

Title: 'Mini' U6 Pol III promoter exhibits nucleosome redundancy and supports multiplexed coupling of CRISPR/Cas9 effects.

Running title: Mini U6 promoter for multiplexed CRISPR/Cas9 effects.

Authors: Roland Preece^{1*}, Christos Georgiadis^{1*}, Soragia Athina Gkazi¹, Aniekan Etuk¹, Abraham Christi¹, Waseem Qasim^{1,2}

¹Molecular and Cellular Immunology Unit, UCL Great Ormond Street Institute of Child Health, London, WC1N 1EH

² NIHR Great Ormond Street Hospital Biomedical Research Centre, 30 Guilford Street, London WC1N 1EH

***These authors contributed equally to this work**

Abstract

RNA polymerase III (Pol III) promoters express short non-coding RNAs and have been adopted for expression of microRNA, interfering RNA, and CRISPR single guide RNA (sgRNA). Vectors incorporating H1 and U6 Pol III promoters are being applied for therapeutic genome editing, including multiplexed CRISPR/Cas9 effects. We report a nucleosome-depleted, minimal U6 promoter, which when embedded within lentiviral long terminal repeat (LTR) regions, supports high level transcriptional activity. Furthermore, duplex minimal H1 & U6 promoters transcribed dual sgRNAs for simultaneous disruption of T cell receptor (TCR) and human leukocyte antigen (HLA) molecules, supporting efficient generation of 'universal' CAR T cells.

Introduction

Transcription of short RNA sequences is mediated by type III RNA polymerases (Pol III), and Pol III promoters are widely employed for the expression of microRNAs, interference RNAs or CRISPR single guide RNA (sgRNA) in biomedical applications (1-6). Architecturally, many human Pol III promoters such as H1 and U6 comprise a proximal sequence element (PSE), a TATA box and an upstream distal sequence element (DSE) comprising an octamer (OCT) and SPH sequence (6-8). Functionally, the PSE attracts snRNA activating protein complex (SNAPc) binding, and the transcription factor Oct-1 binds the highly conserved OCT sequence in the DSE (9). The human H1 promoter has a compact structure with adjacent DSE and PSE, and a minimal H1 promoter of around 100bp in size has previously been described (10).

In contrast, the human U6 promoter, the DSE and PSE are normally separated by a ~150bp spacer sequence which is looped around a positional nucleosome (9, 11, 12). Although previous reports have demonstrated increased transcriptional activity from both the h7SK and hU6 promoters after deletion of this spacer region in the context of naked DNA, it has been considered critical for co-localisation and interaction of associated transcription factors in the presence of chromatin (11-13). However, we demonstrate the U6 nucleosomal region is redundant for transcriptional activity using a therapeutically applicable lentiviral platform. This enabled a minimised U6 to operate in tandem with a mini-H1 promoter for multiplexed CRISPR effects and the efficient generation of 'universal' CAR T cells.

Materials & Methods

CRISPR guide RNA design:

Guide sequences compatible with wild type spCas9 targeting TRAC, and B₂M, were designed using the online CRISPR design tool Benchling (<https://benchling.com>).

TRAC sgRNA: TCTCTCAGCTGGTACACGGC (anti-sense, on-target score 51.8, off-target score 85.6
*higher is better)

B₂M sgRNA: AGTAGCGCGAGCACAGCTA (anti-sense, on-target score 56.3, off-target score 88.6
*higher is better)

Cloning strategy for vector expressed sgRNA:

CRISPR expression cassettes consisted of a Pol III promoter (U6/ H1), a stuffer sequence (~20bp flanked by either *BbsI* or *BsmBI* restriction cut sites), and a sgRNA scaffold sequence. Vector plasmid

DNA (0.5µg) was linearised with either *BbsI* or *BsmBI* type IIS restriction enzymes (FD1014 and FD0454, ThermoFisher Scientific). Vector was dephosphorylated using FastAP thermosensitive alkaline phosphatase (EF0651, ThermoFisher Scientific). After digestion DNA was purified using the QIAquick PCR purification kit (28104, QIAGEN).

Guide sequences ordered as single stranded DNA oligos incorporated either a +1 G or A transcription start site, depending upon the Pol III promoter. Oligo annealing, phosphorylation by T4 Polynucleotide Kinase (M0201S, NEW ENGLAND BioLabs), and ligation (EL0011, ThermoFisher Scientific) were performed as detailed by the Zhang lab GeCKO protocol (<http://genome-engineering.org/gecko/wp-content/uploads/2013/12/lentiCRISPRv2-and-lentiGuide-oligo-cloning-protocol.pdf>).

Generation minimised U6 variants:

Deletions were made within the hU6 Pol III promoter, removing either essential promoter elements or the positional nucleosome sequence. Mini U6 and U6 variant sequences are detailed (**Figure S1**). Primers were designed using the NEB base changer online tool (<http://nebasechanger.neb.com/>). Amplification was performed using Q5® high-fidelity DNA polymerase (M0491S, NEW ENGLAND BioLabs) and PCR products were gel extracted (28704, QIAGEN) and purified (28104, QIAGEN). Purified DNA (50ng) was phosphorylated by T4 Polynucleotide Kinase (M0201S, NEW ENGLAND BioLabs) and ligated (EL0011, ThermoFisher Scientific) prior to bacterial transformation. This protocol was also used to generate the minimal H1 sequence (10).

Insertion of a second CRISPR expression cassette:

A terminal-U6CRISPR-CAR19 third generation SIN lentiviral vector configuration as described in Georgiadis *et al* (2018) was subjected to In-Fusion HD Cloning Plus (638909, Takara Bio Europe, Saint-Germain-en-Laye, France) to insert a second CRISPR expression cassette in tandem with the first (5). The second expression cassette remained identical to the first apart from the incorporation of a H1 Pol III promoter instead of hU6 and with *BsmBI* restriction sites flanking the stuffer sequence as opposed to *BbsI* restriction sites. CRISPR expression cassettes were synthesized by GeneART (ThermoFisher Scientific). Amplifications were performed by Q5® high-fidelity DNA polymerase (M0491S), prior to In-Fusion HD Cloning Plus reaction using a 1:1 molar ratio of insert to vector.

