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Multistep allelic conversion in mouse pre-implantation embryos by AAV vectors

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Site-specific recombinases (SSRs) are critical for achieving precise spatiotemporal control of engineered alleles. These enzymes play a key role in facilitating the deletion or inversion of loci flanked by recombination sites, resulting in the activation or repression of endogenous genes, selection markers or reporter elements. However, multiple recombination in complex alleles can be laborious. To address this, a new and efficient method using AAV vectors has been developed to simplify the conversion of systems based on Cre, FLP, Dre and Vika recombinases. In this study, we present an effective method for ex vivo allele conversion using Cre, FLP (flippase), Dre, and Vika recombinases, employing adeno-associated viruses (AAV) as delivery vectors. AAVs enable efficient allele conversion with minimal toxicity in a reporter mouse line. Moreover, AAVs facilitate sequential allele conversion, essential for fully converting alleles with multiple recombination sites, typically found in conditional knockout mouse models. While simple allele conversions show a 100% efficiency rate, complex multiple conversions consistently achieve an 80% conversion rate. Overall, this strategy markedly reduces the need for animals and significantly speeds up the process of allele conversion, representing a significant improvement in genome engineering techniques.

Keywords AAV, 3R, Gene delivery, Flp/FRT, IVF, Site-specific recombinase

Site-specific recombinase (SSRs) systems represent powerful tools in genetic engineering and molecular biology, enabling precise spatiotemporal manipulation of DNA sequences. The two most widely employed systems are the Cre/*loxP* system, where Cre recombinase binds *loxP* sites, and the FLP/*FRT* system, which involves Flpo recombinase (a mammalian codon-optimized thermostable version of the FLP gene) in the conversion of FRT sites in mammalian genomes. In addition, alternative systems such as Dre/*rox* and Vika/*vox* are part of this SSR toolbox. All these systems facilitate site-specific recombination, allowing for the excision, inversion, insertion, or exchange of DNA segments[1].

The Cre/*loxP* system is the major genetic tool used for various genetic manipulations, including gene knockout, conditional gene inactivation, and transgenic cassette rearrangements[2,3]. The Cre/*loxP* system, in combination with FLP/*FRT*, Dre/*rox* or Vika/*vox,* allows for complex genetic manipulation[4]. The inclusion of Dre and Vika systems has further enriched the toolkit for combinatorial use, offering enhanced flexibility and precision in genetic manipulations[5]. Despite its limited application, the Vika/*vox* system has demonstrated suitability as an alternative or a combinatorial partner with other site-specific recombinase (SSR) systems in vivo, as demonstrated by Karimova et al.[6,7]. The Cre/*loxP* and Dre/*rox* systems are widely combined for various purposes, as shown, for example, in vivo lineage tracing[8,9]. Similarly, the combination of Cre/*loxP* and FLP/*FRT* systems serves as the foundation for gene manipulation using the EUCOMM/KOMP knockout first alleles[3].

The EUCOMM/KOMP "knockout-first" allele utilizes strategic placing of recognition sequences in the vicinity of critical exons and gives the gene of interest conditional properties. This enables functional ablation of a target gene in a spatiotemporal manner. EUCOMM/KOMP alleles find extensive use in generating genetically modified rodents with specific gene reporters, knockouts, or conditional knockouts based on the applied SSR[3,10]. EUCOMM/KOMP employs promoter-driven targeting selection cassettes to create a versatile 'Knockout-first allele' in C57BL/6N embryonic stem cells[3,10]. This innovative strategy hinges on identifying a 'critical' exon

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present in all transcript variants. In its final form (tm1b or tm1d), the deletion of the exon induces a frame-shift mutation and thus forms the KO-first allele. Notably, the allele exhibits flexibility, allowing for the generation of reporter knockouts (tm1a), conditional knockouts (tm1c), and null alleles through exposure to site-specific recombinases such as Cre and FLP.

The 'knockout-first' allele (tm1a) contains an *IRES:lacZ* trapping cassette and a floxed promoter-driven neo cassette inserted into the intron of a gene, disrupting gene function. FLP converts the 'knockout-first' allele to a conditional allele (tm1c), restoring gene activity. Cre deletes the promoter-driven selection cassette and floxed exon of the tm1a allele to generate a *lacZ*-tagged allele (tm1b) or deletes the floxed exon of the tm1c allele to generate a frameshift mutation (tm1d), triggering nonsense mediated decay of the deleted transcript[1,3].

In our previous study, we demonstrated the efficient allele conversion facilitated by electroporation of Cre protein in both zygotes and primary cells. The method was proven to be also compatible with in vitro fertilization procedures without toxic effects on zygotes[11]. To advance the recombinase technology toolbox, we sought to expand it with FLP/*FRT*, Dre/*rox*, and Vika/*vox*, systems and establish a straightforward allele conversion protocol based on AAV delivery without the time and animal consumption, associated with multiple crossings.

Persistent difficulties in the synthesis of FLP (specifically Flpe) and Vika proteins led us to explore alternative recombinase delivery methods. We packaged Flpo and Vika CDS under the control of the *CMV* promoter in recombinant Adeno-Associated Virus vectors (AAV). The delivery in the form of AAV (serotype 1) relies on the virus's natural tropism to zygote's membrane moieties, eliminating the need for electroporation[12,13], and circumvents the issues with protein expression, folding, and overall function. Therefore, this approach does not subject the embryos to physical stress.

In this study, we present a new efficient approach to allele conversion using recombinases delivered via AAVs, representing an attractive alternative to electroporation or mRNA pronuclear microinjection. AAV vectors demonstrated high conversion efficiency in both embryos and adult animals, derived from the MuX reporter mouse. For single-allele conversion, all tested recombinases can reach 100% conversion rate, if optimal titer is used. In addition, we introduce a new approach to multi-level conversion of the EUCOMM/KOMP allele. Our method enables the conversion from tm1a to tm1d in a single animal during IVF-based reanimation/rederivation from sperm, resulting in 80% of fully-converted animals with relatively low AAV vector persistence. These results underscore the spatiotemporal potential of AAV SSR-based conversion and emphasize the reduction of animal consumption in line with 3R principles.

Materials and methods

Construction of mRNA template and AAV SSR transfer plasmids

Vectors were constructed using In-Fusion cloning (Takara Bio) method. Oligonucleotides are shown in Table of Primers (supplementary file) and cloning details are available upon request. In brief, codon-optimized Dre sequence was amplified from pCAG-NLS-HA-Dre (Addgene plasmid #51272) with CDS Dre fw and rv primers, and cloned into pET-Cre-6xhis using *NcoI* and *BamHI* restriction sites. The same approach was used with Flpo and Vika sequence. Vika sequence was amplified form pNPK-NLS-Vika (Addgene plasmid #79969) using CDS Vika fw and rv primers. Flpo sequence was amplified form pCAG-Flpo (Addgene plasmid #60662) using CDS Flpo fw and rv primers. Cloning of the corresponding sequences into pET-Cre-6xhis gave rise to pET-Dre-6xhis, pET-Vika-6xhis pET-Flpo-6xhis, respectively.

