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Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity

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Transcription activator-like effector (TALE) nuclease (TALEN) is a site-specific nuclease, which can be freely designed and easily constructed. Numerous methods of constructing TALENs harboring different TALE scaffolds and repeat variants have recently been reported. However, the functionalities of structurally different TALENs have not yet been compared. Here, we report on the functional differences among several types of TALENs targeting the same loci. Using HEK293T cell-based single-strand annealing and Cel-I nuclease assays, we found that TALENs with periodically-patterned repeat variants harboring non-repeat-variable di-residue (non-RVD) variations (Platinum TALENs) showed higher activities than TALENs without non-RVD variations. Furthermore, the efficiencies of gene disruption mediated by Platinum TALENs in frogs and rats were significantly higher than in previous reports. This study therefore demonstrated an efficient system for the construction of these highly active Platinum TALENs (Platinum Gate system), which could establish a new standard in TALEN engineering.

Recent advances in genome-editing technology have paved the way for genomic engineering in cells and organisms[1]. Several varieties of customized transcriptional activator-like effector (TALE) nucleases (TALENs) have been developed by numerous groups, with various N- and C-terminal domains and DNA-binding modules for TALE. Regarding the N- and C-terminal domains, referred to as the TALE scaffold, the +136/+63 architecture has been the most commonly used[2–6]. However, minor truncation variants have also been reported by several groups[7,8]. Among these reports, we previously demonstrated that customized TALENs with a +153/+47 scaffold could work in human cells[9], flies[9], zebrafish[9,10], medaka[11], frogs[9,12], and rats[13]. The DNA-binding module, referred to as the TALE repeat, represents another important component of TALENs. In general, all TALE repeats have a similar 34 amino acid sequence, excluding the 12th and 13th residues, repeat-variable di-residue (RVD). However, TALE repeats occurring in nature have other variations, such as in the 4th and 32nd residues[14]. The frequencies of amino acid appearances in these residues are approximately aspartic acid (D): glutamic acid (E): Alanine (A) = 2 : 1 : 1 and D : A = 1 : 1, respectively[14]. The most widely used TALEN construction kit, Golden Gate TALEN and TAL Effector Kit (Addgene, Cambridge, MA, USA), includes only the common repeat backbone, harboring constant 4th and 32nd residues reflecting their most common forms (4th: D and 32nd: D)[15]. However, Miller *et al.* reported TALENs with non-RVD variations[2] and Sander *et al.* arranged them in a simple repeating pattern, according to their natural appearance frequency[3]. Despite these studies regarding various TALE scaffolds and repeats, the functional differences and optimal combinations remain to be investigated. To clarify this issue, we comprehensively analyzed two types of TALE scaffolds and two types of repeat backbones, and demonstrated that TALENs with variable repeats (VRs) harboring non-RVD variations, called Platinum TALENs, showed higher activity than TALENs with constant repeats (CRs) without non-RVD variations, not only in cells, but also in frog and rat embryos. These results confirmed the Platinum Gate system as an efficient construction system for highly active Platinum TALENs.

Results

Platinum Gate TALEN construction system: establishment of variable-repeat TALEN assembly method using Golden Gate cloning. We previously established a 6-module assembly system for the construction of

TALENs with CRs[9] by modifying the Golden Gate TALEN and TAL Effector Kit (Addgene)[15]. In the current study, we established a new 4-module assembly system for the construction of TALENs with VRs (Platinum Gate TALEN construction system; Figure 1) to assess the importance of the previously reported repeating pattern of non-RVD variations[3,4]. The basic principle of the system involves 2-step Golden Gate cloning using *Bsa*I and *Esp*3I, as in the previous systems[9,15]. However, the smaller number of modules in the first assembly step compared with the previous systems (4 versus 6[9] or 10[15]) meant that we required fewer one-module plasmids (p1HD-p4HD, p1NG-p4NG, p1NI-p4NI, and p1NN-p4NN) compared with the conventional Golden Gate kits (16 versus 24[6] or 40[15]). In addition, the fewer modules enabled more robust construction of TALENs. On average, success rate of 10-module assembly was approximately 10%, meanwhile success rate of 6-module assembly and newly established 4-module assembly was almost 100% in our groups. Although the maximum repeat number of Platinum TALENs is smaller than that of previous Golden Gate TALENs (21 versus 31[9,15]), it is enough to produce fully functional TALENs; 15- to 20-repeat TALENs have been demonstrated to have activities in many previous studies[4,7–9,16]. In contrast, TALENs with more than 22 repeats have rarely been reported. This new method therefore represents a highly efficient construction system for TALENs with VRs, referred to as Platinum TALENs.

