

Supplementary material for:

Activin-A impedes the establishment of CD4⁺ T cell exhaustion and enhances anti-tumor immunity in the lung

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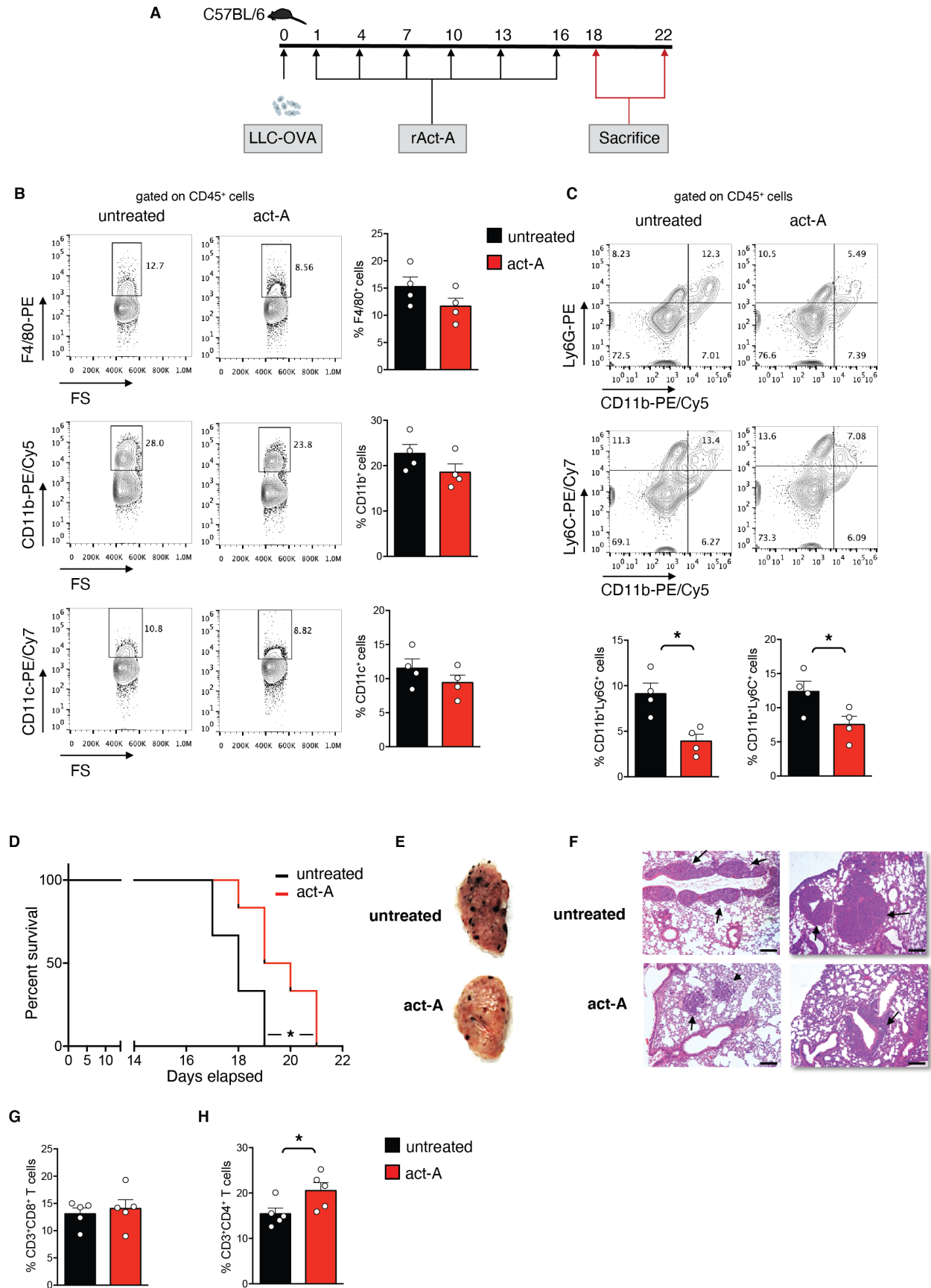
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