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Unlocking LoxP to Track Genome Editing *In Vivo*

William A.C. Gendron¹, Jeffrey D. Rubin¹, Michael Hansen¹, Rebecca Nace¹, Brandon W. Simone², Stephen C. Ekker² and Michael A. Barry³*

¹Virology and Gene Therapy Graduate Program, Mayo Clinic Graduate School of Biomedical Sciences,
²Department of Biochemistry and Molecular Biology
³Department of Medicine, Division of Infectious Diseases, Department of Immunology, Department of Molecular Medicine, Mayo Clinic, Rochester, MN 55905
*Correspondence: Michael A. Barry, Email: mab@mayo.edu

Abstract: 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 and quantitatively monitor gene editing activity non-invasively in living animals that also facilitates confocal microscopy and nucleotide level analyses. Here we report a new CRISPR "footprinting" approach to activate luciferase and fluorescent proteins in mice as a function of gene editing. This system is based on experience with our prior Cre-detector system and is designed for Cas editors able to target LoxP including gRNAs including SaCas9 and ErCas12a [1, 2] . 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 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 recombinase demonstrating high effective delivery and activity in a whole animal. This work establishes genome editing tools and models to track CRISPR editing *in vivo* non-invasively and to fingerprint the identity of targeted cells. This approach also enables similar utility for any of the thousands of previously generated LoxP animal models.

1. Introduction

CRISPR-Cas technology has revolutionized the gene editing space, enabling access to the field for many new users[3]. 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*[4]. For example, somatic *in vivo* CRISPR–Cas9 gene editing was recently performed in humans as a treatment for Leiber's congenital amaurosis 10 (LCA10)[5].

Previous methods to monitor *in vivo* CRISPR editing have commonly relied on nucleotide sequencing of edited target biopsies or by invasive monitoring of the tissues for fluorescent markers[6]. 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.
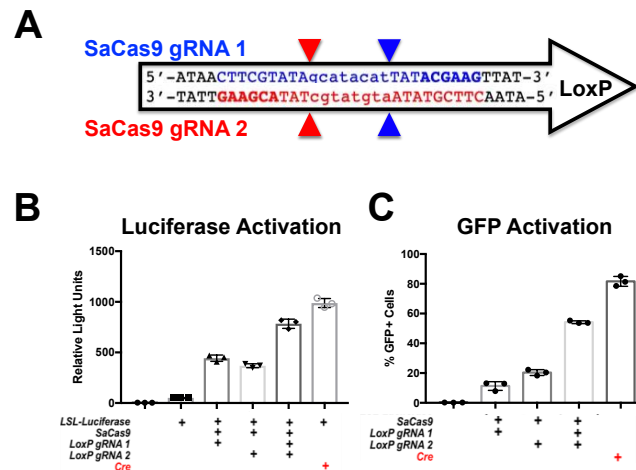
Figure 1. CRISPR-mediated gene activation through targeting LoxP in vitro. A) The loxP sequence is shown with key features and target sites for CRISPR SaCas9. The capitalized base pairs are the 13 base pair palindromic regions flanking the 8 base pair core that gives loxP its directionality. gRNA 1 homologous 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) Cells were plated into a 6 well plate and transfected at 60-80% confluency with Xfect. 2.5ug of the reporter plasmid was transfected to be targeted by Cre or SaCas9. By Anova, all of the groups were significant except between the untransfected control group and the P133 transfected group and between the individual gRNA treated groups. 95% confidence intervals are shown. (n=3). C) The Red/Green HEK293 reporter cells were transfected with 5ug of plasmid. The single gRNA groups were significant compared to untransfected and the combination of gRNAs and Cre were significant compared to all groups.

The Cre-LoxP system is a powerful tool for animal and cell models, providing robust and specific editing of DNA [7]. 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[8]. Thousands of animal models have been generated using Cre-LoxP technologies, 320 of which are specifically mouse reporter models[9]. 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 polyA "stop" sequence or another gene[10, 11]. When the cells of these animals are exposed to Cre, the floxed sequence is excised, this activates reporter gene expression.

We previously made use of these floxed reporter mice to "fingerprint" gene delivery *in vivo* by adeno-associated virus (AAV) vectors [12]. 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) (**Figure 5**). 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[10].