Site directed mutagenesis for addition of alternative cr2 scaffold sequence:

Alternative cr2 scaffold sequence described by Adamson et al (2016), was incorporated into terminal-U6CRISPR-CAR19 configurations (14). NEB base changer online tool (<http://nebasechanger.neb.com/>) was used to design a set of primers with appropriate substitutions to change a cr1 scaffold to a cr2 scaffold sequence. PCR amplification, phosphorylation and ligation were carried out as described above.

Lentiviral vector production:

Lentiviral vector stocks were produced by transient transfection of 293T cells with third generation packaging plasmids as well as a transfer plasmid. Packaging plasmids include pMDLg/pRRE containing the gag-pol and accessory proteins, pMDG2 incorporating the vesicular stomatitis virus envelope, and pRSV-Rev encoding the nuclear exporter rev. 293T cells were transfected with plasmid using 1×10^{-7} mol/l of polyethylenimine (PEI). Media containing lentiviral particles was harvested at 48 hours post transfection, and passed through a 0.22 μ m pore cellulose acetate filter. This was then concentrated by ultracentrifugation for 2 hours at 100,000g. Virus was resuspended in reduced serum OPTI-MEM (31985062, ThermoFisher Scientific) and incubated on ice for 1 hour before virus was pooled and stored at -80°C for further use. Assessment of infectious units was undertaken in 293T cells and analysed by flow cytometry.

Amplification of proviral LTR sequences:

Genomic DNA was isolated using DNeasy Blood and Tissue Kit (69504, QIAGEN) from cells transduced with terminal-CRISPR configurations. Primers were designed to amplify both 3' and 5' proviral LTRs. The 3'LTR was amplified using a WPRE forward primer (5' GGACGTCCTTCTGCTACGTC 3') and a U5 reverse primer (5' GGGCACACACTACTTGAAGC 3'), while the 5'LTR was amplified using a U3 forward primer (5'GGGCTAATTCCTCCCAACG 3') and a Psi reverse primer (5' CTCTCGCACCCATCTCTCTC 3'). Q5® high-fidelity DNA polymerase (M0491S, New England Biolabs) was used for the PCR reactions.

Next Generation Sequencing of proviral LTR sequences:

Amplified products were library prepped for NGS using the Nextera XT kit (Illumina, Cambridge, UK). After the library preparation, individually barcoded samples were pooled together and loaded in a MiSeq

instrument using a 500-V2 nano-cartridge. Deletions were detected with Pindel (<https://github.com/genome/pindel>) and figures were created in Rstudio. Deletions >300bp were characterised as “Large Deletions” and those <300bp (were characterised) as “Small Deletions”.

mRNA spCas9:

CleanCap™ spCas9 mRNA (TriLink biotechnologies,) incorporating two nuclear localisation signals (N and C terminus), and Cap 1 structure was used. Electroporation was conducted with the Neon Transfection System (ThermoFisher Scientific) in accordance with manufacturer’s instructions; using protocol 24 to deliver mRNA. Cells were incubated at 30°C overnight after electroporation before restoration to 37°C.

Primary human lymphocyte culture and modification:

Peripheral blood mononuclear cells (PBMC) were isolated by ficoll density gradient centrifugation and subsequently activated with TransACT reagent (130-111-160, Miltenyi Biotech). Lymphocytes were cultured in TexMACS medium (130-097-196, Miltenyi Biotech) with 3% human AB serum (GEM-100-512-HI, Seralabs) and 100U/ml Proleukin IL-2 (Novartis). Transduction with lentiviral vector was performed 24hr post activation at a multiplicity of infection (MOI) of 5 and mRNA electroporation performed at day 4 post activation. Lymphocytes were cultured for 11 days post activation and magnetically depleted using anti-HLA-ABC-biotin (130-101-463, Miltenyi Biotech), anti-TCR α/β -biotin (130-098-219, Miltenyi Biotech) or a combination of both followed by incubation with anti-biotin microbeads ultrapure (130-105-637, Miltenyi Biotech) and separation through LD columns (130-042-901, Miltenyi Biotech) as per manufacturer’s instructions. Cells were rested overnight before cryopreservation.

Flow cytometry:

Cells were acquired on a 4-laser BD LSRII (BD Biosciences) with FACS analysis performed using FlowJo v10. Fluorochrome-conjugated antibodies used were: CD3-VioBlue (130-114-519, Miltenyi Biotech), CD3-APC (130-113-135, Miltenyi Biotech), TCR $\alpha\beta$ -APC (130-091-237, Miltenyi Biotech), and MHC Class I-APC (311410, BioLegend). CAR19 transduction was assessed using a Biotin-SP (long spacer) AffiniPure F(ab') Fragment Goat Anti-Mouse IgG, F(ab') Fragment Specific antibody (115-066-072-JIR, Stratech Scientific Limited) followed by Streptavidin- APC (405207, BioLegend) or Streptavidin-PE (130-106-789, Miltenyi Biotech).

Detection of non-homologous end joining events:

Genomic DNA extraction was performed using DNeasy Blood and Tissue Kit (69504, QIAGEN) and PCR amplified products over the predicted spCas9 scission site were sequenced and analysed using Tide protocols (<https://tide.nki.nl/>).

Primers were designed for TRAC and B₂M as shown below (all shown as 5' to 3').

TRAC forward: TTGATAGCTTGTGCCTGTCCC, TRAC reverse: GGCAAACAGTCTGAGCAAAGG

B₂M forward: CCTCCAGCCTGAAGTCCTAG, B₂M reverse: GACGAAGTCCACAGCTCTCC

Statistics:

One-way ANOVA, comparing the mean from all groups against each other, followed by a Tukey's multiple comparison test was used to determine significance between groups for TCR $\alpha\beta$ and B₂M knockout when using different promoter or vector configurations. Mann-Whitney U test was used for the comparison of two independent groups of B₂M knockout using wild type U6 and minimal U6 promoters. Area under the curve (AUC) analysis followed by an unpaired t test was used to assess effects of spCas9 titration. Statistical analysis was performed using GraphPad Prism software, version 8.0.0.

Results & Discussion

Redundancy of hU6 positional nucleosome

The native lentiviral U3 region includes strong promoter/enhancer elements required for RNA Pol III mediated transcription of viral genomes, but to reduce the risk of vector mobilisation or transactivation effects, these regions are deleted in SIN vectors (15-17). The tactical placement of RNA expression cassettes within the 3'LTR may circumvent possible interference effects with internal transgene promoters while also ensuring duplication to the 5'LTR following reverse transcription (5, 18). We have previously described the application of such vectors for the generation of genome edited CAR T cells where CAR expression and U6 mediated CRISPR effects were coupled (5). This model was now used to dissect and investigate the sequence and architectural anatomy of the hU6 promoter in detail.

