**Supplemental Information**

**Epistatic and Combinatorial Effects**

**of Pigmentary Gene Mutations**

**in the Domestic Pigeon**

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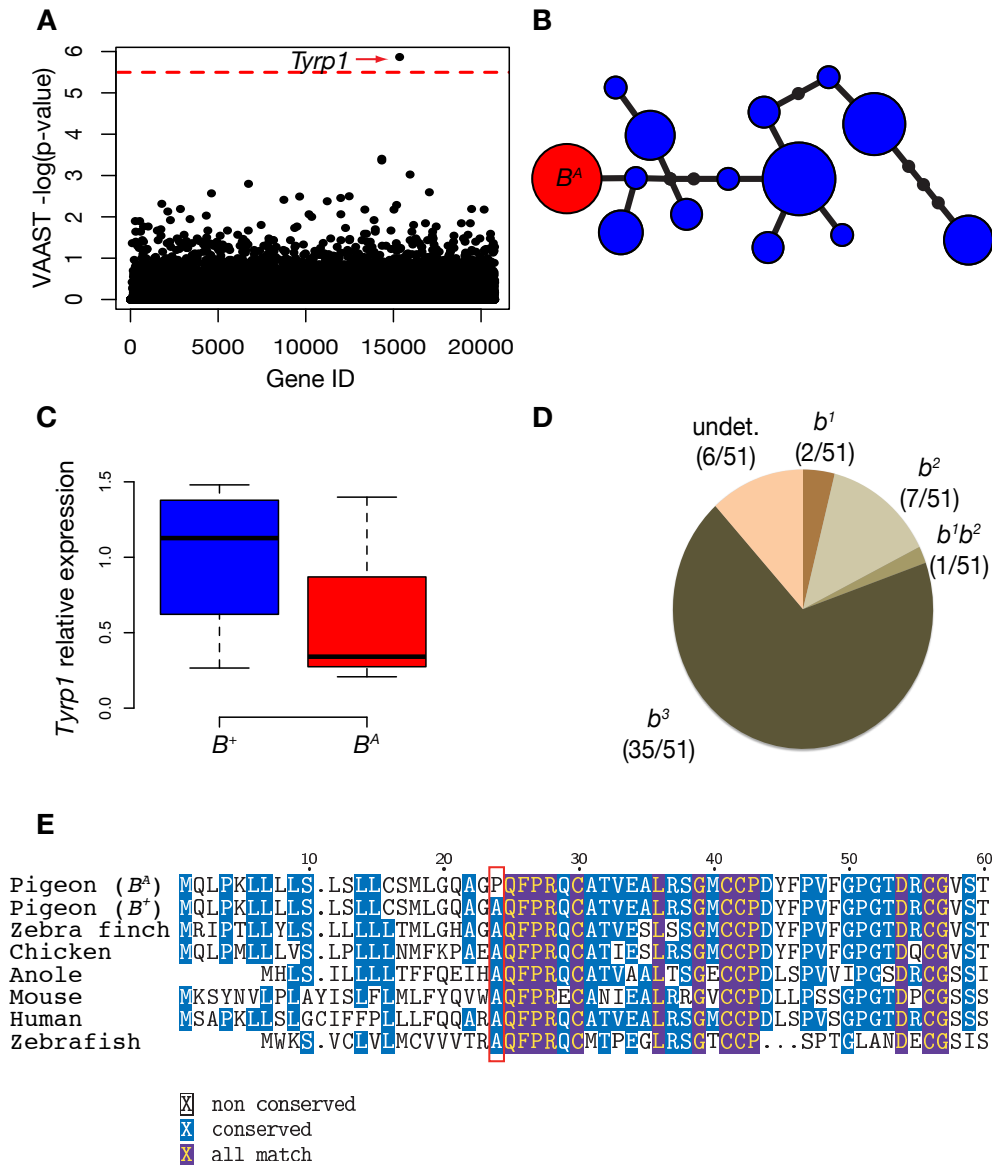
**Figure S1, related to Figure 2. Mutations in *Tyrp1* are associated with major color phenotypes in rock pigeons.** (**A**) VAAST analysis identified a variant in *Tyrp1* associated with the ash-red phenotype. Red dashed line indicates genome-wide significance threshold ($P < 2.77$ x $10^{-6}$). (**B**) Haplotype network diagram of the *B* locus in birds from the whole-genome resequencing panel [S1]. All chromosomes with the $B^A$ mutation are identical at adjacent markers in a 20-kb haplotype, indicating a single origin of the $B^A$ allele. (**C**) qRT-PCR analysis of *Tyrp1* expression in $B^+$ and $B^A$ feathers. Boxes span first to third quartiles; black line, median. ($B^+ = 1 \pm 0.52$, $B^A = 0.64 \pm 0.65$, n = 4 each; $P = 0.39$). (**D**) Pie chart of genotypes of brown