Supplementary Information for

# mTORC1 upregulates B7-H3/CD276 to inhibit antitumor T cells and drive tumor immune evasion

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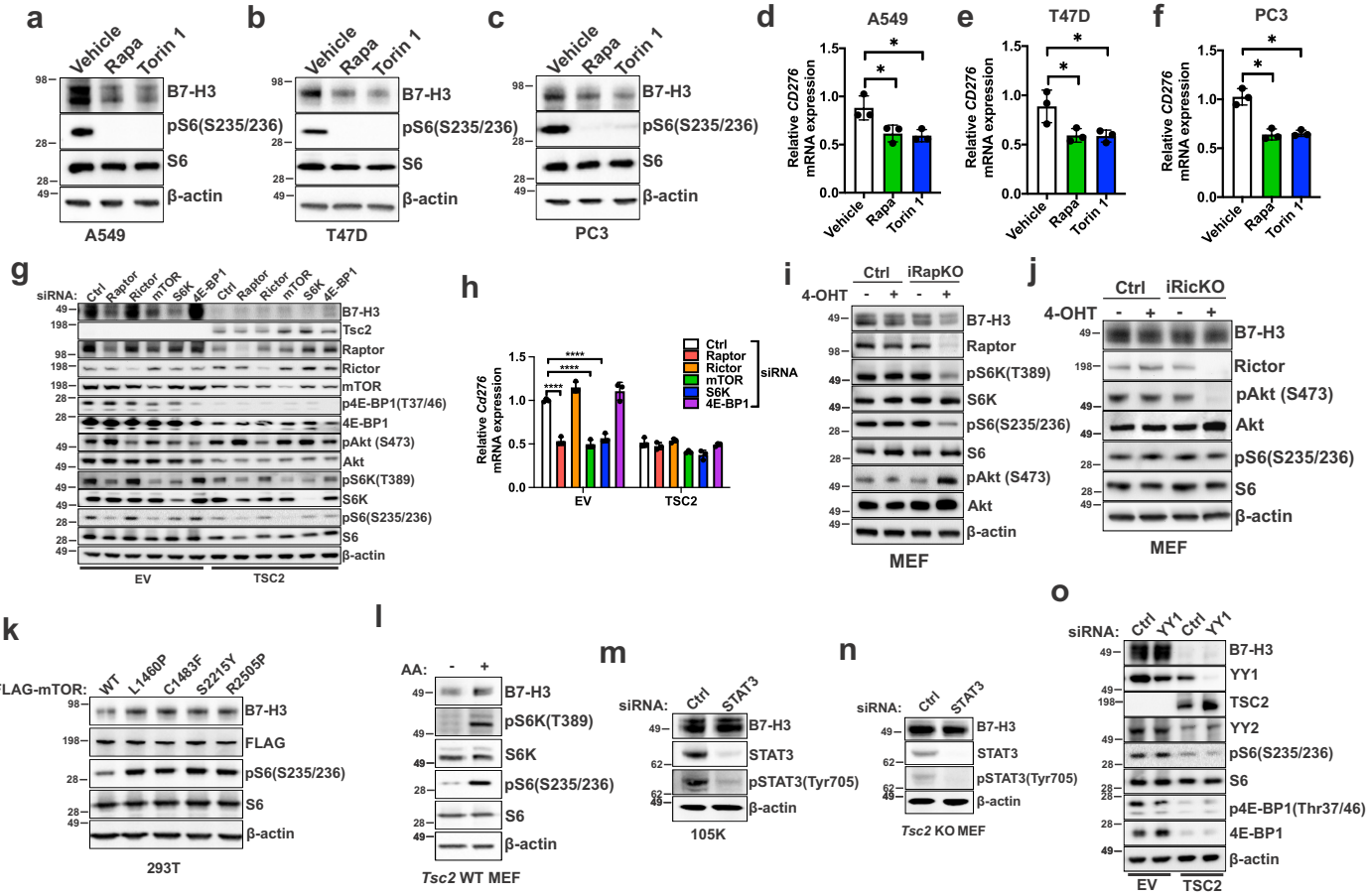
# Supplementary Fig. 1



**Supplementary Fig. 1. B7-H3 expression is regulated by mTORC1, but not mTORC2, STAT3 or YY1.**

**a-c,** Suppression of B7-H3 protein expression in A549 cells **(a)**, T47D cells **(b)**, and PC3 cells **(c)** after treatment with Rapamycin (Rapa) or Torin 1 for 24 hrs (n = 3).

**d-f,** Suppression of B7-H3 mRNA expression in A549 cells **(d)**, T47D cells **(e)**, and PC3 cells **(f)** (n = 3) treated as in **a-c**. Means ± SD, one-way ANOVA with Dunnett's test, * $p < 0.05$.

**g,** Suppression of B7-H3 with downregulation of Raptor, mTOR, and S6K. *Tsc2-/-* 105K cells with stable reconstitution of TSC2 or empty vector (EV) were transfected with non-targeting control siRNA (Ctrl) or SMARTpool siRNA targeting Raptor, Rictor, mTOR, S6K, or 4E-BP1 for 48 hr (n = 3).

**h,** B7-H3 mRNA expression in the cells from **(g)** (n = 3). Means ± SD, two-way ANOVA with Holm-Sidak's multiple comparisons test, **** $p < 0.0001$.

**i,** Knockout of Raptor in iRapKO MEFs decreases B7-H3 expression. iRapKO or control (Ctrl) MEFs were treated with 1 μM 4-hydroxytamoxifen (4-OHT) for 72 hr (n = 3).

**j,** Knockout of Rictor in iRicKO MEFs does not affect B7-H3 expression. iRicKO or control (Ctrl) MEFs were treated with 1 μM 4-hydroxytamoxifen (4-OHT) for 72 hr (n = 3).

**k,** Immunoblot analysis of HEK293T cells transfected with wild-type mTOR (FLAG-mTOR-WT), constitutive active mutant mTOR in expression vectors followed by whole-cell-lysis 48 hr after transfection (n = 3).

