Upgraded CRISPR/Cas9 tools for tissue-specific mutagenesis in *Drosophila*

Gabriel T. Koreman^{a,b,1}, Yineng Xu^{a,b,1} , Qinan Hu^{a,1,2} , Zijing Zhang^{a,3}, Sarah E. Allen^a , Mariana F. Wolfner^a , Bei Wang^{a,b,4} , and Chun Han^{a,b,4}

^aDepartment of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853; and ^bWeill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY 14853

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CRISPR/Cas9 has emerged as a powerful technology for tissue-specific mutagenesis. However, tissue-specific CRISPR/Cas9 tools currently available in *Drosophila* remain deficient in three significant ways. First, many existing gRNAs are inefficient, such that further improvements of gRNA expression constructs are needed for more efficient and predictable mutagenesis in both somatic and germline tissues. Second, it has been difficult to label mutant cells in target tissues with current methods. Lastly, application of tissue-specific mutagenesis at present often relies on Gal4-driven Cas9, which hampers the flexibility and effectiveness of the system. Here, we tackle these deficiencies by building upon our previous CRISPR-mediated tissue-restricted mutagenesis (CRISPR-TRiM) tools. First, we significantly improved gRNA efficiency in somatic tissues by optimizing multiplexed gRNA design. Similarly, we also designed efficient dual-gRNA vectors for the germline. Second, we developed methods to positively and negatively label mutant cells in tissue-specific mutagenesis by incorporating co-CRISPR reporters into gRNA expression vectors. Lastly, we generated genetic reagents for convenient conversion of existing Gal4 drivers into tissue-specific Cas9 lines based on homology-assisted CRISPR knock-in. In this way, we expand the choices of Cas9 for CRISPR-TRiM analysis to broader tissues and developmental stages. Overall, our upgraded CRISPR/Cas9 tools make tissue-specific mutagenesis more versatile, reliable, and effective in *Drosophila*. These improvements may be also applied to other model systems.

CRISPR/Cas9 | CRISPR-TRiM | *Drosophila* | gRNA | co-CRISPR

The ability to characterize gene function in a tissue-specific manner has been critical for studying developmental and disease mechanisms of essential genes. The CRISPR/Cas9 system has recently provided powerful tools for inducing tissue-specific gene loss of function (LOF). In this system, the endonuclease Cas9 is directed by a small guide RNA (gRNA) to a specific DNA sequence to create double-strand breaks (DSBs) (1). In the absence of homologous repair templates, DSBs are primarily repaired by nonhomologous end joining, an error-prone process that often introduces mutations in the form of insertions or deletions (indels) (2, 3). Because the protospacer adjacent motif required for Cas9 action is ubiquitous in genomes (1, 4), by targeting the expression of Cas9 and gRNAs to specific tissues, mutations can be induced at virtually any gene in a tissue-specific manner. However, current tissue-specific CRISPR/Cas9 tools in *Drosophila* are still deficient in three areas, limiting the power of CRISPR/Cas9 in analyzing gene functions in broad tissues and biological processes.

Method of Tissue-Specific Cas9 Delivery

In *Drosophila*, CRISPR/Cas9-mediated tissue-specific mutagenesis is generally achieved by two approaches that differ in the method of Cas9 delivery. The first approach uses a tissue-specific Gal4 to drive *UAS-Cas9* expression and expresses gRNAs using either a ubiquitous or a upstream activating sequence (UAS) promoter (5, 6). The vast number of available tissue-specific Gal4 lines (1, 7–9) makes adoption of this method relatively easy. For this

reason, Gal4-driven Cas9s have been successfully used to elucidate gene functions, such as in circadian rhythm (10, 11), and to screen for new genes involved in neuronal remodeling (12).

The second method, CRISPR-mediated tissue-restricted mutagenesis (CRISPR-TRiM), relies on enhancer-driven Cas9 for tissue specificity and employs ubiquitously expressed gRNAs (13). Compared with the *Gal4/UAS-Cas9* approach, CRISPR-TRiM has several advantages. First, enhancer-driven Cas9 involves only one transcription step and thus requires less time for expression than Gal4-driven Cas9, reducing the chance of perduring gene products masking defects of mutant cells (13). Therefore, CRISPR-TRiM is more effective for studying early phenotypes of mutant cells. Second, enhancer-driven Cas9 is usually expressed at much lower levels than Gal4-driven Cas9, alleviating cytotoxicity associated with high Cas9 expression (13). Third, CRISPR-TRiM is a simpler system that requires only two genetic components, facilitating the construction of tissue-specific knockout (KO) strains with fewer time-consuming crosses. Lastly, CRISPR-TRiM allows for the simultaneous use of Gal4/UAS for manipulating other tissues. This flexibility of CRISPR-TRiM was demonstrated by simultaneous neuronal gene KO and Gal4-dependent labeling of phosphatidylserine exposure in neurodegeneration (14). Until

Significance

Understanding the mechanisms of development and disease often requires the ability to remove gene function from specific tissues. The CRISPR/Cas9 system can mutate any gene by Cas9-mediated DNA cutting, but current tissue-specific CRISPR/Cas9 tools still have much room for improvement. Here, we report a set of tools that significantly improve the efficiency, flexibility, and analytic power of tissue-specific CRISPR/Cas9 in *Drosophila*. Proper components of these tools can be adopted to achieve the highest mutagenic efficiency in the germline or in somatic tissues, to label mutant cells genetically, or to convert existing Gal4 lines into tissue-specific Cas9 lines. These tools can be adapted easily to other organisms.

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¹G.T.K., Y.X., and Q.H. contributed equally to this work.

²Present address: Department of Biology, Southern University of Science and Technology, Shenzhen, Guangdong 518055, China.

³Present address: Winthrop P. Rockefeller Cancer Institute, University of Arkansas for Medical Sciences, Little Rock, AR 72205.

⁴To whom correspondence may be addressed. Email: bw447@cornell.edu or chun.han@cornell.edu.

