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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.

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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 muta-

genesis 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 TALENdesigner 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*^{Δ9.3} 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*^{Δ9.3} 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*^{Δ9.3} allele using the PCR primer pair P-for/P-del. As indicated by the presence of the 316 bp band, the *Rab38*^{Δ9.3} 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*^{Δ9.3} 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 F₀ 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

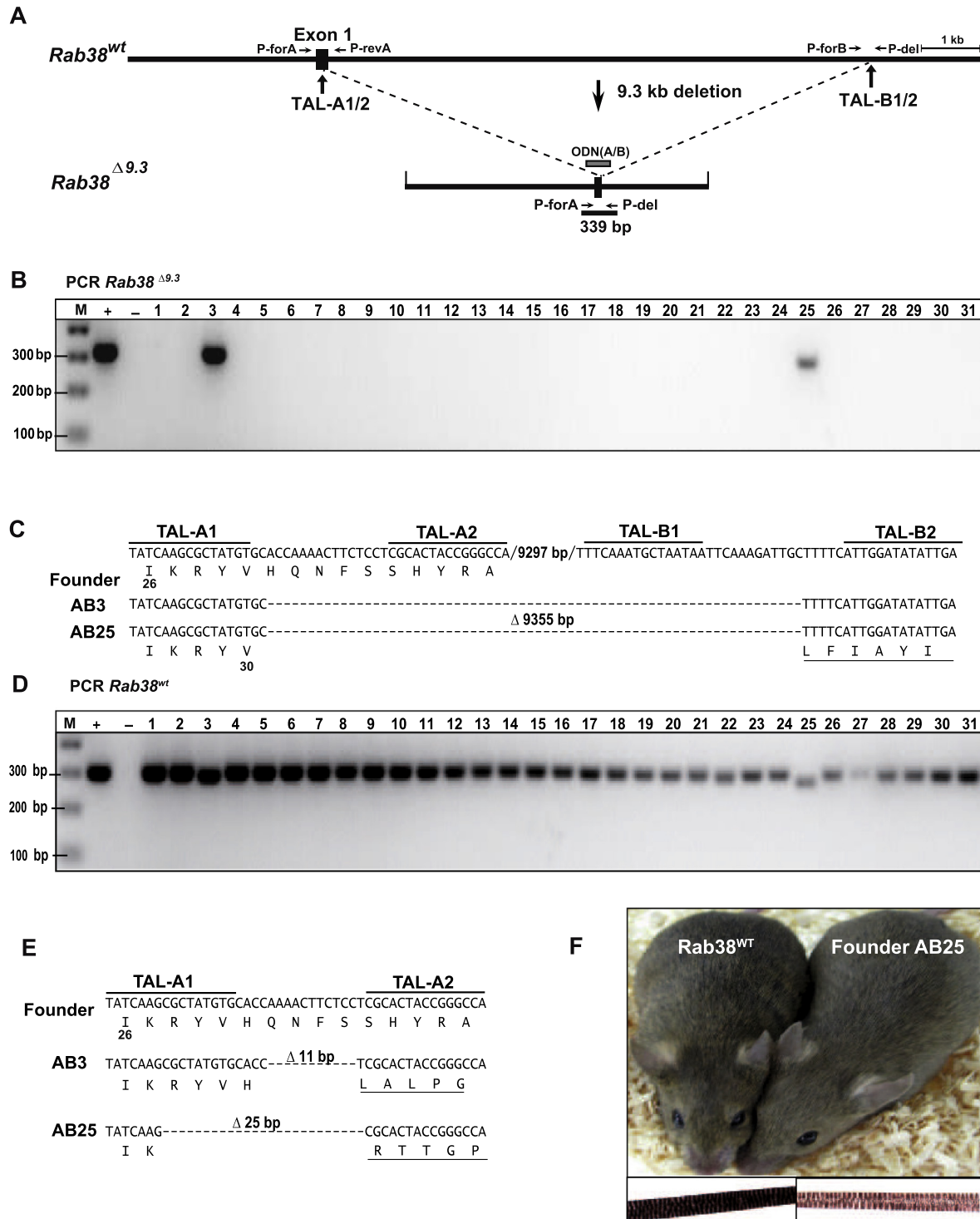


Fig. 1. Deletion of a 9.3 kb *Rab38* gene segment in one-cell embryos using two pairs of TALEN. (A) Schematic diagram of the *Rab38* gene and the planned deletion of 9.3 kb (*Rab38*^{Δ9.3} allele), indicating the position of the first exon, of the TALEN recognition sites and PCR primer pairs. (B) PCR detection of *Rab38*^{Δ9.3} alleles using primers P-forA and P-del using tail DNA from 31 pups derived from embryos microinjected with TAL-A1/2 and TAL-B1/2 mRNAs. M: size marker, +: positive control, -: negative control. (C) Sequence comparison of cloned PCR products from founders AB3 and AB25 with the *Rab38* wildtype locus, indicating identical deletions of 9355 bp in both founders. The deletion endpoints are located within the TALEN spacer regions. The upstream deletion endpoint disrupts codon 31, followed by a random translational frame. (D) PCR analysis of the TAL-A1/2 target region with tail DNA from 31 pups derived from microinjected embryos using primers P-forA and P-revA. The PCR products amplified from the second *Rab38* allele of founders AB3 and AB25 show reduced size, indicating the presence of small deletions. (E) Sequence analysis of cloned PCR products (see D) showing the deletion of 11 bp or of 25 bp within the TAL-A1/2 target region of the second *Rab38* allele of founder AB3 or AB25, respectively. The translation of the TAL-A1/2 target sequence within the first exon of *Rab38* shows reading frameshifts after codon 31 (AB3) or 27 (AB25). (F) Comparison of the coat color of founder AB25 with an agouti colored littermate control (*Rab38*^{wt}) and of dorsal awls showing the reduced pigmentation of hairs in the mutant (20× magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

