Validating a Novel CRISPR/Cas9 System for Simultaneous Gene Modification and Transcriptional Regulation

by

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ABSTRACT

A novel clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) tool for simultaneous gene editing and regulation was designed and tested. This study used the CRISPR-associated protein 9 (Cas9) endonuclease in complex with a 14-nucleotide (nt) guide RNA (gRNA) to repress a gene of interest using the Krüppel associated box (KRAB) domain, while also performing a separate gene modification using a 20-nt gRNA targeted to a reporter vector. DNA Ligase IV (LIGIV) was chosen as the target for gene repression, given its role in nonhomologous end joining, a common DNA repair process that competes with the more precise homology-directed repair (HDR).

To test for gene editing, a 20-nt gRNA was designed to target a disrupted enhanced green fluorescent protein (EGFP) gene present in a reporter vector. After the gRNA introduced a double-stranded break, cells attempted to repair the cut site via HDR using a DNA template within the reporter vector. In the event of successful gene editing, the EGFP sequence was restored to a functional state and green fluorescence was detectable by flow cytometry. To achieve gene repression, a 14-nt gRNA was designed to target LIGIV. The gRNA included a com protein recruitment domain, which recruited a Com-KRAB fusion protein to facilitate gene repression via chromatin modification of LIGIV. Quantitative polymerase chain reaction was used to quantify repression.

This study expanded upon earlier advancements, offering a novel and versatile approach to genetic modification and transcriptional regulation using CRISPR/Cas9. The overall results show that both gene editing and repression were occurring, thereby

providing support for a novel CRISPR/Cas system capable of simultaneous gene modification and regulation. Such a system may enhance the genome engineering capabilities of researchers, benefit disease research, and improve the precision with which gene editing is performed.

DEDICATION

This thesis is dedicated to my husband, Leon Chapman, who has been by my side since I was 16. From the very start, you encouraged and supported my growth and advancement. Thank you for the best thing I have ever experienced, your love and friendship, and for always insisting that I fulfill my potential.

ACKNOWLEDGMENTS

I would like to take this opportunity to extend my sincerest gratitude to Dr. Kiani for offering me this opportunity. Thank you for welcoming me into your lab and offering indispensable support, guidance and expertise over the past few years. You always made time for me, cared about my progress and sought to further my success. I sincerely appreciate all of your efforts on my behalf.

I would also like to thank my other committee members, Dr. Ugarova and Dr. Marchant, whose input and encouragement were greatly appreciated, as well as Dr. Ebrahimkhani who continuously offered valuable ideas and insights.

To all of those in the Kiani and Ebrahimkhani labs with whom I have shared many hours over the past few years, thank you for all that you did to make this experience fulfilling and rewarding. I am so grateful to have been part of such a wonderful group of intelligent and hard-working individuals, and I will not soon forget your teamwork and willingness to freely offer your time and knowledge.

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LIST OF ABBREVIATIONS

bp	Base pair
Cas	CRISPR-associated
Cas9	CRISPR-associated protein 9
Cas9-VPR	Cas9 fused to VPR
CDS	Coding domain sequence
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CRISPRi	CRISPR interference
DI	Deionized
DMEM	Dulbecco's Modified Eagle's Medium
DSB	Double-stranded break
FBS	Fetal bovine serum
GFP	Green fluorescent protein
EBFP	Enhanced blue fluorescent protein
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence-Activated Cell Sorting
gRNA	Guide RNA
HDR	Homology-directed repair
hCas9	Human codon-optimized Cas9

HEK	Human embryonic kidney
hU6	Human U6
indel	Insertion/deletion of base pairs
KRAB	Krüppel associated box
LB	Luria Broth
LIGIV	DNA Ligase IV
NanoDrop™	NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer
NEB	New England Biolabs
NHEJ	Nonhomologous end joining
NLS	Nuclear localization signal
nt	Nucleotide
PAM	Protospacer adjacent motif
PEI	Polyethylenimine
pre-crRNA	Precursor CRISPR RNA
pX330	pX330-U6-Chimeric_BB-CBh-hSpCas9
qPCR	Quantitative polymerase chain reaction
RNAi	RNA interference
TALEN	Transcription activator-like effector nuclease
tracrRNA	Trans-activating RNA
ZFN	Zinc-finger nuclease

1. INTRODUCTION

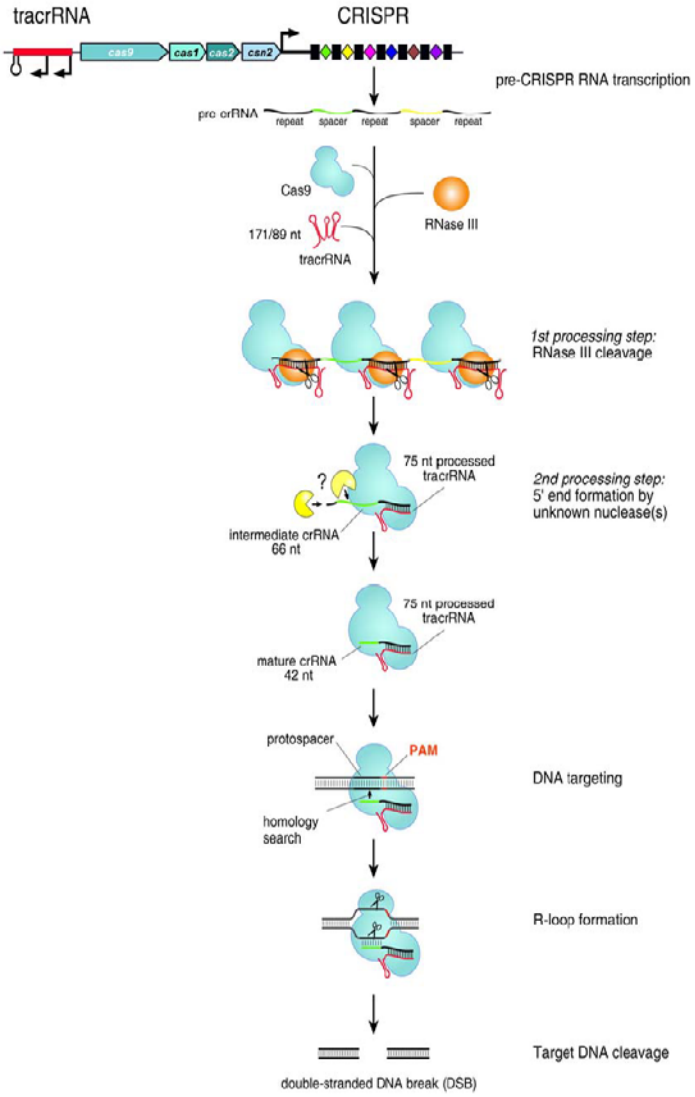
1.1 CRISPR/Cas Genome Editing

Clustered regularly interspaced short palindromic repeats/CRISPR associated (CRISPR/Cas) technology encompasses a revolutionary genome editing technology with tremendous potential for curing human diseases through the development of novel gene therapies. Diseases that have long plagued humanity, causing untold suffering, impaired quality of life, and early death, now face a formidable opponent. Transformed from an adaptive immune system utilized by many bacteria and archaea to defend against foreign nucleic acids, such as viruses or plasmids (Jinek et al., 2012; Wiedenheft, Sternberg, & Doudna, 2012) CRISPR/Cas, or simply CRISPR, is rapidly becoming a powerful tool, not only in clinical medicine, but in microbiology, synthetic biology, agricultural product development, fundamental genetic research and more (Barrangou & Doudna, 2016; Hsu, Lander, & Zhang, 2014).

CRISPR immunity is conferred through a genetic, chronological record of previous infections by foreign nucleic acids, which is embodied in a CRISPR locus within the genome of the host microorganism (Jinek et al., 2012; Wiedenheft et al., 2012). *See* **Fig. 1.** The genomic CRISPR locus generally includes sequences for trans-activating RNA (tracrRNA), an operon of *cas* genes, and a CRISPR array (Doudna & Charpentier, 2014; Jinek et al., 2012). When challenged by foreign genetic material, a microorganism with CRISPR immunity cleaves and integrates fragments of the transgressing DNA into the CRISPR array to protect against future invasions (Doudna & Charpentier, 2014). These acquired fragments of foreign DNA, or spacers, are separated

in the CRISPR array by short repeat sequences (Doudna & Charpentier, 2014; Jinek et al., 2012). The type II CRISPR system, which was utilized in this study and is referenced throughout this text, uses CRISPR-associated protein 9 (Cas9) as a DNA endonuclease, or cutting enzyme, to cut and destroy the invading DNA (Jinek et al., 2012).

The CRISPR system targets and destroys invading DNA through a series of steps. *See* **Fig. 1.** The CRISPR array is transcribed, resulting in a precursor CRISPR RNA (pre-crRNA) molecule (Deltcheva et al., 2011). Each repeat sequence within the pre-crRNA molecule has complementarity to, and base pairs with, tracrRNA (Deltcheva et al., 2011). The resulting duplex RNA undergoes cleavage and multiple stages of processing, resulting in individual mature CRISPR RNAs (crRNAs) paired with mature tracrRNAs (Deltcheva et al., 2011). Each crRNA:tracrRNA duplex is bound to Cas9, forming a ribonucleoprotein complex that patrols the microorganism's intracellular environment for foreign genetic material (Jinek et al., 2012). If Cas9 recognizes a specific protospacer adjacent motif (PAM) site in the DNA being surveyed, the 20-nucleotide (nt) crRNA sequence interrogates the adjacent DNA for a sequence complementary to its own (Sternberg, Redding, Jinek, Greene, & Doudna, 2014). If the crRNA identifies and base pairs with complementary DNA, Cas9 induces a double-stranded break (DSB) in the DNA, thereby destroying the invading genetic material (Jinek et al., 2012). As such, the crRNA:tracrRNA duplex acts as a guide for Cas9, targeting the endonuclease to the site of desired DNA cleavage.

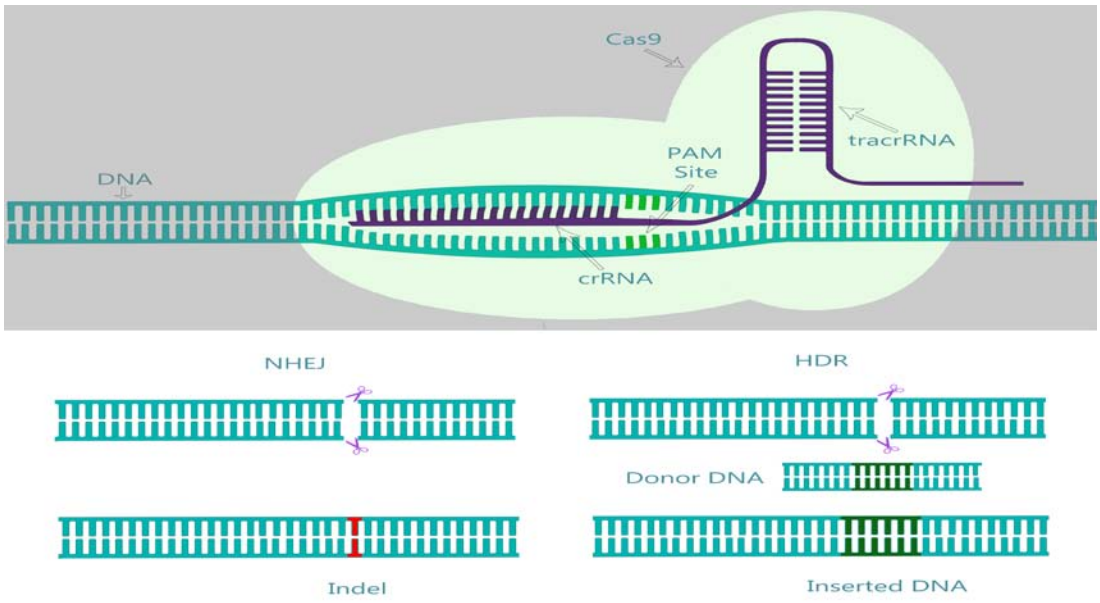


From Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., & Charpentier, E. (2012). A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity. *Science, 337*(6096), 816-821. doi:10.1126/science.1225829. Reprinted with permission from AAAS.

Fig. 1 Type II CRISPR/Cas Locus. The CRISPR locus contains a sequence for tracrRNA, an operon of *cas* genes, and a CRISPR array made up of repeat sequences (black rectangles) and unique spacer sequences (colored diamonds), which is transcribed into a pre-crRNA. Each repeat sequence within the pre-crRNA has complementarity to tracrRNA, which binds to the pre-crRNA. After undergoing multiple stages of processing, individual mature crRNA:tracrRNA structures, each in a ribonucleoprotein complex with Cas9, seek to identify and destroy foreign DNA (Jinek et al., 2012).

Jinek et al. (2012) streamlined this DNA targeting and cutting mechanism by engineering a single chimeric RNA guide, commonly referred to as a single guide RNA or simply a guide RNA (gRNA), composed of a programmable crRNA sequence fused to tracrRNA. (The term gRNA is used interchangeably throughout this text to refer to either the entire crRNA:tracrRNA duplex or just the programmable crRNA sequence alone.) The gRNA can be designed to target any DNA of interest and Cas9 will introduce a DSB at the sequence targeted by the gRNA three base pairs (bps) upstream of the PAM site (Jinek et al., 2012). This powerful, two-component gRNA:Cas9 system has been shown to successfully edit DNA in a variety of cells and organisms (Doudna & Charpentier, 2014; Hsu et al., 2014; Sander & Joung, 2014).

When a DSB is introduced, cells typically attempt to repair the cut site by initiating one of two distinct DNA repair mechanisms: nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (Maruyama et al., 2015). During NHEJ, cells attempt to ligate the cut ends back together (Davis & Chen, 2013). This process is imperfect, however, and often introduces insertions or deletions (indels), rather than restoration of the broken DNA back to the original sequence (Maruyama et al., 2015). During HDR, however, cells use a sequence with homology to the cut site, such as a sister chromatid, as a repair template to precisely repair the cut site (Davis & Chen, 2013). In the laboratory, researchers can use the imprecise NHEJ repair pathway to disrupt gene function (Su et al., 2016). Alternatively, researchers can use custom-designed, exogenous donor DNA with homology to the flanking ends of a desired cut site to facilitate precise genome editing in the event of HDR (Chu et al., 2015). *See* **Fig. 2.**



From Chapman, J.E., Gillum, D., & Kiani, S. (2017). Approaches to reduce CRISPR off-target effects for safer genome editing. *Applied Biosafety*, *22*(1), 7-13. doi:10.1177/1535676017694148

Fig. 2 CRISPR/Cas9 Gene Editing. Top: Induction of a DSB. If Cas9 detects the PAM site (green) and the 20-nt crRNA (purple) base pairs with complementary DNA, Cas9 cleaves 3 bps upstream of the PAM site, inducing a DSB. **Bottom: DNA repair mechanisms.** Following induction of a DSB, the cell attempts to repair the broken DNA. During NHEJ, key molecules work in conjunction with one another to ligate the cut DNA strands back together, often causing indels (red) that may disrupt the gene's expression. During HDR, cells can use donor DNA as a homologous template to repair the DSB. Researchers may include a custom-designed DNA sequence (dark green) in the donor DNA to precisely modify the targeted gene.

1.2 The Need for Simultaneous Gene Editing and Regulation

Along with the ability to modify the genome, the advancement of gene therapies will benefit from effective *regulation* of the genome, such as through the controlled activation and/or repression of genes. Indeed, *simultaneous* genome modification and regulation offers researchers even greater versatility when interrogating and engineering

the genome. Correction of some forms of genetic diseases may only require gene editing, while other diseases may require a sophisticated combination of gene modification and regulation.

As described by Li et al. (2016), cancer cells exhibit elevated metabolic demands to support their rapid growth and proliferation. The knockout of a key gene involved in glucose metabolism, *PDHA1*, caused prostate cancer cells to compensate by relying on an alternative glutamine-dependent metabolic pathway for survival and proliferation (Li et al., 2016). To promote glutamine metabolism, cells increased their expression of two key enzymes, GLS1 and GLUD1 (Li et al., 2016). Targeted inhibition of these enzymes, which are over-expressed in many cancer types, has been shown to stunt tumor growth and proliferation (Wang et al., 2010; Yang et al., 2009). Knockdown of other genes, such as *FOXK1*, has been similarly shown to suppress the proliferation and invasion of cancer cells (Chen, Xiong, Dou, & Ran, 2017). Thus, the ability to modify genes involved with glucose metabolism in cancer cells, while repressing genes involved with alternative metabolic pathways or other mechanisms of proliferation, is one potential application of simultaneous gene modification and transcriptional control.

Simultaneous control over gene modification and regulation may also help improve the precision with which genome editing is performed. HDR and NHEJ are competitive DNA repair processes (Chu et al., 2015). Repressing key molecules associated with NHEJ, for instance, increases the efficiency of HDR (Chu et al., 2015; Maruyama et al., 2015). During NHEJ, DNA Ligase IV (LIGIV), in complex with other NHEJ factors, ligates the broken DNA strands back together (Conlin et al., 2017). Chu et

al. (2015) showed that introducing short hairpin RNAs to suppress key NHEJ proteins, such as LIGIV, either alone or in conjunction with suppression of KU70, resulted in a concomitant increase in HDR. HDR has also been increased by suppressing LIGIV using small molecule inhibitors or certain proteins that mediate its degradation (Chu et al., 2015; Maruyama et al., 2015). Similarly, *ligIV*-deficient *Drosophila* embryos exhibited increased frequency of HDR (Beumer, Trautman, Mukherjee, & Carroll, 2013; Bozas, Beumer, Trautman, & Carroll, 2009). Thus, a genome engineering tool that can be utilized to regulate key genes involved with NHEJ while also performing genome modification at distinct genomic loci offers the potential to increase HDR at the site(s) of modification, thereby bolstering the efficiency with which precise genome edits are performed.

