

Supporting Information

Ren et al. 10.1073/pnas.1318481110

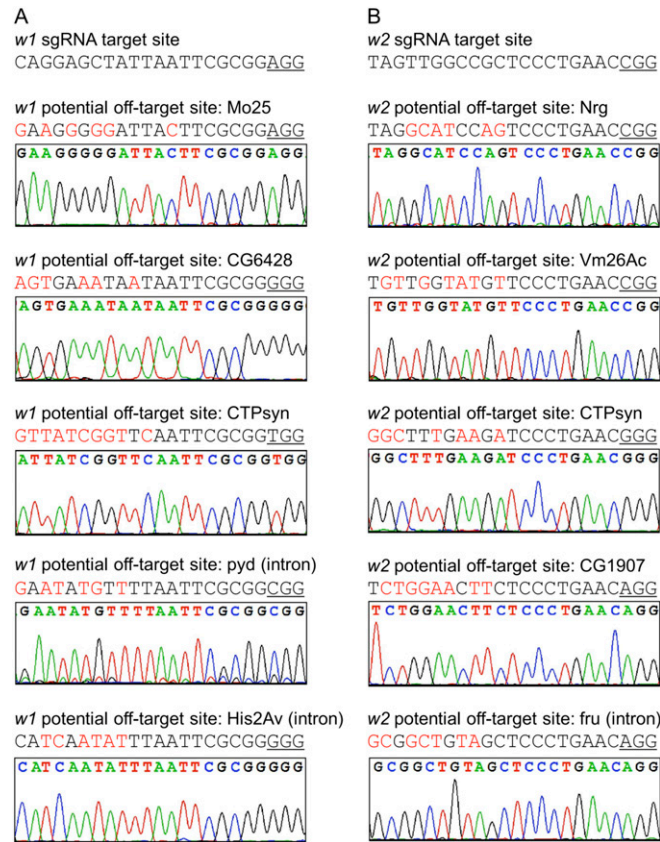


Fig. S1. Off-target analysis of w1 and w2 sgRNAs by sequencing. Representative sequencing results of the region spanning the potential off-target sites are shown. Five sites each for w1 (A) and w2 (B) sgRNA were tested, and no obvious double peaks were observed.

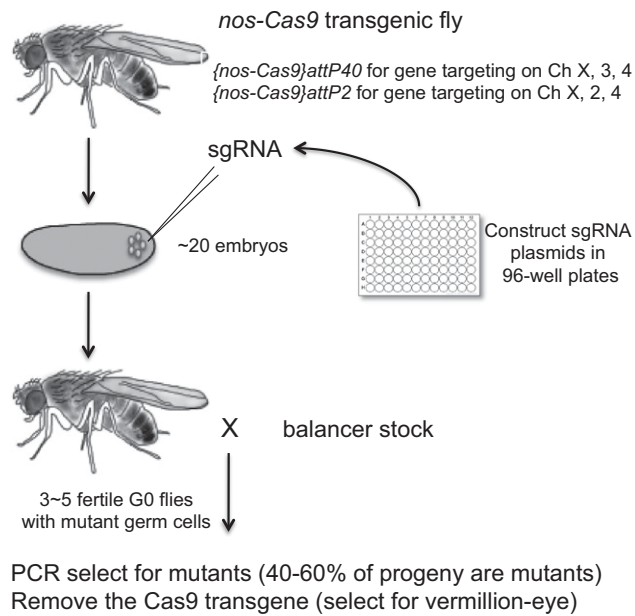


Fig. S3. Experimental design for application of the optimized *Drosophila* Cas9/sgRNA system to generate targeted gene mutation collections. A series of sgRNAs were designed to target endogenous loci. The synthetic 20 nt-sequences were cloned into the sgRNA plasmid, and each sgRNA plasmid was injected either into {*nos-Cas9*}*attP40* embryos if the locus was not on chromosome 2 or into {*nos-Cas9*}*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.

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TCTCCCTTCGGGAAGCGTGGCGTTCCTCATAGCTCAGCTGTAGTATCTCAGTTTCGGTGTAGGTGTTCTGCCTCCAGCTGGGCTG
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CGGGCTCTCGCTATTACGCCAGTGGCGAAAAGGGGATGT

Fig. 54. 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.

A.

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B.

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C.

