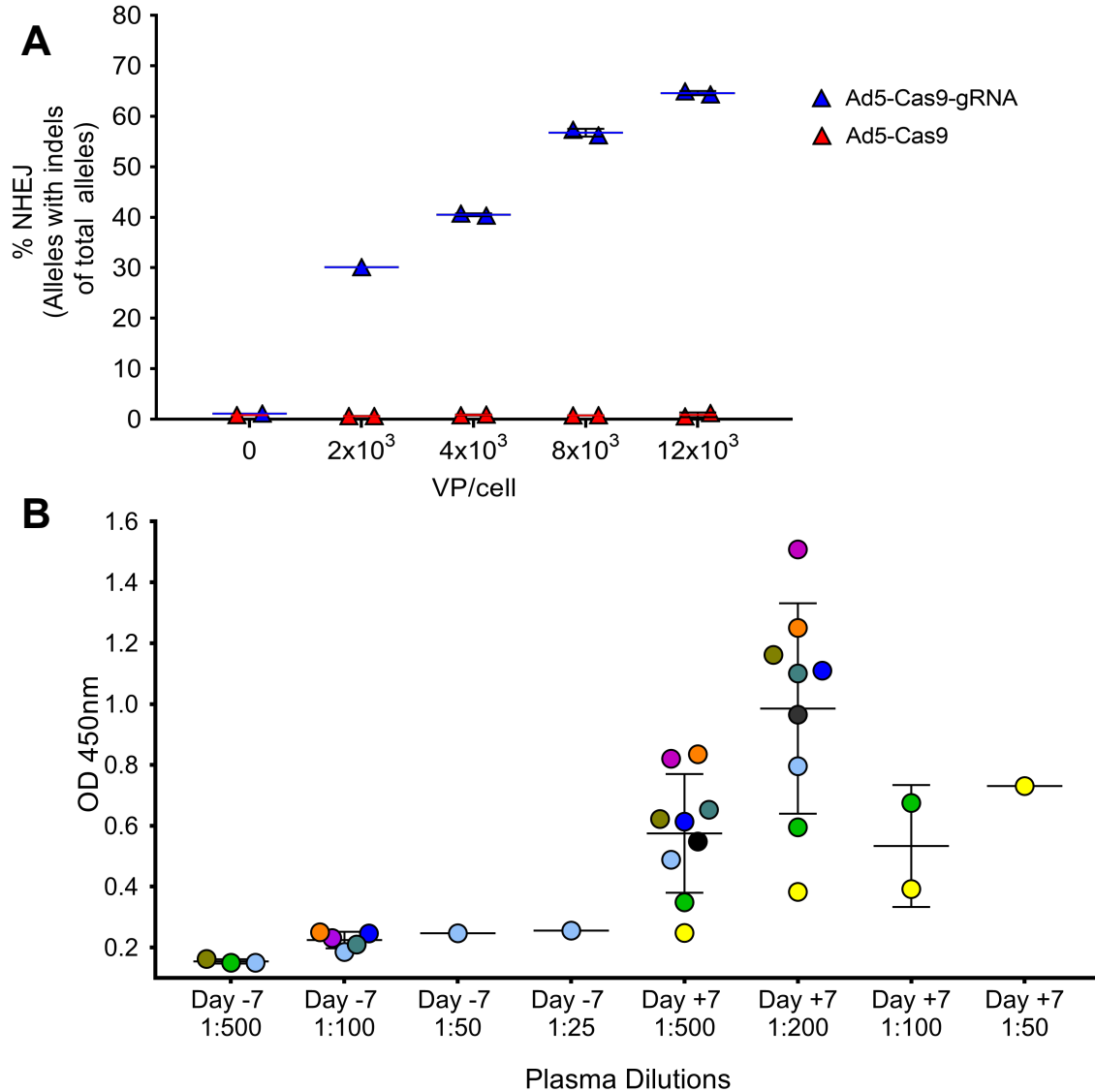


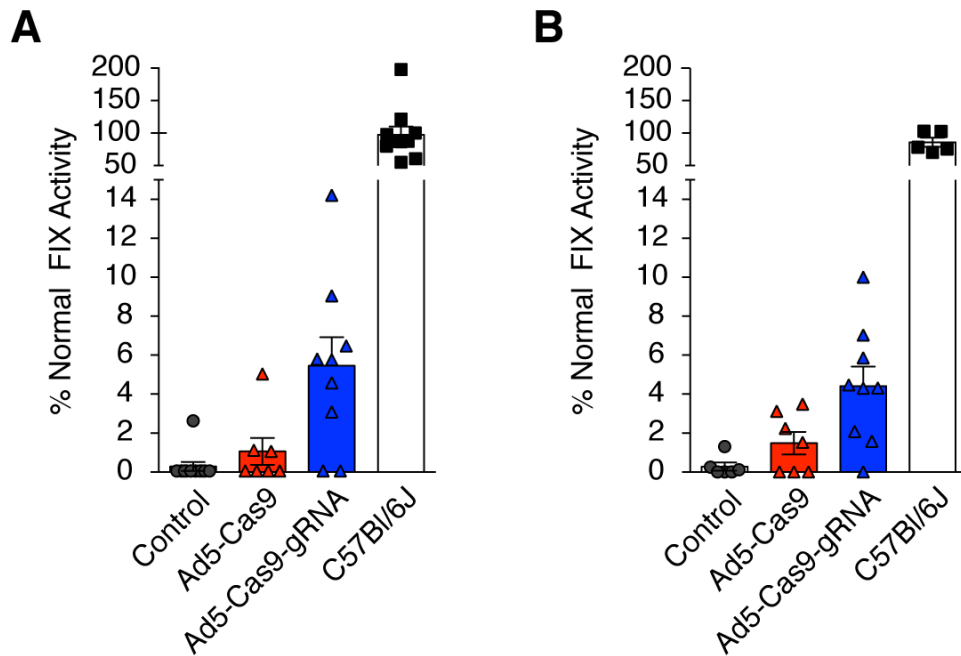
Supplemental Figures- Stephens et al

Supplemental Figure 1



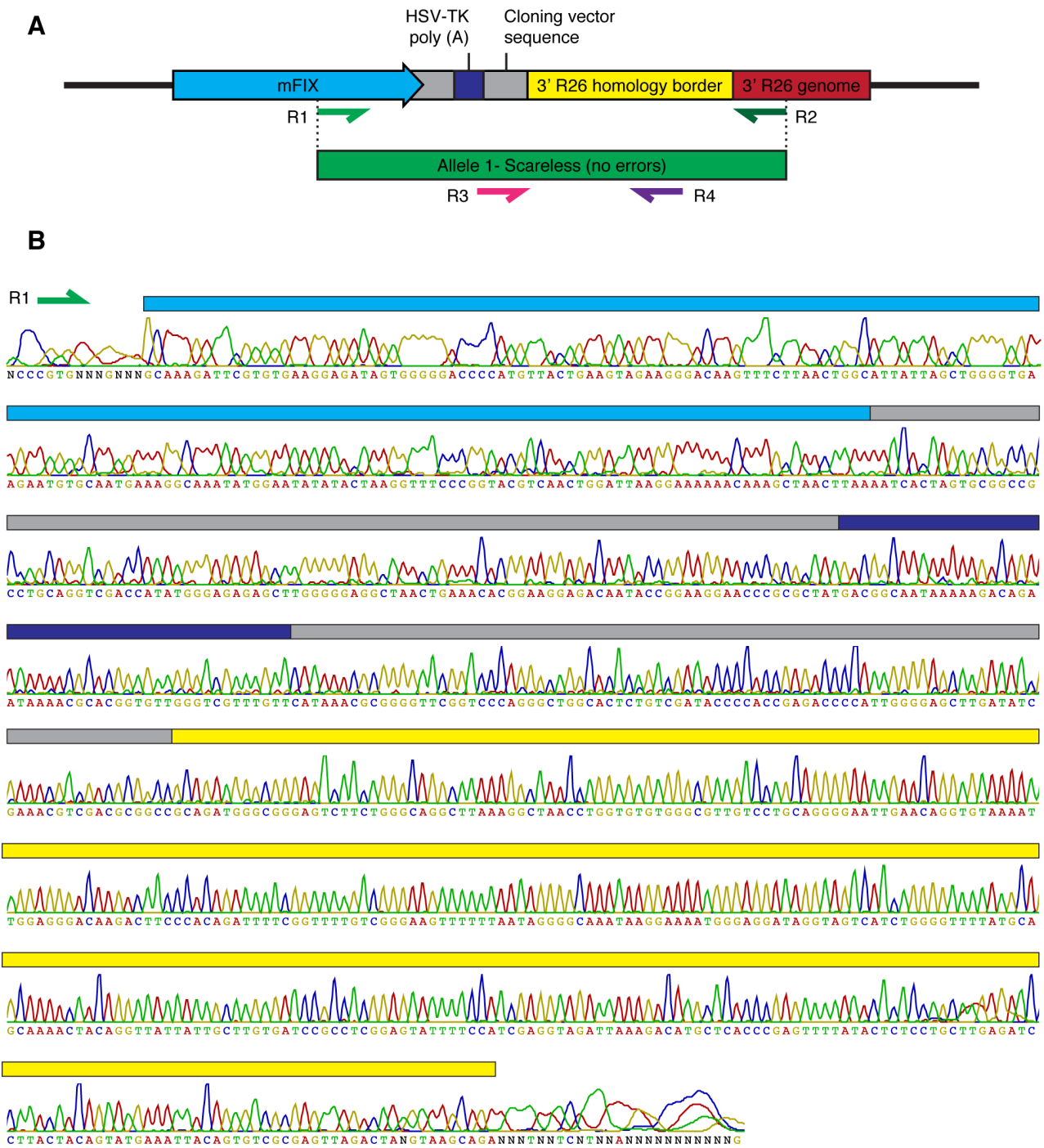
Supplemental Figure 1. Ad5-Cas9 does not introduce DSBs at *ROSA26* *in vitro*. Murine BNL-1NG liver cells were infected with increasing amounts of Ad5-Cas9-gRNA (blue) or Ad5-Cas9 (red). 96 hours later, cells were collected for DNA extraction followed by Illumina targeted deep sequencing of the *ROSA26* locus. Results are from two independent experiments. (B) ELISA OD values are shown from mice plasmas before and after injection with Ad5-EF1 α -mFIX. Plasma was drawn from mice seven days before (n=3) (day -7) and seven days after injection (n=7) (day +7) with 7.5×10^{10} viral particles (VP) of Ad5-EF1 α -mFIX and 2.5×10^{10} VP of Ad5-Cas9-gRNA. An ELISA was used to determine OD readings at 450nm of various plasma dilutions. Each individual color represents data from one animal. Multiple same-colored circles are different dilutions of plasma from a single animal. OD readings after injection were greater than readings prior to injection, even when plasma was more dilute.