A mini hU6 promoter devoid of the 138bp spacer sequence was generated, bringing DSE and PSE into juxtaposition (**Figs. 1a, b and S1 i & ii**). Along with further mutant hU6 variants devoid of PSE and/or DSE (**Figs. 1c i-v and S1 iii-v**), functionality was assessed by incorporation into the 3'LTR. A single sgRNA expression cassette targeting the T Cell Receptor alpha constant (TRAC) locus was employed to disrupt TCR $\alpha\beta$ expression in the first instance. Primary human T cells (n=4) were transduced after 24hrs activation with TransACT (anti-CD3/CD28) and were then electroporated with spCas9 mRNA (50 μ g/ml) after a further 72 hours. Disruption of TCR $\alpha\beta$ by flow cytometry was comparable between native U6 and mini hU6 devoid of its nucleosomal spacer region (p= 0.8937), (**Fig. 1d**), whereas removal of both PSE and DSE resulted in significant loss of CRISPR effects compared to wild type U6 (p<0.0001). These effects were also confirmed in HEK 293T cells (**Figure S2**) indicating activity in alternative cell lineages. Thus the nucleosomal sequence was redundant for U6 transcriptional activity while both the PSE and DSE were confirmed to be essential.

Minimal Pol III promoters support efficient LV-sgRNA multiplexing

Initial attempts to multiplex sgRNA effects at two different loci while maintaining coupling to CAR expression, employed tandem full length promoter/guide cassettes targeting Beta-2 microglobulin (B₂M) and TRAC genes respectively (**Fig. 2a**). The resulting disruption of both TCR $\alpha\beta$ and class I human leukocyte antigen (HLA) aimed to generate immunologically stealthy and non-alloreactive CAR T cells (4). In order to avoid recombination events during reverse transcription due to repetitive sequences, wild type hH1 Pol III promoter was used to drive expression of the second sgRNA (19, 20). However, in primary T cells, levels of HLA class I disruption (75%) were notably higher than TCR disruption (25%) in the CAR19+ expressing cells (**Fig. 2b**). Sequencing of both 5' and 3' proviral LTRs revealed frequent large deletions >300bp (31% and 18% respectively) within the H1-TRAC sgRNA cassette (**Fig. 2c-f**). We hypothesised that homology driven recombination events during reverse transcription may have compromised the dual cassette design (19, 20). To minimise such homology variant guide scaffold sequences (76bp) (cr1, cr2) were employed with no more than 20bp of continuous shared sequences (14) (**Fig. 3a**). This increased knockout effects at the TRAC locus (69%) to levels comparable to B₂M (59%) in vector transduced, CAR19 expressing cells (**Fig. 3b**). Importantly, examination by Next Generation Sequencing (NGS) now revealed a greatly reduced frequency of both large and small deletions within both the 5' and 3' LTRs (6% and 3%) respectively (**Fig. 3c**).

In order to minimise capacity constraints within the lentiviral LTRs (21), we reduced the tandem cassette size using miniU6 and miniH1 to substitute their full length counterparts (**Fig. 3d**). Now, human T cells exhibited 73% TCR and 66% HLA knockout within the CAR19 expressing population, and NGS of 5' and 3' LTRs again detected <5% large deletions (**Fig. 3e, f**). Comparisons undertaken in primary T cells from multiple donors showed no significant difference in multiplexed editing between wild type and minimal tandem cassettes across a gradient of spCas9 mRNA concentrations (**Fig. S3 and 4a, b**).

Dual enriched CAR19+ cells following simultaneous depletion

The multiplexed CRISPR-CAR configuration offers an important advantage over uncoupled CRISPR delivery, in allowing for highly stringent purification for two cell surface molecules using magnetic bead based depletion of residual non-disrupted populations.

Thus in cells transduced with lentiviral CAR vectors expressing guides against both TRAC and B₂M, it was possible to elicit depletion of residual HLA⁺ and TCRαβ⁺ cells using biotin conjugated anti-HLA and anti-TCRαβ antibodies and anti-biotin beads (**Fig. 4c**). CAR T cells that were HLA⁻TCRαβ⁻ were enriched from 24.7% ± 4.6% (mean ± SD) to 99.4% ± 0.5% (mean ± SD) in the total CD45⁺ population further corroborated by the frequency of genomic aberrations at the molecular level (**Fig. 4d, e**). The coupled nature of the vector ensured that transgene expression also underwent enrichment, from 48.3% ± 6% (mean ± SD) CAR19⁺ pre-depletion increasing to 95% ± 2.2% (mean ± SD) (**Fig. 4f**). Taken together, this system offers versatile, efficient and coupled delivery of transgene and CRISPR effects. Stringent removal of residual TCRαβ⁺ cells is particularly important to minimise the risk of alloreactive GVHD in the HLA mismatched setting, and ensuring HLA depletion and CAR expression in the same populations enables the generation of a homogenous and well defined cell therapy product.

In summary, we describe a minimal U6 Pol III promoter devoid of nucleosomal activity but as effective as its full length counterpart and permissive for multiplexing activity in combination with divergent guide RNA scaffold sequences. Early therapeutic applications are envisaged in the context of lentiviral engineered T cells, but minimalisation may also be helpful in alternative vector systems such as AAV where cargo size may be limiting.

Acknowledgments

Supported by National Institute of health research (NIHR) and Great Ormond Street Biomedical Research Centre, (BRC) and Children with Cancer. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. Flow cytometry was performed at the UCL Great Ormond Street Institute of Child Health Flow Cytometry Core Facility, supported by the Great Ormond Street Children's Charity (GOSHCC), grant reference U09822 (October 2007), and UCL Capital Equipment Funding, School of Life and Medical Sciences (September 2012).

Disclosures

IP filed for Minimal U6 promoter N415912GB (RP, CG and WQ). WQ holds interests unrelated to this project in Autolus Ltd and Orchard Therapeutics.

WQ received unrelated research funding from Collectis, Servier, Miltenyi, Bellicum.