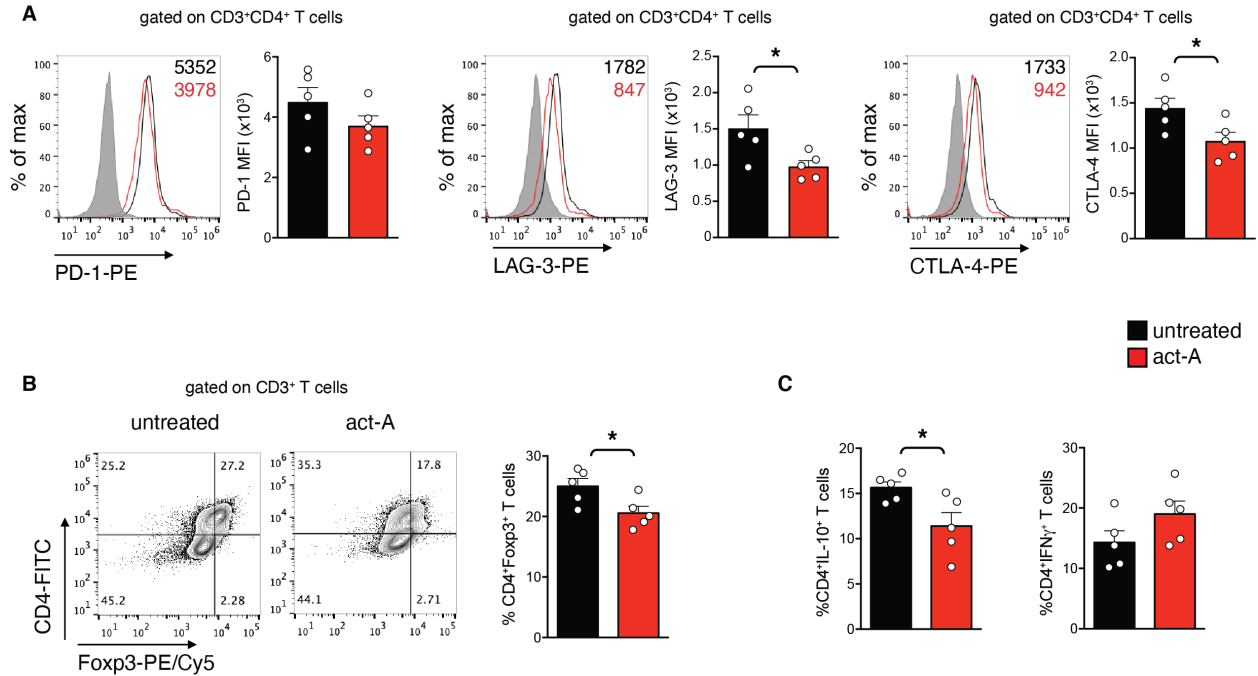
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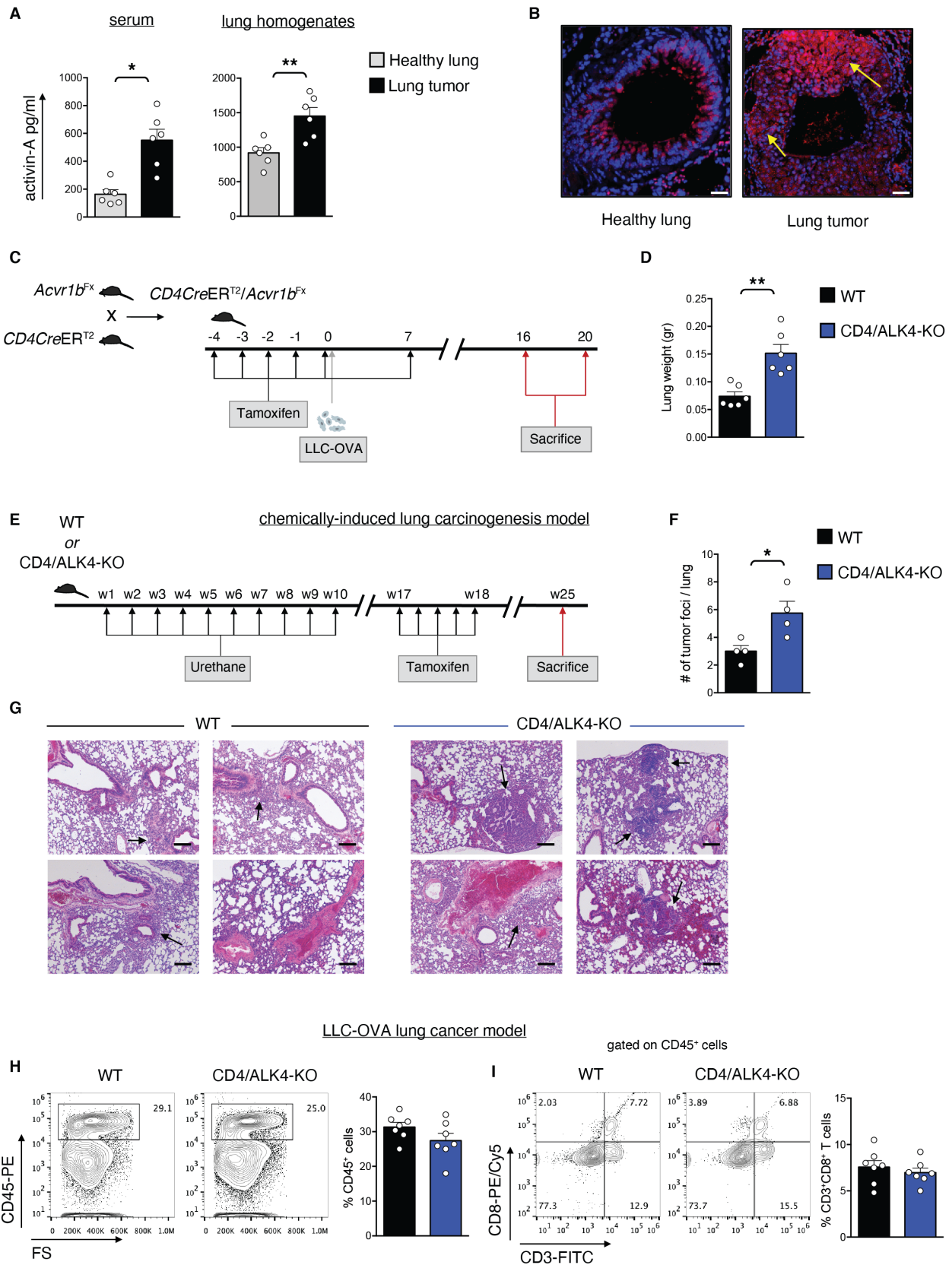
Supplemental figure 1. *In vivo* administration of activin-A attenuates the development and progression of melanoma lung metastases.

