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Ubiquitous and Tissue-specific RNA Targeting in Drosophila melanogaster Using CRISPR/CasRx

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TITLE:

Ubiquitous and Tissue-specific RNA Targeting in *Drosophila melanogaster* Using CRISPR/CasRx

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KEYWORDS:

CRISPR, Cas enzyme, Ribonuclease, CasRx, *Drosophila melanogaster*, ubiquitous RNA targeting, tissue-specific RNA targeting

SUMMARY:

This article outlines a detailed protocol for using the RNA-targeting Cas13D enzyme (RfxCas13D) in flies.

ABSTRACT:

CasRx, a member of the RNA-targeting Cas13 family, is a promising new addition of the CRISPR/Cas technologies in efficient gene transcript reduction with an attractive off-target profile at both cellular and organismal levels. It is recently reported that the CRISPR/CasRx system can be used to achieve ubiquitous and tissue-specific gene transcript reduction in *Drosophila melanogaster*. This paper details the methods from the recent work, consisting of three parts: 1) ubiquitous in vivo endogenous RNA targeting using a two-component CasRx system; 2) ubiquitous in vivo exogenous RNA targeting using a three-component CasRx system; and 3) tissue-specific in vivo RNA targeting using a three-component CasRx system. The effects of RNA targeting observed include targeted gene specific phenotypic changes, targeted RNA transcript reduction, and occasional lethality phenotypes associated with high expression of CasRx protein and collateral activity. Overall, these results showed that the CasRx system is capable of target RNA transcript reduction at the organismal level in a programmable and efficient manner, demonstrating that in vivo transcriptome targeting, and engineering is feasible and lays the foundation for future in vivo CRISPR-based RNA targeting technologies.

INTRODUCTION:

Since the advent of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technologies, much of the focus in this field has been on DNA editing, which offers transformative applications in medicine and biotechnology[1]. Permanent alteration of DNA sequences, however, is not always desired due to ethical considerations. In light of this, recent studies began developing CRISPR-based tools for targeting RNA and demonstrated that CRISPR technologies can indeed be used for RNA-targeting in a variety of biological systems[2-7]. In many of these systems tested, the current widely used approach for targeting RNA and transcript reduction is RNA interference (RNAi), which is far from perfect, often exhibiting varied efficacy and high off-target activity when used in vivo[8-17]. Therefore, given the status of these technologies, it is worth further exploring the potentials of CRISPR-based tools for RNA targeting.

One notable recent study reported that ribonuclease CasRx, a member of the Cas13d class, can efficiently reduce gene transcript levels in human cell culture and possesses an attractive off-target profile[4]. This finding led to the question of whether this new ribonuclease can maintain its efficacy and low off-target rate for RNA targeting at the organismal level. A recent study addressed this question by showing that the CasRx system can be used to achieve ubiquitous and tissue-specific gene transcript reduction in *Drosophila melanogaster*[5].

To streamline usability of this recently published approach, this protocol details the methods from this recent work, which consists of three main parts: 1) ubiquitous in vivo RNA targeting using a two-component CasRx system; 2) ubiquitous in vivo exogenous RNA targeting using a three-component CasRx system; and 3) tissue-specific in vivo RNA targeting using a three-component CasRx system.

Guide RNAs (gRNA) targeting different target genes under the control of a ubiquitous promoter were designed and fly lines expressing these gRNA-containing constructs were generated. CasRx constructs under the control of either a ubiquitous promoter, or a conditional upstream activation sequence (UASt) promoter activatable by the GAL4 transcription factor, were also designed and fly lines harboring these CasRx-containing constructs generated. Catalytically inactive CasRx constructs, dCasRx, were designed and used as negative controls. Ubiquitous RNA targeting in flies is achieved by crossing gRNA-expressing fly lines with ubiquitously CasRx-expressing fly lines. The progeny expressing both the gRNA construct targeting a specific gene transcript and the CasRx protein has a ubiquitous reduction of targeted gene transcripts. Tissue-specific RNA targeting in flies is achieved by first crossing gRNA-expressing flies with UASt-CasRx expressing flies, obtaining transheterozygous flies carrying both gRNA and UASt-CasRx constructs. Such flies in turn are crossed with tissue-specific GAL4-expressing flies, resulting in the generation of tissue-specific CasRx expression and RNA-targeting in flies.

The programmable nature of the CasRx system offers the possibility of customization and optimization to help achieve high efficacy and low off-target activity for in vivo RNA targeting. Potential applications of CRISPR-based RNA-targeting are numerous, including replacing RNAi in the laboratory and contributing to insect vector control in the wild. Of the latter, one of the global unmet needs is the development of efficient tools to combat infections of RNA viruses

transmitted via mosquitos. Many RNA viruses, such as dengue, Zika, and chikungunya virus, are transmitted via mosquitoes, affecting human health, and contributing mortality. Many proposals for engineering mosquito populations with virus resistance for disease prevention have been made; however, no current technology is able to make mosquitoes simultaneously resistant to all significant RNA viruses[18–23]. RNA-targeting Cas systems may provide a starting point for such a technology by enabling a programmable platform for targeting all mosquito-borne RNA viruses.

PROTOCOL:

1. Ubiquitous in vivo RNA Targeting Using a Two-Component CasRx System

1.1. Generating Ubiq-CasRx and Ubiq-dCasRx expression vector

1.1.1. Amplify the CasRx sequence using a polymerase chain reaction (PCR) with primer 1050E.C3 and 1050E.C4 and the original CasRx construct pNLS-RfxCas13d-NLS-HA (pCasRx); and amplify the dCasRx sequence using PCR with primer 1050E.C3 and 1050E.C4 and the original dCasRx construct pNLS-dRfxCas13d-NLS-HA (pdCasRx)[4] (**Table 1**). Gel-purify the amplified CasRx and dCasRx fragments afterwards using a gel purification kit.

1.1.2. Digest base vector (Addgene plasmid #112686) with restriction enzymes SwaI and PacI[24]. In the resulting products, use a kit to gel-purify the larger fragment, which is called the base vector backbone.

1.1.3. Assemble the Ubiq-CasRx vector with the base vector backbone and the CasRx fragment using the Gibson assembly method; assemble the Ubiq-dCasRx vector with the base vector backbone and the dCasRx fragment using the Gibson assembly method[25].

NOTE: The Addgene ID of Ubiq-CasRx vector (OA-1050E) is #132416, and the Addgene ID of Ubiq-dCasRx vector (OA-1050R) is #132417.

1.2. Generating gRNA expression vector

1.2.1. Design each gRNA fragment based on the following criteria: target sequence being 30 nucleotides in length; the maximal length of poly-U stretches in the target sequence being 4 base pairs; the target sequence GC content being in the range of 30% - 70%; the target sequence predicted not to form strong RNA hairpin structures; and the target sequence containing minimal predicted RNA secondary or tertiary structure[5].

NOTE: This study designed each gRNA as 4 tandem sequences each 30 nucleotides long, spaced by 36 nucleotide long direct repeats, and with a 7-thymine terminator on both ends[5]. For the exogenous target gene, GFP, the same criteria as above was followed with an addition of a OpIE2-GFP fragment[5].

1.2.2. Amplify the U6:3 promoter sequence using PCR with primers 1043.C1 and 1043.C23 and the Addgene plasmid #112688 (**Table 1**)[26]. Gel-purify the amplified U6:3 fragments using gel purification kit.

1.2.3. Digest Addgene plasmid #112688 with restriction enzyme AscI and XbaI[24]. In the resulting products, use a kit to gel-purify the larger fragments, which is called the pre-base vector backbone.

1.2.4. Assemble the base vector with the pre-base vector backbone and the U6:3 fragment using the Gibson assembly method[25]. The base vector hereafter is named OA-1043.

NOTE: The plasmid OA-1043's Addgene ID is #164586.

1.2.5. Synthesize target gene's gRNA fragment using external gene synthesis service.

1.2.6. Digest the base vector OA-1043 with restriction enzyme PstI and NotI[24]. Keep the entire digestion product, which is called digested OA-1043.

1.2.7. Assemble gRNA expression vector with the digested OA-1043 and target gene gRNA fragment using the Gibson assembly method[25].

NOTE: Four target genes were studied: three were endogenous (*white*, *Notch*, *yellow*), one was exogenous (*GFP*). Their Addgene IDs are: #132420 (gRNA^{w}), #132421(gRNA^{N}), #132425 (gRNA^{y}), and #133304 (gRNA^{GFP}).

1.3. Generating transgenic flies

1.3.1. Inject expression vectors into fly embryos using external fly embryo injection service and embryos from flies containing ϕC31 integration sites. Rear the injected embryos at 26 °C.

NOTE: The attp40w (with integration sites on the 2nd chromosome) line was used to generate CasRx lines and 8622 (with integration sites on 3rd chromosome) line was used to generate various gRNA lines.

1.3.2. Keep the flies either as homozygous lines or as balanced heterozygous lines.

NOTE: Ubiq-CasRx and Ubiq-dCasRx flies were kept as heterozygous balanced lines with the CyO as the balancer. In addition, both Ubiq-CasRx and Ubiq-dCasRx vectors contain a dsRed marker. As a result, the Ubiq-CasRx and Ubiq-dCasRx flies have the following three phenotypes: dsRed-positive, curly wings, and white eyes. The gRNA-expressing flies were kept as homozygous lines. Their Bloomington Drosophila Stock Center (BDSC) fly stock numbers are: #84118 (Ubiq-CasRx), #84119 (Ubiq-dCasRx), #84124 (gRNA^{w}), #84122 (gRNA^{N}), #84123 (gRNA^{y}), #84986 (gRNA^{GFP}).

1.4. Fly genetics (**Figure 1A**)

1.4.1. Collect 10 virgin adult female flies from the homozygous gRNA line and collect 5 adult male flies from the balanced heterozygous Ubiq-CasRx/CyO line. Put the collected female and male flies, which are called the parental flies, in a vial supplemented with dry yeast powder (**Figure 1A**).

1.4.2. Repeat the previous step 3 times to generate 3 replicates. For the control group, use the Ubiq-dCasRx/CyO line while keeping everything else the same.

NOTE: For a regular glass vial of food, 0.1 g of dry yeast powder is sufficient. The BDSC's fly food recipe is used.

1.4.3. Rear the vials containing the parental flies at 26 °C for 48 hours. Then remove all parental flies from every vial. Then keep the vials at 26 °C for at least 20 days.

1.4.4. Observe the vials every day to see if any new adult progenies emerged from pupae of F1 generation. If so, anesthetize them with carbon dioxide by inserting a tube connected to a carbon dioxide tank inside the fly vials, then turning the flow switch on for 10 seconds.

1.4.5. Once the flies become immobile, empty them from the vial onto a fly-pad, which is also connected to the carbon dioxide tank and carbon dioxide continuously flows out through the fly pad.

1.4.6. Score the flies' phenotypes and image them using color camera-connected to a fluorescent stereomicroscope. Count the numbers of progenies with different phenotypes. Use image processing software for image post-processing and compilation (**Figure 2A - 2D**).

NOTE: Based on Mendelian genetics, two types of flies are expected among the progenies for each cross (**Figure 1A**).

1.5. RNA-Seq (**Figure 2E – 2G**)

1.5.1. Sample collection

NOTE: Choose an appropriate sample collection method from the 3 examples below; 3 replicates for each distinct sample type are required.

