SUPPLEMENTARY INFORMATIONS

CCL3 Predicts Exceptional Response to TGFβ Inhibition in Basal-Like Pancreatic Cancer Enriched in LIF-Producing Macrophages

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LIST OF SUPPLEMENTARY MATERIALS

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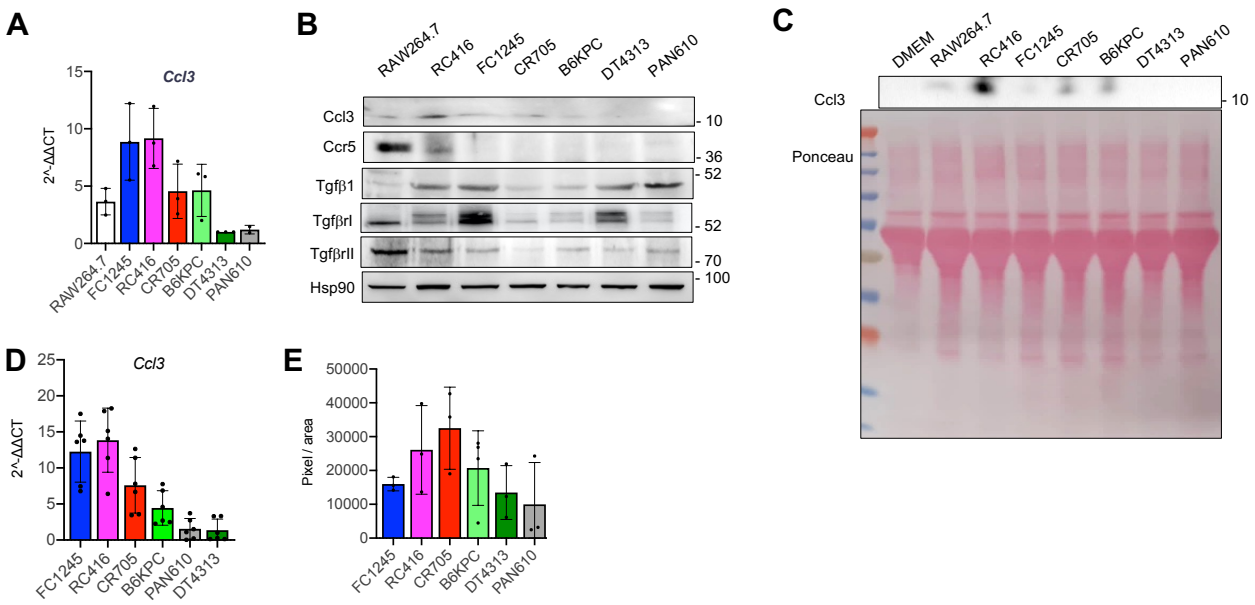
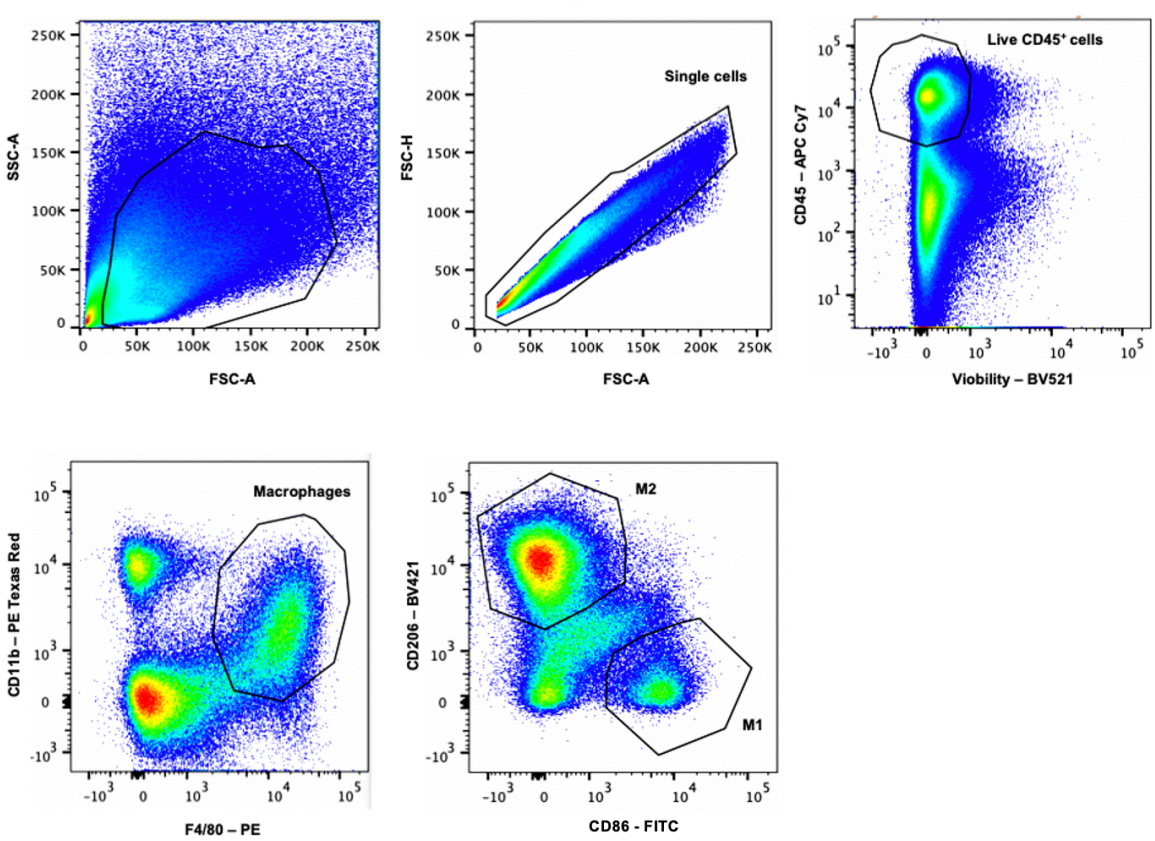
Supplementary Table 1. Baseline characteristics of patients enrolled in the H9H-MC-JBAJ clinical trial.

Supplementary Table 2. List of primers used in this study.

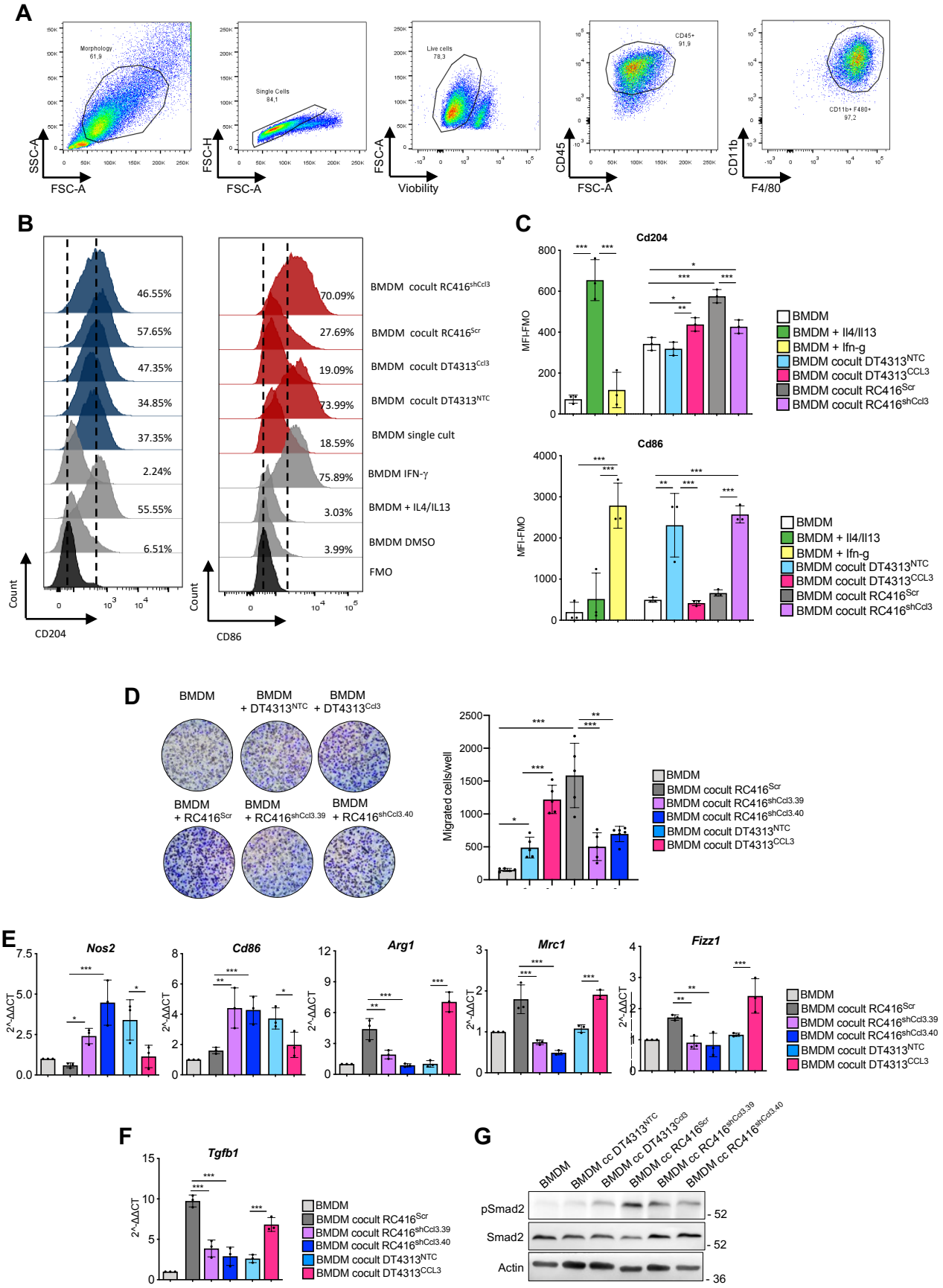
Supplementary Table 3. List of antibodies used in this study.



Supplementary Figure 1. Expression of Ccl3 in PDAC. (A) Quantitative real-time PCR (qPCR) of Ccl3 in RAW264.7 macrophages and in a panel of PDAC cell lines (FC1245, RC416, CR705, B6KPC, DT4313 and PAN610). Data are shown as mean ± s.d (n=3). (B) Representative western blot of Ccl3, Ccr5, Tgfb1, Tgfbr1 and Tgfbr2 in the whole cell extracts of RAW264.7, RC416, FC1245, CR705, B6KPC, DT4313 and PAN610 cell lines. Hsp90 was used as loading control. (C) Representative western blot of Ccl3 in the conditioned medium of RAW264.7, RC416, FC1245, CR705, B6KPC, DT4313 and PAN610 cell lines. Ponceau served as loading control. (D) qPCR analysis of Ccl3 in tumors from mice bearing FC1245, RC416, CR705, B6KPC, DT4313 and PAN610 cells. Data are shown as mean ± s.d (n=6). (E) Pixel intensity measurement of RNA in situ hybridization (ISH) images of Ccl3 in tumor sections from mice bearing FC1245, RC416, CR705, B6KPC, DT4313 and PAN610 cells. Data are shown as mean ± s.d (n=3).

