

Unlocking loxP to Track Genome

Subjects: **Genetics & Heredity**

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The development of CRISPR-associated proteins, such as Cas9, has led to increased accessibility and ease of use in genome editing. However, additional tools are needed to quantify and identify successful genome editing events in living animals. We developed a method to rapidly quantify and monitor gene editing activity non-invasively in living animals that also facilitates confocal microscopy and nucleotide level analyses. Here we report a new CRISPR “fingerprinting” approach to activating luciferase and fluorescent proteins in mice as a function of gene editing. This system is based on experience with our prior cre recombinase (cre)-detector system and is designed for Cas editors able to target loxP including gRNAs for SaCas9 and ErCas12a. These CRISPRs cut specifically within loxP, an approach that is a departure from previous gene editing in vivo activity detection techniques that targeted adjacent stop sequences. In this sensor paradigm, CRISPR activity was monitored non-invasively in living cre reporter mice (FVB.129S6(B6)-Gt(ROSA)26Sortm1(Luc)Kael/J and Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, which will be referred to as LSL-luciferase and mT/mG throughout the paper) after intramuscular or intravenous hydrodynamic plasmid injections, demonstrating utility in two diverse organ systems. The same genome-editing event was examined at the cellular level in specific tissues by confocal microscopy to determine the identity and frequency of successfully genome-edited cells. Further, SaCas9 induced targeted editing at efficiencies that were comparable to cre, demonstrating high effective delivery and activity in a whole animal.

CRISPR

SaCas9

ErCas12a

loxP

Cre

luciferase reporter

fluorescent reporter

gene editing

naked DNA injection

in vivo

1. Introduction

CRISPR-Cas technology has revolutionized the gene editing space, enabling access to the field for many new users [1]. Due to its versatility and ease of use, it is being deployed for usage in vivo as a method to treat genetic disorders requiring novel delivery methods and a means to monitor gene editing in vivo [2]. For example, somatic in vivo CRISPR–Cas9 gene editing was recently performed in humans as a treatment for Leiber’s congenital amaurosis 10 (LCA10) [3].

Previous methods to monitor in vivo CRISPR editing have commonly relied on nucleotide sequencing of edited target biopsies or invasive monitoring of the tissues for fluorescent markers [4]. These methods require the destruction of the tissue of interest and prevent long term serial monitoring of CRISPR editing. Additionally, without non-invasive monitoring, unpredicted off-target tissue activity may not be detected, unless each tissue is tested

directly. Therefore, a method with the ability to monitor CRISPR activity non-invasively is an important tool for developing gene editing delivery systems.

The cre-*loxP* system is also a powerful tool for animal and cell models, providing robust and specific editing of DNA [5]. Cre recombinase is derived from bacteriophage P1. Cre recombines DNA contained within *loxP* sequences, creating inversions, deletions, and insertions depending on the orientation of the *loxP* sites [6]. Thousands of animal models have been generated using cre-*loxP* technologies, 320 of which are specifically mouse reporter models [7]. Many cre-*loxP* animal models have also been developed in which fluorescent or luminescent reporter genes are inactivated by upstream “floxed” sequences, which are two *loxP* sites surrounding a polyadenylation (polyA) “stop” sequence or another gene [8][9]. When the cells of these animals are exposed to cre, the floxed sequence is excised, this activates reporter gene expression.

The Barry lab previously made use of these floxed reporter mice to “fingerprint” gene delivery *in vivo* by adeno-associated virus (AAV) vectors [10]. In this approach, AAV-cre was injected into mice that are transgenic with different cre-activated reporter genes. In LSL-luciferase mice, luciferase’s expression is blocked by a floxed polyA cassette upstream of luciferase (**Figure 1**). In the absence cre, no luciferase is expressed. When cre is delivered, the recombination process results in the net deletion of the floxed stop cassette consisting of neomycin and a polyA transcriptional stop, resulting in an activated luciferase reporter. In mT/mG mice, a floxed membrane-targeted red fluorescent protein mTomato (mT) is followed by membrane-targeted GFP (mG). In the absence of cre, mT is expressed in all cells of the mouse and is membrane targeted. When cre is delivered, mT is deleted and mG is expressed. At higher magnifications, these membrane-targeted reporter proteins provide substantial cell discrimination [8].

