**Bachelor Thesis**

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# Creating Conditional CRISPR Lines for Prominent Learning Mutants in *Drosophila melanogaster*

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# Abstract

Investigating learning and memory mutants in *Drosophila melanogaster* emphasizes the importance of genetic influences on brain functions, particularly considering observed performance variations in operant self-learning experiments with *rutabaga* and *radish* mutants (Brembs and Plendl, 2008; Brembs and Sun, 2017), possibly indicating an adaptation to their learning disabilities. To understand the interplay of mutations and developmental processes, generating flies receptive to conditional mutagenesis of those genes with precise tools like CRISPR/Cas9 is crucial for comparative experiments. This project aimed to create conditional CRISPR lines targeting the genes *rsh* and *rut*, while also verifying the presence of the mutations $rsh^{1}$ and $rut^{2080}$ in existing fly stocks for data comparability. Therefore, we utilized a system of multiple CRISPR gRNAs flanked by tRNAs cloned into a vector capable of integrating into the genome to regulate the targeted gene disruption (Port and Bullock, 2016). We confirmed the presence of the $rsh^{1}$ mutation. However, challenges such as the creation of plasmid constructs require further attention due to time constraints.

# Zusammenfassung

Die Forschung an *Drosophila melanogaster* Lern- und Gedächtnismutanten betont die Bedeutung genetischer Einflüsse auf Gehirnfunktionen, insbesondere unter Berücksichtigung beobachteter Leistungsvariationen in operanten Selbstlernexperimenten mit *rutabaga* und *radish* Mutanten (Brembs and Plendl, 2008; Brembs and Sun, 2017), was möglicherweise auf eine Anpassung an ihre Lernbehinderung hindeutet. Um das Zusammenspiel von Mutationen und Entwicklungsprozessen zu verstehen, ist die Erzeugung von Fliegen, die empfänglich für bedingte Mutagenese dieser Gene sind, mithilfe von präzisen Werkzeugen wie CRISPR/Cas9 entscheidend für vergleichende Experimente. Das Ziel dieses Projekts war das Erschaffen konditionaler CRISPR-Linien, welche die Gene *rsh* und *rut* betreffen und gleichzeitig die Anwesenheit der Mutationen $rsh^{1}$ und $rut^{2080}$ in vorhandenen Fliegenstämmen zur Datenvergleichbarkeit zu überprüfen. Daher haben wir ein System von mehreren CRISPR gRNAs flankiert von tRNAs verwendet, welches in einen Vektor kloniert wurde, der in der Lage ist, in das Genom zu integrieren, um die gezielte Genzerstörung regulieren zu können (Port und Bullock, 2016). Wir haben die Anwesenheit der $rsh^{1}$ Mutation bestätigt. Herausforderungen wie die Erstellung von Plasmidkonstrukten erfordern jedoch weitere Aufmerksamkeit aufgrund von Zeitbeschränkungen.

# 1. Introduction

Extensive research is devoted to the study of learning and memory mutants in *Drosophila melanogaster*. Understanding the genetic basis of learning and memory is essential to elucidate the principles underlying these processes. This requires precise genetic tools for targeted modulation of specific genes. Establishing a reliable system to introduce conditional gene disruption of these leaning mutants at any life stage of *Drosophila* will provide valuable insights into the complexities arising from both developmental processes and the mutations themselves. This is where a groundbreaking method, for which Jennifer A. Doudna and Emmanuelle Charpentier were awarded the 2020 Nobel Prize, comes into play. They found that utilizing a specific arrangement of clustered regularly interspaced palindromic repeats (CRISPR) alongside an enzyme called Cas9, which is derived from the bacterial immune system, enabled them to develop a powerful genetic tool for precise DNA editing (Doudna and Charpentier, 2014).

In 2016, Port and Bullock adapted this method for use in *Drosophila*. Their approach is based on releasing multiple CRISPR sgRNAs from a single precursor transcript using flanking tRNAs positioned between the sgRNAs. The endogenous tRNA processing machinery then separates the functional gRNAs from the tRNAs. These separated gRNAs cooperating with the Cas9 enzyme identify the specific sequence in the *Drosophila* genome and induce targeted mutagenesis. By utilizing the plasmid pCFD6 as vector for the transcript, they enabled its integration into the genome of recombinant fly embryos containing an integrase and attP sites (Groth et al., 2000), facilitated by the presence of attB sites in pCFD6. Additionally, the tRNA-gRNA transcript inserted into the pCFD6 vector is under the control of a UAS promoter, a crucial element of the GAL4/UAS system (Brand and Perrimon, 1993). This system enables the creation of conditional CRISPR lines by crossbreeding transgenic flies with the GAL4 driver line, allowing tissue specific gene disruption to be induced at any time. Therefore, the system provided by

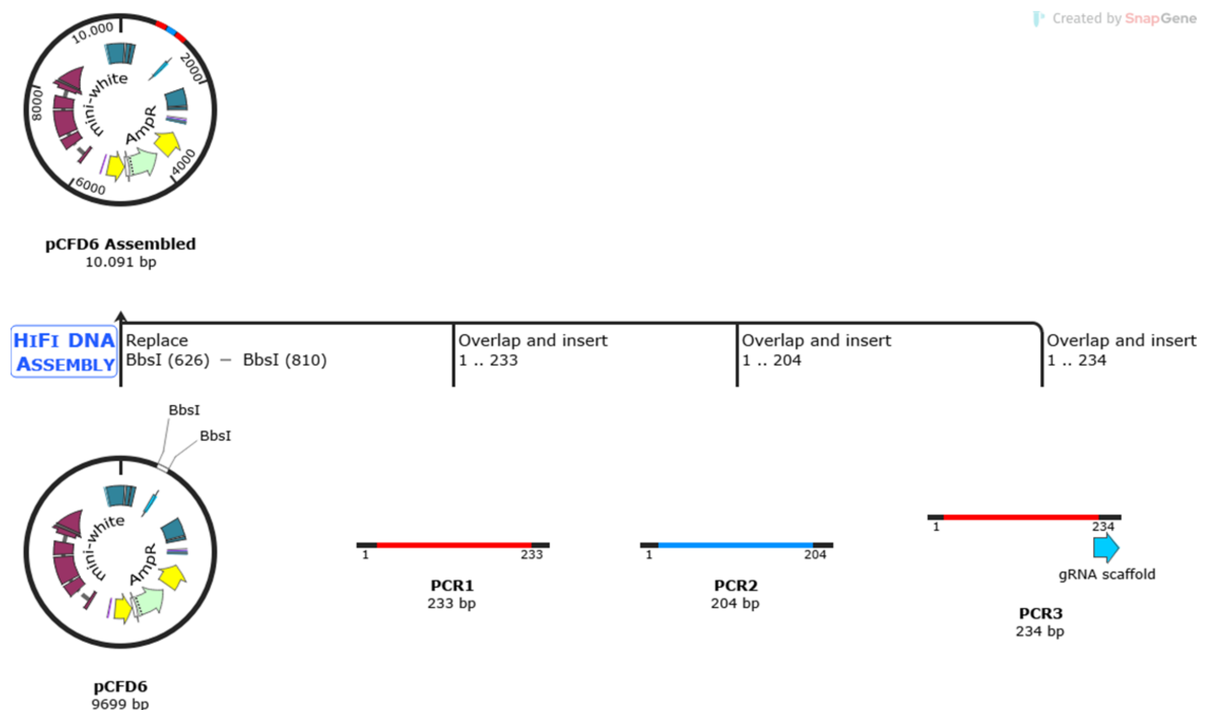


**Figure 1: DNA Assembly of pCFD6 BbsI and three Inserts.** PCR1, PCR2 and PCR3 contain gRNAs targeting the desired gene.

Port and Bullock at www.crisprflydesign.org for generating the desired plasmid construct targeting specific genes was used for this project (**Figure 1**). Following their protocol, numerous scientists have successfully cloned up to four gRNAs into the pCFD6 vector, including Rose et al. (2022), Rallis et al. (2020), Delventhal et al. (2019) and Sheng et al. (2022).

Mutations affecting the genes *rutabaga* (*rut*) and *radish* (*rsh*) are well-known for their impact on reduced learning abilities. In particular, the $rut^{2080}$ and $rsh^{1}$ mutations have long been known to have this effect (Tully and Quinn, 1985; Zars et al., 2000; Folkers et al., 1993; Tully et al., 1994). However, there are studies suggesting that these mutants show enhanced learning compared to wild-type flies in certain experiments focused on operant self-learning (Brembs and Plendl, 2008; Brembs and Sun, 2017). This phenomenon may stem from their experience of growing up with their learning disability resulting from the mutations and adapting to it. Even so, it may be entirely attributed to the mutation itself, regardless of developmental effects. Therefore, it is imperative to generate flies capable of undergoing tissue specific mutagenesis targeting these genes at any time. Furthermore, conducting identical experiments focusing on operant self-learning with these CRISPR mutants and comparing the results with those of the mutants of $rsh^{1}$ and $rut^{2080}$ is essential to gain comprehensive understanding.

