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Knockout rats generated by embryo microinjection of TALENs

To the Editor:

The recent description of highly active transcription activator-like effector nucleases (TALENs)[1] prompted us to explore their utility for *in vivo* genetic engineering in the laboratory rat. The rat is a valuable experimental animal because of its suitability for modeling human disease and toxicology. Zinc-finger nuclease (ZFN) technology and the isolation of rat embryonic stem cells have enabled targeted modifications of the rat genome[2–5]. Recently, *Xanthomonas*-derived transcription activator-like (TAL) effector proteins have elicited much interest because of their apparently simple rules for sequence-specific DNA recognition[6,7]. Several investigators have fused the FokI nuclease domain to TAL effector proteins to create TALENs[1,8–12]. However, only optimal truncation of the TAL effector protein allowed high-frequency gene disruption of endogenous loci and targeted DNA integration[1,13,14]. Here we use TALENs to disrupt the rat *IgM* locus, creating heritable mutations that eliminate *IgM* function. Our results establish the use of TALEN technology for *in vivo* gene knockout in mammals.

We designed and assembled TALENs to exon 2 of rat *IgM* and tested their ability to alter the *IgM* locus in rat S16 cells (**Fig. 1a** and **Supplementary Sequences**). The TALEN pair modified ~21% of chromosomes when delivered as DNA and up to 13% when delivered as mRNA (**Fig. 1b**). We injected titrations of these nucleic acids into one-cell rat embryos[2] and assayed the resulting pups for alteration of the *IgM* locus (**Fig. 1c**). Over all doses, 7/74 (9.5%) of rat one-cell embryos injected with DNA and 51/88 (58%) of rat embryos injected with mRNA were modified at *IgM* (**Table 1**). Of the *IgM*-mutated mRNA-injected rats, 13/51 (25%) were biallelically modified in this single step, several containing frame-shifting mutations predicted to eliminate *IgM* function on both alleles (**Supplementary Table 1**). *IgM* mutation frequency was a function of TALEN dose, with the highest percentage of modified rats (75%) derived from injection of 10 ng/μl and 4 ng/μl mRNA (**Table 1**). The frequency of biallelically modified rats was also dose-dependent: 8/15 (53%) of rats injected with 10 ng/μl mRNA, 5/27 (19%) at 4 ng/μl, and none at 0.8 ng/μl. No biallelically modified rats were obtained from DNA injections in this study, consistent with the lower overall frequency of *IgM* modification in this founder population.

We bred rat 3.4 with a wild-type rat and used PCR to assay the resulting F1 animals for the 90-bp deletion present in

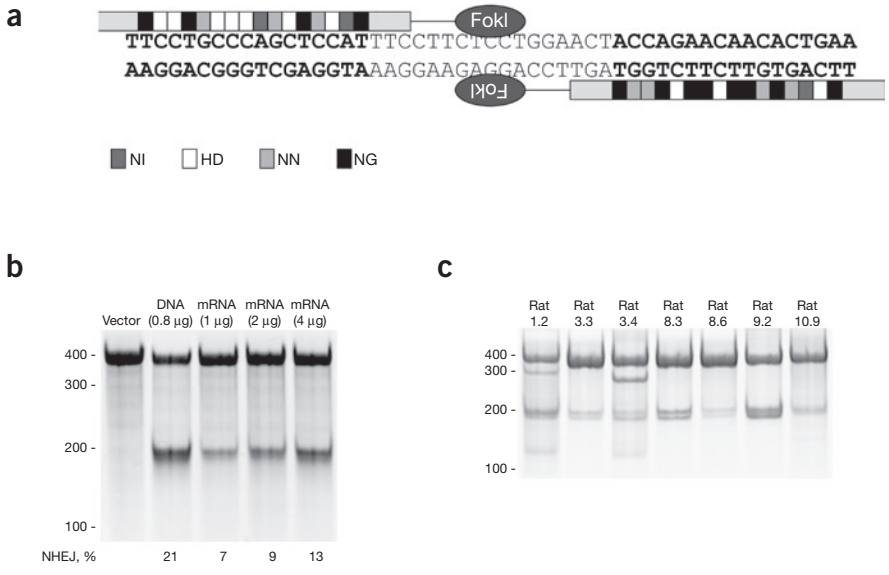

Figure 1 Design and assay of TALENs that target the rat *IgM* locus. (**a**) The DNA binding sites and spacing of the SBS 101187/SBS 101188 TALENs. The specific repeat-variable di-residue used to recognize each base is indicated by shading, as defined in the key. A thymidine nucleotide (T) is present to the 5′ of each binding site. (**b**) TALEN modification of the *IgM* gene in rat S16 cells when transfected as DNA expressed with the CAG promoter or as capped, polyadenylated mRNA. Surveyor nuclease cleavage produces the expected ~181 and ~191 bp bands. NHEJ, %, percentage of cleaved alleles. (**c**) TALEN modification of the *IgM* gene in individual rats derived from DNA injection. Of 74 rats assayed, these 7 were found to be mutated at *IgM*. The extent of Surveyor nuclease cleavage is dependent on the severity of the TALEN-induced allele. Molecular size markers are indicated in base pairs.

