

Supplementary Information for RhoA Y42C Drives Cell Plasticity and PD-1-Mediated Immune Evasion of Diffuse Gastric Cancer

Supplementary Figures 1-6:

Supplementary Fig. 1 | Proliferation of genetically engineered organoids

Supplementary Fig. 2 | Gene expression patterns of RKP and EKP tumors

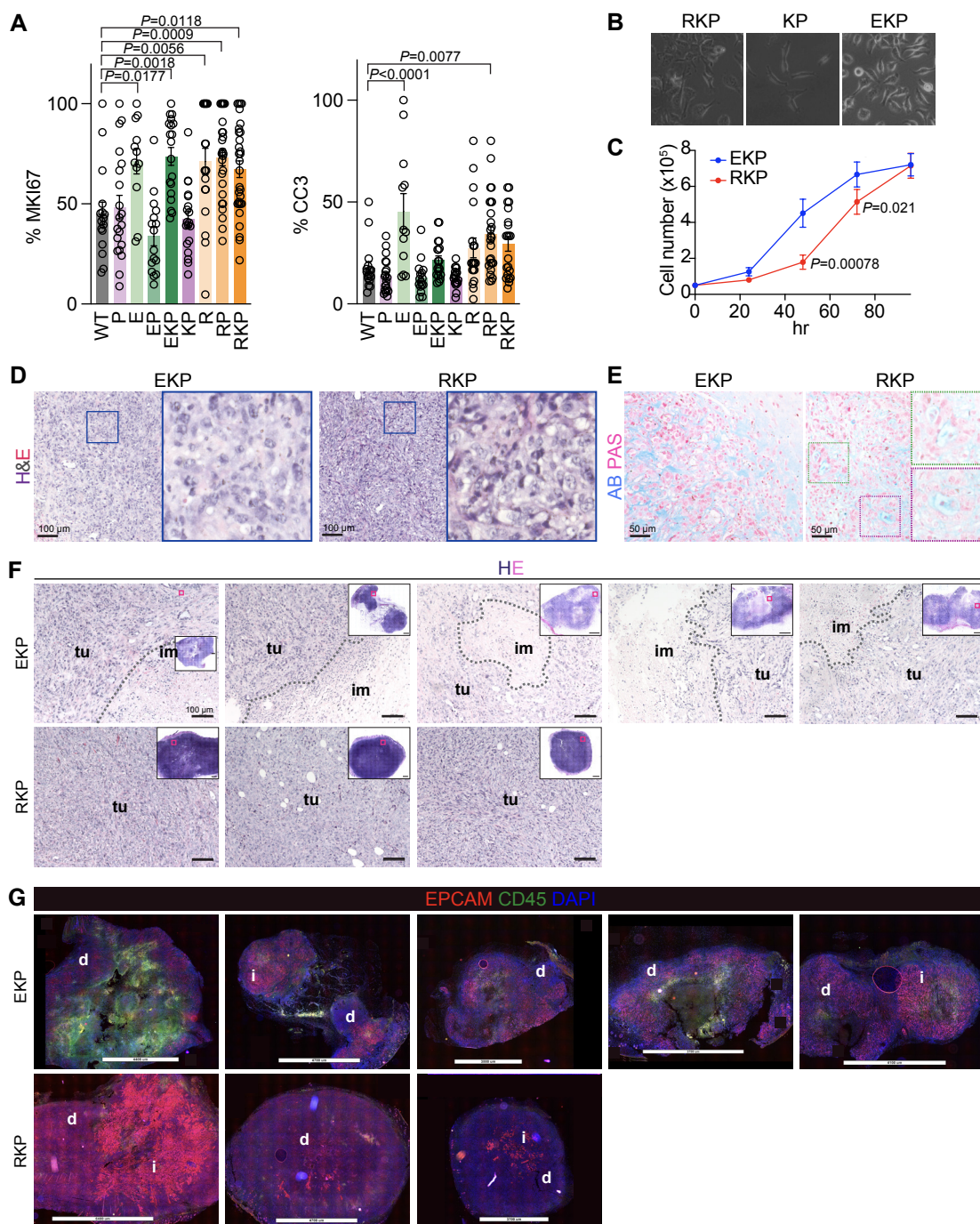
Supplementary Fig. 3 | Comparison of gene expression patterns in EKP and RKP tumor cells

