Expansion of the CRISPR toolbox in an animal with tRNA-flanked Cas9 and Cpf1 gRNAs

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Abstract We present vectors for producing multiple CRISPR gRNAs from a single RNA polymerase II or III transcript in Drosophila. The system, which is based on liberation of gRNAs by processing of flanking tRNAs, permits highly efficient multiplexing of Cas9-based mutagenesis. We also demonstrate that the tRNA-gRNA system markedly increases the efficacy of conditional gene disruption by Cas9 and can promote editing by the recently discovered RNA-guided endonuclease Cpf1.

The clustered regularly-interspaced short palindromic repeats (CRISPR) system greatly facilitates genome engineering in many species (Doudna and Charpentier, 2014). The method involves site-specific DNA cleavage by a bacterial endonuclease – such as the prototypical CRISPR-associated 9 (Cas9) protein – which is directed to a specific genomic target site by a short, single guide RNA (gRNA). Imprecise repair of the DNA break can create loss-of-function insertion/deletion (indel) mutations, whereas homology-directed repair in the presence of an experimentally provided donor DNA can generate specific sequence alterations.

Despite the power of current CRISPR approaches, improvements are necessary to realise the full potential of this technology in basic research, as well as in applied settings. One current focus is the development of robust methods for simultaneous expression of several gRNAs in an organismal setting. Multiplexed gRNA expression would, for example, facilitate the generation of non-functional mutations in a single target gene, as well as the creation of models of polygenic human diseases. The use of multiple gRNAs is also expected to significantly reduce the risk of natural resistance to gene drives designed to combat insect-borne diseases such as malaria or Zika virus (Champer et al., 2016).

Multiplexing methods that liberate gRNAs from a single precursor RNA in the nucleus have the added advantage of allowing expression from RNA polymerase (pol) II promoters (Nissim et al., 2014), as opposed to the RNA pol III promoters that are conventionally used to avoid the modification of gRNAs and subsequent export to the cytoplasm. Coupling of gRNA production to RNA pol II transcription gives scope to regulate production of guide sequences in time and space, thus affording additional control of CRISPR mutagenesis. Strategies to excise multiple gRNAs from a single precursor transcript have been described (Nissim et al., 2014; Tsai et al., 2014; Xie et al., 2015). However, they have not yet been adopted for use in animal models.

Here we describe novel vectors for multiplexed gRNA expression in Drosophila. These plasmids are inspired by a tRNA-gRNA expression system recently described for genome engineering in rice (Xie et al., 2015), in which the endogenous tRNA processing machinery excises multiple functional gRNAs from a single precursor transcript in the nucleus (Fig. 1a). We first demonstrated that a flanking tRNA does not interfere with production of functional gRNAs in Drosophila. The Drosophila tRNA$^{Gly}$ was inserted between the strong, ubiquitously active RNA pol III promoter U6:3 and a previously characterised gRNA, which targets the eye pigmentation gene sephia (se) (Port et al., 2015). The U6:3-tRNA-gRNA (U6:3-t::gRNA) transgene was transformed into the...
Figure 1: Multiplexed Cas9 gRNA expression in Drosophila with the tRNA-gRNA expression system. (a) Principle of the tRNA-gRNA system. gRNAs are excised from a long precursor transcript by the endogenous tRNA processing machinery (Xie et al., 2015). (b) Comparison of rates of target gene mutagenesis of gRNAs in the presence (−) or absence (+) of a single 5' tRNA in the expression vector. y-axis represents rates of germline transmission of non-functional alleles of se or e by flies that are transgenic for both act-cas9 and the indicated gRNA expression vector (five crosses per condition). ns, not significant; error bars, sem. (c) Comparison of rates of mutagenesis between individual gRNA transgenes targeting se and all four gRNAs expressed simultaneously from a pCFD5 U6:3-t::gRNA transgene. Data show proportions of eyes (10% bins) of act-cas9/gRNA animals that are sepia, which indicates non-functional mutations in both se alleles. N, number of eyes scored. (d) The use of pCFD5 to generate mutations in multiple genes in a single step. Percentages of offspring of act-cas9/U6:3-t::gRNA-se<sup>558</sup>-e<sup>549</sup>-cu<sup>801</sup>-f<sup>801</sup> transgenic animals that inherit mutations in se, e, cu and f are shown. Mutation frequencies were determined by sequencing PCR products from the target loci in 48 offspring. The data therefore do not discriminate between functional and non-functional CRISPR alleles.
Drosophila genome and combined through a genetic cross with an act-cas9 transgene, which expresses Cas9 ubiquitously from the actin5C promoter (Port et al., 2014). The rate of germline transmission of loss-of-function se mutations by the act-cas9/U6:3-t:gRNA animals was similar to a control in which no tRNA was present in the gRNA expression construct (Fig. 1b). The presence of the tRNA in the construct also did not influence the rate of germline mutagenesis by a second gRNA, which targeted the pigmentation gene ebony (e) (Fig. 1b).