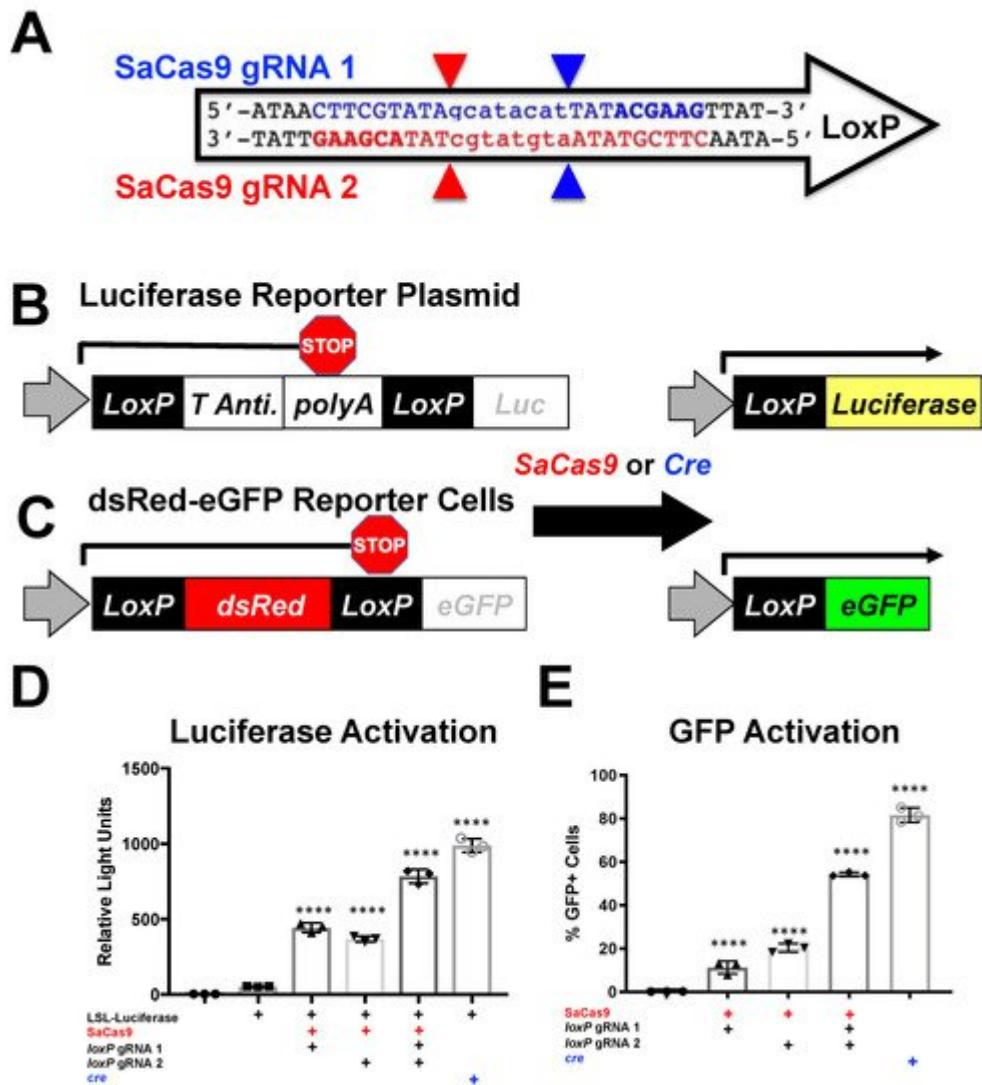


Figure 1. Gene activation through targeting *loxP* in vitro. (A) The *loxP* sequence is shown with key features and target sites for CRISPR SaCas9. The capitalized base pairs are the 13-base-pair palindromic regions flanking the 8-base-pair core that gives *loxP* its directionality. The gRNA 1 homologous strand to the guide RNA is depicted in light blue with the PAM-binding region in dark blue and bold letters and a triangle to indicate the cleavage site. gRNA 2 depicts the same in red. (B) This depicts the reporter plasmid p133, the LSL-Luc reporter plasmid used in D, and the general outcome when treated with SaCas9 with targeting *loxP* gRNAs or cre recombinase. (C) The lentiviral vector is used to make the Red/Green reporter cells used in (E) and similarly depicts the outcome when gene edited by SaCas9 or Cre. (D) Cells were plated into a 6-well plate and transfected at 60–80% confluence with Xfect and 2.5 µg of the reporter plasmid was transfected to be targeted by cre or SaCas9. Groups were compared by one-way Anova and Tukey's multiple comparison. Using this, all the groups were significant compared to other groups except between the untransfected control group and the P133 transfected group and between the individual gRNA treated groups; 95% confidence intervals are shown and the significance against the control is shown (n = 3). (E) The Red/Green HEK293 reporter cells were transfected with 5 µg of plasmid. The single gRNA groups were significant compared to untransfected and the combination of gRNAs and cre was significant compared to all groups by one-way ANOVA with Tukey's multiple comparisons. Individual gRNAs were not significant compared to each other (n = 3) (**** p < 0.00001).

More recently, a similar “footprinting” system was used to track gene delivery with cre recombinase and notably Cas9 by monitoring fluorescent protein activation [11]. This approach lacked the ability to track editing in living animals by luciferase imaging but had the added value of tracking Cas9 in vivo post-mortem by tissue sectioning. In this case, Cas9 was targeted by gRNA to a polyA “stop” cassette near *loxP* sites in mice bearing floxed inactivated fluorescent protein genes. This design is limited to the specific stop cassette in this mouse model and is not widely applicable to other mouse reporter models. Cas9 cleavage followed by host cell DNA repair was able to activate reporter gene expression to monitor in vivo editing [11].

While this footprinting approach was novel, its targeting of the polyA sequence will work only in a small set of 10 fluorescent and neural mouse models that have the same stop cassette [12]. Mice that have stop cassettes that express a gene rather than repeated polyAs as the stop cassette, like the LSL-luciferase and mT/mG mice (see Graphical Abstract), cannot use a polyA based gRNA. This is because the gRNAs will not create a large deletion and can even have off-target editing of the similar target sites. Additionally, if making a hybrid mouse with 2 different *loxP* cassettes, the only site guaranteed to be an editable target on both chromosomes would be *loxP*.

Given this, CRISPR-Cas9 was targeted to *loxP* sequences and tested in the combined luciferase and mT/mG mouse model for combined live imaging and post-mortem evaluation of genome editing. By doing this, it was shown that SaCas9 activation of these reporter genes has near equivalent activity to that of cre recombinase, in vitro and in vivo. Through sequencing, a primary pathway was not conclusively determined due to ambiguity of the repair outcomes, although non-homologous end-joining events were amongst the top reads. Finally, the targeting of *loxP* with SaCas9 and other CRISPR nucleases has opened the door to using the plethora of established *loxP* models to test new delivery methods and monitoring for CRISPR gene editing activity in vivo.

