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Highly Efficient Mouse Genome Editing by CRISPR Ribonucleoprotein Electroporation of Zygotes*[S]

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The CRISPR/Cas9 system has been employed to efficiently edit the genomes of diverse model organisms. CRISPR-mediated mouse genome editing is typically accomplished by microinjection of *Cas9* DNA/RNA and single guide RNA (sgRNA) into zygotes to generate modified animals in one step. However, microinjection is a technically demanding, labor-intensive, and costly procedure with poor embryo viability. Here, we describe a simple and economic electroporation-based strategy to deliver Cas9/sgRNA ribonucleoproteins into mouse zygotes with 100% efficiency for *in vivo* genome editing. Our methodology, designated as CRISPR RNP Electroporation of Zygotes (CRISPR-EZ), enables highly efficient and high-throughput genome editing *in vivo*, with a significant improvement in embryo viability compared with microinjection. Using CRISPR-EZ, we generated a variety of editing schemes in mouse embryos, including indel (insertion/deletion) mutations, point mutations, large deletions, and small insertions. In a proof-of-principle experiment, we used CRISPR-EZ to target the tyrosinase (*Tyr*) gene, achieving 88% bi-allelic editing and 42% homology-directed repair-mediated precise sequence modification in live mice. Taken together, CRISPR-EZ is simple, economic, high throughput, and highly efficient with the potential to replace microinjection for *in vivo* genome editing in mice and possibly in other mammals.

Classic mouse genome engineering techniques utilize homologous recombination to introduce precise modifications in embryonic stem cells (1–4). Modified embryonic stem cells are then injected into recipient blastocysts to generate chimeric animals that are subsequently bred to establish mutant mouse lines (5–7). Although this "gold standard" methodology enables a variety of genome editing schemes and generates numerous mouse models for studying gene function and human disease, it remains laborious, costly, and time-consuming. Modern genome editing technologies employ programmable endonucleases to create double strand breaks (DSBs) at defined loci, the repair of which can yield insertion or deletion (indel) mutations through the non-homologous end joining (NHEJ)[4] pathway (8, 9) or donor-mediated precise modifications through the homology-directed repair (HDR) pathway (10, 11). Early iterations of such technologies include zinc finger nucleases and transcription activator-like effector nucleases (12–14). More recently, the simplicity and efficiency of the CRISPR/Cas9 system have led to its widespread application as the preferred method for mouse genome editing (15).

CRISPR (clustered regularly interspaced short palindromic repeats) RNAs and CRISPR-associated proteins are part of an adaptive immune system in bacteria and archaea to defend against phage infection (16, 17). Derived from the bacterial type II CRISPR/Cas system, the Cas9 nuclease can be directed by a programmable single guide RNA (sgRNA) to induce double strand breaks at specific loci, triggering the NHEJ or HDR repair pathways to yield desired sequence modifications (18–21). Using CRISPR, a variety of simple and sophisticated genomic modification schemes have been generated in mouse models, including precise sequence replacement (22–24), genome insertions/deletions (25, 26), and chromosomal translocations (27). The standard practice for CRISPR editing in mice relies on microinjection of Cas9 DNA/mRNA and sgRNAs into one-cell zygotes (15, 28), a process that remains laborious, costly, and low throughput with considerable technical barriers. More importantly, physical damage caused by microinjection significantly reduces embryo viability (29), further decreasing efficiency in generating viable and germ line-competent mice with desired genetic modifications. Electroporation-based approaches have been reported to deliver Cas9 mRNA and sgRNAs into mouse zygotes, yet such methods often require customized apparatuses, exhibit compromised editing efficiency, and are prone to founder mosaicism (30–32).

As implicated by structural studies (33–35), delivery of pre-assembled Cas9/sgRNA ribonucleoproteins (RNPs) facilitates highly efficient editing in mammalian cell culture and in several model organisms (36–39). To overcome the costly and laborious nature of microinjection, we developed CRISPR-EZ (CRISPR RNP Electroporation of Zygotes), an electroporation-based method to deliver Cas9/sgRNA RNPs into mouse zygotes for *in vivo* genome editing with high efficiency, high throughput, and high viability (Fig. 1*A*). CRISPR-EZ greatly simplifies

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[4] The abbreviations used are: NHEJ, non-homologous end joining; RNP, ribonucleoprotein; HDR, homology-directed repair; sgRNA, single guide RNA; nt, nucleotide; ssDNA, single-stranded DNA; RFLP, restriction fragment length polymorphism; oligo, oligonucleotide; PAM, protospacer adjacent motif.

TABLE 1
sgRNA, ssDNA HDR donor, and PCR primer sequences in CRISPR-EZ experiments

sgRNA sequences	
sgTyr	GGGTGGATGACCGTGAGTCC
sgCdh1	TATGACTGGAGTCCCGGGCG
sgCdk8	AGACAGAAACACCTTCAGAA
sgKif11	CGTGGAATTATACCAGCCAG
sgMecp2 L	CCCAAGGATACAGTATCCTA
sgMecp2 R	AGGAGTGAGGTCTAGTACTT
sgSox2	TGCCCCTGTCGCACATGTGA
Nested primers for RLFP and genotyping	
sgTyr F1	TCTTTTCGGAGACACTCAAATCA
sgTyr F2	TCTGTACAATTTGGGCCCCC
sgTyr R1	GCTTTCAGGCAGAGGTTCCT
sgCdh1 F1	TCTCCGGGTAGGGTTGTTCA
sgCdh1 F2	CCTGTCTGTGATCTGTCCACTT
sgCdh1 R1	CCAACAAGTCCCCAGTGCTA
sgCdk8 F1	CACTTCCAAGCAGCCAGGTA
sgCdk8 F2	GGCCGTGGCATATCCTTGTA
sgCdk8 R1	GGTGACTCCTAGTGCAGTGG
sgKif11 F1	GGATGGGAGGTGTAGCTGAG
sgKif11 F2	GGATCAGTCCTCAGTGTTGCA
sgKif11 R1	CCTTGTTCCGGGGATCATCAA
sgMecp2 F1	GGCCAGATGCATGGGTAGAA
sgMecp2 F2	TGAAAACAGAGGACCTGCCG
sgMecp2 WT R1	CCTTGCCTGAAGGTTGGACA
sgMecp2 KO R1	TTGTCATGTGGCAAGCCCA
sgSox2 F1	ACATGATCAGCATGTACCTCC
sgSox2 V5 F2	ACATGGGCAAGCCCATCC
sgSox2 R1	TAATTTGGATGGGATTGGTGG
ssDNA HDR donor oligos	
Tyr ssDNA	GTGCACCATCTGGACCTCAGTTCCCCTTCAAAGGGGTGGATGACCGTGAATTCCTGGCCCTCTGT-GTTTTATAATAGGACCTGCCAGTGCTC
Sox2 V5 ssDNA	TACCAGAGCGGCCCGGTGCCCGGCACGGCCATTAACGGCACACTGCCCCTGTCGCACATGGGCA-AGCCCATCCCCAACCCCCTGCTGGGCCTGGACAGCACCTGAGGGCTGGACTGCGAACTGGAGAA-GGGGAGAGATTTTCAAAGAGATACAAGGGAATTG