The Flpo gene is a mammalian codon-optimized version of the Flpe gene (enhanced flippase), which is derived from the original FLP protein isolated from *Saccharomyces cerevisiae*. Modifications to the FLP coding sequence produced an 'enhanced', more thermostable FLP enzyme, Flpe. Flpe works more efficiently in mice and mammalian cells, but it is generally less efficient than Cre-lox. Further sequence changes to the Flpe recombinase produced 'Flpo', a mouse codon-optimized version whose recombination efficiency approaches that of Cre. In this work, we used molecules/vectors carrying the mammalian codon-optimized variant of the Flpe gene (Flpo), and therefore, we refer to it as such[14]. To clone pAAV-CMV version of recombinase plasmids using In-Fusion cloning (Takara Bio) method, codon-optimized Cre sequence was amplified from pCAG-Cre-IRES2-GFP (Addgene plasmid #26646) with AAV CDS Cre fw and rv primers and cloned into pAAV-GFP Control Vector (AAV-400, Cellbiolabs Inc, San Diego, CA) using *EcoRI* and *XbaI* restriction sites. Similarly, but using *EcoRI* and *HindIII* restriction sites, pAAV CMV-Dre, pAAV CMV-Vika and pAAV CMV-Flpo were constructed. Dre sequence was amplified from pET-Dre-6xHis using AAV CDS Dre fw and rv. Vika sequence was amplified from pET-Vika-6xHis using AAV CDS Vika fw and rv. Flpo sequence was amplified from pET-Flpo-6xHis using AAV CDS Flpo fw and rv. Sequence of used primers is available in Table of Primers (supplementary file). All assembled plasmids generated in this study will be deposited in the Addgene repository to facilitate their availability to the scientific community.

pET-Cre-6xHis was a gift from David Liu (Addgene plasmid #62939). pAAV-EF1a-Flpo was a gift from Karl Deisseroth (Addgene plasmid #55637, Addgene viral prep #55637-AAV1). pCAG-NLS-HA-Dre (Addgene plasmid #51272) was a gift from Pawel Pelczar. pCAG-Flpo (Addgene plasmid #60662) was a gift from Massimo Scanziani. pNPK-NLS-Vika (Addgene plasmid #79969) was a gift from Frank Buchholz. pCAG-Cre-IRES2-GFP (Addgene plasmid #26646) was a gift from Anjen Chenn.

Production of adeno-associated virus and titration

The production of recombinant AAV SSR vectors [AAV-CMV-Cre (Cre), AAV-CMV-Dre (Dre), AAV-CMV-Flpo (CFlpo), AAV-CMV-Vika (Vika)] involved steps as follows: First, 293AAV cells (AAV-100, Cellbiolabs) were cultivated in DMEM (Sigma Aldrich, D6429) supplemented with 10% fetal bovine serum (Invitrogen, 10437028) and 1% penicillin/streptomycin (GIBCO, 15140122) at 37 °C with 5% CO_2. To generate recombinant AAV vectors, the 293AAV cells underwent a triple transfection process with a Helper plasmid (340202, Cellbiolabs), Rep/

Cap plasmid pAAV2/1 (Addgene plasmid #112862), and a transfer plasmid. The transfection was carried out using polyethylenimine (PEI) (Polysciences, 24765-1) following established protocols[15]. Purity of AAV particles was assessed by Ag-staining using the PierceTM Silver Stain Kit (24612, Thermo Scientific) following the manufacturer's protocol. The DNase-resistant viral genomic titers were determined via qPCR using universal AAV ITR primers (AAV ITR fw and rv) in combination with LightCycler® 480 SYBR Green I Master (Roche) and the LightCycler® 480 System (Roche) following the method described in Aurnhammer[16]. pAAV-CMV-Cre plasmid was used to prepare a standard curve for the absolute quantification of AAV genome copies of purified samples.

Production of mRNA

Cre, Dre, Flpo, and Vika mRNA were transcribed and tailed using the mMessage mMachine Kit (AM1344, Invitrogen) following the manufacturer's protocol from pET-Cre-6xHis (Addgene plasmid #62939), pET-Dre-6xHis, pET-Flpo-6xHis, pET-Vika-6xHis plasmids after *XhoI* (ER0692, Thermo Scientific) linearization. Further purification was performed using the MEGAclearTM Kit (AM1908, Invitrogen) following the manufacturer's protocol. To assess the efficiency of tailing and mRNA integrity, non-tailed and polyA tailed mRNA were separated on a hydrogen peroxide agarose gel following the method described by Pandey and Saluja[17].

Production of Cre and Dre proteins

The Cre (5000 ng/µL) protein was expressed and purified as described by Jenickova[11]. The Dre-6xHis fusion protein was heterologously expressed in *E. coli* BL21-Codon Plus (DE3)-RIPL cells. Briefly, cells were grown in the LB medium supplemented with 0.2% glucose (Sigma) and 100 µM Ampicillin (Sigma) at 37 °C overnight. The following day, cells were diluted 1000-fold into LB medium + Ampicillin and cultured to the OD600 = 0.6 at 37 °C. Subsequently, incubation temperature was lowered to 18 °C, and the recombinant protein expression was induced by the addition of 0.5 mM IPTG; cultivation continued at 18 °C overnight. After the incubation period, cells were harvested by centrifugation (5500G, 12 min) and resuspended in the lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, 20 mM imidazole, pH 8.0) supplemented with EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Cells were disrupted using a high-pressure homogenizer (Avestin) and the lysate cleared by centrifugation steps at 7200G (15 min) and 40,000G (30 min). The supernatant was loaded onto a Ni–NTA column (Ni–NTA Superflow, IBA, Germany), washed with the lysis buffer and the Dre-6xHis fusion eluted with the lysis buffer supplemented with 200 mM imidazole. To minimize the presence of contaminating DNA, the pooled elution fractions were mixed with the equal volume of the HA buffer (100 mM sodium phosphate, 300 mM NaCl, 10% glycerol, 100 mM imidazole, pH 8.0) and loaded onto a hydroxyapatite column (BioRad). The flow-through fraction comprising the Dre-6xHis fusion was pooled, dialyzed against PBS and concentrated to 5.2 mg/mL.

Animals

C57Bl/6NCrl (donor) females between the ages of 3–5 weeks were purchased from Charles River. Rosa26-VFRL-EGFP (also known as Multi-reporter mouse line for loci-of-recombination MuX) strain was imported from Max Planck Institute of Molecular Cell Biology and Genetics (Dresden). Crl:CD1 (ICR) surrogate females were purchased from Charles River.

Ethics and compliance statement

Mice were bred in our specific pathogen-free facility (Institute of Molecular Genetics of the Czech Academy of Sciences; IMG). All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, approved by the Animal Care and Use Committee of the Academy of Sciences of the Czech Republic. All experiments in this study were carried out in compliance with the ARRIVE guidelines and the laws of the Czech Republic. Animal protocol (93/2020) was approved by the Resort Professional Commission for Approval of Projects of Experiments on Animals of the Czech Academy of Sciences, Czech Republic.

All mice were euthanized using carbon dioxide (CO_2) inhalation followed by cervical dislocation. CO_2 inhalation was performed at a flow rate that displaced 30% of the chamber volume per minute to minimize distress.

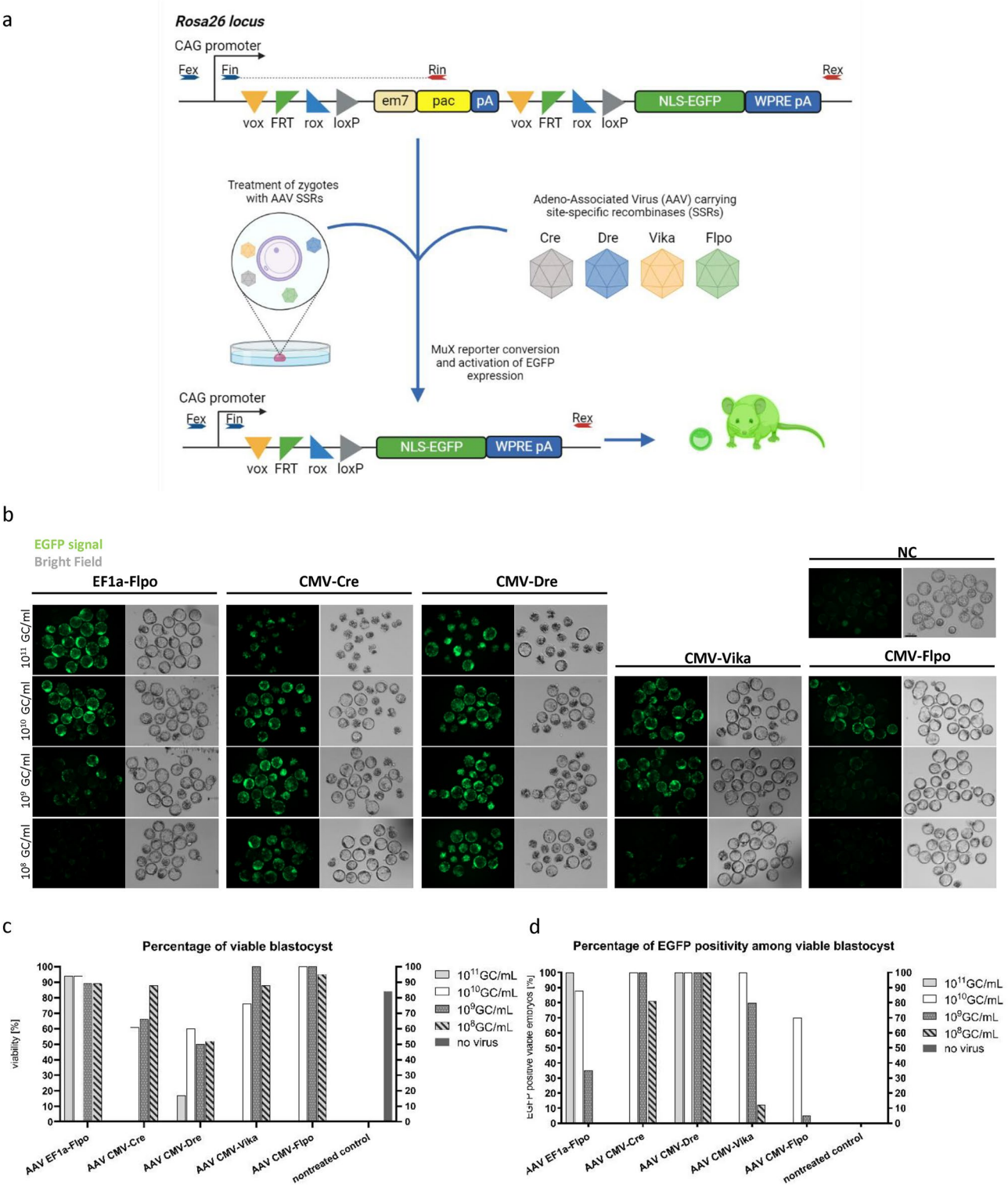
Mouse MuX zygote isolation and AAV treatment