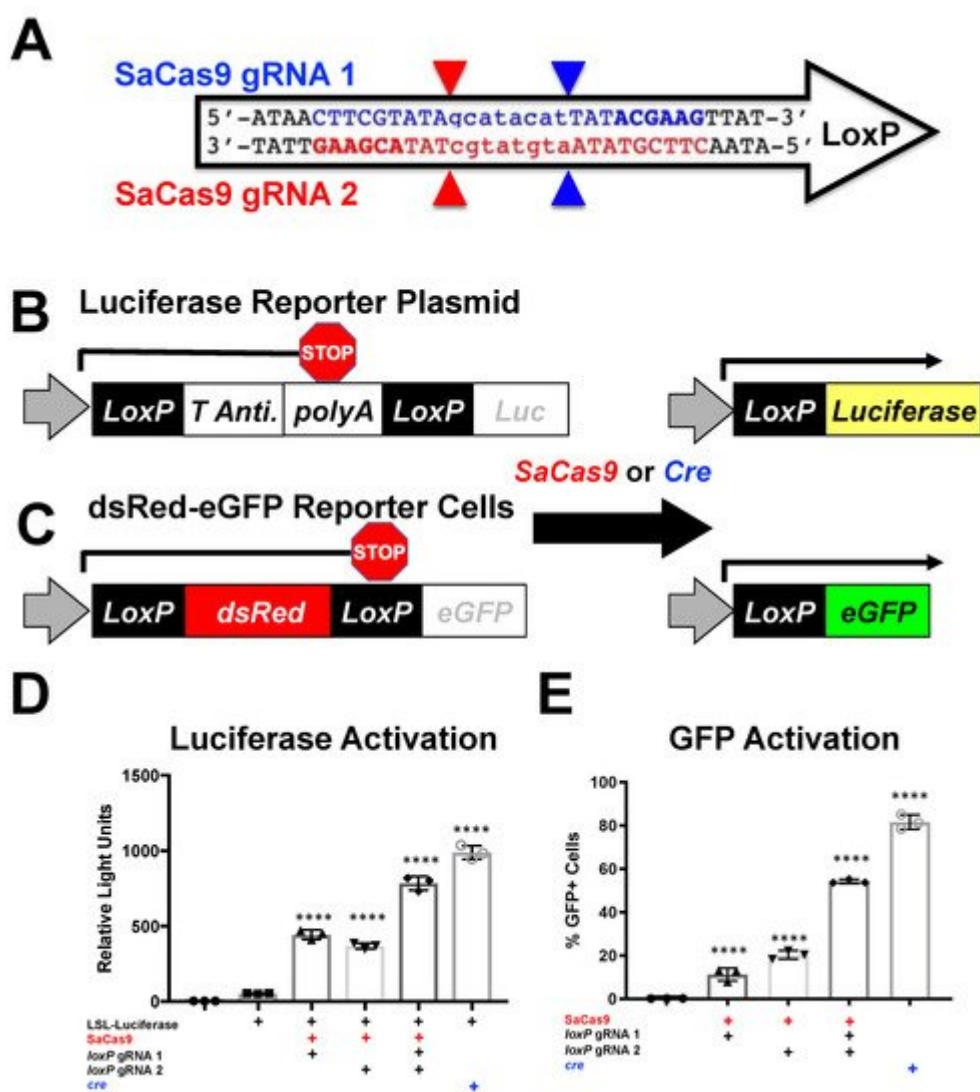
2. Discussion

This study was performed to enable a facile system for monitoring genome editing in living animals as well as to identify edited tissue at a cellular resolution. This work shows that established cre-*loxP* reporter systems can be used to monitor CRISPR activity. This paper showed that *loxP* cleavage by SaCas9 can be comparable to cre recombinase activity in deleting stop signals in vitro or in vivo. By using the previously established mT/mG:LSL Luc, it was demonstrated that CRISPR activity could be detected non-invasively in living mice along with a quantitative approach upon dissection of edited tissues.

This system also establishes the first *loxP* specific reporter for fluorescence monitoring. Previous work has used gRNAs to target adjacent to the *loxP* site or within the polyA region ([Supplementary Figure S2A](#)). While these have shown the ability to monitor CRISPR activity via tissue sectioning and DNA analysis, these systems are specific to Ai9 mice and a few related strains and would not be broadly applicable to other reporter systems such as the LSL-mice or the mT/mG target loci [13]. Other work has shown in vitro targeting with partial overlap with *loxP* and complete coverage with the gRNA in the *loxP* mutants *lox71* and *lox66* ([Supplementary Figure S2B](#)) [14]. These previous gRNAs would not be applicable to most mouse models that likely will not have convenient adjacent sequences that allow for targeting and most mouse reporter models do not use *loxP* mutants. The system shown

herein can be used with any wildtype LoxP site and is therefore capable of being used with any mouse model that uses *loxP* to activate or deactivate genes by cre excision. This system could also be used in cases where *loxP* is positioned for gene inversion to delete a region or disrupt this process.

Cre recombinase has evolved to not only cut *loxP* sequences, but to also repair the resulting genomic lesion. In contrast, Cas editors simply cleave DNA. Any Cas cleavage event must rely on the host cell to repair the cut DNA. Therefore, one might assume that Cas-mediated deletion of a floxed locus would likely be less efficient than the evolved cut and repair cre recombinase system. However, when using SaCas9 and the dual guide system, SaCas9 was like cre in efficiency for reporter activation in vitro and in vivo (Figure 1, Figure 2 and Figure 3). This activity level can be partially explained by SaCas9, an efficient DNA editor that can show higher activity than SpCas9 and a Cas12a variant when using similar or identical targets [15].



