Appendix to

Targeting the mevalonate or Wnt pathways to overcome CAR T-cell resistance in *TP53*-mutant AML cells

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Running Title: CAR T-cell resistance in *TP53*-mutant AML

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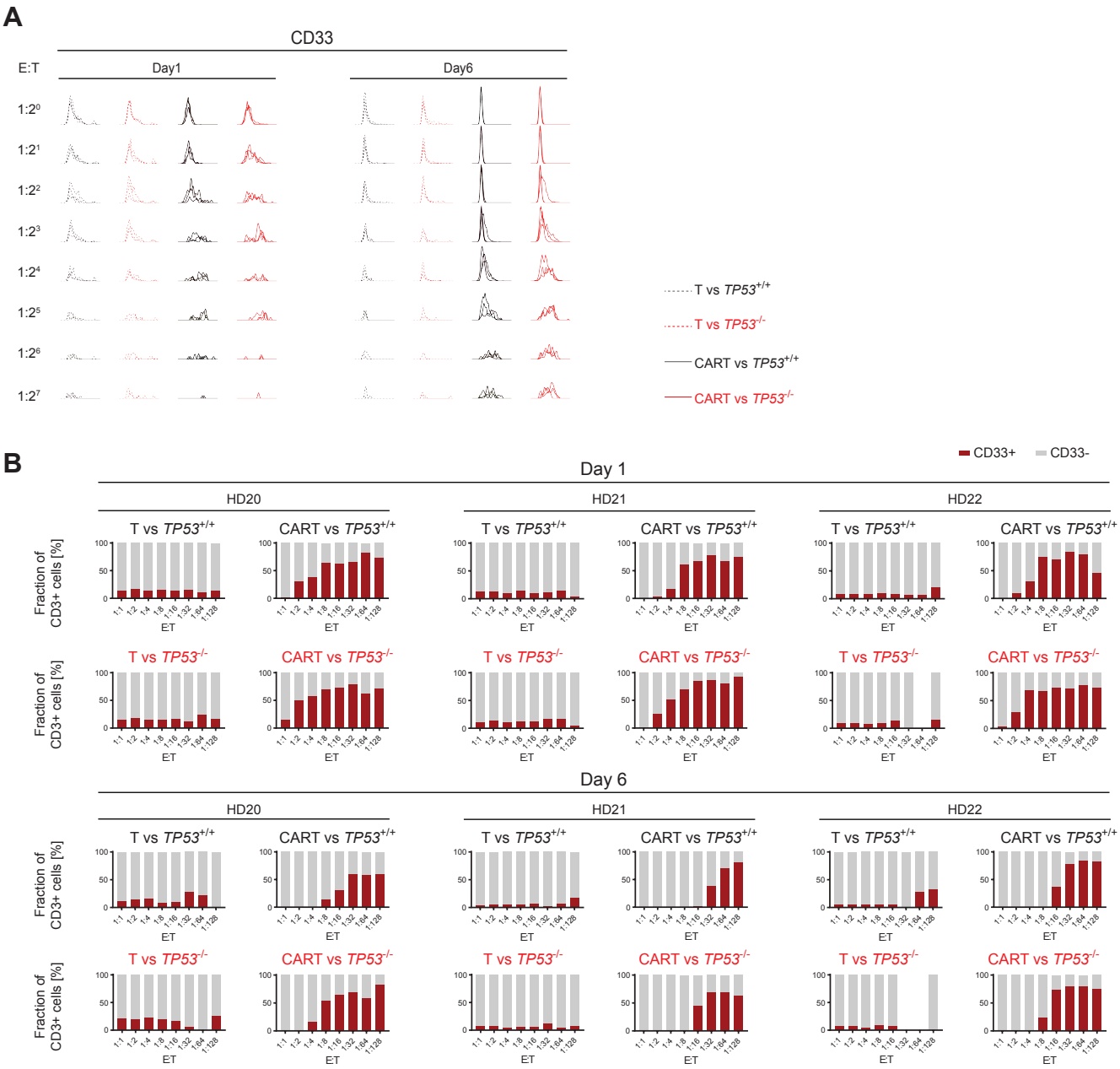
APPENDIX FIGURES AND TABLES

