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Report

Epistatic and Combinatorial Effects of Pigmentary Gene Mutations in the Domestic Pigeon

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Summary

Understanding the molecular basis of phenotypic diversity is a critical challenge in biology, yet we know little about the mechanistic effects of different mutations and epistatic relationships among loci that contribute to complex traits. Pigmentation genetics offers a powerful model for identifying mutations underlying diversity and for determining how additional complexity emerges from interactions among loci. Centuries of artificial selection in domestic rock pigeons (*Columba livia*) have cultivated tremendous variation in plumage pigmentation through the combined effects of dozens of loci. The dominance and epistatic hierarchies of key loci governing this diversity are known through classical genetic studies [1–6], but their molecular identities and the mechanisms of their genetic interactions remain unknown. Here we identify protein-coding and *cis*-regulatory mutations in *Tyrp1*, *Sox10*, and *Slc45a2* that underlie classical color phenotypes of pigeons and present a mechanistic explanation of their dominance and epistatic relationships. We also find unanticipated allelic heterogeneity at *Tyrp1* and *Sox10*, indicating that color variants evolved repeatedly though mutations in the same genes. These results demonstrate how a spectrum of coding and regulatory mutations in a small number of genes can interact to generate substantial phenotypic diversity in a classic Darwinian model of evolution [7].

Results and Discussion

In the domestic rock pigeon (*Columba livia*), hundreds of years of accumulated experience by amateur and professional geneticists provide strong evidence that many complex color traits can be partitioned into combined effects of multiple loci, and that the same loci control similar traits across breeds [6]. The classical major color locus (*B*) is a sex-linked gene that confers one of three "base" colors [1–5]: wild-type blue/black (B^+), ash-red (B^A), and brown (*b*) (Figures 1A–1C). The B^A allele is dominant to B^+ and *b*, and *b* is recessive to the others. Blue/black and brown phenotypes result from high amounts of eumelanin and low amounts of pheomelanin; melanin ratios are reversed in ash-red birds [8]. In addition, the autosomal recessive mutation *recessive red* (*e*) acts epistatically to the *B* locus to elevate pheomelanin production, generating red plumage color irrespective of *B* locus genotype [2, 8] (Figure 1D). Mutant alleles of a third locus, the sex-linked recessive *dilute* (*d*), interact additively with *B* and *e* to lighten plumage color and further enrich pigmentation diversity [1, 2, 8] (Figures 1E–1H). This detailed Mendelian understanding of key phenotypes provides a robust foundation to investigate how genes and alleles interact to generate color variation. However, the molecular basis of this diversity—including the identities of genes underlying major pigmentation variants and a mechanistic explanation for their intra- and interlocus interactions—remains unknown [9, 10].

Multiple Mutations in *Tyrp1* Underlie Base Color Variation in Pigeons

Previously, we reported whole-genome sequences for 41 rock pigeons [11] with diverse color phenotypes. To investigate the molecular identity of the *B* color locus, we compared the genomes of 6 ash-red pigeons to 26 blue/black pigeons for coding changes associated with pigmentation phenotypes using the Variant Annotation, Analysis, and Search Tool (VAAST) [12]. A single gene achieved genome-wide significance: tyrosinase-related protein 1 (*Tyrp1*) ($p = 1.3 \times 10^{-6}$; see Figure S1A available online), which encodes a key enzyme in the melanin synthesis pathway. All blue/black pigeons were homozygous G on the *Tyrp1* sense strand at position 214991 on genomic scaffold 6 (B^+ allele), whereas ash-red pigeons were hetero- or homozygous for C (B^A allele), consistent with the dominant mode of inheritance of ash-red. The B^A mutation causes an alanine-to-proline substitution at codon 23 (A23P), corresponding to the cleavage site of the signal peptide (Figure 2A). In addition to finding a single haplotype containing the B^A allele in our whole-genome panel (Figure S1B), we found a perfect association between the dominant B^A mutation and the ash-red phenotype in an additional 49 ash-red birds from 20 breeds, and 105 blue/black or brown birds from 36 breeds (Figure 2B). These results suggest that the ash-red mutation occurred only once and spread species-wide through selective breeding, similar to our previous finding that the same mutation in *EphB2* underlies the head crest phenotype in multiple pigeon breeds [11].