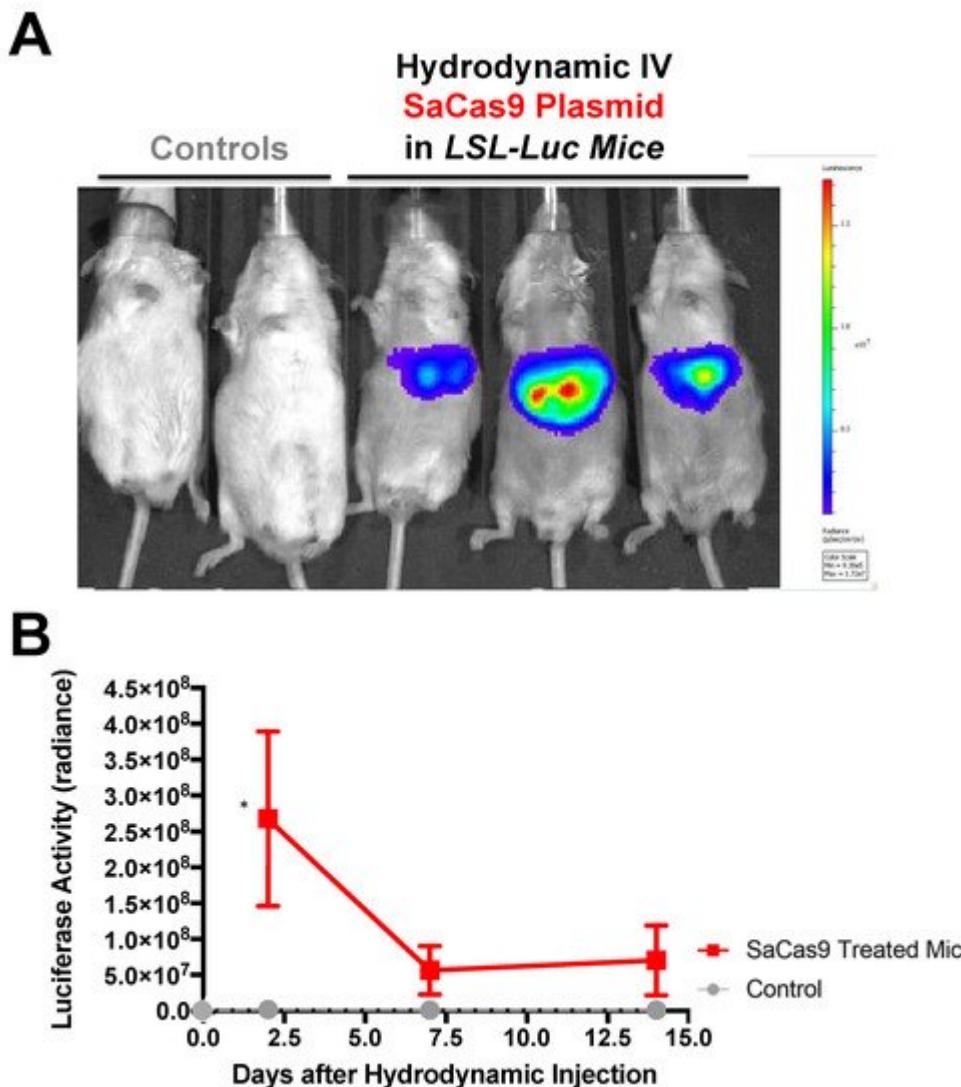


Figure 2. Hydrodynamic delivery in luciferase reporter mice. (A) LSL mice contain a floxed stop cassette blocking luciferase expression. Gene editing by CRISPR gene editing excises the stop cassette exposing luciferase to the promoter allowing for expression. LSL mice were injected hydrodynamically with SaCas9 gene editing plasmids. Control mice received a 2.5 mL PBS injection and the SaCas9 mice received 12.5 μ g of each plasmid in the 2.5 mL of PBS. (B) Mice were monitored for luciferase activity on days 2, 7, and 14. Using a *t*-test for each day, the SaCas9-treated groups were significant compared to the control on Day 2; 95% confidence intervals are shown ($n = 3$) ($* p < 0.01$)