Mix and match analysis of two types of scaffold and repeat. To compare the activities of TALENs constructed using the conventional Golden Gate kit and our novel Platinum Gate kit, we constructed TALENs targeting the same locus using these two systems. In addition to the repeat variations, we also adopted two different types of TALEN scaffolds, +136/+63[2–4,6] and +153/+47[9] (Figure 2A). The human *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*) locus has previously been demonstrated as a locus for TALEN targeting[9,15]. We therefore constructed 32 TALENs with 14, 16, 18 and 20 modules for left and right each using two scaffolds and two repeats (Figure 2B). Among all the combinations of left and right TALENs, the minimum spacer length was 12 bp and the maximum spacer length was 24 bp (Figure 2B). Comparative analysis of every combination of TALENs by reporter-based single-strand annealing (SSA) assay[9] revealed that TALENs with the +136/+63 scaffold were capable of inducing double-strand breaks (DSBs) in a wide range of spacer lengths (Figure 2C), while the DSB-forming activities of TALENs with the +153/+47 scaffold were restricted to shorter spacers (Figure 2C). The activities of +136/+63 TALENs were globally high, and the effect of the VR was thus less apparent in this assay. In contrast, the activities of +153/+47–VR TALENs were clearly higher than those of +153/+47–CR TALENs (Figure 2C).

Repeating pattern of non-RVD variants in TALE repeat is critical for TALEN activity. To evaluate the activities of four types of TALENs investigated in the SSA assay at the *HPRT1* locus, we subsequently constructed TALENs targeting the *ataxia telangiectasia mutated* (*ATM*), *adenomatous polyposis coli* (*APC*), and *enhanced green fluorescent protein* (*eGFP*) genes, and performed SSA and Cel-I assays. The spacer lengths of these three independent loci were 18, 16, and 15 bp, respectively (Figure 3A). HEK293T-based SSA assays revealed that VR

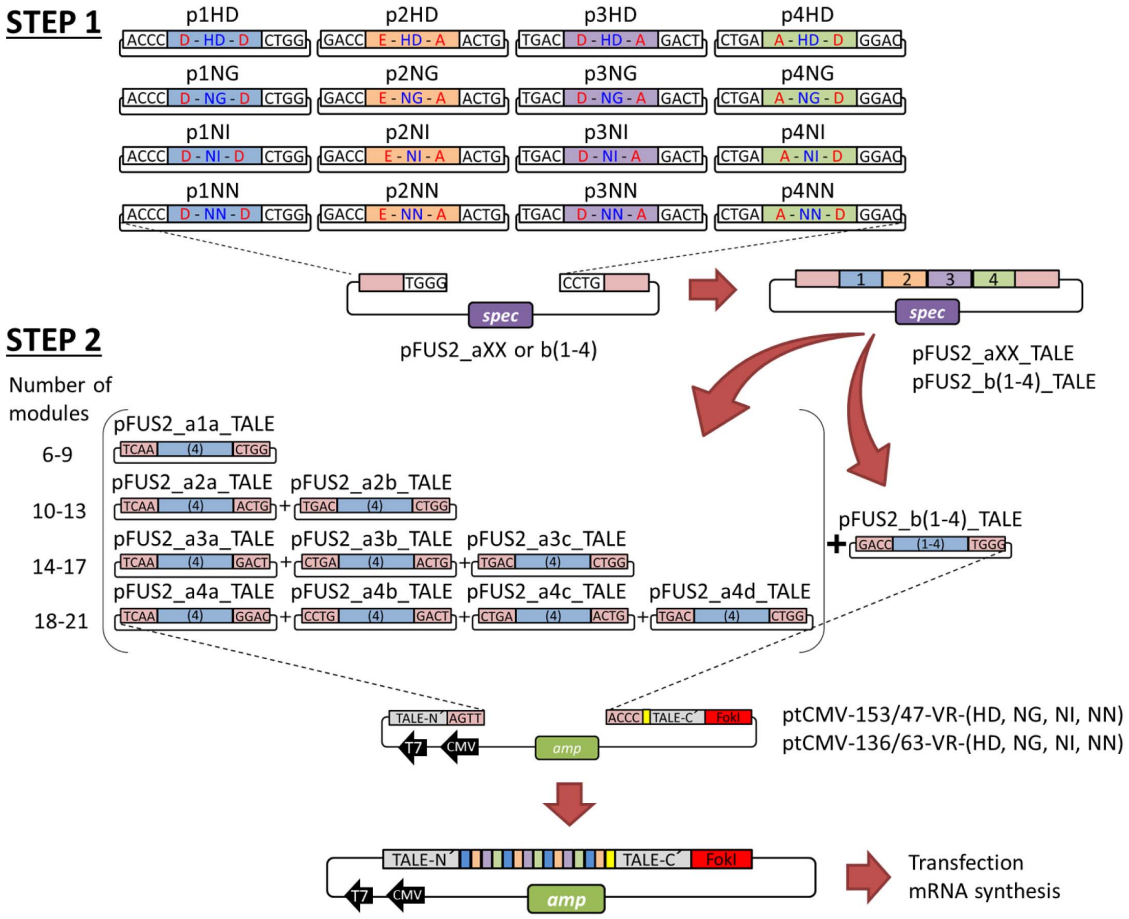
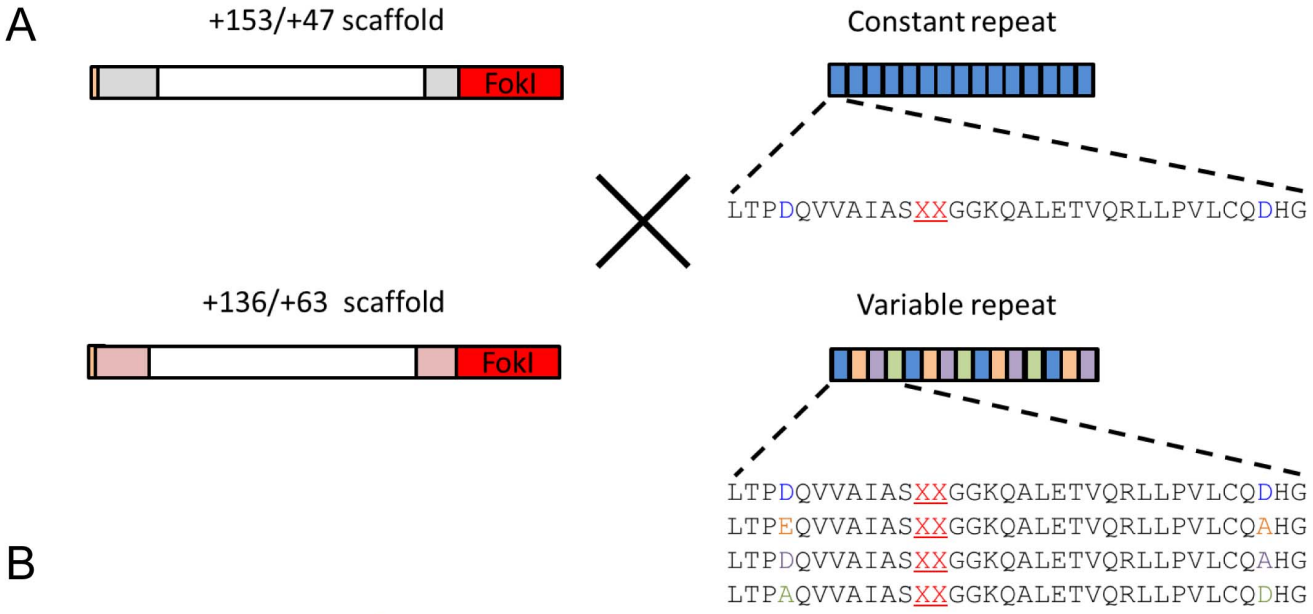
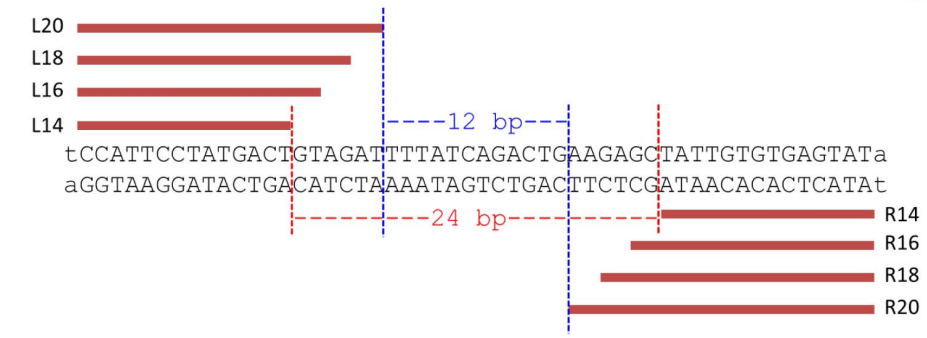