The main objective of this project was to generate conditional CRISPR lines targeting the genes *rsh* and *rut*, respectively. Additionally, verifying the presence of the $rsh^{1}$ and $rut^{2080}$ mutations in existing fly stocks was another goal to ensure comparable data.

# 2. Materials

## 2.1. Experimental Models and Corresponding Medium

Table 1: Experimental models.

| Organism/Strain | Genotype | Description | Company |
|---|---|---|---|
| ***E. coli:* DH5α competent** | F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 ($r_K^-$, $m_K^+$) *pho*A *sup*E44 λ⁻*thi*-1 *gyr*A96 *rel*A1 | Heat shock competent *E. coli* for transformation | Thermo Fisher Scientific Invitrogen |
| ***E. coli:* NEB® 5-alpha competent (High Efficiency)** | *fhu*A2 Δ(*arg*F-*lac*Z)U169 *pho*A *gln*V44 Φ80Δ(*lac*Z)M15 *gyr*A96 *rec*A1 *rel*A1 *end*A1 *thi*-1 *hsd*R17 | Heat shock competent *E. coli* for transformation | New England Biolabs GmbH |
| ***D. melanogaster*: *rsh*$^{1}$** | Nucleotide substitution: C→T | gDNA analysis | Laboratory of Thomas Preat, Mines ParisTech |
| ***D. melanogaster*: *rut*$^{2080}$** | P{1ArB}*rut*$^{2080}$ | gDNA analysis | Laboratory of Thomas Preat, Mines ParisTech |

Table 2: Medium for bacteria.

| Medium | Application | Composition | Company |
|---|---|---|---|
| **$LB_{Amp}$ medium** | Liquid culture for *E. coli* growth with ampicillin resistance | $LB_0$ medium with 100 µg/ml ampicillin | This project |
| **$LB_{Amp}$ plates** | Culture plates with ampicillin for | $LB_{Amp}$ medium with 1.5 % Bacto Agar | Lab-intern production |

| Medium | Application | Composition | Company |
|---|---|---|---|
| | selection of ampicillin resistant *E. coli* | | |
| **Lysogeny Broth medium (LB0)** | Liquid culture for *E. coli* | 0.5% Yeast extract<br>1.0% Tryptone<br>1.0% NaCl<br>0.3% 1N NaOH<br>pH 7, autoclaved | Lab-intern production |
| **SOC outgrowth medium** | Outgrowth medium for freshly transformed *E. coli* | 2.0% Vegetable Peptone<br>0.5% Yeast extract<br>10 mM NaCl<br>2.5 mM KCl<br>10 mM MgCl2<br>10 mM MgSO4<br>20 mM Glucose | New England Biolabs GmbH |

## 2.2.Plasmids, Primers and Oligonucleotides

Table 3: Plasmids used for cloning.

| Plasmid | Size | Resistance | Application | Company |
|---|---|---|---|---|
| **pCFD6** | 9,699 bp | Ampicillin, 100 µg/ml | Expression of multiple gRNAs under Gal4/UAS system | Gift from Simon Bullock (Addgene plasmid #73915) |

Table 4: Primers and oligonucleotides for cloning.

| Primer | 5’ to 3’ Sequence | Application |
|---|---|---|
| **pcr1 rsh fwd** | CGG CCC GGG TTC GAT TCC CGG CCG ATG CAG AGC ACG AGG ACA TTC GGG CGT TTC AGA GCT ATG CTG GAA AC | Creating gRNAs |
| **pcr1 rsh rev** | ATT TGG AGC GCG AGT GCA GCT GCA CCA GCC GGG AAT CGA ACC | Creating gRNAs |
| **pcr2 rsh fwd** | GCT GCA CTC GCG CTC CAA ATG TTT CAG AGC TAT GCT GGA AAC | Creating gRNAs |

| Primer | 5' to 3' Sequence | Application |
|---|---|---|
| **pcr2 rsh rev** | TGC TGG TCC CAC TGC TCC TCT GCA CCA GCC GGG AAT CGA ACC | Creating gRNAs |
| **pcr3 rsh fwd** | GAG GAG CAG TGG GAC CAG CAG TTT CAG AGC TAT GCT GGA AAC | Creating gRNAs |
| **pcr3 rsh rev** | ATT TTA ACT TGC TAT TTC TAG CTC TAA AAC TGC ACC GTG TAG GAA CCA GCT GCA CCA GCC GGG AAT CGA ACC | Creating gRNAs |
| **pcr1 rut fwd** | CGG CCC GGG TTC GAT TCC CGG CCG ATG CAG CTG CAG TGT TCC GTG ATC AGT TTC AGA GCT ATG CTG GAA AC | Creating gRNAs |
| **pcr1 rut rev** | AAT GTG GGC ATC GAC ACC ACT GCA CCA GCC GGG AAT CGA ACC | Creating gRNAs |
| **pcr2 rut fwd** | GTG GTG TCG ATG CCC ACA TTG TTT CAG AGC TAT GCT GGA AAC | Creating gRNAs |
| **pcr2 rut rev** | CGC TTA CGC CAT GAT GCC GCT GCA CCA GCC GGG AAT CGA ACC | Creating gRNAs |
| **pcr3 rut fwd** | GCG GCA TCA TGG CGT AAG CGG TTT CAG AGC TAT GCT GGA AAC | Creating gRNAs |
| **pcr3 rut rev** | ATT TTA ACT TGC TAT TTCTAG CTC TAA AAC GCC ACC GGC GAC AGG ATG TCT GCA CCA GCC GGG AAT CGA ACC | Creating gRNAs |
| **pcfd6 colony new fwd** | AGA GCA TCA GTT GTG AAT GAA | Colony PCR |
| **pcfd6 colony rev** | TTA GAG CTT TAA ATCTCT GTA GGT AG | Colony PCR |

Table 5: Primers and oligonucleotides for gDNA analysis.

| Primer | 5' to 3' Sequence | Application |
|---|---|---|
| **rsh fwd** | CAC CGA GGA GAT ACT GAT CGC | gDNA analysis of *rsh*$^1$ fly stock |
| **rsh rev** | CTG CCA CGA TAA CTG GAA GTA CA | gDNA analysis of *rsh*$^1$ fly stock |

| Primer | 5' to 3' Sequence | Application |
|---|---|---|
| **rut2080 geno fwd** | ACA GTT AAG ATC GCC GCG TTA | gDNA analysis of *rut*$^{2080}$ fly stock |
| **rut2080 geno rev** | GCT GCA AGG CGA TTA AGT TGG | gDNA analysis of *rut*$^{2080}$ fly stock |

## 2.3. Chemicals, Enzymes and Commercial Kits

Table 6: Buffers, solutions and chemicals.

| Substance | Application | Company | Composition |
|---|---|---|---|
| **Agarose, universal** | Agarose gel electrophoresis | VWR International, LLC. | / |
| **Ampicillin** | $LB_{Amp}$ medium | Lab-intern production | 100 mg/ml |
| **Antarctic Phosphatase Reaction Buffer (10x)** | Dephosphorylation of DNA 5'- and 3'-ends | New England Biolabs GmbH | 10x |
| **DNA Ladder 100bp** | Agarose gel electrophoresis | New England Biolabs GmbH | 500 µg/ml |
| **DNA Ladder 1kb** | Agarose gel electrophoresis | New England Biolabs GmbH | 500 µg/ml |
| **dNTPs** | PCR | Lab-intern production | 10 mM |
| **Gel Red 30x** | Agarose gel electrophoresis | Lab-intern production | 30x |
| **Gibson Assembly Master Mix** | DNA assembly cloning | New England Biolabs GmbH | 2x |
| **Loading Dye Purple 6x** | Agarose gel electrophoresis | New England Biolabs GmbH | 6x |
| **NEBuilder HiFi DNA Assembly Master Mix** | DNA assembly cloning | New England Biolabs GmbH | 2x |
| **NEBuilder Positive Control** | DNA assembly cloning | New England Biolabs GmbH | / |
| **PBS (1X)** | gDNA isolation | Lab-intern production | 1x |

| Substance | Application | Company | Composition |
|---|---|---|---|
| **Phusion HF Reaction Buffer** | PCR using Phusion DNA polymerase | New England Biolabs GmbH | 5x |
| **Q5 High GC Enhancer** | PCR using Q5 DNA polymerase | New England Biolabs GmbH | 5x |
| **Q5 Reaction Buffer** | PCR using Q5 DNA polymerase | New England Biolabs GmbH | 5x |
| **rCutSmart Buffer** | Restriction digestion and dephosphorylation | New England Biolabs GmbH | 10x |
| **TAE 1x** | Agarose gel electrophoresis | Lab-intern production | 1x |