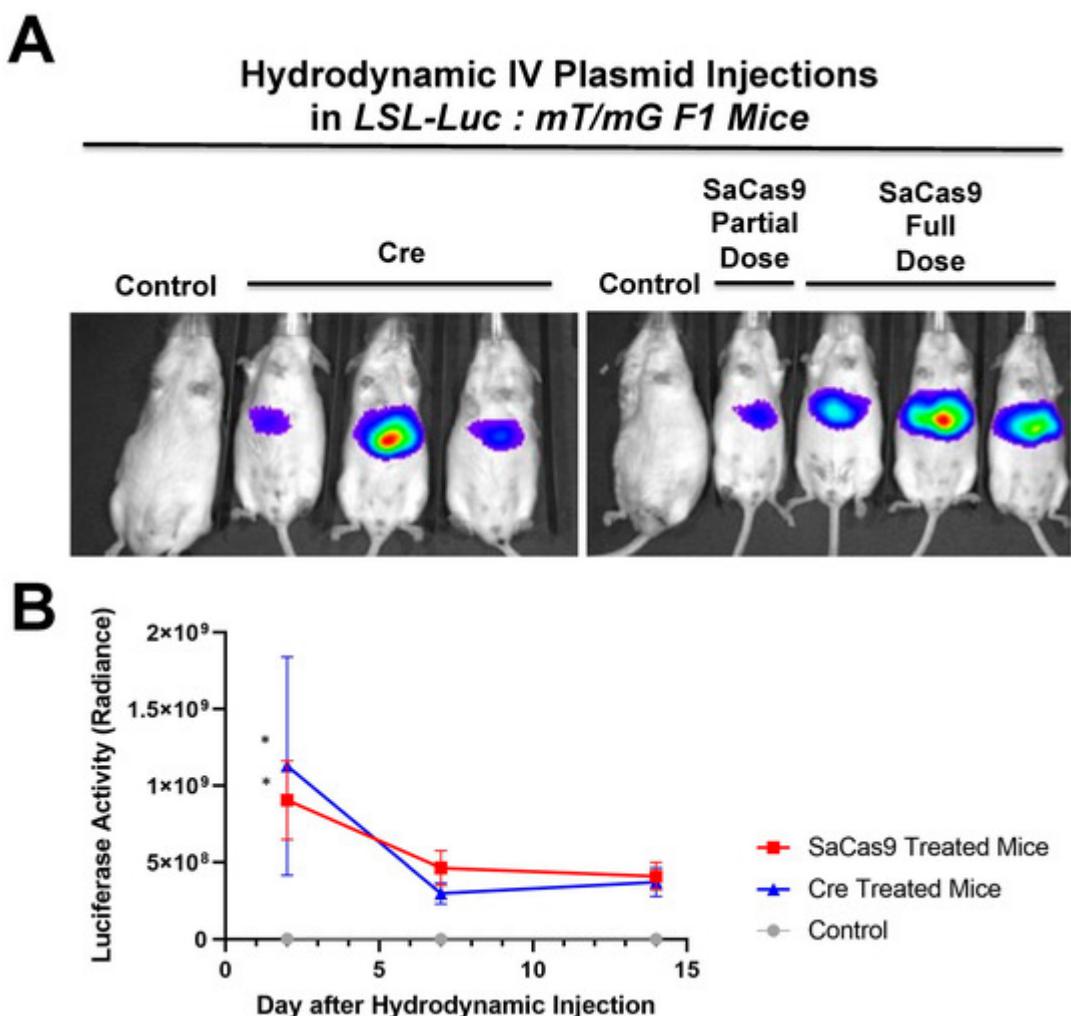


Figure 3. Hydrodynamic delivery in hybrid reporter mice. **(A)** LSL mice were crossed with RG mice to produce a crossed reporter mouse. These mice are sensitive to cre recombinase or CRISPR activity leading to luciferase expression and GFP expression. Mice were hydrodynamically injected with cre recombinase or SaCas9 expressing plasmids. Control mice received a 2.5 mL PBS injection, the SaCas9 plasmid mice received 12.5 μ g of each plasmid in the 2.5 mL of PBS, and the cre groups received 25 μ g of cre plasmid in 2.5 mL of PBS. **(B)** Luciferase activity was monitored within the mice on days 2, 7, and 14 and *t*-tests were carried out. Comparing via *t*-tests showed that the editing groups were significant on Day 2 compared to the control. The lines denote a 95% confidence interval (n = 3) (* p < 0.05).

Hydrodynamic delivery functions by rupturing the cell membranes under pressure, pushing the plasmid DNA into the cell. This pressure and resulting damage could be responsible for the decline in luciferase activity from Day 2 to Day 7 as seen in **Figure 4**, **Figure 2** and **Figure 5**. This coupled with the introduction of neo-antigens in the form of SaCas9 or cre could lead to immunological responses to transduced cells as well. Additionally, there is a discrepancy between the SaCas9 hydrodynamically injected mice in **Figure 2**; **Figure 3**. Older mice are not as effective for hydrodynamic delivery and older mice were used in **Figure 2**. Different maxi-preps were used between the experiments so this may have also contributed.

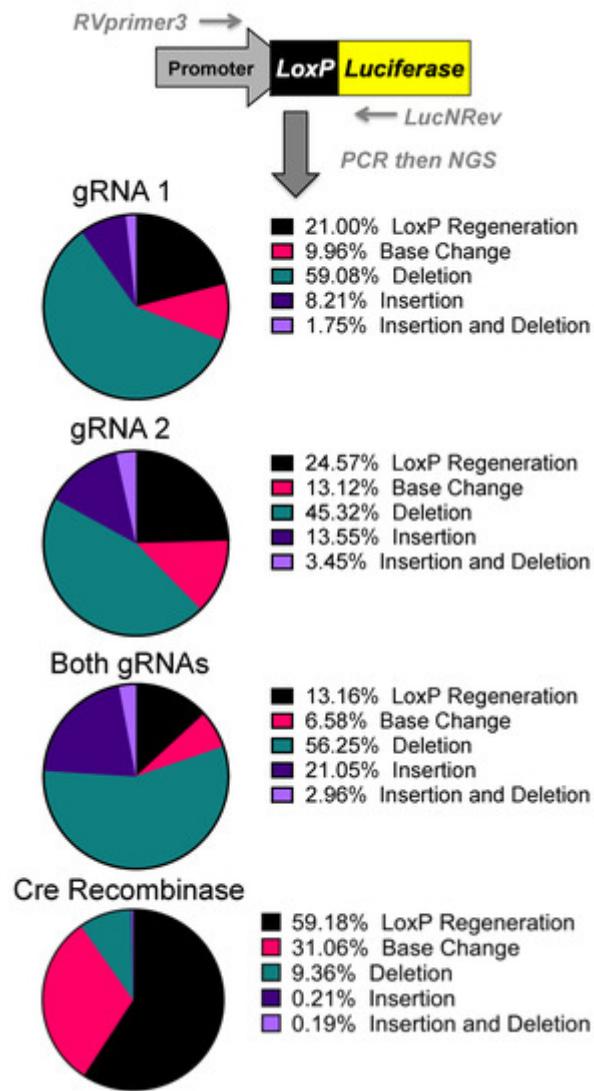


Figure 4. Sequencing of Repair Juncture. DNA was harvested from the previous transfections and amplified using primers in the SV40 promoter and luciferase with next generation sequencing (NGS) sequencing adapters. Using Amplicon-EZ through Genewiz, the deletion results were analyzed and quantified ($n = 1$).