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now, CRISPR-TRiM has been limited by the small number of tissue-specific Cas9 lines currently available. Wide applications of CRISPR-TRiM in *Drosophila* require efficient ways of generating new Cas9 lines that are specific to various tissues and developmental stages.

gRNA Efficiency

Successful tissue-specific mutagenesis requires efficient transgenic gRNAs, as inefficient gRNAs would result in uneven LOF in the target tissue and complicate the analysis. A sound general strategy for improving gRNA efficiency is to optimize the design of gRNA expression vectors. So far, optimizations have been made mainly in two areas. First, since expressing multiple gRNAs targeting a single gene can increase the likelihood of mutagenesis (5, 15), considerable efforts have been devoted to making multiplexed gRNA (multi-gRNA) expression vectors (5, 6, 16). Studies in rice, *Drosophila*, and yeast have demonstrated the effectiveness of transfer RNA (tRNA)-gRNA designs for the efficient expression and processing of multi-gRNAs (5, 17, 18). In these designs, multiple gRNAs are interspaced by glycine (G) tRNAs (tRNA^{Gly}) in a single transcript under the control of a single promoter. Endogenous tRNA-processing enzymes cut out tRNAs from the transcript, simultaneously releasing individual gRNAs.

A second aspect of gRNA optimization concerns the scaffold sequence that forms hairpin loops to complex with Cas9 (1, 19). In an early study, a modified scaffold containing a flip of A-U positions and a stem-loop extension (F+E) was found to improve the targeting of Cas9 to the intended locus (20). More recently, an additional extension of the second stem loop (gRNA2.1) was found to further increase the mutagenic efficiency of gRNAs in human cells (21). To develop general strategies for making highly efficient gRNAs for tissue-specific mutagenesis in *Drosophila*, we previously combined tRNA^{Gly}-gRNA with the (F+E) gRNA scaffold in a transgenic gRNA vector. This vector performed much more efficiently than previous gRNA vector designs in somatic tissues (13). However, there is still room to further improve the design of gRNA vectors toward higher gRNA efficiency and more reliable tissue-specific mutagenesis. Moreover, the germline differs from the soma in important ways that often impact transgene expression (22, 23). It has thus been unknown which design is the most efficient in the *Drosophila* germline for use in germline mutagenesis and gene replacement through homology-directed repair.

Labeling of Mutagenized Cells

An unsolved caveat of all current methods of CRISPR-mediated mutagenesis is the inability to label mutant cells in the target tissue. This is particularly problematic for data analysis when Cas9 activity is not evenly distributed across all cells in the tissue of interest. This problem cannot be solved simply by fusing Cas9 to a fluorescent protein because the presence or absence of Cas9 protein at the time of analysis does not necessarily correlate with the presence or absence of mutations. Therefore, the ability to label mutant cells in the target tissue is an unmet need.

Here, we present our strategies to tackle these challenges. We report further improvements in the design of gRNA vectors that lead to higher gRNA efficiency and more reliable, tissue-specific mutagenesis in somatic tissues. We also address germline performance of various constructs and report efficient vectors for germline mutagenesis. Moreover, to label mutant cells, we developed a co-CRISPR reporter system and demonstrate its applications in mutagenizing the *Drosophila* epidermis in conjunction with positive and negative labeling. Lastly, we generated genetic tools for convenient conversion of existing Gal4 lines into tissue-specific Cas9 lines. These new tools significantly increase the power of tissue-specific mutagenesis in *Drosophila* and make more reliable and more sophisticated CRISPR/Cas9 manipulations available for the study of broader biological questions.

Results

New Multi-gRNA Designs Greatly Improve the Efficiency of Somatic Mutagenesis. We previously identified an efficient multi-gRNA design (tgFE) that employs both tRNA^{Gly} as spacers to separate gRNAs and the (F+E) gRNA scaffold to enhance gRNA/Cas9 interaction (13). We have continued to optimize this design to further increase its mutagenic efficiency. Reasoning that tRNA processing could affect the rate and level of gRNA production, we first tested alternative tRNAs in conjunction with the (F+E) gRNA scaffold. We compared six *Drosophila* tRNAs in a dual-gRNA design, in which a *Drosophila* tRNA^{Gly} is followed by an irrelevant gRNA (targeting the blue fluorescent protein [BFP]), and a second variable tRNA is followed by a gRNA targeting *Syntaxin 5* (*Syx5*) (Fig. 1C). Knocking down *Syx5* in *Drosophila* class IV dendritic arborization (C4da) neurons resulted in severe (81%) dendritic reduction (*SI Appendix, Fig. S1 A–C*), consistent with *Syx5*'s expected role in endoplasmic reticulum (ER) to Golgi vesicle trafficking (24). Therefore, the most efficient gRNA construct should yield the most robust and consistent dendrite reduction (Fig. 1A and B). For comparison, we used a tgFE-based dual-gRNA construct that expresses two gRNAs against *Syx5* (designated as GG). When combined with a C4da-specific Cas9 (*ppk-Cas9*), only the construct containing glutamine (Q) tRNA (tRNA^{Gln}) (designated as GQ) consistently caused strong (59%) reduction of dendrite length, while other versions caused much more variable reductions as indicated by the deviation of each sample from the mean dendrite length (Fig. 1C and D). These data suggest that tRNA^{Gln} may result in more efficient processing of multi-gRNAs. We further compared GQ and GG using *SOP-Cas9* (13), which expresses Cas9 in precursor cells of C4da neurons and thus would result in earlier KO than *ppk-Cas9*. In larvae of both genotypes, we found neurons exhibiting much stronger dendritic reduction (89% for GG and 87% for GQ) (*SI Appendix, Fig. S1 D–F*) as well as wild type-looking neurons. These data suggest that while both gRNAs failed to produce LOF mutations in all C4da neurons, the LOF mutations they induced led to comparable degrees of dendrite defects. Interestingly, GQ-expressing larvae exhibited 60% lethality before the third instar stage, likely due to nonspecific expression of *SOP-Cas9* in some epidermal cells (13). In contrast, GG did not cause significant larval lethality (*SI Appendix, Fig. S1G*). Given that GG contains one additional gRNA targeting *Syx5* compared with GQ, the above data together suggest that tRNA^{Gln} is likely a better choice than tRNA^{Gly} for constructing multi-gRNA constructs.

We also compared tRNA variants in a quadruple-gRNA design to knock out *Nsf1* and *Nsf2* simultaneously (*SI Appendix, Fig. S1 H–J*); these genes act redundantly to permit dendrite growth of C4da neurons (13). However, with *ppk-Cas9*, including tRNA^{Gln} in various combinations did not significantly enhance dendrite reduction (*SI Appendix, Fig. S1I*). Because *Nsf1* and *Nsf2* exhibit perdurance in C4da neurons (13), the generally mild dendrite reductions with these gRNAs are likely due to the late expression of *ppk-Cas9* rather than inefficient tRNA processing (13).