Table 1 Disruption of the rat *IgM* locus via TALEN cleavage

Injection/route	Dose (ng/μl)	Injected	Transferred	Newborns	Founders
DNA/PNI	10	166	98 (59%)	13 (13%)	3 (23%)
DNA/PNI	2	236	150 (63%)	53 (35%)	4 (8%)
DNA/PNI	0.4	84	48 (57%)	8 (17%)	0 (0%)
mRNA/IC	10	200	146 (73%)	20 (14%)	15 (75%)
mRNA/IC	4	187	127(68%)	36 (28%)	27 (75%)
mRNA/IC	0.8	86	73 (85%)	32 (44%)	9 (28%)
Plasmid 1 DNA/PNI	2	402	235 (58%)	47 (20%)	4 (9%)
Plasmid 2 DNA/PNI	2	353	256 (73%)	51 (20%)	5 (10%)

TALENs were injected into the pronucleus as DNA or into the cytoplasm as mRNA at three concentrations each. Survival statistics from injection of two unrelated plasmid DNAs are shown for comparison. The percentages shown in the right three columns were derived using the number in each column as the numerator and the number in the column to the left as the denominator, times 100%. PNI, pronuclear injection; IC, intracytoplasmic.

the F_0 animal. Two of fourteen F_1 animals contained the mutant allele, demonstrating that TALEN-modified alleles can be transmitted through the germline (**Supplementary Fig. 1**).

We confirmed the elimination of *IgM* function in F_0 animals by flow-cytometric profiling of B cells and assay of circulating IgM. TALEN knockout animals lacked both mature B cells and secreted immunoglobulin (**Supplementary Fig. 2**).

To assay the specificity of TALEN cleavage, we determined the *in vitro* binding properties of the TALEN monomers and assembled a list of the most likely off-target cleavage sites based upon homology to this experimentally derived, consensus DNA binding preference (**Supplementary Tables 2** and **3**). Cleavage at potential off-target sites was assayed in the seven *IgM*-modified rats from DNA injection (**Supplementary Fig. 2**). No off-target activity was detected across nine loci (**Supplementary Fig. 3**). TALEN activity at *IgM* was higher in rats injected with mRNA. We therefore assayed off-target cleavage in nine *IgM*-modified rats from mRNA injection, finding modification of one off-target site in three rats (Site 1, **Supplementary Fig. 4**).

We occasionally observed mosaic animals. Although mosaicism might occur owing to a delay in TALEN activity until later embryogenesis, given the very high frequency of *IgM* modification in this population of rats and the broad TALEN monomer spacing range compatible with cleavage, it is possible that mosaicism in these animals could result from recleavage of already-modified *IgM* alleles.

Our optimally truncated TALENs cleave well when the TALEN dimer has a 12–23 bp intrapair spacing[1]; the *IgM* TALENs described here have a 17-bp spacer. We recovered no modified alleles with a small gain of nucleotides and many with deletions larger than typically seen with ZFNs. The potential of the *IgM* TALENs to recleave alleles with small insertions and deletions in the spacer region likely accounts in part for the observed spectrum of alleles. Additionally, two specific alleles, Δ6 and Δ12, were unexpectedly commonly recovered (the specific instances of which are both present in rat 17.5). Both of these alleles were likely formed by microhomology-mediated reclosure of the double-strand break using TCCT and CT, respectively.

Whereas ZFNs have been used to disrupt >25 rat genes[15], this is the first report of rat gene knockout using TALENs. This relative lack of experience with TALEN technology prevents a definitive comparison of ZFNs and TALENs with respect to rat gene disruption. However, the prior use of ZFNs to disrupt the rat *IgM* locus[2] permits a limited comparison of TALENs and ZFNs designed to target *IgM*. The overall frequency of mutated animals was identical (9%) when the nucleases were microinjected as DNA (7/74 for TALENs, 18/198 for ZFNs), whereas mRNA injection yielded a higher frequency of modified animals with TALENs (59% versus 19%). The overall frequency of newborn animals recovered after *IgM* TALEN injection was 25% (162/642) versus 13% (273/2079) with *IgM* ZFN injection[2]. The use of the enhanced FokI heterodimer mutations for the *IgM* TALENs (which increase cleavage activity and eliminate residual homodimerization leading to increased specificity) instead of the original FokI heterodimers on the *IgM* ZFNs may account for this difference (**Supplementary Methods**). Regardless, it is clear that the *IgM* TALENs afforded efficient targeted gene disruption—especially when injected as mRNA—and behaved comparably to our previously described work with ZFNs.

Together, our results suggest that TALE nucleases, when optimally designed and constructed[1,13,14], are compatible with *in vivo* gene disruption in rats. TALENs therefore represent a validated tool for the targeted genetic modification of this important experimental animal.

Note: Supplementary information is available on the Nature Biotechnology website.

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