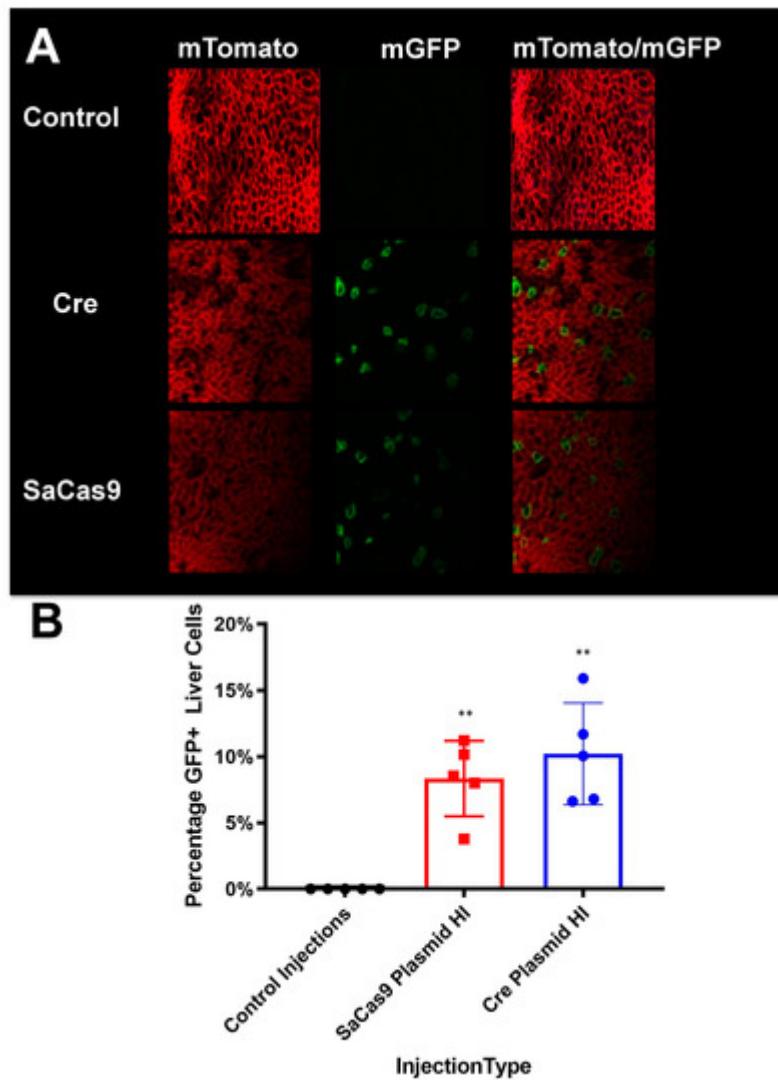


Figure 5. Liver sectioning from hybrid mice. **(A)** Mouse livers were harvested, fixed, and imaged for tdTomato and GFP activity. Representative microscopy shows cre- and SaCas9-treated mouse livers. **(B)** Converted cells were counted as a percentage of total cells in representative images across the gene-edited mice. The bars are 95% confidence intervals and were found significant compared to the control by one-way ANOVA and Tukey's multiple comparisons. The edited groups were not significant compared to each other ($n = 5$) (** $p < 0.005$).

Additionally, **Figure 6** shows that there is a dose response to differing amounts of SaCas9. This is of value because it shows that this system could be used to monitor variant levels of delivery via luciferase activity. Whether SaCas9 is delivered by nanoparticle, virus, ribonucleoprotein, or some novel method, this provides a method to detect variations in delivery efficiency non-invasively and with cellular specificity. It should also be noted that the luciferase levels showed a significant decrease compared to previous experiments. We believe this to be the result of imaging differences between the Xenogen and the Lumina. [Supplementary Figure S3](#) also shows that this reporter system works in tissues beyond the liver. The Barry lab has also previously published in Hillestad et al. that they were able to detect cre recombinase activity using this reporter mouse in the liver, heart, lungs, muscles, brain, kidney, and spleen [10].