More recently, a similar "footprinting" system was used to track gene delivery with Cre recombinase and notably Cas9 by monitoring fluorescent protein activation[13]. 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 poly adenylation (polyA) "stop" cassette near LoxP sites in mice bearing floxed inactivated fluorescent protein genes. This design is limited to the

specific 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 [13].

While this footprinting approach was novel, its targeting of the polyA sequence will work only in a small set of mouse models. Mice that have expression cassettes rather than repeated Poly-As as the stop region, like the LSL and mG/mT mice, cannot use a poly-A 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, we instead targeted CRISPR-Cas9 to LoxP sequences and tested this in our combined luciferase and mT/mG mouse model for combined live imaging and post-mortem evaluation of genome editing. By doing this, we have shown that SaCas9 activation of these reporter genes has near equivalent activity to that of Cre Recombinase, *in vitro* and *in vivo*. Through sequencing, we have not conclusively determined a primary pathway 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 have 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. Materials and Methods

2.1. Plasmids and Cloning

The reporter plasmid p133 pSV-STOP-luc was a gift from Jeffrey Green (Addgene plasmid # 8390 ; http://n2t.net/addgene:8390 ; RRID:Addgene_8390) consists of an SV40 promoter upstream of a floxed set of SV40 polyadenylation signals with firefly luciferase downstream of floxed region. px601 was purchased from Addgene and consists of a CMV expressed SaCas9 and gRNA expression cassette flanked by AAV ITRs (pX601-AAV-CMV::NLS-SaCas9-NLS-3xHA-bGHpA;U6::BsaI-sgRNA was a gift from Feng Zhang (Addgene plasmid # 61591 ; http://n2t.net/addgene:61591 ; RRID:Addgene_61591)). Annealed oligos were inserted into the gRNA cassette after px601 was digested with BsmBI. This method was previously described by Ran et al[14]. The plasmids that were successfully cloned with the LoxP gRNAs 1 and 2 were named px601 LoxP1 and px601 LoxP2 respectively. pSC-CMV-Cre was previously developed by the Barry lab and consists of a CMV driven Cre flanked by AAV ITRs[12]. ErCas12a plasmid was cloned as previously described in Wierson et al[2].

2.2. Cell Culture

HEK293 cells purchased from ATCC (ATCC® CRL-1573™) were grown with Dulbecco's modified Eagle's medium completed with 10% fetal bovine serum (Thermo Fisher Scientific) and penicillin-streptomycin at 100U/mL (Thermo Fisher Scientific). Plasmid transfections were done with Xfect Transfection Reagent from Takara Bio into 6 well plates of HEK293 cells when the cells were at 70% confluency following manufacturer's instructions. Transfections consisted of 2.5 µg of editing plasmids (px601 gRNA1, px601 gRNA2 and pSC-CMV-Cre) and 2.5 µg of reporter plasmids. Transfections that use 2 gRNAs used 1.25 µg of px601 LoxP1 and px601 LoxP2 each. Cells were harvested 48 hours post transfection for luciferase assays. HEK293 cells were transduced with a lentivirus constructed from pLV-CMV-LoxP-DsRed-LoxP-eGFP and selected for using the puromycin selection marker in it. Once these were established, the cells were used in Figure 1C. The cells were transfected with 5ug of px601 LoxP1, 5ug of px601 LoxP2, 2.5 ug of each or with 5ug of Cre reporter plasmid. For the ErCas12a analysis, cells were plated into a 6 well plate and transfected at 60-80% confluency with Xfect. 2.5ug of the reporter plasmid was transfected to be targeted by ErCas12a. The ErCas12a plasmid co-expressed either gRNA 1 or 2 and was co-transfected at 2.5ug. For the cells transfected with both gRNAs, 1.25ug of each was transfected into that well. Cells were harvested for a luciferase assay. By

Anova, all of the groups were significant except between the untransfected control group and the P133 transfected group and between the individual gRNA treated groups. 95% confidence intervals are shown. (n=3).