Table 7: Enzymes.

| Enzyme | Application | Composition | Company |
|---|---|---|---|
| **Antarctic Phosphatase** | Dephosphorylation of DNA 5'- and 3'-ends | 5,000 U/ml | New England Biolabs GmbH |
| **BbsI-HF restriction enzyme** | Restriction digest | 20,000 U/ml | New England Biolabs GmbH |
| **FastAP Thermosensitive Alkaline Phosphatase** | Dephosphorylation of DNA 5'- and 3'-ends | 1 U/µl | Thermo Scientific |
| **HindIII-HF restriction enzyme** | Restriction digest | 20,000 U/ml | New England Biolabs GmbH |
| **NheI-HF restriction enzyme** | Restriction digest | 20,000 U/ml | New England Biolabs GmbH |
| **Phusion DNA Polymerase** | PCR | 2,000 U/ml | New England Biolabs GmbH |
| **Q5 High-Fidelity DNA Polymerase** | PCR | 2,000 U/ml | New England Biolabs GmbH |
| **Quick CIP** | Dephosphorylation of DNA 5'- and 3'-ends | 5,000 U/ml | New England Biolabs GmbH |

| Enzyme | Application | Composition | Company |
|---|---|---|---|
| **RNase** | gDNA isolation | 12.5 µg/ml | Lab-intern production |
| ***Taq* DNA Polymerase** | cPCR | 1,250 U/ml | Lab-intern production |

Table 8: Commercial kits.

| Kit | Application | Company |
|---|---|---|
| **E.Z.N.A. Gel Extraction Kit** | DNA extraction/purification | Omega Bio-tek, Inc. |
| **NucleoSpin Plasmid Mini Kit** | Plasmid DNA purification | MACHEREY-NAGEL GmbH & Co.KG |
| **QIAamp DNA Micro Kit (50)** | Isolation of genomic DNA | QIAGEN |

## 2.4.Laboratory Materials

Table 9: Laboratory materials.

| Material | Application | Company |
|---|---|---|
| **Eppendorf tubes 1,5 ml/2 ml** | Miscellaneous | Eppendorf Corporate |
| **Falcon tubes 15ml/50ml** | Miscellaneous | Sarstedt |
| **PCR tubes** | Miscellaneous | Kisker Biotech GmbH & Co. KG |
| **Pipette tips 1000 µl/200 µl/10 µl** | Miscellaneous | Sarstedt |
| **QIAamp MinElute Column** | Isolation of gDNA | QIAGEN |

## 2.5. Instruments, Appliances and Software

Table 10: Instruments and appliances.

| Instrument/Appliance | Application | Company |
|---|---|---|
| **Biometra TOne 96, 230 V Thermocycler** | PCR and incubation | Analytik Jena GmbH+Co. KG |
| **E-Box VXS** | Gel documentation | VWR PEQLAB |
| **Gel Electrophoresis System PerfectBlue** | Horizontal gel electrophoresis | VWR PEQLAB |
| **Mini-Centrifuge** | Miscellaneous | Sunlab Instruments |
| **NanoDrop 1000 Spectrophotometer** | Quantifying DNA in solution | Thermo Fisher Scientific Inc. |
| **Pico 17 Microcentrifuge** | Miscellaneous | Thermo Fisher Scientific Inc. |
| **Pipettes** | Miscellaneous | Gilson Inc. |
| **Thermomixer Compact** | Incubation | Eppendorf Corporate |
| **Vortex Mixer VV3** | Miscellaneous | VWR |

Table 11: Software.

| Software | Application | Provider | Version |
|---|---|---|---|
| **ChatGPT** | Paraphrasing during writing process | OpenAI | August 3 and September 25 |
| **Flybase** | Resource for genetic and genomic data of *Drosophila melanogaster* | Flybase | FB2023_03 |
| **Microsoft 365 (Office)** | Writing and image processing | Microsoft Corporation | 18.2306.1061.0 |
| **NEB TM Calculator** | Estimating optimal annealing temperature for PCR with NEB polymerases | New England BioLabs Inc. | v1.16.5 |

| Software | Application | Provider | Version |
| --- | --- | --- | --- |
| **NEBioCalculator®** | Calculating required amount of insert for DNA assembly reaction | New England Biolabs GmbH | v1.15.4 |
| **SnapGene** | Visualizing reactions and figures | Dotmatics | 7.0 |

# 3. Methods

## 3.1.Gel Electrophoresis and DNA Extraction

Gel electrophoresis separates DNA molecules by length of base pairs, enabling analysis and purification of specific DNA fragments. In this project, gel electrophoresis was performed using 0.8% agarose gel and 1 kb DNA Ladder or 1% agarose gel and 100 bp DNA Ladder depending on the size of DNA fragments. The voltage ranged from 75 to 120V. To achieve the desired concentration of agarose gel, 100 ml of 1x TAE was boiled along with either 0.8 g or 1.0 g of agarose powder. For every DNA sample, Gel Red Loading Dye 5x was used in a 1x concentration. 5x concentration was prepared by combining 500 µl Loading Dye 6x with 100 µl Gel Red 30x. After the gel run, the DNA fragments were visualized by long-wavelength UV light using the E-Box VXS for analysis.

To obtain purified DNA of interest, the specific fragments were extracted from agarose gel slices using the E.Z.N.A. Gel Extraction kit – Spin Protocol according to manufacturer's guidelines. Final step was to measure the concentration of eluted DNA using the NanoDrop Spectrophotometer.

## 3.2. DNA Sequencing by Sanger

To determine if the plasmid construct had the correct inserts in correct order, or if amplicons of $rsh^{1}$ or $rut^{2080}$ contained the desired mutation, DNA sequencing by Sanger was used. Samples were prepared (see Table 12) with either forward or reverse primers. The choice of primer was based on the project. For plasmid samples, either pcfd6 colony new fwd or rev was utilized, while for gDNA samples, the choice was between rsh fwd or rev, and rut2080 geno fwd or rev depending on the gene. The samples were sent to Eurofins Genomics LLC for fast DNA sequencing.

Table 12: Preparation for sequencing by Sanger.

| Component | Plasmid Sample | gDNA Sample |
|---|---|---|
| **DNA** | 500 ng | 10 ng |
| **10 µM forward or reverse primer** | 2.5 µl | 2.5 µl |
| **Nuclease-free water** | ad 7.5 µl | ad 7.5 µl |

## 3.3. Gradient PCR

Determining the ideal annealing temperature through gradient PCR is crucial for better PCR outcomes in some cases. The reaction set up and program are identical to qualitative PCR. The only difference is the variable annealing temperatures applied to each reaction tube (see Table 13).

Table 13: Varying temperatures for gradient PCR.

| Reaction | ***rsh*[1] Amplicon** | ***rut*[2080] Amplicon** |
|---|---|---|
| **1** | 58.0°C | 58.0°C |
| **2** | 60.6°C | 59.5°C |
| **3** | 63.9°C | 60.6°C |
| **4** | 65.4°C | 63.9°C |
| **5** | 66.5°C | 66.5°C |
| **6** | 68.0°C | 68.0°C |

## 3.4. Generation of Tissue Specific CRISPR Lines

### 3.4.1. Creating gRNAs

To generate the intended gRNAs for cloning, specifically designed primer pairs (pcr1rsh fwd and rev, pcr2 rsh fwd and rev, pcr3 rsh fwd and rev; pcr1 rut fwd and rev, pcr2 rut fwd and rev, pcr3 rut fwd and rev) were used to amplify the gRNAs targeting the genes *rsh* and *rut* by PCR. 640 pg undigested pCFD6 was used as template DNA per 50 µl reaction. For every primer pair, PCR was prepared on ice employing Q5 High-Fidelity DNA Polymerase (see Table 14) and an increment program with an annealing temperature increasing by 0.5°C per cycle (see Table 15) was run in Biometra TOne Thermocycler. Modifications for this procedure are documented in Table 16.

To purify PCR products, gel electrophoresis was performed on a 1% agarose gel at 120V followed by gel extraction and concentration measurement (see 3.1. Gel Electrophoresis and DNA Extraction).

Table 14: PCR for gRNA generation using Q5 High-Fidelity DNA Polymerase.

| Component | 50 µl Reaction | Final Concentration |
|---|---|---|
| **5x Q5 Reaction Buffer** | 10 µl | 1X |
| **10 mM dNTPs** | 1.0 µl | 200 µM |
| **10 µM forward primer** | 2.5 µl | 0.5 µM |
| **10 µM reverse primer** | 2.5 µl | 0.5 µM |
| **Template DNA: pCFD6** | 1.0 µl | 640 pg |
| **Q5 High-Fidelity DNA Polymerase** | 0.5 µl | 1.0 unit |
| **Nuclease-free water** | ad 50 µl | |

Table 15: PCR increment program for gRNA generation. 56°C ΔT: Starting at 56°C, annealing temperature increases 0.5°C per cycle until 72°C is reached.

