CRISPR/Cas-mediated gene editing of retinal cells *in vivo*.

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ABSTRACT:

PURPOSE: CRISPR/Cas has recently been adapted to enable efficient editing of the mammalian genome, opening novel avenues for therapeutic intervention of inherited diseases. In seeking to disrupt Yellow Fluorescent Protein (YFP) in a Thy1-YFP transgenic mouse, we assessed the feasibility of utilising the adeno-associated virus 2 (AAV2) to deliver CRISPR/Cas for genome modification of retinal cells *in vivo*.

METHODS: sgRNA plasmids were designed to target *YFP* and after *in vitro* validation, selected guides were cloned into a dual AAV system. One AAV2 construct was used to deliver SpCas9 and the other delivered sgRNA against *YFP* or *LacZ* (control) in the presence of mCherry. Five weeks after intravitreal injection, retinal function was determined using electroretinography and CRISPR/Cas-mediated gene modifications were quantified in retinal flat mounts.

RESULTS: AAV2-mediated *in vivo* delivery of SpCas9 with sgRNA targeting *YFP*, significantly reduced the number of YFP fluorescent cells of the inner retina of our transgenic mouse model. Overall, we found an 84.0% (95% CI: 81.8-86.9) reduction of YFP-positive cells in *YFP*-sgRNA infected retinal cells compared to eyes treated with *LacZ*-sgRNA. Electroretinography profiling found no significant alteration in retinal function following AAV2-mediated delivery of CRISPR/Cas components compared to contralateral untreated eyes.

CONCLUSIONS: Thy1-YFP transgenic mice were used as a rapid quantifiable means to assess the efficacy of CRISPR/Cas-based retinal gene modification *in vivo*. We demonstrate that genomic modification of cells in the adult retina can be readily achieved by viral mediated delivery of CRISPR/Cas.

INTRODUCTION:

Many ophthalmic diseases manifest due to well-defined genetic mutations, and inherited retinal diseases now comprise the leading cause of blind registrations in working-aged individuals.[1] Although several genetic variants across many loci have been found to cause inherited retinal dystrophies, all of these conditions are as yet currently untreatable. To-date, much work has focused on viral-mediated gene-replacement therapy, where gene expression is augmented by ectopic replacement of a normal gene product.[2] However, emerging data suggests that the efficacy of such viral-mediated gene-replacement therapy reduces over time.[3,4] Additionally, genetic heterogeneity and restrictions in viral payloads limit the broad application of such an approach for many inherited retinal diseases.[5-7]

The Clustered Regularly Interspersed Short Tandem Repeat (CRISPR) and CRISPR associated protein (Cas) system used by bacteria to counter viral intrusion, has recently been adapted to allow efficient editing of the mammalian nuclear genome.[8,9] CRISPR/Cas-based technology is particularly attractive for treating inherited diseases caused by genes with very specific spatial and stoichiometric expression.[2]

There have been a small number of studies, which have demonstrated the potential of *in vivo* gene editing for therapeutic applications.[10] Although the first reported *in vivo* application of CRISPR/Cas used the endogenous homology directed repair pathway to correct the precise disease causing mutation,[11] the majority of subsequent reports have used CRISPR/Cas for gene knockout.[12-14] In postmitotic cells, such as those found in the adult retina, double stranded DNA breaks are preferentially repaired through non-homologous end joining, and as such, diseases caused by gain-of-function, dominant negative or increased copy number variants could be readily amenable to preemptive intervention whereby CRISPR/Cas technology is used to disrupt the mutant allele.

CRISPR/Cas gene editing has been applied to the retina *in vivo* using electroporation.[15,16] Wang and colleagues electroporated constructs expressing *Streptococcus pyogenes* Cas9 (SpCas9) to disrupt the function of *Blimp1* and thereby dissect the molecular pathways involved in the regulation of murine retinal rod and bipolar cell development *in vivo*.[15] More recently, Bakondi *et al.* reported the use of a electroporation to transfect photoreceptors with a plasmid containing SpCas9 and a single guide RNA (sgRNA) targeting the *Rho* gene in rats harbouring the dominant *Rho* S334ter mutation.[16] Strikingly,

this intervention was found to prevent retinal degeneration and improve visual function.[16] Nonetheless, despite these promising applications it is clear that such approaches for CRISPR/Cas delivery *in vivo* are not currently feasible as a human therapy.

