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Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases

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서울대학교 대학원
화학과 생화학 전공
김 종 민

Abstract

Highly efficient gene knockout in mice and zebrafish with RNA-guided endonucleases

Jong Min Kim

Biochemistry, Department of Chemistry

The Graduate School

Seoul National University

Zinc finger nucleases(ZFNs) and transcription activator-like effector nucleases (TALENs) , engineered nucleases, are composed of designable DNA-binding domains and a non-specific nuclease domain and enable a broad range of genomic modification by inducing double-strand breaks (DSBs) that stimulate intrinsic cellular repair mechanisms such as non-homologous recombination (NHEJ) and homologous recombination (HR)

at specific genomic locations. This technology has been described earlier as promising tools for targeted genome engineering in cells and many organisms. Recently, RNA-guided endonucleases (RGENs) derived from bacterial type-II CRISPR/Cas system, have been described as site-specific endonucleases whose specificities are programmed by small RNA components. RGEN also has been applied in cells and organisms as genome engineering tool. Here in this study, injection of RGENs as Cas9 protein: guide RNA complexes or *Cas9* mRNA plus guide RNA into one-cell stage embryos of mice and zebrafish efficiently disrupts a target gene in both species. RGENs efficiently generated germ-line transmittable mutations in up to 93% of newborn mice with minimal toxicity. RGEN-induced mutations in the mouse *Prkdc* gene that encodes an enzyme critical for DNA double strand break repair resulted in immunodeficiency both in F_0 and F_1 mice. I propose that RGEN-mediated mutagenesis in animals will greatly expedite the creation of genetically-engineered model organisms accelerating functional genomic research.

Keywords: RNA-guided endonucleases (RGENs),Cas9, guide RNA, Knockout

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I. Introduction

The clustered regularly interspaced short palindromic repeat (CRISPR)–CRISPR-associated (Cas) system induces an adaptive immune response against invading phages and plasmids in bacteria and archea (Wiedenheft et al. 2012). Cas9, a protein derived from *Streptococcus pyogenes*, is complexed with CRISPR RNA (crRNA), whose sequence is originated from phages and plasmids, and trans-activating crRNA (tracrRNA) to form an active DNA-cleaving endonuclease whose specificity is determined by Watson-Crick base-pairing between crRNA and target DNA of 20 base pairs (bp) in length and by the NGG-trinucleotide protospacer adjacent motif (PAM) recognized by Cas9. A single-guide RNA (sgRNA) generated by fusing crRNA and tracrRNA is also functional and can reprogram Cas9 protein to cleave a specific DNA sequence (Figure. 1) (Jinek et al. 2012). Thus, Cas9 is an RNA-guided endonuclease (RGEN) that protects microbial cells from invading phages and plasmids by digesting their DNA site-specifically, reminiscent of restriction endonuclease-mediated protection of host cells from foreign genetic elements. Unlike restriction endonucleases, however, the Cas9

specificity is readily reprogrammed by replacing crRNA, making these nucleases ideal tools for genome engineering in higher eukaryotic cells and organisms.

Recent studies have shown that Cas9-derived RGENs is, indeed, a new member in the growing family of genome editing nucleases (Bassett et al. 2013; Chang et al. 2013; Cho et al. 2013a; Cong et al. 2013; DiCarlo et al. 2013; Friedland et al. 2013; Hwang et al. 2013b; Jiang et al. 2013; Jinek et al. 2013; Mali et al. 2013; Shen et al. 2013; Wang et al. 2013; Xiao et al. 2013), which include zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). These programmable nucleases induce site-specific DNA double-strand breaks (DSBs) in cells, whose repair via high-fidelity homologous recombination or error-prone non-homologous end-joining (NHEJ) gives rise to targeted mutations (Bibikova et al. 2003; Kim et al. 2009; Kim et al. 2010; Miller et al. 2010) or chromosomal rearrangements (Brunet et al. 2009; Lee et al. 2009; Lee et al. 2011). For example, Shen et al. (2013) demonstrated that RGENs induced mutations in a reporter gene in transgenic mouse lines. Wang et al. (2013) used RGENs to induce multiple mutations in a single step in mice. Although these initial pioneering results are encouraging, it is unknown whether and how efficiently the RGEN-induced mutations detected in embryos or pups can be transmitted to the next generation, a process required for the establishment of gene-knockout (KO) animals.

Here, This studies show that the injection of Cas9 protein complexed with guide RNA or *Cas9* mRNA plus guide RNA into one-cell embryos gives rise to germline-transmittable mutations in vertebrate animals.

II. Materials and Methods

1. RGEN components.

Cas9 mRNA and sgRNAs were synthesized *in vitro* from linear DNA templates using the mMESSAGE mMACHINE T7 Ultra kit (Ambion) and MEGAshortscript T7 kit (Ambion), respectively, according to the manufacturers' instructions, and were diluted with appropriate amounts of diethyl pyrocarbonate (DEPC, Sigma)–treated injection buffer (0.25 mM EDTA, 10 mM Tris, pH 7.4). Templates for sgRNA synthesis were generated using oligonucleotides listed in Supplemental Table 5 as described previously (Cho et al. 2013a). Recombinant Cas9 protein (Cho et al. 2013a) was obtained from ToolGen, Inc.

2. In vitro cleavage reactions.

Restriction enzyme-treated linearized plasmid (5nM) was incubated for 60 min at 37 ℃ with Cas9 protein (20 nM), and sgRNA (40 nM) in 1X NEB 3 buffer. Reactions were stopped with 6X stop solution containing 30% glycerol, 1.2% SDS, and 100 mM EDTA. Products were resolved with 1% agarose gel electrophoresis and were visualized with ethidium bromide (EtBr) staining.

3. Microinjection of RGENs into mouse embryos.

All animal experiments were performed in accordance with the Korean Food and Drug Administration (KFDA) guidelines. Protocols were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) of the Laboratory Animal Research Center at Yonsei University (Permit Number: 2013-0099). All mice were maintained in the specific pathogen–free facility of the Yonsei Laboratory Animal Research Center.

FVB/NTac (Taconic) and ICR (DBL, Korea) mouse strains were used as embryo donors and foster mothers, respectively. Female FVB/NTac mice (7–8 weeks old) were super-ovulated by intra-peritoneal injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma) and 5 IU human

chorionic gonadotropin (hCG, Sigma) at 48-hour intervals. The super-ovulated female mice were mated to FVB/NTac stud males, and fertilized embryos were collected from oviducts.

Cas9 mRNA and sgRNAs in M2 medium (Sigma) were injected into the cytoplasm of fertilized eggs with well-recognized pronuclei using a Piezo-driven micromanipulator (Prime Tech). The recombinant Cas9 protein: *Foxn1*-sgRNA complex was diluted with DEPC-treated injection buffer (0.25 mM EDTA, 10 mM Tris, pH 7.4) and injected into male pronuclei using a TransferMan NK2 micromanipulator and a FemtoJet microinjector (Eppendorf). The manipulated embryos were transferred into the oviducts of pseudopregnant foster mothers to produce live animals, or were cultivated *in vitro* for further analyses.

4. Fluorescent PCR.

100 ng of genomic DNA were amplified by PCR using 5′-6-carboxyfluorescein (6-FAM)-labeled primers (F2) (Supplemental Table 6). PCR products were resolved using ABI 3730xl DNA analyzer, and fPCR results were analyzed using Peak scanner v1.0.

5. Genotyping, sequence analyses, and phenotyping of mutant mice.

To screen F_0 mice and *in vitro* cultivated mouse embryos with RGEN-induced mutations, T7E1 assays were performed as previously described using genomic DNA samples from tail biopsies and lysates of whole embryos (Cho et al. 2013a). Briefly, the genomic region encompassing the RGEN target site was PCR-amplified, melted, and re-annealed to form heteroduplex DNA, which was treated with T7 endonuclease 1 (New England Biolabs), and then analyzed by agarose gel electrophoresis. For the newborn mice that were negative in the T7E1 assays, additional T7E1 assays were conducted by mixing equal amounts of wild-type PCR products. Potential off-target sites were identified by searching with Bowtie 0.12.9 (Langmead et al. 2009) and were also similarly monitored by T7E1 assays. The primer paired used in T7E1 assays were represented on Supplemental Tables 6 and 7.

Mutant founders identified by the T7E1 assay were further analyzed by fPCR. For routine PCR genotyping of F_1 progenies, the following primer pairs were used for both wild-type and mutant alleles: 5'-

CTACTCCCTCCGCAGTCTGA-3' and 5'-CCAGGCCTAGGTTCCAGGTA-3' for the *Foxn1* gene, 5'-CCCCAGCATTGCAGATTTCC-3' and 5'-AGGGCTTCTTCTCTACAATCACG-3' for *Prkdc* gene.

6. Characterization of immune cells by flow cytometry.

BALB/c mice were purchased from DBL (Korea), and C.B.-17 scid mice (C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid}) were imported from Taconic. Lymphocytes were prepared and processed as previously described (Park et al. 2000). PerCP-, APC-, V605-, V421-, PE-Cy7- and PerCP-Cy5.5-conjugated antibodies specifically detecting CD3e (145-2C11), CD19 (1D3), CD4 (RM4-5) and CD8 (53-6.7), respectively were purchased from BD Biosciences or Biolegend. Flow cytometry was performed with FACS Calibur and FACS CantoII (BD Biosciences), and the data was analyzed using the FlowJo software (Tree Star).

