Appendix

MLLT6 Maintains *PD-L1* Expression and Mediates Tumor Immune Resistance

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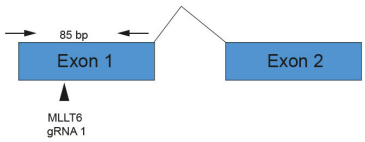
A

gRNA-ID	Exon	sgRNA	Chr. (hg38)	Target site location	
MLLT6-1	1	CTGCGTATGTTCGGACGAGA	chr17	38705656	38705675
MLLT6-2	4	AGGCACGTACTGCAGCACGA	chr17	38707835	38707854
MLLT6-3	4	GCCTCATGATCGCTTCAACA	chr17	38707851	38707870
MLLT6-4	5	TCGACATCCATGGCGGTTAC	chr17	38709237	38709256



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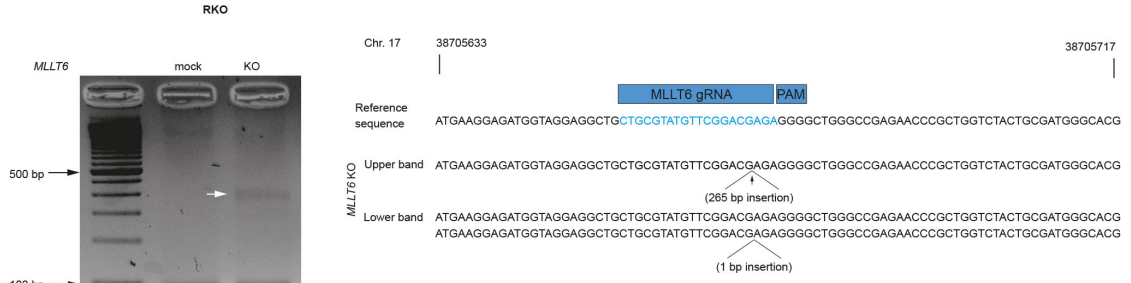