Funding

This work was supported by the NIH Research (NIHR) (RP-2014-05-007), NIHR Blood and Transplant Research Units (BTRU) and Great Ormond Street Biomedical Research Centre (IS-BRC-1215-20012). The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

Figure legends

Figure 1: Generation and evaluation of a minimal hU6 promoter devoid of nucleosome spacer sequence. **a)** DNA loop model of hU6 RNA pol III promoter. Positional nucleosome brings DSE bound transcription factors Oct-1 into juxtaposition with PSE bound SNAPc. Total size of the wild type hU6 is 249bp. **b)** Schematic of minimal hU6 promoter with deletion of positional nucleosome spacer, reducing promoter size by >50% (111bp promoter size). **c)** Illustrative representation of wild type hU6 **(i)**, and minimal hU6 **(ii)**. Additional controls were generated with deletions of essential promoter elements including both DSE and PSE **(iii)**, or deletions of either DSE **(iv)** or PSE **(v)**. **d)** Graph detailing TCR $\alpha\beta$ knockout results from four donors transduced with terminal U6TRAC-CAR19 (TT-CAR19) configurations containing either wild type hU6 promoter or mutant hU6 promoters shown in **c.**, seven

days post electroporation with 50µg/ml spCas9 mRNA. TCRαβ knockout is given as a percentage of transduced CAR19+ cells. No significant difference was observed between knockout using wild type hU6 and the minimal hU6 (p=0.8937 by one-way ANOVA with Tukey's post hoc test). Mutant hU6 promoters missing DSE, or PSE sequences showed significantly reduced CRISPR knockout compared to wild type hU6 (p<0.0001 by one-way ANOVA with Tukey's post hoc test). Lines shown in **d.** represent the mean of the group with the error bars showing 1x standard error of the mean (SEM).

Figure 2: Multiplex terminal CRISPR-CAR19 configuration with tandem CRISPR expression cassettes. **a)** Schematic representation of multiplex terminal CRISPR-CAR19 with two CRISPR expression cassettes placed in tandem within the ΔU3 region of the 3'LTR. These CRISPR expression cassettes contain either a hU6 or H1 promoter with +1G or +1A added respectively, as a transcription start site. This configuration was used to express guides for B₂M knockout (U6 promoter) and TRAC knockout (H1 promoter), both containing cr1 scaffold sequences. **b)** FACS analysis of cells transduced with multiplex terminal U6B₂M(cr1)>H1TRAC(cr1)>CAR19 at day seven post spCas9 mRNA delivery. Individual knockout of both B₂M (HLA class I used as a surrogate marker) and TRAC (CD3 used as a surrogate marker) can be seen coupled to CAR19+ transduction. Double knockout population represents 5.82% of the total population. **c)** PCR amplification of proviral 3'LTR using Woodchuck Hepatitis virus posttranscriptional regulatory element (WPRE) forward and U5 reverse primers. DNA from untransduced (UnTD) cells (lane 1), TT-CAR19 cells (lane 2), pCCL-CAR19 cells (lane 3), and multiplex terminal U6B₂M(cr1)>H1TRAC(cr1)>CAR19 (lane 4). UnTD lane 1 acts as a blank control. **d)** Alignment of Sanger sequencing data obtained from the top band (1137bp) in lane 4. **e)** Sanger sequencing data obtained from the bottom band (~750bp) in lane 4. Alignment of sequence data was carried out using SnapGene software. Arrows indicate direction of sequencing read. Red regions indicate gaps/deletions in the read. **f)** Next generation sequencing of proviral 5' and 3' LTRs of multiplex terminal U6B₂M(cr1)>H1TRAC(cr1)>CAR19 DNA. Alignment to reference sequence allows quantification of large deletions (>300bp) and small deletions (<300bp).

Figure 3: Multiplex terminal configuration incorporating divergent scaffold sequences and minimal Pol III RNA promoters. **a)** Schematic representation of the multiplex terminal cassette

U6B₂M(cr1)>H1TRAC(cr2), containing both cr1 and cr2 scaffold sequences **b)** FACS-based phenotyping of primary human T cells transduced with multiplex terminal U6B₂M(cr1)>H1TRAC(cr2)-CAR19 at day 7 post electroporation with 100µg/ml spCas9 mRNA. **c)** Next generation sequencing (NGS) of proviral 5' and 3' LTRs of multiplex terminal U6B₂M(cr1)>H1TRAC(cr2)>hPGK-CAR19. Alignment to reference allows quantification of large deletions (>300bp) and small deletions (<300bp). **d)** Multiplex terminal minimal cassette miniU6B₂M(cr1)>miniH1TRAC(cr2) configuration. Use of minimal promoters reduces total cassette size to 426bp. **e)** FACS based phenotyping of miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19. Restoration in skewing with similar levels of B₂M and TRAC knockout seen. Increase in double negative HLA-TCRαβ⁻ population. **f)** NGS of proviral 5' and 3' LTRs of miniU6B₂M(cr1)>miniH1TRAC(cr2)>hPGK-CAR19 DNA.

Figure 4: Minimal multiplex Terminal configuration yields highly purified double HLA-TCRαβ⁻ depleted population with CAR19⁺ enrichment. **a)** Comparison of three vector configurations U6B₂M(cr1)>H1TRAC(cr1)>CAR19, U6B₂M(cr1)>H1TRAC(cr2)>CAR19 and miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19 in n=4 primary T cell donors. FACS-based measurement of B₂M and TCR knockout in CAR19⁺ population at 7 days post electroporation. One-way ANOVA with Tukey's multiple comparison test show no significant difference in B₂M knockout (p=0.3753). TCR knockout was significantly higher in U6B₂M(cr1)>H1TRAC(cr2)>CAR19 (*p=0.0102) and minimal miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19 (*p=0.0230) configurations compared to U6B₂M(cr1)>H1TRAC(cr1)>CAR19. **b)** Histogram of individual or double knockout in CAR19⁺ cells across n=4 donors treated with the three vector configurations. **c)** Representative FACS plots of pre-depletion and post HLA Class I and TCRαβ combined microbead depletion of miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19 cells. HLA Class I and TCRab knockout shown in total CD45⁺ population. **d)** Histogram of single and double B₂M and TCRαβ knockout percentages in total CD45⁺ population pre and post simultaneous HLA Class I and TCRab depletion across n=4 donors treated with miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19 vector. **e)** Frequency of aberrant sequences at B₂M and TRAC genomic loci in pre-depletion and post-depletion PCR products from n=4 donors treated with miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19 vector. Frequency determined by TIDE analysis of Sanger sequencing reactions. Error bars represent SEM. **f)** Histogram of CAR19 expression

in total CD45+ population pre and post simultaneous HLA Class I and TCR $\alpha\beta$ depletion across n=4 donors treated with miniU6B₂M(cr1)>miniH1TRAC(cr2)>CAR19 vector.

