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Supplemental Information

An aptamer-mediated base editing platform for simultaneous knockin and multiple gene knockout for allogeneic CAR-T cells generation

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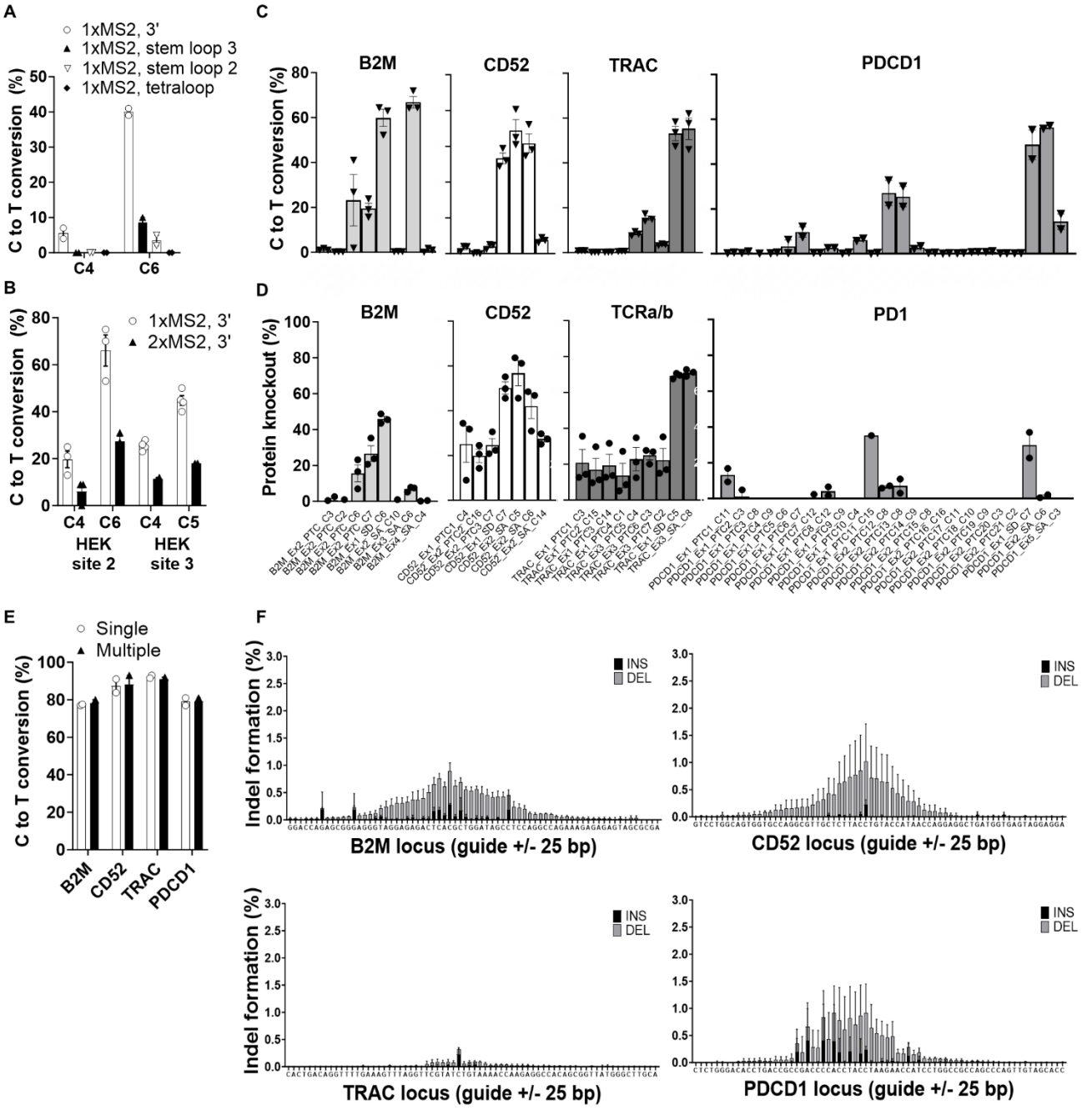


Figure S1. Optimisation and validation of Pin-point platform guide RNAs. Related to Figure 1. **A)** Activity of tracrRNAs incorporating one copy of the MS2 aptamer at either the 3' terminus or at different stem-loop positions within the gRNA scaffold. HEK293 cells were electroporated with mRNAs expressing the nCas9-UGI-UGI and rAPOBEC1-MCP components, and crRNA:tracrRNA complexes targeting site 2. Data is reported as mean (SEM) from one

individual experiment with three replicates. **B)** Activity of tracrRNAs incorporating one or two copies of the MS2 aptamer at the 3' terminus of the gRNA scaffold. HEK293T cells stably expressing the nCas9-UGI-UGI component were transfected with rAPOBEC1-MCP mRNA and crRNA:tracrRNA complexes targeting site 2 or site 3. Data are reported as mean (SEM) from one individual experiment with two replicates. In A) and B) editing was analyzed by Sanger sequencing three days post-electroporation. Activity for all the Cs in the editing windows is reported. **C)** C to T conversion of the target C for each of the gRNAs tested at *B2M, CD52, TRAC* and *PDCD1* gene targets. Individual crRNAs were delivered in combination with the tracrRNA containing one copy of the MS2 aptamer at the 3' end, nCas9-UGI-UGI mRNA, and rAPOBEC1-MCP mRNA to T cells by electroporation. Editing was analyzed by amplicon sequencing three days post electroporation. Data represented as mean (SEM), n = 3 independent T cell donors. **D)** Frequency of B2M, CD52, TCRa/b, and PD1 protein knock-out measured by flow cytometry three days after delivery of Pin-point mRNAs and crRNA/tracrRNA complexes targeting individual genes. Data represented as mean (SEM), n = 3 independent T cell donors. **E)** Comparison of C to T conversion of the target C following individual or combined delivery of optimal sgRNAs targeting *B2M, CD52, TRAC* and *PDCD1* loci by Sanger sequencing seven days post electroporation. Data represented as mean (SEM), n = 2 independent T cell donors. **F)** Levels of insertions and deletions at *B2M, CD52, TRAC* and *PDCD1* loci following co-delivery of Pin-point mRNAs and four target sgRNAs analyzed by NGS seven days post electroporation. Data represented as mean (SD), n = 3 independent T cell donors.

