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Tyrp1 is the mendelian determinant of the Axolotl (*Ambystoma mexicanum*) copper mutant

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Several dozen Mendelian mutants have been discovered in axolotl (*Ambystoma mexicanum*) populations, including several that affect pigmentation. Four recessive mutants have been described in the scientific literature and genes for three of these have been identified. Here we describe and genetically dissect *copper*, a mutant with an albino-like phenotype known only from the pet trade. We performed a cross segregating *copper* and wildtype color phenotypes and used bulked segregant RNA-Seq to identify a region on chromosome 6 that was enriched for single-nucleotide polymorphisms (SNPs) between the color phenotypes. This region included *Tyrosinase-like Protein 1* (*Tyrp1*), a melanin synthesis protein that when mutated, is associated with lighter than black melanin coloration in animal models and oculocutaneous albinism in humans. Inspection of RNA-Seq reads identified a single nucleotide deletion that is predicted to change the coding frame, introduce a premature stop codon in exon 6 and yield a truncated *Tyrp1* protein in *copper* individuals. Using CRISPR-Cas9 editing, we show that wildtype *Tyrp1* crispants exhibit *copper* pigmentation, thus confirming *Tyrp1* as the *copper* locus. Our results suggest that commercial and hobbyist axolotl populations may harbor useful mutants for biological research.

The axolotl (*Ambystoma mexicanum*) has a long and storied history in biological research. Axolotls provide models for studies of embryonic and post-embryonic development, including most famously the study of whole organ regeneration[1]. The primary stock center for axolotl research is a captive bred population that has been maintained for almost 100 years[2,3]. Many mutants have been maintained in this population over time, including four different color mutants that are determined by single genes: *white*, *melanoid*, *albino*, and *axanthic*. Each of these mutants has an interesting history. The *white* mutant was presumably collected from Mexico in 1862 along with 33 wildtype individuals[4]. These axolotls were transported to Paris to establish the first laboratory axolotl population. The *white* mutant is caused by a *Edn3* splicing defect[3]. The *melanoid* mutant was originally identified from laboratory crosses made using wild-caught axolotls from Mexico, and like *white*, *melanoid* is presumably also a natural color variant[5]. Subsequent analyses revealed that *melanoid* is genetically associated with *Ltk*[6]. Similarly, *albino* was originally identified in a wild captured tiger salamander and crossed into axolotl stocks[7]. In contrast, axanthic appears to have arisen spontaneously within a laboratory axolotl strain[8]. Although the gene for *axanthic* has not been identified, Woodcock et al.[3] showed that *albino* is caused by a deletion in the *Tyr* coding sequence.

For as long as axolotls have been studied in research, they have also been highly prized as aquarium pets. All axolotl color mutants known to biological research, and even transgenic axolotls, are available in the pet trade. Additionally, pet breeders have identified color variants that have not received biological study, including a recessive Mendelian mutant called *copper*. Pet breeders describe *copper* axolotls as having copper-colored bodies with yellow xanthophores, brownish melanophores, and iridescent iridophores. The brownish and not black color of melanophores suggests *copper* is a form of albinism, perhaps involving mutation of a melanin synthesis protein. Given the importance of animal models in the study of albinism diseases, we used bulked segregant RNA-Seq (BSR-Seq)[9] and CRISPR-Cas9[10] to identify *Tyrp1* as the *copper* locus. Our results suggest that commercial and private axolotl collections may harbor genetic variants that may prove useful as animal models in biological research.

