Materials and Methods

Mice - intrasplenic inoculations and drug treatments

The AAALAC-accredited Institutional Animal Care and Use Committees of the Veteran's Administration Pittsburgh Health System approved all animal studies and procedures. Seven-week-old male NOD/SCID gamma mice (00557, The Jackson Laboratory, Bar Harbor, ME) were used. After anesthetizing with ketamine/xylazine and suppressing pain with long-acting buprenorphine, sterile surgery exposed the spleen. Half a million viable PCa cells were injected into the spleen using a 27-gauge needle. The omentum was closed with a running stitch of absorbable suture and the skin wound with metal wound clips. Paclitaxel (Fresenius Kabi, Lake Zurich, IL) was administered at 10mg/kg body weight by i.p. every 2 days for a total 5 rounds from 2.5 weeks post-injections. After 5 weeks the mice were euthanized using a carbon dioxide chamber consistent with AVMA Guidelines on Euthanasia.

Cell lines, fresh human hepatocytes and human colon tumor tissues

DU145 (both variants herein termed DU-L and DU-H) and PC-3 human PCa cell lines were purchased from American Type Culture Collection (Manassas, VA, USA) and transfected with red fluorescent protein (RFP) as previously described (48). DU145 (both DU-L and DU-H) and PC-3 were maintained in DMEM and F-12k media, respectively. Human primary hepatocytes were from excess pathological specimens from therapeutic partial hepatectomies, sourced from the NIDDK-funded Liver Tissue and Cell Distribution System (LTCDS) with the procurement core directed by Dr. David Geller at the University of Pittsburgh, (NIH Contract # HHSN276201200017C). Paired colon cancer and liver metastases tissues were obtained from University of Pittsburgh, Tissue and Research Pathology Services/Health Sciences Tissue Bank which receives funding from P30CA047904. The use of these cells and slides was approved by the University of Pittsburgh IRB as exempted.

Co-culture of prostate cancer cells and human hepatocytes

On the same day as liver resection (day 0), the human primary hepatocytes (1×10^6 cells/well) were seeded into 6-well plates coated with 1% (~3.5µg/ml) rat tail collagen I and fitted with 22×22mm coverslips (for imaging) in HMM™ Hepatocytes Maintenance Medium supplement with gentamicin (50µg/ml), amphotericin (50µg/ml), insulin (0.1µM) and dexamethasone (0.1µM) (Lonza, Malpleton, IL). On day 1, RFP labeled DU145 or PC3 cells were harvested with trypsin and suspended in HMM™. PCa cells (2×10^4 cells/well) were then seeded onto the hepatocytes monolayer or alone. Medium was replenished on day 1, 2 and 4. On day 5, co-cultures were harvested for flow assay or fixed for immunostaining.

PD153035-induced prostate cancer epithelial transition

DU145 cells were harvested with trypsin and seeded at a density of 3×10^5 cells/well onto the 6-well plates, fitted with 22×22mm coverslips, in complete growth medium. Cells were cultured overnight and then incubated with media containing 500nM PD153035 (EMD Millipore) for 48-72 hours to induce an epithelial transition. Cells were rinsed off PD153035 and incubated with growth medium for 2 hours to recovery EGFR activity prior to further treatment.

Immunofluorescence microscopy

Cells on coverslips were fixed on the indicated days (see legends) in 3.7% formaldehyde/PBS, and assessed using standard immunofluorescence procedures. Confocal images were obtained on an Olympus upright Fluoview 2000 confocal microscope (Center for Biologic Imaging, University of Pittsburgh, supported by NIH #1S10OD019973-01) using a 60x (UPlanApo NA=1.42) or 20x (UPlanSApo NA=0.85) objective. The primary antibodies used were rabbit anti-human cleaved caspase-3 (9661, Cell Signaling Technology), mouse anti-human E-cadherin (13-5700, Life Technology). Secondary antibodies used were goat anti-mouse Alexa Fluor® 488 or goat anti-rabbit Alexa Fluor® 594 or 647 (Life Technologies). DAPI was applied for nuclei staining. The level of cleaved caspase-3 in tumor cells was determined using the MetaMorph software (Molecular Devices, LLC). The fluorescence intensity of red (cleave caspase-3) was normalized to DAPI.

Click-iT EdU proliferation assay

Proliferation was assessed using the Click-iT EdU Alexa Fluor® 647 Imaging Kit (Life Technologies). Cells were exposed to 10 μM EdU for 4 hours or indicated duration (PD153035 induced cells) or 16 hours (co-culture cells) prior to drug exposure or fixation. EdU was detected according to the manufacturer's instructions.

Flow cytometry

Cells were rinsed with warm PBS and harvested with a non-enzyme cell dissociation buffer (Life Technology). After centrifugation, cells were fixed with 3.7% formaldehyde in PBS for 15 minutes, rinsed with 0.1% PBSA, blocked with 2% BSA/PBS for 30 minutes. Five microliter Alexa Fluor® 647 anti-human CD324/E-cadherin (324112, Biolegend) was added per 100μl cell suspension for 30 minutes. For cleaved caspase-3 staining, fixed cells were permeablized in 0.1% Triton X-100 for 15 minutes, blocked and incubated with goat anti-human cleaved caspase-3 antibody (Cell Signaling Technology) for 30 minutes. A goat anti-rabbit Alexa Fluor® 488 secondary antibody (Life Technology) was then applied for 30 minutes. All procedures were performed at the room temperature and data was analyzed with FlowJo.

Western blotting

Cells were lysed with RIPA buffer containing protease inhibitors cocktail and phosphatase inhibitor cocktail 2 and 3 (Sigma Aldrich). Protein concentrations were measured with BCA™ protein assay kit (Fisher Scientific) and 30μg total protein was loaded for each well. Primary antibodies used were E-cadherin (24E10) rabbit monoclonal antibody (mAb), cleaved capase-3 (Asp175) rabbit polyclonal (pAb) cleaved PARP (Asp214) (D64E10) XP® rabbit mAb, phospho-Akt (Ser473) (D9E) XP® rabbit mAb, Akt(pan) (11E7) rabbit mAb, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® rabbit mAb, p44/42 MAPK (Erk1/2) (137F5) rabbit mAb, phospho-SAPK/JNK (Thr183/Tyr185) (G9) mouse

mAb, APK/JNK (56G8) rabbit mAb, phospho-p38 MAPK (Thr180/Tyr182) (12F8) rabbit mAb, p38α MAP Kinase (7D6) rabbit mAb, phospho-stat3 (Tyr705) (D3A7) XP® rabbit mAb, phospho-Jak2 (Tyr1007/1008) (C80C3) rabbit mAb, Jak2 (D2E12) XP® rabbit mAb, GAPDH (D16H11) XP rabbit mAb (Cell Signaling Technology).

