Published in final edited form as:

Nat Methods. 2016 October ; 13(10): 852–854. doi:10.1038/nmeth.3972.

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 repression^{2–5} 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 sites^{7,8}. 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

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.

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 rice⁸, 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 *sepia* (*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 (*act-cas9*). 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 product⁹. 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^{10,11}. 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 findings¹¹, 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-wls^{2x}*) 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-wls^{2x}* 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-wls^{2x}* in the target tissue is likely to be because of the combined effects of multiple sgRNAs. The finding that *UAS-t::gRNA-wls^{2x}* 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-wls^{2x}* 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 applications¹². However, genome engineering by Cpf1 had thus far only been evaluated in mammalian systems^{12–16}.

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 cases⁹. 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 target^{18,19}.

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^{cas9})₁₋₆)—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^{Gly}, a spacer with two BbsI sites, an sgRNA core sequence, a rice tRNA^{Gly} and appropriate homology arms (Supplementary Fig. 5). The gBlock was assembled with the *pCFD3* backbone by a Gibson Assembly reaction. Rice tRNA^{Gly} was chosen as the downstream tRNA since in preliminary experiments a construct with *Drosophila* tRNA^{His} 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^{2x}—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_{tRNA}fwd1

TGAATCACAAGACGCATACCAAACGA ATTCGGGCTTTGAGTGTGTGTAGACA with UAS_{tRNA^{Gly}}rev1 *CTCAGGTTCTCCAGTATGGTTGCATCGGCCGGAATCGAACC* to generate a fragment encoding *Drosophila tRNA^{Gly}*; UAS_{tRNA^{fwd}}2 *ACCATACTGGAGAACCTGAGGTTTTAGAGCTAGAAATAGCAAG* and UAS_{tRNA^{rev}}2 *TGGCGAATATCACTTAGCAGTGCACCAGCCGGG AATCGAACCCGG* to generate a fragment encoding the first *gRNA-wls* and rice *tRNA^{Gly}*; and UAS_{tRNA^{fwd}}3 *CTGCTAAGTGATATTCGCCAGTTTTAGAGCTAGAAATAGCAAG* and UAS_{tRNA^{rev}}3 *ACAGAAGTAAGGTTCCCTTCACAAAGATCCTCTAGATGCACCAGCCGGGAATCGAA CCCGG* to generate a fragment encoding the second *gRNA-wls* and rice *tRNA^{Gly}*. The three inserts and the *pBID-UASC* backbone were assembled by Gibson Assembly.

pCFD6—To streamline cloning of sgRNAs into a *pUAS-t::gRNA* vector, *pUAS-t::gRNA-wls^{2x}* was modified to contain a BbsI cloning cassette. *pUAS-t::gRNA-wls^{2x}* 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: *AGACCCTGCATCGGCCGGAATCGAACCCG* with template *pUAS-t::gRNA-wls^{2x}*; pCFD6fwd2: *CGGGTTCGATTCCCGGCCGATGC* and pCFD6rev2: *GCTATTTCTAGCTCTAAAACAGGTCTTCTGCACCAGCCGG GAATCGAA* with template *pCFD5.2* (where the first sgRNA core sequence is modified according to Ref. 21); pCFD6fwd3: *GAAGACCTGTTTTAGAGCTAGAAATAGCAAGT* and pCFD6rev3: *ACACCACAGAAGTAAGGTTCCCTC* with template *pUAS-t::gRNA-wls^{2x}*. The three inserts and the *pBID-UASC* backbone were assembled by Gibson Assembly. In a second step, the three BbsI sites present in the vector backbone were removed by site-directed mutagenesis of the *white⁺* marker and by exchanging the *SV40 3'UTR* with the *p10 3'UTR* present in plasmid *pJFRC81* (gift from Gerald Rubin²³, Addgene 36432). Plasmids *pCFD6* and *pUAS-t::gRNA-wls^{2x}* should become available from Addgene in October 2016.

pU6:3-hAsCpf1-gRNA—Expression plasmids for Cpf1 sgRNAs 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 *TCGATTCCCGGCCGATGCATAATTTCTA CTCTGTAGAT-N23(target)-AACAAAGCACCAAGTGGTCTAG* and Cpf1gRNAPcr1rev *TGCACCAGCCGGAATCGAAC* to generate a fragment encoding the Cpf1 sgRNA and a rice *tRNA^{Gly}*; and Cpf1gRNAPcr2fwd *ACAGACCCGGGTTTCGATTCCCGGCTGGTGCATTTT TTTGCCTACCTGGAGCCTGAGA* and Cpf1gRNAPcr2rev *TATACTGTTG CCGAGCACAATTGTCTAGAATGCATACGCATTAAGCGAAC* to generate a fragment encoding the genomic *U6:3* terminator sequence directly downstream of rice *tRNA^{Gly}*.

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: Cpf1gRNABbsIfwd *TCGATTCCCGGCCGATGCATAATTTCTA*

CTCTTGTAGATGGGTCTTCGTTATCAGAAGACCTAACAAAGCACCAGTGGTCTAG and Cpf1gRNA_{Pcr1rev} *TGCACCAGCCGGGAATCGAAC* to generate a fragment encoding the Cpf1 sgRNA core sequence followed by the BbsI cloning cassette and a rice tRNA^{Gly}; and Cpf1gRNA_{Pcr2fwd} *ACAGACCCGGGTTTCGATTCCCGGCTGGTGCATTTT* *TTTGCCTACCTGGAGCCTGAGA* and Cpf1gRNA_{Pcr2rev} *TATACTGTTG* *CCGAGCACAATTGTCTAGAATGCATACGCATTAAGCGAAC* 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 activity¹¹. 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* plasmid¹¹. 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 *CAACTTTGAATCACAAGACGCATACCAAACGAATTCATGGACAAGAAGTACTCCA* *TTG* and UAScas9P2rev *AGAAGTAAGGTTCTTCACAAAGATC* *CTCTAGATCACACCTTCCTCTTCTTCTTGGG*. 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 *GCTTACAGGATCGATCCCCGGGAATTCACCATGACACAGTTCGAGGGCTTTACC* and AsCpf1rev *TCACAAAGATCCTCTAGAGGTACCCTCGAGTTAGGCATAGTCGGGGACATCAT*. 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 procedures¹¹. 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 Bloomington *Drosophila* Stock Centre in October 2016. *act-cas9* has been described previously¹¹. 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 (*se*⁻⁵) 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 sequencing¹¹. Heterozygosity for indels could be reliably determined from mixed sequencing traces near the target site¹¹. Evaluation of somatic targeting of *se* in flies (Fig. 1c) was performed as described⁹.

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 epitope²⁵) 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 Fiji²⁶. 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.

Acknowledgements

We thank N. Muschalik for extensive input and discussions, as well as other members of the Bullock lab and users of www.crisprflydesign.org for feedback. We are also grateful to M. Boutros (DKFZ, Germany) for support during revision of this article and B. Ewen-Campen, D. Yang-Zhou and N. Perrimon (Harvard Medical School, USA) for sharing the unpublished *nub-Gal4 UAS-cas9* stock. This study was supported by a Marie-Curie IntraEuropean Fellowship (to F.P.) and core funding from the MRC (File reference number MC_U105178790 (to S.B.)).

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Figure 1. Multiplexed Cas9 sgRNA expression in *Drosophila* with the tRNA-sgRNA expression system.

(a) Principle of tRNA-mediated sgRNA excision. (b) *U6:3-t::gRNA-se:e:c:-f* construct and percentage of offspring of *act-cas9/U6:3-t::gRNA-se:e:c:-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 μ m. 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 $\pm$ 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).