Highly Improved Gene Targeting by Germline-Specific Cas9 Expression in *Drosophila*

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ABSTRACT We report a simple yet extremely efficient platform for systematic gene targeting by the RNA-guided endonuclease Cas9 in *Drosophila*. The system comprises two transgenic strains: one expressing Cas9 protein from the germline-specific *nanos* promoter and the other ubiquitously expressing a custom guide RNA (gRNA) that targets a unique site in the genome. The two strains are crossed to form an active Cas9–gRNA complex specifically in germ cells, which cleaves and mutates the target site. We demonstrate rapid generation of mutants in seven neuropeptide and two microRNA genes in which no mutants have been described. Founder animals stably expressing Cas9–gRNA transmitted germline mutations to an average of 60% of their progeny, a dramatic improvement in efficiency over the previous methods based on transient Cas9 expression. Simultaneous cleavage of two sites by co-expression of two gRNAs efficiently induced internal deletion with frequencies of 4.3–23%. Our method is readily scalable to high-throughput gene targeting, thereby accelerating comprehensive functional annotation of the *Drosophila* genome.

PHENOTYPES of mutant animals have provided deeper insight into gene function than any other means. In *Drosophila melanogaster*, hundreds of genetic screens have been conducted over the last century aiming to isolate new mutants. Even today, however, loss-of-function mutants in the vast majority of genes are yet to be described, calling for a breakthrough technology that allows systematic gene targeting to complete our understanding of the *Drosophila* genome. Targeted gene disruption in *Drosophila* has been conventionally achieved by deletion of flanking sequences by imprecise excision of transposons or, more recently, by gene replacement using homologous recombination (Rong and Golic 2000; Ryder and Russell 2003). Each of these techniques, however, has its own limitations: Although imprecise excision of transposons is moderately efficient, it is limited by its requirement of an existing transposon near the target locus, which is missing for quite a few loci (Ryder and Russell 2003). Homologous recombination suffers from extremely low efficiency, often requiring the screening of more than 1×10^6 flies (Huang *et al.* 2009).

Designer nucleases are a promising new technology that offers an alternative route to gene targeting. They represent genetically encoded nucleases that can be programmed to target an arbitrary sequence. Heterologously expressed designer nucleases cause a double-strand break at a specified target sequence in the genome, which gives rise to an insertion-deletion (indel) mutation through inaccurate DNA repair involving nonhomologous end joining. Zinc-finger nucleases (ZFNs) and TALE nucleases (TALENs) are heterodimeric nucleases with a programmable DNA-binding domain assembled from DNA-binding peptides (Kim *et al.* 1996; Miller *et al.* 2011). They have been widely used to mutate genes in a broad range of animal species. Despite their demonstrated high efficiency (Bibikova *et al.* 2002; Liu *et al.* 2012), ZFNs and TALENs have not received as much enthusiasm in *Drosophila* research as they have in other model organisms, most likely due to the difficulty of assembling DNA-binding domains and microinjecting nuclease messenger RNA into embryos.

CRISPR/Cas9 is the latest addition to the designer nuclease family. Originally identified as a component of the CRISPR bacterial innate immunity system, Cas9 was found to encode a novel class of sequence-specific endonuclease whose target specificity is determined by its guide-RNA (gRNA) component (Jinek *et al.* 2012; Cong *et al.* 2013; Mali *et al.* 2013). Cas9 can be easily reprogrammed

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to target a new sequence simply by replacing the specificity-determining 20-bp sequence of the gRNA. Constraints to selecting suitable 20-bp targets are minimal, the only requirements being that they must be followed by an “NGG” sequence known as the protospacer adjacent motif (PAM) (Jinek *et al.* 2012). The ease and flexibility of the design make Cas9 an ideal tool for genome-scale applications. Cas9 has been reported to be extremely effective when injected into zygotes of zebrafish and mice, with somatic mutation frequencies exceeding 50% (Hwang *et al.* 2013; Wang *et al.* 2013). Attempts to adapt Cas9 to genome engineering in *Drosophila* were recently reported, in which plasmid vectors or *in vitro*-transcribed RNAs encoding Cas9 and gRNA were injected into fertilized eggs to transiently express Cas9–gRNA (Bassett *et al.* 2013; Gratz *et al.* 2013; Yu *et al.* 2013). The reported procedures, however, resulted in somewhat variable germline mutation frequencies, calling for a more robust system in which any gene can be mutated with high efficiency. In the present study, we describe a method of deriving mutant progeny from transgenic flies stably expressing Cas9–gRNA in germ cells, with a dramatic improvement in consistency and efficiency.

Materials and Methods

Plasmid construction

Standard molecular biology techniques were used to construct plasmid vectors. We first constructed a general transformation vector pBFv, which has an attB sequence for site-specific integration by phiC31 integrase and a *vermillion* (*v*) gene as a visible marker. A 1264-bp promoter sequence and a 965-bp, 3′-UTR-containing sequence of the *nanos* gene, which have been shown to drive highly specific germline expression (Van Doren *et al.* 1998), were cloned into pBFv to create the germline-expression vector pBFv-nosP. A Cas9 cDNA fragment was cut from hCas9 (Mali *et al.* 2013) by *Xba*I/*Age*I and cloned into pBFv-nosP to create pBFv-nosP-Cas9. The general gRNA expression vector pBFv-U6.2 was constructed by cloning a 399-bp promoter sequence of the *Drosophila* *snRNA:U6:96Ab* gene into pBFv. To construct a gRNA expression vector for each target gene, two complementary 24-bp oligonucleotides with a 20-bp target sequence were annealed to generate a double-strand DNA with 4-bp overhangs on both ends and cloned into *Bbs*I-digested pBFv-U6.2 (Figure S2). Six gRNA vectors targeting the *white* gene and seven gRNA vectors targeting different neuropeptide genes were generated. They were named pBFv-U6.2-“gRNA ID” (pBFv-U6.2-w-ex3-1, pBFv-U6.2-w-ex6-1, pBFv-U6.2-Ast-1, etc.).

To construct a double-gRNA vector in which two different gRNAs are separately expressed from their own U6 promoters, a first gRNA was cloned into pBFv-U6.2 and a second gRNA into pBFv-U6.2B, a variant of pBFv-U6.2 that has a dummy sequence flanked by *Eco*RI and *Not*I sites upstream of the U6 promoter (Figure S1). A fragment containing the U6

promoter and the first gRNA was cut from pBFv-U6.2-gRNA#1 by *Eco*RI and *Not*I and ligated with pBFv-U6.2B-gRNA#2 linearized with *Eco*RI and *Not*I. Double-gRNA vectors targeting *white*, *mir-219*, and *mir-315* were constructed. They were named pBFv-U6.2x2-w, pBFv-U6.2x2-mir-219, and pBFv-U6.2x2-mir-315.

The sequences of the oligonucleotides used to construct each gRNA vectors are shown in Table S1.

