CRISPR with independent transgenes is a safe and robust alternative to autonomous gene drives in basic research

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CRISPR/Cas technology allows rapid, site-specific genome modification in a wide variety of organisms. CRISPR components produced by integrated transgenes have been shown to mutagenise some genomic target sites in Drosophila melanogaster with high efficiency, but whether this is a general feature of this system remains unknown. Here, we systematically evaluate available CRISPR/Cas reagents and experimental designs in Drosophila. Our findings allow evidence-based choices of Cas9 sources and strategies for generating knock-in alleles. We perform gene editing at a large number of target sites using a highly active Cas9 line and a collection of transgenic gRNA strains. The vast majority of target sites can be mutated with remarkable efficiency using these tools. We contrast our method to recently developed autonomous gene drive technology for genome engineering (Gantz & Bier, 2015) and conclude that optimised CRISPR with independent transgenes is as efficient, more versatile and does not represent a biosafety risk.

Introduction

The Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR associated (CRISPR/Cas) genome engineering system is currently revolutionising experimental and applied biology (Doudna and Charpentier, 2014; Hsu et al., 2014). The system consists of the bacterial endonuclease Cas9 and a small chimeric guide RNA (gRNA), which directs Cas9 to a genomic target site 5’ to an NGG ‘protospacer adjacent motif’ (PAM) (Jinek et al., 2012). Repair of CRISPR/Cas-mediated DNA double strand breaks (DSBs) by endogenous machinery can result in mutagenic insertions and deletions (Indels) or – together with a supplied piece of donor DNA – precise sequence alterations. This methodology has been used successfully to edit the genome of a variety of organisms, although mutagenesis efficiencies have varied widely between different target sites, even in individual studies.

It has been proposed that inserting a cassette encoding Cas9 and gRNA into the endogenous target site of the gRNA will efficiently autocatalyse its integration into the homologous allele, thus converting heterozygous into homozygous cells (Esvelt et al., 2014; Oye et al., 2014). Such CRISPR/Cas ‘gene drive’ systems could potentially be used to efficiently spread traits within wild populations of plants and animals to address global problems in public health, sustainable agriculture, and environmental management (Burt, 2003; Esvelt et al., 2014; Sinkins and Gould, 2006). Gantz and Bier recently demonstrated such a ‘gene drive’ system using Drosophila melanogaster, a major model organism in biomedical research (Gantz and Bier, 2015). They propose that by initiating a ‘mutagenic chain reaction’ (MCR) their technology can be used to accelerate laboratory genome engineering and reveal homozygous mutant phenotypes in genetic screens. However, there are major biosafety concerns about the use of autonomous gene drive technology in the laboratory because of the risk of accidental infiltration of autocatalytic alleles into wild populations (Esvelt et al., 2014; Oye et al., 2014).

Here we describe the systematic evaluation of Drosophila CRISPR/Cas tools and experimental designs that do not create gene drives. Our work reveals that optimised CRISPR with independent, integrated Cas9 and gRNA transgenes can achieve remarkably efficient germ line and somatic gene targeting at a very large proportion of genomic target sites. The performance of these methods is comparable to that reported for Gantz and Bier’s MCR technology. We conclude that transgenic CRISPR/Cas is a safe and consistently efficient method to modify the fly genome and encourage others to apply similar technology to other experimental model organisms.
Results

We and others have shown that transgenic cas9 strains can in principle be used to rapidly and efficiently modify the Drosophila genome in somatic and germ line cells (Chen et al., 2015; Gratz et al., 2014; Kondo and Ueda, 2013; Port et al., 2014; Ren et al., 2013, 2014; Sebo et al., 2013; Xue et al., 2014; Zhang et al., 2014). gRNAs can be delivered into cas9 strains by injection of plasmids or mRNA, or can be encoded by a previously integrated transgene (which we now refer to as CRISPR with independent transgenes (CRISPR-it)). There is significant uncertainty in the fly community about which of the many published cas9 strains are most effective for genome engineering experiments.

We evaluated the performance of all transgenic cas9 lines available at the time of study. The collection consisted of six lines that we assessed previously (Port et al., 2014) and eight others (Suppl. Table 1). The transgenes differed in regulatory elements, cas9 codon usage and genomic integration sites (Suppl. Table 1). We crossed cas9 flies to a previously validated, ubiquitously expressed transgene with independent transgenes (vasa-cas9) or cas9 mRNA, or can be encoded by a previously integrated transgene (which we now refer to as CRISPR with independent transgenes (CRISPR-it)). There is significant uncertainty in the fly community about which of the many published cas9 strains are most effective for genome engineering experiments.

Figure 1: Large variation of somatic and germ line activity in publicly available transgenic cas9 strains. (A) Representative examples of female flies expressing one copy of the different cas9 transgenes and one copy of U6-3-gRNA-e transgene (> 100 adults of each genotype were examined). Darker body coloration indicates CRISPR/Cas-mediated mutagenesis of e in epidermal cells (arrowheads: sporadic biallelic targeting of e). (B) Assessment of germ line transmission of non-functional e alleles from animals expressing different cas9 transgenes and the U6-3-gRNA-e transgene. In B – D, dots show data from individual crosses with mean ± standard deviation (SD) represented by the underlying bar chart. In B, typically between 30 and 140 progeny were analysed for each cross (C) Frequency of pupal lethality caused by cas9 transgenes with somatic activity in combination with the U6-3-gRNA-wls transgene. Flies that eclosed from the pupal case had leg and wing defects consistent with reduced wls function and died shortly afterwards. In C and D, control flies were of the genotype act-cas9 (U6-3-gRNA-e, which have widespread induction of DSBs. Typically, 50 - 150 animals were examined for each cross. (D) Percentage of non-viable embryos laid by females expressing different germ line restricted nos-cas9 transgenes and the U6-3-gRNA-wls transgene following mating to wild-type males. Typically, between 100 and 300 embryos were examined for each cross. (E) Assessment of germ line transmission rates of CRISPR/Cas-induced wls mutations. Offspring from nos-cas9 U6-3-gRNA-wls male flies crossed to wild-type females were genotyped by PCR of the wls locus followed by sequencing. Each square represents an individual genotyped fly. Many mutations were out-of-frame, presumably disrupting wls function. For the nos-cas9 CAS series of lines there was little correlation between the rate of embryonic lethality induced by U6-3-gRNA-wls and the rate of germ line transmission of wls alleles to the viable offspring. Germ line transmission depends on a mutation of wls in the transcriptionally quiescent oocyte nucleus, whereas embryonic lethality arises from mutagenesis of the gene in the auxiliary nurse cells, which supply protein products to the egg through cytoplasmic bridges. These nos-cas9 lines may therefore be differentially active in targeting wls in the nurse cells and oocyte within the female germ line.
gRNA transgene (U6:3-gRNA-e), which targets the 5' end of the coding sequence (CDS) of the pigment gene ebony (e) (Port et al., 2014). Phenotypic assays using this gRNA typically report only on out-of-frame Indels, as most in-frame mutations at the gRNA-e target site retain protein function (Port et al., 2014). All adult progeny expressing U6:3-gRNA-e and cas9 under the control of either the actin5c (act) or vasu promoter had a large proportion of cuticle that was dark, demonstrating efficient biallelic disruption of e in somatic cells (Figure 1A). In contrast, seven of the nine lines tested expressing cas9 under nanos (nos) regulatory elements did not have detectable somatic activity when crossed to U6:3-gRNA-e (Figure 1A). These findings confirm and extend our previous findings on the expression patterns of cas9 driven by these classes of regulatory elements (Port et al., 2014). Phenotypic analysis of the progeny of cas9 U6:3-gRNA-e flies and e mutant partners revealed a wide range of germ line transmission rates of CRISPR/Cas-induced non-functional e alleles, varying between 0.5% and 49% (Figure 1B). act-cas9, vasa-cas9 BL51323 and nos-cas9 TH00787.N (which we refer to as TH_attP2) gave rise to similar, high rates of mutagenesis.