Quantitative RT-PCR analysis revealed that *Tyrp1* mRNA levels from developing feathers of B^+ and B^A pigeons were indistinguishable (Figure S1C); however, the location of the B^A mutation at the highly conserved cleavage site of the signal peptide (Figure S1E) suggested that cleavage efficiency might be affected. We therefore expressed N- and C-terminally tagged B^+ and B^A versions of TYRP1 protein in cell culture, and we found that cleavage efficiency was dramatically reduced by the B^A mutation (relative efficiency: $B^+ = 1 \pm 0.18$, $B^A = 0.14 \pm 0.04$; n = 4 independent transfections each;

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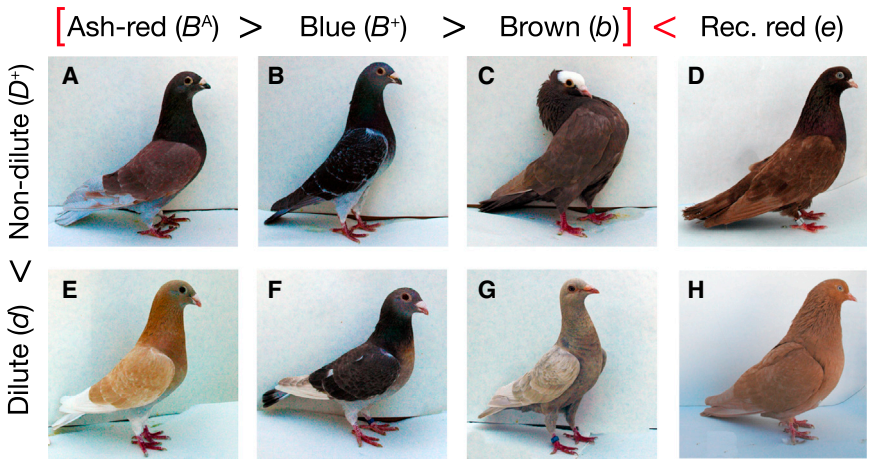

Figure 1. Common Color Phenotypes of Domestic Rock Pigeons

(A–C) Allelic variation at sex-linked major color locus (*B*).

(D) Recessive autosomal mutation *recessive red* (*e*).

(E–H) Recessive allele at another sex-linked locus, *dilute* (*d*), reduces color intensity to generate ash-yellow (E), dun (F), khaki (G), or recessive yellow (H) phenotypes.

Black chevrons (>) indicate order of dominance among alleles; red chevron indicates epistasis between loci. Breeds shown are show-type racing homer (A, B, and E–G), mookee (C), parlor roller (D), and Birmingham roller (H).

$p < 0.002$) (Figure 2C). Furthermore, spatial organization of pigment synthesis differed between *B* genotypes: premelanosomes in regenerating blue/black (B^+) feathers had a well-organized, lamellar matrix, and melanosomes were darkly pigmented, whereas ash-red (B^A and B^+B^A) feathers had a disorganized matrix and only lightly pigmented melanosomes (Figure 2D). After incubation with the melanin precursor L-DOPA, melanosomes from both wild-type and ash-red birds became darkly pigmented, indicating normal catalytic activity of the melanogenic enzyme tyrosinase (TYR) in ash-red birds. However, pigment synthesis in B^+ feathers showed strongest staining localized to the limiting membrane of the melanosome (Figure 2D), whereas staining was diffuse in melanosomes from B^A and B^+B^A feathers. Thus, the striking reduction in TYRP1 cleavage efficiency may disrupt the spatial organization of pigment synthesis activity, providing insight into the molecular basis of dominance of the B^A allele. The dominant *Light* (B^{lt}) *Tyrp1* allele of mice, a missense mutation near the same cleavage site, causes melanocyte death, probably through the accumulation of cytotoxic pigment intermediates [13]. Whether a similar accumulation of cytotoxins contributes to the pheomelanic phenotype of ash-red pigeons is unknown. However, unlike the mouse B^{lt} allele, the pigeon B^A allele results in a different kind and localization of melanin production rather than abrogation of melanogenesis.

