Protocol

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

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.
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\textsuperscript{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 OxoGlutrate 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.
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 doOgdh (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/doOgdh*), 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).
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' end 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).

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**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.

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**Figure 3. Snapshot of the Synthego Knockout Guide Design webpage**
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 (10x) | 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 Bbsl restriction enzyme (RE) reaction in a microcentrifuge tube:

| Bbsl RE Reaction | Reagent                        | Amount |
|------------------|--------------------------------|--------|
| pCFD3.1-w-dU6:3gRNA (1 µg/µL) | 2 µL                      |
| NEB Buffer r2.1 (10x)    | 5 µL                        |
| Bbsl              | 2 µL                        |
| Nuclease-free water  | 41 µL                       |
| **Total**         | **50 µL**                   |

Note: The Bbsl 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 Bbsl-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:
**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 (10x)                               | 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.

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**Figure 4. Snapshot of the results of gRNAs search for dOgdh/Nc73EF from the Synthego Knockout Guide Design tool**
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.
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 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.coli 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, y1 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 pUASTtattB-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 pUASTtattB vector.
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).

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 | Amount |
|-------------------------|--------|
| Reagent | 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 x iProof HF buffer | 10 µL |
| | Nuclease-free water | 35 µL |
| | Total | 50 µL |

Note: The iProof Taq, 5 x 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 | Temperature | Time | Cycles |
|------------------------|-------------|------|--------|
| Initial Denaturation   | 98°C        | 3 min| 1      |
| Denaturation           | 98°C        | 30 s | 30 cycles |

(Continued on next page)
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.

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 BclII 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 (10x)     | 5 µL   |
| BglII                     | 1 µL   |
| XbaI                      | 1 µL   |
| Nuclease-free water       | 41 µL  |
| Total                     | 50 µL  |

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        |      |        |

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.

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 BclII 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
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                          | 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                          | 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).
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.

| Oligo Name         | Sequence                                    |
|--------------------|---------------------------------------------|
| 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).

   | 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 x iProof HF buffer          | 10 µL  |
   | Nuclease-free water           | 35 µL  |
   | Total                         | 50 µL  |

   **Note:** The iProof Taq, 5 x 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.

   | 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.

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

   **Note:** The DpnI enzyme and 10 x 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).
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 x iProof HF Buffer | Bio-Rad                  | Cat# 1725391 |

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## Protocol

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Nuclease-free water (H₂O) | 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 (10X) | New England Biolabs | Cat# B0202S |
| NEBuffer Set (r1, r2, r3, and rCutSmart) | New England Biolabs | Cat# B7030S |
| BclI | New England Biolabs | Cat# R0160S |
| XbaI | New England Biolabs | Cat# R0145S |
| NolI | New England Biolabs | Cat# R0189S |
| EcoRI | New England Biolabs | Cat# R0101S |
| BgIII | New England Biolabs | Cat# R0144S |
| OneTaq Hot Start 2X 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# 53014-5KG |
| BD Bacto Agar Solidifying Agent | VWR | Cat# 90000-760 |
| Ampicillin sodium salt | Sigma-Aldrich | Cat# P0756S |
| Red Star Active Yeast | Genesee | Cat# 62-103 |
| Agarose LE, Molecular Biology Grade | Denville Scientific Inc. | Cat# GR1140-500 |
| Ethanol, Pure + Denatured 200 Proof | Decon Labs Inc. | Cat# 2701 |
| Molasses Unshaphured 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 (MAI) | 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
- Zippy 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<sup>C115-Gal4</sup>
  - BDSC RIRD:BDSC_458 BDSC# 458
- Drosophila melanogaster: UAS-Cas9 P2
  - BDSC RIRD:BDSC_60786 BDSC# 60786
- Drosophila melanogaster: UAS-empty (v437)
  - From Hugo Bellen lab N/A
- Drosophila melanogaster: y[1] w[1+P] y+t7.7=mos-phiC31\int.NLS]X; P (y+t7.7)=CaryPattP40
  - BDSC RIRD:BDSC_79604 BDSC# 79604
- Drosophila melanogaster: y[1] w[1118]; PBac[y[+] attrP-38]Vr00037
  - BDSC RIRD:BDSC_9752 BDSC# 9752

### Oligonucleotides

- See Table 1 for Oligonucleotides: This paper N/A

### Recombinant DNA

- pUASTatatb-dOgdh DNA template: Yoon et al., (2017), Yap et al., (2021) N/A
- pCFD3.1-w-dU6.3gRNA: Addgene, gift from Simon Bullock RIRD: 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/#/

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MATERIALS AND EQUIPMENT

Fly food preparation

| Material                          | 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.

