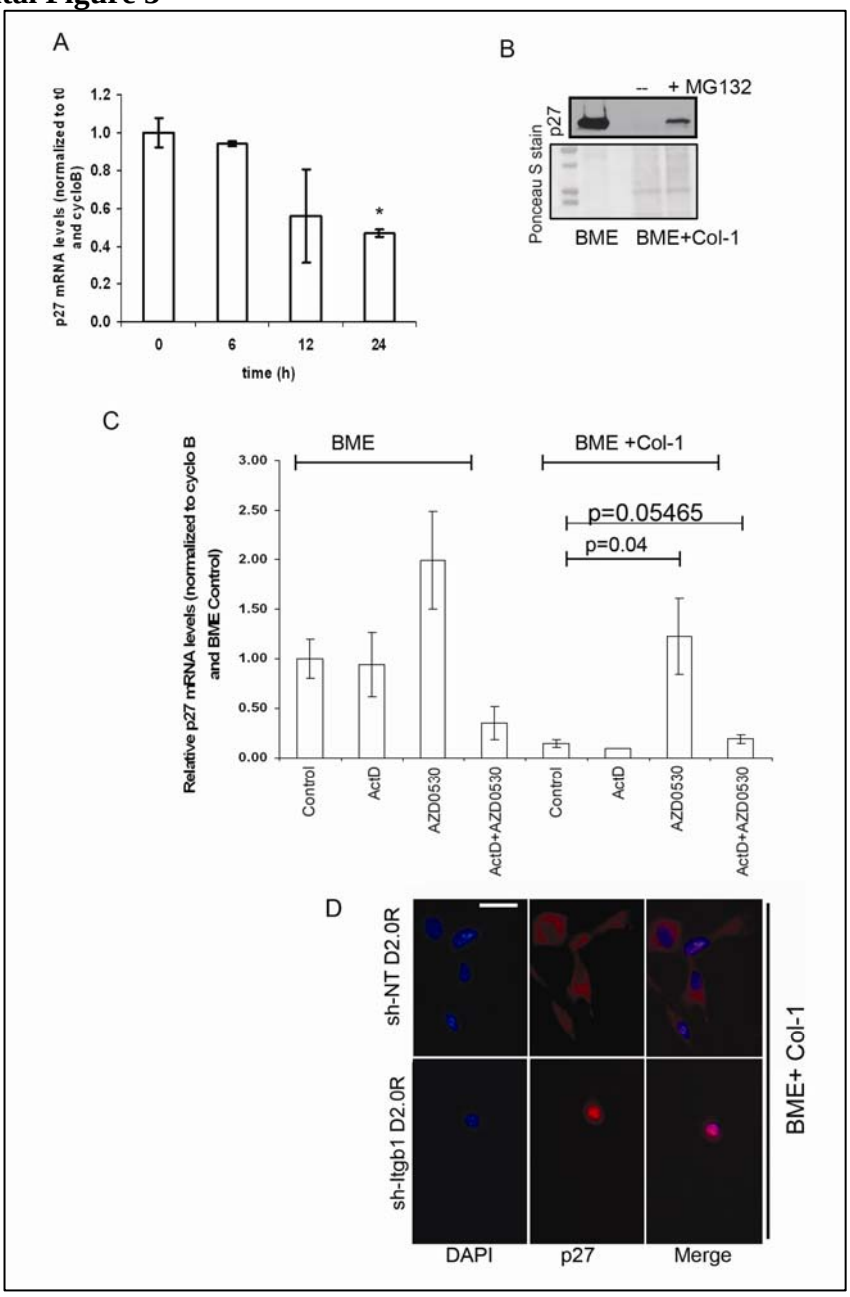
Supplemental Figure 1: (A) Calcein AM staining (green) of AZD0530 treated and sh-Src D2.0R cells seeded on BME+Col-1 for 72h and mounted with DAPI (blue) containing medium. Scale bar: 25um. (B) mRNA and protein levels (right) of SFK members in D2.0R cells seeded on BME+Col-1 for 48 hrs, HEK-293 and Jurkat cells serving as positive controls. (C) mRNA levels of SFKs in D2.0R, D2.0R sh-NT and D2.0R sh-Src (Clones #3 and #4).