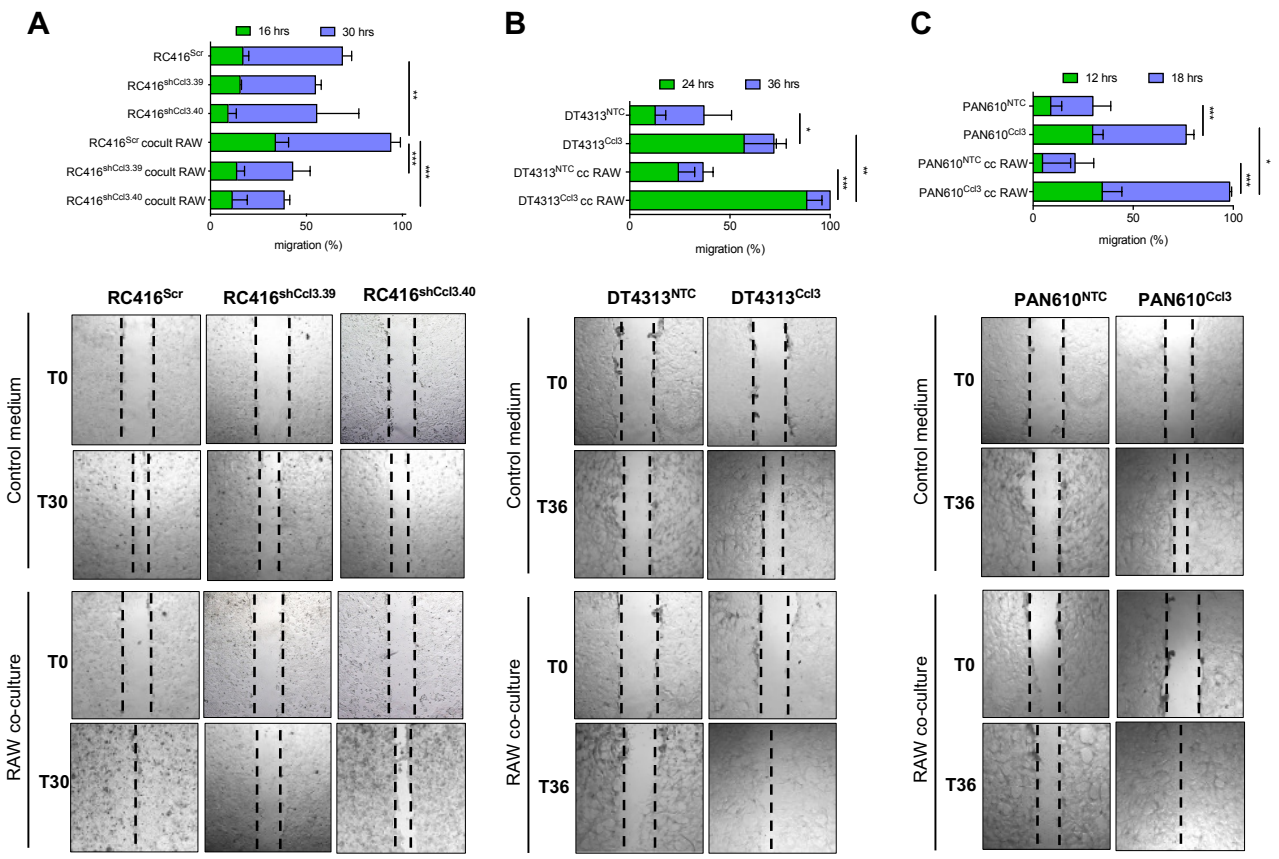


Supplementary Figure 2. Gating strategy. Single-cell suspensions were first gated for physical parameters, including forward scatter (FSC) for size, and side scatter (SSC) for granularity. TAMs were gated based on $CD45^{+}/Cd11b^{+}/F4/80^{+}$ positivity, and evaluated for the expression of Cd86 and Cd204 (M1 TAMs: $Cd86^{+}/Cd204^{-}$; M2 TAMs: $Cd86^{-}/Cd204^{+}$). Sample FACS plots of RC416-bearing C57BL/6J mice are shown.

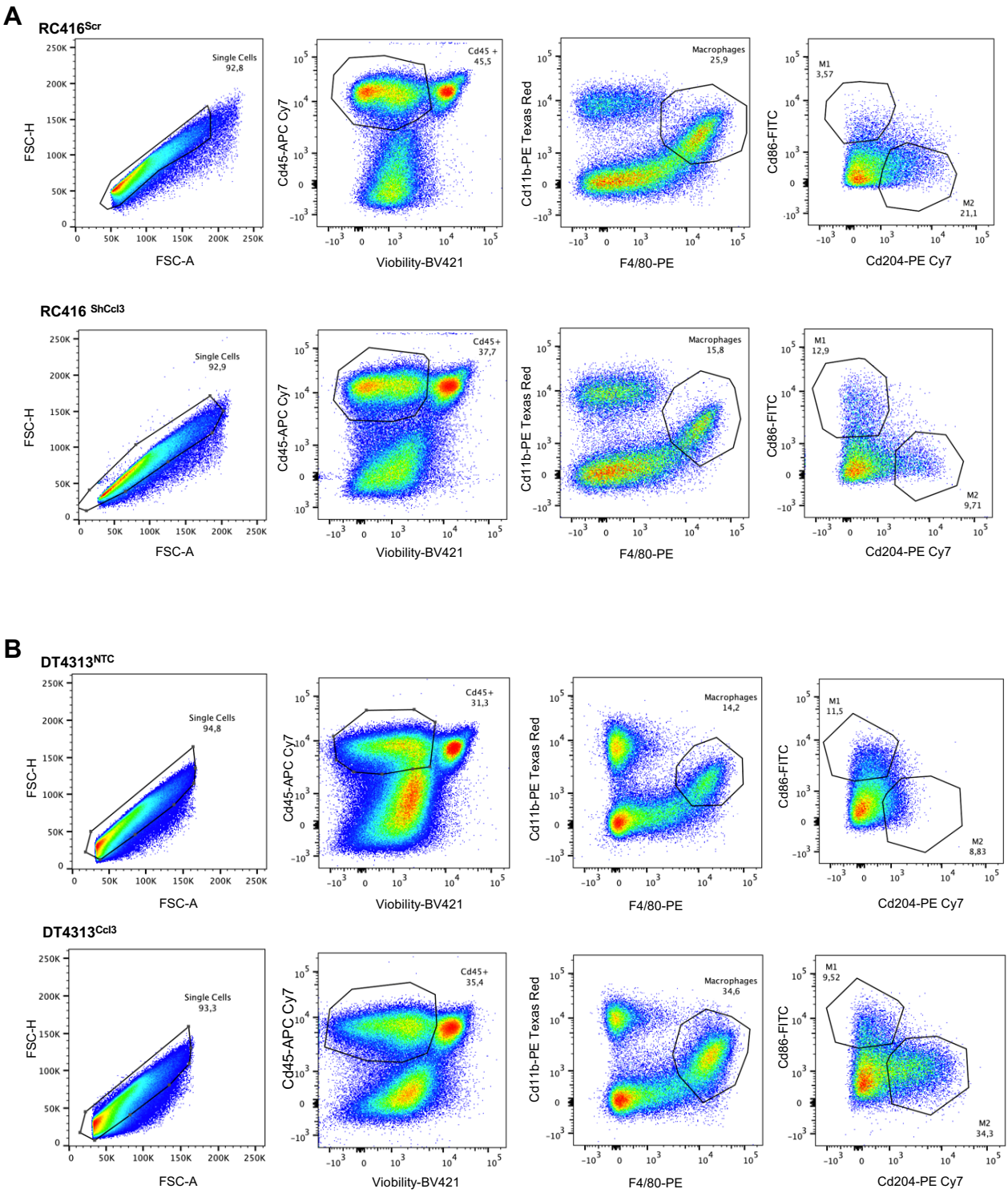


Supplementary Figure 3: Ccl3 favors M2-polarization and TGFβ pathway activation in BMDM. (A) Gating strategy. (B) Representative FCM plots of Cd204 and Cd86 in BMDM as single

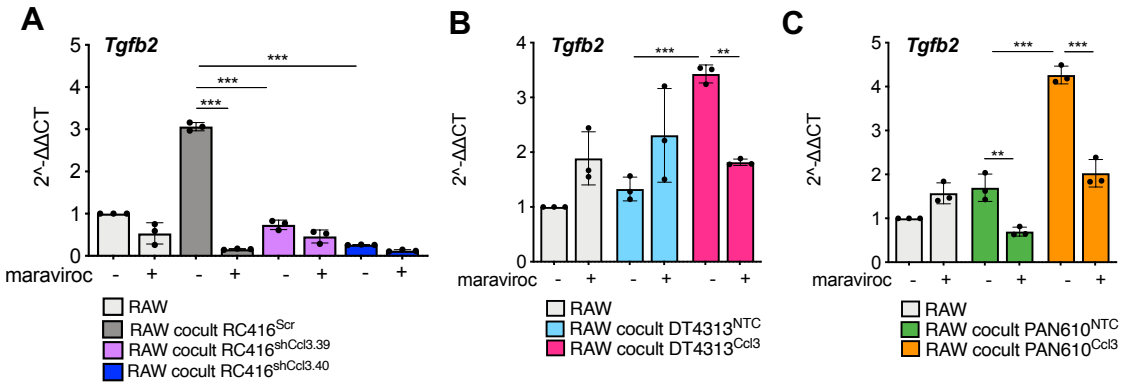
culture or co-cultured with RC416^{Scr}, RC416^{shCcl3}, DT4313^{NTC} or DT4313^{Ccl3} PDAC cells for 48h. IFN-γ or IL-4/IL-13 were used as positive control for M1 or M2 polarization, respectively. (C) Flow cytometry analysis of Cd204 and Cd86 expression of BMDM as single-culture or co-cultured as in (B). Data are shown as mean ± s.d (n=3). (D) Transwell migration assay of BMDM as single culture or co-cultured with RC416^{Scr}, RC416$^{shCcl3.39}$, RC416$^{shCcl3.40}$, DT4313^{NTC} or DT4313^{Ccl3} PDAC cells for 48h. Results show mean ± s.d of 5 biological replicates. P value was calculated by ANOVA and Tukey's test. (E) qPCR analysis of M1 markers *Nos2* and *Cd86*, and of M2 markers *Arg1, Mrc1* and *Fizz1* in BMDM as single cultures or co-cultured with RC416 or DT4313 transduced as indicated. Data are shown as mean ± s.d (n=3). P value was calculated by ANOVA and Tukey's test. (F) qPCR of *Tgfb1* in BMDM as single cultures or co-cultured with RC416 or DT4313 transduced as indicated. Data are shown as mean ± s.d (n=3). P values in C-F were calculated by ANOVA and Tukey's test. *p<0.05; **p<0.01; ***p<0.001. (G) Representative western blot of pSmad2 and Smad2 in BMDM as single culture or in co-culture with RC416 or DT4313 transduced as indicated. Actin was used as loading control.



