STAR Protocols

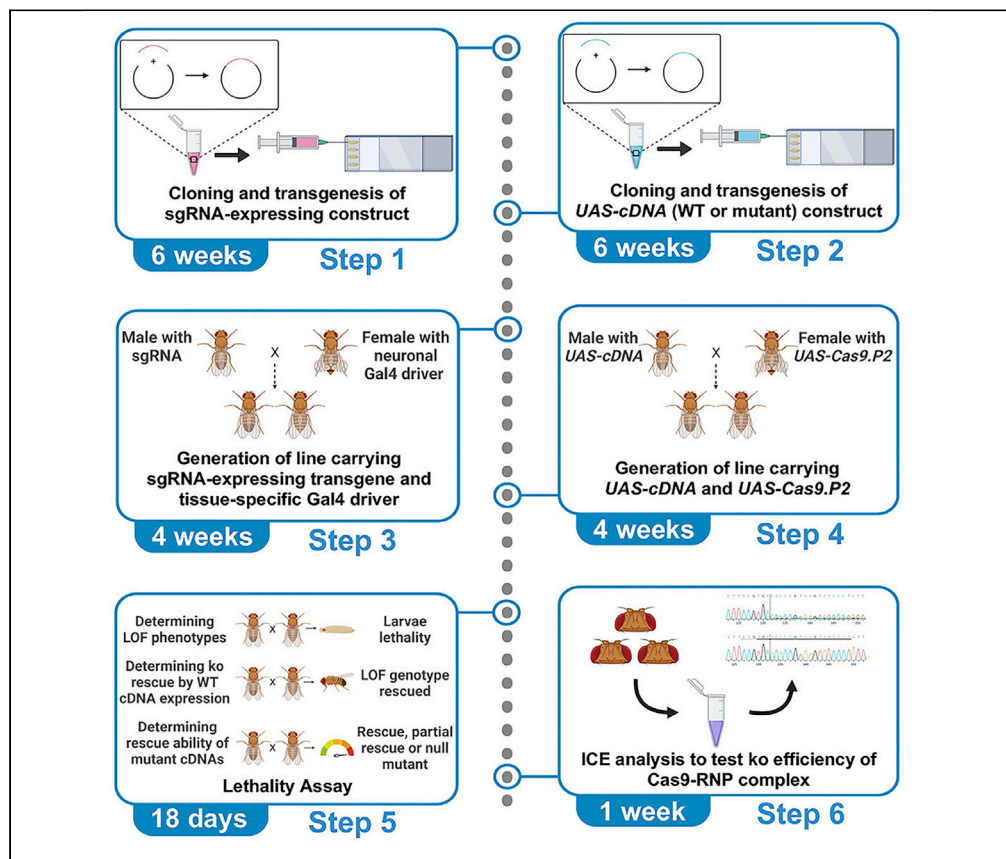### Protocol

CRISPR/Cas9-mediated tissue-specific knockout and cDNA rescue using sgRNAs that target exon-intron junctions in *Drosophila melanogaster*

Cloning and transgenesis of sgRNA-expressing construct — 6 weeks — Step 1

Cloning and transgenesis of *UAS-cDNA* (WT or mutant) construct — 6 weeks — Step 2

Male with sgRNA X Female with neuronal Gal4 driver — Generation of line carrying sgRNA-expressing transgene and tissue-specific Gal4 driver — 4 weeks — Step 3

Male with *UAS-cDNA* X Female with *UAS-Cas9.P2* — Generation of line carrying *UAS-cDNA* and *UAS-Cas9.P2* — 4 weeks — Step 4

Determining LOF phenotypes X Larvae lethality; Determining ko rescue by WT cDNA expression X LOF genotype rescued; Determining rescue ability of mutant cDNAs X Rescue, partial rescue or null mutant — Lethality Assay — 18 days — Step 5

ICE analysis to test ko efficiency of Cas9-RNP complex — 1 week — Step 6

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Highlights

Selective knockout for target genes without affecting UAS-cDNA transgenes

Tissue-specific knockout by CRISPR-Cas9 in *Drosophila*

Detailed protocols for DNA vector generation for sgRNA and cDNA expression in *Drosophila*

Efficient determination of functional effects of disease-associated variants

In this protocol, we take CRISPR/Cas9 and Gal4/UAS approaches to achieve tissue-specific knockout in parallel with rescue of the knockout by cDNA expression in *Drosophila*. We demonstrate that guide RNAs targeting the exon-intron junction of target genes cleave the genomic locus of the genes, but not *UAS-cDNA* transgenes, in a tissue where Gal4 drives Cas9 expression. The efficiency of this approach enables the determination of pathogenicity of disease-associated variants in human genes in a tissue-specific manner in *Drosophila*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Protocol

CRISPR/Cas9-mediated tissue-specific knockout and cDNA rescue using sgRNAs that target exon-intron junctions in *Drosophila melanogaster*

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SUMMARY

In this protocol, we take CRISPR/Cas9 and Gal4/UAS approaches to achieve tissue-specific knockout in parallel with rescue of the knockout by cDNA expression in *Drosophila*. We demonstrate that guide RNAs targeting the exon-intron junction of target genes cleave the genomic locus of the genes, but not *UAS-cDNA* transgenes, in a tissue where Gal4 drives Cas9 expression. The efficiency of this approach enables the determination of pathogenicity of disease-associated variants in human genes in a tissue-specific manner in *Drosophila*.
For complete details on the use and execution of this protocol, please refer to Yap et al. (2021).

BEFORE YOU BEGIN

Functional studies of genetic variants in undiagnosed patients in humans require model organisms such as *Drosophila* (Bellen et al., 2019). Studies of disease-associated variants in *Drosophila* often need to be performed in a tissue-specific manner, especially in cases where human genes have multiple paralogs, one of which is expressed in a specific tissue, while *Drosophila* single orthologs are expressed ubiquitously. The recent development of genetic tool kits for CRISPR/Cas9 in *Drosophila* provided an additional genetic means to efficiently achieve tissue-specific gene knockout (ko) via the UAS/Gal4 system (Brand and Perrimon, 1993; Kondo and Ueda, 2013; Port et al., 2014, 2020). The majority of guide RNAs (gRNAs) in the Weizmann Knock Out project and the Transgenic RNAi Project were designed to target coding exons shared by all mRNA isoforms and target sites that were located in the 5' half of the open reading frame (Port et al., 2014, 2020). Hence, these single guide RNAs (sgRNAs) together with Cas9 can lead to efficient gene ko by cutting endogenous target sites in genomic loci, but they can also cleave *UAS-cDNA* transgenes. Functional studies of biallelic and recessive variants require gene ko in parallel with ko rescue by *UAS-cDNA* expression by Gal4. In this protocol, we describe a new approach using sgRNAs that target the exon-intron junction of target genes and provide detailed protocols for *Drosophila Ogdh* (*dOgdh*) gene as a proof-of-principle study (Figure 1). We demonstrated that neuronal-specific expression of Cas9 with the sgRNA that targets the exon-intron junction of *dOgdh* (sgRNA^{dOgdh}) leads to ko of the endogenous *dOgdh* gene, but not *UAS-dOgdh* cDNA transgene (Figure 1). This approach has enabled us to determine the pathogenicity of missense variants in *OxoGlutarate DeHydrogenase-Like* (*OGDHL*), the human ortholog of *dOgdh* (Yap et al., 2021; Yoon et al., 2017). We anticipate that this new approach will be widely used to study the tissue-specific roles of numerous human disease-associated variants as well as gene function studies in *Drosophila*.
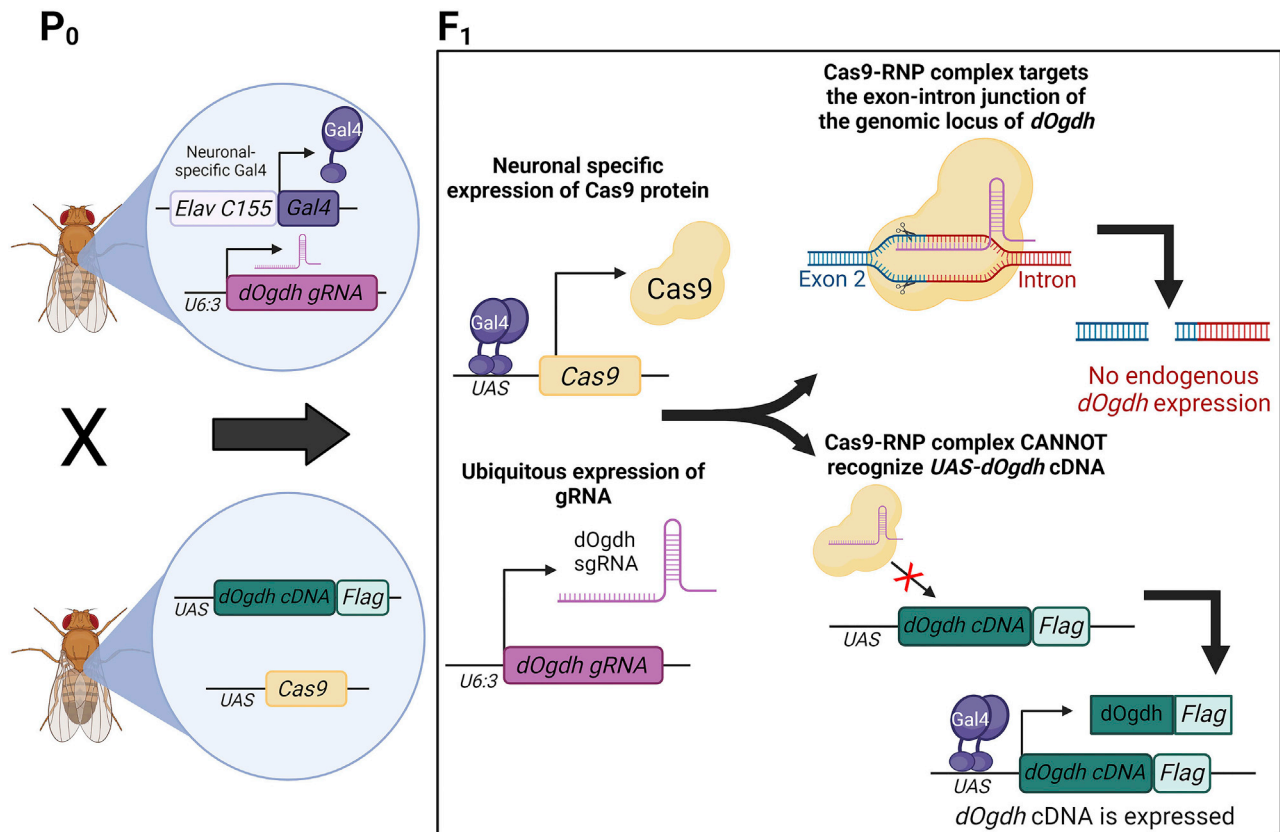
Figure 1. Schematic of CRISPR/Cas9-mediated neuronal ko and cDNA rescue for *dOgdh*

(Left, P0) Fruit fly on the top carries neuronal Gal4 driver and *dOgdh*-targeting sgRNA under the control of *dU6:3* promoter (*elav^C155^-Gal4; dU6:3-sgRNA^dOgdh^*). Fruit fly on the left bottom carries 2 transgenes – *dOgdh* and *Cas9* under the control of UAS (*UAS-dOgdh-Flag; UAS-Cas9*). Cross of these flies gives rise to progenies carrying all 4 components. (Right, F1) *elav^C155^-Gal4* drives expression of Cas9 in neurons where Cas9/sgRNA^dOgdh^ RNP targets the exon-intron junction of *dOgdh*, which results in indel mutations in *dOgdh*. Exon 2 is indicated by the blue DNA strand, and the intron is indicated by the red DNA strand. In contrast, Cas9/sgRNA^dOgdh^ RNP cannot target *dOgdh* cDNA in the *UAS-dOgdh* transgene. Hence, *elav^C155^-Gal4*-driven Cas9 expression leads to *dOgdh* ko in neurons, while Gal4 drives wild-type or mutant *dOgdh* cDNA expression. The schematic was created with BioRender.com.

The method involves the generation of *Drosophila* expression vectors for gRNA, pUASTattB-cDNA, and mutagenesis of cDNA. We described an overview of the cloning procedures in Figure 2.