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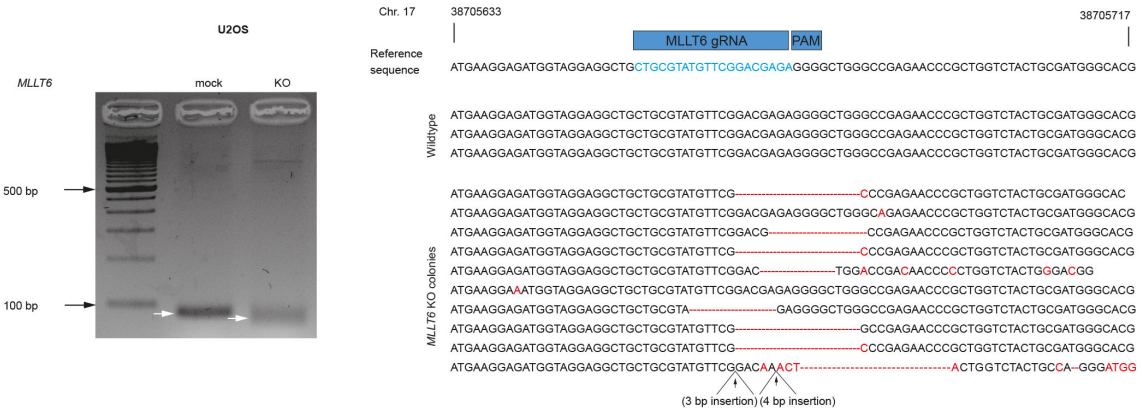
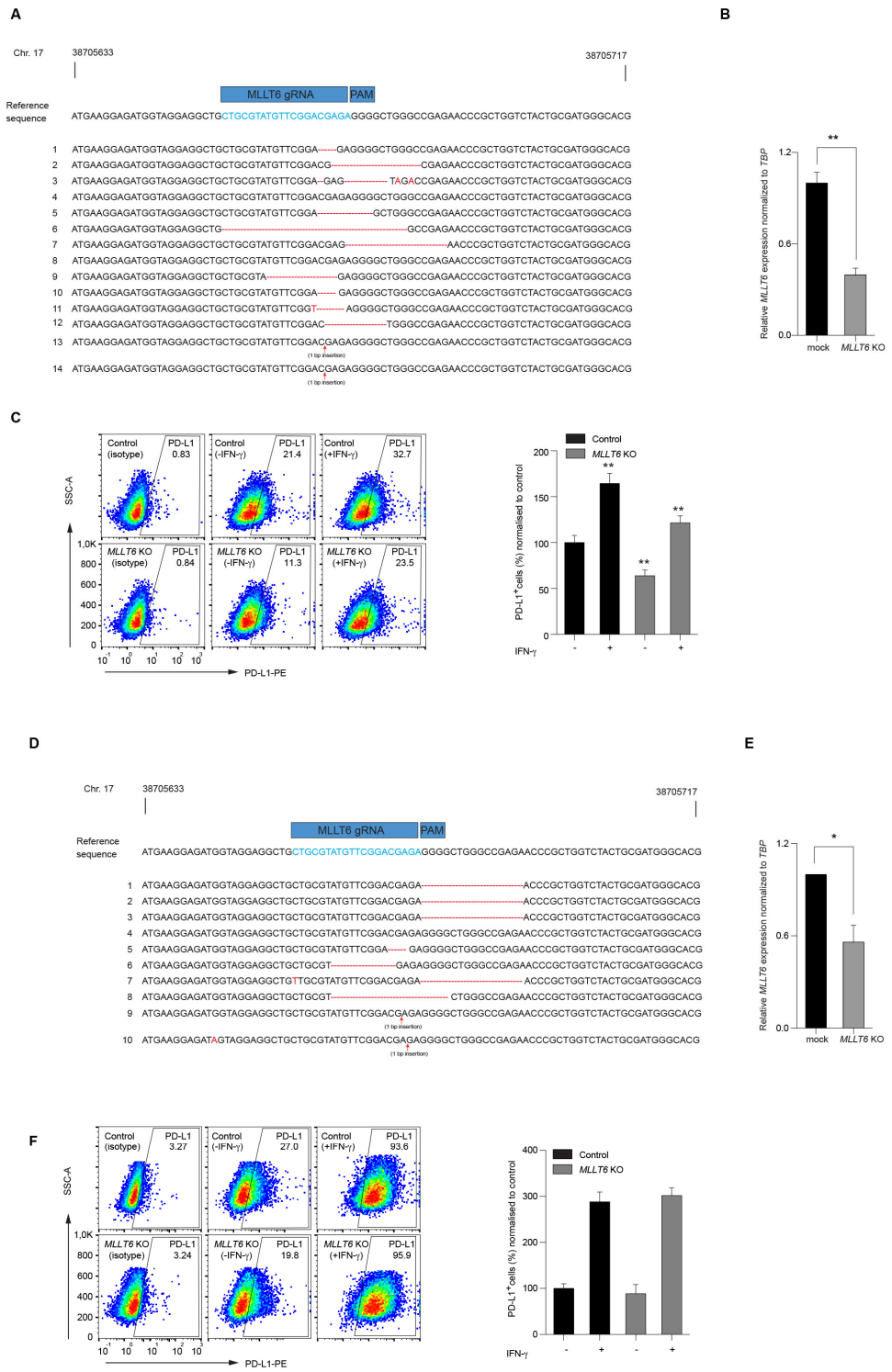
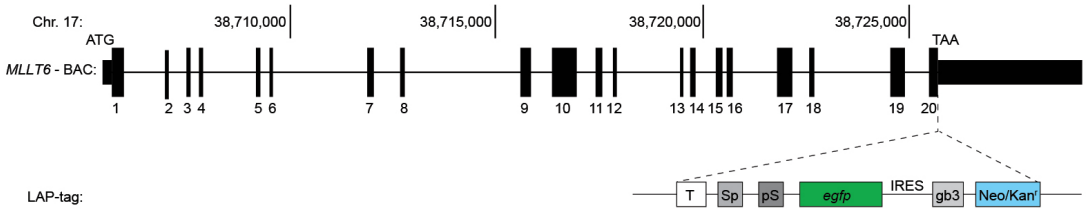