the Cas9/sgRNA delivery into mouse zygotes to enable high efficiency and high-throughput genome editing *in vivo*. Compared with the microinjection-based technology, CRISPR-EZ significantly improves embryo/pup survival following Cas9 and sgRNA delivery. Using CRISPR-EZ, we consistently obtained high efficiency mouse genome editing for multiple genes, and we successfully generated mouse embryos with a variety of editing schemes, including indel mutations, point mutations, genomic deletions, and small precise insertions. Taken together, CRISPR-EZ is a simple, economic, high throughput, and highly efficient technique for genome editing *in vivo*, which can replace the traditional microinjection-dependent genome editing technique in mice and possibly in other mammalian species.

Experimental Procedures

In Vitro Synthesis of sgRNAs—Candidate sgRNA designs were selected from a number of algorithms, including the sequence scan for CRISPR (40), the Gene Perturbation Platform (41), Chop-Chop (42), and CRISPR Design (43). For most experiments, we selected three to four candidate sgRNAs based on the predicted scores from multiple sgRNA design algorithms and the proximity to desired target sites. We then experimentally determined the best sgRNA design by measuring targeted DNA cleavage efficiency using the Surveyor assay (44) in a Cas9-overexpressing 368T1 mouse lung cancer cell line that harbors a $Kras^{G12D}$ mutation and a p53 deletion.

To synthesize sgRNAs *in vitro*, a DNA oligonucleotide template that contained a T7 promoter, a 20-nucleotide (nt) guide sequence, and an sgRNA scaffold (45) was generated by overlapping polymerase chain reaction (PCR) (37). Specifically, we performed PCRs using Phusion® high fidelity DNA polymerase (New England Biolabs, catalog no. M0530), with the annealed product from a uniquely designed oligo (5′-GGA TCC TAA TAC GAC TCA CTA TAG– guide sequence–GTT TTA GAG CTA GAA-3′, 0.02 μM) and a common oligo T7RevLong (5′-AAA AAA GCA CCG ACT CGG TGC CAC TTT TTC AAG TTG ATA ACG GAC TAG CCT TAT TTT AAC TTG CTA TTT CTA GCT CTA AAA C-3′, 0.02 μM) as the template, and T7FwdAmp (5′-GGA TCC TAA TAC GAC TCA CTA TAG-3′, 1 μM) and T7RevAmp (5′-AAA AAA GCA CCG ACT CGG-3′, 1 μM) as two common primers (supplemental Fig. S1*A*). All sgRNA sequences used in this analysis are listed in Table 1. The thermocycler setting consisted of 30 cycles of 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 10 s. A 20-μl *in vitro* transcription reaction consisting of 25 ng/μl PCR-amplified DNA template, 10 mM NTPs, and 1 unit of T7 RNA polymerase (New England Biolabs, catalog no. E2040S) was incubated at 37 °C for more than 18 h, followed by a brief treatment of RNase-Free DNase I (New England Biolabs, catalog no. M0303S, 2 units) at room temperature for 20 min. The *in vitro* synthesized sgRNAs were cleaned up by magnetic beads that allowed solid-phase reversible immobilization of RNAs (46). The *in vitro* transcription reaction was first brought to 150 μl in volume with 100% ethanol, followed by gentle mixing of 100 μl of SeraMeg Speedbeads magnetic carboxylate-modified particles (GE Healthcare, catalog no. 65152105050250) for 10 times before a 5-min room temperature incubation. The reaction was subsequently placed on a magnetic stand (Invitrogen, catalog no. 12321D) for 5 min under room temperature to allow the formation of compact RNA/bead pellets. After the supernatant was carefully aspi-

TABLE 2
NHEJ editing efficiency and embryo viability under multiple CRISPR-EZ conditions

Gene	CRISPR-EZ conditions	Zygotes treated	Embryos survived to morula	Embryos assayed by RFLP	Editing		
					Bi-allelic	Partial	Unedited
Tyr	16 μM Cas9 RNPs, 1 ms	29	8 (28%)	8	1 (13%)	7 (87%)	0
	16 μM Cas9 RNPs, 3 ms	24	6 (25%)	6	5 (83%)	1 (17%)	0
	16 μM Cas9 RNPs, 10 ms	28	5 (18%)	5	5 (100%)	0	0
	8 μM Cas9 RNPs, 1 ms	30	19 (63%)	12	2 (16%)	9 (75%)	1 (8%)
	8 μM Cas9 RNPs, 3 ms	30	18 (60%)	12	8 (67%)	4 (33%)	0
	8 μM Cas9 RNPs, 10 ms	30	12 (40%)	12	11 (92%)	1 (8%)	0
Cdh1	8 μM Cas9 RNPs, 3 ms	35	ND[a]	25	0	14 (56%)	11 (44%)
Cdk8	8 μM Cas9 RNPs, 3 ms	35	ND	22	3 (14%)	19 (86%)	0
Kif11	8 μM Cas9 RNPs, 3 ms	35	ND	24	1 (4%)	12 (50%)	11 (46%)

[a] ND means not determined.

rated by pipette, we washed the pellets gently with 80% ethanol three times (2 min wash each time, without pipetting) and air-dried the pellets for 10 min. sgRNAs bound to the beads were eluted by incubating with 20 μl of RNase-free H_2O (Ambion, catalog no. AM9937) and stored at −80 °C.

Assembly of Cas9/sgRNA RNPs—To assemble the Cas9/sgRNA RNPs, we incubated purified Cas9 protein (QB3 Macrolab, University of California at Berkeley) in a 1:1.5 molar ratio with sgRNAs to obtain a final concentration of 8 or 16 μM Cas9/sgRNA RNPs in a 10-μl solution containing 20 mM HEPES, pH 7.5 (Sigma, catalog no. H3375), 150 mM KCl (Sigma, catalog no. P9333), 1 mM $MgCl_2$ (Sigma, catalog no. M8266), 10% glycerol (Fisher, catalog no. BP229), and 1 mM reducing agent tris(2-carboxyethyl)phosphine, Sigma, catalog no. C4706), at a final concentration of 8 or 16 μM Cas9/sgRNA RNPs. Whenever appropriate, 200 pmol of HDR single-stranded DNA (ssDNA) oligo donor (up to 162 nt in length) was also included in the 10-μl reaction by diluting a concentrated ssDNA stock solution in distilled water. All donor oligo sequences used in this analysis are listed in Table 1. The Cas9/sgRNA RNP complex was prepared by incubating the mixture at 37 °C for 10 min immediately before electroporation.