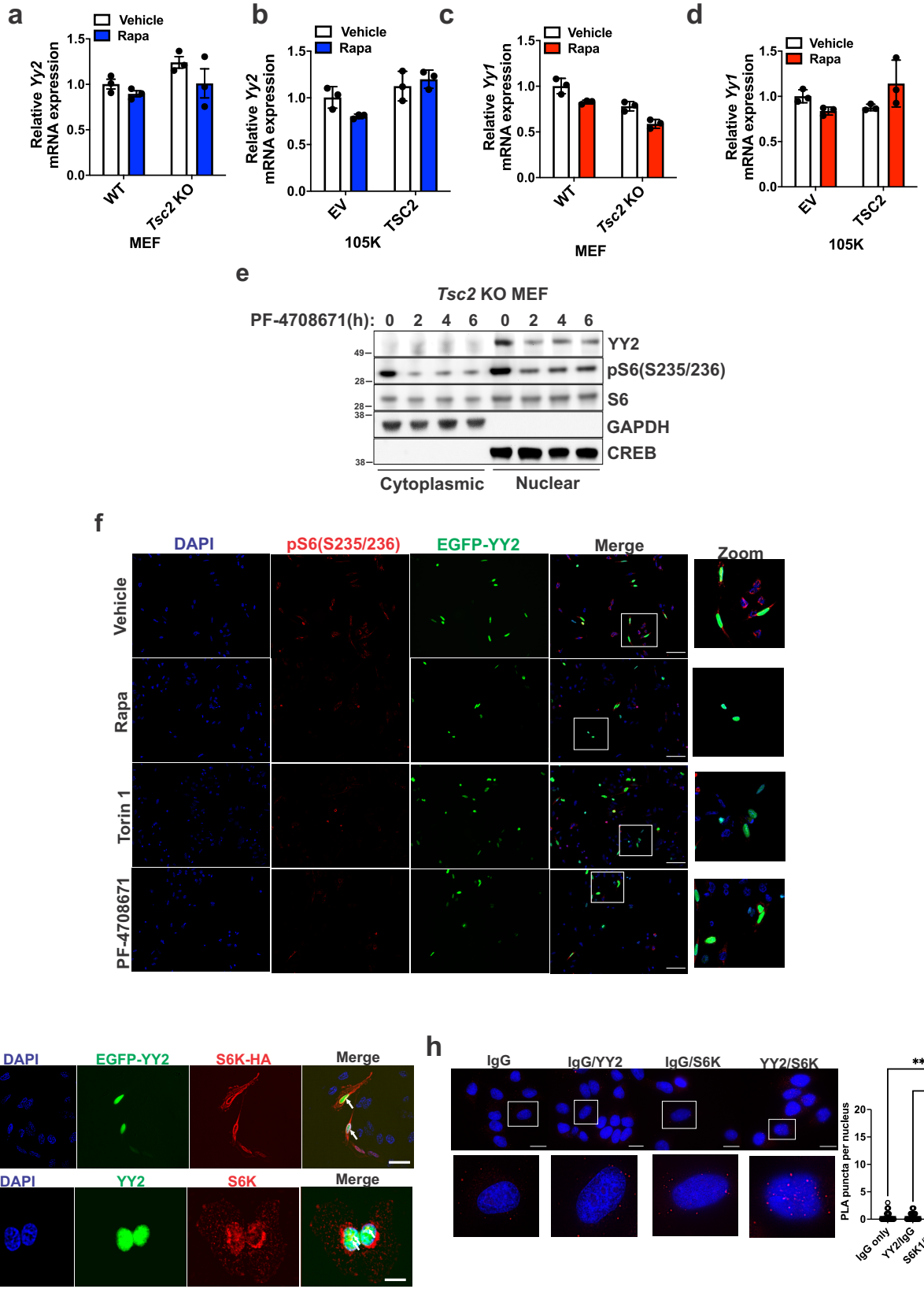
**l,** Immunoblot analysis of WT MEFs that were starved of amino acid for 16 hr and re-stimulated with MEM amino acid for 24 hr (n = 3).

**m,n,** B7-H3 protein expression in *Tsc2-/-* 105K cells **(m)** and *Tsc2* KO MEFs **(n)** following transfection with control siRNA or SMARTpool siRNA targeting STAT3 for 48 hr (n = 3).

**o,** B7-H3 mRNA expression in *Tsc2-/-* 105K cells with stable reconstitution of TSC2 or empty vector (EV) following transfection with control siRNA (Ctrl) or SMARTpool siRNAs targeting YY1 for 48 hr (n = 3).

Source data and exact p values are provided in the Source data file.

# Supplementary Fig. 2



**Supplementary Fig. 2. S6K forms a complex with YY2 in the nucleus.**

**a,b,** YY2 mRNA expression in *Tsc2*-WT and *Tsc2* KO MEFs **(a)** and *Tsc2-/-* 105K cells with stable reconstitution of TSC2 or empty vector (EV) **(b)** following 20 nM Rapamycin (Rapa) or vehicle control for 24 hr (n = 3). Means ± SD, two-tailed unpaired student's t-test.

**c,d,** YY1 mRNA expression in *Tsc2*-WT and *Tsc2* KO MEFs **(c)** and *Tsc2-/-* 105K cells with stable reconstitution of TSC2 or empty vector (EV) **(d)** following 20 nM Rapamycin (Rapa) or vehicle control for 24 hr (n = 3). Means ± SD, two-tailed unpaired student's t-test.

**e,** Cytoplasmic and nuclear fractions of *Tsc2* KO MEFs following 10 μM PF-4708671 treatment for the indicated time points (n = 3).

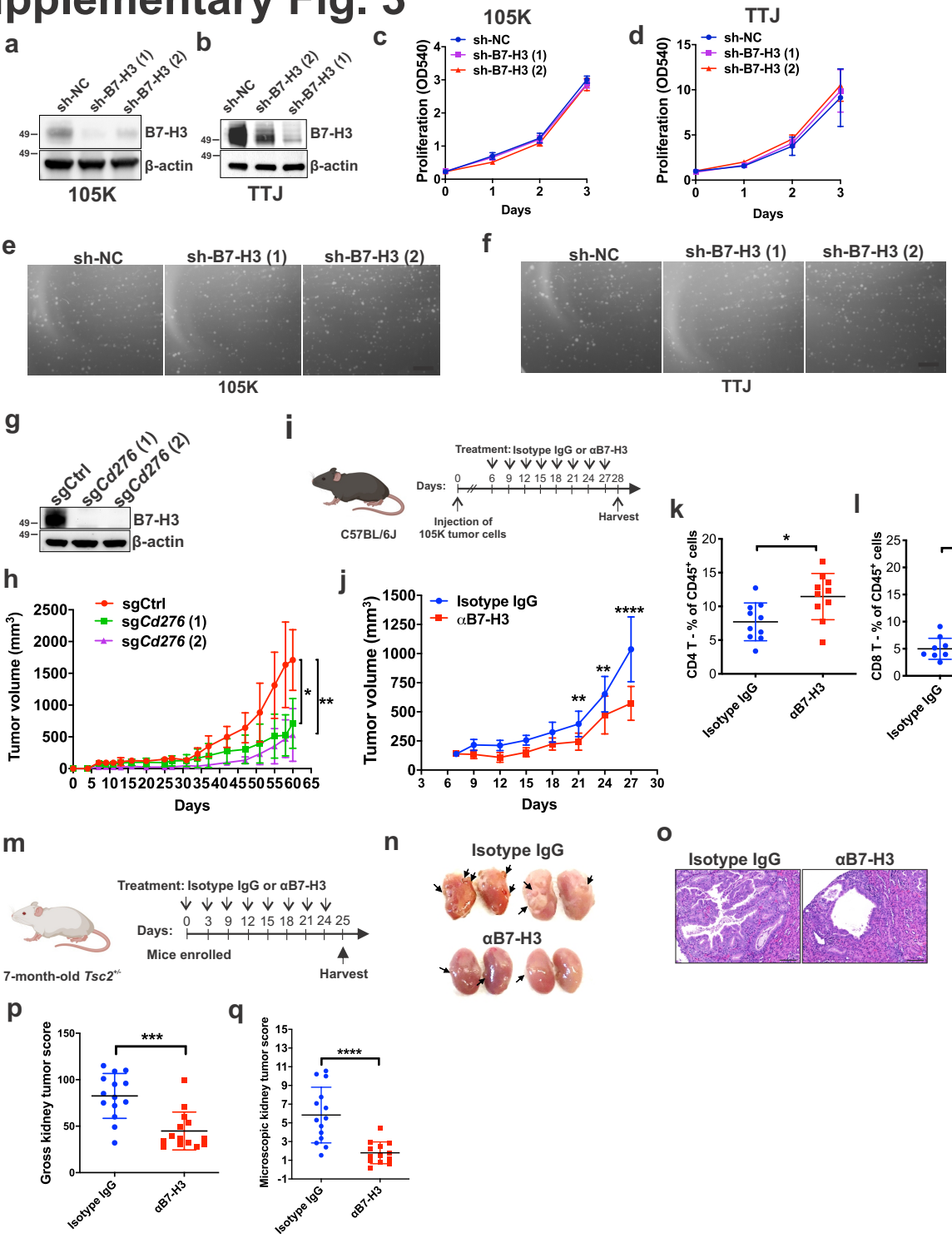
**f,** Immunofluorescent staining of HeLa cells stably expressing EGFP-YY2 and treated with 20 nM rapamycin (Rapa), 500 nM Torin 1, 30 μM PF-4708671 or vehicle for 2 hr. Cells were fixed and stained with pS6 (S235/S236) and DAPI (n = 3). Scale bar = 100 μm.

