Video Article

Manipulation of Gene Function in Mexican Cavefish

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Abstract

Cave animals provide a compelling system for investigating the evolutionary mechanisms and genetic bases underlying changes in numerous complex traits, including eye degeneration, albinism, sleep loss, hyperphagia, and sensory processing. Species of cavefish from around the world display a convergent evolution of morphological and behavioral traits due to shared environmental pressures between different cave systems. Diverse cave species have been studied in the laboratory setting. The Mexican tetra, *Astyanax mexicanus*, with sighted and blind forms, has provided unique insights into biological and molecular processes underlying the evolution of complex traits and is well-poised as an emerging model system. While candidate genes regulating the evolution of diverse biological processes have been identified in A. mexicanus, the ability to validate a role for individual genes has been limited. The application of transgenesis and gene-editing technology has the potential to overcome this significant impediment and to investigate the mechanisms underlying the evolution of complex traits. Here, we describe a different methodology for manipulating gene expression in *A. mexicanus*. Approaches include the use of morpholinos, *Tol2* transgenesis, and gene-editing systems, commonly used in zebrafish and other fish models, to manipulate gene function in *A. mexicanus*. These protocols include detailed descriptions of timed breeding procedures, the collection of fertilized eggs, injections, and the selection of genetically modified animals. These methodological approaches will allow for the investigation of the genetic and neural mechanisms underlying the evolution of diverse traits in *A. mexicanus*.

Video Link

The video component of this article can be found at https://www.jove.com/video/59093/

Introduction

Since Darwin's *Origin of Species*[1], scientists have gained profound insights into how traits are shaped evolutionarily in response to defined environmental and ecological pressures, thanks to cave organisms[2]. The Mexican tetra, *A. mexicanus*, consists of eyed ancestral 'surface' populations that inhabit rivers throughout Mexico and southern Texas and of at least 29 geographically isolated populations of derived cave morphs inhabiting the Sierra del Abra and other areas of Northeast Mexico[3]. A number of cave-associated traits have been identified in *A. mexicanus*, including altered oxygen consumption, depigmentation, loss of eyes, and altered feeding and foraging behavior[4,5,6,7,8,9]. *A. mexicanus* presents a powerful model for investigating mechanisms of convergent evolution due to a well-defined evolutionary history, a detailed characterization of ecological environment, and the presence of independently evolved cave populations[10,11]. Many of the cave-derived traits that are present in cavefish, including eye loss, sleep loss, increased feeding, loss of schooling, reduced aggression, and reduced stress responses, have evolved multiple times through independent origins, often utilizing different genetic pathways between caves[8,12,13,14,15]. This repeated evolution is a powerful aspect of the *A. mexicanus* system and can provide insight into the more general question of how genetic systems may be perturbed to generate similar phenotypes.

While the application of genetic technology for the mechanistic investigation of gene function has been limited in many fish species (including *A. mexicanus*), recent advances in the zebrafish provide a basis for genetic technology development in fish[16,17,18,19,20]. Numerous tools are widely used in zebrafish to manipulate gene expression, and the implementation of these procedures have long been standardized. For example, the injection of morpholino oligos (MOs) at the single-cell stage selectively blocks RNA and prevents translation for a brief temporal window during development[21,22]. In addition, gene-editing approaches, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and transcription activator-like effector nuclease (TALEN), allow for the generation of defined deletions or, in some cases, insertions through a recombination in genomes[19,20,23,24]. Transgenesis is used to manipulate stable gene expression or function in a cell-type specific manner. The *Tol2* system is used effectively to generate transgenic animals by coinjecting transposase mRNA with a *Tol2* DNA plasmid containing a transgene[25,26]. The *Tol2* system utilizes the *Tol2* transposase of medaka to generate stable germline insertions of transgenic construct17. Generating *Tol2* transgenics involves coinjecting a plasmid containing a transgene flanked by *Tol2* integration sites and mRNA for *Tol2* transposase[17]. This system has been used to generate an array of transgenic lines in zebrafish and its use has recently expanded to additional emergent model systems, including cichlids, killifish, the stickleback, and, more recently, the Mexican cavefish[27,28,29,30].

While the cavefish is a fascinating biological system for elucidating mechanisms of trait evolution, its full capability as an evolutionary model has not been fully harnessed. This has partially been due to an inability to manipulate genetic and cellular function directly[31]. Candidate genes regulating complex traits have been identified using quantitative trait loci (QTL) studies, but the validation of these candidate genes has been difficult[32,33,34]. Recently, transient knockdown using morpholinos, gene editing using CRISPR and TALEN systems, and the use of *Tol2*-mediated transgenesis have been used to investigate the genetic basis underlying a number of traits[35,36,37,38]. The implementation and standardization of these techniques will allow for manipulations that interrogate the molecular and neural underpinnings of biological traits, including the manipulation of gene function, the labeling of defined cell populations, and the expression of functional reporters. Whereas the successful implementation of these genetic tools to manipulate gene or cellular function has been demonstrated in emergent model systems, detailed protocols are still lacking in *A. mexicanus*.

A. mexicanus provide critical insight into the mechanisms of evolution in response to a changing environment and present the opportunity to identify novel genes regulating diverse traits. A number of factors suggest that *A. mexicanus* is an extremely tractable model for applying established genomic tools currently available in established genetic models, including the ability to easily maintain fish in the laboratories, large brood size, transparency, a sequenced genome, and defined behavioral assays[39]. Here, we describe a methodology for the use of morpholinos, transgenesis, and gene editing in surface and cave populations of *A. mexicanus*. The broader application of these tools in *A. mexicanus* will allow for a mechanistic investigation into the molecular processes underlying the evolution of developmental, physiological, and behavioral differences between cavefish and surface fish.

Protocol

1. Morpholino oligo design

NOTE: Sequences for *A. mexicanus* are available through National Center of Biotechnology Information (NCBI) Gene and NCBI SRA (https://www.ncbi.nlm.nih.gov), as well as from the Ensembl genome browser (https://www.ensembl.org). When designing a morpholino for use in both surface- and cave-dwelling forms, it is critical to identify any genetic variation between the morphs at this stage, so these genetic regions can be avoided as targets for morpholinos. Any polymorphic variation within a morpholino target site can lead to ineffective binding. The design is similar to other fish systems, such as zebrafish, and has previously been shown to work effectively in *A. mexicanus*[21,36,40].

