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Corresponding Author:	Erik Rolando Duboue, Ph.D. Florida Atlantic University Jupiter, FL UNITED STATES
Corresponding Author's Institution:	Florida Atlantic University
Corresponding Author E-Mail:	eduboue@fau.edu
Order of Authors:	Bethany A. Stahl
	James B. Jaggard
	Jacqueline S.R. Chin
	Johanna E. Kowalko
	Alex C. Keene
	Erik Rolando Duboue, Ph.D.
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1 **TITLE:**
2 Manipulation of Gene Function in Mexican Cavefish
3
4 **AUTHORS AND AFFILIATIONS:**
5 Bethany A. Stahl[1,2], James B. Jaggard[1,2], Jacqueline S.R. Chin[1,2], Johanna E. Kowalko[2,3], Alex C.
6 Keene[1,2], Erik R. Duboué[2,3]
7
8 [1]Department of Biological Sciences, Florida Atlantic University, Jupiter, FL, USA
9 [2]Jupiter Life Science Initiative, Florida Atlantic University, Jupiter, FL, USA
10 [3]Harriet L. Wilkes Honors College, Florida Atlantic University, Jupiter, FL, USA
11
12 Corresponding author:
13 Erik R. Duboué   (eduboue@fau.edu)
14
15 Email addresses of co-authors:
16 Bethany A. Stahl   (bstahl@fau.edu)
17 James B. Jaggard   (jjaggard2015@fau.edu)
18 Jacqueline S.R. Chin   (chin@fau.edu)
19 Johanna E. Kowalko   (jkowalko@fau.edu)
20 Alex C. Keene   (keenea@fau.edu)
21
22 **KEYWORDS:**
23 cavefish, CRISPR, gene-editing, transgenesis, morpholino, knockdown
24
25 **SUMMARY:**
26 We describe approaches for the manipulation of genes in the evolutionary model system
27 *Astyanax mexicanus*. Three different techniques are described: Tol2-mediated transgenesis,
28 targeted manipulation of the genome using CRISPR/Cas9, and knockdown of expression using
29 morpholinos. These tools should facilitate the direct investigation of genes underlying the
30 variation between surface- and cave-dwelling forms.
31

32 **ABSTRACT:**
33 Cave animals provide a compelling system for investigating the evolutionary mechanisms and
34 genetic bases underlying changes in numerous complex traits, including eye degeneration,
35 albinism, sleep loss, hyperphagia, and sensory processing. Species of cavefish from around the
36 world display a convergent evolution of morphological and behavioral traits due to shared
37 environmental pressures between different cave systems. Diverse cave species have been
38 studied in the laboratory setting. The Mexican tetra, *Astyanax mexicanus,* with sighted and blind
39 forms, has provided unique insights into biological and molecular processes underlying the
40 evolution of complex traits and is well-poised as an emerging model system. While candidate
41 genes regulating the evolution of diverse biological processes have been identified in *A.*
42 *mexicanus*, the ability to validate a role for individual genes has been limited. The application of
43 transgenesis and gene-editing technology has the potential to overcome this significant
44 impediment and to investigate the mechanisms underlying the evolution of complex traits. Here,

45 we describe a different methodology for manipulating gene expression in *A. mexicanus*.
46 Approaches include the use of morpholinos, *Tol2* transgenesis, and gene-editing systems,
47 commonly used in zebrafish and other fish models, to manipulate gene function in *A. mexicanus*.
48 These protocols include detailed descriptions of timed breeding procedures, the collection of
49 fertilized eggs, injections, and the selection of genetically modified animals. These
50 methodological approaches will allow for the investigation of the genetic and neural mechanisms
51 underlying the evolution of diverse traits in *A. mexicanus*.
52
53 **INTRODUCTION:**
54 Since Darwin's *Origin of Species*[1], scientists have gained profound insights into how traits are
55 shaped evolutionarily in response to defined environmental and ecological pressures, thanks to
56 cave organisms[2]. The Mexican tetra, *A. mexicanus,* consists of eyed ancestral 'surface'
57 populations that inhabit rivers throughout Mexico and southern Texas and of at least 29
58 geographically isolated populations of derived cave morphs inhabiting the Sierra del Abra and
59 other areas of Northeast Mexico[3]. A number of cave-associated traits have been identified in *A.*
60 *mexicanus*, including altered oxygen consumption, depigmentation, loss of eyes, and altered
61 feeding and foraging behavior[4–9]. *A. mexicanus* presents a powerful model for investigating
62 mechanisms of convergent evolution due to a well-defined evolutionary history, a detailed
63 characterization of ecological environment, and the presence of independently evolved cave
64 populations[10, 11]. Many of the cave-derived traits that are present in cavefish, including eye loss,
65 sleep loss, increased feeding, loss of schooling, reduced aggression, and reduced stress
66 responses, have evolved multiple times through independent origins, often utilizing different
67 genetic pathways between caves[8,12–15]. This repeated evolution is a powerful aspect of the *A.*
68 *mexicanus* system and can provide insight into the more general question of how genetic systems
69 may be perturbed to generate similar phenotypes.
70
71 While the application of genetic technology for the mechanistic investigation of gene function
72 has been limited in many fish species (including *A. mexicanus*), recent advances in the zebrafish
73 provide a basis for genetic technology development in fish[16–20]. Numerous tools are widely used
74 in zebrafish to manipulate gene expression, and the implementation of these procedures have
75 long been standardized. For example, the injection of morpholino oligos (MOs) at the single-cell
76 stage selectively blocks RNA and prevents translation for a brief temporal window during
77 development[21,22]. In addition, gene-editing approaches, such as clustered regularly interspaced
78 short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and transcription
79 activator-like effector nuclease (TALEN), allow for the generation of defined deletions or, in some
80 cases, insertions through a recombination in genomes[19,20,23,24]. Transgenesis is used to
81 manipulate stable gene expression or function in a cell-type specific manner. The *Tol2* system is
82 used effectively to generate transgenic animals by coinjecting transposase mRNA with a *Tol2* DNA
83 plasmid containing a transgene[25,26]. The *Tol2* system utilizes the *Tol2* transposase of medaka to
84 generate stable germline insertions of transgenic construct[17]. Generating *Tol2* transgenics
85 involves coinjecting a plasmid containing a transgene flanked by *Tol2* integration sites and mRNA
86 for *Tol2* transposase[17]. This system has been used to generate an array of transgenic lines in
87 zebrafish and its use has recently expanded to additional emergent model systems, including
88 cichlids, killifish, the stickleback, and, more recently, the Mexican cavefish[27–30].

89

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

103

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

114

115 **PROTOCOL:**

116

117 **1. Morpholino oligo design**

118

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

127

128 **1.1. Design of translation-blocking morpholinos**

129

130 NOTE: Translation-blocking morpholinos block translation by binding to the endogenous start site
131 and impede translational machinery from binding the mRNA sequence through steric hinderance.

132

133   1.1.1. Identify the coding region of the target gene starting with the ATG start site.

134

135   1.1.2. Record the first 25 base pairs of the target sequence by copy-and-pasting the sequence in
136   a text editor or lab notebook.

137

138   1.1.3. Using either online software (e.g., http://reverse-complement.com) or manual translation,
139   generate the reverse complement of the target sequence. Save the resulting reverse
140   complement in a text editor or lab notebook.

141

142   1.1.4. Order a morpholino with the reverse complement sequence from a company that
143   generates morpholino oligonucleotides. See **Table of Materials** for companies.