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| **1. Initial Denaturation** | 98°C | 2 min | |
| **2. Denaturation** | 98°C | 10 sec | 32x |
| **3. Annealing** | 56°C ΔT | 15 sec | |
| **4. Extension** | 72°C | 15 sec | |
| **5. Final Extension** | 72°C | 2 min | |
| **6. Hold** | 10°C | ∞ | |

Table 16: Modifications in gRNAs generation.

| Modification | Amount | Results No. |
|---|---|---|
| **Template DNA pCFD6** | ⅒ of 640 pg/µl | 1.1 |
| **Q5 High GC Enhancer 5x** | 10 µl added (1x) to reaction | 1.2 |
| **Template DNA pCFD6** | ⅕ of 640 pg/µl for PCR1 *rsh* and PCR1 *rut* | 1.3 |
| **DNA polymerase** | Phusion DNA Polymerase (see Attachments) | 1.4 |
| **Template DNA pCFD6** | pCFD6 digested with restriction enzymes HindIII and NheI as template DNA (see Attachments) | 1.5 |

| Modification | Amount | Results No. |
|---|---|---|
| **Gel extraction** | Cut off as much gel as possible and second wash step with SPW buffer to reduce guanidine thiocynate, which can reduce efficiency of assembly reaction | 1.6 |

3.4.2. Restriction Digest of pCFD6

For cloning, it was necessary to digest plasmid pCFD6 with BbsI-HF Type IIS restriction enzyme. Additionally, the 5'- and 3'-ends of the DNA had to be dephosphorylated to prevent religation of the empty plasmid. This was executed in two different ways. Modifications for both procedures are documented in Table 20.

The initial approach was utilizing restriction enzyme BbsI and phosphatase Quick CIP in a single reaction (see Table 17). The mixture was incubated at 37°C for at least 3 h, with the phosphatase being added one hour after the start of incubation. To inactivate enzymes and isolate pCFD6 backbone, the reaction was run on a 0.8% agarose gel at 100V, extracted and the DNA concentration of pCFD6 BbsI AP was quantified (see 3.1. Gel electrophoresis and DNA Extraction).

The second method was to implement a two-step preparation with separate restriction digest and dephosphorylation. Restriction digest was prepared in a higher volume (see Table 18) and incubated at 37°C for 3 h. The digested DNA was separated on a 0.8% agarose gel at 100 V, extracted and measured (see 3.1. Gel Electrophoresis and DNA Extraction). Dephosphorylation was set up with Fast Antarctic Phosphatase (FastAP) (see Table 19) and E.Z.N.A. Gel Extraction Kit – Enzymatic Reaction Protocol was utilized according to manufacturer's guidelines and resulting concentration was assessed with NanoDrop Spectrophotometer.

Table 17: Restriction digest of pCFD6 with BbsI-HF restriction enzyme and Quick CIP.

| Component | 30 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6** | variable | 1 µg |
| **rCutSmart Buffer (10x)** | 3.0 µl | 1x |
| **BbsI-HF** | 1.0 µl | 20 units |
| **Quick CIP** | 1.0 µl | 5 units |
| **Nuclease-free water** | ad 30 µl | |

Table 18: Separate restriction digest reaction of pCFD6 with BbsI-HF restriction enzyme.

| Component | 80 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6** | variable | 8 µg |
| **rCutSmart Buffer (10x)** | 8.0 µl | 1x |
| **BbsI-HF** | 4.0 µl | 80 units |
| **Nuclease-free water** | ad 80 µl | |

Table 19: Dephosphorylation of pCFD6 BbsI with FastAP.

| Component | 35 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6 BbsI** | 30 µl | variable |
| **FastAP Buffer (10x)** | 3.5 µl | 1x |
| **FastAP** | 1.5 µl | 1.5 units |

Table 20: Modifications of restriction digest of pCFD6.

| Modification | Amount | Results No. |
|---|---|---|
| **BbsI and QuickCIP** | Higher amount of pCFD6: 8µg | 2.1. |
| **Antarctic Phosphatase** | See Attachments | 2.2 |
| **Two-step preparation** | Two agarose gels used for inactivation | 2.3 |

### 3.4.3. DNA Assembly Cloning

To obtain the desired DNA constructs, it is essential to assemble the vector pCFD6 BbsI AP with the three gRNA inserts targeting either *rsh* or *rut* in correct order. For efficiency purposes, fragments were designed with 20–25 bp overlaps. The required quantity of inserts when utilizing 100 ng of digested pCFD6 BbsI AP with a two-fold molar excess of each insert was calculated (see Table 21). DNA mix was prepared on ice for use with NEBuilder HiFi DNA Assembly Master Mix following manufacturer's guidelines. An extended incubation time of 60 minutes was implemented to improve efficiency, as recommended. NEBuilder Positive Control was incubated likewise to assess the quality of the master mix and effectiveness of the procedure.

Table 21: DNA mix used for assembly reaction with a 1:2 vector:insert ratio.

| Component | bp | pmol | ng |
|---|---|---|---|
| **pCFD6 BbsI AP** | 9,600 | 0.016 | 100 |
| **Fragment PCR1** | 233 | 0.032 | 4.85 |
| **Fragment PCR2** | 204 | 0.032 | 4.24 |
| **Fragment PCR3** | 234 | 0.032 | 4.87 |
| **Nuclease-free water** | | | ad 10 µl |

For heat shock transformation, we followed the Chemically Competent Cells Transformation Protocol from NEBuilder HiFi DNA Assembly Cloning Kit to a certain extent. 10 µl of assembly reaction was mixed to 100 µl of DH5α competent *E. coli.* Heat shock was extended to 40 seconds. 900 µl of room tempered SOC medium was added and incubated at 500 rpm in a Thermomixer. 125 µl of cells were spread onto pre-warmed $LB_{Amp}$ selection plates. Remaining solution was centrifuged at 5,000 *g* for 2 minutes to remove most of supernatant. The cell pellet was resuspended in remaining supernatant and spread onto $LB_{Amp}$ plates. Same process was executed for both the NEBuilder Positive Control and the negative control. The latter control was performed to monitor number of background colonies, which contained re-ligated vector. For that, the same amount of digested pCFD6 BbsI AP was filled with nuclease-free water to 20 µl and 10 µl of it was transformed to 100 µl cells likewise. All $LB_{Amp}$ plates were incubated at 37°C for approximately 16-18 h overnight. Modifications for the complete procedure are documented in Table 22.

Table 22: Modifications of DNA assembly cloning.

| Modification | Amount | Results No. |
|---|---|---|
| **Rotation during incubation** | 300-700 rpm | 4.1 |
| **Competent *E. coli*** | NEB 5-alpha competent (High Efficiency) | 4.2 |
| **Assembly mix** | Transformation of 5 µl | 4.3 |
| **DNA mix** | Transformation of DNA mix for background check | 4.4 |
| **DNA assembly cloning** | NEBuilder HiFi DNA Assembly Cloning Kit was completely followed. | 4.5 |

| Modification | Amount | Results No. |
|---|---|---|
| **Scanlan et al. (2022)** | 3:1 molar ratio and 4 h incubation | 4.6 |
| **Master mix** | Gibson Assembly Master Mix was used | 4.7 |
| **Cell check** | Non-transformed DH5α competent cells were plated | 4.8 |
| **Vector** | 50 ng of pCFD6 BbsI AP for DNA mix | 4.9 |
| **Transformation** | 5 µl assembly reaction to 70 µl DH5α competent *E. coli* | 4.10 |
| **Transformation** | 2 µl assembly reaction to 70 µl DH5α competent *E. coli* | 4.11 |

### 3.4.4. Colony PCR

To determine whether inserts targeting *rsh* or *rut* were present or absent in the plasmid construct of grown transformants, colony PCR (cPCR) was performed. Individual colonies were picked with a 10 µl pipette tip. Initially, they were spread onto a fresh $LB_{Amp}$ plate, and subsequently, they were added directly into the cPCR reaction prepared on ice (see Table 23). Samples were transferred to Biometra TOne and cPCR program was run (see Table 24). The released DNA construct serves as template for cPCR specific primers (pcfd6 colony new fwd and pcfd6 colony rev), which are designed to target vector DNA flanking insert area. Presence or absence is determined by molecular size of resulting PCR amplicon on an 1% agarose gel (see 3.1. Gel Electrophoresis and DNA Extraction).

To confirm the presence and correct order of inserts by utilizing Sanger DNA sequencing, colonies that seemed to carry the desired plasmid construct were inoculated from $LB_{Amp}$ plate into 3.0 ml $LB_{Amp}$ medium and kept at 37°C and 225 rpm for approximately 16 h. $LB_{Amp}$ medium was prepared by adding 50 µl ampicillin (100mg/ml) to 50 ml $LB_0$ under sterile conditions to achieve a final concentration of 100 µg/ml. Plasmid construct was miniprepped from liquid culture with NucleoSpin Plasmid Mini Kit according to manufacturer's guidelines. Concentration of received product was measured and prepared for DNA sequencing by Sanger (see 3.2.).

