Spring 4-27-2018

Genotype-Specific Insertion of Cytotoxic Genetic Elements Into Cancer Cells

Ryan Englander
ryan.englander@gmail.com

Genotype-Specific Insertion of Cytotoxic Genetic Elements Into Cancer Cells

A Novel Method for Specific Modification of Malignant Cells in a Tumor

Ryan Englander

A University Scholar Thesis
Submitted in Partial Fulfillment of the
Requirements for the Bachelor of Science Degree

May 2018
University of Connecticut

Department of Molecular and Cellular Biology, University of Connecticut
Center for Immunotherapy of Cancer and infectious Diseases, UConn Health

Committee Chair: Pramod K. Srivastava, MD/PhD _____

Committee Member: J. Peter Gogarten, PhD _____

Committee Member: David Daggett, PhD _____

ABSTRACT

The new gene editing system CRISPR/Cas9, composed of a complex composed of a guide RNA and the Cas9 endonuclease, promises to revolutionize biological research and potentially allow clinicians to directly modify patient DNA *in vivo*. While its applications in the treatment of genetic diseases and in modifying immune cells for immunotherapy are currently being explored, CRISPR/Cas9's potential utility as a modular system for targeting tumor-specific mutated sequences has not as of yet been explored. While CRISPR/Cas9 is specific enough to target small insertions and deletions or gross chromosomal rearrangements, it is not specific enough to reliably restrict editing to single nucleotide variants (SNVs), which compose the majority of cancer-associated mutations. By searching for tumor-specific SNVs that generate new protospacer adjacent motifs (neoPAMs), a short sequence that must be present next to the target sequence in order for Cas9 to cleave at the target site, gene editing can theoretically be restricted to tumor cells bearing the mutation of interest. This capability could permit the insertion of suicide genes, pro-inflammatory cytokines, or immunogenic epitopes in a tumor-specific manner. The results shown here demonstrate the importance of taking into account guide RNA efficiency when selecting a target site. New target sites in the tumor cell line FABF with high predicted guide RNA efficiencies are identified and discussed. Finally, strategies for maximizing the antitumor effect of neoPAM-restricted gene editing are described and compared.

ACKNOWLEDGEMENTS

It has been said it takes a village to raise a child, and while that was meant to refer to the support systems required to move a young person through the trials and tribulations of adolescence, I have found that it has applied to my undergraduate education just as well. I have often relied on the wonderful people around me to guide me through the darkest times. It was through them that I have always managed to find the light at the end of each tunnel, and I would like to thank them all here.

First and foremost I would like to thank my family, specifically my parents Elle and Mark and my siblings Hanna and Kyle. They have been my rock, anchoring me when the storm of life threatened to sweep me away. They believed in me even when I did not have faith in myself, and they pushed me to be the best version of me that I could be. I would not be where I am today without them. They are more important to me than words on a page can ever express.

Next, I would like to thank my business and research partners Matthew Lin and Nandan Tumu, with whom this project has been built and who I share a company, CrisprX, with. These two men are honest, brilliant, and hard working. Without them, this project would not be where it is today and likely would not exist at all. I want to thank them for their continued dedication to this idea that we all share and hope that together we can turn the concepts and results presented here into tangible products that can benefit the human race.

I would also like to thank Dr. David Daggett for his advice throughout this project. He urged me to file for a patent on the technique and is a large part of the

reason that we are where we are today. His support and deep reflection has been instrumental to our success so far.

I would like to thank the members of the Gogarten Lab, Matthew Fullmer and Dr. Peter Gogarten in particular, for their support over the 3.5 years I spent there. They both taught me a great deal about computer science and bioinformatics that I have been able to translate to my work in immunology. Those skills have made me a more balanced thinker and scientist. While my project did not conclude as I hoped it would, I am grateful to have had the opportunity to learn and grow with the fantastic individuals in the Gogarten Lab. I consider myself in that regard truly blessed.

In addition, I would like to thank the members of the Srivastava Lab for their incredible support over the past year. They helped teach me everything I know now about laboratory techniques and have made me a much more patient and disciplined individual as well as scientists. I have never met busier people who are as willing to help when I needed it. I would not have been able to obtain the results that I have without their continued assistance, and for that I am immeasurably grateful.

Finally, I would like to thank Dr. Pramod Srivastava for his incredible generosity and support throughout the year that I have been pursuing this project. He gave me, an untested and young undergraduate, access to all the resources I needed to complete this project without any hesitation. Words cannot express how grateful I am for that gift, as it has allowed me to do everything that I have done so far. Without Dr. Srivastava's kindness, I would be nowhere near where I am today.

He has been an incredible mentor and a better role model than I could ever dream of asking for.

TABLE OF CONTENTS

INTRODUCTION

Tumors contain, and are driven by, somatic mutations[1]. Chief among these are the single nucleotide variations (SNVs), point mutations found in all known human cancers[1,2]. While some somatic mutations are acquired later in neoplastic development and hence are not shared by all cancer cells in a tumor (the so-called branch mutations), a subset are acquired before or during the process of neoplastic transformation and hence are shared by almost all cancer cells (the so-called trunk mutations)[3]. These trunk mutations are unique identifiers of tumor cells, but no methods are presently available to use trunk mutations as a basis to therapeutically target tumor cells for destruction.

Sequence-specific gene editing may potentially allow such a capability. Nucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) provide sufficient sequence specificity to in theory permit the selective modification of cancer-specific mutated sequences, but inefficiencies in nuclease design and production have severely limited their use in a clinically relevant context[4,5]. The new CRISPR/Cas9 gene editing system is both more efficient and far easier to use than these older systems, and hence may soon be widely employed in human therapeutic gene editing[6-8].

Two RNA molecules, the CRISPR RNA (crRNA) and trans-acting CRISPR RNA (tracrRNA) interact to form the complete guide RNA (gRNA) molecule. The gRNA then forms a complex with the Cas9 endonuclease to form the complete Cas9 ribonucleoprotein (Cas9 RNP). Biochemical manipulations permit the use of a single guide RNA (sgRNA) composed of a single sequence that folds on itself to mimic the

crRNA:tracrRNA interaction, vastly simplifying gene editing[6-9]. The approximately 20 nucleotides at the 5' end of the gRNA restrict the nuclease activity of Cas9 to target DNA with sufficient sequence complementary as determined by Watson-Crick base pairing[8,9]. Sequence complementarity is insufficient to mediate double-stranded break (DSB) induction, however - a short motif, called a protospacer adjacent motif (PAM), must be present immediately next to the target sequence to permit nuclease activity[8,9]. The canonical PAM of the most commonly used Cas9 isoform from *Streptococcus pyogenes* (SpCas9) is 5'-NGG-3', where N can be any nucleotide[6-9]. A guanine in the first position is generally preferred, but not strictly required[10].

The specificity of wild-type Cas9 is not perfect – mismatches between the target sequence and gRNA are sometimes tolerated[6,7]. PAM proximal mismatches and strings of mismatches are not well tolerated, while isolated, PAM distal mutations do not often affect Cas9 activity[10-12]. Further, off-target PAM sequences have been observed for wild-type SpCas9, with 5'-NAG-3' constituting the vast majority of the off-target PAM sequences[10,11].

The Cas9-induced DSB can be repaired via two main pathways. The first is error-prone non-homologous end joining (NHEJ) whereby the blunt ends of the DNA are directly ligated together, often generating gene-inactivating small insertions and deletions (indels)[6,8,9]. Alternatively, DSBs may be repaired by homology-directed repair (HDR) whereby a DNA strand homologous to the damaged strand is used as a template for precise repair. Transfection with exogenous DNA sequences containing homology arms at the 5' and 3' ends matching either side of the Cas9 target site

permits the insertion, mediated by HDR, of virtually any DNA sequence at any locus with high fidelity[6,8,9].

Multiple groups have made modifications to SpCas9 to enhance its specificity and versatility. Foremost among these are the nickases and nuclease-null variants, which knock out either one or both of the catalytic subunits responsible for strand breakage respectively[13]. Single or paired nickases can be used to generate either single-stranded breaks (SSBs) or DSBs with large overhangs, which both severely limit the frequency of NHEJ and thereby bias editing towards HDR[14,15]. Further, nuclease-null variants, referred to as dead Cas9 or dCas9, can be used to activate or repress transcription or visualize genomic loci[16,17]. Other groups have rationally modified SpCas9 to limit its non-specific contacts with DNA, thereby improving its specificity[18,19]. Finally, recent work has shown that the use of truncated guide RNAs can significantly reduce off-target DSB events without sacrificing on-target activity[20]. Sufficiently truncating the guide RNA can eliminate DSB induction without interfering with Cas9's ability to bind target sites, permitting multiplexed gene editing and transcriptional modulation from the same protein[21].

Recent work has shown that an SNP that generates a novel PAM sequence can restrict Cas9-mediated gene editing to the SNP-containing chromosome, with no detectable off-target editing at the other chromosome[22]. This suggests a cancer-specific point mutation that generates a new PAM, a neoPAM, can restrict Cas9-mediated genome editing to cancer cells alone. While the target sequence complementary to the gRNA would be present with zero mismatches in both tumor

and healthy tissue, only tumor cells would have the necessary adjacent neoPAM sequence to permit recognition and DSB induction by Cas9.

The cancer-specific DSB generated by neoPAM restriction can be used to mediate the insertion of new genes via HDR. Even if neoPAM restriction is truly tumor-specific, it is unlikely that all cancer cells will be targeted due to the low efficiencies of *in vivo* delivery and HDR[23,24]. Cytotoxic elements that exert potent bystander effects whereby successfully transformed tumor cells induce cell death or growth inhibition in nearby cells may solve this problem. One well-studied class of genes that carry this property is the suicide genes. These genes convert non-toxic prodrugs to toxic metabolites, and their selective expression in cancer cells permits tumor-specific cytotoxicity[25-28]. The best studied of these are the herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) and cytosine deaminase/5-fluorocytosine (CD/5-FC) systems, though other effective systems exist[28].

In the HSV-TK/GCV system, the thymidine kinase from herpes simplex virus monophosphorylates nucleoside analogs like ganciclovir, an analog of 2'-deoxyguanosine bound to an acyclic sugar. The monophosphorylated intermediate is fully phosphorylated by endogenous enzymes and incorporated into DNA by endogenous polymerases, whereupon its incomplete sugar ring induces termination of chain elongation and eventually cell death through ligand-independent death receptor aggregation and subsequent apoptosis[29-31]. Because ganciclovir triphosphate is triply negatively charged, it cannot diffuse across cell membranes and hence relies on gap junctions to exert a bystander effect[32]. In tumor cells that

are gap junction deficient, the bystander effect of the HSV-TK/GCV system can be restored through pharmacological induction of gap junctions[35].

Multiple attempts have been made to modify the HSV-TK enzyme to both increase its sensitivity to ganciclovir and reduce its affinity for its intended substrate[29]. These efforts have produced mutants that dramatically increase transduced tumor cell sensitivity to ganciclovir, occasionally by over two orders of magnitude[34]. One rationally designed mutant in particular, TK007, introduces a targeted A to H mutation at position 168 that markedly reduces the enzyme's affinity for deoxythymidine while retaining its affinity for ganciclovir, thereby enhancing its efficiency[35]. This modification dramatically improves TK007's killing efficiency and its bystander effect both *in vitro* and *in vivo* in a murine xenograft model[36].

The CD/5-FC system uses cytosine deaminase from either *E. coli* or *S. cerevisiae* to convert the nontoxic prodrug 5-fluorocytosine into the widely used chemotherapeutic 5-fluorouracil (5-FU). 5-FU is readily converted *in situ* by endogenous enzymes to its monophosphorylated form 5-fluoro-2'-deoxyuridine-5'-monophosphate (5-FdUMP) which irreversibly inhibits the essential enzyme thymidylate synthetase or to its triphosphorylated form 5-fluorouridine-5'-triphosphate which can be incorporated into RNA and subsequently inhibits nuclear RNA processing[28]. While these phosphorylated molecules are restricted to the cells in which they were produced, 5-FU can freely diffuse across cell membranes due to its neutral charge and small size. Thus, the CD/5-FU system mediates a significantly more potent bystander effect than the HSV-TK/GCV system[37]. Further, yeast CD,

which has a significantly lower K_m value for 5-FC than bacterial CD, is a far smaller gene than either HSV-TK or bacterial CD[28,38].

Both of these systems are somewhat limited in that the suicide enzyme is localized to successfully transduced tumor cells. Intercellular trafficking mechanisms by which the enzymes could be transferred between neighboring cells after expression have the potential to markedly increase the efficacy of these systems. One such modification, a fusion of the viral intercellular trafficking protein VP22 to HSV-TK, permitted the transfer of the fusion protein from a donor transfected cell line to co-cultured gap junction-negative neuroblastoma cells, mediating extensive cell death in this untransfected, bystander effect-impaired cell line[39]. This fusion protein was later demonstrated to modestly increase sensitivity to ganciclovir in a human epithelial cell line[40]. A similar fusion protein was constructed using bacterial CD that also mediated increased sensitivity to its prodrug substrate compared to its wild type counterpart[41].

In contrast to gene therapy strategies that rely on enzymes and their cytotoxic metabolites to mediate tumor cell death, tumor-targeted immune gene therapies modify tumor cells to express cytokines or ligands that stimulate an antitumor immune response[42]. These techniques can initiate a new immune response against tumor-associated antigens or can help overcome tumor-mediated immunosuppression. The stunning effectiveness of immune checkpoint blockade supports the notion of leveraging the immune system to fight cancer. These antibody-based therapeutics function by unleashing preexisting immune responses

against tumor neoantigens, lending support to the notion that enhancing tumor immunogenicity through gene therapy may be an effective therapeutic modality[43-46].

The earliest studies into immunoactivatory gene therapy focused on the transfection of inflammatory Th1 cytokines known to be crucial to the adaptive antitumor immune response. The most prominent of these cytokines are interleukin-2 (IL-2) and interferon-γ. IL-2 is essential for CD8+ and CD4+ T cell proliferation, differentiation, and effector function acquisition[47,48]. The acquisition of effector functions *in vitro* impairs IL-2 secretion and limits a sustained antitumor immune response *in vivo*[49], while IL-2 secretion by tumor cells overcomes the need for CD4+ T cell help in the generation of an MHC I-restricted, cytotoxic lymphocyte (CTL)-mediated antitumor response[50]. In a murine fibrosarcoma model, IL-2 secretion by CMS-5 tumor cells induced a CTL response against both the parental and IL-2 secreting cell lines. Mice that rejected IL-2 secreting CMS-5 were protected against rechallenge with a tumorigenic dose of parental CMS-5, indicating the antitumor immune response was specific for tumor antigens[51]. These results have relevance for human patients, as immunization of neuroblastoma-bearing children with autologous unirradiated tumor cells transduced with adenoviral DNA expressing IL-2 magnified CTL killing of autologous tumor cells and generated a local CD4+ helper cell-mediated inflammatory environment[52]. IL-2, while clearly beneficial for the establishment and maintenance of an antitumor immune response, is also responsible for the activation and expansion of powerfully immunosuppressive T regulatory cells (Tregs) in response to self-antigen[53,54]. Treg activity and induction from naïve CD4+ T cells are known to be key mechanisms by

which tumors escape the immune response[55,56], and hence IL-2 secretion by tumor cells is unlikely to be broadly sufficient for immune-mediated tumor rejection. Interferon-γ, meanwhile, is crucial for cellular immunity and hence has a central role in immune-mediated tumor control[57]. It is secreted by activated NK, NKT, Th1 helper cells, and CTLs and has a broad array of functions, including inducing MHC Class I and Class II expression, promoting a Th1 cell phenotype, activating resident macrophages, promoting tumor cell apoptosis and the release of pro-inflammatory cytokines, and inhibiting angiogenesis[57-65]. It has been shown to augment the expression of viral antigens in the nonimmunogenic sarcoma MCA 101, thereby aiding in the generation of tumor-specific CTLs[66]. Further, interferon-γ may be involved in the suppression of Treg proliferation mediated in part by interleukin-12 (IL-12), a cytokine secreted by activated Th1 and NK cells[67], potentially limiting Treg expansion driven by IL-2. These results provide a strong rationale for *in vivo* tumor modification to induce intratumoral secretion of both interferon-γ and IL-2.