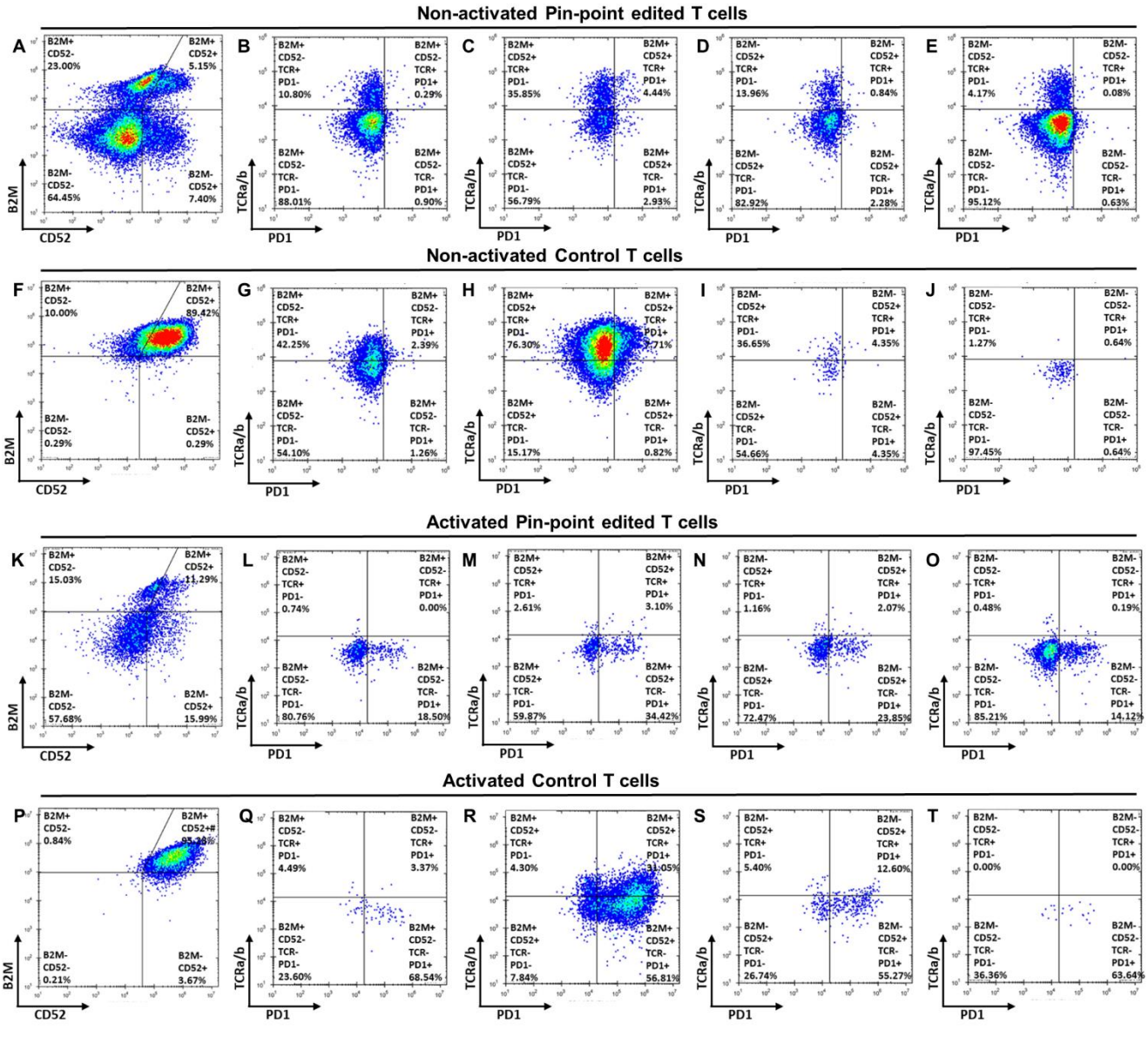
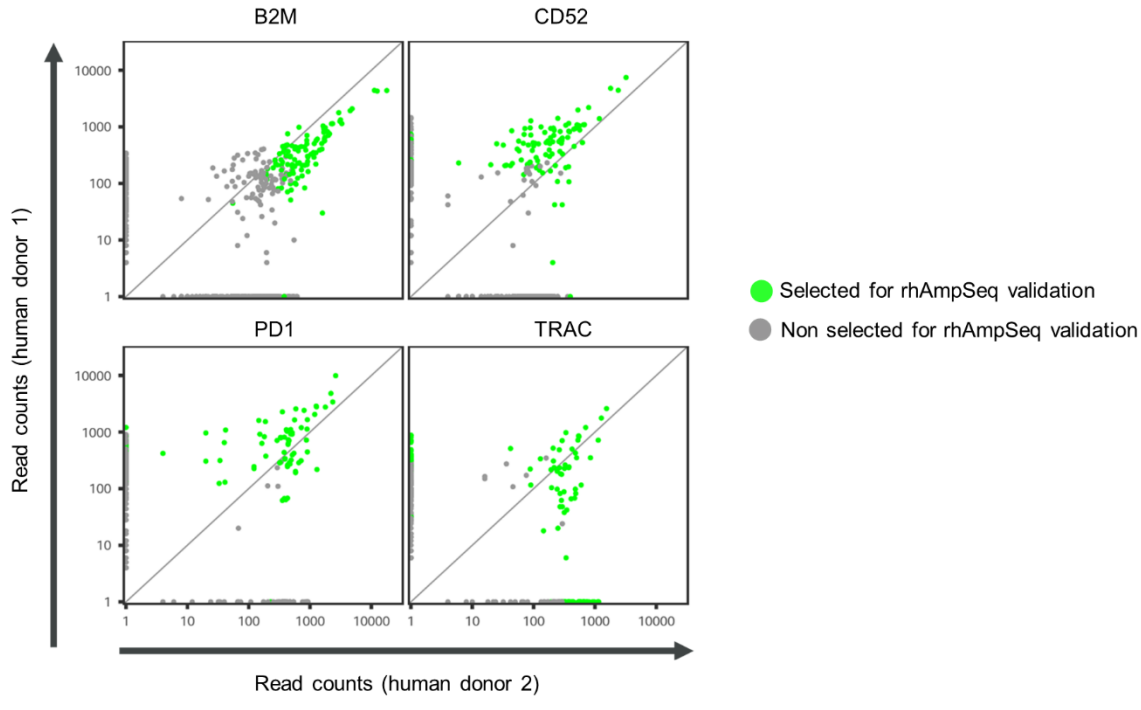


Figure S2 Representative flow cytometry gating strategy for quadruple edited T cells.

Related to Figure 2. A-E and K-O) Quadruple knockout edited cells. F-J and P to T) Unedited cells. A-J) Non-activated T cells. K-T) PMA/ionomycin stimulated cells. A, F, K and P) B2M and CD52 expression within the live population. B, G, L and Q) TCRa/b and PD1 expression within the B2M+ CD52- population. C, H, M and R) TCRa/b and PD1 expression within the B2M+

CD52+ population. D, I, N and S) TCRa/b and PD1 expression within the B2M- CD52+ population. E, J, O and T) TCRa/b and PD1 expression within the B2M- CD52- population.

A



B

Target site	On/Off-target	Site Sequence	PinPoint, % base editing (average)	Cas9, % indels (average)
B2M_Ex1_SD_C6 sgRNA				
B2M	On-target	ACTCACGCTGGATAGCCTCC	**40**	**47.3**
chr12_64682768_64682791	Off-target	**T**CTCAC**A**GCTGGATAGTCTCC	**0.93**	0.02
chr20_22332541_22332564	Off-target	ACTCAC**T**GCTGGA**C**AGCCTCC	**0.68**	**1.18**
PDCD1_Ex1_SD_C7				
PDCD1	On-target	CACCTACCTAAGAACCATCC	**41.3**	**46.5**
chr15_92446125_92446147	Off-target	**ACTG**ACCTAAGAACCATCC	**2.2**	**20.2**
TRAC_Ex3_SA_anti_C8				
TRAC	On-target	TTCGTATCTGTAAAACCAAG	**56.4**	**41.3**
CD52_Ex1_SD_anti_C7				
CD52	On-target	CTCTTACCTGTACCATAACC	**47.4**	**52.7**
chr10_79064348_79064370	Off-target	**AC**CTTA**T**CTG**C**ACCATAACC	**0.75**	0.02

Figure S3 Nomination of CHANGE-seq predicted off-target sites for rhAmpSeq validation. Related to Figure 3. **A)** ~100 predicted off-target sites per target gene (B2M, CD52, PDCD1 and TRAC) were selected for rhAmpSeq validation based on the following hierarchal site selection criteria: i) sites present in both donors and all replicates, ii) sites in all replicates of one donor, iii) sites in at least two replicates of either donor, and iv) sites in at least one replicate from one donor. In cases where we had more than 100 predicted sites we prioritized based on the nuclease-read counts reported in y-axis for donor 1 and in x-axis for donor 2. Additionally, any predicted

site with up to 4 miss-matches discovered by CHANGE-seq was also taken forward for off-target validation by rhAMPSeq. See Table S2 for CHANGE-Seq data summary. **B)** Summary of rhAMPSeq confirmed off-target sites in primary human T cells. Mismatches to the on-target site are highlighted.

