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Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism

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The genetic basis of vertebrate morphological evolution has traditionally been very difficult to examine in naturally occurring populations. Here we describe the generation of a genome-wide linkage map to allow quantitative trait analysis of evolutionarily derived morphologies in the Mexican cave tetra, a species that has, in a series of independent caves, repeatedly evolved specialized characteristics adapted to a unique and well-studied ecological environment. We focused on the trait of albinism and discovered that it is linked to *Oca2*, a known pigmentation gene, in two cave populations. We found different deletions in *Oca2* in each population and, using a cell-based assay, showed that both cause loss of function of the corresponding protein, OCA2. Thus, the two cave populations evolved albinism independently, through similar mutational events.

The relatively closed, often nutrient-poor, and lightless environment of caves represents a marked change in ecological conditions to which several entrapped species have adapted. Obligate cave-dwelling animals, called troglobites or troglodytes, are characterized by a remarkable convergence of eye and pigment loss across diverse species such as spiders, isopods, salamanders and fish[1].

There are 86 known troglodytic species of fish[2]. The best studied is the Mexican tetra, identified by some authors as *Astyanax mexicanus* and others as *Astyanax fasciatus*; the two names should be considered synonymous in the present context and the species will be referred to herein as *Astyanax*. This species has 29 cave populations in the karst region of the Sierra de El Abra of northeast Mexico and one additional population in Guerrero (**Fig. 1a**)[3,4]. A surface, or river-dwelling, sister population of the cave morph lives in southern Texas and northeastern Mexico and can still interbreed with the cave morph. Phenotypically, the cave and surface morphs are very different; among other characteristics, the cave morph has a greater weight per unit length, less pigment, regressed eyes, larger nostrils, more maxillary teeth, more cranial neuromasts and more taste buds, as well as differences in feeding, schooling and aggressive behaviors (**Fig. 1b–d**)[4,5]. Molecular phylogenetic studies indicate that several cave populations independently evolved these characteristics[6–8].

To provide a framework in which to study the genetics of this species, we made a microsatellite linkage map. We have isolated and

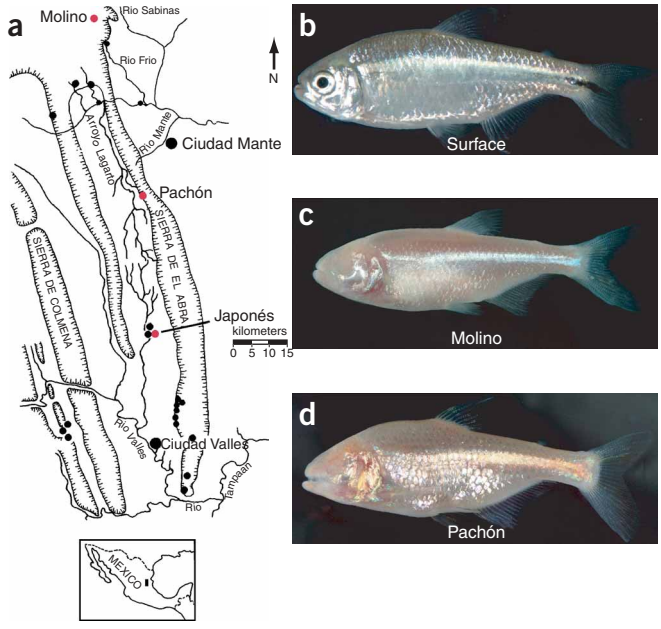

Figure 1 Phenotype and locations of albino cave populations of *Astyanax mexicanus*. (**a**) Map of the area in Mexico where the different cave populations are found. Dots represent cave populations. Caves with red dots are Molino, Pachón and Japonés, all of which contain a majority of albino individuals. Inset map at bottom shows the location of the region within Mexico. (**b**) A representative surface fish. (**c**) A representative Molino cavefish. (**d**) A representative Pachón cavefish.