Fly transformation

All vectors were integrated into the *attP40* landing site on the second chromosome by phiC31 integrase (Markstein *et al.* 2008). Plasmid DNA (100 ng/μl) was injected into the *y¹ v¹ nos-phiC31; attP40* host (Bischof *et al.* 2007). Surviving *G₀* males were individually crossed to *y² cho² v¹* virgins. In a *cho² v¹* background, a *v⁺* transgene turns the eye color from light orange to dark brown, making it easier to identify transformants than in a *v¹* background. A single male transformant from each cross was mated to *y² cho² v¹*; *Sp/CyO* virgins. Offspring in which the transgene was balanced were collected to establish a stock.

Two vectors, pBFv-nos-Cas9 and pBFv-U6.2-w-ex3-1, were individually injected. The other vectors were injected in three pools of multiple vectors: The first pool contained the remaining five gRNA vectors targeting the *white* gene. The second pool contained the seven vectors targeting neuropeptide genes. The third pool contained the three double-gRNA vectors. After establishing lines from individual *F₁* transformants, PCR analysis was performed to identify which vector was integrated in each line. For all three pools, screening of 16 lines was sufficient to recover at least one transgenic line for each of the pooled vectors.

Fly genetics

All the U6-gRNA lines used in this study have the *y² cho² v¹*; *attP40{U6-gRNA}/CyO* genotype. The *nos-Cas9* line has the *y² cho² v¹*; *attP40{nos-Cas9}/CyO* genotype.

Seven U6-gRNA lines—*U6.2-w-ex3-1*, *U6.2-w-ex3-2*, *U6.2-w-ex3-3*, *U6.2-w-ex3-4*, *U6.2-w-ex3-5*, *U6.2-w-ex6-1*, and *U6.2x2-w*—were used to induce mutations at the *white* locus. Females carrying a U6-gRNA transgene were crossed to *nos-Cas9* males to obtain founder animals that have both the U6-gRNA and the *nos-Cas9* transgenes. Each male founder was crossed to four virgin females carrying the compound-X chromosome. We used the compound-X chromosome strain *Bx³/C(1)DX y¹ w¹ f¹*. Female founders were individually put in a vial after being allowed to mate with the siblings. Sixteen to 18 crosses were set up for each experimental condition. The number of *white* mutants in the total progeny from each cross was counted to estimate mutation frequency. Crosses that produced <10 offspring were excluded from analysis. The female germline was not examined for *U6.2x2-w*.

To induce mutations at the neuropeptide and microRNA (miRNA) genes, the following U6-gRNA lines were used: *U6.2-Ast-1*, *U6.2-cap-1*, *U6.2-Ccap-1*, *U6.2-Crz-1*, *U6.2-Eh-1*, *U6.2-Mip-1*, *U6.2-npf-1*, *U6.2x2-mir-219*, and *U6.2x2-mir-315*.

Founder animals were obtained by crossing U6-gRNA females to *nos-Cas9* males. Fifteen to 20 male founders were crossed *en masse* to 15–20 *w*; *Dr/TM6B* virgins. Genomic DNA was extracted from each of the resultant offspring and used for molecular characterization.

PCR amplification of target loci

To molecularly characterize induced mutations, target loci were first amplified by PCR using genomic DNA extracted from individual flies. Each fly was crushed in 50 μ l of DNA extraction buffer (50 mM NaOH, 0.5% Triton-X). The lysate was heated at 95° for 5 min, cooled down to 4°, and immediately neutralized with 50 μ l of 10 mM Tris (pH 8.0). Using 0.5 μ l of the crude DNA extract as a template, a DNA sequence surrounding the target site was amplified by PCR for 35–40 cycles in a 10- μ l reaction with 0.2 U of KOD FX Neo (Toyobo). The PCR product was analyzed by agarose gel electrophoresis, the T7 endonuclease I (T7EI) assay, and DNA sequencing. Primers used for PCR and DNA sequencing are listed in Table S1.

T7EI assay

To identify animals with a heterozygous mutation, we used T7EI, a structure-specific nuclease that cleaves DNA heteroduplexes at mismatch sites. First, genomic DNA of each fly was amplified by PCR for 40 cycles as described. If a fly carries a heterozygous mutation at the target site, the PCR product forms heteroduplex DNA with a mismatch in later cycles of PCR after DNA amplification levels off. Two microliters of the PCR product was directly treated with 1 U of T7 endonuclease I (New England Biolabs) in a total volume of 10 μ l. After incubation for 15 min at 37°, the sample was immediately put on ice to terminate enzyme reaction. Timely termination is critical, as prolonged incubation results in degradation of cleaved DNA fragments. The sample was directly analyzed by electrophoresis on a 2–3% agarose gel in Tris-acetate-EDTA buffer. Of 54 samples analyzed by both the T7EI assay and DNA sequencing, 29 had an indel mutation detected by both assays. Three mutations identified by DNA sequencing were not detectable in the T7EI assay.

Estimation of off-target mutation frequency

Male animals of the genotype *y cho v/Y*; *nos-Cas9/U6-gRNA* (G_0) were crossed to **FM7* virgins. Female offspring (F_1) of the genotype *y cho v/FM7* were individually mated to *FM7/Y* males in separate vials. Absence of *y cho v/Y* males in their progeny (F_2) indicates that a lethal mutation was induced on the X chromosome in the G_0 germline. Approximately 200 F_1 females were scored to estimate the frequency of *de novo* lethal mutations on the X chromosome. Four gRNAs targeting autosomal genes (*capa*, *Ccap*, *Mip*, *npf*) were tested. The results are shown in Table S2.

Results

To express Cas9–gRNA in germ cells of *Drosophila*, we chose to establish transgenic flies carrying genomic sources of the

Figure 1 Schematic overview of the transgenic Cas9–gRNA system. For each gene of interest, a transgenic strain that ubiquitously expresses a gRNA targeting the gene is established. The gRNA strain is crossed to the *nos-Cas9* strain, which expresses Cas9 protein specifically in germ cells. The founder animals obtained from the cross express active Cas9–gRNA nuclease complex specifically in germ cells. Mutation is induced at a certain frequency in the founder germline and transmitted to the next generation.

enzyme components. While transgenes could be transiently expressed by microinjection of plasmid DNA or *in vitro*-transcribed RNA, previous observations indicate that genomic sources of various DNA-modifying enzymes produce significantly higher activity than injected DNA or RNA in *Drosophila* (Robertson *et al.* 1988; Keeler *et al.* 1996; Bischof *et al.* 2007; Bateman and Wu 2008; Holtzman *et al.* 2010). We constructed two transformation vectors that expressed Cas9 protein or a custom gRNA (Figure 1 and Supporting Information, Figure S1). Cas9 is expressed specifically in germ cells by the promoter of the *nanos* (*nos*) gene (Van Doren *et al.* 1998), while gRNA is expressed from a ubiquitous pol III promoter derived from the upstream sequence of a *Drosophila* U6 small nuclear RNA gene (Wakiyama *et al.* 2005). The gRNA vector has a cloning site that allows seamless cloning of a 20-bp specificity-determining sequence by accepting annealed oligonucleotides (Figure S2). We separately integrated the Cas9 and gRNA vectors into a fixed landing site in the *Drosophila* genome by phiC31 integrase (Bischof *et al.* 2007; Markstein *et al.* 2008). Transgenic animals carrying *nos-Cas9* or U6-gRNA were fully viable and fertile, indicating that neither Cas9 nor gRNA alone have a deleterious effect on cells. The *nos-Cas9* and U6-gRNA lines are crossed to obtain founder animals that expressed an active Cas9–gRNA complex specifically in the germline, thereby inducing mutations in target genes. Once a mutant is obtained,