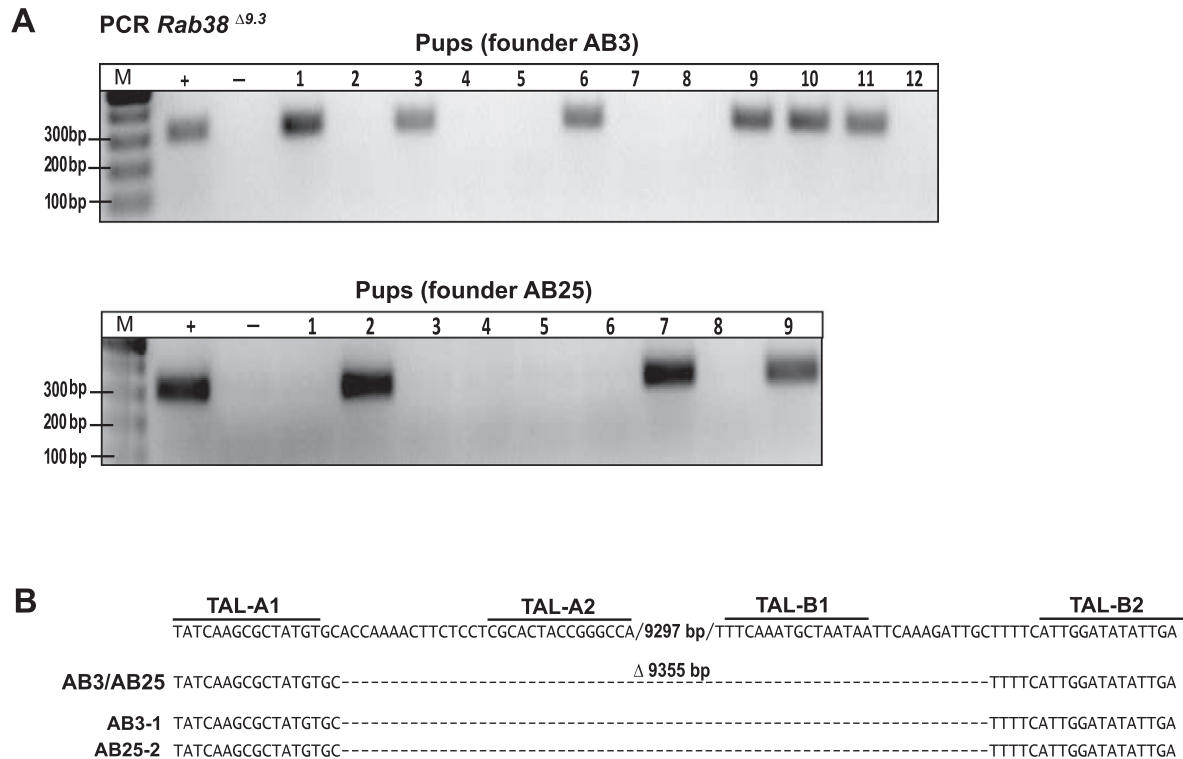


Fig. 2. Germline transmission of *Rab38*^{Δ9.3} alleles. (A) PCR detection of *Rab38*^{Δ9.3} alleles using primers P-forA and P-del with tail DNA from pups derived from founder AB3 or AB25. M: size marker, +: positive control, -: negative control. (B) Sequence comparison of cloned PCR products (see A) derived from pups AB3-1 and AB25-2 with the parental *Rab38*^{Δ9.3} allele.

(Supplementary Fig. 1B). We then tested the proficiency of the paired CRISPR/Cas system by cotransfecting Cas9 and pairs of sgRNA expression vectors into Neuro2A cells, followed by PCR analysis for the detection of genomic deletions. To induce genomic deletions four pairs of sgRNA vectors were cotransfected with a Cas9 expression vector into Neuro2A cells and two days later genomic DNA was isolated from the transfected samples. These were then qualitatively analyzed for deletion events using primer pairs positioned at both sides of the intended breakpoints for the amplification of 300–400 bp fragments. For all tested combinations of Cas9 and the sgRab38-2/-3, -2/-10, -2/-32 and -2/-52 vectors we detected PCR products indicating the presence of genomic deletions of 3.2 kb, 10.4 kb, 31.8 kb or 51.7 kb, respectively; these were absent in the nontransfected negative control (Fig. 3B). These PCR products were cloned and the sequence analysis confirmed that all intended deletions occurred in between the known cleavage site of Cas9, 3 bp upstream of each PAM sequence, without the removal or insertion of additional nucleotides (Fig. 3C). To introduce such deletions into the mouse genome, we microinjected Cas9 mRNA and the sgRNA pairs sgRab38-2/-3 or sgRab38-2/-32 into one-cell embryos. In addition, to enable DSB repair by HR, we included a single-stranded oligodeoxynucleotide (ODNΔ2/3) covering 65 bp upstream of the sgRab38-2 site and 61 bp downstream of the sgRab38-3 site, bridging the deletion endpoints. From the microinjection of Cas9 and sgRab38-2/-32 we obtained 38 pups that were genotyped by PCR analysis of tail DNA for the presence of the intended deletion using the primer pair P-for/P-del32. None of the pups showed the predicted 383 bp PCR band, indicating that the frequency of a >30 kb genomic deletion falls below the level of 3%. From the microinjection of Cas9 and sgRab38-2/-3 we obtained 27 pups that were genotyped by PCR analysis of tail DNA for the presence of the intended 3.2 kb deletion using the primer pair P-for/P-del3. In this case ten of the 27 pups (37%) were

identified as mutant founders by the presence of the expected ~326 bp PCR band in eight samples or by larger PCR fragments in founder #9 and 23, representing noncanonical recombination events (Fig. 4A). The cloning and sequence analysis of up to five PCR products per founder showed the presence of a single deleted *Rab38* allele (*Rab38*^{Δ3.2}) in four founders and of two divergent mutant alleles in six founders (Fig. 4B). Only one of these alleles (founder #23) resulted from HR with ODNΔ2/3 as indicated by the presence of an indicative, additional BamHI site. Among the 15 alleles repaired by NHEJ, four junctions were precisely joined at the Cas9 cleavage sites, whereas 11 alleles lost 1–19 bp, with a 2 bp insertion in one case. Founder #9 and #23 showed one allele resulting from the predicted repair of Cas9 induced DSBs by NHEJ or HR, despite the fact that only larger products were visibly amplified (Fig. 4A). The other mutant alleles of these founders showed the intended processing at the sgRab38-2/3 target sites but the unexpected insertion of sequences identical to genomic regions located upstream (397 bp insertion, #9b) or downstream (163 bp insertion, #23b) of the sgRab38-3 target site.

