Introduction

Customized nucleases (Zinc Finger Nucleases, TALENs and CRISPRs (Clustered Regularly Interspaced Short Palindromic Repeats) [1–4]) have revolutionized the genetic engineering field. TALENs are DNA binding nucleases that can be tailored to bind almost any sequence. It has been reported that a TALEN cleavage site can be found every 35 bp of genomic DNA [5]. TALENs are versatile molecules that can be easily designed, cloned, assembled and tested in a molecular biology laboratory. With the description [6,7], and assembly [8,9] of TALENs, the genetic engineering field has made important progress. Today, there are several web-accessible software available for the design and assembly of customized TALEN arrays. The most used are: TALE-NT or the accessible software available for the design and assembly of TALENs and designed a linear donor insert targeting the 3 TALENs into heterodimeric FokI expressing vectors selected 3 TALENs: TAL 3, TAL 6 and TAL 13. We assembled guidelines for TALEN-site selection in Cermak et al. [5], we obtained a set of 18 TALEN pairs using two query sequences corresponding to the pA458T mutation in mouse, that inhibits GR dimerization, namely GRdim [25]. Following the guidelines for TALEN-site selection in Cermak et al. [5], we selected 3 TALENs: TAL 5, TAL 6 and TAL 13. We assembled the 3 TALENs into heterodimeric FokI expressing vectors described in [24] following the REAL and REAL-Fast standard-cloning assembly method from TAEngineering.org (see Table S1 in File S1 for detailed TALEN binding sequences).
are listed in Table S2 in File S1. Resulting bands were 177 and 288 bp long (Fig. 1b). T7 endo I assay indicated high specific TALEN activity of 20 and 16%, for TAL 3 and TAL 6 respectively (Fig. 1b). We continued with TAL 3. To further determine the “indels” (insertions and deletions) generated by TAL 3 in C6 cells, we amplified the genomic DNA of the TAL 3-transfected C6 cells and performed a screening of the amplified \( \text{Nr3c1} \) gene. Screening results indicated a rate of 22.5% of NHEJ (Non Homologous End Joining, results not shown), which confirmed the results of the T7 endo I assay in cells. Cells transfected with TAL 3 presented mostly deletions ranging from 1 to 16 nucleotides (Fig. 1c).

Donor molecule design and linearization

To generate GR\textsuperscript{dim} KI rats, we designed a common donor plasmid for TALENs 3 and 6, bearing the pA476T mutation along with 4 silent point mutations in each TALEN binding site to prevent further nuclease activity in the targeted alleles (Fig. 2 and Fig. S2 in File S1). The donor plasmid sequence had also 500 bp homology arms on 3' and 5' sides of the pA476T site (Fig. 2 and Fig. S2 in File S1).

Figure 1. TALEN design and evaluation of cutting efficiency in rat glioma C6 cells. (a) Schematic of the rat \( \text{Nr3c1} \) (GR) gene. Zoom on the area of the mutation pA476T in exon 3. The first nucleotide of the 476 codon is highlighted in blue. TALEN binding sites of TAL 3 are highlighted in green. Detailed sequences of TAL 6 binding sites can be found in Table S1, File S1. (b) T-endo1 assay results. Pooled DNA from C6 cells transfected with either Right, Left or Right and Left TALEN monomers (marked with R, L or RL, respectively) was amplified and treated with T7 endo 1 enzyme. Cut bands of 288 and 177 bp indicate TALEN activity. Mcells are mock transfected cells, GFP: GFP transfected cells were used as a positive transfection control. Intensity of the cut bands are indicated for TAL 3 and TAL 6 pairs. (c) TAL 3 transfected cells screening. PCR amplicons of the region around the pA76T mutation were subcloned into TOPO vector. Clones were isolated and analyzed individually. Four point mutations and insertions are marked in red. doi:10.1371/journal.pone.0088146.g001
Generation of GR knockout and knockin rats by TALEN mRNA injection in one-cell stage embryos

TALEN mRNA and excised linearized double-stranded donor DNA containing point mutations and diagnostic restriction sites were co-injected into fertilized one-cell stage embryos at two concentrations (20 ng/µl or 10 ng/µl of each TALEN monomer mRNA) and at 5 ng/µl for donor DNA. As previously described [26] we performed a two-step microinjection procedure: we first injected the mixture TALEN/DNA into the male pronucleus and then into the cytoplasm during the withdrawal of the injection pipette. Data are summarized in Table 1.