We also evaluated the cas9 lines with a second gRNA transgene (U6:3-gRNA-wls) targeting the essential gene wntless (wls) (Port et al., 2014). As expected, cas9 transgenic strains that had somatic activity in combination with U6:3-gRNA-e resulted in non-viable progeny when crossed to U6:3-gRNA-wls(Figure 1C). The progeny of the other seven nos-cas9 lines were viable in combination with U6:3-gRNA-wls, although a small proportion of adults from these crosses had wing defects indicative of gene targeting in a small subset of somatic cells (Figure 1; Suppl. Figure 1). Targeting of wls in the germ line was assessed by crossing nos-cas9 U6:3-gRNA-wls females to wild-type males. All but two of the nos-cas9 transgenes resulted in a high proportion of embryos arresting with defective segmentation (Figure 1D), a phenotype associated with biallelic disruption of wls in the female germ line (Bänzig et al., 2006; Bartscherer et al., 2006). All crosses gave rise to some viable offspring, with genotyping of the wls locus from these flies revealing substantial variation in the frequency of germ line transmission of CRISPR/Cas-induced mutations (Figure 1C). In two cases, all analysed offspring (10/10) received a modified wls allele. Together these results reveal that the cas9 lines differ substantially in their somatic and germ line activity. Whereas act-cas9 and vasa-cas9 lines can directly reveal null mutant phenotypes in the soma when combined with gRNA transgenes, a subset of nos-cas9 lines (e.g. CFD2, TH00788.N (referred to as TH_attP40) and TH_attP2 (inserted on the X, 2nd and 3rd chromosomes, respectively)) can efficiently generate Indel mutations in both essential and non-essential genes in the germ line.

Another important application of CRISPR/Cas is the precise modification of the genome by homology directed repair (HDR). This involves Cas9-mediated induction of DSBs at the target site in the presence of exogenous donor DNA. Three strategies are particularly appealing in Drosophila because of their relative simplicity. Embryos that are transgenic for both cas9 and gRNA can be injected with donor DNA (Port et al., 2014), transgenic cas9 embryos can be injected with a mixture of donor DNA and gRNA-encoding plasmid.

| Strategy | nos-cas9 CDF2 | nos-cas9 CDF2 | nos-cas9 TH_attP2 |
|----------|---------------|---------------|-----------------|
| A        |               |               |                 |
| cas9     | gRNA          |               |                 |
|          | Donor: 500 nM | Founders: 88% | Founders: 88%   |
|          |                | Non-Founders: | Non-Founders:   |
|          | N=8            | 10%           | 10%             |
|          | RFP+ Files: 23%|                |                |
|          | RFP- Files:   |                |                |
| B        |              |               |                 |
| cas9     | gRNA: 500 nM  | Founders: 88% | Founders: 88%   |
|          | Donor: 500 nM | Non-Founders: | Non-Founders:   |
|          | N=180         | 10%           | 10%             |
|          | RFP+ Files: 9% |                |                |
|          | RFP- Files:   |                |                |
| C        |               |               |                 |
| any      | W118          | Founders: 25% | Founders: 25%   |
| genotype | Cas9-gRNA: 75 nM | Non-Founders: | Non-Founders:   |
|          | Donor: 100 nM | 10%           | 10%             |
|          | N=502         | RFP+ Files:   | RFP- Files:     |
|          |               | 10%           | 10%             |

Figure 2: Assessing strategies for generating knock-in alleles with CRISPR/Cas-mediated HDR. (A) nos-cas9 female flies of the indicated strain were crossed to U6:3-gRNA-wg males and a donor plasmid encoding RFP was injected into the embryonic progeny at the indicated concentrations. Due to a pre-existing X-linked RFP insertion in the CFD2 stock only male G0 and F1 flies could be analysed. For consistency we only analysed male flies in all HDR experiments. G0 males that give rise to RFP positive offspring were designated ‘founders’. The percentage of male progeny with RFP expression relative to all offspring (i.e. from fertile founder and non-founder G0 males) is indicated below. N, total number of males analysed. (B) Donor and U6:3-gRNA-wg plasmids were injected into nos-cas9 embryos. In B and C, plasmids were mixed to give an injection mix containing the concentrations shown. (C) Non-transgenic W118 embryos were injected with donor DNA and a single plasmid containing both hp70-cas9 and U6:2-gRNA-wg. The attention sign indicates that unintended integration of this plasmid at the gRNA target site could create an autonomous gene drive. Propagation of a gene drive is not possible in our experiment as integration at the gRNA-wg target site would create a lethal allele. Suppl. Table 2 contains detailed results for all HDR experiments.
(Gratz et al., 2014; Ren et al., 2014; Zhang et al., 2014) or non-transgenic embryos can be injected with a donor plasmid together with a plasmid encoding Cas9 and gRNA (Gokcezade et al., 2014). No study has directly compared the efficiency with which all three methods facilitate HDR with the same gRNA and donor construct. To do so we constructed a donor plasmid designed to knock-in an expression cassette containing red fluorescent protein (RFP) under an eye specific promoter into the essential wg gene (Figure 2 – Suppl. Figure 2). The plasmid, which contained wg homology arms of 1.4 and 1.7 kb, was used in circular form in combination with a previously validated gRNA (gRNA-wg) (Port et al., 2014). For technical reasons (see Figure 2 legend) we only followed integration of donor DNA using male flies.

For the experiments involving transgenic cas9 supply we used different plasmid concentrations and two different nos-cas9 strains (CFD2 and TH_attP2) (Figure 2). Injection of the donor plasmid into cas9 gRNA-wg double transgenic embryos resulted in 19 – 25% of all offspring from G0 flies having integration of the RFP construct, compared to 5 – 15% when the U6:3-gRNA-wg plasmid was injected with donor DNA into nos-cas9 embryos (Figure 2A, B and Suppl. Table 2).

We assessed the efficiency of plasmid-based delivery of Cas9 and gRNA using a published vector that encodes both components (Gokcezade et al., 2014). Although expected to be a rare event, accidental integration of such a plasmid at the gRNA target site would create an autonomous gene drive. We were able to safely test this plasmid as insertion at the gRNA-wg target site would be homozygous lethal, preventing the allele from being propagated. Upon co-injection of the cas9/gRNA plasmid and donor plasmid into non-transgenic embryos, only 3% of all progeny from G0 flies had genomic integration of the RFP cassette (Figure 2C and Suppl. Table 2). Integration of the donor DNA within the wg locus could be confirmed for 87 of 98 RFP positive flies tested from our set of HDR experiments (Figure 2 – Suppl. Figure 2 and Suppl. Table 2). In summary, the methods using a transgenic cas9 source resulted in the highest frequency of knock-in alleles, with injection into embryos that are also transgenic for the gRNA giving the most efficient targeting.

