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CRISPR-Cas9 Mediated Depigmentation in frogs

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Abstract

The CRISPR–Cas9 genome-editing workflow was established and standardized in the Laboratory for the Theory of Evolution (LADE) at the Universidad de Concepción. An end-to-end pipeline spanning bioinformatic guide design (Xenbase, CRISPRdirect, GGGenome, InterPro, Indelphi), PCR-based template generation, in vitro transcription, in-vitro fertilization of Xenopus, and calibrated microinjection with a fluorescent tracer to verify delivery was implemented. Guides targeting tyrosinase and oca2 were designed across Xenopus laevis subgenomes and Xenopus tropicalis; after troubleshooting, several sgRNAs reached concentrations suitable for injection (>1 µg/µL), while remaining bottlenecks were identified and mitigated primarily by improving template concentration. Microinjections were performed and delivery confirmed visually; early observations revealed mosaic, light pigmentation consistent with injections at the 2-cell stage. In parallel, a comparative analysis showed that the S-subgenome copy of Oca2 in X. laevis is a pseudogene, supported by the absence of RNA expression and the presence of deleterious frameshifts together with a deleted exon affecting a transmembrane domain. This work delivers a documented, reproducible protocol adapted to the local infrastructure, establishes feasibility of CRISPR editing in Xenopus at LADE, and delineates immediate next steps—genotyping-based editing quantification and systematic phenotypic scoring—to transition from feasibility to routine reverse-genetics in the lab.

Acknowledgment

I am deeply grateful to Sylvain, my internship referee in Chile. His unwavering support, insightful advice, and encouragement were essential to the success of my internship experience and have significantly contributed to my academic journey.
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Thanks to all the lab as well, we all worked together, they helped me countless times without looking at the clock.

1 Preamble

This report details an internship undertaken in Concepción, Chile, at the Laboratory for the Theory of Evolution (LADE) at the Universidad de Concepción (UdeC). The work focused on cellular biology, a field that was a significant component of our bioengineering curriculum. The primary aim of this research was to contribute to the standardization of the CRISPR gene-editing process. This standardization is intended to facilitate its use by researchers in future projects. The successful induction of albinism in frogs was utilized as a proof-of-concept, offering a readily observable phenotype to validate the optimized CRISPR methodology.
Beyond the scientific objectives, this internship also provided a valuable opportunity to develop intercultural skills through immersion in Chilean (and broader Spanish-speaking) culture and dedicated efforts to improve my Spanish language proficiency.

2 Introduction

This internship implemented and standardized an end-to-end CRISPR–Cas9 workflow at the Laboratory for the Theory of Evolution (LADE), Universidad de Concepción, with three practical goals: (i) convert in-silico guide designs into reliably edited Xenopus embryos using locally available infrastructure; (ii) identify and mitigate operational bottlenecks that limit sgRNA yield and injection readiness; and (iii) validate delivery and early phenotypic consequences with rapid, scorable assays.
The pipeline integrates target selection and computational design (Xenbase, InterPro, CRISPRdirect, GGGenome, Indelphi), PCR-based template synthesis and in vitro transcription of sgRNAs, controlled in vitro fertilization to produce synchronously staged embryos, and calibrated microinjection of Cas9/sgRNA complexes with a fluorescent tracer to confirm delivery prior to downstream genotyping and phenotyping. Emphasis was placed on reproducibility, yield optimization, and documenting decision points that affect cost, time, and data quality when adapting the protocol to local laboratory constraints.
For rapid assessment pigmentation genes (*tyr*, *oca2*) were targeted in *Xenopus laevis* (L/S subgenomes) and *Xenopus tropicalis*; these loci provide early, easily scored phenotypes and serve as practical testbeds for guide specificity and editing efficiency. A parallel comparative genomic analysis showed that the *Oca2* copy on the *X. laevis* S-subgenome is a pseudogene, a result that informs both target selection and interpretation of pigmentation outcomes.
The present report therefore documents: (1) a reproducible, lab-adapted CRISPR pipeline from computational design to microinjection; (2) empirical identification of yield-limiting steps together with straightforward mitigations to improve sgRNA availability; and (3) initial feasibility evidence—fluorescent delivery confirmation

and early pigmentation changes—complemented by genomic context for target choice. The following sections present methods, results, discussion of limitations, and a prioritized roadmap to move from proof-of-concept to routine reverse-genetic experiments at LADE.

3 Objectives

The primary objective of this internship was to implement and standardize an end-to-end CRISPR–Cas9 workflow at the Laboratory for the Theory of Evolution (LADE), Universidad de Concepción, and to adapt this pipeline to the constraints of the local infrastructure. The work aimed to provide a reproducible protocol and clear operating procedures so the method can be reliably used by other researchers in the laboratory.
Specific aims were:

1. Establish a reproducible pipeline from computational guide design through sgRNA synthesis, in vitro fertilization, and calibrated microinjection.
2. Identify and mitigate operational bottlenecks that limit sgRNA yield and injection readiness.
3. Validate delivery and obtain preliminary, scorable phenotypic evidence (pigmentation changes) to guide subsequent genotyping and protocol optimization.

4 Preliminary Comparative Study of the *Oca2* Gene in *Xenopus*

4.1 Introduction

The Oculocutaneous Albinism II (*Oca2*) gene is a key gene involved in melanin synthesis, and its mutation is associated with albinism in many species[9]. In the allotetraploid frog *Xenopus laevis*, the genome is composed of two subgenomes, L (long chromosomes) and S (short chromosomes), which are homeologs. This study aimed to compare the *Oca2* gene sequence and expression from the *X. laevis* L and S subgenomes, using the diploid *Xenopus tropicalis* as a functional reference. Our findings indicate that while the L-subgenome contains a functional *Oca2* gene, the S-subgenome counterpart has become a pseudogene.

4.2 RNA Expression Analysis Reveals Loss of Transcription in the S-Subgenome

To assess the functionality of the *Oca2* gene in both *X. laevis* subgenomes, first was examined RNA sequencing data available on Xenbase.
As shown in Figure 1, the *Oca2* gene on the L-subgenome exhibits clear and consistent RNA expression across various tissues, including the eye, intestine, and spleen. This pattern is consistent with a functional, transcribed gene.