References

1. White MD, Farmer M, Mirabile I, Brandner S, Collinge J, Mallucci GR. Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease. *Proc Natl Acad Sci U S A*. 2008;105(29):10238-43.
2. McBride JL, Boudreau RL, Harper SQ, Staber PD, Monteys AM, Martins I, et al. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A*. 2008;105(15):5868-73.
3. Boudreau RL, McBride JL, Martins I, Shen S, Xing Y, Carter BJ, et al. Nonallele-specific silencing of mutant and wild-type huntingtin demonstrates therapeutic efficacy in Huntington's disease mice. *Mol Ther*. 2009;17(6):1053-63.
4. Ren J, Liu X, Fang C, Jiang S, June CH, Zhao Y. Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. *Clin Cancer Res*. 2017;23(9):2255-66.
5. Georgiadis C, Preece R, Nickolay L, Etuk A, Petrova A, Ladon D, et al. Long Terminal Repeat CRISPR-CAR-Coupled "Universal" T Cells Mediate Potent Anti-leukemic Effects. *Mol Ther*. 2018;26(5):1215-27.
6. Gao Z, Herrera-Carrillo E, Berkhout B. RNA Polymerase II Activity of Type 3 Pol III Promoters. *Mol Ther Nucleic Acids*. 2018;12:135-45.
7. Helbo AS, Lay FD, Jones PA, Liang G, Gronbaek K. Nucleosome Positioning and NDR Structure at RNA Polymerase III Promoters. *Sci Rep*. 2017;7:41947.
8. Schramm L, Hernandez N. Recruitment of RNA polymerase III to its target promoters. *Genes Dev*. 2002;16(20):2593-620.
9. Didychuk AL, Butcher SE, Brow DA. The life of U6 small nuclear RNA, from cradle to grave. *RNA*. 2018;24(4):437-60.
10. Myslinski E, Ame JC, Krol A, Carbon P. An unusually compact external promoter for RNA polymerase III transcription of the human H1RNA gene. *Nucleic Acids Res*. 2001;29(12):2502-9.
11. Stunkel W, Kober I, Seifart KH. A nucleosome positioned in the distal promoter region activates transcription of the human U6 gene. *Mol Cell Biol*. 1997;17(8):4397-405.
12. Zhao X, Pendergrast PS, Hernandez N. A positioned nucleosome on the human U6 promoter allows recruitment of SNAPc by the Oct-1 POU domain. *Mol Cell*. 2001;7(3):539-49.
13. Murphy S, Yoon JB, Gerster T, Roeder RG. Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. *Mol Cell Biol*. 1992;12(7):3247-61.
14. Adamson B, Norman TM, Jost M, Cho MY, Nunez JK, Chen Y, et al. A Multiplexed Single-Cell CRISPR Screening Platform Enables Systematic Dissection of the Unfolded Protein Response. *Cell*. 2016;167(7):1867-82 e21.
15. Houzet L, Jeang KT. MicroRNAs and human retroviruses. *Biochim Biophys Acta*. 2011;1809(11-12):686-93.
16. Kao SY, Calman AF, Luciw PA, Peterlin BM. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature*. 1987;330(6147):489-93.
17. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol*. 1998;72(10):8150-7.
18. Curtin JA, Dane AP, Swanson A, Alexander IE, Ginn SL. Bidirectional promoter interference between two widely used internal heterologous promoters in a late-generation lentiviral construct. *Gene Ther*. 2008;15(5):384-90.
19. An W, Telesnitsky A. Frequency of direct repeat deletion in a human immunodeficiency virus type 1 vector during reverse transcription in human cells. *Virology*. 2001;286(2):475-82.
20. Brake OT, Hooft K, Liu YP, Centlivre M, Jasmijn von Eije K, Berkhout B. Lentiviral Vector Design for Multiple shRNA Expression and Durable HIV-1 Inhibition. *Mol Ther*. 2008;16(3):557-64.
21. Urbinati F, Arumugam P, Higashimoto T, Perumbeti A, Mitts K, Xia P, et al. Mechanism of reduction in titers from lentivirus vectors carrying large inserts in the 3'LTR. *Mol Ther*. 2009;17(9):1527-36.