Other cytokines, in particular tumor necrosis factor, interferon-α, and IL-12, are intimately involved in the antitumor innate and adaptive immune responses to varying degrees[68-76]. Thus, immunoactivatory *in vivo* tumor gene modification strategies may also induce the secretion of these cytokines either in combination with interferon-γ, IL-2, and/or each other. However, as homologous recombination efficiency decreases with increasing insert size[77], the additional efficacy associated with the expression of additional cytokines would need to be balanced against the decreased number of successfully transformed cancer cells.

A number of immunoactivatory ligands can also be used to enhance the immunogenicity of tumors. Co-stimulatory ligands, namely B7-1, B7-2, and B7h, are expressed on activated antigen-presenting cells (APCs) and bind either CD28 (in the case of B7-1 and B7-2) or ICOS (in the case of B7h) on T cells. The CD28-B7 interaction is a necessary costimulatory signal for T cell activation; without it, TCR engagement with the target peptide-MHC complex leads to anergy instead of activation[78-81]. Hence, a paucity of costimulatory B7 expression on APCs may impair the antitumor immune response by preventing T cell priming. In a variety of tumor models, transfection of tumor cells with B7-1 or B7-2 leads to the activation of CD8+ and/or CD4+ T cell responses that can eradicate established tumors and provide protection against the parental tumor cell line[82-84]. Hence, selective *in vivo* modification to induce costimulatory ligand expression in tumor cells either alone or in combination with pro-inflammatory cytokines may have clinical relevance.

MATERIALS AND METHODS

Identification of FABF-specific neoPAMs – The murine tumor cell line FABF, a cell line identified in the Srivastava lab, was used for the following studies. The FABF exome had been sequenced previously and the data generously provided by Cory Brennick. A program was written to identify point mutations that produced GG or CC dinucleotides excluding GG dinucleotides where the wild type sequence was 5'-AG-3' or CC dinucleotides where the wild type sequence was 5'-CT-3'. If the mutation produced a GG dinucleotide, the putative target sequence was retrieved from the 21 nucleotides 5' of the dinucleotide on sense strand in the UCSC *Mus musculus* version mm10 reference genome. The first 20 nucleotides of this sequence were converted to the corresponding RNA sequence and ordered as an sgRNA from Sigma Aldrich. In contrast, if the mutation produced a CC dinucleotide, the putative target sequence was retrieved by reconstructing the antisense strand from the 21 nucleotides 3' of the dinucleotide in the mm10 reference genome[85].

NeoPAM efficiency screen – The cleavage efficiency of putative sgRNAs was predicted with the online CRISPR Efficiency Predictor tool. Any putative sgRNA with a score above 7.5 were considered fit for further analysis.

Design and synthesis of donor DNA – The 500 nucleotides 5' and 3' of the chosen target site were obtained from the mm10 reference genome[85]. The minimal cytomegalovirus early/intermediate enhancer and promoter elements were obtained from the sequence data of Plasmid #80802 in the Addgene database[86]. The SV40 polyadenylation signal was obtained from the sequence data of Plasmid #55764 in the Addgene database[87]. The chicken ovalbumin sequence was retrieved

from entry P01012 in the Uniprot database[88]. Finally, an ER insertion sequence was retrieved from the literature[89]. Donor DNA sequences were constructed *in silico*, synthesized by GenScript, and quantified by a NanoDrop spectrophotometer.

Cell culture – FABF cells were grown in RPMI (Life Technologies) supplemented with 10% heat-shocked fetal bovine serum, penicillin/streptomycin, sodium pyruvate, non-essential amino acids, and β-mercaptoethanol (complete RPMI). Cells were grown until they became 90% confluent, after which they were trypsinized and either used in downstream applications or seeded into larger flasks. When stocks needed to be made, cells were resuspended to between $2*10^6$ and $8*10^6$ cells/mL in complete RPMI supplemented with an additional 10% heat-shocked fetal bovine serum and 10% DMSO and stored in cryopreservation tubes at -80 °C. Cells were thawed by placing the cryopreservation tubes in a 37 °C water bath for 1 minute or until fully thawed directly from the -80 °C freezer.

Cas9 RNP transfection – Lipofectamine CRISPRMAX (Thermofisher) was used for all transfections. Manufacturer protocols were modified as follows: one day before transfection, $5*10^4$ FABF cells were seeded in the requisite number of wells of a 24 well plate in 500 µL complete RPMI media such that the plates were between 30% and 70% confluent at the time of transfection. On the day of transfection, Cas9 v2 protein (ThermoFisher Scientific), sgRNA (Sigma Aldrich, custom synthesized), and donor DNA (GenScript, custom synthesized) were thawed on ice. Opti-MEM reduced serum media, Cas9 Plus reagent, and Lipofectamine CRISPRMAX (all ThermoFisher Scientific) were moved from 4 °C and kept on ice until use. 25 µL of Opti-MEM media was then added to two sterile Eppendorf tubes. 1250 ng Cas9 v2

protein and 240 ng sgRNA were added to one of these tubes (Tube 1) while concurrently 1.5 µL of Lipofectamine CRISPRMAX was added to the second tube (Tube 2). Both of these tubes were briefly vortexed and 1 µL of Cas9 Plus reagent was added to Tube 1. Both tubes were incubated at room temperature for 5 minutes, after which 300 ng of linear donor DNA was added to Tube 1. Tube 1 was then briefly vortexed and its contents added to Tube 2, which was then vortexed briefly and incubated at room temperature for 15 minutes. 50 µL of this mixture was then added to the cells in a single well and mixed well. Cells were incubated for 1 day before the addition of an additional 750 µL of complete RPMI. On the second day after transfection, media was removed, wells were washed with 1 mL PBS twice, and 500 µL of 37 °C of trypsin EDTA was added to each well and allowed to incubate at 37 °C for approximately 1 minute. Wells were then washed with 2 mL complete RPMI and transferred to 14 mL Eppendorf tubes. The tubes were then diluted with 10-14 mL complete RPMI and spun down at 300 g for 5 minutes, resuspended with 10 mL complete RPMI, and plated.

sgRNA cleavage validation assay – Approximately $1*10^6$ cells were lysed and digested with Cell Lysis Buffer and Protein Degrader from the GeneArt® Genomic Cleavage Detection Kit (Thermofisher). 2 µL of cell lysate was added to a PCR reaction with 0.2 µM of each primer and the PCR master mix provided by the Cleavage Detection Kit according to manufacturer protocols and cycled as follows: 10 min. @ 95 °C, 1 cycle; 30 sec. @ 95 °C and 30 sec. at the lowest primer T_m – 5 °C and 30 sec. at 72 °C, 40 cycles; 7 min. at 72 °C, 1 cycle; 4 °C hold. The PCR product was concentrated and contaminating proteins and chaotropic salts removed with

the PCR Clean-Up and Gel Extraction Kit (Takara Bio) according to manufacturer protocols. Purified PCR product was quantified with a NanoDrop spectrophotometer. 100 ng of DNA was then denatured, re-annealed, and digested according to manufacturer protocols. 10 µL of the resulting solution was run on a 2% agarose gel at 50 V for 1 hour and visualized with a GelDoc imager (Bio-Rad Laboratories).

B3Z coculture assay – FABF cells were trypsinized, resuspended, and counted with an automatic hemocytometer and AO/PI staining according to standard protocols. 200,000 FABF cells per group were then added to a 96 well round bottom plate in 100 µL of complete RPMI. Semi-adherent B3Z cells were then resuspended and counted via AO/PI staining with an automatic hemocytometer. 200,000 B3Z cells were then added to the plates previously seeded with FABF in 100 µL of complete RPMI such that each well contained 200 µL of media. Plates were incubated at 37 °C for 20 hours. Plates were then spun at 931 g for 2 min and the supernatant was decanted. Wells were gently washed twice with 200 µL PBS such that the cells at the bottom of the well were not disturbed. 150 µL of a pre-prepared solution of chlorophenol red-β-D-galactopyranoside (45.5 mg chlorophenol red-β-D-galactopyranoside, 2.5 mL NP40, and 4.5 mL of 1 M $MgCl_2$ in 500 mL of PBS) was then added to each well and allowed to incubate at room temperature for 15 minutes. Each well was gently resuspended, avoiding the creation of air bubbles, and 120 µL was transferred from each well to one of the wells of a 96 well flat bottom plate. This plate was then incubated at 37 °C for 1 hour. This plate was then transferred to a dark place shielded from light and incubated at room temperature

for 24 hours. The plate was then read at 570 nm on an iMark microplate reader (Bio-Rad Laboratories).

Peptide pulsing – 800,000 FABF cells were resuspended in 500 µL and pulsed with either 1 or 10 µM of the peptide SIINFEKL in DMSO for one hour at 37 °C. After pulsing, cells were washed three times with 10 mL of PBS.

Crude DNA extraction and PCR – 700,000 FABF cells were resuspended in 180 µL of 50 mM NaOH and moved into a PCR tube which was then incubated at 95 °C for 10 minutes. 25 µL of 1 M Tris-HCl was then added to neutralize the solution. 5 µL of this lysate was added to a Terra™ Direct Red Dye Premix (Takara Bio) reaction with a 0.5 µM of each primer according to manufacturer recommendations. For positive controls, the higher volume of either 10 ng of DNA or 0.1 µL of stock solution was mixed in a Terra™ Direct Red Dye Premix reaction according to manufacturer protocols with 0.5 µM of each primer. The reactions were held in hot start mode for 10 minutes and subsequently cycled as follows: 2 min. @ 98 °C, 1 cycle; 10 sec. @ 98 °C and 15 sec. @ 56 °C and 2:30 @ 68 °C, 30 cycles; 4 °C hold. 5 µL of each reaction was then run on a gel with an agarose quantity sufficient to resolve bands of the expected size(s) at 90 V for an hour.

High quality DNA extraction and PCR – The DNA of between $1*10^6$ and $2*10^6$ FABF cells was extracted with a DNeasy® Blood and Tissue Kit (Qiagen) according to manufacturer protocols. The quantity of DNA was measured using a NanoDrop spectrophotometer. 400 ng of extracted genomic DNA or 10 ng of purified plasmid DNA was mixed with 0.5 µM of each primer and the remaining PCR components according to established Long Amplification AccuTaq manufacturer protocols

(Sigma Aldrich). Annealing temperatures were set at 5 °C below the lowest annealing temperature for any given set of reactions. The reactions were then cycled as follows: 2 min @ 98 °C, 1 cycle; 10 sec @ 96 °C and 15 sec @ annealing temperature and 2:30 @ 68 °C, 30 cycles; 4 °C hold, indefinitely. 5 µL of each reaction was then mixed with 5 µL of loading buffer and run on a gel with an agarose quantity sufficient to resolve bands of the expected size(s) at 90 V for an hour.

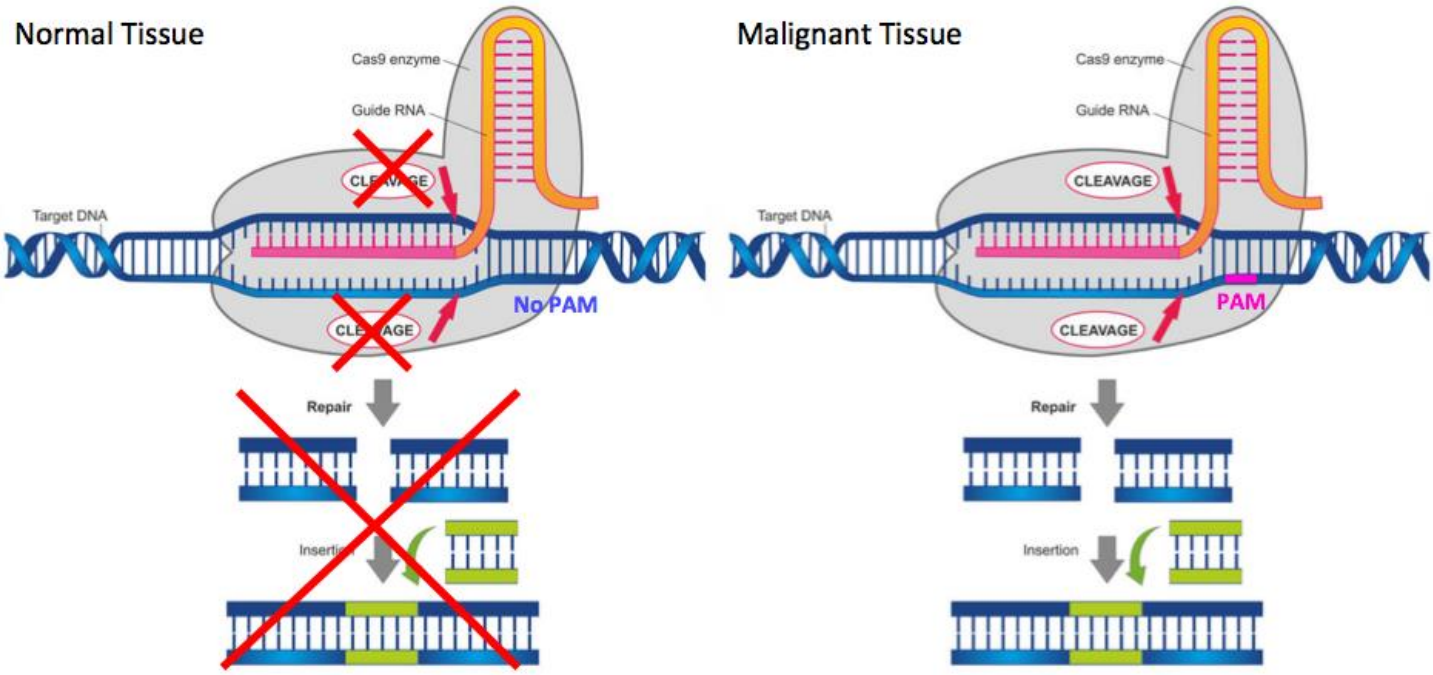


Figure 1: Strategy for Tumor-Specific Gene Insertion. In normal tissue, the sgRNA-Cas9 complex cannot cleave the target sequence because there is no PAM immediately 3' of the target site in the genomic DNA. In contrast, a tumor-specific mutation creates a neoPAM immediately 3' of the target site, allowing the same sgRNA-Cas9 complex to cleave the DNA and generate a DSB. An exogenous donor DNA sequence flanked with arms of homology to either side of the Cas9-generated DSB is used by the cell as a template for repair via homologous recombination, thereby precisely inserting the exogenous DNA into the malignant cell at the target locus. Of the available target sites, Target Sequence 17 (Target17) was chosen because of its low predicted levels of off-target effects (data not shown).