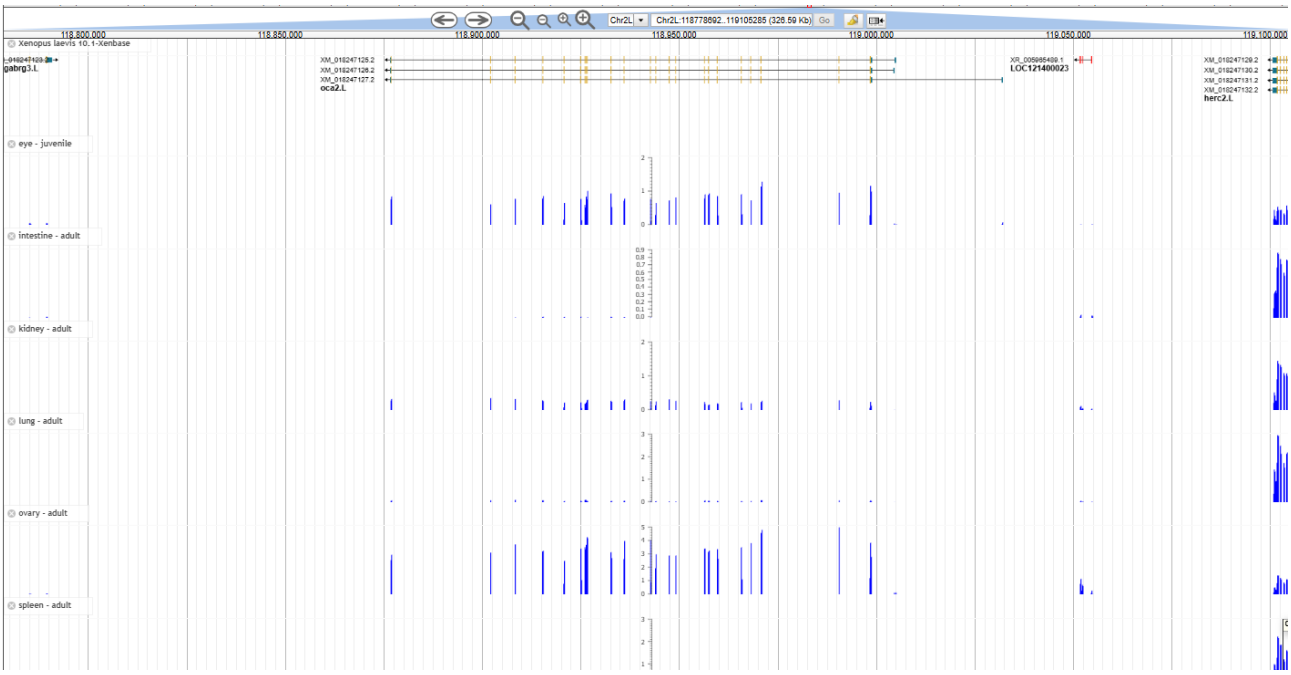


Figure 1: RNA-seq data from Xenbase for the *Oca2* locus in the *Xenopus laevis* L-subgenome. Significant expression is visible across multiple tissues.

In stark contrast, the corresponding locus on the S-subgenome shows a complete lack of RNA expression (Figure 2). This absence of transcripts strongly suggests that the gene has been silenced and is likely a pseudogene.

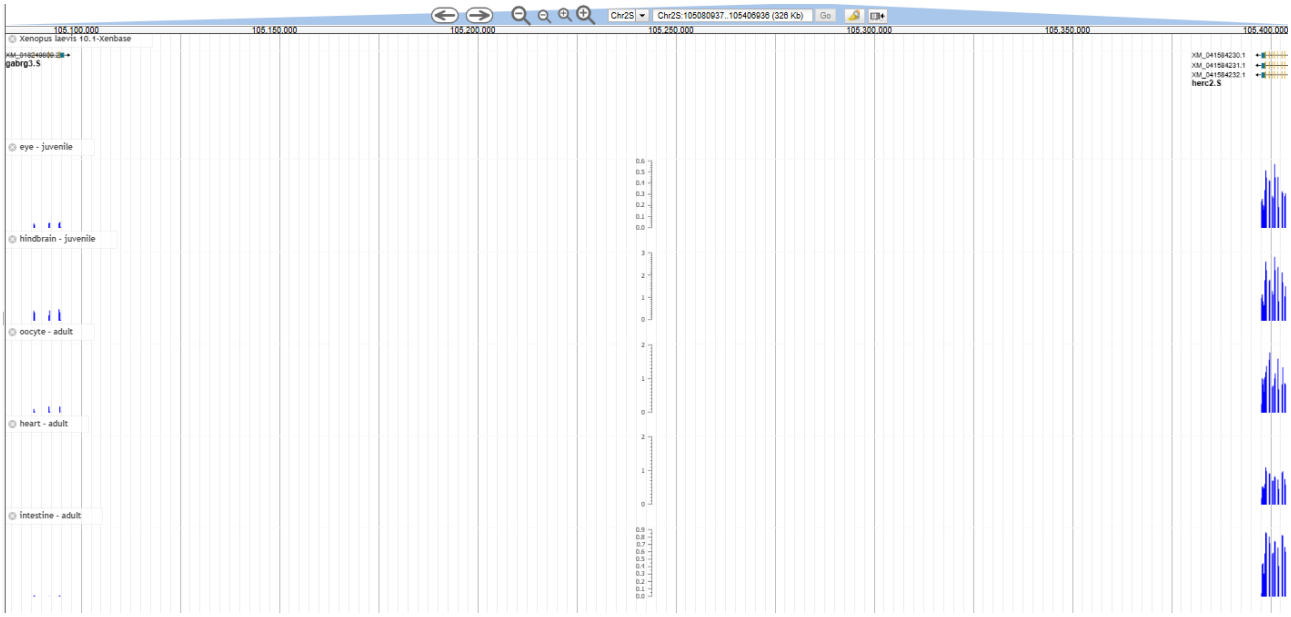


Figure 2: RNA-seq data from Xenbase for the *Oca2* locus in the *Xenopus laevis* S-subgenome. No significant RNA expression is detected, indicating a loss of function.

4.3 Genomic Analysis Uncovers Frameshift Mutations and a Major Deletion

To understand the genomic basis for the loss of expression and function in the S-subgenome's *Oca2* locus, a BLAST alignment was performed. A comparison was made between the coding sequences (CDS) of the functional *Oca2* from *X. laevis* L and *X. tropicalis* against the genomic locus of *Oca2* in the *X. laevis* S-subgenome.

The alignment revealed numerous mutations in the S-subgenome sequence. Crucially, multiple small insertions and deletions (indels) of 1 or 2 base pairs scattered throughout the coding region were identified. These indels result in frameshift mutations, which alter the downstream amino acid sequence and inevitably lead to the introduction of premature stop codons. Such mutations would produce a truncated and non-functional protein, even if transcription were to occur.

Sequence analysis reveals a one-base insertion on the third exon (Figure 4), causing a significant frameshift. A second one-base insertion on the sixth exon (Figure 5) prevents reading frame recovery at that point. The reading frame is finally restored following two separate deletions: one in exon thirteen (Figure 6) and another in exon nineteen (Figure 7). Nevertheless, this initial frameshift alters approximately 80 % of the resulting protein.