Supplemental Figure 2

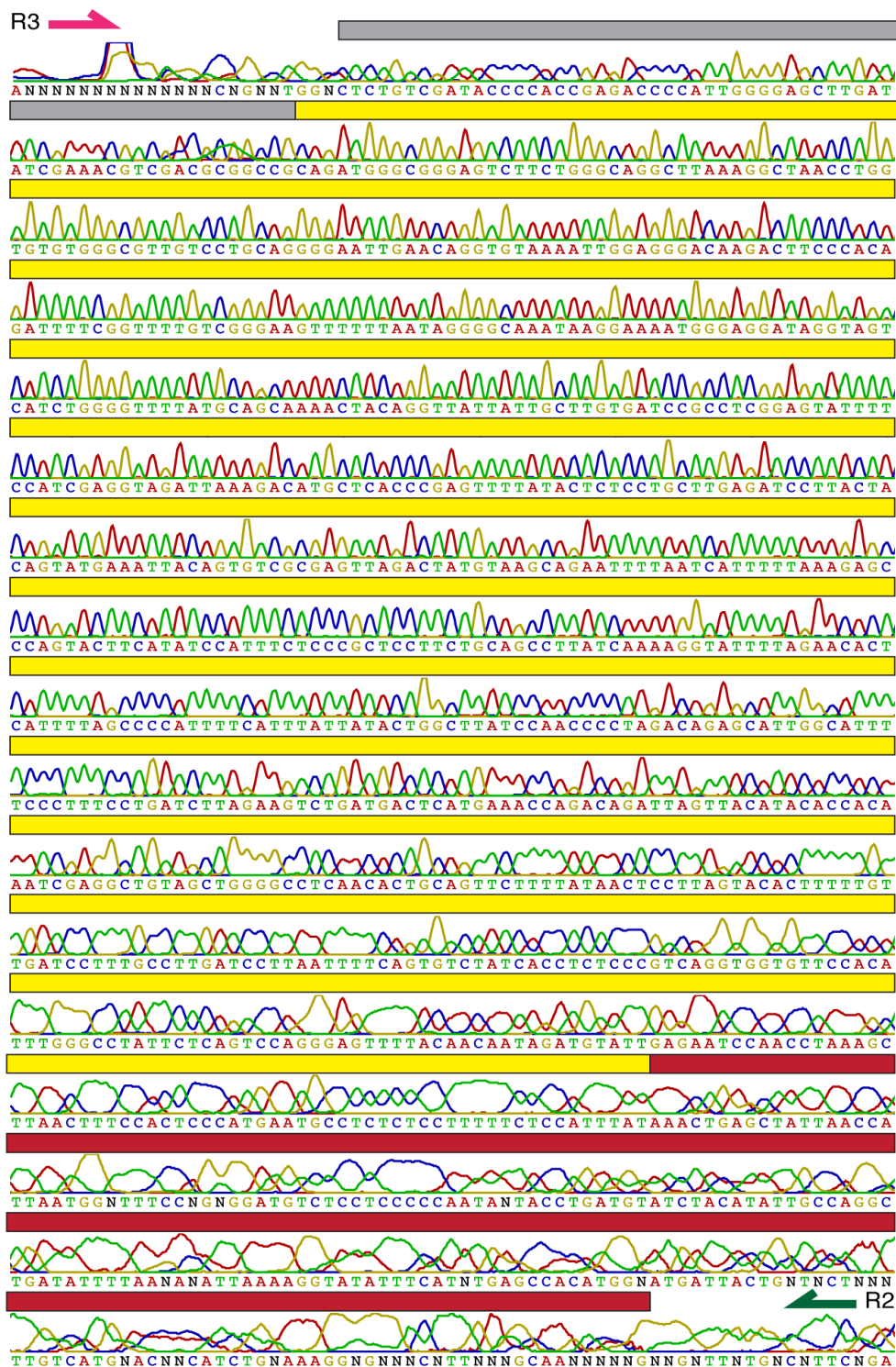


Supplemental Figure 2. FIX activities in plasma collected from two different draw sites. (A) FIX enzymatic activity in citrated plasma, drawn from the submandibular vein at 238 dpi, was determined using a chromogenic assay. FIX activity in untreated R333Q control animals (gray circles) and wild-type C57Bl/6J (black squares) animal plasma are shown. Animals treated with Ad5-EF1 α -mFIX and either Ad5-Cas9 (red triangles) or Ad5-Cas9-gRNA (blue triangles) show differential residual FIX activities. Each point represents data from an individual animal. Error bars are SEM. (B) FIX activity in the same animals' plasma, drawn from the tail at 245 dpi during the tail clip assay, for comparison to FIX activity in plasma drawn from the submandibular vein.