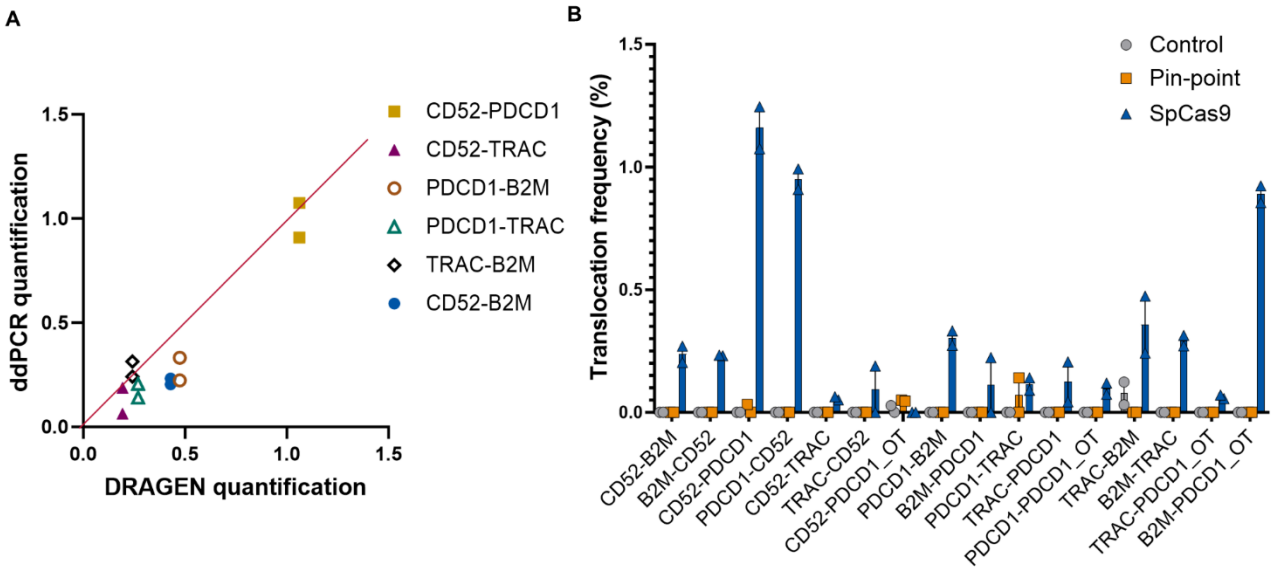


Figure S4 ddPCR validation of translocations identified by Capture-seq. Related to Figure 4. **A)** Correlation of translocation frequencies between sgRNA target sites calculated from Capture-seq and ddPCR analysis of a single SpCas9 edited sample three days post electroporation. The ddPCR data points are quantifications of the two outcomes of a reciprocal translocation between two sgRNA targets. **B)** Individual frequencies of the two outcomes of each predicted reciprocal translocation quantified by ddPCR. Control is mock electroporated T cells without RNA. Samples were analyzed at three days post electroporation. Data are reported as mean (SEM), n=2 independent T-cell donors.

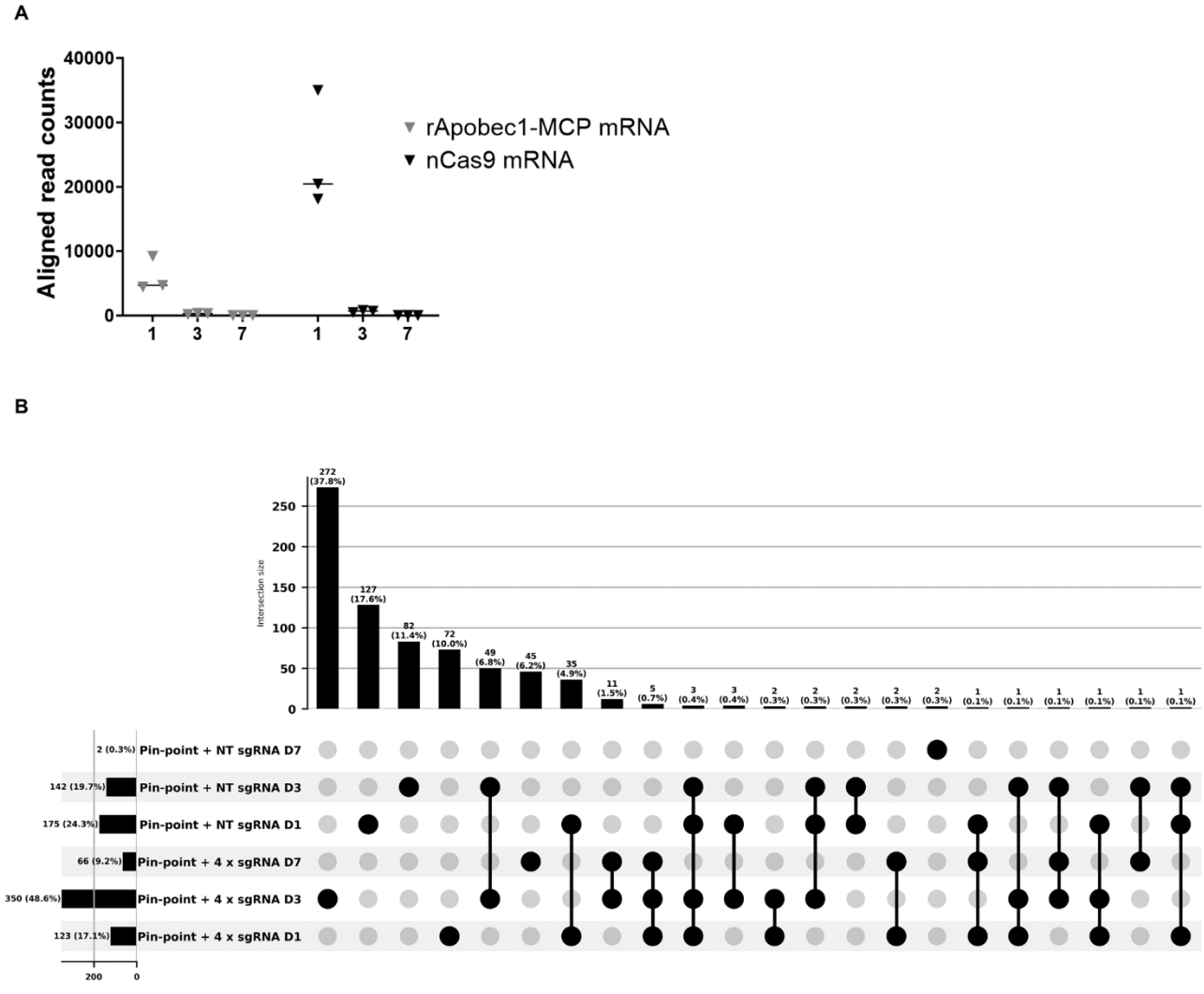


Figure S5 Gene expression analysis. Related to Figure 5. **A)** Reads aligned to the Pin-point mRNA sequences (nCas9-UGI-UGI and rAPOBEC-MCP) in RNA samples from T cells electroporated with Pin-point mRNAs and the four targeting sgRNAs (*TRAC*, B2M, *CD52*, *PDCD1*) at different time points post electroporation. Individual samples were run through the GATK Best Practice for RNA-Seq Pipeline, where instead of aligning against the transcriptome, reads are aligned against the reference sequences (i.e. rAPOBEC1-MCP or nCas9-UGI-UGI) corresponding to that sample. As a result, a filtered alignment file (in BAM-format) and Variant Call Format (VCF) file was generated for each sample. Using the BAM files, read counts were

determined for each component aligned against. **B)** UpSet plot of the differentially expressed genes (DEGs) of RNA-Seq. This panel summarizes the deregulated genes ($p < 0.05$ and log2 FC ≥ 1.5) overlap between human T cells electroporated with Pin-point mRNAs and either a non-targeting (NT) sgRNA or the four targeting sgRNAs and analyzed at day 1, 3 and 7 post electroporation.