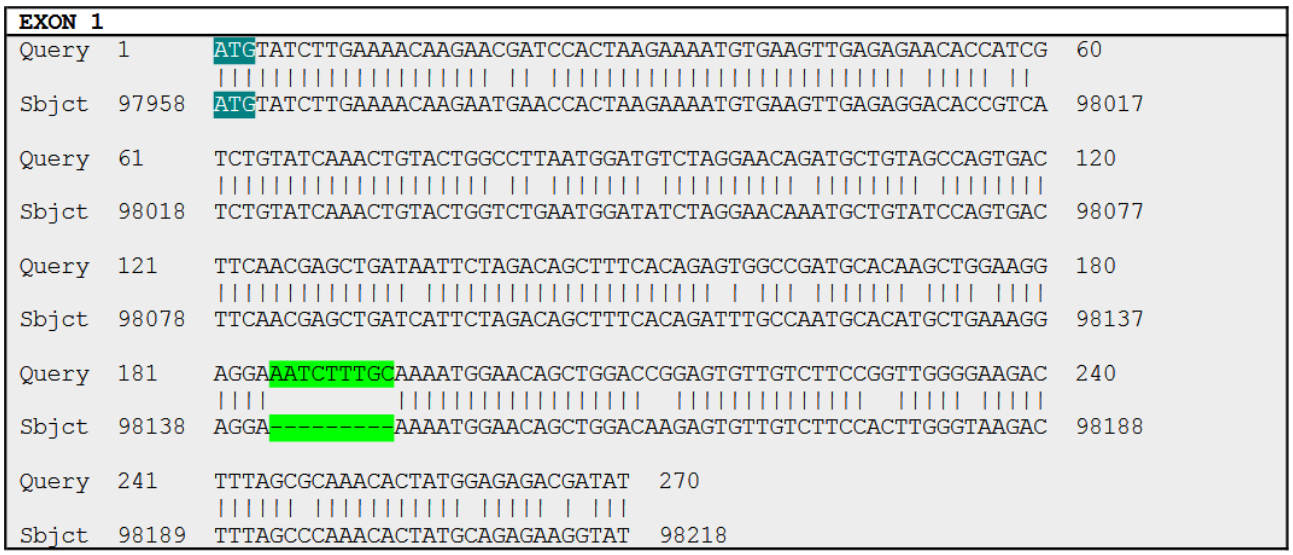


Figure 3: First *Oca2* exon in the *Xenopus laevis* S-subgenome. No significant alteration.



Figure 4: Third *Oca2* exon in the *Xenopus laevis* S-subgenome.



Figure 5: Sixth *Oca2* exon in the *Xenopus laevis* S-subgenome.



Figure 6: Thirteenth *Oca2* exon in the *Xenopus laevis* S-subgenome.



Figure 7: Nineteenth *Oca2* exon in the *Xenopus laevis* S-subgenome.

Furthermore, our analysis identified a significant structural mutation: the complete deletion of an exon. This deletion is clearly visualized when comparing the alignment of the Oca2-L CDS against its own genomic locus (Figure 8, top) versus its alignment against the Oca2-S locus (Figure 9, middle and bottom), where a break in alignment is evident.

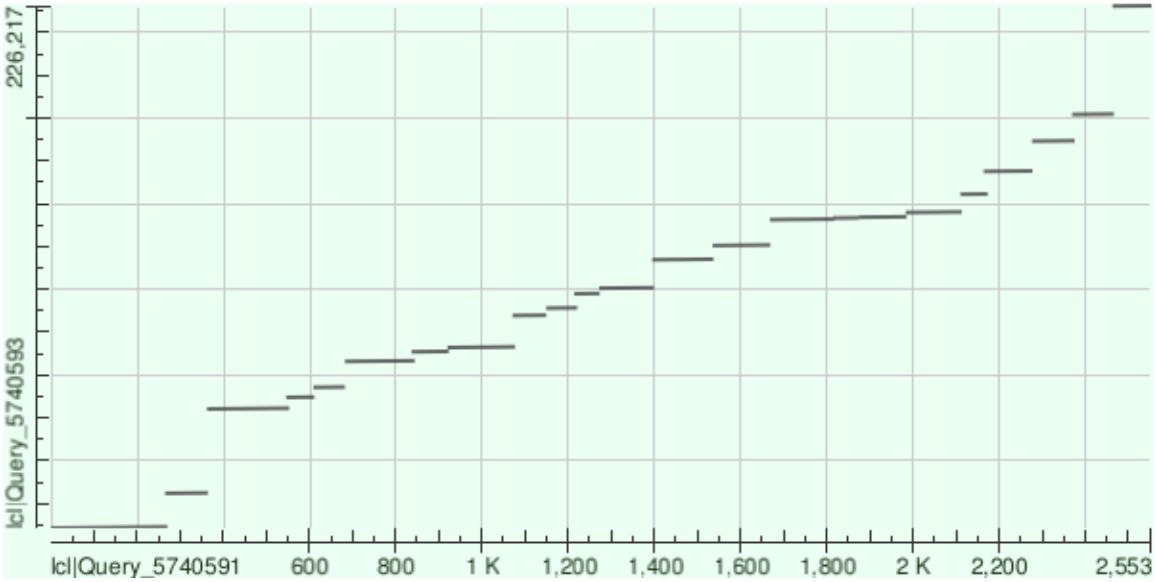


Figure 8: Dotplot of the Oca2-L CDS aligned against its own genomic locus (positive control).

