Supplemental Methods

Peptide immunization of mice. H3.3K27M-specific T cells were generated in ABabDII mice expressing a diverse human TCR repertoire [1]. Mice were immunized by subcutaneous injection of 100 µg H3.3K27M (RMSAPSTGGV) peptide in a 1:1 solution of incomplete Freund's adjuvant and PBS containing 50 µg CpG. After priming, mice received the same immunization twice as boosts in a three weeks interval. To assess $CD8^+$ T cell responses, peripheral T cells were restimulated *in vitro* with either 10^{-6} M peptide, PBS as a negative control, or 10^6 Dynabeads mouse T activator CD3/CD28 (Gibco) as a positive control. After 2h, Brefeldin A (BD) was added to the cultures and after overnight culturing, specific $CD8^+$ T cells were measured by intracellular IFNγ staining (PE anti-mouse IFNγ XMG1.2, Biolegend).

Isolation and cloning of TCRs. To isolate specific TCRs, immunized mice were sacrificed, splenocytes and lymphocytes from inguinal lymph nodes prepared and $CD4^+$ T cells depleted using microbeads (Miltenyi Biotec). $1x10^6$ splenocytes were cultured in T cell media (TCM, RPMI (Gibco™) containing 10% FCS (Pan Biotech), 1 mM HEPES (Gibco™), 100 IU/ml PenStrep (Gibco™), 50 µM 2-Mercaptoethanol (Gibco™)) supplemented with 100 IU/ml IL-2 (Peprotech) for 10 days in the presence of 10^{-8} M H3.3K27M peptide. Four hours prior to the in vitro assessment of IFNγ secretion (mouse IFNγ secretion assay, Miltenyi), cells were stimulated with a peptide concentration of 10^{-6} M. To sort IFNγ-secreting $CD8^+$ T cells, cells were stained with anti-mouse CD3-APC (145-2C11, Biolegend) and anti-mouse CD8-PerCP (53-6.7, Biolegend) at 4°C for 30 minutes before the cells were sorted (BD FACS Aria III) into RTL lysis buffer for RNA isolation with RNeasy Micro Kit (QIAGEN). SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories) was used to synthesize first-strand cDNA synthesis and 5'-RACE PCR. The TCR sequence was specifically amplified using 0.1 µM hTRAC (5'-cggccactttcaggaggaggattcggaac-3') or hTRBC (5'-ccgtagaactggacttgacagcggaagtgg-3') specific primer in a 1-2 µl reverse transcriptase reaction together with 1U Phusion® HotStart II polymerase (Thermo Scientific). The amplicons were analyzed on an agarose gel and specific bands were cut out and cloned using a Zero Blunt® TOPO® PCR cloning kit (Life Technologies). A T3 primer (5'-aattaaccctcactaaaggg-3') was used to sequence plasmids from isolated individual clones (Eurofins Genomics). The dominant TCR-α/β chains were selected and paired as follows: 27633 TCR: TRAV13*01 – CAVRGLTQGGSEKLVF - TRAJ57*01, TRBV27*01 – CASSWGGFPYEQYF - TRBJ2-7*01). For 1H5 TCR we combined TRAV19*01 – CALSEENDMRF – TRAJ43*01, TRBV27*01 – CASGWGGPYEQYF - TRBJ2-7*01) [2]. Corresponding TCR-α/β chains were linked using a P2A element and constant regions of both, the human-derived and mouse-derived TCRs were exchanged with mouse constant

regions. The codon optimized TCR cassettes were synthesized by GeneArt (Thermo Fisher Scientific) and cloned into a MP71 vector as NotI/EcoRI fragments.

