Genetics of Pigmentation - From Axolotls to Humans

This learning block is designed to build upon knowledge gained in HS-LS1-1 (Genes, Proteins, and Tissues) to accomplish learning objectives under HS-LS3-1 (Chromosomal Inheritance), HS-LS3-2 (Inheritable Genetic Variation), and HS-LS3-3 (Variation and Distribution of Traits). The block has three lessons, teach at your own pace. Using axolotls is a great way to bring life to biology terms and concepts learned in class.

Lesson 1: Introduction to axolotl skin pigmentation

Materials Needed: Axolotls (*wildtype, white, melanoid, white/melanoid, albino, copper*) and bowls with water for housing. If you do not have axolotls, you can use pictures/images of the different axolotls.

This lesson introduces students to axolotl salamanders and the biology of skin pigmentation. Axolotls present a variety of different pigment patterns, each of which is determined by a single gene. Students will answer questions that are designed to make them think about the genetics of pigmentation, including chromosomal basis and inheritance of traits. Additionally, students will develop a model to make connections between genes, proteins, and pigmentation phenotypes.

1) Conceptual review. Review central dogma, emphasizing connections between DNA, genes, proteins, and phenotype.

2) Small group activity. Students will break up into groups of 2. Without any introduction to axolotl pigment phenotypes, each group will make observations of 6 different axolotl pigment types (wildtype, white, melanoid, white melanoid, albino, and copper) and answer questions about pigment variation they observe among the different axolotls.

1. Write a short description of each axolotl's pigmentation.

2. Why do you think there are different axolotl pigment phenotypes? Are there advantages and disadvantages associated with having a specific pigment phenotype?

3. If you had to predict, what do you think the parents of each axolotl phenotype looked like? Is it possible that one or both parents had a different pigment phenotype? Explain.

4. Is it possible that axolotls with different color phenotypes are siblings and share the same parents?

5. Is it possible for an axolotl to have two different pigment phenotypes?

6. Do you think skin pigmentation is determined by different pigment cell types or by enzymes that synthesize different pigments? Explain.

7. Develop a model for how axolotl skin pigmentation is determined. Your model should include DNA, proteins, genes, and cells. It should be able to explain the different axolotl pigment phenotypes.

3) Class discussion. Each group shares their answers with the class, as well as their models. This discussion time can be used to educate about axolotls, genetics, and pigment biology.

Answer Key and Teaching Points:

Question 1. The following is a brief review of the 5 different axolotl pigment types:

A) **Wildtype**: The wildtype axolotl is a greenish color. It is the typical pigment pattern seen among the few remaining axolotls in the natural Mexican population. Wildtype axolotls are green because of the intermingling of three different pigment cell types in the skin: blackish melanophores, yellowish xanthophores, and rare, sparsely distributed iridescent iridophores. When black and yellow colored pigment cells intermingle, the resulting color is green. Wildtype is a dominant pigment pattern.

B) **White**: The white axolotl is characterized by few to no pigment cells, with iridescent iridophores present only in the iris of the eye. Some white axolotls develop patches of dark pigmentation after sexual maturity. The white phenotype is seen in axolotls that inherit two recessive (non-functiona) alleles for *Endothelin 3* (*Edn3*). It is a recessive pigment pattern.

C) **Melanoid**: The melanoid axolotl has a dark black pigment pattern that is characterized by increased numbers of melanophores, fewer xanthophores, and an absence of iridophores. During larval development, melanophore numbers greatly increase in *melanoid* embryos and larvae as they age and become distributed throughout the skin to yield a uniform dark coloration that largely masks the yellow pigment of xanthophores. The melanoid phenotype is seen in axolotls that inherit two recessive (non-functional) alleles for *Leukocyte Tyrosine Kinase* (*Ltk*). It is a recessive pigment pattern.

D) **White**-**Melanoid**: Individuals that are both white and melanoid look very similar to white axolotls, except for one distinguishing characteristic - they do not appear to have a circular iris because they lack iridophores in the eye. The white-melanoid phenotype is seen in axolotls that inherit two recessive alleles for both *End3* and *LTK*. It is a double recessive pigment pattern.

E) **Copper**: The copper axolotl originated in the pet trade. Copper axolotls having copper-colored bodies with all three pigment cell types, however the melanophores are brownish in color, not the typical black. The copper phenotype is seen in axolotls that inherit two recessive (non-functional) alleles for *Tyrosinase Related Protein 1* (*Tyrp1*). It is a recessive pigment pattern.