The above results, together with previous literature (Kondo and Ueda, 2013; Port et al., 2014), provides evidence that transgenic supply of both Cas9 and gRNA can lead to very high rates of mutagenesis. An important outstanding question is whether the high

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**Figure 3:** Almost all transgenic gRNAs are highly active with a strong transgenic source of Cas9. Male flies transgenic for individual gRNAs were crossed to act-cas9 females. Somatic phenotypes of act-cas9 gRNA progeny are summarised for gRNAs targeting Dhc64C, Lis1, wg, wls and se. The gRNA-se with no detectable activity was found to contain a polymorphism. Activity in the germ line is shown for gRNAs targeting sep, Lis1 and Wg (data show mean percentage of progeny that inherited a non-functional allele from three independent crosses (see Suppl. Table 3 for more details and somatic phenotypes of these gRNAs)). gRNAs highlighted by black diamonds frequently give rise to functional in-frame mutations (Suppl. Table 3), suggesting that their efficiency in creating indel mutations often approaches 100%. The gRNA additionally highlighted by an asterisk gives rise to an unexpected large number of in-frame mutations in functional alleles (8/8 flies analysed), suggesting a micro-homology mediated bias in non-homologous end joining. Since transcription initiation from U6 promoter is proposed to require a G nucleotide, we extended gRNAs by a mismatched G where necessary (lower case). gRNAs with spacer sequences of 18 nt, 19 nt and 20 nt can often give rise to functional in-frame mutations (Suppl. Table 3), suggesting that their efficiency in creating indel mutations often approaches 100%. The gRNA additionally highlighted by an asterisk gives rise to an unexpected large number of in-frame mutations in functional alleles (8/8 flies analysed), suggesting a micro-homology mediated bias in non-homologous end joining. Since transcription initiation from U6 promoter is proposed to require a G nucleotide, we extended gRNAs by a mismatched G where necessary (lower case). gRNAs with spacer sequences of 18 nt, 19 nt and 20 nt can
rates of efficiency observed for a small number of genomic target sites in these studies are a general feature of this system. Previous studies have found that gRNAs delivered by injection of RNA or a plasmid can differ substantially in their activity (Bassett et al., 2013; Lee et al., 2014; Ren et al., 2014; Zhang et al., 2014), prompting some to recommend screening for active gRNAs in cell culture before performing whole animal experiments (Zhang et al., 2014).

To determine what fraction of gRNAs are functional in the CRISPR-it system we generated a set of 66 transgenic fly strains each expressing a different gRNA. The gRNAs were designed to target seven genes including essential genes (wingless (wg), wls, Lissencephaly-1 (Lis1), Dynemin heavy chain at 64C (Dhc64C)) and non-essential pigmentation genes (e, yellow (y; also controlling coloration of the cuticle), and sepias (se; controlling coloration of the eye)). gRNA target sites were based on the reference sequence of the Drosophila genome (Flybase release 6.02). They were located in the 5′ half of the coding sequence of each gene, such that out-of-frame Indels are likely to create loss-of-function alleles, but were otherwise selected at random. gRNAs were expressed under the control of the strong, ubiquitous U6:3 promoter (Port et al., 2014) and integrated at the same genomic location to ensure comparable expression of all transgenes.

The 66 individual gRNA strains were crossed to the act-cas9 line, which has ubiquitous, high activity (Figure 1A,B). Strikingly, analysis of the progeny revealed that all but one of the gRNAs gave rise to a somatic mutant phenotype (Figure 3 and Suppl. Table 3). Sequencing of our strains revealed a single nucleotide polymorphism in the target site of the inactive gRNA, designed to target se, providing a likely explanation for its inactivity. Most of the gRNAs targeting the essential genes caused lethality at the same developmental stage as reported for the respective homozygous null-mutant animals, with the remainder leading to developmental arrest at later stages (Figure 3). With the exception of the single inactive gRNA-se transgene, all of the gRNAs targeting non-essential genes gave adults with regions of tissue with the null-mutant pigmentation phenotype (Figure 3, Figure 3 – Suppl. Figure 3 and Suppl. Table 3). For almost all gRNAs, biallelic gene disruption was usually observed in over 50% of the affected tissue, with half of the gRNAs, including all five targeting y, leading to more than 80% mutant tissue (Suppl. Table 3). Non-specific phenotypes were not observed in act-cas9 adults co-expressing any of the gRNA transgenes targeting y, e or se, consistent with previous evidence that CRISPR/Cas operates with substantial fidelity in Drosophila (Bassett et al., 2013; Gratz et al., 2014; Ren et al., 2014). The above results demonstrate that the vast majority of randomly selected gRNAs efficiently induce biallelic loss-of-function mutations in target genes, thereby directly revealing the mutant phenotype. There was, however, variation in the strength of the somatic phenotypes observed for gRNAs targeting the same gene (Figure 3 and Suppl. Table 3). Weaker phenotypes could arise either through a reduced frequency of induced Indel mutations or because functional in-frame mutations at the specific gRNA target site provide a significant fraction of cells with functional protein.

To directly test the hypothesis that in-frame mutations can mask high rates of Indel induction by some gRNAs, we characterised CRISPR/Cas-induced mutations transmitted to the next generation. Flies expressing act-cas9 and a transgenic gRNA targeting y or e were crossed to y or e mutant flies, respectively (Figure 3). The rates of transmission of non-functional alleles across target sites was generally higher than reported when gRNAs were delivered by plasmid injection into transgenic cas9 embryos (Ren et al., 2014) (Figure 3 – Suppl. Figure 4A,B), providing further evidence of the relatively high activity of CRISPR-it. All five gRNA-y and six of 18 gRNA-e transgenes transmitted non-functional alleles to >93% of their offspring (Figure 3). The high frequency of non-functional alleles strongly suggests that in-frame mutations at these target sites usually disrupt protein function. Transmission rates for non-functional mutations ranged from 13 – 90% for the other 12 gRNA-e lines (Figure 3). To explore the basis of this variation, we determined the sequence of e around the target sites in some of the phenotypically wild-type progeny from the 12 lines. For nine of the gRNAs, in-frame Indel mutations were detected in all, or almost all, analysed animals (Suppl. Table 3; Figure 3). Thus these gRNAs appear to create Indels in the vast majority of e alleles, with differences in the functional effects of in-frame mutations at their target sites influencing the outcome of phenotypic assays. We confirmed that this effect was not specific for target sites in e by molecular analysis of the se locus in offspring of act-cas9 gRNA-se flies, which again revealed in-frame mutations in the majority of functional alleles (Suppl. Table 3; Figure 3).

Indel mutations were not readily found in functional alleles inherited from flies expressing act-cas9 and the three gRNA-e transgenes that were the least efficient in the phenotypic assays (transmission of non-functional alleles in 13 – 35% of cases) or one poorly active se gRNA (7% of phenotypically mutant eye tissue) (Suppl. Table 3; Figure 3). Thus, these gRNAs induce Indels with relatively low frequency. Sequencing of the target site for these gRNAs excluded the possibility that polymorphisms were responsible for their reduced activity. gRNAs with lower activity were not readily predicted by available online gRNA design tools and their target sites did not have a low GC content in the region proximal to the PAM (Figure 3 – Suppl. Figure 4C – J; Suppl. Table 4), a parameter which strongly predicted less active gRNAs encoded by plasmids injected into transgenic cas9 embryos (Ren et al., 2014). Several gRNAs targeting sites with relatively low PAM proximal GC content had very high activity in our experiments (Figure 3 – Suppl. Figure 4A, F, J). Collectively, our results demonstrate that optimised CRISPR-it is a very robust genome engineering system, leading to highly effective mutagenesis at the vast majority of target sites.