Analysis of the HR events in Fo founders by Alu and Haell enzyme digestion and sequencing

To reveal homologous recombination events located in the correct locus, we amplified the genomic DNA of the founders with the forward primer designed outside of the homology region and reverse primer inside of the homology region (“outside-in”) and digested them with AluI and HaellIII enzymes. Primers used are described in Table S2 in File S1. Gel digestion of the Nr3c1 exon 3 revealed one KI female founder (namely 3.4) from the first injection series of higher TALEN mRNA out of 225 zygotes (KI 3). Nine of the 10 founder rats named 11.4, 6.1 and 5.5, were heterozygous for the Nr3c1 exon 3 allele: KI (44%), KO (17%) and wt (39%). Three founder rats (11.4, 6.1 and 5.5) showed deletions between 5 and 527 bp (data not shown). Three founder rats named 11.4, 6.1 and 5.5, were heterozygous for the Nr3c1 exon 3 allele: KI (44%), KO (17%) and wt (39%), as analyzed by subcloning of the amplified Nr3c1 exon 3 (results not shown). Offspring of the KI rat 3.4 died before giving rise to birth. NHEJ events were observed in 9 rats. Three analyzed and bred rats transmitted the mutated allele to the next generation confirming germline transmission. The 3 founders analyzed where

Analysis of the NHEJ events in Fo founders by AluI enzyme digestion and sequencing

To reveal NHEJ events in other founders, we digested the Nr3c1 exon amplified with “inside-out” primers with AluI enzyme. Nine NHEJ events were found resulting from both injection series (Table 1 and Fig. S5 in File S1). Microinjection with each ratio TALEN/DNA resulted in both cases in high embryo survival (76% and 79% of injected embryos, respectively). All 9 founders showed Nr3c1 deletions between 5 and 527 bp (data not shown). Three founder rats named 11.4, 6.1 and 5.5, were heterozygous and likely presented in-frame deletions of 6, 18 and 309 bp, respectively (Fig. 4). These mutations correspond to deletions of the dimerization and/or DNA-binding domain of the glucocorticoid receptor. We kept these 3 rats for further breeding.

Discussion

To our knowledge, here we report the first KI rat made by TALEN-mediated homology-derived recombination with a linear donor. In this study, we used linear donor since in previous injections, we were unable to obtain KI animals with a supercoiled donor. We observed 10 targeted mutations out of 58 pups born alive, representing 17% of the offspring. These results indicate high TALEN efficiency. Nine of the 10 Nr3c1 gene modifications were knockouts and 1 was a knockin. Our single KI founder 3.4 harbored three Nr3c1 alleles: KI (44%), KO (17%) and wt (39%); as analyzed by subcloning of the amplified Nr3c1 exon 3 (results not shown). Offspring of the KI rat 3.4 died before giving rise to birth. NHEJ events were observed in 9 rats. Three analyzed and bred rats transmitted the mutated allele to the next generation confirming germline transmission. The 3 founders analyzed where

**Table 1.** Injections of TAL 3 mRNA and donor plasmid DNA in rat one-cell embryos.

| Dose TALEN mRNA/DNA (ng/µl) | No. Injected eggs/No. Transferred eggs (% of survival) | Offspring (% of injected eggs) | No. NHEJ pups (% of offspring) | No. KO pups (% of offspring) | Targeting frequency (% of offspring) |
|-------------------------------|------------------------------------------------------|--------------------------------|---------------------------------|-------------------------------|-------------------------------------|
| 40 (20+20)/5                  | 225/171 (76)                                         | 32 (14)                        | 4 (13)                          | 1 (3)                         | 16                                  |
| 20 (10+10)/5                  | 293/228 (79)                                         | 271 (9)                        | 5 (19)                          | 0                             | 19                                  |

*One pup was born dead.

Two doses of TAL 3 mRNA were used (20+20 or 10+10 ng/µl of each TALEN). The egg survival rate is shown in percentage. NHEJ indicates the number of pups that had a gene disruption event in the sequence around pA476T. The percentages were calculated within each set of TALEN mRNA amount injected.

doi:10.1371/journal.pone.0088146.g002

Figure 2. Donor plasmid design for the generation of GRdim KI rat. Upper DNA indicates the wild type sequence of the exon 3 of the rat Nr3c1 GR. In blue the residue A476. TAL 3 and TAL 6 binding sites are indicated. DP, donor plasmid sequence. Donor plasmid was synthesized with 500 pb homology arms on both ends. The pA476T point mutation is highlighted in blue. Silent mutations of the DP are shown in red bold letters and are located in the overlap of TAL 3 and Tal 6 binding sites. Haelli site is highlighted in yellow. AluI site is not shown in this figure.

doi:10.1371/journal.pone.0088146.t001
heterozygous and had a deletion of several base pairs that are likely in-frame mutations of the glucocorticoid receptor, within the dimerization and/or DNA-binding domain. In 2 of the 3 founder lines, we obtained homozygous mutant offspring (Sofia Verouti, personal communication). They will be used for further physiological experiments.