F) **Albino**: The albino axolotl has a yellowish color because melanophores are not capable of producing a dark melanin. Interestingly, an albino tiger salamander was crossed to an axolotl to

move the albino phenotype into a laboratory axolotl population. The albino phenotype is seen in axolotls that inherit two recessive (non-functional) alleles for *Tyrosinase* (*Tyr*). It is a recessive pigment pattern.

Question 2: *This question is meant to bridge the proximate level, genetic focus of the learning block with ultimate level thinking that guides ecology and evolution.* Some of the axolotl pigment phenotypes may provide camouflage in nature, allowing them to blend-in with their surroundings. Camouflage helps prey elude predators. The melanoid phenotype may be advantageous in dark or murky water and the white phenotype may be advantageous in lightly colored water. In support of this idea, melanoid and white phenotypes were observed in axolotls collected from the natural source population in Mexico and thus may have originated by natural selection. In contrast, *albino* and *copper* axolotls have never been collected from nature. A recessive (non-functional) *Tyr* allele was moved from an albino tiger salamander to an axolotl through hybridization, somatic cell nuclear transfer, and tissue grafting. The *copper* phenotype presumably arose spontaneously in an axolotl hobbyist's aquarium. Thus, *albino* and *copper* were artificially selected by humans.

Question 3. *This question requires students to think about the connection between genes and inheritance.* It is possible that parents of each axolotl pigment type look exactly like their offspring. It is also possible that parents of each of the axolotl pigment types do not look like their offspring. This is because axolotl pigment phenotypes are determined by recessive alleles. An individual must inherit two recessive alleles, one from each parent, to express a non-wildtype pigment phenotype. If you cross two wildtype parents that are heterozygous for a pigment gene (they have one recessive allele and one dominant allele), approximately 25% of their offspring will inherit two recessive alleles and express a non-wildtype pigment phenotype. *This can be shown by diagraming the cross and using a Punnett square to show how haploid alleles from each parent combine to form genotypes.* If an individual inherits one or no recessive alleles, and thus one or two dominant alleles, it will express wildtype coloration.

Question 4. *This question introduces the chromosomal basis of inheritance.* Axolotl pigment genes are located on different chromosomes. If individuals inherit two recessive alleles for two different pigment genes, they will express both pigment phenotypes. *This can be shown by diagraming the cross and using a Punnett square to show how haploid alleles at 2 loci combine to form genotypes.*

Question 5. *This question connects genes to proteins and raises an interesting question - how are cells programmed to transcribe specific genes? The answer lies in a related field of inquiry called epigenetics.* Axolotl pigmentation is determined by the distribution of three pigment cell types in the skin and iris of the eye. Melanophores synthesize a dark pigment called melanin, Xanthophores synthesize yellow pigments called pteridines, and Iridophores synthesize iridescent pigments called purines. Although all three pigment cell types share the same DNA sequence, each pigment cell type only makes proteins necessary for synthesizing pigments that are specific to that cell type.

Question 6. Here is an example model. Each of the different pigment phenotypes is determined by a different gene. Each of the genes express a different pigment molecule. The pigment genes are expressed in different pigment cell types that are distributed throughout the skin.

Lesson 2: Genetics of Pigmentation and Polymerase Chain Reaction (PCR)

Materials Needed: PCR machine, gel electrophoresis rig, 1-10 or 2-20 μl pipettor(s), pipette tips, PCR amplification kit (e.g. Genescript Cat. No. L00342), *Tyrp1* oligonucleotides (Forward Primer axTyrp1_5.1: AAGGTGGTTGAATCTTGTCTCCTT; Reverse Primer axTyrp1_3.1 TTTTAAGACAGGTTACCCCCGAG synthesized from a commercial vendor like IDT), 100 BP ladder (e.g. Stellar Scientific SKU:SMOB-DM2100) DNA from wildtype and *copper* axolotls (obtained from the AGSC), agarose (e.g. Alkali Scientific SKU:A7700) to make a gel, and TAE gel buffer (e.g. Alkali Scientific SKU: PB0288). If you don't have PCR reagents, a PCR machine, agarose, TAE, or a gel electrophoresis rig you can describe PCR and gel electrophoresis processes, and/or watch online videos about these techniques.