Generation of DNA construct carrying sgRNA that targets gene of interest

⏱ **Timing: 1 week**

1. Design and select for gRNAs that target your gene of interest.
   a. We used a knockout guide design tool from Synthego to find guide RNAs that target the exon-intron junction of target genes. Here we will show how to find gRNAs for *dOgdh* (*Nc73EF*, FBgn0010352) as an example.
   b. Go to the "Synthego knockout guide design" webpage (https://design.synthego.com/). Choose Genome for *Drosophila melanogaster* and type your gene of interest (for example, *Nc73EF/dOgdh*), and click the 'Search' button (Figure 3).
   c. The web tool typically shows 4 top-ranked gRNAs that target early coding region, common exons, higher on-target activity and minimal off targets (Doench et al., 2016). To see all other gRNAs, click the "All Guides" button on the top right corner (red arrow in Figure 4).
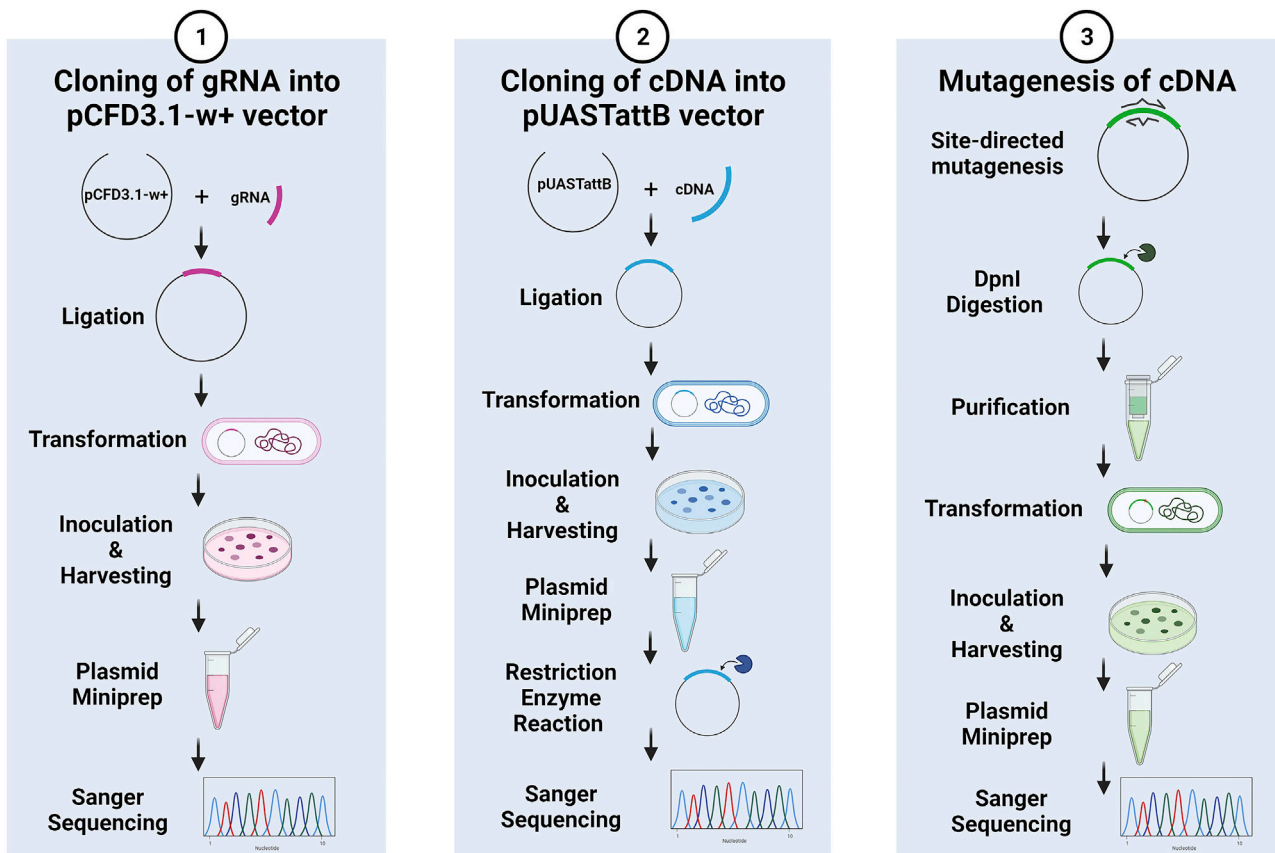
Figure 2. Schematics of cloning and mutagenesis strategies
An overview of the procedures described in the protocol that result in the generation of the Drosophila constructs carrying cDNA or gRNA that are utilized to produce transgenic fruit flies. The schematic was created with BioRender.com.

    d. Recommended gRNAs are shown with green color. In contrast, bad gRNAs with lower on-target scores, are shown with gray color (Figure 4). Do not select gRNAs targeting exons for specific splicing isoforms.

    e. Select gRNAs that target the exon-intron junction shared by all mRNA isoforms in the 5′ of the open reading frame with a higher on-target score and minimum off-targets (Figure 5).

2. Synthesize a pair of oligo DNAs (sense and anti-sense) for gRNA that includes 4 bp overhangs for BbsI restriction enzyme (Figure 6).
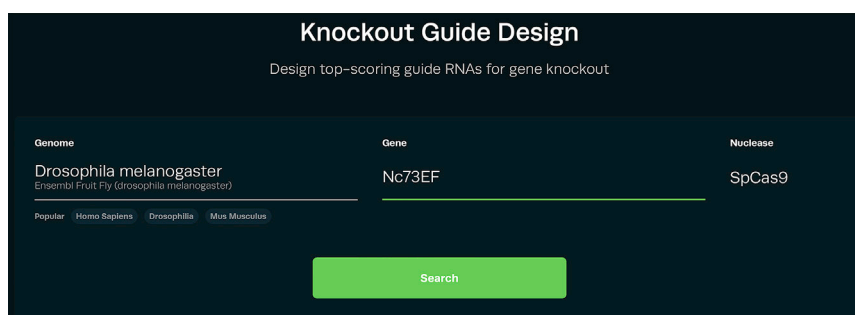

Figure 3. Snapshot of the Synthego Knockout Guide Design webpage

dOgdh gRNA-F	5′-<u>GTCG</u> AGGGATACTTACGGTGTGCA-3′
dOgdh gRNA-R	5′-<u>AAAC</u> TGCACACCGTAAGTATCCCT-3′

Note: The G at position 4 in the sense oligo is the first nucleotide that is transcribed and necessary for U6 promoter-driven transcription. If the target site has a 5′G, then the target sequence to be introduced will be 19 nt long. If it does not start with a G, the target sequence will be all 20 nt behind the GTCG overhang.

3. Phosphorylate and anneal the pair of oligos.
   a. Set-up the following phosphorylation reaction in a PCR tube on ice:

Phosphorylation Reaction

Reagent	Amount
T4 PNK Reaction Buffer (10×)	1 μL
ATP (10 mM)	1 μL
Sense oligo 1 (100 μM)	1 μL
Anti-sense oligo 2 (100 μM)	1 μL
T4 PNK	0.5 μL
Nuclease-free water (Invitrogen)	5.5 μL
Total	10 μL

Note: The T4 PNK, T4 PNK reaction buffer, and ATP should be stored at −30°C to −20°C.

   b. Incubate at 37°C for 30 min in a thermocycler.
   c. Heat the phosphorylated oligos at 95°C for 5 min in a thermocycler. Then cool the oligos to 25°C at 5°C/min.
   d. Dilute annealed and phosphorylated oligos at 1:100 dilution into sterile water.
4. Digest 2 μg of the pCFD3.1-w-dU6:3gRNA. pCFD3.1-w-dU6:3gRNA was a gift from Simon Bullock (Addgene plasmid # 123366 ; http://n2t.net/addgene:123366 ; RRID:Addgene_123366).
   a. Set-up the following BbsI restriction enzyme (RE) reaction in a microcentrifuge tube:

BbsI RE Reaction

Reagent	Amount
pCFD3.1-w-dU6:3gRNA (1 μg/μL)	2 μL
NEB Buffer r2.1 (10×)	5 μL
BbsI	2 μL
Nuclease-free water	41 μL
Total	50 μL

Note: The BbsI enzyme and 10×NEBuffer r2.1 should be stored at −30°C to −20°C.

   b. Incubate at 37°C for 3 h.
   c. Gel purify BbsI-digested pCFD3.1-w-dU6:3gRNA using QIAquick Gel Extraction Kit and elute DNA in 30 μL EB buffer.
5. Ligation of the annealed and phosphorylated oligo duplex and digested plasmid.
   a. Set up the following ligation reaction in a microcentrifuge tube:

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Ligation Reaction	
Reagent	Amount
pCFD3.1-w-dU6:3gRNA (BbsI-digested) from step 4	1 μL (50 ng)
Diluted oligo duplex from step 3	3 μL
T4 DNA Ligase Buffer (10×)	2 μL
T4 DNA Ligase	1 μL
Nuclease-free water	13 μL
Total	20 μL

Note: The T4 DNA Ligase and T4 DNA Ligase buffer should be stored at −30°C to −20°C.

   b. Incubate at 20°C–22°C for 30 min to 1 h.
   c. Use 5 μL of the ligation mix for transformation in the next step, and store the remaining reaction in a −30°C freezer.
6. Transformation of the ligation reaction with DH5α chemical competent cells.
   a. Collect the chemical competent cells from −80°C and allow them to melt for 15–20 min on ice. While this is occurring, pre-chill 1.5 mL microcentrifuge tubes on ice.
   b. Aliquot 50 μL of competent cells to each tube.
   c. Add 5 μL of the ligation reaction to the competent cells.
   d. Incubate the tubes containing the ligation reaction and competent cells on ice for 15 min.
   e. Perform heat shock on tubes of DNA/competent cells mixture in a water bath set to 42°C for 90 s.
   f. After heat shock, incubate the tubes on ice for 2–5 min.
   g. Add 250 μL of LB medium to each sample.
   h. Incubate each transformed *E.coli* with LB medium in the shaking incubator at 37°C for 45–60 min. While incubating the samples, pre-warm LB plates containing ampicillin (100 μg/mL) in 37°C incubator.
   i. Spin down the cells at 8,000 rpm (~6,100 × g) for 4 min, then discard 200–250 μL of supernatant and leave less than 100 μL of LB in the tube.
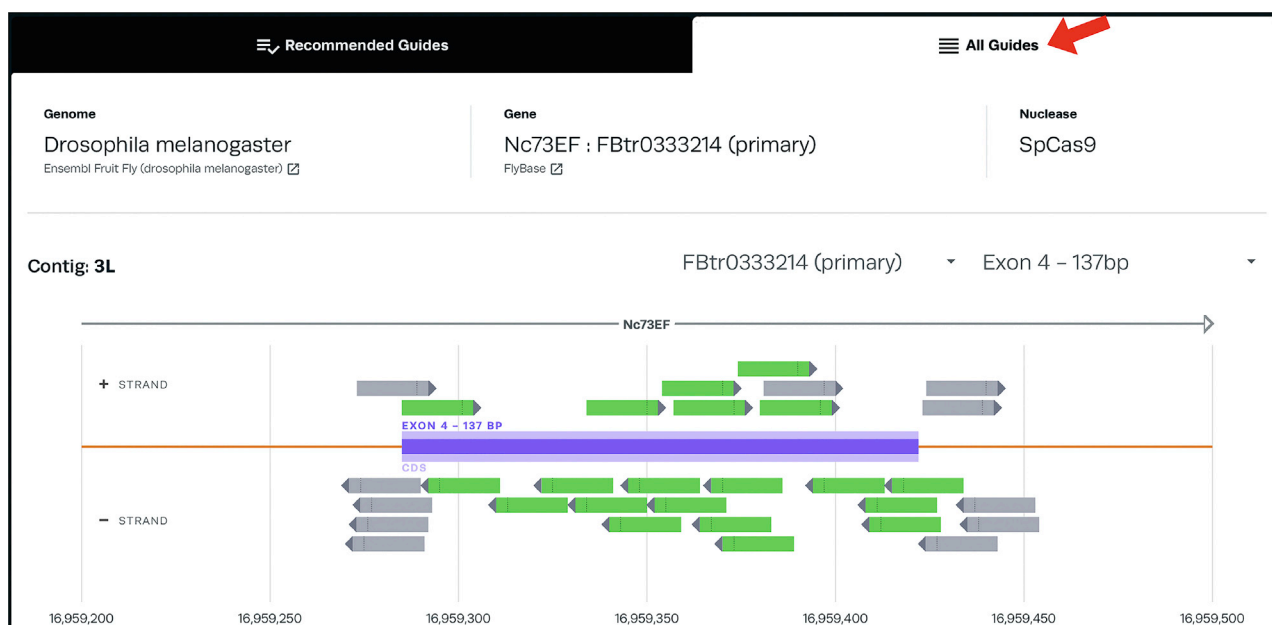