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GC

D.

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Fig. 55. (Continued)

E.
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F.
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AGGAAACAATGTCCTCTTCG

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 *Bbs* I cloning sites in yellow. The sgRNA targeting sequences are inserted into the *Bbs* I cloning site. The red-colored sequences are for convenience of cloning procedure and are removed after *Bbs* I 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.

<i>w1</i> on-target Sample ¹	Replicate ²	Area ³	p-value ⁴
1	1	0.834547	0.002135797
1	2	1.186813	6.67E-09
1	3	1.190249	5.67E-09
1	4	1.519788	<E-20
2	1	1.029308	4.96E-06
2	2	0.880238	0.000632709
2	3	1.639626	<E-20
2	4	0.959659	5.66E-05
3	1	1.150081	3.58E-08
3	2	1.618299	<E-20
3	3	2.082795	<E-20
3	4	1.987664	<E-20
4	1	1.492092	2.22E-16
4	2	1.986695	<E-20
4	3	1.981135	<E-20
4	4	2.31944	<E-20
5	1	0.260193	0.040266397
5	2	0.53769	0.316584971
5	3	0.868042	0.000886232
5	4	0.325677	0.110745215
6	1	1.573759	<E-20
6	2	1.765667	<E-20
6	3	1.929172	<E-20
6	4	1.739432	<E-20
7	1	0.379689	0.214903639
7	2	0.626037	0.117888211
7	3	0.38999	0.239802623
7	4	0.629644	0.112272182
8	1	0.400438	0.266587007
8	2	0.567553	0.236779204
8	3	0.404778	0.278140964
8	4	0.313748	0.09371609
9	1	0.319604	0.101816621
9	2	0.247821	0.032388428
9	3	0.794189	0.00564176
9	4	3.268847	<E-20
10	1	0.518309	0.373771121
10	2	2.584695	<E-20
10	3	0.621754	0.12481009
10	4	0.590012	0.184918956

11	1	0.385953	0.229862108
11	2	0.293296	0.06914424
11	3	0.342194	0.137823386
11	4	0.185113	0.009397499
12	1	0.60772	0.149459866
12	2	0.710507	0.031240773
12	3	0.419151	0.318058613
12	4	0.547516	0.289085401
13	1	0.166759	0.00626728
13	2	0.416422	0.310294924
13	3	0.293316	0.06916587
13	4	0.68931	0.045235801
14	1	0.312713	0.092334504
14	2	0.626416	0.117288842
14	3	0.368425	0.189474259
14	4	0.678823	0.053826554
15	1	0.255372	0.037028947
15	2	0.527339	0.346686981
15	3	0.45202	0.416989105
15	4	0.191856	0.010852144

w2 on-target Sample¹	Replicate²	Area³	p-value⁴
1	1	0.354288	0.270956792
1	2	0.623993	0.372377427
1	3	0.505617	0.326221532
1	4	0.603866	0.36437674
2	1	0.36459	0.274566152
2	2	0.248921	0.235450526
2	3	0.493266	0.321541374
2	4	0.497941	0.32330935
3	1	0.743176	0.420777405
3	2	0.552546	0.344249834
3	3	0.476516	0.315239267
3	4	0.567247	0.349973353
4	1	0.6173	0.369710586
4	2	0.629764	0.374681768
4	3	0.322639	0.260017705
4	4	0.752711	0.424712728
5	1	0.360847	0.273252055
5	2	0.515975	0.330167673
5	3	0.649009	0.382398199
5	4	0.30141	0.252810198
6	1	0.657626	0.38586868
6	2	0.394083	0.285029554

6	3	0.508743	0.327410309
6	4	0.376634	0.278816023
7	1	0.460619	0.309307625
7	2	0.635721	0.377064998
7	3	0.636109	0.377220471
7	4	0.519612	0.33155782
8	1	0.798805	0.443834639
8	2	1.047632	0.451808793
8	3	0.637572	0.377806636
8	4	0.801107	0.444793725
9	1	0.565232	0.34918688
9	2	0.75434	0.425385605
9	3	0.784239	0.437775598
9	4	0.267746	0.241599774
10	1	2.454954	0.054260426
10	2	0.666513	0.389457464
10	3	0.492217	0.321145105
10	4	0.689529	0.398795359
11	1	0.590055	0.35892065
11	2	0.631956	0.375558224
11	3	0.511416	0.328428326
11	4	0.693868	0.400562426
12	1	0.962473	0.487512613
12	2	0.757173	0.426556657
12	3	0.65023	0.382889271
12	4	0.656254	0.385315303
13	1	0.680487	0.395119727
13	2	0.850317	0.46535869
13	3	0.706948	0.405901075
13	4	1.023121	0.462058127
14	1	0.493948	0.321798885
14	2	0.474323	0.314418163
14	3	0.426528	0.296754891
14	4	0.394447	0.285159673
15	1	0.368143	0.275816835
15	2	0.40567	0.28919126
15	3	0.779508	0.435810883