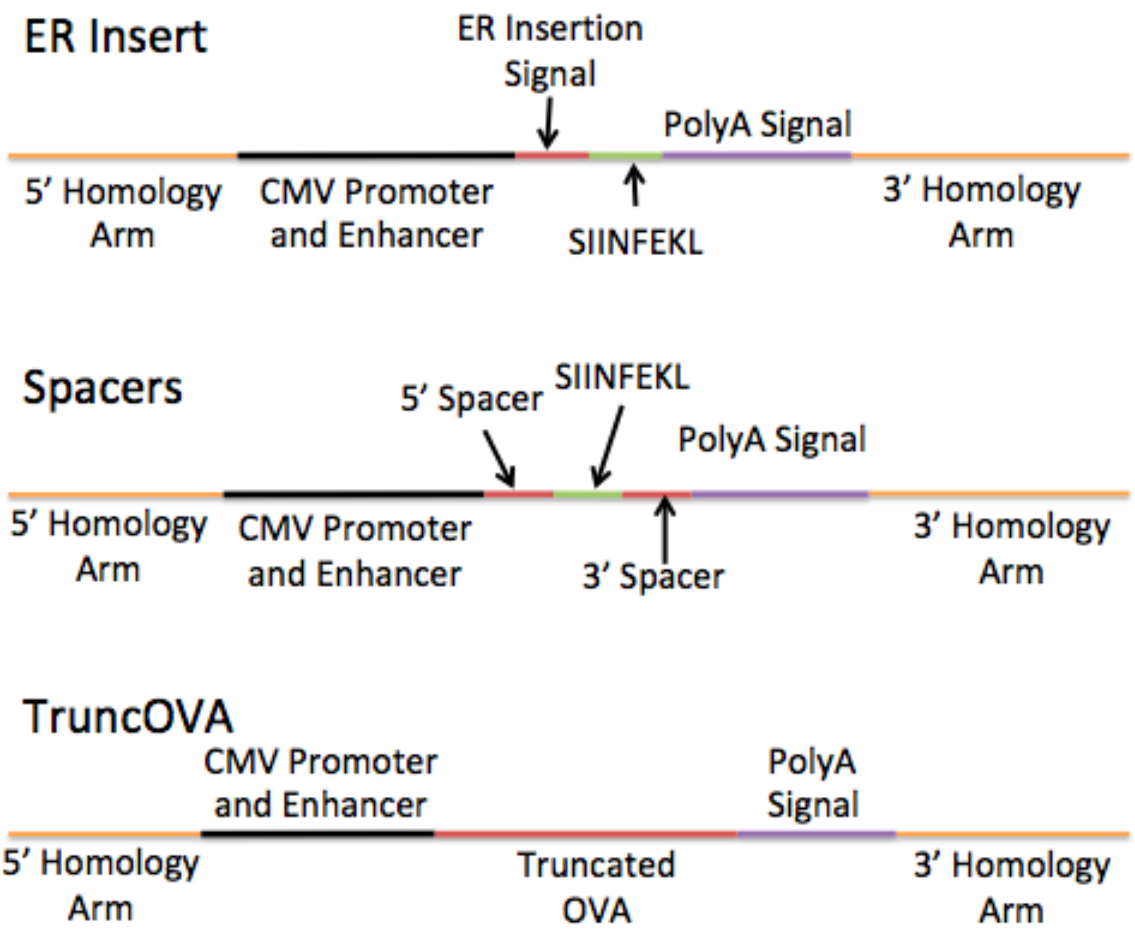
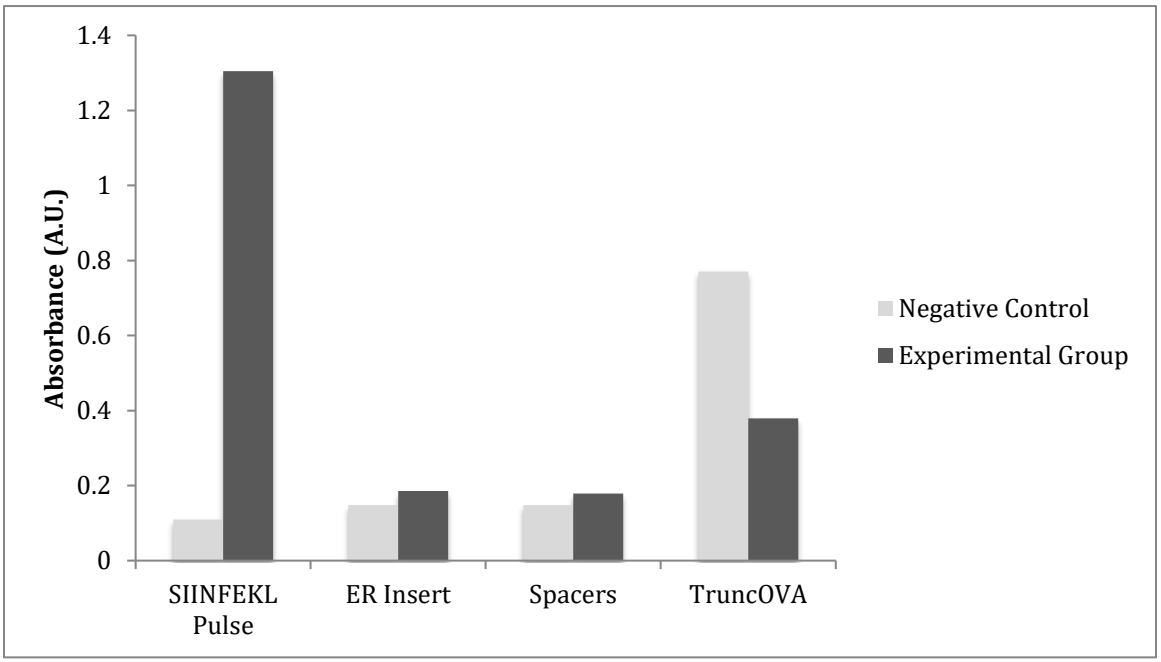


Figure 2: Composition of Donor DNA Constructs. Three different donor DNA constructs were designed and synthesized to identify an expression strategy that would maximize the expression of SIINFEKL, an immunogenic peptide from chicken ovalbumin that is recognized by a wide variety of well-characterized T cell hybridomas[89-91]. SIINFEKL was chosen as a model antigen because the wide array of SIINFEKL-specific hybridomas and transgenic T cell lines makes the detection of SIINFEKL expression in an immunologically relevant context methodologically simple[90,91].

In each construct, homology arms obtained from the sequences surrounding the sgRNA target site flank the minimal cytomegalovirus early/intermediate enhancer/promoter element and the SV40 polyadenylation signal. In the ER Insert sequence, the ER insertion signal from the E3/19K adenovirus protein was attached to the N-terminus of SIINFEKL to promote the insertion of the recombinant polypeptide into the ER where MHC Class I loading occurs and thereby enhance loading of SIINFEKL onto MHC Class I[92,93]. The ER insertion signal is not required for peptide loading onto MHC Class I, as in its absence cytosolic peptide will be transported to the lumen of the ER by the transporter associated with antigen processing (TAP)[94]. In the Spacers sequence, the 15 amino acids N-terminal to SIINFEKL and 11 amino acids C-terminal to SIINFEKL from the endogenous ovalbumin sequence were included in the minigene sequence to account for the possibility that the flanking sequences assist in peptide processing. Finally, the TruncOVA sequence is a truncated ovalbumin sequence without the first 100 amino acids. This polypeptide will not fold and will be degraded, releasing SIINFEKL into the cytosol where it should be transported into the ER, loaded on MHC Class I, and presented on the cell surface.

(a)



(b)

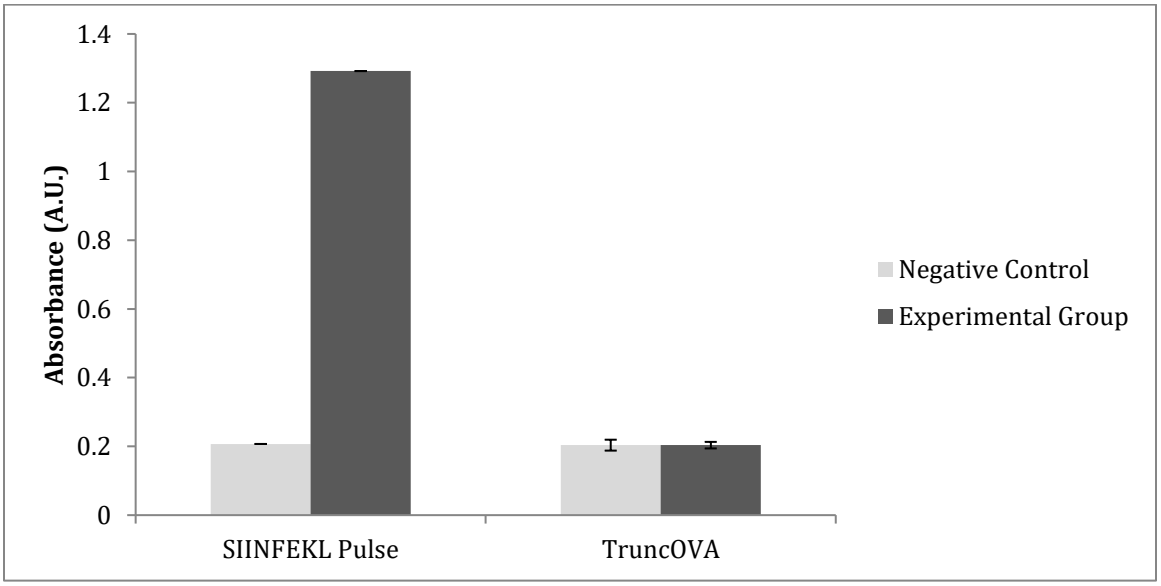
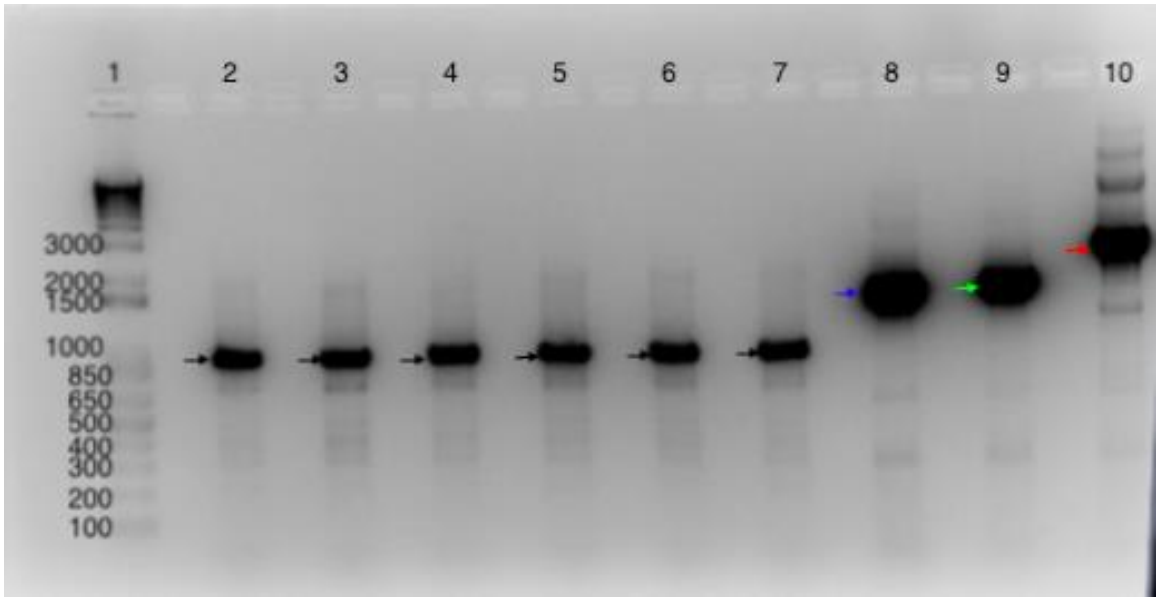


Figure 3: No Donor DNA Construct Drives SIINFEKL Presentation. (a) B3Z, a T cell hybridoma specific for SIINFEKL that produces LacZ upon TCR engagement[90], was cocultured with FABF cells transfected two days prior with

Target17 sgRNA, Cas9, and the indicated donor DNA constructs. Negative controls were FABF cells transfected with donor DNA alone. Naïve FABF was also pulsed with SIINFEKL and cocultured with B3Z cells as a positive control as tumor cells will take up peptide from the medium and present it on MHC Class I[94]. After a day of coculture, cells were lysed and the lysate incubated with CRPG, a yellow compound that is a substrate for β-galactosidase. CRPG turns red after cleavage by β-galactosidase, allowing detection of LacZ expression and hence TCR engagement by measuring the lysate's absorbance at 570 nm. While TruncOVA produced what appears to be a moderate positive signal, its corresponding negative control group's signal is higher. To account for the possibility the negative control group's signal was artifactual, this experiment was repeated. **(b)** The same experiment as in (a) except only TruncOVA was included in the assay and both the negative control and experimental groups were assayed in five replicates. SIINFEKL-pulsed and naïve FABF were only assayed in one replicate each. No signal was detected in either the TruncOVA negative control or experimental group, demonstrating that the signal seen in (a) was artifactual and no donor DNA constructs drive the presentation of SIINFEKL at the FABF cell surface.

