Augmenting CRISPR applications in *Drosophila* with tRNA-flanked Cas9 and Cpf1 sgRNAs

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Abstract

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

RNA-guided CRISPR endonucleases have revolutionised genome engineering in many species1, but improvements are necessary to realise the full potential of this technology. One current focus is the development of robust methods for simultaneous expression of several single guide RNAs (sgRNAs). This would facilitate the generation of non-functional mutations in a single target gene, as well as models of polygenic human diseases. Using multiple sgRNAs simultaneously will also augment CRISPR-based gene activation and repression2–5 and significantly reduce the emergence of alleles resistant to gene drives that combat insect-borne diseases6.

Expressing multiple sgRNAs on a single unprocessed transcript leads to very low activity of the prototypical CRISPR endonuclease Cas9 (Ref. 7). Excising sgRNAs from a precursor RNA in the nucleus can, however, be an effective strategy for targeting multiple genomic sites7,8. Such a strategy has the added advantage that RNA polymerase (pol) II transcription – which would otherwise result in export of RNA to the cytoplasm – can be used to produce active sgRNAs7. The use of RNA pol II promoters gives scope to control CRISPR mutagenesis by regulating sgRNA synthesis in time and space, a feature not afforded by the ubiquitously expressed RNA pol III-based promoters typically used for sgRNA expression.

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Resource Availability

*pCFD5, pCFD7 and act-hAsCpf1* are available from Addgene (https://addgene.org/crispr/crispr-fly-design/; Plasmid numbers 73914, 73916 and 73917, respectively). *UAS-cas9.P2* flies are available from the Bloomington *Drosophila* Stock Center (Stock numbers 58985 and 58986).

Author Contributions

FP conceived the study, designed experiments, performed experiments, analysed data and wrote the manuscript. SB designed experiments, analysed data and wrote the manuscript.

Competing Interests Statement

FP and SB are inventors of Cas9-expressing fly strains that have been licensed by the MRC to commercial providers of *Drosophila* injection services.
However, strategies to excise multiple sgRNAs from a single precursor transcript have not been implemented in whole animal models.

Here we describe vectors for multiplexed sgRNA expression in *Drosophila*. These plasmids are inspired by a tRNA-sgRNA expression system in rice, in which the endogenous tRNA processing machinery liberates multiple functional sgRNAs from a single precursor transcript in the nucleus (Fig. 1a). We first demonstrated that a flanking tRNA does not interfere with production of functional sgRNAs in *Drosophila* (Supplementary Fig. 1a). We next created pCFD5, a vector that facilitates cloning of several Cas9 sgRNAs flanked by *Drosophila* or rice tRNA Gly downstream of a single U6:3 promoter (see Supplementary Methods for cloning protocol). Transgenic flies with a pCFD5 construct containing four sgRNAs targeting the *sepias* (se), *ebony* (e), *curled* (cu) and *forked* (f) genes (Fig. 1b; U6:3-t::gRNA-se:e:cu:f) were crossed to flies expressing Cas9 ubiquitously from the actin5C promoter (Fig. 1b). Animals with both transgenes had a high penetrance of the visible phenotypes associated with biallelic disruption of each gene (Supplementary Fig. 1b, c) and frequently transmitted indel mutations in multiple target genes to their offspring (Fig. 1b). We found no evidence that the position of a sgRNA in the array affects mutagenesis rates (Supplementary Fig. 1d). As expected, robust gene targeting was dependent on the tRNA sequences in the expression vector (Supplementary Fig. 1e). Our results demonstrate that tRNA-sgRNA arrays allow rapid generation of complex genotypes.

We also created transgenic flies harbouring a pCFD5 construct containing four different sgRNAs targeting se. When used individually with act-cas9, these sgRNAs resulted in biallelic disruption of the target gene in only a fraction of tissue (Ref. 9 and Fig. 1c). This is due to either suboptimal cleavage by Cas9 or in-frame mutations at the target sites resulting in a functional protein product. Strikingly, expressing all four sgRNAs 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). We confirmed that mutations at multiple target sites were frequently induced by this construct (Supplementary Fig. 1f). Thus, using the tRNA-sgRNA system to simultaneously target several sites within a gene circumvents the problems of functional in-frame mutations and sgRNAs with low activity.