2.3. Luciferase Assay

Cells were harvested from each condition via trypsin. The cells were spun down at 500xG for 10 minutes and the supernatant was removed. The cells were resuspended in 400ul of PBS and pipetted vigorously to have a homogenous mixture of cells. 100 µl of cells were pipetted into a clear bottom, black 96 well plate. This was done in triplicate for each group of cells. 100 µl of room temperature Bright-Glo was then applied to each well. Wells were then analyzed via the BioTek Synergy H1 Microplate Read and the BioTek Gen5 Microplate Software.

2.4. Flow Cytometry

Cells were harvested as with the luciferase assay. The cells were passed through a 100um filter to avoid clumping of cells. The cells were then assayed by the Microscopy and Cell Analysis Core at Mayo Clinic Rochester (Rochester, Minnesota) using a BD FACSCanto. The data was then processed via FloJo.

2.5. Mice

LSL Luc mice (a.k.a. FVB.129S6(B6)-GT(ROSA)26Sor$^{tm1(Luc)Kael}$/J) have a luciferase expression cassette behind a floxed polyA signal domain with the ROSA26 promoter. The mT/mG mice (a.k.a. GT(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J mice) have a floxed mTomato behind the pCA promoter with membrane bound EGFP behind mTomato. All mice were purchased from Jackson Laboratories and all animals were treated according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, and NIH Guide.

2.6. Hydrodynamic Injections

Mice were injected by hydrodynamic tail vein injections [15]. Injections consisted of 25 µg of gene editing plasmids, either px601 LoxP1 and px601 LoxP2 mixed in a 50:50 ratio or pSC-CMV-Cre. The plasmids were suspended in 2.5 mL (~10% body weight) of PBS and the injection was completed within 8-10 seconds. Control mice were injected with PBS only. Mice were monitored post injection for tolerance of hydrodynamic injection. For the dose escalation hydrodynamic injections, 7.5ug, 25ug and 83.3ug of plasmid were used for the groups low, medium and high respectively.

2.7. In Vivo Bioluminescent Imaging

Mice were anesthetized with 2% isofluorane and maintained at 2% isofluorane. Mice were intraperitoneally injected with 150ul of 20mg/ml D-Luciferin (RR Labs, Inc., San Diego, CA). Xenogen IVIS 200 was used to image the mice ten minutes after injection of the D-Luciferin. The dose escalation imaging experiments were done using the IVIS Lumina S5 Imaging System due to the Xenogen being retired. Living Image software was used to quantify the luminescence.

2.8. Liver Sectioning and Confocal Microscopy of Mouse Liver

Livers were harvested and fixed overnight in 4% paraformaldehyde (PFA)-phosphate buffered saline (PBS) at 4°C. Livers were transferred to 15% sucrose-PBS overnight and then moved to 30% sucrose PBS at 4°C until the tissue sunk in the solution to cryoprotect the tissue. Tissues were trimmed and flash frozen in optimal cutting temperature (OCT) medium (Sakura Finetek). Leica CM1860 UV cryostat (Leica Biosystems) was used to create cryosections (18um thickness) which were mounted on slides (SuperFrost Plus; Thermo Fisher Scientific, Waltham, MA). VECTASHIELD with 4',6-diamidino-2-phenyl-indole (DAPI) (Vector Laboratories, Burlingame, CA) was applied and a CytoSeal-60 coverslip sealant (Thermo Fisher Scientific). The confocal microscopy was performed at the

Microscopy and Cell Analysis Core facility at Mayo Clinic Rochester (Rochester, MN) using a Zeiss LSM790 laser confocal microscope (Carl Zeiss Jena, Jena, Germany). Representative images were selected from the confocal microscopy and used to count GFP+ cells and total cells.