In addition, we amplified from all 27 pups genomic segments spanning the sgRab38-2 (Fig. 4A) or sgRab38-3 regions (data not shown) to assess the frequency of small deletions occurring at single target sites. The sequence analysis of these PCR products revealed that small deletions (3–138 bp) or insertions (1 bp) occurred at both, the sgRab38-2 and sgRab38-3 target regions on the second *Rab38* locus in 5 of 7 of founders (#7, #9, #12, #26, #27) harboring a *Rab38*^{Δ3.2} allele. In the founders #18 and #19 as well as the 17 pups without a *Rab38*^{Δ3.2} allele, both sgRab38 target regions were found intact. As found in the TALEN induced *Rab38*^{Δ9.3} mutants, these results point to a high nuclease activity in founders harboring a *Rab38*^{Δ3.2} allele, frequently leading to the co-processing of the second *Rab38* locus. From the tail DNA of founders #6, #8, and #23 we could not obtain the predicted PCR

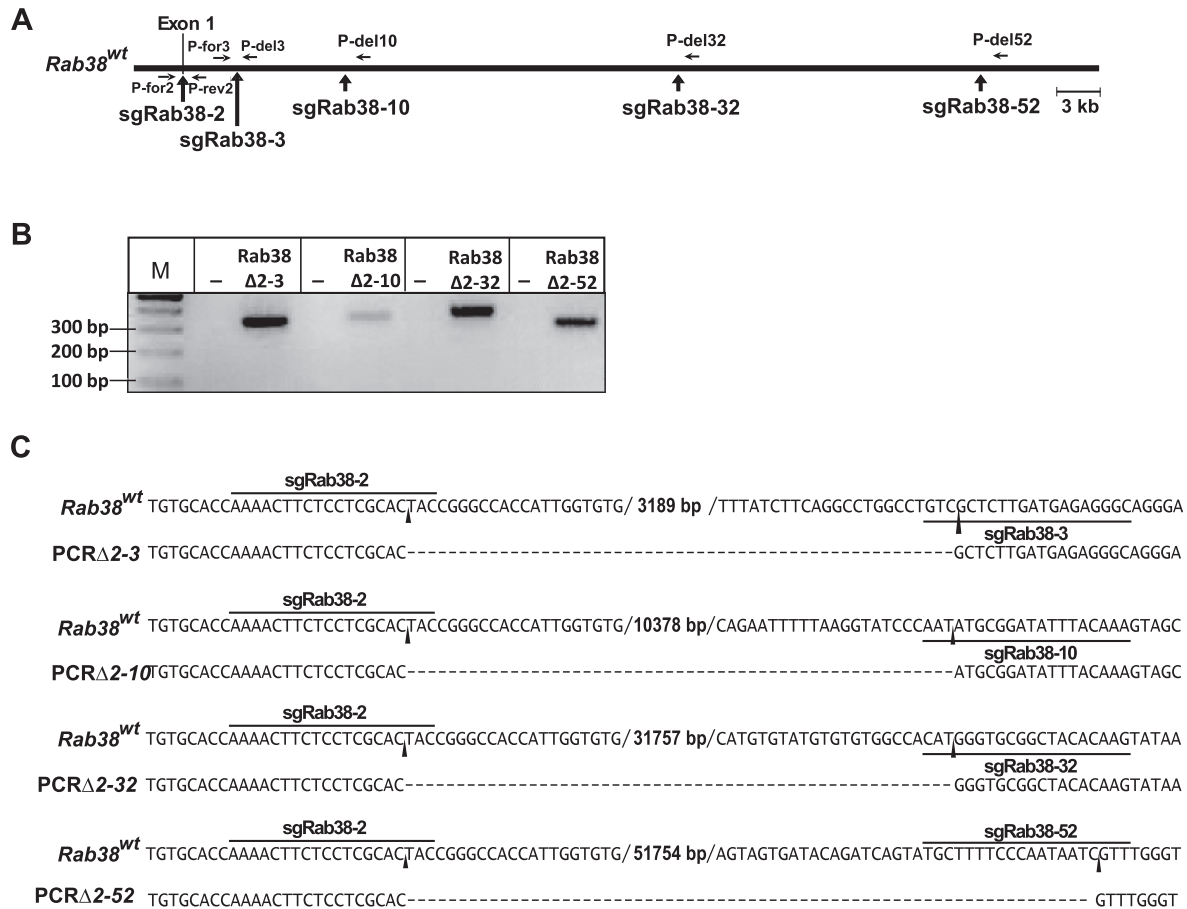


Fig. 3. Deletion of *Rab38* gene segments in Neuro2A cells using the CRISPR/Cas system. (A) Schematic diagram of the *Rab38* gene and the planned deletions of 3.2–51.7 kb, indicating the position of the first exon, of sgRNA target sites and of PCR primers. (B) Detection of deleted *Rab38* alleles 2 days after transfection of Neuro2A cells with expression vectors for Cas9 and sgRab38-2 together with sgRab38-3 (Δ 2-3), sgRab38-10 (Δ 2-10), sgRab38-32 (Δ 2-32), or sgRab38-52 (Δ 2-52). M: size marker, -: negative control. (C) Sequence comparison of the *Rab38* gene with PCR products derived from transfected Neuro2A cells (see B), confirming the authenticity of deleted alleles. In all cases the deletion endpoints were located at the predicted DSB sites 3 bp upstream of the target's PAM sequences (arrowheads).

bands from both sgRNA target regions, suggesting that in these founders both *Rab38* loci were processed by Cas9, resulting into a homozygous *Rab38* ^{Δ 3.2} genotype. From the cloning of five products of the *Rab38* ^{Δ 3.2} PCR reaction from each of these founders we identified two mutant alleles in founder #6 and #23 but only one deleted allele in founder #8. By this analysis we either missed a potential second mutant allele of founder #8 or both mutant alleles show identical sequences. In the absence of *Rab38* wildtype alleles in the melanocyte population, the homozygous mutants #6, #8 and #23 can be expected to exhibit a coat color phenotype, as seen in the TALEN induced *Rab38* mutants. Indeed these founders exhibited a lighter coat as compared to agouti colored, *Rab38*^{wt} controls (Fig. 4C). The microscopic inspection of dorsal awls from founder #8 confirmed the reduced pigmentation of the mutant (Fig. 4C).