pigeons. One pigeon was trans-heterozygous $b^1b^2$, indicating that interbreeding between lineages with different *b* alleles has occurred. (**E**) Multi-species alignment of N-terminus of TYRP1, with the site of the $B^A$ mutation indicated (red box).
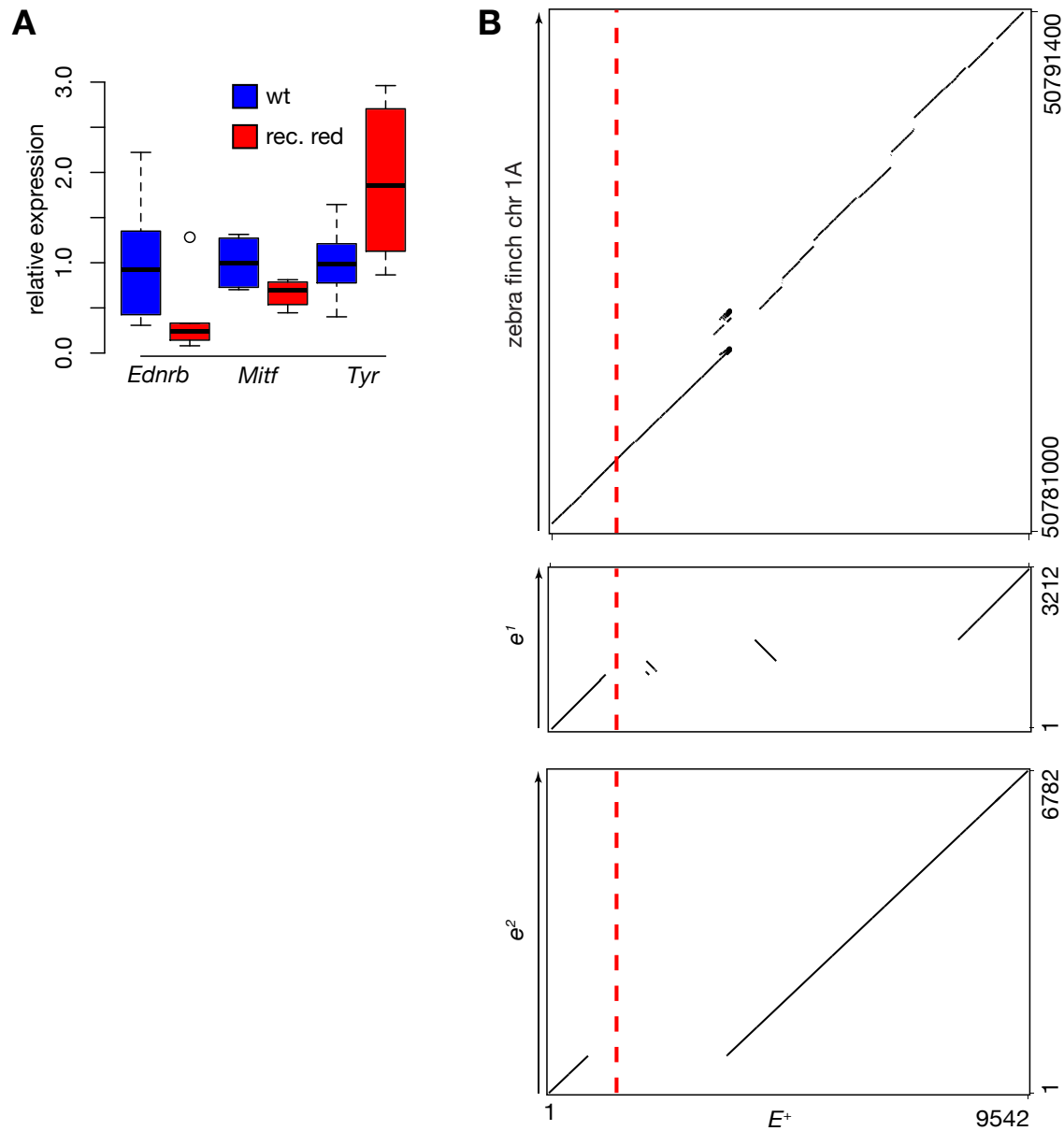
**Figure S2, related to Figure 3. Expression of pigmentation genes and dotplots of genome alignments in recessive red pigeons.** (**A**) Quantitative RT-PCR analyses of melanogenic gene expression in wild-type and *recessive red* feathers. *Ednrb* relative expression: wt = 1 ± 0.72, recessive red = 0.38 ± 0.48, $P = 0.07$; *Mitf* relative expression: wt = 1 ± 0.31, recessive red = 0.66 ± 0.16, $P = 0.19$; *Tyr* relative expression: wt = 1 ± 0.41, recessive red = 1.90 ± 0.93, $P = 0.07$, n = 6 each. Boxes span first to third quartiles; black line, median. (**B**) Recessive red is associated with two deletions upstream of *Sox10*. Dotplot alignment of pigeon $E^+$ allele of *Sox10* (scaffold974) to zebra finch *Sox10* upstream region (top), pigeon $e^1$ (middle), and pigeon $e^2$ alleles (bottom). Location of melanocyte enhancer indicated with red dashed line. In addition to deletions, the $e^1$ allele has several inversions relative to $E^+$. Alignments generated using PipMaker [S2].
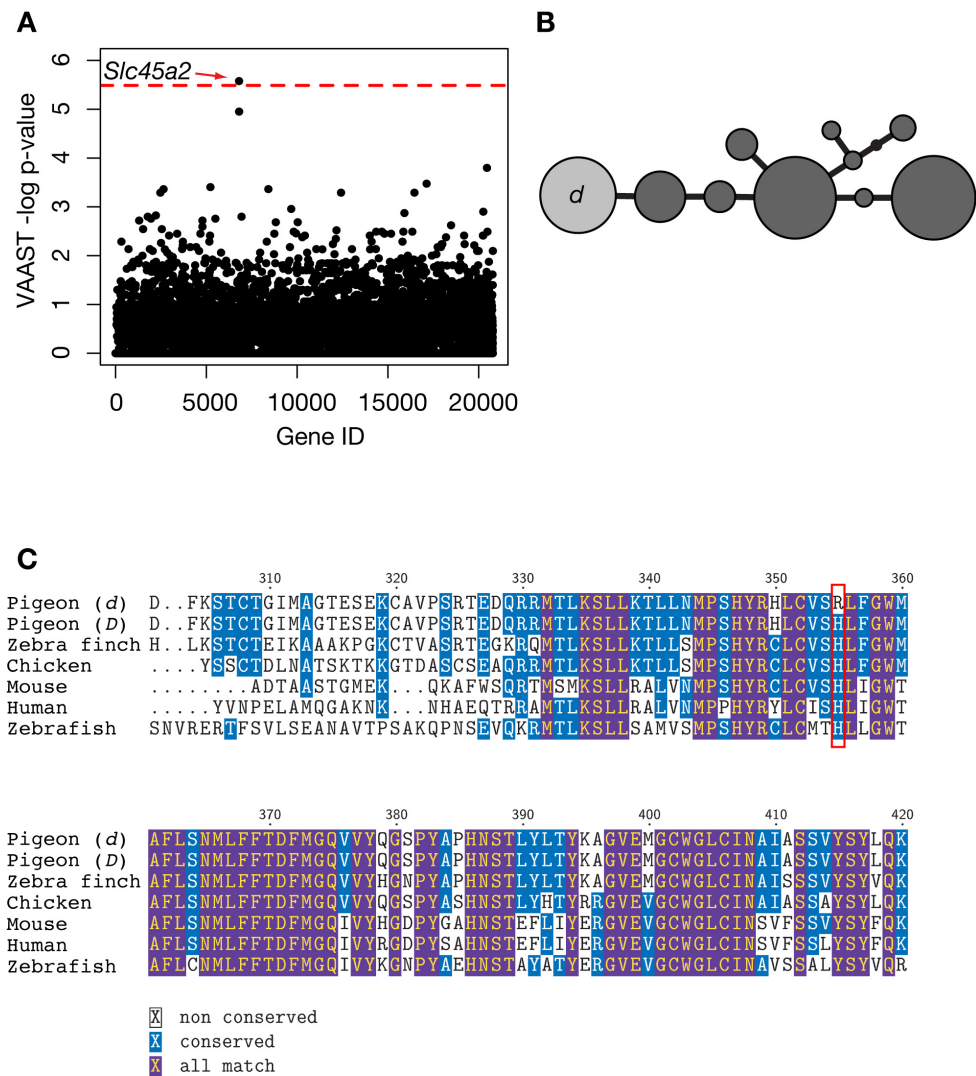
**Figure S3, related to Figure 3. *Slc45a2* is associated with the classical *dilute* locus.**
(**A**) VAAST analysis identifies a variant in *Slc45a2* associated with *dilute* phenotype (A to G nucleotide substitution at scaffold227, position 769369). Red dashed line indicates genome-wide significance threshold ($P < 2.77 \times 10^{-6}$). (**B**) Haplotype network diagram of the *D* locus in birds from the whole-genome resequencing panel [S1]. All chromosomes with the *d* mutation are identical at adjacent markers in a 2-kb haplotype, indicating a single origin of the *d* allele. (**C**) Multi-species alignment of segment of SLC45A2, with the site of the *d* mutation indicated (red box).