7. Procedures of RGEN-mediated mutagenesis in zebrafish embryos.

Wild-type and *Tg[huC:GFP]* transgenic zebrafish were maintained at 28.5 ℃ on a 14-hour light/10-hour dark cycle. Embryonic stages were determined by the post-fertilization hour and microscopic observation. Animal work was approved by the internal animal ethics committee at Chungnam National University (CNU-00191).

One-cell stage zebrafish embryos were injected with the indicated doses of Cas9 protein solution (7 g/ l) containing crRNA (1 g/ l), tracrRNA (2 g/ l), or sgRNA (2.5 g/ l). The injected embryos were incubated at 28.5 ℃ for the indicated points, and their phenotypes and genotypes were examined. Genomic DNA was extracted from developing zebrafish embryos using a genomic DNA purification system (Promega, USA) following the manufacturer's instructions. Targeted genomic loci were amplified from five pooled embryos using primers designed to flank the Cas9 complex target site and cloned into the pGEM T-easy vector (Promega, USA); the cloned segments were then sequenced (Genotech, Korea). T7E1 assays were performed as described above.

III. Results

1. RGEN Design and Production.

To examine the gene knockout potential of RGENs in pronuclear (PN)-stage mouse embryos, I selected the *forkhead box N1 (Foxn1)* gene, which is important for thymus development and keratinocyte differentiation (Nehls et al. 1996), and the *protein kinase, DNA activated, catalytic polypeptide (Prkdc)* gene, which encodes an enzyme critical for DNA DSB repair and recombination (Taccioli et al. 1998). I designed several sgRNAs specific to exon 2 in the *Foxn1* gene (Figure. 2a) and exon 2 in the *Prkdc* gene (Figure. 3aB). First, I tested the *in vitro* DNA-cleavage activities of RGENs composed of synthetic sgRNA and recombinant Cas9 protein expressed in and purified from *E. coli* (Figure. 2b and Figure. 3b). The *Foxn1*- and *Prkdc*-specific sgRNAs that manifested the highest activities were chosen for the subsequent *in vivo* experiments (#3 and #2, respectively, Figure. 2b and Figure. 3b).

2. Generation of Founder Mice with RGEN-induced Mutations.

To evaluate the genome-editing activity of the *Foxn1*-RGEN, I injected *Cas9* mRNA (10 ng/μl solution) with various doses of the sgRNA into the cytoplasm of PN-stage mouse embryos, and conducted T7 endonuclease I (T7E1) assays (Kim et al. 2009) using genomic DNAs obtained from *in vitro* cultivated embryos (Figure. 4a). Mutant fractions (the number of mutant embryos/the number of total embryos) were dose-dependent, ranging from 31% (1 ng/μl sgRNA) to 87% (100 ng/μl) (Figure. 4b). Sequence analysis confirmed mutations in the *Foxn1* gene; most mutations were small deletions (Figure. 4c), reminiscent of those induced by ZFNs and TALENs (Kim et al. 2013b). Notably, approximately 90% of the RGEN-injected embryos developed to blastocysts and then hatched from the zona pellucida, suggesting that RGENs were not cytotoxic under these experimental conditions (Figure. 4b).

Encouraged by the high mutant frequencies and low cytotoxicity

induced by RGENs, I produced live animals by transferring the mouse embryos into the oviducts of pseudo-pregnant foster mothers (Figures. 5a-c and Table 1). Notably, the birth rates were very high, ranging from 58% to 73%, and were not affected by the increasing doses of *Foxn1*-sgRNA (Table 1). Out of 147 newborns, I obtained 99 mutant founder mice (Table 1). Consistent with the results observed in cultivated embryos (Figure. 4b), mutant fractions were proportional to the doses of *Foxn1*-sgRNA, and reached up to 93% (100 ng/μl *Foxn1*-sgRNA) (Table 1). To generate *Prkdc*-targeted mice and to test whether higher doses of RGENs are tolerated in embryos, I applied a 5-fold higher concentration of *Cas9* mRNA (50 ng/μl) with increasing doses of *Prkdc*-sgRNA (50, 100, and 250 ng/μl). Again, the birth rates were very high, ranging from 37% to 60%, enough to produce a sufficient number of newborns for the analysis (Fig. 6 and Table 1). The mutant fraction was 57% (21 mutant founders among 37 newborns) at the maximum dose of *Prkdc*-sgRNA (Table 1). These birth rates obtained with RGENs were approximately 2- to 10-fold higher than those obtained with TALENs targeting two unrelated genes, which were reported in our previous study (Sung et al. 2013), on par with those of transgenic mouse production (Nagy et al. 2003). These results demonstrate that RGENs are potent gene-disrupting reagents with minimal toxicity.

Fluorescent PCR (Schuelke 2000) (fPCR; Figure. 5b and Figure.

6b, Tables 2 and 3) and Sanger sequencing analyses (Figure. 5c and Figure. 6c, Tables 4-5) revealed distinct characteristics of RGEN-induced mutations in founder mice. Most *Foxn1* mutants were mosaic (22 out of 33 founders, 67%), and exhibited up to 5 alleles (founder #66), indicating that the RGEN activity persisted later than the first cleavage of one-cell embryos (Table 2). Notably, 9 mice (27%) harbored bi-allelic mutations with no wild-type allele. Similar mosaic patterns were also observed in *Prkdc* mutant mice (20/39 founders, 51%; Table 3). In addition, identical mutant alleles were often observed for both *Foxn1* and *Prkdc* genes (Tables 5-6). For example, I identified 3 founder mice with homozygous mutations in the *Foxn1* gene (Table 2).

3. Gene Disruption by Cas9 protein-sgRNA Complex in Mice and Zebrafish.

Alternatively, I directly injected the RGEN in the form of recombinant Cas9 protein harboring a nuclear localization signal (NLS) (0.3 to 30 ng/μl) complexed with *Foxn1*-specific sgRNA (0.14 to 14 ng/μl) into the cytoplasm or pronucleus of one-cell mouse embryos, and analyzed mutations in the *Foxn1* gene using *in vitro* cultivated embryos

(Figure. 7). These injection doses and methods minimally affected the survival and development of mouse embryos *in vitro*: over 70% of RGEN-injected embryos hatched out normally in both experiments. Again, mutant fractions obtained with Cas9 protein injection were dose-dependent, and reached up to 88% at the highest dose via pronucleus injection and to 71% via intra-cytoplasmic injection (Figure. 7a and b). Similar to the mutation patterns induced by *Cas9* mRNA plus sgRNA (Figure. 4c), those induced by the Cas9 protein-sgRNA complex were mostly small deletions (Figure. 7c). These results clearly demonstrate that the proteinaceous RGEN has high gene-disrupting activity in mouse embryos.

I also tested whether proteinaceous RGENs could induce mutations in zebrafish embryos. I designed RGENs that target the exogenous *GFP* reporter gene or an endogenous gene, *microphthalmia-associated transcription factor a* (*mitfa*), which encodes a master regulator of melanocyte development in vertebrates (Figure. 8a and b) (Lister et al. 1999; Park et al. 2000). Injection of up to 8 ng of Cas9 protein complexed with *mitfa*-specific sgRNA did not perturb normal development in >80% of the embryos (Figure. 9). When embryos were injected with Cas9 protein plus *mitfa*- or *GFP*-specific sgRNA, mutations were detected at target sites in a dose-dependent manner (Figure. 8a and

b). A time-course experiment revealed that the Cas9 protein:sgRNA complex triggered mutations as early as at 4 hour post-injection stage, 2 hours faster than did *Cas9* mRNA plus sgRNA (Figure. 8c). A subset of zebrafish embryos co-injected with Cas9 protein:*mitfa*-specific sgRNA manifested defects in melanocyte development (Figure. 11), demonstrating that some of their somatic tissues were nullizygous for the *mitfa* gene. These results indicate that RGENs injected as Cas9 protein:sgRNA complexes can exert sufficient gene-disrupting activities to induce null mutations at both endogenous and exogenous genomic loci at an early developmental stage in zebrafish embryos.

4. Analysis of Off-target Effects of RGENs.

I examined whether the RGENs generated off-target mutations in the mouse genome. I searched for possible off-target sites that included 1- to 3-bp mismatches with the sgRNA target sequence. A total of 4 and 63 such sites were found for the *Foxn1* and *Prkdc* target sequences, respectively, in the entire mouse genome. T7E1 assays were conducted using genomic DNA samples from the founder mice obtained with the highest doses of *Foxn1*- and *Prkdc*-sgRNAs. These founder mice were bi-

allelic or mosaic mutants (Tables 2 and 3). Even though these mutant founders were produced using high levels of RGENs in one-cell embryos, no off-target activities were detected in any of the 4 genomic loci that were highly homologous with the target sequence of *Foxn1*-RGEN or any of the 6 such sites of the *Prkdc*-RGEN (Figures. 12 and 13). Of note is the absence of T7E1-detectable mutations at sites with a single-base mismatch in the putative 11-base seed region near the GG-dinucleotide PAM (e.g., *Prkdc* Off5 and *Foxn1* Off3 in Figure. 13 and Figure. 12) or with a perfect match in the seed region and 2- or 3-base mismatches upstream of the seed region (*Prkdc* Off3, Off4, and Off6 in Figure. 13).

5. Phenotype Analysis and Germ-line Transmission of *Prkdc*-mutant Mice.

Targeted deficiency of *Prkdc* gene in mice results in severe combined immunodeficiency (Taccioli et al. 1998). For the functional validation of RGEN-induced mutations of *Prkdc* gene in mice, I evaluated the immunological phenotypes of F_0 mutant founders using peripheral

blood mononuclear cells (PBMC). The mosaic founder #1 carried a wild-type allele (Table 3), and showed comparable B and T cell populations to those of the wild-type BALB/c mouse (Figure. 14). On the other hand, the mutant founder #47 possessed bi-allelic null mutations in the *Prkdc* gene, and these immune cell populations were significantly lowered in the PBMC (Figure. 14), indicating the functional loss of *Prkdc* gene functions.

