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Efficient gene targeting by TAL effector nucleases coinjected with exonucleases in zygotes

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TAL Effector Nucleases (TALENs) are versatile tools for targeted gene editing in various species. However, their efficiency is still insufficient, especially in mammalian embryos. Here, we showed that combined expression of Exonuclease 1 (*Exo1*) with engineered site-specific TALENs provided highly efficient disruption of the endogenous gene in rat fibroblast cells. A similar increased efficiency of up to ~30% with *Exo1* was also observed in fertilized rat eggs, and in the production of knockout rats for the albino (*Tyr*) gene. These findings demonstrate TALENs with *Exo1* is an easy and efficient method of generating gene knockouts using zygotes, which increases the range of gene targeting technologies available to various species.

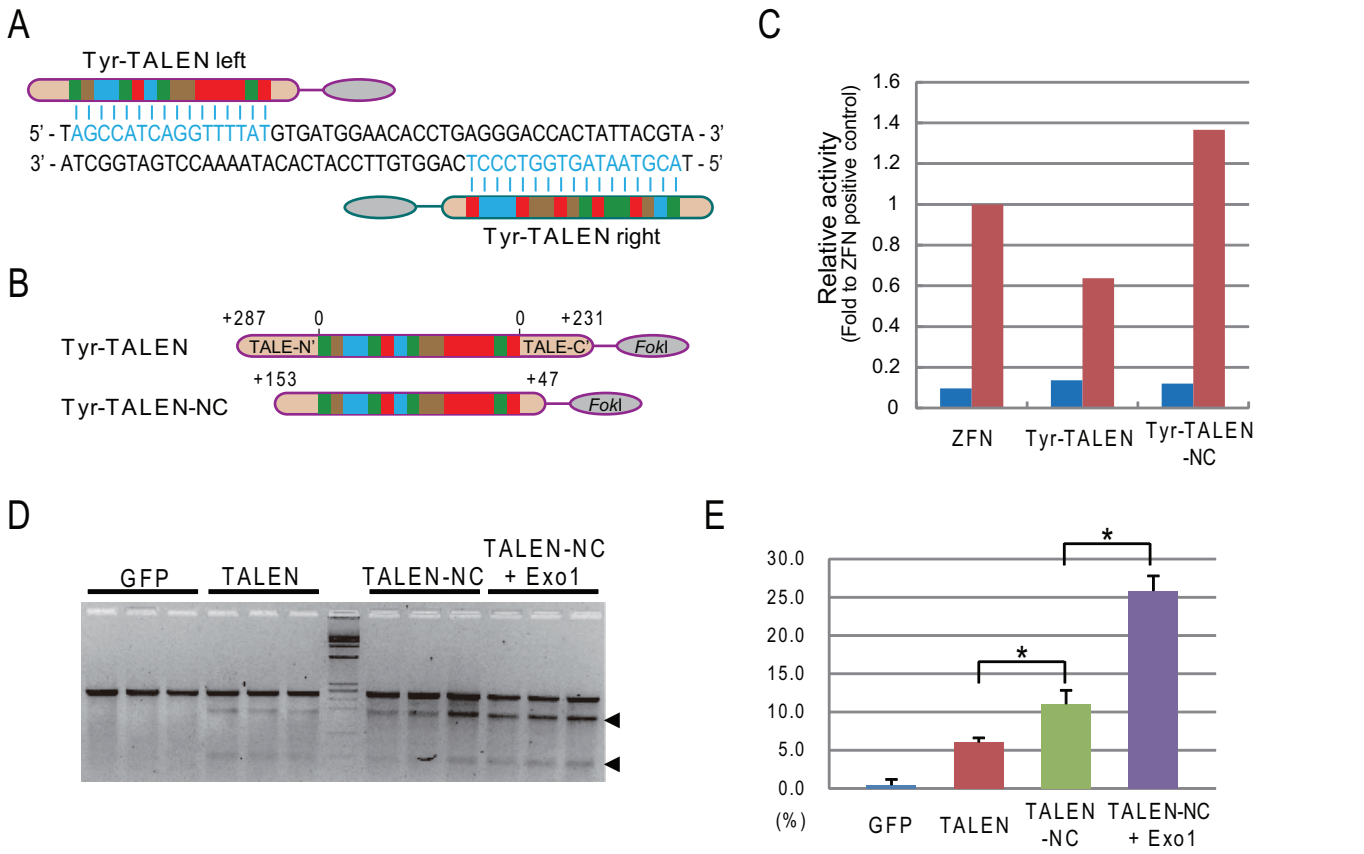
Genetically engineered animals provide a powerful tool for the functional annotation of genes and for modeling human genetic diseases. Advances over the past 20 years in mouse embryonic stem cells and homologous recombination (HR)-mediated targeting have made this species the first choice for modeling human diseases. However, these technologies were only available in mice, and not in other species. Recently, new technologies have been developed and tested for gene disruption, and HR could prove an invaluable tool for the rapid generation of genetically modified animals. These new approaches include zinc-finger nucleases (ZFNs), comprising the DNA-binding domain of zinc-finger proteins fused with the non-specific DNA endonuclease *Fok*I[1–4], and transcription activator-like effector nucleases (TALENs), comprising an engineered array of TAL effector repeats fused to the *Fok*I nuclease domain[5–8]. These engineered nucleases can recognize long stretches of DNA sequences and introduce DNA double-strand breaks (DSBs). DSBs are mainly restored via non-homologous end-joining (NHEJ), a process that introduces small insertions or deletions (indels) at the repair junction, thereby generating mutations at the targeted sequences[1–8]. They are especially useful in previously non-permissive model organisms, such as sea urchins[9], crickets[10], medaka fish[11], or rats[12–14].

