

# CRISPR mutagenesis confirms the role of *oca2* in melanin pigmentation in *Astyanax mexicanus*



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## ABSTRACT

Understanding the genetic basis of trait evolution is critical to identifying the mechanisms that generated the immense amount of diversity observable in the living world. However, genetically manipulating organisms from natural populations with evolutionary adaptations remains a significant challenge. *Astyanax mexicanus* exists in two interfertile forms, a surface-dwelling form and multiple independently evolved cave-dwelling forms. Cavefish have evolved a number of morphological and behavioral traits and multiple quantitative trait loci (QTL) analyses have been performed to identify loci underlying these traits. These studies provide a unique opportunity to identify and test candidate genes for these cave-specific traits. We have leveraged the CRISPR/Cas9 genome editing techniques to characterize the effects of mutations in *oculocutaneous albinism II (oca2)*, a candidate gene hypothesized to be responsible for the evolution of albinism in *A. mexicanus* cave populations. We generated *oca2* mutant surface *A. mexicanus*. Surface fish with *oca2* mutations are albino due to a disruption in the first step of the melanin synthesis pathway, the same step that is disrupted in albino cavefish. Hybrid offspring from crosses between *oca2* mutant surface and cavefish are albino, definitively demonstrating the role of this gene in the evolution of albinism in this species. This research elucidates the role *oca2* plays in pigmentation in fish, and establishes that this gene is solely responsible for the evolution of albinism in multiple cavefish populations. Finally, it demonstrates the utility of using genome editing to investigate the genetic basis of trait evolution.

## 1. Introduction

Identifying the genes that underlie the evolution of traits in different organisms is key to understanding the evolutionary basis of diversity in nature. Towards this end, in a wide range of organisms, candidate genes for the evolution of many traits have been identified (for example Hoekstra et al., 2006; Linnen et al., 2013; Steiner et al., 2007; Chan et al., 2010; Shapiro et al., 2004; Colosimo et al., 2005; Greenwood et al., 2016; Rebeiz et al., 2009; Gross et al., 2009). The recent advent of genome-engineering technologies now provides genetic access to many evolutionarily interesting, but previously genetically inaccessible, organisms, allowing for functional tests of the role of candidate genes in the evolution of traits. Thus, functional genetic studies can now be used to elucidate how and why particular traits evolve.

*Astyanax mexicanus* is a species of fish that exists in two forms, a sighted river-dwelling surface form and multiple blind cave-dwelling forms that inhabit at least 29 caves in central Mexico (Mitchell et al.,

1977). *A. mexicanus* is an excellent system for studying evolutionary genetics. The polarity of evolution is known; both extant surface fish and cavefish evolved from a surface fish ancestor. The evolutionary history of these fish has been well studied, and multiple extant cave populations evolved independently (reviewed in (Gross, 2012)). Cavefish have evolved a number of morphological, behavioral, and physiological traits. These include loss or reduction of eyes and melanin pigmentation, enhancement of sensory systems including an increase in the number of taste buds and the sensory organs of the lateral line, a reduction in sleep and loss of schooling, and altered feeding behavior and metabolism (Wilkens, 1988; Teyke, 1990; Schemmel, 1974, 1980; Duboue et al., 2011; Parzefall, 1983; Aspiras et al., 2015). Importantly, cave and surface fish are interfertile, allowing for the study of the genes underlying the evolution of cave traits (Wilkens, 1988).

Utilizing crosses and quantitative trait loci (QTL) analysis, the genetic bases for many cavefish traits have been explored (Gross et al., 2009; Kowalko et al., 2013a, b; O'Quin et al., 2013; Protas et al., 2007,