144

145   **1.2. Design of splice-blocking morpholinos**

146

147   NOTE: Splice-blocking morpholinos block splicing and, thus, prevent the formation of a mature
148   mRNA molecule. This provides an alternative method for knockdown when the start sites are not
149   well-defined, or a more optimal approach when validation of knockdown via polymerase chain
150   reaction (PCR) is desired. This benefit of splice-blocking morpholinos (over ATG-blocking MOs) is
151   that exon exclusion or intron inclusion can be readily assessed with reverse transcriptase (RT)-
152   PCR and size differences visualized on a gel. RT-PCR and gel electrophoresis to determine
153   morpholino efficacy should be done using standard laboratory procedures.

154

155   1.2.1. Identify the pre-mRNA sequence of the target gene. Utilize available information from the
156   *A. mexicanus* genome via NCBI or Ensembl to determine intron-exon boundaries[33].

157

158   1.2.2. Target exon-intron (splice donor) or intron-exon boundary (splice acceptor) sites for intron
159   inclusion or exon exclusion.

160

161   NOTE: The spliceosome normally targets a "GU" sequence (U1 target) in the intron at the 5' splice
162   site and an "AG" sequence at the 3' (U2 target) intronic splice site. Under normal conditions, the
163   spliceosome U1 and U2 subunits bind these target sites on the pre-mRNA sequence for proper
164   splicing to occur. However, if either of these target sequences is blocked by a morpholino, the
165   spliceosome will move on to the next available U1 or U2 site, causing either an intron exclusion
166   or exon in the mRNA sequence. This will involve planning/optimization, depending on the nature
167   of the target gene[21]. Generally, blocking an internal U1 site redirects the splice to the next
168   available U1 site, causing an exon excision. Alternatively, blocking the first or last splice junction
169   causes an intron inclusion because there is no other site to redirect the splice to. Use sequence
170   software to predict the effect of various exclusions versus inclusions. Predictions can indicate
171   potential frameshifts or premature stop codons, indicating a more effective target site for mRNA
172   disruption.

173

174   1.2.3. Once the target site is identified, record its sequence in a text editor or lab book. Make
175   sure the target site is 25 base pairs (bp) long.

176

177 1.2.4. Using either online software (e.g., http://reverse-complement.com) or manual translation,
178 generate the reverse complement of the target sequence. Record its sequence in a text editor or
179 lab book.
180
181 1.2.5. Order a morpholino with the reverse complement sequence from a company that
182 generates morpholino oligonucleotides. See **Table of Materials** for sample companies.
183
184 **2. Morpholinos for injection**
185
186 NOTE: Several concentrations or volumes of MO injection will need to be performed to establish
187 the optimal concentration to inject. Typical injections quantities are 400–800 pg of MO. The
188 effect of morpholino knockdown can persist for up to 6 days postinjection.
189
190 2.1. Obtain the stock morpholino. The stock morpholino arrives lyophilized. Hydrate it with sterile
191 H_2O prior to use at the desired stock concentration (e.g., 4 mM). Store at -20 °C until use.
192
193 **3. CRISPR gRNA design, in vitro transcription, and preparation**
194
195 **3.1. CRISPR gRNA design**
196
197 NOTE: gRNAs were generated using previously published research by Varshney et al.[40] and
198 Wierson et al.[41].
199
200 3.1.1. Using a genome browser, identify the coding region of the gene of interest. Using the
201 genomic sequence, identify the gRNA target sequence within an exon by searching for a 20 bp
202 nucleotide target sequencing beginning with GG and followed by a PAM sequence (NGG). A
203 region of the gene after the start (ATG) will be targeted.
204
205 NOTE: If a target sequence with GG at the 5' end cannot be found in the desired exon of the gene,
206 one or both of the G's can be substituted for the first and second nucleotides at the 5' end of the
207 sequence. However, the two G's must be incorporated in the oligo, as these are required for T7
208 transcription.
209
210 3.1.2. Design and order a gene-specific oligonucleotide (oligo A: 5'-
211 TAATACGACTCACTATA**GGNNNNNNNNNNNNNNNNNNNN**GTTTTAGAGCTAGAAATAGC-3'). Add the
212 gene-specific 20 bp gRNA target sequence (bolded) without the PAM sequence between a T7
213 promoter sequence (red) and an overlap sequence used to anneal to a second oligonucleotide
214 (blue). Anneal and amplify (see step 3.2.1) this oligo A and a second oligo (oligo B: 5'-
215 GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTT
216 AACTTGCTATTTCTAGCTCTAAAAC-3') to generate the gRNA template used for transcription.
217
218 NOTE: Oligo B is the same for every reaction and need only be ordered 1x.
219
220 **3.2. gRNA preparation and transcription**

221

222 3.2.1. Anneal and amplify the oligos. Include the following primers to amplify the gRNA in order
223 to increase yield: 5'-TAATACGACTCACTATA-3' (T7 primer) and 5'- GATCCGCACCGACTCGGTG-3'
224 (3' gRNA primer). Perform a PCR using *Thermococcus kodakaraensis* (KOD) polymerase.

225

226 NOTE: For both primers in oligo A and oligo B, 10 cycles is recommended for a good yield. A
227 detailed protocol can be found in Wierson et al.[41].

228

229 3.2.2. Transcribe the gRNA using commercially available in vitro transcription kits (see **Table of**
230 **Materials**). This is done through modifications to the manufacturer's protocol published by
231 Klaassen et al.[42].

232

233 3.2.3. Precipitate, wash, and resuspend the gRNA as described by Klaassen et al.[42].

234

235 NOTE: The gRNA must be resuspended in RNase-free water to prevent degradation.

236

237 3.2.4. Record the concentration of the gRNA, which can be determined using a
238 spectrophotometer. Assess the quality of the RNA by running 2 µL on an agarose gel. Aliquot RNA
239 to avoid freeze/thaw and store it at -80 °C until immediately before the injections.

240

241 **3.3. Cas9 preparation and transcription**

242

243 3.3.1. Cas9 mRNA can be transcribed using commercially available in vitro transcription kits (see
244 **Table of Materials**) as described previously[43]. Use the nls-Cas9-nls version[43].

245

246 3.3.2. Record the concentration of the gRNA and assess its quality by running 2 µL on an agarose
247 gel. Aliquot RNA to avoid decomposition that arises through multiple freeze-thaw cycles, and
248 store the aliquots at -80 °C.

249

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

251

252 4.1. Prepare Tol2 transgene constructs for injection.

253

254 NOTE: We have successfully utilized published/available zebrafish and medaka constructs in *A.*
255 *mexicanus*. These constructs are fully functional in *A. mexicanus*, likely because of the high level
256 of sequence homology (refer to the AddGene repository and the Zebrafish Information Network
257 [ZFIN] databases for available constructs). The zebrafish promoter fragments have expressed the
258 transgenes in the expected tissues when used in *A. mexicanus.*

259

260 4.1.1. Acquire Tol2 constructs or generate plasmid with a tissue-specific promoter, the desired
261 transgene, and Tol2 arms (see Kwan et al.[44]). Upon receipt, sequence the construct to validate
262 the plasmid.