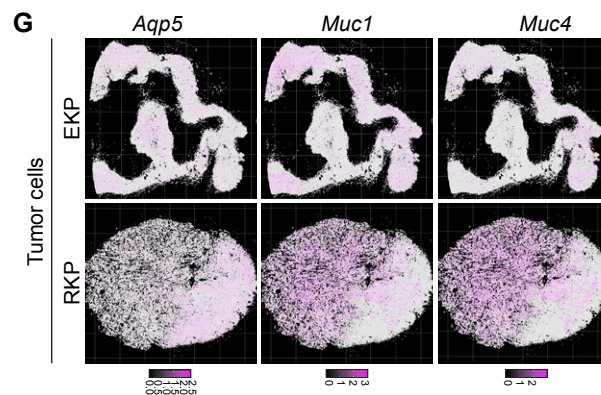
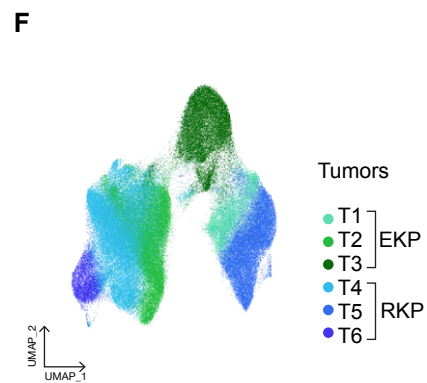
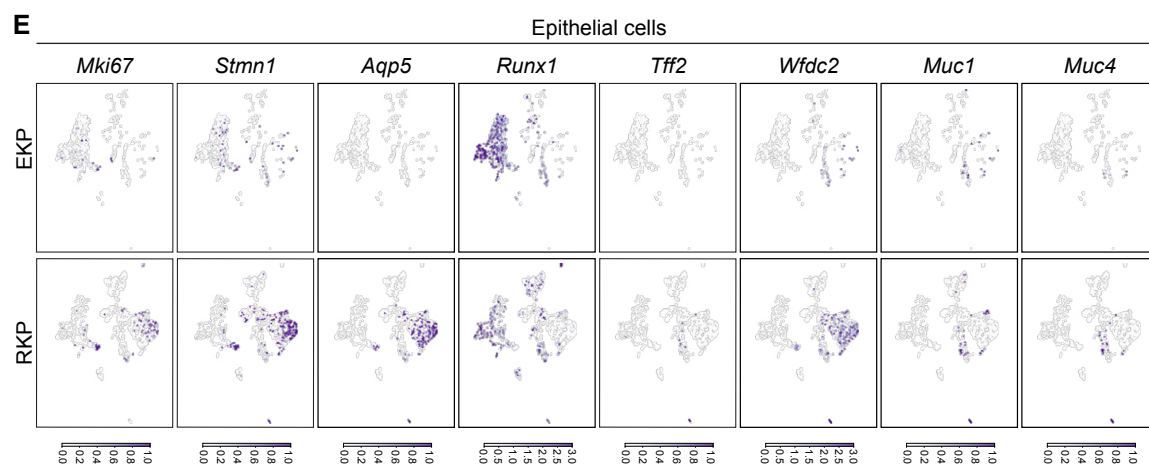
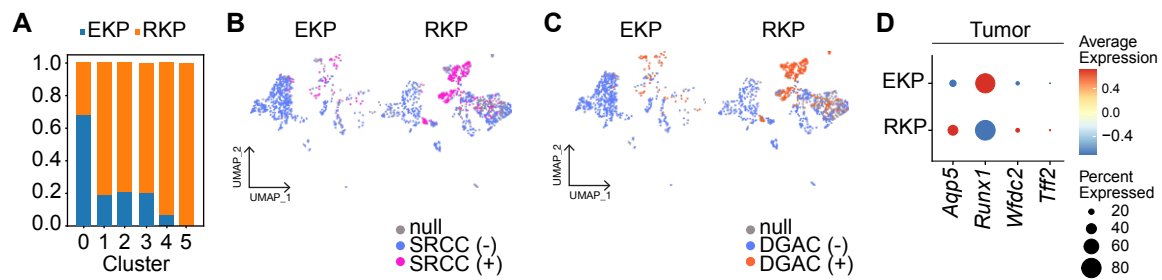
Supplementary Fig. 4 | Hippo signaling pathway relevance in RKP-specific cell lineage