Figure 2 Mutagenesis of the *white* locus by transgenic Cas9–gRNA. (A) Schematic of the *white* locus. Exons are shown as boxes. Coding regions are depicted in black and the 5'- and 3'-UTRs in gray. Locations and sequences of the gRNA targets are indicated with the PAM in green. (B) Frequencies of mutations induced by Cas9–gRNA. For each gRNA, the mutation frequency is shown as a percentage of *white* mutants in total progeny. The percentage of founders producing at least one mutant offspring (yielders) is also shown. Male and female germlines were separately scored. (C) Sequences of mutations induced by the gRNA *w-ex3-1*. The wild-type sequence is shown at the top with the gRNA target sequence highlighted by a top line. The cleavage site is indicated by an arrowhead. Deleted residues are shown as dashes. Inserted residues are shown in blue lowercase letters. The indel size and the number of occurrences are shown on the right of each mutant sequence. (D) PCR analysis of internal deletions induced by simultaneous expression of two gRNAs. Locations of primers and cleavage sites are shown in E. The wild type produces a band of 2.3 kb, while complete deletion between the two target sites produces a band of 0.7 kb. (E) The extent of deletions in D was determined by sequencing the breakpoints. Primer locations and cleavage sites are indicated by arrowheads and arrows, respectively.

the integrated *nos-Cas9* and U6-gRNA transgenes can be conveniently removed by crossing the mutant into a *yellow* or *vermillion* mutant background, as they are marked with *yellow* and *vermillion* transgenes.

To test the efficacy of our system, we first attempted to mutate the sex-linked *white* locus, in which mutants have white eyes due to complete lack of pigment. We established transgenic lines each expressing one of six gRNAs designed to target different locations of the *white* gene (Figure 1). They were crossed to *nos-Cas9* to obtain founder animals carrying both the *nos-Cas9* and U6-gRNA transgenes. We estimated mutation frequency in the germline of these founder animals. The male and female germlines were individually examined because of potential sex-dependent differences in choice of DNA repair pathways because the X

chromosome is hemizygous in *Drosophila*. We crossed individual male founders to females with a compound-X chromosome, such that their male progeny inherit the X chromosome from the father. Female founders were individually crossed to male siblings. The male progeny from each cross were scored for loss of eye color.

Each of the six gRNAs was highly effective in inducing *white* mutants. All founder males expressing Cas9–gRNA produced at least one *white* mutant offspring. Germline mutation frequencies, calculated as a percentage of *white* mutants in total progeny, exceeded 85% in four of the gRNAs. The other two were less effective with mutation frequencies of 12 and 57%. In the female germline, mutation frequencies were consistently lower than in the male germline, ranging from 3.4 to 93% (Figure 2B). In the

the two miRNA genes, two gRNAs were designed to delete 129- and 369-bp sequences, respectively. Complete gene deletion was observed in 7 of 39 and 4 of 94 offspring, respectively (Figure 3, D and E; Figure S6).

A major concern in mutagenesis by designer nucleases is off-target effect, whereby unintended sequences are hap-hazardly cleaved and mutated. It has been shown that Cas9 is capable of cleaving sequences that have multiple mismatches with the gRNA sequence with moderate efficiency (Fu *et al.* 2013; Hsu *et al.* 2013; Pattanayak *et al.* 2013). To estimate the extent of off-target effect in our system, we chose four gRNAs targeting different autosomal genes and tested if they induced unintended sex-linked lethal mutations. For each gRNA, we scored X chromosomes in female offspring of founder animals carrying *nos-Cas9* and U6-gRNA. We found no sex-linked lethal mutations in a total of 766 X chromosomes examined (Table S2), suggesting that off-target mutations that disrupt gene function are rare.

Discussion

Here we have demonstrated that transgenic germline expression of Cas9–gRNA has the capacity to induce targeted mutations with extremely high efficiencies. In addition to the X-linked *white* gene in which mutation leads to a visible phenotype, we demonstrated that our method could indeed be applied to mutating autosomal genes whose mutant phenotypes were unknown. The overall mutation frequencies obtained using our system were much higher than the previously reported procedures based on transient expression of Cas9 and gRNA from injected DNA or RNA: The average mutation frequency of all gRNAs in our study was 57%, while those in the previous studies were 19% (Bassett *et al.* 2013), 29% (Yu *et al.* 2013), and 1% (Gratz *et al.* 2013). It should be noted, however, that direct comparison between the previous studies and ours is difficult as gRNAs with different sequences were used.

We further showed that simultaneous expression of two gRNAs efficiently induced internal deletion between the two target sites. The technique is useful not only for making large deletions but also for targeting very small genes, such as miRNAs, in which no gRNAs can be designed due to sequence constraints. Interestingly, the effect of the simultaneous expression of two gRNAs on mutation frequency was more than additive. While the gRNAs *w-ex3-1* and *w-ex6-1* induced *white* mutants at frequencies of 12 and 57%, respectively, in the male germline (Figure 2B), simultaneous expression of the two resulted in a mutation frequency of 91% (Figure 2D). Thus the double-gRNA approach will also be useful in cases where utmost mutation rates are required.

Differences in the male and female germlines should also be noted from a practical standpoint. Whatever their underlying molecular mechanisms, mutation frequencies in the female germline were often much lower than in the male germline (Figure 2B). In addition, somatic mosaic mutants were frequently produced by maternally deposited Cas9–

gRNA, which would complicate identification of true mutants derived from parental germline mutations. In actual gene-targeting experiments using our system, it is therefore desirable to induce mutations in the male germline.

Recent studies have shown that Cas9 accommodates multiple mismatches in the 20-bp target sequence, raising concerns about off-target effect (Fu *et al.* 2013; Hsu *et al.* 2013; Pattanayak *et al.* 2013). We showed that none of the four gRNAs targeting autosomal genes were able to induce any sex-linked lethal mutations in ~800 gametes examined. Given that the spontaneous mutation rate for sex-linked lethals is 0.2% (Wallace 1970), nonspecific deleterious mutations induced by Cas9 are extremely rare. Nonetheless, it should be borne in mind that possible existence of off-target mutations cannot be entirely excluded. Fortunately, *Drosophila* genetics offers various tools to confirm mutant phenotypes, such as chromosomal deficiencies and transgenic rescue.

Another major application of the designer nuclease technologies is precise modification of the genome using donor templates by homology-directed repair. Gap repair of transposon-induced double-strand breaks has been successfully used for targeted gene replacement (Gloor *et al.* 1991; Keeler *et al.* 1996; Lee *et al.* 2006). More recently, it has been shown that simple injection of a template DNA together with zinc-finger nucleases or Cas9–gRNA induces incorporation of the template sequence into the genome, allowing introduction of single-base-pair changes, deletions with defined breakpoints, and insertion of foreign DNA sequences (Beumer *et al.* 2008; Gratz *et al.* 2013). It would be possible to induce homology-directed repair by injecting a donor template into fertilized eggs that carry the *nos-Cas9* and gRNA transgenes.