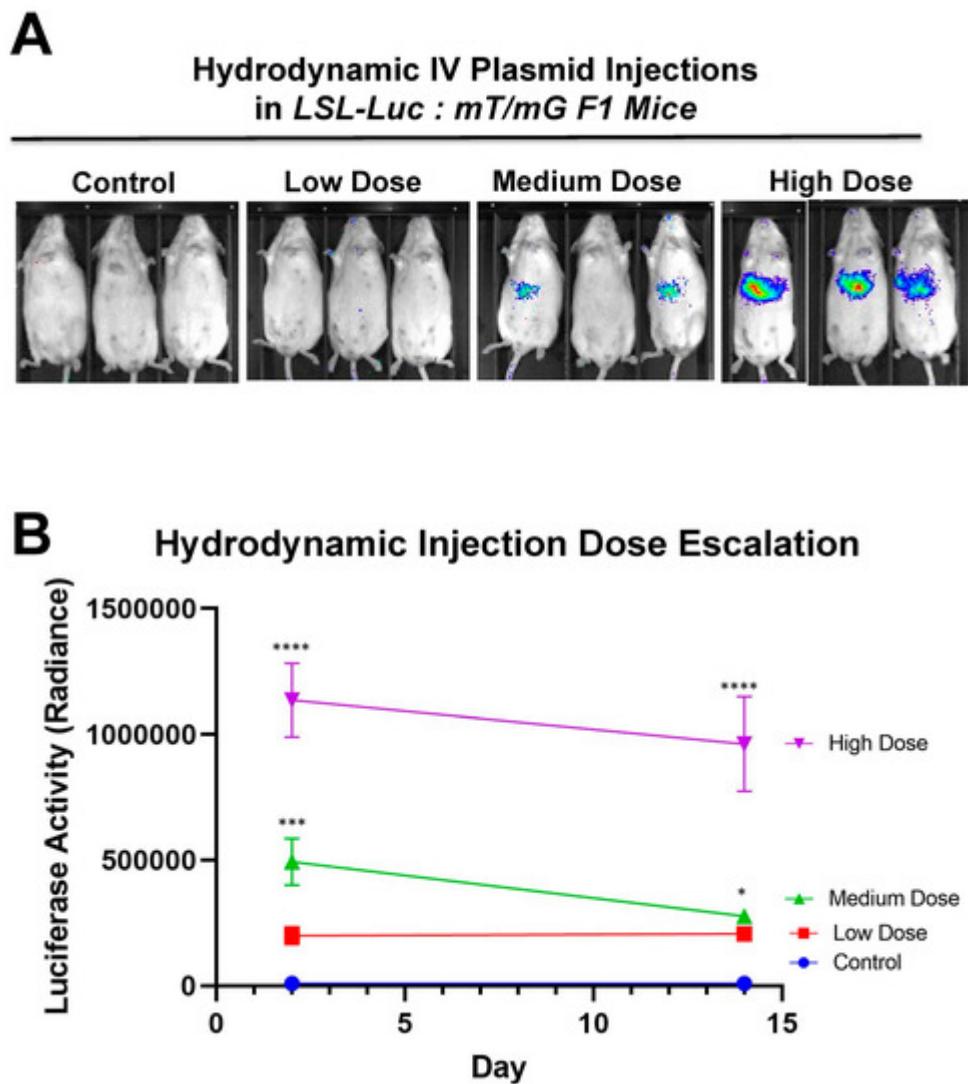


Figure 6. Hydrodynamic dose variation injections. (A) Mice were hydrodynamically injected at varying doses of 7.5 μ g, 25 μ g, and 83.3 μ g of plasmid. Day 2 mouse images are shown. (B) Radiance levels emitting from the mouse livers were measured and compared. Each day was analyzed by one-way ANOVA and Tukey's multiple comparison. High and medium doses were significant compared to the control on both days while low dose was not significant compared to the control on either day via one-way ANOVA with multiple comparison. Via one-way ANOVA, high, medium, and low doses were significant compared to each other on Day 2. On Day 14 and by one-way ANOVA with Tukey's multiple comparisons high dose was significant compared to all other groups. Medium dose was only significant compared to the control dose on Day 14. All other comparisons on Day 14 were not significant by one-way ANOVA with Tukey's multiple comparisons ($n = 3$) (* $p < 0.05$, ** $p < 0.0005$, *** $p < 0.0001$).

Next generation sequencing analyses of the gene editing outcomes provide some insight into how the cell repairs these DNA breaks prior to reporter activation. Three repair pathways were non-homology-induced repairs, small local homology (microhomology), and homology-based outcomes, especially considering that deletions occur between two identical loxP sequences. Analyses of deletions induced by individual gRNAs demonstrated that most

of the edits result in the recreation of a *loxP* site. Unfortunately, these can be the result of either NHEJ or homology-based repair when using individual gRNAs (Supplementary Figure S1).

Analysis of the dual gRNA-induced deletions demonstrated a different mix of outcomes (Supplementary Figure S1). After the repair pathway-ambiguous *loxP* recreation, the next two top reads consisted of NHEJ-based events that are caused by different gRNAs targeting each *loxP* site. Although the top reads are ambiguous, the top reads that are capable of being definitively linked to a pathway are NHEJ. Depending on which gRNA edits which *loxP* site will determine whether an 8bp region would be duplicated or deleted. That being said, these cuts have the potential to cause microhomology-based events, only one of which is distinguishable as such. The strong prevalence of NHEJ-specific events strongly suggests that this is a major repair mechanism, but with the top detected outcome being ambiguous and repeated cutting of regenerated *loxP* sites confusing the data further, it cannot be determined at this time what is the primary repair pathway. Further parsing of the mechanism may be done in future work by specifically knocking down proteins related to these pathways.

The sequencing was initially done to explore the possibility of alternative DNA repair pathways being responsible for the increased efficacy of both gRNAs over individual gRNAs. While repair mechanisms cannot be determined conclusively, the increases in efficacy seen with both gRNAs are potentially connected with CRISPR gene editing selecting for mutations that prevent further cutting. With a single gRNA, there is the potential for the gRNA target site to be cut and result in an insertion or deletion (indel) rather than result in a large deletion and preclude the possibility for a subsequent DSB generating a large deletion. An indel near the cut site of the gRNA target site would greatly reduce, if not inhibit, SaCas9 cutting as seen by some of the top reads in NGS [16]. If a *loxP* site is mutated to prevent CRISPR cutting at both *loxP* sites, large deletion of the stop cassette would be prevented. With two potential gRNA targets, the potential indel would be further away from the PAM and less likely to inhibit SaCas9 binding [17]. This may potentially explain the increased efficacy seen with two gRNAs rather than one.

By targeting Cas9 to the *loxP* site, there is also the opportunity to enable further manipulation of these sites. These models can be used in conjunction with targeted insertion technology to deliver genes of interest at *loxP* sites. This could be done to modify the sites to express a different gene or to reconstitute stop cassettes with mutant *loxP* sites that are resistant to CRISPR cutting but available to cre recombinase. There is also the potential of creating a three-outcome cassette: starting cassette, cre recombinase-treated expression cassette, and CRISPR-treated cassette. This would give greater control over animal models that would be able to turn on defective genes via cre recombinase and then deactivate them by CRISPR. There is also the potential of using the deactivated CRISPR enzyme as an inhibitor of cre recombinase, binding *loxP* and preventing binding and recombination. This could act to prevent cre recombinase activity in specific cell populations.