(a)



(b)

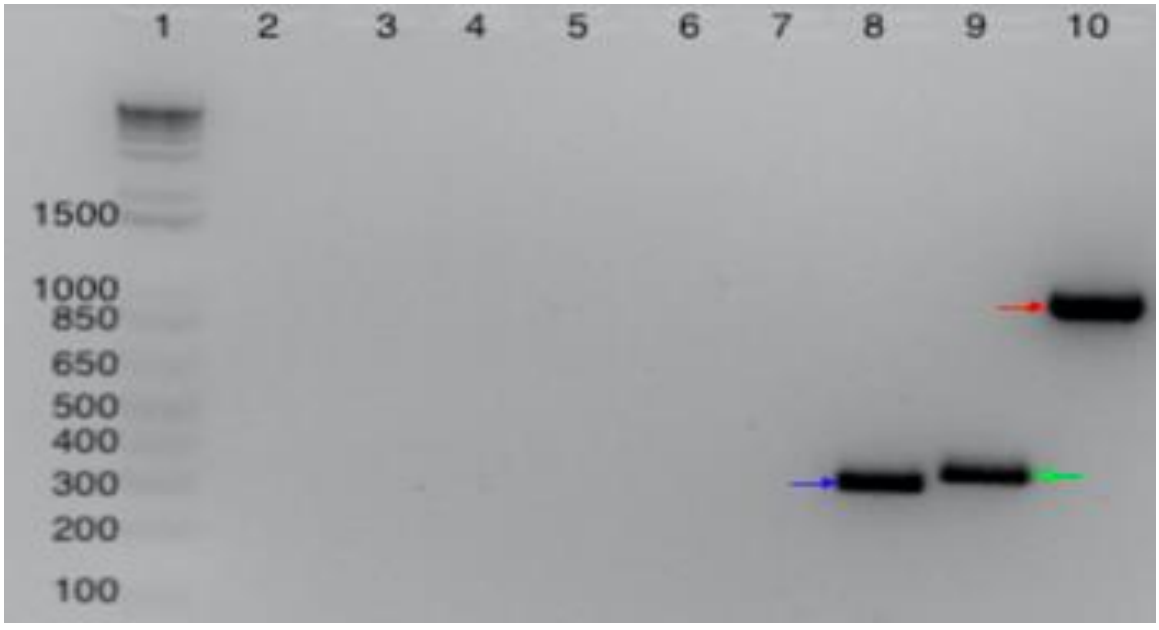


Figure 4: NeoPAM Knock-In with Target17 Does Not Mediate Construct Insertion at the Target Site. (a) A lack of presentation could have been because of no construct insertion, no expression, or a defect in presentation. To identify which

of these was the cause of the negative results in Figure 3, the homology arms included in the donor DNA constructs were amplified from crude extract of ER Insert, Spacers, and TruncOVA negative controls and experimental groups and from purified donor DNA as a positive control. Since the homology arms are present in both the donor DNA and FABF itself, amplification was expected to occur regardless of the success of construct insertion. No insertion was expected to produce a 927 bp band (black arrows), while insertion of ER Insert, Spacers, or TruncOVA was expected to produce a 1623 bp band (blue arrow), a 1650 bp band (green arrow), or a 2412 bp band (red arrow), respectively. In both the negative controls and experimental groups for each individual construct, no band above 927 bp in size can be detected, while positive controls produce bands of the expected size. Nonspecific bands are likely due to the crude extract and hypersensitive polymerase used for these experiments. (Lanes 1, 2, 3: ER Insert, Spacers, and TruncOVA negative controls, respectively; Lanes 4, 5, 6: ER Insert, Spacers, and TruncOVA experimental groups, respectively; Lanes 7, 8, 9) **(b)** To ensure that small amounts of inserted construct was not outcompeted by the more numerous endogenous sequence for polymerase, primers were designed to amplify from the 3' end of the CMV promoter to the 5' end of the SV40 polyadenylation signal. Because this sequence is only present in the donor DNA, successful amplification from FABF genomic DNA could only occur if the donor DNA construct was successfully inserted. No bands were observed for both the negative controls and experimental groups for each individual construct. In contrast, bands of 341 bp (blue arrow), 368 bp (green arrow), and

1130 bp (red arrow) are observed for amplification with ER Insert, Spacers, and TruncOVA donor DNA as template, respectively.

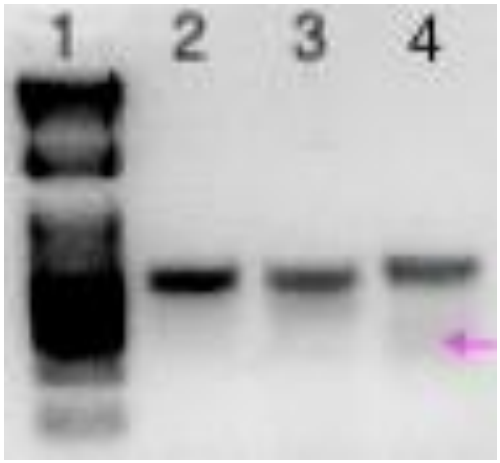


Figure 5: Target17 Is an Inefficient sgRNA. The Surveyor nuclease cleaves mismatched DNA, allowing quantification of the proportion of indels produced by Cas9 cleavage at a given target site[95,96]. The Target17 target site was PCR amplified and digested with the Surveyor nuclease. A distinct, smaller band alongside the parental band therefore indicates the presence of indels produced by Cas9-mediated target site cleavage. Greater intensity of the band representing the smaller cleavage product indicates higher sgRNA efficiency. No band indicative of cleavage by the Surveyor nuclease was observed in either a mock transfected control or FABF cells transfected with Cas9 and Target17. A smaller band indicative of cleavage (purple arrow) was observed in a positive control that came with the Surveyor nuclease kit. Taken together, these results demonstrate that Target17 is an inefficient sgRNA that does not significantly cleave its target site. Further, this result explains why no insertion was observed at the target site – without target site cleavage, the donor DNA cannot be used as a template for homology-directed repair.

Identifier	Wild Type PAM	NeoPAM	Target Sequence	Predicted Efficiency
TARGETSEQUENCE122	CAG	CGG	CAAGTTCAACGTGTGGGACA	9.0919
TARGETSEQUENCE11	AGA	AGG	GAGGCCAGCCTGGTCTATAG	9.07017
TARGETSEQUENCE29	TAG	TGG	CATCGCCAACTTCTCTGAGC	8.96595
TARGETSEQUENCE47	TGA	TGG	TCATGCAGGCATCCGACGAG	8.90553
TARGETSEQUENCE8	TTG	TGG	TCTCTATACCACTTCTCTAC	8.70791
TARGETSEQUENCE97	GAG	GGG	CGGCTGCGGCGAGCGGGGAG	8.67553
TARGETSEQUENCE209	GGT	GGG	TGCCGGCGACGGCTGGGTAT	8.61632
TARGETSEQUENCE15	CCG	CGG	AGAGTCCTGCCGCTTCAAGG	8.55223
TARGETSEQUENCE202	TGC	TGG	GTCCAAGAGCATGCAGTGAC	8.42117
TARGETSEQUENCE60	CGA	CGG	TTCTGGGGTCTGATGAGCGT	8.25255
TARGETSEQUENCE172	CTG	CGG	GTTTCCCGGAAGCTGCCCGG	8.17607
TARGETSEQUENCE134	TTG	TGG	TGATGTTTCCAGTTATTTAT	8.15388
TARGETSEQUENCE87	CTG	CGG	GGCGTCCCGCGGGCCGGCGC	8.14489
TARGETSEQUENCE115	GGC	GGG	TGGAGGCTGCACAGTGCACA	8.12777
TARGETSEQUENCE113	GTG	GGG	TTTTTTTGGTGTGTGTGTGT	8.10467
TARGETSEQUENCE61	TTG	TGG	CTATGCTTACTGTTCTGTTT	8.08406
TARGETSEQUENCE64	TGC	TGG	TGAAGAGGTCGTGGGGAATG	7.98259
TARGETSEQUENCE168	CAG	CGG	ATTCCCAAGCAATCCGACTC	7.97552
TARGETSEQUENCE111	AGA	AGG	GGAGCCTTCCTCTTTCTGTT	7.90524
TARGETSEQUENCE31	CAG	CGG	CCAGGACTGAGCCTGGGGAC	7.90438
TARGETSEQUENCE25	CAG	CGG	ACATCCATGGAGCTGTCAGC	7.88473
TARGETSEQUENCE152	AGC	CGG	ACAGGGAAGTGCCTGTCGTG	7.82484
TARGETSEQUENCE138	CTG	CGG	CGCCAGAAGCGAGAGCCCCT	7.8206
TARGETSEQUENCE46	CAG	CGG	GCAGTTAAACATGGGTCAGT	7.78494
TARGETSEQUENCE114	AGA	AGG	CTTTCAGAACGGGACCGCCG	7.59664
TARGETSEQUENCE49	CGC	CGG	GGTGGAAGGCCACGAGCGCT	7.59237
TARGETSEQUENCE193	TGC	TGG	CGGCCAGCTCCTGATGTTTC	7.57186
TARGETSEQUENCE39	CGA	CGG	GCCGGCTTGCCCGATTTCCG	7.56243
TARGETSEQUENCE109	AGT	AGG	CAAGGTCACCCTGGCTTACA	7.51026
TARGETSEQUENCE17	AGC	AGG	TCGAGGCCGTCTCTATAAGT	6.3988

Table 1: 29 sgRNAs Have High Predicted Cleavage Efficiencies. 29 putative sgRNAs have predicted efficiency scores above the high efficiency threshold of 7.5 according to the DRSC/TRiP CRISPR Efficiency Predictor tool. In addition, Target Sequence 17, an sgRNA designed as part of an older pipeline that did not take efficiency into account and did not mediate measurable gene knock-in

(data not shown), has a score of 6.3988, well below the high efficiency threshold. The six highest-scoring putative sgRNAs, hereafter referred to as sg1, sg2, sg3, sg4, sg5, and sg6 in order of decreasing efficiency score, were chosen to have their cleavage efficiencies evaluated *in vitro*.

DISCUSSION

The results here demonstrate the critical importance of incorporating sgRNA efficiency prediction and/or validation into sgRNA selection pipelines for any therapeutic modality involving personalized gene editing with CRISPR/Cas9. When efficiency is not taken into account, as was not the case with Target17 here, the likelihood of a sufficient number of editing events occurring to induce a phenotypic alteration or, presumably, a therapeutic response drops dramatically. Since both PCR and CRPG cleavage by B3Z-produced β-galactosidase are capable of massively magnifying small signals, it is highly unlikely that successful construct insertion and expression off that inserted construct occurred but was at too low of a level to be detected.

The immediate goal of this project moving forward will be to validate the cleavage efficiencies of sg1, sg2, sg3, sg4, sg5, and/or sg6. Once it has been demonstrated that neoPAMs are targetable with CRISPR/Cas9, designing a construct that can be used as a template in homologous recombination and can express SIINFEKL will be more of a technical challenge than a scientific one.

Therefore, the question of what effector polypeptide to express off the donor DNA construct is worth considering. Suicide genes seem the obvious candidate, as they are the best characterized and are capable of producing powerful bystander effects that can help overcome the low efficiency of any *in vivo* gene editing protocol. However, homologous recombination efficiency decreases markedly as insert size increases[97]. Because suicide genes tend to be large enzymes, any insert containing a suicide gene will be approaching the size limits of the CRISPR/Cas9 system. Thus,

the efficiency of suicide gene insertion may be too low to mediate a significant therapeutic effect, since small numbers of cells transfected will quickly be eliminated before they can generate enough toxic metabolites to exert a bystander effect.

Cytokines, in contrast, tend to be small genes that retain a potent antitumor effect when released intratumorally by broadly activating immune cells and promoting an inflammatory microenvironment. They are small enough that it may be possible to express multiple cytokines divided by self-cleaving peptides off a single promoter, thereby taking advantage of the combinatorial effects of multiple pro-inflammatory cytokines being released into the local milieu. In addition, because cytokines exert most of their cytotoxicity through immune cells that may or may not attack the cell from which the cytokines were initially secreted, these proteins are toxic to tumors overall but may not be as powerfully cytotoxic to individual cells. This effect, if real, may magnify the bystander effect by allowing cytokine-producing cells to remain in the tumor population longer.

B7 costimulatory ligands are also much smaller than suicide genes. Because their expression on tumor cells can permit naïve T cells to become primed by tumor cells directly without the need for an intermediary APC, they may be capable of unleashing powerful CTL- or NK cell-mediated responses that can go on to eradicate tumors locally and systemically. However, because they rely on CTLs and/or NK cells to mediate their antitumor effects (except for tumors with MHC Class II expression which can be controlled with CD4+ T cells alone), their insertion into tumor cells may only be effective for tumors with functional MHC Class I

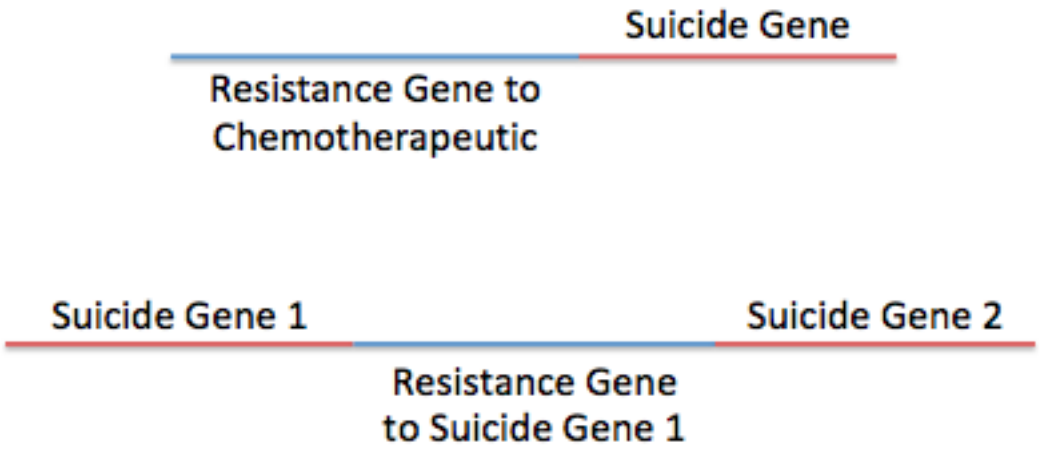
presentation machinery. Further, because they are membrane-bound proteins, they likely will induce primed CTLs and activated NK cells to quickly and efficiently kill successfully edited tumor cells, severely limiting how many tumor-specific CTLs can be primed by a single B7 costimulatory ligand-expressing tumor cell.

The two most significant challenges facing any *in vivo* gene editing strategy are delivery and efficiency[98]. Techniques associated with Cas9 delivery are outside the current scope of this project and hence will not be discussed here. The problem of efficiency, however, is potentially tractable. Because the efficiency of gene knock-in is inversely correlated with insert size, when choosing between two otherwise equivalently effective strategies, the one that involves a smaller insert should be chosen. More broadly, the problem associated with modifying tumor cells to express cytotoxic genes is that too few cells in the tumor may be modified to produce a bystander effect strong enough to destroy the tumor before the modified cells themselves are eliminated. In other words, modifying tumor cells to express cytotoxic genes produces a selective pressure that acts to remove those same tumor cells, thereby allowing the tumor to escape. A gene editing strategy that reversed the direction of the selective pressure to *favor* modified cells within the tumor might therefore overcome low insertion efficiency by gradually increasing the allele fraction of the modified locus in the tumor population.

There is, in fact, an analogous method used ubiquitously in molecular and cellular biology to purify strains with a desired modification – selection of antibiotic resistant clones. By linking the expression of a desired gene with an antibiotic resistance gene, antibiotic selection creates selective pressure to express the

desired gene. Thus, a construct that contains a resistance gene to a chemotherapeutic compound and a suicide gene that exerts cytotoxicity through a different mechanism than the chemotherapy might therefore enrich the tumor in cells expressing the suicide gene. Inducing the suicide gene in a tumor enriched in cells expressing the suicide gene would produce a significantly stronger bystander effect far more likely to be capable of completely destroying the tumor.