To test the germ-line transmission of the mutant alleles of the *Prkdc* gene, I crossed the male founder #25 (Δ269/Δ61/WT) with a female founder #33 (Δ4/+2/WT; Table 3), and monitored the genotypes of F_1 offspring (Figure. 15a). As expected, all the progenies were wild-type, or inherited one or two mutant alleles from their parents (Figure. 15a). I also confirmed the germ-line transmission of *Foxn1* mutant alleles from the *Foxn1* founder #108, and observed the segregation of the mutant alleles in the progenies (Figure. 16).

As expected, the bi-allelic *Prkdc* mutant progeny showed null phenotype (Figure. 15a). Compared to the wild-type BALB/c mouse and the progeny #1 carrying a wild-type allele, the bi-allelic mutant progeny (#7) exhibited defects in B and T cell populations (Figure. 15b and c). Thus, lymphocytes isolated from PBMC and from the primary and secondary lymphoid organs including thymus, spleen, and lymph node were defective in B and T cell populations (Figure. 15b). Compared to

severe combined immunodeficiency (scid) mice, the mutant mouse exhibited phenotypic leakiness. For example, the F_1 progeny had 1.79% $CD4^+$ and 1.07% $CD3^+$ T cells, whereas the T cell populations examined were less than 1% in the scid mouse (Figure. 15b). These small differences between scid and the bi-allelic mutant mice might reflect the differences in their genetic backgrounds, ages, or genotypes. Overall, these results clearly proved the functional loss of the *Prkdc* gene by RGEN-mediated gene knockout in mice.

IV. Discussion

This study provides strong evidence that RGENs hold great promise for generation of knock-out mice and zebrafish. Although there were recent reports describing the generation of mutant mice using RGENs (Shen et al. 2013; Wang et al. 2013), germline transmission of the mutations induced by RGENs has not been confirmed. I showed that RGEN-induced mutant alleles were stably transmitted to F_1 progenies in vertebrate animals, and confirmed the mutant phenotypes using both founder mice and F_1 mutant progenies. In addition, I also showed that recombinant Cas9 protein complexed with sgRNA induced mutations in both mice and zebrafish, which potentially broadens the range of the RGEN application for *in vivo* mutagenesis. Nuclease proteins might have different kinetics and half-lives in cells, when delivered directly, as compared to being produced by gene- or mRNA-mediated expression, potentially alleviating off-target effects (Izmiryan et al. 2011; Gaj et al. 2012). Unlike recombinant ZFNs and TALENs whose purification is cumbersome, time-consuming, and laborious, new proteinaceous RGENs are prepared simply by mixing Cas9 protein with different sgRNAs or

dualRNAs. These features strongly support that RGENs are convenient and efficient tools for gene knockout in model organisms.

The specificity of RGENs is of concern. Because RGENs are derived from prokaryotes, whose genomes are much smaller than those of higher eukaryotes, RGENs may have off-target effects in animals, plants, and cultured human cells such as stem cells. Furthermore, unlike ZFNs and TALENs that function as dimers, which enhance specificity, RGENs act as monomers. Recent *in vitro* studies conducted using cell lines have provided the evidence of off-target mutations induced by RGENs (Cong et al. 2013; Fu et al. 2013) (Hsu et al. 2013; Pattanayak et al. 2013). Fortunately, however, In this study, analyses of off-target effects suggest that RGENs might not be associated with highly frequent off-target mutations *in vivo* or in clones. Unlike *in vitro* experiments using cell populations, *in vivo* experiments analyzed individual offspring or clones. Off-target mutations present in cell populations can be diluted out in individual clones. In addition, some of the rare off-target mutations in the founders can be removed during mating. Although it is possible that the RGENs induced off-target mutations at sites that are not analyzed in this study, our results suggest that RGENs are specific enough to allow targeted mutagenesis in the mouse. A careful choice of target sites that lack highly homologous sequences elsewhere in the genome should be useful for avoiding or minimizing unwanted off-target mutations as much

as possible (Hsu et al. 2013; Kim et al. 2013a).

On the basis of these results, This studies propose that RGEN-mediated *in vivo* mutagenesis will greatly expedite the creation of genetically-engineered model organisms, and thereby accelerate functional genomic research. Recently, three groups (Fujii et al. 2013; Hwang et al. 2013a; Li et al. 2013) have independently reported germline transmission of RGEN-induced mutations in zebrafish, mice and rats, demonstrating broad utility of RNA-guided genome editing in vertebrate model organisms. I also note that recent study used proteinaceous RGENs to disrupt genes in C. elegans (Cho et al. 2013b).

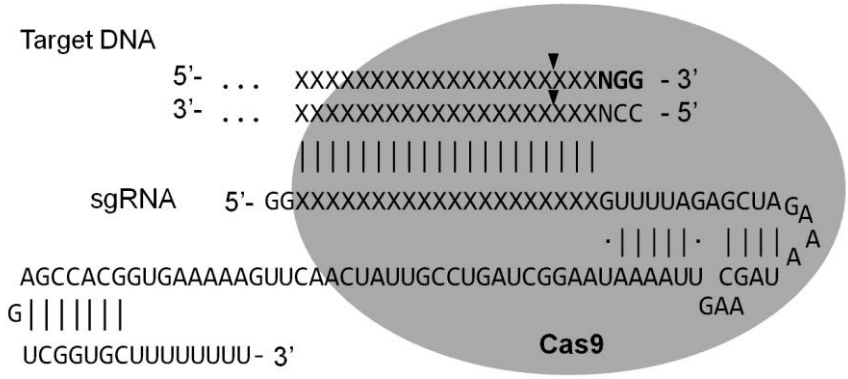
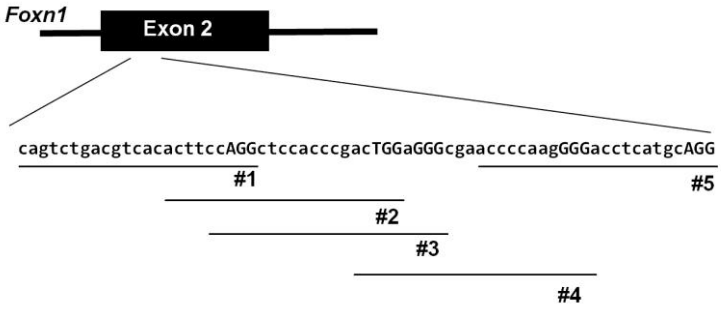


Figure 1. A schematic diagram depicting a target-specific single guide RNA (sgRNA).

a



b

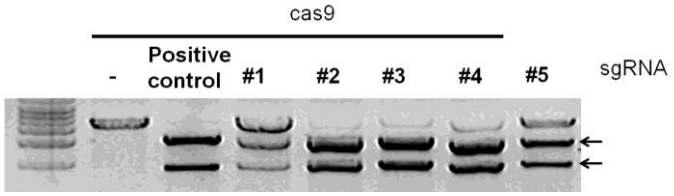
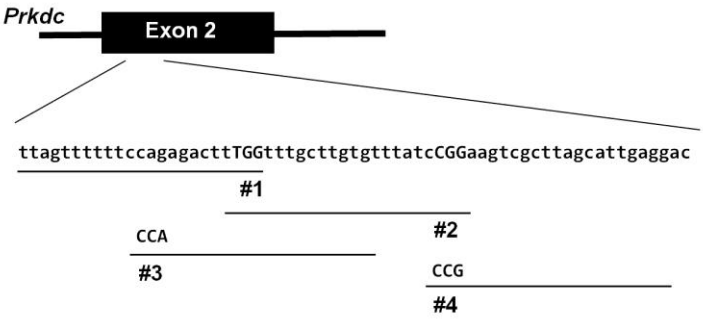


Figure 2. Generation of sgRNAs targeted to exon 2 of the mouse *Foxn1* gene. (a) RGEN target sites in the mouse *Foxn1* gene. PAMs are capitalized, and targets are denoted by black lines. (b) *In vitro* cleavage assays evaluating *Foxn1*-sgRNAs complexed with recombinant Cas9 protein against a linearized plasmid containing target sequences as a substrate. Arrows indicate bands cleaved by RGEN.

a



b

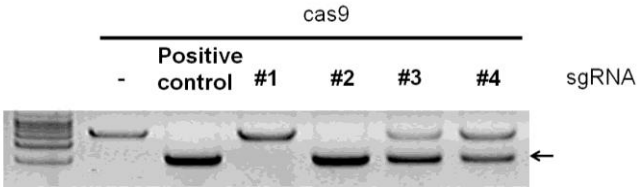
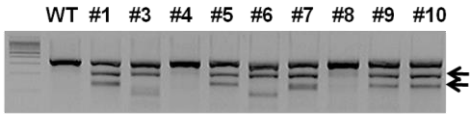


Figure 3. Generation of sgRNAs targeted to exon 2 of the mouse *prkdc* gene. (a) RGEN target sites in exon 2 of the mouse *Prkdc* gene. PAMs are capitalized, and targets are denoted by black lines. (b) *In vitro* cleavage assays evaluating *Prkdc*-RGEN activity. The arrow indicates bands cleaved by RGENs.