Figure 4. Snapshot of the results of gRNAs search for *dOgdh/Nc73EF* from the Synthego Knockout Guide Design tool
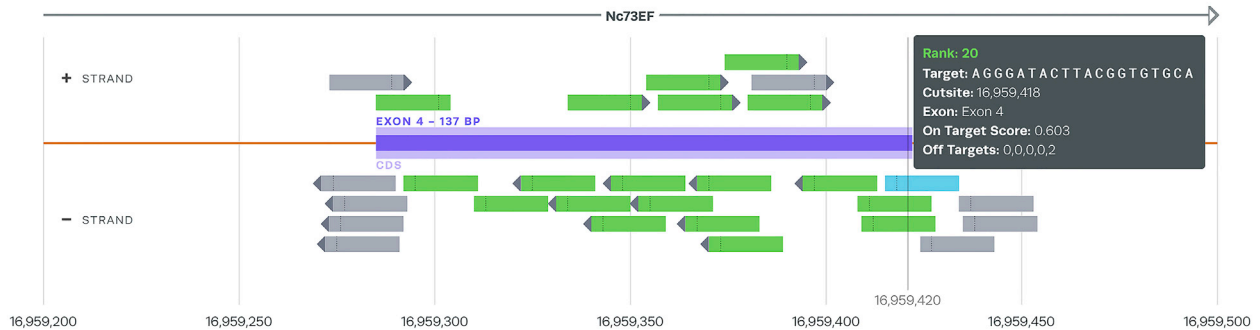
Figure 5. Snapshot of the results of *dOgdh*-targeting gRNAs
Find the gRNA that targets the exon-intron junction of *dOgdh*.

  j. Suspend cells with LB by pipetting gently.
  k. Take the pre-warmed plates containing ampicillin out of the incubator, and plate *E.coli* using sterile glass beads (Zymo Research S1001).
  l. Incubate the plates in a 37°C incubator for 15–18 h.
7. Next day, check the plates, and you will observe several dozens of colonies.
  a. Add 3 mL of LB medium with ampicillin (100 µg/mL) to each individual 15 mL cap-tube.
  b. Use a sterile P200 pipette tip to pick 6 to 10 single colonies, and suspend them into the LB medium of the culture tubes. Place the tubes with LB medium and colonies in a shaking incubator for 15–16 h at 37°C.
8. Next day, harvest cultured *E.coli* by centrifugation at 15,000 rpm (∼11,000 × *g*) (Eppendorf 5424). Discard LB medium properly, being careful to not disturb the pellet.

> ⏸ **Pause point:** The *E.coli* pellets can be stored at −80°C.

9. To prepare the plasmid DNA from the *E.coli* pellet, use the Zyppy Plasmid Miniprep Kit (Zymo, Cat# D4020) and follow the manufacturer's step-by-step instruction.
10. Send the DNA constructs to sequencing companies for Sanger sequencing to read the region of sgRNA using pCFD3.1w-seqF (Table 1).
  a. Determine which clones have the desired gRNA. Typically, 70%–80% of clones have the desired gRNA insert.
  b. Select correct DNA clones to continue to the next step.
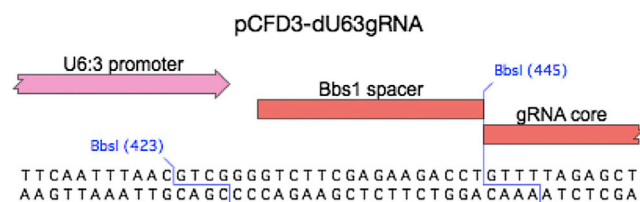
Figure 6. Snapshot of gRNA oligo design for pCFD3 from the CRISPR Fly Design website (http://www.crisprflydesign.org/wp-content/uploads/2014/05/Cloning-with-pCFD3.pdf)

Table 1. Oligonucleotides

Oligonucleotides	Source	Identifier
pCFD3.1w-seqF: 5′-GACTCGCGAAAAAACCGTCTG-3′	Sigma-Aldrich	N/A
dOgdh-F1: 5′-GACTCGCGAAAAAACCGTCTG-3′	Sigma-Aldrich	N/A
dOgdh -R1: 5′-CGATCCATGTCCTGCTCGCC-3′	Sigma-Aldrich	N/A
dOgdh -F2: 5′-TCAACGGAGTTGGGAGTGGAG-3′	Sigma-Aldrich	N/A
dOgdh -F3: 5′-TCGGTTTCACCACCGATCCC-3′	Sigma-Aldrich	N/A
dOgdh -F4: 5′-CCATTGAAGGTAGCGCCCAC-3′	Sigma-Aldrich	N/A
dOgdh -F5: 5′-GAGACTTCAGCAACACGGCCC-3′	Sigma-Aldrich	N/A
dOgdh -F6: 5′-AAGGTCGTCTTCTGCTCGGG-3′	Sigma-Aldrich	N/A
pUAST-F: 5′-AGTGCAAGTTAAAGTGAATC-3′	Sigma-Aldrich	N/A
pUAST-R: 5′-GTAGTTTAACACATTATACAC-3′	Sigma-Aldrich	N/A
dOgdh-gF-1: 5′-CCAACTAATGAGCCCCCAATC-3′	Sigma-Aldrich	N/A
dOgdh-gR-1: 5′-TTTTTTATTGCAGAGAAGTTTGGTTCCC-3′	Sigma-Aldrich	N/A
pUAST-F2: 5′-CAACTGCAACTACTGAAATCTGC-3′	Sigma-Aldrich	N/A
dOgdh-cDNA #R1: 5′-TGGTAGCTCCTGATGATGGCC-3′	Sigma-Aldrich	N/A

Generation of flies expressing sgRNA

⏲ Timing: 6 weeks

11. Perform chemical transformation of the correct DNA clone with competent cells as described in step 6 and obtain transformed *E.coli* carrying correct pCFD3.1-w-dU6:3-gRNA[dOgdh (or your gene of interest)].

12. Use a sterile P200 pipette tip to select 2–3 colonies and resuspend into an Erlenmeyer flask containing 50 mL of LB medium with ampicillin (100 µg/mL). Incubate flask in a shaking incubator (250 rpm) at 37°C for 15–16 h.

13. After 16 h, transfer the cultured *E.coil* to a 50 mL conical tube and centrifuge at 3,220 × *g* (4,000 rpm) at 4°C for 20 min (Eppendorf 3810 R).

14. Use the PureLinkTM HiPure Plasmid Midiprep Kit (Invitrogen, Cat# K210005). Follow step-by-step instructions to complete the Midiprep procedure, and purify the plasmid DNA.

15. For site-specific transgenesis of the pCFD3.1-w-dU6:3-gRNA vector, use PhiC31-mediated transgenesis (Venken et al., 2006). We chose to inject the DNA constructs into the attP40 docking site on the 2nd chromosome (BDSC #79604, y[1] w* P{y[+t7.7]=nos-phiC31\int.NLS}X; P{y[+t7.7]=CaryP}attP40). The DNA was injected by injection companies and transgenic flies were selected and balanced.

Alternatives: You can use other docking sites in any chromosomes such as VK37 (2nd chromosome), VK5 and VK33 (3rd chromosome).

Generation of pUASTattB-cDNA

⏲ Timing: 1 week

16. Find and obtain cDNA clones from DGRC.
    a. Go to FlyBase website (http://flybase.org) and find your gene of interest.
    b. Go to "Stocks and Reagents" and find fully sequenced cDNA clones from "BDGP DGC clones" (Figure 7).
    c. Check whether the clones contain full-length cDNA sequence and no point mutations.
    d. Obtain cDNA clones from Drosophila Genomics Resource Center (DGRC). https://dgrc.bio.indiana.edu/Home.

17. Design primers for PCR of cDNA for cloning into pUASTattB vector.

⊞ Stocks (10)			
⊞ Genomic Clones (23)			
⊟ cDNA Clones (256)			
	List GenBank IDs		Clones Consistent with Transcripts
	ⓘ **Please Note** This section lists cDNAs and ESTs that fall within the genomic extent of the gene model, which may include cDNAs and ESTs of genes within introns, or of overlapping genes. Please see GBrowse for alignment of the cDNAs and ESTs to the gene model.		
cDNA clones, fully sequenced			
BDGP DGC clones	RE42354	RH09189	SD10782

Figure 7. Snapshot of cDNA Clones for *dOgdh* from the FlyBase website

a. The multicloning site (MCS) in pUASTattB vector contains EcoRI, BglII, NotI, KpnI, XhoI, and XbaI (Bischof et al., 2007). Check your cDNA and choose REs that do not exist in the cDNA.

b. Synthesize a pair of PCR primers that include 6 bp overhangs for restriction enzymes. For example, we designed the PCR primers for *dOgdh* as shown below (Yoon et al., 2017).

dOgdh (BclI) -F	5′-attttgTGATCA CAAA ATGCATAGAGCCCACACAGCC-3′
dOgdh (XbaI)-R	5′-agtaaTCTAGA TTACTTGTCGTCATCGTCCTTGTAATC GCCGCTTCCGGTCGAAATCGCGTCGTTGAG-3′

Note: We added a Flag tag in the C-terminus as the N-terminus of dOgdh protein functions as a mitochondrial targeting sequence. You can choose N- or C-terminal tag depending on the nature of your protein of interest.

18. Perform PCR reaction.
    a. Set up the following PCR reaction on ice or at a cooler (−20°C).

PCR reaction master mix

Reagent	Amount
DNA template (50–100 ng) / μL	1 μL
Taq (iProof, BioRad)	1 μL
Primer Forward (10 μM)	1 μL
Primer Reverse (10 μM)	1 μL
dNTP (10 mM)	1 μL
5 × iProof HF buffer	10 μL
Nuclease-free water	35 μL
Total	50 μL

Note: The iProof Taq, 5×iProof HF buffer, dNTP, and PCR primers should be stored at −30°C to −20°C.

b. Use the following cycling conditions for mutagenesis PCR.

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	30 cycles

(Continued on next page)

Continued			
Steps	Temperature	Time	Cycles
Annealing	55°C	30 s	
Extension	72°C	2 min	
Final extension	72°C	7 min	1
Hold	12°C	Forever	

Note: The speed of iProof taq polymerase (Bio-Rad) is 15–30 s/kb. Adjust the extension time depending on the length of the PCR product.

Alternatives: You can use other high-fidelity taq polymerases from other vendors. Note that other proof-reading taq polymerases may have different speed for elongation (e.g., 1 min/kb for PfuTurbo from the QuickChange Site-Directed Mutagenesis kit, Cat#200518, Stratagene).

19. Run 1% agarose gel to confirm the size of the PCR product. For example, we expect to see approximately 3 kb size of PCR band for *dOgdh* cDNA.
20. Purify the PCR sample by using QIAquick PCR Purification Kit (QIAGEN, Cat# 28106). Elute DNA with 48 μL EB buffer.
21. Perform RE digestion for your PCR product. For the digestion of the PCR product for *dOgdh* cDNA, set up the following reaction for XbaI and DpnI and leave on the heat block at 37°C for over 1 h.

RE Digestion	
Reagent	Amount
Purified PCR Product	47.5 μL
XbaI	1 μL
DpnI	1 μL
10 × NEBuffer r2.1	5.5 μL
Total	55 μL

Note: The restriction enzymes including XbaI and DpnI and 10×NEBuffer r2.1 should be stored at −30°C to −20°C.

Note: Use DpnI to remove the template plasmid DNA from the PCR reaction.

22. Heat inactivate the reaction at 65°C for 20 min.
23. Add 1 μL of BclI to the reaction and incubate at 50°C for over 1 h.
24. Purify the RE-digested PCR sample by using QIAquick PCR Purification Kit (QIAGEN, Cat# 28106). Elute DNA with 30 μL EB buffer.
25. Digestion of the pUASTattB vector.
    a. Set-up the following RE reaction in a microcentrifuge tube:
    RE Reaction

Reagent	Amount
pUASTattB (1 μg/μL)	2 μL
NEB Buffer r3.1 (10×)	5 μL
BglII	1 μL
XbaI	1 μL
Nuclease-free water	41 μL
Total	50 μL

Note: The BglII and XbaI enzymes and 10×NEBuffer r3.1 should be stored at −30°C to −20°C.

Note: Choose the appropriate REs that are compatible with your PCR product.

b. Incubate at 37°C for 3 h.
c. Gel purify RE-digested pUASTattB using QIAquick Gel Extraction Kit and elute DNA in 50 μL EB buffer.

Alternatives: You can use DNA purification kits from other vendors.