We next asked whether incorporating the gRNA2.1 scaffold could further augment gRNA mutagenic efficiency. We generated a dual-gRNA construct against (enhanced) green fluorescent protein (EGFP/GFP), with gRNA2.1 as the scaffold and only one tRNA^{Gln} as the spacer between the two gRNAs [Fig. 1E, ACMG(2.1)], and compared it with two earlier versions of gRNA-EGFP/GFP [Fig. 1E, ACDG and ACMG(FE)]. Each construct expresses two gRNAs targeting EGFP and GFP coding sequences separately. ACDG uses two U6 promoters and the original scaffold, while ACMG(FE) is based on the tgFE design. To separate the effects of tRNA and the scaffold, we also made ACDG(FE) (Fig. 1E), which is similar to ACDG, except that the original scaffold was replaced by the (F+E) scaffold. Using a ubiquitous nuclear EGFP and *Act5C-Cas9*, we assayed the efficiency of gRNAs in

Fig. 1. A new multi-gRNA design significantly improves the efficiency of somatic mutagenesis. (A and B) C4da neurons in *ppk-Cas9* control (A) and *ppk-Cas9 gRNA-BFP-Syx5(GQ)* (B). (Scale bars, 50 μm .) (C and D) Total dendrite lengths (C) and residuals (differences from the mean) of dendrite lengths (D) of C4da neurons using various *Syx5* gRNAs. All gRNA constructs contain a tRNA^{Gly} after the U6:3 promoter and use the (F+E) scaffold. Asterisks indicate that both gRNAs in GG target *Syx5*. The significance level above each column indicates comparison with the control. Welch's ANOVA and Welch's *t* tests with Bonferroni correction (C) and one-way ANOVA and Tukey's honestly significant difference (HSD) test (D) were used. Each circle represents one neuron. *n* = number of neurons: GG (*n* = 15), GK (*n* = 14), GL (*n* = 17), GN (*n* = 17), GQ (*n* = 17), GS (*n* = 15), GV (*n* = 15), and Control (*n* = 14). (E) Nine variants of gRNA constructs targeting EGFP/GFP. Each construct contains one gRNA targeting EGFP and one targeting GFP. The identities of tRNAs are indicated, and the gRNA scaffolds are color-coded. (F) EGFP patterns in larvae expressing a ubiquitous EGFP marker, *Act5C-Cas9*, and a *gRNA-EGFP/GFP*, whose identity is indicated by the number defined in E. The control larva has no gRNA construct. (G–I) The ratios of EGFP(+) areas in larvae. The datasets for the same constructs in the plots are identical. Welch's ANOVA and Welch's *t* tests with Bonferroni correction were used. Numbering corresponds to gRNAs in E. *n* = number of larvae: control (*n* = 10), 1 (*n* = 10), 2 (*n* = 14), 3 (*n* = 9), 4 (*n* = 10), 5 (*n* = 9), 6 (*n* = 12), 7 (*n* = 9), 8 (*n* = 12), and 9 (*n* = 14). Each circle represents one larva. (J and K) EGFP and Repo staining in representative optical lobes of *+EGFP; repo-Cas9/gRNA* larvae. The control has no gRNA. (Scale bars, 20 μm .) (L) Quantification of glial nuclei that were EGFP positive. The significance level above each column indicates comparison with the control. Welch's ANOVA and Welch's *t* tests with Bonferroni correction. Each circle represents one brain. *n* = number of brains; Control (*n* = 9), ACDG (*n* = 9), ACMG(FE) (*n* = 6), and ACMG(2.1) (*n* = 6). For all quantifications, ****P* $\leq$ 0.001; ***P* $\leq$ 0.01; and **P* $\leq$ 0.05; ns, not significant; black bar, mean; red bars, SD.

knocking out EGFP in larvae. Consistent with our previous comparisons in da neurons (13), ACMG(FE) is significantly more efficient than ACDG. While ACDG removed EGFP from most cells, there were still many EGFP-positive nuclei (6.28% area) (Fig. 1 F and G). ACMG(FE) further reduced EGFP signals to only some muscle stripes and the larval brain (1.49% area) (Fig. 1 F and G). ACDG(FE) gave an intermediate level of EGFP reduction (3.68%

area) between those of ACDG and ACMG(FE) (Fig. 1 F and G), suggesting that both tRNA^{Gly} and the (F+E) scaffold contribute to the higher efficiency of ACMG(FE). Strikingly, ACMG(2.1) almost completely eliminated EGFP, leaving only occasional EGFP-positive cells (0.60% area) (Fig. 1 F and G). These data suggest that tRNA^{Gln} and gRNA2.1 together contribute to the most complete KO. We conclude that tRNA^{Gln} -gRNA2.1

(referred to as Qtg2.1) is a superior gRNA design over previous generations.

It was unclear whether increasing the number of gRNAs in the multiplex design would impact the efficiency of individual gRNAs. To answer this question, we added an extra irrelevant gRNA, along with a ubiquitous reporter (described later), to ACMG(FE) and ACMG(2.1) constructs [Fig. 1E, ACMG(FE)-BR and ACMG(2.1)-8R, respectively]. These two triple-gRNA constructs performed worse than their dual-gRNA counterparts, although the new Qtg2.1 design was still consistently better than the tgFE version (with 2.10 and 5.42% areas, respectively) (Fig. 1F and H). The reduced efficiencies of triple-gRNA constructs were not due to the ubiquitous reporters, as adding only a reporter to ACMG(2.1) did not change the mutagenic efficiency (0.61% area) (Fig. 1F and H). These data suggest that adding more gRNAs could reduce the efficiency of each gRNA in a multi-gRNA design, perhaps due to competition for Cas9.

The RNA polymerase III (pol III) promoter that drives gRNA expression impacts mutagenic efficiency by controlling the gRNA expression level. The U6:1 promoter that drives gRNA-EGFP expression in our dual-promoter constructs was previously reported to exhibit a weaker activity than the U6:3 promoter (6). Hoping to also improve dual-promoter designs, we made two more versions of gRNA-EGFP/GFP (Fig. 1E). The first version, CR7T-U63(FE), is otherwise identical to ACDG(FE) except that U6:1 was replaced by CR7T, a pol III promoter reported to be potent in both the soma and the germline (15, 25). The second version, CR7T-U63(2.1), additionally adopted the gRNA2.1 scaffold. In larval-body EGFP KO, CR7T-U63(FE) performed similarly to ACDG(FE) (Fig. 1F and I), leaving 3.60% GFP area, suggesting that CR7T is comparable to U6:1 in the soma. CR7T-U63(2.1) was statistically indistinguishable from ACMG(2.1) (Fig. 1F and I), with 0.78% GFP area, suggesting that gRNA2.1 also boosts the efficiency of dual-promoter designs.