Figure 1 | Schematic overview of the Platinum Gate TALEN construction system. Four or fewer modules were ligated into array plasmids in the first step. Constructed arrays were subsequently joined into a mammalian expression vector in the second step. Bases in white and pink boxes represent overhangs left by *Bsa*I and *Esp*3I, respectively. Blue letters indicate RVDs. Red letters indicate non-RVD variations. Yellow boxes represent last half repeats. Spec, spectinomycin; Amp, ampicillin; CMV, cytomegalovirus promoter.





Spacer length

	R20	R18	R16	R14
L20	12	14	16	18
L18	14	16	18	20
L16	16	18	20	22
L14	18	20	22	24

C

Constant repeat

	R20	R18	R16	R14
+153/+47 scaffold				
L20	1.067±0.148	1.429±0.225	0.647±0.051	0.062±0.006
L18	1.160±0.200	1.450±0.308	0.770±0.098	0.101±0.047
L16	0.370±0.060	0.233±0.066	0.557±0.149	0.085±0.019
L14	0.089±0.014	0.089±0.031	0.328±0.065	0.057±0.012
+136/+63 scaffold				
L20	0.578±0.069	2.216±0.144	2.566±0.497	1.924±0.567
L18	1.681±0.359	2.082±0.350	2.355±0.155	2.284±0.352
L16	1.921±0.236	1.802±0.213	2.315±0.600	2.792±0.642
L14	1.430±0.173	1.453±0.085	2.545±0.183	1.668±0.123

Variable repeat

	R20	R18	R16	R14
+153/+47 scaffold				
L20	2.108±0.210	2.669±0.144	1.079±0.076	0.168±0.025
L18	1.803±0.301	1.086±0.279	0.681±0.109	0.418±0.101
L16	0.492±0.123	0.176±0.020	1.000±0.267	0.720±0.264
L14	0.240±0.063	0.267±0.036	0.990±0.119	0.280±0.071
+136/+63 scaffold				
L20	2.065±0.186	1.849±0.327	2.675±0.239	1.888±0.463
L18	1.962±0.064	1.729±0.009	2.412±0.573	1.511±0.181
L16	0.971±0.161	1.116±0.159	2.028±0.159	2.199±0.295
L14	1.989±0.389	1.676±0.300	2.184±0.520	1.206±0.286