1. **Design of translation-blocking morpholinos**
   NOTE: Translation-blocking morpholinos block translation by binding to the endogenous start site and impede translational machinery from binding the mRNA sequence through steric hinderance.
   1. Identify the coding region of the target gene starting with the ATG start site.
   2. Record the first 25 base pairs of the target sequence by copy-and-pasting the sequence in a text editor or lab notebook.
   3. Using either online software (e.g., http://reverse-complement.com) or manual translation, generate the reverse complement of the target sequence. Save the resulting reverse complement in a text editor or lab notebook.
   4. Order a morpholino with the reverse complement sequence from a company that generates morpholino oligonucleotides. See **Table of Materials** for companies.

2. **Design of splice-blocking morpholinos**
   NOTE: Splice-blocking morpholinos block splicing and, thus, prevent the formation of a mature mRNA molecule. This provides an alternative method for knockdown when the start sites are not well-defined, or a more optimal approach when validation of knockdown via polymerase chain reaction (PCR) is desired. This benefit of splice-blocking morpholinos (over ATG-blocking MOs) is that exon exclusion or intron inclusion can be readily assessed with reverse transcriptase (RT)-PCR and size differences visualized on a gel. RT-PCR and gel electrophoresis to determine morpholino efficacy should be done using standard laboratory procedures.
   1. Identify the pre-mRNA sequence of the target gene. Utilize available information from the *A. mexicanus* genome via NCBI or Ensembl to determine intron-exon boundaries[33].
   2. Target exon-intron (splice donor) or intron-exon boundary (splice acceptor) sites for intron inclusion or exon exclusion.
      NOTE: The spliceosome normally targets a “GU” sequence (U1 target) in the intron at the 5' splice site and an **“**AG**”** sequence at the 3' (U2 target) intronic splice site. Under normal conditions, the spliceosome U1 and U2 subunits bind these target sites on the pre-mRNA sequence for proper splicing to occur. However, if either of these target sequences is blocked by a morpholino, the spliceosome will move on to the next available U1 or U2 site, causing either an intron exclusion or exon in the mRNA sequence. This will involve planning/optimization, depending on the nature of the target gene[21]. Generally, blocking an internal U1 site redirects the splice to the next available U1 site, causing an exon excision. Alternatively, blocking the first or last splice junction causes an intron inclusion because there is no other site to redirect the splice to. Use sequence software to predict the effect of various exclusions versus inclusions. Predictions can indicate potential frameshifts or premature stop codons, indicating a more effective target site for mRNA disruption.
   3. Once the target site is identified, record its sequence in a text editor or lab book. Make sure the target site is 25 base pairs (bp) long.
   4. Using either online software (e.g., http://reverse-complement.com) or manual translation, generate the reverse complement of the target sequence. Record its sequence in a text editor or lab book.
   5. Order a morpholino with the reverse complement sequence from a company that generates morpholino oligonucleotides. See **Table of Materials** for sample companies.

2. Morpholinos for injection

NOTE: Several concentrations or volumes of MO injection will need to be performed to establish the optimal concentration to inject. Typical injections quantities are 400–800 pg of MO. The effect of morpholino knockdown can persist for up to 6 days postinjection.

1. Obtain the stock morpholino. The stock morpholino arrives lyophilized. Hydrate it with sterile H_2O prior to use at the desired stock concentration (e.g., 4 mM). Store at -20 °C until use.

3. CRISPR gRNA design, in vitro transcription, and preparation

1. **CRISPR gRNA design**
   NOTE: gRNAs were generated using previously published research by Varshney et al.[40] and Wierson et al.[41].
   1. Using a genome browser, identify the coding region of the gene of interest. Using the genomic sequence, identify the gRNA target sequence within an exon by searching for a 20 bp nucleotide target sequencing beginning with GG and followed by a PAM sequence (NGG). A region of the gene after the start (ATG) will be targeted.
      NOTE: If a target sequence with GG at the 5' end cannot be found in the desired exon of the gene, one or both of the G's can be substituted for the first and second nucleotides at the 5' end of the sequence. However, the two G's must be incorporated in the oligo, as these are required for T7 transcription.
   2. Design and order a gene-specific oligonucleotide (oligo A: 5'-TAATACGACTCACTATA**GGNNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGC-3'). Add the gene-specific 20 bp gRNA target sequence (bolded) without the PAM sequence between a T7 promoter sequence (red) and an overlap sequence used to anneal to a second oligonucleotide (blue). Anneal and amplify (see step 3.2.1) this oligo A and a second oligo (oligo B: 5'-GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTT AACTTGCTATTTCTAGCTCTAAAAC-3') to generate the gRNA template used for transcription.
      NOTE: Oligo B is the same for every reaction and need only be ordered 1x.

2. **gRNA preparation and transcription**
   1. Anneal and amplify the oligos. Include the following primers to amplify the gRNA in order to increase yield: 5'-TAATACGACTCACTATA-3' (T7 primer) and 5'- GATCCGCACCGACTCGGTG-3' (3' gRNA primer). Perform a PCR using *Thermococcus kodakaraensis* (KOD) polymerase.
      NOTE: For both primers in oligo A and oligo B, 10 cycles is recommended for a good yield. A detailed protocol can be found in Wierson et al.[41].
   2. Transcribe the gRNA using commercially available in vitro transcription kits (see **Table of Materials**). This is done through modifications to the manufacturer's protocol published by Klaassen et al.[42].
   3. Precipitate, wash, and resuspend the gRNA as described by Klaassen et al.[42].
      NOTE: The gRNA must be resuspended in RNase-free water to prevent degradation.
   4. Record the concentration of the gRNA, which can be determined using a spectrophotometer. Assess the quality of the RNA by running 2 µL on an agarose gel. Aliquot RNA to avoid freeze/thaw and store it at -80 °C until immediately before the injections.

3. **Cas9 preparation and transcription**
   1. Cas9 mRNA can be transcribed using commercially available in vitro transcription kits (see **Table of Materials**) as described previously[43]. Use the nls-Cas9-nls version[43].
   2. Record the concentration of the gRNA and assess its quality by running 2 µL on an agarose gel. Aliquot RNA to avoid decomposition that arises through multiple freeze-thaw cycles, and store the aliquots at -80 °C.