Because Qtg2.1 is more convenient to use than multipromoter designs when cloning more than two gRNAs, we further tested Qtg2.1 in quadruple/quintuple gRNA constructs against *Nsf1/Nsf2*. However, neither the Qtg2.1 design nor the addition of an irrelevant gRNA significantly affected the level of dendrite reduction in C4da neurons when *ppk-Cas9* was used to induce KO (SI Appendix, Fig. S1I). Switching to *SOP-Cas9* strongly enhanced dendrite reductions caused by these gRNAs, but no statistical differences were detected among the gRNAs (SI Appendix, Fig. S1J). These results are consistent with the idea that Cas9 expression timing, rather than the rate of gRNA production, is the limiting factor in this particular case (13).

Lastly, we tested whether the various gRNA designs also show different efficiencies in tissue-specific mutagenesis. By making a glial-specific *repo-Cas9*, we compared ACDG, ACMG(FE), and ACMG(2.1) in tissue-specific KO of EGFP in larval brains (Fig. 1I–K). We chose glia because certain types of glial cells are polyploid (26) and are thus more difficult to mutate. Consistent with the results of larval-body EGFP KO, ACDG was the least efficient among the three, and ACMG(2.1) removed EGFP from glia the most completely (Fig. 1L).

In summary, we found that the gRNA2.1 scaffold significantly enhances mutagenic efficiency of multi-gRNAs and that tRNA^{Gln} is a better tRNA choice in tRNA–gRNA designs.

Co-CRISPR Visualizes Mutant Cells in Epithelial Tissues. The inability to label mutant cells has limited the analytic power of current methods of tissue-specific CRISPR mutagenesis. A possible solution is to incorporate a ubiquitous reporter, as well as a gRNA targeting the reporter, into the gRNA vector (Fig. 2A). In such a co-CRISPR design, LOF of the gene of interest (GOI) could be correlated with the loss of reporter expression. As a proof of concept, we tested an EGFP/GFP gRNA construct that also carries a BFP gRNA and a ubiquitous nuclear BFP (nBFP) reporter

[ACMG(FE)-BR in Fig. 1E]. This construct, theoretically, could visualize EGFP mutant cells by the absence of BFP signal. Pairing this construct with ubiquitous nuclear EGFP, we examined the correlation of EGFP and BFP KO in larval epidermal cells (Fig. 2C). We measured the ratio of EGFP and BFP double-positive cells in all EGFP-positive epidermal cells (overlap/EGFP ratio), which should have a value of 1 when mutagenesis of the two genes is correlated exactly. Deviations from this optimal ratio would be caused by false positives, where cells reporting CRISPR/Cas9 activity (the lack of BFP) still express functional EGFP.

We compared two Cas9 lines, *hh-Cas9* and *zk-Cas9*, which have distinct spatiotemporal expression patterns in the larval epidermis, in co-CRISPR labeling of EGFP KO cells. *hh-Cas9* is expected to be expressed in the posterior half of each segment from early embryogenesis to late larval stages (13, 27). While EGFP and BFP were completely knocked out in posterior hemisegments by *hh-Cas9*, some anterior cells also lost either EGFP or BFP (Fig. 2D), amounting to an overall overlap/EGFP ratio of 0.85 (Fig. 2F). We also tested *hh-Cas9* in co-CRISPR in the wing imaginal disk, as it is expressed in the posterior compartment of the wing pouch (13). Similarly, we saw complete KO of both EGFP and BFP in the posterior wing disk as expected and sporadic KO of either EGFP or BFP in small, random patches in the anterior wing disk (Fig. 2G). The *hh-Cas9* activity in the anterior epidermal hemisegment and the anterior wing disk is likely due to leaky expression during development, which should be transient and low. In comparison, the *zen-kr* enhancer in *zk-Cas9* is expected to drive transient expression in precursor cells of epidermal cells during early embryogenesis (28–30). It knocked out EGFP and BFP in some, but not all, epidermal cells (Fig. 2E), generating an overlap/EGFP ratio of 0.51 (Fig. 2F). These results suggest that reliable co-CRISPR requires (moderately) high and persistent expression of Cas9 in the cell lineage, such as *hh-Cas9* in the posterior epidermal hemisegment and in the posterior compartment of the wing disk.

In addition to visualizing mutant cells by the absence of the BFP reporter, we also designed a positive-labeling reporter construct that carries a gRNA against *Gal80* and a ubiquitously expressed Gal80 reporter. As Gal80 suppresses Gal4 transcription factor activity (31), the loss of *Gal80* can be visualized by Gal4-driven expression of a fluorescent marker, thus enabling positive labeling of mutant cells. We paired a Gal80 reporter construct [ACMG(2.1)-8R in Fig. 1E] with a BFP reporter construct [ACMG(FE)-BR] and examined their labeling in the wing disk (Fig. 2H). The loss of Gal80 was visualized by *Act5C-Gal4 UAS-tdTom*. In most wing discs, *hh-Cas9* removed BFP and induced tdTom expression in the entire posterior wing pouch, resulting in minimally overlapping expression domains of BFP and tdTom (Fig. 2I). The combined area of BFP-only and tdTom-only cells, which reflects consistent actions of Cas9 on both reporters, correlated highly with total disk area (Fig. 2J). These data suggest that both positive and negative reporter designs are effective.

Co-CRISPR Enables Labeling of Mutant Cells with Both Positive and Negative Reporters in Dendrite Development and Epithelial Morphogenesis.