2.9. Sequencing of LoxP Junctions

DNA was isolated from the transfected human 293 cells via the DNeasy Blood and Tissue Kit's protocol. Using the primers RVprimer3 (5'-CTAGCAAAATAGGCTGTCCC-3') and LucNRev (5'-CCTTATGCAGTTGCTCTCC-3') were used to PCR amplify from the SV40 promoter across the poly-A region or the deleted region. Shortening the extension time to 30 seconds restricts the amplified product to only edited plasmids. This band was then gel excised and Stratacloned to isolate individual sequences. Individual clones were grown up, miniprepped and sent for Sanger sequencing at Genewiz. Additionally, gel isolated bands were also sent for Next Generation Sequencing (NGS) via the Genewiz's Amplicon EZ sequencing using the extended primers RVprimer3 NGS (5'-ACAC-TCTTTCCCTACACGACGCTCTTCCGATCTCTAGCAAAATAGGCTGTCCC-3') and LucNRev NGS (5'- GACTGGAGTTCAGACGTGTGCTCTTCCGATCT CCTTATGCAG-TTGCTCTCC-3'). Read outcomes were compiled and rank from most common to least. These reads were then aligned to the predicted LoxP regeneration that one would expect from Cre Recombinase or a one gRNA NHEJ event. The reads were then analyzed for clear signs of homology or non-homology-based editing. It was taken into account that reads could be ambiguous due to the chance of LoxP sites being regenerated and lead to repeated cutting, obscuring what the initial event was. Reads were determined to likely be NHEJ when the repair outcome showed no signs of homology-based repair at the juncture. Reads were labeled as homology based if it was clear that the homology seen there was the result of the large deletion. Reads that have a chance of being caused by either pathway in the primary large deletion event were labeled as ambiguous. Many events were labeled as ambiguous due to them showing signs either non-homologous end joining or homology directed repair but could have been the result of secondary cleavage of the regenerated LoxP site.

2.10. Statistical analysis

T-test and one-way ANOVA were using GraphPad Prism™. A 95% confidence interval was considered significant.

3. Results

3.1. CRISPR gRNA Design and In Vitro Evaluation.

The ability to target various Cas enzymes to different sequences is commonly limited by their need to bind specific Protospacer Adjacent Motifs (PAMs) (**Table 1**)[1, 2, 16-20]. LoxP is a 34-base pair (bp) target sequence in bacteriophage P1 for Cre recombinase. The conventional LoxP sequence ATAACTTCGTATAgcatacatTATACGAAGTTAT has two palindromic 13 bp sequences (upper case) separated by an asymmetric 8 bp "core" sequence (lower case) (**Fig. 1A**). The PAMs of different Cas proteins were evaluated for their ability to target the wild-type LoxP sequence. For example, the type 1 CRISPR from *Staphylococcus aureus* Cas9 (SaCas9) has a general PAM requirement of NNGRRT where "N" indicates any nucleotide, "R" indicates any purine (A or G)[21]. When this PAM sequence was used to scan LoxP, it initially appeared that SaCas9 could not target LoxP. However, the "T" in NNGRR(T) is actually optional[22]. When T was excluded when scanning the site, two different SaCas9 gRNAs (gRNA1 and 2) were identified that target either the top or bottom strand of LoxP (**Fig. 1A**).

Table 1. Example CRISPRS for LoxP activation. List of**.** CRISPRs with compatible PAMs that enable targeting within a conventional loxP.

CRISPR Variants	PAM Sequence	CRISPR Class
SaCas9	NNGRR(T)	I
ErCas12a	YTTN	II
xCas9	NG, GAA	I
SpCas9-NG (SpG)	NGN	I
SpRY	NRN>NYN	I
AsCpf1 RR-Variant	TYCV	II
LbCpf1 RR-Variant	TYCV	II
AsCpf1 RVR Variant	TATV	II

When the gRNAs of several other type I and type II CRISPRs were identified for targeting LoxP, gRNAs for ErCas12a, xCas9, AsCas12a RR-Variant, LbCas12a RR-Variant, and AsCas12a RVR Variant (**Table 1**)[1, 16, 17, 22, 23].

*3.2. LoxP-targeted Genome Editing with SaCas9*

Genes can be inactivated by placing a LoxP-flanked (floxed) polyA stop sequence between the gene's promoter and its open reading frame (**Fig. 1B**). Cre recombinase can activate expression from such a gene by deleting the stop sequence**.**

In theory, if SaCas9 can cleave both LoxP flanking the polyA sequence, the host cell may be able to repair these breaks resulting in deletion of the stop sequence. If so, we hypothesized that SaCas9 might be able to activate luciferase expression in a manner similar to Cre recombinase to delete the same stop polyA sequence (**Fig. 1B**).