Although other technologies exist that can regulate gene expression, CRISPR/Cas9 has several advantages. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are more expensive and challenging to design and test (Qi et al., 2013), and RNA interference (RNAi) can exhibit substantial off-target effects and irregular on-target efficiency (Evers et al., 2016). Although CRISPR also results in off-target effects, several innovative strategies for addressing this limitation are being actively developed by a rapidly growing field of researchers (Chapman, Gillum, & Kiani, 2017). Additionally, the use of a single genome engineering technology, CRISPR/Cas9, for both editing and regulating the genome simplifies the testing environment, as compared to using a variety of technologies that require multiple skill sets, a more expansive knowledgebase, and a broader inventory of materials and

resources. It is anticipated that reliance on a single technology may also help pave the way for regulatory approval in the event of downstream clinical applications.

1.3 Gene Repression Using CRISPR Interference

With the development of CRISPR/Cas technology, the world was presented with a powerful new genome editing system. Not long after CRISPR's gene editing capabilities became widely known, however, researchers began repurposing its components in a variety of ways to further harness its capabilities. This included strategies for using CRISPR/Cas technology to regulate the genome through targeted activation and repression of genes.

As it relates to gene repression, which is the focus of this study, CRISPR interference (CRISPRi) is a transcription modulation system that uses a catalytically inactive form of Cas9, known as dCas9, to repress gene expression through inhibition of transcription (Gilbert et al., 2013; Qi et al., 2013). dCas9 is a noncleaving mutant of Cas9, which researchers modified to silence its RuvC1 and HNH endonuclease domains (Jinek et al., 2012; Qi et al., 2013). Pairing dCas9 with a gRNA targeted to a specific sequence in the genome results in the gRNA binding to its target site without dCas9 inducing a DSB (Qi et al., 2013). Instead, this gRNA:dCas9 complex represses gene expression by blocking transcription initiation and elongation (Qi et al., 2013). Additionally, CRISPRi can be used to inhibit transcription by fusing effector domains, such as the Krüppel associated box (KRAB) domain, to dCas9 to repress the targeted gene through chromatin modification (Gilbert et al., 2013). With either approach,

CRISPRi uses dCas9 as the platform for gene regulation. Due to the silenced endonuclease domains in dCas9, neither a DSB nor gene editing occur at the target site.

1.4 Cas9 as the Platform for Simultaneous Gene Editing and Regulation

CRISPRi attempted to address the need for a simpler, cheaper, and more efficient approach to genome regulation, as compared to ZFNs, TALENs, and RNAi (Qi et al., 2013). The repurposing of Cas9 into a non-cleaving dCas9 enzyme resulted in an innovative tool capable of reversible genome regulation. However, dCas9's inability to perform targeted genomic modifications restricted its functionality, leaving open the question of whether the catalytically-active Cas9 could be used to both edit and regulate the genome.

Building on a prior study showing that gRNAs ≤ 16-nts resulted in significantly reduced Cas9 nuclease activity as compared to gRNAs 17 to 20-nts in length (Fu, Sander, Reyon, Cascio, & Joung, 2014), Kiani et al. (2015) further exploited Cas9's potential as a multifunctional protein by demonstrating that when Cas9 was fused to VPR (Cas9-VPR), a potent transcriptional activator, and introduced into cells contemporaneously with 14-nt and 20-nt gRNAs targeted to separate endogenous genes, simultaneous gene activation (via the 14-nt gRNA) and mutation (via the 20-nt gRNA) occurred. Thus, Cas9 could be directed to perform either genome editing or activation by simply varying the length of the gRNA. As it relates specifically to repression, Kiani et al. (2015) used 14-nt gRNAs bound to Cas9-VPR or dCas9 to target specially engineered CRISPR-repressible

promoters (Kiani et al., 2014) contained within plasmids, thereby sterically blocking the cell's transcription machinery and resulting in tenfold repression.

The relevant advancement by Kiani et al. (2015) was to show proof-of-concept that 14-nt gRNAs in complex with Cas9 can be used to regulate, rather than edit, the genome. By modifying the length of the gRNA, genome modification and activation were performed simultaneously at distinct genomic loci (Kiani et al., 2015). Although the study by Kiani et al. (2015) also demonstrated repression using 14-nt gRNAs, the gRNAs were targeted in close proximity to a TATA box contained within a plasmid. In the genome, an ideal gRNA target site, with minimal off-target effects and limited mismatches, may not always exist in close proximity to a TATA box. Therefore, an opportunity existed for further studies drawing on this earlier foundational work.

1.5 Aim of Project

The purpose of this project was to design and test a novel CRISPR/Cas9 tool capable of performing simultaneous gene modification and repression. Gene modification was carried out using a fluorescence-based assay. The ideal tool would use 14-nt gRNAs targeted to an endogenous gene to achieve repression without requiring precise targeting in close proximity to a TATA box. The 14-nt gRNAs used in this study targeted an endogenous gene, as opposed to a plasmid-based assay, and used a KRAB effector domain to effectuate repression, as compared to the previous study utilizing steric blocking (Kiani et al., 2015). The gene of interest selected for repression in this study was *LIGIV*, given its central role in DNA repair.

2. MATERIALS & METHODS[1]

2.1 Overview of Vectors

2.1.1 pDRGFP

pDRGFP, a gift from Maria Jasin (Addgene plasmid # 26475), is a fluorescence-based assay (Pierce, Johnson, Thompson, & Jasin, 1999). It contains an enhanced green fluorescent protein (*EGFP*) gene, which was mutated through the inclusion of the 18-bp recognition sequence of the endonuclease I-SceI containing two stop codons, thereby disrupting expression of green fluorescent protein (GFP) (Pierce et al., 1999). Introduction of I-SceI into cells containing pDRGFP (or introduction of Cas9 and a gRNA targeting the I-SceI recognition sequence, as was performed in this study) results in a DSB at the site of mutation. Cells can then use a donor template also contained within pDRGFP to repair the DSB during HDR, thereby restoring the *EGFP* gene. The resulting GFP^+ cells can be quantified by flow cytometry, which acts as a reporter for HDR and successful gene editing. *See* **Fig. 3.**

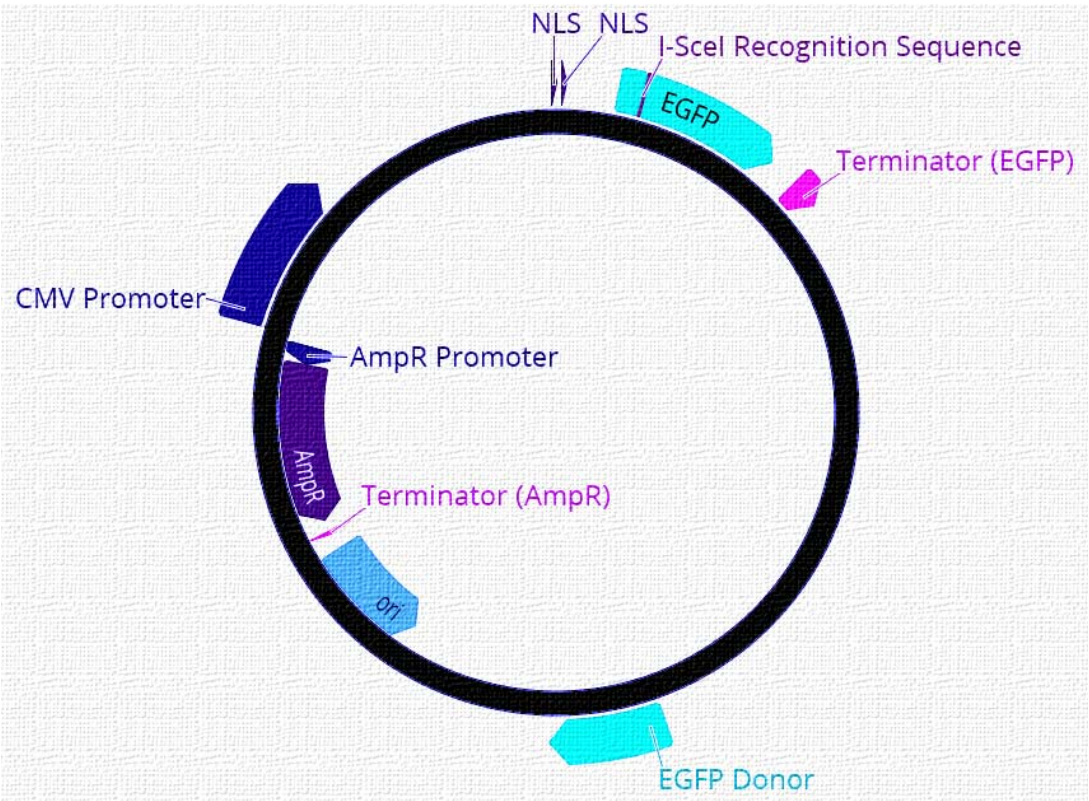


Fig. 3 pDRGFP.[2] The pDRGFP vector contains an *EGFP* gene modified to include the 18-bp recognition sequence for the endonuclease I-SceI containing two stop codons (Pierce et al., 1999). During HDR, cells repair a DSB at the I-SceI recognition sequence using pDRGFP's supplied donor template, thereby restoring the *EGFP* gene. The resulting GFP^+ cells, signifying successful gene editing, can be measured by flow cytometry.

2.1.2 pX330

pX330-U6-Chimeric_BB-CBh-hSpCas9 (pX330), a gift from Feng Zhang (Addgene plasmid # 42230), is a Type II CRISPR/Cas system, containing human codon-optimized Cas9 (hCas9) and a gRNA insert site (Cong et al., 2013). After a gRNA of interest is inserted into pX330, its expression is driven by a human U6 promoter (hU6). A nuclear localization signal (NLS) on each side of hCas9 aids with importation into the nucleus. *See* **Fig. 4.** In the present study, pX330 was modified to include a gRNA targeting the mutated *EGFP* gene in pDRGFP.

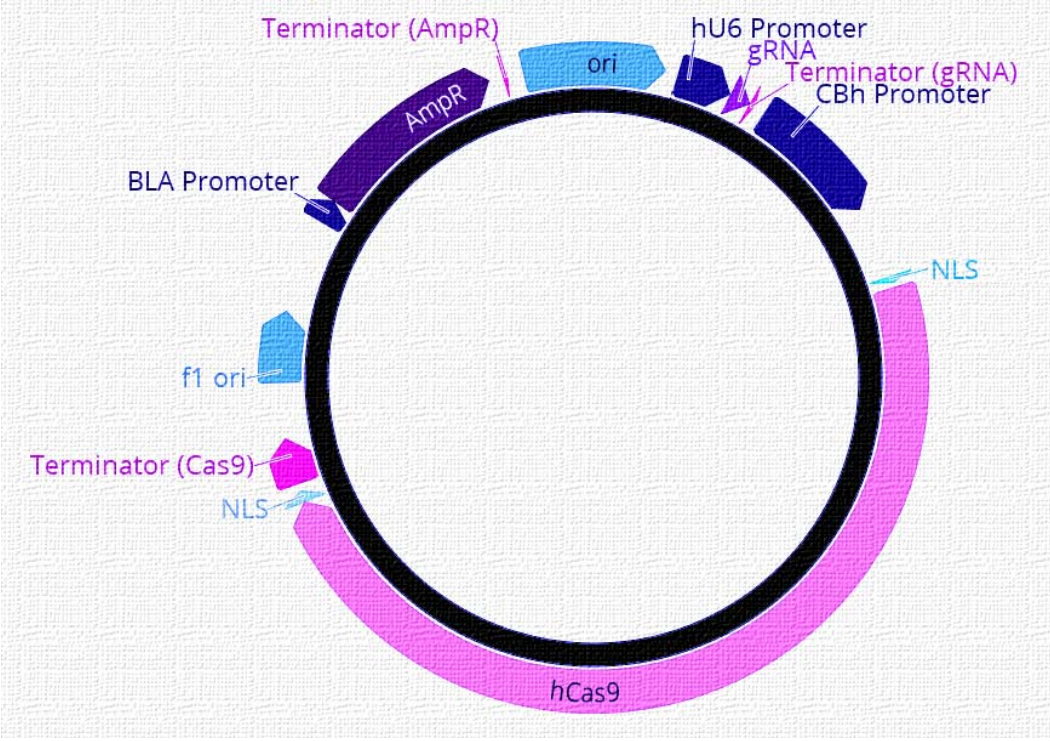


Fig. 4 pX330.[2] pX330 contains the sequence for hCas9 and a gRNA sequence that can be modified to target a gene of interest (Cong et al., 2013).

2.1.3 pJZC74

pJZC74 was a gift from Wendell Lim & Stanley Qi (Addgene plasmid # 62342) (Zalatan et al., 2014). *See* **Fig. 5.** As described by Zalatan et al. (2014), pJZC74's gRNA was extended to include a 3' RNA hairpin sequence for com, which is connected to the gRNA by a short, 2-bp linker. This com protein recruitment domain is recognized by Com, a RNA binding domain that is fused to a KRAB effector domain (Zalatan et al., 2014). With the Com-KRAB fusion protein bound to pJZC74's gRNA, the effector domain is recruited to the gene targeted by the gRNA, and KRAB inhibits the gene's transcription via chromatin modification (Groner et al., 2010). Although pJZC74 was designed for use with dCas9 as a single master controller of transcriptional regulation (Zalatan et al., 2014), this study achieved repression using Cas9 in complex with 14-nt gRNAs. *See* **Fig. 6**.

pJZC74 does not contain ideal restriction enzyme sites immediately flanking its gRNA which, if present, would allow a desired gRNA to be easily inserted. For its use in this study, pJZC74 was modified to include a customizable gRNA insert site, which was then utilized to insert a variety of gRNAs targeting *LIGIV*.

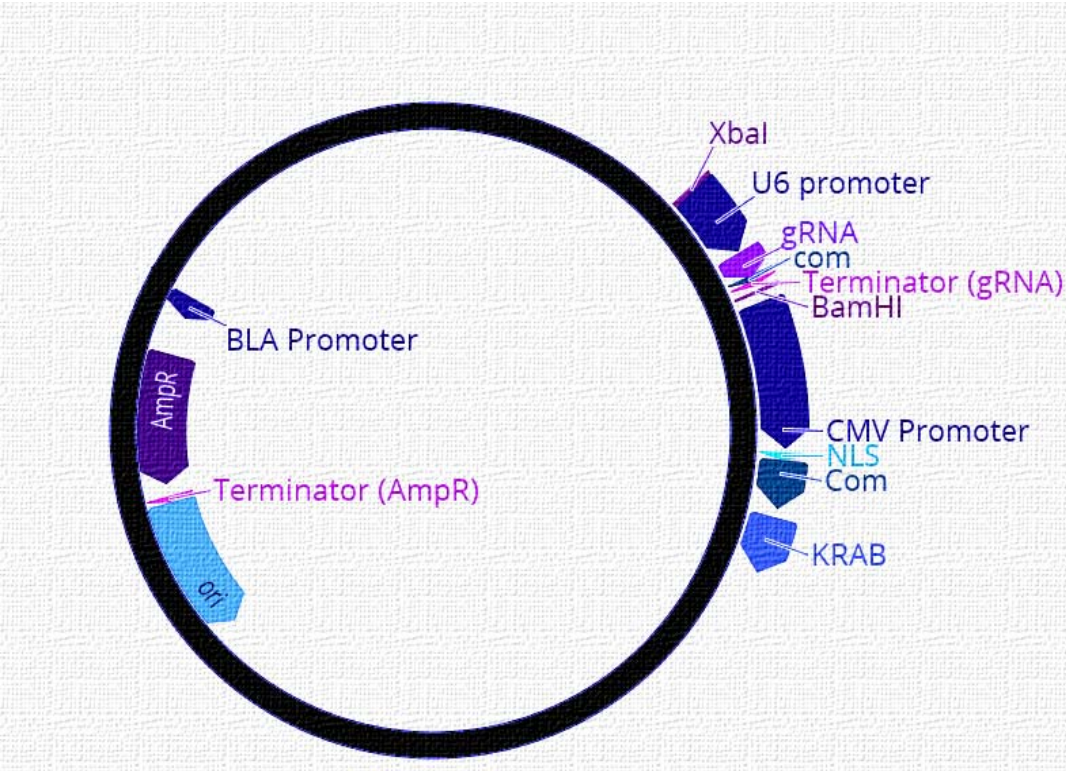


Fig. 5 pJZC74.[2] pJZC74 expresses the Com-KRAB fusion protein, which is recruited to pJZC74's gRNA by the com protein recruitment domain (Zalatan et al., 2014). The KRAB effector domain uses chromatin modification to inhibit transcription of the gene targeted by the gRNA, thereby repressing its expression (Groner et al., 2010).