To test if co-CRISPR can be used to positively report mutant cells through correlations between monoallelic mutations of the reporter and biallelic mutations of endogenous genes, we designed a Gal80-reporter gRNA construct to target *sulfateless* (*sfl*), which encodes a heparan sulfate–glucosamine N-sulfotransferase required by epidermal cells to support growth of C4da dendrites cell-autonomously (30). We paired this construct with *hh-Cas9*, *UAS-tdTom*, and a pan-epidermal Gal4 driver. Accordingly, we observed that tdTom-positive epidermal cells in the posterior hemisegment lacked coverage by high-order dendritic branches of C4da neurons (Fig. 3B), a phenotype associated with epidermal *sfl* LOF (30). Although some tdTom-positive cells were also observed in the anterior hemisegment next to the segment boundary,

Fig. 2. Co-CRISPR visualizes mutant cells in epithelial tissues. (A) General strategy of labeling mutant cells for a GOI using a ubiquitous co-CRISPR reporter. (B) The design of negative labeling via co-CRISPR mutagenesis of EGFP and a nBFP reporter (*gRNA-EGFP/GFP[ACMG(Fe)-BR]*). (C–E) Epidermal expression patterns of BFP (magenta) and EGFP (green) in larvae expressing a ubiquitous EGFP marker, *gRNA-EGFP/GFP[ACMG(Fe)-BR]*, and no Cas9 (C), *hh-Cas9* (D), or *zk-Cas9* (E). The anterior (a) and posterior (p) hemisegments are indicated in D. (Scale Bar, 100 μm .) (F) The ratio of cells expressing both EGFP and BFP over all cells expressing EGFP in larvae carrying the various Cas9 lines. The significance level above each column indicates comparison with the control. *** $P \leq 0.001$ and * $P \leq 0.05$; contrasts of estimated marginal means (EMMs) based on a generalized linear mixed-effects model with a binomial response. Each circle represents one segment. n = number of segments; control ($n = 16$), *hh-Cas9* ($n = 16$), and *zk-Cas9* ($n = 16$); biological replicates = 4 larvae per genotype; black bar, mean; red bars, SD. (G) BFP (magenta) and GFP (green) expression pattern in a wing disk of a larva expressing the ubiquitous EGFP marker, *gRNA-EGFP/GFP[ACMG(Fe)-BR]*, and *hh-Cas9*. The anterior (a) and posterior (p) compartments are indicated. (Scale Bar, 100 μm .) (H) Diagram for comparing BFP- and Gal80-reporter constructs. (I) A representative wing disk of Act-Gal4 UAS-tdTom/*hh-Cas9*; *gRNA-EGFP/GFP[ACMG(Fe)-BR]*/*gRNA-EGFP/GFP[ACMG(2.1)-8R]*. (Scale Bar, 100 μm .) (J) Correlation between disk area and combined area of BFP-only and tdTom-only cells in each wing disk. The solid line shows the linear regression; the shaded area is 95% CI; the dotted line indicates a perfect correction (slope = 1). Pearson's correlation coefficient: $r = 0.98$, $P < 2.4 \times 10^{-16}$. Each dot represents one wing disk; $n = 17$.

reflecting leaky expression of *hh-Cas9*, high-order branches of C4da neurons were also absent from these tdTom patches. The reliability of positive labeling of *sfl* mutant cells is demonstrated by a tight correlation (Pearson's correlation coefficient: $r = 0.96$) between labeled areas with dendrite reduction and all labeled areas (Fig. 3C). Overall, $95 \pm 4\%$ ($n = 29$) labeled areas showed strong dendrite reduction. While we occasionally observed unlabeled cells that showed dendrite reduction, which would have resulted from false-negative labeling, $95 \pm 3\%$ unlabeled areas showed no dendrite reduction (Fig. 3D), suggesting that false-negative labeling is infrequent. These data demonstrate that the positive-labeling reporter is an effective approach for visualizing mutant epidermal cells.

We next further validated the effectiveness of the nBFP reporter by targeting *Nsf1/Nsf2* (Fig. 3E). Epidermal cells lacking both *Nsf1* and *Nsf2* delaminate from the epidermal sheet and become round (13). Because this phenotype requires successful KO of all four alleles in the cell, reliable labeling of double mutant cells is expected to be more challenging. To increase the mutagenic efficiency, we used the Qtg2.1 design in this construct, as opposed to the tgFE design in EGFP/GFP and *sfl* co-CRISPR reporter constructs. When paired with *hh-Cas9*, this construct generated many BFP-negative cells in the posterior epidermal hemisegment, all of which showed deformed morphology, such as delamination and round shape (Fig. 3F).

Together, our results suggest that both positive- and negative-labeling co-CRISPR constructs reliably report mutagenic events in the larval epidermis when paired with a proper Cas9.

gRNA Mutagenic Efficiency Is Governed by Different Rules in the Germline and the Soma of *Drosophila*. While optimizing multi-gRNA designs for somatic mutagenesis, we also tried to improve gRNA mutagenic efficiency for the germline. We compared multiple versions of gRNA constructs to knock out three genes known to play important roles in germline development and/or early embryogenesis of the progeny. *bam* is required for early steps of gametogenesis in both males and females and its loss blocks oogenesis in females and suppresses spermatogenesis in males (15, 32). *cid* is required for centromere identity in meiosis (33), and its LOF reduces male fertility (15). The third gene, *gnu*, is maternally required for early embryogenesis (34); thus, eggs derived from the germline of *gnu* mutant females show reduced hatchability (35). For each gene, we initially designed two constructs based on the tgFE design, with one containing the CR7T promoter (15) and the other containing the U63 promoter [CRMG(Fe) and ACMG(Fe) in Fig. 4A, respectively]. As a comparison, for each gene, we also generated a dual-gRNA construct driven by separate CR7T and U63 promoters [CR7T-U63(Fe) in Fig. 4A].

We carried out KO experiments using a germline-specific Cas9, *nos-Cas9* (6), and measured “male fertility,” which is the

Fig. 3. Co-CRISPR enables positive and negative labeling of mutant cells in dendrite development and epithelial morphogenesis. (A) Design of positive-labeling of *sfl* mutant cells using co-CRISPR mutagenesis of a ubiquitous *Gal80* co-CRISPR reporter [*gRNA-sf1(BR)*]. The labeling of *Gal80*-mutant cells is enabled by *Gal4*-driven *UAS-tdTom* expression. (B) Dendrite morphology and *tdTom* expression in a larva expressing *ppk-CD4-tdGFP* (green), *A58 > tdTom* (47) (magenta), *gRNA-sf1(BR)*, and *hh-Cas9*. *tdTom*-labeled areas are shaded in the dendrite panel. Red asterisks indicate muscle attachment cells, which *C4da* neurons do not innervate. (Scale Bar, 100 μm .) (C and D) Correlation between *tdTom*-labeled areas showing dendrite reduction and all *tdTom*-labeled areas (C) and correlation between unlabeled areas showing no dendrite reduction and all unlabeled areas (D). Solid lines show linear regression, and shaded areas are 95% CI. The dotted lines indicate perfect corrections (slope = 1). Pearson's correlation coefficients (r) are indicated. Each dot represents a segment; $n = 29$, biological replicates = 15. (E) Design of negative labeling of *Nsf1/Nsf2* double mutant cells by a *nBFP* co-CRISPR reporter (*gRNA-NSF1-NSF2[QQQ(2.1)-BR]*). (F) A representative image of epidermal cell morphology in larvae expressing *gRNA-NSF1-NSF2[QQQ(2.1)-BR]*, *hh-Cas9*, and *Nrg-GFP* (Green). (Scale Bar, 100 μm .) n = number of segments = 5, biological replicates = 3.