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2008, 2006; Yoshizawa et al., 2012, 2015). These mapping studies, as well as candidate gene approaches, have led to the identification of a number of candidate genes for the evolution of cave traits (Gross et al., 2009; Aspiras et al., 2015; O'Quin et al., 2013; Protas et al., 2006; McGaugh et al., 2014; Yamamoto et al., 2004; Ma et al., 2014). However, testing these candidate genes functionally has been difficult. While transient overexpression and morpholino knock-down has been used in cavefish (Yamamoto et al., 2004, 2009; Ma et al., 2014), these approaches have drawbacks. Most notably, their transient nature makes *in vivo* analysis of events that occur beyond embryonic development difficult. While transgenic approaches have been used in *A. mexicanus* (Elipot et al., 2014), these approaches do not allow for analysis of loss of function alleles. Thus, until recently, testing the effects of the loss of function of a particular gene in *A. mexicanus* beyond early developmental stages was not feasible. Recently, genome-editing techniques were applied in *A. mexicanus* in a study in which two candidate pigmentation genes were targeted using Transcription activator like effector nucleases (TALENs) (Ma et al., 2015). While phenotypes were examined in mosaic F<sub>0</sub> fish, stable mutant lines were not examined, and the full genetic power of *A. mexicanus*, the ability to hybridize cave and surface fish, was not exploited in this study.

Here, we capitalize on the powerful genetic analysis possible in *A. mexicanus* to examine the role of loss of the *oculocutaneous albinism type 2* (*oca2*) gene in cavefish. The *oca2* gene lies under the QTL for albinism in an albino cavefish population, and two independently evolved albino cave populations have deletions in this gene (Protas et al., 2006), strongly suggesting that this gene underlies the evolution of albinism in cavefish. We examine the role of OCA2 in *Astyanax mexicanus* by analyzing *oca2* mutants of surface *A. mexicanus* and hybrids derived from these fish. Loss of *oca2* in surface fish results in albinism, the inability to produce melanin pigment throughout the body. Although melanin pigment is not produced, this loss is not due to the absence of the cells producing this pigment. Melanoblasts can be induced to produce melanin pigment following treatment with L-DOPA in mutant albino fish, confirming that loss of *oca2* affects the first step of melanin synthesis. Finally, we definitively demonstrate that loss of *oca2* underlies albinism in multiple, independently evolved *A. mexicanus* cavefish populations by revealing a lack of complementation in *oca2*-mutant surface/albino cavefish hybrid crosses. Together, this work illustrates the utility of using genome-editing methods to test candidate genes in an evolutionary model system and provides the first definitive proof that a candidate *A. mexicanus* cavefish allele underlies a cave phenotype.