4. Preparation of Tol2 constructs, Tol2 transposase, and transgenesis

1. Prepare Tol2 transgene constructs for injection.
   NOTE: We have successfully utilized published/available zebrafish and medaka constructs in *A. mexicanus*. These constructs are fully functional in *A. mexicanus*, likely because of the high level of sequence homology (refer to the AddGene repository and the Zebrafish Information Network [ZFIN] databases for available constructs). The zebrafish promoter fragments have expressed the transgenes in the expected tissues when used in *A. mexicanus.*
   1. Acquire Tol2 constructs or generate plasmid with a tissue-specific promoter, the desired transgene, and Tol2 arms (see Kwan et al.[44]). Upon receipt, sequence the construct to validate the plasmid.
   2. Perform a midiprep for constructs according to the manufacturer's guidelines. Elute the final plasmid in RNase-free H_2O, determine the concentration with a spectrophotometer, dilute the concentration to 100–300 ng/µL, and aliquot and store the constructs at -20 °C.

2. Digest Tol2 transposase plasmid, and synthesize mRNA.
   1. Obtain a copy of the Tol2 transposase plasmid (pCS-zT2TP) as a template for generating Tol2 mRNA[45].
   2. Midiprep the pCS-zT2TP construct according to the manufacturer's guidelines. Elute it in a low volume of RNase-free water or buffer (~50 µL). Store the aliquots at -20 °C.
   3. Digest the Tol2 plasmid using a restriction enzyme.
      1. Perform a restriction digest on 10 µg of circular pCS-zT2TP plasmid using **Table 1**.
      2. Split the reaction into 2–50 µL reactions and incubate the reactions overnight at 37 °C in a thermal cycler.
      3. On the following day, inactivate the enzyme by heating it to 65 °C for 20 min.
      4. Purify linearized plasmid immediately following the digest, using commercially available PCR purification kits (see **Table of Materials**) per the manufacturers' guidelines. Elute the plasmid in 15 µL of RNase-free H_2O and determine the concentration of the product using a spectrophotometer.
      5. Run 1 µL of digested plasmid and 1 µL of uncut plasmid on a 1.5% agarose gel to confirm linearized plasmid.
   4. Perform an in vitro transcription of Tol2 mRNA.
      1. Utilize 1 µg of linearized pCS-zT2TP plasmid as a template for transcription. Follow the manufacturer's guidelines for in vitro transcription (see **Table of Materials**) as described in **Table 2**.

2. Incubate at 37 °C in a thermal cycler per the manufacturer's guidelines.
3. Add 1 µL of DNase, incubate it at 37 °C in a thermal cycler per the manufacturer's guidelines.
4. Perform lithium chloride precipitation per transcription kit protocol. Resuspend the RNA pellet in ~20–30 µL of RNase-free H_2O.
5. Determine the concentration of the product by using a spectrophotometer and record the RNA quality.
6. Dilute the product to ~100–300 ng/µL and aliquot it into 5–10 µL samples to avoid repeated freeze-thaw. Store them at -80 °C until use.
   NOTE: It is possible to check 1–2 µL of purified Tol2 mRNA for smear/band with gel electrophoresis.

5. Microinjections

1. **Preparation of general tools for injections**
   NOTE: The procedures in this section have been described in detail by Kowalko et al.[46], and an overview with minor modifications is presented here.
   1. Generate injection plates by pouring warm 3% agarose dissolved in fish system water into a 100 mL Petri dish. Carefully place an egg injection mold in the freshly poured agarose to make wells for the fish eggs. Place the side of the mold in agar at a 45° angle and, then, slowly lower it into the agarose; slowly lowering the mold at an angle avoids air getting trapped underneath the mold. Gently remove the mold once the agarose is solidified. The plates can be stored, sealed, at 4 °C for up to 1 week.
   2. Pull needles from borosilicate glass capillaries for injection in an electrode/needle puller according to the manufacturer's guidelines. This protocol will vary per pipette puller; however, a sample needle-pulling program can be found in **Table 3**.
      NOTE: Optimizing the needle is important for injections, as needles that are too long will bend rather than cleanly penetrate the egg.
   3. Make large-bore glass pipettes for the egg transfer by breaking standard glass pipettes so the opening is large enough for the eggs. Using a Bunsen burner, polish the broken end of the glass by exposing the end of the broken pipettes to the flame until it is smooth.

2. **Breeding setup**
   NOTE: There are many different protocols used for breeding *A. mexicanus*. For a detailed protocol, see Borowsky[39]. Start the breeding setup 1 week prior to the injections.
   1. On day 1, place two to three females and three to four males in a single 10 gallon tank maintained at 24 ± 1 °C.
   2. On days 1–7, increase the feeding to ~3x a day. Ensure the diet includes live food, such as black worms and brine shrimp.
   3. On day 6, add a single tank heater set to 27 °C. Lab tank temperatures can vary; therefore, this will be an increase of 2–3 °C relative to the normal tank temperature.
   4. On the evening of day 7, which is the evening (zeitgeber [ZT] 9–11) of injection night, thoroughly clean the tanks with a water-soaked sponge and remove any excess food or debris using a fine mesh net or a siphon.
   5. On the night of day 7, start checking for surface fish eggs at ZT15 and continue to check every 15–30 min until ZT18. For cavefish, start checking for eggs at ZT17 and continue to check every 15–30 min until ZT20.
      NOTE: The times are based on a 14:10 h light:dark cycle using zeitgeber time. Breeding times are estimates and individual labs must determine exact times. It is critical to collect eggs soon after they are released/fertilized in order to inject them at the single-cell stage.

3. **Collection of single-cell stage eggs**
   1. The night in which breeding is expected, examine the tanks every 15–30 min and monitor for eggs at the bottom of the tank. Eggs appear translucent, measuring approximately 1 mm in diameter.
   2. Use a fine mesh fish net to collect eggs and transfer them to a glass bowl filled with fresh fish system water. Examine the eggs under a microscope to confirm that the eggs are at the one-cell stage.
   3. Using glass pipettes, transfer single-cell eggs to the injection plates. Glass pipettes are required at this stage as the eggs will stick to plastic.
   4. Using a pipette, carefully release the eggs into the wells of the agarose injection plate from section 5.1. Fill the rows of the prewarmed (at room temperature) injection plate with the maximum number of eggs (30–40 per row, and up to five rows). Full rows help keep the eggs from moving during injections. Keep the eggs hydrated on the injection plate with a small amount of fish system water until the performance of the injections.