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Results

BSR-Seq[9] was used to identify SNPs linked to the *copper* locus. Embryos from a cross that segregated *copper* and wildtype individuals were used to create two *copper* and two wildtype RNA pools ($N = 36$ and 27 for *copper* and *wildtype* respectively, per pool) that were each subjected to Illumina short-read RNA sequencing to identify single nucleotide polymorphisms (SNPs). Analysis of SNPs across the axolotl genome revealed a region on chromosome 6p with dissimilar frequencies between the pools (Fisher's exact test F = 686, $p = 2.93E^{-123}$ at peak association) (Fig. 1a; Supplementary File 1). Examination of genes within this region identified *Tyrosinase-like protein 1* (*Tyrp1*) as a candidate gene. *Tyrp1* encodes an enzyme that functions in melanin synthesis. *Tyrp1* mutations generate lighter than black melanin shades of color in animal models[11–13] and Oculocutaneous Albinism 3 (OCA3) in humans[14]. Additionally, significantly more *Tyrp1* transcripts were identified from the wild-type vs. *copper* RNA-Seq pools (Dseq2 Wald Test = 20.03, adjusted P-value = 1.06E-84) (Fig. 1b). The axolotl *Tyrp1* locus (AMEX60DD301043361.1) is distributed across 8 exons and the 1663 bp coding sequence encodes a 524 amino acid protein. A single nucleotide deletion (chr6p:1,225,541,490) was identified in RNA-Seq reads generated for the *copper* RNA pool (Fig. 1c; Supplementary File 2). This deletion, in exon 6, was confirmed by sequencing genomic DNA isolated from two *copper* and two wildtype individuals that were used to construct the RNA BSR-Seq pools (Fig. 1c). The deletion in the *copper Tyrp1* sequence is predicted to change the reading frame and introduce a premature stop codon at amino acid position 416 (Supplementary File 3).

CRISPR-CAS9[10] was performed to determine if disruption of the *Tyrp1* coding sequence would yield individuals that presented *copper* pigmentation. Two guide RNAs targeting exons 2 and 6 of the *Tyrp1* coding sequence were injected into 100, one-cell stage wildtype embryos and visually assessed for color at developmental stage 42[15]. Crispant individuals were characterized by having fewer melanophores than non-injected controls and presented a yellowish overall color, as is typical of *copper* larvae in the pet trade. PCR and DNA sequencing of *Tyrp1* regions targeted by CRISPR-Cas9 confirmed that crispant embryos ($N = 12$) were edited at both gRNA-target sites (Fig. 2). Five *Tyrp1* crispants (that were edited at both gRNA1 and gRNA2) were reared to approximately 17 cm total body length (1.2 years old) and photographed to document pigmentation. Relative to the yellowish color observed during the larval stage, all five *Tyrp1* crispants presented a darker copper skin color that was very similar to the color of adult *copper* axolotls (Fig. 3). The pigmentation phenotypes resulting from CRISPR-Cas9 genome editing strongly implicate *Tyrp1* as the gene for *copper*.