We next created pCFD5, a vector that facilitates cloning of several Cas9 gRNAs flanked by Drosophila or rice tRNA flanked by a single U6:3 promoter (see Supplementary Methods for cloning protocol). Transgenic flies were generated that harbour a pCFD5 construct containing four different gRNAs targeting se. We had previously shown that expression of the individual gRNAs in the presence of act-cas9 results in biallelic disruption of se in only a fraction of eye tissue (Port et al., 2015). This was confirmed in the current study, with the mean amount of se mutant tissue per eye ranging from 11 to 58% for the single gRNAs (Fig. 1c). Incompletely penetrant mutagenesis with these gRNAs is due to either suboptimal cleavage by Cas9 or in-frame mutations at the target sites resulting in a functional protein product (Port et al., 2015). Strikingly, expressing all four gRNAs simultaneously with pCFD5 in the presence of act-cas9 resulted in biallelic disruption of se in 100% of eye tissue in almost all cases (Fig. 1c). Thus, using the tRNA-gRNA system to simultaneously express several gRNAs can circumvent the problems of functional in-frame mutations and gRNAs with low activity.

We also created transgenic flies harbouring a pCFD5 construct containing four gRNAs that each target a different gene: se, e, curled (cu) and forked (f). Animals expressing both act-cas9 and U6:3-t:gRNA-se-e:cu:f had a high penetrance of the visible phenotypes associated with biallelic disruption of each

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**Figure 2:** The UAS-tRNA-gRNA system increases mutagenesis efficiency and tissue specificity of conditional CRISPR. (a) Cartoon of a third-instar wing imaginal disc showing the expression pattern of hh-Gal4. Dotted rectangle shows area imaged. (b – d) Wing imaginal discs of indicated genotypes stained for endogenous Wls. (b) Wls is found throughout hh-Gal4 UAS-cas9 control discs (control genotype is the same in f and g). Image is representative of 15 discs analysed. (c, c’) Introduction of a ubiquitously expressed gRNA-wls transgene results in incomplete mutagenesis in the posterior compartment and unwanted mutagenesis in the anterior compartment. c and c’ show, respectively, examples of complete and partial loss of Wls immunoreactivity in the posterior compartment (arrow in c’ indicates a large posterior clone that retains Wls signal). The disc in e also contains a large Wls-negative clone in the anterior compartment (arrow). (e) Mutagenesis in anterior compartment of disc. Statistical significance was evaluated with a Mann Whitney test.

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gene (Supplementary Fig. 1). Of the progeny of act
-cas9/U6:3:t-gRNA-se-e:cu:tf animals, 17% inherited
indel mutations at all four loci (Fig. 1d). 48% of all
progeny had mutations in se, e, and cu, which are
located on the same chromosome. Creating a triple
mutant chromosome by recombining three single
mutations would be time consuming, especially for
genes without a visible phenotype. Our results
demonstrate that pCDF5 allows efficient generation
of complex genotypes in a short amount of time.

We next tested the performance of tRNA-gRNA
vectors in another important application: the disruption
of gene function in specific cell types in multicellular
organisms. The use of CRISPR for such applications
is desirable because, in principle, null mutations can
be induced that produce more penetrant effects than
RNAi knockdown approaches. Conditional CRISPR
mutagenesis has been attempted in Drosophila
by tissue specific expression of Cas9 with the binary,
RNA pol II-based UAS/Gal4 system in combination
with individual gRNAs expressed from ubiquitously
active RNA pol III promoters (Port et al., 2014; Xue
et al., 2014). However, in our hands these approaches
lead to poorly penetrant phenotypes in the target
tissue or mutagenesis outside the desired domain
(Port et al., 2014 and unpublished observations). Both
these factors can complicate the interpretation of
experimental findings significantly.

