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## Supplemental information

# Anti-PD-1 immunotherapy with androgen deprivation

#### therapy induces robust immune infiltration

### in metastatic castration-sensitive prostate cancer

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Figure S1: Gene Expression Clustering, Related to Figure 1.

A) UMAP plot showing clustering of all cells in tumor microenvironment across all patients, clustered on gene expression instead of VIPER-inferred protein activity. Cell types are inferred by SingleR. Total number of clusters is smaller than clustering in Figure 1 on VIPER-inferred protein activity. B) UMAP plot from A, split by metastatic tissue site.



Figure S2: Top Gene Expression Cluster Markers, Related to Figure 2

Heatmap of top 5 most differentially upregulated genes for each cell type cluster from aggregate single-cell RNA-Sequencing data across all patient samples, with clusters corresponding to Figure S1. Each row represents a protein, grouped by cluster in which they are the most active, with cluster labels on the x and y-axes.



Figure S3: Identification of Tumor Cells by Marker Expression and Copy Number Variation, Related to Figure 6 A) log10 normalized expression of prostate cancer tumor marker protein KLK3 in each cell cluster identified by VIPER, such that expression is non-zero only in Epithelial cell clusters. B) InferCNV plot of cell-by-cell copy number variations, where all immune-lineage cells are taken as a copy-number-normal reference for inference of variations in copy number in Epithelial cell clusters and Endothelial cell cluster as a control. Each epithelial cell cluster is notable copy number aberrant across multiple chromosomes, while endothelial cells are grossly copy number normal.



Figure S4: Inferred Cell Type Frequencies in bulkRNA-Seq Bone Metastasis Data, Related to Figure 3 Cell Type frequencies inferred by CIBERSORTx from a cohort of n=17 hormone naïve castrate-sensitive bone metastases and n=21 short-term castrated samples 94. As in Figure 3A-B, significant increase in myeloid infiltrate is observed with ADT (p = 0.028), with trend toward decrease in CD4 non-Tregs (p = 0.37) and Tumor cells (p = 0.14). Statistically significant results (p<0.05 by Wilcox test) comparing inferred frequency in hormon