Figure 2 | Comprehensive analysis of four types of TALEN framework using the SSA assay. (A) Schematic drawing of scaffold and module swapping analysis. Underlined red letters indicate RVDs. Four types of non-RVD variant are indicated in blue, orange, purple, and green letters, respectively. Repeating pattern of VRs is represented using blue, orange, purple, and green boxes. (B) Schematic design of TALENs used in this assay. Seven TALENs with different numbers of TALE repeats (red bars) were constructed for both left (L14–L20) and right (R14–R20) target sequences. Minimum spacer region is indicated by blue lines and letters. Maximum spacer region is illustrated by red lines and letters. Spacer lengths for all the combinations of left and right TALENs are represented in the table. (C) Relative activities (fold to positive control ZFN[9]) of all the TALENs are shown in the tables. Red, black, and blue letters indicate high (>2), intermediate (1–2), and low (<1) activities, respectively. Data are expressed as means ± SEM (n = 3).

TALENs with either scaffold were more active than CR TALENs for all three genes (Figure 3B). In addition, the activities of +136/+63–VR TALENs were greater in longer spacers (ATM > APC > eGFP). Conversely, the activities of +153/+47–VR TALENs were greater in shorter spacers (ATM < APC < eGFP) (Figure 3B). These characteristics were in accord with the *HPRT1* results (Figure 2C). We further tested the ability of VR to enhance the TALEN activity in targeting endogenous human genes, using the Cel-I assay. TALEN vectors were transfected into HEK293T cells and genomic polymerase chain reaction (PCR) was performed directly using cell pellets. Purified

and re-annealed PCR products were then treated with Cel-I nucleases to digest heteroduplex DNAs. Consistent with the result of the SSA assay, Platinum TALENs could induce mutations more efficiently than CR TALENs (Figure 3C).

Platinum TALENs are effective in frogs and rats. To evaluate the gene-targeting efficiency of Platinum TALENs in zygotes, we first demonstrated tyrosinase (tyr) disruption in *Xenopus laevis* embryos. The spacer length of the *tyr* TALEN target sequence in our previous study[12] was 13 bp (Figure 4A), and we therefore constructed a Platinum *tyr* TALEN with a +153/+47 scaffold suitable for this short spacer. Microinjection of Platinum *tyr* TALEN mRNAs resulted in almost full albino phenotypes in many individuals (Figure 4B). Although the injection dose was lower than in the previous report[12], these phenotypes were stronger than those of conventional TALEN-injected embryos[12] (Figure 4B). In addition, there was no dramatic increase in developmental defects, even though more than half of the injected embryos showed strong phenotypes (Figure 4C). Restriction fragment length polymorphism (RFLP) analysis and DNA sequencing revealed that the mutation rate of Platinum *tyr* TALEN-injected embryos was almost 100% (Figure 4D and Supplementary Fig. S3), which was much higher than in the previous study[12].