To test this, SaCas9 gRNA1 and 2 were cloned separately into a plasmid expressing the SaCas9 and a gRNA. These plasmids were transfected into HEK293 cells with a plasmid p133 that has a floxed SV40 polyA signal upstream of the firefly luciferase cDNA (**Fig. 1B**). Under these conditions, both single gRNAs enabled SaCas9 to activate luciferase expression (**Fig. 1B**). When the two gRNAs were co-transfected together with the luciferase plasmid, reporter gene expression increased nearly two-fold. This level of gene activation was notable as it approached the level of luciferase activation that was mediated by Cre recombinase itself.

To test the editing efficiency, dsRed/eGFP stable 293 cells were created using the pLV-CMV-LoxP-DsRed-LoxP-eGFP (**Fig. 1C**). These cells were then transfected with px601 LoxP1, px601 LoxP2, a combination of the two gRNA-SaCas9 plasmids or Cre plasmid (**Fig. 1C**). These cells were then assayed by flow cytometry. These data show a similar trend to the luciferase assay.

To determine if other genome editors (**Table 1**) might also mediate this effect, ErCas12a plasmids were also transfected along with p133 reporter plasmid. Under these conditions, both were able to induce luciferase activity, albeit at lower levels than SaCas9 (**Fig. 2**).
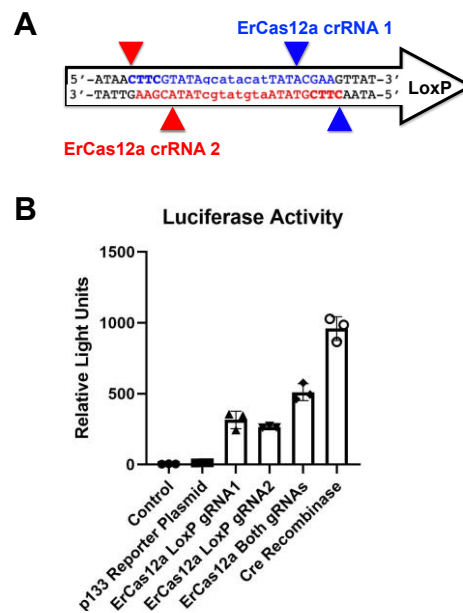
Figure 2. ErCas12a-mediated gene activation through targeting LoxP in vitro. A) The loxP sequence is shown with key features and target sites for CRISPR ErCas12a. The capitalized base pairs are the 13 base pair palindromic regions flanking the 8 base pair core that gives loxP its directionality. gRNA 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 at 2.5ug. By Anova, all of the groups were significant except between the untransfected control group and the P133 transfected group and between the individual gRNA treated groups. 95% confidence intervals are shown. (n=3).

3.3. *Host Cell Repair of SaCas9-Cleaved LoxP Sites*

DNA was purified from cells that were transfected with the p133 luciferase reporter and gene editing plasmids. A 500 base pair fragment containing the LoxP sites was PCR amplified and assessed by next generation sequencing. These sequences were aligned against the floxed sequence and the outcomes of recombination or DNA repair were evaluated (**Fig. 3 and Supplementary Fig. 1**). When Cre-modified fragments were evaluated, more than half accurately deleted the polyA sites and regenerated an intact LoxP site (**Fig. 3**). Interestingly, nearly half of the sites were inaccurately recombined by LoxP generating mutations, deletions, or insertions. The same evaluation of SaCas9 treated fragments revealed markedly lower regeneration of the wild-type LoxP sequence.

About a quarter of the reactions mediated by single gRNA1 or gRNA2 generated an intact LoxP and the rest were a variety of mutations. When both gRNAs were used, this reduced regeneration of LoxP to only 13% of outcomes. Analysis of the specific repair junctions suggested that most were resolved by non-homologous end-joining rather than by homology-directed repair or microhomology-mediated repair (**Figure 3 and Supplementary Figure 1**).
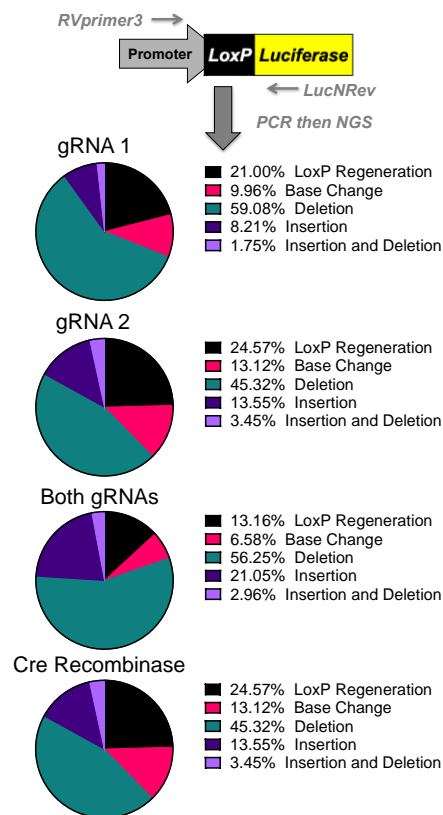
Figure 3.: Sequencing of Repair Juncture. DNA was harvested from the previous transfections and amplified using primers in the SV40 promoter and luciferase with Next Generation Sequencing (NGS) sequencing adapters. Using Amplicon-EZ through Genewiz, the deletion results were analyzed and quantified. (n=1).