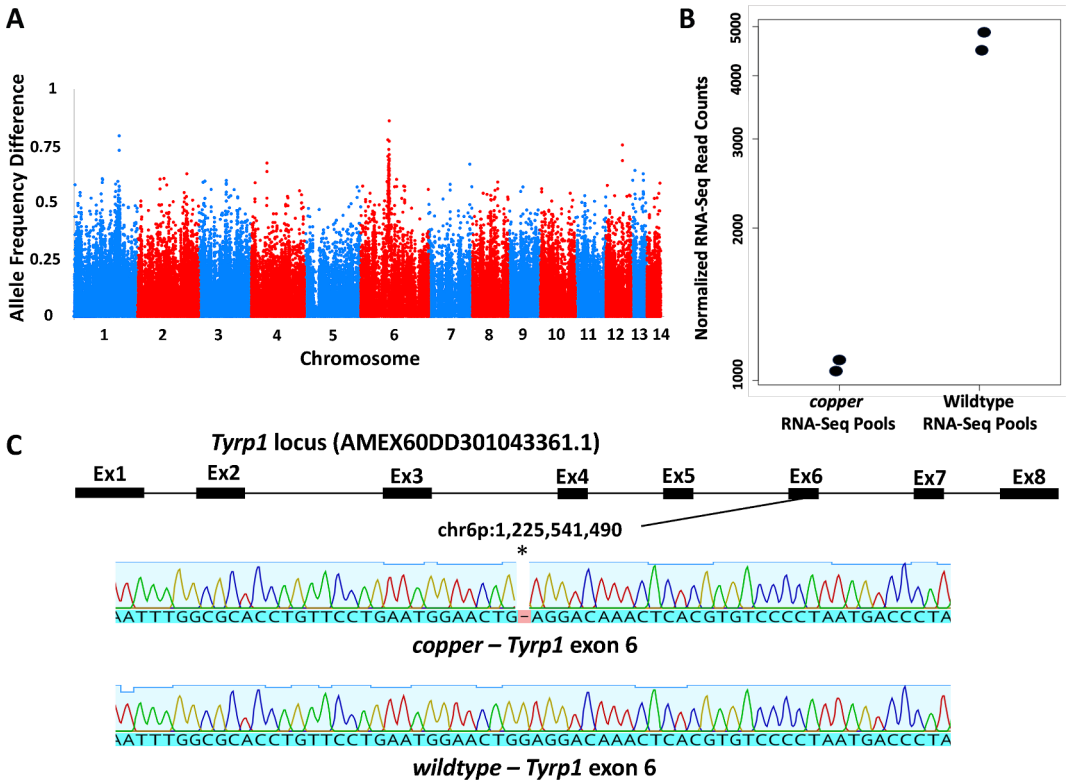

Fig. 1. Identification of *Tyrp1* as a candidate gene for *copper*. (**A**) Plot showing genome wide SNP (allele) frequency differences between *copper* and wildtype BSA-RNA-Seq pools. (**B**) Transcript counts identified for *Tyrp1* between *copper* and wildtype BSA-RNA-Seq pools. The difference between pools is statistically significant (W = 20.03, adjusted P-value = 1.06E-84). (**C**) Genomic map of the axolotl *Tyrp1* locus and deletion detected in exon 6 between *copper* and wildtype alleles.

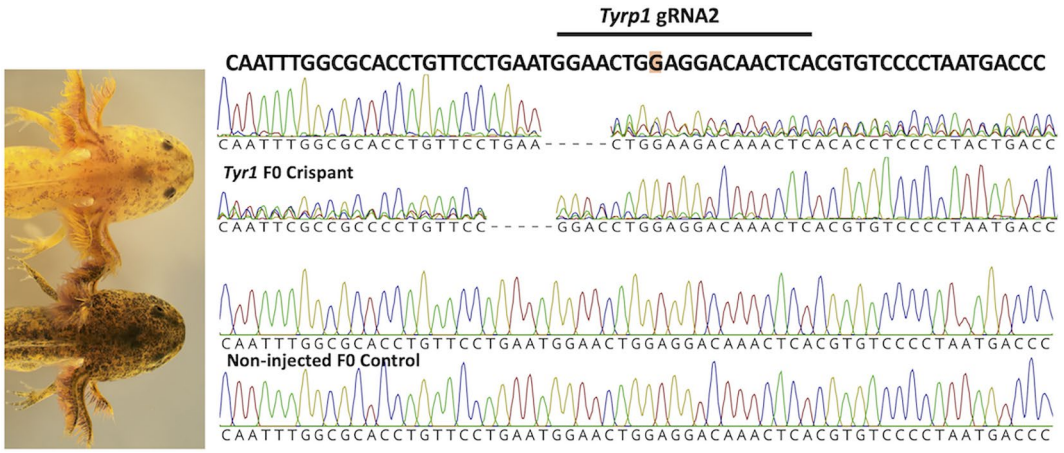

Fig. 2. CRISPR-CAS9 knockdown of *Tyrp1*. Melanin pigmentation was greatly reduced in F0 *Tyrp1* crispant larvae relative to F0 non-injected control siblings. F0 *Tyrp1* crispant electropherograms showed overlapping sequence traces at the gRNA target site for forward and reverse DNA sequencing reactions, consistent with genome editing. F0 non-injected control DNA sequence does not show evidence of genome editing. The gRNA target sequence (underlined) overlaps the deleted nucleotide in the *copper Tyrp1* allele.

Discussion

Several different axolotl pigmentation variants are present in laboratories and households around the world. Several of these variants, including *white*, *albino* and *melanoid*, have received considerable attention in biological research. Previously, we identified genes for these variants to increase their value as research models[3,5]. In this study we identified a new gene in an axolotl pigmentation variant known only from the pet trade. Specifically, we show that *copper*, an axolotl mutant with copper coloration, is likely determined by a single nucleotide deletion in the *Tyrp1* coding sequence. *Tyrp1* encodes an enzyme that functions in melanophores to produce a black pigment called melanin. Mutations in *Tyrp1* are associated with decreased production of melanin and structural alterations that result in lighter than expected coloration, for example brown coat color in mice[11] and blonde hair in Melanesian humans[16]. *copper* axolotls in the larval stage have fewer dark pigmented melanophores than those observed in wildtype animals, and in absence of melanophores pigmentation is primarily determined by yellow xanthophores. As *copper* axolotls age, a brownish pigment emerges and individuals developed a uniform copper skin color, as is typical of animal models with *Tyrp1* mutations. To functionally validate *Tyrp1* for *copper*, we generated *copper*-like pigmentation in wildtype individuals using CRISPR-Cas9 genome editing of *Tyrp1*. The combination of SNP genome association data with CRISPR-Cas9 functional genomics results strongly implicates *Tyrp1* as the *copper* locus.