**g,** Immunofluorescent staining of parental HeLa cells with anti-YY2 and S6K antibodies (n = 3). White arrows indicate co-localization of YY2 and S6K. Top panel: scale bar = 100 μm, bottom panel: scale bar = 20 μm.

**h,** Left panel: Representative images of in situ proximity ligation assay between S6K and YY2 (red) in HeLa cells. Data are representative of three independent experiments. Scale bar = 30 μm. Right panel: Quantification of the number of PLA puncta per nucleus. Column 1, IgG; column 2, mouse anti-YY2 and IgG; column 3, rabbit anti-S6K antibody and IgG; column 4, mouse anti-YY2 and rabbit anti-S6K antibodies. n = 30 cells. Means ± SD, one-way ANOVA with Dunnett's multiple comparisons test, **** $p < 0.0001$.

Source data are provided in the Source data file.

# Supplementary Fig. 3



**Supplementary Fig. 3. The effects of B7-H3 inhibition *in vitro* and *in vivo*.**

**a,b,** Immunoblot of sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* 105K cells **(a)** or *Tsc2-/-* TTJ cells **(b)** (n = 3).

**c,d,** Cell proliferation assessed by crystal violet staining of the cells in **(c)** and **(d)** (n = 8). Means ± SD, one-way ANOVA with Dunnett's multiple comparisons test.

**e,f,** Representative images of the cells in **a, b** grown in soft agar for 3 weeks (n = 3).

**g,** Immunoblot analysis of whole-cell lysates derived from sgCtrl, sg*Cd276* (1), and sg*Cd276* (2) *Tsc2-/-* 105K cells (n = 3).

**h,** Subcutaneous growth of sgCtrl, sg*Cd276* (1), and sg*Cd276* (2) *Tsc2-/-* 105K cells in wild-type C57BL/6J mice. n = 7 mice for sgCtrl and sg*Cd276* (1), n = 8 mice for sg*Cd276* (2). Means ± SD, nonparametric Kruskal-Wallis's test with Dunn's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$.

**i,** Experimental design for treatment of *Tsc2-/-* 105K tumors with isotype control antibody (Isotype IgG) or anti-B7-H3 antibody (α-B7-H3) (Created with Biorender.com).

**j,** Growth of *Tsc2-/-* 105K tumors treated as depicted in i. n = 9 mice for Isotype IgG, n = 10 mice for anti-B7-H3 antibody. Means ± SD, Mann-Whitney U Test, ** $p < 0.01$, **** $p < 0.0001$.

**k,l,** Flow cytometry analysis of tumor-infiltrating $CD4^+$ **(k)** and $CD8^+$ **(l)** T cells from *Tsc2-/-* 105K tumors treated as depicted in (I). 10 mice per treatment group. Means ± SD, Mann-Whitney U Test, * $p < 0.05$.

**m,** Experimental design for $Tsc2^{+/-}$ mice treated with isotype control antibody (Isotype IgG) or anti-B7-H3 antibody (α-B7-H3) (Created with Biorender.com).

**n,** Representative kidneys of $Tsc2^{+/-}$ mice treated as depicted in **m**.

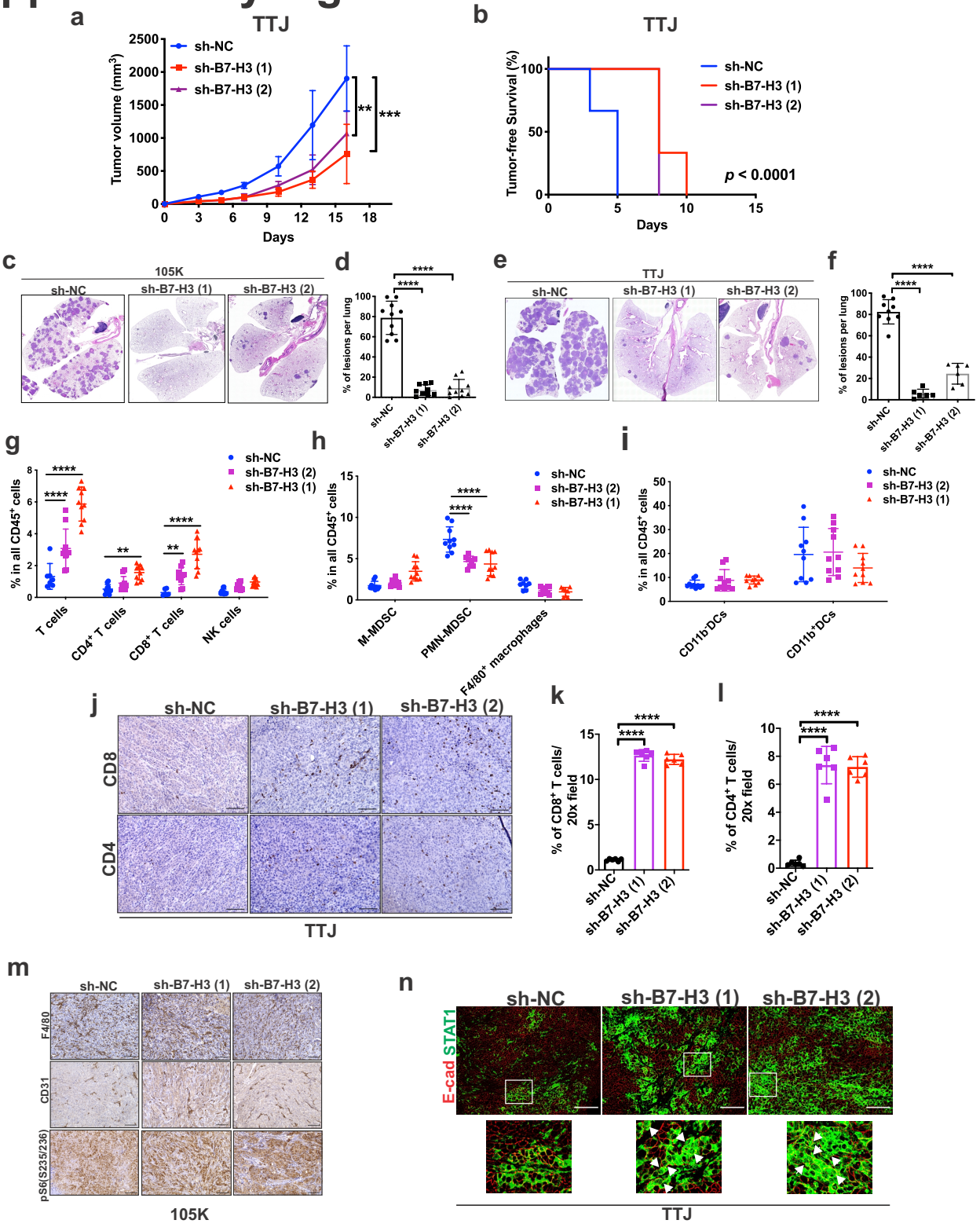
**o,** Representative H&E images of $Tsc2^{+/-}$ mice treated as depicted in **m**.

