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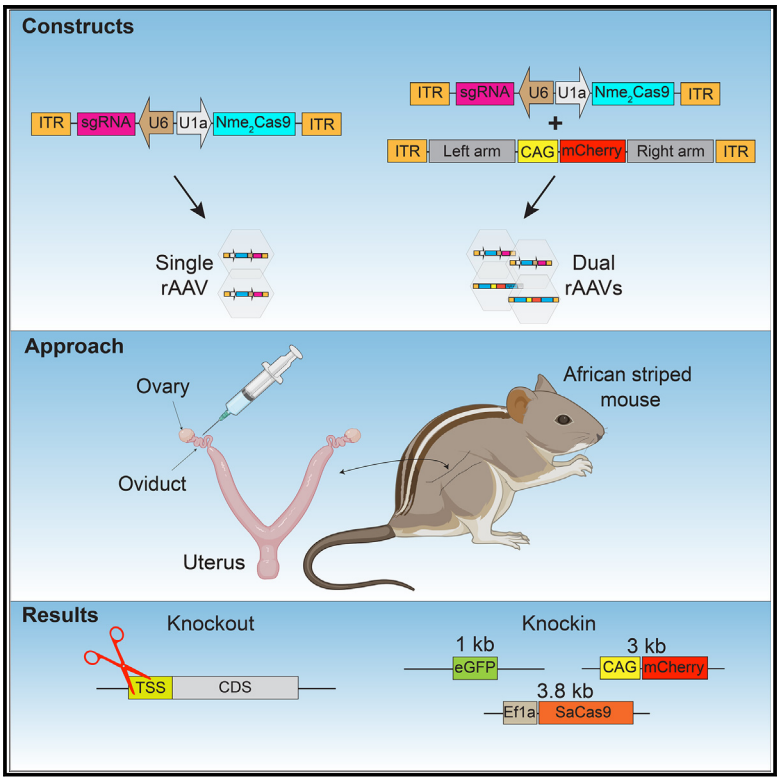
TIGER: Single-step *in vivo* genome editing in a non-traditional rodent

Graphical abstract



Highlights

- An *in vivo* rAAV-based genome-editing method (TIGER) was developed in *R. pumilio*
- TIGER generates knockout and knockin striped mouse lines with high efficiency
- A double-cleaving repair rAAV template increases knockin rate and transmission
- An oversized double-cleaving rAAV template leads to an insertion of 3.8 kb

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In brief

Li et al. develop TIGER (targeted *in vivo* genome editing in rodents), a single-step *in vivo* genome-editing method. Using African striped mice (*Rhabdomys pumilio*), they show that TIGER generates knockout and large knockin lines with efficiency. Due to its simplicity, TIGER can potentially be extended to other rodent species.

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TIGER: Single-step *in vivo* genome editing in a non-traditional rodent

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SUMMARY

Rodents are taxonomically diverse and have evolved a variety of traits. A mechanistic understanding of such traits has remained elusive, however, largely because genome editing in non-traditional model species remains challenging. Here, using the African striped mouse (*Rhabdomys pumilio*), we describe TIGER (targeted *in vivo* genome editing in rodents), a method that relies on a simple intraoviductal injecting technique and uses recombinant adeno-associated viruses (rAAVs) as the sole vehicle to deliver reagents into pregnant females. We demonstrate that TIGER generates knockout and knockin (up to 3 kb) lines with high efficiency. Moreover, we engineer a double-cleaving repair rAAV template and find that it significantly increases knockin frequency and germline transmission rates. Lastly, we show that an oversized double-cleaving rAAV template leads to an insertion of 3.8 kb. Thus, TIGER constitutes an attractive alternative to traditional *ex vivo* genome-editing methods and has the potential to be extended to a broad range of species.

INTRODUCTION

Rodents are a highly diverse group of animals, comprising more than 2,000 species.[1,2] Because rodents occupy such a wide range of habitats, having colonized most terrestrial and aquatic environments, different species have evolved a remarkable array of morphological, behavioral, and physiological traits.[1–3] Such traits offer a fascinating opportunity to increase our understanding of the natural world and, in many cases, can be harnessed for biomedical research. To list but a few examples, thirteen-lined ground squirrels have a cone-dominant retina that resembles the structural organization of the human retina[4]; African striped mice have evolved diurnality[5] and, like prairie voles,[6] oldfield mice,[7] and California mice,[8] provide paternal care,[9–11] whereas most other mammals do not; naked mole rats are eusocial, have a low incidence of cancer, and live up to 20 years[12]; Alston singing mice display sophisticated vocal communication[13]; and spiny mice menstruate[14] and have an exceptional ability to regenerate skin tissue after injury.[15] A mechanistic understanding of such traits has remained elusive, however, largely due to the difficulty of editing the genomes of wild-derived rodent species with precision and ease.

CRISPR-Cas9 genome editing has revolutionized molecular biology and has opened the door to manipulating genomes and understanding gene function in an unprecedented way. To achieve genome editing in laboratory mice (*Mus musculus*), the premier mammalian model species, CRISPR-Cas9 reagents are usually delivered into early pre-implantation embryos. Unfortunately, however, the methods used for gaining access to early-stage embryos and for the delivery of genome-editing reagents have not evolved at the same pace as the genome-editing molecular tools themselves. Although there are variations of this approach, laboratory mouse females are typically given a cocktail of hormones to induce superovulation and then mated with males, and isolated pre-implantation embryos are microinjected or electroporated *ex vivo* with CRISPR-Cas9 reagents. Once this occurs, pre-implantation embryos are transferred into a pseudo-pregnant female, which is produced by mating a female with a vasectomized male.[16] This *ex vivo* approach is cumbersome and has several drawbacks: (1) it uses specialized microinjection or electroporation equipment; (2) it needs to be performed by highly skilled personnel because it is technically challenging; (3) it requires a deep knowledge of the reproductive physiology of the species in question because it relies on hormonal cocktails that need to be customized; (4) vasectomized males have to be surgically prepared, which, together with the need for having pseudo-pregnant females, increases the number of animals needed for each experiment and complicates logistics; (5) it requires embryo transfer, which typically involves a surgical procedcure on the recipient animal; and (6) the need for manipulating zygotes restricts genome editing to a small group of species for which early embryo isolation, culture, and manipulation have been optimized. As a result, with some notable recent exceptions,[17,18] *ex vivo* approaches are not easily transferred to other rodent species and are difficult for individual laboratories to develop and implement.

Recent studies have reported *in vivo* genome-editing strategies in laboratory mice and rats by intraoviductal recombinant adeno-associated virus (rAAV) delivery of Cas9/gRNA (for knockout) or electroporation of Cas9/gRNA RNP and a rAAV repair template (for knockin).[19,20] Despite these major

achievements, *in vivo* gene editing—particularly insertions—remains challenging. Moreover, these approaches are either limited to generating knockouts or are dependent on electroporation. Here, using the African striped mouse (*Rhabdomys pumilio*) (Figure 1A) as a proof-of-concept species, we develop and describe TIGER (targeted *in vivo* genome editing in rodents), a genome-editing platform that relies on a simple injecting technique and that uses rAAVs as the only vehicle to deliver all genome-editing reagents into the oviducts of pregnant females (Figure 1B). By successfully establishing multiple African striped mice (hereafter striped mice/mouse) genome-edited lines, we demonstrate that TIGER can be used to generate both knockouts and large knockins in a wild-derived rodent with high efficiency. Due to its simplicity, effectiveness, and versatility, we suggest that TIGER can be used to edit the genomes of a broad range of rodent species.

RESULTS

TIGER leads to efficient *in vivo* knockout in striped mice

Recent studies have identified several rAAV serotypes that can penetrate the zona pellucida and transduce pre-implantation mouse embryos.[20–22] Among these, rAAV6 has been used to deliver transgenes in embryos of a broad range of mammalian species, including mice, rats, bovines, and primates.[20,22,23] We recently found that rAAV6 can penetrate the zona pellucida and readily transduce striped mouse pre-implantation embryos.[24] Specifically, we showed that intraoviductal injections of an rAAV6 co-expressing *Nme$_2$Cas9* and a single guide RNA (*sgRNA*) targeting either the striped mouse *Tyrosinase* (*Tyr*) (sg*Tyr*) or *Sfrp2* (sg*Sfrp2*) gene led to the production of striped mouse knockout embryos.[24] We used *Nme$_2$Cas9* because it is smaller in size than the more widely used SpCas9 (1,082 vs. 1,368 amino acids), a feature that allows its co-packaging with a sgRNA in one single rAAV.[25] Here, we sought to systematically characterize this simple method of rAAV-mediated *in vivo* genome editing, to evaluate its editing efficiency and frequency, and to analyze the DNA repair outcomes. To this end, we first analyzed F0 animals for both *Tyr* and *Sfrp2* knockout experiments.[24] Out of 18 pups derived from 10 females injected with the sgRNA targeting exon 1 of the *Tyr* gene, we obtained 11 F0 founder animals carrying on-target mutant alleles (61.1% efficiency), including 1 biallelic homozygous knockout (Figures 1C and S1A; Table 1).[24] Using PCR cloning and Sanger sequencing, we analyzed all gene-editing outcomes in F0 animals and found that 8 (72.7%) of them carried insertion or deletion (indel) alleles at more than 50% frequency (Figure S1B). Moreover, 6 mosaic F0 animals (54.5%) carried at least two mutant alleles. Similarly, out of 53 pups obtained from 21 females injected with a sgRNA targeting exon 1 of the *Sfrp2* gene, we obtained 14 F0 founder animals carrying on-target mutant alleles (26.4% efficiency), including 2 biallelic homozygous mutants, one of which was a complete knockout (Figure S1C; Table 1). Analysis of F0 animals indicated that 8 of them (57.1%) carried >50% frequency of indel alleles and that 6 of them (42.9%) carried at least two different mutant alleles (Figure S1D). Lastly, by breeding selected male and female *Tyr* and *Sfrp2* F0 animals with wild-type animals and genotyping all derived F1 animals, we confirmed that knockout alleles were consistently transmitted through the germline at high frequencies (Table 1).

To further evaluate the potential of our method, we targeted an additional locus: *Alx3*. *Alx3* has been previously implicated in regulating differences in stripe patterns[26] and therefore represents an attractive candidate to knock out for detailed follow-up studies. First, we used *in silico* approaches[27] to design multiple candidate sgRNAs targeting exon 2 of *Alx3* (Table S1). Next, we cloned each candidate into the AAV.*sgRNA.Nme$_2$Cas9* plasmid[20] and tested their ability to induce indels by performing transient transfections into striped mouse fibroblast cells followed by a T7 Endonuclease I (T7E1) assay (Figure S2A). While the T7E1 assay does not provide information on the specific edits, it is a useful tool to assess editing rates. The sgRNA that consistently showed high targeting activity, sg*Alx3*G9, was chosen for rAAV production and *in vivo* injections into oviducts of 0.75 days postcoitum (dpc) striped mouse females. At 0.75 dpc, most zygotes (66.7%) are dissociated from surrounding cumulus cells and are thus permeable for rAAV transduction, as evidenced by the strong GFP expression observed after we injected 3×10^8 viral genomes (vg) of an rAAV expressing EGFP (rAAV6.*EF-1α::EGFP*) into females at this stage (Figures 1D and S3; Table S2).

Among the 33 pups derived from 19 females injected with 1×10^9 vg rAAV6.sg*Alx3G9.Nme$_2$Cas9*, we obtained 10 F0 founder animals carrying on-target mutant alleles (30.3% efficiency), including 1 biallelic homozygous knockout (Figure 1E; Table 1). Using PCR cloning and Sanger sequencing, we analyzed the composition and frequency of indels in F0 mosaic animals and found that 6 of them (66.7%) carried >50% frequency of indel alleles (Figure 1F). Moreover, 4 animals (44.4%) carried at least two mutant alleles. Lastly, like what we observed for *Tyr* and *Sfrp2*, *Alx3* mutant alleles were consistently transmitted through the germline at high frequencies (Table 1).

Taken together, our three independent experiments show that TIGER is a highly effective method of generating knockouts in the striped mouse, with derived F0 animals carrying mutant alleles at high frequencies and transmitting them to their descendants at high rates.