To assess the processing of off-target sites in microinjected embryos, we amplified and sequenced three genomic regions each related to the sgRab38-2 or sgRab38-3 target sequence using tail DNA of all founder mutants. Besides the identification of a known single nucleotide polymorphism in one of the regions of founder #9, we did not find evidence for the processing of these sites by CRISPR/Cas (Supplementary Fig. 2). To demonstrate the germline transmission of the *Rab38* ^{Δ 3.2} alleles and for the establishment of breeding colonies, five mutant founders were bred to wildtype females. Tail DNA of the resulting progeny was analyzed for the presence of mutant *Rab38* alleles using the PCR primer pair P-for/P-del. Only about half of the pups derived from the putative

biallelic-mutated founders #6 (7/15 pups) and #8 (4/8 pups) were PCR positive (Fig. 5A), indicating a mosaic distribution of mutant alleles in the germline of these founders. The sequence analysis of five (#6) or three (#8) PCR products confirmed their identity with the founder's allele #6a and #6b or #8, respectively (Fig. 5B). From founder #23 all of the eight pups analyzed harbored either the allele #23a recombined with OD Δ 2/3 (5 pups) or allele #23b (3 pups), confirming the homozygous mutant state in this founder's germline (Fig. 5A and B). Founder #9 transmitted only the aberrantly recombined allele #9b to 4 of 6 pups whereas founder #26 transmitted allele #26a to one pup and allele #26b to six of nine pups (Fig. 5A and B). These results show that a pair of sgRNAs can be used for the deletion of a 3.2 kb segment in one-cell embryos at high rate, but that the frequency of a 30 kb deletion falls below 3%. Some founders represent homozygous compound mutants in the tail and exhibit a coat color phenotype but may be mosaic mutants in the germline. As observed for the mutant alleles induced by TALEN, we found that mutant alleles are readily germline transmitted and that the presence of an ODN bridging the deletion endpoints could not further support recombination.

3. Discussion

Sequence-specific nucleases such as TALEN and the CRISPR/Cas system are increasingly used for editing of single target sites in the mouse genome for the introduction of small deletions or point