Delivery of the Cas9/sgRNA RNPs to Mouse Zygotes by CRISPR-EZ—Three-to-five-week old female C57BL/6J mice (The Jackson Laboratory, catalog no. 000664) were superovulated by intraperitoneal (i.p.) administration of 5 IU of pregnant mare serum gonadotropin (Calbiochem, Millipore, catalog no. 367222), and 46–48 h later, 5 IU human chorion gonadotropin (Calbiochem, Millipore, catalog no. 230734). Superovulated females were mated at a 1:1 ratio with 3–8-month-old C57BL/6J males to generate one-cell zygotes at 0.5 days post-coitum. Under a stereomicroscope (Nikon SMZ-U or equivalent), the ampulla of oviduct was nicked, releasing fertilized zygotes and oocytes associated with surrounding cumulus cells into 50 μl of M2 + BSA media, consisting of M2 media (Zenith, catalog no. ZFM2–100) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma, catalog no. A3311). Using a handheld pipette set to 50 μl, zygotes were subsequently dissociated from cumulus cells after the cell clumps were transferred into a 200-μl droplet of hyaluronidase/M2 solution (Millipore, catalog no. MR-051-F), incubated for 1 min, and passed through five washes in the M2 + BSA media to remove cumulus cells. All embryos from this point on were manipulated by mouth-pipetting with the use of a 15-inch aspirator tube (Sigma, catalog no. A5177) and a handmade glass needle fashioned by glass pulling of capillary tubes (Sigma P0674) over an open flame. Embryo are passed through five washes of M2 + BSA to remove cumulus cells. With as little additional volume as is reasonable, embryos were transferred to a 200-μl droplet of acid Tyrode's solution (Sigma, catalog no. T1788). Subsequently, zygotes were transferred to a 200-μl droplet of acidic Tyrode's solution (Sigma, catalog no. T1788) to weaken the zona pellucida in preparation for the electroporation. Because of batch-to-batch variation of the acidic Tyrode's solution, the exact timing of the acidic Tyrode's treatment needs to be determined empirically by observing the thinning of zona pellucida under the stereomicroscope. Typically, we incubated the zygotes in the acidic Tyrode's solution until ~15–20% zona pellucida was digested by visual inspection, which typically took 30–60 s. Untreated embryos served as a useful control for determining the appropriate timing for acidic Tyrode's treatment. The proper weakening of the zona is critical for the efficient electroporation of the Cas9/sgRNA RNPs into zygotes, yet prolonged zygote exposure to the acidic Tyrode's solution can lead to reduced embryo viability. Treated zygotes were subsequently washed four times in M2 + BSA droplets to remove acidic Tyrode's solution. Subsequently, zygotes can be temporarily cultured in M2 + BSA in a water-jacketed CO_2 incubator (5% CO_2, 37 °C and 95% humidity) until electroporation.

During electroporation, ~30–40 zygotes were pooled and washed once with Opti-MEM reduced serum media (Thermo Fisher Scientific, catalog no. 31985062) to remove the M2 + BSA media. Subsequently, 30–40 zygotes in 10 μl of Opti-MEM reduced serum media were combined with 10 μl of freshly made Cas9/sgRNA RNP solution with or without the corresponding HDR oligos. Using a standard handheld pipette, the 20-μl embryo and RNP mixture was pipetted into a 1-mm electroporation cuvette (Bio-Rad, catalog no. 1652089) and loaded into the Gene Pulser XCell electroporator (Bio-Rad). A standard square wave electroporation was performed using two pulses at 30 V for 3 ms, which were separated by a 100-ms interval. Immediately following electroporation, zygotes were recovered from the cuvette by flushing with 100 μl of KSOM + AA media (KCl-enriched simplex optimization medium with amino acid supplement, Zenith Biotech, catalog no. ZEKS-050) once or twice. Embryos were then washed once in KSOM + BSA media that was equilibrated at least 3–4 h prior to the start of the CRISPR-EZ experiment, and then cultured in 20-μl droplets of KSOM + BSA in 35 × 10-mm culture dishes (CellStar Greiner Bio-One 627160) in a water-jacketed CO_2 incubator (5% CO_2, 37 °C and 95% humidity). The embryos that successfully developed into two-cell embryos were transferred into

the oviduct of CD1 pseudopregnant females, with ~10 embryos per oviduct. The viable pups were then subjected to genotyping and phenotyping analyses.

Delivery of Cas9 mRNA and sgRNA to Mouse Zygotes by Microinjection—The transgenic facility (University of California at Berkeley) performed all microinjection experiments

