Supplementary Figures and Tables

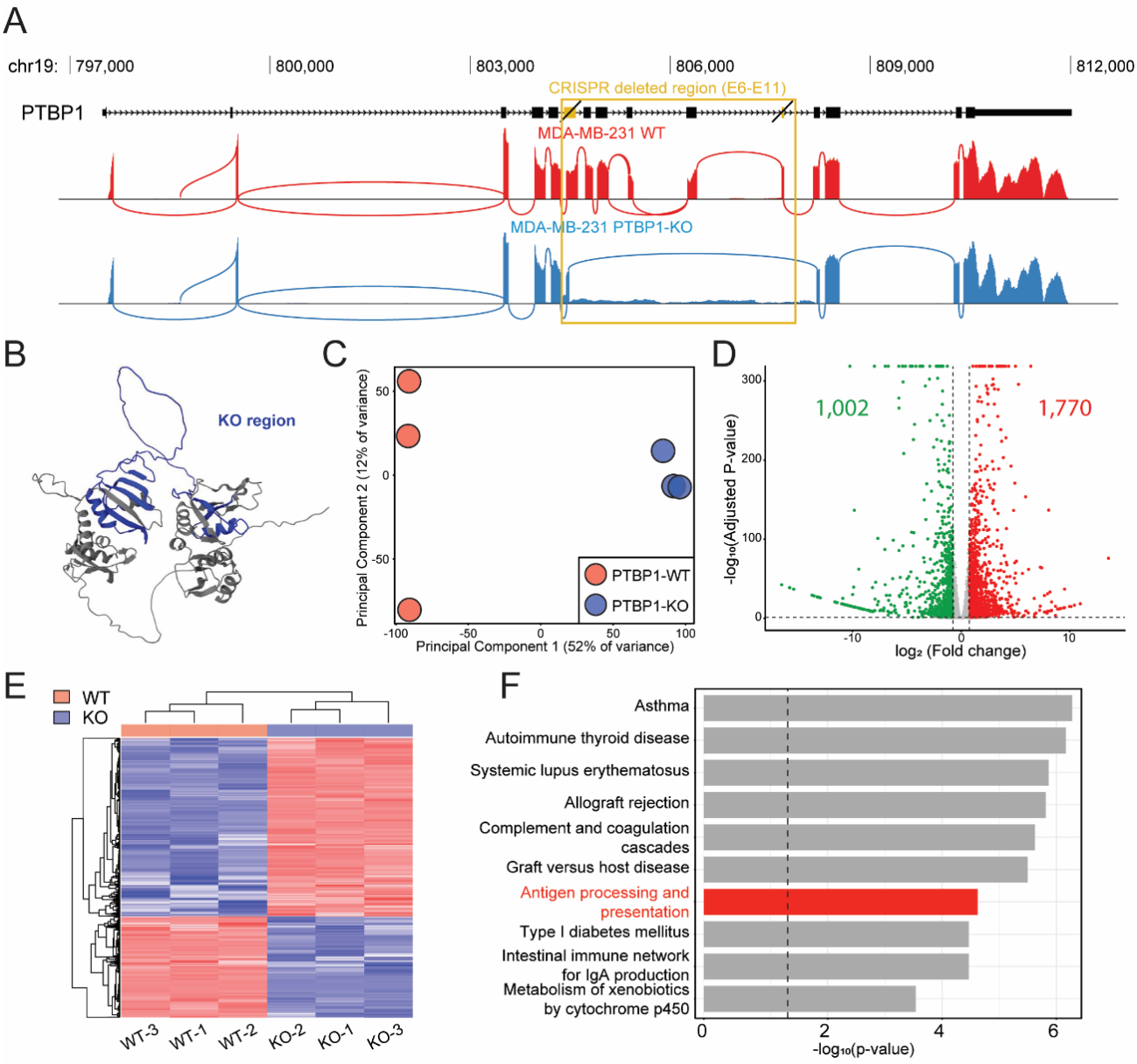
Title: PTBP1 drives immune dysfunction and predicts immunotherapy response in metastatic triple-negative breast cancer