Supplemental Figure 2



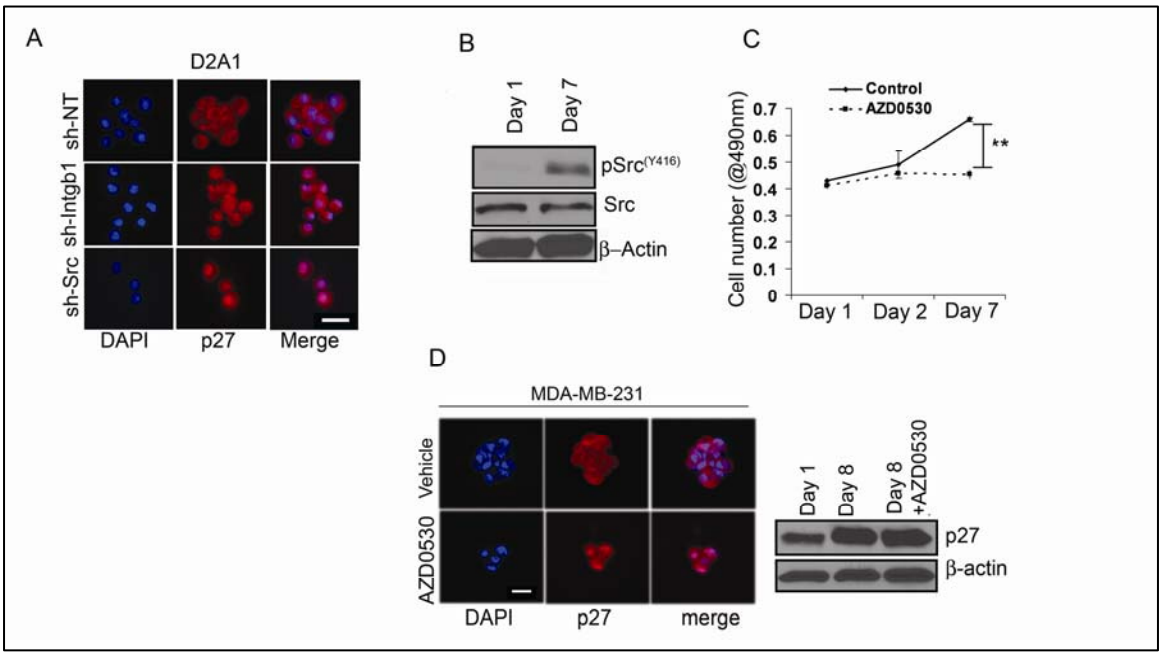
Supplemental Figure 2: (A) quantification of collagen deposition by trichrome staining in lungs sections from CD1/NU/NU mice instilled with Ad-TGF$^{223/225}$ and tail vein injected with 1 x 10^6 D2.0R-GFP cells and gavaged 24 hrs later with vehicle or AZD0530 (50mg/kg) once daily for 21 days. (B) Tumor burden of ex-vivo lung slices from CD1/NU/NU mice receiving Ad-empty (no fibrosis) or Ad-TGFb $^{223/225}$ (fibrosis) and tail vein-injected with 2x 10^5 D2.0R-GFP cells, plated on gelfoam and treated with 250 nM AZD0530 or vehicle for 21d. ***, $p<0.001$ and ns: not significant.

