Report

Current Biology

Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish

Highlights

- Analysis of surface-cave hybrids reveals a relationship between albinism and sleep
- Albinism and reduced sleep are observed in *oca2* mutant surface fish
- The *oca2* gene may be under selection in multiple cavefish populations

Authors

Morgan O'Gorman, Sunishka Thakur, Gillian Imrie, ..., Suzanne E. McGaugh, Alex C. Keene, Johanna E. Kowalko

Correspondence

akeene@bio.tamu.edu (A.C.K.), jok421@lehigh.edu (J.E.K.)

In brief

O'Gorman et al. find that the *oca2* gene, which underlies albinism in *A. mexicanus* cavefish, plays a role in the evolution of sleep loss in cavefish and that *oca2* alleles are under positive selection in multiple cavefish populations. These results reveal a pleiotropic function of *oca2* underlying the adaptive evolution of albinism and sleep loss.

O'Gorman et al., 2021, Current Biology *31*, 1–8
August 23, 2021
https://doi.org/10.1016/j.cub.2021.06.077

CellPress

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

Current Biology

CellPress

Report

Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish

Morgan O'Gorman,[1,8] Sunishka Thakur,[1,8] Gillian Imrie,[1] Rachel L. Moran,[2] Stefan Choy,[1] Itzel Sifuentes-Romero,[1] Helena Bilandžija,[3] Kenneth J. Renner,[4] Erik Duboué,[1,7] Nicolas Rohner,[5] Suzanne E. McGaugh,[2] Alex C. Keene,[1,6,*] and Johanna E. Kowalko[1,7,9,10,*]

[1]Jupiter Life Science Initiative, Florida Atlantic University, Jupiter, FL 33458, USA
[2]Department of Ecology, Evolution, and Behavior. University of Minnesota, St. Paul, MN 55108, USA
[3]Department of Molecular Biology, Rudjer Boskovic Institute, 10000 Zagreb, Croatia
[4]Department of Biology, University of South Dakota, Vermillion, SD 57069, USA
[5]Stowers Institute, Kansas City, MO 64110, USA
[6]Department of Biology Science, Florida Atlantic University, Jupiter, FL 33458, USA
[7]Harriet L. Wilkes Honors College, Florida Atlantic University, Jupiter, FL 33458, USA
[8]These authors contributed equally
[9]Twitter: @JohannaKowalko
[10]Lead contact
*Correspondence: akeene@bio.tamu.edu (A.C.K.), jok421@lehigh.edu (J.E.K.)
https://doi.org/10.1016/j.cub.2021.06.077

SUMMARY

Adaptation to novel environments often involves the evolution of multiple morphological, physiological, and behavioral traits. One striking example of multi-trait evolution is the suite of traits that has evolved repeatedly in cave animals, including regression of eyes, loss of pigmentation, and enhancement of non-visual sensory systems.[1,2] The Mexican tetra, *Astyanax mexicanus*, consists of fish that inhabit at least 30 caves in Mexico and ancestral-like surface fish that inhabit the rivers of Mexico and southern Texas.[3] Cave *A. mexicanus* are interfertile with surface fish and have evolved a number of traits, including reduced pigmentation, eye loss, and alterations to behavior.[4–6] To define relationships between different cave-evolved traits, we phenotyped 208 surface-cave F2 hybrid fish for numerous morphological and behavioral traits. We found differences in sleep between pigmented and albino hybrid fish, raising the possibility that these traits share a genetic basis. In cavefish and other species, mutations in *oculocutaneous albinism 2* (*oca2*) cause albinism.[7–12] Surface fish with mutations in *oca2* displayed both albinism and reduced sleep. Further, this mutation in *oca2* fails to complement sleep loss when surface fish harboring this engineered mutation are crossed to independently evolved populations of albino cavefish with naturally occurring mutations in *oca2*. Analysis of the *oca2* locus in wild-caught cave and surface fish suggests that *oca2* is under positive selection in 3 cave populations. Taken together, these findings identify *oca2* as a novel regulator of sleep and suggest that a pleiotropic function of *oca2* underlies the adaptive evolution of albinism and sleep loss.

RESULTS

Multiple populations of cave *A. mexicanus* have evolved a suite of traits that distinguish them from surface-dwelling counterparts, including regression of eyes, albinism, elevated energy stores, and sleep loss.[6,13–16] To investigate the relationship between evolved traits in cave *A. mexicanus*, we generated hybrids from surface fish and Pachón cavefish and quantified numerous morphological and behavioral traits associated with cave evolution (Figure 1A). At 20 days post-fertilization (dpf), surface fish, cavefish, and surface-cave F1 and F2 hybrids were assayed for sleep over a 24-h period, followed by measurements of food consumption. Fish were then stained with Nile Red, which labels adipocytes in larval fish,[15,17] and imaged to score pigmentation, adiposity, and eye diameter (Figure 1B). Because individual fish were followed throughout all phenotyping steps, this experimental design allowed for examination of the genetic relationship between traits in F2 hybrid fish.

A comparison of hybrids to cave and surface populations revealed variability suggestive of both monogenic and polygenic inheritance of the various traits, largely in agreement with previous findings.[16,18,19] Sleep duration was significantly reduced in Pachón cavefish compared with sleep duration in surface fish. F1 hybrids slept more than Pachón cavefish and less than surface fish (Figure 1C; Figure S1A). F2 individuals were variable in the amount they slept, with some F2 fish sleeping little, similar to Pachón cavefish, and other F2 fish sleeping at surface-like levels (Figure 1C). Additionally, sleep architecture was different between the groups. Sleep loss in Pachón cavefish was due to reductions in the number and duration of sleep bouts relative

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

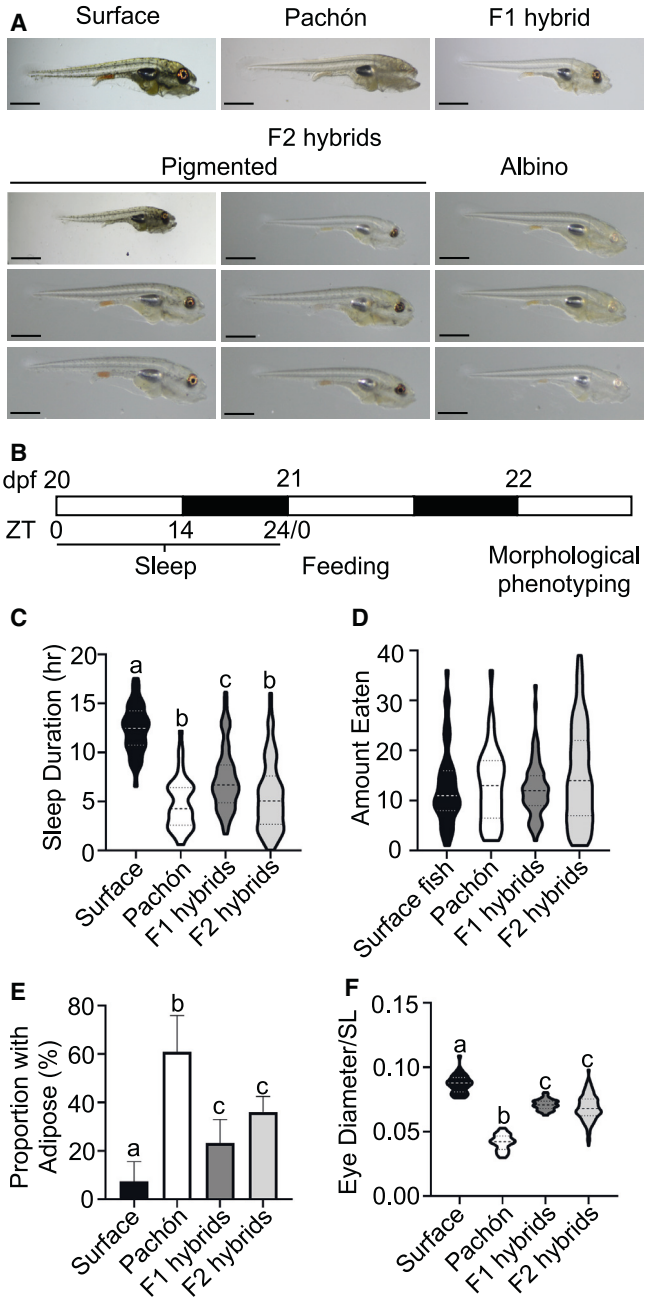

Figure 1. Genetic analysis of multiple traits evolved in cave *A. mexicanus*

(A) Images of 22-dpf fish (scale, 1 mm).