Authors: Miquel Ensenyat-Mendez, Pere Llinàs-Arias, Javier I.J. Orozco, Andrés F. Bedoya-López, Ayla Manughian-Peter, Betsy Valdez, Sandra Íñiguez-Muñoz, Paula Gonzalez-Ericsson, Matthew P. Salomon, Silvia González-Martínez, Maria Gion, Javier Cortés, Marleen Kok, Maggie L. DiNome, Diego M. Marzese[#]

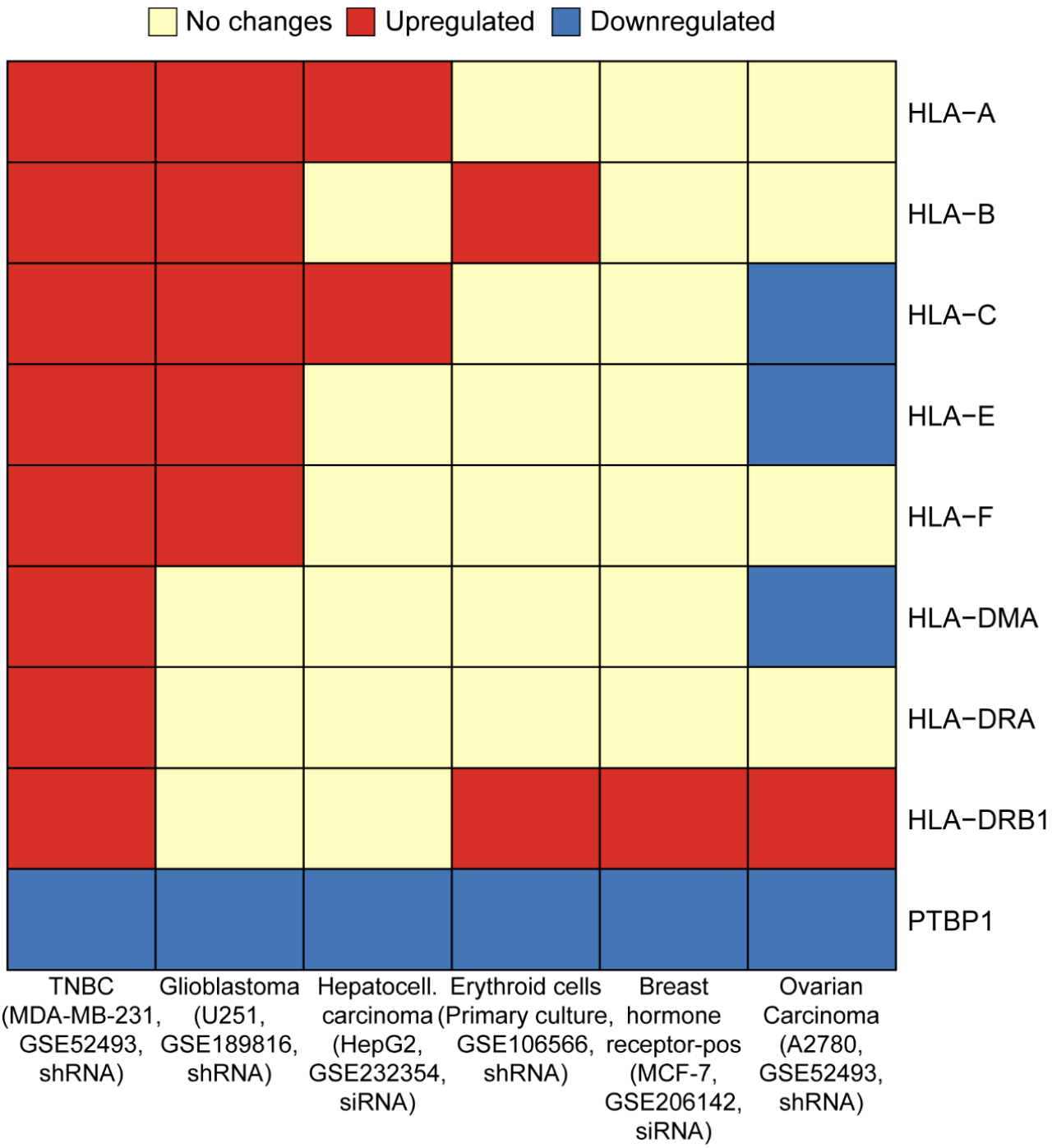
[#]Correspondence and requests for materials should be addressed to:

Diego M. Marzese (diego.marzese@duke.edu)

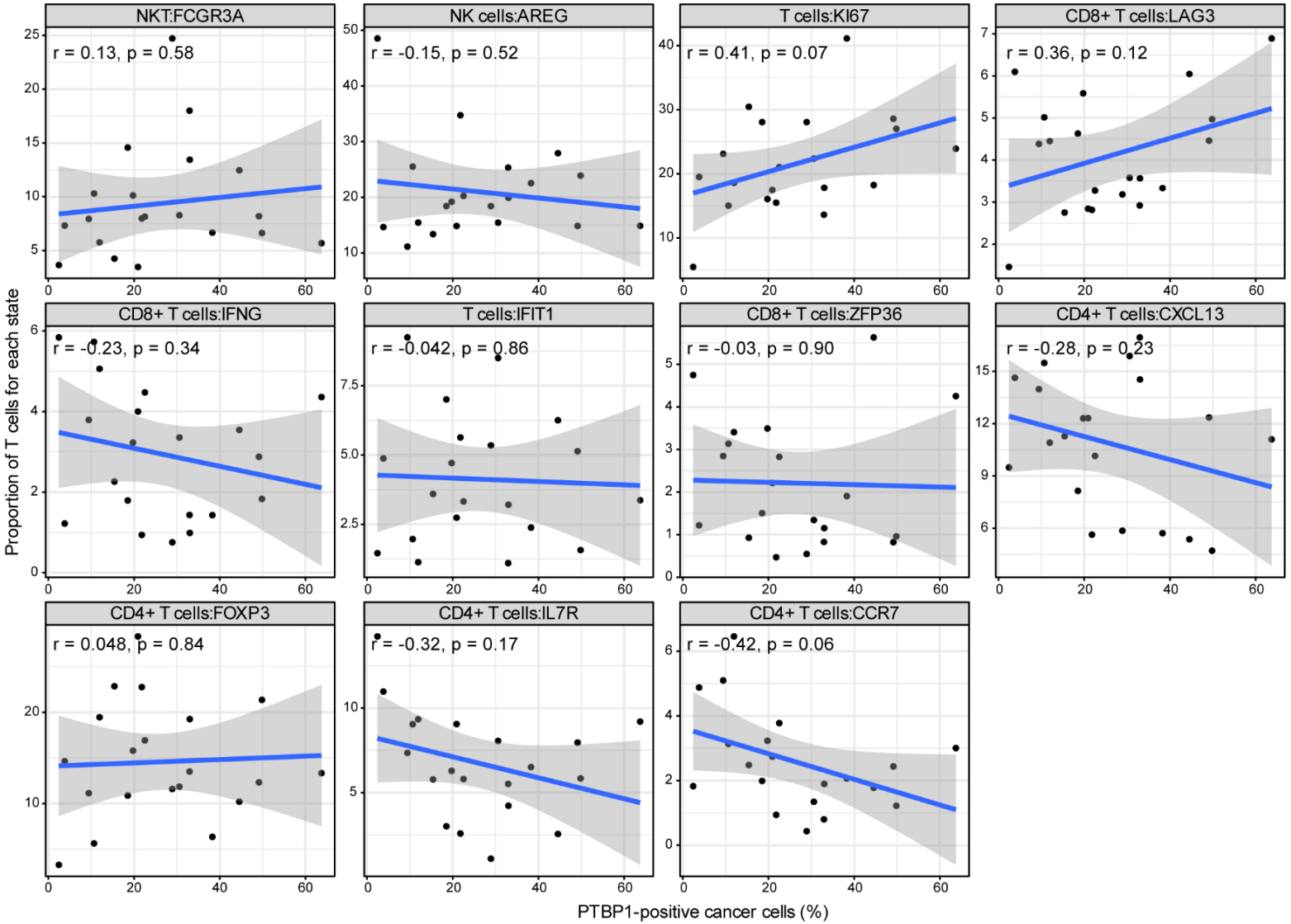
Keywords: Immunotherapy, RNA splicing, metastatic breast cancer, Triple-negative breast cancer, immune evasion, antigen presentation, immune checkpoint blockade, predictive biomarkers