1.5.1.1. Adult fly head sample collection

1.5.1.1.1. Collect 10 virgin adult female flies from the homozygous gRNA line. Collect 5 adult male flies from the balanced heterozygous Ubiq-CasRx/CyO line. Put the collected female and male flies, which are the parental flies, in a vial supplemented with dry yeast powder.

1.5.1.1.2. Repeat the previous step 3 times for 3 replicates. For the control group, use the Ubiq-dCasRx/CyO line while keeping everything else the same.

1.5.1.1.3. Rear the vials containing the parental flies at 26 °C for 48 hours. Then remove all parental flies from every vial. Then keep the vials at 26 °C until progenies emerge from pupae.

1.5.1.1.4. Collect 10 1-day old adult flies with the correct phenotype. Anesthetize the flies with carbon dioxide, then cut off the fly head and put the heads in a 1.5 mL centrifuge tube on dry ice. Store the centrifuge tube at -80 °C. Repeat this step 3 times for 3 replicates.

1.5.1.2. 17 – 20 hour-old embryo sample collection

1.5.1.2.1. Collect 8-10 virgin adult female flies from the homozygous gRNA line. Collect 4-5 adult male flies from the balanced heterozygous Ubiq-CasRx/CyO line. Put the collected female and male flies, which are the parental flies, in a vial supplemented with dry yeast powder.

1.5.1.2.2. Repeat the previous step 3 times for 3 replicates. For the control group, use the Ubiq-dCasRx/CyO line while keeping everything else the same.

1.5.1.2.3. Rear the vials containing the parental flies at 26 °C for 48 hours.

1.5.1.2.4. Prepare one grape-juice embryo collection chamber for each replicate following this recipe: 376 mL of water, 126 mL of grape juice, 15 g of agar, and 6 g of sucrose. Put the media in a 1 L beaker and microwave it on high for 5-6 minutes while keeping a close eye on the media in the beaker to check if bubbles/foam appears. If so, stop the microwave and let the bubble/foam to settle. Continue microwaving this way until the bubble becomes clear. Do not swirl until all bubbles are clear. Finally, add 10 mL of 100% alcohol and 5 mL of acetic acid. Mix well, then pipet the media into 35 mm Petri dishes with a 25 mL serological pipet. When media solidifies in the Petri dish, it is ready for use.

1.5.1.2.5. At the end of the 48-hour incubation, transfer parental flies to the grape-juice embryo collection chambers and incubated them at 26 °C for 3 h. Then remove the adult flies while keeping the freshly laid embryos on the grape-juice plates for another 17 h at 26 °C.

1.5.1.2.6. After incubation, collect the 50 – 100 embryos from the grape-juice plates, clean the embryo surface by submerging them in deionized water, then transfer them to a 1.5 mL centrifuge tube on ice. Store them at -80 °C. Repeat this step 3 times for 3 replicates.

1.5.1.3. First instar larvae sample collection

1.5.1.3.1. Collect 8-10 virgin adult female flies from the homozygous gRNA line. Collect 4-5 adult male flies from the balanced heterozygous Ubiq-CasRx/CyO line. Put the collected female and male flies, which are the parental flies, in a vial supplemented with dry yeast powder. Repeat

this step 3 times for 3 replicates. For the control group, use the Ubiq-dCasRx/CyO line while keeping everything else the same.

1.5.1.3.2. Rear the vials containing the parental flies at 26 °C for 48 hours. Then transfer the adult flies to another new regular food vial for overnight incubation (16 h) at 26 °C. Then, remove the adult flies.

1.5.1.3.3. Keep the embryo-containing vial at 26 °C for 24 h, then score the transheterozygous first instar larvae under the microscope using based distinct markers. Collect 15-30 larvae with correct phenotypes and put them into a 1.5 mL centrifuge tube and store them at -80 °C. Repeat this step 3 times for 3 replicates.

1.5.2. Sequencing

1.5.2.1. RNA extraction: use a commercially available RNA extraction kit and follow the kit's instruction to process all samples. Then, incubate the extracted RNA samples with commercially available deoxyribonuclease and follow its instruction to remove any contaminating DNA from the samples.

1.5.2.2. Measure RNA concentration using commercially available UV-vis spectrophotometer. Measure RNA integrity in the samples using commercially available RNA integrity assay setup.

1.5.2.3. Construct the RNA-seq libraries using commercially available RNA library preparation kit.

1.5.2.4. Use external sequencing service for library sequencing with the following settings: single read mode; read length: 50nt, depth: 20 million reads per library. Perform base calls with RTA 1.18.64 and then converted the data to FASTQ using bcl2fastq 1.8.4.

NOTE: Raw sequencing data can be found in the National Center for Biotechnology Information Sequencing Read Archive (submission ID: SUB6818910 [BioProject: PRJNA600654]).

1.5.3. Bioinformatics

1.5.3.1. Map reads from the sequencing data to Release 6 Drosophila melanogaster genome from the Berkeley Drosophila Genome Project (GenBank accession number: GCA_000001215.4) and the exogenous *CasRx* and *GFP* sequences using the default parameter setting of STAR aligner[28] with the addition of the "-outFilterType BySJout" filter option and "-sjdbGTFfile Drosophila_melanogaster.BDGP6.22.97.gtf" gene transfer format file from ENSEMBL.

1.5.3.2. Determine the raw transcript counts for each annotated transcript with the feature Counts35 using the "-t exon -g gene_id -M -O --fraction" options. Then, normalize raw

transcript counts using total transcript counts using the "addTpmFpkmToFeatureCounts.pl" Perl script.

1.5.3.3. Use the maximum posteriori method with the original shrinkage estimator in the DESeq2 pipeline to estimate each gene's transcripts' logarithmic fold change (LFC).

2. Ubiquitous in vivo exogenous RNA Targeting Using a Three-Component CasRx System

2.1. Generating exogenous target ubiquitous expression vector

2.1.1. PCR amplify the Ubiq promoter fragment using primers 1052B.C1 and 1052B.C2 and the Addgene plasmid #112686[26]. Then, PCR amplify the T2A-eGFP fragment amplified from Addgene plasmid #112686 with primers 908A.1 and 908A.2 (**Table 1**)[26]. Then, PCR amplify the Ubiq promoter fragment as reversed sequence using Addgene plasmid #112686 with primers 908A.3 and 908A.4 (**Table 1**)[26]. Gel-purify the Ubiq promoter fragment, the T2A-eGFP fragment, and the reversed Ubiq promoter fragment using gel purification kit.

2.1.2. Order a custom firefly luciferase coding sequence and a custom fragment containing a p10 3'UTR fragment, reversed renilla luciferase followed by an SV40 3'UTR fragment.

2.1.3. Digest Addgene plasmid #112688 with restriction enzyme AscI and XbaI[24]. In the resulting products, gel-purify the larger fragments using gel purification kit, which is called the base vector backbone.

2.1.4. Use the Gibson assembly method to assemble base vector with the base vector backbone and the following fragments: Ubiq promoter fragment, the T2A-eGFP fragment, the reversed Ubiq promoter fragment, the firefly luciferase coding sequence, and the reversed renilla luciferase followed by an SV40 3'UTR fragment[25].

NOTE: The Addgene ID of the resulting dual-luciferase expression vector (OA-1052B) is #132426.

2.2. Generating gRNA expression vector

2.2.1. PCR amplify the U6:3 promoter sequence using primers 1043.C1 and 1043.C23 and the Addgene plasmid #112688 (**Table 1**)[26]. Gel-purify the amplified U6:3 fragments using gel purification kit.

2.2.2. Digest Addgene plasmid #112688 with restriction enzyme AscI and XbaI[24]. In the resulting products, gel-purify the larger fragments, which is called the pre-base vector backbone, using gel purification kit.

2.2.3. Assemble base vector with the pre-base vector backbone and the U6:3 fragment using the Gibson assembly method[25]. The base vector hereafter is named OA-1043.

2.2.4. Synthesize target gene's gRNA fragment using external gene synthesis service.

2.2.5. Digest the base vector OA-1043 with restriction enzyme PstI and NotI[24]. Keep the entire digestion product, which iscalled digested OA-1043.

2.2.6. Assemble gRNA expression vector with the digested OA-1043 and target gene gRNA fragment using the Gibson assembly method[25].

NOTE: The Addgene ID of the resulting plasmid (OA-1052K) is #132422.

2.3. Generating transgenic flies

2.3.1. Inject OA-1052B vector into fly embryos using embryos from flies containing ϕC31 integration site on the 3rd chromosome, BDSC fly stock number 9744, via external fly embryo injection service. Similarly, inject OA-1052K vector into fly embryos using embryos from flies containing ϕC31 integration site on the 3rd chromosome, BDSC fly stock number 8622. Rear the injected embryos at 26 °C.

2.3.2. Keep the dual-luciferase-expressing flies and the gRNA flies as homozygous lines; keep the Ubiq-CasRx lines as double-balanced heterozygous lines by outcrossing the single-balanced heterozygous Ubiq-CasRx line generated in section 1 to balancer lines carrying TM6 balancer chromosome with stubble (Stb) marker and retain only the double-balanced progenies with white-eyed, curly wings, and dsRed-fluorescent phenotypes simultaneously.

NOTE: The BDSC fly stock numbers are: #84127 (Ubiq-Fluc-Rluc), #84125 (gRNA^{Fluc}).

2.4. Fly Genetics (**Figure 1B and Figure 3A**)

2.4.1. Collect 8-10 virgin adult female flies from the dual-luciferase-expressing line. Collect 4-5 adult male flies from the balanced heterozygous Ubiq-CasRx/CyO; +/TM6, Stb line that show white-eyed, curly wings, and dsRed fluorescence simultaneously. Put the collected female and male flies, which are the parental flies, in a vial supplemented with dry yeast powder (hereafter called Step 1 Cross).

2.4.2. Repeat the previous step 3 times for 3 replicates. For the control group, use the Ubiq-dCasRx/CyO; +/TM6, Stb line while keeping everything else the same.

2.4.3. Rear the Step 1 Cross vials containing the parental flies at 26 °C for 48 hours. Then remove all parental flies from every vial. Then keep the vials at 26 °C for at least 14 days. During this time, collect 8-10 female virgins from the homozygous firefly luciferase-targeting gRNA line. Repeat this step 3 times for 3 replicates.

2.4.4. Observe the Step 1 Cross vials every day to see if any new adult progenies emerged from pupae. If so, anesthetize them with carbon dioxide, collect 5 male flies expressing both the Ubiq-

CasRx (or Ubiq-dCasRx) and the dual-luciferase reporter from the progenies and put them into a new vial along with 10 virgin females from the firefly luciferase-targeting gRNA line (hereafter called Step 2 Cross). Repeat this step 3 times for 3 replicates.

2.4.5. Collect another 5 one-day old male expressing both the Ubiq-CasRx (or Ubiq-dCasRx) and the dual-luciferase reporter from the Step 1 Cross vials and incubate them for 2 – 4 days at 26 °C. Then, transfer them into 1.5 mL centrifuge tube and store them at -80 °C. Repeat this step 3 times for three replicates.

2.4.6. Rear the Step 2 Cross vials containing the parental flies at 26 °C for 48 hours. Then remove all parental flies from every vial. Then keep the vials at 26 °C for at least 20 days.

