Generation of targeted mouse mutants by embryo microinjection of TALENs

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Abstract

Gene engineering for generating targeted mouse mutants is a key technology for biomedical research. Using TALENs as nucleases to induce targeted double-strand breaks, the mouse genome can be directly modified in zygotes in a single step, without the need for embryonic stem cells. Thereby, Knockout and Knockin alleles can be generated fast and efficiently by embryo microinjection of TALEN mRNAs and targeting vectors. In this article we present an introduction into the TALEN technology and provide protocols for the application of TALENs in mouse zygotes.

Keywords

Knockin, Knockout, one-cell embryo, Gene targeting, TALEN, Mouse

1. Introduction

Engineering of the mouse germline to create targeted mutants is a key technology for biomedical research. The ascent of the mouse as genetic model organism is based on gene targeting in embryonic stem (ES) cells that relies on the rare transfer of vector encoded knockout and knockin mutations by homologous recombination (HR) [1]. To increase the rate of HR it was significant to recognize that targeted double-strand breaks (DSB), initially induced with the I-Sce restriction enzyme and later with zinc-finger nucleases, enhance DNA repair by several orders of magnitude at the damaged site [2][3][4][5]. DSBs can be repaired by the HR pathway using the sister chromosome as template or using gene targeting vectors that provide sequence homology regions flanking a desired genetic modification [6][7][8]. In the recombination process gene conversion extends from the vector´s homology regions into the heterologous sequence and transfers the modification into the genome (knockin) (**Fig. 1A)**. Alternatively, DSBs can be closed by the non-homologous end joining (NHEJ) pathway that religates the open DNA ends without repair templates [9]. By this means the DNA ends are frequently edited by the removal of one or more nucleotides, leading within coding regions to frameshift (knockout) mutations (**Fig. 1A**). Based on the utility of targeted DSBs at genomic sites of interest, further reseach focused on the construction of customized sequence-specific nucleases. Amenable for engineering is the family of zinc finger transcription factors that exhibits a modular structure such that individual Cys_2His_2-type elements composed of ~ 30 amino acids recognize 3 - 4 nucleotides of DNA sequence. To create zinc-finger based artificial nucleases, 3 - 6 elements can be fused and combined with the nuclease domain of the restriction enzyme FokI [10][11][12][4][13]. To induce DSBs two zinc-finger nuclease (ZFN) fusion proteins are combined such that each protein binds to 9 - 18 bp of a bipartite target sequence, separated by a spacer region of 6 bp. Upon binding of each protein to its target sequence both nuclease domains interact and create a DSB within the spacer region. Since individual zinc-finger domains jointly interact, each element must be selected from a large pool of modules and tested for optimal compatibility before the next component can be added [14][15]. Furthermore, the zinc-finger recognition code does not cover all nucleotide triplets, restricting the choice of target sequences. Therefore, the construction of highly active ZFNs is a tedious task that lies beyond the horizon of typical research laboratories. Withstanding these challenges a number of ZFNs were derived over the last decade and demonstrated the power of customized nuclease technology in a variety of biological systems, including zygotes of the mouse.

The use of ZFN provided the first validation of the direct, single step mutagenesis of the mouse germline in one-cell embryos, independent of ES cells [16][17].

The application of nucleases for genome engineering was strongly stimulated by the discovery of the novel DNA recognition code of the transcription activator-like (TAL) proteins of the plant pathogenic bacteria *Xanthomonas* in 2009 [18][19]. Based on the experience with ZFNs, the TAL-derived system could be readily adapted for the use as sequence-specific TAL nucleases (TALEN) [20][21]. Within the last years TAL proteins and TALENs were further characterized and applied for gene editing in mice and other species. These efforts showed that TALENs represent a versatile tool for gene editing in various species equal or superior to ZFNs. In particular, the majority of *in silicio* designed TALENs exhibit considerable specific nuclease activity [21][22]. Importantly the off-target profiles of both systems, *i.e.* the number of unintendend target sites, were found comparable [21][23][24]. Taking advantage of the modular nature of the four basic TAL components a growing number of cloning protocols that enable to construct TALEN expression vectors within short time were reported. Thereby, the TALEN approach matches the skills of typical research laboratories and has been elected in 2011 as `Method of the year´ [25][26]. In this article we present an overview of the TALEN technology and provide a protocol for generating targeted mouse mutants by the microinjection of TALEN mRNAs into zygotes.

- FIGURE 1 –

1.1 TAL DNA recognition code and TALEN target sites

TAL proteins comprise a class of > 100 members found in pathogenic bacteria of the genus *Xanthomonas* and *Ralstonia* that colonize important crops [27][28][29]. TAL proteins are secreted and injected via the bacterial type III secretion system into the plant cells where they activate the promoters of genes that support pathogen proliferation. These natural TAL proteins are composed of a N-terminal translocation domain, a central repeat domain that mediates binding to the specific DNA target sequence, and a C-terminal transactivation domain. The central repeat domain of TAL proteins contains an array of 13 - 28 peptide motifs each comprising 34 residues that are strictly conserved except for positions 12 and 13 [27][28][29]. The latter residues are involved in the recognition of a single nucleotide of the target DNA. The TAL recognition code was deciphered by the finding that