ZFNs/TALENs provide a straightforward strategy for targeted gene disruption in zygotes, resulting in rapid and cost-effective knockouts. Although both commercial and open resources are available for the design of ZFN/TALEN reagents, ZFNs present hurdles in terms of cost and protocols, making it difficult to establish ZFNs as a routine laboratory process. The sequences recognized by ZF domains are also limited, whereas TAL effectors can recognize almost any sequence, except T at position 0[5,7,8]. Simple and straightforward design and assembly strategies have been developed for rapid construction of TALENs, providing a cost-effective targeted nuclease platform. Although TALEN technologies have advantages, the technology remains uncertain because its introduction is so recent[6]. Since the original discovery of TAL effectors in the plant pathogenic bacteria, *Xanthomonas*, the system appears to be less effective in rodent embryos, such as mice and rats (unpublished data, personal communication). The larger size of the proteins may also impede the gene targeting efficiency compared with ZFNs, even though the recently reported truncation of the N- and C-terminal regions of the native proteins has increased the TALEN DSB-activity[15]. Improved methodology is needed to further exploit the utility of this technology. For example, a recent report showed that coupling designer endonucleases with DNA end-processing enzymes could improve gene disruption rates in mouse and human cells[16]. To address this issue, we co-expressed Exonuclease 1 (*Exo1*) with engineered TALENs to enhance gene targeting efficiency in rat zygotes.

Results

Construction of TAL effecter nucleases and the single-strand annealing (SSA) assay. Over 100 strains of albino rats deposited into the National Bio Resource Project – Rat (http://www.anim.med.kyoto-u.ac.jp/nbr/) have the same missense mutation (Arg299His) in the Tyrosinase (*Tyr*) gene[17]. The coat-color phenotype is easily and observably distinguishable for TALEN-induced mutations; therefore, we targeted the rat *Tyr* gene. A pair of TALENs was constructed using a two-step assembly method[18] with a Golden Gate TALEN kit, originally established from the Voytas lab[19] (Figure 1A). For the subsequent use of TALENs in the SSA assay, transfection into cultured cells, and mRNA synthesis, the backbone vectors for the second-step assembly were replaced with the mammalian expression vector pcDNA-TAL vector, which has CMV and T7 promoters[18]. The TALEN-based constructs were also replaced with deletion frameworks of +153 N- and +47 C-terminal domains, termed the NC scaffold, as previously reported[15] (Figure 1B).

We then performed a validation test of the mammalian cell-based SSA assay in human embryonic kidney 293T (HEK293T) cells for designed Tyr-TALENs and Tyr-TALENs-NC (Figure 1C).