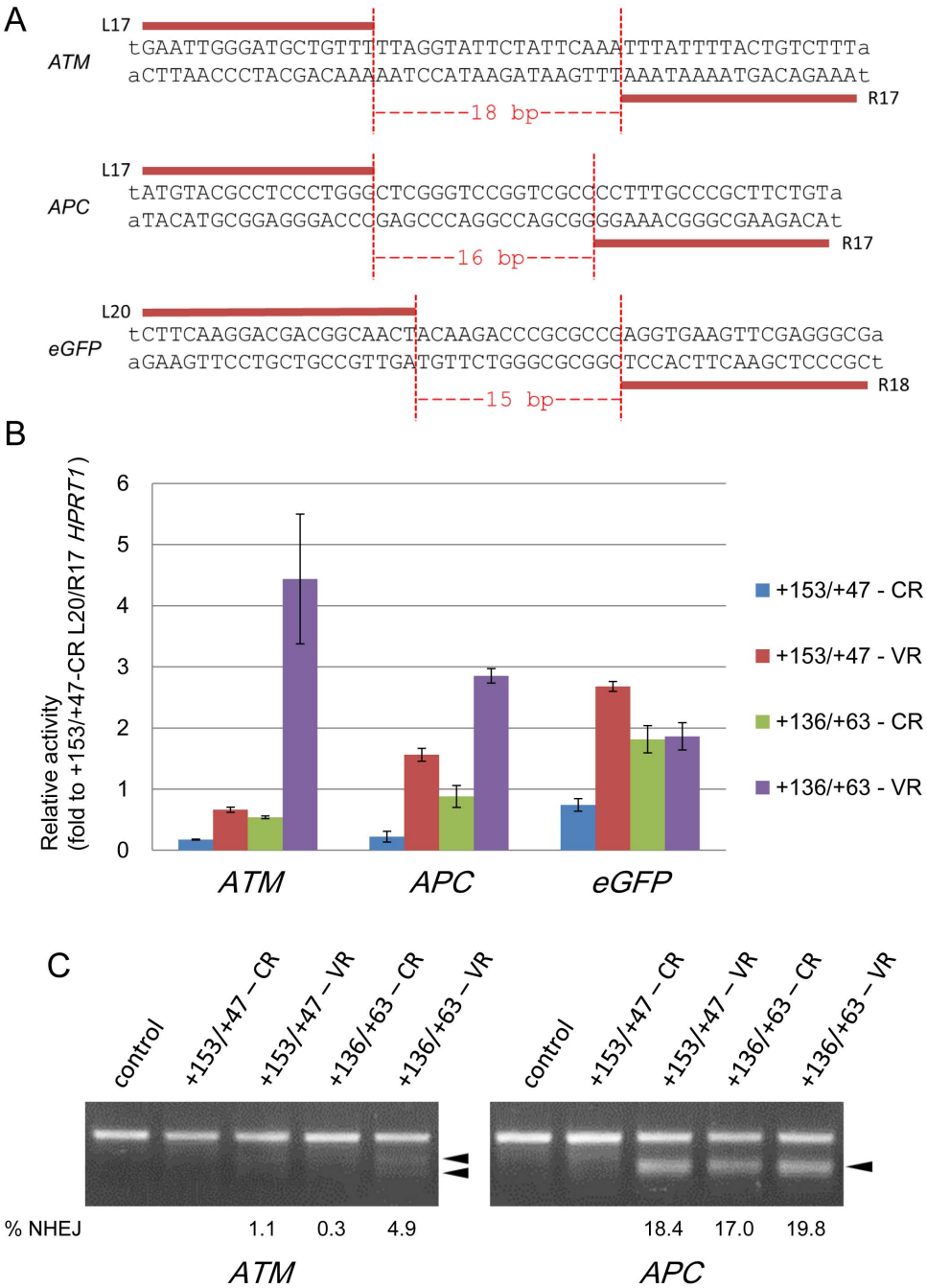


Figure 3 | Repeating pattern of non-RVD variations enhances the activity of TALENs. (A) Schematic design of TALENs used in this assay. Red bars indicate left and right TALENs. Red lines and letters indicate spacer regions. The target sequences of *ATM* and *APC* were originally described by Reyon *et al*[4]. The target sequence of *eGFP* was originally described by Sakuma *et al*[9]. (B) SSA assay for four types of TALEN targeting three genes. Data are expressed as means ± SEM (n = 2). (C) Cel-I assay for four types of TALEN targeting *ATM* and *APC*. Arrowheads indicate the expected positions of the digested products. % NHEJ (nonhomologous end joining) was estimated using ImageJ software as previously described[29].

To elucidate the targeting efficiency of Platinum TALENs in mammalian zygotes, we next applied Platinum TALENs against the *interleukin-2 receptor gamma chain* (*Il2rg*) gene to rat zygotes. *Il2rg* TALENs were designed as shown in Figure 5A, with a +136/+63 scaffold. We first validated the Platinum *Il2rg* TALEN using Rat-1 fibroblast cells (Figure 5B). A negative control GFP-expressing plasmid, *Il2rg* zinc-finger nuclease (ZFN) plasmid[17,18] or Platinum *Il2rg* plasmid was transfected into Rat-1 cells, and Cel-I assays were performed. Platinum *Il2rg* TALENs had a greater mutagenic effect than previously reported *Il2rg* ZFNs (Figure 5B). We then microinjected Platinum *Il2rg* TALEN mRNAs into rat zygotes. Of 52 Platinum TALEN-injected eggs, 20 oocytes were transferred into the oviducts of pseudopregnant Wistar female rats. All of the resulting six pups demonstrated biallelic mutations (Figure 5C, D). This mutant-generating efficiency was greater than those of both CR TALENs[13] and also ZFNs (Figure 5C).

Discussion

To the best of our knowledge, the current report provides the first evidence to indicate that non-RVD variations in the TALE repeat greatly enhance TALEN activity. To date, the Golden Gate TALEN and TAL Effector Kit has been adopted extensively in various cells and organisms including plants[19], flies[9,20], zebrafish[5,10,21,22], medaka[11], frogs[9,12,23], rats[13], and pigs[24]. However, although Golden Gate TALENs have been validated in these organisms, further scope for improvements remains. Indeed, although we recently performed gene disruption in rats using the Golden Gate Kit[13], the efficiency was lower than that reported by Tesson *et al*[25]., in which TALENs with non-RVD variations were used. However, the relationship between TALEN activity and non-RVD variations has yet to be proven.