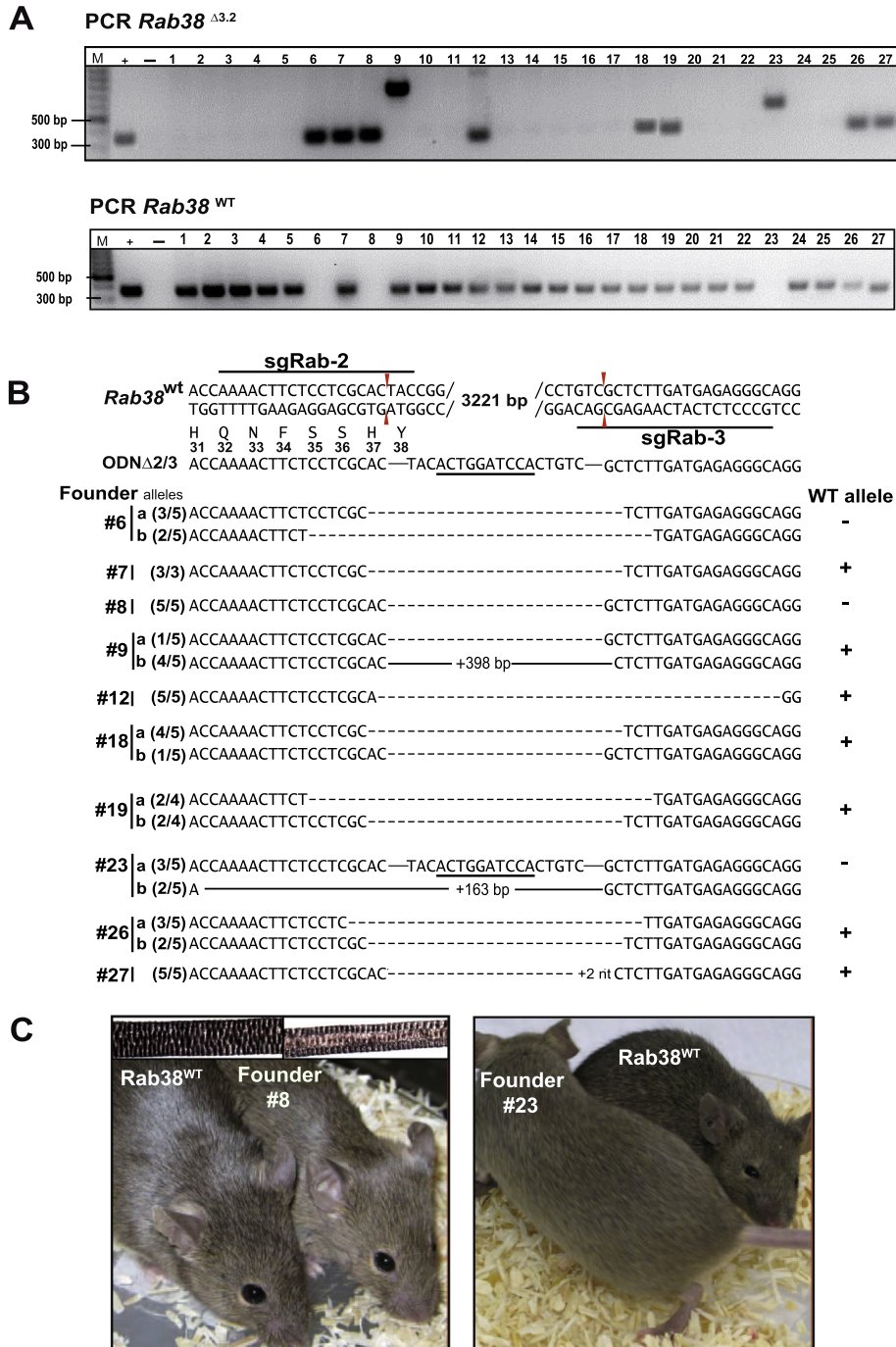


Fig. 4. Deletion of a 3.2 kb *Rab38* gene segment in one-cell embryos using Cas9 and two sgRNAs. (A) PCR detection of *Rab38*^{Δ3.2} alleles (primers P-for2 and P-del3) and of *Rab38*^{WT} alleles (primers P-for2 and P-rev2, spanning exon 1) with tail DNA from 27 pups derived from embryos microinjected with sgRab38-2, -3 and Cas9 RNAs. Upper gel image: Eight founders show PCR bands of ~326 bp (primers P-forA/P-del3) indicating the presence of *Rab38*^{Δ3.2} alleles; founders #9 and #23 exhibit unexpected, larger PCR products. Lower gel image: from the founders #6, #8 and #23 the region covering exon 1 of *Rab38* could not be amplified, suggesting that both gene copies were processed by Cas9. M: size marker, +: positive control, -: negative control. (B) Sequence comparison of cloned PCR products derived from mutant founders (see A) with the genomic sgRNA target regions of *Rab38* and the ODN^{Δ2/3} (sequence insert underlined). The sequencing of 3–5 clones from each founder revealed in six founders the presence of two mutant alleles. The number of clones classified as type a or b allele is shown in brackets. The deletion endpoints are either located at the DSB site 3 bp upstream of the sgRNA's PAM sequence (red arrows) or show the loss of additional nucleotides, leading to the disruption of the *Rab38* reading frame between codon 34 and 37. In the aberrant alleles #9b and #23b the deleted 3.2 kb region was replaced by sequence inserts of 398 bp (#9b) or 163 bp (#23b) that are derived from the *Rab38* gene, located upstream (#9b) or downstream (#23b) of the sgRab38-3 target sequence. (C) Comparison of the coat color of founder #8 and #23 with an agouti colored littermate control (*Rab38*^{WT}) and of dorsal awls showing the reduced pigmentation of hairs in founder #8 (right insert; 40× magnification). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutations, e.g. for modeling human disease mutations. Here we tested the possibility of targeting two distant sites in a single step using two TALEN or sgRNAs for the generation of genomic deletions in the *Rab38* gene in one cell embryos. Since a typical day

of embryo microinjection yields 20–30 pups, a deletion frequency of >3% is required to obtain at least one mutant. We found that a 10 kb deletion could be generated in 6% of pups derived from microinjections of two TALEN pairs and that two sgRNAs enabled the