a



b

Cas9 mRNA (ng/μl)	sgRNA (ng/μl)	Injected Embryos	Hatched Blastocysts (%)	Genotyped Embryos	Mutants (%)
10	1	20	18 (90)	13	4 (31%)
10	10	29	26 (90)	18	11 (61%)
10	100	19	17 (89)	15	13 (87%)

c

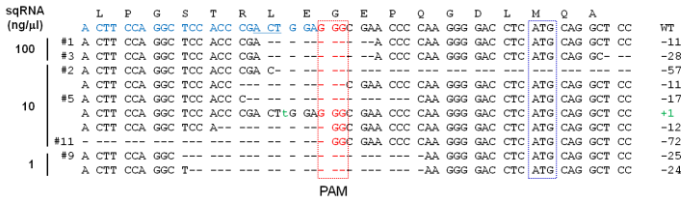
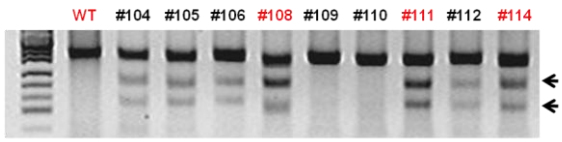


Figure 4. *Foxn1* gene knockout in mouse embryos by intra-cytoplasmic injection of *Cas9* mRNA and *Foxn1*-sgRNA. (a) A representative result of a T7E1 assay monitoring the mutation rate after injecting the highest dose. Arrows indicate bands cleaved by T7E1. (b) A summary of T7E1 assay results. Mutant fractions among *in vitro* cultivated embryos obtained after intra-cytoplasmic injection of the indicated RGEN doses are indicated. The percentages were calculated using the number in the column to its left as the denominator. (c) DNA sequences of *Foxn1* mutant alleles identified from a subset of T7E1-positive mutant embryos. The target sequence of the wild-type allele is denoted in blue.

a



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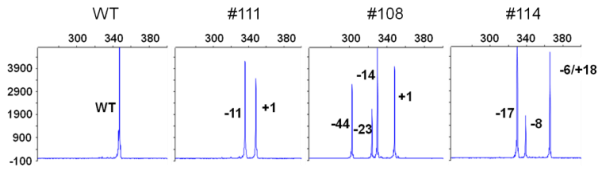
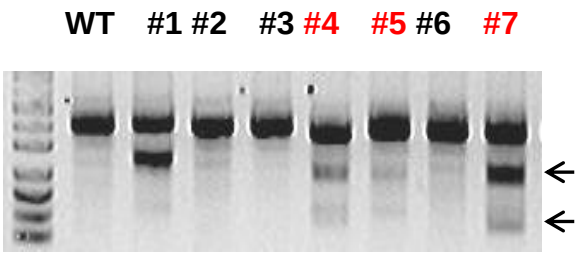


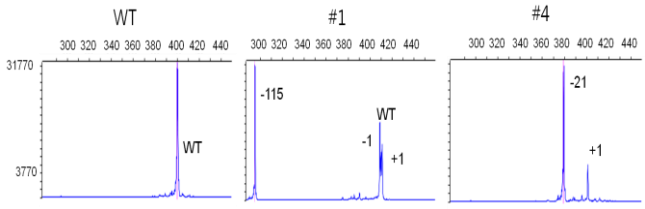
c

```
WT   CCAGGCTCCACCCGACTGGAGGGCGAACCCCAAGGGGACCTCATGCAGG

#108 CCAGGCTCC-------------44bp-----------------------     -44
     CCAGGCTCCACCCGAC----------------------CTCATGCAGG     -23
     CCAGGCTCCACCC-----------------CAAGGGGACCTCATGCAGG     -17
     CCAGGCTCCACCCGACTTGGAGGGCGAACCCCAAGGGGACCTCATGCAGG         +1

#111 CCAGGCTCCACCCGACTTGGAGGGCGAACCCCAAGGGGACCTCATGCAGG         +1
     CCAGGCTCCACCCG-----------AACCCCAAGGGGACCTCATGCAGGG     -11

#114 CCAGGCTCCACCCGACT------CACTATCTTCTGGGCTCCTCCATGTC      -6   +25
     CCAGGCTCCACCC-----------------CAAGGGGACCTCATGCAGG     -17
     CCAGGCTCCACCCGAC--------GAACCCCAAGGGGACCTCATGCAGG     -8
```

Figure 5. RGEN-induced *Foxn1* gene knockout in mice. (a) Representative T7E1 assays demonstrating gene-disrupting efficiencies of *Cas9* mRNA plus *Foxn1*-specific sgRNA that were delivered via intra-cytoplasmic injection into one-cell stage mouse embryos. Numbers indicate independent founder mice generated from the highest dose. Arrows indicate bands cleaved by T7E1. (b) fPCR analysis showing mosaic mutations in the founder mice. (c) DNA sequences of mutant alleles observed in these *Foxn1* mutant founders.

a





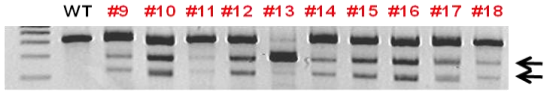
c

```
WT   TTGGTTTGCTTGTGTTTATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT

#1   TTGGTTTGCTTGT------------------------------------------  -115
     TTGGTTTGCTTGTGTTT-TCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATG    -1
     TTGGTTTGCTTGTGT---CTTCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATG  -3  +2
     TTGGTTTGCTTGTGTTTAATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA      +1
     TTGGTTTGCTTGTGTTTATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT   WT

#4   TTGGTTTGCTTGTGTTTCATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA      +1
     TTGGTTTGCTT----------------------AGCATTGAGGACGTAAGTATGA  -21
```

Figure 6. RGEN-induced Prkdc gene disruption in mice. (a) Representative T7E1 assays demonstrating mutagenesis efficiencies of *Cas9* mRNA plus *Prkdc*-specific sgRNA that were delivered via intra-cytoplasmic injection into one-cell stage mouse embryos. Numbers indicate independent founder mice generated from the highest dose. Arrows indicate bands cleaved by T7E1. fPCR results (b) and DNA sequences of mutant alleles (c) observed in three *Prkdc* mutant founders denoted in red in *A*. The red arrowhead in *C* indicates the cleavage site by the RGEN.

a

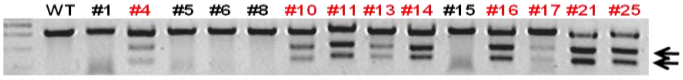
Pronucleous injection



Cas9 protein (nM)	sgRNA (nM)	Tested embryos	Mutants (%)
2	4	20	3 (15%)
20	40	15	5 (33%)
200	400	17	15 (88%)

b

Intra-cytoplasmic injection



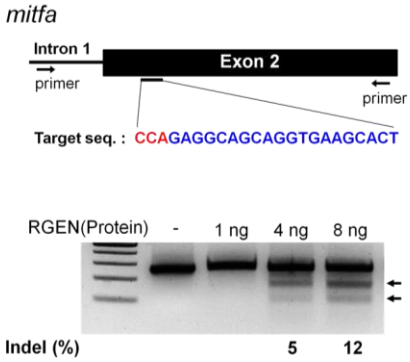
Cas9 protein (nM)	sgRNA (nM)	Tested embryos	Mutants (%)
2	4	18	1 (6%)
20	40	19	6 (32%)
200	400	14	10 (71%)

c

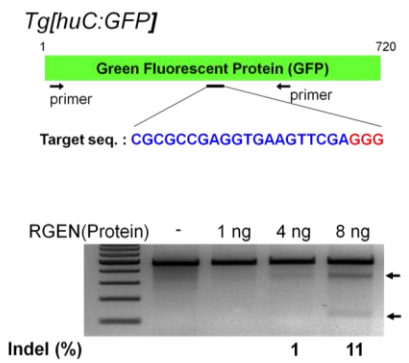
Sequence	Indels	Embryo no.
ACTTCCAGGCTCCACCCGACTGGAGGGCGAACCCCAAGGGGACCTCATGCAG	WT	
ACTTCCAGGCGAACCCC----------AAGGGGACCTCATGCAG	-18	2
ACTTCCAGGCTCCAC----------AAGGGGACCTCATGCAG	-20	1
ACTTCCAGGCTCCACCC----------AAGGGGACCTCATGCCC	-19	1
ACTTCCAGGCTCCACCC----------CAAGGGGACCTCATGCAG	-17	1
ACTTCCAGGCTCCACCCGA----------ACCCCAAGGGGACCTCATGCAG	-11	3
ACTTCCAGGCTCCACCCGAA--GGAGGGCGAACCCCAAGGGGACCTCATGCA	-3+1	1
ACTTCCAGGCTCCACCCGACT--AGGGCGAACCCCAAGGGGACCTCATGCAG	-2	1
ACTTCCAGGCTCCACCCGACTGGGAGGGCGAACCCCAAGGGGACCTCATGCA	+1	1
ACTTCCAGGCTCCACCCGACTTGGAGGGCGAACCCCAAGGGGACCTCATGCA	+1	10
ACTTCCAGGCTCCACCCGA------GGCGAACCCCAAGGGGACCTCATGCAG	-6	1
ACTTCCAGGCTCCACCCGA-----GGGCGAACCCCAAGGGGACCTCATGCAG	-5	2
ACTTCCAGGCTCCACC----------TCATGCAG	-28	1
----------AGGGCGAACCCCAAGGGGACCTCATGCAG	-126	1
Total		26

Figure 7. *Foxn1* gene disruption in mouse embryos using the recombinant Cas9 protein: *Foxn1*-sgRNA complex. (a and b) Representative T7E1 assay results and their summaries. Embryos were cultivated *in vitro* after they underwent pronuclear (a) or intra-cytoplasmic (b) injection. Numbers in red indicate T7E1-positive mutant founder mice. (c) DNA sequences of *Foxn1* mutant alleles identified from the *in vitro* cultivated embryos that were obtained by the pronucleus injection of recombinant Cas9 protein: *Foxn1*-sgRNA complex at the highest dose. The target sequence of the wild-type allele is denoted in blue.