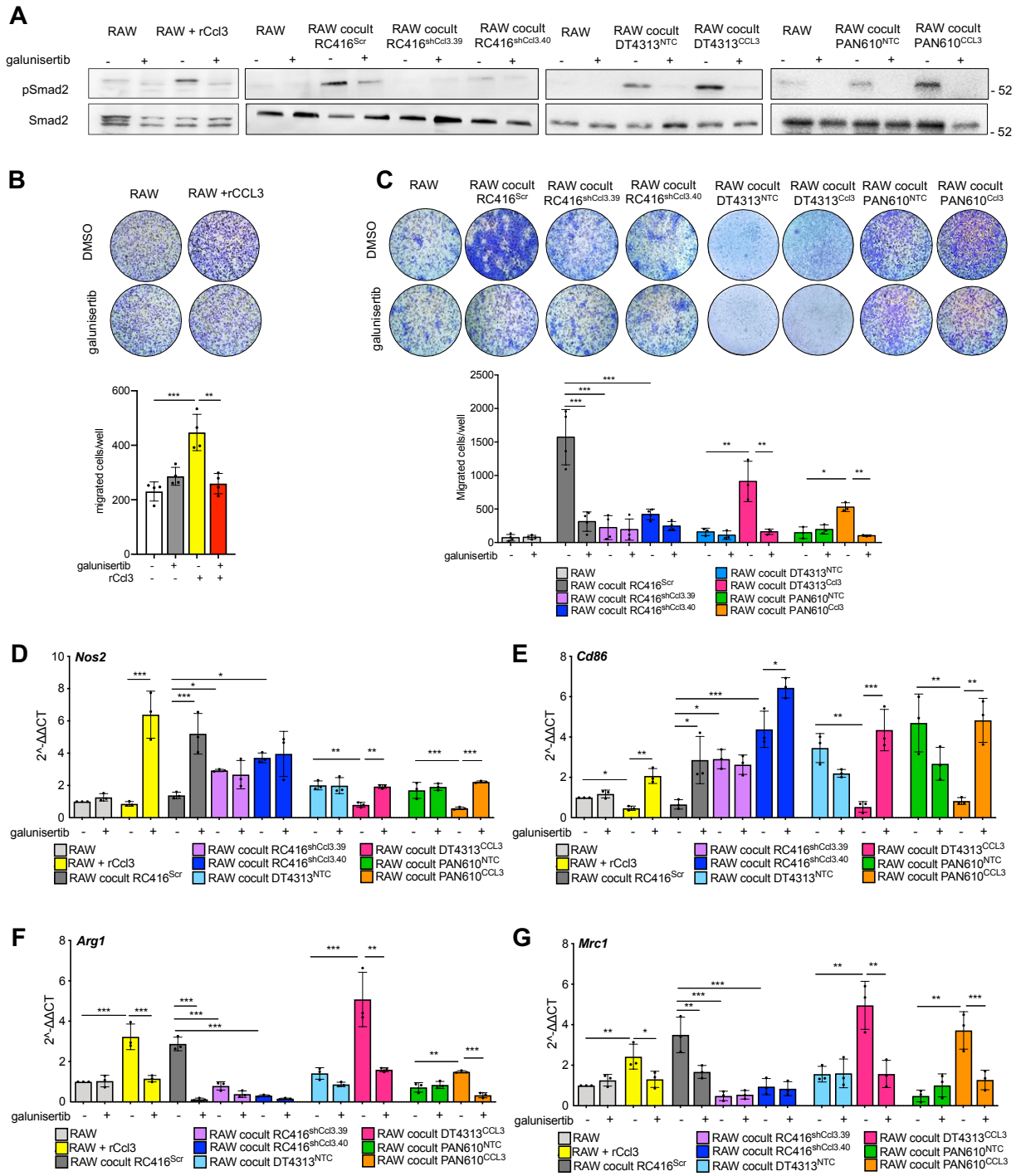
Supplementary Figure 4. RAW264.7 macrophages sustain the migratory ability of PDAC cells induced by Ccl3. (A) Scratch assay (wound closure) in RC416 (A), DT4313 (B) and PAN610 (C) cells transduced as indicated, as single cultures or co-cultured with RAW264.7 macrophages. Data are represented as mean ± s.d. P value was calculated by ANOVA and Tukey's test (n=3 biological independent experiments). *p<0.05; **p<0.01; ***p<0.001.



Supplementary Figure 5. Flow cytometry analysis of macrophages in high- and low-Ccl3 PDAC tumors. Single-cell suspensions were gated for physical parameters (FSC and SSC). Live TAMs were gated based on $CD45^{+}/Cd11b^{+}/F4/80^{+}$ positivity, and analyzed as M1 ($Cd86^{+}/Cd204^{-}$) or M2 ($Cd86^{-}/Cd204^{+}$) TAMs. Representative plots at 4-weeks after treatment of tumors from mice bearing $RC416^{Scr}$ or $RC416^{shCcl3}$ (A) and $DT4313^{NTC}$ or $DT4313^{Ccl3}$ (B) cells are shown.

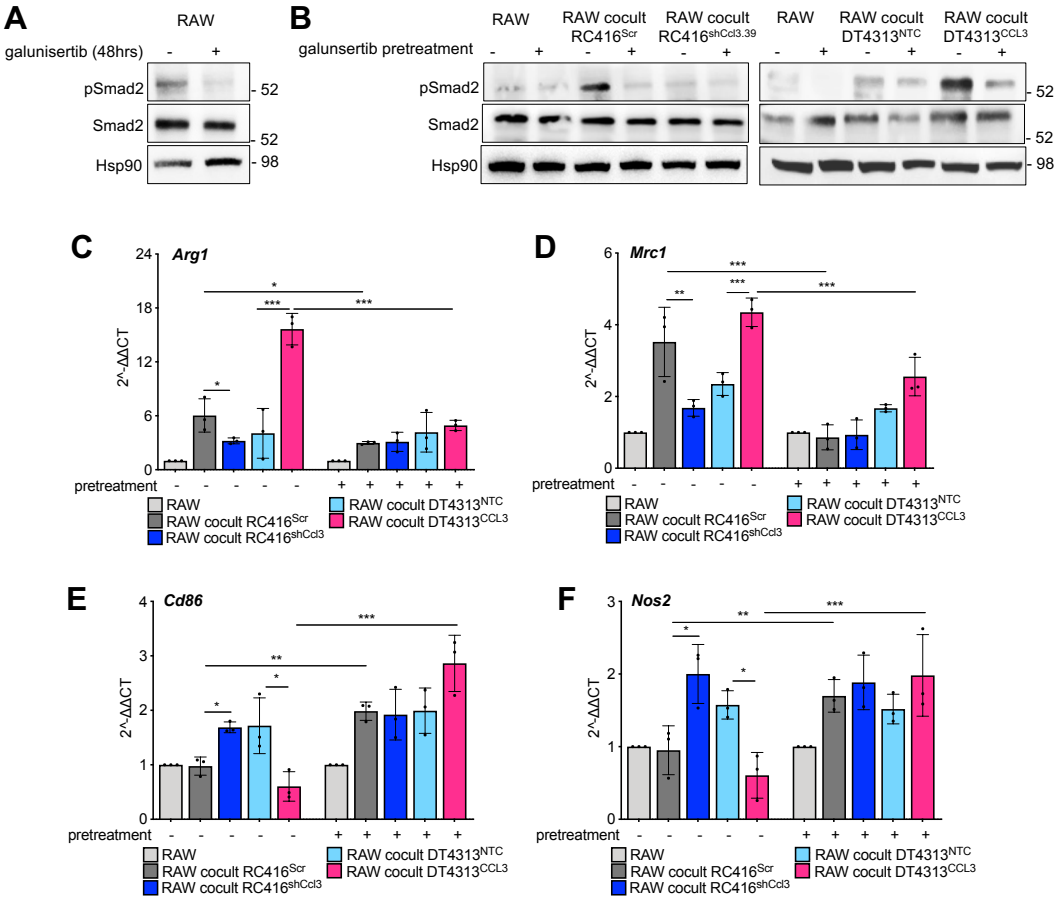


Supplementary Figure 6: Effect of Ccr5 inhibition in RAW264.7 macrophages on Tgfb2 expression. qPCR analysis of *Tgfb2* in RAW264.7 as single culture or co-cultured with RC416 (A), DT4313 (B) or PAN610 (C) transduced as indicated and treated with DMSO or 5μM maraviroc for 24h. Data are shown as mean ± s.d (n=3). P value was calculated by ANOVA and Tukey's test. **p<0.01; ***p<0.001.

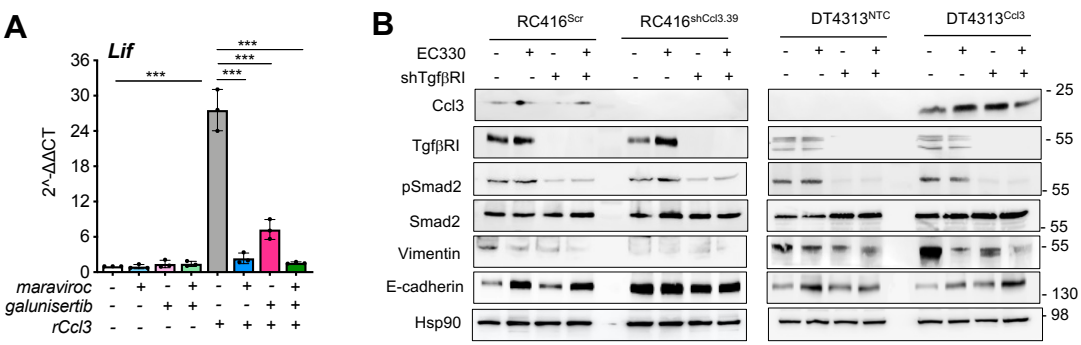


Supplementary Figure 7. Inhibition of TGFβ signaling suppresses recruitment and M2-polarization of macrophages. (A) Representative western blot of pSmad2 and Smad2 in RAW264.7 upon rCcl3 addition, as single cultures or co-cultured with RC416, DT4313 or PAN610 transduced as indicated and treated with 5μM galunisertib for 24h. (B,C) Transwell migration assay of RAW264.7 macrophages unstimulated or stimulated with rCcl3 (B), and as single culture or co-

cultured with RC416^{Scr}, RC416$^{shCcl3.39}$, RC416$^{shCcl3.40}$, DT4313^{NTC}, DT4313^{Ccl3}, PAN610^{NTC} or PAN610^{Ccl3} PDAC cells, upon treatment with DMSO or 5μM galunisertib for 48h. Results show mean ± s.d of 4 (rCcl3 and RC416) or of 3 (DT4313 and PAN610) biological replicates. P value was calculated by ANOVA and Tukey's test. (D-G) qPCR analysis of M1 markers *Nos2* (D) and *Cd86* (E), and of M2 markers *Arg1* (F) and *Mrc1* (G) in RAW264.7 macrophages treated with rCcl3, as single cultures or co-cultured with RC416, DT4313 or PAN610 transduced and treated as indicated. Data are shown as mean ± s.d (n=3). P value was calculated by ANOVA and Tukey's test. *p<0.05; **p<0.01; ***p<0.001.

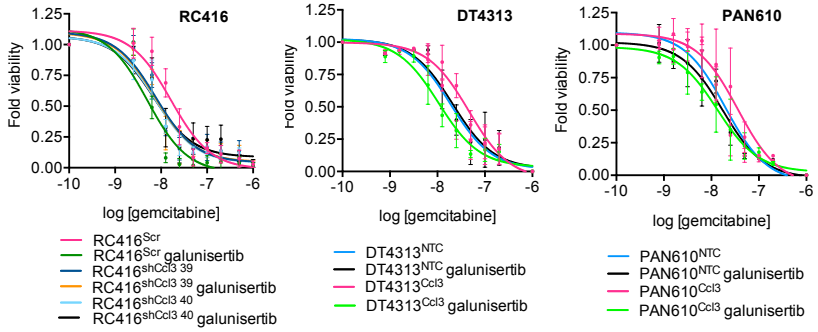


Supplementary Figure 8: Pre-treatment of RAW264.7 macrophages with galunisertib prevents their M2-polarization when co-cultured with high-Ccl3 PDAC cells. (A) Representative western blot of pSmad2 and Smad2 in RAW264.7 cells after treatment with DMSO or 5μM galunisertib for 48h. (B) RAW264.7 cells pre-treated with DMSO or galunisertib as in (A) were seeded as single culture or in co-culture with RC416 and DT4313 PDAC cells transduced as indicated. Representative western blot of pSmad2 and Smad2 are shown. Hsp90 in A and B was used as loading control. (C-F) qPCR of M2 markers *Arg1* (C) and *Mrc1* (D), and of M1 markers *Cd86* (E) and *Nos2* (F) in RAW264.7 cells pre-treated as in (A), as single culture or in co-culture with RC416 and DT4313 PDAC cells transduced as indicated. Data are shown as mean ± s.d (n=3). P value was calculated by ANOVA and Tukey's test. *p<0.05; **p<0.01; ***p<0.001.