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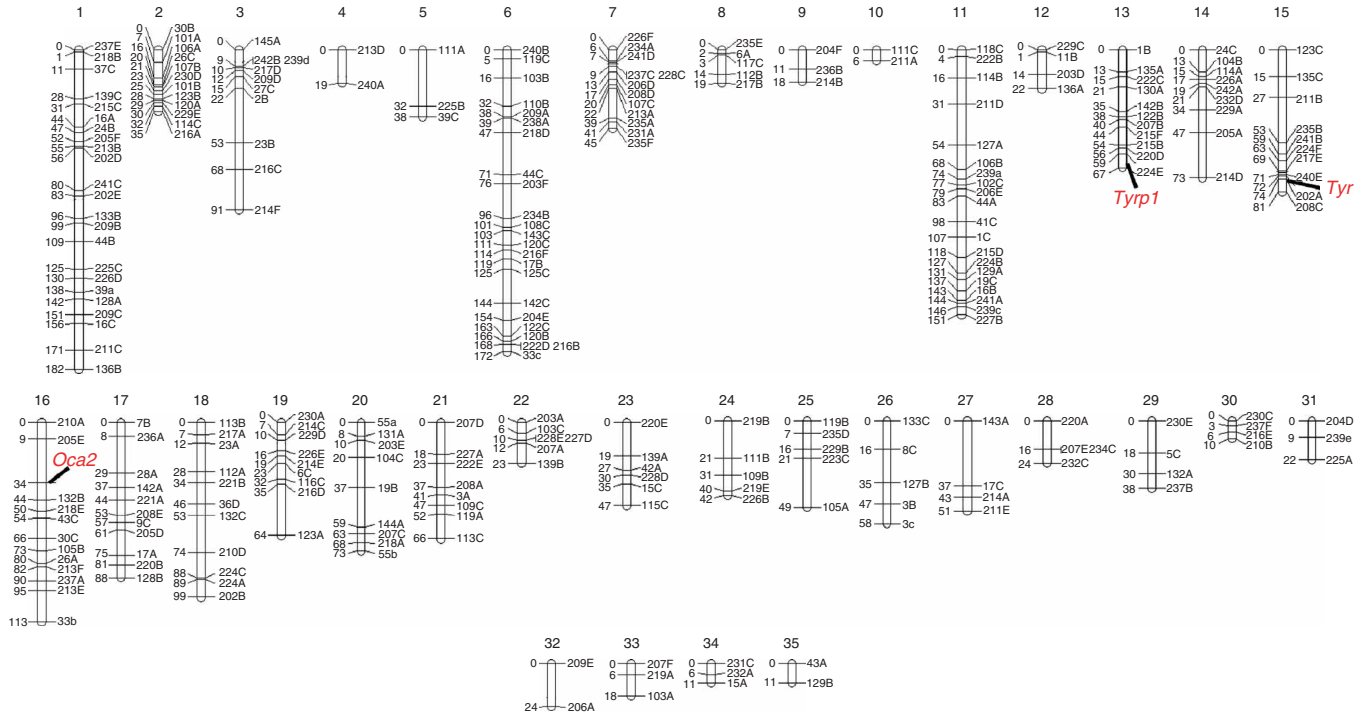

Figure 2 Microsatellite linkage map of *Astyanax mexicanus*. The names of the microsatellite markers are to the right of each linkage group and the positions of the markers (in cM) are on the left. Markers in three genes (*Oca2*, *Tyr* and *Tyrp1*) are labeled in red.

designed primers corresponding to over 600 microsatellites. Using a backcross from the Molino cave population with 111 progeny, we obtained 35 linkage groups composed of 267 markers, out of 300 markers genotyped, with a coverage of 1,916 cM (**Fig. 2** and **Supplementary Table 1** online). *Astyanax* has 25 chromosomes, suggesting that with the addition of more markers, some of the linkage groups would collapse. We are also genotyping a larger F_2 cross from another cave, Pachón, which should coalesce some of the linkage groups and allow for comparisons between the two cave populations.

We identified a number of statistically significant, quantitative loci for different traits present in the cave form, most of which will be described elsewhere. Here we focus on one such trait, albinism. Previous genetic studies have indicated that albinism in the Pachón cave is caused by a single recessive mutation[9,10]. In the Molino backcross, albinism mapped to a single locus in linkage group 16 with a LOD score of 17.29 at microsatellite marker 218E, accounting for 49.4% of the variance in this trait (**Fig. 3a**). A similar analysis of the Pachón F_2 cross mapped the locus for albinism to the same location with a LOD score of 17.98 at marker 218E, accounting for 42.6% of the variance in this trait (data not shown). This coincidence of loci responsible for albinism raises the following three possibilities: the two cave populations could have the same mutation in the same gene, different mutations in the same gene or mutations in distinct but closely linked genes. To address the latter possibility, we performed a complementation test between a Molino individual and a Pachón individual, which yielded only albino offspring (**Fig. 3b**). Thus, albinism in these two cave populations is caused by mutations in the same gene.