F

TALEN

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TATAATAGCCATCAGGTTTTATGTGA----ACACCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGG----------------ACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGA-------CCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGATGGAAACACCTGAGGGACCACTATTACATAATCCT
TATAATAGCCA----------------------------CTATTACATAATCCT
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G

TALEN-NC

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TATAATAGCCATCAG----------CTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGA---------GAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGG----------(Δ53)----------GACAA
TATAATAGCCATCAGGTTTTATGTGATG--ACACCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGATGAAACACCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTT----------ACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGT----------TGAGGGACCACTATTACATAATCCT
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H

TALEN-NC + Exo1

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TATAATAGCCATCAGGTTTTATGTGATGG--------AGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGA----------GGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGATG---------AGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGT----------CTGAGGGACCACTATTACATAATCCT
TAT----------TACATAATCCT
TATAATAGCCATCA----------T-GAACACCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTG--------CCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGATG-------TGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGA-------CCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTG---GAA-----GAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATG---TGG-ACACCTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGT----------ATTACATAATCCT
TATAATAGCCATCAGGTTTTATGTGA--GAA---CTGAGGGACCACTATTACATAATCCT
TATAATAGCCATCAGGTTTTATG---------ACCTGAGGGACCACTATTACATAATCCT
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Figure 1 | TALEN constructs and the validation of their activity in rat fibroblasts. (A) Structure of engineered TALENs binding to exon 2 of rat Tyrosinase (*Tyr*) gene. (B) TALEN scaffolds (upper) and N- and C-terminal truncated scaffolds (lower), respectively. (C) Relative TALEN activity measured by a single-strand annealing (SSA) assay in human embryonic kidney 293T (HEK293T) cells. (D) Surveyor (Cel-I) nuclease assay for TALEN-induced mutations in *Tyr*. Arrowheads indicate the expected positions of the digested products. (E) Surveyor assay showing that increased frequency of TALEN-induced mutations by NC truncation and co-expression of *Exo1*. Data are expressed as means ± SEM (n = 3). *P<0.01 by Student's t-tests. (F–H) Sequence analyses showing increased mutation efficiency by NC truncation and *Exo1* expression. Microhomologous sequences adjacent to the breakpoint are underlined for TALEN (F), TALEN-NC (G), and TALEN-NC + *Exo1* (H).

Compared with the positive control (ZFN transfected cells[9]) and negative controls (reporter vectors with unrelated sequences), the cells transfected with Tyr-TALENs showed marked activation of the *luciferase* gene at about 60% of the value of the ZFN positive control. The cells transfected with Tyr-TALENs-NC showed 40% higher activity than the ZFN treated cells, indicating that the custom-engineered TALENs have the cleavage activity and that the NC truncation of TALENs increases the cleavage efficiency in the SSA assay (Figure 1C).

Combination of exonucleases with TALENs increased the efficiency of targeted gene disruption in rat fibroblasts. To assess the activity of the TALEN architectures against an endogenous gene, we electroporated Tyr-TALEN or Tyr-TALEN-NC expression vectors, and the GFP expression vector as a negative control, into Rat-1 fibroblast cells (Table 1). After 24 h, the control cells showed more than 90% GFP-positive cells, indicating sufficient transformation rates. After 72 h, cell numbers were counted, and genomic DNA was extracted and screened for TALEN-induced mutations using the Surveyor (Cel-I) nuclease assay (Figure 1D). Similar to the results of the SSA assay, TALENs-NC showed higher activity than TALENs (11.0% *vs.* 6.1%) in Rat-1 cells (Figure 1E). However, compared with our previous results using ZFNs (mutation rates ~25%[13,20]), the TALEN activity was significantly lower.

To increase the frequency of TALEN-induced mutations, we added vectors expressing the *Exo1* gene. Co-transfection of the *Exo1* vectors and TALEN-NC showed significantly higher activity (25.9%) compared with TALEN-NC alone in the Surveyor assay (Figure 1D, E). Sequence analyses of the *Tyr* loci revealed similar mutation rates to the results of the Surveyor assay: 5.7%, 7.3%, and 17.7% in TALEN, TALEN-NC, and TALEN-NC + *Exo1* transfected cells, respectively (Table 1). There was no difference in the types of indel mutation or their sizes, which ranged from a 1-bp insertion to a 53-bp deletion centered over the TALEN recognition sites (Figure 1F–H).