Supplementary Figure 9: The Ccl3/Ccr5/TGFβ/Lif axis sustains EMT in PDAC cells. (A) qPCR analysis of *Lif* in RAW264.7 macrophages unstimulated or stimulated with rCcl3 for 48hrs, and treated with maraviroc, galunisertib, their combination or DMSO as control. Data are shown as mean ± s.d (n=3). P value was calculated by ANOVA and Tukey's test. ***p<0.001. (B) Representative western blot of Ccl3, TgfβR1, pSmad2, Smad2, vimentin and E-cadherin in RC416 and DT4313 transduced as indicated, co-cultured with RAW264.7 macrophages and treated with 1μM of the Lif inhibitor EC330 or DMSO as vehicle control. Hsp90 was used as loading control.

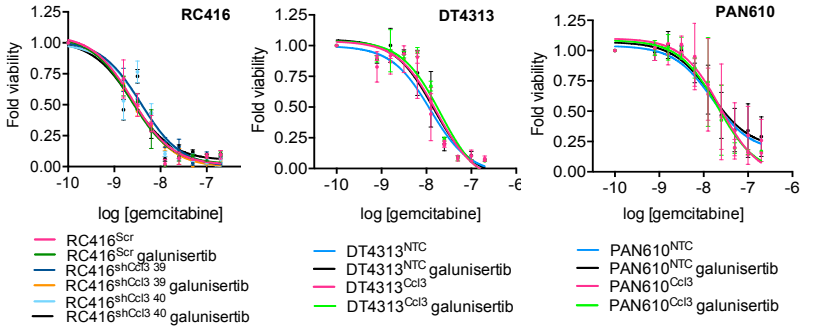
A



B

PDAC cells co-cultured with RAW264.7 macrophages	
Treatments	**EC50 gemcitabine (nM)**
RC416^{Scr}	16.28 (95% CI: 11.19 – 23.68)
RC416^{Scr}+ GAL	5.07 (95% CI: 3.37 – 7.64)
RC416$^{shCcl3.39}$	7.65 (95% CI: 5.19 – 11.28)
RC416$^{shCcl3.39}$+GAL	7.37 (95% CI: 5.04 – 10.79)
RC416$^{shCcl3.40}$	7.51 (95% CI: 5.35 – 10.54)
RC416$^{shCcl3.40}$+ GAL	6.75 (95% CI: 4.27 – 10.68)
DT4313^{NTC}	17.65 (95% CI: 10.93 – 28.48)
DT4313^{NTC}+ GAL	19.34 (95% CI: 10.82 – 24.59)
DT4313^{Ccl3}	49.25 (95% CI: 30.21 – 80.29)
DT4313^{Ccl3}+ GAL	9.97 (95% CI: 6.77 – 14.67)
PAN610^{NTC}	21.10 (95% CI: 11.70 – 38.05)
PAN610^{NTC}+ GAL	17.59 (95% CI: 9.86 – 31.36)
PAN610^{Ccl3}	41.22 (95% CI: 18.74 – 90.67)
PAN610^{Ccl3}+ GAL	11.96 (95% CI: 6.80 – 21.01)

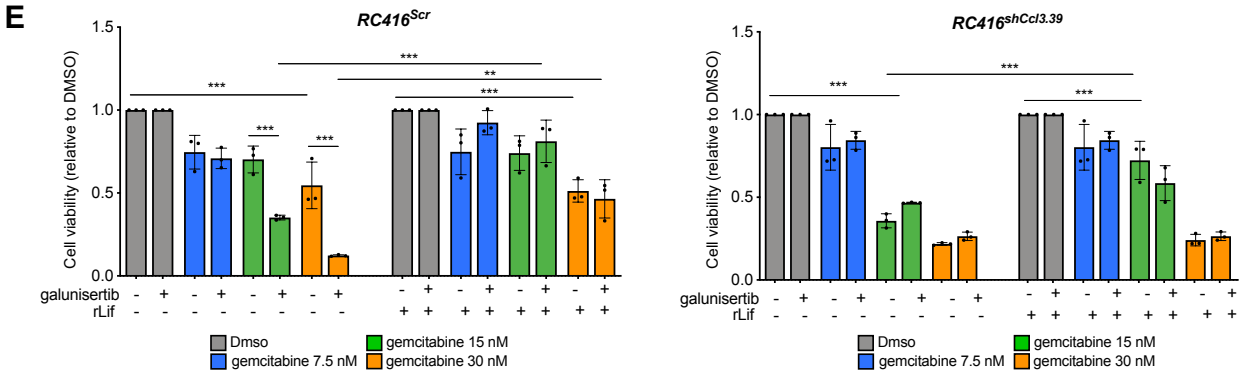
C



D

PDAC cells single cultures	
Treatments	**EC50 gemcitabine (nM)**
RC416^{Scr}	2.51 (95% CI: 1.65 – 3.79)
RC416^{Scr}+ GAL	2.27 (95% CI: 1.49 – 3.45)
RC416$^{shCcl3.39}$	3.79 (95% CI: 2.99 – 4.80)
RC416$^{shCcl3.39}$+GAL	2.63 (95% CI: 2.18 – 3.17)
RC416$^{shCcl3.40}$	4.04 (95% CI: 2.22 – 7.34)
RC416$^{shCcl3.40}$+ GAL	2.38 (95% CI: 1.26 – 4.49)
DT4313^{NTC}	12.23 (95% CI: 7.34 – 20.34)
DT4313^{NTC}+ GAL	14.38 (95% CI: 8.89 – 2.32)
DT4313^{Ccl3}	16.93 (95% CI: 11.69 – 24.5)
DT4313^{Ccl3}+ GAL	20.67 (95% CI: 13.21 – 32.33)
PAN610^{NTC}	15.41 (95% CI: 6.47 - 36.65)
PAN610^{NTC}+ GAL	14.95 (95% CI: 6.87 - 32.58)
PAN610^{Ccl3}	23.94 (95% CI: 9.97 - 57.47)
PAN610^{Ccl3}+ GAL	20.6 (95% CI: 9.91 - 42.82)

E



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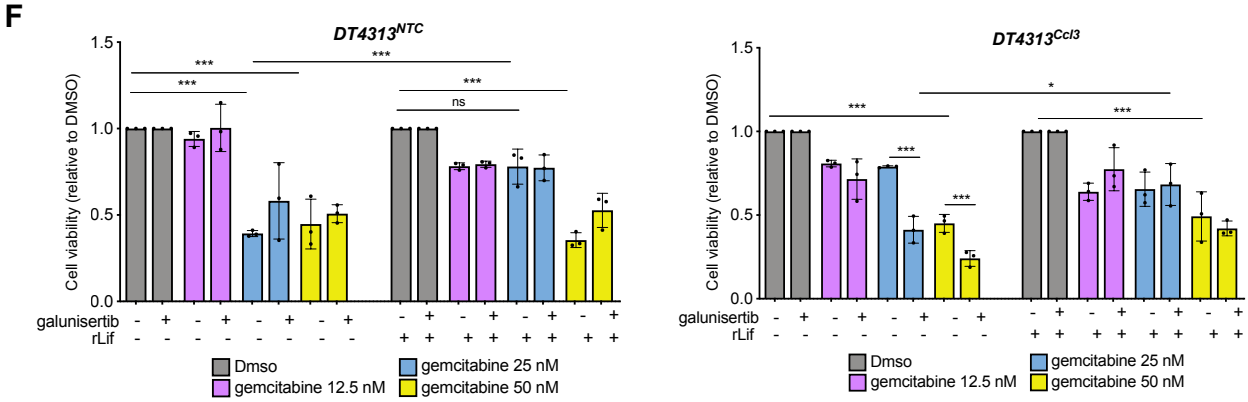
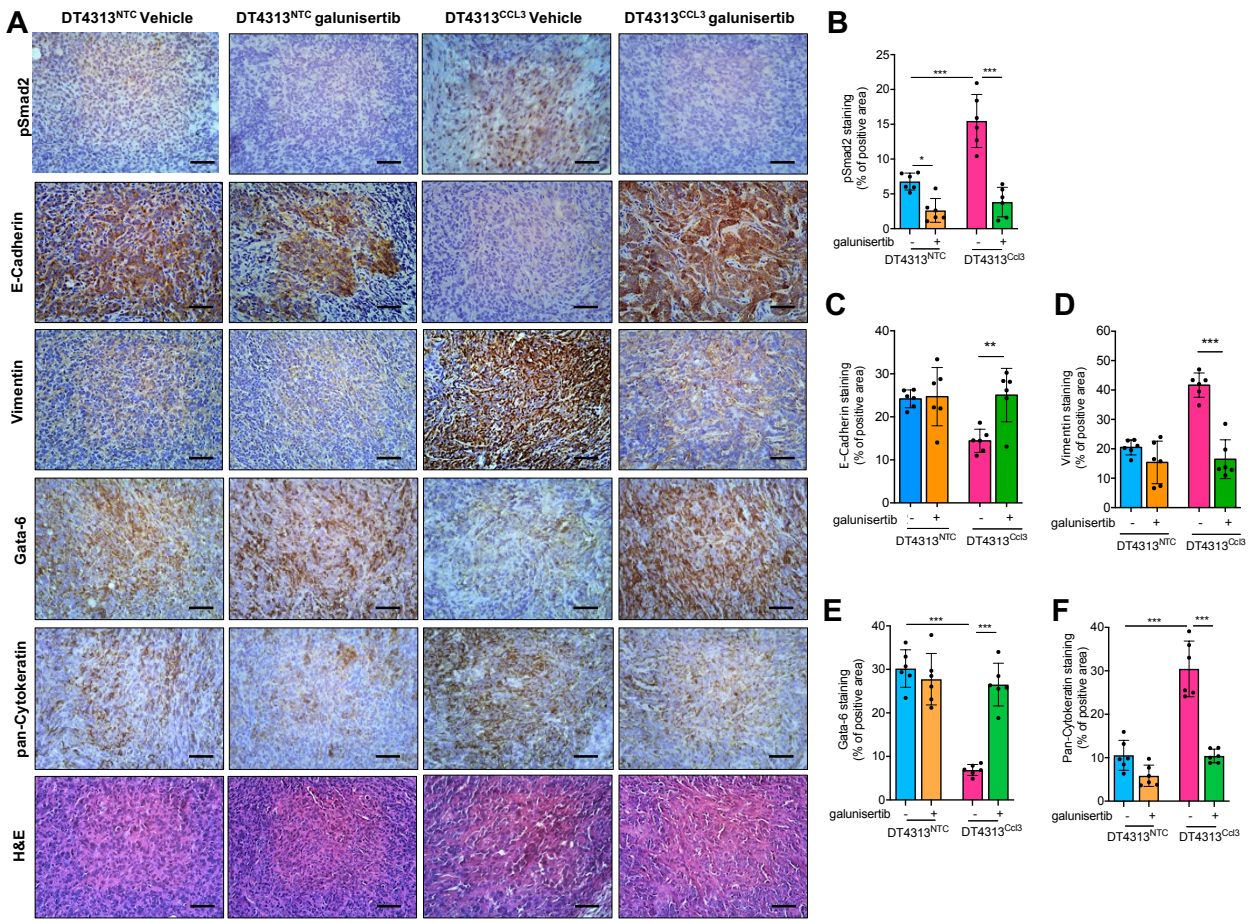


Supplementary Figure 10: Inhibition of TGFβ in macrophages sensitizes Ccl3-high PDAC cells to gemcitabine-based chemotherapy. Dose-response curves of RC416, DT4313 or PAN610 co-cultured with RAW264.7 macrophages (A,B) or as single cultures (C,D) after 72h of treatment with increasing doses of gemcitabine alone or in combination with 2μM galunisertib. Tables in B and D indicates EC50 values and 95% CIs of gemcitabine. (E,F) Cell viability of RC416 (E) or DT4313 (F) co-cultured with RAW264.7 macrophages transduced as indicated, after 72h of treatment with gemcitabine alone or in combination with 2μM galunisertib, in presence or absence of 100 ng/mL recombinant Lif (rLif). GAL: galunisertib; rLif: recombinant Lif.