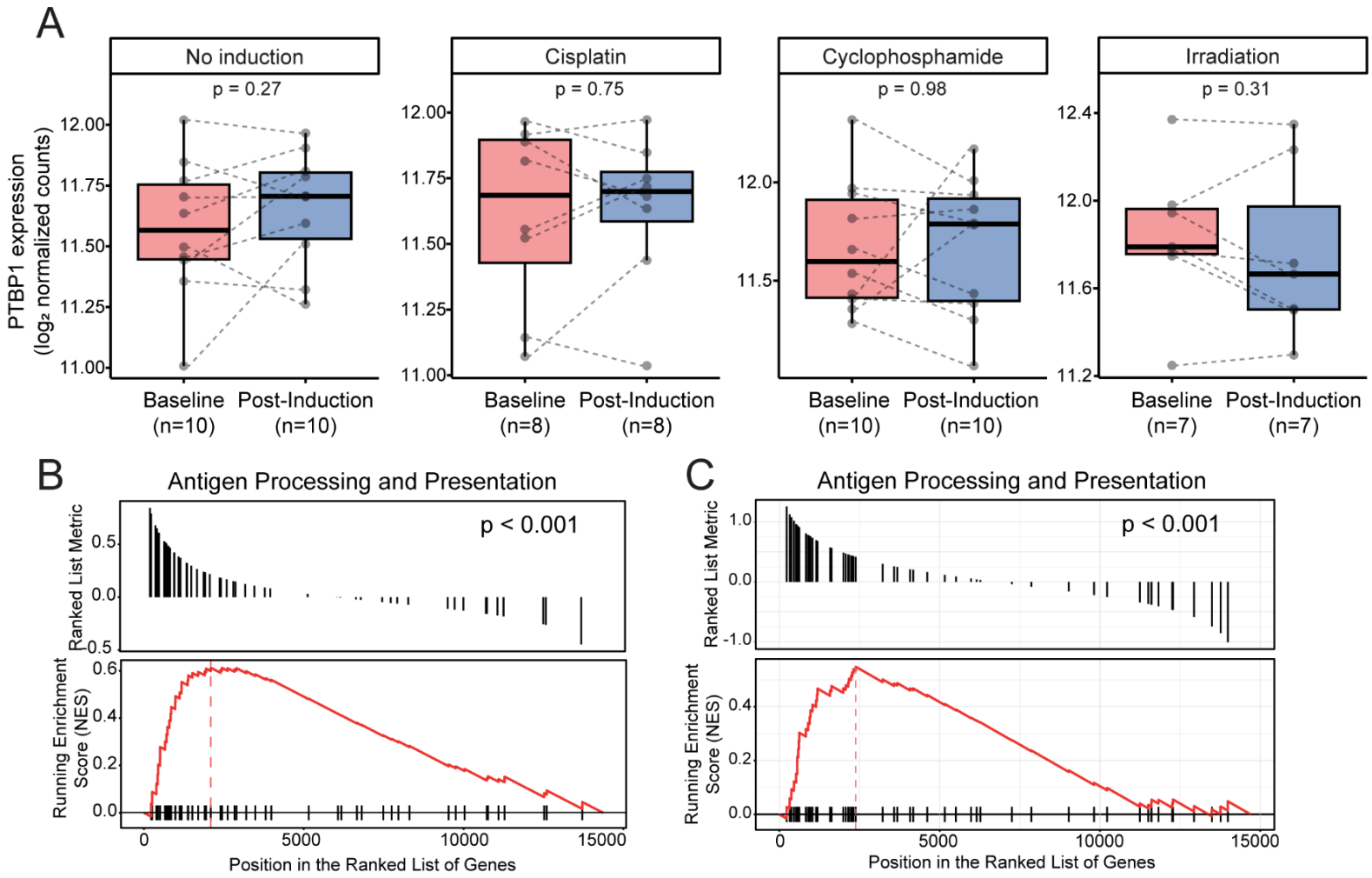
Supplementary Figure 1: Transcriptomic changes in TNBC cellular model after PTBP1 knockout. A) Sashimi plot representing the PTBP1 CRISPR-based knockout (KO) model. RNA-seq data highlights the depletion of Exons 6 to 11, which are targeted by the CRISPR guides. **B)** PTBP1 structure. Amino acids in the deleted region are highlighted in blue, which includes the four RNA recognition motifs of the protein. **C)** PCA representation of the transcriptome of PTBP1-KO and WT cells, characterized by RNA-seq. **D)** Volcano plot displaying the differentially expressed genes (DEGs) in PTBP1-KO and WT TNBC cells. **E)** Heatmap showing the differential clustering of PTBP1-KO and WT cells according to the DEGs. **F)** Pathway enrichment analysis using the DEGs between PTBP1-KO and WT cells, highlighting the changes in the antigen processing and presentation pathway.