Plasmid constructs and cDNA synthesis. All retroviral packaging plasmid vectors were based on plasmid MP71 [3]. Plasmids MP71-A2 encoding HLA-A*02:01, and MP71-CDK4-R24L-i-GFP encoding the full-length cDNA of CDK4 harboring the R24L mutation and co-expressing EGFP through an IRES element, were a kind gift from M. Leisegang. In order to construct MP71_H3.3K27M-CDS-i-GFP and MP71_H3.3-CDS-i-GFP, expressing H3F3A mutant and wild type full-length cDNA respectively, H3F3A coding sequences were cloned by RT-PCR from cDNA samples synthesized from RNA isolated from SF8628 cells. In brief, RNA was isolated from $5x10^5$ cells using the RNeasy Isolation Plus Mini Kit (QIAGEN). cDNA synthesis was performed with 1 µg of total RNA as template and random hexamers, using the Advantage RT-for-PCR Kit (Takara Bio) according to manufacturer's protocol. H3F3A coding sequences were amplified by PCR using primers GP001_H3F3A (5'-aaagcggccgcaccatggctcgtacaaagcag-3') and GP002_H3F3A (5'-aaactcgagattcttaagcacgttctccacgta-3') conferring NotI and XhoI resrtiction sites respectively. The IRES-EGFP element was cloned by PCR using primers GP003_IRES (5'-aaactcgagacgtggcggctagtactcc-3') and GP004_GFP (5'-aaagaattcttgtacagctcgtccatgc-3') conferring XhoI and EcoRI restriction sites respectively. Fragments were digested with the respective restriction enzymes (NEB) and cloned in a two-fragment ligation (DNA ligation mix, Mighty Mix, Takara Bio) into NotI/EcoRI digested MP71. Clones were Sanger sequenced and both K27M and wild type clones were selected.

In order to construct MP71-CDK4R24L-P2A-GFP, MP71_H3.3K27M-CDS-P2A-GFP and MP71_H3.3-CDS-P2A-GFP, a P2A-EGFP fragment was PCR amplified from plasmid pcDNA3.1-Hygro(+)-M7PG (kind gift V. Anastasopoulou) using either primer H3.3-P2A_F (5'-acgccgcatacgtggagaacgtgctggcagcggcgccaccaac-3'), or CDK4-P2A_F (5'-acataaggatgaaggtaatccggagggcagcggcgccaccaac-3') combined with GFP-PRE_Rev (5'-aatggcggtaagatgctcgaatttcatttgtacagctcgtccatgc-3'). Produced amplicons bear homologies to the above plasmids and can serve to prime overlap extention PCR (OE-PCR), replacing the IRES-EGFP elements with P2A-EGFP. In brief, the respective OE-PCR amplicon was mixed in a 200 molar excess ratio to 10 ng of either one of MP71-CDK4-R24L-i-GFP, MP71_H3.3K27M-CDS-i-GFP or MP71_H3.3-CDS-i-GFP, in 25 µl PCR reactions and were cycled, using 52°C annealing temperature and a 7-minute extention time at 72°C, for 21 cycles. Reactions were subsequently digested with DpnI and transformed into competent E. coli.

H3.3K27M and H3.3 triple-epitope minigenes, encoding three tandem copies of the respective decamer epitope followed by EGFP, separated by the preferred proteasome

cleavage site AAY, were synthesized (GeneArt Gene Synthesis, ThermoFischer Scientific) and were cloned into MP71 as NotI/EcoRI fragments (MP71_[triple-H3.3K27M]-GFP and MP71_[triple-H3.3]-GFP).
All PCRs described were performed using Q5, High fidelity 2x Master mix (NEB).