This lesson introduces students to PCR, gel electrophoresis, and DNA sequencing, techniques that are widely used in applied science (e.g. forensics) and biological research. PCR is used to amplify or increase the number of DNA molecules so that they can be visualized by gel electrophoresis. Gel electrophoresis an important quality control step prior to performing DNA sequencing. Axolotl pigment phenotypes are determined by DNA sequence variations which alter mRNA sequences and the triplet code for translating proteins. Students will learn about the genes that make proteins for synthesizing melanin, and in particular *tyrosinase-related protein 1* (*Tyrp1*) which is known to cause variation in skin and hair pigment in many different animals, including humans.

1) Introduction to pigmentation biology emphasizing genetics, and PCR and gel electrophoresis. Review genetics of pigment pattern variation focusing on DNA/gene mutation, protein function, and melanin synthesis. Introduce *Tyrp1* and the many pigment phenotypes attributable to *Tyrp1* in the animal kingdom. Pose and briefly discuss the following hypothesis: The c*opper* axolotl phenotype is caused by a mutation in the *Tyrp1* gene. Then ask the class how they might test this hypothesis: If *Tyrp1* causes the *copper* pigment type, then *copper* axolotls must have a *Tyrp1* DNA sequence that is different from the the *Tyrp1* sequence carried by wildtype axolotls. Introduce PCR and explain how it has revolutionized approaches in forensic, environmental, and health sciences. Explain how PCR and DNA sequencing use enzymes called polymerases that can read a single stranded DNA template and make an exact copy in the presence of nucleotides and a primer sequence. Explain how PCR reactions can be setup in the lab using pipettors to dispense small volumes of fluid into tubes. Finally, explain how a PCR machine works to amplify the DNA and how gel electrophoresis works to visualize DNA.

3) Laboratory activity. Working in groups of two, students will set-up their own PCR reactions. After explaining how to use a pipette, students will follow instructions in the PCR amplification kit to set up reactions. After setting up reactions, students will amplify their samples in the PCR

machine using the following protocol: 1 cycle at 94 C for 3 m; 34 cycles of 94 C for 45 s, 60 C for 45 s, and 72 C for 30 s; and 1 cycle at 72 C for 7 min.

4) Gel electrophoresis. After performing PCR, it is important to determine if the method worked as expected. Gel electrophoresis is a technique that allows a scientist to visualize DNA. Students or the instructor can make a 1% agarose gel following instructions provided with the gel electrophoresis rig or by downloading an online protocol. Students will pipette ~10 ul of their PCR samples into wells of the agarose gel and then perform gel electrophoresis. A sample of the 100 bp DNA ladder should be included in one of the lanes of the gel to serve as a reference for evaluating the sizes of PCR products. While the gel is running, the instructor will explain the principle of gel electrophoresis and how the method allows visualization of DNA fragments.

Lesson 3: DNA Sequence Analysis of a Pigmentation Mutation

Materials needed: Computer(s) with access to the internet.

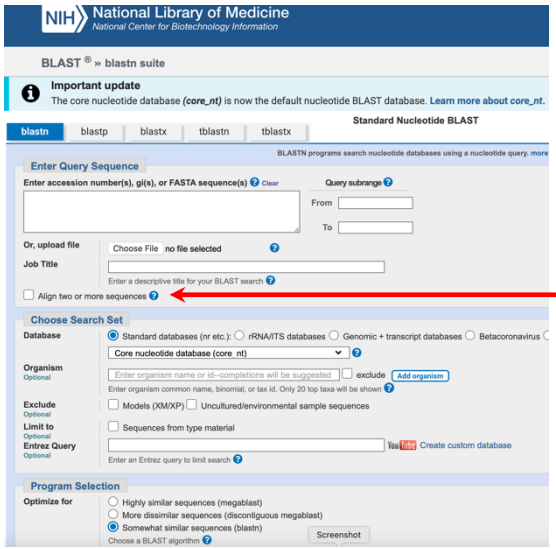
This lesson introduces students to DNA sequence analysis. In Lesson 2, students performed PCR to amplify a DNA fragment of the *Tyrp1* gene from wildtype and copper axolotls. You can submit the PCR samples to a commercial vendor for sequencing (e.g. Eurofins) or move forward under the assumption that the PCR samples were sequenced. In this lesson, students will compare the nucleotide sequences obtained after DNA sequencing commercial vendor to identify any differences. Then students will translate the nucleotide sequences to determine if there is a change in the expected protein coding sequences. Students will use computers to access online DNA sequence analysis programs.