The aim of our work was to assess the feasibility and efficiency of CRISPR/Cas-mediated gene editing in the retina using a viral delivery system that could be readily adapted for use in a clinical setting. In order to rapidly quantify a phenotypic outcome for *in vivo* gene editing, we designed sgRNA constructs to disrupt a yellow fluorescent protein (*YFP*) in a transgenic mouse. As such, we report the use of a rapid, quantifiable means to explore the efficacy of a CRISPR/Cas system for retinal modification and demonstrate clear proof-of-concept evidence for gene modification *in vivo*.

METHODS:

Ethics Approval and Colony Maintenance:

Ethics approval for this work was obtained from the Animal Ethics Committee of the University of Tasmania (A14827) and the St Vincent's Hospital (AEC 014/15), in accordance with the requirements of the National Health and Medical Research Council of Australia (Australian Code of Practice for the Care and Use of Animals for Scientific Purposes). We adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Thy1-YFP transgenic mice [B6.Cg-Tg(Thy1-YFP)16Jrs/J], which express YFP in all retinal ganglion cells, as well as amacrine cells and bipolar cells in the retina, were obtained from the Jackson Laboratory and bred at the mouse facility of the Menzies Institute for Medical Research (Hobart, Australia).[17] Mice were housed in standard conditions (20°C, 12–12 hours light-dark cycle) with access to food and water *ad libitum*.

sgRNA design and construct generation:

sgRNA targeting the 5' region of the *YFP* gene were designed using the CRISPR design tool (http://crispr.mit.edu/)(Supplementary Table 1).[18] The mouse genome assembly MM9 was used as the reference genome and three sgRNAs were selected for subsequent profile testing. YFP overexpressing 3T3-L1 (ATCC® CL-173; ATCC, Manassas, VA, USA) and HEK293A (Life Technologies, Mulgrave, Victoria, Australia) cell lines were used for indel analysis and *in vitro* validation, respectively (Figure 1). Control sgRNA sequence targeting *LacZ* was based on the work by Swiech and colleagues.[19]

sgRNAs were initially cloned into the pSpCas9(BB)purov2.0 vector (a gift from Feng Zhang; Addgene plasmid #62988),[18] and the best performing sgRNAs were subsequently cloned into pX552-mCherry (a gift from Feng Zhang; Addgene plasmids #60957 and #60958).[19] pX552-mCherry was generated by replacing the *GFP* with *mCherry* using SalI and BspEI restriction sites.

Cell culture and transfection:

Stable cell lines of HEK293A and mouse 3T3-L1 expressing YFP were generated using pAS2.EYFP.puro lentivirus (RNAiCore, Academia Sinica, Taipei, Taiwan) and selected using puromycin or FACS sorting. Cell lines were maintained in DMEM supplemented with 10% FBS, 2mM L-glutamine and 50U/mL Penicillin-Streptomycin (Life Technologies) and cultured at 37^{o}C with 5% CO_2 incubation. Transfection of CRISPR/Cas constructs was performed using Lipofectamine 2000 (Life Technologies), with 2.5µg plasmid per well of 6-well plate, according to the manufacturer's instructions. Fluorescence images were visualised and captured with a Nikon Eclipse TE2000 inverted microscope (Nikon, Melville, NY, USA).