We next tested the ability of tRNA-sgRNA vectors to support gene disruption in specific cell types in multicellular organisms. Expression of RNAi constructs with the binary Gal4/UAS system is traditionally used for this purpose in *Drosophila*, but can result in incomplete knockdowns that fail to reveal protein function. Conditional CRISPR mutagenesis has been attempted in *Drosophila* by tissue specific expression of Cas9 in combination with individual, ubiquitously expressed sgRNAs. However, in our hands these approaches often lead to poorly penetrant phenotypes and mutagenesis outside the desired domain (Ref. 11 and unpublished observations).

To test if the tRNA-sgRNA system can overcome these problems, we evaluated sgRNA transgenes in combination with UAS-cas9 and hedgehog-Gal4 (hh-Gal4), which induces expression of UAS transgenes in the posterior compartment of wing imaginal discs (Fig. 2a). Consistent with our earlier findings, a U6:3-sgRNA construct targeting the ubiquitously expressed Wnt secretion factor Wntless (Wls) led to frequent ectopic mutagenesis in the
anterior compartment (Fig. 2b, c, e; 19/30 discs analysed (9 clones with no Wls immunoreactivity; 12 clones with partially reduced immunoreactivity)) and incompletely penetrant gene disruption in the posterior compartment (Fig. 2c’; 11/30 discs analysed). Mutagenesis outside the target compartment was not dependent on Gal4 expression (Supplementary Fig. 2), indicating that it results from ‘leaky’ expression of Cas9 in combination with ubiquitous expression of sgRNA from the strong U6:3 promoter. In contrast, the use of a UAS-tRNA-sgRNA transgene containing two different sgRNAs targeting wls (UAS-t::gRNA-wls2x) with UAS-cas9 and hh-Gal4 resulted in biallelic disruption of the target gene throughout the posterior compartment in all cases (22 discs analysed), as well as very infrequent mutagenesis in the anterior compartment (Fig. 2d, e; 2/22 discs with very small clones with reduced Wls immunoreactivity).

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-wls2x were mirrored by the strength and location of wls mutant phenotypes in the adult wing (Fig. 2f, g). Increased gene disruption by UAS-t::gRNA-wls2x in the target tissue is likely to be because of the combined effects of multiple sgRNAs. The finding that UAS-t::gRNA-wls2x results in much less mutagenesis outside the target tissue is presumably because of the low probability of simultaneous expression of UAS-cas9 and UAS-tRNA-sgRNA transgenes in the absence of Gal4.

The UAS-t::gRNA-wls2x construct also outperformed the U6:3-gRNA-wls transgene in combination with two other Gal4 drivers tested, which are active in either the pouch of the wing disc or the germline (Supplementary Fig. 3a–p). The experiments with the germline driver also revealed that UAS-tRNA-sgRNA constructs can efficiently induce heritable mutations in an essential gene (Supplementary Fig. 3q). Together, these results demonstrate that UAS-tRNA-sgRNA vectors make it possible to perform conditional CRISPR mutagenesis with high efficiency and tight spatial control.

Next, we explored whether the tRNA-sgRNA system can augment genome engineering by the recently discovered RNA-guided endonuclease Cpf1. Cpf1 has a number of properties distinct from Cas9 that have the potential to broaden CRISPR applications. However, genome engineering by Cpf1 had thus far only been evaluated in mammalian systems.