The detailed procedure for hormonal stimulation in mice was previously outlined by Luo[18] and is considered a standard practice at transgenic core facilities. In summary, matured C57Bl/6NCrl females aged 28–35 days underwent hormonal stimulation with 5 IU PMSG. After 46 h, they received an additional 5-IU hCG injection and were subsequently mated with homozygote MuX adult males. Plugged females were selected for zygote isolation from the oviducts using M2 medium. The zygotes were briefly cultured in a drop of EmbryoMax KSOM media, covered with paraffin oil, and placed in an incubator at 37 °C with 5% CO_2.

For zygotes treatment, a specific titer of AAV SSR vectors was added to KSOM media and incubated overnight at 37 °C with 5% CO_2. The next day, 2-cell embryos were transferred to fresh KSOM (without virus) media and later into Crl:CD1(ICR, Charles River) surrogate females or further cultivated until the blastocyst stage (E4.5).

Mouse zygote electroporation and pronuclear microinjection

Zygotes were electroporated using the NEPA21 type II machine (NEPAGENE) and an electroporation slide (CUY501P1-1.5). Only zygotes with two visible pronuclei were selected for electroporation. The electroporation process involved the use of M2 and OptiMEM medium. The Cre or Dre proteins were diluted to a final concentration of 1000 ng/µL in OptiMEM medium prior to electroporation.



Microinjection of SSR mRNA was performed under the microinjection microscope (Leica DMI6000 B) equipped with FemtoJet 4i microinjector (Eppendorf). Only zygotes with visible pronuclei were selected for microinjection. Each mRNA was injected in the concentration 100 ng/μL.

Mouse in vitro fertilization and AAV treatment with Thawed sperm of tm1a strains

The protocol for mouse IVF has been described previously by Luo[18]. In our study, tm1a heterozygous sperms were utilized in conjunction with C57Bl/6N oocytes. The sperm was frozen previously according to the protocol by Takeo and Nakagata[19].

Briefly, C57Bl/6NCrl females (28–35 days old) were hormonally stimulated with 5-IU PMSG, followed by 5-IU hCG 50 h later. Fourteen to fifteen hours after the hCG application, females were sacrificed, and cumulus-oocyte

◂**Fig. 1.** Titration of AAV SSRs on MuX derived zygotes. (**a**) Conversion of Rosa26-VFRL-EGFP (MuX) reporter using AAV Cre, Dre, Vika and Flpo vector: AAV vectors deliver SSRs into MuX derived zygotes, resulting in conversion of the reporter cassette and activation of EGFP expression. Fully-converted embryos and animals show expression of the reporter in all cell types and tissues. Listed SSRs, Cre, Dre, Vika, Flpo recognize and recombine corresponding specific sites *loxP, rox, vox, FRT*, respectively. Fin and Rin detect the presence of the *pac-pA* cassette preventing *EGFP* expression. Fex and Rex primers amplify wild-type allele *Rosa26* without the MuX reporter and serve as internal control. (**b**) MuX-derived zygotes were subjected to decreasing concentrations of AAV SSRs, spanning from 1E + 11 to 1E + 8 GC/mL. Viability and the presence of enhanced green fluorescent protein (EGFP) signal at the blastocyst stage, indicative of stop cassette recombination, were evaluated for each condition. AAV CMV-Vika and CMV-Flpo treatments at a concentration of 1E + 11 GC/mL are not included due to inadequate vector titers. NC: non-treated control. The scale bar (100 µm) in the image of the non treated control serves as a universal reference applicable to all images within this scheme. Table 1 provides an overview of treatment efficiency based on the observed EGFP signal and embryo viability for each tested condition. (**c**) Graph depicting percentage of blastocyst viability based on used titer. (**d**) Percentage of EGFP blastocysts among viable blastocyst. Graphs (**c**) and (**d**) are based on Tab.1. Blastocysts from each treated group were genotyped, see Supplementary Fig. S1 (supplementary data).

complexes were isolated into a fertilization drop (90 µL of high calcium human tubal fluid (HTF) medium with 1 mM L-glutathione (GSH; Sigma-Aldrich)) containing AAV SSR at a concentration of 5E + 10 GC/mL.

Thawed/extracted sperm was capacitated in MBCD medium for 15–20 min. The capacitated sperm was then transferred into the fertilization drop and incubated with oocytes at 37 °C and 5% CO_2 atmosphere for 4 h. Residual sperm cells were washed out, and fertilized oocytes were transferred into the incubation drop of HTF without GSH (total volume 150 µL) enriched by AAV SSR at a concentration of 5E + 10 GC/mL. Fertilized oocytes were incubated with the virus overnight at 37 °C and 5% CO_2 atmosphere.

The next day, 2-cell stage embryos were collected and transferred into the oviduct of a pseudo-pregnant surrogate female. For tm1a-to-tm1d conversion, viable 2-cell stage embryos were transferred into HTF(-GSH) with the AAV CMV-CRE vector (titer: 1E + 10 GC/mL) and incubated for 4 h or until embryo transfer. The whole procedure is summarized in Fig. 3b.

Genotyping

Blastocysts were collected and lysed in lysis buffer (50 mM Tris HCl pH 8, 1 mM EDTA, 0.5% Triton X-100) with proteinase K (R75282, P-LAB) for 30 min at 55 °C; then inactivated at 95 °C for 15 min.

Tissue samples (tail/ear) of all mentioned strains were lysed in lysis buffer (100 mM Tris HCl pH 8, 200 mM NaCl, 5 mM EDTA, 10% SDS) with proteinase K (R75282, P-LAB) overnight at 55 °C, then inactivated at 95 °C for 15 min. Lysates were further processed by phenol:chlorophorm based isolation using Phenol/Chloroform/Isoamylalcohol reagent (A156.2, ROTI) following the manufacturer's protocol.

Multi-reporter mouse line for loci-of-recombination (X) embryos/mice were genotyped using a two primer pairs reaction. The first pair (Fex and Rex) amplifies the *Rosa26* locus without MuX reporter cassette (wild-type allele) with a corresponding product band at 466 bp, the second pair (Fin and Rin) amplifies the non-converted form of the MuX allele generating a 643 bp product. To detect recombination of the MuX allele with these primers, we always used heterozygote animals in the final analysis in order to distinguish the presence of both alleles (wild-type and MuX allele).