Table 23: cPCR of transformant colonies.

| Component | 20 µl Reaction | Final Concentration |
|---|---|---|
| **10x ThermoPol Reaction Buffer** | 2.0 µl | 1x |
| **10 mM dNTPs** | 0.4 µl | 200 µM |
| **10 µM forward primer** | 0.4 µl | 0.2 µM |
| **10 µM reverse primer** | 0.4 µl | 0.2 µM |
| ***Taq* DNA polymerase** | 0.4 µl | 0.5 units |
| **Nuclease-free water** | ad 20 µl | |

Table 24: PCR program for cPCR with *Taq* DNA polymerase.

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| **1. Initial Denaturation** | 95°C | 5 min | |
| **2. Denaturation** | 95°C | 20 sec | 32x |
| **3. Annealing** | 50°C | 40 sec | |
| **4. Extension** | 68°C | 1 min | |
| **5. Final Extension** | 68°C | 5 min | |
| **6. Hold** | 16°C | ∞ | |

## 3.5. Verifying Mutations *rsh*$^{1}$ and *rut*$^{2080}$ in Fly Stocks

### 3.5.1. Genomic DNA Isolation from Adult Flies

Five mutant flies of each sex were collected in a 1.5 ml cup while anaesthetized with carbon dioxide gas and immediately shock frosted with liquid nitrogen. 175 µl of 1x PBS and 20 µl of RNase were added and mixed by pipetting, to continue with manually homogenizing the flies using a sterile pestle on ice. For the following steps QIAamp DNA Micro Kit was utilized. 20 µl of Proteinase K was gently blended into the sample and left for 1 min at room temperature (RT). The mixture was lysed with 200 µl buffer ATL while incubating in a Thermomixer at 56°C for 10 min. After cooling down to RT, 200 µl of 100% ethanol was blended in for optimal binding of gDNA to the silica-gel membrane of a QIAamp MinElute Column, which had been stored at 4°C. Centrifugation at 6,000 *g* for 1 min removes unbound components. To purify the bound gDNA while removing unwanted components, two buffers are introduced successively,

first 500 µl of AW1 buffer, followed by 500 µl of AW2 buffer. Both solutions get centrifuged at 6,000 *g* for 1 min and each flowthrough is discarded. Centrifugation at 17,000 *g* for 3 min eliminates leftover ethanol, preventing interference with gDNA. The column is transferred to a new tube and 50 µl of AE buffer is left on the silica-gel membrane at RT for 35 min. Centrifuging at 6,000 *g* for 1 min releases the eluted gDNA from the membrane. 1µl of gDNA was run on a 0.8% agarose gel (see 3.1. Gel Electrophoresis and DNA Extraction) for quality assessment.

#### 3.5.2. Augmenting Gene Region of Supposed Mutation

To verify the mutation in the specific gene region utilizing Sanger sequencing, it was necessary to first generate the desired amplicon of mutated area via PCR. For that, the gDNA served as template and the reaction was set up on ice with specifically designed primer pairs (rsh fwd/rev or rut2080 geno fwd/rev) and Phusion DNA Polymerase (see Table 25). The annealing temperature employed in the PCR program on a Biometra TOne Thermocycler (see Table 26) was calculated using NEB TM calculator and further optimized through gradient PCR (see 3.3.). Elongation time was dependent on the size of amplicon and the chosen DNA polymerase with an estimated duration of 30 sec per 1 kb. PCR products were purified by running them on a 1% agarose gel at 100V followed by gel extraction. After measuring the achieved concentration (see 3.1. Gel Electrophoresis and DNA Extraction), the cleaned-up amplicon was DNA sequenced by Sanger (see 3.2.).

Table 25: gDNA PCR Reaction with Phusion DNA polymerase.

| Component | 50 µl Reaction | Final Concentration |
|---|---|---|
| **5x Phusion HF Reaction Buffer** | 10 µl | 1X |
| **10 mM dNTPs** | 1.0 µl | 200 µM |
| **10 µM forward primer** | 2.5 µl | 0.5 µM |
| **10 µM reverse primer** | 2.5 µl | 0.5 µM |
| **Template DNA: gDNA** | variable | 100 ng |
| **Phusion DNA Polymerase** | 0.5 µl | 0.02 U/µl |
| **Nuclease-free water** | ad 50 µl | |

Table 26: PCR program for gDNA. Extension time depends on amplicon size, for *rsh*[1] 15 sec and for *rut*[2080] 35sec.

| Step | Temperature | Time | Cycles |
|---|---|---|---|
| **1. Initial Denaturation** | 98°C | 5 min | |
| **2. Denaturation** | 98°C | 15 sec | 36x |
| **3. Annealing** | 65°C | 15 sec | |
| **4. Extension** | 72°C | 15\|35 sec | |
| **5. Final Extension** | 72°C | 2 min | |
| **6. Hold** | 16°C | ∞ | |

# 4. Results

## 4.1. Generation of Tissue Specific CRISPR Lines

Generating conditional CRISPR lines for the genes *rsh* and *rut* in *Drosophila melanogaster* requires plasmids containing CRISPR gRNAs capable of integrating into the fly genome. Subsequent crossbreeding with GAL4-Cas9 flies enables the CRISPR/Cas9 complex to act on the specific genes under the GAL4/UAS system. gRNAs were designed to target the individual splice forms of the genes *rsh* and *rut* prior to the functional protein domains enabling the

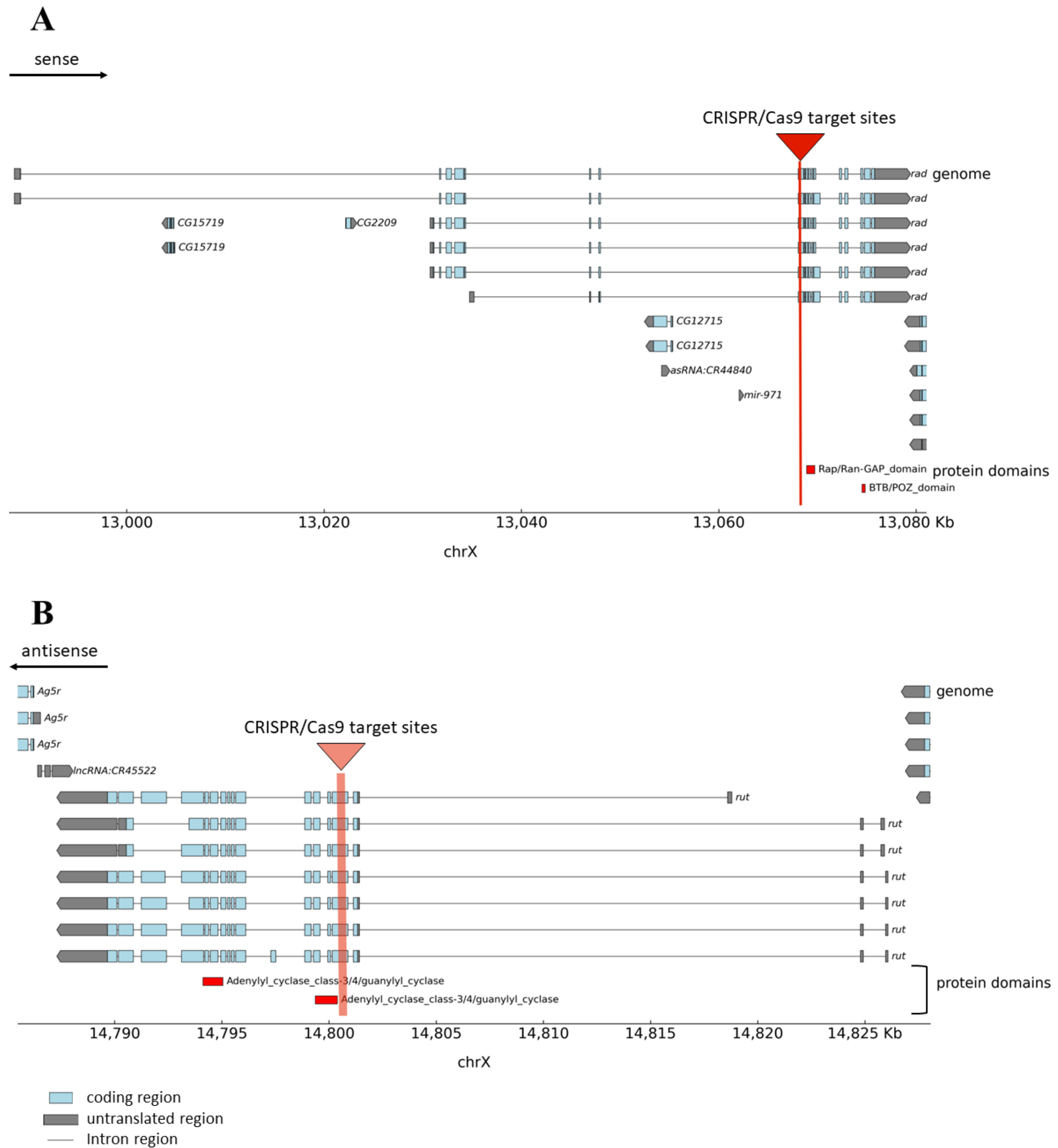


**Figure 2: Schematic representation of the CRISPR/Cas9 target sites. A, B,** CRISPR/Cas9 disrupts the individual splice forms of genes **(A)** *rsh* (=*rad*) and **(B)** *rut*, which results in non-functional protein domains.