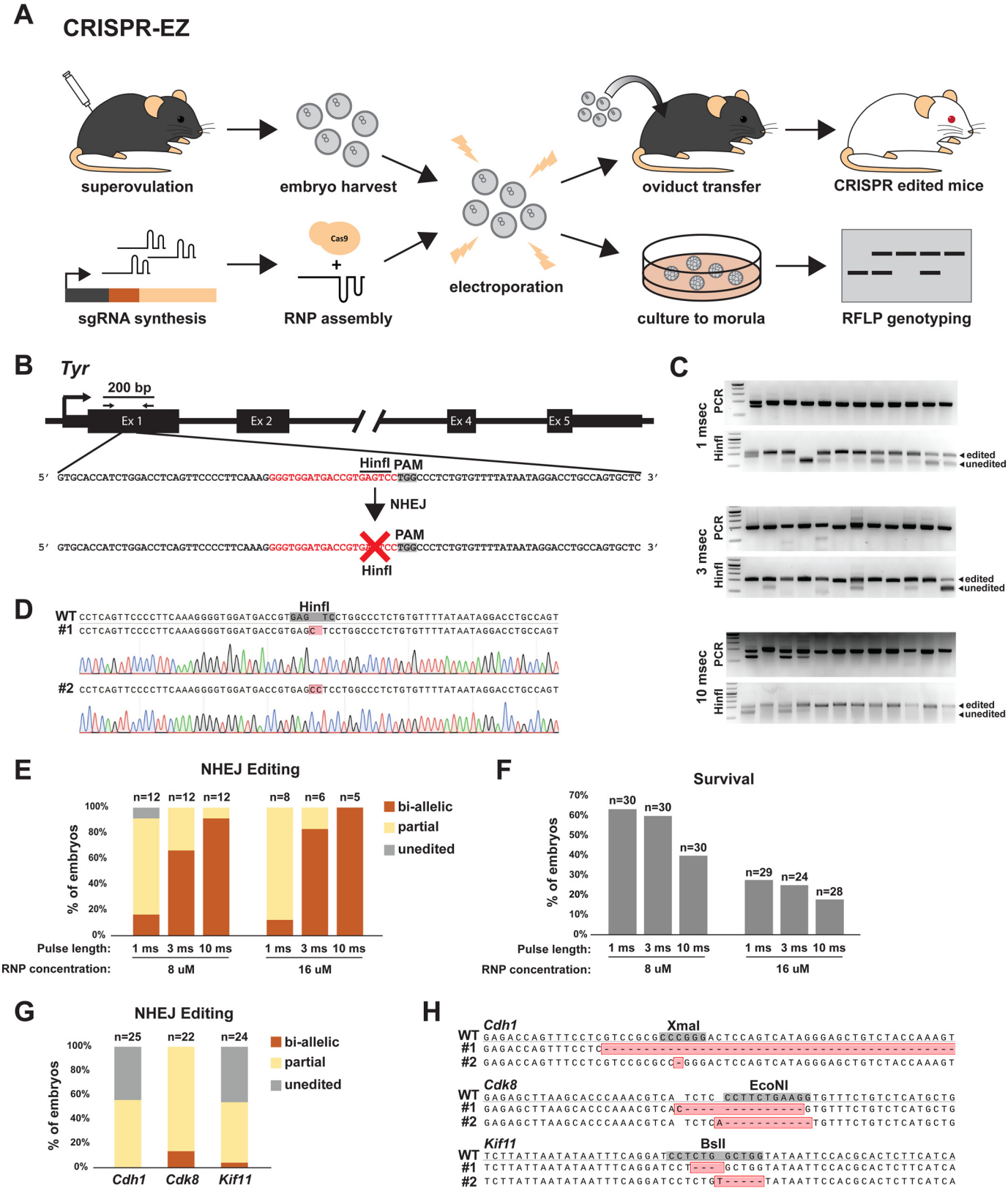


TABLE 3
Phenotype and viability of *Tyr*-edited embryos and mice by CRISPR-EZ

Genome editing method	Embryos treated	Embryos transferred	Mice born	Coat color		
				Black	Albino	Mosaic
mRNA injection	136 (93)[a]	60	5	1 (20%)	3 (60%)	1 (20%)
CRISPR-EZ, 1 ms	140	85	44	23 (53%)	12 (27%)	9 (20%)
CRISPR-EZ, 3 ms	120	90	33	1 (3%)	29 (88%)	3 (9%)

[a] 93 of 136 collected embryos were injected after screening for presence of pronuclei. In contrast, CRISPR-EZ was performed on all collected embryos.

under the standard protocol. Pronucleus embryos were preselected from collected superovulated embryos by visual inspection for the presence of the second pronuclei. Microinjection was performed in M2 media (Sigma, catalog no. M7176) using an inverted microscope (Nikon Corp., Tokyo, Japan) and micromanipulators (Narishige, Tokyo, Japan). A solution containing 100 ng/μl Cas9 mRNA (Life Technologies, Inc., catalog no. A25640) and 50 ng/μl *in vitro* transcribed sgRNAs were injected into pronucleus embryos by microinjection. After microinjection, the embryos were cultured in a KSOM in a CO_2 incubator (5.0% CO_2 at 37 °C) overnight, and the surviving two-cell stage embryos were transferred to 0.5 days post-coitum CD1 pseudopregnant mothers via oviduct transfer.

RFLP and Genotyping Analyses—To extract DNA from cultured morula embryos, embryos were washed twice with PBS, and 1 μl of PBS solution containing a single embryo was transferred into 10 μl of embryo lysis buffer containing 50 mM KCl (Fisher, catalog no. P217-3), 10 mM Tris-HCl, pH 8.5 (Fisher, catalog no. BP1531), 2.5 mM $MgCl_2$ (Fisher, catalog no. M33-500), 0.1 mg/ml gelatin (Fisher, catalog no. G7–500), 0.45% Nonidet P-40 (Fluka, catalog no. 74385), 0.45% Tween 20 (Sigma, catalog no. P7949-500), and 0.2 mg/ml proteinase K (Fisher, catalog no. BP1700-100). Lysis was performed in a thermocycler with the following conditions: 55 °C for 4 h, 95 °C for 10 min, and 10 °C hold. To extract DNA from mouse tails, we used a standard chloroform extraction protocol.

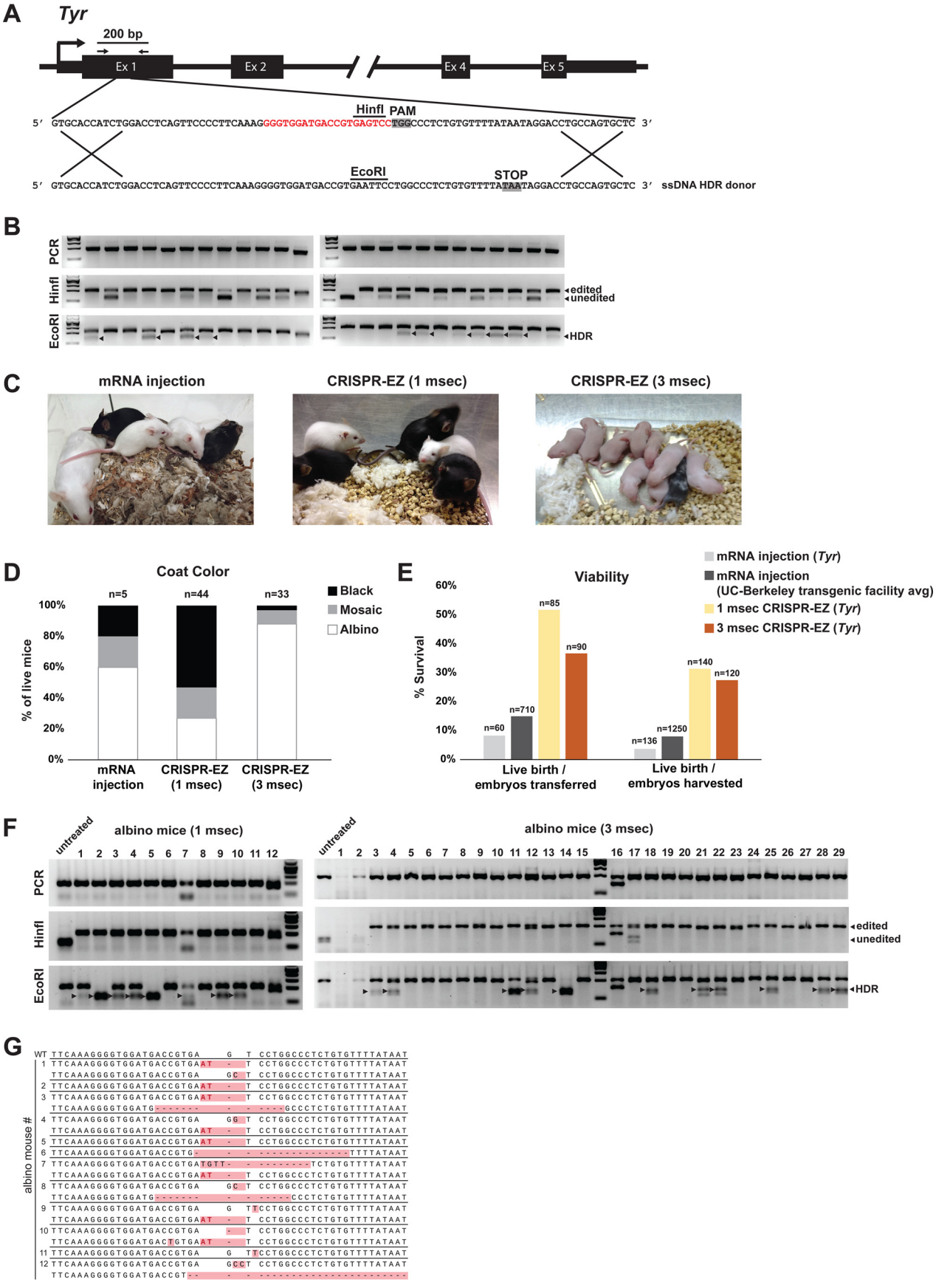
Following DNA isolation, PCR was performed using GoTaq (Promega, catalog no. M712). 3 μl of the embryo lysis solution and 20 ng of tail DNA were used as the PCR templates for embryo and mouse genotyping, respectively, to generate an amplicon containing the edited region. Nested PCRs were then performed using 1 μl of 1:10 diluted primary PCR product. For *Tyr, Cdh1, Cdk8,* and *Kif11* editing experiments, nested PCR products were further subjected to RFLP analyses for genotyping (see below); for the *Mecp2* deletion, PCR genotyping was performed using nested primers flanking the deleted sequences; for the V5 insertion into *Sox2*, PCR genotyping was performed using nested primers, with two primers residing outside the V5 sequence and one primer residing within the V5 sequence.