Appendix Figure S1. Genotyping RKO and U2OS *MLLT6* knockout cells.
A Sequences of gRNAs targeting *MLLT6* and their genomic locations (left). Schematic representation (right) of genomic targeting site and primer location employed for genotyping RKO and U2OS *MLLT6* knockout cells. Monoclonal RKO (B) and polyclonal U2OS (C) *MLLT6* knockout cells genotyping results. Agarose gel images of PCR products obtained from genotyping PCR reactions of mock and *MLLT6* knockout cells. White arrows indicate bands that were subjected to Sanger sequencing. Sequencing reads (right) aligned to reference sequence of *MLLT6* (top row), with gRNA target site (blue) and insertion-deletion mutations as indicated (red).



Appendix Figure S2. *MLLT6* and *PD-L1* expression in HeLa and SW480 cancer cells.
A and D Genotyping from HeLa (A) and SW480 (D) *MLLT6* polyclonal knockout cell lines. Sequencing reads from several clones aligned to reference sequence of *MLLT6* (top row), with gRNA target site (blue) and insertion-deletion (indel) mutations (red) as indicated.
B and E *MLLT6* transcript levels in HeLa (B) and SW480 (E) *MLLT6* polyclonal knockout lines normalized to mock control (± SD (n=3); **p* < 0.05; ***p* < 0.01; df=2).
C and F Representative flow cytometry plots (left) of PD-L1 cell surface presentation in the presence and absence of IFN-γ in HeLa (C) and SW480 (F) mock and *MLLT6* knockout cells. Percentages of cells in the PD-L1$^+$ gate after staining with anti-PD-L1 antibody or isotype controls. Bar plots represent percentage of PD-L1$^+$ cells in mock (black) and *MLLT6* knockout (grey) cells with and without IFN-γ treatment normalized to mock control from four (HeLa) and three (SW480) independent experiments (± SD; **p* < 0.05).