All EUCOMM/KOMP converted strains were genotyped for the presence of the wild-type allele, tm1 allele, tm1c allele, and residual *LacZ* cassette. In the case of *Ube2t* and *C4bp* Cre-converted animals, genotyping focused on the tm1d allele too. Further, all AAV SSR treated mice were analyzed for potential AAV genome persistence (episome/genome integration). The sequence of the used primers is available in the Table of primers (supplementary file).

For detailed description of the genotyping of EUCOMM/KOMP strains and the AAV persistence assay protocol, see the Supplementary Method file and Supplementary Fig. S26. Selected founders for each strain were further bred with wild-type C57Bl/6NCrl animals. Genotyping product of these founders were subjected to Sanger sequencing, data available in supplementary file: Supplementary Data Sanger Sequencing of Founder Mice.

Tissue samples imaging

Organs were captured with a Zeiss Axio Zoom V.16 microscope (200 ms exposure; intestine for 100 ms), using a 450–490 nm excitation wavelength and a 500–550 nm emission wavelength, at a total magnification of 11.2×. E4.5 embryos were captured with an exposure time of 400 ms, using a 480 nm excitation wavelength and a 509 nm emission wavelength, at a total magnification of 179.2×. All images were processed in the ZEN 3.0 (blue edition) software.

Results

Optimal viral titer for efficient AAV-mediated conversion with reduced toxicity in reporter mouse zygotes

Heterozygote MuX reporter mice (Fig. 1a) were used to evaluate the delivery efficiency and potential toxicity of AAV-packaged recombinases on zygotes. We assessed recombination efficiency via imaging of EGFP signal, and toxicity based on the calculation of viable embryos. Treated blastocysts were collected and genotyped for the presence or absence of the *pac-pA* cassette (Supplementary Fig. S1).

AAV vector	Titer (GC/mL)	EGFP positive blastocysts		Viable blastocysts		Total no. of blastocysts
EF1a-Flpo	1E + 11	18	100.00%	18	94.70%	19
	1E + 10	16	88.80%	18	94.70%	19
	1E + 09	6	35.30%	17	89.50%	19
	1E + 08	0	0.00%	17	89.50%	19
CMV-Cre	1E + 11	0	0.00%	0	0.00%	18
	1E + 10	11	100.00%	11	61.10%	18
	1E + 09	12	100.00%	12	66.60%	18
	1E + 08	13	81.30%	16	88.80%	18
CMV-Dre	1E + 11	3	100.00%	3	17.60%	17
	1E + 10	9	100.00%	9	60.00%	15
	1E + 09	9	100.00%	9	50.00%	18
	1E + 08	9	100.00%	9	52.90%	17
CMV-Vika	1E + 10	13	100.00%	13	76.50%	17
	1E + 09	16	80.00%	20	100.00%	20
	1E + 08	2	12.51%	16	88.80%	18
CMV-Flpo	1E + 10	14	70.00%	20	100.00%	20
	1E + 09	1	5.00%	20	100.00%	20
	1E + 08	0	0.00%	19	95.00%	20
Negative control	-	0	0.00%	16	84.20%	19

Table 1. Titration of AAV SSRs on MuX derived zygotes.

Distinct viral titers of each AAV recombinase were applied to zygotes, starting at 1E + 11 genomes per milliliter (GC/mL) and descending to 1E + 8 GC/mL. For AAV CMV-Flpo (CFlpo) and Vika, the highest concentration was limited to 1E + 10 GC/mL due to the lower yield of virus titer during production compared to Cre and Dre (as can be referenced in Supplementary Fig. S24).

Despite their substantial conversion efficiency, the high titers of Cre and Dre had a detrimental impact on the development of treated embryos, as depicted in Fig. 1b–d, and quantified in Table 1. The highest conversion efficiency with AAV Vika and CFlpo vectors was observed at a concentration of 1E + 10 GC/mL.

Ready-to-use AAV1 particles carrying the EF1a-Flpo (EFlpo) were used to enhance conversion efficiency with Flpo. This purchased virus allowed us to use 1E + 11 GC/mL titer and assess the efficiency-to-toxicity ratio of the treatment as shown in Table 1. Overall, both AAV Flpo vectors show low toxicity towards treated embryos. Application of commercial EFlpo proved that the efficiency of conversion depends on titer (Table 1, Fig. 1b–d).

Titration screening in blastocysts showed that a viral concentration of 1E + 10 GC/mL yields the optimal efficiency-to-toxicity ratio for CMV-Cre, CMV-Dre, and CMV-Vika, as shown in Table 1 and Fig. 1b–d. In contrast to the Cre or Dre vectors, for Flpo vectors, particularly EFlpo, a higher titer enhances conversion efficiency without a negative impact on the viability of embryos (Fig. 1b–d). Treated blastocysts were collected and genotyped to confirm conversion on the genomic level (Supplementary Fig. S1).

AAV-SSRs have minimal impact on embryo development and show reduced AAV genome persistence potential

After determining the optimal titer for efficient allele conversion with AAV recombinases, we conducted experiments to assess whether AAV treatment at relatively high titers (1E + 10 GC/mL) had any adverse effects on late-stage embryo development following embryo transfer into surrogate females. Utilizing the identified optimal titers for AAV recombinases, we achieved a remarkable 100% efficiency of conversion with AAV EF1a-Flpo, AAV CMV-Cre, -Dre, and -Vika vectors. Notably, in the case of the AAV CMV-Flpo vector, we observed a 92.6% conversion rate among the born animals (total of 27), with 2 (7.4%) exhibiting mosaicism and 2 (7.4%) animals without recombination (Fig. 2c, Table 2). To increase the efficiency of Flpo conversion, we used the EF1a-Flpo vector at 1E + 11 GC/mL titer to convert MuX embryos. Using the EFlpo vector, we reached 100% conversion rate. Interestingly, both CFlpo and EFlpo treated groups produced the highest number of born animals, as shown in Table 2. These findings indicate that AAV-Flpo treatment does not interfere with the viability of embryos in later stages of development, and treated zygotes have the potential to give rise to fully converted animals. We selected a fully-converted male from each AAV treated group and analyzed EGFP signal in liver, lung, skin (ear), heart, intestine and testes. All converted animals were EGFP positive in analyzed organs (Fig. 2a). The remaining animals of the treatment group underwent a biopsy of fingers, which were used to detect the EGFP signal to confirm correspondence to the genotype (Supplementary Figs. S2–S6). Based on the genotype and phenotype analysis, we quantified the number of fully-converted, partially-converted (mosaics) and non-converted animals within each treated group (Table 2, Fig. 2c).

Additionally, we conducted an analysis of treated animals for the persistence of the AAV-based recombination system in either an integrative or episomal state. Specifically, we genotyped converted mice for the AAV genome