Cells and cell culture. The human diffuse intrinsic pontine glioma (DIPG) cell line SF8628 (RRID:CVCL_IT46, SCC127 Sigma-Aldrich) was cultured in DMEM (Gibco™) supplemented with 10% FCS (Pan Biotech) and 100 IU/ml PenStrep (Gibco™). The human DIPG cell line SF7761 (RRID:CVCL_IT45, SCC126 Sigma-Aldrich) was grown as neurospheres in suspension culture and cultured in ReNcell NSC Maintenance Medium (Cat. No. SCM005), containing 20 ng/mL EGF (Cat. No. GF001), 20 ng/mL FGF-2 (Cat. No. GF003) and 100 IU/ml PenStrep (Gibco™). The retroviral packaging cell line 293GP-GLV (producing amphotropic retroviral vectors) was cultured in DMEM supplemented with 10% FCS [4]. TAP deficient T2 cells (RRID:CVCL_2211, ATCC: CRL-1992) were cultured in TCM. The melanoma cell lines Mel624 and Mel21a were cultured in RPMI (Gibco™) supplemented with 10% FCS (Pan Biotech) and 100 IU/ml PenStrep (Gibco™). U87MG cells were cultured in MEMα (Gibco™) supplemented with 10% FCS (Pan Biotech) and 100 IU/ml PenStrep (Gibco™). U87MG cells were authenticated by STR profiling (Cell Line Authentication Service of Eurofins Genomics, Ebersberg, Germany).

Retroviral transduction. TCR gene transfer was carried out as described before [3 5]. In brief, for retrovirus generation, 293GP-GLV cells were transfected with MP71 vector carrying the respective TCR cassettes using Lipofectamine 3000 (ThermoFisher Scientific). On the same day, PBMCs from healthy donors were seeded on plates coated with 5 µg/ml anti-CD3 (OKT3, Invitrogen) and 1 µg/ml anti-CD28 antibodies (CD28.2, Invitrogen) in TCM supplemented with 100 IU/ml IL-2 (Peprotech). 48 hours later, the virus supernatant was harvested, filtered and supplemented with 8 µg/ml protamine sulfate (Sigma-Aldrich) and 100 IU/ml IL-2, before spinoculation with the activated T cells at 800g for 90 minutes at 32°C was performed. The next day, a second supernatant was harvested from the same 293GP-GLV cells, transferred to a RetroNectin (Takara Bio) coated plate and centrifuged at 3200g for 90 minutes at 4°C. The PBMCs were harvested, supplemented with 100 IU/ml IL-2 and 8 µg/ml protamine sulfate and spinoculated with the virus-containing plates at 800g for 30 minutes at 32°C. After the second transduction, T cells were expanded for 10 days, before being transferred to low IL-2 (10 IU/ml). After 48 hours, transduced T cells were harvested, analyzed for TCR expression by flow cytometry and frozen for future experiments. To detect the transduction rate of the TCRs transduced into primary T cells, following antibodies were

used in a 1:100 dilution at 4°C for 30 min: anti-human CD3-PerCP (UCHT1, Biolegend), anti-human CD8-APC (HIT8a, Biolegend) and anti-mouse TCR β chain-PE (H57-597, Biolegend). Since both DIPG cell lines are HLA-A*02:01 negative, SF8628-A2 and SF7761-A2 cells were generated by transfecting MP71-HLA-A*02:01 into 293GP-GLV cells using Lipofectamine 3000 (ThermoFisher Scientific). 48 hours after transfection, 293GP-GLV virus-containing supernatant was harvested, supplemented with 8 μg/ml protamine sulfate and transferred to the respective glioma cell line. Cells and virus supernatant were spinoculated at 800g for 90 minutes at 32°C. Medium was changed to the respective growth medium 6 hours later. The same transduction protocol was used to overexpress full-length cDNAs H3.3K27M, H3.3 wild type, CDK4R24L, wildtype and mutant triple epitopes in the SF8628-A2 DIPG cells and as control in HLA-A*02:01 positive 624-Mel (RRID:CVCL 8054) and SK-Mel-21 (Mel21a, RRID:CVCL_3877) melanoma cells and U87MG ATCC (RRID:CVCL_0022) glioblastoma cells[6]. To analyze successful transduction, flow cytometry was used to determine the fraction of GFP-positive cells. HLA-A*02:01 transduction was confirmed by flow cytometry using an anti-human HLA-A2-APC (BB7.2, Biolegend) specific antibody and an APC mIgG2b, k isotype control (MPC-11, Biolegend).