**Discussion**

We set out to provide a systematic evaluation of tools and design parameters for transgenic CRISPR/Cas genome engineering in Drosophila melanogaster. We show that selection of the cas9 strain is critical for the successful application of this methodology, as publicly available lines vary widely in their activity in somatic and germ line cells. Success rates also vary substantially between different protocols for HDR-mediated knock-ins. High efficiency is particularly important for HDR without selectable markers, which introduce unwanted sequences into the target locus, as in most cases integration must be detected by PCR-based analysis of genomic DNA. Most importantly, we show that use of transgenic gRNAs allows highly efficient mutagenesis across a large proportion of target sites, with our analysis of germ line transmission of mutations in pigmentation genes suggesting that the vast majority of gRNAs cause close to maximal rates of Indel induction. Incomplete penetrance of phenotypes induced by some transgenic gRNAs is
due to functional in-frame mutations; co-expression of more than one gRNA using a dual gRNA vector (Port et al., 2014) should alleviate this problem.

Importantly, our study highlights that CRISPR-it is a safe, efficient and robust alternative to the recently reported MCR, which uses an autonomous gene drive for functional studies (Gantz and Bier, 2015) (Table 1). MCR has so far only been demonstrated at a single target site in the Drosophila y gene, a locus often used in pioneer studies and which appears to have an unusually high susceptibility for gene targeting (Gratz et al., 2013; Rong and Golic, 2000). MCR can rapidly reveal recessive somatic phenotypes as heterozygous target sites in the genome (shown for many target sites) efficiently reveal recessive mutant phenotypes of essential genes and can be employed in a tissue specific manner (Port et al., 2014; Xue et al., 2014), applications that are not easy to achieve with MCR. The ability of CRISPR-it to efficiently reveal recessive mutant phenotypes associated with a large proportion of genomic target sites also makes it a highly attractive system for large scale F1 mutagenesis screens.

Gene drive technology has great promise for applications such as ecosystem management and pest control and may well be adopted for basic research applications (Burt, 2003; Esvelt et al., 2014; Sinkins and Gould, 2006). However, responsible use of this technology will require development of robust molecular containment strategies (Esvelt et al., 2014). We therefore urge researchers not to apply gene drives without these safeguards in place. Our experiments illustrate safe and effective genome engineering strategies that are already available for basic research and can be applied to any genetically tractable organism.

Table 1: Comparison of MCR and CRISPR-it technology

| Mutagenic chain reaction | CRISPR-it |
|--------------------------|-----------|
| Work environment         |           |
| Physical containment     | Regular fly room and working procedures |
| • Triple contained flies |           |
| • Locked facilities at all times |           |
| • Accounting for individual flies |           |
| • Single experimenter    |           |
| CRISPR components        |           |
| On one construct         | Separate, integrated transgenes |
| Multiplexing*             | Yes       |
| Directly reveals recessive phenotypes | Yes |
| (so far shown for a single target site) | (shown for many target sites) |
| Efficiency of germ line transmission of mutations | Up to 100% |
| CRISPR inactivated by crossing | No |
| Risk of infiltrating wild and lab populations | Yes |
| Genotype at target site  |            |
| Mosaic in all generations | F1: Mosaic (mostly biallelic for Indels) |
| (mostly homozygous for MCR allele) | F2: heterozygous |
| Time from cloning to recessive phenotype | ~25 days |
| Targeting essential genes | No* |
| Tissue specific           | Yes |

a: Generation of multiple gRNA plasmids can be multiplexed during cloning and transgenesis. Multiplexing is not possible during MCR. b: One additional generation. c: Would require complex rescue construct (Gantz and Bier, 2015).

Working with flies containing an MCR cassette has to be performed using very strict biosafety procedures (Table 1) (Gantz and Bier, 2015). No such precautions are necessary for transgenic CRISPR work, meaning that experiments can be performed much more conveniently and rapidly. Furthermore, CRISPR-it can readily reveal mutant phenotypes of essential genes and can be employed in a tissue specific manner (Port et al., 2014; Xue et al., 2014), applications that are not easy to achieve with MCR. The ability of CRISPR-it to efficiently reveal recessive mutant phenotypes associated with a large proportion of genomic target sites also makes it a highly attractive system for large scale F1 mutagenesis screens.

Methods

Plasmid construction

All primer sequences (purchased from Integrated DNA Technologies (IDT)) are listed in Suppl. Table 5. Unless stated otherwise, enzymatic reactions were performed according to the manufacturers’ guidelines. To generate gRNA expression vectors, pCDF3 (Port et al., 2014) (Addgene 49410) or pDC6 (Gokcezade et al., 2014) (Addgene 59985) were cut with BbsI and dephosphorylated with alkaline phosphatase, followed by gel purification of the linear plasmid. To introduce the target specific spacer sequence, two oligonucleotides containing the spacer sequence and reverse complement spacer sequence, as well as appropriate overhangs, were mixed (1µl each oligo (100µM stock solutions)) together with 1µl 10x T4 Ligation Buffer (New England Biolabs (NEB)), 6.5µl dH₂O and 0.5 µl T4 polynucleotide kinase (NEB). Phosphorylation and annealing of oligos was performed in a thermocycler (30 min at 37°C, 5 min at 95°C, followed by ramping down to 25°C at 5°C/min). Annealed oligos were diluted 1:200 in dH₂O and ligated into the linear expression plasmids with T4 DNA ligase (NEB), followed by transformation into chemically competent bacteria. A step-by-step protocol is available from www.crisprflydesign.org.

Drosophila culture

Flies were maintained at 25°C and 50% humidity at a 12h light/dark cycle.
Assessing activity of cas9 lines

To assess cas9 line performance in targeting, virgin females from the various cas9 lines were mated to U6:3-grNA-wls transgenic males. The resulting double transgenic offspring was examined for CRISPR/Cas-induced phenotypes, and virgins heterozygous for both cas9 and U6:3-grNA were crossed to e mutant males (w, y wls/TM3) (66b). The number of progeny from each cross was recorded. At least five independent crosses were performed for each cas9 line.

To analyse the ability of the various cas9 lines to mediate mutagenesis of wild-type (wls), virgin cas9 females were crossed to U6:3-grNA-wls transgenic males. At least three independent crosses were performed for each genotype. In cases where the resulting cas9 U6:3-grNA-wls animals had significant rates of pupal lethality the number of dead versus total pupae was recorded. Males from the remaining, viable cas9 U6:3-grNA-wls genotypes were crossed to y w hs-tFLP/M力还是/Th recombinase. To determine the rate of germ line transmission, genomic DNA was isolated from some of the offspring, using microlysis-Plus (Microzone) according to the manufacturer’s instructions, and used for PCR analysis. Primers wls_genotype_fwd and wls_genotype_rev were used to amplify the regions flanking the grNA-wls target site. The genomic sequence from 10 to 12 flies from two independent crosses was analysed for each genotype. To monitor embryonic viability of the offspring, virgins heterozygous for both cas9 and U6:3-grNA-wls were crossed to wild-type males, and the cross was transferred to cages mounted on apple juice agar plates. All embryos from a 1–2 h egg collection were counted and kept at 25°C for 48 h to allow for embryonic development to be completed. After 48 h the number of embryos that hatched was recorded. Where inspected, arrested embryos had segmentation defects. This analysis was carried out in quadruplicate for each genotype. Intercrosses of act-cas9 U6:3-grNA-e flies were used as a control.

Embryo injections and transgenesis

Microinjection into embryos were performed using standard procedures as described previously (Port et al., 2014). To generate transgenic grNA lines, plasmids were often injected in pools (Bischof et al., 2013). Equal amounts of grNA plasmids (5–20 µg per pool) were mixed and diluted to a final concentration of 150 ng/µl in dH2O and injected into y w nos-PhsC31, attP40 (Bloomington Stock (BL)25709) embryos. Single transgenic offspring, selected by the v marker encoded by the grNA plasmid, were mated with v; SpCyO flies for several days and then squashed in 10 µl microlysis-Plus (Microzone) to extract genomic DNA. To identify the integrated grNA plasmid the transgene was amplified by PCR using primers U6:3 responsible for the CRISPR/Cas-induced Indel and are consequently heterozygous at the grNA target site contained an overlay of chromatograms from both allelic. Chromatograms were analysed with Tide (http://tide.nki.nl/; Brinkman et al., 2014) and re-examined manually to correct for possible software mistakes, which occur occasionally for alleles involving insertions.