Indel analysis:

Genomic DNA was extracted using QIAamp DNA mini kit (Qiagen, Chadstone, Victoria, Australia). Indel analysis was performed with the SURVEYOR assay kit (Integrated DNA Technologies, Inc. Coralville, USA) using 200-400ng of PCR products generated from genomic DNA of transfected cells. SURVEYOR primers flanking the cleavage sites are listed in Supplementary Table 1. PCR cleaved products were resolved on 1.8% TBE agarose gel and visualised using GelRed nucleic acid gel stain (Biotium, Hayward, CA, USA). The DNA band intensity was quantitated using ImageJ v1.49.[20] The percentage of indels for each sample was calculated using the method described by Ran *et al.* 100 x (1-1-(b+c)/(a+b+c))^{½}), where a is the band intensity of the undigested PCR product, and b and c are the combined intensity for the cleavage products.[21]

Virus production:

Recombinant AAV2 vectors were produced in HEK293 cells packaging either pX551, containing SpCas9 or pX552-mCherry with the respective sgRNAs, and pseudo-serotyped with the AAV2 capsid (pXX2; courtesy of Ian Alexander, Children's Medical Research Institute, Westmead, NSW, Australia) as previously outlined.[22] Viral vectors were purified by AAV2pro® Purification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) and vector genomes were titred by real-time quantitative polymerase chain reaction using the following primer sets: (pX551-F:

CCGAAGAGGTCGTGAAGAAG, pX551-R: GCCTTATCCAGTTCGCTCAG, pX552-F: TGTGGAAAGGACGAAACACC, pX552-R: TGGTCCTAAAACCCACTTGC) with the SYBR Green Master Mix (Life Technologies).

Intraocular Injection of AAV vectors:

A total of 22 adult mice, aged between 14 and 16 weeks, were randomly allocated to receive YFP-sgRNA or LacZ-sgRNA (n=11 per group). Mice were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg). Intravitreal injections were performed using a surgical microscope similar to that previously described.[23] In brief, after making a guide track through the conjunctiva and sclera at the superior temporal hemisphere using a 30-gauge needle, a hand-pulled glass micropipette was inserted into the mid-vitreal cavity. A total of 1 μL of dual-viral suspension (AAV2-SpCas9 $3x10^9$ vg/μL with AAV2-LacZ-sgRNA $2.5x10^9$ vg/μL or AAV2-YFP-sgRNA $3x10^9$ vg/μL) or Balanced Salt Solution was injected into each eye at a rate of 100 nL/s using a UMP3-2 Ultra Micro Pump (World Precision Instruments, Inc. Sarasota, USA). Patency was confirmed following needle removal.

Electrophysiology Assessment and Retinal morphology:

Mice were euthanized five weeks after intraocular injection. Immediately prior to terminal anesthesia, electroretinography was used to assess retinal function in six mice treated with SpCas9/YFP-sgRNA and six mice treated with SpCas9/LacZ-sgRNA.

Our methods for electroretinogram recordings have been well described previously.[24-26] In brief, overnight dark-adaptation (minimum 12 hours) was performed and all animals were prepared for recording under dim red illumination. Following general anesthesia (intramuscular ketamine 80 mg/kg, xylazine 10 mg/kg), pupil dilation and corneal anesthesia were obtained with 0.5% tropicamide and 0.5% proxymetacaine, respectively. A Ganzfeld sphere (Photometric Solutions International, Oakleigh, Victoria, Australia) was used to delivery luminous energy ranging from −6.26 to 2.07 log $cd \cdot m^{-2} \cdot s$ calibrated with an IL1700 integrating photometer (International Light Technologies, Peabody, MA, USA). Electrical signals were recorded with a chlorided silver electrode on the corneal apex and referenced to a ring electrode placed on the conjunctiva posterior to the limbus. A ground needle electrode was placed in the tail. Full-field flash electroretinograms were recorded using standard settings. Response amplitudes of the scotopic a-wave, scotopic b-wave, pSTR, photopic negative response and photopic b-wave were measured. Signals were amplified (×1000) over a band-pass of 0.3

to 1000 Hz (−3 dB) and digitized using an acquisition rate of 4 kHz. Ganglion cell response was measured as the peak-to-trough amplitude of the scotopic threshold response (STR) recorded at −5.25 log cd·m^{-2}·s.[24-26]

Retinal morphology was assessed *in vivo* using spectral domain optical coherence tomography (OCT)(Micron III; Phoenix Research Labs, Pleasanton, CA, USA). As described previously, immediately following ERG recordings, a total of ten horizontal scans across the retina centered at the optic nerve head were obtained and averaged.[27] Total retinal thickness, retinal nerve fibre layer and ganglion cell layer thickness were measured.[27]