The *copper* deletion is predicted to alter the *Tyrp1* coding sequence and introduce a premature stop codon in exon 6, yielding a truncated protein with an altered function. We note that the exon 6 deletion in *copper Tyrp1* parallels an exon 6 deletion identified in the first human case report of OCA3[14], with both deletions leading to a premature stop codon. No *Tyrp1* mRNA or protein was detected in the human case while *Tyrp1* transcripts were recovered by BSR-Seq in *copper* axolotls, albeit at lower transcript abundances. We speculate that *Tyrp1* transcript abundances are lower in *copper* axolotls because the premature stop codon causes nonsense-mediated mRNA decay. Although it remains to be determined if a *copper Tyrp1* protein is generated in axolotl, a nonsense mutation in the catalytic, tyrosinase-like domain of *Tyrp1* is expected to affect protein function and melanogenesis[17].

Domestic plant and animal populations have long served as reservoirs for phenotypes of commercial, biological, and biomedical relevance[18–20]. While the pet trade has gained access to axolotls from research labs, including for example GFP transgenics, axolotl researchers have not assessed commercial and domestic populations for new research models. Now that *Tyrp1* has been identified as the causative gene for *copper*, this new axolotl model can be used to study molecular functions underlying OCA3 phenotypes. As is observed in humans with OCA3, *copper* is characterized by a reduction in melanin. A reduction in melanin could trace to many different cellular mechanisms as *Tyrp1* functions in multiple pathways that directly or indirectly regulate melanin biosynthesis, as well as affecting melanophore cell division and death[21]. In addition to *copper*, other axolotl pigment variants in the pet trade include hypomelanistic, which is characterized by a reduction in melanin and UV-light excitable face/cranium fluorescence, and "starburst" which presents increased numbers of iridophores in *albino* axolotls. These and other phenotypic variants in the axolotl pet trade may provide useful models for biological research.

Materials and methods

Approval for animal experiments

Animal care procedures were approved under University of Kentucky IACUC protocol 2017–2580 and performed in accordance with ARRIVE[22] and AAALAC[23] guidelines and standards.