We constructed a plasmid for production of AsCpf1 (Ref. 12) from the actin5c promoter, as well as plasmids expressing a Cpf1 sgRNA targeting e (Supplementary Fig. 4a) in the presence or absence of flanking tRNAs from the U6:3 promoter. When these constructs were provided by either plasmid injection into embryos or genomically integrated transgenes, the flanking tRNAs resulted in significantly increased rates of germline transmission of non-functional e alleles (Fig. 3). Sequencing of the e locus confirmed the transmission of indel mutations at the target site (Supplementary Fig. 4b). Most deletions were 10–20 bp-long, significantly larger than those typically induced by Cas9 (Ref. 17 and Supplementary Fig. 4c). However, even with the flanking tRNAs, the germline transmission rate for e mutations was only ~5% (Fig. 3). In contrast, transgenic supply of Cas9 and its sgRNAs results in mutagenesis of most target alleles in the vast majority of cases. These observations indicate that Cpf1 does not perform as efficiently as Cas9 in Drosophila. Consistent with this notion,
germline transmission of non-functional mutations was not detected using act-cpf1 with three other Cpf1 sgRNAs, even in the presence of flanking tRNA sequences in the expression vector (Supplementary Fig. 4d). Thus, improvements are necessary to transform Cpf1 into a robust genome editing tool. Our data indicating that flanking active Cpf1 sgRNAs with tRNAs can enhance genome editing in vivo is a first step in this direction. Future experiments will investigate how tRNAs can increase the activity of Cpf1 sgRNAs.

Given the conservation of tRNA processing mechanisms, we envisage that this system will be a useful in a wide range of animal cells, including mammalian systems. In addition to the applications discussed above, the use of flanking tRNAs will also increase the repertoire of genomic sequences that can be targeted by CRISPR, as target site selection is no longer constrained by the need for a specific nucleotide at the 5' of the guide sequence for efficient transcriptional initiation by RNA pol III. This is particularly relevant for high-fidelity Cas9 variants, which are unable to tolerate mismatches between the sgRNA and the genomic target18,19.

Online Methods

Additional information and updates are available from our website www.crisprflydesign.org.

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. sgRNA sequences and other details of sgRNA expression plasmids are presented in Supplementary Table 1.

pCFD5 (U6:3-(t::gRNA<sup>cas9</sup>)<sub>1-6</sub>)—The plasmid pCFD3 (Ref. 11) containing the strong U6:3 promoter was cut with BbsI and gel purified using standard procedures. A gBlock was ordered from Integrated DNA Technologies (IDT) that contained Drosophila tRNA<sup>Gly</sup>, a spacer with two BbsI sites, an sgRNA core sequence, a rice tRNA<sup>Gly</sup> and appropriate homology arms (Supplementary Fig. 5). The gBlock was assembled with the pCFD3 backbone by a Gibson Assembly reaction. Rice tRNA<sup>Gly</sup> was chosen as the downstream tRNA since in preliminary experiments a construct with Drosophila tRNA<sup>His</sup> at this position had reduced activity of 3’ sgRNAs. A cloning protocol to generate pCFD5 plasmids encoding one to six tRNA-flanked sgRNAs is provided in the Supplementary Methods. Recombinations in bacteria between repetitive tRNA and sgRNA sequences are rare in pCFD5 but it is likely that their incidence could be reduced in the future by using recently described sequence divergent sgRNA core sequences20. The sgRNA mutations that disrupt potential RNA pol III termination signals but retain genome editing activity (Supplementary Fig. 2d) are described in Ref. 21.

pUAS-t::gRNA-wls<sup>2x</sup>—Two tRNA-flanked wls sgRNAs were cloned into the pBID-UASC backbone (gift from Brian McCabe22, Addgene 35200). pBID-UASC was digested with EcoRI and XbaI and the linear plasmid purified from a gel. Inserts were generated by PCR using pCFD5 as the template and the following primers: UAS<sub>f</sub>tRNAfwd1
TGAATCACAAAGACGCATACCAAACGA ATTCGGGCTTTGAGTGTGTGACAC to generate a fragment encoding *Drosophila tRNA*<sup>Gly</sup>; UASrRNArev1 CTCAGGTTCTCCAGTATGGTGCATCGGCCGGGAATCGAACC to generate a fragment encoding the first gRNA-wls and rice *tRNA*<sup>Gly</sup>; and UASrRNAfwd2 ACCATACTGGAGAACCTGAGGTTTTAGAGCTAGAAATAGCAAG and UASrRNArev2 TGGCGAATATTCGCTTCACCAGGCCGGAATCGAACC to generate a fragment encoding the second gRNA-wls and rice *tRNA*<sup>Gly</sup>. The three inserts and the pBID-UASC backbone were assembled by Gibson Assembly.