(a)



(b)

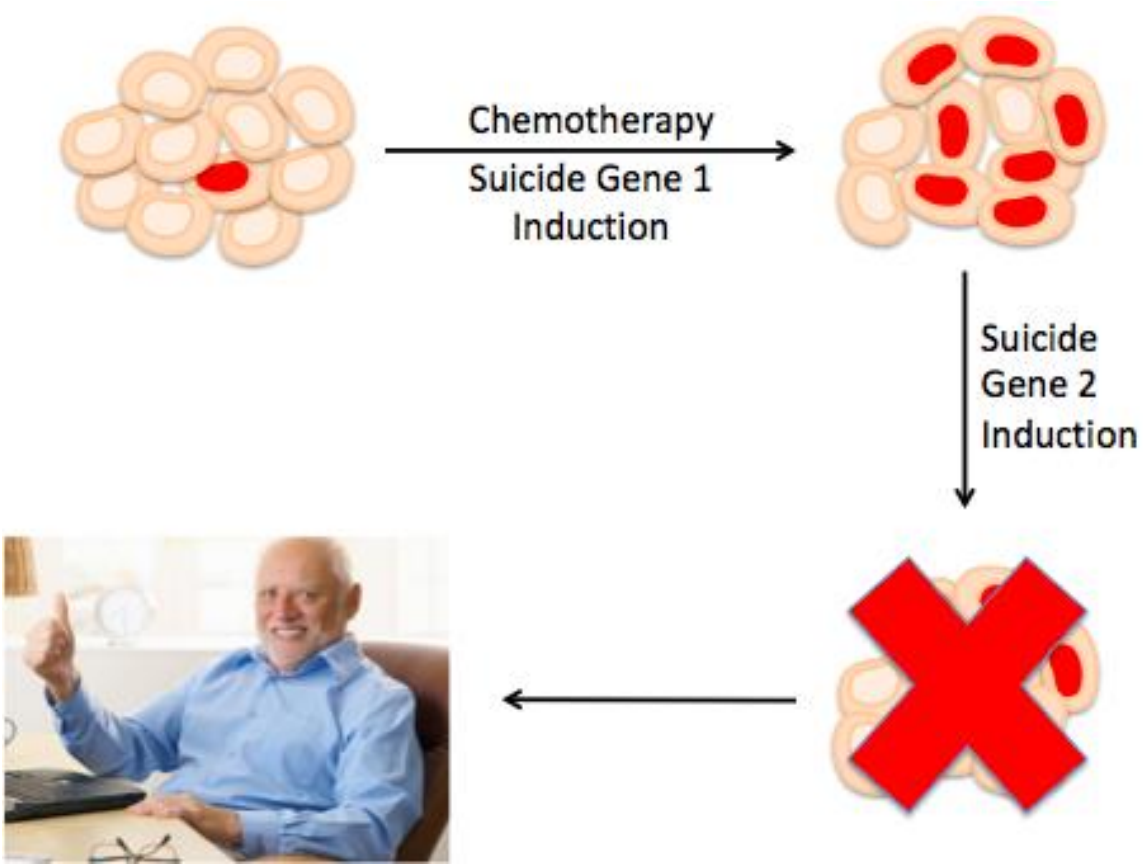


Figure 6: Model for Combining Resistance and Suicide Genes in Single Constructs to Direct Tumor Evolution. (a) Two different constructs potentially capable of directing tumor evolution by simultaneously providing resistance to one cytotoxic agent and sensitizing tumor cells to another are shown. The order of these genes in the construct is likely not relevant. These proteins could be expressed off the same promoter by linking the polypeptides with self-cleaving viral 2A peptides[99,100] or internal ribosome entry site (IRES) elements[101,102]. **(b)** At first, not many cells in a tumor are successfully edited. After the induction of the suicide gene to which resistance has been provided or the start of chemotherapy, cells expressing polypeptides from the construct grow unabated while the growth of unedited cells

is significantly inhibited. Once a sufficient proportion of the tumor contains the construct, the second suicide gene is induced, producing an overwhelming bystander effect that wipes out the tumor.

FUTURE DIRECTIONS

The most immediate priority is validating the cleavage efficiency of the six sgRNAs with the highest predicted cleavage efficiency. Cleavage efficiency validation will also be repeated with Target17 (sg7) to confirm it is inefficient, as the positive control cleavage band intensity was lower than expected. Once cleavage efficiency has been validated for the six sgRNAs, the highest efficiency sgRNA will be ordered. New homology arms will be identified from the sequences flanking the target site in the mm10 reference genome and used to design new donor DNA sequences as previously described.

The ability of these donor DNA constructs to generate SIINFEKL that can be presented on the FABF cell surface will be assayed as previously described and compared. Once the best donor DNA construct has been identified, the ability of edited FABF cells to elicit anti-SIINFEKL responses *in vivo* will be investigated. The magnitude of the bystander effect whereby anti-SIINFEKL responses lead to a generalized antitumor immune response will be quantified. These experiments will be repeated in a different tumor cell line that does not contain the targeted neoPAM to demonstrate that the editing event is truly restricted to cells harboring the mutation.

After demonstrating the effectiveness of neoPAM knock-in by inserting a SIINFEKL-producing construct into FABF, multiple different antitumor strategies outlined above will be investigated. In particular, TK007 and a bicistronic construct expressing interferon-γ and IL-2 will be knocked into tumor cells separately. Bulk edited cells will be used to form tumors in mice and their growth will be monitored

to assess the magnitude of the constructs' antitumor effects. Finally, a polycistronic construct will be generated that will express cytosine deaminase, the 5-FU metabolizing enzyme DPYD[103], and TK007. These cells will be used to form tumors in mice that will be treated with first with 5-FC and later with ganciclovir.

APPENDIX I: PREDICTED GUIDE RNA EFFICIENCIES OF TARGETABLE SNVS IN FABF EXOME

Identifier	Wild Type PAM	NeoPAM	Target Sequence	Predicted Efficiency
TARGETSEQUENCE122	CAG	CGG	CAAGTTCAACGTGTGGGACA	9.0919
TARGETSEQUENCE11	AGA	AGG	GAGGCCAGCCTGGTCTATAG	9.07017
TARGETSEQUENCE29	TAG	TGG	CATCGCCAACTTCTCTGAGC	8.96595
TARGETSEQUENCE47	TGA	TGG	TCATGCAGGCATCCGACGAG	8.90553
TARGETSEQUENCE8	TTG	TGG	TCTCTATACCACTTCTCTAC	8.70791
TARGETSEQUENCE97	GAG	GGG	CGGCTGCGGCGAGCGGGGAG	8.67553
TARGETSEQUENCE209	GGT	GGG	TGCCGGCGACGGCTGGGTAT	8.61632
TARGETSEQUENCE15	CCG	CGG	AGAGTCCTGCCGCTTCAAGG	8.55223
TARGETSEQUENCE202	TGC	TGG	GTCCAAGAGCATGCAGTGAC	8.42117
TARGETSEQUENCE60	CGA	CGG	TTCTGGGGTCTGATGAGCGT	8.25255
TARGETSEQUENCE172	CTG	CGG	GTTTCCCGGAAGCTGCCCGG	8.17607
TARGETSEQUENCE134	TTG	TGG	TGATGTTTCCAGTTATTTAT	8.15388
TARGETSEQUENCE87	CTG	CGG	GGCGTCCCGCGGGCCGGCGC	8.14489
TARGETSEQUENCE115	GGC	GGG	TGGAGGCTGCACAGTGCACA	8.12777
TARGETSEQUENCE113	GTG	GGG	TTTTTTTGGTGTGTGTGTGT	8.10467
TARGETSEQUENCE61	TTG	TGG	CTATGCTTACTGTTCTGTTT	8.08406
TARGETSEQUENCE64	TGC	TGG	TGAAGAGGTCGTGGGGAATG	7.98259
TARGETSEQUENCE168	CAG	CGG	ATTCCCAAGCAATCCGACTC	7.97552
TARGETSEQUENCE111	AGA	AGG	GGAGCCTTCCTCTTTCTGTT	7.90524
TARGETSEQUENCE31	CAG	CGG	CCAGGACTGAGCCTGGGGAC	7.90438
TARGETSEQUENCE25	CAG	CGG	ACATCCATGGAGCTGTCAGC	7.88473
TARGETSEQUENCE152	AGC	AGG	ACAGGGAAGTGCCTGTCGTG	7.82484
TARGETSEQUENCE138	CTG	CGG	CGCCAGAAGCGAGAGCCCCT	7.8206
TARGETSEQUENCE46	CAG	CGG	GCAGTTAAACATGGGTCAGT	7.78494
TARGETSEQUENCE114	AGA	AGG	CTTTCAGAACGGGACCGCCG	7.59664
TARGETSEQUENCE49	CGC	CGG	GGTGGAAGGCCACGAGCGCT	7.59237
TARGETSEQUENCE193	TGC	TGG	CGGCCAGCTCCTGATGTTTC	7.57186
TARGETSEQUENCE39	CGA	CGG	GCCGGCTTGCCCGATTTCCG	7.56243
TARGETSEQUENCE109	AGT	AGG	CAAGGTCACCCTGGCTTACA	7.51026
TARGETSEQUENCE48	AGT	AGG	TTCCATGGACCGATGGACTG	7.49501
TARGETSEQUENCE165	GCG	GGG	CACGAGCGGCGCCGGGGAGT	7.48202
TARGETSEQUENCE118	GGC	GGG	AAGACAAGGGCTCTGATGGG	7.47718
TARGETSEQUENCE28	GGA	GGG	GTCCTGTTCCCATTGATGGT	7.35056
TARGETSEQUENCE184	AGC	AGG	AGCAAGCAGCCTACCAGGCT	7.329
TARGETSEQUENCE207	AGA	AGG	TGGACAGTGGCCATGGAAGT	7.31052
TARGETSEQUENCE18	TGT	TGG	CATGGAAGACTCGATGGATA	7.24548
TARGETSEQUENCE99	TTG	TGG	TCTGTTGGCCGAGGTGAGTC	7.21284

TARGETSEQUENCE179	AGC	AGG	CATACCAGGCTTTGAAAGGC	7.18283
TARGETSEQUENCE176	TGA	TGG	CCTCCTGTTATTATGGGGTC	7.17312
TARGETSEQUENCE86	CGT	CGG	GGGGTGGGGGGTGGGGGACC	7.16004
TARGETSEQUENCE57	CCG	CGG	GGCAAGAAGCGCAAGCGCAG	7.14979
TARGETSEQUENCE7	TGC	TGG	ACAAGTGTACTAGCAGTCTG	7.13917
TARGETSEQUENCE90	CTG	CGG	CTAGGCATTCCTCCCTCCCT	7.13758
TARGETSEQUENCE12	GTG	GGG	CTCTGGAGGACAGTCCATCA	7.12872
TARGETSEQUENCE27	AGC	AGG	CATGGTCTCCAGCCTGGCCC	7.10614
TARGETSEQUENCE100	GGC	GGG	GGGCGGGCGGAGGCCGCAGC	7.0806
TARGETSEQUENCE156	TGT	TGG	CTTCCGGCTCTCTACATGCT	7.04131
TARGETSEQUENCE131	AAG	AGG	CCACCAAGTCACGCACCAAA	7.01241
TARGETSEQUENCE162	CGC	CGG	TCCCGTGGCCATCATACCCC	6.99684
TARGETSEQUENCE145	TGC	TGG	TTGCTAATTACCGTCTCTTC	6.98089
TARGETSEQUENCE91	CAG	CGG	CGCTGGCAGAACGAGAAGAG	6.96332
TARGETSEQUENCE82	AGC	AGG	TTCTCCCCGATGCTGGACAC	6.95848
TARGETSEQUENCE101	GGA	GGG	CCGGGGACGGAGGGGAGAGC	6.90135
TARGETSEQUENCE70	TGC	TGG	GCTCTCCTCGGGGTCAATGA	6.85213
TARGETSEQUENCE20	CAG	CGG	GAGCCAGGGATAATCTGAGT	6.84996
TARGETSEQUENCE53	AGC	AGG	TTCTTCTTTGATTTCTTTTC	6.81477
TARGETSEQUENCE112	GGC	GGG	GAAGCGGCCCATAAAGGGGG	6.81466
TARGETSEQUENCE85	GGA	GGG	GTGGGGGACCCGTCGTCTGT	6.77051
TARGETSEQUENCE169	GGA	GGG	GCTCGGCTCTTCCTGGAGTC	6.74216
TARGETSEQUENCE79	AAG	AGG	TCTGTTACGCAGAGTTAAGA	6.73532
TARGETSEQUENCE35	ATG	AGG	GCACGCACACAGTCCATGGC	6.73034
TARGETSEQUENCE208	CAG	CGG	AGTTGTTACACACTCCTTAG	6.69514
TARGETSEQUENCE158	TTG	TGG	TATTAAGATGGTCTCCCAGC	6.68536
TARGETSEQUENCE30	AGC	AGG	ACCTTATCCCCAGGCGGAAC	6.67683
TARGETSEQUENCE210	CAG	CGG	TGGAGCGTGGGGACCATACC	6.57391
TARGETSEQUENCE181	TTG	TGG	CATTATCTGCCCTAAGATGT	6.54838
TARGETSEQUENCE124	CAG	CGG	CTGGTGGGCGACGGCAGCAC	6.52535
TARGETSEQUENCE155	CCG	CGG	CGAGGAGCCCGACCTCCCCC	6.51818
TARGETSEQUENCE10	AAG	AGG	AAGTGGATCTGGCTGAAGTG	6.49663
TARGETSEQUENCE175	CTG	CGG	GTCACTGTCGCCTTGATTTC	6.49193
TARGETSEQUENCE201	GGC	GGG	TCCCTCCTCAGGGTTCCCCT	6.48417
TARGETSEQUENCE133	CAG	CGG	AACGGAATGGATGAAGGTTA	6.48375
TARGETSEQUENCE166	CAG	CGG	GGCGCGCCGGGGGCCGCGAC	6.47819
TARGETSEQUENCE105	GTG	GGG	CCGTGTCCCCGCCGCCGCCA	6.46101
TARGETSEQUENCE38	GAG	GGG	AGTCGCCCAAGAGGGCTGAG	6.43311
TARGETSEQUENCE17	AGC	AGG	TCGAGGCCGTCTCTATAAGT	6.3988
TARGETSEQUENCE40	AGA	AGG	AACCCGCGACCGGCACCGAC	6.36686
TARGETSEQUENCE192	CGA	CGG	TGCGGCGACCGCTCTTGTGA	6.35608
TARGETSEQUENCE147	GGA	GGG	CGTGGCGGCGCTGCTGGCCG	6.34633
TARGETSEQUENCE174	AGC	AGG	GTACTGGTTTTCCTCCCAGC	6.31684
TARGETSEQUENCE76	CAG	CGG	GGGAGACTGAGGGCCCGGCC	6.28109