To identify the gene responsible for albinism in *Astyanax*, we genotyped individuals of the Molino backcross for a series of candidate genes, based on known albinism loci in mouse and humans: tyrosinase (*Tyr*), tyrosinase-related protein-1 (*Tyrp1*) and ocular and cutaneous albinism-2 (*Oca2*) (**Fig. 2**). One of these genes, *Oca2*, mapped to the albino locus and increased the LOD score in the Molino backcross to 68.6 (**Fig. 3c**), now accounting for 93.1% of the variance of this trait, and the LOD score in the Pachón F_2 cross to 60.66, now accounting for 71.6% of the variance of this trait (data not shown). Furthermore, there is a perfect association between the genotype of the *Oca2* marker and the phenotype of albinism in all successfully genotyped individuals of both the Molino backcross (105 individuals) and the Pachón F_2 cross (215 individuals).

Although the function of *Oca2* is unknown, it is the most commonly mutated gene in cases of human albinism[11] and is also responsible for pigmentation phenotypes in mouse and medaka[12,13]. To test whether *Oca2* mutations are responsible for albinism in cavefish, and, more importantly, to identify the specific genetic lesions in *Oca2* responsible for albinism, we compared the sequence of the *Oca2* cDNA in surface, Pachón and Molino individuals (**Supplementary Fig. 1** online). We found numerous differences in the *Oca2* sequences present in the two cave populations as compared to their surface counterparts (**Fig. 4a** and **Supplementary Fig. 1**). The Pachón cave population had three polymorphisms that could affect *Oca2* function: two were amino acid changes in conserved residues, and the last was a deletion extending from within intron 23 through most of exon 24, such that the cDNA includes part of intron 23 fused to the last nine base pairs of exon 24 and the 3′ UTR (**Fig. 4a** and **Supplementary Fig. 1**). The Molino cave population had only one major difference: exon 21 was missing (**Fig. 4a** and **Supplementary Fig. 1**).

The missing exons observed in the Molino and Pachón *Oca2* sequence could, in principle, be explained by either alterations in splicing or deletions of genomic DNA. Amplifying from genomic DNA, we found that in both cases, the observed losses of exonic sequence in the Molino and Pachón *Oca2* cDNAs were attributable to genomic deletions (**Supplementary Fig. 2** online).