26. Ligation of the purified PCR DNA and digested plasmid.
    a. Set up the following ligation reaction in a microcentrifuge tube:

Ligation Reaction	
Reagent	**Amount**
pUASTattB (BglII/XbaI-digested) from step 25	1 μL (50 ng)
Purified PCR from step 24	3 μL
T4 DNA Ligase Buffer (10×)	2 μL
T4 DNA Ligase	1 μL
Nuclease-free water	13 μL
Total	20 μL

Note: Use appropriate amount of purified DNA as an insert. Typical molar ratio of vector: insert is 1:3.

b. Incubate at room temperature for 30 min to 1 h.
c. Use 5 μL of the ligation mix for transformation in the next step, and store the remaining reaction at −30°C freezer.
27. Perform transformation and mini-prep as described in steps 6–9.
28. Use the miniprep DNA to prepare the following restriction enzyme reaction (RE) in order to confirm the construct has the right size of insert and vector.

RE Reaction	
Reagent	**Amount**
EcoRI	0.5 μL
XbaI	0.5 μL
Miniprep DNA (200–300 ng/μL)	2 μL
NEBuffer r3.1	2 μL
Nuclease-free water	15 μL
Total	20 μL

Note: The EcoRI and XbaI enzymes and 10×NEBuffer r3.1 should be stored at −30°C to −20°C.

a. Keep the reaction tubes on the heat block at 37°C for 1–2 h.
b. Run 10 μL of the RE reactions in a 1% agarose gel to check the size of the products. There should be 2 different sized bands (pUASTattB (vector) approximately 9 kb and *dOgdh* cDNA (insert) approximately 3 kb) after the RE Reaction (Figure 8).
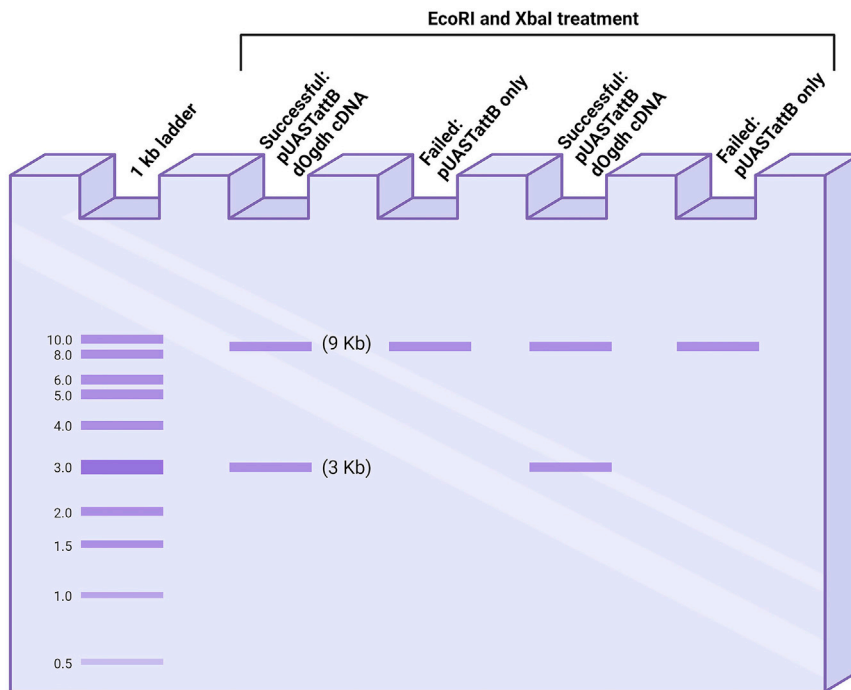
Figure 8. A schematic of screening of mini-prep DNA by RE digestion using EcoRI and XbaI
The lanes with 2 bands (one at 9 kb and one at 3 kb) were successful, whereas the lanes with one band at 9 kb failed.
Created with BioRender.com.

Alternatives: You can perform colony PCR to save restriction enzymes and time if you need to pick many colonies due to the low cloning efficiency.

29. Send the purified mini-prep DNAs for Sanger sequencing using sequencing primers for *dOgdh* (Table 1).
    a. Confirm the clones have no other unwanted mutations.
    b. Select the correct DNA clone to continue to the next step.

Mutagenesis of pUASTattB-cDNA carrying disease candidate variants

⏱ **Timing: 1 week**

30. Design a pair of primers for mutagenesis PCR in order to create pUASTattB-cDNA that carries homologous mutations to human pathogenic variants in your gene of interest. For example, we created 9 missense mutations in *dOgdh* cDNA that are homologous to pathogenic variants in *OGDHL* (Yap et al., 2021; Yoon et al., 2017). Design a pair of 45 bp-length oligos (sense and anti-sense) that are complementary to the *dOgdh* cDNA, but have a homologous missense mutation identified from human patients. The point mutation should be located in the center of your primers. For example, the primers below are used for creating dOgdh (P867A) mutation.

dOgdh (wild-type)	5′-cccaaatcgctgctgcgtcacCccgaggctaagagtcctttcagc-3′
dOgdh(P867A)-F	5′-cccaaatcgctgctgcgtcacGccgaggctaagagtcctttcagc-3′
dOgdh(P867A)-R	5′-gctgaaaggactcttagcctcggCgtgacgcagcagcgatttggg-3′

31. Perform mutagenesis PCR to generate pUASTattB-dOgdh (P867A)-Flag.
    a. Set up the following PCR reaction on ice or at a cooler (−20°C). For the template DNA, we use pUASTattB-dOgdh-Flag (wild-type) construct from the previous study (Yoon et al., 2017).

PCR reaction master mix	
Reagent	Amount
DNA template (100–200 ng) / μL	1 μL
Taq (iProof, Bio-Rad)	1 μL
Primer Forward (10 μM)	1 μL
Primer Reverse (10 μM)	1 μL
dNTP (10 mM)	1 μL
5 × iProof HF buffer	10 μL
Nuclease-free water	35 μL
Total	50 μL

Note: The iProof Taq, 5×iProof HF buffer, dNTP, and PCR primers should be stored at −30°C to −20°C.

b. Use the following cycling conditions for mutagenesis PCR.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	22 cycles
Annealing	55°C	30 s	
Extension	72°C	4 min	
Final extension	72°C	7 min	1
Hold	12°C	Forever	

Note: The speed of the iProof taq polymerase is 15–30 s/kb. Adjust the extension time based on the length of the vector and insert.

Alternatives: You can use mutagenesis kits from other vendors.

32. To remove the template plasmid DNA from the PCR reaction, set up the following reaction for DpnI Digestion and leave on the heat block at 37°C for over 1 h.

DpnI Digestion	
Reagent	Amount
PCR Product	50 μL
DpnI	2 μL
10 × Smart Buffer (NEB)	10 μL
Nuclease-free water	38 μL
Total	100 μL

Note: The DpnI enzyme and 10×Smart Buffer (NEB) should be stored at −30°C to −20°C.

33. Purify the DpnI-digested PCR sample by using QIAquick PCR Purification Kit (QIAGEN, Cat# 28106). Elute DNA with 30 μL EB buffer.
    a. Run the purified DNA in 1% agarose gel. If the PCR reaction went successfully, you will see a linear DNA with around 12 kb size (approximately 9 kb for the vector plus approximately 3 kb for the insert) (Figure 9).
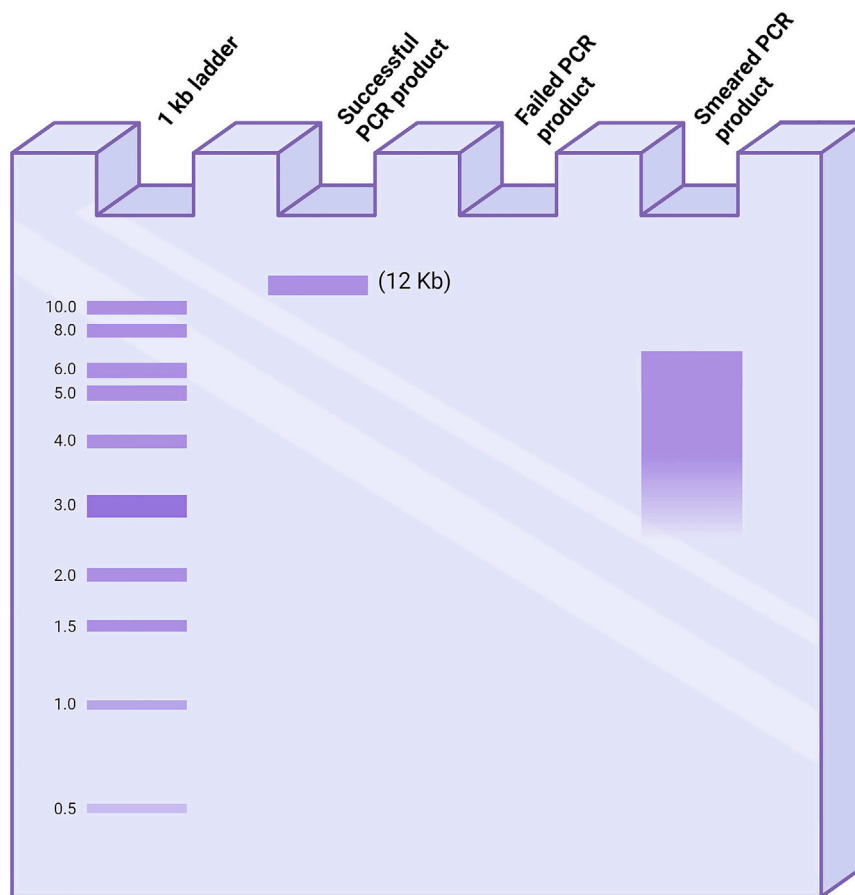
Figure 9. A schematic of successful and failed reactions of mutagenesis PCR shown by electrophoresis in agarose gel

An example of a successful PCR reaction is in lane 2, with a single band at 12 kb. There are 2 examples of failed PCR reactions in lane 3 and lane 4, which show no band or a smear, respectively. Created with BioRender.com.

    b. If the reaction was successful, then move to the next step for chemical transformation. However, if the reaction was unsuccessful, repeat the PCR reaction, altering the conditions (e.g., adjust annealing temperature and/or adding 1%–3%DMSO) to optimize the reaction.

34. Perform transformation for 5 μL of the purified PCR DNA as described in step 6.
35. Perform mini-DNA preparation as described in steps 7–9.
36. Send the purified mini-prep DNAs for Sanger sequencing to read the mutant region.
    a. Determine which clones have the desired mutation and confirm that the clones have no other unwanted mutations. Typically, 50%–80% of clones have the desired mutation.
    b. Select the correct DNA clone to continue to the next step.

Generation of flies carrying *UAS-cDNA* with disease candidate variants

⏱ Timing: 6 weeks

37. Perform chemical transformation of the correct mutant plasmid DNA with competent cells as described in step 6. Incubate plates in 30°C incubator for 15–20 h.
38. Inoculate transformed colonies into 50 mL LB with ampicillin (100 μg/mL) in an Erlenmeyer flask. Incubate the flask in a shaking incubator at 250 rpm at 30°C for 16–17 h.

Note: We sometimes found that spontaneous mutations occurred in the coding sequence of mitochondrial genes including *dOgdh*, and human *OGDH* cDNA when using large-scale cultures (50–100 mL). If this occurs, culture *E.coli* at lower temperature (30°C) to reduce the likelihood of natural mutation.

39. After 16 h, transfer the *E.coli* culture to a 50 mL conical tube and centrifuge at 3220 cfu (4,000 rpm) at 4°C for 20 min (Eppendorf 3810 R).
40. Use the PureLinkTM HiPure Plasmid Midiprep Kit (Invitrogen, Cat# K210005). Follow step-by-step instructions to complete the Midiprep procedure, and purify the plasmid DNA.
41. Use the Midiprep DNA to prepare the following restriction enzyme reaction (RE) in order to confirm the construct has the right size of insert and vector.

RE Reaction	
Reagent	Amount
EcoRI (20,000 units/mL)	1 μL
NotI (10,000 units/mL)	1 μL
pUASTattB-dOgdh(P867A) (200 ng/μL)	1 μL
NEBuffer 3.1	2 μL
Nuclease-free water	15 μL
Total	20 μL

Note: The EcoRI and NotI enzymes and 10×NEBuffer r3.1 should be stored at −30°C to −20°C.

a. Keep the reaction tubes on the heat block at 37°C for 1–2 h.
b. Run 10 μL of the RE reaction in a 1% agarose gel to check the size of the products. There should be 2 different sized bands (pUASTattB (vector) approximately 9 kb and dOgdh cDNA (insert) approximately 3 kb) after the RE Reaction based on which restriction enzymes you use to cut the DNA construct.

42. Send the Midiprep DNA constructs to sequencing companies for Sanger sequencing to read the whole coding region of *dOgdh.*
a. Check that the sequences are correct including the start codon, location of the desired mutation, stop codon, location of the tag sequence, and that there are no background mutations.