Supplemental Figure 3



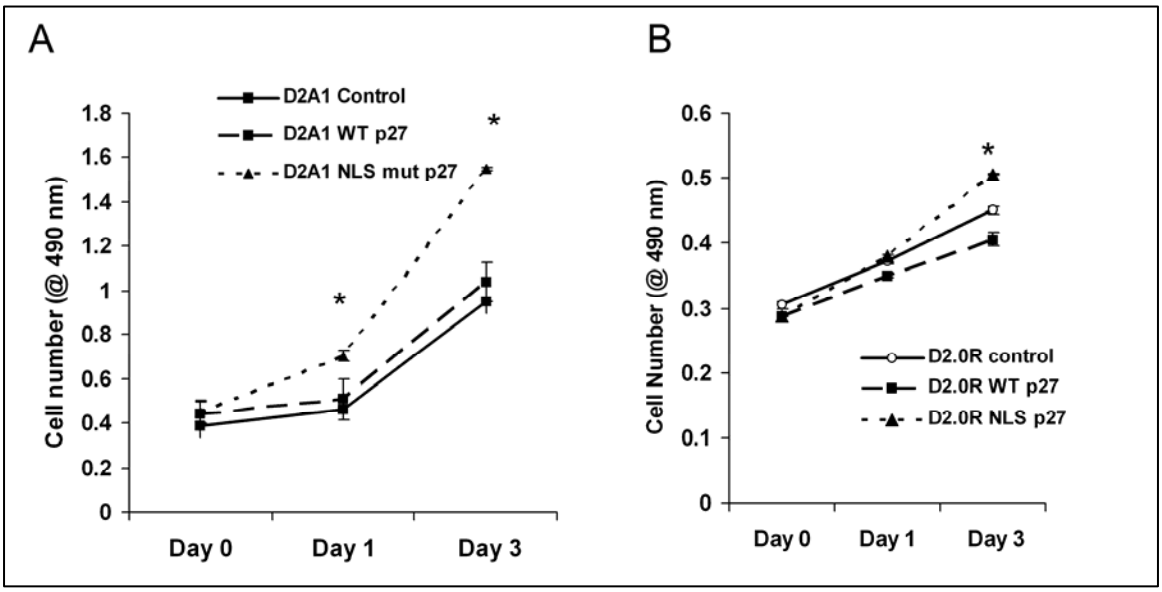
Supplemental Figure 3: (A) p27 mRNA levels in D2.0R cells determined by Q-PCR at 0, 6, 12 and 24h post-seeding on BME+Col-1 *, p= 0.0204 compared to time 0. (B) p27 protein levels in D2.0R cells extracted 18h post-seeding on BME, and D2.0R cells seeded for 12h on BME+Col-1 and treated with MG132 for 6h, Ponceau S stain shown as loading control. (C) p27 mRNA levels of D2.0R cells seeded on BME or BME+Col-1 for 24h and treated ± 250 nM AZ0530 ± Actinomycin D. Values are normalized to p27 levels in untreated D2.0R cells seeded on BME for 24h. (D) p27 localization in sh-NT and sh-Itgb1 D2.0R cells seeded on BME+Col-1 for 72h and counterstained with DAPI (20x magnification), Scale bar: 25um

