and co-wrote the manuscript. M.G. cultured and differentiated the cells used in this study, carried out gene-expression studies and proofread the manuscript. S.S. and K.S. supervised the experimental work and proofread the manuscript.

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Lindsey Van Haute[1,3], Claudia Spits [1,3], Mieke Geens[1], Sara Seneca[1,2] & Karen Sermon[1]

[1]Research Group Reproduction and Genetics, Vrije Universiteit Brussel, Jette, Brussels, Belgium. [2]Centre for Medical Genetics, Universitair Ziekenhuis Brussel, Jette, Brussels, Belgium. [3]These authors equally contributed to this work.
e-mail: lindsey.van.haute@vub.ac.be

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Knockout mice created by TALEN-mediated gene targeting

To the Editor:
Phenotypic analysis of gene-specific knockout mice has transformed our understanding of *in vivo* gene functions. Generation of knockout mice, however, remains a time-consuming and expensive process. Transcription activator-like (TAL) effector nucleases (TALENs) are highly effective in inducing mutations at specific genomic loci[1,2], and consequently TALEN-mediated mutagenesis in zygotes is a potential alternative to conventional gene targeting in mice. However, to the best of our knowledge, gene knockout mice have yet to be created using TALENs. Here, we report the generation of mice with a genetic knockout of the *progesterone immunomodulatory binding factor 1(Pibf1)* or *selenoprotein W, muscle 1 (Sepw1)* gene using TALENs.

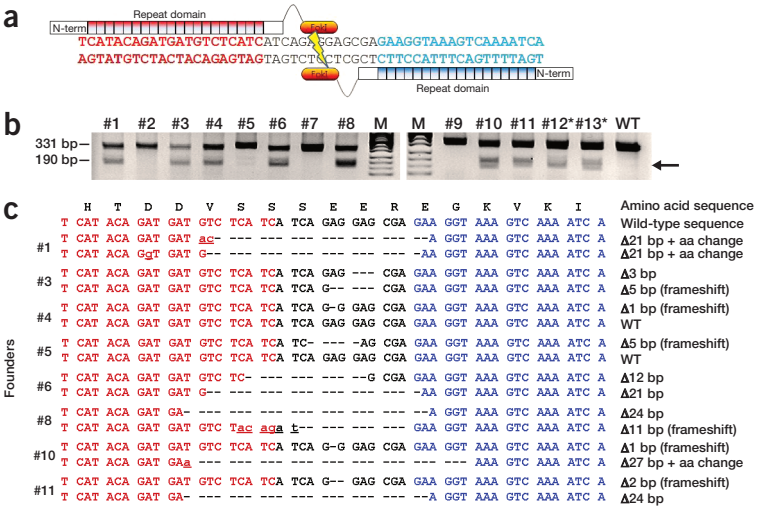

Figure 1 Generation of TALEN-mediated *Pibf1* mutant mice. (**a**) DNA-binding sequences (in red or blue) and the spacer region for Pibf1-TALEN. (**b**) T7 endonuclease I (T7E1) assays were conducted using genomic DNA from pronuclear-stage embryos intra-cytoplasmically injected with *Pibf1-TALEN* mRNA (50 ng/μl). The arrow indicates the size (~190 bp) of T7E1-digested DNA fragments. *Cannibalized mice. (**c**) DNA sequences of the*Pibf1* locus from live F_0 mice identified by T7E1 assays in **b**. '-' denotes deleted nucleotides. Underlined sequences in lower case represent nucleotide substitutions.

To target these genes in the mouse genome, we designed and synthesized highly active TALENs specific to exon 2 of *Pibf1* (Pibf1-TALEN; **Fig. 1a**, **Supplementary Figs. 1** and **2**) and exon 1 of *Sepw1* (Sepw1-TALEN, **Supplementary Figs. 3** and **4**). When tested in mouse NIH3T3 cells, Pibf1-TALEN efficiently induced small deletions at the target site (**Supplementary Fig. 1b**). Each *TALEN* mRNA pair was injected into the cytoplasm of mouse pronuclear-stage embryos to produce mutant founders (F_0) with mutations in *Pibf1* (**Fig. 1**, **Table 1** and **Supplementary Fig. 5**) or *Sepw1* (**Supplementary Table 1** and **Supplementary Fig. 6a**,**b**).