# Hallmarks of Cancer Pathway Enrichment

MSigDB Hallman	rk 2020	MSigDB Hallmark 2020				
Androgen Response *5.e-09		C2 M Charles *2.808-30				
Adipogenesis *7.26e-09		G2-M Checkpoint *8.54e-13				
Hypoxia *1.17e-06		Myc Targets V1 *4.72e-08				
Glycolysis *1.17e-06		DNA Repair *9.07e-05				
mTORC1 Signaling *5.58e-06		heme Metabolism *4.31e-04				
Fatty Acid Metabolism *8.68e-06		Reactive Oxygen Species Pathway 2.36e-02				
G2-M Checkpoint *2.49e-05	REF-EPI1	Myc Targets V2 3.23e-02	REF-EPI₂			
Xenobiotic Metabolism *2.49e-05		mTORC1 Signaling 7.35e-02				
p53 Pathway *2.49e-05		p53 Pathway 7.35e-02				
IL-2/STAT5 Signaling *9.77e-05		Unfolded Protein Response 1.04e-01				
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MSigDB Hallmar	rk 2020	MSigDB Hallmark 2 TNF-alpha Signaling via NF-kB *5.3e-17	020			
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Myc largets V1 *1.95e-05		Apoptosis #1 070-05				
G2-M Checkpoint *1.3e-04		Mitotic Spindle #2 99e 05-				
mTORC1 Signaling *1.3e-04		Huppyin #2.060.05				
heme Metabolism *1.3e-04		Hypoxia -3.062-05				
Hypoxia *3.91e-03		U 2/STATE Size 12 00- 01				
p53 Pathway *3.91e-03	RFF-FPI₃	IL-2/STATS Signaling *3.99e-04	RFF-FPI			
Unfolded Protein Response 5.25e-02		Interferon Gamma Response *4.18e-04				
		IL-6/JAK/STAT3 Signaling *5.76e-04				
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Giycolysis 6.27e-02 Allograft Rejection 6.27e-02 0 1 2 3 4 MSigDB Hallmar Apoptosis *1.10e-09 Interferon Alpha Response *2.06e-09 Interferon Gamma Response *2.84e-09 Reactive Oxygen Species Pathway *1.89e-06 TNF-alpha Signaling via NF-kB *5.92e-06 Unfolded Protein Response *9.28e-06 IL-2/STAT5 Signaling *3.11e-05 UV Response Up *1.30e-04 Protein Secretion *1.62e-04 Complement *1.63e-04 0 1 2 3 4 -log=0 MSigDB Hallmar Unfolded Protein Response *2.49e-11 Androgen Response *1.70e-05 TNF-alpha Signaling via NF-kB *1.07e-04 mTORC1 Signaling *1.07e-04 Hypoxia *7.62e-04 Reactive Oxygen Species Pathway *2.36e-03 Myc Targets V2 *3.81e-03 heme Metabolism 2.32e-02	ue) 5 6 7 rk 2020 REF-EPI5 k 2020 REF-EPI7	UV Response Up *6.48e-04 0 2 4 6 8 -logue(p-value) MSigDB Hallmark 21 MSigDB Hallmark 21 IL-2/STAT5 Signaling *6.78e-05 mTORC1 Signaling *7.00e-05 Reactive Oxygen Species Pathway *1.09e-04 Fatty Acid Metabolism *1.24e-03 Adipogenesis *3.47e-03 Complement *3.47e-03 Complement *3.47e-03 UV Response Up *8.46e-03 0 8 16 24 22 -logue(p-value) MSigDB Hallmark Reactive Oxygen Species Pathway *1.63e-08 Fatty Acid Metabolism *2.43e-06 Oxidative Phosphorylation *2.98e-06 Estrogen Response Late *1.94e-05 Androgen Response *4.29e-05 Hypoxia *1.14e-04	10 12 14 16 D20 REF-EPI6 40 48 56 64 C 2020 REF-EPI8			
Glycolysis 6.27e-02 Allograft Rejection 6.27e-02 1 2 3 4 -log1o(p-value Apoptosis *1.10e-09 Interferon Alpha Response *2.06e-09 Interferon Gamma Response *2.84e-09 Reactive Oxygen Species Pathway *1.89e-06 TNF-alpha Signaling via NF-kB *5.92e-06 Unfolded Protein Response *9.28e-06 IL-2/STAT5 Signaling *3.11e-05 UV Response Up *1.30e-04 Protein Secretion *1.62e-04 Complement *1.63e-04 0 1 2 3 4 -log1o(p-value MSigDB Hallmar Unfolded Protein Response *2.49e-11 Androgen Response *1.70e-05 TNF-alpha Signaling via NF-kB *1.07e-04 mTORC1 Signaling *1.07e-04 Hypoxia *7.62e-04 Reactive Oxygen Species Pathway *2.36e-03 Myc Targets V2 *3.81e-03 heme Metabolism 2.32e-02	ue) 5 6 7 REF-EPI5 4 2020 k 2020	UV Response Up *6.48e-04 0 2 4 6 8 -logue(p-value) MSigDB Hallmark 20 MSigDB Hallmark 20 IL-2/STAT5 Signaling *6.78e-05 mTORC1 Signaling *7.00e-05 Reactive Oxygen Species Pathway *1.09e-04 Fatty Acid Metabolism *1.24e-03 Adipogenesis *3.47e-03 Complement *3.47e-03 VV Response Up *8.46e-03 0 8 16 24 32 -logue(p-value) MSigDB Hallmart Reactive Oxygen Species Pathway *1.63e-08 Fatty Acid Metabolism *2.43e-06 Oxidative Phosphorylation *2.98e-06 Estrogen Response Late *1.94e-05 mTORC1 Signaling *1.94e-05 Androgen Response *4.29e-05 Hypoxia *1.14e-04 Adipogenesis *1.14e-04	10 12 14 16 D20 REF-EPI6 40 48 56 64 C2020 REF-EPI8			

Figure S5: Hallmarks of Cancer Enriched Pathways in Tumor Cell Subclusters, Related to Figure 6 For each tumor cell subcluster identified in Figure 6, plots of the top 10 enriched pathways from Hallmarks of Cancer. Pathway enrichment is computed on genes differentially expressed in each tumor subcluster relative to other tumor subclusters. -Log10(p-values) are plotted on the x-axes, such that statistically significant enriched pathways are shaded in blue.



AIC: 498.44; Concordance Index: 0.61



Figure S6: Tumor Single-Cell Subcluster Signatures and Outcome in West Coast SU2C, Related to Figure 7 A) Forest plot of Cox regression hazard ratios testing association in West Coast Stand Up to Cancer (SU2C) dataset of patient-by-patient Normalized Enrichment Score for each tumor subcluster gene set with overall survival. B) Kaplan-Meier curve testing association of binarized REF-EPI 7 gene set enrichment (greater than 0 = high, less than 0 = low) with survival, such that REF-EPI 7 enrichment significantly associates with improved survival. Kaplan-Meier curves are not shown for the remaining clusters as log-rank p-values for these were not statistically significant (p>0.05).