2.4.7. Observe the Step 2 Cross vials every day to see if any new adult progenies emerged from pupae. If so, anesthetize them with carbon dioxide, score their phenotypes and image them using color camera equipped with a fluorescent stereomicroscope. Count the numbers of progenies with different phenotypes. Use image processing software for image post-processing and compilation (**Figure 3B – 3C**).

NOTE: Mendelian genetics suggest that, if flies are all viable, 4 types of flies are expected among the progenies from Step 2 Cross, each accounting for 25% of the population (**Figure 1B** and **Figure 3A**).

2.5. Luciferase assay (**Figure 3D**)

2.5.1. Generate the triple transheterozygous flies as well as the control flies by repeating steps 2.4.1-2.4.5. Collect male flies at birth and age them until 3 days old.

2.5.2. Transfer the 3-day old flies into 1.5 mL centrifuge tubes and lyse them using a pestle and the luciferase lysis buffer of commercially available luciferase assay kit.

2.5.3. Use 5 µL of lysed tissue from each sample to measure both firefly and renilla luciferase activity using commercially available luciferase assay kit and luminometer.

3. Tissue-specific in vivo RNA Targeting Using a Three-Component CasRx System

3.1. Generating UASt-CasRx and UASt-dCasRx Expression Vector

3.1.1. PCR amplify the UASt promoter sequence using plasmid pJFRC81 and primers 1041.C9 and 1041.C11; then, PCR amplify CasRx fragment using plasmid OA-1050E (Addgene ID #132416) and primers 1050L.C1 and 1050E.C4; and then PCR amplify dCasRx fragments using plasmid OA-1050R (Addgene ID #132417) and primers 1050L.C1 and 1050E.C4 (**Table 1**)[26]. Gel-purify the amplified UASt promoter sequence, CasRx, and dCasRx fragments using gel purification kit.

3.1.2. Digest base vector (Addgene plasmid #112686) with restriction enzymes NotI and PacI[24]. In the resulting products, gel-purify the larger fragment, which is called the base vector backbone, using gel purification kit.

3.1.3. Assemble the UASt-CasRx vector with the base vector backbone, the UASt promoter sequence, and CasRx fragment using the Gibson assembly; then assemble the UASt-dCasRx vector with the base vector backbone, the UASt promoter sequence, and the dCasRx fragment using the Gibson assembly method[25].

NOTE: The UASt-CasRx vector is Addgene plasmid #132418, and the UASt-dCasRx vector is Addgene plasmid #132419

3.2. Generating transgenic flies

3.2.1. Inject UASt-CasRx vector into fly embryos using fly embryo injection service and embryos from flies ϕC31 integration site 8621 on their 2nd chromosomes; then inject UASt-dCasRx vector into fly embryos using fly embryo injection service and embryos from flies ϕC31 integration site 8621 on their 2nd chromosomes. Rear the injected embryos at 26 °C.

3.2.2. Keep the flies as double balanced heterozygous lines with CyO and Sb markers. NOTE: The IDs of the fly lines in BDSC are 84121 (UASt-CasRx) and 84120 (UASt-dCasRx).

3.3. Fly genetics (**Figure 1C**)

3.3.1. Order desired GAL4 lines from BDSC; Obtain relevant gRNA lines from step 3.2.2 (or from BDSC).

NOTE: The following 2 GAL4 flies from BDSC were used: GAL4-GMR (BDSC ID: #29967), GAL4-y (BDSC ID: #44373). The same 3 gRNA lines generated in the first section were used: gRNA^{w} (BDSC ID: #84124), gRNA^{N} (BDSC ID #84122), gRNA^{y} (BDSC ID: #84123).

3.3.2. Collect 5-10 virgin adult female flies from the gRNA line. Collect 2-4 adult male flies from the double balanced heterozygous UASt-CasRx/CyO; +/TM6, Sb line that show white-eyed, curly wings, and dsRed fluorescence simultaneously. Put the collected female and male flies, which are the parental flies, in a regular food vial (hereafter called Step 1 Cross). Repeat this step 3 times for 3 replicates. For the control group, use the UASt-dCasRx/CyO; +/TM6, Sb line while keeping everything else the same.

3.3.3. Rear the Step 1 Cross vials containing the parental flies at 26 °C for 48 hours. Then remove all parental flies from every vial. Then keep the vials at 26 °C for at least 14 days. During this time, collect 5-10 female virgins from the GAL4 line. Repeat this step 3 times for 3 replicates.

3.3.4. Observe the Step 1 Cross vials every day to see if any new adult progenies emerged from pupae. If so, anesthetize them with carbon dioxide, collect 2-4 male flies expressing both the

UASt-CasRx (or UASt-dCasRx) and the gRNA vector from the progenies which simultaneously have dsRed-fluorescent and stubble phenotypes. Put the collected males from Step 1 Cross into a new vial along with 5-10 collected virgin female from the GAL4 line (hereafter called Step 2 Cross). Repeat this step 3 times for 3 replicates.

3.3.5. Rear the Step 2 Cross vials containing the parental flies at 26 °C for 48 hours. Then remove all parental flies from every vial. Then keep the vials at 26 °C for at least 20 days.

3.3.6. Observe the Step 2 Cross vials every day to see if any new adult progenies emerged from pupae. If so, anesthetize them with carbon dioxide, score their phenotypes and imaged them using color camera equipped with a fluorescent stereomicroscope. Count the numbers of progenies with different phenotypes. Use image processing software for image post-processing and compilation (**Figure 4**).

NOTE: Mendelian genetics suggest that, if flies are all viable, 4 types of flies are expected among the progenies from Step 2 Cross, each accounting for 25% of the population (**Figure 1C**).

REPRESENTATIVE RESULTS:

Ubiquitous in vivo RNA Targeting Using a Two-Component CasRx System

The F_1 transheterozygous flies expressing both the Ubiq-CasRx and the gRNA (targeting both endogenous and exogenous genes) constructs showed marked phenotypes compared to the control flies expressing the Ubiq-dCasRx and gRNA constructs (**Figure 2** and **Figure 4**). Specifically, the transheterozygous CasRx flies have significantly lower levels of survival rate compared to the transheterozgyous dCasRx flies, indicating toxicity of the Ubiq-CasRx system (**Figure 2A** and **Figure 4A**). It is worth noting that both transheterozygous CasRx and dCasRx flies have less than 50% inheritance rate, which is the expected ratio based on Mendelian genetics. Of the three target genes, the Ubiq-CasRx/+; U6-gRNA^N/+ flies and Ubiq-CasRx/+; U6-gRNA^y/+ flies are non-viable (0% inheritance) and did not grow beyond the second instar larvae stage (**Figure 2A-2B**). The surviving Ubiq-CasRx/+; U6-gRNA^w/+ flies, the inheritance of which was 12.9%, showed a distinct fully-penetrant white-eyed phenotype (**Figure 2B**). In addition to observable traits associated with CasRx, we were able to confirm significant reduction of target gene transcripts for 3 target genes: *Notch*, *yellow*, and *GFP* (**Figure 2E-2G**). Reduction of *white* gene transcripts was observed in Ubiq-CasRx/+, U6-gRNA^w/+ flies, compared to the control Ubiq-dCasRx/+, U6-gRNA^w/+ flies, though the reduction was not statistically significant (**Figure 2E - 2F**). Evidence of off-target activity induced by CasRx was found when comparing the differentially expressed transcripts between samples from CasRx-expressing flies and samples from dCasRx-expressing flies (**Figure 2E, 2G**). The number of non-target transcripts significantly differentially expressed are as follows: *white*, 253 (1.4% of total transcripts); *Notch*, 300 (1.7%); *yellow*, 41 (0.23%); *GFP*, 5,880 (33%) (**Figure 2G**)**.** Out of the total 17,779 different transcripts, 6 non-target transcripts were significantly differentially expressed in all 4 groups of samples. One of the 6 transcripts identified was Gadd45, a gene involved in apoptosis and cellular arrest in flies, raising the possibility that the enzymatic action of CasRx may either directly trigger cellular apoptosis or indirectly trigger misexpression of other genes, which in turn leads to apoptosis. Finally, it is worth noting that the Ubiq-CasRx and Ubiq-dCasRx flies were not established as homozygous

stocks, presumably due to toxicity conferred by high ubiquitous expression. As a result, heterozygous Ubiq-CasRx/CyO and Ubiq-dCasRx/CyO flies were used for crossing with homozygous gRNA fly lines. In sum, the two-component Ubiq-CasRx system is able to achieve ubiquitous RNA targeting for both endogenous and exogenous targets resulting in observable phenotypes and transcript reduction. These results also showed that CasRx-mediated RNA targeting may introduce toxicity in vivo.

Ubiquitous in vivo exogenous RNA Targeting Using a Three-Component CasRx System

The results from the two-step cross showed that despite the exogenous nature of the target gene (i.e., Fluc), expressing all three transgenes in F_2 triple transheterozygotes (Ubiq-CasRx/+; gRNA^{Fluc}/Ubiq-Fluc-Ubiq-Rluc) resulted in 100% lethality compared to control crosses involving Ubiq-dCasRx, where no lethality was observed in the F_2 triple transheterozygotes (Ubiq-dCasRx/+; gRNA^{Fluc}/Ubiq-Fluc-Ubiq-Rluc) (**Figure 3B-C**). More specifically, only the combination of all three transgenes (Ubiq-CasRx/+; gRNA^{Fluc}/Ubiq-Fluc-Ubiq-Rluc) resulted in 100% lethality (**Figure 3B and D**), while (Ubiq-CasRx/+; gRNA^{Fluc}/TM6) and (Ubiq-CasRx/+; Ubiq-Fluc-Ubiq-Rluc/TM6) genotypes were viable and lacked phenotypes with their inheritance rates matching the expected Mendelian transmission rates, suggesting that the availability of the target sequence (i.e., *firefly luciferase*) in combination with Ubiq-CasRx/+ and the gRNA^{Fluc} is what resulted in the observed lethality phenotypes, presumably stemming from the collateral activity of Cas13 enzymes[2, 8]. In addition, no distinguishable phenotypes or dramatic influence on inheritance in F_1 transheterozygotes (Ubiq-CasRx/+; gRNA^{Fluc}/+ or Ubiq-CasRx/+; Ubiq-Fluc-Ubiq-Rluc/+) were observed compared to Ubiq-dCasRx controls (Ubiq-dCasRx/+; gRNA^{Fluc}/+ or Ubiq-dCasRx/+; Ubiq-Fluc-Ubiq-Rluc/+) (**Figure 3B**), indicating that a catalytically active enzyme is essential to obtain the lethality phenotypes observed. Furthermore, Fluc and Rluc expression levels in flies of all viable genotypes did not show significant reduction in Fluc expression in the Ubiq-dCasRx triple transheterozygotes (Ubiq-dCasRx/+; gRNA^{Fluc}/Ubiq-Fluc-Ubiq-Rluc) compared to dual luciferase reporter controls. This suggests that Fluc protein expression levels were not reduced by dCasRx targeting (**Figure 3D**). Taken together, the common lethality phenotype in the two different CasRx-mediated ubiquitous RNA targeting experiments indicate that when used on tissues ubiquitously, CasRx-mediated RNA targeting can be toxic to the organism.