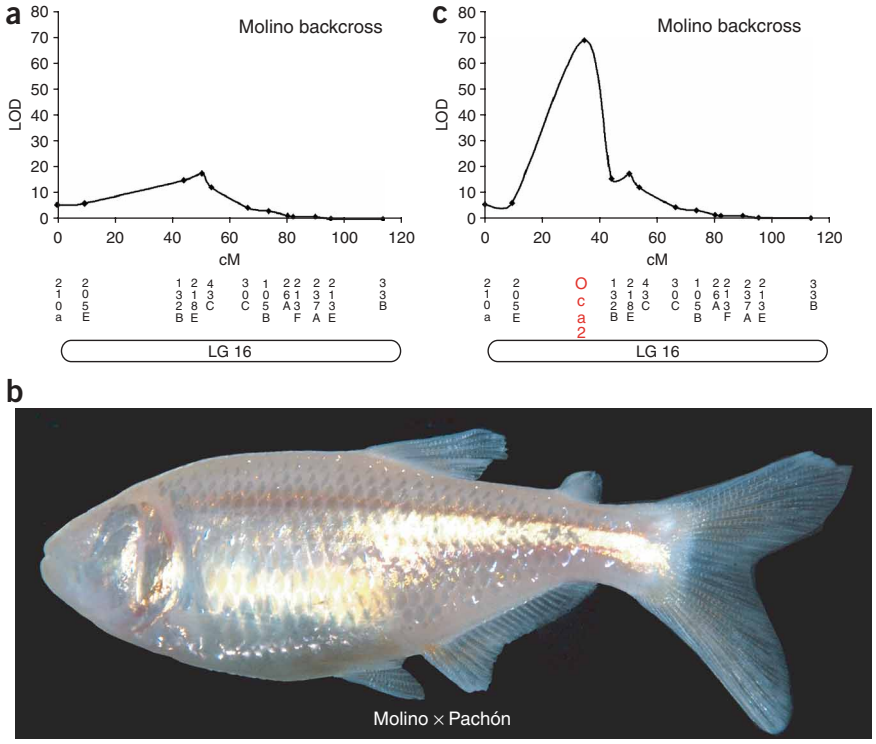

Figure 3 *Oca2* is linked to albinism in both the Pachón and Molino cave populations. (**a**) The albino locus in the Molino backcross is in linkage group (LG) 16. The LOD score is plotted against the distance (in cM) across this linkage group. (**b**) An albino Pachón × Molino hybrid, showing noncomplementation. (**c**) The marker in *Oca2* is located at the albino locus in the Molino backcross and its inclusion in the analysis greatly increases the LOD score.

To test whether the polymorphisms in the cave populations' *Oca2* sequences cause albinism through loss of function of *Oca2*, we used the melan-p cell line, which is a melanocyte cell line generated from an *Oca2*-deficient mouse[14]. We made constructs expressing wild-type surface-fish *Oca2* and surface-fish *Oca2* modified with each of the cavefish polymorphisms driven by the human ubiquitin promoter. We first transfected the surface-fish *Oca2* construct to ascertain whether wild-type *Astyanax Oca2* could complement *Oca2* function in mouse cells. Indeed, melan-p cells transfected with the surface-fish *Oca2* showed high levels of pigmentation (**Fig. 4b–d**). We then transfected cells with constructs encoding the different amino acid substitutions seen in the Pachón cave. Both point mutations caused rescue of pigmentation (**Fig. 4e–j**). However, neither the deletion found in the Molino cave nor the deletion found in the Pachón cave caused rescue of pigmentation (**Fig. 4k–p**). Therefore, the deletions cause loss of function of OCA2, whereas the two point mutations do not drastically affect the function of OCA2 in this cell line, strongly suggesting that the exon 21 deletion is the mutation that causes albinism in the Molino cave population and the exon 24 deletion is the mutation that causes albinism in the Pachón population.

The identification of different inactivating mutations in *Oca2* in the Molino and Pachón populations suggests that albinism evolved independently in these two caves, by convergent evolution in the same gene. The independent evolution of *Oca2* in the two caves is further supported by analysis of amino acid changes and neutral base changes; these polymorphisms do not group the Molino and Pachón populations together to the exclusion of the surface population (**Supplementary Table 2** online).

We also examined another cave population, Japonés, that contains albino individuals (**Fig. 1a**). In a complementation test between an albino Japonés individual and an albino Pachón individual, only albino offspring resulted, suggesting that albinism in the Japonés population also arose through mutations in the *Oca2* gene (data not shown). Sequencing of *Oca2* from one albino Japonés individual showed that neither the Molino nor the Pachón deletion was present (data not shown), though the two point mutations found in the Pachón cave were found. Together with the complementation data, this result suggests that a third, independent mutation in the *Oca2* gene, perhaps in a regulatory sequence, is responsible for the convergent albinism phenotype in the Japonés cave.

The parallel evolution of loss of pigmentation and loss of eyes within the species *Astyanax* is mirrored in many other cave dwelling organisms. The most debated issue about cave animals is why this loss of eyes and pigmentation occurs. There are three main theories[1,8,9,15]. First, the genes responsible for structures or pathways that are not advantageous in a dark environment, such as eyes or pigmentation, might accumulate deleterious mutations and, over time, completely degenerate. Second, it might be advantageous to lose eyes and pigmentation to conserve the energy or space that these structures consume. Third, the genetic changes that cause loss of eye and pigmentation might cause changes in other structures or pathways that might be advantageous in the cave environment.