4. **Pico-injection setup and general injection optimization guidelines**
   1. Backfill the injection needles using either gel-loading pipette tips that fit inside the capillary or using standard micropipette tips and adding a 2–4 µL bolus to the end. Once the needle is filled, use forceps to trim the excess length from the injection needle.
   2. Perform microinjections using a needle mounted in a micromanipulator, connected to a picoliter microinjector.
   3. Set the injection time to 0.03 s and the pressure out at ~0.0 psi. The injection pressure will vary accordingly with minor differences between needles, so optimize to achieve a ~1.0 nL injection bolus.
      NOTE: The injection pressure is often in the range of ~10–30 psi.
   4. Standardize the injection bolus by injecting into mineral oil and measuring the bolus size with a slide micrometer to achieve a ~1–1.5 nL injection volume. Adjust the injection pressure (psi) to increase or decrease the bolus volume.
   5. Draw water off of the very top of the eggs using a lab tissue.
      NOTE: The *Astyanax* egg chorion is slightly tougher to penetrate than zebrafish eggs. We find that drawing the water off of the top of the eggs helps facilitate needle penetration into the egg. Plate optimization can allow for ~200 eggs on a single plate.
   6. Use the micromanipulator to penetrate each egg with the needle, and inject directly into the yolk. Once positioned in the yolk, inject the egg by pressing the inject button or injection foot pedal.
      NOTE: A full plate can be injected within ~15 min. The single-cell stage lasts for ~40 min.

5. **Injection of morpholinos**
   NOTE: The amount of morpholino necessary for knockdown without causing toxicity will need to be optimized per gene target; however, a concentration of 400 pg is a good place to start.

1. Prepare morpholino so that 400 pg of morpholino will be injected per egg. Thaw morpholino on ice. The injection solution is comprised of morpholino (at the desired concentration), RNase-free H_2O or Danieau's solution, and phenol red (10% of the final volume). For an example, see **Table 4**.
2. Inject 1 nL per embryo.

6. **CRISPR injections**
   1. Prepare RNA so that 25 pg of gRNA and 300 pg of Cas9 mRNA total will be injected per embryo. For a sample CRISPR/Cas9 injection mixture, see **Table 5**.
   2. Inject 2 nL of gRNA/Cas9 mRNA per embryo directly into the embryo.

7. **Injection of Tol2 transposase and Tol2-flanked plasmid for transgenesis**
   1. Thaw transposase mRNA and Tol2 plasmid on ice. Combine Cas9 mRNA (at 25 ng/µL), desired Tol2 construct (at 25 ng/µL), and phenol red (≤10% of the final volume) in RNase-free water. For a sample Tol2 transgenesis injection mixture, see **Table 6**.
   2. Keep the injection solution and needles on ice to avoid the degradation of mRNA. Inject at 1 nL in volume per embryo.

6. Rearing and screening injected fish

1. **Injected fish husbandry**
   1. After the eggs are injected, immediately transfer them to glass bowls (10 x 5 cm) filled with ~200 mL of fish system water. Eggs are easily rinsed by dipping the injection plates into bowls filled with fish water and rinsing the eggs with a pipette.
   2. Place ~50–80 injected embryos per bowl and rear them at 22–24 °C.
   3. Clean the bowls with injected fish 2x per day to remove dead embryos and change ~20% of the water on a daily basis.
   4. Additional rearing is performed in accordance with previously published protocols[39].

2. **Screening of morpholino-injected individuals**
   1. Visualize the animals under a stereomicroscope to screen for phenotypes. The effect of morpholinos can persist up to ~5 days postinjection[21].
   2. Measure behavioral phenotypes at 4 days postinjection.

3. **Screening for CRISPR indels**
   1. Design primers to amplify genomic DNA around the target site. Design primers so that the target PCR product is approximately 100–125 bp.
      NOTE: For example, for the *oca2* locus, the region surrounding the gRNA target site was amplified using the forward primer 5'-CTCCTCTGTCAGGCTGTGC-3' and the reverse primer 5'- GAAGGGGATGTTGTCTATGAGC-3' for a PCR product length of 105 bp.
   2. Sacrifice embryos or fin clip adult fish according to the institutional animal protocol.
   3. Collect embryos or dissected fins into PCR tubes and extract DNA and perform PCRs. Sample PCR protocols for gene-specific primers can be found in Ma et al.[35].
   4. To assess for mutagenesis, run 5 µL of PCR product on a 3% agarose gel at 70 V for 3 h. Wild-type (nonmutagenized) DNA will result in a PCR product as a distinct band. Mutant DNA will result in a smeary band on the gel.
   5. To determine the sequence of mutant alleles, TA clone the PCR product according to the manufacturer's instructions, pick colonies, and miniprep cultures. Send the resulting DNA for sequencing.
   6. To establish and maintain lines of fish transmitting mutant alleles, cross adult injected fish to wild-type fish, and screen 5–10 embryos to determine if any of the progeny carry a mutant allele of the gene following steps 6.3.1-6.3.6.
      NOTE: Different F_1 individuals from the same F_0 founder fish can carry different mutations. Ensure mutant lines are sequenced to obtain mutations predicted to produce alleles that are out of frame.
   7. Identify fish carrying a mutant allele by PCR using the smeary band assay (steps 6.3.1-6.3.6) or by designing allele-specific PCR primers that will amplify mutant and wild-type bands (**Figure 2C**).
   8. Once a line of fish is established, homozygose mutant alleles to test for recessive phenotypes.