Tissue-specific in vivo RNA Targeting Using a Three-Component CasRx System

The high level of toxicity observed in ubiquitous RNA targeting experiments prompted us to explore the tissue-specific RNA targeting using a three-component CasRx system design detailed in the methods section. Indeed, the level of toxicity observed was reduced when the overall CasRx expression was lowered using the UASt promoter compared to that of the Ubiq promoter, this is exemplified in three aspects: 1) the UASt-CasRx and UASt-dCasRx lines were maintained as homozygous lines, though based on the two-step cross scheme double balanced UASt-CasRx and UASt-dCasRx lines were used to perform the crosses, 2) all F_2 generation dCasRx triple transheterozygous inheritance rates matched the expected 25% Mendelian inheritance rate, and 3) the F_2 generation CasRx triple transheterozygous lethality phenotype was moderately reduced. In the *white* targeting experiment, of the 25% Mendelian inheritance rates expected in the F_2 triple transheterozygotes, only 0.57% viable adult flies (UASt-CasRx/+; gRNA^{w}/GMR-Gal4) were observed, all of which displayed severe eye specific pigmentation and morphology phenotypes

(**Figure 4A and 4B**). For the *white*-targeting cross, the CasRx-expressing triple transheterozygous F_2 inheritance rate was significantly lower than that of the dCasRx-expressing triple transheterozygous control group (27.6%) (**Figure 4A**). In the *Notch* targeting experiment, CasRx-expressing triple tranheterozygous carrying all three transgenes were 100% lethal, while the dCasRx control inheritance rate was 29.3% (**Figure 4A**). In the *yellow* targeting experiment, F_2 triple transheterozygous CasRx-expressing, gRNA^{y}, and *y*-GAL4 showed marginal chitin pigment reduction as small patches of yellow cuticle on the thorax and abdomen with an inheritance rate of 2.67%, much lower than that of the dCasRx control group (25.2%) (**Figure 4A**). All dCasRx control triple transheterozygous flies did not present obvious phenotypes as the CasRx-expressing flies, indicating that catalytic activity of CasRx contributed to the phenotypes observed. The low inheritance rate in the CasRx triple transheterozygous group suggested that two sources of toxicity exist in CasRx RNA targeting: one is associated with high expression of CasRx, the toxicity of which was reduced by restrictive CasRx expression, the other is associated with the collateral activity. Taken together, these results showed that the CasRx system can achieve tissue-specific in vivo RNA targeting by leveraging the classical Gal4/UASt system and in the meantime reduce the toxicity. However, toxicity and occasional lethality phenotypes were still observed at a lower level of severity compared to that of the ubiquitous approaches, indicating that collateral cleavage activity is associated with toxicity.

FIGURE AND TABLE LEGENDS:

Figure 1: General overview of RNA targeting using a Cas13D system. (A) Schematics of the one-step genetic cross in the ubiquitous in vivo RNA targeting using a two-component CasRx system. (B) Schematics of a two-step genetic cross in the ubiquitous in vivo exogenous RNA targeting using the three-component CasRx system. (C) Schematics of a two-step genetic cross in the tissue-specific in vivo RNA targeting using a three-component CasRx system.

Figure 2: Ubiquitous in vivo RNA targeting using a two-component CasRx system (reprinted[5]). (A) Total inheritance percentages of transheterozygous flies inheriting Ubiq-CasRx (or Ubiq-dCasRx) and gRNAs. Blue shading in the box plot indicates phenotype penetrance. (B) Phenotypes of transheterozygous flies. Arrows indicate tissue necrosis in the eye. Black and white fly marked with ''X'' represents lethality. (C) Total inheritance percentages of transheterozygous flies of bidirectional crosses between Ubiq-CasRx (or Ubiq-dCasRx) and gRNA^{GFP}-OpIE2-GFP flies. M, maternal inheritance of CasRx; P, paternal inheritance of CasRx. (D) F1 larvae progenies in the paternal cross. (E) Transcripts' maximum a posteriori estimates for the logarithmic fold change. DESeq2 pipeline was used. (F) Transcripts per million (TPM) targeted with CasRx or dCasRx. (G) CasRx-depentent differentially expressed transcript percentage of transcripts.

Figure 3: Ubiquitous in vivo exogenous RNA targeting using a three-component CasRx system. (A) Schematics of the two-step genetic cross. (B) Total inheritance percentages for all genotypes emerging in the F_2 generation. Inheriting all three transgenes (Ubiq-CasRx, Ubiq-Fluc-Ubiq-Rluc, and gRNA^{FLuc}) in F_2 progeny resulted in 100% lethality and was significantly lower compared to the Ubiq-dCasRx triple transheterozygotes control group ($p = 0.001$, t-test). (C) Carrying Ubiq-CasRx/gRNA^{Fluc} alone or Ubiq-CasRx and Ubiq-Fluc-Ubiq-Rluc alone did not lead to severe lethality, and inheritance ratios between Ubiq-CasRx and Ubiq-dCasRx transheterozygotes were

not significantly different (p = 0.41 and p = 0.51, respectively, t-test). (D) Luciferase ratios normalizing Fluc readings to Rluc readings. Triple transheterozygous flies expressing Ubiq-CasRx, Ubiq-Fluc-Ubiq-Rluc, gRNA^{FLuc} were embryonic lethal, which was represented by a fly with an "X", and as a result luciferase expression was not measured. Fluc/Rluc ratio of Ubiq-CasRx/+, Ubiq-Fluc-Ubiq-Rluc/TM6, Stb transheterozygotes was significantly lower than that of the other Ubiq-Fluc-Ubiq-Rluc-expressing groups (p = 1.2e-06 or lower, t-test). The results from the gRNA^{FLuc}-only group were significantly lower than that of all other groups (p = 1.2e-06 or lower, t-test).

Figure 4: Tissue-specific in vivo RNA targeting using a three-component CasRx system (reprinted[5]). (A) Total inheritance percentage of triple transheterozygous flies carrying three transgenes (UASt-CasRx or UASt-dCasRx, gRNAs, and Gal4-driver. (B) Phenotypes of the triple transheterozygous flies. The white arrow indicates chitin pigment reduction in the thorax. Black and white fly marked with ''X'' represents lethality.

Table 1: List of molecular construct and primers used in this study. This list includes all constructs (both the ID and description) and each construct's associated primers (both the ID and sequences (5' to 3')) and templates used.

DISCUSSION:

With three different application designs of the CasRx system, this work demonstrated in vivo programmable RNA targeting in flies. The different strategies cater to different project needs, such as endogenous versus exogenous gene targeting and ubiquitous versus tissue-specific RNA targeting. The effects of RNA targeting included target gene specific phenotypic changes, target RNA transcript reduction, and occasional lethality phenotypes associated with high expression of CasRx protein and collateral activity. Overall, these results showed that the CasRx system is capable of target RNA transcript reduction at the organismal level in a programmable and efficient manner.

One of the key factors in successful customizing of the CasRx system is the design of gRNAs. Specifically, the following advice shall be heeded: the target sequence is around 30 nucleotides in length, the length of poly-U stretches in the target sequence is 4 base pairs or less, the target sequence GC content is in the range of 30% - 70%, the target sequence is not predicted to form strong RNA hairpin structures, and the target sequence contains minimal predicted RNA secondary or tertiary structure[5].

In addition to the gRNA designs, the fly genetics step in each protocol is also critical in a successful implementation. The presence or lack of the defined phenotypes passed down from the parents in the progenies are important for identifying and quantifying phenotypes induced by the CasRx system in the transheterozygous progenies. Also, setting up control crosses using the dCasRx flies in parallel are also helpful in the ruling out non-specific phenotypes in the transheterozygous progenies.

It is worth noting that these results revealed toxicity issue introduced by ubiquitously expressing CasRx and dCasRx protein in the fly, a limitation of CasRx system. Ubiquitous expression of CasRx

or dCasRx under the Ubiq promotor alone, without gRNAs, came with nontrivial fitness costs, as neither Ubiq-CasRx nor Ubiq-dCasRx flies could be established as homozygous lines. On the contrary, UASt-CasRx and UASt-dCasRx flies can be established as healthy homozygous stocks, though due to the design of the cross scheme they were kept as double-balanced stocks, a fact that supports the existence of toxicity induced by ubiquitous CasRx protein expression. Another piece of supporting evidence is that in control experiments involving dCasRx, which is catalytically inactive, the percentages of flies carrying both dCasRx and gRNA constructs out of the total number of flies in the F_1 generation were consistently lower than 50%, the ratio expected based on Mendelian genetics if no dCasRx-associated toxicity was present. This indicated that ubiquitously expressing dCasRx, along with gRNAs, induces toxicity in the fly, resulting less than expected inheritance ratio. The inheritance ratios of transheterozygous UASt-dCasRx, gRNA, GAL4 flies followed Mendelian genetics, which again suggests the toxicity induced specifically by ubiquitous expression of CasRx and dCasRx proteins. Toxicity in CRISPR/Cas system is not new. High amounts of Cas9 protein has been shown to be toxic in several organisms, including flies[29-32]. A recent study has developed a customized GAL4/UAS system that can tune the amount of Cas9 protein expressed in flies by adding an open-reading frame of varying length between the UAS sequence and the Cas9 sequence in the UAS-Cas9 construct[33]. Therefore, it is worth exploring ways to reduce CasRx-induced toxicity by tuning the CasRx protein expression level.

Other than the toxicity induced by ubiquitous expression of CasRx and dCasRx proteins, the results also showed lethality linked to CasRx system's non-specific collateral off-target effects, a feature of many CRISPR systems[1, 2, 7, 34]. In some of the CasRx and non-essential gene gRNA-expressing double or triple transheterozygous flies, for example when targeting *Notch*, the transheterozygous CasRx flies have significantly lower levels of survival rate compared to the transheterozgyous dCasRx flies. In the RNA-seq analysis of these CasRx and gRNA-expressing transheterozygous flies, both the reduction of target gene transcript levels and the reduction of non-target gene transcripts were observed. These collateral effects were CasRx-dependent and target-dependent, as they were only observed in transheterozygous flies expressing both the CasRx protein and the gRNA. It is worth pointing out that one of the target genes, *white*, showed only a limited, non-statistically significant reduction in transcripts when the *white* gene was targeted by CasRx, which was in contrast to the clear pigment reduction phenotype. It is hypothesized that this may be due to the fact that 1) the timing of RNA-seq sample collection was not well aligned with the timing when the *white* gene reach its peak expression during early development, and 2) the localized expression of the white gene in the eyes makes it challenging to collect the relevant tissues during early development phase when only whole-body sample collection is feasible. To reduce collateral activity in the CasRx system, future studies are called for to fully understand the mechanisms underlying the off-target phenomenon system at the organismal level.

Interestingly, a recent study[35] describing RNA-targeting Cas13 tools in flies appeared to ameliorate the general toxicity associated with CasRx expression, for several possible reasons. Firstly, the authors recoded the Cas13 transgenes to optimize expression in *Drosophila* and utilized a more weakly-expressing promoter (actin 5C) as compared to the ubiquitin promoter used in the present study, likely leading to lower levels of Cas13 expression and thus less toxicity.