Assessing different HDR strategies

Primers pBSwg3'HA_fwd and pBS-wsg5'HA_rev were used to amplify the 5' and 3' homology arms and the pBluescript SK (+) vector backbone from the previously described Wg: GFP donor plasmid (Port et al., 2014). The 3xP3-RFP-putb-3' UTR (gift from Nick Lowe and Daniel St Johnston) sequence was amplified by PCR using primers 3xP3-RFP_fwd and 3xP3-RFP_rev. Both fragments were then joined by Gibson assembly to generate the RFP donor construct. Sequence-verified plasmid DNA was purified using the MinElute PCR purification kit from Qiagen. To test HDR efficiency using transgenic cas9 and grNA, the purified circular donor plasmid was injected into embryos resulting from crosses between nos-cas9 CFD2 or nos-cas9 TH; attP2 virgin females and U6:3-grNA-wg males. For the protocol based on transgenic act9 stocks, both the donor and U6:3-grNA-wg plasmid were injected into embryos either hemizygous (CFD2 males) or homozygous (CFD2 females or TH; attP2 males and females) for the nos-cas9 transgene. To test HDR efficiency using the pDCC6 plasmid encoding cas9 and U6:2-grNA-wg (giGokezade et al., 2014; hereafter called pDCC6-wg), both donor and pDCC6-wg were injected into embryos of an isogenised w1118 stock (Bloomington Stock Centre (BL)905) (5). The concentrations of injected plasmids for the HDR experiments are given in Figure 2. Injected embryos were kept at 18°C for ~48 h and then moved to 25°C. G3 males were crossed to wv hs-tFLP; SpCyO females and the resulting male offspring was screened for the presence of RFP. To test whether the 3xP3-RFP cassette was inserted into the wg locus, genomic DNA was isolated from some of the RFP positive male offspring for PCR analysis as described above, with primer pairs wgHRgeno_fwd1/wgHRgeno_rev1 and wgHRgeno_fwd2/wgHRgeno_rev2 used to amplify the regions flanking each homology arm.

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- Preprint design based on template by Denis Eckmeier www.eckmeier.de/

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Supplementary Figure 1: Assessing somatic activity of nos-cas9 lines in combination with U6:3-gRNA-wls. Flies expressing the indicated nos-cas9 line and U6:3-gRNA-wls were examined and the number of flies with notches of the wing margin counted. Dots represent data from individual crosses with mean ± SD indicated as lines. Notches were always small (<10% of wing area) and only present in the minority of animals, demonstrating low levels of somatic activity. Typically, 30 - 50 flies were examined for each cross.
**Supplementary Figure 2 : Targeted donor integration by HDR.** (A) Schematic of the RFP donor vector in relation to the \( wg \) locus on chromosome arm 2L. The donor plasmid is based on a previous plasmid (Port et al., 2014), which was used to create a functional GFP knock-in in the \( wg \) locus. In the current plasmid, GFP was replaced by an autonomous RFP expression cassette. Integration of the donor plasmid causes a disruption of the \( wg \) gene and removes the protospacer adjacent motif (PAM) that is essential for cleavage by \( gRNA-wg \), thus preventing recutting of the targeted allele. (B) Flies with genomic insertions of the donor plasmid can be easily identified by strong red fluorescence in the eye compared to control animals. (C) Schematic showing the \( wg \) locus after successful ends-out integration of the donor sequence. The indicated primers were used to analyse whether donor integration in RFP positive flies occurred at the correct location. Note that primers \( wgHRgenofwd1 \) and \( wgHRgenorev2 \) anneal outside the homology arms that are present in the donor plasmid. (D) A representative example of diagnostic PCRs from 10 flies from experiments designed to integrate the donor plasmid at the \( wg \) locus. Eight out of 10 flies gave rise to a product of the expected size with both \( wgHRgeno \) primer pairs S2
and were scored as positive for RFP integration in wg. Only one PCR product was amplified from fly number 7, suggesting a complex integration event. Genomic DNA from control flies did not result in any PCR product with primers diagnostic for donor insertion in the \textit{wg} locus (four lanes to the left of 1–10). Amplification of part of the \textit{wls} locus was used as a positive control for DNA quality (bottom panel). A summary of all HDR genotyping experiments can be found in Suppl. Table 2.

**Supplementary Figure 3: Assessing activity of gRNAs targeting se.** Eyes from flies heterozygous for \textit{act-cas9} and \textit{gRNA-se} transgenes were examined by three independent researchers. Example images are shown of eyes with various degree of \textit{se} mutant tissue, which has darker coloration than wild type tissue. Mean values from the independent observations are shown in Figure 3 (see also Suppl. Table 3).
Supplementary Figure 4: gRNA activity in CRISPR-Cas9 is not reliably predicted by online design tools or PAM proximal GC content of the target site. (A - B) Comparison of data from transgenic gRNAs from this study (A) and injected gRNAs from Ren et al. 2014 (B) relating the PAM proximal GC content of the target site (3’ most 6 nt of the spacer) to the rate of germ line transmission of non-functional alleles in phenotypic assays. Note that transmission of non-functional alleles was plotted as Ren et al. did not investigate in-frame functional mutations in their study. act-cas9 was used in our study and nos-cas9 (TH_attP2) was used by Ren et al.. These cas9 sources had very similar activities when compared with the same gRNA transgene (Figure 1). The mean (± SD) rate of germ line transmission of non-functional alleles in non-essential genes was 80% ± 25 (N = 22) for our study and 57% ± 26 (N = 39) for Ren et al. (P = 0.0014, two-tailed unpaired t-test). (C – F) Prediction of gRNA activity with popular design tools. Activity scores for gRNA targeting se, y and e were generated with each online gRNA design tool (C – E; see Suppl. Table 4 for URLs); the GC content of the 3’ most 6 nt of the gRNA spacer was calculated manually (F). Values were plotted for gRNAs assigned to three classes (colour coded as in Figure 3). Whereas class I gRNAs mediate suboptimal mutagenesis, class II and III gRNAs mediate near maximal mutagenesis, but differ in phenotypic penetrance due to in-frame mutations. None of the methods can reliably predict reduced activity of the class I gRNAs. (G – J) gRNA activity scores plotted for individual pigmentation genes shows that lack of correlation in C – F is not caused by a single target gene. The four evaluated prediction methods for gRNA activity also failed to discriminate between those gRNAs targeting essential genes that gave a completely penetrant phenotype and those that did not. It should be noted, however, that the contribution of functional in-frame mutations to incompletely penetrant phenotypes is not known for these gRNAs.
| Construct(s) | Name(s) | Cas9 coding sequence | Integration site of cas9 construct | Reference |
|-------------|---------|----------------------|-------------------------------------|-----------|
| act-cas9*   | CFD1; BL54590 | Hs_Cas9 (41815, 62209) | attP-ZH2a (X) | Port et al. (2014) |
| vasa-cas9   | BL56552 | Hs_Cas9 (42230) | attP-VK00037 (2L) | Gratz et al. (2014) |
| vasa-cas9*  | BL51324 | Hs_Cas9 (42230) | attP-VK00027 (3R) | Gratz et al. (2014) |
| vasa-cas9*  | BL51323 | Hs_Cas9 (42230) | attP-ZH2a (X) | Gratz et al. (2014) |
| vasa-cas9   | BL52669 | Hs_Cas9 (41815) | attP-ZH2a (X) | Sebo et al. (2014) |
| nos-cas9*   | CFD2; BL54591 | Hs_Cas9 (41815, 62208) | attP-ZH2a (X) | Port et al. (2014) |
| nosG4VP16 UAS-cas9* | CFD3_nos; BL54593 | Dm-Cas9 | attP2 (3L) | Port et al. (2014) |
| nos-cas9    | CFD8 | Hs_Cas9:GFP (42234) | attP-ZH2a (X) | Port et al. (2014) |
| nos-cas9    | TH00787.N | Dr_Cas9 | attP2 (3L) | Ren et al. (2013) |
| nos-cas9    | TH00788.N | Dr_Cas9 | attP40 (2L) | Ren et al. (2013) |
| nos-cas9    | CAS-0001 | Hs_Cas9 (41815) | attP40 (2L) | Kondo and Ueda (2013) |
| nos-cas9    | CAS-0002 | Hs_Cas9 (41815) | Random insertion (X) | Kondo and Ueda (2013) |
| nos-cas9    | CAS-0003 | Hs_Cas9 (41815) | Random insertion (3) | Kondo and Ueda (2013) |
| nos-cas9    | CAS-0004 | Hs_Cas9 (41815) | Random insertion (2 (CyO balancer)) | Kondo and Ueda (2013) |