Co-culture experiments. All co-culture experiments were conducted using $1x10^4$ transduced T cells with $1x10^4$ target cells for 24 hours in a 96 well plate. As a positive control for the T cell activation, 50 ng/ml PMA (Phorbol-12-myristat-13-acetat, (Calbiochem) and 1 μg/ml Ionomycin (Calbiochem) were added to the transduced T cells. T2 cells were loaded with the H3.3K27M (R<u>M</u>SAPSTGGV) or the H3.3 wild type (R<u>K</u>SAPSTGGV) peptide (JPT Peptide Technologies GmbH) at the indicated concentrations. Secreted IFNγ, TNF and IL-2 amounts in the supernatant were measured by ELISA (BD OptEIA; BD Biosciences).

Western blot. To determine the endogenous expression and overexpression of the H3.3K27M mutation in the SF8628 and Mel624 cells, we lysed $2x10^6$ tumor cells using a lysis buffer containing 2% SDS and 0,05 M TrisHCl for 10 minutes at 95°C. Subsequently, the protein concentration was measured using the Total Protein Kit, Micro-Lowry (Sigma). 25 μg of protein in LDS sample buffer and DTT was loaded onto a NuPage 4-12% Bis-Tris 1,5 mm gel (Invitrogen) next to a Full Range Rainbow Protein ladder (Sigma-Aldrich) and ran at 200V, 250mA for 42 minutes. The separated proteins were transferred onto a PVCF membrane for 1h at 30V and 250mA. After blocking in 5% milk in TBST, the membrane was incubated in either H3.3K27M specific antibody (RM192, Rabbit mAb, Abcam) or a beta actin specific antibody (Rabbit mAb, Cell Signaling) diluted 1:1000 in 5% milk shaking at 4°C overnight. The membrane was washed three times for 15 minutes in TBST and stained with a secondary HRP coupled anti-rabbit IgG (Cell Signaling) diluted 1:2000 in 5% milk at room

temperature for one hour. After washing, the membrane was soaked in ELC substrates (ThermoFisher) and exposed for 10 minutes on a ChemiDoc XRS+ Gel Imaging System (Biorad).

Quantitative analysis of cDNA overexpression. cDNA synthesis from all samples was performed as described above. Samples were then diluted 1:5 and 5 µl used as template in a 20 µl qPCR reaction using SYBR Green PCR Master Mix (Thermo), with 500 nM primer concentration. Following primers were used for real-time PCR. H3.3: F:5'-taaagcacccaggaagcaac-3', R:5'-gtcttcaaaaaggccaacca-3', GAPDH: F:5'-gtgaaggtcggagtcaacg-3', R:5'-tgaggtcaatgaaggggtc-3' Samples were run on the QuantStudio 3 Real-Time PCR system (ThermoFisher) and analyzed according to the comparative ΔΔCt method. Samples were sent for Sanger Sequencing (Eurofins Genomics) to confirm presence of wildtype and mutant H3.3 genes.

ERAP1 allotype identification. To identify the presence of ERAP1 polymorphisms, gDNA was extracted from cell lines using QIAamp DNA Mini Kit (Qiagen) and targeted ERAP1 loci amplified and sequenced using the following primers: E56K/R127P 5'-TCTGTGAATGCTGGGTGGAT-3' and 5'-CTCAGTTCCCCTTCCTTGGT-3'; G346D/M349V_5'-AAGGCCTGAGATGCAAGTCT-3' and 5'-CCATAGTGACCAGGTTCCCA-3'; K528R 5'-GTGTTATTGCCAGCCCCAAA-3' and 5'-AGGAGCATTACCCAGTGTCC-3'; D575N_5'-GCTGACACTCCCTACAAAGC-3' and 5'-GCAACTACATCTCTGGCCAT-3'; R725Q/Q730E 5'-GGTCCCTGTTTCCCTGTACA-3' and 5'-GCATGGCTGTCACCGTTTAA-3'.