## 2. Results and discussion

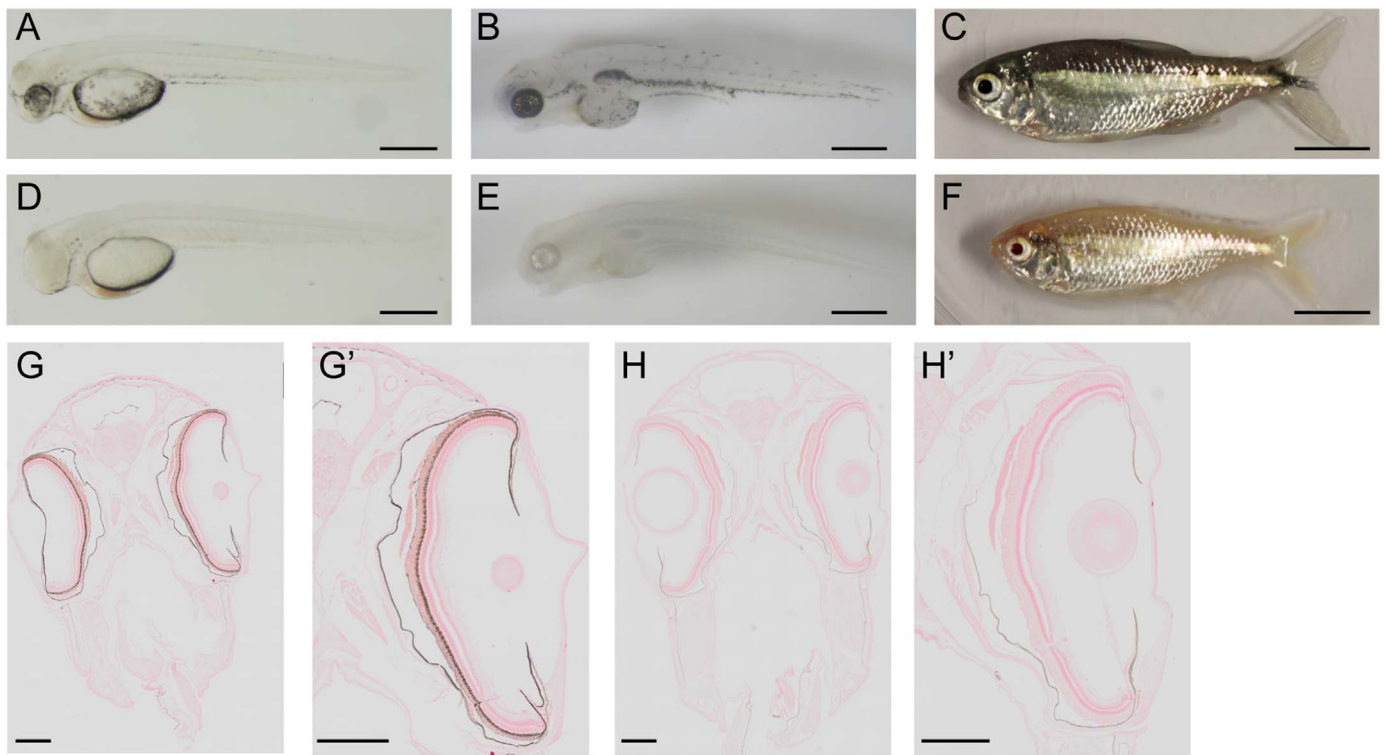
To investigate the role of *oca2* in *A. mexicanus*, Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 was used to produce mutations in the *oca2* gene in surface *A. mexicanus*. Exon 21 was targeted, as this exon is deleted in fish from the albino Molino cave population (Protas et al., 2006). Founder fish were injected with a gRNA targeting *oca2* and Cas9 mRNA. To obtain germline mutants, all injected fish raised to adulthood were screened for transmission of mutant alleles. A high percentage, 64% of founder fish (9/14 fish), transmitted mutant alleles of *oca2* (Supplemental Table 1). Further, transmission rates, the percentage of mutant alleles transmitted, were also high at 29–82% (Supplemental Table 1). Given the high percentage of transmission, we were able to assess phenotypes of *oca2* mutant fish in the first generation by incrossing *oca2* CRISPR/Cas9-injected F<sub>0</sub> founder fish. As these injected fish are mosaic, they transmit both wild-type and mutant *oca2* alleles. These incrosses produced surface fish that have a typical pigmentation pattern, producing melanin pigment in the retinal pigment epithelium (RPE) of the eye and throughout the body (Fig. 1 A–C). Additionally, albino surface fish are produced from these crosses, which do not produce melanin during development or as adults (Fig. 1D–F). To confirm the absence of melanin pigmentation in

these albino fish, we sectioned pigmented and albino adult surface *A. mexicanus*. Melanin producing cells were observed under the skin and in the RPE of the eye in pigmented fish (Fig. 1 G and 1G'). In contrast, no melanin was observed in the albino fish (Fig. 1H and 1H'). To determine whether these results were indeed due to mutations in *oca2*, we isolated an individual mutant line of surface fish containing a 2 base pair deletion predicted to lead to a frameshift. We incrossed heterozygous F<sub>2</sub> fish from this line (see Methods), and analyzed the F<sub>3</sub> progeny. At 1.5 days post fertilization (dpf), 28% (65 of the 232) progeny from this cross were albino and identical to individuals we observed by incrossing founder fish (Fig. 1, data not shown). Thus, this cross demonstrated the expected Mendelian ratio of individuals with a recessive phenotype. This was expected, as albinism in cavefish is a recessive trait (Sadoglu, 1957). Further, we genotyped 12 albino and 12 pigmented fish from this cross and found that 12/12 albino fish were homozygous mutant for the *oca2*<sup>2bpdel</sup> allele, 9/12 pigmented fish were *oca2*<sup>2bpdel/+</sup> and 3/12 pigmented fish were wild-type, *oca2*<sup>+/+</sup>. These results are consistent with homozygous mutant *oca2* alleles causing the albino phenotype in surface fish rather than off-target mutations. Further, they confirm that phenotyping of F<sub>1</sub> individuals is a valid method for rapid functional analysis of candidate genes hypothesized to be involved in cavefish evolution.