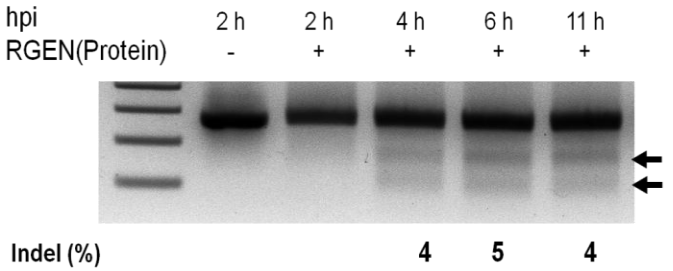
a


mitfa
Intron 1
Exon 2
primer
primer
Target seq. : CCAGAGGCAGCAGGTGAAGCACT
RGEN(Protein) - 1 ng 4 ng 8 ng
Indel (%) 5 12

b


Tg[huC:GFP]
1
720
Green Fluorescent Protein (GFP)
primer
primer
Target seq. : CGCGCCGAGGTGAAGTTCGAGGG
RGEN(Protein) - 1 ng 4 ng 8 ng
Indel (%) 1 11

c


hpi 2 h 2 h 4 h 6 h 11 h
RGEN(Protein) - + + + +
Indel (%) 4 5 4

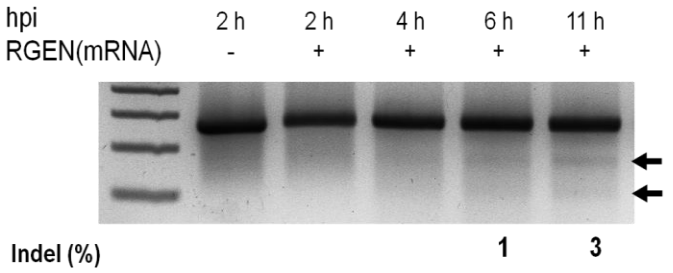

hpi 2 h 2 h 4 h 6 h 11 h
RGEN(mRNA) - + + + +
Indel (%) 1 3

Figure 8. RNA-guided mutagenesis in zebrafish embryos. (a and b) Target sequences (upper) and RGEN-induced indels detected by T7E1 assays (lower). The Cas9 protein-sgRNA complex was microinjected into one-cell stage embryos with concentration of 1 ng, 4 ng, or 8 ng per embryo. (c) Time courses of Cas9 protein:sgRNA and Cas9 RNA:sgRNA injection into embryos during early development. Arrows indicate bands cleaved by T7E1. hpi, hours post-injection.

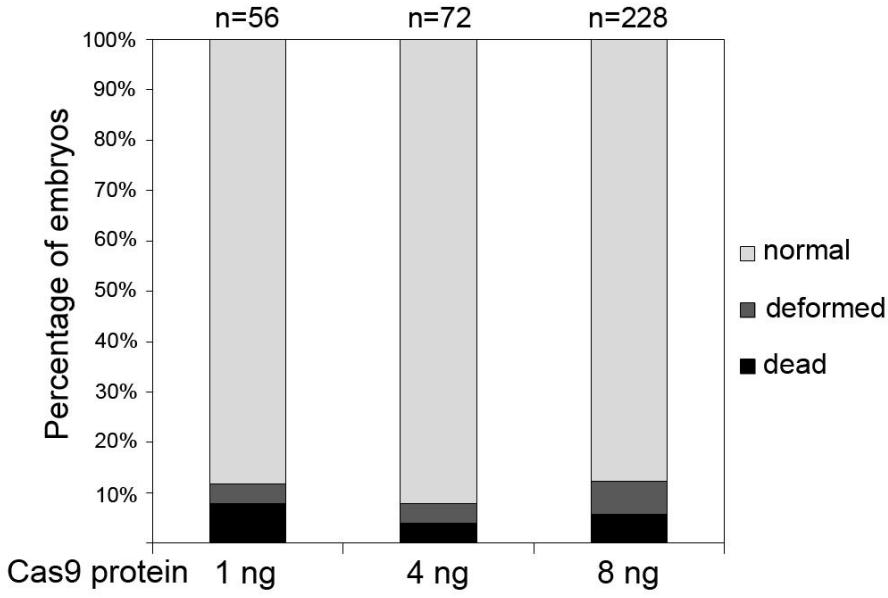
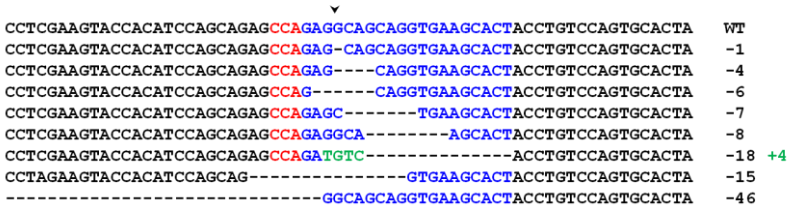


Figure 9. Toxicities of Cas9 protein complexed with *mitfa*-specific sgRNA in zebrafish embryos. The Cas9 protein-sgRNA complex was microinjected into one-cell stage embryos with concentration of 1 ng, 4 ng, or 8 ng per embryo. After one day of injection, the number of normal, deformed, or dead embryos was scored.

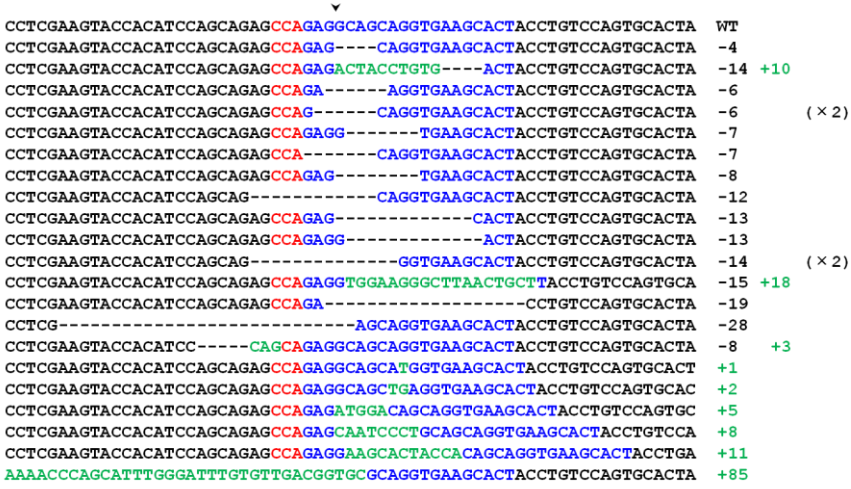
mifta specific sgRNA plus Cas9 protein (4 ng/ embryo)

Mutations in 8 out of 14 sequenced alleles (57%)



mifta specific sgRNA plus Cas9 protein(8 ng/ embryo)

Mutations in 23 out of 32 sequenced alleles (72%)



GFP specific sgRNA plus Cas9 protein in *Tg[huC:GFP]* transgenic fish (4 ng/ embryo)

Mutations in 5 out of 6 sequenced alleles (83%)

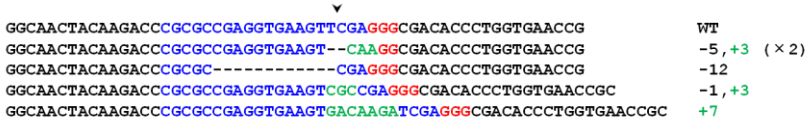


Figure 10. Targeted indel mutations induced by Cas9 protein complex at *mitfa* and GFP reporter gene. The wild-type sequence is shown at the top, and target site in blue, and the PAM motif in red; Inserted bases highlighted as green. The column on the right indicates the number of inserted or deleted bases. Arrowheads indicate the expected RGEN cleavage site.

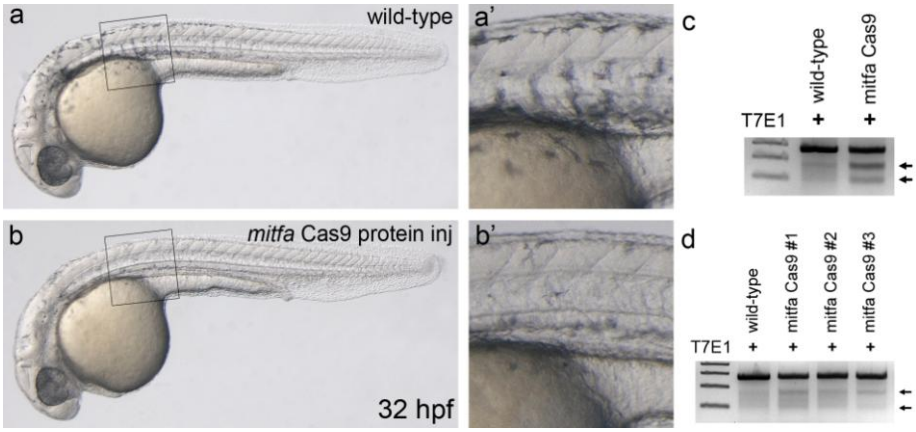


Figure 11. Induction of somatic *mitfa* mutations by the Cas9 protein complex. (a) Un-injected wild-type embryo at 32 hpf. (b) Injection of *mitfa* Cas9 protein complex causes a defect in melanocyte development. (a') and (b') is a high-magnification in black box in (a) and (b), respectively. (c) T7E1 assay of wild-type (a) or *mitfa* Cas9 protein (b)-injected embryo. (d) T7E1 assay of *mitfa* Cas9 protein-injected one-month old embryos. The somatic *mitfa* mutations remain constant in Cas9 protein-injected embryos (#1-#3) by one-month. Arrows indicate the cleavage bands by T7E1.