**p,** Gross tumor score of kidneys of $Tsc2^{+/-}$ mice treated as depicted in **m**. n = 14 kidneys per treatment group, each dot represents one kidney. Means ± SD, Mann-Whitney U Test, *** $p < 0.001$.

**q,** Microscopic tumor score of $Tsc2^{+/-}$ mice treated as depicted in **m**. n = 14 kidneys per treatment group, each dot represents one kidney. Means ± SD, Mann-Whitney U Test, **** $p < 0.0001$.

Source data and exact p values are provided in the Source data file.

# Supplementary Fig. 4



**Supplementary Fig. 4. B7-H3 deficiency in *Tsc2-/-* TTJ tumor cells suppresses subcutaneous tumor growth and lung tumor burden in models of metastasis.**

**a,** Subcutaneous tumor growth of sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* TTJ tumors in WT C57BL/6J mice. n = 6 mice per group, means ± SD, one-way ANOVA with Dunnett's multiple comparisons test, ** $p < 0.01$, *** $p < 0.001$.

**b,** Tumor-free survival curve of mice in **a**. Log-rank analysis.

**c,** Representative H&E images of lungs with sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* 105K tumors in WT C57BL/6J mice.

**d,** Percentage of lung area occupied by tumor per lung in **(c)**. n = 9 mice for sh-NC and sh-B7-H3 (2), n = 10 mice for sh-B7-H3 (1), means ± SD, one-way ANOVA with Dunnett's multiple comparisons test, **** $p < 0.0001$.

**e,** Representative H&E images of lungs with sh-NC, sh-B7-H3 (1), or sh-B7-H3 (2) *Tsc2-/-* TTJ tumors in WT C57BL/6J mice.

**f,** Percentage of lung area occupied by tumor per lung in (E). n = 6-9 mice per group, means ± SD, one-way ANOVA with Dunnett's multiple comparisons test, **** $p < 0.0001$.

**g-i,** Percentage of indicated cell types within $CD45^+$ TILs from sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* TTJ tumors. n = 7 tumors for T cells, n = 10 tumors for $CD4^+$ T and NK cells, n = 6 tumors for $CD8^+$ T cells in sh-NC, n = 10 tumors for all cell types in sh-B7-H3 (1), and sh-B7-H3 (2), means ± SD, two-way ANOVA with Holm-Sidak's multiple comparisons test, ** $p < 0.01$, **** $p < 0.0001$.

**j-l,** Representative images of CD4 and CD8 immunohistochemical staining on sections from *Tsc2-/-* sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) TTJ subcutaneous tumors **(j)**. Scale bar = 100 μm. Quantification of $CD8^+$ **(k)** or $CD4^+$ **(l)** T cells in each group (n = 6/group). Means ± SD, one-way ANOVA with Dunnett's multiple comparisons test, **** $p < 0.0001$.

**m,** Representative images of F4/80, CD31, and pS6 (S235/236) immunohistochemical staining on tumor sections from *Tsc2-/-* 105K subcutaneous tumors transduced with sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) (n = 3). Scale bar = 100 μm.

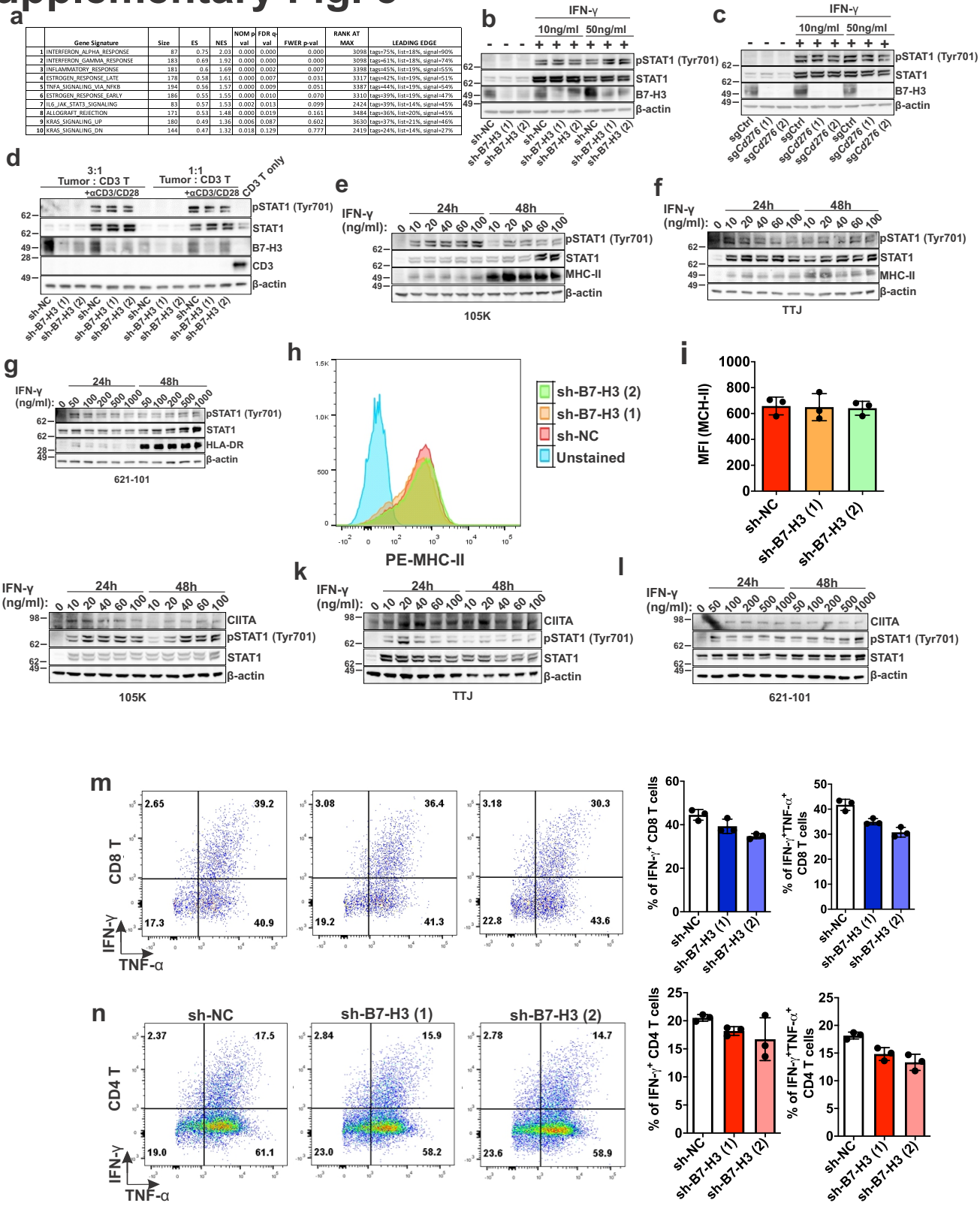
**n,** Representative images of E-cadherin and STAT1 immunofluorescent staining on subcutaneous TTJ tumor sections of the indicated groups (n = 3). Scale bar = 100 μm. White arrows indicate cells with nuclear STAT1.