43. For site-specific transgenesis of the pUASTattB vector, use PhiC31-mediated transgenesis (Venken et al., 2006). We chose to inject the DNA constructs into the VK37 docking site on the 2nd chromosome (BDSC #9752; (PBac{y[+]-attP-3B}VK00037). The DNA was injected by injection companies and transgenic flies were selected and balanced.

Alternatives: You can use other docking sites in any chromosomes such as attP40 (2nd chromosome), and VK5 or VK33 (3rd chromosome).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5α competent cells	Thermo Fisher Scientific	Cat# 18258-012
Chemicals, peptides, and recombinant proteins		
iProof High Fidelity DNA Polymerase	Bio-Rad	Cat# 1725302
DMSO	Bio-Rad	Cat# 1725302
Thermo Scientific dNTP Set, 100 mM Solutions	Fisher Scientific	Cat# FERR0181
5 × iProof HF Buffer	Bio-Rad	Cat# 1725391

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Nuclease-free water (H$_2$O)	Invitrogen (Thermo Fisher Scientific)	Cat# 10977-015
DpnI	New England Biolabs	Cat# R0176S
T4 Polynucleotide Kinase (PNK)	New England Biolabs	Cat# M0201S
T4 PNK Reaction Buffer	New England Biolabs	Cat# B0201S
ATP	New England Biolabs	Cat# P0756S
BbsI	New England Biolabs	Cat# R0539S
T4 DNA Ligase	New England Biolabs	Cat# M0202L
T4 DNA Ligase Buffer (10×)	New England Biolabs	Cat# B0202S
NEBuffer Set (r1,1, r2.1, r3.1 and rCutSmart)	New England Biolabs	Cat# B7030S
BclI	New England Biolabs	Cat# R0160S
XbaI	New England Biolabs	Cat# R0145S
NotI	New England Biolabs	Cat# R0189S
EcoRI	New England Biolabs	Cat# R0101S
BglII	New England Biolabs	Cat# R0144S
OneTaq Hot Start 2× Master Mix with Standard Buffer	New England Biolabs	Cat# M0484S
BD Bacto Yeast Extract, Technical	VWR	Cat# 90004-092
BD Bacto Trypton	VWR	Cat# 90000-286
Sodium chloride	Sigma-Aldrich	Cat# S3014-5KG
BD Bacto Agar Soldifying Agent	VWR	Cat# 90000-760
Ampicillin sodium salt	Sigma-Aldrich	Cat# A9518-25G
Red Star Active Yeast	Genesee	Cat# 62-103
Agarose LE, Molecular Biology Grade	Denville Scientific Inc.	Cat# GR140-500
Ethanol, Pure + Denatured 200 Proof	Decon Labs Inc.	Cat# 2701
Molasses Unsulphured Original, 1 Gallon	Genesee	Cat# 62-117
Red Star Basic Yeast, 60 MHF Inactive Dry Primary Grown	Genesee	Cat# 62-106
Yellow Corn Meal	Genesee	Cat# 62-100
Gelidium Agar	MoorAgar (MA)	Cat# 41054
Propionic Acid (Certified ACS), Fisher Chemical	Thermo Fisher Scientific	Cat# A258-500
Methyl 4-hydroxybenzoate	Sigma-Aldrich	Cat# H5501-1KG
Critical commercial assays		
QIAquick PCR Purification Kit	QIAGEN	Cat# 28106
Zyppy Plasmid Miniprep Kit	Zymo Research	Cat# D4020
PureLinkTM HiPure Plasmid Midiprep Kit	Invitrogen (Thermo Fisher Scientific)	Cat# K210005
PureLink Genomic DNA Mini Kit	Invitrogen (Thermo Fisher Scientific)	Cat# K1820-02
QIAquick Gel Extraction Kit	QIAGEN	Cat# 28705
Experimental models: Organisms/strains		
Drosophila melanogaster: yw; spif / CyO; TM2 / TM6B	From Hugo Bellen lab	N/A
Drosophila melanogaster: elav$^{C155-Gal4}$	BDSC	RRID:BDSC_458 BDSC# 458
Drosophila melanogaster: UAS-Cas9.P2	BDSC	RRID:BDSC_67086 BDSC# 67086
Drosophila melanogaster: UAS-empty (vk37)	From Hugo Bellen lab	N/A
Drosophila melanogaster: y[1] w[*] P{y[+t7.7]=nos-phiC31\int.NLS}X; P {y[+t7.7]=CaryP}attP40	BDSC	RRID:BDSC_79604 BDSC# 79604
Drosophila melanogaster: y[1] w[1118]; PBac{y[+]-attP-3B}VK00037	BDSC	RRID:BDSC_9752 BDSC# 9752
Oligonucleotides		
See Table 1 for Oligonucleotides	This paper	N/A
Recombinant DNA		
pUASTattB-dOgdh DNA template	Yoon et al., (2017), Yap et al., (2021)	N/A
pCFD3.1-w-dU6:3gRNA	Addgene, gift from Simon Bullock	RRID:Addgene_123366 Cat# 123366
Software and algorithms		
Synthego ICE Analysis	N/A	https://ice.synthego.com/#/
Synthego Knockout Guide Design	N/A	https://design.synthego.com/#/

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Steam-Jacketed Kettle	Groen	Cat# TDB-40
Wide Drosophila vials, polypropylene	Genesee	Cat# 32-121
Rattler Plating Beads	Zymo Research	Cat# S1001

MATERIALS AND EQUIPMENT

Fly food preparation	
Materials	Amount
Water	4,500 mL
Cornmeal	2,250 mL
Yeast (inactive)	900 mL
Water	13,000 mL
Agar	175 g
Molasses	1,600 mL
Propionic acid	85 mL
Tegosept (20% in EtOH)	230 mL
Food can be stored at 4°C for up to 1 month.	

Note: Propionic acid needs to be stored at 20°C–22°C in an acid storage cabinet.

Note: Tegosept (20% in EtOH) can be store at 20°C–22°C for 2–3 weeks. However, we prefer to use freshly-prepared one.

Mix 13,000 mL of water and agar into a steam-jacketed kettle, and before boiling, add molasses and turn down the heat. Allow the mixture in the kettle to simmer for 15 min. While the mixture is simmering, mix together the cornmeal, yeast, and 4,500 mL of water in a separate container until smooth. At 15 min, add the smooth cornmeal, yeast, and water mixture to the kettle and turn off the heat. Stir food every 15 min until cooled to 55°C - 60°C. Then, add propionic acid and tegosept mixture. Before food cools below 55°C, disperse into approximately 1,800 polypropylene vials. Allow food to solidify and cool for 18–24 h before sealing in plastic wrap and storing.

⚠ CRITICAL: Tegosept has long term effects on aquatic life, avoid releasing into the environment.

⚠ CRITICAL: Propionic acid can cause eye damage, severe skin burns and respiratory irritation. Use protective clothing, gloves, eye protection, and face protection.

LB medium		
Reagent	Final concentration	Amount
BD Bacto Yeast Extract, Technical	0.5%	2.5 g
BD Bacto Trypton	1%	5 g
NaCl	1%	5 g
miliQ water	N/A	To 500 mL
Total	**N/A**	**500 mL**
Store at either 4°C or 20°C–22°C temperature for 2–3 months.		

Gently mix the reagents in a 500 mL glass bottle and autoclave at 121°C (15 psi) for 20 min.

LB agar plates		
Reagent	Final concentration	Amount
BD Bacto Yeast Extract, Technical	0.5%	2.5 g
BD Bacto Trypton	1%	5 g
NaCl	1%	5 g
BD Bacto Agar	1.5%	7.5 g
Milli-Q water	N/A	To 500 mL
Ampicillin	100 ug/mL	500 uL
Total	N/A	500 mL

Store in 4°C for 1 month.

Gently mix the Yeast extract, Trypton, NaCl, Bacto Agar, and Milli-Q water in a 500 mL glass bottle and autoclave at 121°C (15 psi) for 20 min. After the autoclaved media is cooled down to around 60°C, add 0.5 mL of ampicillin (100 mg/mL), and pour 15 mL of the LB/Agar solution into the petri-dishes. Allow the LB/Agar solution to solidify, keep LB Agar plates in 4°C for storage.

STEP-BY-STEP METHOD DETAILS

To achieve a tissue-specific ko in parallel with cDNA expression, four genetic components are required, including a tissue-specific *Gal4*, a *dU6:3-sgRNA*$^{gene\ of\ interest}$, *UAS-Cas9.P2*, and *UAS-cDNA*$^{gene\ of\ interest}$ (WT or mutant). We suggest you generate two fly lines – one carrying a Gal4 driver and *sgRNA*$^{gene\ of\ interest}$, and another carrying *UAS-Cas9.P2* and *UAS-cDNA (your gene of interest)*. These parental lines are genetically stable as they lack at least one component for Cas9 or sgRNA expression. Progenies arising from crosses of the parental lines have all four components, which permit tissue-specific knockout in the genome, as well as cDNA expression from the *UAS* transgene by Gal4 (Figure 1). Any Gal4 driver can be used for this system. In this protocol, we describe a method regarding a neuronal Gal4 (*elav*^{C155}-*Gal4*) to determine the functional effects of human *OGDHL* variants in neurons as human *OGDHL* is mainly expressed in the human brains (Bunik et al., 2008; Consortium, 2013).

Generation of flies carrying *sgRNA*^{dOgdh} and neuronal Gal4 driver

⏱ **Timing: 6 weeks**

To establish a *Drosophila* line with a neuronal Gal4 driver on the 1st chromosome and *sgRNA*^{dOgdh} on the 2nd chromosome, a series of crosses will be performed. Genetic balancers and markers will be used to track the presence of the desired genes and transgenes in the progeny (Figure 10).

1. Cross 3 male flies carrying sgRNA (*y,w/Y chromosome; U6:3-sgRNA*^{dOgdh}) with 3–6 virgin female flies having a neuronal Gal4 driver as well as a balancer and a maker on the 2nd chromosome (*elav*^{C155}-*Gal4; Bl*^{1}*/CyO, Tub>Gal80*). Two crosses will provide enough progenies for down-stream experiments.
2. Select 3 male progeny with *CyO, Tub>Gal80*, making sure to exclude those with *Bl*^{1}. Cross these males (*elav*^{C155}-*Gal4/Y chromosome; U6:3-sgRNA*^{dOgdh}*/CyO, Tub>Gal80*) with 3–6 virgin female flies having a neuronal Gal4 driver and 2nd chromosome balancer and maker (*elav*^{C155}-*Gal4; Bl*^{1}*/ CyO, Tub>Gal80*).
3. Select 3 male and 3–6 virgin female progenies with *CyO, Tub>Gal80*, making sure to exclude those with *Bl*^{1}. Make a cross to produce a genetic line containing *CyO, Tub>Gal4* and *sgRNA*^{dOgdh} on the 2nd chromosome (*elav*^{C155}-*Gal4; U6:3-sgRNA*^{dOgdh}*/CyO, Tub>Gal80*).
4. Select and cross 3 male and 3–6 virgin female progenies that are homozygous for *sgRNA*^{dOgdh} on the 2nd chromosome (*elav*^{C155}-*Gal4; U6:3-sgRNA*^{dOgdh}*/CyO, Tub>Gal80*).

$$\male \frac{yw}{\diagup} ; \frac{dU6:3sgRNA^{dOgdh}}{dU6:3sgRNA^{dOgdh}} \quad X \quad \female \frac{P\{GawB\}elav^{C155}w^{1118}}{P\{GawBela\}v^{C155}w^{1118}} ; \frac{Bl^{1}}{CyO, Tub>Gal80}$$

Select males with curly wings not short bristles

$$\male \frac{P\{GawB\}elav^{C155}w^{1118}}{\diagup} ; \frac{dU6:3sgRNA^{dOgdh}}{CyO, Tub>Gal80} \quad X \quad \female \frac{P\{GawB\}elav^{C155}w^{1118}}{P\{GawB\}elav^{C155}w^{1118}} ; \frac{Bl^{1}}{CyO, Tub>Gal80}$$

Select males & virgin females with curly wings & without short bristles

$$\male \frac{P\{GawB\}elav^{C155}w^{1118}}{\diagup} ; \frac{dU6:3sgRNA^{dOgdh}}{CyO, Tub>Gal80} \quad X \quad \female \frac{P\{GawB\}elav^{C155}w^{1118}}{P\{GawB\}elav^{C155}w^{1118}} ; \frac{dU6:3sgRNA^{dOgdh}}{CyO, Tub>Gal80}$$

Select males & virgin females without curly wings

$$\male \frac{P\{GawB\}elav^{C155}w^{1118}}{\diagup} ; \frac{dU6:3sgRNA^{dOgdh}}{dU6:3sgRNA^{dOgdh}} \quad X \quad \female \frac{P\{GawB\}elav^{C155}w^{1118}}{P\{GawB\}elav^{C155}w^{1118}} ; \frac{dU6:3sgRNA^{dOgdh}}{dU6:3sgRNA^{dOgdh}}$$