***Exo1* increases the frequency of TALEN-induced gene disruption in rat zygotes.** To evaluate the gene targeting efficiency of TALENs in zygotes, we microinjected mRNA of the assembled Tyr-TALEN-NC with and without *in vitro* transcribed *Exo1* mRNA into fertilized rat eggs (Figure 2A, B). After 24 h, 30–40% of the TALENs-injected embryos differentiated normally into two-cell embryos with or without *Exo1*. PCR and sequence analyses on the two-cell embryos detected a mutation rate of 5.6% (1/18) in TALEN-NC and 28.6% (4/14) in TALEN-NC with *Exo1*, indicating that *Exo1* increases the frequency of TALEN-induced mutations in zygotes (Figure 2A). Interestingly, PCR and sequence analyses revealed that TALEN-injection into fertilized eggs could introduce homozygous mutations, which could not be detected by the Surveyor assay (Figure 2C, D). This means TALEN-induced mutations created by NHEJ could be induced at the one-cell stage, presumably during the S-phase (the DNA synthesis phase) of the cell cycle, in fertilized eggs.

Coinjection of TALENs mRNA with *Exo1* mRNA into embryos provides efficient generation of knockout rats. To generate knockout rats for the albino locus by TALENs, we again microinjected mRNA for TALENs or TALENs-NC into fertilized eggs of agouti DA rats. Of 328 TALEN-injected eggs, 126 two-cell embryos (38.4%) were transferred into the oviducts of pseudopregnant Wistar female rats, and 29 (23.0%) of these embryos were successfully carried to term (Figure 3A). However, no mutant pup was detected by sequence analyses of the targeted *Tyr* locus. Microinjection of TALENs commercially obtained from Cellectis (Paris, France) delivered two mutant pups among 30 born (6.7%) (Figure 3A). When we coinjected *Exo1* mRNA with TALENs-NC, 29 two-cell embryos were obtained from 68 injected eggs (42.6%) without obvious toxicity, and of 12 pups delivered (41.4%), three founders possessed mutations comprising 5- to 29-bp deletions (25.0%) (Figure 3B, C).

One male founder showed the albino coat-color phenotype over its entire body (Figure 3B), and sequence analyses revealed that this founder carried a homozygous mutation (a 29-bp deletion) at the *Tyr* locus (Figure 3C). When we crossed the founders with the DA strains, the TALEN-induced mutations were faithfully transmitted to the next generation. For example, a male G0 founder showing a mosaic coat-color phenotype was crossed with a DA female to obtain G1 heterozygotes. Subsequent intercrossing between G1 heterozygous males and females produced homozygous albino G2 offspring (Supplementary Fig. S1).

Discussion

As far as we know, this is the first report to show that coinjection of exonuclease *Exo1* with engineered nucleases, such as TALENs, could enhance the frequency of targeted gene disruption in zygotes, and could improve the targeting efficiency to generate knockout animals. This approach provides an easy, rapid and efficient method of generating knockout animals (Supplementary Fig. S2). For example, the custom design and assembly of TALENs and their validation by the SSA assay took 1–2 weeks. Direct injection of the engineered TALENs with pre-transcribed *Exo1* into embryos, transplantation into pseudopregnant foster mothers, and identification of TALEN-induced mutants took 1 month. A homozygous mutation producing an albino G0 founder was observed 3 weeks after birth. It will be interesting to determine how TALENs targeted both alleles to produce the homozygous mutation in zygotes, as has been observed in our previous experiments with ZFNs[13]. In addition, in our *in vitro* and *in embryo* experiments, no overt toxicity was observed when overexpressing *Exo1* in conjunction with TALENs. We also observed no effect of *Exo1* on the cell cycle and cell growth, or on embryo survival and embryo development. Although *Exo1* could increase the number of mutations at off-target sites, TALENs seem to have fewer off-target effects than ZFNs[21]. Although off-target effects might play a role in cell-based experiments, in animals such unknown mutations will be "washed out" by repetitive subsequent backcrossing with the parental strain.