Source data and exact p values are provided in the Source data file.

# Supplementary Fig. 5

**a**

| | Gene Signature | Size | ES | NES | NOM p-val | FDR q-val | FWER p-val | RANK AT MAX | LEADING EDGE |
|---|---|---|---|---|---|---|---|---|---|
| 1 | INTERFERON_ALPHA_RESPONSE | 87 | 0.75 | 2.03 | 0.000 | 0.000 | 0.000 | 3098 | tags=75%, list=18%, signal=90% |
| 2 | INTERFERON_GAMMA_RESPONSE | 183 | 0.69 | 1.92 | 0.000 | 0.000 | 0.000 | 3098 | tags=61%, list=18%, signal=74% |
| 3 | INFLAMMATORY_RESPONSE | 181 | 0.6 | 1.69 | 0.000 | 0.002 | 0.007 | 3398 | tags=45%, list=19%, signal=55% |
| 4 | ESTROGEN_RESPONSE_LATE | 178 | 0.58 | 1.61 | 0.000 | 0.007 | 0.031 | 3317 | tags=42%, list=19%, signal=51% |
| 5 | TNFA_SIGNALING_VIA_NFKB | 194 | 0.56 | 1.57 | 0.000 | 0.009 | 0.051 | 3387 | tags=44%, list=19%, signal=54% |
| 6 | ESTROGEN_RESPONSE_EARLY | 186 | 0.55 | 1.55 | 0.000 | 0.010 | 0.070 | 3310 | tags=39%, list=19%, signal=47% |
| 7 | IL6_JAK_STAT3_SIGNALING | 83 | 0.57 | 1.53 | 0.002 | 0.013 | 0.099 | 2424 | tags=39%, list=14%, signal=45% |
| 8 | ALLOGRAFT_REJECTION | 171 | 0.53 | 1.48 | 0.000 | 0.019 | 0.161 | 3484 | tags=36%, list=20%, signal=45% |
| 9 | KRAS_SIGNALING_UP | 180 | 0.49 | 1.36 | 0.006 | 0.087 | 0.602 | 3630 | tags=37%, list=21%, signal=46% |
| 10 | KRAS_SIGNALING_DN | 144 | 0.47 | 1.32 | 0.018 | 0.129 | 0.777 | 2419 | tags=24%, list=14%, signal=27% |



**Supplementary Fig. 5. The effects of B7-H3 suppression *in vivo* and *in vitro* relating to IFN-γ.**

**a,** Top 10 activated gene signatures identified by gene set enrichment analysis in B7-H3 knockdown *Tsc2-/-* 105K subcutaneous tumors compared to controls (n = 3). ES = Enrichment Score, NES = Normalized Enrichment Score, NOM = Nominal, FDR = False Discovery Rate.

**b,** Immunoblot analysis of sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* 105K cells treated with IFN-γ for the indicated time points and concentrations (n = 3).

**c,** Immunoblot analysis of sgCtrl, sg*Cd276* (1), and sg*Cd276* (2) *Tsc2-/-* 105K cells treated with IFN-γ for the indicated time points and concentrations (n = 3).

**d,** Immunoblot analysis of cells in **(b)** co-cultured with CD3/CD28 activated splenic $CD3^+$ T cells at the indicated tumor: T-cell ratio. CD3 T only was used as a control (n = 3).

**e-g,** Immunoblot analysis of *Tsc2-/-* 105K cells **(e)**, *Tsc2-/-* TTJ cells **(f)**, and *TSC2-/-* 621-101 cells **(g)** treated with IFN-γ for the indicated concentrations and time durations (n = 3).

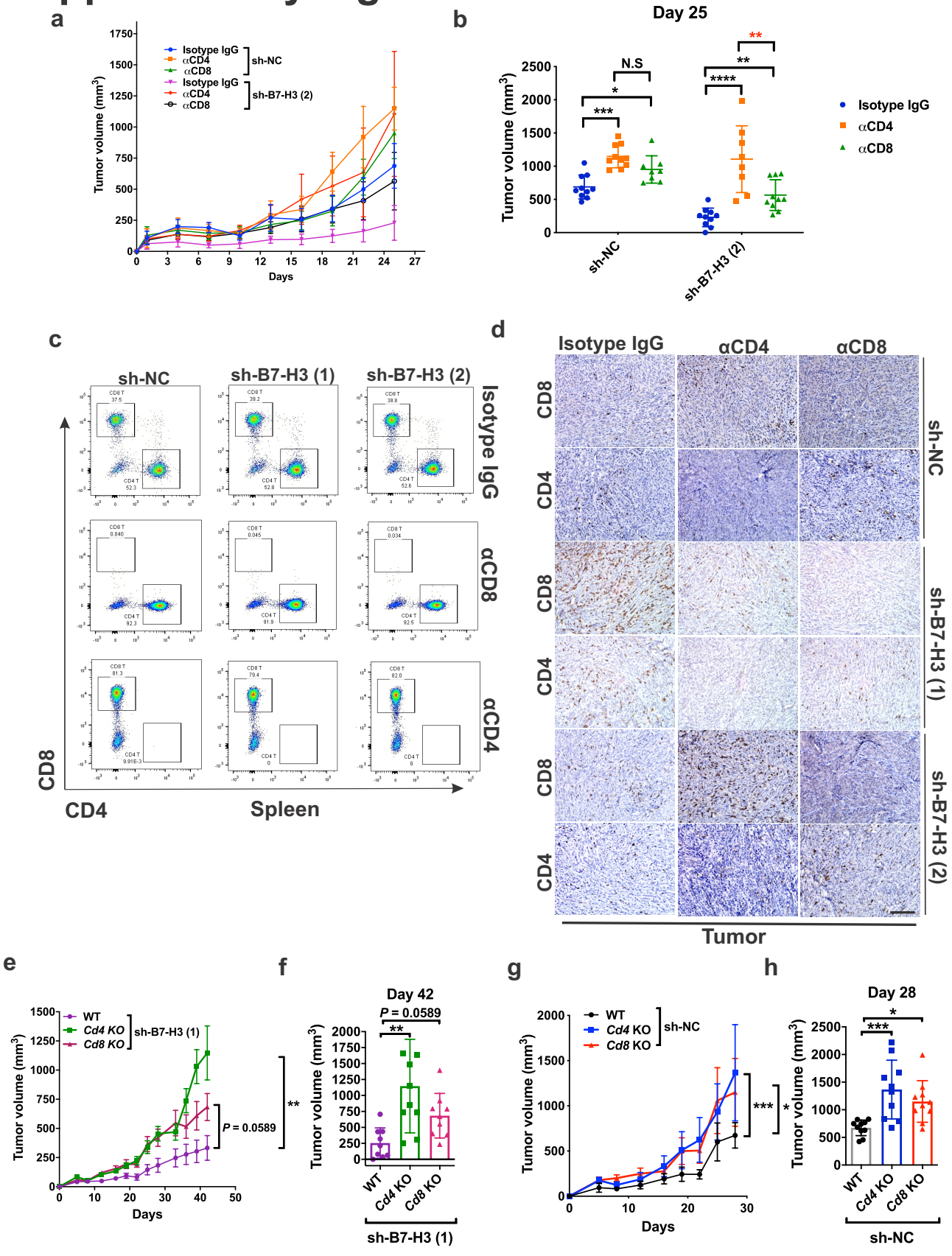
**h,i,** Flow cytometry analysis of MHC-II expression on sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* 105K cells. Cells were treated with 10ng/ml of IFN-γ for 48 hr. **(h)**. Mean fluorescent intensity (MFI) of MCH-II measured from each group of cells (n = 3) **(i)**. Means ± SD, one-way ANOVA with Dunnett's multiple comparisons test.