The melanin synthesis pathway is a biosynthetic pathway during which L-tyrosine is converted to melanin through a number of enzyme catalyzed steps. The first step of this pathway, the conversion of L-tyrosine to L-DOPA, is disrupted in albino cavefish, and exogenous L-DOPA can rescue pigment production in these fish (McCauley et al., 2004). To determine if L-DOPA is sufficient to rescue *oca2* mutant albino surface fish, pigmented and albino surface fish produced from incrosses of *oca2* CRISPR/Cas9-injected founder fish were treated with L-DOPA (Fig. 2). Albino surface fish produce melanin pigment following L-DOPA treatment (Fig. 2D). Thus, albinism is produced by a defect in the first step of the pigmentation pathway in these mutant fish, replicating what is observed in albino cavefish populations.

Albinism can be caused by mutations in a number of genes (reviewed in (Kamaraj and Purohit, 2014)). Thus, it is possible that while mutation of *oca2* can cause albinism in surface *A. mexicanus*, another gene may be responsible for the evolution of albinism in cavefish. While both genetic crosses and QTL analysis suggests that albinism is monogenic (Protas et al., 2006; Sadoglu, 1957), neither of these analyses can rule out the possibility that another gene under the QTL is responsible for albinism, or that mutant alleles of two closely linked genes are responsible together for albinism in cavefish. Mutations in *oca2* in zebrafish (Beirl et al., 2014) and in humans (reviewed in (Gronskov et al., 2007)) can reduce melanin pigmentation without leading to the complete loss of melanin. Additionally, non-coding variants in the *herc2* locus are associated with eye, hair, and skin pigmentation variation in humans, and can affect *oca2* expression levels (Mengel-From et al., 2010; Sturm et al., 2008; Sulem et al., 2007; Kayser et al., 2008; Han et al., 2008; Visser et al., 2012; Eiberg et al., 2008; Branicki et al., 2009). The *herc2* gene is located upstream of *oca2* in humans and a search of the cavefish genome (McGaugh et al., 2014) revealed that *herc2* is adjacent to *oca2* in cavefish as well, raising the possibility that a combination of coding and noncoding mutations is required in cavefish for albinism. Thus, it is critical to determine whether coding mutations in *oca2* alone are responsible for albinism in cavefish.

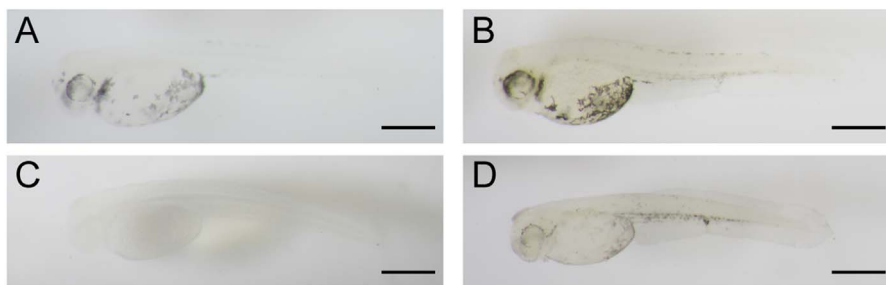
To determine if mutations in *oca2* are responsible for the entire albinism phenotype in cavefish, *oca2* CRISPR/Cas9-injected surface fish were crossed to fish from two cave populations and hybrid offspring were evaluated. Fish from both the Pachón and the Molino populations have different coding mutations in the *oca2* gene (Protas et al., 2006). Pachón/*oca2* CRISPR/Cas9-injected surface fish hybrids are either pigmented (Fig. 3A & B), as observed in crosses between Pachón and wild-type surface fish, or albino (Fig. 3C & D). Similar results are observed from crosses between *oca2* CRISPR/Cas9-injected