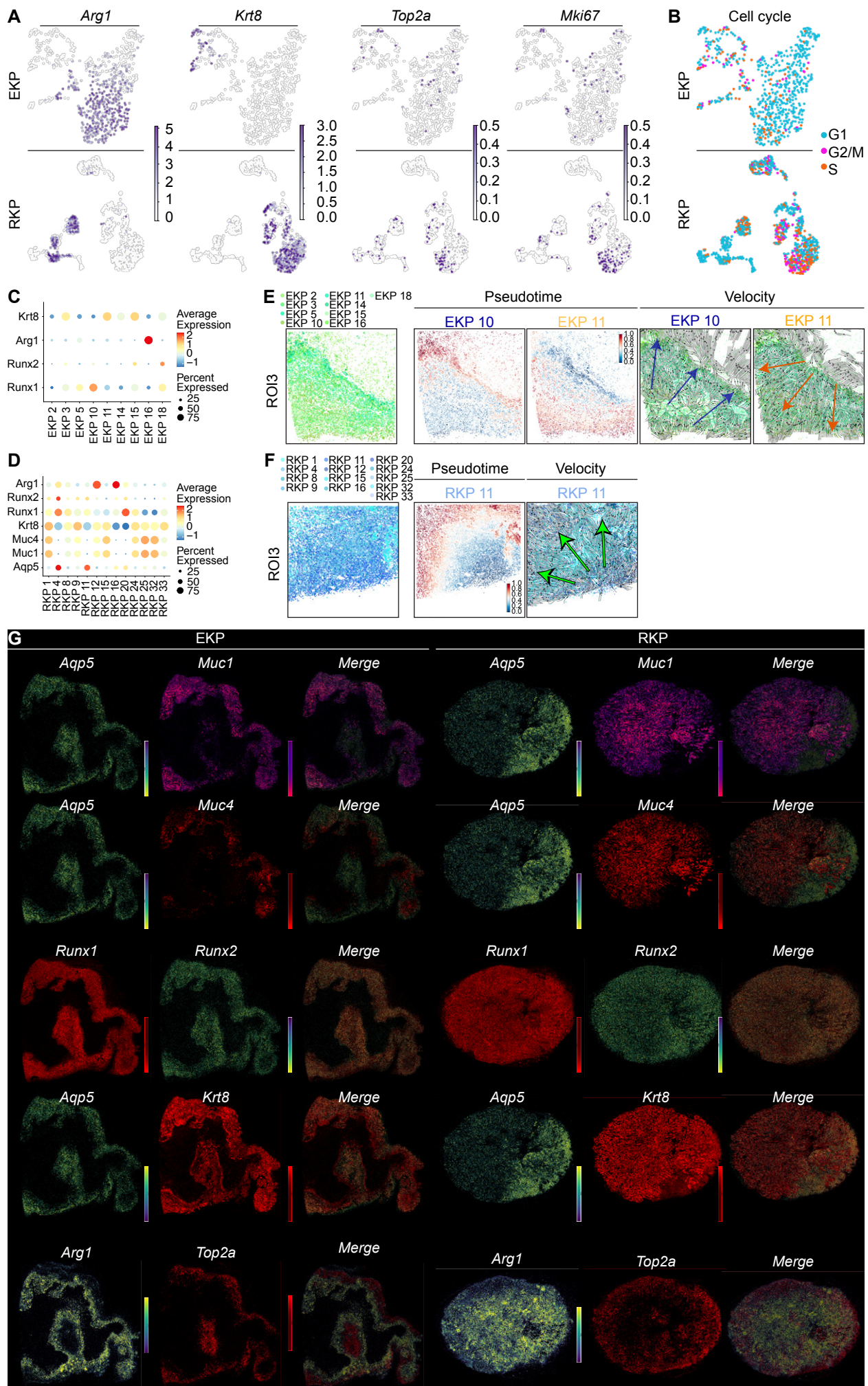
Supplementary Fig. 5 | Gene expression comparison in immune cells from EKP and RKP tumors