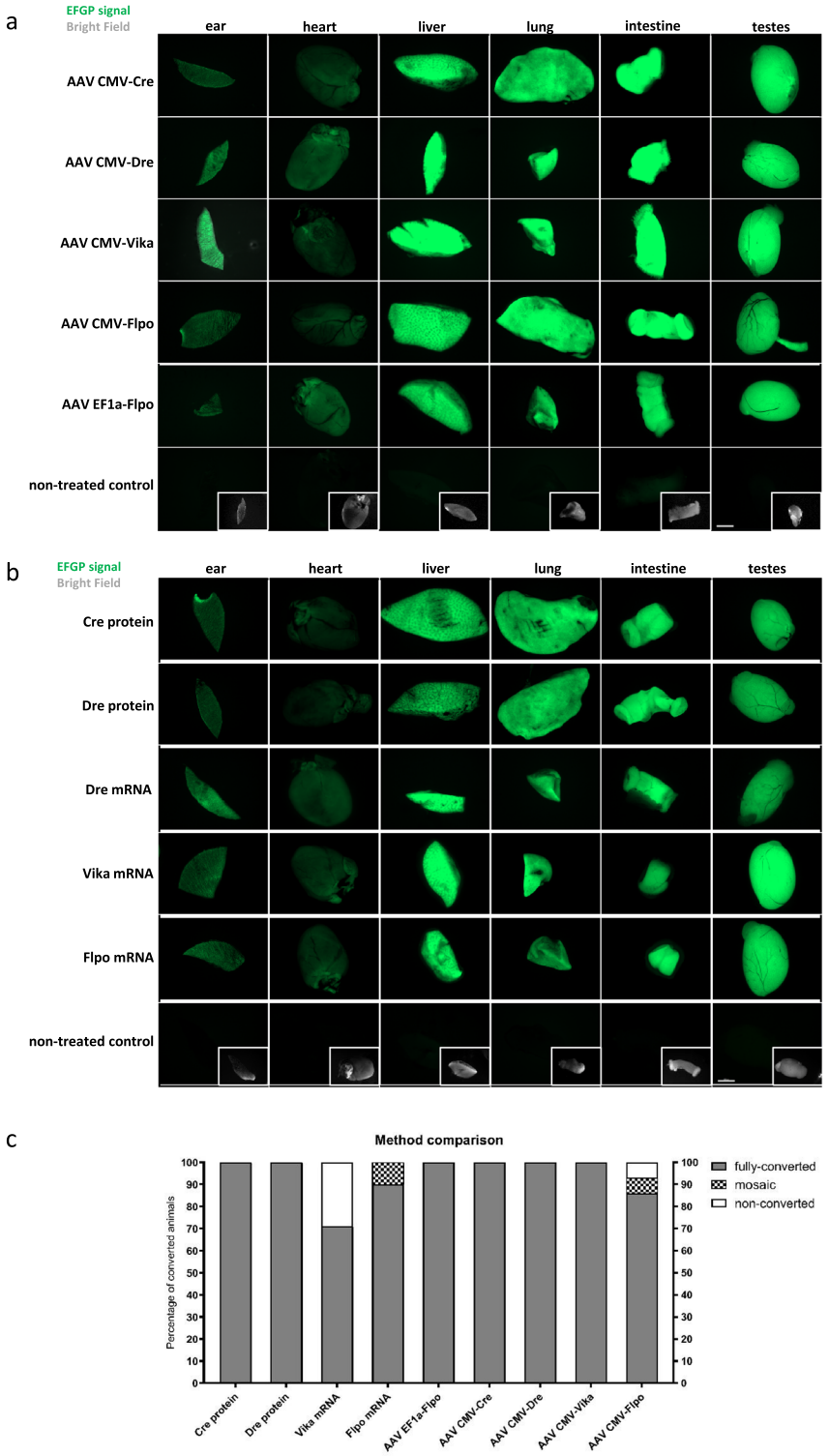


Fig. 2. Comparison of conversion efficiency using SSR protein or mRNA with AAV delivered SSRs. MuX heterozygote zygotes underwent conversion by treatment with either AAV or protein/mRNA. (**a**) Imaging of EGFP signal in fully-converted animals by AAV-SSR vectors (Cre, Dre, Vika and CMV-Flpo, EF1a-Flpo). (**b**) Imaging of EGFP signal in fully-converted animals by electroporated Cre or Dre proteins, and microinjection of Vika or Flpo mRNA molecules. (**c**) Graph depicting recombination efficiency throughout delivery methods, based on Table 2 data. The scale bar (2000 µm) in the image of non treated testes serves as a universal reference applicable to all images within this figure.

Delivery	Protein		mRNA		AAV				
Molecule/construct	Cre	Dre	Vika	Flpo	Cre	Dre	Vika	CFlpo	EFlpo
Transferred embryos	91	60	65	60	87	164	82	97	156
Born mice (*)	20 (22%)	13 (21.6%)	7 (10.8%)	10 (16.7%)	16 (18.4%)	13 (7.9%)	8 (9.8%)	27 (27.8%)	36 (23.1%)
Fully recombined	20 (100%)	13 (100%)	4 (57.1%)	9 (90%)	16 (100%)	13 (100%)	8 (100%)	23 (85.2%)	36 (100%)
Mosaics	0	0	1 (14.3%)	1 (10%)	0	0	0	2 (7.4%)	0
Non-recombined	0	0	2 (28.6%)	0	0	0	0	2 (7.4%)	0
AAV genome persistence rate	–	–	–	–	1 (6.3%)	2 (15.4%)	1 (12.5%)	1 (3.7%)	4 (11.1%)

Table 2. Comparison of conversion efficiency using SSR protein or mRNA and AAV delivered SSRs. *Percentage of born pups from transferred embryos. The table presents a summary of genotype and phenotype data for animals in the respective treatment groups, as depicted in supplementary data Supplementary Figs. S2–S10.

of the corresponding SSR. The analysis revealed that AAV SSRs have the potential to persist in treated animals at an average rate of 9.8%. For instance, with CMV-Cre, CMV-Vika and CMV-Flpo vectors, we detected residual AAV genome presence in one animal for each vector out of more than 8 animals (Supplementary Figs. S2c,d, S4c,d and S5c,d). In the case of CMV-Dre we detected 2 animals positive for AAV CMV-Dre genome and in EF1a-Flpo treated animals, 4 were Flpo positive (Supplementary Figs. S3c,d and S6c,d). A detailed summary is provided in Table 2.

AAV-mediated allele conversion is suitable alternative to other conventional (in vitro) methods

To compare AAV-based SSR delivery with more established methods, MuX reporter was converted by using electroporation of Cre and Dre proteins, and microinjection of Vika and Flpo mRNA. Fully-converted males were dissected and selected organs were sampled to confirm their genotype by the presence of EGFP signal (Fig. 2b). The remaining animals of studied groups were analyzed using finger samples (Supplementary Figs. S7–S10). The genotype and phenotype of treated animals are summarized in Table 2 and compared in Fig. 2c.

When employing protein-based conversion, the conversion rate reached 100% in born animals (Table 2, Fig. 2c). However, this method is not available for Flpo and Vika, as Flpo and Vika proteins could not be produced in sufficient quantities despite the use of various (bacterial, baculovirus, mammalian) expression systems (unpublished data). To compare the AAV system with other systems enabling Flpo- and Vika-mediated conversion, we proceeded with the microinjection of Vika and Flpo mRNA. Both Cre and Dre proteins consistently demonstrated 100% conversion efficiency. Flpo mRNA microinjection exhibited a 100% conversion rate with 10% of animals displaying mosaicism, while Vika mRNA application resulted in a 71.4% conversion rate with 14.3% of animals exhibiting mosaic characteristics (Table 2). Thus, the AAV-based delivery SSR system emerges as a fully functional alternative to select established methods of in vitro allele conversion (Fig. 2c).