Seven out of 58 live born founders (representing 12% of the offspring) presented off-site-target events. Of the 7 pups presenting off-target events, 2 had already on-target modifications. In some cases, off-targeted insertions were observed in up to three different loci in the same pup.

The rate of NHEJ found in rats was predicted efficiently by our assay in rat C6 cells and confirmed by screening (20% cutting efficiency determined by T7 endo I assay and 22.5% NHEJ by screening). Screening assays in cells indicated the generation of indels ranging from 1 to 16 bp, compared to deletions ranging from 5 to 527 bp in rats. 50% of the Nr3c1-targeted rats carried various Nr3c1 alleles, including wild type sequences and deletions of several nucleotides.

This report serves as a proof of concept that TALENs are efficient tools to generate targeted and specific mutagenesis of the rat with linear donor molecules. They are affordable, convenient, freely designed and assembled in a molecular biology laboratory. We demonstrate the first gene editing in the rat by homology-derived recombination (HDR) using oocyte microinjection of TALENs mRNA with a linear donor molecule. This report might encourage further TALEN-mediated gene targeting in the rat to apply these models in physiological and genetic research.

Materials and Methods

GR Nr3c1 gene sequencing

The GR sequence around the pA476 site was sequenced from amplified genomic DNA pools of 10^6 rat C6 cells (CCL-107, ATCC) and 6 adult Sprague-Dawley rats (Charles River Laboratory SAS SD rats): 3 males and 3 females. The primers used for the sequencing are listed in Table S2 in File S1.

TALEN design and construction

TALEN pairs were designed using the free software from the website http://zifit.partners.org/ZiFiT/ and protocols for the standard cloning method and REAL assembly are available at http://www.talengineering.org/platforms-real.htm. The query sequence for TALEN design and the TALEN binding sites are available in Table S1 in File S1. TAL 3 was cloned by standard cloning according to instructions in http://zifit.partners.org/ZiFiT/Program_use.aspx#. TAL 6 and 13 sequence were ordered for synthesis at Eurofins MWG Operon with 5' overhang bearing the BbsI site and the 3' bearing the BsaI site and then cloned into nuclease backbone vectors from the REAL protocol [12].

Donor plasmid design

The donor plasmid had the pA476T site and homology arms made of 500 bp on the 3' and 5' ends. The AluI site in intron 3 was suppressed and the silent mutation giving the HaeIII site in exon 3 was added to test the insertion efficiency of the TALENs. Each TALEN binding site also carried 4 extra silent point mutations.

Cell transfection, gene amplification and DNA preparation for the T7 endo I assay

10^5 rat C6 cells were cultured in F-12 GlutamaX (Invitrogen, now Life Technologies), 10% FBS heat-inactivated and seeded in 6 well plates. Cells were transfected the next morning with plasmids encoding for TALENs at 0.6 mg total DNA per well and Lipofectamine 2000 (Invitrogen, now Life Technologies) following manufacturer’s instructions. They were kept at 37°C for 4 hours in F-12 medium without FBS and Lipofectamine 2000. Then the medium was replaced by complete medium (F-12 and FBS), and cells were left for 72 hours at 30°C. Then cells were harvested and genomic DNA was extracted using Phire Animal Tissue Direct PCR Kit (Thermo Scientific) and amplified. Primers used are listed in Table S2, File S1.

Materials and Methods

GR Nr3c1 gene sequencing

The GR sequence around the pA476 site was sequenced from amplified genomic DNA pools of 10^6 rat C6 cells (CCL-107, ATCC) and 6 adult Sprague-Dawley rats (Charles River Laboratory SAS SD rats): 3 males and 3 females. The primers used for the sequencing are listed in Table S2 in File S1.