percentage of hatched eggs from individual wild-type females mated with the KO males; “female egg number,” which is the number of eggs laid by individual KO females; and “female fertility,” which is the percentage of hatched eggs from individual KO females mated with wild-type males. We only measured female egg number and female fertility for the *gnu* KO, as *gnu* does not play a role in the male germline. All three constructs for *bam* yielded strong reduction of male fertility and female egg number but, surprisingly, with CR7T-U63(FE) showing the highest percentage of complete loss of fertility in both males and females (100 and 90%, respectively) (Fig. 4 B and C). This suggests that the efficiency of *bam* gRNAs is high with all three designs. For *cid*, we found that the CR7T-U63(FE) design was also much more efficient than tgFE designs in reducing male fertility and female fertility (Fig. 4 B and D): While CR7T-U63(FE) reduced 79% of male fertility and 63% of female fertility, the tgFE constructs did not affect male fertility and reduced 11 to 23% of female fertility. For *gnu*, although differences among the three constructs were not statistically significant for female fertility, CR7T-U63(FE) appeared to perform better than tgFE constructs (Fig. 4D). We then wondered whether gRNA2.1 can enhance gRNA mutagenic efficiency in the germline. Since the *bam* gRNAs were already very efficient, we made another dual-promoter dual-gRNA construct using the gRNA2.1 scaffold [CR7T-U63(2.1) in Fig. 4A] for *cid* and *gnu* only. While this version appeared to increase the efficiency for *gnu* slightly (Fig. 4D), it was not as efficient as CR7T-U63(FE) for *cid* (Fig. 4 B and D).

The above data indicate that including tRNAs in the gRNA construct may negatively impact mutagenic efficiency in the germline. To further test this hypothesis, we examined germline KO of EGFP/GFP by four gRNA-EGFP/GFP constructs.

We crossed *nos-Cas9/Y; ubi-GFPnls/ubi-EGFP; gRNA/+* males to wild-type females and quantified the percentage of EGFP/GFP-positive larvae in ~2,000 of their progeny. While ACMG(FE) resulted in 3.01% EGFP/GFP-positive offspring, ACDG(FE) produced only 0.10% EGFP/GFP-positive offspring, supporting the idea that including tRNA negatively impacts the efficiency of gRNA constructs in the germline. Lastly, both CR7T-U63(FE) and CR7T-U63(2.1) resulted in GFP/EGFP loss in all progeny (Fig. 4E). Together, our data suggest that both CR7T-U63(FE) and CR7T-U63(2.1) are efficient gRNA designs for germline KO. Importantly, efficient mutagenesis requires different design principles for multi-gRNAs in the germline and the soma, perhaps due to differences in RNA processing.

Gal4-to-Cas9 Conversion Expands the Options for Tissue-Specific Cas9.

Our CRISPR-TriM strategy relies on tissue-specific Cas9 lines, the limited availability of which is currently the bottleneck for wide applications of CRISPR-TriM. Meanwhile, a large number of tissue-specific *Gal4* lines are available for virtually every tissue and all developmental stages of *Drosophila*. To take advantage of the *Gal4* resources and eliminate the need for cloning and transgenesis for making new tissue-specific Cas9 lines, we developed a fast and reliable way to convert existing *Gal4* lines into

Fig. 4. gRNA mutagenic efficiency is governed by different rules in the germline and the soma of *Drosophila*. (A) Design of gRNA constructs tested in the germline. Numbering of gRNAs corresponds to that in B–D. (B) Hatchability of eggs derived from crosses using males expressing gRNAs targeting *bam* or *cid*. The control had no gRNAs. For *bam*, pairwise Fisher exact tests with Bonferroni corrections were used to analyze categorical results (no progeny versus with progeny); for *cid*, contrasts of estimated marginal means (EMMs) based on proportion of hatched/unhatched eggs using a generalized linear mixed-effects model with a binomial response were used. *n* = number of single-male × single-female crosses. Control (*n* = 22), *bam*-1 (*n* = 20), *bam*-2 (*n* = 10), *bam*-3 (*n* = 10), *cid*-1 (*n* = 20), *cid*-2 (*n* = 20), *cid*-3 (*n* = 7), and *cid*-4 (*n* = 10). (C) The number of eggs laid by females expressing gRNAs targeting *bam*, *cid*, or *gnu*. The control had no gRNAs. For *bam*, pairwise Fisher exact tests with Bonferroni corrections were used to analyze categorical results (no eggs versus with eggs); for *cid* and *gnu*, contrasts of EMMs using a generalized linear model with a negative binomial response were used. *n* = number of single-male × single-female crosses. Control (*n* = 22), *bam*-1 (*n* = 20), *bam*-2 (*n* = 10), *bam*-3 (*n* = 10), *cid*-1 (*n* = 20), *cid*-2 (*n* = 20), *cid*-3 (*n* = 10), *cid*-4 (*n* = 10), *gnu*-1 (*n* = 19), *gnu*-2 (*n* = 10), *gnu*-3 (*n* = 20), and *gnu*-4 (*n* = 10). (D) Hatchability of eggs derived from females expressing gRNAs targeting *bam*, *cid*, or *gnu*. The control had no gRNAs. Contrasts of EMMs are based on proportion of hatched/unhatched eggs using a generalized linear mixed-effects model with a binomial response. Statistics were not shown for *bam* because the eggs produced (due to incomplete *bam* mutagenesis in early gametogenic germline cells) are expected to be fertile, and because females that laid eggs were too few to be statistically meaningful. *n* = number of single-male × single-female crosses. Control (*n* = 22), *bam*-1 (*n* = 5), *bam*-2 (*n* = 3), *bam*-3 (*n* = 1), *cid*-1 (*n* = 16), *cid*-2 (*n* = 17), *cid*-3 (*n* = 6), *cid*-4 (*n* = 8), *gnu*-1 (*n* = 19), *gnu*-2 (*n* = 10), *gnu*-3 (*n* = 19), and *gnu*-4 (*n* = 7). (E) Percentage of EGFP/GFP-positive larvae derived from the germline of *nos-Cas9/Y*; *ubi-GFP*ns/*ubi-EGFP*; *gRNA*/+ males. Kruskal–Wallis test and pairwise comparisons with Bonferroni correction. Each circle represents one cross, with six crosses for each genotype. Total larvae number *n*: ACMG(FE) (*n* = 2091), ACDG(FE) (*n* = 1946), CR7T-U63(FE) (*n* = 2028), and CR7T-U63(2.1) (*n* = 2108). “n/a” indicates that there is no *P* value for the comparison of CR7T-U63(FE) and CR7T-U63(2.1) because all values are zero. For all quantifications, the significance level above each column indicates comparison with the control. ****P* ≤ 0.001; ***P* ≤ 0.01; and **P* ≤ 0.05; ns, not significant. Black bar, mean; red bar, SD.