Chemoresistance assay

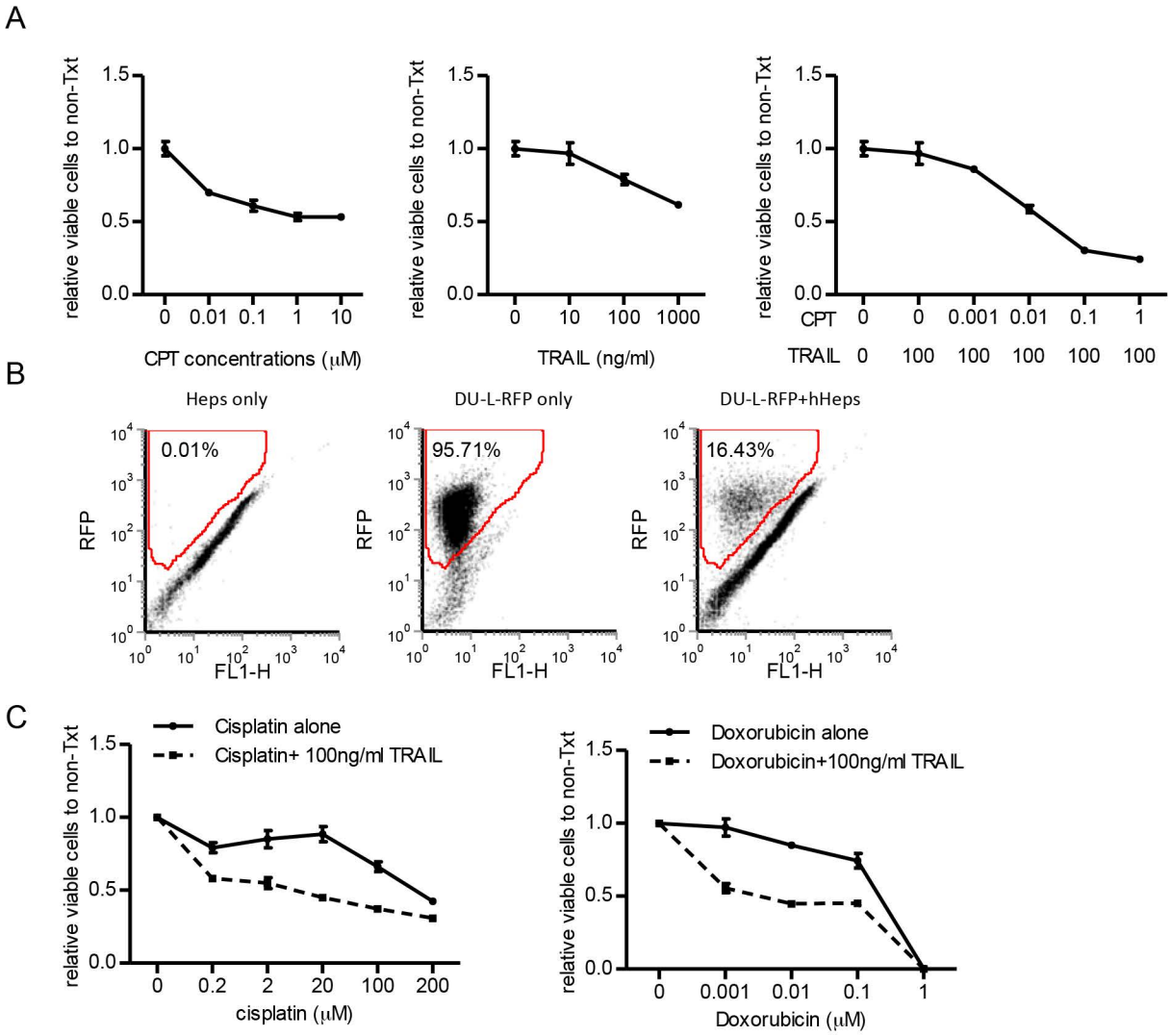
DU145 cells were treated with PD153035 to re-express E-cadherin and then starved overnight. Cells were treated with camptothecin (Sigma-Aldrich), doxorubicin or cisplatin (APP Pharmaceuticals, LLC) alone or in combination with recombinant human TRAIL (Life Technologies, PH1634) in serum-free medium. For co-cultures, cells were treated with chemotherapeutic drugs on day 5 in HMM medium.

Cell cycle analysis using propidium iodide staining

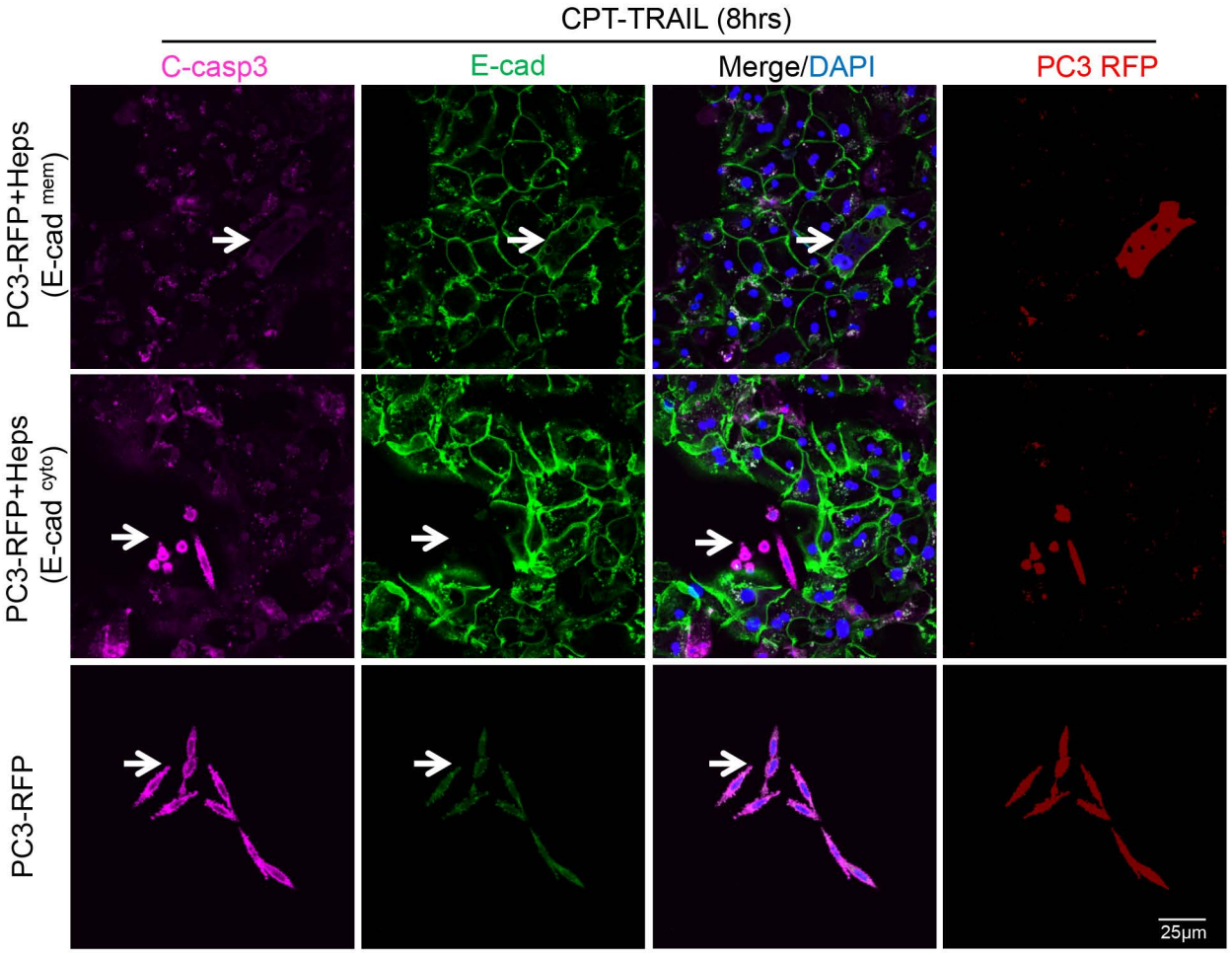
Cells were trypsinized and rinsed with PBS, fixed with 3.7% formaldehyde, left in absolute -20°C ethanol for 15 minutes. After pelleting, RNase A and PI (Invitrogen, P3566) were added to a final concentration of 5µg/ml and 10µg/ml respectively. For cleaved-caspase3 and PI double staining, cells were permeablized with 0.1% Triton X-100, and stained for cleaved-caspase3 prior to PI staining.