**SUPPLEMENTAL EXPERIMENTAL PROCEDURES**

**Determination of feather color phenotype**

Feather color phenotypes of individual birds were assigned by their respective breeders. Photographs of each bird were also taken during sample (blood or feather) collection as an archive and to verify breeder-assigned phenotypes against standard references [S3, S4]. For $F_1$ and $F_2$ birds generated in the laboratory cross, photographs were taken of each bird, and compared to photographs of breeder-phenotyped birds and standard references.

**Genomic analyses**

Reads from a whole-genome resequencing panel [S1] were aligned to the rock pigeon reference genome using Bowtie 2 (end-to-end) [S5]. Duplicate reads (identical outer coordinates) were removed using Samtools "rmdup" [S6]. The Genome Analysis Toolkit (GATK) was used to identify and polish regions with insertions/deletions [S7]. Variants were called for each sample individually using Samtools, and variants of low quality ($Q \leq 20$) were excluded from all downstream analyses. Effects of variants on coding sequences were annotated using VAT in the Variant Annotation, Analysis, and Search Tool (VAAST) pipeline [S8]. All variants were joined into a single "condenser file" (CDR), the input format for VAAST. Missing genotypes, due to low coverage or low quality were flagged as "no-calls" [S1]. Genomic association analyses were then performed using VAAST. Results were considered statistically significant if $P < 2.77 \times 10^{-6}$. Haplotype network analyses were performed as described previously [S1].

**Genotyping assays**

DNA was extracted from blood and feather samples as described previously [S9], following protocols approved by University of Utah Institutional Animal Care and Use Committees of University of Utah (protocols 09-04015 and 10-05007) and UT Arlington (protocol 09-009). Primers used to amplify exons of *Tyrp1* for sequencing and for genotyping *Sox10* upstream deletions are listed in Table S1. To genotype birds for $B^A$, $b^3$, and $d$ alleles, TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA) were performed as described [S1] (Table S1). Sixteen brown birds that were not hemi- or homozygous $b^3$ were genotyped for $b^1$ and $b^2$ alleles by Sanger sequencing. Breeds used for association testing are as follows:

*ash-red vs. blue/black or brown*

*ash-red:* American roller, domestic show flight, Egyptian Baghdad, English carrier, exhibition homer, Franconian trumpeter, frillback, Indian fantail, medium-faced crested helmet, Modena, mookee, Old German owl, racing homer, saint, show-type racing homer, Spanish little friar tumbler, starling, Voorburg shield cropper, West of England tumbler.