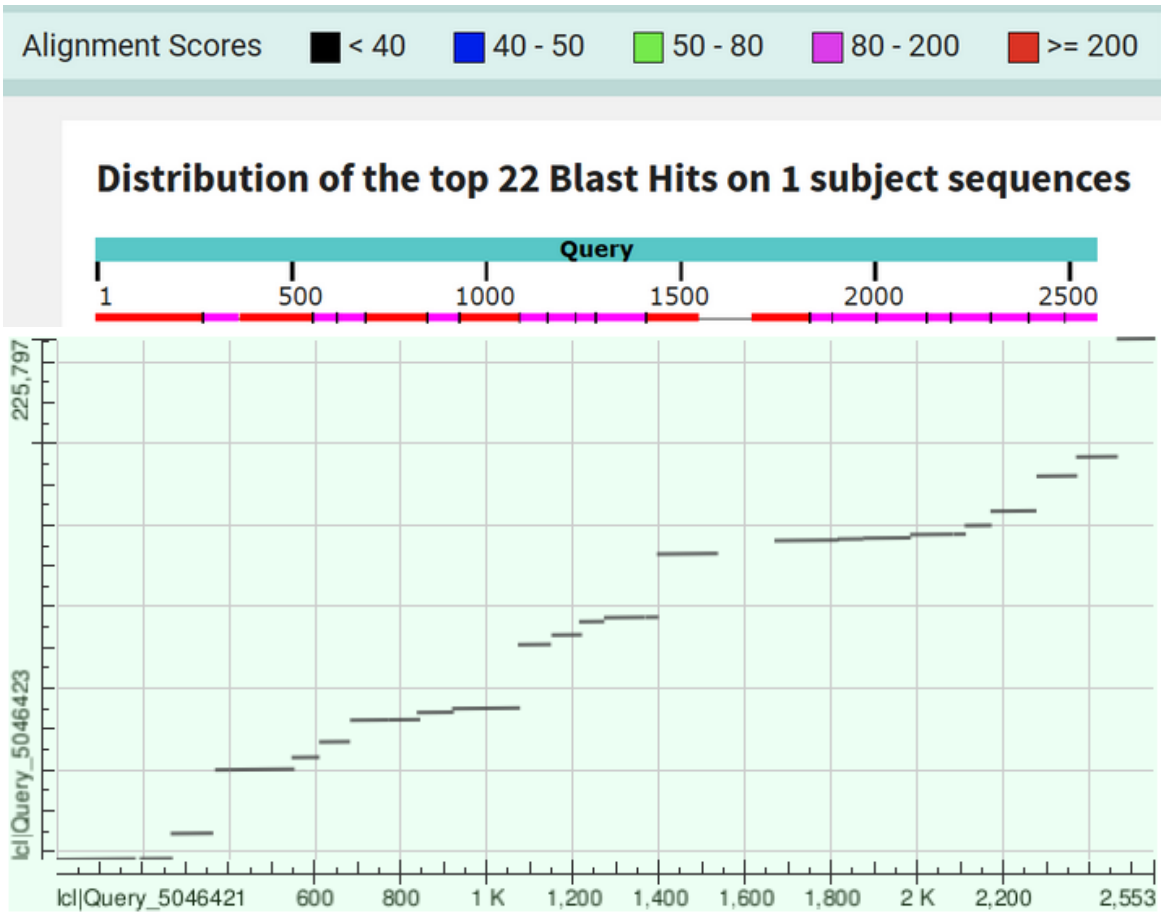


Figure 9: Visualization of the exon deletion on the S-subgenome. **Top:** BLAST result of the Oca2-L CDS on the Oca2-S locus, showing the alignment break. **Bottom:** Dotplot of the Oca2-L CDS on the Oca2-S locus, illustrating the missing exon.

Analysis of the exon deletion visualization: The top panel (Figure 8) shows the alignment of the Oca2-L

CDS against its own genomic locus, serving as a positive control and demonstrating a continuous alignment. The bottom two panels (Figure 9) visualize the exon deletion in the S-subgenome. The top of these two shows the BLAST result of the Oca2-L CDS aligned against the Oca2-S locus, where a clear break in the alignment indicates a missing section. The bottom of these two is a dotplot further illustrating this missing exon in the Oca2-S locus when compared to the Oca2-L CDS.
The specific exon lost in the S-subgenome is located after base 1,547 in the L-subgenome CDS. Its nucleotide sequence and the resulting lost polypeptide are shown in Figure 10.

Figure 10: Deleted sequences from the S-subgenome *Oca2* gene.

(A) Deleted nucleotide sequence (141 bp)

```
GGGATTGGACTTTGCAGGATTTACTGGACATATGTTCTTAGGAATTTGCCTGGTTCTCCTAGTATCTTTTCCTTTTCTCAGGATTTTATATTGGAACA
AGAAACTTTATAATAAGGAACCCAGTGAAATTGTTG
```

(B) Resulting lost polypeptide (47 aa)

```
GLDFAGFTGHMFLGICLVLLVSFPFLRILYWNKKLYNKEPSEIV
```

The loss of this entire functional domain, which corresponds to a critical transmembrane domain of the Oca2 protein (Figure 11), would fundamentally compromise the protein's structure and its ability to function as a transporter [9].

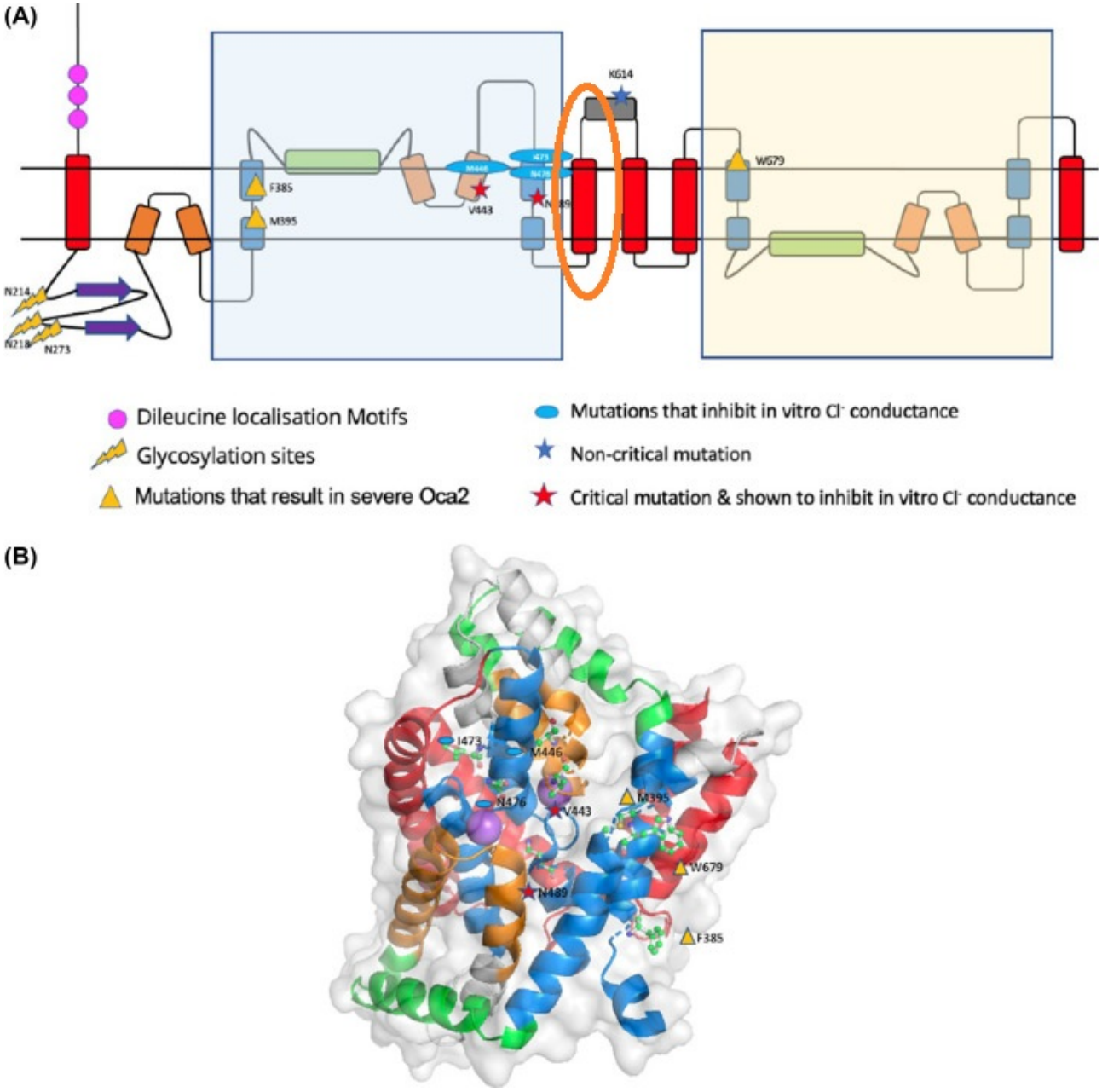


Figure 11: Schematic representation of the Oca2 protein. The transmembrane domain lost in the S-subgenome version is highlighted in red, illustrating the major structural impact of the deletion.