Abbreviations: act – actin5C; nos – nanos; Hs – Homo sapiens; Dm – Drosophila melanogaster; Dr – Danio rerio; BL – Bloomington Drosophila Stock Center stock number.

a, species refers to codon optimisation; all constructs express Streptococcus pyogenes Cas9 protein. *, lines that were also evaluated in Port et al. 2014.
**Supplementary Table 2: Summary of HDR results**

| Genotype of injected embryos | Injected plasmid(s) | DNA concentration gRNA/donor (ng/µl) | Independent experiments | No. of injected embryos | Embryo survival rate (%) | No. of G₀ males crossed | No. of sterile or dead G₀ males | No. of male founders/fertile G₀ males screened (%) | No. of RFP-positive F₁ males/total screened (%) | No. of confirmed knock-ins into wg/total screened (%) |
|-----------------------------|---------------------|-------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-----------------------------|---------------------------------------------|-------------------------------------------------|--------------------------------------------------|
| nos-cas9 CFD2, U6:3-gRNA-wg | pDonor wg-3P3-RFP   | 500                                 | Expt 1                  | 125                     | nd                      | 10                      | 2                          | 7/8 (88)                                    | 41/180 (23)                                    | 35/36 (97)                                      |
| nos-cas9 CFD2, U6:3-gRNA-wg | pDonor wg-3P3-RFP   | 100                                 | Expt 1                  | 151                     | 30                      | 13                      | 0                          | 9/13 (69)                                    | 46/242 (19)                                    | 7/8 (88)                                        |
| nos-cas9 TH_attP2, U6:3-gRNA-wg | pDonor wg-3P3-RFP   | 100                                 | Expt 1                  | 131                     | 18                      | 9                       | 2                          | 7/7 (100)                                    | 43/123 (35)                                    | 7/8 (88)                                        |
|                               |                     |                                     | Expt 2                  | 149                     | 32                      | 10                      | 1                          | 5/9 (56)                                     | 13/97 (13)                                     | 5/6 (83)                                        |
|                               |                     |                                     | Total                   | 280                     | 25                      | 19                      | 3                          | 12/16 (75)                                   | 56/220 (25)                                    | 12/14 (86)                                      |
| nos-cas9 CFD2                 | pU6:3-gRNA-wg/pDonor wg-3P3-RFP | 500/500                             | Expt 1                  | 91                      | nd                      | 10                      | 2                          | 7/8 (88)                                    | 19/222 (9)                                     | 14/18 (78)                                      |
| nos-cas9 CFD2                 | pU6:3-gRNA-wg/pDonor wg-3P3-RFP | 75/100                              | Expt 1                  | 136                     | 32                      | 16                      | 0                          | 8/16 (50)                                    | 18/232 (8)                                     | 5/6 (83)                                        |
|                               |                     |                                     | Expt 2                  | 117                     | 34                      | 8                       | 0                          | 3/8 (38)                                     | 9/270 (3)                                      | 4/8 (67)                                        |
|                               |                     |                                     | Total                   | 253                     | 33                      | 24                      | 0                          | 11/24 (46)                                   | 27/502 (5)                                     | 9/12 (75)                                       |
| nos-cas9 TH_attP2             | pU6:3-gRNA-wg/pDonor wg-3P3-RFP | 75/100                              | Expt 1                  | 134                     | 22                      | 11                      | 3                          | 6/8 (75)                                     | 33/217 (15)                                    | 4/4 (100)                                       |
| w¹¹²                         | pDCC6-gRNA-wg/pDonor wg-3P3-RFP | 75/100                              | Expt 1                  | 268                     | 7                       | 3                       | 1                          | 2/2 (100)                                    | 6/46 (13)                                      | 3/3 (100)                                       |
|                               |                     |                                     | Expt 2                  | 123                     | 52                      | 26                      | 5                          | 2/21 (10)                                    | 6/322 (2)                                      | 3/3 (100)                                       |
|                               |                     |                                     | Total                   | 391                     | 22                      | 29                      | 6                          | 4/23 (17)                                    | 12/368 (3)                                     | 6/6 (100)                                       |

Abbreviations: nd – not determined; Expt – experiment.

a. In total, 97/98 RFP-positive flies were confirmed to have integration at the expected site in the wg locus using two diagnostic PCRs (Figure 2 – figure supplement 1). For the other 11 flies, PCRs resulted in either a product for only one of the reactions (six flies; indicating a potential complex insertion event in the wg locus), or no products for either reaction (five flies; indicating potential insertion at another genomic location).
Supplementary Table 3: Assessment of somatic and germ line mutagenesis mediated by U6:3-gRNA transgenes targeting pigmentation genes