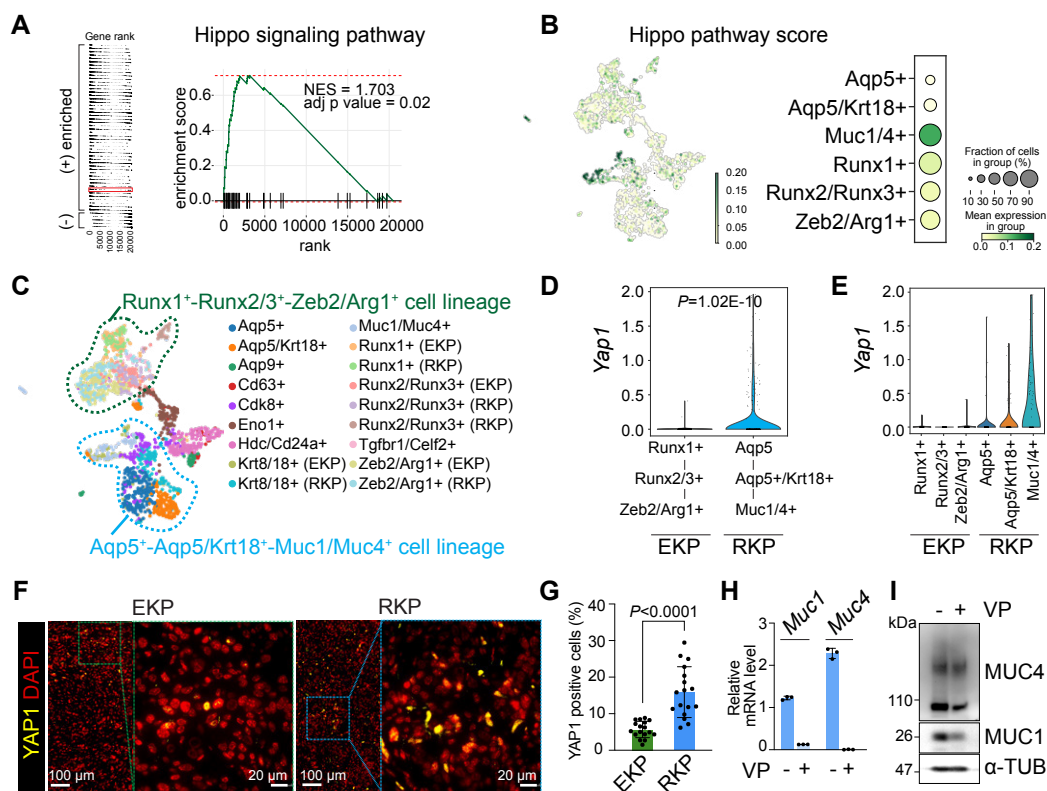
Supplementary Fig. 6 | AREG-induced immunosuppression in RKP tumors