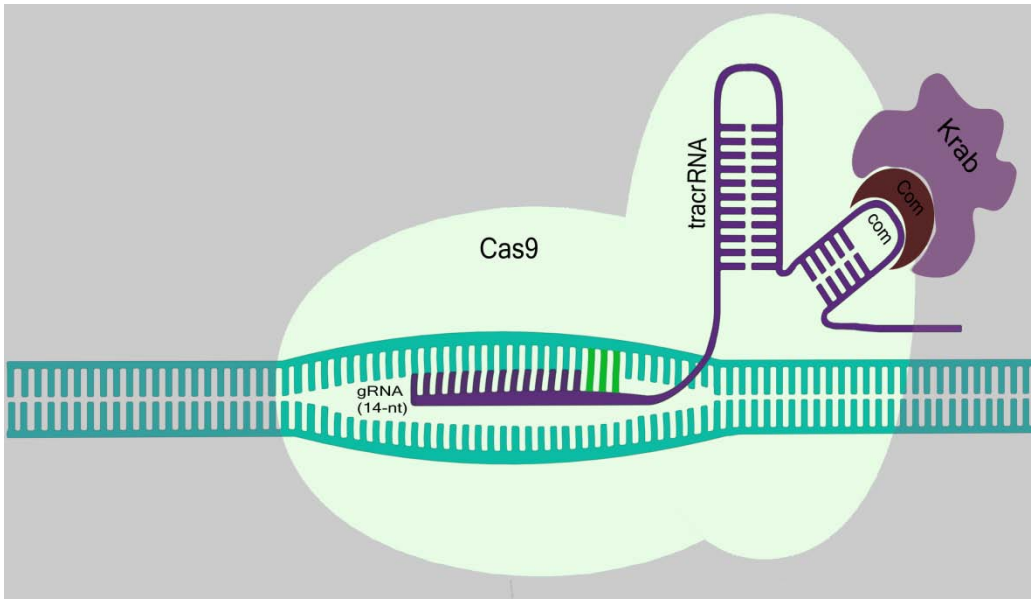


Fig. 6 Com-KRAB Bound to pJZC74's gRNA. pJZC74's gRNA was extended to include a com protein recruitment domain that recruits the Com-KRAB fusion protein (Zalatan et al., 2014). KRAB then facilitates repression of the gene targeted by pJZC74's gRNA. Shown here is the strategy utilized in this study of using a 14-nt gRNA in complex with Cas9 to target the gene of interest for repression without inducing a DSB.

2.1.4 hCas9

hCas9, a gift from George Church (Addgene plasmid # 41815), contains a human codon-optimized Cas9 driven by a cytomegalovirus (CMV) promoter and followed by a NLS to aid in nuclear transport (Mali et al., 2013). *See* **Fig. 7.** hCas9 was used in this study to supplement the Cas9 expressed by pX330.

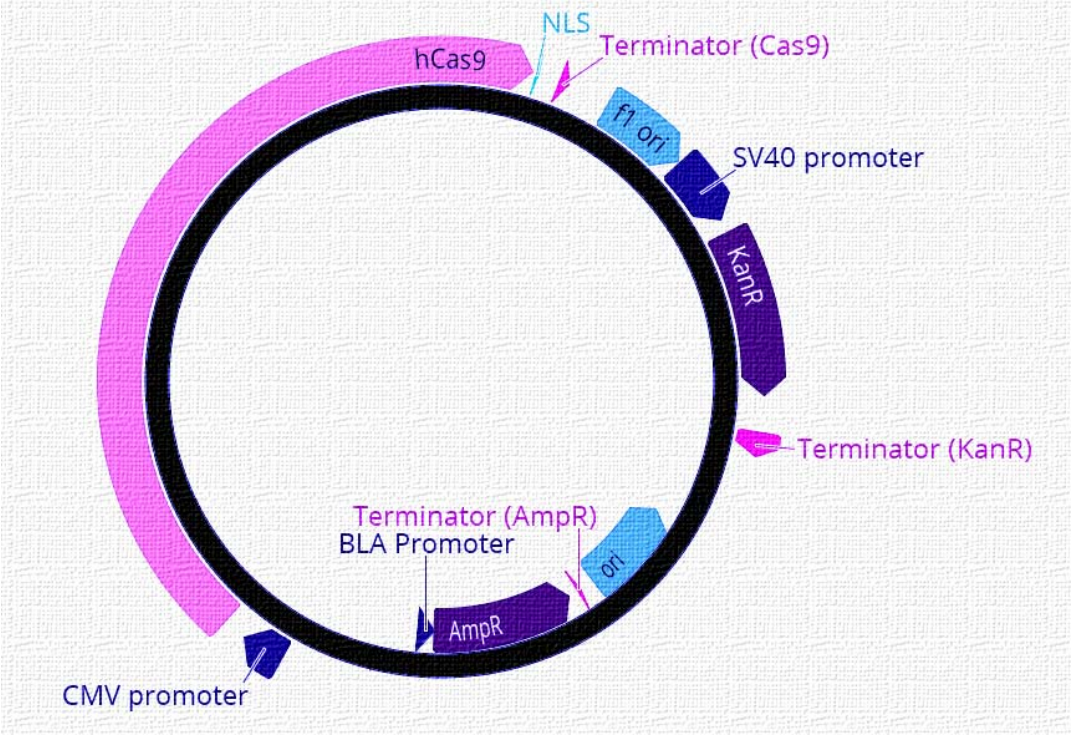


Fig. 7 hCas9.[2] The hCas9 vector expresses human codon-optimized Cas9 (Mali et al., 2013).

2.2 Modification of pX330

2.2.1 Design of gRNA Targeting Mutated *EGFP* in pDRGFP

The gRNA inserted into pX330 was designed to induce a DSB in the mutated *EGFP* gene in pDRGFP. In the event of HDR, cells repaired the DSB using pDRGFP's donor template, resulting in green fluorescence. The 20-nt gRNA was designed with appropriate overhangs for insertion into pX330. An extra guanine nucleotide was included at the 5' end to promote optimal expression by the hU6 promoter in pX330 (Graham & Root, 2015). *See* **Fig. 8**.

(A) Sequence in pDRGFP targeted by gRNA

(B) gRNA targeting pDRGFP

Fig. 8 Design of gRNA Targeting Mutated *EGFP* in pDRGFP. (A) Sequence in pDRGFP targeted by gRNA. The sequence shown here is the mutated portion of the *EGFP* gene in pDRGFP, which disrupts its expression. The I-SceI recognition sequence (gray shaded box) inactivates expression of the *EGFP* gene. Arrows (▲▼) indicate I-SceI cut sites. When Cas9 recognizes the PAM site (NGG; green underline) and the gRNA binds to the target sequence (blue underline), Cas9 induces a DSB (dashed blue line). **(B) gRNA targeting pDRGFP.** A 20-bp gRNA (blue underline) complementary to the target sequence in pDRGFP was designed to include overhangs (red underline) complementary to the overhangs in digested pX330 and an extra guanine nucleotide (pink shaded box) to promote optimal expression by the hU6 promoter expressing the gRNA in pX330 (Graham & Root, 2015).

2.2.2 Anneal and Kinase of pX330 gRNA Oligos

Top and bottom single-stranded oligos for the gRNA to be inserted into pX330 were ordered from Integrated DNA Technologies and received as dried down DNA. Each oligo was centrifuged upon receipt at 12000g for 60 seconds to concentrate the dried DNA in the bottom of the tube. The oligos were individually reconstituted to a concentration of 100µM using nuclease-free water. To anneal and kinase the top and bottom oligos, a 20µL reaction was setup on ice in a PCR tube, including 2µL of the top oligo (100µM), 2µL of the bottom oligo (100µM), 2µL of T4 DNA Ligase Buffer, 1µL of T4 PNK, and 13µL of nuclease-free water. After all components were added, the tube was tapped gently to mix and incubated in a thermocycler at 37°C for 30 minutes, 95°C for 5 minutes, then 25°C for 2 minutes.

2.2.3 Golden Gate Assembly of gRNA Into pX330

The gRNA for targeting the mutated *EGFP* gene in pDRGFP was ligated into pX330 using Golden Gate Assembly. The gRNA insert site in pX330 is flanked by two BbsI Type II restriction enzyme recognition sequences on opposing DNA strands. *See* **Fig. 9A**. BpiI, an isoschizomer of BbsI, was used to digest pX330, leaving 4-bp overhangs at the gRNA insert site. *See* **Fig. 9B**. The annealed and kinased gRNA oligo, designed to include overhangs complementary to the overhangs in the digested pX330, was then ligated into pX330 using Golden Gate Assembly, removing the BbsI recognition sequences. *See* **Figs. 9C-D**.

To ligate the gRNA into pX330 using Golden Gate Assembly, the gRNA insert and vector were diluted to establish a 6:1 ratio of insert-to-vector. A 20µL reaction was setup in a PCR tube on ice, including 1µL of the diluted pX330 vector (100ng), 1µL of the diluted gRNA insert, 2µL of T4 10X ligase buffer, 1µL of T4 DNA ligase, 1.5µL of the restriction enzyme BpiI, and 13.5µL of nuclease-free water. After all components were added, the tube was tapped gently to mix and incubated in a thermocycler for 50 cycles of 37°C for 10 minutes followed by 16°C for 5 minutes. After these 50 cycles were complete, the temperature was raised to 50°C for 5 minutes, then 80°C for 5 minutes.

Before experimental use, the modified pX330 vector was transformed, inoculated, and the resulting DNA was extracted and purified by Miniprep, then digested and analyzed by gel electrophoresis to confirm appropriate band sizes were present. *See* **Section 2.4** for protocols. The vector was also sequenced to confirm the gRNA was successfully ligated into pX330. *See* **Appendix A.**

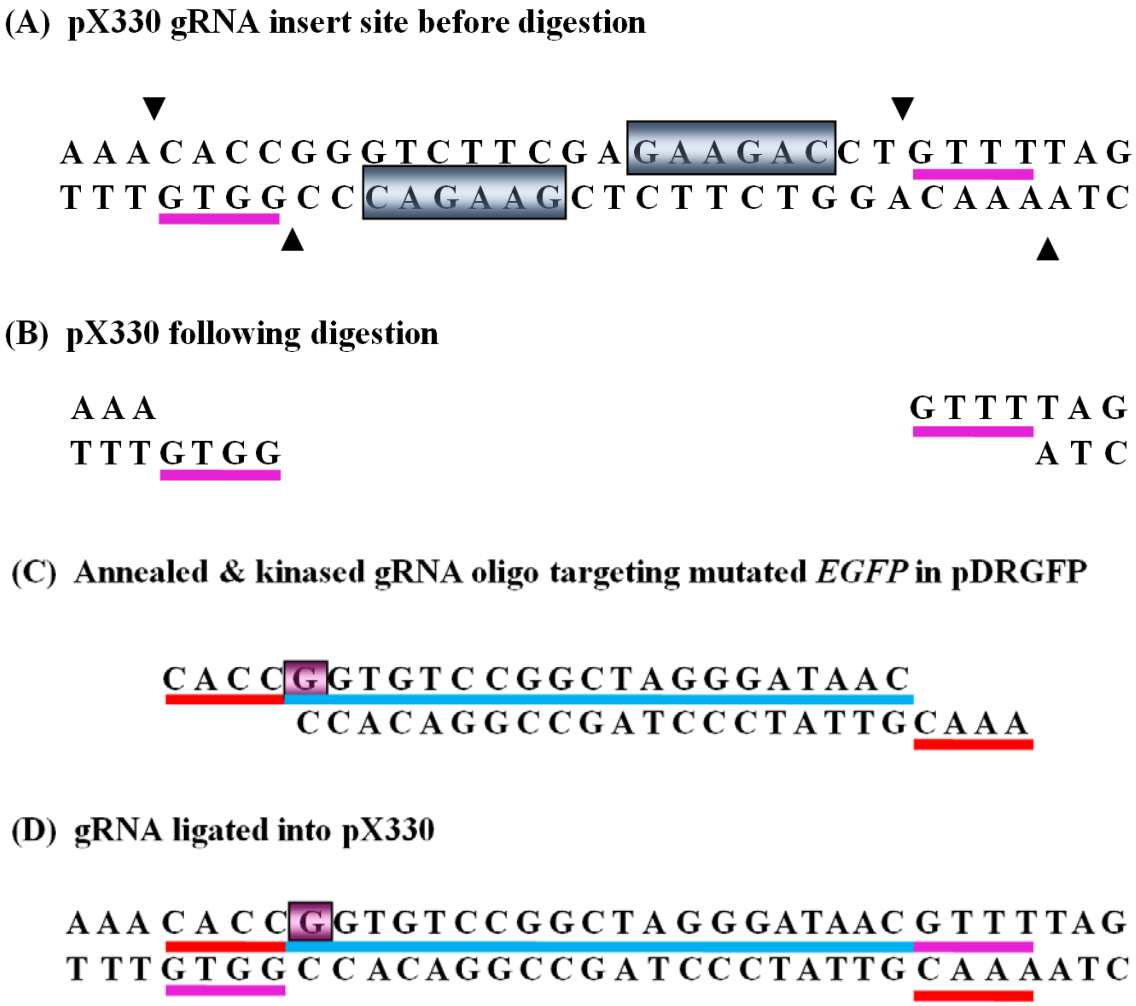


Fig. 9 Insertion of gRNA Targeting pDRGFP Into pX330. (A) pX330 gRNA insert site before digestion. The pX330 gRNA insert site included BbsI recognition sites (gray shaded boxes). BbsI cut sites are indicated by arrows (▲▼). **(B) pX330 following digestion.** After digestion with BbsI (or one of its isoschizomers, such as BpiI), the gRNA insert site is removed leaving 4-bp overhangs (purple underline). **(C) Annealed & kinased gRNA oligo targeting pDRGFP.** The 20-bp gRNA (blue underline) was designed to include overhangs (red underline) complementary to the overhangs in the digested pX330 vector. The gRNA also included an extra guanine nucleotide (pink shaded box) to promote optimal expression by pX330's hU6 promoter (Graham & Root, 2015). **(D) gRNA ligated into pX330**. The gRNA was inserted into pX330 using Golden Gate Assembly.

2.3 Modification of pJZC74

2.3.1 Design of gBlock with Customizable gRNA Insert Site

To enhance the modularity of pJZC74, a customizable gRNA insert site was designed and inserted into the vector. This gRNA insert site was then utilized to insert a variety of gRNAs targeting *LIGIV*. To facilitate insertion of the customizable gRNA insert site, a 550-bp gBlock was designed to be identical to a 550-bp section of pJZC74, with the exception of the customizable gRNA insert site that would replace pJZC74's original gRNA sequence. The gBlock was flanked on its ends by recognition sequences for the restriction enzymes XbaI and BamHI, which were used to create proper overhangs for insertion of the gBlock into pJZC74. The customizable gRNA insert site was designed to include BsmBI recognition sequences, such that when the vector was digested with BsmBI, the customizable gRNA insert site was cut away, leaving overhangs complementary to the overhangs in any custom-designed gRNA to be inserted into pJZC74. *See* **Fig. 10**.

2.3.2 Digestion of gBlock for Insertion Into pJZC74

Upon receipt, the gBlock was spun down and reconstituted in 10µL of nuclease-free water, then briefly vortexed and spun down again. Prior to insertion of the gBlock into pJZC74, a small excess of nucleotides were digested off both outer ends of the gBlock using XbaI and BamHI-HF. This created overhangs in the gBlock that were complementary to the overhangs in linearized pJZC74. The gBlock was digested using a 50µL reaction, including 10µL of diluted gBlock, 2µL of XbaI, 2µL of BamHI-HF, 5µL

of CutSmart® Buffer, and 31µL of nuclease-free water. This reaction was incubated for 2 hours at 37°C.

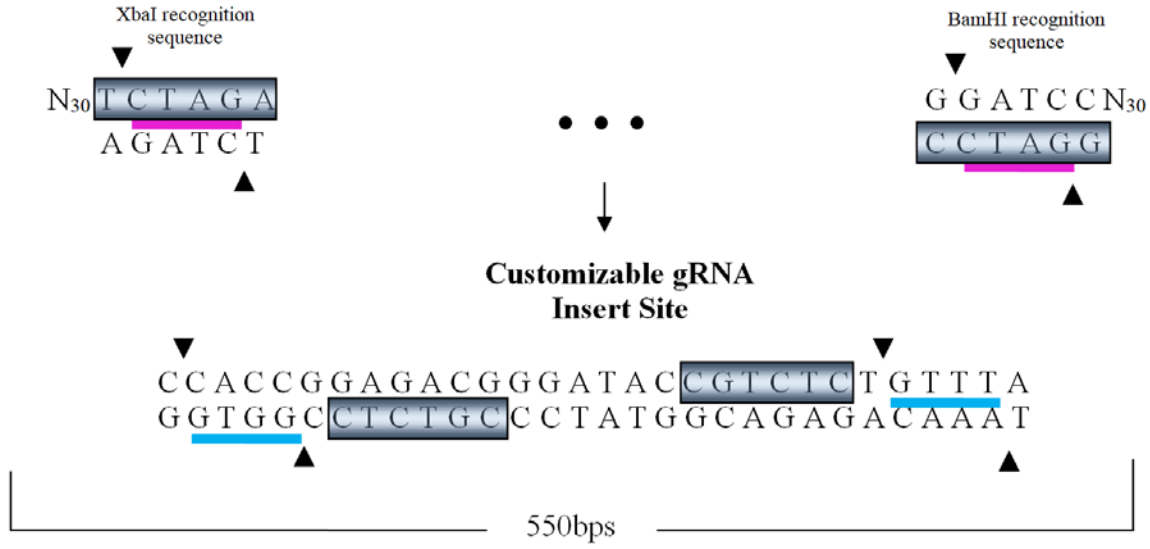


Fig. 10 gBlock with Customizable gRNA Insert Site. The entire gBlock is 550 bps. Restriction enzyme cut sites are indicated by arrows (▲▼). **Top**: A small excess of base pairs flank both ends of the gBlock (N_{30}) to allow proper binding of XbaI and BamHI on their recognition sequences (gray shaded boxes) (Green & Sambrook, 2012). Overhangs remaining after digestion with XbaI and BamHI-HF are underlined in pink. **Bottom**: Customizable gRNA insert site includes BsmBI recognition sequences (gray shaded boxes). When digested with BsmBI, 4-bp overhangs (blue underline) remain for insertion of a gRNA of interest.