263

264   4.1.2. Perform a midiprep for constructs according to the manufacturer's guidelines. Elute the
265   final plasmid in RNase-free H_2O, determine the concentration with a spectrophotometer, dilute
266   the concentration to 100–300 ng/μL, and aliquot and store the constructs at -20 °C.
267
268   4.2. Digest Tol2 transposase plasmid, and synthesize mRNA.
269
270   4.2.1. Obtain a copy of the Tol2 transposase plasmid (pCS-zT2TP) as a template for generating
271   Tol2 mRNA[45].
272
273   4.2.2. Midiprep the pCS-zT2TP construct according to the manufacturer's guidelines. Elute it in a
274   low volume of RNase-free water or buffer (~50 μL). Store the aliquots at -20 °C.
275
276   4.2.3. Digest the Tol2 plasmid using a restriction enzyme.
277
278   4.2.3.1. Perform a restriction digest on 10 μg of circular pCS-zT2TP plasmid using **Table 1**.
279
280   4.2.3.2. Split the reaction into 2–50 μL reactions and incubate the reactions overnight at 37 °C in
281   a thermal cycler.
282
283   4.2.3.3. On the following day, inactivate the enzyme by heating it to 65 °C for 20 min.
284
285   4.2.3.4. Purify linearized plasmid immediately following the digest, using commercially available
286   PCR purification kits (see **Table of Materials**) per the manufacturers' guidelines. Elute the plasmid
287   in 15 μL of RNase-free H_2O and determine the concentration of the product using a
288   spectrophotometer.
289
290   4.2.3.5. Run 1 μL of digested plasmid and 1 μL of uncut plasmid on a 1.5% agarose gel to confirm
291   linearized plasmid.
292
293   4.2.4. Perform an in vitro transcription of Tol2 mRNA.
294
295   4.2.4.1. Utilize 1 μg of linearized pCS-zT2TP plasmid as a template for transcription. Follow the
296   manufacturer's guidelines for in vitro transcription (see **Table of Materials**) as described in **Table
297   2**.
298
299   4.2.4.2. Incubate at 37 °C in a thermal cycler per the manufacturer's guidelines.
300
301   4.2.4.3. Add 1 μL of DNase, incubate it at 37 °C in a thermal cycler per the manufacturer's
302   guidelines.
303
304   4.2.4.4. Perform lithium chloride precipitation per transcription kit protocol. Resuspend the RNA
305   pellet in ~20–30 μL of RNase-free H_2O.
306

307 4.2.4.5. Determine the concentration of the product by using a spectrophotometer and record
308 the RNA quality.

310 4.2.4.6. Dilute the product to ~100–300 ng/μL and aliquot it into 5–10 μL samples to avoid
311 repeated freeze-thaw. Store them at -80 °C until use.

313 NOTE: It is possible to check 1–2 μL of purified Tol2 mRNA for smear/band with gel
314 electrophoresis.

316 **5. Microinjections**

318 **5.1. Preparation of general tools for injections**

320 NOTE: The procedures in this section have been described in detail by Kowalko et al.[46], and an
321 overview with minor modifications is presented here.

323 5.1.1. Generate injection plates by pouring warm 3% agarose dissolved in fish system water into
324 a 100 mL Petri dish. Carefully place an egg injection mold in the freshly poured agarose to make
325 wells for the fish eggs. Place the side of the mold in agar at a 45° angle and, then, slowly lower it
326 into the agarose; slowly lowering the mold at an angle avoids air getting trapped underneath the
327 mold. Gently remove the mold once the agarose is solidified. The plates can be stored, sealed, at
328 4 °C for up to 1 week.

330 5.1.2. Pull needles from borosilicate glass capillaries for injection in an electrode/needle puller
331 according to the manufacturer's guidelines. This protocol will vary per pipette puller; however, a
332 sample needle-pulling program can be found in **Table 3**.

334 NOTE: Optimizing the needle is important for injections, as needles that are too long will bend
335 rather than cleanly penetrate the egg.

337 5.1.3. Make large-bore glass pipettes for the egg transfer by breaking standard glass pipettes so
338 the opening is large enough for the eggs. Using a Bunsen burner, polish the broken end of the
339 glass by exposing the end of the broken pipettes to the flame until it is smooth.

341 **5.2. Breeding setup**

343 NOTE: There are many different protocols used for breeding *A. mexicanus*. For a detailed
344 protocol, see Borowsky[39]. Start the breeding setup 1 week prior to the injections.

346 5.2.1. On day 1, place two to three females and three to four males in a single 10 gallon tank
347 maintained at 24 ± 1 °C.

349 5.2.2. On days 1–7, increase the feeding to ~3x a day. Ensure the diet includes live food, such as
350 black worms and brine shrimp.

351

5.2.3. 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.

5.2.4. 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.

5.2.5. 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.

5.3. Collection of single-cell stage eggs

5.3.1. 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.

5.3.2. 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.

5.3.3. 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.

5.3.4. 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.

5.4. Pico-injection setup and general injection optimization guidelines

5.4.1. 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.

5.4.2. Perform microinjections using a needle mounted in a micromanipulator, connected to a picoliter microinjector.

395 5.4.3. Set the injection time to 0.03 s and the pressure out at ~0.0 psi. The injection pressure will
396 vary accordingly with minor differences between needles, so optimize to achieve a ~1.0 nL
397 injection bolus.
398
399 NOTE: The injection pressure is often in the range of ~10–30 psi.
400
401 5.4.5. Standardize the injection bolus by injecting into mineral oil and measuring the bolus size
402 with a slide micrometer to achieve a ~1–1.5 nL injection volume. Adjust the injection pressure
403 (psi) to increase or decrease the bolus volume.
404
405 5.4.6. Draw water off of the very top of the eggs using a lab tissue.
406
407 NOTE: The *Astyanax* egg chorion is slightly tougher to penetrate than zebrafish eggs. We find that
408 drawing the water off of the top of the eggs helps facilitate needle penetration into the egg. Plate
409 optimization can allow for ~200 eggs on a single plate.
410
411 5.4.7. Use the micromanipulator to penetrate each egg with the needle, and inject directly into
412 the yolk. Once positioned in the yolk, inject the egg by pressing the inject button or injection foot
413 pedal.
414
415 NOTE: A full plate can be injected within ~15 min. The single-cell stage lasts for ~40 min.
416
417 **5.5. Injection of morpholinos**
418
419 NOTE: The amount of morpholino necessary for knockdown without causing toxicity will need to
420 be optimized per gene target; however, a concentration of 400 pg is a good place to start.
421
422 5.5.1. Prepare morpholino so that 400 pg of morpholino will be injected per egg. Thaw
423 morpholino on ice. The injection solution is comprised of morpholino (at the desired
424 concentration), RNase-free H_2O or Danieau's solution, and phenol red (10% of the final volume).
425 For an example, see **Table 4**.
426
427 5.5.2. Inject 1 nL per embryo.
428
429 **5.6. CRISPR injections**
430
431 5.6.1. Prepare RNA so that 25 pg of gRNA and 300 pg of Cas9 mRNA total will be injected per
432 embryo. For a sample CRISPR/Cas9 injection mixture, see **Table 5**.
433
434 5.6.2. Inject 2 nL of gRNA/Cas9 mRNA per embryo directly into the embryo.
435
436 **5.7. Injection of Tol2 transposase and Tol2-flanked plasmid for transgenesis**
437