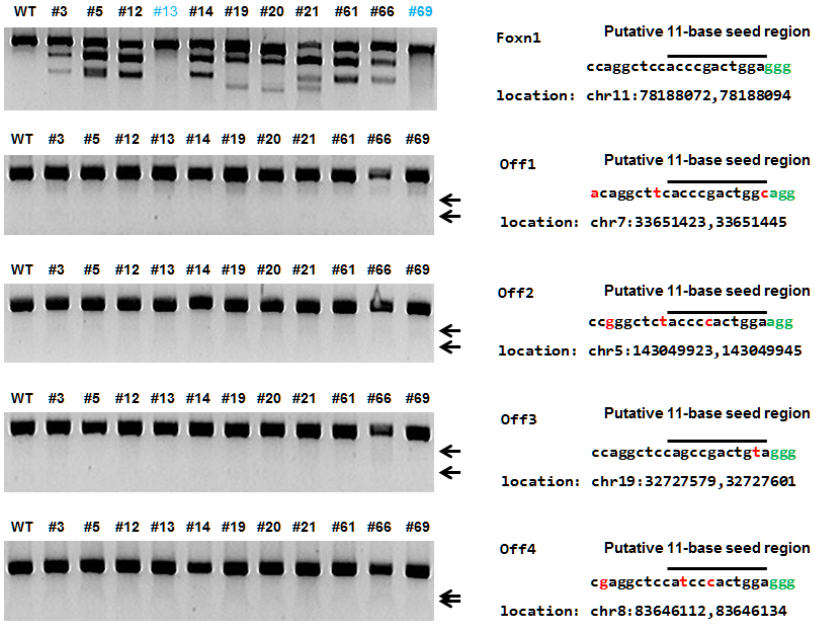


Figure 12. Analysis of off-target activity of *Foxn1*-RGEN. T7E1 assays were conducted using DNA from the indicated *Foxn1* mutant founders. The *Foxn1* gene and putative off-target sites bearing 1~3-bp mismatches that were selected with bowtie 0.12.9 (25) were examined. Homozygous mutants or bi-allelic mutants containing large insertion are shown in blue (Table 2), mismatched bases in red, and PAM in green; black lines indicate putative 11-bp seed regions.

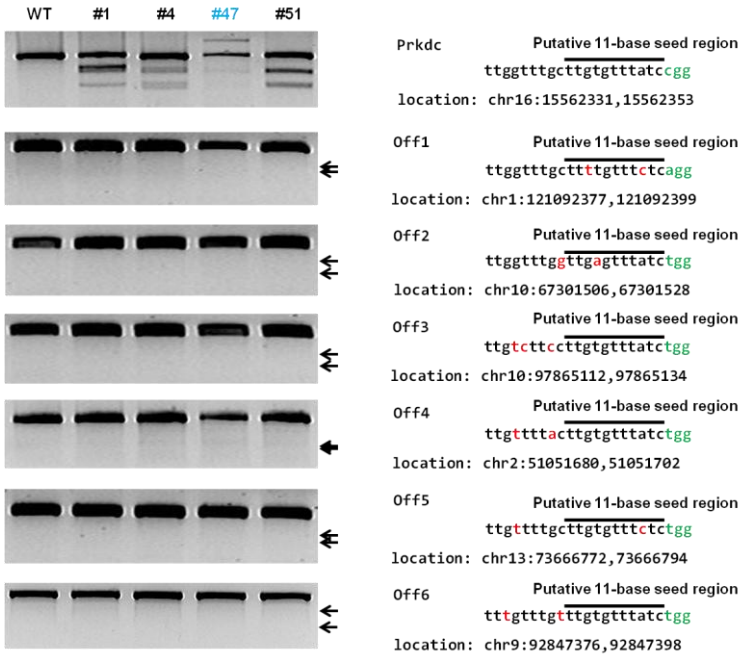
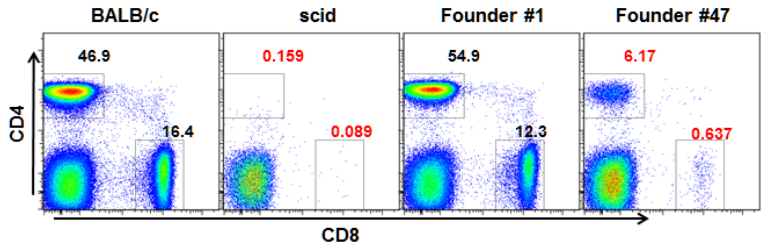


Figure 13. Analysis of off-target activity of RGENs in *Prkdc*-mutant founders. T7E1 assays were conducted using genomic DNA samples from the indicated *Prkdc* mutant founders. The *Prkdc* gene and putative off-target sites bearing 1~3-bp mismatches that were selected with Bowtie 0.12.9 (Langmead et al. 2009) were examined. Bi-allelic mutants containing large insertion are shown in blue (Table 3), mismatched bases in red, and PAM in green; black lines indicate putative 11-bp seed regions.

a



b

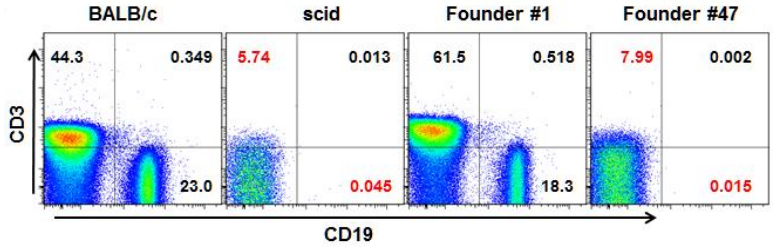


Figure 14. Defective immune cell populations in PBMC from a bi-allelic *Prkdc* mutant founder #47. (a) Frequency of $CD4^+$ and $CD8^+$ T cells. (b) Frequencies of T cells ($CD3^+$) and B cells ($CD19^+$). The abnormal frequencies of immune cells were denoted in red. BALB/c and scid mice, positive and negative controls for B and T lymphocytes, respectively; Founder #1, mosaic mutation (Δ115/Δ3+2/+1/wild-type); founder #47, bi-allelic mutation (Δ14/ +455).

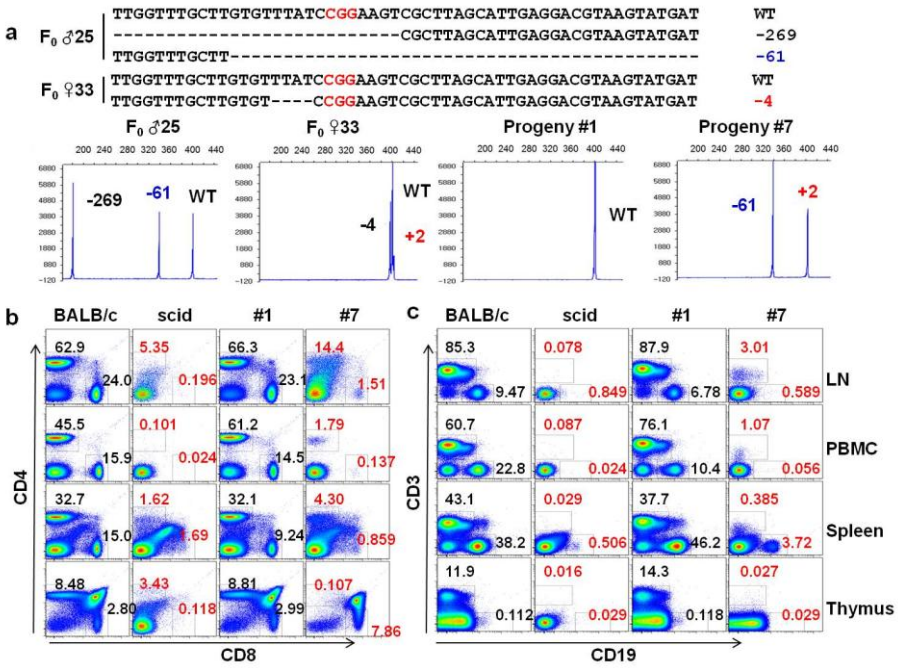
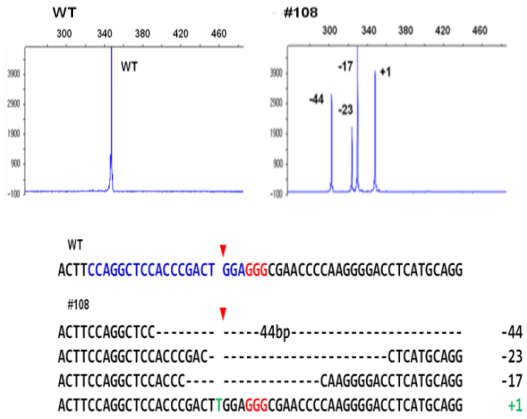


Figure 15. Germ-line transmission and mutant phenotypes of the *Prkdc* mutant mouse. (a) Detailed genotypes of F_0 mutants (♂25 and ♀33, upper panel) and determination of genotypes of their F_1 progenies (#1 and #7) by fPCR (lower panel). Using lymphocytes isolated from peripheral blood (PBMC), lymph node (LN), spleen, and thymus, frequencies of $CD4^+$ and $CD8^+$ T cells (b) and those of $CD3^+$ T cells and $CD19^+$ B cells (c) were determined. The abnormal frequencies of immune cells were denoted in red. BALB/c and scid mice, positive and negative controls for B and T lymphocytes, respectively.

a



b

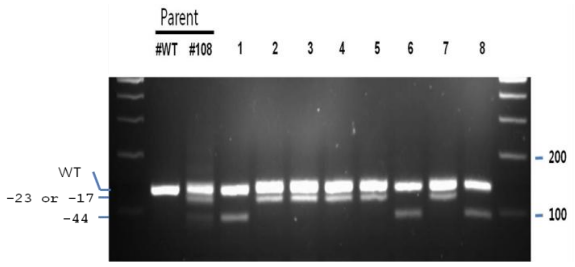


Figure 16. Germ-line transmission of the mutant alleles found in *Foxn1* mutant founder #108. (a) fPCR results of wild-type and the founder mice (upper panel) and the sequences of mutant alleles (lower panel). (b) PCR genotyping of F_1 progenies derived from crossing *Foxn1* founder #108 and wild-type FVB/NTac. Note the segregation of the mutant alleles found in *Foxn1* founder #108 in the progenies. The number of occurrences of the sequences is shown in parentheses.