Most TALEN-induced mutations were deletions of variable lengths that induced frameshifts in the *Pibf1* and *Sepw1* genes (**Fig. 1b**, **Supplementary Figs. 5b** and **6b**). In-frame mutations, as a result of deletions and substitutions of specific amino-acid residues, were also frequent (**Fig. 1c**). Such mutations will be beneficial for studying putative domain- or amino acid residue–specific functions of the gene products. Insertional mutations were only observed in two instances (**Supplementary Fig. 5b**,**c**).

To investigate the dose-dependent effects of the *TALEN* mRNAs, two different concentrations of *Pibf1-TALEN* mRNA (50 ng/μl and 20 ng/μl) were used, yielding 29 mutants (55.8%) from 52 newborns (**Fig. 1b**, **Table 1** and **Supplementary Fig. 5**). Bi-allelic mutations were observed in seven mutant mice (**Fig. 1c** and **Supplementary Fig. 5b**). The mutation rate was approximately proportional to the injection dose of *Pibf1-TALEN* mRNA. Injection of a high concentration (50 ng/μl) of *Pibf1-TALEN* mRNA yielded 10/13 (76.9%) mutant F_0 mice, whereas injection of a lower dose (20 ng/μl) yielded 19/39 (48.7%) F_0 mutant mice (**Table 1**). Bi-allelic mutations were also found more frequently in the high-dose (6/8 F_0) than in the low-dose (1/19 F_0; **Fig. 1c** and **Supplementary Fig. 5**) group, indicating that Pibf1-TALEN activity was dose-dependent. Furthermore, relatively large deletions were more frequently observed in mutant founders obtained by high-dose injection than in those by low-dose injection (**Fig. 1c** and **Supplementary Fig. 5b**).

In contrast to the mutation rate, the number of mutant mice produced by the low-dose injection (19 mutants/176 transplanted embryos, 10.8%) was ~2.5-fold larger than

Table 1 TALEN-mediated *Pibf1* gene targeting in C57BL/6J mice.

Dose of *TALEN-Pibf1* mRNA (ng/μl)	Number of injected zygotes	Number of surviving zygotes	Two-cell embryos	Transferred embryos	Newborns	Founders*
50	276	263 (95.3%)	262 (99.6%)	243	13 (5.3%)†	10 (76.9%)††
20	183	176 (96.2%)	176 (100%)	176	39 (22.2%)†	19 (48.7%)

Percentages were calculated using the number in each column as the numerator and the number in the column to its left as the denominator. *Determined by T7E1 assays. †Two pups were cannibalized at birth. ††T7E1 assays were conducted using genomic DNA samples from newborns, including the two cannibalized pups.

the number produced by the high-dose injection (10 mutants/243 transplanted embryos, 4.1%; **Table 1**). This phenomenon is reminiscent of the toxicity of zinc-finger nucleases (ZFNs) induced by generation of nonspecific double-strand breaks at off-target sites[3]. Indeed, *IgM* mutant rats obtained by intracytoplasmic injection of *IgM-TALEN* mRNA had modifications at an off-target site[4]. Although we did not detect off-target effects of Pibf1-TALEN by T7E1 assays (**Supplementary Table 2** and **Supplementary Fig. 7**), we cannot rule out the possibility that nonspecific effects might have caused embryonic toxicity.

To validate TALEN-induced mutations, we measured the level of Sepw1 protein in tail biopsies. No expression of *Sepw1* protein was detected in a *Sepw1* mutant founder possessing bi-allelic null mutations at their start codon (**Supplementary Fig. 6c**).