It remains unclear how exonucleases enhance the efficiency of TALEN-induced gene disruption in zygotes. *Exo1* is a 5′–3′ exonuclease that has a major role in DSB-repair by 5′-strand resection of DSB-ends[22–26]. There are two major pathways for DSB-repair: NHEJ and HR. Recently, a third pathway, alternative NHEJ (altNHEJ), or microhomology mediated end-joining (MMEJ), was shown to repair DSBs in the absence or failure of the classical NHEJ (cNHEJ)

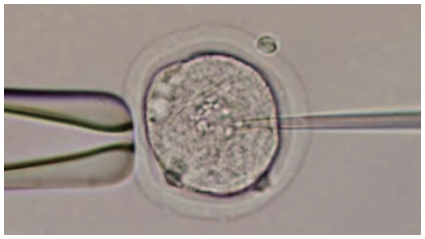
Table 1 | Surveyor assay and sequencing for TALEN-induced mutations in Rat-1 fibroblasts

			Sequence analysis		
Plasmid(s)	Cell No. (1×10^5)	Surveyor assay (%)	Mutations/Colonies	Mutation Rate (%)	Average del size (bp)
GFP	2.44 ± 0.58	0.44 ± 0.71	—	—	—
TALEN	2.81 ± 0.23	6.06 ± 0.56	5/96	5.2	14.2 ± 6.5
TALEN-NC	2.72 ± 0.33	11.04 ± 1.77	7/96	7.3	17.4 ± 6.8
TALEN-NC + Exo1	3.02 ± 0.35	25.86 ± 1.91	17/96	17.7	11.9 ± 2.6

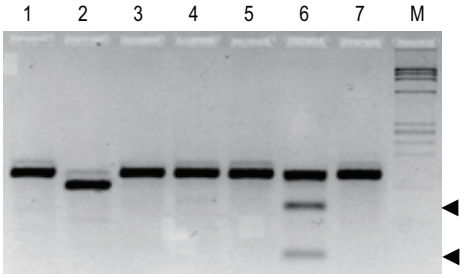
A

Injected mRNA	Injected embryos	Two-cell embryos (%)	PCR-amplified (%)	Mutations (%)
TALEN-NC	70	21 (30.0)	18 (85.8)	1 (5.6)
TALEN–NC + Exo1	59	21 (35.6)	14 (66.7)	4 (28.6)

B



C



D

eT-NC-#8
TATAATAGCCATCAGGTTTT------TGGAAC---TGAGGGACCACTATTACGTAATCCT

eT-NC-Exo1-#2
TATAATAGCCATC--------(Δ24bp)---------AGGGACCACTATTACGTAATCCT

eT-NC-Exo1-#6
TATAATAGCCATCAGGTTTTATGTGATGGAACACCTGAGGGACCACTATTACGTAATCCT
TATAATAGCCATCAGGTTTTATGTG---------CTGAGGGACCACTATTACGTAATCCT

eT-NC-Exo1-#10
TATAATAGCCATCAGGTTTTATGTGATGGAACACCTGAGGGACCACTATTACGTAATCCT
TATAATAGCCATCAGGTTTTATG-----GAACACCTGAGGGACCACTATTACGTAATCCT

eT-NC-Exo1-#14
TATAATAGCCATCAGGTTTTATGTGATGGAACACCTGAGGGACCACTATTACGTAATCCT
TATAATAGCCATCAGGTT----------(Δ29bp)----------ATTACGTAATCCT

Figure 2 | Targeted gene disruption by engineered TALENs in rat embryos. (A) Injection of TALEN-NC with or without *Exo1*mRNA into rat fertilized eggs. *Exo1* increased the efficiency of TALEN-induced mutations in zygotes by ~5×. (B) Microinjection of TALENs mRNA into male pronuclei of a fertilized egg. (C) Surveyor assay on the PCR products shows a TALEN-induced mutation as the digested products (arrowheads) in lane 6, but could not detect a homozygous mutation in lane 2. (D) Sequence analyses of the PCR products showed a 24-bp deletion in the homozygous alleles (eT-NC-*Exo1*-#2). Microhomologous sequences adjacent to the breakpoint are underlined.