diresidues of histidine/aspartate (HD) at position 12/13 specify binding to cytidine, of asparagine/asparagine (NN) specify guanosine and adenosine recognition, of asparagines/isoleucin (NI) specify adenosine and of asparagine/glycin (NG) specifiy thymidine recognition (**Fig. 2A**) [18][19]. Other diresidues are found in natural TAL proteins but are less frequent and less specific. An array of nucleotide-specific TAL repeats is flanked by an invariable first and a last "half" repeat that also comprise 34 residues but only half of these amino acids are sequence conserved. In natural TAL proteins the N-terminal half repeat (repeat 0.5) is positioned towards a thymidine that defines the first nucleotide of a target sequence. In contrast, the C-terminal half repeat does not participate in sequence recognition [18][27]. The assembly of TAL repeats into a functional DNA binding domain requires additional flanking N- and C-terminal TAL-derived sequences of ~ 110 and ~ 45 residues [21]. In natural TAL proteins the C-terminal end of the DNA binding domain extends into a transactivation effector region. This functional domain can be replaced in engineered TAL proteins with a FokI nuclease domain [20]. Importantly, it has been found that TAL repeats can be assembled into new arrays that bind to the target sequence as predicted by the four letters code [18][20][21][30]. Utilizing the TAL four letters code it is now possible to customize protein domains to address any DNA sequence that is preceded by a thymidine. Since only the HD and NN repeats contribute strongly to DNA binding but NI and NG repeats exhibit lower affinity, adenosine and thymidine rich sequences should be avoided as target [31]. The molecular basis of this observation may be explained by the chemical interaction between TAL repeats and nucleotides. The crystal structures of the TAL proteins PthXo1 and dHax3 bound to DNA revealed how TAL repeats recognize DNA sequences and helices [32][33]. Each 34 residue repeat folds into a two-helix bundle that presents residues 12 and 13 as a loop towards the major groove of the DNA helix. This conformation privileges residue 13 to contact a specific nucleotide whereas residue 12 stabilizes the repeat backbone. In the HD and NN repeats residue 13 establishes a hydrogen bond with cytidine or guanosine while NI and NG repeats exhibit weaker, nonpolar van der Waals contacts to adenosine and thymidine. To bind the DNA helix the neighbored repeats of PthXo1 self associate into a superhelix that wraps around the DNA [33].

TALEN proteins bind to one strand of the DNA helix in a 5'-3' direction. By combination of TAL elements following the four letters recognition code it is possible to design TALENs against virtually any target sequence. As reported, the only requirement is that a target sequence has to be preceded by a 5'-thymidine [18]. Functional TALENs have been build with a varying length of the binding site of

9 - 21 bp [34][35], however, commonly used is a target length of 15 - 18 bp, which corresponds to 14.5 - 17.5 TAL repeats. Due to the obligate dimerization of the FokI nuclease domains, a complete TALEN target site includes two TALEN binding sites (**Fig. 1A**). In order to position the two C-terminal FokI domains in the correct position, the two binding sites must be located on the opposite DNA strands. Moreover, the distance between the TALEN binding sites, the spacer region, is a crucial factor for the dimerization of the FokI domains. The ideal length of the spacer to enable dimerization is described as 14 - 16 bp [21], but TALEN pairs with longer spacers of up to 20 bp show similar nuclease activity. The construction of TALEN expression vectors and the selection of TALEN target sites are described in Methods sections 3.1 and 3.2 of this article.

1.2 Gene editing of the mouse genome using TALENs

Following the ZFN paradigm TALENs were readily adopted to achieve genome modifications in mouse embryos. For delivery into the embryo´s cytoplasm or pronucleus the TALEN coding regions are first *in vitro* transcribed into mRNA. Upon the microinjection of mRNA, the nucleases are translated, imported into nuclei, and cause DSBs in the target gene (**Fig. 1B**). Knockout mutations can be obtained by targeting coding regions that are repaired by NHEJ. The religated DSB ends frequently exhibit the random loss and addition of nucleotides, leading to frameshift mutations in the majority of events. In the presence of a coinjected targeting vector or oligonucleotide as repair templates, DSBs can be fixed by HR, enabling the introduction of precisely targeted mutations such as codon replacements or reporter gene insertions. A substantial fraction of the founder mutants obtained from such manipulated embryos harbor heterozygous mutations of the target gene, but mosaic patterns are also found. Since the modified alleles are also present in the founder´s germ cells heterozygous mutant progeny can be obtained by natural breeding. TAL nucleases were applied up to now in seven studies for the targeting of 20 coding or miRNA genes using zygotes derived from various inbred or outbred mouse strains (**Table 1**). Knockout alleles were obtained at high frequency upon cytoplasmic TALEN microinjections such that up to 70% of the pups represent founder mutants [36]. A single day of microinjection and embryo transfer is therefore sufficient to obtain a variety of knockout alleles. The production of Knockin alleles upon the pronuclear microinjection of TALEN mRNAs and targeting molecules relies on HR that occurred at a rate of up to6.8% for the *Fus* gene [37]. The overall mutagenesis rate certainly depends on critical parameters such as the concentration and volume of the injected RNA/DNA solution, the

TALEN nuclease activity and, possibly the differential susceptibility of various target genes for nucleases and repair proteins. The protocol presented in this article is based on our work on the pronuclear injection of TALEN mRNAs and targeting molecules, resulting into 41% Knockout alleles for *C9orf72* and 6.8% of Knockin alleles for the *Fus* gene [36]. Although the hitherto published studies cover only a relatively small number of target genes, the results suggest that TALENs provide a new standard tool enabling the routine manipulation the mouse germline.