Indeed, this is supported by the observations that UASt-driven CasRx and dCasRx expression was not, by itself, toxic, as this study (and the authors in [35]) did not observe any obvious lethality in UASt-CasRx flies. Furthermore, these authors encoded their gRNAs differently compared to this study, which may have affected their expression and reduced the toxicity of the system in transheterozygous Cas13/gRNA flies. For example, in their study two gRNAs were expressed using the U6:3 promoter and flanked by tRNAs to enable gRNA processing upon tRNA maturation without requiring CasRx[35]. Conversely, in this study, the gRNAs were encoded as arrays targeting up to 4 locations per gene and mimicking the endogenous Cas13 array structure found in bacteria, which requires the Cas13 enzyme to process each gRNA. These different approaches may have led to differences in gRNA expression levels and other factors that may have inherent effects on the toxicity of the whole system. Finally, Huynh et al. targeted different genes than those targeted in the present study, which result in differences in target-Cas/gRNA interaction and collateral activity and may have effects on the observed levels of lethality. These differences in observed toxicity warrant further investigation to identify ways that the overall systems can be improved.

Overall, this study is the first demonstration of a functional genetically encoded programmable RNA-targeting Cas system in *D. melanogaster*, albeit further optimization of the CasRx system (in line with what is reported[35]) will be required to further reduce the off-target-associated lethality and increase the efficacy of CasRx on-target cleavage. RNA-targeting with Cas enzymes is a rapidly evolving field with many potential applications ranging from insect vector control to therapeutic usages[1-7], and this protocol offer a starter package for anyone interested in designing their first CasRx system in flies, while being compatible with customization and further optimization of the system. The examples presented here demonstrate a range of results one may encounter during implementation of this system in vivo and may serve as benchmarks for other users in evaluating the performance of CasRx system in their applications.

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DISCLOSURES:

O.S.A is a founder of Agragene, Inc., has an equity interest, and serves on the company's Scientific Advisory Board. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict-of-interest policies. All other authors declare no competing interests.

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Figure 1

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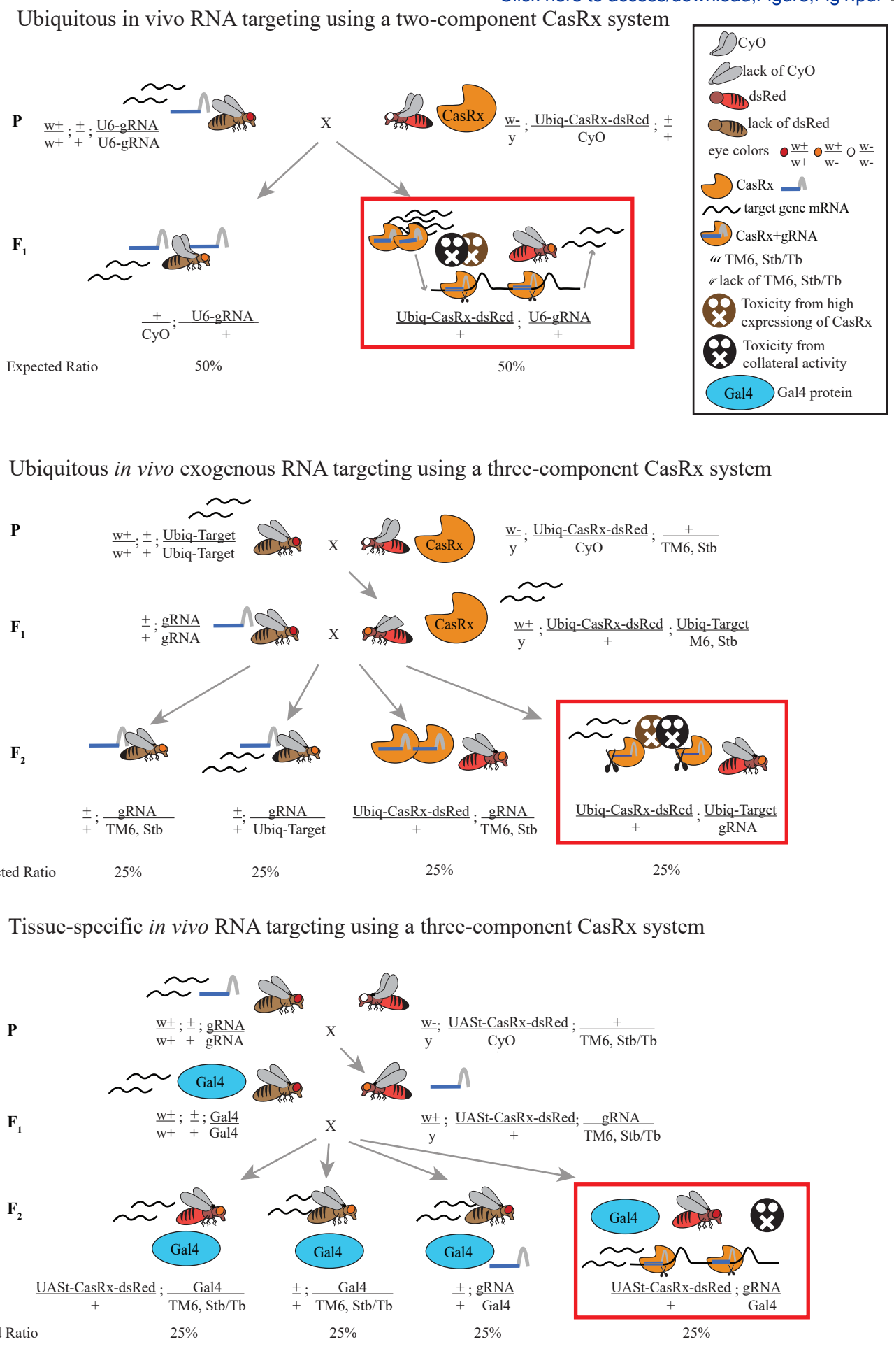

Figure 2

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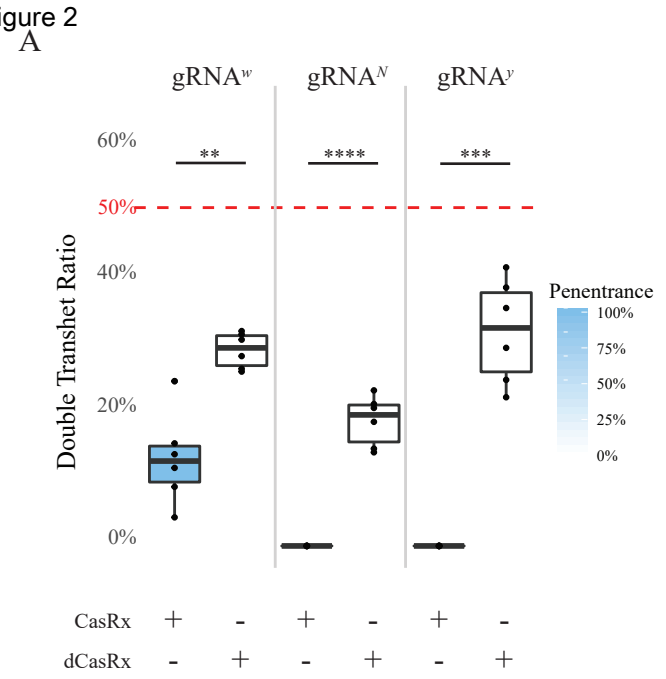