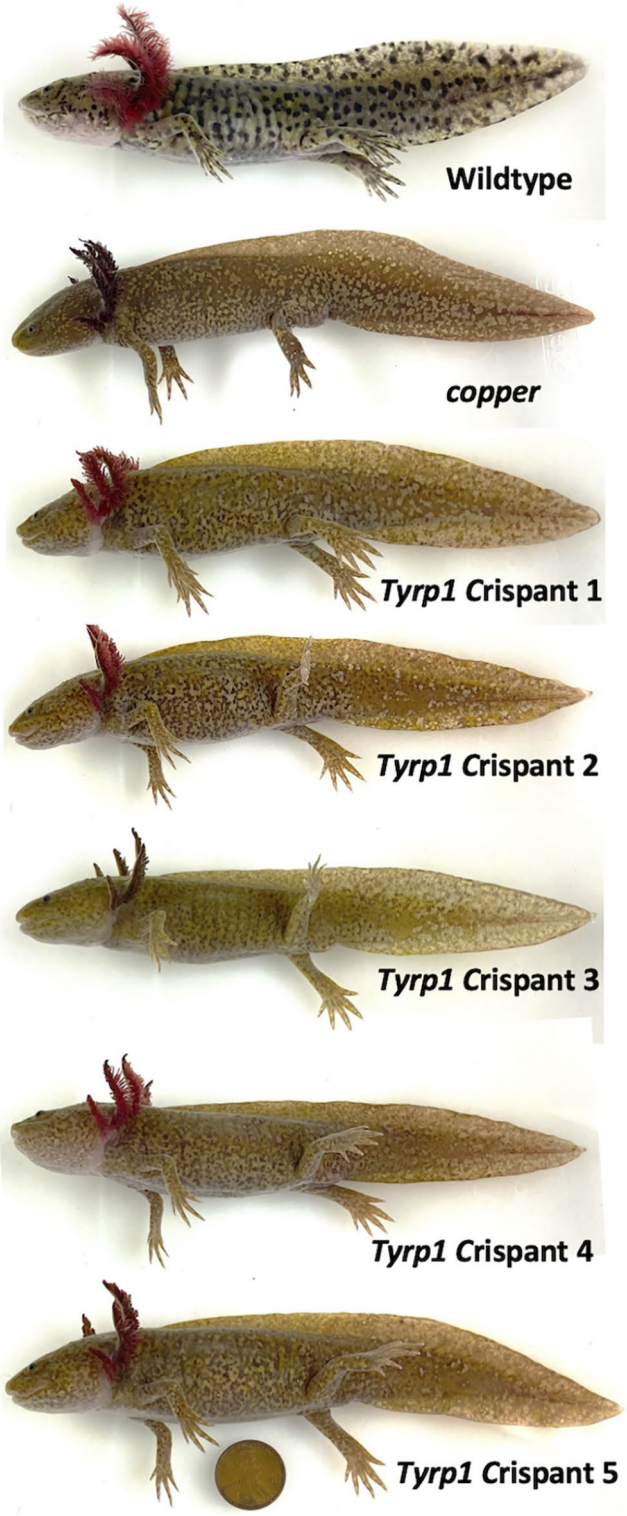

Fig. 3. Images of F0 *Tyrp1* crispants relative to a wildtype axolotl and *copper* mutant. Salamanders are 15–18 cm total body length and 1-1.2 years old. A USA copper penny (1.9 cm diameter) is provided as a color and size reference.

Animal procedures

Axolotls used in this study were obtained from a commercial population (Strohls Herptiles: wildtype and *copper* sibling larvae) and the Ambystoma Genetic Stock Center (AGSC RRID: SCR_006372; wildtype axolotl embryos RRID: AGSC_100E). All experiments were performed using either 50% (pre-hatching) or 100% (post-hatching) axolotl rearing water (ARW: 1.75 g NaCl, 100 mg MgSO4, 50 mg CaCl2, and 25 mg KCl per liter, buffered with

NaHCO3 to pH 7.3–7.5) in a room maintained at 17–18 °C. Larvae were housed in 1 L glass or 2 L polypropylene bowls at either low density (8–10 per bowl) or one per container. After larvae reached 5 cm they were transferred to 9 L boxes on an Aquatic Enterprises recirculating system. Larvae were initially fed newly hatched brine shrimp until 3 cm total body length and then transitioned to California Black worms (J&R Enterprises) until large enough to be fed fish pellets (Rangen). Animals were anesthetized using a 0.02% benzocaine solution. Benzocaine was first dissolved in 4 ml 100% EtOH and then the chemical and solvent were diluted in 1 L of ARW.

Bulked segregant RNA-Seq

A total of 36 *copper* and 27 wildtype siblings were sampled from a cross between a homozygous *copper* male and a heterozygous female carrier and reared to approximately 3 cm. Tail tissue was collected from each while under benzocaine anesthesia. The resulting tail tips were pooled into separate 1.7 ml plastic tubes and flash frozen with dry ice to create *copper* and wildtype bulk segregant pools for RNA isolation. Tail tissue samples were dissociated with 23- and 26-gauge needles, and RNA was isolated with TRIzol and then further purified using a QIAGEN RNeasy Mini Kit with DNase treatment. The resulting RNA pools were used to generate outsourced Poly(A) RNA-seq libraries that were sequenced on an Illumina NovaSeq X Plus (150 bp paired end reads) by Novogene. Reads from each pool of *copper* or wildtype individuals were mapped to the axolotl genome assembly[24] using HiSat2[25] v. 2.2.0 (http://daehwankimlab.github.io/hisat2). SNPs were identified using BCFtools[26] v. 1.12-57-g0c2765b (https://samtools.github.io/bcftools) and SNP frequencies at polymorphic sites were compared between copper and wildtype pools using Popoolation2[27] v. 1.201 (https://sourceforge.net/projects/popoolation2). Additionally, DEseq2[28] v. 1.44.0 (https://www.bioconductor.org/packages/release/bioc/html/DESeq2.html) was used to compare *Tyrp1* transcript abundances between *cooper* and *wildtype* pools. RNA sequence data will be published in the Short Read Archive at the National Center for Biotechnology Information.