(A) Experimental protocol utilized. (B) Representative flow cytometry plots (left) and cumulative percentages of F4/80⁺ macrophages, CD11b⁺ myeloid and CD11c⁺ dendritic cells among CD45⁺ lung tumor infiltrating cells (right). (C) Representative flow cytometry plots (left) and cumulative percentages of CD11b⁺Ly6G⁺ granulocytic and CD11b⁺Ly6C⁺ monocytic myeloid derived suppressor cells among CD45⁺ lung tumor infiltrating cells (right). (D) Survival plot of activin-A-treated or untreated B16F10-tumor bearing mice ($n=6$). (E) Representative macroscopic images of B16F10-tumor bearing lung lobes. (F) Representative photomicrographs of H&E-stained lung sections (scale bars: 100 μ m). (G) Cumulative percentages of CD3⁺CD8⁺ T cells among CD45⁺ lung tumor infiltrating cells. (H) Cumulative percentages of CD3⁺CD4⁺ T cells among CD45⁺ lung tumor infiltrating cells. Data are representative of 4-5 independent experiments. Statistical significance was obtained by Log-rank (Mantel-Cox) test and unpaired Student's t-test; * $P < 0.05$.



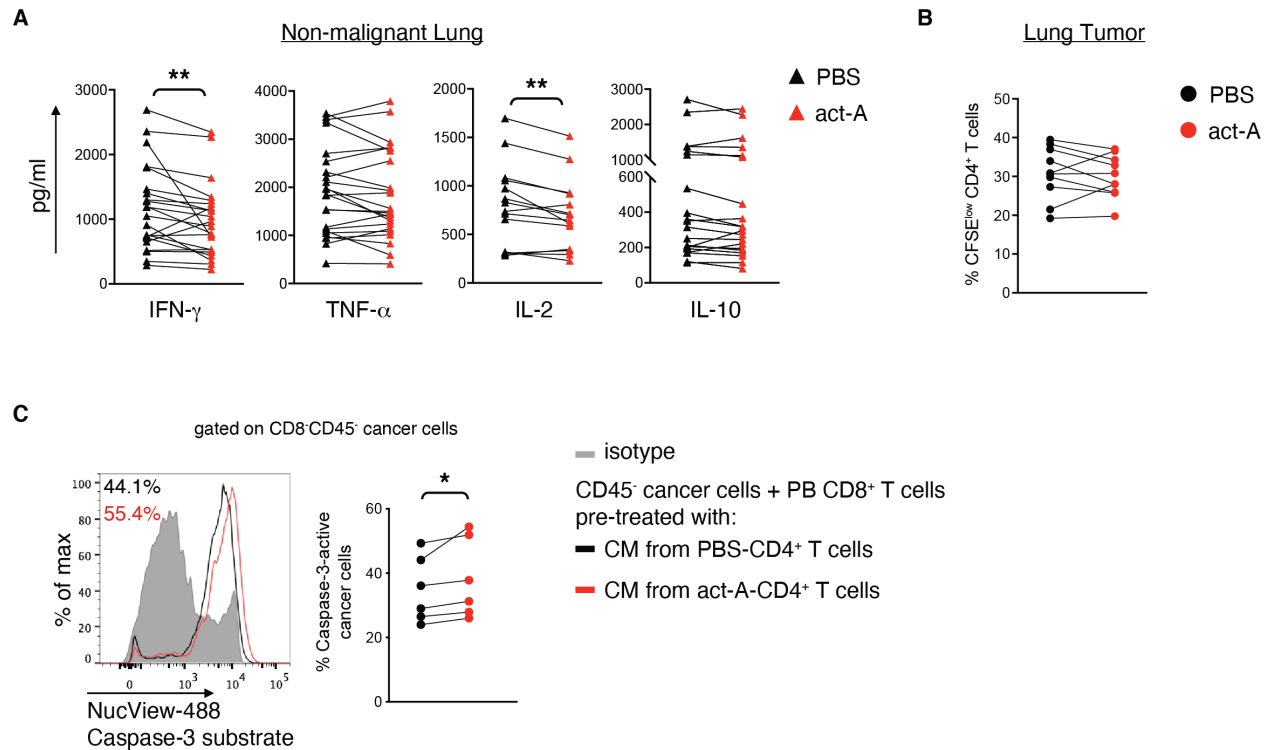
Supplemental figure 2. Activin-A represses the expression of inhibitory receptors on melanoma lung metastases-infiltrating CD4⁺ T cells.

(A) Representative flow cytometry plots (left) and cumulative MFI values of PD-1, LAG-3 or CTLA-4 expression by CD3⁺CD4⁺ T cells infiltrating lung tumors (right) from activin-A-treated or untreated B16F10-tumor bearing mice. Numbers in plots indicate frequencies of positively stained cells. Shaded histograms represent isotype controls. (B) Representative flow cytometry plots (left) and cumulative percentages of Foxp3-expressing cells among CD3⁺CD4⁺ T