(B–F) (B) Timeline for phenotypic analysis. Surface fish (n = 40), Pachón cavefish (n = 41), surface-Pachón F1 hybrids (n = 73), and surface-Pachón F2 hybrid fish (n = 208) were phenotyped for: (C) total sleep duration over 24 hrs (Kruskal-Wallis: $H_2 = 92.76$, $p < 0.0001$; Dunn's multiple comparison: Pa versus F1: $z = 3.55$, $p = 0.002$, F1 versus F2: $z = 3.95$, $p = 0.0016$; All other: $p < 0.0001$), (D) total brine consumed (Kruskal-Wallis: $H_2 = 3.49$, $p = 0.3221$), (E) proportion of individuals with adipose deposits (error bars were calculated using z* value of 1.96 and denote the margin of error of the sample proportion. Fishers exact tests: SF v Pa $p < 0.0001$, SF v F1 $p = 0.0408$, SF v F2 $p = 0.0002$, Pa v F1 $p = 0.0001$, Pa v F2 $p = 0.0048$, F1 v F2 $p = 0.0590$), and (F) eye diameter, corrected for standard length (ANOVA: $F = 200.7$, $p < 0.0001$; Tukey's: F1 versus F2 $q = 2.802$, $p = 0.1970$; all others $p < 0.0001$).

Statistical differences are indicated by different letters in (C)–(F). In (C), (D), and (F), the dotted lines represent the median and ± the quartiles.

See also Figure S1.

to surface fish (Figures S1B and S1C). The average duration of each sleep bout in F1 hybrid fish was similar to average duration of sleep bouts in cavefish, whereas bout number in F1 hybrids was intermediate between surface fish and cavefish (Figures S1B and S1C). This raises the possibility that different components of sleep architecture are independently regulated. Indeed, bout number and bout duration were weakly correlated in F2 fish (Spearman's rho = 0.36, $p < 0.0001$), consistent with some different genetic factors contributing to these different components of sleep architecture (Figure S1D).

One hypothesis for the evolution of sleep loss in cavefish is that altered foraging and/or metabolic demands associated with living in a food-poor environment could drive the evolution of sleep loss.[20] We observed no significant differences in total food consumption between surface fish and Pachón cavefish, nor were there significant differences between F1 hybrids or F2 hybrids and either parental population (Figure 1D), in agreement with previous findings establishing that feeding does not differ between surface fish and Pachón cavefish.[14,21] The initial production of adipose deposits is ontogenically regulated and occurs earlier in Pachón cavefish than in surface fish.[15] Within all groups, some fish developed adipose deposits by 22 dpf, while others did not (Figure 1E; Figures S1E and S1F). However, a significantly larger percentage of cavefish developed adipose deposits than did surface fish. An intermediate percentage of F1 and F2 hybrid fish had developed adipose deposits at this stage relative to both cave and surface populations (Figure 1E), suggesting that differences in adiposity have a genetic basis and that regulation of adiposity shows partial or incomplete dominance.

Cave animals are characterized by loss of eyes and pigmentation.[1] Similarly to what was found in previously reported studies,[22,23,6] we found that Pachón cavefish eyes were significantly smaller than eyes in surface fish. Further, eyes in F1 hybrid fish were intermediate in size and significantly different from the eyes in both parental populations (Figure 1F). Eye size ranged in F2 hybrid fish from sizes that were surface-like to cave-like (Figure 1F). Therefore, nearly all traits analyzed with differences between surface fish and cavefish displayed intermediate phenotypes in F1 hybrids and variability in F2 fish consistent with a polygenic basis for evolved differences between *A. mexicanus* cave and surface populations.

Finally, we analyzed albinism. All surface and F1 fish exhibited robust melanin pigmentation, whereas all Pachón cavefish were albino, consistent with the recessive nature of this trait (Figure 1A).[7,13] In our F2 population, 61 of 208 tested fish were albino (Figure 1A). To examine segregation of albinism in F2 individuals precisely, we quantified the number of albino F2 fish from a single clutch by collecting a random cohort of fish pre-pigmentation and allowing these fish to develop until 5 dpf, when albinism can be easily scored by eye as complete absence of melanin pigmentation. Of these fish, 66 out of 283 were albino (23.3%), which was not significantly different from the expected ratios for a recessive trait controlled by a single gene (Chi square:

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

CellPress

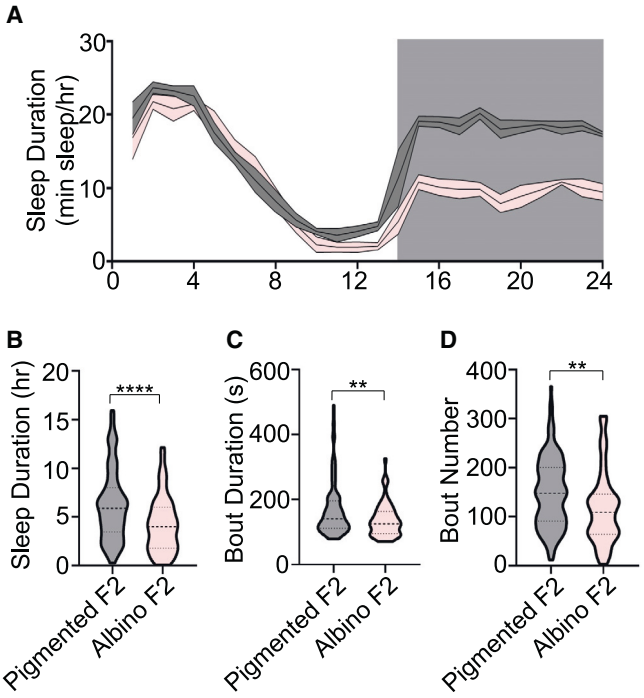

Figure 2. Relationship between sleep and pigmentation in cave-surface hybrid fish

(A) 24-h sleep profile of F2 hybrid crosses depicting the amount slept at 1-h intervals over a 24-h time frame. The gray area of each graph represents laboratory night, when the lights were off. The gray line represents pigmented F2s, and the pink line represents albino F2s.

(B) Total sleep duration over 24 hours in albino compared with that in pigmented F2 hybrids during the 24-h period (Mann-Whitney, U = 3,379, $p < 0.0001$).

(C) Average bout duration of albino compared with that in pigmented F2 hybrids (t test, t = 2.67, p = 0.0079).

(D) Total number of bouts over 24 hours in albino versus pigmented F2 hybrids (t test, t = 3.24, p = 0.0043).

For (A)–(D), pigmented F2 hybrids (n = 147), and albino F2 hybrids (n = 61). Graph in (A) represents the average and standard deviation. For (B)–(D), the dotted lines represent the median and ± the quartiles. **$p < 0.01$, ****$p < 0.0001$.

p = 0.69), consistent with the monogenic inheritance of albinism previously reported.[7,13]

Cave traits could have evolved independently from each other or through a shared genetic or functional basis. To determine whether there is a relationship between cave-evolved traits, we performed pairwise comparisons between the traits that were significantly different between cavefish and surface fish: sleep, eye size, pigmentation, and the presence of adipose tissue, reasoning that traits that were co-inherited would be significantly correlated with one another. We found 3 significant relationships from this analysis. F2 fish with adipose deposits had significantly smaller eyes than did F2 fish without adipose (Cohen's d = 0.31; Table S1). Further, albino F2 fish had significantly smaller eyes than did pigmented fish (Cohen's d = 0.61; Figure S1G; Table S1). Additionally, total sleep duration was significantly reduced in albino F2 fish in comparison with pigmented F2 fish (Cohen's d = 0.55; Figures 2A and 2B). In albino F2 hybrid fish, both bout duration and bout number were significantly reduced (Figures 2C and 2D). These results suggest that albinism is associated with multiple other traits that are present in Pachón cavefish, raising the possibility that albinism could have evolved due to selection for another trait that is important for cave adaptation. Because the molecular basis of albinism is known,[7,8] these results provide a rare opportunity to investigate the relationship between morphological and behavioral evolution. Thus, we examined the relationship between albinism and sleep loss to determine if the co-inheritance of these traits is due to closely linked loci or pleiotropy.

In 2 independently evolved populations of cavefish, Pachón and Molino, albinism is caused by deletions in the coding sequence of the *oculocutaneous albinism 2* (*oca2*) gene.[7,8] To explore the role of *oca2* in behavioral evolution, we examined whether *oca2* is expressed in the brain. Previous studies found that *oca2* is expressed in melanophores and the retinal pigmented epithelium in developing surface fish.[24] We performed qPCR on fins and brains from adult surface fish and found that expression of *oca2* is detectable in adult surface-fish brains and melanophore-containing fins (Figure S2A). In addition, we performed RNA fluorescent *in situ* hybridization (FISH) on larval surface fish and Pachón cavefish and found that *oca2* is expressed in the brain during development in *A. mexicanus* (Figure S2B). These results suggest that *oca2* could play a role in both pigmentation and behavior.