Ex vivo knockin in laboratory mouse embryos by short exposure to dual rAAVs

To generate precise on-target genomic integration mediated by homology-directed DNA repair (HDR), Cas9/sgRNA and the DNA repair template need to be simultaneously delivered into the nucleus. While plasmids, synthetic single-stranded oligonucleotides, and linearized double-stranded DNA (dsDNA) are commonly used repair templates,[28] recent studies have shown that knockins can be obtained after prolonged and repeated exposure (e.g., single exposure for 16–24 h[22] and two exposures for a total of 24 h[21]) of cultured mouse embryos to rAAVs carrying a DNA template.[20–22] Such long exposure times may have been deliberately chosen under the premise that they were required, considering that HDR-dependent knockin is largely restricted to the S/G2 phase in early pre-implantation embryos. Long rAAV exposures, however, may result in reduced embryo viability and may posit a challenge for *in vivo* scenarios. We therefore sought to determine whether exposing zygotes to

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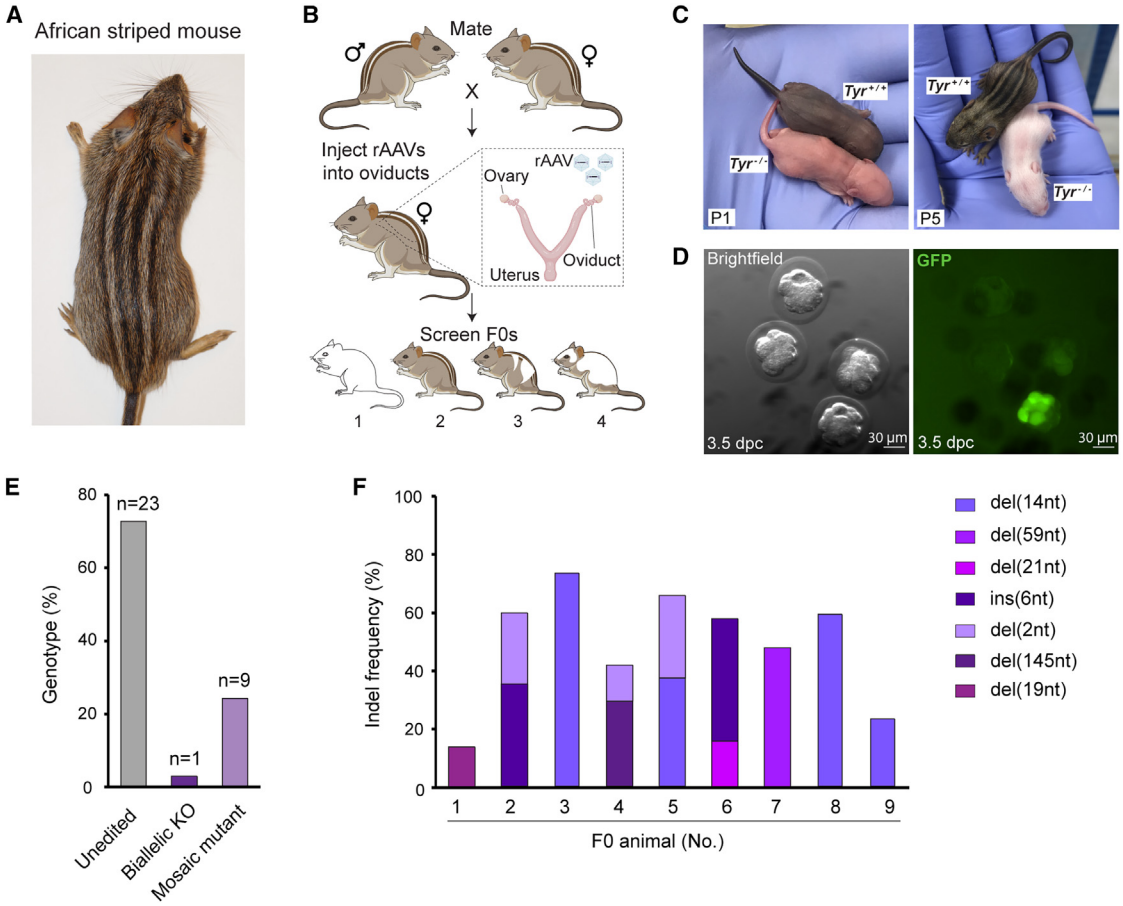

Figure 1. TIGER enables simple and efficient gene knockout in the African striped mouse

(A) An adult African striped mouse.

(B) Schematic illustration of the one-step *in vivo* strategy for generating genome-edited striped mice via injection of rAAV(s) into the oviducts of pregnant females. Depicted for illustration purposes are different genome-editing outcomes, including a pup with a homozygous biallelic *Tyrosinase* (*Tyr*) knockout (white pup, #1), an unedited pup (wild-type pup, #2), and mosaic *Tyr* pups (pups with white patches, #3 and #4).

(C) Representative images of the F0 homozygous *Tyr* biallelic knockout albino pup (20 bp deletion) and its unedited sibling (wild-type looking). Shown are both individuals at postnatal day 0 (P0) and P5. See the main text for description and reference to the original study.

(D) Representative bright-field (left) and fluorescent (right) images of embryos collected at 3.5 days postcoitum (dpc) from a striped mouse female injected with rAAV.*EF-1α::EGFP* at 0.75 dpc.

(E) Bar graph showing the percentage distribution and number of unedited, *Alx3* biallelic mutant, and mosaic mutant pups derived from female striped mice injected with rAAV6.sg*Alx3*.*Nme$_2$Cas9*.

(F) Stacked histogram showing the frequency (percentage distribution) of mutant alleles detected in *Alx3* mutant mosaic F0 striped mice. Each bar represents an individual F0 striped mouse. Mutant alleles of distinct sequences are color coded differently. For example, del (14 nt) denotes the mutant allele with a 14 bp on-target deletion. Striped mouse cartoon adapted from BioRender.

See also Figures S1–S3 and Tables 1, 2, S1, and S2.

rAAVs for shorter periods of time would be sufficient to induce efficient knockin events. To this end, we first evaluated whether we could knockin an *EGFP* cassette into the *Tyr* locus by *ex-vivo*-exposing laboratory mouse embryos to rAAVs for a short period of time. We produced two rAAVs carrying all required CRISPR-Cas9 reagents for knockin. The first rAAV carried the *Nme$_2$Cas9* and sg*Tyr* expression cassettes (rAAV6.sg*Tyr*.*Nme$_2$Cas9*). The second, which provided its genome as the repair template, contained a P2A-*EGFP*-polyA fragment (997 bp) flanked by 919 bp left and 925 bp right homology arms (rAAV6.*Tyr_EGFP*Donor) (Figure 2A). The length of the homology arms (~1 kb) was chosen based on previous knockin studies in mouse embryos.[21,29] We incubated laboratory mouse zygotes for either 2 or 4 h in three different concentrations of rAAV mixtures (2×10^8, 5×10^8, or 1×10^9 vg/μL; 1:1 ratio). Treated embryos were cultured until the blastocyst stage and analyzed for the presence of the on-target insertion using junction PCR and Sanger sequencing (Figures 2B and 2C). While all conditions led to on-target knockin, the highest dose yielded the highest knockin rate (for both exposure times), while survival rate showed only a slight reduction compared to the untreated controls (Figure 2D). Specifically, the 2 h exposure to 1×10^9 vg/μL rAAV yielded a

Table 1. Summary of knockout experiments in striped mice

Genomic locus	Injected females	Injected females that delivered pups (%)	Pups born	F0 carrying mutant alleles (%)	Biallelic KO F0 (%)	F0 tested, no.	Germline transmission, %
Tyr	26	10 (38.5)	18	11 (55.6)	1 (5.6)	12[a]	100.0 (11/11)
Tyr	26	10 (38.5)	18	11 (55.6)	1 (5.6)	8[b]	44.4 (4/9)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	15[a]	100.0 (17/17)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	2[a]	52.4 (11/21)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	3[b]	80 (4/5)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	7[a]	46.2 (6/13)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	9[b]	57.2 (8/14)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	10[b]	100.0 (12/12)
Sfrp2	45	21 (46.7)	53	14 (26.4)	2 (3.8)	12[b]	33.3 (2/6)
Alx3	40	19 (47.5)	33	10 (30.3)	1 (3.0)	10[a]	100.0 (16/16)
Alx3	40	19 (47.5)	33	10 (30.3)	1 (3.0)	4[a]	53.8 (7/13)
Alx3	40	19 (47.5)	33	10 (30.3)	1 (3.0)	7[b]	40.4 (2/5)

See also Figures 1 and S1.
[a]Male F0 paired with wild-type female striped mouse.
[b]Female F0 paired with wild-type male striped mouse.

similar insertion efficiency (37.5%) compared with 4 h exposure (39.3%), while maintaining a higher survival rate (83% for 2 h, 77% for 4 h, and 90% for control) (Figure 2D). Thus, we chose this condition for all subsequent *ex vivo* experiments. Next, we evaluated whether varying the ratio of the two rAAVs would influence knockin efficiency and embryo survival. We exposed zygotes to 4:1, 2:1, 1:1, 1:2, and 1:4 mixtures of rAAV6.sg*Tyr*.*Nme$_2$Cas9* and rAAV6.*Tyr_EGFP*Donor, and found that the 1:1 ratio led to the highest knockin efficiency without impacting survival (Figure 2E). Thus, in contrast to previous reports that relied on long incubation periods,[20–22] our *ex vivo* experiments indicate that a short exposure (i.e., 2 h) to a mix of dual rAAVs is sufficient to achieve efficient knockin in laboratory mouse embryos.

TIGER efficiently generates *in vivo* knockin in striped mice

Encouraged by the results from our *ex vivo* experiments in laboratory mouse, we next sought to establish whether our dual-rAAV strategy could be used to generate *in vivo* knockin in striped mice. To this end, we used the *sgTyr* that we validated through our knockout experiments[24] and tested whether we could insert the P2A-*EGFP*-polyA sequence into the striped mouse *Tyr* locus. Starting from stripe mouse genomic DNA, we amplified 1,157 bp left and 925 bp right homology arms, cloned them into the rAAV plasmid flanking the P2A-*EGFP*-polyA insert, and produced the striped mouse-specific donor rAAV (rAAV6.*Tyr_EGFP*Donor). We then injected a mixture of rAAV6.sg*Tyr*.*Nme$_2$Cas9* and rAAV6.*Tyr_EGFP*Donor (2×10^9 vg total; 1:1 ratio) into the oviducts of striped mouse females. Out of 15 pups derived from 7 injected females, 6 were found to carry the knockin allele with 100% accuracy (40% knockin efficiency) (Figures 2F, S4A, and S4B). Using PCR cloning and Sanger sequencing, we found that all derived mosaic F0 animals carried the knockin allele at a moderate frequency (4%–46%) (Figure 2G; Table 2). Moreover, we confirmed germline transmission of the knockin allele by breeding representative F0 animals of both sexes with wild-type striped mice (Table 2). Lastly, by analyzing F1 animals using droplet digital PCR (ddPCR), we confirmed that the insert was present in a single copy (Figure S5). Taken together, our data show that *in vivo* intraoviductal injection of dual rAAVs efficiently induces ~1 kb fragment insertions in striped mice, though the knockin allele frequency was moderate.