Using the nested PCR products amplified from embryo or mouse DNA, we designed an RFLP-based strategy to distinguish unedited, NHEJ, or HDR events for the *Tyr* editing experiments. All primer sequences used in this analysis are listed in Table 1. We first identified a unique restriction site within the unedited amplicon near the Protospacer Adjacent Motif, and we designed sgRNAs that were predicted to disrupt the endogenous restriction site by a successful NHEJ or HDR editing. For HDR editing of the *Tyr* gene, we designed the donor oligo to replace the endogenous HinfI restriction site with a new EcoRI restriction site while simultaneously disrupting the target sequence to prevent secondary editing. For restriction digest, 3 μl of PCR product was added to a 10-μl reaction containing 1 unit of the restriction enzyme and incubated at 37 °C for 4 h. Digested PCR products were electrophoresed on a 2% agarose gel and imaged on a Gel Doc XR+ (Bio-Rad). The following restriction enzymes were used: HinfI (New England Biolabs, catalog no. R0155S), EcoRI (New England Biolabs, catalog no. R0101S), XmaI (New England Biolabs, catalog no. R0180S), EcoNI (New England Biolabs, catalog no. R0521S), and BslI (New England Biolabs, catalog no. R0555S).

To validate the CRISPR editing by Sanger sequencing, nested PCR products were gel-purified using the GeneJET gel extraction kit (Thermo Scientific, catalog no. K0691) and cloned into pGEM-T Easy vector (Promega, catalog no. A1360) by TA cloning. Plasmid DNAs were submitted for Sanger sequencing to determine the DNA sequences in edited embryos or mice.

Embryo Immunofluorescence—Blastocyst embryos were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, catalog no. 19202) for 15 min at room temperature and then transferred to PBS containing 0.1% bovine serum albumin (BSA, Sigma, catalog no. A3311). Permeabilization was performed by incubating the embryos with PBS containing 0.1% Triton X-100 and 0.1% BSA for 5 min. Subsequently, embryos

FIGURE 1. **CRISPR-EZ efficiently generates NHEJ-mediated indel mutations in mouse embryos**. *A,* diagram illustrating the workflow of the CRISPR-EZ technology in mouse genome editing. Fertilized embryos were combined with pre-assembled Cas9/sgRNA RNPs for electroporation and then transferred to pseudopregnant mothers to generate edited mice. *B,* gene schematic illustrating the NHEJ-mediated editing design that targets *Tyr* exon 1. The HinfI restriction site within *Tyr* exon 1, located 2 nt upstream of the PAM, will be disrupted upon successful NHEJ editing. *Arrows* indicate the positions of primers that amplify a DNA fragment for RFLP genotyping analyses. *C* and *D,* representative RFLP genotyping analyses (*C*) and sequencing confirmation (*D*) of *Tyr* NHEJ editing in mouse morula embryos using 8 μM Cas9/sgRNA RNPs under different electroporation conditions. *C,* presence of an undigested PCR product (200 bp) indicates successful NHEJ editing. *Top,* nested PCR amplicons from morula embryos following CRISPR-EZ; *bottom,* HinfI digestion using nested PCR amplicons in RFLP analyses. *D,* chromatograms and alignment of sequences from two edited mouse morula embryos compared with the wild type *Tyr* sequence. *Red boxes* indicate edited sequences. *E* and *F,* key experimental conditions in CRISPR-EZ were optimized to achieve high editing efficiency on *Tyr* and favorable embryo viability in culture. Three electroporation conditions and two Cas9 RNP concentrations were compared for *Tyr* NHEJ editing efficiency (*E*) and for embryo viability (*F*). *F,* percent survival was calculated as the ratio between the number of embryos that developed to the morula stage and the total number of zygotes electroporated. *G,* CRISPR-EZ achieved efficient NHEJ editing of *Cdh1*, *Cdk8,* and *Kif11*. Following CRISPR-EZ using optimized conditions (8 μM Cas9/sgRNA RNPs, two pulses of electroporation at 3-ms pulse length), the efficiency of NHEJ editing on *Cdh1*, *Cdk8,* and *Kif11* was measured by RFLP analyses. *H,* sequence validation is shown for representative *Cdh1*, *Cdk8,* and *Kif11* editing events.