To directly test whether mutation of *oca2* contributes to the evolution of other cave evolved traits, we examined eye size, adipose deposition, feeding, and sleep in surface fish with a CRISPR-Cas9-engineered 2 base-pair deletion in exon 21 of the *oca2* gene ($oca2^{\Delta 2bp}$). A deletion of this entire exon is found in Molino cavefish,[7] suggesting the engineered mutation could phenocopy the naturally occurring mutation. This engineered mutation, when homozygous, results in complete loss of melanin pigmentation in surface fish, phenocopying albino cavefish (Figures 3A and 3B).[8] Eye size, proportion of individuals with adipose tissue, and amount of brine shrimp consumed were all not significantly different in $oca2^{\Delta 2bp/\Delta 2bp}$ surface fish when compared with wild-type $oca2^{+/+}$ siblings (Figures S2D–S2F), suggesting that variation at the *oca2* locus does not contribute to the evolution of these traits.

Sleep was significantly reduced in $oca2^{\Delta 2bp/\Delta 2bp}$ surface fish in comparison with pigmented, wild-type $oca2^{+/+}$ control siblings (Cohen's d = 1.3; Figures 3C and 3D). This reduction in sleep derives from reduced bout duration, which is significantly reduced in $oca2^{\Delta 2bp/\Delta 2bp}$ surface fish in comparison with wild-type $oca2^{+/+}$ siblings (Figure 3E). Mutation of *oca2* did not result in a statistically significant change in bout number (Figure 3F). To determine whether sleep differences resulting from mutations in the *oca2* gene were dependent on light, we examined sleep in these fish under standard light:dark (L:D) conditions and dark:dark (D:D) conditions. Sleep was reduced in $oca2^{\Delta 2bp/\Delta 2bp}$ surface fish in comparison with pigmented, wild-type $oca2^{+/+}$ control siblings under both L:D and D:D conditions, suggesting that the effects of mutations of *oca2* on sleep were independent of light and not due to vision impairment or changes in light sensitivity (Figures S2G and S2H). Together, these findings demonstrate that mutations in the *oca2* gene can affect both pigmentation and sleep as well as raise the possibility that pleiotropy of the *oca2* gene

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

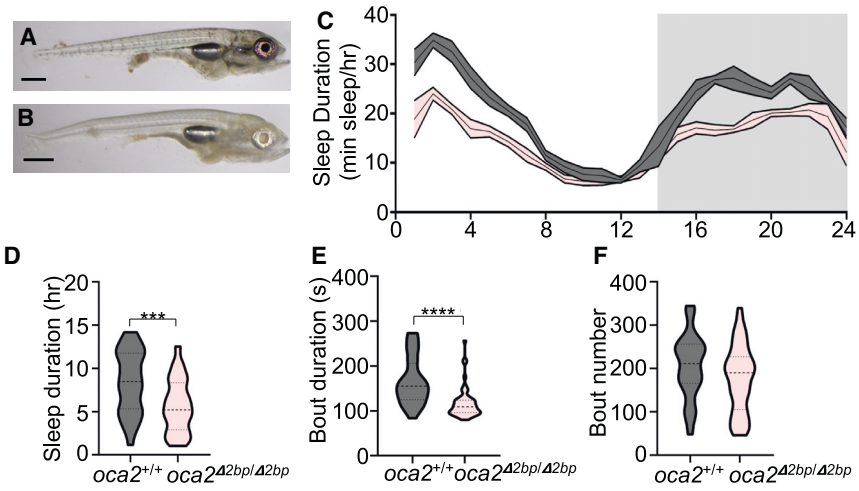

Figure 3. *oca2* mutant surface fish sleep less than wild-type surface fish

(A and B) Images of 22-dpf (A) pigmented wild-type and sibling (B) albino $oca2^{\Delta 2bp/\Delta 2bp}$ surface fish (scale, 1mm).

(C) 24-h sleep profile of $oca2^{+/+}$ and $oca2^{\Delta 2bp/\Delta 2bp}$ surface fish siblings depicting the amount slept at 1-h intervals over a 24-h time frame. The shaded area represents laboratory night, when the lights were off.

(D) Total sleep duration over 24 hours in $oca2^{\Delta 2bp/\Delta 2bp}$ compared with that in wild-type $oca2^{+/+}$ siblings (t test, t = 3.71, p = 0.0004).

(E) Average bout duration in $oca2^{\Delta 2bp/\Delta 2bp}$ compared with that in wild-type $oca2^{+/+}$ siblings (Mann-Whitney, U = 309, $p < 0.0001$).

(F) Total bout number over 24 hours in $oca2^{\Delta 2bp/\Delta 2bp}$ compared with that in wild-type $oca2^{+/+}$ siblings (Mann-Whitney, U = 662, p = 0.092).

For (C)–(F), pigmented wild-type n = 32, and albino $oca2^{\Delta 2bp/\Delta 2bp}$ n = 51. Graph in (C) represents the average and standard deviation. For (D)–(F), the dotted lines represent the median and ± the quartiles. ***$p < 0.001$, ****$p < 0.0001$.

See also Figure S2.

contributed to the evolution of both albinism and reduced sleep in cavefish.

Surface fish heterozygous at the *oca2* locus ($oca2^{\Delta 2bp/+}$) are pigmented, and they presented an intermediate total sleep-duration phenotype, which did not differ significantly from sleep in wild-type or *oca2* mutant siblings (wild-type and homozygous mutant surface fish rerun with heterozygous individuals included average total sleep duration for $oca2^{+/+}$ = 8.42 h [n = 32], $oca2^{\Delta 2bp/+}$ = 7.03 h [n = 47], $oca2^{\Delta 2bp/\Delta 2bp}$ = 5.59 h [n = 51]; one-way ANOVA: F = 6.89, p = 0.0014; Tukey's posthoc test: $oca2^{+/+}$ versus $oca2^{\Delta 2bp/+}$ [p = 0.1674], $oca2^{+/\Delta 2bp}$ versus $oca2^{\Delta 2bp/\Delta 2bp}$ [p = 0.1104], and $oca2^{\Delta 2bp/\Delta 2bp}$ versus $oca2^{+/+}$ [p = 0.0010]). We next investigated whether our engineered loss-of-function *oca2* alleles in surface fish complemented naturally occurring deletions in *oca2* in albino cavefish. We crossed surface fish heterozygous at the *oca2* locus ($oca2^{\Delta 2bp/+}$) to Pachón or Molino cavefish, both of which harbor coding mutations in *oca2*.[7] The presence of the engineered $oca2^{\Delta 2bp}$ allele resulted in albino offspring in surface-Pachón and surface-Molino crosses (genotypes $oca2^{\Delta 2bp/\Delta Pa}$ and $oca2^{\Delta 2bp/\Delta Mo}$, respectively), whereas offspring that inherited the wild-type allele from the surface parent (genotypes $oca2^{+/\Delta Pa}$ and $oca2^{+/\Delta Mo}$) were pigmented (Figures 4A, 4B, 4D, and 4E), confirming this mutation is sufficient to induce albinism in 2 cave populations, a finding consistent with previous studies.[8] Further, albino $oca2^{\Delta 2bp/\Delta Pa}$ and $oca2^{\Delta 2bp/\Delta Mo}$ hybrid fish slept significantly less than their $oca2^{+/\Delta Pa}$ and $oca2^{+/\Delta Mo}$ hybrid pigmented siblings (Figures 4C and 4F). These findings demonstrate that the engineered *oca2* mutant allele fails to complement the sleep phenotype in Pachón and Molino cavefish, supporting the notion that loss of *oca2* contributes to sleep loss in 2 independently evolved cavefish populations. Bout duration, but not bout number, was reduced in surface-Pachón $oca2^{\Delta 2bp/\Delta Pa}$ hybrid fish in comparison with $oca2^{+/\Delta Pa}$ siblings (Figure S3A; Figure S3D). Both bout duration and bout number in surface-Molino $oca2^{\Delta 2bp/\Delta Mo}$ hybrid fish were lower than what was seen in siblings that inherited 1 wild-type surface allele, but they did not reach significance (Figures S3B and S3E). Finally, we crossed $oca2^{\Delta 2bp/+}$ surface fish to Tinaja cavefish, which are not albino, and do not harbor known loss-of-function mutations in *oca2* but do show a reduction of *oca2* expression and exhibit a reduced sleep phenotype.[16,25] We found no visible difference in melanin pigmentation between $oca2^{\Delta 2bp/Ti}$ and $oca2^{+/Ti}$ hybrid fish, confirming a lack of effect of coding mutations in *oca2* on the presence of melanin pigmentation in this population (Figures 4G and 4H). Further, sleep in the $oca2^{\Delta 2bp/Ti}$ hybrid fish was not significantly different from sleep in the $oca2^{+/Ti}$ siblings, suggesting *oca2* mutations do not play a role in sleep loss in this population (Figure 4I; Figures S3C and S3F).