A double-cleaving rAAV increases knockin allele frequency

The moderate knockin allele frequency observed in our mosaic animals may be explained by factors inherent to the biology of rAAVs. For example, upon nuclear entrance, the rAAV single-stranded DNA (ssDNA) is converted to dsDNA, while the T-shaped palindromic hairpin inverted terminal repeat (ITR) structures found on both ends of the rAAV genome remain temporally unresolved.[30,31] This likely hinders homologous recombination between the template rAAV and the targeted genomic regions. Moreover, the linear dsDNA of rAAV tends to form intramolecular circularization and intermolecular concatemers of higher stability,[32,33] which may further lower its efficacy as a repair template. To circumvent these issues and potentially increase the knockin allele frequency in F0 mosaics, we engineered an rAAV that incorporated a sgRNA hybridization sequence and a PAM site flanking the homology arms. We predicted that such strategy would generate a double-cleaving event upon Cas9/sgRNA recognition, releasing a linear dsDNA repair template free of ITR ends (Figure 3A). While previous studies have used similar approaches to produce a linearized donor template from a circular DNA plasmid,[34,35] our strategy involves removing the ITR ends from a sequence that is already linearized. We first sought to determine the feasibility of using this approach by *ex-vivo*-exposing laboratory mouse zygotes to a 1×10^9 vg/μL mixture of rAAV6.sg*Tyr*.*Nme$_2$Cas9* and the double-cleaving template rAAV (rAAV6.*Tyr_EGFP*DonorDC). We tested different ratios of our viral mix (i.e., 4:1, 2:1, 1:1, 1:2, and 1:4), as we expected the rAAV6.*Tyr_EGFP*DonorDC

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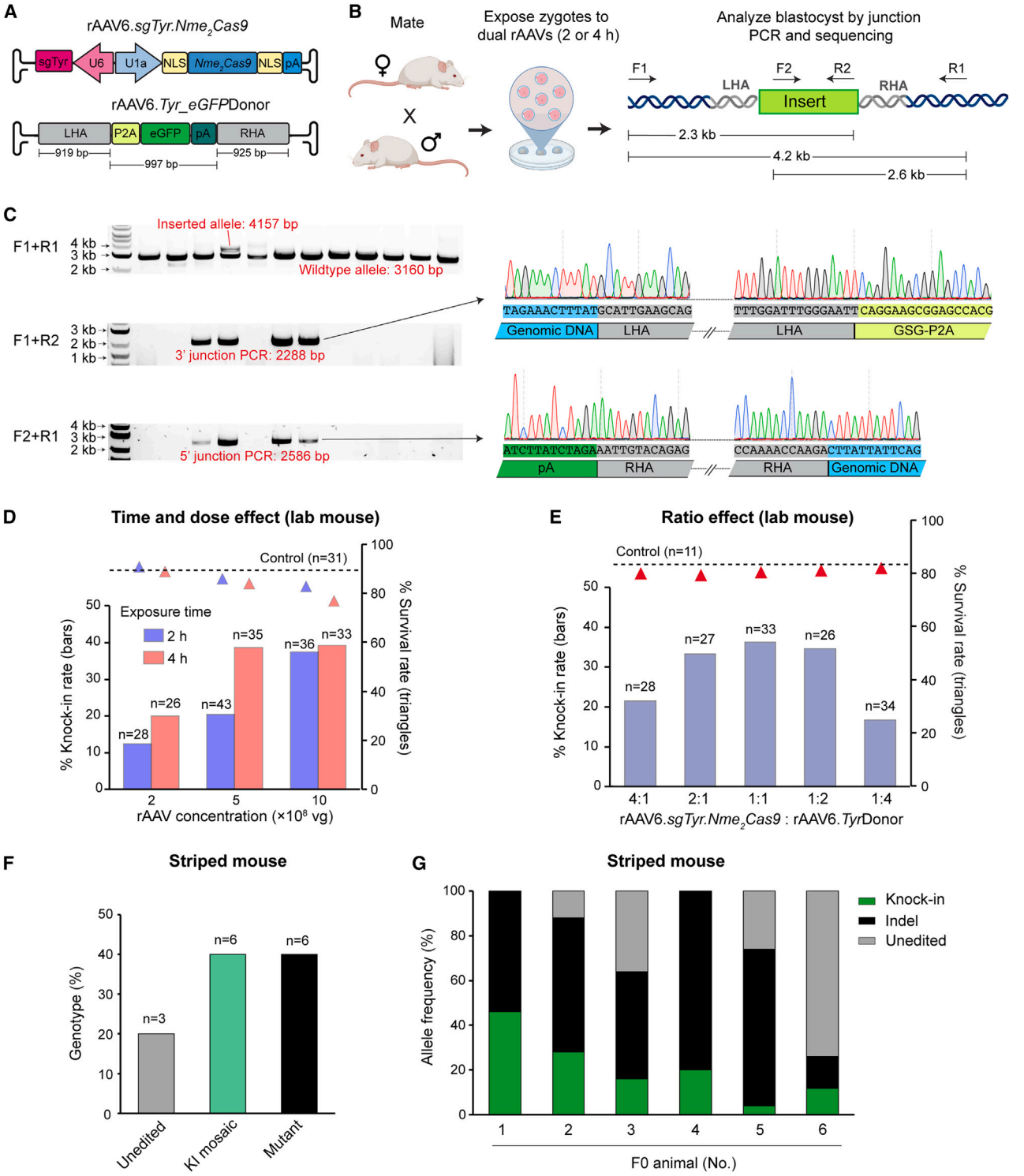

Figure 2. Efficient knockin in laboratory mouse blastocysts and striped mice

(A) The dual-rAAV system comprises an rAAV that expresses sg*Tyr* and *Nme*$_2$*Cas9* driven by the U6 and U1a promoters, respectively (rAAV6.sg*Tyr*.*Nme*$_2$*Cas9*) and an rAAV used as a repair template (rAAV6.*Tyr_EGFP*Donor).

(B) Schematic of the workflow used to analyze the knockin efficacy and efficiency in laboratory mouse *ex vivo* experiments. Arrows show annealing sites of primers used in junction PCR.

(legend continued on next page)

template to compete with the genomic target site for *Nme$_2$Cas9*/sg*Tyr* binding. We found that the engineered rAAV6.*Tyr_EGFP*DonorDC achieved similar knockin efficiency compared with the regular rAAV6.*Tyr_EGFP*Donor when each of them were used at their respective optimal ratio (i.e., 37.5% efficiency at 1:1 ratio for the rAAV6.*Tyr_EGFP*Donor vs. 40% efficiency at 1:2 ratio for the rAAV6.*Tyr_EGFP*DonorDC) (Figures 2E and 3B). However, contrary to what we observed with the regular rAAV6.*Tyr_EGFP*Donor, which only produced knockin mosaics, the modified rAAV6.*Tyr_EGFP*DonorDC generated biallelic knockin embryos when used at 2:1 (7.4% rate) and 1:1 (7.5% rate) ratios (Figure 3B), suggesting an improvement in the knockin allele frequency.

Having established that our engineered double-cleaving rAAV led to knockins *ex vivo*, we next tested whether it would improve the frequency of the knockin allele *in vivo*. To this end, we injected a mixture of rAAV6.sg*Tyr.Nme$_2$Cas9* and a stripe mouse-specific rAAV6.*Tyr_EGFP*DonorDC (2×10^9 vg total; 1:2 ratio) into striped mouse oviducts. Out of 21 pups derived from 9 injected females, 10 carried the on-target precise knockin allele, revealing a similar efficiency (47.6%) to that achieved using the regular rAAV6.*Tyr_EGFP*Donor (40%) (Figure 3C; Table 2). Importantly, however, F0 mosaics generated by using the rAAV6.*Tyr_EGFP*DonorDC had a significantly higher knockin allele frequency compared with animals generated using the conventional rAAV template (t test, $p = 0.0402$) (Figure 3D; Table 2). Consistent with this result, we observed a significant increase in the germline transmission rate (t test, $p = 0.0060$) (Table 2). Like with the conventional rAAV template, we confirmed that F1 descendants had a single copy of the insert (Figure S5). In addition to the 10 F0 animals carrying the on-target precise integration with 100% accuracy, we also obtained 1 pup (9%) with a truncated on-target insertion allele, likely due to repair errors or the presence of a truncated repair template originating from rAAV cleavage (Figure S6A).

Altogether, these results show that the double-cleaving rAAV repair template significantly increases the knockin frequency in F0 animals and the overall germline transmission rate. These properties are highly desirable in genome-editing platforms, as they facilitate the isolation of knockin alleles through subsequent breeding of F0 edited animals.

TIGER enables efficient large knockins in striped mice

Our experiments demonstrate that TIGER is an effective strategy for generating targeted deletions and insertions of ~1 kb fragments. However, there are many instances in which introducing larger DNA fragments, including fluorescent reporters and genes, into specific parts of the genome may be desirable. Thus, we next set out to establish whether TIGER could be used to obtain larger DNA insertions. To achieve this, we attempted to insert a 3 kb *CAG*-driven *mCherry* expression cassette into the striped mouse genome. First, we used *in silico* approaches to design multiple candidate sgRNAs targeting a region homologous to the XbaI element found in intron 1 of the laboratory mouse *Rosa26* locus, a site commonly used as the safe harbor site for insertions in rodents. Next, we screened the different candidate sgRNAs by cloning them into the AAV.*sgRNA.Nme$_2$Cas9* plasmid, transiently transfecting them into striped mouse fibroblasts, and performing T7E1 assays. The sgRNA that consistently showed high targeting activity (i.e., sgR26G1) was chosen to produce virus (rAAV6.sg*R26G1.Nme$_2$Cas9*) (Figures 4A and S2B). We then constructed the rAAV donor template by flanking the *CAG*::*mCherry* cassette with 641 bp left and 700 bp right homology arms and produced the rAAV6.*CAG::mCherry.R26*Donor (Figure 4A). Notably, this represents a reduction in the length of the homology arms compared with the length used in the experiments described above (~1 kb vs. ~650 bp). We injected a mixture of the two rAAVs (3×10^9 vg total; 1:1 ratio) into the oviducts of striped mouse females and used junction PCR and Sanger sequencing to analyze F0 animals (Figures S4C and S4D). Out of 61 animals derived from 26 injected females, 14 carried the knockin allele (20.3% efficiency) (Figure 4B). In addition, we assessed the frequency of the knockin allele present in F0 animals by PCR cloning and Sanger sequencing and further confirmed germline incorporation by breeding selected F0 female and males with wild-type animals (Figure 4C; Table 2). As in all our other experiments, F1 animals had only one copy of the transgene (Figure S5). Analysis of whole-body images under a fluorescence source and of cryosections of multiple tissues from a selected F0 animal (animal #14 in Figure 4C) revealed strong and ubiquitous a *mCherry* expression (Figures 4D and 4E).

The limited packaging size of rAAVs (~4.5 kb excluded ITRs) restricts their ability to deliver large cargos. While oversize packaging is reported to result in truncated rAAV genomes, it has also been suggested that partially packaged rAAVs may complement one another through recombination and may express oversized transgenes in the host nucleus.[36–38] We therefore asked whether we could further push the limits of TIGER and knock in an *EF-1α*-driven *SaCas9* expression cassette (3.8 kb) using an

(C) Representative images showing amplification of the wild-type allele (F1 + R1, top image) and junction PCR amplicons (F1 + R2, middle image, and F2 + R1, bottom image) analyzed on agarose gels (left) and corresponding Sanger sequencing chromatograms (right). Junction PCR primers detect the presence of the knockin (KI).

(D) Effect of rAAV concentration and incubation time on survival (triangles) and KI rate (bars) in mouse pre-implantation embryos.

(E) Effect of rAAV ratio (rAAV6.sg*Tyr.Nme$_2$Cas9* and rAAV6.*Tyr_EGFP*Donor) on survival (triangles) and KI rate (bars) in mouse pre-implantation embryos.

In (D) and (E), the dashed line corresponds to the survival rate of untreated control embryos, and "n" represents the number of embryos used in each treatment group.

(F) Bar graph showing the percentage distribution and number of unedited, KI, and mutant pups derived from female striped mice injected with rAAV6.sg*Tyr.Nme$_2$Cas9* and rAAV6.*Tyr_EGFP*Donor.

(G) Stacked histogram showing the frequency (percentage distribution) of KI, indel mutant, and unedited alleles detected in KI mosaic F0 striped mice. Each bar represents an individual F0 striped mouse. ITR, inverted terminal repeat; NLS, nuclear localization sequence; LHA, left homology arm; RHA, right homology arm; pA, polyA signal sequence.

See also Figures S4–S6 and Table 2.