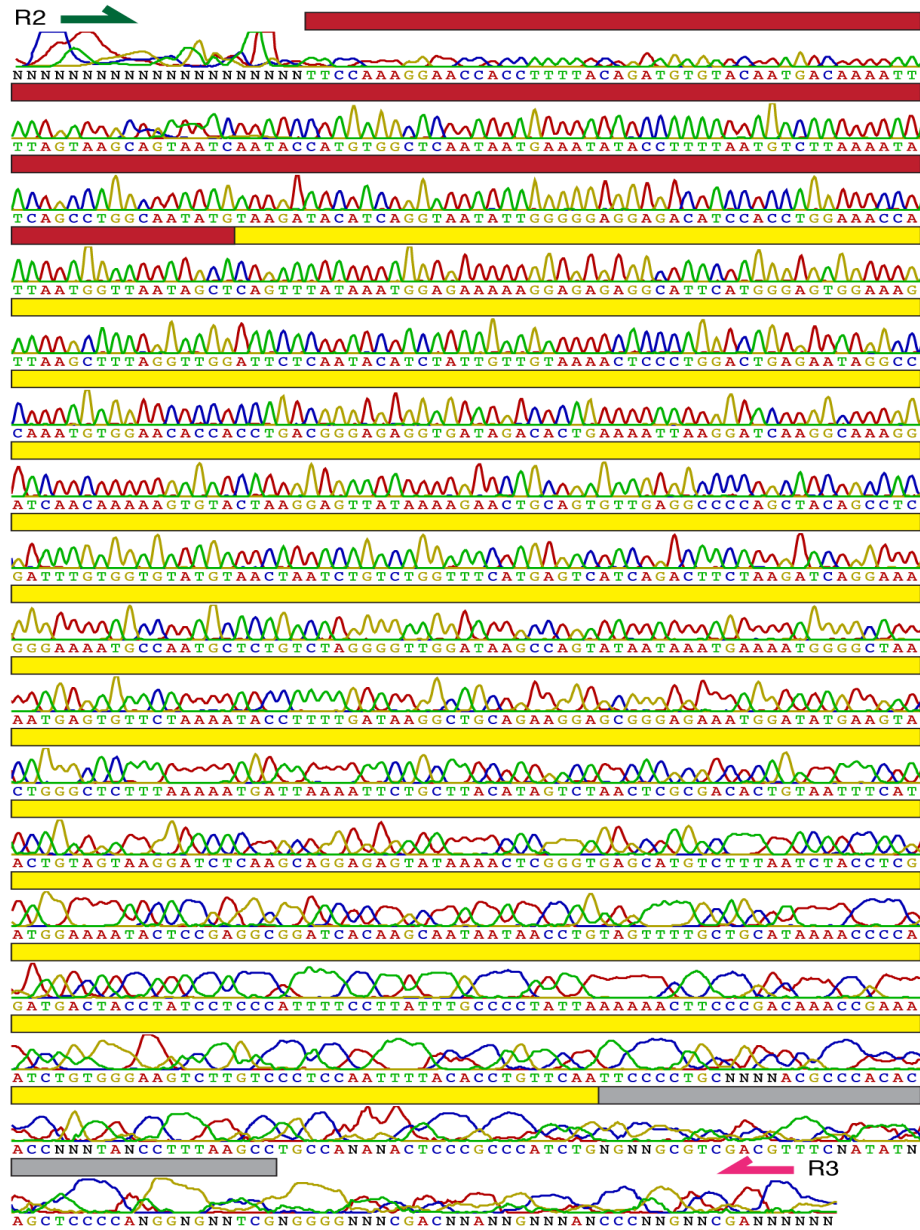
Supplemental Figure 3



C

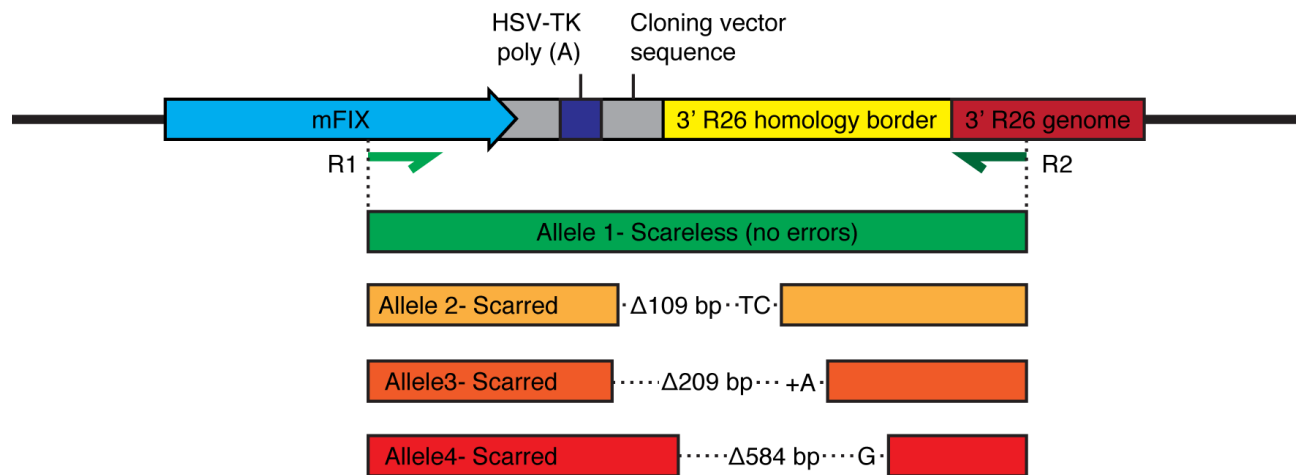


D



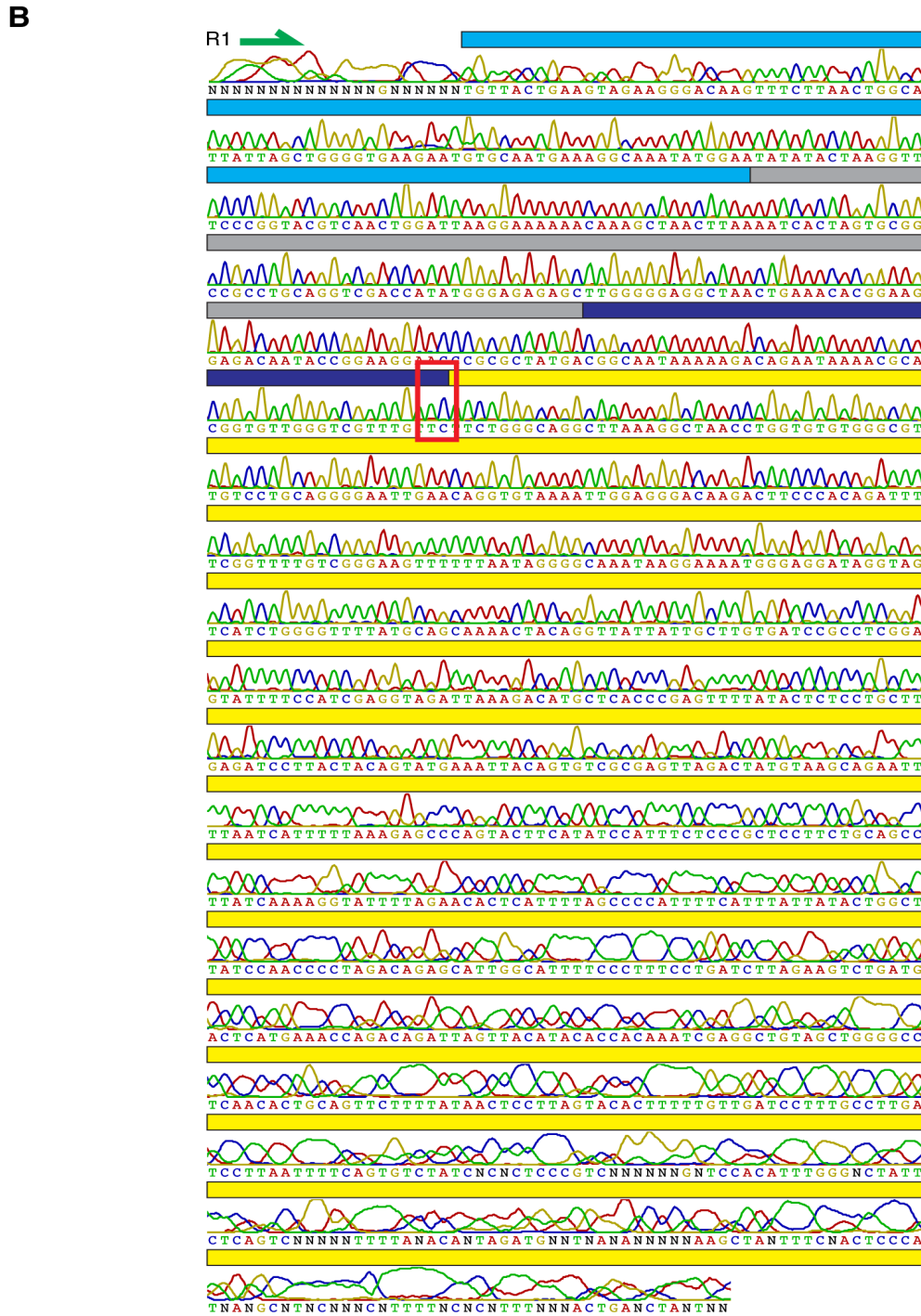
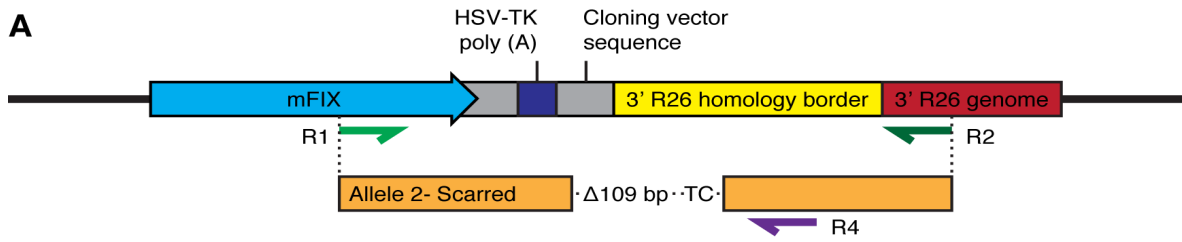
Supplemental Figure 3. Identification of error-free *mFIX* integration at *ROSA26*. (A) Schematic representing the full-length and error-free insertion (allele 1, green bar) of the *mFIX* expression cassette at *ROSA26*, generated by junction capture PCR. Green arrows (R1, R2) represent primers used for the generation of amplicons in junction capture PCR and in Sanger sequencing. Pink and purple arrows (R3, R4) represent the location and direction of primers used for Sanger sequencing. (B-D) Chromatograms of Sanger sequencing reads used to characterize the allele and validate correct amplification from genomic origins. Colored bars overlaid on the chromatogram represent the origin of the sequences from mFIX cDNA (light blue), sequences remaining from cloning plasmids (gray), HSV-TK poly(A) regulatory sequences (dark blue), *ROSA26* homology sequences (yellow), or *ROSA26* genomic sequences past the homology arms (red).

Supplemental Figure 4

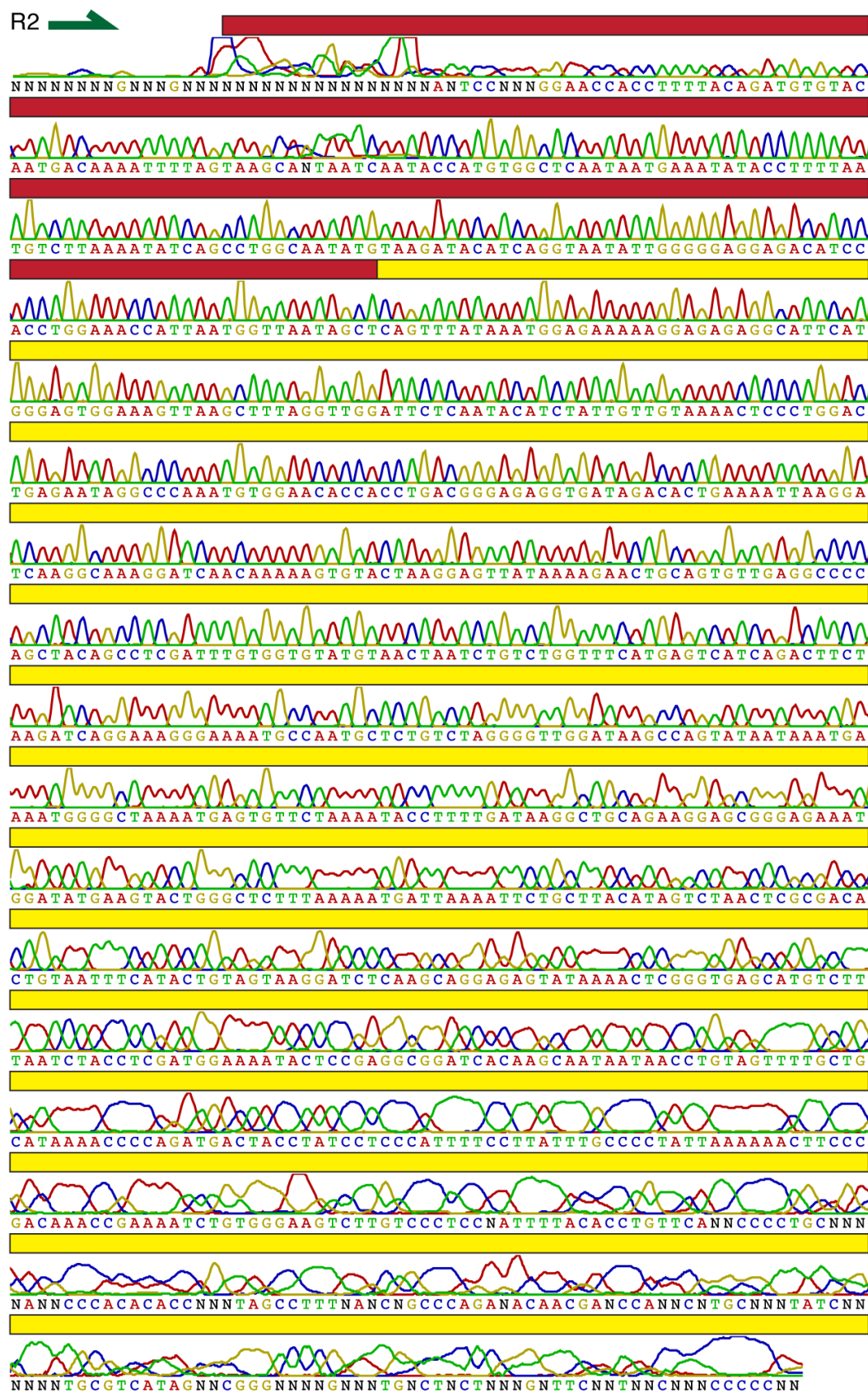


Supplemental Figure 4. A schematic summary of identified knock-in alleles. A schematic showing *ROSA26* containing various *mFIX* integration configurations. Each allele was generated using junction capture PCR with primers (green arrows) binding within *mFIX* and in the *ROSA26* genomic region, past the homology border. Four various sized alleles, similar to the multiple amplicons seen in Figure 4 junction capture PCR, were characterized from one animal treated with Ad5-EF1 α -*mFIX* and Ad5-Cas9-gRNA using Sanger sequencing. The knock-in alleles consisted of a full-length integration without errors (allele 1, green bar) and three alleles (alleles 2, 3, and 4, orange/red bars) containing deletions in or near the homology border. One allele also contained a single bp insertion at the break point, represented by the '+A' nucleotide in the deletion gap (allele 3). Homologous bp's at the deletion breakpoint site are shown in the deletion gap (alleles 2 and 4).