Our findings that a single gene contributes to both albinism and sleep loss in multiple populations in which albinism has evolved independently raises the possibility that selection for one or both of these traits leads to evolution of both traits in cave populations harboring *oca2* mutations. To test directly whether *oca2* is under selection in cavefish, we used a convolutional neural network (CNN) approach implemented in diploS/HIC[26] to determine whether the region of the genome containing *oca2* has experienced a selective sweep in each population. We analyzed whole-genome resequencing data of Molino (n = 9), Pachón (n = 10), and Tinaja (n = 8) cavefish as well as surface *A. mexicanus* populations from 2 different localities: Rascón (n = 7) and the Río Choy (n = 9). This analysis suggests *oca2* experienced soft sweeps in each of the 3 cave populations, as expected when selection acts on standing genetic variation. In both surface populations, diploS/HIC suggests *oca2* follows neutral evolution. These results were largely supported by a second analysis using hapFLK (see STAR Methods). Together, these analyses reveal that *oca2* alleles are under positive selection across *A. mexicanus* cave populations and support that selection for loss of *oca2* could be a critical contributor to the evolution of sleep loss and albinism in cave habitats.

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

CellPress

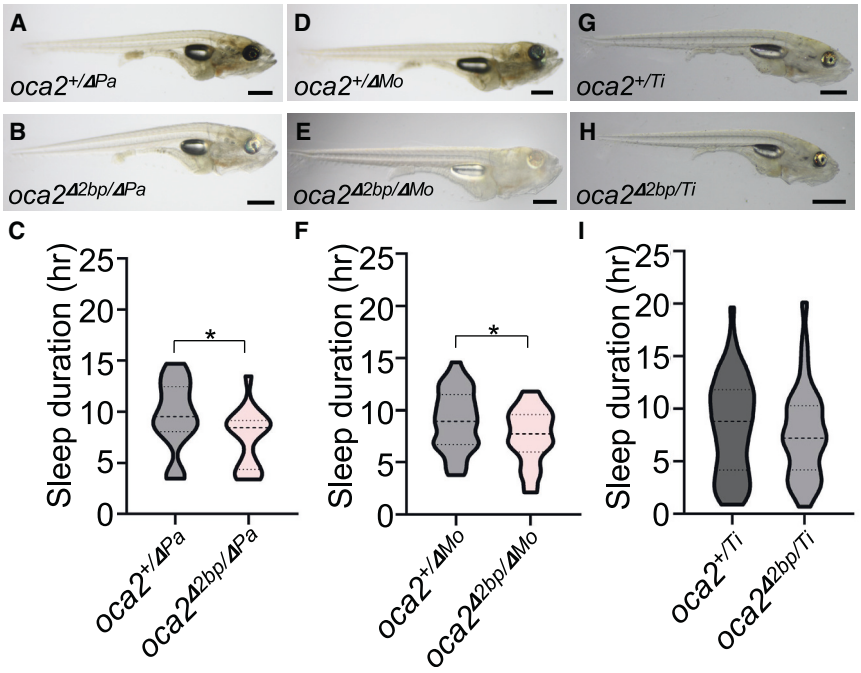

Figure 4. Lack of complementation in cave-surface F1 hybrid fish with two mutant *oca2* alleles

Melanin pigmentation (A, B, D, E, G, and H) and sleep duration (C, F, and I) were assessed in Pachón-surface F1 hybrid fish (A, B, and C), Molino-surface F1 hybrid fish (D, E, and F) and Tinaja-surface F1 hybrid fish (G, H, and I). Images of 22-dpf (A) $oca2^{+/\Delta Pa}$, (B) $oca2^{\Delta 2bp/\Delta Pa}$, (D) $oca2^{+/\Delta Mo}$, (E) $oca2^{\Delta 2bp/\Delta Mo}$, (G) $oca2^{+/Ti}$, and (H) $oca2^{\Delta 2bp/Ti}$ F1 hybrid fish.

Total sleep duration over 24 hours in (C) $oca2^{+/\Delta Pa}$ (n = 21) compared with that in $oca2^{\Delta 2bp/\Delta Pa}$ (n = 17) siblings (t test, t = 2.210, p = 0.033), (F) $oca2^{+/\Delta Mo}$ (n = 38) compared with that in $oca2^{\Delta 2bp/\Delta Mo}$ (n = 32) siblings (t test, t = 2.231, p = 0.029), and (I) $oca2^{+/Ti}$ (n = 56) compared with that in $oca2^{\Delta 2bp/Ti}$ (n = 33) siblings (t test, t = 0.7128, p = 0.478). In (A), (B), (D), (E), (G), and (H), the scale bar is 1mm. In (C), (F), and (I), the dotted lines represent the median and ± the quartiles. *p < 0.05.

See also Figures S3 and S4 and Table S2.

DISCUSSION

Understanding the relationships between evolved traits is a central goal of evolutionary biology. The interfertility of *A. mexicanus* populations provides a system to investigate the genetic and functional relationships between traits. Previous studies of trait evolution in *A. mexicanus* found pleiotropy contributing to some regressive and constructive traits[27,24] and quantitative trait loci (QTL) for many seemingly unrelated morphological traits cluster in the genome more than expected by chance.[19,28] Further, evolved differences in sensory systems are thought to be critical drivers of the behavioral changes observed in cavefish, and in some cases, QTL for sensory traits and behaviors overlap.[29,30] However, it is still unclear whether these observed trait relationships are due to linkage, pleiotropy, or both. Here, we present an in-depth genetic analysis of the relationship between multiple cave-evolved traits in *A. mexicanus* and identify several traits that could share genetic underpinnings. Our finding that *oca2* underlies albinism and contributes to the reduction in sleep in 2 independently evolved cave populations of *A. mexicanus* definitively demonstrates a pleiotropic role of a naturally occurring genetic variant in a morphological and a behavioral trait. To our knowledge, these findings represent the first association between monoallelic albinism and the evolution of an ecologically relevant behavior in any cave animal.

Our analysis reveals *oca2* expression in the brain in *A. mexicanus*, raising the possibility that *oca2* plays a role in the central nervous system to regulate behavior. These results, along with previously published reports that demonstrated that *oca2* is expressed in melanophores and the retinal pigmented epithelium of the eye,[24] suggest that *oca2* is expressed in multiple tissues. Thus, loss of *oca2* in either the brain or in pigment-producing cells could contribute to reductions in sleep in cavefish. For example, pigmentation loss is associated with visual defects (reviewed in Creel[31]), raising the possibility that the cave alleles of *oca2* impact circadian regulation of locomotor activity. Due to technical limitations, we were not able to extend recordings over multiple days. However, the recent development of methods for transgenesis, in which lines of fish have been established that express cell-type-specific transgenes,[32–34] will allow for further analysis of the tissue-specific requirements for *oca2* in these phenotypes in future studies.

There is evidence to suggest that OCA2 modulates behavior by regulating catecholamine levels. Multiple identified cave alleles of *oca2* cause albinism through disruptions of the first step of the melanin-synthesis pathway in *A. mexicanus*, the conversion of L-tyrosine to L-DOPA.[8,35] Further, morpholino knockdown of *oca2* in larval surface fish increases levels of dopamine, and albino cavefish have evolved higher levels of the catecholamines dopamine and norepinephrine.[24,36,37] Therefore, OCA2 could modulate behaviors through regulating catecholamine levels.[24,37] Catecholamines regulate a number of behaviors, including sleep, feeding, and social behaviors[38–40] that have evolved in cavefish,[14,16,30,41] and reductions in schooling behavior, anesthesia resistance, and loss of sleep have all been linked to catecholamines in *A. mexicanus* through pharmacological analyses.[30,37,42] Thus, our observations that *oca2* contributes to sleep loss in cavefish could be due to effects of *oca2* on catecholamine levels. Future analyses are needed to determine whether loss-of-function mutations in *oca2* are sufficient to alter catecholamine levels in regions of the brain that modulate sleep.

For decades, it has been argued that loss of pigmentation in cave animals is due to reduced selective pressure to maintain pigmentation within the dark cave environment.[6] Alternatively, it has been argued that loss of pigmentation could be due to direct or indirect selection.[24,37,43] Here, we provide genomic evidence that *oca2* is under selection in 3 cave populations of

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

A. mexicanus. This is consistent with previous studies that determined that *oca2* contains F_{ST} outlier SNPs between surface fish and fish from multiple cave populations, suggesting albinism via *oca2* could be adaptive in *Astyanax* cavefish populations.[44] Together, these results support the hypothesis that albinism has evolved through selection in cave populations and raise the possibility that selection could have acted on another trait, distinct from albinism: sleep.