Table 1. RGEN-mediated gene knockout in FVB/NTac mice.

Target Gene	*Cas9* mRNA + sgRNA (ng/μl)	Injected embryos	Transferred embryos (%)	Total newborns (%)	Live newborns* (%)	Founders† (%)
Foxn1	10 + 1	76	62 (82)	45 (73)	40 (65)	13 (33)
	10 + 10	104	90 (87)	52 (58)	49 (54)	32 (65)
	10 + 100	100	90 (90)	62 (69)	58 (64)	54 (93)
	Total	280	242 (86)	159 (66)	147 (61)	99 (67)
Prkdc	50 + 50	73	58 (79)	35 (60)	33 (57)	11 (33)
	50 + 100	79	59 (75)	22 (37)	21 (36)	7 (33)
	50 + 250	94	73 (78)	37 (51)	37 (51)	21 (57)
	Total	246	190 (77)	94 (49)	91 (48)	39 (43)

Percentages were calculated using the number in each column as the numerator and the number in the column to its left as the denominator. *After eliminating the numbers of pups that were cannibalized at birth, the percentages were calculated using the numbers of transferred embryos as the denominator. †Determined by T7E1 assays.

Table 2. Genotypes of *Foxn1* mutant mice.

Founder NO.	sgRNA (ng/μl)	Genotyping Summary	Detected alleles
58*	1	not determined	-11
19	100	bi-allelic	-60/+1
20	100	bi-allelic	-67/Δ19
13	100	bi-allelic	-18/+455
32	10	bi-allelic	-13/-15+1
115	10	bi-allelic	-18/Δ5
111	10	bi-allelic	-11/+1
110	10	Homozygote	-8/-8
120	10	Homozygote	+2/+2
69	100	Homozygote	-11/−11
81	100	Heterozygote	+1/WT
55	1	Mosaic	-18/-1/+1/+3
56	1	Mosaic	-127/-41/-2/+1
127	1	Mosaic	-18/+1/WT
53	1	Mosaic	-11/-5/-4+1/WT
27	10	Mosaic	-17/-5/WT
29	10	Mosaic	-18/-20+1/+1
95	10	Mosaic	-18/-14/-8/Δ4
108	10	Mosaic	+1/-17/-23/Δ44
114	10	Mosaic	-17/-8/-6+25
124	10	Mosaic	-11/-15+2/+1
126	10	Mosaic	-17/Δ2+3/Δ12+6
12	100	Mosaic	-30/-28/-17/+1
5	100	Mosaic	-28/-11/-2+6/+1
14	100	Mosaic	-17/-11/-10
21	100	Mosaic	-127/-41/-2/-6+25
24	100	Mosaic	-17/+1/WT
64	100	Mosaic	-31/-21/+1/WT
68	100	Mosaic	-17/-11/+1/WT
79	100	Mosaic	-22/-5/+2/WT
61	100	Mosaic	-21+4/-6/+1/+9
66**	100	Mosaic	-17/-8/-11+6/+1/WT
3	100	Mosaic	-11/-8/+1

Underlined alleles were sequenced.

Alleles in red, detected by sequencing, but not by fPCR.

*only one clone sequenced.

**Not determined by fPCR.

Table 3. Genotypes of *Prkdc* mutant mice.

Founder no.	sgRNA (ng/µl)	Genotyping Summary	Detected alleles
7*	250	not determined	-21/WT
4	250	bi-allelic	-21/+1
47	250	bi-allelic	-14/ +455
37	50	Heterozygote	+2/WT
39	50	Heterozygote	+3/WT
70	50	Heterozygote	-11/WT
71	50	Heterozygote	-12/WT
72	50	Heterozygote	-12/WT
90	50	Heterozygote	-2/WT
53	100	Heterozygote	-10/WT
67	100	Heterozygote	-2/WT
8	250	Heterozygote	+2/WT
14	250	Heterozygote	-6/WT
16	250	Heterozygote	-8/WT
17	250	Heterozygote	-21/WT
23	250	Heterozygote	-13/WT
44	250	Heterozygote	-2/WT
45	250	Heterozygote	-21/WT
48	250	Heterozygote	-7/WT
76	50	Mosaic	+1/+2/WT
80	50	Mosaic	-4/-4+252/WT
81	50	Mosaic	-4/-12/WT
85	50	Mosaic	-21/-3+1/WT
86	50	Mosaic	-4/+1/WT
33	100	Mosaic	-4/+2/WT
54	100	Mosaic	-7+28/-12+30/WT
64	100	Mosaic	-3+97/-4/WT
66	100	Mosaic	-3/+1/WT
55	100	Mosaic	-7/-6+2/WT
1	250	Mosaic	-115/-3+2/+1/WT
5	250	Mosaic	-4/+5/+9/WT
11	250	Mosaic	-2/+2/WT
12	250	Mosaic	-14/+1/WT
15	250	Mosaic	-5+1/+7/+12/WT
18	250	Mosaic	-1+32/-4/WT
25	250	Mosaic	-269/-61/WT
26	250	Mosaic	-6+2/-2/WT
50	250	Mosaic	-13/-2/-1/WT
51	250	Mosaic	-15/Δ12/-4

Underlined alleles were sequenced.

Alleles in red, detected by sequencing, but not by fPCR.

*Not determined by fPCR.

Table 4. DNA sequences of *Foxn1* mutant alleles identified from a subset of T7E1-positive mutant founders.

ACTTCCAGGCTCCACCCGACTGGAGGGCGAACCCCAAGGGGACCTCATGCAGG	del+ins	#	Founder mice
ACTTCCAGGC----------AACCCCAAGGGGACCTCATGCAGG	-19	1	20
ACTTCCAGGC----------GAACCCCAAGGGGACCTCATGCAGG	-18	1	115
ACTTCCAGGCTCC----------	-60	1	19
ACTTCCAGGCTCC----------	-44	1	108
ACTTCCAGGCTCC----------CAAGGGGACCTCATGCAGG	-21	1	64
ACTTCCAGGCTCC----------TTAGGAGGCGAACCCCAAGGGGACCTCA	-12+6	1	126
ACTTCCAGGCTCCACC----------TCATGCAGG	-28	1	5
ACTTCCAGGCTCCACCC----------CCAAGGGACCTCATG	-21+4	1	61
ACTTCCAGGCTCCACCC----------AAGGGGACCTCATGCAGG	-18	2	95, 29
ACTTCCAGGCTCCACCC----------CAAGGGGACCTCATGCAGG	-17	7	12, 14, 27, 66, 108, 114, 126
ACTTCCAGGCTCCACCC----------ACCCAAGGGGACCTCATGCAG	-15+1	1	32
ACTTCCAGGCTCCACCC----------CACCCAAGGGGACCTCATGCA	-15+2	1	124
ACTTCCAGGCTCCACCC----------ACCCCAAGGGGACCTCATGCAGG	-13	1	32
ACTTCCAGGCTCCACCC--------GGCGAACCCCAAGGGGACCTCATGCAGG	-8	1	110
ACTTCCAGGCTCCACCCT----------GGGGACCTCATGCAGG	-20+1	1	29
ACTTCCAGGCTCCACCCG----------AACCCCAAGGGGACCTCATGCAGG	-11	1	111
ACTTCCAGGCTCCACCCGA----------ACCTCATGCAGG	-22	1	79
ACTTCCAGGCTCCACCCGA----------GGGGACCTCATGCAGG	-18	2	13, 127
ACTTCCAGGCTCCACCCCA----------AGGGGACCTCATGCAGG	-17	1	24
ACTTCCAGGCTCCACCCGA----------ACCCCAAGGGGACCTCATGCAGG	-11	5	14, 53, 58, 69, 124
ACTTCCAGGCTCCACCCGA----------GACCCCAAGGGGACCTCATGCAGG	-10	1	14
ACTTCCAGGCTCCACCCGA-----GGGCGAACCCCAAGGGGACCTCATGCAGG	-5	3	53, 79, 115
ACTTCCAGGCTCCACCCGAC----------CTCATGCAGG	-23	1	108
ACTTCCAGGCTCCACCCGAC----------CCCCAAGGGGACCTCATGCAGG	-11	1	3
ACTTCCAGGCTCCACCCGAC----------GAAGGGCCCCAAGGGGACCTCA	-11+6	1	66
ACTTCCAGGCTCCACCCGAC--------GAACCCCAAGGGGACCTCATGCAGG	-8	2	3, 66
ACTTCCAGGCTCCACCCGAC-----GGCGAACCCCAAGGGGACCTCATGCAGG	-5	1	27
ACTTCCAGGCTCCACCCGAC--GTGCTTGAGGGCGAACCCCAAGGGGACCTCA	-2+6	2	5
ACTTCCAGGCTCCACCCGACT------CACTATCTTCTGGGCTCCTCCATGTC	-6+25	2	21, 114
ACTTCCAGGCTCCACCCGACT----TGGCGAACCCCAAGGGGACCTCATGCAG	-4+1	1	53
ACTTCCAGGCTCCACCCGACT--TGCAGGGCGAACCCCAAGGGGACCTCATGC	-2+3	1	126
ACTTCCAGGCTCCACCCGACTTGGAGGGCGAACCCCAAGGGGACCTCATGCAG	+1	15	3, 5, 12, 19, 29, 55, 56, 61, 66, 68, 81, 108, 111, 124, 127
ACTTCCAGGCTCCACCCGACTTTGGAGGGCGAACCCCAAGGGGACCTCATGCA	+2	2	79, 120
ACTTCCAGGCTCCACCCGACTGTTGGAGGGCGAACCCCAAGGGGACCTCATGC	+3	1	55
ACTTCCAGGCTCCACCCGACTGGAG(+455)GGCGAACCCCAAGGGGACCTCC	+455	1	13

Table 5. DNA sequences of *Prkdc* mutant alleles.

TTGGTTTGCTTGTGTTTATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	del+ins	#	founders
TTGGTTT----------GGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-14	1	47
TTGGTTT----------TCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-11	1	70
TTGGTTTG----------AAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-15	1	51
TAGGTTTG----------TCCGTGTCGTTTGGCATGGAGGACGTTCAACGGAA	-12+30	1	54
TTGGTTTG----TGTTTATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-4	1	81
TTGGTTTGC----------GGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-12	1	72
TTGGTTTGCTT----------AGCATTGAGGACGTAAGTATGAT	-21	4	4, 7, 17, 85
TTGGTTTGCTT----------AGTCGCTTAGCATTGAGGACGTAAGTATGAT	-13	1	23
TTGGTTTGCTT--------CCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-8	1	16
TTGGTTTGCTT------TGATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATG	-6+2	1	26
TTGGTTTGCTTG-----AATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA	-5+1	1	15
TTGGTTTGCTT----------	-61	1	25
TTGGTTTGCTTGT----------	-115	1	1
TTGGTTTGCTTGT----------CGCTTAGCATTGAGGACGTAAGTATGAT	-14	1	12
TTGGTTTGCTTGT-//-ATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-4+252	1	80
TTGGTTTGCTTGTG---ATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-3	1	66
TTGGTTTGCTTGTGT----------CGCTTAGCATTGAGGACGTAAGTATGAT	-12	2	71, 81
TTGGTTTGCTTGTGT-------CGCTTAGCATTGGGGACGCTCAATTGAAGAAGT	-7+28	1	54
TTGGTTTGCTTGTGT----CCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-4	3	33, 64, 86
TTGGTTTGCTTGTGT---GTCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA	-3+1	1	85
TTGGTTTGCTTGTGT---CTTCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATG	-3+2	1	1
TTGGTTTGCTTGTGT--ATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-2	1	44
TTGGTTTGCTTGTGT-ACATATCCGTGTACATATCCGTGTACAGTACATATCCGG	-1+32	1	18
TTGGTTTGCTTGTGTT-/-CCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-3+97	1	64
TTGGTTTGCTTGTGTT--TCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-2	1	26
TTGGTTTGCTTGTGTT/ATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-1+220	1	47
TTGGTTTGCTTGTGTTT--CCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGAT	-2	2	50, 67
TTGGTTTGCTTGTGTTA------AGTCGCTTAGCATTGAGGACGTAAGTATGAT	-6	1	14
TTGGTTTGCTTGTGTTTAATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA	+1	1	1
TTGGTTTGCTTGTGTTTTATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA	+1	3	12, 66, 86
TTGGTTTGCTTGTGTTTCATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATGA	+1	2	4, 76
TTGGTTTGCTTGTGTTTTTATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATG	+2	1	76
TTGGTTTGCTTGTGTTTCCATCCGGAAGTCGCTTAGCATTGAGGACGTAAGTATG	+2	1	8
----------CGCTTAGCATTGAGGACGTAAGTATG	-269	1	25

Table 6. Oligonucletide for preparation of *in vitro* RNA transcription template.

RNA Name	Direction	Sequence (5' to 3')
Foxn1 #1 sgRNA	F	GAAATTAATACGACTCACTATAGGCAGTCTGACGTCACACTTCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Foxn1 #2 sgRNA	F	GAAATTAATACGACTCACTATAGGACTTCCAGGCTCCACCCGACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Foxn1 #3 sgRNA	F	GAAATTAATACGACTCACTATAGGCCAGGCTCCACCCGACTGGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Foxn1 #4 sgRNA	F	GAAATTAATACGACTCACTATAGGACTGGAGGGCGAACCCCAAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Foxn1 #5 sgRNA	F	GAAATTAATACGACTCACTATAGGACCCCAAGGGGACCTCATGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Prkdc #1 sgRNA	F	GAAATTAATACGACTCACTATAGGTTAGTTTTTTCCAGAGACTTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Prkdc #2 sgRNA	F	GAAATTAATACGACTCACTATAGGTTGGTTTGCTTGTGTTTATCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Prkdc #3 sgRNA	F	GAAATTAATACGACTCACTATAGGCACAAGCAAACCAAAGTCTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
Prkdc #4 sgRNA	F	GAAATTAATACGACTCACTATAGGCCTCAATGCTAAGCGACTTCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
eGFP sgRNA	F	GAAATTAATACGACTCACTATAGGCGCGCCGAGGTGAAGTTCGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG
sgRNA Reverse	R	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAAC
mitfa crRNA	F	GAAATTAATACGACTCACTATAGGAGTGCTTCACCTGCTGCCTCGTTTTAGAGCTATGCTGTTTTG
mitfa crRNA	R	CAAAACAGCATAGCTCTAAAACGAGGCAGCAGGTGAAGCACTCCTATAGTGAGTCGTATTAATTTC
TracrRNA	F	GAAATTAATACGACTCACTATAGGAACCATTCAAAACAGCATAGCAAGTTAAAATAAGGCTAGTCCG
TracrRNA	R	AAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATG

[a] F, forward; R, reverse

[b] Sequences complementary to target DNA are shown in blue. The T7 promoter sequence is underlined.

Table 7. Primer sequences used in T7E1 assay and fPCR analysis.

Gene	Direction	Sequence(5' to 3')
Foxn1	F1	GTCTGTCTATCATCTCTTCCCTTCTCTCC
	F2	TCCCTAATCCGATGGCTAGCTCCAG
	R1	ACGAGCAGCTGAAGTTAGCATGC
	R2	CTACTCAATGCTCTTAGAGCTACCAGGCTTGC
Prkdc	F1	GACTGTTGTGGGGAGGGCCG
	F2	GGGAGGGCCGAAAGTCTTATTTTG
	R1	CCTGAAGACTGAAGTTGGCAGAAGTGAG
	R2	CTTTAGGGCTTCTTCTCTACAATCACG
eGFP	F	CAAGGGCGAGGAGCTGTTCACC
	R	CGGCCATGATATAGACGTTGTGG
mitfa	F	GTAGAATGTGAGCTTATTGGCGT
	R	TCTCACAGTTGAGGGTGAGAAGG

V. Reference

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국문 초록

ZFN 및 TALEN은 특정한 DNA 염기서열에 결합하는 단백질 도메인과 비특이적인 뉴클레아제 도메인으로 구성된 효소로써 유전체의 원하는 위치를 절단을 유도하여 세포 내부의 유전자 교정 메커니즘인 Non-homologous recombination (NHEJ)과 Homologous recombination (HR)을 통해 돌연변이를 유발할 수 있다. 최근에 개발된 RNA-guided endonucleases (RGENs) 기술은 박테리아 발견된 뉴클레아제 효소로써 작은 RNA 분자에 의해 서열 특이성을 조절 할 수 있는 유전자 가위이고, 지금까지 다양한 cell과 organism에 대해 적용되어 왔다.

이번 연구에서는 RGEN을 Cas9 단백질과 guide RNA 복합체 또는 Cas9 mRNA와 guide RNA를 mice와 zebrafish의 수정란에 주입함으로써 원하는 유전자를 성공적으로 knockout 할 수 있는 것을 확인하였다. RNA 가위를 이용한 방법 유전자 교정 방법은 최대 태어난 mice의 93%가 돌연변이를 가지고 있는 정도로 높은 유전자 교정효율을 보였을 뿐만 아니라 수정란에서 독성을 보이지 않는 것을

확인하였다. 또한 RNA 가위를 통해 세포내의 유전자교정 기작에 관여하는 효소를 암호화하고 있는 유전자인 *prkdc*를 손상시킴으로써 태어난 mice와 자손 mice에 면역결핍증상이 나타나는 것을 확인하였다. 또한 유전자 가위를 통해 얻어진 돌연변이가 유전자가위를 주입을 통해 태어난 mice 뿐만 아니라 교배를 통해 다음세대로 전달이 가능한 것을 보였다. 이러한 연구 결과를 통해 RNA가위를 이용한 돌연변이 유발 방법이 동물에서도 효과적인 유전자 교정 기술로 적용될 수 있을 것이고, 앞으로 유전체 연구를 좀 더 가속화 할 수 있는 수단으로 활용 될 것으로 전망할 수 있다.

주요어: RNA-guided endonucleases(RGENs), Cas9, guide RNA, Knockout

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