Our method requires generation of transgenic lines for each target gene. Although it takes an additional time of at least 1 month compared to direct injection of DNA or RNA encoding Cas9 and gRNA genes, we presume that the high mutation frequency of our method more than compensates for the extra time. Large-scale applications will further benefit from the transgenic approach as the time-consuming injection process can be drastically reduced by pooling multiple plasmid vectors (Bischof *et al.* 2013). Our approach can even obviate the need for microinjection experiments altogether by outsourcing transgenic production, which is currently the standard method of choice in *Drosophila* research.

The extremely high mutagenesis efficiency and the ease with which U6-gRNA vectors and transgenic lines are constructed make our methodology an ideal platform for generating genome-scale mutant resources in *Drosophila*. We and others have successfully generated genome-wide collections of transgenic RNA interference lines and distributed them to the community (Leulier *et al.* 2002; Dietzl *et al.* 2007; Ni *et al.* 2011). Likewise, it is highly feasible to generate transgenic U6-gRNA lines targeting all genes and derive mutants from them in a reasonable time frame.

The transgenic strains and vectors used in the present study will be made available through the NIG-Fly Stock Center (<http://www.shigen.nig.ac.jp/fly/nigfly>). We have set up a dedicated website for our Cas9 method (<http://www.shigen.nig.ac.jp/fly/nigfly/cas9>).

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Highly Improved Gene Targeting by Germline-Specific Cas9 Expression in *Drosophila*

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pBFv-nosP-Cas9

attB: 7-290

vermillion: 469-1962

nos promoter: 2207-3470

Cas9: 3516-7655,

nos 3'UTR: 7677-8641

Amp^R: 9911-10771

>pBFv-nosP-Cas9

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pBFv-U6.2

attB: 7-290

vermilion: 469-1962

U6 promoter: 2207-2605

gRNA: 2627-2707

Amp^R: 3987-4847

>pBFv-U6.2

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attB: 7-290
vermilion: 469-1962
 U6 promoter: 2845-3243
 gRNA: 3265-3345
 Amp^R: 4617-5477

GGTACATCGCAGATGCCCGCGTGCACCGTTCGAGAACCCCGCTGACGCTGCCCGCGGTATCCGCAACCCGCCAGCGCGTTCGACAGTCCCGGTGCTCACCCTGACC
ACCGCGCCCGACCGGTTTCGAGGGGCGAGGGCTTCCCGGTGCGCGCGCGGTTTCGCGGGATCAACATACGCCACCTCGACCCGTTTCATCATGATGGACCCAGAT
GGGTGAGGTGGAGTACGCGCGCGGGGAGGCCAAGGCGACCCCTGCGCCGCGCTTCGACAGCCGTGACCTACATCTGTCGACAGCTTGGATT
TTTTGTTATGTTATATGTTATATATGTCAGACATAAAGAAAAGGAACACATCAAAATGTGATAACAAGACATAAACAGTAATTTTATTACACAAAACGAC
AAAACATAGGCGAGAACAAACAACGCATAGCCAAACATATTGACGAATTTGGATACCTTCCGATTGTCAGACACATTTTGTGTGATCAGTTTCTTTCGGAATGGTC
TCGTCAGAGCGTGGAAATCGCCTCGCGGGGAATCAGAAAAGTGGAACAGATTGAACAGATCCGAGAACACCTTGTACCGTACTGAAACCCAAAACAAAAACAA
AGGGAAGACAGTTTGGATTCTATTGATCCCCGATATAATACATCTCGCATGATACCTTGAGAGTGGAGCGCAGATATTGATATCCAGACGAGCCACAGGT
CCCAACTGTTGGGATCCAATCATGCGTTGCACCATGATCAGTGTATTGTCTCGCGCGGGAATAGAAAGTATTTGGTTAGGAAAACACAGTCTTAAACATAAA
ATATATTTTAAAGAGATTCAAAGAAATGCAATCATCTACATCTCCACTTGGTTATTAAAGAGTCGATGTCCATGAGCAGGGTGAACCACTGGTGGTGTG
TGAACCTGGGTTCATCCCTATAGAAGGTGATCATGATGGCTCCCGAAGGCGCAGTATGGCTTAAACCGGCGATCCCCACGACGCAACAGGTGCATCTGTCGAC
TGC CGGATCAAAGATGGAGCGATACACCTCGCGCTCGCTTCTCAATGTCCATGAGCGGTAGTTTTTTCGCTTCTCCACGGGCTCTCCATGGCGCTCTGTA
CTCGCGCTCGCAGGAATCGATCGACGCTCTCTGAACTTGGCCGAGAAGTTGAAGCCACTCTCTCCAGTCCGGGCTCTCTCCAGCGCATCTGCTGCAT
AGCTCAGTAGCGAGGAGTCTTCTCCGAGTTGCGAATCAGTTCCGCGCCTCTCTGCTGCTTAAAGACATCCGAGTACTTCTGTTGATCTCACCCGCTG
CTCTGTCAGAACTCCACGCTTGTCTCGATCAAACGGAAGTGCAGCGACTGAAAACAGATGCGGGTGCCAGGTACTTGCAGGAGTCCATGAAGTCTAGCG
GGGTCTATGGTCTCGAAGATGGGCACCTTGGTCCACGAGGAGTGTAACAAGAGGATTATAACCGGATTTTGGTAAAGAGTTCAGAAAGCACTACTTTTAGA
ATCAGAACCACTCGTTTCAGTTCGTTGCAACTCTCCAGCTCTGGTTTTCATCATGATCACTTGCATCCAACATGTCTGATGGAGTGAACATCAAAGAT
GATCTGCTTGAACCAAAGCTCGTAGGCTGTGGCGAAGGTACTTAAATGCCATTGAGTGTGTGTCATCAAAGTTGTAACCTACTCACCTGGTGGTGTGATGA
TGAACAGATGCTCATCTGTCGACGGGTGCTGTCTCTCCGACGACATACATGGGCATCCAGAGTTTGTCCAGCATCAGATACTCTCCATGATATTGTC
CCCACTTCCTGGTTTAAATGGCAGCCCGCAATCATCTGATCGTCTTCTGATGGGTTTGAATTCGATTCGAGATCAAGATCGATTGGCATCTCTGTCGAC
CAGCTGCTGGTGTCTCACCCGTTTCTGTCATAGGGACAGCTCATGGTGCACAGCTCAGATCAGATCGTGACTCTCTCGAGCGCGGATGCTGGCGAACTGATC
TCCGCAGCGGACCGGAGATGAGACCCGACGCCAAGCAGTAACGAGCGGACGAGAGCTCCAGTTCCGATGATTCAGTCCAGTCCGTTGATCTGGGCGGCGAC
CTCAGATAGGCTGGGAATGATTAACATCACTGAGGTGAAAGTCGGGCGACCAAACTCAAGCTTGTATCGAATTCAGTCCAGTGGCATTTGGTTGGCAT
CATCTCGGCCATCGTCTTATCTCTCTCGTGGCCTTTGTTACCGGAGCGGCTATGTTGGGCGCGGTGGAGCCCGGAAGCTGAAAGGTTAATCGAATGCGTT
AACTTTTCTGAACCTCGAAGATTTTGCCGCTTTTGTTGCGACTGCCAATAACTGTTGATTGCAAAATTCGAATCGAAGCGGTGAAATTCGTAGGTTGGCCAA
CTACACCAAAGTTTCGCCGCGGTGTACATGCTGTTTTTTTTTTTTTTCGTCGATGAGAAATGGCTCTTAAATCTACTAGATAAAAAAATTTCTATTA
TTTCTATGCTGCTGGAACGCTTCATTAATCTTAAAAATCTAAATCTCGGTTACCAATGAGAACTTCGACGCAACATGTAGATTTTGGATAGAAATTAAGAGAGA
AAATGGCGAGAGAGATAAAATTCGGCGCTCGGCAAGTAGAGCAAAAAAATCAGTATACCAATTTAGCTACCTCTCTCACTCGCACGCACTGCGCGCTCAAGT
TGGCGCGGCTCTGCAATTCATGATTTCTTGGGGTGTGTAACATATCATCTCGTTTTCCTCTCTCTCACTCCACAGCGTGAAGCGCGCGGATCCACTGA
GTTCTAGAGCGCGCGCTTCGACTTGCAGCTCGAATACGGCACAGTAGGAAAAGCGGAGTCAAAATGCCGAATGCAGAGTCTCATTCAGACCAATCAACT
CAAGAAAACCTTCGACACTTTTTTACCAATTTGCACTTTAACTCTTTTTTATTCTGTTATGTATGATCTTTTTTGGTCCCTAACCAAAACAAACCAAACTCTC
TAGTCTGCTCTCTATATTAAAAACTATCAATTTATATAGCTAAATAACGAACTGTGTTTTCAACAAACGAACATAGGACACTTTGATTTCTAAAGGAAA
TTTTGAAATCTTAAGCAGAGGGTTCTTAAGACATTTGCCAATCTTATAATTTCAACTGCTCTTCTCTGATTTGATCATTATATAGGTTATGTTTTTCT
CTCAATACTTCTCGGTTCTTCGAGTTGAAGACTGTTTTTAGAGCTAGAAATAGCAGATTAAAAATAGGCTAGTCCGTTATCAACTTGA AAAAGTGGCACCAG
TCGGTCTCTTTTTTGCATGCACTTGGCTTTCCAGCTTTGATTCGATTTCTTCCGTCATGAGTCCAGCTCCAGCTTTGTTCCCTTTAGTGGGGTTAGT
TGGCGCTTTGGCGTAATCATGTGCTATAGTGTCTTTCTGTTGAAATTTCTTATCCGCTCACAATTCACACAACATACGAGCGGGAAGCATAAAGTGAAG
CCTGGGGTGCCTAATGAGTGAGCTAATCTACATTAATTCGTTTGGCTTGCCTCACTGCCCGCTTTCCAGTTCGGGAACCTGTGCTGCCAGCTGCATTAATGAATC
GGCTAACCGCGGGGAGAGGCGGTTTTCGCTATTGGGCGCTCTTCCGCTTCTCTGCTCACTGACTCGTCTGCGCTCGGTCGTTCTGGCTGCGGCGAGCGGTAA
AGCTCACTCAAAGCCGTAATACGTTTATCACAGAAATCAGGGATAACGAGAAAGAACATGTGAGCAAAAGGCGACAAAAGGCCAGGAACCTGATAAA
AGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAA
AGATACACCGGCTTTCCCCCTGGAAGACTCCTCTCGCGCTCTCTGTTCCGACCTCGCCCTTACCGGATACCTGTCGCGCTTTCTCCTCTCGGGAAGCGGT
GGCGCTTTCTCATAGCTACGCTGTAGGTATCTCAGTTTCGTTGAGTGTCTGCTCTGCTCAACGCTGGGCTGTGTGTCGACGAAACCCCGCTTACGCCAGCGCT
GCGCTTATCCGGTAACATATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTAT
GTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCTTAACATCGGCTACATAGAGAGCAGATTGTTGGTATCTGCGCTCTGCTGGAAGCGAGTTACCTCGG
AAAAAGAGTTTGGTGAATCTTTGATCCCGCAAAACCAACCCGCTGGTAGCGGTGTTTTTTTTTTTTCGACGAGCAGATTACGCGCAGAAAAGGAGGATCT