1) Introduction to DNA sequencing and DNA sequence analysis. Review PCR amplification and then introduce DNA sequencing without going into too much technical depth. The end-product of a DNA sequencing run is a file called an electropherogram. Show students pictures of DNA electropherograms to show good and bad DNA sequencing outcomes, and how an electropherogram provides a colorful visualization of DNA sequence quality and the linear DNA sequence.

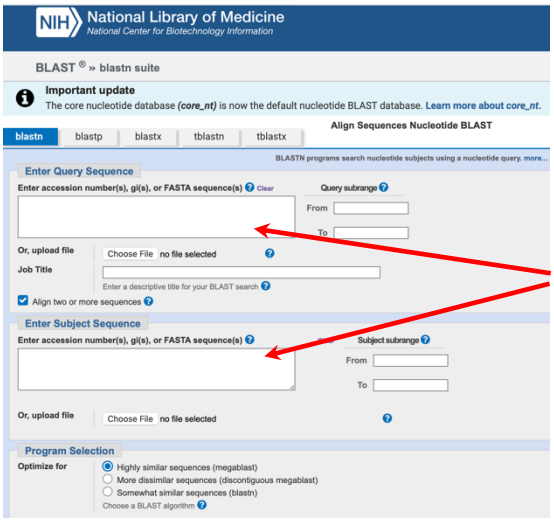
2) Small group activity. The instructor will give students a text file that contains DNA sequences obtained from the wildtype and *copper* axolotl PCR/sequencing samples. Students are handed an activity sheet that provides instructions on how to analyze the sequences using online tools. The activity sheet also has questions for students to answer.

Activity Sheet

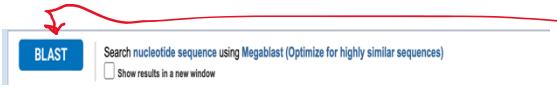
1. One of the first steps in the analysis of two or more DNA sequences to compare them for similarities and differences. Go to the following website: https://blast.ncbi.nlm.nih.gov/Blast.cgi and click on the Nucleotide Blast icon to access the Blast server. You should see the following:



Click the box for aligning two or more sequences.



This will open two boxes, one for entering a Query Sequence (*copper* sequence) and one for entering a Subject Sequence (wildtype sequence).
Use cut and paste to move the sequences from the text file to these boxes and then select the BLAST icon at the bottom. This will initiate alignment of the two sequences.



The text file will have two sequences for the PCR products that were amplified, see below. The primer sequences are underlined to show their locations in the amplified pieces of DNA. The PCR products include both intronic (non-*Tyrp1* protein coding) and exonic (*Tyrp1* protein coding sequence). The protein coding sequence is highlighted. Cut and paste only the exonic sequence into the NCBI Blast windows.

