

Supplementary Information

Targeting IRE1 α reprograms the tumor microenvironment and enhances anti-tumor immunity in prostate cancer

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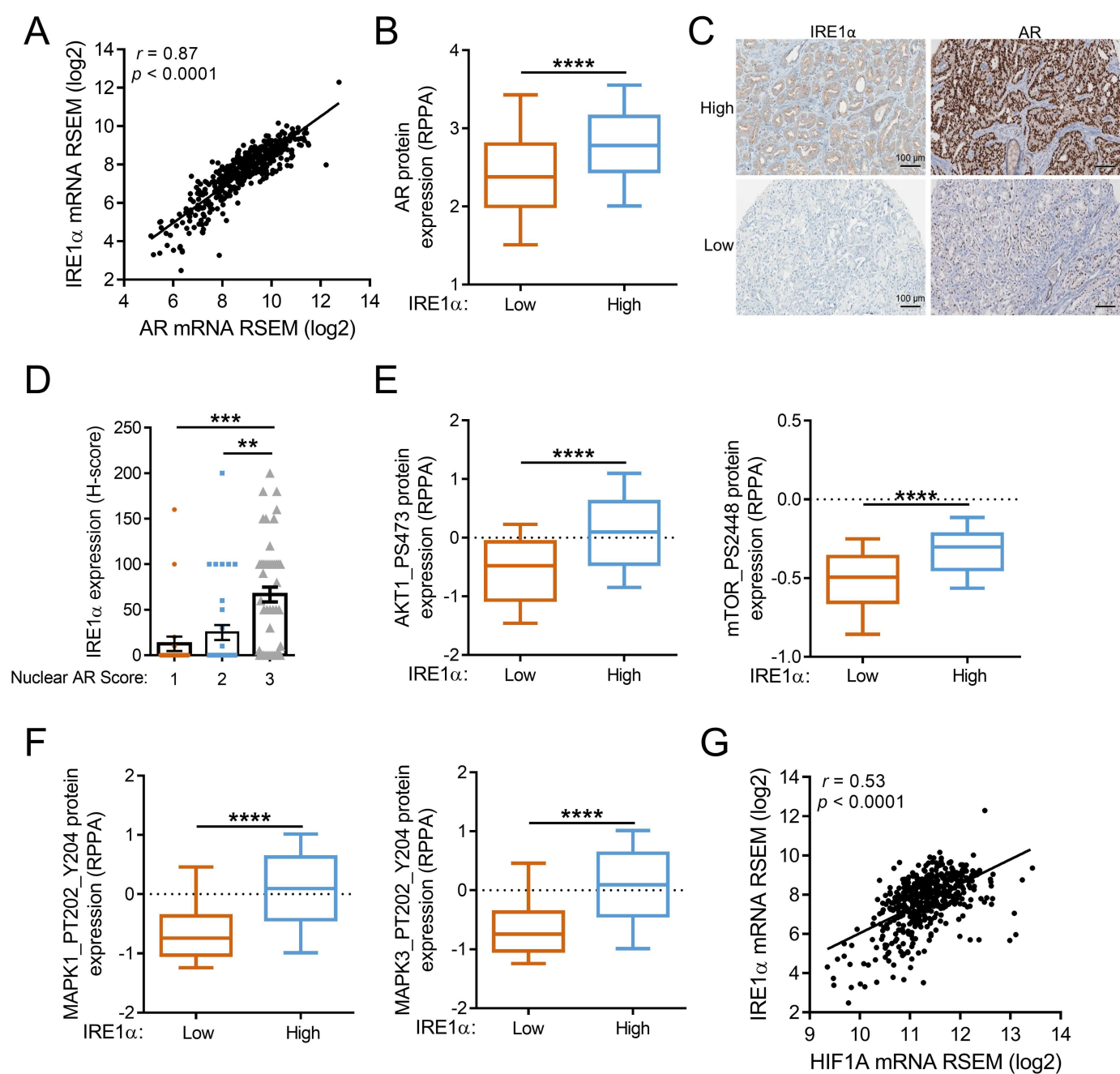
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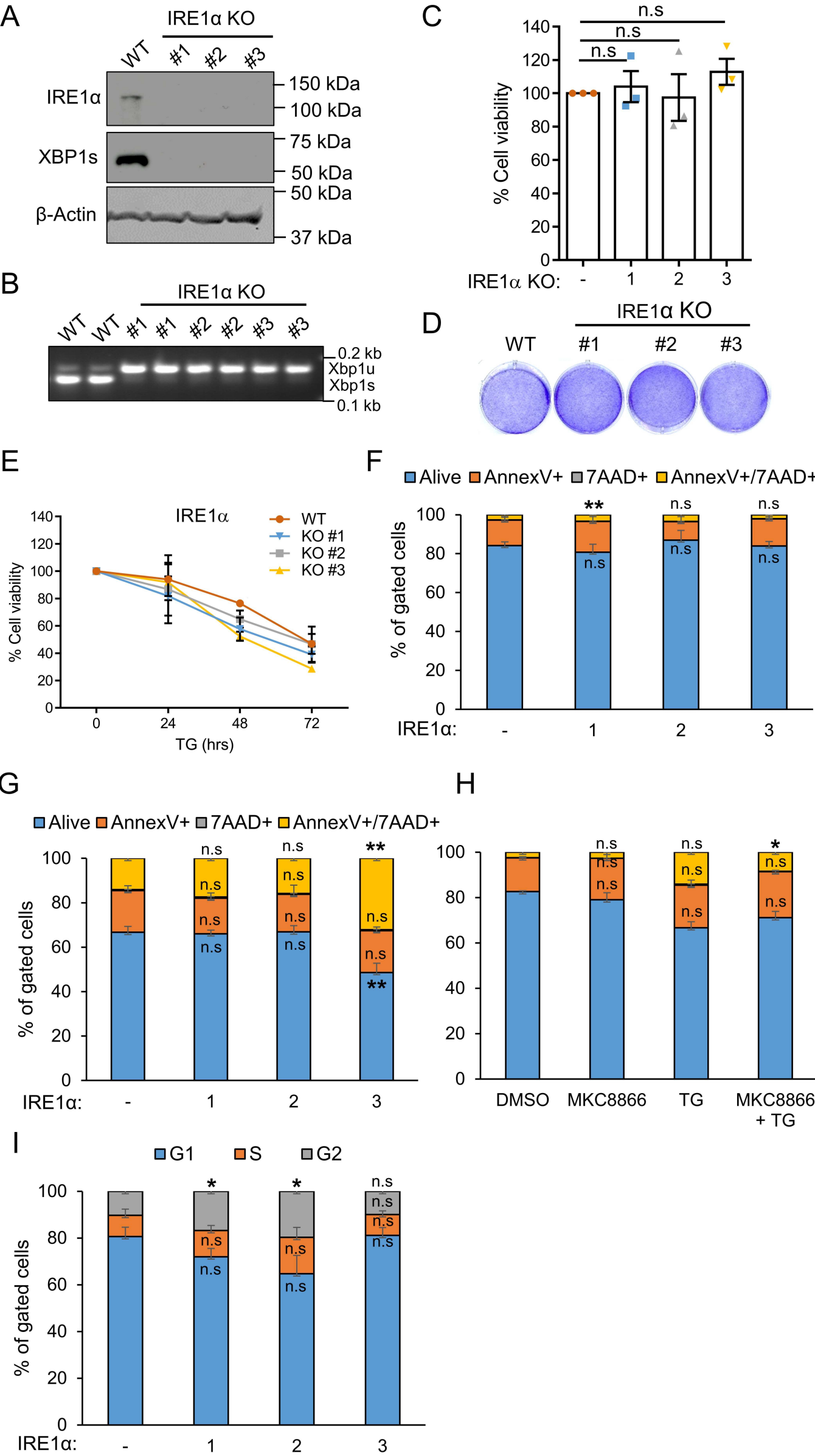
Supplementary Figure 1