Based on our previous findings with se we reasoned
that the expression of multiple guides from tRNA-
gRNA vectors should increase the penetrance of loss-
of-function phenotypes in the Gal4 expression domain.
We also hypothesised that our approach would
address the issue of mutagenesis outside the intended
domain. Such mutagenesis is caused by stochastic
expression of the UAS-cas9 transgenes in the
presence of the ubiquitously expressed gRNA.
Expressing both Cas9 and gRNA under UAS control
should increase tissue-specificity, because it is
unlikely that sufficient ‘leaky’ expression of both
transgenes would occur at the same time in the same
cell. Previous attempts to express functional gRNAs
from RNA pol II promoters in Drosophila using
conventional vectors have been unsuccessful (Ren et
al., 2013), presumably due to modification of the RNA
and export to the cytoplasm. As described above,
tRNA-mediated excision of gRNAs from larger
transcripts in the nucleus should allow expression of
active guides from this class of promoters.

We integrated into the genome a UAS-tRNA-gRNA
construct (UAS-t:gRNA-wls\textsuperscript{2x}), which contains two
different Cas9 gRNAs targeting the ubiquitously
expressed Wnt secretion factor \textit{wntless} (\textit{wls/evi}). We
compared the activity of this transgene to \textit{U6:3-gRNA-
wls}, which has one of the \textit{wls} gRNAs under the control
of the \textit{U6:3} promoter (Port et al., 2014). This was
achieved by combining each transgene with an
optimised UAS-cas9 transgene (see Methods), and
the \textit{hedgehog-Gal4 (hh-Gal4)} driver (Tanimoto et al.,
2000), which activates expression of UAS transgenes
in the posterior compartment of wing imaginal discs
(Fig. 2a). Only 60% of hh-Gal4 UAS-cas9 U6:3-gRNA-
wls wing discs had no detectable Wls protein in cells
of the posterior compartment (n = 30; Fig. 2b – c’). In
contrast, all hh-Gal4 UAS-cas9 UAS-t:gRNA-wls\textsuperscript{2x}
discs had lost Wls immunoreactivity throughout the
posterior compartment (n = 22; Fig. 2d). Thus, the
UAS-tRNA-gRNA construct induced much more
efficient biallelic mutagenesis within the target domain.

Moreover, the U6:3-gRNA-wls transgene caused
extensive mutagenesis outside the hh-Gal4
expression domain. In 60% of the hh-Gal4 UAS-cas9
U6:3-gRNA-wls wing discs, there was anterior
compartment tissue that had no (9 clones) or reduced
levels (12 clones) of Wls protein (Fig. 2c, e). These
patches ranged in size between a few cells to
approximately half the size of the anterior
compartment (Fig. 2e). Despite more efficient
mutagenesis in the posterior compartment than
observed with \textit{U6:3-gRNA-wls}, only 10% of the hh-
Gal4 UAS-cas9 UAS-gRNA-wls\textsuperscript{2x} discs had patches
of cells in the anterior compartment with reduced Wls
levels, and these were very small (Fig. 2e). The
effects we observed on disruption of Wls expression in
the anterior and posterior compartment of the wing
disc with U6:3-gRNA-wls and UAS-t:gRNA-wls\textsuperscript{2x}
were mirrored by the strength and location of \textit{wls}
mutant phenotypes in the adult wing (Fig. 2f, g). Together,
these results demonstrate that UAS-t:gRNA
expression vectors make it possible to perform
conditional CRISPR mutagenesis with high efficiency
and tight spatial control.