438 5.7.1. Thaw transposase mRNA and Tol2 plasmid on ice. Combine Cas9 mRNA (at 25 ng/µL),
439 desired Tol2 construct (at 25 ng/µL), and phenol red (≤10% of the final volume) in RNase-free
440 water. For a sample Tol2 transgenesis injection mixture, see **Table 6**.
441
442 5.7.2. Keep the injection solution and needles on ice to avoid the degradation of mRNA. Inject at
443 1 nL in volume per embryo.
444
445 **6. Rearing and screening injected fish**
446
447 **6.1. Injected fish husbandry**
448
449 6.1.1. After the eggs are injected, immediately transfer them to glass bowls (10 x 5 cm) filled with
450 ~200 mL of fish system water. Eggs are easily rinsed by dipping the injection plates into bowls
451 filled with fish water and rinsing the eggs with a pipette.
452
453 6.1.2. Place ~50–80 injected embryos per bowl and rear them at 22–24 °C.
454
455 6.1.3. Clean the bowls with injected fish 2x per day to remove dead embryos and change ~20%
456 of the water on a daily basis.
457
458 6.1.4. Additional rearing is performed in accordance with previously published protocols[39].
459
460 **6.2. Screening of morpholino-injected individuals**
461
462 6.2.1. Visualize the animals under a stereomicroscope to screen for phenotypes. The effect of
463 morpholinos can persist up to ~5 days postinjection[21].
464
465 6.2.2. Measure behavioral phenotypes at 4 days postinjection.
466
467 **6.3. Screening for CRISPR indels**
468
469 6.3.1. Design primers to amplify genomic DNA around the target site. Design primers so that the
470 target PCR product is approximately 100–125 bp.
471
472 NOTE: For example, for the *oca2* locus, the region surrounding the gRNA target site was amplified
473 using the forward primer 5'-CTCCTCTGTCAGGCTGTGC-3' and the reverse primer 5'-
474 GAAGGGGATGTTGTCTATGAGC-3' for a PCR product length of 105 bp.
475
476 6.3.2. Sacrifice embryos or fin clip adult fish according to the institutional animal protocol.
477
478 6.3.3. Collect embryos or dissected fins into PCR tubes and extract DNA and perform PCRs.
479 Sample PCR protocols for gene-specific primers can be found in Ma et al.[35].
480
481 6.3.4. To assess for mutagenesis, run 5 µL of PCR product on a 3% agarose gel at 70 V for 3 h.

<mark>Wild-type (nonmutagenized) DNA will result in a PCR product as a distinct band. Mutant DNA will</mark>
<mark>result in a smeary band on the gel.</mark>

485 6.3.5. To determine the sequence of mutant alleles, TA clone the PCR product according to the
486 manufacturer's instructions, pick colonies, and miniprep cultures. Send the resulting DNA for
487 sequencing.

489 6.3.6. To establish and maintain lines of fish transmitting mutant alleles, cross adult injected fish
490 to wild-type fish, and screen 5–10 embryos to determine if any of the progeny carry a mutant
491 allele of the gene following steps 6.3.1-6.3.6.

493 NOTE: Different F_1 individuals from the same F_0 founder fish can carry different mutations. Ensure
494 mutant lines are sequenced to obtain mutations predicted to produce alleles that are out of
495 frame.

497 6.3.7. Identify fish carrying a mutant allele by PCR using the smeary band assay (steps 6.3.1-6.3.6)
498 or by designing allele-specific PCR primers that will amplify mutant and wild-type bands (**Figure**
499 **2C**).

501 6.3.8. Once a line of fish is established, homozygose mutant alleles to test for recessive
502 phenotypes.

504 **6.4. Screening for transgenic positive individuals**

506 NOTE: Using constructs containing a fluorescent maker is recommended to streamline the
507 screening for transgenic positive individuals. However, standard PCR screening methods can be
508 used to screen for transmission.

510 6.4.1. Visualize tissue-specific fluorescent proteins in F_0 fish as early as 2 days postinjection, with
511 an epifluorescence dissecting scope.

513 NOTE: The expression in F_0 larvae is mosaic, and positive individuals may have a range of
514 expression phenotypes.

516 6.4.2. Keep positive individuals as F_0 founder fish.

518 6.4.3. When F_0s reach maturity, backcross founding fish to nontransgenic individuals derived
519 from the same population/lab stock. Screen F_1 offspring using the same protocol as described in
520 step 6.2.

522 NOTE: The expression in F_1 larvae is uniform and ensures consistency among F_1 siblings.

523

524   6.4.4. Since Tol2 integration is not site-mediated and integration can vary among founders, select
525   F_1 siblings derived a single F_0 founder and interbreed positive-expressing F_1 siblings to generate
526   F_2s. This offspring will be the basis for a stable line.
527
528   **REPRESENTATIVE RESULTS:**
529   Multiple populations of cave-dwelling *A. mexicanus* show reduced sleep and increased
530   wakefulness/activity relative to their surface-dwelling conspecifics[14]. Hypocretin/orexin (HCRT)
531   is a highly conserved neuropeptide, which acts to increase wakefulness, and aberrations in the
532   HCRT pathway cause narcolepsy in humans and other mammals[47,48]. We have previously
533   demonstrated that cave *A. mexicanus* have increased expression of HCRT peptide, suggesting
534   that an increased expression of this peptide may underlie the loss of sleep in cavefish[38]. The MO
535   knockdown of *hcrt* expression provides a powerful approach for directly examining the effect of
536   increased *hcrt* expression mediating the loss of sleep in cavefish.
537
538   To examine the relationship between *hcrt* expression and sleep, we designed a translation-
539   blocking morpholino. The MO targets the first 25 bp of exon one, including the ATG start site
540   (**Figure 1A,B**). As a control, we utilized a commercially available scrambled MO control (**Figure**
541   **1B**). Using the BLAST algorithm on NCBI, we confirmed that there are no off-target effects for
542   either MO throughout the genome (data not shown). *A. mexicanus* surface fish and Pachón
543   cavefish were bred and their eggs were collected and then injected with 400 pg of MO in a 1 nL-
544   volume at the one-cell stage (**Figure 1C,D**). The fish were raised to 4 dpf and then measured for
545   activity and sleep behavior.
546
547   Cave *A. mexicanus* injected with the control MO exhibited significantly more locomotor activity
548   and reduced sleep over a 24 h period compared to surface fish also injected with the scrambled
549   MO (**Figure 1E,F**), suggesting no effect of the control morpholino injections as the results are
550   consistent with previously published activity and sleep patterns in each morphotype (t = 5.021,
551   df = 88, $p < 0.0001$). The injection of the HCRT-MO had little effect on sleep in surface fish
552   compared to control-injected fish (t = 0.17, df = 88, $p > 0.99$). In contrast, *hcrt* knockdown via MO
553   injection had a significant effect on sleep in cave-dwelling fish. Pachón cavefish larvae showed
554   less than a fourfold reduction in locomotor activity, and sleep nearly two times more than control
555   Pachón larvae (**Figure 1E,F**; t = 2.694, df = 88, $p < 0.05$). A comparison of locomotor activity and
556   sleep in MO-knockdown surface- and cave-dwelling fish revealed comparable locomotion and
557   sleep amounts (**Figure 1E,F**). These data provide a direct link between *hcrt* expression and sleep
558   loss and provide a method to interrogate the biological mechanisms for loss of sleep in the
559   evolutionarily derived cave-dwelling morphs.
560
561   Pigmentation loss is a hallmark of cave organisms, and multiple cave-dwelling *Astyanax*
562   populations demonstrate loss of pigmentation. Albinism in Molino and Pachón cavefish has been
563   mapped using QTL mapping to a genomic region containing the gene, *ocular albinism 2* (*oca2*),
564   suggesting mutations in *oca2* underlie albinism in cavefish[6].
565
566   To validate *oca2* as the causative locus for albinism in Pachón cave morphs, we utilized
567   CRISPR/Cas9 gene editing to mutate this region in surface fish populations. Since exon 21 is