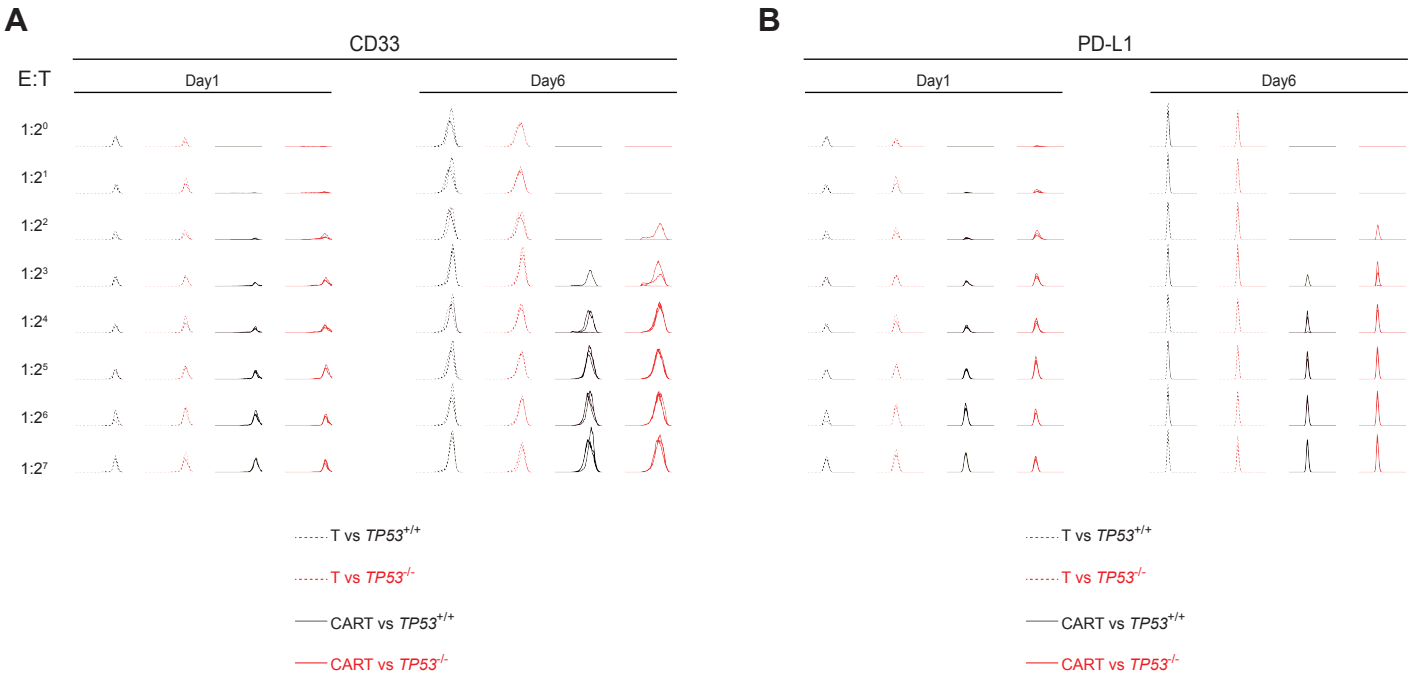
Appendix Figure S1

Appendix Figure S2

Appendix Figure S3



Appendix Figure S4



Appendix Figure S5

A

MOLM13-*TP53*$^{+/+}$(resting) over MOLM13-*TP53*$^{-/-}$(resting)

p53 Pathway
Interferon Gamma Response
Inflammatory Response
Bile Acid Metabolism
Allograft Rejection
Apoptosis
Xenobiotic Metabolism
Myogenesis
TGF-beta Signaling
Pperoxisome