The data above demonstrated that the tRNA-gRNA
system enhances Cas9-based CRISPR applications in
Drosophila. Next, we explored whether this system
can augment genome engineering by the RNA-guided
endonuclease Cpf1. Cpf1 has a number of properties
distinct from Cas9 that have the potential to broaden
genome engineering applications (Zetsche et al.,
2015). Because Cpf1 uses a poly-T protospacer
adjacent motif (PAM) in the target DNA (Zetsche et al.,
2015), as opposed to Cas9’s G-rich PAM (Jinek et al.,
2012; Kleinstiver et al., 2015), the number of genomic
sites for CRISPR is increased. Furthermore, the
staggered cleavage of Cpf1 relatively far from the
PAM (Fig. 3a) could conceivably facilitate CRISPR-
induced homology-directed repair (Zetsche et al.,
2015). However, genome engineering by Cpf1 had
thus far only been demonstrated in cultured mammalian
cells.

We constructed a Drosophila expression plasmid for
production of AsCpf1 – one of the active Cpf1 variants
in mammalian cells (Zetsche et al., 2015) – from the
\textit{actin5c} promoter. We also produced plasmids with a
Cpf1 gRNA targeting e with or without flanking tRNAs under the control of the U6.3 promoter. Co-injection of embryos with the act-cpf1 plasmid and the Cpf1 gRNA-e plasmid without the tRNA resulted in transmission of non-functional e mutations to 1.6% of the progeny (Fig. 3b). Interestingly, the germline transmission rate was increased to 6% after injecting the act-cpf1 plasmid and the plasmid encoding the e gRNA flanked by tRNAs (Fig. 3b). Targeted sequencing of the e locus confirmed the transmission of indel mutations at the gRNA target site (Fig. 3c). The vast majority of deletions were 10 – 20 bp in size (Fig. 3d). This is significantly larger than the deletions typically induced by Cas9, which rarely exceed 10 bp (Moreno-Mateos et al., 2015; Fig. 3d). Analysis of more target sites will be necessary to confirm whether the creation of relatively large indels, which are more likely to be mutagenic, is a general feature of Cpf1-mediated mutagenesis.

To test if increased mutagenesis with tRNA-flanked guides is a consistent feature of mutagenesis by Cpf1 in Drosophila, we generated flies with stable genomic integrations of act-cpf1 and the U6.3-gRNA plasmids targeting e. We again observed a significant increase in germline transmission rates with the tRNA-flanked gRNA transgene compared to a transgene expressing the gRNA alone (Fig. 3b). Surprisingly, the germline transmission rates were similar for the same gRNA when the CRISPR components were provided by transgenes or by injected plasmids (Fig. 3b). This contrasts to the situation for Cas9 gRNAs, where mutagenesis is significantly increased by supply of the endonuclease and gRNA by transgenes (Kondo and Ueda, 2013; Port et al., 2014). This method results in the majority of alleles being mutagenised by Cas9 using the vast majority of gRNAs (Port et al., 2015). These observations suggested that Cpf1 might not perform as efficiently as Cas9 in Drosophila genome engineering experiments. Consistent with this notion, in plasmid injection experiments we did not detect any germline transmission of non-functional mutations using act-cpf1 and three other Cpf1 gRNAs (targeting either se or the pigmentation gene yellow (y)), even in the presence of flanking tRNAs (Supplementary Fig. 2). We conclude that Cpf1 is able to act as an RNA-guided endonuclease in Drosophila. However, improvements will be necessary to transform Cpf1 into
a robust genome editing tool. Our data indicating that flanking active Cpfl gRNAs with tRNAs can enhance genome editing in vivo is a first step in this direction. Future experiments will investigate the mechanism by which tRNAs can increase the activity of Cpfl gRNAs.

In summary, our data demonstrate that the tRNA-gRNA system can enhance Cas9 and Cpfl-mediated CRISPR mutagenesis in an animal model. In addition to the applications discussed above, we envisage that multiplexed gRNA expression with this method will prove valuable in vivo for disrupting the function of non-coding regulatory elements and for robust transcriptional activation (CRISPRa) and repression (CRISPRi), which often requires the action of multiple guides (Lin et al., 2015; Maeder et al., 2013; Mali et al., 2013a; Perez-Pinera et al., 2013). Furthermore, the use of flanking tRNAs will increase the repertoire of genomic sequences that can be targeted with novel high-fidelity Cas9 variants (Kleinstitver et al., 2016; Slaymaker et al., 2015). Because these endonucleases are unable to tolerate mismatches between the gRNA and genomic DNA, target site selection has been constrained by the need for a 5' G in the guide sequence for optimal transcriptional initiation by RNA pol III. This is not the case when using tRNA-gRNA vectors with this polymerase, as transcription is initiated before the 5' tRNA.