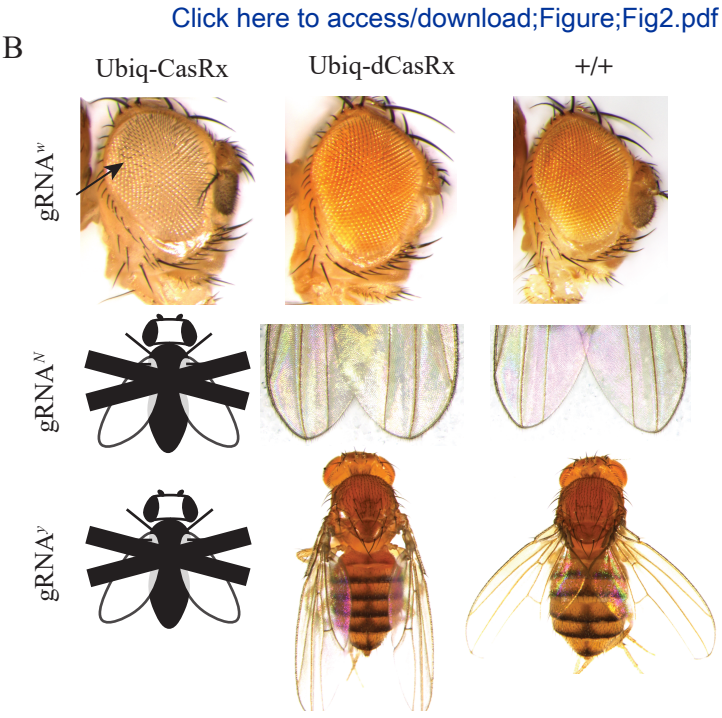

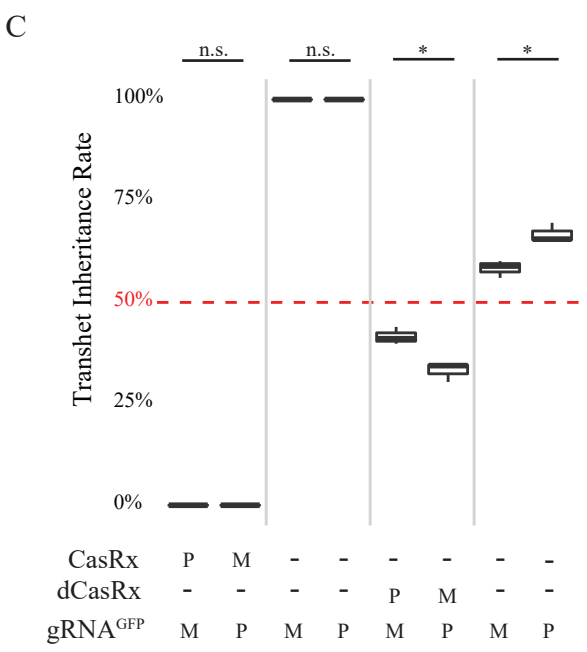

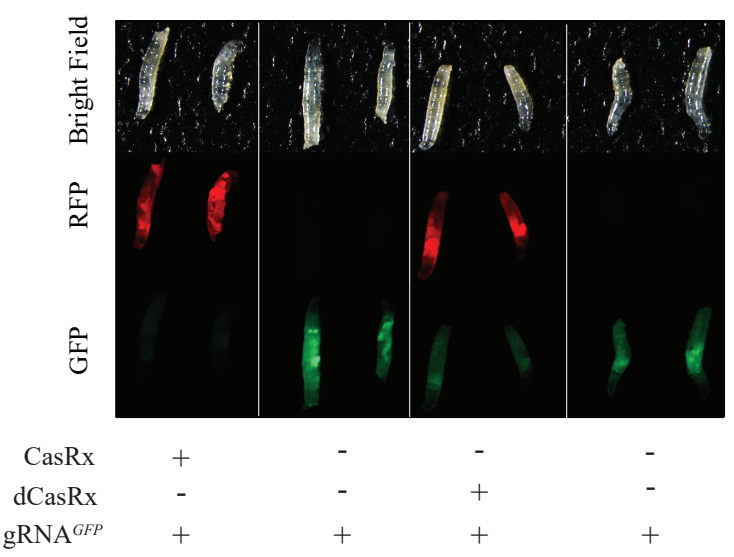

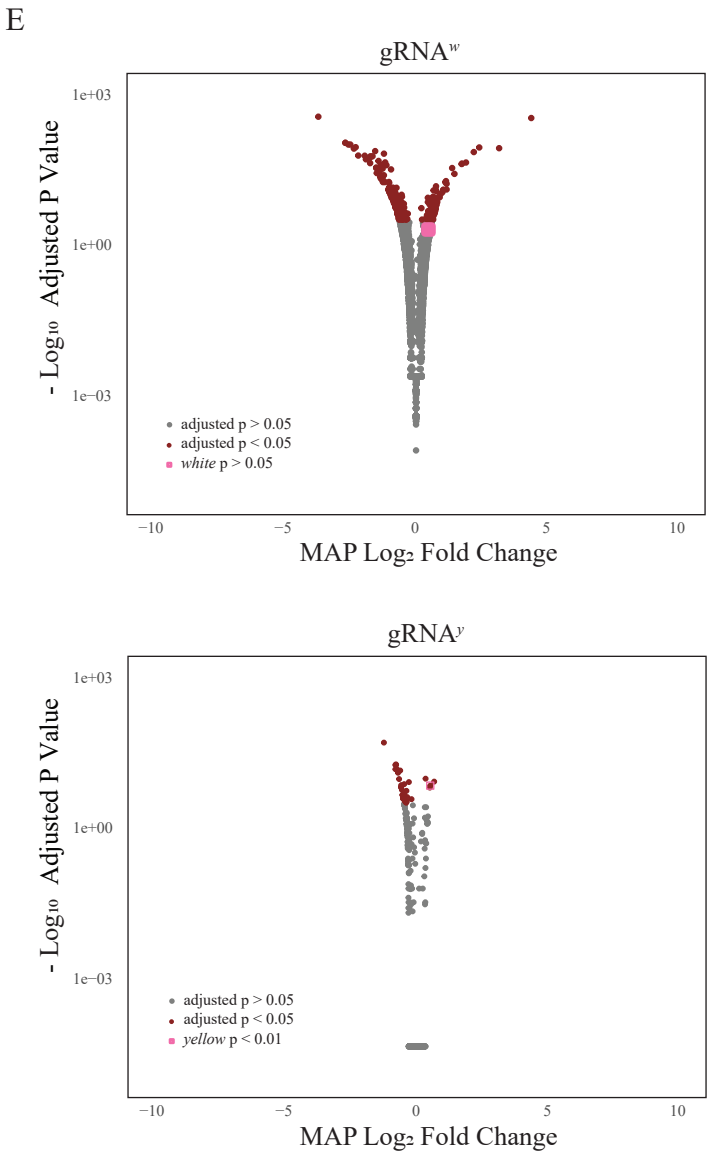

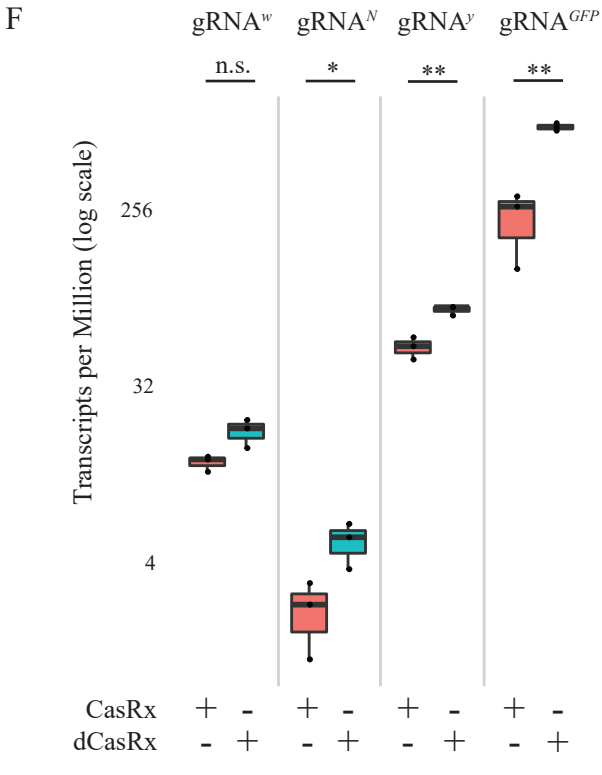

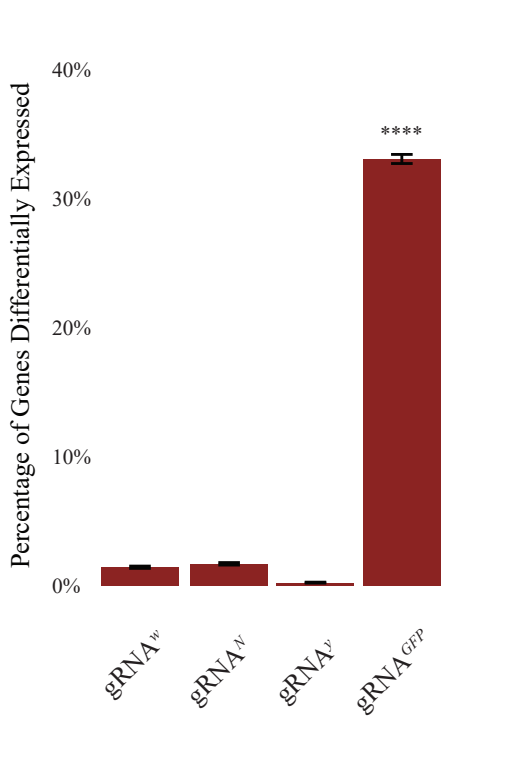

Figure 3

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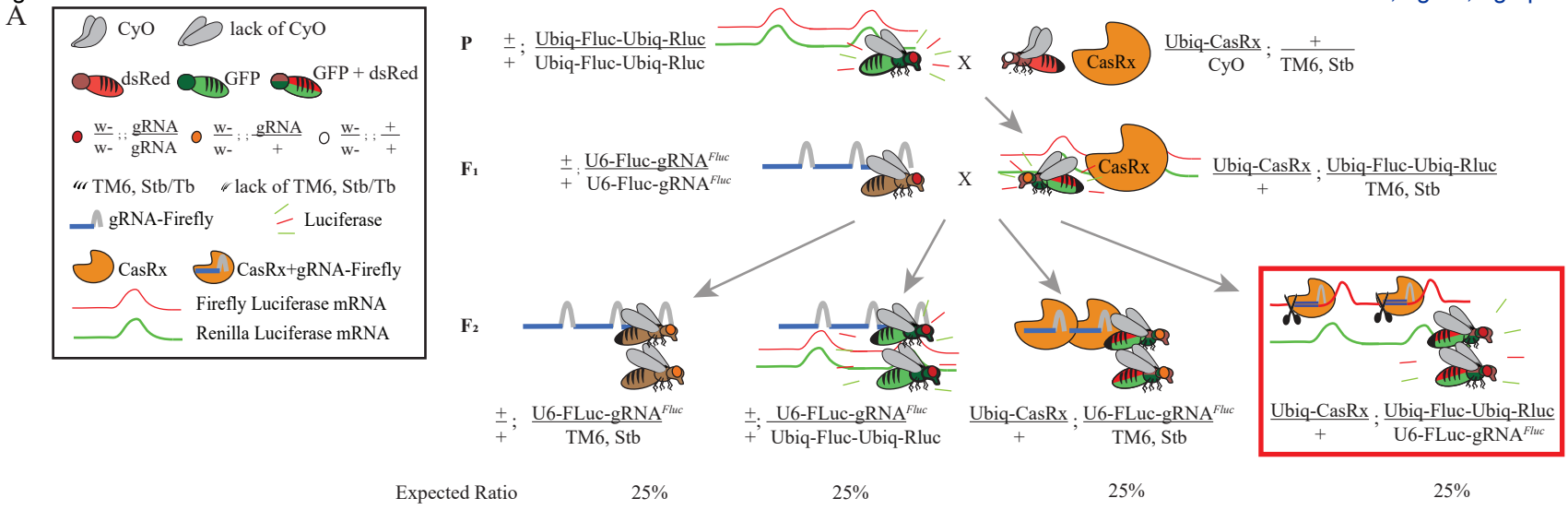