The identification of the mutations responsible for loss of pigmentation in *Astyanax* allows us to consider the molecular nature of the variation facilitating this change in response to the cave environment. It is notable that *Oca2* has repeatedly mutated in the cave populations we examined. It is possible that in the cave environment, loss of *Oca2* function is actually advantageous, for some as yet unknown reason. Alternatively, *Oca2* might be mutated more often than other pigmentation pathway genes in *Astyanax* simply because *Oca2* mutations do not seem to have any deleterious effects aside from loss of pigmentation and problems with vision. It is possible that some of the other pigmentation genes have more pleiotropic effects and that those mutations are not as viable; for example, all of the mutations in zebrafish *Tyr* that cause complete loss of pigmentation are only semiviable[16]. A final explanation is that *Oca2* is the most frequently targeted pigmentation gene in *Astyanax* for the same reasons that it seems to be in humans: first, *Oca2* presents a very large target size for mutagenesis, being 345 kb in humans, with 24 exons[11]. Second, human *Oca2* maps to a region characterized by repetitive sequences, which are often associated with chromosomal rearrangements and deletions[17]. Although it is not yet known whether the *Astyanax Oca2* locus is similarly characterized by a large size and a large number of associated repeat sequences, the most parsimonious explanation seems to be a combination of a lack of deleterious pleiotropic effects in conjunction with the structure of *Oca2* itself. These sorts of features may, in general, predispose certain loci to be targets for evolutionary

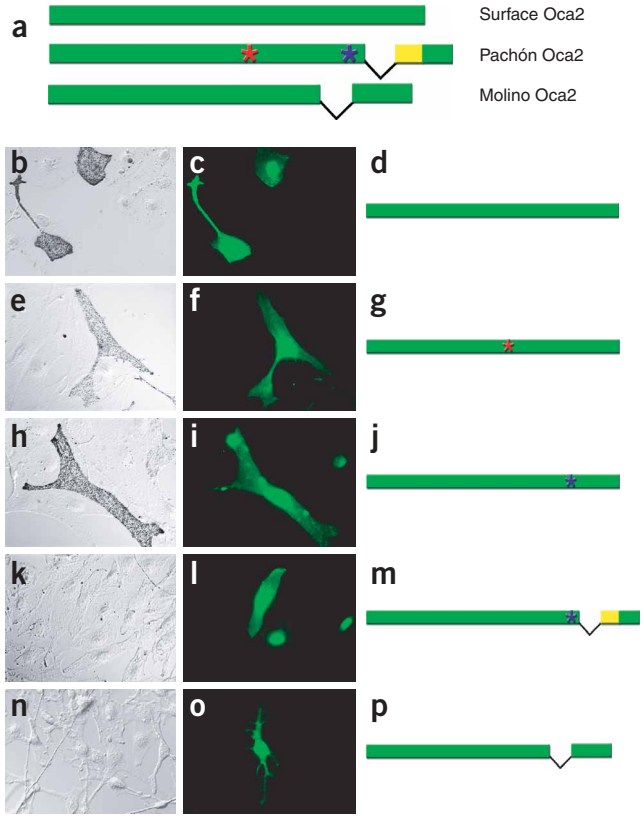

Figure 4 Different *Oca2* exon deletions in the Molino population and the Pachón population cause loss of function of the OCA2 protein. (**a**) Schematics of the surface, Pachón and Molino *Oca2* coding regions. Asterisks in the Pachón *Oca2* represent changes in conserved amino acid residues: red asterisk, methionine to valine; blue asterisk, proline to serine (see **Supplementary Figs. 1** and **2**). In the Pachón coding sequence, exon 24 is almost completely deleted. Following exon 23 are additional sequence (intron 23), the last few amino acids of exon 24, and the 3′ UTR. The Molino coding sequence is identical to that of the surface-fish *Oca2* except that exon 21 is missing. (**b**–**d**) Transfection of the unmodified surface-fish *Oca2* construct results in highly pigmented cells. (**e**–**g**) Transfection of the construct encoding the methionine-to-valine substitution, found in the Pachón cave, results in pigmented cells, although they are slightly less pigmented than the cells transfected with the surface-fish sequence. (**h**–**j**) Transfection of the construct encoding the proline-to-serine substitution results in highly pigmented cells. (**k**–**p**) Transfection of the construct with the extra intron 23 sequence and exon 24 deletion (**k**–**m**) or the construct with the exon 21 deletion (**n**–**p**) does not rescue pigmentation. **b**,**e**,**h**,**k**,**n** show bright-field pictures of the melan-p cell line transfected with different constructs; **c**,**f**,**i**,**l**,**o** show the corresponding transfected cells visualized by fluorescence (all constructs were co-injected with pUB-GFP[26]); and **d**,**g**,**j**,**m**,**p** provide schematics of the constructs transfected in each experiment.

forces effecting morphological change. The existence of at least 86 species of cavefish[2], a subset of which show albinism, will allow for further examination of the parallel evolution of this trait in the cave environment.