TARGETSEQUENCE62	CTG	CGG	AATGCAGCATCTGCGATGTG	6.25101
TARGETSEQUENCE203	GGC	GGG	GTCACTAGGGATGCAATGGG	6.23212
TARGETSEQUENCE160	CAG	CGG	TTTCCCATGAACAAAAGAAC	6.22627
TARGETSEQUENCE4	TTG	TGG	CATCCACTTCCTTCAGTGTA	6.22058
TARGETSEQUENCE66	AAG	AGG	ACCTGAAACCCCAAAGCAAA	6.20832
TARGETSEQUENCE173	TTG	TGG	GTAGCCTCTTGCATCAGCTC	6.18684
TARGETSEQUENCE65	AAG	AGG	TGAGAACCAACTTTCCCAAC	6.18632
TARGETSEQUENCE125	CAG	CGG	CTCGTCCTGGTGGGCGACGG	6.17893
TARGETSEQUENCE96	CGC	CGG	GGGGCCGTCGTGTTTCGGGT	6.12515
TARGETSEQUENCE80	CAG	CGG	GCTCTAAGGGCTGGGTCTGA	6.12091
TARGETSEQUENCE13	CCG	CGG	TGAGTGTCCCATCCTGAGCT	6.11728
TARGETSEQUENCE44	CAG	CGG	CAAGGCACGTGCCCCTCTCT	6.09946
TARGETSEQUENCE170	CTG	CGG	TGAAACCGTTAAGAGGTAAA	6.0761
TARGETSEQUENCE144	GGC	GGG	CTCACTAGTCTTCCCCAAGT	6.06224
TARGETSEQUENCE83	CCG	CGG	GCATGACTGGAGAGGCCAGC	6.05279
TARGETSEQUENCE204	ACG	AGG	CAGCGTGCACTGAAAAGAGG	6.02901
TARGETSEQUENCE187	TAG	TGG	TTCTTCCTCTTCATTCGCCC	5.99791
TARGETSEQUENCE127	CGC	CGG	AAGGACTCCTGCTTCTGCTT	5.9431
TARGETSEQUENCE129	CAG	CGG	CGCGGCCCGGACCGTCGCGG	5.9013
TARGETSEQUENCE69	CAG	CGG	GCCACCTCCATCTTGTTGTG	5.88761
TARGETSEQUENCE74	ATG	AGG	TTAATGAGCCTCCGAAGAGT	5.86874
TARGETSEQUENCE58	AGC	AGG	ACTTCTTGGAACAATGTGTC	5.85862
TARGETSEQUENCE67	AGC	AGG	CCAGTTTAAGAGTTGGGTGA	5.78629
TARGETSEQUENCE183	TCG	TGG	TAGGTGGAAGTCTTCTACTC	5.77796
TARGETSEQUENCE81	GCG	GGG	GTAGATGGACCGAAGGATCA	5.74471
TARGETSEQUENCE104	GAG	GGG	ACACACAAGACGGGGAGAGC	5.71338
TARGETSEQUENCE77	CTG	CGG	CACGTCTGAACTTTGGGAGA	5.70015
TARGETSEQUENCE6	TAG	TGG	CTGCAATCAGCACTGGCTCT	5.67779
TARGETSEQUENCE199	AAG	AGG	TGTTGGTTGATATAGACAGC	5.62602
TARGETSEQUENCE157	CTG	CGG	TTTGAGTTTGCTGGGGGAAG	5.62123
TARGETSEQUENCE45	CAG	CGG	CGGGTTCAGATCCCCAAATC	5.5684
TARGETSEQUENCE102	CAG	CGG	CGGGTTTTGTCGGACCCCGA	5.5511
TARGETSEQUENCE23	CGC	CGG	AGCTCTTACCGTTGTCACAC	5.53652
TARGETSEQUENCE151	TCG	TGG	AAAGGACAGGGAAGTGCCTG	5.52926
TARGETSEQUENCE191	CCG	CGG	CGAAGTTGGTGGGGAAAAAG	5.52594
TARGETSEQUENCE185	CTG	CGG	GGCAGCTACTCTCTTCACAT	5.49995
TARGETSEQUENCE136	CAG	CGG	AACGGGCTTGGCGGAATCAG	5.44758
TARGETSEQUENCE188	CAG	CGG	CCTGAGTTCGTAGGCGCAGC	5.43718
TARGETSEQUENCE182	TGC	TGG	CATCTTGGCTCCGCTCTGAC	5.37512
TARGETSEQUENCE68	TGA	TGG	CTGCTCCTCGTCTCGGCCTC	5.25009
TARGETSEQUENCE132	CCG	CGG	GAAGACGGGTCAGCTGAGGT	5.21881
TARGETSEQUENCE72	ATG	AGG	TCATGGCGCACAGCCTCTGG	5.21703
TARGETSEQUENCE200	GGA	GGG	AGGAAGTCTCTTTAAGAAAA	5.20918
TARGETSEQUENCE205	AAG	AGG	CTTTCCCTTTTTTCCAAAGG	5.2061

TARGETSEQUENCE141	GGC	GGG	TAGGCAGAATTTGCTCCCCT	5.1969
TARGETSEQUENCE51	TCG	TGG	CGTGCATGAGAAAAGAGCCG	5.18665
TARGETSEQUENCE56	CGC	CGG	TTTGAGGACACCAACCTGTG	5.13792
TARGETSEQUENCE164	ACG	AGG	GGCGGTCTTGGCCAGGTGGC	5.13594
TARGETSEQUENCE5	GGA	GGG	AACCATGCGGTAGCTCTTGC	5.13309
TARGETSEQUENCE78	TGT	TGG	CGGGAAAACGCAGGGTTGTA	5.13107
TARGETSEQUENCE110	AGC	AGG	TGTGGCGTTATGTATACTAA	5.12674
TARGETSEQUENCE137	GAG	GGG	CCGACCCCGACGCGAGGACG	5.11605
TARGETSEQUENCE161	CCG	CGG	TCTGAAGCAGATAGACCAGG	5.08964
TARGETSEQUENCE94	CTG	CGG	GAACTCGGAGCGGGGAGGCG	5.08578
TARGETSEQUENCE36	ACG	AGG	GTCCCCAGGTTCCCAGGGTC	5.0781
TARGETSEQUENCE92	GGA	GGG	CGGAGGCACACCAGGGAATG	5.0759
TARGETSEQUENCE98	GAG	GGG	GGTCAGTCAGAGGAGAGGGG	5.06516
TARGETSEQUENCE143	CAG	CGG	CCCAGAAAGGCAACGTGACT	5.06346
TARGETSEQUENCE108	TAG	TGG	TTCAGTCATAATCCCACAGA	5.02782
TARGETSEQUENCE189	CCG	CGG	AGGAATGCCAGTCGCAGCTG	5.01306
TARGETSEQUENCE128	CAG	CGG	GGGGGCATCGGCAGAAGGGG	5.00173
TARGETSEQUENCE153	TGC	TGG	ATGGTTCCGATGCCCCACAT	4.98881
TARGETSEQUENCE89	AGA	AGG	AGCGGCCGCGTGCGGCGCAA	4.93993
TARGETSEQUENCE171	CAG	CGG	AACGATGCCGACTGGTGATG	4.89998
TARGETSEQUENCE194	AAG	AGG	TTCAGCTCATACTCGGGTGG	4.89753
TARGETSEQUENCE34	CGC	CGG	GAACTCCTCGAAGCCCAGCA	4.89131
TARGETSEQUENCE54	CCG	CGG	GCTCCTGCGACCTGCTGCTA	4.82327
TARGETSEQUENCE148	CAG	CGG	CACCTAGTGGTGACAAGTTT	4.80408
TARGETSEQUENCE52	AGC	AGG	CTTCTTCTATTTCCTTTTCC	4.77708
TARGETSEQUENCE32	GCG	GGG	TTACCATCACCATCGGAGCA	4.77594
TARGETSEQUENCE146	CCG	CGG	CTAGAAGCAAGTGAAGCCGA	4.75457
TARGETSEQUENCE135	GGT	GGG	GAGAAAGACATAGATGTGCC	4.73426
TARGETSEQUENCE71	CCG	CGG	GCCACCTCTTCTGCCTGCAG	4.72597
TARGETSEQUENCE21	CGC	CGG	GACACACTGAAGTCCAAGTA	4.72112
TARGETSEQUENCE139	GGC	GGG	AGAGGGGAAGAGTAGAAAGA	4.71344
TARGETSEQUENCE19	AAG	AGG	GGAAAGCGATCTGCTCCTGG	4.70543
TARGETSEQUENCE163	CTG	CGG	CTGGTCGTTGCGACTTCGTC	4.6899
TARGETSEQUENCE24	TGC	TGG	GGATATGCAGGAGCTGGAAG	4.68434
TARGETSEQUENCE59	AGA	AGG	CTTCTACTATGACCAGTGCG	4.67399
TARGETSEQUENCE149	AGC	AGG	ACCTAGTGGTGACAAGTTTC	4.65943
TARGETSEQUENCE197	TGA	TGG	ATTTTCACAGCTAGTTGATT	4.65266
TARGETSEQUENCE121	AGC	AGG	GTCTCTCCCTGGGGACTCCG	4.62262
TARGETSEQUENCE117	AAG	AGG	ACCATACACTCATTAAAAGA	4.60744
TARGETSEQUENCE93	TCG	TGG	GTCTCTCCCGAATGGTCCCC	4.60179
TARGETSEQUENCE42	AGC	AGG	CCCTTGAAAATCCGGGGGAG	4.58036
TARGETSEQUENCE22	TTG	TGG	GCGCATTTTTGTGGTGGGTT	4.50944
TARGETSEQUENCE107	CAG	CGG	GAGCTTTGGAAATCCCAGCG	4.49144
TARGETSEQUENCE167	GGC	GGG	GCCCCCGGCGCGCCGGGCTC	4.46064

TARGETSEQUENCE154	GGT	GGG	CACATGAACGGGCGGTCTCC	4.44428
TARGETSEQUENCE73	TGC	TGG	ATGATGGTTTTCCGAAGCTT	4.43532
TARGETSEQUENCE130	CCG	CGG	CACCCACTCTCTCGCTGCTC	4.43252
TARGETSEQUENCE177	CGC	CGG	AGGTTTTTCTCCGATTCAGA	4.43023
TARGETSEQUENCE195	AGC	AGG	GCTCGTGGAAGATGTCTTCC	4.4277
TARGETSEQUENCE9	TGA	TGG	GGCCAGTGTCCCTCTCACAC	4.42154
TARGETSEQUENCE95	AAG	AGG	CACGGACCCTCTCGACCCCG	4.37757
TARGETSEQUENCE26	TGA	TGG	AGCCTGACCTCCCAGGAGAG	4.33787
TARGETSEQUENCE159	TGC	TGG	GGGAGATGGGGCCCAAATCA	4.33521
TARGETSEQUENCE41	CCG	CGG	GGTTCGGGAGCGGAAAAAGA	4.31398
TARGETSEQUENCE190	GCG	GGG	CTCCTTGATGGCTGGTGTAA	4.31297
TARGETSEQUENCE178	GCG	GGG	ACCTTCTTCACCCAGCCTAA	4.31239
TARGETSEQUENCE33	TCG	TGG	GAGAGCCTTGGGCACCAGAA	4.25377
TARGETSEQUENCE84	GGC	GGG	CTGTGGATTGTGCGTCCCGG	4.24152
TARGETSEQUENCE150	TGA	TGG	CAAAGCTCCTGGTCCCCGGT	4.23858
TARGETSEQUENCE43	CGT	CGG	CCATGACGCTTTCCAAGGCA	4.22658
TARGETSEQUENCE180	TCG	TGG	GGATCCCGTACTTCTCCAGC	4.20347
TARGETSEQUENCE1	TGA	TGG	GAATAACATTGAGAATTTAA	4.20293
TARGETSEQUENCE119	TGC	TGG	CCAGCCTGAGAGTATCTCCC	4.15624
TARGETSEQUENCE103	CAG	CGG	AACGGGAGAGTGCATGCGGC	4.12085
TARGETSEQUENCE37	TGC	TGG	AAGCCTGCAGTGAACCTCCA	4.06205
TARGETSEQUENCE196	CAG	CGG	TCCAGCACCATCCATTTTCA	4.0254
TARGETSEQUENCE14	TGC	TGG	GGCCAGCCAGGGAGGCTCCC	4.00327
TARGETSEQUENCE123	TAG	TGG	GAACTTGATGGGTCCTCTGT	3.93871
TARGETSEQUENCE126	CGC	CGG	GTGATTCCCATCAAAAGTGC	3.9386
TARGETSEQUENCE63	CAG	CGG	TGTCTTTTTGGATGGCGTCA	3.92324
TARGETSEQUENCE120	CCG	CGG	TCCCCCTCCTCCACGTGAGT	3.88766
TARGETSEQUENCE140	TGA	TGG	GTGGGTGATGTCTGTGAAGA	3.84887
TARGETSEQUENCE198	TGT	TGG	GGTTGATATAGACAGCAAGA	3.77935
TARGETSEQUENCE116	TAG	TGG	ATGCTCCCTTCCCCTTACTG	3.76858
TARGETSEQUENCE88	GGC	GGG	CAGAGATGAAGAAAAGCCAA	3.76649
TARGETSEQUENCE106	ATG	AGG	GCTTCTTATACTTGAGGTCC	3.75764
TARGETSEQUENCE75	GAG	GGG	TGGTGAACTATGCCTGGGCA	3.72089
TARGETSEQUENCE142	CAG	CGG	TAGTCTGGTGCTTCTGAGGC	3.68784
TARGETSEQUENCE2	AAG	AGG	TGGCTTTAATCTCCTTCAGC	3.64992
TARGETSEQUENCE186	TGC	TGG	TCCCAGGCTGGGAGTCCCAT	3.64314
TARGETSEQUENCE16	CCG	CGG	GCCAATGGTGCCAGAATTCA	3.54109
TARGETSEQUENCE3	TAG	TGG	GGAATTGAATGAACACAGCC	3.4996
TARGETSEQUENCE55	CAG	CGG	GCAGAACTTATTTGAGAGGC	3.43438
TARGETSEQUENCE50	AAG	AGG	AAAAAAAAAATCCAAAGCCG	3.33545
TARGETSEQUENCE206	CAG	CGG	TAAAAATCATTAAAAATGGA	2.67133

APPENDIX II: DONOR DNA, SGRNA, AND PRIMER SEQUENCES

ER Insert:

>5' homology arm (482)
GTTACCAGGCTATCCCTAGACCTTGCCATAACTCCTGGCACATAGGGGATGCTCACACAA
GCCTATTAACTTTGTAAAGCAAAATTTCATGGGACGTTTCATAATGTGAATTTCTCCTA
AAATAAGTCAGAAGCAAAACCAGCAAAAGAGGCAGCATTTCTTTATTACCTGCAATTAG
TCATTTGCATGGTACAAAGAGGTGAAAAGTTTCAGATACATGATTTGGATGAATTGATT
TTTAAAGATTTTACTTTTATTATTTTTACTGTGTGTGTGCATACATGTGTGTGTATGCAC
TCCTGTGCATGTGTGTGCACACACAGGCACCTACTTGAGTATAGGTGCCTACAAAATCTG
GAAGACAACACTGGATTCCTGGTAGCTGGAATCACCGAAGGCTGTGATACAGGTGCTGG
GGAGTCCTTTACCAATGAGCTGCATCTCAGGATCTGGGGCACGCCTTCTCACAGAGGACA
CTGTCC

>CMV enhancer (304)
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATT
GACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCC
AAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTA
CATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC
CATG

>CMV promoter (204)
GTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATT
TCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGA
CTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACG
GTGGGAGGTCTATATAAGCAGAGCT

>Kozak sequence (9)
GCCGCCACC

>E3/19K ER insertion signal with N-terminal Met (51)
ATGAGGTACATGATCCTGGGCCTGCTGGCCCTGGCCGCCGTGTGCAGCGCC

>SIINFEKL (24)
AGCATCATCAACTTCGAGAAGCTG

>Stop codon (3)
TGA

>SV40 PolyA Signal (122)
AACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCAC
AAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATC
TTA

>3' homology arm (424)
GGTTTCTGATGTACTCCCGGTACATGGCAGTGTCCTCTTTGCCAAGGGGCTGCATGATAC
CTTGGCTAGCTTGGATATGGGCCTGGAAGATCTCTGTAGACTTTCTGTCTACTCTTGGAG
GCTGAACTTCATAAATGTTGTCTTGCGAGAAAGCTGAGATGGGAGTTCTGTAAAAGAGG
AAAGACAGGACTGTGTTATGGAACTTCTCACCACAACATGGGCTTCACCAACCAAGGGCA
ACCTCTGCAGTTGTTACTTCCTGGGAGGCCAAAGGATAGAAGCTGTCCCTGATACCCAGG
CAGGGCAGAGGTCCTGTCCCTTATAGAATGTCAGCTCTATAGTTGCGTGTCCTGCTGGCT
CCCACTGGAAGAAGCTTCAGGTGGTACTATACCTAAGTTTTGTTCCTTGTCCTTGCAGGC
TCTTG