Notably, although many populations of *A. mexicanus* cavefish display reduced pigmentation, albinism has been reported in just a subset of *A. mexicanus* cavefish populations.[7,43,45] Moreover, many other cavefish species produce melanin pigmentation.[46] Thus, the relationship between pigmentation reduction and sleep loss could be specific to the subset of cave animals that are completely albino. Tinaja cavefish are not albino and do not harbor the coding mutations reported in Molino and Pachón but do show reduced expression of *oca2* relative to surface fish.[25] Our results, which indicate that *oca2* is under positive selection in Tinaja cavefish, suggest that different *oca2* alleles could lead to different adaptive advantages in different cave habitats. Future studies characterizing the ecological differences between different cavefish populations will further resolve these questions.

In conclusion, we report 3 complementary findings that suggest *oca2* contributes to sleep loss in cavefish. First, in F2 hybrid fish, sleep is reduced in albino fish in comparison with pigmented fish from the same brood. Second, CRISPR-mediated mutation of *oca2* in surface fish reduces sleep. Third, these CRISPR-mediated mutations fail to complement the sleep phenotypes of Pachón and Molino cavefish that harbor endogenous *oca2* mutations. Finally, we find that *oca2* is under selection in 2 albino cavefish populations, Pachón and Molino, as well as the hypopigmented Tinaja population. These findings raise the possibility that selection drove the evolution of albinism and sleep loss observed in multiple independently evolved cavefish populations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Husbandry
  - *oca2* mutant fish
  - Hybrid fish
- METHOD DETAILS
  - Phenotyping
  - Dark:Dark sleep
  - RNA extraction, reverse transcription and qPCR to quantify *oca2* expression
  - Hybridization chain reaction (HCR) *in situ* hybridization and imaging
  - Test for selection
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistical analysis for trait comparisons

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.06.077.

ACKNOWLEDGMENTS

This work was supported by funding from NIH awards 1R01GM127872-01 to S.E.M., A.C.K., and N.R. and 1R21NS122166-01 to A.C.K and J.E.K. and NSF awards DEB1754231 to J.E.K. and A.K.; IOS165674 to A.C.K., and IOS1923372 to J.E.K., S.E.M., E.D., and N.R.; and IOS1933076 to J.E.K., S.E.M., and N.R. H.B. is supported by TTP-2018-07-9675 (HRZZ, EPFL). All authors are grateful to Arthur Lopatto and Peter Lewis (FAU) for assistance with cavefish husbandry and to Yongfu Wang and Brian Slaughter for help with the *in situ* hybridization.

AUTHOR CONTRIBUTIONS

Conceptualization, J.E.K. and A.C.K.; investigation and analysis, M.O., S.T., G.I., R.L.M., S.C., I.S., H.B., K.J.R., E.D., N.R., S.E.M., A.C.K., and J.E.K.; writing, review, and editing, M.O., S.T., G.I., R.L.M., S.C., I.S., H.B., K.J.R., E.D., N.R., S.E.M., A.C.K., and J.E.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 27, 2020
Revised: March 22, 2021
Accepted: June 25, 2021
Published: July 21, 2021

REFERENCES

1. Culver, D.C., and Pipan, T. (2009). The biology of caves and other subterranean habitats. (Oxford University Press).
2. Jeffery, W.R. (2001). Cavefish as a model system in evolutionary developmental biology. Dev. Biol. *231*, 1–12.
3. Mitchell, R., Russell, W., and Elliot, W. (1977). Mexican eyeless characin fishes, genus Astyanax: Environment, distribution and evolution*Volume 89* (Texas Tech Press).
4. Keene, A.C., Yoshizawa, M., and McGaugh, S.E. (2015). Biology and Evolution of the Mexican Cavefish. (Elsevier).
5. Kowalko, J. (2020). Utilizing the blind cavefish *Astyanax mexicanus* to understand the genetic basis of behavioral evolution. J. Exp. Biol. *223* (Pt, Suppl 1), jeb208835.
6. Wilkens, H. (1988). Evolution and genetics of epigean and cave Astyanax fasciatus (Characidae, Pisces) support for the neutral mutation theory. Evolutionary Biology, Volume 23 (Springer), pp. 271–367.
7. Protas, M.E., Hersey, C., Kochanek, D., Zhou, Y., Wilkens, H., Jeffery, W.R., Zon, L.I., Borowsky, R., and Tabin, C.J. (2006). Genetic analysis of cavefish reveals molecular convergence in the evolution of albinism. Nat. Genet. *38*, 107–111.
8. Klaassen, H., Wang, Y., Adamski, K., Rohner, N., and Kowalko, J.E. (2018). CRISPR mutagenesis confirms the role of oca2 in melanin pigmentation in Astyanax mexicanus. Dev. Biol. *441*, 313–318.
9. Brilliant, M.H., King, R., Francke, U., Schuffenhauer, S., Meitinger, T., Gardner, J.M., Durham-Pierre, D., and Nakatsu, Y. (1994). The mouse pink-eyed dilution gene: association with hypopigmentation in Prader-Willi and Angelman syndromes and with human OCA2. Pigment Cell Res. *7*, 398–402.
10. Grønskov, K., Ek, J., and Brondum-Nielsen, K. (2007). Oculocutaneous albinism. Orphanet J. Rare Dis. *2*, 43.
11. Fukamachi, S., Asakawa, S., Wakamatsu, Y., Shimizu, N., Mitani, H., and Shima, A. (2004). Conserved function of medaka pink-eyed dilution in melanin synthesis and its divergent transcriptional regulation in gonads among vertebrates. Genetics *168*, 1519–1527.

12. Saenko, S.V., Lamichhaney, S., Martinez Barrio, A., Rafati, N., Andersson, L., and Milinkovitch, M.C. (2015). Amelanism in the corn snake is associated with the insertion of an LTR-retrotransposon in the OCA2 gene. Sci. Rep. *5*, 17118.

13. Şadoğlu, P. (1957). A mendelian gene for albinism in natural cave fish. Experientia *13*, 394.

14. Aspiras, A.C., Rohner, N., Martineau, B., Borowsky, R.L., and Tabin, C.J. (2015). Melanocortin 4 receptor mutations contribute to the adaptation of cavefish to nutrient-poor conditions. Proc. Natl. Acad. Sci. USA *112*, 9668–9673.

15. Xiong, S., Krishnan, J., Peuß, R., and Rohner, N. (2018). Early adipogenesis contributes to excess fat accumulation in cave populations of Astyanax mexicanus. Dev. Biol. *441*, 297–304.

16. Duboué, E.R., Keene, A.C., and Borowsky, R.L. (2011). Evolutionary convergence on sleep loss in cavefish populations. Curr. Biol. *21*, 671–676.

17. Flynn, E.J., 3rd, Trent, C.M., and Rawls, J.F. (2009). Ontogeny and nutritional control of adipogenesis in zebrafish (Danio rerio). J. Lipid Res. *50*, 1641–1652.

18. Protas, M., Conrad, M., Gross, J.B., Tabin, C., and Borowsky, R. (2007). Regressive evolution in the Mexican cave tetra, Astyanax mexicanus. Curr. Biol. *17*, 452–454.

19. Protas, M., Tabansky, I., Conrad, M., Gross, J.B., Vidal, O., Tabin, C.J., and Borowsky, R. (2008). Multi-trait evolution in a cave fish, Astyanax mexicanus. Evol. Dev. *10*, 196–209.

20. Keene, A.C., and Duboue, E.R. (2018). The origins and evolution of sleep. J. Exp. Biol. *221*, jeb159533.

21. Alie, Alexandre, et al. (2018). Developmental evolution of the forebrain in cavefish, from natural variations in neuropeptides to behavior. eLIFE15, doi:https://elifesciences.org/articles/32808.

22. Ma, L., Strickler, A.G., Parkhurst, A., Yoshizawa, M., Shi, J., and Jeffery, W.R. (2018). Maternal genetic effects in Astyanax cavefish development. Dev. Biol. *441*, 209–220.

23. Sifuentes-Romero, I., Ferrufino, E., Thakur, S., Laboissonniere, L.A., Solomon, M., Smith, C.L., Keene, A.C., Trimarchi, J.M., and Kowalko, J.E. (2020). Repeated evolution of eye loss in Mexican cavefish: Evidence of similar developmental mechanisms in independently evolved populations. J. Exp. Zoolog. B Mol. Dev. Evol. *334*, 423–437.

24. Bilandžija, H., Ma, L., Parkhurst, A., and Jeffery, W.R. (2013). A potential benefit of albinism in Astyanax cavefish: downregulation of the oca2 gene increases tyrosine and catecholamine levels as an alternative to melanin synthesis. PLoS ONE *8*, e80823.

25. Stahl, B.A., and Gross, J.B. (2017). A Comparative Transcriptomic Analysis of Development in Two Astyanax Cavefish Populations. J. Exp. Zoolog. B Mol. Dev. Evol. *328*, 515–532.