cells infiltrating lung tumors (right). (C) Cumulative percentages of IL-10- or IFN- γ -expressing cells among CD3⁺CD4⁺ T cells infiltrating lung tumors. Data are representative of 5 independent experiments. Statistical significance was obtained by unpaired Student's t-test; *P < 0.05.



Supplemental figure 3. Disruption of activin-A's signaling on CD4⁺ T cells accelerates tumor progression in a chemically-induced model of lung cancer.

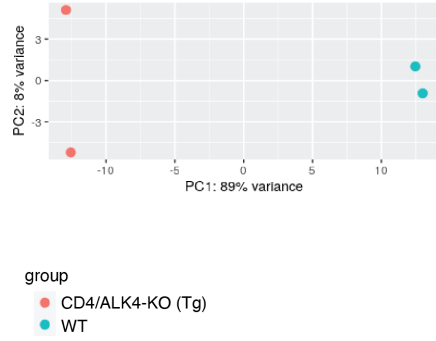
(A) Activin-A levels in serum and lung homogenates from healthy and LLC tumor-bearing mice. Data are mean $\pm$ SEM of triplicate wells and are representative of 6 independent experiments. (B) Representative confocal microscopy images of lung sections from healthy and LLC tumor-bearing mice stained with an antibody against activin-A (red). Nuclei are stained blue with DAPI (scale bars: 20 μ m). (C) Experimental protocol utilized. (D) Lung weight measurements of CD4/ALK4-KO and WT LLC-tumor bearing mice ($n=6$). (E) Experimental protocol utilized. (F) Tumor foci measurements of CD4/ALK4-KO and WT lung-tumor bearing mice ($n=4$). (G) Representative photomicrographs of H&E-stained lung sections (scale bars: 100 μ m). (H) Representative flow cytometry plots (left) and cumulative percentages of CD45⁺ lung tumor infiltrating cells (right). Data are representative of 7 independent experiments. (I) Representative flow cytometry plots (left) and cumulative percentages of CD3⁺CD8⁺ T cells among CD45⁺ lung tumor infiltrating cells (right). Data are representative of 7 independent experiments. Statistical significance was obtained by unpaired Student's t-test; *P < 0.05 and **P < 0.01.