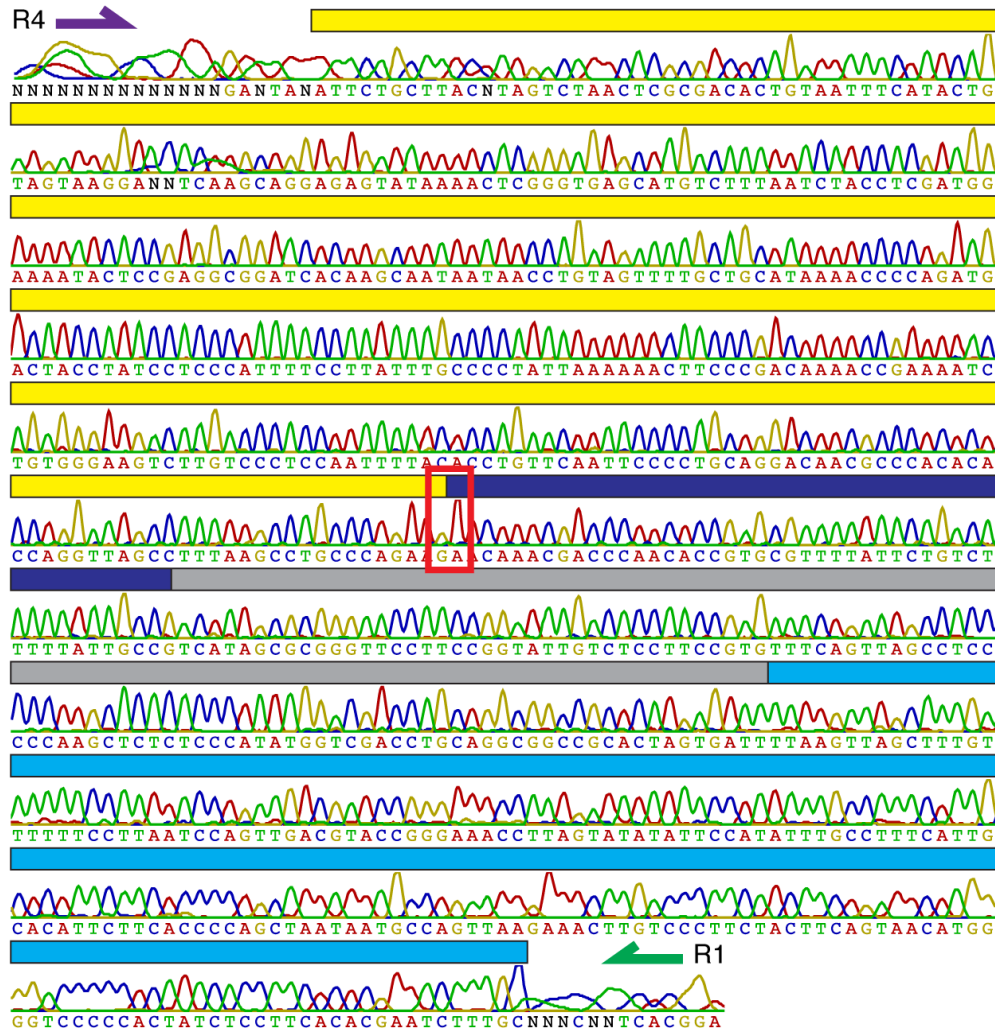
Supplemental Figure 5



C

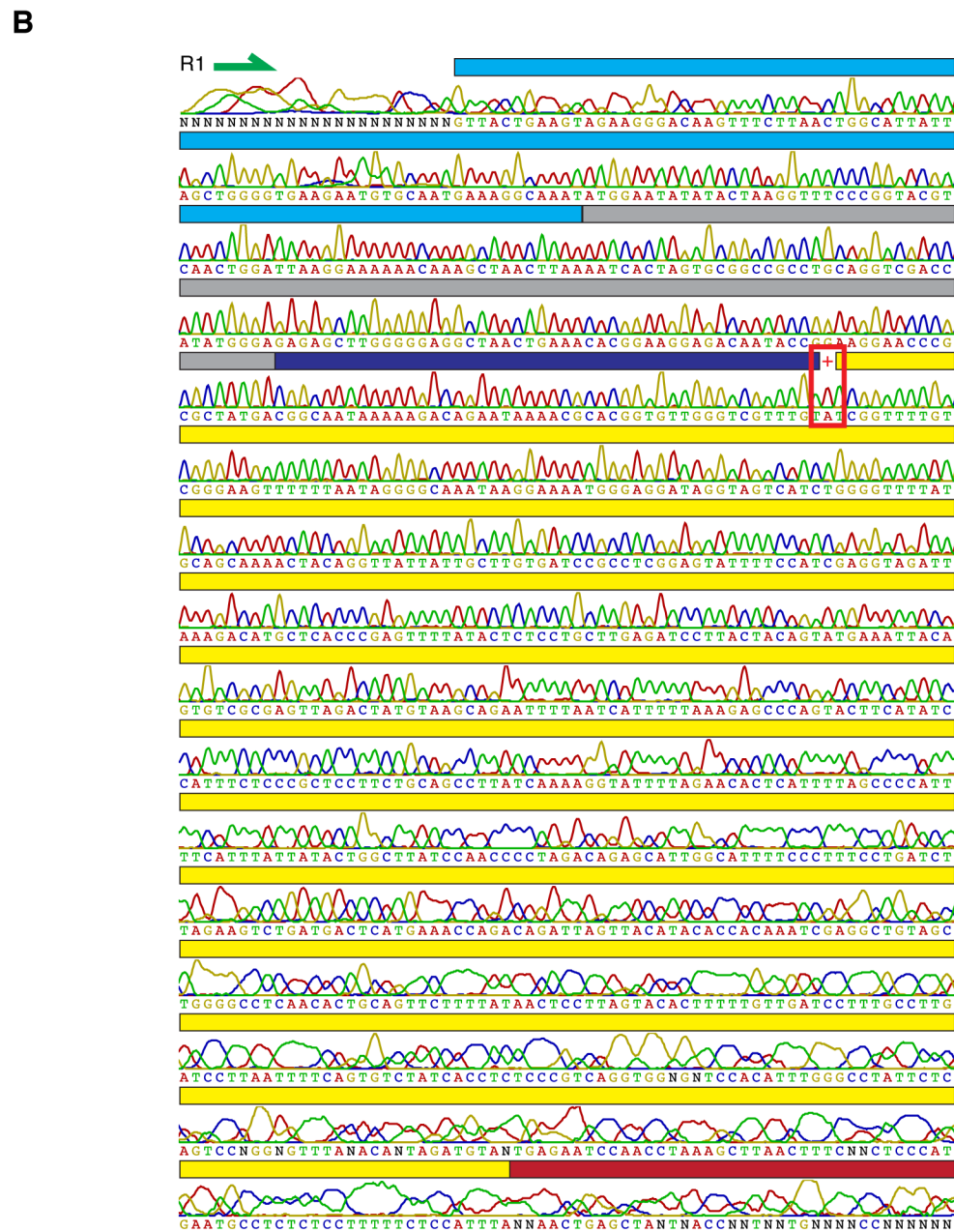
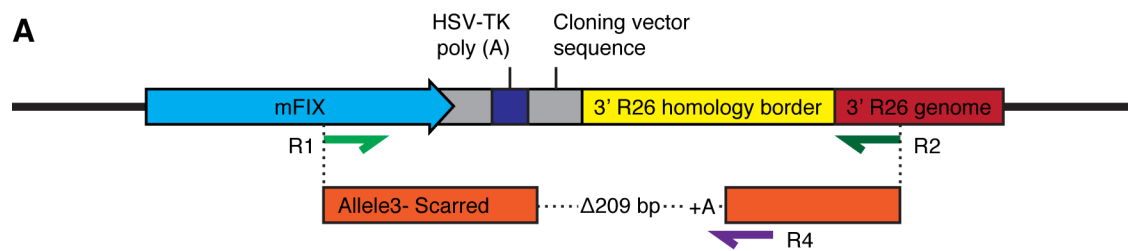