pathway[27–29] (Figure 4). DSBs created by ZFNs or TALENs are generally repaired by cNHEJ with precise DNA-ends joining. When precise repair fails, or after the recession of DNA-ends by DNA exonucleases, DSBs would be repaired by altNHEJ, typically using closer microhomology sequences at both ends, thereby driving mutagenic end processing. Overexpression of *Exo1* probably increases DNA-end resections at the DSB-ends created by TALENs, thereby increasing the frequency of mutagenic DSB-repair via altNHEJ. To examine the effects of overexpression of *Exo1* on the NHEJ and HR pathways, we performed an NHEJ assay that measures the repair of I-SceI-generated DSBs via the NHEJ pathway[20,30] and an HR assay that measures the repair of I-SceI-generated DSBs via the HR pathway[20,31] (Supplementary Fig. S3). Those assays revealed that the *Exo1* significantly inhibited both the NHEJ pathway and the HR pathway in a wide variety of human cells (U2OS, HeLa, and MRC5), suggesting that the altNHEJ pathway would be enhanced in compensation, which might increase the frequency of the targeted gene disruption.

In conclusion, coinjection of exonucleases *Exo1* with TALENs provides an easy and efficient approach for gene knockouts in zygotes, and represents a promising breakthrough for gene targeting technologies applicable to various species.

Methods

Animals. All animal care and experiments conformed to the Guidelines for Animal Experiments of Kyoto University, and were approved by the Animal Research Committee of Kyoto University. Novel developed DA-*Tyr*^{em1Kyo} albino rats (NBRP-Rat No.0666) were deposited into the National Bio Resource Project – Rat in Japan (www.anim.med.kyoto-u.ac.jp/nbr).

Construction of TALEN plasmids and single-strand annealing (SSA) assay. The protocol for TALEN assembly was as previously reported[18,19]. Repeat assembly was conducted using a Golden Gate reaction, transformed into XL1-Blue competent cells and screened for precisely assembled clones by colony PCR using the pCR8_F1 and pCR8_R1 primers[19]. Constructed array plasmids and the appropriate last repeat were joined directly into the mammalian expression vectors, pcDNA-TAL or

A

Injected mRNA	Injected embryos	Two-cell embryos (%)	Pups (%)	♂	♀	Mutants (%)
TALEN	127	40 (31.5)	9 (23.0)	5	4	0 (0)
TALEN-NC	201	86 (42.8)	20 (23.3)	10	10	0 (0)
TALEN (Cellectis)	290	92 (31.7)	30 (32.6)	14	16	2 (6.7)
TALEN–NC + Exo1	68	29 (42.6)	12 (41.4)	7	5	3 (25.0)

B

C

gT-NC-Exo1-#1

TATAATAGCCATCAGGTT---------(Δ29bp)----------ATTACGTAATCCT

gT-NC-Exo1-#7

TATAATAGCCATCAGGTTTTATGTGATGGAACACCTGAGGGACCACTATTACGTAATCCT
TATAATAGCCATCAGG---------(Δ24bp)---------GACCACTATTACGTAATCCT

gT-NC-Exo1-#8

TATAATAGCCATCAGGTTTTATGTGATGGAACACCTGAGGGACCACTATTACGTAATCCT
TATAATAGCCATCAGGTTTTATGTGATG-----CCTGAGGGACCACTATTACGTAATCCT

Figure 3 | Efficient generation of knockout rats using TALENs with *Exo1*. (A) Microinjection of TALENs, TALENs-NC, TALENs from Cellectis, and TALENs-NC with *Exo1* into fertilized eggs of agouti DA rats. Coinjection of TALEN-NC mRNA with *Exo1* mRNA provided higher mutational efficiency (25%) in pups. (B) The white coat-color of an albino male rat (right) obtained by coinjection of TALEN-NC and *Exo1*. An agouti male (left) is a littermate. (C) Sequence analyses on founder rats showed a homozygous 29-bp deletion in *Tyr* (gT-NC-*Exo1*-#1). Microhomologous sequences adjacent to the breakpoint are underlined.

pcDNA-TAL-NC[18]. Final assembly, transformation and colony PCR screening for the second Golden Gate reaction were performed as previously reported[18,19].