A



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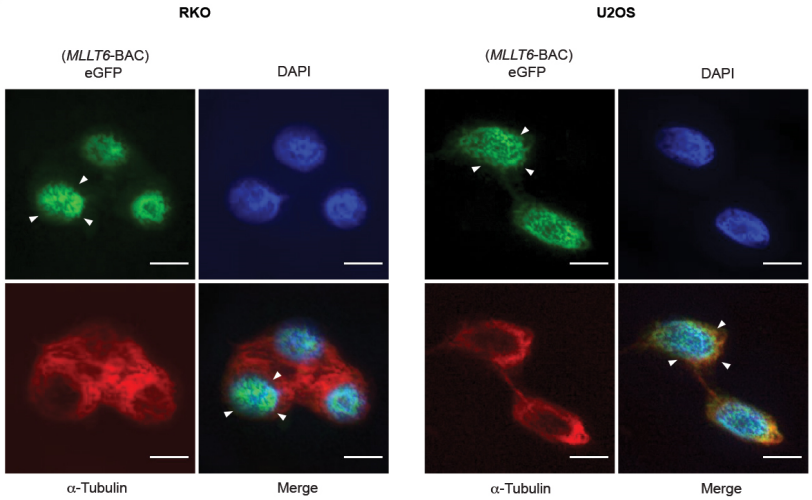
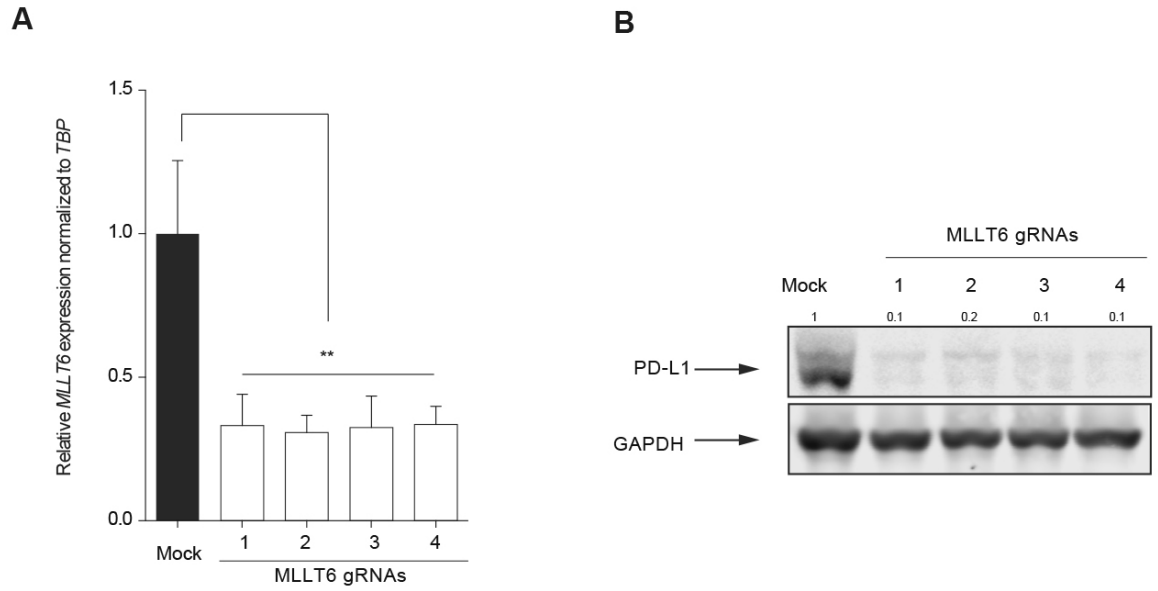
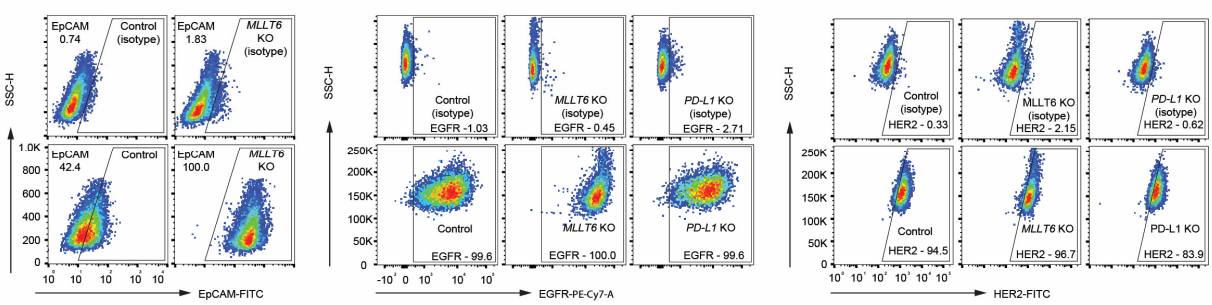