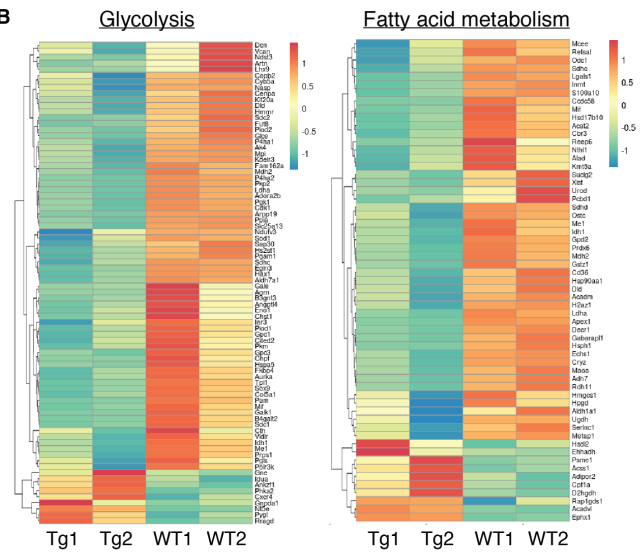
Supplemental figure 4. Activin-A unveils different effects on T cells infiltrating lung tumors compared to adjacent healthy lung tissue.

(A) Primary lung infiltrating leukocytes were isolated from, adjacent to tumors, non-malignant tissues from NSCLC patients and stimulated with antibodies against CD3/CD28 in the presence of activin-A or PBS. Cytokine levels in culture supernatants of activin-A- or PBS-treated non-malignant lung infiltrating leukocytes ($n=20-26$ donors). Data are mean $\pm$ SEM of triplicate wells. (B) Activin-A- or PBS-treated human lung tumor infiltrating CD4⁺ T cells were generated as in Fig. 6A and labelled with CellTrace-CFSE. Cumulative data of CD4⁺ T cell proliferation, measured by flow cytometry are depicted ($n=10$ donors). (C) CD45⁻ lung cancer cells were co-cultured with autologous PB CD8⁺ T cells pre-treated with CM from act-A- or PBS-CD4⁺ T cell cultures. Representative flow cytometry plot (left) and cumulative percentages (right) depicting caspase-3 activity of CD45⁻ lung cancer cells ($n=6$). Statistical significance was obtained by Wilcoxon matched-pairs signed rank test; * $P < 0.05$ and ** $P < 0.01$.

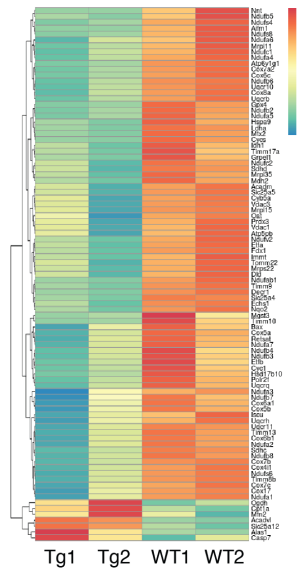
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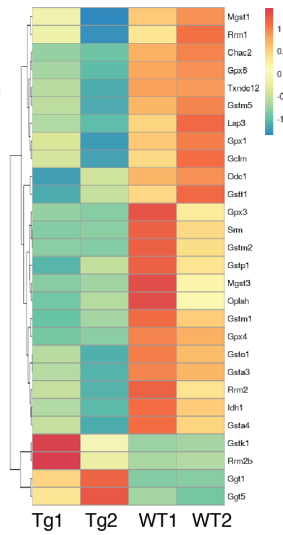
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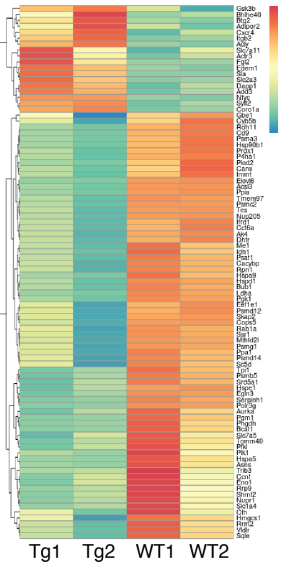
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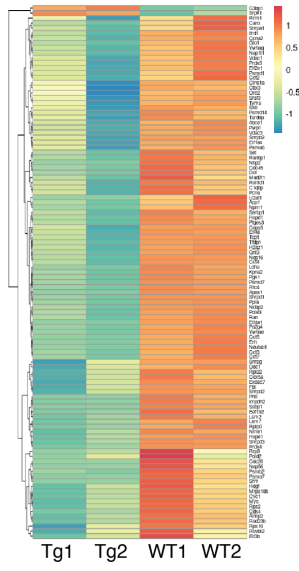
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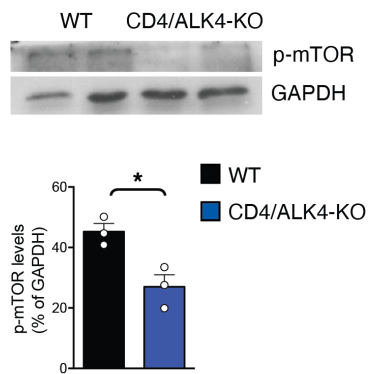
C mTOR pathway