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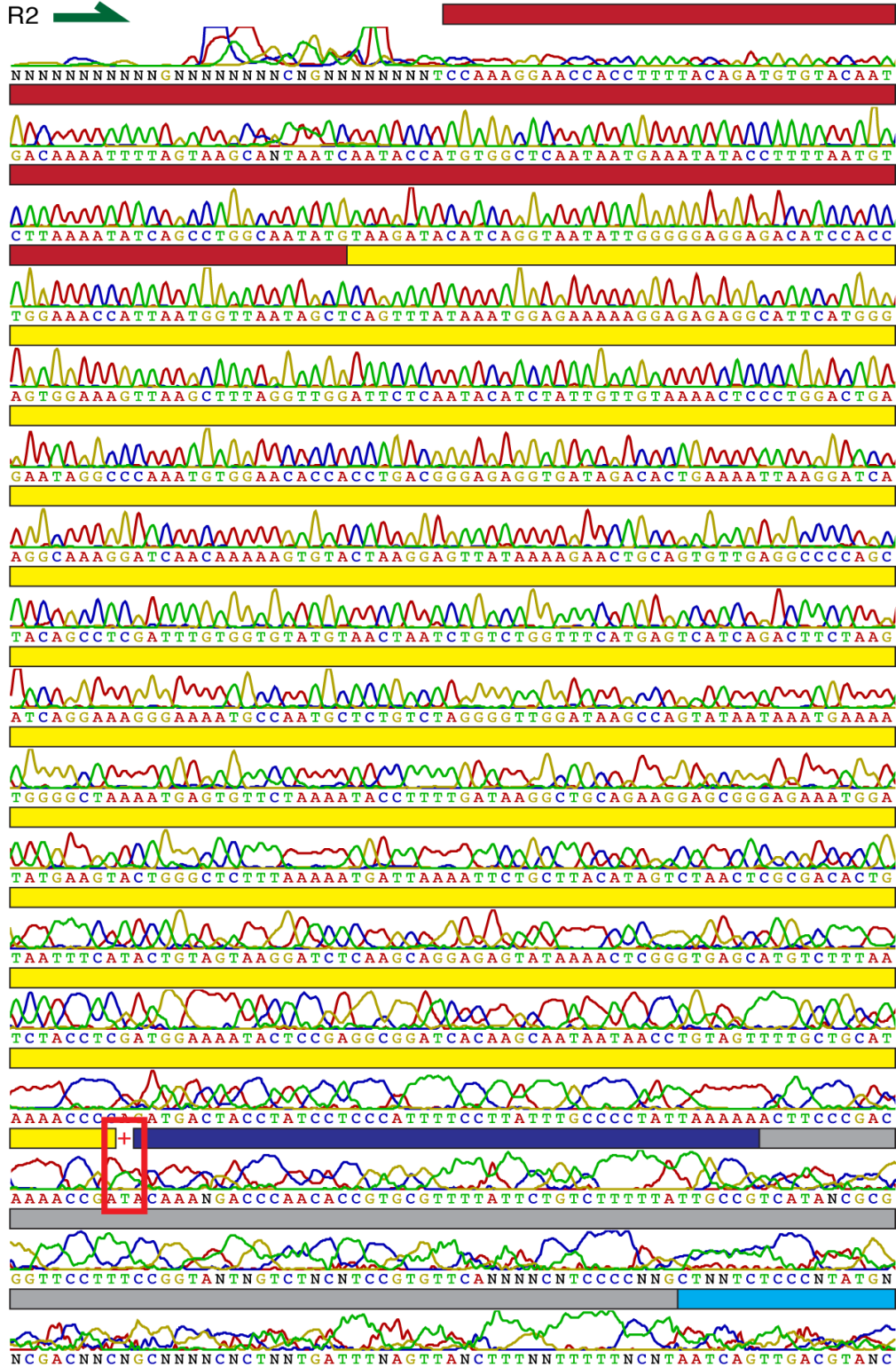


Supplemental Figure 5. Characterization of an error-containing knock-in allele. (A) A schematic of a *ROSA26* allele integrated with *mFIX* cDNA, containing a deletion in the homology border (allele 2, orange bar). Junction capture PCR of the 3' end of *ROSA26* generated a reduced-MW *mFIX* knock-in amplicon, which was characterized by Sanger sequencing. Green arrows represent primers used to generate the junction PCR amplicon and in Sanger sequencing. The purple arrow (R4) represents the location of a primer used for Sanger sequencing. (B-D) Chromatograms of Sanger sequencing reads used to identify the allele. This allele contained a 109 bp deletion occurring at the last two base pairs of the poly(A) sequence (TC, red square) and around the beginning of the *ROSA26* homology sequence (TC, red square). As TC nucleotides were originally present at both ends of the deletion site, but only one TC present in the resultant error containing allele, it is unclear the origin of these nucleotides at the break-point. Colored bars overlaid on the chromatogram represent the origin of the sequences from *mFIX* cDNA (light blue), sequences remaining from cloning plasmids (gray), HSV-TK poly(A) regulatory sequences (dark blue), *ROSA26* homology sequences (yellow), or *ROSA26* genomic sequences past the homology arms (red).

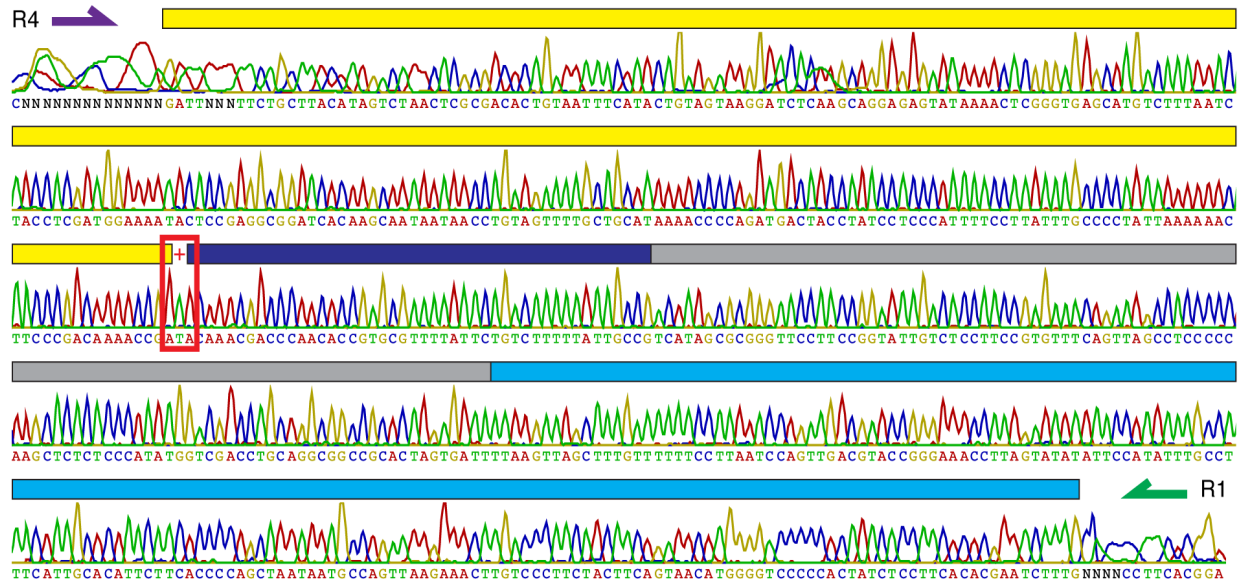
Supplemental Figure 6



C



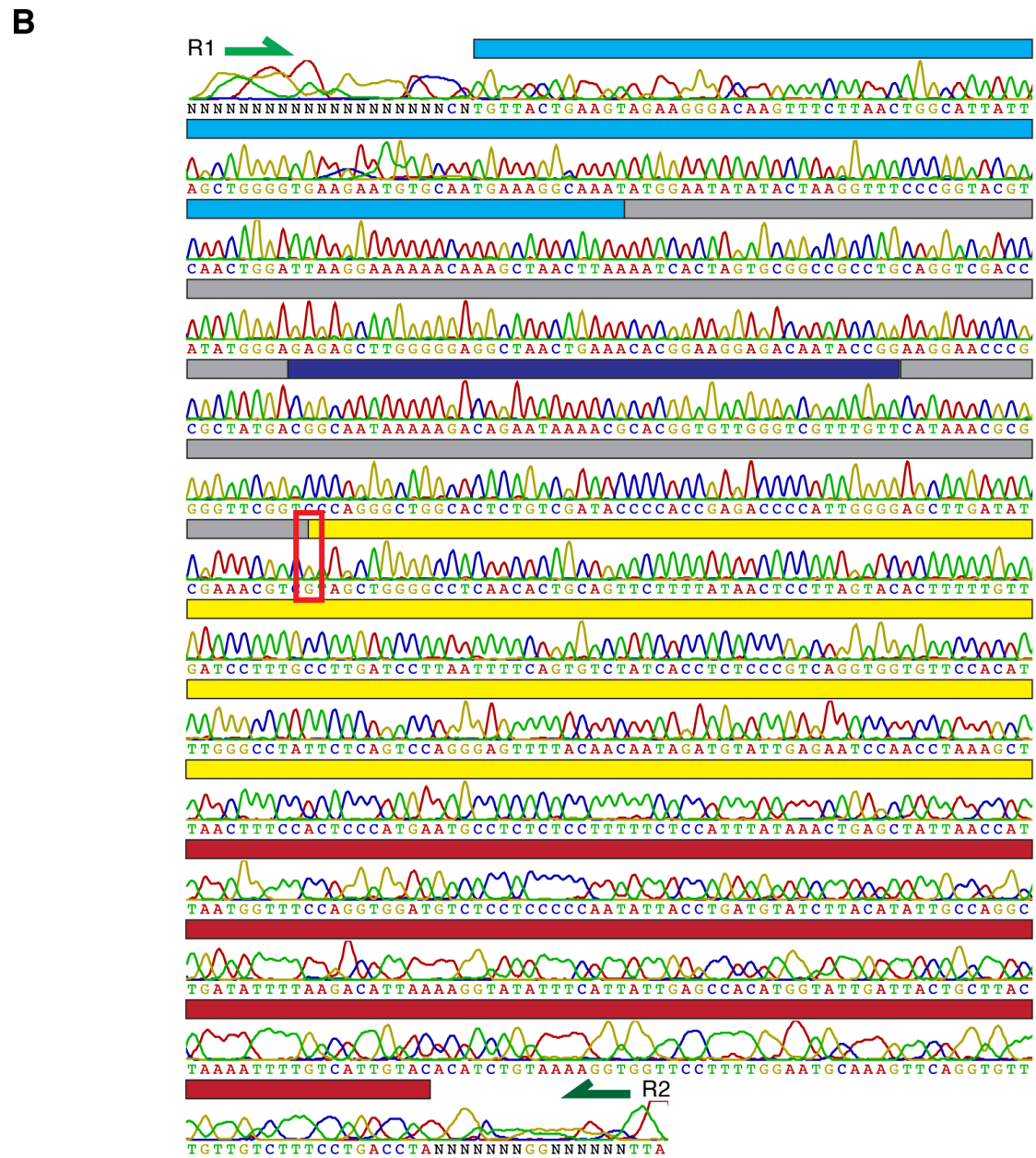
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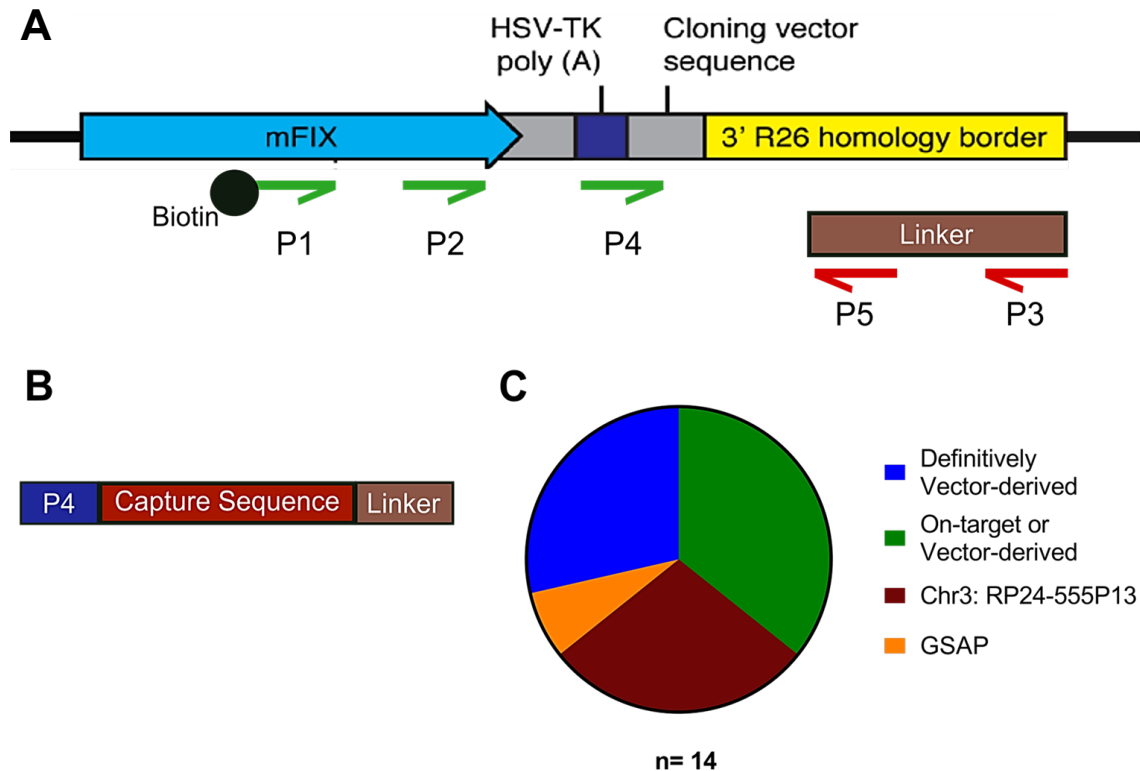
Supplemental Figure 6. Characterization of an error-containing knock-in allele. (A) A schematic of a *ROSA26* allele integrated with *mFIX* cDNA, containing a deletion in the homology border (allele 3, orange bar). Junction capture PCR of the 3' region *ROSA26* generated a reduced-MW *mFIX* knock-in amplicon, which was characterized by Sanger sequencing. Green arrows (R1, R2) represent primers used to generate the junction PCR amplicon and in Sanger sequencing. The purple arrow (R4) represents the location of a primer used for Sanger sequencing. (B-D) Chromatograms of Sanger sequencing reads used to characterize the allele. This allele contains a 209 bp deletion occurring at the end of the intact poly(A) sequence and within the *ROSA26* homology sequences. At the deletion site, a +1bp A/T insertion occurred (red '+' and box). This inserted bp did not appear at either end of the break point and its origin is unknown. Colored bars overlaid on the chromatogram represent the origin of the sequences from mFIX cDNA (light blue), sequences remaining from cloning plasmids (gray), HSV-TK poly(A) regulatory sequences (dark blue), *ROSA26* homology sequences (yellow), or *ROSA26* genomic sequences past the homology arms (red).