-Table 1-

2. Materials

2.1 TALEN construction

1. TALEN construction kit (www.addgene.org/TALEN)

2.2 Targeting molecules

1. single-stranded oligodeoxynucleotide (ssODN), “gene synthesis” grade
2. *or*: cloned gene targeting vector

2.3 Validation of nuclease activity

2.3.1 Transfection of mammalian cell lines

1. DMEM (Life Technologies)
2. 10% FCS (PAN)
3. 100x Glutamine (Life Technologies)
4. 0.1 mM non-essential amino acids (Life Technologies)
5. Culture plates, 48 wells
6. X-tremeGENE HP DNA Transfection Reagent (Roche)
7. OptiMEM (Life Technologies)

2.3.2 Validation by mismatch assay

1. Murine cell line (e.g. Neuro2A)
2. Genomic DNA extraction Kit (e.g. Promega)
3. PCR thermocycler
4. Proof-reading DNA Polymerase (e.g. Herculase II, Stratagene)
5. Gene-specific PCR primers
6. PCR purification kit (Qiagen)
7. T7 endonuclease I (New England Biolabs)
8. Agarose or polyacrylamide gel electrophoresis

2.3.3 Validation by cellular repair assay

1. HEK293 cell line
2. Generic reporter vector pCMV-TALEN-Rep (deposited at Addgene)
3. Synthetic target oligos
4. β-Gal Reporter Gene Assay, chemiluminescent (Roche)
5. Luciferase assay reagent (Promega)
6. White measurement plates, 96 wells
7. Plate luminometer

2.4 *In vitro* transcription and preparation of injection aliquots

1. Restriction enzyme for linearization of TALEN plasmids
2. MinElute gel extraction kit (Qiagen)
3. mMessage mMachine T7 Ultra kit (Life Technologies)
4. MEGAclear Kit (Life Technologies)
5. Embryo-tested water (Sigma)
6. Microinjection buffer (10 mM Tris, 0.1 mM EDTA pH 7.4; use high-purity reagents and embryo-tested water to prevent embryo toxicity)
7. Dialysis membranes (e.g. 0.025 µm, VSWP; Millipore)
8. Centrifugal filters (e.g. Ultrafree filters PTFE membrane; Millipore)

2.5 Embryo microinjection

See reference 51

3. Methods

3.1 TALEN construction

The first step in gene targeting using TALENs – even before checking your gene of interest – is to think about the way how to generate the TALEN expression vectors as this will affect the later search for suitable target sites. The construction of such TALEN expression vectors can be achieved most conveniently by using one of the publicly available TALEN construction kits. Currently, five different kits can be found and ordered at the non-commercial plasmid repository Addgene (www.addgene.org/TALEN). While the construction kits of Voytas [22], Zhang [43], and Hornung [39] are meant for the low through-put production of just some TALEN pairs, the kits from Joung [40][34] and Church [35] are specialized on the medium to high through-put production using liquid-handling workstations. We recommend using either the “Golden Gate TALEN 2.0” Kit from Voytas’ lab (http://www.addgene.org/TALeffector/goldengateV2/) or the “Zhang Lab TALE Toolbox” (http://www.addgene.org/TALE_Toolbox/), as these are easy to set up and are used most commonly.

For the generation of TALENs for microinjection, follow the instructions of the chosen construction kit. In general, all of these kits are based on the assembly of predesigned DNA modules into sequence-specific TAL repeat arrays. These array are subsequently inserted into generic TALEN expression vectors which carry promoter sequences and the coding sequences of the N-terminal and C-terminal

TAL domains and of the FokI nuclease domain. All construction kits offer different generic TALEN expression vectors for various applications; therefore be cautious to clone the TAL repeat array into an expression vector that carries a strong mammalian promoter (e.g. CMV or CAG) for the validation of TALEN activity in cells and a T7 promoter for the *in vitro* transcription of TALEN mRNA. In the standard procedure the TALEN coding region is first transcribed *in vitro* and polyadenylated in a second step, as described in section 3.5. Alternatively, we also described a single step procedure using a TALEN vector that includes a plasmid coded polyA sequence [36].

3.2 Target site selection

The positioning of TALEN target regions primarily depends on the type of the desired mutation. For the generation of knockout alleles, the TALEN target region should be located within the first critical exon of the targeted gene. Small insertions or deletions (Indels, mostly 1-50 bp), generated by the error-prone repair mechanism NHEJ that closes the TALEN-mediated DSB, randomly cause frame-shifts in the coding sequence, which ultimately lead to premature stop codons.

For the generation of directed mutations, such as small sequence insertions or substitutions, the TALEN target region should be located in close vicinity to the desired mutation. As the frequency of sequence conversion by homologous recombination decreases with distance, this is especially important for targeting molecules with short homology regions like single-stranded oligonucleotides. In these cases, we recommend a maximum distance of 30 bp between DSB and mutation site. For the directed insertion, deletion or substitution of larger sequences (e.g. for the generation of humanized alleles, insertion of cDNAs and so on), the TALEN binding sites are ideally located near to the upstream or downstream end of the targeted region.