Supplementary Figure 1: IRE1α expression is associated with hallmarks of PCa growth.

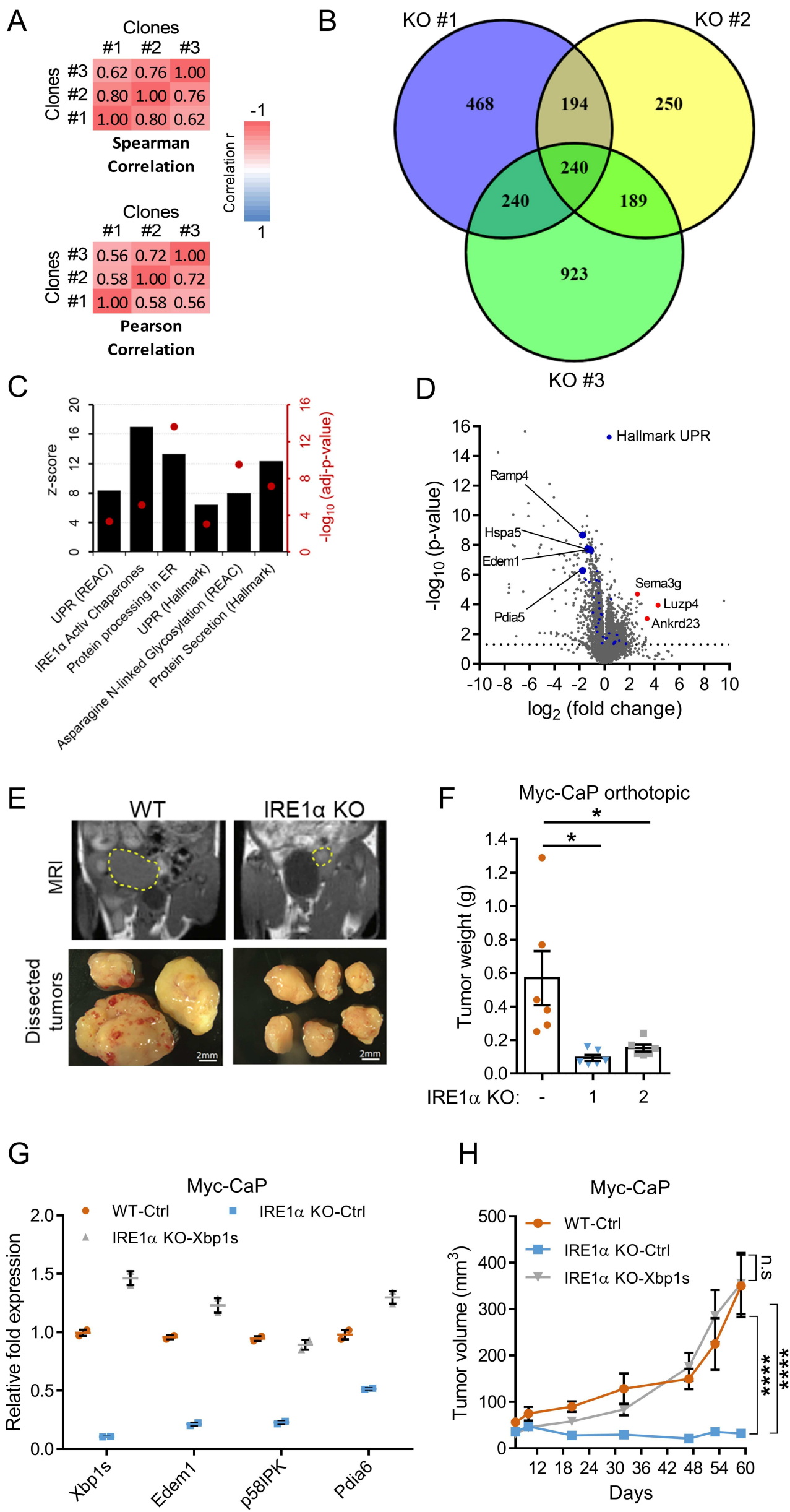
A) mRNA levels of AR and IRE1α are highly correlated in the TCGA PCa dataset (n = 493). **B)** The AR protein levels were increased in PCa patients with higher IRE1α mRNA low (n = 112) or high (n = 106) expression in the TCGA PCa dataset. **C)** Representative images are shown for IRE1α and AR IHC staining for PCa tumor samples. **D)** Quantification of corresponding IRE1α and nuclear AR scores (1: n = 23; 2: n = 34; 3: n = 52) are presented from figure C. **E)** Same as in B, but phosphorylated AKT1 and mTOR, and **F)** phosphorylated ERK1/2 are increased with higher IRE1α mRNA expression in the TCGA PCa dataset. **G)** mRNA levels of HIF1A and IRE1α are highly correlated in the TCGA PCa dataset. Mean ± standard error by unpaired two-tailed Mann Whitney t-test is presented for figures B, E, and F; one-way Anova is used for D (source data and exact p values are provided as a Source Data); ** p<0.01, *** p<0.001, **** p<0.0001. In box-plots, whiskers represent 10-90 percentile and middle lines indicate median of the data. r represents Spearman correlation where indicated.