Spacers:

>5' homology arm (482)
GTTACCAGGCTATCCCTAGACCTTGCCATAACTCCTGGCACATAGGGGATGCTCACACAA
GCCTATTAACTTTGTAAAGCAAAATTTCATGGGACGTTTCATAATGTGAATTTCTCCTA
AAATAAGTCAGAAGCAAAACCAGCAAAAGAGGCAGCATTTCTTTATTACCTGCAATTAG
TCATTTGCATGGTACAAAGAGGTGAAAAGTTTCAGATACATGATTTGGATGAATTGATT
TTTAAAGATTTTACTTTTATTATTTTTACTGTGTGTGTGCATACATGTGTGTGTATGCAC
TCCTGTGCATGTGTGTGCACACACAGGCACCTACTTGAGTATAGGTGCCTACAAAATCTG
GAAGACAACACTGGATTCCTGGTAGCTGGAATCACCGAAGGCTGTGATACAGGTGCTGG
GGAGTCCTTTACCAATGAGCTGCATCTCAGGATCTGGGGCACGCCTTCTCACAGAGGACA
CTGTCC

>CMV enhancer (304)
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATT
GACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCC
AAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTA
CATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC
CATG

>CMV promoter (204)
GTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATT
TCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGA
CTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACG
GTGGGAGGTCTATATAAGCAGAGCT

>Kozak sequence (9)
GCCGCCACC

>SIINFEKL plus spacer (102)
ATGGCCGTGCTGCTGCCCGACGAGGTGAGCGGCCTGGAGCAGCTGGAGAGCATCATCAAC
TTCGAGAAGCTGACCGAGTGGACCAGCAGCAACGTGATGGAG

>Stop codon (3)
TGA

>SV40 PolyA Signal (122)
AACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCAC
AAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATC
TTA

>3' homology arm (424)
GGTTTCTGATGTACTCCCGGTACATGGCAGTGTCCTCTTTGCCAAGGGGCTGCATGATAC
CTTGGCTAGCTTGGATATGGGCCTGGAAGATCTCTGTAGACTTTCTGTCTACTCTTGGAG
GCTGAACTTCATAAATGTTGTCTTGCGAGAAAGCTGAGATGGGAGTTCTGTAAAAGAGG
AAAGACAGGACTGTGTTATGGAACTTCTCACCACAACATGGGCTTCACCAACCAAGGGCA
ACCTCTGCAGTTGTTACTTCCTGGGAGGCCAAAGGATAGAAGCTGTCCCTGATACCCAGG
CAGGGCAGAGGTCCTGTCCCTTATAGAATGTCAGCTCTATAGTTGCGTGTCCTGCTGGCT
CCCACTGGAAGAAGCTTCAGGTGGTACTATACCTAAGTTTTGTTCCTTGTCCTTGCAGGC
TCTTG

TruncOVA:

>5' homology arm (482)
GTTACCAGGCTATCCCTAGACCTTGCCATAACTCCTGGCACATAGGGGATGCTCACACAA
GCCTATTAACTTTGTAAAGCAAAATTTCATGGGACGTTTCATAATGTGAATTTCTCCTA
AAATAAGTCAGAAGCAAAACCAGCAAAAGAGGCAGCATTTCTTTATTACCTGCAATTAG
TCATTTGCATGGTACAAAGAGGTGAAAAGTTTCAGATACATGATTTGGATGAATTGATT
TTTAAAGATTTTACTTTTATTATTTTTACTGTGTGTGTGCATACATGTGTGTGTATGCAC
TCCTGTGCATGTGTGTGCACACACAGGCACCTACTTGAGTATAGGTGCCTACAAAATCTG
GAAGACAACACTGGATTCCTGGTAGCTGGAATCACCGAAGGCTGTGATACAGGTGCTGG
GGAGTCCTTTACCAATGAGCTGCATCTCAGGATCTGGGGCACGCCTTCTCACAGAGGACA
CTGTCC

>CMV enhancer (304)
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATT
GACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA
ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCC
AAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTA
CATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC
CATG

>CMV promoter (204)
GTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATT
TCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGA

CTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACG
GTGGGAGGTCTATATAAGCAGAGCT

>Kozak sequence (9)
GCCGCCACC

>Truncated Ovalbumin (864)
ATGGGCAGCCTGGCCAGCAGACTGTACGCCGAGGAGAGATACCCCATCCTGCCCGAGTAC
CTGCAGTGCGTGAAGGAGCTGTACAGAGGCGGCCTGGAGCCCATCAACTTCCAGACCGCC
GCCGACCAGGCCAGAGAGCTGATCAACAGCTGGGTGGAGAGCCAGACCAACGGCATCATC
AGAAACGTGCTGCAGCCCAGCAGCGTGGACAGCCAGACCGCCATGGTGCTGGTGAACGCC
ATCGTGTTCAAGGGCCTGTGGGAGAAGGCCTTCAAGGACGAGGACACCCAGGCCATGCCC
TTCAGAGTGACCGAGCAGGAGAGCAAGCCCGTGCAGATGATGTACCAGATCGGCCTGTTC
AGAGTGGCCAGCATGGCCAGCGAGAAGATGAAGATCCTGGAGCTGCCCTTCGCCAGCGGC
ACCATGAGCATGCTGGTGCTGCTGCCCGACGAGGTGAGCGGCCTGGAGCAGCTGGAGAGC
ATCATCAACTTCGAGAAGCTGACCGAGTGGACCAGCAGCAACGTGATGGAGGAGAGAAA
GATCAAGGTGTACCTGCCCAGAATGAAGATGGAGGAGAAGTACAACCTGACCAGCGTGC
TGATGGCCATGGGCATCACCGACGTGTTCAGCAGCAGCGCCAACCTGAGCGGCATCAGCA
GCGCCGAGAGCCTGAAGATCAGCCAGGCCGTGCACGCCGCCCACGCCGAGATCAACGAGG
CCGGCAGAGAGGTGGTGGGCAGCGCCGAGGCCGGCGTGGACGCCGCCAGCGTGAGCGAGG
AGTTCAGAGCCGACCACCCCTTCCTGTTCTGCATCAAGCACATCGCCACCAACGCCGTGCT
GTTCTTCGGCAGATGCGTGAGCCCC

>Stop codon (3)
TGA

>SV40 PolyA Signal (122)
AACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCAC
AAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATC
TTA

>3' homology arm (424)
GGTTTCTGATGTACTCCCGGTACATGGCAGTGTCCTCTTTGCCAAGGGGCTGCATGATAC
CTTGGCTAGCTTGGATATGGGCCTGGAAGATCTCTGTAGACTTTCTGTCTACTCTTGGAG
GCTGAACTTCATAAATGTTGTCTTGCGAGAAAGCTGAGATGGGAGTTCTGTAAAAGAGG
AAAGACAGGACTGTGTTATGGAACTTCTCACCACAACATGGGCTTCACCAACCAAGGGCA
ACCTCTGCAGTTGTTACTTCCTGGGAGGCCAAAGGATAGAAGCTGTCCCTGATACCCAGG
CAGGGCAGAGGTCCTGTCCCTTATAGAATGTCAGCTCTATAGTTGCGTGTCCTGCTGGCT
CCCACTGGAAGAAGCTTCAGGTGGTACTATACCTAAGTTTTGTTCCTTGTCCTTGCAGGC
TCTTG

sgRNAs:

sgRNA Name	Target Sequence	NeoPAM
sg1	CAAGTTCAACGTGTGGGACA	CGG
sg2	GAGGCCAGCCTGGTCTATAG	AGG
sg3	CATCGCCAACTTCTCTGAGC	TGG
sg4	TCATGCAGGCATCCGACGAG	TGG
sg5	TCTCTATACCACTTCTCTAC	TGG
sg6	CGGCTGCGGCGAGCGGGGAG	GGG
sg7	TCGAGGCCGTCTCTATAAGT	AGG

Primers:

Experiment	Forward Primer	Reverse Primer
Donor DNA Amplification (Fig. 4a)	GGGTGGCCTATGGAGGT	CAAGAGCCTGCAAGGACAA
SIINFEKL-Specific Amplification (Fig. 4b)	GTGATGCGGTTTTGGC	GTGATGCTATTGCTTTATTTGTAACC
Cleavage Validation (sg1)	GTATGTAGCCACCCTGGG	GGCAACAAACTCCAAGTTAGG
Cleavage Validation (sg2)	TAATAGGTCGCCGGGC	TCAGGTCTTAGCAAATGCAA
Cleavage Validation (sg3)	GGATACCAGACACCGTGAAC	GCCCCTCTGAATCACATC
Cleavage Validation (sg4)	GCTGAAGCCCAGCAAAGA	TGACGCTTTCCAAGGCA
Cleavage Validation (sg5)	TACACATCGACAGCCCAA	GCACAAGAACAGGACACACT
Cleavage Validation (sg6)	TTCCCGAAGGACGACAG	CAGTGTGATTCCCGCC
Cleavage Validation (sg7)	CCGAAGGCTGTGATACAGG	GCTTCTTCCAGTGGGAGC

REFERENCES

1. Nowell, P. C. (1976). The clonal evolution of tumor cell populations. *Science*, *194*(4260), 23-28.

2. Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G., ... & Edkins, S. (2007). Patterns of somatic mutation in human cancer genomes. *Nature*, *446*(7132), 153-158.

3. Swanton, C. (2012). Intratumor heterogeneity: evolution through space and time. *Cancer research*, *72*(19), 4875-4882.

4. Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., & Gregory, P. D. (2010). Genome editing with engineered zinc finger nucleases. *Nature Reviews Genetics*, *11*(9), 636.

5. Joung, J. K., & Sander, J. D. (2013). TALENs: a widely applicable technology for targeted genome editing. *Nature reviews Molecular cell biology*, *14*(1), 49.

6. Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols*, *8*(11), 2281.

7. Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, *157*(6), 1262-1278.

8. Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*, *346*(6213), 1258096.

9. Anderson, E. M., Haupt, A., Schiel, J. A., Chou, E., Machado, H. B., Strezoska, Ž., ... & van Brabant Smith, A. (2015). Systematic analysis of CRISPR–Cas9 mismatch tolerance reveals low levels of off-target activity. *Journal of biotechnology*, *211*, 56-65.

10. Kuscu, C., Arslan, S., Singh, R., Thorpe, J., & Adli, M. (2014). Genome-wide analysis reveals characteristics of off-target sites bound by the Cas9 endonuclease. *Nature biotechnology*, *32*(7), 677.

11. Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., ... & Cradick, T. J. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature biotechnology*, *31*(9), 827.

12. Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. A., & Liu, D. R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature biotechnology*, *31*(9), 839.

13. Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nature biotechnology*, *32*(4), 347.

14. Mali, P., Aach, J., Stranges, P. B., Esvelt, K. M., Moosburner, M., Kosuri, S., ... & Church, G. M. (2013). CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nature biotechnology*, *31*(9), 833.

15. Shen, B., Zhang, W., Zhang, J., Zhou, J., Wang, J., Chen, L., ... & Skarnes, W. C. (2014). Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature methods*, *11*(4), 399.

16. Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., & Lim, W. A. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*, *152*(5), 1173-1183.

17. Chen, B., Gilbert, L. A., Cimini, B. A., Schnitzbauer, J., Zhang, W., Li, G. W., ... & Huang, B. (2013). Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell*, *155*(7), 1479-1491.

18. Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. A., Yan, W. X., & Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. *Science*, *351*(6268), 84-88.

19. Kleinstiver, B. P., Pattanayak, V., Prew, M. S., Tsai, S. Q., Nguyen, N. T., Zheng, Z., & Joung, J. K. (2016). High-fidelity CRISPR–Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*, *529*(7587), 490.

20. Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M., & Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature biotechnology*, *32*(3), 279.

21. Kiani, S., Chavez, A., Tuttle, M., Hall, R. N., Chari, R., Ter-Ovanesyan, D., ... & Buchthal, J. (2015). Cas9 gRNA engineering for genome editing, activation and repression. *Nature methods*, *12*(11), 1051.

22. Li, Y., Mendiratta, S., Ehrhardt, K., Kashyap, N., White, M. A., & Bleris, L. (2016). Exploiting the CRISPR/Cas9 PAM constraint for single-nucleotide resolution interventions. *PloS one*, *11*(1), e0144970.

23. Cox, D. B. T., Platt, R. J., & Zhang, F. (2015). Therapeutic genome editing: prospects and challenges. *Nature medicine*, *21*(2), 121-131.

24. Gori, J. L., Hsu, P. D., Maeder, M. L., Shen, S., Welstead, G. G., & Bumcrot, D. (2015). Delivery and specificity of CRISPR/Cas9 genome editing technologies for human gene therapy. *Human gene therapy*, *26*(7), 443-451.

25. McCormick, F. (2001). Cancer gene therapy: fringe or cutting edge?. *Nature Reviews Cancer*, *1*(2), 130-141.

26. Mullen, C. A. (1994). Metabolic suicide genes in gene therapy. *Pharmacology & therapeutics*, *63*(2), 199-207.

27. Freeman, S. M., Whartenby, K. A., Freeman, J. L., Abboud, C. N., & Marrogi, A. J. (1996, February). In situ use of suicide genes for cancer therapy. In *Seminars in oncology* (Vol. 23, No. 1, pp. 31-45).

28. Portsmouth, D., Hlavaty, J., & Renner, M. (2007). Suicide genes for cancer therapy. *Molecular aspects of medicine*, *28*(1), 4-41.

29. Fillat, C., Carrio, M., Cascante, A., & Sangro, B. (2003). Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Current gene therapy*, *3*(1), 13-26.

30. Hamel, W., Magnelli, L., Chiarugi, V. P., & Israel, M. A. (1996). Herpes simplex virus thymidine kinase/ganciclovir-mediated apoptotic death of bystander cells. *Cancer research*, *56*(12), 2697-2702.

31. Beltinger, C., Fulda, S., Kammertoens, T., Meyer, E., Uckert, W., & Debatin, K. M. (1999). Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. *Proceedings of the National Academy of Sciences*, *96*(15), 8699-8704.

32. Elshami, A. A., Saavedra, A., Zhang, H., Kucharczuk, J. C., Spray, D. C., Fishman, G. I., ... & Albelda, S. M. (1996). Gap junctions play a role in the'bystander effect'of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. *Gene therapy*, *3*(1), 85-92.

33. Robe, P. A., Princen, F., Martin, D., Malgrange, B., Stevenaert, A., Moonen, G., ... & Bours, V. (2000). Pharmacological modulation of the bystander effect in the herpes simplex virus thymidine kinase/ganciclovir gene therapy system: Effects of dibutyryl adenosine 3′, 5′-cyclic monophosphate, α-glycyrrhetinic acid, and cytosine arabinoside. *Biochemical pharmacology*, *60*(2), 241-249.

34. Qiao, J., Black, M. E., & Caruso, M. (2000). Enhanced ganciclovir killing and bystander effect of human tumor cells transduced with a retroviral vector carrying a herpes simplex virus thymidine kinase gene mutant. *Human gene therapy*, *11*(11), 1569-1576.