The SSA assay was carried out as previously described[9]. The pGL4-SSA reporter vector was generated, containing inactive fragments of the luciferase gene, which bear 700-bp regions of homologous overlap and are driven by a cytomegalovirus (CMV) immediate-early enhancer/promoter. For the addition of TALEN target sequences, sense and antisense oligonucleotides, Sense: 5'- GTCGGATATAATAGCCATC-AGGTTTTATGTGATGGAACACCTGAGGGACCACTATTACGTAATCCTGG-AGGT-3', and Antisense: 5'- CGGTACCTCCAGGATTACGTAATAGTGGTCC-CTCAGGTGTTCCATCACATAAAACCTGATGGCTATTATATC-3', were annealed and inserted into *Bsa*I sites between the dissected luciferase elements of pGL4-SSA. For the ZFN positive control, a ZFN expression vector, pSTL-ZFA36, was constructed, as previously described[9].

Rat *Exo1* cDNA cloning. To clone the rat *Exo1* cDNA, first strand cDNA was isolated using the oligo(dT)12-18 primer and SuperscriptII reverse transcriptase (Life Technologies, Carlsbad, CA, USA) synthesized from total RNA extracted from the brain of F344 rats by the Isogen reagent (Nippon Gene, Tokyo, Japan). RT-PCR was performed with the primers: 5'-GGGCCATGCCTGTTTATTC-3' and 5'-TGTTACCAGTGTGTTACCAGTCG-3'. RT-PCR products were inserted into pGEM-T Easy (Promega, Fitchburg, USA) and sequenced to confirm the sequence. For expression *in vitro*, the full-length cDNA was subcloned into pcDNA6.2/V5/GW/D-TOPO (Life Technologies).

Cell culture and transfection. Rat fibroblast-like (Rat-1) cells were obtained from the RIKEN BRC Cell Bank (Tsukuba, Japan, http://www.brc.riken.jp/lab/cell/english). The Rat-1 cells were cultured in DMEM (Invitrogen), supplemented with 10% FBS (fetal bovine serum, CBB) in a humidified atmosphere containing 5% CO_2 at 37°C. The cells (1×10^5) were suspended in 10 μl R buffer (supplied as part of the Neon Transfection System, Invitrogen), given 0.5 μg of each plasmid, and electroporated under the following conditions: pulse voltage, 1300 V; pulse width, 20 ms; and pulse

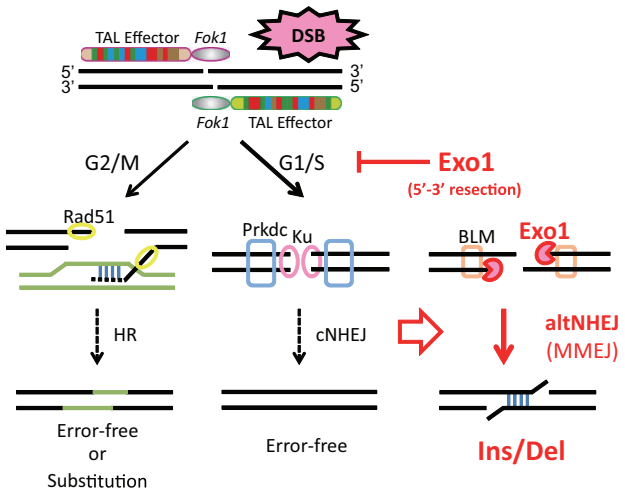


Figure 4 | Schematics of exonucleases (*Exo1*) functions in double-stranded break (DSB) repair pathways. Alternative NHEJ (altNHEJ), or microhomology mediated end-joining (MMEJ), generally repairs DSBs in the absence or failure of the classical NHEJ (cNHEJ) pathway[27–29]. Overexpression of *Exo1* induces increased recessions of DNA-ends at the DSB created by TALENs, which inhibits cNHEJ (See Supplementary Fig. S3) and enhances the mutagenic DSB-repair via altNHEJ, resulting in more indel mutations at the targeted gene.

number, 2 (program #15). Following electroporation, the cells were cultured in the medium described above without antibiotics for 24 h and then in the medium with antibiotics for 48 h. The *in vitro* transfer experiment was replicated three times.