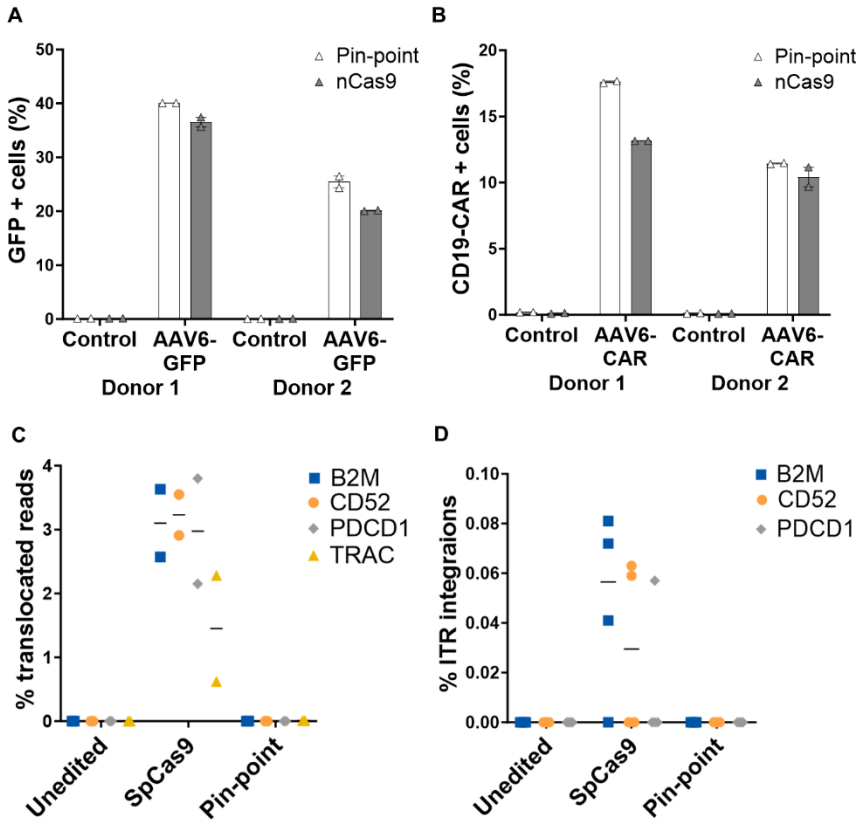


Figure S6 Efficacy and safety of aptamer-less sgRNAs for locus specific knock-in. Related to Figure 7. Pin-point mRNAs (nCas9-UGI-UGI and rAPOBEC1-MCP) or nCas9-UGI-UGI alone have been co-delivered with 2 aptamer-less gRNAs designed to target the exon 1 of TRAC locus. Shortly after electroporation, cells have been transduced with AAV6 carrying the GFP or CD19-CAR transgene flanked by the homology arms to the TRAC locus. **A**) Frequency of GFP positive cells in the T cell population after delivery of either Pin-point or nCas9-UGI-UGI mRNA and transduction with the AAV6-GFP in two donors. **B)** Frequency of CD19-CAR positive cells in the T cell population after delivery of either Pin-point or nCas9-UGI-UGI mRNA and transduction with the AAV6-CAR in two donors. **C)** Quantification of translocations due to dual nicking using Capture-seq. Pin-point mRNAs (nCas9-UGI-UGI and rAPOBEC1-MCP) were delivered with three aptamer-containing sgRNAs targeting B2M, CD52 and PDCD1 loci for base-editing in combination with two paired aptamer-less gRNAs targeting the TRAC locus. SpCas9

mRNA was delivered with three sgRNAs targeting B2M, CD52 and PDCD1 loci in combination with one of the two paired gRNAs targeting the TRAC locus. Control is mock electroporated T cells without RNA. Samples were analysed three days post electroporation. n=2 independent T cell donors. D) Quantification of homology-independent integration of AAV donor ITR sequence at sgRNA target loci. Pin-point mRNAs (nCas9-UGI-UGI and rAPOBEC1-MCP) were delivered with three aptamer-containing sgRNAs targeting B2M, CD52 and PDCD1 loci for base-editing in combination with two paired aptamer-less gRNAs targeting the TRAC locus. SpCas9 mRNA was delivered with three sgRNAs targeting B2M, CD52 and PDCD1 loci in combination with either one of the two paired gRNAs targeting the TRAC locus. Control is mock electroporated T cells without RNA. Shortly after electroporation, cells were transduced with AAV6 carrying the CD19-CAR transgene flanked by sequence homologous to the TRAC locus. Samples were analysed fourteen days post electroporation. n=2 independent T cell donors. Results for the two spCas9 gRNAs targeting TRAC are pooled. For C and D translocation frequency is represented by the percentage of Capture-seq sequencing reads marked as translocations by the DRAGEN Structural Variant (SV) Caller mapping to each sgRNA target site.

PKKKRKVDKKYSIGLAIGTNSVGWAVITDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGET
AEATRLKRTARRRYTRRKNRICYLQEIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIV
DEVAYHEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQ
LVQTYNQLFEENPINASGVDAKAILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNF
KSNFDLAEDAKLQLSKDTYDDDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAP
LSASMIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILE
KMDGTEELLVKLNREDLLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTF
RIPYYVGPLARGNSRFAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPK
HSLLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIE
CFDSVEISGVEDRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTY
AHLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSL
TFKEDIQKAQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARE
NQTTQKGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELD
INRLSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLI
TQRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVI
TLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYDV
RKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATV
RKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVV
AKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFELENGRK
RMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHYLDEIIEQISE
FSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTK
EVLDATLIHQSITGLYETRIDLSQLGGDSGGSGGSGGSTNLSDIIEKETGKQLVIQESILMLPEEVE
EVIGNKPESDILVHTAYDESTDENVMLLTSDAPEYKPWALVIQDSNGENKIKMLSGGSGGSGGS
TNLSDIIEKETGKQLVIQESILMLPEEVEEVIGNKPESDILVHTAYDESTDENVMLLTSDAPEYKP
WALVIQDSNGENKIKMLSGGSKRTADGSEFEPKKKRKV

Figure S7 Protein sequence of Cas9^{D10A}. Color key: Nuclear Localization Signal (NLS), CAS9^{D10A}, UGI.

PKKKRKVSSETGPVAVDPTLRRRIEPHEFEVFFDPRELRKETCLLYEINWGGRHSIWRHTSQNTN
KHVEVNFIEKFTTERYFCPNTRCSITWFLSWSPCGECSRAITEFLSRYPHVTLFIYIARLYHHADP
RNRQGLRDLISSGVTIQIMTEQESGYCWRNFVNYSPSNEAHWPRYPHLWVRLYVLELYCIILGL
PPCLNILRRKQPQLTFFTIALQSCHYQRLPPHILWATGLKELKTPLGDTTHTSPPCPAPELLGGPM
ASNFTQFVLVDNGGTGDVTVAPSNFANGIAEWISSNSRSQAYKVTCSVRQSSAQNRKYTIKVE
VPKGAWRSYLNMELTIPIFATNSDCELIVKAMQGLLKDGNPIPSAIAANSGIY

Figure S8 Protein sequence of rAPOBEC1-MCP. Color key: NLS, rApobec1, MCP

AACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCG
AGUCGGUGCGCGCACAUGAGGAUCACCCAUGUGCUUUUmU*mU*U

Figure S9 Sequence of aptamer containing tracrRNA. Color key: gRNA scaffold, MS2 aptamer, mN* nucleotides containing 2′-O-methyl 3′phosphorothioate modifications.

mN*mN*NNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG
CUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGCGCACAUGAGGAUCAC
CCAUGUGCUUUUmU*mU*U

Figure S10 Sequence of aptamer containing sgRNA. Color key: Spacer sequence, gRNA scaffold, MS2 aptamer, mN* nucleotides containing 2′-O-methyl 3′phosphorothioate modifications.

mN*mN*NNNNNNNNNNNNNNNNNNGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG
CUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUmU*mU*U

Figure S11 Sequence of aptamer-less sgRNA. Color key: Spacer sequence, gRNA scaffold, mN* nucleotides containing 2′-O-methyl 3′phosphorothioate modifications.