Cas9 lines through several genetic steps (SI Appendix, Fig. S2) based on homology-assisted CRISPR knock-in (HACK) (36). This conversion utilizes a Cas9-donor transgenic construct that carries a 2A-Cas9 coding sequence flanked by Gal4 homology arms, a CR7T-U63(2.1) dual-gRNA cassette targeting the Gal4 coding sequence, and a ubiquitous nBFP marker for separating the Cas9-donor insertion from the converted Cas9 (Fig. 5A). When combined with *nos-Cas9* and the Gal4 line of interest, the gRNAs generate DSBs in the middle of the Gal4 coding sequence and allow in-frame incorporation of 2A-Cas9 via homology-directed repair. The “self-cleaving” 2A peptide will release Cas9 and a truncated and nonfunctional Gal4 as two separate proteins after translation. To conveniently screen for the converted Cas9, we generated a Cas9 reporter based on gRNA-induced single-strand annealing (SSA) (16). This GFP SSA reporter (GSR) was built on the ACMG(2.1) design and carries an interrupted EGFP-2A-nGFP coding sequence driven by a *ubi* enhancer (Fig. 1B). Cas9 activity will trigger DSBs at the site of interruption and result in reconstitution of the reading frame and expression of both a cytosolic EGFP and a nuclear GFP.

As a proof of principle, we converted three Gal4 lines specific to different tissues into Cas9 lines. We compared activity patterns of the Cas9 lines, as revealed by GSR or the Cas9 positive tester *Act5C-Gal4 UAS-GFP*; *tub-Gal80 gRNA-Gal80* (13), to those of the original Gal4 lines. Interestingly, Cas9s and the corresponding Gal4s do not always show identical activity patterns. *esg-Gal4* is active in stem cell populations, including larval histoblasts (Fig. 5C) (37), but we detected a broad activity pattern of *esg-Cas9* in larval epidermal cells and somatic muscles (Fig. 5D). *HmlΔ-Gal4* is specific to larval hemocytes (Fig. 5F) (38), but the activity of *HmlΔ-Cas9* was detected in both hemocytes and a small number of random epidermal cells (Fig. 5G). Lastly, *OK371-Gal4* is specific to glutamatergic motor neurons in the third instar ventral nerve cord (VNC) (Fig. 5I) (39), while *OK371-Cas9* activity was also detected in sporadic glial cells in optical lobes and in some neural clusters in the VNC (Fig. 5J and J'). The discrepancies between Gal4 and Cas9 activity patterns could reflect differences in detection; whereas Gal4 reporters only reflect the current Gal4 activity, the Cas9 positive tester reports accumulated Cas9 activity in the entire cell lineage

Fig. 5. Gal4 to Cas9 conversion expands the choices of tissue-specific Cas9. (A) Diagram of Gal4-to-Cas9 conversion using a homology-assisted CRISPR knock-in (HACK) donor line. The donor expresses two gRNAs (TS1 and TS2) targeting the tissue-specific (ts) Gal4, which results in in-frame incorporation of 2A-Cas9 into the Gal4 locus through homology-directed repair. The donor expresses *ubi-nBFP* that can be selected against when screening for Cas9 convertants. (B) Diagram of GSR. (C–H) Activity patterns of *esg-Gal4* (C and E), *esg-Cas9* (D), *HmlΔ-Gal4* (F and H), and *HmlΔ-Cas9* (G) in whole larvae using indicated reporters. (I–L') Activity patterns of *OK371-Gal4* (I and K), *OK371-Cas9* (J and J'), and *OK371-Gal4 UAS-Cas9* (L and L') in the larval brain using indicated reporters. NC82 staining shows brain neuropils. mGFP:CD4-tdGFP (F) and mCD8::GFP (E, H, and K); nRFP: RedStinger; PT: Cas9 positive tester. (Scale Bars, 250 μ m in C–H, 100 μ m in I–L').

during development, potentially leading to broader Cas9 patterns. To test this hypothesis, we examined Gal4 drivers using *tubP(FRT.stop)Gal4 UAS-Flp UAS-mCD8::GFP*, which irreversibly marks Gal4-expressing cell lineages based on “Flp-out” (40). Indeed, *esg-Gal4* showed activity in epidermal cells and muscles (Fig. 5E), and *HmlΔ-Gal4* also labeled random epidermal cells (Fig. 5H). However, *OK371-Gal4* activity pattern remained unchanged (Fig. 5K), which could be due to a relatively higher threshold for the Flp-out detection system. Lastly, we examined *OK371-Gal4 UAS-Cas9* using GSR (Fig. 5L and L'), which resulted in a similar activity pattern as *OK371-Cas9*. This suggests that the nonspecific activity of *OK371-Cas9* is also likely due to leaky activity of the OK371 enhancer. Overall, our method of Gal4-to-Cas9 conversion simplifies the generation of new tissue-specific Cas9 lines and opens up broader opportunities for using CRISPR-TRiM to study gene function.

Discussion

CRISPR/Cas9-mediated mutagenesis holds great promise in advancing genetic analysis in *Drosophila* and is currently undergoing rapid development (41–43). However, existing tools for tissue-specific mutagenesis can still be improved to increase their power for discovery and functional analysis of genes. First, essential to the reliability of LOF analysis, gRNA efficiency can be further improved for both somatic and germline mutagenesis. Second, methods for labeling mutant cells within the tissue of interest are still missing. Lastly, although Gal4/UAS-Cas9 can be adopted for tissue-specific mutagenesis, convenient ways of applying CRISPR/Cas9 independent of Gal4/UAS are needed to maximize the simplicity, flexibility, and effectiveness of the system. In this study, we present solutions to these deficiencies. The tools we developed will likely be useful for *Drosophila* researchers to address broad biological questions and can be adapted to improve CRISPR/Cas9 approaches in other systems.