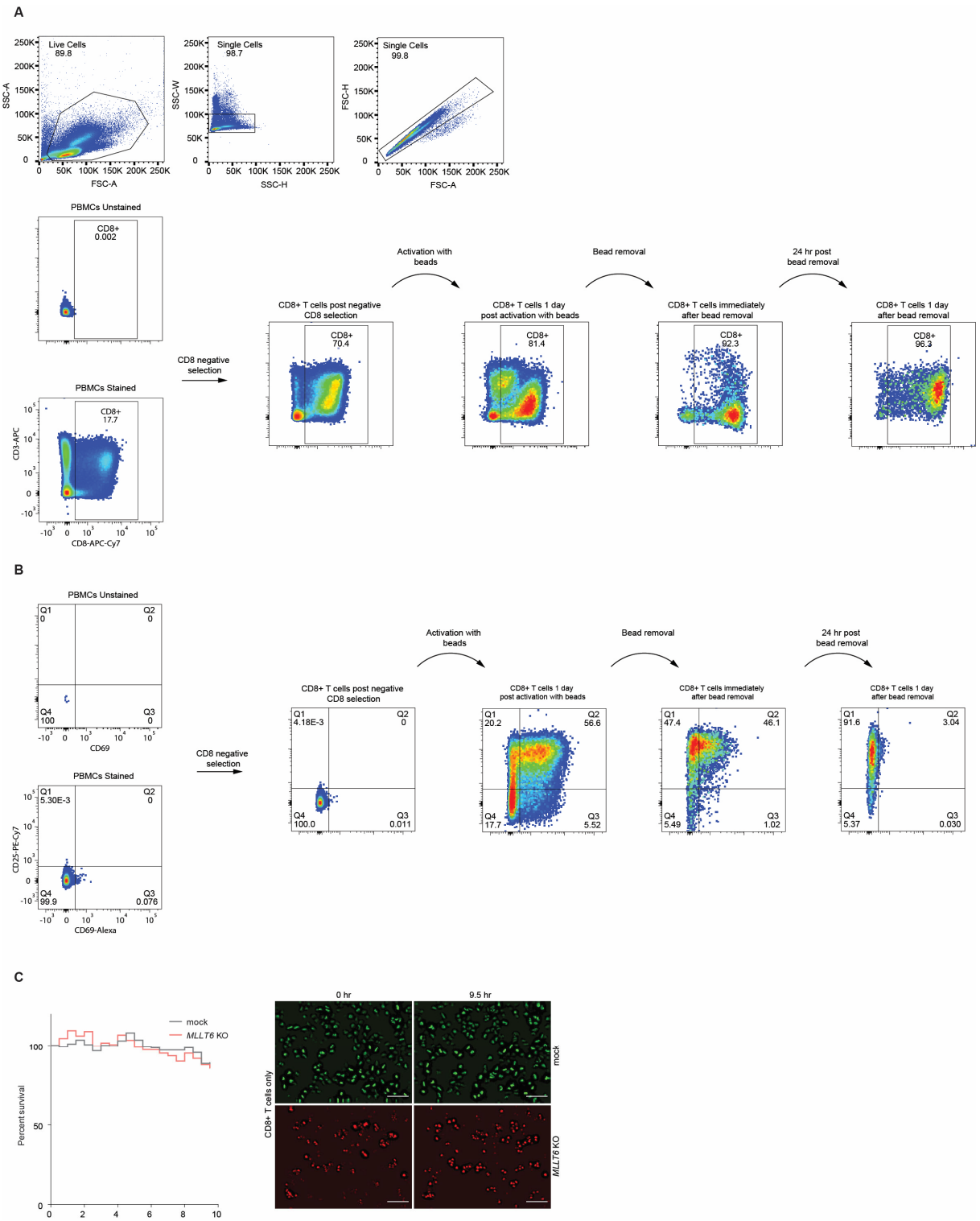
Appendix Figure S3. Characterization of MLLT6-LAP reporter cells using a tagged BAC.
A Schema of *MLLT6* genomic locus outlining the exon-intron arrangement on chromosome 17 (numbers: chromosome position) with structure of the C-terminal LAP-tag in a bacterial artificial chromosome (BAC) (T: TEV cleavage site, Sp: S-peptide, pS: PreScission cleavage site, eGFP: enhanced green fluorescent protein, IRES: internal ribosomal entry site, gb3: bacterial promoter, Neo/Kan^r: neomycin/kanamycin resistance gene).
B Immunofluorescence microscopy images of RKO (left) and U2OS (right) *MLLT6* knockout cell lines harboring a MLLT6-LAP BAC stained with α-eGFP, α-tubulin antibodies and DAPI. Arrowheads indicate MLLT6-eGFP protein localized to the nucleus (scale bars: 10 μm).