Supplemental Figure 4



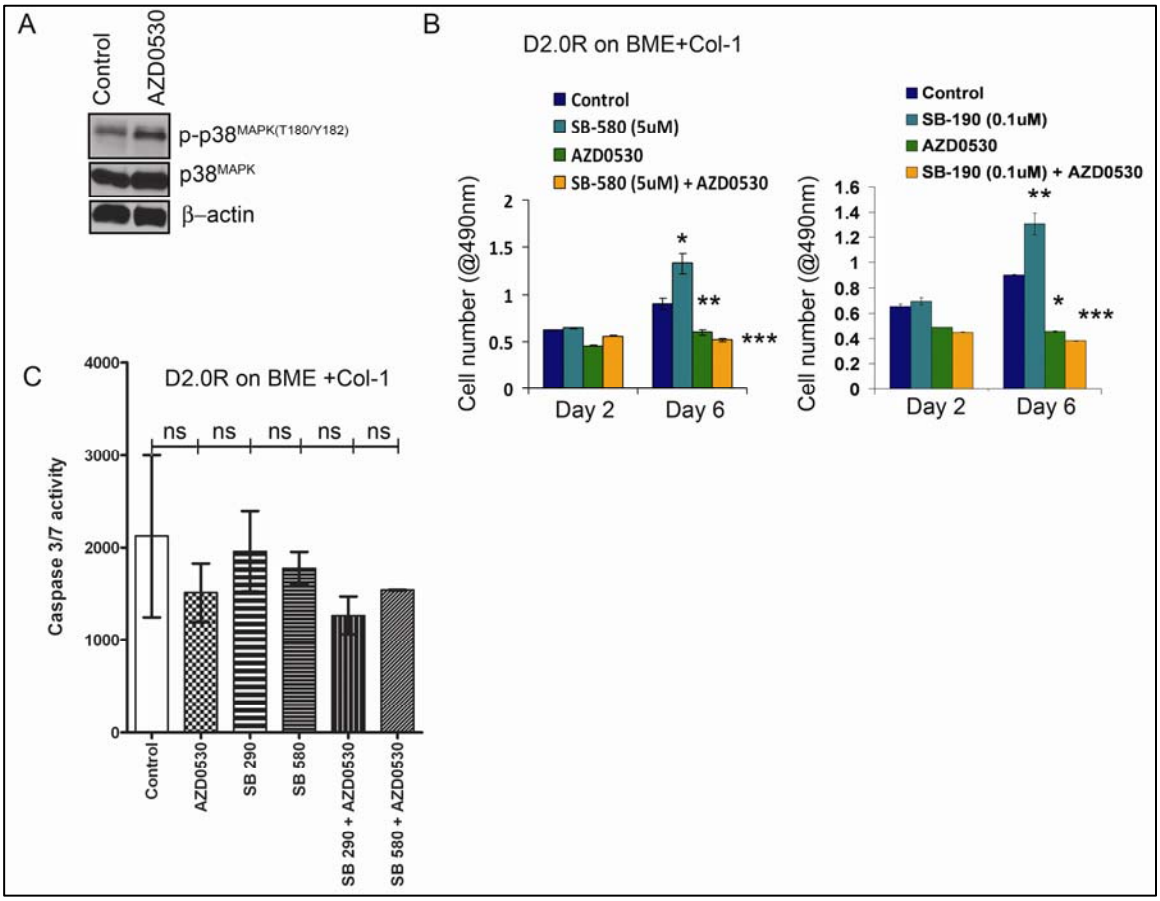
Supplemental Figure 4: (A) p27 immunofluorescence staining of sh-NT, sh-Intgβ1 and sh-Src #1 D2A1 cells seeded for 12d on BME, scale bar= 25um; (B) Phospho and total Src levels of MDA-MB231 cells seeded on BME on d1 and d8. (C) MTS assay of MDA-MB231 cells seeded on BME and treated with 250 nM AZD0530 or vehicle.**, p=0.0062; (D) p27 immunofluorescence staining and protein levels by western blot of MDA-MB231 cells seeded on BME treated with 250nM AZD0530 or vehicle for 8 days, scale bar=25um