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Table 2. Summary of knockin experiments in striped mice

Modified allele	rAAV donor template	Injected females	Injected females that delivered pups (%)	Pups born	Pups with the KI allele (%)	F0 tested, no.	Germline transmission, %
Tyr_EGFP	conventional	20	7 (35.0)	15	6 (40.0)	1[a]	41.7 (5/12)
Tyr_EGFP	conventional	20	7 (35.0)	15	6 (40.0)	2[b]	11.1 (1/9)
Tyr_EGFP	conventional	20	7 (35.0)	15	6 (40.0)	4[b]	18.2 (2/11)
Tyr_EGFP	double cleaving	20	9 (45.0)	21	10 (47.6)	4[b]	66.7 (12/18)
Tyr_EGFP	double cleaving	20	9 (45.0)	21	10 (47.6)	5[b]	63 (5/8)
Tyr_EGFP	double cleaving	20	9 (45.0)	21	10 (47.6)	8[a]	55.5 (10/18)
Rosa26_CAG::mCherry	conventional	41	26 (63.4)	61	14 (20.3)	6[b]	16.7 (1/6)
Rosa26_CAG::mCherry	conventional	41	26 (63.4)	61	14 (20.3)	13[a]	28.6 (2/7)
Rosa26_EF-1α::SaCas9	oversized double cleaving	45	22 (48.9)	73	2 (2.7)	1[b]	33.3 (2/6)
Rosa26_EF-1α::SaCas9	oversized double cleaving	45	22 (48.9)	73	2 (2.7)	2[b]	7.7 (1/13)

See also Figures 2, 3, 4, and S4–S6.
[a]Male F0 paired with wild-type female striped mouse.
[b]Female F0 paired with wild-type male striped mouse.

oversized rAAV repair template. We constructed a 5.2 kb rAAV6 containing the *EF-1α-SaCas9* insert flanked by 600 bp left and right homology arms (Figure 4F). To increase the knockin allele frequency, we added a sgR26G1 hybridization sequence and a PAM site at the ends of the two homology arms (rAAV6.*EF-1α*:*SaCas9*DonorDC). We injected the mixture of rAAV6.sg*R26G1.Nme$_2$Cas9* and rAAV6.*EF-1a*::*SaCas9.R26* DonorDC (2×10^9 vg total; 1:2 ratio) into the oviducts of striped mouse females. Out of 73 pups derived from 22 injected females, we obtained 2 F0 pups carrying the on-target knockin allele (2.7% efficiency) and 1 pup that had a truncated insertion (Figures 4G–4I and S6B; Table 2). For both F0 animals, the knockin allele was transmitted to F1 offspring, and each of the F1 animals carried only a single copy (Table 2; Figure S5).

Taken together, our results indicate that TIGER is an effective platform for generating large knockins (up to 3 kb) with high efficiency. Moreover, we show that the length of the homology arms can be reduced (from ~1 to ~650 kb) and that the size of the insertion can be further expanded to 3.8 kb by using an oversized double-cleaving rAAV template.

DISCUSSION

Here, we describe and characterize TIGER, a method that uses rAAV-mediated intraoviductal delivery of genome-editing reagents to achieve highly efficient genome editing in African striped mice. First, by analyzing animals from three independent knockout experiments, we demonstrate that our method produces striped mouse lines carrying mutant alleles at high frequencies and that such alleles are transmitted to subsequent generations at high rates. Moreover, using a dual-rAAV strategy, we show that TIGER generates knockin animals containing inserts up to 3 kb in length with high efficiency. Lastly, by further engineering the rAAV genome, we demonstrate that knockin frequencies significantly increase and that an insertion of 3.8 kb can be achieved. To the best of our knowledge, this is the largest fragment that has been inserted into mammalian genomes using *in vivo* approaches. Moreover, it constitutes an example of how knockin can be achieved in a non-traditional rodent.

Compared with the commonly used gene editing strategies, which depend on microinjection or electroporation of Cas9/sgRNA ribonucleoprotein (RNP) and synthetic template DNA into early embryos, TIGER uses rAAVs both as the delivery vehicle and the templates providing all gene-editing components. Thus, it is likely that the timing and window of active gene editing differs between the two approaches. In particular, Cas9/sgRNA RNP and synthetic template DNA allow for immediate and transient gene editing in zygotes containing 2–4 alleles, which leads to a high likelihood of biallelic editing in F0 animals. In contrast, TIGER likely features a delayed window of active gene editing because the rAAV genome relies on the host cell both for converting the ssDNA genome into the dsDNA form and for transcribing and translating the Cas9/sgRNA. These rate-limiting steps may postpone gene-editing events until a later phase in pre-implantation embryos, which may explain why we observed a high percentage of mosaic F0 animals carrying at least two mutant alleles. This disadvantage, however, is compensated by a prolonged editing window. Unlike synthetic Cas9/sgRNA and DNA templates, rAAV genomes reside in the nucleus in relatively stable forms of linear (short-term) or concatemerized (long-term) dsDNA. Thus, the rAAV genomic DNA may continuously provide Cas9/gRNA and repair template to blastomeres until eventually becoming diluted and degraded following embryo cleavage. This notion is consistent with our data showing that TIGER has high editing efficiencies, including reproducibly generating a homozygous biallelic knockout at all three tested loci.

HDR-dependent knockin events primarily occur at the S/G2 phase of the cell cycle, consistent with studies reporting improved knockin efficiency when targeting this stage.[29,39,40]

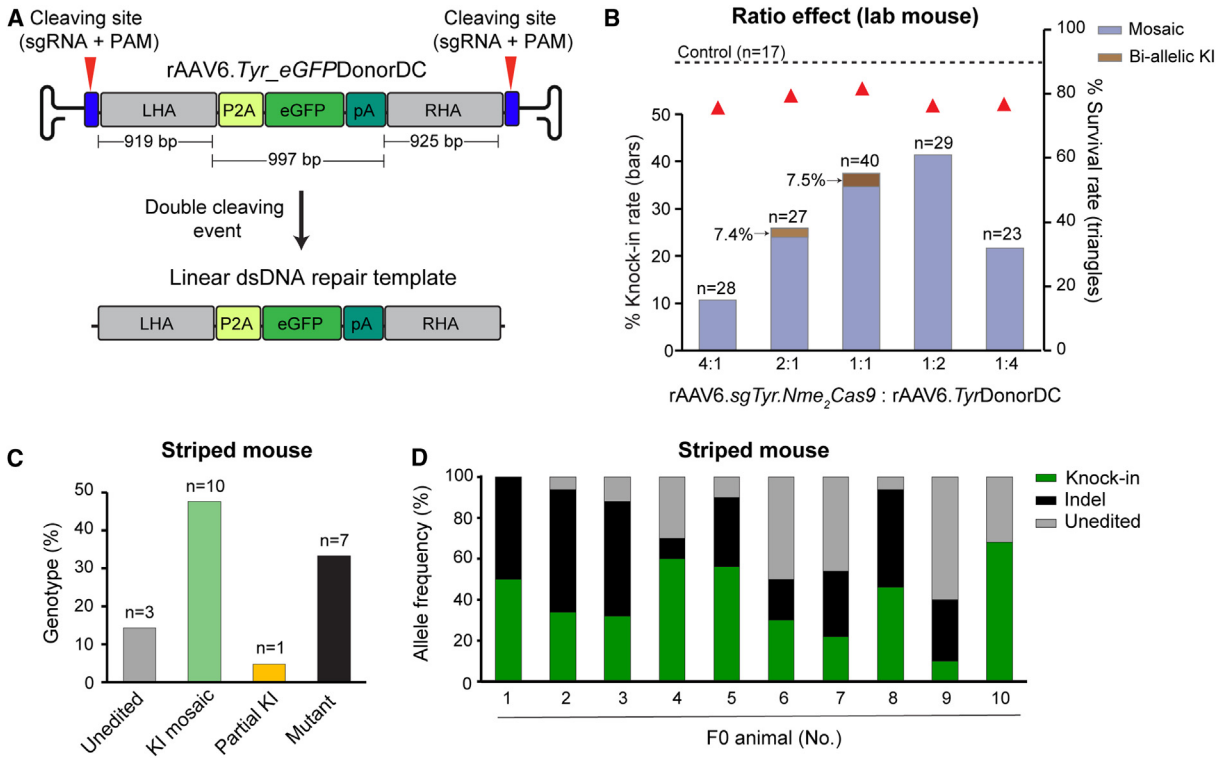

Figure 3. A double-cleaving rAAV template improves KI allele frequency in lab mouse blastocysts and in striped mice

(A) Schematic of the double-cleaving rAAV template. When present with *Nme$_2$Cas9*/sgRNA in the nucleus, the double-cleaving rAAV is excised, and the linear dsDNA free of ITR is released. ITR, inverted terminal repeat; LHA, left homology arm; RHA, right homology arm.

(B) Effect of rAAV ratio (rAAV6.sg*Tyr.Nme$_2$Cas9* and rAAV6.*Try_EGFP*DonorDC) on survival (triangles) and KI rate (bars) in mouse pre-implantation embryos. The dashed line corresponds to the survival rate of untreated control embryos, and "n" refers to the number of embryos used in each treatment group.

(C) Bar graph showing the percentage distribution and number of unedited, KI mosaic, partial KI, and mutant pups derived from female striped mice injected with rAAV6.sg*Tyr.Nme$_2$Cas9* and rAAV6.*Tyr-EGFP*DonorDC.

(D) Stacked histogram showing the frequency of the KI, indel mutant, and unedited alleles found in KI mosaic F0 striped mice. Each bar represents an individual F0 mouse.

See also Figures S4–S6 and Table 2.

Moreover, as evidenced by reports in mice,[41] rats,[42] and hamsters,[43] rodents have a major wave of zygotic genome activation (ZGA) that occurs in 2-cell embryos, which slows the cell cycle and introduces a long G2 pause. Thus, we hypothesize that there are two factors contributing to the high knockin efficiency we observed with TIGER: (1) ZGA causes a burst of Cas9/sgRNA transcription and translation from rAAV dsDNA, and (2) the 2-cell G2 pause provides a long and active window (<12 h in laboratory mouse) during which ample gene-editing components are available for robust HDR-dependent knockin events to occur. Further characterization of the length and repair mechanisms active in different cell-cycle phases of early rodent embryos will yield key insights that will improve gene-editing outcomes.

By engineering a double-cleaving rAAV vector, we show that we can considerably increase the frequency of knockin alleles in mosaic animals. This demonstrates that TIGER is a flexible tool that is amenable to optimization. Moving forward, we envision that TIGER can be further expanded and used for different applications, including multiplex genome editing via delivery of multiple gRNAs, epigenetic modifications through the delivery of small dCas9 fused with transactivating/inhibitory domains[44], andprecise genome editing without inducing double-stranded breaks via delivery of compact base/prime editors.[45,46]

Altogether, TIGER constitutes a simple, effective, and hence attractive alternative to traditional *ex vivo* genome-editing methods widely employed in laboratory mice and rats because (1) it circumvents the need to culture and manipulate embryos *ex vivo*, (2) it relies on natural breeding and therefore does not require vasectomized males or donor/recipient females, and (3) it does not involve specialized equipment and can be performed in under 30 min in any animal procedure room by personnel trained in basic surgical techniques. In addition, TIGER represents an improvement over previously reported *in vivo* gene-editing methods used in laboratory mice and rats because it generates the largest knockin reported so far (3.8 kb). Lastly, unlike methods requiring preparation and delivery of both rAAV and pre-assembled RNP (or synthetic mRNA or DNA) followed by electroporation, it uses rAAV as the only delivery vehicle, which simplifies the overall procedure and reduces the amount of tissue manipulation. For all these reasons, TIGER has the potential

