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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_0 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_0 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_2 fish from this line (see Methods), and analyzed the F_3 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 $oca2^{2bpdel}$ allele, 9/12 pigmented fish were $oca2^{2bpdel/+}$ and 3/12 pigmented fish were wild-type, $oca2^{+/+}$. 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_1 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