GACGCTGTGGCTCTGCATGACTCACTAGCACTCTATCACGGCCATATTCTGGCAGGGTCAGT
GGCTCCAACTAACATTTGTTTGGTACTTTACAGTTTATTAAATAGATGTTTATATGGAGAAG
CTCTCATTTCTTTCTCAGAAGAGCCTGGCTAGGAAGGTGGATGAGGCACCATATTCATTTTG
CAGGTGAAATTCCTGAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATATCGAGTA
AACGGTAGTGCTGGGGCTTAGACGCAGGTGTTCTGATTTATAGTTCAAAACCTCTATCAAT
GAGAGAGCAATCTCCTGGTAATGTGATAGATTTCCCAACTTAATGCCAACATACCATAAAC
CTCCCATTCTGCTAATGCCCAGCCTAAGTTGGGGAGACCACTCCAGATTCCAAGATGTACA
GTTTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTACTCTGCCAGAGTTATATTGCTGGGG
TTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGTATTATTAAGTAGCCCTGCATTTC
AGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTT
GGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCT
AAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCC
CTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCA
TGTCCTAACCCTGATCCTCTTGTACCACAGATATCCAGAACCCTGAACCTGCCGTGTACCAG
CTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTAggaagcggagctactaacttcagcctgctgaagca
ggctggagatgtggaggagaaccctggacctATGCTGCTGCTGGTCACATCTCTGCTGCTGTGCGAGCTGCCC
CATCCTGCCTTTCTGCTGATCCCCGACATCCAGATGACCCAGACCACAAGCAGCCTGTCTGC
CAGCCTGGGCGATAGAGTGACCATCAGCTGTAGAGCCAGCCAGGACATCAGCAAGTACCT
GAACTGGTATCAGCAAAAGCCCGACGGCACCGTGAAGCTGCTGATCTACCACACCAGCAG
ACTGCACAGCGGCGTGCCAAGCAGATTTTCTGGCAGCGGCTCTGGCACCGACTACAGCCTG
ACAATCAGCAACCTGGAACAAGAGGATATCGCTACCTACTTCTGCCAGCAAGGCAACACCC
TGCCTTACACCTTTGGCGGAGGCACCAAGCTGGAAATCACCGGCTCTACAAGCGGCAGCGG
CAAACCTGGATCTGGCGAGGGATCTACCAAGGGCGAAGTGAAACTGCAAGAGTCTGGCCC
TGGACTGGTGGCCCCATCTCAGTCTCTGAGCGTGACCTGTACAGTCAGCGGAGTGTCCCTG
CCTGATTACGGCGTGTCCTGGATCAGACAGCCTCCTCGGAAAGGCCTGGAATGGCTGGGAG
TGATCTGGGGCAGCGAGACAACCTACTACAACAGCGCCCTGAAGTCCCGGCTGACCATCAT
CAAGGACAACTCCAAGAGCCAGGTGTTCCTGAAGATGAACAGCCTGCAGACCGACGACAC
CGCCATCTACTATTGCGCCAAGCACTACTACTACGGCGGCAGCTACGCGATGGATTATTGG
GGCCAGGGCACCAGCGTGACCGTTTCTTCTGCCGCCGCTATCGAAGTGATGTACCCTCCTCC
TTACCTGGACAACGAGAAGTCCAACGGCACCATCATCCACGTGAAGGGCAAGCACCTGTGT
CCTTCTCCACTGTTCCCCGGACCTAGCAAGCCTTTCTGGGTGCTCGTTGTTGTTGGCGGCGT
GCTGGCCTGTTACAGCCTGCTGGTTACCGTGGCCTTCATCATCTTTTGGGTCCGAAGCAAGC

GGAGCCGGCTGCTGCACTCCGACTACATGAACATGACCCCTAGACGGCCCGGACCAACCA
GAAAGCACTACCAGCCTTACGCTCCTCCTAGAGACTTCGCCGCCTACCGGTCCAGAGTGAA
GTTCAGCAGATCCGCCGATGCTCCCGCCTATCAGCAGGGCCAAAACCAGCTGTACAACGAG
CTGAACCTGGGGAGAAGAGAAGAGTACGACGTGCTGGACAAGCGGAGAGGCAGAGATCCT
GAAATGGGCGGCAAGCCCAGACGGAAGAATCCTCAAGAGGGCCTGTATAATGAGCTGCAG
AAAGACAAGATGGCCGAGGCCTACAGCGAGATCGGAATGAAGGGCGAGCGCAGAAGAGG
CAAGGGACACGATGGACTGTACCAGGGACTGAGCACCGCCACCAAGGATACCTATGACGC
CCTGCACATGCAGGCCCTGCCTCCAAGATAAtgagtttaaacccgctgatcagcctcgactgtgccttctagttgccagcc
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tggGATTCTCAAACAAATGTGTCACAAAGTAAGTATTCTGATGTGTATATCACAGACAAAAC
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AGGTTCTGCCCAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCT
TATCCATTGCCACCAAAACCCTCTTTTTACTAAGAAACAGTGAGCCTTGTTCTGGCAGTCCA
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TGCTCTTCTAGGCCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCT
GCCAAAAAATCTTTCCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATTAACCCACCAA
TCACTGATTGTGCCGGCACATGAATGCACCAGGTGTTGAAGTGGAGGAATTAAAAAGTCAG
ATGAGGGGTGTGCCCAGAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCAGCTGGGA
AAAGTCCAAATAACTTCAGATTGGAATGTGTTTTAACTCAGGGTTGAGAAAACAGCTACCT
TCAGGACAAAAGTCAGGGAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTA
CCAAGGGCAGGGAGAGGACCCTATAGAGGCCTGGGACAGGAGCT

Figure S12 Sequence of the CAR template. Color key: NNNNN TRAC homology arms, nnnnnn GSG+P2A, <u>NNNNN</u> 1928Z-CAR, nnnnn bGH poly(A) signal.

Table S1. Base editing guide RNA information. crRNAs exhibiting the highest level of target C>T conversion and associated protein loss for each gene are underlined.