A

The diagram illustrates the mFIX construct and its integration into the R26 locus. The mFIX construct is shown as a blue arrow pointing right, containing the mFIX gene. It is flanked by a grey box labeled 'HSV-TK poly (A)' and a blue box labeled 'Cloning vector sequence'. The construct is integrated into the R26 locus, which is shown as a red box. The R26 locus contains the '3' R26 homology border' (yellow box) and the '3' R26 genome' (red box). The integration is mediated by the 'R1' and 'R2' regions, indicated by green arrows. Below the R26 locus, a red box labeled 'Allele4- Scarred' is shown, indicating the presence of a scar sequence. The distance between the scarred allele and the R26 locus is indicated as 'Δ584 bp'.

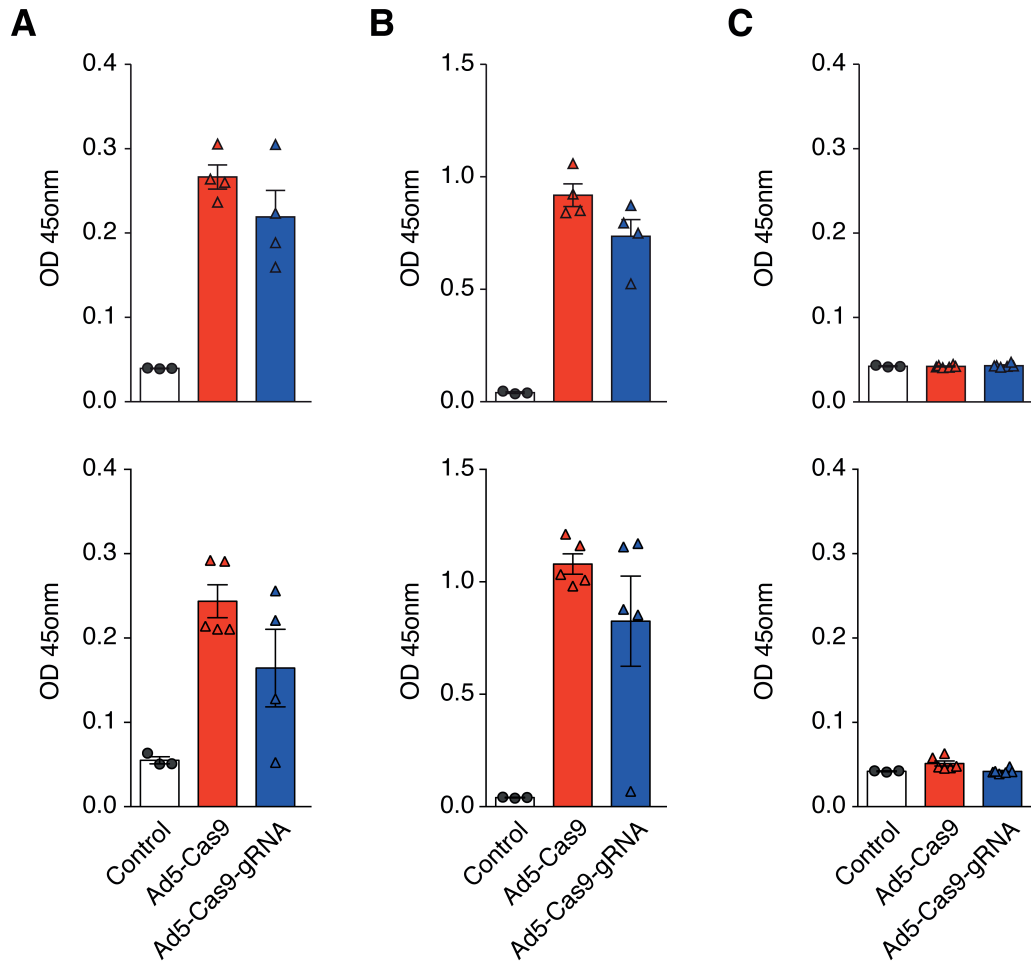


Supplemental Figure 8



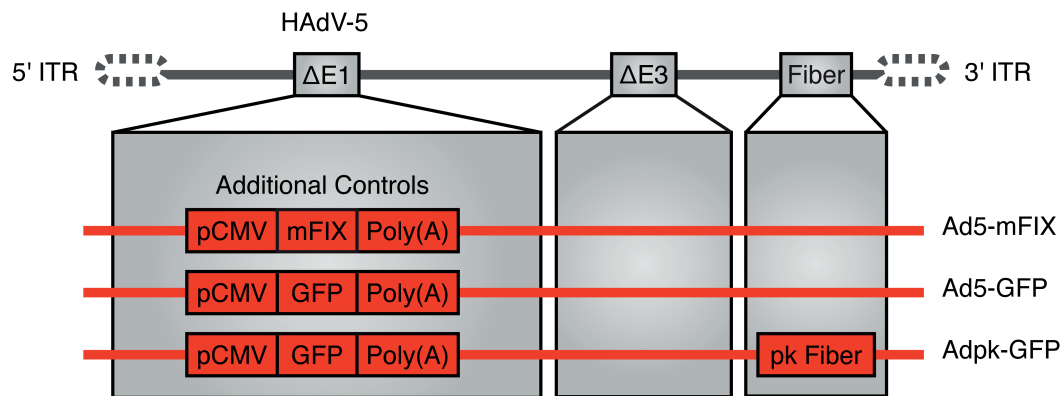
Supplemental Figure 8. Identification of two off-target integration sites. (A) A schematic showing a biotinylated primer (P1) specific to *mFIX* cDNA that was used to generate linear ssDNA amplicons of surrounding sequences adjacent to the *mFIX* cDNA. The linear amplification was performed using whole liver extracted DNA from two mice injected with 7.5×10^{10} VP of Ad5-EF1 α -*mFIX* and 2.5×10^{10} VP of Ad5-Cas9-gRNA at 245 dpi. Following second strand synthesis, amplicons were digested with NheI, AvrII, XbaI, and NcoI and ligated to linkers with corresponding sticky ends. A nested PCR using primers P2 (in *mFIX* cDNA) and P3 (in linker), followed by a second nested PCR with primers P4 in the expression cassette and P5 (in linker), generated sufficient DNA for clonal analysis. (B) A schematic depicting a typical captured sequence amplicon generated by nested PCR with a primer in the known sequence (P4) and a primer in the linker (P5). These amplicons were topo-cloned into bacteria for clonal analysis. (C) Graphic showing the represented reads from 14 clones analyzed by Sanger sequencing.

Supplemental Figure 9



Supplemental Figure 9. Antibody formation in plasma of treated animals. (A) Empty adenoviral particles were immobilized on 96-well plates. 35 (top row) or 189 days (bottom row) after injection, incubation of plasma dilutions (1:25) from untreated R333Q control animals (gray circles), animals treated with Ad5- EF1 α -mFIX and either Ad5-Cas9 (red triangles) or Ad5-Cas9-gRNA (blue triangles) on the plates tested for the presence of IgG antibodies reactive to the adenoviral particle. OD450 nm values were determined using an anti-mouse IgG antibody conjugated to HRP. (B) The same plasma samples were also incubated on wells coated with recombinant Cas9 for reactivity to recombinant Cas9 protein. All treated animals' plasma contained IgG antibodies reactive to the nuclease. (C) The plasma samples were tested for reactivity to recombinant mFIX protein. No detection of IgG's recognizing mFIX occurred using untreated and treated animals' plasma. Individual data points represent results from one animal.