3.4. Monitoring In vivo Genome Editing in Living Animals

To determine if SaCas9 with gRNAs targeting LoxP would be effective *in vivo*, LoxP-Stop-LoxP Luciferase (LSL Luc) mice with a LoxP-inactivated luciferase gene were injected by hydrodynamic intravenous injection with the SaCas9 gRNA1 and gRNA2 plasmids and the animals were imaged for luciferase activity up to 14 days after injection (**Fig. 4**).

Hybrid mice generated from crossing LSL mice with mT/mG mice have exactly one gene copy of Cre-activatable luciferase and exactly one gene copy of the mT/mG cassette (**Fig. 5**). Therefore, these hybrid mice provide an "on/off" system to detect vector transduction and pharmacodynamics. The presence of three reporter genes enables 1) *in vivo* imaging, 2) cell-specific transduction monitoring via mG expression, and 3) on/off confirmation of transduction by coordinated loss of mT with activation of mG[12]. This system can also be applied to the vast repertoire of mice engineered for tissue-specific Cre expression (https://www.jax.org/research-and-faculty/resources/cre-repository).

Under these conditions, the Cas9 and gRNA plasmids appeared to active the genome copies of luciferase as evidenced by the generation of photons of light from the liver (**Fig. 4A**). Sustained luciferase activity was observed for over 2 weeks after a single SaCas9 and gRNA plasmid injection (**Fig. 4B**). SaCas9 activity was next compared to Cre recombinase in LSL-Luc:mT/mG hybrid mice. Mice were hydrodynamically injected with Cre plasmids or a combination of Cas9 gRNA1 and 2 plasmids and luciferase imaging was performed as in **Fig. 4**.
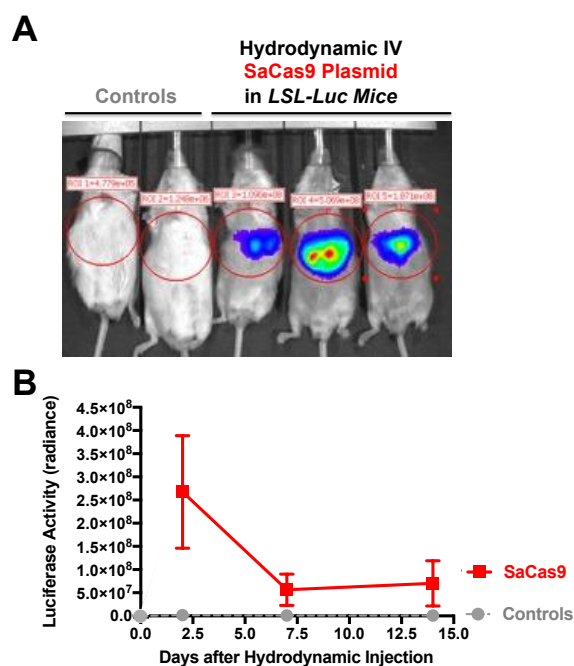
Figure 4. Hydrodynamic Delivery in Luciferase Reporter Mice. A) LSL mice contain a floxed stop cassette blocking luciferase expression. Gene editing by CRISPR gene editing excises the stop cassette exposing luciferase to the promoter allowing for expression. LSL mice were injected hydrodynamically with SaCas9 gene editing plasmids.   Control mice received a 2.5 mL PBS injection and the SaCas9 plasmid mice received 12.5 ug 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. 95% confidence intervals are shown. (n=3).