Once the suitable target region is determined, a TALEN target sequence has to be identified. The actual demands and limitations on the TALEN target sequence are given by the respective TALEN construction kit. In general, two opposing TALEN binding sites (each 15 to 20 bp long and preceded by a mandatory 5'-T) with a spacer distance of 14-16 bp are required (**Fig. 2A**). Several web-based tools like the "TALENdesigner" (http://www.talen-design.de/, [38], the "TAL Effector Nucleotide Targeter 2.0" (https://tale-nt.cac.cornell.edu/, [46], or the "ZiFiT Targeter Version 4.2" (http://zifit.partners.org/ZiFiT/ [45], can be used to scan user-specified or genomic DNA templates for TALEN binding sites. Moreover, the first two tools offer the analysis of closely related sequences within the mouse genome, which can represent potential TALEN off-target sites. Although the tolerance of TALENs to mismatches cannot yet be fully predicted, TALENs with 3 or more mismatches are supposed to be specific to their on-target site.

Finally, the TALEN target sites should be analyzed for the presence of genomic variations (e.g. SNPs), especially if zygotes derived from other genetic backgrounds than C57BL/6 are used. Mismatches due to SNPs can prevent TALEN binding and thereby hamper gene targeting. Hence, use the online SNP databases, like Ensembl's "Genetic Variation – Resequencing" option (http://www.ensembl.org) to identify detrimental SNPs.

3.3 Targeting molecules

As mentioned above, the generation of knockout alleles is based on the error-prone repair of DSBs, whereas the introduction of directed genome modifications is achieved by the homologous recombination of a mutagenic repair template. This repair template can either be a traditional gene targeting vector containing long 5'- and 3'-homology arms flanking the desired mutation, or, being more convenient, a short single-stranded oligodeoxynucleotide (ssODN). ssODNs can be synthesized rapidly and low priced and they supersede the laborious cloning of targeting vectors. To generate a targeting ssODN, design two short homology regions (typically 50-70 bp each) that flank the desired mutation, which can be a deletion or a short insertion or substitution. However, due to the limited size of ssODNs of up to 200 bp, modifications of longer sequences (e.g. insertion of cDNAs) can only be achieve using targeting vectors. For the generation of a targeting vector, two long homology regions (700-3000 bp) flanking the target site are PCR-amplified from a genomic wild-type template and ligated into a standard cloning vector. Then, the desired mutation is inserted in between the two homology arms. Detailed instructions for the generation of targeting vector can be found in the guidelines for ES cell-based gene targeting from Hasty [47], except that selection marker genes are not required.

3.4 Validation of nuclease activity

Prior to the generation of TALEN mRNA for microinjection, we recommend testing all TALEN pairs *in vitro* for nuclease activity to ensure their potential to induced DSBs. Here, we present two options to validate the TALEN activity in transient cellular assays, which either rely on the enzymatic digestion of mismatch heteroduplex alleles or on the HR-mediated repair of a reporter construct.

3.4.1 Mismatch-sensitive assay

To determine nuclease activity on endogenous loci, the TALEN expression plasmids are transiently transfected into a murine cell line (here: Neuro2A). Due to the error-prone NHEJ repair, heterozygous or biallelic indel mutations occur in a fraction of the cell population which can be determined by the mismatch-sensitive assay.

1. Preparation of Neuro2A cells: Grow cells in culture. 24 h prior to the transfection, seed 5 x 10^4 cells into 48 well plates.
2. Transfection of cells: Add 150 ng plasmid DNA of each TALEN expression vector into a sterile tube and fill up to 30 µl using OptiMEM. Add 0.9 µl X-treme GENE HP transfection reagent and incubate the mixture for 20 min at RT. Meanwhile, aspirate the medium from the culture plates and replace it by 300 µl of fresh complete DMEM. After incubation, mix the DNA/OptiMEM/Reagent mixture and add to the fresh medium in a dropwise manner. Incubate culture plates for 48 h at standard conditions.
3. Isolate genomic DNA from the transfected cells using the DNA extraction kit.
4. Use gene-specific primers to PCR-amplify the respective locus from the genomic DNA template. The resulting amplicon should be 200-1000 bp in length.
5. Purify the PCR product using a PCR purification kit.

6. Add 200 ng purified PCR product and 2 µl NEB Buffer 2.1 to a PCR tube and adjust volume to 19 µl with H_2O.
7. Use a PCR thermocycler for the formation of heteroduplexes using following program: 95°C for 5 min, cool to 85°C (cooling rate -2°C/sec), cool to 25°C (cooling rate -0.1°C/sec).
8. Add 1 µl (10 U) T7 endonuclease I and incubate at 37 °C for 15 min.
9. Add 2 µl of 0.25 M EDTA to stop the reaction.
10. Analyze the reaction products by agarose or polyacrylamide gel electrophoresis and quantify the band intensities e.g. by using ImageJ.
11. Nuclease activity can be quantified by the proportion of digested and undigested PCR products following the formula [48]:

$$Indels[\%] = 100 \times (1 - \sqrt{1 - \frac{b+c}{a+b+c}})$$

a: fraction of undigested PCR product

b, c: fraction of each cleavage product

3.4.2 HR-mediated reporter repair

The HR-mediated reporter repair is based on the transient co-transfection of the two TALEN expression plasmids with a TALEN-specific β-Gal-reporter construct into HEK293 cells. The β-Gal reporter construct contains of a partly duplicated, nonfunctional β-galactosidase gene followed by the TALEN binding site and an intact β-galactosidase coding region (**Fig. 2B**). Upon nuclease-mediated linearization of the reporter plasmid, the two homologous β-galactosidase regions act as homology arm for homologous recombination (**Fig. 2C**) and thereby, the functional coding cassette is reconstituted (**Fig. 2D**). Quantification of the TALEN activity is achieved by a chemiluminescent assay 48 hours post-transfection and compared to a control transfection lacking the TALEN expression plasmids.

1. Preparation of the reporter construct: design and order two complementary oligonucleotides that contain the entire TALEN target site, a CG overhang at one side and a blunt end on the other side. Add 0.5 µl of each oligo (100 µM) to 99 µl TE buffer and incubate the mixture at 95 °C for 5 min. Anneal the oligos by slow cooldown to RT. Ligate the annealed oligos into the BstBI and NruI sites of the generic reporter vector pCMV-TALEN-Rep.
2. Preparation of cells: Grow cells in culture. 24 h prior to the transfection, seed 5 x 10^4 cells into 48 well plates. Use triplicates for every sample.
3. Transfection of cells: For each well, add plasmid DNA to a sterile tube following the pipetting plan (**Table 2**) and fill up to 30 µl using OptiMEM. Use pBluescript or other, inert cloning vector DNA to equalize DNA content in all samples. Add 0.9 µl X-treme GENE HP transfection reagent and incubate mixture for 20 min at RT. Meanwhile, aspirate the medium from the culture plates and replace it by 300 µl of fresh complete DMEM. After incubation, mix the

DNA/OptiMEM/Reagent mixture and add to the fresh medium in a dropwise manner. Incubate culture plates for 48 h at standard conditions.

4. Cell lysis and chemiluminescent assay: For lysis of transfected cells, use 300 µl Lysis buffer per well from the β-Gal reporter gene assay kit. Perform in parallel a β-Galactosidase and a Luciferase chemiluminescent assay following the manufacturers' instructions. Quantify the reporter activity in a plate luminometer and calculate relative ratios of β-Galactosidase and Luciferase values.

-FIGURE 2-

-TABLE 2-

3.5 mRNA production

1. Linearize 10 µg of each TALEN expression plasmid using a restriction enzyme that cuts downstream of the TALEN coding region
2. Gel purify the linearized DNA fragments using the MinElute gel extraction kit and elute DNA in 10 µl EB
3. Perform the vitro transcription and polyadenylation reaction using the mMessage mMachine T7 Ultra kit: add 10 µl T7 2x NTP/ARCA, 2 µl 10x reaction buffer, 6 µl linearized DNA (~1 µg), and 2 µl T7 enzyme in a 1.5 ml tube. Mix reaction and incubate for 2 h at 37 °C. Then, add 36 µl nuclease-free water, 20 µl 5x E-PAP buffer, 10 µl 25 mM $MnCl_2$, 10 µl 10 mM ATP solution, and 4 µl E-PAP enzyme. Mix reaction and incubate for 1 h at 37 °C.
4. Purify mRNA using the MEGAclear kit: add 350 µl binding buffer and 250 µl ethanol to the transcription reaction. Apply sample to MEGAclear spin column, centrifuge for 15 sec, 10,000 x g and discard flow-through. Wash column twice with 500 µl wash solution and then elute mRNA from column twice with each 50 µl pre-warmed elution solution (95 °C).
5. Precipitate mRNA using the MEGAclear kit: to the eluted mRNA add 10 µl 5 M ammonium acetate and 275 µl ethanol. Mix sample and incubate for at least 30 min at -20 °C. Spin down mRNA by centrifugation for 15 min, 16,000 x g, discard supernatant and wash pellet with 500 µl 70 % ethanol. Centrifuge for 1 min, 16,000 x g, discard supernatant and let the mRNA pellet air dry for 5 min. Then, resuspend mRNA in 40 µl microinjection buffer for 5 min at 37 °C. Finally, determine mRNA concentration using a spectrophotometer und store the mRNA at -80 °C.
6. Recommended: check the integrity of the mRNA samples by using denaturing RNA polyacrylamide gel electrophoresis (e.g. BioAnalyzer RNA Chip)

3.6 Preparation of injection aliquots

For the generations of knockout mutants by NHEJ, the two mRNAs of the TALEN pair are mixed in equimolar ratios. Usually, RNA concentrations of 5 to 45 ng/µl (each mRNA) [37][38][39] are used for embryo microinjection. As we did neither observe embryo toxicity nor unspecific genome modifications when using high RNA concentrations, we recommend using 45 ng/µl to maximize Indel formation

efficiency. For the generation of directed mutations using a targeting vector or ssODN, injection aliquots with 45 ng/µl of each TALEN mRNA and 15 ng/µl targeting molecule are prepared.
The targeting molecule has to be either purified by ethanol precipitation and resuspension in injection buffer (gene targeting vectors) or by dialysis against embryo-tested water using a dialysis membrane (ssODNs). For the preparation of the mRNA/DNA solution, mix the purified mRNA and DNA samples and adjust the concentration using injection buffer. Filter the mRNA/DNA solution with a centrifugal filter and prepare single-use aliquots of 30 µl for each day of microinjection. Store aliquots at -80°C until usage.