In this study, we demonstrated the importance of non-RVD variations in the TALE repeat in highly active Platinum TALENs. Furthermore, we established a simple and efficient construction

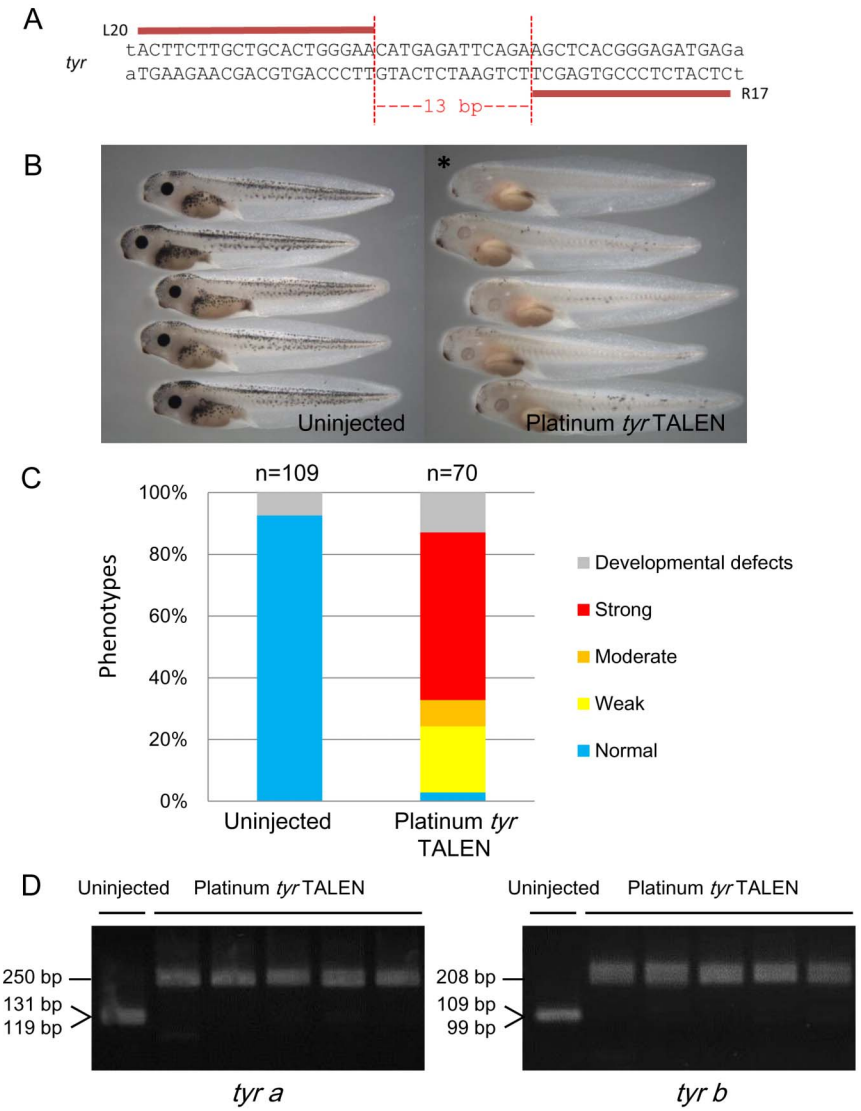
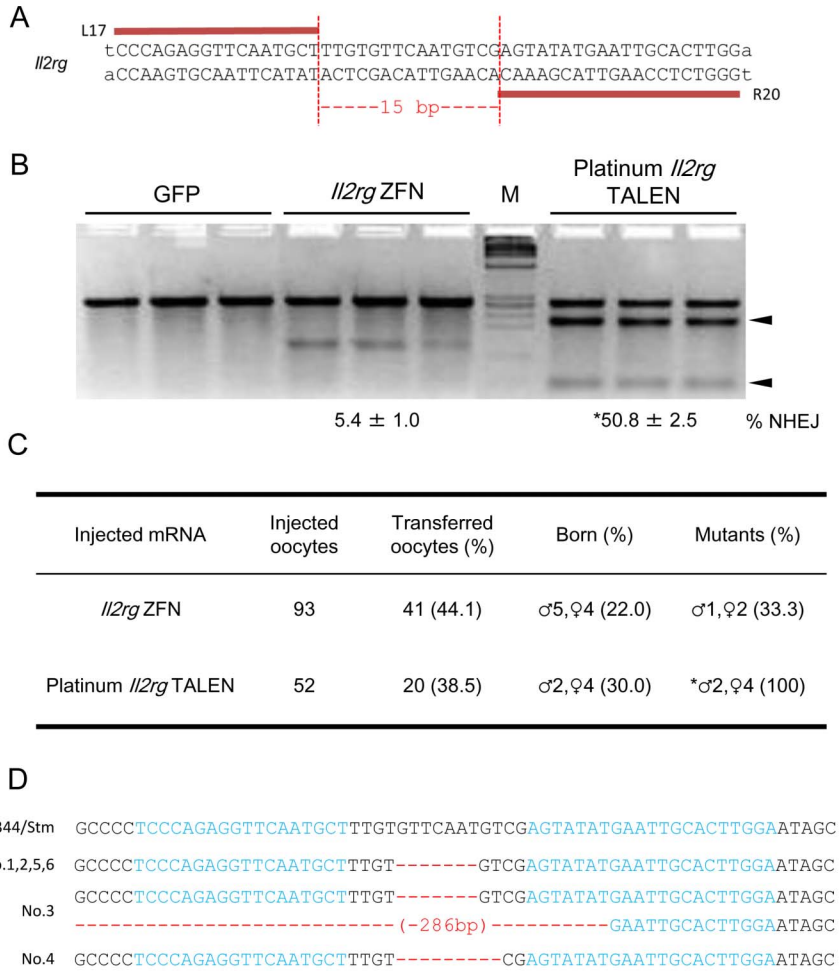