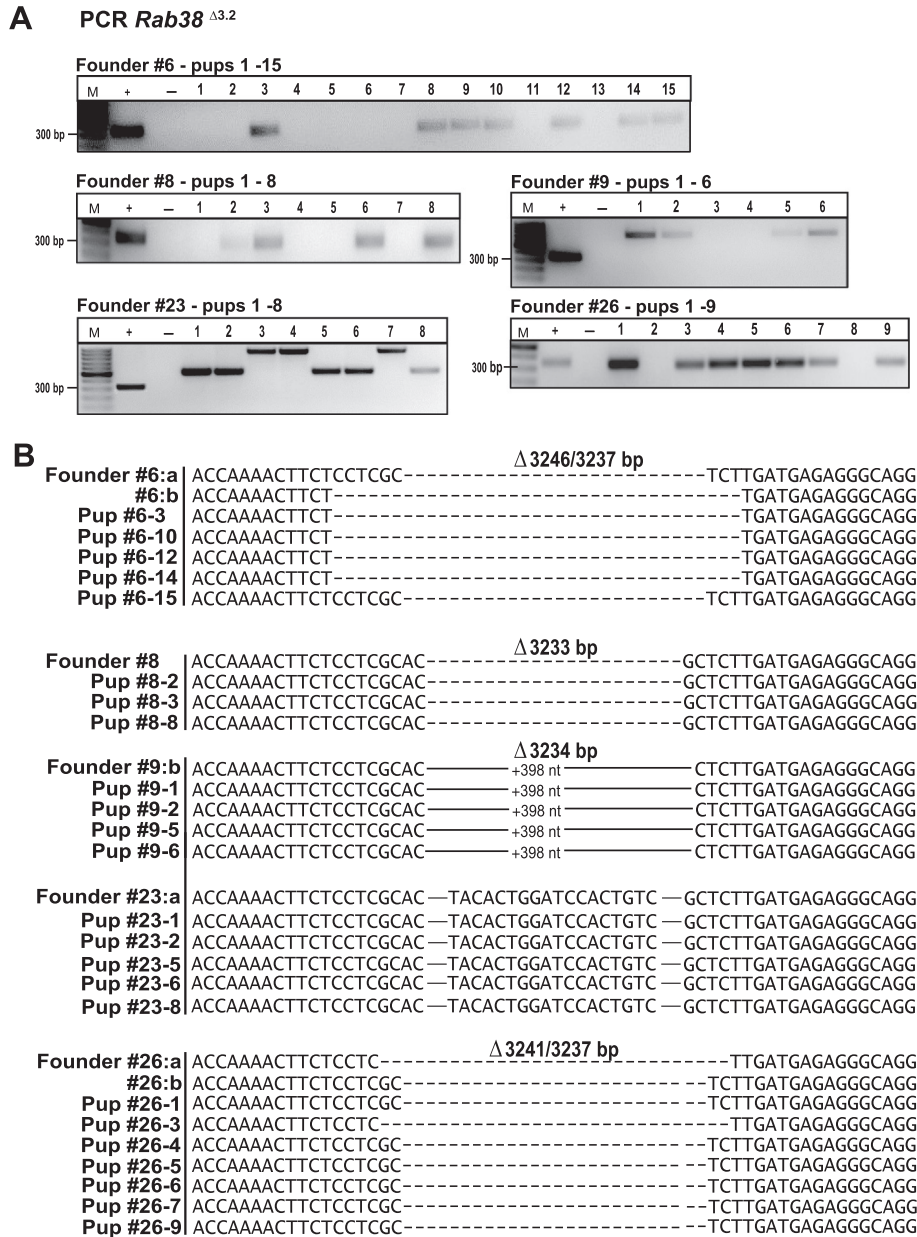


Fig. 5. Germline transmission of *Rab38*^{Δ3.2} alleles. (A) PCR detection of *Rab38*^{Δ3.2} alleles (primers P-for/P-del3) using tail DNA from pups derived from matings of the indicated mutant founders with wildtype mice. M: size marker, +: positive control, -: negative control. (B) Sequence comparison of PCR products (see A) obtained from the indicated pups with the parental *Rab38*^{Δ3.2} alleles. The number of deleted basepairs is indicated.

deletion of a 3.2 kb gene segment in 37% of mice, but a 30 kb deletion was not obtained among 38 mice.

These deletions reflect the spontaneous joining between the distant ends of two DSBs by NHEJ repair. We reasoned that a reduced frequency can be expected with increasing DSB distances and that the junction of such ends may be further promoted by HR repair and ODNs including homology sequences to the DSB ends. We found that ODNs did not promote the rejoining of such ends, indicating that NHEJ but not HR is the predominant DSB repair mechanism in mammalian cells. Nevertheless, we observed the deletion of 30 kb or 50 kb in *Neuro2A* cells. This indicates that larger deletions can be obtained, but likely occur at a reduced frequency. In a recent study using Mel cells it has been found that clones with deletions of up to 1 Mb can be readily obtained if the cells transfected with CRISPR/Cas plasmids are enriched [16]. In

this study the deletion frequency is inversely related to its size, predicting 25% efficiency for the deletion of 3 kb and 10% efficiency for the deletion of 30 kb. Nevertheless, this relation was delineated from the top 3% population of transfected cells. Therefore, the selection of microinjected embryos showing high levels of nuclease expression may further increase the yield of mutant founders. This could be achieved in future by the expression of a Cas9-venus fusion protein, culture of microinjected zygotes to 2-cell embryos and the selection of embryos showing highest fluorescence for transfer into foster mothers. Furthermore, it may be possible to increase the rate of larger deletions. This could be achieved by targeting genomic sites for which long range interactions are known to occur within topological domains. Thus, the DSB ends can meet at a higher probability [17]. Alternatively, the joining of distant DSB ends could be promoted by the selective dimerization of

nuclease molecules that are bound to their target sequence. This, however, will require the development of new technology that builds on controlled dimerization that depends on conformational changes that occur upon specific DNA binding of TALEN or Cas9 [18].

As previously reported for TALEN- and CRISPR/Cas-induced mutations [19,11], many of our founders harbor two modified alleles, together with or without a wildtype *Rab38* allele, as identified by the analysis of tail DNA. Five founders appeared as homozygous compound mutants in the tail tissue and skin melanocytes leading to a coat color phenotype. Since two of the three CRISPR/Cas derived homozygous mutants identified by tail DNA analysis transmitted a wildtype allele to some of their pups, individual founders harbor variable patterns of *Rab38* alleles in different tissues. Nevertheless, all of the mutant alleles identified in the tail were also transmitted to the founders' progeny, validating the analysis of tail DNA, in qualitative terms, as predictive of the founders' germline. The use of single sgRNAs for DSB induction can lead to the processing of related off-target sites in the genome [20–22]. We used target sequences predicted for high specificity and analyzed the sequence of three potential off-target sites predicted for sgRab38-2 and sgRab38-3 using tail DNA of 10 founder mice. Although we found no evidence for the processing of these sites in any of these founders, the recognition of other sites can presently not be excluded.

We showed that both TALEN and sgRNA pairs can be used to induce genomic deletions in one-cell embryos. Since gene segments of different lengths were deleted, we could not directly compare the efficiency of both systems. The advantage of CRISPR/Cas mutagenesis is the simplicity of programming sgRNAs to new target sequences by cloning of short oligodeoxynucleotides, as compared to the construction of 1.5 kb coding regions for TALEN proteins. Nevertheless, TALEN pairs spanning a 30 bp recognition sequence may be advantageous for excluding off-target effects that are hard to control for sgRNAs and wildtype Cas9 [20–22]. The use of shortened sgRNAs [23] and of sgRNA pairs together with a Cas9 nickase mutant [24] have been described as strategies for increasing the specificity of DSB induction by CRISPR/Cas. Since the nickase approach has been recently validated for the targeting of single sites in one-cell mouse embryos [25], the use of such sgRNA pairs should be explored for the future generation of targeted deletions by CRISPR/Cas. Our results show that each of these nuclease systems can be used to create genomic deletions. Therefore, the choice of TALEN or CRISPR/Cas may depend on the specifics of individual experiments.

Using TALEN or CRISPR/Cas in one cell embryos enables the identification of mutant founders, just 7 weeks after microinjection [26,27]. It is therefore considerably faster in comparison to ES cell technology. Moreover, creating large deletions in ES cells requires the construction of gene targeting vectors and the manipulation of two distant genomic sites by sequential gene targeting steps [28]. These constraints are released by the application of nuclease technology, enabling the expedite generation of deletions in the mouse germline. Targeted deletions are of use to study structural genome variation that occurs between individuals of the same species or between related species. In mice targeted deletions could be applied for modeling of human disease associated genomic deletions [13] or to explore the consequences of deletions found specifically in the human genome [29]. Furthermore, it will be possible to derive new, improved inbred mouse strains, e.g. by the removal of deleterious retroviral insertions [30–32]. Besides the straightforward deletion of genomic sequences it will be interesting to explore in future whether genomic deletions can be combined with the insertion of new genes or gene segments to achieve single step gene replacements.