Figure 1

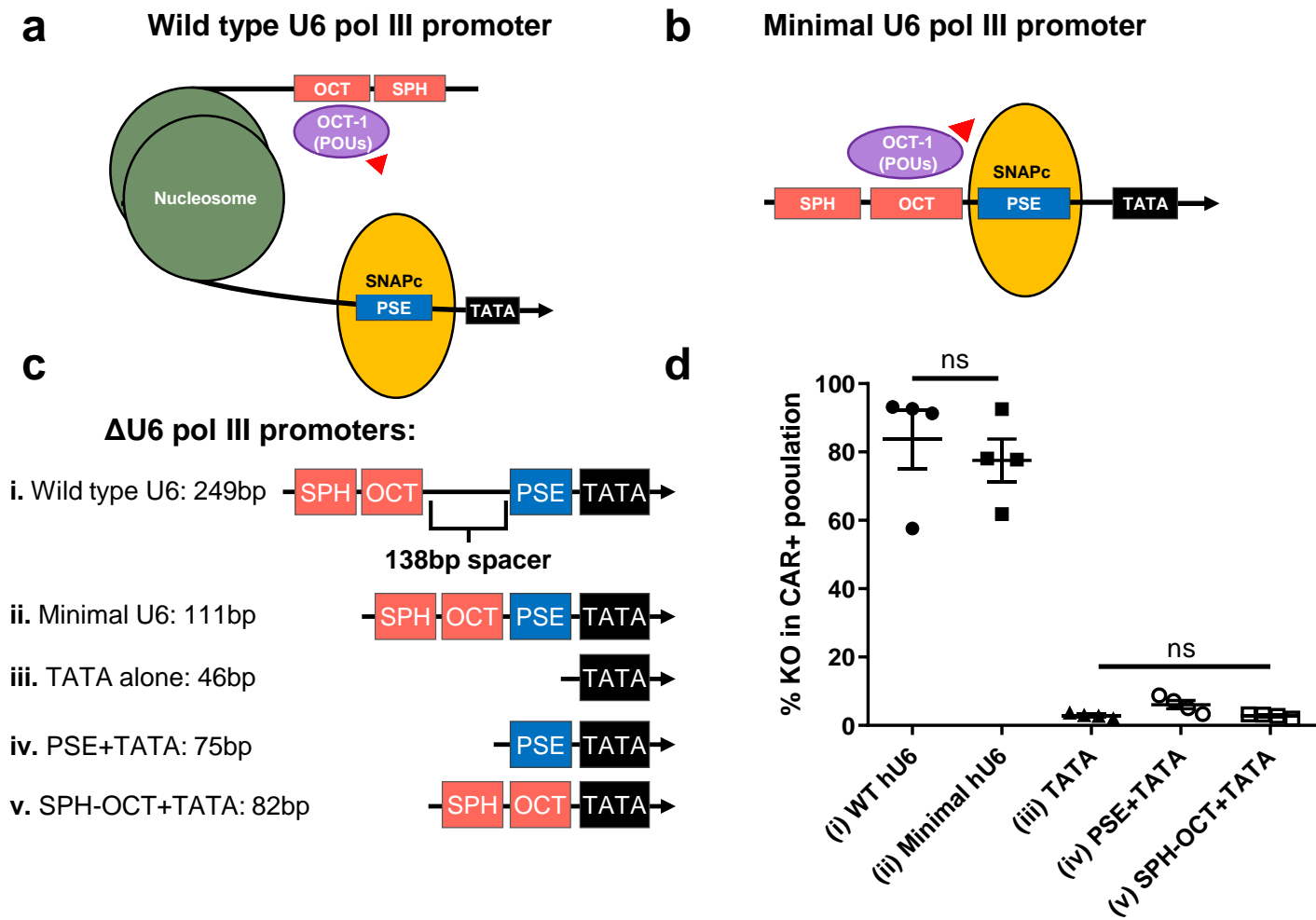


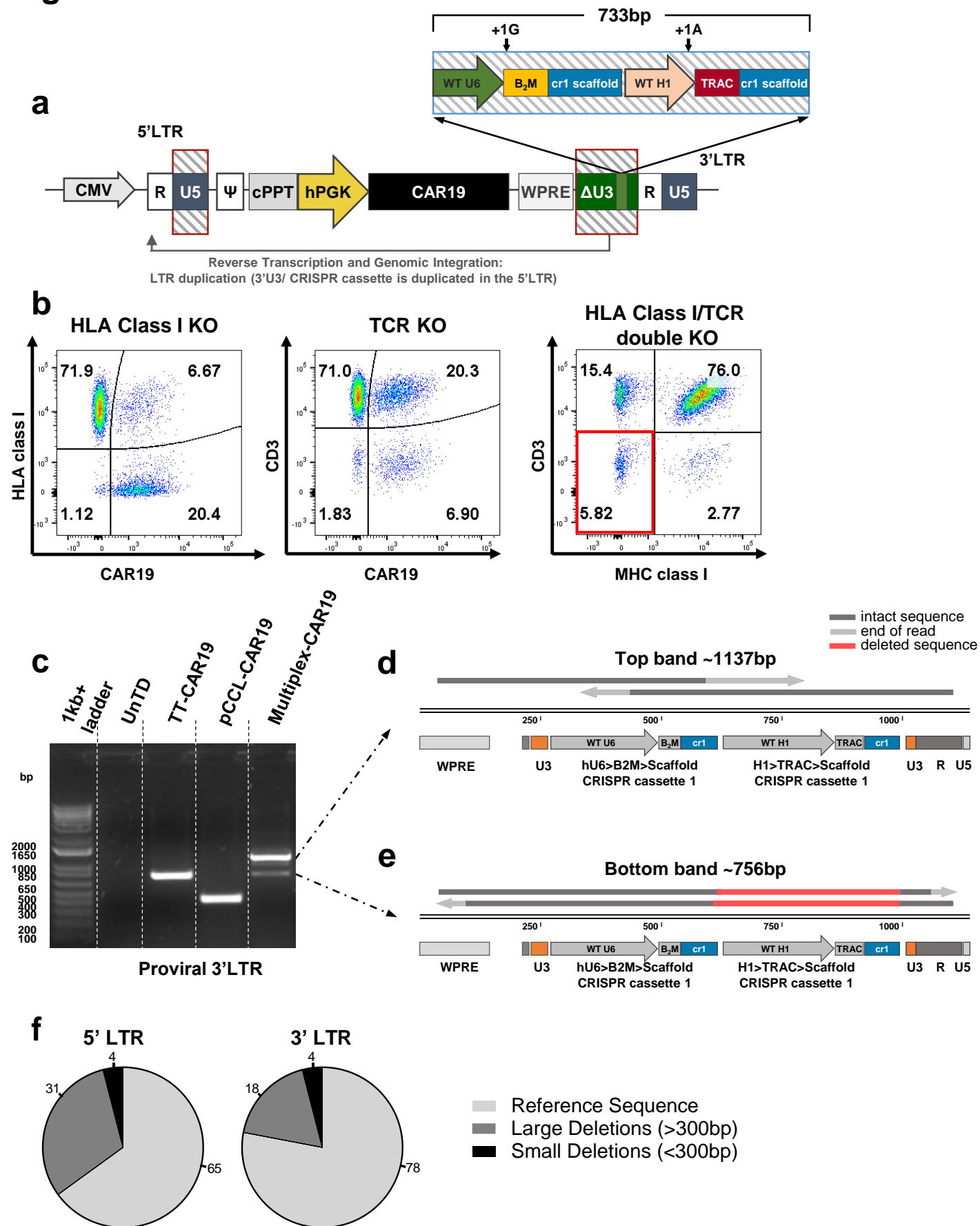