Statistical analyses

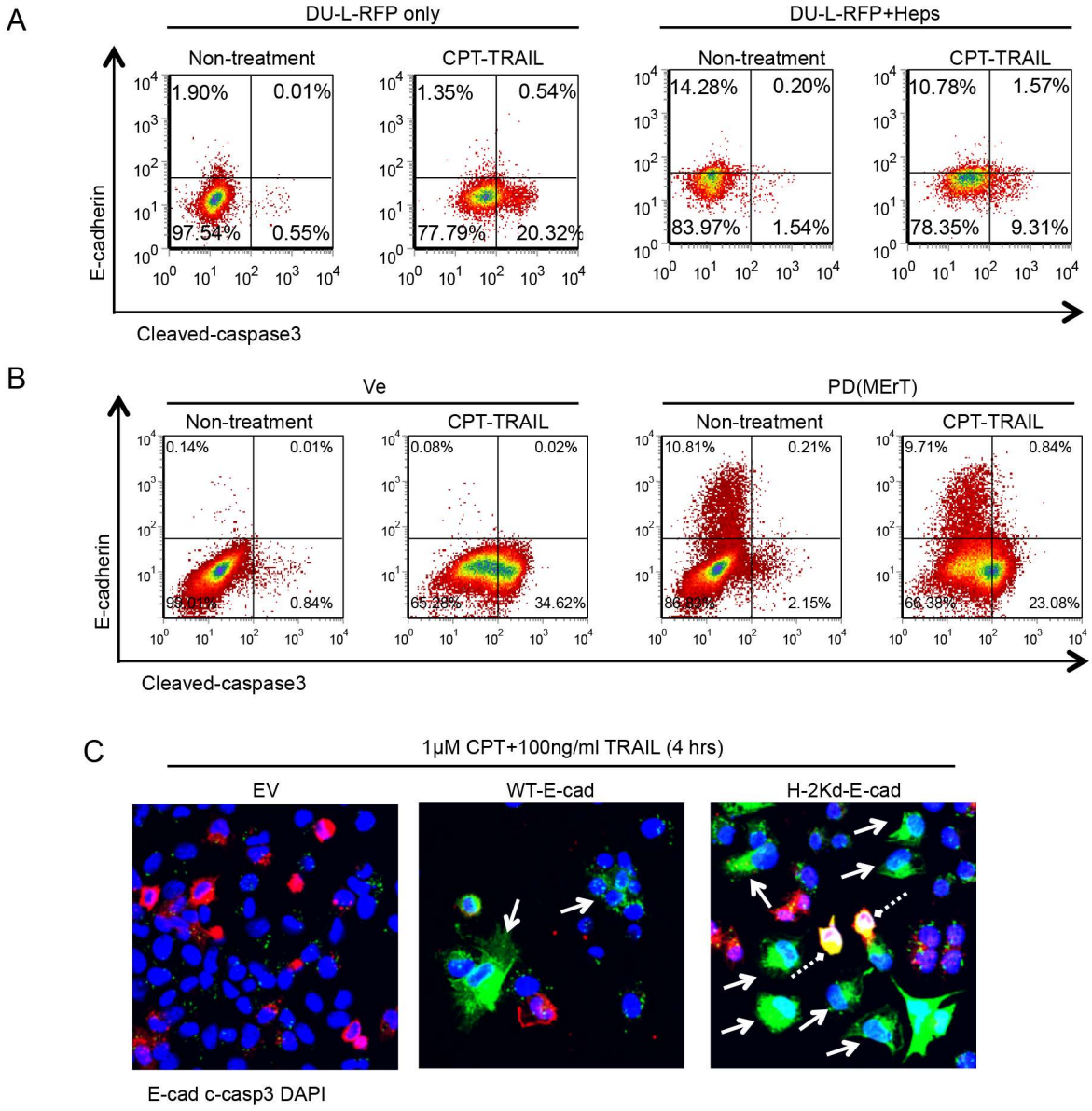
Two-way ANOVA analyses, Chi-squared tests, or Student's t-tests were used. Graphs and statistics were generated using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).