**pCFD6**—To streamline cloning of sgRNAs into a pUAS-<i>t</i>::gRNA vector, pUAS-<i>t</i>::gRNA-wls<sup>2x</sup> was modified to contain a BbsI cloning cassette. pUAS-<i>t</i>::gRNA-wls<sup>2x</sup> was cut with EcoRI and XbaI and the linear backbone purified from a gel. Inserts were generated by PCR using the following primers and templates: pCFD6fwd1: GCCAACTTTGAATCACAAGACGC and pCFD6rev1: AGACCCTGATCGCCGGGAATCGAACC with template pUAS-<i>t</i>::gRNA-wls<sup>2x</sup>; pCFD6fwd2: CCGGGTTCGATTCCCGGCCGATGC and pCFD6rev2: GCTATTTCTAGCTCTAAAACAGGTCTTCTGCACCAGCCGGGAATCGAACC with template pCFD5.2 (where the first sgRNA core sequence is modified according to Ref. 21); pCFD6fwd3: GAAAGCTGTTTTAGAAGCTAGAAATAGCAAG and pCFD6rev3: ACACCAAGAAGTATAGGTGCATTTTC to generate a fragment encoding the genomic U6:3 terminator sequence directly downstream of rice *tRNA*<sup>Gly</sup>.

**pCFD7**—To make cloning of Cpf1 sgRNAs more simple, we constructed pCFD7, which contains a BbsI cassette in place of the sgRNA 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: Cpf1gRNAfwd TCGATTCCCGGCCGGATGCATAATTTCTACCTGACCAGCCGGGAATCGAACC to generate a fragment encoding the genomic U6:3 terminator sequence directly downstream of rice *tRNA*<sup>Gly</sup>.
and Cpf1gRNAper1rev TGCACCAAGCCGGAATCGAAC to generate a fragment encoding the Cpf1 sgRNA core sequence followed by the BbsI cloning cassette and a rice tRNA Gly;
and Cpf1gRNAper2fwd ACAGACCCGGGTTCGATTCCCGGCTGGTGCATTTTTTTTGCTTACCTGGAGCCTGAGA and Cpf1gRNAper2rev TATACTGTTGCCAGCACAAATTGCTAATGCATACGCATTAAGCGAAC to generate a fragment encoding the genomic U6:3 terminator sequence directly downstream of rice tRNA Gly.

**Cas9 and Cpf1 expression plasmids**

**UAS-cas9.P2**—We previously found that expressing high levels of Cas9 with the Gal4/UAS system resulted in toxicity in Drosophila, and that this was independent of endonuclease activity. To attempt to reduce toxicity, we generated UAS-cas9.P2. This vector was designed to express lower levels of Cas9 compared to our previous UAS-cas9.P1 plasmid. To this end we used a cas9 codon-optimised for expression in human cells (hCas9; gift from George Church24, Addgene 41815) instead of the fly codon-optimised cas9 present in UAS-cas9.P1. We also used the pBIC-UASC backbone (gift from Brian McCabe22, Addgene 35200) instead of pJFRC081 (gift from Gerald Rubin23, 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 (Ref. 11) with primers UAScas9P2fwd CAACTTTGAATCACAAGACCATACCAAACGAATTCATGGACAAGAAGTACTCCA TTG and UAScas9P2rev AGAAGTAAGGTTCCTTCACAAAGATCCTCTAGATCACACCTTCCTTCTTGGG. The backbone and insert were combined by Gibson Assembly. Toxicity of UAS-cas9.P2 was markedly reduced. 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 in this system when Cas9 levels are high. Plasmid UAS-cas9.P2 should become available from Addgene in October 2016.