Immunoglobulin Production Involved In Immunoglobulin-Mediated Immune Response (GO:0002381)
Immunoglobulin Mediated Immune Response (GO:0016064)
Positive Regulation Of Lymphocyte Activation (GO:0051251)
MHC Class II Protein Complex Assembly (GO:0002399)
Peptide Antigen Assembly With MHC Class II Protein Complex (GO:0002503)
Regulation Of T Cell Mediated Cytotoxicity (GO:0001914)
Antigen Processing And Presentation Of Exogenous Peptide Antigen Via MHC Class II (GO:0019886)
Antigen Processing And Presentation Of Peptide Antigen Via MHC Class II (GO:0002495)
Regulation Of Interleukin-6 Production (GO:0032675)
Peptide Antigen Assembly With MHC Protein Complex (GO:0002501)

MOLM13-*TP53*$^{-/-}$(resting) over MOLM13-*TP53*$^{+/+}$(resting)

IL-2/STAT5 Signaling
Epithelial Mesenchymal Transition
Estrogen Response Early
Apoptosis
Estrogen Response Late
Myogenesis
TNF-alpha Signaling via NF-kB
KRAS Signaling Up
KRAS Signaling Dn
Cholesterol Homeostasis

Negative Regulation Of MAP Kinase Activity (GO:0043407)
Positive Regulation Of Cell Differentiation (GO:0045597)
Negative Regulation Of MAPK Cascade (GO:0043409)
Negative Regulation Of Protein Serine/Threonine Kinase Activity (GO:0071901)
Negative Regulation Of Ras Protein Signal Transduction (GO:0046580)
Cardiac Epithelial To Mesenchymal Transition (GO:0060317)
Negative Regulation Of Small GTPase Mediated Signal Transduction (GO:0051058)
Positive Regulation Of Ossification (GO:0045778)
Mononuclear Cell Differentiation (GO:1903131)
Negative Regulation Of Cell Differentiation (GO:0045596)

B

CART(resting) over CART(MOLM13-*TP53*$^{+/+}$)

Wnt-beta Catenin Signaling
KRAS Signaling Up
Allograft Rejection
TGF-beta Signaling
Bile Acid Metabolism
Estrogen Response Early
IL-2/STAT5 Signaling
TNF-alpha Signaling via NF-kB
Myogenesis
Inflammatory Response

Regulation Of MAP Kinase Activity (GO:0043405)
Dendritic Cell Chemotaxis (GO:0002407)
Regulation Of GTPase Activity (GO:0043087)
Gamma-Delta T Cell Activation (GO:0046629)
Dendritic Cell Migration (GO:0036336)
T Cell Activation (GO:0042110)
Regulation Of Glomerular Filtration (GO:0003093)
Regulation Of Hydrolase Activity (GO:0051336)
Positive Regulation Of Hydrolase Activity (GO:0051345)
Positive Regulation Of Toll-Like Receptor 3 Signaling Pathway (GO:0034141)

CART(resting) over CART(MOLM13-*TP53*$^{-/-}$)

Allograft Rejection
KRAS Signaling Up
Wnt-beta Catenin Signaling
Estrogen Response Early
IL-2/STAT5 Signaling
TGF-beta Signaling
Interferon Gamma Response
UV Response Dn
PI3K/AKT/mTOR Signaling
Mitotic Spindle

T Cell Activation (GO:0042110)
Gamma-Delta T Cell Activation (GO:0046629)
Regulation Of GTPase Activity (GO:0043087)
Lysosomal Transport (GO:0007041)
Immunoglobulin Mediated Immune Response (GO:0016064)
Positive Regulation Of Ras Protein Signal Transduction (GO:0046579)
Dendritic Cell Migration (GO:0036336)
Antigen Processing And Presentation Of Exogenous Peptide Antigen (GO:0002478)
Positive Regulation Of Rho Protein Signal Transduction (GO:0035025)
Immunoglobulin Production Involved In Immunoglobulin-Mediated Immune Response (GO:0002381)