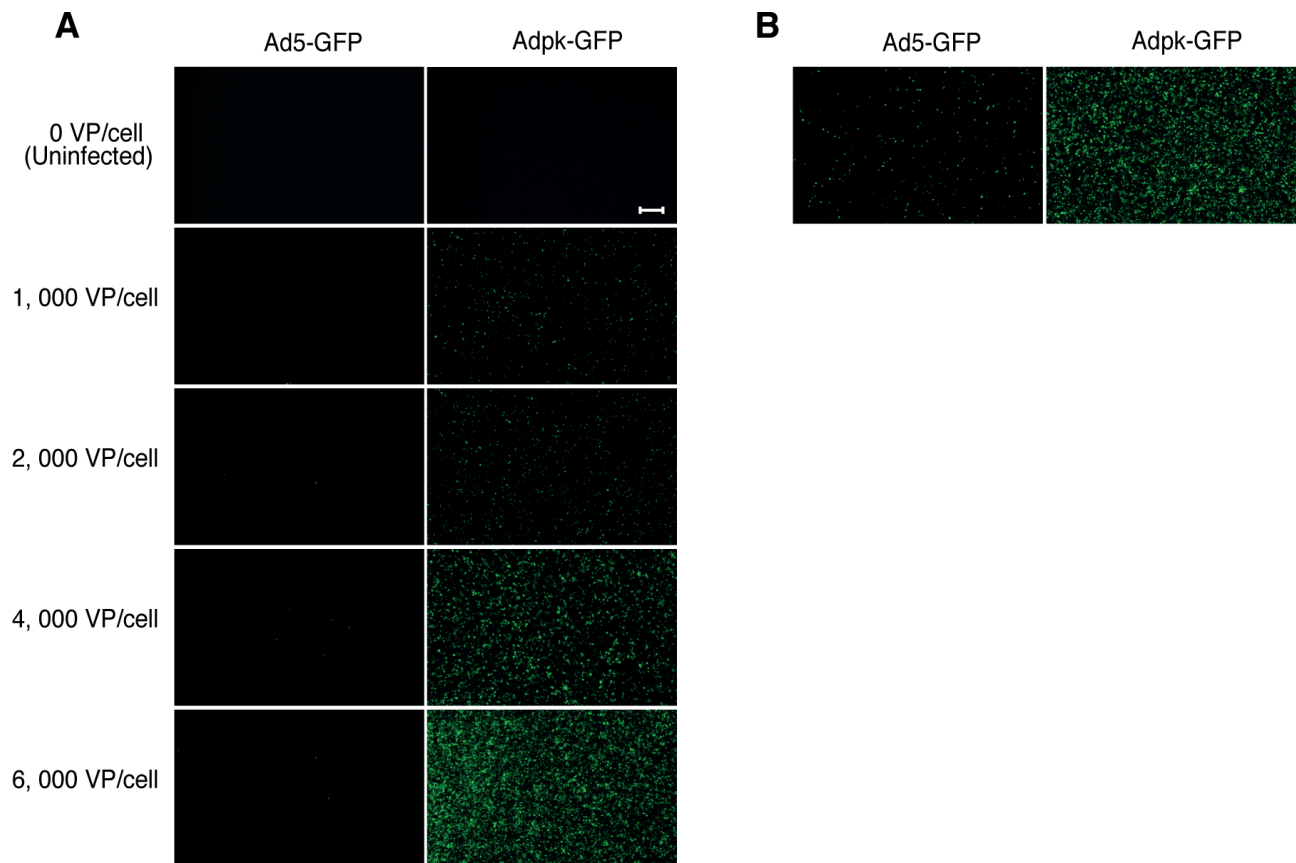
Supplemental Figure 10



Supplemental Figure 10. Standard control adenovirus vectors used in these studies.

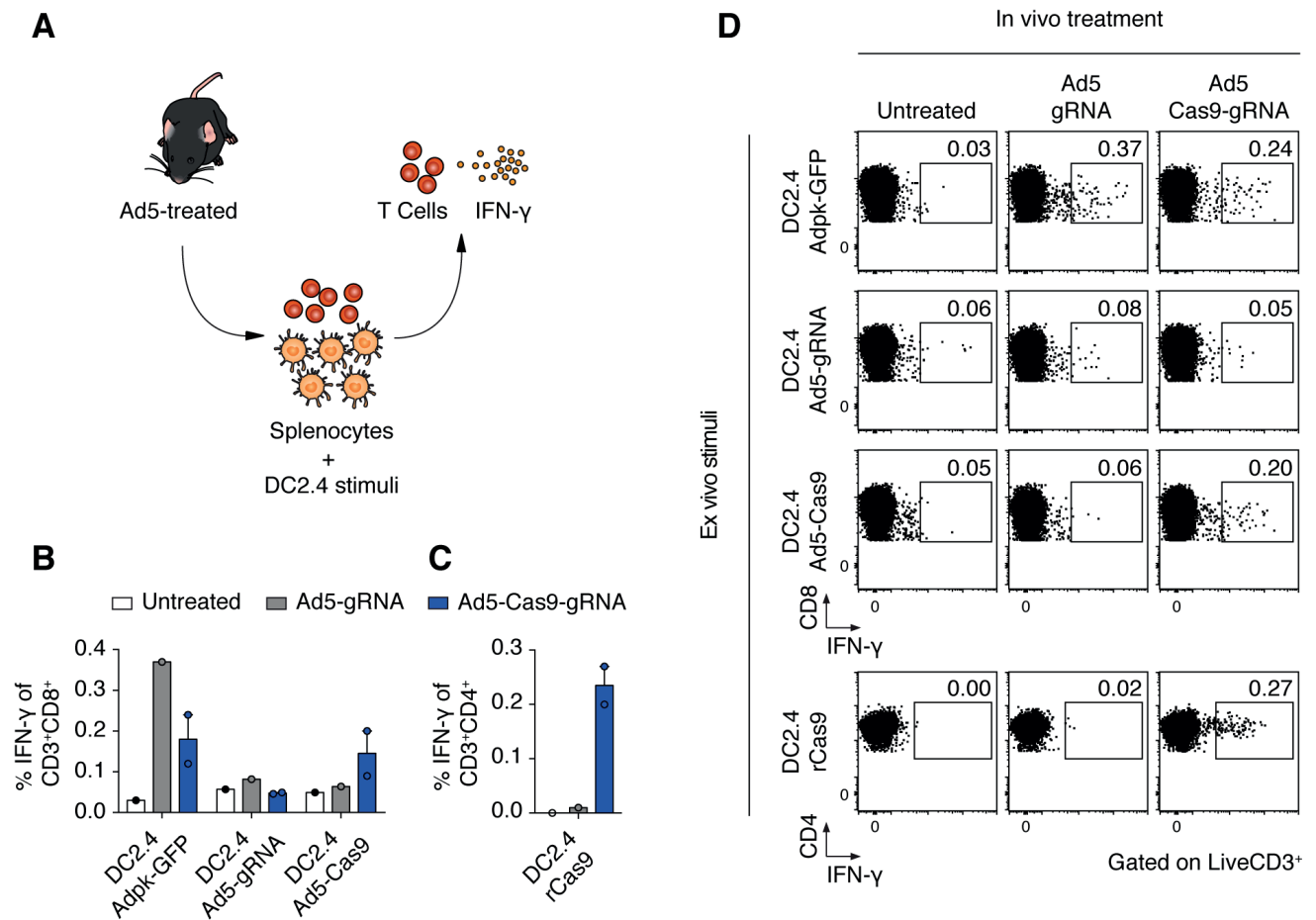
Supplemental control vectors used in this study include standard Ad5 vectors Ad5-mFIX and Ad5-GFP, expressing mFIX or GFP from the CMV promoter, respectively. Additionally, Adpk-GFP is an Ad5-based vector expressing GFP, with a fiber modification. The fiber modification allows for increased adenoviral transduction of the DC2.4 cell line for *in vitro* stimulation assays.

Supplemental Figure 11



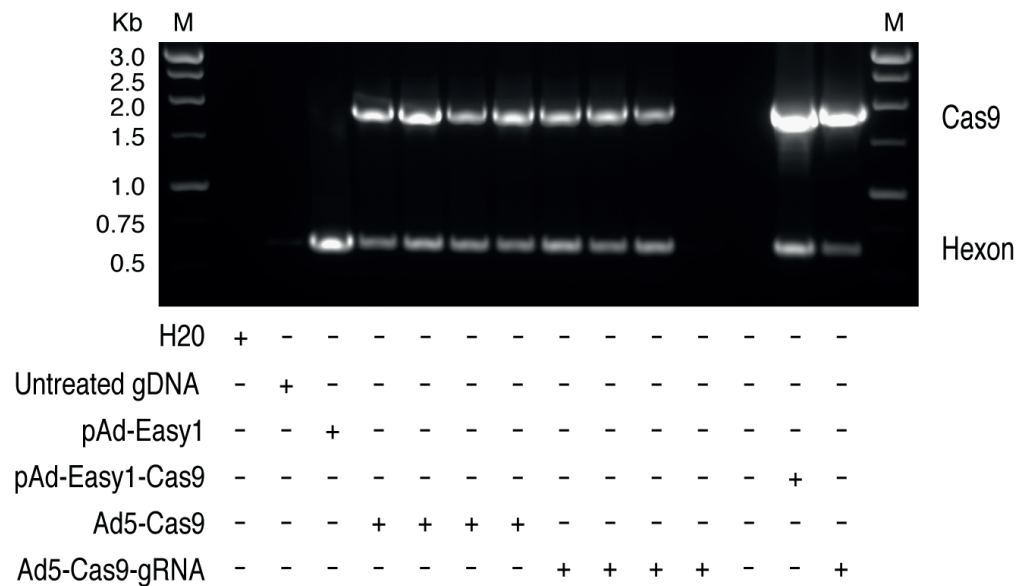
Supplemental Figure 11. Infectivity of Ad5-GFP and Adpk-GFP on DC2.4 cells. (A) To determine adenoviral vector infectivity and optimal MOI, DC2.4 cells were infected with Ad5-GFP or Ad5pk-GFP (columns) at increasing amounts of viral particles (VP) per cell (rows). One day after infection, GFP expression was imaged using florescent microscopy. Adpk-GFP readily infects the DC2.4 cells, whereas Ad5 transduction is refractory. Scale bar represents 200 μ m. (B) To determine if viral-based transgene expression was still detectable several days after infection, DC2.4 cells were infected with 2,000 VP/cell of Ad5-GFP or Adpk-GFP. Three days later GFP expression was imaged, showing continued presence of transgene expression, a necessary consideration for the viral transduction timeframe used to stimulate DC2.4s in T cell assays.