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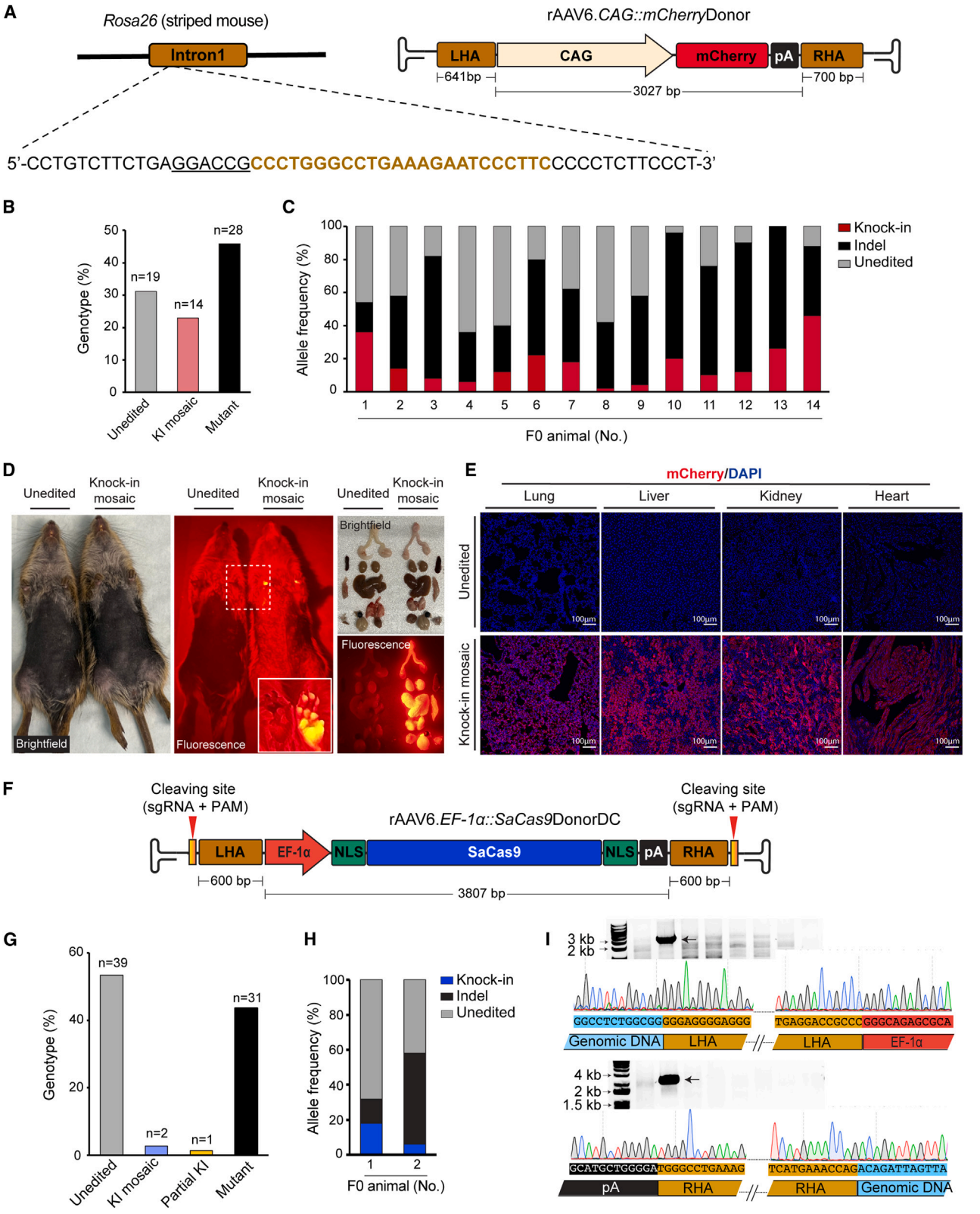

(legend on next page)

to be readily extended to a broad range of rodent species that can be bred in a controlled environment. This will open the door to understanding the molecular basis of a wide array of phenotypic traits and will impact a wide range of fields—from basic research to biomedical sciences.

Limitations of the study

Although TIGER allows simple, efficient, and flexible genome editing in striped mice and potentially other rodents, there are important aspects that need to be considered. For example, while our method reduces the amount of tissue manipulation compared with approaches that rely on electroporation, production of rAAVs is usually associated with higher costs relative to methods that use pre-assembled RNP or synthetic mRNA/DNA. In addition, since TIGER relies on the *in vivo* delivery of genome-editing reagents into 1-cell stage pre-implantation embryos, key hurdles may need to be overcome before our approach can be extended to other species. These include accurately timing the pregnancy of females and determining the optimal *in vivo* delivery window. Importantly, these obstacles are not necessarily specific to TIGER, as they must be solved when extending practically any genome-editing approach into a new species.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112980.

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AUTHOR CONTRIBUTIONS

S.L. and R.M. conceived the project and designed experiments. S.L. and S.A.M. performed genome-editing experiments, genotyping, and strain characterization. M.R. did ddPCRs. M.R.J. obtained confocal images from mouse tissue. M.R.J. and B.J.B. provided logistical support. S.L. analyzed data. S.L. and R.M. wrote the paper. All authors approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Figure 4. TIGER enables efficient KI of large inserts into striped mice

(A) Schematic of the sgRNA targeting site in intron 1 of *Rosa26* (left) and the rAAV.*CAG::mCherry R26*Donor template used for KI (right). The PAM sequence is underlined, and the sgRNA hybridization sequence is highlighted in brown. ITR, inverted terminal repeat; LHA, left homology arm; RHA, right homology arm; pA, polyA signal sequence.

(B) Percentage distribution and number of unedited, *CAG::mCherry* KI mosaic, and mutant pups derived from female striped mice injected with rAAV6.*sgR26G1*.*Nme$_2$Cas9* and rAAV6.*CAG*::*mCherry*.*R26*Donor.

(C) Stacked histogram showing the frequency of the KI, indel mutant, and unedited alleles detected in KI mosaic F0 striped mice. Each bar represents an individual F0 mouse.

(D) Bright-field and fluorescent images of a mosaic *CAG::mCherry* KI F0 (#14 in C) and its wild-type (unedited) sibling.

(E) Immunofluorescence showing mCherry in all analyzed tissues.

(F) Schematic of the rAAV6.*EF-1a*::*SaCas9*.*R26*DonorDC template. NLS, nuclear localization sequence.

(G) Percentage distribution and number of unedited, mosaic *EF-1a*::*SaCas9* KI, partial KI, and mutant pups delivered by female striped mice injected with rAAV6.*sgR26*.*Nme$_2$Cas9* and rAAV6.*EF-1a*::*SaCas9*.*R26*DonorDC.

(H) Stacked histogram showing the frequency of KI, indel mutant, and unedited alleles detected in mosaic F0 striped mice. Each bar represents an individual F0 mouse.

(I) Representative images showing junction PCR amplicons analyzed on agarose gels. Arrowheads indicate positive amplification. Corresponding sequencing chromatograms are shown below.

See also Figures S2 and S4–S6 and Table 2.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-mCherry	Novus biologicals	Cat#NBP2-25157, RRID:AB_2753204
Donkey polyclonal anti-Rabbit IgG, Alexa Fluor™ 555	Thermo Fisher Scientific	Cat#A-31572, RRID:AB_162543
Bacterial and virus strains		
Escherichia coli K12	New England Biolabs	Cat#C3040
Recombinant AAV serotype 6	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Pregnant mare serum gonadotropin	ProSpec Bio	Cat#HOR-272
Human chorionic gonadotropin	EMD Millipore	Cat#230734
T7E1 Endonuclease I	New England Biolabs	Cat#E3321
CloneAmp™ HiFi PCR Premix	Takara Bio	Cat#639298
DMEM	Thermo Fisher Scientific	Cat#11965092
RPMI	Thermo Fisher Scientific	Cat#11875093
Lipofectamine 3000	Thermo Fisher Scientific	Cat#L3000001
Proteinase K	New England Biolabs	Cat#P8107
M2	Cytospring	Cat#M2114
KSOM^{AA}	Cytospring	Cat#K0104
Critical commercial assays		
StrataClone PCR Cloning Kit	Agilent Technologies	Cat#240205
In-fusion HD Cloning Kit	Takara Bio	Cat#102518
Site-directed mutagenesis Kit	New England Biolabs	Cat#E0554S
QX200 ddPCR EvaGreen Supermix	BioRad	Cat#1864034
Experimental models: Cell lines		
B16 cells	ATCC	ATCC Cat# CRL-6324, RRID:CVCL_U240
Immortalized *Rhabdomys* fibroblast cells	Johnson et al.[22]	N/A
Experimental models: Organisms/strains		
Laboratory mouse	Charles River	Strain 022; RRID: IMSR_CRL:22
African striped mouse (*Rhabdomys pumilio*)	This paper	N/A
Oligonucleotides		
Oligonucleotides used for all our experiments	This paper	Table S1
Recombinant DNA		
Nme$_2$Cas9_AAV	Edraki et al.[25]	Gift from Dr. Jaime Rivera; Addgene: Cat#119924
pAAV EF1a eGFP	Savolainen et al.[47]	Addgene: Cat# 60058
pX600-AVV-CMV::NLS-SaCas9-NLS-3XHA-bGHpA	Ran et al.[48]	Addgene: Cat# 61592
pBRPB CAG-mCherry-IP	Yamaji et al.[49]	Addgene: Cat#106333
pAAV-nEFCas9	Suzuki et al.[50]	Addgene: Cat#87115
pEGF-puro	Abbate et al.[51]	Addgene: Cat#45561
Software and algorithms		
CRISPOR	Concordet and Haeussler[27]	http://crispor.tefor.net
ImageJ	Schindelin et al.[52]	https://ImageJ.nih.gov/ij/
ICE (Inference of CRISPR Edits)	Conant et al.[53]	github.com/synthego-open/ice
Other		
Thin Wall Glass Capillaries	World Precision Instruments	Cat#TW100-4

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ricardo Mallarino (rmallarino@princeton.edu).

Materials availability

All sequencing traces, animals, and plasmids generated in this study are available upon request.

Data and code availability

- Original data can be requested from the lead contact.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and Animal Welfare Act and approved by Princeton University's Institutional Animal Care and Use Committee (IACUC). Wild-derived striped mice (*Rhabdomys pumilio)* native to the Goegap Nature Reserve, South Africa, S 29° 41.56′, E 18° 1.60′, were originally obtained from a captive colony at the University of Zurich (Switzerland). F10 descendants and beyond are housed at Princeton University at 12:12 light-dark cycle, 68–78°F and provided with standard a daily diet of rodent diet (4 g/animal) supplemented with sunflower seeds. CD-1 IGS (strain 022) mice were purchased from Charles River Laboratories.

METHODS DETAILS

Pregnancy timing and embryo collection

Four-to six-month-old female striped mice (~55–65 g) were paired with age and weight matched males. We examined females daily for the presence of post coitum plugs or vaginal sperm. Vaginal plugs were usually found within one week after setting up breeding pairs.

We considered 12 p.m. of the day in which vaginal plugs or vaginal sperm were observed, as 0.5 days postcoitum (dpc). To time and characterize the development of pre-implantation striped mouse embryos, we euthanized plugged females at 0.375 dpc, 0.5 dpc, 0.625 dpc, 0.75 dpc, 0.875 dpc, 1.5 dpc, 2.5 dpc, 3.5 dpc, and 4.5 dpc. Oviducts and their attached uterine horns were immediately dissected and flushed with pre-warmed M2 media (CytoSpring, M2114). Flushed embryos were observed under microscope and imaged using a Nikon SMZ18 stereoscope equipped with a DS Qi2 camera.

Cell culture and transfection

B16 cells (American Type Culture Collection) were cultured at 37°C and 5% CO_2 in RPMI-1640 (Thermo Fisher Scientific, 11875093), supplemented with 10% fetal bovine serum (FBS) (Corning Life Science, 35-010-CV) and 100 U ml^{-1} penicillin-streptomycin (pen-strep, Thermo Fisher Scientific, 15140122) (Thermo Fisher Scientific). Immortalized striped mouse fibroblast cells have been previously described[24] and were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, 11965092), supplemented with 10% FBS and pen-strep. For transient transfections, we used lipofectamine 3000 (Thermo Fisher Scientific, L3000001) to deliver 1.5 μg total plasmid(s) into 400,000 cells/well in a 12-well plate format. Transfected and mock control cells were collected 96 h post transfection, and total genomic DNA was extracted using Zymo Quick DNA Miniprep Plus Kit (Zymo Research, D4068).

Molecular cloning

The AAV.*sgTyr.Nme$_2$Cas9.Tyr_sgRNA* plasmid was generously provided by Dr. Jaime Rivera (Frederick National Laboratory for Cancer Research) and has been previously described[25] (Addgene 119924). For all our experiments, we chose to use Nme$_2$Cas9 because it is smaller in size than the more widely used saCas9 (1,082 amino acids vs. 1,364 amino acids), a feature that allows its co-packaging with an sgRNA in one single rAAV.[25] Our previous study showed that the sg*Tyr* targets the *Tyr* gene in both laboratory mouse and striped mouse. AAV.*sgSfrp2.Nme$_2$Cas9*, AAV.*sgAlx3.Nem$_2$Cas9* and AAV.*sgRosa26.Nem$_2$Cas9* were constructed by substituting the Tyr spacer sequence from the AAV.*sgTyr.Nme$_2$Cas9.Tyr_sgRNA* plasmid[25] with the desirable sgRNA sequence using a site-directed mutagenesis kit (New England Biolabs, E0554S). The NLS-SaCas9-NLS-3XHA-bGHpA coding sequence was PCR amplified from pX600-AVV-CMV::NLS-SaCas9-NLS-3XHA-bGHpA (Addgene 61592). CAG and EF-1α promoters were PCR amplified from pBRPB CAG-mCherry-IP (Addgene 106333) and pAAV-nEFCas9 (Addgene 87115), respectively. EGFP and mCherry coding sequences were PCR amplified from pEGF-puro (Addgene 45561) and pBRPB CAG-mCherry-IP, respectively. PCR primers are listed in Table S1.