2.3.3 Linearization of pJZC74

pJZC74 was linearized using restriction enzymes XbaI and BamHI-HF, whose recognition sequences flank the region of pJZC74 where the gBlock containing the customizable gRNA insert site was to be placed. *See* **Fig. 5**. A 40µL reaction was setup on ice using 10µL of pJZC74, 2µL of XbaI, 2µL of BamHI-HF, 4µL of CutSmart® Buffer, and 22µL of nuclease-free water. Digestion was performed at 37°C for 3 hours.

When 30 minutes was remaining in the incubation period, calf-intestinal alkaline phosphatase was added. The digested vector was then gel purified, along with the digested gBlock.

2.3.4 Gel Purification of Linearized pJZC74 and gBlock

Gel purification of linearized pJZC74 and the gBlock containing the customizable gRNA insert was performed using the QIAQuick Gel Extraction Kit, according to the kit instructions. A 1% agarose gel was prepared using the protocol in **Appendix G**, substituting UltraPure™ Low Melting Point Agarose for ME Agarose. The gel was cast and a wide-toothed comb was placed to allow for adequate separation of samples.

After ~1 hour, 8µL of 6X purple loading dye was added to linearized pJZC74 and the digested gBlock, which were then loaded into alternate wells in the gel. The gel electrophoresis apparatus was run at 150V. After an hour and a half, the samples were visualized using an Invitrogen Safe Imager™ 2.0, a blue light transilluminator. *See* **Appendix B**. Based on the band sizes visualized, the desired DNA fragments were identified and extracted using a clean spatula. The gel fragments were transferred to individual, sterile microcentrifuge tubes and weighed. Buffer QG was added to the gels in an amount equal to 3X the weight of the gel. The tubes were then incubated in a 58°C heat block for approximately 10 minutes to allow the gel to completely dissolve.

After briefly vortexing the samples, 100% isopropyl alcohol was added to each tube in a volume equal to 1X the weight of the gel in grams and mixed gently by pipette. 750µL of this gel-solution was transferred to a spin column and centrifuged. (All

centrifugation steps were performed at 12000g for 1 minute.) The flow-through was discarded and the remaining gel-solution was added to the spin column and centrifuged. After again discarding the flow-through, 500µL of Buffer PE was added to the spin column, the columns were centrifuged, and the flow-through was discarded. This was repeated two additional times, then a final dry spin centrifugation cycle was performed. The spin column was placed into a sterile microcentrifuge tube and 30µL of nuclease-free water was added directly to the membrane. After 3-5 minutes, the tubes were centrifuged a final time to elute the DNA.

2.3.5 Ligation of gBlock Containing Customizable gRNA Insert Site Into pJZC74

Following gel purification, the gBlock containing the customizable gRNA was ligated into pJZC74. *See* **Fig. 11**. Ligation was performed using the New England Biolabs (NEB) Quick Ligation™ Kit. A 3-fold molar excess of the gBlock was combined with 50ng of pJZC74. A 20µL reaction was setup on ice with 1.2µL of linearized pJZC74 (50ng), 3µL of the gBlock, 10µL of 2X Quick Ligation Buffer, 1µL of Quick T4 DNA Ligase, and 4.8µL of nuclease-free water. Tubes were spun down and incubated at 25°C for 10 minutes. The ligation reaction was held at -20ºC until ready for transformation, inoculation, and DNA purification. *See* **Section 2.4** for protocols. Successful ligation was confirmed by diagnostic digestion and sequencing. *See* **Appendix C**. Following ligation, any gRNA targeting a gene of interest could be ligated into pJZC74 using the customizable gRNA insert site.

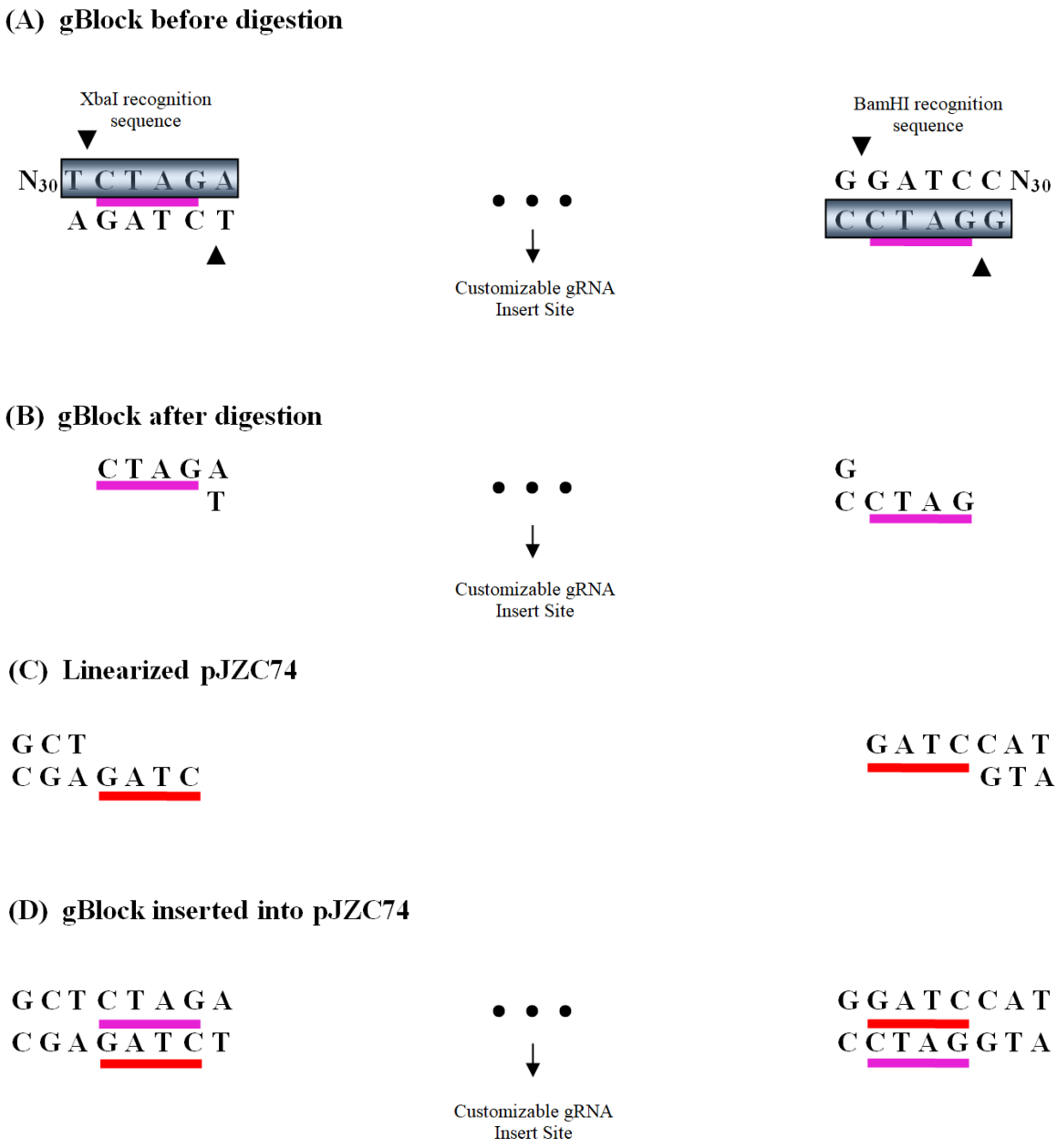


Fig. 11 Insertion of gBlock Containing Customizable gRNA Insert Site Into pJZC74. (A) gBlock before digestion. A 550-bp gBlock included restriction enzyme recognition sequences for XbaI and BamHI (gray shaded boxes) and a small excess of base pairs (N_{30}) on its ends. Restriction enzyme cut sites are indicated by arrows (▲▼). **(B) gBlock after digestion.** After digestion of the gBlock with XbaI and BamHI-HF, overhangs remain (pink underline). **(C) Linearized pJZC74.** The overhangs in linearized pJZC74 (red underline) are complementary to the overhangs in the gBlock. **(D) gBlock inserted into pJZC74.** The gBlock containing the customizable gRNA insert site was inserted into pJZC74 using the NEB Quick Ligation™ Kit.

2.3.6 Design of gRNAs Targeting *LIGIV*

Various gRNAs were inserted into pJZC74 for repression of *LIGIV*. The gRNAs were designed for robust testing of key regions of the *LIGIV* gene. *See* **Table 1** and **Fig. 12**. This includes targeting *LIGIV*'s promoters and exons, as well as regions nearby and within the coding domain sequence (CDS) of exon 4 (Zerbino et al., 2018). Several gRNAs were tested on both DNA strands to assess whether there was any impact on repression efficiency. *See* **Appendix D** for gRNA sequences.

gRNA(s)	Region of *LIGIV* targeted
1, 2	• Within promoter 1 • Within 100 bps of start of exon 1
3, 4	• Opposite strand of gRNA-1 & gRNA-2
5, 6	• Within promoter 2 & promoter 3 • Within 25 bps of start of exon 2
7	• Opposite strand of gRNA-5 & gRNA-6
8, 9*	• Within promoter 3 • Within 250 bps of start of exon 3
10, 11	• Opposite strand of gRNA-8 & gRNA-9 (first set)
12, 13	• Opposite strand of gRNA-8 & gRNA-9 (second set)
14, 15	• Within first 50 bps of exon 4 • At start of CDS of exon 4
16, 17	• Opposite strand of gRNA-14 & gRNA-15
18, 19	• Within CDS of exon 4
20	• Near end of CDS of exon 4 • Region previously targeted for knockout of *LIGIV* (Shalem et al., 2014)

***** Complementary sequences for gRNA-8 and gRNA-9 are present twice in *LIGIV*.

Table 1 Description of Regions of *LIGIV* Targeted by gRNAs. *See* **Fig. 12** for visual representation of targeted areas.

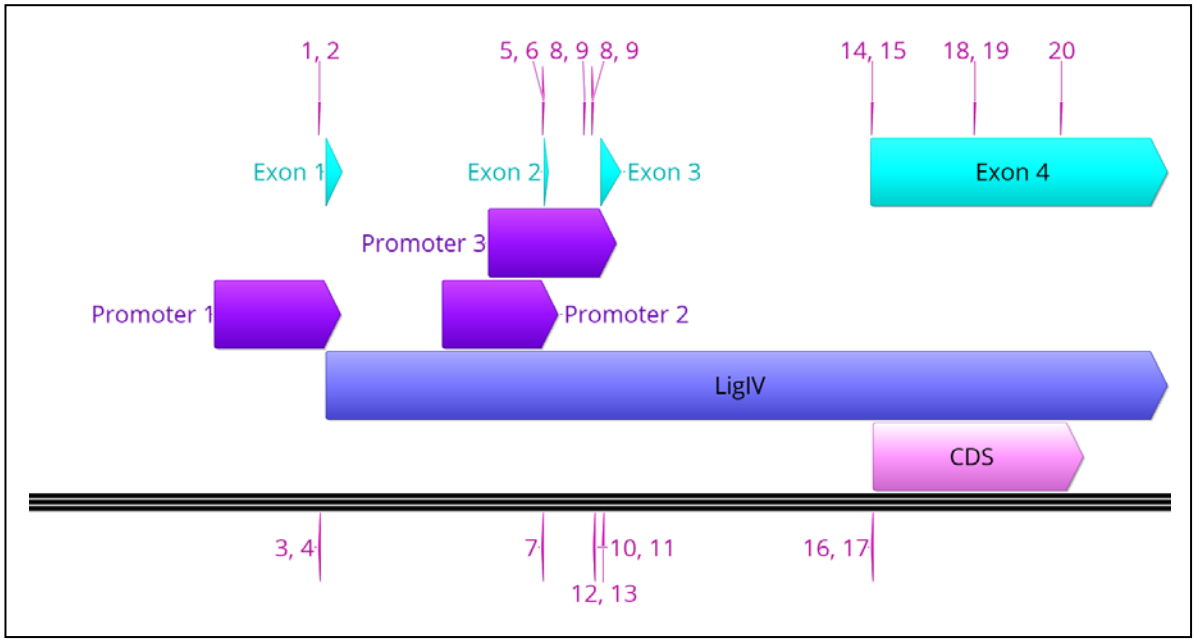


Fig. 12 Key Regions of *LIGIV* Targeted by gRNAs.[2] gRNAs target an array of regions within *LIGIV*, including its promoters, exons and the CDS of exon 4 (Zerbino et al., 2018). Complementary sequences for gRNAs 8 and 9 are each present twice in *LIGIV*.

2.3.7 Golden Gate Assembly of gRNAs Targeting *LIGIV* Into pJZC74

Top and bottom gRNA oligos designed to target *LIGIV* were previously annealed and kinased, according to the protocol in **Section 2.2.2**. To ligate each gRNA insert into pJZC74 using Golden Gate Assembly, individual 20µL reactions were setup in PCR tubes on ice, including 6.5µL of pJZC74 (100ng), 6µL of the gRNA insert, 3µL of nuclease-free water, 2µL of T4 10X ligase buffer, 1µL of T4 DNA ligase, and 1.5µL of the restriction enzyme BsmBI. BsmBI was used to digest the customizable gRNA insert site in pJZC74, creating overhangs for insertion of the gRNA. After the reactions were setup, PCR tubes were added to a thermocycler and run for 50 cycles of 37°C for 10 minutes followed by 16°C for 5 minutes. Following completion of these 50 cycles, the temperature was raised to 50°C for 5 minutes, then 80°C for 5 minutes.

Before experimental use, the modified vectors were transformed using high-fidelity 5-alpha *E. coli* cells, inoculated, and the resulting DNA was extracted and purified by Miniprep, then digested and analyzed by gel electrophoresis to confirm appropriate band sizes were present. After a gRNA of interest was inserted, the BsmBI sites were no longer present. *See* **Fig. 13**.

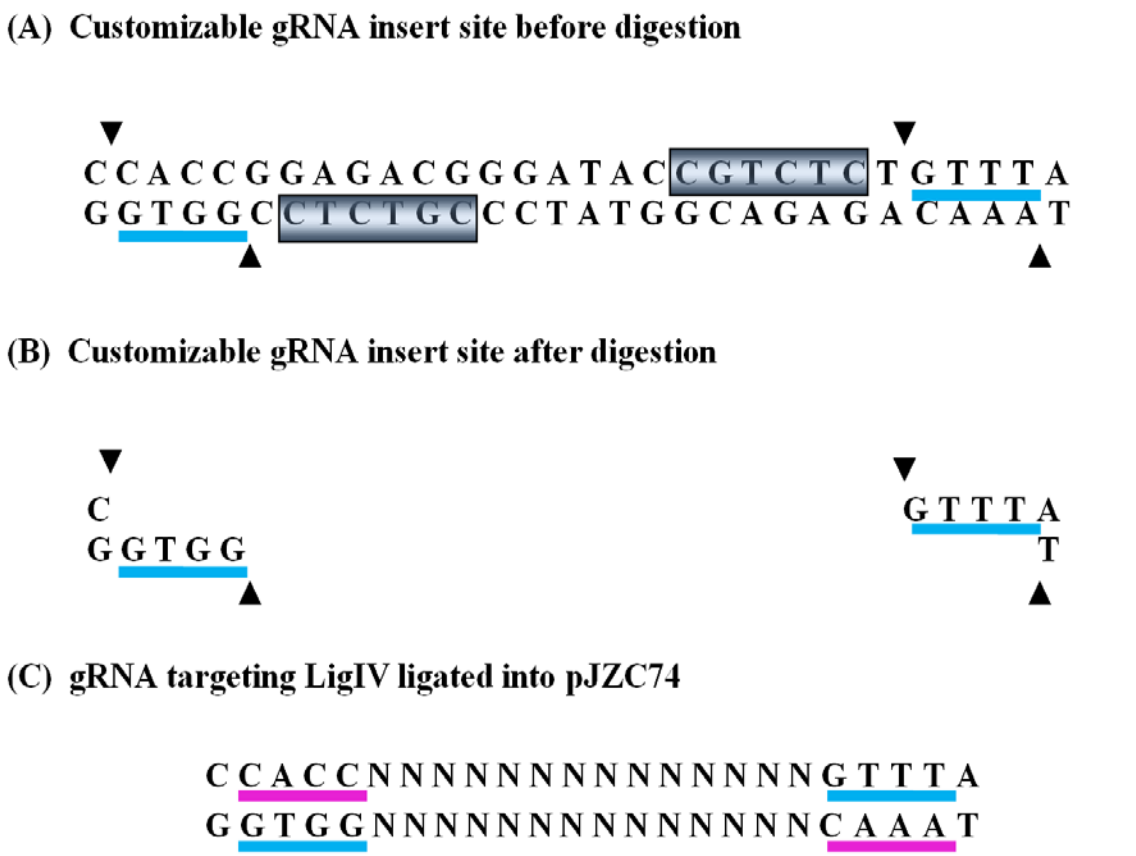


Fig. 13 Insertion of gRNA of Interest Into pJZC74. (A) Customizable gRNA insert site before digestion. BsmBI recognition sequences (gray shaded boxes) were used to digest pJZC74. BsmBI cut sites are indicated by arrows (▲▼). **(B) Customizable gRNA insert site after digestion.** Following digestion with BsmBI, the customizable gRNA insertion site was cut away and overhangs remained (blue underline). **(C) gRNA targeting *LIGIV* ligated into pJZC74.** A 14-bp gRNA (N_{14}) targeting *LIGIV* was then ligated into pJZC74 using Golden Gate Assembly.

2.4 Preparation of Vectors

2.4.1 Streaking Plates

All vectors used in this study were initially received as transformed bacteria in stab culture from Addgene. Stab cultures were stored at 4ºC for up to two weeks prior to streaking on 10cm growth plates prepared with Luria Broth (LB) agar, according to the protocol in **Appendix G**. For each vector, a clean bacterial loop was inserted into the stab culture and used to gently streak the bacteria containing the vector on the surface of one third of a plate. The initial streak was then spread across an additional third of the plate with a clean bacterial loop, and this was repeated a final time. Plates were inverted and incubated overnight (12-18 hours) at 37°C. After the incubation period, plates were removed and checked for bacterial colonies sufficient for inoculation.