Supplementary Figure 11. Galunisertib suppresses the mesenchymal/basal-like phenotype in mice bearing Ccl3-high tumors. (**A**) Representative images for DT4313^{NTC} or DT4313^{Ccl3} tumors treated with either vehicle or galunisertib, stained with pSmad2, E-cadherin, vimentin, Gata-6 and cytokeratin antibodies and H&E. Scale bar=60 µm. **(B-F)** Quantification of paraffin sections from orthotopic murine PDAC tumors stained as indicated in (A). Data in B-F are expressed as mean ± s.d. (n=6). P values were calculated by two-tailed unpaired Student's t test. **p<0.01; ***p<0.001.

Supplementary Table 1. Baseline Characteristics of Patients enrolled in the H9H-MC-JBAJ randomized clinical trial.

Baseline Characteristics	Galunisertib+Gemcitabine N = 104, n (%)	Placebo+Gemcitabine N = 52, n (%)
Age, ≥65 years	68 (65.4)	31 (59.6)
Sex, male	57 (54.8)	28 (53.9)
ECOG performance status		
0	34 (32.7)	19 (36.5)
1	60 (57.7)	28 (53.9)
2	10 (9.6)	5 (9.6)
Disease stage IV	94 (90.4)	46 (88.5)
Liver metastasis, Yes	60 (57.7)	26 (50)
Number metastatic sites		
0	17 (16.4)	10 (19.2)
1	62 (59.6)	34 (65.4)
≥2	25 (24.0)	8 (15.4)

Supplementary Table 2. List of primers used in this study.

Primer	Sequence (5' to 3')
Ccl3 Fwd	CAACCAAGTCTTCTCAGCGC
Ccl3 Rev	CTTTGGAGTCAGCGCAGATC
Tgfb1 Fwd	TTACCTTGGTAACCGGCTGC
Tgfb1 Rev	AGCCCTGTATTCCGTCTCCT
Hprt Fwd	AGTCCCAGCGTCGTGATTAG
Hprt Rev	GCCTCCCATCTCCTTCATGA
Arg1 Fwd	CTGAGCTTTGATGTCGACGG
Arg1 Rev	TCCTCTGCTGTCTTCCCAAG
iNos2 Fwd	CCCCGCTACTACTCCATCAG
iNos2 Rev	CCACTGACACTTCGCACAAA
Lif Fwd	CGGGACAGAGAAGACCAAGT
Lif Rev	GGTACTTGTTGCACAGACGG
Mrc1 Fwd	ATGGGCAACATCGAGCAGAA
Mrc1 Rev	AAACCAATGCAACCCAGTGC
Cd86 Fwd	CCGGATGGTGTGTGGCATAT
Cd86 Rev	TGAGCAGCATCACAAGGAGG
Fizz1 Fwd	CCTGCTGGGATGACTGCTA
Fizz1 Rev	TGGGTTCTCCACCTCTTCAT

Supplementary Table 3. List of antibodies used in this study.

Antibody	Source	Cat. No.	Dilution
Ccl3	R&D System	AF-450-NA	1mg/ml (WB)
E-Cadherin (24E10)	Cell Signaling Technology	#3195	1:1000 (WB) 1:200 (IHC)
Gapdh	Thermo Fisher Scientific	G9545	1:50000 (WB)
Gata-6	ProteinTech	55435-1-AP	1:200 (IHC)
Pan-cytokeratin	Novus Biologicals	NB600-579	1:200 (IHC)
Phospho-Smad2 (Ser465/467) (138D4)	Cell Signaling Technology	#3108	1:1000 (WB) 1:200 (IHC)
Smad2	Cell Signaling Technology	#3103	1:1000
Vimentin	Abcam	ab92547	1:1000 (WB) 1:800 (IHC)
Ccr5	Santa Cruz Technologies	sc-17833	1:1000 (WB)
Tgfβ1	RayBiotech	102-11520	1:1000 (WB)
Tgfβr1	Abcam	ab31013	1:1000 (WB)
Tgfβr2	Santa Cruz Technologies	sc-17799	1:500 (WB)
Actin	Santa Cruz Technologies	sc-47778	1:5000 (WB)
Hsp90	Santa Cruz Technologies	sc-13119	1:10000 (WB)
CD45-APC-Vio770	Miltenyi Biotec	REA737	1:100 (FC)
Cd11b-PE-Vio615	Miltenyi Biotec	REA592	1:100 (FC)
F4/80-PE	Miltenyi Biotec	REA126	1:100 (FC)
CD86-Vio Bright B515	Miltenyi Biotec	REA1190	1:100 (FC)
CD204-PE-Vio770	Miltenyi Biotec	REA148	1:200 (FC)

WB: Western blotting; IHC: Immunohistochemistry; FC: Flow cytometry.