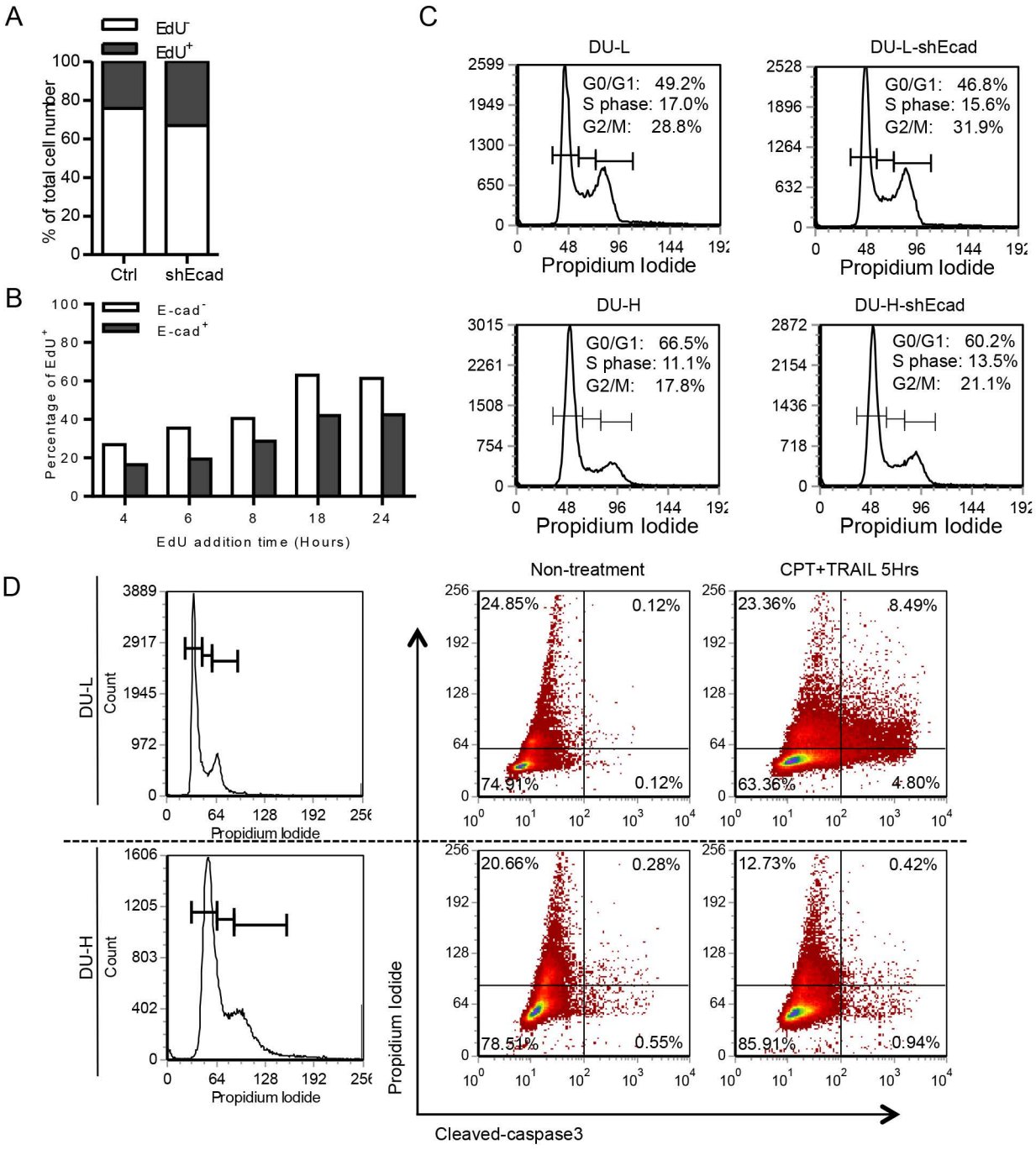
Supplemental Figure 1. TRAIL sensitizes PCa cells to chemotherapeutics. (A) Flow cytometry assay of living/ dead cells. DU-L cells were treated with CPT or TRAIL or both collaboration for 24 hours. (B) Representative of 2-D dot plot of flow cytometry assay for Heps only, DU-L-RFP only and co-cultured cells. Gated cells were RFP^+ population for the further analysis. (C) DU-L cells were treated with cisplatin or doxorubicin and TRAIL for 24 hours and then living/dead cells were analysis by flow cytometry. All cultures were collected and stained with Annexin V/PI kit followed by manufacture's instruction.



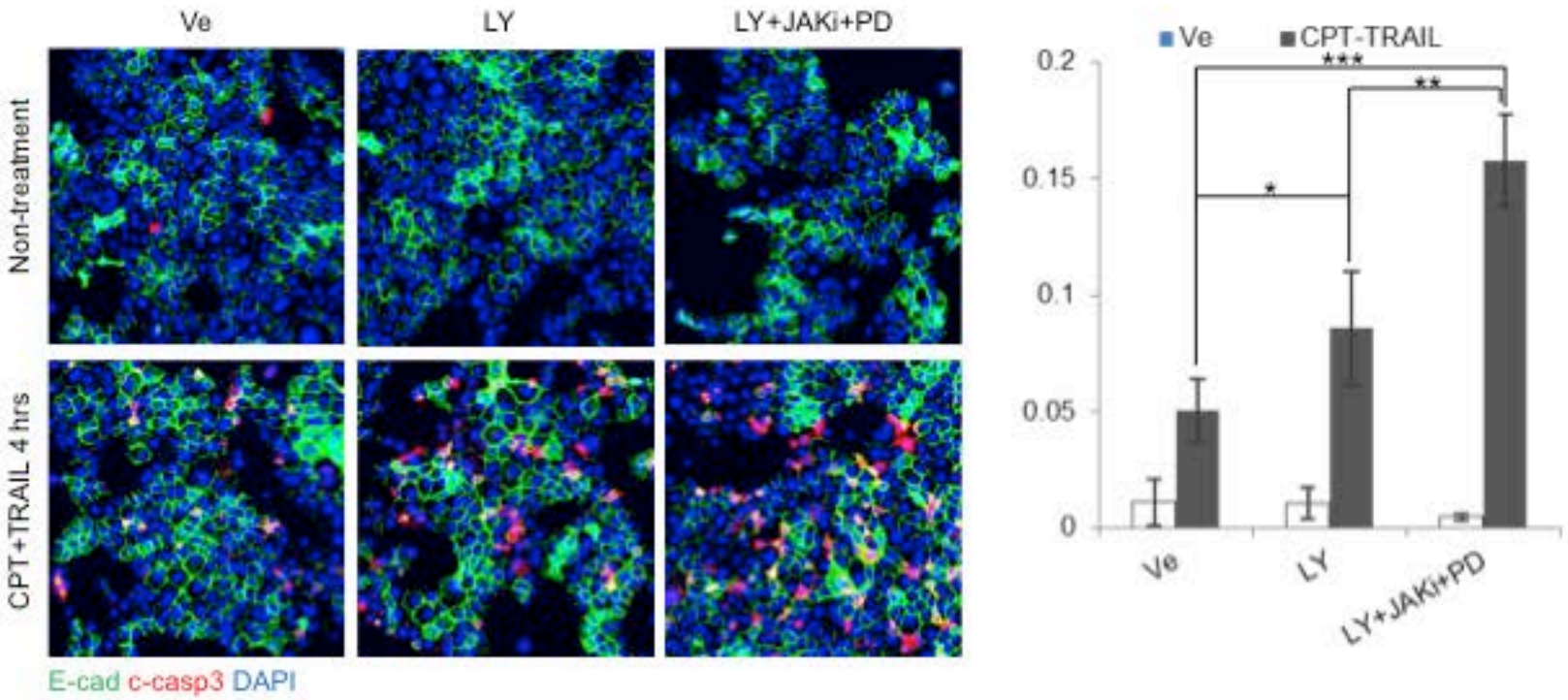
Supplemental Figure 2. PC3 with membrane-bound E-cadherin co-cultured with human hepatocytes are chemoresistant. Representative co-staining pictures of cleaved-caspase-3 (c-casp3, magenta), E-cadherin (E-cad, green), nucleus (DAPI, blue) in RFP labled PC3 cells ± hepatocytes, which treated with CPT-TRAIL for 8 hours.