Figure 3. Founder KI female 3.4 genotyping from subcloned PCR amplicons of tail biopsies. Wt: Wild type; DP: donor plasmid. Point mutations in the DP are indicated in red bold letters. The pA476T mutation is highlighted in blue. doi:10.1371/journal.pone.0088146.g003

Figure 4. Fo KO rat genotyping. Wt, wild type sequence. TALEN binding sites are shown in green. The pA476T mutation is highlighted in blue. Longer deletions are marked with double slash. Rats 11.4, 6.1 and 5.5 (underlined) were kept for breeding. All rats beared the wt allele of the Nr3c1 gene. Primers used are listed in Table S2, File S1. doi:10.1371/journal.pone.0088146.g004
bacteria. Colonies were seeded in 200 mL of LB medium in 96 well plates and sequenced.

**DNA was cloned into blunt vector using the TOPO cloning kit.**

Endo I nuclease (New England Biolabs) were added to the PCR mixture, after incubation for 30 minutes at 37°C. Dehybridization with exon 3-derived probe. Indicated is the 3.6 kb exon 3 sequence was amplified by PCR using primers listed in Table S2 in File S1. 

**Microinjection into rat zygotes**

Prepubescent females (4–5 weeks old) were injected with 30 IU pregnant mare serum gonadotropin (Intervet) and followed 48 hours later with 20 IU human chorionic gonadotropin (Intervet) before breeding as previously described [27]. Fertilized one-cell stage embryos were collected for subsequent microinjection using a previously published procedure [27]. Briefly, both TALEN mRNAs and donor DNA were microinjected into the male pronucleus and into the cytoplasm of fertilized one-cell stage embryos. Two ratios of diluted TALEN mRNA and donor DNA have been tested. Surviving embryos were implanted on the same day in the oviduct of pseudo-pregnant females (0.5 dpc) and allowed to develop to full term.

**Cell lysis and Western blot analysis**

C6 cells were harvested and lysed with 1% Triton buffer (1 M Tris, 5 M NaCl, 1% Triton X-100 (Sigma), 1:500 vol/vol of the following: leupeptin, apro tinin and pepstatin (Sigma) and 1:1000 vol/vol of PMSF (Sigma)) for 30 minutes at 4°C in a roller. Then cell lysis was centrifuged 15 minutes at 15,000 rpm to remove cell membrane debris. The supernatant containing the proteins was loaded on a 10% acrylamide gel with denaturing sample buffer after incubated at 95°C for 5 minutes. Proteins were transferred to a nitrocellulose membrane and blocked with 5% milk TBS. After washing, the membrane was incubated with 1:1000 monoclonal antibody against flag protein produced in mouse (Sigma) and secondary antibody was anti-mouse IgG linked to horseradish peroxidase (GE Healthcare). The film was exposed 5 minutes.

**In vitro transcription of TALEN mRNA**

For the pA476T gene targeting, TALEN-encoding expression plasmids were linearized with PmeI. Messenger RNA was in vitro transcribed and polyadenylated using the Message mMachine T7 M1 ultra kit (Ambion) following the manufacturer protocol and purified using the MegaClear Kit (Ambion), quantified using a NanoDrop-1000 (Thermo Scientific), and stored at −80°C until use. Messenger RNAs encoding pA476T TALENs were mixed to a final total concentration of 10 ng/μl, or 20 ng/μl of each TALEN monomer in TE 5/0.1 (5 mM Tris-Cl pH 7.5, 0.1 mM EDTA in RNase DNase free water) and stored at −80°C until use. mRNAs were kept on ice during all micro-injection procedures.

**Animals**

Sprague-Dawley rats (SD/Crl, Charles River) were housed in standard cages and protocols were conducted in accordance with the guidelines for animal experiments of the Veterinary Services and were performed by officially authorized personnel in a certified animal facility. All animal experiments were compliant with the Animal Protection Law of the French republic (article R-214-89), which is in compliance with the European Community (ref: CEEA-2011-45).

**Southern blot analysis of founders following HinII digestion of tail DNA and hybridization with a duplicated PCR-amplified exon 3 probe.**

**Supporting Information**

**File S1** Supplementary Data are available at PLoS ONE Online and include Table S1–S2 and Figure S1–S5.
DNA1, clone 1, DNA2, clone 2, DNA5, clone 5. TALEN binding sites from the donor are in green, bold letters indicate the mutated nucleotides. HauIII site is present in the donor sequence only; Adh1 is present in the wild type sequence only. Figure S5 Gel digestion analysis of “indels” in Fo founders rats. “Inside-out” primers were used in this assay (Table S2 in File S1). Wt indicates expected fragments of wild type animals upon digestion with Adh1 enzyme. All pups have variable size bands on the lower molecular weight zone, indicating possible indels. The KI rat 3.4 upper band (marked with a * of 469 bp) indicates absence of the Adh1 site close to the pA476T. KI fragment sizes expected: 125, 250 and 469 bp. Wt fragment sizes expected: 125, 175, 250 and 294 bp.