The success of tissue-specific mutagenesis depends vitally on gRNA efficiency. Although many algorithms have been developed to predict gRNA efficiency based on the target sequence (44), optimized gRNA expression vectors are still needed for achieving the maximal gRNA efficiency. The design of the gRNA expression vector can affect the rate of gRNA production and gRNA–Cas9 complex formation. We previously found that the tgFE design that incorporates tRNA^{Gly} spacers and the (F+E) gRNA scaffold for making multi-gRNAs was more efficient than other designs in knocking out genes in neurons (13). Now, we show that the combination of tRNA^{Gln} and the gRNA2.1 scaffold (the Qtg2.1 design) further improves gRNA efficiency in broad somatic tissues. This increase of gRNA efficiency is especially important for knocking out genes in polyploid tissues, like muscles and glia, or when more gRNAs are expressed simultaneously. It will also likely facilitate unmasking LOF phenotypes of genes expressed early in the cell lineage.

Our data suggest that including tRNAs in multi-gRNA constructs may be detrimental for the efficiency of germline mutagenesis. This unexpected result may reflect differences of tRNA-processing mechanisms in somatic and germline tissues. In addition, although gRNA(F+E) seems to be more efficient than gRNA2.1 for *cid*, our data for *gnu* and EGFP/GFP suggest that both scaffolds work well in the germline. Therefore, different gRNA expression vectors may be selected depending on whether somatic or germline mutagenesis is more important. For maximal efficiency of tissue-specific mutagenesis in the soma, we recommend the Qtg2.1 design, especially when more than two gRNAs are required (such as in co-CRISPR reporter constructs). For making dual gRNAs, we recommend CR7T-U63(2.1), as it offers the best trade-off for both the soma and the germline.

To solve the challenge of labeling mutant cells in tissue-specific mutagenesis, we incorporated co-CRISPR systems in gRNA vectors to report Cas9 activity in the tissue of interest both positively and negatively. Due to the nature of CRISPR/Cas9-mediated mutagenesis, mutations in the targeted cells are inherently heterogeneous. Therefore, a reporting system that can truly reflect the nature of mutations in every cell is unlikely to be feasible. However, we show that a practical approach is to correlate the loss of the target gene with that of a co-CRISPR reporter. Because this approach requires reliable mutagenesis of both the GOI and the reporter, its success depends on both the Cas9 and the gRNAs for the GOI. First, Cas9 needs to be expressed evenly and at a relatively high level in the intended tissue so that both the GOI and the reporter have ample opportunities to be mutated. For example, the high and persistent expression of *hh-Cas9* in the posterior wing disk results in reliable labeling of mutant cells, while its low and transient leaky expression in the anterior wing disk cannot be faithfully reported. It should be noted, however, that very high levels of Cas9 could be cytotoxic in some cell types (13, 42) and should also be avoided. Second, as gRNAs for co-CRISPR reporters are already chosen to be highly efficient, gRNAs for the GOI also need to be adequately efficient to minimize false-positive reporting. In practice, both Cas9s and gRNA transgenes need to be validated before use in labeled mutagenesis, such as by using our EGFP-BFP reporter line and the Cas9-induced lethal effect through the absence of Lig4 (Cas9-LEThAL) assay (13), respectively. It is important to note that it is not appropriate to predict

the quantitative degree of GOI LOF by measuring expression levels of co-CRISPR reporters, as incomplete removal of co-CRISPR reporters indicates weak or late Cas9 activity and hence poor correlation between mutagenesis of GOI and the reporters. Therefore, phenotypic analysis should be conducted only in cells that show robust co-CRISPR labeling.

To overcome the limited availability of tissue-specific Cas9 lines, we adopted the HACK approach (36) and developed reagents for convenient conversion of existing Gal4 lines into Cas9 lines, requiring no cloning or transformation steps. This method adds to the existing options of enhancer-driven Cas9 lines (13) and puts CRISPR-TRiM analysis within reach of the broader *Drosophila* community. As a proof of principle, we generated three tissue-specific Cas9s. Interestingly, converted Cas9s showed broader activity patterns than the original Gal4s due to the difference in the ways their activities are visualized. It is worth noting that the option of Gal4-driven Cas9 does not alleviate this discrepancy because Gal4-driven Cas9 could also have leaky expression (42) and mutagenesis by Gal4-driven Cas9 suffers even more from perdurance (13). Nevertheless, our results emphasize that it is important to validate the activity patterns of converted Cas9s before using them in tissue-specific mutagenesis.

Excitingly, large-scale transgenic gRNA libraries are currently in production and being made available to the *Drosophila* community (12, 42, 43). However, the mutagenic efficiencies of existing gRNAs vary significantly (42, 43). While progress has been made to improve gRNA efficiency in *Drosophila* and mammalian systems (13, 21), many optimizations have not been incorporated into these libraries. With the designs we present here, future libraries could be developed to fit specific screening needs, for example, in the germline, in somatic tissues, or for marked mutagenic analysis.

Methods

Fly Stocks. See *SI Appendix, Table S1* (Key Resource Table) for details of fly stocks used in this study. All flies were cultured on standard yeast–glucose medium in a 12:12 light/dark cycle at 25 °C. We used *ppk-CD4-tdGFP* (45) and *ppk > CD4-tdTom* (45) to visualize C4da neurons; *Gal4²¹⁻⁷ ppk-CD4-tdTom*; *UAS-Dcr-2* (46) for RNA interference; *CyO*, *Wee-P20* balancer as ubiquitous nuclear EGFP; *Nrg-GFP* to visualize epidermal cell shape; *UAS-CD4-tdGFP*, *UAS-RedStinger*, and *UAS-EGFP* to visualize Gal4 activity in third instar larvae; and *tubP(FRT.stop)Gal4 UAS-Flp UAS-mCD8::GFP* (40) to visualize expression histories of Gal4 drivers. To visualize activity patterns of *Hm1Δ-Cas9*, we used Cas9 positive tester because the *ubi* enhancer that drives GSR expression is not active in hemocytes.

See *SI Appendix, SI Methods* for details of molecular cloning, genetic assays, imaging, immunohistochemistry, image analysis and quantification, and statistical analysis. See *SI Appendix, Table S2* for gRNA cloning vectors generated for this study. See *SI Appendix, Table S3* for gRNA PCR template vectors. See *SI Appendix, Table S4* for tRNA sequences. See *SI Appendix, Table S5* for gRNA expression vectors. See *SI Appendix, Table S6* for gRNA target sequences. See *SI Appendix, Table S7* for primer designs for making gRNA expression constructs.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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