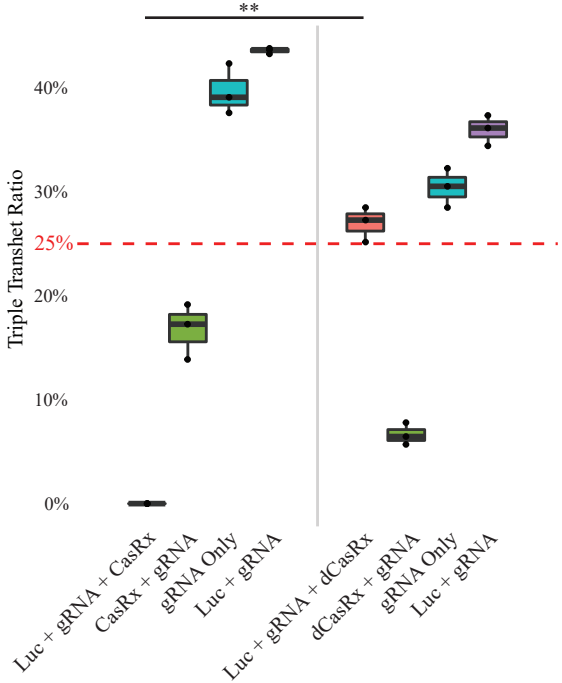

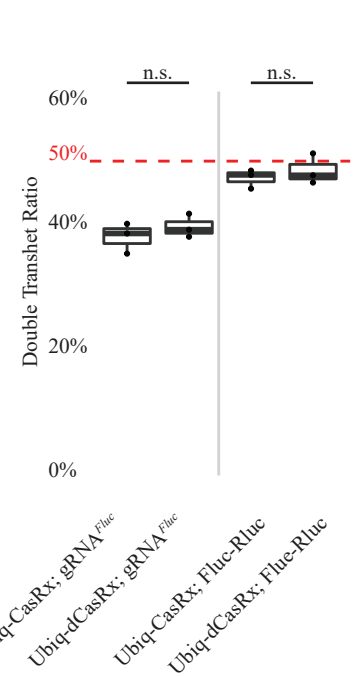

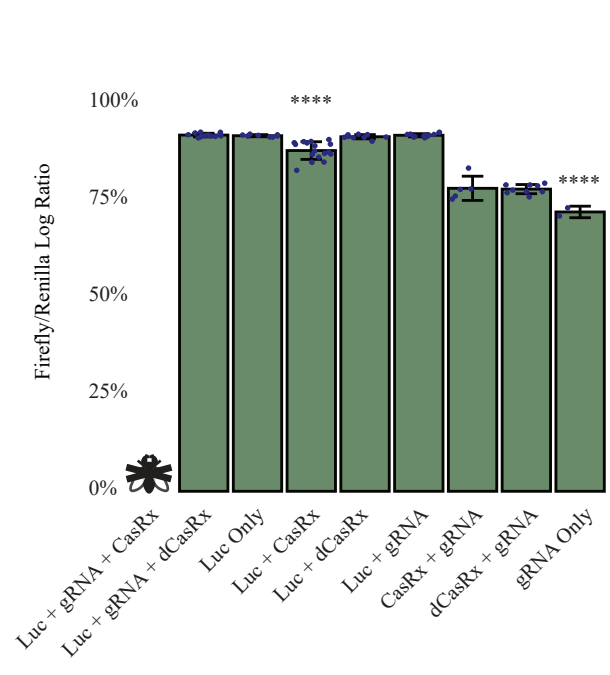

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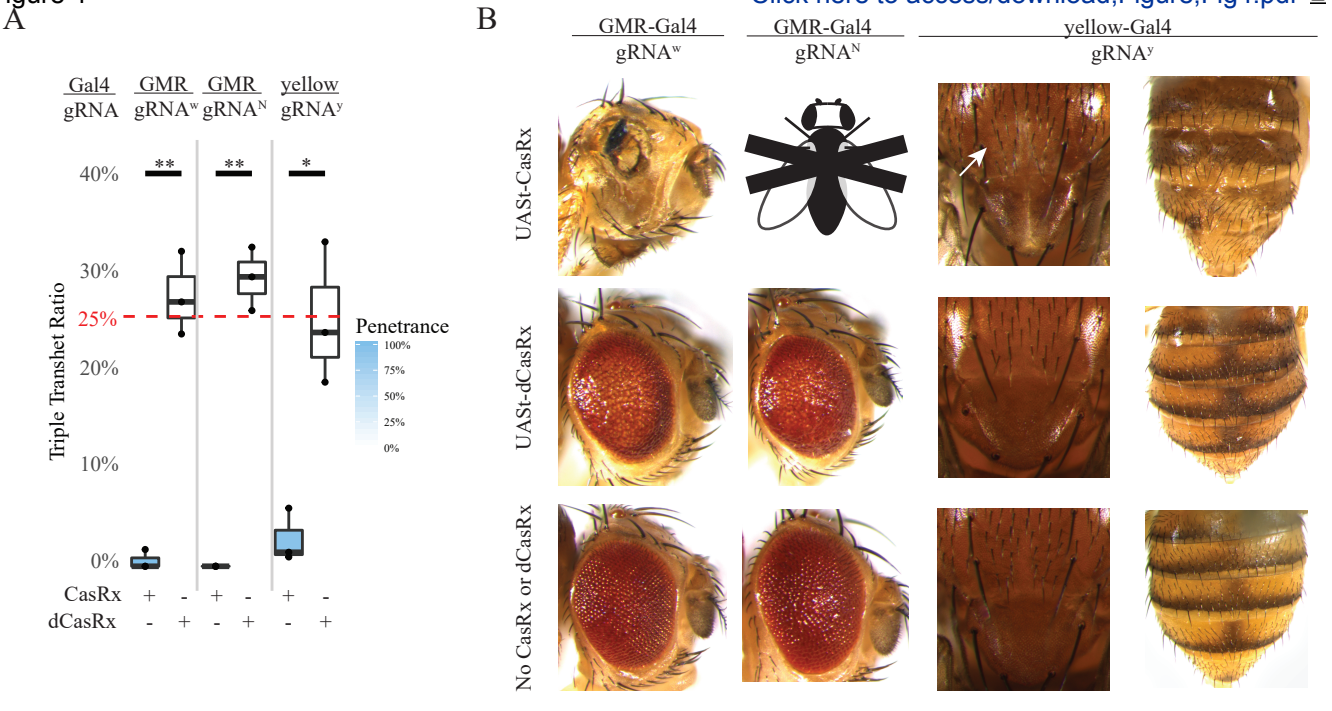

Table

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Construct	Description	Primer
OA-1050E	CasRx	1050E.C3
		1050E.C4
OA-1050R	dCasRx	1050E.C3
		1050E.C4
OA-1050L	UASt promoter	1041.C9
		1041.C11
	CasRx	1050L.C1
		1050E.C4
OA-1050S	UASt promoter	1041.C9
		1041.C11
	dCasRx	1050L.C1
		1050E.C4
OA-1043	U6:3 promoter	1043.C1
		1043.C23
OA-1052B	Ubiq promoter	1052B.C1
		1052B.C2
	T2A-eGFP	908A1
		908A2
	Reversed Ubiq promoter	908A3
		908A4

Primer Sequence (5’ to 3’)	PCR Template
TACTAATTTTCCACATCTCTATTTTGACCCGCAGATTAA TTAATGAGCCCCAAGAAGAA CAATTGATTTGTTATTTTAAAAACGATTCATTCTAGCTA GCTTAAGCGTAATCTGGAACA	pNLS-RfxCas13d-NLS-HA (pCasRx)
TACTAATTTTCCACATCTCTATTTTGACCCGCAGATTAA TTAATGAGCCCCAAGAAGAA CAATTGATTTGTTATTTTAAAAACGATTCATTCTAGCTA GCTTAAGCGTAATCTGGAACA	pNLS-dRfxCas13d-NLS-HA (pdCasRx)
GCGGGTTCTCGACGGTCACGGCGGGCATGTCGACGCGG CCGCAACCAACAACACTAGTAG CTGGCCTCCACCTTTCTCTTCTTCTTGGGGCTCATGTTTA AACCCAATTCCCTATTCAGA	pJFRC81
AATACAAGAAGAGAACTCTGAATAGGGAATTGGGTTTA AACATGAGCCCCAAGAAGAA CAATTGATTTGTTATTTTAAAAACGATTCATTCTAGCTA GCTTAAGCGTAATCTGGAACA	pCasRx
GCGGGTTCTCGACGGTCACGGCGGGCATGTCGACGCGG CCGCAACCAACAACACTAGTAG CTGGCCTCCACCTTTCTCTTCTTCTTGGGGCTCATGTTTA AACCCAATTCCCTATTCAGA	pJFRC81
AATACAAGAAGAGAACTCTGAATAGGGAATTGGGTTTA AACATGAGCCCCAAGAAGAA CAATTGATTTGTTATTTTAAAAACGATTCATTCTAGCTA GCTTAAGCGTAATCTGGAACA	pdCasRx
GGGAATTGGGAATTGGGCAATATTTAAATGGCGGCGCG CCGAATTCTTTTTTGCTCACCT	Addgene plasmid #164586
ACACTAGTGGATCTCTAGAGGTACCGTTGCGGCCGCAA AAAAGTTGTAATAGCCCCTCAAAACTGGACCTTCCACA ACTGCAGCCGACGTTAAATTGAAA	Addgene plasmid #112686
GGGAATTGGGCAATATTTAAATGGCGGCTGCAGCGCGC AGATCGCCGAT	Addgene plasmid #112686
TTTCTTTATGTTTTTGGCGTCTTCCATCCTAGGTCTGCGG GTCAAAATAGAGATG	Addgene plasmid #112686
ATAAAGGCCAAGAAGGGCGGAAAGATCGCCGTGGAGG GCAGAGGAAGTCTTCTAACATGC	Addgene plasmid #112686
TTGTTATTTTAAAAACGATTCATTCTAGGCGATCGCTTA CTTGTACAGCTCGTCCATGCC	Addgene plasmid #112686
ACCGTGACCTACATCGTCGACACTAGTGGATCTCTAGA CGCGCAGATCGCCGATG	Addgene plasmid #112686
GGATCATAAACTTTCGAAGTCATGCGGCCGCTCTGCGG GTCAAAATAGAGATGT	Addgene plasmid #112686

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

Name of Material/Equipment
100% Grape Juice
Active Dry Yeast (908g)
DMC4500 color camera
Dual-Luciferase Reporter Assay System
GAL4-GMR flies
GAL4-y flies
Glomax 20/20 Luminometer
Illumina HiSeq2500 Sequencer
M165FC fluorescent stereomicroscope
Nanodrop One^{C} UV-vis spectrophotometer
NEBNext Ultra II RNA Library Prep Kit
plasmid # 112686
plasmid # 112688
plasmid # 132416
plasmid # 132417
plasmid # 132419
plasmid # 132420
plasmid # 132421
plasmid # 132422
plasmid # 132425
plasmid # 132426
plasmid # 133304
plasmid # 164586
plasmid #132418
plasmid pJFRC81
Qiagen RNeasy Mini Kit
Restriction endonucleases AscI
Restriction endonucleases NotI
Restriction endonucleases PacI
Restriction endonucleases PstI
Restriction endonucleases SwaI

Restriction endonucleases XbaI
RNA 6000 Pico Kit for Bioanalyzer
Turbo DNase
U6-3:4-gRNA-Fluc flies
U6-3:4-gRNA-GFP; OpIE2-GFP flies
U6-3:4-gRNA-N flies
U6-3:4-gRNA-w flies
U6-3:4-gRNA-y flies
UASt-CasRx flies
UASt-dCasRx flies
Ubiq-CasRx flies
Ubiq-dCasRx flies
Ubiq-Firefly-T2A-eGFP-Ubiq-Renilla flies
Zymoclean Gel DNA Recovery Kits

Company	Catalog Number	Comments/Description
Welch Foods Inc.	N/A	
Red Star Yeast Company, LLC	N/A	
Leica Microsystems	DMC4500	
Promega	E1910	
Bloomington Drosophila Stock Center	29967	
Bloomington Drosophila Stock Center	44373	
Promega	E5331	
Illumina, Inc.	HiSeq2500	
Leica Microsystems	M165FC	
ThermoFisher	NDONEC-W	
New England Biolabs, Inc.	E7770	
Addgene	112686	
Addgene	112688	
Addgene	132416	
Addgene	132417	
Addgene	132419	
Addgene	132420	
Addgene	132421	
Addgene	132422	
Addgene	132425	
Addgene	132426	
Addgene	133304	
Addgene	164586	
Addgene	132418	
Addgene	36432	
Qiagen	74104	
New England Biolabs Inc.	R0558L	
New England Biolabs Inc.	R0189L	
New England Biolabs Inc.	R0547L	
New England Biolabs Inc.	R0140L	
New England Biolabs Inc.	R0604L	

New England Biolabs Inc.	R0145L
Agilent Technologies	5067-1513
Invitrogen	AM2238
Bloomington Drosophila Stock Center	84125
Bloomington Drosophila Stock Center	84986
Bloomington Drosophila Stock Center	84122
Bloomington Drosophila Stock Center	84124
Bloomington Drosophila Stock Center	84123
Bloomington Drosophila Stock Center	84121
Bloomington Drosophila Stock Center	84120
Bloomington Drosophila Stock Center	84118
Bloomington Drosophila Stock Center	84119
Bloomington Drosophila Stock Center	84127
Zymo Research Corporation	D4007

Rebuttal Letter
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Authors In-Line Responses to All Revision Comments

Dear JOVE editors:

Thank you for reviewing our manuscript. Please find our in-line response below (in blue).

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Authors' Response: We have made the suggested change.

2. Please revise your title to "Ubiquitous and Tissue-specific RNA Targeting in Drosophila melanogaster Using CRISP/CasRx".

Authors' Response: We have made the suggested change.

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Authors' Response: We have made the suggested change.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: GenScript USA, Inc.; Rainbow Transgenic Flies, Inc.; Bloomington Drosophila Stock Center (BDSC); Leica DMC4500; Leica M165FC; Leica Application Suite X; Helicon Focus 7; Eppendorf; Welch's Grape Juice; Qiagen RNeasy Mini kit; Invitrogen Turb™ DNase; NanoDrop Onec; ThermoFisher NDONEC-W; RNA 6000 Pico kit for BioAnalyzer (Agilent Technologies 5067-1513); NEB Next Ultra II RNA Library Prep Kit; Illumina HiSeq2500; NEB $#; STAR aligner34; gBlocks®; Integrated DNA Technologies, Coralville, Iowa;

Dual-Luciferase® Reporter Assay System (Promega E1910); Glomax 20/20 Luminometer (E5331) etc

Authors' Response: We have removed all commercial product info from the manuscript with the exception of Bloomington Drosophila Stock Center (BDSC) and Addgene. BDSC is a non-profit fly stock center for fly researchers globally and we have deposited all fly lines generated in this study there for other researchers to use. Similarly, Addgene serves as a repository of molecular constructs for researchers globally and we have deposited our plasmids there for others to use.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Authors' Response: We have made the suggested change.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? **Alternatively, add references to published material specifying how to perform the protocol action**. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Authors' Response: We have made the suggested change.