3.7 Embryo microinjection

The complete procedure of embryo microinjection (including zygote preparation, microinjection, and reimplantation) is a highly sophisticated method which requires specialized skills and equipment. Therefore, we recommend collaborating with institutional mouse service units experienced in performing embryo microinjections. If no service unit is available, Ittner and Götz provided a detailed and complete protocol covering all aspects of the pronuclear injection into mouse zygotes [49].
For the generation of targeted mouse mutants using TALENs, we recommend the injection into the pronucleus instead of cytoplasmic microinjection, as it is done for the generation of NHEJ-derived knockout mutants. The pronuclear injection ensures that the co-injected targeting molecule is directly delivered to the genomic DNA for HR.
Another important consideration before starting with the microinjection is the selection of the genetic background of the zygotes. Using the commonly used inbred strain C57BL/6 as embryo donor usually results in low embryo numbers and in low microinjection survival. Therefore, if a pure inbred background is not required for your experimental aims, we recommend using hybrid embryos, like (FVBxC57BL/6)-F_1. If a pure genetic background is mandatory, extra injection dates should be scheduled to compensate for the low yield.

3.8 Genotyping

At the age of 3 weeks, the founders of the F_0 generation can be genotyped for the presence of genetic modifications by analyzing genomic DNA from ear or tail tip biopsies. The recommended method for genotyping depends on the type of mutation and on the availability of specialized equipment. Here, we will briefly introduce four common genotyping strategies and discuss their possibilities and limitations.
The most common genotyping strategy for mutagenized alleles is the mismatch-sensitive assay, which we already introduced for the *in vitro* analysis of nuclease activity (section 3.4.1). The target locus is PCR-amplified using gene-specific primers and the resulting PCR amplicon is then analyzed for the presence of mismatched heterodimers, which are formed by the annealing of wildtype and mutant alleles. Using either T7 endonuclease I or the Cel-I enzyme (“Surveyor assay”), the mismatched DNA is digested and the reaction products can be analyzed by gel electrophoresis.
For the genotyping of just a few animals, the direct sequencing of the PCR-amplified locus offers a very fast and convenient alternative to the mismatch-sensitive assay. The occurrence of superimposed sequencing traces, starting near or at the mutation site, indicates heterozygous gene targeting. These

mixed traces can be automatically de-convoluted (e.g. using Indelligent or Mutation Surveyor; SoftGenetics) to determine the exact sequence of the mutant allele.

The third, very common genotyping strategy is the restriction fragment length polymorphism (RFLP) analysis, which is often used to identify larger mutations like knockins. A prerequisite of this technique is either the introduction or depletion of a restriction site upon gene modification or the significant change in fragment length due to insertion or depletion of a DNA sequence. Restriction analysis can then either be done by enzymatic digestion of the PCR-amplified locus and subsequent agarose gel electrophoresis or by the more accurate, but more sophisticated Southern blot analysis of genomic DNA samples [50].

Finally, the HRM (high-resolution melt) analysis offers a cheap and fast genotyping strategy which is especially suitable for the high-throughput screening of founders. HRMA is based on the characteristic melting profile of short PCR products that are generated from genomic templates [51]. HRMA can be used to detect small deletions, insertions or basepair substitutions with a very high sensitivity. However, the requirement of a specialized instrument (e.g. LightScanner, BioFire Diagnostics or Rotor-Gene Q, Qiagen) restricts the general application of HRMA.

Independent of the genotyping method, we recommend to further analyze the genotypes of putative founders by subcloning and sequencing of PCR products. Mosaicism and compound heterozygocity occur often in F_0 founders and therefore, a thorough analysis of genotypes is essential for the establishment of a mutant mouse line.

4. Conclusions

Classical gene targeting in embryonic stem cells and the generation of germ line chimaeras is a time- and labor- consuming procedure that often requires one year for the establishment of a mutant line. The direct mutagenesis of the germline in mouse zygotes using TALENs provides an expedite alternative since founder mutants can be obtained within 7 weeks upon a single or a few days of microinjection. For the successful implementation of a new technology it is important to develop standard protocols to facilitate its routine application. Following the protocol described in this chapter we anticipate that at least one Knockout or Knockin allele is found within a group of 50 pups derived from embryo microinjections. If the mutagenesis rate observed in specific microinjection experiments falls below this threshold of 2%, the nuclease activity of TALENs and the qualitity and concentration of the mRNA preparation used are the most critical factors for troubleshooting. Nevertheless, the application of TALENs in zygotes has just emerged and it is possible that in future further improvements will be reported. In addition, we anticipate that the range of TALEN applications will further extended, e.g. by the use of two TALEN pairs that address distant genomic loci to enable large deletions and duplications for the modeling of copy number variations.