Supplementary Figure 2



Supplementary Figure 2: IRE1 α does not significantly affect cell viability, proliferation, apoptosis or cell-cycle in Myc-Cap cells in vitro. **A)** Western analysis of IRE1 α wild type (WT) and three independent CRISPR-Cas9 knockout (KO) Myc-CaP cell lines that were treated with 100 nM TG for 12 hours. Data are representative of at least two independent experiments. **B)** mRNA expression of XBP1u or XBP1s in IRE1 α WT and KO cells after 100 nM TG treatment for 12 hours. **C)** Viability of WT (n = 3 biologically independent replicates) and IRE1 α KO clones (n = 3 biologically independent replicates each) were determined by the CCK8 assay. **D)** IRE1 α WT and KO clones were cultured for four days and the colonies were stained with crystal violet. **E)** IRE1 α WT (n = 2 biologically independent replicates) and KO clones (n = 2 biologically independent replicates each) were treated with 100 nM TG for the indicated times and cell viability was measured. Cell death rates in IRE1 α WT (n = 3 biologically independent replicates) or KO clones (n = 3 biologically independent replicates each) in the **F)** absence or **G)** presence of TG and **H)** in Myc-CaP cells either treated with DMSO (n = 3 biologically independent replicates) or MKC8866 (n = 4 biologically independent replicates) alone or in MKC8866 + TG combination (n = 5 biologically independent replicates), were measured by flow cytometry upon Annexin V and 7-AAD staining. **I)** Cell cycle in IRE1 α WT (n = 2 biologically independent replicates, one sample was lost due to a technical problem, thus were excluded from the final analysis) and KO clones (n = 3 biologically independent replicates) cultured in vitro was measured by flow cytometry upon Hoechst staining. Mean $\pm$ standard error by two-tailed student's t test is presented for C, F (IRE1 α WT vs KO #1 AnnexV+/7AAD+: p = 0.001) G (IRE1 α WT vs KO #3 Alive: p = 0.003; AnnexV+/7AAD+: p = 0.007), H (AnnexV+/7AAD+: p = 0.02), and I (IRE1 α WT vs KO #1 or #2 G2: p = 0.01 or p = 0.04 respectively); * p<0.05, ** p<0.01, n.s, non-significant.

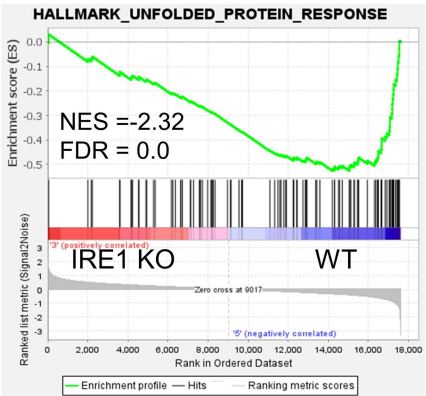
Supplementary Figure 3



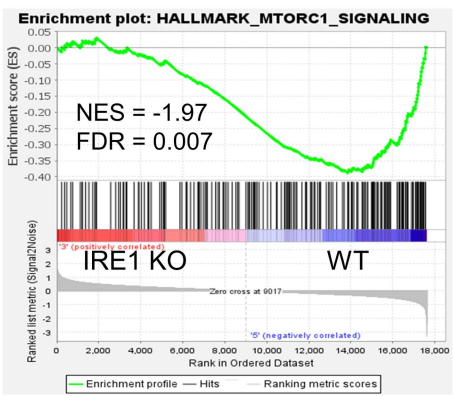
Supplementary Figure 3: UPR signaling is downregulated in IRE1 α KO Myc-CaP cells and XBP1s restores target gene expression and tumor growth. A-D) IRE1 α WT and three independent Myc-CaP KO clones were cultured in vitro and subjected to RNA-seq analysis. **A)** Spearman and Pearson correlation heatmaps for expression fold changes of 711 genes that were significantly deregulated in at least one of the three IRE1 α KO clones compared to WT cells. **B)** Venn diagram summarizes the number of differentially downregulated genes (fold-change ≥ 1.3 and $p < 0.05$, WT vs KO) for the indicated IRE1 α KO clones. **C)** GSEA results of 240 genes that are common among the different IRE1 α KO groups in B. **D)** Volcano plot indicating the up- and down-regulated genes; UPR Hallmark genes are color coded as blue and some notable genes are colored in red. **E)** IRE1 α WT and KO cell lines were orthotopically injected into the prostates of FVB mice. After four weeks, MRI images were captured, tumors were dissected, and **F)** tumor weights were determined ($n = 6$ mice / group). **G)** Myc-CaP IRE1 α KO clone #1 cells were transduced with either an empty lentiviral vector or one expressing Xbp1s. Expression of indicated genes was analyzed by qRT-PCR ($n = 2$ biologically independent replicates, consistent with RNA-seq data) from in vitro cultured cells. **H)** WT-Ctrl ($n = 2$ mice, 4 tumors), IRE1 α KO-Ctrl ($n = 6$ mice, 12 tumors), and IRE1 α KO-XBP1s ($n = 5$ mice, 10 tumors) were injected subcutaneously into FVB mice and tumor volumes were measured at indicated time points. For the WT-Ctrl group, three mice were sacrificed at earlier time points due to tumors reaching ethical endpoints and were thus excluded from the final data analysis. Mean $\pm$ standard error by two-tailed student's t test is presented for Figure F ($p = 0.016$) and H (WT-Ctrl vs IRE1 α KO-Ctrl: $p = 4.4E-07$; IRE1 α KO-Ctrl vs IRE1 α KO-Xbp1s: $p = 2.9E-05$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, n.s, non-significant.