Line Established

$$\frac{P\{GawB\}elav^{C155}w^{1118}}{P\{GawB\}elav^{C155}w^{1118}/\rightarrow} ; dU6:3sgRNA^{dOgdh}$$
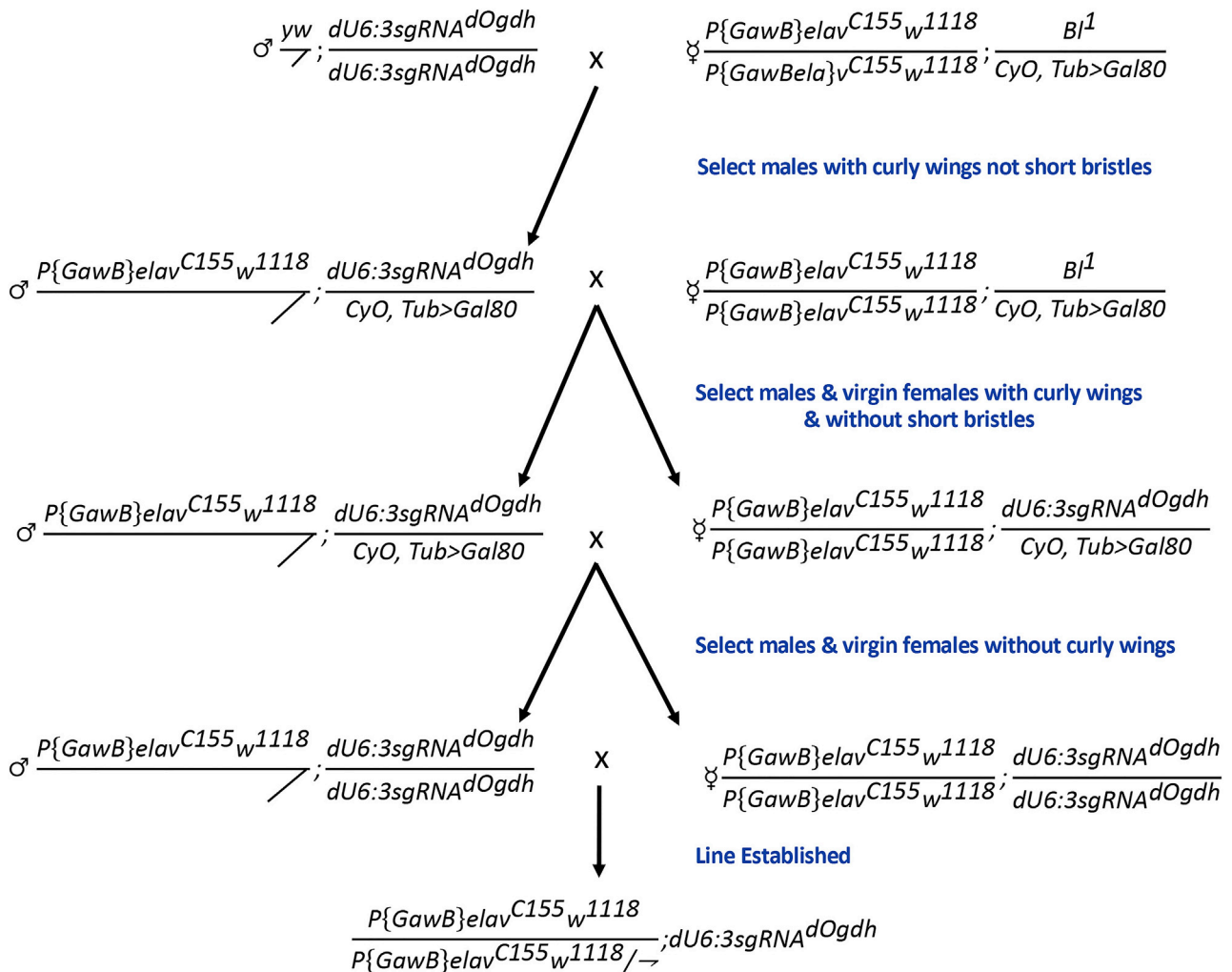
Figure 10. Crossing scheme for the generation of files carrying neuronal Gal4 driver and *sgRNA^{dOgdh}*

⚠ CRITICAL: It is important to use the correct combination of male and female genotypes in crosses. For example, you should use female virgin flies having *elav^{C155}-Gal4; Bl^{1}/CyO, tub-Gal80*, and male flies of *yw/Y chromosome; dU6:3-sgRNA^{dOgdh}* for the first cross. As *elav^{C155}-Gal4* is located on the X chromosome, the Y chromosome in males will be used to track the presence of the neuronal Gal4 on X chromosome. In this instance, the Y chromosome acts as a balancer.

⏸ Pause point: Once a line is established, it can be maintained indefinitely.

Generation of flies carrying *UAS-dOgdh (WT* or mutant) and *UAS-Cas9.P2*

⏱ Timing: 4 weeks

5. Cross 3 male flies carrying *UAS-dOgdh (WT or mutant)* on 2nd chromosome with 3–6 virgin female flies having balancers and makers in the 2nd and 3rd chromosomes (*yw; sp, if/CyO; TM2/TM6B*) (Figure 11). Two crosses will provide enough progenies for downstream experiments.
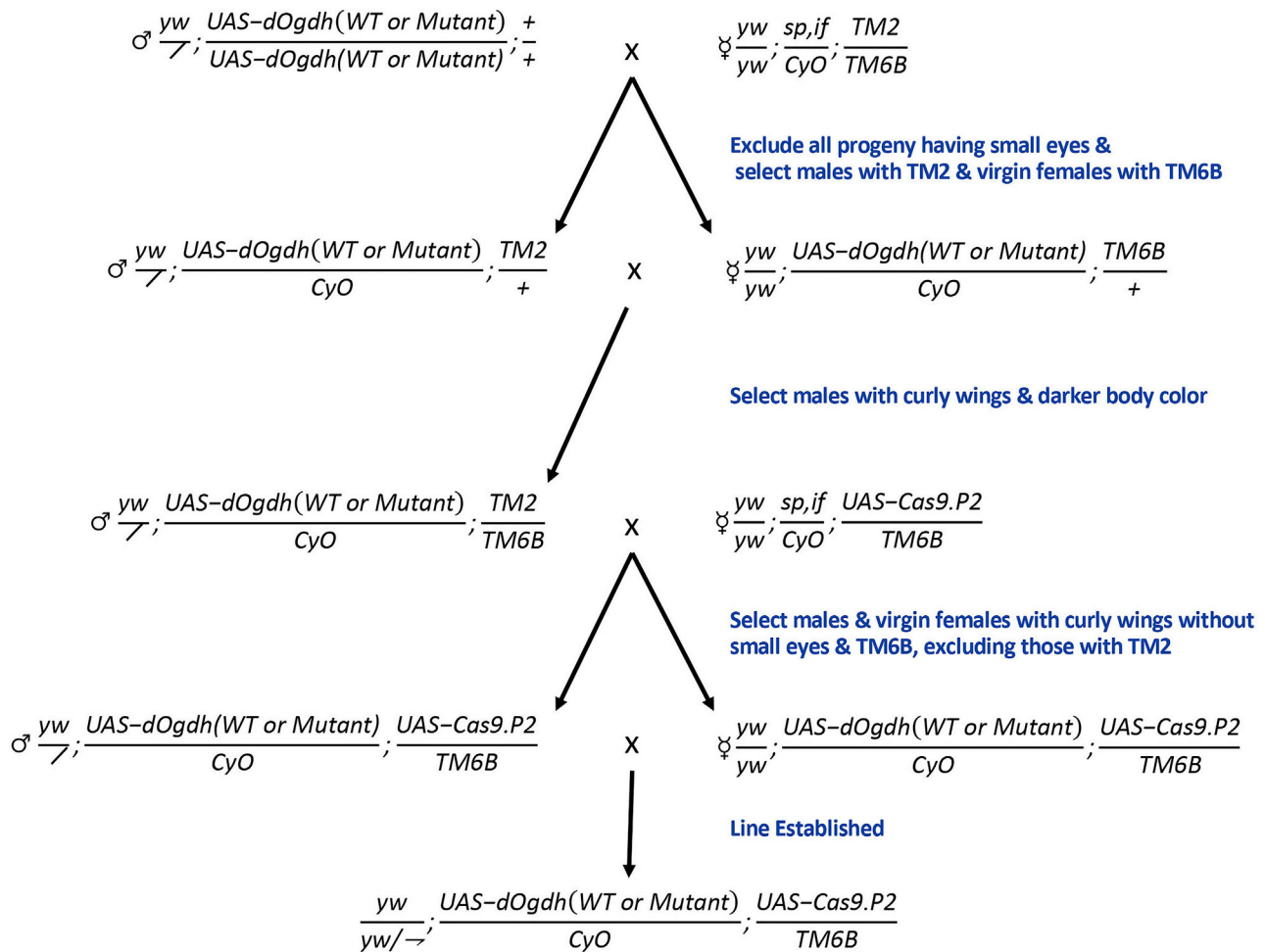
$$\male \; \frac{yw}{Y} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{UAS-dOgdh(WT\ or\ Mutant)} ; \frac{+}{+} \qquad \times \qquad \female \; \frac{yw}{yw} ; \frac{sp, if}{CyO} ; \frac{TM2}{TM6B}$$

Exclude all progeny having small eyes & select males with TM2 & virgin females with TM6B

$$\male \; \frac{yw}{Y} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{CyO} ; \frac{TM2}{+} \qquad \times \qquad \female \; \frac{yw}{yw} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{CyO} ; \frac{TM6B}{+}$$

Select males with curly wings & darker body color

$$\male \; \frac{yw}{Y} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{CyO} ; \frac{TM2}{TM6B} \qquad \times \qquad \female \; \frac{yw}{yw} ; \frac{sp, if}{CyO} ; \frac{UAS-Cas9.P2}{TM6B}$$

Select males & virgin females with curly wings without small eyes & TM6B, excluding those with TM2

$$\male \; \frac{yw}{Y} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{CyO} ; \frac{UAS-Cas9.P2}{TM6B} \qquad \times \qquad \female \; \frac{yw}{yw} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{CyO} ; \frac{UAS-Cas9.P2}{TM6B}$$

Line Established

$$\frac{yw}{yw/\to} ; \frac{UAS-dOgdh(WT\ or\ Mutant)}{CyO} ; \frac{UAS-Cas9.P2}{TM6B}$$

Figure 11. Crossing scheme for the generation of flies carrying *UAS-dOgdh (WT)* and *UAS-Cas9.P2* line

6. Select 3 male progeny having *CyO* on the 2nd chromosome and *TM6B* on the 3rd chromosome, Making sure to exclude *sp,if* from the 2nd chromosome and *TM2* from the 3rd chromosome. Cross with 3–6 virgin female flies selected to have *CyO* on the 2nd chromosome and *TM2* on the 3rd chromosome, Making sure to exclude *sp,if* from the 2nd chromosome and *TM6B* from the 3rd chromosome.

7. Collect male progeny having *CyO* on the 2nd chromosome and both *TM6B* and *TM2* on the 3rd chromosome (*y, w/Y; UAS-dOgdh (WT or mutant)/CyO; TM2/TM6B*). The 3rd chromosome balancers (*TM2* and *TM6B*) have a recessive marker *ebony* (*e* - black body color). Cross these 3 males with 3–6 virgin female flies having balancers and makers on the 2nd chromosome as well as *UAS-Cas9.P2* on the 3rd chromosome (*y, w; sp, if/CyO; UAS-Cas9.P2*).

8. Collect 3 male and 3–6 virgin female progeny with *CyO* and *TM6B*, excluding *sp,if* and *TM2* and cross to establish the line (*y,w; UAS-dOgdh (WT or mutant)/CyO; UAS-Cas9.P2/TM6B*).

Note: The male and female genotypes in step 6 can be switched and still produce the same results.

Note: Once a line is established, it is possible that after several generations, balancers such as *CyO* and *TM6B* will no longer be present in every fly as they become homozygous for the inserted transgenes.