4.4 Conclusion and Future Directions

The combined evidence from RNA expression and genomic sequence analysis conclusively demonstrates that the *Oca2* gene in the *Xenopus laevis* S-subgenome is a pseudogene. The lack of transcription, coupled with the accumulation of deleterious mutations—including multiple reading frame disruptions and the deletion of a critical exon—confirms its non-functional status.

While the functional gene in the L-subgenome has been conserved, the S-subgenome copy has degenerated since the allopolyploidization event that formed the *X. laevis* genome.

An initial investigation into the promoter region of the S-subgenome *Oca2* was considered to determine if its decay contributed to the silencing of the gene. However, the sequence was found to be highly fragmented and difficult to analyze. Given the conclusive evidence of fatal mutations within the coding sequence itself, an in-depth analysis of the promoter was deemed non-essential to the conclusion of this study.

5 Materials and Methods

5.1 Design of Single Guide RNA (sgRNA)

5.1.1 Gene Background Research and Sequence Retrieval

Comprehensive background research on the target gene was conducted according to our internal protocol by examining literature for mutations, understanding gene function, identifying isoforms or alternate start sites, and comparing the *Xenopus* genome sequence with orthologs (human, mice, zebrafish) [7]. Public databases such as Xenbase [12], OMIM [5], and NCBI [10] were utilized to gather information on gene variants, conserved regions, and potential alternative start sites. The gene sequence of interest was located and retrieved from Xenbase using J-browse to view and select the longest feature sequence annotation, following our established procedure [7].

5.1.2 Generation and Annotation of MacVector Files

Following our protocol [7], the retrieved gene sequence was used to create new nucleic acid files in MacVector [8]. A genomic DNA (gDNA) file was saved, and a separate cDNA file was created using the highlighted CDS regions from Xenbase. Both gDNA and cDNA files were annotated by creating features for CDS and UTRs, using consistent visual cues for easy identification [7].

5.1.3 Protein Sequence Analysis and Domain Mapping

The annotated cDNA file was translated into an amino acid sequence using the translate option in MacVector [8], and this wild-type protein sequence was saved [7]. The final asterisk was excluded from the copied amino acid sequence as per our protocol [7]. Protein domains were identified by submitting the amino acid sequence to the InterPro database [3]. The identified domains and their positions were then mapped as features onto the translated MacVector file and subsequently onto the cDNA and gDNA files. This process involved adjusting coordinates for nucleotide sequences by multiplying amino acid positions by three for the cDNA, while for gDNA, domain start and end points were searched separately due to potential exon spanning [7].

5.1.4 sgRNA Target Site Selection and Off-Target Analysis

Target regions for guide design were selected upstream of a target domain, early within the gene, or early within a domain, while avoiding proximity to the N-terminus [7]. Potential sgRNA sequences within the target CDS, specific to *X. laevis* or *X. tropicalis* with an NGG PAM, were identified using CRISPRdirect [1]. Guide specificity was assessed by examining the '12mer + PAM' column to check for off-target sites. Each potential guide's 12mer + PAM was then checked using GGGenome to list all genomic hits [2]. Guides were selected only if they showed no hits in exons of other genes (indicated by a red bar in GGGenome's primary transcription box) and ideally had only one hit in the desired gene. Selected guides were then located and annotated as a 'misc_feature' in the cDNA and gDNA MacVector files [7].

5.1.5 Prediction of sgRNA-Induced Mutations

The potential mutations and frameshift frequency of selected guides were predicted using the Indelphi tool [11]. For each guide, sequences were prepared from the gDNA file as detailed in our protocol, ensuring they were in the positive direction (taking the reverse complement if necessary). Guides predicted to cause in-frame mutations (multiples of 3) were generally avoided [7].

5.1.6 Oligonucleotide Design for sgRNA Synthesis

Guides were ordered as single-stranded DNA oligos from Integrated DNA Technologies (IDT) [4]. The design followed a specific set of rules from our internal protocol: the PAM sequence was removed, the first two nucleotides opposite the PAM were changed to 'GG' to promote binding, and a T7 or Sp6 promoter sequence was added upstream of the guide, with a universal reverse sequence added downstream. This resulted in a final oligo length of 60 bases [7].

5.2 Synthesis of sgRNA

5.2.1 sgRNA Template Preparation via PCR

Steps

1. **Rehydrate** the *sgRNA forward primer* to 100 µM (nuclease-free H_2O).
2. **Prepare the PCR mix** (100 µL):
   - 74.5 µL nuclease-free H_2O
   - 20 µL Q5 buffer 5 x
   - 1 µL target forward primer (sgRNA)
   - 2 µL 50 µM universal reverse primer
   - 2 µL 10 µM dNTPs
   - 0.5 µL Q5 polymerase *(keep on ice)*

Tip: prepare a master mix for n+10% reactions and add the specific primers last.

Thermocycler program

Temp.	Time	Cycles
98 °C	30 s	
98 °C	10 s	
62 °C	20 s	×30
72 °C	20 s	
72 °C	5 min	
4 °C	*Hold*	

The green rows represent the **30 cycle loop**.

5.2.2 Purification of PCR Product

The PCR product was purified using the Qiagen QIAquick PCR and Gel Cleanup Kit following the manufacturer's protocol[7]. The DNA concentration of the purified product was measured using a Nanodrop, aiming for an ideal template DNA concentration of 100-300 ng/μL[7].

5.2.3 In Vitro Transcription of sgRNA

RNA synthesis was performed using the Ambion Megascript Kit, selecting the T7 polymerase as per the promoter used in the sgRNA forward primer design. The 20 μL reaction included the PCR product (300ng), NF H2O, 10X Buffer, ATP, CTP, GTP, UTP, and RNA Polymerase, with buffers and NTP solutions fully thawed and polymerase kept on ice[7]. For the T7 MEGAshort script, incubation was carried out at 37°C overnight (or for 4-6 hours)[7].