Supplementary Figure 4

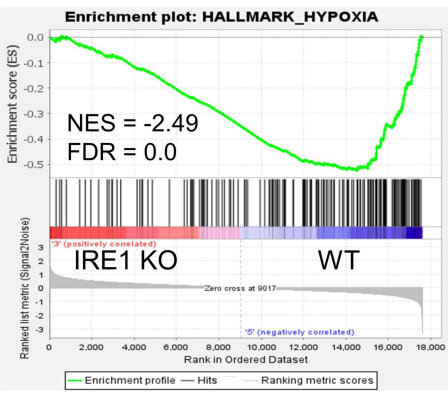
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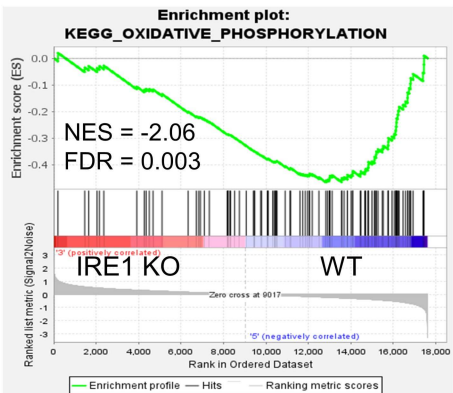
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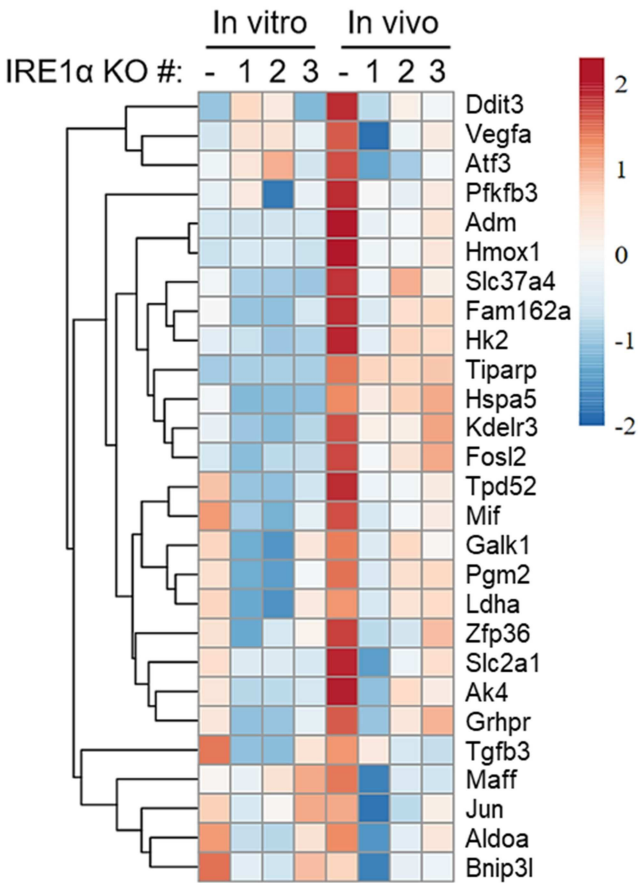
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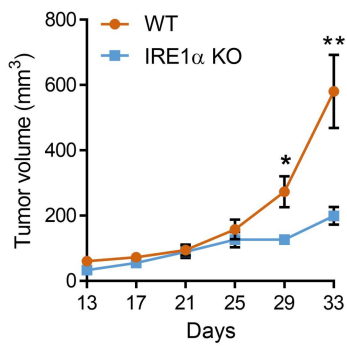
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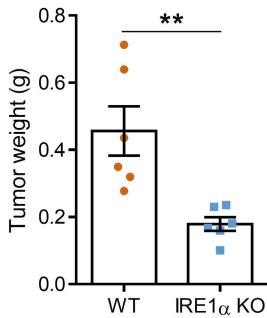
Supplementary Figure 4: IRE1α loss reduces cancer hallmark transcriptional programs in the PCa TME. A-D) GSEA plots comparing IRE1α KO (1-3) vs WT tumor samples for the indicated hallmark gene sets. **E)** The heatmap indicates the expression of hallmark hypoxia genes in the three independent IRE1α KO tumor samples as well as in cells cultured in vitro.