Supplementary Figure 2: PTBP1 modulates MHC gene expression across different types of cancer. Heatmap displaying the changes in gene expression of various MHC Class I and Class II genes from different studies disrupting *PTBP1* expression in cancer cells.

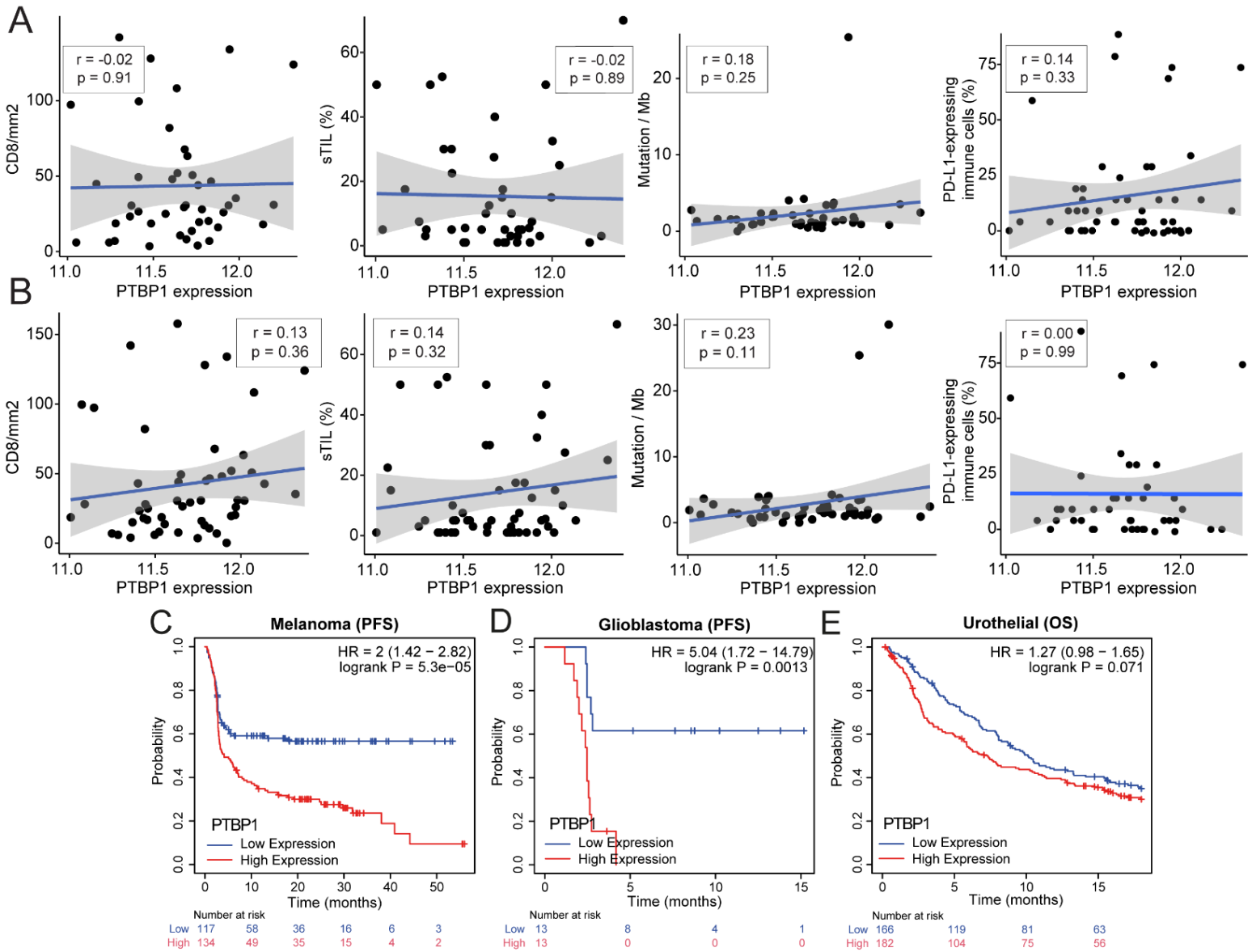

NKT:FCGR3A
r = 0.13, p = 0.58
NK cells:AREG
r = -0.15, p = 0.52
T cells:KI67
r = 0.41, p = 0.07
CD8+ T cells:LAG3
r = 0.36, p = 0.12
CD8+ T cells:IFNG
r = -0.23, p = 0.34
T cells:IFIT1
r = -0.042, p = 0.86
CD8+ T cells:ZFP36
r = -0.03, p = 0.90
CD4+ T cells:CXCL13
r = -0.28, p = 0.23
CD4+ T cells:FOXP3
r = 0.048, p = 0.84
CD4+ T cells:IL7R
r = -0.32, p = 0.17
CD4+ T cells:CCR7
r = -0.42, p = 0.06
Proportion of T cells for each state
PTBP1-positive cancer cells (%)

Supplementary Figure 3: Associations between PTBP1 expression and specific T cell subsets in TNBC tumors. Correlation analyses between the proportion of PTBP1-positive tumor cells and the abundance of individual T cell subsets associated with several functional states in TNBC tumors in scRNA-seq data.