**act-hAsCpf1**—The plasmid act-cas9 (Ref. 11) was digested with EcoRI and XhoI and gel purified. AsCpf1, which encodes one of the active Cpf1 variants in mammalian cells12, was amplified from pY010 (gift from Feng Zhang12, Addgene 69982) using primers AsCpf1fwd GCTTACAGGATCCATCCCAAGTACCCCGGAATTCTAGCTTCACATGACACAGTTCGAGGGCTTTACC and AsCpf1rev TCACAAAGATCCTCAGGACTGCCCTCCCTCGGCGGAGGAGATCACATCAT. The backbone and insert were assembled by Gibson Assembly.

**Fly transgenesis and culture**

In all cas9/gRNA strains, transgenes were present in one copy. Transgenic fly lines were generated by standard procedures.11 All transgenes used in this study were integrated at the same genomic landing site (P[y(+t7.7)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 sgRNAs. An independent integration of UAS-cas9.P2 was generated at
(P[y(+7.7)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 sgRNAs. act-cpf1 flies should become available from the Bloomingon Drosophila Stock Centre in October 2016. act-cas9 has been described previously. Additional fly strains used in this study are presented in Supplementary Table 2.

Evaluating mutagenesis rates
Germline transmission of non-functional CRISPR alleles was evaluated by crosses to e, se or y homozygous mutants (the se mutant allele (seΔ5) used for this purpose was generated in a previous series of CRISPR experiments (F. Port, unpublished)). Progeny with ebony, sepia or yellow 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 the indicated crosses, PCR of the target site and Sanger sequencing. Heterozygosity for indels could be reliably determined from mixed sequencing traces near the target site. Evaluation of somatic targeting of se in flies (Fig. 1c) was performed as described.

Immunohistochemistry
Wing imaginal discs and brains were dissected from third instar larvae 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. Samples were washed three times in PBT for 10 min each at room temperature and then incubated in primary antibody (rabbit anti-Wls 1:1000, recognising a C-terminal epitope) in PBT at 4°C overnight. Samples 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 tissue.

Image Acquisition
Wing imaginal discs and brains 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. 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. 2.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


Figure 1. Multiplexed Cas9 sgRNA expression in *Drosophila* with the tRNA-sgRNA expression system.
(a) Principle of tRNA-mediated sgRNA excision.8 (b) U6:3-t:gRNA-se:e:cu:f construct and percentage of offspring of act-cas9/U6:3-t:gRNA-se:e:cu:f transgenic animals (N=48) that inherited indels in the target genes. (c) Somatic mutagenesis (in combination with act-cas9) by transgenes expressing individual sgRNAs targeting *se* or all four sgRNAs expressed simultaneously using a U6:3-tRNA-sgRNA array. Sepia eye coloration indicates non-functional mutations in both *se* alleles. N, number of eyes scored.
Figure 2. The UAS-tRNA-sgRNA system increases mutagenesis efficiency and tissue specificity of conditional CRISPR.

(a) Drawing of third-instar wing imaginal disc showing hh-Gal4 expression pattern. Rectangle shows area imaged. A, anterior compartment; P, posterior compartment. (b–d) Wing imaginal discs of the indicated genotypes immunostained for Wls. Scale bar, 50 μm. Arrowhead in c indicates large anterior clone with disrupted Wls expression. Arrow in c’ indicates large posterior clone that retains Wls signal. (e) Quantification of ectopic mutagenesis in the anterior compartment of discs. (f) Representative examples of adult wing phenotypes caused by disruption of Wls. Arrows and arrowheads indicate, respectively, loss of posterior wing margin tissue and loss of bristles at the anterior wing margin. Dotted lines indicate anterior-posterior boundary. (g) Quantification of adult wing phenotypes induced by sgRNA transgenes in combination with hh-Gal4 and UAS-cas9. Control, no sgRNA transgene. In e and g, horizontal cyan lines indicate mean ± s.e.m. per wing imaginal disc or wing, respectively. Statistical significance was evaluated with a Mann-Whitney test.
Figure 3. Flanking tRNAs can enhance Cpf1 genome editing in vivo.
Comparison of editing of e by Cpf1 in the presence or absence of flanking tRNA sequences in the sgRNA expression vector. N, number of flies analysed. P values are for the comparison to the equivalent parameter in the absence of tRNA (two-tailed Fisher’s exact test).