2.4.2 Transformation of Modified Vectors Into *E. coli*

After vectors were modified, such as to contain a custom gRNA, transformations were performed using chemically competent 5-alpha *E. coli*. For high efficiency transformations, chemically competent *E. coli* cells were purchased from NEB in single-use 50µL vials. Chemically competent *E. coli* were also prepared by lab personnel. All competent cells were stored at -80°C until use.

For each transformation, one vial of chemically-competent bacteria was thawed on ice for 10 minutes. During this time, a heat block was set to 42°C and its wells were filled with deionized (DI) water. After 10 minutes, 4µL of the ligation reaction was

added to the thawed cells, and each tube was tapped 3-4 times to mix then placed back on ice for 15 minutes.

After 15 minutes, tubes were placed into the heat block for 30 seconds, then returned to ice for 5 minutes. LB agar plates with Ampicillin were removed from the 4°C refrigerator and placed inverted into a bacterial incubator set at 37°C for pre-warming. After 5 minutes, each tube received 500µL of LB broth. Tubes were then placed into an incubator shaker set at 37°C and agitated at 250 rpm for 1 hour.

After 1 hour, 100µL of the bacterial mix was added in droplets onto a pre-warmed plate and streaked across the plate using a bacterial loop. Plates were inverted and placed into a 37°C incubator for 12-14 hours to allow for colony growth. Colonies were then inoculated and the DNA was extracted and purified by Miniprep and/or Midiprep. Diagnostic digestion, gel electrophoresis, and sequencing were subsequently performed to confirm the vector was successfully modified.

2.4.3 Inoculation for Miniprep

A serological pipette was used to pipette 3mL of LB media containing Ampicillin into 15mL polypropylene culture tubes with aerated lids. A single, isolated bacterial colony was removed by gently passing a clean pipette tip over the colony and ejecting the tip into the culture media. After 12-16 hours of agitation in an incubator shaker set at 37°C and 250 rpm, tubes were removed and the DNA was extracted and purified by Miniprep.

2.4.4 Inoculation for Midiprep

Certain modified vectors that were validated by diagnostic digestion and gel electrophoresis were inoculated in preparation for Midiprep. Fifty µL of bacterial culture was added to 50mL of LB containing Ampicillin in a sterile, vented culture flask. The cultures were agitated for 12-16 hours in a 37°C / 250 rpm incubator shaker, and the DNA was subsequently extracted and purified by Midiprep.

2.4.5 Miniprep DNA Extraction and Purification

Minipreps were performed using the QIAprep Spin Miniprep Kit, according to kit instructions. All centrifugation steps were performed at 4°C. Prior to initial use of the kit, RNAse and LyseBlue reagents were added to Buffer P1 and molecular biology grade 100% ethanol was added to Buffer PE.

Following inoculation, 1.5µL of each overnight bacterial culture was transferred to a sterile microcentrifuge tube and centrifuged at 2000g for 5 minutes. Following centrifugation, the bacterial media was discarded. The pelleted cells remaining were resuspended by adding 250µL of Buffer P1 and sweeping the tube across an empty microcentrifuge rack multiple times. To lyse the cells, 250µL of Buffer P2 was added and tubes were inverted gently 3-4 times to mix. After no more than 5 minutes, 350µL of Buffer N3 was added to halt lysis, and tubes again were inverted gently to mix. Tubes were centrifuged at 12000g for 10 minutes, and the resulting supernatant was transferred to a spin column. (All subsequent centrifugation steps were performed at 12000g for 1 minute.) Three centrifugation cycles were performed, the first following addition of

500µL of Buffer PB to the supernatant, and the next two after adding 750µL of Buffer PE with ethanol. After each centrifugation cycle, the flow-through was discarded before the next buffer was added. A final centrifugation spin was performed, and the spin columns were placed into sterile 1.5µL microcentrifuge tubes. Forty µL of nuclease-free water was added directly to the spin column. After 5 minutes, tubes were then centrifuged a final time to elute the DNA. The DNA concentration was determined using the NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (NanoDrop™).

2.4.6 Midiprep DNA Extraction and Purification

Midiprep was performed for samples previously confirmed via diagnostic digestion and/or sequencing. Bacterial cultures were obtained from cold storage and inoculated for Midiprep. Following overnight agitation, Midiprep DNA extraction and purification was performed using the Qiagen Plasmid *Plus* Midi Kit, according to the kit instructions. All centrifugation steps were performed at 4°C.

The Midiprep culture was poured from its overnight culture flask into a sterile 50mL Falcon tube and centrifuged at 3000g for 15 minutes. The bacterial media was discarded, leaving the pelleted cells, and 4mL of Buffer P1 was added. The cells were resuspended in Buffer P1 using a serological pipette. To lyse the cells, 4mL of Buffer P2 was added and tubes were inverted gently 3-4 times to mix. After no more than 3 minutes, 4mL of Buffer S3 was added to halt lysis and tubes were gently inverted 3-4 times. Each reaction was poured into a filter cartridge placed into a sterile 50mL Falcon tube and allowed to stand for 10 minutes. Using a plunger, the filtrate was filtered into

the 50mL Falcon tube, and 2mL of Buffer BB was added. Tubes were inverted to mix. After pouring this solution into a tube extender that had been placed into a spin column, the solution was filtered through the spin column using a vacuum.

A series of three vacuum filtration steps were subsequently performed, the first following addition of 700µL of Buffer ETR, and the next two following addition of 700µL of Buffer PE containing molecular biology grade 100% ethanol. A dry centrifugation spin was subsequently performed at 10000g for 1 minute, and the spin columns were transferred to sterile 1.5µL microcentrifuge tubes. To elute the DNA, 200µL of nuclease-free water was added to the spin column membrane and, after 7 minutes, tubes were centrifuged at 10000g for 1 minute.

2.4.7 Diagnostic Digestion

All reactions were performed in PCR tubes on ice. For each diagnostic digestion, a 20µL reaction was prepared, including 300-600ng of the DNA to be digested, 2µL of an enzyme-compatible buffer, 1µL of 1-2 restriction enzymes, and sufficient nuclease-free water to establish a 20µL total reaction volume. If multiple enzymes were used, a buffer compatible with both enzymes was selected. A master mix containing buffer, restriction enzymes, and nuclease-free water was prepared and thoroughly mixed by pipette. An appropriate amount of master mix and DNA was added to each PCR tube. Tubes were briefly tapped and spun down to mix, then added to the thermocycler using appropriate time and temperature conditions for the particular restriction enzymes used.

After the reaction was complete, gel electrophoresis was performed to assess whether the expected band sizes were present.

2.4.8 Agarose Gel Electrophoresis

A gel was prepared according to the procedure in **Appendix G**. Fifteen µL of a 1kb DNA ladder was added to one or more wells, as appropriate. Four µL of 6X purple loading dye was added to each digested DNA sample. Samples were mixed once by pipette and loaded into individual wells. The gel electrophoresis apparatus was run at 150V for at least 30 minutes. Band sizes were subsequently visualized under UV or blue light.

2.5 Transfection Using Human Embryonic Kidney Cells

2.5.1 Passaging Human Embryonic Kidney Cells for Transfection

All procedures were performed using sterile technique within a biosafety cabinet. Human embryonic kidney (HEK) cells were maintained on 10cm growth plates and passaged approximately every 3 days when the cells approached confluency. Four mL of 1X PBS (-/-), 2mL of trypsin diluted with PBS (0.05%) , and an appropriate volume of Dulbecco's Modified Eagle's Medium (DMEM) were pre-warmed in a water bath. Using a vacuum, the existing DMEM on the growth plate was aspirated with a glass Pasteur pipette. Using a serological pipette on the gravity setting, 4mL of pre-warmed 1X PBS was added gently along the side of the plate. After briefly swirling to distribute, the 1X

PBS was aspirated and 2mL of trypsin was added to detach the cells. The plate was incubated in a 37ºC / 5% CO_2 incubator for 5 minutes.

After incubation, the plate was returned to the biosafety cabinet and 10mL of DMEM was added. The plate was washed down and cells were resuspended using a serological pipette. The cell suspension was placed into a sterile 15mL Falcon tube and centrifuged at 300g for 5 minutes. Following centrifugation, the supernatant was aspirated from the pelleted cells. Four mL of pre-warmed DMEM was added and the cells were re-suspended. One mL of the cell suspension was added to a separate 15mL Falcon tube containing 3mL of pre-warmed DMEM, pipetting to mix. After adding 13mL of pre-warmed DMEM to a sterile growth plate, 1mL of the cell suspension was added to the new growth plate in drops using a serological pipette on the gravity setting. The plate was swirled to distribute and placed into a 37ºC / 5% CO_2 incubator. The DMEM was changed daily as described in **Appendix G**.

2.5.2 Collagen-Coating 24-Well Plates for Transfection

All procedures were performed using sterile technique within a biosafety cabinet. To collagen-coat a 24-well plate in preparation for transfection, 12mL of PBS was aliquoted into a 15mL Falcon tube, along with 40µL of 3000X collagen. Each well of the 24-well plate received 0.5mL of this solution, which was allowed to coat the wells for an hour or more. After coating, the 24-well plate was seeded with HEK cells.

2.5.3 Seeding 24-Well Plates for Transfection

The initial steps of this protocol are the same as the steps described in **Section 2.5.1** through the centrifugation step at 300g for 5 minutes to pellet the cells. Following centrifugation and aspiration of the supernatant, 3mL of pre-warmed DMEM was added to the pellet and the cells were resuspended by serological pipette. Twelve mL of pre-warmed DMEM was added to a clean Falcon tube, along with 1mL of the resuspended cells, and the solution was mixed well. To seed the 24-well plate, the collagen solution was thoroughly aspirated from the 24-well plate using a glass pipette within the biosafety cabinet. To aliquot the cells, 0.5mL of the cell suspension was added to the coated wells using a serological pipette set to gravity. The plate was then returned to the 37ºC / 5% CO_2 incubator until ready for transfection.

2.5.4 Performing Transfection

Transfections were generally performed when HEK cells were 60-90% confluent, as confirmed by microscope analysis. DNA samples were prepared in duplicate for fluorescence-activated cell sorting (FACS) analysis and in quadruplicate if quantitative polymerase chain reaction (qPCR) analysis was also to be performed. All transfections were performed using sterile technique within a biosafety cabinet.

DNA samples were diluted to the desired concentrations by mixing DNA with an appropriate volume of nuclease-free water in sterile 1.5µL microcentrifuge tubes. A DNA mix was then prepared by adding an appropriate volume of the desired DNA

components to sterile 1.5µL microcentrifuge tubes. The total DNA concentration per well was normalized across all tubes by adding non-coding DNA, as appropriate.

To prepare for transfection, polyethylenimine (PEI) was removed from the -80°C freezer and allowed to thaw. The thawed PEI was vortexed and an appropriate amount was aliquoted to a sterile 1.5µL microcentrifuge tube in a volume necessary to achieve a 2:1 ratio of PEI-to-DNA per well. An appropriate volume of serum-free DMEM was added to the PEI, such that a total volume of 25µL of the DMEM-PEI solution would be available for each well.

An appropriate volume of serum-free DMEM was added to each DNA mix sufficient to bring the total volume of the DMEM-DNA solution to 25µL for each well designated to receive the DNA components. The aliquoted DMEM-PEI solution was vigorously vortexed, and the appropriate volume was added to each DMEM-DNA mix. Each reaction was vortexed twice for two seconds immediately after adding the DMEM-PEI solution. All reactions were allowed to sit for 30 minutes within the biosafety cabinet. During this time, the DMEM was changed on the previously-seeded 24-well plates using the protocol in **Appendix G**. After 30 minutes, each reaction was individually pipetted once to mix, and 50µL of each reaction was added to each designated well in drops. At appropriate intervals, the transfection plate was tilted and swirled gently to distribute.

After all transfection components were added to the 24-well plate, the plate was returned to the 37ºC / 5% CO_2 incubator. DMEM was changed every 24 hours using the protocol in **Appendix G**. FACS analysis was generally performed after 72 hours. qPCR

analysis was also performed after 72 hours or RNA was extracted and stored at -80ºC until ready for use.

2.6 Analytical Methods

2.6.1 Fluorescence Microscope Imaging

Images of cells were collected using an EVOS® FL Cell Imaging System set at 4x magnification. Images were viewed on appropriate color channels, such as DAPI and GFP, to visualize transfection efficiency and repair of the mutated *EGFP* gene in pDRGFP, respectively, as compared to controls. Imaging was done before transfection or qPCR analysis and during the cell culturing process.

2.6.2 Flow Cytometry with Fluorescence-Activated Cell Sorting Workstation

Flow cytometry was performed using the BD FACSCelesta™ Flow Cytometer, generally 72 hours after transfection. For each well of a transfection plate to be analyzed by FACS, 100µL of PBS-diluted trypsin (0.05%) and 450µL of 1X HBSS (Ca-/Mg-) / 2% fetal bovine serum (FBS), or HBSS/FBS media, was pre-warmed at room temperature. Working on a sterile bench, the media in each 24-well plate to be analyzed was discarded. To dissociate the cells, 100µL of trypsin was added to each well and the plate was secured in a compact digital waving rotator set to 100rpm for 3-5 minutes. After this short incubation period, 200µL of HBSS/FBS media was added to each well. The contents of each well were gently resuspended by pipette and placed into individual wells on a 96-well plate. After centrifugation at 1500rpm for 2 minutes, the supernatant

was aspirated from each well using a glass pipette, leaving pelleted cells in the bottom of each well. Each well then received 250µL of HBSS/FBS media, and the cells were gently resuspended a final time. A calibration bead suspended in 200µL of HBSS/FBS was added to a single well on the 96-well plate. The plate settings were input into the BDS FACSDiva™ Software, and FACS was performed using a high-throughput sampler.

2.6.3 Flow Cytometry Analysis

FACS data was analyzed using FlowJo® v10. Appropriate gates were added to omit debris and dead cells and to isolate cells that had been successfully transfected (i.e., those containing enhanced blue fluorescence protein, or EBFP). Color compensation was performed, and the geometric mean of GFP^+ cells for each well was determined.

2.6.4 RNA Extraction

RNA extraction was either automated using the QIAcube robotic workstation, described below, or manually performed using the RNeasy Plus Mini Kit, per kit instructions.

To begin preparing the cells for analysis in the QIAcube workstation, the DMEM was completely aspirated from the 24-well plates from which the RNA was to be extracted. To lyse the cells, 350µL of Buffer RLT Plus, previously prepared with β-mercaptoethanol, was added to each well, and the wells were scraped with a pipette tip to thoroughly dissociate the cell lysate. The cell lysate solution was added to a microcentrifuge tube, for a total volume of 700µL per conditions prepared in duplicate,

and placed on ice. Tubes were centrifuged at 17rpm for 3 minutes and promptly returned to ice.

QIAcube rotor adapters were prepared, each with a QIAshredder spin column placed in a 2mL collection tube, a gDNA Eliminator spin column placed in a 2mL collection tube, and a 1.5mL elution tube. Bottles containing all appropriate reagents, including Buffer RW1, Buffer RPE, 70% ethanol, and RNA-free water were filled to the maximum levels. For each condition, 350µL of the cell lysate solution was added to a 1.5mL microcentrifuge tube and loaded into the QIAcube workstation. The remaining cell lysate was stored at -80ºC.

Following RNA purification by the QIAcube workstation, the RNA concentration of each sample was measured using the NanoDrop™, while keeping the tubes on ice. RNA was stored at -80ºC until ready for cDNA synthesis.

2.6.5 cDNA Synthesis

All RNA samples and cDNA synthesis kit components were kept on ice throughout this protocol. RNA samples stored at -80ºC following RNA purification were thawed on ice and briefly spun down. Each RNA sample was diluted in PCR tubes to 100ng/µL using nuclease-free water. Tubes were tapped briefly and spun down to mix. The appropriate volume of a master mix was prepared such that each reaction would receive 2µL of 10X RT Buffer, 0.8µL of 25X dNTP Mix, 2µL of 10X RT Random Primers, 1µL of Reverse Transcriptase, and 9.2µL of nuclease-free water. The master mix was tapped briefly to mix and spun down.

Fifteen µL of the master mix solution was combined with 5µL of RNA (100ng/µL) diluted to 100ng/µL in fresh PCR tubes on ice, such that each tube contained 500ng of RNA along with the master mix components. Tubes were tapped gently, spun down, and run on the thermocycler at 25ºC for 10 minutes, 37ºC for 2 hours, then 85ºC for 5 minutes. Following cDNA synthesis, samples were held briefly at 4ºC in the thermocycler.

All RNA was assumed to have been converted to cDNA. Thus the total cDNA concentration was assumed to be 500ng following cDNA synthesis. Samples were stored at -20ºC or analyzed immediately by qPCR.

2.6.6 qPCR Analysis

The gene of interest in this study was *LIGIV*. The endogenous control used was *18S rRNA*. Primers and cDNA (if previously stored at -20ºC) were thawed on ice. cDNA was diluted to 5ng/µL by resuspending 5µL of cDNA (at 500ng) in 20µL of nuclease-free water. Master mix solutions were prepared for the gene of interest, *LIGIV*, and the endogenous control gene, *18S rRNA*. The master mix for *LIGIV* included 10µL of SYBR® Green Mastermix, 1µL of *LIGIV* Primetime primer, and 5µL of nuclease-free water. The master mix for *18S rRNA* included 10µL of SYBR® Green Mastermix, 1µL of *18S rRNA* forward primer, 1µL of *18S rRNA* reverse primer, and 4µL of nuclease-free water. Each master mix was tapped gently to mix and spun down before use.

