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Method

# Creation of targeted genomic deletions using TALEN or CRISPR/Cas nuclease pairs in one-cell mouse embryos

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ABSTRACT

**The use of TALEN and CRISPR/CAS nucleases is becoming increasingly popular as a means to edit single target sites in one-cell mouse embryos. Nevertheless, an area that has received less attention concerns the engineering of structural genome variants and the necessary religation of two distant double-strand breaks. Herein, we applied pairs of TALEN or sgRNAs and Cas9 to create deletions in the *Rab38* gene. We found that the deletion of 3.2 or 9.3 kb, but not of 30 kb, occurs at a frequency of 6–37%. This is sufficient for the direct production of mutants by embryo microinjection. Therefore, deletions up to ~10 kb can be readily achieved for modeling human disease alleles. This work represents an important step towards the establishment of new protocols that support the ligation of remote DSB ends to achieve even larger rearrangements.**



## 1. Introduction

Since 1988, gene targeting in embryonic stem (ES) cells has enabled the generation of mouse mutants and the study of gene function by reverse genetics [1]. It has also transpired that sequence-specific nucleases can be used as an alternative to induce targeted double-strand breaks (DSB) and enhance local DNA repair. Thus, it is possible to produce mutants from one cell embryos, independent of ES cells [2]. The proof-of-principle for this mutagenesis approach was provided by the Zinc-finger nucleases (ZFN). Nevertheless, this system was not convenient since ZFNs cannot be easily programmed for the recognition of new target sequences. This problem was circumvented with the determination that transcription activator-like (TAL) proteins of *Xanthomonas* follow a simple modular code for DNA recognition [3] and were suitable for gene editing by fusion with the FokI nuclease domain into TAL effector nucleases (TALEN) [4]. The latest, third generation of nucleases is provided by the CRISPR/Cas9 bacterial defense system. This approach uses short, single guide (sg) RNAs for DNA sequence recognition and can be programmed towards new targets by adaption of the sgRNA first 20 nucleotides that determine the specificity of the system [5]. The sgRNAs are bound by the generic, two domain nuclease Cas9 and will guide the binary complex to the complementary DNA sequence for DSB induction. These DSBs are then either repaired by homologous recombination (HR) together with gene targeting vectors [6,7] or become, in the absence of repair templates, religated by non-homologous end joining (NHEJ). This is frequently accompanied by the loss of multiple nucleotides [8]. HR-mediated repair of nuclease-induced DSBs enables the insertion of preplanned sequence alterations into the

*Abbreviations:* DSB, double-strand break; ES, embryonic stem (cell); HR, homologous recombination; NHEJ, non-homologous end joining; sg, single guide; TAL, transcription activator-like; TALEN, TAL effector nucleases; ZFN, zinc-finger nucleases

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genome, whereas small deletions caused by NHEJ repair are often used for the generation of frameshift mutations.

Both, TALEN and CRISPR/Cas nucleases were successfully applied for creating germline mutations in a variety of species [5]. In mice nuclease expression in one-cell embryos has been used for the generation of knockout alleles, codon replacements and the insertion of reporter genes or loxP sites [9–12]. Besides mutations addressing single target genes, structural genome rearrangements such as large deletions and duplications, as well as inversions and translocations, represent an important subset of mutations associated with epilepsy, autism, schizophrenia and cancer [13,14]. For engineering of such alleles in ES cells sequential steps of gene targeting are required. The generation of structural variants via nuclease technology requires the simultaneous processing of two distant sites in a single cell. This is an area that is yet poorly explored by the use of TALEN or CRISPR/Cas in mouse embryos.

Here we applied two pairs of TALEN or two sgRNAs to generate genomic deletions in one cell mouse embryos. Since the simultaneous processing of two unrelated DSBs and the sealing of their distant ends are required for deletion, it was an open question whether such events occur at a reasonable frequency. This is given that a limited number of embryos can be handled in microinjection experiments. Furthermore, we explored whether NHEJ or HR provides the most efficient pathway for the sealing of distant DSB ends. As a model system, we used two TALEN pairs or sgRNAs specific to the *Rab38* gene that we previously targeted at a single site using ZFNs or TALEN [11,12]. We found that the deletion of 3.2 or 9.3 kb, but not of 30 kb occurs by NHEJ repair at a frequency (6–37%), sufficient for the direct production of mutants by embryo microinjection. Thus, genomic deletions in the scale of up to ~10 kb can be readily achieved using established nuclease technology. Nevertheless, the construction of larger rearrangements requires the development of new techniques and protocols supporting the end joining between distant DSBs.