Surveyor assay and DNA sequencing. For the Surveyor assay to detect TALEN-induced mutations, the SURVEYOR Mutation Detection Kit (Transgenomic, Omaha, NE, USA) was used in accordance with the manufacturer's protocol. Briefly, 72 h after electroporation, genomic DNA was extracted from the Rat-1 cells using Nucleospin (Macherey-Nagel, Düren, Germany). PCR was then performed using PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan), a high-fidelity enzyme, under the following conditions: 95°C for 1 min; followed by 95°C for 30 s, 68°C for 30 s, and 72°C for 1 min for 35 cycles. The PCR primers were as follows: 5′-TTGCATAAATTGGTTTTCACAGA-3′ and 5′-ATTTAAACATGAAAATAT-TACCTTCCA-3′. The PCR amplification products were heat denatured, digested by the Surveyor nuclease, and subjected to agarose gel electrophoresis to confirm TALEN-induced mutations.

For DNA sequencing analysis, the PCR products were subcloned into pCR4Blunt-TOPO plasmid vector (Life Technologies). Plasmids were extracted from the resultant *Escherichia coli* colonies for DNA sequencing. Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit and an ABI PRISM 3130 Genetic Analyzer (Life Technologies).

Rat embryo culture. Pronuclear stage embryos were collected from DA/Slc females at 7 weeks of age that had undergone super-ovulation by injection with PMSG (Serotropin, Aska Pharmaceutical Co., Tokyo, Japan) and hCG (Gonatropin, Aska Pharmaceutical Co.). The GenomPlex Single Cell Whole Genome Amplification Kit (WGA4; Sigma Aldrich, St. Louis, MO, USA) was used to generate proprietary amplification of genomic DNA with universal oligonucleotide primers from a two-cell embryo. After purification, the single cell WGA products were analyzed the Surveyor assay and DNA sequencing analysis.

Microinjection of TALENs mRNA. To prepare mRNA of TALENs, TALEN-encoding expression plasmids were linearized with *Xho*I and extracted with phenol-chloroform by standard methods. To prepare the mRNA for *Exo1*, the *Exo1* plasmids were linearized with *Spe*I. Messenger RNA was transcribed *in vitro* using a MessageMax™ T7 mRNA transcription kit (Illumina, San Diego, CA, USA) and polyadenylated using a A-Plus™ Poly(A) polymerase tailing kit (Illumina). The resultant mRNA was purified using a MEGAClear™ kit (Illumina) and finally resuspended in RNase-free water at 10 ng/μl for each TALEN or *Exo1*. Approximately 2-3 pL of capped mRNA were injected into the male pronuclei of zygotes by microinjection[13]. The injected embryos were cultured in mKRB at 37°C with 5% CO_2 and 95% humidified air to promote their recovery. Surviving embryos were transferred to the oviducts of pseudopregnant Wistar females.

NHEJ and HR assays. NHEJ and HR assays were performed as previously reported[20,30,31]. MRC5SV-pEJ cells were used for the NHEJ assay. U2OS-DRGFP, HeLa-DRGFP and MRC5SV-DRGFP were used for the HR assay. To measure the repair of I-SceI-generated DSBs, 50 μg of the I-SceI expression vector (pCBASce) with or without a human *Exo1* expression plasmid (30 μg) was introduced into 5×10^6 cells by electroporation (GenePulser; Bio-Rad, Hercules, CA, USA). To determine the level of NHEJ or HR repair, the percentage of GFP-positive cells was quantified by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA) 3 days after electroporation.

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Author contributions

T.M. designed the work, produced all the data, and wrote the paper. T.K. and B.V. performed microinjection of TALENs into rat embryos. T. Sakuma and T.Y. constructed the TALENs. J.K. and Y.K. helped with *in vitro* experiments. T. Serikawa supervised the work. All authors read and corrected the manuscript before submission.

Additional information

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