Note: We also generated a control fly line carrying *UAS-empty; UAS-Cas9.P2* to equalize the number of UAS in flies.
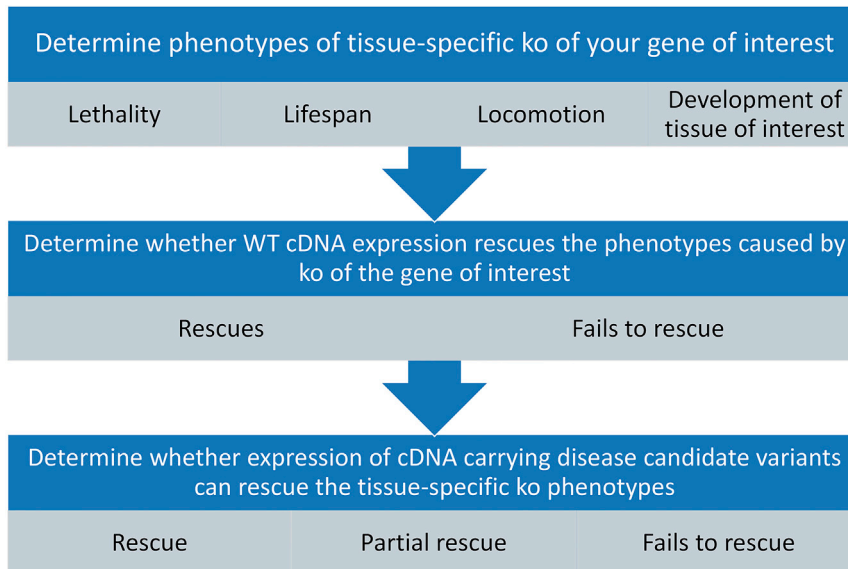
Figure 12. A schematic of functional studies of disease candidate variants in Drosophila

⏸ **Pause point:** Once a line is established, it can be maintained indefinitely.

Functional studies of disease candidate variants in *Drosophila*

⏱ **Timing: 1 month**

To perform the functional studies of disease candidate variants from patients, conduct the following experiments. First, determine phenotypes of tissue-specific ko of your gene of interest. Second, determine whether WT cDNA expression rescues the phenotypes caused by ko of your gene of interest. Third, determine whether the expression of cDNA carrying disease candidate variants can rescue or fails to rescue the tissue-specific ko phenotypes (Figure 12). The genomic DNA analysis by Inference of CRISPR Edits (ICE) will show the efficiency of ko in the samples (described in the next section – ICE analysis). As an example, here, we describe how to perform functional analysis for disease candidate variants in *OGDHL* by using neuronal-specific *dOgdh* ko flies.

9. In order to determine phenotypes of tissue-specific loss of your gene, cross flies carrying *tissue-specific-Gal4; dU6:3sgRNA*[gene of interest] with flies carrying *UAS-empty; UAS-Cas9.P2/TM6B*. By counting flies with and without balancers (e.g., *TM6B*), you could determine if the loss of your gene in given tissues leads to lethality. For example,
   a. Cross virgin female with elav[C155]-Gal4; dU6:3sgRNA[dOgdh] with males carrying UAS-empty; UAS-Cas9.P2/TM6B.
   b. After 10–14 days of incubation at a 25°C, progenies will begin to arrive. Count and sort the progenies according to the presence of balancers (e.g., TM6B).
   c. From the numbers of flies with or without balancers, determine whether progenies are born as Mendelian ratio or not. For example, we found that no flies with neuronal ko of dOgdh can reach to the adult stage as all flies have the TM6B balancer. Careful examination revealed that neuronal loss of dOgdh results in developmental lethality at 3rd instar larval and pupal stages (elav[C155]-Gal4; dU6:3sgRNA[dOgdh] / UAS-empty; UAS-Cas9.P2/+) (Yap et al., 2021).

Note: If tissue-specific ko of your gene does not cause lethality, examine and find phenotypes associated with loss of your gene by performing additional assays such as life-span, climbing, or flight assays.

10. To determine whether wild-type (WT) cDNA expression rescues the phenotype caused by tissue-specific loss of your gene,
    a. Cross flies carrying UAS- WT cDNA; UAS-Cas9.P2 with flies having tissue-specific-Gal4; dU6:3sgRNA$^{gene\ of\ interest}$.
    b. After 10–14 days of incubation at a 25°C fly incubator, progenies will begin to arrive. Count and sort the progenies according to the presence of balancers (e.g., TM6B).
    c. From the numbers of flies with or without balancers, determine whether progenies are born as Mendelian ratio or not.

Note: Complete rescue of the phenotypes by expressing WT cDNA indicates that the phenotype is caused by the loss of your gene, and the off-target effects of sgRNA/Cas9 are negligible. For example, *dOgdh (WT)* expression fully rescued the developmental lethality caused by neuronal loss of *dOgdh* (*elav^{C155}-Gal4; dU6:3sgRNA^{dOgdh} / UAS-empty; UAS-Cas9.P2/ UAS-dOgdh (WT)*) (Yap et al., 2021).

Note: If tissue-specific loss of your gene leads to phenotypes in the later life of flies (e.g., neuro-degeneration in photoreceptor neurons), examine the phenotype in appropriate time points.

11. To determine whether disease-associated mutations affect gene function *in vivo*,
    a. Cross flies carrying UAS-(mutant) cDNA; UAS-Cas9.P2 with flies having tissue-specific-Gal4; dU6:3sgRNA$^{gene\ of\ interest}$.
    b. After 10–14 days of incubation at a 25°C fly incubator, progenies will begin to arrive. Count and sort the progenies according to the presence of balancers (e.g., TM6B).
    c. From the numbers of flies with or without balancers, determine whether progenies are born as Mendelian ratio or not.

Note: Results will enable you to determine whether tested mutations affect gene function or not. If expression of mutant cDNA fails to rescue the phenotypes caused by loss of your gene, we can conclude that the mutation is a severe loss of function allele. Partial rescue indicates that mutants are benign alleles, and complete rescue indicates that mutants do not affect gene function (Figure 9).

ICE analysis

⊙ **Timing: 1 week**

This section explains how to use Inference of CRISPR Edits (ICE) analysis on *in vivo* samples to test the efficiency of the CRISPR-Cas9-mediated ko of the genomic locus of your gene of interest. Here we provide detailed protocols of ICE analysis for *dOgdh* case as an example. The protocol includes how to use ICE analysis to determine the ko efficiency of *dOgdh* as well as determine whether the sgRNA/ Cas9-RNP targets *UAS-dOgdh* cDNA transgene or not.

12. Collect the progeny from the lethality assay as well as control flies:

Fly genotype	Experimental group	Description
elav^{C155}-Gal4; dU6:3-gRNA^{dOgdh} / UAS-empty	Control	**NO** gene editing
elav^{C155}-Gal4; dU6:3-gRNA^{dOgdh} / UAS-dOgdh(WT)-Flag; UAS-Cas9.P2 /+	Wild-type rescue (Experiment)	ko for *dOgdh* by gRNA^{dOgdh}/ Cas9.P2 & rescue by *dOgdh (WT)* cDNA expression
elav^{C155}-Gal4; dU6:3-gRNA^{dOgdh} / UAS-dOgdh(P867A)-Flag; UAS-Cas9.P2 /+	Viable mutant flies (Experiment)	ko for *dOgdh* by gRNA^{dOgdh}/ Cas9.P2 & rescue by *dOgdh* (mutant) cDNA expression

Note: Collected F1 flies carrying wild-type or mutant cDNA will be the experiment, and flies carrying *UAS-empty(VK37)* and no *UAS-Cas9.P2* will serve as the control.

13. Chop and collect 10–20 heads of the flies, keeping the collected sample on ice.

⏸ **Pause point:** Fly heads can be stored in −30°C for 3 months.

14. Purify genomic DNA from the fly heads using PureLink Genomic DNA Mini Kit (Invitrogen, Cat# K1820-02) according to the manufacturer's instructions.

⏸ **Pause point:** Purified genomic DNA can be stored in −30°C.

15. Set up the following reaction for genomic PCR on ice or in a cooler (−20°C).

PCR reaction master mix	
Reagent	Amount
Genomic DNA	2 μL
OneTaq Hot Start 2× Master Mix with Standard Buffer (NEB)	25 μL
Primer forward (10 μM)	1 μL
Primer reverse (10 μM)	1 μL
Nuclease-free water (Invitrogen)	21 μL

Note: The One*Taq* Hot Start 2× Master Mix and PCR primers should be stored at −30°C to −20°C.

a. Set the PCR cycling conditions as follows.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	33 cycles
Annealing	56°C	30 s	
Extension	68°C	1 min	
Final extension	68°C	5 min	1
Hold	12°C	Forever	

Note: The genomic PCR reaction will need to be completed for two different sets of primers for each sample. One set is for targeting the genomic locus and the second set is for targeting the *UAS-cDNA* transgene locus. For example, the sgRNA/Cas9-tageting site of the *dOgdh* in the genome was amplified by a primer pair (dOgdh-gF-1 and dOgdh-gR-1, Table 1). Amplification for *UAS-dOgdh cDNA* transgenes in the genome was done by a primer pair (pUAST-F2 and dOgdh-cDNA-R1, Table 1).

16. Run the PCR products in 1% agarose gel to confirm the bands are the correct size.
17. Purify the PCR product with QIAquick PCR Purification Kit (QIAGEN, Cat# 28106) following the manufacturer's instructions.

Alternatives: You can also use the QIAquick Gel Extraction Kit (QIAGEN, Cat# 28706) if there are any non-specific bands.
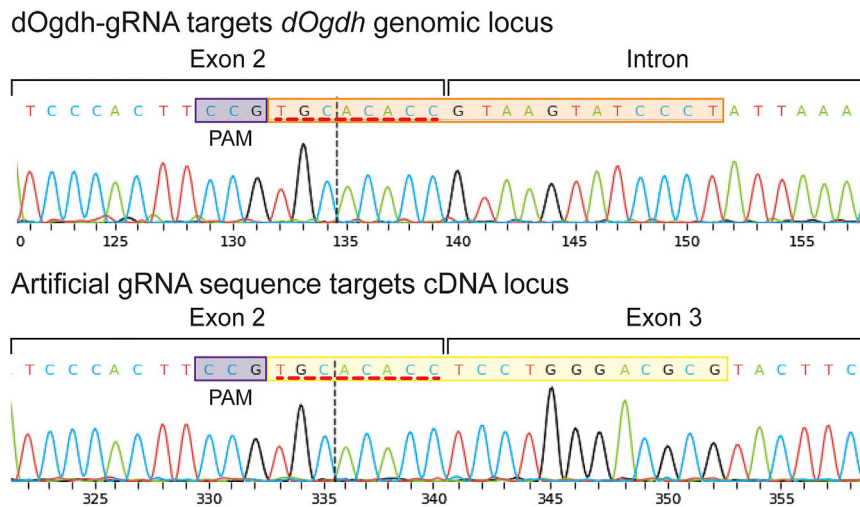
Figure 13. Sanger sequencing chromatograms for the junction of Exon2 (coding exon) and intron of *dOgdh* and *UAS-dOgdh* (WT) cDNA transgene

The orange box of the top image depicts the sequence of the exon-intron junction in the *dOgdh* genome that is targeted by the gRNA^dOgdh. The yellow box of the bottom image shows the sequence of the *UAS-dOgdh* cDNA transgene that the artificial gRNA targets. The purple boxes indicate the PAM sequence. Note that the last 8 nucleotides of the artificial gRNA (red dashed lines) are the same as the last 8 nucleotides of the gRNA. The two gRNAs differ in the first 12 nucleotides.

18. Send the purified PCR products to a sequencing service for Sanger sequencing.
19. Fill in the blanks from Synthego website as described below to complete ICE analysis for the genomic locus (Synthego Performance Analysis, ICE Analysis. 2019. V2.0. Synthego; [9.22.2021].).
    a. Write the sample name in the 'Label' and gRNA sequence in the 'Guide Sequences.' (Figure 14).
        i. To verify that the sgRNA/Cas9-RNP complex targets at the genomic locus, use the gRNA that you designed in "generation of DNA construct carrying sgRNA that targets gene of interest'', excluding the PAM sequence. For example, we used the sequence derived from dOgdh-gRNA-F (step 2 in before you begin) (Figure 13).
        ii. To perform ICE analysis for the *UAS-cDNA* locus that is not targeted by the sgRNA/Cas9-RNP complex, use an artificial gRNA sequence that targets the cDNA. For example, we used an artificial gRNA that is reverse complementary to the end of Exon 2 and beginning of Exon 3 (yellow box in Figure 13).
    b. Skip 'Donor Sequence (optional).'