35. Preuß, E., Treschow, A., Newrzela, S., Brücher, D., Weber, K., Felldin, U., ... & Fehse, B. (2010). TK. 007: A novel, codon-optimized HSVtk (A168H) mutant for suicide gene therapy. *Human gene therapy*, *21*(8), 929-941.

36. Preuß, E., Muik, A., Weber, K., Otte, J., von Laer, D., & Fehse, B. (2011). Cancer suicide gene therapy with TK. 007: superior killing efficiency and bystander effect. *Journal of molecular medicine*, *89*(11), 1113.

37. Huber, B. E., Austin, E. A., Richards, C. A., Davis, S. T., & Good, S. S. (1994). Metabolism of 5-fluorocytosine to 5-fluorouracil in human colorectal tumor cells transduced with the cytosine deaminase gene: significant antitumor effects when only a small percentage of tumor cells express cytosine deaminase. *Proceedings of the National Academy of Sciences*, *91*(17), 8302-8306.

38. Kievit, E., Bershad, E., Ng, E., Sethna, P., Dev, I., Lawrence, T. S., & Rehemtulla, A. (1999). Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts. *Cancer research*, *59*(7), 1417-1421.

39. Dilber, M. S., Phelan, A., Aints, A., Mohamed, A. J., Elliott, G., Smith, C. E., & O'hare, P. (1999). Intercellular delivery of thymidine kinase prodrug activating enzyme by the herpes simplex virus protein, VP22. *Gene therapy*, *6*(1), 12.

40. Liu, C., Kong, B., HuiQin Xia, H., Ellem, K. A., & Wei, M. Q. (2001). VP22 enhanced intercellular trafficking of HSV thymidine kinase reduced the level of ganciclovir needed to cause suicide cell death. *The journal of gene medicine*, *3*(2), 145-152.

41. Wybranietz, W. A., Gross, C. D., Phelan, A., O'Hare, P., Spiegel, M., Graepler, F., ... & Lauer, U. M. (2001). Enhanced suicide gene effect by adenoviral transduction of a VP22-cytosine deaminase (CD) fusion gene. *Gene Therapy*, *8*(21), 1654.

42. Roth, J. A., & Cristiano, R. J. (1997). Gene therapy for cancer: what have we done and where are we going? *Journal of the National Cancer Institute*, *89*(1), 21-39.

43. Sharma, P., & Allison, J. P. (2015). The future of immune checkpoint therapy. *Science*, *348*(6230), 56-61.

44. Postow, M. A., Callahan, M. K., & Wolchok, J. D. (2015). Immune checkpoint blockade in cancer therapy. *Journal of clinical oncology*, *33*(17), 1974-1982.

45. Topalian, S. L., Drake, C. G., & Pardoll, D. M. (2015). Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer cell*, *27*(4), 450-461.

46. McGranahan, N., Furness, A. J., Rosenthal, R., Ramskov, S., Lyngaa, R., Saini, S. K., ... & Watkins, T. B. (2016). Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science*, aaf1490.

47. Ma, A., Koka, R., & Burkett, P. (2006). Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis. *Annu. Rev. Immunol.*, *24*, 657-679.

48. Liao, W., Lin, J. X., & Leonard, W. J. (2011). IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Current opinion in immunology*, *23*(5), 598-604.

49. Gattinoni, L., Klebanoff, C. A., Palmer, D. C., Wrzesinski, C., Kerstann, K., Yu, Z., ... & Restifo, N. P. (2005). Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *Journal of Clinical Investigation*, *115*(6), 1616.

50. Fearon, E. R., Pardoll, D. M., Itaya, T., Golumbek, P., Levitsky, H. I., Simons, J. W., ... & Frost, P. (1990). Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell*, *60*(3), 397-403.

51. Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R., & Gilboa, E. (1990). Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity. *Journal of Experimental Medicine*, *172*(4), 1217-1224.

52. Bowman, L., Grossmann, M., Rill, D., Brown, M., Zhong, W. Y., Alexander, B., ... & Woods, D. (1998). IL-2 adenovector-transduced autologous tumor cells induce antitumor immune responses in patients with neuroblastoma. *Blood*, *92*(6), 1941-1949.

53. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., & Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *The Journal of Immunology*, *155*(3), 1151-1164.

54. Setoguchi, R., Hori, S., Takahashi, T., & Sakaguchi, S. (2005). Homeostatic maintenance of natural Foxp3+ CD25+ CD4+ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *Journal of Experimental Medicine*, *201*(5), 723-735.

55. Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., ... & Zhu, Y. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature medicine*, *10*(9), 942-949.

56. Liu, V. C., Wong, L. Y., Jang, T., Shah, A. H., Park, I., Yang, X., ... & Lee, C. (2007). Tumor evasion of the immune system by converting CD4+ CD25− T cells into CD4+ CD25+ T regulatory cells: role of tumor-derived TGF-β. *The Journal of Immunology*, *178*(5), 2883-2892.

57. Schoenborn, J. R., & Wilson, C. B. (2007). Regulation of interferon-γ during innate and adaptive immune responses. *Advances in immunology*, *96*, 41-101.

58. Schroder, K., Hertzog, P. J., Ravasi, T., & Hume, D. A. (2004). Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*, *75*(2), 163-189.

59. Pace, J. L., Russell, S. W., Torres, B. A., Johnson, H. M., & Gray, P. W. (1983). Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. *The Journal of Immunology*, *130*(5), 2011-2013.

60. Xie, Q. W., Whisnant, R., & Nathan, C. (1993). Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *Journal of Experimental Medicine*, *177*(6), 1779-1784.

61. Lowenstein, C. J., Alley, E. W., Raval, P., Snowman, A. M., Snyder, S. H., Russell, S. W., & Murphy, W. J. (1993). Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proceedings of the National Academy of Sciences*, *90*(20), 9730-9734.

62. Yasumoto, K., Okamoto, S. I., Mukaida, N., Murakami, S., Mai, M., & Matsushima, K. (1992). Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene. *Journal of Biological Chemistry*, *267*(31), 22506-22511.

63. Tsujimoto, M., Yip, Y. K., & Vilcek, J. (1986). Interferon-gamma enhances expression of cellular receptors for tumor necrosis factor. *The Journal of Immunology*, *136*(7), 2441-2444.

64. Barth, R. J., Mulé, J. J., Spiess, P. J., & Rosenberg, S. A. (1991). Interferon gamma and tumor necrosis factor have a role in tumor regressions mediated by murine CD8+ tumor-infiltrating lymphocytes. *Journal of Experimental Medicine*, *173*(3), 647-658.

65. Arenberg, D. A., Kunkel, S. L., Polverini, P. J., Morris, S. B., Burdick, M. D., Glass, M. C., ... & Strieter, R. M. (1996). Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *Journal of Experimental Medicine*, *184*(3), 981-992.

66. Restifo, N. P., Spiess, P. J., Karp, S. E., Mulé, J. J., & Rosenberg, S. A. (1992). A nonimmunogenic sarcoma transduced with the cDNA for interferon gamma elicits CD8+ T cells against the wild-type tumor: correlation with antigen presentation capability. *Journal of Experimental Medicine*, *175*(6), 1423-1431.

67. Cao, X., Leonard, K., Collins, L. I., Cai, S. F., Mayer, J. C., Payton, J. E., ... & Ley, T. J. (2009). Interleukin 12 Stimulates IFN-γ–Mediated Inhibition of Tumor-Induced

Regulatory T-Cell Proliferation and Enhances Tumor Clearance. *Cancer research*, *69*(22), 8700-8709.

68. Carswell, E. A., Old, L. J., Kassel, R., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences*, *72*(9), 3666-3670.

69. Old, L. J. (1985). Tumor necrosis factor. *Science*, *230*, 630-633.

70. Brunda, M. J., Bellantoni, D., & Sulich, V. (1987). In vivo anti-tumor activity of combinations of interferon alpha and interleukin-2 in a murine model. Correlation of efficacy with the induction of cytotoxic cells resembling natural killer cells. *International journal of cancer*, *40*(3), 365-371.

71. Wussow, P. V., Block, B., Hartmann, F., & Deicher, H. (1988). Intralesional interferon-alpha therapy in advanced malignant melanoma. *Cancer*, *61*(6), 1071-1074.

72. Borden, E. C., Holland, J. F., Dao, T. L., Gutterman, J. U., Wiener, L., Chang, Y. C., & Patel, J. (1982). Leukocyte-derived interferon (alpha) in human breast carcinoma: the American Cancer Society phase II trial. *Annals of internal medicine*, *97*(1), 1-6.

73. Belardelli, F., Ferrantini, M., Proietti, E., & Kirkwood, J. M. (2002). Interferon-alpha in tumor immunity and immunotherapy. *Cytokine & growth factor reviews*, *13*(2), 119-134.

74. Tahara, H., & Lotze, M. T. (1995). Antitumor effects of interleukin-12 (IL-12): applications for the immunotherapy and gene therapy of cancer. *Gene therapy*, *2*(2), 96-106.

75. Colombo, M. P., & Trinchieri, G. (2002). Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine & growth factor reviews*, *13*(2), 155-168.

76. Del Vecchio, M., Bajetta, E., Canova, S., Lotze, M. T., Wesa, A., Parmiani, G., & Anichini, A. (2007). Interleukin-12: biological properties and clinical application. *Clinical Cancer Research*, *13*(16), 4677-4685.

77. Li, K., Wang, G., Andersen, T., Zhou, P., & Pu, W. T. (2014). Optimization of genome engineering approaches with the CRISPR/Cas9 system. *PloS one*, *9*(8), e105779.

78. Gimmi, C. D., Freeman, G. J., Gribben, J. G., Sugita, K., Freedman, A. S., Morimoto, C., & Nadler, L. M. (1991). B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proceedings of the National Academy of Sciences*, *88*(15), 6575-6579.

79. Koulova, L., Clark, E. A., Shu, G., & Dupont, B. (1991). The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of CD4+ T cells. *Journal of Experimental Medicine*, *173*(3), 759-762.

80. Reiser, H., Freeman, G. J., Razi-Wolf, Z., Gimmi, C. D., Benacerraf, B., & Nadler, L. M. (1992). Murine B7 antigen provides an efficient costimulatory signal for activation of murine T lymphocytes via the T-cell receptor/CD3 complex. *Proceedings of the National Academy of Sciences*, *89*(1), 271-275.

81. Coyle, A. J., & Gutierrez-Ramos, J. C. (2001). The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. *Nature immunology*, *2*(3), 203.

82. Baskar, S., Ostrand-Rosenberg, S., Nabavi, N., Nadler, L. M., Freeman, G. J., & Glimcher, L. H. (1993). Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules. *Proceedings of the National Academy of Sciences*, *90*(12), 5687-5690.

83. Yang, G., Hellström, K. E., Hellström, I., & Chen, L. (1995). Antitumor immunity elicited by tumor cells transfected with B7-2, a second ligand for CD28/CTLA-4 costimulatory molecules. *The Journal of Immunology*, *154*(6), 2794-2800.

84. Cayeux, S., Beck, C., Aicher, A., Dörken, B., & Blankenstein, T. (1995). Tumor cells cotransfected with interleukin-7 and B7. 1 genes induce CD25 and CD28 on tumor-infiltrating T lymphocytes and are strong vaccines. *European journal of immunology*, *25*(8), 2325-2331.

85. Mouse Genome Sequencing Consortium. Initial sequencing and comparative analysis of the mouse genome. *Nature*. 2002 Dec 5;420(6915):520-62. PMID: 12466850

86. Fowler, D. K., Stewart, S., Seredick, S., Eisen, J. S., Stankunas, K., & Washbourne, P. (2016). A multisite gateway toolkit for rapid cloning of vertebrate expression constructs with diverse research applications. *PloS one*, *11*(8), e0159277.

87. Lee, Y. H., Andersen, J. B., Song, H. T., Judge, A. D., Seo, D., Ishikawa, T., ... & Woo, H. G. (2010). Definition of ubiquitination modulator COP1 as a novel therapeutic target in human hepatocellular carcinoma. *Cancer research*, *70*(21), 8264-8269.

88. UniProt Consortium. (2016). UniProt: the universal protein knowledgebase. *Nucleic acids research*, *45*(D1), D158-D169.

89. Rötzschke, O., Falk, K., Stevanovic, S., Jung, G., Walden, P., & Rammensee, H. G. (1991). Exact prediction of a natural T cell epitope. *European journal of immunology*, *21*(11), 2891-2894.

90. Karttunen, J., Sanderson, S., & Shastri, N. (1992). Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proceedings of the National Academy of Sciences*, *89*(13), 6020-6024.

91. Clarke, S. R., Barnden, M., Kurts, C., Carbone, F. R., Miller, J. F., & Heath, W. R. (2000). Characterization of the ovalbumin-specific TCR transgenic line OT-I: MHC elements for positive and negative selection. *Immunology and cell biology*, *78*(2), 110.

92. Restifo, N. P., Bacik, I., Irvine, K. R., Yewdell, J. W., McCabe, B. J., Anderson, R. W., ... & Bennink, J. R. (1995). Antigen processing in vivo and the elicitation of primary CTL responses. *The Journal of Immunology*, *154*(9), 4414-4422.

93. Porgador, A., Yewdell, J. W., Deng, Y., Bennink, J. R., & Germain, R. N. (1997). Localization, quantitation, and in situ detection of specific peptide–MHC class I complexes using a monoclonal antibody. *Immunity*, *6*(6), 715-726.

94. Elliott, T. (1997). Transporter associated with antigen processing. *Advances in immunology*, *65*, 47-110.

95. Qiu, P., Shandilya, H., D Alessio, J. M., O Connor, K., Durocher, J., & Gerard, G. F. (2004). Mutation detection using Surveyor™ nuclease. *Biotechniques*, *36*(4), 702-707.

96. Vouillot, L., Thélie, A., & Pollet, N. (2015). Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. *G3: Genes, Genomes, Genetics*, *5*(3), 407-415.

97. Kung, S. H., Retchless, A. C., Kwan, J. Y., & Almeida, R. P. (2013). Effects of DNA size on transformation and recombination efficiencies in Xylella fastidiosa. *Applied and environmental microbiology*, *79*(5), 1712-1717.

98. Cox, D. B. T., Platt, R. J., & Zhang, F. (2015). Therapeutic genome editing: prospects and challenges. *Nature medicine*, *21*(2), 121.

99. Szymczak, A. L., Workman, C. J., Wang, Y., Vignali, K. M., Dilioglou, S., Vanin, E. F., & Vignali, D. A. (2004). Correction of multi-gene deficiency in vivo using a single'self-cleaving'2A peptide–based retroviral vector. *Nature biotechnology*, *22*(5), 589.

100. Provost, E., Rhee, J., & Leach, S. D. (2007). Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis*, *45*(10), 625-629.

101. Tsukiyama-Kohara, K., Iizuka, N., Kohara, M., & Nomoto, A. (1992). Internal ribosome entry site within hepatitis C virus RNA. *Journal of virology*, *66*(3), 1476-1483.

102. Martínez-Salas, E. (1999). Internal ribosome entry site biology and its use in expression vectors. *Current opinion in biotechnology*, *10*(5), 458-464.

103. Yoo, B. K., Gredler, R., Vozhilla, N., Su, Z. Z., Chen, D., Forcier, T., ... & Sarkar, D. (2009). Identification of genes conferring resistance to 5-fluorouracil. *Proceedings of the National Academy of Sciences*, *106*(31), 12938-12943.