Luciferase activity generated by the introduction of SaCas9 was similar to that generated by injection of Cre recombinase plasmid (**Fig. 5**). Following this live animal imaging, the animals were sacrificed, and their livers were sectioned for confocal microscopy to observe mT and mG at cellular resolution.
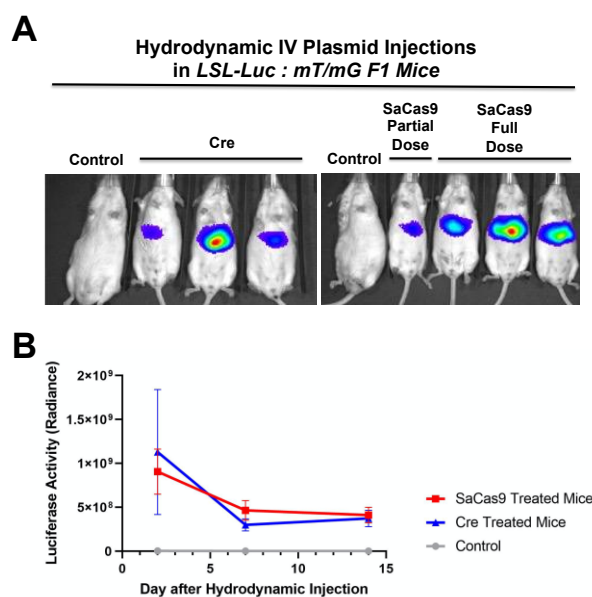
Figure 5. Hydrodynamic Delivery in Hybrid Reporter Mice. A) LSL mice were crossed with RG mice to produce a crossed reporter mouse. These mice are sensitive to Cre recombinase or CRISPR activity leading to luciferase expression and GFP expression. Mice were hydrodynamically

injected with Cre recombinase or SaCas9 expressing plasmids. Control mice received a 2.5 mL PBS injection, the SaCas9 plasmid mice received 6.25 ug of each plasmid in the 2.5 mL of PBS and the Cre groups received 12.5ug of Cre plasmid in 2.5mL of PBS. **B)** Luciferase activity was monitored within the mice on days 2, 7 and 14. An Anova test showed that the editing groups were significant compared to the control but not between each other. The lines denote a 95% confidence Interval. (n=3).

These analyses showed that Cre and SaCas9 plasmids mediated conversion of cells from mTomato to mGFP expression in a subset of cells (**Fig. 6A**). When GFP positive cells were counted on representative slides this indicated that SaCas9 and Cre mediated similar conversion of approximately 10% of cells from mT to mG expression after hydrodynamic injection of these plasmids (**Fig. 6B**).
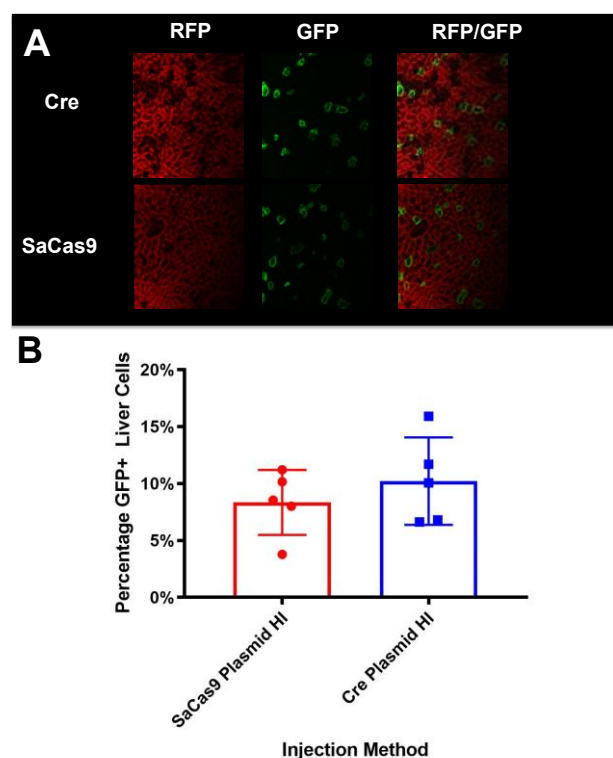
Figure 6. Liver Sectioning from Hybrid Mice. A) Mouse livers were harvested, fixed imaged for dsRed and GFP activity. Representative microscopy of Cre and SaCas9 treated mouse livers. **B)** Converted cells were counted as a percentage of total cells in representative images across the gene edited mice. The bars are 95% confidence intervals. (n=5).