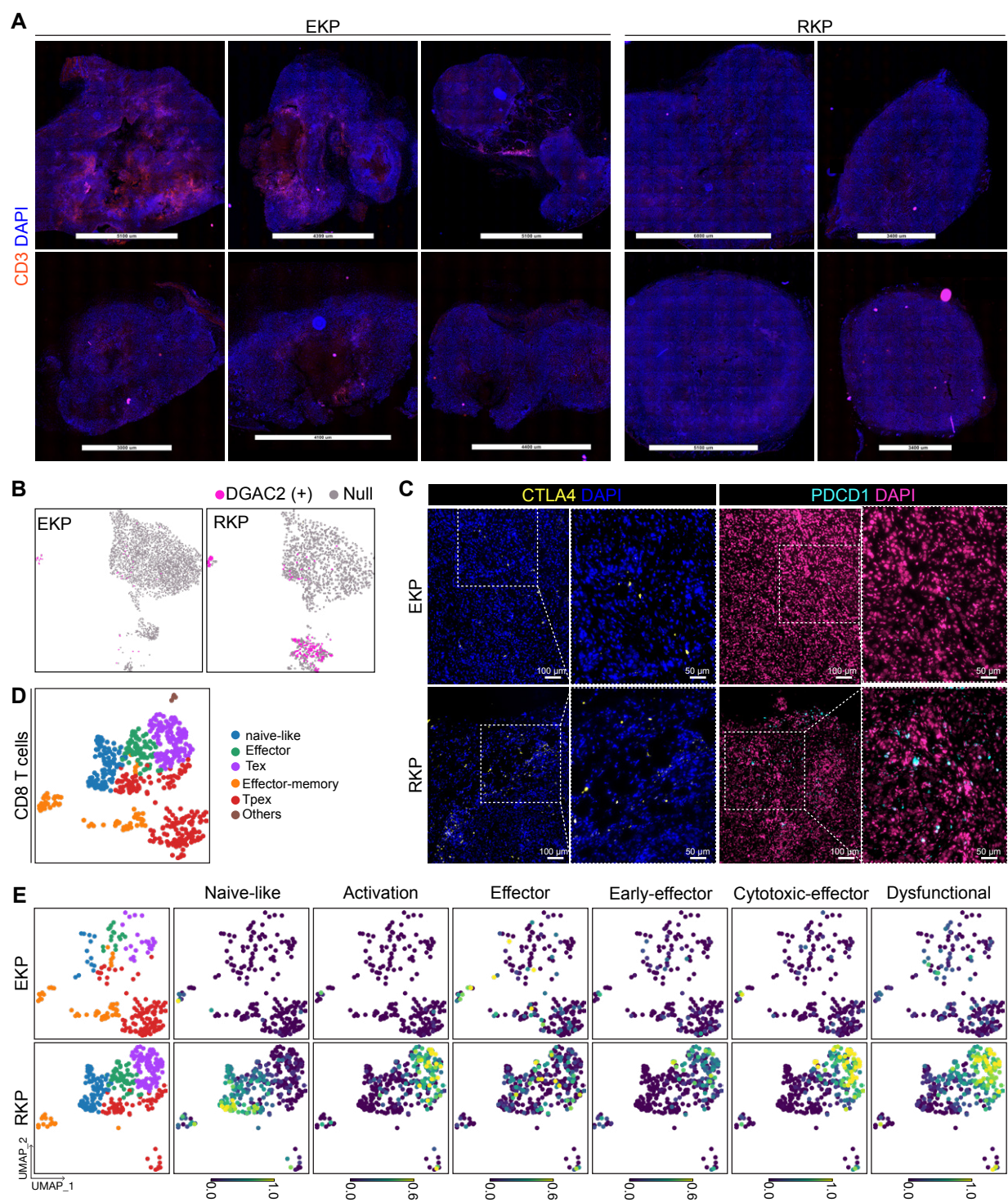
References 1-80.