Supplementary Figure 5

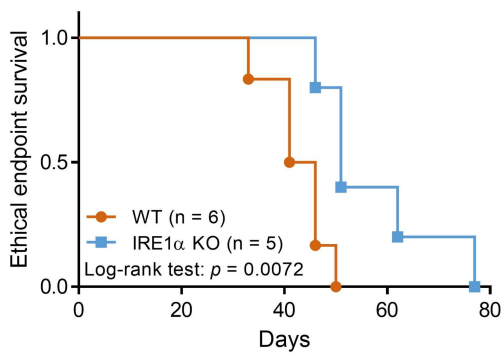
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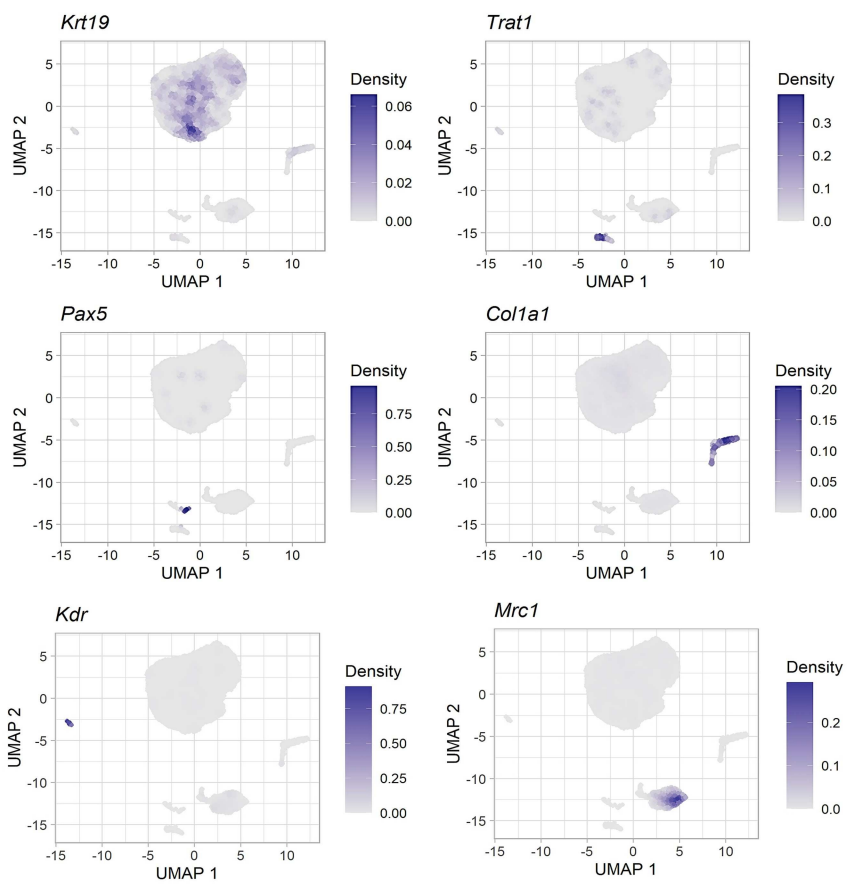
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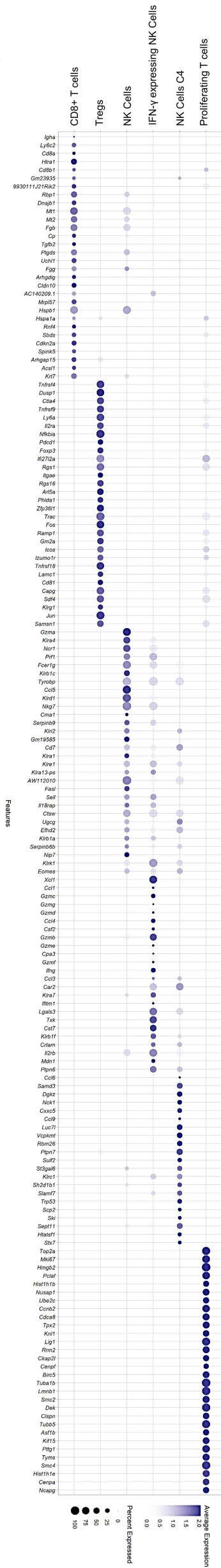
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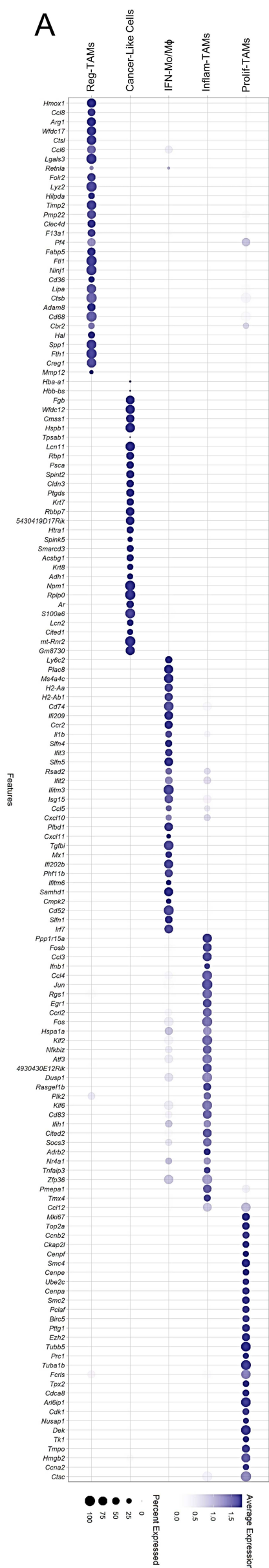


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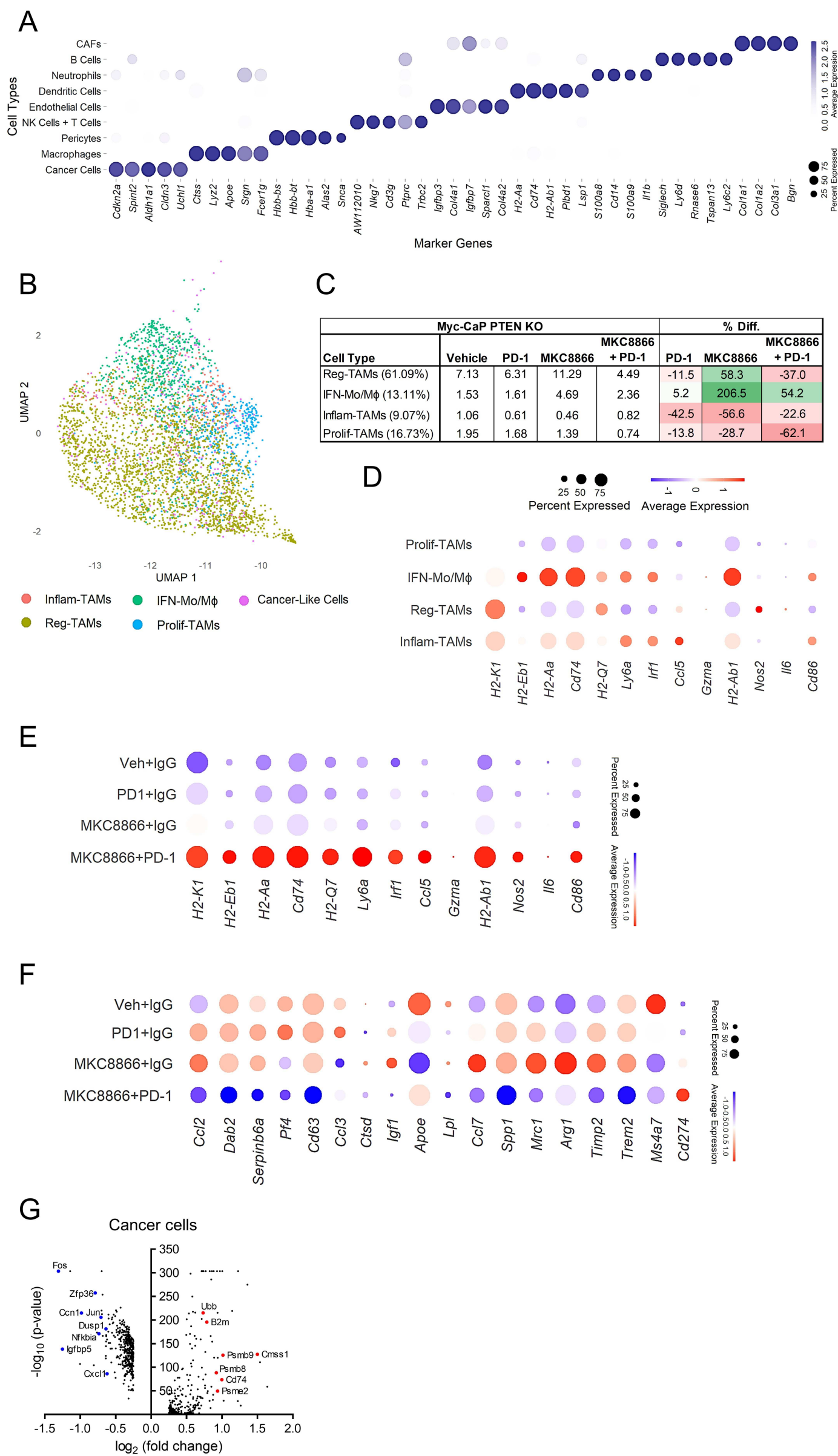
Supplementary Figure 5: Annotation of NK and T Cell subtypes in the scRNA-seq data from IRE1 α WT and KO Myc-CaP tumors. **A, B)** WT and IRE1 α KO (clone #2 cell lines were subcutaneously injected into two flanks of FVB mice. **A)** Tumor sizes were measured at the indicated time points for WT (n=3 mice, 6 tumors) and IRE1 α KO (n=3 mice, 6 tumors). **B)** Same as in A, but tumor weights measured at the end of the experiment. **C)** Kaplan-Meier survival analysis of the WT (n =6 mice) and IRE1 α KO (n = 5 mice) (clone #2) tumor-bearing mice is presented. **D, E)** scRNA-seq was performed from WT and IRE1 α KO (clone #2) tumors (six tumor samples per group were pooled for scRNA-seq) and analyzed. **D)** The two-dimensional UMAP plots display individual cells (represented as cluster points), where different shades of blue indicate varying levels of cell type-specific gene expression in each cell cluster that were employed to annotate different cell types in the TME. **E)** Dot plot illustrating the marker genes that define six subtypes of NK and T Cell main cluster. The intensity of each dot's color corresponds to the average gene expression observed across all cells within each indicated lymphocyte population. The size of the dots indicate the percentage expression of the indicated marker genes in the sub-clusters. Mean $\pm$ standard error by two-tailed student's t-test is presented in figures A (p = 0.01 for day 29 and p = 0.008 for day 33) and B (p = 0.005); * p<0.05, ** p<0.01.