Retinal Tissue processing:

Enucleated eyes were fixed in 4% paraformaldehyde (PFA; ProSciTech, Australia) for 1 hour, and retinal flat mounts were prepared and stained with a 4',6-diamidino-2-phenylindole (DAPI; 0.2 ug/mL, Sigma-Aldrich, Sydney, NSW, Australia) counterstain. For histological assessment, enucleated eyes were fixed in 4% PFA for 1 hour and embedded in optimal cutting temperature compound prior to frozen sectioning on a microtome-cryostat. Serial 10 μm thick sections were cut, mounted on silanated glass slides, and then stained with DAPI.

Retinal imaging, cell counting and statistical analysis:

A fluorescence microscope (Zeiss Axio Imager Microscope, Carl-Zeiss-Strasse, Oberkochen, Germany) equipped with a charge-coupled device digital camera (Axiocam MRm, Zeiss) and image acquisition software (ZEN2, Zeiss) was used. Following whole mounting, each retinal quadrant was photographed using an appropriate filter for the fluorescence emission spectra of mCherry (605nm, Zeiss Filter set 64HE) and YFP (495nm, Zeiss Filter set 38HE). Confocal images were taken using a Nikon C1 confocal laser scanning microscope using a 20x 0.75NA lens and images were tile stitched using the NIS Elements AR program (Nikon, Melville, NY, USA). Retinal cell quantification was performed using individual fluorescent images captured at 400x magnification. A total of 18 images from four eyes treated with SpCas9/LacZ-sgRNA, and 26 images from five eyes treated with SpCas9/YFP-sgRNA were quantified manually using using ImageJ v1.49, by an experienced grader (AFL), masked to treatment status.[20] Sample group decoding was performed only once data were quantified and analyzed.

The efficiency of YFP knockout was determined by the proportion of YFP negative cells among mCherry expressing cells. Specifically, the mean proportion of YFP-knockout for all eyes treated with SpCas9/YFP-sgRNA was calculated by $\Delta YFP^{-ve}/(\Delta YFP^{-ve} + YFP^{+ve})$, where ΔYFP^{-ve} was the number of mCherry transfected cells in SpCas9/YFP-sgRNA treated eyes, which did not express YFP, minus the average number of mCherry expressing cells in SpCas9/LacZ-sgRNA treated eyes, which did not express YFP (YFP^{-ve}); and YFP^{+ve} was the number of cells which expressed both YFP and mCherry.

Comparisons between continuous traits were made using the paired *t*-test, whilst categorical data were analysed using the Chi-square or Fisher exact test. Unless otherwise specified, data are presented as mean ± SD. A total of 14 images were processed twice to determine intra-grader variability. The intraclass correlation coefficient for YFP^{+ve} cell counts was 0.945 (95% CI: 0.842-0.982) and for YFP^{-ve} cell counts was 0.954 (95% CI: 0.867-0.985). Analyses were performed in the R statistical environment (version 3.2.2).

RESULTS:

sgRNA Selection and in vitro Validation:

We assessed three sgRNAs targeting the *YFP* sequence, together with a LacZ-sgRNA control,[19] for cleavage efficiency in 3T3-L1-YFP cells (Figure 1A). Indel analysis, for the quantification of sgRNA-induced nucleotide mismatches revealed that one of our sgRNAs (designated "YFP KO sgRNA2") targeted *YFP* most effectively (Figure 1B). With approximately 13% cleavage activity this YFP-sgRNA was used for all subsequent experiments (Figure 1B). When transfected into HEK293A-YFP cells, our selected YFP-sgRNA resulted in almost complete reduction in yellow fluorescence compared with untransfected and LacZ sgRNA controls (Figure 1C).