AAGAAGATCCTTTGATCTTTCTACGGGGTCTGACGCTCAGTGGAACGAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACC
 TAGATCCTTTTAAATTAAAAATGAAGTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTAT
 CTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAA
 TGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACAGCCAGCCGGAAGGGCCGAGCGCAGAAAGTGGTCTGCAACTTTATCCGCC
 TCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC
 ACGCTCGTCGTTTGGTATGGCTTCATTTCAGTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
 GTCCTCCGATCGTTGTGCAAGTAAGTTGGCCGCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGC
 TTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGCGTCAATACGGGATAATACCGCGCC
 ACATAGCAGAACTTTAAAGTGCTCATCATTTGGAAAACGTCTCTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCA
 CTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCG
 ACACGGAATGTTGAATACTCATACTCTTCTTTTCAATATTATGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA
 GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTAAAAATTCGCGTTAAATTTTGTTA
 AATCAGCTCATTTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGAACA
 AGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCTAATCAAGTTTT
 TTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCTTAAAGGGAGCCCCGATTTAGAGCTTGACGGGAAAGCCGGCGAAGTGGCGAGAAAGGAAGG
 GAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTACGCTGCGCGTAACCAACACACCCGCGCGCTTAATGCGCCGCTACAGGGCG
 CGTCCCATTGCGCAATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGATGTGCTGCAAGG
 CGATTAAAGTTGGGTAACGCCAGGGTTTTCCAGTACAGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTG

Figure S1 Plasmid maps and sequences. Full sequences of the germline Cas9 expression vector pBFv-nosP-Cas9 and the gRNA expression vectors pBFv-U6.2 and pBFv-U6.2B are shown along with their maps. Useful restriction enzyme sites are underlined.

1. Identify a targetable 20-bp sequence

Genome sequence (*white* locus)

```

... GGCCTATCCGGGCGAACTTTTGGCCCTGATGGGCAGTTCCGGTGC PAM CGGAAAAGACGACCCCTGCTGAATGCCCTT...
      |||
... CCGGATAGGCCCGCTTGAAAACCGGCAC TACCCGTC AAGGCCACG GCC TTTCTGCTGGGACGACTTACGGGAA...