5.2.4 Purification and Quantification of sgRNA

Following incubation, the synthesized RNA was treated with 1μL of Turbo DNAse (from the Megascript kit) per reaction at 37°C for 15 minutes. RNA purification was then performed using the Genejet protocol, including a heat shock step at 70°C for 3 minutes. The purified RNA was kept on ice. The RNA concentration was measured by Nanodrop, and aliquots were prepared for storage at -80°C with a final concentration of 500 pg/nL for XT or 750 pg/nL for XB and XL, calculated using an online dilution calculator[7].

5.3 In-vitro Fertilization (IVF) of *Xenopus* Embryos

5.3.1 Adult *Xenopus* Preparation

The night before fertilization, male and female *Xenopus* were primed by injecting 20U of hCG into the dorsal lymph sac, keeping sexes separate overnight[12]. On the morning of fertilization, males and females were boosted with 200U of hCG into the dorsal lymph sac[12].

5.3.2 Gamete Collection

Starting 3 hours after boosting, females were gently squeezed to collect eggs, which were then rinsed with 0.1X MR and drained[12]. Testes were isolated from males prior to squeezing females[12]. Males were euthanized, and testes were located near the aorta, gently removed, cleaned of connective tissue and blood, and stored in L15+10% FBS on ice[12].

5.3.3 Fertilization and De-jellying

Half a testis was transferred to 200 μL of 0.1X MR in a 1.5 mL Eppendorf and macerated with a sterile pestle[7]. The macerated testis solution was immediately applied to the eggs and mixed. The dish was left tilted until signs of fertilization were observed (approx. 3 min), then flooded with 0.1X MR[7]. For de-jellying, the 0.1X MR was poured off, and 3% cysteine was added to the petri dish. Embryos were incubated in cysteine for

5-10 minutes with occasional swirling until eggs just touched each other. Embryos were then rinsed four times with 0.1X MR and transferred to L15+serum-coated 110mm petri dishes with 3% Ficoll, removing any bad embryos[6].

5.4 Micro-injection of *Xenopus* Embryos

5.4.1 Embryo Preparation for Injection

Healthy de-jellied eggs were placed in injection dishes containing 3% Ficoll, with eggs fitting into mesh squares[12]. Excess liquid was removed until eggs were just covered and slightly pressed into the mesh[12].

5.4.2 Preparation of CRISPR/Cas9 Injection Solution

The injection mixes used for embryo microinjection were prepared as 10 µL master mixes and differed slightly depending on whether one or two sgRNAs were co-delivered. The recipes below reproduce the lab notes (volumes) and show the resulting payload per injected volume (2 nL). Calculations assume Cas9 stock = 1 µg/µL and sgRNA stocks as indicated (example: 500 ng/µL for XT); adjust numbers if different stocks are used[7].

For 1 sgRNA

- 4 µL nuclease–free H_2O
- 2 µL Cas9
- 2 µL sgRNA
- 2 µL Texas Red

For 2 sgRNA

- 2 µL nuclease–free H_2O
- 2 µL Cas9
- 2 µL sgRNA1
- 2 µL sgRNA2
- 2 µL Texas Red

Notes: (i) when using different sgRNA stock concentrations, re-calculate final ng/µL and pg per injection; (ii) keep all reagents on ice during preparation and aliquot mixes to avoid freeze–thaw; (iii) Texas Red (or other tracer) volume may be adjusted to modulate fluorescence intensity but should be kept consistent across experimental groups.

5.4.3 Micro-injection Procedure

A volume of 2 nL of the CRISPR/Cas9 injection solution was injected into each egg or into each blastomere at the 2-cell stage.

5.4.4 Assess the Success of Injection via Bacterial Transformation

In order to be able to inject properly and to see the results, we need to first find a way to be certain that the injection worked before letting the frogs grow. Letting them grow without knowing if the injection was properly done will lead to a tremendous time loss in case of failure. To exhibit the injection, the idea is to do a bacterial transformation to generate great quantities of plasmids that will induce florescence in the cells membrane when injected in frog's eggs. This fluorescence will last a day, but is enough to screen out the one that were not injected properly. The complete protocol is in the protocol part of this report. The idea is to use competent bacterias ability to absorb and recombine plasmid to generate more plasmids. The injected plasmid has a double interest, first coding for the fluorescent protein, second to induce antibio-resistance. This allows to kill all the cells that didn't recombine efficiently. Then steps are taken to make sure cells multiply. The cells are in the end destroyed and everything is filtered out except the plasmids which is our end targeted product.

5.4.5 Post-Injection Embryo Culture and Monitoring

Embryos were allowed to recover for approximately 30 minutes post-injection and then transferred to 0.1X MR + gentamycin[12]. After 1-3 hours, good embryos were sorted, with no more than 110 per 110mm petri dish, and incubated overnight at 26-28°C[12]. Dead and dying embryos were removed, and the medium was replaced if fouled[12]. Screening for fluorescence could begin on day 2[12]. Once embryos reached the feeding stage, they were moved to frog water.

6 Results

6.1 sgRNA Design and Synthesis Verification

Sequences of designed sgRNAs targeting *tyr* and *oca-2* across *Xenopus* species, with exon positions and PAM sites.
Guide sequences were selected using genome data from Xenbase and verified via NCBI BLAST alignment to ensure on-target specificity and reading frame disruption likelihood.

Nom	Séquence (5' → 3')
tyr_XT_ex1	aattTAATACGACTCACTATAGGTGTTTGCAGGGGCCAG
	TGTTTTAGAGCTAGAAATAG,25nm
tyr_XL-L_ex1	aattTAATACGACTCACTATAGGCTCGATGATAGAAGGAC
	GTTTTAGAGCTAGAAATAG,25nm
tyr_XL-S_ex1	aattTAATACGACTCACTATAGGACCAGCTCTGCTACGGG
	CGTTTTAGAGCTAGAAATAG,25nm

Guides d'intérêt

Nom	Séquence (5' → 3')
oca2_XT_ex6	AATTTAATACGACTCACTATAGGTGTTGGCCCTATCATCTA
	GTTTTAGAGCTAGAAATAG,25nm
oca2_XT_ex9	aattTAATACGACTCACTATAGGCTGATGAGATAAAGACAA
	GTTTTAGAGCTAGAAATAG,25nm
oca2_XT_ex14	aattTAATACGACTCACTATAGGAAGATGACTTCGGCAAT
	AGTTTTAGAGCTAGAAATAG,25nm
oca2_XT_ex19	aattTAATACGACTCACTATAGGTCCTACATCGGGTTGAA
	TGTTTTAGAGCTAGAAATAG,25nm
oca2_XL_ex7	aattTAATACGACTCACTATAGGTGTTGGCCCTATCGTCT
	AGTTTTAGAGCTAGAAATAG,25nm
oca2_XL_ex15	aattTAATACGACTCACTATAGGAGCAATAAGGACGTGTC
	TGTTTTAGAGCTAGAAATAG,25nm
oca2_XL_ex20	aattTAATACGACTCACTATAGGGCCCATTCAACCCGATG
	TGTTTTAGAGCTAGAAATAG,25nm
CRISPR_UNIV_R	CTATTTCTAGCTCTAAAAC,25nm

Initial DNA amplification using Q5 polymerase yielded concentrations 3–5 times below the target (typical values: 10–40 ng/μL vs. 100–300 ng/μL). RNA transcription efficiencies were also below expectations during first attempts, producing concentrations up to 100 times lower than the desired 2000 ng/μL.
After optimization, several sgRNAs achieved sufficient yields for downstream applications (Table 1).