Supplementary Figure 6



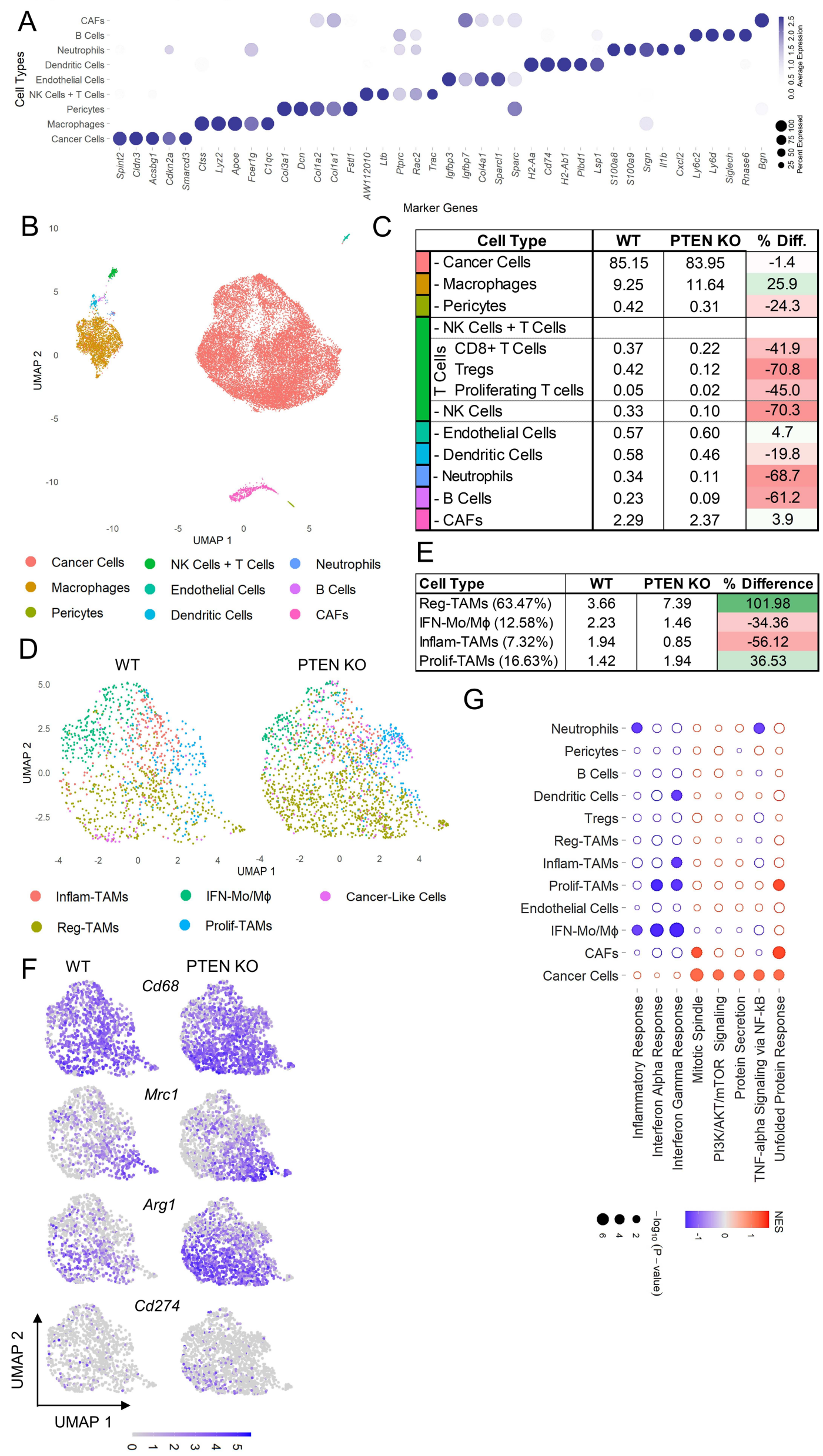
Supplementary Figure 6: Annotation of TAM subtypes and GSEA from the scRNA-seq data of IRE1 α WT and KO Myc-CaP tumors. A, B) scRNA-seq was performed from WT and IRE1 α KO (clone #2) tumors (six tumor samples per group were pooled for scRNA-seq) and analyzed. **A)** Dot plot shows the marker genes that define TAM subtypes. The intensity of each dot's color corresponds to the average gene expression observed across all cells within each indicated cell population. The size of the dots indicate the percentage expression of the indicated marker genes in the sub-clusters. **B)** The dot plot represents the result of the GSEA performed on all annotated cell types. The normalized enrichment score (NES) is depicted, with up- and down-regulated pathways color-coded in red and blue, respectively. The size of each dot represents the $-\log_{10}$ (p-value) of the indicated pathway for corresponding cell types. Details of the statistical test used for figure B was previously described ¹.