```

2. Design oligonucleotides

w-ex3-3-F: CTTTCGTGATGGGCAGTTCCGGTGC

w-ex3-3-R: AAACGCACCGGAAGTCCCCATCAC

3. Anneal oligonucleotides

```

CTTCGTGATGGGCAGTTCCGGTGC
      |||
GCACCGGAAGTCCCCATCACAAA

```

4. Digest vector and clone annealed oligos

Figure S2 Overview of the cloning strategy.

w-ex3-2

```
AACTCAGTTTGC GGCGTGG CCTATCCGGGCGAACTTTTGGCCGTGATGGG Wild type
AACTCAGTTTGC GG-----GCGAACTTTTGGCCGTGATGGG -14
AACTCAGTTTGC GGCGTGG-----GCGAACTTTTGGCCGTGATGG -10
AACTCAGTTTGC GGCGTGC-----GGGCGAACTTTTGGCCGTGATGG -8
AACTCAGTTTGC GGCGTGGCCTA----GCGCGAACTTTTGGCCGTGATGG -4
AACTCAGTTTGC GGCGTGGCGg----CGGGCGAACTTTTGGCCGTGATGG +1,-5
AACTCAGTTTGC GGCGTGGCG gacttgCGGGCGAACTTTTGGCCGTGATGG +6,-7
```

w-ex3-3

```
TTTTGGCC GTGATGGGCAGTTCCGGTGCCGGAAAGACGACCCTGCTGAAT Wild type
TTTTGGC-----AAAGACGACCCTGCTGAAT -24
TTTTGGCCG-----GAAAGACGACCCTGCTGAAT -21 [x2]
TTTTGGCCGTGATGGGCAG-----ACGACCCTGCTGAAT -16
TTTTGGCCGTGATGGGCAGT-----GCCGAAAGACGACCCTGCTGAAT -6 [x2]
TTTTGGCCGTGATGGGCAGTTCCG-----AAAGACGACCCTGCTGAAT -6 [x2]
```

w-ex3-4

```
CCGGTGCCGGAAGACGAC CCTGCTGAATGCCCTTGCCTTTCGATCGCCG Wild type
CCGGTGCCGGAAGACGACCTTGC-----CTTTCGATCGCCG -13 [x3]
CCGGTGCCGGAAGACGACCCT-----TGCCTTTCGATCGCCG -12
CCGGTGCCGGAAGACGACCCTGC-----CCTTGCCTTTCGATCGCCG -7 [x2]
CCGGTGCCGGAAGACGACCCTGC aag--GCCCTTGCCTTTCGATCGCCG +3,-5
CCGGTGCCGGAAGACGACCCTGCT ttgacctgCGCCCTTGCCTTTCG +10,-3
```

w-ex3-5

```
TGCCCTTGCCCTTTCGATCG CCGCGAGGCATCCAAGTATCGCCATCCGGGA Wild type
TGCCCTTGCCCTTTCG-----GA -33
TGCCCTTGCCCTTTCGATCGCCGCA----TCCAAGTATCGCCATCCGGGA -5 [x3]
TGCCCTTGCCCTTTCGATCGCCGAGG--CATCCAAGTATCGCCATCCGGGA -1
TGCCCTTGCCCTTTCGATCGCCGAG tatcaaGCATCCAAGTATCGCCATC +6,-1
TGCCCTTGCCCTTTCGATCGCCG gatccgATCCAAGTATCGCCATCCGGG +6,-5
```

w-ex6-1

```
GCCGGAGTGCTGCACTTCTTCAACTGCC TGGCGCTGGTCACTCTGGTGGC Wild type
GCCGG----- -153
GCCGGAGTGCTGCACTTCTTCAACTG----GCGCTGGTCACTCTGGTGGC -4
GCCGGAGTGCTGCACTTCTTCA---GCCTGGCGCTGGTCACTCTGGTGGC -3
GCCGGAGTGCTGCACTTCTTCAA--TGCTGGCGCTGGTCACTCTGGTGGC -1 [x2]
GCCGGAGTGCTGCACTTCTTCAg--GCCTGGCGCTGGTCACTCTGGTGGC +1,-3
GCCGGAGTGCTGCACTTCT gc--CTGCCTGGCGCTGGTCACTCTGGTGGC +2,-4
GCCGGAGTGCG ggggggacca-----GCCTGGCGCTGGTCACTCTGGTGGC +10,-15
```

Figure S3 Cas9-induced mutations at the *white* locus. The *white* locus in Cas9-induced mutants was PCR-amplified and sequenced. At least six mutants were examined for each gRNA. The wild-type sequence is shown at the top of each set of sequences as a reference. The Cas9-gRNA target sequence is underlined with the PAM indicated in green. Deleted nucleotides are shown as dashes. Inserted nucleotides are indicated in blue lowercase letters. The indel size and the number of occurrences are shown next to each sequence.

>#2
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGGCGAA---[Δ35bp]---AGACGACCCTGCTGAATGCCCTTGCCCTTTCGATCGCCGAGGGCATCCAAGTATCGCCATCCGGGAa
 ga---[Δ1497bp]---CTGGCGCTGGTCACTCTGGTGGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCAGCTCCTCGA
 CCTCGATGGCGCTGTCTGTGGGTCCGCCGTTATCATACCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTAT

>#3
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGGCGAACTTTTGGCCGTGATGGGCAGTTCCGGTGCCGAAAGACGACCCTGCTGAATGCCCTTGCCCTTTCGATCGCCGAGGGCAT
 CCAGTAATCGCCATCCGGGATGCGACTGCTCAATGGCCAACCTGTGGACGCCAAGGAGATGCAGGCCAGGTGCGCCTATGTCCAGCAGGA
 TGACCTCTTTATCGGCTCCCTAACGGCCAGGGAACACCTGATTTTCCAAGCCATGGTGGCGATGCCACGACATCTGACCTATCGGCAGCG
 AGTGGCCCGCTGGATCAGGTGATCCAGGAGCTTTTCGCTCAGCAAATGTCAGCACACGATCATCGGTGTGCC---[Δ1265bp]---TG
 GCGCTGGTCACTCTGGTGGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCAGCTCCTCGACCTCGATGGCGCTGTCTGTG

>#4
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGT[GGCCT
 ATCCGGGCGAAaaa---[Δ949bp]---TGGCTGTGGTGCTCAAGGAACCACTCCTCGTAAAAGTGCGACTTATTAGACAACGGTGAG
 TGGTTCAGTGGAACAATGATATAACGCTTACAATTTTGGAAACAAATTCGCTAGATTTTAGATAGAATTGCCTGATTCCACACCCT

>#5
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGGCG---[Δ1600bp]---CTGGCGCTGGTCACTCTGGTGGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCAGCTC
 CTCGACCTCGATGGCGCTGTCTGTGGGTCCGCCGTTATCATACCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCC

>#6
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGG---[Δ1658bp]---TCACCTCTGGTGGCCAATGTGTCAACG
 TCCTTCGGATATCTAATATCCTGCGCCAGCTCCTCGACCTCGATGGCGCTGTCTGTGGGTCCGCCGTTATCATACCATTCCTGCTCTTT

>#7
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGGCGAT---[Δ689bp]---AGCGTGGGTGCCAGTGTCTTACCAACTACAATCCGGCGGACTTTTACGTACAGGTGTGGCCGTT
 GTGCCCCGACGGGAGATCGAGTCCCGTGATCGGATCGCCAAGATATGCGACAATTTTGCCATTAGCAAAGTAGCCCCGGGATATGGAGCAG

>#8
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGGCG---[Δ1600bp]---CTGGCGCTGGTCACTCTGGTGGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCAGCTC
 CTCGACCTCGATGGCGCTGTCTGTGGGTCCGCCGTTATCATACCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCC

>#9
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCC---[Δ1722bp]---TGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTATACCTCAAATGGTTGTCTGACCTCTCAT
 GGTTCGGTTACGCCAACGAGGGTCTGCTGATTAACCAATGGGCGGACGTGGAGCCGGGCGAAATTAGCTGCACATCGTCAACACACGT