4. Materials and methods

4.1. TALEN construction and expression

TALEN target sites were selected using the “TALENdesigner” (www.talen-design.de) as described [26]. Selected target sites cover two recognition sequences of 15 bp, preceded by a T and separated by a spacer of 15 bp. TALEN construction and expression was carried out as described using the expression vectors pCAG-TALEN-pA [11] and pT7-95A [33]. For sequence recognition of the TAL RVDs see Table 1. The targeting oligodeoxynucleotide ODN (A/B) was synthesized and HPLC purified by Metabion (Martinsried, Germany). ODN (A/B) sequence (BamHI site underlined): tggtgatcg gcgacctgggtgtgggcaagaccagcattatcaacgctatgtgcaccaaaaactggatcc actattgcttttcattggatatattgagctaattatctag tgaacctatttaggatg ttaatt.

4.2. CRISPR/Cas target sites and vector construction

CRISPR target sites were identified using <http://crispr.mit.edu> as described [34]. In order to create differently sized genomic deletions in Neuro2A cells, four different sgRNAs target sequences of 20 bp located upstream of a NGG PAM-sequence were selected. Selected target sequences (underlined) were cloned as complementary oligonucleotides in between two BbsI sites of a Bluescript plasmid containing a U6 promoter (pBS-U6) and the sgRNA backbone [34] for the production of RNA from the template sequence of sgRab38-2 cloned into pBS-U6-sgRab38-2: AAAACCTCTCCTCCG ACTACGTTTTAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGTCTTTTTT, of sgRab38-3 cloned into pBS-U6-sgRab38-3:

GGCCCTCTCATCAAGAGCGACGCTTTTAGAGCTAGAAATAGCAAGTT AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG TGCTTTTTT, of sgRab38-10 cloned into pBS-U6-sgRab38-10:

GTTTGTAATATCCGCATATGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT GCTTTTTT, of sgRab38-32 cloned into pBS-U6-sgRab38-32:

CTTGTGTAGCCGCACCCATGTTTTAGAGCTAGAAATAGCAAGTTA AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT GCTTTTTT, and of sgRab38-52 cloned into pBS-U6-sgRab38-52:

TGCTTTTCCCAATAATCGTTGTTTTAGAGCTAGAAATAGCAAGTTAA AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTG CTTTTTT.

For the production of sgRNAs by in vitro transcription a T7 promoter sequence (TAATAATACGACTACTATAG) was added upstream of the target sequence. To assess potential CRISPR off-target activity, three high-scored off-target sites (see Supplementary Fig. 2) were amplified by the locus-specific primer pairs shown in Table 2. For analysis the PCR products were purified, sequenced (GATC Biotech, Konstanz, Germany) and compared to wild type using the Vector NTI Advance 11.5 (Life Technologies) and Chromas (Technelysium) software. For the expression of Cas9 we replaced the TAL sequences of our pTALEN-pA plasmid with a T7 promoter and a codon optimized Cas9 coding region [35] purchased from Genscript (Piscataway, USA) to derive the pCAG-Cas9-pA expression vector. For the production of Cas9 mRNA we inserted the Cas9 coding region into our pT7-95A plasmid that was further modified by the addition of 67 A residues to derive the plasmid pCAG-Cas9-162A.

The targeting oligodeoxynucleotide ODN Δ 2/3 was synthesized and HPLC purified by Metabion (Martinsried, Germany). ODN Δ 2/3 sequence (BamHI site underlined): ACTGGGTGTGGCAAGACC AGCATTATCAAGCGCTATGTGCACCAAACTTCTCCTCGACTACTACT GGATCCACTGTGCTCTTGATGAGAGGGCAGGGATTCCCTGACTCT ATAGGATATTAGCCTACTGCAA.

Table 1
TAL RVDs used for DNA sequence recognition.

Rab38 TAL-A1															
TALEN RVD sequence	NI	NG	HD	NI	NI	NN	HD	NN	HD	NG	NI	NG	NN	NG	
Target sequence (5'–3')	A	T	C	A	A	G	C	G	C	T	A	T	G	T	
Rab38 TAL-A2															
TALEN RVD sequence	NN	NN	HD	HD	HD	NN	NN	NG	NI	NN	NG	NN	HD	NN	
Target sequence (5'–3')	G	G	C	C	C	G	G	T	A	G	T	G	C	G	
Rab38 TAL-B1															
TALEN RVD sequence	NG	NG	HD	NI	NI	NI	NG	NN	HD	NG	NI	NI	NG	NI	
Target sequence (5'–3')	T	T	C	A	A	A	T	G	C	T	A	A	T	A	
Rab38 TAL-B1															
TALEN RVD sequence	HD	NI	NI	NG	NI	NG	NI	NG	HD	HD	NI	NI	NG	NN	
Target sequence (5'–3')	C	A	A	T	A	T	A	T	C	C	A	A	T	G	

Table 2
Primer pairs used for the amplification of high-scored off-target sites.