CART(MOLM13-*TP53*$^{+/+}$) over CART(resting)

TNF-alpha Signaling via NF-kB
E2F Targets
IL-2/STAT5 Signaling
mTORC1 Signaling
Interferon Gamma Response
Inflammatory Response
Hypoxia
Glycolysis
Myc Targets V2
Epithelial Mesenchymal Transition

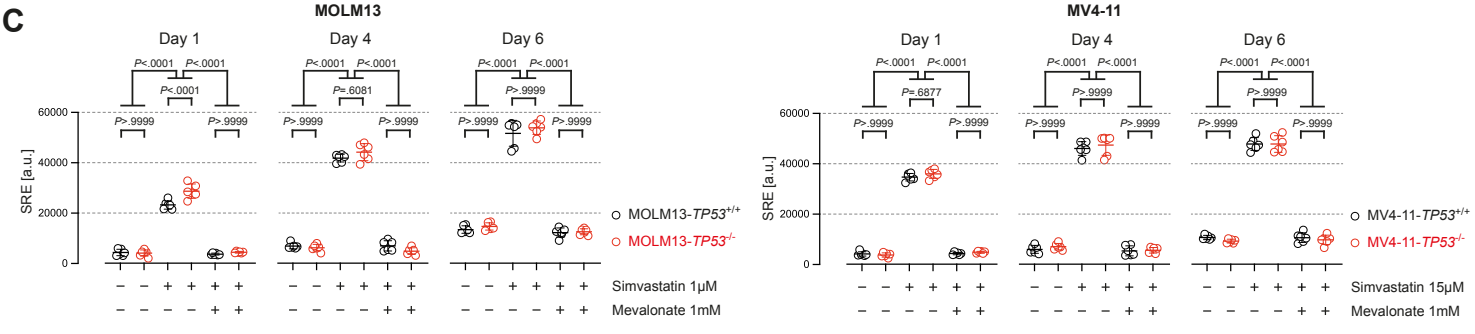
DNA Replication (GO:0006260)
Positive Regulation Of DNA-directed DNA Polymerase Activity (GO:1900264)
Regulation Of DNA-directed DNA Polymerase Activity (GO:1900262)
DNA-templated DNA Replication (GO:0006261)
Mitotic DNA Replication (GO:1902969)
Cytokine-Mediated Signaling Pathway (GO:0019221)
DNA Unwinding Involved In DNA Replication (GO:0006268)
Response To Type II Interferon (GO:0034341)
Positive Regulation Of Cytokine Production (GO:0001819)
Myeloid Leukocyte Differentiation (GO:0002573)

CART(MOLM13-*TP53*$^{-/-}$) over CART(resting)