EXTENDED DATA: Uncropped and unprocessed scans of blots

Figure 3A

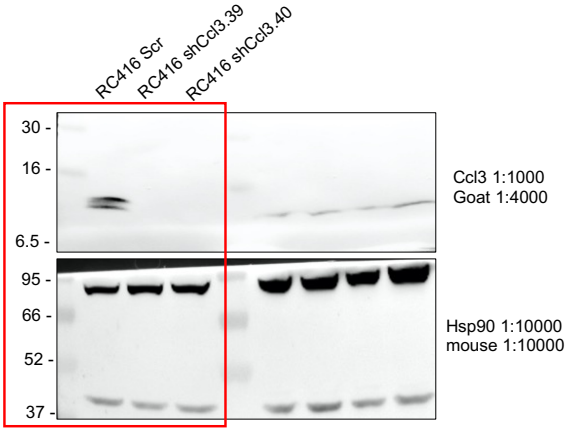


Figure 3D

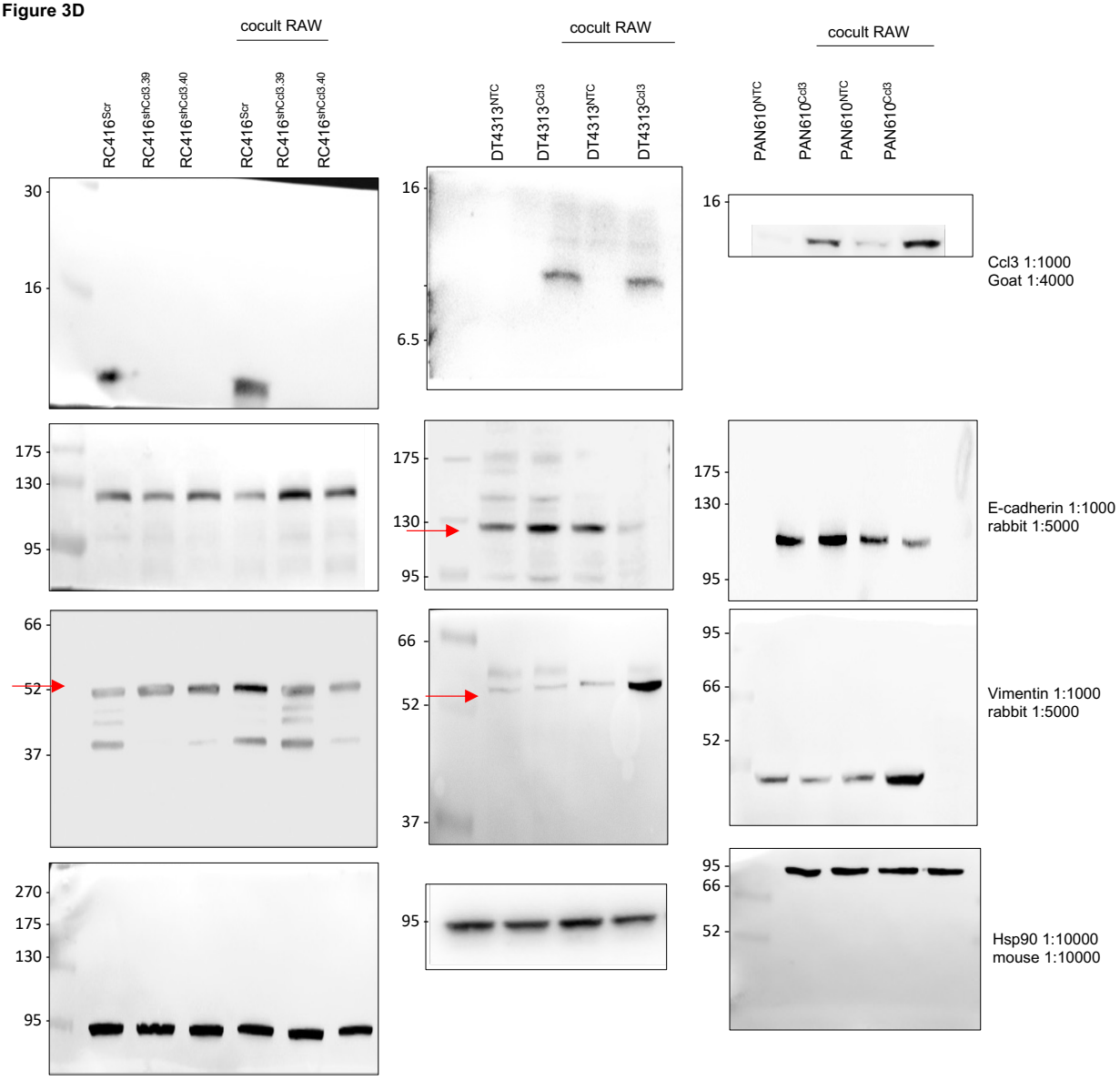


Figure 4D

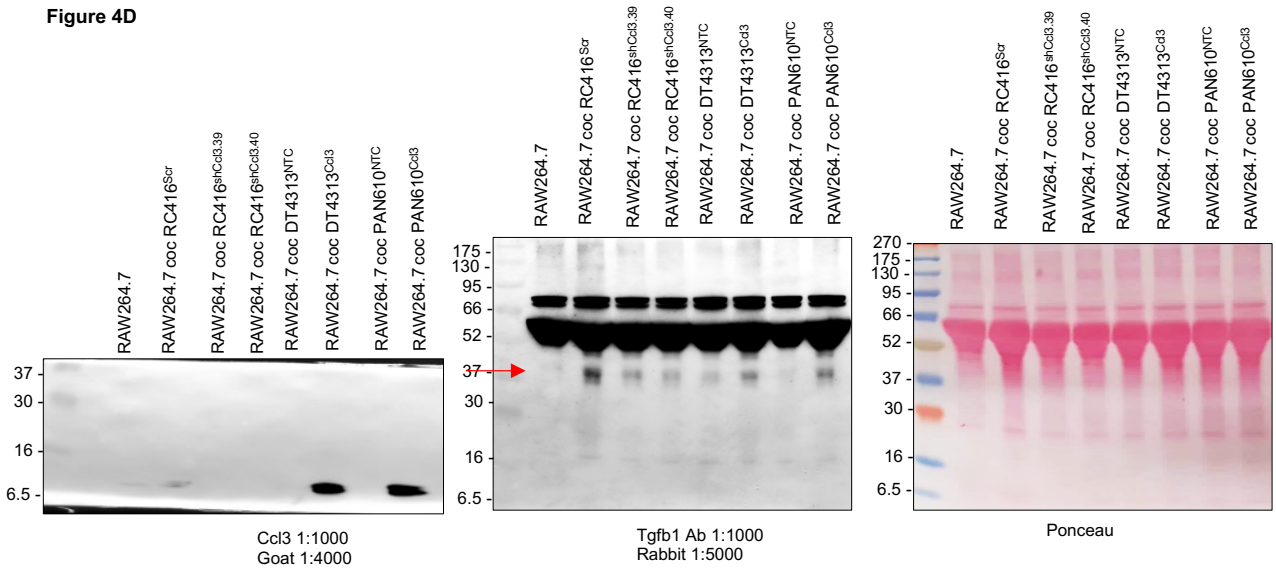


Figure 4E

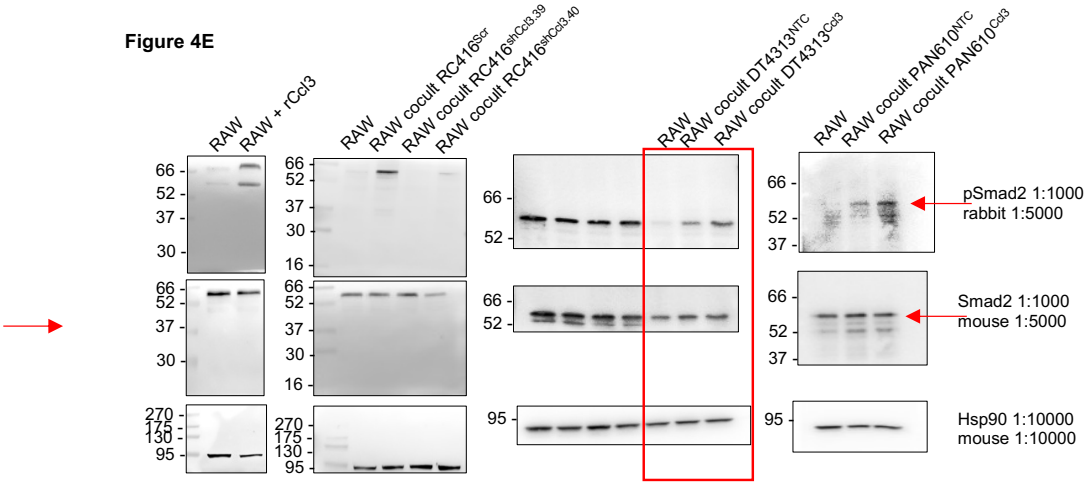


Figure 4G

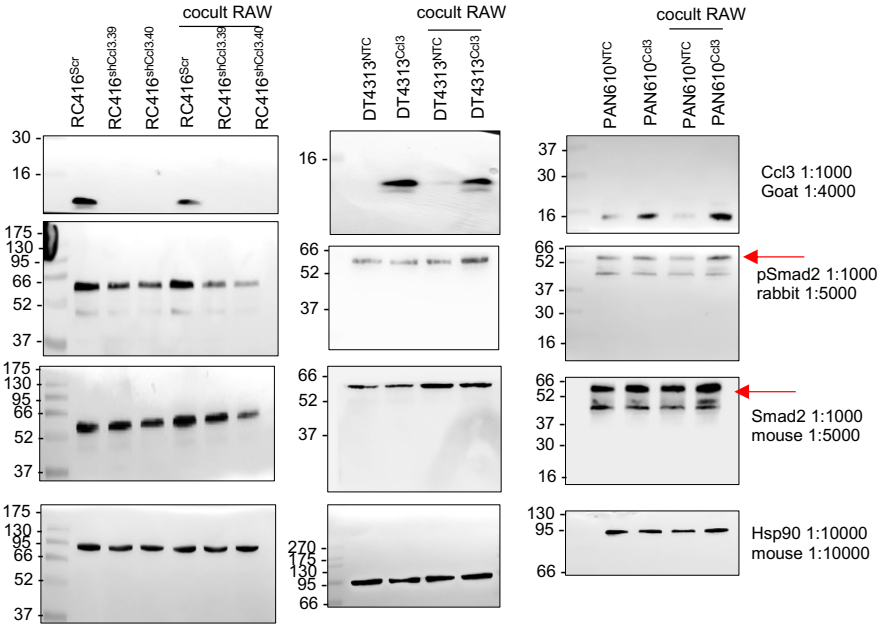


Figure 5C

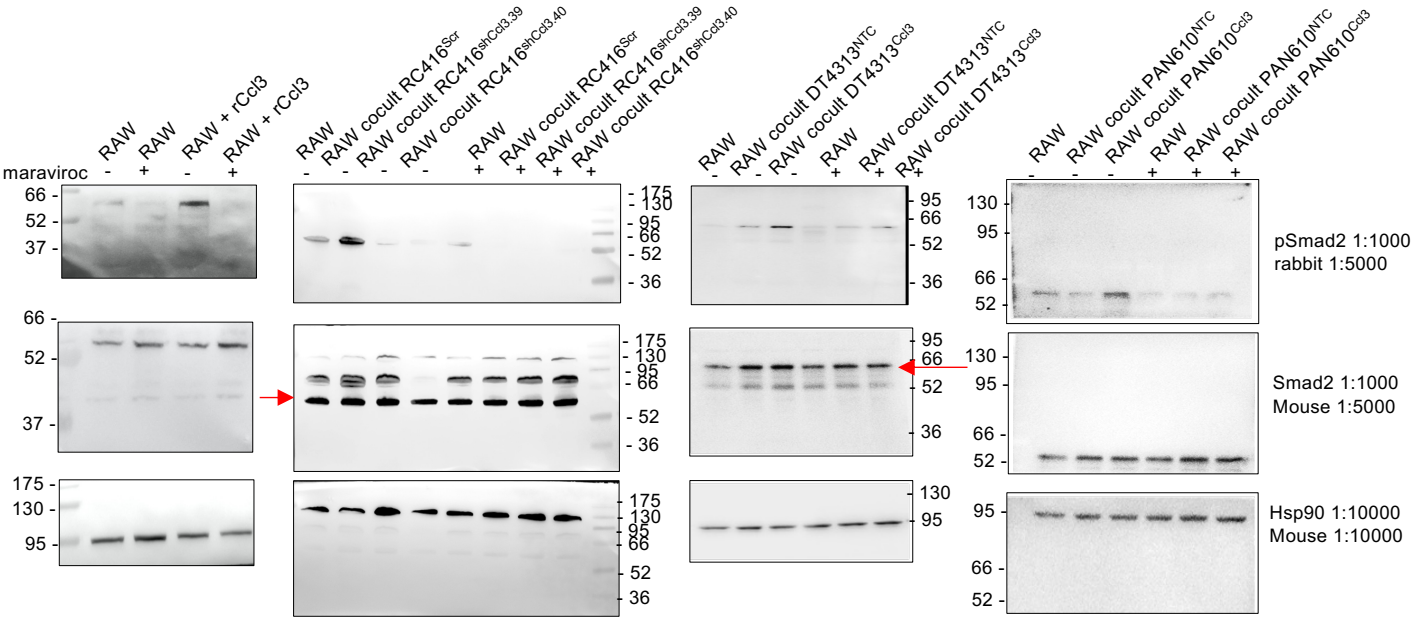


Figure 5F

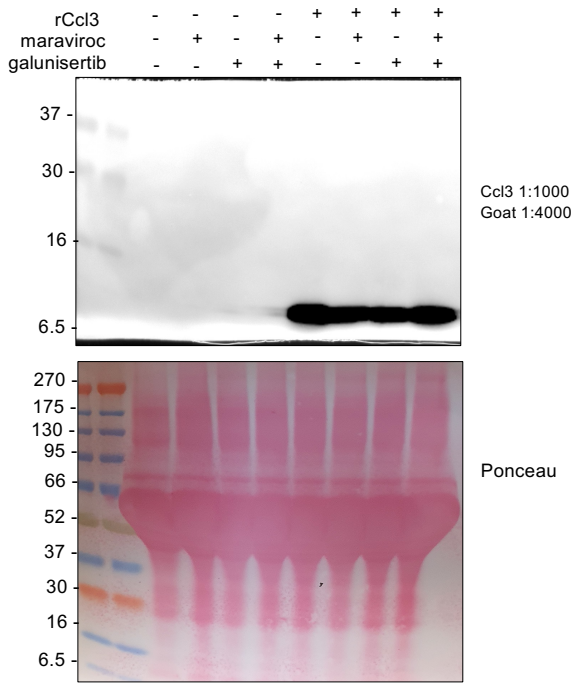


Figure 5I

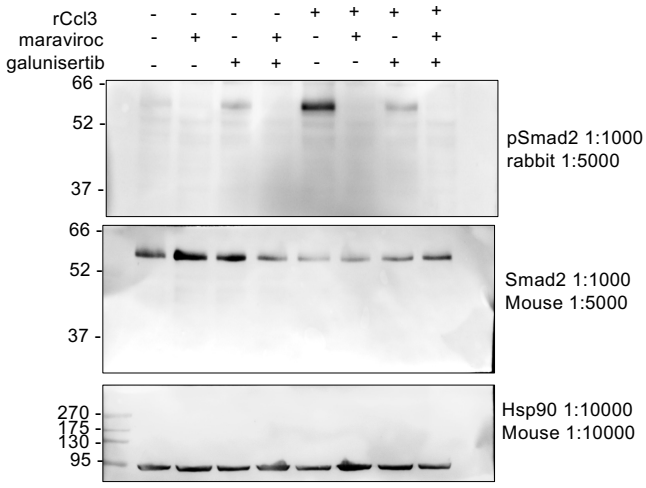


Figure 6A

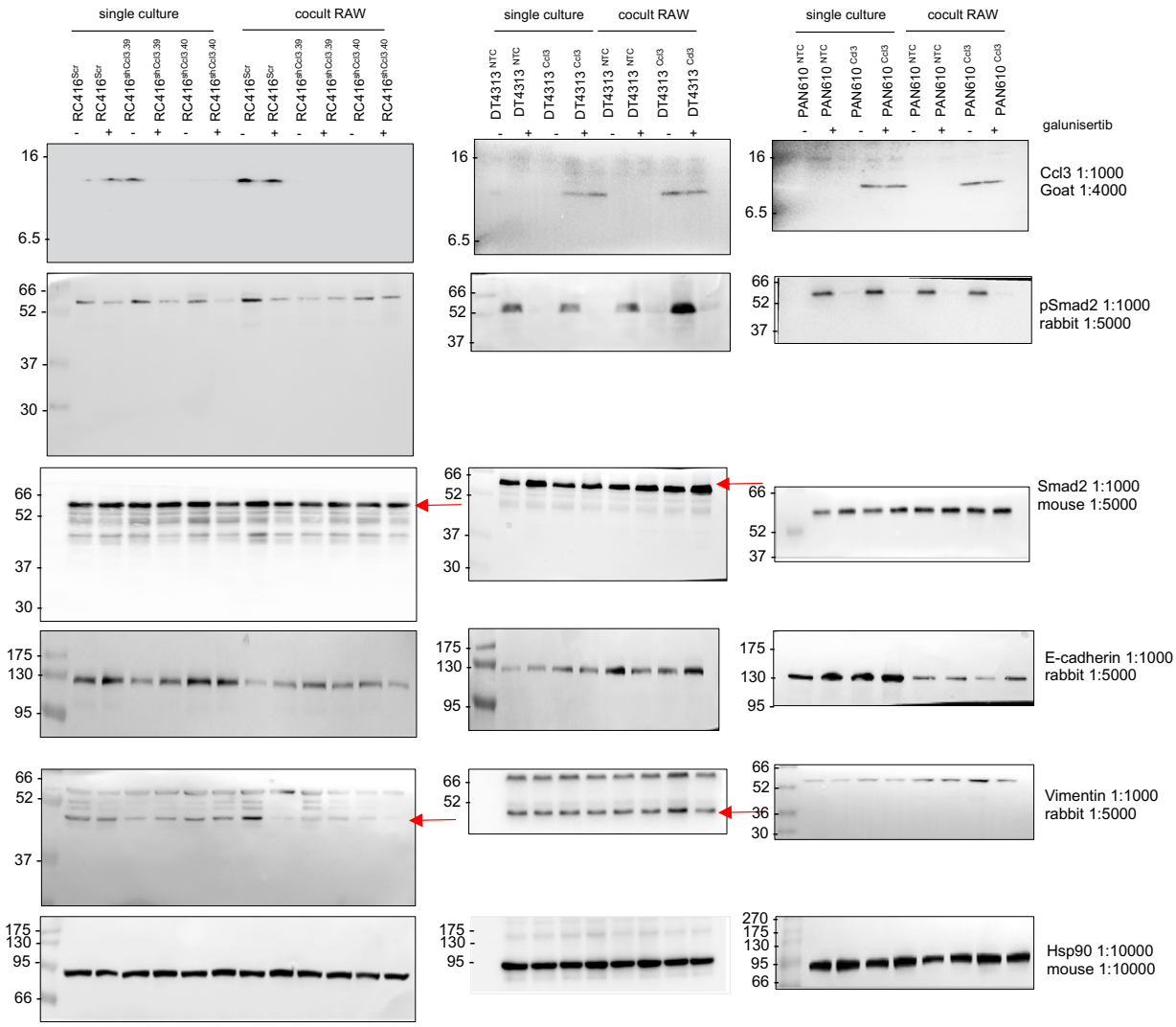