Acknowledgments
We thank K. Joung and talengineering.org for providing the web-accessible software and protocols. We thank Séverine Ménoret, Sérène Remy and Claire Usal for TALEN mRNA and donor plasmid DNA injections; Simona Frateschi for rat maintenance; Jérémie Canonica for invaluable comments, Laura Battista and Sandra Ramirez for figure editing and David Largaespada for critically reading this manuscript.

Author Contributions
Conceived and designed the experiments: VPdL, EH. Performed the experiments: VPdL. Analyzed the data: VPdL, LT. Wrote the paper: VPdL, EH. Conceived and supervised the experiments: IA.

References
1. Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, et al. (2001) Stimulation of Homologous Recombination through Targeted Cleavage by Chimeric Nucleases. Society 21: 289–7. doi:10.1128/MCB.21.1.289
2. Sun N, Zhao H (2013) Transcription activator-like effector nucleases (TALENs): A highly efficient and versatile tool for genome editing. Biotechnol Bioeng 100: 1811–1821. doi:10.1002/bit.24980
3. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna J, et al. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337: 1666–1670. doi:10.1126/science.1225829
4. Yang H, Wang H, Shivalila CS, Cheng AW, Shi L, et al. (2013) One-Step Generation of Mice Carrying Reporter and Conditional Alleles by CRISPR/Cas-Mediated Genome Engineering. Cell 154: 1370–1379. doi:10.1016/j.cell.2013.08.022
5. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, et al. (2011) Efficient Designer TAL Effectors by Golden Gate cloning. PLoS One 6: e19722. doi:10.1371/journal.pone.0002016
6. Davies B, Davies G, Preece C, Palyriadis R, Szmukka D, et al. (2013) Site Specific Mutation of the Zic2 Locus by Microinjection of TALEN mRNA in Mouse CD1, C3H and C57BL/6j Oocytes. PLoS One 8: e60216. doi:10.1371/journal.pone.0060216
7. Tesson L, Usai C, Ménoret S, Leung E, Niles BJ, et al. (2011) Knockout rats generated by embryo microinjection of TALENs. Nat Biotechnol 29: 693–696. doi:10.1038/nbt.1940
8. Mashimo T, Kaneko T, Sakuma T, Kobayashi J, Kunihiro Y, et al. (2013) Efficient gene targeting by TAL effector nucleases cojected with exonucleases in zygotes. Sci Rep 3: 1253. doi:10.1038/srep01253
9. James M (1997) Why map the rat? Trends Genet 13: 171–173.
10. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, et al. (1998) Zic2 is essential for normal heart development and establishment of the neural plate. Genes Dev 12: 121–132. doi:10.1016/j.cell.2013.08.022
11. Parker CC, Chen H, Flagel SB, Geurs AM, Richards JB, et al. (2013) Rats are the smart choice: Rationale for a renewed focus on rats in behavioral genetics: Neuropharmacology 76: 250–250. doi:10.1016/j.neuropharm.2013.05.047
12. Huang G, Ashton C, Kumbhari DS, Ying Q-L (2011) Genetic manipulations in the rat: progress and prospects. Curr Opin Neuroporpheresis Hypertension 20: 391–399. doi:10.1097/MNH.0b013e328347768a
13. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, et al. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93: 531–541.
14. Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, et al. (2007) An improved zinc-finger nuclease architecture for highly specific genome editing. Nature Biotechnol 25: 778–785. doi:10.1038/nbt1319
15. Bourner KJ, Trautman JK, Mukherjee K, Carroll D (2013) Donor DNA Utilization during Gene Targeting with Zinc-finger Nucleases. G3 (Bethesda). doi:10.1534/g3.112.004393
16. Cost GJ, Remy S, Remy S, Cui X, Tesson L (2010) Generation of gene-specific mutated rats using zinc-finger nucleases. Methods Mol Biol 597: 211–225. doi:10.1007/978-1-60327-389-3_15
17. Ménoret S, Fontaniere S, Janitz D, Tesson L, Thannard R, Remy S, et al. (2013) Generation of Rag2 knockout immunodeficient rats and mice using engineered meganucleases. FASEB J 27: 705–711. doi:10.1096/fj.12-219907