**j-l,** Immunoblot analysis of *Tsc2-/-* 105K cells **(j)**, *Tsc2-/-* TTJ cells **(k)**, and *TSC2-/-* 621-101 cells **(l)** treated with IFN-γ for the indicated concentrations and time durations (n = 3).

**m,n** Left panels: Representative flow cytometry plots showing the percentage of IFN-γ/TNF-α-positive splenic $CD8^+$ T cells **(m)** or $CD4^+$ T cells **(n)** incubated with conditioned media isolated from cells in **(b)**. Middle and right panels: Quantification of the left panel. Means ± SD, one-way ANOVA with Dunnett's multiple comparisons test (n = 3).

Source data are provided in the Source data file.

# Supplementary Fig. 6



**Supplementary Fig. 6. Anti-tumor effect of B7-H3 inhibition depends more on $CD4^+$ T cells compared to $CD8^+$ T cells.**

**a,** Growth of sh-NC or sh-B7-H3 (2) *Tsc2-/-* 105K tumors treated with isotype control, α-CD4 or α-CD8 antibodies (n = 10 mice/group).

**b,** Tumor volume of (A) at 25-day post-cell injection. n = 10 tumors for sh-NC treated with isotype control, sh-NC treated with α-CD4, and sh-B7-H3 (2) treated with α-CD8, and n = 8 tumors for the remaining groups, means ± SD, two-way ANOVA with Holm-Sidak's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

**c,** Representative flow cytometry plots showing that splenic $CD8^+$ and $CD4^+$ T cells were successfully depleted. (n = 5/group).

**d,** Representative immunohistochemical staining of CD8 and CD4 on tissue sections of sh-NC, sh-B7-H3 (1), or sh-B7-H3 (2) *Tsc2-/-* 105K tumors treated with the indicated antibody. Scale bar = 100 μm. (n = 5/group).

**e,** Growth of sh-B7-H3 (1) *Tsc2-/-* 105K tumors in WT, *Cd4* KO, or *Cd8* KO mice. n = 9 mice for WT, *Cd8* KO, n = 9 mice for *Cd4* KO, means ± SD, nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test, ** $p < 0.01$.

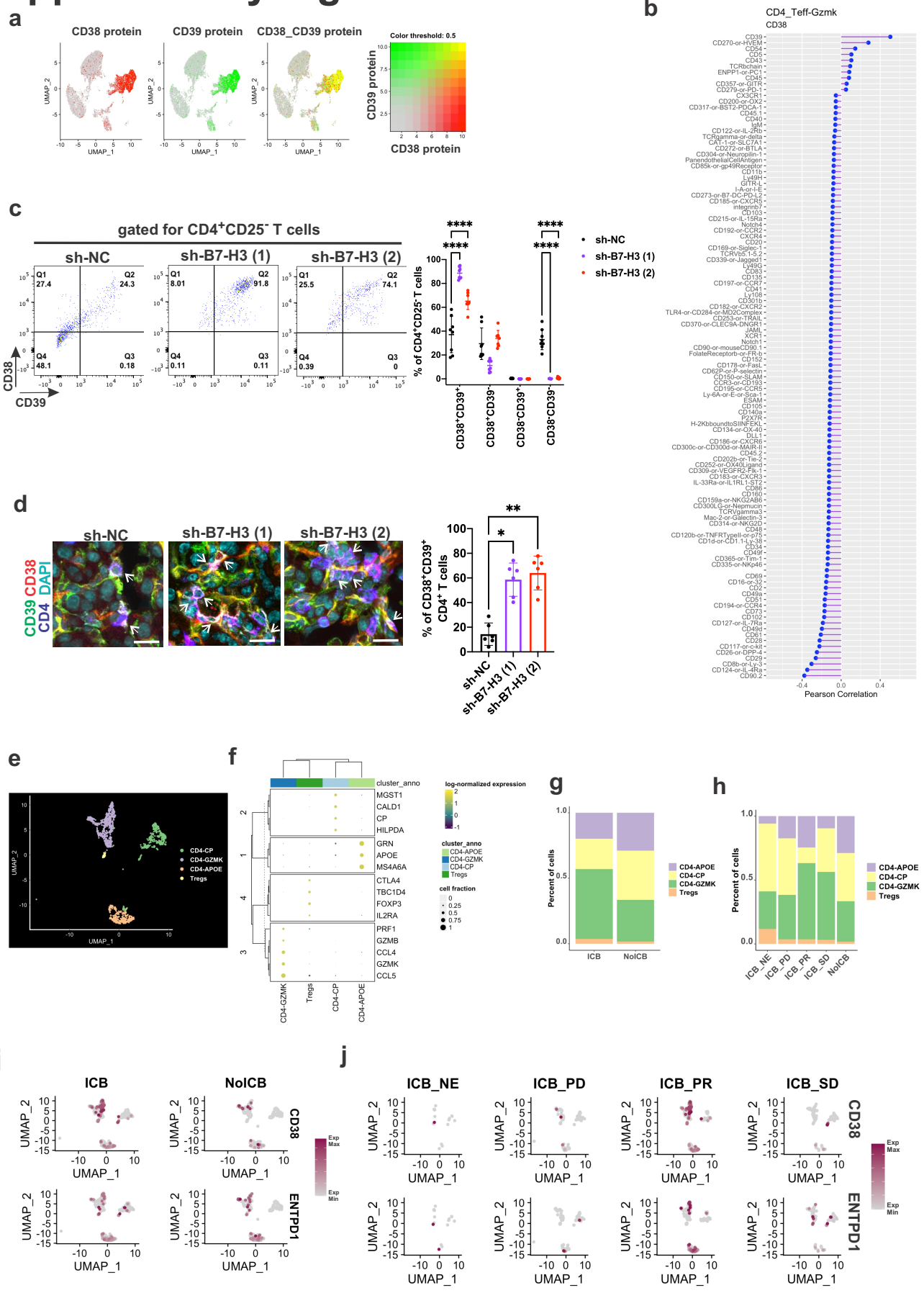
**f,** Tumor volume of **e** at 42-day post-cell injection. n = 9 mice for WT, *Cd8* KO, n = 9 mice for *Cd4* KO, means ± SD, nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test, ** $p < 0.01$.