It should be noted that despite the great activity shown by SaCas9 targeting *loxP*, results may vary depending on the DNA site being targeted or the CRISPR being used. This SaCas9 reporter system is highly efficient while the ErCas12a has lower activity. (**Figure 1** and **Figure 7**). This can be attributed, at least partially, by differential binding and cleavage kinetics demonstrated by Cas12a type effectors compared to Cas9. It would be of interest in the future to determine if similar Cas12a effectors such as AsCas12a or LbCas12a can improve upon this

foundational work with SaCas9. It is important to note that this may overestimate the activity when using a gRNA relevant to a gene therapy application or a CRISPR other than SaCas9.

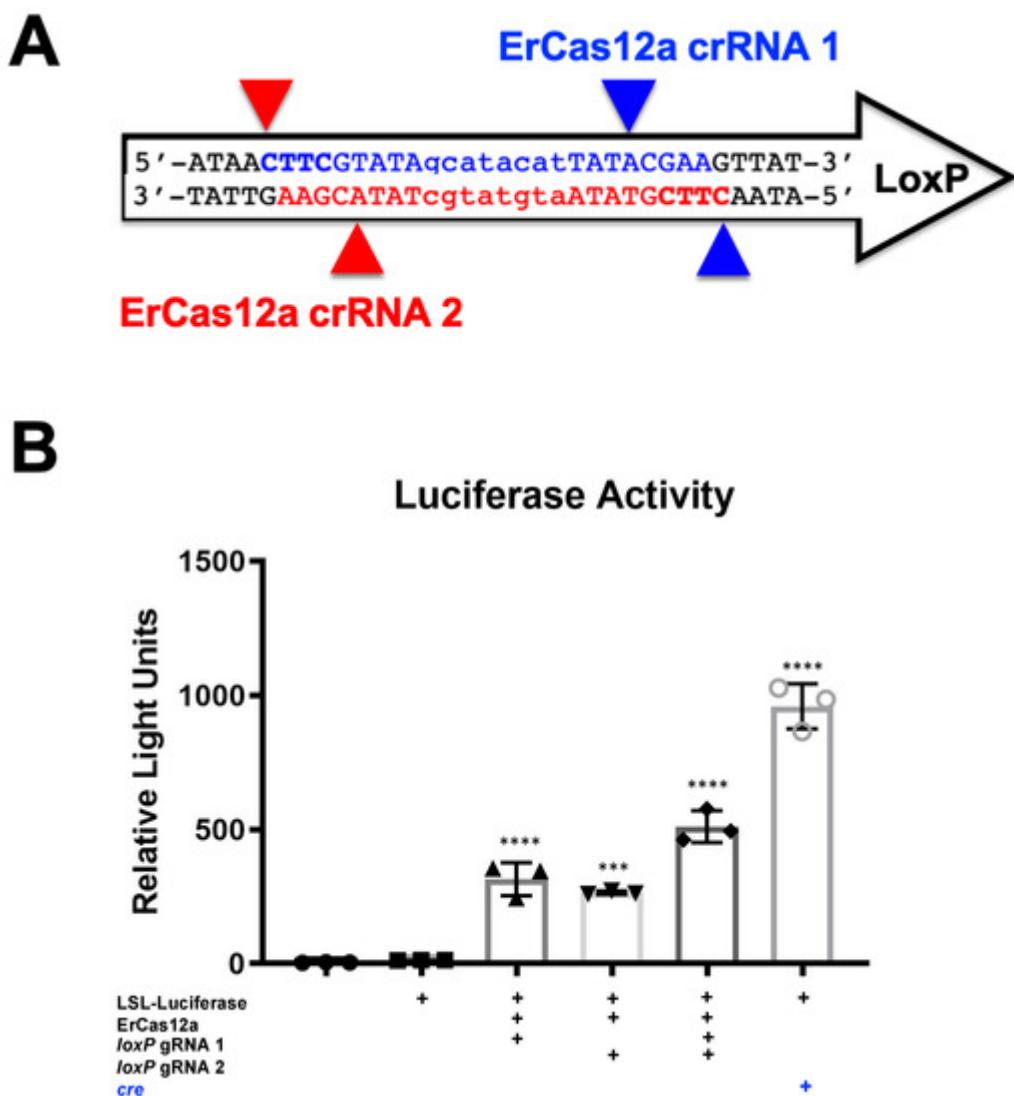


Figure 7. ErCas12a-mediated gene activation through targeting *loxP* in vitro. (A) The *loxP* sequence is shown with key features and target sites for CRISPR ErCas12a. The capitalized base pairs are the 13-base-pair palindromic regions flanking the 8-base-pair core that gives *loxP* its directionality. gRNA 1 homologous strand to the guide RNA is depicted in light blue with the PAM-binding region in dark blue and bold letters and a triangle to indicate the cleavage site. gRNA 2 depicts the same in red. (B) The ErCas12a plasmid co-expressed either gRNA 1 or 2 and was co-transfected with the LSL-Luc reporter plasmid at 2.5 ug. By one-way ANOVA with Tukey's multiple comparisons, all the groups were significant except between the untransfected control groups (untransfected versus reporter plasmid only) and between the individual gRNA-treated groups (*loxP* gRNA 1 vs. *loxP* gRNA 2); 95% confidence intervals are shown (n = 3) (** p < 0.0005 and **** p < 0.0001).

This three-way reporter system can be applied to in vivo delivery of CRISPR systems to assess the tropism of the delivery system on a broad and narrow level along with a timeline of CRISPR editing. More broadly speaking, this

system can be used in combination with any *loxP* system that relies on deletion for its activity, for which there are over 3000 mice on JAX Laboratories website related to the *cre-lox* system.

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