In vivo AAV delivery and gene targeting of CRISPR/Cas9 in the mouse retina:

To evaluate the YFP disruption *in vivo* by CRISPR/Cas-mediated gene-editing, Thy1-YFP mice received a single intravitreal injection of our dual viral suspension of AAV2-SpCas9 and AAV2 sgRNA-mCherry (Figure 2A). Five weeks following treatment, inspection of retinal whole mount images, captured under a stereomicroscope, revealed a lower number of YFP positive cells from SpCas9/YFP-sgRNA treated eyes compared to SpCas9/LacZ-sgRNA treated (Figure 2) or contralateral control eyes. To quantify the efficiency of YFP disruption following AAV2 delivery of SpCas9 and sgRNA, high magnification flat mount images were obtained and the proportion of YFP positive and

negative, mCherry expressing cells was calculated. A marked decrease in the number of YFP positive cells in the inner retina was found in SpCas9/YFP-sgRNA treated eyes, compared SpCas9/LacZ-sgRNA treated eyes. The proportion of YFP/mCherry expressing cells in eyes treated with SpCas9/YFP-sgRNA was 0.106 ± 0.022, compared to SpCas9/LacZ-sgRNA treated eyes where the proportion of YFP/mCherry expressing cells was 0.664 ± 0.050 (Figure 2D). Overall there was an 84.0% (95% CI: 81.8-86.9) reduction of YFP-positive cells in SpCas9/YFP-sgRNA transfected retinal cells compared to eyes treated with SpCas9/LacZ-sgRNA. As expected, there was no significant variation in the number of YFP cells in untreated control eyes of both groups. Similarly, retinal cross-sections showed a clear disruption of YFP expression in the ganglion cell layer (GCL) of the SpCas9/YFP-sgRNA-treated eyes, but not SpCas9/LacZ-sgRNA-treated eyes (Figure 2F&H).

Electroretinography assessment of retinal function.

Electrophysiological assessment of mice treated with SpCas9/YFP-sgRNA or SpCas9/LacZ-sgRNA, confirmed no adverse effects on photoreceptoral (a-wave: YFP p=0.64; LacZ p=0.35) or bipolar cell (b-wave: YFP p=0.38; LacZ p=0.40) function (Figure 3; Supplementary Figures 1-3). Importantly, given the AAV2 transfection profile following intravitreal injection, inner retinal function as indicated by the oscillatory potentials (YFP p=0.42; lacZ p=0.68) was also not disrupted (Figure 3; Supplementary Figure 3). The scotopic threshold response amplitude, indicative of ganglion cell activity, did not significantly differ between YFP-sgRNA treated and contralateral control eyes (p=0.48), or between lacZ-sgRNA treated and their contralateral eyes (p=0.87)(Supplementary Figure 3).

There was no statistically significant difference in retinal morphological profiles *in situ*, between treated or contralateral controls eyes (Supplementary Figure 4). In particular the retinal nerve fibre layer thickness and ganglion cell complex thickness as measured by OCT did not significantly differ between eyes treated with YFP-sgRNA and contralateral control eyes (RNFL p=0.30, GCC p=0.80) or between LacZ-sgRNA and their control eyes (RNFL p=0.70, GCC p=0.39).

DISCUSSION:

We have successfully demonstrated viral-mediated delivery of essential CRISPR/Cas components to retinal cells *in vivo*. Using a transgenic fluorescent mouse model, we have definitive evidence for CRISPR/Cas-mediated gene-editing of *Thy1* expressing retinal cells. With our dual-viral SpCas9/sgRNA suspension we observed a reduction of YFP-positive cells of approximately 84.0%

(95% CI: 81.8-86.9). This level of gene modulation *in vivo* is similar to that reported for other tissues, such as brain (~68% using AAV) and liver (80-90% using adenovirus).[19,28]

Our study builds on recent work, where CRISPR/Cas system was introduced into rodent retinas using an electroporation delivery method.[15,16] By demonstrating that CRISPR/Cas can also cause substantial gene modification activity when introduced by a viral delivery method in the retina, we are closer to translating gene-editing technology for therapeutic purposes. Importantly we found that AAV2-delivered SpCas9 was not retinotoxic over a five week treatment period. Nonetheless, it is clearly possible that adverse effects from ongoing or prolonged CRISPR/Cas expression could arise. As such it will be important to continue to develop CRISPR/Cas systems that could be tightly regulated and thereby be able to create an optimal window for gene modification while reducing the chance for potential off-target activity.[29,30]