CRISPR/Cas9 complex to disrupt the region and making them non-functional (**Figure 2**). These gRNAs generated by increment PCR were analyzed by gel electrophoresis and bands were observed at 233 bp for PCR1 *rsh*/*rut*, at 204 bp for PCR2 *rsh*/*rut* and at 234 bp for PCR3 *rsh*/*rut* as anticipated (**Figure 3**). BbsI-HF digested and dephosphorylated plasmid pCFD6 was analyzed by gel electrophoresis and bands were expected at approximately 9.6 kb and detected between 8.0 and 10.0 kb (**Figure 4**).

pCFD6 BbsI AP was assembled with products PCR1, PCR2 and PCR3 targeting either *rsh* or *rut* and heat-shock transformed into competent *E. coli*. Resulting colonies were examined on the $LB_{Amp}$ plates. As expected, positive control plates exhibit colony growth. Surprisingly, on negative control plates, which contain cells transformed with digested pCFD6, nearly the same number of colonies grew compared to the experimental plates. Colonies transformed with pCFD6 construct were analyzed via cPCR followed by gel electrophoresis (**Figure 5**). Control PCR of intact pCFD6 is meeting expectations of 623 bp. The amplified area of vector with all three inserts in correct order has an expected size of 1,015 bp. This was the estimated value for colony c5 (**Figure 5A**). Most bands of cPCR products are at approximately 300 bp. Constructs

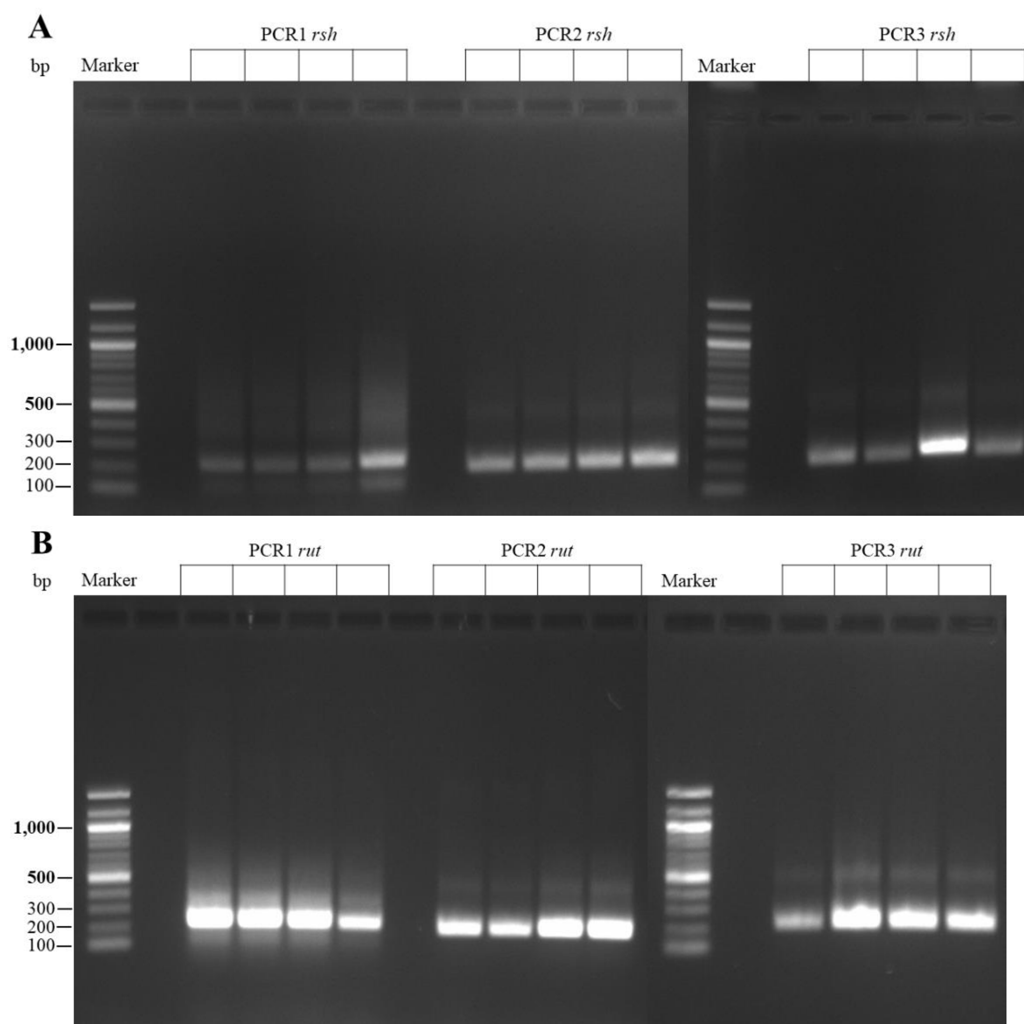


**Figure 3: Gel electrophoresis analysis of PCR products targeting either *rsh* or *rut*. A, B,** 1% agarose gel loaded with 100 bp DNA Ladder and PCR products amplified with different primer pairs: **(A)** PCR1 *rsh*: pcr1 rsh fwd/rev; PCR2 *rsh*: pcr2 rsh fwd/rev; PCR3 *rsh*: pcr3 rsh fwd/rev. **(B)** PCR1 *rut*: pcr1 rut fwd/rev; PCR2 *rut*: pcr2 rut fwd/rev; PCR3 *rut*: pcr3 rut fwd/rev.

with absence of all inserts are expected at 439 bp, which was observed for colony c8. A size of roughly 600bp was received for c10, which represents constructs including one insert. The band of colony c7 was at about 850 bp, as anticipated for vectors with two inserts (**Figure 5B**). Most promising findings of c5 for the presumed pCFD6-*rsh* construct and c7 for the putative pCFD6-*rut* were subjected to Sanger sequencing. The analysis revealed that the included inserts were either fragmented or disordered.

Modifications that resulted in no grown colonies were 2.1 and 2.2 for restriction digest (see Table 18), 4.4, 4.6 and 4.8 for DNA assembly cloning (see Table 20). Colonies without three inserts were obtained with modifications of gRNA creation: 1.1 to 1.6 (see Table 14), modifications of restriction digest: 2.3 (see Table 18), and modifications of DNA assembly cloning: 4.1 to 4.3, 4.5, 4.7, 4.9 to 4.11 (see Table 20). All modifications did not lead to any positive transformants. Therefore, the creation of pCFD6-*rsh* and pCFD6-*rut* constructs was not possible.

## 4.2. Verifying Mutations *rsh*$^{1}$ and *rut*$^{2080}$ in Fly Stocks

Verification of the mutants required extraction of gDNA from present fly stocks and subsequent amplification of mutation sites by PCR. Annealing temperature was calculated to be 65°C for *rsh*$^{1}$ and 60°C for *rut*$^{2080}$, respectively. Gradient PCR confirmed that the optimal temperature for *rsh*$^{1}$ was 65.4°C. For *rut*$^{2080}$, it was not possible to determine the required annealing temperature. GradPCR resulted in multiple bands, none of which matched the expected size of 810 bp (**Figure 6A**). Therefore, clearly verifying the *rut*$^{2080}$ mutation in present fly stocks was not applicable. Via PCR, an about 200bp large amplicon of *rsh*$^{1}$ mutation area was detected (**Figure 6B**) and Sanger sequenced, which displayed the desired point mutation of cytidine to thymidine. This finding confirms that present fly stocks of *rsh*$^{1}$ mutants still carry the relevant mutation.

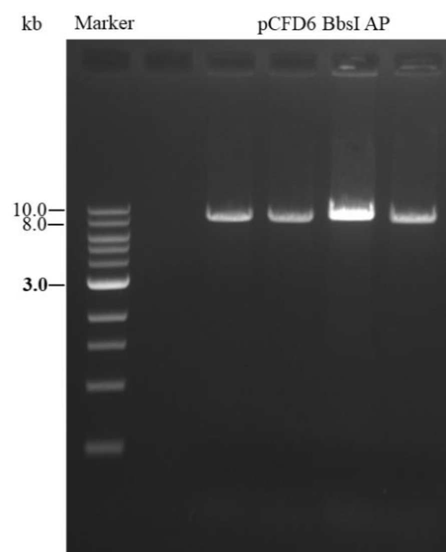


**Figure 4: Gel electrophoresis analysis of pCFD6 restriction digest.** 0.8% agarose gel loaded with 1 kb DNA Ladder and digested, dephosphorylated pCFD6 BbsI AP.