We have seen that albinism has evolved in two different cave populations through independent changes in the same gene. Other studies have also shown that one gene is responsible for the same morphological change in multiple populations or species: ectodysplasin (*Eda*) in body armor and *Pitx1* in pelvic reduction in the three-spine stickleback[18,19], *ovo* (shaven-baby) in trichome loss in different drosophilids[20], and *MC1R* in pigmentation in pocket mice, jaguars and several avian species[21,22]. Thus, the same morphology often evolves by mutation in the same gene, possibly because it is the most efficient or best way for a certain phenotype to evolve.

Another issue often discussed regarding the evolution of morphological change is whether coding changes or regulatory changes generate new traits. Regulatory mutations have an advantage as agents of morphological change in that they can alter a gene's activity in a subset of the regions in which it is expressed. Regulatory mutations have been identified or implicated in morphological change in plate armor and pelvic reduction in sticklebacks[18,19], wing-spot pigmentation in *Drosophila biarmipes*[23], muscle mass in pigs[24] and trichome loss in drosophilids[20]. Often it is difficult to confirm that a gene has a specific regulatory mutation, as these mutations can span large areas of sequence. In contrast, coding differences are easier to find. It is notable that many of the examples of evolutionary change involving coding mutations that have previously been described, including our example of *Oca2* in albinism in *Astyanax*, involve overall changes in pigmentation[21,22]. This could be because pleiotropic effects of pigmentation genes are minimal and usually do not affect the viability of the organism.

We have identified specific genetic lesions responsible for the parallel evolution of albinism in different cave populations of *Astyanax*, and found that they represent convergent genetic events in separate populations. The genetic tools we have developed will facilitate further investigation into the molecular bases for the evolution of specialized morphological characteristics in the unique and well-studied cave environment.

METHODS

Crosses. We used five crosses in this study: a Molino backcross, a Pachón F_2 cross, a Pachón backcross, a Molino × Pachón complementation cross and a Pachón × Japonés complementation cross (see **Supplementary Methods** online).

Microsatellite identification. To make a genomic library, we digested genomic surface-fish DNA with *Sau*3AI and cloned fragments of 500–700 bp into *Bam*H1-linearized pBluescriptSK$^+$ (Stratagene). We then electroporated the library into SURE electroporation-competent cells (Stratagene). A ^{32}P- or digoxigenin-end-labeled $(CA)_{12}$ probe was used to hybridize colony lifts and colonies hybridizing to the probe were sequenced using T3 and T7 primers. Primers to detect microsatellites that had ten or more repeats were designed using Primer3. The following tag sequence was added to the 5′ end of every forward primer: 5′-CACGACGTTGTAAAACGAC-3′ (Y.Z. and L.I.Z., unpublished data).

Genotyping and linkage map construction. We genotyped 111 individuals for 300 microsatellite markers in the Molino backcross and 235 individuals with 254 microsatellites in the Pachón F_2 cross. PCR reactions were 10 μl in volume and contained 0.1 mM $MgCl_2$, 6 mM Tris-HCl, pH 8.3, 30 mM KCl, 0.006% glycerol, 0.25 mM dNTP mix (Roche), 0.06% Tween, 0.06% Nonidet P-40, 0.25 units of *Taq* DNA polymerase (Roche), 5 nM forward primer, 200 nM reverse primer and 200 nM of the fluorescent tag primer 5′-CACGACGTTGTAAAACGAC-3′ labeled with one of two phosphoramidite conjugates, Hex and Fam, using a PCR program as described[25]. Each PCR reaction was primed with the specific forward primer. The majority of cycles used the forward primer in excess—the fluorescently labeled tag (Y.Z. and L.I.Z., unpublished data). Three Fam-labeled PCR products and three Hex-labeled PCR products were pooled and run on an ABI 3700 with GeneScan-500 ROX size standard (Applied Biosystems). Genotyper software (version 3.6) was used to analyze the genotyping results. The program JoinMap was used to make the microsatellite linkage map using the Kosambi mapping function, a jump threshold of 5.0 and

a ripple function. For the Molino backcross, 267 of 300 markers were grouped into 35 linkage groups using a LOD threshold of 4.0.