To construct rAAV donor templates, homology arms flanking the sgRNA targeting site were PCR amplified from genomic DNA extracted from animal tissues using primers listed in Table S1. Since striped mice is wild-derived, this species will show higher heterozygosity than inbred species, and potential polymorphism may affect the targeting efficacy of gene editing molecules and HDR-dependent DNA repair. Thus, to examine the extent of heterozygosity surrounding sgRNA regions, we extracted genomic DNA from ~5 to 7 animals of different breeding background in the colonies and sequenced 2 kb genomic regions upstream and downstream of the sgRNA targeting site. None of the sequenced genomic regions contained polymorphisms. The left and right homology arms and their flanked insert were cloned into the pAAV-nEFCas9 backbone by replacing the nEFCas9 insert using the In-fusion HD Cloning Kit (Takara Bio, 102518). To construct the double cleaving rAAV donor plasmid, we use PCR to introduce the corresponding sgRNA recognition sequence and the PAM (N_4GG for *Nme_2Cas9*) on each end of the left and right homology arms. We used *stbl3 Escherichia coli* bacteria (New England Biolabs, C3040) for all rAAV plasmid transformations and confirmed all rAAV plasmids used in our study both by Sanger sequencing (Genewiz) and long read sequencing (Plasmidsaurus). All PCRs were carried out using the PrimeSTAR max PCR master mix (Takara Bio), using primers listed in Table S1.

Guide-RNA design and screening

The striped mouse genome was previously sequenced and annotated.[24] For each genomic locus of interest, we designed 5–10 sgRNAs using the CRISPOR tool (http://crispor.tefor.net).[27] Only sgRNAs with zero predicted off-target sites were selected. Off-target sites are defined by genomic sequences that contains zero mismatches with the on-target sites over the 10 bp PAM-proximal end, and 4 or less mismatches spanning over the 14 bp PAM-distal end. This stringent definition allows selection of sgRNAs that are highly specific and have a low tolerance for off-target hybridization. To screen and select the sgRNA with highest targeting efficacy, we cloned each candidate into the AAV.*Nme_2Cas9*.sgRNA plasmid and performed transient transfections into immortalized fibroblast striped mouse cells, followed by T7 Endonuclease I (T7E1) assays (described below).

T7E1 assay

Target-specific primers (listed in Table S1) were used to PCR amplify genomic regions of 600–1200 bp length containing sgRNA recognizing site, using genomic DNA extracted from cells or animal tissues. PCR amplicons were reannealed by heating to 95°C and then gradual cooled at a rate of −0.1 °C/s. Re-annealed PCR products were treated with T7E1 (New England Biolabs, E3321) at 37°C for 15 min before terminating the reaction by adding 2M EDTA (per 20 μl reaction). The final products were purified and resolved on 1.5% agarose gels containing SYBR safe (Thermo Fisher Scientific, S33102). Gel images were taken for band analyses.

Production of rAAV6

All rAAV6 were produced by the Princeton Neuroscience Institute (PNI) viral core facility at Princeton University. HEK293 cells were co-transfected with three plasmids (transfer plasmid, Rep/Cap serotype plasmid, and adenovirus helper plasmid) using PEIpro (Polyplus-transfection, 101000033). After 48 h, rAAVs were collected, concentrated, and purified by iodixanol gradient ultracentrifugation. Concentrated rAAVs were quantified by quantitative-PCR using previously optimized probes.[54] Purity was confirmed by silver staining of viral samples after SDS-gel electrophoresis.

Ex vivo experiments in laboratory mouse embryos

CD-1 (IGS) (Charles River Laboratories) female mice of 6–8 weeks were superovulated by intraperitoneally injecting 5 IU of pregnant mare serum gonadotropin (ProSpec, HOR-272) and, 47 h later, 5 IU human chorionic gonadotropin (EMD Millipore, 230734). Immediately following the second injection, females were paired with CD-1 males. We collected zygotes from the oviducts at 0.375 dpc and briefly incubated them in M2 media (CytoSpring, M2114) containing 300 μ g ml^{-1} hyaluronidase (EMD Millipore, HX0514) to dissociate from cumulus cells. Zygotes were then rinsed three times in KSOM media supplemented with Amino Acids and BSA ($KSOM^{AA+BSA}$) (CytoSpring, K0104). Subsequently, zygotes were incubated in droplets of 20 μ l $KSOM^{AA+BSA}$ containing rAAVs, covered with mineral oil (EMD Millipore, ES-005-C), and incubated for 2 or 4 h at 37°C and 5% CO_2. rAAV exposed zygotes were then rinsed in $KSOM^{AA+BSA}$ media seven times and incubated until the blastocyst stage (~4 days) in fresh $KSOM^{AA}$ at 37°C and 5% CO_2.

Single blastocyst analysis

We used a micropipette to collect a single blastocyst in less than 0.5 μl $KSOM^{AA+BSA}$ and placed it in 5 μl embryo lysis buffer containing 125 μ g ml^{-1} proteinase K (New England Biolabs, P8107), 100 mM Tris–HCl (pH 8.3), 100 mM KCl, 0.02% gelatin, 0.45% Tween 20, and 60 μ g ml^{-1} yeast tRNA. The blastocyst was lysed by incubating at 56°C for 10 min followed by proteinase K inactivation at 95°C for 10 min.[55] To analyze the presence of targeted knock-in in each blastocyst, we carried out junction PCR using two primer pairs. For each pair, one primer annealed to the distal region flanking the 3' or 5' homology arms and the other primer annealed to the inserted fragment. Note that an additional third PCR using the two distal genomic primers was conducted as a control to detect the wild-type allele. This PCR, however, is not necessarily capable of detecting the presence or the percentage of the inserted allele due to allelic dropout bias. Therefore, a total three PCR amplification reactions were carried out for the analyzing each blastocyst. We used 0.5–1 μl crude DNA extract per 10 μl PCR reaction. PCR amplicons were resolved on 0.8–1.2% agarose gels. Gel bands were

cut, purified, and Sanger sequencing was used to verify precise and on-target knock-in. All PCRs were conducted using PrimeSTAR max PCR master mix (Takara Bio, R045A) using primers listed in Table S1.

In vivo intra-oviductal delivery of rAAVs

The intra-oviductal injection procedure used here was adapted from a technique that has been used in laboratory mouse[20] and implemented in striped mice as previously described.[24] Briefly, we made incisions to the skin/muscle tissue, exposed the oviduct, and injected 0.5–1 μ L rAAVs (1-3× 10^9 viral genomes (vg)) into each oviduct using a glass micropipette with tip diameter of 15–30 μ m. After injection, we gently returned the oviduct into coelom, sutured the overlying musculature, and closed the incision using surgical staples. Females were closely observed until they were awake, placed back into the cage, and monitored for subsequent days until they gave birth.

Striped mice genotyping and mosaicism analysis

We collected ear punches from each striped mouse and extracted genomic DNA using the Zymo Quick DNA Miniprep Plus Kit (Zymo Research, D4069). To analyze the composition and frequency of targeted indels, we used forward and reverse primers annealing to genomic regions located at least 600 bp upstream and downstream of the sgRNA targeting site. This allowed us to PCR-amplify genomic regions potentially containing large deletions. PCR amplicons were purified using the DNA Clean and Concentrator Kit (Zymo Research, D4014) and analyzed by using two approaches: First, using Inference of CRISPR Edits (ICE),[53] we analyzed the Sanger trace data to predict potential indels as well as their likely identity and frequency in each animal. Second, purified PCR products were cloned into a pSC-amm/kan vector using the StrataClone PCR Cloning Kit (Agilent Technologies, 240205). We picked and sequenced 50 single colonies per F0 animal to determine the presence, composition, and frequency of targeted mutations. To analyze the presence of targeted KI, we extracted genomic DNA and performed junction PCR. PCR amplicons were analyzed by agarose gel electrophoresis and bands were recovered from gels, purified, and Sanger sequenced. To analyze the frequency of on-target inserted alleles as well as the potential presence of indels in the same F0 animals, purified PCR products were cloned into the pSC-amm/kan vector and at least 50 single colonies per F0 individual were screened by Sanger sequencing.

Germline transmission of knock-out and knock-in alleles

Genotype-confirmed F0 animals were bred with age and weight matched wild-type animals. Derived offspring was genotyped and analyzed for germline transmission rates by calculating the percentage of F1 heterozygous pups in all derived F1 animals. F1 heterozygous females and males were then crossed and the F2 progeny was genotyped by sequencing to confirm that edited alleles were transmitted following expected Mendelian ratios.

Digital droplet PCR

To assess transgene copy number in F1 transgenic mice, we performed digital droplet PCR (ddPCR) on genomic DNA from representative F1 mice, as well as their wild type littermates. ddPCR reactions were assembled using 60ng gDNA template in QX200 ddPCR EvaGreen Supermix (Bio-Rad) and droplet formation was performed using an AutoDG Automated Droplet Generator (Bio-Rad). Upon droplet formation, samples were transferred to a C1000 Touch Thermal Cycler (Bio-Rad) and cycled for 40 cycles. Following amplification, DNA target copies were measured and analyzed using the QX200 Droplet Reader and QuantaSoft Software (Bio-Rad). Target copy number was determined by dividing the insertion concentration (number of copies per uL of reaction) by the concentration of the reference gene *Axin2*, which is present as two copies per cell. One F1 pup and one wildtype littermate were analyzed for each insertion.

Primers are listed in Table S1.

Immunofluorescence

Striped mice were anesthetized with 100 mg kg^{-1} ketamine (Covetrus) and 10 mg kg^{-1} xylazine (Covetrus) and administered with 0.1 mg kg^{-1} buprenorphine (Covetrus) and 5 mg kg^{-1} ketoprofen (Covetrus) analgesics. We transcardially perfused the mice with PBS followed by ice-cold 4% paraformaldehyde (PFA) (Thermo Fisher Scientific, 23-730-571). Organs were collected, additionally fixed in 4% PFA for 30 min, allowed to sink overnight in 30% sucrose, and embedded in Tissue-Plus O.C.T. compound (Fisher Healthcare). Embedded blocks were cryosectioned at a thickness of 12 μ m in a Leica 350S cryostat. Tissue sections were permeated with PBS containing 0.1% Triton X-100 for 10 min, blocked with 3% BSA in PBS containing 0.01% Triton X-100 (PBST), and incubated in mCherry antibody (1:500 dilution in PBST; Novus biologicals, NBP2-25157, RRID: AB_2753204) overnight at 4°C. Sections were washed three times with PBST, incubated in PBST containing 1:500 diluted Alexa 555 donkey anti-rabbit secondary antibody (Thermo Fisher Scientific, A-31572, RRID: AB_162543) and 0.2 μ g ml^{-1} DAPI (Thermo Fisher Scientific, D1306) for 1 h at room temperature, washed three times with PBST, mounted with Fluoromount-G (Southern Biotech, 0100-01) and imaged using a Nikon AX/AXR Confocal microscope.