Figure 2

Figure 3

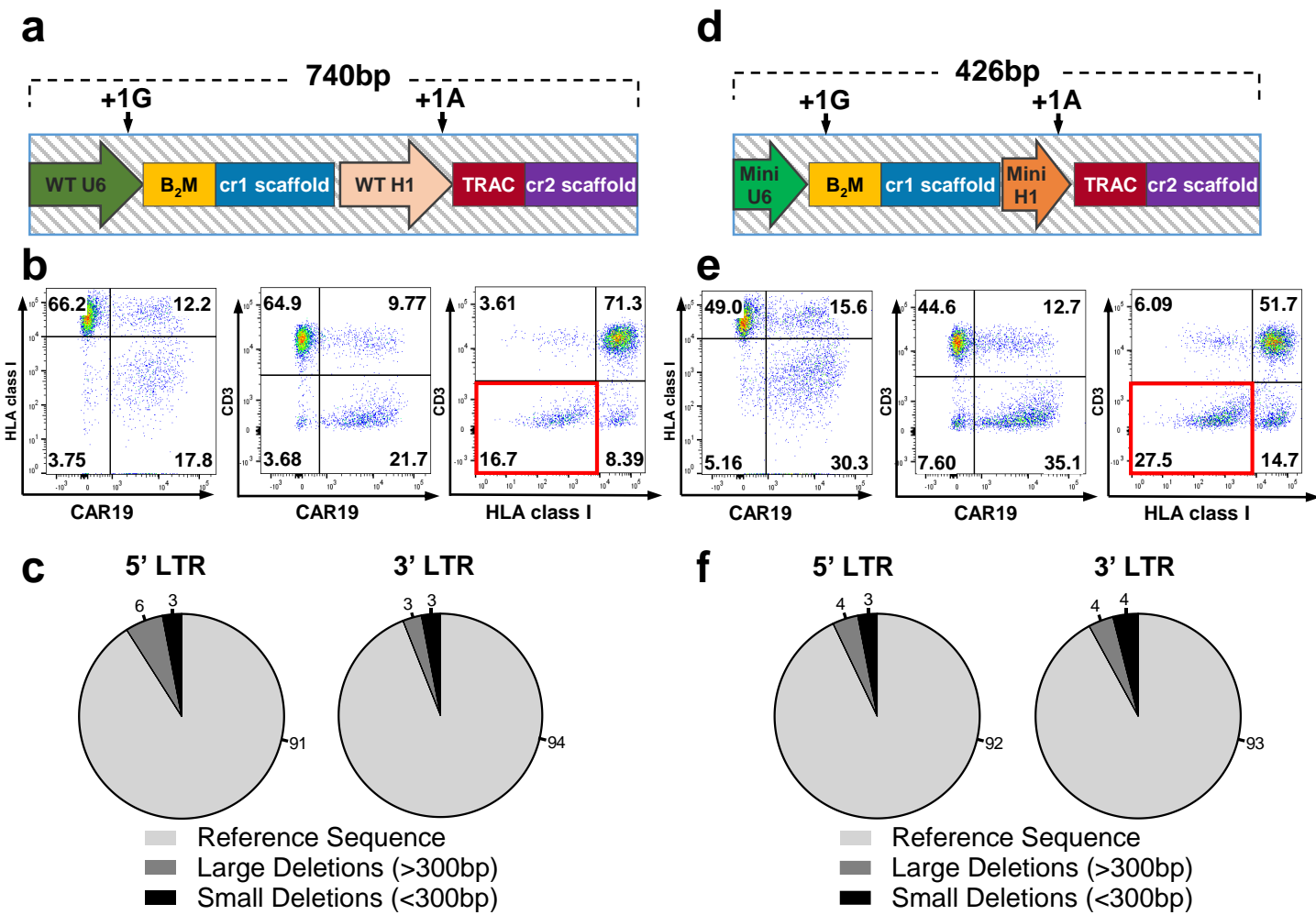
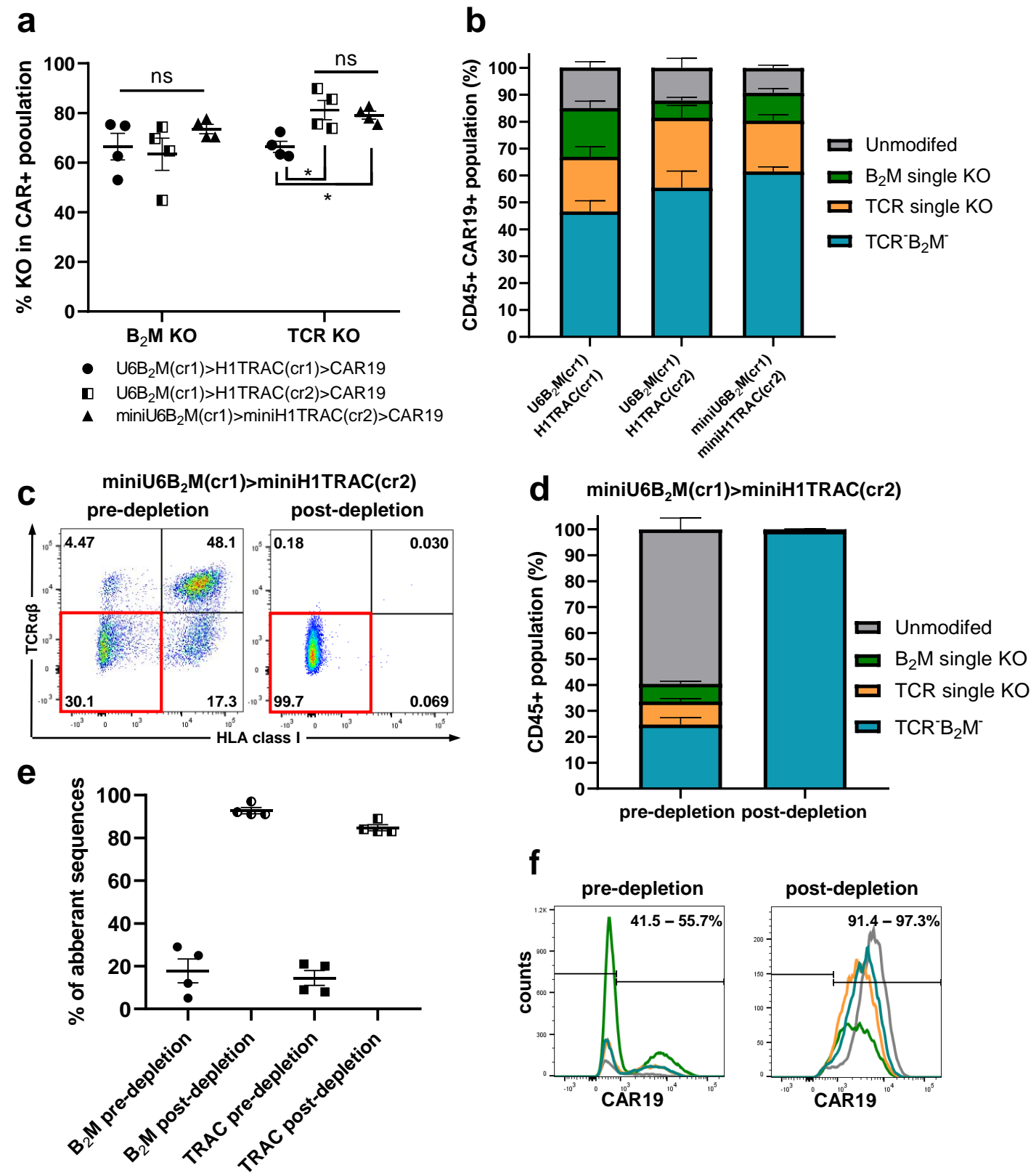