In contrast to the single ash-red mutation, *Tyrp1* sequences from 51 brown pigeons from 30 breeds revealed three different nonsense and frameshift mutations (R72X, b^1;*411-418 del*, b^2;*893 delA*, b^3) (Figures 2A and S1D), predicted to be null alleles. Indeed, *Tyrp1* mRNA abundance in b^3 pigeons—the most common *b* allele in our sample—is greatly reduced or absent (relative expression: $B^+ = 1 \pm 0.53$, n = 4; $b^3 = 0.009 \pm 0.005$, n = 3; p = 0.05) (Figure 2E), consistent with the activation of nonsense-mediated decay. This indicates that, in contrast to the single origin of the ash-red phenotype, brown color has evolved multiple times in pigeons. Several brown pigeons did not have any of the identified *b* alleles, raising the possibility that additional mutations might also cause brown feather color (Figure S1D).

Together, these results demonstrate distinct effects of different mutations in the same gene and also confirm the predicted orthology of the classical mouse and pigeon *B* loci [5, 14]. Our analyses suggest a model in which B^A is a neomorphic allele that alters processing of the mutant TYRP1 protein within the cell. Since TYRP1 can modulate TYR activity [15–18], we postulate that the B^A version of TYRP1 protein alters normal TYR functionality, resulting in an increased ratio of pheomelanin to eumelanin production. In contrast, *Tyrp1* loss-of-function alleles b^1–b^3 cause brown pigment production, consistent with findings in other vertebrates [19].

Recurrent Deletions of a *Sox10* Enhancer Underlie Recessive Red

In addition to the dominant, sex-linked B^A allele, the autosomal mutation *recessive red* (*e*) acts epistatically to *B* to increase pheomelanogenesis and decrease eumelanogenesis (Figure 1D). VAAST scans for the *e* locus did not identify a strong candidate, suggesting that an unannotated structural variant, such as a large insertion or deletion, might underlie this phenotype. To identify candidates for *e*, we compared expression of several genes involved in melanin biosynthesis and found that the transcription factor *Sox10* and one of its target genes, *Tyrp1* (the *B* locus), were significantly downregulated in feathers of recessive red birds (Figure 3A) (*Sox10* relative expression: blue/black = 1 ± 0.62; recessive red = 0.14 ± 0.07, p = 0.001; *Tyrp1* relative expression: blue/black = 1 ± 0.556, recessive red = 0.0001 ± 0.00006, p = 0.002; n = 6 for each). Other melanin biosynthesis genes did not show altered transcript levels (Figure S2A), suggesting that a mutation directly or indirectly affecting *Sox10* expression might underlie the recessive red phenotype.

Deletions of a conserved *Sox10* enhancer result in pigmentation defects in other vertebrates, including a lack of pigmentation in mouse and increased pheomelanin production in chicken [20, 21]. Alignment of the pigeon reference genome assembly (a recessive red Danish tumbler [11]) upstream of *Sox10* to the orthologous regions of the chicken and zebra finch genomes identified a 7.5 kb deletion in the pigeon genome (Figures 3B and S2B). Furthermore, four recessive red birds in our genome resequencing panel—but no wild-type birds—were homozygous for this deletion. Importantly, the deletions in pigeon, chicken, and mouse all span a conserved enhancer element that drives *Sox10* expression in melanocytes [20, 23].

To test for broader association between the pigeon *Sox10* enhancer deletion and recessive red, we genotyped 41 recessive red pigeons from 19 breeds and 103 wild-type pigeons from 45 breeds. We found that 21 recessive red birds (but no wild-type birds) were homozygous for the deletion harbored by the reference genome (e^1 allele; Figures 3B and 3C). An additional 17 of the recessive red birds (but no