#### **GSEA Hazard Ratio**

REF-EPI1       (N=81)       1.03 (0.79 - 1.3)       0.85         REF-EPI2       (N=81)       0.99 (0.67 - 1.5)       0.953         REF-EPI3       (N=81)       (0.87 - 3.0)       0.953         REF-EPI4       (N=81)       (0.35 - 1.3)       0.205         REF-EPI5       (N=81)       (0.86 (0.35 - 1.3)       0.205         REF-EPI5       (N=81)       (0.78 (0.89 - 1.9)       0.172         REF-EPI6       (N=81)       (0.78 (0.46 - 1.3)       0.356         REF-EPI7       (N=81)       (0.56 - 1.8)       0.975         REF-EPI8       (N=81)       (0.71 (0.45 - 1.1)       0.157							:		
REF-EPI2 $(N=81)$ $0.999$ $0.67 - 1.5)$ $0.953$ REF-EPI3 $(N=81)$ $0.666$ $0.35 - 1.3)$ $0.131$ REF-EPI4 $(N=81)$ $0.666$ $0.35 - 1.3)$ $0.205$ REF-EPI5 $(N=81)$ $0.689 - 1.9)$ $0.172$ REF-EPI6 $(N=81)$ $0.78$ $0.356$ REF-EPI7 $(N=81)$ $0.56 - 1.8)$ $0.975$ REF-EPI8 $(N=81)$ $0.71$ $0.975$ REF-EPI8 $(N=81)$ $0.71$ $0.157$ x: 47; Global p-value (Log-Rank): 0.19701 $0.2$ $0.5$		REF-EPI1	(N=81)	1.03 (0.79 - 1.3)		·			0.85
REF-EPI3       (N=81)       1.62 (0.87 - 3.0)       0.131         REF-EPI4       (N=81)       0.666 (0.35 - 1.3)       0.205         REF-EPI5       (N=81)       0.130 (0.89 - 1.9)       0.172         REF-EPI6       (N=81)       0.78 (0.46 - 1.3)       0.356         REF-EPI7       (N=81)       0.566 - 1.8)       0.975         REF-EPI8       (N=81)       0.71 (0.45 - 1.1)       0.157		REF-EPI <sub>2</sub>	(N=81)	0.99 (0.67 - 1.5)					0.953
REF-EPI4       (N=81)       0.666 (0.35 - 1.3)       0.205         REF-EPI5       (N=81)       (0.89 - 1.9)       0.172         REF-EPI6       (N=81)       (0.46 - 1.3)       0.356         REF-EPI7       (N=81)       (0.566 - 1.8)       0.375         REF-EPI8       (N=81)       (0.45 - 1.1)       0.157         s: 47; Global p-value (Log-Rank): 0.19701       0.2       0.5		REF-EPI₃	(N=81)	1.62 (0.87 - 3.0)		H-			0.131
REF-EPIs       (N=81)       1.30 (0.89 - 1.9)       0.172         REF-EPI6       (N=81)       0.78 (0.46 - 1.3)       0.356         REF-EPI7       (N=81)       0.071 (0.56 - 1.8)       0.975         REF-EPI8       (N=81)       0.71 (0.45 - 1.1)       0.157         S: 47; Global p-value (Log-Rank): 0.19701       0.5       0.5		REF-EPI4	(N=81)	0.66 (0.35 - 1.3)	<b></b>	-			0.205
REF-EPI6         (N=81)         0.78 (0.46 - 1.3)         0.356           REF-EPI7         (N=81)         1.01 (0.56 - 1.8)         0.975           REF-EPI8         (N=81)         0.71 (0.45 - 1.1)         0.157           s: 47; Global p-value (Log-Rank): 0.19701         0.2         0.5		REF-EPI₅	(N=81)	1.30 (0.89 - 1.9)		۰	-		0.172
REF-EPI7         (N=81)         1.01 (0.56 - 1.8)         0.975           REF-EPI8         (N=81)         0.71 (0.45 - 1.1)         0.157           s: 47; Global p-value (Log-Rank): 0.19701         0.5         1         0		REF-EPI6	(N=81)	0.78 (0.46 - 1.3)	-	-			0.356
REF-EPIs         (N=81)         0.71 (0.45 - 1.1)         0.157           s: 47; Global p-value (Log-Rank): 0.19701         0.5         0.5         0.5		REF-EPI7	(N=81)	1.01 (0.56 - 1.8)				-	0.975
s: 47; Global p-value (Log-Rank): 0.19701		REF-EPI8	(N=81)	0.71 (0.45 - 1.1)		-			0.157
	S	: 47; Global p-value	(Log-Rank): 0.19701	2	0	5		2	

# Event AIC: 323.48; Concordance Index: 0.65



Figure S7: Tumor Single-Cell Subcluster Signatures and Outcome in East Coast SU2C, Related to Figure 7 A) Forest plot of Cox regression hazard ratios testing association in East Coast Stand Up to Cancer (SU2C) dataset of patient-by-patient Normalized Enrichment Score for each tumor subcluster gene set with overall survival. B) Kaplan-Meier curve testing association of binarized REF-EPI 3 gene set enrichment (greater than 0 = high, less than 0 = low) with survival, such that REF-EPI 3 enrichment significantly associates with worse survival. C) Kaplan-Meier curve testing association of binarized REF- EPI 7 gene set enrichment (greater than 0 = high, less than 0 = low) with survival, such that REF-EPI 7 enrichment significantly associates with improved survival. D) Kaplan-Meier curve testing association of binarized REF-EPI 8 gene set enrichment (greater than 0 = high, less than 0 = low) with survival, such that REF-EPI 8 enrichment significantly associates with improved survival. Kaplan-Meier curves are not shown for the remaining clusters as log-rank p-values for these were not statistically significant (p>0.05).