# 5. Discussion

## 5.1.Generation of Tissue Specific CRISPR Lines

To create CRISPR lines for the *rsh* and *rut* learning mutants, the generation of plasmid constructs containing gRNAs directed towards these specific genes was essential. Generating the inserts via PCR along with pCFD6 digestion was apparently successful according to gel electrophoresis analysis. Interestingly, negative control plates showed a remarkable number of colonies transformed with pCFD6 BbsI AP. Despite various approaches, DNA assembly of these components was not possible. Port and Bullock (2016) provided the system available at www.crisprflydesign.org utilized in this project to perform CRISPR-based gene disruption in *Drosophila*. They successfully created a plasmid construct containing two sgRNAs suitable for microinjecting into fly embryos. Although they used four gRNAs for their cloning, our efforts

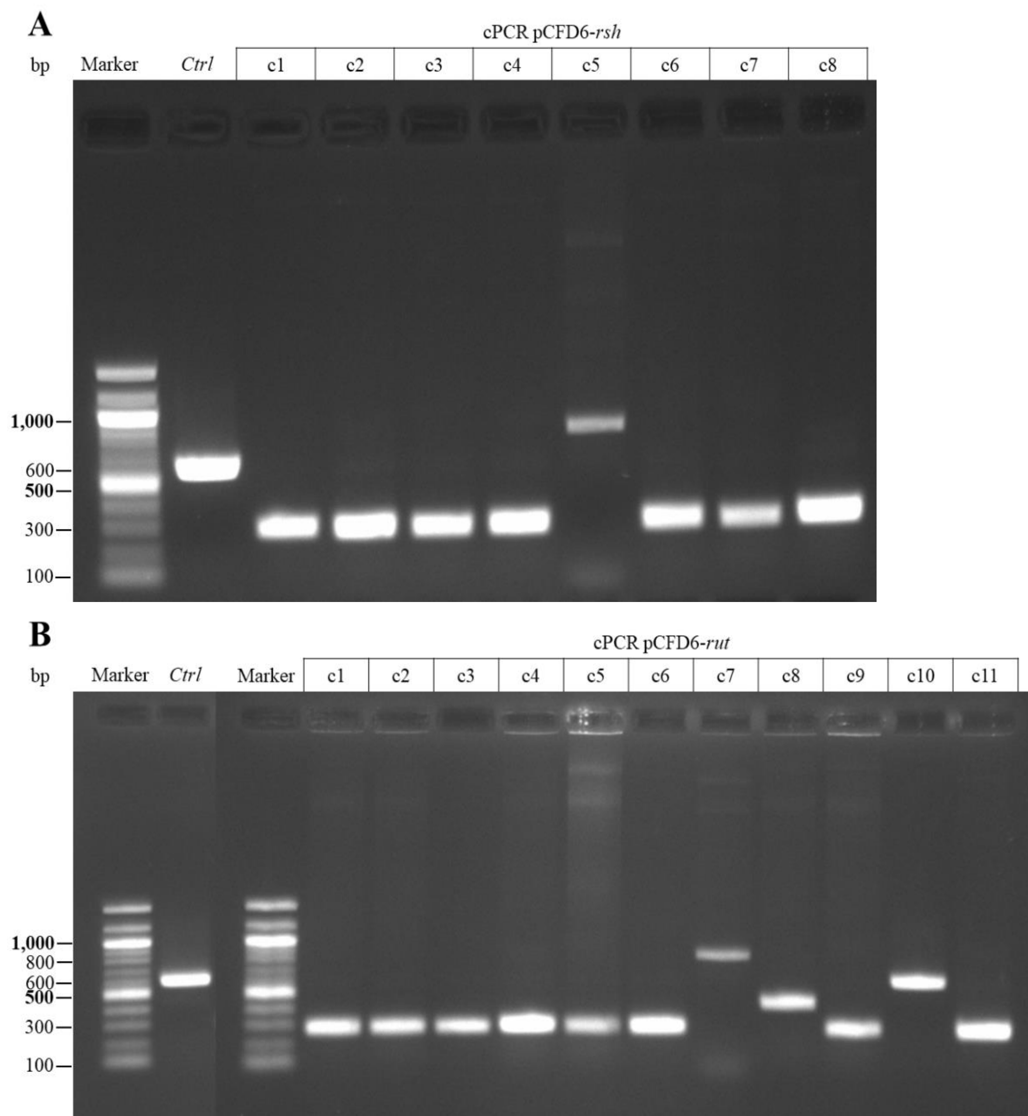


**Figure 5: Gel electrophoresis analysis of cPCR products. A, B,** 1% agarose gel loaded with 100 bp DNA Ladder, control PCR (ctrl) of intact pCFD6 and cPCR products of *E. coli* colonies transformed with DNA assembly mix containing either inserts **(A)** PCR1 *rsh*, PCR2 *rsh* and PCR3 *rsh* for the putative construct pCFD6-*rsh* or **(B)** PCR1 *rut*, PCR2 *rut* and PCR3 *rut* for the putative construct pCFD6-*rut*.

to replicate the successful DNA assembly achieved by Scanlan et al. (2022) using a 3:1 molar ratio and incubation time of 4 h for the DNA assembly were ineffective. These are surprising results that were beyond our initial expectations. Especially considering the numerous studies with successful results, such as Rose et al. (2022), Rallis et al. (2020) or Delventhal et al. (2019) cloning four gRNAs using three inserts and Sheng et al. (2022) cloning two gRNAs into the pCFD6 vector.

One plausible explanation for these differing results could be the possible re-ligation of digested pCFD6, despite the use of gel electrophoresis to isolate and purify the desired DNA fragment prior to further manipulations. This theory is supported by the number of colonies observed on the negative control plates and the count of cPCR amplicons that lacked any inserts. These results suggest potential problems with the restriction digest of pCFD6 or its re-ligation before or during DNA assembly. However, we cannot exclude the possibility that other procedural issues may also play an important role in interaction during DNA assembly. Further investigation and optimization of the assembly process and preceding steps are crucial to overcome these challenges. Interestingly, Port et al. (2020) generated the plasmid constructs for their recent cloning by subjecting all inserts to digestion with the appropriate restriction enzyme after PCR amplification and prior to the DNA assembly reaction. This may hold potential as a future strategy.

After successful construct generation, further steps would involve microinjecting constructs into fly embryos to obtain transformants with CRISPR-mediated gene mutations in either *rsh* or *rut*. These CRISPR lines are anticipated to provide deeper insights into experiments focusing on enhanced self-learning in flies with notable learning mutations, such as $rut^{2080}$ and $rsh^{1}$ (Brembs and Plendl, 2008; Brembs and Sun, 2017). The CRISPR-mediated disruption of these genes will help determining whether increased self-learning is developmental in flies born with mutations, or if this effect can be observed with the conditional CRISPR lines induced with those mutations. Unfortunately, the time constraints of this bachelor's thesis prevented the generation of tissue specific CRISPR lines.

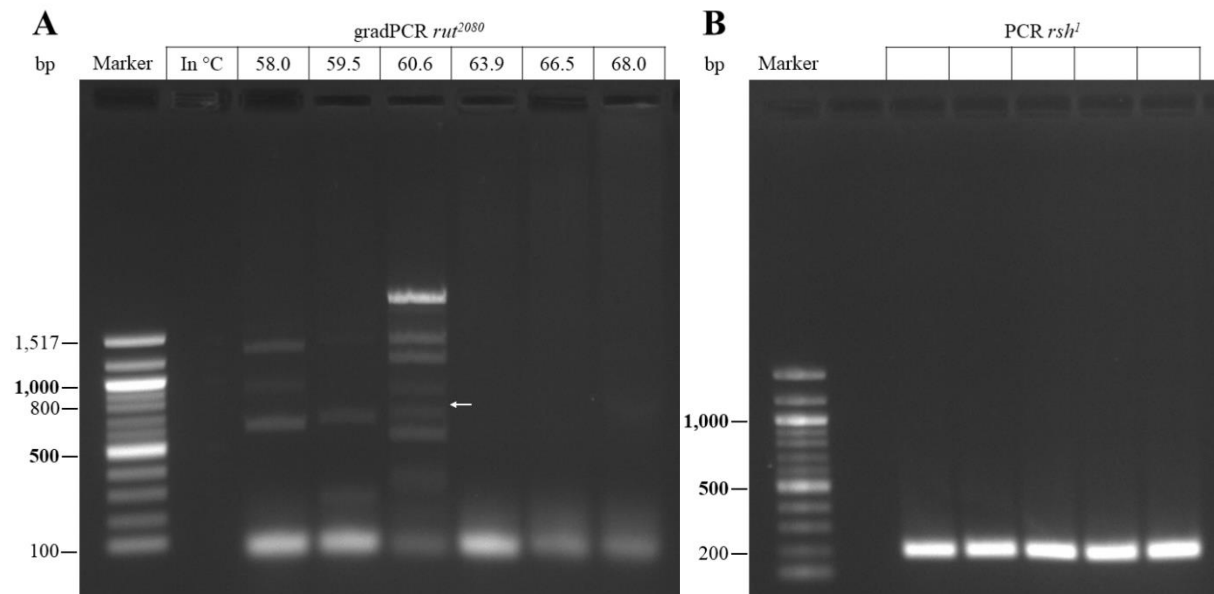


**Figure 6: Gel electrophoresis analysis of gDNA gradPCR and PCR products. A, B,** 1% agarose gel loaded with 100 bp DNA Ladder and either **(A)** gradient PCR products of $rut^{2080}$ mutant gDNA or **(B)** PCR products of $rsh^{1}$ mutant gDNA.