Gene	spacer sequence (5'-3')	PAM	Target exon	Target C position	Base editing outcome	gRNA ID
B2M	CACAGCCCAAGATAGTTAAG	TGG	Exon 2	3	PTC	B2M_Ex2_PTC1_C3
B2M	ACAGCCCAAGATAGTTAAGT	GGG	Exon 2	2	PTC	B2M_Ex2_PTC2_C2
B2M	TTACCCCACTTAACTATCTT	GGG	Exon 2	6, 7	PTC	B2M_Ex2_PTC3_C6
B2M	CTTACCCCACTTAACTATCT	TGG	Exon 2	7, 8	PTC	B2M_Ex2_PTC4_C7
B2M	ACTCACGCTGGATAGCCTCC	AGG	Exon 1 SD	6	SD disruption	B2M_Ex1_SD_C6
B2M	TTGGAGTACCTGAGGAATAT	CGG	Exon 2 SA	10	SA disruption	B2M_Ex2_SA_C10
B2M	TCGATCTATGAAAAAGACAG	TGG	Exon 3 SA	6	SA disruption	B2M_Ex3_SA_C6
B2M	AACCTGAAAAGAAAAGAAAA	AGG	Exon 4 SA	4	SA disruption	B2M_Ex4_SA_C4
CD52	GTACAGGTAAGAGCAACGCC	TGG	Exon 1	4	PTC	CD52_Ex1_PTC1_C4
CD52	CTCCTCCTACAGATACAAAC	TGG	Exon 2	16	PTC	CD52_Ex2_PTC2_C16
CD52	CAGATACAAACTGGACTCTC	AGG	Exon 2	7	PTC	CD52_Ex2_PTC3_C7
CD52	CTCTTACCTGTACCATAACC	AGG	Exon 1 SD	7	SD disruption	CD52_Ex1_SD_C7
CD52	GTATCTGTAGGAGGAGAAGT	GGG	Exon 2 SA	5	SA disruption	CD52_Ex2_SA_C5
CD52	TGTATCTGTAGGAGGAGAAG	TGG	Exon 2 SA	6	SA disruption	CD52_Ex2_SA_C6
CD52	GTCCAGTTTGTATCTGTAGG	AGG	Exon 2 SA	14	SA disruption	CD52_Ex2_SA_C14
TRAC	AACAAATGTGTCACAAAGTA	AGG	Exon 1	3	PTC	TRAC_Ex1_PTC1_C3
TRAC	CTTCTTCCCCAGCCCAGGTA	AGG	Exon 1	15	PTC	TRAC_Ex1_PTC2_C15
TRAC	TTCTTCCCCAGCCCAGGTAA	GGG	Exon 1	14	PTC	TRAC_Ex1_PTC3_C14
TRAC	AGCCCAGGTAAGGGCAGCTT	TGG	Exon 1	5	PTC	TRAC_Ex1_PTC4_C5
TRAC	TTTCAAAACCTGTCAGTGAT	TGG	Exon 3	4	PTC	TRAC_Ex3_PTC5_C4
TRAC	TTCAAAACCTGTCAGTGATT	GGG	Exon 3	3	PTC	TRAC_Ex3_PTC6_C3
TRAC	CCGAATCCTCCTCCTGAAAG	TGG	Exon 3	2	PTC	TRAC_Ex3_PTC7_C2
TRAC	CTTACCTGGGCTGGGGAAGA	AGG	Exon 1 SD	5	SD disruption	TRAC_Ex1_SD_ C5
TRAC	TTCGTATCTGTAAAACCAAG	AGG	Exon 3 SA	8	SA disruption	TRAC_Ex3_SA_C8
PDCD1	TCCAGGCATGCAGATCCCAC	AGG	Exon 1	11	PTC	PDCD1_Ex1_PTC1_C11

PDCD1	TGCAGATCCCACAGGCGCCC	TGG	Exon 1	3	PTC	PDCD1_Ex1_PTC2_C3
PDCD1	CGACTGGCCAGGGCGCCTGT	GGG	Exon 1	8, 9	PTC	PDCD1_Ex1_PTC3_C8
PDCD1	ACGACTGGCCAGGGCGCCTG	TGG	Exon 1	9, 10	PTC	PDCD1_Ex1_PTC4_C9
PDCD1	ACCGCCCAGACGACTGGCCA	GGG	Exon 1	6, 7	PTC	PDCD1_Ex1_PTC5_C6
PDCD1	CACCGCCCAGACGACTGGCC	AGG	Exon 1	7, 8, 19, 20	PTC	PDCD1_Ex1_PTC6_C7
PDCD1	TGTAGCACCGCCCAGACGAC	TGG	Exon 1	12, 13	PTC	PDCD1_Ex1_PTC7_C12
PDCD1	GGGCGGTGCTACAACTGGGC	TGG	Exon 1	12	PTC	PDCD1_Ex1_PTC8_C12
PDCD1	CGGTGCTACAACTGGGCTGG	CGG	Exon 1	9	PTC	PDCD1_Ex1_PTC9_C9
PDCD1	CTACAACTGGGCTGGCGGCC	AGG	Exon 1	4	PTC	PDCD1_Ex1_PTC10_C4
PDCD1	CACCTACCTAAGAACCATCC	TGG	Exon 1	15, 16	PTC	PDCD1_Ex1_PTC11_C15
PDCD1	GGGGTTCCAGGGCCTGTCTG	GGG	Exon 2	7, 8	PTC	PDCD1_Ex2_PTC12_C8
PDCD1	GGGGGTTCCAGGGCCTGTCT	GGG	Exon 2	8, 9	PTC	PDCD1_Ex2_PTC13_C8
PDCD1	GGGGGGTTCCAGGGCCTGTC	TGG	Exon 2	9, 10	PTC	PDCD1_Ex2_PTC14_C9
PDCD1	CAGCAACCAGACGGACAAGC	TGG	Exon 2	8	PTC	PDCD1_Ex2_PTC15_C8
PDCD1	CCCGAGGACCGCAGCCAGCC	CGG	Exon 2	16	PTC	PDCD1_Ex2_PTC16_C16
PDCD1	GGACCGCAGCCAGCCCGGCC	AGG	Exon 2	11	PTC	PDCD1_Ex2_PTC17_C11
PDCD1	CGTGTCACACAACTGCCCAA	CGG	Exon 2	10	PTC	PDCD1_Ex2_PTC18_C10
PDCD1	GTGTCACACAACTGCCCAAC	GGG	Exon 2	9	PTC	PDCD1_Ex2_PTC19_C9
PDCD1	CGCAGATCAAAGAGAGCCTG	CGG	Exon 2	3	PTC	PDCD1_Ex2_PTC20_C3
PDCD1	GCAGATCAAAGAGAGCCTGC	GGG	Exon 2	2	PTC	PDCD1_Ex2_PTC21_C2
PDCD1	CACCTACCTAAGAACCATCC	TGG	Exon 1 SD	7	SD disruption	PDCD1_Ex1_SD_C7
PDCD1	GGAGTCTGAGAGATGGAGAG	AGG	Exon 2 SA	6	SA disruption	PDCD1_Ex2_SA_C6
PDCD1	TTCTTTGAGGAGAAAGGGAG	AGG	Exon 5 SA	3	SA disruption	PDCD1_Ex5_SA_C3

Table S2. CHANGE-seq off-targets nomination.

The average and individual nuclease read counts (replicates 1-3) for each amplified site are reported, for two human donors evaluated (identified as D71 and D73). Sites selected for rhAmpSeq validation are identified in column L. These sites have been selected based on hierarchical selection criteria (column M) as defined in the table and in methods section. Genomic coordinates and sequence for each amplified site are reported. Mismatches to the on-target site are reported in lowercase.

Provided as separate file.

Table S3. Summary of rhAmpSeq on-target and off-target editing outcomes in T cells edited with the Pin-point system or SpCas9 for sites predicted by the CHANGESeq off-target discovery.

Percentage editing in edited samples (raw editing), control samples (control), and corrected editing calculated by subtracting the value of the control sample from the edited sample (background corrected editing), together with matched read depth at each interrogated site. Data is representative of either base editing (BE) or Insertions/Deletions (INDEL) events in cells edited with the Pin-point system (PnP) or SpCas9 for indicated on-target (TRAC/ B2M/ PDCD-1/ CD52) and predicted off-target sites. Bayesian statistics has been performed on the dataset, citing the evidence ratio, posterior probability and * as measures of statistical significance. On-targets are highlighted in green and validated off-targets in red.

Provided as separate file.

Table S4: Summary of rhAmpSeq on-target and off-target editing for additional sites with up to four miss-matches.

Percentage editing in edited samples (raw editing), control samples (control), and corrected editing calculated by subtracting the value of the control sample from the edited sample (background corrected editing), together with matched read depth at each interrogated site. Data is representative of either base editing (BE) or Insertions/Deletions (INDEL) events in cell edited with the Pin-point system (PnP) or SpCas9 for indicated on-target (TRAC/ B2M/ PDCD-1/ CD52) and predicted off-target sites. Bayesian statistics has been performed on the dataset, citing the evidence ratio, posterior probability and * as measures of statistical significance. On-targets are highlighted in green and validated off-targets in red.

Provided as separate file.