Name of guide	**Concentration (ng/μL)**	**A260**	**A260/A280**
Xt-tyr	1860	46.50	2.03
Xla-tyr-S	1247	50.00	0.51
Xla-tyr-L	2010	31.22	1.16
Xt-oca2-ex6	93.0	2.20	1.77
Xt-oca2-ex9	5.8	0.36	1.65
Xla-oca2-ex7	1380	34.51	2.03
Xla-oca2-ex15	579	14.4	1.99
Xla-oca2-ex20	1048	26.23	2.14

Table 1: RNA concentrations after optimized transcription.

6.2 Micro-injection Efficiency and Embryo Viability

Injection mixes were prepared by combining sgRNA with Cas9 protein and Texas Red for visualization. Hormone priming protocols for egg collection were successfully performed. Fertilization rates were within expected ranges. Embryo injection and survival data are still being compiled; however, injection calibration and delivery were confirmed visually.

6.3 Genotyping and Assessment of Editing Efficiency

At this stage, genotyping results are pending. Molecular assays (PCR/sequencing) will be used to confirm editing efficiency. Preliminary visual checks post-injection indicate successful delivery of injection mix.

6.4 Phenotypic Analysis

Phenotypic assessment will be based on loss of pigmentation (albinism) in developing embryos. Observation windows have been defined but results are not yet available.

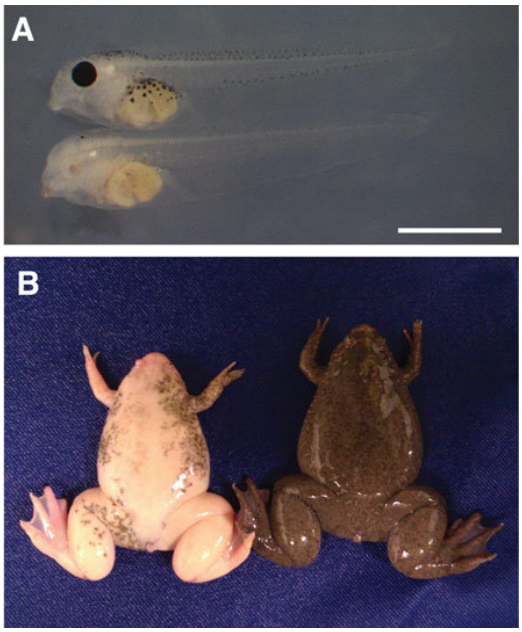


Figure 12: Expected pigmentation phenotypes in control vs CRISPR-edited *Xenopus* larvae. From Shoko Ishibashi, Rebecca Cliffe, and Enrique Amaya, "Highly Efficient Bi-Allelic Mutation Rates Using TALENs in Xenopus tropicalis," Biology Open

In order to have a visual look of our results, here are some pictures of the larvas that were injected with the crispr mix:

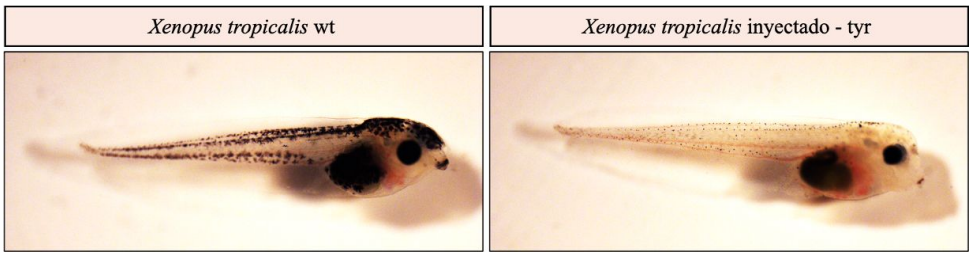


Figure 13: Injected *Xenopus* larvae.

It can be seen that the injected larvae are not depigmented, but have a very light pigmentation. This is probably due to the fact that the injection was done at the 2 cell stage, and that the sgRNA was not present in all the cells of the larvae. It could also be due to the fact that other genes are involved in pigmentation, such as Oca2, which is not targeted by the sgRNA. This will be further investigated in the future.

7 Discussion

Time limit

The time constraints of the internship limited the scope of troubleshooting and optimization that could be performed. Future experiments should allocate sufficient time for each stage of the protocol, particularly for critical steps like amplification and transcription. Furthermore the fact that the QIAquick purification kit was significantly delayed impacted the timeline. In the end it wasn't possible to fully standardize the whole process.

Amplification and Transcription Challenges

Initial amplification attempts produced DNA yields significantly below expectations, limiting the amount of template available for transcription. Transcription efficiency was also very low, with yields insufficient for injections. Multiple troubleshooting steps were explored:

- Switching from Q5 polymerase to Q5 High Fidelity.
- Adjusting elution volume from 50 μL to 20 μL.
- Primer dilution tests (1:10) to assess efficiency and cost-effectiveness.
- Modifications to thermocycler programs.
- Variation of dNTP concentrations.
- Altered elution protocols, including heated (70°C) buffers.

Most of these adjustments produced marginal improvements. The most impactful change was reducing elution volume, which increased DNA concentration sufficiently for transcription. This experiment aims to standardize the CRISPR protocol, prioritizing overall efficiency by maintaining an optimal elution volume should have been prioritized. However no other choice was possible given the time given for the internship.

sgRNA Yield and Quality

RNA concentration varied greatly between guides. While several (*Xt-tyr*, *Xla-tyr-L*, *Xla-oca2-ex7*) reached concentrations above 1000 ng/μL, others (*Xt-oca2-ex9*) remained below 10 ng/μL, likely due to inefficiencies in transcription or template quality.

Implications for CRISPR Efficiency

Sufficient RNA yield is a prerequisite for high-efficiency editing. Guides with low RNA output will require either:

- Further optimization of template synthesis and transcription.
- Replacement by alternative sgRNAs targeting the same exon.

The variability in yield underlines the importance of parallel guide preparation and redundancy in sgRNA design.

Limitations and Future Directions

At present, phenotypic and genotypic validations are incomplete. Final assessment of CRISPR efficiency will depend on pigmentation scoring across injected and control groups. Future protocol iterations should integrate yield optimization steps earlier, avoid ineffective parameter changes, and adopt reduced elution volumes by default. They will need sequencing to quantify indel frequency since phenotype schoring will not always be possible depending on the targeted gene.