4. **Screening for transgenic positive individuals**
   NOTE: Using constructs containing a fluorescent maker is recommended to streamline the screening for transgenic positive individuals. However, standard PCR screening methods can be used to screen for transmission.
   1. Visualize tissue-specific fluorescent proteins in F_0 fish as early as 2 days postinjection, with an epifluorescence dissecting scope.
      NOTE: The expression in F_0 larvae is mosaic, and positive individuals may have a range of expression phenotypes.
   2. Keep positive individuals as F_0 founder fish.
   3. When F_0s reach maturity, backcross founding fish to nontransgenic individuals derived from the same population/lab stock. Screen F_1 offspring using the same protocol as described in step 6.2.
      NOTE: The expression in F_1 larvae is uniform and ensures consistency among F_1 siblings.
   4. Since Tol2 integration is not site-mediated and integration can vary among founders, select F_1 siblings derived a single F_0 founder and interbreed positive-expressing F_1 siblings to generate F_2s. This offspring will be the basis for a stable line.

Representative Results

Multiple populations of cave-dwelling *A. mexicanus* show reduced sleep and increased wakefulness/activity relative to their surface-dwelling conspecifics[14]. Hypocretin/orexin (HCRT) is a highly conserved neuropeptide, which acts to increase wakefulness, and aberrations in the HCRT pathway cause narcolepsy in humans and other mammals[47,48]. We have previously demonstrated that cave *A. mexicanus* have increased expression of HCRT peptide, suggesting that an increased expression of this peptide may underlie the loss of sleep in cavefish[38]. The MO

knockdown of *hcrt* expression provides a powerful approach for directly examining the effect of increased *hcrt* expression mediating the loss of sleep in cavefish.

To examine the relationship between *hcrt* expression and sleep, we designed a translation-blocking morpholino. The MO targets the first 25 bp of exon one, including the ATG start site (**Figure 1A**,**B**). As a control, we utilized a commercially available scrambled MO control (**Figure 1B**). Using the BLAST algorithm on NCBI, we confirmed that there are no off-target effects for either MO throughout the genome (data not shown). *A. mexicanus* surface fish and Pachón cavefish were bred and their eggs were collected and then injected with 400 pg of MO in a 1 nL-volume at the one-cell stage (**Figure 1C**,**D**). The fish were raised to 4 dpf and then measured for activity and sleep behavior.

Cave *A. mexicanus* injected with the control MO exhibited significantly more locomotor activity and reduced sleep over a 24 h period compared to surface fish also injected with the scrambled MO (**Figure 1E**,**F**), suggesting no effect of the control morpholino injections as the results are consistent with previously published activity and sleep patterns in each morphotype (t = 5.021, df = 88, p < 0.0001). The injection of the HCRT-MO had little effect on sleep in surface fish compared to control-injected fish (t = 0.17, df = 88, p > 0.99). In contrast, *hcrt* knockdown via MO injection had a significant effect on sleep in cave-dwelling fish. Pachón cavefish larvae showed less than a fourfold reduction in locomotor activity, and sleep nearly two times more than control Pachón larvae (**Figure 1E**,**F**; t = 2.694, df = 88, p < 0.05). A comparison of locomotor activity and sleep in MO-knockdown surface- and cave-dwelling fish revealed comparable locomotion and sleep amounts (Figure 1E,F). These data provide a direct link between *hcrt* expression and sleep loss and provide a method to interrogate the biological mechanisms for loss of sleep in the evolutionarily derived cave-dwelling morphs.

Pigmentation loss is a hallmark of cave organisms, and multiple cave-dwelling *Astyanax* populations demonstrate loss of pigmentation. Albinism in Molino and Pachón cavefish has been mapped using QTL mapping to a genomic region containing the gene, *ocular albinism 2* (*oca2*), suggesting mutations in oca2 underlie albinism in cavefish[6].

To validate *oca2* as the causative locus for albinism in Pachón cave morphs, we utilized CRISPR/Cas9 gene editing to mutate this region in surface fish populations. Since exon 21 is deleted in Molino fish[6], we designed a gRNA to target this region of the gene (**Figure 2A**). The genomic sequence, including the gRNA target sequence and the PAM (bolded), is 5'-GGTCATGTGGGTCTCAGCTT**TGG**-3'. This target sequence (without the PAM sequence) was used to generate a gRNA for targeted mutagenesis. Surface breeders were made to mate, and the resulting embryos were collected at the one-cell stage. One-cell stage embryos were injected with Cas9 mRNA and gRNA-targeting *oca2*, and the injected animals were raised to adulthood[43]. The injected adults were made to mate to wild-type surface fish, and the embryos from these crosses were genotyped to determine germ-line transmission, using primers from step 6.3.1. We sequenced a mutant *oca2* allele and identified a germ-line-transmitted 2 bp deletion in *oca2* (**Figure 2B**,**C**). For ease of genotyping, we designed allele-specific primers to identify the wild-type and mutant alleles (**Figure 2D**) and genotyped fish, using PCR followed by gel electrophoresis (**Figure 2E**). We incrossed surface fish heterozygous for this oca2 mutant allele. The resulting progeny were pigmented or albino (**Figure 2F**-**I**). Pigmented individuals were wild-type or heterozygous at the oca2 locus, while albino individuals were homozygous mutant (**Figure 2E**). These data provide a direct link between the *oca2* gene locus and albinism in *A. mexicanus* cavefish.

Myriad behaviors such as sleep, feeding, and stress differ in *A. mexicanus* cavefish relative to surface conspecifics, yet the underlying neuronal determinants between morphs remain unclear. Whole-brain calcium imaging provides a powerful unbiased approach for examining correlations between altered neural activity and modified behavior. We generated surface fish and cavefish with a near-ubiquitous neuronal expression of the genetically encoded calcium indicator, GCaMP6s, using reagents widely used in zebrafish research. *ELAV-like neuron-specific RNA-binding protein 3* (*elavl3*) is expressed endogenously in newly differentiated neurons throughout the central nervous system[49] and has been used in zebrafish to drive the expression of proteins throughout the majority of the nervous system[50]. We obtained a Tol2 construct containing ~2.8 kb zebrafish *elavl3* promoter transcription upstream of the genetically encoded calcium indicator GCaMP6s (a fusion of green fluorescent protein [GFP], calmodulin, and M13, a peptide sequence from myosin light-chain kinase), flanked at the 5' and 3' ends with Tol2 sites (Tol2-*elavl3:GCaMP6s*-Tol2)[51].