Table S5. Identity of translocations in 4 target knockout samples.

Total reads assigned to individual translocation clusters identified by the DRAGEN Structural Variant Caller (MANTA). Read depth at breakpoints on each chromosome (Chr A and Chr B) are shown for translocations involving at least one gRNA target.

Provided as separate file.

Table S6. Quantification of translocations in 4 target knockout samples

Total number of reads mapping to intervals +/-1000bp of gRNA target sites on each chromosome (Chr A and Chr B) for groups of translocations involving similar breakpoints. In cases where a breakpoint on either ChrA or ChrB is not adjacent to a captured gRNA target site the total number of reads mapping to the breakpoint is reported. Cluster reads reports the sum of reads supporting each group of translocations.

Provided as separate file.

Table S7 Quantification of translocations at each target site in 4 target knockout samples

Total number of reads mapping to intervals +/-1000bp of each gRNA target site. Cluster reads reports the total number of reads assigned to translocation clusters by MANTA mapping within +/-1000bp of each captured gRNA target site.

Provided as separate file.

Table S8. Lists of downregulated genes in T cells electroporated with Pin-point mRNAs and the 4 targets gRNAs *B2M, CD52, PDCD1* and *TRAC* or the scramble gRNA

Provided as separate file.

Table S9 – Identity of translocations following multi target editing with paired nicking guide RNAs.

Total reads assigned to individual translocation clusters identified by the DRAGEN Structural Variant Caller (MANTA). Read depth at breakpoints on each chromosome (Chr A and Chr B) are shown for translocations involving at least one gRNA target.

Provided as separate file.

Table S10 – Quantification of translocations following multi target editing with paired nicking guide RNAs.

Total number of reads mapping to intervals +/-1000bp of gRNA target sites on each chromosome (Chr A and Chr B) for groups of translocations involving similar breakpoints. In cases where a breakpoint on either ChrA or ChrB is not adjacent to a captured gRNA target site the total number of reads mapping to the breakpoint is reported. Cluster reads reports the sum of reads supporting each group of translocations.

Provided as separate file.

Table S11 – Quantification of translocations at each target site following multi target editing with paired nicking guide RNAs.

Total number of reads mapping to intervals +/-1000bp of each gRNA target site. Cluster reads reports the total number of reads assigned to translocation clusters by MANTA mapping within +/-1000bp of each captured gRNA target site.

Provided as separate file.

Table S12 – Identity of AAV off target integration sites following simultaneous multi target editing and CAR knock-in.

Total reads assigned to individual clusters identified by the DRAGEN Structural Variant Caller (MANTA). Read depth at AAV ITR integration sites +/-1000bp of captured gRNA targets are shown.

Provided as separate file.

Table S13 – Quantification of AAV off target integration events following simultaneous multi target editing and CAR knock-in.

Total number of reads mapping to intervals +/-1000bp of captured gRNA target sites with AAV ITR integrations. Cluster reads reports the sum of reads supporting each integration.

Provided as separate file.

Table S14. Guide RNAs utilised with SpCas9 optimal for indels formation.

Gene	spacer sequence (5'-3')	PAM	Target exon
B2M	GAGTAGCGCGAGCACAGCTA	AGG	Exon 1
CD52	CAGCCTCCTGGTTATGGTAC	AGG	Exon 1
PDCD1	CTGCAGCTTCTCCAACACAT	CGG	Exon2
TRAC	CAGGGTTCTGGATATCTGT	GGG	Exon 1

Table S15. Guide RNAs utilized for the knock-in in the TRAC locus.

Gene	spacer sequence (5'-3')	PAM	Target exon
TRAC	GAGAATCAAAATCGGTGAAT	AGG	Exon 1
TRAC	AACAAATGTGTCACAAAGTA	AGG	Exon 1

Table S16. Gene specific sequences for primers used for NGS genomic DNA amplification to detect base editing and indel events.

Gene	spacer sequence (5'-3')	gRNA name	Forward Primer (5'-3')	Reverse Primer (5'-3')
B2M	CACAGCCCAAGATAGTTAAG	B2M_Ex2_PTC1_C3	ACTCACGTCATCCAGCAGAGA	TGGGACTCATTCAGGGTAGT
B2M	ACAGCCCAAGATAGTTAAGT	B2M_Ex2_PTC2_C2	ACTCACGTCATCCAGCAGAGA	TGGGACTCATTCAGGGTAGT
B2M	TTACCCCACTTAACTATCTT	B2M_Ex2_PTC3_C6	ACTCACGTCATCCAGCAGAGA	TGGGACTCATTCAGGGTAGT
B2M	CTTACCCCACTTAACTATCT	B2M_Ex2_PTC4_C7	ACTCACGTCATCCAGCAGAGA	TGGGACTCATTCAGGGTAGT
B2M	ACTCACGCTGGATAGCCTCC	B2M_Ex1_SD_C6	GGCCTTGTCCTGATTGGCTG	CGCTTCCCCGAGATCCAG
B2M	TTGGAGTACCTGAGGAATAT	B2M_Ex2_SA_C10	AGGTGGAAGCTCATTTGGCC	ACCAGTCCTTGCTGAAAGAC
B2M	TCGATCTATGAAAAAGACAG	B2M_Ex3_SA_C6	TCTGAGGCTAGTAGGAAGGGC	TCCTCAGGACAGTGAAACA
B2M	AACCTGAAAAGAAAAGAAAA	B2M_Ex4_SA_C4	GGGAGCACCAAAGGGATACAC	TAAGTTGCCAGCCCTCCTA
CD52	GTACAGGTAAGAGCAACGCC	CD52_Ex1_PTC1_C4	AAGCTGCTACCAAGACAGCC	CAGGTTTCTCTCAGGGCAGC
CD52	CTCCTCCTACAGATACAAAC	CD52_Ex2_PTC2_C16	GAGTTCGAGACCAGCCTGAC	AGGAAAATGCCTCCGCTTAT
CD52	CAGATACAAACTGGACTCTC	CD52_Ex2_PTC3_C7	GAGTTCGAGACCAGCCTGAC	AGGAAAATGCCTCCGCTTAT
CD52	CTCTTACCTGTACCATAACC	CD52_Ex1_SD_C7	AAGCTGCTACCAAGACAGCC	CAGGTTTCTCTCAGGGCAGC
CD52	GTATCTGTAGGAGGAGAAGT	CD52_Ex2_SA_C5	GAGTTCGAGACCAGCCTGAC	AGGAAAATGCCTCCGCTTAT
CD52	TGTATCTGTAGGAGGAGAAG	CD52_Ex2_SA_C6	GAGTTCGAGACCAGCCTGAC	AGGAAAATGCCTCCGCTTAT
CD52	GTCCAGTTTGTATCTGTAGG	CD52_Ex2_SA_C14	GAGTTCGAGACCAGCCTGAC	AGGAAAATGCCTCCGCTTAT
TRAC	AACAAATGTGTCACAAAGTA	TRAC_Ex1_PTC1_C3	GCCGTGTACCAGCTGAGAGA	AAGGCCGAGACCACCAATCA
TRAC	CTTCTTCCCCAGCCCAGGTA	TRAC_Ex1_PTC2_C15	GCCGTGTACCAGCTGAGAGA	AAGGCCGAGACCACCAATCA
TRAC	TTCTTCCCCAGCCCAGGTAA	TRAC_Ex1_PTC3_C14	GCCGTGTACCAGCTGAGAGA	AAGGCCGAGACCACCAATCA
TRAC	AGCCCAGGTAAGGGCAGCTT	TRAC_Ex1_PTC4_C5	GCCGTGTACCAGCTGAGAGA	AAGGCCGAGACCACCAATCA
TRAC	TTTCAAAACCTGTCAGTGAT	TRAC_Ex3_PTC5_C4	CTGCAAGGGACAGGAGGTG	CTCACCTCAGCTGGACCAC
TRAC	TTCAAAACCTGTCAGTGATT	TRAC_Ex3_PTC6_C3	CTGCAAGGGACAGGAGGTG	CTCACCTCAGCTGGACCAC
TRAC	CCGAATCCTCCTCCTGAAAG	TRAC_Ex3_PTC7_C2	CTGCAAGGGACAGGAGGTG	CTCACCTCAGCTGGACCAC
TRAC	CTTACCTGGGCTGGGGAAGA	TRAC_Ex1_SD_anti_C5	GCCGTGTACCAGCTGAGAGA	AAGGCCGAGACCACCAATCA
TRAC	TTCGTATCTGTAAAACCAAG	TRAC_Ex3_SA_anti_C8	GGGGATATGCACAGAAGCTGC	CTCAGAGCTTAGGATGCACCC
PDCD1	TCCAGGCATGCAGATCCCAC	PDCD1_Ex1_PTC1_C11	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	TGCAGATCCCACAGGCGCCC	PDCD1_Ex1_PTC2_C3	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG

PDCD1	CGACTGGCCAGGGCGCCTGT	PDCD1_Ex1_PTC3_C8	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	ACGACTGGCCAGGGCGCCTG	PDCD1_Ex1_PTC4_C9	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	ACCGCCCAGACGACTGGCCA	PDCD1_Ex1_PTC5_C6	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	CACCGCCCAGACGACTGGCC	PDCD1_Ex1_PTC6_C7	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	TGTAGCACCGCCCAGACGAC	PDCD1_Ex1_PTC7_C12	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	GGGCGGTGCTACAACTGGGC	PDCD1_Ex1_PTC8_C12	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	CGGTGCTACAACTGGGCTGG	PDCD1_Ex1_PTC9_C9	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	CTACAACTGGGCTGGCGGCC	PDCD1_Ex1_PTC10_C4	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	CACCTACCTAAGAACCATCC	PDCD1_Ex1_PTC11_C15	CTGAGCAGTGGAGAAGGCG	CACACAGCTCAGGGTAAGGG
PDCD1	GGGGTTCCAGGGCCTGTCTG	PDCD1_Ex2_PTC12_C8	GAAGAGGCTCTGCAGTGGAG	TGGAGAAGCTGCAGGTGAAG
PDCD1	GGGGGTTCCAGGGCCTGTCT	PDCD1_Ex2_PTC13_C8	GAAGAGGCTCTGCAGTGGAG	TGGAGAAGCTGCAGGTGAAG
PDCD1	GGGGGGTTCCAGGGCCTGTC	PDCD1_Ex2_PTC14_C9	GAAGAGGCTCTGCAGTGGAG	TGGAGAAGCTGCAGGTGAAG
PDCD1	CAGCAACCAGACGGACAAGC	PDCD1_Ex2_PTC15_C8	GGGACAACGCCACCTTCA	CAGGCTCTCTTTGATCTGCG
PDCD1	CCCGAGGACCGCAGCCAGCC	PDCD1_Ex2_PTC16_C16	GGGACAACGCCACCTTCA	CAGGCTCTCTTTGATCTGCG
PDCD1	GGACCGCAGCCAGCCCGGCC	PDCD1_Ex2_PTC17_C11	GGGACAACGCCACCTTCA	CAGGCTCTCTTTGATCTGCG
PDCD1	CGTGTCACACAACTGCCCAA	PDCD1_Ex2_PTC18_C10	GGGACAACGCCACCTTCA	CAGGCTCTCTTTGATCTGCG
PDCD1	GTGTCACACAACTGCCCAAC	PDCD1_Ex2_PTC19_C9	GGGACAACGCCACCTTCA	CAGGCTCTCTTTGATCTGCG
PDCD1	CGCAGATCAAAGAGAGCCTG	PDCD1_Ex2_PTC20_C3	CAACGGGCGTGACTTCCA	GAGCTCCTGATCCTGTGCAG
PDCD1	GCAGATCAAAGAGAGCCTGC	PDCD1_Ex2_PTC21_C2	CAACGGGCGTGACTTCCA	GAGCTCCTGATCCTGTGCAG
PDCD1	CACCTACCTAAGAACCATCC	PDCD1_Ex1_SD_C7	GGCACCCTCCCTTCAACCT	CTCCAGACCCCTCGCTCC
PDCD1	GGAGTCTGAGAGATGGAGAG	PDCD1_Ex2_SA_C6	GAAGAGGCTCTGCAGTGGAG	TGGAGAAGCTGCAGGTGAAG
PDCD1	TTCTTTGAGGAGAAAGGGAG	PDCD1_Ex5_SA_C3	GAAGAGGCTCTGCAGTGGAG	TGGAGAAGCTGCAGGTGAAG

Table S17. Probes used for Capture-Seq experiments.

Provided as separate file.

Table S18. Primers and probes used to detect translocation events by ddPCR

Translocation	Primer 1	Probe	Primer 2
B2M-CD52	GGGCATTCCTGAAGCTGAC	AGAAAGAGAGAGTAGCGCGAGC	GAGTCCTGGCAGTGGTG
B2M-PDCD1	GGGCATTCCTGAAGCTGAC	CCTTAGCTGTGCTCGCGCTACT	AGGGACTGAGGGTGGAAG

B2M-TRAC	ATGTCTCGCTCCGTGGCCTTAG	CCTGTCAGTGATTGGGTTCCGAATCCTCCTCC	CATGAGCAGATTAAACCCGGCCAC
CD52-B2M	GCCACGAAGATCCTACCAAA	TTCCTCTTCCTCCTACTCACCATC	GGGAGAGGAAGGACCAGA
CD52-PDCD1	AGACAGCCACGAAGATCCTA	TTCCTCTTCCTCCTACTCACCATC	AGGGACTGAGGGTGGAAG
CD52-TRAC	CCTCTTCCTCCTACTCACCATCA	AACCTGTCAGTGATTG GGTTCC	CATGAGCAGATTAAACCCGGCCAC
PDCD1-B2M	GGCATGCAGATCCCACAG	AAGTCACGGAGCGAGAGCAC	GGCCACCAAGGAGAACTTG
PDCD1-CD52	GGCATGCAGATCCCACAG	TCTGGGCGGTGCTACAACTG	CCTCCATGCCAAGCAACT
PDCD1-TRAC	GGCATGCAGATCCCACAG	CCTGTCAGTGATTGGGTTCCGAATCCTCCTCC	CATGAGCAGATTAAACCCGGCCAC
TRAC-B2M	CAGCCTGCTCTGCCTTG	CATGCAAGCCCATAACCGCTGTG	AAGTCACGGAGCGAGAGAG
TRAC-CD52	AAACCGTGGGTGTGTCC	CTGGGACATGCAAGCCCATAA	CCTCCATGCCAAGCAACT
TRAC-PDCD1	CAGCCTGCTCTGCCTTG	CATGCAAGCCCATAACCGCTGT	AGGGACTGAGGGTGGAAG
TRAC-PDCD1_OT1	CAGCCTGCTCTGCCTTG	CATGCAAGCCCATAACCGCTGTG	AGAGAGAGAGACGCATGGTCAACC
B2M-PDCD1_OT1	GGGCATTCCTGAAGCTGAC	CCTTAGCTGTGCTCGCGCTACT	AGAGAGAGAGACGCATGGTCAACC
CD52-PDCD1_OT1	GCCACGAAGATCCTACCAAA	TTCCTCTTCCTCCTACTCACCATC	AGAGAGAGAGACGCATGGTCAACC
PDCD1-PDCD1_OT1	GGCATGCAGATCCCACAG	TCTGGGCGGTGCTACAACTGG	AGAGAGAGAGACGCATGGTCAACC
PPIA	CTCTGAGCACTGGAGAGAAAG	TAAGGGTTCCTGCTTTCACAGAAT	TGAAGGGAGCAACCCAAATA