<i>wI</i> off-targets	Sample ¹	Replicate ²	Area ³	p-value ⁴
<i>wI</i> CG6428	1	1	0.995176	0.474435181
	1	2	1.417724	0.390732469
	2	1	1.234206	0.448648321
	2	2	0.787725	0.408345468
	3	1	1.004227	0.477348454

	3	2	0.751132	0.396899724
	4	1	0.658174	0.368240774
	4	2	0.776126	0.404708559
	5	1	0.797618	0.41145379
	5	2	0.683402	0.375953796
	6	1	0.517243	0.326201257
	6	2	0.650057	0.365770359
	7	1	0.797961	0.411561604
	7	2	0.945771	0.458561646
	8	1	0.484122	0.316610642
	8	2	0.918486	0.449821601
wI CTPsyn				
	1	1	0.648255	0.493330359
	1	2	0.976337	0.158857068
	2	1	0.818325	0.305063584
	2	2	1.586962	0.001926642
	3	1	0.842249	0.279619497
	3	2	0.477413	0.292628549
	4	1	0.95574	0.174794011
	4	2	0.574291	0.402939741
	5	1	1.092612	0.087040744
	5	2	2.835977	7.02E-12
	6	1	1.254613	0.031383998
	6	2	0.292679	0.131839185
	7	1	0.871456	0.250025847
	7	2	0.846776	0.274923414
	8	1	0.502099	0.319434038
	8	2	0.686234	0.459822615
wI His2Av				
	1	1	2.214064	0.296737612
	1	2	0.405221	0.185093478
	2	1	2.025275	0.350279879
	2	2	1.480965	0.481765345
	3	1	1.194116	0.392624845
	3	2	0.847479	0.292358175
	4	1	1.793075	0.420347019
	4	2	0.741661	0.264295068
	5	1	2.03334	0.347920541
	5	2	1.870838	0.396476051
	6	1	1.679508	0.455717451
	6	2	1.876225	0.394835364
	7	1	1.456777	0.474148749
	7	2	2.151307	0.314123373
	8	1	1.949697	0.372659079

	8	2	2.619776	0.196405217
w1 Mo25	1	1	0.538598	0.335835859
	1	2	0.394541	0.439250272
	2	1	1.322622	0.000183567
	2	2	0.911486	0.027638727
	3	1	0.128459	0.111591
	3	2	0.723169	0.122460537
	4	1	0.662661	0.178646746
	4	2	0.84075	0.051181646
	5	1	0.408805	0.461854437
	5	2	1.311037	0.000218829
	6	1	0.390388	0.432703528
	6	2	1.150383	0.002032345
	7	1	0.50531	0.38568348
	7	2	0.946846	0.019783126
	8	1	0.334064	0.346426303
	8	2	0.491279	0.407331396
w1 pyd	1	1	0.482296	0.419937449
	1	2	0.845976	0.03410002
	2	1	0.560777	0.407068541
	2	2	0.791204	0.064431967
	3	1	0.384127	0.226968007
	3	2	0.5673	0.39303057
	4	1	0.225138	0.051081624
	4	2	0.55193	0.426296412
	5	1	0.257586	0.073012204
	5	2	0.689286	0.170826364
	6	1	0.377218	0.215537767
	6	2	0.58165	0.362660646
	7	1	0.461999	0.376338511
	7	2	0.56484	0.398310768
	8	1	0.431764	0.314360714
	8	2	0.740192	0.108516737

w2 off-targets	Sample ¹	Replicate ²	Area ³	p-value ⁴
w2 CG1907	1	1	1.365789	0.186622653
	1	2	2.29158	0.004083753
	2	1	1.082818	0.361634187
	2	2	2.090067	0.011813594
	3	1	1.084639	0.360341446
	3	2	1.617468	0.085747014