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Supplemental information

TIGER: Single-step *in vivo* genome editing in a non-traditional rodent

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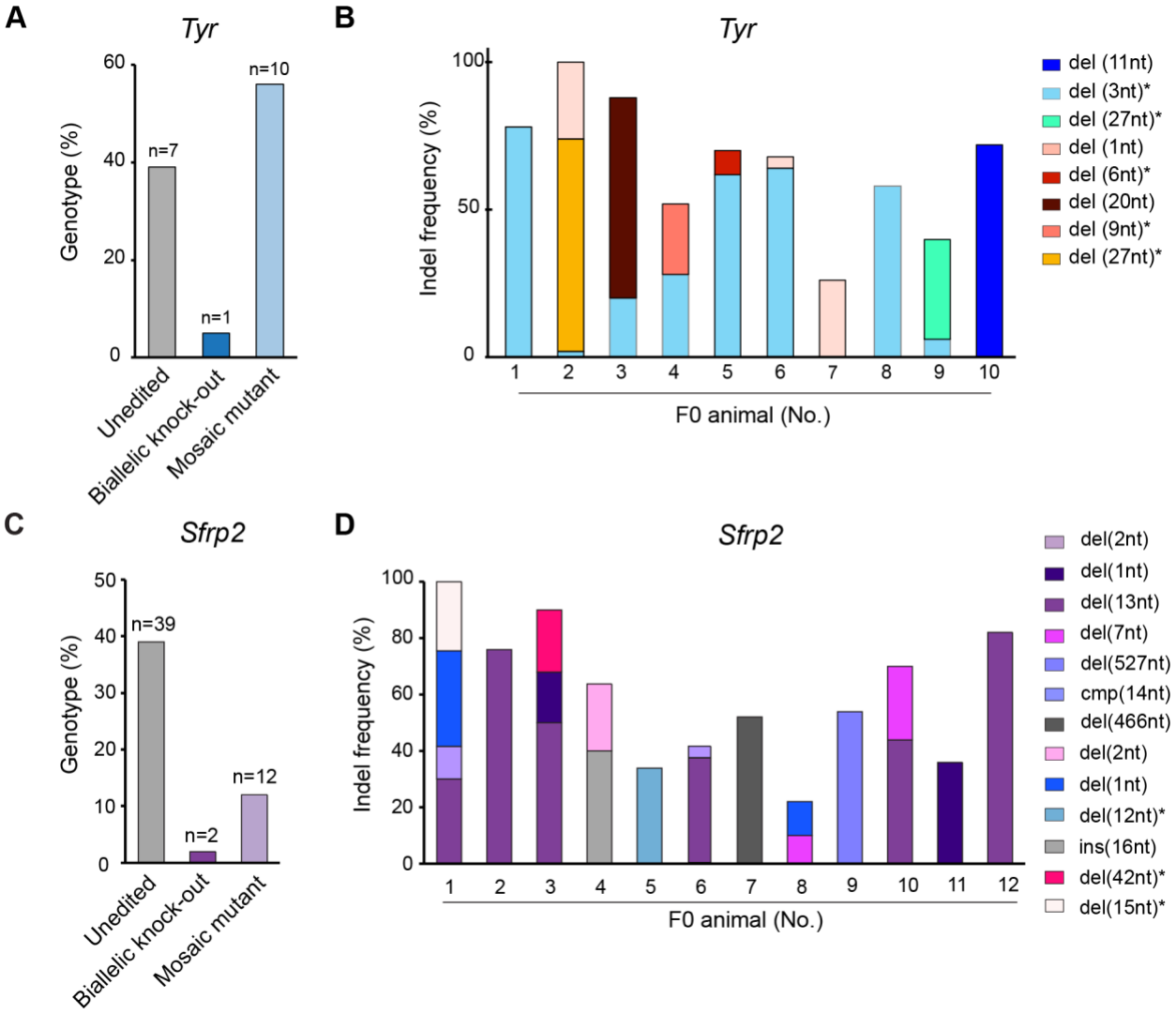
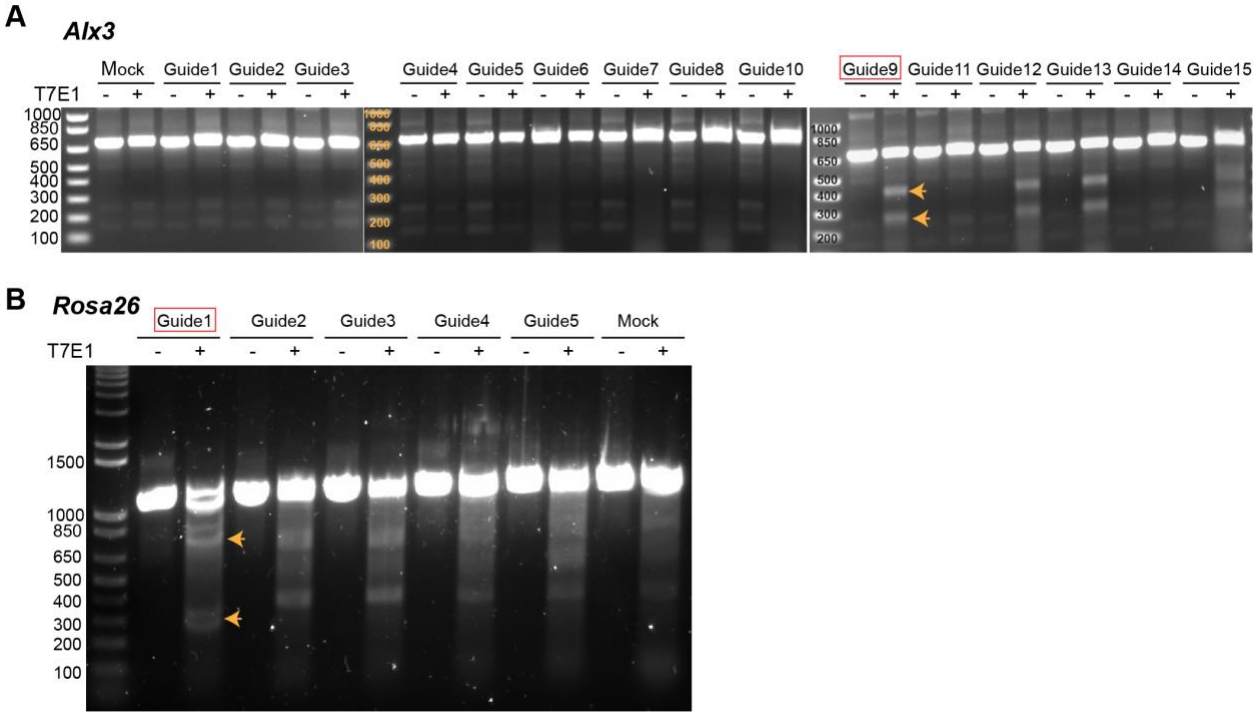
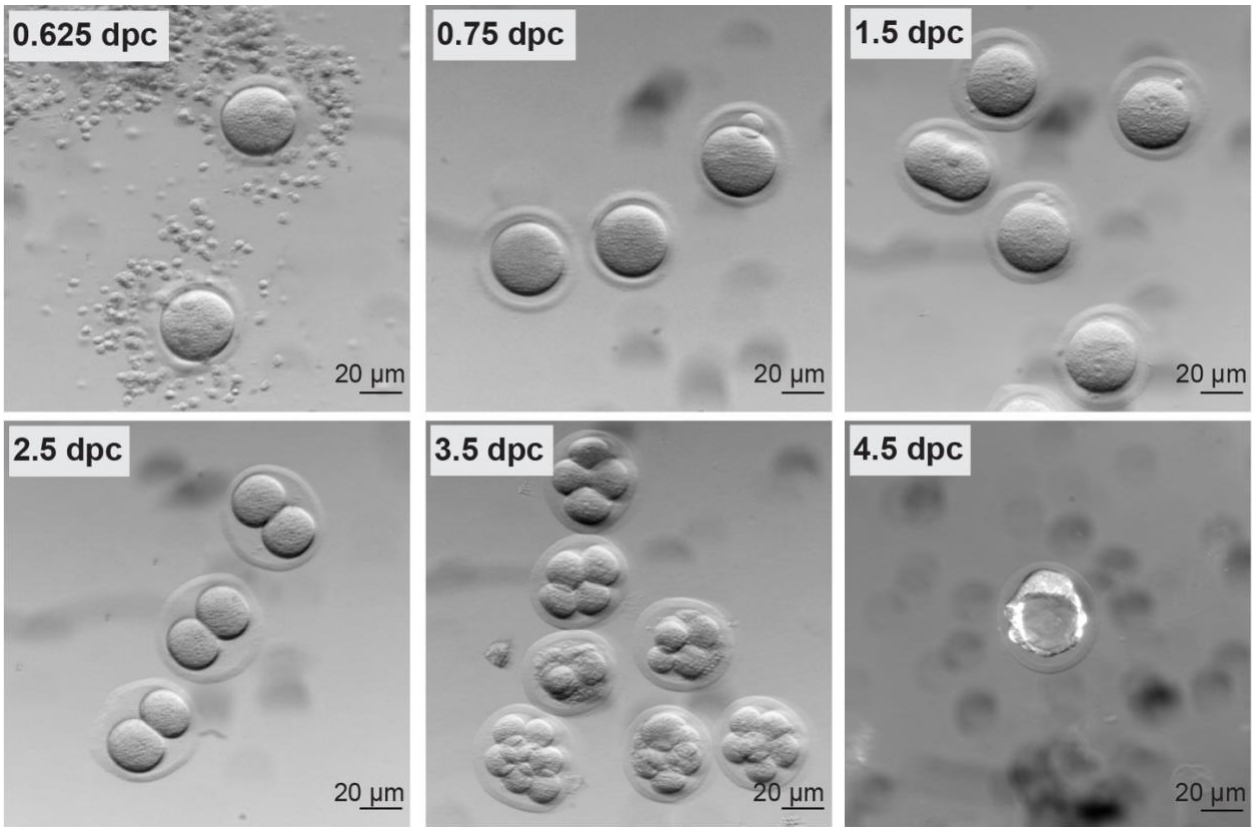


Supplementary Fig. 1. Knockout (KO) in striped mice using TIGER. Related to Figure 1. (A-F) Experimental design and results for the *Tyr* and *Sfrp2* KO experiments in striped mice. **(A), (C),** Bar graphs showing the percentage distribution and number of unedited, bi-allelic mutant, and mosaic mutant pups delivered by injected female striped mice. **(B), (D),** Stacked histogram showing the frequency (percentage distribution) of mutant alleles detected in mutant mosaic F0 stripe mice. Each bar represents an individual F0 striped mouse. Distinct indel mutant alleles are color-coded. For example, del (2nt), ins (16nt), and cmp (14nt) in (**D**) denote respectively a 2 bp deletion (del), a 16 bp insertion (ins), and a 14 bp compound (cmp) mutation. A compound mutation is a combination of a nucleotide substitution, deletion, and/or insertion at the targeting site. Asterisks in (B) denote cases in which the mutation does not lead to a frame shift.

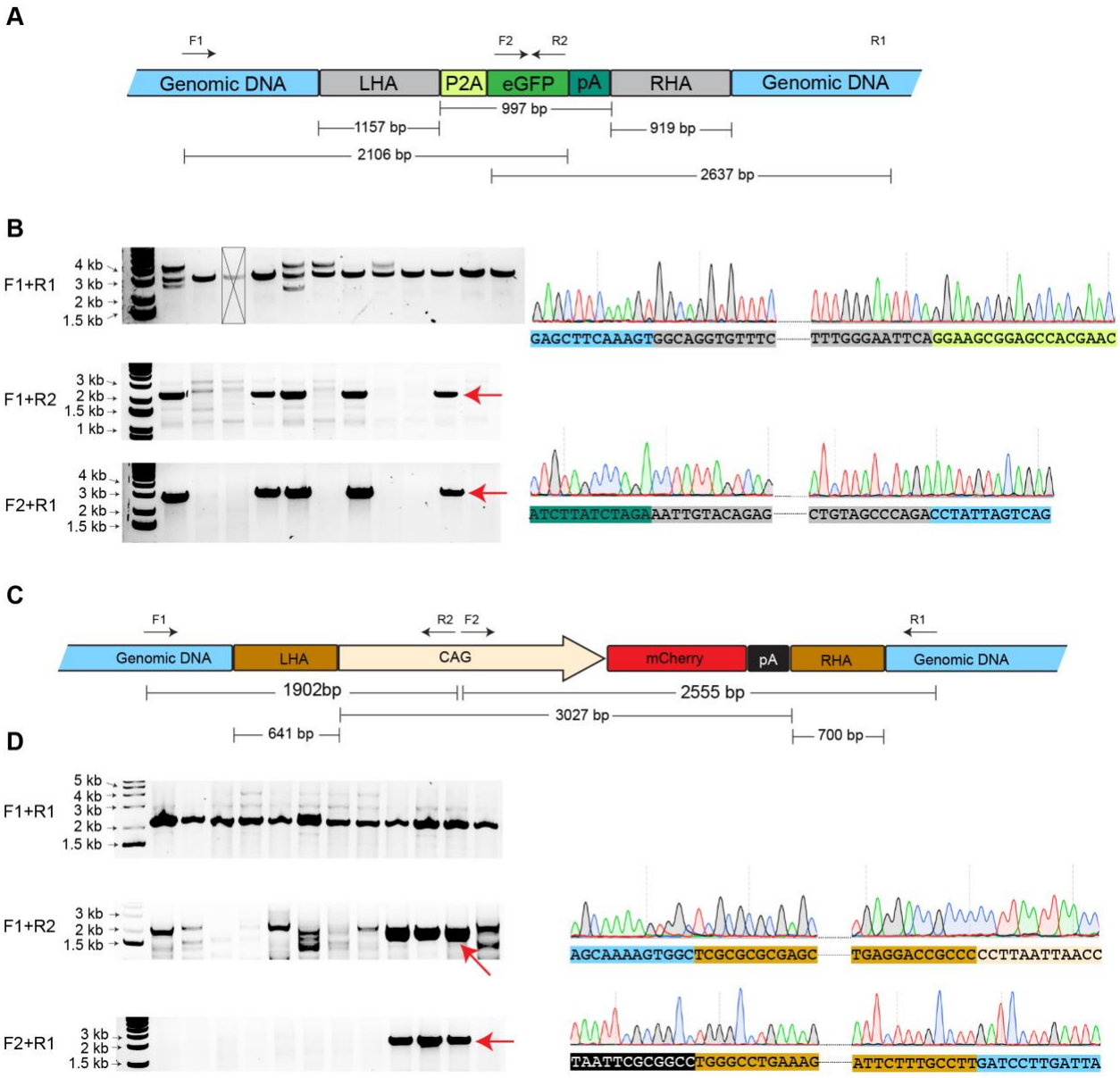


Supplementary Fig. 2. T7E1 assays in striped mouse dermal fibroblasts. Related to Figure 1 and Figure 4.