A
Tyr
200 bp
Ex 1
Ex 2
Ex 4
Ex 5
HinfI PAM
5′ GTGCACCATCTGGACCTCAGTTCCCCTTCAAAGGGGTGGATGACCGTGAGTCCTGGCCCTCTGTGTTTTATAATAGGACCTGCCAGTGCTC 3′
EcoRI
STOP
5′ GTGCACCATCTGGACCTCAGTTCCCCTTCAAAGGGGTGGATGACCGTGAATTCCTGGCCCTCTGTGTTTTATAATAGGACCTGCCAGTGCTC 3′ ssDNA HDR donor
B
PCR
HinfI
EcoRI
edited
unedited
HDR
C
mRNA injection
CRISPR-EZ (1 msec)
CRISPR-EZ (3 msec)
D
Coat Color
n=5
n=44
n=33
Black
Mosaic
Albino
% of live mice
100%
80%
60%
40%
20%
0%
mRNA injection
CRISPR-EZ (1 msec)
CRISPR-EZ (3 msec)
E
Viability
mRNA injection (Tyr)
mRNA injection (UC-Berkeley transgenic facility avg)
1 msec CRISPR-EZ (Tyr)
3 msec CRISPR-EZ (Tyr)
% Survival
60%
50%
40%
30%
20%
10%
0%
n=60
n=710
n=85
n=90
n=136
n=1250
n=140
n=120
Live birth / embryos transferred
Live birth / embryos harvested
F
albino mice (1 msec)
albino mice (3 msec)
untreated
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
PCR
HinfI
EcoRI
edited
unedited
HDR
G
albino mouse #
WT
1
2
3
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were blocked for 1 h at room temperature in blocking solution (PBS containing 10% goat serum, Fisher catalog no. 31872, and 0.1% BSA). Embryos were then incubated with anti-V5 primary antibody (1:100, ThermoFisher, catalog no. R960-25) in blocking solution at 4 °C overnight and subsequently in Alexa Fluor 594 goat anti-mouse IgG (1:400, ThermoFisher, catalog no. A21125) in blocking solution at 4 °C overnight. Embryos were imaged using a Zeiss Observer A1 fluorescent microscope.

Results

The CRISPR/Cas9 system provides a powerful yet simple platform for genome editing in mice, yet the current technology requires a microinjection-based procedure, which is technically demanding, laborious, and costly. To improve CRISPR genome editing in mice, we developed CRISPR-EZ, an electroporation-based technology, to deliver Cas9/sgRNA RNPs into mouse zygotes to engineer genome editing.

To optimize experimental conditions for efficient genome editing by CRISPR-EZ, we targeted exon 1 of the *Tyr* gene (Fig. 1*B*), an essential enzyme for pigment synthesis (24). We generated the *Tyr* sgRNA using a cloning-free *in vitro* transcription method (supplemental Fig. S1*A*) (37, 45), which was then assembled with purified Cas9 protein to form Cas9/sgRNA RNPs. Prior to electroporation, we collected C57B6/J mouse zygotes from superovulated females and subjected them to a brief treatment with acid Tyrode's solution to weaken the zona pellucida and facilitate Cas9 RNP delivery. We then combined 30–40 pre-treated mouse zygotes with assembled Cas9/sgRNA RNPs for each electroporation and cultured the electroporated zygotes to the morula stage for genotyping analysis or to the two-cell stage for oviduct transfer to pseudopregnant mothers (Fig. 1*A*). Successful NHEJ-mediated *Tyr* editing was predicted to ablate a HinfI restriction site 1-nt upstream of the PAM (protospacer adjacent motif) (Fig. 1*B*), allowing us to design an RFLP assay to distinguish among bi-allelic editing, partial editing, and unedited mouse embryos. Partially edited embryos could contain either mono-allelic or mosaic editing.

To optimize experimental conditions for CRISPR-EZ, we determined the NHEJ editing efficiency and embryo survival rate at different RNP concentrations (8 or 16 μM) using multiple electroporation conditions (two pulses of electroporation at 1-, 3-, and 10-ms pulse length) (Fig. 1*C*). Electroporated embryos were cultured to the compacted morula stage and subjected to RFLP and sequencing analyses for genotyping (Fig. 1, *C* and *D*). From these experiments, we determined that electroporation pulse length was the critical parameter that impacted the efficiency of genome editing in mouse embryos (Fig. 1*E*). CRISPR-EZ at 1-ms pulse length yielded mostly partially edited embryos, but the 3- and 10-ms pulse length conditions mostly generated bi-allelic editing (83 and 100%, respectively, Fig. 1, *C* and *E*, and Table 2), indicating that a longer electroporation pulse facilitated greater CRISPR editing in mouse embryos. It is possible that a longer pulse permits greater Cas9/sgRNA RNP delivery, hence improving NHEJ editing efficiency. Notably, all embryos exhibited partial or complete editing at 3- or 10-ms pulse length conditions, demonstrating 100% delivery of Cas9/sgRNA RNPs into mouse zygotes at these conditions (Fig. 1*E*). Not surprisingly, a higher concentration of Cas9/sgRNA RNPs in CRISPR-EZ experiments also yielded improved NHEJ editing efficiency (Fig. 1*E*), possibly due to increased Cas9/sgRNA RNP delivery. Although a longer pulse length and/or a greater RNP concentration significantly enhanced the editing efficiency, they compromised embryo viability as fewer embryos developed to the morula stage post-electroporation (Fig. 1*F* and Table 2). This decreased embryo survival rate is possibility due to off-target Cas9 cleavage when excessive Cas9/sgRNA RNPs were delivered to mouse zygotes. Based on our optimizations, CRISPR-EZ experiments using 8 μM Cas9/sgRNA RNP with two pulses of 3-ms electroporation at 30 V achieved an ideal balance between efficient editing and optimal embryo survival (67% bi-allelic editing and 60% survival to morula embryo). However, longer pulse conditions or increased pulse numbers can be used to enable better CRISPR editing efficiency at the cost of embryo viability, when the sgRNA design is suboptimal.

To evaluate the robustness of the CRISPR-EZ technology, we targeted three additional genes, *Cdh1, Cdk8,* and *Kif11,* for NHEJ-mediated editing in mouse embryos. In each case, sgRNAs were designed to target a restriction site 3–4 nt upstream of the PAM, thus allowing us to diagnose NHEJ editing efficiency by RFLP analyses. Although CRISPR-EZ editing efficiency varied across different sgRNA designs, at least 50% of mouse embryos exhibited editing for each experiment, as demonstrated by RFLP analyses and sequencing validation (Fig. 1, *G*