Supplementary Figure 7



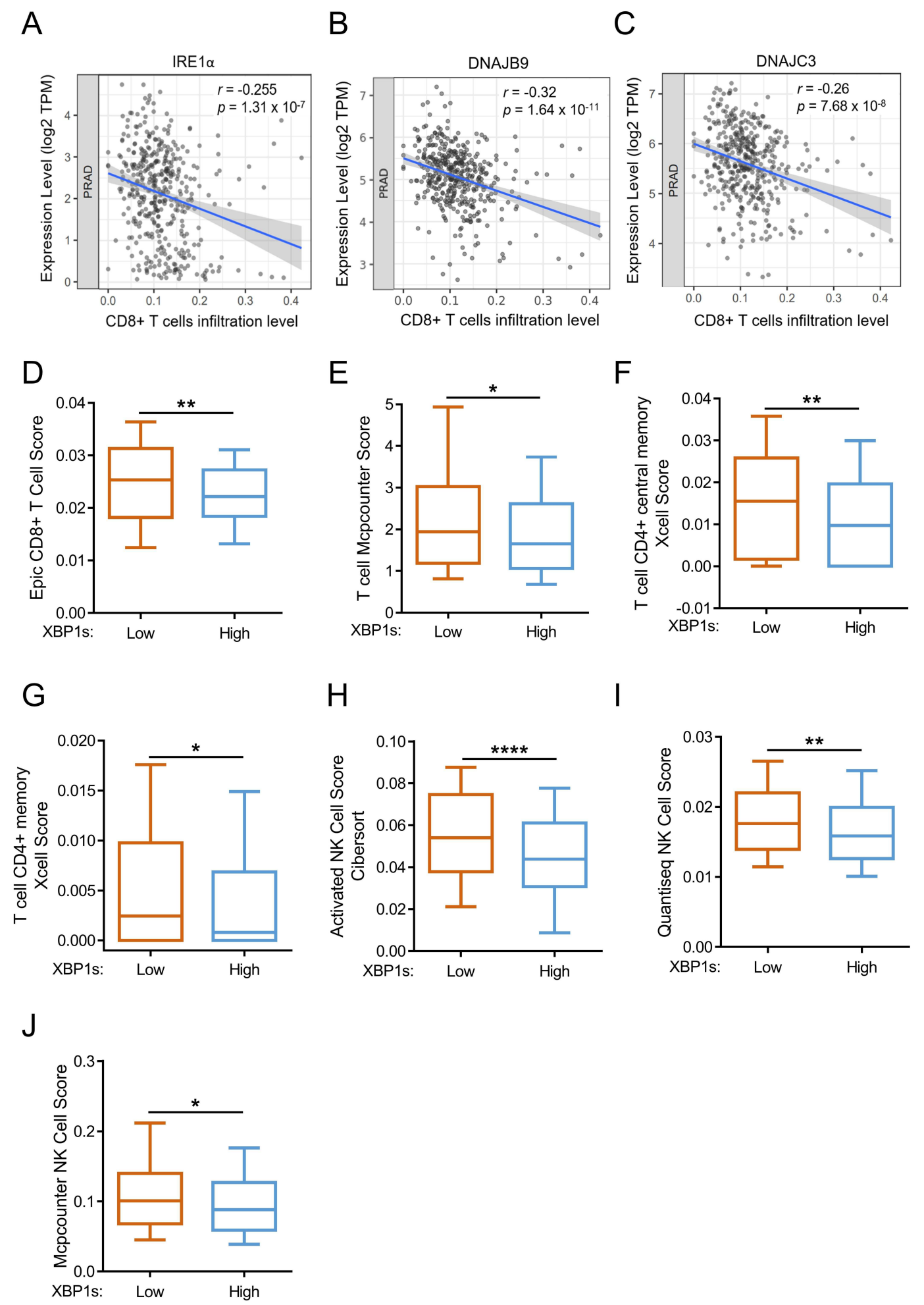
Supplementary Figure 7: MKC8866 and anti-PD-1 combination therapy remodels the TME. A-G) scRNA-seq was performed on tumor samples from the experiment presented in Figure 6D and 6E (six tumor samples per group were pooled for scRNA-seq) were analyzed. **A)** The dot plot illustrates top marker genes associated with indicated cell types from figure 7. **B)** Macrophages from vehicle, anti-PD-1 alone, MKC8866 alone, and MKC8866 + anti-PD-1 combination treatments were sub-clustered. The UMAP plot displays individual cells as points in two-dimensional space, with colors indicating different cell types. **C)** The table shows the percentage abundance of TAM subtypes within TME and the observed percentage changes for anti-PD-1, MKC8866, or combination treatments compared to vehicle. The percentage of each TAM subtype among the total TAM population in PTEN KO tumors is indicated within parentheses. **D)** The dot plot illustrates the expression of indicated genes in the various TAM subtypes. **E)** The dot plot shows the expression of M1 macrophage markers and **F)** M2 macrophage markers in the macrophage clusters for the indicated samples. **G)** The volcano plot illustrates the differentially expressed genes in cancer cells from the combination therapy scRNA-seq data compared to vehicle. The Wilcoxon rank-sum test implemented in Seurat was used for G.