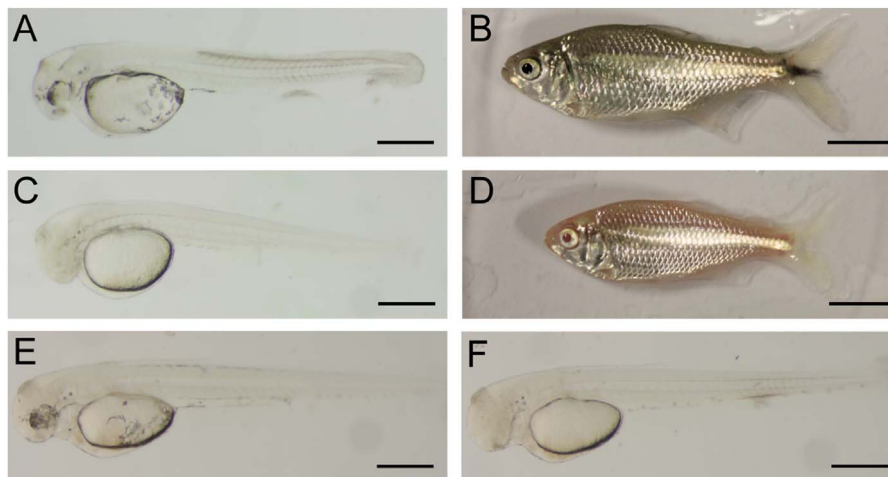
**Fig. 1.** *oca2* mutant *Astyanax mexicanus* surface fish lack melanin pigmentation. (A–C, G, G') Pigmented surface fish and (D–F, H, H') albino surface fish siblings from an incross of *oca2* CRISPR-injected surface fish that transmit *oca2* mutant alleles. These fish were imaged at 2.5 dpf (A & D), 4.5 dpf (B & E) and at 4 months (C & F). H & E stained sections of pigmented (G, G') and albino (H, H') adult surface *Astyanax mexicanus* heads. The scale bars indicate 0.5 mm (A, B, D, E, G–H') and 1 cm (C & F).

surface fish and Molino cavefish. Hybrids from this cross are also either pigmented (Fig. 3E) or albino (Fig. 3F). Thus, these albino offspring reveal a lack of complementation in fish harboring our engineered *oca2* mutations and the natural cavefish *oca2* mutant alleles, demonstrating that *oca2* is indeed solely responsible for albinism in these cave populations. These data complement the results of earlier work that demonstrated that cave alleles of *oca2* from Pachón and Molino populations cannot rescue pigmentation in an albino mouse cell line (Protas et al., 2006). Further, these results provide definitive proof that the evolution of albinism in two populations was the result of different mutations in the *oca2* gene. Finally, these fish can be used in the future to further elucidate the role of *oca2* in cavefish evolution. For example, embryonic fish injected with morpholinos to knock-down *oca2* have enhanced levels of the catecholamine dopamine compared to wild-type surface fish, raising the intriguing possibility that *oca2* plays a role in cavefish evolution beyond pigmentation (Bilandzija et al., 2013). The stable line of *oca2* mutant fish generated here can be used in future studies to examine connections between *oca2* and other traits, such as catecholamine-regulated behaviors.

The development of genome editing technologies such as CRISPR/Cas9 has the potential to revolutionize the field of evolutionary biology. These technologies make it possible to modify the genome of virtually any organism. A wide range of organisms have been genetically manipulated with these technologies, including model organisms such as *Drosophila melanogaster* and zebrafish as well as organisms as diverse as the axolotl, tilapia, butterflies and the amphipod *Parhyale hawaiiensis* (Bassett et al., 2013; Jao et al., 2013; Flowers et al., 2014; Li et al., 2014, 2015; Martin et al., 2016; Markert et al., 2016). Use of these technologies has already led to important insights about which genes are involved in the evolution of traits, including pigmentation patterns in butterfly wings (Mazo-Vargas et al., 2017; Zhang et al., 2017a,b; Zhang and Reed, 2016) and appendage specification in crustaceans (Martin et al., 2016). Here, we demonstrate the utility of these tools to evaluate the contribution of an individual gene, *oca2*, known to contain mutations in derived populations, to the albinism phenotype in the cavefish *A. mexicanus*. *A. mexicanus* is a particularly powerful system for evaluating evolutionary genetics because ancestral-like surface fish and derived cavefish are extant and interfertile.



**Fig. 2.** Exogenous L-DOPA administration in albino surface *Astyanax mexicanus* is sufficient to lead to melanin pigment production. Pigmented 2.5 dpf surface fish from an incross of *oca2* CRISPR-injected surface fish that transmit *oca2* mutant alleles were either not treated with L-DOPA (control) (A) or treated with L-DOPA (B). Albino 2.5 dpf surface fish from an incross of *oca2* CRISPR-injected surface fish that transmit *oca2* mutant alleles were either not treated with L-DOPA (control) (C) or treated with L-DOPA (D). All scale bars indicate 0.5 mm.