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.

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**STE-P-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 (elavC155-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<sup>dOgdh</sup> and neuronal Gal4 driver**

© Timing: 6 weeks

To establish a Drosophila line with a neuronal Gal4 driver on the 1<sup>st</sup> chromosome and sgRNA<sup>dOgdh</sup> on the 2<sup>nd</sup> 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<sup>dOgdh</sup>) with 3–6 virgin female flies having a neuronal Gal4 driver as well as a balancer and a maker on the 2<sup>nd</sup> chromosome (elav<sup>C155</sup>-Gal4; Bl<sup>1</sup>/CyO, Tub>Gal80). Two crosses will provide enough progenies for downstream experiments.

2. Select 3 male progeny with CyO, Tub>Gal80, making sure to exclude those with Bl<sup>1</sup>. Cross these males (elav<sup>C155</sup>-Gal4/Y chromosome; U6:3-sgRNA<sup>dOgdh</sup>/CyO, Tub>Gal80) with 3–6 virgin female flies having a neuronal Gal4 driver and 2<sup>nd</sup> chromosome balancer and maker (elav<sup>C155</sup>-Gal4; Bl<sup>1</sup>/CyO, Tub>Gal80).

3. Select 3 male and 3–6 virgin female progenies with CyO, Tub>Gal80, making sure to exclude those with Bl<sup>1</sup>. Make a cross to produce a genetic line containing CyO, Tub>Gal4 and sgRNA<sup>dOgdh</sup> on the 2<sup>nd</sup> chromosome (elav<sup>C155</sup>-Gal4; U6:3-sgRNA<sup>dOgdh</sup>/CyO, Tub>Gal80).

4. Select and cross 3 male and 3–6 virgin female progenies that are homozygous for sgRNA<sup>dOgdh</sup> on the 2<sup>nd</sup> chromosome (elav<sup>C155</sup>-Gal4; U6:3-sgRNA<sup>dOgdh</sup>/CyO, Tub>Gal80).
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<sup>C155</sup>-Gal4; *B<sup>1</sup>* /CyO, *tub-Gal80*, and male flies of *yw/Y* chromosome; *dU6:3-sgRNA<sup>dOgdh</sup>* for the first cross. As *elav<sup>C155</sup>-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 2<sup>nd</sup> chromosome with 3–6 virgin female flies having balancers and makers in the 2<sup>nd</sup> and 3<sup>rd</sup> chromosomes (*yw; sp, il/CyO; TM2/TM6B*) (Figure 11). Two crosses will provide enough progenies for downstream experiments.
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; UAS-dOgdh(WT or mutant)/CyO; UAS-Cas9.P2). 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.
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: dU6:3sgRNAgene 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 elavC155-Gal4: dU6:3sgRNAdOgdh 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 (elavC155-Gal4; dU6:3sgRNAdOgdh / 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 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 (elavC155-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., neurodegeneration 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 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                                                                 |
|---------------------------------------------------|--------------------|-----------------------------------------------------------------------------|
| elavC155-Gal4; dU6:3-gRNA/dOgdh / UAS-empty       | Control            | NO gene editing                                                            |
| elavC155-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 |
| elavC155-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 OneTaq 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.

| 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-targeting 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.
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 %, R² 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
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 R² value measures how well the dependent variable’s variation fits the independent variable’s linear regression model. A higher R² 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 14. Snapshot of the ICE analysis webpage from Synthego
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 F-element (Rorth, 1998). Enhanced germline expression from UAST was shown in Hsp70 Δ mutant flies because Hsp 70 piRNAs repress UAST.
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 x iProof HF buffer                 | 10 μL  |
| ddH2O                                | 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\textsuperscript{C155}\textsuperscript{-Gal4}; dU6:3sgRNA\textsuperscript{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\textsuperscript{C155}\textsuperscript{-Gal4}; dU6:3sgRNA\textsuperscript{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\textsuperscript{C155}\textsuperscript{-Gal4/+ or Y;
dU6:3sgRNA\textsuperscript{dC\textsubscript{49h}/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   |
| 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)                  | 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.

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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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