26. Kern, A.D., and Schrider, D.R. (2018). diploS/HIC: An Updated Approach to Classifying Selective Sweeps. G3 (Bethesda) *8*, 1959–1970.

27. Yamamoto, Y., Byerly, M.S., Jackman, W.R., and Jeffery, W.R. (2009). Pleiotropic functions of embryonic sonic hedgehog expression link jaw and taste bud amplification with eye loss during cavefish evolution. Dev. Biol. *330*, 200–211.

28. O'Quin, K., and McGaugh, S.E. (2016). Mapping the Genetic Basis of Troglomorphy in Astyanax: How Far We Have Come and Where Do We Go from Here?. Biology and Evolution of the Mexican Cavefish (Elsevier), pp. 111–135.

29. Yoshizawa, M., Yamamoto, Y., O'Quin, K.E., and Jeffery, W.R. (2012). Evolution of an adaptive behavior and its sensory receptors promotes eye regression in blind cavefish. BMC Biol. *10*, 108.

30. Kowalko, J.E., Rohner, N., Rompani, S.B., Peterson, B.K., Linden, T.A., Yoshizawa, M., Kay, E.H., Weber, J., Hoekstra, H.E., Jeffery, W.R., et al. (2013). Loss of schooling behavior in cavefish through sight-dependent and sight-independent mechanisms. Curr. Biol. *23*, 1874–1883.

31. Creel, D.J. (2015). Visual and Auditory Anomalies Associated with Albinism. In Webvision: The Organization of the Retina and Visual System [Internet], H. Kolb, E. Fernandez, and R. Nelson, eds. (University of Utah Health Sciences).

32. Stahl, B.A., Jaggard, J.B., Chin, J.S.R., Kowalko, J.E., Keene, A.C., and Duboué, E.R. (2019). Manipulation of Gene Function in Mexican Cavefish. J. Vis. Exp. (146)

33. Elipot, Y., Legendre, L., Père, S., Sohm, F., and Rétaux, S. (2014). Astyanax transgenesis and husbandry: how cavefish enters the laboratory. Zebrafish *11*, 291–299.

34. Stahl, B.A., Peuß, R., McDole, B., Kenzior, A., Jaggard, J.B., Gaudenz, K., Krishnan, J., McGaugh, S.E., Duboué, E.R., Keene, A.C., and Rohner, N. (2019). Stable transgenesis in Astyanax mexicanus using the Tol2 transposase system. Dev. Dyn. *248*, 679–687.

35. Ma, L., Jeffery, W.R., Essner, J.J., and Kowalko, J.E. (2015). Genome editing using TALENs in blind Mexican Cavefish, Astyanax mexicanus. PLoS ONE *10*, e0119370.

36. Elipot, Y., Hinaux, H., Callebert, J., Launay, J.M., Blin, M., and Rétaux, S. (2014). A mutation in the enzyme monoamine oxidase explains part of the Astyanax cavefish behavioural syndrome. Nat. Commun. *5*, 3647.

37. Bilandžija, H., Abraham, L., Ma, L., Renner, K.J., and Jeffery, W.R. (2018). Behavioural changes controlled by catecholaminergic systems explain recurrent loss of pigmentation in cavefish. Proc. Biol. Sci. *285*, 20180243.

38. Baik, J.H. (2013). Dopamine signaling in reward-related behaviors. Front. Neural Circuits *7*, 152.

39. Scerbina, T., Chatterjee, D., and Gerlai, R. (2012). Dopamine receptor antagonism disrupts social preference in zebrafish: a strain comparison study. Amino Acids *43*, 2059–2072.

40. Singh, C., Oikonomou, G., and Prober, D.A. (2015). Norepinephrine is required to promote wakefulness and for hypocretin-induced arousal in zebrafish. eLife *4*, e07000.

41. Yoshizawa, M., Robinson, B.G., Duboué, E.R., Masek, P., Jaggard, J.B., O'Quin, K.E., Borowsky, R.L., Jeffery, W.R., and Keene, A.C. (2015). Distinct genetic architecture underlies the emergence of sleep loss and prey-seeking behavior in the Mexican cavefish. BMC Biol. *13*, 15.

42. Duboué, E.R., Borowsky, R.L., and Keene, A.C. (2012). β-adrenergic signaling regulates evolutionarily derived sleep loss in the Mexican cavefish. Brain Behav Evol *80*, 233–243.

43. Jeffery, W.R., Ma, L., Parkhurst, A., and Bilandžija, H. (2016). Pigment Regression and Albinism in Astyanax Cavefish. In Biology and Evolution of the Mexican Cavefish, A.C. Keene, M. Yoshizawa, and S.E. McGaugh, eds. (Elsevier), pp. 155–174.

44. Bradic, M., Teotónio, H., and Borowsky, R.L. (2013). The population genomics of repeated evolution in the blind cavefish Astyanax mexicanus. Mol. Biol. Evol. *30*, 2383–2400.

45. Gross, J.B., and Wilkens, H. (2013). Albinism in phylogenetically and geographically distinct populations of Astyanax cavefish arises through the same loss-of-function Oca2 allele. Heredity *111*, 122–130.

46. Romero, A., and Green, S.M. (2005). The end of regressive evolution: examining and interpreting the evidence from cave fishes. J. Fish Biol. *67*, 3–32.

47. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

48. Fariello, M.I., Boitard, S., Naya, H., SanCristobal, M., and Servin, B. (2013). Detecting signatures of selection through haplotype differentiation among hierarchically structured populations. Genetics *193*, 929–941.

49. Herman, A., Brandvain, Y., Weagley, J., Jeffery, W.R., Keene, A.C., Kono, T.J.Y., Bilandžija, H., Borowsky, R., Espinasa, L., O'Quin, K., et al. (2018). The role of gene flow in rapid and repeated evolution of cave-related traits in Mexican tetra, Astyanax mexicanus. Mol. Ecol. *27*, 4397–4416.

50. Borowsky, R. (2008). Breeding Astyanax mexicanus through natural spawning. CSH Protoc *2008*, t5091.

51. Kowalko, J.E., Ma, L., and Jeffery, W.R. (2016). Genome Editing in Astyanax mexicanus Using Transcription Activator-like Effector Nucleases (TALENs). J. Vis. Exp. *20*.

52. Jaggard, J.B., Lloyd, E., Lopatto, A., Duboue, E.R., and Keene, A.C. (2019). Automated Measurements of Sleep and Locomotor Activity in Mexican Cavefish. J. Vis. Exp. (145)

53. Campbell, S.S., and Tobler, I. (1984). Animal sleep: a review of sleep duration across phylogeny. Neurosci. Biobehav. Rev. *8*, 269–300.

54. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. *3*, H0034.

55. Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

56. Choi, H.M.T., Schwarzkopf, M., Fornace, M.E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., and Pierce, N.A. (2018). Third-generation *in situ* hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. Development *145*, dev165753.

57. Kern, A.D., and Schrider, D.R. (2016). Discoal: flexible coalescent simulations with selection. Bioinformatics *32*, 3839–3841.

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

CellPress

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Nile red	Sigma Aldrich	Sigma Aldrich 19123
Experimental Models: Organisms/Strains		
Astyanax mexicanus: Rio Choy surface fish	This paper	N/A
Astyanax mexicanus: cave fish, Pachon	This paper	N/A
Astyanax mexicanus: F2 hybrids, Rio Choy surface fish X Pachon cave fish	This paper	N/A
Astyanax mexicanus: Surface fish containing engineered *oca2* mutation (*oca2*^{42bp})	This paper	N/A
Oligonucleotides		
Primer: *oca2* WT forward 5¢-CTGGTCAT GTGGGTCTCAGC- 3¢	Klaassen et al.[8]	N/A
Primer: *oca2* 2bp del forward 5¢-TCTGGTCA TGTGGGTCTCATT-3¢	Klaassen et al.[8]	N/A
Primer: *oca2* WT and 2 bp del reverse 5¢- TTTC CAAAGATCACATATCTTGAC−3¢	Klaassen et al.[8]	N/A
Software and Algorithms		
VirtualDub (Version 1.10.4)	N/A	virtualdub.org
Ethovision XT 13.0	Noldus, IT	https://www.noldus.com/ethovision-xt/
Fiji	Schindelin et al.[47]	https://imagej.nih.gov/ij/
Graphpad Prism 8.4.3	N/A	www.graphpad.com
hapFLK (v1.4)	Fariello et al.[48]	https://forge-dga.jouy.inra.fr/projects/hapflk/documents
Other		
Whole genome resequencing data	Herman et al.[49]	N/A

Lead contact

Further information, questions about materials and methods, and requests for resources can be directed to and will be fulfilled by the lead contact, Johanna Kowalko (jok421@lehigh.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The source data for the behavioral analysis in the current study is published with the study in the supplemental material files (Data S1). Original data underlying the images in Figure S2 of this manuscript can be accessed from the Stowers Original Data Repository at http://www.stowers.org/research/publications/libpb-1622.
- The Perl script for sleep analysis is available online: https://doi.org/10.17632/rg2ykygyp4.2.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Husbandry