Note: You do not need to include the donor sequence for ko analysis. You will need to provide donor sequences for gene edition by exogenous templates.

    c. Upload an .abi file of Sanger sequencing results of control flies to 'Control File,' and upload an .abi file of Sanger sequencing results of WT rescue or viable mutants to the 'Experiment File' (Figure 14).
    d. Click 'Add Sample to Analysis' (red arrow in Figure 14). Repeat steps a – d for each sample that you want to analyze. Once you have added all of the samples, click 'Analyze.'
    e. Then the web will show your results, including: ko score, indel %, R^2 value, the relative contribution of each sequence, indel plot, discordance plot, and the trace for the experimental and control samples (Figure 15). To determine if the ICE program was able to analyze your data, look at the status on your results page. There are three different levels of status: green, yellow, and red. A green means that there were no problems. Yellow indicates that the ICE

Figure 14. Snapshot of the ICE analysis webpage from Synthego

program was able to analyze the data, but it sensed issues of quality of Sanger sequencing. Lastly, the red status means that the Sanger sequences were poor quality and that the program could not analyze the data.

i. Indel % or ICE score refers to the overall editing efficiency of the sgRNA/Cas9-RNP. This value indicates the percent of the sample with insertions or deletions (indels) that make the sequence different from the wild-type. This information describes how well the Cas9 can cleave the target site. An increase in ICE score indicates that more cells are targeted by the CRISPR/Cas9 complex; however, it doesn't tell us if these indels result in the ko of the protein.

ii. The R^2 value measures how well the dependent variable's variation fits the independent variable's linear regression model. A higher R^2 value signifies that you can be more confident in the ICE score.

iii. The ko score refers to the proportion of sequences edited by the sgRNA/Cas9-RNP complex resulting in the protein ko. The program determines if the protein has been knocked out by detecting if the sequence has a frameshift-inducing indel or 21 bp or longer indel in the protein-coding region.

iv. The relative contribution of each sequence also shows the percent of the different indel sizes present in your edited sequences. However, it also displays the inferred sequence for each indel size when lined up with the control sequence (Figure 15A).

v. The indel plot uses a bar graph to illustrate the percent of the edited sequences with a specific indel size (Figure 15B).

Figure 15. Snapshot of the ICE analysis results for *dOgdh* ko from the Synthego webpage
(A) Contributions include Status, Indel %, Model Fit (R^2), Knockout-Score, and relative contribution of each sequence from ICE analysis for *dOgdh* ko sample.
(B) Indel distribution includes a histogram illustrating the percent of edited sequences and a discordance plot.
(C) Traces show Sanger chromatograms for control and experimental samples.

    vi. The discordance plot shows how much the edited samples deviate from the control sample by measuring the discordance at each base pair. The black dashed line indicates where the Cas9 is expected to cut (Figure 15B).

    vii. The trace plot gives a Sanger sequencing chromatogram of your control and experimental samples. The black dashed line on the plot distinguishes where the Cas9 is expected to cut, so by looking at the peaks before and after this dashed line you can determine the success of the editing. For the genomic locus that the sgRNA/Cas9-RNP targets, the peaks after the cut should decrease in height (Figure 15C).

  f. Click 'Download Analysis Data' (red arrow in Figure 15A) to receive your analysis through email.

EXPECTED OUTCOMES

We anticipate that the method described here will lead to efficient tissue-specific ko, as well as a cDNA-mediated rescue in the same tissue. We also expect that some mutations (variants) identified from human patients lead to failure of the rescue of ko of your gene of interest, and the other mutations may partially rescue the ko phenotypes. In the study by Yap et al. (Yap et al., 2021), of 9 *OGDHL* recessive variants identified from individuals with neurological features, we found that four missense variants completely failed to rescue the lethality caused by neuronal ko of *dOgdh*, indicating that these variants are severe loss of function alleles. In contrast, the expression of five missense variants rescued the lethality, but mutant adult flies exhibit behavioral defects, suggesting that those variants are partial loss of function mutations (Yap et al., 2021).

From the ICE analysis, we expect to see that the sgRNA/Cas9-RNP complex can target the genomic locus, effectively knocking out the desired gene while the complex fails to target cDNA. This should be true for both the WT and mutant cDNA. The ko efficiencies of the sgRNA/Cas9-RNP complex at each loci will be measured by the ICE analysis based on the Sanger sequencing chromatogram for the experimental and control samples. The ICE analysis results from the study by Yap et al. (Yap et al., 2021) revealed that the sgRNA[dOgdh]/Cas9-RNP complex leads to ko at the *dOgdh* genomic locus (ko score 21, Table A in Figure 16). Note that the genomic PCRs were performed from whole fly heads. As fly heads contain many other tissues and cells including muscles and glial cells, we expect significant fractions of neurons are subjected to *dOgdh* ko. The Sanger sequencing chromatogram for the genomic *dOgdh* locus displays that the nucleotide peaks are much shorter and have a lot of noise peaks starting from the nucleotide that the sgRNA/Cas9-RNP complex is expected to cut (second chromatogram in Figure 16). In contrast, when we analyze the ko efficiency at the *UAS-dOgdh* cDNA transgene, the ko-score is zero (Table A in Figure 16), and the Sanger sequencing chromatogram shows no change to the sequence at the expected cut point of the artificial sgRNA/Cas9-RNP complex (the third chromatogram in Figure 16). Therefore, the ICE analysis results demonstrate that the sgRNA[dOgdh]/Cas9-RNP complex was effective in knocking out the genomic *dOgdh*, but was unable to target the *dOgdh* cDNA.

LIMITATIONS

The primary advantage of this protocol is that the system permits cDNA expression for your gene of interest in tissues that are subjected to ko of the gene. However, the protocol has several limits. First, the protocol is designed with the pUAST vector (Brand and Perrimon, 1993), which enables efficient expression of transgenes in the somatic cells, but not in the female germline cells; thus, the expression of cDNA from the pUAST vector would not be sufficient for rescue when using germline Gal4 drivers. Hence, to express your gene in the germline cells, you need to use the pUASp vector with a germline-compatible promoter derived from the *P*-element (Rorth, 1998). Enhanced germline expression from UAST was shown in *Hsp70* Δ mutant flies because *Hsp 70 piRNAs* repress *UAST*

A Table of ICE results for *UAS-dOgdh(WT)* rescue of neuronal *dOgdh* ko

Label	ICE	KO - Score	KI - Score	ICE d	R Squared	Mean Discord Before	Mean Discord After	Guide Sequences
ko in the *dOgdh* genomic locus	27	21	None	42	0.95	0.175	0.341	AGGGATACTT ACGGTGTGCA
UAS-dOgdh cDNA transgene	0	0	None	1	1	0.079	0.064	CGCGTCCCAG GAGGTGTGCA

B Sanger sequencing chromatograms from ICE analysis

Figure 16. ICE analysis results show that sgRNA-Cas9 complex targets the genomic *dOgdh* locus but has no effect on *UAS-dOgdh* cDNA

(A) Table of the scores received after doing ICE analysis on the genomic PCR from fly heads carrying *elav[C155]-Gal4; U6:3-gRNA[dOgdh] / UAS-dOgdh(WT); UAS-Cas9.P2* using Synthego's website. The first row describes results using primers to target the genomic *dOgdh* locus and the second row indicates results from primers amplifying the wild-type *dOgdh* cDNA transgene.

(B) Sanger sequencing chromatograms of the genomic PCR indicate that the sgRNA-Cas9 complex targets at the *dOgdh* genomic locus, but does not target the *UAS-dOgdh(WT)* cDNA. The first chromatogram was obtained from flies with *elav[C155]-Gal4; U6:3-gRNA[dOgdh] / UAS-empty(VK37)*, but without *UAS-Cas9.P2*. The second and third chromatograms were obtained from flies that have *elav[C155]-Gal4; U6:3-gRNA[dOgdh] / UAS-dOgdh(WT); UAS-Cas9.P2*. The purple boxes indicate the PAM sequence of the guide RNAs, and the yellow boxes indicate the target site of the guide RNA. The black dashed lines show the location where sgRNA/Cas9-RNP complex cuts.

(DeLuca and Spradling, 2018). Hence, you can use *UAST* for germline expression in the *Hsp70 Δ* mutant background. Hsp70 Δ mutant flies have no significant defects in viability or egg production in the absence of heat shock (Gong and Golic, 2004). Alternatively, you would choose pUASz vector that shows superior expression in the germline to those of pUASp, and comparable expression in the somatic cells to those from UAST (DeLuca and Spradling, 2018). Second, given that the protocol is designed to express a single isoform of cDNA, the rescue may not occur in the case that the selected tissues need the expression of more than one isoform of a gene.

TROUBLESHOOTING
Problem 1
Your agarose gel for mutagenesis PCR reveals there are non-specific bands or not the expected size of band. (Related to steps 18 and 31 in before you begin).

Potential solution

If you do not have a band, then you can repeat the PCR, following the reaction master mix in step 31 but changing the cycling conditions to optimize the reaction.

Optimized PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	5 cycles
Annealing	55°C	30 s	
Extension	72°C	4 min	
Second Denaturation	98°C	30 s	22 cycles
Second Annealing	53°C	30 s	
Second Extension	72°C	4 min	
Final extension	72°C	7 min	1
Hold	12°C	Forever	

However, if there are non-specific bands in your agarose gel, then you can add 1%–3% DMSO to your reaction master mix, while still using the original PCR cycling conditions in step 31.

PCR reaction master mix with DMSO

Reagent	Amount
DNA template (100–200 ng) / μL	1 μL
Taq (iProof)	1 μL
Primer Forward (10 μM)	1 μL
Primer Reverse (10 μM)	1 μL
dNTP (10 mM)	1 μL
5 × iProof HF buffer	10 μL
ddH$_2$O	33.5 μL
DMSO	1.5 μL

Problem 2

There are not enough males or virgin females to make a cross. This problem may be encountered in step-by-step method details steps 1–8.

Potential solution

It is best practice to make multiple replicates of the same cross in order to ensure there will be enough progeny to make the next cross.

Problem 3

How do you determine the difference between embryonic lethality and a failed gRNA targeting? What if progeny production is reduced or absent due to gene KO? This problem is related to step 9 in the step-by-step method.

Potential solution

To determine whether gRNA/Cas9-mediated gene editing causes embryonic lethality or defective growth, performed lethal staging of the flies with gRNA/Cas9. Instead of using flies having balancer (i.e., *UAS-empty; UAS-Cas9.P2/TM6B* in step 9 in the step-by-step method), use flies homozygous for all transgenes. Using grape (or apple) juice plates, perform lethal staging for the flies with a tissue-specific ko of your gene. For example, we did a cross for ~50 female virgin flies with *elav^{C155}-Gal4; dU6:3sgRNA^{dOgdh}* with ~20 males carrying *UAS-empty; UAS-Cas9.P2*. For the control group, we made a cross for ~50 female virgin flies with *elav^{C155}-Gal4; dU6:3sgRNA^{dOgdh}* and ~20 males having *UAS-empty* only. The flies were incubated with grape juice plates for ~12–24 h at 25°C. The collected embryos with the neuronal ko for *dOgdh* (*elav^{C155}-Gal4/+ or Y;*

dU6:3sgRNA^dOgdh^/UAS-empty; UAS-Cas9.P2/+) exhibited lethality at the 3rd instar larvae and pupa stages, whereas the control flies showed normal growth up to the adult stage.

Problem 4

The status of the ICE program will show a yellow or red rating if the quality of the Sanger sequencing is poor with high background peaks. Genomic PCR with under-optimized conditions could lead to poor quality Sanger chromatograms, which the ICE cannot analyze. This problem is related to steps 15–18 in the step-by-step method.

Potential solution

Mixed peaks in the Sanger sequencing are usually due to the PCR reaction's non-specific amplification of the genomic DNA. To fix this, perform the PCR using DMSO. DMSO prevents non-specific binding between primers and DNA. Therefore, the following master mix will reduce the non-specific amplification during the PCR reaction. Follow the genomic PCR reaction master mix with DMSO below, and use the same PCR cycling conditions in step 15a. Alternatively, you can optimize the PCR condition by increasing annealing temperature of the PCR cycle.

> *Note:* DMSO can be 1%–5%. The master mix below includes 3% DMSO, which we found the optimal percentage for our PCR reaction. You can optimize DMSO concentration.

Genomic PCR reaction master mix with DMSO	
Reagent	Amount
Genomic DNA	2 μL
One*Taq* Hot Start 2× Master Mix with Standard Buffer (NEB)	25 μL
Primer forward (10 μM)	1 μL
Primer reverse (10 μM)	1 μL
Nuclease-free water (Invitrogen)	19.5 μL
DMSO	1.5 μL

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wan Hee Yoon (wanhee-yoon@omrf.org).

Materials availability

Plasmids and *Drosophila* lines will be available upon request.

Data and code availability

This study did not generate new datasets or codes.

AUTHOR CONTRIBUTIONS

M.C. and K.V.P. carried out the experiments and wrote the manuscript. A.S. and J.R. prepared tables and provided technical help. W.H.Y. conceived of the approach and experimental design, secured funding, and wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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