AAV vectors have been widely exploited as a promising tool for gene delivery *in vivo* and it is clear that combining alternate AAVs and sgRNA promoters will extend the CRISPR/Cas therapeutic repertoire. Specifically, different AAV serotypes have the ability to transfect distinct cell populations within the retina,[31] and whilst the AAV2 capsid used in our study is known to primarily transduce retinal ganglion cells when injected intravitreally,[32] a different AAV2 variant, 7m8, can infect photoreceptors following intravitreal delivery.[33] Additionally, to overcome the packaging size limitation of AAV vectors,[34] we used a dual-vector system to package SpCas9 and sgRNA expression cassettes in two separate viral vectors. Although, a single vector that packages a smaller Cas9 ortholog, such as *Staphylococcus aureus* Cas9 (SaCas9), together with a sgRNA, could achieve greater knockout efficiencies *in vivo*,[14,35] dual-vector systems may still be required for mutation correction by enabling the cellular delivery of additional donor templates and appropriate promoter elements. For example, a recent study from Yang and colleagues demonstrated that a two-vector approach with SaCas9 resulted in mutation correction in approximately 10% of hepatocytes in ornithine transcarbamylase-deficient newborn mice.[36] With ongoing advances in viral engineering and the development of new serotypes, future research investigating the efficacy of different viral-CRISPR/Cas systems in the retina, is warranted.

An important limitation of our work, is the fact that off-target effects were not directly quantified. Nonetheless, to some extent this is a minor consideration for our initial proof-of-concept work which sought to primarily assess “on-target” efficacy of virally delivered CRISPR/Cas in the adult retina.

Additionally, it is well appreciated that CRISPR/Cas technology is advancing rapidly and although a major limitation of gene editing has been the prospect of off-target effects, this issue is being addressed.[37,38] For example, with careful design of the guide RNA (using a double-nick; truncated sgRNA) it is already possible to avoid most off-target cuts and recently, two independent studies report modifications to SpCas9, which can dramatically reduced off-target activity.[37,38] Regardless, it is clear that safety will be paramount prior to CRISPR/Cas being used in mainstream medical care, and off-target profiling will need to be considered before current research is translated into clinical trials.

In summary, this work suggests that a relatively high-efficiency of gene editing in the retina can be achieved by a dual AAV2-mediated CRISPR/Cas system. Whilst *ex vivo* TALEN-based approaches are now in clinical trials, given the versatility and relative ease of design, CRISPR/Cas techniques appear to be more clinically applicable. Inherited eye diseases share several features that make them appealing for the application of *in vivo* gene editing. For example, non-syndromic retinal diseases generally have a specific and well-defined pathogenesis, whereby only a specific subpopulation of cells would need to be targeted. Additionally, following any intervention, the eye can be directly examined for the development of any adverse effects and enucleated if necessary. Nonetheless, any *in vivo* gene editing therapies will require a number of functionally active cells, and it is possible that the threshold for viable cells will vary across genes and diseases.

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FIGURES:

Figure 1. sgRNA design, plasmid construction and validation for knockout (KO) of Yellow-Fluorescent Protein (YFP). (**A**) YFP target sequence used for sgRNA design. Selected sgRNA ideograms display the PAM sequence (red) and putative cut site (green triangle). Indel analysis using a SURVEYOR assay identified "YFP KO sgRNA2" as having the best KO efficiency in our 3T3-L1-YFP line (**B**), and this was confirmed *in vitro* using the HEK293A-YFP line (**C**). Scale bar = 100μm.