Supplemental Figure 5



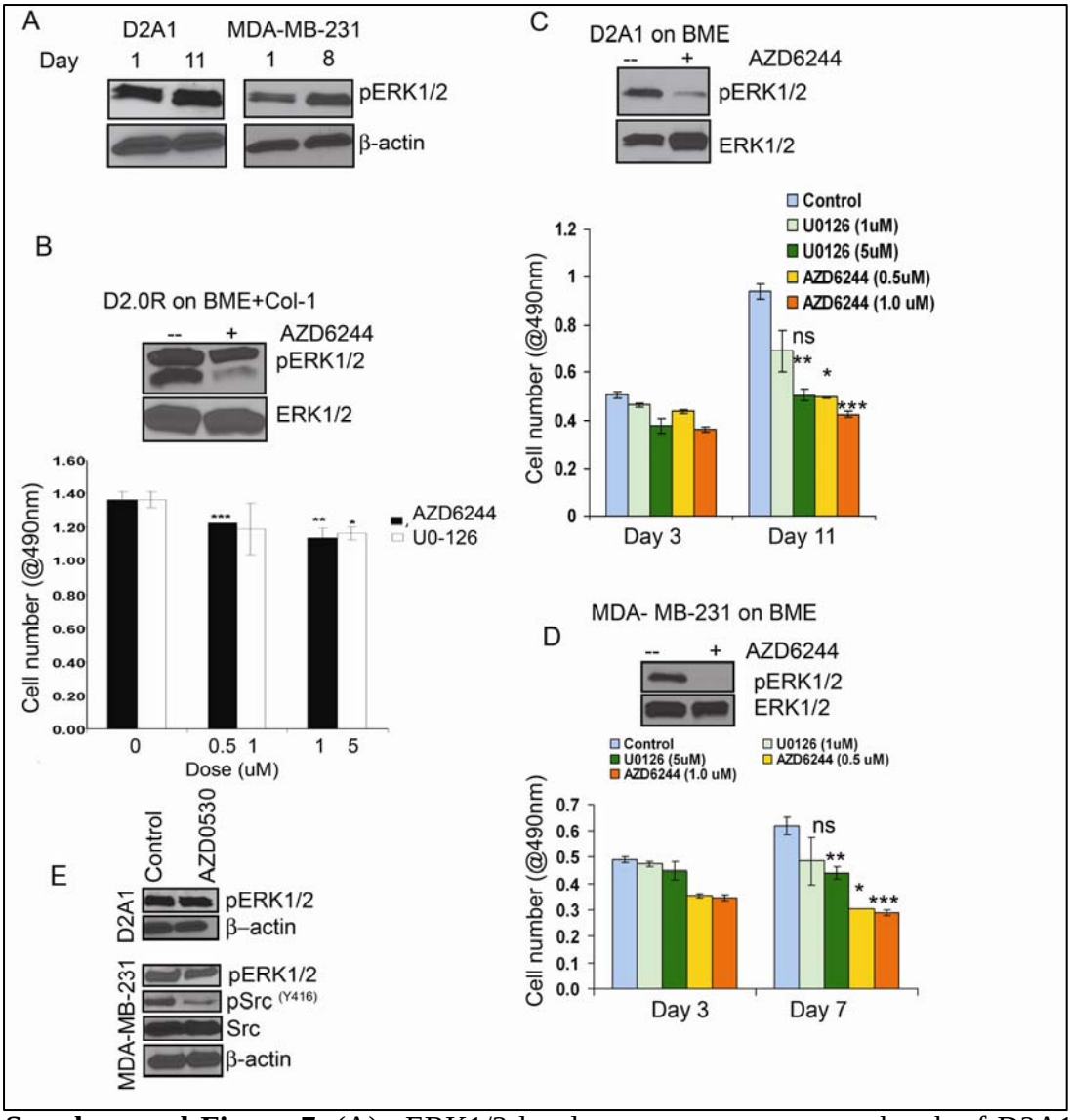
Supplemental Figure 5: MTS assay of D2A1 (A) and D2.0R (B) cells transfected with empty vector (control), wt p27 or p27 deleted-NLS and seeded on BME+Col-1 24h later. * indicates $p<0.05$, compared to control cells.