8 Conclusion

This project established and documented a complete CRISPR–Cas9 workflow in *Xenopus* at LADE, from bioinformatic design through sgRNA synthesis, IVF, and calibrated microinjection with fluorescent verification. Several guides achieved injection-ready concentrations after targeted troubleshooting, and early observations of mosaic, light pigmentation are consistent with effective but distributed editing due to injections at the 2-cell stage. In parallel, comparative analyses demonstrated that the *Oca2* gene on the *Xenopus laevis* S-subgenome is a pseudogene, supported by absent transcription, multiple frameshifts, and a deleted exon affecting a transmembrane domain. This result informs target selection and interpretation for pigmentation assays and underscores the value of integrated genomic context in experimental design.

Current limitations are primarily confirmatory: quantitative genotyping (indel spectra and frequencies) and systematic phenotypic scoring remain to be completed. Yield variability across guides also suggests that template quality and purification steps should be prioritized during routine preparation. Despite these open items, the feasibility of the pipeline has been demonstrated, the main operational bottlenecks have been identified, and practical remedies have been incorporated into the standard operating procedure.

Immediate next steps are: (i) complete genotyping of injected embryos to quantify editing efficiency per guide; (ii) standardize injection at the 1-cell stage when feasible to reduce mosaicism; (iii) retire persistently low-yield sgRNAs in favor of alternative designs; and (iv) formalize phenotype scoring against appropriate controls. With these steps, the workflow will transition from proof-of-implementation to a robust, routinely deployable platform for reverse genetics in *Xenopus*, enabling future projects in development, disease modeling, and gene–function studies within the laboratory.

9 Appendix

9.1 detailed protocoles

9.1.1 Bacterial Transformation Protocol

Materials

- Competent cells (e.g., DH5α, BL21)
- Plasmid DNA (10–20 ng)
- Luria-Bertani (LB) medium
- LB-agar plates with Ampicillin (150 µg/mL)
- Ampicillin stock solution (100 mg/mL)
- Sterile water (dH_2O)
- Ice
- Microcentrifuge tubes

Downstream Applications

The purified plasmid DNA is now ready for:

- In vitro transcription
- Microinjection
- Long-term storage

Storage and Safety

- **Bacteria:** Store competent cells at −80 °C. Do not re-freeze once thawed.
- **Media:** Store sterile LB medium and prepared plates at 4 °C.
- **Disposal:** All liquid waste and used plates are biohazardous. Autoclave all materials before disposal according to institutional guidelines.

Step	Stage	Details
1	Day 1 – Thaw Cells	Thaw a tube of competent cells on ice for 5–10 minutes.
2	Day 1 – Add Plasmid	Add 10–20 ng plasmid DNA to the competent cells; mix gently by flicking the tube 4–5 times.
3	Day 1 – Incubate on Ice	Incubate the cell/DNA mixture on ice for 30 minutes.
4	Day 1 – Heat Shock	Transfer the tube to a 42°C water bath for exactly 45 seconds. Do not exceed this time.
5	Day 1 – Recovery on Ice	Immediately return the tube to ice for 2 minutes.
6	Day 1 – Add Medium	Add 200 µL pre-warmed LB medium (without antibiotics).
7	Day 1 – Recovery Incubation	Incubate at 37°C for 1 hour with shaking (220 rpm).
8	Day 1 – Plate Bacteria	Spread 150 µL of the cell suspension onto pre-warmed LB-Agar plates containing 150 µg/mL Ampicillin. Plate the main transformation on 4 plates. Optional: prepare a 1:10 dilution (100 µL + 900 µL LB) and plate 150 µL on 3 additional plates. Include a non-transformed control plate.
9	Day 1 – Incubate Plates	Incubate plates inverted at 37°C for 16 hours.
10	Day 2 – Check Plates	Confirm colonies on transformed plates and absence of growth on the control plate.
11	Day 2 – Inoculate Cultures	Pick 4 single colonies and inoculate each into 5 mL LB + 150 µg/mL Ampicillin (one colony per tube).
12	Day 2 – Overnight Culture	Incubate cultures at 37°C for 16 hours with shaking (220 rpm).
13	Day 2 – MiniPrep	Isolate plasmid DNA from overnight cultures using a commercial MiniPrep kit.
14	Day 2 – Quantify DNA	Measure concentration and purity (A260/280) by Nanodrop and compare to the original plasmid.

Table 2: Consolidated transformation and plasmid preparation workflow (Day 1–2).

Transformation and Plasmid Preparation (Day 1–2)

9.1.2 Purification Process Table

DNA Purification

Step #	Action DNA	Details DNA
1	Prepare Sample	Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix. If the mixture is orange or violet, add 10 µL of 3 M sodium acetate (pH 5.0) until it turns yellow.
2	Bind DNA	1. Place a QIAquick spin column in a provided 2 mL collection tube. 2. Apply the sample from Step 1 to the column. 3. • Centrifuge for 30 —-60 s. 4. Discard the flow-through and place the column back in the same tube.
3	Wash Column	1. Add 750 µL of Buffer PE to the column. 2. • Centrifuge for 30 —-60 s. 3. Discard the flow-through and place the column back in the same tube.
4	Dry Column	• Centrifuge the column for an additional 1 min to remove any residual wash buffer. *This step is critical.*
5	Elute DNA	1. Place the QIAquick column in a clean 1.5 mL microcentrifuge tube. 2. Add 50 µL of Buffer EB (10 mM Tris-Cl, pH 8.5) or water directly to the center of the membrane. 3. Let the column stand for 1 min. 4. • Centrifuge for 1 min to collect the purified DNA.

RNA Purification

Step #	Action	Details
1	Prepare & Bind	1. Adjust your sample volume to 200 µL with nuclease-free water. 2. Add 100 µL of Binding Buffer and mix by pipetting. 3. Add 300 µL of ethanol (96–100%) and mix by pipetting. 4. Transfer the mixture to an RNA Purification Micro Column. 5. Centrifuge for 30 —-60 s. Discard the flow-through.
2	First Wash	1. Add 700 µL of Wash Buffer I to the column. 2. Centrifuge for 30 —-60 s. Discard the flow-through.
3	Second Wash (x2)	1. Add 700 µL of Wash Buffer II to the column. 2. Centrifuge for 30 —-60 s. Discard the flow-through. 3. Repeat this wash step one more time.
4	Dry Spin	1. Centrifuge the empty column for 1 min to remove all residual ethanol. *This step is essential.*
5	Elute RNA	1. Place the column into a new, clean 1.5 mL tube. 2. Add 10 µL of nuclease-free water directly onto the membrane. 3. Centrifuge for 1 min to collect your concentrated RNA. 4. Discard the column; your RNA is in the tube.

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