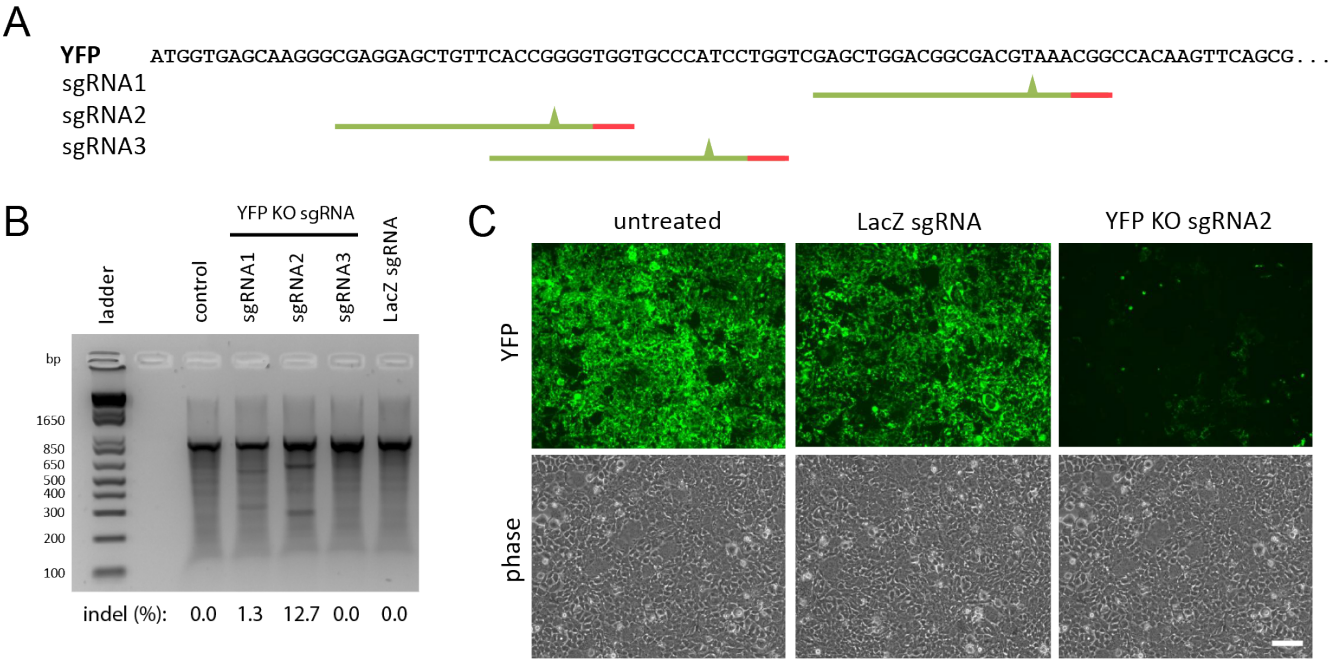

Figure 2. CRISPR/Cas mediated gene-editing of retinal cells *in vivo*. Dual viral suspension of AAV2-SpCas9 and AAV2 sgRNA were used (**A**). sgRNA plasmids also expressed mCherry. Representative retinal montages from Thy1-Yellow-Fluorescent Protein (YFP) mice exposed *in vivo* to our dual AAV2 plasmid-system carrying SpCas9 and either control (LacZ) sgRNA (**B**) or sgRNAs targeting YFP (**C**) (Scale bar = 500µm). Overall, the proportion of mCherry expressing cells (mCherry+), which lacked YFP (YFP-) was higher in SpCas9/YFP-sgRNA treated eyes (**D**). Higher magnification of flat mount images, displaying differences in YFP expression following AAV2-mediated delivery of SpCas9/LacZ-sgRNA (**E**) or SpCas9/YFP-sgRNA (**G**) (Scale bar = 10µm). Cross sections displaying AAV2 infection of the inner retina confirm YFP knockout in SpCas9/YFP-sgRNA treated eyes (**H**), compared to SpCas9/LacZ-sgRNA treated eyes (**F**) (Scale bar = 50µm). White arrows indicate AAV2-sgRNA infected cells. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