Figure 4

Supplementary Figure 1

Red= SPH

Blue= OCT

Green= PSE

Pink= TATA box

Purple= +1 transcription start (G)

i. Wild type U6: 249bp

GAGGGCCT**ATTTCCCATGATTCCTTCATATTTGCAT**ATACGATACAAGGCTGTTAGAGAG
ATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAA
AGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAATATGTTTTAAATGGACTATCATATG
CTTACCGTAACTTGAAAGTATTTTCGATTTCTTGGCTT**TATA**TATCTTGTGGAAAGGACGA
AACACCG

ii. Minimal U6: 111bp

GAGGGCCT**ATTTCCCATGATTCCTTCATATTTGCAT**ATACGATAG**CTTACCGTAACTTGA**
AAGTATTTTCGATTTCTTGGCTT**TATA**TATCTTGTGGAAAGGACGAAACACCG

iii. TATA alone: 46bp

TTTCGATTTCTTGGCTT**TATA**TATCTTGTGGAAAGGACGAAACACCG

iv. PSE+TATA: 75bp

ATACGATAG**CTTACCGTAACTTGAAGT**ATTTTCGATTTCTTGGCTT**TATA**TATCTTGTGG
AAAGGACGAAACACCG

v. SPH-OCT+TATA: 82bp

GAGGGCCT**ATTTCCCATGATTCCTTCATATTTGCAT**TTTCGATTTCTTGGCTT**TATA**TATC
TTGTGGAAAGGACGAAACCG

Figure S1. Generation of U6 variant sequences. Annotated sequences of wild type (wt) hU6 sequence (**i**). Minimal U6 sequence with spacer region removed (**ii**). U6 variants with critical elements removed; (**iii**) TATA alone, (**iv**) PSE+TATA, (**v**) SPH-OCT+TATA.

Supplementary Figure 2

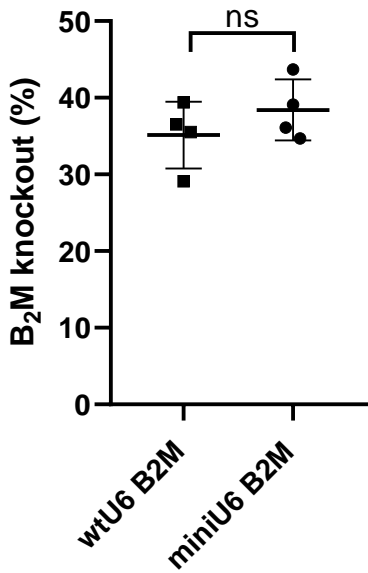


Figure S2. Comparison of B₂M knockout using wild type U6 and minimal U6 promoters in HEK 293T cells. Cells were transduced at an MOI 10 with lentiviral vectors expressing either the *U6B2M(cr1)>H1TRAC(cr2)* or *miniU6B2M(cr1)>miniH1TRAC(cr2)* cassette within the LTR and subsequently electroporated with 50µg/ml spCas9 mRNA using the Lonza 4D nucleofector system (program DG130). No significant difference was observed in B₂M knockout across four replicate experiments between wild type or minimal U6 promoters (p= 0.686) by Mann-Whitney U statistical analysis.

Supplementary Figure 3

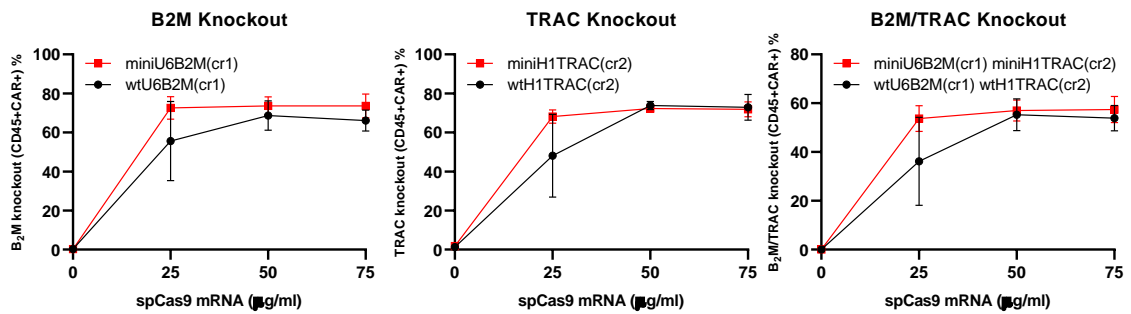


Figure S3. B₂M and TRAC knockout in primary human T cells across a gradient of spCas9 mRNA concentrations. Primary human T cells from (n=4) donors transduced with either *U6B2M(cr1)>H1TRAC(cr2)* or *miniU6B2M(cr1)>miniH1TRAC(cr2)* CAR19 expressing vectors were electroporated with spCas9 mRNA at 25, 50, or 75µg/ml. Knockout of B₂M and TRAC within the CAR19 population was quantified by flow cytometry and plotted. Area under the curve (AOC) analysis showed no significant difference between knockout achieved from sgRNAs expressed by either wild type or minimal promoters for knockout of B₂M (p= 0.138), TRAC (p= 0.114) or dual (B₂M/TRAC) (p= 0.170). Student's t-test performed at the lowest concentration of 25µg/ml similarly showed no significant difference between wild type and minimal promoters; B₂M (p= 0.343), TRAC (p= 0.114) or dual (B₂M/TRAC) (p= 0.200).