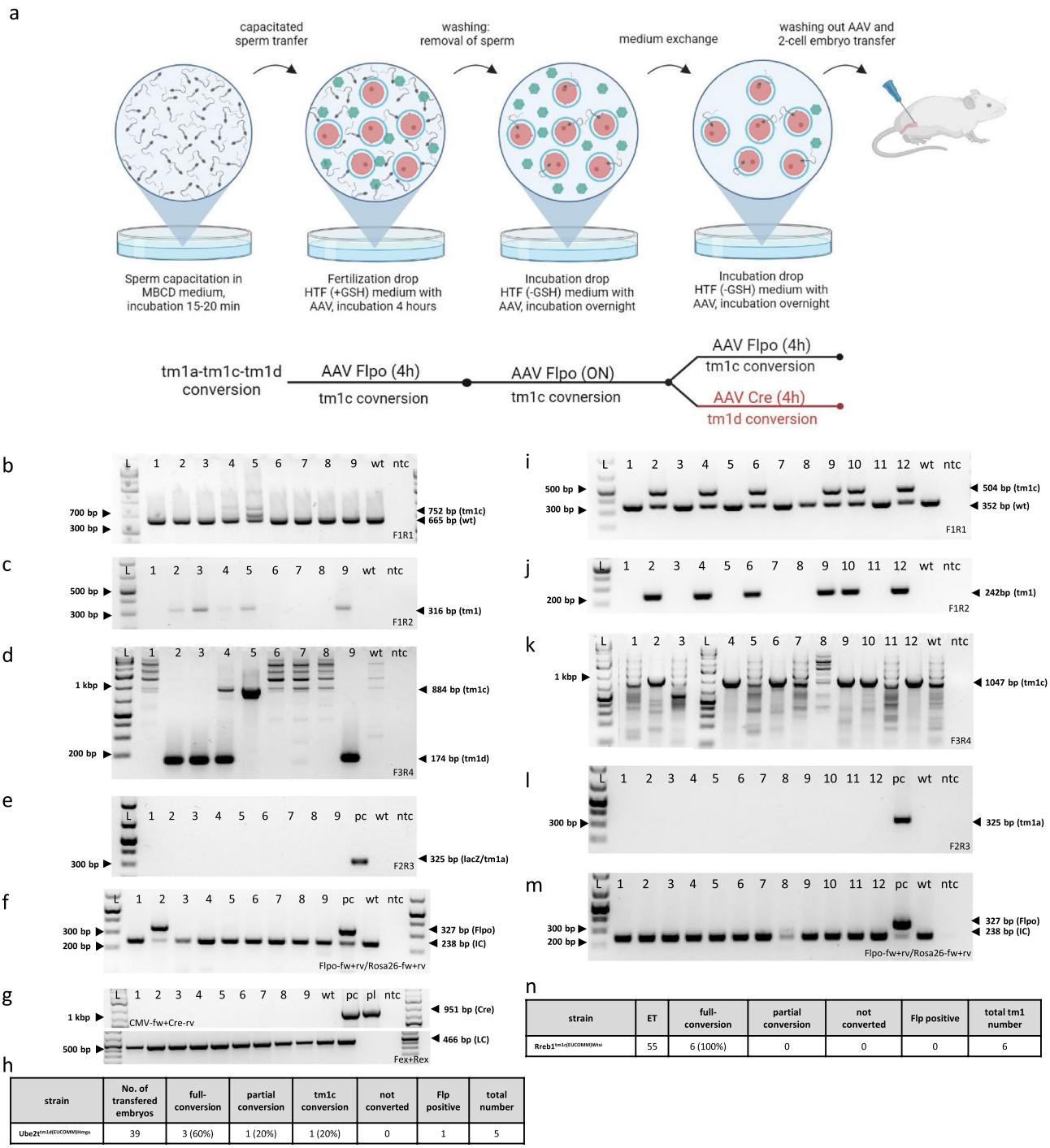
AAV-Cre and AAV-Flpo vectors enable single or double conversion during mouse line reanimation

To explore the potential of AAV-Flpo recombinase, we attempted to treat presumptive mouse zygote produced by in vitro fertilization from cryopreserved sperm with the AAV-Flpo recombinase. Our protocol was evaluated on selected EUCOMM/KOMP alleles, converting tm1a (KO first allele, reporter-tagged insertion allele) to tm1c (tm1c: conditional allele, post-Flpo) or even tm1d (deletion allele, post-Flpo and Cre with no reporter). The schematic description of the treatment is depicted in Fig. 3a. To convert $Aatk^{tm1a(KOMP)Wtsi}$ and $Cdh26^{tm1a(KOMP)Wtsi}$ lines to tm1c form, the AAV CMV-Flpo vector (at 1E + 10 GC/mL) was applied. Despite the anticipated reduction in efficiency at the highest attainable titer, the conversion resulted in 3 tm1c-positive out of 4 Aatk tm1-positive pups, where all 3 pups exhibited complete conversion to the tm1c allele (Supplementary Fig. S11). The conversion of Cdh26 zygotes led to the birth of 7 Cdh26 tm1 cassette positive animals, among which 4 were completely converted and 3 were mosaics (Supplementary Fig. S12).

To maximize the yield of fully converted animals, we tested the commercial AAV EF1a-Flpo vector for recombination in the following lines: $Rreb1^{tm1a(EUCOMM)Wtsi}$, $Atf2^{tm1a(EUCOMM)Hmgu}$ and $Ube2t^{tm1a(EUCOMM)Hmgu}$. The application of the commercial vector yielded high conversion efficiency, with Rreb1 tm1c conversion occurring in all 6 born tm1-positive animals (Fig. 3i–n). Atf2 tm1c conversion was observed in all 15 born tm1-positive pups, where one pup showed a partial conversion (Supplementary Fig. S13). Ube2t tm1c conversion was confirmed in all 7 tm1-positive animals (Supplementary Fig. S14).

To test the limits of AAV-based delivery, the direct in vitro conversion of tm1a alleles to tm1d was performed. This involved the sequential treatment of $Ube2t^{tm1a(EUCOMM)Hmgu}$ and $C4bp^{tm1a(EUCOMM)Hmgu}$ embryos with AAV EF1a-Flpo and CMV-Cre vectors, respectively. We achieved tm1d conversion in 4 out of 5 tm1-positive pups for Ube2t (Fig. 3b–h), and 4 out of 5 for the C4bp strain (Supplementary Fig. S15). Selected founders of all converted strains were sequenced to confirm specific conversions (Supplementary Data Sanger sequencing of founder mice).

All treated strains and the number of converted animals are summarized in Table 3. One or two of the fully converted and SSR genome negative founders for each strain were subsequently bred with a C57Bl/6NCrl wild-type counterpart. The progeny inherited the fully converted tm1c allele, for Aatk (Supplementary Fig. S16), Cdh26 (Supplementary Fig. S17), Atf2 (Supplementary Fig. S18), Rreb1 (Supplementary Fig. S19), Ube2t



h

strain	No. of transfered embryos	full-conversion	partial conversion	tm1c conversion	not converted	Flp positive	total number
Ube2t$^{tm1d(EUCOMM)Hmgu}$	39	3 (60%)	1 (20%)	1 (20%)	0	1	5

n

strain	ET	full-conversion	partial conversion	not converted	Flp positive	total tm1 number
Rreb1$^{tm1c(EUCOMM)Wtsi}$	55	6 (100%)	0	0	0	6

Fig. 3. EUCOMM/KOMP allele conversion using AAV Flpo and/or AAV Cre vectors. (**a**) Initially, thawed or extracted sperm undergo capacitation. The capacitated sperm are transferred to a fertilization drop containing AAV SSRs and are incubated. Residual sperm are washed out and fertilized oocytes are transferred to incubation drop with AAV, where they are incubated overnight. On the following day, 2-cell embryos are harvested and transferred into the oviduct of a pseudopregnant recipient female. For tm1a-to-tm1d conversion, viable zygotes, following the overnight treatment, are introduced into an incubation drop with AAV CMV-CRE vector and incubated for 4 h. (**b**–**e**) Ube2t$^{tm1d(EUCOMM)Hmgu}$ genotyping with corresponding primers to detect tm1d allele. (**f**,**m**) AAV Flpo persistence assay: four primers reaction, Flpo FR primers amplify AAV Flpo genome; internal control (Rosa26 FR primers). wt: C57Bl/6NCrl genomic DNA, pc: positive control—Gt(Rosa)26Sor(CAG-Flpo,-EYFP) genomic DNA. (**g**) AAV Cre persistence assay: detection of residual AAV Cre genome, CMV-F and Cre-R primers detect presence of AAV CMV-Cre genome; Fex and Rex primers amplify a DNA loading control reaction; wt:wild-type sample, pc: wild-type DNA and 1 ng/µ of AAV CMV-Cre plasmid, pl: AAV CMV-Cre plasmid, ntc: non-template control. (**h**) Ube2t$^{tm1d(EUCOMM)Hmgu}$ genotyping summary. (**i**–**l**) Rreb1$^{tm1c(EUCOMMWtsi}$ genotyping with corresponding primers to detect tm1c allele. (**n**) Rreb1$^{tm1c(EUCOMMWtsi}$ genotyping summary. L(ladder): GeneRuler 1 kb Plus DNA. Presented figures have been cropped for clarity, complete gel images can be found in Supplementary data Fig. S25.