METHODS

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gRNA expression plasmids

Unless noted otherwise, PCRs were performed with the Q5 Hot-start 2x master mix (New England Biolabs (NEB)) and cloning was performed using the Gibson Assembly 2x Master Mix (NEB) following the manufacturer's instructions. The sequence of each insert was verified by Sanger sequencing. gRNA expression plasmids are presented in Supplementary Table 1.

To streamline cloning of gRNAs into a pUAS-t::gRNA vector, pUAS-t::gRNA-wls² was modified to contain a BbsI cloning cassette. pUAS-t::gRNA-wls² was cut with EcoRI and XbaI and the linear backbone gel purified. Inserts were generated by PCR using pCFD5 as the template and the following primers: pCFD6fwd1: TGAATTCAGACGCTATCAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGT
CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAG
ACCATACTGGAGAAACTGAGGTATTAGAGCTAGA
ATAGCAAG and pCFD6rev1: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG with UASIRNAfwd1:CTCAGGTTCTTCCAGATTGTTCAATCGGCGCCGGGAATCGAACCCGG
ATCGAACCAG and UASIRNArev1: ATAGCAAG and UASIRNArev2: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG to generate a fragment encoding the first gRNA-wls and rice tRNA⁵⁰; and UASIRNAfwd3:CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAGA
ATAGCAAG and UASIRNArev3: ACAGAAGTGATATTCGCCAGTTTTAGAGCTAGA
ATAGCAAG and UASIRNArev4: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG to generate a fragment encoding the second gRNA-wls and rice tRNA⁵⁰. The three inserts and the pBID-UASC backbone were assembled by Gibson Assembly.

pCFD6. To streamline cloning of gRNAs into a pUAS-t::gRNA vector, pUAS-t::gRNA-wls² was modified to contain a BbsI cloning cassette. pUAS-t::gRNA-wls² was cut with EcoRI and XbaI and the linear backbone gel purified. Inserts were generated by PCR using the following primers and templates: pCFD6fwd1:GCCAATTTTGAATCACAGGCGC and pCFD6rev1:AGACCCCTGATTCGGCGGGAATCGAACCAG with template pUAS-t::gRNA-wls²; pCFD6fwd2:CGGGTTCGATTCCCGGCCGATGC and pCFD6rev2:GCCAACTTTGAATCACAAGACGCATACCAAACGA with template pCFD5.2 (the first gRNA core sequence is modified according to (Dang et al., 2015)); pCFD6fwd3:GAAGACCTGTTTTAGAGCTAGAATAGCAAGT and pCFD6rev3:ACACACAGGAATCACAAGGCTAGAATTCC at this position had reduced activity of 3' gRNAs. A cloning protocol to generate pCFD5 plasmids encoding one to six tRNA-flanked gRNAs is provided in the Supplementary Methods. pCFD5 is available from Addgene (Plasmid 73914).