Additionally, to determine the system's ability to have a dose-based response, mice were hydrodynamically injected with varying doses of SaCas9 plasmid **(Fig.7A)**. This shows that the dose of SaCas9 does affect the luciferase reporter activity as seen by the varying activity levels measured **(Fig. 7B)**. Mice were shown to have a dose response with significance between the high dose and the lower doses and all doses vs the control.

A

Hydrodynamic Plasmid Injections

Control   **Low Dose**   **Medium Dose**   **High Dose**

B
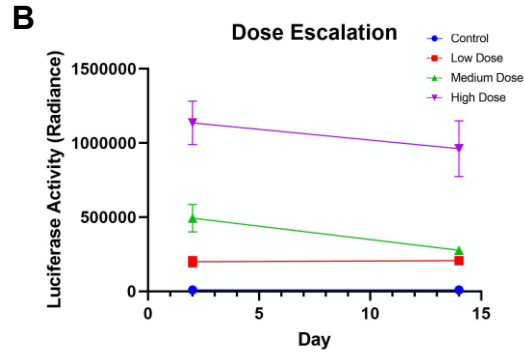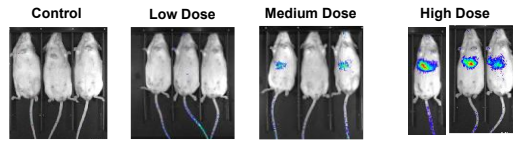
Figure 7. Hydrodynamic Dose Variation Injections: A) Mice were hydrodynamically injected at varying doses of 7.5 ug, 25 ug and 83.3 ug of plasmid. B) Radiance levels emitting from the mouse livers were measured and compared.

4. 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. We 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, we have been able to demonstrate the ability to detect CRISPR activity 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 Poly-A region (**Supplementary Fig. 2**) . While these have shown the ability to monitor CRISPR activity via tissue sectioning and DNA analysis, these systems are specific to Ai9 mice and would not be broadly applicable to other reporter systems such as the LSL-mice or the mT/mG target loci[24]. 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 similar to Cre in efficiency for reporter activation *in vitro* and *in vivo* (**Figures 1, 4, and 5**). 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[20].

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-Day 7 as seen in **Figures 3, 4 and 6**. 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 **Figures 4 and 5**. Older

mice are not as effective for hydrodynamic delivery and older mice were used in **Figure 4**. Different maxi-preps were used between the experiments so this may have also contributed.

Additionally, **Figure 7** 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 3** also shows that this reporter system works in tissues beyond the liver. The Barry lab has also previously published in Hillestadt 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[12].

Next generation sequencing analyses of the gene editing outcomes provide some insight into how the cell repairs these DNA breaks prior to reporter activation. We focused on three likely potential outcomes – non-homology induced repairs, small local homology (microhomology), or homology-based outcomes, especially considering the deletions occur between two identical LoxP sequences. Analyses of deletions induced by individual gRNAs demonstrated that the majority 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 1**).

Analysis of the dual gRNA-induced deletions demonstrated a different mix of outcomes (**Supplementary Figure 1**). After the repair pathway-ambiguous LoxP recreation, the next 2 top reads consist 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 we are unable to parse the exact repair mechanism, the increases in efficacy seen with both gRNAs is 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 resulting 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[25]. If a LoxP site is mutated to prevent CRISPR cutting at both LoxP sites, large deletion of the stop cassette would be prevented. With 2 potential gRNA targets, the potential indel would be further away from the PAM and less likely to inhibit SaCas9 binding[26]. This may potentially explain the increased efficacy seen with two gRNAs rather than one.

By targeting Cas9 to the LoxP site, we have also created 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 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 3-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 as a way 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. (**Fig. 1 and 2**). 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.

This 3-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 3,000 mice on JAX Laboratories website related to the Cre-lox system.

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