FIGURE 2. **CRISPR-EZ generates HDR-mediated genome modifications in mice.** *A,* diagram illustrating the HDR editing scheme targeting *Tyr* exon 1. A synthesized 92-nt ssDNA donor oligo directs HDR-mediated editing, which replaces the endogenous HinfI restriction site with an EcoRI site, and causes a frameshift mutation to create a premature stop codon in *Tyr* exon 1. *Arrows* indicate the positions of primers that amplify a 200-bp DNA fragment for RFLP genotyping analyses. *B,* RFLP genotyping analyses revealed the successful NHEJ and HDR editing in morula embryos. Nested PCR amplicons (*top row*) were digested with both HinfI (*middle row*) and EcoRI (*bottom row*) to assay for NHEJ editing and HDR editing, respectively. HDR-specific digestion products (~100 bp, migrate as one band) are marked with *black arrowheads*. *C,* representative *Tyr*-edited mouse litters for a microinjection experiment (*left*), a CRISPR-EZ experiment at 1-ms pulse length (*middle*), and a CRISPR-EZ experiment at 3-ms pulse length (*right*). *D,* quantification of coat color phenotypes of live *Tyr*-edited mice generated by microinjection and CRISPR-EZ experiments. *E,* CRISPR-EZ significantly improves mouse viability after genome editing compared with microinjection-based experiments. The University of California at Berkeley transgenic facility averages were calculated based on data collected across recent five CRISPR experiments that inject *cas9* mRNA and sgRNAs for genome editing. *F* and *G,* albino mice obtained from CRISPR-EZ experiments were subjected to RFLP genotyping analyses (*F*) to demonstrate NHEJ and/or HDR editing, and select albino mice were sequence-confirmed (*G*). HDR-specific digestion products (~100 bp, migrating as one band) are marked with *black arrowheads* (*F*). *Red boxes* indicate edited sequences, and *red letters* indicate the HDR-mediated precise modification (*G*). *H,* diagram illustrating the editing scheme to delete exon 3 of the *Mecp2* gene by CRISPR-EZ. Two sgRNAs were designed to direct Cas9 cleavage in *Mecp2* intron 2 and intron 3 to generate the ~720-bp deletion of exon 3. *Arrows* indicate the positions of primers used for PCR genotyping that amplifies across the deleted region. *I* and *J,* representative PCR genotyping analyses (*I*) and sequencing confirmation (*J*) for assessing the editing efficiency of *Mecp2* in mouse morula embryos. *Red boxes* indicate deleted sequences. *K,* diagram illustrating our HDR editing scheme that inserts a V5 epitope tag (42 bp in length) at the 3′ of the *Sox2* open reading frame via a 162-nt ssDNA donor oligo using CRISPR-EZ. *Arrows* indicate the positions of primers that amplify a 180-bp DNA fragment across the edited genomic region. *L* and *M,* PCR genotyping analysis (*L*) and immunofluorescence staining (*M*) for assessing the editing efficiency of the *sox2* gene in mouse morula and blastocyst embryos, respectively. *Scale bar,* 20 μm.

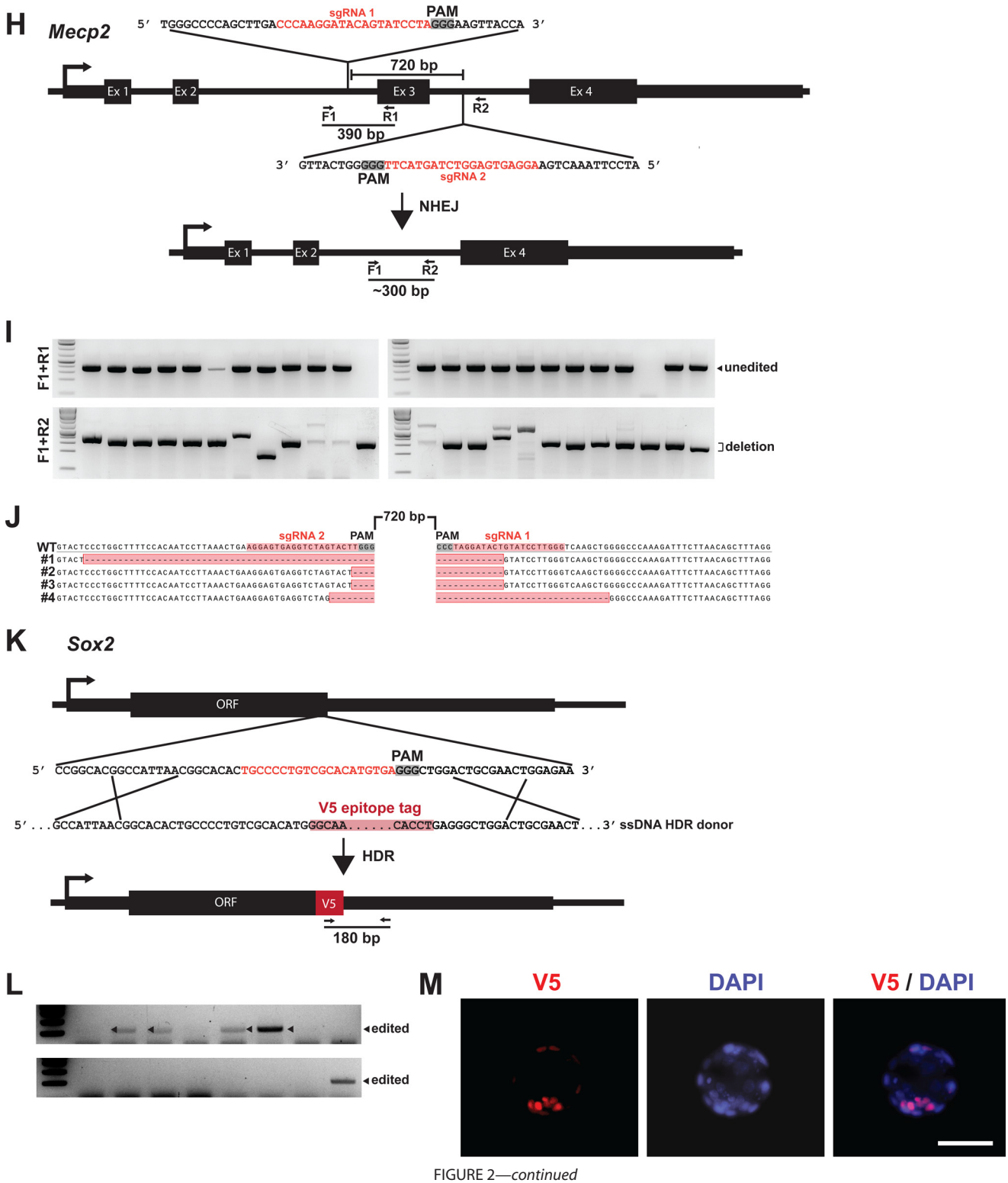

FIGURE 2—*continued*

and *H*, Table 2, and supplemental Fig. S1, *C–D*). Thus, CRISPR-EZ efficiently delivers Cas9/sgRNA RNPs to broadly induce indel mutations through the NHEJ pathway in mouse embryos. Because Cas9/sgRNA RNPs can be delivered to mouse zygotes with 100% delivery efficiency in CRISPR-EZ experiments (Fig. 1*E*), varying editing efficiencies can likely be attributed to sgRNA design.