>#10
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGGCGAACTTTTGGCCGTGATGGGCAGTTCCGGTGCCGAAAGACGACCCTGCTGAATGCCCTTGCCCTTTCGATCGCCGAGGGCAT
 CCAAGTATCGCCATCCGGGATGCGACTGCTCAATGGCCAACCTGTGGACGCCAAGGAGATGCAGGCCAGGTGCGCCTATGTCCAGCAGGA
 TGACCTCTTTATCGGCTCCCTAACGGCCAGGGAACACCTGATTTTCCAAGCCATGGTGGCGATGCCACGACATCTGACCTATCGGCAGCG
 AGTGGCCCGCTGGATCAGGTGATCCAGGAGCTTTTCGCTCAGCAAATGTCAGCACACGATCATCGGTGTGCCCGGAGGGTGAAg---[Δ
 1254bp]---TGGCGCTGGTCACTCTGGTGGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCAGCTCCTCGACCTCGATG
 GCGCTGTCTGTGGGTCCGCCGTTATCATACCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGGTGCCAGTATACCTCAA

>#11
 GTGAGTTTCTATTTCGCAGTCGGCTGATCTGTGTGAAATCTTAATAAAGGGTCCAATTACCAATTTGAAACTCAGTTTGCGGCGTGGCCTA
 TCCGGcgctggata---[Δ1605bp]---GCGCTGGTCACTCTGGTGGCCAATGTGTCAACGTCCTTCGGATATCTAATATCCTGCGCCA
 GCTCCTCGACCTCGATGGCGCTGTCTGTGGGTCCGCCGTTATCATACCATTCCTGCTCTTTGGCGGCTTCTTCTTGAACTCGGGCTCGG

Figure S4 Breakpoint sequences of deletions induced by simultaneous cleavage of two sites in the *white* locus. Sequences on the left and right sides of the cleavage sites are shaded in yellow and green, respectively. Inserted nucleotides are shown lowercase. Deletion sizes are shown in brackets.

Ast locus (6/8)

ATAGCATCACAATGAATCCCTTCACGCCACCTCCTACTGCTTGGCAGTT Wild type
ATAGCATCACAATGAATCCCTTC-----TGCTGGCA -18
ATAGCATCACAATGAATCCCTTCA-----GCTGGCAGTT -15
ATAGCATCACAATGAATCC-----ACCTCCTACTGCTGGCAGTT -10
ATAGCATCACAATGAATCCCACC-----TCCTACTGCTGGCAGTT -9
ATAGCATCACAATGAATCCCTTgaACGCCACCTCCTACTGCTGGCAGT +2,-1
ATAGCATCACAATGAATCCCTTCACtgcctggCACCTCCTACTGCTGGCA +6,-3

capa locus (1/6)

CAGAGACGGGACCACGACAAGAACCGACGAGGTGCCAACATGGGGCTCTAT Wild type
CAGAGACGGACCACGA-----GGTGCCAACATGGGGCTCTAT -13

Ccap locus (1/8)

GCACCTCTGGCTTGCGCCATTGCTCTCAGGCTTCGCTGGAAAGGGAGAA Wild type
GCACCTCTGGCTTGCGCA-----GGAAAGGGAGAA -19

Crz locus (5/8)

TCTGCCGAAACATGTTGCGCCTCCTGCTGCTGCCCTCTTCCTCTTCACGCTCTCCATGTG Wild type
TCTGCCGAAACATGTTGCGCCTCC-----ATGTG -32
TCTGCCGAAACATGTTGCGCCTC-----TCCTCTTCACGCTCTCCATGTG -15
TCTGCCGAAACATGTTGC-----TGCCCTCTTCCTCTTCACGCTCTCCATGTG -12
TCTGCCGAAACATGTTGCGCCTCCTGC-----CCCTCTTCCTCTTCACGCTCTCCATGTG -6
TCTGCCGAAACATGTTGCGCCTCCTGCTGC---CCCTCTTCCTCTTCACGCTCTCCATGTG -3

Eh locus (8/8)

CATTTTGGAAATGCATTGCGCCGCATAAGTCATTATACGCACAAAGAGATT Wild type
CATTTTGGAAATGCATTGCCCC-----ATTATACGCACAAGAGATT -10
CATTTTGGAAATGCATTGCCCCGCAT-----TATACGCACAAGAGATT -7
CATTTTGGAAATGCATTGCCCCGCATta-----TATACGCACAAGAGATT +2,-7
CATTTTGGAAATGCATTGCCCCGCATaAGTCATTATACGCACAAGAGA +2
CATTTTGGAAATGCATTGCCCCattgcattGTCATTATACGCACAAGAGAT +8,-7
CATTTTGGAAATGCATTGCCCCGCATttattatCATTATACGCACAAGAG +6,-3
CATTTTGGAAATGCATTGCCCCGCAGtcataagtccgcaTAAGTCATTAT +12

Mip locus (6/8)

ACTAAGACGCGGAGAACTTACGGCTTTCTGATGGTGCTCCTCATCCTGGG Wild type
ACTAAGACGCGGAGAACTTACGG-----TGCTCCTCATCCTGGG +11
ACTAAGACGCGGAGAACTTACGGCTTT-----GCTCCTCATCCTGGG +8
ACTAAGACGCGGAGAACTTACGGCT-----GGTGCTCCTCATCCTGGG +7
ACTAAGACGCGGAGAACTTACc-----ATGGTGCTCCTCATCCTGGG +1,-9
ACTAAGACGCGGAGAACTTACGGCTTTacgGATGGTGCTCCTCATCCTGG +3,-1
ACTAAGACGCGGAGAACTTACGGCTTTcatggtGGTGCTCCTCATCCTG +6,-4

npf locus (5/8)

TTGCCTGTGTGGCCCTTGCCCTCCTAGCCGCCGGCTGCCGAGTGAGGCG Wild type
TTGCCTGTGTGGCCCTTGCCCTC-----GCCGGCTGCCGAGTGAGGCG -6
TTGCCTGTGTGGCCCTTGCCCTCCT---CGCCGGCTGCCGAGTGAGGCG -3 [x2]
TTGCCTGTGTGGCCCTTGCCCTaa---CCGCCGGCTGCCGAGTGAGGCG +2,-6
TTGCCTGTGTGGCCCTTGCCCTCCTAGCgccc-GCTGCCGAGTGAGGCG +4,-5
TTGCCTGTGTGGCCCTTGCCCTCCTAGgctCCGCCGGCTGCCGAGTGAGG +3

Figure S5 Sequences of Cas9-induced mutations in neuropeptide genes. Number of identified mutations/total number of animals sequenced is shown next to each locus name. Symbols and colors are the same as in **Figure S3**.