Figure 4 | Highly efficient targeted gene disruption in *Xenopus laevis* using Platinum TALENs. (A) Schematic design of TALEN used in this assay. Red bars indicate left and right TALENs. Red lines and letters indicate spacer region. (B) Phenotypes of uninjected and Platinum *tyr* TALEN-injected embryos. Embryos were reared to the hatching stage. An asterisk indicates sequenced embryo related to Supplementary Fig. S3. (C) Percentages of phenotypes in the uninjected and Platinum *tyr* TALEN groups. Total numbers of individuals in this experiment are shown at the top of each graph. Strong, near complete loss of pigmentation in retina pigmented epithelium (RPE); Moderate, more than half loss of pigmentation in RPE; Weak, less than half loss of pigmentation in RPE; Normal, no alteration of pigmentation. Representative phenotypes were shown in a previous report[12]. (D) RFLP analysis of *tyr* paralogs. Due to its allotetraploid genome, there are two paralogs, *tyr a* and *tyr b*, in *X. laevis*. PCR products of *tyr a* (left panel) and *tyr b* (right panel) paralogs were purified, digested by *Hinf*I and analyzed by agarose gel electrophoresis.



Injected mRNA	Injected oocytes	Transferred oocytes (%)	Born (%)	Mutants (%)
Il2rg ZFN	93	41 (44.1)	♂5,♀4 (22.0)	♂1,♀2 (33.3)
Platinum *Il2rg* TALEN	52	20 (38.5)	♂2,♀4 (30.0)	*♂2,♀4 (100)

Figure 5 | Highly efficient targeted gene disruption in rats using Platinum TALENs. (A) Schematic design of TALEN used in this assay. Red bars indicate left and right TALENs. Red lines and letters indicate spacer region. (B) Cel-I assay for ZFN- or TALEN-induced mutations in rat *Il2rg* gene. Arrowheads indicate the expected positions of the TALEN-digested products. % NHEJ (nonhomologous end joining) was estimated using ImageJ software as previously described[29]. Data are expressed as means ± SEM (n = 3). *P < 0.01 by Student's t-test: *Il2rg* ZFN vs. Platinum *Il2rg* TALEN. (C) Microinjection of ZFNs or TALENs targeting *Il2rg* into fertilized eggs of F344 rats. *P < 0.01 by chi-square test: *Il2rg* ZFN vs. Platinum *Il2rg* TALEN. (D) Sequence analyses of TALEN-induced mutant rats. Blue letters indicate TALEN target sites. Gaps generated by deletion are shown as dashes in red.

system for Platinum TALENs (Platinum Gate system). Because TALE repeats in Platinum TALENs need to be assembled in increments of four blocks, we used a 4-module Golden Gate cloning method for the first assembly. This modification means that it might become possible to skip the first assembly step by completing 4-module libraries. For example, 17-repeat Platinum TALENs require four types of 4-module plasmids, pFUS2_a3a, a3b, a3c, and b4. If we try to complete these plasmid libraries, 256 plasmids are needed for each pFUS2 vector. Several groups have reported plasmid libraries for Golden Gate TALEN assembly[8,16], but none of these have included a repeated pattern of TALE repeats with non-RVD variations. REAL[3,26]-, REAL-Fast[26]-, and FLASH[4,27]-assembly systems, developed by Joung's lab, can construct VR TALENs similar to our Platinum TALENs, but the necessary fragmentation and purification of TALE repeat arrays means that these methods are less convenient than the Platinum Gate construction system.

It remains unclear how the repeating pattern of non-RVD variations affects TALEN activity. However, we speculate that it might affect protein folding or the binding affinity between the DNA and TALEN protein. Further studies are needed to clarify the detailed mechanisms.

In conclusion, we successfully established the Platinum Gate TALEN construction system, which allowed the construction of Platinum TALENs that demonstrated high efficiency *in vitro* and *in vivo*. We have deposited the 'TALEN Construction and Evaluation Accessory Pack' in Addgene as a supplemental package to the 'Golden Gate TALEN and TAL Effector Kit'. In addition, we are currently preparing to submit materials for our novel Platinum Gate system. We anticipate that Platinum TALENs will provide a valuable contribution to genome editing research.

Methods

Plasmid construction. For module plasmids harboring non-RVD variations, p1HD-p4HD, p1NG-p4NG, p1NI-p4NI, and p1NN-p4NN, single TALE repeat sequences with *Bsa*I restriction sites at both ends were synthesized and cloned into pBluescript SK vector. Each repeat sequence is described in Supplementary Fig. S1. pFUS2 vectors were used as the capture vectors for the first assembly step in the Platinum Gate system, and were constructed using PCR and In-Fusion cloning (Takara Bio, Shiga, Japan) using pFUS_B6 (Addgene) as a template. The final ptCMV capture vectors were constructed using pTALEN_v2 plasmids (Addgene)[6] as backbones. The N- and C-terminal domains of TALE and the FokI nuclease domain in ptCMV vectors are described in Supplementary Fig. S2. Each TALEN expression plasmid was constructed using the Golden Gate cloning method, as described previously[9,15]. Target sequences for each gene are shown in Figures 2B, 3A, and 4A. Reporter plasmids for the SSA assay were constructed as described previously[15].