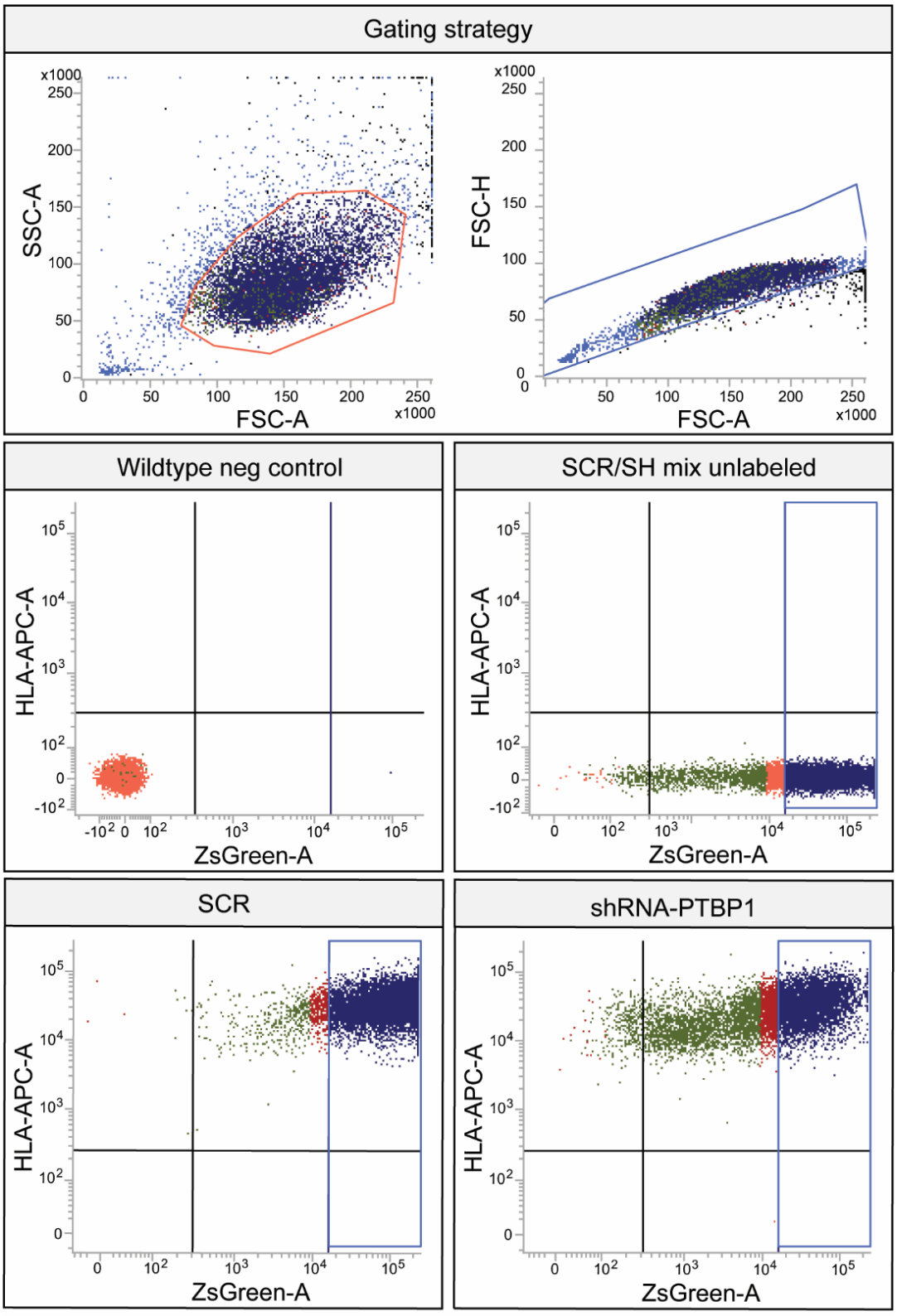


Supplementary Figure 4: Induction treatments other than doxorubicin do not reduce *PTBP1* expression. A) *PTBP1* expression in paired tumor biopsies before and after induction across non-doxorubicin arms of the TONIC trial. No significant changes in *PTBP1* levels were observed

following cisplatin, cyclophosphamide, or irradiation induction strategies ($p>0.05$ for all). **B)** GSEA of antigen processing and presentation pathway in tumors exposed to doxorubicin induction treatment. **C)** GSEA of antigen processing and presentation pathways in tumors from TNBC patients who responded to nivolumab compared to non-responders.