single culture
cocult RAW
RC416Scr
RC416shCcl3.39
RC416shCcl3.40
DT4313 NTC
DT4313 Ccl3
PAN610 NTC
PAN610 Ccl3
galunisertib
Ccl3 1:1000
Goat 1:4000
pSmad2 1:1000
rabbit 1:5000
Smad2 1:1000
mouse 1:5000
E-cadherin 1:1000
rabbit 1:5000
Vimentin 1:1000
rabbit 1:5000
Hsp90 1:10000
mouse 1:10000

Figure 6G

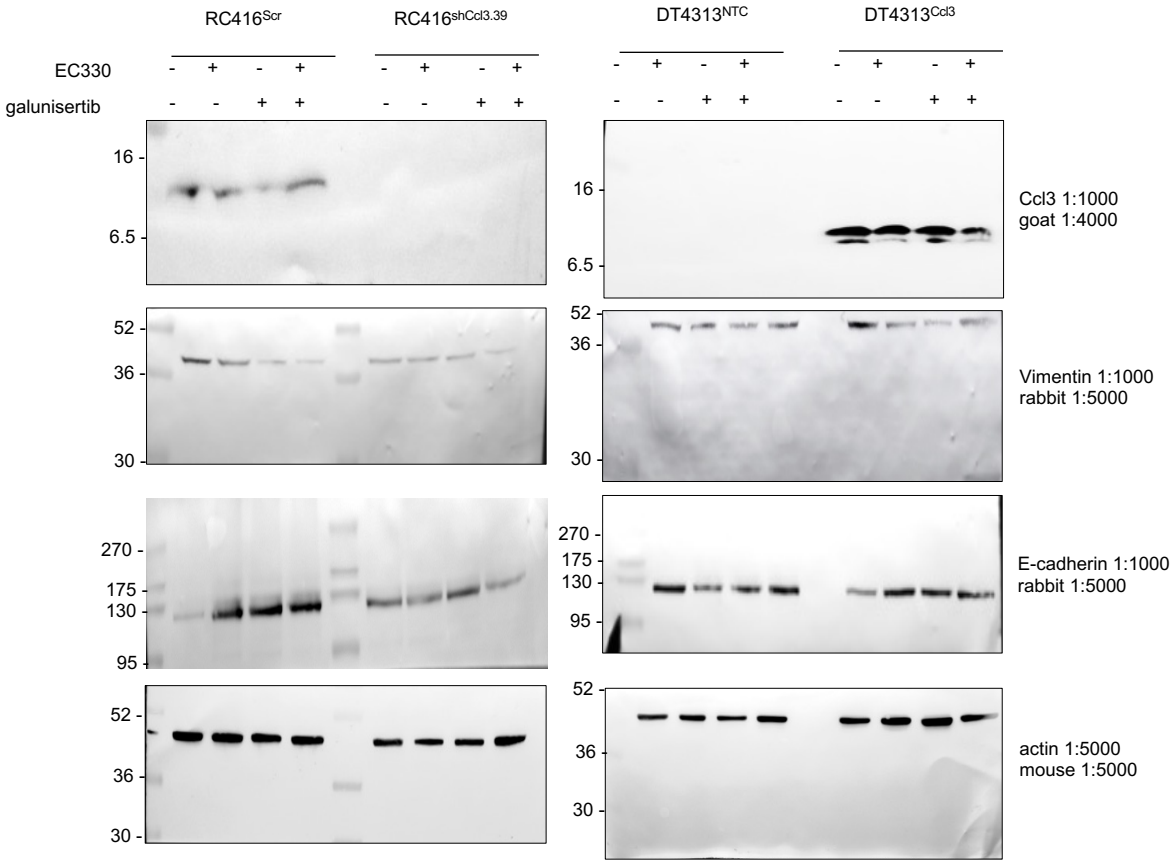

RC416Scr
RC416shCcl3.39
DT4313NTC
DT4313Ccl3
EC330
galunisertib
16
6.5
52
36
30
270
175
130
95
Ccl3 1:1000
goat 1:4000
Vimentin 1:1000
rabbit 1:5000
E-cadherin 1:1000
rabbit 1:5000
actin 1:5000
mouse 1:5000