Off target name	Forward primer (5'–3')	Reverse primer (5'–3')
sgRab38-2: OT2-1	TTGAGGAGGATTTCTGTATGTGTGTGTG	CTAGAACATTAACCATGCAAGTCTTATTGAC
sgRab38-2: OT2-2	TGGGTGAGAGACAGGGACCTCATG	GCCTTACCCTGGATCTGGCAGCAG
sgRab38-2: OT2-3	CCTGGACAAGGAGGAGGAGGAAGAG	CATTCGGCTCTTAACGCAAATGAC
sgRab38-3: OT3-1	CATGAGTGACCTAAGATACCCAGCACCTG	GCACCACATGAAAGCTGGTCTCAAG
sgRab38-3: OT3-2	AATCAAAATCATCTGCCTCCAAATGCTC	GATGTCTGTCCACAGGGATGTCTGTG
sgRab38-3: OT3-3	AGCTCGGACTTCAGTTCAAGTCACTTG	TGACCAGCAGAAGCACCATCACCCAC

4.3. Nuclease reporter plasmids and activity assay

Nuclease reporter plasmids were generated by the insertion of annealed sense and anti-sense oligonucleotides (Metabion, Martinsried, Germany), harboring TALEN and sgRNA target sequences, into the generic reporter plasmid pTAL-Rep, as described [11]. The integrity of all reporter plasmids was confirmed by DNA sequencing. For the assessment of nuclease activities, expression vectors were cotransfected with the corresponding reporter plasmid into Neuro2A cells (No. ACC 148, DSMZ, Braunschweig, Germany). Two days after transfection the cells were lysed and β -galactosidase was determined by chemiluminescence, as described [11].

4.4. Deletions in Neuro2A cells

To create deletions in Neuro2A cells, pairs of sgRNA vectors and pCAG-Cas9-pA plasmid DNA were cotransfected using the X-tremGENE reagent (Roche). Two days later genomic DNA was isolated using the Wizard Genomic DNA Purification Kit and used for the detection of deleted alleles as described below.

4.5. Microinjection of one-cell embryos

The injection of TALEN mRNA and targeting molecules (ODNs) was performed as described [33], except that injections were done only into pronuclei. Briefly, capped TALEN mRNA was prepared in a single step by in vitro transcription from pT7-TALEN-95A plasmids (linearized with XbaI and AclI) or from pCAG-Cas9-162A (linearized with AsiSI and XbaI) using the mMessage mMachine T7 Ultra kit (Ambion, AM1345, Life Technologies, Carlsbad, USA) (omitting the polyadenylation step) and the MEGAclear kit (Ambion, #1908). sgRNAs were prepared using templates amplified by PCR from pBS-U6-sgRNA plasmids (primer T7-tracr1: GTACAAAATACGTGACGTAGAAAG; T7-tracr2: AAAAAAAGCACCGACTCGGTG) for in vitro transcription using the Megashortscript kit (Ambion, #AM1354) and purification with the MEGAclear kit. The quality of mRNAs (TALEN, Cas9) was controlled by agarose gel electrophoresis under denaturing conditions using the NorthernMax-Gly system and the RNA Millennium size marker (Life Technologies) or for

sgRNAs using a Bioanalyzer and the RNA 6000 Nano chip (Agilent, Santa Clara, CA, USA). TALEN mRNAs were diluted in microinjection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.2) to a working concentration each of 45 ng/ μ L; Cas9 mRNA and sgRNAs were used at concentrations of 67 ng/ μ L or 30 ng/ μ L, respectively. The targeting oligodeoxynucleotides were dissolved in water and diluted with injection buffer to a working concentration of 30 ng/ μ L. For microinjections, one-cell embryos were obtained by mating of (DBA/2 \times C57BL/6) F₁ males with superovulated FVB/N females (Charles River, Sulzbach, Germany). One-cell embryos were injected into the larger pronucleus, but not into the cytoplasm. Injected zygotes were transferred into pseudopregnant CD1 female mice to obtain live pups. All mice showed normal development and appeared healthy. Mice were handled according to institutional guidelines approved by the animal welfare and used committee of the government of upper Bavaria and housed in standard cages in a specific pathogen-free facility on a 12 h light/dark cycle with *ad libitum* access to food and water.

4.6. DNA isolation and genotyping of mutant founders and their offspring

Genomic DNA was isolated from tail tips of founder mice and biopsies of their progeny, using the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany), following the manufacturer's instructions. For the genotyping of founder mice and their progeny, PCR reactions using 1 μ L genomic DNA (~100 ng) and 1 μ L of each appropriate primer (10 μ M) was carried out in a total volume of 25 μ L using the 5 PRIME Mastermix (5 PRIME GmbH, Hilden, Germany) and PCR steps of: 94 °C – 5 min; {94 °C – 40 s; 60 °C – 40 s; 72 °C – 60 s} for 30 cycles; 72 °C – 10 min. For the detection of TALEN induced deletions we used the primer pair P-forA (AAGCTCCAGGCTCCGCAAGAC) and P-revA (CCGAACCTCTC ACTGGCTCAC) to amplify the TALEN-A1/2 region, the primer pair P-forB (AATGCTACTGTGTTGCCTTGG) and P-del (CATCTCAAATGTT GGGATCACAAG) to amplify the TALEN-B1/2 region and the primers P-forA and P-del to detect *Rab38*^{A9.3} alleles. For the detection of CRISPR/Cas induced deletions we used the primer pairs P-for2 (CACATAGAGCTCCGGTCTCC) and P-rev2 (CGAACTCCTACTGGCT

CAC) to amplify the sgRab38-2 region, the primer pair P-for3 (AACTTCTGTTCTTCTTCTTCTGTC) and P-del3 (TACGAATCAGACC TTACATCAGTG) to amplify the sgRab38-3 region, and the primer pairs P-for2 and P-del3, or P-del10 (GTACCTAGAGCCTCGAGCTG), P-del32 (CGCTAGGAAATCTGGTTAGTG), or P-del52 (GGTCATATT TACAGTCACACCT) for the detection of large deletions. For the analysis of gene editing PCR products were sequenced and analyzed using the TIDE tool [36]. For the subcloning of mutant alleles, PCR reactions were carried out using Herculase II Fusion polymerase (Agilent, Waldbronn, Germany) in a total volume of 50 μ L with 30 cycles of {98 °C, 20 s; 60 °C, 20 s; 72 °C, 20 s}. PCR products were purified using the Qiaquick PCR purification kit (QIAGEN, Hilden, Germany), subcloned using the StrataClone Blunt PCR Cloning Kit (Agilent) and sequenced (GATC Biotech, Konstanz, Germany). Sequences were compared to wild type, using the Vector NTI Advance 11.5 software suite (Life Technologies).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fob.2014.11.009>.

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