A. mexicanus surface fish and Molino cavefish were bred, and the resulting embryos were coinjected at the single-cell stage with 25 ng/µL of the Tol2-*elavl3:GCaMP6s*-Tol2 construct and 25 ng/µL Tol2 transposase mRNA (**Figure 3A**). At 24–48 dpf, larvae were screened for a transient neuronal expression of GCaMP6s. Those injected (F0) embryos with an expression of GCaMP6s were raised to adulthood and backcrossed with wild-type adults derived from the same lineage of surface fish or cavefish. The resulting F1 adults were screened for a stable expression of GCaMP6s expression, and those larvae were maintained to generate stable lines (**Figure 3B**,**C**). Because each F1 adult with a stable expression has likely integrated Tol2-*elavl3*:GCaMP6s-Tol2 at different genomic sites, each F1 is designated a different allele. Using this approach, we have generated stable F1s for surface fish and Molino cavefish populations (**Figure 3B**,**C**), thus enabling live calcium imaging to uncover differences in neuronal activity mediating behavioral changes in the cave environment (**Figure 3C**,**D**). Further, this approach lays the groundwork for the expression of many additional transgenes to characterize and manipulate the gene function in A. mexicanus.

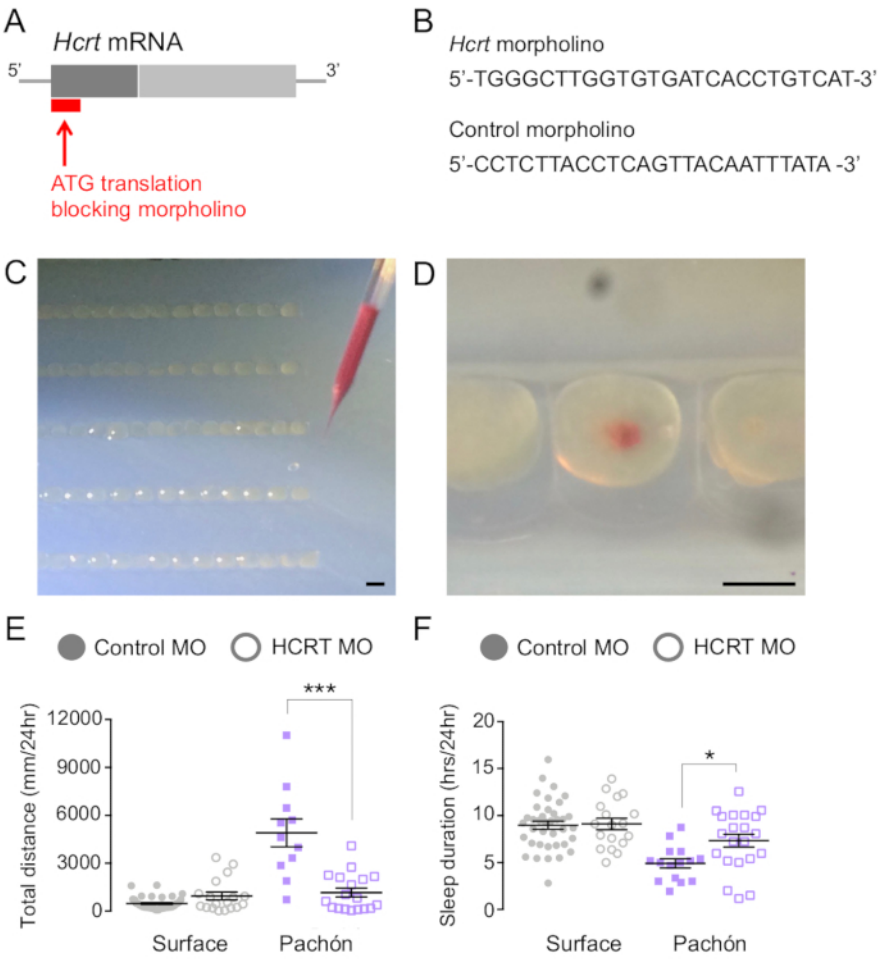

Figure 1: Morpholino knockdown of *Hcrt* reduces activity and increases sleep in cavefish. (**A**) Translation-blocking morpholino targets the first 25 bp of the Hcrt coding sequence, including the ATG start site. (**B**) *Hcrt* morpholino oligo (MO) and control sequences. (**C**) Alignment of ~200 single-cell eggs on agarose egg molds for injection. (**D**) Microinjection of 1.0 nL of injection mixture with phenol red indicator for visualization. The scale bar = 0.5 mm. (**E**) Morpholino knockdown of *Hcrt* reduces the activity (total distance traveled) of Pachón cavefish (t = 5.021, df = 88, $p < 0.0001$) but not of surface fish (t = 1.318, df = 88, $p > 0.72$). (**F**) Knockdown increases sleep in Pachón cavefish (t = 2.694, df = 88, $p < 0.05$) but has no effect on surface fish (t = 0.17, df = 88, $p > 0.99$). The scale bar = 1 mm. The error bars in panels **E** and **F** denote the standard error of the mean. Please click here to view a larger version of this figure.