Converted to	No. of tranfered embryos	AAV vector	Full-conversion (tm1c + and LacZ -)	Partial conversion (tm1c + and tm1a: LacZ +)	Not converted (tm1a: LacZ +)	Flp positive	Total number (tm1 cassete positive)			GLT*
Aatk $^{tm1c(KOMP)Wtsi}$	57	CMV-Flpo	3 (75%)	0	1 (25%)	0	4			100%
Cdh26$^{tm1c(KOMP)Wtsi}$	88	CMV-Flpo	4 (57.1%)	3 (42.8%)	0	0	7			100%
Rreb1$^{tm1c(EUCOMM)Wtsi}$	55	EF1a-Flpo	6 (100%)	0	0	0	6			100%
Atf2$^{tm1c(EUCOMM)Hmgu}$	51	EF1a-Flpo	14 (93.4%)	1 (6.6%)	0	0	15			100%
Ube2t$^{tm1c(EUCOMM)Hmgu}$	56	EF1a-Flpo	7 (100%)	0	0	0	7			100%
Converted to	**No. of tranfered embryos**	**AAV vector**	**Full-conversion (tm1d + and tm1c-)**	**Partial conversion (tm1d + and tm1c +)**	**tm1c conversion (LacZ-)**	**Not converted (LacZ +)**	**Flp positive**	**Cre positive**	**Total number (tm1 cassete positive)**	**GLT**
Ube2t$^{tm1d(EUCOMM)Hmgu}$	39	EF1a-Flpo/ CMV-Cre	3 (60%)	1 (20%)	1 (20%)	0	1	0	5	100%
C4bp$^{tm1a(EUCOMM)Hmgu}$	54	EF1a-Flpo/ CMV-Cre	4 (80%)	0	1 (20%)	0	1	0	5	100%

Table 3. EUCOMM/KOMP allele conversion using AAV Flpo and/or AAV Cre vectors. germ-line transmission of target allele form in animals positive for tm1 allele. The table provides a summary of genotyping data for the corresponding strain and treatment, as illustrated in Supplementary data Figs. S11–S19.

(Supplementary Fig. S20) or tm1d for Ube2t (Supplementary Fig. S21) and C4bp (Supplementary Fig. S23) without any residual presence of AAV genome. This pioneering strategy underscored the potency of AAV recombinases in the direct ex vivo conversion of tm1a alleles, resulting in rapid conversion to tm1c and/or tm1d in the first generation of transgenic mouse line immediately after fertilization. This approach not only significantly reduces the consumption of animals but also seamlessly aligns with the principles of the 3Rs concept (Replacement, Reduction, Refinement) accepted in the scientific community. All treated strains and numbers of converted animals are summarized in Table 3.

Discussion

Application of complex transgenic alleles, exemplified by EUCOMM/KOMP alleles, often requires multiple recombination events to understand specific biological processes fully. To achieve this level of genetic engineering sophistication, an efficient combinatorial method based on site-specific recombinases (SSRs) is needed. In this context, we introduce a new method utilizing AAV vectors to facilitate such an approach.

As shown with Cre protein[11], we attempted to produce and purify the recombinant Dre, Flpe, and Vika proteins in multiple cellular heterologous systems, including BL21-DE3-RIPL bacterial (BL21-DE3-RIPL), Hi5 insect cells, and HEK293T mammalian cells (HEK293). Nevertheless, Flpe and Vika proteins were not sufficient for experiments in large-scale workflows (unpublished data). To overcome this limitation, SSR coding sequences were packaged in AAV serotype 1 vectors, and their conversion efficiency was compared to established SSR delivery techniques in the form of protein electroporation and/or mRNA microinjection.

Titers of the vectors varied. Cre and Dre containing vectors could be prepared in high-titers, while Vika and CMV-Flpo vectors achieved relatively low-titer (Supplementary Fig. S24). The reason for this discrepancy is unclear. The constructs fall below the AAV packaging limit of 4.7 kb (Cre: 3167 bp, Dre: 3156 bp, Vika: 3159 bp, CMV-Flpo: 3369 bp), thus, the size of the vector should not influence packaging. Despite employing identical procedure for the production and purification protocols, our data suggest that the expression of Vika and Flpo may influence the AAV replication process in a mammalian system. However, it is important to highlight that our data did not indicate a significant presence of empty capsids in the Vika and CMV-Flpo vectors. If such capsids were prevalent, a disproportionately higher amount of protein relative to the GC (genome copy) count would be expected (Supplementary Fig. S24). Therefore, it is more plausible that the efficiency of AAV-SSRs production is negatively affected by the overexpression of Vika and Flpo in mammalian producer cells.

Different AAV SSR titers were applied to MuX heterozygote (het) zygotes to determine the most effective and least toxic viral concentration (Fig. 1b,c). Once the optimal titer was identified, the efficiency and scale of conversion were evaluated in adult mice. Performance of AAV vectors was compared with standard delivery methods, such as electroporation of protein (Cre and Dre), or microinjection of mRNA (Vika and Flpo).

Despite the relatively low-yield in production of CMV-Flpo and Vika vectors (reaching a maximum titer of 7E + 10 GC/mL and 3.3E + 11 GC/mL, respectively), the titration screen identified the minimum effective titers for CMV-Flpo and Vika as 1E + 10 GC/mL and 1E + 9 GC/mL, respectively (Fig. 1b–d). Reduced embryo viability at 1E + 11 GC/mL was observed in the case of Cre and Dre vectors (Fig. 1b,c). The optimal titers were determined based on the viability and conversion efficiency ratio, as quantified in Table 1. However, it is important to note that Cre and Dre vectors can be used at concentrations one order of magnitude lower while still ensuring efficient conversion and low toxicity. In contrast, CMV-Flpo and Vika titration experiments indicated that concentrations below 1E + 10 GC/mL significantly reduce conversion efficiency. A similar trend was observed with the commercial EF1a-Flpo vector. However, unlike Cre and Dre, the highest titer of this vector did not adversely affect embryo viability (Fig. 1b,c).

While AAV vectors are generally considered non-integrative vectors, recent evidence has introduced some uncertainty regarding their potential integration following double-stranded breaks (DSB)[20,21]. SSRs, unlike site-specific endonucleases, do not induce open DSB. SSR-mediated recombination represents a more isolated and tightly regulated process compared to the machinery involved in DSB repair[22]. Furthermore, the AAV vectors used in this study do not carry any recombination sites recognized by the given site-specific recombinases. This factor might considerably reduce the likelihood of AAV genome integration during SSR-mediated recombination. Therefore, we hypothesize that the observed persistence of the AAV genome is associated with the episomal state of the AAV genome or spontaneous integration into the genome, rather than with SSR activity[20].

The presence of expression-competent fragments of the AAV vector within the host cells was analyzed, since AAVs are associated with low integrative potential[21]. Our data suggest that all used recombinases in AAV form can persist in the host tissue. Based on AAV persistence assay in MuX mice, the frequency of residual AAV genome is on average 9.8%, which is relatively high by standards for gene therapy (Supplementary Figs. S2–S6). Due to this persistence potential, newborn animals should be screened for Flpo and Cre AAV genomes after AAV-mediated conversions. Based on this selection, there is no need for further back-cross breeding to eliminate the active *Flpo* gene from the genome, as is often case with conventional Flpo driver lines.

Notably, the AAV vector can integrate into the genome, frequently at the AAVS1 site, or it can remain in the nucleus in an episomal state[20]. Flpo-positive animals converted by AAV can generate progeny both with and without Flpo integration, as demonstrated in Supplementary Fig. S22. This observation suggests that while AAV-converted animals can test positive for Flpo, the recombinase gene is not necessarily passed to the next generation. This is likely due to the potential episomal state of the AAV vector, mosaic integration in germ cells, or allelic crossover during meiosis. One of the practical applications of the AAV-based SSRs delivery is in vitro fertilization. A single treatment with AAV Flpo or Cre facilitates the direct conversion of the previously mentioned EUCOMM/KOMP alleles without a significant risk of recombinase vector persistence. This approach offers a simple and effective method for Cre or Flpo-mediated conversion. The AAV system not only enables the conversion from tm1a to tm1c, but also provides the opportunity to perform tm1a to tm1d conversion in the whole animal, as shown in Fig. 3b–n. Furthermore, we verified that AAV-mediated conversion is heritably transmitted to the next generation. Since AAV vectors can remain present in either episomal or integrated states, we suggest the preference for AAV SSR-free animals for further work (Supplementary Figs. S16–S23).