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Tables

Table 1: Gene editing in mice with TALEN				
Target genes	**Genotype**	**Founder mutants Knockout (%)**	**Founder mutants Knockin (%)**	**Reference**
Pibf1, Sepw1	**C57BL/6**	**56 - 77**	n.a.	[36]
Rab38	**C57BL/6 x FVB**	**6**	**2**	[41]
Zic2	**CD1, C3H, C57BL/6**	**10 - 46**	n.a.	[39]
Lepr & nine others	**FVB/N, C57BL/6**	**13 - 67**	n.a.	[40]
Fus, C9orf72	**(C57BL/6xBS) x FVB/N**	**41**	**6.8**	[37]
Fats	**C57BL/6**	**62**	n.a.	[41]
miRNAs	**C57BL/6 x DBA2**	**3 – 30**	n.a.	[42]
n.a. – not applied				

Table 2: Pipetting plan for transient transfections			
Plasmid DNA	**Positive control**	**Sample 1**	**Sample 2**
TALEN expression vector A	-	-	75 ng
TALEN expression vector B	-	-	75 ng
TALEN reporter vector	-	75 ng	75 ng
pCMV-beta	75 ng	-	-
pCMV-Luc	75 ng	75 ng	75 ng
pBluescript	150 ng	150 ng	-

Figure Legends:

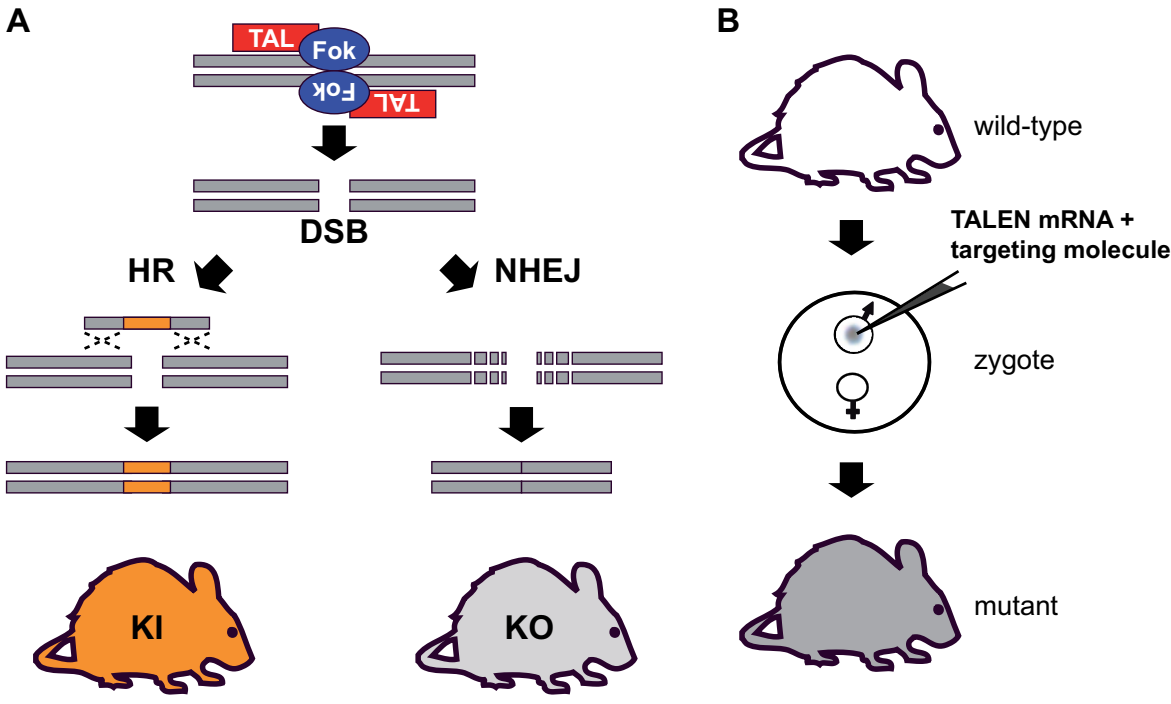
Fig. 1: Gene targeting in one-cell embryos using TALENs

(**A**) TAL-Fok fusion proteins (TALENs) recognize their binding sites on opposing strands of the target site. Upon dimerization of the two Fok monomers, a double-strand break (DSB) is generated. The DSB is repaired by one of the two main repair mechanisms: homologous recombination (HR) or non-homologous end-joining (NHEJ). In the presence of a repair template harboring homologous regions to the target site and the desired mutation, HR enables the replacement or insertion of a given sequence, leading to a knockin (KI) mutant. Alternatively, the error-prone NHEJ repair closes the DSB, leading to small nucleotide insertions or deletions that randomly cause frame-shift knockout (KO) mutations. (**B**) For microinjection, one-cell embryos are collected from wild-type female mice. TALEN mRNA together with a targeting vector, which can either be a single-stranded oligodeoxynucleotide or a double-stranded targeting vector, is injected into the larger male pronucleus. The embryos are then transferred to pseudopregnant foster mice and develop to founder animals that, if gene targeting occurred, carry the mutant allele.