SSA and Cel-I assay for human cells. HEK293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The SSA assay was carried out as described previously[9]. Briefly, 50,000 cells were cotransfected with 200 ng of each of the TALEN expression plasmids, 100 ng of the SSA reporter plasmid, and 20 ng of the pRL-CMV reference vector in a 96-well plate. After 24 h, dual-luciferase assays

were conducted using the Dual-Glo luciferase assay system (Promega, Madison, WI, USA).

Transfection for the Cel-I assay was carried out as follows: 30,000 HEK293T cells were transfected with 200 ng of each of the TALEN expression plasmids using Lipofectamine LTX (Life Technologies, Carlsbad, CA, USA) in a 96-well plate. At 48 h post-transfection, cells were collected and the cell pellets were used directly for genomic PCR. PCR was carried out using KOD FX Neo (Toyobo, Tokyo, Japan) with the primers listed in Supplementary Table S1. The Cel-I assay was performed as described previously[15] and the products were analyzed by electrophoresis in agarose gels and ethidium bromide staining.

mRNA synthesis, manipulation of *X. laevis* eggs, and mutation analysis. TALEN mRNAs were synthesized using an mMessage mMachine T7 Ultra Kit (Life Technologies) according to manufacturer's instructions. Fertilized *X. laevis* eggs were obtained from breeding pairs injected with human chorionic gonadotropin[28]. Eggs were de-jellied by treatment with 2% cysteine and then moved into 3% Ficoll in 0.36 X Marc's modified Ringer's (MMR). Approximately 250 pg of each left and right TALEN mRNA was injected into eggs at the one-cell stage in a volume of 4.6 nl using a Nano-ject II (Drummond, Broomall, PA, USA). Injected embryos were cultured at 20°C in 0.16 X MMR containing gentamycin from the blastula to the swimming tadpole stages. The animals were handled in accordance with the guidelines of Hiroshima University for the use and care of experimental animals.

Genomic DNA samples were extracted from individual embryos using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Genomic regions containing the TALEN target site were amplified with specific primer sets (Supplementary Table S1). The efficiency of target mutations in the injected embryos was examined by restriction enzyme digestion of PCR products using *Hinf*I. To confirm the presence of TALEN-mediated mutations, PCR products were subcloned into pCR2.1/TOPO (Life Technologies), and positive clones were then selected by colony PCR. Colony PCR products were sequenced using BigDye Terminator Ver. 3.1 (Life Technologies).

Rat-1 cell culture and Cel-I assay. The protocols for cell culture and transfection were performed as reported previously[13]. Briefly, 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 cells (1×10^5) were suspended in 10 ml R buffer (supplied as part of the Neon Transfection System, Life Technologies) with 0.5 mg of each plasmid, and electroporated under the following conditions: pulse voltage, 1300 V; pulse width, 20 ms; and pulse number, 2 (program #15). Following electroporation, the cells were cultured in DMEM supplemented with 10% FBS without antibiotics for 24 h, followed by medium with antibiotics for 48 h.

Genomic DNA was extracted from Rat-1 cells using Nucleospin (Macherey-Nagel, Düren, Germany) 72 h after electroporation. PCR was then performed using PrimeSTAR HS DNA polymerase (Takara Bio) with the primers listed in Supplementary Table S1. The PCR amplification products were heat denatured, digested as in the Cel-I assay described above, and subjected to agarose gel electrophoresis to confirm TALEN-induced mutations.

Microinjection of TALENs into rat embryos. All rat care and experiments were carried out according to the Guidelines for Animal Experiments of Kyoto University, and were approved by the Animal Research Committee of Kyoto University. The newly developed F344-*Il2rg*^{em7Kyo} albino rats (NBRP-Rat No.0694) were deposited into the National Bio Resource Project – Rat in Japan (www.anim.med.kyoto-u.ac.jp/nbr).

The microinjection of TALEN mRNA into F344/Stm rat embyos was carried out as described previously[13]. Briefly, mRNA was transcribed *in vitro* using a MessageMax™ T7 mRNA transcription kit (Cellscript Inc., Madison, WI, USA) and polyadenylated using a A-Plus™ Poly(A) polymerase tailing kit (Cellscript). The resultant mRNA was purified using a MEGAClear™ kit (Cellscript) and finally resuspended in RNase-free water at 10 ng/μl for each TALEN. Approximately 2–3 pl of capped mRNA were injected into the male pronuclei of zygotes by microinjection, and surviving embryos were transferred to the oviducts of pseudopregnant Wistar female rats.

Genomic DNA was extracted from the tail using a GENEXTRACTOR TA-100 automatic DNA purification system (Takara Bio). The PCR products amplified with specific primer sets (Supplementary Table S1) were directly sequenced using the BigDye terminator v3.1 cycle sequencing mix and the standard protocol for an Applied Biosystems 3130 DNA Sequencer (Life Technologies).

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Author contributions

T.S. designed the work, performed the experiments, and wrote the manuscript. H.O. supported the creation of TALEN modules and vectors. T.K. and T.M. performed rat experiments. D.T. supported human cell experiments. Y.S. and K.I.S. performed *Xenopus* experiments. T.M., N.S. and S.M. provided instructions. T.Y. supervised the work. All authors reviewed the manuscript.

Additional information

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