For both *LIGIV* and *18S rRNA*, 16µL of the appropriate master mix and 4µL of cDNA was added to individual wells of a 96-well tray. Each cDNA sample was tested in

duplicate. The tray was covered with film and centrifuged at 1500g for 2 minutes, then loaded into QuantStudio® 3. Comparative CT ($\Delta\Delta C_T$) was performed to analyze *LIGIV* gene repression.

3. RESULTS

3.1 Initial Transfection and qPCR Analysis of gRNAs Targeting *LIGIV*

An initial transfection in HEK cells was performed, with samples receiving the components listed in **Table 2.** Twenty gRNAs, each in its own pJZC74 vector, were initially tested at both 250ng and 25ng with a corresponding amount of hCas9. Each sample received 20ng of EBFP as a transfection control. Following transfection, RNA extraction and cDNA synthesis, qPCR was performed to measure the expression of *LIGIV*. Several gRNAs achieved robust repression of *LIGIV*. *See* **Fig. 14.**

Sample	**Components**
Control	20ng EBFP + 250ng pJZC74 with customizable gRNA insert site
1	20ng EBFP + 250ng gRNA-1 + 250ng hCas9
2	20ng EBFP + 25ng gRNA-1 + 25ng hCas9
3	20ng EBFP + 250ng gRNA-2 + 250ng hCas9
4	20ng EBFP + 25ng gRNA-2 + 25ng hCas9
5	20ng EBFP + 250ng gRNA-3 + 250ng hCas9
6	20ng EBFP + 25ng gRNA-3 + 25ng hCas9
7	20ng EBFP + 250ng gRNA-4 + 250ng hCas9
8	20ng EBFP + 25ng gRNA-4 + 25ng hCas9
9	20ng EBFP + 250ng gRNA-5 + 250ng hCas9
10	20ng EBFP + 25ng gRNA-5 + 25ng hCas9
11	20ng EBFP + 250ng gRNA-6 + 250ng hCas9
12	20ng EBFP + 25ng gRNA-6 + 25ng hCas9
13	20ng EBFP + 250ng gRNA-7 + 250ng hCas9
14	20ng EBFP + 25ng gRNA-7 + 25ng hCas9
15	20ng EBFP + 250ng gRNA-8 + 250ng hCas9
16	20ng EBFP + 25ng gRNA-8 + 25ng hCas9
17	20ng EBFP + 250ng gRNA-9 + 250ng hCas9

18	20ng EBFP + 25ng gRNA-9 + 25ng hCas9
19	20ng EBFP + 250ng gRNA-10 + 250ng hCas9
20	20ng EBFP + 25ng gRNA-10 + 25ng hCas9
21	20ng EBFP + 250ng gRNA-11 + 250ng hCas9
22	20ng EBFP + 25ng gRNA-11 + 25ng hCas9
23	20ng EBFP + 250ng gRNA-12 + 250ng hCas9
24	20ng EBFP + 25ng gRNA-12 + 25ng hCas9
25	20ng EBFP + 250ng gRNA-13 + 250ng hCas9
26	20ng EBFP + 25ng gRNA-13 + 25ng hCas9
27	20ng EBFP + 250ng gRNA-14 + 250ng hCas9
28	20ng EBFP + 25ng gRNA-14 + 25ng hCas9
29	20ng EBFP + 250ng gRNA-15 + 250ng hCas9
30	20ng EBFP + 25ng gRNA-15 + 25ng hCas9
31	20ng EBFP + 250ng gRNA-16 + 250ng hCas9
32	20ng EBFP + 25ng gRNA-16 + 25ng hCas9
33	20ng EBFP + 250ng gRNA-17 + 250ng hCas9
34	20ng EBFP + 25ng gRNA-17 + 25ng hCas9
35	20ng EBFP + 250ng gRNA-18 + 250ng hCas9
36	20ng EBFP + 25ng gRNA-18 + 25ng hCas9
37	20ng EBFP + 250ng gRNA-19 + 250ng hCas9
38	20ng EBFP + 25ng gRNA-19 + 25ng hCas9
39	20ng EBFP + 250ng gRNA-20 + 250ng hCas9
40	20ng EBFP + 25ng gRNA-20 + 25ng hCas9

Table 2 Components of Transfection for Initial Screening of *LIGIV* gRNAs. All cells received 20ng of EBFP as a transfection control. Each sample received pJZC74 containing a single gRNA targeting *LIGIV*. Each gRNA was tested at both 250ng and 25ng of pJZC74, along with a corresponding amount of hCas9. An appropriate amount of non-coding DNA was added to each well to normalize the total amount of DNA per well to ~520ng.

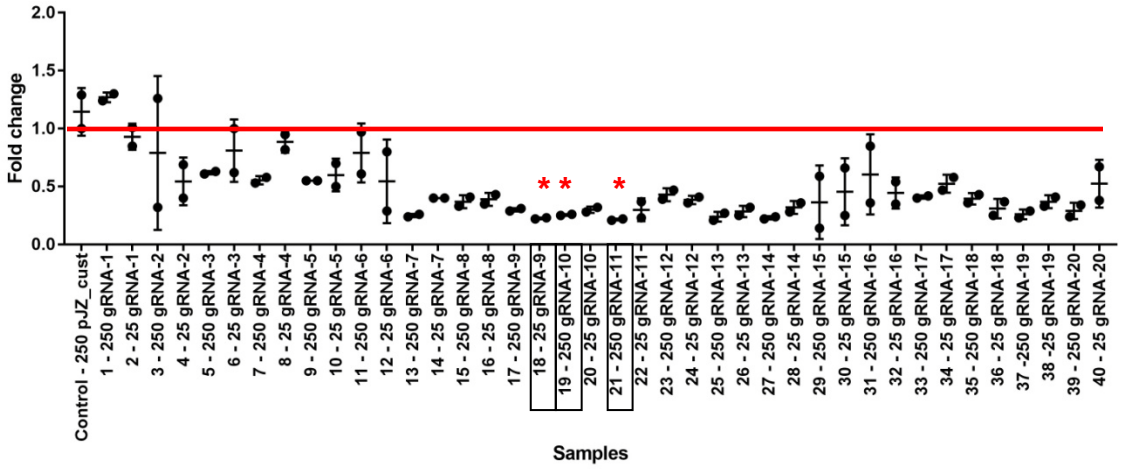


Fig. 14 Initial qPCR Results Showing Repression of *LIGIV* by gRNAs. Samples received the components listed in **Table 2**. The control sample contained pJZC74 with the customizable gRNA insert site, but no gRNA. Anything below the control (1.0) indicates *LIGIV* repression. Samples 18, 19 and 21, containing gRNA-9, gRNA-10 and gRNA-11 respectively, showed significant repression of *LIGIV* and were selected for further analysis.

3.2 Further qPCR Analysis of Select gRNAs Targeting *LIGIV*

gRNAs 9, 10 and 11 all achieved robust repression of *LIGIV* following initial qPCR analysis and were selected for further testing. These gRNAs all fall within promoter 3 and within ~250 bps of the start of exon 3. *See* **Fig. 15**. Successful ligation of these select gRNAs into pJZC74 was confirmed by diagnostic digestion and sequencing. *See* **Appendix E.**

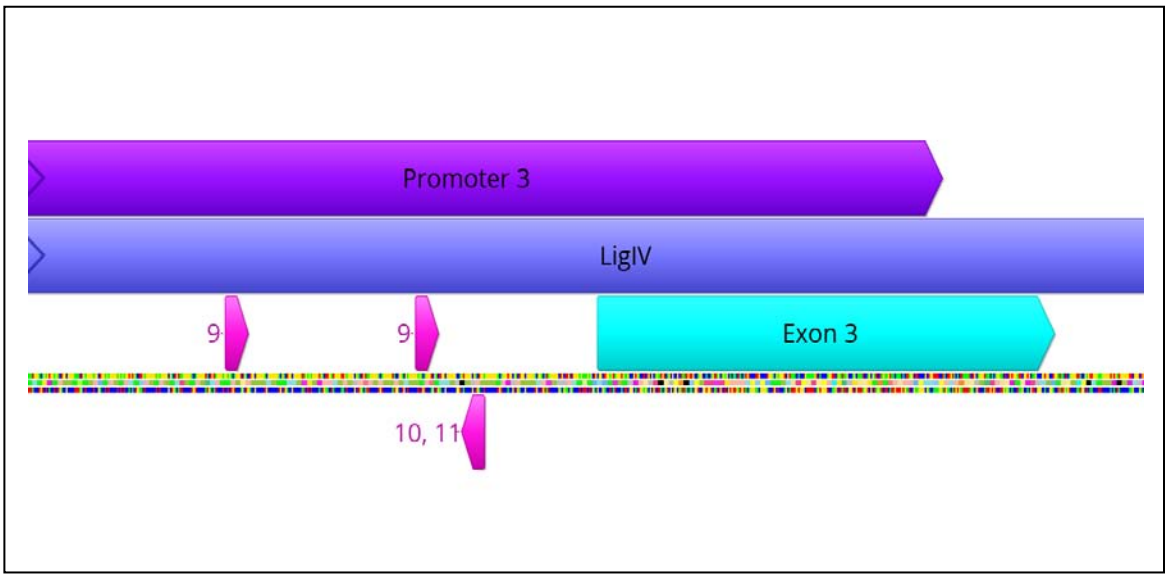


Fig. 15 Region of *LIGIV* Targeted by gRNAs Selected for Further Testing.[2]

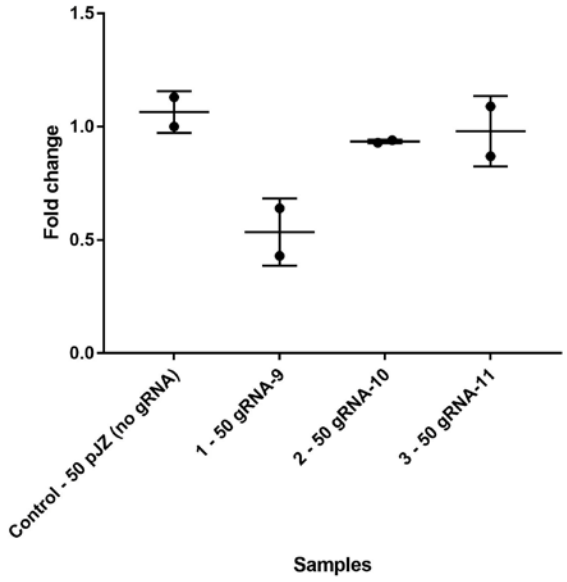
An additional transfection in HEK cells was performed, with each sample receiving the components listed in **Table 3** and **Table 4.** Each gRNA was tested at 50ng and 100ng of pJZC74 (containing the indicated gRNA) with a corresponding amount of pX330 (containing the gRNA targeting pDRGFP) and hCas9 (50ng or 100ng) and 250ng of pDRGFP. All samples received 20ng of EBFP as a transfection control. Following transfection, RNA extraction and cDNA synthesis, qPCR was performed to assess the resulting repression of *LIGIV*. *See* **Fig. 16.**

Sample	Components
Control	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng pJZC74 (no gRNA) + 50ng hCas9
1	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng **pJZC74-gRNA-9** + 50ng hCas9
2	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng **pJZC74-gRNA-10** + 50ng hCas9
3	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng **pJZC74-gRNA-11** + 50ng hCas9

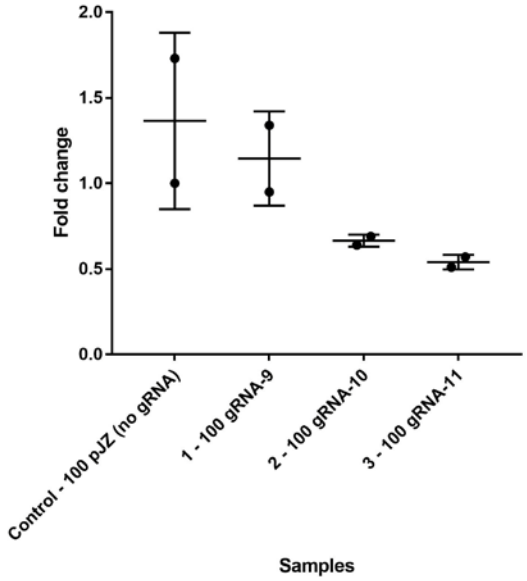
Table 3 Components of Transfection with 50ng of pJZC74 for qPCR Analysis.

Sample	Components
Control	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng pJZC74 (no gRNA) + 100ng hCas9
1	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng **pJZC74-gRNA-9** + 100ng hCas9
2	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng **pJZC74-gRNA-10** + 100ng hCas9
3	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng **pJZC74-gRNA-11** + 100ng hCas9

Table 4 Components of Transfection with 100ng of pJZC74 for qPCR Analysis.



(A) qPCR results (50ng of pJZC74 with gRNA)



(B) qPCR results (100ng of pJZC74 with gRNA)

Fig. 16 qPCR Results Showing Repression of *LIGIV* Using Select gRNAs. Control samples at 50ng and 100ng received the same components as the other samples, except pJZC74 only contained the customizable gRNA insert site. An appropriate amount of non-coding DNA was added, as needed, to normalize the total amount of DNA per well to ~570ng. At 50ng of pJZC74 (**top**), gRNA-9 achieved the most repression of *LIGIV*. At 100ng of pJZC74 (**bottom**), gRNA-11 was the best-performing gRNA.

3.3 FACS Analysis of Select gRNAs Targeting *LIGIV*

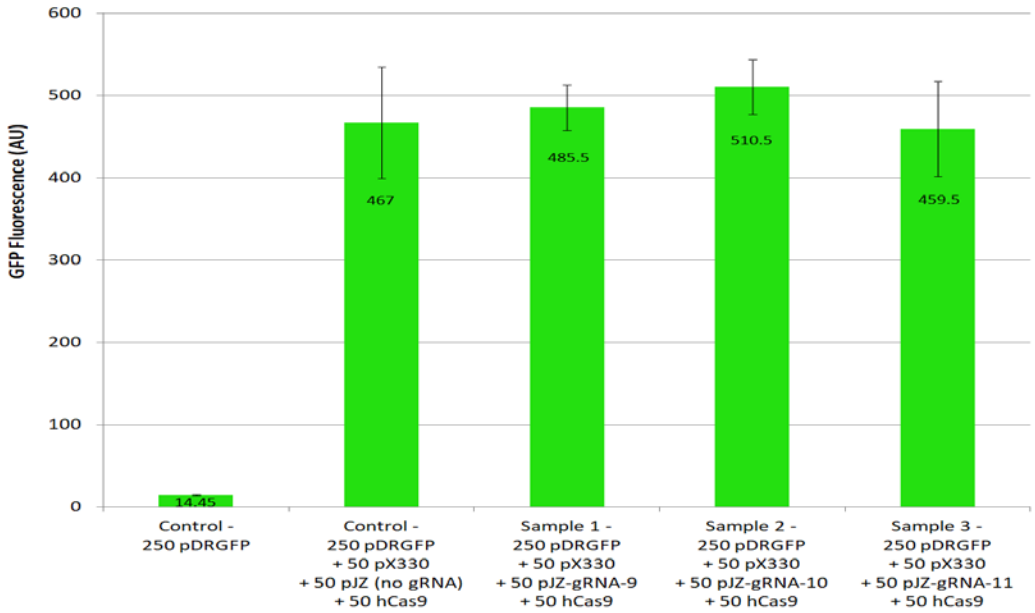
The same components transfected into HEK293FT cells to test for repression of *LIGIV* using qPCR analysis were also transfected to test for gene editing using FACS analysis. Each sample received the components listed in **Table 5** and **Table 6.** Each gRNA was tested at 50ng and 100ng of pJZC74 (containing the indicated gRNA) with a corresponding amount of pX330 targeting pDRGFP and hCas9 (50ng or 100ng), as well as 250ng of pDRGFP. Results by FACS analysis show high levels of GFP compared to a control containing only pDRGFP, indicating that HDR repair and, therefore, gene editing occurred. *See* **Fig. 17.** Fluorescence images of transfected HEK cells before FACS analysis was performed are shown in **Appendix F.**

Sample	Components
Control	20ng EBFP + 250ng pDRGFP
Control	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng pJZC74 (no gRNA) + 50ng hCas9
1	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng **pJZC74-gRNA-9** + 50ng hCas9
2	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng **pJZC74-gRNA-10** + 50ng hCas9
3	20ng EBFP + 250ng pDRGFP + 50ng pX330 + 50ng **pJZC74-gRNA-11** + 50ng hCas9

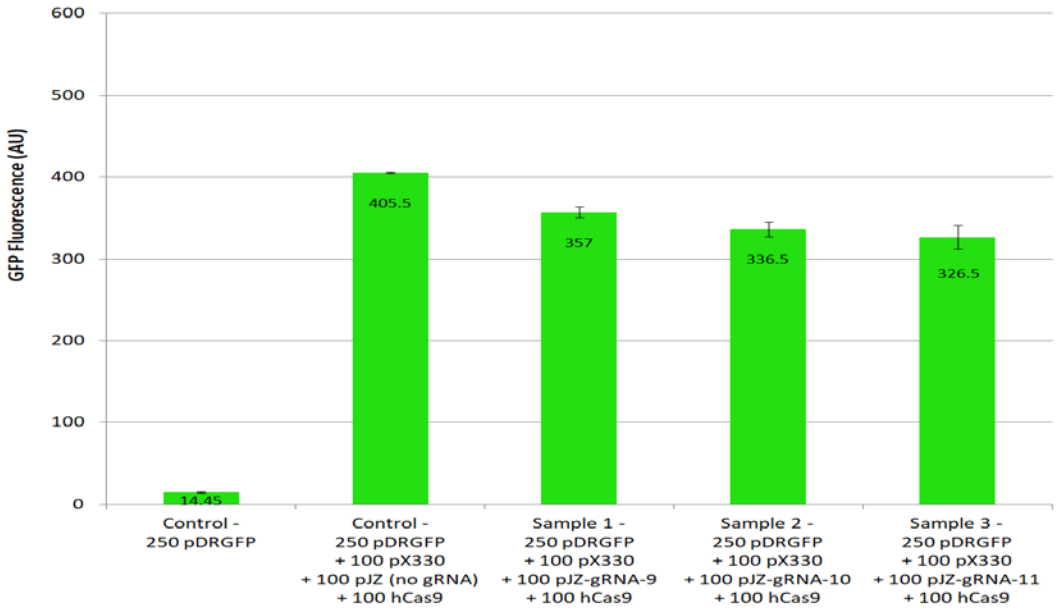
Table 5 Components of Transfection with 50ng of pJZC74 for FACS Analysis.