Animal husbandry and breeding were carried out as described previously.[50] All procedures were done in accordance with the IACUC committee at Florida Atlantic University and the Stowers Institute. Adult fish were housed in a fish facility with an Aquaneering flow-through system maintained at 23°C with a 14:10 h light: dark cycle. Fish were bred by feeding frozen blood worms 2-3 times for the

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

duration of breeding, and heating the water to 26- 28°C to induce spawning. Larvae were raised in groups of 50 in stand-alone tanks, and fed brine shrimp once daily until 20 days post fertilization (dpf) for all assays described here. Sex cannot be determined by this age in *Astyanax mexicanus,* and was therefore not assessed in this study.

oca2 mutant fish

The *oca2*$^{\Delta 2bp}$ allele was previously isolated following CRISPR-Cas9 induced mutagenesis at the *oca2* locus.[8] Founder fish from this cross were from a surface fish population originated in Texas. These fish were outcrossed for 1-2 generations to surface fish from Mexico (Río Choy). For the experiments described here, we crossed heterozygous (*oca2*$^{\Delta 2bp/+}$) F2 or F3 fish to one another, or crossed *oca2*$^{\Delta 2bp/+}$ F2 or F3 fish to cavefish. All crosses were performed using pairs of fish, with each assay being performed on fish from multiple crosses. All experiments were performed using sibling control fish.

Genotyping was performed as described previously.[8,32,51] DNA was isolated from whole larval fish or from adult fin clips by incubating larvae or fin clips in 50 mM NaOH for 30 min at 95°C. Following addition of 1/10th volume 1 M Tris-HCl pH 8.0, PCRs were run using forward primers specific to the alleles: 5'-CTGGTCATGTGGGTCTCAGC-3' was used for the wild type surface *oca2* allele and 5'-TCTGGTCATGTGGGTCTCATT-3' was used for the 2 base pair mutant allele. The same reverse primer, 5'-TTTCCAAAGATCAC ATATCTTGAC-3' was used for both reactions.

Hybrid fish

To obtain F2 hybrid fish, a single surface fish female from a Mexican population was crossed with a single Pachón cavefish male to obtain cave-surface F1 hybrids. A single pair of F1 hybrid fish was crossed to produce all of the F2 hybrids described here. Wild-type surface and cave larvae were produced by group crosses of surface fish and Pachón cavefish. F1 fish for behavioral assays were progeny of a single pairwise cross between a female Río Choy surface fish and a male Pachón cavefish.

METHOD DETAILS

Phenotyping

Sleep experiments were carried out as described previously.[16,52] Briefly, 20 dpf larvae were acclimated in 24-well plates for ~15 h prior to recording. Following feeding with artemia for 10 min, fish were recorded for 24 h (14L:10D) starting at ZT0, lit from the bottom with LED white and IR lights. Videos were recorded at 15 frames per second using the video capturing software, VirtualDub (Version 1.10.4). Videos were then subsequently tracked using Ethovision XT 13.0 (Noldus, IT) software. Sleep behavior parameters were defined from raw data using a custom-written Perl script and MS Excel macros. Sleep is defined by periods of inactivity during which individuals experience an increased arousal threshold.[53] Periods of inactivity that were of 60 s or greater were defined as sleep, as this period of immobility is associated with an increase in arousal threshold in *A. mexicanus*.[16] Total sleep duration per 24 hours, total number of sleep bouts per 24 hours, and average sleep bout length were quantified for each fish.

At 21 dpf, following the 24 h sleep recording, fish were analyzed for feeding behavior. They were transferred to a 24-well plate prefilled with approximately 70 *Artemia* nauplii per well and recorded for two hours. Total feeding was quantified by counting artemia before and after the feeding using Fiji[47] and subtracting to get the total number of artemia consumed.

Following feeding, fish were fasted for 20 h (to limit autofluorescence), and at 22 dpf, fish were stained with Nile red (Sigma Aldrich 19123). No mortality was observed during fasting or staining with Nile red. The stock solution was prepared by dissolving Nile red in acetone at a concentration of 1.25 mg/mL and stored in the dark at −20°C. Prior to staining, the stock solution was diluted with fish water to a final working concentration of 1/1000. Fish were stained in a 24-well plate with 1mL of the working solution in each well, and placed in a 28°C incubator for 30 min, as previously described.[15] Following staining, fish were euthanized in 100 ug/mL MS-222 and imaged on a Nikon SMZ25 stereoscope using an GFP filter. Fish were scored for presence or absence of Nile red staining.

Measurements of standard length and eye diameter were taken from these images, using Fiji.[47] Eye diameter was measured from anterior edge of the eye to the posterior edge. Standard length was measured from snout to caudal peduncle. Eye diameter throughout the paper was corrected for standard length by dividing eye diameter by standard length.

Color images of a subset of representative larval fish were taken at 21 dpf using a Canon Rebel t6i camera on a clear background, illuminated from above and below.

Dark:Dark sleep

To compare sleep under different lighting conditions, fish were reared in identical conditions to what is described above, with all individuals reared under a light:dark cycle, and sleep experiments were then conducted in dark:dark (D:D) as well as in light:dark (L:D), using similar methods to those described above. 20 dpf larvae were acclimated in 24-well plates for ~15 h in dark conditions (D:D) or standard conditions (L:D) prior to recording. Following feeding with artemia for 10 min, fish were recorded for 24 h starting at ZT0, lit from the bottom with IR lights. During the time of recording, the only light within the D:D set up was from IR lights. Video capture and analysis were completed as described above.

RNA extraction, reverse transcription and qPCR to quantify *oca2* expression

Fins and brains were dissected from adult surface fish (3 male and 3 female) and immediately stored in Trizol at −80C. Total RNA was extracted the next day using a modified protocol from the RNeasy extraction kit (QIAGEN). Briefly, the tissues were lysed using Trizol

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

(Invitrogen), centrifuged and the supernatant transferred to a Maxi-extract Phaselock (QIAGEN) to reduce carryover of organic solvents. Nucleic acids were then extracted with chloroform and precipitated with isopropanol. Genomic DNA was digested using the RNase free-DNase (QIAGEN) and the RNA was finally purified using the RNeasy kit (QIAGEN) according to the manufacturer's instructions. cDNA was reverse-transcribed using iScript Reverse Transcription Supermix for RT-qPCR (BioRad) according to the manufacturerś protocol. Primers used to assay *oca2* gene expression were designed from the coding sequence of *Astyanax mexicanus oca2* gene (Ensembl: ENSAMXT00000049368.1; Forward: 5'-CAT GAA TTT GAC AGA CTT CCG TGAT-3'; Reverse: 5'-ACA CGT TTC CTG CGC GAT CC-3') amplifying a fragment of 150 bp. Gene expression was quantified with a CFX96 Touch Deep Well Real-Time PCR Detection System (BioRad) using Sybr Green as an intercalating dye (SsoAdvanced Universal SYBR Green Supermix, BioRad). Individual samples were analyzed in triplicate. *A. mexicanus elf1* was used as an internal control (Forward: 5'-CTG ACT GTG CTG TGC TGA TTG T-3'; Reverse: 5'-CGC TGA CTT CCT TTG TGA TTT CCT-3') in order to normalize raw *oca2* CT data (ΔCT).[54] PCR conditions were as follows: one cycle at 95°C 30 s, 40 cycles at 95°C 15 s, and 60°C 60 s. PCR specificity was confirmed through melting curve analysis. An end point PCR visualized in a 1.5% agarose gel was used to confirm presence of bands. Two samples were removed from the dataset (a female fin tail and a male brain) after running an outlier analysis (ROUT Method, Q = 1%). Fold change in gene expression levels was calculated through the comparative $2^{-\Delta\Delta CT}$ method according to.[55] To look for differences in *oca2* expression levels, ΔΔCT values were converted to the linear form using the $2^{-\Delta\Delta CT}$ equation.[55] Statistical analyses were performed using Graph-Pad Prism (version 8.2.1). Data were first tested for normality (Shapiro–Wilk test) and to identify statistical differences between tissues an unpaired t test was performed ($\alpha<0.05$).