## 2. Results

### 2.1. Generation of mice harboring a 9.3 kb deletion in Rab38 using TALENs

To explore whether genomic deletions can be generated using two pairs of TALEN, each defining one endpoint of the intended deletion, we used a previously described TALEN against the first exon of *Rab38* [11] (TAL-A1/2, Fig. 1A). We then constructed a second TALEN recognizing a sequence within the first intron, at a distance of 9.3 kb (TAL-B1/2, Fig. 1A). TAL-B1/2 was designed using our TALEN*designer* software and cloned by a modular construction protocol into an expression vector, as described [11]. Upon cotransfection of HEK 293 cells with the A1/2 or B1/2 pair of TALEN expression and nuclease reporter plasmids [11] harboring the selected target sequences both TALEN pairs were found to exhibit specific nuclease activity (Supplementary Fig. 1A). The presence of two neighboring DSBs may be sufficient for removal of the intervening genomic segment at a reasonable frequency. Alternatively, each DSB could be independently closed by NHEJ repair such that the distant ends of both DSBs may be rarely connected. To further support targeted deletions, we sought to provide a template for HR repair and designed the single-stranded oligodeoxynucleotide ODN(A/B) covering 62 bp of sequence upstream of the TAL-A1 site and 61 bp downstream of the TAL-B2 site, bridging the ends of the intended deletion in between an additional BamHI restriction site (Fig. 1A). For deletion of the 9.3 kb *Rab38* gene segment flanked by the A1/2 and B1/2 recognition sites, four TALEN mRNAs and ODN (A/B) were microinjected into the pronuclei of mouse one-cell embryos. Upon embryo transfer we obtained 33 pups that were genotyped by PCR analysis of tail DNA using the primer pair P-for/P-del for the detection of deleted alleles (Fig. 1A). PCR products of 324 bp are predicted in case the deletion endpoints reach exactly the center of the TALEN spacer sequences. Two of these mice (6%) were identified as mutant founders (AB3, AB25) by the presence of the predicted PCR bands (Fig. 1B). Subcloning and sequence analysis of these PCR products proved the deletion of 9355 bp in both founders, covering sequences located 3 bp downstream of the TALEN site A1 and 6 bp upstream of the site B2 ($Rab38^{\Delta 9.3}$ allele, Fig 1C). Both alleles were not recombined with ODN (A/B), as indicated by the absence of the new BamHI site, but are likely generated by NHEJ between the DSBs at the A1/2 and B1/2 target sites. To further characterize the frequency of small deletions occurring at the TALEN target sites we amplified the regions spanning the target site A1/2 or B1/2 from all 33 pups. The sequence analysis of these PCR products showed that heterozygous, small deletions (4–11 bp) occurred in three pups (#18, #21, #22) at the target site A1/2 and in one pup (#21; 1 bp deletion) at the B1/2 target site, altogether confirming the activity of TALENs in 6 of 33 (18%) of pups. In addition, PCR products spanning the A1/2 target site from the founders AB3 and AB25 showed reduced size (Fig. 1D), suggesting the presence of small deletions in their second *Rab38* allele. Subcloning and sequence analysis of these PCR products revealed the loss of 11 bp in founder AB3 and of 25 bp in founder AB25, respectively, within the TALEN target site A1/2 (Fig. 1E). Since the target site A1/2 is located within the first exon of *Rab38*, both alleles of founders AB3 and AB25 were predicted to be inactivated either by the 9.3 kb targeted deletion or the translational frameshift within exon 1. Since the G19V replacement of RAB38 leads to impaired pigment production and chocolate fur color on the black C57BL/6 background [15], we reasoned that the knockout of *Rab38* may also lead to a lighter coat on the agouti background we used for embryo microinjection. Upon macroscopic inspection the coat of founders AB25 and AB3 exhibited a lighter agouti color as compared to $Rab38^{wt}$ littermates (Fig. 1F). The reduced pigmentation of the *Rab38* mutants was further confirmed by the microscopic comparison of dorsal awl hairs (Fig. 1F). To demonstrate the germline transmission of the $Rab38^{\Delta 9.3}$ allele and for the establishment of breeding colonies, both male founders were bred to wildtype females. The progeny was analyzed for the presence of the $Rab38^{\Delta 9.3}$ allele using the PCR primer pair P-for/P-del. As indicated by the presence of the 316 bp band, the $Rab38^{\Delta 9.3}$ allele was identified in 6 of 12 pups and in 3 of 9 pups derived from founder AB3 or AB25, respectively (Fig. 2A). Subcloning and sequencing of the PCR products from the pups AB3#1 and AB25#2 confirmed the identity of the transmitted and the parental $Rab38^{\Delta 9.3}$ alleles (Fig. 2B). These results show that two pairs of TALEN can be used for the single step deletion of a 9.3 kb gene segment by NHEJ in one-cell embryos and that the mutant alleles are germline transmitted. In addition, we found that the presence of an ODN bridging the deletion endpoints does not further support recombination and that homozygous compound mutants exhibiting a mutant phenotype can be obtained in the $F_0$ generation.

### 2.2. Generation of genomic deletions using the CRISPR/Cas system

As an alternative to the use of TALEN, we explored generating genomic deletions by CRISPR/Cas mediated mutagenesis using pairs of sgRNAs. Within the *Rab38* gene we used the upstream target site Rab38-2 located within the first exon of *Rab38* together with each one of four distant sgRNA sites at a distance of 3.2 kb, 10.4 kb, 31.8 kb or 51.8 kb (Fig. 3A). To confirm the activity of our CRISPR/Cas vectors, nuclease reporter plasmids were constructed and cotransfected into mouse neuroblastoma (Neuro2A) cells. This analysis showed that both TALEN pairs and sgRNAs exhibit high nuclease activity against the respective target sequences