pUAS-t::gRNA-wls². Two tRNA-flanked wls gRNAs were cloned into the pBID-UASC backbone (gift from Brian McCabe (Wang et al., 2012), Addgene 35200). pBID-UASC was digested with EcoRI and XbaI and the linear plasmid gel purified. Inserts were generated by PCR using pCFD5 as the template and the following primers: UASIRNAfwd1: TGAATTCAGACGCTATCAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGT
CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAG
ACCATACTGGAGAAACTGAGGTATTAGAGCTAGA
ATAGCAAG and UASIRNArev1: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG with UASIRNAfwd1:CTCAGGTTCTTCCAGATTGTTCAATCGGCGCCGGGAATCGAACCCGG
ATCGAACCAG and UASIRNArev1: ATAGCAAG and UASIRNArev2: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG to generate a fragment encoding the first gRNA-wls and rice tRNA⁵⁰; and UASIRNAfwd3:CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAGA
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CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAG
ACCATACTGGAGAAACTGAGGTATTAGAGCTAGA
ATAGCAAG and UASIRNArev1: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG with UASIRNAfwd1:CTCAGGTTCTTCCAGATTGTTCAATCGGCGCCGGGAATCGAACCCGG
ATCGAACCAG and UASIRNArev1: ATAGCAAG and UASIRNArev2: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
ATCGAACCAG to generate a fragment encoding the first gRNA-wls and rice tRNA⁵⁰; and UASIRNAfwd3:CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAGA
ATAGCAAG and UASIRNArev3: ACAGAAGTGATATTCGCCAGTTTTAGAGCTAGA
ATAGCAAG and UASIRNArev4: TGGCGAATATCACTTAGCAGTGCACCAGCCGGGAATCGAACCCGG
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pU6-3: hAsCpf1-gRNA. Expression plasmids for Cpf1 gRNAs were generated by Gibson Assembly using pCFD5 digested with BbsI and XbaI and inserts generated by PCR using the following primers and pCFD5 as a template: Cpf1gRNAPcr1fwd TGGACACCGGGGAATCGAC to generate a fragment encoding the Cpf1gRNA and and Cpf1gRNAPcr1rev TTGCCATCCCTTGACATTGCTCTAG and TGACACCGGGGAATCGAC to generate a fragment encoding the Cpf1gRNA and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr2fwd ACAGACCGGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev TATACTGTGCGGACACAATTTGCTCTAGATGACCCGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr1rev TTGCCATCCCTTGACATTGCTCTAG and TGACACCGGGGAATCGAC to generate a fragment encoding the Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr2fwd ACAGACCGGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev TATACTGTGCGGACACAATTTGCTCTAGATGACCCGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr1rev TTGCCATCCCTTGACATTGCTCTAG and TGACACCGGGGAATCGAC to generate a fragment encoding the Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr2fwd ACAGACCGGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev TATACTGTGCGGACACAATTTGCTCTAGATGACCCGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr1rev TTGCCATCCCTTGACATTGCTCTAG and TGACACCGGGGAATCGAC to generate a fragment encoding the Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr2fwd ACAGACCGGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev TATACTGTGCGGACACAATTTGCTCTAGATGACCCGGGTTCGATTCCCGGCTGGTGCATT and Cpf1gRNAPcr2rev Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr1rev TTGCCATCCCTTGACATTGCTCTAG and TGACACCGGGGAATCGAC to generate a fragment encoding the Cpf1gRNA core sequence followed by the BbsI cloning cassette and a rice tRNA (Wang et al., 2012) and Cpf1gRNAPcr2fwd ACAGACCGGGGTTCGATTCCCGGCTGGTGCATT

pCFD7. To make cloning of Cpf1 gRNAs more simple, we constructed pCFD7, which contains a BbsI cassette in place of the gRNA target site in pU6-3: hAsCpf1-gRNA. pCFD7 was generated by Gibson Assembly using pCFD5 digested with BbsI and XbaI and inserts generated by PCR using the following primers and pCFD5 as a template: Cpf1gRNA backbone (gift from Brian McCabe (Wang et al., 2012), Addgene 35200) instead of pJFRC081 (gift from Gerald Rubin (Pfeiffer et al., 2012), Addgene 36432), as the latter plasmid is optimised for high protein expression levels. pBIC-UASC was digested with EcoRI and XbaI and gel purified. hCas9 was amplified from plasmid act-cas9 (Port et al., 2014) with primers UAScas9P2wd CAACATTTGAATTCAAGAGCGCATACAAAACGAATT CATGGAAAAGGAGTACCTCCATTG and UAScas9P2rev AGAAGTAGGTCTTCTCACAAGATCTCTAGATC ACACCTGCTTCTTCTTCTTCCGG. The backbone and insert were assembled by Gibson Assembly. Toxicity of endonuclease activity of high levels of Cas9 with the UAS-cas9.P2, Whereas expression of a single copy of UAS-cas9.P1 with act-Gal4 or hh-Gal4 resulted in lethality, flies with act-Gal4 or hh-Gal4 and a single copy of UAS-cas9.P2 flies were viable and fertile. Some loss of posterior wing margin tissue was observed when two copies of UAS-cas9.P2 were expressed from a hh-Gal4 driver at 25°C, suggestive of residual toxicity. Plasmid UAS-cas9.P2 should become available from Addgene in April or May 2016.

act-hAsCpf1. The plasmid act-cas9 (Port et al., 2014) was generated at 25°C, suggestive of residual toxicity. Plasmid UAS-cas9.P2 should become available from Addgene (Plasmid 73917).