Supplementary Figure 8



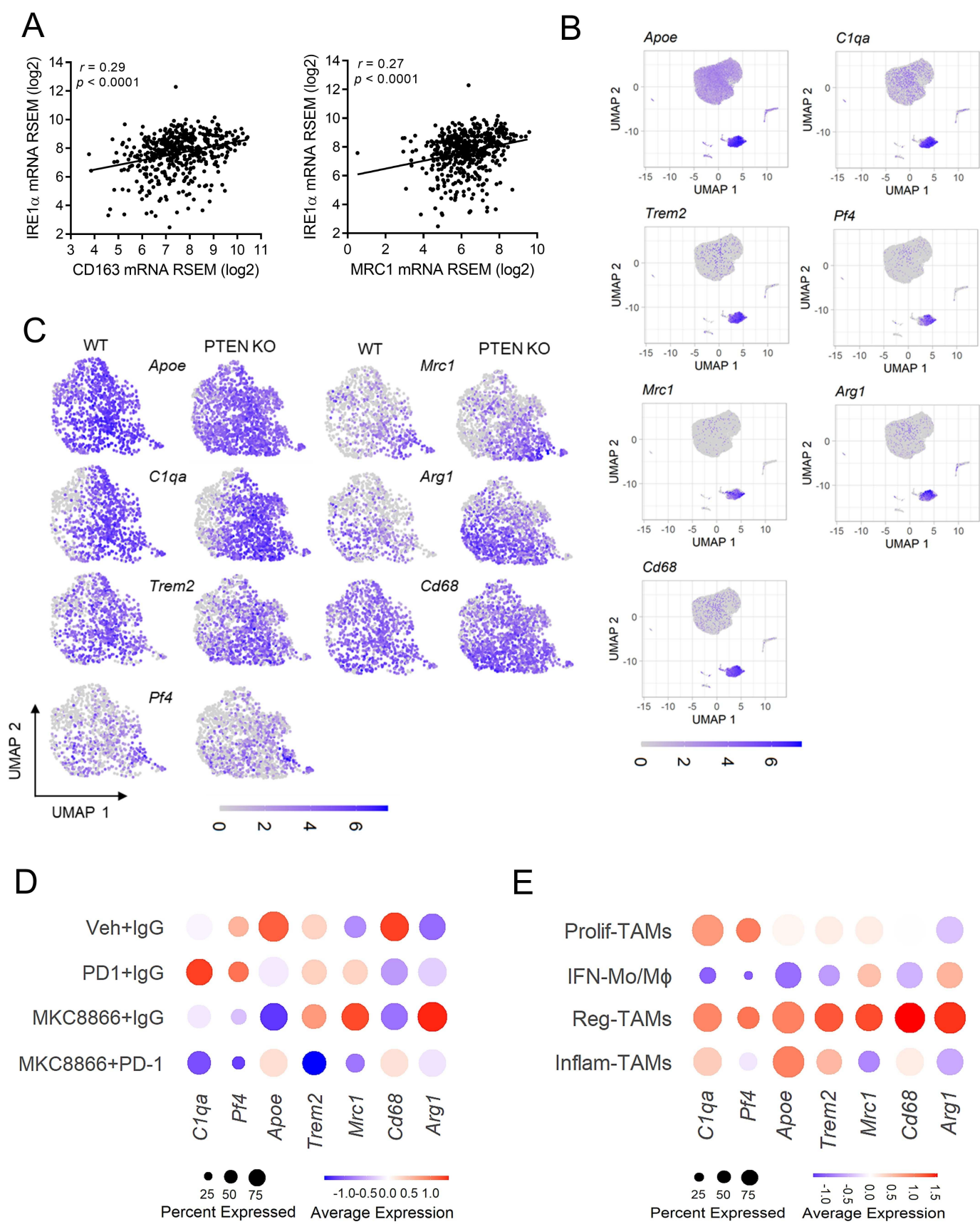
Supplementary Figure 8: PTEN loss in Myc-CaP tumors results in an immunosuppressive TME. A-G) scRNA-seq performed on tumor samples from Myc-CaP WT (from figure 4) and PTEN KO (Vehicle + anti-IgG treated) tumor samples (from figure 7) (six tumor samples per group were pooled for scRNA-seq) were analyzed and cell types were identified and annotated by clustering based on the expression of their top marker genes. **A)** The dot plot illustrates top marker genes associated with indicated cell types. **B)** The UMAP plot represents individual cells as points in a two-dimensional space. Distinct colors denote the assigned cell types, which are determined by the markers depicted in the dot plot in panel A. **C)** The table shows the percentage abundance of each indicated cell type within the TME, along with the observed percentage changes. **D)** The UMAP plots illustrate the TAM subtypes in WT and PTEN KO tumors, with different colors representing each distinct TAM subtype. **E)** The percentage abundance of TAM subtypes in WT and PTEN KO TME is shown, along with the observed percentage changes in PTEN KO compared to WT tumors. The percentage of each TAM subtype among the total TAM population in PTEN KO tumors is indicated within parentheses next to TAM subtypes. **F)** UMAP plots showing expression of indicated genes in the macrophage cluster from PTEN WT and KO tumors. **G)** The dot plot displays GSEA results from scRNA-seq data PTEN KO vs WT tumors, showing NES for the indicated cell types. Up- and down-regulated pathways are depicted in red and blue, respectively. Details of the statistical test used for figure G was previously described ¹.

Supplementary Figure 9



Supplementary Figure 9: IRE1 α -XBP1s pathway expression is negatively associated with T and NK cell infiltration in PCa patient samples. A-C) Correlation analysis between expression of the indicated genes and estimation of CD8⁺ T cell infiltration (based on Cibersort) in the TCGA PCa dataset (n =498). **D-J)** Box plots showing infiltration scores for specified immune cells, analyzed using indicated tools, in PCa tumors with low (n = 200) vs high XBP1s (n = 200) expression in the TCGA PCa dataset. Mean $\pm$ standard error by unpaired two-tailed Mann Whitney t-test is presented for figures D (p = 0.004), E (p = 0.02), F (p = 0.002), G (p = 0.04), I (p = 0.004), and J (p = 0.01); * p<0.05, ** p<0.01, **** p<0.0001. Infiltration estimation data for figures A-C was obtained from ². In box-plots, whiskers represent 10-90 percentile and middle lines indicate median of the data.

Supplementary Figure 10



Supplementary Figure 10: TAM gene signature derived from scRNA-seq data. A) IRE1α mRNA levels positively correlate with CD163 and MRC1 expression in the TCGA PCa dataset (n = 493). **B)** UMAP plots display expression of indicated genes in each cluster. **C)** UMAP plots display the expression of the TAM gene signature marker genes in the macrophage cluster from WT and PTEN KO tumors. **D)** The dot plot illustrates the expression of the TAM gene signature genes in the macrophage cluster for each indicated sample from PTEN KO syngeneic model. **E)** Same as in D, but expression is shown in each indicated TAM subtype for MKC8866 + anti-PD-1 treatment. r represents Spearman correlation where indicated.

References

- 1 Gennady Korotkevich, V. S., Nikolay Budin, Boris Shpak, Maxim N. Artyomov, Alexey Sergushichev. Fast gene set enrichment analysis. *bioRxiv* (2021).
- 2 Li, T. *et al.* TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res* **48**, W509-W514 (2020). <https://doi.org/10.1093/nar/gkaa407>