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

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

599 *A. mexicanus* surface fish and Molino cavefish were bred, and the resulting embryos were
600 coinjected at the single-cell stage with 25 ng/μL of the Tol2-*elavl3:GCaMP6s*-Tol2 construct and
601 25 ng/μL Tol2 transposase mRNA (**Figure 3A**). At 24–48 dpf, larvae were screened for a transient
602 neuronal expression of GCaMP6s. Those injected (F_0) embryos with an expression of GCaMP6s
603 were raised to adulthood and backcrossed with wild-type adults derived from the same lineage
604 of surface fish or cavefish. The resulting F_1 adults were screened for a stable expression of
605 GCaMP6s expression, and those larvae were maintained to generate stable lines (**Figure 3B,C**).
606 Because each F_1 adult with a stable expression has likely integrated Tol2-*elavl3*:GCaMP6s-Tol2 at
607 different genomic sites, each F_1 is designated a different allele. Using this approach, we have
608 generated stable F_1s for surface fish and Molino cavefish populations (**Figure 3B,C**), thus enabling
609 live calcium imaging to uncover differences in neuronal activity mediating behavioral changes in
610 the cave environment (**Figure 3C,D**). Further, this approach lays the groundwork for the

611 expression of many additional transgenes to characterize and manipulate the gene function in *A.*
612 *mexicanus.*
613
614 **FIGURE AND TABLE LEGENDS:**
615
616 **Figure 1: Morpholino knockdown of *Hcrt* reduces activity and increases sleep in cavefish.** (**A**)
617 Translation-blocking morpholino targets the first 25 bp of the *Hcrt* coding sequence, including
618 the ATG start site. (**B**) *Hcrt* morpholino oligo (MO) and control sequences. (**C**) Alignment of ~200
619 single-cell eggs on agarose egg molds for injection. (**D**) Microinjection of 1.0 nL of injection
620 mixture with phenol red indicator for visualization. The scale bar = 0.5 mm. (**E**) Morpholino
621 knockdown of *Hcrt* reduces the activity (total distance traveled) of Pachón cavefish (t = 5.021, df
622 = 88, p < 0.0001) but not of surface fish (t = 1.318, df = 88, p > 0.72). (**F**) Knockdown increases
623 sleep in Pachón cavefish (t = 2.694, df = 88, p < 0.05) but has no effect on surface fish (t = 0.17,
624 df = 88, p > 0.99). The scale bar = 1 mm. The error bars in panels **E** and **F** denote the standard
625 error of the mean.
626
627 **Figure 2: CRISPR gene-editing of *oca2* introduces albinism in surface-dwelling *A. mexicanus*.** (**A**)
628 Schematic of the oca2 coding regions, and guide RNA (gRNA) targeting exon 21 for CRISPR-
629 mediated gene editing. (**B**) Sequencing chromatogram of wild-type *Oca2$^+$* and (**C**) mutant *Oca2$^-$*
630 alleles. The red box in panel **B** indicates the 2 bp sequence, which is missing in the mutant *Oca2$^-$*
631 allele depicted in panel **C**. (**D**) Targeted CRISPR introduces a 2 bp deletion, disrupting *Oca2*
632 function. Primers are designed for the genotypic screening of 2 bp deletion in F_0 offspring. (**E**)
633 PCR and gel electrophoresis of *Oca2* in wild-type surface fish (band size = 134 bp) and F_0 CRISPR-
634 injected offspring (band size = 133 bp). Individuals homozygous or heterozygous for the wild-type
635 variant of *Oca2* (*Oca2$^+$*) are pigmented, while F_0s homozygous for the 2 bp deletion (*Oca2$^-$*) harbor
636 albinism. (**F**) Whole-body images of wild-type surface fish and (**G**) of surface fish with CRISPR-
637 mediated albinism. The scale bar = 5 mm. (**H** and **I**) Magnified view of the heads of individuals
638 depicted in panels **F** and **G**, respectively. The scale bar = 2 mm.
639
640 **Figure 3: Tol2 transgenesis of pan-neuronal GCAMP6s enables the live imaging of brain activity**
641 **in *A. mexicanus*.** (**A**) Tol2-mediated transgenesis in *A. mexicanus.* Coinjection of Tol2 mRNA and
642 Tol2-*elav3l*:GCAMP6s-Tol2 plasmid integrates GCAMP6s transgene in F_0 founders. Backcrossing
643 to the original stock of wild-type fish yields F_1 individuals with a stable pan-neuronal expression
644 of GCAMP6s. (**B** and **C**) The resulting offspring are screened for a stable expression using
645 dissection and confocal microscopy. Stable *TgAsty*(*elav3l*:GCaMP6s) F_1 individuals have been
646 established for both surface- (depicted in panel **B**) and cave-dwelling morphotypes (depicted in
647 panel **C**). The scale bar = 200 μm.
648
649 **Table 1: Restriction digest of Tol2 plasmid.**
650
651 **Table 2: In vitro synthesis of Tol2 mRNA.**
652
653 **Table 3: Sample pipette-pulling protocol.**
654

655 **Table 4: Morpholino injection mixture.**

656

657 **Table 5: Sample CRISPR/Cas9 injection mixture.**

658

659 **Table 6: Sample Tol2 transgenesis injection mixture.**

660

661 **DISCUSSION:**

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

668

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

677

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

690

691 The transgenesis approaches described in this protocol provide a simple and powerful method
692 for gain-of-function studies and for generating tools to alter biological processes genetically. The
693 Tol2 system is widely used in zebrafish research, and we have shown that it is similarly powerful
694 in *A. mexicanus*. Moreover, we demonstrated a transgenic construct generated in zebrafish that
695 utilizes a zebrafish promoter and recapitulates endogenous expression in *A. mexicanus*. We have
696 found four other promoters isolated from zebrafish that drive tissue-specific expression as
697 expected in *A. mexicanus* (data not shown). Since zebrafish promoters recapitulate their
698 conserved expression patterns in *A. mexicanus,* this suggests that many of the genetic tools can

699   be transferred directly from zebrafish to *A. mexicanus* without the need for modification with *A.*
700   *mexicanus* promotors. Moreover, with the advance in sequencing technologies in *A. mexicanus*[33],
701   the transgenic approaches described here will permit a powerful future for the investigation of
702   enhancers and promoters that may play a role in the variation between surface and cave forms.
703   Lastly, the powerful tools for the genetic manipulation of biological processes that have made
704   zebrafish valuable are equally important in *A. mexicanus*[60–63]. Differences in diverse behavioral
705   traits, such as sleep, feeding, stress, and aggression, between *A. mexicanus* surface- and cave-
706   dwelling forms have been extensively documented[12,14,15,38,64], yet the underlying neuronal
707   correlates are not well understood. Tools such as *Tg(elavl3:GCaMP6s)* will permit a dissection of
708   how differences in neuronal activity brain-wide correlate with differences in behavior and offer
709   a unique insight into how the brain has be modified evolutionarily.
710
711   Taken together, *A. mexicanus* is poised to become a leading model for investigating the evolution
712   of a variety of morphological and behavioral traits. The diverse differences in complex biological
713   processes in *A. mexicanus* provide a platform for investigating genetic mechanisms of trait
714   evolution. The application of tools for manipulating gene function may help develop this
715   organism into a model that can be applied to investigate biological diseases related to eye
716   degeneration, neurodevelopmental abnormalities, and insomnia.
717