Figure S1B

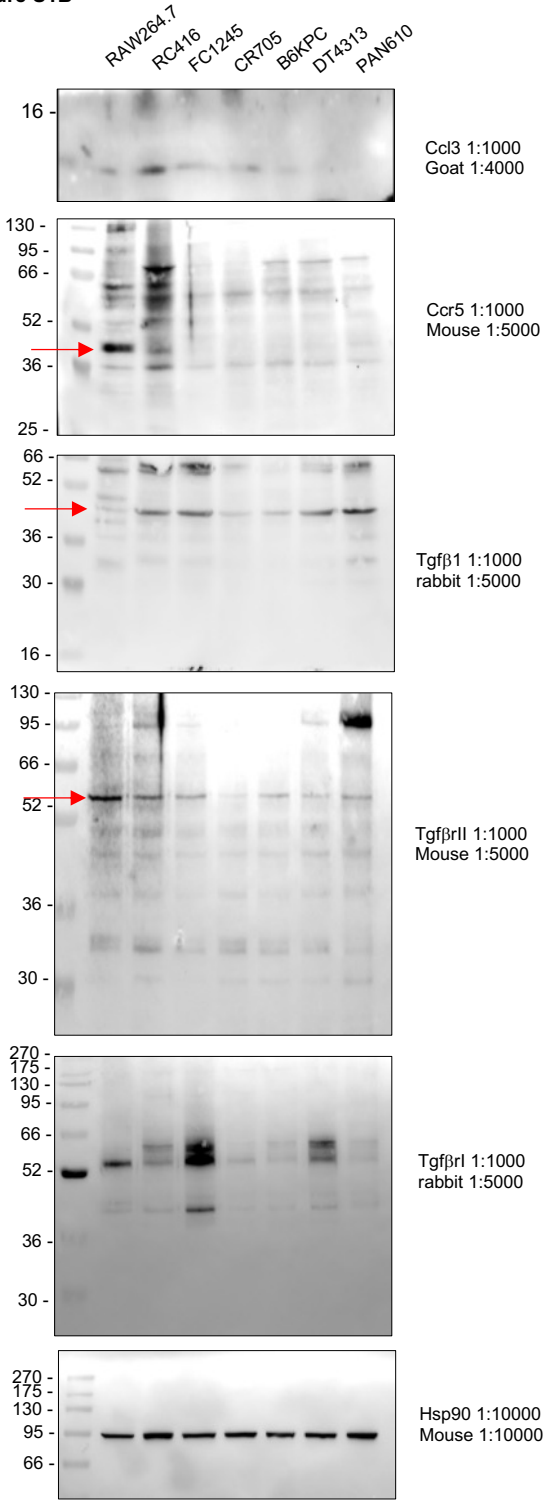


Figure S1C

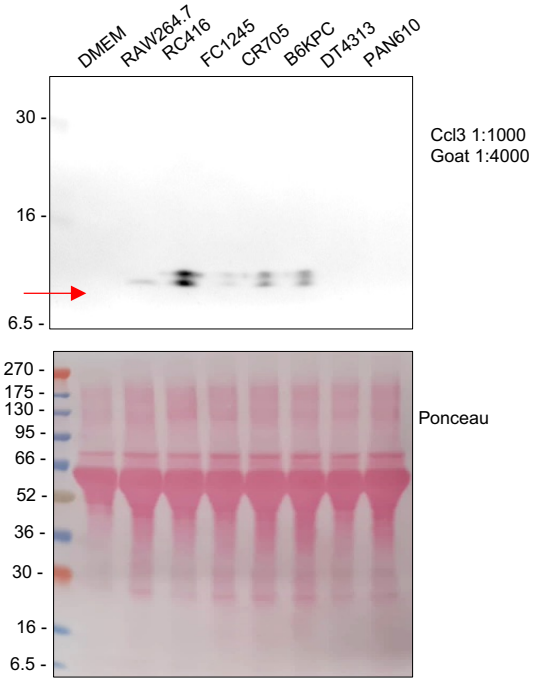


Figure S3G

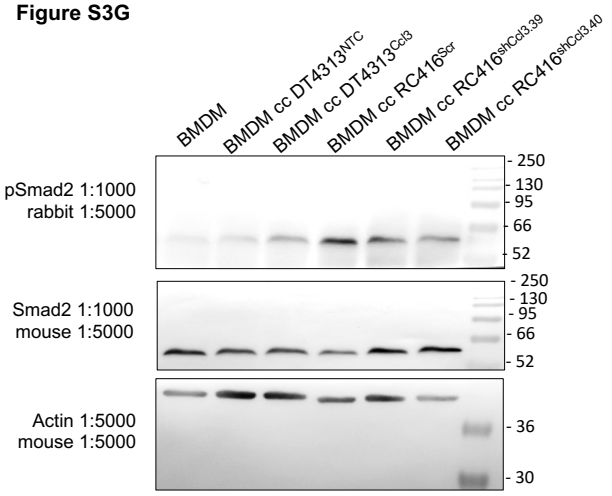


Figure S7A

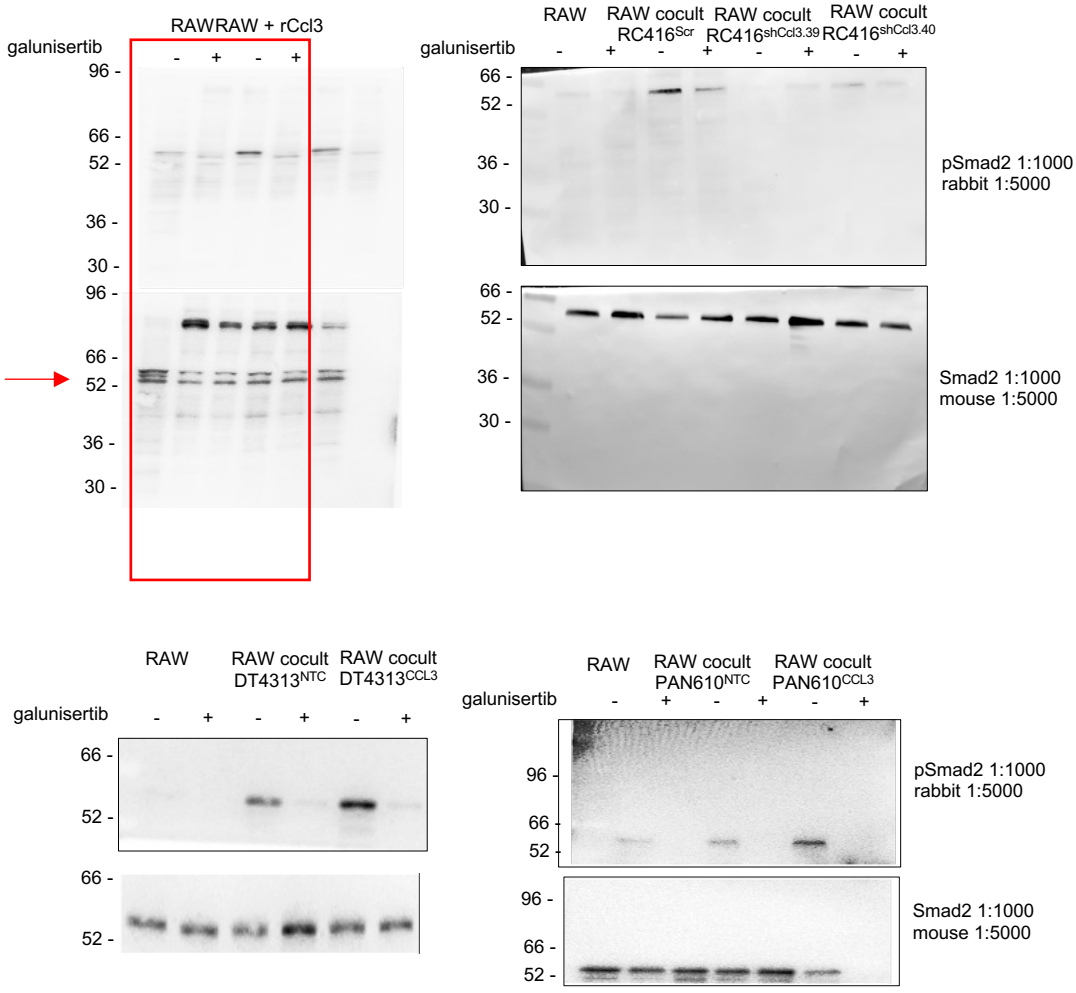


Figure S8A,B

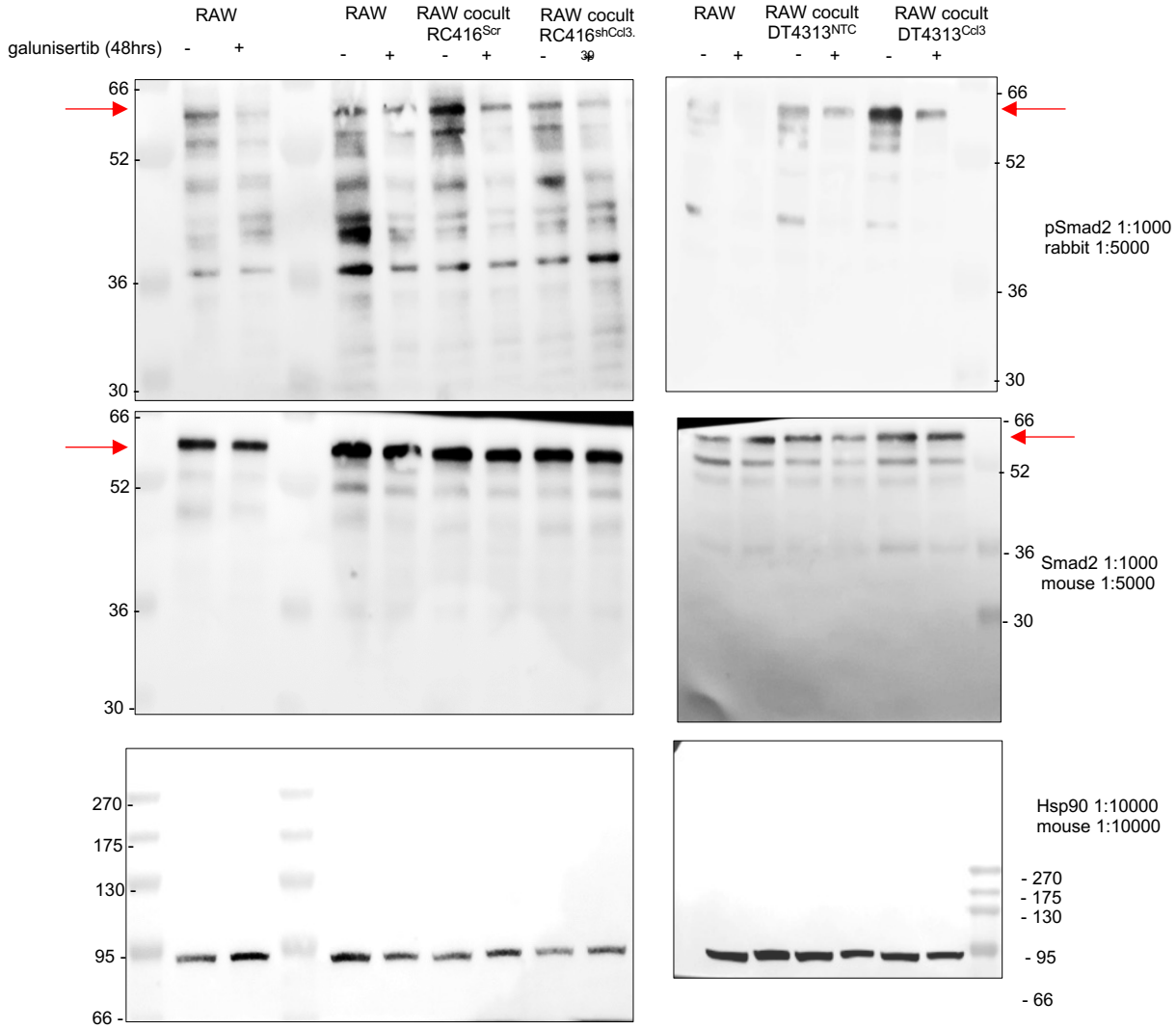
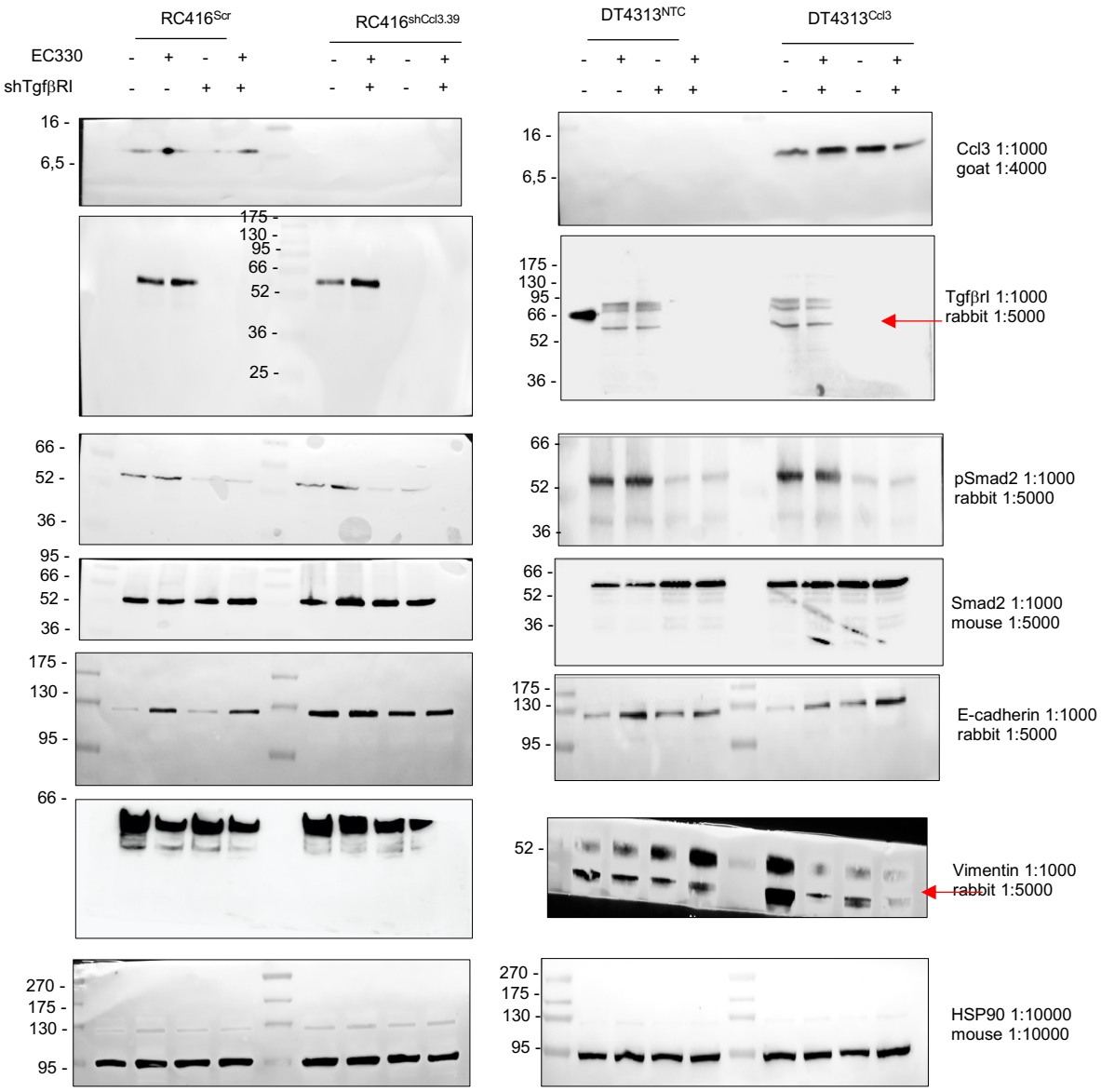

RAW
RAW
RAW cocult RC416Scr
RAW cocult RC416shCcl3.
RAW
RAW cocult DT4313NTC
RAW cocult DT4313Ccl3
galunisertib (48hrs) - + - + - + - + - + - +
66
52
36
30
pSmad2 1:1000
rabbit 1:5000
Smad2 1:1000
mouse 1:5000
270
175
130
95
66
Hsp90 1:10000
mouse 1:10000

Figure S9B


RC416Scr
RC416shCcl3.39
DT4313NTC
DT4313Ccl3
EC330
shTgfβRI
Ccl3 1:1000
goat 1:4000
Tgfβrl 1:1000
rabbit 1:5000
pSmad2 1:1000
rabbit 1:5000
Smad2 1:1000
mouse 1:5000
E-cadherin 1:1000
rabbit 1:5000
Vimentin 1:1000
rabbit 1:5000
HSP90 1:10000
mouse 1:10000