**Fig. 3.** Engineered mutations in *oca2* do not complement naturally-occurring cavefish albinism alleles. Crosses between *oca2* CRISPR-injected surface fish and Pachón cavefish produced hybrid fish that were pigmented both during development, as shown at 2.5 dpf (A) and as adults (B). These crosses between *oca2* CRISPR-injected surface fish and Pachón cavefish also produced hybrid fish that were albino both during development, as shown at 2.5 dpf (C) and as adults (D). Crosses between *oca2* CRISPR-injected surface fish and Molino cavefish produced hybrid fish that were pigmented (E) and albino (F) at 2.5 dpf. In A & C, B & D, and E & F, pigmented and albino fish are siblings. Scale bars indicate 0.5 mm in larval photographs and 1 cm in adult photographs.

Applying genome editing technologies in *A. mexicanus* allowed us to exploit these features of the system, and through hybridization experiments definitely link specific genetic lesions to the phenotypes they modify. This is notable, as few evolutionary genetics studies have been able to perform analyses to definitely demonstrate the contributions of single genes to the naturally occurring phenotypes. The application of the CRISPR/Cas9 system to *A. mexicanus* will also expand the types of genetic manipulations possible in this system. For example, it is possible to obtain targeted and precise integration of exogenous DNA at the site of a CRISPR/Cas9 double-stranded break (for example (Irion et al., 2014; Hisano et al., 2015; Hoshijima et al., 2016)). Thus, these methods could be further developed in future studies to knock-in a gene encoding a fluorescent protein for monitoring of endogenous gene expression, or to swap alleles between surface fish and cavefish. These results highlight the utility of genome editing for understanding the genetic basis of the evolution of traits, and these methods apply beyond *Astyanax mexicanus*, to other naturally occurring populations that can now be manipulated using genome editing methods.

### 3. Materials and methods

#### 3.1. Fish maintenance and breeding

All animal procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University. *A. mexicanus* were maintained on a 14–10 light-dark cycle at 23 °C. *A. mexicanus* were bred by increasing the frequency and quantity of feeding prior to breeding attempts, and increasing the temperature by 2° F each day in the evening prior to breeding for 2 evenings. Eggs were collected immediately after spawning for injections, in the dark, as described previously (Kowalko et al., 2016).

#### 3.2. Generation of *oca2* mutant *A. mexicanus*

Mutant surface *A. mexicanus* were generated using the CRISPR/Cas9 system. The CRISPR gRNA was targeted to exon 21, and was designed such that it contains two five prime guanine nucleotides for transcription: 5'-GGTCATGTGGGTCTCAGCTT-3'. The gRNA was generated as described previously (Varshney et al., 2015). Briefly, two oligos, oligo A containing the gRNA target sequence (underlined) between the T7 promoter sequence and a sequence that overlaps with a second oligo, oligo B, containing the gRNA sequence, were synthesized (IDT). Oligo A: 5'-TAATACGACTCACTATAGGTCATGTGGG-