724   **DISCLOSURES:**
725   The authors have nothing to disclose.
726

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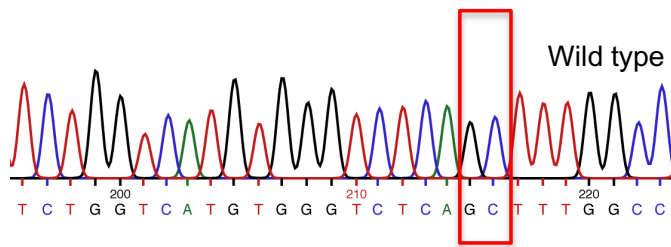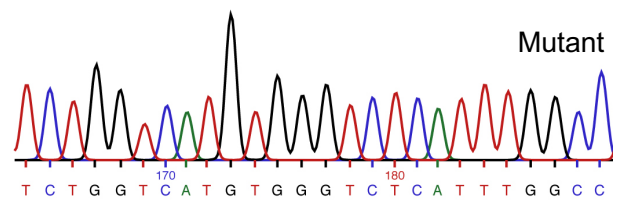883

Figure 1

A *Hcrt* mRNA

5'   3'

ATG translation
blocking morpholino

B *Hcrt* morpholino

5'-TGGGCTTGGTGTGATCACCTGTCAT-3'

Control morpholino

5'-CCTCTTACCTCAGTTACAATTTATA -3'

C

D

E ● Control MO ○ HCRT MO

Total distance (mm/24hr)

***

12000

9000

6000

3000

0

Surface   Pachón

F ● Control MO ○ HCRT MO

Sleep duration (hrs/24hr)

*

20

15

10

5

0

Surface   Pachón

Figure 2

A **Oca2 DNA**

gRNA target exon 21

5'   3'

B   Wild type

TCTGGTCATGTGGGTCTCAGCTTTGGCC

C   Mutant

TCTGGTCATGTGGGTCTCATTTGGCC

D

Forward primer 1
(for genotyping wild type allele)

```
5'-CTGGTCATGTGGGTCTCAGC-3'
5'-CATTCTGGTCATGTGGGTCTCAGCTTTGGCCT-3'   Oca2⁺

5'-CATTCTGGTCATGTGGGTCTCA--TTTGGCCT-3'   Oca2⁻
5'-TCTGGTCATGTGGGTCTCATT-3'
```

Forward primer 2
(for genotyping mutant allele)

Reverse primer
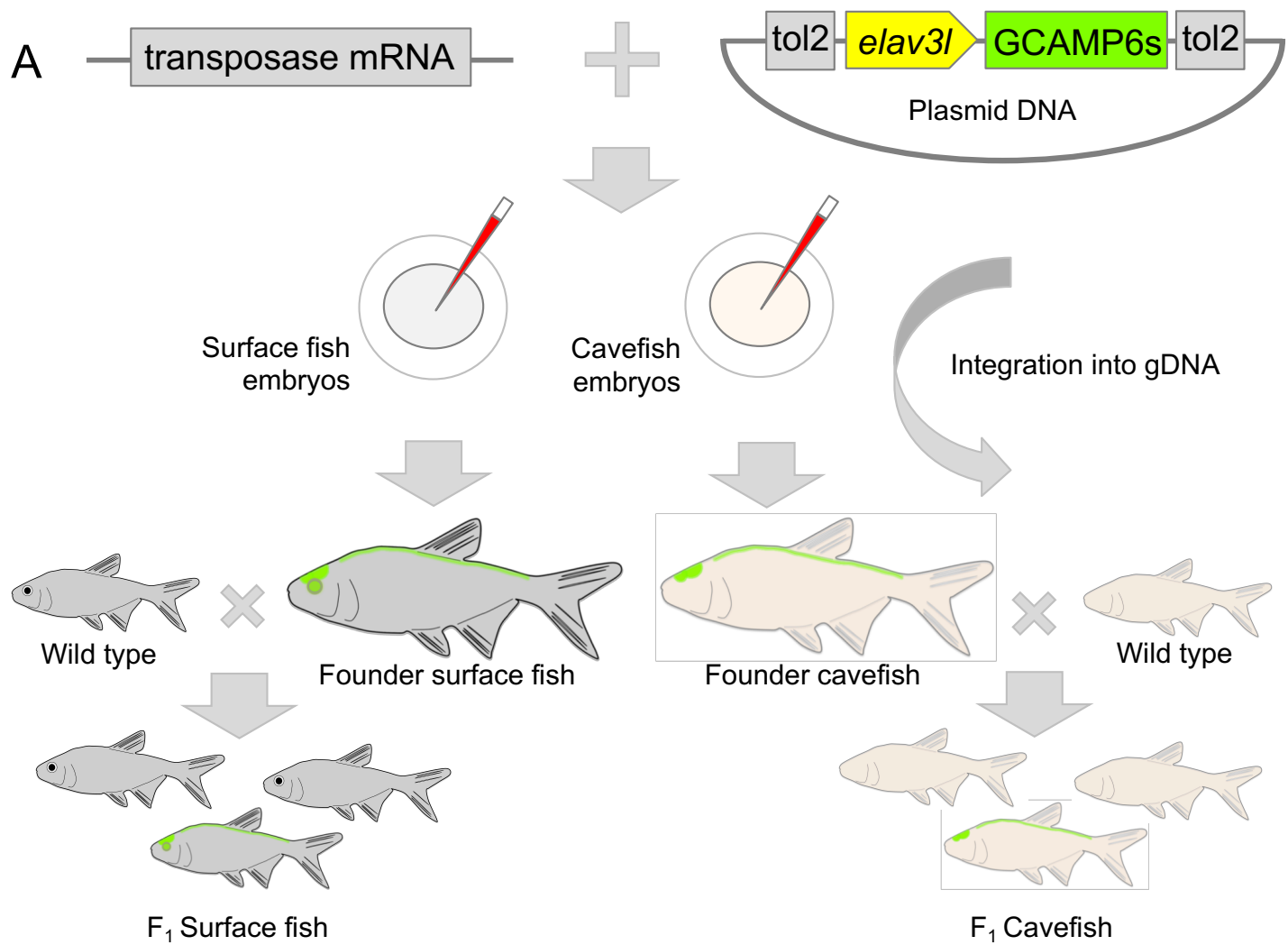(for both reactions)

```
5'-GAAGGGGATGTTGTCTATGAGC-3'
```