A
r = -0.02
p = 0.91
CD8/mm2
r = -0.02
p = 0.89
sTIL (%)
r = 0.18
p = 0.25
Mutation / Mb
r = 0.14
p = 0.33
PD-L1-expressing immune cells (%)
PTBP1 expression
B
r = 0.13
p = 0.36
r = 0.14
p = 0.32
r = 0.23
p = 0.11
r = 0.00
p = 0.99
C
Melanoma (PFS)
HR = 2 (1.42 − 2.82)
logrank P = 5.3e−05
D
Glioblastoma (PFS)
HR = 5.04 (1.72 − 14.79)
logrank P = 0.0013
E
Urothelial (OS)
HR = 1.27 (0.98 − 1.65)
logrank P = 0.071
Probability
Time (months)
PTBP1
Low Expression
High Expression
Number at risk
Low 117 58 36 16 6 3
High 134 49 35 15 4 2
Low 13 8 4 1
High 13 0 0 0
Low 166 119 81 63
High 182 104 75 56

Supplementary Figure 5: *PTBP1* expression is not associated with conventional immune biomarkers in metastatic TNBC. A-B) Correlation of **(A)** baseline (up) and **(B)** pre-nivolumab (bottom) *PTBP1* expression with $CD8^+$ cells per mm^2, the percentage of TILs, the tumor mutational burden, and the percentage of PD-L1 expressing immune cells in the TONIC clinical trial. **C-D)** Kaplan-Meier analysis of Progression-Free Survival (PFS) in **(C)** melanoma and **(D)** glioblastoma patients treated with IT stratified by *PTBP1* expression levels. **E)** Kaplan-Meier analysis of Overall Survival (OS) in urothelial carcinoma patients treated with IT stratified by *PTBP1* expression levels.



Supplementary Figure 6

Supplementary Figure 6: Gating strategy used in the flow cytometry analysis to analyze HLA expression. For the cells in the knockdown model, green cells were selected as the 10% of cells with the highest ZsGreen fluorescence.

Supplementary Table 1. List of study cohorts and databases, including clinical specimens analyzed in the study, with accession codes and number of patients.

Cohort	Accession Code	Total Samples	Total TNBC	Total TNBC after filtering
TCGA-BRCA	Genomic Data Commons portal	1009	152	95
SCAN-B	doi: 10.17632/yzxtxn4nmd.4	9206	778	655
AURORA-US	GSE209998	129	52	52
NCT03366844	GSE246613	36	36	20
TONIC	NA (DTA)	54	54	53
I-SPY2	GSE173839; GSE194040	140	90	50

Supplementary Table 2. Sequence of nucleic acids used across the project.

Primer name (application)	Sequence
SCR_S (KD)	gatccCGCAGAACAAATTCGTCCATTCAAGAGATGGACGAATTTGTTCTGCGTTTTTTACGCGTg
SCR_AS (KD)	aattcACGCGTAAAAAACGCAGAACAAATTCGTCCATCTCTTGAATGGACGAATTTGTTCTGCGg
shRNA-PTBP1_S (KD)	gatccGCACAGTGTTGAAGATCATTTCAAGAGAATGATCTTCAACACTGTGCTTTTTTACGCGTg
shRNA-PTBP1_AS (KD)	aattcACGCGTAAAAAAGCACAGTGTTGAAGATCATTCTCTTGAAATGATCTTCAACACTGTGCg
PTBP1_Fd (qPCR)	CAAGTTCGGCACAGTGTTGA
PTBP1_Rv (qPCR)	TACTTGACGTTGAGGCTGGT
SDHA_Fd (qPCR)	TCAGCATGCAGAAGTCAAT
SDHA_RV qPCR)	GAACGTCTTCAGGTGCTTT
sgPTBP1_Ex6 (KO)	GCGGUGAACUCGGUCCAGUCGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU
sgPTBP1_Ex11(KO)	GCCGUCCGCCAUCUGCACUAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU

KD: knockdown, KO: CRISPR-mediated knockout. Lower case bases in KD primers generate restriction enzyme-compatible cohesive ends. Underlined bases in KO primers correspond to sgRNA scaffold.