We crossed three F_0 *Pibf1* mutants to wild-type mice and determined the genotypes of the F_1 offspring. All the mutations observed in F_0 mice were transmitted through the germline (**Supplementary Fig. 8**). These results indicate that TALEN-introduced mutant alleles were stably inherited by their F_1 progeny.

Mosaicism was rarely detected in both *Pibf1* (**Supplementary Fig. 5b**, **c**) and *Sepw1* mutants (**Supplementary Fig. 6b**), indicating that TALENs are primarily active at the one-cell stage. To demonstrate that Pibf1-TALEN is active at this stage, we amplified the whole genomes of one-cell embryos before initiation of the first cell division and assessed the alteration of the target region in the *Pibf1* locus. PCR genotyping and sequencing revealed deletions in the *Pibf1* locus, even in one-cell embryos (**Supplementary Fig. 9**). We conclude that TALEN activity in one-cell embryos is sufficient to induce mutations.

The occurrence of mosaicism in F_0 would be predicted to result from sustained TALEN activity during later embryogenesis or re-cleavage of already-modified alleles[4]. Because the first round of DNA replication begins after pronuclei become visibly evident under a stereomicroscope[5], the presence of mosaic mutants would not necessarily prove that TALENs act at the two-cell stage or later. If TALEN activity were sustained or delayed until the late one-cell stage, when the embryo possesses 4N DNA content before cell division, up to four different mutant alleles would be produced. No more than three different alleles were found among the *Pibf1* (**Fig. 1** and **Supplementary Fig. 5**) or *Sepw1* mutant founders (**Supplementary Fig. 6b**). These results support the hypothesis that TALEN-induced mosaic mutations can occur at the one-cell stage.

If Pibf1-TALEN protein is still active after the first cleavage of one-cell embryos, it should mutate the target locus independently in each nucleus of the two blastomeres, thereby frequently generating mosaic animals. However, predominantly mono-allelic *Pibf1* mutations were produced in the low-dose experiment (**Supplementary Fig. 5b**), indicating that the activity of Pibf1-TALEN was not sustained in blastomeres at the two-cell stage. This pattern is reminiscent of the maternal-to-zygotic transition (MZT) that actively eliminates maternally-provided gene products[6]. Although more detailed studies should be conducted that are designed to provide more direct evidence for TALEN activity at different developmental stages, our results suggest that TALEN activity is not likely to be maintained after the first cleavage of one-cell embryos.

Our study establishes that TALEN-mediated gene targeting is an efficient method for creating heritable null mutations in a specific locus of the mouse genome. We also provide evidence that TALEN activity in one-cell embryos is sufficient to induce mutations. Taken together, these data suggest that TALEN-mediated *in vivo* mutagenesis might expedite the creation of genetically engineered mouse models and thereby help to accelerate functional genomic research.

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AUTHOR CONTRIBUTIONS

H.-W.L. and J.-S.K. wrote the manuscript. All the other authors performed the experiments.

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Young Hoon Sung[1,6], In-Jeoung Baek[2,6], Duk Hyoung Kim[3,6], Jisun Jeon[1], Jaehoon Lee[1], Kyunghee Lee[4], Daewon Jeong[4], Jin-Soo Kim[3] & Han-Woong Lee[1,5]

[1]Department of Biochemistry, College of Life Science and Biotechnology, Yonsei University, Seoul, Republic of Korea. [2]Asan Institute for Life Sciences, University of Ulsan College of Medicine, Seoul, Republic of Korea. [3]Department of Chemistry, Seoul National University, Seoul, Republic of Korea. [4]Department of Microbiology and Aging-Associated Vascular Disease Research Center, Yeungnam University, Daegu, Republic of Korea. [5]Laboratory Animal Research Center, Yonsei University, Seoul, Republic of Korea. [6]These authors contributed equally to this work. e-mail: hwl@yonsei.ac.kr or jskim01@snu.ac.kr

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