CRISPR/Cas9 knockout of endogenous TCRs. To knock out the endogenous TCR in transduced T cells, we used the CRISPR/Cas9 system with TRAC-gRNA: agagucucucagcugguaca [7] and gRNA2: uggcucaaacacagcgaccu [8] targeting the endogenous human TCR alpha and beta constant regions, respectively. Before RNP complexing, transduced cells were thawed and cultured for one hour in TCM and 10 IU/ml IL-2. Subsequently, cells were washed 2x in TCM and resuspended at $5x10^7$/ml in OptiMEM. 150 pmol of Alt-R tracrRNA (IDT) were annealed to 150 pmol of the respective Alt-R crRNAs (IDT) according to manufacturer's instructions. The resulting TRAC-gRNA and gRNA2 were complexed with 50 pmol Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT) and the RNP complexes were nucleofected into 10^6 T cells in 20 µl OptiMEM, using pulse EO-115 on Lonza 4D-Nucleofector™ Core Unit in Opti-MEM (Gibco™). The cells were cultured for 4 days in TCM containing 10 IU/ml IL-2 and the knockout efficiency of the endogenous TCR was measured by flow cytometry. To analyze the expression of endogenous and transduced TCR, the T

cells were first stained with 10 µl of HLA-A*02:01 H3.3K27M Dextramer-PE (Immudex) per 50 µl PBS containing 50% FCS at room temperature for 30 minutes. After washing, the following antibodies were added in a 1:100 dilution at 4°C for 30 minutes in PBS containing 5% FCS: anti-human CD3-PerCP (UCHT1, Biolegend), anti-human CD8-BV421 (HIT8a, Biolegend), anti-human TCR α/β-FITC (BW242/412, Miltenyi Biotec) and anti-mouse TCR β chain-APC (H57-597, Biolegend).

Cytotoxicity assay. The cytotoxic potential of transduced T cells was analyzed using the live cell imaging system IncuCyte Zoom (Essen Bioscience). Target cells were transduced with either MP71-CDK4R24L-P2A-GFP, or MP71_H3.3K27M-CDS-P2A-GFP and 3-5x10^3 GFP positive target cells were resuspended in TCM without phenol red and seeded into flat-bottom 96 well plates. The following day, for a 5:1 (effector : target) ratio 1,4-2,5x10^4 transduced T cells were added to the respective wells in triplicates. GFP expression in target cells was determined every hour over a time period of 72 hours at 37°C and 5% CO2. For analysis, the average of GFP total area (µm²/image) in the target cells co-cultured with the respective TCR-transduced T cells was calculated and normalized to the average of GFP total area (µm²/image) of the same target cells co-cultured with mock-transduced T cells (% of mock T cells).

Immunoprecipitation and LC-MS/MS analysis of HLA class I bound peptides. 5x10^6 U87MG cells overexpressing either full-length cDNAs H3.3K27M or CDK4R24L were injected into NSG mice (EPO GmbH, Berlin). At a size of 1.5-2 cm^3 tumors were isolated and 500 mg tumor pieces were snap frozen in liquid nitrogen. Samples were dry cryopulverized immediately using the cryoPREP (Covaris) device. Next, quadruplicate samples of each cell line were lysed using 2xCHAPS buffer containing 400mM TrisCl pH 8.0, 300mM NaCl, 2%CHAPS (Serva) and 2x protease inhibitor (ThermoFisher Scientific) in HPLC grade ultrapure water. To avoid unspecific binding to the beads, the samples were incubated at 4°C for 1 hour with 50µl/ml ProteinA-Sepharose beads (Merck). Next, samples were incubated with 60µg/ml HLA binding antibody (W6/32, Biolegend) at 4°C for 1 hour. For immune-affinity purification, 100µl/ml ProteinA-Sepharose beads were added to the precleared and stained samples overnight at 4°C. Samples were washed three times with HPLC grade ultrapure water and eluted with 10% acetic acid. Acidified samples were desalted on stage tips [9]. A mix of heavy synthetic versions of the mutated peptides (CDK4R24L: 25 fmol; H3-3K27M: 5 fmol; source JPT Peptide Technologies) was spiked into each sample and injected into liquid chromatography coupled to mass spectrometry on an orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). A 44 min gradient was applied using an EASY-nLC 1200 system (Thermo Fisher Scientific) with an in-house packed column (C18-AQ 1.9 µm