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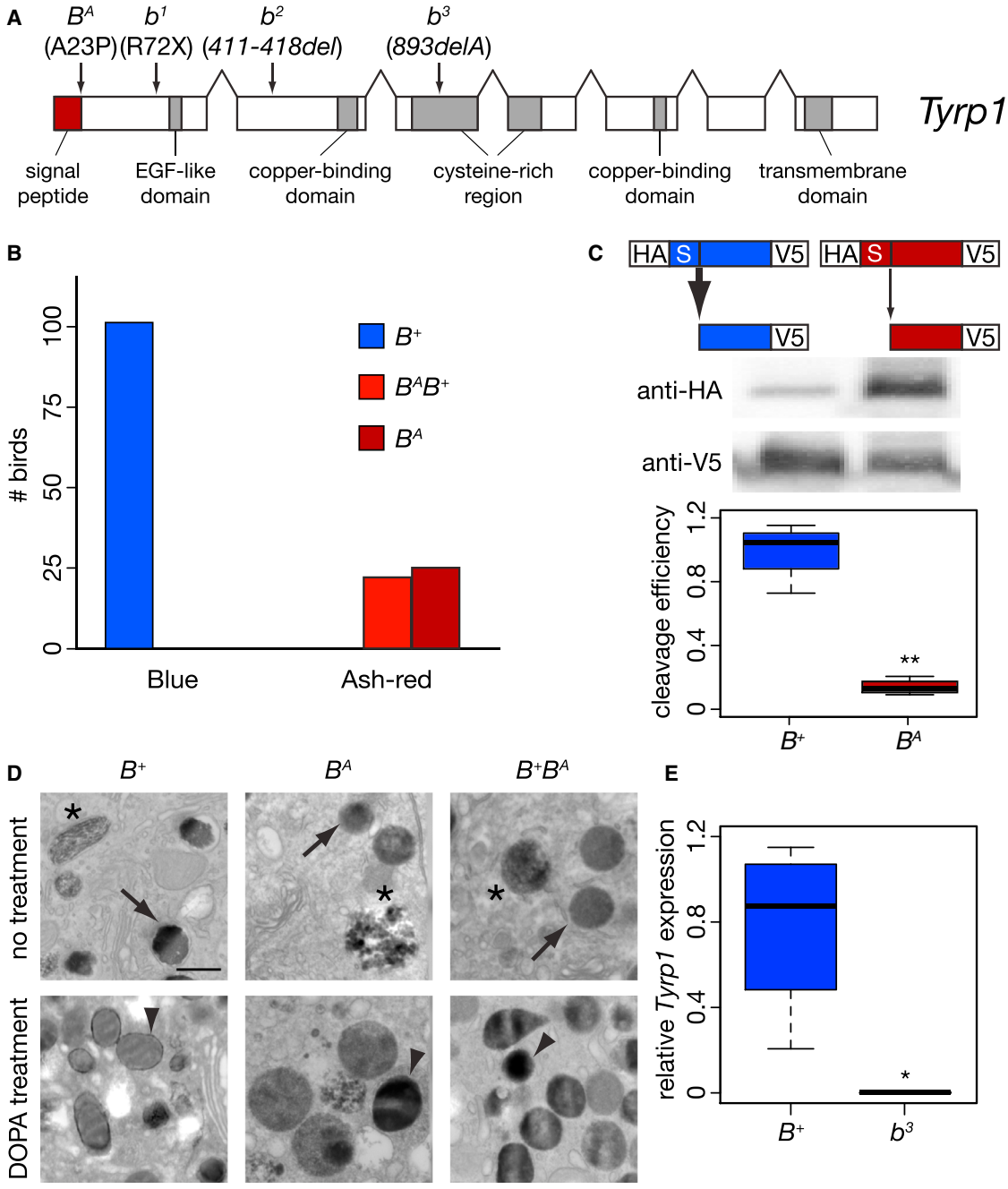

Figure 2. *Tyrp1* Is the Major Color Locus *B* in Domestic Pigeons

(A) Schematic of the genomic *Tyrp1* locus with putative B^A and *b* mutations.

(B) Histogram of genotypes of pigeons displaying wild-type or ash-red phenotypes.

(C) Schematic and western blot analysis of cleavage of TYRP1 proteins encoded by B^+ and B^A alleles, demonstrating reduced cleavage efficiency of the B^A allele. HA, N-terminal hemagglutinin epitope tag; V5, C-terminal V5 epitope tag. Boxes in (C) and (E) span first to third quartiles; bars extend to minimum and maximum observed values; black line indicates median. **p < 0.002.

(D) Ultrastructural analysis of melanocytes from B^+, B^A, and B^+B^A feathers. Asterisks indicate premelanosomes, arrows indicate untreated melanosomes, and arrowheads indicate DOPA-treated melanosomes. Scale bar represents 500 nm.