*blue/black or brown:* American roller, American show racer, Berlin long-faced tumbler, Bohemian pouter, Budapest short-faced tumbler, Crested Saxon field color, domestic show flight, dragoon, Egyptian Baghdad, Egyptian swift, English short-faced tumbler, English trumpeter, exhibition homer, fantail, feral, French mondain, German beauty homer, Holle cropper, homer, horseman pouter, Italian owl, king, Modena, mookee, Norwich cropper, Old Dutch Capuchine, Old German owl, oriental frill, racing homer, saint, Scandaroon, Spanish little friar tumbler, Syrian Baghdad, Thuringer clean-legged tumbler, Vienna medium-faced tumbler, West of England tumbler.

<u>*brown vs. wild-type*</u>

*brown:* African owl, American roller, Arabian trumpeter, Berlin long-faced tumbler, Berlin short-faced tumbler, Birmingham roller, Chinese owl, domestic show flight, English carrier, English long-faced tumbler, fantail, Hungarian piedhen, Indian fantail, Lahore, Marchenero pouter, Modena, mookee, Norwich cropper, Old Dutch Capuchine, old-style oriental frill, oriental frill, oriental roller, Pica pouter, Polish Lynx, Portuguese tumbler, racing homer, show-type racing homer, starling, Swiss Mondain, West of England tumbler.

*wild-type:* Altenburg trumpeter, Arabian trumpeter, archangel, Birmingham roller, Bohemian pouter, cauchois, domestic show flight, dragoon, Egyptian swift, English carrier, English long-faced tumbler, English magpie, English trumpeter, fantail, Franconian Trumpeter, French Mondaine, frillback, Holle cropper, Indian fantail, Italian owl, Jacobin, Little Spanish friar tumbler, medium-faced crested helmet, Modena, mookee, nun, Old Dutch Capuchine, oriental frill, Pomeranian pouter, racing homer, Rhineland ringbeater, Russian tumbler, saint, West of England tumbler.

<u>*recessive red vs. wild-type*</u>

*recessive red:* American roller, Backa tumbler, Birmingham roller, Budapest short-faced tumbler, carneau, cauchois, domestic show flight, English long-faced tumbler, English carrier, English trumpeter, Franconian trumpeter, Indian fantail, king, Maltese, medium-faced crested helmet, parlor roller, Scandaroon, Spanish barb, Stargarder Zitterhals.

*wild-type:* American giant homer, American roller, American show racer, Armenian tumbler, Backa tumbler, Bokara trumpeter, Berlin long-faced tumbler, Budapest short-faced tumbler, cauchois, dragoon, Egyptian Baghdad, Egyptian swift, English long-faced tumbler, English trumpeter, fairy swallow, fantail, feral, frillback, German double-crested trumpeter,

Holle cropper, horseman pouter, Indian fantail, Italian owl, Jiennese pouter, king, Lahore, Maltese, mookee, Norwich cropper, nun, Old Dutch Capuchine, oriental frill, oriental roller, Polish lynx, Pomeranian pouter, Portuguese tumbler, racing homer, Russian tumbler, saint, Scandaroon, Spanish barb, starling, Syrian Baghdad, Voorberg shield cropper, Stargarder Zitterhals.

*dilute vs. non-dilute*

*dilute:* Altenburg trumpeter, American roller, Berlin long-faced tumbler, Budapest short-faced tumbler, domestic show flight, Egyptian swift, English carrier, English magpie, English trumpeter, fantail, Franconian trumpeter, Indian fantail, Italian owl, Jacobin, mookee, Old Dutch Capuchine, racing homer, Rhineland ringbeater, Russian tumbler, Scandaroon, Shaksharli, show-type racing homer, Spanish little friar tumbler, starling, Thuringer clean-legged tumbler, West of England tumbler.

*non-dilute:* African owl, American roller, Arabian trumpeter, Birmingham roller, Bohemian pouter, cauchois, Chinese owl, domestic show flight, dragoon, English carrier, English long-faced tumbler, fantail, French mondaine, frillback, German beauty homer, Holle cropper, Hungarian piedhen, Italian owl, Lahore, Marchenero pouter, medium-faced crested helmet, Modena, mookee, Norwich cropper, nun, Old Dutch Capuchine, oriental frill, oriental roller, pica pouter, Polish lynx, Pomeranian pouter, Portuguese tumbler, racing homer, saint, Scandaroon, show-type racing homer, Spanish pouter, Swiss mondain, Syrian Baghdad, Vienna medium-faced tumbler, West of England tumbler.