Although NHEJ-mediated CRISPR editing is a powerful means to engineer loss of function mutations, HDR-mediated CRISPR editing enables more precise and sophisticated editing schemes. To determine the efficiency of HDR-mediated editing in CRISPR-EZ experiments, we designed a 92-nt ssDNA donor oligo that substitutes the endogenous HinfI site in *Tyr* exon 1 for an EcoRI site, causing a frameshift mutation and early

TABLE 4
Efficiency of NHEJ and HDR editing in CRISPR-EZ edited mice

		Type of editing events detected by RFLP analyses					
CRISPR-EZ pulse length	Mice assayed by RFLP	Unedited	Unedited, NHEJ	Unedited, HDR	NHEJ, HDR	Bi-allelic NHEJ	Bi-allelic HDR
1 ms	44	18 (41%)	6 (14%)	2 (5%)	8 (18%)	8 (18%)	2 (5%)
3 ms	31	0	2 (6%)	1 (3%)	11 (35%)	16 (52%)	1 (3%)

termination of the open reading frame (Fig. 2*A*). Using CRISPR-EZ, we co-electroporated Cas9/sgRNA RNPs with the ssDNA donor oligo into mouse zygotes, and we obtained 46% morula embryos that harbored the precise sequence modification we engineered (Fig. 2*B*). Thus, Cas9/sgRNA RNPs, together with an ssDNA HDR donor, can be delivered into mouse zygotes by CRISPR-EZ to achieve highly efficient HDR-mediated editing.

As Tyr is the rate-limiting enzyme in pigment synthesis (47, 48), the extent of albino coat color in edited mice can be used as a proxy for bi-allelic *Tyr* inactivation. Mosaicism in bi-allelic editing, which was otherwise difficult to determine by RFLP, can be estimated by the extent of coat color mosaicism in the edited animals. To assess *Tyr* editing in live mice, we performed CRISPR-EZ using both 1- and 3-ms pulse length conditions to deliver Cas9/sgRNA RNPs and the ssDNA HDR donor into mouse zygotes, cultured electroporated zygotes to the two-cell stage, and transferred viable two-cell embryos to the oviducts of pseudopregnant mothers. The 3-ms pulse length condition resulted in 100% efficiency in Cas9 RNP delivery, as all live pups exhibited evidence of HDR and/or NHEJ editing in RFLP analyses (Fig. 2*C* and supplemental Fig. S2*A*). Notably, 88% (29/33) of animals appeared completely albino, indicating bi-allelic *Tyr* editing; 9% (3/33) were mosaic with an ~50% albino coat, suggesting that bi-allelic *Tyr* editing occurred after S-phase initiation in one-cell zygotes; and 3% (1/33) had a black coat yet carried an edited *Tyr* allele (Fig. 2*D* and Table 3), suggesting complete or partial mono-allelic *Tyr* editing. We speculate that the timing of Cas9/sgRNA delivery is crucial for efficient gene editing in mice, as any editing events occurring after S-phase initiation in one-cell zygotes could lead to partial editing and less efficient germ line transmission of the edited allele(s). Although slightly increasing the live birth rate (Fig. 2*E*), the 1-ms pulse length condition yielded less efficient editing, with 27% (12/44) albino mice and 27% (12/44) efficiency in HDR-mediated editing. Remarkably, when compared with a standard microinjection-based CRISPR experiment using the same *Tyr* sgRNA, both CRISPR-EZ pulse conditions offered a significant improvement on the live birth rate of edited mice (Fig. 2*E* and Table 3). To better appreciate the exact molecular outcome of these editing events, we then performed RFLP analysis on DNA isolated from tail samples of the mice. For the 3-ms pulse condition, 42% (13/31) of the assayed animals harbored the HDR-mediated precise modification (Fig. 2*F*, supplemental Fig. S2*A* and Table 4), one of which was homozygous HDR-edited. Using sequencing confirmation, all tested albino mice harbored NHEJ and/or HDR editing as determined by RFLP (Fig. 2*G*), including some that appeared to be bi-allelic HDR edited. Six albino mice were tested for germ line transmission, and all tested animals gave rise to progenies with edited *Tyr* alleles confirmed by RFLP analyses (supplemental Fig. S2*B*).

In addition to indel mutations and small sequence modifications, we also successfully applied CRISPR-EZ to generate a genomic deletion (~720 bp in length), and a precise sequence insertion (42 bp in length) in mouse embryos. By co-electroporating RNPs containing two sgRNAs flanking exon 3 of the *Mecp2* gene (Fig. 2*H*) (28), we generated 71% (17/24) morula embryos harboring an ~720-bp deletion of the intervening sequence, as determined by PCR analyses and validated by sequencing (Fig. 2, *I* and *J*). Additionally, using CRISPR-EZ, we introduced Cas9/sgRNA RNPs and a 162-nt ssDNA donor oligo that directed the insertion of a V5 tag sequence (42 bp) to the 3′ end of the *Sox2* ORF (Fig. 2*K*) (28), achieving 31% insertion efficiency (5/16) (Fig. 2*L*). Successful integration of the V5 epitope tag to the endogenous *Sox2* ORF was demonstrated by PCR and by immunofluorescence staining in blastocyst mouse embryos (Fig. 2, *L* and *M*). Thus, CRISPR-EZ allows efficient delivery of Cas9/sgRNA RNPs and ssDNA donors into mouse zygotes to mediate a variety of editing schemes. Taken together, the CRISPR-EZ technology generates both NHEJ- and HDR-edited mice with unprecedented ease, speed, throughput, and efficiency, while significantly improving animal viability.

Discussion

A significant advantage of the CRISPR/Cas9 system is the possibility to bypass embryonic stem cell manipulation by directly engineering the genome of zygotes. The current standard practice requires microinjection of Cas9 DNA or mRNA together with sgRNAs into zygotes; yet this procedure remains rate-limiting due to its laborious and costly nature and high technical barriers. In contrast, CRISPR-EZ utilizes commonly available reagents and equipment, and it can be performed by laboratory personnel with basic training in embryo manipulation. With our optimized conditions, CRISPR-EZ delivers Cas9/sgRNA RNPs into mouse zygotes with 100% efficiency, allowing for a variety of genome editing schemes with unprecedented efficiency, while significantly improving embryo viability compared with microinjection. Finally, electroporation is a rapid procedure that can be applied to many zygotes simultaneously, hence enabling high-throughput production. Given the lengthy, costly, and laborious nature of the microinjection procedure, and its negative impact on embryo/pup viability, CRISPR-EZ is superior to microinjection, and can become the primary methodology for *in vivo* CRISPR editing in mice, and possibly other mammals.

While our manuscript was in preparation, Wang *et al.* (49) described a similar zygote electroporation technology to deliver Cas9/sgRNA RNPs to achieve successful genome editing in mice. These investigators employed different electroporation conditions.

Author Contributions—S. C. designed and performed most CRISPR-EZ experiments, optimized the experimental conditions, analyzed data, generated the figures, and drafted the manuscript. B. L. and A. Y. L. performed experiments and provided essential technical assistance. A. J. M. conceived the initial idea of electroporating Cas9/sgRNA RNPs into mouse zygotes for genome editing, worked with S. C. to optimize the CRISPR-EZ experimental conditions, and drafted the methods of the manuscript. L. H. guided the design of the experiments, established collaborations, and drafted and revised the manuscript.

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