	4	1	1.073367	0.368366575
	4	2	1.576346	0.098615588
	5	1	2.211016	0.006344651
	5	2	1.407534	0.166140508
	6	1	1.415142	0.162569904
	6	2	1.587884	0.094870177
	7	1	1.889729	0.029820593
	7	2	1.837965	0.037104074
	8	1	1.445804	0.148690986
	8	2	2.046731	0.014591596
w2 CTPsyn				
	1	1	0.893817	0.489474465
	1	2	0.268079	0.088007932
	2	1	2.052331	0.007549794
	2	2	0.620501	0.272293054
	3	1	1.965027	0.012388123
	3	2	0.413014	0.147821495
	4	1	1.932708	0.014764614
	4	2	0.639569	0.285879599
	5	1	0.618116	0.270616789
	5	2	0.891199	0.487260847
	6	1	1.502879	0.102936925
	6	2	0.417654	0.150104874
	7	1	1.976908	0.01160185
	7	2	1.474582	0.114102646
	8	1	1.879376	0.019543495
	8	2	0.39706	0.140149382
w2 fru				
	1	1	1.627069	0.082002378
	1	2	2.987025	4.73979E-06
	2	1	0.560624	0.161134663
	2	2	1.931883	0.01911289
	3	1	1.118601	0.398881278
	3	2	1.610092	0.087897329
	4	1	0.898765	0.407226521
	4	2	1.859085	0.028075971
	5	1	0.741566	0.279027344
	5	2	1.659181	0.071673609
	6	1	0.892346	0.401673356
	6	2	1.480321	0.143660385
	7	1	1.327827	0.234693442
	7	2	1.564856	0.105139498
	8	1	0.710557	0.256240245
	8	2	0.79346	0.319232596

w2 Nrg	1	1	1.967933	0.139996511
	1	2	0.378219	0.201463842
	2	1	1.493797	0.305494257
	2	2	0.550325	0.264709538
	3	1	1.013617	0.471979142
	3	2	3.299625	0.003615929
	4	1	0.615289	0.290965911
	4	2	0.836361	0.388198948
	5	1	0.77984	0.362357464
	5	2	1.545595	0.283960062
	6	2	1.231177	0.423863934
	7	1	0.874529	0.405941866
	7	2	0.748345	0.348217218
	8	1	1.537592	0.287239392
	8	2	0.835918	0.38799429
w2 Vm26Ac	1	1	1.427707	0.027435614
	1	2	0.900827	0.467006437
	2	1	1.195764	0.149572232
	2	2	1.066394	0.292350948
	3	1	1.398714	0.035171769
	3	2	1.115337	0.231912066
	4	1	1.299192	0.076157205
	4	2	0.846226	0.385779652
	5	1	1.420624	0.029180888
	5	2	1.205374	0.141232153
	6	2	1.202503	0.143689879
	7	1	1.041463	0.32571857
	7	2	0.99772	0.387631236
	8	1	1.346142	0.053713782
	8	2	1.10608	0.242783429

¹**Sample:** biological replicates

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.