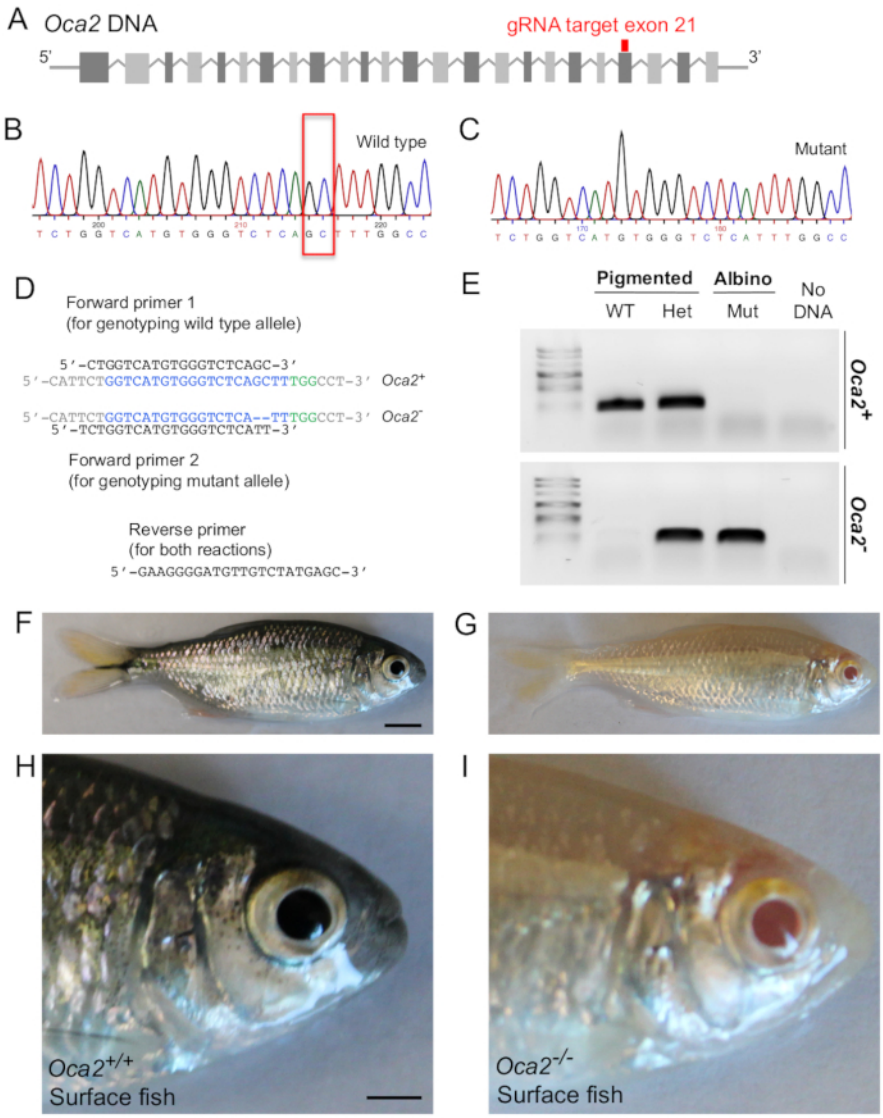

Figure 2: CRISPR gene-editing of *oca2* introduces albinism in surface-dwelling *A. mexicanus*. (**A**) Schematic of the oca2 coding regions, and guide RNA (gRNA) targeting exon 21 for CRISPR-mediated gene editing. (**B**) Sequencing chromatogram of wild-type *Oca2*+ and (**C**) mutant *Oca2*- alleles. The red box in panel **B** indicates the 2 bp sequence, which is missing in the mutant *Oca2*- allele depicted in panel **C**. (**D**) Targeted CRISPR introduces a 2 bp deletion, disrupting *Oca2* function. Primers are designed for the genotypic screening of 2 bp deletion in F0 offspring. (**E**) PCR and gel electrophoresis of *Oca2* in wild-type surface fish (band size = 134 bp) and F0 CRISPR-injected offspring (band size = 133 bp). Individuals homozygous or heterozygous for the wild-type variant of *Oca2* (*Oca2*+) are pigmented, while F0s homozygous for the 2 bp deletion (*Oca2*-) harbor albinism. (**F**) Whole-body images of wild-type surface fish and (**G**) of surface fish with CRISPR-mediated albinism. The scale bar = 5 mm. (**H** and **I**) Magnified view of the heads of individuals depicted in panels **F** and **G**, respectively. The scale bar = 2 mm. Please click here to view a larger version of this figure.

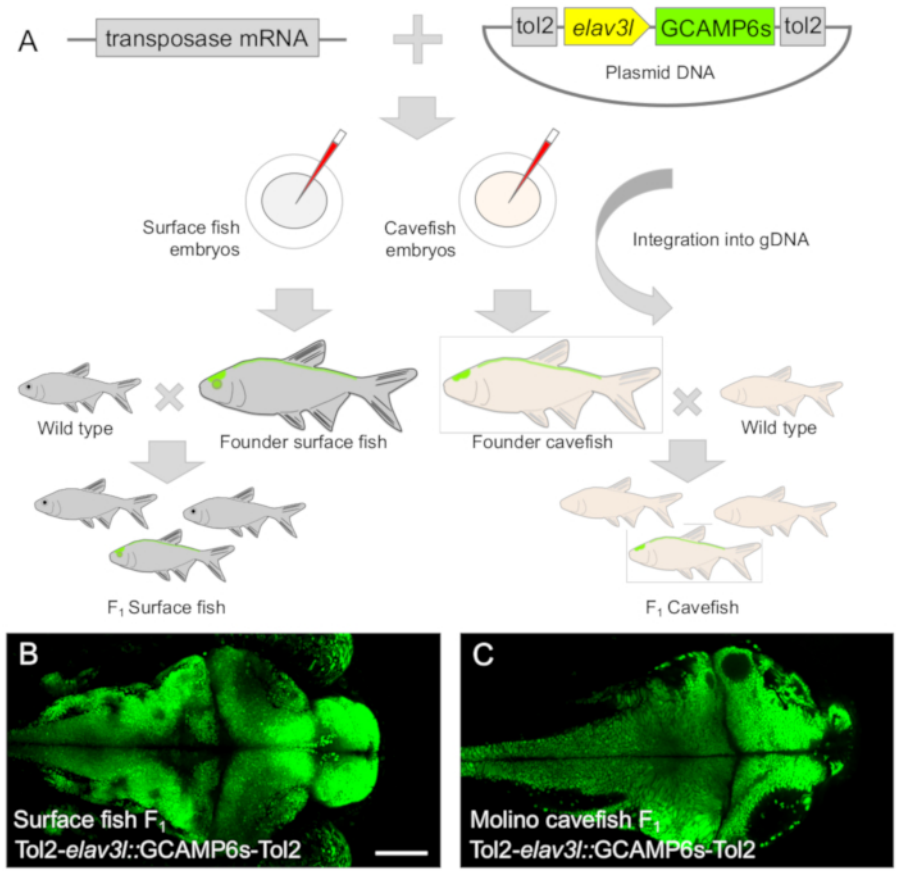

Figure 3: Tol2 transgenesis of pan-neuronal GCAMP6s enables the live imaging of brain activity in *A. mexicanus*. (**A**) Tol2-mediated transgenesis in *A. mexicanus*. Coinjection of Tol2 mRNA and Tol2-*elav3l*:GCAMP6s-Tol2 plasmid integrates GCAMP6s transgene in F0 founders. Backcrossing to the original stock of wild-type fish yields F1 individuals with a stable pan-neuronal expression of GCAMP6s. (**B** and **C**) The resulting offspring are screened for a stable expression using dissection and confocal microscopy. Stable *TgAsty*(*elav3l*:GCaMP6s) F1 individuals have been established for both surface- (depicted in panel B) and cave-dwelling morphotypes (depicted in panel C). The scale bar = 200 µm. Please click here to view a larger version of this figure.