(E) *Tyrp1* mRNA abundance in B^+ and b^3 feathers by qRT-PCR. *p = 0.05.

wild-type birds) were homozygous for a second, 2.5 kb deletion (e^2) that partially overlaps e^1, and the remaining three birds were heterozygous e^1e^2 (Figures 3B and 3C). Since both pigeon deletions span the *Sox10* melanocyte enhancer, we predicted that the reduction in *Sox10* expression in recessive red birds was due to a *cis*-regulatory change. We therefore assayed allele-specific expression of E^+ and e^2 alleles of *Sox10* in E^+e^2 heterozygous birds, and we found that the e^2 allele was expressed at only ~10% of E^+ levels (SNP1 = 0.126 ± 0.055, SNP2 = 0.056 ± 0.043, SNP3 = 0.127 ± 0.059; p < 0.0001 for each, n = 10 E^+e^2 birds) (Figure 3D). Since in heterozygotes both the E^+ and e^2 alleles

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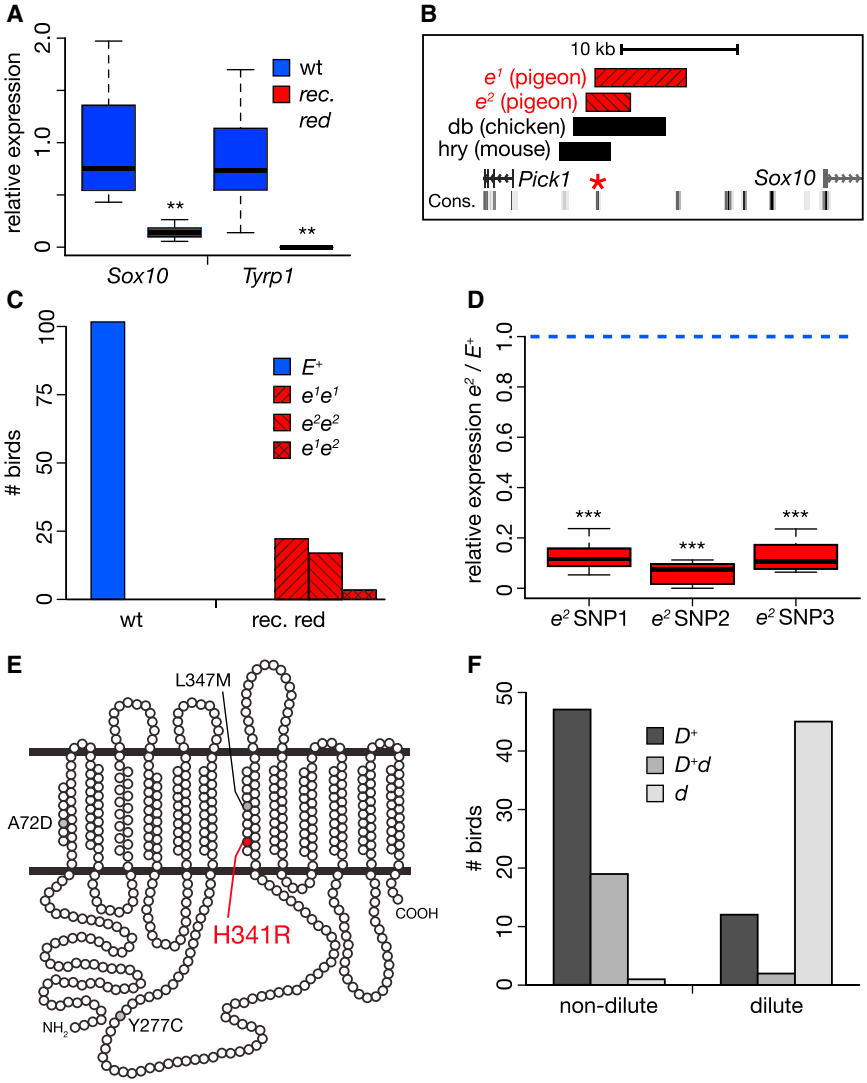

Figure 3. *Sox10* and *Slc45a2* Are the *recessive red* (*e*) and *dilute* (*d*) Loci of Domestic Pigeons

(A) qRT-PCR analysis of *Sox10* and *Tyrp1* in wild-type versus recessive red feathers (see Figure S2 for additional genes). Boxes in (A) and (D) span first to third quartiles; bars extend to minimum and maximum observed values; black line indicates median. **$p \leq 0.002$.