**RNA isolation and cDNA synthesis**

To assay gene expression, several covert wing feathers were plucked to stimulate regeneration. Seven to ten days later, regenerating feathers were plucked, the proximal 4 mm of each feather collected, cut in half lengthwise, and placed in RNAlater (Qiagen, Valencia, CA) at $4°C$ overnight. Next, the dermis of each follicle was removed by dissection under a microscope, and the remaining collar cells (composed primarily of melanocytes and keratinocytes) were harvested. Total RNA was extracted using a previously described protocol [S10], then cleaned and DNase-treated using an RNeasy kit (Qiagen). mRNA was reverse-transcribed to cDNA using oligo-dT and M-MLV RT (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

**TYRP1 processing analysis**

$B^+$ *Tyrp1* coding sequence was PCR-amplified from feather cDNA using primers listed in Table S1, cloned into *pcDNA-V5-HisA* (Invitrogen) using Gibson assembly [S11], then subcloned into *pcDNA-Flag-HA* (Addgene plasmid 10792) to generate an N- and C-terminal tagged *Tyrp1* construct (*pcDNA-Flag-HA-Tyrp1-V5-His*). The $B^A$ A23P mutation was generated by site-directed mutagenesis (Quick-Change Mutagenesis Kit; Stratagene, La Jolla, CA). $B^+$ and $B^A$ *Tyrp1* constructs were transfected into B16F10 cells using Lipofectamine 2000 (Invitrogen). Cells were lysed in RIPA buffer after 48 h. Western blot analyses were performed on cell lysates, using mouse anti-V5 (Invitrogen, 1:500 dilution) and mouse anti-HA (Sigma, 1:2000 dilution) antibodies to detect total and uncleaved TYRP1 protein, respectively. Signal intensity was quantified using ImageJ, and analyzed using Student's t-test. Results are presented as mean +/- S.E., and differences were considered statistically significant if $P < 0.05$.

**qRT-PCR analyses**

cDNA was amplified using intron-spanning primers for the appropriate targets using a CFX96 qPCR instrument and iTaq Universal Sybr Green Supermix (Bio-Rad) (Supplemental Table S1). Results are presented as mean +/- S.E., and were compared by Mann-Whitney U test. Differences were considered statistically significant if $P < 0.05$.

**Allele-specific expression assay**

Three SNPs in *Sox10* were identified by Sanger sequencing in the parents of a cross between $E^+E^+$ and $e^2e^2$ birds, and PyroMark Custom Assays (Qiagen) for each SNP were designed using the manufacturer's software. Pyrosequencing was performed on cDNA and gDNA derived from $E^+e^2$ feathers using a PyroMark Q24 instrument. The $e^2/E^+$ signal intensity ratio from cDNA samples was normalized to ratios obtained from corresponding gDNA samples to control for allele-specific amplification bias. Normalized ratios were analyzed by Mann-Whitney U test, and considered significant if $P < 0.05$. Primers used for each assay are listed in Table S1.

**Sequencing of $E^+$ and $e^2$ alleles**

Breakpoint-flanking sequences defined by the pigeon reference genome sequence (derived from an $e^1$ homozygote) were used to seed de novo assemblies of the $E^+$ and $e^2$ alleles from whole-genome shotgun libraries derived from wild-type and recessive red birds [S1]. Additionally, primers spanning the $e^1$ deletion in the reference genome were used to amplify

genomic sequence in birds with the $E^+$ and $e^2$ alleles. These products were cloned and Sanger sequenced.

**Ultrastructural analyses**

Feather regeneration in birds with different *Tyrp1* genotypes was stimulated as described above. Seven to ten days later, the regenerating feathers were plucked, the proximal 4 mm of each feather was collected and cut in half lengthwise, and placed in Karnovsky's fixative overnight at 4°C. Samples were then washed in 0.2M sodium cacodylate buffer, postfixed in 2% osmium tetroside, dehydrated, and embedded in Epon 813. Thin sections were cut using a RMC-MT6000XL ultramicrotome, stained with uranyl acetate and lead citrate, and viewed using a JEOL JEM-1230 transmission electron microscope. For DOPA histochemistry, tissues were incubated in 0.1% L-DOPA twice for 2 hours each at 37ºC prior to postfixing.

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