Fly transgenesis and culture

In all experiments, transgenes were present in one copy. Transgenic fly lines were generated by standard procedures (Port et al., 2014). All transgenes used in this study were integrated at the same genomic landing site (P[y]+1.7[CaryP]attP40) on the second chromosome using the PhiC31/attP/attB system. The use of a single landing site allows direct comparison of the activity of different gRNAs. A second independent integration of UAS-cas9.P2 was generated at (P[y]+1.7[CaryP]attP2) on the third chromosome. All crosses were performed at 25°C with 50 ± 5% relative humidity and a 12-h light/12-h dark cycle. Virgin females transgenic for Cas9 or Cpf1 were crossed to males expressing transgenic gRNAs. UAS-cas9.P2 flies are available from the Bloomington Stock Center (Stock numbers 58985 and 58986) and act-cpf1 flies should become available there in April or May 2016. Additional fly strains used in this study are presented in Supplementary Table 2.
Evaluating mutagenesis rates

For the data in Fig. 1b and 3b, germline transmission of non-functional CRISPR alleles was evaluated by crosses to e or se homozygous mutant adults (the se mutant allele (se<sup>45</sup>) was generated in a previous series of CRISPR experiments (F. Port, unpublished)). Progeny with ebony or sepia pigmentation phenotypes indicated transmission of a non-functional CRISPR allele. In other cases, germline transmission of CRISPR-induced mutations was evaluated by extraction of genomic DNA from progeny of act-cas9/gRNA flies x OR-R wild-type flies, PCR of the target site and Sanger sequencing (Port et al., 2014). Heterozygosity for indels could be reliably determined from mixed sequencing traces near the target site (Port et al., 2014). Evaluation of somatic targeting of se in flies (Fig. 1c) was performed as described (Port et al., 2015).

Immunohistochemistry

*Drosophila* wing imaginal discs were dissected from third instar larva in ice cold PBS and immediately fixed in 4% formaldehyde in PBT (PBS containing 0.3% Triton-X 100) for 25 min at room temperature. Discs were washed three times in PBT for 10 min each at room temperature and then incubated in primary antibody (rabbit anti-Wls 1:1000, (Port et al., 2008)) in PBT at 4°C overnight. Discs were then washed three times (20 min, room temperature) in PBT containing 1% heat-inactivated goat serum, followed by incubation with secondary antibody (Alexa Fluor 555 goat anti-rabbit (Invitrogen), 1:500 in PBT) (2 h, room temperature). Samples were washed three times in PBT and once in PBS before mounting in Vectashield containing DAPI (Vector Laboratories). Double-sided tape was used as a spacer between the microscopy slide and the coverslip to avoid compression of the wing discs.

Image Acquisition

Wing imaginal discs were imaged on a Zeiss LSM 780 laser-scanning confocal microscope using the sequential scanning mode and a 40x/1.3NA oil objective. Adult wings were mounted in 50% glycerol / 50% ethanol and imaged on a Nikon Eclipse TS100 microscope with a 5x objective and a Nikon Coolpix digital camera.

Image Analysis

Image analysis was performed in Fiji (Schindelin et al., 2012). Brightness and contrast was adjusted to a comparable extent in all images in one series. The “measure” tool was used to determine the areas of wing imaginal discs and adult wings that displayed the phenotypes documented in Fig. 2c, d and f.

AUTHOR CONTRIBUTIONS

F.P. conceived the study, designed experiments, performed experiments, analysed data and wrote the manuscript. S.B. designed experiments, analysed data and wrote the manuscript.

COMPETING INTERESTS STATEMENT

F.P. and S.B. are inventors of Cas9-expressing fly strains that have been licensed by the MRC to commercial providers of *Drosophila* injection services.

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