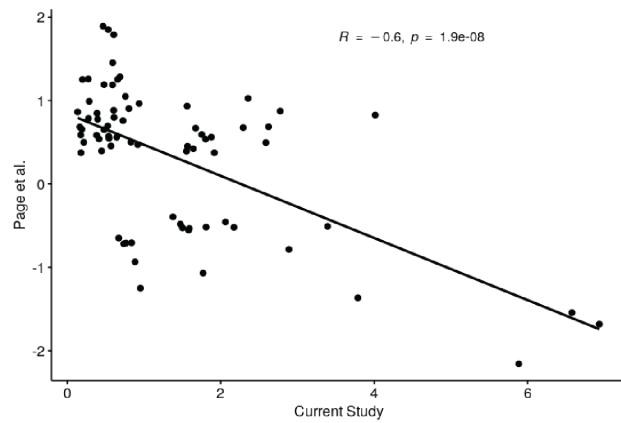
Myc targets



D



E



Supplemental figure 5. Ablation of activin-A signaling imposes metabolic dysfunction on lung tumor infiltrating CD4⁺ T cells.

(A) Principle Component Analysis (PCA) for normalized gene counts in lung tumor infiltrating CD4⁺ T cells (each dot represents an individual CD4⁺ T cell sample derived from either CD4/ALK4-KO or WT mice). Counts were normalized to log2 scale. (B) Heatmap depicting z-score differences among the analyzed samples regarding glycolysis, fatty-acid metabolism, oxidative phosphorylation, glutathione metabolism genes. (C) Heatmap depicting z-score differences among the analyzed samples regarding mTOR and Myc signaling pathways genes. (D) Representative immunoblots showing p-mTOR expression. Quantification of relative p-mTOR protein levels is depicted. Data are mean $\pm$ SEM and are representative of 3 independent experiments. (E) Correlation analysis regarding fold changes of Tox-regulated genes according to Page *et al.*, obtained from the respective publication and our RNA-Seq analysis. Applied is Pearson's correlation coefficient analysis. Statistical significance was obtained by unpaired Student's t-test; *P < 0.05.

Supplemental table 1. NSCLC patient characteristics (n=38)		
Age	Mean	70.2
	Range	51-86
Gender (%)	Female	11 (28.9)
	Male	27 (71.1)
Histology (%)	Adenocarcinoma	21 (55.3)
	Squamous cell carcinoma	16 (42.1)
	Other*	1 (2.6)
Smoking status (%)	Current	27 (71.1)
	Former	5 (13.1)
	Never	6 (15.8)
TNM Stage (%)	IA (T1a/b/cN0M0)	13 (34.2)
	IB (T2aN0M0)	12 (31.6)
	IIA (T2bN0M0)	3 (7.9)
	IIB (cT3N0M0, cT1N1M0)	5 (13.15)
	IIIA (T2bN2M0, T4N1M0, T4N0M0, T3cN1M0)	5 (13.15)
Concomitant Disease (%)	COPD**	11 (28.9)
	Heart Disease	16 (42.1)
	Diabetes	6 (15.8)
	None	12 (31.6)

*Other refers to Neuroendocrine tumors

**Chronic Obstructive Pulmonary Disease