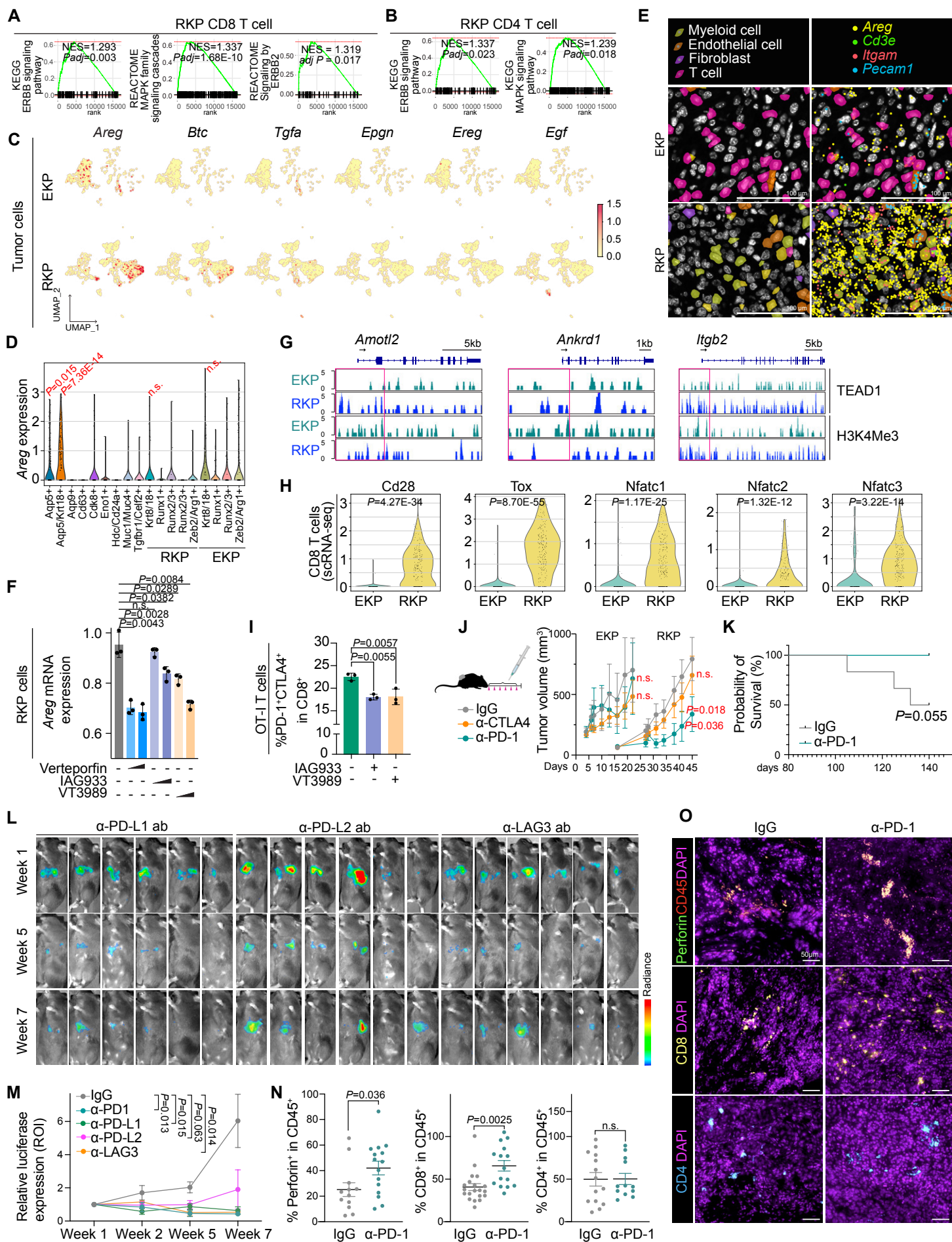












Supplementary Figure Legends

Supplementary Figure 1. Proliferation of genetically engineered organoids.