Two guide RNAs (gRNAs) (CTGGCCACTGCGGAGAGCCT, TTTGTCCTCCAGTTCCATTC) were designed to target the 2nd and 6th exons, respectively, of axolotl *Tyrp1* (TYRP1|AMEX60DD301043361.1). To generate targeted mutations, gRNAs were first duplexed with Alt-R tracrRNA, aliquoted and stored at -80^{0} C. All RNA products were synthesized by Integrated DNA Technologies. Guide RNAs and Alt-R tracrRNA were mixed with Cas9 to generate ribonucleoproteins that were injected into 100, 1-cell stage wildtype AGSC embryos as described previously [29]. A total of 12 injected embryos were reared to approximately 3 cm and tail tissue was collected from each while under benzocaine anesthesia. During animal rearing, larvae were fed brine shrimp. The resulting tail tips were placed into separate 1.7 ml plastic tubes and placed on ice for DNA isolation. DNA isolations were performed using a New England Biolabs (NEB) Monarch genomic DNA isolation kit. DNA concentrations were determined using a Nanodrop (Thermo Scientific) and all samples were diluted to 30 ng / ul for PCR. PCR primers were designed to amplify DNA amplicons spanning gRNA target sites. The flanking PCR primers for gRNA1: Tyrp1_gRNA1_5. 1 TTGGTTTTATAGTTCCAGGTCCCG and Tyrp1_gRNA1_3.1 AGAC AGAAGCCATTCATCGACTG. The flanking PCR primers for gRNA2: Tyrp1_gRNA2_5.1 AAGGTGGTTGAA TCTTGTCTCCTT and Tyrp1_gRNA2_3.1 TTTTAAGACAGGTTACCCCCGAG. PCR amplicons were treated with NEB Exo-CIP and shipped to Eurofins for Sanger sequencing. The resulting sequences were compared using Geneious Prime software to evaluate CRISPR editing.

Homozygous *copper* and wildtype (axolotls not carrying *copper* alleles) larvae were sampled from two separate spawns. PCR was performed using Tyrp1_gRNA2_5.1 and Tyrp1_gRNA2_3.1 primers to amplify *Tyrp1* exon 6 genomic sequence from 4 individuals (2 *copper*, 2, *wildtype*) and amplicons were sequenced as described above. The resulting sequences were aligned using Geneious Prime software and searched for polymorphisms.

Data availability

Raw DNA sequence reads and transcript abundance estimates may be found under GEO GSE269079.

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Author contributions

R.F.C. performed animal care procedures, collected tissues for RNA and DNA isolation, isolated RNA and DNA, designed PCR primers and gRNAs for CRISPR-CAS9, performed PCR, prepared samples for RNA and DNA sequencing, analyzed and summarized results from PCR and DNA sequencing, and contributed to writing of the manuscript. L.S. contributed to study design, supplied copper and wildtype larvae for RNA-Seq, provided information about axolotl stocks in the pet trade, assessed candidate genes for association to copper, and contributed to writing of the manuscript. M.T. and J.S. performed animal care procedures and took pictures of salamanders. N.T. developed and applied methods and pipelines for bioinformatic analyses and contributed to writing of the manuscript. J.J.S. contributed to study design, developed and applied methods and pipelines for bioinformatic analyses, summarized results from bioinformatic analyses, and contributed to writing of the manuscript. S.R.V. contributed to study design, performed animal care procedures, collected tissues for RNA and DNA isolation, performed embryo microinjections, photographed salamanders, analyzed, and summarized results from the study, and drafted the original manuscript.

Supplementary files

Competing interests

The authors declare no competing interests.

Supplementary file 1

Manhattan plots showing the degree of association of segregating genotypes with *copper* phenotype vs. wildtype in BSR-Seq pools. (A) Values shown are -log10(p-values) from Fisher's exact tests. (B) Values are Z-scores from statistical tests of allele frequency difference.

Supplementary file 2

Nucleotide sequence for wildtype *Tyrp1* showing where a nucleotide deletion (nonsense mutation at 1156 bp) occurs in the *copper Tyrp1* allele.

Supplementary file 3

Predicted amino acid sequence for wildtype Tyrp1 showing where the *copper* amino acid sequence is altered by a nonsense mutation that results in a stop codon at amino acid residue 415.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-73283-1.

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