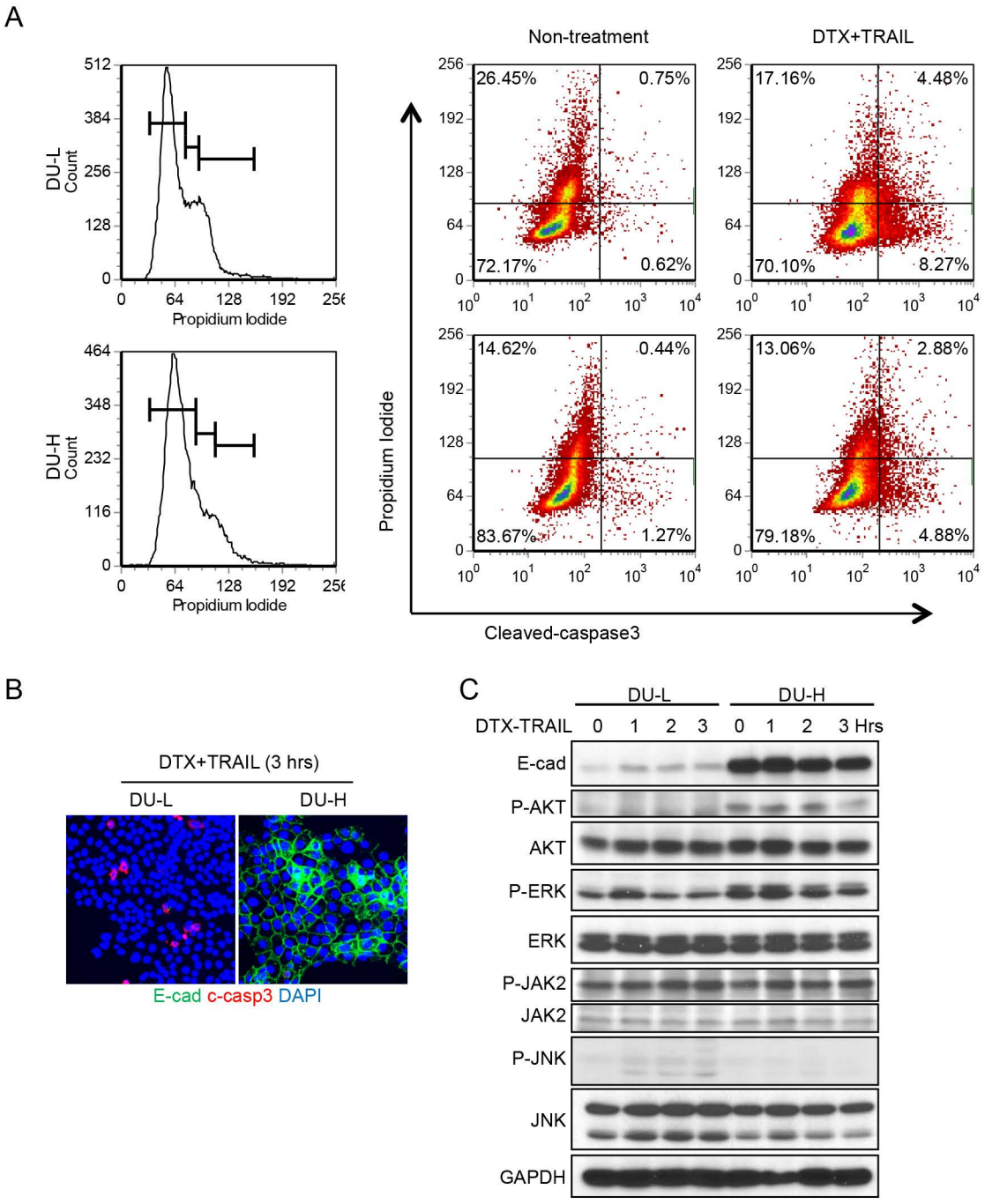
Supplemental Figure 3. Functional E-cadherin protects from induced cell death. (A) Dot plot of E-cadherin and cleaved-caspase3 double staining in RFP$^+$ cell population with flow cytometry assay in DU-L-RFP ± Heps treated with or without CPT-TRAIL for 5 hours. (B) Dot plot of E-cadherin and cleaved-caspase3 double staining in Vehicle or PD153035 induced MErT DU-L cells with flow cytometry assay. (C) DU-L transfected with empty vector or wildtype E-cadherin (WT-E-cad), H-2Kd and E-cadherin cytoplasmic tail fusion plasmids for 48 hours, subsequently treated with CPT-TRAIL for 4 hours. E-cadherin (green) and cleaved-caspase3 (red) immuno-staining were performed. Solid arrow, Ecad$^+$ casp3$^-$; dotted arrow, Ecad$^+$casp3$^+$.



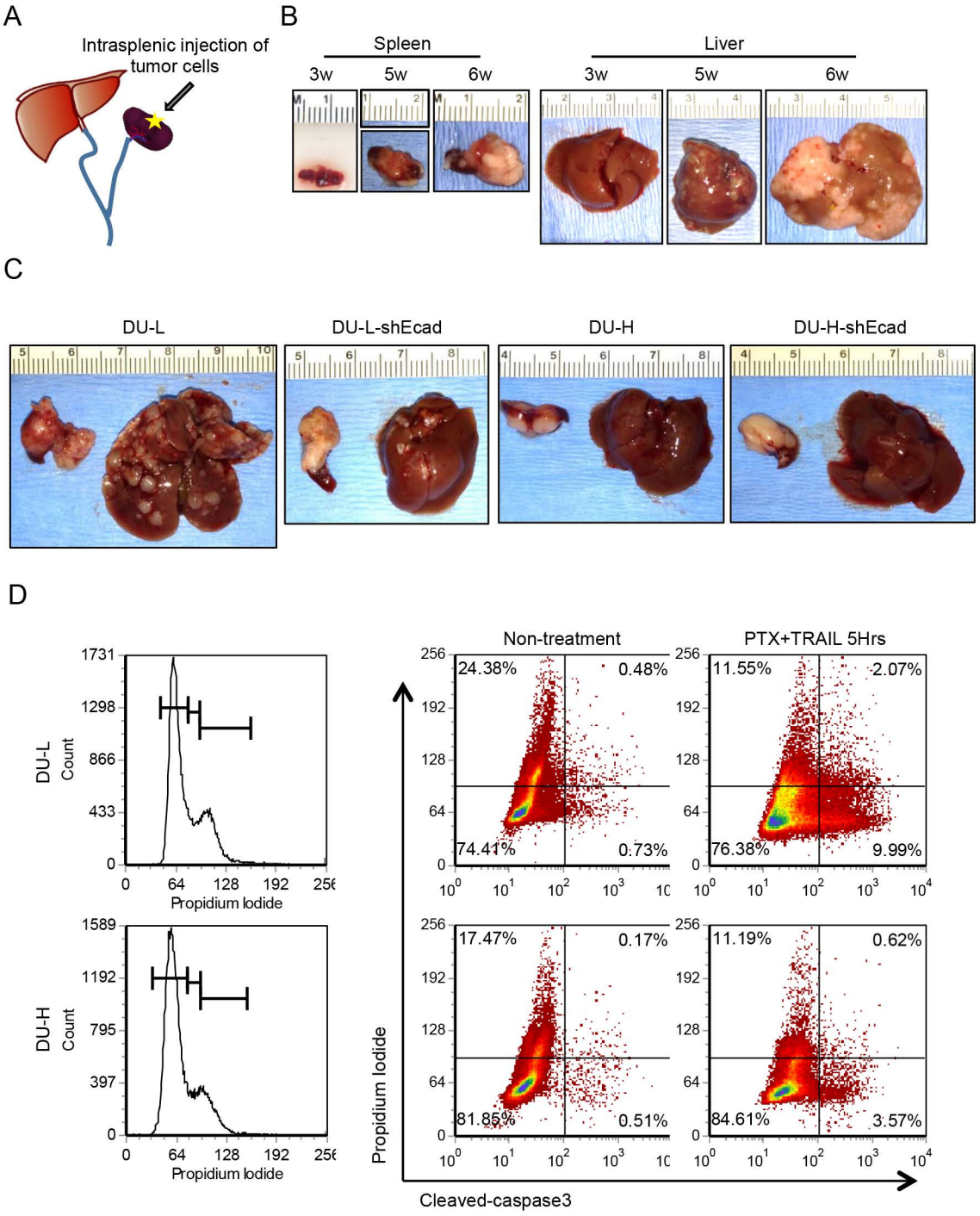
Supplemental Figure 4. (A) The percentage of EdU± cells in control or E-cadherin shRNA treated DU-H cells. 10μM EdU was added for 4 hours prior to fixation. (B) Ve or PD(MErT)-DU-L cells were incubated with 10μM EdU for 4-24 hour time course. The percentage of EdU^+ cells in DU-L with cytoplasmic and null ($E\text{-}cad^-$) or membrane-bound ($E\text{-}cad^+$) subgroup were enumerated for 5 fields per stained slide. (C) DU-L ± shEcad and DU-H ± shEcad cells were seed for 24 hours (~80% confluence). Propidium iodide (PI) cell cycle analyses were performed by flow cytometry. (D) Histogram of PI staining (left panel). Dot plot of PI and cleaved-caspae3 staining of DU-L and DU-H cells treated with or without CPT-TRAIL for 5 hours (right panel).