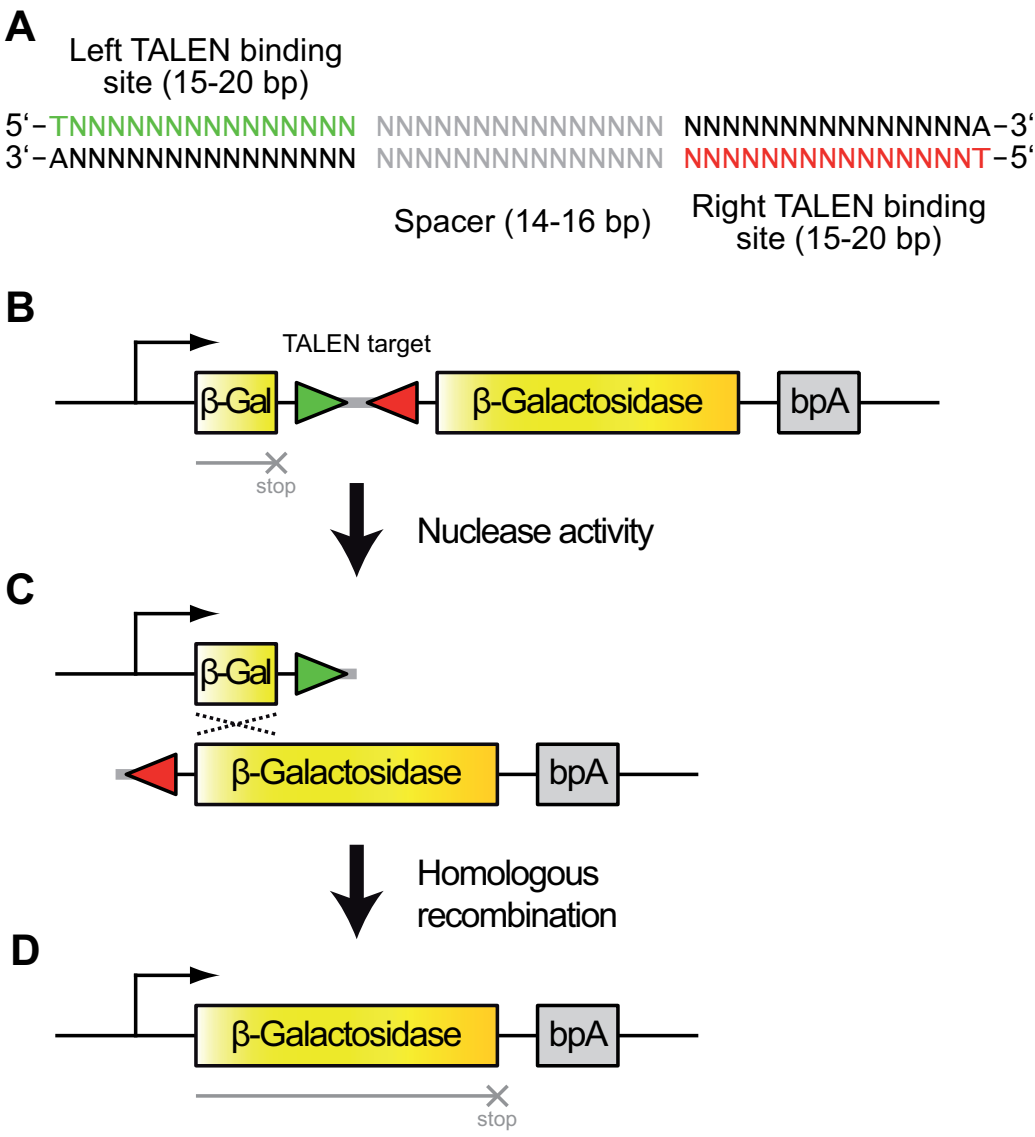
Fig. 2: Structure of a TALEN target site and the TALEN reporter plasmid

(**A**) A TALEN target site is composed of a left (shown in green) and a right (shown in red) TALEN binding site on opposing strands. Each binding site is preceded by an invariant 5'-T and has a length of 15-20 bp. The two binding sites are separated by a spacer region of 14-16 bp. (**B**) The TALEN reporter plasmid contains of a CMV promoter (CMV), a nonfunctional segment of the β-galactosidase gene (β-Gal) followed by a stop codon, a TALEN-specific target site, the complete coding sequence of the β-galactosidase gene, and a polyA signal (bpA). (**C**) Upon co-transfection of the TALEN reporter and TALEN expression plasmids into HEK293 cells, the reporter plasmid is linearized by the TALEN-mediated creation of a double-strand break in the target site. (**D**) The double-strand break is repaired by the homologous recombination of the analogous β-galactosidase segments and thereby, the functional coding sequence is reconstituted.

Figure


A
TAL
Fok
Fok
TAL
DSB
HR
NHEJ
KI
KO
B
wild-type
TALEN mRNA +
targeting molecule
zygote
mutant

Figure


A
Left TALEN binding site (15-20 bp)
5‘–TNNNNNNNNNNNNNNN NNNNNNNNNNNNNNN NNNNNNNNNNNNNNNA–3‘
3‘–ANNNNNNNNNNNNNNN NNNNNNNNNNNNNNN NNNNNNNNNNNNNNNT–5‘
Spacer (14-16 bp)
Right TALEN binding site (15-20 bp)
B
TALEN target
β-Gal
β-Galactosidase
bpA
stop
Nuclease activity
C
β-Gal
β-Galactosidase
bpA
Homologous recombination
D
β-Galactosidase
bpA
stop