| gRNA spacer sequence  | Plasmid ID | Target gene | % phenotypically mutant cuticle per animal | % germ line transmission of non-functional alleles, mean ± SD | No. in-frame mutations /functional alleles analysed |
|-----------------------|------------|-------------|------------------------------------------|-----------------------------------------------------------|--------------------------------------------------|
| GCGATAAGTGGAGCCAGGC   | pFP407     | y           | 100                                      | 100                                                       | nd                                               |
| gATTCTGCACTGTTCCCGGC  | pFP570     | y           | 100                                      | 100                                                       | nd                                               |
| gCTTGGAGATGGCAACTATT  | pFP566     | y           | 80 - 100                                 | 99 ± 2                                                   | nd                                               |
| gCAAAGTGTCCAGTCCACAC  | pFP553     | y           | 100                                      | 100                                                       | nd                                               |
| gAGGACCAAGCTCTGGCTAG   | pFP552     | y           | 80 - 100                                 | 93 ± 7                                                   | nd                                               |
| gTGGCCATCTGGAAGCTGG   | pFP545     | e           | 100                                      | 100                                                       | nd                                               |
| gATCGATCTACAGAAAGCTTA | pFP578     | e           | 100                                      | 100                                                       | nd                                               |
| gCAGCAATATGTTGGATAGT  | pFP507     | e           | 80 - 100                                 | 99 ± 1                                                   | nd                                               |
| gTCTCACCTCGGGCAGTAC   | pFP573     | e           | 100                                      | 99 ± 1                                                   | nd                                               |
| gACAGAGAAGCATCTCAT    | pFP576     | e           | 100                                      | 97 ± 5                                                   | nd                                               |
| gTTGGAGCCTTATAAGATC   | pFP574     | e           | 40 - 80                                  | 96 ± 6                                                   | nd                                               |
| gGTGGGCTTCCGGCCACAGG  | pFP527     | e           | 40 - 80                                  | 90 ± 13                                                  | 2/3                                              |
| gAGTGCGCTGAGGACGTCT   | pFP529     | e           | 80 - 100                                 | 87 ± 12                                                  | 3/3                                              |
| gGTGTCATGACGGCTCGG    | pFP548     | e           | 80-100                                   | 84 ± 10                                                  | 2/3                                              |
| gGCACGACCATGCTCAG     | pFP575     | e           | 80 - 100                                 | 81 ± 7                                                   | 2/2                                              |
| gCTGACTGGGCCCATCTCC   | pFP514     | e           | 40 - 80                                  | 80 ± 15                                                  | 3/3                                              |
| gTCTTCGACACAAACAGCA   | pFP530     | e           | 40 - 80                                  | 75 ± 5                                                   | 3/3                                              |
| gCCTGACATGCAGAATTC    | pFP547     | e           | 40 - 80                                  | 70 ± 23                                                  | 3/3                                              |
| gTCTTCGGCCTCCGGATGCT  | pFP510     | e           | 40 - 80                                  | 65 ± 21                                                  | 3/3                                              |
| gCTTCAGGACAGACAGCT    | pFP546     | e           | 40 - 80                                  | 45 ± 31                                                  | 8/8*                                             |
| gCGACACGTGGCTGATGCT   | pFP519     | e           | 80 - 100                                 | 40 ± 4                                                   | 0/6                                              |
| gACATCGCTGTTGGCTGC    | pFP549     | e           | 40 - 80                                  | 35 ± 9                                                   | 0/6                                              |
| gCACAATTTGCTCAGTCA    | pFP544     | e           | 5 - 40                                   | 13 ± 4                                                   | 0/7                                              |
| % area of eye with sepia pigmentation, mean ± SD |

g, gRNAs for each gene are listed in the same order as in Figure 3; nucleotides in lower case are mismatched to the genomic target; b, estimate based on observation of > 20 flies of each genotype; c, from scoring pigmentation of progeny of three independent crosses of act-cas9 gRNA flies to a homozygous e mutant strain. At least 125 progeny were analysed for each genotype; nd = not determined; d, gRNA-γ transgene first described in Port et al. 2014; e, mean of estimates by three independent observers blind to the genotype of each fly (based on observation of > 20 flies per genotype per researcher); *, gRNA target site with suspected microhomology bias in non-homologous end joining; all eight analysed flies had deletions of either six or nine base pairs, consistent with the microhomology around the cut site. **, gRNA (designed using Drosophila reference genome) contained a single nucleotide polymorphism to the se sequence in our experimental strains.
## Supplementary Table 4: Computed gRNA efficiency scores and 3′ 6nt GC content

| gRNA spacer sequence | Plasmid ID | Target gene | E-CRISPR efficiency score | DRSC efficiency score | Broad Institute gRNA score | 3′ 6nt GC content |
|----------------------|-----------|-------------|--------------------------|----------------------|---------------------------|-------------------|
| GTATCTGTATCAAGGACA   | pFP505    | Dhc64C      | 36.7                     | 3.91                 | 0.584                     | 50                |
| GATAAGCTGGAGGACCCGC   | pFP508    | Dhc64C      | 35.0                     | 4.99                 | 0.108                     | 83                |
| GCTTGAAGCACCACCTGGAT  | pFP511    | Dhc64C      | 34.8                     | 7.72                 | 0.004                     | 50                |
| GACTACAATCTCTGATAG    | pFP516    | Dhc64C      | 49.3                     | 2.89                 | 0.073                     | 33                |
| GAAAGGCGGAGTGCCAGC    | pFP535    | Dhc64C      | 40.0                     | 9.90                 | 0.449                     | 83                |
| GATCTGCTGCCACACAGGT   | pFP541    | Dhc64C      | 28.1                     | 3.57                 | 0.581                     | 67                |
| GCGAGGTTTGGCAGCTCCTTA | pFP504    | Dhc64C      | 42.9                     | 3.64                 | 0.013                     | 17                |
| GACTGCTGCTGATATACCA   | pFP532    | Dhc64C      | 41.8                     | 2.07                 | 0.741                     | 50                |
| GATGCTACGCTATCGAGG    | pFP518    | Dhc64C      | 36.9                     | 5.70                 | 0.411                     | 67                |
| gTTGGAGACCTTCGCAGAG   | pFP551    | Lis1        | 33.0                     | 7.08                 | 0.397                     | 67                |
| GATGCGCTCCTGCCAGGA    | pFP554    | Lis1        | 31.7                     | 7.90                 | 0.272                     | 67                |
| GGGAGATTACACCTCAGC    | pFP555    | Lis1        | 32.1                     | 4.62                 | 0.089                     | 83                |
| gCAGAGAAGTGTTCTCCTGAC | pFP569    | Lis1        | 55.3                     | 5.06                 | 0.222                     | 67                |
| gCAAAGGAGCCTGCCTGCAG  | pFP550    | Lis1        | 33.1                     | 8.77                 | 0.301                     | 83                |
| gCAGTCTCAGCAGATCAGA   | pFP513    | wg          | 47.3                     | 3.96                 | 0.529                     | 50                |
| gAGAAATCGGGAGGGGCGG    | pFP515    | wg          | 42.5                     | 3.68                 | 0.353                     | 100               |
| GGGAGATACAAATCCCAGAG  | pFP533    | wg          | 47.8                     | 5.94                 | 0.636                     | 67                |
| GAGCACTGCTGACGAGATCG   | pFP411    | wg          | 44.3                     | 4.98                 | 0.201                     | 50                |
| gTTTCTGTTTGGCCTCAGT   | pFP506    | wg          | 38.1                     | 4.46                 | 0.157                     | 50                |
| gGGCCAAAGAGAATCCCGAG  | pFP521    | wg          | 55.3                     | 3.84                 | 0.449                     | 67                |
| GACTCTGCTGCCCGCTGCAC  | pFP542    | wg          | 47.6                     | 6.40                 | 0.021                     | 67                |
| gACAGAAATCGGGAGGCCG    | pFP538    | wg          | 49.5                     | 5.95                 | 0.068                     | 83                |
| gACCATACTGGAAGACCTGAG | pFP410    | wls         | 27.8                     | 6.04                 | 0.414                     | 67                |
| GCTGTAAATGTACCTGCCA   | pFP412    | wls         | 40.5                     | 5.09                 | 0.627                     | 67                |
| gGGCCCAAGCAGTCCATAT   | pFP512    | wls         | 40.5                     | 6.77                 | 0.018                     | 33                |
| gGACATGCGGCGGGCAGAAG   | pFP525    | wls         | 45.4                     | 6.62                 | 0.071                     | 83                |
| gACCTGCGAGAGCAGAAGAG   | pFP522    | wls         | 44.6                     | 4.13                 | 0.391                     | 50                |
| gAGCAAAGTGGCCACCAATA  | pFP526    | wls         | 39.0                     | 3.35                 | 0.065                     | 33                |
| gGGACAGTCTGGCGGCACCTC  | pFP531    | wls         | 19.1                     | 5.07                 | 0.056                     | 67                |
| gTTTCTGCTGTACTACG     | pFP534    | wls         | 54.1                     | 7.93                 | 0.886                     | 67                |
| gCCACATATGGACAGGCTTCG  | pFP509    | wls         | 38.5                     | 4.00                 | 0.512                     | 50                |
| gATCCAGTGAGCCGGAGAGG   | pFP523    | wls         | 51.1                     | 6.00                 | 0.434                     | 67                |
| gCCAGTGCCGCCAGGGTCAGG  | pFP520    | wls         | 41.1                     | 7.12                 | 0.396                     | 67                |
| gTTTCTGCCATTTGCCACCA   | pFP556    | se          | 55.8                     | 4.94                 | 0.115                     | 67                |
| gATGCACCGCGTTGGCAAAT  | pFP567    | se          | 64.2                     | 5.62                 | 0.433                     | 33                |
| GACGCGACATCATGAGTAA    | pFP564    | se          | 43.7                     | 6.17                 | 0.537                     | 33                |
| gGGCCAGGATGATCCTTCCT  | pFP563    | se          | 41.6                     | 4.98                 | 0.019                     | 33                |
| gTCTGGGAAACATCCGGCA    | pFP557    | se          | 41.8                     | 7.49                 | 0.034                     | 83                |
| GCACATGATGAGTAACGGC    | pFP562    | se          | 42.9                     | 5.77                 | 0.270                     | 67                |
| gTGGCCAGATGTTCCGGAGAG  | pFP558    | se          | 40.1                     | 4.10                 | 0.107                     | 50                |
| gCGAAGCCGCCATGAGTAC    | pFP561    | se          | 53.9                     | 4.30                 | 0.115                     | 50                |
| gCCACTAGGCTCACCCATGC   | pFP565  | se  | 42.9 | 6.54 | 0.024 | 67 |
|-------------------------|---------|-----|------|------|-------|----|
| gATTCGTCACTGTTCCCCGC   | pFP570  | y   | 53.7 | 7.28 | 0.304 | 100|
| gCAAAGTGTTCGACTCCAAC   | pFP553  | y   | 40.7 | 3.04 | 0.716 | 50 |
| gAGGACCAAGCTCTGCTTAG    | pFP552  | y   | 47.7 | 6.90 | 0.029 | 67 |
| gTGCCATCTGGAAGGCTGGG   | pFP545  | e   | 48.8 | 5.47 | 0.240 | 83 |
| gATCGAGTCAGGAAGGTAT    | pFP578  | e   | 35.3 | 7.67 | 0.163 | 33 |
| gCAGCGGTATGTGGTGAATG   | pFP507  | e   | 32.2 | 5.13 | 0.508 | 33 |
| gCTACACCTCGGGGCGATAC   | pFP573  | e   | 32.9 | 4.99 | 0.023 | 50 |
| GCACGAGACATCTCTCAAT     | pFP576  | e   | 34.6 | 3.96 | 0.164 | 33 |
| GTTGGACGCTTATAAGATC     | pFP574  | e   | 37.4 | 6.64 | 0.113 | 33 |
| gTTGCTGCTGACCCCTGCGG   | pFP548  | e   | 44.0 | 5.42 | 0.045 | 83 |
| GGCTCAACCTGCTCTCAG      | pFP575  | e   | 29.5 | 5.21 | 0.175 | 50 |
| gCTGACGTGGGCCAATTCC     | pFP514  | e   | 39.6 | 4.39 | 0.018 | 50 |
| gTCTGTGCGACAAACGCAGA   | pFP530  | e   | 41.6 | 6.24 | 0.332 | 67 |
| gCCTGAGATCTGACCAATTTG   | pFP547  | e   | 29.4 | 5.45 | 0.097 | 33 |
| gTTCCTGCGTGGCCTGCT    | pFP510  | e   | 42.5 | 9.58 | 0.074 | 50 |
| gCTCGAGAGCAGCAGCTG     | pFP546  | e   | 29.2 | 5.70 | 0.114 | 67 |
| gGCACAGCTCCTCCATCTG    | pFP519  | e   | 45.1 | 6.08 | 0.545 | 50 |
| gACCACGCTCCTGCTCTG     | pFP549  | e   | 52.2 | 6.18 | 0.027 | 83 |
| gCAATTGTCGATCGTCAA      | pFP544  | e   | 43.8 | 5.99 | 0.535 | 50 |