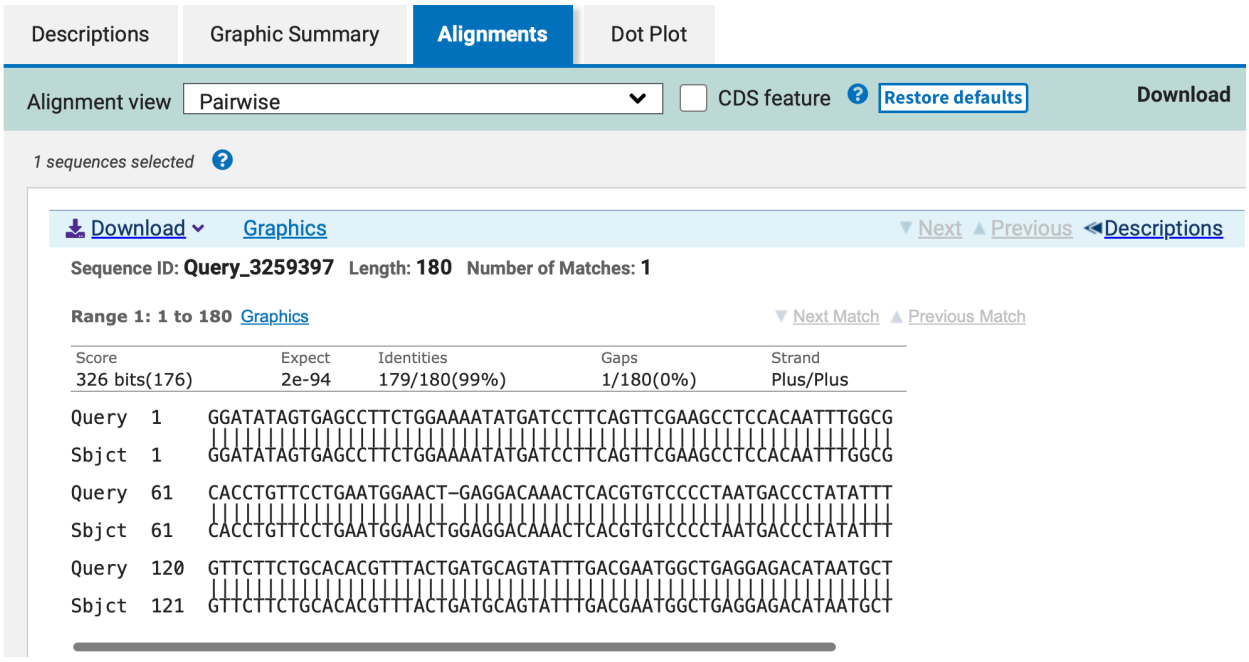
copper Tyrp1

AAGGTGGTTGAATCTTGTCTCCTTGTATGATTCGTGCGATTGTTAGACTT
GCTCTAAACATCTAAAGCATAGTTTGCGGGCTCTTCACAGAACGTTCTTA
GACTTTGTCAATGAAGACTTGCTGATGTTTGATTTTGAGGGTGTGGGTGG
AATGAAGAAACATAGATCGTACTAATTGCCTCACGTTCGACTTGCTTCAG
GATATAGTGAGCCTTCTGGAAAATATGATCCTTCAGTTCGAAGCCTCCAC
AATTTGGCGCACCTGTTCCTGAATGGAACTGAGGACAAACTCACGTGTC
CCCTAATGACCCTATATTTGTTCTTCTGCACACGTTTACTGATGCAGTAT
TTGACGAATGGCTGAGGAGACATAATGCTGGTAAGTTGGCATGCCTTTGT
TCTACTGTCTTTTTCTAGCGACGGTGCAGGCCGGCTCGGGGGTAACCTGT
CTTAAAA

Wildtype *Tyrp1*

AAGGTGGTTGAATCTTGTCTCCTTGTATGATTCGTGCGATTGTTAGACTT
GCTCTAAACATCTAAAGCATAGTTTGCGGGCTCTTCACAGAACGTTCTTA
GACTTTGTCAATGAAGACTTGCTGATGTTTGATTTTGAGGGTGTGGGTGG
AATGAAGAAACATAGATCGTACTAATTGCCTCACGTTCGACTTGCTTCAG
GATATAGTGAGCCTTCTGGAAAATATGATCCTTCAGTTCGAAGCCTCCAC
AATTTGGCGCACCTGTTCCTGAATGGAACTGGAGGACAAACTCACGTGTC
CCCTAATGACCCTATATTTGTTCTTCTGCACACGTTTACTGATGCAGTAT
TTGACGAATGGCTGAGGAGACATAATGCTGGTAAGTTGGCATGCCTTTGT
TCTACTGTCTTTTTCTAGCGACGGTGCAGGCCGGCTCGGGGGTAACCTGT
CTTAAAA

Below is the BLAST result showing a DNA sequence alignment of the two sequences.



There is a single nucleotide deletion at position 82 in the *copper Tyrp1* sequence.

The single base pair deletion disrupts the *Tyrp1* protein-coding sequence. You can show this by translating the *Tyrp1* sequence with an Expasy tool: https://web.expasy.org/translate/ . Simply paste the sequences (one at a time) into the box and click translate.