(A-B) Representative gel images for *in vitro* assays used to evaluate the efficacy of different candidate sgRNAs for *Alx3* (**A**) and *Rosa26* (**B**). Arrowheads show bands of expected size following T7E1 cleavage at the on-target site. Guide9 for *Alx3* (**A**), and Guide1 for *Rosa26* (**B**) were chosen for downstream *in vivo* experiments (red boxes). Note that in (**A**), two bands (~150 bp and 250 bp) corresponding to non-specific PCR amplicons are present in all T7E1 treated and untreated samples.

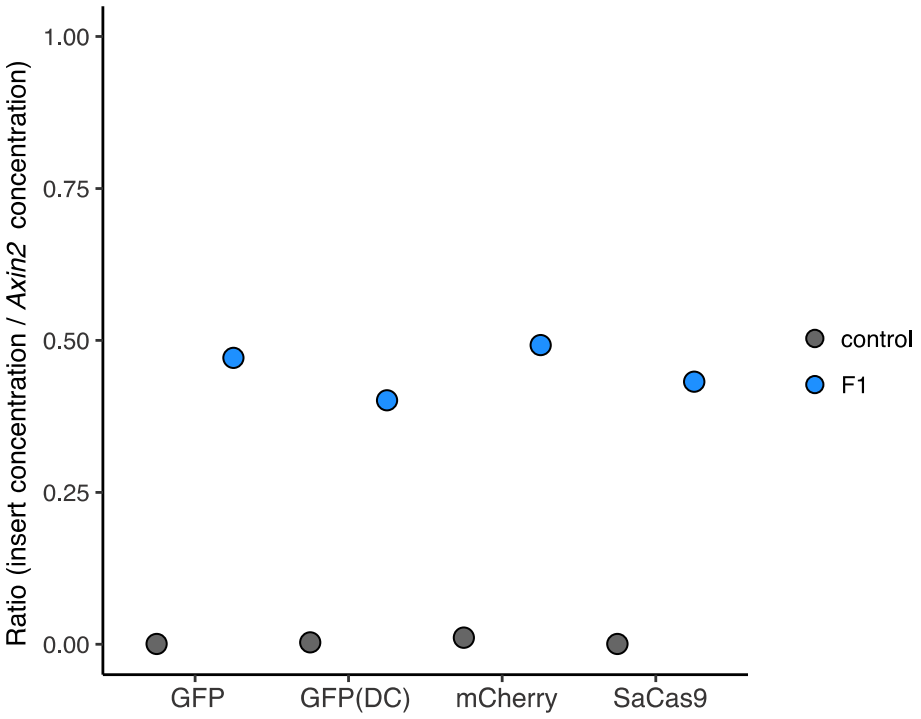


Supplementary Fig. 3. Characterization of pre-implantation embryos in African striped mice. Related to Figure 1 and Table S2.
Representative images of pre-implantation striped mouse embryos collected from multiple developmental stages. dpc = days post coitum.



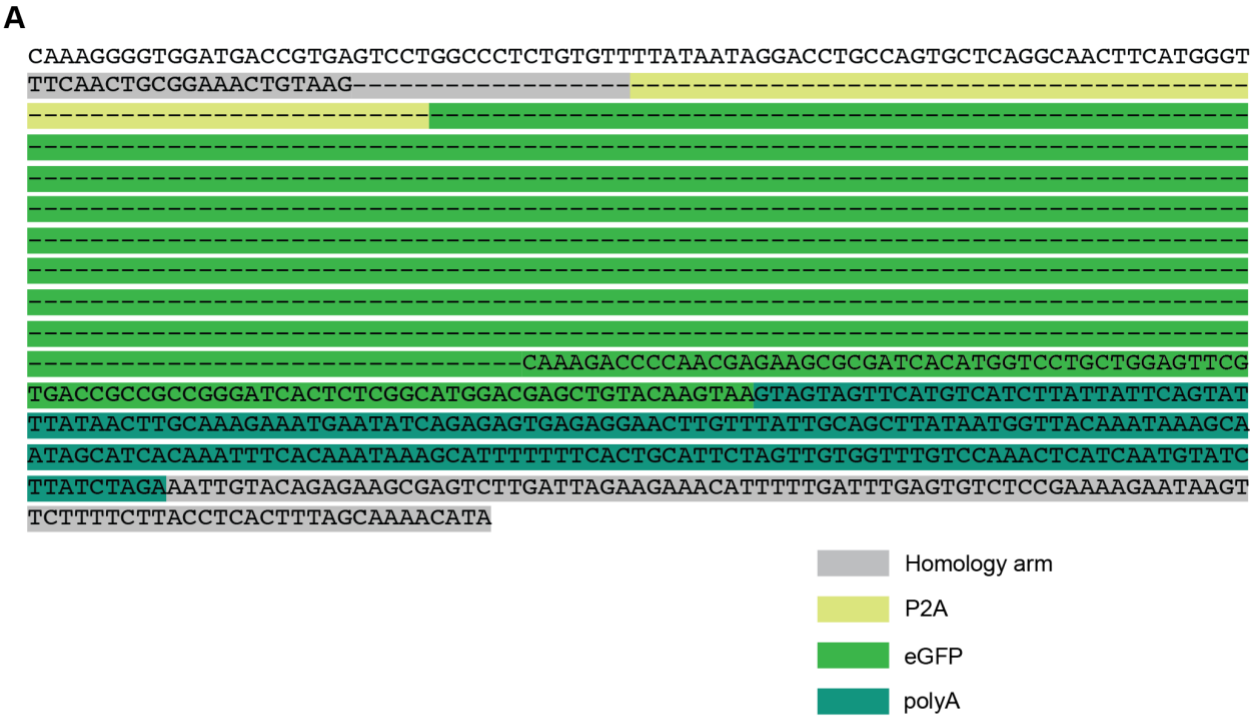
Supplementary Fig. 4. TIGER-mediated on-target integration in striped mice. Related to Figure 2 and Figure 4.

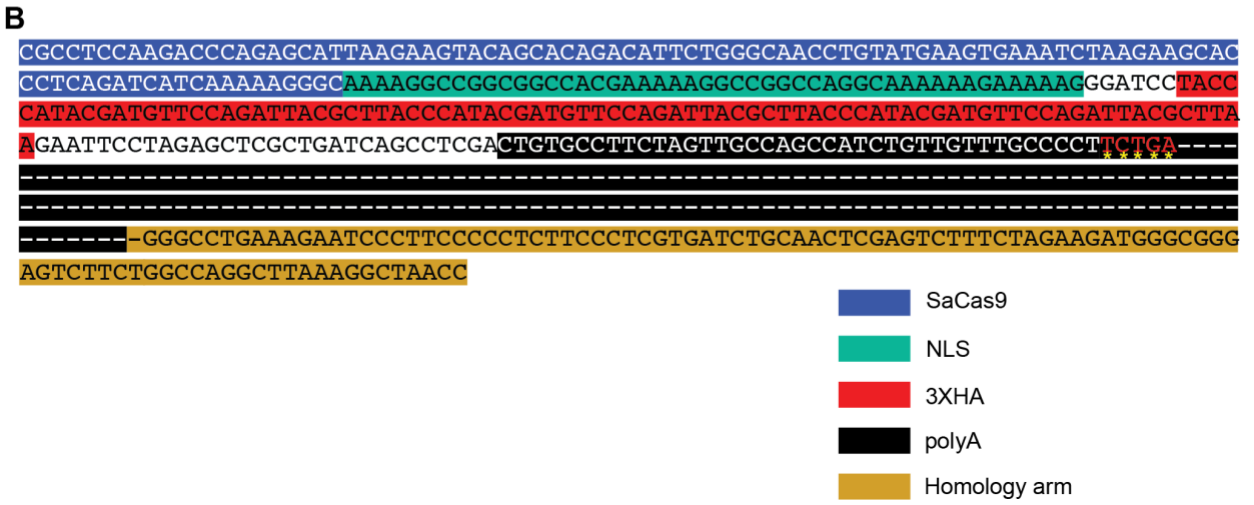
A-D, Analyses of two different on-target integration experiments. **(A), (C),** Schematic of the *eGFP* expressing cassette integrated into the *Tyr* locus **(A)** and the *CAG::mCherry* expressing cassette integrated into the *Rosa26* locus **(B)**. Shown are the sizes of the cassettes, the right and left homology arms (RHA and LHA), as well as primer locations (arrows) and expected product sizes for the different Forward (F) and Reverse (R) primers used in junction PCR. **(B)**, **(D),** Detection of knock-in in pups derived from injected females via junction PCR and Sanger sequencing. Crossed lane on the gel shown in **(B)** represents a loading error. In **(B)** and **(D)**, junction PCR amplicons resolved on agarose gels are shown on the left. Red arrows indicate positive amplification. Corresponding sequencing chromatograms are shown on the right.



Supplementary Fig. 5. Copy number analysis of the four insertions performed in our study. Related to Figure 2, Figure 3, and Figure 4.

Digital droplet PCR on representative F1 individuals and wildtype control littermates was used to determine the concentration of the insert relative to the concentration of *Axin2*, a gene that is present in two copies in the striped mouse genome. In all cases, the copy number of the insertion is approximately half of the concentration of *Axin2*, confirming the presence of a single copy in F1 animals.





Supplementary Fig. 6. Sanger sequencing identified truncated insertions in F0 striped mice generated using the double cleaving rAAV template. Related to Figure 3 and Figure 4. **(A)** Insertion of a truncated P2A-eGFP-polyA sequence into the *Tyr* locus. **(B)** Insertion of a truncated EF-1α-NLS-SaCas9-NLS-polyA sequence into the *Rosa26* locus. Asterisks (*) denote indel mutations and different portions of the sequences are highlighted in color.

Supplementary Table 1. Primers used in this study. Related to STAR Methods.

Supplementary Table 1 is provided as an excel spreadsheet.

Supplementary Table 2. Characterization of the developmental stages seen in striped mouse pre-implantation embryos. Related to Figure S2.

Collecting time[1]	Plugged females	Recovered embryos[2]	Cumulus-enclosed embryos[2]	Developmental status
0.375 dpc	2	9	100% (9/9)	1-cell embryo
0.5 dpc	3	14	100% (14/14)	1-cell embryo
0.625 dpc	5	18	72.2% (13/18)	1-cell embryo
0.75 dpc	5	15	33.3% (5/15)	1-cell embryo
0.875 dpc	3	16	13.3% (2/15)	1-cell embryo
1.5 dpc	3	16	0% (0/16)	1-cell to 2-cell transition
2.5 dpc	3	7	0% (0/7)	2-cell embryos
3.5 dpc	3	8	0% (0/8)	4-cell or 8-cell embryos
4.5 dpc	3	5	0% (0/5)	morulae or blastocysts

[1]12:00am on the day plug detected is designated as 0 dpc, e.g., 0.375 dpc corresponds to 9 am on the plug day.
[2]All fertilized and unfertilized ova, as well as empty zona pellucida were included.

References

1. Edraki, A. *et al.* A Compact, High-Accuracy Cas9 with a Dinucleotide PAM for In Vivo Genome Editing. *Mol. Cell* **73**, 714-726.e4 (2019).