Supplemental Figure 6



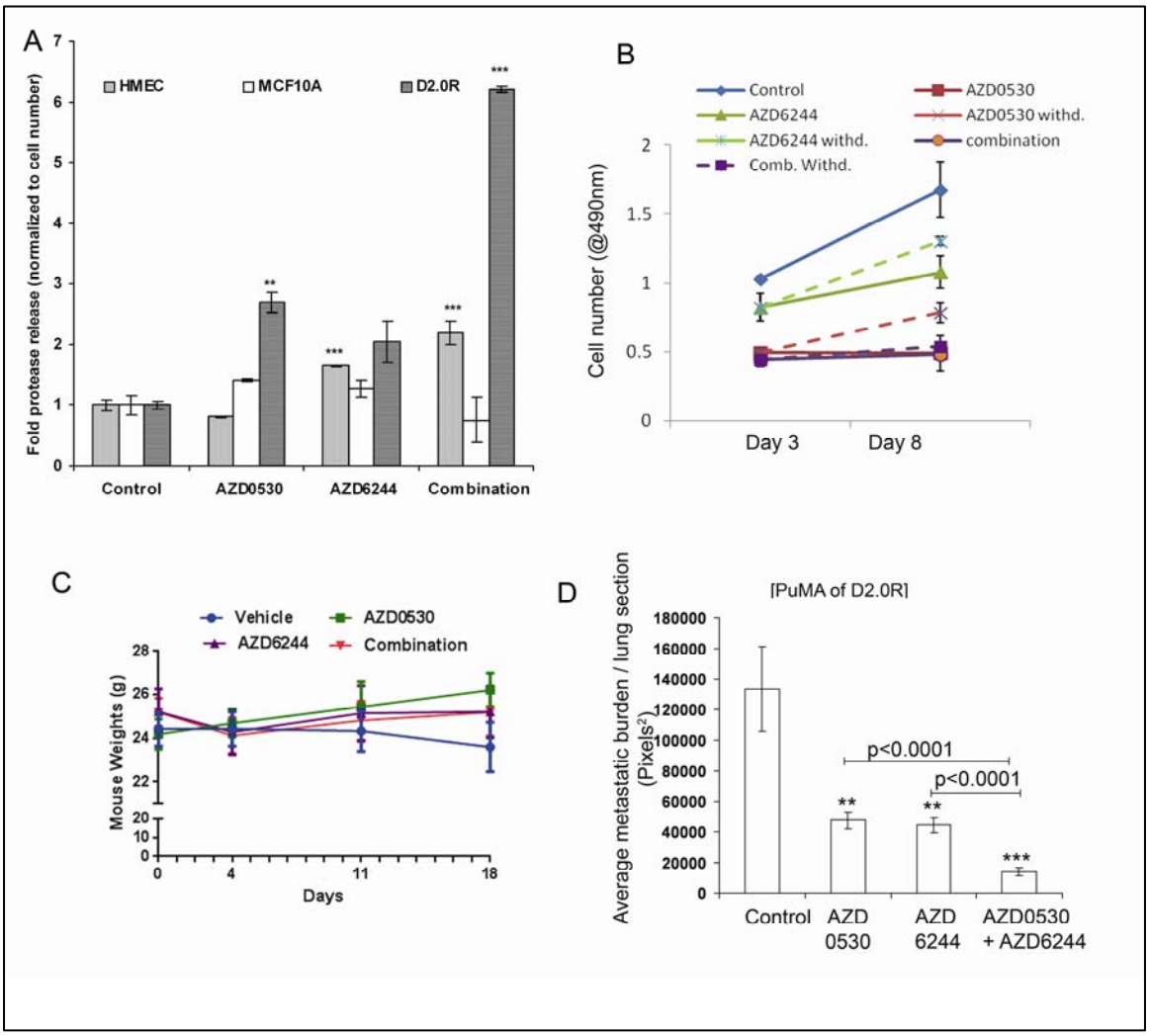
Supplemental Figure 6: (A) Phospho and total p38MAPK levels in control and AZD0530-treated D2.0R cells (250nM) on BME+Col-1 at 72h. (B) MTS values of D2.0R cells on BME+Col-1 treated with SB203580 (left) (*, p=0.0134, **, p=0.0089 and ***, p=0.0005 compared to vehicle-treated D2.0R cells), and SB202190 (right) ± AZD0530 (250 nM) (*, p=0.0294, **, p=0.0127 and ***, p=0.005 compared to vehicle-treated D2.0R cells) (C) Caspase 3/7 activity of D2.0R cells treated as in (B) at 48h.