a, gRNAs for each gene are listed in the same order as in Figure 3; nucleotides in lower case are mismatched to the genomic target; b, accessed in January 2015; c, www.e-crispr.org; d, http://www.flyrnai.org/evaluateCrispr/; e, http://www.broadinstitute.org/maipublic/analysis-tools/sgrna-design; f, gRNA transgene first described in Port et al.
### Supplementary Table S5: Oligonucleotides used in this study (5' – 3')

#### Primers used for cloning:
- pBSwg3'HAfwd: CAATTA GGCAAAACCATGCGAAGCTTCCATGTGGTGGTAAGTTCATTG
- pBS-wg5'HArev: CCTTCCGGATTTCTGTTTGCCTTCGACTTGGCTGAGACT
- 3xP3-RFPfwd: CGAAGGCAAACGAGAATCCGGAAGGGGATCTAATTCAATTAGAGAC
- 3xP3-RFPrev: AGCTTCGCAATGTTTTCCTAATTTGTTTCAGATTTATGGG

#### Primers used to identify CRISPR/Cas induced mutations:
- ebonygenofwd: GATATCGAATTCCTGCAGCCCGGGGGATCCAAATCGTATTACCCTGAAACCCTT
- ebonygenorev: GGAGCTCCACCGCGGTGGCGGCCGCTCTAGAGATGAAGTCACCATCGCTGTTTG
- egenotyperev2: GCATCTCCTCGAGACGAGATTG
- egenotypefwd2: CATCGAGCCGGCTTCTCCAAGGG
- egenotyperev3: GATAGGGGTCTCCGGAGACGAGACC
- segenofwd: CTCGAAATGGCAATCCGATATCACC
- segenorev: CGACTCTGATCGGAGTTATATCC
- segenotypew2: GATAAGCGGAACGCCGAGCGCCGAAAG
- segenotypewrev2: CTGCTGAGCCGGCCGATCCGCGGTGGCGGCCGCTCTAGATCACCCAAAACCCCATTCATATCT
- wggenofwd: GATATCGAATTCCTGCAGCCCGGGGGATCCACAAATGAGCAGCAATATCGGC
- wggenorev: GGAGCTCCACCGCGGTGGCGGCCGCTCTAGATCACCCAAAACCCCATTCATATCT
- wlsgenofwd: GATATCGAATTCCTGCAGCCCGGGGGATCCTCACACTGCTTTGTTGATTCTTGA
- wlsgenorev: GGAGCTCCACCGCGGTGGCGGCCGCTCTAGATCGAGTTATATCC
- wgHRgenofwd1: GAAGCGGCCAAGCAATGGATGAGG
- wgHRgenorev1: GAATTGTCGCTCCGTAGACGAAGC
- wgHRgenofwd2: GTGTCAGTCCTGCTTACAGGAACG
- wgHRgenorev2: TCGCTGGGTCCATGTACATGATGGG

#### Primers used to identify gRNA transgenes from pooled injections:
- U63fwd1: TGTATGCTATACGAAGTTATGCTAGCGGATCCTTGCTCACCTGTGATTGCTCC
- CFD4seqrev3: GCCGGACGAAATTTGCTAGAAATGC

#### Primers used to diagnose insertion of the 3xP3-RFP cassette into the wg locus:
- wgHRgenofwd1: GAAGCGGCCAAGCAATGGGAAGG
- wgHRgenorev1: GAATTGTCGCTCCGTAGACGAAGC
- wgHRgenofwd2: GTGTCAGTCCTGCTTACAGGAACG
- wgHRgenorev2: TCGCTGGGTCCATGTACATGAGTGG