Supplemental Figure 5. Abrogation of multiple survival signaling pathways sensitizes the E-cadherin-expressing cells. IF staining of Ecadherin (green) and cleaved-caspase3 (red) of DU-H cells pretreated with DMSO (Vehicle, Ve), LY294002 (LY), or LY, JAKi and PD98059 collaboration for 1 hour. All inhibitors were washed out thoroughly with serum free medium and subsequently treated with CPT-TRAIL for 4 hours. The quantification is in the right panel. C-casp3 (Red) fluorescence density, measured by MetaMorph software, was normalized by DAPI. Data showed average ± SD. Paired t-test , two tailed p value, *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

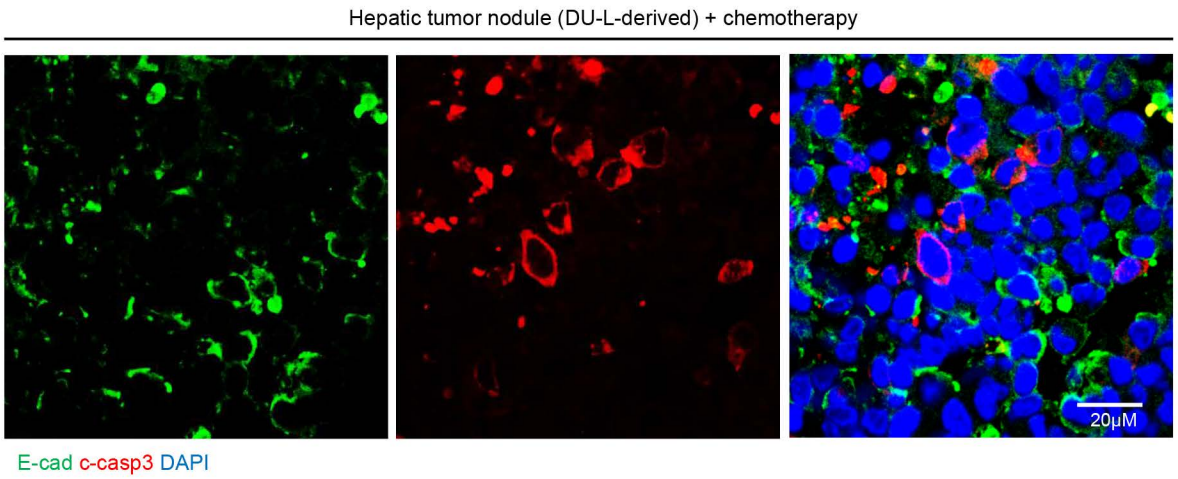


Supplemental Figure 6. Docetaxel treatment has similar efficacy as other chemotherapies. (A) Histogram of PI staining (left panel). Dot plot of PI and cleaved-caspase3 staining of DU-L and DU-H cells treated with or without Docetaxel (DTX, 1μM)-TRAIL for 3 hour (right panel). (B) IF staining of E-cadherin (green) and cleaved-caspase3 (red) of DU-L and DU-H cells treated with Docetaxel-TRAIL for 3 hours. (C) Western blot of total and phospho-AKT, ERK, JAK2, JNK and E-cadherin expression levels in DU-L and DU-H treated with DTX-TRAIL for indicated hours.

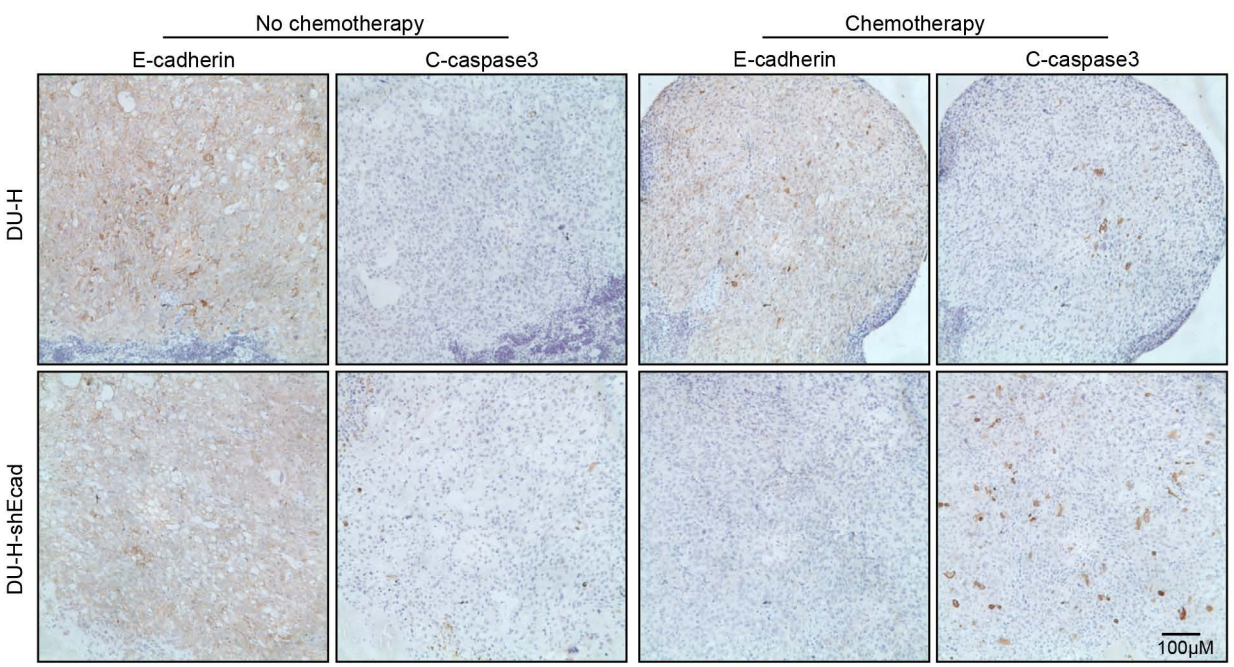


Supplemental Figure 7. (A) Schematic of intrasplenic injection. (B) Representative images of spleen and liver from DU-L inoculated mice on post-intrasplenic injection week 3, 5 and 6. (C) Representative images of spleen and liver from DU-L, DU-L-shEcad, DU-H, DU-H-shEcad cell lines inoculated mice on post-intrasplenic injection week 5. (D) Histogram of PI staining (left panel). Dot plot of PI and cleaved-caspae3 staining of DU-L and DU-H cells treated with or without PTX-TRAIL for 5 hours (right panel).