Appendix Figure S4. *MLLT6* knockout by four independent gRNAs and PD-L1 expression.
A *MLLT6* transcript levels (± SD, n=3) in U2OS mock and polyclonal *MLLT6* knockout cells generated by four independent gRNAs, normalized to expression in mock cells (** $p < 0.01$; df =3).
B Total cellular protein levels of PD-L1 (top) and GAPDH (bottom) in U2OS mock and polyclonal *MLLT6* knockout cells generated by four independent gRNAs. Numbers indicate relative band intensities of PD-L1 protein normalized to GAPDH.



Appendix Figure S5. Antigen presentation in *MLLT6* and *PD-L1* knockout cells.
Cell surface presentation of Ep-CAM (left), EGFR (middle) and HER2 (right) in U2OS control or polyclonal *MLLT6* or *PD-L1* knockout cells. Numbers indicate percentage of cells in the respective gates.

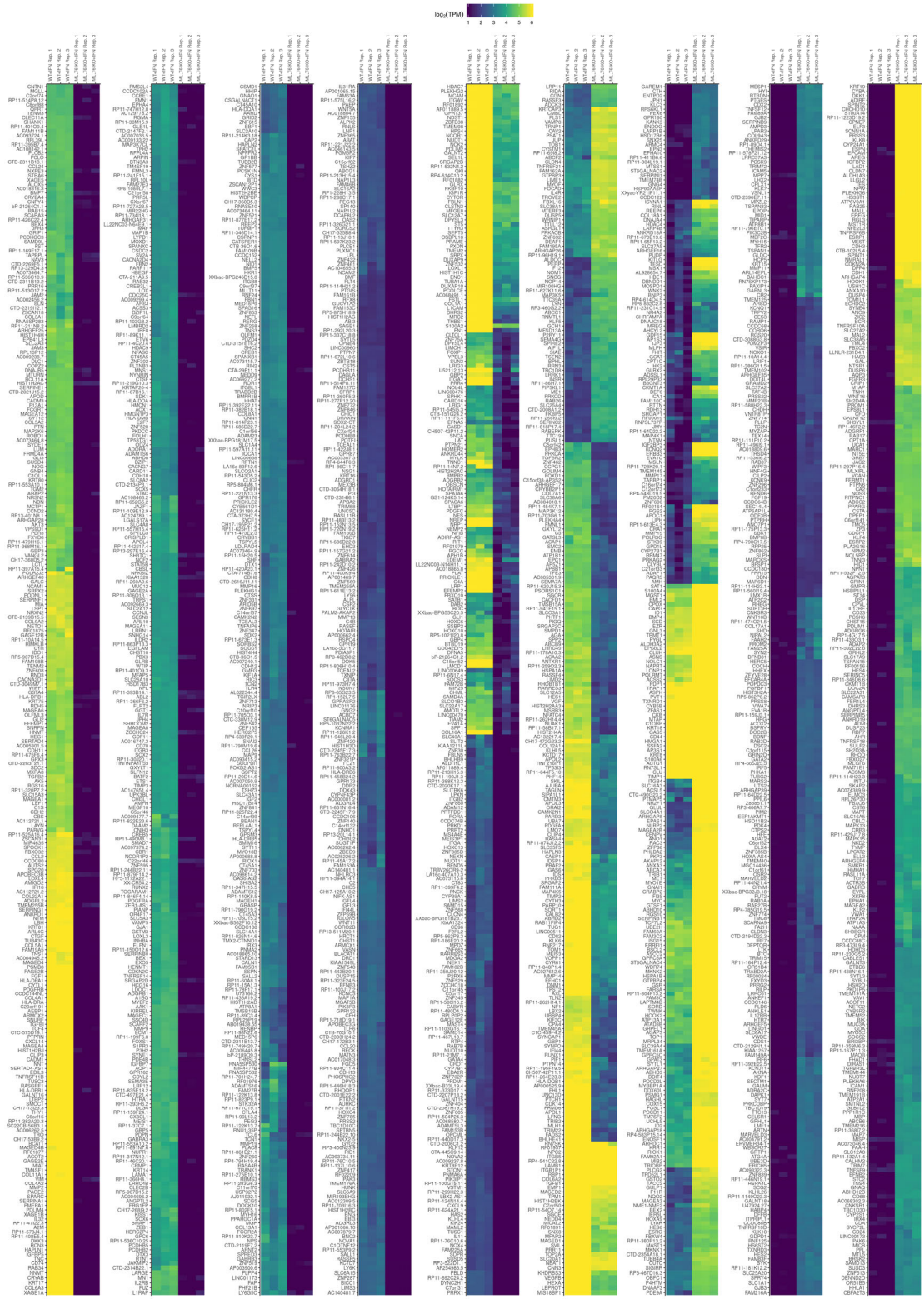


Appendix Figure S6. CD8⁺ T cell isolation and activation from PBMCs.

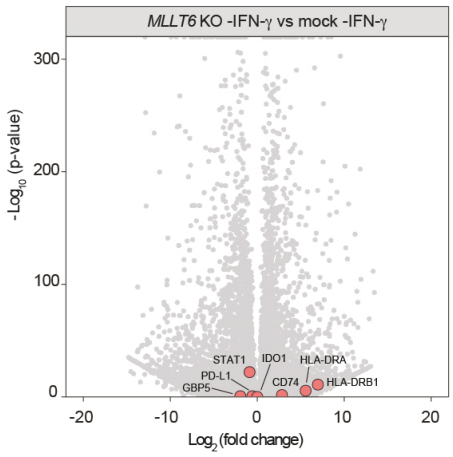
A Workflow outlining the steps involved in PBMC and CD8⁺ T cell isolation from human blood. Representative plots showing gating strategies followed to distinguish live and dead cells (top left) and to separate doublets from single cells (top middle and top right). Flow cytometry plots show cells stained with anti-CD3 and anti-CD8 antibodies to assess purity and efficiency of T cell isolation.

B Scatter plots showing PBMCs and CD8⁺ T cells stained with anti-CD25 and -CD69 antibodies to determine activation status of CD8⁺ T cells. CD25 and CD69 expression correlate with late and early activation of CD8⁺ T cells respectively.