QTL mapping. We used the interval mapping function of MapQTL[26] to determine the albino locus and determined significant LOD scores using a permutation test.

Cloning and mapping of albinism candidate genes. We obtained fragments of *Astyanax Tyr*, *Trp1* and *Oca2* by degenerate PCR. For primers and specifics, see **Supplementary Methods**. The fragment that we initially amplified by degenerate PCR of *Oca2* contained exons 11–23. We were able to amplify exon 1 of *Oca2* from the DNA of surface fish by designing primers to the area of exon 1 that is most conserved between zebrafish and fugu. Using a forward primer in exon 1, 5′-GAGCCCAGGGTCATCAGG-3′, we obtained the sequence through exon 11. To obtain the 5′ and 3′ ends, we used the SMART Race cDNA kit (Clontech).

Genomic analysis of missing *Oca2* exons in Pachón and Molino populations. We carried out PCR to exons 21 and exons 14 for genomic DNA from all individuals of the Molino backcross using the following primers: exon 21F, exon 21R, exon 14F and exon 14R (see **Supplementary Table 1** online for all primer sequences). For the Pachón backcross, we amplified intron 23 and exon 24 to the 3′ untranslated region using the following primers: intron 23F, intron 23R, exon 24F and 3′ UTR.

Sequencing of full-length *Oca2* coding region for Surface, Molino and Pachón fish. We extracted RNA from fin clips and whole fish using Trizol (Invitrogen), and we made cDNA using AMV reverse transcriptase (Roche). To amplify the full-length cDNA, we used primers from the 5′ and 3′ untranslated regions of *Oca2*. In some cases, we amplified *Oca2* in two pieces for ease of amplification, using a reverse primer in exon 12 and a forward primer in exon 10. We compared *Oca2* sequences from five surface fish, five Molino fish and two Pachón fish.

Making overexpression constructs. We cloned surface, Molino and Pachón full-length sequences into pGEM-T Easy (Promega). We cloned the missing exon 21 (Molino) and missing exon 24 plus extra sequence (Pachón) cloned into the surface-fish construct using *Mlu*I and *Sma*I. pUB-GFP[26], a vector that encodes GFP under the control of the human ubiquitin promoter, was used as the backbone for the overexpression constructs. pUB-GFP was blunted with *Sma*I and *Msc*I, removing the GFP, and pGEM-T Easy surface-fish *Oca2*, surface-fish *Oca2* minus exon 21 and surface-fish *Oca2* minus exon 24 were blunted with *Pvu*II. The different *Oca2* fragments and the pUB fragment were ligated together (Takara), putting surface-fish *Oca2* under the control of the ubiquitin promoter. To make the point mutations found in the Pachón cave, we carried out site-directed mutagenesis using the QuikChange site directed mutagenesis kit (Stratagene) on the pUB-surface *Oca2* construct.

Cell line assay. We obtained melan-p cells from D. Bennett's group and cultured them as described[14]. We then transfected the cells on 3-cm plates using Fugene (Roche) and 1 μg of each construct. The transfections were as follows: pUB-GFP alone[26], pUB-GFP with pUB-surface *Oca2*, pUB-GFP with pUB-surface minus exon 21, pUB-GFP with pUB surface minus exon 24 and the proline-to-serine amino acid substitution, pUB-GFP with pUB-surface *Oca2* with the methionine-to-valine substitution, and pUB-GFP with pUB-surface *Oca2* with the proline-to-serine substitution. After transfection, we waited 4 d for pigmentation to develop.

Accession numbers. GenBank: *A. mexicanus* microsatellite markers, BV678703–BV678968; *A. mexicanus Oca2*, DQ232591.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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