7. In the protocol, wherever you state "gel-purify" and "digest the …", please provide details about the digestion (amounts of enzymes used, total volume of mixture, digestion conditions (temperature, time etc), gel and electrophoresis as well as expected fragment sizes OR cite references in which this has been described.

Authors' Response: We have made the suggested change.

8. Line 175: How do you anesthetize the flies with CO2?

Authors' Response: We have made the suggested change (see protocol 1 section d step iii).

9. Line 184: How much yeast (what kind) is present in the vial? What else is there in the vial?

Authors' Response: We have added the requested information about yeast and fly food recipe in protocol 1 section d step i.

10. Instead of only writing "highlighted for filming", please highlight the text in yellow for filming the video after leaving one-line spacing after every step and every sub-heading and heading. Notes need not be highlighted. The total length of highlighted text should not exceed 3 pages. This is a hard limit to ensure that filming can take place in one day.

Authors' Response: We have highlighted the relevant sections as suggested.

11. In representative results section, please ensure that in-text references such as Buchman et al. are cited in the correct format. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s), but before punctuation.

Authors' Response: We have made the suggested change.

12. . As we are a methods journal, please ensure that the Discussion covers the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Authors' Response: We have made the suggested change in the discussion section.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Please include volume and issue numbers for all references, and do not abbreviate journal names.

Authors' Response: We have made the requested changes in the references format.

14. Please sort the Materials Table alphabetically by the name of the material.

Authors' Response: We have revised the Materials Table and sorted the items alphabetically by the name of the material.

Reviewers' comments:

Reviewer # 1:

Manuscript Summary:

this manuscript demonstrates the use of the CRISPR/CasRx system for ubiquitous and tissue-specific transcription reduction in Drosophila. Authors showed three different applications, the protocols are clear and well written.

Major Concerns:

It would be great if authors provide a clearer protocol about how to design the gRNA for the specific RNA targeting. A figure demonstration would be useful to facilitate understanding of the gRNA design strategy by readers.

Minor Concerns:

None.

Authors' Response: Thank you for your comment. We have added the guide RNA design strategy in both the protocol design *1* section *b* step *i* and the discussion section per your suggestions.

Reviewer #2:

Manuscript Summary:

In this manuscript Sun and colleagues report on the characterization of the CRISPR/CasRx system in Drosophila melanogaster. It has been previously shown that the ribonuclease CasRx is able to induce highly efficient and specific RNA depletion in human cell culture. To test the efficacy of this system in Drosophila, the authors chose 3 endogenous (white, yellow, Notch) and 2 exogenous (GFP, luciferase) target genes. First, they generated gRNA constructs allowing tandem expression of 4 gRNA designed for different target sites of each target genes. They established transgenic flies expressing CasRx and catalytically inactive dCasRx under the control of Ubiq or UASt promoter.

The authors demonstrated that, the ubiquitous expression of both CasRx and dCasRx with Ubiq promoter results toxicity as generation of homozygous stains was not possible. Transheterozygous flies expressing Ubiq-CasRx and gRNAs targeting both endogenous and exogenous genes were non-viable, with the exception of gRNA targeting the white gene, which resulted the expected white-eyed phenotype. The RNA-seq analysis revealed significant reduction of mRNA levels of the target genes. However, off-target activity induced by CasRx was also demonstrated.

Tissue-specific RNA targeting using the UAS-Gal4 system resulted less toxicity and allowed the observation of expected phenotypes for each target transcript. However, RNA-seq data was not provided for these experiments.

Overall, I think this study conclusively demonstrates that the CRISPR/CasRx system is a potent method to manipulate mRNA levels in D. melanogaster.

Authors' Response: Thank you for your comments. RNA-seq on flies from tissue-specific RNA targeting experiment was beyond the scope of this protocol due to the following reasons: tissue-specific in vivo RNA targeting resulted in either lethality or localized phenotype available only in 25% of the triple transheterozygous progenies. Also, target gene transcripts are not expressed at the same developmental stage, and for gene targets with high transcript expression levels during larval stages, it is difficult to identify the correct triple transheterozygous progenies among all progenies as markers are not all visible. All of these rendered collecting sufficient tissue mass for RNA sequencing a challenging task.

Reviewer #3:

Manuscript Summary:

In the past few years, the CRISPR/Cas methodology has been extensively used for genomic manipulations at the DNA level. Recently, studies also began to demonstrate the potential application of CRISPR technologies for targeting at the RNA level, but this was not yet attempted in Drosophila. In addition to offering an attractive alternative to the widely used RNAi technique, CRISPR-mediated RNA targeting could provide an extremely useful tool in the combat against vector-borne viral infections.

In this paper, Ruichen et al. summarize findings from their recent publication (Buchman et al. 2020), and provide a step-by-step protocol for the use of the CasRx ribonuclease for either ubiquitous or tissue-specific targeting of both endogenous and exogenous RNA.

The provided protocol is detailed and thorough and should, given certain changes/additions as detailed below, be successfully implemented by other labs. The presented technology is very preliminary and, at its present stage, still suffers from some major limitations - mainly concerning lethality and off-target effects (therefore at the moment, its advantages over RNAi are still far from evident). Still, I found this work interesting and important, since it sets the stage for further optimization of this promising technology in future studies. It is therefore important that the current limitations are further highlighted and appropriately addressed in a more coherent manner (see point number 6). This way future work can focus on resolving these limitations, and in addition, readers can make an informed decision if they wish to attempt the protocol at this stage, or wait for a more optimized version.

Major Concerns:

1. The abstract should briefly mention the lethality obstacle, as this is a main issue and conclusion of this paper (raising the need for further optimization in future studies)

Authors' Response: We have added the lethality issue in the abstract. Since the discussion/conclusion section has discussed this issue at length, we decide to keep it as is.

2. Unless I missed something, throughout the paper, almost no information is given about the characteristics of CasRx and its gRNA requirements - what is the desired length of the gRNA? Is there a requirement for a PAM sequence? Are there any other specific requirements of limitations? Is there any online tool/algorithm to help with the gRNA design? All of these are highly important factors to take into consideration when designing CRISPR-based experiments. If readers are to test this system on other genes of their own interest (beyond the 4 genes shown in this paper), this is a critical part of the protocol. Moreover, looking at Buchman et al. 2020, I see that this information is actually present ("To select the CasRx target sites, target genes were analyzed to identify 30-nucleotide regions that had no poly-U stretches greater than four base pairs, had GC base content between 30% and 70%, and were not predicted to form strong RNA hairpin structures. Care was also taken to select target sites in RNA regions that were predicted to be accessible…"). I advise including this important information in this publication as well.

Authors' Response: We have added the guide RNA design strategy in both the protocol design *1* section *b* step *i* and the discussion section per your suggestions.

3. In the introduction, perhaps highlight additional potential advantages of RNA targeting over permanent alteration of the DNA, as well as over RNAi (especially since at the moment, CasRx does not seem to have less off-targets than RNAi)

Authors' Response: We have modified the first paragraph of the introduction section to incorporate your comments.

4. Did they deposit the gRNA backbone plasmid as well (OA-1043)? Unless I missed it, they only write that they deposited the ones that already have the gRNAs cloned into them, but the backbone is important for cloning gRNAs for other genes.

Authors' Response: OA-1043 plasmid has been deposited in Addgene (ID: 164586).

5. In the RNA-seq experiments, how can they in fact distinguish between transcripts that were reduced because they were direct off-targets, and those that were reduced due to downstream consequences of the on-target gene disruption? Worth saying something about that.

Authors' Response: We believe that the transcript reduction observed is due to direct off-target activity and not downstream consequences of on-target gene disruption. This is suggested by the RNA-seq result from the GFP RNA targeting set of the experiment. In this data, expression of 33% of the transcripts were reduced when the transcripts of GFP, an exogenous gene for fruit flies, were targeted.

6. The whole lethality issue, which is a major point in the paper, I feel is not explained clearly enough. There are a few different sources described for this lethality, and to me it remains unclear if they all stem from the same origin or are these independent effects. They mention toxicity of the CasRx protein itself; off target effects; collateral activity; induction of apoptosis-related genes (either direct or indirect)… Are these all part of the same phenomenon or unrelated causes? Moreover, results sometimes seem contradictory. For example, on the one hand it does not seem that the mere presence of high CasRx levels in the cells is toxic, but only if it is catalytically active (since toxicity was gRNA dependent); on the other hand, even the non active dCasRx cannot be kept as a homozygous stock (even when the gRNA is not there). Also, there was some level of toxicity for dCasRx with gRNAs (less than 50% inheritance), although it is catalytically inactive. I understand of course that none of this is known and that the authors are raising possible hypotheses, but still, I think at the moment things are confusing, and readers could benefit from a more coherent explanation of the lethality findings (even if they sometimes contradict each other - maybe even in table form). The hypothesized lethality causes should be appropriately addressed, especially when the ultimate goal is overcoming them.

Authors' Response: Based on your comment, we have revised the discussion section where the lethality issue was mentioned.

7. In the case of the white gene, the use of UAS-CasRx was in fact significantly more lethal than with Ubiq-CasRx, no? What could be a possible explanation for that?

Authors' Response: The higher lethality of transheterozygous flies carrying UASt-CasRx, GAL4-GMR, and gRNA^{w} compared to flies carrying Ubiq-CasRx and gRNA^{w} is likely due to the GAL4-GMR presence. The few surviving UASt-CasRx, GAL4-GMR, and gRNA^{w} flies showed a distinct eye-specific complete cell death phenotype, which is different from the white-eyed phenotype observed

in Ubiq-CasRx and gRNA^{w} flies. It would be interesting to explore other eye-targeting GAL4 drivers in the future.

8. Since lethality/toxicity is such a major issue, I think that it is worth discussing potential future ways to control the levels of CasRx in order to reduce toxicity (not sure that this could be valid here but just as an example, Port et al. 2020 [elife] developed a series of transgenes utilizing upstream open reading frames of different length to fine tune Cas9 expression).

Authors' Response: We have added a discussion of results from Port et al 2020 in the discussion section and added a citation of it in the references section.

Minor Concerns:

10. Line 137 - why do the flies have white eyes? I assume they are on a w- background on the X chromosome, but this is not mentioned in the text nor in the crosses schemes.

Authors' Response: Yes, the white eyes are from the w- marker on the x chromosome. We have modified the Fig 1 and added the eye color specifications.

11. Line 191 - from personal experience, Welch's grape juice is very challenging/impossible to obtain outside the US, and another brand of grape juice does not necessarily work well… Maybe try to provide an alternative for non-US labs.

Authors' Response: We removed the Welch's grape juice commercial name from the manuscript. We believe any concentrated grape juice should work for this protocol.

12. Line 411 - why did they write Ubiq-CasRx? This specific section is about the UAS variant.

Authors' Response: It was a typo and we have revised it to UASt-CasRx.

Best,

Ruichen Sun and Omar Akbari

UCSD