Fig. S1. Off-target analysis of w1 and w2 sgRNAs by sequencing. Representative sequencing results of the region spanning the potential off-target sites are shown. Five sites each for w1 (A) and w2 (B) sgRNA were tested, and no obvious double peaks were observed.
Fig. S2. Visual display of sgRNA designs. All potential CRISPR designs were extracted from genome sequences and uploaded to JBrowse for visual display. Shown are screenshots from the JBrowse-based online tool. At the top left, the user can click to view options and select specific tracks to appear. Clicking on a specific sgRNA design (green bars) brings up a detailed information page for that design, including off-target potential. To view designs for another gene, the user can scroll or zoom to another location (top center, arrows and + or – signs) or enter a new FBgn, CG number, gene symbol, or genome position (top center, text box).
Fig. S3. Experimental design for application of the optimized *Drosophila* Cas9/sgRNA system to generate targeted gene mutation collections. A series of sgRNAs were designed to target endogenous loci. The synthetic 20 nt-sequences were cloned into the sgRNA plasmid, and each sgRNA plasmid was injected either into \((\text{nos-Cas9})\text{attP40}\) embryos if the locus was not on chromosome 2 or into \((\text{nos-Cas9})\text{attP2}\) embryos if the targeted locus was not on chromosome 3. We estimated that injection of 20 embryos should yield between three and five deletion lines. G0-injected flies were crossed to a balancer stock. Two or three G0 flies should produce mutant progeny, and two F1 progeny from each of these G0 crosses should yield at least one deletion line. Removal of the Cas9 transgene can be readily detected by the absence of red eye color marker encoded by the *vermillion* gene.
Fig. S4. (Continued)
Fig. S4. Full-length sequence of the nos-Cas9 plasmid. The key components of the plasmid are color-coded, with the vermillion reporter gene in magenta, the attB site in brown, the nos promoter 5' UTR and 3' UTR in cyan, and the Cas9 gene in green.
Fig. S5. Partial sequences of the six different sgRNA constructs used in this study: U6a-sgRNA-short (A), U6b-sgRNA-short (B), nos-minisgRNA-short (C), U6a-sgRNA-long (D), U6b-sgRNA-long (E), and nos-minisgRNA-long (F). The sgRNA scaffolds are shown in cyan, the regulatory sequences in green, and the BbsI cloning sites in yellow. The sgRNA targeting sequences are inserted into the BbsI cloning site. The red-colored sequences are for convenience of cloning procedure and are removed after BbsI digestion.

Other Supporting Information Files

Table S1 (DOC)
Table S2 (DOC)
Table S1. HRMA analysis data for w1 and w2 on-target sites using genomic DNA prepared from G0 injected embryos or off-target sites using genomic DNA from F1 adult flies.

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2 Replicate: technical replicates of HRM assay
3 Area: the area between the sample melting curve and a null curve generated from control sample data
4 p-value: indicating the probability likelihood that samples curves are significantly different to control curves.
Differences between melting curves can be due to changes in template sequence or differences in reaction conditions such as salt concentrations. Therefore, samples were considered as mutants only when all replicates generated p-values <0.01.
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<td>HRM His2av right</td>
<td>GGCCTAGTGGTGTCACCTTTC</td>
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<tr>
<td>Potential off-target sites of w2 sgRNA (for sequencing)</td>
<td>Nrg-OT-F</td>
<td>ccggaattcGTAAAGTGAGGAAATCGGTGCAGC</td>
</tr>
<tr>
<td></td>
<td>Nrg-OT-R</td>
<td>ttgctctagaCCTCAACCGGATGTCGTC</td>
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<td>Vm26Ac-OT-F</td>
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<td>ttgctctagaTGGAAGCCTCGATCGTTTG</td>
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<td>CTPsyn(w2)-OT-F</td>
<td>ccggaattcCAGAAGGAGACTGGGAGTTCG</td>
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<td>CG1907-OT-R</td>
<td>ttgctctagaCGTGGAATTCCCTCCACAGC</td>
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<tr>
<td>Potential off-target sites of <em>w2</em> sgRNA (for HRM)</td>
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<tr>
<td>fru-OT-F</td>
<td>ceggaattcAATATGGCAAGCGAGTGCTTC</td>
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</tr>
<tr>
<td>fru-OT-R</td>
<td>tgtcttagAAGCGGAAATGTCTCAAAAGC</td>
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<tr>
<td>HRM Nrg left</td>
<td>ATACTCCGGAGGGTGATACCA</td>
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</tr>
<tr>
<td>HRM Nrg right</td>
<td>TTCTTCCAGTGAGCATGAA</td>
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<tr>
<td>HRM Vm26Ac left</td>
<td>GGCAAGCTGGAGAAGGAACT</td>
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<tr>
<td>HRM Vm26Ac right</td>
<td>CAGCTACGACCAGGATGAGG</td>
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<tr>
<td>HRM CTPsyn left</td>
<td>CATTCAAGGAATGGGTGGAG</td>
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<tr>
<td>HRM CTPsyn right</td>
<td>ATGCTTTCGATGTCAACCAAT</td>
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<tr>
<td>HRM CG1907 left</td>
<td>TGGGCATGTACACGTATCTGA</td>
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<tr>
<td>HRM CG1907 right</td>
<td>CAATAAAGGAGCCCACAGG</td>
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<tr>
<td>HRM fru left</td>
<td>ACACCTGAGAAAGCCAGTG</td>
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<td>CAAGCGCAGCTGAACAAGCT</td>
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<tr>
<td>ftz-R</td>
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