## 5.2. Verifying Mutations *rsh*[1] and *rut*[2080] in Fly Stocks

Another critical step in this project was to validate mutations in current fly stocks used for studies of enhanced self-learning in mutant flies (Brembs and Plendl, 2008; Brembs and Sun, 2017). To ensure consistency with the anticipated CRISPR lines, it was essential to confirm the presence of the respective mutations, *rsh*[1] and *rut*[2080]. We attempted to generate amplicons containing the specific mutation region from the flies' gDNA and confirm the presence of the mutations via Sanger sequencing. For *rsh*[1], this attempt was successful and led to the verification of the cytidine-to-thymidine point mutation (**Figure** 7) first described by Folkers et al. (2006), who also prepared the DNA in a similar manner.

The transposable P element insertion at the *rut* locus (*rut*[2080]) was first isolated via plasmid rescue (Levin et al., 1992 and Han et al., 1996). This P element is contained by the used primers for the *rut*[2080] amplicon. For this region, determining the required annealing temperature proved challenging because gradient PCR yielded multiple bands in gel electrophoresis analysis at the calculated temperature. Indeed, multiple bands in PCR can have different interpretations. Before considering explanations such as the insertion being found in a heterozygous state, it's crucial to address the PCR conditions as they can significantly contribute to this phenomenon. Adjustments of DNA polymerases, implementing additional purification steps for the gDNA, and use of negative controls, such as gDNA from wild-type flies, to validate the PCR results should be primary steps in troubleshooting. Unfortunately, the time constraints of this bachelor's thesis prevented the attainment of satisfactory results for verification of *rut*[2080].

**Figure 7: Sanger sequencing of *rsh*[1] amplicon**. Verification of the cytidine-to-thymidine point mutation.

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# 7. Index of Abbreviations

| | |
|---|---|
| % | Percent |
| µ | Mikro |
| Δ | Delta |
| °C | Degree Celcius |
| Amp | Ampicillin |
| AP | Alkaline phosphatase |
| bp | Base pair |
| c | Colony |
| Cas | CRISPR-associated |
| CIP | Calf intestinal alkaline phosphatase |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleotide triphosphates |
| *E. coli* | *Escherichia coli* |
| fwd | Forward |
| g | Gram |
| *g* | Standard gravity |
| grad | Gradient |
| gDNA | genomic DNA |
| gRNA | guide RNA |
| h | Hour |
| HF | High Fidelity |
| kb | Kilo bases |
| l | Liter |
| LB | Luria Bertani medium |
| LSB | Low salt buffer |
| m | Mili |
| M | Molar |
| min | Minutes |
| ml | Mililiter |

| | |
|---|---|
| mod | Modification |
| n | Nano |
| p | Pico |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pH | “power of hydrogen”, negative decimal cologarithm |
| rev | Reverse |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| rpm | Rounds per minute |
| *rsh* | *radish* |
| RT | Room temperature |
| *rut* | *rutabaga* |
| sec | Seconds |
| sgRNA | single guide RNA |
| T | Temperature |
| TAE | Tris-acetate-EDTA |
| *Taq* | Polymerase from *Thermus aquaticus* |
| tRNA | transfer RNA |
| U | Units |
| UAS | Upstream activating sequence |
| UV | Ultra violette |
| V | Volt |

# 8. Acknowledgements

I want to thank…

…	my supervisor Prof. Dr. Björn Brembs for the opportunity to do my research in his lab.

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…	my family for always supporting me.

# 9. Attachments

## *Creating gRNAs*

Modifications of the gRNA creation included the change to Phusion DNA Polymerase (Mod. 1.4) with the corresponding PCR reaction (see Table 27). The PCR program (see Table 15) was altered to suit the requirements of the polymerase. This involved increasing both the extension time and final extension to 30 seconds and 5 minutes, respectively.

Table 27: PCR Reaction with Phusion DNA Polymerase (Mod. 1.4).

| Component | 20 µl Reaction | Final Concentration |
|---|---|---|
| **Phusion HF Reaction Buffer (5x)** | 4.0 µl | 1X |
| **10 mM dNTPs** | 0.4 µl | 200 µM |
| **10 µM forward primer** | 1.0 µl | 0.5 µM |
| **10 µM reverse primer** | 1.0 µl | 0.5 µM |
| **Template DNA pCFD6** | 0.5 µl | 320 pg |
| **Phusion DNA Polymerase** | 0.2 µl | 0.4 units |
| **Nuclease-free water** | ad 20 µl | |

## *Restriction Digest of pCFD6*

Table 28: Restriction digest of 8 µg of pCFD6 (Mod. 2.1).

| Component | 40 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6** | variable | 8 µg |
| **rCutSmart Buffer (10x)** | 4.0 µl | 1x |
| **BbsI-HF** | 2.0 µl | 40 units |
| **Quick CIP** | 2.0 µl | 10 units |
| **Nuclease-free water** | ad 40 µl | |

Restriction digest of 5 µg pCFD6 (see Table 29) was incubated at 37°C for 3h followed by 20 min inactivation at 65°C. Dephosphorylation was performed with Antarctic Phosphatase (see Table 30) at 37°C for 30 min and inactivated at 70°C for 5 min (Mod. 2.2).

Table 29: Restriction digest of 5µg pCFD6 (Mod. 2.2).

| Component | 100 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6** | variable | 5 µg |
| **rCutSmart Buffer (10x)** | 10.0 µl | 1x |
| **BbsI-HF** | 5.0 µl | 100 units |
| **Nuclease-free water** | ad 100 µl | |

Table 30: Dephosphorylation of pCFD6 BbsI with Antarctic Phosphatase (Mod. 2.2).

| Component | 110 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6 BbsI** | 93.5 µl | variable |
| **Antarctic Phosphatase Reaction Buffer (10x)** | 11 µl | 1x |
| **Antarctic Phosphatase** | 5.5 µl | 27.5 units |

*Double Digest of pCFD6 with HindIII and NheI*

To minimize the risk of template DNA interfering with downstream applications, pCFD6 was double digested with HindIII-HF and NheI-HF (Mod. 1.5). The reaction (see Table 31) was incubated at 37°C for 3 h and inactivated with a 1% agarose gel at 100V, extracted and measured (see 3.1. Gel Electrophoresis and DNA Extraction). Aim was to decrease transformant background from template residue from gRNA creation with substituting the intact pCFD6 with a digested option.

Table 31: Double digest of pCFD6 with HindIII and NheI (Mod. 1.5).

| Component | 50 µl Reaction | Final Concentration |
|---|---|---|
| **pCFD6** | variable | 1 µg |
| **rCutSmart Buffer (10x)** | 5.0 µl | 1x |
| **HindIII-HF** | 1.0 µl | 20 units |
| **NheI-HF** | 1.0 µl | 20 units |
| **Nuclease-free water** | ad 50 µl | |

# 10. Declaration of authorship

The submitted printed copies and the submitted electronic version of the thesis are identical. I have written the thesis independently, have not used any sources or tools other than those indicated and have not already submitted the thesis to another university for the purpose of obtaining an academic degree. Furthermore, I confirm that I am aware of the legal consequences provided in § 27 (5) of the current examination regulations.

Die vorgelegten Druckexemplare und die vorgelegte elektronische Version der Arbeit sind identisch. Ich habe die Arbeit selbstständig verfasst, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und die Arbeit nicht bereits an einer anderen Hochschule zur Erlangung eines akademischen Grades eingereicht. Weiterhin bestätige ich, dass ich von den in § 27 Abs. 5 der geltenden Prüfungsordnung vorgesehenen Rechtsfolgen Kenntnis habe.

________________________ ________________________

Date Signatur