Reagent	Volume or Amount
10.0 µg of pCS-zT2TP plasmid DNA	X µl
NEB CutSmart Buffer	10.0 µl
NotI-HF Enzyme	2.0 µl
Nuclease-free H_2O	X µl
BSA	1.0 µl
Total	**100 µl**

Table 1: Restriction digest of Tol2 plasmid.

Reagent	Volume or Amount
1.0 µg of *linearized* pCS-zT2TP plasmid DNA	X µl
10x Reaction buffer	2.0 µl
2x NTP/CAP	10.0 µl
SP6 enzyme mix	2.0 µl
RNase-free H_2O	X µl
Total	**≤20 µl**

Table 2: In vitro synthesis of Tol2 mRNA.

Setting name	Setting value
Heat	510
Pull	55
Velocity	100
Time	40
Pressure	500
Ramp	534

Table 3: Sample pipette-pulling protocol.

Reagent	Volume or Amount
Morpholino (Freezer stock @ 4mM)	1.0 µl
Nuclease-free H_2O or Danieau's solution	17.0 µl
Phenol Red	2.0 µl
Total	**20 µl**

Table 4: Morpholino injection mixture.

Reagent	Volume of Amount
gRNA (final working concentration @ 100ng/µl)	1 µl
Cas9 mRNA (final working concentration @ 1200ng/µl)	1 µl
RNase-free H_2O	2 µl
Total	**4 µl**

Table 5: Sample CRISPR/Cas9 injection mixture.

Reagent	Volume of Amount
Tol2 plasmid (final working concentration @ 25ng/µl)	X µl
Tol2 mRNA (final working concentration @ 25ng/µl)	X µl
Phenol Red	1.0 µl
RNase-free H_2O	X µl
Total	**15 µl**

Table 6: Sample Tol2 transgenesis injection mixture

Discussion

Here, we provided a methodology for manipulating gene function using morpholinos, CRISPR/Cas9 gene editing, and transgenesis methodology. The wealth of genetic technology and the optimization of these systems in zebrafish will likely allow for the transfer of these tools into *A. mexicanus* with ease[52]. Recent findings have used these approaches in *A. mexicanus*, but they remain underutilized in the investigation of diverse morphological, developmental, and behavioral traits in this system[30,36,42,53].

Morpholinos have been widely used in zebrafish research to knockdown the expression of genes. The approach is facile and results in a robust knockdown of expression. However, off-target effects have been widely documented[22,53]; thus, animals in which morpholinos have been injected to should be carefully monitored for any unexpected phenotypes[22,55]. When possible, results obtained from morpholino knockdown should be validated through the use of other methods, such as CRISPR/Cas9-mediated knockout approaches. While morpholinos allow for the robust knockdown of gene expression, morpholino-mediated knockdown is transient. Thus, analyzing adult phenotypes is not possible when using this method.

CRISPR/Cas9-mediated mutagenesis offers direct manipulation of specific genes. Further, unlike morpholino-mediated knockdown, CRISPR/Cas9 mutagenesis allows for the analysis of mutant phenotypes into adulthood. To prevent off-target effects of CRISPR/Cas9, mutant lines should be outcrossed several generations, and when possible, more than one allele should be obtained and tested. The CRISPR/Cas9 system also provides the potential for utilizing gene-editing approaches to knock-in alleles or to produce specific genetic changes. The CRISPR/Cas9 system has been used in zebrafish to produce precise integrations of exogenous DNA and to generate precise point mutations[19,20,56,57,58,59]. With the sequencing of the cavefish genome, it is now possible to identify single nucleotide polymorphisms (SNPs) or other subtle genetic changes between surface fish and cavefish populations[33]. The application of CRISPR/Cas9 gene editing provides the opportunity to exchange alleles between surface fish and cavefish, or between different populations of cavefish, to examine the role of these genetic changes in different developmental processes.

The transgenesis approaches described in this protocol provide a simple and powerful method for gain-of-function studies and for generating tools to alter biological processes genetically. The Tol2 system is widely used in zebrafish research, and we have shown that it is similarly powerful in *A. mexicanus*. Moreover, we demonstrated a transgenic construct generated in zebrafish that utilizes a zebrafish promoter and recapitulates endogenous expression in *A. mexicanus*. We have found four other promoters isolated from zebrafish that drive tissue-specific expression as expected in *A. mexicanus* (data not shown). Since zebrafish promoters recapitulate their conserved expression patterns in *A. mexicanus*, this suggests that many of the genetic tools can be transferred directly from zebrafish to *A. mexicanus* without the need for modification with *A. mexicanus* promotors. Moreover, with the advance in sequencing technologies in *A. mexicanus*[33], the transgenic approaches described here will permit a powerful future for the investigation of enhancers and promoters that may play a role in the variation between surface and cave forms. Lastly, the powerful tools for the genetic manipulation of biological processes that have made zebrafish valuable are equally important in *A. mexicanus*[60,61,62,63]. Differences in diverse behavioral traits, such as sleep, feeding, stress, and aggression, between *A. mexicanus* surface- and cave-dwelling forms have been extensively documented[12,14,15,38,64], yet the underlying neuronal correlates are not well understood. Tools such as *Tg(elavl3:GCaMP6s)* will permit a dissection of how differences in neuronal activity brain-wide correlate with differences in behavior and offer a unique insight into how the brain has be modified evolutionarily.

Taken together, *A. mexicanus* is poised to become a leading model for investigating the evolution of a variety of morphological and behavioral traits. The diverse differences in complex biological processes in *A. mexicanus* provide a platform for investigating genetic mechanisms of trait evolution. The application of tools for manipulating gene function may help develop this organism into a model that can be applied to investigate biological diseases related to eye degeneration, neurodevelopmental abnormalities, and insomnia.

Disclosures

The authors have nothing to disclose.

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