Sample	Components
Control	20ng EBFP + 250ng pDRGFP
Control	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng pJZC74 (no gRNA) + 100ng hCas9
1	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng **gRNA-9** + 100ng hCas9
2	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng **gRNA-10** + 100ng hCas9
3	20ng EBFP + 250ng pDRGFP + 100ng pX330 + 100ng **gRNA-11** + 100ng hCas9

Table 6 Components of Transfection with 100ng of pJZC74 for FACS Analysis.



(A) FACS results (50ng of pJZC74 with gRNA)



(B) FACS results (100ng of pJZC74 with gRNA)

Fig. 17 FACS Results Showing Geometric Mean of GFP$^+$ Cells. Non-coding DNA was added to normalize the total amount of DNA per well to ~570ng. **(A) FACS results at 50ng.** Significant GFP was present for all gRNAs as compared to pDRGFP alone. gRNA-10 showed the highest geometric mean of GFP$^+$ cells at 50ng of pJZC74. **(B) FACS results at 100ng.** gRNA-9 showed the highest geometric mean of GFP$^+$ cells at 100ng of pJZC74.

4. DISCUSSION

4.1 Summary of Study Outcomes

An initial screen of gRNAs was performed to test which gRNAs were the most effective at repressing *LIGIV*. Twenty gRNAs were designed to target key regions of the *LIGIV* gene. *See* **Table 1** and **Fig. 12**. These gRNAs were screened for repression of *LIGIV*, with cells receiving 20ng of EBFP as a control for transfection, 250ng or 25ng of pJZC74 with a gRNA targeting *LIGIV*, and a corresponding amount (250ng or 25ng) of hCas9. *See* **Table 2**. If successful, each 14-nt gRNA targeting *LIGIV* with the Com-KRAB effector domain attached forms a ribonucleoprotein complex with hCas9. When the gRNA binds to its targeted region of the *LIGIV* gene, repression of *LIGIV* results due to the chromatin-modifying effects of KRAB (Groner et al., 2010). *See* **Fig. 6**. Cas9 does not induce a DSB because the gRNAs are truncated at 14 nts. Several gRNAs showed significant repression of *LIGIV*. *See* **Fig. 14**.

An additional experiment was performed to corroborate the earlier findings of *LIGIV* repression and to further test whether simultaneous gene editing was occurring. Three high-performing gRNAs (9, 10 and 11) were selected for further study. *See* **Fig. 15**. The 20-nt gRNA in pX330 targeted the mutated *EGFP* in pDRGFP and the 14-nt gRNA in pJZC74 targeted *LIGIV*, each in complex with hCas9. Because pX330's gRNA was 20-nt, hCas9 induced a DSB in the *EGFP* gene, prompting cells to repair the cut site. If cells performed HDR using the donor template present in pDRGFP, GFP^+ cells resulted. Alternatively, if cells performed NHEJ, indels likely occurred and the *EGFP* sequence remained disrupted. As with the initial screen, the 14-nt gRNAs in pJZC74

bind to *LIGIV* and Cas9 does not induce a DSB. This allows the KRAB effector domain to repress *LIGIV* without any genome modification.

HEK cells were transfected and analyzed contemporaneously by FACS and qPCR. For qPCR, cells received 250ng of pDRGFP, 50ng or 100ng of pJZC74 (containing a gRNA targeting *LIGIV*), and a corresponding amount (50ng or 100ng) of both pX330 and hCas9. A control received the same components in the same concentrations as the remaining samples, except that pJZC74 only contained the customizable gRNA insert site (no gRNA targeting the human genome was present). *See* **Tables 3-4.** qPCR analysis of gRNAs 9, 10 and 11 showed that all three gRNAs repressed *LIGIV*. *See* **Fig. 16**.

For FACS, cells received the same components as were transfected for qPCR analysis. *See* **Tables 5-6**. Two controls were included, one containing only 250ng of pDRGFP and another with all the same components in the same concentrations as the remaining samples, except pJZC74 did not contain a gRNA. The results showed abundant GFP^+ cells compared to the control without pX330, demonstrating that gene editing was occurring. *See* **Fig. 17**.

4.2 Advancements Presented by This Study

The overall results indicate that a novel CRISPR/Cas9 tool for simultaneous gene editing and transcriptional regulation was designed and tested. Such a tool has important implications for the development of gene therapies, whereby the repression of key genes during genome editing may aid with the study and implementation of a host of disease

treatments. Cancer, for instance, is a complex disease process that will benefit from an arsenal of strategies. With the ability to repress genes that promote cancer proliferation, migration and invasion, while simultaneously performing targeted genome edits, researchers are endowed with an additional level of genomic control that could mitigate the disease process.

Furthermore, repressing key genes involved with NHEJ during genomic editing may benefit the efficiency of HDR, thereby improving the efficiency with which desired genomic edits are performed. The ability to precisely control HDR has widespread implications, not only for clinical medicine, but for fundamental genomic research, synthetic biology, agriculture, and other applications (Barrangou & Doudna, 2016; Hsu, et al., 2014).

By expanding upon earlier advancements, the results of this study broadened the versatility of pJZC74 and the modularity of Cas9 as a platform for simultaneous gene editing and transcriptional regulation. Similar to other repression vectors available on Addgene at the initiation of this study, pJZC74 was designed for use with the catalytically-inactive dCas9 to achieve transcriptional regulation (Zalatan et al., 2014). However, pJZC74 was unique compared to other repression vectors, in that it was designed to recruit RNA-binding effector domains (e.g., Com-KRAB) directly to the *gRNA* (via the com recruitment domain), rather than fusing the effector domain directly to dCas9 (Zalatan et al., 2014). This key design element enabled the use of catalytically-active Cas9 as the platform for transcriptional regulation, rather than dCas9, in conjunction with 14-nt gRNAs (Kiani et al., 2015). With Cas9 as the platform, genome

editing could be simultaneously initiated by introducing a 20-nt gRNA targeted to the desired site of gene editing.

In addition to corroborating earlier findings showing that truncated gRNAs can be used with Cas9 to regulate gene expression (Kiani et al., 2015), this study expanded upon those findings by testing repression using 14-nt gRNAs targeted to the genome, rather than a plasmid-based assay. This study also extended the pool of 14-nt gRNAs available for repression. Whereas gene repression through steric blocking alone using 14-nt gRNAs had been tested on gRNAs targeted in close proximity to an engineered TATA box (Kiani et al., 2014; Kiani et al., 2015), the approach in this study appears to expand beyond those initial design constraints due to Com-KRAB's effectuation of gene silencing through long-range distribution of repressive chromatin modifiers along the target gene (Groner et al., 2010). As a result of this more extensive transcriptional regulation, a broader selection of target sites is now available when designing repression experiments using CRISPR/Cas9.

5. CONCLUSION & FUTURE WORK

Improving the efficiency of HDR is critical to the precision of genome editing. More comprehensive studies are recommended to test whether this tool can increase the efficiency of HDR by repressing a variety of key genes involved with NHEJ. Such genes may include *LIGIV* in combination with *XRCC4*, *XRCC5,* and *XRCC6,* among others (Chu et al., 2015; Maruyama et al., 2015).

Furthermore, it may be desirable to utilize an alternative reporter system to substantiate findings developed with pDRGFP. For instance, Glaser, McColl, and Vadolas (2016) designed a simple system for quantifying HDR. In GFP cell lines, gRNAs were targeted to the *EGFP* gene to induce a DSB (Glaser et al., 2016). A single-stranded donor template was used to convert GFP to BFP, in the event of HDR (Glaser et al., 2016). BFP, therefore, acted as a reliable quantifier of HDR and loss of blue fluorescence indicated the occurrence of NHEJ (Glaser et al., 2016). This reporter system would be a useful addition to further studies.

Expansion of the CRISPR toolkit is vital to pushing forward downstream medical advancements and other important applications of CRISPR/Cas technology. This study offered foundational support for an innovative approach to simultaneous gene modification and transcriptional regulation with the potential to benefit a variety of future research endeavors.

FOOTNOTES

[1] Materials and methods described herein are based on standard operating procedures issued for Dr. Kiani's laboratory.

[2] Image credit: Kearse, et al. (2012).

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APPENDIX A

VALIDATION THAT PX330 CONTAINS

GRNA TARGETING MUTATED EGFP IN PDRGFP

After Golden Gate Assembly of the gRNA targeting pDRGFP's mutated *EGFP* sequence into pX330, diagnostic digestion on the modified vector was performed using a 20µL reaction: 2µL of the modified pX330 vector, 1µL of BbsI-HF, 1µL of AgeI-HF, 2µL of CutSmart® Buffer, and 14µL of nuclease-free water. Samples were run at 37°C for 30 minutes. Expected band size for vectors that successfully integrated the gRNA, thereby eliminating the BbsI recognition sequences, was ~8.5kb. Two reactions from those tested showed the correct band sizes. Successful insertion of the gRNA sequence was confirmed by Sanger sequencing.

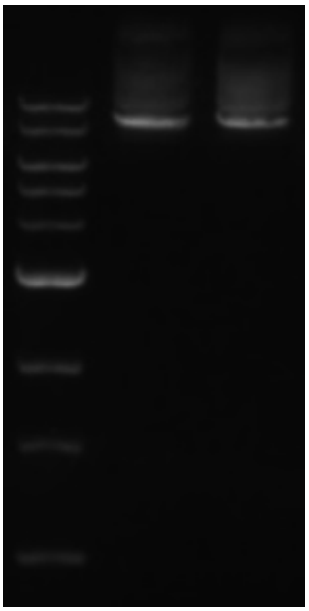


Fig. 18 Diagnostic Digestion of pX330 Confirming Insertion of gRNA. The expected ~8.5kb band was observed following insertion of the gRNA targeting the mutated *EGFP* in pDRGFP into pX330.

Sample 1	ATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTG GGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCA TATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT ATATCTTGTGGAAAGGACGAAACACCGGTGTCCGGCTAGGGA TAACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG TCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT TGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG TTTTTAGCGCGTGCGCCAATTCTGCAGACAAATGGCTCTAGAG GTACCCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGAC CGCCCAACGACCCCCGCCCATTGACGTCAATAGTAACGCCAAT AGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAA ACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTA CGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCA TTGTGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAG TACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGGTGA GCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCA CCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAGCG AT
Sample 2	TGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTA GAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATG TTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTAT TTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACA CCGGTGTCCGGCTAGGGATAACGTTTTAGAGCTAGAAATAGC AAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGG CACCGAGTCGGTGCTTTTTTGTTTTAGAGCTAGAAATAGCAAG TTAAAATAAGGCTAGTCCGTTTTTAGCGCGTGCGCCAATTCTG CAGACAAATGGCTCTAGAGGTACCCGTTACATAACTTACGGTA AATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGA CGTCAATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATG GGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAA GTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG GTAAATGGCCCGCCTGGCATTGTGCCCAGTACATGACCTTATG GGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCT ATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTCC CCATCTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATT TTTTAATTATTTTGTGCAGCGAT

Table 7 Sequencing Results Confirming Insertion of gRNA Into pX330. Sequencing results confirm successful insertion into pX330 of the gRNA targeting pDRGFP's mutated *EGFP* (red underline).

APPENDIX B

GEL PURIFICATION OF LINEARIZED PJZC74 & GBLOCK WITH CUSTOMIZABLE GRNA INSERT SITE

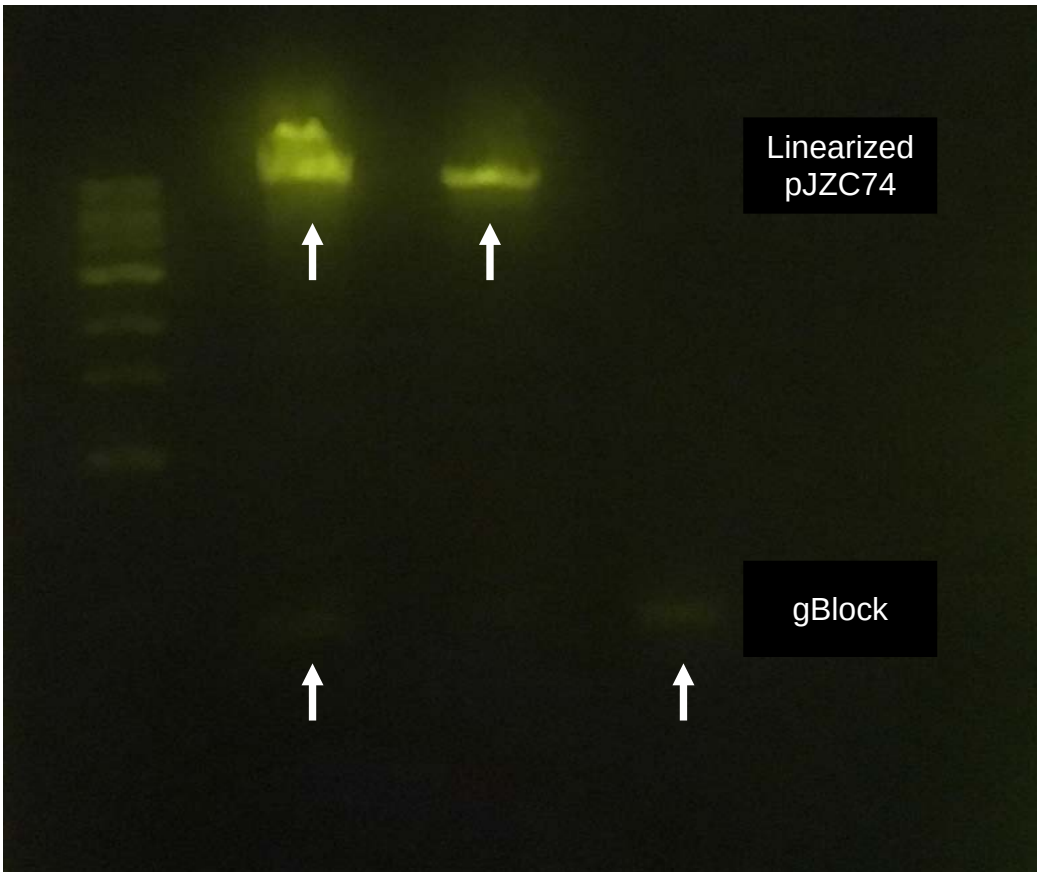


Fig. 19 Images of Linearized pJZC74 and gBlock Containing Customizable gRNA. Images on blue transilluminator taken before gel purification showed bands for linearized pJZC74 at ~8.2kb and the digested gBlock containing the customizable gRNA insert site at ~550 bps, as expected.

APPENDIX C

VALIDATION THAT GBLOCK WITH CUSTOMIZABLE GRNA INSERT SITE LIGATED INTO PJZC74

After Golden Gate Assembly of the customizable gRNA insert site into pJZC74, diagnostic digestion was performed on the modified vector using a 20µL reaction: 4µL of the modified pJZC74 vector, 1µL of PciI, 2µL of BsmBI, 2µL of NEBuffer^{TM} 3.1, and 11µL of nuclease-free water. After adding 1µL of PciI initially, samples were run at 37°C for 1 hour. Thereafter, 2µL of BsmBI was added, and samples were run at 55°C for 2 hours. Expected band size for vectors that successfully integrated the gRNA insert site was ~900 bps, 1.8kb and 6.1kb. Successful insertion of the customizable gRNA insert site was confirmed by Sanger sequencing.

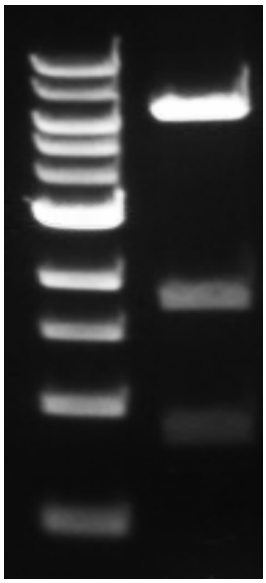


Fig. 20 Diagnostic Digestion of pJZC74 Confirming Insertion of gBlock. The expected band sizes of ~900 bps, 1.8kb and 6.1kb were observed following insertion of the gBlock containing the customizable gRNA insert site into pJZC74.