TCTCAGCTTGTTTTAGAGCTAGAAATAGC-3'. Oligo B: 5'-AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTATTTTAACTTGCTATTCTAGCTCTAAAAC-3'. Oligos were annealed and amplified. The gRNA was transcribed using the T7 Megascript Kit (Ambion) with the following modifications to the manufacturers protocol: For each reaction, 1 µL 10 × transcription buffer, 4 µL NTP, 1 µL of T7 polymerase, 5 µL of gDNA template and 10 µL of RNase-free water were combined. The gRNA was precipitated in 300 µM sodium acetate pH 5.2 and 1 vol of isopropanol at -20C, centrifuged and washed with 70% ethanol, and precipitated gRNA was resuspended in RNase-free water. The gRNA was co-injected with nls-Cas9-nls (Jao et al., 2013) mRNA, transcribed using the MESSAGE MACHINE T3 kit (Life Technologies) as described previously (Jao et al., 2013). 25 pg of gRNA and 300 pg Cas9 mRNA were coinjected into 1-cell stage *A. mexicanus* surface fish embryos. Microinjections were performed as described previously (Ma et al., 2015; Kowalko et al., 2016). Founder fish were screened for those transmitting mutant alleles by either in-crossing and looking for albino progeny, or by genotyping. Genotyping was performed on DNA from whole embryos and fin clips from adults, as described previously (Ma et al., 2015). Mutagenesis was detected by amplifying the region surrounding the gRNA target site by PCR using the forward primer 5'-CTCCTCTGTGAGGCTGTGC-3' and the reverse primer 5'-GAAGGGGATGTTGTCTATGAGC-3'. Alleles with indels were distinguished from wild-type alleles by gel electrophoresis, as described previously (Kowalko et al., 2016). Fish carrying mutant alleles were identified by the presence of more than one band. Fourteen founder fish were screened for transmission. Of these, nine fish transmitted mutant alleles that resulted in albino progeny in offspring (Supplemental Table 1). Transmission rates were calculated by quantifying the number of embryos containing mutant alleles by PCR and gel electrophoresis. Four to thirty-eight individuals were assessed for each cross reported for genotyping by PCR. *A. mexicanus* analyzed here were from incrosses between *oca2* CRISPR/Cas9-injected F<sub>0</sub> surface fish or hybrid fish from crosses between *oca2* CRISPR/Cas9-injected F<sub>0</sub> surface fish and a Molino or Pachon cavefish. Additionally, a line of surface fish containing individual mutant *oca2* alleles were identified by outcrossing *oca2* CRISPR/Cas9-injected F<sub>0</sub> surface fish to wild-type surface fish. The *oca2* mutation was identified by PCR amplification of the region (as described above) followed by TA cloning and sequencing. A line of fish containing an engineered 2 base pair deletion (predicted to result in a frameshift) was evaluated by outcrossing engineered *oca2* mutant surface fish to wild-type surface fish for two generations. Heterozygous F<sub>2</sub> fish from this cross were incrossed to confirm the

albino phenotype. These fish were genotyped with allele specific primers: wild-type forward primer: 5'-CTGGTCATGTGGGTCACG-3', mutant allele forward primer: 5'-TCTGGTCATGTGGGTCATT-3', reverse primer (for both): 5'-TGTCAAGATATGTGATCTTTGAAA-3'. The annealing temperature for this PCR reaction was 58 °C to obtain specificity.

Larval fish were imaged on a dissection scope and adult fish were imaged using a Canon Rebel (Canon) camera. 2.5 dpf fish were illuminated from below. 4.5 dpf and adult fish were illuminated from above on a white background.

### 3.3. Histology

Samples were fixed in 4% paraformaldehyde overnight at room temperature and rinsed well with 1 × PBS after fixation was complete. For adult fish with calcified bones, treatment began with decalcification in 19% EDTA for 2 days at room temperature. Both embryos and decalcified adult fish were dehydrated through graded ethanol (30%, 50% and 70%) and stored in 70% ethanol at 4. Tissue processing was carried out using a **PATHOS Delta hybrid tissue processor (Milestone Medical Technologies, Inc, MI)**. Individual fish and embryos were embedded with paraffin and sectioned at 5 µm thickness using a Leica RM2255 microtome (Leica Biosystems Inc. Buffalo Grove, IL). Sections were dewaxed and counterstained with nuclear fast red solution (ScyTek Laboratories, Utah) followed by coverslipping and imaging with a VS120 Slide Scanner system (Olympus, PA).

### 3.4. L-DOPA treatment

L-DOPA treatment was performed as in (McCauley et al., 2004) with a few modifications. Briefly, embryos were sacrificed by submerging in tricaine, and fixed in 4% paraformaldehyde for 1 h at room temperature. Following fixation, embryos were washed five times in PBS and once in L-DOPA solution, and then stained with L-DOPA (Sigma-Aldrich). L-DOPA solution was composed of 25 mL L-DOPA stock solution (0.1% L-DOPA), 6 mL disodium phosphate dehydrate solution (11 g of Na<sub>2</sub>HPO<sub>4</sub> \*2H<sub>2</sub>O in 1000 mL water) and 2 mL monopotassium phosphate solution (9 g of KH<sub>2</sub>PO<sub>4</sub> in 1000 mL water). Following staining, embryos were rinsed with deionized water and imaged. Control embryos were fixed and rinsed, but not placed into L-DOPA solution. Fish were imaged on a dissection scope illuminated from above on a white background.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.03.014.

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