<table>
<thead>
<tr>
<th>Title</th>
<th>Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Sakuma, Tetsushi; Ochiai, Hiroshi; Kaneko, Takehito; Mashimo, Tomoji; Tokumasu, Daisuke; Sakane, Yuto; Suzuki, Ken-Ichi; Miyamoto, Tatsuo; Sakamoto, Naoaki; Matsuura, Shinya; Yamamoto, Takashi</td>
</tr>
<tr>
<td>Citation</td>
<td>Scientific reports (2013), 3</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2013-11-29</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/179554">http://hdl.handle.net/2433/179554</a></td>
</tr>
<tr>
<td>Rights</td>
<td>This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit <a href="http://creativecommons.org/licenses/by-nc-sa/3.0/">http://creativecommons.org/licenses/by-nc-sa/3.0/</a></td>
</tr>
<tr>
<td>Type</td>
<td>Journal Article</td>
</tr>
<tr>
<td>Textversion</td>
<td>publisher</td>
</tr>
</tbody>
</table>

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

Tetsushi Sakuma¹, Hiroshi Ochiai¹, Takehito Kaneko², Tomoji Mashimo³, Daisuke Tokumasu¹, Yuto Sakane¹, Ken-ichi Suzuki¹, Tatsuo Miyamoto³, Naoaki Sakamoto¹, Shinya Matsuura³ & Takashi Yamamoto¹

¹Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan, ²Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan, ³Department of Genetics and Cell Biology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima 734-8553, Japan.

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.

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\(^6\) by modifying the Golden Gate TALEN and TAL Effector Kit (Addgene)\(^7\). 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\(^9\) 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 and +153/+47 (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.
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.

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) 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).
and re-annealed PCR products were then treated with Cel-I nuclease 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 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, these phenotypes were stronger than those of conventional TALEN-injected embryos (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.

![Figure 3](https://www.nature.com/scientificreports/)

**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*. The target sequence of eGFP was originally described by Sakuma *et al*. (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.
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) plasmid17,18 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 TALENs13 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 plants19, flies9,20, zebrafish5,10,21,22, medaka11, frogs9,12,23, rats13, and pigs24. 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 Kit13, the efficiency was lower than that reported by Tesson et al25, 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...
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. REAL3,26-, REAL-Fast26-, and FLASH4,27-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 BsaI restriction sites at both ends were synthesized and cloned into pBluescript 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 are described in Supplementary Fig. S2. Each TALEN expression plasmid was constructed using the Golden Gate cloning method, as described previously15. Target sequences for each gene are shown in Figures 2B, 3A, and 4A. Reporter plasmids for SSA assay were constructed as described previously15.

**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 previously29. 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-1 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 previously19 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 mRNA was synthesized using an mMessage mMachine 77 Ultra kit (Life Technologies) according to manufacturer’s instructions. Fertilized *X. laevis* eggs were obtained from breeding pairs injected with human chorionic gonadotropin20. Eggs were de-jellied by treatment with 2% cysteine and then moved into 3% Ficoll in 0.36X Marc’s modified Ringer’s (MMR). Approximately 250 pg of each left and right TALEN RNA transcription kit (inTec, USA) was used, and gel purified using a Nano-ject II (Drummond, Broomall, PA, USA). Injected embryos were cultured at 20°C in 0.16X 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 HinfI. To confirm the presence of TALEN-mediated mutations, PCR products were subcloned into pcCR2.1/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-1 assay.** The protocols for cell culture and transfection were performed as reported previously21. Briefly, rat fibroblast-like (Rat-1) cells were obtained from the RIKEN BC Cell Bank (Tsukuba, Japan, http://www.rrc.brc.u-tokyo.ac.jp/lab/). (1 x 10^6) were suspended in 10 ml R buuffer (supplied as part of the Neo 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 amplification products were then sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing products were purified using a MEGAclear kit (m) (Sangon Biotech, China) and sequenced using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). The primers were designed to amplify a fragment spanning the target sequence.

**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 P344/STm male embryos (NBRP Rat No.9694) 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 embryos was carried out as described previously22. Briefly, mRNA was transcribed in vitro using a MessageMax MAXI T7 kit (Ambion) and gel purified using a A-Plus Poly(A) polymerase tailing kit (Clontech). The resultant mRNA was purified using a MEGAclear kit (Clontech) 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 kit (Applied Biosystems). The sequences were then analyzed for the presence of TALEN-induced mutations.

**Acknowledgments**

We would like to thank the members of Yamamoto’s laboratory for their assistance in TALEN construction. We also thank the authors of Life Technologies (USA) and Agilent Technologies for supplying the Golden Gate TALEN and TALE Effector Kit (Addgene, TALEN kit #1000000016) and TALE Toolbox Kit (cat#1000000019), respectively, and the Cytogenetic Center of Hiroshima University for supplying liquid nitrogen. This study was supported by a Grant-in-Aid for Challenging Exploratory Research Grant Number 24675176 (to T.Y.).

**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 care and experiments. T.M., N.S. and S.M. provided instructions. T.Y. supervised the work. All authors reviewed the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/srep
How to cite this article: Sakuma, T. et al. Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity. Sci. Rep. 3, 3379; DOI:10.1038/srep03379 (2013).

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported license. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0