GAGATCCAGTTTGGTTAGTACCGGGCCCGCTCTAGAGATCCGACGCGCC
ATCTCTAGGCCCGCGCCGGCCCCCTCGCACAGACTTGTGGGAGAAGCTC
GGCTACTCCCCTGCCCCGGTTAATTTGCATATAATATTTCCTAGTAACTA
TAGAGGCTTAATGTGCGATAAAAGACAGATAATCTGTTCTTTTTAATAC
TAGCTACATTTTACATGATAGGCTTGGATTTCTATAACTTCGTATAGCAT
ACATTATACGAAGTTATAAACAGCACAAAAGGAAACTCACCCTAACTG
TAAAGTAATTGTGTGTTTTGAGACTATAAGTATCCCTTGGAGAACCACC
GGAGACGGGATACCGTCTCTGTTTAAGAGCTATGCTGGAAACAGCATA
GCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG
AGTCGGTGCCTGAATGCCTGCGAGCATCTTTTTTTGTTTTTTATGTCTCT
GCAGAGTTCGGTCTCGAGTACTAGGATC*ATTAGGCGGCCGCGTGGATA
ACCGTATTAC

Table 8 Sequencing Results Confirming Insertion of gBlock Into pJZC74.
Sequencing results confirm successful insertion into pJZC74 of the gBlock containing the customizable gRNA insert site (red underline).

APPENDIX D

SEQUENCES OF GRNAs TARGETING LIGIV

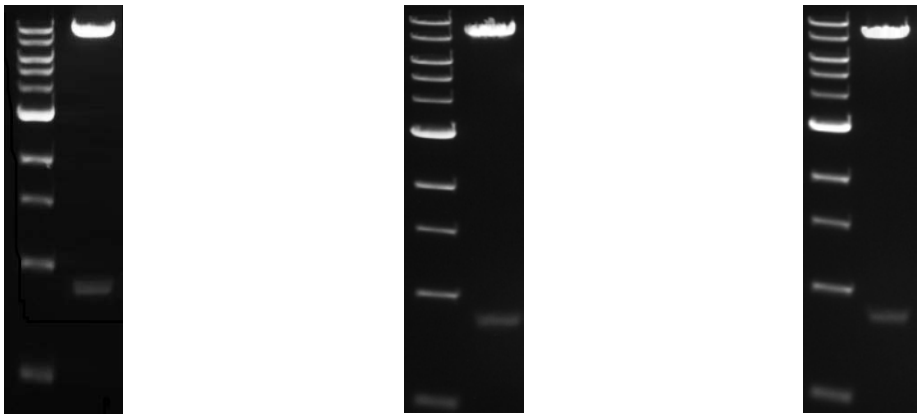
1	GGCAAATGCCCCCGC
2	GGCAAATGCCCCCGC
3	GGCGGGGGCATTTGC
4	GGCGGGGGCATTTGC
5	GCGGCGAGCAGCTGG
6	GCGGCGAGCAGCTGG
7	GGCTGCTCGCCGCGC
8*	GGTGTCTGGGACGTC
9*	GGTGTCTGGGACGTC
10	GACCTGACGCCCCTC
11	GACCTGACGCCCCTC
12	GAGTCTACAGCGCTG
13	GAGTCTACAGCGCTG
14	GCATCACCGCTTTGA
15	GCATCACCGCTTTGA
16	GGGCAGCCATCAAAG
17	GGGCAGCCATCAAAG
18	GGACAAAAGAGGTGA
19	GGACAAAAGAGGTGA
20	GTCGACGCCACACCGTTTATT

Table 9 Sequences of gRNAs Targeting *LIGIV*. gRNAs 1, 3, 5, 8, 10, 12, 14, 16, 18, and 20 were placed into a pJZC74 vector that contained an extra TTGTT sequence, which followed the gRNA. The purpose of this short sequence was unclear from a review of relevant literature. Therefore, certain gRNAs were tested with and without this sequence. * Complementary sequences for gRNAs 8 and 9 each appear twice in *LIGIV*.

APPENDIX E

VALIDATION THAT SELECT GRNAS TARGETING LIGIV LIGATED INTO PJZC74

After Golden Gate Assembly of gRNAs 9, 10 and 11 into pJZC74, diagnostic digestion was performed. Each digestion included 1-1.2µL of pJZC74 with the gRNA, 1µL of PciI, 1µL of BsmBI, 2µL of NEBuffer^{TM} 3.1, and sufficient nuclease-free water to bring the reaction to 20µL. After adding 1µL of PciI initially, samples were run at 37°C for 1 hour, then 2µL of BsmBI was added and samples were run at 55°C for an additional 2 hours. Expected band size for vectors that successfully integrated the gRNA, thereby eliminating the BsmBI recognition sequences, was ~900 bps and 8.0kb. Successful insertion of each gRNA was confirmed by Sanger sequencing.



gRNA-9 gRNA-10 gRNA-11

Fig. 21 Diagnostic Digestion of pJZC74 Confirming Insertion of Select gRNAs Targeting *LIGIV*. The expected band sizes of ~900 bps and 8.0kb were observed following insertion of the gRNAs targeting *LIGIV* into pJZC74.

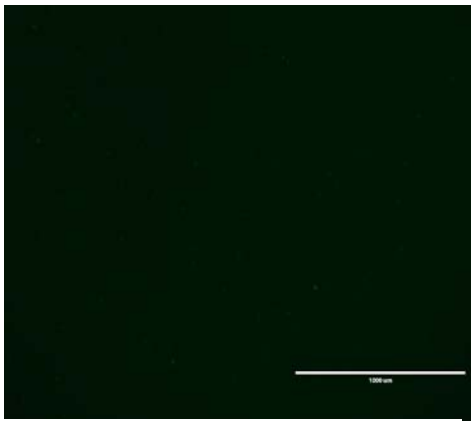
gRNA	Sequencing results
gRNA-9	TATCCCTTGGAGAACCACCGGTGTCTGGGACGTCGTTTAAGAGC TATGCTGGAA
gRNA-10	CTTGGAGAACCACCTTGTTGACCTGACGCCCCTCGTTTAAGAGC TATGCTGGAA
gRNA-11	TATCCCTTGGAGAACCACCGACCTGACGCCCCTCGTTTAAGAGC TATGCTGGAA

Table 10 Sequencing Results Confirming Insertion of Select gRNAs Into pJZC74. Sequencing results confirm successful insertion into gRNAs 9, 10 and 11 targeting *LIGIV* into pJZC74 (red underline).

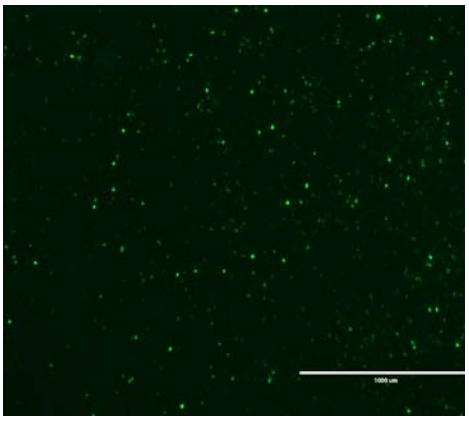
APPENDIX F

FLUORESCENCE MICROSCOPE IMAGES OF HEK CELLS

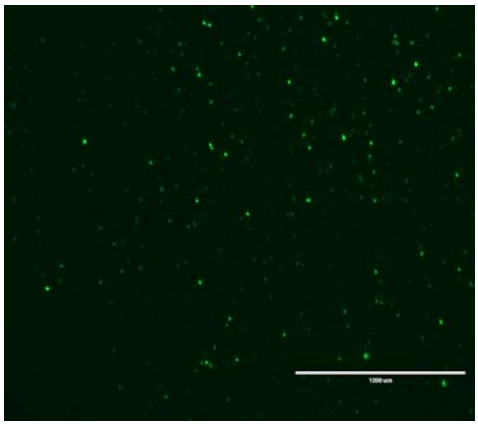
BEFORE FACS ANALYSIS



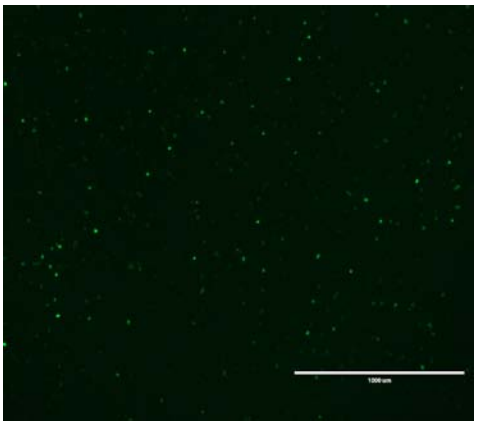
Control
250 pDRGFP



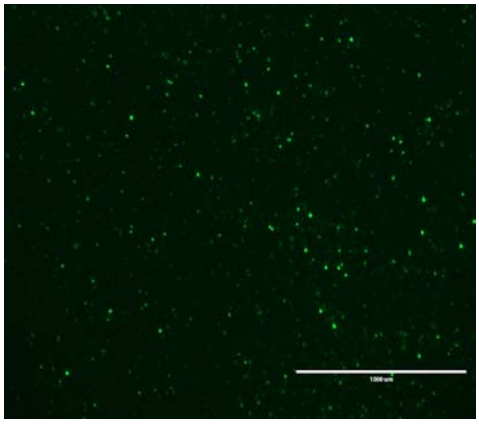
Control
250 pDRGFP + 50 pX330 + 50 pJZ_cust + 50 hCas9



Sample 1
250 pDRGFP + 50 pX330 + **50 gRNA-9** + 50 hCas9

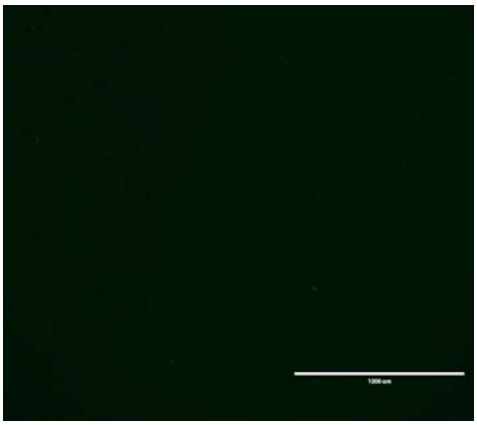


Sample 2
250 pDRGFP + 50 pX330 + **50 gRNA-10** + 50 hCas9

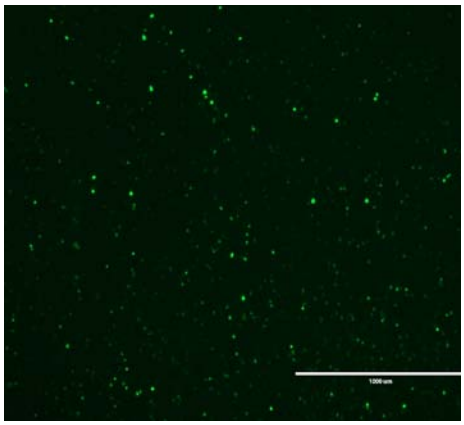


Sample 3
250 pDRGFP + 50 pX330 + **50 gRNA-11** + 50 hCas9

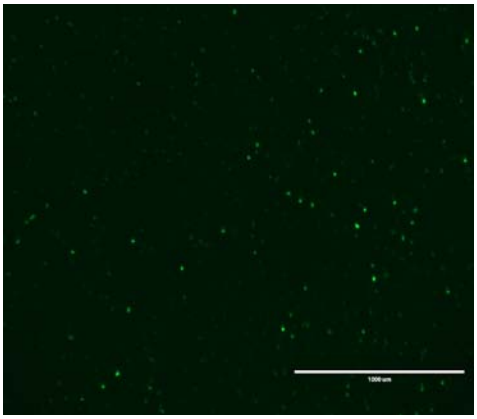
Fig. 22 GFP Images of HEK293FT Cells Receiving 50ng of pJZC74 with gRNAs.



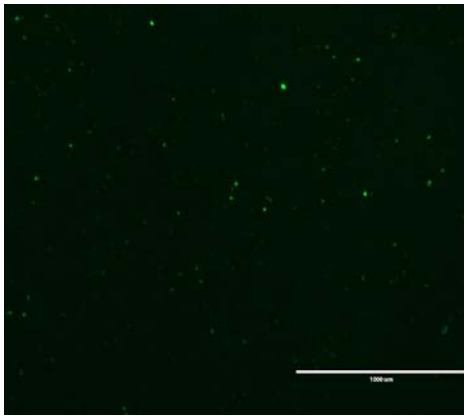
Control
250 pDRGFP



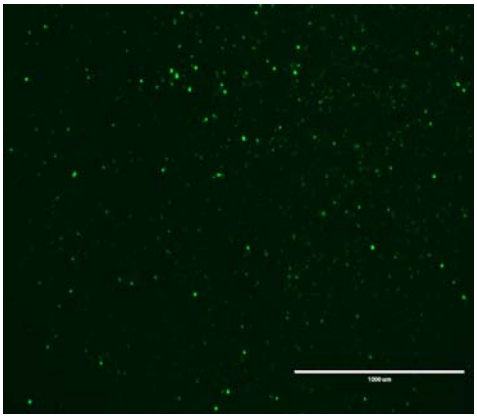
Control
250 pDRGFP + 100 pX330 +100 pJZ_cust + 100 hCas9



Sample 1
250 pDRGFP + 100 pX330 + **100 gRNA-9** + 100 hCas9



Sample 2
250 pDRGFP + 100 pX330 + **100 gRNA-10** + 100 hCas9



Sample 3
250 pDRGFP + 100 pX330 + **100 gRNA-11** + 100 hCas9

Fig. 23 GFP Images of HEK293FT Cells Receiving 100ng of pJZC74 with gRNAs.

APPENDIX G

SELECT PROTOCOLS[1]

Preparation of Agarose Gel

To prepare a 1% gel, 1.5g of powdered ME Agarose and 150mL of 1X TAE were added to a 250mL glass bottle. (Note: UltraPure™ Low Melting Point Agarose was substituted for ME Agarose when a gel purification was to be performed). The solution was mixed by gently shaking, then microwaved until the gel dissolved, approximately 2-3 minutes. After cooling for ~10 minutes, 15µL of 10,000X SYBR® Safe, a nucleic acid stain, was added and swirled vigorously to mix. The gel was poured into an electrophoresis tray and an appropriately-sized comb was placed. The gel was allowed to solidify for at least 30 minutes, after which the comb was removed, the tray was properly positioned in the gel electrophoresis apparatus, and 1X TAE was poured into the chambers to cover the gel. The desired samples and a DNA ladder were then loaded, as appropriate.

Preparation of DMEM

In a biosafety cabinet, 450mL of DMEM (with sodium pyruvate, 4.5g/L glucose), 50mL of pre-warmed FBS, and 5mL of non-essential amino acids, Glutamine, and Penicillin Streptomycin was filter sterilized using a 500µL filter flask.

Changing Media on HEK Cell Growth Plates

To change the media on a growth plate, 14mL of DMEM was pre-warmed in a water bath. Within the biosafety cabinet, the existing DMEM on the plate was aspirated using a glass pipette under vacuum. Using a serological pipette on the gravity setting,

14mL of pre-warmed DMEM was added carefully along the sides of the plate. The plate was then returned to the 37ºC / 5% CO_2 incubator.

Changing Media on 24-Well Transfection Plates

For each 24-well plate to receive media, 12mL of DMEM was pre-warmed. Within a biosafety cabinet, the existing DMEM was aspirated from each well using a sterile glass pipette. Using a serological pipette, 0.5mL of pre-warmed DMEM was added to each well along the side of the plate using the gravity setting. The plate was promptly returned to the 37ºC / 5% CO_2 incubator.

Preparation of Luria Broth Agar Growth Plates with Antibiotic

To prepare the plates, 800mL of DI water and 32g of powdered LB agar were added to a 1000mL glass bottle. After shaking to distribute, the lids were loosened slightly and covered with aluminum foil. The bottles were labeled with autoclave tape and autoclaved in a tray containing water. After cooling, 800µL of 1000x concentrated Ampicillin antibiotic was added and the bottles were swirled briefly to thoroughly distribute. Sterile, 10cm polystyrene plates were placed on a sterile bench, and media was poured into each plate to partially fill. Plates were covered with their lids and allowed to solidify. Plates were inverted and allowed to continue cooling overnight, then placed into sealed plastic bags and stored at 4°C until use. Any remaining LB agar media was also stored at 4°C.

Quantification of DNA or RNA Concentration

To measure DNA or RNA, the appropriate sample type was selected on the NanoDrop™ (DNA or RNA). The instrument was blanked with nuclease-free water and both arms of the instrument were cleaned with DI water using a Kimwipe™. The desired sample was pipetted onto the probe (1-1.2µL) and the upper arm was lowered to measure the concentration.

Glycerol Stock Preparation

Long-term storage of vectors was done using a glycerol stock solution composed of equal parts of DI water and 100% glycerol, making a 50% glycerol solution. The solution was vortexed until well-mixed and stored at room temperature until ready to use.

To prepare the glycerol stock of a desired sample, 500µL of the 50% glycerol solution was aliquoted into a sterile 1.5µL microcentrifuge tube, to which 500µL of the bacterial culture was added. The tube was vortexed and placed on ice before prompt storage in a -80°C freezer.

APPENDIX H

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Subject: RE: Use of Figure for Thesis

Hi David!

That's a good question – author reuse policies can be quite confusing!

Here's a quick guide for the author regarding ways she can reuse parts or all of her manuscript:

https://us.sagepub.com/en-us/nam/journal-author-archiving-policies-and-re-use

Her figure would fall under "include up to one full article in my unpublished dissertation or thesis" – in which case she is welcome to reuse the figure, as long has she properly attributes it to the journal article.

Thanks for reaching out!

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BIOGRAPHICAL SKETCH

Jennifer joined Dr. Kiani's lab in 2016 to study CRISPR/Cas technology and genome engineering. She is an attorney with a certification in Genomics & Biotechnology Law from Arizona State University's Center for Law, Science & Innovation. She is interested in the application of gene therapy to aging, the diseases of aging, and cancer. She also has a strong interest in stem cell technology, as well as tissue engineering for the development of replacement organs and tissues. She plans to use her legal, scientific, and business management background to advance the life sciences.