Supplemental Figure 12



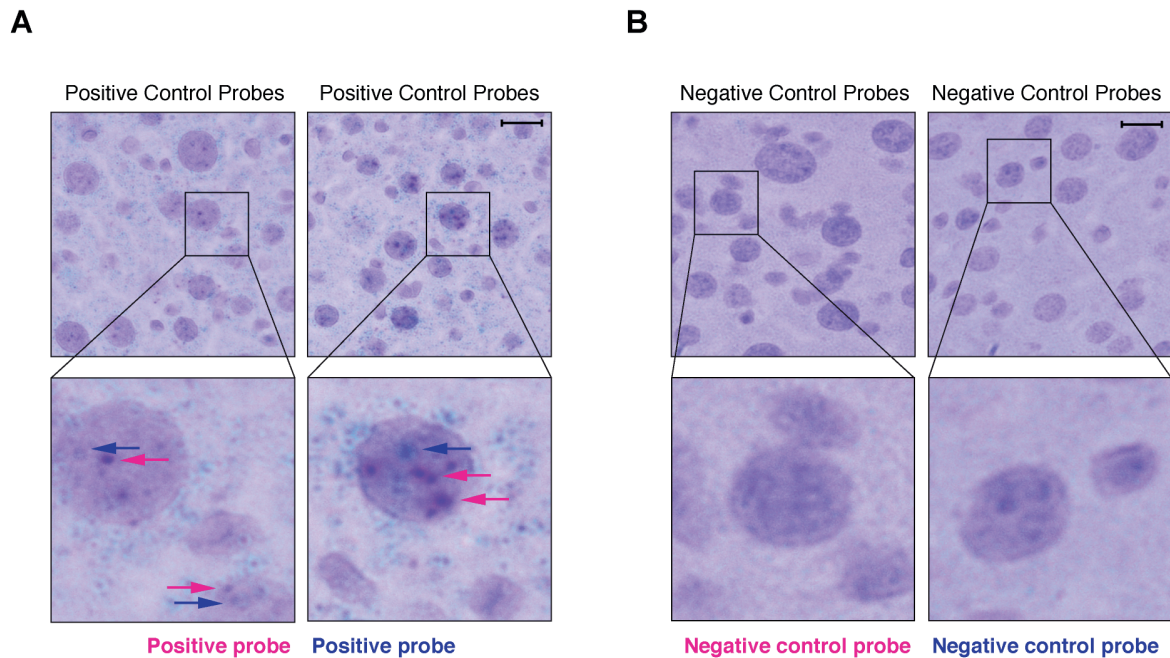
Supplemental Figure 12. Long-term T cell reactivity to Cas9 and vector particles. (A) T cell stimulation assays are comprised of *in vivo* vector injection, followed by harvesting of splenocytes for co-culturing with DC2.4s which had been treated *in vitro* with various stimuli. Cognate recognition of antigen presentation leads to stimulation of IFN γ production in T cells measurable by flow cytometry. (B) Animals were exposed to therapy vectors by sub-optimal injection (confirmed exposure to viral vectors but insufficient expression, see 'Methods') with PBS (untreated, n=1, white bars), Ad5-Cas9-gRNA (n=3, blue bars), or Ad5-gRNA (n= 2, gray bars). 210 days later, splenocytes were harvested and used to assay for long-term T cell responsiveness to potential antigens. DC2.4 cells were treated with Ad5pk-GFP, Ad5-gRNA, Ad5-Cas9, or recombinant Cas9 (x-axis). After co-incubation of treated DC2.4 cells and splenocytes, quantification of IFN γ producing CD8⁺ T cells was performed. (C) Quantification of IFN γ producing CD4⁺ T cells in response to DC2.4 treated with rCas9 is shown. (D) Representative flow plots shows T cells responding to various antigens (rows), according to vector exposure (columns). Results are from one experiment with two technical replicates.

Supplemental Figure 13



Supplemental Figure 13. PCR amplification of an internal sequence of Cas9 CDS. Liver-extracted DNA was used to test for the presence of Cas9 CDS in animals treated with Ad5-Cas9 or Ad5-Cas9-gRNA at 245 days post-infection. PCR amplification of the internal Cas9 sequences yields a 1.88 Kb amplicon. Amplification of a 0.62 kb sequence of the adenoviral capsid gene *hexon* was used as a loading control. Negative controls consisted of liver extracted DNA from an untreated R333Q animal's genomic DNA (gDNA) and the unmodified parental adenoviral genomic plasmid, pAd-Easy1. Positive controls consisted of liver extracted DNA from an animal injected with Ad5-Cas9-gRNA three days after injection, and the adenoviral plasmid (pAdEasy1-Cas9) used to generate Ad5-Cas9. Each lane is data from one animal or plasmid control. Gel is representative of two duplicate gels.

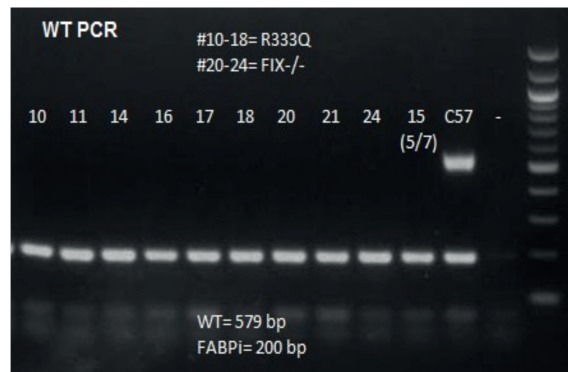
Supplemental Figure 14



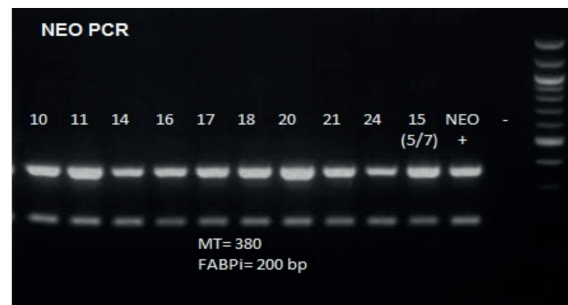
Supplemental Figure 14. *In-situ* hybridization of control probes to test sample quality. Liver sections from virally treated animals were used with positive and negative control probes, for determination of sample quality and assay functionality. (A) Positive control probes (red and blue dots) targeting murine genes *PPIB* and *POLR2A* were used to validate assay targeting and detection of genes present in the sample. Red and blue arrows denote probe signals in individual nuclei. Ubiquitous detection ensured sufficient sample quality of the animals' liver tissue. (B) Negative control probes (red and blue) targeted the bacterial gene *dapB* ensured assay specificity, witnessed by the absence of signal detection in the samples. Scale bars are 20 μm . Inserts show individual cell nuclei.

Supplemental Figure 15

A

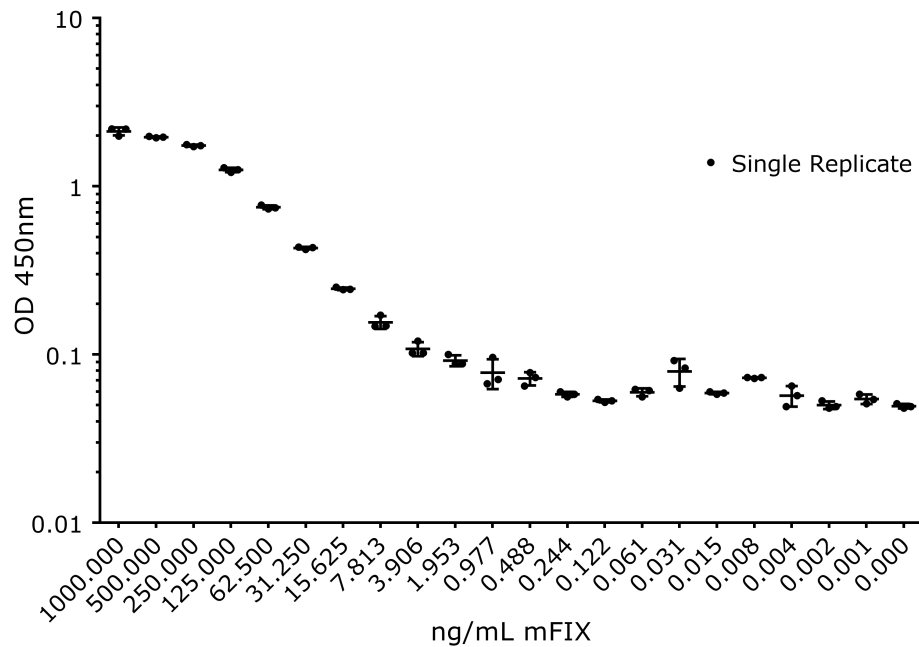


B



Supplemental Figure 15. Genotyping of R333Q hemophilia animals. (A) Genotyping PCR and gel for wild-type *mFIX* gene using forward primers located in intron 1 region and a reverse primer located in intron 2 of *mFIX*. *mFIX* amplicons (579 bp) are only seen in wild-type (WT) controls. Amplification of 200 bp of the *FABPI* gene served as a loading control. Numbers on the gels refer to animal IDs. (B) Genotyping PCR and gel based upon amplification of the *neo* gene, part of the R333Q (ID#s 10-18) strain's knock-in construct, shows amplicons (380 bp) in R333Q breeding animals.

Supplemental Figure 16



Supplemental Figure 16. mFIX standard curve for use in ELISAs. A standard curve was generated using recombinant mFIX protein for determination of mFIX plasma levels. A linear range of the dynamic curve occurs from approximately 62.5 ng/mL to 3.096 ng/mL. All mFIX plasma levels were calculated by sample dilutions' OD values within the linear range of the standard curve and at least two standard deviations greater than the zero value. Each dot represents data from one replicate well. Error bars are standard deviation from the mean.