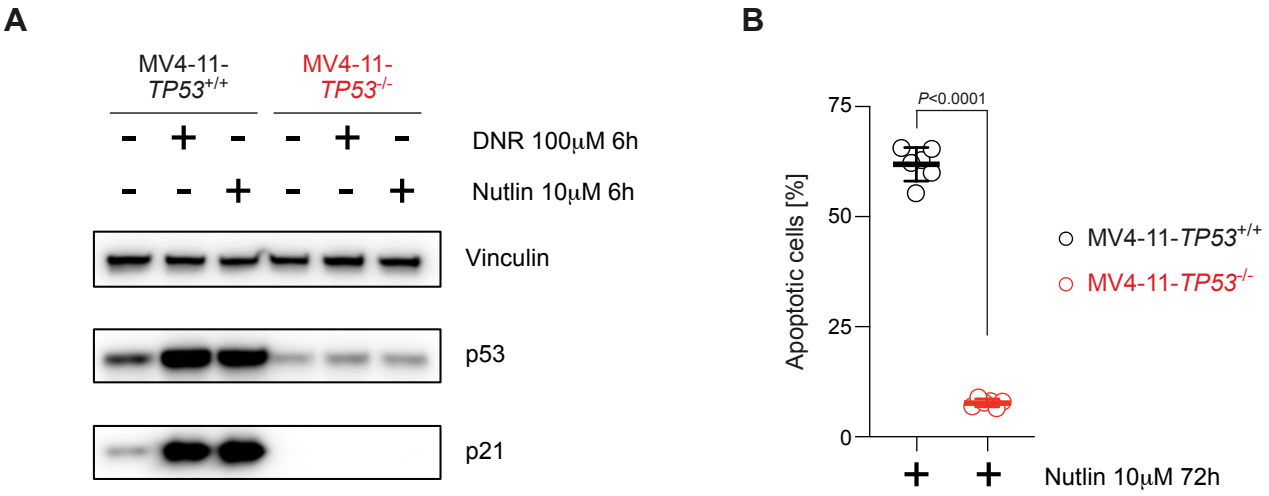
TNF-alpha Signaling via NF-kB
Hypoxia
IL-2/STAT5 Signaling
mTORC1 Signaling
Glycolysis
Inflammatory Response
E2F Targets
Interferon Gamma Response
Epithelial Mesenchymal Transition
Estrogen Response Late

Enzyme-Linked Receptor Protein Signaling Pathway (GO:0007167)
Cytokine-Mediated Signaling Pathway (GO:0019221)
Positive Regulation Of Cytokine Production (GO:0001819)
Dendritic Cell Differentiation (GO:0097028)
Regulation Of Interleukin-1 Beta Production (GO:0032651)
Glycolytic Process (GO:0006096)
Positive Regulation Of Membrane Protein Ectodomain Proteolysis (GO:0051044)
Negative Regulation Of Smooth Muscle Cell Proliferation (GO:0048662)
Negative Regulation Of Cell Population Proliferation (GO:0008285)
Positive Regulation Of Interleukin-1 Beta Production (GO:0032731)

C



Appendix Figure S6



Appendix Table S1: List of antibodies and reagents used for flow cytometry

Target	Clone	Fluorochrome	Supplier	Dilutions
hCD3	OKT3	PE/Dazzle™ 594	BioLegend®	1:600
hCD3	OKT3	APC	BioLegend®	1:400
hCD33	WM53	Brilliant Violet™ 711	BioLegend®	1:600
hCD33	WM53	APC	BD Pharmingen™	1:200
hCD123	6H6	APC	BioLegend®	1:400
hCD279(PD-1)	EH12.2H7	Brilliant Violet421™	BioLegend®	1:300
hCD279(PD-1)	eBioJ205	APC-eFluor™	eBioscience™	1:200
hCD366(TIM3)	F38-2E2	PE	eBioscience™	1:300
hCD366(TIM3)	F38-2E2	PerCP/Cyanine5.5	BioLegend®	1:200
hCD223(LAG3)	11C3C65	Brilliant Violet™ 605	BioLegend®	1:400
hCD274(PD-L1)	29E.2A3	PE/Cyanine7	BioLegend®	1:800
hLDLR	C7	PE	BD Pharmingen™	1:400
hLDLR	301	APC	ThermoFischer	1:200
L/D	NA	Hoechst 33342	Thermo Scientific	1:5’000
L/D	NA	FVD eFluor™ 780	eBioscience™	1:2’000
L/D	NA	DAPI	BioLegend®	1:2’500

Appendix Table S2: Oligos/Primers for introducing Regnase-1 deficiency in CAR T-cells

Name/Target	Sequence (5’ → 3’)	Locus	Direction	Purpose
Z3H12A	AAG GAG GTC TTC TCC TGC CG	Exon 3	NA	crRNA
Z3H12A	GGA ACT GGC ACT GGG AAT GGA	Exon 3	Forward	PCR
Z3H12A	AAT GAC CAC CAT TCA GAG CAG G	Exon 3	Reverse	PCR