beads; Dr. Maisch Reprosil-Pur 120). MS1 resolution was set to 120'000, a MIPS peptide filter with relaxed restrictions was applied, the minimum intensity threshold was specified to 50'000, dynamic exclusion occurred for 20 s and charge states 1-5+ were allowed as precursors. A mixed mode of data-dependent MS2 scans and PRM was applied with an isolation width of 1.2 Th, an MS2 resolution of 15'000 and a maximum injection time of 100 ms. The cycle time was set to 3 s. For PRM scans, a resolution of 30'000 and a maximum injection time of 150 ms was chosen. The inclusion list contained the mutated H3.3K27M and CDK4R24L peptides in their heavy and light versions at charges 2 and 3 which were identified as most suitable in pre-experiments. For database search, MaxQuant version 2.0.3.0 [10] was used. Peptide and site FDRs were specified to 1% while switching protein FDR off. The heavy amino acids of the mutated sequences were set as variable modifications (proline 6 and valine 6) along with oxidized methionine (H3.3K27M peptide) and acetylated protein N-termini. Digestion mode was set to unspecific with a maximum peptide length of 25 amino acids. Match-between runs was switched on. Andromeda search was done against a human Uniprot database (2021) plus the mutated CDK4R24L and H3.3K27M peptide sequences. Downstream analysis was done in R. Quantitation of the peptides of interest was done at MS2 level using the msms.txt table from the MaxQuant output. Fragment ions for the heavy and light versions of both sequences were used for quantitation. For the final quantitation, only charge 2 precursors of the mutated peptides were included for a more robust and consistent quantitation.

Supplementary references

1. Li L-P, Lampert JC, Chen X, et al. Transgenic mice with a diverse human T cell antigen receptor repertoire. *Nature Medicine* 2010;16(9):1029-34. doi: 10.1038/nm.2197 PMID - 20693993
2. Okada H, Hou Y. H3.3 CTL peptides and uses thereof. WO2016179326A1. 2016.
3. Engels B, Cam H, Schler T, et al. Retroviral Vectors for High-Level Transgene Expression in T Lymphocytes. *Human Gene Therapy* 2003;14(12):1155-68. doi: 10.1089/104303403322167993 PMID - 12908967
4. Ghani K, Wang X, Campos-Lima POd, et al. Efficient Human Hematopoietic Cell Transduction Using RD114- and GALV-Pseudotyped Retroviral Vectors Produced in Suspension and Serum-Free Media. *Human Gene Therapy* 2009;20(9):966-74. doi: 10.1089/hum.2009.001 PMID - 19453219
5. Uckert W, Becker C, Gladow M, et al. Efficient Gene Transfer into Primary Human CD8+ T Lymphocytes by MuLV-10A1 Retrovirus Pseudotype. *Human Gene Therapy* 2000;11(7):1005-14. doi: 10.1089/10430340050015310 PMID - 10811229

6. Allen M, Bjerke M, Edlund H, et al. Origin of the U87MG glioma cell line: Good news and bad news. *Science Translational Medicine* 2016;8(354):354re3-54re3. doi: 10.1126/scitranslmed.aaf6853 PMID - 27582061
7. Ren J, Zhang X, Liu X, et al. A versatile system for rapid multiplex genome-edited CAR T cell generation. *Oncotarget* 2017;8(10):17002 - 11. doi: 10.18632/oncotarget.15218
8. Legut M, Dolton G, Mian AA, et al. CRISPR-mediated TCR replacement generates superior anticancer transgenic T cells. *Blood* 2018;131(3):311 - 22. doi: 10.1182/blood-2017-05-787598
9. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 2007;2(8):1896-906. doi: 10.1038/nprot.2007.261
10. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 2008;26(12):1367-72. doi: 10.1038/nbt.1511