We have effectively demonstrated the practical application of AAV SSRs in the context of EUCOMM/KOMP allele conversion, specifically transitioning from the tm1a state to tm1c, and further to tm1d in a single animal (Fig. 3b–h and Supplementary Fig. S15). A distinctive feature of the AAV-based approach is the ability to convert tm1a alleles to tm1d through sequential treatment with two distinct vectors. The current practice for achieving tm1d allele involves converting the tm1a allele to tm1c using either a Flpo driver or mRNA microinjection[23,24]. Subsequent progeny of tm1c mice are then converted either through a Cre driver or mRNA/protein microinjection/electroporation[5,11,23–25]. As a result, the conventional approach requires more time and animals until the final model is established, which can be at odds with the principles outlined in the 3Rs guidelines.

The main objective of this study is to underscore the versatility of AAV SSRs and improve conversion methods employing these enzymes. We focused on the FLP/*FRT* system, whose protein synthesis is complicated, and mRNA microinjection is invasive and less efficient. The utilization of AAV Flpo construct offers an efficient and straightforward tool for effective tm1c conversion without inducing significant adverse effects. Additionally, it enables the potential for combinatorial treatment with AAV Cre vectors to achieve the tm1d allele, resulting in rapid conversion compared to conventional methods. Furthermore, combinatorial treatment enables a reduction in the number of required animals.

Moreover, the use of recombinant AAVs (rAAVs) can be conducted within a biosafety level 1 (BSL-1) facility, provided that the AAV vector does not carry an oncogenic or toxic payload and is produced in a helper virus-free manner. rAAVs loaded with SSRs adhere to the BSL-1 criteria, making the vector readily applicable in laboratories lacking BSL-2 facilities[26]. Furthermore, rAAV vectors can be purchased in a high-purity grade and ready-to-use form, making AAV-mediated conversion a broadly available method. To facilitate the availability of the method, all plasmids generated in this study will be deposited in a non-profit plasmid repository.

Conclusion

In summary, this study highlights the potential of AAV Site-Specific Recombinases (SSRs) for precise allele conversion. Although the dominant Cre/*loxP* system provides a robust framework for achieving spatiotemporal control over alleles through recombination events, Flpo, Dre, and Vika, are not as commonly used for direct ex vivo allele conversion. The delivery of Cre, Dre, Flpo, and Vika recombinases via AAV1 particles resulted in efficient conversion at various target sites while minimizing risk of AAV genome persistence and ensuring the safety of treated embryos. This approach seemlessly aligns with in vitro fertilization (IVF) procedures applied on EUCOMM/KOMP lines. The AAV vectors demonstrate excellent conversion capabilities with a reduced risk of AAV genome persistence. Incorporating these findings, we provide a safe and efficient pathway for allele conversion, with the specific focus on the remarkable potential of AAV Flpo vectors. Our research underscores the versatility and safety of recombinant AAVs within research facilities, irrespective of biosafety level, and highlights AAV Flpo as a standout candidate for efficient and reliable allele conversion.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). Knock-out first (EUCOMM/KOMP) mouse lines, used in this study, are available in the European Mouse Mutant Archive (EMMA).

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References

1. Coleman, J. L. J. *et al.* Rapid knockout and reporter mouse line generation and breeding colony establishment using EUCOMM conditional-ready embryonic stem cells: A case study. *Front. Endocrinol. (Lausanne)* **6**, 105 (2015).
2. Loulier, K. *et al.* Multiplex cell and lineage tracking with combinatorial labels. *Neuron* **81**, 505–520 (2014).
3. Skarnes, W. C. *et al.* A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**, 337–344 (2011).
4. Sket, T., Falcomatà, C. & Saur, D. Dual recombinase-based mouse models help decipher cancer biology and targets for therapy. *Cancer Res.* **83**, 2279–2282 (2023).
5. Kim, H., Kim, M., Im, S. K. & Fang, S. Mouse Cre-LoxP system: General principles to determine tissue-specific roles of target genes. *Lab. Anim. Res.* **34**, 147–159 (2018).
6. Karimova, M. *et al.* A single reporter mouse line for Vika, Flp, Dre, and Cre-recombination. *Sci. Rep.* **8**, 1–12 (2018).
7. Karimova, M. *et al.* Vika/vox, a novel efficient and specific Cre/loxP-like site-specific recombination system. *Nucleic Acids Res.* **41**, e37 (2013).
8. Kouvaros, S. et al. A CRE/DRE dual recombinase transgenic mouse reveals synaptic zinc-mediated thalamocortical neuromodulation. *Sci Adv* **9**, (2023).
9. Wang, H. et al. Dual Cre and Dre recombinases mediate synchronized lineage tracing and cell subset ablation in vivo. *J. Biol. Chem.* **298** (2022).
10. Pettitt, S. J. *et al.* Agouti C57BL/6N embryonic stem cells for mouse genetic resources. *Nat. Methods* **6**, 493–495 (2009).
11. Jenickova, I. *et al.* Efficient allele conversion in mouse zygotes and primary cells based on electroporation of Cre protein. *Methods* **191**, 87–94 (2021).
12. Chen, S. *et al.* CRISPR-READI: Efficient generation of knockin mice by CRISPR RNP electroporation and AAV donor infection. *Cell Rep.* **27**, 3780-3789.e4 (2019).
13. Mizuno, N. *et al.* Intra-embryo gene cassette knockin by CRISPR/Cas9-mediated genome editing with adeno-associated viral vector. *iScience* **9**, 286–297 (2018).
14. Kranz, A. *et al.* An improved Flp deleter mouse in C57Bl/6 based on Flpo recombinase. *Genesis* **48**, 512–520 (2010).
15. Grieger, J. C., Choi, V. W. & Samulski, R. J. Production and characterization of adeno-associated viral vectors. *Nat. Protoc.* **1**, 1412–1428 (2006).
16. Aurnhammer, C. *et al.* Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. *Hum. Gene Ther. Methods* **23**, 18–28 (2012).
17. Pandey, R. & Saluja, D. Hydrogen peroxide agarose gels for electrophoretic analysis of RNA. *Anal. Biochem.* **534**, 24–27 (2017).
18. Luo, C. *et al.* Superovulation strategies for 6 commonly used mouse strains. *J. Am. Assoc. Lab. Anim. Sci.* **50**, 471 (2011).
19. Takeo, T. & Nakagata, N. Reduced glutathione enhances fertility of frozen/thawed C57BL/6 mouse sperm after exposure to methyl-beta-cyclodextrin. *Biol. Reprod.* **85**, 1066–1072 (2011).
20. Deyle, D. R. & Russell, D. W. *Adeno-Associated Virus Vector Integration*.
21. Wolter, J. M. *et al.* Cas9 gene therapy for Angelman syndrome traps Ube3a-ATS long non-coding RNA. *Nature* **587**, 281–284 (2020).
22. Grainge, I. & Jayaram, M. The integrase family of recombinases: Organization and function of the active site. *Mol. Microbiol.* **33**, 449–456 (1999).
23. Ludwig, D. L., Stringer, J. R., Wight, D. C., Doetschman, T. C. & Duffy, J. J. FLP-mediated site-specific recombination in microinjected murine zygotes. *Transgenic Res.* **5**, 385–395 (1996).
24. Wu, Y., Wang, C., Sun, H., LeRoith, D. & Yakar, S. High-efficient FLPo deleter mice in C57BL/6J background. *PLoS One* **4**, 8054 (2009).
25. Luckow, B. *et al.* Microinjection of Cre recombinase protein into zygotes enables specific deletion of two eukaryotic selection cassettes and enhances the expression of a DsRed2 reporter gene in Ccr2/Ccr5 double-deficient mice. *Genesis* **47**, 545–558 (2009).
26. Collins, D. E., Reuter, J. D., Rush, H. G. & Villano, J. S. Viral vector biosafety in laboratory animal research. *Comp. Med.* **67**, 215 (2017).

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Author contributions

P.N. and I.J. conceived the experiments, cloning work conducted by J.E., P.K. and P.N., production of AAVs conducted by P.N. and I.J., animal work planned and performed by P.K., P.N. I.J. and J.K., production of recombinant proteins C.B. and P.K., P.N. analyzed the results. Writing manuscript, review, editing; P.N., P.K., R.S. Funding acquisition, supervision, administration; RS. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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