(B) Schematic of deletions upstream of *Sox10* in recessive red pigeons (e^1, e^2), dark-brown (db) chicken [20], and *Hry* mutant mouse [21]. Red asterisk denotes a conserved element deleted in all three species. Conservation track is based on Multiz alignment to chicken, human, mouse, rat, opossum, *Xenopus tropicalis*, and zebrafish in UCSC Genome Browser (http://genome.ucsc.edu/; chicken assembly v2.1 used as framework).

(C) Histogram of genotypes of pigeons displaying wild-type or recessive red phenotypes.

(D) Expression of SNPs in the e^2 allele relative to the E^+ allele of *Sox10* in feathers of E^+e^2 heterozygous pigeons. Blue dashed line indicates normalized expression level of E^+ allele. ***$p < 0.0001$.

(E) Schematic of SLC45A2 protein with putative *d* mutation in red. Mutations in chicken and quail associated with lightened feather color are indicated in gray. Adapted from [22].

(F) Histogram of genotypes of pigeons displaying wild-type or dilute phenotypes.

are in the same cellular environment, this experiment confirmed that the reduction in *Sox10* expression from the e^2 allele is due to a *cis*-acting mutation. Together, these genetic and expression results implicate the deletion of a *Sox10* melanocyte enhancer as the molecular basis of recessive red in domestic pigeons (Figure 3B). These results also demonstrate that the *E* (*extension*) loci of mammals (*Mc1r*) and pigeons (*Sox10*) are not orthologous [5, 9, 24–26]. Moreover, similar to the brown phenotype, recessive red appears to have evolved more than once in pigeons. While we do not observe obvious phenotypic distinctions between e^1 and e^2 homozygotes, it is possible that the different deletions generate subtly different effects on color by altering other unidentified regulatory elements [27].

The epistatic relationship of *e* to *B* is now easily reconciled in light of their molecular identities and mutations: *Sox10* directly regulates *Tyrp1* expression in melanocytes [28] (Figure 3A), explaining how loss of *Sox10* expression abrogates phenotypic effects of *Tyrp1* genotypes. Interestingly, the recessive red phenotype caused by *Sox10* downregulation is distinct from the brown phenotype of *Tyrp1* loss-of-function mutants, possibly owing to contributions of additional *Sox10* regulatory targets or residual *b* allele activity.

Missense Mutation in *Slc45a2* Is Associated with Color Dilution

While the *B* and *E* loci affect pigment color, the sex-linked recessive *dilute* (*d*) reduces pigment quantity, further enriching pigmentation diversity [8] (Figures 1E–1H). To identify candidates for *d*, we compared the genomes of 5 birds with diluted feather color and 31 birds with nondiluted pigment intensity using VAAST. A single gene achieved genome-wide significance: solute carrier family 45 member 2 (*Slc45a2*, $p = 2.65 \times 10^{-6}$; Figure S3A), which is associated with pigmentation phenotypes in diverse vertebrates, including other birds [22, 29–32], but is not orthologous to the *dilute* locus in mouse (*Myo5a*) [33]. In pigeons, the *d* mutation causes a histidine-to-arginine substitution (H341R) at a highly conserved intramembrane residue of SLC45A2 (Figures 3E and S3C). We genotyped an additional 59 diluted birds from 26 breeds and 67 nondiluted birds from 41 breeds and found a strong (but not perfect) association between *d* genotypes and color intensity under a recessive model (Fisher's exact test, $p < 2.2 \times 10^{-16}$) (Figure 3F). Fourteen birds not homozygous for *d* had diluted feather color, and one homozygote was reported to have nondiluted color. However, several other loci can cause either lightened (e.g., *milky*, *reduced*, and *faded*) or darkened (e.g., *dirty*, *sooty*, and *smoky*) pigmentation in pigeons [6], and it is expected that a broad hobbyist-identified sample should include birds with varied genetic bases for color intensity.