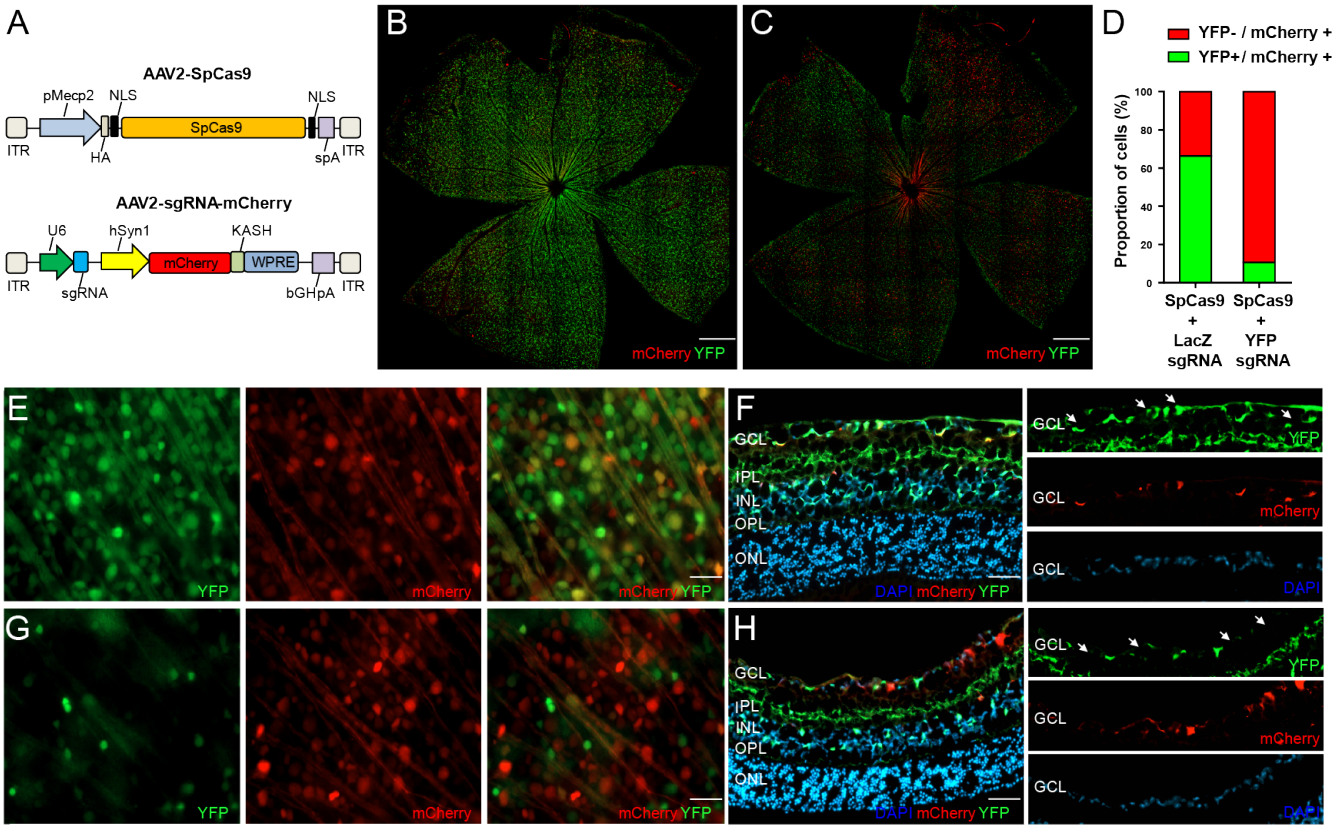

Figure 3. Effect of SpCas9 on retinal function. Averaged ERG waveforms at selected intensities for YFP-sgRNA treated (n=6, red) and contralateral (n=6, black) eyes (**A**). The average photoreceptoral (a-wave), bipolar cell (b-wave), amacrine cell (oscillatory potentials, OPs) and ganglion cell (scotopic threshold response, STR) amplitude in YFP-sgRNA treated relative to contralateral control eyes (%, ±SEM) is displayed (**B**). Panel (**C**) displays the averaged ERG waveforms at selected intensities in LacZ-sgRNA treated (n=6, blue) and contralateral eyes (n=6, black). The LacZ-sgRNA group's average a-wave, b-wave, OPs and STR amplitude relative to contralateral control eyes (%, ±SEM)(**D**).