**g,** Growth of sh-NC *Tsc2-/-* 105K tumors in WT, *Cd4* KO, or *Cd8* KO mice. n = 10 mice/group, means ± SD, nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test, * $p < 0.05$, *** $p < 0.001$.

**h,** Tumor volume of **g** at 28-day post-cell injection. n = 10 mice/group, means ± SD, nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test, * $p < 0.05$, *** $p < 0.001$.

Source data and exact p values are provided in the Source data file.

# Supplementary Fig. 7



**Supplementary Fig. 7. Inhibition of B7-H3 increases the number of intratumoral $CD38^+CD39^+CD4^+$ TILs and analysis of $CD4^+$ T cells from advanced metastatic RCC patients.**

**a,** UMAP plots showing co-expression of CD38 and CD39 proteins in CD4_Teff-Gzmk cells.

**b,** Spearman correlation analysis of CD38 protein expression versus other proteins detected by CITE-seq in CD4_Teff-Gzmk cells.

**c,** Representative flow cytometry plots (left panel) and quantification of the percentage (right panel) of CD38 and CD39 on $CD4^+CD25^-$ TILs in sh-NC, sh-B7-H3 (1), and sh-B7-H3 (2) *Tsc2-/-* 105K tumors. n = 8 mice/group, mean ± SD, two-way ANOVA with Holm-Sidak's multiple comparisons test, **** $p < 0.0001$.

**d,** Representative immunofluoresent images (left panel) and quantification (right panel) of $CD38^+CD39^+CD4^+$ TILs in lungs of WT mice with sh-NC, sh-B7-H3 (1), or sh-B7-H3 (2) *Tsc2-/-* 105K tumors. n = 6 mice/group, mean ± SD, nonparametric Kruskal-Wallis test with Dunn's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$. White arrows showing $CD38^+CD39^+CD4^+$ TILs.

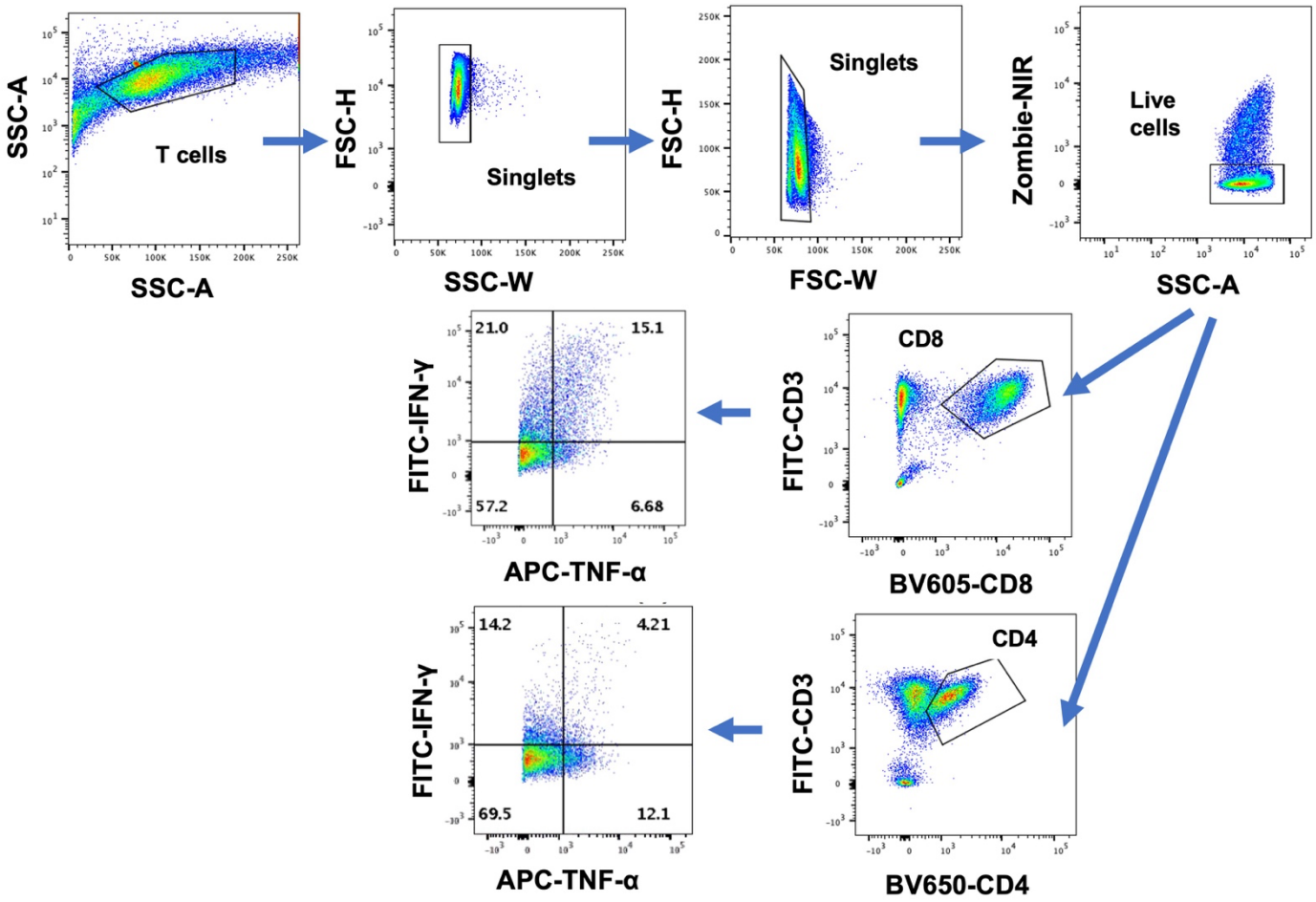
**e,** UMAP plot of $CD4^+$ T cells combined from 8 metastatic RCC patients. 3 from kidneys, 3 from lymph nodes, 2 from visceral metastases.