Supplemental Figure 7



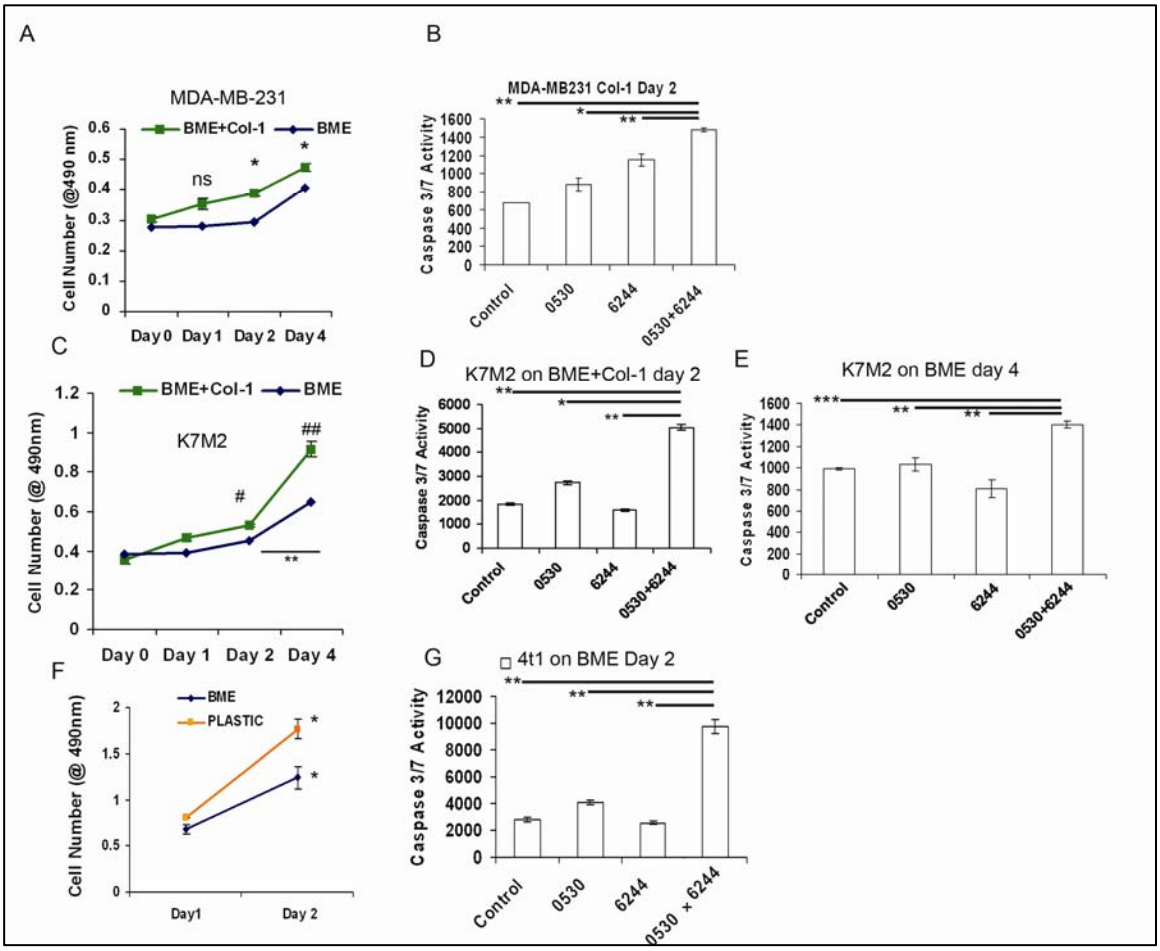
Supplemental Figure 7: (A) pERK1/2 levels upon spontaneous outbreak of D2A1 and MDA-MB231 cells on BME. (B) MTS values of D2.0R cells on BME+ Col-1 ± U0-126 or ±AZD6244 (*, p=0.0441, **, p=0.0226 and ***, p=0.0003 compared to vehicle-treated D2.0R cells); inset: pERK1/2 levels in D2.0R cells on BME+Col-1 upon treatment with AZD6244 (1000nM) for 72h. (C) MTS values of D2A1 (ns=not significant, *, p= 0.0463, **, p=0.0270 and ***, p=0.0161 compared to vehicle-treated D2A1 cells) and (D) MDA-MB231 on BME ± U0-126 or ±AZD6244 (ns=not significant, *, p=0.0463, **, p=0.0270 and ***, p=0.0161 compared to vehicle-treated MDA-MB231) with respective insets representative of pERK1/2 levels in above cells treated with AZD6244 (1000nM) for 11 (D2A1) or 7 (MDA-MB-231) days, respectively. (E) Unaltered pERK1/2 levels in AZD0530-treated D2A1 (11 days) and MDA-MB231 cells (8 days) on BME. Note: the lane for control day 11 D2A1 cells in panel E is the same as the lane for D2A1 on day 11 in panel A; panel E contains the additional contiguous lane of the AZD0530 treated cells (see suppl. figure 10 for full unedited gel).