Appendix Table S3: Effector-to-target ratios for FACS-sorting and RNA isolation

Effectors	Targets	E :T
CD33 CAR T-cell	MOLM13-*TP53*	1 :8
CD33 CAR T-cell	MV4-11-*TP53*	1 :4
CD123 CAR T-cell	MOLM13-*TP53*	1 :4
CD123 CAR T-cell	MV4-11-*TP53*	1 :1

Appendix figure legends

Appendix Figure S1: Flow cytometry analysis of activation marker surface expression on CD3$^+$ T-cells.

(A) CD25 signal on CD3+ cells for the indicated E:T ratios and days co-incubated with MOLM13-*TP53*$^{+/+}$ AML cells (black) or MOLM13-*TP53*$^{-/-}$ AML cells (red). Y-axes are scaled for every individual measurement.

(B) CD25 signal summary data of individual biological replicates.

Appendix Figure S2: Flow cytometry analysis of exhaustion marker surface expression on CD3$^+$ T-cells.

(A) PD-1, **(B)** LAG3 and **(C)** TIM3 signal on CD3+ cells for the indicated E:T ratios and days co-incubated with MOLM13-*TP53*$^{+/+}$ AML cells (black) or MOLM13-*TP53*$^{-/-}$ AML cells (red). Y-axes are scaled for every individual measurement.

(D) Summary data of exhaustion marker expression of individual biological replicates.

Appendix Figure S3: Flow cytometry analysis of the MOLM13 surface marker CD33 surface signal on CD3$^+$ T-cells.

(A) CD33 signal on CD3+ cells for the indicated E:T ratios and days co-incubated with MOLM13-*TP53*$^{+/+}$ AML cells (black) or MOLM13-*TP53*$^{-/-}$ AML cells (red). Y-axes are scaled for every individual measurement.

(B) CD33 signal summary data of individual biological replicates.

Appendix Figure S4: Flow cytometry analysis of CD33 and PD-L1 on CD3- leukemia cells.

(A) CD33 and **(B)** PD-L1 signal on CD3- leukemia cells for the indicated E:T ratios and days co-incubated with MOLM13-*TP53*$^{+/+}$ AML cells (black) or MOLM13-*TP53*$^{-/-}$ AML cells (red). Y-axes are set at the same maximum for all CD33 and PD-L1 samples, respectively.

Appendix Figure S5: Further details of mRNA-seq data and SREBP gene reporter assays.

(A) Enriched pathways and GO terms relating to BPs for the indicated cell populations in resting MOLM13-*TP53* and **(B)** in T-cells resting or co-incubated with MOLM13-*TP53*.

(C) Luminescent signal of MOLM13-*TP53* and MV4-11-*TP53* AML cells transduced with SREBP-responsive luciferase gene reporter upon incubation with either simvastatin 1μM (for

MOLM13-*TP53*), simvastatin 15μM (for MV4-11-*TP53*) and/or mevalonate 1mM for 1, 4 or 6 days(biological replicates, n=3; 2 technical replicates per biological replicate; symbols indicate individual replicates; thickened lines indicate means and error bars indicate SD; two-way ANOVA).

Appendix Figure S6: Validation of functional p53-deficiency in MV4-11-*TP53* AML cell lines.

(A) Immunoblot for vinculin, p53 and p21 of MV4-11-*TP53* AML cell lines with $TP53^{+/+}$ or $TP53^{-/-}$ treated with DMSO, Daunorubicin (DNR) 100μM or Nutlin 10μM for 6 hours (3 independent experiments; 1 representative image is shown).

(B) Total percent apoptotic cells of MV4-11-*TP53* AML cell lines treated with DMSO or Nutlin for 72 hours (biological replicates, n=3; 2 technical replicates per biological replicate; symbols indicate individual replicates; thickened lines indicate means and error bars indicate SD; student's t-test).