E

| | Pigmented | | Albino | No |
| | WT | Het | Mut | DNA |

$Oca2^+$

$Oca2^-$

F

G

H

$Oca2^{+/+}$
Surface fish

I

$Oca2^{-/-}$
Surface fish

Figure 3

A

transposase mRNA

tol2 *elav3l* GCAMP6s tol2
Plasmid DNA

Surface fish embryos

Cavefish embryos

Integration into gDNA

Wild type × Founder surface fish

Founder cavefish × Wild type

F₁ Surface fish

F₁ Cavefish

B Surface fish F₁
Tol2-*elav3l*::GCAMP6s-Tol2

C Molino cavefish F₁
Tol2-*elav3l*::GCAMP6s-Tol2
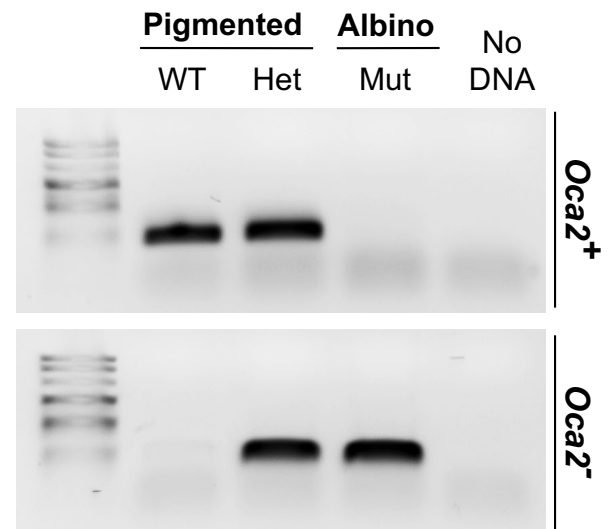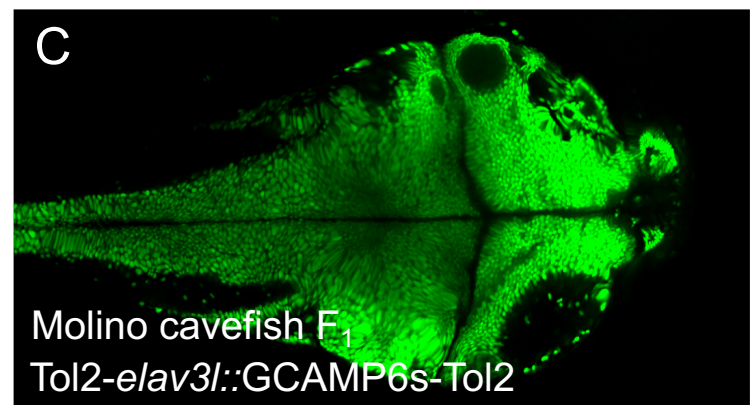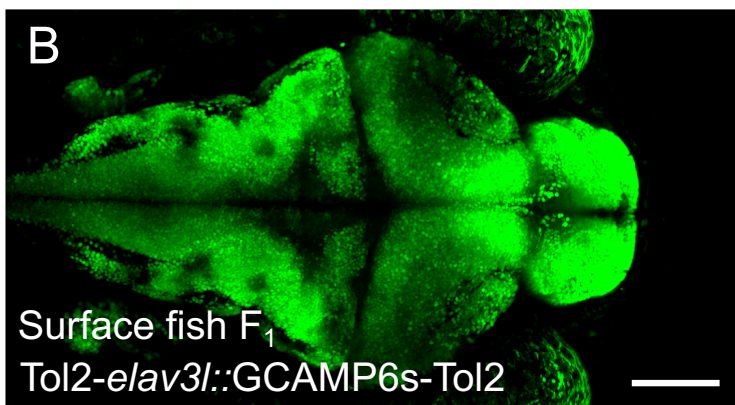
Table 1

Table #1. Restriction digest of Tol2 plasmid.

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 2

Table #2. *In vitro* synthesis of Tol2 mRNA.

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$_2$O	X µl
Total	**≤20 µl**

Table 3

Table #3. Sample pipette-pulling protocol.

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

Table 4

Table #4. **Morpholino injection mixture.**

Reagent	Volume or Amount
Morpholino (Freezer stock @ 4mM)	1.0 µl
Nuclease-free H$_2$O or Danieau's solution	17.0 µl
Phenol Red	2.0 µl
Total	**20 µl**

Table #5. Sample CRISPR/Cas9 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$_2$O	2 µl
Total	**4 µl**

Table #6. Sample Tol2 transgenesis 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**

Reagent	Volume of Amount

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Fish breeding & egg supplies		
Fine mesh fish net	Penn Plax	BN4
Fish tank heater	Aqueon	100106108
Egg traps	Custom made	NA
Glass pipettes	Fisher Scientific	13-678-20C
Pipette bulbs	Fisher Scientific	03-448-21
Agarose	Fisher Scientific	BP160-500
Egg molds	Adaptive Science Tools	TU-1
Morpholino supplies		
Control Morpholino	Gene Tools, LLC	Standard control olio
Custom Morpholino	Gene Tools, LLC	NA
Phenol Red	Sigma Aldrich	P0290-100ML
CRISPR supplies		
Cas9 Plasmid	AddGene	46757
GoTaq DNA Polymerase	Promega	M3001
KOD Hot Start Taq	EMD Millipore	71-842-3
Primers	Integrated DNA Technologies	Custom
T7 Megascript Kit	Ambion/Thermofisher	AM1333
miRNeasy Kit	Qiagen	217004
mMessage mMachine T3 kit	Ambion/Thermofisher	AM1348
MinElute Kit	Qiagen	28204
Tol2 transgenesis supplies		
pCS-zT2TP plasmid	Kawakami et al., 2004	Request from senior author
CutSmart Buffer	New England Biolabs	B7204
NotI-HF Restriction Enzyme	New England Biolabs	R3189
PCR purification Kit	Qiagen	28104

SP6 mMessenger Kit	Ambion/Thermofisher	AM1340

Microinjection supplies		
Glass Capillary Tubes	Sutter Instruments	BF100-58-10
Pipette puller	Sutter Instruments	P-97
Picoinjector	Warner Instruments	PLI-100A
Micromanipulator	World Precision Instruments	M3301R
Micromanipulator Stand	World Precision Instruments	M10
Micmanipulator Base	World Precision Instruments	Steel Plate Base, 10 lbs

Comments/Description

Design and create plastic grate to place at bottom of tank to protect eggs

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Institution: Florida Atlantic University

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> All steps in the protocol section have been revised to state action phrases. We have included notes sparsely, where we felt appropriate. Finally, the discussion has been removed from the protocol section and places in the appropriate place.

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> The protocol has been revised to include more detail, which we hope better guides the reader. We have also included a citation for the CRISPR/Cas9 generation methods. This citation will point the reviewer to a detailed overview of CRISPR/Cas9 design and implementation.

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> We have added the requested details. Primer sequences for gene manipulation using CRISPR/Cas9 are provided in the text, as well as in the main figure.

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16. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

> Done

17. Tables: Please change "µl" to "µL". Please remove commercial language (CutSmart, etc.)

Done

18. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
19. References: Please do not abbreviate journal titles.

We have modified the discussion to highlight the above points.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors provided detailed protocols for manipulation of gene expression in a special cavefish, Mexican tetra (Astyanax mexicanus). These methods are critical for further genetic and functional investigation.

We thank the author for reviewing the manuscript, and for their feedback. Each point below has been addressed.

Major Concerns:
Lines 254-255: Why didn't use the published genome sequence of this cavefish for designing Tol2 constructs?

We initially decided to use well-established Tol2 vectors, which are widely used in zebrafish research. This allowed us to optimize the protocol using reagents that we knew worked well. However, since establishing this protocol in *Astyanax mexicanus*, subsequent studies can use the published genome sequences to target specific genes of interest.

Lines 252-298: Where is the description of Table #2 in the main text?