Mutations and Color Traits Cosegregate in a Controlled Cross

As an independent test of our association analyses, we examined cosegregation of pigmentation phenotypes and our three

A

	Blue	Dun	Ash-red	Ash-yellow	Recessive red	Recessive yellow
Tyrp1 B^A	0/28	0/21	19/19	6/6	5/11	0/5
Sox10 e^2e^2	0/28	0/21	0/19	0/6	11/11	5/5
Slc45a2 dd	0/28	21/21	0/19	6/6	0/11	5/5

B

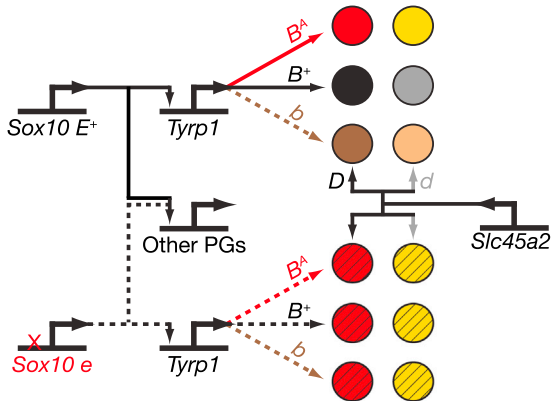

Figure 4. Segregation Analysis and Mechanistic Model of Common Color Phenotypes

(A) Representative feathers from F_1 and F_2 offspring in a cross segregating B^A, e^2, and *d* mutations; numbers of birds with a given genotype are listed below each phenotype.

(B) Schematic illustrating common feather colors in pigeons and mutations responsible for their production. Other pigmentation genes (PGs) are probably also affected by a decrease in *Sox10* expression, thereby causing differences between *b* (*Tyrp1* loss of function) and *e* (*Sox10* loss of function) phenotypes.

candidate loci in progeny of a male pigeon doubly heterozygous for B^A and *d* alleles (B^AB^+,D^+d,E^+E^+) mated to two recessive yellow females (B^+,*d*,e^2e^2; females have heterogametic sex chromosomes and are therefore hemizygous at the sex-linked *B* and *D* loci). As anticipated, segregation of the ash-red (dominant) and dilute phenotypes was observed in the F_1 and F_2 generations, whereas recessive red was observed only in the F_2. We genotyped all three generations for the candidate mutations in *Tyrp1*, *Sox10*, and *Slc45a2* and found complete cosegregation between alleles at these loci and their respective pigmentation phenotypes (Figure 4A). Coupled with our genetic association results, these transmission genetics data strongly support the molecular identities of the classical B^A (and *b*), *e*, and *d* alleles as mutations in *Tyrp1*, *Sox10*, and *Slc45a2*, respectively.

Few Loci Generate Many Phenotypes

Similar to the genetic architecture of dog coat morphology [34], a relatively small number of loci generate a wide range of plumage color phenotypes in pigeons (Figure 4B). We found that coding changes at the base color locus *B* result in a neomorphic dominant allele (B^A, ash-red) that interferes with melanosome formation and localization of melanogenesis to cause one derived phenotype, and multiple recessive, putative null alleles (b^1–b^3, brown) that underlie another phenotype. The *e* mutation is epistatic to *B* genotypes due to a regulatory mutation in *Sox10*, which is a transcriptional regulator of *Tyrp1*. A mutation at the *d* locus influences the color of birds of all genotypes at *B* and *e* by reducing the quantity of pigment produced, generating an additional layer of genetic complexity and phenotypic diversity. We find evidence that some color phenotypes, such as ash-red, appear to have a single origin whereas others, such as brown and recessive red, originated multiple times.

Many color phenotypes in pigeons and other domestic animals result from artificial selection [35]. Nevertheless, the combination of coding and regulatory changes, combined effects of multiple loci on a common phenotypic output, and single and multiple origins of derived alleles is reminiscent of the genetic architecture of a variety of adaptive traits in the wild (e.g., [27, 36–38]). Additionally, the specific genes that we have implicated in plumage color phenotypes in pigeons also contribute to both natural pigmentation diversity and skin disease in humans, including melanoma risk [39, 40]. Thus, by elucidating the complex interactions among these loci, we enrich our mechanistic understanding of adaptive and nonadaptive variation across species.

Accession Numbers

Sequence data reported in this paper have been deposited in GenBank with the accession numbers KJ023250–KJ023253.

Supplemental Information

Supplemental Information includes three figures, Supplemental Experimental Procedures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.01.020.

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