C Kaplan Meier survival plot (left) showing percentage survival of mock (grey) and *MLLT6* knockout (red) cells treated with activated CD8⁺ T cells (CTLs) in the absence of BiTE or bi-specific antibodies over a course of 9.5 hours. Representative images (right) of time-lapse fluorescence microscopy following U2OS mock (eGFP tagged, green) and *MLLT6* knockout (mCherry tagged, red) cells immediately after addition of CTLs (top left and bottom left) in the absence of BiTE or bi-specific antibodies and after 9.5 hours (top right and bottom right) (scale bars: 100 μm).



Appendix Figure S7. Gene expression heat map of cells stimulated by IFN-γ.
Genes differentially expressed comparing IFN-γ treated mock and IFN-γ treated *MLLT6* knockout cells in nine split panels. Columns represent replicates. Each row represents expression levels of individual genes across the six samples. The cell color represents $\log_2$ of TPM values for genes significantly modulated (FDR < 0.05, fold-change > 4) and expressed in at least one of the two conditions (TPM > 3). Gene order represents hierarchical clustering of the gene expression matrix, calculated using Euclidean distance and complete linkage.



Appendix Figure S8. Transcriptome changes in U2OS *MLLT6* knockout cells.
Scatter plot of pairwise comparison of transcript expression in U2OS mock or polyclonal *MLLT6* knockout cells without IFN-γ treatment. Horizontal axis shows $\log_2$ fold change in transcript expression and vertical axis the statistical significance ($\log_{10}$ of *p*-value, n=3). Transcripts nominated for further characterization are highlighted (red).