mir-219 locus
 CGGGGCCAGGATGAAATAGCTTGCGCCGCGG (367bp) CCGAATGAACATCGTACTGCTGCCTTAAAT Wild type
 CGGGGCCAGGATGAAATAGCTT**ac**-----GAACATCGTACTGCTGCCTTAAAT
 CGGGGCCAG-ATGAAATA-----GAACATCGTACTGCTGCCTTAAAT
 CGGGGCCAGGATGAAATAGCTTGC-----TGCCTTAAAT
 CGGGGCCAGGATGAAATAGCTTGC**tg**-----ATGAACATCGTACTGCTGCCTTAAAT [x2]
 CGGGGCCAGGATGAAATAG**gccaggata**-----GAACATCGTACTGCTGCCTTAAAT
 CGGGGCCAGGATGAAATAGCTT**cgtacatcgatgtacgatgaata**-----GAACATCGTACTGCTGCCTTAAAT

mir-315 locus
 TGT**GTTAAGCCATTGGCTAAGTTTGG** (129bp) **GATTTCAAAGCACTTCGTTTGG**TAAGTTAAGATTTA Wild type
 TGTGTTAAGCCATTGGCT-----TTGGTAAGTTAAGATTTA
 TGTGTTAAGCC**tttggtataacttacca**-----TTTGGTAAGTTAAGATTTA

Figure S6 Breakpoint sequences of Cas9-induced deletions at *miRNA* loci. Wild-type sequences around the two target sites are shown at the top, followed by junction sequences of individual deletions. Symbols and colors are the same as in **Figure S3**.

Table S1 Oligonucleotides used in this study

Oligonucleotides used to construct gRNA expression vectors

Target locus	gRNA ID	Top oligonucleotide	Bottom oligonucleotide
<i>white</i> exon 3	<i>w-ex3-1</i>	CTTCGGCCTATCCGGGCGAACTTT	AAACAAAGTTCGCCCGGATAGGCC
<i>white</i> exon 3	<i>w-ex3-2</i>	CTTCGGCCAAAAGTTCGCCCGGAT	AAACATCCGGGCGAACTTTTGCC
<i>white</i> exon 3	<i>w-ex3-3</i>	CTTCGTGATGGGCAGTTCCGGTGC	AAACGCACCGGAAGTGGCCATCAC
<i>white</i> exon 3	<i>w-ex3-4</i>	CTTCGAAAGGCAAGGGCATTTCAGC	AAACGCTGAATGCCCTTGCCCTTC
<i>white</i> exon 3	<i>w-ex3-5</i>	CTTCGGCGATACTTGGATGCCCTG	AAACAGGGCATCCAAGTATCGCC
<i>white</i> exon 6	<i>w-ex6-1</i>	CTTCGCTGCACCTCTTCAACTGCC	AAACGGCAGTTGAAGAAGTGCAGC
<i>mir-219</i>	<i>mir219-1</i>	CTTCGGATGAAATAGCTTGCGCCG	AAACCGGCGCAAGCTATTTTCATCC
<i>mir-219</i>	<i>mir219-2</i>	CTTCGCAGCAGTACGATGTTTCATT	AAACAATGAACATCGTACTGCTGC
<i>mir-315</i>	<i>mir315-1</i>	CTTCGTTAAGCCATTGGCTAAGTT	AAACAACCTAGCCAATGGCTTAAC
<i>mir-315</i>	<i>mir315-2</i>	CTTCGATTTCAAAGCACTTCGTTT	AAACAAACGAAGTGCTTTGAAATC
<i>Ast</i>	<i>Ast-1</i>	CTTCGCAGTAGGAGGTGGGCGTGA	AAACTCACGCCCACCTCCTACTGC
<i>capa</i>	<i>capa-1</i>	CTTCGACCACGACAAGAACCGACG	AAACCGTCGGTTCTTGCTGCTGGTC
<i>Ccap</i>	<i>Ccap-1</i>	CTTCGGCTTGTGCCATTTGCTCTC	AAACGAGAGCAAATGGCACAAGCC
<i>Crz</i>	<i>Crz-1</i>	CTTCGGAAGAGGGGCGAGCAGCAGG	AAACCCTGCTGCTGCCCCCTCTCC
<i>Eh</i>	<i>Eh-1</i>	CTTCGTGCGTATAATGACTTATGG	AAACCCATAAGTCATTATACGCAC
<i>npf</i>	<i>npf-1</i>	CTTCGCCCTTGCCCTCCTAGCCGC	AAACGCGGCTAGGAGGGCAAGGGC
<i>Mip</i>	<i>Mip-1</i>	CTTCGAGAAGTTACGGCTTTCTGA	AAACTCAGAAAGCCGTAAGTTCTC

Oligonucleotides used for T7 endonuclease I analysis and DNA sequencing.

Target locus	Primer ID	Primer sequence
<i>white</i> exon 3	<i>w-ex3-CHK-F</i>	CAGAGCTGCATTAACCAGGGCTTCG
	<i>w-ex3-CHK-R</i>	GTTAGAGCCTCGGAGGCGAATGCCAG
<i>white</i> exon 6	<i>w-ex6-CHK-F</i>	GGTTGCCATCTTGATTGGCCTCATC
	<i>w-ex6-CHK-R</i>	GAGAAGTTAAGCGTCTCCAGGATGAC
<i>mir-219</i>	<i>mir219-CHK-F</i>	CATCCCACTGCTGGGTGTGTTTGAC
	<i>mir219-CHK-R</i>	CTGACACTGATGGTAATCGAGATG
<i>mir-315</i>	<i>mir315-CHK-F</i>	CCAGGACTCTCATTGGTCGCCATCG
	<i>mir315-CHK-R</i>	CGATTGAATTTTCAGTGAGTAGTGG
<i>Ast</i>	<i>Ast-CHK-F</i>	CGCTAGAAGGTACGCATTAGGGTGG
	<i>Ast-CHK-R</i>	CCCAGTCCGAAGGAGTAGGGACGAG
<i>capa</i>	<i>capa-CHK-F</i>	CTTAAAGCACTTCTTGAAGAGGCATGC
	<i>capa-CHK-R</i>	CAGCACCTGTTGCAGAGCCAGAAGGTG
<i>Ccap</i>	<i>Ccap-CHK-F</i>	CACTCGGCAAGGGCAAGGACCATCC
	<i>Ccap-CHK-R</i>	GAGGGATACGTACGCTTTCGTCCAC
<i>Crz</i>	<i>Crz-CHK-F</i>	GCAGCCAGCTGTCGTTGGTACAAGG
	<i>Crz-CHK-R</i>	CGTTGGAGCTGCGATAGACAGCTGG
<i>Eh</i>	<i>Eh-CHK-F</i>	GTGTCGACTTGTCTCGCATCTGCGAC
	<i>Eh-CHK-R</i>	CACTGGACGCAGTTGTTAAGGCACAC
<i>npf</i>	<i>npf-CHK-F</i>	CGGTAATATGTGTGTACGTATTTGG
	<i>npf-CHK-R</i>	GAGTTAGTGACGTTGCCATGGTCGTC
<i>Mip</i>	<i>Mip-CHK-F</i>	CATTCCGCGAGTGCGGTTGTGCTGGC
	<i>Mip-CHK-R</i>	GTGCCATCCGTGATTACCAGCGGAAC

Table S2 Frequency of f-target lethal mutations on the X chromosome. See Materials and Methods for details on the experimental procedures.

gRNA	Number of F1 parents examined	Number of sex-linked lethals
<i>capa-1</i>	196	0
<i>Ccap-1</i>	184	0
<i>Mip-1</i>	194	0
<i>npf-1</i>	192	0