A. Quantification of MKI67⁺ proliferating cells and cleaved Caspase-3 (CC3)⁺ apoptotic cells. Relative percentages were calculated and plotted. Statistical significance was assessed using Student's *t*-test ($n \geq 3$ independent biological replicates). **B.** Bright-field images of RKP cells [*Rhoa*^{Y42C}, *Kras*^{G12D}, *Trp53*^{-/-}], KP cells [*Kras*^{G12D}, *Trp53*^{-/-}], and EKP cells [*Cdh1*^{-/-}, *Kras*^{G12D}, *Trp53*^{-/-}] cultured in two dimensions. **C.** Cell counting assay of RKP and EKP cells. Mean values are shown ($n = 4$ independent assays); error bars: SD. **D-E.** IHC staining of EKP and RKP tumors with hematoxylin and eosin (E) and Alcian blue and periodic acid-Schiff (F). **F.** Low- and high-magnification images of H&E-stained sections from each allograft tumor. Tumor (tu) and immune cell-infiltrated (im) regions are indicated.. **G.** IHC staining for epithelial cells (EPCAM) and immune cells (CD45) in EKP and RKP tumors. Diffuse (d) and intestinal-like (i) epithelial cell populations are annotated.

Supplementary Figure 2. Gene expression patterns of RKP and EKP tumors.

A. Cell proportion of EKP and RKP tumor cells within each cell cluster (scRNA-seq data). **B, C.** Scissor analysis from scRNA-seq data. Transcriptomic similarities of each cell in RKP tumors [*Rhoa*^{Y42C}, *Kras*^{G12D}, *Trp53*^{-/-}] and EKP [*Cdh1*^{-/-}, *Kras*^{G12D}, *Trp53*^{-/-}] tumors to human signet ring cell cancer (SRCC) (B) and diffuse gastric adenocarcinoma (DGC) (C) were analyzed and visualized on uniform manifold approximation and projection (UMAP) plots. **D, E.** Expression of markers of proliferating, isthmus stem, and spasmodic polypeptide-expressing metaplastic (SPEM) cells from scRNA-seq are displayed in dot plots (D) and feature plots (E). **F.**

Tumor cells isolated and normalized from Xenium In Situ results. **G.** Normalized gene expression of *Aqp5*, *Muc1*, and *Muc4* from Xenium In Situ data.

Supplementary Figure 3. Comparison of gene expression patterns in EKP and RKP tumor cells.

A. Feature plots of representative cell cluster markers and proliferative cell markers (scRNA-seq data). **B.** Cell cycle analysis of EKP [*Cdh1*^{-/-}, *Kras*^{G12D}, *Trp53*^{-/-}] and RKP [*Rhoa*^{Y42C}, *Kras*^{G12D}, *Trp53*^{-/-}] tumor cells from scRNA-seq data. **C.** Dotplots showing selection of EKP10 and EKP11 clusters as starting cells based on Runx1 and Krt8 expression. **D.** Dotplots showing selection of RKP11 cluster as the starting cell population based on its exclusive expression of *Aqp5* and low expression of *Arg1*. **E-F.** Spatial maps of cell clusters, pseudotime, and velocity are shown to illustrate EKP (E) and RKP (F) cell lineage trajectories in the ROI3. **G.** Spatial gene expression patterns of representative cell cluster markers from Xenium In Situ results.

Supplementary Figure 4. Hippo signaling pathway relevance in RKP-specific cell lineage.

A. Gene set enrichment analysis of the Hippo signaling pathway in EKP [*Cdh1*^{-/-}, *Kras*^{G12D}, *Trp53*^{-/-}] cells versus RKP [*Rhoa*^{Y42C}, *Kras*^{G12D}, *Trp53*^{-/-}] cells. **B.** Hippo pathway scores in each cell cluster of RKP cells (scRNA-seq data). **C.** Uniform manifold approximation and projection (UMAP) plot showing major cell lineages of EKP and RKP integrated tumor dataset (scRNA-seq data). **D-E.** *Yap1* expression in cell lineages (D) and clusters (E) of EKP and RKP cells (scRNA-seq data). **F-G.** Representative immunofluorescence images (F) of YAP1 in EKP and RKP tumors and their quantification ($n \geq 3$) (G). **H-I.** *Muc1* and *Muc4* mRNA (H) and protein (I)

expression in RKP cells with or without verteporfin (n=3 independent biological replicates).