Below is the wildtype translation of *Tyrp1* coding sequence

5'3' Frame 1

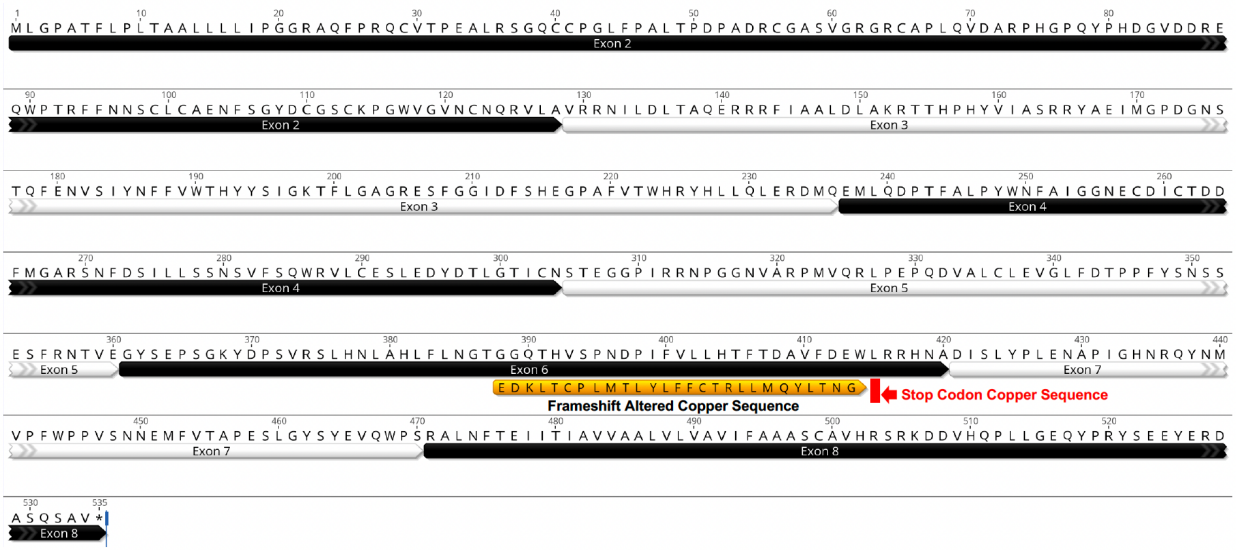
GYSEPSGKYDPSVRSLHNLAHLFLNGTGGQTHVSPNDPIFVLLHTFTDAVFDEWLRRHNA

Below is the *copper* translation of *Tyrp1* coding sequence

5'3' Frame 1

GYSEPSGKYDPSVRSLHNLAHLFLNGTEDKLTCPLMTLYLFFCTRLLMQYLTNG-GDIM

Notice that the amino acid sequence is altered in the *copper* translation and there is also a hyphen in one position indicating the position of a premature stop codon. When we look below at the PCR amplified sequence relative to complete *Tyrp1* protein coding sequence we can see that the nucleotide deletion and altered protein coding sequence occurs in Exon 6.



Questions

1. What can we conclude from our analyses of the wildtype and *copper Tyrp1* nucleotide and protein coding sequences?

2. How might the differences we observe between the sequences explain the pigment difference between wildtype and *copper* axolotls?

3. Do you think mutations in *Tyrp1* might explain skin color differences in other organisms, including humans?

For further information about axolotl pigment variation

Cecil R, Strohl L 2nd, Thomas ML, Schwartz JL, Timoshevskaya N, Smith JJ, Voss SR. 2024. *Tyrp1* is the mendelian determinant of the axolotl copper mutant. *Sci Rep* 14:22399.

Kabangu M, Cecil R, Strohl L 2nd, Timoshevskaya N, Smith JJ, Voss SR. 2023. *Leukocyte tyrosine tinase* (*Ltk*) is the mendelian determinant of the axolotl melanoid color variant. *Genes* (Basel) 14:904.

Woodcock MR, Vaughn-Wolf J, Elias A, Kump DK, Kendall KD, Timoshevskaya N, Timoshevskiy V, Perry DW, Smith JJ, Spiewak JE, Parichy DM, Voss SR. 2017. Identification of mutant genes and introgressed tiger salamander DNA in the laboratory axolotl, *Ambystoma mexicanum. Scientific Reports,* 7:6.

For further information about *Tyrp1* mutations in other organisms, including humans.

Boissy, R. E. et al. Mutation in and lack of expression of tyrosinase-related protein-1 (TRP-1) in melanocytes from an individual with brown oculocutaneous albinism: a new subtype of albinism classified as OCA3. Am. J. Hum. Genet.58, 1145–1156 (1996).

J Jackson, I. A cDNA encoding tyrosinase-related protein maps to the brown locus in mouse. Proc. Nat. Acad. Sci. U S A. 85, 4392–4396 (1988).

Peterson, S. M. et al. Genetic variants in melanogenesis proteins TYRP1 and TYR are associated with the golden rhesus macaque phenotype. G3 13, jkad168 (2023).

Schmidt-Küntzel, A., Eizirik, E., O'Brien, S. J. & Menotti-Raymond, M. Tyrosinase and tyrosinase related protein 1 alleles specify domestic cat coat color phenotypes of the albino and brown loci. J. Hered. 96, 289–301 (2005).