Hybridization chain reaction (HCR) *in situ* hybridization and imaging

Both surface and cavefish embryos (5dpf) were collected and fixed in glyoxal fixative at room temperature for 1 h, followed by 1x PBS rinsing and graded ethanol (30%, 50%, 70%, 100%) dehydration. Embryos were stored in 100% ethanol at −20°C until use. The single molecule HCR3.0 FISH assay was conducted based on previous published protocol by Choi et al. (2018)[56] with modifications. In brief, embryos were rehydrated with graded ethanol and then rinsed with PBS. Embryos were pre-hybridized with HCR probe hybridization buffer (Molecular Instruments Inc) for 30min at 37°C. HCR3.0 probe pairs were designed and purchased from the Molecular Instruments Inc. Hybridization was conducted with 4 pmol per probe mixture in HCR probe hybridization buffer at 37°C overnight. After hybridization, the embryos were washed with HCR wash buffer for 16x15 min at 37°C, rinsed with 5x SSCTw (SSC with 0.1% Tween-20) for 8x15 min at room temperature, and then incubated with HCR amplification buffer for 60min. HCR hairpins were annealed at 95°C for 90 s, then cooled down to room temperature in dark and diluted into HCR amplification buffer with a final concentration of 60nM. For FISH signal detection, we used the prepared HCR hairpin solution to incubate embryos at room temperature overnight in the dark. Embryos were then washed with 5x SSCTw for 4x15 min at room temperature, dehydrated in 30% sucrose solution at 4°C overnight, embedded in OCT, and sectioned with a cryostat at 15μm-thickness. Sections were stained with DAPI before coverslipping with Prolong gold antifade mounting medium.

Three v3HCR probe sets were obtained from Molecular Instruments. https://www.molecularinstruments.com/. Control sets for brain expression in Astyanax mexicanus were HCRTR2 https://www.ncbi.nlm.nih.gov/nuccore/XM_007239619.3, and tyrosine-3-hydrodxylase https://www.ncbi.nlm.nih.gov/nuccore/XM_007228063. A third set was designed for OCA2 https://www.ncbi.nlm.nih.gov/nuccore/NM_001320209.1.

V3HCR images of 5 dpf cavefish and surface fish sections were acquired using a Nikon spinning disc, with a W1 disc, on a Nikon Ti Eclipse base. A CFI Plan Apochromat LWD 40x 1.15 NA water objective was used. Emission was collected onto an ORCA-Flas 4.0 V2 SCMOS camera. With a 405/488/561/640-nm min dichroic, AF647, AF594, AF488, and dapi were excited with 640 nm, 561 nm, 488 nm, and 405 nm laser lines, respectively. Emission filters were 665-735 nm, 579-631 nm, 507-543 nm, and 430-480 nm, for far red, red, green, and blue channels. Z-step spacing was 1 μm. Nikon Elements software controlled all acquisition. Identical camera exposure time and laser power were used across samples.

Image processing was done with an open source version of Fiji.[47] A blur of radius 1 pixel was applied, followed by a rolling ball background subtraction of radius 35 pixels, in all channels with the exception of the DAPI channel. A max projection was applied over all z slices. Channel unmixing was used to remove autofluorescence from the presented images. Briefly, a ratio of intensity of red and green channels was calculated from the autofluorescent cells around the eye, and this ratio was multiplied by the red channel. A subsequent subtraction of channels was done prior to final analysis of the images. Finally, spectral data was acquired on a Zeiss LSM 780, using a 40x 1.2 NA water objective, and linear unmixing was performed using in house, custom built FIJI plugins https://research.stowers.org/imagejplugins/ (not shown). Analysis of linear unmixed data further verified the presence of signal relative to autofluorescence. All v3HCR transcript data was confirmed by comparison to a no-probe control.

Test for selection

To test for signatures of selection on the *oca2* gene we used hapFLK (v1.4)[48] and diploS/HIC[26] on *A. mexicanus* whole genome resequencing data from two surface populations, Rascón (n = 7) and Río Choy (n = 9), and three cave populations, Tinaja (n = 8), Molino (n = 9), and Pachón (n = 10). We also included a single *Astyanax aeneus* surface fish as an outgroup. Details on sequencing and genotyping can be found in Herman et al. (2018).[49] Briefly, samples were sequenced as 100 bp paired end reads on an Illumina HiSeq2000 at The University of Minnesota Genomics Center. Raw sequencing data for these samples was downloaded from NCBI (available under SRA Accession Numbers SRP046999, SRR4044501, and SRR4044502). Three samples (Rascon_6, Tinaja_6, and Tinaja_E) that were included in[49] were excluded from this analysis due to putative recent hybrid ancestry.

Please cite this article in press as: O'Gorman et al., Pleiotropic function of the *oca2* gene underlies the evolution of sleep loss and albinism in cavefish, Current Biology (2021), https://doi.org/10.1016/j.cub.2021.06.077

The haplotype-based hapFLK statistic is an extension of the SNP-based FLK statistic.[48] hapFLK provides a powerful approach to detect regions of the genome under selection by using a model that incorporates linkage disequilibrium to test for differentiation in haplotype clusters among populations. Unlike F_{ST}, the hapFLK statistic accounts for hierarchical population structure and for the effects of recombination.[48] We first ran hapFLK in SNP mode to calculate FLK tests and to generate a kinship matrix based on the entire genome. The whole genome kinship matrix was then used to calculate haplotype-based hapFLK statistics on a 5 Mb region of chromosome 13 (35 Mb - 40 Mb) containing *oca2* (37,904,635-38,048,888 bp). For the haplotype-based tests, we used a K of 10 based on cross validation with fastPHASE (v1.4.8) and ran 20 expectation maximization iterations. hapFLK P values were calculated using a chi-square distribution with the script scaling_chi2_hapflk.py. We used the R package qvalue to set a q-value cutoff of 0.01 and apply for a 1% false discovery rate (FDR) correction based on p values present within the 35 Mb - 40 Mb regions of chromosome 13. To identify population-specific signatures of selection at *oca2*, we built local population trees using Reynolds distances based on haplotype frequencies with the script local_reynolds.py. We visualized changes in haplotype clusters across *oca2* in each population using the script hapflk-clusterplot.R. P values were computed with the script local_trees.R by comparing the Reynolds distances among populations for the local tree compared to those from the whole genome tree. R and python scripts used in this analysis were downloaded from the hapFLK developers' website https://forge-dga.jouy.inra.fr/projects/hapflk/documents.

We observed statistically significant hapFLK values (1% FDR cutoff, p values < 4.11e-05) within the *oca2* region, consistent with positive selection at this locus (Figure S4A, see Materials and Methods). To identify the populations that experienced positive selection, branch lengths were re-estimated by building a local population tree for *oca2* using Reynolds distances based on haplotype frequencies. We observed significant p values in branches corresponding to both surface and all three cave populations, Pachón, Molino, and Tinaja, (Figure S4B, Table S2), indicative of selection.

In both surface populations, *oca2* was classified as putatively under selection by hapFLK but as neutral by diploS/HIC. The diploS/HIC analysis indicating neutrality in the surface populations is likely more robust because it incorporates multiple metrics of selection and provides a model-free estimation of neutrality or selective sweeps through a CNN, whereas hapFLK assumes a hierarchical population structure and tries to fit a model of neutral evolution on that structure. Thus, diploS/HIC offers a more powerful approach to detect selection compared to hapFLK when populations have experienced complex demographic events (e.g., recent admixture or extreme differences in effective population size).

diploS/HIC uses a supervised machine learning approach to identify windows in the genome that have undergone "soft" sweeps (selection on standing genetic variation) or "hard" sweeps (selection on new mutations). We first simulated selective sweeps using discoal and then used the simulated data to train diploS/HIC.[26,57] We generated feature vectors for each population using the default settings of 11 sub-windows across a 1,100,000 Mb region of the genome. diploS/HIC ran predictions using the feature vectors to classify each window as neutral (no evidence of a selection), linkedSoft (loci near a window that has undergone a soft sweep), linkedHard (loci near a window that has undergone a hard sweep), Soft (loci that have undergone a soft sweep), or Hard (loci that have undergone a hard sweep).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis for trait comparisons

All statistical analyses were performed using Graphpad Prism 8.4.3. All data was tested for normality using Shapiro-Wilk tests. Data which did not pass the normality test were analyzed using the nonparametric Kruskal-Wallis test. Where statistical significance was indicated, post hoc comparisons were completed using Dunn's multiple comparisons test. Normally distributed data with multiple groups were analyzed using an ANOVA, with post hoc comparisons completed using a Tukey post hoc test. For continuous traits which did not pass the normality test, relationships between these continuous traits were analyzed using the Spearman's rank correlations. Relationships between binary and continuous traits were analyzed for normality, and then analyzed using a t test if they passed the normality test, or a Mann-Whitney test if they did not pass the normality test. For adipose proportion, data error bars were calculated using z*-value of 1.96 and denote the margin of error of the sample proportion and data was analyzed using Fisher's Exact Test, and a post hoc of pairwise Fisher's Exact Test was performed where significance was indicated. Effect size tests were also performed on F2 and *oca2* comparisons, an odds ratio effect size test was performed for adipose proportion data, all other effect size tests for comparisons were Cohen's D-test. Statistical details can be found in the figure legends.