Supplemental Figure 8

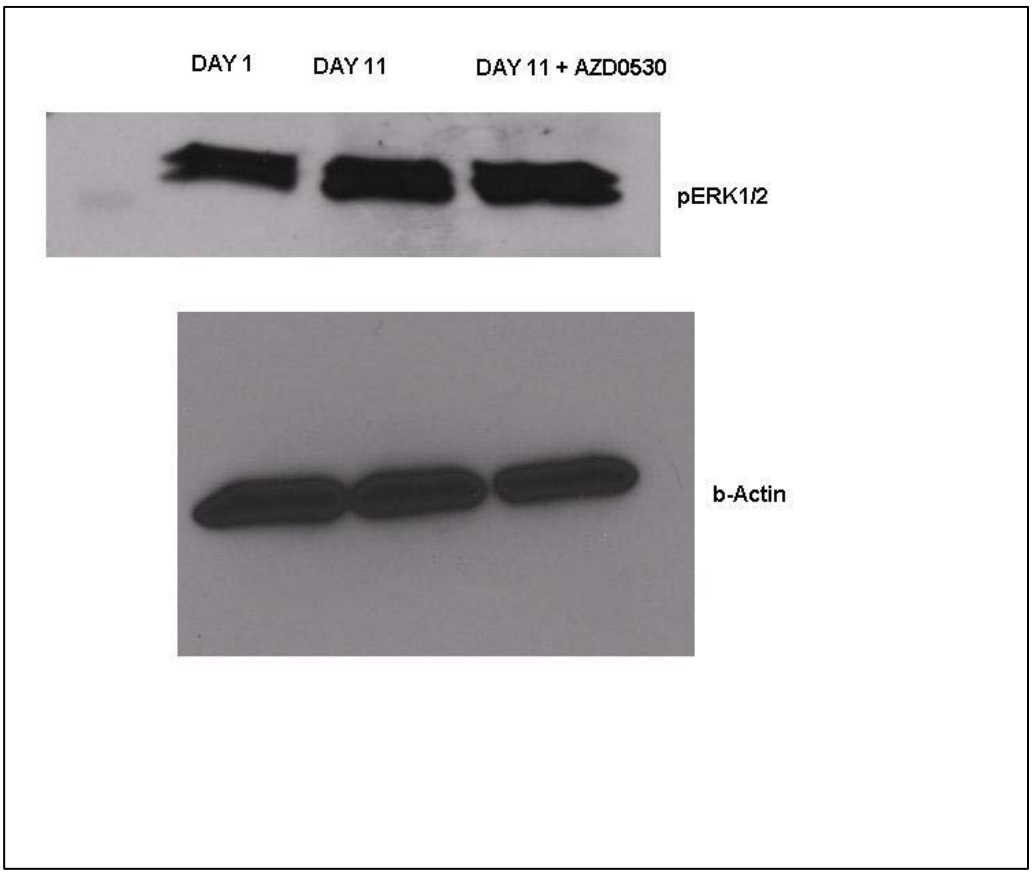


Supplemental Figure 8: (A) Cytotoxicity assay of AZD0530, AZD6244 or their combination for HMEC and MCF10A cells compared to D2.0R cells on BME+Col-1 at 48hrs, ** and ***, indicate p<0.01 and 0.001, respectively compared to control vehicle-treated cells (B) MTS values for D2.0R cells treated with AZD0530, AZD6244 or AZD0530+AZD6244 either for either 8 days, or for 3 days followed by vehicle for an additional 5 days (withdrawal). (C) Weights of CD1/NU/NU mice instilled with Ad-TGF$^{223/225}$, injected with 1x10^{6} D2.0R GFP cells and gavaged with AZD0530, AZD6244 or their combination once daily for 21 days. (D) Metastatic burden per lung section of CD1/NU/NU mice instilled with Ad-TGF$^{223/225}$, injected with 2x10^{5} D2.0R GFP cells and lungs were removed 15 min later, and sections were floated on gelfoam and treated 5 hrs later with AZD0530 (250nM), AZD6244 (500 nM) or their combination for 21 days. ** and ***, indicate p<0.01 and p<0.001, compared to control vehicle treated lung sections.

Supplemental Figure 9



Supplemental Figure 9: MTS values for MDA-MB-231 (A) and K7M2 (C) cells seeded on BME or BME +Col-1 and highly metastatic 4T1 (F) seeded on plastic or BME where they readily proliferate. Caspase 3/7 activity levels in MDA-MB231 (B), K7M2 (D) cells seeded on BME+Col-1, or K7M2 seeded on BME (E), or 4T1 cells seeded on BME (G) and treated with vehicle, AZD0530, AZD6244 or both. For MTS panels (ns: not significant *, $p<0.05$ on Col-1 compared to day 0). **, $p=0.0019$ for K7M2 on BME only comparing day 4 to day 2; # and ##, $p<0.05$ and $p<0.01$ compared to day 0 on BME+Col-1, *, $p<0.05$ in (F) comparing day 2 to day 1. For caspase 3/7 panels, (* and **, indicate $p<0.05$ and 0.01, respectively).



Supplemental Figure 10: Western blot of pERK1/2 and beta actin (loading control) levels in D2A1 cells seeded on BME at day 1, day 11 and day 11+AZD0530 (250 nM) showing activation of ERK1/2 upon spontaneous outbreak and no change to these levels upon AZD0530 treatment (lanes 1 and 2 are in suppl. Figure 7A; lanes 2 and 3 are in suppl. Figure 7E).