We reference the Table in Step #4.2.4

Minor Concerns:
Please don't use abrreviations for certain normal terms, such as spec on lines 264 & 280.
The authors are recommended to polish the manuscript carefully, since it is not a note records of experiments.

Abbreviations have been removed, and the manuscript has been proof-read.

Reviewer #2:

Manuscript Summary:
The author described the procedure to generate the gain of function and loss of function in A. mexicanus with detailed parameters and impressive pictures. In overall, the description and protocols were clear, which is ideally to extent the protocols to other fish species. However, there are more data should be

provided as the requirement for Journal of Visualized Experiments, the efficacy of the protocol must be demonstrated. Therefore, a minor reversion need be made before accept.

> We thank the reviewer for providing thoughtful comments. We have modified the figures and the text to address most of the concerns raised. Unfortunately, we did not systematically keep track of survival for either transgenesis or MO-injection, but we have not observed notable number of deaths when compared to controls. All other points have been addressed fully.

1. In figure 1, the author obtained the morpholino-fish and showed the final effect, how many embryos (n=?) they used for Surface fish and Pachon fish? How many embryos show effect?

> We did not systematically track this. On average, the MO does not seem to affect larvae grossly.

2. In Figure 2, the Oca2 gene was disrupted by Crispr/Cas9, and evidently proved by PCR and final mutant fish. Is it possibly and necessary to provide the original sequencing photo/data to show the deletion of 2-bp near PAM? And how many mutants they obtained from the sequenced clones? Another question is: Crispr/Cas9 always give more than one type of deletion in the target region, whether the author observe other type of deletion in the PAM region?

> We focused on sequencing of alleles that were transmitted through the germline, and which we could use to establish lines. These sequencing results have been added to the manuscript, notably in Figure 2B-C. Regarding the differences in mutations among individuals: we established 2 lines from two different founder fish, each of which contained a different mutation.

3. In figure 3, they already obtained the transgenic lines and looked great. What is number of injected embryos and the ratio to get the transgenic line?

> We also did not track this systematically. We do see that more death following injection in cavefish, but it is not severe. We also expect that this will vary with different constructs.

Reviewer #3:

Manuscript Summary:
The authors describe a protocol for three approaches for studying and manipulating gene functions in A. mexicanus. The protocol included the use of morpholino, tol2 transgenesis and CRISPR-Cas9 gene editing. Morpholinos were used to knock down the neuropeptide hcrt to study its role in sleep regulation. Oca2 gene that is responsible for albinism was knocked out using the CRISPR-Cas9 system. Tol2 transgenic approach was used to generate transgenic fish expressing the GCaMP6s gene.

> We thank this reviewer as well for their thoughtful feedback. We have addressed the concerns raised, with in the manuscript or through modified figures. A detailed list of the changes is found below.

Minor Concerns:

Where did the authors microinject the embryos? in the cytoplasm of the one-cell stage or in the yolk? This should be clarified.

> We inject into one-cell stage individuals, and we target the yolk. This has been stated explicitly in 5.4.8

Line 187: morpholino

    Thanks. This has been corrected.

Line 197-201: It appears that the authors designed their gRNA manually? There are several online tools that are time-saving and usually design more efficient oligos. Those tools generate several oligos for the target genes and rank them by a quality score. Some of those tools also check for potential off targets against some available genomes.

    We did design our gRNAs manually, as many of the online tools do not include the cavefish genome. Some online tools now include cavefish, such as https://crispr.cos.uni-heidelberg.de/, but we have never used this tool, and cannot assess whether it produces better results.

Line 203-206: the first sentence of the note is confusing to me as it appears that the authors suggest that a gRNA can be designed for a genomic target that doesn't have a PAM sequence (NGG)?

    This refers to the GGs at the 5' end of the gRNA, not the PAM sequence. We have clarified within the text that this refers to the 5' end.

Line 231: check the sequence of the 3' gRNA primer for mismatches (at least 4 or 5 nucleotides). Is there a reason for this mismatch? Also, why does the 3' gRNA primer begin after 5 nucleotides from the end (the first 5 nucleotides are not included)? Is this a requirement of the primer design, and will not affect the binding of the final oligo with Cas9 protein?

    The sequence of the oligo B in the text was incorrect. We have updated this, and thank the reviewer for noticing.

Line 233: what are the PCR conditions? How much do you add of each oligo and other PCR components? How do you check the yield? This should be described, or a reference should be provided.

    For PCR, we followed previously reported conditions, Kowalko et al. (2016), and have cited this paper. We have made this clear in the manuscript.

Line 278-283: Step f. is a repeat for step d.

    Thank you. We have omitted the duplicated step.

Line 289-290: how long is the incubation time?

    For all incubation time throughout the protocol, we follow the guidelines provided by the manufactures of whatever kit we use. For Tol2 step, we use 2 hours, as suggested by the mMessage machine protocol.

Line 329: how much feed do you regularly add so that the 3X can be estimated (% of body weight?)

    3x here refers to number of feedings per day, not 3x as much food. We regularly feed fish 1-2x per day. We have tried to make this clear in the manuscript.

Line 334: ZT is defined at line 341 and should be defined at the first time you mention it.

    Thank you for bringing this to our attention. The definition has been added earlier.

Line 337: the two statements are confusing regarding the time of check for eggs. If the first one is for surface fish, this should be mentioned.

> For reasons that we do not understand, surface fish and cavefish spawn at different times of the day. We have tried to clarify this point in the manuscript.

Line 356: I guess you warm the agarose plates to room temperature after you get them from the refrigerator and before placing the embryos. If so, it is important to mention that here to avoid the harmful effects of temperature fluctuations on embryos.

> Yes, the reviewer is correct – plates are warmed. We have added this detail.

Line 363: how do you backfill the injection needles? it should be described.

> Needles are backfilled using a standard gel-loading pipet tip. This has been added to section 5.4.1.

Line 374: this step can be deleted.

> Thanks. We've removed it.

Line 383: how long does the one-cell stage last? This will help researchers to plan a head so that they inject before the embryos reach the two-cell stage.

> The one-cell stage lasts for approximately 40 min. We have added this to step 5.4.9.

Line 399: Transposase mRNA not Cas9 mRNA.

> Thanks. This has been corrected.

Line 408: what's the size of the glass bowl? Or what's the embryo stocking density, per liter for example?

> The size of the glass bowl is 10 x 5 cm. This has been added to step 6.1.1.

Line 423: check the step numbering (2.2.A, 2.2.A)

> Thanks. This has been corrected.

Line 444-449: Do you generate lines of fish that are mutant for one or both alleles? The procedures mentioned here can generate mutant fish for one allele but not both. It should be clarified here.

> Yes, the reviewer is correct. For the results given here, we have generated a 'Molino-specific' allele. We have clarified that this procedure is to establish lines of fish transmitting mutant alleles

Line 452: state the figure number.

> This has been fixed.

Line 460-461: a reference for a PCR screening protocol may be provided here.

Whereas this is commonly done in zebrafish, a detailed protocol describing the approach is typically not provided. We have looked for one, but as far as we can see, there is not standard reference for it.

Line 520: "25 pg of gRNA was injected into 1-cell stage surface fish embryos, along with 300 pg Cas9 mRNA" is already mentioned in the methods and can be deleted.

Done.

Line 747: SB should be defined here and in other figures.

Scale bars have been included.

Table of materials
Morpholino supplies: Standard control oligo.

In compliance with data protection regulations, please contact the publication office if you would like to have your personal information removed from the database.