Supplementary Figure 5. Gene expression comparison in immune cells from EKP and RKP tumors.

A. IHC staining results of CD3 in EKP and RKP tumors. **B.** Scissor analysis of immune cells from EKP [*Cdh1*^{-/-}, *Kras*^{G12D}, *Trp53*^{-/-}] and RKP [*Rhoa*^{Y42C}, *Kras*^{G12D}, *Trp53*^{-/-}] scRNA-seq data to compare transcriptomic similarity with human diffuse gastric adenocarcinoma (DGC). **C.** Immune cells from Xenium data were isolated and displayed with UMAP. **D.** Subclusters of CD8 T cells. **E.** CD8 T cell states in EKP tumors and RKP tumors by gene scoring (scRNA-seq data).

Supplementary Figure 6. AREG-induced immunosuppression in RKP tumors.

A, B. RKP [*Rhoa*^{Y42C}, *Kras*^{G12D}, *Trp53*^{-/-}] tumor-enriched signaling pathways in CD8 (A) and CD4 (B) T cells of scRNA-seq data by gene set enrichment analysis (GSEA). **C.** Ligands of EGFR expression in EKP and RKP tumor cells (scRNA-seq data). **D.** *Areg* expression in EKP [*Cdh1*^{-/-}, *Kras*^{G12D}, *Trp53*^{-/-}] and RKP tumors is shown by cell clusters (scRNA-seq data). **E.** Xenium In Situ analysis of *Areg* expression in the EKP and RKP with non-epithelial cell visualization. **F.** *Areg* mRNA expression in RKP cells with verteporfin, IAG933, and VT3989 treatment. Verteporfin (1.0 and 3.0 μ M), IAG933 (0.47 and 470 mM), VT3989 (6.4 and 640 nM) were treated for 48h. n.s.: Not significant. *P* values were calculated by Welch's t-test. (n=3 independent biological replicates) **G.** CUT&RUN peaks of known YAP1 target genes in EKP and RKP cells. **H.** Expression of key genes associated with *Pdcd1* induction in CD8 T cells from RKP and EKP, shown as violin plot (scRNA-seq). **I.** Effects of TEAD

inhibitors, IAG933 and VT3989, on T-cell exhaustion. OT-I T cells were cultured RKP cells and treated with TEAD inhibitors. Exhausted T cells were evaluated by FACS. (n=3 independent biological replicates) **J.** Immunotherapy responses in subcutaneously transplanted EKP (nIgG, n = 6 mice; anti-CTLA, n = 6 mice; anti-PD-1, n = 8 mice) and RKP (nIgG, n = 11 mice; anti-CTLA, n = 8 mice; anti-PD-1, n = 6 mice) tumors. Mean values are shown from at least six replicates; *P* values were calculated with Student's *t*-test. **K.** Kaplan-Meier survival analysis of orthotopically transplanted RKP tumors treated with or without anti-PD-1. *P* value was calculated by log-rank (Mantel-Cox) test. **L-M.** Luciferase activity of orthotopically transplanted tumors was measured using the S2 LagoX system (L) and quantified luciferase intensity was plotted (M). *P* value was calculated by Welch's *t*-test. **N-O.** Characterization of infiltrating T cells in RKP tumors. Effector T cell (Perforin+), CD8 T cell, and CD4 T cell populations within RKP subcutaneous tumors were detected by IHC (O) and quantitatively analyzed from independent images (N). n.s.: Not significant (n ≥ 3)