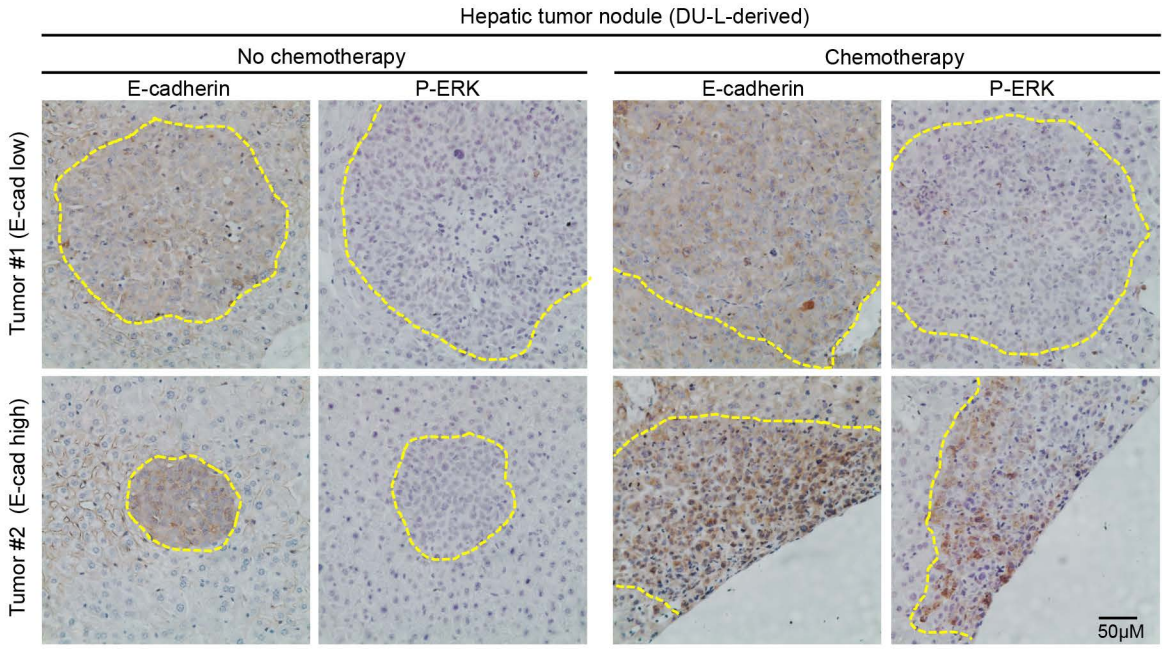
A


Hepatic tumor nodule (DU-L-derived) + chemotherapy
20μM
E-cad c-casp3 DAPI

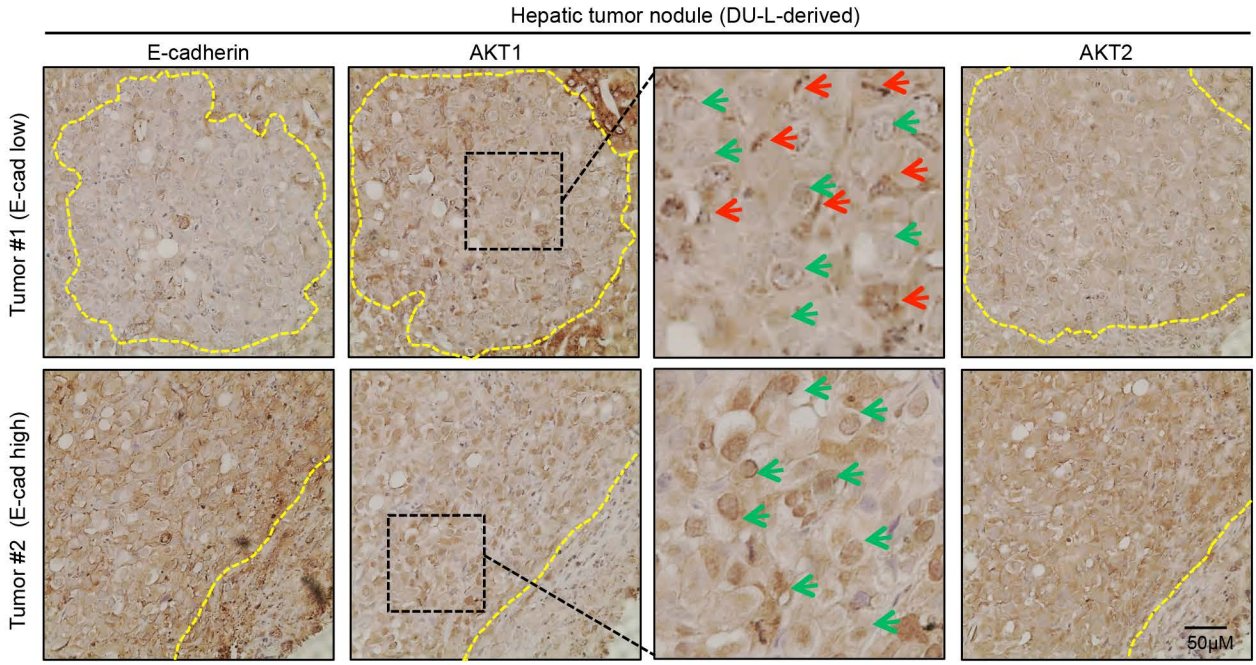
B


No chemotherapy
Chemotherapy
E-cadherin
C-caspase3
E-cadherin
C-caspase3
DU-H
DU-H-shEcad
100μM