Table S2. Sequences of the oligonucleotides used in this study

Use	oligonucleotides	Sequence (5' to 3')
<i>w1</i> sgRNA target site (for detection of indels)	W1-F	AAGCTCCAAGCGGTTTACGC
	W1-R	GCAGTTCTCACGCCGCAG
<i>w2</i> sgRNA target site (for detection of indels)	W2-F	TATCATTGCAGGGTGACAGCG
	W2-R	GGCATTTCAGCAGGGTTCGTC
<i>w1</i> sgRNA target site (for HRM)	HRM W1 left	GCAGGGGAAAGTGTGAAAAA
	HRM W1 right	TGTTTCAGATGCTCGGCAGAT
<i>W2</i> sgRNA target site (for HRM)	HRM W2 left	TCGCAGAGCTGCATTAACC
	HRM W2 right	TATTGTGCCAGGCATAGGTG
Defined deletion detection	W-deletion-F	AAGCTCCAAGCGGTTTACGC
	W-deletion-D	CCATTGAGCAGTCGCATCCC
RT-PCR for Cas9 transgene detection	Actin5c-RT-F	ATACTCCTCCCGACACAAAGC
	Actin5c-RT-R	CAGGTAGTCGGTCAAATCGC
	Cas9-RT-F	GCATAAAGAAGAACCTCATTGG
	Cas9-RT-R	GAAAGAGTCATCCACCTTAGC
Potential off-target sites of <i>w1</i> sgRNA (for sequencing)	Mo25-OT-F	ccggaattcGGACGAGCAGTTCAACGACG
	Mo25-OT-R	tgctctagaCATGCCGAAGGATGCACAGAC
	CG6428-OT-F2	ccggaattcGAGCTGTAGTCGTCCATCAAGTCG
	CG6428-OT-R2	tgctctagaCGGGCCTCTTTGACATCCTG
	CTPsyn (<i>w1</i>)-OT-F	ccggaattcGGGAAAGTAATACGAATTATAGGCC
	CTPsyn (<i>w1</i>)-OT-R	tgctctagaCTTACACTCGCACAGATGGGC
	pyd-OT-F	ccggaattcGTTTCGGATCTTGGGCGTGAG
	pyd-OT-R	tgctctagaCATACTTTCATGCGGTTGATGGTC
	His2Av-OT-F	ccggaattcGGGCTGTTGCCGAGTCATTG
	His2Av-OT-R	tgctctagaGATGACGATGGATGCGACCC
Potential off-target sites of <i>w1</i> sgRNA (for HRM)	HRM Mo25 left	GGGCCTAGAGATCCGCTATC
	HRM Mo25 right	CAACTAGTTAAATGGATATCGAAAAA
	HRM CG6428 left	TGCGATAGAGTCCCAATACCA
	HRM CG6428 right	TTTGGCCAACCTCGTTATCGT
	HRM CTPsyn left	TAAATTGTGGCTCGGATTGC
	HRM CTPsyn right	CCTTTCGTCTCGAATGTCAA
	HRM pyd left	CGTGAAGTGAATTCAAGTTTGG
	HRM pyd right	CCTCCCTTATTTAACTGATGAAATG
	HRM His2av left	ATTGCGGTGAATTTTTGAGC
HRM His2av right	GCCTACGTGGTGTCCACTTT	
Potential off-target sites of <i>w2</i> sgRNA (for sequencing)	Nrg-OT-F	ccggaattcGTAAGTGAGGAATCGGTGCGC
	Nrg-OT-R	tgctctagaCCTCAACGGTGTTCGTGTCG
	Vm26Ac-OT-F	ccggaattcGTCTTAGCAATGATTAAGCGGCAC
	Vm26Ac-OT-R	tgctctagaTTGGAAGCCTCGACCTGTGG
	CTPsyn(<i>w2</i>)-OT-F	ccggaattcCCAAGGAAAGCTGGGAGTGC
	CTPsyn(<i>w2</i>)-OT-R	tgctctagaCTCCATCAGCAGCGGAACATG
	CG1907-OT-F	ccggaattcGCCACCAATGCCATCAAGTTCC
CG1907-OT-R	tgctctagaGCGTGAATCCCTTCCACAGC	

	fru-OT-F	ccggaattcAATATGGCAAAGCAGAGTGCTTC
	fru-OT-R	tgctctagaAGCGGAAATGTCTCAACAAGC
Potential off-target sites of <i>w2</i> sgRNA (for HRM)	HRM Nrg left	ATACTCCGGAGGGTGTACCA
	HRM Nrg right	TTCTTCCAGTGGAGCATGAA
	HRM Vm26Ac left	GGCAAGCTGGAGAAGGAACT
	HRM Vm26Ac right	CAGCTACGACCAGGATGAGG
	HRM CTPsyn left	CATTCAGGAATGGGTGGAG
	HRM CTPsyn right	ATGCCTTCGATGTCACCAAT
	HRM CG1907 left	TGGGCATGTACACGTATCTGA
	HRM CG1907 right	CAATAAAGGCGCCACAGG
	HRM fru left	ACACCTGAGAAAGCCCAGTG
	HRM fru right	TGGCTGGTAACTGTGACGTG
Sequencing primers	Hsp70-F	CAAGCGCAGCTGAACAAGCT
	ftz-R	CGGCTCTAGTTCTTTGCAATC