**f,** Dotplot showing expression of selected genes from $CD4^+$ T cells in **e**.

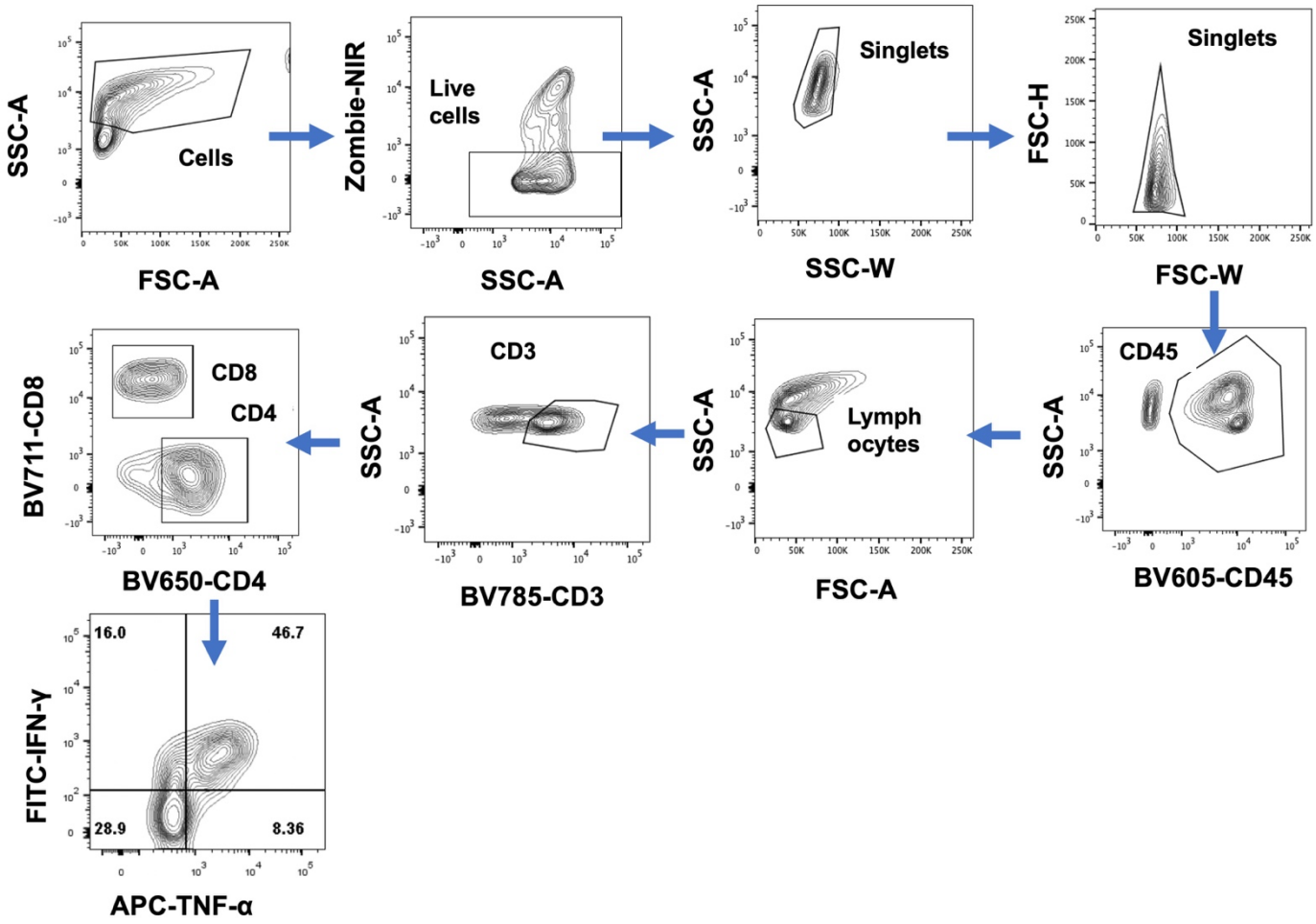
**g,h,** Proportions of each $CD4^+$ T-cell clusters. ICB = Immune checkpoint blockade, NoICB = No Immune checkpoint blockade. ICB Response: PR = partial response, SD = stable disease, PD = progressive disease, and NE = not evaluable.

**i,** UMAP plots displaying expression of *CD38* and *ENTPD1* (CD39 gene name) in tumors according to their ICB exposure.

**j,** UMAP plots displaying expression of *CD38* and *ENTPD1* in tumors according to their ICB response. Source data and exact p values are provided in the Source data file.




**Supplementary Fig 8.** Gating strategy for *in vitro* CD3/CD28 activated T-cell IFN-γ and TNF-α analysis.




**Supplementary Fig 9.** Gating strategy for *ex vivo* tumor-infiltrating T-cell IFN-γ and TNF-α analysis.

**Supplementary Table 1. List of antibodies used for CyTOF staining**

| Target | Clone | Supplier | Dilution |
|---|---|---|---|
| CD8α | 53-6.7 | Lederer Lab CyTOF Core | 1:100 |
| CD4 | RM4-5 | Lederer Lab CyTOF Core | 1:100 |
| CD11c | N418 | Lederer Lab CyTOF Core | 1:100 |
| TCRβ | H57-597 | Lederer Lab CyTOF Core | 1:100 |
| CD3 | 145-2C11 | Lederer Lab CyTOF Core | 1:100 |
| CD19 | 6D5 | Lederer Lab CyTOF Core | 1:100 |
| NK1.1 | PK136 | Lederer Lab CyTOF Core | 1:100 |
| CD45 | 30-F11 | Lederer Lab CyTOF Core | 1:100 |
| CD11b | M1/70 | Lederer Lab CyTOF Core | 1:100 |
| Singlec-F | E50-2440 | Lederer Lab CyTOF Core | 1:100 |
| Ly6G | 1A8 | Lederer Lab CyTOF Core | 1:100 |
| Ly6C | HK1.4 | Lederer Lab CyTOF Core | 1:100 |

**Supplementary Table 2. List of antibodies used for flow cytometry staining**

| Target | Clone | Supplier | Dilution |
|---|---|---|---|
| CD45 | 30-F11 | BioLegend | 1:100 |
| IFNγ | XMG1.2 | BioLegend | 1:50 |
| TNF-α | MP6-XT22 | BioLegend | 1:100 |
| CD4 | RM4-5 | BioLegend | 1:100 |
| CD8 | 53-6.7 | BioLegend | 1:100 |
| CD3 | 17A2 | BioLegend | 1:100 |
| CD38 | 90 | BioLegend | 1:100 |
| CD39 | Y23-1185 | BD Biosciences | 1:100 |

**Supplementary Table 3. List of primers used for ChIP-qPCR**

| Primer | FW | RV |
|---|---|---|
| Cd276 promoter (mouse) | GAGTCCCTTCTTTTCCTTGAGTC | CCCTTCACCTGTGTTTGTATCT |

**Supplementary Table 4. List of siRNAs used for transfection**

| siRNA | Cat # | Company |
|---|---|---|
| SMARTpools mTOR siRNA | L-065427-00-0005 | Dharmacon |
| SMARTpools Raptor siRNA | L-058754-01-0005 | Dharmacon |
| SMARTpools Rictor siRNA | L-064598-01-0005 | Dharmacon |
| SMARTpools S6K siRNA | L-040893-00-0005 | Dharmacon |
| SMARTpools 4E-BP1 siRNA | L-058681-01-0005 | Dharmacon |
| SMARTpools YY2 siRNA | L-171481-00-0005 | Dharmacon |
| SMARTpools control siRNA | D-001810-10-05 | Dharmacon |

**Supplementary Table 5. List of probes used for qRT-PCR**

| Probe | Cat # | Company |
|---|---|---|
| Cd276 (mouse) | Mm00506020_m1 | ThermoFisher Scientific |
| CD276 (human) | Hs00987207_m1 | ThermoFisher Scientific |
| Yy2 (mouse) | Mm03059489_sH | ThermoFisher Scientific |
| YY2 (human) | Hs02597954_sH | ThermoFisher Scientific |
| Yy1 (mouse) | Mm00456392_m1 | ThermoFisher Scientific |
| YY1 (human) | Hs00998747_m1 | ThermoFisher Scientific |