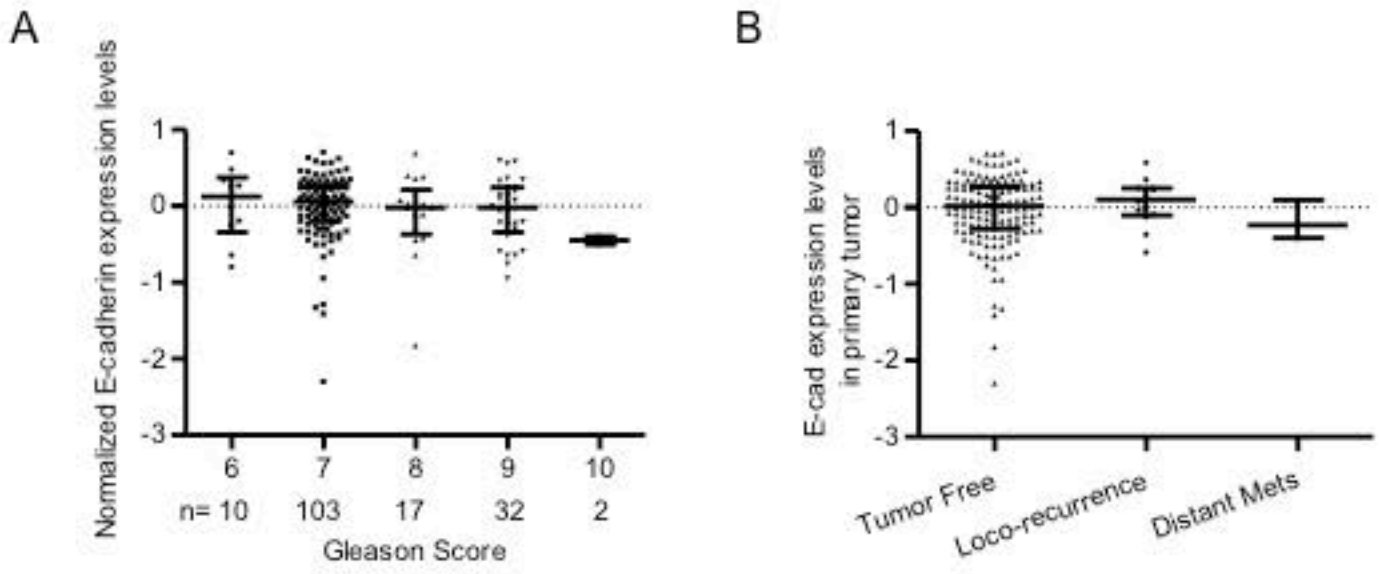
C


Hepatic tumor nodule (DU-L-derived)
No chemotherapy
Chemotherapy
E-cadherin
P-ERK
E-cadherin
P-ERK
Tumor #1 (E-cad low)
Tumor #2 (E-cad high)
50μM

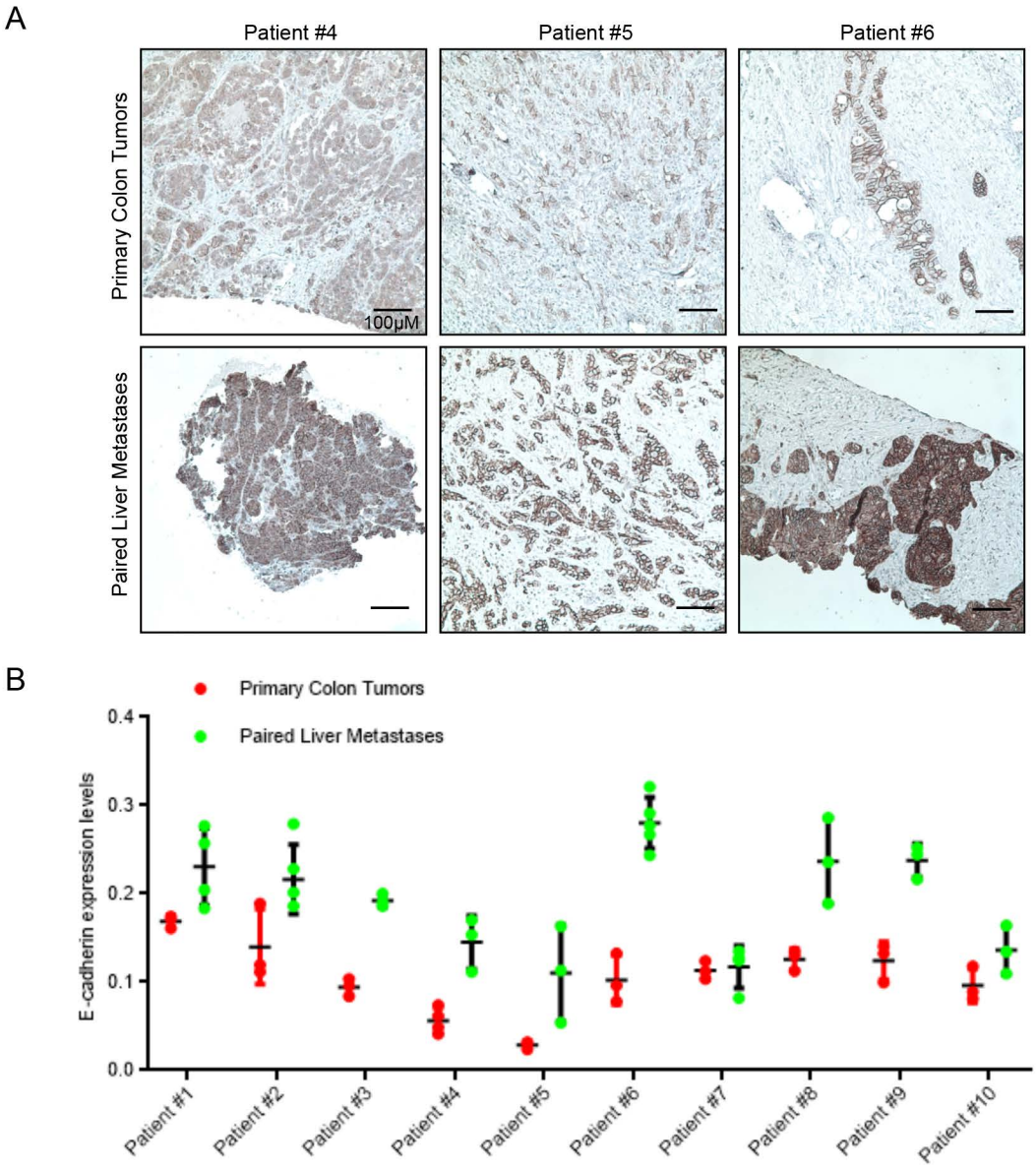
D



Supplemental Figure 8. (A) Co-staining of E-cadherin and cleaved-caspase3 in hepatic tumor nodule derived from DU-L intrasplenic inject NOD-SCID mouse with chemotherapy treatment. DAPI (blue) is nuclear. Entire field is tumor tissue. (B) IHC staining of E-cadherin and cleaved-caspase3 in spleen tumor nodules derived from DU-H or DU-H-shEcad cells ± chemotherapy. (C) IHC staining of E-cadherin and p-ERK in DU-L derived hepatic tumor nodules with or without chemotherapy. (D) IHC staining of E-cadherin, AKT1 and AKT2 in DU-L derived hepatic tumor nodules without chemotherapy. Red arrow: mouse liver non-parenchymal cells; green arrow: human tumor cells.



Supplemental Figure 9. E-cadherin expression levels in the TCGA database. (A) E-cadherin expression and Gleason score in the TCGA prostate cancer data set. (B) E-cadherin expression and tumor-recurrence in primary tumors in the TCGA prostate cancer data set.



Supplemental Figure 10. E-cadherin IHC staining of primary colon tumors and paired liver metastases. (A) Representative images of E-cadherin staining from 3 patients. (B) Quantification of E-cad expression with DAB in tumor area. Each dot represents the mean grey value density of tumor nodules in one image. P=0.002 with two-way ANOVA.